A Novel Link between Integrins, Transmembrane-4 Superfamily Proteins (CD63 and CD81), and Phosphatidylinositol 4-Kinase*  

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Enzymatic and immunochemical assays show a phosphatidylinositol 4-kinase in novel and specific complexes with proteins (CD63 and CD81) of the transmembrane 4 superfamily (TM4SF) and an integrin (αβ). The size (55 kDa) and other properties of the phosphatidylinositol 4-kinase (PI 4-K) (stimulated by nonionic detergent, inhibited by adenosine, inhibited by monoclonal antibody 4CG5) are consistent with PI 4-K type II. Notably, TM4SF proteins may link PI 4-K activity to the αβ integrin. The αβ integrin, which does not associate with TM4SF proteins, was not associated with PI 4-K. Notably, αβ-C63-CD81-PI 4-K complexes are located in focal complexes at the cell periphery rather than in focal adhesions. The novel linkage between integrins, transmembrane 4 proteins, and phosphoinositide signaling at the cell periphery may play a key role in cell motility and provides a signaling pathway distinct from conventional integrin signaling through focal adhesion kinase.

Cell adhesion mediated by transmembrane receptors in the integrin family triggers signal transduction cascades that regulate cell proliferation, apoptosis, morphology, and motility (1, 2). Activation of Rho, a small GTP-binding protein, and focal adhesion kinase (FAK) may be central events in signaling cascades initiated by most integrins (3–5). On the other hand, distinct functions for integrins expressed in the same cellular environment are suggestive of additional integrin-specific signaling pathways not yet elucidated (6–9).

Specific association between membrane proteins in the transmembrane-4 superfamily (TM4SF; tetraspan proteins) and certain β integrins, including αβ, αβ, and αβ, was previously demonstrated (10–14). A role for TM4SF proteins in signaling is suggested by their modulation of intracellular calcium, tyrosine phosphorylation, and cell proliferation (15–17). However, there has been little understanding of the mechanisms whereby TM4SF proteins might signal. Here we found that phosphatidylinositol 4-kinase is associated with αβ integrin and TM4SF proteins. This supports our hypothesis that integrin-TM4SF complexes could be a point of convergence for integrin and TM4SF protein signaling.

MATERIALS AND METHODS

Antibodies—Anti-integrin mAbs used were: anti-α, A3-X8 (18), A3-IIV5 (18), and A3-IIIF5 (18); anti-α, A5-PUI2 (19); anti-α, A6-ELE (19); anti-β, A-1AS (20). Anti-TM4 mAbs used were anti-CD63, 6H1 (10), and RUU.SP. 2.28 (21); anti-CD81, M38 (22), and SAG (23). Anti-vinculin mAb hVIN-1 was from Sigma, and mAb 8G6 to a 47-kDa cell surface protein named emerin (24) will be described. The mAb 4CG5 specifically immunoprecipitates type II PI 4-K and inhibits lipid kinase activity (25).

Immunoprecipitation and Lipid Kinase Assays—For immunoprecipitation, HT1080 cells were lysed in buffer containing 1% Brij 99, 20 mM Hepes (pH 7.5), 200 mM NaCl, 5 mM MgCl2, 200 μM Na3VO4, 2 mM NaF, 10 mM Na2P2O7, 2 mM phenylmethylsulfonyl fluoride, 10 mg/ml apro- tinin, 10 mg/ml leupeptin, and immunoprecipitates were prepared as described (11). Prior to phosphoinositide assay, immunoprecipitates were washed once in HNE buffer (20 mM Hepes (pH 7.5), 100 mM NaCl, 1 mM EDTA), and assays were performed on the beads as described (26). Briefly, samples were incubated in 20 mM Hepes (pH 7.5), 10 mM MgCl2, 50 μM ATP, 200 μM sonicated phosphoinositides (40% PS, 20% PtdIns, 20% PtdIns 4-phosphate, 20% PtdIns 4,5-diphosphate), and 50 μM γ[32P]ATP (8 mCi/nmol) for 10 min at 25 °C. Reactions were stopped with 50 μl of 1 N HCl, and lipids were extracted with 160 μl of 1:1 (v/v) chloroform:methanol and analyzed by thin layer chromatography (26).

RESULTS AND DISCUSSION

Immunofluorescent Staining of Integrins and TM4SF Proteins—To gain initial clues regarding the functional importance of integrin-TM4SF complexes, we analyzed their cellular distribution by immunofluorescence. Previous studies showed that standard fixation and permeabilization procedures removed substantial amounts of TM4SF proteins from the cell surface, thereby precluding detailed analysis of the complex distribution (11). To overcome this problem, we pretreated cells with chemical cross-linker prior to fixation and permeabilization. HT1080 cells plated on laminin in serum-free medium could assemble structures strongly resembling classical integrin focal adhesions (27, 28) as indicated by staining with anti-integrin α (Fig. 1a) or anti-vinculin (Fig. 1b) mAbs. In addition, integrin α (Fig. 1a) and vinculin (Fig. 1b) were dis-
Inability of TM4SF proteins to cluster into focal complexes and excluded from the focal adhesions (data not shown). In comparison, two TM4SF proteins (CD63 and CD81) that associate with the α6 integrin (10, 11) were detected in focal complexes but excluded from focal adhesions (Fig. 1, c and d). The αβ1 integrin (Fig. 1e) also showed peripheral focal complex-type staining but no focal adhesion staining, whereas another prominent membrane protein, emmprin (24), showed uniform punctate distribution and was not present in either focal adhesions or focal complexes (Fig. 1f). Notably, in α6-transfected RD cells (18) plated on laminin-1, fibronectin, or a 40-kDa fragment of fibronectin, both αβ1 integrin and TM4SF proteins were again detected in focal complexes and excluded from the focal adhesions (data not shown). Inability of TM4SF proteins to cluster into focal adhesions even when an appropriate integrin is present (e.g. αβ1 in Fig. 1a) suggests that function of the integrin-TM4SF complexes may be specifically relevant to focal complexes rather than focal adhesions. In subsequent experiments we focused on the αβ1-C63-CD63 complex because it is far more abundant in HT1080 cells than αβ1-CD63-CD81. ![](image)

**Fig. 1. Distribution of integrin-TM4SF complexes.** HT1080 cells in synthetic buffer (11) were allowed to spread on laminin for 90 min. Then cells were treated with a chemical cross-linker (250 μM dithiobis(succinimidyl propionate)) at 25°C for 30 min. After three washes with PBS, cells were then stained with mAbs against integrin α6 (a), vinculin (b), CD63 (c), CD81 (d), integrin α5 (e), or emmprin (f). Staining was visualized with rhodamine-conjugated goat anti-mouse Ig (b–f) or fluorescein isothiocyanate-conjugated goat anti-rat Ig (a). Focal adhesion-like structures are indicated by arrowheads (a and b).

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Contributed in complexes throughout the cell periphery in a pattern strongly resembling plasma membrane “focal complexes” that were recently described (29). In comparison, two TM4SF proteins (CD63 and CD81) that associate with the α6 integrin (10, 11) were detected in focal complexes but excluded from focal adhesions (Fig. 1, c and d). The αβ1 integrin (Fig. 1e) also showed peripheral focal complex-type staining but no focal adhesion staining, whereas another prominent membrane protein, emmprin (24), showed uniform punctate distribution and was not present in either focal adhesions or focal complexes (Fig. 1f). Notably, in α6-transfected RD cells (18) plated on laminin-1, fibronectin, or a 40-kDa fragment of fibronectin, both αβ1 integrin and TM4SF proteins were again detected in focal complexes and excluded from the focal adhesions (data not shown). Inability of TM4SF proteins to cluster into focal adhesions even when an appropriate integrin is present (e.g. αβ1 in Fig. 1a) suggests that function of the integrin-TM4SF complexes may be specifically relevant to focal complexes rather than focal adhesions. In subsequent experiments we focused on the αβ1-C63-CD63 complex because it is far more abundant in HT1080 cells than αβ1-CD63-CD81.

**Integrin-TM4SF Protein Association with Phosphatidylinositol 4-Kinase—**Lamellipodial and filopodial focal complexes may trigger signals leading to the reorganization of actin cytoskeleton and focal adhesion assembly (29, 30). Because phosphoinositides may be potent effectors of actin polymerization (31–33), we investigated whether phosphoinositide kinase activity could be co-purified with the αβ1-TM4SF complex. Integrin and TM4SF immunoprecipitates prepared from HT1080 cells were assayed for phosphoinositide kinase activity. The reaction products co-migrated on TLC plates with standard PIP but not PIP2 or PIP3 (Fig. 2A). Incorporation of [32P] into PIP was observed with α3, β1, CD63, and CD81 immunoprecipitates (Fig. 2A, lanes a and c–e) but not with α5 or negative control P3 immunoprecipitates (Fig. 2A, lanes b and f). This result is consistent with previous results showing that even when α5β1 is abundantly expressed (e.g. on HT1080 and K562 cells), it is not associated with TM4SF proteins (10–12). Notably, an immunoprecipitate of NAG2, another TM4SF protein associated with αβ1 integrin, did not exhibit associated phosphatidylinositol kinase activity (data not shown). This provides additional evidence for the specificity of interaction between αβ1-CD63-CD81 and phosphatidylinositol kinase.

Although TLC analysis separated PIP from PIP2 and PIP3, it did not discriminate between different PtdIns phosphates species, e.g. PtdIns 3-phosphate and PtdIns 4-phosphate (and PtdIns 5-phosphate, if such a product exists). To determine the position of phosphorylation, the [32P]PtdIns phosphatase product generated by the CD63 immunoprecipitate was extracted from the TLC plate, deacylated, and analyzed by HPLC. This deacylated product co-migrated identically with authentic deacylated [3H]-labeled phosphatidylinositol 4-phosphate but apart from standard phosphatidylinositol 3-phosphate (Fig. 2B). Thus, there is PI 4-K activity in the αβ1-TM4SF complex.

To determine the type of PI 4-K associated with the αβ1-TM4 complexes, lipid kinase reactions on α6 and CD63 immunoprecipitates were carried out in the presence of Triton X-100 (0.3%), adenosine (200 nM) or mAb 4C5G (5 μg/ml). Previous data showed that activity of PI 4-K type II can be stimulated by nonionic detergent and inhibited by adenosine and the 4C5G mAb, whereas all three reagents have little or no effect on PI 4-K type III (25, 34). Adding adenosine and 4C5G mAb to the reactions decreased the activity of the enzyme by 70–80%, whereas Triton X-100 had a stimulatory effect (15–20-fold) (data not shown), thus indicating that αβ1-TM4SF complex is associated with a PI 4-kinase with type II properties. This conclusion was extended by Western blotting with an anti-PI 4-K polyclonal antibody that detected a protein of 55 kDa, characteristic of PI 4-K type II. Notably, the 55-kDa protein was present (Fig. 2C) in anti-α6 (lane a) and anti-CD63 (lane c) but not in anti-α5 or negative control immunoprecipitates (lanes b and d). Compared with the total lysate sample (Fig. 2C, lane e), comparable levels of 55-kDa protein were detected in CD63 and α5 lanes that were derived from 20-fold more cell equivalents. Thus, approximately 5% or more of the 55-kDa PI 4-K protein may be present in a complex with α5 integrin and/or CD63.

The amount and the activity of PI 4-K co-immunoprecipitated with anti-CD63 mAbs was consistently greater than that detected with anti-integrin or anti-CD63 mAbs (Fig. 2, A and C), suggesting that the CD63 interaction with PI 4-K may not require αβ1 integrin. Indeed, PI 4-K could be co-purified with CD63 protein from K562 cells, which do not express appreciable levels of αβ1 integrin (Fig. 2D, lane b). As expected for these cells, β1 integrins (predominantly αβ1) lacked associated phosphoinositide kinase activity (Fig. 2D, lane c). However, when the αβ2 integrin heterodimer was expressed in K562 cells (after transfection of α2 subunit cDNA), PI 4-K was then co-immunoprecipitated with β1 integrins (Fig. 2D, lane g). Together these results suggest that TM4SF proteins may link αβ1 integrin to PI 4-K.

Adhesion-dependent stimulation of phosphatidylinositol 4,5-bisphosphate production is an established biological phenomenon that may be controlled by members of the Rho family of...
small GTPases (35, 36). The present demonstration of physical association between a3b1 integrin and PI 4-K, an intracellular enzyme that controls the first step in biosynthesis of PIP2, suggests another link between integrin activation and metabolism of phosphoinositides. The a3b1-CD63-CD81-PI 4-K-linked complex is distinct from the conventional FAK-related pathway insofar as its specificity for a particular b1 integrin (e.g. a3b1 but not for a5b1). Moreover, triggering of the a3b1-CD63-CD81-PI 4-K complex with anti-TM4 mAbs (to either CD63 or CD81) failed to induce tyrosine phosphorylation of 120–130-kDa cellular proteins (Fig. 3, lanes e and f). In contrast, tyrosine phosphorylation of 120–130-kDa cellular proteins that probably correspond to FAK and Cas (37–40) was induced by all three anti-integrin mAbs (Fig. 3, lanes a, b, and d). Thus, we hypothesize that the fraction of a3b1 in a3b1-CD63-CD81-PI4-K complexes may be distinct from that which signals through FAK or Cas.

What could be the function of the a3b1-CD63-CD81-PI 4-K complex in cells? The formation of an integrin-TM4SF-PI 4-K complex is not adhesion-dependent, because it is observed in K562 cells grown in suspension (e.g. see Fig. 2D, lane g). Rather, given its prominent clustering at the periphery of spread cells, it is possible that an a3b1-CD63-CD81-PI 4-K complex may direct lamellipodial and filopodial protrusions during cell migration. Indeed, some properties of the complex may be well suited for this purpose. First, the a3b1-CD63-CD81-PI 4-K complex can be easily extracted from the cell membrane, thus suggesting that its interaction with ECM substrate is not very strong (11). These weak and transient interactions are particularly important at the leading edge of lamellipodia because they allow a cell to sample the substrate before deciding where to move. In this regard, the presence in

indicated. C, integrins or CD63 were immunopurified and probed by Western blotting with a rabbit polyclonal antibody raised against recombinant PI 4-Ka, a 97-kDa protein that shares similar enzymatic characteristics with type II PI 4-K (46). D, integrins or CD63 were immunopurified from K562 or a5-transfected K562 (18) cells, and the presence of PI 4-K activity in the immunoprecipitates was tested as in A. The lanes marked PI 3-K show PIP, PIP2, and PIP3 standards generated by a PI 3-K reaction.
the complex of PI 4-K, an enzyme implicated in vesicular transport (41, 42), and CD63, a protein that has a YXXM internalization signal (43), could help to perpetuate the process of sampling through the recycling of $\alpha_2\beta_1$ within the leading edge. Second, the magnitude of the biochemical signal (synthesis of phosphoinositides) produced by activated complex could be a decisive factor in determining the degree of actin polymerization potency of the signal.

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