The urokinase plasminogen activator (uPA) interacts with its cell surface receptor (uPAR), providing an inducible, localized cell surface proteolytic activity, thereby promoting cellular invasion. Evidence is provided for a novel function of cell surface-associated uPA-uPAR. Specifically, induction of cell surface expression of uPA-uPAR by growth factors or phorbol ester was necessary for vitronectin-dependent carcinoma cell migration, an event mediated by integrin αvβ5. Cell migration on vitronectin was blocked with either a soluble form of uPAR, an antibody that disrupts uPA binding to uPAR, or a monoclonal antibody to αvβ5. Moreover, plasminogen activator inhibitor type 2 blocked this migration event but did not affect adhesion, suggesting a direct role for uPA enzyme activity in this process and that migration but not adhesion of these cells is regulated by uPA-uPAR. Growth factor-mediated induction of uPA-uPAR on the carcinoma cell surface promotes a specific motility event mediated by integrin αvβ5, since cells transfected with the β3 integrin subunit expressed αvβ3 and migrated on vitronectin independently of growth factors or uPA-uPAR expression. This relationship between αvβ3 and the uPA-uPAR system has significant implications for regulation of motility events associated with development, angiogenesis, and tumor metastasis.

Urokinase-type plasminogen activator (uPA) is a serine protease that, when bound to its cell surface receptor (uPAR), converts plasminogen into plasmin, which is known to degrade various matrix glycoproteins (1, 2). The expression of uPA and its receptor is induced by a variety of growth factors known to promote cell motility such as basic fibroblast growth factor, epidermal growth factor, transforming growth factor-α (TGF-α), and hepatocyte growth factor (HGF) (3–6) as well as by the phorbol ester phorbol 12-myristate 13-acetate (PMA) (7). The simultaneous expression of uPA and its receptor has been associated with localized plasminogen activation and pericellular matrix degradation during directed cell migration of normal and tumor cells. In support of this concept, receptor-bound uPA has been associated with neuronal cell migration, keratinocyte migration, and endothelial cell migration during tissue remodeling, wound healing, and angiogenesis, respectively (3, 5, 8). In addition, a variety of neoplastic cells depend on cell surface-associated proteolytic activity, mediated by receptor-bound uPA, to degrade matrix proteins during in vivo and in vitro invasion (9–11).

The integrin family of cell adhesion receptors mediates cell attachment to extracellular matrix proteins and is known to play a critical role in cell motility (12–15), thus contributing to a variety of biological processes including angiogenesis, wound healing, and tumor cell invasion and metastasis (16–18).

We previously demonstrated that FG human pancreatic carcinoma cells utilize integrin αvβ5 to attach to vitronectin yet require growth factor or phorbol ester-mediated activation of a protein kinase C-dependent signaling pathway for migration on this ligand (12). This vitronectin-directed motility required a late activation event involving de novo gene transcription and protein synthesis (14). We now present evidence that growth factor activation of FG cells leads to induction of cell surface uPA-uPAR that appears to be required for the αvβ5-dependent FG cell motility on vitronectin. The specificity of this migration response is demonstrated, since αvβ3 or αvβ1-directed migration of these cells is independent of uPA-uPAR expression. Thus, we define a novel mechanism regulating cell migration involving specific functional cooperation between uPA-uPAR and the integrin αvβ5.

**EXPERIMENTAL PROCEDURES**

**Cells and Cell Culture**—FG is a human pancreatic carcinoma cell line that fails to express mRNA for the β3 integrin subunit (19). FG-B is a subline stably transfected with a full-length cDNA encoding the human β3 gene and expresses functional αvβ3 integrin (19). M21 human melanoma cells were a gift from Dr. Donald Morton (Department of Surgery, University of California, Los Angeles, CA). WM35 human melanoma cells were kindly provided by Dr. Meenhard Herlyn (Wistar Institute, Philadelphia, PA). All cell lines were grown in RPMI 1640 (Life Technologies) supplemented with 10% fetal bovine serum and 50 µg/ml gentamycin and tested free from mycoplasma during these studies.

**Antibodies and Reagents**—All chemicals and reagents were purchased from Sigma unless otherwise specified. Integrin-specific mAbs P1F6 (anti-αvβ5) (17) and LM609 (anti-αvβ3) (16) and the mouse mAb W6/32 (anti-HLA class I) (20) were affinity-purified from ascites on protein A-Sepharose. The mouse anti-human uPAR mAb 3936 (21), the

Mayra Yebra‡, Graham C. N. Parry‡, Staffan Strömblad§, Nigel Mackman, Steven Rosenberg®, Barbara M. Mueller†, and David A. Cheresh**

From ‡The Scripps Research Institute, Departments of Immunology and Vascular Biology, IMM24, La Jolla, California 92037. Tel.: 619-554-8281; Fax: 619-554-8926.

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Requirement of Receptor-bound Urokinase-type Plasminogen Activator for Integrin αvβ5-directed Cell Migration*

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polyclonal rabbit anti-human uPAR antibody 399R (11), the mouse anti-uPAR antibodies 394 (22), the appropriate secondary antibodies (at a 1:3,000 dilution) conjugated with peroxidase (Bio-Rad), washed several times in TBS-T, and analyzed using the enhanced chemiluminescence detection system (Amersham Corp.).

Flow Cytometric Analysis—Serum-starved FG cells were harvested as for the migration assay and were stimulated with 5 ng/ml PMA in FBM-BSA for 6 h in the presence or absence of mouse anti-uPAR mAb 3936 (50 μg/ml). Cells were then rinsed twice in ice-cold FACs buffer (phosphate-buffered saline with 0.1% BSA and 0.02% sodium azide, pH 7.4), and incubated with polyclonal rabbit anti-uPAR (399R) or rabbit anti-uPA (389) at 10 μg/ml for 1 h on ice. Cells were washed three times with excess FACs buffer and then incubated with secondary antibody (FITC-conjugated goat anti-rabbit IgG; Southern Biotechnology, Birmingham, AL) diluted 1:100 for 1 h on ice. Cells were washed and analyzed with a Becton-Dickinson FACScan flow cytometer. Cell analysis was gated on forward and size scatter intensities, and the results are presented as histograms.

Determination of Secreted and Cell Surface uPA Levels—FG cells were serum-starved for 24 h before stimulation with 5 ng/ml PMA for 1, 4, 8, or 24 h. At each time point, spent culture medium and cell surface acid eluates were collected as described previously (27) and analyzed for the amount of secreted and surface-bound uPA antigen by an anti-uPA enzyme-linked immunosorbent assay, according to the manufacturer’s instructions (American Diagnostica, Greenwich, CT).

Analysis of uPA and uPAR mRNA—Cells were incubated in serum-free RPMI for 20–24 h before stimulation with 10 ng/ml PMA or 100 ng/ml TGF-α for 1, 4, 5, 6, or 24 h. Total cellular RNA (10 μg) isolated from these cultures was subjected to electrophoresing electrophoresing in 1% agarose formaldehyde gels (28) and transferred to a GeneScreen membrane (DuPont NEN). Membranes were hybridized with either a uPA or a uPAR cDNA fragment labeled using [α-32P]dCTP (>3,000 Ci/mmol, Amersham) as described (29). To control for variations in RNA loadings, membranes were rehybridized with radiolabeled cDNA fragment from the human glyceraldehyde-3-phosphate dehydrogenase gene. Membranes were exposed to Kodak BioMax film (Eastman Kodak Co.) at −80°C. The intensity of hybridization signals was determined directly using a PhosphorImage and ImageQuant software (Molecular Dynamics Inc., Sunnyvale, CA).

RESULTS

PMA and TGF-α Increase uPA and uPAR Expression on FG Cells—Exposure of cells to growth factors or phorbol esters is known to promote their migration on extracellular matrix proteins (12, 30). We recently showed that induction of αβ3-dependent FG pancreatic carcinoma cell migration on vitronectin but not adhesion on this substrate requires activation of protein kinase C with either PMA or growth factors (12) and that this event requires gene transcription and protein synthesis (14). However, FG cell motility on collagen was constitutive and mediated by integrin α2β1, suggesting the induction of αβ3-directed cell motility was specific (12).

Induction of cell invasive behavior has been linked to expression of uPA and uPAR on the cell surface (9–11). Thus, we examined whether activation of FG cell migration on vitronectin was associated with the expression of uPA and uPAR on these cells. As shown in Fig. 1, stimulation of serum-starved FG cells with either PMA or TGF-α promoted cell motility toward vitronectin within 4–6 h and induced a similar time-dependent increase in uPA mRNA levels (Fig. 2A). PMA treatment resulted in a dramatic increase in the steady-state levels of uPA mRNA. Maximal levels of uPA mRNA were observed at 4 h, and levels remained elevated at 24 h. Following TGF-α treatment, the steady-state levels of uPA mRNA increased after 1 h and remained elevated between 1 and 24 h. Both PMA and TGF-α increased the steady-state levels of uPAR mRNA (Fig. 2B) with kinetics similar to those observed for uPA mRNA. Maximal levels of uPAR mRNA were observed 4 h after PMA treatment and remained elevated at 24 h. In TGF-α-treated cells, maximal levels of uPAR mRNA were observed at 1 h and remained elevated through 24 h.

No cell-associated uPA or uPAR protein was detected by
Western blotting in unstimulated cells or cells stimulated for 1 h with PMA. However, a high level of surface-bound uPA and uPAR protein was first detected at 4 h post-PMA stimulation (Fig. 2C) and remained elevated at 24 h. The kinetics of induction of uPA and uPAR proteins were similar to those observed for the induction of FG cell migration on vitronectin (Fig. 1). TGF-α stimulation of FG cells resulted in a similar but smaller increase in cell-bound uPA and uPAR protein levels that was first detected after 2 h and remained elevated at 24 h (Fig. 2C). In addition, activation of protein kinase C induced a time-dependent increase in both cell surface-associated uPA and uPA secreted into the cell-conditioned medium as measured by uPA enzyme-linked immunosorbent assay. Secreted uPA steadily increased between 4 and 24 h post-PMA stimulation, whereas surface-bound uPA greatly increased between 4 and 8 h, reached a peak at 8 h, and remained elevated 24 h post-PMA stimulation (Fig. 3), in accordance with the time course of induction of uPAR protein observed above (Fig. 2C) and the motility of FG cells on vitronectin (Fig. 1).

Binding of uPA to Its Receptor Promotes FG Cell Migration on Vitronectin but Not on Collagen—The observation that exposure of FG cells to PMA or TGF-α promoted uPA and uPAR expression and induced αvβ5-directed cell migration prompted us to examine whether these events were functionally related. Thus, we evaluated whether the binding of uPA to its receptor might contribute to the αvβ5-dependent FG cell motility on vitronectin. As shown in Fig. 4, inhibition of uPA binding to its receptor with a function-blocking monoclonal antibody to uPAR (mAb 3936) or by the addition of excess soluble uPAR results in a >50% inhibition of FG cell motility on vitronectin, a level of inhibition comparable with that obtained with the anti-αvβ5 mAb P1F6. In contrast, these reagents had no effect on FG cell motility on collagen, suggesting that α2β1-dependent migration of FG cells is not influenced by uPA or uPAR. Furthermore, when either anti-uPAR or soluble uPAR was used together with anti-αvβ5, the level of inhibition of migration was the same as that seen when either antagonist was used alone, suggesting a functional cooperation between αvβ5 and uPA-uPAR. In addition, we demonstrated by FACS analysis (Fig. 5) that when FG cells were stimulated with PMA in the presence of a function-blocking antibody to uPAR (mAb 3936, panel C), the levels of receptor-bound uPA, detected using a polyclonal anti-uPA antibody (389), were similar to the back-
uPAuPAR Is Required for Integrin-directed Migration

**DISCUSSION**

Growth factors or chemokines influence cell migration, which contributes to wound healing, development, and tumor cell invasion. To this end, we previously demonstrated that αvβ5-mediated motility but not adhesion of carcinoma cells depends on prior exposure of cells to growth factors or phorbol esters (12, 14). Here, we provide evidence for a novel mechanism that accounts for the induction of such motility, based on the functional expression of uPAuPAR and its cooperation with the integrin αvβ5. Specifically, exposure of cells to TGF-α or PMA induces the expression of uPA and its receptor and concomitantly stimulates cell migration on vitronectin, which is inhibited with antagonists of αvβ5. This αvβ5-dependent motility is also significantly abrogated by specific antagonists of both the interaction between uPA and its receptor and uPA enzymatic activity. In fact, three distinct competitors of uPAuPAR interaction significantly reduced αvβ5-dependent motility including: soluble uPAR, an antibody to the uPARuPA binding site, and a 17-mer peptide (11, 21, 25). In addition, the specific inhibitors of uPA enzymatic activity, PAI-1, PAI-2, and the neutralizing anti-uPA mAb 394, were also found to block the integrin-dependent motility, suggesting a role for uPA enzymatic activity in this migration response. Importantly, although inhibitors of both αvβ5 and uPAR were effective in abrogating migration by themselves, when used in combination there was no additional inhibition, implying a true functional cooperation or formation of a complex between these molecules. While it is evident from these findings that expression of uPAuPAR is a prerequisite for αvβ5-dependent motility, it is

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also clear that such cooperation is not required for αvβ3-dependent migration of these same cells to vitronectin or α2β1-dependent migration on collagen (12, 19).

The functional cooperation between αvβ5 and uPA-uPAR may operate at a number of levels. The uPA-uPAR system could promote migration via multiple processes including proteolysis, signal transduction, and/or direct ligation to vitronectin (10, 31, 35). A requirement for proteolysis appears to play some role in our migration system, since the inhibitors of uPA enzymatic activity block migration. While a requirement for uPA catalytic activity appears to be surprising, given the absence of exogenous plasminogen in the assays and the lack of inhibition by either aprotinin or tranexamic acid, several explanations may be given. First, it is conceivable that uPA enzymatic activity may be required for the activation of a latent growth factor that can concomitantly promote cell motility. In this respect, it has been shown that uPA directly cleaves and activates latent hepatocyte growth factor/scatter factor (36), a factor known to promote motility and matrix invasion of epithelial cells (37). Second, uPA enzymatic activity may be required for the initial cleavage of vitronectin so that it becomes more vulnerable to proteolysis by additional enzymes, as has been shown to be the case for fibronectin (38, 39), or it may be required for the remodeling and/or exposure of additional epitopes on vitronectin. Third, uPA enzymatic activity may be necessary for the association of the catalytically active uPA-uPAR complex with other cell surface proteins, as has been shown for vitronectin and thrombospondin (40).

While it is evident that uPA-uPAR proteolytic activity can promote migration, it has also been established that uPA enzymatic activity is not always required for cell migration. For example, it has been shown that the binding of the uPA receptor by the enzymatically inactive amino-terminal fragment of uPA is sufficient to promote human epidermal cell motility (41). In another report, human monocyte chemotaxis was prevented by blocking uPA binding with an anti-uPAR monoclonal antibody but not with an antibody that neutralizes uPA catalytic activity (42). In a recent study Busso et al. (35) suggest that ligation of uPAR by uPA leads to enhanced epithelial cell migration as a result of uPAR-mediated signal transduction. In this regard it has been shown in human monocytes that uPAR is a component of a large receptor complex consisting of Src-family protein-tyrosine kinases and β2 integrins (43). Furthermore, in this study, it was shown that activation of monocytes with either active or enzymatically inactivated uPA resulted in induction of tyrosine phosphorylation of several proteins. In light of these observations and our findings that competitors of the interaction between uPA and its receptor inhibit αvβ5-dependent motility, it is possible that uPAR-mediated signal transduction plays a role in the functional cooperation between αvβ5 and uPA-uPAR during cell migration on vitronectin.

Importantly, it was recently shown that the uPA-uPAR complex can bind to vitronectin (31, 44), which raises the possibility that uPA-uPAR can potentiate αvβ5-dependent motility by providing an additional receptor for attachment to vitronectin. Thus, the migration of tumor cells to vitronectin may require dual recognition of this ligand by αvβ5 and the uPA-uPAR complex. In this regard, following induction of uPA-uPAR expression by PMA, we observed some contribution (25–30%) of uPA-uPAR to FG cell adhesion to vitronectin (data not shown),
whereas prior to such induction, adhesion was mediated solely by αvβ5. While direct ligation between uPA-zuPAR and vitronectin may contribute to migration, it cannot account for all the migration observed, because PAI-2 and the anti-uPA mAb 394 significantly abrogated migration and yet had no effect on adhesion. Thus, the dependence of αvβ5-directed migration on uPA-zuPAR cannot be attributed to an enhancement of vitronectin-mediated adhesion.

While it is clear that the ability of PAI-2 to inhibit migration cannot be attributed to abrogation of adhesion, this cannot be said of PAI-1. Thus, it is evident from our data that the ability of PAI-1 to inhibit migration may be due, at least in part, to its ability to block the adhesion of FG cells to vitronectin. In fact, it is well documented that PAI-1 can bind to vitronectin, although the binding site is controversial (32, 33). It is interesting to note that these sites are adjacent to both the RGD integrin binding site (45) and to the uPA-zuPAR binding site (46). This said, PAI-1 could prevent adhesion and, concomitantly, migration to vitronectin by sterically blocking one or both of these ligation sites.

A number of studies have demonstrated co-localization between uPA-zuPAR and a variety of integrins including β2 integrins (43, 47) and αvβ3 (48). Recently, Reinartz et al. (49) demonstrated the localization of uPA, its receptor, and αvβ5 in focal contacts formed by human keratinocytes. Ciambrone and McKeown-Longo (50) showed that both uPA and uPAR were localized to focal contacts in human fibrosarcoma cells and fibroblasts plated on vitronectin but not in cells plated on fibronectin. Thus, vitronectin may regulate the synthesis of uPA and direct the localization of uPA and uPAR into focal contacts. By immunofluorescence analysis, we were able to confirm a partial colocalization of αvβ5 and uPAR in approximately 10% of the PMA-stimulated FG cells plated on vitronectin (data not shown). However, such co-localization was relatively rare, with both uPA and uPAR localizing to pseudopod extensions and membrane ruffles in addition to focal contacts. This supports previous studies that have demonstrated uPAR localized to the leading edge of migrating cells at lamellipodia and pseudopod extensions (42). Significantly, such structures are highly reversible, likely leading to cell detachment from the substrate. These structures may then
reattach or fold back upon themselves to produce membrane ruffles, thereby explaining their localization to the leading edge of migrating cells (51). A recent study by Kindzelskii et al. (52) shows that individual cells undergo multiple cycles of β2 integrin-uPA coupling and uncoupling during cellular polarization that precedes migration. Interestingly, following dissociation or uncoupling, uPA accumulates at the leading edge of the cell or lamellipodia, whereas the β2 integrins distribute to the trailing edge or uropod. Such dynamic interreceptor interactions may be an important component of the functional cooperation between uPA-uPAR and αvβ5 and may account for transient co-localization in some cells.

The link established in this study among TGF-α, uPA-uPAR, and integrin-mediated cell motility has significant implications for the regulation of cell migration events associated with processes as diverse as development, angiogenesis, wound healing, and tumor metastasis. Our observations may account for a variety of cell motility events described by others. For example, the migration of keratinocytes has been linked to the induction of uPA expression by TGF-α (5). Interestingly, it has recently been shown that both TGF-α and PMA induce angiogenesis via an αvβ5-dependent mechanism (17). This finding is consistent with the possibility that these agonists promote αvβ5-dependent migration of endothelial cells. Our observations also have significant implications for the spread of epithelial tumors that commonly express αvβ5 (53). In this regard, it is particularly interesting that the autocrine production of TGF-α has been linked to both the expression of malignancy and motility in carcinomas (54). While the precise mechanism of uPA-uPAR in cell migration is not completely understood, it appears that this ligand-receptor complex functionally cooperates with integrin αvβ5 to promote the migration of carcinoma cells.

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