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Technical Advance

Epitope-Mapped Monoclonal Antibodies as Tools for Functional and Morphological Analyses of the Human Urokinase Receptor in Tumor Tissue

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uPAR (CD87), the receptor for the urokinase-type plasminogen activator (uPA) facilitates tumor cell invasion and metastasis by focusing uPA proteolytic activity to the cell surface. As uPAR exists in various molecular forms, it is desirable to use well defined antibodies for analyses of uPAR antigen expression in human malignant tumors by immunological methods. Therefore, twelve monoclonal antibodies (MAbs) directed against uPAR were generated by using nonglycosylated, recombinant human uPAR (spanning amino acids 1 to 284), expressed in* Escherichia coli*, as the immunogen. The reaction pattern of these MAbs with the immunogen and a series of carboxyl-terminally truncated versions of uPAR demonstrated that at least six different epitopes of uPAR are recognized. All MAbs reacted under reducing conditions in immunoblot analyses with* E. coli*-expressed uPAR and also with highly glycosylated, functionally intact, recombinant human uPAR expressed in Chinese hamster ovary (CHO) cells. Seven of the MAbs recognized CHO uPAR under nonreducing conditions as well. By flow cytofluorometric analyses, three of these MAbs were shown to bind to native human uPAR present on the cell surface of monocytoid U937 cells with MAb IIIF10 being the best. Saturation of uPAR with uPA on U937 cells completely blocked interaction of MAb IIIF10 with uPAR (mapped epitope, amino acids 52 to 60 of domain I of uPAR). In turn, preincubation of U937 cells with MAb IIIF10 efficiently reduced binding of uPA to uPAR, indicating that the epitope detected by MAb IIIF10 is located within or closely to the uPA-binding site of uPAR, and thus, this site may be a target to influence uPA/uPAR-mediated proteolysis in tumors. Binding of MAbs IID7 or IIIB11 (mapped epitope, amino acids 125 to 132 of domain II of uPAR) to uPAR is not affected when uPAR is occupied by uPA. As these MAbs reacted strongly with cellular uPAR antigen in formalin-fixed paraffin-embedded tumor sections, the domain-II-specific antibodies IID7 and IIIB11 may be useful for immunohistochemical studies of uPAR expression in tissue remodeling processes in tumor invasion. In conclusion, we have devised well defined and epitope-mapped MAbs to uPAR that are highly specific tools for detection and targeting of uPAR in tumor tissue. (Am J Pathol 1997, 150:1231–1244)

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Tumor cell invasion is the active translocation of neoplastic cells across host cellular and extracellular matrix barriers.[1] Mechanisms of invasion used by tumor cells are not unique to tumor cells; they may equal those used by nonmalignant cells to invade neighboring tissue under normal physiological situations, eg, in embryogenesis and trophoblast invasion during pregnancy, wound healing, angiogenesis, and tissue remodeling.[2] Tumor cell invasion, however, in contrast to physiological invasion is considered to be an uncontrolled, deregulated event concurrent with the expression of the malignant phenotype.

While traversing tissue boundaries, various proteins on the surface of tumor cells mediate attachment to and degradation of the basement membrane and components of the extracellular matrix (tumor stroma). The penetrating tumor cell focuses proteolytic activity to its cell surface through receptors for the serine proteases plasmin and uPA, the urokinase-type activator of plasminogen. The receptor for uPA, uPAR (CD87), is a highly glycosylated 45- to 60-kd protein that is attached to the cell membrane *via* a glycosylphosphatidylinositol anchor.[3] It consists of three homologous domains, of which the amino-terminal domain (domain I) represents the uPA-binding domain. uPAR plays a critical role in cancer invasion and metastasis due to its ability to bind uPA secreted by tumor cells or surrounding stroma cells, thus facilitating extracellular matrix degradation, cell proliferation, invasion, and metastasis.[4,5] In fact, overexpression of uPAR in tumor cells results in increased tumor invasion and growth *in vitro* and *in vivo*.[6] Internalization of uPAR by the cell is achieved by formation of a ternary complex after binding of uPA and the inhibitor plasminogen activator inhibitor type-1 (PAI-1) to uPAR. Upon internalization the uPA/PAI-1 complex is degraded and at least part of uPAR reshuttled back to the cell surface. Internalization and recycling of uPAR may restore focalized surface-associated proteolytic activity at the invasive front.[7] Thus it appears that both the protease uPA and its inhibitor PAI-1 have to be present in the tumor to achieve effective uPAR-mediated limited proteolysis required for tumor cell invasion.[8]

Clinical studies have shown that in various solid malignant cancers uPA, PAI-1, and/or uPAR may serve as valuable, statistically independent prognostic factors to predict disease recurrence and survival probability (reviewed in Ref. 2). Significant evidence has accumulated that the increase of proteolytic factors in tumors is correlated with an increase in tumor aggressiveness. Intervention directed against proteolytic action in tumors by targeting the uPAR on tumor cells has been proposed.[1] Targeting the uPAR by uPA analogues or antibodies to uPAR may significantly reduce tumor cell proliferation and migration and may interfere with uPA-mediated tumor cell invasion and metastasis (reviewed in Refs. 9 and 10). In view of this, we have produced new types of epitope-mapped monoclonal antibodies (MAbs) to uPAR as highly specific tools for the analysis of the role of uPAR in malignant tumors.

Materials and Methods

Recombinant Human uPAR Variants from Escherichia coli and Chinese Hamster Ovary (CHO) Cells

Expression and synthesis of uPAR variants in *E. coli* and CHO cells has been described previously.[11,12] Briefly, recombinant uPAR from *E. coli*, containing an amino-terminal histidine tag insertion, was purified by nickel chelate affinity chromatography, whereas soluble uPAR, produced by CHO cells (CHO-uPAR$_{1-277}$, corresponding to amino acids (aa) 1 to 277 of human uPAR), was isolated by ligand (uPA) affinity chromatography. The *E. coli* uPAR variants cover aa 1 to 284 (Ec-uPAR$_{1-284}$), 1 to 196 (Ec-uPAR$_{1-196}$), 1 to 178 (Ec-uPAR$_{1-178}$), 1 to 154 (Ec-uPAR$_{1-154}$), 1 to 130 (Ec-uPAR$_{1-130}$), or 1 to 94 (Ec-uPAR$_{1-94}$) of human uPAR.

Monoclonal Antibodies

Female Balb/c mice were immunized by four 50-μg intraperitoneal injections of Ec-uPAR$_{1-284}$ that was dialyzed against phosphate-buffered saline (PBS)[13] before injection. Hybridomas were produced according to established methods.[14] Spleen cells were fused with mouse myeloma cells P3x63Ag8653.1 by use of polyethylene glycol (Boehringer Mannheim, Mannheim, Germany). Hybridoma supernatants were screened by an enzyme-linked immunosorbent assay (ELISA) using microtiter plates coated with Ec-uPAR$_{1-284}$. Positive hybridomas were cloned twice. Hybridomas were expanded and the MAbs purified from the culture supernatants by protein G chromatography (Pharmacia, Freiburg, Germany). All established MAbs are of IgG1κ isotype.

Dot Blot Analyses

Reduced *E. coli*-derived uPAR variants, CHO-uPAR$_{1-277}$ (reduced and nonreduced), and bovine serum albumin, respectively, were spotted onto a

nylon membrane (PALL, Dreieich, Germany) and residual protein binding sites blocked with 5% milk powder in PBS. The strips were then probed with the MAbs (2 μg/ml PBS). The detection system consisting of peroxidase-conjugated rabbit anti-mouse IgG (Dianova) as the secondary antibody and 3,3′,5,5′-tetramethylbenzidine (Kirkegaard & Perry Laboratories, Gaithersburg, MD) as the developer was used as recommended by the suppliers.

Western Blots

Proteins were separated by SDS-PAGE on 12% gels under reducing conditions and transferred to a nylon membrane (PALL) by semi-dry blotting. For detection of the peroxidase-coupled antibody either tetramethylbenzidine or a chemoluminescence kit (Amersham, Braunschweig, Germany) was used. Treatment of CHO-$uPAR_{1-277}$ with chymotrypsin (37°C, 8 ng of chymotrypsin per 10 μg of CHO-$uPAR_{1-277}$, pH 8.0) was performed for 1 hour. For the preparation of total protein extracts, approximately 10^6 cells of the Raji lymphoblastoid B-cell line or the promyeloid cell line U937 were washed once with PBS and then incubated for 10 minutes at 96°C in 50 μl of SDS-PAGE sample buffer (reducing conditions).

Peptide Synthesis

Linear peptides were synthesized on a 2-chlorotrityl resin[15] using a multiple peptide synthesizer, model Syro II (MultiSynTech). Applying an orthogonal Fmoc-strategy,[16,17] the amino acid side chains were protected with trityl (Asn, Cys, Gln, and His), *tert*-butyloxycarbonyl (Lys and Trp), *tert*-butyl (Asp, Glu, Ser, Thr, and Tyr), or 2,2,4,6,7-pentamethyldihydro-benzofuran-5-sulfonyl (Arg) groups. Coupling was performed at room temperature in dimethylformamide using a threefold excess of *O*-(1*H*-benzotriazol-1-yl)-*N,N,N′,N′*-tetra-methyluroniumtetra-fluoroborate/1-hydroxy-benzotriazol/Fmoc amino acid with 2.5 equivalents of *N*-ethyl-diisopropylamine in *N*-methyl-pyrrolidone. The Fmoc group was removed by sequential treatment of the resins with an excess of 40 and 20% piperidine in dimethylformamide, respectively. Cleavage of the peptides and removal of the side chain protecting groups were performed simultaneously by treatment with 82.5% trifluoroacetic acid/5% phenol/2.5% ethandithiol/5% thioanisol/5% H_2O (0°C for 2 hours). The crude peptides were precipitated in diethylether at −30°C, dissolved in methanol, precipitated as before, redissolved in *tert*-butanol and lyophilized. Tryptophan-containing peptides were additionally treated with 5% acetic acid for 2 hours before lyophilization.[18]

Enzyme-Linked Immunosorbent Assay

Synthetic uPAR-derived peptides, dissolved in 50 mmol/L HCl (1 mg/ml), were diluted 50-fold in coating buffer (100 mmol/L Na_2CO_3, pH 9.5), applied in 100-μl aliquots to wells of a polystyrene microtiter plate (MaxiSorb, Nunc, Wiesbaden, Germany) and incubated overnight at 4°C. After three washes with Tris-buffered saline (TBS)[10] supplemented with 0.05% Tween 20 (TBS-T), residual protein-binding sites were saturated by incubating each well with 200 μl of TBS containing 2% bovine serum albumin (BSA) (2 hours at room temperature). The blocking solution was aspirated, the wells were washed once with TBS-T, and then 100 μl of the MAb sample (2 μg/ml TBS-T plus 1% BSA) was placed into each well (overnight at 4°C). After three washes with TBS-T, the plates were incubated (1 hour at room temperature) with biotinylated goat anti-mouse IgG (B7264, Sigma, Deisenhofen, Germany; 1:5000 in TBS-T/1% BSA; 100 μl/well). After another wash with TBS-T, 100 μl of avidin-peroxidase (Sigma 7282; 1:4000 in TBS-T/0.5 mol/L NaCl) were added (1 hour at room temperature). After still another wash, binding of avidin-peroxidase was detected at room temperature by using 100 μl of a solution containing *o*-phenylene-diamine (1 mg/ml) and 0.003% H_2O_2 in 0.1 mol/L KH_2PO_4, pH 6.0, as the substrate for peroxidase. The reaction was stopped after 6 to 10 minutes by the addition of 100 μl of 1 mol/L H_2SO_4. The absorbance of the brownish color was measured at 492 nm on a multichannel Titertek microtiter plate reader. Recombinant *E. coli*- or CHO-cell-derived uPAR variants served as positive controls.

Flow Cytofluorometry

U937 cells, stimulated for 48 hours with phorbol-12-myristate-13-acetate (PMA; 1 mmol/L), were used to investigate binding of MAbs to cell-associated uPAR. Approximately 2.5×10^5 cells were washed several times with PBS supplemented with 0.1% BSA and then subjected to acidic buffer (50 mmol/L glycine/HCl, pH 3.0, for 1 minute at 22°C) to remove receptor-bound endogenous uPA, followed by a neutralization step with 0.5 mol/L HEPES/NaOH, pH 7.5.[12,19] The still viable U937 cells were subsequently incubated with increasing amounts of MAbs (0 to 8 μg) for 30 minutes (250 μl total volume) and washed again with PBS/0.1% BSA. uPAR-bound MAbs were reacted with 2 μg of fluorescein isothio-

cyanate (FITC)-labeled anti-mouse IgG and the cell-associated fluorescence determined. To test whether binding of MAbs to uPAR is affected by cell-bound uPA, 250,000 U937 cells per 250 μl were incubated for 30 minutes with 2 μg of pro-uPA before the addition of the MAbs. To analyze the capacity of MAbs to interfere with uPA/uPAR interaction, U937 cells were incubated for 30 minutes with 8 μg of MAb directed to uPAR before addition of FITC-labeled pro-uPA (16 ng).

Preparation of U937 Cells for Immunocytochemistry

Approximately 10×10^6 U937 cells, either resting or stimulated with PMA (1 mmol/L for 48 hours), were incubated with and without FITC-labeled pro-uPA (100 μg/10 ml for 30 minutes at room temperature). After several washes with PBS, the cell pellets were incubated in 300 μl of recalcified, initially citrated human plasma at 37°C until clot formation was performed. The cell-fibrin clots were fixed overnight in phosphate-buffered 4% formaldehyde and subsequently embedded in paraffin.

Immunocytochemistry and Immunohistochemistry

For immunohistochemical assays, routinely processed paraffin-embedded as well as frozen tissues from invasive ductal breast carcinomas were obtained from patients who had undergone primary surgery. The uPAR antigen concentration in tumor tissue extracts was determined by ELISA (American Diagnostica, Greenwich, CT).

Paraffin sections of the embedded U937 cells and of the breast cancer tissues were mounted on silane-coated slides, dried overnight at 37°C, dewaxed, and irradiated twice (5 minutes each at 750 W) in a microwave oven (Micro-Chef oven FM 3910Q, type 151, Moulinex, Bagnolet, France) in 10 mmol/L sodium citrate buffer (pH 6.0) as previously described.[20] Frozen sections corresponding to the largest cross-sectional area of the tumor were cut, placed on glass slides, and air dried. After several washes with PBS, both types of sections were treated with 0.3% H_2O_2 for 30 minutes and incubated with 30% fetal calf serum in PBS for 20 minutes.

For detection of human uPAR, the immunoperoxidase procedure was performed as described previously with a Vectastain Elite kit (Vector Laboratories, Burlingame, CA).[21] Briefly, MAbs directed to uPAR (4 to 10 μg of IgG/ml) were applied overnight at 4°C. Subsequently, the slides were washed with PBS and incubated with biotinylated horse anti-mouse IgG (15 minutes at 37°C). After several washes with PBS, the Vectastain Elite ABC reagent was applied (15 minutes at 37°C) and the washing steps repeated. Color was developed with diaminobenzidine (Aldrich Chemical Co., Milwaukee, WI) for 10 minutes at room temperature. Counterstaining of nuclei was performed with hematoxylin. As a negative control, the primary antibody was replaced by PBS or an irrelevant monoclonal IgG. Preincubation of the MAbs directed to uPAR with synthetic peptides (20 μg of peptide/ml) was performed for 30 minutes at 37°C) before applying the antibody/peptide-containing solution to the slides.

Results

Generation of MAbs Directed to uPAR and Immunodecoration Analyses

To generate MAbs to human uPAR, mice were immunized with nonglycosylated recombinant human uPAR produced in *E. coli* (Ec-$uPAR_{1-284}$; corresponding to aa 1 to 284 of human uPAR). A panel of 12 $uPAR_{1-284}$-specific antibodies were selected for further analyses. As the antigen used for immunization represented a rather denatured form of uPAR compared with functionally active, native uPAR, we expected that several of the MAbs generated would react with linear uPAR peptide sequences.

For initial characterization, the reactivity of the MAbs to uPAR was tested by dot and Western blot analyses using various nonglycosylated carboxyl-terminally truncated versions of human uPAR (produced in *E. coli*) as well as a soluble, highly glycosylated recombinant form of human uPAR synthesized by CHO cells, CHO-$uPAR_{1-277}$.[12,19] CHO-$uPAR_{1-277}$ was shown to efficiently interact with uPA.[11] All MAbs reacted with the reduced and denatured (linear) form of the antigen used for immunization, Ec-$uPAR_{1-284}$. The reaction pattern obtained with carboxyl-terminally truncated uPAR versions demonstrated that some of the MAbs are directed to different epitopes of uPAR (Table 1 and Figure 1A). All of the MAbs also interacted with reduced and denatured CHO-$uPAR_{1-277}$, indicating that the binding regions of the MAbs in uPAR are not masked by glycosylation. On the other hand, only 7 of the 12 MAbs recognized CHO-$uPAR_{1-277}$ under nonreducing conditions. We observed that 2 of these 7 MAbs, IIID10 and IIIB6,

Table 1. *Characteristics of MAbs Directed to uPAR*

Antibody	Western/dot blot				FACS	Immunohistochemistry	Epitope	uPAR domain[§]
	E. coli (minimal),*	CHO[†]		U937,[‡]				
	r	r	nr	r				
IIID10	1–94	++	+	+	−	+	4–15	I
IIIB6	1–94	++	+	+	−	+	4–15	I
IIIF10	1–94	++	++	−	++	−	52–60	I
IG7	1–130	++	++	ND	−	−	ND	II
IB5	1–130	++	++	ND	−	−	ND	II
IID7	1–154	++	++	++	+	++	125–132	II
IIIB11	1–154	++	++	++	+	++	125–132	II
IIG1	1–154	+	−	ND	−	+	125–132	II
IIIG2	1–154	+	−	ND	−	+	125–132	II
IIIB4	1–196	+	−	−	−	−	ND	III
IIIB5	1–196	+	−	ND	−	−	ND	III
IIIH9	1–284	+	−	+	−	−	ND	III

r, reducing conditions; nr, nonreducing conditions; ND, not determined. ++, strong reaction; +, moderate reaction; −, no reaction detectable.

*The reaction pattern of the MAbs were tested with different *E. coli*-derived nonglycosylated carboxyl-terminally truncated versions of uPAR (for details see Figure 1). The shortest fragment still reacting with the respective antibody is indicated.

[†]Purified recombinant CHO-cell-derived uPAR (CHO-uPAR$_{1-277}$).

[‡]Total protein from PMA-stimulated promyeloid cell line U937.

[§]The analysis of the exon/intron boundaries of the uPAR gene indicate that domain I is likely to consist of aa 1 to 81, domain II of aa 82 to 180, and domain III of aa 181 to 283[33]; MAbs IIIB4 and IIIB5 do not react with Ec-uPAR$_{1-178}$ and, therefore, very likely react with an epitope located on domain III.

interacted relatively weakly with CHO-uPAR$_{1-277}$ in the immunoblots under nonreducing, nondenaturing conditions. Binding was, however, significantly increased when CHO-uPAR$_{1-277}$ was briefly incubated at 96°C before binding to nitrocellulose (for a summary of the characteristics of the MAbs see Table 1). For comparison, we included MAb HD13.1 in the analysis, which is derived from mice immunized with functionally intact CHO-uPAR$_{1-277}$.[22] MAb HD13.1 reacts only with nonreduced CHO-uPAR$_{1-277}$, not with *E. coli*-derived uPAR variants or with reduced CHO-uPAR$_{1-277}$, which indicates that binding of MAb HD13.1 to uPAR is conformationally dependent (data not shown).

Mild treatment of uPAR with chymotrypsin liberates two proteolytic fragments, covering domain I (aa 1 to 87) and domains II plus III.[23] In Western blot analyses of chymotrypsin-treated CHO-uPAR$_{1-277}$, the three MAbs IIID10, IIIB6, and IIIF10 detected the domain-I-containing fragment ($M_r \approx 18{,}000$). The remaining nine MAbs interacted with the proteolytic fragment containing domains II plus III of uPAR ($M_r \approx 35{,}000$). The reaction patterns of MAbs IID7 and IIIF10 are exemplified in Figure 1B. In agreement with these results, by means of using carboxyl-terminally truncated *E. coli*-derived uPAR fragments in dot as well as Western blot analyses, only the epitopes of MAbs IIID10, IIIB6, and IIIF10 mapped to domain I of uPAR (Table 1 and Figure 1A).

In addition, total protein extracts of two human cell lines, the lymphoblastoid B-cell line Raji and the promyeloid cell line U937, were analyzed by Western blotting with some of the MAbs directed to uPAR. Raji cells do not synthesize uPAR, whereas U937 cells produce considerable amounts of cell-surface-associated uPAR upon stimulation with PMA.[19] MAb IID7 reacted strongly with several proteins from U937 cells (in the range of $M_r \approx 40{,}000$ to 60,000) which most likely represent glycosylation variants of uPAR.[23,24] Raji cell proteins did not react with MAb IID7 (Figure 1C). MAb IIIB11 revealed identical reactivity, whereas MAbs IIID10, IIIB6, and IIIH9 showed comparable but less intense signals. MAbs IIIF10 and IIIB4 reproducibly failed to detect human uPAR under the conditions applied (Table 1).

Precise Epitope Mapping of Domain-I-Specific MAbs to uPAR

The Western blot data indicated that three of the MAbs characterized (IIID10, IIIB6, and IIIF10) are directed to epitopes located on domain I of uPAR. Domain I mediates binding of uPA to uPAR.[20] To narrow down the binding sites of these MAbs, 17 different 15-meric peptides overlapping by 10 amino acids and covering the sequence uPAR$_{1-95}$ were synthesized. The overlaps ensured that any sequential epitope up to 11 residues in length would be present in at least one of the uPAR-based peptides. Figure 2, A and B, shows the reaction pattern of MAbs IIIF10 and IIIB6 with the different peptides

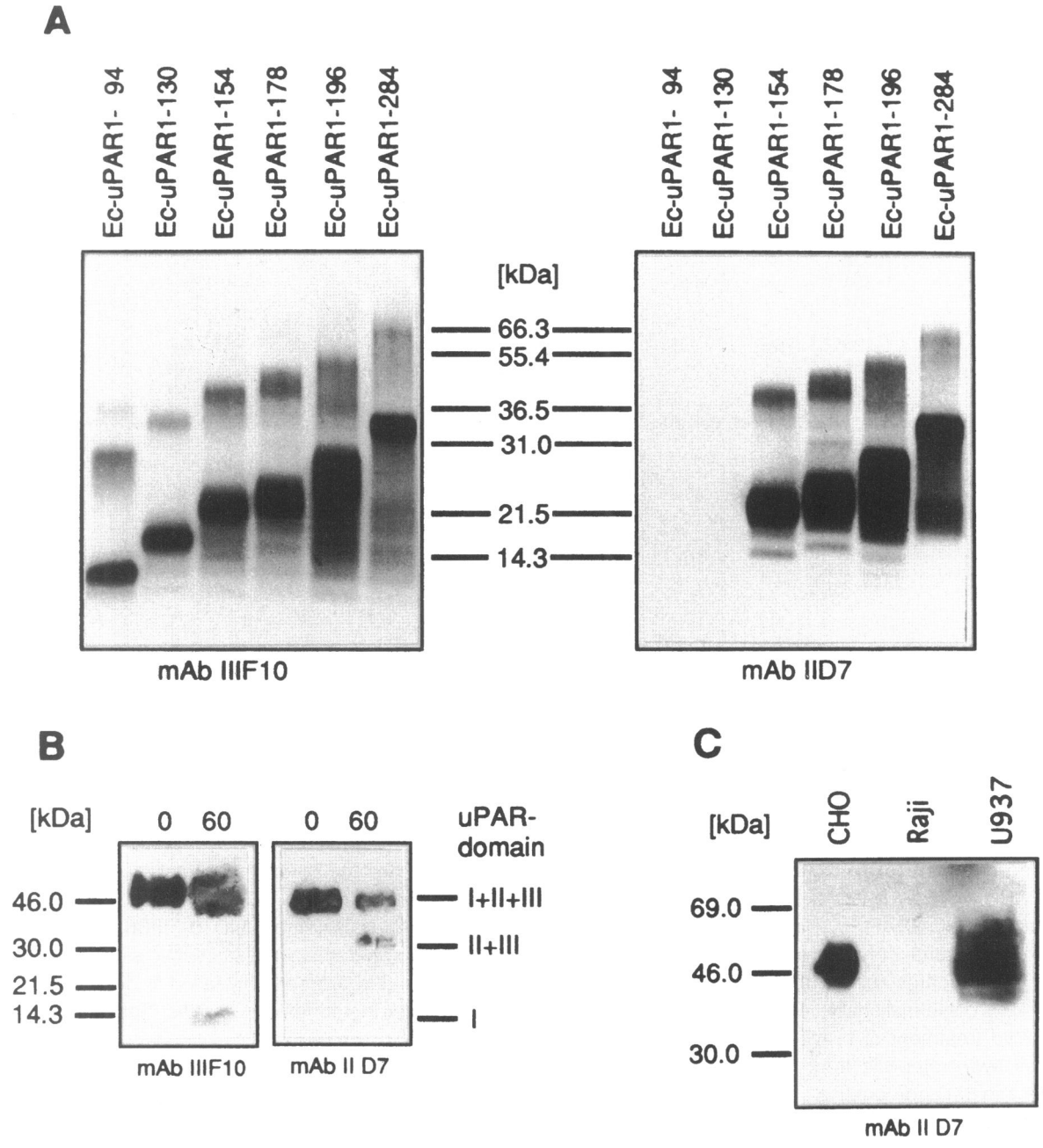

Figure 1. *Reaction pattern of MAbs directed to uPAR in Western blot analyses.* A: *Recombinant uPAR variants were separated by 14% SDS-PAGE and then transferred to a nylon membrane by semi-dry blotting. Subsequently, the blots were probed with MAbs IIIF10 or IID7, respectively, which both are directed against Ec-uPAR$_{1-284}$. The (reduced)* E. coli*-derived uPAR fragments contain aa 1 to 284 (Ec-uPAR$_{1-284}$), aa 1 to 196 (Ec-uPAR$_{1-196}$), aa 1 to 178 (Ec-uPAR$_{1-178}$), aa 1 to 154 (Ec-uPAR$_{1-154}$), aa 1 to 130 (Ec-uPAR$_{1-130}$), and aa 1 to 94 (Ec-uPAR$_{1-94}$) of human uPAR, respectively.*[12] B: *Purified CHO-uPAR$_{1-277}$, either untreated (0) or treated under mild conditions for 60 minutes (60) with chymotrypsin, was separated in duplicate by 12% SDS-PAGE and then transferred to a nylon membrane. The Western blot was cut into halves that were then probed with either MAb IIIF10 or IID7. Native uPAR is cleaved into two fragments by the chymotrypsin treatment, harboring domain I and domains II plus III, respectively.*[23] C: *Approximately 60 μg of protein from human cell homogenates was separated by 10% SDS-PAGE, transferred to a nylon membrane, and immunodecorated with MAb IID7. CHO, purified CHO-uPAR$_{1-277}$; Raji, total protein from the uPAR-negative lymphoblastoid B-cell line Raji; U937, total protein from the uPAR-positive promyeloid cell line U937.*

tested by ELISA. MAb IIIF10 reacted with two partially overlapping peptides (aa 46 to 60 and 51 to 65 of uPAR) whereas MAb IIIB6 interacted with peptide 1–15 only. To further cut down the minimal binding sequence of these MAbs, additional peptides were tested. MAb IIIF10 reacted strongly with uPAR peptides 49–60, 50–61, 51–62, and 52–63 but not with other 12-meric peptides, such as peptides 48–59 or

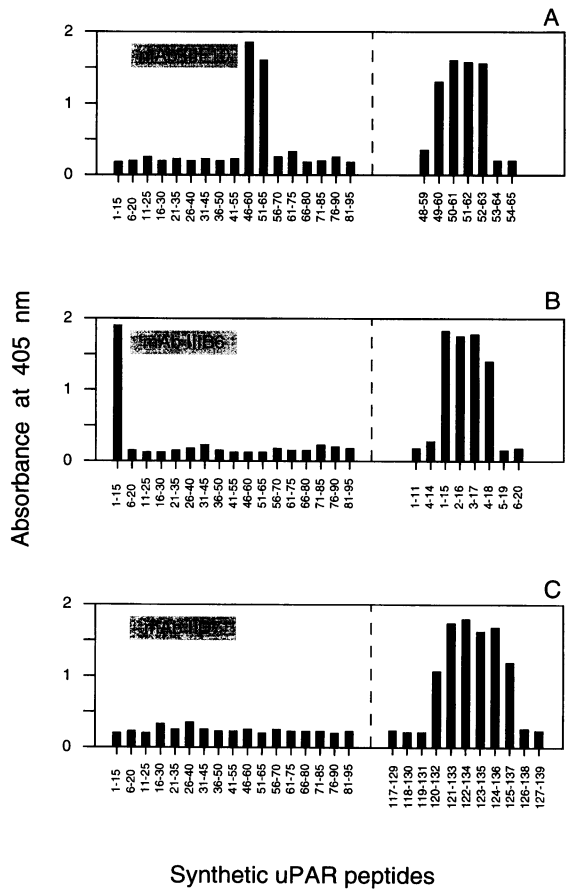

Figure 2. *Epitope mapping of MAbs IIIF10, IIIB6, and IID7. First, antibodies were tested for binding to 15-meric uPAR peptides overlapping each other by 10 residues* (left half of panels). *Additional uPAR peptides were employed for the precise mapping of the MAbs* (right half of panels). *Peptides were adsorbed to wells of a microtiter plate and tested for MAb binding by ELISA (for details see Materials and Methods).*

53–64. MAb IIIB6 reacted strongly with uPAR peptides 2–16, 3–17, and 4–18 but not with 5–19, 4–14, or 1–11. MAb IIID10 revealed a very similar pattern as MAb IIIB6 (data not shown). Taken together, the reaction patterns suggest that the sequence covering aa 52 to 60 of uPAR is sufficient for binding of MAb IIIF10, whereas MAbs IIIB6 and IIID10 bind to an epitope at the amino terminus of uPAR (aa 4 to 15). The other nine MAbs (eg, IID7; Figure 2C) did not react with any peptide of domain I of uPAR.

Precise Epitope Mapping of Domain-II-Specific MAbs to uPAR

As MAbs IID7 and IIIB11 reacted strongly with human uPAR in Western blot analyses (Figure 1C), we also determined the target sequence on uPAR for these two MAbs. Both MAbs reacted with the uPAR variants Ec-uPAR$_{1-154}$, Ec-uPAR$_{1-196}$, and Ec-uPAR$_{1-284}$ but not with Ec-uPAR$_{1-94}$ and Ec-uPAR$_{1-130}$. To precisely localize the MAb epitopes, a series of domain-II-derived peptides were synthesized covering the sequence uPAR$_{117-154}$, and the reaction patterns of the MAbs were tested by ELISA. Both MAbs, IID7 and IIIB11, reacted strongly with uPAR peptides 120–132, 121–133, 122–134, 123–135, 124–136, and 125–137 but not with other peptides, such as 119–131 and 126–138 (Figure 2C). These results indicate that both MAbs react with a similar epitope located between aa 125 and 132 of uPAR. Interestingly, MAbs IIG1 and IIIG2 also reacted with an epitope between aa 125 and 132. In contrast to IID7 and IIIB11, these two antibodies did not react with nonreduced uPAR from CHO cells or with cell surface human uPAR (for a summary see Table 1). The other eight MAbs did not bind to any of the domain-II-derived peptides spanning uPAR$_{117-154}$ (data not shown).

Flow Cytofluorometric Analyses

We next investigated whether the 12 MAbs can detect human uPAR on intact cells. For this purpose, PMA-stimulated U937 cells, which express uPAR on their surface, were first acid washed to dissociate endogenous receptor-bound uPA and then incubated with increasing amounts of the MAbs. Only 3 MAbs (IIIF10, IID7, and IIIB11) were found to interact in saturable kinetics with U937 cells with MAb IIIF10 being the best (Figure 3A and Table 1). These 3 MAbs did not bind to uPAR-negative Raji cells.

The epitope of MAb IIIF10 resides in domain I of uPAR, the uPA-binding domain. Therefore, we next analyzed whether saturation of cell-surface-associated uPAR by uPA before incubation with the MAb has an effect on the binding efficiency of MAb IIIF10 to U937 cells. For comparison, we used MAb IID7, the target epitope of which was mapped to domain II of uPAR. As can be seen in Figure 3A, preincubation of cells with pro-uPA completely blocks binding of MAb IIIF10 to U937 cells whereas binding of MAb IID7 is only marginally affected. If U937 cells were preincubated with either MAb IIIF10 or IID7 and then tested for binding of fluorescently labeled pro-uPA (FITC-pro-uPA), MAb IIIF10 strongly inhibited binding of FITC-uPA to U937 cells whereas MAb IID7 had no significant effect on uPA/uPAR interaction (Figure 3B). These results indicate that the epitope of MAb IIIF10 (aa 52 to 60 of uPAR) is involved, at least in part, in binding of uPAR to its ligand uPA.

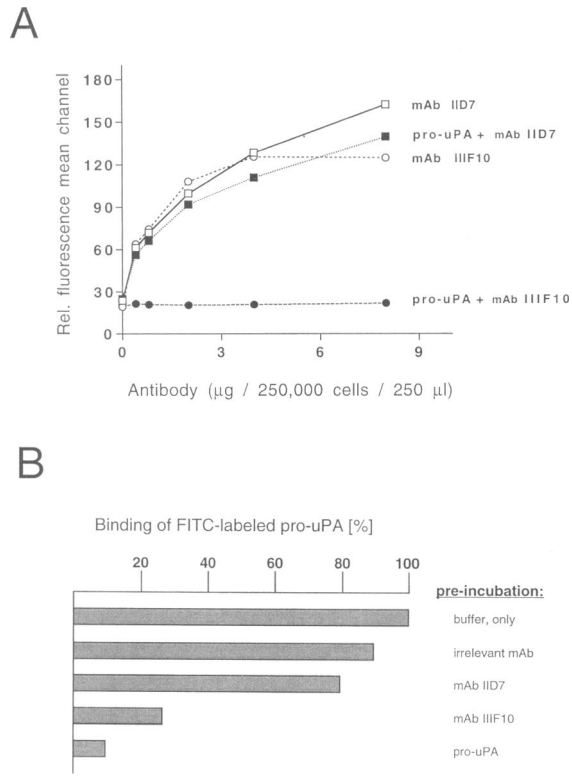

Figure 3. *Detection of uPAR on U937 cells by MAbs IIIF10 and IID7 applying flow cytofluorometry.* **A:** *To study the influence of uPAR occupancy on binding of MAbs IIIF10 or IID7 directed to uPAR, acid-washed, PMA-stimulated U937 cells were preincubated with an excess of pro-uPA (2 μg per 2.5 × 10^5 cells) to occupy all functional uPAR. Controls received buffer only. Second, U937 cells were incubated with different concentrations of the MAbs (in the range of 0 to 8 μg per 2.5 × 10^5 cells). U937 cells were stained with FITC-conjugated anti-mouse IgG, and after several washes, cell-associated fluorescence was determined by flow cytofluorometry. Relative fluorescence intensity is expressed as fluorescence mean channel. Values are not corrected for autofluorescence of the cells (autofluorescence = 22).* **B:** *Acid-washed, PMA-stimulated U937 cells were preincubated with either pro-uPA (2 μg per 2.5 × 10^5 cells), MAb directed to β-galactosidase (isotype-matched negative control MAb), MAb IID7, or MAb IIIF10 (8 μg each per 2.5 × 10^5 cells). Controls received buffer only. FITC-uPA (16 ng) was then added to the samples and the cells incubated further. Finally, the relative amount of cell-associated FITC-uPA was determined by flow cytofluorometry. Binding of FITC-pro-uPA to the buffer-only control was defined as 100% binding, and the autofluorescence of the cells was taken as 0% binding.*

Immunocytochemical Assessment of U937 Cells by MAb IID7

To develop an immunocytochemical assay for detection of uPAR, U937 cells, either resting or stimulated with PMA, were incubated with or without FITC-labeled pro-uPA and collected by centrifugation, and the cell pellets were fixed with formaldehyde and then embedded in paraffin. Six of the 12 MAbs analyzed (IIID10, IIIB6, IID7, IIIB11, IIG1, and IIG2) reacted with cell-surface-associated uPAR in sections of PMA-stimulated U937 cells with MAbs IID7 and IIIB11 being the best (Table 1). A moderate staining only was obtained by incubation of these antibodies with resting U937 cells (compare Figure 4, A and C). As a control, resting and PMA-stimulated cells were incubated with FITC-labeled pro-uPA. Again, a strong induction of uPAR expression was seen in the presence of PMA (compare Figure 4, B and F). Preincubation of PMA-stimulated cells with (FITC-labeled) pro-uPA before addition of the uPAR domain-II-specific MAbs IID7 (compare Figure 4, C and E) or IIIB11 (data not shown) did not interfere with the uPAR/MAb reaction.

Immunohistochemical Staining of Breast Cancer Sections with MAb IID7

Immunostaining of paraffin sections of invasive ductal carcinomas with MAb IID7 revealed a staining pattern that varied from case to case. In all of the sections, staining was confined to fibroblast-like or histiocytic cells within the stroma (Figure 5), but in 12 of 20 tumors examined, cancer cells also displayed a staining for uPAR (Figure 5, A and D) similar to findings by others.[25,26] In both cell types, uPAR-specific staining is associated with the cell membrane and the cytoplasm.

uPAR antigen content of the 20 carcinomas was assessed in tissue extracts by ELISA (range, 1.2 to 16.8 ng/mg protein) and compared with the uPAR reactivity of MAb IID7 in immunohistochemistry. Intense (weak) staining reaction corresponded to an elevated (low) uPAR antigen content (for examples see Figure 5, A and B). Eight of the 10 tumors above and 4 of the 10 below the median value of the 20 specimens showed a positive reaction for cancer cells in addition to stromal cell staining.

To further characterize MAb IID7, we compared paraffin sections with cryostat sections obtained from the same tumors. The patterns of immunostaining of IID7 on paraffin sections were superimposable with those of cryostat sections; staining, however, was more pronounced in paraffin sections (data not shown). The staining pattern of MAb IID7 was comparable with that obtained with another anti-uPAR MAb, HD13.1,[22] which detects uPAR in cryostat sections but not in paraffin-embedded material. Staining of cells with MAb IID7 was completely suppressed by preincubation of the specimens with the epitope-specific synthetic peptide $uPAR_{123-132}$. Preincubation of MAb IID7 with an irrelevant peptide, eg, $uPAR_{1-15}$, did not affect the staining reaction (compare Figure 5, E and F). Substitution of MAb IID7 by MAb IIIB11 (also directed to $uPAR_{125-132}$) in the above described experiments gave indistinguishable results.

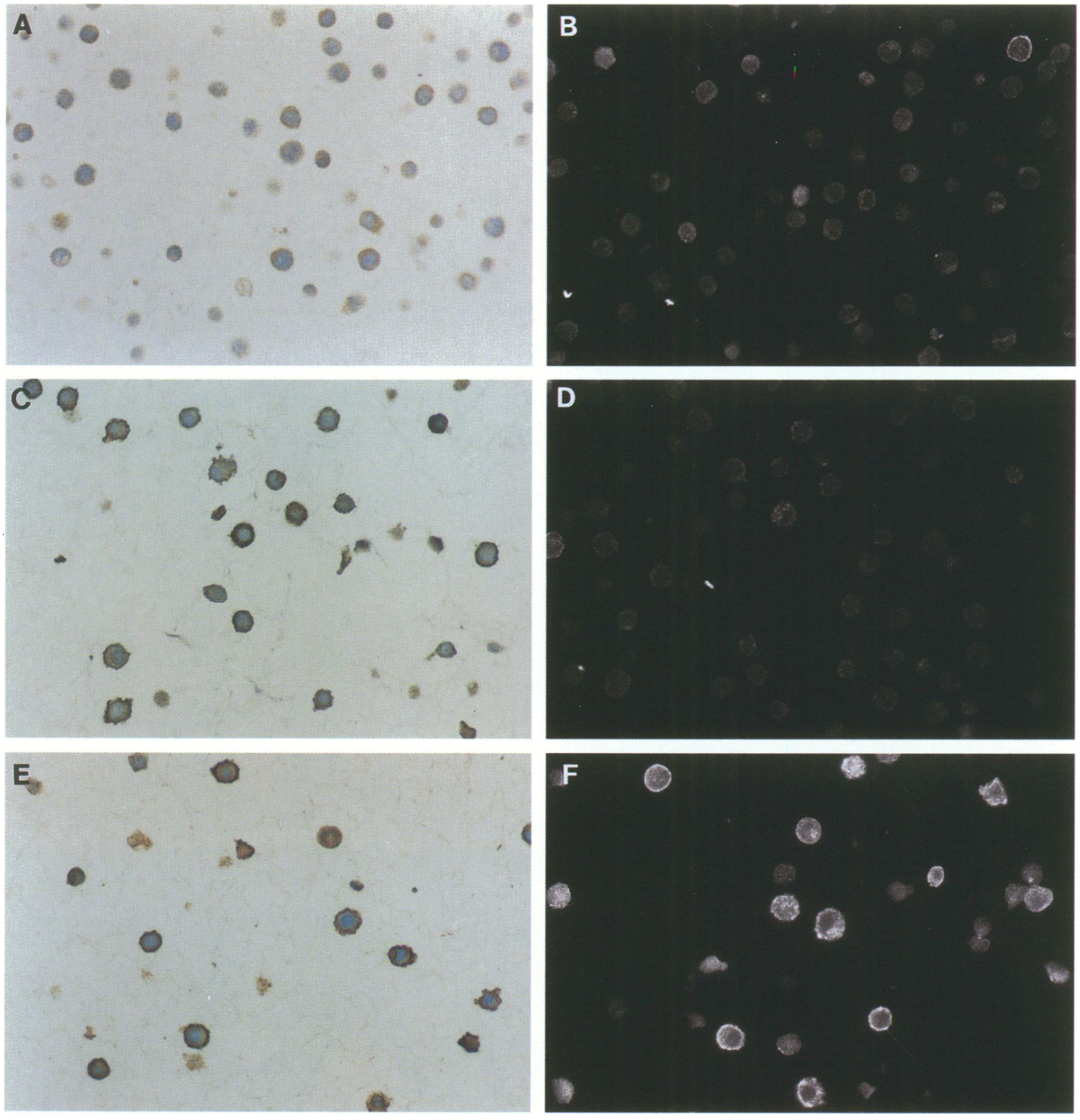

Figure 4. *Immunocytochemical detection of uPAR by MAb IID7. Resting or PMA-stimulated U937 cells were either incubated with or without fluorescently labeled pro-uPA, incorporated into a clot, fixed with 4% formaldehyde and subsequently embedded in paraffin. uPAR was detected by immunocytochemistry using MAb IID7 as described in Materials and Methods.* **A** *and* **B**: *Weak uPAR staining reaction in resting U937 cells* (**A**), *which display little cell-membrane-associated fluorescence after incubation with FITC-labeled pro-uPA* (**B**). **C** *and* **E**: *Strong cell-surface-associated uPAR staining reaction in PMA-stimulated U937 cells* (**C**). *The reaction pattern is indistinguishable from the pattern obtained using cells that which have been incubated with (FITC-)pro-uPA before the paraffin-embedding procedure* (**E**). **D** *and* **F**: *Cell-associated fluorescence of PMA-stimulated U937 cells reacting with FITC-pro-uPA* (**F**) *or with an irrelevant fluorescent probe* (**D**). *Positive staining in* **A**, **C**, *and* **E**: *brownish color; staining of nuclei: blue color; fluorescence in* **B**, **D**, *and* **F** *appears in white; magnification, ×380* (**A** *to* **F**).

Discussion

The specific surface receptor for uPA, uPAR, is present in human tissues and body fluids and exists in various molecular forms. 1) uPAR is heavily glycosylated as reflected by a substantial heterogeneity of the apparent molecular mass of uPAR. The extent of glycosylation of uPAR may result in differences in the affinity for uPA.[24,27] 2) A cleaved form of uPAR has been identified that results from proteolytic cleavage thereby releasing the uPA-binding domain and leaving domains II and III of uPAR attached to the cell surface.[28–30] 3) In ascitic fluid of ovarian cancer patients, a soluble form of uPAR lacking the glycosyl-

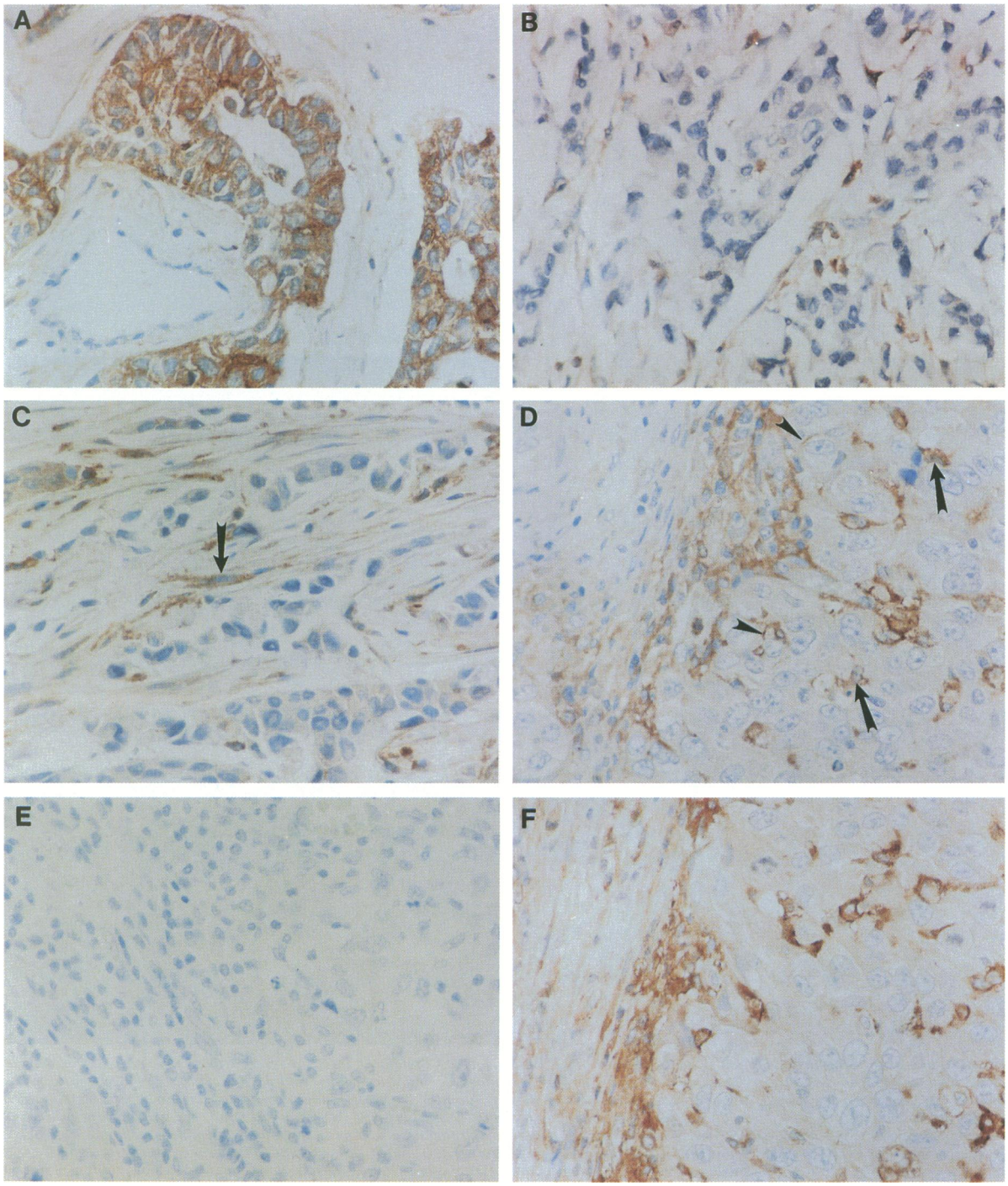

Figure 5. *Immunohistochemical staining of invasive ductal breast carcinoma specimens with domain-II-specific anti-uPAR MAb IID7. Routinely processed paraffin-embedded tissues from invasive ductal breast carcinomas were obtained from patients undergoing primary surgery, and uPAR was detected by immunohistochemistry using MAb IID7 as described in Materials and Methods. No staining was obtained when the anti-uPAR IgGs were substituted by irrelevant monoclonal IgGs.* A: *Strong uPAR staining in tumor cells of a carcinoma with a high uPAR antigen content (16.8 ng/mg total protein).* B: *Weak uPAR staining in stromal cells in a carcinoma with a low uPAR antigen content (2.9 ng/mg total protein).* C *and* D: *Comparison of two tumors with similar uPAR antigen levels (13.8 ng/mg protein* (C) versus *10.7 ng/mg protein* (D)) *but different uPAR expression patterns.* Arrows *in* C *and* D *indicate uPAR-positive stromal cells.* Arrowheads *in* D *indicate cancer cells, which display surface staining at the site of invasion.* E *and* F: *Preincubation of MAb IID7 with either its epitope-specific synthetic peptide (covering aa 123 to 132 of human uPAR) or an irrelevant peptide (covering aa 1 to 15 of human uPAR); slides derived from the same tumor as in* D *were stained. Note that preincubation of MAb IID7 with peptide 123 to 132 completely abolishes binding of the antibody to the cells. Magnification, ×380* (A *to* F).

phosphatidylinositol moiety (GPI anchor) was detected.[31] 4) Analyses of uPAR cDNAs have revealed alternatively spliced forms of uPAR lacking either exon 5 or exchanging exon 7 for another final exon.[32–34] In addition to the uPAR variants described above, uPAR may occur in an unoccupied state, it may be bound to uPA, or it may be part of a trimeric complex consisting of uPA, uPAR, and one of the uPA inhibitors (PAI-1, PAI-2, or PN-1) or interact with vitronectin.[35–39] Taking these reflections into consideration, it seems desirable to use well defined antibodies for analysis of uPAR by immunological methods.

MAbs to uPAR used so far were usually generated using glycosylated, recombinant, or native uPAR as the antigen,[19,22,40,41] and only indirect methods (such as measuring the influence of the MAb on uPA/uPAR interaction or analysis of binding to uPAR fragments generated by chymotrypsin-treatment) were applied to narrow down the epitope on uPAR recognized by the MAbs. In many cases, it is also not clear whether the antibodies are directed to the protein or to the glycosyl moiety of uPAR and, if so, whether reduced reactivity of the MAb in question with uPAR is different because of the varying extent of glycosylation. Moreover, as MAbs to uPAR often do not react with unfolded uPAR, it is likely that the binding sites of these MAbs are dependent on the three-dimensional structure of uPAR and may be composed of noncontiguous peptide sequences. To overcome these attributes, we have developed a protocol to generate MAbs to uPAR by using nonglycosylated, recombinant human uPAR produced in *E. coli* as the immunogen. By this approach, domain-specific antibodies were produced and the epitopes these MAbs are directed to mapped by reaction with carboxyl-terminally truncated uPAR variants and synthetic uPAR-derived peptides.

Three of the 12 MAbs analyzed were directed against the uPA-binding domain I of uPAR; 2 (MAbs IIID10 and IIIB6) reacted with the amino terminus of uPAR (aa 4 to 15), and MAb IIIF10 interacted with aa 52 to 60 of uPAR. Interestingly, both uPAR regions were shown previously to be involved in species-specific ligand recognition. Pollänen[42] constructed a mouse/human uPAR chimera carrying as few as the first 13 amino acids of the amino terminus of the murine uPAR instead of the corresponding human sequence and demonstrated that binding of the chimeric protein to human uPA was completely abolished. In contrast, a complementary chimera (with the first 13 amino acids of the amino terminus of the human uPAR instead of the corresponding murine sequence) reacted with human uPA. Wild-type murine or human uPAR does not interact with its respective cross-species counterpart.

MAbs IIID10 and IIIB6 reacted with all uPAR forms tested (glycosylated and nonglycosylated and full-length and truncated versions) in Western and dot blot analyses but surprisingly did not detect uPAR on intact, nonfixed cells. This feature points to that the amino terminus of uPAR is not exposed on the surface of the uPAR protein and may, thus, not directly be involved in uPAR/uPA interaction. It is tempting to speculate that the amino-terminal region of uPAR constitutes a structural determinant for species-specific recognition of uPA.

MAb IIIF10, directed to aa 52 to 60 of human uPAR, did not react with uPAR occupied by uPA. Furthermore, preincubation of uPAR with MAb IIIF10 strongly inhibited interaction of uPA with uPAR. Thus, this region of uPAR seems to be critical for binding of uPA. By alanine scanning mutagenesis, Pollänen[42] demonstrated that a uPAR mutant with four amino acid substitutions (His47, Glu49, Lys50, and Arg53 exchanged by alanine) no longer interacted with uPA, whereas other triple or quadruple mutations in other regions of uPAR did not significantly affect binding to uPA. In addition, chemical modification of uPAR with tetranitromethane, which resulted in efficient and specific nitration of solvent-accessible tyrosine residues, identified Tyr57 of uPAR to be intimately engaged in the interaction with uPA.[43] The binding epitope of IIIF10 also covers the only glycosylation site, Asn52, of domain I of human uPAR. Exchange of Asn52 to Gln52 by *in vitro* mutagenesis resulted in a nonglycosylated ligand-binding domain of uPAR with considerably reduced affinity for uPA compared with wild-type uPAR.[44] The inhibitory properties of MAb IIIF10, together with the other findings described above, strongly suggest that the MAb IIIF10 epitope, aa 52 to 60 of human uPAR, is located at or close to the uPA-binding site of uPAR. Together with the results of the previously performed mutational analyses of the uPAR-binding region of uPA,[45] the present mapping of the uPA interaction site in domain I of uPAR may further facilitate the development of specific and efficient inhibitors of uPA/uPAR interaction that may serve as potential therapeutic agents to block tumor-cell-associated uPA/uPAR interaction.

We further tested the MAbs for their suitability for immunohistochemical studies. Six of the 12 MAbs reacted with cellular uPAR in paraffin-embedded breast cancer specimens. Distinct staining was obtained with 2 of the MAbs, IID7 and IIIB11, both directed against the domain II epitope aa 125 to 132. The specificity of staining of uPAR with these MAbs

was supported by several controls: 1) staining was completely abolished after competitive inhibition of the immunohistochemical reaction with an epitope-specific, synthetic uPAR peptide; 2) the intensity of staining of uPAR determined in resting U937 cells was increased after PMA stimulation; and 3) uPAR staining reaction corresponded to the uPAR antigen content measured by ELISA in matching breast cancer extracts. In addition, with both MAbs, identical staining patterns in both paraffin and cryostat sections of breast cancer tissues were obtained. Furthermore, the staining reaction in cryostat sections was comparable to another anti-uPAR IgG, HD13.1.[22] HD13.1 does not react with paraffin-embedded specimens.

Using the domain-II-specific MAb IID7, we could demonstrate that in breast cancer cells uPAR antigen is associated with both the cell membrane and the cytoplasm. Similar findings have been reported by Pyke et al[46] for colon cancer cells and Wagner et al[47] for kidney cancer cells. The observed staining pattern for uPAR antigen in tumor and stromal cells in breast cancer confirms previous findings by Pyke et al[25] and Bianchi et al[26] using different but less characterized MAbs to uPAR. The most intense staining was located in tumor cells present in the invasive front of the malignant epithelium. Interestingly, Pyke et al[46] observed on human colon cancer cells that many tumor cells at the invasive foci displayed prominent *in situ* hybridization signals for uPAR mRNA but no detectable immunoreactivity with MAbs to uPAR. This might be explained by an interference of the interaction of the MAbs used with cellular uPAR by receptor-bound uPA or by the extent of glycosylation.[25,37] In fact, many solid cancers produce uPAR as well as uPA,[26,37,48] and the extent of glycosylation may vary in malignant cells compared with other cells.[24,49] For both MAbs, IID7 and IIIB11, binding to uPAR, as shown in various immunological tests in the present study, is not affected by uPA or by the presence or lack of the glycosyl moiety of the protein. Thus, MAbs IID7 and IIIB11 are useful tools for immunohistochemical detection of cellular uPAR especially in formalin-fixed, paraffin-embedded tissue specimens. In this context, it is worth mentioning that, using MAb IID7, we detected uPAR antigen in tumor cells of invasive ductal carcinoma with high frequency (12 of 20 tumors analyzed). In previous studies, Pyke et al[25] and Bianchi et al,[26] using different antibodies directed to uPAR, detected uPAR-expressing tumors in 5 of 40 and 16 of 59 cases, respectively. Whether the uPAR positivity of tumor cells in breast cancer is of clinical use is presently being analyzed with a larger series of specimens by correlating uPAR staining with other prognostic variables (such as uPAR-, PAI-1, and uPA antigen content) and patient outcome.

In conclusion, we have devised well defined and epitope-mapped MAbs to uPAR that are highly specific tools for the detection and targeting of uPAR in tumor tissue. The protocol employed may also prove useful for other receptor proteins that are involved in similar complex interactions.

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