Regulation of Urokinase Receptor Proteolytic Function by the Tetraspanin CD82*

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The high affinity interaction between the urokinase-type plasminogen activator (uPA) and its glycolipid-anchored cellular receptor (uPAR) promotes plasminogen activation and the efficient generation of pericellular proteolytic activity. We demonstrate here that expression of the tetraspanin CD82/KAI1 (a tumor metastasis suppressor) leads to a profound effect on uPAR function. Pericellular plasminogen activation was reduced by ~50-fold in the presence of CD82, although levels of components of the plasminogen activation system were unchanged. uPAR was present on the cell surface and molecularly intact, but radioligand binding analysis with uPA and anti-uPAR antibodies revealed that it was in a previously undetected cryptic form unable to bind uPA. This was not due to direct interactions between uPAR and CD82, as they neither co-localized on the cell surface nor could be co-immunoprecipitated. However, expression of CD82 led to a redistribution of uPAR to focal adhesions, where it was shown by double immunofluorescence labeling to co-localize with the integrin α5β1, which was also redistributed in the presence of CD82. Co-immunoprecipitation experiments showed that, in the presence of CD82, uPAR preferentially formed stable associations with α5β1, but not with a variety of other integrins, including α2β1. These data suggest that CD82 inhibits the proteolytic function of uPAR indirectly, directing uPAR and α5β1 to focal adhesions and promoting their association with a resultant loss of uPA binding. This represents a novel mechanism whereby tetraspanins, integrins, and uPAR, systems involved in cell adhesion and migration, cooperate to regulate pericellular proteolytic activity and may suggest a mechanism for the tumor-suppressive effects of CD82/KAI1.

Proteolytic enzymes in the pericellular environment can profoundly influence the interaction between cells and the surrounding extracellular matrix that plays a large part in determining cellular behavior (1). The serine protease plasmin is one of the major proteases involved in mediating these rapid and irreversible changes. Plasmin can directly degrade many components of the extracellular matrix, generate bioactive fragments from it, release or activate extracellular matrix-seques-tered growth factors, modify other cell-surface proteins, and activate latent matrix metalloproteases (2–4). It is also emerging that pericellular protease systems, including the plasminogen activation system, communicate closely with cell adhesion systems (5–7).

Plasmin is a powerful protease with a broad substrate specificity and is generated from its abundant precursor plasminogen in the pericellular environment by the highly specific urokinase-type plasminogen activator (uPA),1 which is itself generated by proteolytic activation of pro-uPA. The plasminogen activation system represents an enormous proteolytic potential, which is reflected in the multiple levels at which its function is regulated. A central molecule in this regulation is the uPA receptor (uPAR), the high affinity cell-surface receptor for uPA (8). Binding of uPA to uPAR has two primary effects. First, it greatly amplifies the generation of plasmin activity by promoting the activation of both plasminogen and pro-uPA in a system of reciprocal zymogen activation. Second, as these reactions involve cell-associated plasminogen, uPAR acts to focus the proteolytic activity of this system to the cell surface (9–11). These uPAR-dependent interactions also regulate this system at the level of inhibition both directly by the inhibitors PAI-1 and α2-antiplasmin (10, 12) and by internalization of uPA/PAI-1 complexes (13, 14).

uPAR can also potentially direct proteolytic activity to discrete regions of the cell surface, e.g. during cell migration (15–17). As uPAR is a glycosylphosphatidylinositol-anchored protein, it is thought that this behavior is dependent on its association with transmembrane proteins. Integrins may fulfill this role, as a number of them have been shown to associate with uPAR, including the fibrinogen receptor αMβ2 (18–20), the laminin receptor α5β1, and the fibronectin receptor α5β1 (21–23). The interaction of uPAR with some of these integrins has been shown to modulate their function by influencing either their ligand-binding specificity or affinity (20, 22, 24). However, although it is established that there is communication between these cell adhesion receptors and uPAR, it has yet to be demonstrated that this communication can influence pericellular proteolytic activity.

Integrins also associate with other proteins at the cell sur-

1 The abbreviations used are: uPA, urokinase-type plasminogen activator; uPAR, urokinase-type plasminogen activator receptor; PAI-1, plasminogen activator inhibitor-1; mAb, monoclonal antibody; PI, phosphatidylinositol; PBS, phosphate-buffered saline; RT, reverse transcription; HRP, horseradish peroxidase; CHAPS, N-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonic acid; ERK, extracellular signal-regulated kinase. The ganglioside nomenclature proposed by Svennerholm was followed (Svennerholm, L. (1980) Adv. Exp. Med. Biol. 125, 11).
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CD82, a tetraspanin that engages in many of the interactions mentioned above (35–37), has also been independently identified as the tumor metastasis suppressor gene KAI1. This was originally shown to suppress the metastasis of human prostate cancer cells in animal models and to be reduced in cell lines derived from metastatic prostate tumors (38). Its expression has since been found to be down-regulated in a variety of human cancers, influencing tumor progression and invasion as well as metastasis (39–43). The reduced expression of CD82 in tumors is closely correlated with inactivation of the p53 tumor suppressor (44).

We demonstrate here that the role of tetraspanins extends to the regulation of pericellular proteolytic activity, as we have found that expression of CD82 leads to an ~50-fold reduction in the capacity of the epithelial cell line HB2 to generate plasmin activity. This is due to an effect on uPAR, which, although present on the cell surface, becomes “cryptic,” losing the ability to bind uPA. CD82 achieves this indirectly by promoting stable associations between uPAR and α5β1 integrin concomitant with a redistribution of uPAR to focal adhesions. These data are the first to demonstrate that interaction with cell adhesion systems can regulate uPAR proteolytic function and therefore the existence of bidirectional communication between these two systems fundamentally involved in cellular behavior.

EXPERIMENTAL PROCEDURES

Cell Lines, Proteins, and Antibodies—The HB2/neo and HB2/CD82 cell lines were generated by the respective transfection of pZeoSV (Invitrogen) and pZeoSV/CD82 into the normal mammary epithelial cell line HB2 (45) as described previously (36). Zeocin-resistant colonies were pooled (>30 colonies for HB2/neo cells and >25 colonies for HB2/CD82 cells). The latter pool was subjected to two cycles of fluorescence-activated cell sorting to obtain cells with homogeneous expression of CD82. Cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum, 10 µg/ml insulin, and 10 µg/ml hydrocortisone. All cell culture reagents were from Invitrogen. Anti-uPA monoclonal antibody (mAb) clone 5, anti-uPAR mAbs R3 and R4, and rabbit anti-uPAR polyclonal antibody were kindly provided by Dr. Gunilla Høyer-Hansen (Finsen Laboratory, Copenhagen, Denmark), and anti-uPAR polyclonal antibody 399R was from American Diagnostica Inc. (Greenwich, CT). mAb R4 was biotinylated using the EZ-LinkTM Sulfo-NHS-LC-Biotinylation kit (Pierce) according to the manufacturer’s instructions. Anti-CD82 mAb M104 was provided by Dr. O. Yoshie (Kinki University School of Medicine, Osaka, Japan); anti-α5 integrin mAb SAM-1 was a kind gift from CellTec (Slough, UK); and anti-α1 integrin mAb MCA189 was from Serotec (Oxford, UK). Rabbit polyclonal antibodies to α5 and α6 integrins and goat anti-uPA polyclonal antibody AB776 were from Chemicon (Harrow, UK). Secondary antibodies were from Dako (Ely, UK) and Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA). Lys-plasminogen was from Enzyme Research Laboratories (Swansea, UK). Peptide M25 (24) was prepared by solid-phase synthesis. Phosphatidylserine (PS)-specific phospholipase C was from Roche.

Detection of Cell-surface Plasminogen Activation—Plasminogen activation by uPAR-bound uPA on the surface of HB2 cells was determined as described previously (10). In brief, cells grown to confluence in 48-well plates were washed with phosphate-buffered saline (PBS) to remove unbound uPA and incubated at 37 °C with Lys-plasminogen (200 nM) and the plasmin-specific fluorogenic substrate H-υ-Val-Leu-Lys-aminomethylcoumarin (0.25 mM). Plasmin generated by endogenously bound uPA was measured continuously as change in fluorescence in a SpectraMax Gemini microplate reader (Molecular Devices, Sunnyvale, CA) at A360/440 nm. Plasmin activation was determined as ΔF, and plasmin generation is represented as ΔF versus time by reference to standard curves of active site-titrated plasmin. In some experiments, endogenously bound uPA was removed from the cells by brief treatment in 0.1 M glycine (pH 3) (46) prior to incubation with pro-uPA (2 nM) for 20 min at 37 °C and additional washing. The effect of incorporating exogenous gangliosides into HB2 cell membranes was assayed with purified GM1 and GD1a (Calbiochem) solubilized in N,N-dimethylformamide or PBS with sonication according to the manufacturer’s instructions and incubated with cells at concentrations between 0.5 and 100 µg/ml for 1 or 24 h prior to assay (47). Controls included carrier in the absence of ganglioside.

Radioligand Binding—Dissopropyl fluorophosphate-inactivated uPA and anti-uPAR mAb were labeled with Na125I using IODO-BEADS (Pierce) to a specific activity of ~10 µCi/µg. Radiolabeled ligand binding assays were performed on monolayers of cells in 24-well plates by competitive displacement with unlabeled ligand as described previously (48). In brief, acid-washed cells were incubated with a fixed concentration of labeled ligand (~2000 counts/s) and varying concentrations of unlabeled ligand for 20 min at 37 °C. The supernatant was removed for determination of unbound ligand. The cells were then washed three times with the same buffer and lysed in N,N-dimethylformamide or PBS containing carrier. The bound ligand was determined by γ-counting. Data were analyzed both by nonlinear regression of the primary data and by Scatchard analysis.

Reverse Transcription (RT)-PCR and Enzyme-linked Immunosorbent Assay—Specific oligonucleotide primers and fluorogenic probes were designed for uPA and uPAR to allow measurement by quantitative real-time RT-PCR using an ABI Prism 7700 sequence detection system (Applied Biosystems, Foster City, CA). TaqMan® assay probe oligonucleotides (Pierce) to a specific activity of ~10 µCi/µg. Radiolabeled ligand binding assays were performed on monolayers of cells in 24-well plates by competitive displacement with unlabeled ligand as described previously (48). In brief, acid-washed cells were incubated with a fixed concentration of labeled ligand (~2000 counts/s) and varying concentrations of unlabeled ligand for 20 min at 37 °C. The supernatant was removed for determination of unbound ligand. The cells were then washed three times with the same buffer and lysed in N,N-dimethylformamide or PBS containing carrier. The bound ligand was determined by γ-counting. Data were analyzed both by nonlinear regression of the primary data and by Scatchard analysis.

Chemical Cross-linking and Biotinylation—Cross-linked samples were prepared by the addition of 2 µM disuccinimidyl suberate (Pierce) to PBS to subconfluent cell monolayers after washing twice with PBS. Samples were incubated at room temperature for 30 min with agitation. The cross-linking reaction was quenched by the addition of 20 mM NaHPO4, and plasmin generation is represented as ΔF versus time by reference to standard curves of active site-titrated plasmin. In some experiments, endogenously bound uPA was removed from the cells by brief treatment in 0.1 M glycine (pH 3) (46) prior to incubation with pro-uPA (2 nM) for 20 min at 37 °C and additional washing. The effect of incorporating exogenous gangliosides into HB2 cell membranes was assayed with purified GM1 and GD1a (Calbiochem) solubilized in N,N-dimethylformamide or PBS with sonication according to the manufacturer’s instructions and incubated with cells at concentrations between 0.5 and 100 µg/ml for 1 or 24 h prior to assay (47). Controls included carrier in the absence of ganglioside.

Preparation of Cell Lysates, Immunoprecipitation, and Western Blotting—Cell lysates were prepared from subconfluent monolayers. After washing twice with PBS, cells were harvested in 0.5 ml of lysis buffer containing Complete EDTA-free inhibitors (Roche), and cells were lysed as described below.

Preparation of Cell Lysates, Immunoprecipitation, and Western Blotting—Cell lysates were prepared from subconfluent monolayers. After washing twice with PBS, cells were harvested in lysis buffer containing Complete EDTA-free inhibitors (Roche), and cells were lysed as described below.
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RESULTS

CD82 Decreases Cell-associated Plasminogen Activation—To study the effect of CD82 on uPAR function, the normal human breast epithelial cell line HB2, which expresses essentially undetectable levels of endogenous CD82, was stably transfected with either CD82 (HB2/CD82 cells) or expression vector alone (HB2/zeo cells). CD82 expression in HB2/CD82 cells was >200-fold greater than in HB2/zeo cells, but at a level comparable with other cell lines displaying endogenous expression as determined by quantitative RT-PCR (data not shown).

Casein overlay zymography of cell lysates showed that HB2/CD82 cells had much lower amounts of cell-associated plasminogen activator activity compared with control HB2/zeo cells, and the electrophoretic mobility of the activator was consistent with that of uPA (data not shown). These observations and the cell-surface localization of the uPA activity were confirmed by quantitative measurements of cell surface-associated plasminogen activation. In the presence of CD82, the reduction in plasmin generation by endogenously bound uPA approached 50-fold (Fig. 1A).

This unexpectedly large effect was not due to reduced expression of uPA in the CD82-transfected cells, as the levels of uPA measured in the conditioned medium by both Western blotting (Fig. 1A, upper inset) and specific enzyme-linked immunosorbent assay were equivalent for both cell lines (1.7 ± 0.4 nM versus 1.3 ± 0.3 nM for HB2/CD82 and HB2/zeo, respectively). The expression of PAI-1 was also unchanged (Fig. 1A, lower inset). The expression of these components was also unchanged as determined by quantitative RT-PCR (data not shown). To confirm that endogenous uPA was not limiting in these experiments, endogenously bound uPA was removed from both cell lines by washing at low pH, and the cells were subsequently saturated with exogenously added pro-uPA (data not shown). These observations suggested that HB2/CD82 cells bound less uPA or that binding of uPA to HB2/CD82 cells was reduced by mechanisms other than trans-
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uPAR is latent on the surface of HB2/CD82 cells and, in this case, regains uPA-binding activity after disruption of the cells. CD82 Alters the Accessibility of uPAR—uPAR is a glycosylphosphatidylinositol-anchored protein and as such is susceptible to hydrolysis by PI-specific phospholipase C. However, during these studies, we observed that uPAR in HB2/CD82 cells appeared to be unusually resistant to PI-specific phospholipase C treatment. This was further investigated quantitatively using 125I-labeled uPA, and it was found that, although uPAR in HB2/zeo cells was readily removed by PI-specific phospholipase C, in the presence of CD82, uPAR became almost completely refractory to this treatment (Fig. 4). Similar observations were also made with 125I-labeled mAb R3 (data not shown). These data are consistent with the enzyme being unable to access uPAR due to steric hindrance, possibly because of the association of uPAR with other membrane proteins, e.g. CD82.

The possibility that the effects of CD82 were related to changes in the membrane environment of uPAR was also investigated. We have shown recently that CD82 causes increased surface expression and redistribution of the gangliosides GM1 and GD1a in HB2 cells and other cell types (37). To determine whether these gangliosides affect uPAR, purified GM1 and GD1a were incorporated into the plasma membrane of HB2/zeo cells, and plasminogen activation was determined (Fig. 5). GM1 was found to reduce plasminogen activation in HB2/zeo cells to ~70% of control values, still far above that observed in HB2/CD82 cells, whereas GD1a was without effect. We next tested whether the altered ganglioside composition of HB2/CD82 cells changed the partitioning of uPAR between lipid raft and non-raft plasma membrane compartments (51). Using sucrose density gradient centrifugation, we observed no difference in the partitioning of uPAR between these two compartments in HB2/CD82 cells compared with HB2/zeo cells (data not shown). Therefore, it is unlikely that gangliosides GM1 and GD1a mediate the effect of CD82 on the binding of uPA to uPAR.

CD82 Does Not Interact Directly with uPAR—Tetraspanins are relatively promiscuous in their interactions on the cell surface. To determine whether interactions between CD82 and uPAR could be responsible for the large reduction in uPA binding observed, co-immunoprecipitation experiments were performed with HB2/CD82 cells. However, no evidence for molecular associations was found in cells extracted with either BriJ 97 (Fig. 6) or a range of other detergents (listed under “Experimental Procedures”).

CD82 Leads to a Redistribution of uPAR on the Cell Surface—To further address the potential association of CD82 and uPAR, the localization of these proteins on the cell surface was investigated by immunofluorescence staining. In HB2/CD82 cells, uPAR and CD82 displayed quite distinct localizations (Fig. 7). CD82 was found primarily in fine punctate clusters at the cell periphery and in microvillus-like protrusions (Fig. 7D), as reported for other tetraspanins (32, 33). By contrast, uPAR

**Fig. 2. Reduced binding of uPA, but not mAb R3, to HB2/CD82 cells.** Binding of both uPA (A) and anti-uPAR mAb R3 (B) was determined by radioligand binding analysis using 125I-labeled ligands and competitive displacement by unlabeled ligands. Cells were stripped of endogenously bound uPA by treatment at low pH prior to the addition of the radioligands. Data are shown for HB2/CD82 cells (●) and HB2/zeo cells (○). Binding constants were determined by nonlinear regression analysis of the primary data, which are shown here as Scatchard plots. The constants obtained for uPA binding were as follows: HB2/CD82 cells, $K_d = 1.4$ nM and $B_{\text{max}} = 0.055$ pmol; and HB2/zeo cells, $K_d = 1.5$ nM and $B_{\text{max}} = 0.011$ pmol. The constants for mAb R3 binding were as follows: HB2/CD82 cells, $K_d = 2.3$ µg/ml and $B_{\text{max}} = 4.1$ ng; and HB2/zeo cells, $K_d = 2.3$ µg/ml and $B_{\text{max}} = 3.3$ ng. In control experiments, 125I-labeled uPA binding was inhibited by >90% by anti-uPAR mAb R3. The inset in B shows a Western blot demonstrating equivalent amounts of uPAR in HB2/CD82 and HB2/zeo Triton X-100 cell lysates using mAb R3.
was associated primarily with large focal adhesions (Fig. 7B) and was completely excluded from the peripheral structures containing CD82. The distinct localization of the two proteins was confirmed by double immunofluorescence staining (Fig. 7E).

When the cell-surface localization of uPAR was compared in the presence and absence of CD82, it was found to be markedly different. In the absence of CD82, uPAR was concentrated primarily in small clusters at the cell periphery (Fig. 7A), in contrast to the large focal adhesions in the presence of CD82, as noted above. These observations demonstrate that the presence of CD82 leads to a redistribution of uPAR on the cell surface and that this redistribution correlates with the loss of its uPA-binding capability.

**CD82 Leads to the Stable Association of uPAR with α5β1 Integrin**—These observations point to the effect of CD82 on uPAR ligand-binding function and cell-surface distribution being indirect. The observed relocation of uPAR to focal adhesions, structures rich in integrin cell adhesion molecules, together with the known associations of both uPAR and tetraspanins with integrins, led us to investigate whether the latter were involved in mediating the effects of CD82. We initially focused on integrins that have been shown to associate with both CD82 and uPAR. We have previously observed that α5β1 integrin associates with CD82 (33, 52), and it has also been reported to associate with uPAR (22, 53). However, in co-immunoprecipitation experiments, no interaction was observed between uPAR and the integrin α5β1 chain in either cell type (Fig. 8, upper panel).

We also investigated the potential role of α5β1, as this integrin has also been shown to associate both with uPAR (21, 23) and, in some cell types, with CD82 (54). The immunoprecipitation experiments showed a robust association between α5β1 and uPAR, and furthermore, this was specific for the cells expressing CD82 (Fig. 8, upper panel). This observation was confirmed using cells that had been surface-biotinylated. Immunoprecipitation of these lysates with anti-uPAR antibody, followed by re-immunoprecipitation with anti-α5 antibody and detection with streptavidin, demonstrated biotinylated protein corresponding to both integrin α5- and β1-chains in the HB2/CD82 cells (Fig. 8, center panel). A range of other integrins tested, in addition to α5β1, failed to co-immunoprecipitate with uPAR in either cell type. Therefore, preferential association of uPAR with α5β1 is specifically induced in the presence of CD82, correlating with redistribution of uPAR and loss of uPA binding.

**CD82 Redistributes Both uPAR and α5β1 Integrin**—The association between uPAR and α5β1 in HB2/CD82 cells demonstrated in the co-immunoprecipitation experiments was confirmed by double immunofluorescence staining. Fig. 9 demon-
with HRP-conjugated streptavidin. The integrin was identified by comparison with Western blots for these integrin subunits.

The integrin bands were identified by comparison with Western blots for these integrin subunits in total cell lysates. The lower panel shows control Western blots for $\alpha_5$ and $\beta_1$ integrins in total cell lysates. The first lane in the upper panel shows immunoprecipitation in the absence of cell lysate as a control for cross-reaction of the various detecting antibodies with the immunoprecipitating antibody.

![Co-immunoprecipitation of uPAR with $\alpha_5\beta_1$ integrin.](image)

**FIG. 8.** Co-immunoprecipitation of uPAR with $\alpha_5\beta_1$ integrin. HB2/zeo and HB2/CD82 cells extracted in 1% CHAPS were immunoprecipitated with anti-uPAR polyclonal antibody and subsequently Western-blotted. The upper panel shows blots for uPAR (mAb R4, 10 $\mu$g/ml), $\alpha_5$ integrin (anti-$\alpha_5$ integrin polyclonal antibody, 1:5000 dilution), and $\alpha_5\beta_1$ integrin (anti-$\alpha_5\beta_1$ integrin polyclonal antibody, 1:1000 dilution). In similar experiments, no association of uPAR was found with integrin $\alpha_2$, $\alpha_5$, or $\beta_2$-chains. The center panel shows lysates from surface-biotinylated cells similarly immunoprecipitated with anti-uPAR antibody, re-immunoprecipitated with anti-$\alpha_5$ integrin antibody (as described under “Experimental Procedures”), and Western-blotted with HRP-conjugated streptavidin. The $\alpha_5$ and $\beta_1$ integrin bands were identified by comparison with Western blots for these integrin subunits in total cell lysates. The lower panel shows control Western blots for $\alpha_5$ and $\beta_1$ integrins in total cell lysates. The first lane in the upper and center panels shows immunoprecipitation in the absence of cell lysate as a control for cross-reaction of the various detecting antibodies with the immunoprecipitating antibody.

![Immunofluorescence staining of uPAR and $\alpha_5\beta_1$ integrin.](image)

**FIG. 9.** Immunoﬂuorescence staining of uPAR and $\alpha_5\beta_1$ integrin. The upper panels show staining for $\alpha_5\beta_1$ integrin, which was observed to redistribute from a diffuse staining pattern in the HB2/zeo cells to a highly focal pattern in the HB2/CD82 cells. The center panels show staining for uPAR, with the previously observed redistribution again apparent. The lower panels (merged images) demonstrate extensive co-localization of uPAR (green) and $\alpha_5\beta_1$ (red) in the HB2/CD82 cells.

strates that there was extensive co-localization of the two proteins in the presence of CD82. In the absence of CD82, $\alpha_5\beta_1$ was diffusely distributed on the cell surface, in contrast to the strong focal staining observed in its presence. These observations suggest that the presence of CD82 leads to a redistribution of both uPAR and $\alpha_5\beta_1$, thereby promoting their association.

These experiments suggest that stable associations form between uPAR and $\alpha_5\beta_1$ in the presence of CD82. M25, a phage display-derived, uPAR-binding peptide, has been shown to block uPAR/integrin interactions in other cell types (19, 24). We examined whether this peptide could restore uPAR ligand-binding function by competitively inhibiting the putative uPAR/$\alpha_5\beta_1$ interaction. However, this reagent was found to be ineffective in increasing plasminogen activation in HB2/CD82 cells (data not shown). This is consistent with the high stability of the interactions leading to the profound change in uPAR function.

**DISCUSSION**

The generation of pericellular proteolytic activity is a critical process in the dynamic regulation of cellular behavior (1), and plasmin is a central mediator of this process (2, 4). We have demonstrated here that expression of the tetraspanin CD82 leads to a profound reduction of the functional activity of the pericellular plasminogen activation system and that it does so by a novel mechanism. Although molecularly intact uPAR is available at the cell surface, the presence of CD82 prevents it from binding its high affinity ligand uPA, which is a prerequisite for efficient pericellular plasmin generation. Cell adhesion systems are known to cooperate with pericellular proteases at the molecular level, e.g. to localize proteolytic activity to discrete domains of the cell surface (5, 6, 55), but previously, there had been no evidence that components of cell adhesion systems are involved in directly regulating proteolytic activity. Therefore, the effect of CD82 on uPAR function observed here represents a novel mechanism for the regulation of protease function and, as both CD82 and the uPA/uPAR system are involved in cell motility and migration, may allow for the dynamic regulation of pericellular proteolysis in these processes.

We have rigorously excluded other potential causes for the effect of CD82 on uPAR ligand-binding function. Accordingly, the reduction in plasminogen activation is not due to altered expression of uPA, uPAR, or PAI-1; reduced trafficking of uPAR to the cell surface; proteolytic degradation or shedding of uPAR; or reduced catalytic activity of bound uPA. How then does uPAR lose its ability to bind uPA? In the presence of CD82, although refractory to the action of PL-specific phospholipase C, cell-surface uPAR retains the capacity to bind various monoclonal antibodies. These include mAb R3, whose epitope is coincident with the ligand-binding site, ruling out the possibility that simple steric blocking effects are involved. Therefore, this effect is intimately linked to the characteristics of the uPA/uPAR interaction and possibly involves conformational effects on uPAR.

The mechanism by which uPAR binds uPA with high affinity is not fully understood, but there is evidence to suggest that conformational changes in uPAR are involved in regulating ligand binding (56). The N-terminal domain of the three homologous domains of uPAR is an essential determinant of, but not sufficient for, high affinity binding (57, 58). The C-terminal juxtamembrane domain is also implicated, suggesting that these noncontiguous domains cooperate in forming a composite ligand-binding site (59, 60). The observation that mAb R3 can interact with uPAR on the cell surface, whereas uPA cannot, is consistent with the hypothesis that a conformational change in uPAR underlies the effect observed here. We have previously shown that the binding of mAb R3, which recognizes the N-terminal domain of uPAR and competitively inhibits uPA binding, is unaffected by changes in uPAR that lead to the loss of uPA binding (58). Direct evidence that conformational changes in uPAR can abolish uPA binding comes from the observation that certain anti-uPAR mAbs inhibit uPA binding noncompetitively and can dissociate preformed uPAuPAR complexes (61). Therefore, it is plausible that CD82 causes uPAR to engage in molecular interactions that lead to a similar inhibitory conformational change.
The presence of CD82 leads to the relocation of uPAR to focal adhesions. It is unlikely that this in itself is responsible for the altered ligand-binding behavior of uPAR, as uPA has previously been detected in focal adhesions (62), and in some cases, uPA has been shown to redistribute uPAR to these structures (63). Nevertheless, the redistribution of uPAR from the peripheral clusters to large focal adhesions concomitant with the loss of uPA binding is consistent with the notion that the former are dynamic structures and sites of pericellular proteolysis and the latter are more stable adhesion structures. Molecular clustering or self-association of uPAR in focal adhesions may play a role in the loss of ligand binding. Consistent with this, soluble uPAR has been shown to form dimers and larger oligomers that bind uPA with reduced affinity (64), although uPA-dependent dimerization of soluble uPAR has been observed (65). Dimerization has also been demonstrated on the surface of uPAR-transfected 293 cells, as a relatively small fraction of the protein can be chemically cross-linked (51). This dimerization was uPA-independent, and although dimeric uPAR could bind uPA, minor effects on uPA binding could not be excluded. Therefore, it appears that uPAR can self-associate in multiple ways and that this may lead to effects on uPA binding, possibly by the conformational mechanisms discussed above. Although we found no evidence for uPAR dimers in HB2 cells in chemical cross-linking experiments similar to those shown in Fig. 3, this does not exclude the presence of other self-associated forms.

The effect of CD82 on uPAR may be exerted through integrins (well established tetraspanin partners), as specifically in the presence of CD82, we found strong associations between uPAR and $\alpha_\beta_1$, but not other integrins. The association observed in co-immunoprecipitation experiments was shown by double immunofluorescence staining to be a consequence of the redistribution on the cell surface of both uPAR and $\alpha_\beta_1$. There have been various reports of interactions between uPAR and integrins, and in some cases, these interactions have been proposed to modulate integrin function (23, 24, 66). Our observations point to a fundamentally different consequence of uPAR/integrin interactions, as the CD82-mediated effect is on uPAR function, with an abolition of uPA binding and a consequent down-regulation of pericellular proteolytic activity. Previous studies have suggested that uPA positively influences uPAR/integrin interactions (20, 21, 23), which is in contrast to our observations here with $\alpha_\beta_1$. For the leukocyte integrin $\alpha_M\beta_2$, the molecular basis for this effect has been established, as both uPAR and uPA bind to the $\alpha_c$-chain, uPAR close to the ligand-binding site (24) and uPA with the “inserted” or I-domain. However, $\alpha_\beta_1$ and other uPAR-associated integrins lack an I-domain, and the mechanism by which uPA might influence uPAR interactions with these integrins is not known. Therefore, the function of CD82 is possibly to promote stable interactions between uPAR and $\alpha_\beta_1$, leading to the loss of uPA binding either directly or indirectly by the self-association or clustering of uPAR. The unique mechanism identified here for the regulation of protease function, together with previous observations on uPAR/integrin interactions, suggests bidirectional communication between the systems involved in cell adhesion and pericellular proteolysis. As the observed inhibition of uPAR function is not brought about by any irreversible changes, this communication has the potential to be dynamic.

Our data strongly suggest that the effect of CD82 on the interaction of uPAR with $\alpha_\beta_1$ is indirect because uPAR and CD82 do not co-localize on the cell surface, and we could find no evidence for an association between them in immunoprecipitation experiments. Furthermore, our results indicate that CD82-mediated alterations in ganglioside expression at the plasma membrane are not responsible for altering uPAR behavior. How CD82 exerts its effect on the distribution of uPAR and its binding to uPA in the absence of a physical association is not clear at present. Tetraspanins also engage in diverse interactions with both non-integrin cell-surface and cytoplasmic proteins (67).

For example, a potential link between CD82 and uPAR is protein kinase C, an established partner for CD82 (68). In this regard, it has been reported recently that protein kinase C$\alpha$ specifically affects the function of $\alpha_\beta_1$ integrin (69). We have previously shown that activation of protein kinase C leads to a reduction in the affinity of the uPA/uPAR interaction in the A549 carcinoma cell line concomitant with an increase in expression (48), and similar observations have been made in other cell types (70, 71). These modest changes in affinity, which vary from 4- to 40-fold, do not lead to an absolute loss of uPA binding as observed here, but nevertheless point to a similar lack of correlation between the presence of uPAR at the cell surface and uPA binding.

$\alpha_\beta_1$ has been proposed to be involved in the uPA-mediated signaling events observed in the presence of high levels of uPAR. uPA-dependent adhesion and migration of uPAR-over-expressing Chinese hamster ovary cells are promoted in the presence of $\alpha_\beta_1$ (23), and in some tumor cell types, high levels of uPAR expression promote interactions with $\alpha_\beta_1$ and lead to increased activity of the ERK signaling pathway (66). These effects do not occur in HB2 cells, as the levels of phosphorylated ERK were found to be reduced in the presence of CD82,2 the conditions under which we observed strong uPAR/$\alpha_\beta_1$ interactions and the abolition of uPA binding. It is unlikely that uPAR/$\alpha_\beta_1$-dependent signaling events are responsible for the effects of CD82 observed here. If such signaling events were to occur subsequent to the binding of uPA, as previously observed, they could not occur here, as uPA binding is lost in the presence of CD82. Alternatively, if uPAR/$\alpha_\beta_1$ interactions in the absence of uPA binding can lead to signaling, this signaling would be a consequence of the uPAR/$\alpha_\beta_1$ interaction and the loss of uPA binding and, for that reason, secondary to it. Therefore, in neither of these cases would $\alpha_\beta_1$-mediated signaling be expected to contribute to the observed cryptic behavior of uPAR.

It is apparent that many of the observations reported here on the effect of CD82 on uPA/uPAR run counter to established knowledge of this proteolytic system. However, we have also observed that overexpression of CD82 in ITT-1080 cells, which express uPA, uPAR, and $\alpha_\beta_1$, led neither to a redistribution of uPAR nor to any effect on uPA binding and plasminogen activation. Therefore, other factors must contribute to the effect of CD82 observed here in HB2 cells, possibly the precise integrin or tetraspanin profile of the cells or the known differences between integrin/tetraspanin interactions observed in other cell types (26).

CD82/KAI1 is a known suppressor of tumor invasion and metastasis (38, 43), although the mechanisms underlying this are as yet unknown. By contrast, the uPA/uPAR proteolytic system is known to promote the invasive phenotype, acting to degrade the extracellular matrix and to facilitate invasive cell migration (3, 72). Inhibition of the proteolytic activity of uPA and antagonism of the uPA/uPAR interaction have both been shown to reduce tumor progression in animal models (8, 73). Our observations here were made in a normal mammary epithelial cell line (45), which is noninvasive and therefore cannot be used to test whether the effect of CD82 on uPAR directly influences invasive cell migration. Nevertheless, if CD82 can cause uPAR to become cryptic in vivo, it can be speculated that

2 R. Bass, E. Odintsova, F. Berditchevski, and V. Ellis, unpublished data.
the suppressive role of CD82/KAI1 in cancer involves inhibition of the proteolytic activity of the uPA/uPAR system.

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Regulation of Urokinase Receptor Proteolytic Function by the Tetraspanin CD82
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