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A urokinase-sensitive region of the human urokinase receptor is responsible for its chemotactic activity

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The role of urokinase-type plasminogen activator (uPA) and its receptor (uPAR/CD87) in cell migration and invasion is well substantiated. Recently, uPA has been shown to be essential in cell migration, since uPA$^{-/-}$ mice are greatly impaired in inflammatory cell recruitment. We have shown previously that the uPA-induced chemotaxis requires interaction with and modification of uPAR/CD87, which is the true chemoattracting molecule acting through an unidentified cell surface component which mediates this cell surface chemokine activity. By expressing and testing several uPAR/CD87 variants, we have located and functionally characterized a potent uPAR/CD87 epitope that mimics the effects of the uPA–uPAR interaction. The chemotactic activity lies in the region linking domains 1 and 2, the only protease-sensitive region of uPAR/CD87, efficiently cleaved by uPA at physiological concentrations. Synthetic peptides carrying this epitope promote chemotaxis and activate p56/p59^{hck} tyrosine kinase. Both chemotaxis and kinase activation are pertussis toxin sensitive, involving a $G_{i/o}$ protein in the pathway.
Keywords: chemotaxis/G protein/pertussis toxin/receptor/urokinase-type plasminogen activator

Introduction

Cell recruitment is an essential component in inflammation, neo-angiogenesis and cancer invasiveness. Urokinase-type plasminogen activator (uPA), its receptor (uPAR/CD87) and inhibitor (PAI-1) are directly involved in these mechanisms (Danø *et al*., 1994; Blasi, 1997). An important proof of this statement is the profound impairment of inflammatory cell recruitment in uPA$^{-/-}$ mice which do not respond properly but succumb to infection by *Cryptococcus neoformans*, due to deficient T lymphocyte and monocyte–macrophage recruitement (Gyetko *et al*., 1996). Moreover, uPA-deficient mice fail to support the growth and development of experimental melanomas, while inhibition of the uPA–uPAR/CD87 interaction *in vivo* blocks angiogenesis, growth and spreading of syngeneic tumors (Min *et al.*, 1996; Shapiro *et al.*, 1996). A chemotactic activity of uPA has long been recognized *in vitro* on different cell types in culture (Gudewicz and Bilboa, 1987; Fibbi *et al.*, 1988; Busso *et al.*, 1994; Gyetko *et al.*, 1994; Resnati *et al.*, 1996). The chemotactic activity of uPA strictly depends on binding to its receptor uPAR (CD87): it does not occur in murine cells lacking uPAR/CD87, or containing uPAR/CD87 but not recognizing human uPA; can be restored by transfection of the uPAR/CD87 cDNA; and is inhibited by antibodies that prevent uPA–uPAR interaction (Resnati *et al.*, 1996). Occupancy of uPAR/CD87 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/CD87-mediated chemoattraction (our unpublished data), or by the block of ligand-induced chemotaxis by tyrosine kinase inhibitors in THP-1 cells (Resnati *et al*., 1996).

uPAR/CD87 is a glycophosphatidylinositol (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/CD87, often at rather high levels (Blasi, 1988). In T lymphocytes and monocytes, uPAR/CD87 is an activation antigen and its expression appears to be relevant to the function of these cells, at least in culture (Picone *et al*., 1989; Nykjær *et al.*, 1994; Bianchi *et al.*, 1996).

The membrane attachment of uPAR/CD87 via a GPI anchor (Ploug *et al*., 1991), i.e. the lack of an intracytoplasmic region capable of connecting with the cytoplasmic signal transducers, suggests that transmembrane adaptor(s) must mediate the activation of intracellular transducers. Indeed, uPAR/CD87 cells can respond to a chemotactic stimulus induced by exogenous, soluble uPAR (suPAR), indicating that the binding of uPA to uPAR/CD87 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 *et al.*, 1993; Ploug and Ellis, 1994). The chemotactic properties of uPA and chymotrypsin-cleaved suPAR are identical, i.e. they both induce p56/p59^{hck} 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/CD87 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. Here we demonstrate that the uPAR/CD87 chemotactic

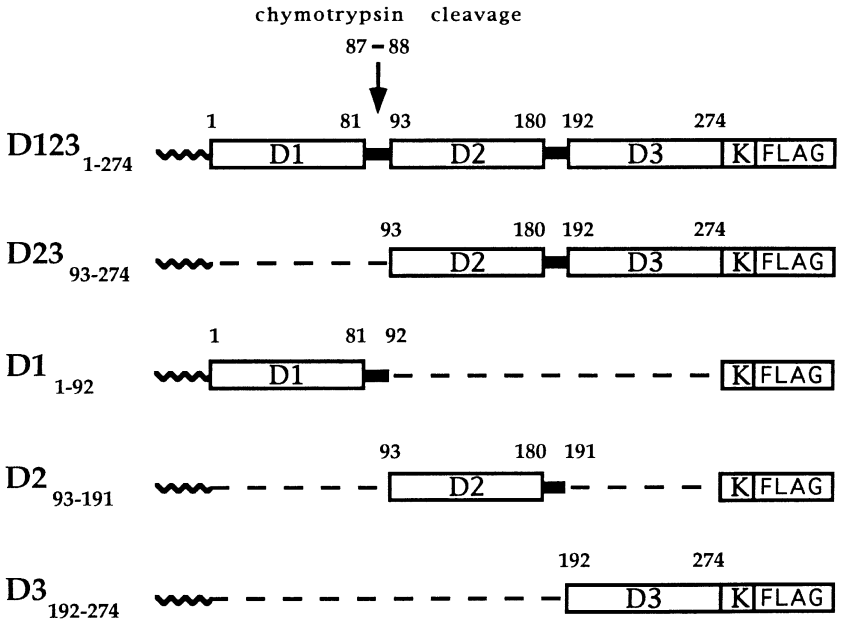

Fig. 1. Scheme depicting the basic structure of suPAR and its mutated derivatives.

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.*, 1997). We also show that a synthetic uPAR/CD87 chemotactic epitope activates p56/p59^{hck} and that both chemotaxis and activation of p56/p59^{hck} are pertussis toxin (PTX) sensitive, implying a connection between G proteins and intracellular protein tyrosine kinases. Here, we indicate with uPAR/CD87 the 'normal' membrane-attached uPA receptor, and with suPAR the exogenous, recombinant receptor.

Results

Identification and mapping of the uPAR/CD87 chemotactic epitope

We employed suPAR truncated mutants to identify the uPAR/CD87 chemotactically active region (see Figure 1 for a scheme, and Materials and methods for terminology). We had shown previously that chymotryptic cleavage of exogenous suPAR induced a very efficient chemotactic activity at picomolar concentrations in cells which did not express this receptor (Resnati *et al.*, 1996). To identify the region of suPAR responsible for the activity, we first purified the FLAG-tagged, full size three-domain suPAR, digested it with chymotrypsin and separated the resulting fragments $D1_{1–87}$ and $D23_{88–274}$ on FLAG™ antibody affinity chromatography columns. The two fragments were tested individually in chemotaxis assays on human THP-1 cells. We chose THP-1 cells, even though they are poor responders to chemotactic signals, because their basal level of tyrosine phosphorylation is low (Resnati *et al.*, 1996) and hence make the subsequent biochemical analysis faster. However, the same results were obtained with several other cells (mouse uPAR/CD87$^{-/-}$, macrophages, NIH-3T3 fibroblasts, rat smooth muscle cells and mouse LB6 tumor cells), which respond ~2-fold better to the chemotactic stimuli of suPAR fragments and other suPAR derivatives (see below). The C-terminal fragment, $D23_{88–274}$, but not the N-terminal $D1_{1–87}$, elicited a dose-dependent chemotactic response (Figure 2E and F). We then tested individual recombinant suPAR mutants. Of these, only $D1_{1–92}$ induced a dose-dependent chemotactic effect (Figure 2A), while $D23_{93–274}$, $D2_{93–191}$ and $D3_{192–274}$ failed to induce chemotaxis through the entire concentration range analyzed (Figure 2B–D). These findings show that only fragments containing the sequence 88– 92 of uPAR/CD87, 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 N-, as in $D23_{88–274,}$ as well as at the C-terminus, as in $D1_{1–92}$.

To confirm the above interpretation, we tested synthetic uPAR/CD87 peptides for their chemotactic activity on THP-1 cells. Peptide 1 [AVTYSRSRYLEC, which corresponds to the amino acid sequence 84–95 of human uPAR/CD87 (Roldan *et al.*, 1990)] and its shorter version, peptide 3 (SRSRYLEC, residues 88–95), exhibited chemotactic activity with a maximal response at concentrations as low as 0.1 pM (Figure 3A and C). On the other hand, peptide 4 (TLVEYYSRASCR), a scrambled version of peptide 1, and peptide 2 (YTARLWGGTLLT), which covers residues 301–313, and thus is absent from processed mature uPAR/CD87 (Ploug *et al.*, 1991), had no effect on cell migration over the entire concentration range analyzed (Figure 3B and D). These results support the conclusion that the sequence SRSRY of human uPAR/CD87 has a potent chemotactic activity. It is important to note that this region is cleaved efficiently by uPAR-bound uPA at chemotactically active concentrations (Høyer-Hansen *et al.*, 1997; see Discussion).

Since THP-1 cells are not very sensitive to chemotactic stimuli, we tested peptide 1 on other cells. Peptide 1 was found to be active also on several other cells, like murine uPAR/CD87$^{-/-}$ fibroblasts, NIH-3T3 and LB6 cells, or on rat aortic smooth muscle cells (data not shown). On some of these cells, the effect is much more pronounced (~4-fold

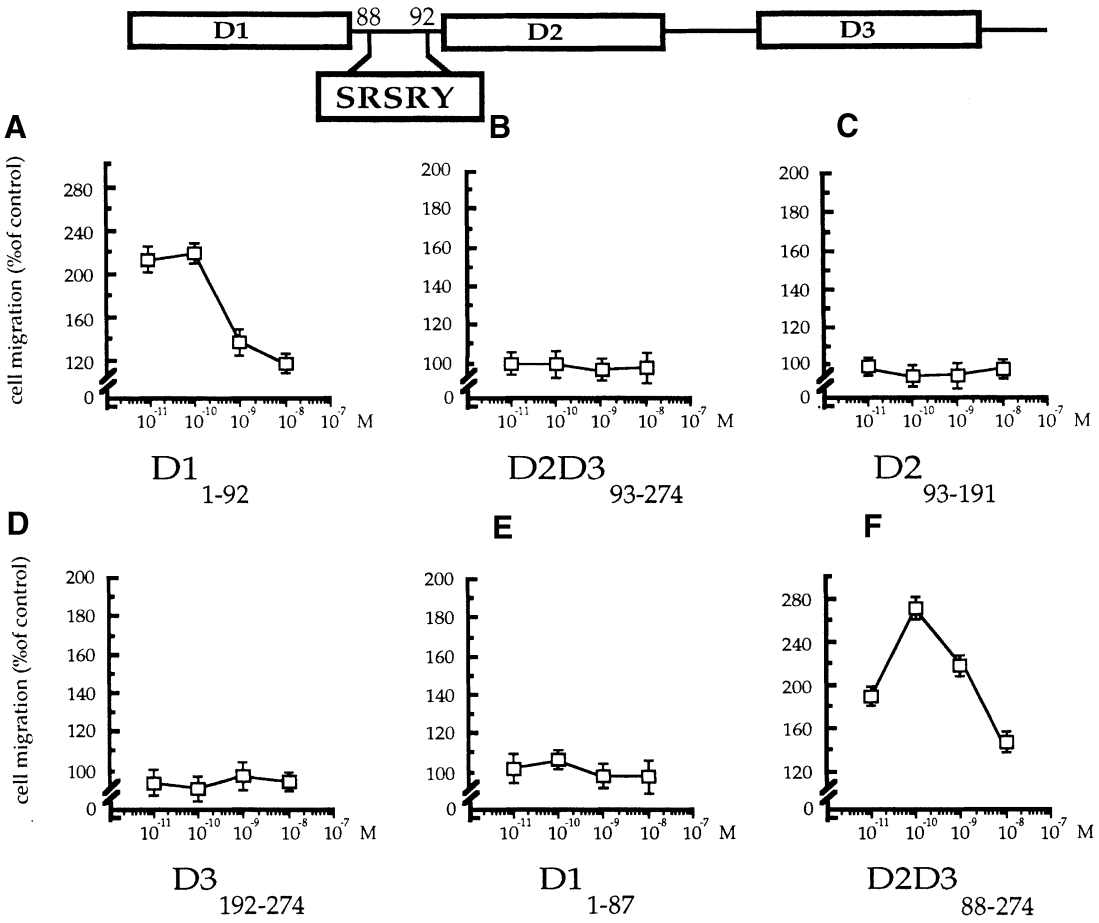

Fig. 2. Chemotactic response of THP-1 cells to different recombinant suPAR fragments. The position of the sequence SRSRY in the linker region between domains 1 and 2 of uPAR/CD87 is shown at the top. (**A–D**) The indicated soluble recombinant products; (**E**) and (**F**) the chymotryptic fragments isolated from wild-type suPAR123$_{1–274}$ were used as attractants. 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.

over basal migration), but has an identical 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 cells) had no effect on migration (data not shown).

Activation of p56/p59^{hck} by chemotactic uPAR/CD87 peptides

Treatment of THP-1 cells with the uPA fragment ATF at chemotactic concentrations induces the activation of p56/p59^{hck} tyrosine kinase. Treatment with exogenous chymotrypsin-cleaved suPAR, that substitutes for uPA in inducing chemotaxis in uPAR/CD87-lacking cells, also activates p56/p59^{hck} (Resnati *et al.*, 1996; unpublished data). We therefore tested the effect of chemotactic concentrations of peptide 1 on the activity of p56/p59^{hck} 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 (Figure 5A). The effect was already apparent after 2 min, reached a maximum after 10 min and returned to the basal level after 30 min. This time course is very similar to that of ATF or chymotrypsin-cleaved suPAR (Resnati *et al.*, 1996). 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/CD87, resembles that of uPA and chymotrypsin-cleaved suPAR not only in stimulating chemotaxis but in the activation of the same tyrosine kinase. This supports the view that peptide 1 is the, or one of the, chemotactic epitope(s) of uPAR/CD87.

Involvement of heterotrimeric G proteins

The activity of many chemokines is mediated through specific receptors by signal–effector coupling GTP-binding proteins (G proteins). PTX inhibits the activity of some chemokines through the α_i subset of G proteins; the inhibitory effect of PTX is therefore an indication of the involvement of heterotrimeric $G_{i/o}$ proteins in the signaling pathway (Baggiolini *et al.*, 1994; Neer, 1995). To test the involvement of G proteins in the uPA/suPAR/synthetic peptides-induced chemotaxis, we tested the effect of PTX and one of its inactive mutants. Chemotaxis of THP-1 cells induced by the N-terminal fragment of uPA (ATF, Stoppelli *et al.*, 1985) also proved to be PTX sensitive (data not shown). As shown in Figure 4, pre-incubation of THP-1 cells with PTX also inhibited chymotrypsin-cleaved suPAR and peptide 1-induced chemotaxis, while

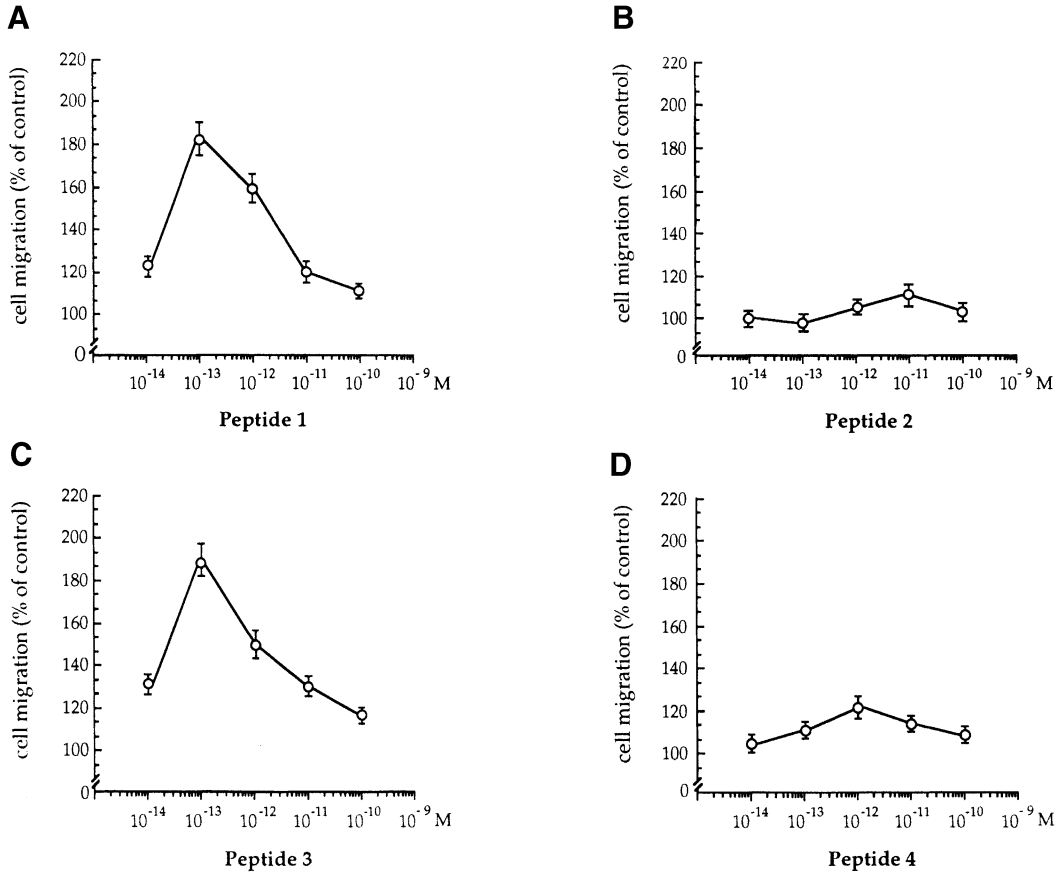

Fig. 3. Chemotactic response of THP-1 cells to different concentrations of the indicated peptides. THP-1 cells migrated towards a solution containing different concentrations of the chemoattractants. Random cell migration of untreated THP-1 cells is referred to as 100% migration.

an inactive mutant had no effect. An identical result was obtained on peptide 1-induced chemotaxis of rat smooth muscle cells. This result indicates that the uPAR/CD87 epitope exerts its chemotactic activity through a G protein-coupled cell surface protein.

Since uPA as well as the chemotactic uPAR/CD87 peptides also activate p56/p59^{hck}, in a separate experiment we also tested the effect of the toxin on the activation of p56/p59^{hck} by peptide 1. As shown in Figure 5B and C, the activation of p56/p59^{hck} activity by peptide 1 was also PTX sensitive. Inhibition of p56/p59^{hck} activation by peptide 1 was observed in the presence of 100 ng/ml wild-type, but not mutant, PTX. This result confirms the G protein dependence of uPAR/CD87 chemotaxis and suggests that the activation of tyrosine kinases is a downstream step from the heterotrimeric $G_{i/o}$ protein.

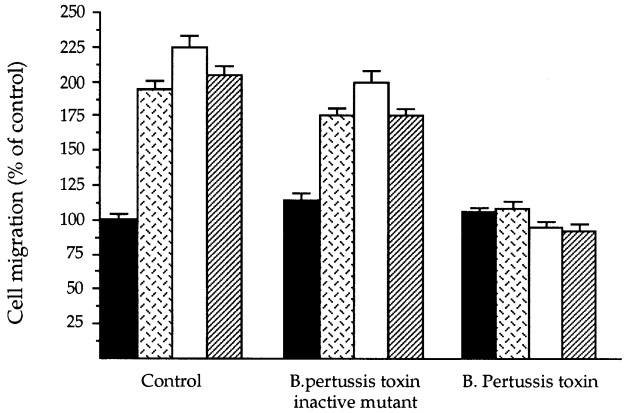

Fig. 4. Effect of PTX on chemotaxis of THP-1 cells in response to peptide 1 or chymotrypsin-cleaved suPAR. THP-1 cells were pre-incubated for 4 h with (100 ng/ml) PTX or its inactive mutant (Pizza *et al.*, 1989), washed and their chemotactic response measured. Medium alone (black bars), 0.1 pM peptide 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.

Discussion

GPI-anchored proteins are potent signaling molecules. However, the lack of a transmembrane domain requires that they interact with another transmembrane 'adaptor', i.e. with the real signaling molecule. This has in fact been shown to be true for several GPI-anchored proteins, including, among others, CD14 (Uhlevitch and Tobias, 1995). In the case of uPAR, the adaptor (Resnati *et al.*, 1996) is not yet known; however, in the present study, by identifying the chemotactic epitope of uPAR, we in fact suggest the nature of the actual ligand for such an adaptor.

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^{hck} in THP-1 and other cells, through a PTX-sensitive signaling system. The concentration of peptide 1 required to elicit the signaling

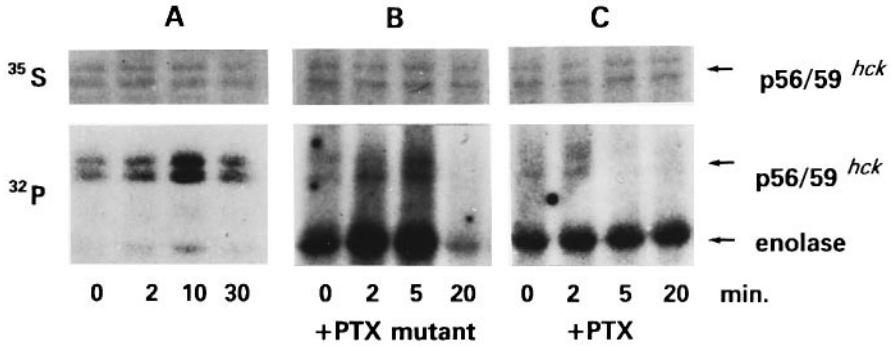

Fig. 5. Peptide 1 activation of p56/p59^{hck} kinase activity and its inhibition by PTX. Metabolically ^{35}S-labeled THP-1 cells were acid washed and treated with 1 pM peptide 1 for the indicated times at 37°C, then lysed and immunoprecipitated with anti-p56/p59^{hck} antibody. ^{35}S: direct analysis by SDS–PAGE of the amount of p56/p59^{hck} employed. ^{32}P: *in vitro* kinase assay in the presence of [γ-^{32}P]ATP and 5 μg of rabbit muscle enolase. (**A**) Effect of peptide 1; (**B**) Effect of peptide 1 on cells pre-treated with mutated PTX (100 ng/ml); (**C**) Same as in (B) in the presence of 100 ng/ml wild-type PTX. The data in (A) and in (B) and (C) are from two different experiments.

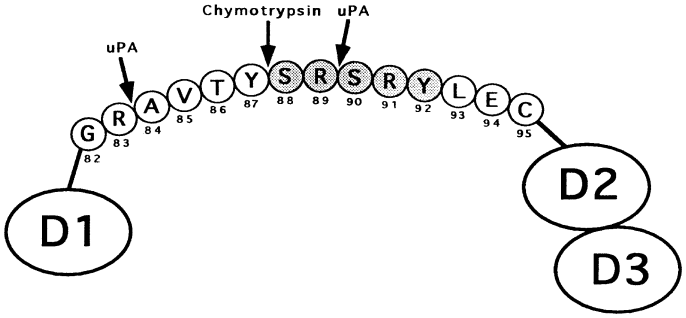

Fig. 6. The uPAR/CD87 linker sequence between domains 1 (D1) and 2 (D2) and the protease-sensitive sites.

effects (~0.1 pM) is far lower than the concentration of uPA or ATF required to generate the same biological response (0.5–5 nM, Resnati *et al*., 1996), but is of the same order of magnitude as the effect obtained with chymotrypsin-cleaved suPAR. This represents an enormous functional amplification which follows or bypasses the uPA–uPAR/CD87 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 can respond as well. This differs from the binding of uPA to uPAR/CD87 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 (see Figure 6). 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 occurs physiologically through proteolytic cleavage. While we cannot exclude that a ligand-induced conformational change of uPAR/CD87 is sufficient to transduce migratory signals, we must take note that a relevant proteolytic event does indeed occur *in vivo*. (i) In different cell lines and human cancer tissues, uPAR/CD87 fragments missing domain D1 have been demonstrated (Høyer-Hansen *et al.*, 1992; Sølberg *et al.*, 1994). (ii) In purified suPAR, the linker region between domains 1 and 2 (Figure 6) is susceptible to cleavage by uPA and other proteases, like chymotrypsin (Høyer-Hansen *et al.*, 1992; Ploug and Ellis, 1994). (iii) Importantly, at chemotactically active concentrations, endogenous or exogenous receptor-bound uPA cleaves 50% of surface uPAR/CD87 at residue 83 or 89 (Høyer-Hansen *et al.*, 1997). The former site would generate a chemotactically active uPAR/CD87, starting with an N-terminal AVTYSR-SRY sequence, identical to that of peptide 1 (see Figure 6). (iv) Binding of uPA to uPAR/CD87 activates cell surface plasminogen to plasmin which also might cleave uPAR/CD87 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/CD87, 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/CD87 by a different protease substituting for uPA. Since the sequence SRSRY is active when positioned both N- and C-terminally, different types of proteases may be involved. Again, we cannot exclude that a conformational change may be sufficient to expose the SRSRY epitope, and that the observed proteolytic event may be unrelated.

A proteolytic event activating the cell surface uPAR chemokine may explain the paradox that the high level of expression of uPAR and uPA in human tumors is a strong negative prognostic factor, independently of whether uPAR is expressed by cancer or stromal cells. While in colon cancer, for example, uPAR/CD87 is expressed by cancer cells, in breast cancer it is confined mostly to stromal

Table I. Primers used in PCR

Primer	Sequence (5′–3′)
FRA18	ATTATACTCGAGGAAGACGTGCAGGGACCCCGCGCA
D1.3′T	TTATCGATGGTAACGGCTTCGGGAATA
D2.3′T	TTATCGATGGCCATTCTGCGGCAGATT
D3.3′T	TTATCGATGTGGGTGGTTACAGCCACT
D2.5′T	AATGCATTCGAGGCCCCAAGAGGCTGGGA
D3.5′T	TCCATGGGTGCTGTTCCCCTTGCAGCTGTAACACTGGCGGCCCCAAGAGGCTGGGA
K/FOcs	CGACGAGCATCTGTCGACTATAAGGATGACGACGACAAGTAA
K/FOas	CGTTACTTGTCGTCGTCATCCTTATAGTCGACAGATGCTCGT
NS1	CCGCGGAAGAACCCATGGGACTCCCAA
46D	CAAGCTTACTTGTCGTCGTCATCC

cells. In both cases, however, high tumor levels of uPAR are predictive of cancer relapse (Pyke *et al.*, 1991, 1993; Bianchi *et al.*, 1994; Grøndahl-Hansen *et al.*, 1995). Depending on the protease involved, cleavage of uPAR might generate either a soluble chemoattracting domain D1 acting on distant cells, or a membrane-attached chemotactic domain D23, which would then act on the very cell which is expressing it. In other words, a cell surface chemokine or a soluble chemokine can be generated, depending on the protease involved.

It is also interesting to note 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 (Figure 6), might destroy the chemotactic activity, as it would cleave within the conserved region. It is interesting to note that the efficiency of cleavage by uPA at this second site is lower (Høyer-Hansen *et al.*, 1997), suggesting that the cleavage might actually occur only at high concentrations of uPA.

Whatever the mechanism, the data reported here show that the uPA–uPAR system is a cell surface-regulated chemokine. The exposed chemotactic epitope must interact with a cell surface (adaptor) protein which can signal through a heterotrimeric G protein. The nature of the adaptor is still unknown: among already known proteins, possible candidates are the chemokine receptors, integrins and caveolin. Chemokine receptors, as members of the seven transmembrane-spanning protein family, may interact with heterotrimeric G proteins (Baggiolini *et al.*, 1994). 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/p59^{hck}, which is specifically prevented by PTX (Figure 4). It appears, therefore, that activation of p56/p59^{hck} occurs downstream from the heterotrimeric G protein. The tyrosine kinase activation appears to be essential for the chemotactic activity of uPAR/CD87 since this can be inhibited by inhibitors of tyrosine kinases (Resnati *et al.*, 1996), and since the peptide 1 effect can be observed in murine wild-type, but not src$^{-/-}$, fibroblasts (B.Degryse, F.Fazioli and F.Blasi, unpublished). The identification of the G protein involved and the interconnection with the intracellular tyrosine kinase will be important future research targets.

Mice lacking the uPA gene are strongly immunodeficient and succumb to bacterial infections (Gyetko *et al.*, 1996). The mechanism outlined in this study is likely to contribute to the role of uPA in cell recruitment. The inability to induce a conformational change and/or to cleave uPAR/CD87 proteolytically may explain the immunodeficiency of uPA$^{-/-}$ mice. If this hypothesis is correct, the uPAR$^{-/-}$ mice will have to be immunodeficient as well. In man, uPAR/CD87 is an activation antigen in T lymphocytes and monocytes, and is required for T cell migration, at least *in vitro* (Nykjær *et al.*, 1994; Bianchi *et al.*, 1996).

Finally, the results described here may have a practical application, as it is conceivable that agents acting on uPAR/CD87 chemotaxis, for example by 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/CD87 interaction step, and might have properties different from those of uPAR/CD87 antagonists, expanding the application of agents inhibiting this functions.

Materials and methods

Materials and cells

Human ATF was a generous gift of Dr Jack Henkin (Abbott Laboratories, IL). Soluble uPAR/CD87 was prepared and chymotrypsin-cleaved as described before (Resnati *et al.*, 1996). Recombinant PTX and its inactive mutant (Pizza *et al.*, 1989) were generous gifts of Dr Maria Grazia Pizza (IRIS, Siena, Italy). Synthetic peptides had the following sequences: peptide 1, AVTYSRSRYLEC; peptide 2, YTARLWGGTLLT; peptide 3, SRSRYLEC; peptide 4 (scrambled version of peptide 1), TLVEYYSRASCR.

THP-1 cells were obtained from the ATCC (Rockville, MD). The uPAR/CD87$^{-/-}$ cells were derived from spontaneous immortalization of embryo fibroblasts prepared from uPAR/CD87$^{-/-}$ mice (Dewerchin *et al.*, 1996), kindly donated by Drs Peter Carmeliet and Desiré Collen.

Construction of plasmids expressing soluble, truncated uPAR/CD87 molecules

uPAR/CD87 has three domains, an N-terminal (D1), an intermediate (D2) and a C-terminal domain (D3), which is connected to the GPI anchor (Ploug *et al.*, 1991; Ploug and Ellis, 1994). All uPAR/CD87 constructs expressed soluble molecules since they employed the basic cDNA modification described previously (Roldan *et al.*, 1990; Masucci *et al.*, 1991). The full size soluble uPAR/CD87 is designated 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. $D1_{1–92}$, domain 1, from residue 1 to 92). Mutant cDNAs encoding soluble uPAR/CD87 were generated by PCR with the following primers (see Table I for sequences): $D1_{1–92}$, oligonucleotides FRA18 and D1.3′T; $D12_{1–191}$, oligonucleotides FRA18 and D2.3′T; $D123_{1–274}$, oligonucleotides FRA18 and D3.3′T. The PCR products were digested with *Bcl*I and *Cla*I, and cloned in pBluescript SK– (Stratagene) digested with *Bam*HI and *Cla*I. To generate the constructs encoding $D2_{93–191}$ and

$D23_{93–274}$, the D1 region in the D12 and D123 constructs was deleted by substituting the *Nru*I–*Nsi*I fragment containing the D1 coding region, with a fragment generated by amplifying the uPAR/CD87 cDNA with the primers FRA18 and D2.5′T and digesting with the same enzymes. To generate $D3_{192–274}$, the D12 region in D123 was deleted by substituting the *Nru*I–*Nco*I fragment containing the D12 coding region, with a fragment generated from uPAR/CD87 cDNA with the primers FRA18 and D3.5′T and digesting with the same enzymes. All mutant receptors were tagged at the C-terminus with the peptide sequence HRRASVDYKDDDDK, which includes a protein kinase substrate and the FLAG™ epitope, by inserting in the C-terminal *Cla*I 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 *Nco*I and transferred to the eukaryotic expression vector pBNSEN (Pallisgaard *et al.*, 1994) digested with *Nco*I and Klenow-treated *Eco*RI.

Purification of recombinant soluble uPAR/CD87 molecules

Semi-confluent COS7 cells were harvested in phosphate-buffered saline (PBS)–1 mM EDTA, washed with RPMI medium and the suspension (0.8 ml, $1–2\times10^7$ cells/ml in RPMI) 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, cells were washed with PBS and supplemented with 50 ml of serum-free medium (Dulbecco's modified Eagle's medium containing 1% Nutridoma NS, Boehringer Mannheim). Every 4–5 days, conditioned medium was collected and fresh medium added. Recombinant proteins were purified from the conditioned medium by passage over an anti-FLAG™ affinity column (M2 Affinity gel, Sigma). Recombinant proteins were eluted with 0.1 M glycine pH 3.0.

Purified $D1_{1–87}$ and $D23_{88–274}$ were prepared by cleavage of $D123_{1–274}$ with chymotrypsin (Resnati *et al*., 1996) followed by passage over the FLAG™ affinity column. $D1_{1–87}$ was recovered in the flow-through while the $D23_{88–274}$ containing the FLAG™ epitope was retained and eluted with 0.1 M glycine pH 3.0.

Chemotaxis assays

Chemotaxis analysis 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), 2×10^5 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% CO_2 in air for 90 min. 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 a high power field (10 fields for each condition) and expressed as a 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.

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

Tyrosine kinase assay

The kinase assay was performed as previously described (Resnati *et al.*, 1996) using THP-1 cells metabolically labeled 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/p59^{hck} antibody (Resnati *et al.*, 1996) and some were analyzed by SDS–PAGE to control the amount of protein. The remainder were used for *in vitro* kinase assay (5–10 μCi of [γ-^{32}P]ATP, ~3000 Ci/mmol, Amersham, 5 μg rabbit muscle enolase substrate, 5 min, room temperature) and resolved by SDS–PAGE.

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