Urokinase receptor cleavage and shedding: occurrence and consequences.

Thesis

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Nicolai Sidenius

UROKINASE RECEPTOR CLEAVAGE AND SHEDDING: OCCURRENCE AND CONSEQUENCES

A thesis submitted in partial fulfilment of the requirements of the Open University for the degree of Doctor of Philosophy

November 1999.

DIBIT

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DATE OF AWARD: 29 JUNE 2000
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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>APS</td>
<td>Ammonium persulfate</td>
</tr>
<tr>
<td>ATF</td>
<td>The aminoterminal fragment of uPA</td>
</tr>
<tr>
<td>D1</td>
<td>Domain 1 of uPAR</td>
</tr>
<tr>
<td>D2</td>
<td>Domain 2 of uPAR</td>
</tr>
<tr>
<td>D2D3</td>
<td>A uPAR fragment composed of domain 2 and 3</td>
</tr>
<tr>
<td>D3</td>
<td>Domain 3 of uPAR</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco's Modified Medium</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular Matrix</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EGF</td>
<td>Epidermal Growth Factor</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>FACS</td>
<td>Flow cytometry</td>
</tr>
<tr>
<td>FAK</td>
<td>Focal adhesion kinase</td>
</tr>
<tr>
<td>FGF</td>
<td>Fibroblast growth factor</td>
</tr>
<tr>
<td>fMLP</td>
<td>formyl-MetLeuPro</td>
</tr>
<tr>
<td>GFD</td>
<td>Growth factor-like domain</td>
</tr>
<tr>
<td>Glu-Plg</td>
<td>Glu-Plasminogen</td>
</tr>
<tr>
<td>GPI</td>
<td>Glycosylphosphatidylinositol</td>
</tr>
<tr>
<td>HGF</td>
<td>Hepatocyte growth factor</td>
</tr>
<tr>
<td>HK</td>
<td>High molecular weight Kininogen</td>
</tr>
<tr>
<td>HUVEC</td>
<td>Human umbilical vein endothelial cells</td>
</tr>
<tr>
<td>ICAM</td>
<td>Intercellular adhesion molecule</td>
</tr>
<tr>
<td>IL-3</td>
<td>Interleukin-3</td>
</tr>
<tr>
<td>LFA</td>
<td>Leukocyte function associated antigen</td>
</tr>
<tr>
<td>LMW-uPA</td>
<td>Low molecular weight uPA</td>
</tr>
<tr>
<td>LRP</td>
<td>Low-density lipoprotein receptor-related protein</td>
</tr>
<tr>
<td>Lys-Plg</td>
<td>Lys-plasminogen</td>
</tr>
<tr>
<td>MMP</td>
<td>Matrix metalloprotease</td>
</tr>
<tr>
<td>MT-MMP:</td>
<td>Membrane-type matrix metalloprotease</td>
</tr>
<tr>
<td>PAGE</td>
<td>Polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>PAI</td>
<td>Plasminogen activator inhibitor</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PIPL-C</td>
<td>Phosphatidyl-inositol specific lipase C</td>
</tr>
<tr>
<td>PIPL-D</td>
<td>Phosphatidyl-inositol specific lipase D</td>
</tr>
<tr>
<td>PLA-2</td>
<td>Phospholipase A2</td>
</tr>
<tr>
<td>PMC</td>
<td>Polymorphic nucleated cells</td>
</tr>
<tr>
<td>PNGase F</td>
<td>Peptide-N-glycosidase F</td>
</tr>
<tr>
<td>PNH</td>
<td>Paroxysmal nocturnal haemoglobinuria</td>
</tr>
<tr>
<td>PTK</td>
<td>Protein tyrosine kinase</td>
</tr>
<tr>
<td>PTX</td>
<td>Pertussis toxin</td>
</tr>
<tr>
<td>TAE</td>
<td>Tris acetate EDTA buffer</td>
</tr>
<tr>
<td>TBS</td>
<td>Tris buffered saline</td>
</tr>
<tr>
<td>TE</td>
<td>Tris-EDTA buffer</td>
</tr>
<tr>
<td>TEMED</td>
<td>N,N,N,N-tetramethylethylene diamine</td>
</tr>
<tr>
<td>v/v</td>
<td>Volume for volume</td>
</tr>
</tbody>
</table>
Publications and manuscripts

Part of the work presented in this thesis is published, submitted for publication, or in preparation for publication. The Results & Discussion sections (Chapter 3-6) present the data described in the following publications / manuscripts, and will be cited throughout the thesis by their chapter number.

Chapter 3:
Sidenius, N., and Blasi, F. (1999). Domain 1 of the urokinase receptor (uPAR) is required for cellular adhesion to vitronectin. FEBS Letters in press

Chapter 4:

Chapter 5:

Chapter 6:
Abstract

The urokinase-type plasminogen activator receptor (uPAR), is a multifunctional protein with an impressive range of distinct, but overlapping functions in the process of tissue remodelling and cell migration: 1) uPAR regulates extracellular proteolysis by promoting plasminogen activation; 2) uPAR regulates cell adhesion as an adhesion receptor for vitronectin and by its capacity to modulate integrin function; 3) uPAR regulates cell migration as a signal transduction molecule and by its intrinsic chemotactic activity. In this thesis I have analysed the consequences, and occurrence, of uPAR cleavage and shedding.

In chapter 3 I analyse the structural requirements for uPAR to promote cellular adhesion to vitronectin. I demonstrate that cell surface expression of intact uPAR is necessary and sufficient to promote binding of the myeloid cell line 32D to vitronectin. In this cell system the uPAR mediated cell binding does not lead to cell spreading and does not require integrin activation. In chapter 4 I demonstrate that the chemotactic activity of uPAR maps to the linker region which connects the first and second domain of uPAR. This chemotactic epitope (the SRSRY motif) is required and sufficient for the chemotactic activity of soluble uPAR fragments and appears to induce signalling similar to that induced by uPAR ligands such as uPA. In chapter 5 I show that uPAR and the uPAR fragments D1 and D2D3 are indeed generated on the cell surface and released to the surroundings by several different cell types. In chapter 6 I describe and characterise the presence of soluble uPAR and uPAR fragments in vivo. I demonstrate that suPAR as well as the suPAR fragments D1 and D2D3 are present in human urine. Using mice xenografted with human model tumours I demonstrate that the tumour tissue is a source of urinary suPAR antigen and that the suPAR fragment pattern in urine correlates with uPAR cleavage in the tumour tissue.
Chapter 1:

INTRODUCTION
Preface

The development and normal functioning of all cell types in the multicellular organism depends upon interactions with molecules present in their environment. The major types of molecules that regulate and determine cell growth and development include growth and differentiation factors, extracellular matrix proteins, and their respective cellular membrane receptors.

The extracellular matrix (ECM) is the intercellular mesh, which provides the mechanical strength, rigidity and elasticity of virtually all tissues. Initially, the ECM was mostly considered important for its mechanical properties, an inert structure through which nutrition and signalling molecules can diffuse. However it has become clear that the extracellular matrix per se plays many fundamental roles in cell function. One example of this is the nervous system in which cell interactions with the ECM play a fundamental role in cell migration, neuronal survival, axon guidance and synapse formation.

Rather than being static, the organism undergoes continuous remodelling throughout development and in the adult phase. Physiological processes that involve extensive tissue remodelling include wound healing, mammary gland involution and embryo implantation. The process of tumour invasion and metastasis in cancer provides an "excellent" example of the extensive tissue remodelling that may occur in pathological processes.

Tissue remodelling is almost synonymous with cell migration; a process in which the ECM plays the dual role of the substratum on which the cells move as well as the physical obstacle that the cells have to surpass. To degrade the physical obstacle, which the ECM poses in the direction of migration, cells use proteolytic enzymes capable of degrading the ECM components. The best studied proteolytic systems responsible for ECM degradation are the plasminogen activation system, which generates the potent serine protease plasmin, and the family of matrix metalloproteases (MMPs).

The subject of this thesis, the urokinase-type plasminogen activator receptor (uPAR), is a multifunctional protein with an impressive range of distinct, but overlapping functions in the process of tissue remodelling and cell migration: 1) uPAR regulates extracellular proteolysis by promoting plasminogen activation; 2) uPAR regulates cell adhesion as an adhesion receptor for vitronectin and by its capacity to modulate integrin function; 3) uPAR regulates cell migration as a signal transduction molecule and by its intrinsic chemotactic activity.


Chapter 1: Introduction

Cell adhesion molecules

The interactions between cells and the ECM play a fundamental role in cell adhesion and migration. Cell-cell and cell-ECM contacts are mediated by cellular adhesion receptors, which may interact with proteins in the ECM or on the surface of other cells. Cell-cell contacts range from the stable interactions between epithelial cells to the transient and highly regulated interactions between cells of the immune system and between leukocytes and the vascular endothelium. The most well characterised adhesion receptors are members of the cadherin, immunoglobulin, selectin and integrin super families.

Cadherins are a family of single-pass transmembrane glycoproteins that play a crucial role in Ca$^{2+}$-dependent cell-cell interactions. The function of these molecules is to establish tight cell-cell associations and to define the adhesive specificity of cells. The expression of the cadherins is both temporally and spatially regulated in a fashion that correlates with (re)organisation of tissues. Proteins from the cadherin family are responsible for the formation of gap junctions and thus for intercellular signalling, and for the formation of desmosomes which contribute to the tensile strength of epithelial tissues.

Cell adhesion receptors from the immunoglobulin (Ig) superfamily have one or more of the typical Ig domains in their extracellular portion. The receptors are either single-pass transmembrane proteins or linked to the cell surface by a glycosylphosphatidyl inositol (GPI) anchor. Adhesion receptors from the Ig superfamily play a multitude of functions in embryonic development and are also expressed by many cells in the adult organism. Many proteins from this superfamily play important functions in the cell-cell interactions of the immune system and between leukocytes and the vasculature. This family includes proteins such as ICAM-1, ICAM-2 and VCAM.

Selectins are single-pass transmembrane proteins that are expressed by leukocytes, platelets and vascular endothelial cells. Through their extracellular lectin-like domains these receptors interact with carbohydrate structures present on other cells. The selectins mediate cell-cell interactions between leukocytes and the vessel wall and participate in platelet aggregation. The selectins play important functions in processes such as neutrophil extravasation and lymphocyte homing.

The integrin family of adhesion receptors plays many important functions in cell-cell and cell-ECM interactions. Integrins are heterodimers composed of one α-subunit and one β-subunit. The existence of at least 15 different α-subunits and 8 different β-subunits gives rise to an immense number of possible combinations, however only a limited number (around 20) seem to exist. Integrins interact with a variety of ECM proteins including vitronectin, fibronectin, collagen, laminin and with a number of different cell surface proteins. Integrins are the major
type of adhesion receptors mediating cell-ECM contacts but also have important functions in cell­
cell contacts and in signal transduction.

The subject of this thesis, the urokinase receptor (uPAR), is not a member of these protein
families. However, uPAR is capable of mediating cell-ECM and cell-cell contacts, and can
therefore also be considered an adhesion receptor, maybe the first of a yet to be defined family.
Chapter 1: Introduction

Extracellular proteolysis

Processes involving tissue remodelling and cell migration require the degradation of ECM components. To accomplish this degradation cells express a variety of proteases with variable activity and substrate specificity. The activity of these proteases is localised and regulated by their interactions with ECM components and a variety of specific inhibitors and cell surface receptors. The two major proteolytic systems responsible for ECM degradation are the matrix metalloproteases (MMPs) and the plasminogen activator system.

Some of the proteases in these two systems appear to be highly specific while others have a broad range of substrates. Proteases from both systems are secreted as inactive zymogens, which require a proteolytic cleavage to gain full activity. The activating cleavage may be executed by a protease from the same system, as exemplified by the activation of pro-uPA by plasmin, or by the action of a protease from another system, as exemplified by the activation of proMMP-2 by plasmin. The redundancy within and between these two protease systems most likely reflects the importance of regulated extracellular proteolysis.

uPAR is intimately connected to the plasminogen activator system which will therefore be discussed in detail. However, to provide a more complete picture I will start with a very brief introduction to the MMP system.

Matrix metalloproteases (MMPs)

MMPs are a family of proteins capable of degrading ECM components under physiological conditions. The family currently consists of 16 members but more may exist. The proteins share a common catalytic domain, which binds the Zn$^{2+}$ necessary for function, and a region responsible for the maintenance of zymogen latency. Various other domains present in the enzymes determine activity, substrate specificity, ECM association and inhibitor binding. Four of the MMPs are integral membrane proteins (membrane-type MMPs, MT-MMPs). The MT-MMPs have a domain structure which is similar to the other MMPs except for the addition of a transmembrane domain and a cytoplasmic tail. The MMPs have been classified based on their substrate preferences: the gelatinases, which degrade non-fibrillar and other types of denatured collagens; the collagenases, which degrade fibrillar collagen; and the stromelysins, which preferentially degrade glycoproteins and proteoglycans.

Four specific inhibitors of matrix metalloproteases (tissue inhibitor of metalloproteases, TIMPs) have been identified. The TIMPs inhibit MMP activity through the formation of complexes with the active MMPs. The TIMPs have in common their inhibitory effect on MMPs but have different expression patterns. TIMPs are found to have other functions and may even promote MMP activation under certain circumstances.
MMP activation occurs by proteolytic cleavage of the propeptide which blocks the active site cleft and is a critical point in the regulation of these enzymes. The activation can be accomplished by other members of the MMP family or by enzymes from other protease systems as for instance by plasmin which is capable of activating several of the MMPs. Furthermore, MT-MMPs may be activated before they reach the cell surface by action of the protease furin.

In parallel to what will be described later for the plasminogen activator system, interesting mechanisms exists for the activation of MMPs on the cell surface. The activation of MMP-2 on the cell surface for example starts with the binding of MMP-2 to a preformed cell surface complex composed of MT1-MMP and TIMP-2. The formation of the trimeric MT1-MMP/TIMP-2/pro-MMP-2 complex localises pro-MMP-2 to the cell surface where it is efficiently activated by flanking MT1-MMP free of TIMP-2. In accordance with this model the concentration of TIMP-2 plays a critical role in the cell surface activation of pro-MMP-2. TIMP-2 is required to create the binding sites for pro-MMP-2 on the cell surface, but too much TIMP-2 will block all the active MT1-MMPs and prevents the concomitant activation of bound pro-MMP-2 (for recent reviews on the interesting field of MMP activation, MT-MMPs and MMPs in angiogenesis see Murphy et al., 1999; Seiki, 1999; Stetler-Stevenson, 1999; Werb et al., 1999.)

The plasminogen activator system

Plasmin is capable of degrading virtually all proteins present in the ECM, either directly or through the activation of pro-MMPs. Plasminogen, the zymogen of plasmin, is present almost everywhere in the organism and can be activated by either of the two known plasminogen activators: urokinase-type plasminogen activator (uPA) and tissue-type plasminogen activator (tPA).

The uPA-type of plasminogen activation is very efficiently exerted on the cell surface in a process which require the concomitant binding of both pro-uPA and plasminogen to the cell surface. The binding of pro-uPA to the cell surface is critical for the acceleration of plasminogen activation and is mediated by a specific uPA-receptor (the subject of this thesis, uPAR). The activity of the enzymes from the plasminogen activator system is regulated by a number of plasmin and plasminogen activator inhibitors, which prevent the potentially disastrous consequences of uncontrolled ECM proteolysis. Plasminogen activation thus provides a highly versatile, directed, and regulated system for the degradation of the ECM.

The plasminogen activator system has for many years been considered the major proteolytic system responsible for ECM degradation in processes involving tissue remodelling and cell migration. However, the accumulation of information about other protease systems, and
in particular about the MMPs, has demonstrated the existence of an extensive functional overlap and complementarity between these systems.

As the different players of the plasminogen system are intrinsically connected to the function of uPAR, I will in this section describe these molecules in some detail.

Plasminogen

Plasminogen is the zymogen for the serine protease plasmin. The major form of plasminogen is a single chain glycoprotein of 92 kDa with an aminoterminal glutamic acid residue and is known as Glu-plasminogen (Glu-Plg). Minor forms of plasminogen are generated by limited proteolysis of the aminoterminal sequence and are collectively known as Lys-plasminogen (Lys-Plg) (Lijnen and Collen, 1982). The different forms of plasminogen have different binding affinities for fibrin and different activation characteristics (Claeys and Vermylen, 1974; Lijnen and Collen, 1982). By its "kringle" domains plasminogen is able to bind many different ECM proteins including fibrin, fibronectin, thrombospondin, vitronectin and laminin (Preissner, 1990; Salonen et al., 1985; Salonen et al., 1984; Silverstein et al., 1990).

Plasminogen activation is catalysed by one of the two known plasminogen activators uPA and tPA and occurs by a specific proteolytic cleavage between aminoacids Arg 560 and Val 561. The cleavage generates the plasmin A and B chains, which remain linked by two disulphide bonds. The generated plasmin is a trypsin-like, broad specificity serine protease that cleaves most of the ECM proteins to which it binds including fibrin, fibronectin, thrombospondin and laminin (Danø et al., 1985; Pöllän en et al., 1991). Plasmin is rapidly inactivated by the serum inhibitors α2-antiplasmin and α2-macroglobulin, which are present abundantly in the organism.

Virtually all cells have high capacity, low-affinity (1 μM), cellular binding sites for plasminogen (reviewed by Felez et al., 1990) and the concomitant binding of uPA to cell surfaces strongly enhances the generation of plasmin (Ellis et al., 1989; Stephens et al., 1989). Cell surface bound plasmin is resistant to inhibition by α2-antiplasmin and α2-macroglobulin (Hall et al., 1991) hereby enhancing and concentrating the plasmin activity to the pericellular space.

Mice with an inactivated plasminogen gene have been generated (Bugge et al., 1995). Plasminogen deficiency results in high mortality, wasting, spontaneous gastrointestinal ulceration, rectal prolapse, and severe thrombosis, but is however compatible with development and reproduction. A combined deficiency of fibrinogen and plasminogen rescues mice from most of the pleiotropic effects of plasminogen-deficiency (Bugge et al., 1996). In fact, mice deficient in both plasminogen and fibrinogen are phenotypically indistinguishable from fibrinogen-deficient mice, suggesting that the fundamental and maybe the only essential physiological role of plasminogen is fibrinolysis. However, plasminogen-deficient mice display impaired wound
healing (Rømer et al., 1996a; Rømer et al., 1996b), arterial neointima formation (Carmeliet et al., 1997d), and monocyte migration (Ploplis et al., 1998), demonstrating the important role of plasminogen in processes involving cell migration.

Plasminogen is capable of activating some pro-MMPs (Mignatti et al., 1986; Murphy et al., 1992; Werb et al., 1977) and may thereby promote the degradation of ECM proteins which plasmin fails to degrade directly. The role of plasmin in the activation of pro-MMPs has been addressed in vivo using Plg-deficient mice (Lijnen et al., 1998). In an experimental system of arterial injury plasminogen appears to be required for the activation of pro-MMP-9 but not for pro-MMP-2 activation. These latter data suggest that plasmin may be important for the activation of some pro-MMP, while others are more dependent upon other activation mechanisms such as the activation by (MT-)MMPs.

Plasmin may also have a function in the activation of precursor forms of cytokines and growth factors (reviewed by Rifkin et al., 1999) as exemplified by the activation of transforming growth factor β (TGFβ)(Nunes et al., 1995).

The important role of plasminogen in cancer is substantiated by the finding that tumour growth and metastasis in Polyoma virus middle T (PymT) antigen-induced mammary cancer is reduced in plasminogen-deficient mice (Bugge et al., 1998). However, in the Lewis-Lung cancer model system plasminogen deficiency has no significant effect on metastasis, and only a mild effect on primary tumour growth and overall survival (Bugge et al., 1997). The latter observation is particularly intriguing as plasminogen is the precursor for the anti-angiogenic molecule angiostatin which has strong effects on metastasis of the Lewis-Lung tumour (O'Reilly et al., 1994).

**Plasminogen activators**

Plasminogen activators (PA) are highly specific serine proteases capable of activating plasminogen to plasmin. In mammals, two different PAs have been identified, urinary-type and tissue-type plasminogen activator (uPA and tPA). Even though the two PAs catalyse the same reaction, uPA is thought to be more important in tissue remodelling and tPA in vascular fibrinolysis. As these enzymes catalyse the same reaction, a significant functional overlap between the tPA and uPA has been observed. This functional overlap is best illustrated by gene inactivation experiments in which the single inactivation of uPA or tPA causes only rather mild phenotypes, while the double deficiency causes extensive spontaneous fibrin deposition and consequent serious effects on growth, fertility and survival (Carmeliet et al., 1994). The consequences of uPA and tPA double deficiency, is similar to the consequences of plasminogen deficiency (Bugge et al., 1995).
Urokinase-type plasminogen activator uPA

The zymogen form of human uPA (pro-uPA) is secreted as a single glycoprotein chain of 52 kDa. The protein is composed of three distinct regions namely the growth factor domain, the kringle domain and the catalytic domain. The growth factor domain has significant homology with epidermal growth factor (EGF), while the kringle domain is homologous to those present in tPA and plasminogen.

Many different cell types secrete pro-uPA and elevated levels of production are in particular associated with transformed phenotypes of cells. In adherent cells uPA is almost exclusively localised at cell-cell contacts and at focal adhesion contacts with the ECM (Pöllänen et al., 1988; Pöllänen et al., 1990).

Both pro-uPA and uPA bind with high affinity (0.1-1 nM) to a glycosylphosphatidyl inositol (GPI) anchored glycoprotein, uPAR. The coincident binding of pro-uPA and plasminogen to the cell surface strongly enhances pro-uPA and plasminogen activation (Ellis et al., 1991; Stephens et al., 1989) by a mechanism which probably involves a constructive alignment of the two pro-enzymes (Ellis and Danø, 1993). Low affinity interactions between uPA and ECM proteins such as thrombospondin, fibrin and vitronectin have also been described (Moser et al., 1995; Pannell and Gurewich, 1986; Silverstein et al., 1990).

Active uPA is generated by plasmin cleavage of pro-uPA between Lys 158 and Ile 159, resulting in a molecule composed of two polypeptides held together by a single disulphide bond. Plasmin is also able to cleave pro-uPA at Lys 135 / Lys 136 generating two separate fragments: the aminoterminal fragment (ATF) composed of the EGF and kringle domains, and the carboxyterminal fragment containing the catalytic domain (often referred to as low molecular weight uPA (LMW-uPA)).

uPA deficient mice have been generated (Carmeliet et al., 1994). The mice have normal life-span and fertility, and the only histological phenotype is the occasional occurrence of fibrin deposits. Experiments with uPA-deficient mice and cells derived from these have identified a number of interesting abnormalities with respect to cell migration, immune response, growth factor activity and activation of MMPs.

uPA-deficiency causes an in-efficient pulmonary inflammatory response to C. neoformans (Gyetko et al., 1996) with concomitant elevated mortality, suggesting that uPA plays important functions in the immune system. Furthermore, T cells isolated from the uPA-deficient mice display impaired activation and proliferation in vitro (Gyetko et al., 1999).

uPA-deficient mice display impaired arterial neointima formation after injury, most likely caused by an impaired migration of smooth muscle cells (Carmeliet et al., 1997a). Analysis of atherosclerotic aortas in mice with a combined deficiency of apolipoprotein E and uPA indicates
that deficiency of uPA protects against media destruction and aneurysm formation, probably by means of reduced plasmin-dependent activation of pro-MMPs (Carmeliet et al., 1997b).

Experiments using smooth muscle cells isolated from uPA-deficient mice show that uPA catalysed plasminogen activation, through the generation of active TGFβ from latent TGFβ, is required for the anti-apoptotic activity of TGFβ on smooth muscle cells (Herbert and Carmeliet, 1997).

Tissue-type plasminogen activator (tPA)

\textit{tPA} is a secreted single chain 70 kDa glycoprotein. The protein is composed of an aminoterminal finger domain, a growth factor domain, two kringle domains, and a carboxyterminal catalytic domain. \textit{tPA} is secreted from vascular endothelial cells and measurable levels of \textit{tPA} are found in the circulation (Holvoet et al., 1985). \textit{tPA} is also secreted from many cultured tumour cells, including melanoma and neuroblastoma (Andreasen et al., 1984). Cellular binding sites exist on many cells including endothelial, neuronal and melanoma cell lines (Bizik et al., 1990; Hajjar et al., 1987), but the function of the cellular binding site for \textit{tPA} has not been finally determined.

Single chain \textit{tPA} has, unlike pro-uPA, notable enzymatic activity (Boose et al., 1989). The enzymatic activity is enhanced by plasmin cleavage at Arg 275, which generates the two-chain form held together by a single disulphide bond. The catalytic activity of both forms of the enzyme is strongly enhanced by the interaction with fibrin (Hoylaerts et al., 1982), which also reduces the rate of inhibition by PAI-1 and PAI-2 (Leung et al., 1987). The fibrin requirement for optimal catalytic activity of \textit{tPA} suggests that this enzyme may predominantly play a functional role in fibrinolysis.

\textit{tPA}-deficient mice have been generated (Carmeliet et al., 1995). The mice are fertile and have a normal life-span. However, the mice display impaired lysis of blood clots supporting the view that \textit{tPA} is involved in the maintenance of vascular fibrinolysis.

Interestingly, \textit{tPA} appears to play a role in neuron function. Mice deficient for \textit{tPA} are resistant to excitotoxin-mediated hippocampal neuronal degeneration (Tsirka et al., 1997) and long-term potentiation in different neuronal cells is also impaired (Huang et al., 1996). The role of \textit{tPA} in the brain has recently been substantiated, as transgenic mice overexpressing \textit{tPA} have enhanced hippocampal long-term potentiation and learning ability (Madani et al., 1999).

Plasminogen activator inhibitor type 1 (PAI-1)

Plasminogen activator inhibitors (PAIs) belong to the family of serine protease inhibitors (serpins). The inhibitors form 1:1 complexes specifically with the active (two chain) forms of the
plasminogen activators (Wun and Reich, 1987). The formed complexes are SDS-PAGE resistant suggesting a covalent binding between the two molecules.

PAI-1 is a 52 kDa glycoprotein (Andreasen et al., 1986; Ny et al., 1986; Pannekoek et al., 1986) secreted by a wide range of cell types including vascular endothelium, platelets, hepatocytes, fibroblasts, and many different tumour cell lines. The protein is present in normal plasma at 10-30 ng/ml but elevated levels are observed in different physiological and pathological conditions. In solution, secreted active PAI-1 rapidly loses activity by a conformational change into a latent form (Levin, 1986). PAI-1 interacts with circulating and ECM forms of vitronectin and this binding stabilises PAI-1 in its active configuration (Declerck et al., 1988; Pöllänen et al., 1987; Salonen et al., 1989; Wiman et al., 1988).

PAI-1 is considered to be the principal regulator of fibrinolysis in the vasculature and recent findings demonstrate that it also plays important roles in processes as cell migration and adhesion. The latter aspects of PAI-1 will be described later in the introduction.

PAI-1-deficient mice are viable and fertile, and show no apparent macroscopic histological abnormalities (Carmeliet et al., 1993). A more detailed analysis of PAI-1-deficiency demonstrates impaired vascular wound healing and arterial neointima formation after injury, probably caused by reduced cell migration (Carmeliet et al., 1997c). Absence of host PAI-1 prevents invasion and vascularisation of transplanted malignant keratinocytes (Bajou et al., 1998), suggesting that PAI-1 may play a role in angiogenesis and tumour invasion. Also the resolution of experimentally induced thrombi is enhanced in PAI-1-deficient mice strengthening the view that PAI-1 is indeed an important determinant of thrombolysis for instance at sites of arterial injury (Farrehi et al., 1998).

**Plasminogen activator inhibitor type 2 (PAI-2)**

PAI-2 exists in a 47 kDa intracellular and in a glycosylated 60 kDa extracellular form (Wohlwend et al., 1987). PAI-2 is secreted from stimulated monocytes, cultured U937 cells and from placental trophoblastic epithelium (Åstedt et al., 1985; Golder and Stephens, 1983; Wun and Reich, 1987). Unlike PAI-1, PAI-2 does not spontaneously lose activity in solution and has no known binding proteins in the extracellular matrix.

PAI-2 is thought to predominantly be an inhibitor of uPA, as its activity towards fibrin bound tPA is strongly reduced (Leung et al., 1987; Wun and Reich, 1987).

The understanding of the role of PAI-2 in extracellular matrix proteolysis is complicated by the fact that induction of PAI-2 expression in fibroblasts and melanoma cells leads to accumulation of an intracellular form, without notable secretion (Pytel et al., 1990).
PAI-2 deficient mice have been generated (Dougherty et al., 1999) and are phenotypically normal.
The Urokinase Receptor (uPAR)

uPAR was discovered in 1985 as a cellular high affinity binding site for uPA present on fibroblasts, monocytes and cultured U937 cells (Bajpai and Baker, 1985; Stoppelli et al., 1985; Vassalli et al., 1985). Utilising its affinity for uPA the receptor was purified, partially sequenced, and a human uPAR cDNA was cloned (Roldan et al., 1990). Since then, uPAR encoding cDNAs from mouse, rat, and bovine cells have been cloned and sequenced (Krätzschmar et al., 1993; Kristensen et al., 1991; Rabbani et al., 1994; Reuning et al., 1993).

uPAR mediated cell binding of uPA is regulated at various levels. The steady state level of uPAR mRNA is regulated on the level of transcription and mRNA stability. Posttranslational glycosylation affects the affinity of the receptor for uPA and sorting of uPAR to secretory organelles provides means for a rapid up-regulation of the cell surface expression. Once uPAR is on the cell surface the capacity to bind uPA is also regulated by receptor shedding, a process in which uPAR is released from the cell surface, and by proteolytic cleavage of uPAR which prevents uPA binding.

uPAR gene structure and expression

The human uPAR gene is composed of seven exons and six introns. (Wang et al., 1995) and in man maps to chromosome 19 (Børglum et al., 1992).

uPAR is present on many cells, including migrating keratinocytes, tumour-infiltrating fibroblasts, T lymphocytes and macrophages, many cancer cells, and most cultured cell lines (Pyke et al., 1991; Rømer et al., 1994a; Rømer et al., 1994b). In leukocytes uPAR is expressed by monocytes, neutrophils and activated T cells, but not B cells, resting T cells, and undifferentiated myeloid cells (Plesner et al., 1997; Ploug et al., 1992b; Vassalli et al., 1985). uPAR expression in T cells can be efficiently induced by co-clustering of the T cell receptor complex and integrins (Bianchi et al., 1996), or by infection with HIV (Nykjaer et al., 1994; Speth et al., 1998), identifying uPAR as a T cell activation antigen. uPAR is also a monocyte activation antigen (Dore-Duffy et al., 1992; Picone et al., 1989).

Basal transcription of the uPAR gene is driven by a TATA-less proximal promoter (Soravia et al., 1995). A number of cytokines, such as TNFα and TGFβ, and phorbol esters may regulate uPAR transcription (Lund et al., 1995; Lund et al., 1991; Niiya et al., 1998; Picone et al., 1989; Wang et al., 1994). The expression level of uPAR is also regulated on the level of mRNA stability and the responsible regions within the uPAR mRNA as well as mRNA binding proteins have been identified (Lund et al., 1995; Lund et al., 1991; Niiya et al., 1998; Shetty and Idell, 1998; Shetty et al., 1997; Wang et al., 1998).
Chapter 1: Introduction

The protein structure of uPAR

The human uPAR cDNA encodes a 335 amino acid polypeptide. The nascent polypeptide chain is posttranslationally modified in several ways to generate the mature receptor. An aminoterminal signal peptide and a carboxyterminal GPI-anchor peptide are removed during the processing for glycosylphosphatidylinositol (GPI) anchoring and cell-surface sorting. Finally, the protein is extensively glycosylated (Møller et al., 1992; Ploug et al., 1991; Roldan et al., 1990).

Glycosylation

The glycosylation of uPAR is highly heterogeneous and responsible for approximately half of uPAR's apparent molecular weight of 50-60 kDa (Behrendt et al., 1990; Roldan et al., 1990). Based on the primary sequence, human uPAR contains five potential N-glycosylation sites (Roldan et al., 1990). However on recombinant human suPAR expressed in Chinese hamster ovary cells (CHO) only four of the five asparagine residues (Asn52, Asn162, Asn172, and, Asn200) are glycosylated. The glycosylation profile of uPAR has recently been determined (Ploug et al., 1998c).

The glycosylation profile of uPAR is dependent on cell-type and differentiation, and may play a role in the regulation of the affinity for uPA (Behrendt et al., 1990; Møller et al., 1993; Picone et al., 1989; Ploug et al., 1998c).

GPI-anchoring

During the post-translational processing of the nascent uPAR polypeptide the ~28 carboxyterminal amino acids are removed and the GPI-moiety covalently attached to the novel carboxyterminus. The exact carboxyterminus, and thus the position of the GPI-anchor on mature uPAR, has not been determined yet but mutagenesis studies strongly suggest that Gly 283 is the GPI-anchorage site (Møller et al., 1992).

The fact that uPAR is anchored to the outer leaflet of the plasma membrane is essential for the understanding of its biology. Lacking a membrane or intracellular protein segment, the highly regulated cell-surface distribution and signal transduction by uPAR must be mediated through the interaction of uPAR with other integral membrane proteins and/or specific lipids. GPI-anchoring of uPAR also renders the protein prone to release from the cell surface by phospholipases, generating soluble forms of uPAR (termed suPAR) which can indeed be found in vitro and in vivo, although the biological relevance of this still has to be clarified.

Much of the biochemical analysis of uPAR has been performed using recombinant soluble forms of uPAR, termed suPAR, generated by carboxyterminal truncation after amino acid
Truncation of uPAR at this position generates a receptor which is correctly folded but secreted rather than GPI-anchored. suPAR apparently possesses the same binding properties as GPI-anchored uPAR purified from eukaryotic cells (Rønne et al., 1994).

**Domain structure of mature uPAR**

Mature uPAR is composed of three similar domains which will be termed D1, D2 and D3 from now on (figure 1.1). Each of these domains consists of approximately 90 amino acids and they are characterised by a particular pattern of Cys residues (Behrendt et al., 1991; Ploug et al., 1993). uPAR belongs to a superfamily of proteins comprising the mouse antigen Ly-6, the complement component CD59, the murine thymocyte / B cell antigen ThB, a human homologue of ThB (Ag E48), the rat bone protein RoBo-1, the herpes virus protein HVS-115, the snake serum protein PLI-1 (Nobuhisa et al., 1997), and the snake venom α-neurotoxins (Albrecht et al., 1992; Brakenhoff et al., 1995; Gumley et al., 1992; Palfree, 1991).

The disulphide pairing pattern of uPAR D1 has been determined (Ploug et al., 1993) and conforms to that of CD59 (Sugita et al., 1993). The structure of CD59 has been determined by NMR-studies and has been used to model the individual uPAR domains. The structure is composed of a disulphide cross-linked core from which three loops extend. The structure is dominated by β sheets and a short α-helical segment in the third loop (Fletcher et al., 1994; Kieffer et al., 1994; Ploug and Ellis, 1994).

**Molecular uPAR interactions**

uPAR is anchored to the outer leaflet of the plasma membrane by a GPI-anchor, has no intracellular domain or transmembrane spanning segment, is devoid of catalytic activity, and therefore must perform all its known functions through its interaction with other proteins. These interactions can be divided into two groups, depending whether they occur with other membrane molecules on the same cell (lateral interactions) or with molecules in solution, in the extracellular matrix, or on other cells (ligand interactions).

Lateral interactions between uPAR and other membrane proteins are crucial for the function of uPAR as a modulator of cell adhesion and migration, and for its chemokine activity. The existence of these uPAR interactions relies so far only on circumstantial evidence. The techniques employed include co-localisation by immunohistochemistry, fluorescence resonance energy transfer (RET) analysis, chemical cross-linking and co-immunoprecipitation. The structural basis for these direct molecular interactions is presently unknown.

The ligand interactions of uPAR include its classical interaction with uPA and vitronectin. The interaction of uPAR with these two molecules forms the molecular basis for many of the
biological roles of uPAR in proteolysis and cell adhesion are they are very well documented in the literature.

The interaction between uPAR and uPA

uPAR was initially identified as the cellular binding site for uPA, and the structural basis for this interaction has now been studied for more than a decade. While the binding determinants within uPA have been mapped to a single epitope, the regions of uPAR critical for binding are multiple and may involve conformational changes of the receptor.

In uPA, the region necessary and sufficient for high-affinity binding to uPAR is located in the aminoterminal growth factor-like domain (GFD, Appella et al., 1987). The binding site is located within the omega-loop (amino acid residues 22-28, Hansen et al., 1994), a region which is critical for binding (Magdolen et al.; 1996; Ploug et al., 1995). Glycosylation of uPA does not seem to be important for binding because uPA expressed in E.coli interacts with uPAR with similar affinity as the glycosylated protein.

Cleavage of uPAR by chymotrypsin in the linker region between D1 and D2 generates the two uPAR fragments D1 and D2D3 (Behrendt et al., 1991). This cleavage dramatically reduces (>2000 fold) the affinity for uPA, and specific uPA binding can only be demonstrated for the D1 fragment (Behrendt et al., 1991; Ploug et al., 1994). The fact that uPAR cleavage reduces the affinity dramatically suggests that determinants within D2 and/or D3 are also important for uPA binding. In fact, a soluble uPAR lacking D3 displays strongly impaired binding of uPA (Behrendt et al., 1996). Photoaffinity labelling experiments using synthetic peptides that mimic the binding characteristics of uPA (Goodson et al., 1994), have indeed demonstrated the existence of a composite binding site involving regions within both D1 and D3 (Ploug, 1998a). In particular Tyr 57 in loop 3 of D1 has been demonstrated to be localised at the interaction interface (Ploug et al., 1995).

The glycosylation of Asn 52 in D1 also appears to be required for high affinity, as an engineered uPAR lacking D1 glycosylation displays moderately reduced binding affinity (Møller et al., 1993). The kinetic explanation for this appear to be an increased dissociation rate (Ploug et al., 1998c).

The interaction between uPAR and vitronectin

The second well-characterised ligand for uPAR is vitronectin. Vitronectin is glycoprotein present at high concentrations in plasma and produced by the liver (Preissner, 1989). It is found in a single-chain form with an apparent molecular weight of 75 kDa, and in a two-chain form (65 and 10 kDa) in which the two domains are held together by a single disulphide bond. Vitronectin
is found in a monomeric form (the native form) and in a denatured multimeric form (the matrix form).

The interaction between uPAR and vitronectin appears to be even more complex than the interaction between uPAR and uPA, and is not yet well understood. Both soluble and cellular uPAR interact with vitronectin (Waltz and Chapman, 1994; Wei et al., 1994; Chapter 3) and the binding takes place with both the native and denatured form (Høyer-Hansen et al., 1997a; Kanse et al., 1996). uPAR binding has been mapped to the aminoterminal somatomedin B domain of vitronectin (Deng et al., 1996), a region which also contains the binding sites for PAI-1 and the vitronectin receptor. Another region in vitronectin, close to the carboxyterminus, has also been demonstrated to bind uPAR but less efficiently (Waltz et al., 1997).

Vitronectin binding requires multiple regions within uPAR, as both the D1 and D2D3 fragments fail to display appreciable affinity (Høyer-Hansen et al., 1997a; Chapter 3). Several antibodies against D1 inhibit the interaction between uPAR and vitronectin suggesting a crucial role for this domain in the binding (Høyer-Hansen et al., 1997a; Kanse et al., 1996; Chapter 3). These data are in contrast to the initial observations which suggested that a fragment containing D2 and D3 was capable of interaction with vitronectin (Wei et al., 1994).

The binding to vitronectin is connected to the occupancy of uPAR by uPA (Høyer-Hansen et al., 1997a; Waltz and Chapman, 1994; Wei et al., 1994; Chapter 3). In most studies a notable increase in binding to vitronectin is observed, and in one case a strict requirement, upon binding of uPA. However, the affinity as assayed by real-time biomolecular analysis is not affected by uPA (Høyer-Hansen et al., 1997a). Taken together, most data are consistent with a model in which uPAR can exist in more than one conformation and that the vitronectin binding conformation is acquired upon uPA binding (Wei et al., 1994).

Other molecular uPAR interactions

Besides the (well-established) interactions with uPA and vitronectin, uPAR has been reported to directly interact with a couple of other proteins.

High molecular weight kininogen (HK) has been shown to interact with both cellular and soluble uPAR in a Zn\(^{2+}\) dependent mechanism through interactions with a region within D2 and/or D3 (Colman et al., 1997). The interaction between uPAR and kininogen, however, still needs more study.

A direct interaction between soluble uPAR and purified immobilised Mac-1, in a reaction which requires the active ligand binding conformation of Mac-1, has been demonstrated (Wei et al., 1996). The modulatory role of uPAR on Mac-1 function, which will be discussed in detail below, gives some support to the existence of this interaction.
Cleaved and soluble forms of uPAR

Cellular uPAR has been shown to undergo two major types of cleavage that profoundly alter the characteristics of the receptor.

The first type is a proteolytic cleavage in the linker region connecting D1 and D2 and results in the release of the D1 fragment from the rest of the receptor. The cleavage changes the biochemical properties of the receptor completely. I will refer to this phenomenon as uPAR cleavage.

The second type of uPAR cleavage has not been characterised at the molecular level, but is either a proteolytic cleavage of the polypeptide chain close to the GPI anchor or hydrolysis of the GPI-anchor by a phospholipase. This cleavage releases the entire receptor from the cell surface, with concomitant functional changes, but does not alter the ligand binding properties of the receptor notably. I will refer to this process as uPAR shedding.

Mechanism

The clues for the domain structure of uPAR relies on the fact that uPAR can be fragmented into two parts using very low concentrations of chymotrypsin and that one of these, D1, is endowed with weak but specific uPA-binding activity (Behrendt et al., 1991). Together with the internal homology revealed by sequence analysis, these data suggested that uPAR is composed of three domains connected by two "linker regions" of approximately 20 amino acids. The linker region connecting D1 and D2 contains a number of potential cleavage sites for trypsin- and chymotrypsin-like proteases and for leukocyte elastase as shown in figure 1.1 (Behrendt et al., 1991; Ploug and Ellis, 1994).

Trypsin and chymotrypsin are not likely candidates for the uPAR cleavage in vivo and it is shown that biologically more relevant proteases, such as plasmin and uPA itself, are capable of cleaving the receptor in this region (Høyer-Hansen et al., 1992). The cleavage of uPAR by uPA in solution does not require binding of uPA to uPAR as the ligand binding domain of uPA can not inhibit the reaction, and because the cleavage is equally well performed by a binding-deficient form of uPA (LMW uPA).

The cleavage of uPAR by uPA is accelerated on the surface of U937 cells in a mechanism that requires the binding of uPA to uPAR (Høyer-Hansen et al., 1997b). The use of anti-catalytic uPA antibodies in cell culture has indeed demonstrated that uPA activity is required for the cleavage of uPAR on cultured U937 cells. The reason for the cell surface acceleration is not clear, but it has been suggested uPA bound to uPAR does not cleave the receptor it is occupying,
but rather nearby receptors on the same cell. The latter implies that efficient receptor cleavage is expected to occur most efficiently in uPAR rich zones on the cell surface, and even in the presence of low concentrations of uPA.

The high affinity between uPAR and uPA makes uPA a good candidate for uPAR cleavage in vivo. However, whether other proteases are also involved still remains to be resolved.

The linker region connecting D2 and D3 does not contain similar cleavage sites and is indeed much less susceptible to cleavage. The D2-D3 linker region is also expected to be less flexible than the D1-D2 linker region as D1 has one disulphide bond less than D2 and D3. Specific cleavage of uPAR between D2 and D3 can however be achieved in solution using pepsin (Behrendt et al., 1996).

Biochemical consequences

As described in the previous section, the cleavage of cellular uPAR in the region between D1 and D2 profoundly alters the biochemical properties of the receptor. The released D1 has a 2000 fold reduced affinity for uPA as compared to the intact receptor and the D2D3 fragment has no measurable affinity at all (Ploug et al., 1994). Also the interaction with vitronectin is lost as none of the generated fragments binds with measurable affinity (Høyer-Hansen et al., 1997a). In this respect the cleavage of uPAR can be seen as an inactivation mechanism. However, as the cleavage of uPAR exposes novel epitopes capable of interacting with so far unidentified membrane proteins (Fazioli et al., 1997; Resnati et al., 1996), the cleavage of uPAR may also be considered an activation event.

The occurrence of receptor cleavage

Cleaved uPAR (D2D3) has been found on the surface of many cells including endothelial cells, lymphocytes, U937 cells, and in several different cancer cell lines (Holst-Hansen et al., 1999; Høyer-Hansen et al., 1992; Ragno et al., 1998; Solberg et al., 1994; Chapter 6). Cleaved uPAR has also been identified in detergent extracts of transplanted human tumours grown in nude mice and in the mouse Lewis Lung tumour (Holst-Hansen et al., 1999; Solberg et al., 1994; Chapter 6). The cleavage of cellular uPAR releases D1 to the surroundings and D1 can indeed be found in the conditioned medium from cells with cleaved uPAR on the surface (Chapter 5).

One of the major obstacles in the analysis of cleaved uPAR is caused by the highly heterogeneous glycosylation of uPAR that often masks the difference between D2D3 and full-length uPAR in ordinary immunoblots. Only the careful use of domain-specific monoclonal antibodies and/or deglycosylated extracts allows this type of analysis to be performed in an unambiguous way (Chapter 5).
uPAR shedding

Soluble forms of uPAR (suPAR) are thought to lack the GPI-anchor, rendering the molecule more soluble in aqueous solutions. The absence of the GPI-anchor has been demonstrated for suPAR isolated from ascitic fluid of ovarian cancer patients (Pedersen et al., 1993). However, I will in the following consider all uPAR molecules found in cell-free liquids such as conditioned medium, serum and urine, as suPAR, even though the absence of the GPI-anchor often has not been confirmed.

Mechanism

The mechanism of uPAR shedding has not been determined yet. Bacterial phosphatidylinositol specific lipase C (PIPL-C) is able to release uPAR from the surface of cells, but no equivalent eukaryotic activity has been described (Ploug et al., 1991). A human cellular phosphatidylinositol specific phospholipase D (PIPL-D) is able to release GPI-anchored proteins from the cell surface (Metz et al., 1994), and this phospholipase has also recently been implicated in the release of uPAR (Wilhelm et al., 1999).

Another possibility is that uPAR shedding is caused by "imperfect" sorting, possibly in cases where uPAR expression is very high or rapidly upregulated.

Biochemical consequences

uPAR shedding does apparently not alter its affinity for uPA and vitronectin (Rønne et al., 1994; Wei et al., 1994).

Occurrence of suPAR and suPAR fragments

suPAR is present in the conditioned medium from endothelial cells, leukocytes, U937 and HT1080 cells (Chavakis et al., 1998; Lau and Kim, 1994; Ploug et al., 1991; Chapter 5) and in biological fluids such as plasma, urine, spinal fluid, ascites, and ovarian cyst fluid (Pedersen et al., 1993; Sier et al., 1999; Stephens et al., 1997; Wahlberg et al., 1998).

Until recently, soluble forms of cleaved uPAR (D2D3) have only been described in ovarian cyst fluid (Wahlberg et al., 1998) while it had been suggested that D2D3, unlike full-length uPAR, was not released from the cell surface (Holst-Hansen et al., 1999). However, data presented in this thesis demonstrate the presence of a soluble D2D3 fragment in the conditioned medium from several different cell types as well as in human and mouse urine (Sier et al., 1999;
Chapter 5; Chapter 6), suggesting that cleaved uPAR is indeed shed from the cell surface both in vitro and in vivo.

Serum and plasma from healthy individuals contain suPAR with a reported median value of 1.2-1.5 ng/ml (Sier et al., 1998; Stephens et al., 1997). Leukocytes and endothelial cells are likely sources for the circulating suPAR as these cells indeed shed uPAR (Chavakis et al., 1998; Chapter 5).

Enhanced levels of suPAR have been observed in plasma from patients with paroxysmal nocturnal haemoglobinuria (PNH) (Ninomiya et al., 1997; Rønne et al., 1995). PNH is a rare acquired defect in the membrane attachment of GPI-anchored proteins on bone marrow derived cells. The reduced GPI-anchoring in leukocytes from PNH patients results in the secretion of suPAR (Ploug et al., 1992; Ploug et al., 1992a), and it is therefore likely that the increase in circulating suPAR present in these patients is caused by suPAR release from leukocytes.

Elevated levels of suPAR in serum have also been described in ovarian, breast and colon cancer patients (Sier et al., 1998; Stephens et al., 1999; Stephens et al., 1997), and recently also in patients with leukaemia (Mustjoki et al., 1999). The excess serum suPAR in these patients has been suggested to derive from malignant tumour cells or surrounding stromal cells (Sier et al., 1998; Stephens et al., 1997). Nude mice xenografted with human tumours have human suPAR in the blood demonstrating that the tumour releases suPAR the circulation (Holst-Hansen et al., 1999; Chapter 6).

suPAR is also found in urine (Sier et al., 1999; Chapter 6). While all suPAR in the circulation is full-length (Holst-Hansen et al., 1999; Chapter 6), urine also contains the D1 and D2D3 fragments (Chapter 6). There is a strong correlation between the levels of suPAR antigen in serum and in urine and it is therefore likely that suPAR present in the urine derives from the circulation (Sier et al., 1999). Leukocytes and endothelial cells release suPAR fragments in vitro (Chapter 5) and similar fragments are present in urine (Chapter 6), suggesting the transient presence of these fragments in the circulation. Urine from ovarian cancer patients contains elevated levels of suPAR antigen and experiments with mice xenografted with human tumours demonstrate that the tumour derived suPAR indeed ends up in the urine (Chapter 6). The pattern of suPAR fragments in urine of xenografted mice correlates with uPAR cleavage on tumour cells. Mice xenografted with a tumour having high degree of cleaved uPAR in the tumour tissue have high levels of the D1 fragment in the urine, while D1 is not present in urine from mice xenografted with a tumour that expresses only intact uPAR. These data indicates that uPAR fragments in urine may reflect uPAR cleavage in distant parts of the organism.
Chapter 1: Introduction

Functions of uPAR

The function of uPAR in plasminogen activation

Acceleration of plasminogen activation

In solution, the binding to purified suPAR does not strongly affect the proteolytic activity of uPA towards plasminogen, or the activation of pro-uPA by plasmin (Behrendt and Danø, 1996; Ellis, 1996; Ellis et al., 1991). Whether uPAR binding affects the zymogenic nature of pro-uPA is still an open question (Behrendt and Danø, 1996; Behrendt and Danø, 1997; Higazi et al., 1995; Higazi, 1997).

The reciprocal activation of pro-uPA and plasminogen is strongly accelerated by the presence of cells and both plasmin-catalysed conversion of pro-uPA to uPA and the uPA-catalysed conversion of plasminogen to plasmin are accelerated (Ellis et al., 1991; Ellis et al., 1989). The cell surface acceleration of plasminogen activation requires both pro-uPA binding to uPAR and plasminogen binding to other cell surface elements (Rønne et al., 1991). It is thus likely that the higher local concentration and the spatial organisation of the reagents caused by the binding to the cell surface is responsible for the acceleration of the reactions. Cell surface acceleration of plasmin formation can almost be abolished by molecules that block the interaction between pro-uPA and uPAR (Stephens et al., 1989).

Cell surface bound plasmin is protected against its abundant plasma inhibitors α2-antiplasmin and α2-macroglobulin, restricting the presence of active plasmin to the surface of cells under conditions where serum proteins are present (Ellis et al., 1991; Stephens et al., 1991). In contrast to plasmin, cell surface bound uPA is not protected from its specific inhibitors PAI-1 and PAI-2, which inactivate both free and uPAR bound uPA with comparable efficiency (Cubellis et al., 1989; Ellis et al., 1990; Stephens et al., 1989).

The reduced capacity of peritoneal macrophages isolated from uPA-deficient mice to promote plasminogen activation in vitro confirms the important role of uPAR in plasminogen activation (Dewerchin et al., 1996).

Internalisation of uPA inhibitor complexes

Enzymatically inactive uPA/PAI complexes are internalised in a process which requires the binding to uPAR (Cubellis et al., 1990; Estreicher et al., 1990; Jensen et al., 1990) and involves cell surface proteins from the family of low-density lipoprotein receptor (Heegaard et al., 1995; Ilczuk et al., 1992; Moestrup et al., 1993; Nykjaer et al., 1992). The process of
internalisation leads to the degradation of both uPA and PAI-1 but uPAR is recycled to the cell surface (Nykjaer et al., 1997).

As the acceleration of plasminogen activation depends upon pro-uPA binding to uPAR, the clearance of inactive uPA/PAI complexes and recycling of uPAR, is a process that maintains the cells capacity to bind pro-uPA and therefore to promote plasminogen activation.

**Localisation of uPA activity**

Besides concentrating pro-uPA to the cell surface, uPAR is also capable of localising the protein to specific sites where plasminogen activation is required. uPAR is usually located to specific compartments of the cell surface such as cell-ECM contacts, cell-cell contacts and in particular to the leading edge of migrating cells (Conese and Blasi, 1995; Conforti et al., 1994; Estreicher et al., 1990; Rømer et al., 1994a; Xue et al., 1997). The localisation of uPAR and of uPA activity is often overlapping or identical, supporting the idea that uPAR plays an important role in the regulation of localised pericellular proteolysis. The mechanism that governs the spatial localisation of uPAR on the cell surface has not been determined yet but may very well depend on the association of uPAR with uPA, integrins and the extracellular matrix protein vitronectin. In polarised MDCK cells uPAR is sorted to the apical surface (Limongi et al., 1995).

uPAR is also found in cellular structures known as caveolae (Stahl and Mueller, 1995) but the significance of this localisation is not known.
Chapter 1: Introduction

The function of uPAR in cell adhesion and migration

Adhesion is the process by which cells make contacts to extracellular matrix proteins (ECM) and other cells. The mechanism of cell adhesion is fundamental to many, if not all, morphogenetic processes occurring throughout the entire life span of multicellular organisms. Adhesive interactions regulate the mechanical strength of cell contacts and thereby determine cell morphology and motility.

It is important to note that increased cell adhesion is not necessarily associated with increased cell migration. In fact, too strong adhesion may block cell migration as the process of cell migration requires repeated cycles of cell attachment and release.

Integrins are ubiquitous transmembrane receptors and represent the major family of adhesion receptors regulating dynamic cell-cell and cell-ECM contacts by providing the physical link between extracellular proteins and the intracellular cytoskeleton. Integrins are heterodimeric membrane proteins composed of an α- and a β-subunit. The difference between the various integrins is mostly determined by their binding selectivity and their affinity for binding sites on the extracellular adhesion ligands such as the versatile integrin-binding RGD-motif located in many extracellular matrix proteins. Integrin occupancy and clustering causes the short intracellular portion of the integrin molecules to connect with the cytoskeleton by the associating with a variety of intracellular proteins such as talin, vinculin, α-actinin, focal adhesion kinase (FAK) and many more. These intracellular proteins provide the regulated physical link between the integrins, actin filaments, and the rest of the cytoskeleton. Signal transduction by integrins plays an important role in the biology of these receptors and can be classified depending on the directionality of the signal flow. Signalling information originating from the ligand binding interactions between the integrins and the ECM proteins may be transduced across the plasma membrane into a variety of signal transduction pathways in a process termed "outside-in" signalling. In addition, intracellular regulators may modify the binding affinity of the integrins in a process termed "inside-out" signalling.

Over the last years evidence has accumulated for an important role of uPAR in cell adhesion in which it appears to play a dual role. First, uPAR may promote cellular adhesion to vitronectin by a direct high affinity interaction. Second, uPAR may modulate cellular adhesion to a number of different ECM proteins by lateral interactions with integrin receptors and regulation of their activity.

uPAR mediated cell adhesion to vitronectin

Stimulation of in suspension-growing HL60 and U937 cells with phorbol esters, or with a combination of vitamin D3 and transforming growth factor TGFβ, leads to differentiation into
macrophage-like cells with an adherent phenotype. Antibodies against uPA block the adherence phenotype and the function of uPA in this adhesion can be traced to a requirement for uPAR occupancy (Nusrat and Chapman, 1991; Waltz and Chapman, 1994). The ECM protein responsible for the observed adhesion is vitronectin (Waltz and Chapman, 1994; Wei et al., 1994; Chapter 3) and it has been shown that both cellular and soluble forms of uPAR bind vitronectin in a reaction which is stimulated by uPA occupancy of uPAR and requires intact, three domain uPAR (Høyer-Hansen et al., 1997a; Waltz and Chapman, 1994; Wei et al., 1994; Chapter 3).

Binding to vitronectin mediated by uPAR most likely depends on a direct interaction between cellular uPAR and vitronectin, because agents blocking the uPAR/vitronectin interaction also block cell adhesion (Chapter 3). Vitronectin adhesion mediated by uPAR has also been observed in smooth muscle and thyroid cells lines (Chang et al., 1998; Ragno et al., 1998), suggesting that it may be a general phenomenon and not only restricted to leukocytes.

Inhibitors of integrin binding function such as RGD-containing peptides and EDTA do not inhibit uPAR mediated adhesion to vitronectin, suggesting that the underlying mechanism does not require direct ligand interactions between integrin receptors and the extracellular matrix (Waltz and Chapman, 1994; Wei et al., 1994; Chapter 3). uPAR does not have any intracellular or transmembrane spanning segments and is therefore incapable of providing a direct protein/protein connection between the ECM and the cytoskeleton. However, it is possible that the GPI-anchor interacts with lipid anchors of intracellular membrane-associated proteins. It appears that uPAR mediated cell spreading on vitronectin requires integrin activation and function as evidenced by the observation that low temperatures or dominant negative integrin molecules (lacking the intracellular domain) inhibit uPAR mediated vitronectin adhesion of uPAR transfected HEK293 kidney cells (Wei et al., 1996).

**uPAR as a regulator of integrin function**

Besides mediating direct cellular adhesion to vitronectin uPAR also associates with and modulates the function of adhesion receptors from the integrin family. The connection between these properties and the vitronectin-binding features are not clear.

**Interactions between uPAR and \( \beta_1 \) and \( \beta_3 \) integrins**

As demonstrated by resonance energy transfer (RET) and immunoprecipitation, uPAR is found associated with \( \beta_1 \) integrins on the surface of HT1080 fibrosarcoma cells and in particular at focal adhesions contacts (Xue et al., 1997). The association between uPAR and \( \beta_1 \) integrins in these cells does not depend on the substrate as it is observed on fibronectin, vitronectin and laminin. In contrast, the pattern of \( \beta_3 \) and \( \alpha \)-subunits which closely associated with uPAR does depend on the substrate. On fibronectin uPAR is associated with the \( \alpha_5 \) subunit, on vitronectin
with $\beta_3$, $\alpha_2$, and $\alpha_\alpha$, and on laminin with $\alpha_3$ and $\alpha_6$. Importantly, uPAR is not closely associated with any of the integrins when the cells are plated on the non-specific poly-D-lysine substrate. Although these data do not provide any information regarding the functional role of uPAR in cell adhesion, they do suggest that the uPAR/integrin interactions are dynamic and that uPAR mostly forms close associations with ECM engaged integrins. In uPAR transfected HEK293 kidney cells, uPAR associates and modulates the function of $\beta_1$ integrins (Wei et al., 1996). Upon uPAR transfection these cells become adherent to vitronectin and lose the $\beta_1$ mediated adhesion to fibronectin. The adhesion to fibronectin can be restored by a synthetic uPAR-binding peptide blocking the interaction between uPAR and integrins.

The functional interaction between uPAR and $\beta_1$ integrins apparently also involves the membrane protein caveolin (Wei et al., 1996). Caveolin has been demonstrated to associate with a number of signalling molecules, including src-family kinases, G proteins, and notably uPAR (Li et al., 1996; Stahl and Mueller, 1995; Wary et al., 1998). Caveolin co-immunoprecipitates with $\beta_1$-integrins and uPAR in transfected HEK293 cells, and the majority of $\beta_1$ /uPAR complexes in these cells appears to contain caveolin (Wei et al., 1996). Evidence for a functional role for caveolin in uPAR and integrin mediated adhesion comes from recent experiments demonstrating that down regulation of caveolin expression (by antisense technology) inhibits both $\beta_1$ and uPAR mediated cell adhesion of HEK293 cells (Wei et al., 1999). The inhibition is on the biochemical level characterised by the disruption of the association between $\beta_1$ integrins, src-kinases and uPAR. If thus appears that caveolin plays an important role for $\beta_1$ and uPAR mediated adhesion, maybe by providing the physical link between uPAR, integrins and the signalling machinery including src-kinases.

**Interaction between uPAR and leukocyte integrins**

The integrin subunit $\beta_2$ is specifically expressed in leukocytes and plays a pivotal role in cellular adhesion and migration in connection with processes such as inflammation, extravasation and in the resolution of fibrin clots. The $\beta_2$ integrin subunit associates with different $\alpha$-subunits forming the $\alpha_M\beta_2$ (Mac-1, CR3), $\alpha_4\beta_2$ (leukocyte function-associated antigen, LFA-1), and $\alpha_X\beta_2$ (complement receptor 4, CR4). These integrins mediate the cell-cell binding of leukocytes to the vascular wall by interactions with cellular integrin ligands such as ICAM-1 and to vascular fibrin deposits. Other important ligands for these integrin receptors include fibrinogen that is bound and degraded through its interaction with Mac-1, and Factor X, which is bound by Mac-1 and may be activated and cause blood clotting. Most of these integrins are expressed at rather low levels in resting leukocytes but can be rapidly up-regulated, both in number and affinity, by stimulation with cytokines and chemokines.
Histological analysis, co-capping and immunoprecipitation experiments have provided extensive documentation for the co-localisation of uPAR and $\beta_2$ integrins in monocytes and neutrophils (Bohuslav et al., 1995; Petty et al., 1997; Simon et al., 1996; Xue et al., 1994).

On monocytes uPAR and Mac-1 form a functional complex with reciprocal stimulation of the two receptor functions (Simon et al., 1996). uPAR mediated cellular adhesion to vitronectin is stimulated by Mac-1 engagement with fibrinogen, while Mac-1 mediated degradation of fibrinogen is stimulated by uPAR mediated vitronectin adhesion. In accordance with these data, monoclonal antibodies against uPAR and antisense-mediated down regulation of uPAR expression partially inhibit Mac-1 mediated adhesion of monocytes to fibrinogen (Sitrin et al., 1996).

In neutrophils, antibodies against uPAR block chemotactic migration (Gyetko et al., 1994) and neutrophils isolated from PNH-patients deficient in cell surface uPAR (and other GPI-anchored proteins!) fail in chemotaxis and trans-endothelial migration (Pedersen et al., 1996). The association between uPAR and $\beta_2$-integrins in neutrophils is a dynamic process, as uPAR is associated with Mac-1 on resting cells, and with CR4 at lamellipodia of migrating cells (Kindzelskii et al., 1997).

The important functional role of uPAR in leukocyte adhesion and migration is evidenced by recent experiments with uPAR-deficient mice. Leukocyte adhesion to endothelial cells and recruitment to an inflamed peritoneum is significantly impaired in uPAR-deficient mice as compared to wild-type control animals (May et al., 1998).

The role of uPA in these processes seems to be inhibitory. In monocytes uPAR engagement by uPA or ATF inhibits the Mac-1 mediated binding of fibrinogen and Factor X (Simon et al., 1996). Adhesion of leukocytes to vascular endothelium is inhibited by binding of inactivated uPA, suggesting that it is the ligand-free form of uPAR that stimulates $\beta_2$ mediated adhesion (May et al., 1998).

The tight functional connection between $\beta_2$-integrins and uPAR is further substantiated by the fact that similar signalling pathways regulate the expression of both receptor types as exemplified by transforming growth factor $\beta$ (TGF-$\beta$), which strongly upregulates both integrins and uPAR (Lund et al., 1991). Co-engagement of $\beta_2$-integrins and the T cell receptor in lymphocytes upregulate uPAR expression (Bianchi et al., 1996), illustrating the cross talk between these systems.

Mac-1 represents the only integrin for which a direct molecular interaction with uPAR has been demonstrated in vitro (Wei et al., 1996). Soluble uPAR binds to purified Mac-1 immobilised on plastic surfaces and the interaction requires the active conformation of the integrin. Freshly isolated monocytes and neutrophils do not express caveolin and the interaction between $\beta_2$,
integrins and uPAR is therefore different from the stable uPAR/β₁/caveolin complexes observed in epithelial cells (Wei et al., 1996; Wei et al., 1999).

**PAI-1 and cell adhesion**

Another member of the plasminogen activator system, PAI-1, plays a pivotal role in cell adhesion and migration (Blasi, 1997). The structural basis for the regulatory function of PAI-1 on cell adhesion is its capacity to interact with both vitronectin and uPA. PAI-1 binds with high affinity to vitronectin in a reaction that stabilises PAI-1 in its active conformation (Knudsen and Nachman, 1988). Vitronectin bound PAI-1 can still bind and inactivate uPA, but the formed uPA/PAI-1 complex has only low affinity for vitronectin and is rapidly released.

The fact that PAI-1 inhibits uPAR mediated cell adhesion was first suggested to be caused by the capacity of PAI-1 to mediate the internalisation of uPAR/uPA/PAI-1 complexes (Waltz and Chapman, 1994). However, later it was demonstrated that the binding sites for PAI-1 and uPAR on vitronectin are overlapping and that the binding of these proteins is competitive and mutually exclusive (Deng et al., 1996; Seiffert and Loskutoff, 1991). Low levels of active PAI-1 are capable of releasing U937 cells adherent to vitronectin and an excess of uPA can revert this process by the formation of uPA/PAI-1 complexes (Deng et al., 1996).

The binding site of PAI-1 in vitronectin is also very close and overlapping with the binding site utilised by the vitronectin receptor (α₅β₃, VNR) (Stefansson and Lawrence, 1996). PAI-1 binding blocks the interaction between α₅β₃ and vitronectin and causes a functional inhibition of α₅β₃ mediated migration of smooth muscle cells, human amnion WISH cells, and human epidermoid carcinoma HEp-2 cells on vitronectin (Kjøller et al., 1997; Stefansson and Lawrence, 1996).

These data demonstrate the central role of PAI-1 in both uPAR and integrin-mediated cell adhesion on vitronectin. An excess of active PAI-1 over uPA inhibits cell adhesion mediated by α₅β₃ and uPAR, whereas an excess of uPA will stimulate this process. Complexes between uPA and PAI-1 are internalised and degraded in a mechanism that involve uPAR and the LRP receptor, adding further complexity to these processes.

**The biological function of soluble and cleaved forms of uPAR**

uPAR plays a fundamental role in pericellular proteolysis by accelerating and localising the process of plasminogen activation. The basis for this function of uPAR is the anchoring of the protein to the cell membrane, and its capacity to bind pro-uPA. Cleavage of uPAR in the linker region between D1 and D2 generates receptor fragments that fail to bind uPA and vitronectin and thus inactivates the receptor with respect to uPA and vitronectin binding. The shedding of uPAR
and uPAR fragments is expected to destroy the receptor functions of uPAR but creates the possibility of novel "paracrine" effects on other cells.

The broad occurrence of uPAR cleavage and shedding suggests that these phenomena play a functional role in the biology of uPAR.

**uPAR cleavage and plasminogen activation**

The shedding of uPAR from the cell surface does not affect the affinity for pro-uPA, but is expected to impair plasminogen activation as the co-localisation between plasminogen and pro-uPA on the cell surface is lost. Soluble uPAR may still associate with the cell surface and the extracellular matrix through its interactions with integrins (Mizukami and Todd, 1998) and the extracellular matrix protein vitronectin (Chavakis et al., 1998). The efficiency of cell associated suPAR in promoting plasminogen activation has not been analysed but is most likely inferior to that of the GPI-anchored protein. First, not all the suPAR is retained at the cell surface, as significant levels of suPAR can be found in the circulation and in urine (Sier et al., 1999; Sier et al., 1998; Stephens et al., 1999; Stephens et al., 1997; Chapter 6). Second, suPAR associated uPA/PAI-1 complexes are not expected to be internalised efficiently, although they might still bind LRP.

It should be noted that recently it has been found that suPAR/pro-uPA complexes may have fibrinolytic activity (Higazi et al., 1998). However the importance of this observation in vivo remain to be established.

Cleavage of uPAR in the linker region between D1 and D2 dramatically reduces the receptors affinity for pro-uPA (Behrendt et al., 1991) and is therefore expected to result in a complete loss of the receptors function in acceleration of plasminogen activation. In fact, the cleavage of uPAR has been suggested to be a negative feedback mechanism occurring at sites of high proteolytic activity (Høyer-Hansen et al., 1997b).

Taken together, it appears likely that the processes of uPAR shedding and cleavage predominantly have a negative effect on plasminogen activation.

**Consequences of uPAR cleavage and shedding in cell adhesion and migration**

The function of uPAR in cell adhesion and migration is mediated through its interactions with uPA, vitronectin and integrins. Cleavage of uPAR destroys the high affinity interaction between uPAR and vitronectin (Høyer-Hansen et al., 1997a; Chapter 3) and also uPAR mediated adhesion to vitronectin requires intact uPAR (Chapter 3).

Impaired adhesion of PIPL-C treated leukocytes to vascular endothelium is reconstituted by pre-incubation with suPAR, suggesting that the function of uPAR in $\beta_2$ mediated cell adhesion
does not require the GPI-anchor (May et al., 1998). The same stimulation was not observed using a suPAR lacking D1, demonstrating that intact uPAR is required for the modulation of integrin function. In another study the exogenous addition of suPAR was shown to have the same inhibitory function as GPI-anchored uPAR on the integrin mediated adhesion of HEH293 cells to fibronectin (Wei et al., 1996).

It thus seems clear that suPAR may affect integrin mediated adhesion even in the absence of the GPI-anchor. However, as uPAR shedding results in the release from the cell surface it is questionable whether sufficiently high concentrations of suPAR occur in vivo.

**uPAR in cell chemotaxis and chemokinesis**

uPA has been known for many years to have chemotactic and chemokinetic effects on the migration of a range of different cell types (Fibbi et al., 1988; Sato and Rifkin, 1988; Sato et al., 1990) (Busso et al., 1994; Fazioli et al., 1997; Nguyen et al., 1998; Resnati et al., 1996). Although binding to uPAR seems to be required for all of the chemotactic effects of uPA, the phenomena can be divided into two groups depending on whether uPA activity is also required.

By proteolysis uPA can directly activate pro-HGF (Naldini et al., 1995) and indirectly, through the activation of plasminogen to plasmin, also basic FGF and TGFβ (Sato and Rifkin, 1988; Sato et al., 1990). The activated cytokines may in these cases be responsible for the observed effects on cell migration.

In most cases there is no apparent requirement for uPA activity, because proteolytically inactive uPA or an uPA fragment lacking the catalytic domain (the aminoterminal fragment, ATF) are able to induce cell migration equally well (Busso et al., 1994; Fibbi et al., 1988; Nguyen et al., 1998; Odekon et al., 1992; Resnati et al., 1996). The protease-independent effects of uPA on chemotaxis, at least in THP-1 cells and fibroblasts, is strictly dependent upon uPAR as they can be inhibited by anti-uPAR antibodies preventing the ligand interaction, and fails to occur on cells lacking uPAR (Resnati et al., 1996).

Taken together, these data suggest that the binding of uPA to uPAR in some cell types causes the transmission of signalling over the cell membrane leading to increased motility. Indeed, ligand engagement of uPAR causes the transient activation of a variety of signalling proteins including src-family members (Chiaradonna et al., 1999; Fazioli and Blasi, 1994; Resnati et al., 1996), ERK-1 and ERK-2, (Nguyen et al., 1998), FAK (Tang et al., 1998), JAK and STAT1 (Dumler et al., 1998), and fos, jun and myc (Rabbani et al., 1997).

Chymotrypsin-cleaved suPAR is a potent chemoattractant for several different cell lines (Fazioli et al., 1997; Resnati et al., 1996). The chemotactic response and kinetics of p56/59ck phosphorylation induced by proteolytically inactive uPA derivatives and that of cleaved suPAR
are similar, suggesting that the same signalling pathway is activated by these molecules (Resnati et al., 1996). Inhibitors of tyrosine kinases and heterotrimeric G proteins block the chemotactic response and the induced phosphorylation of p56/59\textsuperscript{ck}. The fact that the pertussis toxin inhibitor of heterotrimeric G proteins also blocks the phosphorylation of p56/59\textsuperscript{ck}, suggests that the heterotrimeric G protein is upstream of the tyrosine kinase in the signalling pathway.

Experiments using recombinant soluble uPAR mutants and synthetic peptides has mapped the uPAR chemotactic epitope to the linker region connecting D1 and D2 (Fazioli et al., 1997). This linker region is susceptible to cleavage by several different proteases including plasmin and uPA itself (Høyer-Hansen et al., 1997b; Høyer-Hansen et al., 1992; Ploug and Ellis, 1994). Both GPI-anchored and soluble forms of cleaved uPAR have indeed been observed on different cell types and in diverse biological fluids.

The fact that cleaved soluble uPAR and peptides containing the uPAR chemotactic epitope are strong chemokine-like molecules this strongly suggests the existence of one or more membrane "adapter" molecule(s) capable of transmitting the chemotactic signal over the membrane (Fazioli et al., 1997; Resnati et al., 1996). However, this adapter protein still has to be identified.

The strong chemotactic properties of suPAR fragments generated in the laboratory taken together with the cellular release of similar fragments in vivo, strongly suggests that these fragments may play a role in biological processes such as leukocyte recruitment to sites of inflammation.
**uPAR in cancer**

Tumour invasion and metastasis are processes associated with extensive ECM proteolysis and the plasminogen activator system together with the MMPs appear to play a central role in these processes.

In human cancers the components of the plasminogen activator and MMP system are expressed both by tumour and stromal cells. The cellular expression of individual components is often similar between different cases of the same type of cancer, but may vary a lot between different types of tumours. The expression pattern of the components of the plasminogen activator and MMP system has been determined in several tumour types, but I will in the following just give a brief introduction to the expression patterns observed in colon cancer (for a recent review see Danø et al., 1999).

In colon cancer the cells responsible for uPA production are fibroblast-like stromal cells while uPAR is expressed by cancer cells located adjacent to the uPA producing fibroblasts (Pyke et al., 1991). The uPAR-expressing tumour cells might therefore bind the fibroblast-expressed uPA and thus localise plasminogen activation to the surface of the tumour cells. uPAR is not expressed by all the tumour cells but is restricted to the budding cancer cells located at the tip of the invading malignant epithelium (Pyke et al., 1991; Pyke et al., 1994; Pyke et al., 1995). PAI-1 is expressed by stromal endothelial cells surrounding the tumour and PAI-1 may serve to protect the tumour against excessive uPA-mediated plasminogen activation (Pyke et al., 1994). Several members of the MMP family are also expressed in human colon cancer. Stromal fibroblasts express among others MMP-2 and MT1-MMP, while the only MMP expressed by the tumour cells appears to be MMP-7 (McDonnell et al., 1991; Okada et al., 1995; Pyke et al., 1993).

The high levels and the localisation of components of the plasminogen activator system in tumour tissues provides a "smoking gun" link between plasminogen activation and the malignant process of cancer invasion. The correlation's between plasminogen activation and tumour invasion lead to a major interest in the possible usefulness of uPAR as a target for anti-cancer therapy and as a prognostic marker.

**uPAR as a target for anti-cancer therapy**

Urokinase mediated plasminogen activation appears to play an important role in tumour invasion and metastasis, and inhibition of this system is therefore an attractive target for anti-cancer therapy. Inhibition of uPA mediated plasminogen activation can be obtained by a direct inhibition of uPA activity, or by blocking the interaction between uPA and uPAR. Inhibition of uPA activity poses the problem of specificity, because the activity of tPA, which plays important functions in the maintenance of fibrinolytic activity in the vasculature, should not be affected.
Interest has therefore mostly been focused on inhibition of the uPA/uPAR interaction, as this should provide a specific inhibition of uPA-mediated cell surface plasminogen activation associated with tumour invasion and metastasis.

Agents inhibiting the interaction between uPAR and uPA (uPAR antagonists) range from catalytically inactive fragments of uPA, to synthetic peptides constructed on basis of the uPAR binding region of uPA or isolated by phage-display techniques, to low molecular weight compounds isolated from chemical libraries by systematic screening (Appella et al., 1987; Goodson et al., 1994). The efficacy of these compounds in inhibiting tumour invasion and metastasis has been analysed in mouse models using syngeneic mouse tumours or human tumours xenografted in nude mice.

Spontaneous lung metastasis of the murine Lewis-Lung tumour grown subcutaneously in syngeneic mice can be efficiently inhibited by a peptide derived from the binding region of murine uPA (aminoacid 17-34)(Kobayashi et al., 1994) and metastasis of human prostate carcinoma cell line PC3 grown in nude mice is inhibited by a catalytically inactive uPA-IgG fusion protein (Crowley et al., 1993). Inhibition of tumour growth by uPAR antagonists may also work through other mechanisms than on the invasion of tumour cells. In vivo, basic fibroblast growth factor-induced neo-vascularisation and B16 melanoma growth in syngeneic mice is substantially suppressed by uPAR-antagonists suggesting that these compounds may provide additional anti-cancer effects through inhibition of angiogenesis (Min et al., 1996). Tumour cells often express reduced levels of adhesion receptors and also often fail to deposit ECM around themselves. The fact that uPAR is up-regulated in many tumour cells suggests that the cells may use this alternative adhesion pathway as a response to the altered expression of normal cell adhesion proteins. Some (but not all) uPAR antagonists stimulate uPAR mediated cell adhesion to vitronectin (Waltz and Chapman, 1994; Chapter 3) and it is possible that their effects on tumour metastasis may depend also on their effects on cellular adhesion.

The redundancy within the proteolytic system suggests that efficient inhibition of tumour invasion and metastasis will require the simultaneous inhibition of both plasminogen activation and of the MMPs. MMP inhibitors are indeed promising anti-cancer drugs and, in contrast to the uPAR-antagonists, clinical trials are already underway (reviewed by Brown, 1999).

**uPAR as a prognostic marker in cancer patients**

Prognosis in cancer patients typically involves evaluation of tumour size, degree of differentiation, the extent of invasion and the presence or absence of metastasis. However, even patients with similar prognosis based on these parameters may very well have dramatically different disease progression. Identifying patients with good and bad prognosis might provide the
physicians with a tool to tailor the therapy. For example, if it was possible to identify patients with a good prognosis one might avoid unnecessary adjuvant therapy and the associated suffering.

Not surprisingly, members of the plasminogen activator system, including uPA, uPAR and PAI-1, have been found to have prognostic value in several different cancers (reviewed by Schmitt et al., 1997). High levels of these proteins in extracts from the primary tumour are associated with poor survival in different cancers including lung, colon, bladder, ovarian and breast cancer. The fact that high levels of PAI-1, which is a negative regulator of plasminogen activation, is strongly correlated with poor survival may be explained by its capacity to protect the tumour against the high levels of proteolytic activity present at points of invasion. Another possibility is that PAI-1 facilitates tumour dissemination by modulation of cell adhesion (Deng et al., 1996). High levels of uPAR antigen in tumour extracts are associated with poor prognosis in patients with squamous cell lung cancer (Pedersen et al., 1994), breast cancer (Grøndahl-Hansen et al., 1995) and colon cancer (Ganesh et al., 1996).

Measuring tumour markers and prognostic factors in plasma and serum samples of cancer patients provides at least two advantages to the measurement in tumour lysates. First, the measurements do not require tumour biopsies and can be performed prior to the surgical removal of the tumour. Second, the measurements can be performed repeatedly and may be used in the follow-up analysis of patients. The serum level of soluble uPAR (suPAR) is elevated in plasma and serum from patients suffering from ovarian (Sier et al., 1998), non-small-cell lung and colorectal cancer (Stephens et al., 1997). Indeed, it has recently been demonstrated that the plasma suPAR level in colorectal cancer patients is a strong and independent prognostic marker (Stephens et al., 1999).

Recently, we have demonstrated that suPAR is also present in urine, and that the suPAR antigen levels in urine and serum are highly correlated (Sier et al., 1999). The level of suPAR antigen in urine is stable under relevant storage conditions and might therefore substitute for the measurement of suPAR in serum samples (Sier et al., 1999).
Chapter 2:
MATERIALS & METHODS
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**Materials**

**Chemicals**

General chemicals used in this study were obtained from Sigma or Boehringer Mannheim and were of the highest available quality.

**Enzymes**

Restriction enzymes, alkaline phosphatase, bacteriophage T4 ligase, Klenow fragment, Taq polymerase and PNGase F were purchased from Boehringer Mannheim, Italy.

**Antibodies**

Monoclonal mouse antibodies against human uPAR (mAb: R2, R3, R4 and R5) and uPA (mAb: c6) were obtained from the Finsen Laboratory, Denmark. Monoclonal mouse antibody directed against the FLAG™-epitope (mAb: M2) was purchased from Sigma, Italy. Polyclonal rabbit antibody against human uPAR used in ELISA and immunoblotting analysis of native human uPAR, was obtained from the Finsen Laboratory, Denmark. Rabbit polyclonal antibodies against the FLAG-epitope were purchased from Santa Cruz Biotechnology, California, USA. The polyclonal rabbit antibody against human uPAR used in the analysis of reduced and deglycosylated uPAR was previously prepared in our laboratory by immunisation of rabbits with recombinant soluble human uPAR. All polyclonal rabbit antibodies used in this study were purified by affinity chromatography on protein-A Sepharose (Pharmacia Biotech, Sweden) according to manufacturers instructions.

**Radioactive isotopes**

Radioactive isotopes were purchased from Amersham, Italy.

**Cell culture reagents**

Cell culture reagents (RPMI, DMEM, glutamine, penicillin, streptomycin, FCS, trypsin) were purchased from Gibco-BRL, Italy. Serum free growth supplement Nutridoma NS and recombinant murine IL-3 were obtained from Boehringer Mannheim, Italy. Tissue culture plastic ware was from Costar, Italy. ELISA plates.
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Peptides

Synthetic peptides had the following sequences (N-C): Peptide-1: AVTYSRSRYLEC; Peptide-2: YTARLWGGTLLT; Peptide-3: SRSRYLEC; Peptide-4 (scrambled version of peptide 1): TLVEYYSRASC.

Oligonucleotides

Oligonucleotides were purchased from PRIMM (Italy) or GENSET (France) and had the following sequences (3'-5'):

FRA18: ATTATACTCGAGGAAGACGTGCAGGGACCCCGCGCA
FRA19: TATATTGATCATTTAGGTCCAGAGGAGAGTGCCTC
D2.5'T: AATGCATTGAGGCCCAAGAGGCTGGGA
FRA18: ATTATACTCGAGGAAGACGTGCAGGGACCCCGCGCA
D1.3'T: TTATCGATGGTAACGGCTTCGGGAATA
D2.3'T: TTATCGATGGCCATTCTGCGGCAGATT
D3.3'T: TTATCGATGTGGGTGTTACAGCCACT
D3.5'T: TCCATGGGTCTGTTCCTCCCTTGCAGCTGTAACACTGGCGGCCCAAGAGGCTGGGA
K/FOcs: CGACGAGCATCTGTGACTATAAGGATGACGACGACAAGTAA K/FOas: CGTTACTTGTCGTCGTCATCCTTATAGTCGACAGATGCTCGT
NS1: CCGCGGAAGAACCCATGGGACTCCCAA; 46D: CAAGCTTACTTGTCGTCGTCATCC.

Other reagents

Human ATF was a generous gift of Dr. Jack Henkin (Abbott Laboratories, IL, USA). Recombinant Pertussis toxin and its inactive mutant (Pizza et al., 1989) were generous gifts of Dr. Maria Grazia Pizza (IRIS, Siena, Italy). Native human vitronectin was obtained from Molecular Innovations INC, Michigan, USA. Fibronectin was obtained from Boehringer Mannheim, Italy.

Cells

The 32D and WEHI-1 cell lines were obtained from Drs. Jacalyn H. Pierce. U937, THP-1 and COS7 cells were obtained from the ATCC (Rockville, MD, U.S.A.). The uPAR⁻/⁻ cells were derived from spontaneous immortalisation of embryo fibroblasts prepared from uPAR⁻/⁻ mice (Dewerchin et al., 1996) and kindly donated by Drs. Peter Carmeliet and Desiré Collen.
Human urine samples

Urine from twenty-five patients (mean age 62, range 20-78) admitted for surgery of ovarian carcinoma was sampled at the Department of Gynaecology and Obstetrics, San Raffaele Hospital, Milan. The FIGO (International Federation of Gynaecology and Obstetrics) stages of the tumours comprised 14 women with stage I/II, and 11 with stage III/IV respectively. Urine from 20 age-matched healthy women (median age 56, range 36-78) was used as controls. All samples were snap frozen and kept at -80°C. Before analysis, the samples were quickly thawed at 37°C and centrifuged at 10,000 g for 10 minutes. In specific cases urine was 20-fold concentrated using ultrafiltration with a Centricon YM-10 filtration membrane (Amicon), followed by overnight dialysis against PBS.

Human serum samples

Serum from twenty-five patients (mean age 62, range 20-78) admitted for surgery of ovarian carcinoma was sampled at the Department of Gynaecology and Obstetrics, San Raffaele Hospital, Milan. The serum samples were obtained from the same patients, and on the same day, as the urine samples described above.
Methods

Recombinant DNA techniques

Bacterial strains

The *E.coli* strain HB101 was used for the propagation of all plasmids.

Maintenance and media

*E.coli* were grown at 37°C in L-broth or on L-broth agar plates as described elsewhere (Sambrook et al., 1989). For selection of transformants and for plasmid preparations the L-broth was supplemented with 200 µg/ml ampicillin.

Preparation of competent cells

500 ml of L-broth was inoculated with 5 ml of an over night culture of HB101 cells and allowed to grow to until OD₄₃₆ = 0.6. Cells were cooled on ice for 60 minutes and pelleted by centrifugation (3000 rpm, 15 minutes, 4°C). The supernatant was removed by decanting and the cells resuspended in 200 ml ice cold 100 mM CaCl₂ by vortexing. After 20 minutes incubation on ice, the cells were pelleted and resuspended in 45 ml ice cold 100 mM CaCl₂. The cells were incubated at room temperature for 20 minutes, pelleted by centrifugation (at 4°C), resuspended in 5 ml ice cold 100 mM CaCl₂ and added 1.2 ml 50% glycerol (v/v). The competent cells were aliquoted (200 µl), snap-frozen in liquid nitrogen, and stored at -80°C.

Transformations

Aliquots of competent cells were thawed on ice and added the plasmid or ligation mixture (10 µl) to be transformed. The cells were incubated for 30 minutes on ice and heat-shocked for 90 seconds at 42°C before plating on L-broth agar plates containing 200 µg/ml ampicillin.

Mini-preparations of plasmid DNA

Plasmid DNA was isolated by the alkaline lysis method: 1.5 ml overnight culture was pelleted by centrifugation and washed in 1 ml Tris-HCl, EDTA buffer (TE, 1 mM ethylenedinitro-tetraacetic acid disodium salt (EDTA), 10 mM Tris-HCl pH 8). After centrifugation the pellet was resuspended in 50 µl Sol A (50 mM glucose, 10 mM EDTA, 25 mM Tris-HCl pH 8). Cells were lysed by addition of 200 µl Sol B (0.2 M NaOH, 1% sodium dodecyl
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Sulfate (SDS) and incubated 5 minutes on ice. Cell/SDS aggregate was precipitated by adding 150 µl Sol C (3 M KAc pH 4.5) and incubated 5 minutes on ice. After centrifugation, 400 µl of the supernatant was extracted once with phenol mix (PhChlaa: phenol/chloroform/isoamylalcohol (25/24/1) with 1 mg/ml hydroxyquinoline, neutralized with Tris-HCl pH 8 and equilibrated with TE) and precipitated with 2.5 volume 96% ethanol. The DNA was pelleted by centrifugation, washed once with 70% ethanol, dried and resuspended in TE. Plasmid DNA obtained by this method was sufficiently pure to be used in cloning and for sequencing.

Maxi-preparations of plasmid DNA

Plasmid DNA used for transfection of mammalian cells were prepared by CsCl density equilibrium centrifugation as described elsewhere (Sambrook et al., 1989).

Agarose gel electrophoresis

Agarose gels of appropriate percentage were cast using multi purpose agarose (Boehringer Mannheim) dissolved in TAE buffer by boiling in a microwave oven. Before casting, ethidiumbromide (Sigma) was added to 1 µg/ml from. Agarose gels were allowed to solidify and were run under standard conditions using TAE as running buffer.

DNA sequencing

Sequencing was performed using a T7 sequencing kit (Pharmacia) according to the manufacturers directions using $^{35}$S-α-dATP. The extension products were separated by electrophoresis on denaturing 8% acrylamide/urea gels.

General DNA techniques

Standard recombinant DNA techniques were employed (Sambrook et al., 1989). T-tailing of EcoRV digested pBluescript SK- vector was performed as described elsewhere (Holton and Graham, 1991).

Restriction enzyme digestions

Plasmids and PCR products were digested with restriction enzymes in the buffers supplied, and according to the manufacturers instructions. Klenow filling of blunt ends and dephosphorylation using alkaline phosphatase were performed as described (Sambrook et al., 1989).
Purification of DNA fragments

Prior to ligation all DNA fragments were purified by gel electrophoresis on agarose gels of appropriate concentration. Desired DNA fragments were cut out from the gels on a transilluminator and the DNA isolated from the gel pieces using the QiaQuick gel extraction kit (Qiagen).

PCR

PCR reactions were performed using Taq polymerase and the buffer supplied by the manufacturer. All reactions were performed using 1 μM of each oligonucleotide and 0.2 mM deoxynucleotides in a total volume of 50 μl. PCR reactions were generally performed using the following cycle parameters: an initial denaturation (3 min at 94°C) followed by cycles consisting of: denaturation (1 min, 94°C), annealing (1 min, 55°C), extension (2 min, 72°C), and a single final extension step (10 min, 72°C). Preparative PCR reactions were performed using a high concentration of template (typically 5 μg plasmid) and a low number of amplification cycles (typically 12) to minimise the introduction of mutations. Prior to restriction digestions, the PCR products were purified using the QuiaQuick PCR purification kit (Qiagen).

To limit the tedious work of plasmid preparations, PCR was extensively used to identify colonies containing plasmids with the desired structure. In this case, a fraction of fresh colonies were picked with a pipette tip and transferred to PCR reactions containing oligonucleotides generating PCR products informative with respect to the presence and orientation of the insert. Amplifications were performed as before but using 30 cycles.

Ligations

In a total volume of 20 μl were mixed: DNA fragments, water, 10-fold concentrated ligation buffer, and 2 units of bacteriophage T4 ligase (Boehringer Mannheim, Italy). Ligation was allowed to proceed over night at room temperature. Control reactions, receiving no vector or no insert, were always performed and colonies were only screened if the number of colonies in transformations receiving both vector and insert was significantly higher than in controls.

Vector construction

Expression vectors encoding GPI-anchored uPAR

Vectors expressing the wild type receptor (uPAR) and a deletion mutant lacking the first 92 amino acids corresponding to domain 1 (D2D3) were constructed by first amplifying the
entire uPAR cDNA (Roldan et al., 1990) with the primers FRA18 and FRA19 and cloning the product in a EcoRV digested and T-tailed pBluescript SK- vector. The expression vector LTR-2/uPAR was generated by transferring a XhoI-ClaI fragment, containing the entire uPAR-coding region, from the pBluescript sub-clone to the LTR-2 expression vector digested with same enzymes. The LTR-2/D2D3 expression vector was constructed by first replacing the NruI-NsiI fragment (containing the entire D1 coding region) of the pBluescript uPAR sub-clone with a PCR product generated by amplification of the uPAR cDNA with the primers FRA18 and D2.5'T and digested with the same enzymes (thereby restoring the desired signal-peptide/D2D3 junction aminoacid –1/93). Finally, the modified uPAR cDNA was transferred to the LTR-2 vector as described for the wild type cDNA.

Expression vectors encoding soluble uPAR molecules.

Mutant cDNAs encoding soluble uPAR were generated by PCR with the following primers: D1.92: oligonucleotides FRA18 and D1.3'T; D121.191: oligonucleotides FRA18 and D2.3'T; D1231.274: oligonucleotides FRA18 and D3.3'T. The PCR-products were digested with BclII and ClaI, and cloned in pBluescript SK-(Stratagene) digested with BamHI and ClaI. To generate the constructs encoding D293.191 and D2393.274 the D1 region in the D12 and D123 constructs was deleted by substituting the NruI/NsiI fragment containing the D1 coding region, with a fragment generated by amplifying the uPAR cDNA with the primers FRA18 and D2.5'T and digested with the same enzymes. To generate D3192.274, the D12 region in D123 was deleted by substituting the NruI/NcoI fragment containing the D12 coding region, with a fragment generated from uPAR cDNA with the primers FRA18 and D3.5'T and digesting with the same enzymes. All mutant receptors were tagged at the carboxyterminus with the peptide sequence HRRASVDYKDDDDK, which includes a protein kinase substrate and the FLAG™ epitope, by inserting in the carboxyterminal ClaI site a linker made by annealing the two oligonucleotides K/FOcs and K/FOas. All the recombinant coding regions were amplified with the primers NS1 and 46D, digested with NcoI and transferred to the eukaryotic expression vector pBNSEN (Pallisgaard et al., 1994) digested with NcoI and Klenow-treated EcoRI.

Protein techniques

Cell and tissue lysates

Tumour tissue and cell detergent lysates were prepared by Dounce homogenisation (only tissue) in PBS (10 ml / gram of tissue) containing 1% Triton X-100 and protease inhibitors (Complete™, Boehringer, Mannheim) for 1 hour on ice. Lysates were centrifuged at 14.000 x g
for 20 min at 4°C and the supernatants were stored at -80°C until analysis. Total protein was determined by the Dc protein assay (Bio-Rad) with bovine serum albumin as a standard.

**Tyrosine Kinase Assay.**

Kinase assay was performed as previously described (Resnati et al., 1996). THP-1 cells were metabolically labelled with $^{35}$S-TransLabel, acid washed (Stoppelli et al., 1986) and either mock-treated or treated with 1 pM peptide 1 (AVTYSRSRYLEC) for the indicated time at 37°C. Radiolabeled cell lysates were immunoprecipitated with a polyclonal anti-p56/59$^{ck}$ antibody (Resnati et al., 1996) and part analysed by SDS-PAGE to control the amount of protein. The rest was used for *in vitro* kinase assay (5-10 µCi of $\gamma^{32}$P-ATP, ~3000 Ci/mmol, Amersham, 5 µg rabbit muscle enolase substrate, 5 minutes, room temperature) and resolved by SDS-PAGE.

**Biotinylation of antibodies**

Antibodies (concentration 0.5-1 mg/ml) were dialysed against 0.05 M NaHCO$_3$ pH 8.5 prior to biotinylation. The antibody solution was added the biotinylation reagent (EZ-Link™ Sulfo-NHS-LC-Biotin, Pierce Chemicals) dissolved in water (10 mg/ml) immediately before use. The biotinylation was performed at room temperature for two hours using a molar ratio of biotinylation reagent to antibody of 100:1. The biotinylation was stopped by addition of concentrated NH$_4$Cl to a final concentration of 0.01 M and the biotinylation regent removed by extensive dialysis against PBS. The biotinylated antibodies were diluted 1:1 with 100% glycerol and stored at -20°C.

**Immunoprecipitations**

In 1 ml of RIPA/PBS (1:1 mixture of PBS and 2-fold concentrated RIPA buffer (0.2 M TrisHCl pH 7.5, 0.3 M NaCl, 2% Triton X-100, 2% sodium deoxycholate, 0.2% SDS)) 2 µg of biotinylated antibody and 10 µl of a 50% suspension of streptavidin beads (Boehringer Mannheim) were mixed and incubated at 4°C for one hour under gentle agitation. The beads were pelleted by centrifugation (15 seconds at full speed in a micro centrifuge) and the supernatant aspirated. The beads were washed once with 1 ml of RIPA/PBS to remove unbound antibodies. The volume of the samples to be analysed were adjusted to 0.5 ml using PBS and then added 0.5 ml 2-fold concentrated RIPA buffer containing protease inhibitors (Complete™, Boehringer Mannheim). The mixture was transferred to a tube containing the pellet of beads with pre-bound antibodies and binding was allowed to occur for 16 hours at 4°C under gentle agitation. At the end of the incubation, the beads were washed 3 times for 30 minutes with 1 ml aliquots of RIPA/PBS to remove unspecific proteins. For western blotting, the bound proteins were eluted in
15 µl non-reducing sample buffer (0.125 M TrisHCl pH 6.8, 20% glycerol (v/v), 4% (w/v) SDS, 20% (w/v) glycerol, 0.02% (w/v) bromphenol blue) by incubation at 95°C for 3 minutes and 12 µl of the eluate used in 10% SDS-PAGE.

Deglycosylation of uPAR

Immunoprecipitates prepared as described above were deglycosylated in a modified previously described procedure (Behrendt et al., 1990). The washed beads were added 10 µl of PBS containing 0.5% SDS and 1.6 mM DTT and the samples were incubated at 98°C for 3 minutes to denature the proteins under mildly reducing conditions. The samples were added 20 µl of 1.5-fold concentrated deglycosylation buffer (PBS containing 1% Triton X-100 and 15 mM EDTA), 1 unit of peptide-N-glycosidase F (PNGase-F) and incubated at 37°C for two hours. Control samples were treated in the same manner but did not receive PNGase-F. Prior to SDS-PAGE the samples were added 30 µl of 2-fold concentrated reducing sample buffer (0.125 M TrisHCl pH 6.8, 0.1 M DTT, 20% (v/v) glycerol, 4% SDS (w/v), 20% (w/v) glycerol, 0.02% (w/v) bromphenol blue) and the samples were incubated for 5 minutes at 95°C. For Western blotting 15 µl of the samples were analysed on 10% SDS-PAGE gels.

SDS-PAGE

SDS-GELS (0.75 mm thick) were cast and run in a Hoefer Mighty Small SE250 gel apparatus at 20 mA/gel (constant current) until the bromphenol blue front was about to exit the gel. Separation gels (10% acrylamide, 10 ml of solution) were prepared using the following ingredients: 3.33 ml 30% acrylamide monomer solution (acrylamide:bis-acrylamide, 29:1), 2.5 ml 1.5 M Tris.HCl pH 8.8, 0.1 ml 10% SDS, 4.0 ml H₂O, 50 µl of 10% (w/v) ammonium persulfate (APS) and 5 µl of N,N,N,N',tetramethylethylene diamine (TEMED). Separation gels were overlaid with water saturated isobutanol until polymerised. Stacking gels were prepared using the following ingredients (5 ml of solution): 0.67 ml 30% (w/v) acrylamide monomer solution (29:1), 1.25 ml 0.5 M Tris.HCl pH 6.8, 0.05 ml 10% (w/v) SDS, 3 ml H₂O, 25 µl 10% (w/v) APS and 2 µl TEMED. The running buffer used for electrophoresis had the following composition: 0.025 M Tris, 0.192 M glycine and 0.1% SDS, pH 8.3.

Protein blotting

Semi-dry blotting, using a Sigma apparatus, was used to transfer proteins from SDS-PAGE gels to PVDF membranes. The following sandwich was made (bottom to top): Steel anode; three layers of Whatman 3MM filterpaper soaked with transfer buffer (39 mM Glycine, 48 mM Tris, 10% methanol (v/v)); PVDF membrane (Immobilon P, Millipore) activated by soaking
in methanol (20 seconds), equilibrated with water gel (5 minutes), and transfer buffer (5 minutes),
gel, three layers of filter paper soaked with transfer buffer, and finally the platinum coated
cathode. Transfer was allowed to proceed for 90 minutes at 0.8 mA/cm^2 constant current. The
transfer of pre-stained protein molecular weight markers was used to confirm efficient transfer.

Western hybridisation

After transfer the membrane was briefly washed with TBS-T (150 mM NaCl, 50 mM
TrisHCl pH 7.5, 0.2 % Tween-20) and blocked over night at 4°C in blocking solution (5% non-
fat dry milk (Carnation, USA) in TBS-T). Membranes were probed for uPAR antigen by
incubation for 1 hour at room temperature in blocking reagent containing 1 µg/ml of a rabbit
polyclonal anti-uPAR antibody. The antibody solution was recycled for several blots and a
dramatic improvement of the signal/noise ratio was observed after the first use. Membranes were
washed 5 times (2 x 1 minute and 3 x 15 minutes) in TBS-T and probed for bound antibody by
incubation with a horseradish conjugated goat anti-rabbit antibody (Amersham) diluted 1:10.000
in blocking buffer for 1 hour at room temperature. The secondary antibody solution was made
fresh every time. The blots were washed as before and developed using a chemoluminescent
substrate according to the manufactures instructions (SuperSignal Ultra™, Pierce Chemicals).
Briefly, the humid membranes (protein side facing upwards) were overlaid with the
chemoluminescent substrate solution for 5 minutes at room temperature. The membranes were
placed between two sheets of transparency film and exposed in ordinary x-ray cassettes (without
amplifying screens) using Amersham multi-purpose films. Exposure times varied according to
signal strength.

Enzyme-linked immunosorbent assay (ELISA)

Three different ELISA were applied in this study. The first two (from now on termed the
"uPAR" and the "R2" ELISA) were developed at the Finsen Laboratory in Denmark and
measures human uPAR antigen. Although very similar, these two ELISA have different
selectivity and specificity. The third ELISA (the FLAG ELISA) was developed by me and was
used to quantify recombinant FLAG-tagged proteins.

The uPAR ELISA (Stephens et al., 1997) uses a polyclonal rabbit anti-uPAR antibody to
catch, a mixture of three different monoclonal anti-uPAR antibodies (mAb R2, R3 and R5) to
detect, and an alkaline phosphatase conjugated rabbit anti-mouse antibody to report. The ELISA
measures all known forms of uPAR including all known uPAR fragments and complexes. The
ELISA is designed to measure uPAR antigen in biological samples of human origin. The R2
ELISA (Holst-Hansen et al., 1999; Stephens et al., 1999) uses a mouse monoclonal anti-uPAR
antibody (mAb R2) to catch, a polyclonal rabbit anti-uPAR to detect, and an alkaline phosphatase conjugated anti-rabbit IgG monoclonal mouse antibody to report. The R2 ELISA is an "inverted" variant of the uPAR ELISA and was developed to measure human uPAR in serum samples from mice xenografted with human tumours. This ELISA does not detect free D1 but is otherwise similar to the general uPAR ELISA.

I developed the FLAG ELISA to measure the concentration of recombinant proteins expressed in cell culture. The assay uses a mouse monoclonal anti-FLAG antibody (mAb M2) to catch, a rabbit polyclonal anti-FLAG to detect, and a horseradish peroxidase coupled polyclonal donkey anti-rabbit antibody to report.

96 well ELISA plates were coated O.N. at 4°C with 0.1 ml antibody diluted in 0.1 M sodium carbonate buffer (pH 9.7) in the following concentrations; uPAR ELISA: 0.5 µg/ml anti-uPAR rabbit IgG; R2 ELISA: 0.5 µg/ml mAb R2; FLAG ELISA: 2 µg/ml mAb M2. The wells were washed six times with wash solution (PBS containing 0.1% Tween-20, PBS-T) and unspecific binding sites saturated by incubation with 0.2 ml blocking solution (PBS containing 2% BSA) for one hour at room temperature. Wells were washed six times with wash solution. Samples to be analysed were diluted in dilution buffer (PBS containing 1% BSA) and added to the wells in duplicates. The plates were incubated for 1 hour at room temperature and washed six times with PBS-T. The plates were probed with secondary antibodies diluted to the following concentrations; uPAR ELISA: 0.375 µg/ml of a mix of mAb R2, mAb R3, and mAb R5 (mixed in the weight ratio 1:4:1); R2 ELISA: 0.5 µg/ml anti-uPAR rabbit IgG; FLAG ELISA: 2 µg/ml anti-FLAG rabbit IgG. Binding was allowed to proceed for 1 hour at room temperature and followed by six washes using PBS-T. Bound secondary antibodies were detected using enzyme-conjugated antibodies diluted in blocking buffer as follows; uPAR ELISA: alkaline phosphatase conjugated rabbit-anti-mouse IgG 1:1000 (DAKO); R2 ELISA: alkaline phosphatase conjugated mouse-anti-rabbit IgG 1:2000 (SIGMA); FLAG ELISA: horseradish conjugated donkey-anti-rabbit IgG 1:10000 (Amersham). After incubation for 1 hour at room temperature the plates were washed six times with PBS-T and 3 times with water. Colorimetric enzyme substrates (alkaline phosphatase conjugates: 1.7 mg/ml p-nitrophenyl phosphate (pNPP, Sigma) in 100 mM NaCl, 100 mM TrisHCl pH 9.5, 5 mM MgCl2; horseradish peroxidase conjugates: ready-to-use ABTS substrate solution (Boehringer Mannheim). The absorbance at 405 nm (pNPP substrate) and 436 nm (APTS substrate) was measured after 1 hour incubation at room temperature using an ELISA reader.

Concentrations of antigen in the individual samples were calculated by comparison with a standard curve obtained by a two-fold dilution series of purified soluble human uPAR (1.0, 0.5, 0.25, 0.12, 0.06, 0.03, 0.015 ng/ml). Inter-assay variations were, if necessary, corrected by the value obtained for a reference sample included on all plates.
Purification of recombinant soluble uPAR molecules.

Recombinant proteins were purified from COS7 conditioned medium by passage over an anti-FLAG™ affinity column (M2 Affinity gel, Sigma). After washing of the column with PBS, the recombinant proteins were eluted using 0.1 M glycine pH 3.0. The fractions were immediately neutralised using 1/10 volume of 1 M TrisHCl pH 8.0 and analysed by SDS-PAGE and ELISA. Fractions containing the recombinant protein were pooled and dialysed against PBS. Recombinant proteins were stored at -80°C until use.

Purification of chymotryptic fragments of soluble uPAR

Purified soluble uPAR (D123-274) was dialysed against 0.1 M (NH₄)₂CO₃ and digested with chymotrypsin (Boehringer Mannheim) in the molar ratio 1:2000 (chymotrypsin:uPAR) for 16 hours at 37°C. Complete digestion was confirmed by SDS-PAGE and the mixture was added PMSF to 1 mM to stop the digestion. The domain 1 fragment (D1-87) was separated from the domain 2+3 fragment (D2388-274) by chromatography on a small FLAG™ affinity column. The D1-87 was recovered in the flow-through and the FLAG tagged D2388-274 fragment eluted from the column with 0.1 M glycine pH 3.0. Both flow-through and eluate were dialysed against PBS and the concentration of the purified fragments estimated by SDS-PAGE and commassie staining.

Purification and iodination of recombinant suPAR

Recombinant soluble uPAR was purified by affinity chromatography from conditioned medium of Chinese hamster ovary cells transfected with a soluble (non membrane-anchored) mutant uPAR cDNA (Masucci et al., 1991). The purified suPAR was labeled with ¹²⁵I using the Iodogen procedure (Pierce Chemicals), resulting in a specific activity of 80 x 10⁶ cpm/µg. For the analysis of suPAR degradation in urine, aliquots were supplemented with ¹²⁵I-suPAR to a concentration of 2 ng/ml and incubated for 24 hours at 37°C. Non-reducing sample buffer was added, and the samples were boiled and separated by 12% SDS-PAGE. Dried gels were analysed by autoradiography at -80°C using two amplifying screens. The chymotrypsin used in suPAR cleavage experiments was from Boehringer (Mannheim), whereas the uPA was a kind gift from Dr J. Henkin, Abbott Laboratories, Chicago).

In vitro binding assays

ELISA plates (Nunc Maxisorb) were coated with vitronectin (0.1 ml, 5 µg/ml, unless otherwise indicated) in PBS for two hours at 37°C or over night at 4°C. The remaining protein binding sites were blocked using 1% blocking reagent (Boehringer Mannheim) in TBS for one
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hour at room temperature. All further incubations were performed with gentle agitation at room temperature in 0.1 ml TBS containing 1% blocking reagent (Boehringer Mannheim) and followed by extensive washing with TBS containing 0.1% Tween-20. First, wells were incubated with the FLAG™-tagged soluble uPAR molecules diluted to the indicated concentrations for 1 hour at room temperature. In some cases the recombinant suPAR was co-incubated with pro-uPA, ATF and/or antibodies. After washing, wells were probed for the FLAG-epitope by incubation with 0.2 µg/ml rabbit anti-FLAG antibody (Santa Cruz) for one hour. After washing, wells were probed for bound antibody by incubation with a horseradish conjugated goat anti-rabbit antibody (Amersham) diluted 1:1000. After another round of washing, bound horseradish conjugate was quantified by colorimetric development using the chromogenic substrate ABTS (Boehringer Mannheim). The absorption at 436 nm was recorded in an ELISA plate reader and the signal obtained from wells that were coated with blocking reagent subtracted.

Creatinine measurement

Urinary creatinine was measured with a modification of the Jaffé-method according to the manufacturers instructions (Boehringer Mannheim), using a BM/Hitachi 747 analyser.

Cell culture techniques

General cell culture

All cell culture media used were supplemented with glutamine (5 mM), penicillin (100 U/ml), streptomycin (100 µg/ml). WEHI-1 cells were cultured in complete RPMI medium containing 15% FCS. U937 cells were cultured in complete RPMI medium supplemented with 5% FCS and the cells split by dilution. 32D cells were cultured in complete RPMI medium supplemented with 10% FCS and 10% conditioned medium from the cell line WEHI-1 as a source of IL-3. Stable pools and clones of transfected 32D cells were maintained under weak selective pressure in medium containing 0.2 mg / ml G418.

Preparation of conditioned WEHI medium

Cultures of proliferating WEHI-1 cells were split 1:100 in fresh medium and allowed to grow until the medium turned acidic (5-6 days). Cells were pelleted by centrifugation and the conditioned medium sterile filtered (0.2 µm) and stored at 4°C until use.
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Isolation and purification of blood leukocytes

Whole blood was fractionated by Ficoll-Hypaque gradient centrifugation (Seromed-Biochem KG). Monocytes were isolated from the peripheral blood mono-nuclear cells (PBMC) by gradient centrifugation on 46% Percoll (Pharamacia & Upjohn). Polymorph nucleated cells (predominantly neutrophils) were collected from the pellet of the initial Ficoll-Hypaque fractionation and further purified by gradient centrifugation on 62% Percoll.

Transfections

Transfection of COS7 cells

Semi-confluent COS7 cells were harvested in PBS containing 1 mM EDTA, washed 3 times with RPMI medium. The cell suspension (0.8 ml, 1-2x10^7 cells/ml in RPMI) was electroporated in 0.4 cm, Bio-Rad cuvettes containing plasmid DNA (30 µg, 1 mg/ml in water) at 960 µF, 240 V (GenePulser, Bio-Rad). After recovery overnight in complete DMEM medium containing 10% FCS, cells were washed with PBS and supplemented with 50 ml of serum-free medium (DMEM containing 1% Nutridoma NS, Boehringer Mannheim). Every 4-5 days, conditioned medium was collected and fresh medium added. The conditioned medium was stored frozen at -20°C until purification of the recombinant proteins.

Transfection of 32D cells

Growing 32D cells (0.5 x 10^6 / ml) were harvested and washed twice in RPMI medium. Cells were resuspended in RPMI (50 x 10^6 / ml) and 0.2 ml aliquots were added to electroporation cuvettes (0.4 cm, Bio-Rad) already containing 20 µg of the desired expression vector and 2 µg of pRSVneo (1 µg/ml in water). Cells were electroporated using a single 280 V, 960 µF pulse in an electroporation apparatus (Bio-Rad, Gene Pulser II) and transferred to complete medium for recovery. After 48 hours cells were transferred to complete medium containing 0.8 mg/ml G418 for selection of stable transfectants. Single clones of transfectants were obtained by limited dilution of G418 resistant pools into 96 well tissue culture plates.

Chemotaxis Assays.

Chemotaxis analyses were performed in modified Boyden chambers, using polyvinylpyrrolidone-free polycarbonate filters (13 mm diameter, 5 µm pore size) coated with type I collagen (100 µg/ml in PBS pH 7.4). After an acid wash (Stoppelli et al., 1986), 2x105 THP-1 cells in serum-free medium were added in the upper chamber. Attractants were diluted in
serum-free medium at the indicated concentrations and added in the lower chamber. The assays were incubated at 37°C in 5% CO₂ in air for 90 minutes. The upper surface of filters was scraped free of cells, the filters fixed in methanol, stained with crystal violet and cells counted. All experiments were performed in triplicate; data are reported as the number of cells counted for high power field (ten fields for each condition) and expressed as percentage of the control values. Data points represent the mean of three independent experiments (± SEM). When cells of the fibroblast-type were employed for the assay, the time required for migration was longer and the Boyden chambers were usually incubated overnight.

Pertussis toxin sensitivity was carried out by pre-incubating cells with 100 ng/ml wild-type or mutated (Pizza et al., 1989) pertussis toxin in RPMI for 4 hours at 37°C. After treatment, cells were extensively washed, resuspended in RPMI and assayed in chemotaxis, as described above, or in kinase assays (see below).

Adhesion assays

Coating of 96 well tissue culture dishes were performed by incubation with 5 µg/ml vitronectin or 10 mg/ml fibronectin in PBS for 2 hours at 37°C or over night at 4°C. Remaining binding sites were saturated by incubation of the wells with 2 % bovine serum albumin in PBS for 1 hour at 37°C. Immediately before the adhesion assay, cells were washed twice in RPMI containing 0.1% BSA and resuspended in the same medium at 2 x 10⁶/ml. Cells (0.05 ml) were added to wells already containing 50 µl RPMI with 0.1 % BSA and the agonists / antagonists to be tested. The plates were centrifuged at 1000 rpm for one minute to sediment the cells and incubated in a humidified incubator at 37°C for 1 hour. At the end of the incubation the wells were washed with 37°C warm RPMI as follows: to the medium already present in the wells was added 2 x 100 µl medium, at normal pipetting speed, using a multi-well pipettor, row by row, and from two opposite sites of the wells. The medium (containing the suspended non-adherent cells) was then immediately aspirated from one side of the well and 100 µl of fresh medium added. The procedure of washing was repeated, if necessary, until BSA coated wells contained only few remaining cells. Adherent cells were fixed for 10 minutes with 37°C warm PBS containing 3 % (w/v) formaldehyde and the cells stained for 5 minutes with 0.05 ml crystal violet solution (0.5 % (w/v) in 20 % (v/v) methanol). After washing of the plate by immersion in tap-water, the cells were lysed in 1% SDS in water and the absorption at 540 nm was measured in an ELISA plate reader. To obtain a measure for specific adhesion the readings were subtracted the value obtained in BSA coated wells, unless otherwise indicated. All experiments were performed at least twice and the shown values are the mean +/- SD of triplicate or quadruplicate determinations from a representative experiment.
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FACS analysis

32D cells to be analysed (1 x 10⁶) were pelleted by centrifugation and resuspended in 0.1 ml ice-cold wash buffer (PBS containing 5% FCS) containing 10 mg/ml of the R2 monoclonal antibody and incubated on ice for 30 minutes. The cells were washed twice with 3 ml ice cold wash buffer and resuspended in 0.1 ml wash buffer containing a FITC conjugated goat anti-mouse antibody (DAKO) diluted 1:50 in wash buffer and incubated for 20 minutes on ice. After another round of washing, the cells were resuspended in 0.5 ml PBS and analysed in a Becton Dickinson flow cytomter according to manufactures instructions. Relative values for the mean fluorescence were calculated using the Lysis II software package. Non-transfected cells were used as controls for specificity.

Animal experiments

The HOC8 xenografts were established and maintained as ascites in NCr nu/nu mice (Taconic, Germantown, NY) as described previously (Massazza et al., 1989; Pedersen et al., 1993). Urine, serum and tumour cells were obtained four weeks after the interperitoneal injection of a tumour suspension (10 million cells) from nude mice all bearing ascites and metastatic tumours in the peritoneal cavity (Pedersen et al., 1993). Urine collected from 5 mice, kept in a metabolic cage for 24 hours, was centrifuged and stored frozen at -80°C until analysis. Blood was obtained by intracardiac puncture from anaesthetised mice. As control, blood and urine were collected from five non tumour bearing nude mice and processed in the same way.

The MDA-MB-435-BAG (MDA435) cells (Brünner et al., 1992) were cultured in EagleMEM containing glutamax (Life Technologies) and 5% foetal bovine serum. Semi-confluent cell cultures were harvested by scraping and the concentration was adjusted to 10 x 10⁶ cells/ml. Six to eight weeks old male META-Bom-nu/nu mice (Bomholdtgaard, Denmark) were injected subcutaneously and bi-laterally with 0.1 ml MDA435 cell suspension. After 50 days, urine, plasma, and tumor tissue, were collected from two tumour-bearing mice as described for the HOC8 xenografted animals.

Statistics

To evaluate differences between group median values, the data were analysed with the Mann-Whitney rank-test. P values below or equal to 0.05 were considered significant.
Chapter 3:

RESULTS & DISCUSSION (I)

Molecular analysis of uPAR mediated vitronectin adhesion.
Summary

In this chapter I analyse uPAR mediated cellular binding to vitronectin using the murine erythroid progenitor cell line 32D. I show that expression of uPAR in the 32D promotes cellular binding to vitronectin, but fails to support cell spreading. The strength of binding is correlated to the expression level of uPAR and is strongly stimulated by the presence of the uPAR ligand pro-uPA. Using a recombinant truncated variant of uPAR lacking domain 1, and by antibody inhibition experiments, I demonstrate that domain 1 plays a crucial role in uPAR mediated cellular binding. The failure of the mutant uPAR lacking domain 1 to promote cellular binding is paralleled by strong reduction in the affinity for vitronectin in vitro.

Introduction

The presence of a cellular proteolytic variants of uPAR composed of domain 2 and 3 (D2D3), and thus lacking the major uPA binding domain 1 (D1), was first identified in U937 cells (Høyer-Hansen et al., 1992) and has since then been observed in other cell lines and tumour detergent extracts (Ragno et al., 1998; Solberg et al., 1994; Chapter 5; Chapter 6). The formation of the D2D3 uPAR variant may involve the proteolytic cleavage by uPA, which is capable of cleaving uPAR in the linker region between domain 1 and 2 both in vitro and at physiological concentrations in vivo (Høyer-Hansen et al., 1997b; Høyer-Hansen et al., 1992). The cleavage of uPAR in this region results in profound changes in the biological properties of uPAR: First, uPAR looses the capacity to bind uPA with a concomitant loss of potential to enhance uPA mediated cell surface plasmin generation (Ellis et al., 1989; Høyer-Hansen et al., 1997b). Since the cleavage is expected to occur most efficiently under conditions of high uPA activity it might function as a negative feedback mechanism (Høyer-Hansen et al., 1997b). Second, the cleavage in the hinge region unmasks bioactive epitopes of uPAR that have the potential to induce cellular chemotaxis and activation of kinases from the src-family (Fazioli et al., 1997; Resnati et al., 1996).

Cellular binding to vitronectin, mediated by uPAR, was first shown in the human histiocytic ÿmphoma cell line U937. This cell line becomes strongly adhesive to vitronectin when stimulated with a combination of vitamin D3 and transforming growth factor ß1 or phorbol esters in the presence of uPAR ligands (Nusrat and Chapman, 1991; Waltz and Chapman, 1994). This has later been extended to other cell lines and it has been shown that both cellular and soluble uPAR interact directly with certain forms of vitronectin (Høyer-Hansen et al., 1997a; Waltz and Chapman, 1994; Wei et al., 1994). The region of uPAR responsible for the interaction with vitronectin was initially assigned to D2D3 (Wei et al., 1994) but recent data obtained using real-time biomolecular interaction analysis have demonstrated that also D1 is required for the
interaction *in vitro* (Høyer-Hansen et al., 1997a). At present, very few data on the effect of cleavage on the potential of uPAR to mediate cellular binding to vitronectin have been presented (Ragno et al., 1998).

Besides mediating cellular binding to vitronectin uPAR also modulates the function of adhesion receptors from the integrin family (Simon et al., 1996; Sitrin et al., 1996; Wei et al., 1996; Yebra et al., 1996) and data for a direct interaction between uPAR and purified Mac-1 have been presented (Wei et al., 1996).

In this chapter I analyse the consequence of uPAR cleavage on the affinity for vitronectin and on the capacity to mediate cell binding to vitronectin. Using cellular and soluble uPAR mutant receptors I report that D1 plays a crucial role for the capacity of uPAR to mediate cellular binding to vitronectin as well as for the high affinity interaction with vitronectin *in vitro*. The system employed, 32D erythroid progenitor cells, is suitable for this analysis as these cells display low intrinsic binding to the extracellular matrix proteins vitronectin. Thus the uPAR-mediated binding is not confused with possible integrin mediated binding.
Results

Adhesive properties of 32D cells

To analyse the structural requirements of uPAR as an adhesion receptor I wanted to use a cell system that fulfils two criteria. First, the cell line should be easy to transfect in order to introduce the desired recombinant uPAR receptors to test for their properties as adhesion receptors. Second, the cell line should have low basal binding to vitronectin in order to discriminate between binding caused by the transfected molecules and binding mediated by endogenously expressed adhesion receptors.

To evaluate the murine myeloid 32D cell line as a model system for uPAR mediated adhesion I compared the adhesive properties of these cells with those of the well characterised human myeloid U937 cells (figure 3.1, panel A). While U937 cells displayed significant binding to both fibronectin and vitronectin, 32D cells completely failed to adhere to vitronectin and only displayed moderate binding to fibronectin. Stimulation with cytokines or phorbol-esters have been shown to enhance the cellular binding of U937 cells to vitronectin as a result of enhanced expression of uPAR and of its ligand uPA (Waltz and Chapman, 1994). To analyse the effect of phorbol-esters stimulation on the cellular binding of 32D cells I stimulated cultures of 32D and U937 with PMA for 18 hour prior to the adhesion assay (figure 3.1, panel B). As expected, PMA treatment strongly stimulates the binding of U937 cells to vitronectin as previously described (Waltz and Chapman, 1994). However, the PMA treatment fails to modulate the binding of the 32D cells to any of these substrates. The 32D cells express a range of integrin receptors including fibronectin and vitronectin receptors (Bazzoni et al., 1996; Zhang et al., 1996) and it has been shown that the failure of PMA to induce the activation of these receptors can be reverted by the expression of protein kinase C-δ (Mischak et al., 1993). The activation state of integrin receptors and the binding to fibronectin of 32D cells has been reported to be regulated by the IL-3 stimulation state of the cells (Bazzoni et al., 1996). My experimental protocol uses cells grown in medium where the IL-3 is supplied in the form of conditioned medium from the WEHI cell line and the level of IL-3 stimulation is therefore ill defined. To analyse the effect of IL-3 stimulation on binding to fibronectin and vitronectin I performed experiments where the 32D cells had been IL-3 starved for 8 hours prior to the adhesion assay (figure 3.2). Under these conditions the presence of IL-3 stimulate the cellular binding of 32D cells to fibronectin approximately 2-fold, but fails to modulate the binding to vitronectin.

From these experiments I conclude that the 32D cells represent a suitable model system for the analysis of uPAR-mediated cellular binding to vitronectin as these cells fails to display any notable binding to vitronectin under all the conditions analysed.
uPAR expression promotes 32D cell binding to vitronectin

To analyse uPAR-mediated cell binding I transfected 32D cells with an expression vector in which I had engineered the full-length human uPAR cDNA (Roldan et al., 1990). By selection of the transfected cells with G418 I obtained a pool of resistant cells representing a population of clones expressing variable amounts of uPAR on the cell surface as determined by FACS analysis using the anti-uPAR monoclonal antibody R2 (results not shown). I observed that during normal passage, the pool of uPAR transfected cells are notably more adherent to the tissue culture plates than the mock transfected cells (results not shown). To investigate whether this increased binding of the uPAR transfected cells is mediated by the vitronectin present in the culture medium, I performed adhesion assays with the stable pools of transfected 32D cells under serum free conditions, to purified vitronectin immobilised in 96 well tissue culture plates (figure 3.3). Indeed, the pool of uPAR transfected cells binds stronger to vitronectin than the mock-transfected cells over a range of coating concentrations, demonstrating that expression of uPAR in 32D cells causes specific cellular binding to vitronectin.

Binding is proportional to the expression level of uPAR

Only a small fraction of the 32D cells (less than 10%, not shown) in the pool of uPAR transfected cells remain bound after washing, even at the highest vitronectin coating concentration, and I speculated that this is a result of heterogeneous uPAR expression in the pool of transfected cells. To analyse the relationship between the expression level of uPAR and the strength of binding to vitronectin I cloned the pool of transfected cells by end-point dilution. I analysed the cell surface expression of uPAR in 50 clones by FACS analysis using the anti-uPAR monoclonal antibody R2 (figure 3.4, panel B and not shown) and selected a number of clones representing a range of expression levels for analysis of their binding to vitronectin (figure 3.4, panel A). I found that there is a good correlation between the expression level of uPAR and the strength of binding to vitronectin as higher expression levels of uPAR are associated with stronger binding to vitronectin. In the highest expressing clones (clones 22 and 1) more that 90% of the cells remain bound after washing of the plates (not shown), while the lowest expressing clones (clone 21 and 24) display binding comparable to the pool of transfected cells. Some clones (clones 14 and 17) do not express detectable levels of cell surface uPAR and are included in the analysis as negative controls.
Binding is stimulated by uPAR ligands

uPAR mediated binding of U937 cells to vitronectin is stimulated by uPA (Waltz and Chapman, 1994) and I therefore wanted to analyse the effect of uPA on the binding of the uPAR transfected 32D cells. To do this I performed adhesion assays in the presence of saturating levels of pro-uPA (figure 3.4, panel A). Clones expressing high levels of uPAR (clone 22 and 1) bind strongly to vitronectin in the absence of ligand and the addition of pro-uPA does not stimulate this binding further. The binding of clones expressing low and intermediate levels of uPAR are stimulated by pro-uPA and the stimulation is inversely correlated with the expression level of uPAR. Clones expressing low levels of uPAR (clone 24A and 21) are strongly stimulated and clones expressing intermediate to high levels of uPAR (clone 22.7, 8, and 2) are only weakly stimulated. The presence of pro-uPA during the adhesion assays has no significant effect on the binding of mock transfected cells (not shown) or control clones expressing undetectable levels of uPAR (clone 14 and 17) demonstrating that the stimulatory effect of uPAR ligands is indeed mediated by uPAR.

The dose-dependency of the stimulation by pro-uPA analysed in two clones (c21 and c24A) which are strongly stimulated by pro-uPA, is shown in figure 3.5. For both clones the stimulation is half-maximal at 0.1-0.3 nM pro-uPA which is consistent with the Kd for the interaction between uPAR and pro-uPA (0.1-1 nM). Also two-chain uPA and the amino-terminal fragment (ATF) of uPA, which contains the entire uPAR binding site, stimulates the binding with equal efficiency demonstrating that it is the ligand binding capacity, rather than the catalytic activity of uPA, responsible for the stimulation of binding (results not shown).

A uPAR mutant lacking domain 1 fails to promote binding

The occurrence of uPAR cleavage in tumour cell lines and xenografted tumour tissue prompted me to investigate whether cleaved uPAR supports cellular binding to vitronectin. To analyse this I constructed a mutant uPAR cDNA encoding a deletion variant of uPAR where the region encoding domain 1 (amino acid 1-92) was deleted creating a receptor composed of domain 2 and 3 (termed D2D3). This recombinant receptor is equivalent to cellular cleaved uPAR observed in cell culture as it lacks domain 1. I cloned this mutant uPAR cDNA into the expression vector and generated clones of 32D cells expressing the mutant receptor as described for full-length uPAR. I identified several clones expressing the truncated receptor at levels comparable to what I observe with full-length uPAR (figure 3.4, panel B). However, even though the D2D3 receptor is efficiently expressed the cells fail to adhere to vitronectin (figure 3.4, panel A). The failure to adhere to vitronectin is independent of the addition of pro-uPA.
Antibodies against domain 1 inhibit binding

To further analyse which region of uPAR is involved in cellular binding to vitronectin, I tested four monoclonal antibodies against uPAR for their ability to inhibit the binding of uPAR transfected 32D cells (figure 3.6). I used the 32D/uPAR clone 1 cells, as these adhere strongly to vitronectin in the absence of pro-uPA, and I performed the adhesion assay in the absence of pro-uPA to avoid the potential interference between antibody and pro-uPA binding to uPAR. Adhesion assays were performed in the presence of 50 μg/ml of monoclonal antibodies recognising different epitopes on uPAR or an irrelevant control antibody. Two antibodies recognising epitopes within domain 3 of uPAR (mAb R2 and R4) and the control antibody (M2) do not affect the binding to vitronectin. The two antibodies recognising epitopes within domain 1 of uPAR (mAb R3 and R5) strongly inhibit the binding. In line with the data from the mutant D2D3 receptor, these results suggest a fundamental role of domain 1 in uPAR mediated cell binding.

uPAR mediated binding to vitronectin is independent of integrin activation

uPAR mediated vitronectin adhesion of transfected HEK293 cells depends upon integrin activation as it is inhibited at low temperatures and by the introduction of dominant negative integrin molecules (Wei et al., 1996). To analyse the requirement for integrin function and activation in the 32D cell system, I performed adhesion assays in which I regulated the integrin activation state using IL-3 and temperature (figure 3.7). The binding to vitronectin is not significantly affected by IL-3 stimulation and is not reduced at 4°C, suggesting that uPAR mediated cell binding to vitronectin does not require integrin activation or metabolic energy in the 32D cell system. The binding to fibronectin, which is mediated by integrin receptors, is enhanced by IL-3 stimulation at 37°C, and almost completely inhibited at 4°C demonstrating the requirement for metabolic energy. The inhibitors of integrin function EDTA, and RGD containing peptides also fail to inhibit the binding uPAR-expressing cells to vitronectin (not shown).

In support of the lack of integrin involvement in the observed binding, I also fail to observe cellular spreading of the uPAR transfected cells on vitronectin (figure 3.8) as the morphology of the uPAR transfected cells bound to vitronectin or to BSA is indistinguishable.

The data suggest that uPAR mediated binding of the transfected 32D cells to vitronectin occurs independently of integrin function and activation. The observed binding may therefore simply reflect an affinity between the cells and the tissue culture plastic mediated by the interaction between uPAR (on the cell surface) and vitronectin (on the plastic). To investigate this possibility I performed adhesion assays to plates that I had coated with monoclonal antibodies
against uPAR (figure 3.9). In fact, uPAR transfected 32D cells bind strongly to wells coated with antibodies against both domain 1 (mAb R3) and domain 3 (mAb R2). The 32D cells transfected with the mutant D2D3 receptor, which fail to adhere to vitronectin, adhere to the uPAR antibody recognising an epitope within domain 3 (mAb R2). The results suggest that any high affinity interaction between a cell surface molecule (in this case uPAR) and an immobilised protein (in this case vitronectin or anti-uPAR antibodies) can mediate cellular binding of the 32D cells.

Domain 1 and uPAR ligand are required for the high affinity binding of soluble uPAR to vitronectin

Taken together the data suggest that the inability of D2D3 receptors to mediate cellular binding is caused by a reduced or abolished ability to interact with vitronectin. I therefore proceeded to analyse if the D2D3 receptor also has a reduced affinity for vitronectin in vitro. To do this I performed in vitro binding assays to immobilised vitronectin using a soluble form of full-length uPAR and the mutant D2D3 receptor. The GPI-anchoring sequence in these recombinant receptors has been replaced with a short peptide sequence (the FLAG epitope) recognised by specific antibodies and thus represent soluble forms of the cellular receptors used in the 32D cells. The construction, expression and purification of these soluble receptors are described in detail in chapter 4. The in vitro binding assay used in this analysis is in principle similar to the adhesion assay as the molecules are allowed to bind to immobilised vitronectin. After washing, bound protein is quantified by virtue of the FLAG-tag in an ELISA-like assay (see material & methods).

I incubated vitronectin-coated wells with increasing concentrations of the recombinant proteins in the presence or absence of pro-uPA and detected bound proteins using an antibody directed against the FLAG-epitope located at the carboxy-terminus of the recombinant receptors (figure 3.10). In the presence of pro-uPA, I detect specific binding of the full-length receptor down to approximately 1 nM, while I fail to observe any significant binding in the absence of pro-uPA, even at 100-fold higher concentrations. The soluble D2D3 receptor mutant fails to bind notably to vitronectin over the entire concentration range analysed and does so both in the presence or absence of pro-uPA.
Discussion

Firm cellular attachment to the extracellular matrix is a complex process, which involve numerous molecular interactions between extracellular proteins, cell surface receptors and the intracellular cytoskeleton. The process is initiated with the cellular binding to the extracellular matrix, mediated by the interaction between cellular receptors and extracellular matrix proteins, and is followed by a complex process of signalling, receptor activation, and cytoskeleton reorganisation, eventually leading to cell spreading and firm attachment.

The expression of uPAR in 32D cells promotes cellular binding to vitronectin but not promote firm adhesion and cell spreading on the substrate. This is in contrast to the epithelial HEK293 cell line in which expression of uPAR leads to firm cell adhesion and spreading on vitronectin (Wei et al., 1994). The apparent discrepancy between these observations may be found in differences between the two cell lines. HEK293 grow firmly adherent and therefore clearly possesses the necessary cell program and machinery to promote firm cell adhesion and spreading. The 32D cells are highly undifferentiated, grow in suspension, and are only expected to undergo firm adhesion and cell spreading upon differentiation. It is therefore likely that these cells do not possess the required cell program and/or machinery to support cell spreading. Expression of uPAR in 32D cells evidently promotes the initial cellular binding to vitronectin, but fails to initiate the process of cell spreading. It is possible that in the 32D cells uPAR fails to associate to integrin molecules or that the uPAR/integrin complexes fail to connect to the cytoskeleton. The strength of binding of cells to vitronectin is comparable to that of anti-uPAR antibodies suggesting that the only requirement for binding is a sufficiently high number of interactions between the cell and the substrate to resist the hydro-dynamic pressure applied during washing of the plates.

The structural requirement for a high affinity interaction between vitronectin and uPAR has been addressed in a couple of studies (Høyer-Hansen et al., 1997a; Wei et al., 1994). Wei and co-workers have suggested that a region within the D2D3 part of uPAR is responsible for the interaction. This conclusion is based on experiments with a D2D3 fragment purified from chymotrypsin-cleaved suPAR and inhibition by the R4 antibody (recognising an epitope within domain 3). Høyer-Hansen and co-workers have, based on real-time biomolecular analysis, concluded that intact uPAR is required for the high affinity interaction between uPAR and vitronectin. My data are in accordance with the latter, as I do not observe any significant binding of D2D3 to immobilised vitronectin. A likely explanation for the discrepancy between the observations could be the presence of low levels of contaminating full-length soluble uPAR in the D2D3 preparations used by Wei and co-workers. In my experimental set-up this potential
problem has been eliminated using recombinant uPAR fragments expressed in cells that do not produce any full-length suPAR.

I observe a strict requirement for the pro-uPA to obtain significant binding of suPAR to immobilised vitronectin in vitro. This observation is in contrast to data published by others who observe no difference (Høyer-Hansen et al., 1997a) or a moderate to high stimulation (Wei et al., 1994) by uPAR ligands. The reason for this discrepancy probably lies in the different assays, as well as in the different soluble uPAR molecules or vitronectin preparations used in the experiments. The binding of suPAR to vitronectin is not a simple first order binding reaction and probably involves conformational changes of both uPAR and vitronectin (Høyer-Hansen et al., 1997a). This is in accordance with the model proposed by Wei and co-workers who suggest that uPAR, with respect to vitronectin binding, is in an equilibrium between a high and a low affinity conformation which is pushed towards the high affinity state upon ligand binding. Modifications such as radio labelling, biotinylation or the engineered addition of epitopes may potentially affect the equilibrium between the different conformations present in the recombinant soluble uPAR preparations used by different laboratories.

In contrast to my data in vitro, I did not observe a strict requirement for ligand binding in the process of cellular binding to vitronectin in vivo. This apparent difference may have a number of explanations. First, the local concentration of membrane bound uPAR at the interface between the cell and the substratum might be high enough to allow a sufficient number of low affinity interactions between uPAR and vitronectin to obtain the required resistance to the hydrodynamic stress applied in the adhesion assay. Second, the high affinity conformation of uPAR could be stabilised by the GPI-anchor, which is not present on soluble uPAR, or by the interaction with other membrane proteins. Third, the presence of the FLAG-epitope on our soluble uPAR might stabilise the low affinity conformation of the soluble receptor. However, these explanations only apply to cells that express high levels of uPAR. Low-uPAR cells requires ligand to bind to vitronectin.

uPAR antagonist are molecules that inhibit the uPA/uPAR interaction through binding to uPAR. These molecules include non-catalytic derivatives of uPA (ATF, GFD and derivatives), antibodies as R3 and R5, and different low molecular weight compounds. uPAR antagonists are therapeutically interesting as they have been shown to inhibit tumour growth and metastasis in certain model systems. The anti-cancer effect of these molecules has been assigned to the capacity to block the binding of uPA to uPAR with its concomitant reduction in plasminogen activation potential of the cells. However, it is likely that the effect of these compounds might, at least partially, be caused by their pro- or anti adhesive properties. A great deal of work is done in the pharmaceutical industry to develop non-toxic low molecular weight uPAR antagonists and
the 32D/uPAR cell system is perfect to test the pro- or anti adhesive properties of these compounds.

Contributions:

All the experimental work presented in this chapter was planned and performed by me.
Chapter 4:

RESULTS AND DISCUSSION (II)

The chemotactic epitope in uPAR
Summary

Through the expression and testing of several uPAR variants, I have located and functionally characterised a potent chemotactic uPAR epitope that mimics the effects of the uPA/uPAR interaction. The chemotactic activity lies in the region linking domains 1 and 2, which is highly sensitive to proteolytic cleavage. Synthetic peptides carrying this epitope promote chemotaxis and activate p56/p59tyrosine kinase. Both the chemotaxis and kinase activation are pertussis-toxin sensitive and thus involve a G subunit protein in the pathway.

Introduction

The role of urokinase (uPA) and uPAR in cell migration and invasion is well substantiated. It was previously shown that uPA-induced chemotaxis requires interaction with and modification of uPAR, which is the true chemoattracting molecule acting through an unidentified cell surface component which mediates this cell surface chemokine activity (Resnati et al., 1996). Cell recruitment is an essential component in inflammation, neo-angiogenesis and cancer invasiveness. Urokinase-type plasminogen activator (uPA), its receptor (uPAR) and inhibitor (PAI-1) are directly involved in these mechanisms (Blasi, 1997). An important proof of this statement is the profound impairment of inflammatory cell recruitment in uPA−/− mice which do not properly respond but succumb to infection by Cryptococcus neoformans, due to deficient T-lymphocytes and monocytes-macrophages recruitment (Gyetko et al., 1996). Moreover, uPA-deficient mice fail to support the growth and development of experimental melanomas, while inhibition of the uPA/uPAR interaction in vivo blocks angiogenesis, growth and spreading of syngeneic tumours (Min et al., 1996; Shapiro et al., 1996). A chemotactic activity of uPA has long been recognised in vitro on different cell types in culture (Busso et al., 1994; Fibbi et al., 1988; Gudewicz and Gilboa, 1987; Gyetko et al., 1994; Resnati et al., 1996). The chemotactic activity of uPA strictly depends on binding to its receptor uPAR: First, it does not occur in murine cells lacking uPAR; second, it does not occur in cells containing uPAR but not recognising human uPA; third, it can be restored by transfection of the uPAR cDNA; fourth, it is inhibited by antibodies that prevent the uPA/uPAR interaction (Resnati et al., 1996). Occupancy of uPAR transduces a signal that results in the movement of cells; indeed, a rapid and transient activation of intracellular serine and tyrosine kinases (PTK) has been observed in different cells (Busso et al., 1994; Resnati et al., 1996). That tyrosine kinase activation is required for the chemotactic activity of uPA is shown by the ability of 3T3 fibroblasts from wild-type but not from Src−/− mice to respond to uPAR-mediated chemotraction (our unpublished data), or by the block of ligand-induced chemotaxis by tyrosine kinase inhibitors in THP-1 cells (Resnati et al., 1996).
uPAR is a GPI anchored protein made up of three cysteine-rich CD59-like domains connected by short linker regions (Ploug and Ellis, 1994), expressed by a variety of cells including neutrophils, T-lymphocytes, monocytes-macrophages and fibroblasts. In addition, most cancer cells express uPAR, often at rather high levels (Blasi, 1988). In T-lymphocytes and monocytes, uPAR is an activation antigen and its expression appears to be relevant to the function of these cells at least in culture (Bianchi et al., 1996; Nykjaer et al., 1994; Picone et al., 1989).

The membrane attachment of uPAR via a GPI-anchor (Ploug et al., 1991) i.e. the lack of an intra-cytoplasmic region capable of connecting with the cytoplasmic signal transducers, suggests that transmembrane adapter(s) must mediate the activation of intracellular transducers. Indeed, uPAR<sup>-/-</sup> cells can respond to a chemotactic stimulus induced by exogenous, soluble uPAR (suPAR), indicating that the binding of uPA to uPAR transforms the latter into a ligand for another cell surface component. However, this activity of suPAR is apparent only after chymotrypsin cleavage at Tyr87 (Resnati et al., 1996), a modification causing a major conformational change, similar to uPA binding (Ploug and Ellis, 1994; Ploug et al., 1993). The chemotactic properties of uPA and chymotrypsin-cleaved suPAR are identical, i.e. they both induce p56/p59<sub>ck</sub> tyrosine kinase activation with the same time-course (Resnati et al., 1996) indicating that the same mechanism operates in both cases.

The above data show therefore that uPAR is a cell-surface chemokine-like molecule, the activity of which is regulated by the binding of its ligand uPA which in an as yet undefined way uncovers the chemotactic epitope. In this chapter I demonstrate that the uPAR chemotactic epitope is located in the sequence linking domains 1 and 2, a region highly sensitive to cleavage by uPA and other proteases (Høyer-Hansen et al., 1992; Høyer-Hansen et al., 1997b). Indeed, also a synthetic uPAR chemotactic epitope activates p56/p59<sub>ck</sub> and both chemotaxis and activation of p56/p59<sub>ck</sub> are pertussis-toxin sensitive, implying a connection between G-proteins and intracellular protein tyrosine kinases.
Results

Identification and mapping of the uPAR chemotactic epitope.

To identify the chemotactically active region in uPAR, I constructed recombinant uPAR cDNA's encoding different parts of uPAR (figure 4.1). The recombinant receptors were expressed by transient transfection of COS7 cells as this allowed me to obtain sufficient purified protein (0.05-0.5 mg depending on the construct) within one month of transfection. The recombinant receptors were purified from the conditioned medium by affinity chromatography utilising the flag epitope engineered onto the carboxyterminus of all the recombinant receptors.

We had previously shown that chymotryptic cleaved suPAR induced a very efficient chemotactic activity at picomolar concentrations (Resnati et al., 1996), but as the chymotrypsin digestion generates a mixture of the two fragments D11-87 and D2D388-274, it was not possible to determine which of the fragments possessed the chemotactic activity.

To determine which of the two fragments possessed the chemotactic activity, I first purified the FLAG-tagged, full-size three domains suPAR, digested it with chymotrypsin and separated the resulting fragments D11-87 and D2D388-274 by antibody affinity chromatography. The D11-87 fragment was recovered in the flow through and the D2D388-274 fragment eluted from the column by lowering the pH (not shown). The two fragments were individually tested in chemotaxis assays on human THP-1 cells. THP-1 cells were chosen, even though they are poor responders to chemotactic signals, because their basal level of tyrosine phosphorylation is low (Resnati et al., 1996) making the subsequent biochemical analysis easier. The carboxyterminal fragment, D2D388-274, but not the aminoterminal D11-87, elicited a dose-dependent chemotactic response, demonstrating that the chemotactic region in uPAR is located within the D2D388-274 fragment (figure 4.2). To further map the chemotactic region I expressed and purified Flag™ tagged recombinant fragments of uPAR encoding different parts of uPAR (figure 4.1). Of these, only D11-92 induced a dose-dependent chemotactic effect, while D2D393-274, D293-191 and D3192-274 failed to induce chemotaxis through the entire concentration range analysed (figure 4.2). These findings show that only fragments containing the sequence 88 to 92 of uPAR, SRSRY, had a chemotactic effect. Since the presence of this motif correlates with enhanced migration of THP-1 cells it may represent the chemotactically-active epitope. Interestingly, the 88-92 sequence appears to be active both when present at the amino-, as in D2D388-274, as well as at the carboxyterminus, as in D11-92.

To determine whether the identified SRSRY motif was responsible for the observed chemotactic effect, we tested synthetic uPAR peptides for their chemotactic activity on THP-1 cells. Peptide 1 (AVTYSRSRYLEC) which corresponds to the amino acid sequence 84-95 of
human uPAR (Roldan et al., 1990) and its shorter version, peptide 3 (SRSRYLEC, residues 88-95), both contain the SRSRY motif, exhibited chemotactic activity with a maximal response at concentrations as low as 0.1 pM (figure 4.3, panels A and C). On the other hand, peptide 4 (TLVEYYSRASC), a scrambled version of peptide 1, and peptide 2 (YTARLWGGTLLT), which covers residues 301-313, and thus is absent from processed mature uPAR (Ploug et al., 1991), had no effect on cell migration over the entire concentration range analysed (figure 4.3, panels B and D). These results support the conclusion that peptides containing the sequence SRSRY of human uPAR have a potent chemotactic activity. It is important to note that this region is efficiently cleaved by uPAR-bound uPA at chemotactically active concentrations (Høyer-Hansen et al., 1997b).

Since THP-1 cells are not very sensitive to chemotactic stimuli, we also tested peptide 1 on other cells. Peptide 1 was found to be active also on several other cells, including murine uPAR−/− fibroblasts, 3T3 and LB6 cells, and rat aortic smooth muscle cells (data not shown). On some of these cells the effect is much more pronounced (about four-fold over basal migration), but has the same concentration-dependence as in THP-1 cells.

The chemotactic effect of suPAR fragments and synthetic peptides was not a general effect on migration but was dependent on the establishment of a concentration gradient. Also, these agents did not display a chemokinetic effect on THP-1 cells, as addition in the upper well (containing the cells) had no effect on migration (data not shown).

**Activation of p56/p59hck by chemotactic UPAR peptides.**

Treatment of THP-1 cells with the uPA fragment ATF at chemotactic concentrations induces the activation of p56/p59hck tyrosine kinase. Treatment with exogenous chymotrypsin-cleaved suPAR, that substitutes for uPA in inducing chemotaxis, also activates p56/p59hck (Resnati et al., 1996). We therefore tested the effect of chemotactic concentrations of peptide 1 on the activity of p56/p59hck kinase. Treatment of THP-1 cells at 37°C with 1 pM peptide 1, induced increased autophosphorylation as well as phosphorylation of the exogenous substrate enolase (not shown). The effect was apparent after 2 minutes, reached a maximum after 10 minutes and returned to the basal level after 30 minutes and thus has a time-course very similar to that of ATF or chymotrypsin-cleaved suPAR (Resnati et al., 1996)(data not shown). No effect was observed when 1 pM of the corresponding scrambled peptide (peptide 4) was used in the assay (data not shown). Thus, the effect of peptide 1, containing the SRSRY sequence of uPAR, resembles that of uPA and chymotrypsin-cleaved suPAR not only in stimulating chemotaxis but
also in the activation of the same tyrosine kinase. This supports the view that the SRSRY motif present in peptide 1 is indeed the chemotactic epitope of uPAR.

Involvement of heterotrimeric G-proteins.

The activity of many chemokines is mediated through specific receptors and involves GTP-binding proteins (G-proteins) for the signal transduction. Pertussis toxin (PTX) inhibits the activity of some chemokines through the αi subset of G-proteins; the inhibitory effect of PTX is therefore an indicator of the involvement of heterotrimeric Gi/o proteins in the involved signalling pathway (Baggiolini et al., 1995; Neer, 1995). To test the involvement of G proteins in uPAR-induced chemotaxis we tested the effect of PTX using as a control its active site mutant (Pizza et al., 1989). A pre-incubation with PTX-inhibited the chymotrypsin-cleaved suPAR and peptide-1 induced chemotaxis of THP-1 cells, while the inactive PTX mutant had no effect (figure 4.4). An identical result was obtained with peptide-1 induced chemotaxis of rat smooth muscle cells and on THP-1 chemotaxis induced by the amino terminal fragment of uPA (ATE, (Stoppelli et al., 1985)) (not shown). This result indicates that the uPAR epitope exerts its chemotactic activity through a G-protein-coupled cell surface protein.

Since both uPA and the chemotactic uPAR peptides activate the p56/p59hck kinase we also tested whether the action of the PTX toxin could inhibit the p56/p59hck activation. Activation of p56/p59hck by peptide-1 was inhibited by wild-type PTX, but not mutant PTX (not shown), supporting the G-protein dependence of uPAR chemotaxis and suggestive that the activation of tyrosine kinases occurs downstream of the heterotrimeric Gi/o protein.
GPI-anchored proteins are potent signalling molecules. However, the lack of a trans-membrane domain requires that they interact with another trans-membrane "adaptor", i.e. with the real signalling molecule. This has been shown to be true for several GPI-proteins, including, among others, CD14 (Ulevitch and Tobias, 1994). In the case of uPAR, the adapter (Resnati et al., 1996) is not yet known; however by identifying the chemotactic epitope of uPAR, we in fact suggest the nature of the actual ligand for such an adapter. We have shown that peptide-1 can reproduce, with the same time-course, the chemotactic effect of uPA and chymotrypsin-cleaved suPAR, causing cell migration and rapid and transient activation of p56/p59\(^{ck}\) in THP1 and other cells, through a PTX-sensitive signalling system. The concentration of peptide-1 required to elicit the signalling effects (about 0.1 pM) is far lower than the concentration of uPA or ATF required to generate the same biological response (0.5 to 5 nM, (Resnati et al., 1996)), but it is in the same order of magnitude of the effect obtained with chymotrypsin-cleaved suPAR. This represents a strong functional amplification, which follows or bypasses the uPA/uPAR interaction step. The shape of the dose-response curve for the various peptides closely resembles that for the chymotrypsin-cleaved suPAR (Resnati et al., 1996), and appears to be typical of the chemotactic response.

Our data show that the chemotactic effect of peptide-1 is not species-specific, as murine and rat cells also respond as well. This differs from the binding of uPA to uPAR which is species-specific (Appella et al., 1987; Estreicher et al., 1989).

The chemotactic epitope is located within the linker region connecting domains 1 and 2 (figure 4.5). This is the only region that is in fact susceptible to proteases (Behrendt et al., 1991). The question arising from the identification of the chemotactic epitope is whether exposure of this epitope physiologically occurs through proteolytic cleavage. While we cannot exclude that a ligand-induced conformational change of uPAR is sufficient to transduce migratory signals, we must notice that a relevant proteolytic event does indeed occur in vivo: First, uPAR fragments missing domain D1 have been demonstrated in different cell lines and human cancer tissues (Høyer-Hansen et al., 1992; Solberg et al., 1994). Second, in purified suPAR, the linker region between domains 1 and 2 (figure 4.5) is susceptible to cleavage by uPA and other proteases, like chymotrypsin and neutrophil elastase (Høyer-Hansen et al., 1992; Ploug and Ellis, 1994). Third, at chemotactically active concentrations, endogenous or exogenous receptor-bound uPA cleaves 50% of surface uPAR at residue 83 or 89 (Høyer-Hansen et al., 1997b). The former of these cleavage sites generates a chemotactically active uPAR fragment, starting with an N-terminal AVTYSRSRY sequence, identical to that of peptide-1. Fourth, the binding of uPA to uPAR...
activates cell surface plasminogen to plasmin which, too, might cleave uPAR in the same region (Høyer-Hansen et al., 1992; Ploug and Ellis, 1994).

While these data suggest that proteolysis activates the chemotactic activity of uPAR, in cultured cells the uPA protease domain is not necessarily required, since chemotaxis can be stimulated by uPA derivatives lacking protease activity (i.e. ATF or pro-uPA) (Fibbi et al., 1988; Resnati et al., 1996). It is known that ligand binding induces a conformational change (Ploug et al., 1994). Possibly, this may allow cleavage of uPAR by a different protease substituting for uPA. Since the sequence SRSRY is active both when amino- and carboxy-terminally positioned, different types of proteases may be involved. Again, we cannot exclude that a conformational change be sufficient to expose the SRSRY epitope, and that the observed proteolytic event be unrelated.

It is also interesting to notice that uPA has two potential cleavage sites in the sequence of uPAR linking domain D1 to domain D2. The second site, at position 89/90 might destroy the chemotactic activity, as it would cleave within the conserved region.

Whatever the mechanism, our data show that the uPA/uPAR system functions as a cell-surface regulated chemokine. The exposed chemotactic epitope must interact with a cell surface (adaptor) protein, which can signal through an heterotrimeric G protein. The nature of the adapter is still unknown: among already known proteins, possible candidates are chemokine receptors, integrins and caveolin. Chemokine receptors, as members of the seven trans-membrane-spanning protein family, interact with heterotrimeric G proteins (Baggiolini et al., 1995). Integrins and caveolin, on the other hand, have been shown to co-immunoprecipitate with uPAR (Bohuslav et al., 1995; Kindzelskii et al., 1996; Wei et al., 1996).

Our data show that uPAR-mediated chemotaxis requires a connection between intracellular tyrosine kinases of the Src family and heterotrimeric G proteins. In fact, peptide-1 induces an early and transient activation of the p56-59hck, which is specifically prevented by PTX. It appears therefore that activation of p56-59hck occurs downstream from the heterotrimeric G protein. The tyrosine kinase activation appears to be essential for the chemotactic activity of uPAR since it can be inhibited by inhibitors of tyrosine kinases (Resnati et al., 1996) and since peptide-1 effect can be observed in murine wild-type, but not src<sup>−/−</sup> fibroblasts (B. Degryse, F. Fazioli and F. Blasi, unpublished).

Mice lacking the uPA gene are strongly immunodeficient and succumb to bacterial infections (Gyetko et al., 1996). The mechanism outlined in this chapter is likely to contribute to the role of uPA in cell recruitment. The inability to induce a conformational change and/or to proteolytically cleave uPAR may explain the immunodeficiency of uPA<sup>−/−</sup> mice. If this hypothesis is correct, the uPAR<sup>−/−</sup> mice will have to be immunodeficient as well. In man, uPAR is
an activation antigen in T-lymphocytes and monocytes, and is required for T cell migration, at least \textit{in vitro} (Bianchi et al., 1996; Chavakis et al., 1998; Nykjaer et al., 1994; Wei et al., 1999).

Finally, the results described in this chapter may have a practical application, as it is conceivable that agents acting on uPAR chemotaxis, for example blocking the function of the chemotactic epitope, may be employed to modify inflammatory reactions or cancer invasiveness. These agents would be acting downstream of the primary uPA/uPAR interaction step, and might have properties different from those of uPAR antagonists, expanding the application of agents inhibiting these functions.

\textbf{Contributions}

The experimental work presented in this chapter has been published (Fazioli et al., 1997). My contribution was the construction, expression and purification of recombinant receptors and receptor fragments. Massimo Resnati from our laboratory performed all the cell migration assays as well as the biochemical analysis of phosphorylation induced by peptides and recombinant receptors.
Chapter 5:

RESULTS AND DISCUSSION (III)

Cellular uPAR cleavage and shedding
Summary

In this chapter I present data on the expression, molecular structure, and shedding of uPAR and uPAR fragments in monocytic U937 cells, human leukocytes, human umbilical vein endothelial cells (HUVEC), and transfected 32D cells. I have performed this analysis using a combination of ELISA and immunoprecipitation/western blotting techniques which provides accurate and sensitive quantification of the amount of uPAR antigen as well as indications on the molecular structure of the receptor and receptor fragments, even in dilute biological samples such as conditioned medium.

I find that: 1) Stimulation with phorbol esters up-regulates uPAR expression and promotes uPAR cleavage and shedding. 2) uPAR cleavage is associated with release of the D1 fragment to the culture medium. 3) The pattern of soluble full-length uPAR and D2D3 fragment in conditioned medium corresponds to the cleavage state of uPAR on the cell surface.

While non-stimulated U937 cells predominantly express intact uPAR, monocytes, neutrophils and HUVEC also express high levels of cleaved receptor. Stimulation of neutrophils with the chemotactic peptide fMLP, and wounding of HUVEC monolayers, increases the shedding of uPAR antigen.

Introduction

Cellular uPAR can be released from the plasma membrane generating a soluble form of uPAR (suPAR). It has not been determined whether this receptor 'shedding' occurs following proteolytic cleavage close to the carboxy-terminus, or by the action of phospholipases that might cleave the GPI-anchor. The pro-uPA binding properties of suPAR are similar to that of the GPI-anchored protein (Rønne et al., 1994), but the release from the cell surface strongly alters other biological functions of the receptor. suPAR may partially maintain its capacity to promote pericellular proteolysis as it can concentrate uPA on the cell surface and in the extracellular matrix via its interaction with vitronectin (Chavakis et al., 1998). suPAR is not expected to promote cellular adhesion to vitronectin as the linkage to the cell surface is lost but may still be able to modulate adhesion by its interactions with integrin molecules (Wei et al., 1996; Wei et al., 1994).

Soluble uPAR (suPAR) has been identified in cell culture supernatants and in diverse biological fluids such as tumour ascites, cystic fluids, serum, plasma and recently also in urine (Chavakis et al., 1998; Holst-Hansen et al., 1999; Lau and Kim, 1994; Ninomiya et al., 1997; Pedersen et al., 1993; Ploug et al., 1992a; Ploug et al., 1991; Rønne et al., 1995; Sier et al., 1999; Sier et al., 1998; Stephens et al., 1999; Stephens et al., 1997; Stephens et al., 1997; Wahlgberg et al., 1998; Chapter 6).
Plasma, serum and urine from healthy individuals contain detectable levels of suPAR suggesting that uPAR shedding indeed occurs in healthy individuals. Leukocytes, vascular endothelial and smooth muscle cells are good candidates for the source of circulating suPAR as they are intimately associated with the circulatory system and are known to express uPAR. Indeed, it has been shown that cultured human monocytes, endothelial and smooth muscle cells shed full-length uPAR (Chavakis et al., 1998). While healthy individuals have rather constant levels of suPAR in serum and urine, increased levels have been observed in patients with different types of cancer (Sier et al., 1999; Sier et al., 1998; Stephens et al., 1999; Stephens et al., 1997) and in patients suffering from the rare paroxysmal nocturnal hemoglobinuria syndrome (PNH) (Ninomiya et al., 1997; Rønne et al., 1995). The PNH syndrome is at the molecular level characterised by a biochemical defect in biosynthesis of GPI-anchors, and the increased serum levels of suPAR in these patients has been explained by uPAR release from neutrophils (Ploug et al., 1992b; Rønne et al., 1995). Excess serum suPAR in cancer patients has been suggested to derive from the cancer cells and/or tumour-infiltrating cells as these cells express and release high levels of uPAR (Stephens et al., 1997). Recent experiments using xenografted mice carrying human tumours have indeed demonstrated that the tumour tissue indeed releases uPAR and uPAR fragments to the circulation and urine (Holst-Hansen et al., 1999; Chapter 6).

Cleavage in the linker region between domain 1 and 2 greatly reduces the capacity of uPAR to bind uPA and therefore blocks the receptors function in promoting plasminogen activation (Behrendt et al., 1991; Ploug et al., 1994). Cleavage of uPAR in this region also prevents the high affinity interaction with vitronectin (Høyer-Hansen et al., 1997a) and blocks uPAR mediated cell adhesion to vitronectin (Chapter 3). In this context, uPAR cleavage can be considered a loss of function. However, suPAR fragments generated in the laboratory, have strong chemokine-like activity in vitro (Fazioli et al., 1997), and in this respect the uPAR cleavage can be considered a gain of function event, generating molecules with biological activities not present in intact uPAR.

Cleaved forms of uPAR, lacking D1, are indeed present on the surface of several different cultured human cell lines and in detergent tumour extracts (Holst-Hansen et al., 1999; Høyer-Hansen et al., 1992; Ragno et al., 1998; Solberg et al., 1994; Chapter 6). The natural ligand for uPAR, uPA, is able to cleave its receptor and it has been demonstrated that uPA is responsible for the uPAR cleavage on U937 cells. The cleavage of cellular uPAR by uPA is an efficient process that occurs at physiological concentrations of uPA, while the reaction in solution requires high concentrations of uPA (Høyer-Hansen et al., 1997b; Høyer-Hansen et al., 1992). The cleavage of uPAR by uPA has been suggested to serve as a negative feedback mechanism occurring at sites of high uPA activity and high concentrations of uPAR such as at the leading edge of migrating cells and at areas of tumour invasion (Høyer-Hansen et al., 1997b).
Cellular shedding of uPAR fragment has so far not been described, and selective shedding of full-length uPAR has in fact been suggested (Holst-Hansen et al., 1999). Soluble D2D3 fragments have only been identified in fluid from benign and malignant cysts (Wahlberg et al., 1998). The D1 fragments of uPAR, which is supposedly released from the cell surface upon uPAR cleavage, has also failed to be identified in cell culture supernatants or in any biological fluid.

To understand the biological role of soluble and cleaved uPAR molecules it is of great importance to understand how, where and when they are generated. I have developed a sensitive immunoprecipitation/blotting technique with which I have performed a qualitative analysis of the presence of uPAR and uPAR fragments in dilute biological samples such as conditioned medium and urine. In this chapter I describe the expression, cleavage and shedding of uPAR from a number of different cell lines with special focus on leukocytes and endothelial cells.
Results

U937 cells shed soluble uPAR antigen into the growth medium

To investigate cellular shedding of uPAR I analysed cell lysates and conditioned medium from human myeloid U937 cells cultured for variable periods of time in the presence or absence of the inducer PMA (figure 5.1). While the amount of cellular uPAR in untreated cells was relatively constant during the experiment, PMA induced a strong and persistent increase. The PMA induced increase in cellular uPAR antigen was strongest after 24 hours but remained high for at least 72 hours. Throughout the experiment suPAR antigen accumulated in the conditioned medium indicating a continuous shedding of uPAR from the cells to the medium. The amount of uPAR antigen released from non-treated cells during the first 24 hours of culture (0.4 ng / 10^6 cells / 24 hours) corresponded approximately to a complete turnover of cellular uPAR in 24 hours. The release rate was much higher in PMA stimulated cells (6.1 ng / 10^6 cells / 24 hours) corresponding to a turnover rate of cellular uPAR of approximately 3.5 hours.

Conditioned medium from U937 cells contain full-length suPAR, D2D3 and D1 fragments.

To determine the molecular structure of the uPAR antigen present in lysates and in conditioned medium I performed immunoprecipitation experiments using monoclonal antibodies to uPAR followed by western blotting with a polyclonal anti-uPAR antibody (figure 5.2). In non-stimulated cells a prominent band with a molecular weight around 50-55 kDa representing full-length uPAR could be observed both in cell-lysates and in conditioned media. Stimulation with PMA resulted in a strong increase in the intensity of this band and the appearance of two shorter polypeptides. The intense band with a molecular weight around 35-40 kDa suggests a cleaved receptor composed of domain 2 and 3 (D2D3), and could be observed in both cell lysates and in conditioned media. Long exposure of the blots revealed the presence of a fast migrating band in conditioned medium from PMA stimulated cell with molecular weight around 15 kDa representing the domain 1 fragment (D1). Despite long exposures, the D1 band was never observed in cell lysates in accordance with the model that D1 is released from the cell surface upon receptor cleavage (not shown).

To unequivocally identify the different polypeptides present in lysates and conditioned media I immunoprecipitated conditioned medium from PMA-stimulated U937 cells with a panel of different monoclonal antibodies (figure 5.3). Antibodies recognising domain 3 (mAb R2 and R4) precipitated the two larger fragments in accordance with the identity of these bands being full-length suPAR and D2D3. An antibody against D1 (mAb R3) precipitated full-length uPAR.
and the small -15 kDa fragment but not the D2D3 band, confirming the identity of these peptides to be full-length uPAR and the D1 fragment. Full-length uPAR could furthermore be precipitated with a monoclonal antibody against uPA (mAb c6) demonstrating that the receptor in the conditioned medium, at least partially, is associated with uPA. A control antibody (mAb M2) did not precipitate any of these peptides. The highly heterogeneous glycosylation of uPAR causes a "smeary" appearance of uPAR and uPAR fragments in western blotting preventing a good separation of full-length uPAR and the D2D3 fragment. To eliminate this problem I performed western blotting on immunoprecipitates that had been reduced, denatured, and deglycosylated. Indeed, the migration pattern of uPAR, D2D3 and D1 after deglycosylation was in complete accordance with the molecular weights based on the cDNA derived amino acid sequence of uPAR and a cleavage in the region between domain 1 and 2. Also, the separation of full-length uPAR and D2D3 was under these conditions excellent allowing an accurate identification of the different uPAR fragments.

**Cleaved uPAR is efficiently shed from cells**

The uPAR cleavage event generating the D2D3 fragment present in conditioned medium could occur while uPAR is still anchored to the cell membrane or derive from shed uPAR being cleaved in the medium. To determine whether the D2D3 fragment was generated on the cell surface or in the medium an experiment was set up using U937 cells PMA-stimulated for 72 hour to generate a high level of cleaved uPAR on the cell surface. The cells were acid washed to remove any uPAR bound uPA and plated in fresh medium prior to the experiment and after a one-hour incubation cells and medium were collected and analysed by immunoprecipitation. This experimental protocol should prevent further uPAR cleavage as uPA is removed from the cells and the medium. In fact, I observed a good correlation between the ratio of uPAR to D2D3 in the medium and on the cells suggesting that uPAR and D2D3 are shed at comparable speeds (not shown). Further evidence for shedding of cleaved uPAR comes from 32D cells transfected with a recombinant D2D3 uPAR receptor (Chapter 3) that indeed also shed the protein to the growth medium (figure 5.8).

**Cleavage and shedding of uPAR in leukocytes.**

To characterise the molecular forms of uPAR expressed on, and shed from, leukocytes I cultured freshly isolated monocytes and neutrophils for 48 hours in vitro and analysed cell lysates and conditioned medium for the presence of uPAR fragments by immunoprecipitation (figure 5.4A). Cell lysates and conditioned medium from monocytes contained uPAR polypeptides, which could be readily identified as full-length uPAR and the D2D3 fragment. The level of these
peptides was higher in cells that had been treated with PMA but the ratio of full-length uPAR to D2D3 fragment was not altered significantly. Long exposures of the blots also revealed the presence of the D1 fragment in conditioned medium from PMA stimulated cells monocytes. The D1 fragment was never observed in cell lysates. Cell lysates and conditioned medium from neutrophils contained a uPAR peptide with a migration intermediate between full-length uPAR and D2D3 fragment and therefore not readily identifiable. To obtain a better separation and identification of the uPAR peptides the analysis was repeated using deglycosylated material (figure 5.4B). Surprisingly, the pattern of uPAR peptides in lysates and conditioned medium from neutrophils was characterised by a prominent D2D3 fragment, and only a minor amount of full-length uPAR.

Neutrophils have been shown to up-regulate the cell surface expression of uPAR by the mobilisation of intracellular uPAR stores upon stimulation with fMLP. The reaction is fast (a few minutes) and occurs by the fusion between intracellular granules and the plasma membrane (Plesner et al., 1994). To determine if the process was accompanied by a release of soluble uPAR, I performed ELISA on the medium from neutrophils that had been stimulated with fMLP for 30 minutes (figure 5.5). Indeed, I observed that the medium from neutrophils stimulated with fMLP contained twice as much suPAR antigen as from non-stimulated cells.

Cleavage and shedding of uPAR in HUVEC cells.

HUVEC cells have been shown to express and shed full-length suPAR but no information are available regarding the presence and shedding of uPAR fragments. To address this point I cultured confluent monolayers of HUVEC cells for 72 hours in the presence and absence of PMA. Conditioned medium was collected after 24, 48 and 72 hours and cell lysates prepared at the end of the experiment. All the samples were immunoprecipitated and analysed by western blotting (figure 5.6).

Cell lysates from both treated and untreated cells contained uPAR peptides corresponding to intact uPAR and the D2D3 fragment. Soluble full-length uPAR could be observed after 24 hours in the medium from PMA stimulated cells and after 72 hours in untreated cells. The uPAR peptides in the medium accumulated during culture and the appearance of the D2D3 fragment appeared later as it could first be detected after 48 hours in treated cells. The ratio of uPAR to D2D3 in conditioned medium and cell lysates was comparable after 72 hours of culture. As I did not prepare cell lysates at the 24 and 48 time points, it is possible that the cellular D2D3 fragment accumulated during culture as did the soluble form in the medium. As observed both in U937 cells and monocytes, the D1 fragment could be identified in the conditioned medium from PMA stimulated cells after 72 hours.
PMA stimulation is not very physiological and to determine if the shedding of uPAR could be up-regulated under more relevant conditions I analysed the conditioned medium from HUVEC monolayers that had been 'wounded' using a rubber policeman (figure 5.7). The conditioned medium from wounded monolayers contained slightly more uPAR antigen than control plates, suggesting that wounding results in up-regulation of expression and/or shedding of uPAR. These data should however be taken with care as the experiment was performed only once, as the difference is small, and as several factors such as cell proliferation, cell death etc. may contribute to the increased uPAR release.

Urokinase independent cleavage of uPAR

Cleavage of uPAR on U937 cells is likely to occur through the direct cleavage of uPAR by its ligand uPA (Høyer-Hansen et al., 1997b), but whether other biologically relevant proteases can do the same in other cell lines is still not known. To address this point I analysed the cleavage of uPAR in a cell system in which uPA-mediated uPAR cleavage is unlikely. I used the murine 32D cells transfected with human uPAR (Chapter 3) as these cells do not express uPA (not shown) and as the species barrier between uPA and uPAR binding would prevent the binding of any low levels of murine uPA possibly expressed by these cells (Appella et al., 1987; Estreicher et al., 1989).

Indeed, I observed the presence of an uPAR fragment compatible with D2D3 in both cell lysates and conditioned medium from the uPAR transfected cells suggesting the existence of an uPA independent mechanism of uPAR cleavage (figure 5.8).
Discussion

Serum, plasma and urine from healthy individuals contain measurable levels of suPAR and suPAR fragments suggesting that a constant release and cleavage of uPAR occurs in the healthy organism; however the cellular source of suPAR has not been determined. In accordance with other reports (Chavakis et al., 1998) I find that leukocytes and vascular endothelial cells shed full-length suPAR, and I therefore propose that these cells may, at least partially, is the source of circulating suPAR.

Elevated levels of serum suPAR have been observed in individuals suffering from PNH and in serum and urine from cancer patients. In these patients a deficiency in GPI-anchoring (PNH) or uPAR release from tumour cells (cancer) may explain the increased levels of circulating suPAR. I find a good correlation between the amount of released suPAR antigen and the expression level of uPAR on the cell surface. Any physiological or pathological condition involving the up-regulation of uPAR expression may therefore contribute to the serum suPAR levels. Malignant conditions such as cancer, HIV infection, chronic progressive multiple sclerosis, but also inflammation are associated with up-regulation of uPAR expression in leukocytes (Dore-Duffy et al., 1992; Nykjaer et al., 1994; Speth et al., 1998) and vascular endothelial cells (Graham et al., 1998; Washington et al., 1994). I therefore suggest that these pathologies may also be associated with increased serum/urine levels of suPAR antigen.

The close connection between uPAR and uPA with respect to spatial expression and affinity makes uPA a good candidate for the enzyme responsible for uPAR cleavage and it has indeed been suggested that uPAR cleavage preferentially occurs at sites of high uPA activity (Høyer-Hansen et al., 1997b). However, my data suggest that other enzymes may also be involved in uPAR cleavage. First, uPAR cleavage occurs in transfected cells where uPA is absent and very unlikely to be relevant. Second, a high fraction of cleaved uPAR is present on monocytes and neutrophils, which have been isolated from a tissue (blood), characterised by the presence of protease inhibitors in general. The cleavage of uPAR in neutrophils may involve proteases such as neutrophil elastase which is indeed capable of cleaving suPAR in vitro (Ploug et al., 1991).

Rather than being only a reflection of tissular uPAR expression, circulating suPAR and suPAR fragments may actually have important functions in health and disease. Soluble full-length suPAR, can associate with circulating pro-uPA and the generated complex may have a function in the lysis of plasma clots (Higazi et al., 1998). Fragments of suPAR (generated in the laboratory) are endowed with strong chemokine-like activities in vitro, but the function of these fragments in vivo remains speculative (Fazioli et al., 1997; Resnati et al., 1996). I find that leukocytes and vascular endothelial cells shed cleaved forms of uPAR. However, only full length
suPAR can be detected in plasma/serum (Holst-Hansen et al., 1999; Chapter 6). The shedding of uPAR fragments from leukocytes and vascular endothelial cells taken together with the presence of these fragments in urine (Sier et al., 1999; Chapter 6), suggests that these fragments are cleared to the urine at a rate preventing detection in the circulation. However, the transient presence of these fragments in the circulation is very likely. The expression of the uPAR chemotactic peptide on the surface of vascular endothelial cells may have a function in biological processes such as leukocyte extravasation and/or recruitment to points of endothelial damage. The release of chemotactically active suPAR fragments from activated neutrophils may be involved in the recruitment of monocytes and lymphocytes to sites of inflammation.

Contributions:

Simone Orlando from our laboratory performed the separation and purification of leukocytes. Cornelis F.M. Sier (Called "Kees") did the ELISA determinations. All other experimental work was performed by me.
Chapter 6:

RESULTS AND DISCUSSION (IV)

Soluble and cleaved uPAR in urine
Summary

A soluble form of the receptor (suPAR) is present in blood of healthy people and suPAR levels are increased in plasma/serum of cancer patients. Fragments of uPAR, cleaved between domain 1 and domain 2, are found on the surface of carcinoma cell lines and experimental tumours. Cleaved forms of suPAR, generated in the laboratory, are potent inducers of chemotaxis of cultured cells, but their occurrence and possible function in vivo is presently unknown.

In this chapter I demonstrate the presence of full-size as well as cleaved forms of suPAR in urine of healthy volunteers and ovarian cancer patients: 1) The level of urinary suPAR antigen is significantly higher in cancer patient. 2) Immunoprecipitation / immunoblotting experiments show that in human serum suPAR was mostly full-length, whereas in urine a fragment corresponding to domain 2+3 (D2D3) was the most abundant form. 3) Urine also contained a fragment corresponding to domain 1 of suPAR (D1), a fragment that has never been observed in vivo or in vitro before. 4) Urine from mice xenografted with human ovarian tumor cells also contained high levels of human suPAR antigen, and high levels of D1 were found in mice carrying a human xenografted tumor displaying cleaved uPAR on the cell surface, but not in the urine from mice carrying a human xenografted tumor displaying only full-length uPAR.

Although all 3 forms of suPAR were also found in urine from healthy individuals, the distribution pattern of suPAR fragments was clearly different in ovarian cancer patients, with varying D1/D2D3 ratios in different patients. Control experiments demonstrated that the pattern of urinary suPAR fragments did not change after extensive incubation at 37°C. Moreover, incubation of iodinated recombinant suPAR in urine did not lead to significant fragmentation, indicating that cleavage of suPAR does not primarily take place in the urine. The data suggest that the enhanced presence of suPAR fragments in urine might reflect a cancer specific process, and that their assay might be exploited in clinical investigations of certain types of cancer.
The interaction between urokinase-type plasminogen activator (uPA) and its receptor (uPAR), plays a key role in physiological and pathological processes like cell migration, tissue remodeling and cancer invasion and metastasis (Andreasen et al., 1997; Blasi, 1997). Both uPA and uPAR are made up of discrete domains endowed with specific functions. uPA has a protease domain, and an aminoterminal fragment that specifically binds the receptor. In addition to activating plasminogen, uPA is able to induce uPAR-dependent adhesion, proliferation and chemotaxis (Rabbani et al., 1992; Resnati et al., 1996; Waltz and Chapman, 1994).

uPAR is composed of three homologous domains (figure 6.2A) and is linked to the cell surface at the carboxy terminus by a glycosyl-phosphatidylinositol (GPI) anchor (Ploug et al., 1991). The aminoterminal domain of uPAR (D1) contains the main uPA-binding site (Behrendt et al., 1991), whereas the next two domains of uPAR (D2D3) bind the extracellular matrix protein vitronectin (Wei et al., 1994). However, full-size uPAR is required for the high affinity of both interactions (Høyer-Hansen et al., 1997a; Ploug et al., 1998b). Like uPA, uPAR is accessible to proteolytic cleavage, especially in the D1 to D2D3 linker region which is cleaved by trypsin, plasmin, chymotrypsin and uPA itself (Høyer-Hansen et al., 1992; Ploug et al., 1994). Chymotrypsin-cleaved or recombinant soluble uPAR (suPAR) fragments have strong and specific anti-adhesive and chemotactic activities on cells that do not possess endogenous uPAR (Fazioli et al., 1997; Resnati et al., 1996; Wei et al., 1996).

The importance of proteolytic fragmentation of uPA and uPAR in vivo has not yet been addressed. ATF, the amino terminal fragment of uPA, is found in conditioned media of cancer cells (Rabbani et al., 1992). A variant of uPAR lacking D1 has been identified on several tumour cell lines, in experimental tumours and similar fragments are found in the fluid from human ovarian cysts (Høyer-Hansen et al., 1992; Ragno et al., 1998; Solberg et al., 1994; Wahlberg et al., 1998). However, released soluble D1 has never been identified in any cell culture medium or biological fluid.

In the present paper, we show that the urinary levels of suPAR are enhanced in ovarian cancer patients, in accordance to what has been previously shown for serum (Sier et al., 1998). Moreover, urine from healthy women and ovarian cancer patients contains, in addition to full-size suPAR, also two suPAR fragments representing D2D3 and D1. The distribution pattern of suPAR fragments in urine from ovarian cancer patients is clearly different from that of healthy controls. Control experiments show that suPAR fragmentation does not depend on urine storage conditions, in fact it does not occur in the urine. Experiments in xenografted mice show a correlation between the presence of cleaved uPAR on tumour membranes and the appearance of
D1 in urine, indicating that fragmentation most likely occurs on the cell surface in the tumour tissue.
Chapter 6: Results & Discussion (TV)

Results

Urinary suPAR is increased in ovarian cancer patients

The suPAR/creatinine ratios in urine from 25 ovarian carcinoma patients and 20 healthy controls are presented in figure 6.1. The ratio of suPAR and creatinine was used because it eliminate differences due to dilution and sampling time (Sier et al., 1999). The median level of urinary suPAR/creatinine was significantly enhanced in carcinoma patients compared with age-matched healthy women (P=0.004; Mann-Whitney U-test). A cut-off value of 5.5 (the median value determined in the 20 healthy women plus twice the standard deviation) distinguished 11 cancer patients with low and 14 patients with high urinary suPAR values, resulting in a sensitivity of 56% and a specificity of 95%. In particular, high FIGO stage carcinomas (IIIc, closed circles in figure 6.1) showed elevated values, but also some samples of patients with stage I/II carcinomas were enhanced.

Urine contains full-length suPAR as well as suPAR fragments D2D3 and D1

To determine the nature of suPAR antigen in urine I performed immunoprecipitation analysis on concentrated urine obtained from an ovarian carcinoma patient. I immunoprecipitated the sample with monoclonal antibodies recognising epitopes on domain 1 (mAb R3) or domain 3 (mAb R2), and analysed the precipitated material by western blotting using a polyclonal anti-uPAR antibody (figure 6.2B). Three different polypeptides were identified; a ~55 kDa band immunoprecipitated by both mAb R2 and mAb R3, representing the full size receptor; a ~35 kDa band immunoprecipitated only by mAb R2 and hence corresponding to suPAR fragment D2D3; and finally, a ~17 kDa band identified only by mAb R3 and therefore compatible with fragment D1. Another set of monoclonal antibodies having similar recognition specificity (mAb R4 and mAb R5, respectively) displayed an identical pattern, confirming the identity of these fragments (data not shown). Serum from ovarian cancer patients and healthy controls contained only full-length suPAR, as no material with a molecular weight below ~50 kDa could be identified (figure 6.3C and not shown).

The mAb R3 precipitated less full-length suPAR than mAb R2. This might be due to the different efficiency of the two antibodies; however, it might also indicate that urinary suPAR is partially associated with other molecule(s) able to inhibit the binding of mAb R3 (but not mAb R2) to the receptor. Indeed, mAb R3 has been shown to compete with the natural ligand of uPAR, pro-uPA (List et al., 1999; Rønne et al., 1991).
suPAR fragmentation is different in ovarian cancer patients

Figure 6.3 shows an immunoprecipitation/immunoblotting experiment carried out on urine from 7 healthy women (figure 6.3A) and 7 ovarian carcinoma patients (figure 6.3B). The pattern of distribution of suPAR fragments in control urine's was very consistent. The major urinary form of suPAR appeared to be the D2D3 fragment that was twice or more as abundant as full-size suPAR. In these non-concentrated urine's the D1 fragment could be clearly detected in three samples (C1, C4 and C7). The suPAR fragment pattern in ovarian cancer patients, however, was clearly different (figure 6.3B). Some patients showed distribution patterns similar to normal urines (P1, P3, P5, and P6), whereas others had relatively high amounts of D1 (P2, P4, and P7). Urine from patient P7 contained high amounts of all forms, but D1 was most abundant (5 times more than D2D3, estimated from a short time exposure of the blot, data not shown). In contrast to urine, no fragments of suPAR were identified in serum from the same ovarian carcinoma patients (figure 6.3C). Even immunoprecipitation of five times more serum did not reveal any cleaved suPAR fragments (not shown).

suPAR and suPAR fragments are stable in urine.

The difference between the pattern of uPAR fragments in urine (full length, D2D3 and D1) and in serum (only full length) prompted me to investigate whether the urinary suPAR fragments were actually generated in the urine by proteolytic degradation of full-length suPAR. First, I analysed whether the pattern of suPAR fragments in freshly sampled urine was stable when stored for 24 hours at different temperatures (-20°C, 4°C, 20°C and 37°C) or when subjected to repeated freeze/thaw cycles in the presence and absence of protease inhibitors. The pattern of suPAR fragments on immunoblots was found to be largely unaffected by the storage temperature and by the presence or absence of protease inhibitors (not shown). These findings indicate that the suPAR fragmentation pattern was stable in urine independently of storage conditions. To further examine suPAR stability in urine, we added full-length 125I-labelled recombinant suPAR at physiological concentration (2 ng/ml) to urine from seven different ovarian cancer patients. The samples were incubated for 24 hours at 37°C and then analysed by SDS-PAGE. Some degradation of full-size suPAR to the D2D3 and D1 fragments was observed in the urine from one patient (approximately 20%), but not in any of the other samples (not shown). The absence of significant cleavage in those samples was not a consequence of a resistance of the used 125I-labelled suPAR to cleavage, because chymotrypsin and uPA could efficiently cleave this particular suPAR when incubated in buffered saline under the same conditions. Both proteases had no or very little effect when incubated in urine (data not shown). These experiments suggest that suPAR fragmentation most likely does not occur in urine.
Urinary suPAR antigen is partially derived from distant tumour tissue

The increased urinary suPAR levels in cancer patients might derive from malignant cells or tumour infiltrating stromal cells. It is difficult to demonstrate this possibility in humans since it is impossible to discriminate tumour-derived suPAR from non-tumour derived suPAR. We therefore looked for the presence of human suPAR antigen in urine from nude mice xenografted with human carcinomas. The ELISA used for human urine could not be used in this analysis because of the interference with murine antibodies present in mouse urine. We therefore used a recently developed ELISA (Holst-Hansen et al., 1999) which is highly specific for human uPAR and suitable for analysis of murine samples (see materials and methods). We analysed urine samples obtained from mice xenografted with the ovarian carcinoma HOC8 (Giavazzi et al., 1998) and the metastatic breast carcinoma MDA435 (Cailleau et al., 1974). Indeed, we found that pooled urine from HOC8 or MDA435 xenografted mice contained soluble human uPAR (shuPAR) antigen (figure 6.4), whereas no shuPAR was detected in urine from non-xenografted mice. The rather low level of shuPAR antigen in the pooled urine from HOC8 xenografted mice (0.23 ng/ml) was confirmed in a second experiment (not shown) with urine samples from individual xenografted mice (n=5) and control mice (n=6) and the difference was highly significant (P=0.009, Mann-Whitney U-test). This result strongly suggests that the enhanced urinary suPAR levels from cancer patients at least partially could derive from tumour cells.

Urinary suPAR fragments might reflect the cleavage-state of uPAR on tumour tissue in xenografted mice.

Urinary fragmented suPAR may reflect the presence of cleaved uPAR on tumour cells. To examine this possibility we compared uPAR in tumour lysates, blood samples, and urine from the HOC8 and MDA435 xenografts by immunoabsorption/immunoblotting (figure 6.5).

While the HOC8 tumour lysate only contained full-length uPAR, the MDA435 tumour lysate also contained cleaved receptor (D2D3). Both tumours release the full-length receptor since it was detected in the circulation. In both tumours circulating suPAR apparently undergoes proteolytic cleavage during translocation from blood to urine since a prominent D2D3 fragment, but no full-length suPAR, was identified in urine from the animals. However, in addition to the D2D3 fragment, a very abundant D1 fragment was observed in urine from mice bearing the MDA435, but not the HOC8 tumour. From shorter exposures the intensity of the D1 band present in urine from MDA435 xenografts was estimated to be approximately ten fold more intense than the D2D3 fragment (not shown). In HOC8 xenografts, the D2D3 fragment in urine was less abundant than in MDA435. No D1 fragment was detected, even after very long exposures (not shown). The difference between the urinary D1 levels of both tumour models correlates with the
presence of cleaved uPAR on the tumour cells and indicates that urinary D1 could be a reflection of suPAR cleavage on tumour cells.
Discussion

Components of the plasminogen activation system are found in enhanced amounts in various types of tumours (Andreasen et al., 1997). High tissue and serum levels of uPA, uPAR, and inhibitor PAI-1 are associated with bad prognosis in human cancers (Casslèn et al., 1994; Chambers et al., 1998; Mustjoki et al., 1999; Pedersen et al., 1994; Schmitt et al., 1997; Sier et al., 1998; Verspaget et al., 1995). Since we previously showed that urinary and serum suPAR levels are strongly correlated (Sier et al., 1999), we hypothesised that enhanced urinary suPAR levels might also be associated with malignancy. The present study shows that approximately 50-60% of the patients with ovarian carcinoma display an increased urinary concentrations of suPAR, comparable to what was found previously in serum and tissue from ovarian carcinoma patients (Casslèn et al., 1991; Schmalfeldt et al., 1995; Sier et al., 1998; Young et al., 1994).

The source of suPAR in serum or urine from healthy individuals has not been identified yet. Enhanced levels of circulating suPAR might reflect an increase in tissue uPAR, as for instance found in many tumour types. Indeed, plasma levels of human suPAR correlate with tumour burden in nude mice carrying ovarian and breast cancer xenografts (our unpublished data and Holst-Hansen et al., 1999).

We never observed any fragmented suPAR in serum/plasma of healthy volunteers, nor in patients with various types of cancer, nor in serum from mice xenografted with human tumours. In the last case, urinary suPAR must derive from tumour cells as these represents the only human component in the xenografts. Assuming that in cancer patients the enhanced serum suPAR derives from the surface of tumour and/or stromal cells, the absence of fragments implies that either suPAR is not fragmented in the tissue (which is at least not the case in some mouse xenografts) or that the fragments does not enter the circulation. More likely, however, since uPAR uPAR fragments are indeed released from the tissue, their half-life in the circulation maybe too short to generate a detectable steady-state level.

After entering the bloodstream, suPAR may bind circulating molecules that could potentially modify its clearance. Two likely serum ligands for suPAR are pro-uPA and vitronectin. Association of suPAR with these molecules would bring the molecular weight of the complex well above the limit for simple diffusion into the urinary compartment. The uPAR fragments are unable to associate with physiological concentrations of uPA and vitronectin (Høyer-Hansen et al., 1997a; Ploug et al., 1994) or with any other presently known serum protein, and their half-life is therefore not expected to be modulated by these interactions.

In addition to tissue cleavage, one cannot forget that both uPA and uPAR are present in the human kidney. In particular uPA is found ubiquitously and abundantly in all segments of the
tubular epithelium, where it is thought to be essential for the prevention of fibrin clot formation in kidney and urine (Wagner et al., 1996). Therefore, uPA, or other proteases present in the kidney like kallikrein or meprin A (Chen et al., 1995; Walker et al., 1998), might be responsible for the cleavage of full-size suPAR and hence for the presence of suPAR fragments in the urine. Our immunoblot data in urine from healthy volunteers clearly show that in fact most of the suPAR is present as D2D3 fragment. The striking difference with cancer patient's urine is, that while in healthy volunteers the ratio between full-size suPAR and D2D3 is rather stable, this is not the case in urine from cancer patients. Therefore, changes in the urinary suPAR fragment pattern might reflect the cleavage of uPAR in distant parts of the organism. Mice xenografted with human tumours release full-size receptor in the blood, and present suPAR fragments in the urine. The urinary level of the D1 fragment correlates with the extent of surface uPAR cleavage in the xenografted tumour tissue. This result argues for a similar phenomenon in human cancer patients.

Compared to the very regular pattern of suPAR fragments in urine from healthy volunteers, some patients showed relatively high urinary D1 levels. It will therefore be relevant to evaluate the clinical usefulness of this observation. So far, the only easily obtainable human body fluid in which suPAR fragment analysis is possible is urine.

Recently, a number of cleaved extracellular matrix and cell surface proteins have been discovered, and the presence of some of these fragments has been associated with cancer (O'Reilly et al., 1994; Taraboletti et al., 1997). Some of these fragmented proteins have biological or pharmacological activity, like angiostatin. Also cleaved forms of suPAR have biological activity, at least in vitro. First, suPAR cleavage results in fragments possessing a very strong chemotactic activity, provided they contain at the carboxy-terminus (D1) or at the amino-terminus (D2D3) the specific chemotactic epitope of uPAR that lies in the linker region between D1 and D2D3 (Fazioli et al., 1997). Second, D2D3 can affect the interaction of uPAR with integrins, thus modifying cell adhesion (Wei et al., 1996). Third, in addition to gaining chemotactic functions, suPAR fragments lose the properties of the full-size molecule, i.e. the binding to uPA and vitronectin.

In conclusion, the enhanced presence of cleaved fragments of suPAR in urine of cancer patients might provide clinically relevant information about the actual tumour and should therefore be studied in larger groups of patients with various types of cancer. The presence of suPAR fragments in human urine suggests that suPAR release and fragmentation may be biologically relevant processes. In particular, the adherence/chemotactic activities (Fazioli et al., 1997; Wei et al., 1996) of these fragments may influence cell migration and hence the increase in fragments may be related to the degree of aggressiveness and invasiveness of a tumour. This hypothesis still needs to be proven.
Contributions:

Thomas L. Frandsen from the Finsen Laboratory in Copenhagen, Denmark, did the xenograft experiments with the MDA435 tumour. Ines Nicoletti from the Mario Negri laboratory in Bergamo, Italy, did the xenograft experiments with the HOC8 tumour. Andrea Mariani, Giovanni Aletti, Luigi Frigero, Augusto Ferrari and Vittorio Agape from Hospital San Raffaele in Milan, Italy, collected the urine samples and measured the creatinine content. I performed all other experiments and co-ordinated the study.
Chapter 7:

FINAL DISCUSSION
Final discussion

In this thesis I have sought to define the occurrence and biological significance of uPAR cleavage and shedding. The data clearly indicate that uPAR and uPAR fragments are present notably *in vitro*, but also *in vivo* under physiological and pathological conditions. The generation of soluble uPAR and soluble uPAR fragments under natural conditions gives rise to several questions: First, what are the underlying mechanisms? Second, what is the molecular structure. Third, what is the biological activity of these molecules, not only with respect to uPAR binding functions, but especially as integrin-modulators and as chemotactic factors. Finally, how about the measurement of uPAR or its fragments in the clinic for diagnostic or prognostic purposes.

In this last chapter I discuss the possible biological relevance of the data presented in this thesis. I will furthermore give my suggestions for possible experiments that may answer the questions raised by the obtained results.

Mechanism of uPAR shedding

The shedding of uPAR from the cell surface occurs in many different normal and transformed cell lines (Chavakis et al., 1998; Lau and Kim, 1994; Ploug et al., 1991; Chapter 5). No cells have so far been described which express uPAR but do not shed it to the medium. It thus appears that the process of uPAR shedding is an ubiquitous process, which occurs in all cells.

It is possible that uPAR shedding simply reflects uPAR which "escapes" GPI-anchoring. This may in particular occur under conditions of high uPAR expression where the GPI-anchoring machinery of the cell could be saturated. Two lines off evidence suggest that the absence of GPI-anchoring is not the major reason for suPAR production. First, the drug Brefeldin A which blocks the intracellular translocation of secretory proteins between the endoplasmic reticulum and the golgi apparatus (Misumi et al., 1986) does not affect the cellular release of suPAR (Wilhelm et al., 1999). Second, the D2D3 fragment, which is generated on the surface of U937 cells, is also shed (Chapter 5).

Bacterial phospholipase C (PIPL-C) is capable of releasing uPAR from the cell surface, but a similar enzymatic activity has not been described in humans. Purified preparations of the related mammalian enzymes phospholipase D and phospholipase A2 (PIPL-D and PLA-2) fail to catalyse the process (Ploug et al., 1991). Serum contains high levels of PIPL-D but the enzyme requires the presence of detergent in order to hydrolyse GPI-anchors (Low and Huang, 1991). Recently it has been suggested that a cellular form of PIPL-D may be the enzyme which catalyses the release of uPAR (Wilhelm et al., 1999). Cellular PIPL-D co-localises with caveolin suggesting that uPAR shedding could preferentially occur when both uPAR and PIPL-D are associated with caveolin. This would in fact connect the phenomenon of uPAR shedding to the
process of cell adhesion and migration. The fact that 1,10-phenanthroline inhibits uPAR shedding (Wilhelm et al., 1999) also suggests that MMPs may be involved in the process. Although highly speculative, one could imagine a scenario in which MMPs activate serum or cellular PIPL-D which then hydrolys the GPI-anchor of uPAR.

**Mechanism of uPAR cleavage**

Cleavage of uPAR occurs in many different cell types and tumour tissues (Holst-Hansen et al., 1999; Høyer-Hansen et al., 1992; Ragno et al., 1998; Solberg et al., 1994; Chapter 6).

uPA is able to cleave suPAR in solution but the reaction is slow and requires high concentrations of uPA (Høyer-Hansen et al., 1992). The reaction does not depend on binding of uPA to suPAR, as the cleavage is equally well performed by low molecular weight uPA which lack the uPAR binding domain. uPA catalysed uPAR cleavage is more than 10 fold accelerated on the surface of cells (Høyer-Hansen et al., 1997b). The cell-surface acceleration requires the binding of uPA to uPAR as the interaction antagonist ATF inhibits the reaction almost completely. These data clearly demonstrate that uPA is able to cleave uPAR, but that it does not preferentially cleave the receptor to which it may be bound. Taken together a model emerges in which the cell surface acceleration of uPAR cleavage is caused by the concentration of uPA on the cell surface, and that uPAR bound uPA cleaves flanking uPAR molecules (Høyer-Hansen et al., 1997b). One experiment aimed at validating this mechanism could be the "labelling" of cell surface uPAR by incubation with sub-saturating concentrations of $^{125}$I-labelled ATF, followed by immobilisation using a chemical cross-linker. A subsequent release of D1/ATF complexes upon incubation with saturating concentration of uPA would provide evidence for lateral cleavage, as the binding of uPA and ATF to uPAR is mutually exclusive.

From several points of view, uPA is a likely candidate for the enzyme cleaving uPAR. However, it is not likely to be the only mechanism, because cleavage occurs in cell systems where uPA is unlikely to be involved. Cleavage of uPAR occurs in murine 32D cells transfected with human uPAR. The absence of uPA expression by these cells and the species barrier for binding between murine uPA and human uPAR almost completely excludes any role for uPA in the cleavage of uPAR in this cell system (Chapter 5). No information about proteases expressed by 32D cells is available and the first hint to the identity of the responsible enzyme may come from the determination of the uPAR cleavage site.

uPA is also not likely to be responsible for all of the uPAR cleavage observed on monocytes and neutrophils (Chapter 5). The blood is a neutralising environment for proteases as it contains high levels of general protease inhibitors such as $\alpha_2$-antiplasmin and $\alpha_2$-macroglobulin. Resting human monocytes have high levels (~50%) of cleaved uPAR on the
surface in contrast to cultured U937 cells. Monocytes may express low levels of pro-uPA but as serum contains PAI-1, the effective concentration of active uPA on the surface of resting monocytes is expected to be very low. A similar situation is true for neutrophils. Approximately 80% of uPAR on neutrophils is cleaved (Chapter 5) and of the remaining 20% only one tenth is occupied by uPA or pro-uPA (Plesner et al., 1994). Neutrophils have intracellular granules that can be mobilised to the plasma membrane upon different kinds of stimuli. These granules contain different types of pro-inflammatory molecules and a number of proteases including pro-uPA/uPA and neutrophil elastase. These granules also contain uPAR (Plesner et al., 1994). uPAR is distributed equally between the easily mobilised granules (characterised by their mobilisation by fMLP), and in the specific and primary granules which require a stronger stimulation (in experimental conditions TNFα or PMA are used). In contrast to the distribution of uPAR, pro-uPA/uPA is mostly confined to the specific and primary granules (Plesner et al., 1994). It is thus possible that uPAR cleavage in neutrophils may take place before the receptor arrives at the cell surface. In fact, it is possible that uPAR is also "shed" prior to release of the granules as suggested by the rapid accumulation of suPAR antigen in the conditioned medium from fMLP stimulated neutrophils (Chapter 5).

Data not presented in this thesis show that PMA-induced uPAR cleavage in endothelial cells (HUVEC) is almost completely inhibited by the broad-range MMP inhibitor batimastat. The involvement of MMPs in uPAR cleavage is not easy to explain. All the MMPs tested fail to cleave suPAR efficiently in vitro. However, the absence of efficient cleavage in vitro does not exclude that the reaction might be favoured on the cell surface (as is true for several processes described in this thesis). As the linker region in uPAR does not contain any obvious MMP cleavage sites, it is likely that the mechanism is indirect and involves the activation of other proteases. Anti-catalytic uPA antibodies and the plasmin inhibitor trasylol should be tested to establish whether uPA and plasmin are the enzymes actually responsible for the cleavage. Further experiments aimed at identifying the responsible MMP(s) should be initiated by the use of more specific inhibitors.

The uPAR chemotactic epitope

The data presented in chapter 4 strongly suggest that the position of the cleavage site in the linker region determines whether the chemotactic SRSRY epitope (amino acid 88-92) will be located on the D1 or D2D3 fragments, or whether it will be destroyed by the cleavage.

Two relevant proteases in uPAR cleavage appear to be uPA and plasmin. These two proteases are intimately connected to uPAR as their active forms are generated in a process which occurs in the molecular vicinity of uPAR. Trypsin, plasmin and uPA cleave uPAR at the same
two positions in the linker region: Arg83/Ala84 and Arg89/Ser90. Cleavage at Arg83/Ala84 results in the localisation of the chemotactic epitope to the generated D2D3 fragment while cleavage at Arg89/Ser90, localised in the centre of the chemotactic epitope, is expected to generate D1 and D2D3 fragments, which are both devoid of chemotactic activity. Cleavage of uPAR catalysed by uPA on the surface of U937 cells occurs equally well at both sites, whereas there is a weak preference for the Arg83/Ala84 site when the cleavage occurs in solution (Høyer-Hansen et al., 1997b). Thus, cleavage of uPAR by uPA has the potential to generate a chemotactically active D2D3 fragment, two fragments devoid of activity, but never an active D1 fragment. In fact, no protease cleavage sites have been identified carboxyterminal to the chemotactic epitope and the possible existence \textit{in vivo} of D1 fragments carrying the SRSRY motif is therefore speculative. However, a recombinant D1 fragment carrying the SRSRY motif is chemotactically active, demonstrating that the epitope works also in this position (Fazioli et al., 1997). The cleavage sites for chymotrypsin and human neutrophil elastase (NE) are both located aminoterminal to the chemotactic epitope and therefore generate D2D3 fragments expected to be chemotactically active. In fact, the aminoterminal border of the chemotactic epitope is defined based on the chemotactic activity of a D2D3 fragment generated by chymotrypsin cleavage (Fazioli et al., 1997).

So far, only chymotryptic uPAR fragments and various recombinant proteins and peptides have been tested for their chemotactic activity (Fazioli et al., 1997; Resnati et al., 1996). To confirm the data obtained using these physiologically not relevant molecules, experiments testing the chemotactic activity of uPAR fragments generated by uPA, plasmin, and neutrophil elastase cleavage of uPAR should be performed. This analysis is however complicated by the fact that uPA and plasmin posses two cleavage sites in the linker region. One solution to this problem is the production of recombinant suPAR in which one of the cleavage sites has been destroyed by site-directed mutagenesis. In particular the destruction of the Arg83/Ala84 cleavage site would be informative, as this would force uPA and plasmin cleavage to the Arg89/Ser90 site located in the chemotactic epitope. The chemotactic activity of uPA or plasmin generated fragments of this mutant suPAR, compared with that of normal suPAR cleaved with the same enzyme, should determine the respective contributions of the two cleavage sites to the chemotactic activity.

Further experiments are needed to establish more precisely the structural requirements for the uPAR chemotactic epitope. By analogy with the experiments presented in chapter 4 this may be performed using synthetic peptides or recombinant uPAR fragments in which the desired mutations have been engineered.
Chapter 7: Final discussion

The structure and biological activity of suPAR fragments found in vivo

The evaluation of uPAR fragments by immunoprecipitation and western blotting allow for the determination of the domain structure of suPAR fragments, but is not informative with respect to the exact cleavage point in the linker region. In chapter 5 and 6 I showed that uPAR fragments are indeed produced by endothelial cells, leukocytes and tumours, and that these fragments can be found in urine. Determination of the molecular structure and biological activity of these fragments is of high priority, as this may yield information with respect to enzymes responsible for the cleavage and the possible biological function of these fragments. The most interesting sources of these fragments for further characterisation are conditioned medium from freshly isolated leukocytes and urine.

The determination of the chemotactic activity is an attractive starting point as the fragments are expected to be active at sub-nanomolar concentrations (Fazioli et al., 1997; Resnati et al., 1996). Experimentally, the different uPAR fragments could be affinity purified using domain specific monoclonal antibodies such as R2 and R3, and the captured material could be analysed by chemotaxis experiments using responsive cells such as THP-1 or smooth muscle cells. Confirmation of the chemotactic activity of these fragments would strengthen the hypothesis that these fragments play a functional role in vivo.

Determination of the molecular structure of the uPAR fragments also requires purification of the fragments. The method of purification would be the same as for the determination of the biological activity, but requires larger amounts of starting material. To obtain enough urinary D2D3 fragment to perform aminoterminal sequencing, purification from 1-2 litres of urine is required. Human urine is easy to obtain in large quantities and the purification should therefore be rather straightforward. Purification of the D2D3 fragment from conditioned medium from monocytes and neutrophils is complicated by the limited number of cells available, but should also be possible with some patience. Urine from xenografted mice carrying the human MDA435 tumour contains as much as 50-100 ng/ml of the human D1 fragment and is therefore a perfect source of tumour-derived uPAR fragments. The cleavage point of uPAR cannot be determined by aminoterminal sequencing of the D1 fragment, and the analysis should in this case be performed by mass spectroscopy. In fact, mass spectroscopic techniques are becoming so sensitive that both the amino- and carboxyterminal termini may be determine on sub-microgram quantities of purified uPAR fragments.

Neither aminoterminal sequencing, nor mass spectroscopy are suitable techniques for the qualitative analysis of uPAR fragmentation in large numbers of samples or in situations where the amount of sample is limited. The generation of monoclonal antibodies specific for cleaved uPAR will therefore be crucial for the analysis of uPAR cleavage in this type of samples. These
antibodies should recognise epitopes within the linker region but fail to recognise the same epitopes in intact and ligand-associated uPAR.

Identification of the adapter molecule

The fact that suPAR fragments have chemotactic effects on several different cell types strongly suggests the existence of cell surface molecules capable of binding the fragments and transmitting a signal over the cell surface (Fazioli et al., 1997; Resnati et al., 1996). In spite of extensive experimental work in our laboratory this "adapter" molecule has not yet been identified.

The intracellular signalling induced by cleaved suPAR involve the function of G proteins as it is inhibited by Pertussis toxin (Degryse et al., 1999; Fazioli et al., 1997). G-proteins are involved in signalling by chemokine receptors from the seven-transmembrane family. Chemokine receptors are therefore candidate molecules for the uPAR adapter.

uPAR can directly interact with and modulate the function of integrin receptors (Wei et al., 1996; Wei et al., 1999). uPAR can modulate the signalling by β₁-integrins in HEK293 cells in a process which involve the membrane protein caveolin (Wei et al., 1999). Both uPAR and β₁-integrins mediated signal transduction involves scr family kinases (Degryse et al., 1999; Resnati et al., 1996; Wei et al., 1999). Also integrin receptors and caveolin are therefore good candidates for the adapter molecule.

The chemotactic effect of cleaved suPAR is maximal at picomolar levels, suggesting that the cellular binding site has a high affinity for the uPAR chemotactic epitope. However, biochemical methods such as co-immunoprecipitation, cross-linking and in vitro binding assays, have so far failed to consistently identify any of the mentioned molecules as the adapter molecule able to bind the chemotactic uPAR epitope.

Identification of the uPAR adapter molecule may be obtained using biological assays in which the introduction of candidate adapter proteins (or an expression library!) in an appropriate cell system, generates the desired and measurable signal upon incubation with the chemotactic epitope. This approach has so far been hampered by the fact that the uPAR chemotactic epitope seems to induce signalling in most cells (Degryse et al., 1999; Fazioli et al., 1997).

The biological function of uPAR shedding and cleavage

The biological roles of suPAR and suPAR fragments have not been determined. Cleavage and shedding of uPAR irreversibly destroys the well-defined functions of uPAR in plasminogen activation and cell adhesion. In this respect, suPAR and suPAR fragments may be considered inert metabolites. However, the strong biological activity of suPAR fragments in vitro and the
occurrence of these fragments *in vivo* strongly suggest a biological function. suPAR fragments are apparently devoid of affinity for uPA and vitronectin and their possible function *in vivo* is thus expected to be related to their chemotactic activity.

Neutrophils are the first cells to arrive at sites of microbial infection and tissue damage. By the release of pro-inflammatory molecules, present in intracellular granules, neutrophils are thought to promote the concomitant recruitment of monocytes and lymphocytes. Most of uPAR in neutrophils is cleaved and fMLP stimulation caused a rapid release of some of this suPAR material (Chapter 5). It is thus possible that the release of chemotactically active suPAR fragments contributes to the recruitment of inflammatory cells.

A similar situation may be true in cancer. Proteolysis is particularly active at the invasive front of tumours and this process may generate chemotactic suPAR fragments. The presence of these fragments at the invasive front may promote the directed migration of tumour cells towards zones of proteolysis. Release of chemotactic suPAR fragments from tumour cells may recruit and activate stromal cells to produce proteases sustaining the process of invasion. Also endothelial cells may be recruited promoting the process of tumour vascularisation.

Many different experiments aimed at addressing these possibilities could be performed. Subcutaneous implantation of beads, slowly releasing defined suPAR fragments, may provide information about the recruitment of inflammatory cells. Migration of cells into an artificial ECM containing suPAR fragments may provide information about the effects on tumour invasion, stromal cell recruitment and angiogenesis.

**Integrin associations with cleaved uPAR**

Physical and functional interactions between uPAR and integrin receptors have been extensively documented in the literature. uPAR is required for neutrophil migration (Gyetko et al., 1994) and is found dynamically associated with Mac-1 and CR4 depending on the activation state of the cells (Kindzelskii et al., 1997). Approximately 80% of uPAR in neutrophils is cleaved (Chapter 5). Taken together these data raise a number of questions. Does cleaved uPAR associate with integrins? If yes, is the association between integrins and cleaved uPAR an inhibitory or stimulatory interaction?

Non-catalytic uPAR ligands inhibit the adhesion of neutrophils to vascular endothelium (May et al., 1998) suggesting that the ligand-free conformation of uPAR, which is interestingly the non-chemotactic conformation, promotes adhesion. In the same system suPAR, but not soluble D2D3 which is the chemotactically active configuration of cleaved suPAR, restores the adhesion of PIPL-C treated neutrophils to the same endothelium.
Antibodies specifically recognising cleaved uPAR would much facilitate the experimental approach to the analysis of interactions between integrins receptors and cleaved uPAR. Until these antibodies will be available the immunoprecipitation/western blotting system described in chapter 5 may provide the desired information. Any co-immunoprecipitation of Mac-1 or CR-4 with cleaved uPAR would suggest a functional role, inhibitory or activating, of cleaved uPAR in neutrophils. A preferential co-immunoprecipitation between cleaved uPAR and Mac-1 in migrating (fMLP stimulated) neutrophils would suggest an inhibitory function of cleaved uPAR.

**uPAR, cell adhesion and cancer metastasis**

High expression of uPAR in tumour tissue is associated with short survival in different types of cancer. Two critical points in tumour metastasis are cancer cell dissemination and extravasation. Dissemination is the process in which metastatic cancer cells dissociate from the primary tumour and enter the vasculature. Extravasation is the process in which circulating tumour cells leave the vasculature to form a metastasis.

High levels of intact uPAR may inhibit the capacity of cancer cells to disseminate by causing firm cell adhesion to vitronectin in the stromal tissue. However, uPAR mediated effects on cancer cell adhesion may also favour cell migration and thus dissemination. Again, this effect would be inverted by uPAR cleavage. Addressing these possibilities experimentally is not easy as it requires the controlled expression and cleavage of uPAR in suitable experimental tumour systems. However, indirect indications of the involvement of uPAR in the process of dissemination may come from the analysis of the prognostic value of uPAR cleavage in cancer patients.

uPAR expressed by circulating tumour cells may promote adhesion to the vascular endothelium and may therefore facilitate the process of extravasation. On the other hand, strong uPAR mediated cell adhesion to the vascular endothelium may prevent the spreading of the cells in the organism. The effect of uPAR expression on tumour cell extravasation could be addressed experimentally. Tumour cells may be selected, or engineered *in vitro*, to express high levels of uPAR and the extravasation of the cells may then be followed after intravenous injection in mice.

**Metabolism of suPAR and suPAR fragments**

Blood and urine from healthy persons contain measurable levels of suPAR antigen (Sier et al., 1999; Sier et al., 1998; Stephens et al., 1997; Chapter 6). While suPAR found in serum is all full-length (Holst-Hansen et al., 1999; Chapter 6), urine also contains the suPAR fragments D1 and D2D3 (Sier et al., 1999; Chapter 6). The concentrations of suPAR antigen in serum and
in urine are highly correlated suggesting that urinary suPAR antigen derives from the circulation (Sier et al., 1999).

Cells associated with the vasculature produce suPAR and suPAR fragments, suggesting that these cells, at least partially, are the source of circulating and urinary suPAR (Chavakis et al., 1998; Chapter 5). suPAR fragments present in urine may derive from the degradation of full-length suPAR in the kidney and urine, or from the cellular release of these fragments in distant parts of the organism, followed by clearance to the urine. Experiments with xenografted mice demonstrate that a D1 fragment released by a distant tumour tissue ends up in the urine without causing detectable D1 in the circulation (Chapter 6). In contrast, release of full-length suPAR from tumour tissue in xenografted mice causes the appearance of full-length suPAR in the circulation and D2D3 fragment in urine, demonstrating that suPAR may be degraded to the D2D3 fragment upon entry into the urinary compartment. However, degradation of full-length suPAR in human urine is very limited, suggesting that urinary suPAR fragments predominantly derive from the cellular release of these fragments (Chapter 6).

The failure to detect the D1 and D2D3 fragments in the circulation is most likely caused by a rapid clearance of these proteins, and experiments aimed at confirming the transient presence of these fragments in the circulation could be performed. Indirect evidence may be obtained by measuring the serum half-life, and urine accumulation, of human recombinant suPAR and suPAR fragments injected into mice. A large difference in the half-life between full-length suPAR and of the fragments would support the hypothesis. Another approach involves the use of specific monoclonal antibodies to prolong the half-life of suPAR fragments in the circulation. Antibodies have a rather long half-life in the circulation (in the order of days) and may therefore also slow down the clearance of their ligands. Injection of uPAR specific antibodies into tumour bearing mice followed by immunoprecipitation and western blot analysis on serum samples, obtained after a given time interval after injection, might therefore prove the presence of the fragments in the circulation.

**Serum and urinary suPAR as prognostic markers**

The expression level of uPAR in tumour tissue is a prognostic marker in several different tumour types, including squamous cell lung cancer, breast cancer and colon cancer (Ganesh et al., 1996; Grøndahl-Hanssen et al., 1995; Pedersen et al., 1994). Patients suffering from ovarian, colon and breast cancer furthermore have elevated levels of suPAR antigen in serum and urine, and high concentrations may be associated with a bad prognosis (Sier et al., 1999; Sier et al., 1998; Stephens et al., 1999; Stephens et al., 1997; Chapter 6).
The correlation between elevated levels of circulating suPAR and cancer strongly suggests that the tumour tissue is the source of the excess suPAR present in the circulation. Evidence supporting this notion comes from nude mice xenografted with human tumours where the presence of the tumour causes appearance of human suPAR antigen in the blood and urine (Holst-Hansen et al., 1999; Chapter 6). The level of human suPAR in plasma from xenografted mice is strongly correlated with the tumour size and is therefore (at least in mice) a measure of tumour mass (Holst-Hansen et al., 1999). The fact that a large tumour size is associated with poor survival may thus partially explain the prognostic value of serum suPAR. Cleavage of uPAR has been suggested to occur preferentially at sites of high uPA activity and in particular at sites of tumour invasion (Høyer-Hansen et al., 1997b; Solberg et al., 1994). uPAR cleavage may therefore be an indicator of tumour invasiveness. Tumour invasiveness is intrinsically connected to metastasis, which is the major cause of death in cancer patients, and specific markers for tumour invasiveness are thus expected to be highly prognostic.

Even though the potential prognostic value of uPAR cleavage in cancer has been recognised for more than five years, no data have been presented to confirm or reject this hypothesis. Two lines of data presented in this thesis suggest that an evaluation of uPAR cleavage is both possible and feasible.

The first step in the analysis of uPAR cleavage is the development of a reasonably simple assay with the potential to provide a quantitative evaluation of uPAR cleavage in spite of the highly heterogeneous glycosylation of uPAR. In Chapter 5 I have presented an assay by which this type of analysis is possible. The assay is based on the immunoprecipitation of total uPAR antigen from the biological sample followed by enzymatic deglycosylation and sensitive western blotting analysis. Application of this assay on collections of tumour extracts from several different cancer types could reveal the value of uPAR cleavage as a prognostic marker in cancer.

The pattern of suPAR fragments in urine from nude mice carrying xenografted human tumours reflects uPAR cleavage on the tumour cells (Chapter 6) and a similar situation is likely to be true in humans. The identification of tumour-derived uPAR fragments in human urine is currently impossible, as the tumour-derived suPAR fragments cannot be distinguished from the suPAR fragments already present in normal urine. The pattern of suPAR fragments in urine from healthy individuals is rather regular and the altered pattern of suPAR fragments observed in urine from cancer patients (Chapter 6) may thus be a consequence of the presence of tumour-derived suPAR fragments. If uPAR cleavage in tumour-tissue is a strong prognostic marker, and if we are able to "read" the tumour-component of the urinary suPAR fragments, the analysis of urinary suPAR fragments could provide a completely novel approach to the prognosis of cancer patients.

Essentially all diseases that involve inflammatory responses or tissue damage, are expected to be associated with local upregulation of uPAR expression. HIV infection upregulates
uPAR expression in monocytes and lymphocytes (Nykjaer et al., 1994; Speth et al., 1998) and chronic progressive multiple sclerosis is associated with upregulation of uPAR expression in monocytes and vascular endothelial cells (Dore-Duffy et al., 1992; Graham et al., 1998; Washington et al., 1994). Data not presented in this thesis indeed demonstrate that the level of serum and urinary suPAR is elevated in HIV infected individuals, and that high levels are associated with a bad prognosis.

Taken together the available data suggest that many malignant conditions may be associated with elevated levels of serum and urinary suPAR. This fact obviously reduces a possible diagnostic value of suPAR, but does not necessarily affect the value of suPAR in prognosis. The real strength of suPAR measurements in disease is most likely in the monitoring of patients in therapy. Changes in suPAR levels during therapy may for example reflect the efficacy of chemotherapy in cancer patients, or the efficacy of anti-retroviral therapy in HIV patients.
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Chapter 1: Introduction
Figure 1.1: Primary structure of uPAR

The primary aminoacid sequence of human uPAR is shown in single letter code. Disulphide bonding is indicated as thick lines connecting cysteine residues. The disulphide pattern of domain 2 and 3 has not actually been determined and is based on the known pattern in domain 1. Diamonds indicates potential glycosylation sites. Large arrows indicate the position of introns in the uPAR gene. Small arrows indicate protease cleavage sites in the linker region connecting domain 1 and 2. Chym., chymotrypsin; Hne1., human neutrophil elastase; Chym., chymotrypsin. Plasmin and uPA can also cleave uPAR at the same sites as trypsin (Høyer-Hansen et al., 1992).

The figure is reproduced from (Ploug and Ellis, 1994).
Chapter 3: Results & Discussion (I)
Figure 3.1: Adhesive properties of 32D and U937 cells.

Non-stimulated (A) or PMA stimulated (B) 32D cells (closed bars) and U937 cells (open bars) were allowed to adhere to wells coated with fibronectin, vitronectin or BSA under serum free conditions. After 1 hour, the wells were washed and bound cells fixed and stained. The amount (in arbitrary units) of bound cells were calculated by measuring the absorbance at 540 nm and subtracting the value obtained from wells that received no cells.
Figure 3.2: IL-3 stimulation of cell binding to fibronectin.

Binding of IL-3 starved 32D cells to vitronectin and fibronectin in the absence (open bars) or presence (closed bars) of 100 U/ml recombinant mouse IL-3. Bound cells were quantified as before and the value obtained in wells coated with BSA subtracted. The data represent the mean +/- SD of triplicate determinations from a representative experiment.
Figure 3.3: Binding of uPAR transfected 32D cells to vitronectin.

Pools of G418 resistant cells obtained after transfection with an uPAR expression vector (circles) or empty expression vector (squares) were allowed to adhere to wells coated with increasing concentrations of vitronectin and the bound cells were quantified as before.
Appendix I: Figures

Figure 3.4: Binding of 32D uPAR/D23 clones to vitronectin.

(A) Clones of 32D cells transfected with full-length uPAR (c14, c17, c24, c21, c22.7, c8, c2, c22, and c1) or with a D2D3 (c18, c6, c13, and c19) were allowed to adhere to vitronectin under serum free conditions in the presence (filled bars) or absence (open bars) of 5 nM pro-uPA. (B) Surface expression of uPAR and D2D3 on cells was evaluated by flowcytometry using the R2 monoclonal antibody that recognises an epitope on domain 3 of human uPAR. The mean fluorescence (in arbitrary units) is indicated and the clones have been arranged from left to right in order of increasing expression level.
Figure 3.5: Stimulation of binding by pro-uPA.

uPAR transfected 32D cells clone 21 (circles) and clone 24 (squares) were allowed to adhere to vitronectin in the presence of increasing concentrations of pro-uPA. Bound cells were measured as before.
Figure 3.6: Antibodies against uPAR domain 1 inhibit binding.

32D/uPAR clone 1 cells expressing high levels of uPAR were allowed to adhere to vitronectin in the presence of monoclonal antibodies against either uPAR domain 3 (mAb R2 and R4), uPAR domain 1 (mAb R3 and R5), or a negative control antibody (mAb M2). Cellular binding was determined as described before.
Figure 3.7: Requirement for integrin activation.

32D/uPAR clone 1 cells were allowed to adhere to vitronectin or fibronectin in the presence (filled bars) or absence (open bars) of IL-3 (100 U/ml) at 37°C or at 4°C as indicated. The cells had been IL-3 starved for 8 hours prior to the experiment.
Figure 3.8: uPAR mediated vitronectin binding does not lead to cell spreading

uPAR transfected 32D cells (clone 1) were allowed to adhere to plastic dishes coated with BSA (panel A) or vitronectin (panel B) for one hour at 37°C after which the cells were photographed.
Figure 3.9: Cell binding to anti-uPAR antibodies.

32D cells transfected with uPAR (open bars) or the mutant D2D3 receptor (closed bars) were allowed to adhere to wells coated with vitronectin or antibodies against uPAR (5 µg/ml). After washing of the plates bound cells were quantified as before.
Figure 3.10: Binding of soluble uPAR to vitronectin.

ELISA plates were coated with vitronectin (5 μg/ml) and the remaining binding sites blocked with BSA. Wells were incubated 0.15, 1.5, 15 or 150 nM of FLAG tagged soluble uPAR (squares) or D2D3 mutant (circles). The binding was performed in the presence (filled) or absence (open) of 50 nM pro-uPA. After washing, the amount of bound protein was quantified by sequential incubations with an anti-FLAG antibody, a horseradish peroxidase conjugated secondary antibody and a colorimetric peroxidase substrate. The absorbance at 412 nm was measured in an ELISA reader and taken as a measure of bound proteins. The data represent the medium value of duplicate determinations (+/- SD) from a representative experiment. The curves for the D2D3 protein cannot be seen very well as they are superimposed with the curve for soluble uPAR in the absence of pro-uPA.
Chapter 4: Results & Discussion (II)
Figure 4.1: Scheme depicting the basic structure of suPAR and its mutated derivatives.

suPAR has three domains, an amino terminal (D1), an intermediate (D2) and a carboxyterminal domain (D3), which is connected to the GPI-anchor (Ploug et al., 1991; Ploug and Ellis, 1994). All the suPAR constructs express soluble molecules since they employed the basic cDNA modification described before (Masucci et al., 1991; Roldan et al., 1990). The symbol for the full size soluble suPAR is D123. Truncated molecules are identified by the number of the domains (i.e. D1, D2, D3) and by the number of amino acids they express (i.e. D1_92, domain 1, from residue 1 to 92).
Figure 4.2: Chemotactic response of THP-1 cells to different recombinant suPAR fragments.

The position of the sequence SRSRY in the linker region between domain 1 and 2 of uPAR is shown on top. Panels A-D refer to the indicated soluble recombinant uPAR mutants. Panels E and F refer to the chymotryptic fragments isolated from wild type suPAR\textsubscript{1-274}. THP-1 cells migrated towards a solution containing different concentrations of the chemoattractants. Random cell migration of untreated THP-1 cells is referred to as 100% migration.
Figure 4.3: Chemotactic response of THP-1 cells to different concentrations of the indicated peptides.

THP-1 cells migrated towards a solution containing different concentrations of the chemoattractants. Random cell migration of untreated THP-1 cells is referred to as 100% migration.
Figure 4.4 Effect of PTX on THP-1 cells chemotaxis in response to peptide-1 or chymotrypsin-cleaved suPAR.

THP-1 cells were pre-incubated 4 hours with (100 ng/ml) PTX or its inactive mutant (Pizza et al., 1989), washed and their chemotactic response measured. Medium alone (black bars), 0.1 pM peptide-1 (dotted bars), 1 pM peptide-1 (white bars), 10 pM chymotrypsin-cleaved suPAR (striped bars). Random cell migration of untreated THP-1 cells is referred to as 100% migration.
Figure 4.5 Outline of the protease-sensitive uPAR linker located between domain 1 (D1) and 2 (D2)
Chapter 5: Results & Discussion (III)
Figure 5.1: uPAR antigen in U937 cell lysates and in conditioned medium.

U937 cells were plated in the absence (open bars) or presence of PMA (black bars). After 24, 48 and 72 hours, cells and conditioned medium was harvested. uPAR antigen in cell lysates (panel A) and in the conditioned medium (panel B) was measured by ELISA.
Figure 5.2: Full length and cleaved forms of uPAR on U937 cells.

U937 cell lysates (20 μg total protein) and conditioned medium (0.25 ml) obtained as described in the legend to figure 5.1 were immunoprecipitated with a mixture of monoclonal antibodies R2 and R3. The absorbed material was analysed by western blotting using a polyclonal anti-uPAR antibody. The position of molecular weight markers, full-length uPAR, cleaved uPAR (D2D3) and the released domain 1 (D1) is indicated. The right-most panel is a long exposure of the lane containing conditioned medium from U937 treated with PMA for 72 hours.
Figure 5.3: Molecular structure of uPAR fragments in conditioned medium from U937 cells.

(A) Conditioned medium obtained from a 72 hour PMA stimulation of U937 cells was either immunoprecipitated with antibodies recognising uPAR domain 3 (mAb R2 and R4), or uPAR domain 1 (mAb R3), or an anti-uPA antibody (mAb c6), or an irrelevant antibody (mAb M2). After washing, the precipitates were denatured, reduced and treated for two hours without (left panel) or with PNGase-F (right panel).
Figure 5.4: uPAR shedding from monocytes and neutrophils.

Human monocytes and neutrophils were isolated from 'buffy coats' as described in the materials & methods section. Cells were seeded (10^6/ml) in leukocyte culture medium in the absence (Ct.) or presence of PMA (PMA, only monocytes). After 48 hours of incubation, cells were harvested by centrifugation and the conditioned medium collected. Cell lysates (Lys) and conditioned medium (CM) were analysed by immunoprecipitation and western blotting as described in the legend to figure 5.2. (B) Cell lysates and conditioned medium from monocytes and neutrophils were immunoprecipitated with a mixture of monoclonal antibodies R2 and R3 and the absorbed material reduced and treated for two hours with PNGase-F. The deglycosylated material was analysed by western blotting using a polyclonal antibody against uPAR.
Figure 5.5: uPAR shedding from fMLP stimulated neutrophils.

Freshly isolated neutrophils were incubated in serum free medium (20x10^6/ml) for 30 minutes at 37°C in the absence (no) or presence of 5 nM fMLP (fMLP). The cells were pelleted and the amount of uPAR antigen in the conditioned medium measured by ELISA. The indicated values are the mean obtained from neutrophils isolated from two independent buffy coats +/- standard error.
Figure 5.6: uPAR cleavage and shedding in HUVEC cells.

Human umbilical vascular endothelial cells (HUVEC, passage 5) were allowed to grow to confluence. The medium was replaced with fresh medium without (Ct.) or with PMA (PMA). After 24, 48 and 72 hours the conditioned medium was collected and cells lysates were prepared after 72 hours. Lysates and conditioned medium were analysed by immunoprecipitation and western blotting as described in the legend to figure 5.2. Panels labelled with a "#" are short exposures.
Figure 5.7: PMA stimulation and "Wounding" of HUVEC promotes uPAR shedding.

Confluent monolayers of HUVEC cells were "wounded" by "scratching" using the side of a rubber policeman. The total wounded area was small (> 1%) and the wounding was repeated once after 24 hours. As a positive control separate plates received medium containing PMA. After 48 hours of incubation the conditioned medium was harvested and the concentration of uPAR antigen measured by ELISA. The indicated values are the mean of duplicate plates +/- SD. The experiment was performed only once.
Figure 5.8: uPA independent cleavage of uPAR in 32D cells.

Cell lysates (Lys) and conditioned medium (CM) from 32D cells transfected with wild-type uPAR (uPAR) and a mutant lacking domain 1 (D2D3) were immunoprecipitated with a mixture of monoclonal antibodies R2 and R3. The absorbed material was analysed by western blotting using a polyclonal anti-uPAR antibody. The position of full-length uPAR and cleaved uPAR (D2D3) are indicated.
Appendix I: Figures

Figure 6.1:
Urinary suPAR levels normalised for creatinine in ovarian cancer patients (n=25) and age matched healthy women (n=20). The difference is significant (P = 0.004) according to Mann-Whitney's U-test. Patients with FIGO stage IIIc carcinomas are indicated as full circles. The remainder of the patients had FIGO stage I/II carcinomas. The horizontal bar indicates the median value of controls plus two times the standard deviation.
Figure 6.2:

(A) Diagram illustrating the domain structure of uPAR and the proteolytic fragments thereof. The receptor is composed of three domains (D1, D2, and D3) and is linked to the cell-surface by a GPI-anchor attached to D3. Proteolytic cleavage in the region between D1 and D2 results in release of the D1 fragment, leaving behind on the cell surfaces the D2D3 fragment. Soluble forms, lacking the GPI-anchor, exist for both uPAR and D2D3. Monoclonal antibodies specific for D1 (mAb R3) and D3 (mAb R2) are used in this study to identify the different uPAR fragments. (B) Immunoblot demonstrating the presence of suPAR and suPAR fragments in urine. Concentrated sample (corresponding to 1 ml urine) from an ovarian cancer patient was immunoprecipitated with monoclonal antibodies recognising uPAR (mAb R2 and R3) or an irrelevant control antibody (mAb Ct). The absorbed material was analysed by immunoblotting using a polyclonal antibody directed against human uPAR.
Figure 6.3:

Difference in suPAR fragmentation pattern in urine from ovarian cancer patients. Panel A: urine from seven healthy individuals; panel B: urine from seven ovarian cancer patients; panel C: Serum from the same seven patients. Urine, normalised for creatinine content, and serum samples (0.1 ml) were immunoprecipitated with a combination of mAb R2 and R3 and the absorbed material was analysed by immunoblotting with a polyclonal antibody directed against human uPAR.
Figure 6.4:

Human suPAR levels in urine from mice xenografted with the ovarian carcinoma HOC8 and the breast carcinoma MDA435 in comparison with non-tumour bearing control mice. Pooled urine from five (HOC8) or two (MDA435) xenografted mice was collected in metabolic cages and analysed by ELISA as described in the materials and methods section.
Figure 6.5:

Human uPAR fragmentation in mice carrying the xenografted human tumours HOC8 or MDA435. Tumour extracts (20 µg total protein), serum (0.02 ml) and urine (0.05 ml) were subjected to immunoprecipitation with a mixture of mAb R2 and R3. The absorbed material was analysed by immunoblotting with a polyclonal antibody directed against human uPAR.
CHAPTER 7: FINAL DISCUSSION
Figure 7.1: The uPAR chemotactic epitope

Schematic diagram showing the primary sequence of linker region between uPAR domain 1 and 2. Aminoacids are indicated by single letter code and the aminoacid number. Arrows indicate cleavage sites for trypsin, plasmin, uPA, neutrophil elastase (NE) and chymotrypsin. The minimal chemotactic epitope is underlined.