Identification of a Urokinase Receptor-Integrin Interaction Site

PROMISCUOUS REGULATOR OF INTEGRIN FUNCTION*

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The integrin family of adhesion receptors is involved in inflammation, immunity, hemostasis, and tumor metastasis (1). Integrins are heterodimeric proteins composed of an α- and β-subunit, each with a 950–1100-amino acid extracellular domain and short (ranging from 15 to 77 amino acids) cytoplasmic tail (2). The adhesion of integrins to their ligands is not constitutive, but it is dynamically regulated by conformational changes in integrin extracellular domains, by intracellular signal transduction pathways that reorganize intracytoplasmic (especially cytoskeletal) connections, and by up-regulation and/or redistribution of integrins on the cell surface (2, 3). In part, these dynamic aspects of integrin function are regulated by the interaction of integrins with neighboring membrane-associated proteins, including tetraspan-1 superfamily members (CD9, CD63, CD81, CD82, and others) (4, 5), integrin-associated protein (CD47) (6), CD98 (7), caveolin (8, 9), and the glycosylphosphatidylinositol-anchored urokinase receptor (uPAR) (10). Recent studies have focused on the regulation of integrin function by uPAR.

Several different lines of evidence indicate that integrins interact with uPAR both physically and functionally. In monocytic cells, uPAR co-purifies with Mac-1 and Src family kinases under mild denaturing conditions (10). Immunolocalization studies show uPAR and Mac-1 to co-cluster in the presence of antibodies to either receptor and to dissociate as cells polarize in vitro in response to chemotactants, uPAR moving to the lamellipodium and Mac-1 to the uropod (11). Resonance energy transfer microscopy also indicates that uPAR dynamically associates with β2- and β1-integrins and suggests that this association is favored by integrin binding to extracellular matrices (12). Migration of tumor cells on vitronectin using αβ2 is reported to require uPAR (13). Our own studies using co-immunoprecipitation and purified recombinant proteins indicate that uPAR forms complexes with a subset of β1- and β2-integrins (14) and modulates the signaling capacity of these integrins (8).

Although uPAR appears to interact with numerous integrins, the functional consequences of integrin interactions with uPAR have been most extensively studied with β2-integrins. Human monocytic cells rendered deficient in uPAR by treatment with antisense oligonucleotides have defective Mac-1-dependent adhesion to fibronectin and migration on plastic (15). A recent study of uPAR-deficient mice reported defective Mac-1-dependent migration of neutrophils into the peritoneal cavity of mice injected with thioglycollate (16). Mac-1 and uPAR appear to form a bidirectional functional unit on monocytic cells. Induction of Mac-1 and uPAR expression on monocytic cell lines by cytokine stimulation conferred urokinase and uPAR-dependent adhesion to vitronectin, which was further promoted by engagement of Mac-1 (17). In contrast, saturation of uPAR by exogenous urokinase inhibited Mac-1 function, implying uPAR can inhibit as well as promote integrin function. A molecular explanation for this finding as well as the site(s) of interaction between Mac-1 and uPAR remain obscure.

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1 The abbreviations used are: uPAR, urokinase receptor; ATF, aminoterminal fragment of urokinase; FGN, fibronectin; BCECF AM, (2′,7′-bis-(2-carboxyethyl))-5′-(and -6′)-carboxyfluorescein, ace1toxymethyl ester; BSA, bovine serum albumin; PBS, phosphate-buffered saline; PMA, phorbol 12-myristate 13-acetate; suPAR, soluble form of uPAR; SMC, smooth muscle cell(s); CHO, Chinese hamster ovary; mAb, monoclonal antibody.

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In this study, we have used cells stably transfected with uPAR and chimeric CD18 integrins and purified, recombinant forms of these receptors to define the interaction site between uPAR and Mac-1. We identify a sequence corresponding to amino acids 424–440 in the region between the I-domain and highly conserved divergent cation binding repeats of CD11b as a critical Mac-1 binding site for uPAR. By virtue of its uPAR binding properties, a peptide composed of this sequence (M25) disrupts the association of uPAR with a subset of β1- and β2-integrins and, thereby, blocks postreceptor integrin function.

**EXPERIMENTAL PROCEDURES**

**Materials—**Two-chain active urokinase and an amino-terminal fragment (residues 1–143, ATF) of urokinase were gifts of Dr. Jack Henkin (Abbott Laboratories, Abbott Park, IL). Human plasminogen and plasminogen-free fibrinogen (FGN) were purchased from Enzyme Research Laboratories (South Bend, IN). FGN and urokinase were iodinated, as described previously (17). Transforming growth-factor β1 was from Collaborative Research Inc. (Bedford, MA), and 1,25-(OH)2 vitamin D3 was from Calbiochem. LPM19c, a monoclonal antibody (mAb) to the α-subunit of Mac-1 that blocks FGN binding to Mac-1, was purchased from DAKO Corp. (Carpinteria, CA). L2/M1, an anti-CD11b mAb, was a gift of Dr. Lloyd Klickstein (Brigham and Women’s Hospital). Polyclonal anti-CD11b (C-19) was obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). IB4, an anti-CD18 mAb, and M1/70, a rat anti-mouse CD11b mAb, were obtained from American Type Culture Collection (ATCC) (Manassas, VA). The stimulating CD18 mAb (recognizes the αI integrin) was obtained from the American Type Culture Collection. The stimulating CD18 mAb (recognizes the αIIb integrin) was obtained from Molecular Probes, Inc. (Eugene, OR). Fluorescein isothiocyanate-conjugated goat anti-mouse IgG, F(ab’2) antibody was obtained from Roche Molecular Biochemicals.

**Cell Lines and Culture Conditions—**THP-1 monocytic cells (ATCC), 293 cells with and without uPAR co-transfection, and β2-integrin (Mac-1, p150,95, and CD11b/CD11c chimeras)-transfected CHO cells with and without uPAR co-transfection were maintained as described previously (17, 18). CHO cells transfected with human Mac-1 (Mac-1 CHO), p150,95 (p150,95 CHO), or chimeric versions of Mac-1 and p150,95, generated as described previously (19). Fig. 1A schematically illustrates five different chimeric α-subunits composed of various fusions of CD11b (Mac-1) and CD11c (p150,95). For simplicity, we have previously shown that CHO cells transfected with Mac-1 bound, internalized, and degraded FGN and that this degradation by Mac-1 CHO cells is inhibited by LPM19c (percentage inhibition = 90 ± 14%), a mAb that blocks FGN binding to Mac-1, confirming the role of Mac-1 in this degradation pathway (17). For CHO cells transfected with Mac-1, p150,95, or chimeric versions of Mac-1 and p150,95, FGN turnover is inhibited by the anti-CD11b I-domain mAb LPM19c (Mac-1, MP-1–5) or anti-CD18 mAb IB4 (p150,95 and MP-6) was calculated and expressed relative to Mac-1 CHO cells in the absence of ATF.

**Soluble uPAR Binding Experiments—**The binding of biotinylated soluble human uPAR (suPAR) to purified full-length human Mac-1 or LFA-1 was performed as described previously (14). In brief, Nunc Maxisorb 96-well microtiter wells were coated with recombinant Mac-1 or LFA-1 (250 ng/ml) and then blocked with BSA. Biotinylated suPAR (0–250 nM) was added to each well in buffer containing 20 mM Hepes, 150 mM NaCl, 1 mM MnCl2, pH 7.4, and 0.05% Tween 20, and the plates were incubated for 90 min at 25 °C. After washing, avidin peroxidase was added, and biotinylated suPAR binding was quantified by measuring the absorbance at 490 nm after the addition of o-phenylenediamine dihydrochloride. suPAR bound to Mac-1 in a concentration-dependent manner (0–250 nM).

Peptide amides were synthesized and purified by Quality Controlled Biochemicals, Inc. (Hopkinton, MA). All peptides were dissolved in Me2SO at 40 mM. Nunc Maxisorb 96-well microtiter plates were coated with M25 (10 μg/ml) in PBS overnight at 37 °C and then blocked with the PBS containing 1% BSA. Biotinylated suPAR (100 nM), in the presence or absence of M25, scM25, L25, or P25 (1–50 μM), was then added to each well for 1.5 h at 25 °C. After washing, avidin peroxidase was added, and biotinylated suPAR binding was quantified as described above. Relative binding was calculated as the ratio of binding in the presence of peptide to binding in the absence of peptide.

**Immunoprecipitation—**The co-immunoprecipitation of β2 integrins, uPAR, and Src family kinases was performed exactly as previously reported (8). UPAR-transfected 293 cells were lysed on ice for 30 min in radiimmunoprecipitation buffer containing 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% deoxycholate, 1% SDS, 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate, 1 mM benzamidine, and 0.05% Tween 20, and the lysates were incubated for 90 min at 25 °C. After preclarification with protein A-agarose, lysates were incubated with antibody to β2 (JB1A) in the presence or absence of peptide M25 or scM25 (100 μM). The immunoprecipitates were blotted with antibody to uPAR (399R), stripped, and reblotted for Src family kinases (Sck and β5) (AB1937). The co-immunoprecipitation of CD11b and uPAR was performed as above by incubating lysates of wild-type Mac-1/uPAR 293 or mutant Mac-1 (pCX424–440)/uPAR 293 cells with antibody to the I-domain of CD11b (LM2/1). The immunoprecipitates were blotted with antibody to uPAR (399R), stripped, and reblotted for CD11b using a polyclonal antibody (C-19).

**Fibrinogen Binding and Degradation Assays—**The binding of 125I-labeled FGN to THP-1 monocytic cells was investigated as described previously (17), with modification. Briefly, THP-1 cells in RPMI containing 10% fetal bovine serum were added to 96-well microtiter plates (107/well) and stimulated with PMMA (7 ng/ml), a concentration of FGN washed with RPMI containing 0.5% BSA and 125I-labeled FGN (1.0 μM) was added to each well. After 60 min at 4 °C, cell-bound FGN was determined by washing the adherent cells, which were then lysed and counted. Specific binding was calculated by subtracting non-specific binding in the presence of a 20-fold excess of unlabeled FGN from total binding or by incubating cells in the presence of the Mac-1-blocking mAb LPM19c.

The degradation of 125I-labeled FGN by β2-integrin-transfected CHO cells was examined exactly as reported (17). To investigate the effect of uPAR occupancy on FGN binding and degradation, incubations with transfected CHO cells were performed in the presence of ATF (5 μM). We have previously shown that CHO cells transfected with Mac-1 bound, internalized, and degraded FGN and that this degradation by Mac-1 CHO cells is inhibited by LPM19c (percentage inhibition = 90 ± 14%), a mAb that blocks FGN binding to Mac-1, confirming the role of Mac-1 in this degradation pathway (17). For CHO cells transfected with Mac-1, p150,95, or chimeric versions of Mac-1 and p150,95, FGN turnover is inhibited by the anti-CD11b I-domain mAb LPM19c (Mac-1, MP-1–5) or anti-CD18 mAb IB4 (p150,95 and MP-6) was calculated and expressed relative to Mac-1 CHO cells in the absence of ATF.

The region X424–440 was verified by flow cytometry and Western blotting.

**Maturation of the uPAR Binding Site within CD11b—**The region 424–440 of CD11b was switched to its homologous sequence (424–440) of CD11c in THP-1 cells by polymerase chain reaction. Briefly, the fragment containing the mutations was amplified using the following primers (the bases mutated are underlined): 5′-ATCAGCAAGCAAGGTAAAGGCGATTCTTCAGGCCAGTGGCAGTGGGAGGTCC-3′ and 5′-ACATCTGAAATACATCTTGTGCGACTTCTCC3′, purified, and digested with XbaI. The digested fragment containing the mutations was then purified and transferred back into the expression vector (pCIss2M) containing the full-length CD11b with GFP and Apel restriction sites. Stable cell lines expressing the mutated CD11b, together with wild-type CD18 and uPAR were established by transfecting the uPAR-expressing 293 cells with the expression vectors containing CD11b and CD18 according to our described method (20). Expression of uPAR, Mac-1, and the Mac-1 mutant containing ΔX424–440 was verified by flow cytometry and Western blotting.
In the case of neutrophil adhesion experiments, low passage (passage 1–3) human saphenous vein endothelial cells were grown to confluence in 96-well microtiter wells and stimulated with tumor necrosis factor-α (10 ng/ml) for 4 h to up-regulate intercellular adhesion molecule-1 expression (21). Thrombolyte-elicited murine neutrophils, obtained as reported (22), were loaded with BCECF AM for 30 min at 37 °C, washed, and stimulated with formylmethionylleucylphenylalanine (1 μM) prior to adding to endothelial cell monolayers.

**Migration Assay—**Haptotactic smooth muscle cell migration was examined as recently reported (8). In brief, passage 1–3 human saphenous vein smooth muscle cells (SMC) (10²) were seeded into Transwell inserts (Becton Dickinson) precoated on the bottom with fibronectin (10 μg/ml) or collagen type I (20 μg/ml) and cultured overnight with or without peptide M25 (25–100 μM) or scM25 (100 μM). Cells on both sides of the filter were detached and counted. All assays were performed in triplicate, and the data were expressed as percentage of inhibition by the peptides.

**RESULTS**

**Identification of a uPAR Binding Sequence in Mac-1**—Prior observations have demonstrated that biotinylated suPAR binds to purified Mac-1 immobilized on plastic (14). Binding of suPAR to Mac-1 requires “active” Mac-1, being stimulated by Mn²⁺ or the Mac-1-activating antibody KIM127 and completely inhibited by EDTA. However, solid phase adhesion assays similar to that for purified Mac-1 showed much less binding of biotinylated suPAR to purified LFA-1. This raised the possibility that uPAR was restricted in its interaction with Mac-1, i.e. CD11a (LFA-1), CD11b (Mac-1), CD11c (p150,95), and CD11d. To explore this possibility and define the domains within Mac-1 responsible for this interaction, human uPAR was co-transfected into members of the (CD11/CD18) family, i.e. CD11a (LFA-1), CD11b (Mac-1), CD11c (p150,95), and CD11d. To confirm this possibility and define the domains within Mac-1 responsible for this interaction, human uPAR was co-transfected into CHO cells expressing wild-type Mac-1, wild-type p150,95, or chimeric versions of Mac-1 and p150,95. Fig. 1A schematically illustrates six different chimeric α-subunits composed of various fusions of CD11b (Mac-1) and CD11c (p150,95) (19). We have previously shown that FGN binding and degradation by CHO cells transfected with Mac-1 is inhibited by LPM19c, a mAb that blocks FGN binding to Mac-1, confirming the role of Mac-1 in this degradation pathway (17). CHO cells transfected with p150,95, a known fibrinogen receptor (23), also bound and degraded FGN, and this FGN turnover was inhibited by IB4 (inhibition = 87%), an anti-CD18 mAb that blocks CD11/CD18 function. CHO cells transfected with various chimeric integrins internalized and degraded FGN in a CD11/CD18-dependent manner (Table I). Successful co-transfection of uPAR into CD11/CD18 CHO cells was confirmed by Western blotting, urokinase binding, and flow cytometry. The addition of ATF to Mac-1/uPAR CHO cells blocked FGN turnover by 79% (Table I), consonant with prior studies in monocytes and monocytic cell lines; in contrast, the addition of ATF to p150,95/uPAR CHO cells had a reduced effect (34% inhibition) on FGN turnover. Inhibition by ATF was preserved in the Mac-1/p150,95 CHO cell chimeras designated MP-3 (71% inhibition) and MP-6 (67% inhibition). This inhibitory pattern suggested a possible interaction site between uPAR and Mac-1 in a region between the I-domain and the highly conserved divalent cation repeats of CD11b. The CD11b amino acid sequence of a portion of this region is indicated in Fig. 1B. Inspection of this sequence revealed a previously unrecognized homology to peptide 25 (STYHILSLGYMYTLN), a uPAR-binding peptide previously identified in a phage display library (14). We have termed this corresponding Mac-1 sequence (αM424–440, PRYQHIQLVAMFQRQNTG) M25. Homologies to the corresponding sequences in LFA-1 and p150,95 are also shown in Fig. 1B.

To determine if suPAR actually bound to M25, solid-phase binding assays were done with immobilized M25. Biotinylated suPAR (100 nM) bound to M25, and this binding was blocked by soluble M25 (IC₅₀ ~ 2 μM) but not by a scrambled version of M25 (scM25) (Fig. 2A). The specificity of suPAR-M25 interaction was explored further using soluble peptides corresponding to sequences from LFA-1 (L25) or p150,95 (P25). L25 and P25 had no inhibitory effect on suPAR binding to M25, indicating that suPAR interacts preferentially with this Mac-1 sequence. The capacity of uPAR-expressing cells to attach to immobilized M25 was also tested. The monocytic cell line THP-1 expresses uPAR in response to stimulation with cytokines and exhibits urokinase-dependent adhesion to the matrix protein vitronectin (24). Cytokine-stimulated THP-1 cells bound to immobilized M25 in a urokinase-dependent manner but did not attach to scM25 (Fig. 2B), L25, or P25 (data not shown). Attachment to M25 was stimulated by either 10 nm active urokinase (Fig. 2B) or ATF (data not shown). Binding was blocked by prior treatment of the cells with phosphonositol-specific phospholipase C, which removes most cell surface uPAR, and by a polyclonal anti-uPAR antibody, 399R (25) (Fig. 2B). Preferential binding of uPAR-transfected 293 cells as compared with nontransfected 293 cells to immobilized M25 was also observed (data not shown). Together, these observations indicate that uPAR directly binds a linear sequence in Mac-1 and that this binding is promoted by urokinase.

To assess the importance of the linear sequence comprised by M25 on the overall interaction between Mac-1 and uPAR, we created a mutant Mac-1 in which the M25 sequence (αM424–440) was replaced with the homologous sequence of p150,95 (αX424–440). Wild type and mutant Mac-1 cDNAs were co-transfected into uPAR-expressing 293 cells, and stable cell lines expressing both uPAR and Mac-1 or the mutant Mac-1
The effect of urokinase receptor occupancy by ATF on FGN binding and degradation by transfected CHO cells was investigated as described under “Experimental Procedures.” Equivalent uPAR expression in uPAR-transfected Mac-1/p150,95 chimeras was verified by specific 125I-labeled urokinase binding and flow cytometry. ATF (5 nM) was preincubated with the cells for 30 min prior to the addition of 125I-labeled FGN. FGN turnover inhibitable by the anti-CD11b I-domain mAb LPM19c (Mac-1, MP-1–5) or anti-CD18 mAb IB4 (p150,95 and MP-6) was calculated and expressed relative to Mac-1/uPAR CHO cells in the absence of ATF. Values represent mean ± S.E. (n = 3–5).

TABLE I

| α-Subunit | FGN turnover without ATF | FGN turnover with ATF | Inhibition (+ATF vs. −ATF) |
|-----------|--------------------------|-----------------------|---------------------------|
|           | % Mac-1/uPAR CHO         | % Mac-1/uPAR CHO       | %                         |
| Mac-1     | 100 ± 12                 | 21 ± 2*                | 79                        |
| p150,95   | 85 ± 23                  | 56 ± 10                | 34                        |
| MP-1      | 122 ± 13                 | 82 ± 25                | 33                        |
| MP-2      | 95 ± 10                  | 101 ± 18               | 0                         |
| MP-3      | 133 ± 12                 | 38 ± 13                | 71                        |
| MP-4      | 110 ± 10                 | 116 ± 17               | 0                         |
| MP-5      | 95 ± 18                  | 105 ± 14               | 0                         |
| MP-6      | 90 ± 13                  | 30 ± 4*                | 67                        |

*a p < 0.05.

**Fig. 2.** Identification of uPAR binding sequence in Mac-1. A, recombinant uPAR binds M25. Linear peptides spanning amino acids 424–440 of LFA-1, Mac-1, and p150,95 (a region homologous to peptide 25; Fig. 1) were synthesized and designated L25, M25, and P25, respectively. A scrambled version of M25 (HQIPAYRGVNQRFTML, scM25), was used as a control. Biotinylated suPAR (100 nM) was added to microtiter wells coated with M25 (10 μg/ml) and blocked with BSA. The specificity of suPAR binding to M25-coated wells was investigated by preincubating suPAR with M25 ( ), scM25 ( ), P25 ( ), and L25 ( ) (1–50 μM). Binding of suPAR in the presence of peptides is expressed as percentage of suPAR binding in the absence of peptides (% control). Values represent averages of duplicate determinations of two or three separate experiments.

B, uPAR-expressing cells bind M25. Cytokine-stimulated THP-1 cells expressing uPAR were added to microtiter wells coated with M25 and scM25 (80 μg/ml) and blocked with BSA. The role of uPAR in cellular adhesion to M25 was examined by incubating cells with urokinase or ATF (10 μM), polyclonal anti-uPAR antibody, or phosphoinositol-specific phospholipase. After washing, adhesion was quantified by staining cells with Geimsa and measuring absorbance at 540 nm. Data shown are representative of three independent experiments.

were established. Comparable expression of CD11/CD18 was verified by fluorescence-activated cell sorting in wild-type and mutant Mac-1 293 cells co-transfected with uPAR; immuno-

blots of 293 cell lysates and fluorescence-activated cell sorting analysis verified high levels of uPAR expression in all of the co-transfectants (data not shown). As indicated in Fig. 3A, uPAR co-precipitates with CD11b but not with CD11b containing αx2424–440, indicating that αM424–440 is critical to the formation of stable Mac-1/uPAR complexes. We next assessed the importance of this interaction site to Mac-1 function. Mac-1-transfected 293 cells, unlike nontransfected cells, adhere to fibronectin in the presence of manganese (Fig. 3B). Co-transfection of uPAR with Mac-1 in 293 cells inhibits Mac-1-dependent adhesion to fibronectin, consistent with data in Table I indicating that ATF-uPAR blocks Mac-1 function in CHO cell co-transfectants. Interestingly, in 293 cells expressing high levels of uPAR, unlike CHO cell co-transfectants (Table I) or THP-1 cells (17), inhibition of Mac-1 function becomes independent of urokinase. In contrast, although uPAR is present at high levels, the Mac-1 mutant adhered to fibronectin as if unaffected by uPAR (Fig. 3B). Thus, we conclude that the αM424–440 sequence within Mac-1 is a critical site for interaction with and regulation by uPAR.

M25 Disrupts Complex Formation between uPAR and β1- and β3-Integrins—Having demonstrated that M25 binds uPAR, we tested the ability of M25 to compete with β1- and β3-integrin binding to uPAR, as we have previously reported for the phage display-generated peptide 25 (14). As indicated in Fig. 4A, the addition of M25 (75 μM) to lysates of uPAR-transfected 293 cells completely disrupted co-precipitation of uPAR with β1-integrins, whereas a scrambled M25 had no effect. Analogous to peptide 25, M25 blocked co-precipitation of Src family kinases with β1-integrins. In these cells, association of Src-family kinases with β1-integrins is uPAR-dependent (8). In identical experiments, we could not co-precipitate β2-integrins and uPAR in THP-1 cells, possibly because the level of uPAR expression is lower in THP-1 cells than uPAR-transfected 293 cells. Since uPAR/β1-integrin co-precipitation in Triton X-100 lysates requires the presence of caveolin (8), it is also possible that caveolin does not stabilize uPAR/β3-integrin complexes in these cells. Therefore, the effect of M25 on uPAR/β3-integrin interaction was examined with purified proteins. Binding of biotinylated suPAR (0–100 μM) to microtiter wells coated with purified Mac-1 was inhibited by M25 in a dose-dependent manner (IC50 ~ 25 μM) but not by scM25 (Fig. 4B).

Effects of M25 on Integrin Function—Mac-1 and uPAR appear to be functionally as well as physically linked on cells expressing these receptors. Therefore, we tested the effects of M25, which disrupts the physical association between uPAR and Mac-1 (Fig. 4B), on the function of these receptors in monocytic THP-1 cells. M25 blocked Mac-1-dependent adhesion of THP-1 cells to immobilized FGN in a dose-dependent
manner (IC$_{50}$ ~ 25 μM) (Fig. 5A) but did not inhibit soluble FGN binding to adherent THP-1 cells (Fig. 5B). In fact, Mac-1-dependent binding of FGN to THP-1 cells was enhanced several-fold in the presence of M25. M25 also inhibited Mac-1-dependent adhesion of thioglycollate-elicited murine neutrophils to stimulated endothelial cells (Fig. 5C). The control peptide scM25 and the corresponding LFA-1 and p150,95 sequences, L25 and P25, respectively, had no antiadhesive effects.

We have previously shown that mAbs that block Mac-1 function or a uPAR binding phage display peptide 25, which disrupts integrin-uPAR complexes, greatly diminished (>80%) uPAR-dependent adhesion of PMA-stimulated monocytes to serum or vitronectin (14, 17). We tested whether M25 would block such adhesion. PMA-stimulated THP-1 cell adhesion to vitronectin was completely blocked by M25 (Fig. 6). Although scM25 and P25 had no inhibitory effect, weak inhibition of the corresponding LFA-1 peptide, which has the closest homology to M25, is discernible. Interestingly, in these cells M25 had no effect on THP-1 cell attachment to fibronectin (not shown).

In addition to associating with Mac-1 in leukocytes, uPAR physically associates with β$_1$-integrins and regulates their function in epithelial cells and smooth muscle cells (8, 14). Concordant with its ability to disrupt uPAR-β$_1$ complexes (Fig. 4A), M25 also inhibited β$_1$-integrin function. Early passage human vascular SMC, utilizing β$_1$-integrins (14, 26), spread slowly on fibronectin in the presence of 75 μM M25, whereas scM25 had no effect (not shown). Within 2 h, however, spreading and adhesion of SMC on fibronectin were comparable. Based on these observations, we tested the effects of M25 on haptotactic migration of SMC toward fibronectin and collagen type I (Fig. 7). M25 markedly inhibited SMC migration toward either fibronectin or collagen in a dose-dependent manner, whereas scM25 had no effect.

**FIG. 3.** uPAR fails to complex with or influence the function of a Mac-1 mutant containing x424–440. A, uPAR and CD11b were co-immunoprecipitated by incubating lysates from 293 cells co-transfected with uPAR and wild-type Mac-1 (wt-M/U) or the Mac-1 mutant containing x424–440 (mut-M/U) with anti-CD11b mAb (LM2/1). The immunoprecipitates or whole cell lysates were blotted with polyclonal anti-uPAR (399R), stripped, and rebotted for CD11b (C-19). B, Mac-1-expressing 293 cells (wt-M) and 293 cell co-transfectants expressing either Mac-1 or mutant Mac-1 with uPAR were incubated in fibronogen-coated microtiter wells in the presence or absence of 5 mM MnCl$_2$ for 1 h at 37°C and washed, and adherent cells were then revealed by Giemsa staining. Data shown are representative of three independent experiments.

**DISCUSSION**

In this study, we have identified a non-I-domain peptide sequence, M25, within the α-chain of Mac-1 (x424–440) that critically regulates the interaction of Mac-1 to the non-integrin receptor uPAR. On the basis of homology with G protein-coupled receptors, Springer (27) has proposed that the N-terminal region (~450 amino acids) of integrin α-subunits folds into a β-propeller. In this model (see Fig. 1B), repeating units (W1–W7) of anti-parallel β-sheets connected by surface loops (~60 amino acids/unit) arrange into a torus around a small central cavity. The upper surface loops are thought to contain the major ligand-binding sites that synergize with the RGD-binding site on the β-chain to define the specificity and affinity of interactions of integrins with their ligands. The disc-like propeller is connected to and spans the plasma membrane via a C-terminal stalk. In β$_2$-integrins, a 200-amino acid 1-domain, also directly implicated in ligand binding, is inserted between repeats 2 and 3 (19, 28). In the β-propeller model, M25 comprises the entire upper loop sequence of W4 and extends into the third β-strand of this repeat. Since the upper W4 loops of other integrin α-chains have been directly implicated in integrin ligand binding (29), our results indicate that uPAR, which does not contain an RGD sequence, may now be considered as an atypical integrin ligand. As a non-I-domain binding ligand influencing the function of Mac-1, uPAR resembles factor X (30, 31).
recognition that uPAR is a cis-acting integrin ligand may explain several previously obscure features of the interaction between Mac-1 and uPAR: the strong promoting effect of the “active” state of Mac-1 on uPAR/Mac-1 binding (14), the ability of suPAR to block the binding of known ligands such as factor X and FGN to Mac-1 (17), and the co-localization of uPAR and Mac-1 on leukocytes expressing these receptors (10, 12). In addition, the positioning of M25 on Mac-1 may also explain our observation that disruption of uPAR/Mac-1 association on PMA-stimulated THP-1 cells by M25 promotes FGN binding to these cells (Fig. 5B). The I-domain binding site for FGN and the W4 binding site for uPAR may be in close proximity in the folded Mac-1. If so, the additional finding that the urokinase promotes the binding of uPAR to Mac-1, as reflected in M25 binding (Fig. 5B), may explain why saturation of uPAR with urokinase inhibits FGN binding/turnover by Mac-1 (17) (Table I). Finally, our observations may also explain the suggestion of other investigators that uPAR may “activate” integrins (16). Ligand binding to integrins is a recognized mechanism for integrin activation (2). The intriguing possibility that uPAR may activate Mac-1 via trans-type intercellular interactions as well as through the cis-type interactions identified here remains to be explored.

The specificity of the interaction between M25 and uPAR was established using peptides corresponding to analogous sequences from LFA-1 (L25) or p150,95 (P25). Biotinylated suPAR bound immobilized M25, and this binding was blocked efficiently by soluble M25 but not by scM25, L25, or P25. These results indicate that among β3-integrins uPAR appears to react preferentially with Mac-1 in this otherwise homologous (>80%) α-subunit region between the I-domain and highly conserved divalent cation repeats. However, transient physical linkage between uPAR and p150,95 has been reported in activated neutrophils using resonance energy transfer microscopy (32). This is consistent with data reported in Table I that uPAR has a weak effect on p150,95 function in CHO cell co-transfectants. A recent study of uPAR-deficient mice reported defective LFA-1-dependent migration of neutrophils into the peritoneal cavity of mice injected with thioglycollate (16). Although the M25 site on Mac-1 appears to be the most robust interaction site for uPAR among β3-integrins, we cannot exclude additional interaction site(s) between uPAR and Mac-1 or other β3-integrins. Our recent studies and that of other investigators indicate that uPAR interacts not only with Mac-1 but with a class of β3-integrins. Although the binding site(s) for uPAR on these integrins is currently uncertain, the rules of physical interac-

31). The precise region(s) within Mac-1 mediating factor X binding is, however, unknown. Preliminary experiments indicate that factor X fails to compete with suPAR binding to immobilized M25 (data not shown).

Identification of a uPAR-binding sequence in αM and the
tion between uPAR and β₁ integrins appear to be similar to those observed for Mac-1: binding of suPAR to α₅β₁ is promoted by activating TS2/16 antibodies, and matrix engagement of integrins, we favor the view that its primary role in the number of ligand binding sites available. For example, the binding of soluble ligands can be supported by the solubility of the integrin receptors. Whether uPAR and other nonintegrin membrane proteins, which are reported to bind β₁- and/or β₂-integrins (4, 5) (e.g. tetraspan-4 superfamily members), share a common site of interaction with uPAR also remains to be defined.

Although binding of uPAR to Mac-1 and other integrins inhibits additional ligand binding to this integrin, as reflected by dramatically altered adhesion in Fig. 3B, numerous studies document that uPAR promotes normal β₁- and β₂-integrin functions, including Mac-1-dependent adhesion to FGN and endothelial cells in vitro (15, 17) and vascular emigration in vivo (16). The findings reported here that M25 impairs Mac-1-dependent binding of soluble ligand to FGN and stimulated cell signaling, which collectively termed “post-receptor binding events” (3). Because uPAR promotes the association of signaling molecules with integrins, we favor the view that its primary role in integrin clusters is to promote the post-receptor binding events important to β₁- and β₂-integrin function. This hypothesis implies that uPAR is present within integrin clusters in far fewer numbers than the integrin molecules themselves. Viewed in this perspective, the ligand-like binding of uPAR to Mac-1 is a mechanism to enrich and localize integrin clusters with important signaling molecules necessary for efficient integrin signaling, although this may be at the expense of decreasing the total number of ligand binding sites available. For example, the number of FGN binding sites on THP-1 cells is lower when uPAR is associated with Mac-1, and yet THP-1 cells only adhere well to FGN when uPAR is Mac-1-associated (Fig. 5). Available data are consistent with a similar role for uPAR in regulating post-receptor binding events important to β₁-integrin function (Fig. 7; Refs. 8 and 34). In contrast to β₁-integrin- uPAR complexes, however, important signaling molecules interacting with Mac-1 as a consequence of uPAR association remain undefined.

Currently, strategies to inhibit integrin function in settings of deleterious inflammation and tumor progression are based on the possibility that inhibition of integrin/uPAR interactions could complement existing strategies for therapeutic regulation of integrin function. A particularly attractive feature of this strategy is that uPAR is not constitutively expressed on most cells or is expressed only at low levels. Instead, uPAR is strongly up-regulated on migrating inflammatory cells and on tumor cells, where uPAR expression strongly correlates with tumor progression (34, 35). Thus, reagents such as M25 may have the benefit that they should only impair integrin function of the cells most desirable to target.

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Identification of a Urokinase Receptor-Integrin Interaction Site: PROMISSCUOUS REGULATOR OF INTEGRIN FUNCTION
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