Cell-surface transglutaminase undergoes internalization and lysosomal degradation: an essential role for LRP1

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Summary

Tissue transglutaminase functions as a protein crosslinking enzyme and an integrin-binding adhesion co-receptor for fibronectin on the cell surface. These activities of transglutaminase and the involvement of this protein in cell-matrix adhesion, integrin-mediated signaling, cell migration and matrix organization suggest a precise and efficient control of its cell-surface expression. We report a novel mechanism of regulation of surface transglutaminase through internalization and subsequent lysosomal degradation. Constitutive endocytosis of cell-surface transglutaminase depends on plasma membrane cholesterol and the activity of dynamin-2, and involves both clathrin-coated pits and lipid rafts or caveolae. Furthermore, the key matrix ligands of transglutaminase, fibronectin and platelet-derived growth factor, promote its endocytosis from the cell surface. Our results also indicate that transglutaminase interacts in vitro and on the cell surface with the major endocytic receptor, low-density lipoprotein receptor-related protein 1, and demonstrate the requirement for this receptor in the endocytosis of transglutaminase. Finally, a deficiency of this endocytic receptor or blockade of endo-lysosomal function upregulate transglutaminase expression on the cell surface, leading to increased cell adhesion and matrix crosslinking. These findings characterize a previously unknown pathway of transglutaminase internalization and degradation that might be crucial for regulation of its adhesive and signaling functions on the cell surface and reveal a novel functional link between cell-matrix adhesion and endocytosis.

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Introduction

Tissue transglutaminase (tTG or TG2, EC 2.3.2.13) belongs to a multigene family of Ca^{2+}-dependent protein crosslinking enzymes (Lorand and Graham, 2003). tTG is a ubiquitously expressed multifunctional protein that possesses transglutaminase (Folk and Cole, 1966), GTPase (Nakaoka et al., 1994) and protein disulfide isomerase (Hasegawa et al., 2003) activities. tTG is localized in the cytoplasm and is also present on the cell surface and in the extracellular matrix (ECM), where it plays an important role in cell-matrix interactions (Fesus and Piacentini, 2002; Lorand and Graham, 2003).

Cell-surface tTG associates with the major ECM protein fibronectin (Fellin et al., 1988; Turner and Lorand, 1989) and β1 and β3 integrins (Akimov et al., 2000). The high-affinity interaction of tTG with fibronectin enhances cell-matrix adhesion and other adhesion-dependent phenomena, including cell migration, ECM assembly and remodeling and outside-in signaling (reviewed in Zemskov et al., 2006). The non-covalent association of tTG with the β1 and β3 integrin subunits leads to the formation of ternary fibronectin-tTG-integrin complexes and stabilizes the interaction of integrins with fibronectin (Akimov et al., 2000). Importantly, the transamidating- and GTPase-deficient mutants of the protein retain these properties, indicating that this recently defined adhesive function of tTG is independent of its enzymatic activities (Akimov et al., 2000; Akimov and Belkin, 2001a; Janiak et al., 2006). Moreover, the interaction of tTG with integrins promotes their clustering, causing activation of RhoA and its downstream target ROCK by means of inhibition of the Src-p190RhoGAP signaling pathway (Janiak et al., 2006). Taking into account the numerous functions of cell-surface tTG both as a crosslinking enzyme and as an adhesion co-receptor involved in signal transduction, it could be predicted that excessive levels of tTG on the surface would impair cellular functions. To prevent a disproportionate crosslinking of the ECM and to downregulate tTG-dependent cell-matrix adhesion and outside-in signaling, cells should employ an efficient mechanism(s) for its removal from their surfaces.

LRP1 is a member of the LDL receptor superfamily which consists of six structurally related and widely expressed proteins [LRP1, LRP1B, LRP2 (also known as gp330), LDL receptor, very low-density lipoprotein (VLDL) receptor and LRP8 (also known as apolipoprotein E receptor 2)] (Lillis et al., 2005). This large endocytic receptor functions in lipoprotein metabolism, degradation of proteases, activation of

lysosomal enzymes and cellular entry of bacterial toxins and viruses (Herz and Strickland, 2001). Furthermore, recent papers demonstrate the involvement of this protein in several signal-transduction cascades (see Lillis et al., 2005). Importantly, LRP1 has also been shown to function in the turnover of extracellular tTG-binding proteins, β1 and β3 integrins and fibronectin (Czekay et al., 2003; Salicioni et al., 2002; Salicioni et al., 2004).

Here, we demonstrate that downregulation of cell surface tTG is mediated by constitutive endocytosis. Cell-surface tTG interacts directly with LRP1 and promotes the association of LRP1 with β1 integrins and the ECM. The efficient internalization of tTG from the cell surface occurs by means of a dynamin-dependent process that involves clathrin- and caveolin-dependent endocytic pathways and requires LRP1. Finally, we show that accumulation of surface tTG in LRP1-deficient cells or in cells with blocked endo-lysosomal function increases extracellular transglutaminase activity and cell-matrix adhesion. Our findings underscore the crucial role of this endocytic receptor in the internalization and degradation of tTG and in the regulation of its adhesive and signaling functions on the cell surface.

Results

tTG is internalized from the cell surface by a cholesterol-dependent, dynamin-mediated mechanism

Endocytosis is a specific cellular mechanism of internalization of macromolecules and particles by means of vesicles derived from the plasma membrane. To examine whether cell-surface tTG undergoes endocytosis, antibody-uptake experiments were performed with WI-38 fibroblasts that express high surface levels of tTG (Akimov and Belkin, 2001a). As bivalent antibodies cause aggregation of cell-surface tTG and might artificially stimulate its endocytosis, we employed monovalent antigen-binding fragments (Fab) of the 4G3 monoclonal antibody (mAb) against tTG (Akimov and Belkin, 2001b). After binding the 4G3-Fab fragments to the cell surfaces at 4°C, the cells were allowed to internalize the tTG–4G3-Fab complexes at 37°C for 15 or 60 minutes (Fig. 1A). The remaining surface-bound tTG–4G3-Fab complexes were stripped off with a low-pH treatment and the internalized complexes were detected by immunofluorescence after cell permeabilization. The internalized tTG was detected initially in intracellular peripheral vesicles, whereas prolonged incubation increased the amounts of the internalized tTG that resided in numerous perinuclear vesicles. No surface binding or internalization was observed with the 4G3-Fab fragments in cells lacking surface tTG (data not shown).

To confirm that tTG undergoes internalization, the levels of cell-surface tTG were determined in NIH3T3-tTG cells treated with the cholesterol-binding agent M-β-CD, which acutely inhibits endocytosis (Rodal et al., 1999; Subtil et al., 1999). The cells treated with M-β-CD for short intervals displayed sharply increased amounts of tTG on the cell surface, although total tTG expression remained unaffected (Fig. 1B). This increase in cell-surface presentation of tTG caused by the endocytosis-arresting drug indicates that the levels of surface tTG are efficiently controlled by cholesterol-dependent internalization.

The function of the large GTPase dynamin-2 (Altschuler et al., 1998) is required for fission of vesicles from the plasma membrane during endocytic processes (Damke et al., 1994; Gaborik et al., 2001; Henley et al., 1999; Torgersen et al., 2001). Thus, we next compared the internalization of the tTG–4G3-Fab complexes in MRC-5 fibroblasts transiently expressing either wild-type dynamin-2 or a dominant-negative mutant dynamin-2 (K44A), both containing a hemagglutinin (HA) tag. Double immunostaining for the HA tag and the tTG–4G3-Fab complexes following a standard internalization procedure revealed efficient endocytosis of tTG and accumulation of tTG in the vesicular compartments of the cells transfected with wild-type dynamin-2, whereas tTG could not be detected inside the cells expressing the dominant-negative mutant (Fig. 1C). Hence, tTG is internalized from the cell surface, and this process is dependent upon membrane cholesterol and the GTPase activity of dynamin-2.

Internalized tTG is transported through early and late endocytic endosomes to lysosomes for degradation

To determine the endocytic route(s) and destination(s) of internalized tTG, we defined the endosomal compartments where tTG was localized after internalization (Fig. 2). The intracellular localization of internalized tTG–4G3-Fab complexes in WI-38 fibroblasts was compared with those of markers of endocytic compartments. Double immunostaining revealed the internalized tTG co-distributed with EEA1, a marker of early endosomes (Mu et al., 1995) after 5 minutes of internalization, whereas 30 minutes later it appeared colocalized with Rab7-positive compartments, indicative of late endosomes (Chavrier et al., 1990). Co-staining with antibody against Arf1 did not reveal translocation of the internalized tTG to the Golgi complex. However, we observed occasional colocalization of internalized tTG with peripheral Arf1-positive membrane structures, likely to be the endosome carrier vesicles (ECVs) transporting proteins from the early to the late endosomes (Donaldson and Honda, 2005). Importantly, extended (>90 minute) incubations resulted in the appearance of the internalized tTG in Lamp-1-positive lysosomes (Chen et al., 1985).

Double immunofluorescence staining demonstrated colocalization of the internalized tTG and β1 integrins in the WI-38 fibroblasts (Fig. 3A), suggesting that tTG and β1 integrins, interacting on the cell surface (Akimov et al., 2000; Janiak et al., 2006), also undergo endocytosis as a complex and/or follow the same endocytic route(s). To assess the fate of internalized tTG and β1 integrins, the following experiments were performed. First, CHO cells were chilled to 4°C to stop internalization and cell-surface proteins were biotinylated with membrane-impermeable SH-cleavable sulfo-NHS-SS-biotin. Then, the cells were incubated for varying periods of time (Fig. 3B) at 37°C to allow internalization, and the remaining biotin on the cell-surface proteins was removed by reduction, while internalized biotinylated proteins retained their biotin label. As expected, significant levels of biotinylated tTG was detected in cells incubated at 37°C, demonstrating the efficient internalization of tTG from the cell surface (Fig. 3B). At early time points of endocytosis, the intact internalized tTG was detected inside the CHO cells, whereas longer incubations revealed a gradual decline in the amounts of intact tTG, coinciding with the appearance and subsequent accumulation of two proteolytic tTG fragments of molecular mass of ~42-45

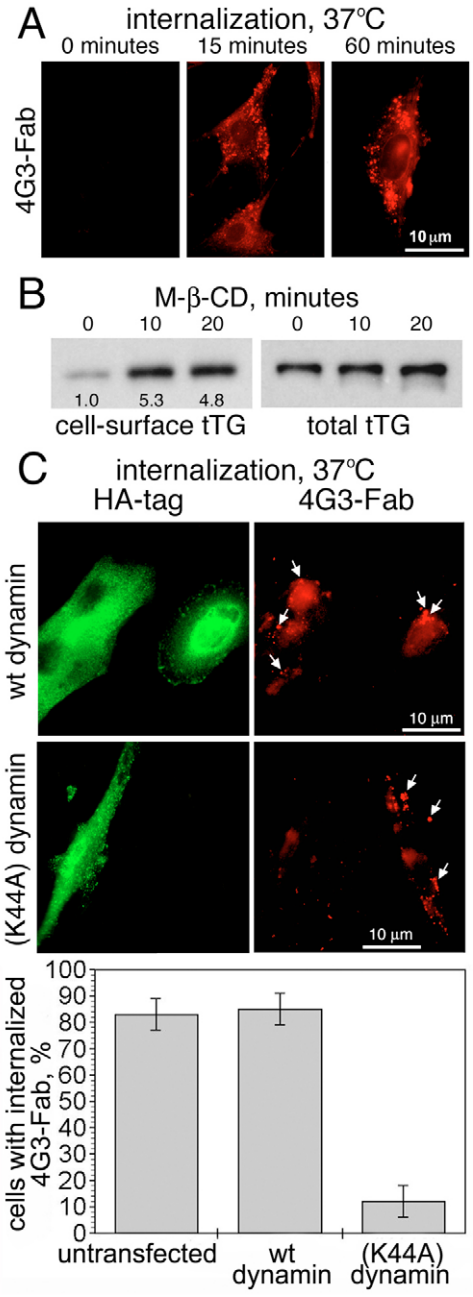

Fig. 1. tTG is internalized from the cell surface by means of a cholesterol- and dynamin-dependent mechanism. (A) Endocytosis of tTG from the surface of WI-38 fibroblasts. The Fab fragments of mouse mAb 4G3 against tTG were incubated with WI-38 fibroblasts at 4°C. Next, the cells were warmed up to 37°C for 15 or 60 minutes and the Fab fragments remaining on the cell surface were stripped by a low-pH treatment. The internalized tTG–4G3-Fab complexes were detected by immunofluorescence after cell permeabilization. (B) Plasma membrane cholesterol is required for internalization of cell-surface tTG. NIH3T3 fibroblasts expressing exogenous tTG were treated with 10 mM M-β-CD and then incubated for 3 hours at 37°C in DMEM-FBS without the inhibitor. Cell-surface proteins were biotinylated with membrane-impermeable sulfo-NHS-LC-biotin. Biotinylated proteins were isolated and total cellular and cell-surface fractions were analyzed for tTG by SDS-PAGE and immunoblotting. The numbers beneath the tTG bands display relative intensities compared with a value of 1.0 assigned to untreated cells. Shown is a representative result of three independent experiments. (C) GTPase activity of dynamin-2 is required for endocytosis of cell-surface tTG. MRC-5 fibroblasts were transiently transfected with either wild-type (wt) or a GTPase-deficient dynamin-2 mutant (K44A) with a hemagglutinin (HA) tag. 40 hours after transfection, antibody-uptake assays with the Fab fragments of mAb 4G3 were performed for 15 minutes as described in the legend to panel 1A. After permeabilization, the cells were co-stained for the transfected dynamin-2 or the dynamin-2 K44A mutant with antibody against HA (left panels) and for the tTG–4G3-Fab complexes (right panels). Arrows mark multiple vesicles containing the internalized tTG–4G3-Fab complexes in the cells expressing the endogenous or transfected wild-type dynamin-2, but not its (K44A) mutant. The percentages of cells with positive staining for the internalized tTG–4G3-Fab complexes (mean pixel intensity within the cell area $\geqslant$30) were calculated for the populations of untransfected and wild-type dynamin- or mutant K44A-dynamin-transfected MRC-5 fibroblasts (n=120, lower panel).

kDa. Therefore, the delivery of internalized tTG to Lamp-1-positive vesicles (Fig. 2) resulted in proteolysis of the internalized tTG in the lysosomal compartments (Fig. 3). The dynamics of β1 integrin internalization from the surface of CHO cells was similar to that of tTG endocytosis. However, endocytosis of β1 integrin did not lead to its degradation, indicating that internalized β1 integrin subunits follow recycling routes rather than undergoing intracellular proteolysis (Caswell and Norman, 2006; Pellinen and Ivaska, 2006). Finally, a similar lysosomal degradation of internalized tTG was observed in WI-38 and NIH3T3 fibroblasts and was blocked by the lysosomal inhibitor bafilomycin, suggesting that this is a general pathway utilized in different types of cells (data not shown).

Constitutive internalization of cell-surface tTG occurs via clathrin- and caveolin-dependent endocytic pathways

The dependence of tTG endocytosis on membrane cholesterol and the GTPase activity of dynamin indicates that it might proceed through clathrin- and/or caveolin-mediated pathways (Conner and Schmid, 2003; Kirkham and Parton, 2005). Co-internalization assays with the tTG–4G3-Fab complexes and a marker of the clathrin-dependent endocytic pathway, transferrin, revealed a significant overlap in their localization patterns in WI-38 fibroblasts (Fig. 4A). Furthermore, co-distribution of the internalized tTG with clathrin-positive vesicles was detected in these cells at early time points during endocytosis (Fig. 4A). To assess the contribution of different endocytic mechanisms in the tTG internalization, we tested the effects of hyperosmotic conditions, which inhibit clathrin-mediated endocytosis (Heuser and Anderson, 1989; Hansen et al., 1993), and those of filipin, which is a specific inhibitor of lipid raft- or caveolae-dependent endocytosis (Orlandi and Fishman, 1998; Schnitzer et al., 1994). Notably, both these inhibitors significantly diminished internalization and degradation of tTG in WI-38 fibroblasts (Fig. 4B).

To assess the spatial relationship of tTG and caveolae on the cell surface, triple staining of live MRC-5 fibroblasts was performed with antibodies against tTG, caveolin-1 and β1 integrin (Fig. 4C). This revealed a partial co-distribution of cell-surface tTG with lipid rafts or caveolae, as defined by

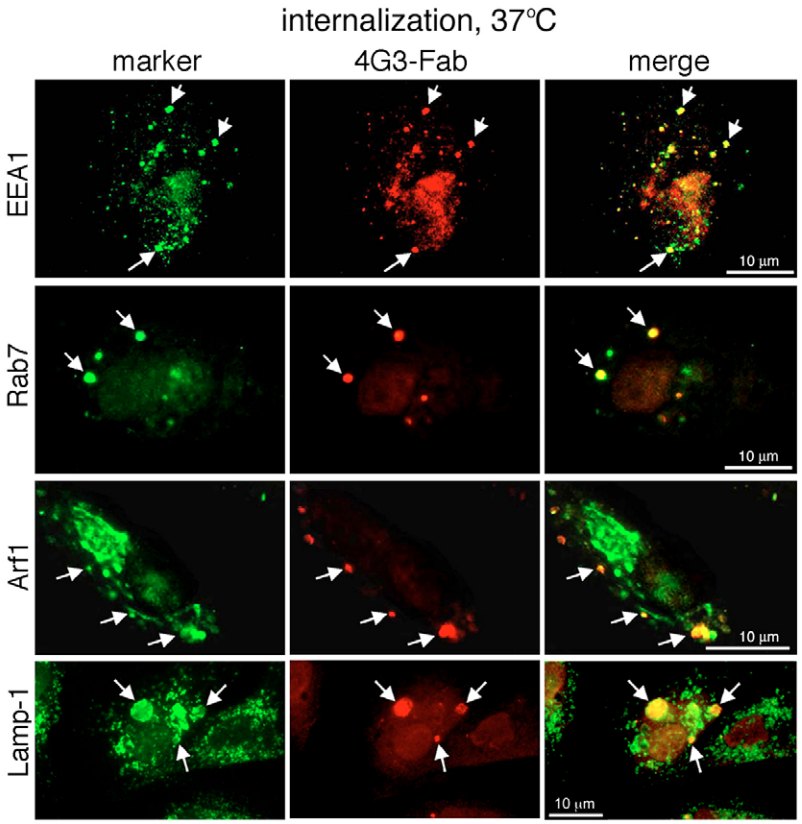

Fig. 2. The internalized cell-surface tTG is transported through endosomal compartments to lysosomes. Antibody-uptake experiments were performed as described above in Fig. 1A. Double-immunofluorescence staining of WI-38 fibroblasts for internalized tTG–4G3-Fab complexes (central panels) and endocytic markers (left panels), including EEA1 (early endosomes, 10 minutes of endocytosis), Rab7 (late endosomes, 30 minutes of endocytosis), Arf1 (ECV/Golgi, 30 minutes of endocytosis) or Lamp-1 (lysosomes, 120 minutes of endocytosis). Merged images are shown in the right panels. Arrows indicate colocalization of the internalized tTG–4G3-Fab complexes with various organelle markers.

colocalization of tTG with caveolin-1. In turn, these membrane structures containing tTG often overlapped with, or were adjacent to, cell-matrix contacts on the dorsal cell surfaces, as defined by localization of β1 integrins.

We further defined the role of lipid rafts or caveolae in the endocytosis of cell-surface tTG by using either a pharmacological inhibitor (filipin) or siRNA approach (downregulation of caveolin-1) to block caveolae-dependent endocytosis (Fig. 4D,E). Treatment with filipin showed a robust elevation of cell-surface tTG in the NIH3T3-tTG fibroblasts without any significant effect on total tTG expression (Fig. 4D). Finally, biochemical endocytosis assays with U251 glioma cells stably transfected with siRNA against caveolin-1 or a control vector showed an increased rate of tTG internalization in the cells that had decreased expression of caveolin-1 (Fig. 4E). As recent studies have shown that caveolin-1 negatively regulates caveolae-dependent endocytosis by stabilizing these structures (Le et al., 2002; Thomsen et al., 2002), our data indicate a significant role for these membrane microdomains in the internalization of tTG from the cell surface. Together, they imply that endocytosis of cell-surface tTG proceeds through both clathrin- and caveolae-mediated pathways.

Platelet-derived growth factor and fibronectin promote endocytosis of cell-surface tTG

We investigated whether endocytosis of cell-surface tTG is regulated by growth factors such as platelet-derived growth factor (PDGF), an important physiological modulator of anchorage-dependent cells (Heldin and Westermark, 1999). Importantly, the PDGF receptor (PDGFRβ) has been shown to

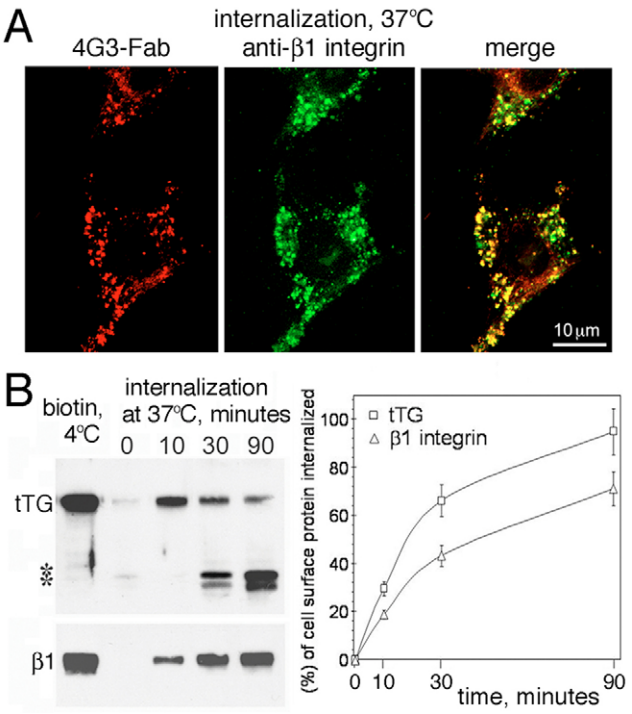

Fig. 3. The internalized cell-surface tTG undergoes proteolytic degradation. (A) tTG colocalizes with its binding partner, the β1 integrin subunit, early after endocytosis from the cell surface. Antibody-uptake experiments were performed for 15 minutes, as described above in Fig. 1A, with WI-38 fibroblasts and the Fab fragments of mouse mAb 4G3 against tTG and rat mAb 9EG7 against β1 integrins. (B) Cell-surface tTG, but not the β1 integrin subunit, is degraded after internalization. Surface biotinylation and endocytosis assays were performed with CHO cells and membrane-impermeable SH-cleavable sulfo-NHS-SS-biotin. Biotinylated proteins were isolated and tTG and β1 integrin were detected by SDS-PAGE and immunoblotting in the fraction of proteins internalized from the cell surface. The intensities of protein bands at various time points of internalization were quantified by densitometry and compared with those of cell-surface tTG and β1 integrins before endocytosis. Shown are the means ± s.d. for three independent experiments. Asterisks indicate proteolytic fragments of tTG.

be involved in regulation of the adhesive functions of β1 and β3 integrins, promoting their disengagement from fibronectin (Greenwood et al., 2000; Berrou and Bryckaert, 2001; Sun et al., 2005) and their internalization and recycling upon PDGF stimulation (Caswell and Norman, 2006). Therefore, cell-surface tTG associated with β1 or β3 integrins and fibronectin might be targeted by PDGF-activated endocytosis. Our experiments with WI-38 fibroblasts indicated that little or no endocytosis of surface tTG was seen in quiescent serum-starved cells, whereas the protein was rapidly internalized and degraded in response to PDGF (Fig. 5A). The inhibitors of clathrin- and caveolin-mediated pathways attenuated internalization of tTG in the cells stimulated with PDGF, indicating the involvement of the same internalization routes as observed for constitutive endocytosis of tTG.

The role of the key binding partner of tTG, fibronectin, in endocytosis from the cell surface was tested biochemically in internalization assays with fibronectin-null mouse embryonic fibroblasts (MEFs) expressing exogenous tTG (Janiak et al., 2006) (Fig. 5B). These cells were grown in medium with fibronectin-depleted serum or in the same medium with fibronectin added 3 hours before the assays. The

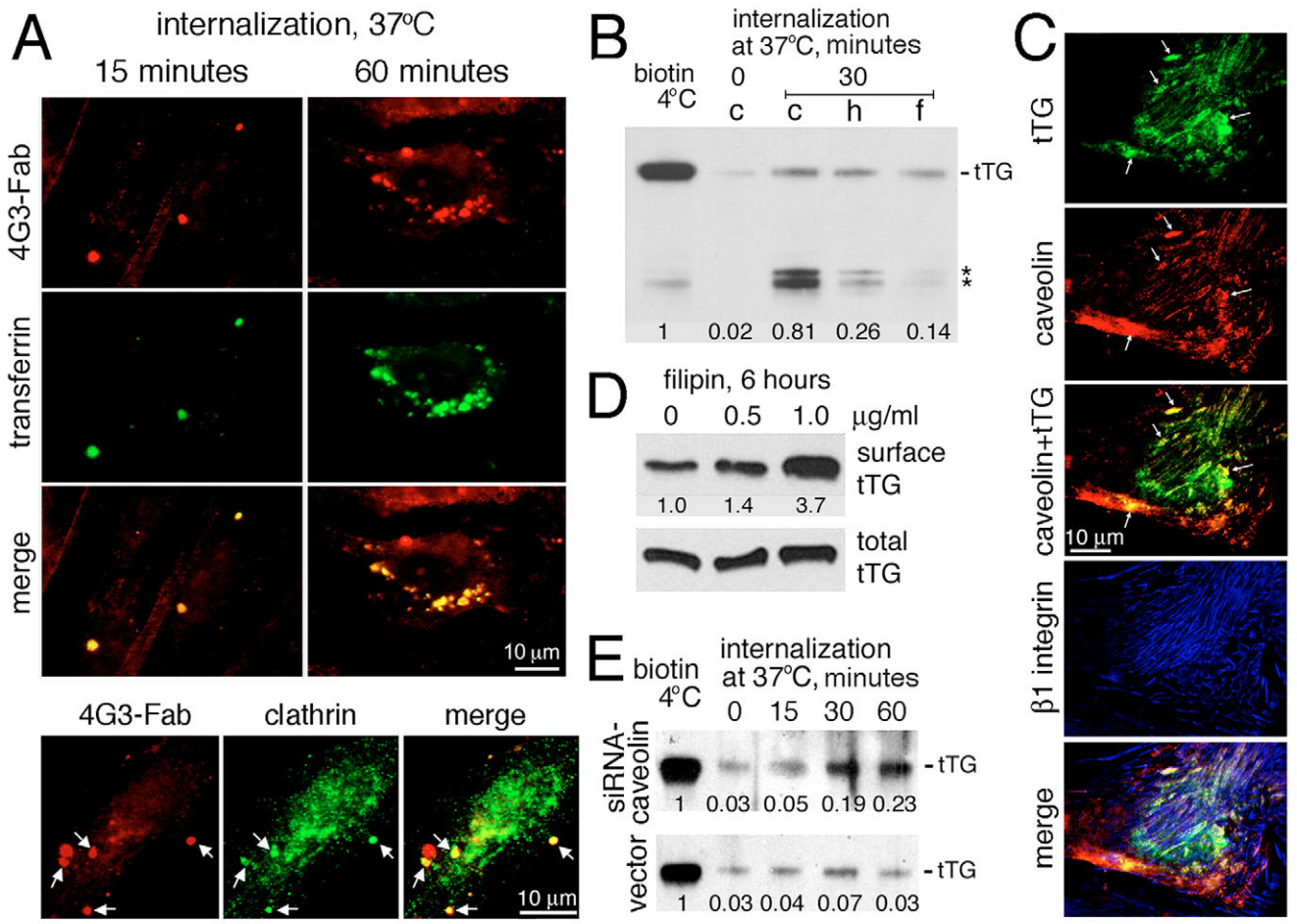

Fig. 4. Cell-surface tTG is internalized by means of clathrin- and lipid-raft- (caveolae-) dependent pathways. (A) Cell-surface tTG colocalizes with transferrin and clathrin during internalization. Antibody-uptake assays were performed with WI-38 fibroblasts as described above in Fig. 1A with the Fab fragments of mouse mAb 4G3 against tTG and FITC-labeled transferrin (upper panels), or the anti-tTG Fab fragments only, and the cells were co-stained for clathrin after 15 minutes of internalization (lower panels). (B) Internalization of cell-surface tTG is attenuated by the inhibitors of clathrin- and caveolae-dependent endocytosis. WI-38 fibroblasts were left (c) untreated or were treated with (h) hyperosmotic 0.45 M sucrose or (f) 5 μg/ml filipin before surface biotinylation with membrane-impermeable SH-cleavable sulfo-NHS-SS-biotin. (C) Co-distribution of tTG with caveolin-1 and β1 integrins on the cell surface. Live non-permeabilized MRC-5 fibroblasts were triple-labeled with antibodies against the three proteins. Arrows mark the sites of colocalization of tTG and caveolin-1 on the cell surface. (D) Inhibition of caveolae-dependent endocytosis increases cell-surface levels of tTG. NIH3T3 fibroblasts expressing tTG were either left untreated or were treated with filipin. Surface labeling of untreated and filipin-treated cells with sulfo-NHS-LC-biotin was followed by isolation of biotinylated (cell surface) tTG. (E) Downregulation of caveolin-1 promotes endocytosis of cell-surface tTG. Surface biotinylation and endocytosis assays were performed with U-251 glioma cells expressing vector only or caveolin-1 siRNA, using membrane-impermeable SH-cleavable sulfo-NHS-SS-biotin. Biotinylated proteins in (B,E) were isolated and tTG was detected by SDS-PAGE and immunoblotting in the fraction of proteins internalized from the cell surface. The numbers beneath the tTG bands show relative intensities compared with the value of 1.0 assigned to untreated cells (D) or cells before internalization (B,E). Shown in (B,D,E) are representative results from three independent experiments.

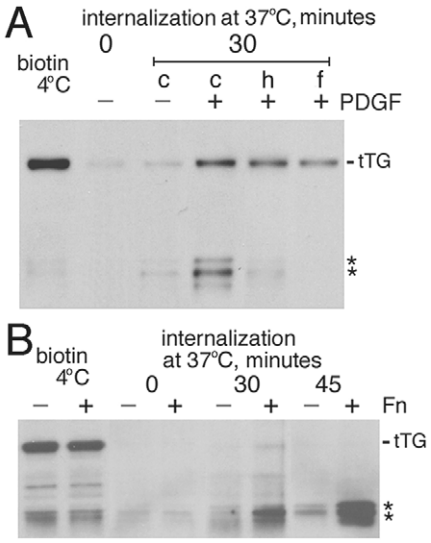

Fig. 5. PDGF and fibronectin promote endocytosis of cell-surface tTG. (A) PDGF accelerates internalization and degradation of cell-surface tTG. Quiescent serum-starved WI-38 fibroblasts surface-labeled with sulfo-NHS-SS-biotin, were either left untreated ['C'], or were treated with (c) PDGF only, or with (h) PDGF and the inhibitors of clathrin-dependent (0.45 M sucrose) or (f) caveolae-dependent (5 μg/ml filipin) endocytosis. (B) The internalization of tTG from the cell surface is stimulated by fibronectin. Fibronectin-null mouse embryonic fibroblasts expressing exogenous tTG were grown in fibronectin-depleted 10% serum with or without 50 μg/ml fibronectin (Fn) and were surface-biotinylated using sulfo-NHS-SS-biotin. Biotinylated proteins were isolated and tTG was detected by SDS-PAGE and immunoblotting in the fraction of proteins internalized from the cell surface (A,B). Proteolytic fragments of tTG are marked with asterisks. Shown in (A,B) are representative results of three independent experiments.

membrane-bound fibronectin strongly promoted endocytosis of cell-surface tTG, demonstrating that association with its major ECM ligand accelerates tTG internalization.

tTG interacts with LRP1 on the cell surface and promotes the association of LRP1 with β1 integrins

To better understand the mechanism of tTG internalization and identify the key players involved in this process, we next focused on a search for a putative endocytic receptor for tTG. LRP1 emerged as a good candidate because this large, ubiquitously expressed, endocytic receptor regulates internalization and/or intracellular trafficking of numerous ligands (Herz and Strickland, 2001), including those of the tTG-binding partners β1 and β3 integrins and fibronectin (Czekay et al., 2003; Salicioni et al., 2002; Salicioni et al., 2004). Several independent approaches were used to test this hypothesis.

Co-endocytosis assays performed with WI-38 fibroblasts and tTG–4G3-Fab and LRP1–anti-LRP1 complexes were followed by immunostaining for internalized cell-surface tTG and LRP1 (Fig. 6A). They revealed an accumulation and colocalization of these proteins in small peripheral vesicles, probably early endosomes, at the initial stages of endocytosis. These results showed the presence of internalized tTG in the LRP1-positive vesicles and suggested an involvement of this endocytic receptor in internalization of cell-surface tTG.

The potential association of tTG with LRP1 was examined by co-immunoprecipitation using the NIH3T3 fibroblasts expressing or lacking exogenous tTG (Fig. 6B). As LRP1 collaborates with PDGFRβ in signaling (Boucher et al., 2002; Newton et al., 2005), the analysis was performed with quiescent and PDGF-stimulated cells. It revealed that tTG associated with LRP1, and the complex was detected in quiescent fibroblasts but was not significantly affected by PDGF treatment. The identified association of tTG with LRP1 suggested that it might affect interactions of LRP1 with tTG-binding proteins, including β1 integrins and fibronectin. Analysis of the immune complexes precipitated with antibodies against β1 integrin revealed a weak association of LRP1 with β1 integrins in the quiescent cells lacking tTG that increased upon short stimulation of cells with PDGF (Fig. 6C, left panels). By contrast, in the quiescent cells expressing tTG, much more prominent association of LRP1 with β1 integrins was detected, and this was not further enhanced by PDGF (Fig. 6C, right panels). These results indicate that tTG associates with LRP1 and mediates the formation of ternary complexes with LRP1 and β1 integrins. Such formation of complexes can modulate the functions of the proteins involved, affecting cell-matrix adhesion and motility, receptor-mediated signaling and endocytosis.

Finally, buoyant-density centrifugation was used to analyze the distribution of LRP1 in lipid raft and non-raft fractions of plasma membrane in cells lacking or expressing tTG (Fig. 6D). Analysis of membrane fractions obtained from the cells without tTG showed an enrichment of LRP1 in low-density lipid raft fractions (lanes 8-10), its presence in non-raft membrane fractions (lanes 4-6), and only traces of cell-surface receptor associated with the ECM fractions containing fibronectin (lanes 1-3). Notably, fractionation of the tTG-expressing cells revealed a significant elevation of the surface LRP1 levels in the fibronectin-positive ECM fractions that were also enriched in cell-surface tTG (lanes 4-9). These data, combined with observations that tTG interacts with the α chain of the extracellular domain of LRP1 (supplementary material Fig. S1), demonstrate the association of tTG with the endocytic receptor LRP1 on the cell surface and suggest that surface tTG can mediate the interaction of LRP1 with cell-matrix adhesive protein complexes containing β1 integrins and fibronectin.

tTG interacts directly with LRP1 by means of the catalytic domain

To further study the interaction of tTG with LRP1, in vitro binding assays were performed with purified proteins (Fig. 7). In solid-phase binding experiments, purified soluble LRP1 displayed a specific concentration-dependent binding to tTG immobilized on polystyrene-coated microtiter wells, while strong binding to the immobilized receptor-associated protein (RAP), which interacts with and antagonises LRP1 (Strickland et al., 1995), and the lack of interaction with BSA served as positive and negative controls, respectively (Fig. 7A). Interestingly, RAP did not inhibit the interaction of LRP1 with tTG in vitro, suggesting the existence of distinct binding sites for tTG and RAP on the LRP1 molecule. Similar results were obtained in reciprocal experiments, in which purified tTG in solution phase was incubated with immobilized LRP1 and was found to bind to this receptor as well as to another member of

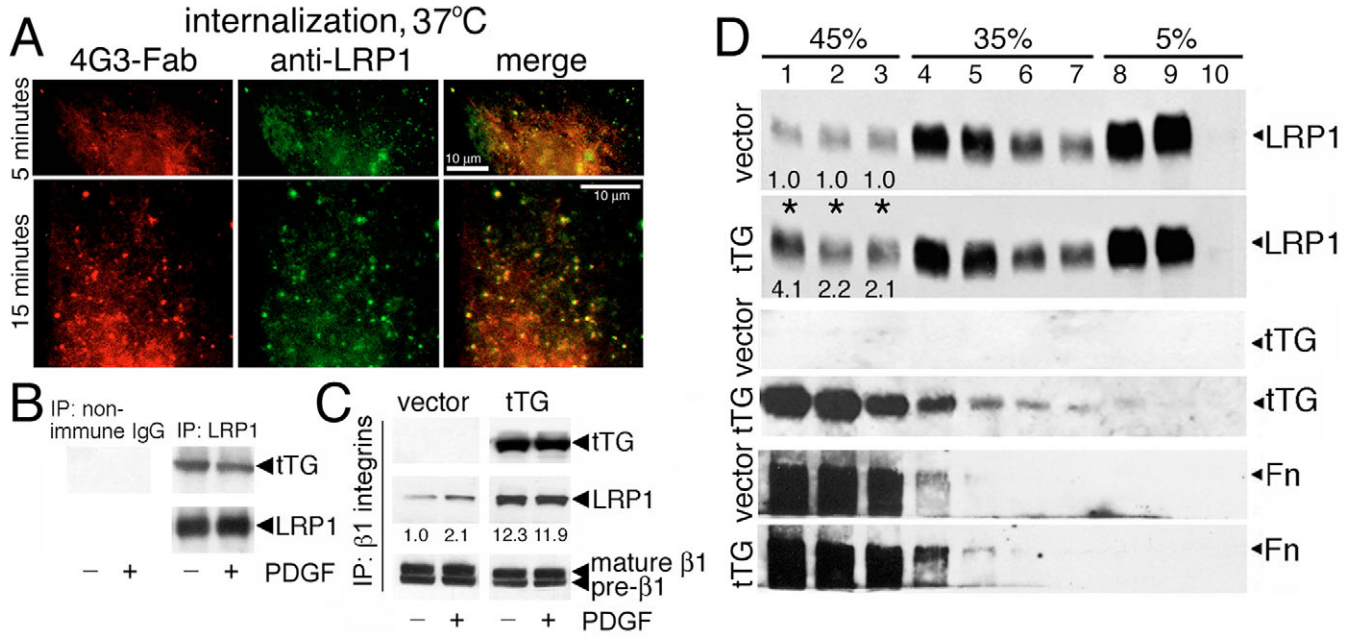

Fig. 6. tTG is colocalized with LRP1 during internalization and the two interact on the cell surface. (A) tTG colocalizes with LRP1 early after endocytosis from the cell surface. Antibody-uptake experiments were performed for 5 or 15 minutes with WI-38 fibroblasts and the Fab fragments of mouse mAb 4G3 against tTG and rabbit antibody Rb2629 against LRP1, as described above in Fig. 1A. The internalized tTG–4G3-Fab and LRP1–anti-LRP1 complexes were detected by immunofluorescence after cell permeabilization. Note the significant colocalization of the internalized proteins in the numerous peripheral endocytic vesicles. (B) tTG is associated with LRP1. Extracts of quiescent (–) and PDGF-treated (+) NIH3T3 cells expressing exogenous tTG were subjected to immunoprecipitation with non-immune IgG or antibody 2629 against LRP1. The resulting immune complexes were examined by SDS-PAGE and immunoblotting for tTG and LRP1. (C) tTG enhances the association of LRP1 with β1 integrins. β1 integrins were immunoprecipitated from the extracts of quiescent (–) or PDGF-treated (+) NIH3T3 cells lacking (vector) or expressing tTG. The resulting immune complexes were analyzed for LRP1, tTG and β1 integrins by SDS-PAGE and immunoblotting. (D) Cell-surface tTG mediates a shift of LRP1 towards high-density membrane or ECM fractions enriched in tTG and fibronectin. NIH3T3 fibroblasts lacking (vector) or expressing tTG were surface-biotinylated with membrane-impermeable sulfo-NHS-LC-biotin. Carbonate extracts at pH 11 of the cells were subjected to ultracentrifugation in a discontinuous (45%-35%-5%) sucrose gradient. Biotinylated (cell surface) proteins from each gradient fraction were isolated and analyzed by SDS-PAGE and immunoblotting with antibodies against LRP1, tTG and fibronectin. The high-density fractions enriched in tTG and fibronectin and expressing increased amounts of LRP1 in the tTG-expressing cells are marked with asterisks. The numbers beneath the LRP1 bands show relative intensities compared with the value of 1.0 assigned to cells lacking tTG in the absence of PDGF. Shown are representative results of three independent experiments.

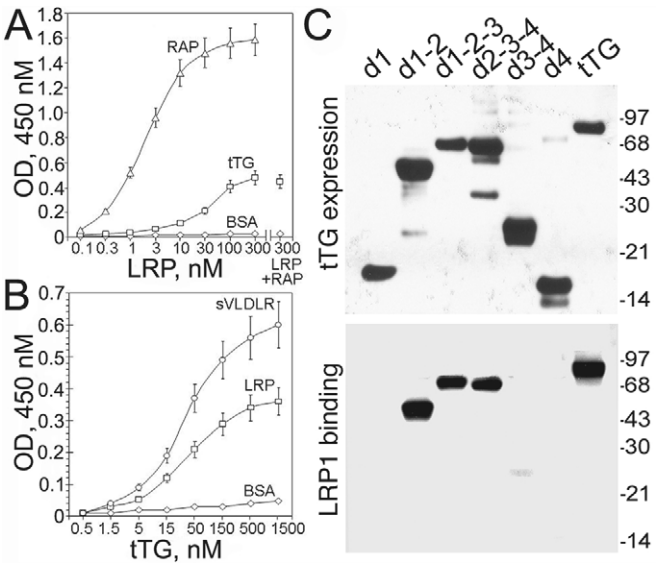

Fig. 7. tTG interacts directly with LRP1 by means of the catalytic domain. (A) The interaction of purified LRP1 with tTG immobilized on plastic wells. Binding assays were performed as described in Materials and Methods in the presence or absence of the LRP1 interactor RAP. Immobilized BSA and RAP were used as negative and positive binding controls, respectively. (B) The interaction of purified tTG with LRP1 and soluble VLDL receptor (sVLDLR) immobilized on plastic wells. Binding assays were performed as described in Materials and Methods with BSA used as a negative binding control. Shown in (A,B) are the means ± s.d. for two independent experiments performed in triplicate. (C) LRP1 interacts with the second (catalytic) domain of tTG. Full-length tTG and its deletion mutants (Hang et al., 2005), all containing the C-terminal c-Myc tag, were expressed in NIH3T3 fibroblasts. Cell extracts were analyzed by SDS-PAGE and immunoblotting with antibody against c-Myc for total expression levels of full-length tTG and its deletion mutants (upper panel). LRP1 was immunoprecipitated from cell extracts with the rabbit antibody Rb2629, and the resulting LRP1 immune complexes were analyzed by SDS-PAGE and immunoblotting with antibody against c-Myc (lower panel). Molecular mass markers (kDa) are shown to the right of the gels.

the LDL receptor superfamily, VLDLR, which is structurally related to LRP1 (Fig. 7B). Together, these in vitro experiments specify a direct interaction between tTG and LRP1.

To define the domain(s) of tTG involved in this interaction, the association of LRP1 with previously characterized tTG deletion mutants (Hang et al., 2005) was studied by co-immunoprecipitation (Fig. 7C). These experiments revealed that, in addition to full-length tTG, only deletion mutants containing the second (catalytic) domain of the protein interact with LRP1. Therefore, the catalytic domain of tTG is involved in the interaction with LRP1.

LRP1 is required for endocytosis of cell-surface tTG

The above observations of co-internalization of tTG and LRP1 (Fig. 6A) and the interaction between these proteins (Figs 6, 7) prompted us to test the role of LRP1 in endocytosis of tTG from the cell surface (Fig. 8). To evaluate whether LRP1 is required for tTG internalization in cells endogenously expressing this protein, we used the original CHO cells that produce significant amounts of LRP1 and their derivatives lacking LRP1 expression (CHO 13-5-1 cell line; Fig. 8A) (Fitzgerald et al., 1995). Cell-surface biotinylation and internalization assays demonstrated a robust endocytosis and intracellular degradation of tTG in the original CHO cells but showed a strong impairment of tTG internalization in their derivatives lacking LRP1.

To prove that the crucial role of LRP1 in endocytosis of surface tTG is not limited to certain cell lines, exogenous human tTG was stably expressed in normal MEFs and their counterparts with a genetically knocked-out *LRP1* gene [*Lrp1*$^{-/-}$ PEA-13 MEFs (Willnow and Herz, 1994)]. Internalization assays were performed with surface-biotinylated MEF and PEA-13 cells expressing exogenous tTG (Fig. 8B). Biochemical analysis with surface biotinylation and endocytosis of tTG revealed that, although cell-surface tTG was efficiently internalized and intracellularly degraded in the LRP1-expressing MEFs, its endocytosis was abolished in the LRP1-deficient PEA-13 cells. These findings show that, in two different cell systems, the endocytic receptor LRP1 is required for internalization of cell-surface tTG.

LRP1 deficiency and blockade of endocytosis upregulate transglutaminase activity and cell-matrix adhesion owing to accumulation of cell-surface tTG

Finally, we tested the effects of LRP1 deficiency and inhibition of endocytosis on steady-state levels of surface tTG and enzymatic and adhesive functions of this protein on the cell surface (Fig. 9). With similar expression levels of exogenous tTG in the MEF and PEA-13 transfectants, increased levels of surface tTG were detected in the latter cells deficient in LRP1 (Fig. 9A, left panels). Likewise, prolonged inhibition of endolysosomal function in wild-type CHO cells by bafilomycin or chloroquine resulted in greater accumulation of cell-surface tTG compared with untreated cells (Fig. 9A, right panels). In turn, the increased levels of surface tTG in the PEA-13 transfectants and CHO cells treated with bafilomycin or chloroquine resulted in elevated transamidation on the cell surface (Fig. 9B). Finally, accumulation of tTG on the surface of these cells led to upregulation of its adhesive function, as judged by the increased adhesion of these cells on the 42-kDa tTG-binding fragment of fibronectin (Fig. 9C). Therefore,

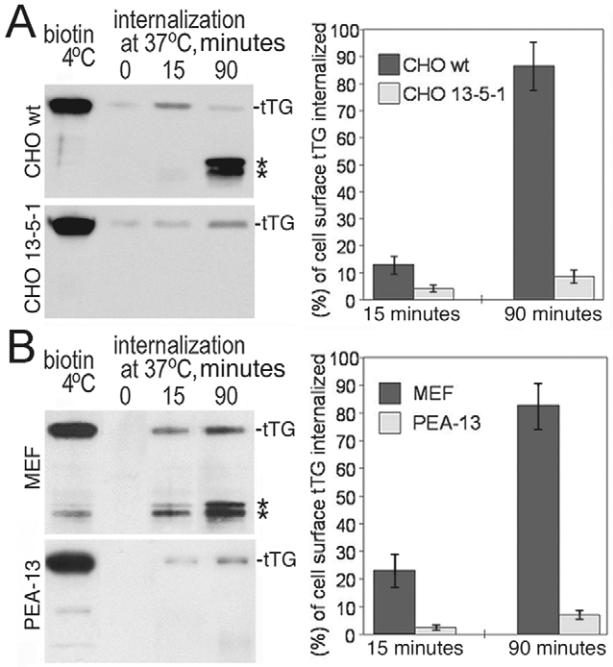

Fig. 8. Internalization of cell-surface tTG requires the endocytic receptor LRP1. LRP1 deficiency inhibits internalization of cell-surface tTG. Wild-type (CHO wt) and LRP1-deficient (CHO 13-5-1) CHO cells (A) or normal (MEF) and *Lrp1*$^{-/-}$ (PEA-13) mouse embryonic fibroblasts expressing exogenous tTG (B) were surface-biotinylated with sulfo-NHS-SS-biotin and subjected to internalization assays. (A,B) Biotinylated proteins were isolated and tTG was detected by SDS-PAGE and immunoblotting in the fraction of proteins internalized from the cell surface. Proteolytic fragments of tTG are marked with asterisks. Graphs on the right show the levels of internalized tTG for each cell type at different time points of internalization. The values presented are expressed as percentages of those obtained for cell-surface tTG before internalization. The results are representative of three independent experiments performed for each cell type (means ± s.d.).

LRP1 deficiency, or inhibition of endolysosomal function, elevates the expression and transglutaminase activity of cell-surface tTG and promotes cell-matrix interactions.

Discussion

Previous studies demonstrated a dual function for cell-surface tTG as a protein crosslinking enzyme and high-affinity integrin-binding co-receptor for fibronectin (reviewed in Zemskov et al., 2006). Therefore, precise regulation of cell-matrix adhesion and adhesion-mediated signaling requires a versatile and efficient regulation of cell-surface tTG. Previous work delineated two major pathways of surface tTG regulation by means of the Ras-Raf-MEK1-ERK1 (ERK2) signaling module (Akimov and Belkin, 2003) and pericellular proteolysis by membrane-anchored and soluble metalloproteases (Belkin et al., 2001; Belkin et al., 2004). Here, we describe a novel means of regulation of tTG functioning on the cell surface that involves its internalization followed by intracellular degradation in lysosomes. We found that internalization of tTG proceeds in a dynamin-mediated cholesterol-dependent manner in a wide range of cell types.

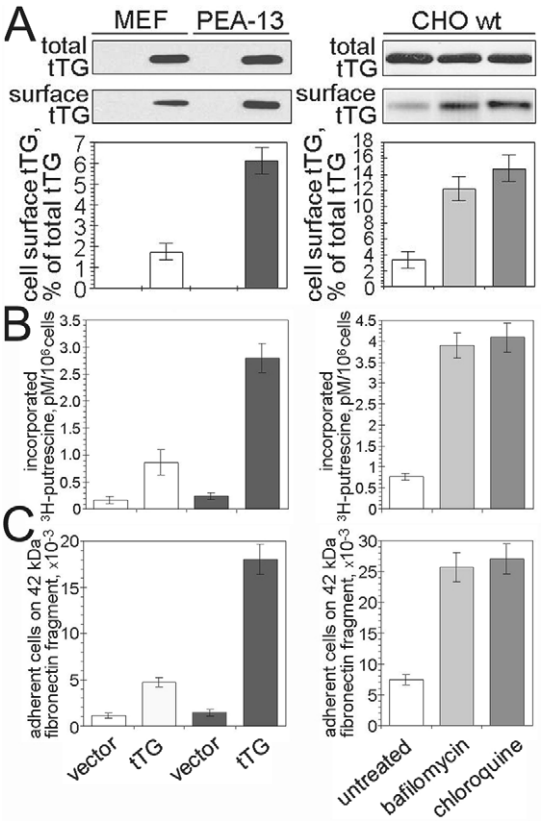

Fig. 9. LRP1 deficiency and impairment of endocytosis increase extracellular transglutaminase activity and cell adhesion by upregulation of cell-surface tTG. Surface tTG levels (A), extracellular transglutaminase activity (B) and adhesion to the 42-kDa fragment of fibronectin (C) were determined for normal (MEF) and *Lrp1*$^{-/-}$ (PEA-13) mouse embryonic fibroblasts lacking or expressing tTG (left panels) and for wild-type CHO cells, either untreated or treated with 2 μM bafilomycin or 35 μM chloroquine for 16 hours (right panels). (A) LRP1 deficiency and inhibition of endocytosis upregulate the levels of cell-surface tTG. The steady-state surface tTG levels were determined by surface labeling with sulfo-NHS-LC-biotin, isolation of biotinylated proteins, SDS-PAGE and immunoblotting, and expressed as percentages of the total tTG levels. The results are representative of three independent experiments performed for each cell type (means ± s.d.). (B) The absence of LRP1 and ablation of endocytosis increase the enzymatic activity of tTG on the cell surface. Cell-mediated incorporation of ^{3}H-putrescine into *N,N*-dimethylcaseine was measured as described previously (Belkin et al., 2001). (C) The lack of LRP1 and treatment with endocytic inhibitors enhance adhesion of cells to the tTG-binding 42-kDa fragment of fibronectin (Radek et al., 1993). Adhesion to the immobilized 42-kDa fragment was measured as described previously (Akimov et al., 2000). The data in (B,C) are means of two independent experiments performed in triplicate.

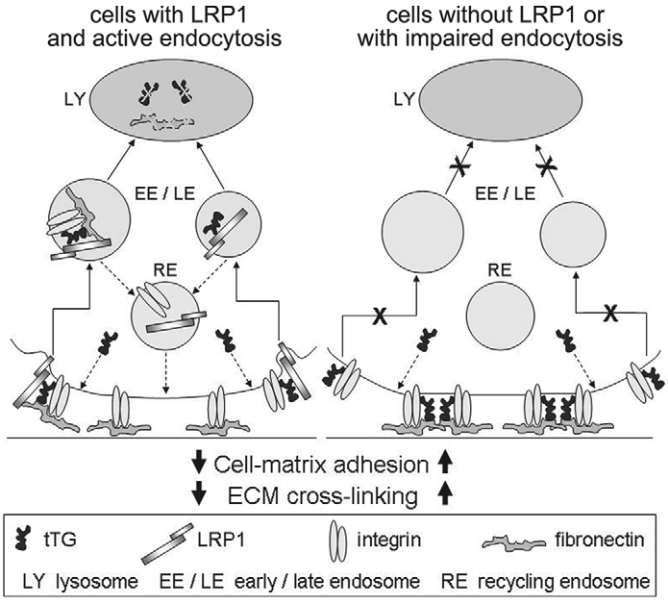

Fig. 10. A scheme summarizing the regulation of tTG functions on the cell surface by LRP1-mediated endocytosis. In LRP1-expressing cells with active endocytosis, the receptor downregulates surface tTG by internalization and targeting it to lysosomes for degradation, thereby decreasing cell-matrix adhesion and ECM crosslinking. In cells with no LRP1 or with impaired endocytosis, tTG accumulates on the surface owing to lack of internalization. For further information, see main text.

The efficient constitutive endocytosis of tTG from the cell surface utilizes both clathrin- and caveolae-dependent mechanisms and is significantly enhanced by PDGF and the major ECM ligand of tTG, fibronectin. We demonstrate that tTG interacts with the major endocytic receptor LRP1 on the cell surface, and LRP1 is required for endocytosis of cell-surface tTG. Moreover, the identified interaction of tTG with the functional homolog of LRP1, the VLDL receptor, suggests that, in certain cell types that lack LRP1, such as endothelial cells, VLDL might be involved in tTG internalization.

Our model indicates that LRP1-mediated endocytosis efficiently downregulates surface tTG by internalization and targeting of the protein to lysosomes for degradation (Fig. 10). In turn, this reduces tTG functionality on the cell surface and decreases cell-matrix interactions and extracellular crosslinking of matrix proteins. By contrast, the absence or functional downregulation of LRP1 (or VLDLR) causes accumulation of tTG on the surface of various cell types, thereby increasing cell-matrix adhesion and extracellular crosslinking. Likewise, cells with impaired endocytosis or endolysosomal function accumulate elevated levels of surface tTG. This suggests that dysfunctions of cellular endocytic systems should promote both extracellular transamidation and tTG-mediated cell-matrix interactions independent of the enzymatic activities of this protein. Furthermore, integrin-associated cell-surface tTG is known to affect other pivotal integrin functions, including outside-in signaling (Janiak et al., 2006) and cell migration (Akimov and Belkin, 2001b). Therefore, LRP1-mediated regulation of tTG on the cell surface can influence a wide range of cellular processes.

Our reported pathway of surface tTG internalization implies a previously unexplored relationship between tTG, and its key binding partners on the cell surface, β1 and β3 integrins and fibronectin. Previous studies revealed the regulation of β3 integrin presentation on the cell surface by means of PDGF-driven endocytosis and recycling (Caswell and Norman, 2006). As all the tTG is associated with the β1 or β3 integrin subunits on the cell surface (Akimov et al.,

2000), it is plausible that tTG is internalized as a complex with integrins and that this process is accelerated by certain growth factors (Fig. 10). Indeed, our experiments revealed similar dynamics of endocytosis for cell-surface tTG and β1 integrins in CHO cells. However, unlike the β1 or β3 integrins that typically are recycled back to the cell surface by means of several distinctive pathways (Powelka et al., 2004; Pellinen and Ivaska, 2006), internalized tTG has a different fate and is targeted to lysosomes for degradation. These findings indicate that the putative internalized integrin-tTG adhesive complexes are probably dissociated in the endocytic compartments.

Notably, previous work showed that fibronectin is internalized by an LRP1-dependent mechanism in CHO cells and MEFs, whereas our findings revealed a significant enhancement of surface tTG endocytosis by membrane-associated fibronectin. Therefore, in the absence of fibronectin, tTG is mostly retained on the cell surface, whereas the formation of tTG-fibronectin complexes promotes their LRP1-mediated endocytosis. As fibronectin interacts directly with LRP1, it might bridge surface tTG to this endocytic receptor and facilitate tTG internalization (Fig. 10). In future work, it will be important to compare the effects of soluble and matrix forms of fibronectin on endocytosis of cell-surface tTG. Although membrane-bound monomeric fibronectin was found to promote this process, polymeric fibronectin (matrix fibrils) might prevent endocytosis of tTG by anchoring the integrin-tTG complexes on the cell surface, thus stabilizing cell-matrix adhesion and promoting fibronectin assembly.

Our findings indicate that a key binding partner and functional antagonist of LRP1, RAP, does not perturb the interaction of tTG with LRP1 or interfere with the endocytosis of cell-surface tTG (data not shown). Therefore, although tTG interacts with LRP1 directly, it might not represent a typical ligand, but instead function as a mediator involved in LRP1-dependent endocytosis through the association with LRP1 ligands such as fibronectin. Furthermore, initial analysis indicates that tTG does not bind to the β chain of LRP1 (supplementary material Fig. S1). Future studies should help to delineate the tTG-binding site(s) on the α chain of the LRP1 molecule. They also should define the relationships between integrins, tTG and fibronectin in the LRP1-dependent endocytic process and determine the role of the identified interactions between cell-surface tTG and LRP1 in internalization of integrin- and fibronectin-containing adhesive complexes.

An emerging general theme in the field highlights the intricate functional relationship between cell-matrix adhesion and endocytosis. A recent study showed that nascent adhesive structures, the focal-adhesion complexes, might be targeted for endocytosis from inside the cell through the interaction of dynamin-2 with the key regulatory component of these complexes, focal adhesion kinase, to modulate cell-matrix adhesion and migration (Ezratti et al., 2005). The present work describes yet-another functional link between a constituent of cell-matrix adhesive structures, cell-surface tTG, and a major endocytic receptor, LRP1. Regulation of surface tTG by LRP1-mediated internalization endows cells with the means to efficiently adjust their interactions with the ECM in response to outside cues. Future investigations will determine the importance of this regulatory mechanism in endocytosis, cell-matrix adhesion, migration and signaling.

Materials and Methods

Cells and plasmids

Human (MRC-5, WI-38) and mouse (NIH3T3) fibroblasts and human U-251 glioma cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% antibiotic antimycotic (100×) (Invitrogen, Carlsbad, CA). Chinese hamster ovarian cells (CHO), wild-type and LRP1-deficient derivatives (13-5-1 CHO) (Fitzgerald et al., 1995), were maintained in DMEM/F-12 (1:1) medium supplemented with 5% FBS. LRP1-deficient mouse embryonic fibroblasts ($Lrp1^{-/-}$ MEF, clone PEA-13) (Willnow and Herz, 1994), fibronectin-null mouse embryonic fibroblasts ($Fn^{-/-}$ MEF, clone 7E) (Tomasini-Johansson et al., 2001) and their wild-type counterparts were maintained in DMEM supplemented with 10% FBS. U251 glioma cells and their stable transfectants expressing caveolin-1 siRNA were provided by A. Strongin (The Burnham Institute, La Jolla, CA). All the cells were grown at 37°C in an atmosphere of 5% CO_2.

Transfections of cells were performed with lipofectamine 2000 (Invitrogen), following the manufacturer's instructions. Wild-type dynamin-2 and a GTPase-deficient (K44A) mutant dynamin-2, both containing an HA tag, were a gift from S. L. Schmid (The Scripps Institute, La Jolla, CA). The plasmids were transiently expressed in MRC-5 cells. Mifepristone (Mfp)-inducible expression of full-length human tTG and deletion mutants in NIH3T3 cells and in $Fn^{-/-}$ MEFs has been described previously (Hang et al., 2005; Janiak et al., 2006).

The cDNA of human LRP1 (Ulery et al., 2000) was used as a template to generate expression vectors for the LRPβ essentially as described previously (Mikhailenko et al., 2001). Briefly, the fragment of cDNA that encodes amino acids 3844-4525 of LRP1 (GenBank™ accession number X13916) was generated by PCR amplification and subcloned into the pSecTag expression vector (Invitrogen) modified to produce a protein with two copies of the Myc epitope at its amino terminus. The mini-receptor contained a portion of the LRP1 extracellular domain (including membrane-proximal YWTD β-propeller and EGF-like repeats), transmembrane domain and cytoplasmic tail. Deletion of a cytoplasmic domain was achieved by mutation of the codon encoding Ala4432 to a termination codon using the Transformer™ site-directed mutagenesis kit (Clontech) and the construct authenticity confirmed by sequencing.

Antibodies, proteins and reagents

Rabbit polyclonal antibodies against EEA1, Rab7, Arf1, Lamp-1, caveolin-1, clathrin heavy chain and rat anti-mouse β1 integrin mAb 9EG7 were all obtained from BD Biosciences (San Jose, CA). Rabbit antibody against the cytoplasmic domain of β1 integrin, anti-rabbit and anti-mouse IgG conjugated with peroxidase were all sourced from Chemicon International (Temecula, CA). Mouse mAbs TG100 and CUB7402 against human tTG were obtained from NeoMarkers (Freemont, CA). Mouse anti-tTG mAb 4G3 has been described previously (Akimov and Belkin, 2001b). Fab fragments of mAb 4G3 were prepared by limited proteolysis using papain immobilized on agarose (Pierce, Rockford, IL). Rabbit affinity-purified antibody Rb2629 and mAbs 11E4 and 5A6 against LRP1 were generated in the laboratory of D.K.S. The polyclonal affinity-purified antibody to fibronectin has been described previously (Akimov and Belkin, 2001a). Mouse mAb 9E10 against a c-Myc tag and a rabbit polyclonal antibody against an HA tag were obtained from Abgent (San Diego, CA).

tTG was purified from human red blood cells as described previously (Radek et al., 1993). LRP1 was purified from bovine placenta as previously reported (Ashcom et al., 1990). The LRP1 antagonist, receptor-associated protein (RAP) was prepared as described earlier (Williams et al., 1992). The soluble VLDL receptor fragment containing ligand-binding repeats was prepared and characterized as reported previously (Ruiz et al., 2005). Purified human plasma fibronectin was obtained from Chemicon.

FITC-labeled transferrin was acquired from Molecular Probes (Eugene, OR). Human recombinant PDGF-BB was sourced from R&D Systems (Minneapolis, MN). Sulfo-NHS-LC-biotin, sulfo-NHS-SS-biotin, NeutrAvidin-agarose and SuperSignal West Pico chemiluminescent substrate were obtained from Pierce. Filipin, methyl-β-cyclodextrin (M-β-CD), bafilomycin and chloroquine were obtained from Sigma (St Louis, MO).

Cell-surface biotinylation and endocytosis assays

To compare the overall levels of cell-surface tTG in Mfp-induced NIH3T3 fibroblasts expressing tTG in the absence or presence of endocytosis inhibitors, the cells were labeled using cell-impermeable sulfo-NHS-LC-biotin as described previously (Janiak et al., 2006).

For endocytosis assays, cell monolayers were labeled with cell-impermeable SH-cleavable sulfo-NHS-SS-biotin. After biotinylation and quenching, the cells were incubated in cell-culture medium at 37°C with or without inhibitors to allow internalization of biotinylated proteins. To remove the remaining biotin residues from the cell-surface proteins, the cells were washed with ice-cold PBS and incubated three times for 10 minutes on ice with 50 mM cell-impermeable reducing reagent MESNA (sodium 2-mercaptoethane sulfonate) in stripping buffer (50 mM Tris-HCl, pH 8.4, 150 mM NaCl). Preparation of cell extracts and isolation of biotinylated (internalized) proteins were performed as described previously (Janiak

et al., 2006). Proteins isolated on NeutrAvidin-agarose were subjected to SDS-PAGE in Novex 4-12% polyacrylamide Bis-Tris gels (Invitrogen) followed by electrotransfer onto Immobilon-P membrane (Millipore, Billerica, MA) and immunoblotting.

To study internalization of cell-surface tTG in fibronectin-null mouse embryonic fibroblasts ($Fn^{-/-}$ MEF, clone 7E), the cells growing in DMEM supplemented with 10% fibronectin-depleted FBS were treated with Mfp for 24 hours. Purified fibronectin (50 μg/ml) was added to some dishes 3 hours before cell biotinylation. The cells were labeled with sulfo-NHS-SS-biotin and used in the endocytosis assay as described above. Protein fractions isolated on NeutrAvidin-agarose were analyzed by SDS-PAGE and immunoblotting.

To inhibit endocytosis, the cell monolayers were treated with the general inhibitor of endocytosis, M-β-CD, or the specific inhibitor of lipid rafts or caveolae, filipin. To block endo-lysosomal function, cells were treated with bafilomycin or chloroquine. To specifically inhibit clathrin-dependent endocytosis by hyperosmotic shock, cells were incubated in 0.45 M sucrose. To block LRP1-mediated endocytosis, cells were incubated with 100 ng/ml RAP.

Ultracentrifugation in discontinuous sucrose gradients

To analyze the membrane distribution of various proteins, cell homogenates were subjected to detergent-free fractionation by ultracentrifugation in a discontinuous sucrose gradient, essentially as described previously (Song et al., 1996). Briefly, NIH3T3 fibroblasts lacking or expressing tTG were labeled with sulfo-NHS-LC-biotin, scrapped in 0.5 M carbonate buffer (pH 11.0) on ice and sonicated. The resulting homogenates were mixed with 80% sucrose in MBS (25 mM MES, pH 6.5, 150 mM NaCl) to a final concentration of 45% sucrose and placed on the bottom of the centrifuge tube. A 5-35% discontinuous sucrose gradient was formed above (4 ml of 35% sucrose and 4 ml of 5% sucrose; both in MBS) and centrifuged at 39,000 rpm for 18 hours in an SW41 rotor (Beckman Instruments, Palo Alto, CA). After centrifugation, 1 ml gradient fractions were collected, adjusted to 1% SDS by addition of 50 μl of 20% SDS, boiled and incubated with NeutrAvidin-agarose to isolate biotinylated proteins. The isolated membrane protein fractions were analyzed by SDS-PAGE and immunoblotting.

Analysis of the interaction of tTG and LRP1 in vitro

To study the interaction between tTG and LRP1 in vitro, purified human tTG in TBS (10 μg/ml) was immobilized in polystyrene-coated microtiter plates by incubation for 3 hours at room temperature. As positive and negative binding controls, RAP and BSA were immobilized in parallel wells. The wells were blocked with 3% BSA in TBS for 1 hour at room temperature, washed and the immobilized proteins incubated with purified LRP1 (0-300 nM) in the binding buffer (1% BSA in TBS) for 1 hour at 37°C. LRP1 bound to the proteins was detected with 0.1 μg/ml mouse mAb 11E4 to human LRP1 and 0.2 μg/ml secondary anti-mouse IgG conjugated with peroxidase. The reaction was developed with a SureBlue TMB microwell peroxidase chromogenic substrate (KPL, Gaithersburg, MD), stopped with 1 M HCl and measured by spectrophotometry at 450 nm. In reciprocal experiments, purified LRP1 and soluble VLDL receptor were immobilized in microtiter plates, and human tTG (0-1500 nM) in the binding buffer (1% BSA in TBS) was incubated with immobilized proteins. Bound tTG was detected with 0.1 μg/ml mouse mAb TG100.

Immunofluorescence microscopy

To analyze the localization of internalized Fab fragments of anti-tTG–4G3-mAb in the antibody-uptake experiments, WI-38 fibroblasts grown on fibronectin-coated glass coverslips were washed with ice-cold PBS and incubated with 10 μg/ml Fab fragments in PBS with 0.1% BSA for 1 hour on ice. Cells with surface-bound Fab fragments were washed with cold PBS-BSA and warmed up for the indicated time periods at 37°C in DMEM-FBS. Next, the cells were rinsed with cold PBS and incubated in cold 0.1 M glycine-HCl (pH 2.5) twice for 5 minutes to strip the remaining Fab fragments from the cell surface. The cells were fixed with 3% paraformaldehyde, permeabilized with 0.5% Triton X-100 in PBS and incubated with antibodies against the endocytic markers or clathrin. The internalized tTG–4G3-Fab complexes were detected with Alexa Fluor 594 goat anti-mouse IgG that reacts with Fab fragments of mouse IgG, whereas clathrin and the endocytic markers were visualized with Alexa Fluor 488 goat anti-rabbit IgG.

To study internalization of anti-tTG–4G3-Fab in MRC-5 fibroblasts expressing HA-tagged dynamin-2 or its inactive K44A mutant, the cells with internalized tTG–4G3-Fab complexes were labeled with a rabbit polyclonal antibody to the HA tag, followed by Alexa Fluor 594 goat anti-mouse IgG and Alexa Fluor 488 goat anti-rabbit IgG.

In double-antibody-uptake experiments, co-internalization of anti-tTG–4G3-Fab and either rat mAb 9EG7 against β1 integrin or rabbit anti-LRP1 antibody Rb2629 was examined. In this case, the latter internalized protein-antibody complexes were detected with either Alexa Fluor 488 goat anti-rat IgG or Alexa Fluor 488 goat anti-rabbit IgG. Also, co-internalization assays were performed with anti-tTG–4G3-Fab and 5 μg/ml FITC-labeled transferrin.

To examine the relationship of cell-surface tTG, β1 integrins and caveolae, triple staining of live non-permeabilized MRC-5 fibroblasts was performed with mouse anti-tTG–4G3-mAb, rat anti-β1 integrin mAb 9EG7 and rabbit anti-caveolin-1 antibody. The cell-surface proteins were visualized, respectively, with Alexa Fluor 488 goat anti-mouse IgG, Alexa Fluor 350 goat anti-rat IgG and Alexa Fluor 594 goat anti-rabbit IgG.

Cells were viewed and photographed with 63× and 100× objectives using a Nikon Eclipse E800 microscope (Nikon, Melville, NY) and SPOT RT digital camera. Images were acquired and digitally merged with Advance Spot software (Diagnostic Instruments, Sterling Heights, MI).

Immunoprecipitation, SDS-PAGE and immunoblotting

To study the interaction of β1 integrins, tTG and LRP1-tTG, β1 integrins or LRP1 were immunoprecipitated from the RIPA extracts of NIH3T3 fibroblasts expressing or lacking tTG (Janiak et al., 2006). To examine an effect of PDGFRβ activation on the LRP1–β1-integrin complexes, quiescent cells were stimulated with PDGF-BB (20 ng/ml) for 5 minutes. The cells were lysed in ice-cold lysis buffer (1% NP-40 in TBS with protease and phosphatase inhibitor cocktails), cell lysates were cleared by centrifugation (15,000 ***g***, 30 minutes, 4°C) and used for immunoprecipitation with 2 μg of rabbit polyclonal antibody against the cytoplasmic domain of β1 integrin, or mouse mAb 11E4 against LRP1, and protein-G–agarose beads. The immune complexes were analyzed by SDS-PAGE and immunoblotting with antibodies against LRP1, tTG and β1 integrin. SDS-PAGE under denaturing conditions was performed in Novex 4-12% gradient polyacrylamide Bis-Tris gels using MOPS running buffer (Invitrogen). Separated proteins were electrotransferred onto PVDF membranes in a mini trans-blot electrophoretic transfer cell. Immunoblots were developed with SuperSignal West Pico chemiluminescent substrate.

Other methods

Transglutaminase activity on the surface of live cells was measured by determining incorporation of ^{3}H-putrescine in *N*,*N*-dimethylcaseine (Belkin et al., 2001). tTG-dependent adhesion of cells on the 42-kDa gelatin-binding domain of fibronectin was determined as described previously (Akimov et al., 2000).

We are grateful to Alex Strongin (The Burnham Institute) for the generous gift of U251 glioma cells expressing caveolin-1 siRNA. We also thank Sandra L. Schmid (The Scripps Institute) for providing the plasmids encoding dynamin-2 and its dominant-negative mutant K44A. This work was supported by the NIH grants GM62895 to A.M.B. and HL50784 to D.K.S.

References

Akimov, S. S. and Belkin, A. M. (2001a). Cell-surface transglutaminase promotes fibronectin assembly via interaction with the gelatin-binding domain of fibronectin: a role in TGFβ-dependent matrix deposition. *J. Cell Sci.* **114**, 2989-3000.

Akimov, S. S. and Belkin, A. M. (2001b). Cell surface tissue transglutaminase is involved in adhesion and migration of monocytic cells on fibronectin. *Blood* **98**, 1567-1576.

Akimov, S. S. and Belkin, A. M. (2003). Opposing roles of Ras/Raf oncogenes and the MEK1/ERK signaling module in regulation of expression and adhesive function of tissue transglutaminase. *J. Biol. Chem.* **278**, 35609-35619.

Akimov, S. S., Krylov, D., Fleischman, L. F. and Belkin, A. M. (2000). Tissue transglutaminase is an integrin-binding adhesion coreceptor for fibronectin. *J. Cell Biol.* **148**, 825-838.

Altschuler, Y., Barbas, S. M., Terlecky, L. J., Tang, K., Hardy, S., Mostov, K. E. and Schmid, S. L. (1998). Redundant and distinct functions for dynamin-1 and dynamin-2 isoforms. *J. Cell Biol.* **143**, 1871-1881.

Ashcom, J. D., Tiller, S. E., Dickerson, K., Cravens, J. L., Argraves, W. S. and Strickland, D. K. (1990). The human alpha 2-macroglobulin receptor: identification of a 420-kD cell surface glycoprotein specific for the activated conformation of alpha 2-macroglobulin. *J. Cell Biol.* **110**, 1041-1048.

Belkin, A. M., Akimov, S. S., Zaritskaya, L. S., Ratnikov, B. I., Deryugina, E. I. and Strongin. A. Y. (2001). Matrix-dependent proteolysis of surface transglutaminase by membrane-type metalloproteinase regulates cancer cell adhesion and locomotion. *J. Biol. Chem.* **276**, 18415-18422.

Belkin, A. M., Zemskov, E. A., Hang, J., Akimov, S. S., Sikora, S. and Strongin, A. Y. (2004). Cell-surface-associated tissue transglutaminase is a target of MMP-2 proteolysis. *Biochemistry* **43**, 11760-11769.

Berrou, E. and Bryckaert, M. (2001). Platelet-derived growth factor inhibits smooth muscle cell adhesion to fibronectin by ERK-dependent and ERK-independent pathways. *J. Biol. Chem.* **276**, 39303-39309.

Boucher, P., Liu, P., Gotthardt, M., Hiesberger, T., Anderson, R. G. and Herz, J. (2002). Platelet-derived growth factor mediates tyrosine phosphorylation of the cytoplasmic domain of the low density lipoprotein receptor-related protein in caveolae. *J. Biol. Chem.* **277**, 15507-15513.

Caswell, P. T. and Norman, J. C. (2006). Integrin trafficking and the control of cell migration. *Traffic* **7**, 14-21.

Chavrier, P., Parton, R. G., Hauri, H. P., Simons, K. and Zerial, M. (1990). Localization of low molecular weight GTP binding proteins to exocytic and endocytic compartments. *Cell* **62**, 317-329.

Chen, J. W., Murphy, T. L., Willingham, M. C., Pastan, I. and August, J. T. (1985). Identification of two lysosomal membrane glycoproteins. *J. Cell Biol.* **101**, 85-95.
Conner, S. D. and Schmid, S. L. (2003). Regulated portals of entry into the cell. *Nature* **422**, 37-44.
Czekay, R. P., Aertgeerts, K., Curriden, S. A. and Loskutoff, D. J. (2003). Plasminogen activator inhibitor-1 detaches cells from extracellular matrices by inactivating integrins. *J. Cell Biol.* **160**, 781-791.
Damke, H., Baba, T., Warnock, D. E. and Schmid, S. L. (1994). Induction of mutant dynamin specifically blocks endocytic coated vesicle formation. *J. Cell Biol.* **127**, 915-934.
Donaldson, J. G. and Honda, A. (2005). Localization and function of Arf family GTPases. *Biochem. Soc. Trans.* **33**, 639-642.
Ezratti, E. J., Partridge, M. A. and Gundersen, G. G. (2005). Microtubule-induced focal adhesion disassembly is mediated by dynamin and focal adhesion kinase. *Nat. Cell Biol.* **7**, 581-590.
Fellin, F. M., Barsigian, C., Rich, E. and Martinez, J. (1988). Binding and cross-linking of rabbit fibronectin by rabbit hepatocytes in suspension. *J. Biol. Chem.* **263**, 1791-1797.
Fesus, L. and Piacentini, M. (2002). Transglutaminase 2, an enigmatic enzyme with diverse functions. *Trends Biochem. Sci.* **27**, 534-539.
Fitzgerald, D. J., Fryling, C. M., Zdanovsky, A., Saelinger, C. B., Kounnas, M., Winkles, J. A., Strickland, D. K. and Leppla, S. (1995). Pseudomonas exotoxin-mediated selection yields cells with altered expression of low-density lipoprotein receptor-related protein. *J. Cell Biol.* **129**, 1533-1541.
Folk, J. E. and Cole, P. W. (1966). Identification of a functional cysteine essential for the activity of guinea pig liver transglutaminase. *J. Biol. Chem.* **241**, 3238-3240.
Gaborik, Z., Szaszak, M., Szidonya, L., Balla, B., Paku, S., Catt, K. J., Clark, A. J. L. and Hunyady, L. (2001). β-Arrestin- and dynamin-dependent endocytosis of the AT_1 angiotensin receptor. *Mol. Pharmacol.* **59**, 239-247.
Greenwood, J. A., Theibert, A. B., Prestwich, G. D. and Murphy-Ullrich, J. E. (2000). Restructuring of focal adhesion plaques by PI-3 kinase: regulation by PtdIns (3,4,5)-P_3 binding to α-actinin. *J. Cell Biol.* **150**, 673-685.
Hang, J., Zemskov, E. A., Lorand, L. and Belkin, A. M. (2005). Identification of a novel recognition sequence for fibronectin within the NH2-terminal beta-sandwich domain of tissue transglutaminase. *J. Biol. Chem.* **280**, 23675-23683.
Hansen, S. H., Sandvig, K. and van Deurs, B. (1993). Clathrin and HA2 adaptors: effects of potassium depletion, hypertonic medium, and cytosol acidification. *J. Cell Biol.* **121**, 61-72.
Hasegawa, G., Suwa, M., Ichikawa, Y., Ohtsuka, T., Kumagai, S., Kikuchi, M., Sato, Y. and Saito, Y. (2003). A novel function of tissue-type transglutaminase: protein disulfide isomerase. *Biochem. J.* **373**, 793-803.
Heldin, C.-H. and Westermark, B. (1999). Mechanism of action and in vivo role of platelet-derived growth factor. *Physiol. Rev.* **79**, 1283-1316.
Henley, J. R., Cao, H. and McNiven, M. A. (1999). Participation of dynamin in the biogenesis of cytoplasmic vesicles. *FASEB J.* **2**, S243-S247.
Herz, J. and Strickland, D. K. (2001). LRP: a multifunctional scavenger and signaling receptor. *J. Clin. Invest*. **108**, 779-784.
Heuser, J. E. and Anderson, R. G. W. (1989). Hypertonic media inhibit receptor-mediated endocytosis by blocking clathrin-coated pit formation. *J. Cell Biol.* **108**, 389-400.
Janiak, A., Zemskov, E. A. and Belkin, A. M. (2006). Cell surface transglutaminase promotes RhoA activation via integrin clustering and suppression of the Src-p190RhoGAP signaling pathway. *Mol. Biol. Cell* **17**, 1606-1619.
Kirkham, M. and Parton, R. G. (2005). Clathrin-independent endocytosis: new insights into caveolae and non-caveolar lipid raft carriers. *Biochim. Biophys. Acta* **1746**, 349-363.
Le, P. U., Guay, G., Altschuler, Y. and Nabi, I. R. (2002). Caveolin-1 is a negative regulator of caveolae-mediated endocytosis to the endoplasmic reticulum. *J. Biol. Chem.* **277**, 3371-3379.
Lillis, A. P., Mikhailenko, I. and Strickland, D. K. (2005). Beyond endocytosis: LRP function in cell migration, proliferation and vascular permeability. *J. Thromb. Haemost.* **3**, 1884-1893.
Lorand, L. and Graham, R. M. (2003). Transglutaminases: crosslinking enzymes with pleiotropic functions. *Nat. Rev. Mol. Cell Biol.* **4**, 140-156.
Mikhailenko, I., Battey, F., Migliorini, M., Ruiz, J. F., Argraves, K., Moayeri, M. and Strickland, D. K. (2001). Recognition of alpha 2-macroglobulin by the low density lipoprotein receptor-related protein requires the cooperation of two ligand binding cluster regions. *J. Biol. Chem.* **276**, 39484-39491.
Mu, F. T., Callaghan, J. M., Steele-Mortimer, O., Stenmark, H., Parton, R. G., Campbell, P. L., McCluskey, J., Yeo, J. P., Tock, E. P. and Toh, B. H. (1995). EEA1, an early endosome-associated protein. *J. Biol. Chem.* **270**, 13503-13511.
Nakaoka, H., Perez, D. M., Baek, K. J., Das, T., Husain, A., Misono, K., Im, M. J. and Graham, R. M. (1994). Gh: a GTP-binding protein with transglutaminase activity and receptor signaling function. *Science* **264**, 1593-1596.
Newton, C. S., Loukinova, E., Mikhailenko, I., Ranganathan, S., Gao, Y., Haudenschild, C. and Strickland, D. K. (2005). Platelet-derived growth factor receptor-β (PDGFR-β) activation promotes its association with the low density lipoprotein receptor-related protein (LRP). Evidence for co-receptor function. *J. Biol. Chem.* **280**, 27872-27878.
Orlandi, P. A. and Fishman, P. H. (1998). Filipin-dependent inhibition of cholera toxin: evidence for toxin internalization and activation through caveolae-like domains. *J. Cell Biol.* **141**, 905-915.
Pellinen, T and Ivaska, J. (2006). Integrin traffic. *J. Cell Sci.* **119**, 3723-3731.
Powelka, A. M., Sun, J., Li, J., Gao, M., Shaw, L. M., Sonnenberg, A. and Hsu, V. W. (2004). Stimulation-dependent recycling of integrin beta1 regulated by ARF6 and Rab11. *Traffic* **5**, 20-36.
Radek, J. T., Jeong, J. M., Murthy, S. N., Ingham, K. C. and Lorand, L. (1993). Affinity of human erythrocyte transglutaminase for a 42-kDa gelatin-binding fragment of human plasma fibronectin. *Proc. Natl. Acad. Sci. USA* **90**, 3152-3156.
Rodal, S. K., Skretting, G., Garred, O., Vilhardt, F., van Deurs, B. and Sandvig, K. (1999). Extraction of cholesterol with methyl-β-cyclodextrin perturbs formation of clathrin-coated endocytic vesicles. *Mol. Biol. Cell* **10**, 961-974.
Ruiz, J., Kouiavskaia, D., Migliorini, M., Robinson, S., Saenko, E. L., Gorlatova, N., Li, D., Lawrence, D., Hyman, B. T., Weisgraber, K. H. et al. (2005). The apoE isoform binding properties of the VLDL receptor reveal marked differences from LRP and the LDL receptor. *J. Lipid Res.* **46**, 1721-1731.
Salicioni, A. M., Mizelle, K. S., Loukinova, E., Mikhailenko, I., Strickland, D. K. and Gonias, S. L. (2002). The low density lipoprotein receptor-related protein mediates fibronectin catabolism and inhibits fibronectin accumulation on cell surfaces. *J. Biol. Chem.* **277**, 16160-16166.
Salicioni, A. M., Gaultier, A., Brownlee, C., Cheezum, M. K. and Gonias, S. L. (2004). Low density lipoprotein receptor-related protein-1 promotes beta1 integrin maturation and transport to the cell surface. *J. Biol. Chem.* **279**, 10005-10012.
Schnitzer, J. E., Oh, P., Pinney, E. and Allard, J. (1994). Filipin-sensitive caveolae-mediated transport in endothelium: reduced transcytosis, scavenger endocytosis, and capillary permeability of select macromolecules. *J. Cell Biol.* **127**, 1217-1232.
Song, K. S., Li, S., Okamoto, T., Quilliam, L. A., Sargiacomo, M. and Lisanti, M. P. (1996). Co-purification and direct interaction of Ras with caveolin, an integral membrane protein of caveolae microdomains. Detergent-free purification of caveolae microdomains. *J. Biol. Chem.* **271**, 9690-9697.
Strickland, D. K., Kounnas, M. Z. and Argraves, W. S. (1995). LDL receptor-related protein: multiligand receptor for lipoprotein and proteinase catabolism. *FASEB J.* **9**, 890-898.
Subtil, A., Gaidarov, I., Kobylarz, K., Lampson, M. A., Keen, J. H. and McGraw, T. E. (1999). Acute cholesterol depletion inhibits clathrin-coated pit budding. *Proc. Natl. Acad. Sci. USA* **96**, 6775-6780.
Sun, Z., Martinez-Lemus, L. A., Trache, A., Trzeciakowski, J. P., Davis, G. E., Pohl, U. and Meininger, G. A. (2005). Mechanical properties of the interaction between fibronectin and $\alpha_5\beta_1$-integrin on vascular smooth muscle cells studied using atomic force microscopy. *Am. J. Physiol.* **289**, H2526-H2535.
Thomsen, P., Roepstorff, K., Stahlhut, M. and van Deurs, B. (2002). Caveolae are highly immobile plasma membrane microdomains, which are not involved in constitutive endocytic trafficking. *Mol. Biol. Cell* **13**, 238-250.
Tomasini-Johansson, B. R., Kaufman, N. R., Ensenberger, M. G., Ozeri, V., Hanski, E. and Mosher, D. F. (2001). A 49-residue peptide from adhesion F1 of Streptococcus pyogenes inhibits fibronectin matrix assembly. *J. Biol. Chem.* **276**, 23430-23439.
Torgersen, M. L., Skretting, G., van Deurs, B. and Sandvig, K. (2001). Internalization of cholera toxin by different endocytic mechanisms. *J. Cell Sci.* **114**, 3737-3747.
Turner, P. M. and Lorand, L. (1989). Complexation of fibronectin with tissue transglutaminase. *Biochemistry* **28**, 628-635.
Ulery, P. G., Beers, J., Mikhailenko, I., Tanzi, R. E., Rebeck, G. W., Hyman, B. T. and Strickland, D. K. (2000). Modulation of beta-amyloid precursor protein processing by the low density lipoprotein receptor-related protein (LRP). Evidence that LRP contributes to the pathogenesis of Alzheimer's disease. *J. Biol. Chem.* **275**, 7410-7415.
Williams, S. E., Ashcom, J. D., Argraves, W. S. and Strickland, D. K. (1992). A novel mechanism for controlling the activity of alpha 2-macroglobulin receptor/low density lipoprotein receptor-related protein. Multiple regulatory sites for 39-kDa receptor-associated protein. *J. Biol. Chem.* **267**, 9035-9040.
Willnow, T. E. and Herz, J. (1994). Genetic deficiency in low density lipoprotein receptor-related protein confers cellular resistance to Pseudomonas exotoxin A. Evidence that this protein is required for uptake and degradation of multiple ligands. *J. Cell Sci.* **107**, 719-726.
Zemskov, E. A., Janiak, A., Hang, J., Waghray, A. and Belkin, A. M. (2006). The role of tissue transglutaminase in cell-matrix interactions. *Front. Biosci.* **11**, 1057-1076.