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Urokinase Receptor Interacts with $\alpha_v\beta_5$ Vitronectin Receptor, Promoting Urokinase-dependent Cell Migration in Breast Cancer[1]

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ABSTRACT

Perturbation of adhesive interactions at cell-substratum and cell-cell contact sites is a critical event in the multistep process of cancer invasion. Recent studies indicate that the urokinase receptor (uPAR) is associated in large molecular complexes with other molecules, such as integrins. To test the possibility that uPAR may physically and functionally interact with vitronectin (Vn) receptors, we determined the expression level of uPAR, $\alpha_v\beta_3$, and $\alpha_v\beta_5$ Vn receptors in 10 human breast carcinomas. Here, we show the ability of uPAR to physically associate with $\alpha_v\beta_5$ in the breast carcinomas examined. The functional effects of this interaction were studied using HT1080 human fibrosarcoma and MCF-7 human breast carcinoma cell lines, both exhibiting a urokinase-dependent physical association between uPAR and $\alpha_v\beta_5$. Both cell lines respond to urokinase or to its noncatalytic amino-terminal fragment by exhibiting remarkable cytoskeletal rearrangements that are mediated by $\alpha_v\beta_5$ and require protein kinase C activity. On the contrary, binding of Vn to $\alpha_v\beta_5$ results in the protein kinase C-independent formation of F-actin containing microspike-type structures. Furthermore, $\alpha_v\beta_5$ is required for urokinase-directed, receptor-dependent MCF-7 and HT1080 cell migration. These data show that uPAR association with $\alpha_v\beta_5$ leads to a functional interaction of these receptors and suggest that uPAR directs cytoskeletal rearrangements and cell migration by altering $\alpha_v\beta_5$ signaling specificity.

INTRODUCTION

The invasive ability of malignant cells requires a complex interplay of various cell surface-associated components participating to the proteolytic disruption of ECM[3] and the modification of cell adhesion properties (1).

A large body of evidence assigns to urokinase [urokinase-type plasminogen activator (uPA)] a key role in tumor progression and invasion, by virtue of its ability to activate plasminogen, which degrades many ECM components, such as fibronectin, laminin, and proteoglycans, and activates latent collagenases (2). The inoculation of metastatic Lewis lung carcinoma cells into plasminogen-deficient mice results in the formation of smaller and less hemorrhagic tumors than in control wild type mice (3). Furthermore, the absence of uPA negatively affects the progression of chemically induced melanocytic neoplasms in mice (4).

In addition to its proteolytic role, uPA can regulate cell mobilization, adhesion, proliferation, and transcription of specific genes through a catalytic-independent mechanism (5–7). A key player in this process is the specific cell surface uPAR, which binds with high affinity the ATF of uPA (8). uPAR is a highly glycosylated 55,000–60,000 M_r protein that includes the N-terminal uPA binding domain, designated D1, a connecting domain (D2), and the COOH-terminal domain bearing a glycosylphosphatidylinositol anchor (D3; Ref. 2). D2 and D3 domains have the property to recognize the matrix-like form of Vn (9). The multiple molecular events following uPAR ligation with uPA include diacylglycerol formation in endothelial cells, activation of PKC, and phosphorylation of cytokeratins 8 and 18 in human epithelial cells (10, 11). A transient modification of the Src-family kinase p56/59^{hck} activation state has been also reported in mielomonocytic cells (12, 13). All of these cell responses raise a question concerning the modality of uPAR signaling transmission, as this is restricted to the outer leaflet of the membranous bilayer and therefore requires a transmembrane "adaptor." Coimmunoprecipitation studies show that uPAR is associated in large molecular complexes with integrins, caveolin, and Src kinases (14–16). The reversible association with other receptors is supported by the uPAR lateral mobility in the plasma membranous bilayer and its focal redistribution upon interaction with uPA (17). The relevance of uPAR lateral mobility to signaling is further sustained by the finding that a nonsignaling uPA variant is also unable to mobilize the receptor (18). Integrin receptors are composed of α and β subunits that heterodimerize to produce more than 20 different receptors, capable of mediating a variety of cell responses, such as spreading and migration, control of gene expression, growth, and differentiation (19–21). Changes in integrin structure and/or expression are frequently associated with malignant transformation and tumor progression (22). Overexpression of α_v integrins occurring in human mammary carcinomas is associated to a widespread deregulated expression of other integrins, such as α_2 and $\alpha_6\beta_4$ (23).

uPAR and integrins share the ability to activate members of the Src family, as well as pp125FAK, further supporting the possibility that uPAR impinges on cell function via integrins (24). Following engagement with uPA, uPAR colocalizes or physically associates with β_1, β_2, β_3, or β_5 integrins both *in vivo* and *in vitro* (15, 25, 26). It is presently known that uPAR association suppresses the normal adhesive function of different integrins, suggesting that they may acquire new functional properties (16).

A shared ligand between integrins and uPAR is Vn that binds and stabilizes plasminogen activator inhibitor PAI-1 (27). Both, uPAR and PAI-1 bind to the somatomedin-like domain of Vn, and PAI-1 prevents Vn binding to the VnR, therefore stimulating cell detachment (28, 29). The physical linkage between uPAR and VnR is supported by the colocalization of uPAR and $\alpha_v\beta_5$ at focal contacts of human keratinocytes (30). Furthermore, Vn-dependent migration of human pancreatic carcinoma cells is inhibited by anti-uPAR or by anti-$\alpha_v\beta_5$ antibody, suggesting a functional coupling between these two receptors (31).

The two known VnRs exhibit different functional properties, as they regulate two distinct pathways in angiogenesis (32, 33). Unlike $\alpha_v\beta_3$, $\alpha_v\beta_5$ can direct cell migration, as well as redistribution of talin, vinculin, and α-actinin only in the presence of PKC activators (31, 34).

We have previously shown the ability of membrane-associated

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[3] The abbreviations used are: ECM, extracellular matrix; uPA, urokinase-type plasminogen activator; uPAR, urokinase receptor; Vn, vitronectin; VnR, vitronectin receptor; ATF, amino-terminal fragment of urokinase; PKC, protein kinase C; mAb, monoclonal antibody.

uPARs to form high-affinity ternary complexes with ATF and Vn both in tumor cell lines and in breast carcinomas (35, 36).

The aim of this study was to investigate whether $\alpha_v\beta_3$ and $\alpha_v\beta_5$ VnRs play a role in the uPA-dependent tumor cell spreading and migration. First, we show that $\alpha_v\beta_5$ copurifies with uPAR in breast carcinoma samples expressing high levels of these receptors. Second, we studied the functional consequences of uPAR-integrins interaction in HT1080 human fibrosarcoma and MCF-7 human breast adenocarcinoma cell lines, both of which express uPAR (36). Here, we report that treatment of both cell lines with uPA promotes the physical association of uPAR with $\alpha_v\beta_5$ and that $\alpha_v\beta_5$ is required for uPAR-directed cell migration. We also show that uPA promotes cytoskeletal rearrangements that are mediated by $\alpha_v\beta_5$ in a PKC-dependent manner. Finally, the finding that PKC inhibitors selectively prevent uPA-dependent and not Vn-dependent effects on cytoskeleton suggest that uPAR association modifies normal $\alpha_v\beta_5$ signaling specificity.

MATERIALS AND METHODS

Reagents. ATF (amino acids 1–135) was a gift of Dr. J. Wang (Abbott Laboratories, Abbott Park, IL). Recombinant single-chain uPA was obtained from Dr. Sarmientos (Farmitalia, Milan, Italy). Native human Vn was purchased from Promega (Florence, Italy). The biotinylated immunoglobulins and the enhanced chemiluminescence detection system were from Amersham Pharmacia Biotech (Milan, Italy). The rodhamine-conjugated phalloidin, FITC-conjugated antibodies, PKC inhibitors, protein G-Sepharose, and cycloheximide were from Sigma Chemical Co. (Milan, Italy). The tissue culture dishes, polycarbonate chemotaxis filters, and Boyden chambers were from Costar, Nucleopore (Milan, Italy). All cell culture reagents were purchased from Life Technologies, Inc. (Gaithersburg, MD).

Antibodies. Anti-uPAR 399 rabbit polyclonal antibody was from American Diagnostica (Greenwich, CT). Anti-uPAR R4 mAb was a gift of Dr. Gunilla Hoyer-Hansen (Copenhagen, Denmark; Ref. 37). Anti-$\alpha_v\beta_5$ mAb (clone P1F6) and anti-αv chain mAb (clones VNR147 and VNR139) were from Life Technologies (Milan, Italy; Refs. 38 and 39). Anti-$\alpha_v\beta_3$ mAb (clone 23C6) was from Pharmigen (San Diego, CA; Ref. 40). Anti-β_5 chain polyclonal antibody was from Chemicon Int. Inc. (Temecula, CA; Ref. 32). Anti-β_3 chain mAb (clone 26; Ref. 41), the positive control human fibroblast, and A431 extracts were from Transduction Laboratories (Lexington, KY). Anti-β_3 chain mAb (clone 61) was from DAKO (Copenhagen, Denmark; Ref. 25).

Cell Lines and Culture Conditions. HT1080 human fibrosarcoma and MCF-7 breast adenocarcinoma cell lines were grown in DMEM supplemented with 10% fetal bovine serum, 100 IU/ml penicillin, and 50 μg/ml streptomycin. The MCF-7/uPAR4 cell line is derived from the MCF-7 cell line, stably transfected with pcDNA3-uPAR, carrying the entire human uPAR cDNA excised from puPAR-1 (42). Briefly, 1×10^7 subconfluent MCF-7 cells were cotransfected with 30 μg of pcDNA3-uPAR and pRSVneo (10:1) by electroporation at 250 V and 960 microfarads. The neomycine-resistant clones were analyzed by radioreceptor binding assay with ^{125}I-ATF, which revealed that MCF-7/uPAR4 clone produces about 5 times more uPAR than the parental cells (8).

Tissue Preparation. Ten tumor biopsy specimens were obtained from patients undergoing surgery for a breast lump. Tumors included a total of seven ductal and three lobular carcinomas, whereas benign breast lesions consisted of five fibrocystic diseases. The specimens were immediately frozen in liquid nitrogen and stored at −80°C until used for immunocytochemistry and crude lysate preparation.

Immunostaining. For immunohistochemical analysis of breast tumor specimens, 5-μm-thick frozen serial sections corresponding to the largest cross-sectional area of the tumor were cut, placed on clean glass slides, air dried, and subjected to immunostaining with the streptavidin-biotin-peroxidase method as described previously (35, 36). For immunocytochemical staining, HT1080 and MCF-7 cells were grown on glass slides in the presence of serum. Before VnR immunostaining, samples were fixed with 3.5 formaldehyde, 0.1% Triton X-100 in PBS for 10 min at 4°C. In all cases, slides were incubated overnight at 4°C with 10 μg/ml R4 anti-uPAR, anti-$\alpha_v\beta_5$, anti-$\alpha_v\beta_3$ or anti-α_v chain clone VNR 147 mAbs. Photographs were taken on Kodak 100 ASA film at ×400 magnification.

The intensity of immunostaining with anti-uPAR and anti-$\alpha_v\beta_5$ mAbs was graded from 1 to 3+ when faint, moderate, or intense staining was observed in epithelial tumor cells.

Western Blot Analysis of uPAR Containing Immunocomplexes. Two hundred mg of breast carcinoma samples were lysed using 600 μl of lysis buffer (10 mM Tris-HCl, pH 8.1, 140 mM NaCl, 2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 0.5% NP-40, 1% Triton X-100) and extracted for 30 min at 4°C. Lysates were cleared by centrifugation at 13,000 rpm for 10 min at 4°C, and the protein content was determined by the Bradford method. Eight hundred μg of each sample were preabsorbed with 20 μl of a 1:1 suspension of protein G-Sepharose for 2 h at 4°C and then immunoprecipitated overnight at 4°C using 10 μg/ml R4 or 399 anti-uPAR antibodies. The immunoprecipitates were recovered by absorption to protein G-Sepharose as described (35).

For analyzing uPAR-containing complexes from cell lines, acid-treated HT1080 cells were incubated for 1 h at 22°C with 10 nM recombinant uPA, with or without 500 nM urea-denatured Vn. Cells (5×10^6 cells/sample) were lysed in 500 μl of 10 mM Tris-HCl, pH 8.1, 140 mM NaCl, 2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 0.5% NP-40, 1% Triton X-100 for 30 min at 4°C. Lysates were then cleared by centrifugation, and 800 μg of each sample were immunoprecipitated with anti-uPAR 399 polyclonal antibody. Alternatively, 50 μg of acid-pretreated cell membrane fractions were incubated with 10 nM ATF in the presence or in the absence of 500 nM urea-denatured Vn and cross-linked using disuccinimidyl suberate according to a previously published procedure (36, 43). The samples were then immunoprecipitated with the indicated anti-uPAR antibodies.

In all cases, the immunoprecipitated proteins were separated by 6% SDS-PAGE under unreducing conditions and transferred to nitrocellulose membranes. Blots were blocked overnight with 5% nonfat dry milk, 3% BSA and then incubated with a 2 μg/ml concentration of the indicated antibody for 2 h at 4°C. After washing with 0.1% Tween-20 in PBS, the filters were incubated with 1:4000 biotinylated antimouse immunoglobulins for 1 h at 22°C. They were extensively washed and finally analyzed using the ECL system, according to the manufacturer's recommendations.

Analysis of Cytoskeleton. HT1080 and MCF-7 cells were harvested by a mild trypsinization and incubated with 10% fetal bovine serum/DMEM for 1 h at 37°C, 5% CO_2. Then, cells were acid treated, washed with PBS, and incubated in serum-free medium with 10 nM recombinant uPA or ATF in the presence or in the absence of 500 nM urea-denatured Vn for 1 h at 22°C. When specified, the cells were pretreated with a 5 μg/ml concentration of the indicated antibodies or with 200 nM calphostin C, 20 μM bisindolylmaleimide (GF109203X), or 10 μg/ml cycloheximide for 1 h at 22°C. The cells were fixed with 3.5% formaldehyde for 10 min on ice, permeabilized with 0.1% Triton X-100, and incubated for 40 min with 0.1 μg/ml rodhamine-conjugated phalloidin. Finally, cell cytoskeleton was examined by a fluorescence microscope (Axioplan Zeiss), and photographs were taken on Kodak 400 ASA film at ×1000 magnification.

Cell Migration Assay. Cell migration assays were carried out in Boyden chambers under serum-free conditions as described previously, with minor modifications (12). The 8 μm pore size polycarbonate filters were coated with either 5 μg/ml Vn or 50 μg/ml collagen. Subconfluent HT1080 or MCF-7/uPAR4 cells were harvested, acid treated, and incubated with or without a 10 μg/ml concentration of the indicated anti-VnR antibodies for 1 h at 22°C in serum-free medium. In all cases, 2×10^5 viable cells/sample were allowed to migrate toward 10 nM recombinant uPA or ATF for 3 h at 37°C. Then, cells were fixed in ethanol and stained with hematoxylin, and 10 random fields/filter were counted at ×200 magnification.

RESULTS

uPAR and VnR Interaction in Breast Carcinomas. We analyzed the expression of uPAR, $\alpha_v\beta_5$, and $\alpha_v\beta_3$ VnRs in 10 human breast carcinomas and in 5 benign breast lesions by immunohistochemical staining with R4 anti-uPAR, P1F6 anti-$\alpha_v\beta_5$, and 23C6 anti-$\alpha_v\beta_3$ mAbs. Table 1 reports the clinical data and pathological findings of 10 human breast carcinomas (7 ductal and 3 lobular carcinomas). The intensity of both uPAR and $\alpha_v\beta_5$ staining of epithelial tumor cells was

Table 1 *Patient's age, pathological findings, uPAR, and $\alpha_v\beta_5$ grading of 10 human breast carcinomas*

Patient no.	Age (yr)	Diagnosis	Stage	Size (cm)	uPAR grading[a]	$\alpha_v\beta_5$ grading[a]
1	42	Ductal	$T_2N_1M_0$[b]	2.5	3	3
2	43	Lobular	$T_2N_1M_0$	2.6	2	2
3	52	Ductal	$T_3N_0M_0$	>5	2	2
4	51	Ductal	$T_1N_1M_0$	2	2	3
5	52	Lobular	$T_2N_0M_0$	2.6	2	2
6	63	Lobular	$T_2N_1M_0$	5	3	2
7	59	Ductal	$T_1N_1M_0$	2	3	3
8	68	Ductal	$T_1N_0M_0$	2	1	1
9	85	Ductal	$T_1N_1M_0$	2	1	1
10	42	Ductal	$T_2N_1M_0$	3	3	3

[a] Immunohistochemical staining of epithelial tumor cells with R4 anti-uPAR and P1F6 anti-$\alpha_v\beta_5$ monoclonal antibodies was graded as faint (grading 1), moderate (grading 2), or intense (grading 3).
[b] TNM, tumor-node-metastasis.

graded as faint (grading 1), moderate (grading 2), or intense (grading 3; Table1). In agreement with our previous findings (35), each individual tumor showed a heterogeneous pattern of staining with anti-uPAR mAb (Table 1). A diffuse staining of the epithelial tumor cells in sections from ductal and lobular carcinomas was obtained using anti-$\alpha_v\beta_5$ mAb. In positive tumor cells, a prominent staining of plasma cell membranes was often observed. Three representative cases are shown in Fig. 1*A*. Both anti-uPAR and anti-$\alpha_v\beta_5$ mAbs were slightly reactive to the five benign breast lesions examined, weakly staining ductal cells (not shown). On the contrary, anti-$\alpha_v\beta_3$ mAb was poorly reactive toward the epithelial tumor cells, showing a strong reaction to the endothelial cells (Fig. 1*A*) as well as to normal ductal cells (not shown).

It has been described that uPAR can physically associate with integrin receptors, possibly forming functional units that impinge on cell transduction pathways. To examine the possibility that α_v chain of VnRs can be copurified with uPAR, crude lysates were prepared from breast tumor samples and subjected to immunoprecipitation with anti-uPAR antibodies. Then, the immunoprecipitated proteins were analyzed by immunoblotting for their α_v content. Anti-α_v VNR139 mAb reacted with a M_r 120,000 protein, indicating a physical association between uPAR and α_v (Fig. 1*B*). Similar results were obtained with 399 anti-uPAR polyclonal antibodies (not shown). Furthermore, we noticed that the relative intensity of the α_v band copurified with uPAR from T_1, T_2, and T_3 tumors is consistent with their relative $\alpha_v\beta_5$ grading. The absence of a specific reaction in samples incubated in the absence of the primary antibody was also observed.

uPA- and Vn-dependent Physical Association of uPAR and $\alpha_v\beta_5$ in HT1080 and MCF-7 Tumor Cell Lines. The results of the previous experiments suggest the occurrence of uPAR-$\alpha_v\beta_5$ physical association in the epithelial tumor cells of breast carcinomas. To test whether this association may lead to a functional interaction, MCF-7 and HT1080 cell lines were preliminarily analyzed, by immunohistochemical staining, for the expression of uPAR, $\alpha_v\beta_3$, α_v, and $\alpha_v\beta_5$ using anti-uPAR R4, anti-$\alpha_v\beta_5$, anti-$\alpha_v\beta_3$, or anti-α_v chain clone VNR147 mAbs (Fig. 2, *top panel*). Both cell lines express, although to a different extents, $\alpha_v\beta_5$, α_v chain and uPAR. Anti-$\alpha_v\beta_3$ mAb did not stain HT1080 cells, whereas it appreciably reacted with MCF-7 cells. Accordingly, when various anti-β_3 mAbs, such as clones 26 and 61, were incubated with HT1080 cells, again, a scarce specific staining was observed (not shown).

Next, we examined the possibility that $\alpha_v\beta_5$ and/or $\alpha_v\beta_3$ VnRs can be copurified with uPAR in an *in vitro* assay. To this end, acid-treated membrane fractions from HT1080 and MCF-7 cells were incubated with ATF in the presence or in the absence of urea-denatured Vn, solubilized, and immunoprecipitated with 399 or R4 anti-uPAR antibodies. Then, these samples were analyzed by immunoblotting for their α_v, β_5, and β_3 content. Anti-α_v VNR139 mAb reacted with a M_r 120,000 protein, thereby suggesting the uPA-dependent physical association of α_v with uPAR, in the presence of Vn (Fig. 2*A*). *In vitro*, α_v does not copurify with uPAR, in the absence of ATF or uPA (not shown). The absence of a specific reaction in samples incubated with a nonspecific rabbit serum and the absence of cross-reactivity between each secondary antibody and anti-uPAR or anti-α_v antibodies were also observed.

Immunoprecipitation of lysates from MCF-7 cells with R4 anti-uPAR mAb, followed by probing with anti-β_5 polyclonal antibodies, showed a specific band at M_r 82,000, in agreement with the β_5 chain M_r (Fig. 2*B*). Conversely, the molecular species immunoprecipitated with 399 anti-uPAR polyclonal antibody from MCF-7 lysates did not react with anti-β_3 mAb (Fig. 2*C*). As a positive control, the reactivity of anti-β_5 and anti-β_3 antibodies was tested employing commercial extracts from either A431 epidermoid carcinoma cell line or human

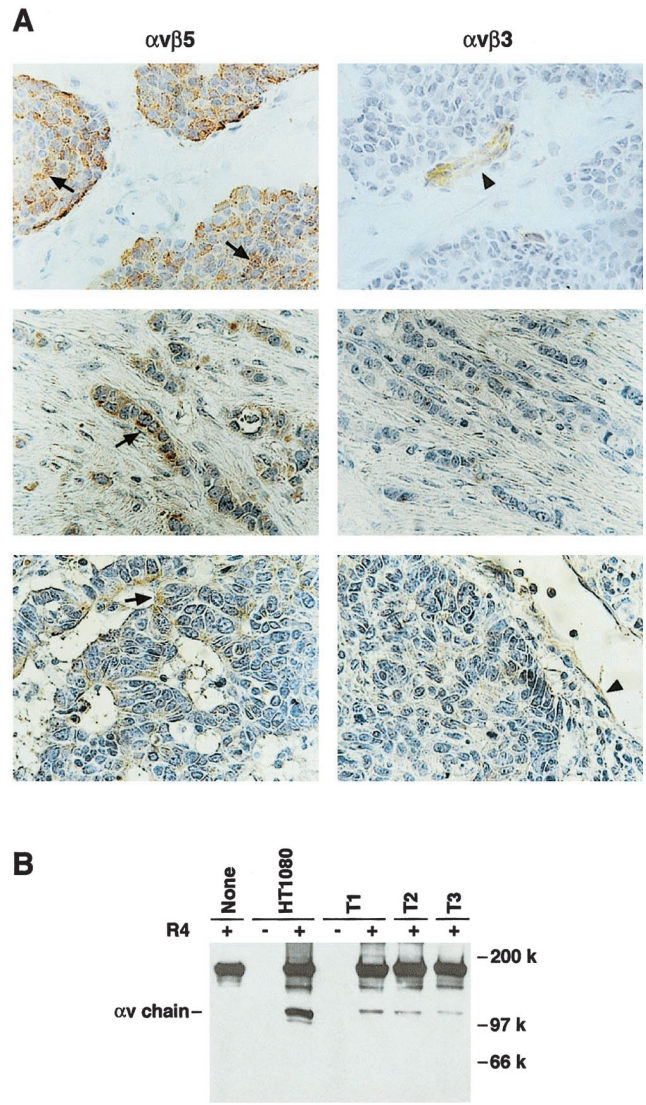

Fig. 1. Expression and association of uPAR and $\alpha_v\beta_5$ in breast carcinomas. *A*, frozen sections from ductal (T_1 and T_3) and lobular (T_2) breast carcinomas were subjected to immunohistochemical staining using anti-$\alpha_v\beta_5$ P1F6 or anti-$\alpha_v\beta_3$ 23C6 mAbs. *Arrows*, positive epithelial tumor cells; *arrowheads*, positive endothelial cells. ×400. *B*, 800 μg of lysates from HT1080 cells, T_1, T_2, and T_3 breast carcinomas, were immunoprecipitated with R4 anti-uPAR mAb (+) or with nonimmune serum (−). 5 μg of R4 mAb was loaded (*None*). The samples were separated by 6% SDS-PAGE under unreducing conditions and transferred to a nitrocellulose membrane. The filter was probed with anti α_v clone VNR139 mAb. The position of the M_r 120,000 α_v band is indicated.

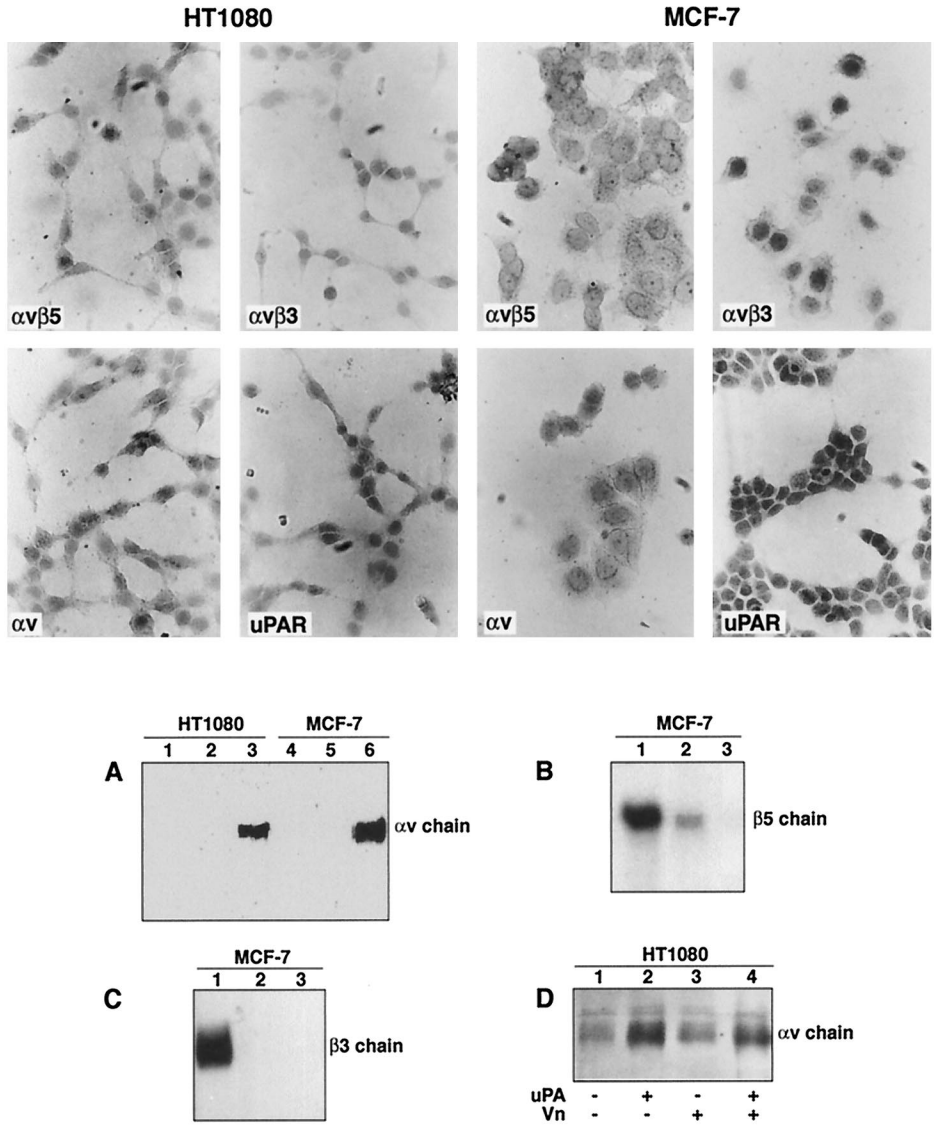

Fig. 2. Expression and association of uPAR and $\alpha_v\beta_5$ in HT1080 and MCF-7 cell lines. *Top panels*, HT1080 and MCF-7 cells were grown on glass slides and subjected to immunohistochemical staining using anti-$\alpha_v\beta_5$ P1F6, anti-$\alpha_v\beta_3$ 23C6, anti-α_v clone VNR147, or anti-uPAR R4 mAbs. ×400. *A*, 50 μg of acid-treated membrane fractions from HT1080 (*Lanes 1–3*) and MCF-7 (*Lanes 4* and *6*) cells were incubated with 10 nM ATF in the presence (*Lanes 2, 3,* and *6*) or in the absence (*Lanes 1* and *4*) of 500 nM Vn, cross-linked, solubilized, and immunoprecipitated with 399 anti-uPAR polyclonal antibody (*Lanes 1, 3, 4,* and *6*) or with nonimmune serum (*Lane 2*). 5 μg of 399 antibody were loaded in *Lane 5*. The samples were separated by 6% SDS-PAGE under unreducing conditions and subjected to Western blot analysis for the α_v content (*Lanes 1–6*). *B*, membrane extracts from MCF-7 cells were incubated with ATF and Vn under the conditions described in *A*, immunoprecipitated with R4 anti-uPAR mAb (*Lane 2*) or with nonimmune serum (*Lane 3*). 5 μg of a control A431 cell extract was loaded in *Lane 1*. The filter was probed with anti-β_5 polyclonal antibodies. *C*, membrane extracts from MCF-7 cells were incubated with ATF and Vn under the conditions described in *A* and subsequently immunoprecipitated with anti-uPAR 399 polyclonal antibody (*Lane 2*) or with nonimmune serum (*Lane 3*). 5 μg of human fibroblast extract were loaded in *Lane 1*. The filter was probed with anti-β_3 mAb. *D*, 800 μg of cell lysate from HT1080 cells, previously stimulated with (*Lanes 2* and *4*) or without (*Lanes 1* and *3*) 10 nM recombinant uPA, 500 nM Vn (*Lanes 3* and *4*), or both (*Lane 4*) were immunoprecipitated with 399 anti-uPAR polyclonal antibody and subjected to Western blot analysis for the α_v content.

fibroblasts, respectively. These results indicate that uPAR associates with α_v and β_5 chains following exposure to ATF, in the presence of Vn. To test whether the interaction between uPAR and $\alpha_v\beta_5$ may occur *in vivo*, coimmunoprecipitation experiments were carried out on intact cells. Acid-treated HT1080 cells were incubated with or without 10 nM recombinant uPA, in the presence or in the absence of 500 nM urea-denatured Vn, under serum-free conditions. Crude lysate extracts were subsequently immunoprecipitated with 399 anti-uPAR polyclonal antibody, and the resulting immunoprecipitates were tested for the α_v content. Fig. 2*D* shows that in the absence of uPA, the low amount of α_v copurifying with uPAR is comparable to that obtained following the addition of Vn alone (*Lanes 1* and *3*). It is evident that cell exposure to uPA greatly enhances the extent of uPAR-associated α_v. The low amount of α_v copurified with uPAR in the absence of exogenous uPA may be due to traces of uPA possibly secreted from HT1080 cells during the 1-h incubation. The combined use of Vn and uPA neither reduced nor enhanced the extent of α_v copurified with uPAR (Fig. 2*D*, *Lane 4*). This experiment suggests that *in vivo* Vn is dispensable for this association to occur. Our previous data indicated that Vn is required for uPAR-α_v association in isolated membranes; these apparently contradictory results may be reconciled considering the possibility that uPAR may exclusively associate with the $\alpha_v\beta_5$ active conformer. Therefore, *in vitro*, Vn may be required to convert $\alpha_v\beta_5$ to its active state, whereas *in vivo*, integrin affinity could be regulated by an inside-out mechanism that depends on cell metabolic activity. This experiment confirms the central role of uPA in triggering uPAR-$\alpha_v\beta_5$ interaction and raises the possibility of a functional cooperation between these two receptors.

Role of VnRs in uPA- and/or Vn-dependent Cytoskeletal Rearrangements. The physical interaction between $\alpha_v\beta_5$ and uPAR prompted us to examine whether this uPA-dependent coupling may indeed affect cytoskeletal arrangement. HT1080 cells were harvested, acid treated, and incubated with uPA and/or Vn in the presence or in the absence of anti-$\alpha_v\beta_5$, VNR147 anti-α_v, anti-β_5, or anti-$\alpha_v\beta_3$ antibodies. Staining with rodhamine-phalloidin showed that exposure to uPA remarkably modified the F-actin distribution in at least 50–60% of the cell population with the appearance of peripheral, filamentous structures, often localized at one pole of the cell and possibly resembling lamellipodia-type structures (Fig. 3*A*). Unlike uPA, Vn treatment resulted in the formation of short, thin, and homogeneously distributed microspike-type structures in about half of the cell population. These effects are combined in cells treated with uPA and Vn, although it is difficult to ascertain the relative contribution of each agonist. The effects promoted by uPA are catalytic-independent, as its

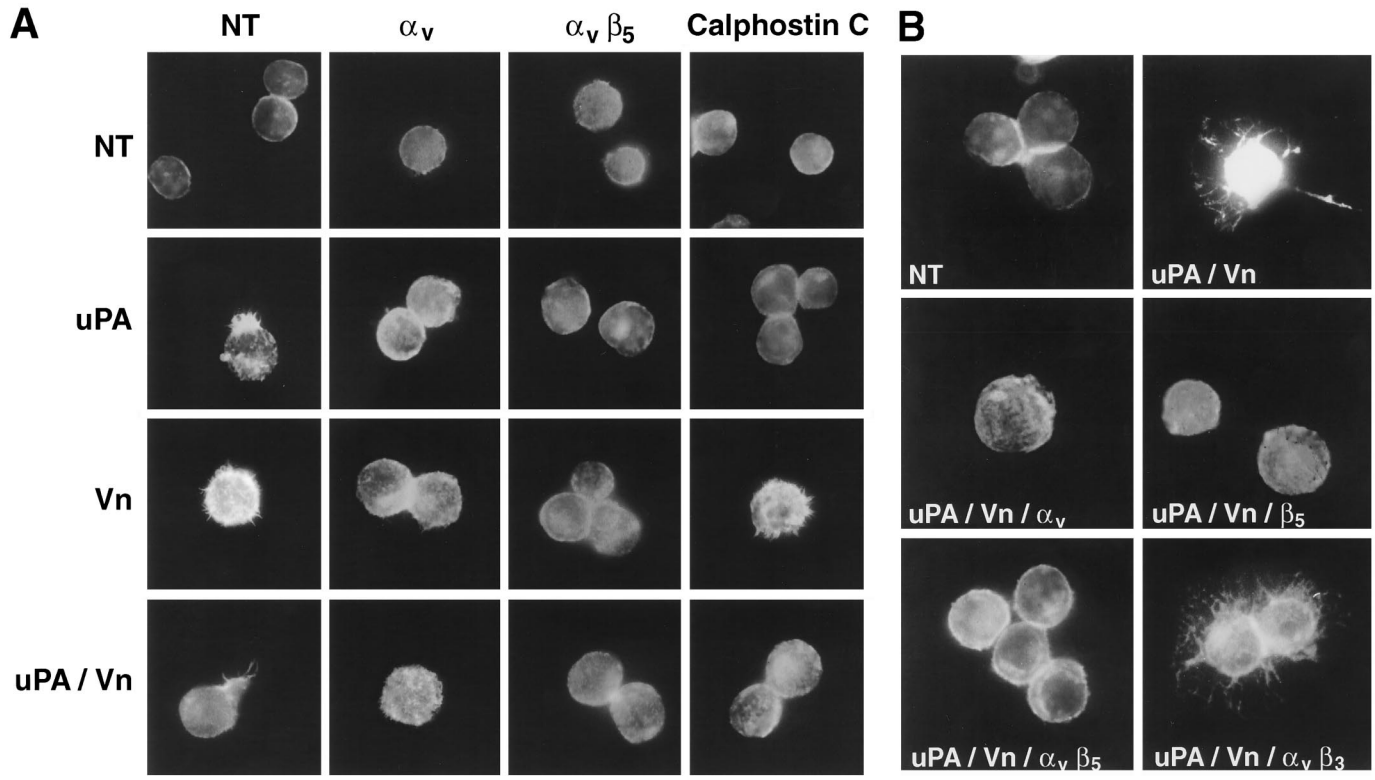

Fig. 3. Effects of uPA and Vn on HT1080 and MCF-7 cell cytoskeleton. HT1080 (*A*) and MCF-7 (*B*) were incubated either with diluents (*NT*) or with 10 nM recombinant uPA and/or 500 nM Vn. When specified, cells were either not treated (*NT*) or treated previously with anti-α_v, anti-β_5, anti-$\alpha_v\beta_5$, or anti-$\alpha_v\beta_3$ antibodies or with 200 nM calphostin C under serum-free conditions. Cells were subsequently stained with rhodamine-conjugated phalloidin. Representative cells for each condition are shown. ×1000.

ATF retains the same ability (not shown). As expected, preincubation of HT1080 cells with 399 anti-uPAR antibodies prevented most of the uPA-dependent cytoskeletal modifications, whereas it did not significantly affect Vn-induced filamentous structures (not shown). In keeping with the previous data, anti-$\alpha_v\beta_3$ mAb prevents neither the uPA nor the uPA/Vn-induced F-actin redistribution (not shown). In control samples, preincubation of unstimulated HT1080 cells with anti-$\alpha_v\beta_5$ or anti-α_v antibodies did not produce any effect on the cytoskeletal organization. On the contrary, preincubation with anti-$\alpha_v\beta_5$ and anti-α_v chain mAbs prevented the uPA-dependent effects, indicating that $\alpha_v\beta_5$ is required by uPAR signaling (Fig. 3*A*). Anti-$\alpha_v\beta_5$ and anti-α_v mAbs also inhibited the effects of Vn, indicating that despite the markedly different rearrangements observed, $\alpha_v\beta_5$ is a mediator of both Vn-dependent and uPA-dependent cell responses. The finding that $\alpha_v\beta_5$ supports uPAR signaling is confirmed by the results of parallel experiments carried out in MCF-7 cells (Fig. 3*B*). Exposure of MCF-7 cells to uPA, in the presence of Vn, produced filipodia and lamellipodia-type structures similar to those observed in HT1080 cells, which were prevented by the addition of anti-α_v anti-β_5 or anti-$\alpha_v\beta_5$ antibodies, whereas anti-$\alpha_v\beta_3$ mAb was ineffective.

Inhibition of uPA- and Vn-dependent Pathways. The qualitative differences between uPA- and Vn-dependent effects on the cellular arrangement of F-actin suggest that, although both are $\alpha_v\beta_5$-dependent, these pathways are somehow divergent. To gain insights in the functional interaction of uPAR and $\alpha_v\beta_5$, we investigated the possibility that uPAR signaling in HT1080 and MCF-7 cell lines is mediated by PKC, as in HEp3 and in U937 cell lines (10, 13). Therefore, urokinase-dependent cytoskeletal rearrangements were analyzed in cells previously treated with specific PKC inhibitors, such as calphostin C or bisindolylmaleimide (GF109203X). Although to a different extent, both inhibitors are effective at preventing uPA-induced rearrangements in both cell lines examined (Fig. 3*A* and Table 2). On the contrary, Vn-induced microspike-type structures were affected neither by calphostin C nor by bisindolylmaleimide treatments, suggesting that Vn acts in a PKC-independent manner. Similar data were obtained by analyzing cells pretreated with the protein synthesis inhibitor cycloheximide, which selectively prevented uPA-dependent and not Vn-dependent effects. This finding suggests that uPAR-dependent signaling requires a short-lived factor that is dispensable for the effects of Vn on cytoskeleton. Quantitative assessment of inhibition was attempted considering the number of cells with uPA-dependent filamentous structures, Vn-dependent microspikes, or uPA- and Vn-dependent combined rearrangements as 100%. The percentage of inhibition of each series of samples is reported as relative to each

Table 2 *Inhibition of uPA and/or Vn-induced cytoskeletal rearrangements in HT1080 and MCF-7 cell lines*

HT1080 and MCF-7 cells were harvested, acid-treated, and incubated with 200 nM calphostin C or 20 μM GF109203X (bisindolylmaleimide) or with 10 μg/ml cycloheximide and then stimulated with 10 nM recombinant uPA and/or 500 nM urea-denatured Vn. Cells were then fixed, stained with rhodamine-conjugated phalloidin, and analyzed by a fluorescence microscope. A total of 200 cells/sample was examined in each experiment, and the extent of their cytoskeletal rearrangements was estimated with respect to untreated cells. The number of cells exhibiting rearrangements induced by each effector was taken as 100%, and the extent of inhibition by calphostin C, GF109203X, or cycloheximide, is reported as relative to that.

Cells	Effector	Calphostin C[a]	GF109203X[a]	Cycloheximide[a]
HT1080	uPA	80 ± 9	ND	53 ± 11
HT1080	Vn	8 ± 6	ND	0
HT1080	uPA/Vn	69 ± 7	ND	52 ± 3
MCF-7	uPA	73 ± 1	54 ± 4	45 ± 11
MCF-7	Vn	1 ± 1	0	0
MCF-7	uPA/Vn	52 ± 10	54 ± 1	35.8 ± 17

[a] Numbers indicate the percentage of inhibition of agonist-induced cytoskeletal rearrangements. SDs are also indicated.

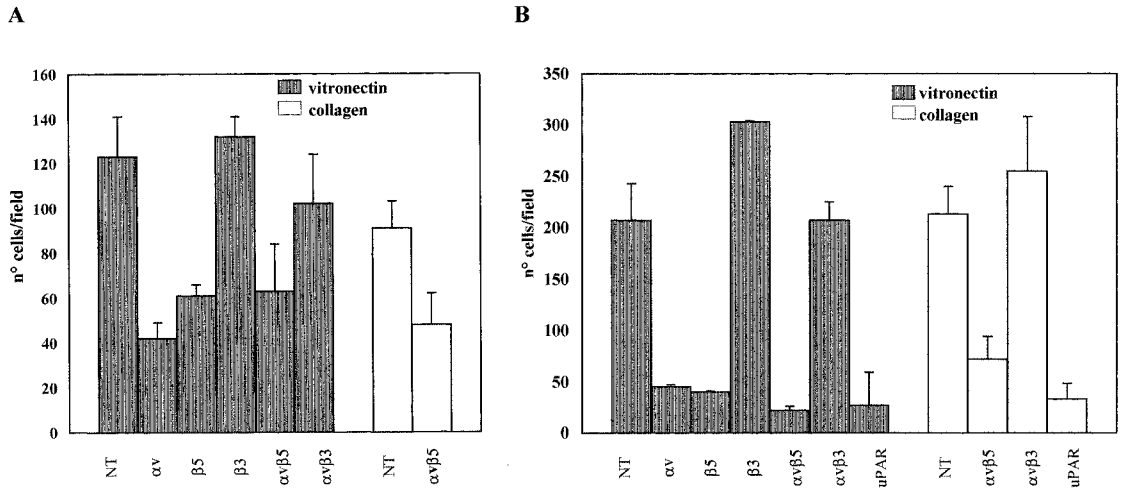

Fig. 4. Effect of anti-VnRs antibodies on the uPA-dependent cell migration. Cell migration assays were performed in Boyden chambers, using Vn- or collagen-coated filters and 10 nM recombinant uPA as a chemoattractant, under serum-free conditions. The average number of the cells migrated/field was subtracted of random cell migration (average number of cells migrated in the absence of chemoattractant). *Columns,* mean of three independent experiments; *bars,* SD. *A,* extent of uPA-dependent migration of HT1080 cells pretreated either with diluents (*NT*) or with anti-α_v, anti-β_5, anti-β_3, anti-$\alpha_v\beta_5$, or anti-$\alpha_v\beta_3$ antibodies. *B,* extent of uPA-dependent migration of MCF-7/uPAR4 cells pretreated with either diluents (*NT*) or with anti-α_v, anti-β_5, anti-β_3, anti-$\alpha_v\beta_5$, anti-$\alpha_v\beta_3$, or anti-uPAR 399 antibodies.

internal positive control (Table 2). Each experiment was repeated at least three times with the same results. In particular, the finding that PKC inhibitors and cycloheximide selectively block uPA-dependent and not Vn-dependent effects on cytoskeleton indicates the existence of two different activation modes of $\alpha_v\beta_5$.

Functional Cooperation between uPAR and $\alpha_v\beta_5$. Because uPAR-bearing cells are able to migrate toward a uPA gradient (27), we tested the possibility that blocking of the VnRs could prevent uPA-dependent cell motility. To this end, directional migration assays of HT1080 (Fig. 4*A*) and MCF-7/uPAR4 (Fig. 4*B*) cells were carried out in Boyden chambers. As expected, preincubation of MCF-7/uPAR4 cells with 399 anti-uPAR antibodies caused a dramatic inhibition of uPA-dependent cell migration. Interestingly, preincubation of HT1080 cells with anti-α_v, anti-β_5, or anti-$\alpha_v\beta_5$ antibodies caused a 66, 51, or 49% inhibition of the uPA-dependent cell migration onto Vn-coated filters, respectively. A 78, 81, or 89% inhibition of uPA-dependent migration of MCF-7/uPAR4 cells onto Vn was found following cell exposure to VNR147 anti-α_v, anti-β_5, or anti-$\alpha_v\beta_5$ antibodies, respectively. These results provide a functional support to the previously observed uPAR-$\alpha_v\beta_5$ physical association and indicate that this integrin is required for ligand-activated, uPAR-dependent cell migration. Similar results were obtained, in the absence of Vn, using collagen-coated filters. In this case, anti-$\alpha_v\beta_5$ mAb caused a 47 and 67% inhibition of HT1080 and MCF-7/uPAR4 cell migration, respectively. Similar results were obtained using ATF as a chemoattractant (not shown). In keeping with previous findings, anti-$\alpha_v\beta_3$ and anti-β_3 mAbs did not significantly modify uPA-dependent cell migration of both cell lines, again suggesting that $\alpha_v\beta_3$ is not involved in uPAR signaling.

DISCUSSION

This study sheds light on the physical and functional interaction between uPAR and VnRs. We determined the expression of uPAR, and VnRs $\alpha_v\beta_3$ and $\alpha_v\beta_5$ in 10 human breast carcinomas. First, we found that uPAR and $\alpha_v\beta_5$ can be copurified in human carcinomas. Then, we show that this association also occurs in HT1080 fibrosarcoma and MCF-7 breast carcinoma cell lines. The functional interaction between uPAR and $\alpha_v\beta_5$ VnR is shown by the inhibitory effect of anti-α_v, anti-β_5, and anti-$\alpha_v\beta_5$ antibodies exerted on uPAR-dependent effects such as cytoskeletal arrangement and cell migration. The data also suggest that the uPA-dependent physical association between uPAR and $\alpha_v\beta_5$ leads to the acquisition of new signaling properties by $\alpha_v\beta_5$.

Changes of integrin expression and subcellular distribution have been reported in mammary tumor cell lines and in tissue sections (44–47). These alterations have been shown to be associated with tissue disorganization, loss of polarity, increased tumor aggressiveness, and metastasis (45–47). Recently, Weaver *et al.* have reported that modification of cell surface β_1 and β_4 integrins influences mammary morphogenesis and regulates cell growth and signal transduction in malignant breast cells (48). These findings raise the hypothesis that integrins may play a crucial role in the expression of malignant phenotype in human epithelial breast cells. $\alpha_v\beta_3$ VnR is considered an endothelial cell marker with significant prognostic value in breast cancer (49), and it promotes cell migration on Vn independently of growth factors or uPA-uPAR expression (31). On the contrary, $\alpha_v\beta_5$ is expressed in a number of breast cancer cell lines (50) and requires exogenous activation of PKC to mediate cell spreading and migration (34). Our data show the occurrence of a physical association between uPAR and α_v chain of VnR in malignant epithelial cells of breast carcinomas expressing high levels of uPAR and $\alpha_v\beta_5$ but not $\alpha_v\beta_3$.

Association of uPAR with α_v, as well as with β_1 and β_3 integrin receptor subunits in HT1080 cells plated on ECM-coated surfaces, has been reported (25). Here, we report the physical and functional association of uPAR with $\alpha_v\beta_5$ in nonadherent cells, to avoid any interference by integrin activation. In addition, we selectively dissected the molecular and cellular effects following uPAR ligation with uPA. Although uPAR may be located in the close proximity of different integrins, the data presented here demonstrate that uPAR-dependent cell responses, such as cytoskeletal rearrangements and migration, are specifically mediated by $\alpha_v\beta_5$. The functional coupling between $\alpha_v\beta_5$ and ligand-activated uPAR is instrumental to gain insights into the role of integrins in uPAR-dependent signaling. Unlike $\alpha_v\beta_3$, $\alpha_v\beta_5$ is a Vn-dependent integrin receptor that can direct cell migration and stimulate a redistribution of talin, vinculin, and α_v-actinin only in the presence of PKC activators (31). In a variety of cases, it has been described the ability of uPAR to activate PKC and DAG formation (10, 11). Our data show that the ligation of $\alpha_v\beta_5$ with

Vn leads to the formation of microspike-type structures that do not require PKC activation. However, the finding that PKC is required for uPAR-dependent, $\alpha_v\beta_5$-mediated effects, raises a question about the mechanism of PKC activation. One possibility is that uPAR triggers PKC activation by influencing the membrane level of diacylglycerol, via a presently unknown mechanism (11). Alternatively, uPAR ligation with uPA may lead to PKC activation through the recruitment of a specific partner in a signaling complex. The occurrence of a functional coupling between uPAR and $\alpha_v\beta_5$ is in agreement with previous findings showing the ability of uPA-uPAR complexes to inhibit the adhesive function of β_1 integrin (16). Here, we show that anti-$\alpha_v\beta_5$ antibodies inhibit cell migration on Vn and collagen-coated filters, suggesting that uPAR signaling is not based on the normal adhesive function of $\alpha_v\beta_5$. On the other hand, uPA does not simply activate $\alpha_v\beta_5$ in a Vn-like manner but triggers unique effects that are PKC-dependent. These data definitely highlight an alternative activation mode of $\alpha_v\beta_5$, triggered by uPAR.

The molecular mechanisms underlying the signaling ability of uPAR are intriguing, as this receptor lacks a transmembrane domain, suggesting the occurrence of a specific "adaptor." In this paper, we provide such evidence: uPA modulates cytoskeleton and cell migration via $\alpha_v\beta_5$, although additional mediators, associated to this signaling complex may be required to fully support these effects. We could hypothesize that uPAR and integrin-containing signaling units recruit other components, such as caveolin, Src kinases, and PKC, thereby triggering a different pathway than integrins stimulated by their cognate ligand. On the other hand, it has been reported that a functional unit of uPAR, integrin, and caveolin regulates integrin function (14). Further work is required to address these issues.

The results presented here extend beyond the uPAR field, as they have important implications for the spatial and temporal regulation of integrin function by other receptor pathways that may be important in adhesion and migration. In this paper, we show the existence of two alternative activation modes of $\alpha_v\beta_5$, either by Vn or by activated uPAR. It is tempting to speculate that the first mode is more appropriate for stable adhesion to the ECM and the latter for a reversible activation required during cell locomotion. The latter possibility is in agreement with previous findings showing a reversible association of uPAR with CR3 during granulocyte locomotion (51). Another example of "lateral" activation of integrins by proteases is provided by the activation of $\alpha_v\beta_3$ by the PEX domain of the matrix metalloprotease 2 on the surface of angiogenic blood vessels (52). Cell migration implies the dynamic formation of adhesive contacts at the leading edge of the cell and disruption at the cell rear: in particular, at the cell front, the formation of cytoskeletal and catalytic signaling protein complexes promotes PKC activation (53). It is possible that during directional migration of HT1080 and MCF-7 cells, uPAR cycles between the cell front and rear, locally regulating adhesion through the local activation/disruption of its interaction with $\alpha_v\beta_5$. The $\alpha_v\beta_5$-uPAR interaction may be activated under both physiological and pathological conditions, due to an altered cell migration: we and others have previously shown a coordinate overexpression of uPA and uPAR on the cell surface of human breast carcinoma cells (35, 54, 55). Other authors claim that epithelial tumor cells bear uPARs and bind uPA produced by fibroblast-like stromal cells in a paracrine manner (2). Overexpression of α_v, in human mammary carcinomas has been reported (23). The finding that uPAR and $\alpha_v\beta_5$ physically associate in breast carcinomas raises the possibility that they may functionally cooperate *in vivo*, thereby favoring the metastatic process. Our results show that epithelial tumor cells of breast carcinomas express considerable amount of $\alpha_v\beta_5$ whereas $\alpha_v\beta_3$ is expressed exclusively by endothelial cells. Blocking uPAR is a major goal of antimetastatic therapy (56, 57). Molecular antagonists include peptides, antibodies, and antisense, as well as the naturally occurring phosphorylated uPA and the relative phosphorylation-like variants, which bind to uPAR but fail to activate receptor signaling (56, 58). In the emerging picture, the tumor cell metastasis can be regulated by a functional cooperation between uPA-uPAR complexes and VnR type $\alpha_v\beta_5$. A detailed molecular analysis of the uPAR and $\alpha_v\beta_5$ domains involved in the interaction may lead to the development of novel drugs, blocking this association and thereby inhibiting tumor invasion.

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REFERENCES

1. Woodhouse, E. C., Chuaqui, R. F., and Liotta, L. A. General mechanism of metastasis. Cancer (Phila.), *80:* 1529–1537, 1997.
2. Dano, K., Behrendt, N., Brunner, N., Ellis, V., Ploug, M., and Pyke, C. The urokinase receptor. Protein structure and role in plasminogen activation and cancer invasion. Fibrinolysis, *8:* 189–203, 1994.
3. Bugge, T. H., Kombrinck, K. W., Xiao, Q., Holmback, K., Daugherty, C. C., Witte, D. P., and Degen, J. L. Growth and dissemination of Lewis Lung carcinoma in plasminogen deficient mice. Blood, *90:* 4522–4531, 1997.
4. Shapiro, R. L., Duquette, J. G., Roses, D. F., Nunes, I., Harris, M. N., Kamino, H., Wilson, E. L., and Rifkin, D. B. Induction of primary cutaneous melanocytic neoplasms in urokinase-type plasminogen activator (uPA)-deficient and wild-type mice: cellular blue nevi invade but not progress to malignant melanoma in uPA-deficient animals. Cancer Res., *56:* 3597–3604, 1996.
5. Kirchheimer, J. C., Woita, J., Christ, J., and Binder, R. R. Functional inhibition of endogenously produced urokinase decreases cell proliferation in a human melanoma cell line. Proc. Natl. Acad. Sci. USA, *86:* 5424–5428, 1989.
6. Gyetko, M. R., Todd, R. F., III, Wilkinson, C. C., and Sitrin, R. G. The urokinase receptor is required for human monocyte chemotaxis *in vitro*. J. Clin. Invest., *93:* 1380–1387, 1994.
7. Waltz, D. A., Sailor, L. Z., and Chapman, H. A. Cytokines induce urokinase-dependent adhesion of human myeloid cells: a regulatory role for plasminogen activator inhibitors. J. Clin. Invest., *91:* 1541–1552, 1993.
8. Stoppelli, M. P., Corti, A., Soffientini, A., Cassani, G., Blasi, F., and Assoian, R. K. Differentiation-enhanced binding of the amino-terminal fragment of human urokinase plasminogen activator to a specific receptor on U937 monocytes. Proc. Natl. Acad. Sci. USA, *832:* 4939–4943, 1985.
9. Wei, Y., Waltz, D. A., Rao, N., Drummend, R. J., Rosenberg, S., and Chapman, H. A. Identification of the urokinase receptor as an adhesion receptor for vitronectin. J. Biol. Chem., *269:* 32380–32388, 1994.
10. Busso, N., Masur, S. K., Lazega, D., Waxman, S., and Ossowski, L. Induction of cell migration by pro-urokinase binding to its receptor: possible mechanism for signal transduction in human epithelial cells. J. Cell. Biol., *126:* 259–270, 1994.
11. Fibbi, G., Caldini, R., Chevanne, M., Pucci, M., Schiavone, N., Morbidelli, L., Parenti, A., Granger, H. J., Del Rosso, M., Ziche, M. Urokinase-dependent angiogenesis *in vitro* and diacylglycerol production are blocked by antisense oligonucleotides against the urokinase receptor. Lab. Invest., *78:* 1109–1119, 1998.
12. Resnati, M., Guttinger, M., Valcamonica, S., Sidenius, N., Blasi, F., and Fazioli, F. Proteolytic cleavage of the urokinase receptor substitutes for the agonist-induced chemotactic effect. EMBO J., *15:* 1572–1582, 1996.
13. Chiaradonna, F., Fontana, L., Iavarone, C., Carriero, M. V., Sholz, G., Barone, M. V., and Stoppelli, M. P. Urokinase receptor-dependent and independent p56/59^{hck} activation state is a molecular switch between myelomonocytic cell motility and adherence. EMBO J., *18:* 3013–3023, 1999.
14. Stahl, H., and Mueller, B. A. The urokinase-type plasminogen activator receptor, a GPI-linked protein, is localized in caveolae. J. Cell Biol., *129:* 335–344, 1995.
15. Bohuslav, J., Horejsi, V., Hansmann, C., Stockl, J., Weidle, U. H., Majdic, O., Bartke, I., Knapp, W., and Stockinger, H. Urokinase plasminogen activator receptor, β_2-integrins, and Src kinases within a single receptor complex of human monocytes. J. Exp. Med., *181:* 1381–1390, 1995.
16. Wei, Y., Lukashev, M., Simon, D. I., Bodary, S. C., Rosenberg, S., Doyle. M. V., and Chapman, H. A. Regulation of integrin function by the urokinase receptor. Science (Washington DC), *273:* 1551–1555, 1996.
17. Myohanen, H. T., Stephens, R. W., Hedman, K., Tapiovaara, H., Ronne, E., Hoyer-Hansen, G., Dano, K., and Vaheri, A. Distribution and lateral mobility of the urokinase-receptor complex at the cell surface. J. Histochem. Cytochem., *41:* 1291–1301, 1993.
18. Franco, P., laccarino, C., Chiaradonna, F., Brandazza, A., Iavarone, C., Mastronicola, M. R., Nolli, M. L., and Stoppelli, M. P. Phosphorylation of human pro-urokinase on Ser$^{138/303}$ impairs its receptor-dependent ability to promote myelomonocytic adherence and motility. J. Cell Biol., *137:* 779–791, 1997.
19. Hynes, R. O. Integrins: versatility, modulation, and signaling in cell adhesion. Cell, *69:* 11–25, 1992.
20. Clark, E. A., and Brugge, J. S. Integrins and signal transduction pathways: the road taken. Science (Washington DC), *268:* 233–239, 1995.

21. Schwartz, M. A., Schaller, M. D., Ginsberg, M. H. Integrins: emerging paradigms of signal transduction. Annu. Rev. Cell Dev. Biol., *11:* 549–599, 1995.
22. Schwartz, M. A. Integrins, oncogenes and anchorage-independence. J. Cell Biol., *139:* 575–578, 1997.
23. Pignatelli, M., Cardillo, M. R., Hanby, A., and Stamp, G. W. H. Integrins and their accessory adhesion molecules in mammary carcinomas: loss of polarization in poorly differentiated tumors. Hum. Pathol., *23:* 1159–1166, 1992.
24. Tang, H., Kerins, D. M., Hao, Q., Inagami, T., and Vaughan, D. E. The urokinase-type plasminogen activator receptor mediates tyrosine phosphorylation of focal adhesion proteins and activation of mitogen-activated protein kinase in cultured endothelial cells. J. Biol. Chem., *273:* 18268–18272, 1998.
25. Xue, W., Mizukami, I., Todd, R. F., III, and Petty, H. R. Urokinase-type plasminogen activator receptors associate with β_1 and β_3 integrins of fibrosarcoma cells: dependence on extracellular matrix components. Cancer Res., *57:* 1682–1689, 1997.
26. Chapman, H. A. Plasminogen activators, integrins, and the coordinated regulation of cell adhesion and migration. Curr. Opin. Cell Biol., *9:* 714–724, 1997.
27. Blasi, F. uPA, uPAR, PAI-1: key intersection of proteolytic, adhesive and chemotactic highways? Trends Immunol. Today, *18:* 415–417, 1997.
28. Deng, G., Curriden, S. A., Wang, S., Rosenberg, S., and Loskutoff, D. J. Is plasminogen activator inhibitor-1 the molecular switch that governs urokinase receptor-mediated cell adhesion and release? J. Cell Biol., *134*:1563–1571, 1996.
29. Steingrimur, S., and Lawrence, D. A. The serpin PAI-1 inhibits cell migration by blocking integrin $\alpha_v\beta_3$ binding to vitronectin. Nature (Lond.), *383:* 441–443, 1996.
30. Reinartz, J., Schafer, B., Batrla, R., Klein, C. E., and Kramer, M. D. Plasmin abrogates $\alpha_v\beta_5$-mediated adhesion of a human keratynocyte cell line (HaCaT) to vitronectin. Exp. Cell Res., *220:* 274–282, 1995.
31. Yebra, M., Parry, G. C. N., Strombland, S., Mackman, N., Rosenberg, S., Mueller, B. M., and Cheresh, D. A. Requirement of receptor-bound urokinase-type plasminogen for integrin $\alpha_v\beta_5$-directed cell migration. J. Biol. Chem., *271:* 29393–29399, 1996.
32. Smith, J. W., and Cheresh, D. A. Integrin ($\alpha_v\beta_3$)-ligand interaction. J. Biol. Chem., *265:* 2168–2172, 1990.
33. Friedlander, M., Brooks, P. C., Shaffer, R. W., Kincaid, C. M., Varner, J. A., and Cheresh, D. A. Definition of two angiogenic pathways by distinct α_v integrins. Science (Washington DC), *270:* 1500–1502, 1995.
34. Lewis, J. M., Cheresh, D. A., and Schwartz, M. A. Protein kinase C regulates $\alpha_v\beta_5$-dependent cytoskeletal associations and focal adhesion kinase phosphorylation. J. Cell Biol., *134:* 1323–1332, 1996.
35. Carriero, M. V., Franco, P., Del Vecchio, S., Massa, O., Botti, G., D'Aiuto, G., Stoppelli, M. P., and Salvatore, M. Tissue distribution of soluble and receptor-bound urokinase in human breast cancer using a panel of monoclonal antibodies. Cancer Res., *54:* 5445–5454, 1994.
36. Carriero, M. V., Del Vecchio, S., Franco, P., Potena, M. I., Chiaradonna, F., Botti, G., Stoppelli, M. P., and Salvatore, M. Vitronectin binding to urokinase receptor in breast carcinoma. Clinical Cancer Res., *3:* 1299–1308, 1997.
37. Ronne, E. Behrendt, N., Ellis, V., Ploug, M., Dano, K., and Hoyer Hansen, G. Cell-induced potentiation of the plasminogen activation system is abolished by a monoclonal antibody that recognizes the NH_2-terminal domain of the urokinase receptor. FEBS Lett., *288:* 233–236, 1991.
38. Wayner, E. A., Orlando, R. A., and Cheresh, D. A. Integrins $\alpha_v\beta_3$ and $\alpha_v\beta_5$ contribute to cell attachment to vitronectin but differentially distribute on the cell surface. J. Cell Biol., *113:* 919–929, 1991.
39. Freed, E., Gailit, J., Van Der Geer, P., Ruoslahti, E., and Hunter, T. A novel integrin β subunit is associated with the vitronectin receptor α subunit (αv) in a human osteosarcoma cell line and is a substrate for protein kinase C. EMBO J., *8:* 2955–2965, 1989.
40. Chuntharapai, A., Bodary, S., Horton, M., and Kim, K. J. Blocking monoclonal antibodies to $\alpha_v\beta_3$ integrin: a unique epitope of $\alpha_v\beta_3$ integrin is present on human osteoclasts. Exp. Cell Res., *205:* 345–352, 1993.
41. Chen, Y. P., Djaffar, I., Pidard, D., Steiner, B., Cieutat, A. M., Caen, J. P., and Rosa, J. P. Ser^{752} –pro mutation in the cytoplasmic domain of integrin β_3 subunit and defective activation of platelet integrin $\alpha_{IIb}\beta_3$ (glycoprotein IIb-IIIa) in a variant of Glanzmann thrombasthenia. Proc. Natl. Acad. Sci. USA, *89:* 10169–10173, 1992.
42. Roldan, A., Cubellis, M. V., Masucci, M. T., Beherendt, N., Lund, L. R., Dano, K., Appella, E., and Blasi, F. Cloning and expression of the receptor for human urokinase plasminogen activator, a central molecule in cell surface, plasmin dependent proteolysis. EMBO J., *9:* 467–474, 1990.
43. Stoppelli, M. P., Tacchetti, C., Cubellis, M. V., Corti, A., Hearing, V. J., Cassani, G., Appella, E., and Blasi, F. Autocrine saturation of pro-urokinase receptors on human A431 cells. Cell, *45:* 675–684, 1986.
44. Zutter, M. M., Krigman, H. R., and Santoro, S. A. Altered integrin expression in adenocarcinoma of the breast. Analysis by in situ hybridization. Am. J. Pathol., *142:* 1439–1448, 1993.
45. Berdichevsky, F., Wetzels, R., Shearer, M., Maringnone, S., Ramaekers, F. C. S., and Taylor-Papdimitriou, J. Integrin expression in relation to cell-phenotype and malignant change in the human breast. Mol. Cell Diff., *2:* 255–274, 1994.
46. Zutter, M. M., Santoro, S. A., Staatz, W. D., and Tsung, Y. L. Re-expression of the $\alpha_2\beta_1$ integrin abrogates the malignant phenotype of breast carcinoma cells. Proc. Natl. Acad. Sci. USA, *92:* 7411–7415, 1995.
47. Tagliabue, E., Ghirelli, C., Squicciarini, P., Colnaghi, M. I., Menard, S. Prognostic value of $\alpha_6\beta_4$ integrin expression in breast carcinomas is affected by laminin production from tumor cells. Clin. Cancer Res., *4:* 407–410, 1998.
48. Weaver, V. M., Petersen, O. W., Wang, F., Larabell, C. A., Briand, P., Damasky, C., and Bissell, M. J. Reversion of the malignant phenotype of human breast cells in three-dimensional culture and in vivo by integrin blocking antibodies. J. Cell Biol., *137:* 231–245, 1997.
49. Gasparini, G., Brooks, P. C., Biganzoli, E., Vermeulen, P. B., Bonoldi, E., Dirix, L. Y., Ranieri, G., Miceli, R., Cheresh, D. A. Vascular integrin $\alpha_v\beta_3$: a new prognostic indicator in breast cancer. Clin. Cancer Res., *4:* 2625–2634, 1998.
50. Gui, G. P. H., Puddefoot, J. R., Vinson, G. P., Wells, C. A., and Carpenter, R. *In vitro* regulation of human breast cancer cell adhesion and invasion via integrin receptors to the extracellular matrix. Br. J. Surg., *82:* 1192–1196, 1995.
51. Kindzelskii, A. L., Laska, Z. O., Todd, R. F., III, and Petty, H. R. Urokinase-type plasminogen activator reversibly dissociates from complement receptor type 3 (aMb2. CD11b/CD18) during neutrophil polarization. J. Immunol., *157:* 297–309, 1996.
52. Brooks, P. C., Silletti, S., Von Schalscha, T. L., Friedlander, M., and Cheresh, D. A. Disruption of angiogenesis by PEX, a non catalytic metalloproteinase fragment with integrin binding activity. Cell, *92:* 391–400, 1998.
53. Huttenlocher, A., Sandborg, R. R., and Horwitz, A. F. Adhesion in cell migration. Curr. Opin. Cell Biol., *7:* 697–706, 1995.
54. Del Vecchio, S., Stoppelli, M. P., Carriero, M. V., Fonti, R., Massa, O., Li, P. Y., Botti, G., Cerra, M., D'Aiuto, G., Esposito, G., and Salvatore, M. Human urokinase receptor concentration in malignant and benign breast tumors by in vitro quantitative autoradiography: comparison with urokinase levels. Cancer Res., *53:* 3198–3206, 1993.
55. Andreasen, P. A., Kjoller, L., Christensen, L., and Duffy, M. J. The urokinase-type plasminogen activator system in cancer metastasis: a review. Int. J. Cancer, *72:* 1–22, 1997.
56. Fazioli, F., and Blasi, F. Urokinase-type plasminogen activator and its receptor: new targets for anti-metastatic therapy? Trends Pharmacol. Sci., *15:* 25–29, 1994.
57. Min, H. Y., Doyle, L. V., Vitt., C. R., Zandonella, C. L., Stratton-Thomas, J. R., Shuman, M. A., and Rosenberg, S. Urokinase receptor antagonists inhibit angiogenesis and primary tumor growth in syngenic mice. Cancer Res., *56:* 2428–2434, 1996.
58. Franco, P., Massa, O., Garcia-Rocha, M., Chiaradonna, F., Iaccarino, C., Correas, I., Mendez, E., Avila, J., Blasi. F., and Stoppelli, M. P. Protein kinase C-dependent *in vivo* phosphorylation of prourokinase leads to the formation of a receptor competitive antagonist. J. Biol. Chem., *273:* 27734–27740, 1998.