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Urokinase Induces Basophil Chemotaxis through a Urokinase Receptor Epitope That Is an Endogenous Ligand for Formyl Peptide Receptor-Like 1 and -Like 2[1]

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Basophils circulate in the blood and are able to migrate into tissues at sites of inflammation. Urokinase plasminogen activator (uPA) binds a specific high affinity surface receptor (uPAR). The uPA-uPAR system is crucial for cell adhesion and migration, and tissue repair. We have investigated the presence and function of the uPA-uPAR system in human basophils. The expression of uPAR was found at both mRNA and protein levels. The receptor was expressed on the cell surface of basophils, in the intact and cleaved forms. Basophils did not express uPA at either the protein or mRNA level. uPA (10^{-12}–10^{-9} M) and its uPAR-binding N-terminal fragment (ATF) were potent chemoattractants for basophils, but did not induce histamine or cytokine release. Inactivation of uPA enzymatic activity by di-isopropyl fluorophosphate did not affect its chemotactic activity. A polyclonal Ab against uPAR inhibited uPA-dependent basophil chemotaxis. The uPAR-derived peptide 84–95 ($uPAR_{84–95}$) induced basophil chemotaxis. Basophils expressed mRNA for the formyl peptide receptors formyl peptide receptor (FPR), FPR-like 1 (FPRL1), and FPRL2. The FPR antagonist cyclosporin H prevented chemotaxis induced by FMLP, but not that induced by uPA and $uPAR_{84–95}$. Incubation of basophils with low and high concentrations of FMLP, which desensitize FPR and FPRL1, respectively, but not FPRL2, slightly reduced the chemotactic response to uPA and $uPAR_{84–95}$. In contrast, desensitization with WKYMVm, which also binds FPRL2, markedly inhibited the response to both molecules. Thus, uPA is a potent chemoattractant for basophils that seems to act through exposure of the chemotactic uPAR epitope $uPAR_{84–95}$, which is an endogenous ligand for FPRL2 and FPRL1. ***The Journal of Immunology,*** **2004, 173: 5739–5748.**

The urokinase plasminogen activator (uPA)[3] is a serine protease that activates plasminogen to plasmin and binds to a specific high affinity cell surface receptor, uPAR (CD87) (1–3). The uPAR is synthesized as a single polypeptide chain of 313 aa residues, preceded by a 21-residue signal peptide. Post-translational events lead to cleavage of the last 30 C-terminal residues and the attachment of a GPI tail to Gly^{283} (4). The mature protein has a three-domain structure: D1 is the N-terminal domain and binds uPA, D2 connects D1 and D3, and D3 is the C-terminal domain that anchors the molecule to the membrane through the GPI tail (4). Domains 1, 2, and 3 are connected by linker regions. The uPAR can be cleaved within the D1/D2 linker region by several proteolytic enzymes, including uPA itself (5, 6). The cleavage causes the release of D1 from the molecule. Therefore, uPAR can exist on the cell surface in either a three-domain form (D1D2D3), which is capable of binding uPA, or a two-domain form (D2D3), which does not bind uPA (5). Despite the lack of a transducing cytoplasmic tail, the uPA receptor is able to activate cell signaling pathways, probably by interacting with other cell surface proteins, such as integrins (7, 8) and FMLP receptors (9, 10), that interact with the cell interior (11).

There is increasing evidence that the uPA/uPAR system plays a role in the chemotaxis of inflammatory cells in vivo and in vitro. Migration of immune cells to tissue lesions is impaired in $uPA^{-/-}$ and $uPAR^{-/-}$ mice, resulting in impairment of host defenses, bacterial spread, and death (12–14). Chemotaxis of inflammatory cells stimulated by uPA in vitro and in vivo requires binding to uPAR (15–17) and the existence of a transmembrane adapter (15, 18). It has been suggested that a cleaved form of uPAR induces chemotaxis of THP-1 monocyte-like cells through the activation of a seven-transmembrane (STM) receptor formyl peptide receptor (FPR)-like 1 (FPRL1) (9).

Several natural *N*-formyl peptides, including the prototype FMLP, have been purified from bacterial supernatants, which suggests that they are biologically relevant ligands for formyl peptide receptors. FMLP binds and activates G protein-coupled STM receptor (19). Three STM receptors expressed by phagocytic leukocytes have been identified and cloned: neutrophils express the high affinity receptor FPR and its homologue FPRL1, whereas monocytes express FPR, FPRL1, and FPRL2 (20, 21). FPR is a high affinity receptor for FMLP, whereas FPRL1 has a much lower affinity (22, 23). FPRL1 is a promiscuous receptor activated by serum amyloid A (24), the prion peptide $PrP_{106–126}$ (25), lipoxin A_4 (26), and various bacterial and synthetic peptides (27). In

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[3] Abbreviations used in this paper: uPA, urokinase plasminogen activator; ATF, N-terminal fragment; CsH, cyclosporin H; DFP, di-isopropyl fluorophosphate; $Hp_{2–20}$, *Helicobacter pylori*-derived peptide; LDH, lactate dehydrogenase; STM, seven transmembrane; uPAR, uPA receptor. FPR, formyl peptide receptor; FPRL1, FPR-like 1.

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addition, we recently demonstrated that two HIV-1 gp41 peptides act as chemoattractants for human basophils by interacting with FPRL1 (28). Cyclosporin H (CsH) and spinorphin are specific antagonists of the FMLP receptor subtype FPR (29–31).

Chemotaxis of human basophils can be stimulated by heterogeneous stimuli such as bacterial and viral peptides (28), PGD_2 (32), various chemokines (33–35), and anaphylatoxins (36). The uPA/uPAR system and its functions have not yet been characterized in human basophils. We therefore investigated the expression of the uPA/uPAR system and its possible interaction with members of the FMLP receptor family in human basophils. Our results demonstrate that human basophils express uPAR, but not uPA. However, uPA is a potent stimulus for basophil chemotaxis that seems to act consequent to the exposure of a uPAR chemotactic epitope that is a ligand of FPRL2 and FPRL1.

Materials and Methods

Reagents

The following were purchased: di-isopropyl fluorophosphate (DFP; Fluka, Buchs, Switzerland); 60% $HClO_4$ (Baker Chemical, Deventer, The Netherlands); human serum albumin, PIPES, and protease inhibitors (Sigma-Aldrich, St. Louis, MO); HBSS, FCS, TRIzol, and SuperScript II (murine Moloney leukemia virus reverse transcriptase (Invitrogen Life Technologies, Grand Island, NY); FMLP (Calbiochem, La Jolla, CA); RPMI 1640 (Invitrogen Life Technologies, Gaithersburg, MD) with 25 mM HEPES buffer, Eagle's MEM (Flow Laboratories, Irvine, U.K.); Dextran 70 and Percoll (Pharmacia Biotech, Uppsala, Sweden); rabbit anti-uPAR 399 polyclonal Ab and the uPA N-terminal fragment (ATF) (American Diagnostica, Greenwich, CT); HRP-conjugated anti-rabbit IgG and protein colorimetric assay (Bio-Rad, Richmond, CA); PE-labeled anti-IgE Abs (Caltag Laboratories, Burlingame, CA); FITC-labeled goat anti-rabbit IgG (Abcam, Cambridge, U.K.); ECL detection kit (Amersham Biosciences, Little Chalfont, U.K.); polyvinylidene fluoride filters (Millipore, Bedford, MA); PCR kit (PerkinElmer, Branchburg, NJ); and chemotaxis polyvinyl pyrrolidone-free filters (NeuroProbe, Cabin John, MD). The hexapeptide WKYMVm was synthesized by Innovagen (Lund, Sweden); the peptide $uPAR_{84–95}$ (AVTYSRSRYLEC) and its scrambled version (TLVEYY SRASCR) were synthesized by PRIMM (Milan, Italy). CsH was obtained from Drs. D. Romer and E. Rissi (Novartis, Basel, Switzerland).

Buffers

The PIPES buffer used in these experiments was made up of 25 mM PIPES (pH 7.4), 110 mM NaCl, and 5 mM KCl. The mixture is referred to as P. PCG contains, in addition to P, 5 mM $CaCl_2$ and 1 g/l D-glucose (37). PACGM contains, in addition to P, human serum albumin 3%, 1 mM $CaCl_2$, 1 g/l dextrose, and 0.25 g/l $MgCl_2 \cdot 6H_2O$ (pH 7.4); PGMD contains 0.25 g/l $MgCl_2 \cdot 6H_2O$, 10 mg/l DNase, and 1 g/l gelatin in addition to P, pH 7.4. PBS contains 8 g/l NaCl, 1.15 g/l Na_2HPO_4, 200 mg/l KCl, and 200 mg/l KH_2PO_4 (pH 7.4).

Purification of peripheral blood basophils

Basophils were purified from the peripheral blood of healthy volunteers, aged 20–39 yr, who were negative for HIV-1 and HIV-2 Abs. Buffy coat cell packs were provided by the Immunohematology Service (University of Naples Federico II). Informed consent, according to the guidelines of the University of Naples Federico II institutional review board for the use of humans in research, was obtained. Cells were reconstituted in PBS containing 0.5 g/l human serum albumin and 3.42 g/l sodium citrate, and loaded onto a countercurrent elutriator (Beckman Coulter, Fullerton, CA). Several fractions were collected, and fractions with $>20 \times 10^6$ basophils and a good purity (>15%) were enriched by discontinuous Percoll gradients. Basophils were further purified to near homogeneity (>98%) by depleting B cells, monocytes, NK cells, dendritic cells, erythrocytes, platelets, neutrophils, eosinophils, and T cells, using a mixture of hapten conjugated CD3, CD7, CD14, CD15, CD16,CD36, CD45RA, and anti-HLA-DR Abs and MACS MicroBeads (Miltenyi Biotec, Bergisch Gladbach, Germany) coupled to an anti-hapten mAb. The magnetically labeled cells were depleted by retaining them on a MACS column in the magnetic field of the MidiMACS (Miltenyi Biotec). Yields ranged from $3–10 \times 10^6$ basophils, with purity >99%, as assessed by basophil staining with Alcian Blue and counting in a Spiers-Levy eosinophil counter (38).

Cell culture

The THP-1 monocyte-like cell line was grown in RPMI 1640 medium supplemented with 10% heat-inactivated FCS (39).

Inactivation of uPA

The uPA was inactivated by incubation with 10 mM DFP for 2 h at 4°C (40).

Flow cytometric analysis of surface molecules

Flow cytometric analysis of cell surface molecules was performed as described previously (38). Briefly, after saturation of nonspecific binding sites with total rabbit IgG, cells were incubated for 20 min at 4°C with specific or isotype control Abs. For indirect staining, this step was followed by a second incubation for 20 min at 4°C with an appropriate anti-isotype-conjugated Ab. Finally, cells were washed and analyzed with a FACSCalibur cytofluorometer using CellQuest software (BD Biosciences, San Fernando, CA). A total of 10^4 events for each sample were acquired in all cytofluorometric analyses.

Western blot

Basophils or THP-1 cells were lysed in 1% Triton X-100/PBS in the presence of protease inhibitors; the protein content was measured by a colorimetric assay. Fifty micrograms of protein was electrophoresed on a 9% SDS-PAGE under nonreducing conditions and transferred onto a polyvinylidene fluoride membrane. The membrane was blocked with 5% nonfat dry milk and probed with 1 μg/ml of an anti-uPAR polyclonal Ab. Finally, washed filters were incubated with HRP-conjugated anti-rabbit Ab and detected by ECL (10).

RT-PCR

Total cellular RNA was isolated by lysing cells in TRIzol solution according to the supplier's protocol. RNA was precipitated and quantitated by spectroscopy. Five micrograms of total RNA was reversely transcribed with random hexamer primers and 200 U of murine Moloney leukemia virus reverse transcriptase. One microliter of reverse transcribed DNA was then amplified, using FPR-specific 5′ sense (ATG GAG ACA AAT TCC TCT CTC) and 3′ antisense (CAC CTC TGC AGA AGG TAA AGT) primers, FPRL1-specific 5′ sense (CTT GTG ATC TGG GTG GCT GGA) and 3′ antisense (CAT TGC CTG TAA CTC AGT CTC) primers, FPRL2-specific 5′ sense (CTG AAA TGT TTC AGG TGT GGG) and 3′ antisense (TGA ACG CAG GGT AGA AAG AGA) primers, uPA-specific 5′ sense (AAA ATG CTA TGT GCT GCT GAC C) and 3′ antisense (CCC TGC CCT GAA GTC GTT AGT G) primers, uPAR-specific 5′ sense (CTG CGG TGC ATG CAG TGT AAG) and 3′ antisense (GGT CCA GAG GAG AGT GCC TCC) primers, and GAPDH-specific 5′ sense (TTC ACC ACC ATG GAG AAG GCT) and 3′ antisense (ACA GCC TTG GCA GCA CCA GT) primers, as a control. Semiquantitative PCR was performed in a thermocycler for the indicated number of cycles at 57°C for FPR and FPRL1, at 64°C for FPRL2, and at 62°C for uPAR and uPA. The reaction products were analyzed by electrophoresis in 1% agarose gel containing ethidium bromide, followed by photography under UV illumination (10).

Chemotaxis assay

Modified Boyden chambers were used for chemotaxis assays. Twenty-five microliters of PACGM buffer with or without the indicated concentrations of chemoattractants was loaded in the lower compartments of a 48-well microchemotaxis chamber (NeuroProbe). The lower compartments were covered with 5-μm pore size polyvinyl pyrrolidone-free polycarbonate membranes. Fifty microliters of the cell suspension (5×10^4/well), resuspended in PACGM, was pipetted in the upper compartment. The chemotactic chamber was incubated for 1 h at 37°C in a humidified incubator with 5% CO_2 (Automatic CO_2 Incubator, model 160IR; ICN Flow Laboratories, McLean, VA). At the end of incubation, the membrane was removed; the upper side was washed with PBS, and the filter was fixed, stained with May-Grünwald/Giemsa, and mounted on a microscope slide with Cytoseal (Stephen Scientific, Springfield, NJ). Basophil chemotaxis was quantitated microscopically by counting the number of cells attached to the surface of a 5-μm pore size cellulose nitrate filter (38). In each experiment, 10 fields/triplicate filter were measured at ×40 magnification. The results were compared with buffer controls. Checkerboard analysis was used to discriminate chemotaxis and nondirect migration (chemokinesis) of basophils. In these experiments basophils were placed in the upper chemotactic chambers, and various concentrations of stimuli or buffer were added to the upper wells, lower wells, or both. Spontaneous migration (chemokinesis) was determined in the absence of chemoattractants or when stimuli were added to

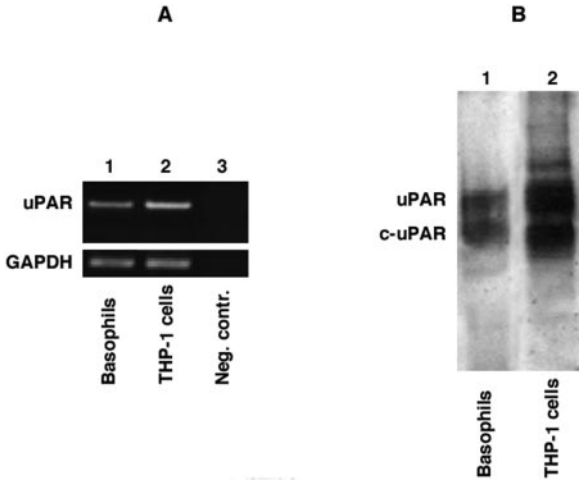

FIGURE 1. *A*, uPAR expression in human basophils. Purified human basophils (*lane 1*) and THP-1 monocyte-like cells, as a positive control (*lane 2*), were lysed in TRIzol. Total RNA was prepared, reverse transcribed, and amplified by 40 PCR cycles in the presence of uPAR-specific primers and GAPDH primers, as a loading control. PCR amplification of buffer represented the negative control (*lane 3*). PCR products were analyzed by electrophoresis in 1% agarose gel containing ethidium bromide, followed by photography under UV illumination. *B*, Basophils (*lane 1*) and THP-1 monocyte-like cells, as a positive control (*lane 2*), were lysed in Triton X-100, and 50 μg of total proteins were analyzed by 9% SDS-PAGE and Western blot with an anti-uPAR polyclonal Ab.

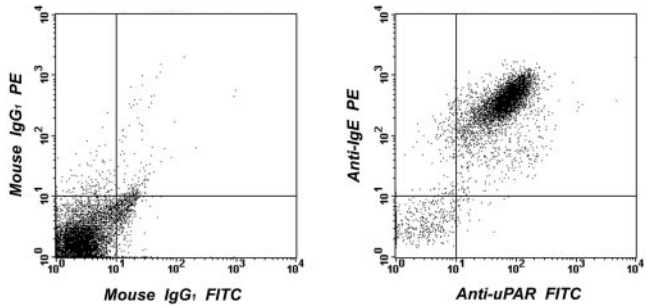

FIGURE 2. Cytofluorometric analysis of uPAR expression on the basophil surface. Basophils were preincubated with anti-IgE PE mAb, anti-uPAR polyclonal Ab, isotype-matched control mAbs, and FITC-conjugated goat anti-rabbit isotype.

either the lower or upper chambers. The basophil migratory response to chemotactic stimuli was largely due to chemotaxis and not to chemokinesis. Indeed, a checkboard analysis in which chemoattractants above and below the filters varied resulted in significant migration only when there was a gradient of the factor below the filters.

Histamine release

Basophils ($\sim 6 \times 10^4$ basophils/tube) were resuspended in PCG, and 0.1 ml of the cell suspension was placed in 12 × 75-mm polyethylene tubes (Sarstadt, Princeton, NJ) and warmed to 37°C; 0.1 ml of each prewarmed releasing stimulus was added, and incubation was continued at 37°C for 45 min (37). At the end of this step, the reactions were stopped by centrifugation (1000 × *g*, 22°C, 2 min), and the cell-free supernatants were assayed for histamine content with an automated fluorometric technique (41). Total histamine content was assessed by lysis induced by incubating the cells with 2% $HClO_4$ before centrifugation. To calculate histamine release as a percentage of total cellular histamine, the spontaneous release of histamine from basophils (2–8% of the total cellular histamine) was subtracted from both the numerator and the denominator (42). All values are based on the means of duplicate or triplicate determinations. Replicates differed in histamine content by <10%.

IL-4 and IL-13 ELISAs

IL-4 and IL-13 release in the culture supernatants of basophils was measured in duplicate determinations with a commercially available ELISA (R&D Systems, Minneapolis, MN).

Lactate dehydrogenase (LDH) assay

LDH release at the end of the incubations served as an index of cytotoxicity. It was measured in cell-free supernatants using a commercially available kit (Sigma-Aldrich) (28).

Statistical analysis

The results are the mean ± SEM. Values from groups were compared using paired Student's *t* test (43). Significance was defined as $p < 0.01$.

Results

Expression of uPA and uPAR in human basophils

We examined uPA and uPAR expression in human basophils at mRNA and protein levels. THP-1 monocyte-like cells were used as a control because the uPA/uPAR system has been characterized in this cell line (18). The analysis of PCR products by electrophoresis in agarose gel showed uPAR-mRNA expression in basophils and in THP-1 monocyte-like cells (Fig. 1*A*).

We then investigated uPAR expression in basophils at the protein level. Western blot analysis with an anti-uPAR polyclonal Ab demonstrated that basophils express uPAR in the intact (D1D2D3; 50 kDa) and cleaved (c-uPAR; D2D3; 35 kDa) forms previously described in other cell types, including monocyte-like THP-1 cells (5, 6) (Fig. 1*B*).

Flow cytometric analysis was performed to confirm that uPAR was expressed on the basophil surface. Basophils were incubated with an anti-uPAR rabbit polyclonal Ab or with purified control IgG, then stained with FITC-conjugated goat anti-rabbit IgG and PE-conjugated anti-IgE. Fig. 2 shows uPAR expression on the vast majority (80–94%) of basophils. These experiments demonstrate

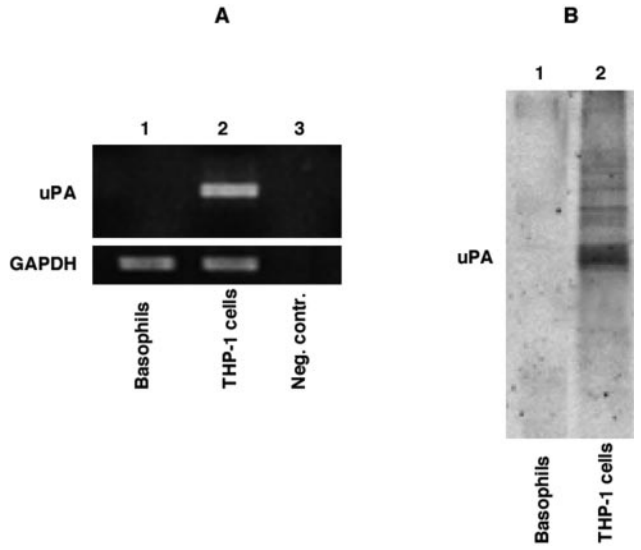

FIGURE 3. The expression of uPA in human basophils. *A*, Purified human basophils (*lane 1*) and THP-1 monocyte-like cells, as s positive control (*lane 2*), were lysed in TRIzol. Total RNA was prepared, reverse transcribed, and amplified by 40 PCR cycles in the presence of uPA-specific primers and GAPDH primers, as a loading control. PCR amplification of buffer represented the negative control (***lane 3***). PCR products were analyzed by electrophoresis in 1% agarose gel containing ethidium bromide, followed by photography under UV illumination. *B*, Basophils (*lane 1*) and THP-1 cells, as a positive control (*lane 2*), were lysed in Triton X-100, and 50 μg of total proteins were analyzed by 9% SDS-PAGE and Western blot with an anti-uPA polyclonal Ab.

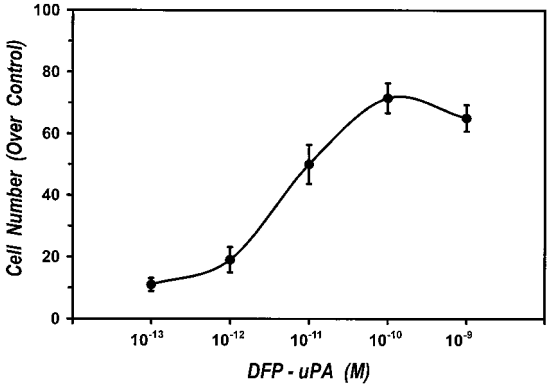

FIGURE 4. Effects of DFP-uPA on human basophil chemotaxis. Basophils were allowed to migrate with the indicated concentrations of DFP-uPA for 1 h at 37°C in a humidified (5% CO_2) incubator. Values are the mean ± SEM of six experiments with different basophil preparations.

that basophils synthesize uPAR RNA and translate it into a protein that is expressed on the cell surface.

uPA mRNA was not detected by RT-PCR analysis of basophil RNA (Fig. 3*A*). Western blot analysis of basophil lysates with an anti-uPA polyclonal Ab also confirmed the absence of cytoplasmic uPA at the protein level (Fig. 3*B*). By contrast, THP-1 cells expressed uPA at both mRNA and protein levels (Fig. 3).

Effect of DFP-uPA on chemotaxis of human basophils

We evaluated the in vitro effects of a wide range of low concentrations (10^{-13}–10^{-9} M) of DFP-inactivated uPA on the chemotaxis of purified basophils (>99%). uPA was inactivated to evaluate the effect mediated by its interaction with uPAR, not the effect due to its enzymatic activity. Subnanomolar concentrations of DFP-uPA caused basophil chemotaxis, which plateaued at 10^{-10} M (Fig. 4). To determine whether DFP-uPA-induced migration of basophils resulted from chemotaxis or chemokinesis, checkerboard analysis was performed and showed that DFP-uPA dose-dependently induced the migration of basophils when added to the lower wells of the chemotaxis chamber. An optimal concentration of DFP-uPA (5×10^{-9} M) added with the cells to the upper wells or to both compartments did not induce directional basophil migration (Table I). Thus, DFP-uPA-induced migration of basophils resulted from chemotaxis, rather than from chemokinesis.

To exclude that inactivation of uPA could influence its chemotactic activity, we performed six experiments with native uPA. Fig. 5*A* shows that DFP inactivation did not modify uPA's chemotactic properties. In addition, experiments with ATF (aa 1–143), which consists only of the uPAR-binding region of uPA and is devoid of enzymatic activity (44), demonstrated that this peptide retains its chemotactic properties (Fig. 5*B*). Taken together, these results indicate that the enzymatic activity of uPA is not primarily responsible for inducing basophil chemotaxis.

Table I. *Checkerboard analysis of basophil migration to DFP-uPA*[a]

Lower Compartment	Upper Compartment	
	Buffer	DFP-uPA
Buffer	12.0 ± 2.2	10.0 ± 3.4
DFP-uPA	80.3 ± 7.5[b]	13.5 ± 2.8

[a] An optimal chemotactic concentration of DFP-uPA (5×10^{-9} M) was placed in the upper and/or lower wells of the chemotaxis chamber; basophils were placed in the upper wells. The upper and lower wells were separated by a polycarbonate filter. Basophils were allowed to migrate for 1 h at 37°C in a humidified incubator with 5% CO_2. Values are the mean ± SEM of migrated basophils from three experiments.

[b] $p < 0.01$ compared with migration in the presence of buffer alone or of DFP-uPA added to the upper well or to both compartments.

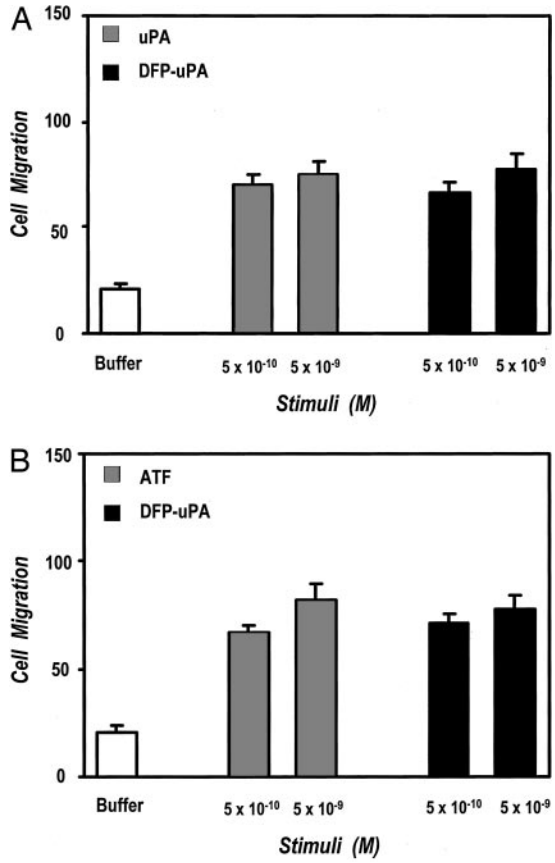

FIGURE 5. *A*, Effects of uPA- and DFP-treated uPA on human basophil chemotaxis. Basophils were allowed to migrate with the indicated concentrations of proteins for 1 h at 37°C in a humidified (5% CO_2) incubator. Values are the mean ± SEM of three experiments with different basophil preparations. *B*, Effects of ATF and DFP-uPA on human basophil chemotaxis. Basophils were allowed to migrate with the indicated concentrations of proteins for 1 h at 37°C in a humidified (5% CO_2) incubator. Values are the mean ± SEM of three experiments with different basophil preparations.

To verify whether the basophil chemotaxis caused by DFP-uPA was mediated by uPAR, a polyclonal Ab against uPAR was used in blocking experiments. Fig. 6 shows that preincubation of basophils with an anti-uPAR polyclonal Ab (5 μg/ml) significantly inhibited (68.2 ± 5.4%) DFP-uPA-dependent basophil chemotaxis. The specificity of this observation was supported by the finding that preincubation of basophils with the anti-uPAR Ab did not modify eotaxin-induced basophil chemotaxis. The magnitude of basophil chemotaxis induced by DFP-uPA was comparable to that caused by eotaxin. These results indicate that binding to uPAR is necessary to mediate the chemotactic activity of uPA.

Expression of the FMLP receptor family in human basophils

FPRL1, a member of the FMLP receptor family, mediates uPA- and uPAR-D2D3-dependent monocyte chemotaxis (9). In addition, uPAR expression is required for FMLP-dependent migration (10, 45, 46). The expression of the FMLP receptor family in human basophils was investigated to study the functional interactions of these receptors with the uPA-uPAR system in these cells. Monocyte-like THP-1 cells were used as a control, because monocytes express all three FMLP receptors (19, 47). Purified basophils and THP-1 cells were lysed in TRIzol for total RNA preparation. RNA was then transcribed and amplified by 40 PCR cycles in the

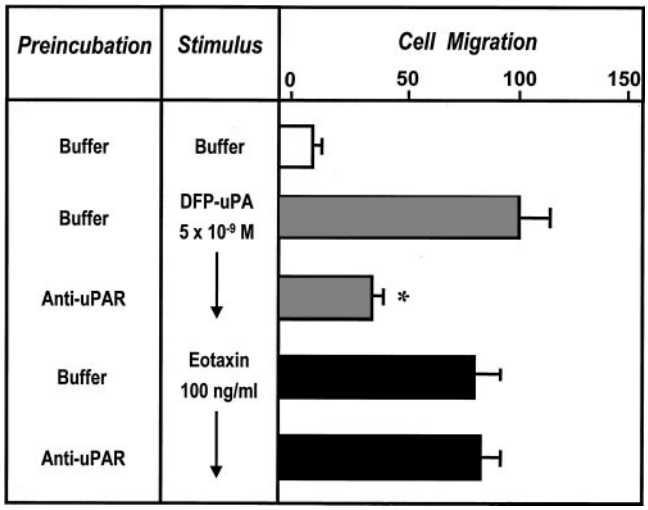

FIGURE 6. Effects of preincubation of basophils with an anti-uPAR polyclonal Ab. Basophils were preincubated (1 h at 37°C) with an anti-uPAR polyclonal Ab. Basophils were then allowed to migrate with the indicated concentrations of DFP-uPA and eotaxin for 1 h at 37°C in a humidified (5% CO_2) incubator. Values are the mean ± SEM of three experiments with different basophil preparations. *, $p < 0.01$ compared with cells stimulated with uPA.

presence of FPR-, FPRL1-, and FPRL2-specific primers and GAPDH primers, as a loading control. The analysis of PCR products by electrophoresis in agarose gel showed the presence of all three receptors in basophils and THP-1 monocyte-like cells (Fig. 7). The levels of PCR products after 30, 35, and 40 cycles, estimated by densitometric scanning and plotted vs the number of cycles, indicated a lower expression of FPRL1 compared with FPR and FPRL2 in basophils. Similar results were found in THP-1 cells (data not shown).

uPA-dependent basophil migration and FMLP receptor family

uPA, upon binding to uPAR, induces monocyte chemotaxis by activating FPRL1, the low affinity FMLP receptor (9). We investigated which member of the FMLP receptor family mediates uPA-dependent basophil chemotaxis. CsH is a specific antagonist of the high affinity FMLP receptor FPR (29–31). In fact, preincubation (15 min at 37°C) of basophils with CsH significantly inhibited chemotaxis of basophils induced by FMLP. By contrast, CsH did not affect basophil migration toward DFP-uPA (Fig. 8*A*). These results are compatible with the hypothesis that FMLP induced basophil chemotaxis by activating FPR, whereas DFP-uPA acted through different subtypes of FMLP receptors.

Preincubation of cells with high concentrations of FMLP is known to cause desensitization of FPR and FPRL1 (19). In our experiments, desensitization of FPR and FPRL1 by pretreatment of basophils with a high concentration (10^{-4} M) of FMLP induced a slight inhibition (23.1 ± 2.1%) of the chemotactic response to DFP-uPA ($p = 0.11$, NS; Fig. 8*B*). In a parallel series of experiments pretreatment of THP-1 cells with 10^{-4} M FMLP caused a marked inhibition (53 ± 4.2%) of the chemotactic response to DFP-uPA, as recently reported (9). We thus investigated the involvement of FPRL2, which does not bind FMLP, but is activated by the synthetic peptide WKYMVm (21). This peptide also binds to FPR and FPRL1, although with different affinities (19). Basophil pretreatment with the WKYMVm peptide strongly reduced uPA-dependent basophil migration (55.6 ± 4.8%; Fig. 8*C*). These results indicate that uPA-dependent basophil migration is mainly mediated by activation of FPRL2. The specificity of this observation is demonstrated by the finding that preincubation of basophils with low or high concentrations of FMLP did not alter the responsiveness to other unrelated chemotactic stimuli such as eotaxin (Fig. 9*A*), PGD_2 (Fig. 9*B*), and C5a (Fig. 9*C*), which exert their effects by activating specific receptors (32, 33, 36).

Effect of $uPAR_{84-95}$ on chemotaxis of human basophils

The uPAR-derived peptide fragment $uPAR_{84-95}$ induces monocyte migration by binding to FPRL1 (9). We investigated the effect of this peptide on basophil chemotaxis and the involvement of the FMLP receptor family. Fig. 10 shows the results of six experiments demonstrating that picomolar concentrations (10^{-13}–10^{-9} M) of $uPAR_{84-95}$ caused basophil chemotaxis that plateaued at 10^{-11} M. No basophil chemotaxis was induced with a wide range of concentrations (10^{-13}–10^{-9} M) of a scrambled $uPAR_{84-95}$ peptide.

The relationship between peptides $uPAR_{84-95}$, FMLP (5×10^{-7} or 10^{-4} M), and WKYMVm was further examined by evaluating the effects of heterologous desensitization between these stimuli on basophil chemotaxis. Purified basophils were treated for 30 min at 37°C with buffer or with low (5×10^{-7} M) or high (10^{-4} M) concentrations of FMLP that activate FPR and FPRL1, respectively (19). At the end of the incubation, cells were washed

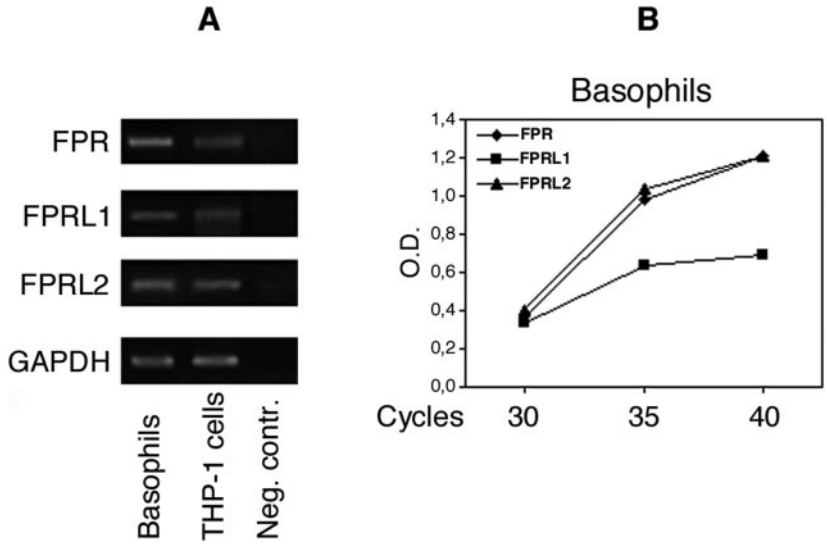

FIGURE 7. FMLP receptor expression in human basophils. *A*, Purified basophils (*lanes 1*) and THP-1 control cells (*lanes 2*) were lysed in TRIzol for total RNA preparation. RNA was then transcribed and amplified by 40 PCR cycles in the presence of FPR-, FPRL1-, and FPRL2-specific primers and GAPDH primers, as a loading control. PCR products were analyzed by electrophoresis in 1% agarose gel containing ethidium bromide, followed by photography under UV illumination. *B*, The levels of PCR products after 30, 35, and 40 cycles were estimated by densitometric scanning. FPR, FPRL1, and FPRL2 levels, normalized to GAPDH levels, were plotted vs the number of cycles (*right*).

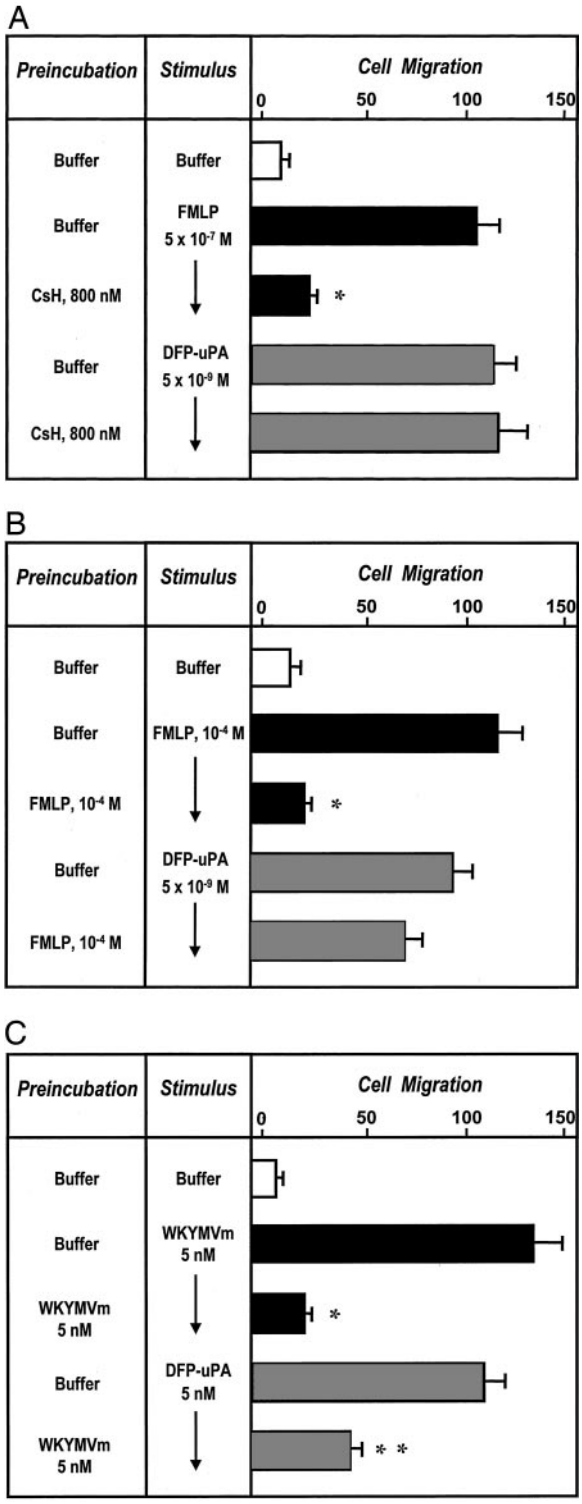

FIGURE 8. *A*, Effects of CsH on human basophil chemotaxis induced by FMLP or DFP-uPA. Basophils were preincubated (5 min at 37°C) with CsH (800 nM) or buffer. Basophils were allowed to migrate toward FMLP (5×10^{-7} M) or DFP-uPA (5×10^{-9} M) for 1 h at 37°C in a humidified incubator with 5% CO_2. Values are the mean ± SEM obtained from three experiments with different basophil preparations. *, $p < 0.01$ compared with cells stimulated with FMLP. *B*, Effects of heterologous desensitization with a high concentration of FMLP (10^{-4} M) on basophil chemotaxis. Basophils were incubated in PIPES buffer containing EDTA (4 mM) or FMLP (10^{-4} M) for 30 min at 37°C. At the end of incubation, cells were washed twice, resuspended in PACGM, and challenged with the chemotactic stimuli (FMLP, 10^{-4} M; uPA, 5×10^{-9} M). Basophils were allowed to migrate for 1 h at 37°C in a humidified incubator with 5% CO_2. Values are the mean ± SEM from five experiments. *, $p < 0.01$ compared with cells stimulated with FMLP. *C*, Effects of heterologous desensitization between WKYMVm (5×10^{-9} M) and DFP-uPA (5×10^{-9} M) on basophil chemotaxis. Basophils were incubated in PIPES buffer containing EDTA (4 mM) or WKYMVm (5×10^{-9} M) for 30 min at 37°C. At the end of incubation, cells were washed twice, resuspended in PACGM, and challenged with the chemotactic stimuli (WKYMVm, 5×10^{-9} M; or DFP-uPA, 5×10^{-9} M). Basophils were allowed to migrate for 1 h at 37°C in a humidified incubator with 5% CO_2. Values are the mean ± SEM from five experiments. *, $p < 0.01$ compared with cells stimulated with WKYMVm; **, $p < 0.01$ compared with cells stimulated with DFP-uPA.

and allowed to migrate in the presence of $uPAR_{84-95}$ (10^{-10} M). Fig. 11 shows the results of nine experiments in which the response to $uPAR_{84-95}$ was slightly, but significantly, inhibited (26.7 ± 2.1%; $p < 0.01$) by preincubation with a high concentration of FMLP. Preincubation of basophils with a low concentration of FMLP did not affect the response to $uPAR_{84-95}$. By contrast, desensitization by preincubation with WKYMVm, which activates FPR, FPRL1, and FPRL2 (19), markedly inhibited (71.6 ± 4.5%) the response to $uPAR_{84-95}$. These results are compatible with the hypothesis that $uPAR_{84-95}$ induces basophil chemotaxis mainly by activating FPRL2 and, to some extent, FPRL1.

Effect of DFP-uPA on histamine and cytokine release from human basophils

We evaluated the effects of increasing concentrations of DFP-uPA on histamine release from basophils purified (>99%) from healthy individuals. The results of the five experiments illustrated in Fig. 12 demonstrate that DFP-uPA did not cause histamine release from basophils. Also, gp41 2019, which activates the formyl peptide receptors FPRL1 and FPRL2 (28), did not induce histamine secretion from basophils. The concentrations of DFP-uPA used did not induce spontaneous LDH release from basophils (data not shown). In these experiments FMLP (10^{-8}–10^{-6} M), which activates the high affinity formyl peptide receptor FPR, (37), and low concentrations (10^{-9}–10^{-7} M) of WKYMVm, which activates FPR, FPRL1, and FPRL2 (19), were potent stimuli for the secretion of histamine from basophils.

In three experiments, incubation (18 h at 37°C) of basophils with DFP-uPA did not induce IL-4 or IL-13 release, whereas anti-IgE induced the release of both IL-4 and IL-13 (data not shown).

Discussion

The uPA-uPAR system is an important and complex cellular recognition system that mediates fibrinolysis, cell adhesion and migration, and tissue remodeling (3, 11, 48–52). In this study we demonstrate that human basophils express uPAR on their surface in both the intact (D1D2D3) and the cleaved (D2D3) form. By contrast, basophils do not express uPA, the ligand of uPAR. We also found that enzymatically inactive uPA and ATF, which is devoid of enzymatic activity, are potent chemoattractants for basophils. The uPAR involvement in uPA-induced chemotaxis is supported by the observation that an anti-uPAR polyclonal Ab inhibited uPA-dependent basophil chemotaxis. Our results are compatible with the hypothesis that uPA-dependent basophil chemotaxis requires the engagement of uPAR and does not require uPA enzymatic activity.

We also found that the uPAR-derived peptide $uPAR_{84-95}$ is a potent basophil chemoattractant. uPAR can be cleaved by several enzymes, including uPA (5, 6), in the linker region connecting the N-terminal domain D1 to the D2 domain, thus inducing the removal of D1 and exposing a chemotactic epitope on D2D3-uPAR

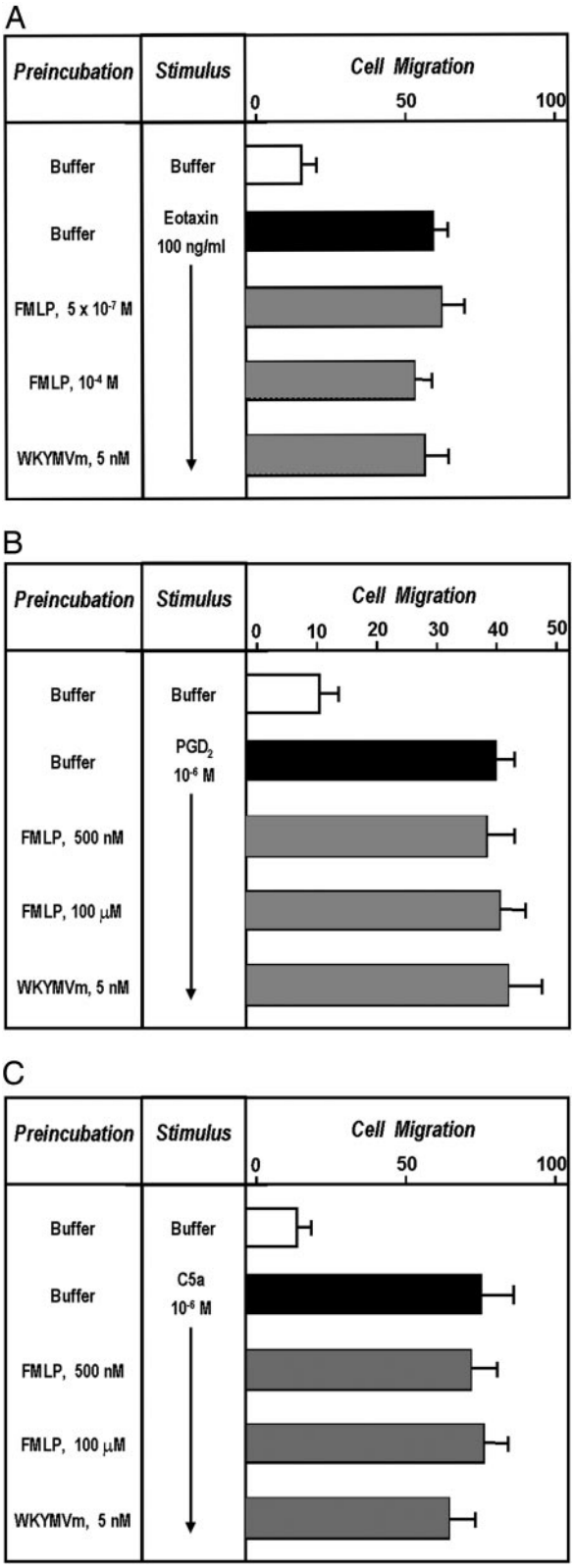

FIGURE 9. *A*, Effects of heterologous desensitization between low (5×10^{-7} M) and high (10^{-4} M) concentrations of FMLP, WKYMVm (5×10^{-9} M), and eotaxin (100 ng/mg) on basophil chemotaxis. Basophils were incubated in PIPES buffer containing EDTA (4 mM), FMLP (5×10^{-7} M), FMLP (10^{-4} M), or WKYMVm (5×10^{-9} M) for 30 min at 37°C. At the end of incubation, cells were washed twice, resuspended in PACGM, and challenged with eotaxin (100 ng/ml). Basophils were allowed to migrate for 1 h at 37°C in a humidified incubator with 5% CO_2. Values are the mean ± SEM from three experiments. *B*, Effects of heterologous desensitization between low (5×10^{-7} M) or high (10^{-4} M) concentrations of FMLP, WKYMVm (5×10^{-9} M), and PGD_2 (10^{-6} M) on basophil chemotaxis. Basophils were incubated in PIPES buffer containing EDTA (4 mM), FMLP (5×10^{-7} M), FMLP (10^{-4} M), or WKYMVm (5×10^{-9} M) for 30 min at 37°C. At the end of incubation, cells were washed twice, resuspended in PACGM, and challenged with PGD_2 (10^{-6} M). Basophils were allowed to migrate for 1 h at 37°C in a humidified incubator with 5% CO_2. Values are the mean ± SEM from three experiments. *C*, Effects of heterologous desensitization between low (5×10^{-7} M) and high (10^{-4} M) concentrations of FMLP, WKYMVm (5×10^{-9} M), and C5a (10^{-6} M) on basophil chemotaxis. Basophils were incubated in PIPES buffer containing EDTA (4 mM), FMLP (5×10^{-7} M), FMLP (10^{-4} M), or WKYMVm (5×10^{-9} M) for 30 min at 37°C. At the end of incubation, cells were washed twice, resuspended in PACGM, and challenged with C5a (10^{-6} M). Basophils were allowed to migrate for 1 h at 37°C in a humidified incubator with 5% CO_2. Values are the mean ± SEM from three experiments.

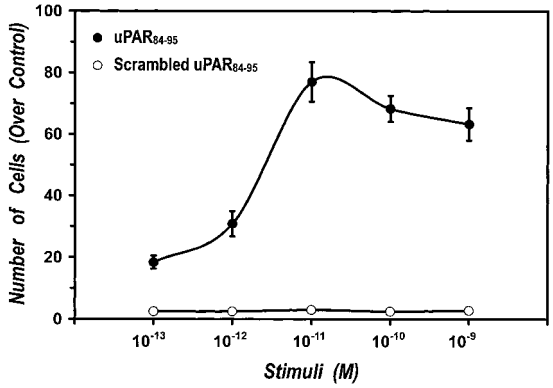

FIGURE 10. Effects of $uPAR_{84-95}$ and its scrambled peptide on human basophil chemotaxis. Basophils were allowed to migrate with the indicated concentrations of $uPAR_{84-95}$ and its scrambled peptide for 1 h at 37°C in a humidified (5% CO_2) incubator. Values are the mean ± SEM from six experiments with different basophil preparations.

anchored on the cell surface (18). This chemotactic epitope (residues 84–95), in a soluble form, also acts as a potent chemoattractant for monocytes by activating FPRL1 (9). Soluble cleaved forms of uPAR, devoid of D1 (D2D3-suPAR) and probably containing the chemotactic epitope, have been found in tissues and biological fluids (53, 54).

Human basophils are responsive to FMLP, which induces their chemotaxis and/or the release of proinflammatory mediators (28, 29), and we have demonstrated that these cells express the three receptors (FPR, FPRL1, and FPRL2) (20, 21). These receptors belong to the STM, a G protein-coupled rhodopsin superfamily (55, 56). The FPR has a high affinity for FMLP and is activated by nanomolar concentrations of FMLP. FPRL1 is a promiscuous receptor activated in response to higher concentrations of FMLP, the prion peptide $PrP_{106-126}$ (25), lipoxin A_4 (26), *Helicobacter pylori*-derived peptide (Hp_{2-20}) (27), serum amyloid A (24), and various synthetic peptides (21). Interestingly, uPA and $uPAR_{84-95}$ do not share sequence homologies with any of the other agonists. Human monocytes and basophils express FPRL2, and Hp_{2-20} exerts its chemotactic effect by activating this receptor subtype (27, 57). Our results are compatible with the hypothesis that FPRL2 is involved in the chemotactic activity induced by urokinase in basophils.

CsH blocks FPR-evoked responses (21, 29, 30). Accordingly, CsH blocked the chemotactic activity of FMLP on basophils, but had no effect on the response evoked by uPA. This indicates that FPR is not involved in uPAR-induced basophil chemotaxis. We

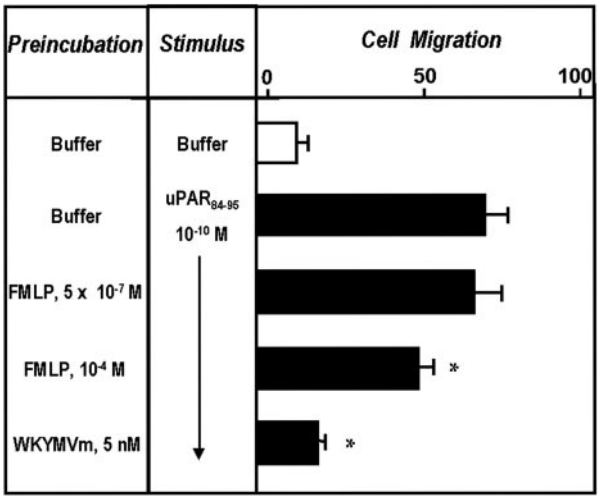

FIGURE 11. Effects of heterologous desensitization between low (5×10^{-7} M) and high (10^{-4} M) concentrations of FMLP, WKYMVm (5×10^{-9} M), and $uPAR_{84-95}$ (10^{-10} M) on basophil chemotaxis. Basophils were incubated in PIPES buffer containing EDTA (4 mM), FMLP (5×10^{-7} M), FMLP (10^{-4} M), or WKYMVm (5×10^{-9} M) for 30 min at 37°C. At the end of incubation, cells were washed twice, resuspended in PACGM, and challenged with $uPAR_{84-95}$ (10^{-10} M). Basophils were allowed to migrate for 1 h at 37°C in a humidified incubator with 5% CO_2. Values are the mean ± SEM from nine experiments. *, $p < 0.01$ compared with cells stimulated with $uPAR_{84-95}$.

also performed heterologous desensitization experiments to verify the specificity of the uPA and FMLP activation routes. Upon binding of FMLP to its FPR receptor, the occupied receptor is phosphorylated (58); thus, cells are desensitized and unable to generate signals through the same receptor. We found that basophils preincubated with FMLP in the absence of Ca^{2+} did not generate a chemotactic response when rechallenged with the same agonist (homologous desensitization). Heterologous desensitization yielded interesting results. Basophil preincubation with a low concentration of FMLP, which desensitizes FPR (55), did not affect the chemotactic responses to uPA and $uPAR_{84-95}$. Preincubation with a high concentration of FMLP, which desensitizes FPR and FPRL1, slightly inhibited the chemotactic responses to uPA and $uPAR_{84-95}$. In contrast, when basophils were exposed to WKYMVm, which binds also to FPRL2 (55), the chemotactic responses to uPA and $uPAR_{84-95}$ were greatly reduced. The results of these two groups of experiments indicate that the potent chemoattractant effect of uPA occurs consequent to the exposure of a uPAR chemotactic epitope, presumably $uPAR_{84-95}$, which is an endogenous ligand for FPRL2. The apparent discrepancy between the abilities of uPA and $uPAR_{84-95}$ to induce basophil chemotaxis through the engagement of FPRL1 might reflect their different accessibilities to and/or affinities for this receptor. Until receptor-specific agonists/antagonists become available, we cannot exclude the possibility that uPA and $uPAR_{84-95}$ might activate another, as yet unknown, receptor(s) on basophils.

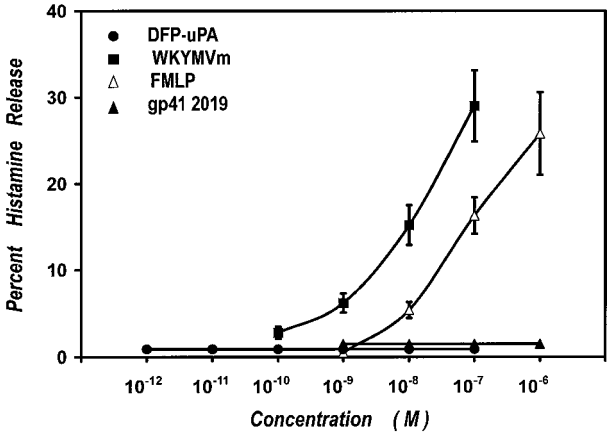

FIGURE 12. Effects of DFP-uPA, WKYMVm, FMLP, and gp41 2019 on histamine release from human basophils. Basophils were incubated with the indicated concentrations of stimuli for 45 min at 37°C. Values are the mean ± SEM obtained from six experiments with different basophil preparations. Error bars are not shown when they are too small to graph.

Our results agree to a large extent with the results obtained with monocytes by Resnati et al. (9) and their interpretation. They demonstrate that uPA and a cleaved form of uPAR induces chemotaxis of THP-1 cells by binding to the FPRL1 receptor. We have extended their observation by showing that even if both basophils and THP-1 cells express mRNA for FPRL1 and FPRL2, uPA and $uPAR_{84-95}$ induce basophil chemotaxis mainly through the engagement of FPRL2. These results suggest that after binding to uPA, uPAR undergoes conformational changes (59), allowing it to interact with FPRL1 in monocytes (9) and with FPRL2 in basophils. We cannot exclude the possibility that FMLP receptor binding of the uPA/uPAR complex triggers additional conformational changes that are important in mediating the transmission of signal from the cell surface to the inner domains involved in basophil chemotaxis.

We did find a significant difference between human basophils and THP-1 cells with respect to the uPA-uPAR system. In fact, we confirmed the presence of mRNA for uPA in THP-1 cells, but we did not detect a specific message for uPA in human basophils. Interestingly, Valent and coworkers (16) have demonstrated that human mast cells express functional receptors for urokinase, but do not produce detectable amounts of uPA. These observations raise the possibility that the lack of an autocrine loop in human basophils and mast cells could play a role in rendering these cells exquisitely sensitive to low concentrations of uPA. The involvement of uPA and uPAR in inflammation regardless of plasmin activity has been demonstrated in vitro and in vivo. uPA is a potent chemoattractant for neutrophils and monocytes (15, 60). uPA also primes neutrophils for superoxide anion release (61), and uPAR expression is required for FMLP-dependent monocyte and granulocyte chemotaxis (45, 46) and for neutrophil degranulation (62). Finally, $uPA^{-/-}$ and $uPAR^{-/-}$ mice show impaired neutrophil recruitment and susceptibility to bacterial infections (12–14). There is compelling evidence that mast cells and basophils are multifunctional effector cells in inflammation (63) and in host defense against bacteria, viruses and parasites (64). The interaction between low concentrations of uPA with the FMLP receptors expressed by basophils could contribute to their recruitment to sites of inflammation.

FPRL2 involvement in $uPAR_{84-95}$-dependent basophil migration raises the possibility that this peptide represents an endogenous ligand for this receptor. Until recently, FPRL2 was considered an orphan receptor despite its sequence homology with the other two formyl receptors, FPR and FPRL1 (19). Betten et al. (27) and our group (57) have demonstrated that the Hp_{2-20} induces monocyte and basophil chemotaxis, respectively, through the activation of FPRL2. Therefore, other endogenous or exogenous ligands may exist, making FPRL2 a promiscuous receptor like FPRL1.

In conclusion, we provide the first evidence that human basophils express intact uPAR and its cleaved form. We also demonstrate that enzymatically inactive uPA and $uPAR_{84-95}$ are potent

chemoattractants for basophils. uPAR has no transmembrane domain and must in large part mediate its action by partnering with additional membrane proteins capable of direct interaction with the cellular interior. Our results are compatible with the hypothesis that uPA-dependent chemotaxis is mediated by the exposure of a chemotactic urokinase receptor epitope that is an endogenous agonist for FPRL2 on human basophils.

Finally, the results described in this study may have practical implications in disorders such as allergic diseases and certain bacterial infections in which basophils infiltrating the sites of inflammation play a prominent role (57, 65–67). In fact, it is conceivable that agents acting on uPAR-mediated chemotaxis (i.e., by blocking the chemotactic epitope) may be used to modify the basophil-driven inflammatory reactions.

Acknowledgments

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