Regulation of α5β1 integrin conformation and function by urokinase receptor binding

Ying Wei,1 Ralf-Peter Czekay,2 Liliane Robillard,1 Matthias C. Kugler,1 Feng Zhang,1 Kevin K. Kim,1 Jian-ping Xiong,3 Martin J. Humphries,4 and Harold A. Chapman1

1Department of Medicine and Pulmonary and Critical Care Division, University of California, San Francisco, San Francisco, CA 94143
2Department of Cell Biology, The Scripps Research Institute, La Jolla, CA 92037
3Structural Biology Program, Massachusetts General Hospital, Charlestown, MA 02129
4Welcome Trust Centre for Cell/Matrix Research, School of Biological Sciences, University of Manchester, Manchester M13 9PT, England, UK

The urokinase-type plasminogen activator receptors (uPARs), up-regulated during tumor progression, associate with β1 integrins, localizing urokinase to sites of cell attachment. Binding of uPAR to the β-propeller of α3β1 empowers vitronectin adhesion by this integrin. How uPAR modifies other β1 integrins remains unknown. Using recombinant proteins, we found uPAR directly binds α5β1 and rather than blocking, renders fibronectin (Fn) binding by α5β1 Arg-Gly-Asp (RGD) resistant. This resulted from RGD-independent binding of α5β1–uPAR to Fn type III repeats 12–15 in addition to type III repeats 9–11 bound by α5β1. Suppression of endogenous uPA by small interfering RNA in tumor cells promoted weaker, RGD-sensitive Fn adhesion and altered overall α5β1 conformation. A β1 peptide (res 224NLDSPEGGF232) that models near the known α-chain uPAR-binding region, or a β1-chain Ser227Ala point mutation, abrogated effects of uPAR on α5β1. Direct binding and regulation of α5β1 by uPAR implies a modified “bent” integrin conformation can function in an alternative activation state with this and possibly other cis-acting membrane ligands.

Introduction

The urokinase-type plasminogen activator receptor (uPAR) plays important roles in cell adhesion and migration and chemotaxis, as well as tumor metastasis, by virtue of its interactions with the urokinase-type plasminogen activator (uPA), vitronectin (Vn), and integrins (Degryse et al., 2001; Ahmed et al., 2003; Sturge et al., 2003). Because of the capacity of uPAR to bind both integrins and uPA, this receptor contributes to matrix remodeling and cell migration in part by focusing plasmin activation to sites of cell attachment (Chapman and Wei, 2001). It has also become clear that uPAR–integrin complexes can transduce intracellular signals. Several groups have reported that the binding of uPA to uPAR stimulates intracellular signaling (Aguirre-Ghiso et al., 2003; Tarui et al., 2003; Sitrin et al., 2004), and much of this signaling is consistent with an integrin-mediated pathway. In addition, uPAR expression by itself, independently of uPA, has been reported to mediate extracellular signal–regulated kinase (ERK) activation. Recently, Czekay et al. (2003) showed that plasminogen activator inhibitor-1 (PAI-1) can detach cells by binding to the uPA present in uPA–uPAR–integrin complexes on the cell surface. Although the mechanism remains uncertain, these investigators found that PAI-1 not only detached cells from Vn, but also from fibronectin (Fn) and type-1 collagen. This influence of PAI-1 on integrin function, via uPAR, may be related to the consistent findings that high PAI-1 is an independent risk factor for tumor metastasis (Andreasen et al., 1997). Together, this body of evidence indicates uPAR–integrin interactions likely have physiological and pathobiological importance.

Previous reports have indicated that uPAR can physically interact with multiple integrins including β1 and β2 integrins (Xue et al., 1997; Pluskota et al., 2003). We previously identified uPAR as an integrin ligand because a binding site for uPAR on the integrin αMβ2 mapped to the ligand-binding region of its β-propeller (Simon et al., 2000). Among β1 integrins, uPAR directly associates with α3β1 via a surface loop within the β-propeller (W4 BC loop), but outside the laminin-5 (Ln-5) binding region. uPAR is also able to associate with α5β1 (Aguirre-Ghiso et al., 1999; Wei et al., 2001) and to

Correspondence to Harold A. Chapman: halchap@itsa.ucsf.edu; or Ying Wei: yingwei@itsa.ucsf.edu

Abbreviations used in this paper: ERK, extracellular signal–regulated kinase; Fn, fibronectin; LIBS, ligand-induced binding site; Ln-5, laminin-5; PAI-1, plasminogen activator inhibitor-1; RGD, Arg-Gly-Asp; siRNA, small interfering RNA; suPAR, soluble uPAR; Tet, tetracycline; uPA, urokinase-type plasminogen activator; uPAR, uPA receptor; Vn, vitronectin.

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regulate α5β1-mediated cell migration to Fn (Yebra et al., 1999), α5β1 signaling (Aguirre-Ghiso et al., 2003; Tarui et al., 2003), and Fn matrix assembly (Monaghan et al., 2004).

Integrin α5β1 is among many members of the integrin family that recognize an Arg-Gly-Asp (RGD) motif within their ligands (Takagi et al., 2003). Peptides containing this motif can efficiently block these integrin–ligand interactions (Aramaout et al., 2002). α5β1 integrin and Fn form a prototypic integrin/ligand pair (Takagi et al., 2003), functionally important because it mediates Fn adhesion and Fn matrix assembly, which is vital to many cell functions in vivo (Cukierman et al., 2001). This integrin is also shown to play a key role in promoting tumor angiogenesis and tumor metastasis (Jin and Varner, 2004). In addition to the RGD sequence present in Fn type III module 10, a set of residues present in the Fn type III module 9 (synergy site) contribute to high affinity recognition by α5β1 (Redick et al., 2000). The COOH-terminal heparin-binding site (HepII) of Fn also plays an important role in regulating cell adhesion, migration, Fn fibrillogenesis, signal transduction, and organization of focal adhesions and cytoskeleton (Huang et al., 2001; Kim et al., 2001). The interaction of cells with the HepII domain is currently thought to operate through proteoglycans such as syndecan 4 (Kim et al., 2001) and integrin α4β1 (Mould and Humphries, 1991).

To date, little is known of the molecular mechanism by which uPAR regulates α5β1-mediated function. In this report, the effect of uPAR on α5β1-mediated adhesion, migration, and Fn matrix assembly was investigated. Surprisingly, using recombinant proteins, we found that direct binding of uPAR to α5β1 does not change overall integrin binding to Fn, but changes integrin conformation, subsequently forming an additional binding site on Fn, which is RGD independent. In the course of this work a β1 peptide sequence was discovered that blocks all uPAR/β1 function. Mapping of this peptide near the known α-chain site of uPAR–β1 interaction confirms this region of β1 integrins as an important regulatory site and suggests a molecular basis for PAI-1–mediated cell detachment. Positioning of the uPAR-binding site near the Fn-binding site of α5β1 not only promotes α5β1 interactions with Fn, but allows PAI-1 to reverse Fn binding, empowering a mechanism of cell migration on Fn in either a protease-rich or protease inhibitor–rich milieu.

Results

PAI-1 mediates cell detachment by reversing ligand binding to β1 integrins

PAI-1 can detach cells from Vn and Fn by binding to uPA present in uPA–uPAR–integrin complexes on the cell surface (Czekay et al., 2003). However, the mechanism of detachment is uncertain. To explore a possible mechanism, we took advantage of a recently described point mutant of the Ln-5 receptor, α3β1, incapable of uPAR interaction but fully capable of Ln-5 adhesion and signaling (Zhang et al., 2003). This mutation,
His245Ala, allowed us to test the hypothesis that PAI-1 acts directly on matrix-engaged α3β1 by binding to uPA–uPAR complexes bound to α3β1 and not through indirect effects mediated by another integrin. Mouse kidney epithelial cells expressing wt α3β1 (wt α3) attach to Ln-5- or Fn-coated surfaces, and this attachment is not reversed by the presence of uPA or uPA–PAI-1 complexes (Fig. 1 A). Co-expression of human uPAR in these cells (wt α3/3) resulted in marked sensitivity of matrix attachment to the presence of uPA–PAI-1, as predicted by prior studies of Czekay et al. (2003). However, epithelial cells bearing the H245A α3 mutation (mut α3), although attaching normally to Ln-5, failed to detach from Ln-5 in the presence of uPA–PAI-1 complexes whether or not there was coexpression of uPAR. In contrast, mut α3 cells expressing uPAR (mut α3/3) adhered to Fn and detached >80% in the presence of uPA–PAI-1. Similar results were obtained in at least three separate experiments. These data indicate that matrix detachment initiated by uPA–PAI-1 requires uPAR interaction with the specific integrin that is matrix engaged. If so, this finding raises the possibility that uPA–PAI-1 directly affects matrix ligand binding to uPAR–integrin complexes, resulting in cellular detachment. This possibility was tested in HT1080 fibrosarcoma cells, cells known to express uPAR and bind Fn through α5β1 (Xue et al., 1997). The binding of biotinylated, soluble III 9-11 (Fn type III repeats 9–11, containing the RGD integrin-binding site) to HT1080 cells was almost completely blocked when cells were pretreated with active uPA and PAI-1 during the 4°C binding assay (Fig. 1 B). These results indicate that uPA–PAI-1 complexes interfere with β1 integrin ligand binding in a uPAR-dependent manner.

To explore this finding further, we performed Fn-binding assays using recombinant purified soluble α5β1 and uPAR. Recombinant α5β1-Fc fusion protein was expressed in HEK 293 cells and purified using protein A–agarose beads (Coe et al., 2001). The α5β1-Fc fusion was found to bind to Fn-coated surfaces (Fig. 1 C) and the binding is Mn2+ dependent (unpublished data). Integrin α5β1 binding to Fn was not affected by the presence of either uPA–PAI-1 complexes or soluble uPAR (suPAR). However, in the presence of suPAR, increasing amounts of uPA–PAI-1 complexes progressively blocked α5β1 binding to Fn (Fig. 1 C). Addition of uPA or PAI-1 separately had no effect on Fn binding. In additional experiments, biotinylated suPAR could also be shown to bind to immobilized α5β1-Fc, as expected (unpublished data). Together, these data indicate that uPAR binds α5β1, and such binding modifies α5β1 integrin binding to Fn and enables PAI-1 to release Fn from α5β1 through uPAR-bound uPA. All of these events can happen on the extracellular domains of the integrin independent of cell signaling.

These observations imply that uPAR can interact with at least two and possibly multiple β1 integrins, suggesting the
involvement of the common β1-chain itself in uPAR binding. Moreover, the direct effect of uPA–PAI-1 on Fn binding to uPAR–α5β1 suggests the uPAR-binding site may be positioned close to the Fn-binding site. As demonstrated in Fig. 2 A, an energy-minimized model of integrin α5β1 structure was generated based on the atomic coordinates of the αvβ3 crystal structure (Xiong et al., 2001). Recently, we have found that the blade 4 BC loop of the proposed β-propeller structure of integrin α3 is important for uPAR association (Wei et al., 2001). The corresponding BC loop in the α5β1 model is highlighted in red. Inspection of the model reveals two loops on the β1-chain (224NLDSPEGGF232 in yellow, 262FHFAGDGKL270 in purple) that are very close to the blade 4 BC loop of the β-propeller (in red). We hypothesized that the two β1-chain loops may also be involved in integrin–uPAR association. The alignment of these β1-chain sequences with that of other integrin β-chains is shown in Fig. 2 B. Interestingly, the N-terminal Asn 224 in β1P1 has been implicated in bonding to the Asp (D) of RGD in the αvβ3 crystal, placing these loops very close to the putative RGD-binding pocket of α5β1. This could potentially explain the direct effect of uPA–PAI-1 on Fn binding to uPAR–α5β1.

uPAR associates with α5β1 and changes Fn adhesion properties

To explore whether the β1 loops discussed above are involved in uPAR and α5β1 interactions, a series of Fn adhesion experiments were performed. Kidney epithelial cells used in Fig. 1 and their uPAR cotransfectants were again used. The adhesion of these cells to Fn is mediated to a large extent by the α5β1 integrin because the adhesion can be completely blocked by an α5β1-blocking antibody 5H10-27 (unpublished data). The observation that uPAR associates with α5β1 integrin (Wei et al., 2001) and the complex mediates RGD-independent cell adhesion to Fn (unpublished data) prompted us to test whether adhesion of uPAR-expressing cells to Fn can be inhibited by RGD peptides. Surprisingly, overexpression of uPAR strikingly increased cell adhesion to Fn in the presence of RGD-containing peptides (Fig. 3 A). Indeed up to 1 mM RGD had no discernible effect on adhesion of uPAR-expressing cells to Fn. By contrast, the adhesion of non-uPAR–expressing cells to Fn was totally abolished by 500 μM RGD peptides. The RGD-resistant adhesion of uPAR-expressing cells was observed at all Fn concentrations supporting adhesion (0.5–5 μg/ml; Fig. S3, available at http://www.jcb.org/cgi/content/full/jcb.200404112/DC1). The β1 peptides had no effect on Fn adhesion of non-uPAR–expressing cells at any concentration tested, but 400 μM of either of the two β1 peptides completely inhibited cell adhesion to Fn in uPAR-expressing cells. The scrambled peptides had no effect (Fig. 3 A). The dose inhibition effect of one of the two β1 peptides (β1P1) on adhesion to Fn is shown in Fig. 3 B.
Because the β1 peptide sequence contains a natural Ser227Ala polymorphism, inviting a mutational analysis, we explored the functional properties of this peptide in more detail. We tested the effect of the β1P1 peptide on the biochemical association of α5β1 and uPAR. HT1080 cell lysates were immunoprecipitated with α5 antibodies in the presence of β1P1 or its scrambled control, and the presence of uPAR was determined by immunoblotting. As indicated in Fig. 3 C, uPAR could be reliably coprecipitated with α5β1 and this association was completely blocked by the functionally active peptide, suggesting residues 224–232 in the α5β1P1 peptide on the biochemical association. To test this point further, a α5β1-Fc fusion protein containing the entire extracellular domains of α5β1 was expressed in which Ser227 was mutated to Ala (α5β1SA-Fc). As shown in Fig. 3 D, the α5β1SA-Fc fusion was found to bind Fn identically to wt. We verified that recombinant biotinylated suPAR bound to α5β1-Fc/Fn, but not appreciably to Fn alone. However, the Ser227 to Ala mutant integrin (α5β1SA-Fc) totally lost interaction with uPAR, indicating that Ser227 on the integrin β1-chain, and by inference the 224–232 β1 peptide loop, is critical for uPAR association.

uPAR binding to α5β1 induces an additional binding site for Fn

Next, we examined whether the apparent switch in the mechanism of α5β1 adhesion to Fn initiated by the presence of uPAR (Fig. 3 A) was evident with purified proteins. Fn was immobilized and α5β1-Fc binding to Fn was measured by protein A–HRP as before. Fn–α5β1 binding in the presence or absence of suPAR is similar (Fig. 4 A), and both can be blocked by an α5-blocking antibody (unpublished data). The binding of α5β1 to Fn in the absence of suPAR was abolished by RGD peptides, as expected, whereas binding in the presence of suPAR was vice versa. Interestingly, the binding of mutant integrin (α5β1SA) to Fn remained RGD sensitive in the presence of suPAR (Fig. 4 A). These findings completely recapitulate the pattern seen with live cells and indicate that the presence of uPAR markedly changes the matrix-binding properties of α5β1. This raises two possibilities: uPAR binding switches the integrin-binding site from the central RGD binding domain (III 10) to a different site on Fn. Or, uPAR binding to α5β1 creates an additional binding site for Fn. To determine which possibility is more likely, we made biotinylated RGD peptides and performed binding assays with purified proteins. Binding of biotin-RGD (closed bars) and α5β1-Fc (open bars) to Fn were measured separately and graphed together (Fig. 4 B). In the absence of uPAR, biotin-RGD (0.5 mM) competed with the RGD-binding site on immobilized Fn, blocking α5β1 binding to the plate. However, in the presence of suPAR, biotin-RGD robustly bound to uPAR–α5β1–Fn unless 10-fold excess unlabeled RGD was added (Fig 4 B), strongly suggesting the existence of an additional RGD-independent binding site for Fn. Nevertheless, the RGD-binding site on α5β1 must still be intact in the presence of suPAR, otherwise biotin-RGD would not be able to bind α5β1.

To determine where the additional binding site interacts on Fn, we examined the effect of uPAR on cell adhesion to Fn fragments in the presence or absence of RGD peptides. Our initial experiments show that adhesion to NH2-terminal 70-kD Fn was RGD-resistant, but unaffected by the presence of uPAR and not blocked by α5 integrin blocking antibodies (unpublished data). Thus, we focused on the Fn type III repeats. Cells with or without uPAR were allowed to attach to immobilized
Fn fragments containing type III repeats 9–11 (III 9-11) or type III repeats 12–15 (III 12-15). Cells without or with uPAR expression adhered strongly to the RGD-containing III 9-11 and the adhesion of both cells was blocked by RGD peptides, confirming that the uPAR-expressing cells maintained an RGD-binding site on Fn for $\beta_1$. As expected, $\beta_1P1$ had no effect on this adhesion. However, only uPAR-expressing cells attached to III 12-15, and this adhesion was resistant to RGD peptides and now sensitive to the $\beta_1P1$ peptide ($\beta_1P1P1$), indicating uPAR induces at least one $\beta_1$-binding site within the COOH-terminal heparin-binding domain of Fn. This additional site conveys RGD resistance to binding of cells to the whole Fn molecule (Fig. 4 C). As expected, the Ser227Ala point mutant peptide ($\beta_1P1SA$), like scrambled $\beta_1P1$, had no effect.

uPAR alters $\alpha_5\beta_1$ conformation and changes $\alpha_5\beta_1$ integrin-dependent adhesion and detachment in tumor cells

The above data indicate that binding of uPAR to $\alpha_5\beta_1$ alters integrin conformation and changes its matrix ligand binding properties. To probe this idea further and determine whether uPAR-mediated changes in $\alpha_5\beta_1$ function are observable in nontransfected cells, several tumor cell lines expressing various amounts of uPAR were evaluated. HT1080 (fibrosarcoma), MDA-MB-231 (breast carcinoma), and Skov-3 (ovarian carcinoma) cells were transfected with a small interfering RNA (siRNA) previously shown to suppress uPAR mRNA (Vial et al., 2003) or control and suppression of surface uPAR expression verified 48 h later by FACS analysis (Fig. 5 A). Suppression of surface uPAR had no effect on total $\beta_1$ integrin expression (JB1A). However, suppression of uPAR had clear effects on integrin conformation as judged by altered binding of the conformation-sensitive mAbs, HUTS-21, and 9EG7, in all of the cell lines examined. Suppression of surface uPAR was accompanied by increased binding of both HUTS-21 and 9EG7 antibodies (Fig. 5 A), confirming that endogenous uPAR expression modifies integrin $\beta_1$-chain conformation.

Are the changes in $\alpha_5\beta_1$ conformation in tumor cells induced by knockdown of uPAR accompanied by changes in mechanism of adhesion? Previous studies show that these cell lines

![Figure 5. Suppression of uPAR expression induces LIBS epitope and changes $\alpha_5\beta_1$-mediated Fn binding in tumor cells.][Figure 5]
express substantial levels of uPAR and α5β1 integrin (Xue et al., 1997; van der Pluijm et al., 2001). Indeed, we confirmed that Fn adhesion was mainly mediated by α5β1 as the α5-blocking antibody (P1D6) totally blocked the Fn adhesion (unpublished data).

To investigate whether increased RGD resistance of Fn adhesion by uPAR expression also exists in nontransfected cells, we tested the relation between uPAR level and Fn adhesion in HT1080, MDA-MB-231, and Skov-3 cells. Because the data from these cell lines are all very similar, we only show results with HT1080 cells (Fig. 5 B). Expression of high levels of endogenous uPAR leads to the expected phenotype when cells are plated on Fn: RGD resistance and β1 peptide susceptibility. siRNA suppression of uPAR in HT1080 cells, like prior studies with transfected epithelial cells, switched the Fn phenotype to RGD sensitive and β1 peptide resistant (Fig. 5 B). Furthermore, the noninvasive breast cancer cells MCF-7 and T47D, expressing little uPAR, showed only RGD-sensitive Fn adhesion (Fig. S2, available at http://www.jcb.org/cgi/content/full/jcb.200404112/DC1). These data support the conclusion that the mechanism of Fn adhesion among tumor cells depends upon uPAR expression level.

**uPAR expression promotes α5β1-mediated cell adhesion, migration, and Fn matrix assembly**

When cells are plated on an Fn-coated surface (5 μg/ml) for 1 h, the difference in Fn adhesion between cells with and without surface uPAR is marginal (Fig. 6 A). However, when cells are seeded onto lower amounts of Fn (0.2–5 μg/ml) for shorter periods of time (20 min), adhesion of uPAR-expressing HT1080 cells to Fn was obviously more robust (Fig. 6 A). We repeated similar experiments using MDA-MB-231 (Fig. S1, available at http://www.jcb.org/cgi/content/full/jcb.200404112/DC1) and Skov-3 cells with or without uPAR suppression and found similar results. These findings indicate that uPAR expression not only changes the conformation of α5β1 and how it engages Fn, but together these changes might promote cell adhesion and migration.

To explore activation of α5β1 by uPAR further, kidney epithelial cells expressing uPAR under a tetracycline (Tet)-inducible promoter were established. In the absence of Tet (−Tet) the cells had little uPAR surface expression, whereas Tet-induced cells (+Tet) showed robust surface uPAR (Fig. 6 B). Initial experiments verified that induction of uPAR produced the same switch in Fn adhesion phenotype observed for stable clones and tumor cells examined above. Although the mechanism of α5β1-mediated Fn attachment changed in the presence of uPAR, immunofluorescence of fixed cells plated on Fn did not reveal discernible changes in the distribution of α5β1 by uPAR induction alone (unpublished data). Nonetheless, +Tet cells produced more cell-associated and deoxycholate-insoluble Fn (Fig. 6 B), indicating that this pool of Fn is much more organized into matrix fibrils in the presence of uPAR.

To test whether uPAR association with α5β1 affects cell migration, we performed wound assays on tumor cells (HT1080 and MDA-MB-231) and the Tet-responsive cells with or without uPAR induction using RGD and β1 peptides (β1P1). The cells were seeded onto Fn-coated wells and allowed to form a monolayer before wounding. Preliminary experiments indicated that as little as 20 μM of either the RGD-containing or the β1P1 peptide suppressed migration. As shown in Fig. 7, both RGD and β1P1 alone blocked wound closure of HT1080 cells, and the combination of both peptides had a statistically significant greater effect. Similar results were obtained from MDA-MB-231 cells. More importantly, Tet-inducible cells with uPAR expression (+Tet) migrated faster in this assay and the migration was blocked by both RGD and β1 peptides, whereas cells without uPAR (−Tet) had little migration (unpublished data). Together, these data confirm our findings that uPAR, through its interaction with β1 integrin(s), promotes cell motility and that this function can be specifically blocked by the β1-chain peptides identified here.
Available evidence indicates that integrin activation involves a global change in integrin conformation, at least part of which is a change in the orientation of the α and β head domains to better accommodate ligand binding. Several lines of evidence also support a model in which the very “bent” integrin conformation found in the αVβ3 crystal structure extends to point the head domains away from the cell under activating conditions (Takagi et al., 2002). However, the full range of conformational changes a ligand-bound integrin may assume is uncertain (Mould and Humphries, 2004). It is especially difficult to envision a fully extended conformation of activated integrins acting in cis to engage the much smaller GPI-anchored uPAR at the integrin upper surface. Rather, our findings suggest uPAR–α5β1 complexes exhibit an activation state involving a modified bent integrin with distinct functional properties. Similarities and differences between models of “extended” integrin activation and a model consistent with results reported here are summarized in Fig. 8. The model raises the more general possibility that some version of an angled integrin configuration, rather than being inactive, actually functions to promote integrin binding to cis-acting membrane ligands, such as uPAR, which coordinate integrin function with specific cellular needs.

The notion that uPAR activates and stabilizes α5β1 has been previously proposed by Ossowski and colleagues based on their studies of human epidermoid carcinoma cell lines. Enhanced adhesion to Fn of tumorigenic (T-HeP3) over dormant (D-HeP3) epidermoid cells was directly related to uPAR levels (Aguirre-Ghiso et al., 1999). The high levels of uPAR in human epidermoid carcinoma cells (HeP-3) resulted in increased α5β1-dependent signaling to ERK as well as increased formation of Fn fibrils (Aguirre-Ghiso et al., 2001). Both α5β1-dependent ERK signaling and Fn matrix assembly were decreased in the presence of a uPAR-binding peptide, P25, which blocks uPAR–integrin association, indicating that in these cells ligation of uPAR with P25 inhibited α5β1 function. Similar to these findings, we observed that induction of uPAR expression with Tet in an epithelial cell line increased Fn fibril formation (Fig. 6 B). Conversely, suppression of uPAR expression by RNA interference in many tumor cell lines decreased adhesion (Fig. 6 A, Fig. S1). Additional observations reported here help provide a physical rationale for these and prior functional studies. The current data indicate that uPAR directly binds and changes the conformation and matrix-binding properties of α5β1. The capacity of short β1-chain peptides to block uPAR–α5β1 functions without affecting α5β1-mediated Fn binding itself point to important conformational differences between free and uPAR-bound α5β1. The finding that β1P1 does not simply convert the function of uPAR–α5β1 to that of free α5β1 by dissociating uPAR also suggests that the conformational change incurred by complex formation with uPAR is distinct and perhaps not readily reversible. This is consistent with the hypothesis that bent and extended conformations of α5β1 can function as distinct activation states (Fig. 8).

This hypothesis is supported by our results, which reveal that suppressing uPAR expression induces ligand-induced binding site (LIBS) epitopes in HT1080, MDA-MB-231, and Skov-3 cells (Fig. 5 A). A recent report also documented in-

Discussion

Here, we report evidence that uPAR directly associates with the head domains of integrin α5β1, modifies α5β1 conformation, and creates an additional binding site for Fn, likely within the second Fn heparin-binding domain. Complexes of uPAR and α5β1 are functionally relevant because uPAR binding promotes α5β1-dependent Fn matrix assembly and migration. Importantly, our observations are not based strictly on transfected cells because these features of uPAR–α5β1 interaction could be demonstrated in several tumor cell lines expressing endogenous uPAR. Collectively, these functional changes imply α5β1 activation by uPAR binding, as suggested previously by Aguirre-Ghiso et al. (1999). Our studies solidify α5β1 as a binding partner of uPAR and further define the uPAR-binding region on the integrin. The positioning of the uPAR-binding site near the integrin RGD-binding site also reveals a potential mechanism whereby uPAR–α5β1 complex formation empowers PAI-1-dependent cell detachment from Fn: while the integrin RGD-binding site remains intact in uPAR–α5β1 (Fig. 4), concurrent binding of urokinase and PAI-1 to uPAR now displaces intact Fn or the Fn cell–binding domain (containing RGD) from the integrin (Fig. 1), presumably by steric hindrance.

The consequences of uPAR–α5β1 complex formation contrast with that of other pathways of integrin activation.

Figure 7. RGD and β1P1 peptides inhibit HT1080 cell wound healing. Serum-starved HT1080 monolayers were wounded and incubated with different peptides (RAD, RGD, scβ1P1, and β1P1; 20 μM) in DME/0.1% BSA. The wounded areas were imaged at 0 and 24 h using a bright-field imaging system (Spot camera). The migration of HT1080 cells was quantified using SimplePCI software. The percent wound closure of each peptide-treated cell is shown on the right. n = 3. Marked pair (*) shows significant difference by t test (P < 0.006).
creased β1-chain LIBS epitopes on human skin fibroblasts exposed to a peptide that disrupts uPAR–integrin interactions (Wei et al., 1996; Monaghan et al., 2004). The LIBS antibodies (HUTS-21, 9EG7) used here map to sites near the hinge region of the integrin but far away from the uPAR interaction site. Although these LIBS antibodies are thought to recognize the “active” conformational state of the β1 subunit that can be induced by ligand binding (e.g., Fn, RGD peptides, by activating antibody TS2/16, or Mn²⁺), their binding is more sensitive to conformational changes in the hinge, knee, or leg domains than changes near the ligand-binding pocket (Bazzoni et al., 1995; Luque et al., 1996; Mould and Humphries, 2004). We postulate that in uPAR-expressing cells the lower LIBS antibody binding reflects integrin angulation resulting from uPAR–α5β1 complex formation. This occurs in spite of “activation” of the integrin as judged by enhanced adhesion and Fn matrix assembly, further supporting the idea that activated integrins could exist far away from the uPAR interaction site. Although these LIBS antibodies are thought to recognize the “active” conformational state of the β1 subunit that can be induced by ligand binding (e.g., Fn, RGD peptides, by activating antibody TS2/16, or Mn²⁺), their binding is more sensitive to conformational changes in the hinge, knee, or leg domains than changes near the ligand-binding pocket (Bazzoni et al., 1995; Luque et al., 1996; Mould and Humphries, 2004). We postulate that in uPAR-expressing cells the lower LIBS antibody binding reflects integrin angulation resulting from uPAR–α5β1 complex formation. This occurs in spite of “activation” of the integrin as judged by enhanced adhesion and Fn matrix assembly, further supporting the idea that activated integrins could exist far away from the uPAR interaction site.

Previous studies have shown that initial cell attachment and spreading on Fn is mediated by the interaction of the RGD-containing Fn cell-binding domain (type III repeats 9–10) with α5β1 (Mould et al., 2000; Redick et al., 2000; Takagi et al., 2003), but that further progression of the cytoskeletal response requires additional signals (Hocking et al., 1998; Tarui et al., 2003). Additional binding sites for cells on Fn provide the necessary signals. For example, interaction of cells with the Fn NH₂-terminal region can trigger integrin-mediated intracellular signals that are distinct from those generated in response to ligation with the RGD sequence (Forsyth et al., 2002). However, in our assays, adhesion of epithelial cells to this 70-kD fragment was not influenced by uPAR expression and not inhibited by α5β1 blocking antibodies (unpublished data). Signals for cytoskeletal reorganization may also be provided by the interaction of Fn fragments containing the heparin-binding domain (Hep II) (type III repeats 12–14) with cell surface proteoglycans (Huang et al., 2001). In fibroblasts, this response requires two cooperative signals provided by interactions of the RGD sequence with α5β1 integrin and the heparin-binding domain with syndecan-4 (Kim et al., 2001). Our data show that both cells with uPAR or without uPAR adhere to Fn III 9–11 in an RGD-dependent manner, whereas only cells bearing uPAR adhere to Fn III 12–15. The latter cannot be blocked by RGD peptides, but can be blocked by β1 peptides that disrupt uPAR–β1 integrin interaction (Fig. 4 C). In most uPAR-expressing cells there are likely to be pools of α5β1 both free and bound to uPAR, suggesting that the incorporation of the heparin binding domain into the uPAR–α5β1 complex results in distinct signals that lead to enhanced integrin function, as our data show (Fig. 6). We cannot be sure whether uPAR–α5β1 complexes possess both Fn-binding sites or binding to both sites in Fn requires free and uPAR-complexed integrin. Future studies may distinguish between these possibilities.

We have previously reported that uPAR expression in kidney embryonic 293 cells both promotes Vn adhesion through association of uPAR with α3β1 and impairs Fn adhesion mediated by α5β1 (Wei et al., 1996, 2001). Impairment of Fn adhesion in 293 cells appears anomalous with respect to all other cells expressing uPAR examined here and by others (Aguirre-Ghiso et al., 1999). Consistent with this difference, expression of uPAR in 293 cells did not decrease binding of HUTS-21 and 9EG7 antibodies (unpublished data), implying that for some reason uPAR interacts, but not in the same manner, with α5β1 in 293 cells as that seen in other transformed cells. The molecular basis for the anomalous behavior of 293 cells remains to be defined.

The discovery of the capacity of α5β1 to undergo a phenotypic switch (i.e., RGD vs. β1P1 dependent; Fig. 8), in Fn attachment may be relevant to attempts to regulate inflammation or tumor progression through integrin inhibition in vivo. uPAR is up-regulated in both inflammatory cells and many tumor cells with a metastatic phenotype. Indeed, uPAR expression is an independent risk factor for tumor metastasis in several clinical studies. RGD-based compounds or peptides have been shown to inhibit integrin function in vivo, but our data imply that one limitation in their use is the complete resistance of β1 integrins complexed with uPAR from RGD-dependent ligand binding. Vn adhesion mediated by uPAR–α3β1 complexes is also RGD-resistant (Wei et al., 1994). Instead, uPAR-bound β1 integrins are sensitive to β1 peptides that map to the region of uPAR–integrin interaction. As these β1 peptides block cell adhesion (Fig. 5 B) and migration of various tumor cells (Fig. 7), it is possible that these reagents, perhaps coupled with RGD-based compounds, have therapeutic potential for suppression of tumor progression.

Figure 8. Model for regulation of α5β1 integrin conformation and function by uPAR binding. Model proposes three basic forms of α5β1 exist on the cell surface: (1) a bent inactive form in the absence of integrin ligand; (2) an extended, active form induced by Fn binding in the absence of uPAR; and (3) a modified bent active form stabilized by uPAR binding, and potentially other cis-acting membrane proteins. In the case of uPAR, the modified bent form engages Fn differently as judged by Fn fragment III 9–11 and III 12–15 binding, altered β1P1 peptide sensitivity, the reversal of Fn binding by the presence of uPA–PAI-1 complexes, and enhanced Fn matrix assembly.
Materials and methods

Reagents and antibodies
Active human uPA and uPAR mAbs were purchased from American Diagnostics. Active PAI-1 was a gift from Dr. Dan Lawrence (American Red Cross, Rockville, MD). suPAR was supplied by Dr. Gary Deng (Berlex Biosciences). Fn, Vn, and peptides GRGDSPK and GRADSPK were purchased from Sigma-Aldrich. F(ab')2 fragments of anti-human Fc antibody were from Jackson Immunoresearch Laboratories, Inc. Mouse anti-human FcγRIII antibody was purchased from Jackson ImmunoResearch Laboratories, Inc. uPA mAb for blotting (R2) was a gift from G. Hoyer-Hansen (Finsen Lab, Copenhagen, Denmark).

Cell culture
Mouse kidney epithelial cells expressing wt α3 (a gift from Dr. Jordan A. Kreidberg, Harvard Medical School, Boston, MA) or mut α3 and their uPAR counterparts were cultured in DME as described previously (Wang et al., 1999; Zhang et al., 2003). HEK293, human fibrosarcoma HT1080, breast cancer MCF-7, T47D, and MDAMB-231 Skov-3 cell lines were obtained from American Type Culture Collection (Rockville, MD) and grown in DME. The Tet-inducible uPAR cells (Tet-uPAR) were maintained in DME supplemented with zeocin, hygromycin, and blasticidin (5 μg/ml) was added to induce uPAR expression. Modified Skov-3 cell line was a gift from Dr. Ernest Lengyel (University of Chicago, Chicago, IL).

Cell detachment assay
Microtiter plates were coated with 5 μg/ml Fn or Ln-5 supernatant (1:100) for 18 h at 4°C. The cell detachment assay was performed as described previously (Czepek et al., 2003). In brief, cells attached were acid washed, resuspended in incubation buffer (RPMI, 20 mM Hepes, and 0.02% BSA), and then incubated in the absence or presence of active uPA followed by PAI-1. After wash, the remaining adherent cells were fixed and stained. The amount of extracted stain was quantified by absorbance at 590 nm.

Cell adhesion assay
The cell adhesion assay was performed as described previously (Wei et al., 2001). In brief, cells were seeded onto Fn (5 μg/ml) or Fn fragment (10 μg/ml)–coated plates and incubated in DME/0.1% BSA with or without RGD or β1 peptides for 1 h at 37°C. After washing, attached cells were fixed and stained with Giemsa. The data were quantified by measuring absorbance at 550 nm.

Biotinylation of suPAR, III 9-11, and RGD peptides
Human suPAR, Fn fragment III 9-11, or RGD peptides were biotinylated at 0.25 mg/ml using FluorReporter Biotin-XX Protein Labeling Kit (Molecular Probes, Inc.) following the manufacturer’s instructions.

Purification of α5β1-Fc and α5β1SA-Fc integrins
Integrin α5-Fc and β1-Fc or Ser227 to Ala mutant β1SA-Fc constructs (α5/β1A2.2HFc and β1/β1V1.66Fc or β1SA/β1V1.66Fc; Cee et al., 2001) were transfected into 293 cells. Culture supernatant was harvested after 48–72 h and passed through a Protein A–agarose column. Soluble integrin was eluted using 0.1 M glycine, pH 3.0, and neutralized in 1 M Tris-HCl, pH 8.0. Protein-containing fractions were dialyzed, concentrated, and identified by SDS-PAGE.

Purified protein binding assay
Nunc high binding microtiter plates were coated with 20 μg/ml Fn and blocked with 1% BSA. 20 μg/ml purified recombinant α5β1-Fc with or without RGD peptide or uPA–PAI-1 mixture were added together with suPAR. After washing, bound α5β1-Fc was detected by protein A–HRP and quantified by measuring absorbance at 490 nm. Data were expressed as specific binding (i.e., total binding minus the binding to wells coated with BSA alone).

To test biotinylated RGD peptides binding to Fn–α5β1–uPAR, Nunc microtiter plates were coated with Fn and incubated with α5β1-Fc with or without suPAR as above. 0.5 mM biotin-RGD was then added to each well for another hour. After washing, avidin peroxidase was added and the bound biotin-RGD was quantified as described above. To test specificity of binding, 10-fold molar excess nonbiotinylated RGD peptides were added. Biotinylated suPAR binding assay was performed similarly to confirm Fn–α5β1–uPAR complex formation.

uPAR RNA interference
HT1080 cells were transfected with siRNAs that specifically target the uPAR gene or nonsilencing control and used within 48–72 h. siRNA duplexes were synthesized as by in vitro transcription. The sequence of the DNA targeting uPAR is 5′-GGTGAAAGAAGGCGTCACA-3′. A nonsilencing siRNA 5′-AACCTGGGGAAGAAGTG-3′ was used as a control (Vial et al., 2003). Synthetic siRNA oligonucleotides were purified with Microspin G-25 columns from Amersham Biosciences.

FACS analysis
Cells with or without siRNA uPAR transfection were incubated with primary antibody to active form β1 integrin (HUTS-21, 9EG7) or to total β1 integrin (J1A1) and secondary FITC-conjugated anti-mouse IgG or anti-rat IgG (for 9EG7; Sigma-Aldrich) and analyzed on a flow cytometer (FACS-Calibur; BD Biosciences). uPAR was detected by a mAb to uPAR.

Biotin-III 9-11 binding assay
All the procedures were done at 4°C. HT1080 cells were acid washed and incubated without or with uPA followed by PAI-1. The cells were then incubated with 50 nM biotinylated Fn fragment III 9-11 in RPMI/0.02% BSA for 1 h. After washing, the cells were lysed and the total protein separated by SDS-PAGE. The bound biotin-III 9-11 was detected by avidin-HRP. The bands were quantified and analyzed by densitometry.

Detection of ECM-associated Fn
Fn fibrils were detected as described previously (Aguirre-Ghizo et al., 2001). In brief, Tet-uPAR cells without or with Tet induction were lysed with 3% Triton X-100 buffer. Triton-insoluble pellets were treated with DNase and then extracted with 2% deoxycholate buffer. The insoluble and soluble fractions were mixed with sample buffer and analyzed by SDS-PAGE and Western blotting using anti–human Fn antibodies and an antibody to β-actin.

Wound healing
HT1080 cells were grown to confluence on Fn-coated surface. Medium was replaced with DME/0.1% BSA 6 h before wounding. The wound was made using a 1-ml pipet tip. The detached cells were removed by washing and the wounded cells were incubated without or with RGD or β1 peptides (20 μM) for 24 h. Cells were imaged at 0 and 24 h by phase-contrast videomicroscopy.

Online supplemental material
The supplemental material (Figs. S1–S3) is available at http://www.jcb.org/cgi/content/full/jcb.200404112/DC1. Fig. S1 shows that suppression of uPAR expression by RNA interference induces LBS epitope and changes α5β1-mediated Fn binding in MDAMB-231 cells. Fig. S2 shows the effect of uPAR expression level on Fn adhesion of different breast cancer cell lines. Fig. S3 shows the RGD-resistant adhesion of uPAR-expressing cells on different concentrations of Fn.
References


