The TGF-β co-receptor, CD109, promotes internalization and degradation of TGF-β receptors

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ABSTRACT

Transforming growth factor-β (TGF-β) is implicated in numerous pathological disorders, including cancer and mediates a broad range of biological responses by signaling through the type I and II TGF-β receptors. Internalization of these receptors via the clathrin-coated pits pathway facilitates SMAD-mediated signaling, whereas internalization via the caveolae pathway is associated with receptor degradation. Thus, molecules that modulate receptor endocytosis are likely to play a critical role in regulating TGF-β action. We previously identified CD109, a GPI-anchored protein, as a TGF-β co-receptor and a negative regulator of TGF-β signaling. Here, we demonstrate that CD109 associates with caveolin-1, a major component of the caveolae. Moreover, CD109 increases binding of TGF-β to its receptors and enhances their internalization via the caveolae. In addition, CD109 promotes localization of the TGF-β receptors into the caveolar compartment in the presence of ligand and facilitates TGF-β receptor endocytosis and degradation to inhibit TGF-β signaling.

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1. Introduction

TGF-β, a multifunctional growth factor with three different subtypes, TGF-β1, β2, β3, controls numerous cellular processes such as growth, differentiation, migration and extracellular matrix deposition, and thus plays a crucial role in development and homeostasis [1]. Dysregulation of its signaling pathway has been implicated in tissue fibrosis and cancer, underscoring the critical importance of a tight regulation of TGF-β action.

TGF-β signaling is transduced by a pair of transmembrane serine/threonine kinases known as type I (TGFBR1) and II (TGFBR2) receptors [2]. The binding of TGF-β to TGFBR1, a constitutively active kinase, results in the phosphorylation of TGFBR1. The activated TGFBR1 then propagates the signal by phosphorylating its intracellular substrates SMAD2 and SMAD3, which in turn form complexes with SMAD4. The complexes accumulate in the nucleus, where they regulate gene expression [3].

In addition to the type I and II receptors, many cell types also express the TGF-β co-receptors betaglycan and endoglin [4]. Our group has recently identified another TGF-β co-receptor, CD109, in skin cells [5]. CD109 is a 180 kDa glycosylphosphatidylinositol (GPI)-anchored protein that belongs to the α2-macroglobulin/complement family [6]. CD109 binds the TGF-β1 subtype with high affinity, forms a heteromeric complex with the TGF-β signaling receptors and inhibits TGF-β signaling and responses in a variety of cell types [5]. Moreover, recent reports have shown that CD109 expression is deregulated in basal-like breast carcinoma [7], squamous cell carcinoma [8–11] and melanoma [5] and that CD109 is mutated in colorectal cancer [12]. Despite its important role in TGF-β signaling and its potential significance in cancer progression, its mechanism of action is not yet understood.

Receptor endocytosis is a pivotal regulatory mechanism in signal transduction. TGF-β receptors are internalized via both clathrin- and caveolae-dependent pathways [13]. Internalization of the TGF-β receptors via the clathrin-coated pits has been linked with signaling via SMAD2/3 and receptor recycling [14–17]. In contrast, TGF-β receptor localization in caveolae is associated with downregulation of SMAD2/3 signaling and receptor degradation following ubiquitination by the E3-ubiquitin ligase Smurf2 [13,18].

Because CD109 is a negative regulator of TGF-β signaling, we investigated whether CD109 exerts this effect by modulating caveolae-mediated internalization of the TGF-β receptors or receptor degradation.

Abbreviations: TGF-β, transforming growth factor-β; Smurf, SMAD ubiquitination regulatory factor; GPI, glycosylphosphatidylinositol; MβCD, methyl-β-cyclodextrin; EFA-1, early endosome antigen-1; HA, hemagglutinin
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2. Materials and methods

2.1. Cell lines

The human keratinocyte cell line HaCaT, kindly provided by P. Boukamp (Heidelberg, Germany), Mv1Lu cells and human embryonic kidney 293 cells purchased from the American Type Culture Collection, were cultured as described previously [5]. HaCaT clones stably expressing CD109 (or its empty vector, EV) were selected and cultured in the presence of 0.5 mg/ml Geneticin (Invitrogen, Carlsbad, CA, USA).

2.2. Transient transfections and siRNA treatment

Transfections of different combinations of the following plasmids: CD109 or its empty vector (pCMVSPORT6), caveolin-1 (gift from C. Hardin, University of Missouri), Dyn2K44A (gift from S. Egan, University of Toronto), TGFBR2 and TGFBR1-WT (gifts from J. Wrana, University of Toronto) were performed using Superfect (Qiagen, Mississauga, ON, Canada). Alternatively, cells were transfected with CD109 siRNA (cat.#129083), caveolin-1 siRNA (cat.#10297) or a negative control siRNA (cat.#4611) (Ambion, Austin, TX, USA) using Lipofectamine 2000 (Invitrogen).

2.3. Affinity labeling

293 cells or HaCaT cells were incubated with 100 pM 125I-TGF-β1 for 3 h at 4 °C, the ligand was cross-linked to the receptors with a non-permeable cross-linker BS3 (Pierce, Rockford, IL) and the cells were incubated at 37 °C for 0 to 8 h prior to lysis as described previously [19]. Protein concentrations were determined using a Lowry protein assay (Bio-Rad, Mississauga, ON, Canada) and the lysates were analyzed by SDS-PAGE/autoradiography. Densitometry was performed using the ImageJ software.

2.4. Immunoprecipitation and western blot

Lysates from 293 cells were immunoprecipitated with a mouse monoclonal anti-caveolin-1 antibody (clone 2297, BD Biosciences, Mississauga, ON, Canada). Lysates from HaCaT cells were immunoprecipitated with mouse monoclonal anti-CD109 (gift from R&D systems, Minneapolis, MN, USA) or a rabbit polyclonal anti-caveolin-1 antibody (BD Biosciences), while a mouse monoclonal anti-histone H1 or a rabbit polyclonal anti-HA antibodies (both from Santa Cruz Biotechnology, Santa Cruz, CA, USA) were used as control IgG. Western blot analyses were conducted with the following antibodies: mouse monoclonal anti-CD109 (TEA 2/16, BD Biosciences), anti-EEA-1 (BD Biosciences), anti-SMAD3 (FL-425) and anti-actin (H-300) antibodies (both form Santa Cruz Biotechnology), rabbit polyclonal anti-TGFBR1 and anti-phospho-SMAD3 antibodies (both from Cell Signaling Technologies, Danvers, MA, USA).

2.5. Internalization assay

Internalization assay was performed as described in Ref. [20] with some modifications. Briefly, cells were treated with 2.5 mM MβCD (Sigma Aldrich, Oakville, ON, Canada) for 1 h at 37 °C prior to internalization assay. Cells were then incubated with 100 pM 125I-TGF-β1 (Perkin Elmer, Waltham, MA, USA) with or without 10 nM unlabeled TGF-β1 (to determine non-specific binding) for 2 h at 4 °C and transferred to 37 °C for the indicated time. Surface ligand was removed with 150 mM NaCl, 0.1% acetic acid, 2 M urea. Cells were then solubilized in order to extract internalized ligand.

2.6. Sucrose gradient fractionation

Preparation of membrane fraction from HaCaT cells was performed as described in Ref. [21]. The twelve fractions collected were analyzed by western blot for EEA-1 and caveolin-1. The fractions containing EEA-1 or caveolin-1 were pooled and equal amounts of protein were analyzed by SDS-PAGE/autoradiography or western blot.

2.7. Immunofluorescence microscopy

Cell surface proteins were extracted using the Hook Cell Surface Protein Isolation kit (G-Biosciences, St. Louis, MO, USA). Briefly, cell surface proteins were labeled with Hook-sulfo-NHS-SS-Biotin and lysed. An aliquot was analyzed by western blot for actin to make sure that equal amount of protein was used. Biotinylated proteins from the remaining lysates were isolated using streptavidin-agaro column, eluted and analyzed by western blot.

2.9. Statistical methods

Numerical results are represented as means of \( n \geq 3 \) independent experiments ± SEM. Statistical significance between two groups was determined by two-tailed Student t test. Comparisons within more than two groups were made by one-way analysis of variance. Multiple comparisons were made by Holm–Sidak test (post-hoc).

3. Results

3.1. CD109 associates with caveolin-1 at endogenous concentration

We sought to determine whether CD109 exerts its negative effects on TGF-β signaling by modulating TGF-β receptor localization into the caveolae, a process shown to enhance TGF-β receptor degradation. We first examined whether CD109, a GPI-anchored protein, can associate with caveolin-1, a major component of the caveolae. Co-immunoprecipitation experiments reveal that CD109 associates with caveolin-1 in 293 cells co-transfected with both CD109 and caveolin-1 (Fig. 1A). In the absence of caveolin-1 transfection, (i.e. when caveolin-1 expression is undetectable [24]), CD109 is not co-immunoprecipitated (Fig. 1A), demonstrating the specificity of the association. In untransfected HaCaT cells, caveolin-1 is co-immunoprecipitated by an anti-CD109 antibody (Fig. 1B), confirming that the interaction between CD109 and caveolin-1 is not an artifact of overexpression and that it occurs at endogenous concentrations of caveolin-1 and CD109 (Fig. 1B). Similarly, an anti-caveolin-1 antibody co-immunoprecipitates small but detectable amounts of CD109 (Supplementary Material, Fig. S1).
Fig. S1 and Fig. 1C). Importantly, the association between CD109 and caveolin-1 is enhanced after TGF-β treatment (Fig. 1C).

Immunofluorescence microscopy of HaCaT cells (Fig. 1D) shows that caveolin-1 (in red) displays a punctate staining pattern at the plasma membrane, with some punctate staining in the cytoplasm, which may represent neo-synthesized or internalized caveolin-1. Endogenous CD109 (blue) localizes mainly at the plasma membrane in both non-caveolar and caveolin-1 positive compartments. The colocalization of CD109 with caveolin-1 (in purple, Fig. 1D) suggests that CD109 is able to associate with caveolin-1 in the same cellular compartment, supporting the results obtained in Fig. 1A–C.

3.2. CD109 and TGF-β receptors colocalize in caveolae

We have previously shown that CD109 binds directly to TGFBR1 [5]. We therefore sought to determine whether TGF-β receptors colocalize with CD109 in caveolae. Because the endogenous levels of TGF-β receptors in HaCaT cells are too low to allow detection of the internalized ligand, 293 cells were transfected with TGFBR1/TGFBR2 and caveolin-1 and labeled with biotin-TGF-β, followed by streptavidin-AF647 treatment. After 30 min at 37 °C to allow receptor and ligand internalization, the cells were immunostained for CD109 and caveolin-1 (Fig. 1E). Importantly, a significant amount of triple colocalization (visualized in white) is observed between the internalized TGF-β (in green), CD109 (in blue), and caveolin-1 (in red) (Fig. 1E). The proportion of internalized biotin-TGF-β that is not associated with CD109 and caveolin-1 (Fig. 1E) may represent molecules that are internalized via the clathrin pathway (associated with SMAD2/3 signaling) or that are in recycling compartments. In the absence of TGF-β treatment, CD109 localizes mainly at the plasma membrane, where it partially co-localizes with caveolin-1 (Fig. 1F), as observed in untransfected HaCaT cells (Fig. 1D). When the cells were incubated at 4 °C instead of 37 °C, no TGF-β staining was detected (data not shown), indicating that at 4 °C, the receptors are not internalized. Interestingly, in the absence of TGF-β receptor transfection, low levels of co-localization between CD109 and caveolin-1 could be observed (Fig. 1G and Supplementary Fig. S2A). It is possible that the interaction between the GPI-anchored CD109 and the integral membrane protein caveolin-1 is mediated via endogenous TGF-β receptors in that case, although the contribution of other transmembrane proteins or lipid molecules cannot be ruled out.

The notion that the biotin-TGF-β staining is specific for biotin-TGF-β bound to TGFBR1/TGFBR2 complex and not to other TGF-β binding molecules under our detection settings is supported by the following results: (1) when TGFBR1/TGFBR2 are not transfected, no staining is observed for biotin-TGF-β, even in the presence of CD109 (Fig. 1G); (2) biotin-TGF-β colocalizes with HA-tagged TGFBR1 (Supplementary Fig. S2C). This suggests that TGF-β follows the same internalization route as TGFBR1, as reported by others [13,20]; (3) HA-TGFBR1 co-localizes with CD109 in caveolar vesicles and at the plasma membrane (Supplementary Fig. S2D). Together, these data indicate that biotin-TGF-β can be used to assess TGF-β receptor localization and suggest that CD109 is able to form a complex with TGF-β-bound TGF-β receptors (activated receptors) in caveolae.

3.3. CD109 enhances binding of TGF-β to TGF-β receptors and TGF-β-bound receptor internalization

We then asked if CD109 affects TGF-β-bound receptor internalization. CD109 overexpression significantly (p < 0.05, n = 5 independent experiments) decreases the number of cells that have internalized biotin-TGF-β (i.e. TGF-β-bound receptors) as compared to empty vector (EV) transfection in 293 cells (Fig. 2A and B). After normalization for transfection efficiency (as determined by caveolin-1 expression), we observe that 64% of caveolin-1 positive cells internalize biotin-TGF-β in the presence of CD109, whereas only 27% incorporate biotin-TGF-β in EV transfected cells (Fig. 2B), suggesting that CD109 increases the internalization of TGF-β-bound receptors. The amount of internalized biotin-TGF-β reflects the levels of receptors that underwent endocytosis, and also any recycling and/or degradation that may have occurred during that time period. Notably, CD109 transfection does not alter the level of TGF-β receptors or caveolin-1, as compared to EV transfection (Fig. 2B and data not shown), suggesting that CD109 only affects the amount of internalized TGF-β-bound receptors.

We next examined the effect of CD109 on the internalization of biotin-TGF-β at endogenous TGF-β receptor concentration in HaCaT cells. The 125I-TGF-β internalization assay has been shown to reliably reflect receptor internalization [20,25]. At early time points (15 and 30 min), CD109 enhances 125I-TGF-β internalization (Fig. 2C). The increase of 125I-TGF-β internalization in CD109 overexpressing cells is followed by a decline after 30 min whereas a decline occurs only after 60 min in EV transfected cells (Fig. 2C). The decline in internalized 125I-TGF-β has been reported previously and might be due to ligand depletion and/or degradation [20]. Importantly, our results suggest that CD109 enhances ligand-bound receptor internalization.

Interestingly, CD109 overexpression is associated not only with an increase of 125I-TGF-β internalization, but also with an increase of surface-bound 125I-TGF-β at 30 min (Fig. 2D). The ratio of internalized/surface-bound 125I-TGF-β (I/S) is higher in CD109 transfected cells, as compared to EV cells at both 15 and 30 min (Fig. 2E), suggesting that CD109 accelerates TGF-β-bound receptor endocytosis. Moreover, knocking down CD109 expression using CD109 siRNA or CD109 antisense morpholino oligos leads to a decrease in both internalized and surface-bound 125I-TGF-β at 30 min (Fig. 2F and Supplementary Fig. S3). These results indicate that endogenous CD109 increases surface binding and internalization of TGF-β.

To determine whether the increase in TGF-β binding to the cell surface is due to an increase in TGF-β binding to the TGF-β receptors and not solely due to an increase in TGF-β binding to CD109, we performed affinity labeling of cell surface receptors at 4 °C followed by SDS-PAGE analysis. Our results show that CD109 is able to enhance TGF-β binding to the receptors (Fig. 3A and B). CD109 siRNA transfection in HaCaT cells decreases and CD109 overexpression in 293 cells increases the amount of cell surface 125I-TGF-β-bound receptors (Fig. 3A and B). Notably, CD109 does not affect the total level of TGFBR1, as demonstrated by western blot (Fig. 3A and B). Moreover, overexpression of CD109 does not alter the cell-surface level of TGF-β receptors (Fig. 3B). These results, together with our previous findings demonstrating that CD109 forms a complex with the TGF-β receptors [5], suggest that such association is an important step in CD109’s ability to increase TGF-β binding to this...
multimeric TGF-β receptor complex and enhances the internalization of this TGF-β bound-complex.

3.4. CD109 enhances TGF-β receptor internalization via the caveolar pathway

Next, we assessed if the CD109-induced increase in TGF-β receptor internalization could be mediated by the caveolar compartment.

Internalization of biotin-TGF-β in EV transfected 293 cells is prevented by co-transfection of the Dynamin2-K44A mutant (Dyn2K44A) (Fig. 4A), which blocks endocytosis via both clathrin and caveolar pathways [26] and is consistent with previous reports demonstrating that TGF-β receptors are internalized via clathrin and/or caveolar pathways [13,27]. Importantly, internalization of biotin-TGF-β is also blocked by co-transfection of the Dynamin2-K44A mutant (Dyn2K44A) when CD109 is overexpressed (Fig. 4A). In the
presence of Dyn2K44A, the biotin-TGF-β staining appears at the plasma membrane in 81 (± 5.2)% of the cells and co-localizes with CD109 staining, whereas the biotin-TGF-β staining is found at the plasma membrane in only 5.1 (± 4.6)% of the cells in the absence of Dyn2K44A (Fig. 4A). This indicates that CD109 promotes dynamin-dependent internalization of the TGF-β-bound receptors.

Because CD109 is an inhibitor of TGF-β signaling [5], we looked more specifically at CD109’s ability to increase internalization via caveolae, a route that has been shown to lead to receptor degradation and signaling downregulation. We demonstrate that pre-treatment with methyl-β-cyclodextrin (MβCD), an inhibitor of the caveolae/lipid raft-dependent endocytosis [17,26] blocks CD109’s effect on 125I-TGF-β internalization in HaCaT cells (Fig. 4B). The increase in TGF-β internalization mediated by CD109 is also reversed by caveolin-1 siRNA (Fig. 4C), confirming that CD109 is able to enhance caveolae-mediated endocytosis. The slight (and non-significant)
TGF-β receptors have been shown to traffic independently of ligand addition [13,17]. Thus, we determined whether the effect of CD109 on TGFBR1 trafficking occurs in a ligand-independent manner. By sucrose gradient centrifugation, the raft fractions (fractions 5 and 6) were separated from the non-raft fractions (fractions 8 to 12). The purity of the raft and the non-raft fraction was verified using the early endosome antigen-1 (EEA-1) and caveolin-1, two markers conventionally used for the non-raft/endosomal and the raft/caveolar compartment, respectively [13,21] (Fig. 5A). The analysis of TGFBR1 and CD109 localization in the pooled raft and pooled non-raft fractions were performed on the same gel by western blot (Fig. 5B). In the absence of exogenous TGF-β, TGFBR1 is present predominantly in the non-raft fraction in both CD109 and EV transfected cells. In contrast, TGF-β treatment leads to a marked increase in the amount of TGFBR1 in the raft fraction of CD109 transfected cells as compared to EV transfected cells (Fig. 5B). This effect is consistent with our previous results in this cell type (Fig. 4B) showing that only a small amount of TGF-β-bound receptors is internalized via the lipid raft/caveolar pathway in EV transfected HaCaT cells. Under basal conditions, CD109 localizes mainly in the non-raft compartment, but is also present in the lipid raft compartment (visualized on a more exposed film), which agrees with our immunofluorescence data (Fig. 1D). The effect of TGF-β on the pattern of CD109 localization is similar to the one observed for TGFBR1: TGF-β treatment increases the localization of CD109 in the raft compartment, which is consistent with our co-immunoprecipitation data (Fig. 1C). Together, these results suggest that CD109 promotes
TGFBR1 entry into the lipid raft and that this effect is enhanced by TGF-β treatment.

We further examined the ability of CD109 to induce TGF-β-bound receptor localization to the caveolar compartment by first affinity labeling the receptors on HaCaT cells with 125I-TGF-β and then performing sucrose gradient fractionation as described above. In CD109 transfected cells, the majority of TGF-β-bound TGFBR1 localizes into the caveolin-1 positive/lipid raft fraction, whereas in EV transfectants, TGFBR1 resides mainly in the non-raft fraction (Fig. 5C). Similar results were obtained with TGF-β-bound TGFBR2 (data not shown). These results indicate that CD109 leads to preferential localization of TGFBR1 into the lipid raft compartment, in the presence of TGF-β.

3.6. Inhibition of TGF-β signaling by CD109 involves caveolae-mediated internalization

The results presented so far show that CD109 promotes TGF-β receptor internalization via the caveolae. We next investigated if this step is involved in mediating CD109’s inhibitory effect on TGF-β signaling. Our results show that overexpression of CD109 inhibits TGF-β-induced SMAD3 phosphorylation (Fig. 6A), as expected [5]. This inhibitory effect of CD109 is abrogated by co-transfection of caveolin-1 siRNA (Fig. 6A), indicating that CD109’s effects on TGF-β signaling involve the caveolar pathway. Similarly, knocking down CD109 expression using siRNA results in an increase in TGF-β-induced SMAD3 phosphorylation, and this effect is blocked by co-transfection

Fig. 4. CD109 enhances TGF-β internalization via the caveolar pathway. (A) Immunofluorescence: 293 cells transfected with TGFBR1/TGFBR2, CD109 (or its EV) and Dyn2K44A (or its EV) were treated with biotin-TGF-β/streptavidin (green), incubated for 30 min at 37 °C and stained for CD109 (blue). Colocalization of CD109 and biotin-TGF-β appears in turquoise (overlay). Representative cells are shown. (B–D) 125I-TGF-β internalization assay: (B) HaCaT cells transfected with CD109 or EV were treated with or without MβCD prior to incubation with 125I-TGF-β. The graph shows the amounts of internalized 125I-TGF-β after 30 min incubation at 37 °C (normalized to EV, no treatment). (C) Left panel: Amount of internalized 125I-TGF-β after 30 min incubation at 37 °C in HaCaT cells co-transfected with CD109 or EV and with caveolin-1 or control siRNA (normalized to EV, control siRNA). Right panel: Western blot showing caveolin-1 siRNA efficacy. (D) Left panel: Mv1Lu cells transfected with CD109 siRNA or control siRNA were treated with or without MβCD prior to incubation with 125I-TGF-β. The graph shows the amounts of internalized 125I-TGF-β after 30 min incubation at 37 °C (normalized to CD109 siRNA, no treatment). Right panel: Western blot showing CD109 siRNA efficacy in Mv1Lu cells. All graphs show the mean of n ≥ 3 independent experiments±SEM, *: p<0.05.
of caveolin-1 siRNA (Fig. 6B). This result suggests that downregulation of TGF-β signaling by endogenous CD109 involves the caveolar pathway.

3.7. CD109 accelerates TGF-β receptor degradation

Localization of TGF-β receptors to caveolae has been shown to be associated not only with a decrease in SMAD2/3 phosphorylation but also with receptor degradation [13]. Therefore, we tested if CD109 modulates TGF-β receptor degradation, by monitoring the levels of affinity labeled receptors (Fig. 7A). Consistent with our previous findings, CD109 overexpression increases the binding of 125I-TGF-β to its receptors, compared to EV transfection (see time 0). TGFBR1, TGFBR2 and CD109 levels decrease over time, suggesting that the CD109-TGF-β-bound-receptor complex is being degraded (Fig. 7A). Quantification of TGFBR1 levels by densitometry (expressed as a percentage of time 0) reveals that CD109 accelerates degradation of TGF-β-bound receptor (Fig. 7A, right panel, p < 0.05). In addition, stable transfection of CD109 in HaCaT cells (clone CD3-3 and CD2-4) accelerates the degradation of endogenous TGFBR1 and TGFBR2, as compared to stable transfection of EV (clone EV3-3 and EV1-6) (Fig. 7B and Supplementary Fig. S4, p < 0.05). Furthermore, the increased rate of degradation is prevented by MG132, a proteasome inhibitor, indicating that CD109 enhances proteasomal degradation of TGF-β receptors (Fig. 7B). Moreover, HaCaT cells transfected with CD109 siRNA and treated with TGF-β display a significant (p < 0.05) increase in TGFBR1 levels, as compared to control siRNA transfection and this effect of CD109 can be blocked by addition of MG132, but not chloroquine, an inhibitor of lysosomal degradation (Fig. 7C). These results indicate that endogenous CD109 facilitates proteasomal degradation of TGF-β receptors and confirm that the previous results (Fig. 7A and B) were not an artifact due to CD109 overexpression. Collectively, our results suggest that CD109 negatively regulates TGF-β action by promoting TGF-β receptor internalization to the caveolar compartment and by enhancing TGFBR1 degradation via the proteasomal pathway (Fig. 8).

4. Discussion

Our group has previously identified CD109, a GPI-anchored protein, as a novel TGF-β co-receptor [5]. CD109 has high affinity for TGF-β1, forms a complex with the TGF-β signaling receptors and negatively regulates TGF-β signaling in vitro [5]. Mutation of CD109 or deregulation of its expression has been reported in many cancers [7–12,28]. Because of CD109’s potential relevance as a regulator of TGF-β action in vivo, we examined the mechanism by which it regulates TGF-
β signaling. Here, we show that CD109 increases TGF-β binding to its receptors, promotes TGF-β receptor compartmentalization and internalization into the caveolae and accelerates TGF-β receptor proteasomal degradation. Together, these findings suggest that downregulation of TGF-β signaling by CD109 involves these mechanisms (Fig. 8). In addition, our results suggest that ligand addition may regulate CD109's effect on TGF-β receptor localization to lipid raft/caveolar compartment.

Caveolae serve as platforms for signaling molecule assembly, endocytosis of receptors, and modulation of signaling [29]. They are a subset of lipid rafts characterized by the insertion of caveolin-1 in the membrane bilayer and are enriched in GPI-anchored proteins. Here, we report that caveolin-1 associates with CD109, suggesting that CD109 can localize to caveolae. This association is likely indirect and may involve lipid molecules or transmembrane proteins, such as TGFBR1, which has been shown to interact directly with both CD109 [5] and caveolin-1 [18]. Interestingly, immunofluorescence and sucrose gradient analyses reveal that CD109 is present in both caveolar and non-caveolar compartments. Whether the non-caveolar compartment represents a storage area for CD109 or whether CD109 has other roles in that compartment is not known.

The ability of CD109 to enhance the binding of TGF-β to its receptors on one hand and to inhibit TGF-β signaling on the other suggests that CD109 may direct the ligand and its receptors into a compartment where they cannot signal. Although our results do not rule out the possibility that CD109 may also play a role in clathrin-mediated endocytosis of TGF-β receptor, our data showing that inhibitors of caveolae-mediated endocytosis reverse CD109's effect on TGF-β internalization suggest that CD109 facilitates TGF-β-bound receptor internalization via the caveolae, a compartment associated with TGF-β receptor degradation and signaling downregulation [13,18]. Accumulation of receptors in caveolae has been shown to trigger the rapid internalization via caveolae [30]. Thus, CD109 may facilitate receptor clustering in the presence of ligand to promote their internalization via the caveolar route. Together, our study demonstrates that activated TGF-β receptors can internalize via the caveolae and that this event is regulated by CD109. Thus, an alteration in CD109 concentration, as seen in many cancers, may lead to a change in the proportion of TGF-β receptor internalizing via caveolae, resulting in aberrant TGF-β signaling and action.

Several molecules have been implicated in the regulation of TGF-β receptor compartmentalization. Hyaluronan has been shown to modulate TGF-β receptor partitioning to caveolae [21], but the question of hyaluronan controlling TGF-β receptor degradation remains open. The TGF-β co-receptors betaglycan and its homologue endoglin have been shown to facilitate TGF-β receptor endocytosis through their interaction with β-arrestin2, a component of the clathrin-coated pits [31,32]. However, there is a discrepancy in the results reported, since internalization via the clathrin pathway leads to inhibition of TGF-β signaling in one study [31], and delay in receptor degradation in another study [33]. The proportion of TGF-β receptors that internalize via the caveolae may vary depending on the cell type (possibly due to a difference in the levels of caveolin, receptors and/or co-receptors), as observed in the current study, and may explain the discrepancy. Here, we demonstrate that, in contrast to other TGF-β co-receptors, CD109 may regulate TGF-β receptor internalization and degradation in a ligand-dependent manner.

Although the events downstream of TGF-β receptor internalization are ligand-dependent [34,35], studies on TGF-β receptor endocytosis have been unable to demonstrate an effect of ligand on TGF-β receptor compartmentalization or trafficking [13,36]. Our results reveal that ligand may play a role in CD109's effect on TGF-β receptor compartmentalization. By sucrose gradient fractionation, we show that CD109 promotes localization of TGFBR1 into the caveolae, in the presence of TGF-β. Our findings are in line with the results obtained for several receptors, such as insulin, β2adrenergic and EGF receptors, that localize into caveolae in a ligand-dependent manner [37–39]. Although the molecular mechanisms involved in TGF-β receptor internalization are less well understood than those of the receptors above, our study provides evidence that ligand addition can modulate TGF-β receptor localization into caveolae in the presence of CD109. These findings implicate a link between ligand-induced TGF-β receptor
compartmentalization/trafficking and ligand-induced receptor degradation. Our results demonstrating that CD109 facilitates TGF-β receptor degradation is consistent with CD109's ability to direct TGF-β receptor localization into the caveolae. Although TGF-β receptors have been shown to be degraded by both lysosomal and proteasomal machineries following receptor ubiquitination [35,40], limited data are available on factors controlling TGFBR1 proteasomal degradation. Our results showing that CD109 enhancement of TGFBR1 degradation is blocked by the proteasome inhibitor MG132 but not by the lysosome inhibitor chloroquine, suggest that CD109 may regulate TGFBR1 proteasomal degradation.

An important finding in the present study is that CD109 inhibits SMAD3 phosphorylation in a caveolin-dependent manner. One potential explanation is that CD109, by promoting TGF-β receptor endocytosis into the caveolae, sequesters the receptors away from SMAD2/3. Furthermore, CD109 promotes TGF-β receptor degradation, which in turn can lead to inhibition of signaling. CD109 may thus dampen TGF-β responses in order to avoid a deleterious effect of excess TGF-β that often leads to many human diseases such as tissue fibrosis and cancer metastasis. Because deregulation of CD109 expression may result in aberrant TGF-β receptor internalization and degradation, CD109 may represent a novel therapeutic target for treatment of diseases in which TGF-β is known to play a pathophysiological role.

5. Conflict of interest

The authors have no competing financial interests in relation to the work described.
Supplementary materials related to this article can be found online at doi:10.1016/j.bbamcr.2011.01.028.

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