GARP regulates the bioavailability and activation of TGFβ

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ABSTRACT Glycoprotein-A repetitions predominant protein (GARP) associates with latent transforming growth factor-β (proTGFβ) on the surface of T regulatory cells and platelets; however, whether GARP functions in latent TGFβ activation and the structural basis of coassociation remain unknown. We find that Cys-192 and Cys-331 of GARP disulfide link to the TGFβ1 prodomain and that GARP with C192A and C331A mutations can also noncovalently associate with proTGFβ1. Noncovalent association is sufficiently strong for GARP to outcompete latent TGFβ-binding protein for binding to proTGFβ1. Association between GARP and proTGFβ1 prevents the secretion of TGFβ1. Integrin αvβ6 and to a lesser extent αvβ8 are able to activate TGFβ from the GARP-proTGFβ1 complex. Activation requires the RGD motif of latent TGFβ, disulfide linkage between GARP and latent TGFβ, and membrane association of GARP. Our results show that TGFβ is a latent TGFβ-binding protein that functions in regulating the bioavailability and activation of TGFβ.

INTRODUCTION
Transforming growth factor-β (TGFβ) is a pleiotropic cytokine with potent immunoregulatory properties, which manifests in TGFβ1 knockout mice as multifocal inflammatory disorders and death within 4 wk of birth (Shull et al., 1992; Kulkarni et al., 1993). TGFβ1 is produced by every leukocyte lineage and controls the differentiation, proliferation, and other functions of immune cells (Li et al., 2006; Yoshimura et al., 2010). For example, TGFβ is involved in the generation and function of T regulatory cells (Treg) and T helper 17 cells (Th17; Nakamura et al., 2004; Veldhoen et al., 2006). TGFβ also induces the expression of αvβ6 integrin in intraepithelial lymphocytes (Kilshaw and Murant, 1991; Cepek et al., 1993). In addition, TGFβ regulates immunoglobulin A isotype expression in B cells (Coffman et al., 1989).

TGFβ1, 2, and 3 are synthesized as precursor polypeptides (pro+TGFβ), which dimerize and are proteolytically cleaved by furin prior to secretion to yield pro-TGFβ. (Here we use pro-TGFβ for furin-cleaved latent TGFβ, pro+TGFβ for uncleaved latent TGFβ, and pro+TGFβ to refer to the cDNA or a mixture of pro-TGFβ and pro+TGFβ protein products.) Pro-TGFβ contains a ~250-residue prodomain known as latency-associated peptide (LAP) and a ~110-residue mature TGFβ growth factor domain. The prodomain remains noncovalently associated with TGFβ after secretion, thereby conferring latency (Gentry et al., 1987; Wakefield et al., 1988; Khalil, 1999). Latent TGFβ does not have biological activity, and the release of TGFβ from LAP therefore is a critical regulatory step for TGFβ function and signaling. The LAPs of TGFβ1, 2, and 3 are denoted LAP1, 2, and 3, respectively.

LAP1 and LAP3 contain an RGD motif, which is recognized by some αv integrins (Rifkin, 2005). αvβ6 and αvβ8 activate TGFβ through binding to the RGD motif; mice lacking both αvβ6 and αvβ8 integrins recapitulate all major phenotypes of TGFβ1 and β3 double-deficient mice (Aluwihare et al., 2009), demonstrating the critical roles of αvβ6 and αvβ8 in TGFβ1 and β3 activation. Furthermore, knock-in mice with the RGD motif of TGFβ1 mutated to RGE phenotypically resemble mice with complete deficiency of TGFβ1 (Yang et al., 2007). Therefore, although multiple mechanisms that include thrombospondin and metalloproteases have been implicated in activation of TGFβ1 and TGFβ3, recognition by αv integrins of the RGD motif has a central role in activation in vivo.

The latent TGFβ-binding proteins (LTBPs) are important in the biosynthesis, storage, and activation of TGFβ (Rifkin, 2005). Association with and disulfide linkage to LTBPs targets proTGFβ to the
extracellular matrix (ECM; Miyazono et al., 1991). There are four different LTBP, and at least three of them bind to proTGFβ. LTBP are large proteins related to fibrillins, which have a large number of calcium-binding epidermal growth factor–like domains and a smaller number of TGFβ-binding (TB) domains. One of these TB domains specifically associates with proTGFβ, and two cysteines in the TB domain disulfide link to Cys-4 in each of the prodomains. One of these TB domains is stored until activation (Rifkin, 2005). The role of LTBP in TGFβ assembly and activation is well established; association of proTGFβ with LTBP and incorporation of LTBP into the ECM are required for activation (Rifkin, 2005). Association between GARP and LAP has been shown by immunoprecipitation (IP) followed by Western blotting, and binding of GARP-Fc to proTGFβ was shown by fluorescence (Tran et al., 2009); however, whether they are covalently linked by disulfide bonds is unknown. Small interfering RNA to GARP has been shown to decrease surface expression of LAP and to moderately decrease Treg-mediated suppression in vitro (Tran et al., 2009). However, whether a proTGFβ complex with GARP can provide a cell-surface reservoir of latent TGFβ for αvβ6 integrin–dependent activation and how GARP coexpression affects secretion and bioavailability of TGFβ remain unknown. Here we address gaps in understanding of the role of GARP in TGFβ function. Our findings support the idea that GARP is a new latent TGFβ-binding protein that regulates the bioavailability of TGFβ and provides a cell surface platform for αvβ6 integrin–dependent TGFβ activation.

RESULTS
GARP associates with proTGFβ
To study their interaction, we transiently expressed GARP and proTGFβ in 293T cells. Consistent with previous findings (Stockis et al., 2009; Tran et al., 2009), the expression level of LAP on the cell surface was greatly elevated in the presence of GARP (Figure 1A, bottom). In addition, GARP and LAP coimmunoprecipitated in
cotransfected cells (Figure 1B). An ~250-kDa species representing the GARP-proTGFB1 complex was detected in the cotransfected cells on a 7.5% nonreduced SDS–PAGE gel (Figure 1C), indicating that GARP forms a disulfide linkage with proTGFB1. ProTGFB1 and LAP secretion was detected in the supernatant of cells transfected with proTGFB1 alone but not in the supernatant of cells cotransfected with GARP and proTGFB1 (Figure 1D), suggesting that GARP blocks direct secretion of pro+TGFB1 and proTGFB1.

Cys-192 and Cys-331 of GARP disulfide linkage to Cys-4 of proTGFB1

Our findings suggested that GARP disulfide links with proTGFB1. Cys-4 in each proTGFB1 disulfide linkage to LTBP, and the proTGFB1 C4S mutant is unable to bind to LTBP (Saharinen et al., 1996). In contrast, we found that GARP was able to noncovalently associate with the proTGFB1 C4S mutant. The C4S mutant increased LAP secretion in cotransfected cells (Figure 1B). An ~250-kDa species representing the GARP-proTGFB1 complex was detected in the cotransfected cells on a 7.5% nonreduced SDS–PAGE gel (Figure 1C), indicating that GARP forms a disulfide linkage with proTGFB1. ProTGFB1 and LAP secretion was detected in the supernatant of cells transfected with proTGFB1 alone but not in the supernatant of cells cotransfected with GARP and proTGFB1 (Figure 1D), suggesting that GARP blocks direct secretion of pro+TGFB1 and proTGFB1.

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GARP competes for TGFβ binding

Both GARP and LTBP disulfide link to Cys-4 of proTGFβ1. To investigate whether GARP and LTBP compete for proTGFβ1 binding, we performed IP experiments using cell lysates or supernatants from cells transfected with proTGFβ1, GARP, and/or short or long alternatively spliced isoforms of LTBP1 (LTBP1S and LTBP1L, respectively; Figure 5A). LTBP1S and LTBP1L complexed with proTGFβ1 were found in both the cell supernatant (Figure 5A, row 1) and lysate (Figure 5A, row 2), in contrast to the GARP complex, which was present only in lysates (Figure 5A, row 3) and not in supernatant, as shown earlier (Figure 1D).

Of interest, GARP outcompeted both LTBP1S and LTBP1L for proTGFβ1. When cells were cotransfected with GARP and either LTBP1S or LTBP1L, proTGFβ1 was found only in association with GARP (Figure 5A, row 3) and not with LTBP (Figure 5A, rows 1 and 2). Moreover, LAP was found on the cell surface only when GARP was present but not when LTBP1S was present; LTBP1S did not diminish GARP-dependent LAP surface expression (Figure 5B).

Furthermore, the GARP C192A/C331A double mutant also outcompeted LTBP1 for proTGFβ1 binding (unpublished data), suggesting that the noncovalent association between GARP and proTGFβ1 is sufficient for GARP to outcompete LTBP.

TGFβ can be activated from the GARP–proTGFβ complex by integrins

We next studied whether the GARP–proTGFβ complex could serve as a source of activated TGFβ. Several αv integrins were shown to activate TGFβ in different settings (Munger et al., 1999; Mu et al., 2002; Ludbrook et al., 2003; Wipff et al., 2007). Stable transfectants of 293 cells expressing αv and each of the five β subunits known to associate with αv (Supplemental Figure S2A) were further transfected with GARP and proTGFβ1 and cocultured with the transformed mink lung TGFβ1–reporter cell line (TMLC; Abe et al., 1994). αvβ6 strongly activated TGFβ from GARP- and proTGFβ1-cotransfected cells (Figure 6A). αvβ3 also activated TGFβ, but to a lesser extent. In contrast, αvβ1, αvβ2, and αvβ5 transfectants showed no more TGFβ activation than did mock transfectants (Figure 6A). Similar results were obtained when αv integrins and the GARP–proTGFβ1 complex were expressed on different cells, demonstrating transactivation (Figure 6B).

GARP and LTBP1 supported αvβ3-mediated TGFβ activation at comparable levels (Figures 6, C and D). αvβ5 also activated TGFβ from cells transfected only with proTGFβ1 (Figures 6, A–C). This may be due to endogenous LTBP expression in 293 cells, since this activation was greatly reduced in proTGFβ1 C4S-transfected cells or in the presence of LTBP1 ECR3E fragment, as previously reported (Annes et al., 2004; Figure 6, F and G).

An αvβ6-dependent release of TGFβ into culture supernatants was also seen. Activation of latent TGFβ associated with endogenous LTBP is consistent with the presence of TGFβ activity in supernatants of cells transfected with proTGFβ1 (Figure 6D). TGFβ activity in supernatants was also seen with cells cotransfected with GARP and proTGFβ1 (Figure 6D). In all cases, release of TGFβ into supernatants was αvβ6 dependent (Figure 6D).

The ECR3E fragment contains the LAP-binding TB domain of LTBP, and the ECR3E fragment has been shown to compete with LTBP1 for proTGFβ1, thereby inhibiting TGFβ activation by αvβ6 (Annes et al., 2004; Figure 6F). However, the ECR3E fragment had little effect on αvβ6-mediated activation of the GARP–proTGFβ1 complex (Figure 6F). Similar results were obtained with αvβ3-mediated TGFβ activation (Figure 6G). This finding is consistent with our IP experiments showing that GARP interacted with proTGFβ1 in the presence of the ECR3E fragment (Supplemental Figure S2B). These results further confirmed our conclusion that GARP outcompetes LTBP for proTGFβ1 binding.

The αvβ6–mediated TGFβ activation from the GARP–pro-TGFβ complex requires the disulfide linkage between GARP and proTGFβ, the RGD motif in LAP, and membrane association of GARP

The C4S mutation in proTGFβ1 greatly reduced TGFβ activation from the GARP–pro-TGFβ complex (Figure 6E). The GARP C192A or GARP C331A single mutants, which supported disulfide linkage
FIGURE 4: Cys-192 and Cys-331 of GARP disulfide link to proTGFβ1. (A) 293T cells were transfected with the indicated plasmids, and the surface FLAG-GARP and LAP1 expressions were measured by FACS. (B) The mutated GARP’s associate with proTGFβ1. The cell lysates were immunoprecipitated with anti-FLAG antibody, subjected to reducing SDS–10% PAGE, and blotted with the indicated antibodies. (C) Cys-192 and Cys-331 of GARP disulfide link to proTGFβ1. 293T cells were transfected with the indicated plasmids. The clarified lysates were immunoprecipitated with anti-LAP1 antibody, subjected to reducing SDS–7.5% PAGE, and blotted with a different anti-LAP1 antibody. (D) C192A/C331A double mutation in GARP reduces the stability of the GARP–proTGFβ complex. 293T cells were transfected with the indicated plasmids. The supernatants were immunoprecipitated with an anti-LAP1 antibody, subjected to reducing SDS–10% PAGE, and blotted with a different anti-LAP1 antibody.

Electron microscopy of complexes with GARP, proTGFβ, and integrin αβ6

The noncovalently associated proTGFβ C45 mutant complex with sGARP was stable to gel filtration and was subjected to negative-stain electron microscopy (EM) with particle alignment and class averaging (Figure 8A). The covalent proTGFβ complex with sGARP was similarly subjected to EM (Figure 8B). ProTGFβ is ring-like, as previously described (Shi et al., 2011; Figure 8C). The noncovalent and covalent proTGFβ complexes with GARP are very similar and show an elongated and more or less linear or slightly curved density for GARP that is associated with the periphery of the proTGFβ ring (Figure 8, A and B).

To better appreciate the mode of association shown by EM, we made a homology model of GARP (Figure 8, G and H). LRR are horseshoe-shaped proteins, as shown for GARP using cryo-EM (Probst-Kepper et al., 2009). Each LRR makes one complete turn around the horseshoe. The cysteines forming the intermolecular disulfides, Cys-192 and Cys-331, locate to one side of the horseshoe, between the concave and convex faces, and near the middle of the horseshoe (Figure 8, G and H). Placing Cys-192 and Cys-331 on the flat side of the GARP model in close opposition to Cys-4 on the outer edge of the proTGFβ ring (Figure 8, F and G) recreates the orientation seen in EM (Figure 8, A and B). Furthermore, the two Cys-4 residues in proTGFβ1 are 40 Å apart (Shi et al., 2011), an appropriate spacing for binding to Cys-192 and Cys-331, which are 35 Å apart from each other in the GARP homology model (Figure 8, G and H).

Complexes between the ectodomain of integrin αβ6 and sGARP–proTGFβ were isolated by gel filtration and subjected to EM (Figure 8D). The two RGD motifs to which integrins bind reside on the shoulders of the proTGFβ1 ring (Figure 8F). Representative class averages showed either one (Figure 8C, 1 and 2) or two (Figure 8C, 3) αβ6 ectodomains bound per proTGFβ1; αβ6b bound with its lower legs extended and its headpiece open, that is, in the high-affinity conformation. The proTGFβ1-binding site in αβ6a was at the interface between large and small densities, corresponding to the αβ6-propeller domain and β6I domain, respectively. This is the crystallographically determined binding site for RGD ligands in αβ6 and αβ6b (Xiong et al., 2002; Xiao et al., 2004). The negative-stain EM class averages clearly demonstrated the relationship between the GARP- and αβ6-binding sites on the proTGFβ1 ring in ternary complexes (Figure 8D). sGARP and αβ6b bound to opposite sides of the ring of proTGFβ1. The spatial relationships on the periphery of the proTGFβ1 ring for integrin binding and GARP binding are as predicted from the positions of the RGD motifs and Cys-4 in the proTGFβ1 crystal structure (Figure 8F). The ring-like structure of proTGFβ was similar in the absence and presence of αβ6 (Figure 8, A, B, and D). Furthermore, SDS–PAGE of the same gel filtration fraction as subjected to EM of the proTGFβ1 complex with sGARP–proTGFβ
showed the presence of TGFβ in the complex (Figure 8E, lane 1), suggesting that binding of αβ6 was not sufficient to induce release of TGFβ.

**DISCUSSION**

The pivotal role of TGFβ in immune regulation emphasizes the need for a better understanding of the mechanisms for TGFβ storage and activation. In the present study, we characterized the structural basis and functional significance of the interaction between GARP and TGFβ and defined a critical role for GARP in regulating bioavailability of TGFβ.

Previous studies demonstrated coassociation of GARP and proTGFβ (Stockis et al., 2009; Tran et al., 2009), and yet the structural basis for this interaction was not clear. Here we present the first evidence that GARP disulfide links with proTGFβ and that noncovalent bonds are also sufficient for association. The disulfide interaction was mediated by Cys-192 and Cys-331 of GARP and Cys-4 of proTGFβ, suggesting that one GARP protein associates with one proTGFβ dimer. Such a complex has an estimated polypeptide molecular mass of 153,200 Da; with 11 N-linked sites at 2500 Da each, the estimated mass is 180,700 Da, close to the mass measured by multicycle light scattering of 176,000 ± 3500 Da.

Although the disulfide linkage is not required for GARP–proTGFβ association, the noncovalent interaction between GARP and proTGFβ alone could not stably present proTGFβ on the cell surface because in the absence of the disulfide linkage, GARP was unable to prevent proTGFβ1 from leaking into the supernatant.

We defined by EM and confirmed with a homology model the structure of the complex between GARP and proTGFβ. Cys-192 and Cy-s331 are located in the 7th and 12th LRR of GARP, respectively. The distance between the two Cα atoms of Cys-192 and Cys-331 in our GARP homology model is ∼35 Å, whereas the distance between the two Cα atoms of the two C4S mutant residues of our proTGFβ1 homodimer crystal structure is ∼40 Å (Shi et al., 2011; Protein Data Bank [PDB] code, 3RJR). Thus disulfide linkage of Cys-192 and Cys-331 in GARP with the two C4-S residues in proTGFβ1 is structurally feasible. Negative-stain class averages showed overall similarity between noncovalent sGARP–proTGFβ1 C4S and covalent sGARP–proTGFβ1 complexes, although the appearance of the sGARP moiety was variable. The class averages and the positions of disulfide-linked cysteines in GARP are consistent with the disulfide linkage of the ring of proTGFβ1 to the side of GARP, with the planes of the proTGFβ1 ring and the GARP horseshoe more normal to one another than coplanar. Thus, with the proTGFβ1 ring lying flat on the EM carbon substrate, the large horseshoe of GARP may collapse at variable orientations onto the substrate. Although GARP may have some flexibility, flexibility was not evident in previous EM studies of proTGFβ1 alone (Probst-Kepper et al., 2009).

Two integrin α6β1 molecules could bind simultaneously to the proTGFβ1–GARP complex. The orientations around the proTGFβ1 ring were as predicted based on locations of RGD motifs and Cys-4 residues in the crystal structure of latent TGFβ. As previously described for latent TGFβ, α6β1 bound in the extended-open, high-affinity conformation, and the affinity for the proTGFβ1–GARP complex is unusually high for an integrin, allowing isolation by gel filtration under nonactivating conditions, that is, in buffer with Ca²⁺ and Mg²⁺ (Shi et al., 2011). Furthermore, there was no evidence for disruption of the ring-like structure of proTGFβ upon α6β1 integrin binding, and TGFβ remained present in the complex, as shown by SDS–PAGE. This suggests that binding of α6β1 is not sufficient to release TGFβ from the GARP–proTGFβ complex, as previously reported for proTGFβ (Shi et al., 2011).

Both GARP and LTBP disulfide link to the same cysteine, Cys-4, in proTGFβ1. We found that GARP strongly outcompetes LTBP1 for associating with proTGFβ1. Several lines of evidence support this conclusion. First, in cells transfected with equal cDNA amounts of GARP, proTGFβ1, and LTBP1, GARP but not LTBP1 became associated with proTGFβ1. Second, whereas GARP presents proTGFβ1 on the cell surface and LTBP localizes proTGFβ1 to the ECM, LAP was
FIGURE 6: Integrins αvβ6 and αvβ8 can activate TGFβ from the GARP–pro-TGFβ1 complex. (A) Mock or different αv integrin-expressing cells were transfected with the indicated plasmids and cocultured with TMLC to measure active TGFβ production. Data represent mean ± SEM of triplicate samples. (B) 293T cells were transfected with indicated plasmids and cocultured with TMLC to measure active TGFβ production. (C, D) GARP and LTBP1 support αvβ6-mediated TGFβ activation at comparable levels. Mock or αvβ6-expressing cells were transfected with indicated plasmids. Cells (C) or the supernatants 24 h posttransfection (D) were cocultured with TMLC to assess active TGFβ production. (E, F) Mock or αvβ6-expressing cells were transfected with indicated plasmids and were cocultured with TMLC to measure active TGFβ production. (E, H) The ECR3E fragment does not interfere with αvβ6- or αvβ8-mediated TGFβ activation from the GARP–pro-TGFβ1 complex. Mock or αvβ6- or αvβ8-expressing cells were transfected with the indicated plasmids. The transfected cells were cocultured with TMLC to measure active TGFβ production.

GARP and regulation of TGFβ | 1135

present on the cell surface in GARP-, proTGFβ1-, and LTBP1-cotransfected cells; furthermore, GARP abolished coassociation of LTBP and LAP in cell supernatants. Third, ECR3E, the LAP-binding motif in LTBP1 that was previously shown to interfere with the interaction between LTBP and proTGFβ1 and block activation by αvβ6 cell contact, which in turn induces a larger Treg pool through the infectious tolerance mechanism. TGFβ also contributes to Th17 generation (Velshnoen et al., 2006). Recently, αvβ6 expressed by DCs was implicated to regulate Th17 differentiation (Melton et al., 2010). Our results may also suggest a role of GARP in Th17 generation.
Previous studies suggested that tensile force exerted by integrin is required for activation of the proTGFβ-LTBP complex (Arnes et al., 2004; Wipff et al., 2007). The crystal structure of the proTGFβ1 homodimer shows that the TGFβ growth factor dimer is sequestered by LAP straightjacket elements (Shi et al., 2011). The α1-helix, latency lasso, and clasp of the straightjacket lock the TGFβ against the prodomain arm domain. These prodomain elements shield TGFβ from recognition by both its type I and type II receptors and also change its conformation. Tensile forces exerted across the proTGFβ ring on the straightjacket would break the noncovalent structural restraints and release mature TGFβ dimer into the extracellular milieu. The conditions required for this activation include the binding of αβ1 integrins to the RGD motif of LAP; the incorporation of proTGFβ into the ECM by LTBP; the C-terminal portion of the β6 cytoplasmic domain; an intact cytoskeleton to generate cell traction forces and/or to provide mechanical resistance; and a mechanically resistant ECM (Wipff and Hinz, 2008).

Here we show that TGFβ can also be activated from the GARP–proTGFβ1 complex by αβ6 and αβ8 integrins. The αβ6-mediated activation also requires the interaction of integrin to the RGD motif of LAP, suggesting that TGFβ is activated via similar mechanisms, whether presented by LTBP in the ECM or GARP on cell surface. Membrane anchoring of GARP is required, as a soluble form of GARP is unable to support αβ6-mediated TGFβ activation despite forming an sGARP–proTGFβ1 complex. Furthermore, the disulfide linkage between GARP and proTGFβ1 is required, as TGFβ could not be activated in the absence of disulfide linkage and presence only of noncovalent association between proTGFβ and GARP. In addition, complex formation between purified αβ6 and GARP–proTGFβ1 did not release TGFβ. These results suggest that αβ6-dependent activation of TGFβ from the GARP–proTGFβ1 complex also requires tensile force. Negative-stain EM class averages showed that in the sGARP–proTGFβ1–αβ6 ternary complex, GARP and αβ6 bind to opposite sides of the proTGFβ1 ring. This arrangement is important for exerting tensile force through this ternary complex for releasing mature TGFβ to bind its receptors.

Although most of our experiments were conducted using cells cotransfected with GARP, proTGFβ1, and αβ integrins, we have no evidence that αβ integrins can activate the GARP–proTGFβ complexes in cis on the same cell, since activation could have occurred in trans in cell culture. We only have evidence for activation in trans, from experiments in which the proTGFβ/GARP and αβ integrins were expressed on different cells.

It is known that some integrin–ligand pairs cannot interact with one another when expressed on the same cell, such as LFA-1 and ICAM-1 (Wang and Springer, 1998).

We propose three mechanisms by which GARP regulates TGFβ bioavailability at cell surfaces (Figure 9). First, GARP prevents release of free and possibly misassembled proTGFβ into the extracellular environment and thereby helps maintain its latency (Figure 9A).
Second, GARP inhibits secretion of proTGFβ in association with LTBP and hence its assembly into fibrils in the ECM (Figure 9B). Third, GARP provides a cell-surface platform for presentation of latent TGFβ to αVβ3 integrins, including αVβ6 integrins on the surface of other cells, for activation of TGFβ in the context of cell–cell adhesive interactions (Figure 9C).

**MATERIALS AND METHODS**

**Subcloning**

Transfection-ready, untagged human GARP cDNA was purchased from Origene (Rockville, MD). Human LTBP1 cDNA was provided by Vesna Todorovic (New York University, New York, NY). TGFβ1 cDNA was provided by Katri Koli (University of Helsinki, Helsinki, Finland). GARP was subcloned into a modified pLEXm vector (Aricescu et al., 2006) with a FLAG tag at the N-terminus. LTBP1S, LTBP1L, and the ECR3E domain of LTBP1 were subcloned into a modified pIRES2-EGFP vector (BD Biosciences, San Diego, CA), which contains a streptavidin-binding peptide (SBP) tag at the C-terminus. sGARP was constructed by fusing the extracellular domain of GARP to a histidine (His)–SBP tag, followed by a 3C protease site (Shi et al., 2011) at the N-terminus. GARP and TGFβ1 point mutations were generated using the QuickChange II XL Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA), following the manufacturer’s instructions. αVβ6 was cloned into a modified pEF1 vector (Invitrogen, Carlsbad, CA) with puromycin resistance. β subunits of αV integrins were cloned into pcDNA3.1 with neomycin resistance (Invitrogen).

**Antibodies and other reagents**

The following antibodies were used in the present study: anti-FLAG antibody (Sigma-Aldrich, St. Louis, MO), mouse anti-LAP1 antibody for IP and fluorescence-activated cell sorting (FACS; 37232; R&D Systems, Minneapolis, MN), biotinylated goat anti-LAP1 antibody for Western blot (BAF246; R&D Systems), anti-LTBP1 antibody (R&D Systems), anti-αv antibody (Santa Cruz Biotechnology, Santa Cruz, CA), anti-β3 antibody (Millipore, Billerica, MA), anti-β6 antibody (a kind gift of Dean Sheppard, University of California, San Francisco), anti-β6 antibody (a kind gift of Stephen Nishimura, University of California, San Francisco), phycoerythrin (PE)-labeled goat anti-mouse immunoglobulin G (lgG; BD Biosciences), and horseradish peroxidase (HRP)-conjugated sheep anti-mouse IgG and streptavidin-HRP (GE Healthcare, Piscataway, NJ).

To generate monoclonal anti-human GARP antibodies, a stable 293S cell line expressing sGARP was generated. After affinity purification of sGARP, the His-SBP tag was removed from sGARP by 3c protease digestion. sGARP was then further purified and used for immunizing mice. Several in-house anti-GARP antibodies (mouse IgG1; GARP2, GARP5 and GARP6) were confirmed to bind GARP in assays, including enzyme-linked immunosorbent assay, flow cytometry, IP, and Western blot analysis (unpublished data). The RGE (GRGESP) and RGD (GRGDSP) peptides were purchased from Bachem Americas (Torrance, CA). All other chemicals and reagents were obtained from Sigma-Aldrich, unless otherwise indicated.

**Cell culture and transfection**

HEK293 and 293T cells were maintained in DMEM supplemented with 10% fetal calf serum (FCS), 4 mM l-glutamine, 1% nonessential amino acids, and penicillin/streptomycin. All cells were cultured at 37°C in a humidified 5% CO2 atmosphere. For transient transfection, cells were transfected using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions. To make stable cell lines expressing αV integrins, HEK293 cells were cotransfected with constructs encoding αV and β subunit. At 40 h posttransfection, cells were selected for the ability to proliferate in medium containing puromycin (1 μg/ml) and G418 (400 μg/ml). Live cells were FACS sorted 1 wk later into single clones based on surface integrin expression. Integrin expression was confirmed via FACS analysis 2 wk postsorting.

**FACS**

Cells were stained and analyzed as described previously (Wang et al., 2009). In brief, cells were incubated with primary antibody in FACS buffer (phosphate-buffered saline [PBS] with 2% FCS and 0.02% NaN3) on ice for 30 min. After washing, the cells were incubated with anti-mouse PE for 30 min and analyzed by FACScan (BD Biosciences).
SDS–PAGE, and immunoblotted with the indicated antibodies. To immunoprecipitate SBP-tagged proteins and their binding partners, streptavidin-conjugated Sepharose (GE Healthcare) was used. Data shown are representative of at least two independent experiments.

**TGFβ bioassay**

The TGFβ reporter cell line TMLC was a kind gift of Daniel Rifkin (New York University). The TGFβ bioassay was performed as previously described (Abe et al., 1994; Annes et al., 2003). In brief, in each well of a 96-well white plate, 15,000 TMLC cells were cocultured with 15,000 293 cells transfected with indicated plasmids for 16–24 h. In some experiments, 10,000 293 cells stably expressing integrins and 10,000 transfected 293T cells were cocultured with 15,000 TMLC cells. For the supernatant experiments, 100 μl of supernatants from transfected cells was cocultured with 15,000 TMLC cells. The cells were then processed using the Luciferase Assay System (Promega, Madison, WI) and analyzed by Synergy 2 Multi-Mode Microplate Reader (BioTek, Winooski, VT). Data are presented as the mean ± SEM of triplicate samples.

**Negative-stain electron microscopy**

Affinity-tagged sGARP was purified from supernatant of 293S cells as described previously for proTGFβ1 (Shi et al., 2011). To purify the sGARP–proTGFβ1 complex, sGARP-stable cells were transiently transfected with proTGFβ1-encoding plasmid. To obtain the sGARP–proTGFβ1–αVβ6 ternary complex, the purified sGARP–proTGFβ1 complex was mixed with purified αVβ6 in the presence of 1 mM CaCl₂ and 1 mM MgCl₂. Peak fractions of the purified proteins or complexes from S200 chromatography were subjected to negative-stain electron microscopy. Data processing was performed as previously described (Shi et al., 2011).

**Model for GARP**

We found no LRR structure with the same number of LRRs as GARP (23 LRRs). Therefore the template was constructed from multiple portions of different LRR proteins, and these were superimposed on TLR3 (PDB code 12IW), which has 24 LRRs. For some LRRs, multiple templates were used. The segments used were the N-cap and LRR1-4 of variable lymphocyte receptor Vlra.R5.1 (PDB code 3M19) for the N-cap and LRR1-4 of GARP; the LRR2-6 of mouse toll-like receptor 3 (PDB code 3CIY) for LRR2-6 of GARP; the LRR10-14 of hagfish variable lymphocyte receptors (PDB code 2O6S) for LRR10-14 of GARP; the LRR8-11 of Lrim1 leucine-rich repeat domain (PDB code 3O53) for LRR8-11 of GARP; the LRR7-10 of GARP; and the IP

Cells were collected, washed once with PBS, and lysed in lysis buffer (1% Triton in Tris-buffered saline with proteinase inhibitor cocktail [Roche; Mannheim, Germany]) at 4°C for 30 min. The lysate was clarified by centrifugation at 12,000 × g for 10 min at 4°C, and the clarified lysate was incubated with antibodies overnight at 4°C on a rocking platform. Protein G–Sepharose (GE Healthcare) was then added and incubated at 4°C for another 1 h. The Sepharose was sedimented and washed three times with lysis buffer. Bound proteins were eluted by heating in SDS sample buffer, separated by
LRR2-8 and C-terminal of neuronal leucine-rich repeat protein Amigo-1 (PDB code 2XOT) for the LRR17-23 and C-terminal of GARP. The model was built using MODELLER (Eswar et al., 2003).

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