Transmembrane-4 Superfamily Proteins Associate with Activated Protein Kinase C (PKC) and Link PKC to Specific β1 Integrins*

Received for publication, March 9, 2001, and in revised form, April 25, 2001
Published, JBC Papers in Press, April 26, 2001, DOI 10.1074/jbc.M102156200

Xin A. Zhang‡, Alexa L. Bontrager, and Martin E. Hemler§

From the Department of Cancer Immunology and AIDS, Dana-Farber Cancer Institute and Department of Pathology, Harvard Medical School, Boston, Massachusetts 02115

Translocation of conventional protein kinases C (PKCs) to the plasma membrane leads to their specific association with transmembrane-4 superfamily (TM4SF; tetraspanin) proteins (CD9, CD53, CD81, CD82, and CD151), as demonstrated by reciprocal co-immunoprecipitation and covalent cross-linking experiments. Although formation and maintenance of TM4SF-PKC complexes are not dependent on integrins, TM4SF proteins can act as linker molecules, recruiting PKC into proximity with specific integrins. Previous studies showed that the extracellular large loop of TM4SF proteins determines integrin associations. In contrast, specificity for PKC association probably resides within cytoplasmic tails or the first two transmembrane domains of TM4SF proteins, as seen from studies with chimeric CD9 molecules. Consistent with a TM4SF linker function, only those integrins (α6β1, αβ1, and a chimeric “X3TC5” α6 mutant) that associated strongly with tetraspanins were found in association with PKC. We propose that PKC-TM4SF-integrin structures represent a novel type of signaling complex. The simultaneous binding of TM4SF proteins to the extracellular domains of the integrin α6 subunit and to intracellular PKC helps to explain why the integrin α6 extracellular domain is needed for both intracellular PKC recruitment and PKC-dependent phosphorylation of the α6 integrin cytoplasmic tail.

Integrin-dependent cell adhesion, through integration of cell signaling pathways and cytoskeletal reorganization, markedly influences cell growth, death, and differentiation (1–3). Signaling through many different integrins causes similar calcium fluxes, pH changes, and activation of focal adhesion kinase. However, specific integrins may also differ markedly from each other in support of cell cycle progression, cell survival, or gene induction (4–6). Consistent with signaling differences, different integrin cytoplasmic domains may interact with a number of specific integrin-associated proteins (7).

Integrin signaling may not only involve cytoplasmic domain associations but also may utilize lateral interactions through integrin transmembrane and ectodomains (4, 8). In this regard, some integrins (e.g. α3β1, α4β1, α6β1, and α10β3) interact specifically with various cell surface TM4SF/tetraspanin proteins (9–12). The TM4SF proteins share 20–30% sequence similarity, and contain four highly conserved transmembrane domains, flanked by short N and C termini. The TM4SF proteins, including CD9, CD53, CD63, CD81, and CD82, may regulate cell signaling, motility, and tumor cell metastasis (13–15). TM4SF proteins tend to assemble into protein complexes at the plasma membrane (14, 15), where they may recruit other molecules (such as growth factor ligands and phosphatidylinositol 4-kinase) into proximity with integrins (11, 16, 17). As shown here, TM4SF proteins may also link specific integrins to protein kinase C (PKC).

The PKC family of phospholipid-dependent serine and threonine kinases participates in a wide spectrum of biological activities (18–20). Activation of cytosolic PKC by phorbol ester or diacylglycerol occurs in parallel with PKC translocation to cellular membranes. Membrane association is largely attributed to specific PKC interactions with phosphatidylserine. Various PKC isoforms also associate with a number of specific binding proteins (20). However, aside from PKC interaction with the transmembrane proteoglycan syndecan-4 (21), a role for specific transmembrane proteins during PKC translocation has not previously been suggested.

Conventional PKC isoforms participate in the “inside-out” activation of cell adhesion mediated by β1, β2, and β3 integrins (22–24). PKC not only appears in focal adhesion complexes, as seen in well spread cells (25), but also is required for cell spreading (26, 27). In addition, PKCα may associate with β1 integrins and regulate their trafficking (28). A subset of integrins (αβ1, α6 integrins) becomes phosphorylated in a PKC-dependent manner (29–32). Phosphorylation of the α6 integrin may regulate cell signaling, morphology, migration, and cytoskeletal organization (31).

Here we demonstrate that upon activation and translocation, conventional PKCs associate closely with several different TM4SF/tetraspanin proteins. Upon PKC activation, those integrins (αβ1, α6 integrins) already constitutively associated with TM4SF proteins then become linked to PKC. Within these PKC-TM4SF-integrin complexes, integrin α6 and α6 tails are phosphorylated in a PKC-dependent manner. The presence of TM4SF linker proteins helps to explain how association of intracellular PKC may be determined by integrin extracellular domains.

* This work was supported by National Institutes of Health Grant CA86712 (to M. E. H.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ Present address: Vascular Biology Center, University of Tennessee Health Science Center, Memphis, TN 38163.
§ To whom correspondence should be addressed: Dana-Farber Cancer Institute, Rm. D-1430, 44 Binney St., Boston, MA 02115. Tel.: 617-632-3410; Fax: 617-632-2662; E-mail: Martin_Hemler@DFCI.Harvard.EDU.

1 The abbreviations used are: TM4SF, transmembrane-4 superfamily; DSP, dithiobis(succinimidyl propionate); PI 4-K, phosphatidylinositol 4-kinase; PI 3-K, phosphatidylinositol 3-kinase; PKA, protein kinase A; PKC, protein kinase C; PKM, protein kinase M; PLC, phospholipase C; PMA, phorbol myristate acetate; mAb, monoclonal antibody; MHC, major histocompatibility complex; PBS, phosphate-buffered saline; RACK, receptor for activated C kinase; PAGE, polyacrylamide gel electrophoresis.
**EXPERIMENTAL PROCEDURES**

**Antibodies**—Anti-integrin antibodies used were as follows: anti-αβ2, A2-H1E10 (33); anti-αβ3, A3-X8, A3-TIVAX, and A3-IIF5 (34); anti-α4, A4-B5G10 (35) and A4-PUJ1 (36); anti-α5, A5-PUJ2 (36); anti-αv, A6-ELE (37); anti-β1, A-Ia5 (38) and TS2/16 (39). Anti-TM4SF mAbs used were as follows: anti-C8, DU-ALL1 (Sigma); anti-CDS3, HD77 (40); anti-CDS2, M104 (Dr. C. Ruddle, DanaFarber Cancer Institute); anti-CDS1, DC15, 5C11 (42); and anti-A15/TALLA1, B2D (43). mAbs to PKCα, PKCγ, and phosphatidylinositol 3-kinase (PI 3-K) were obtained from Transduction Laboratories (Lexington, KY). Control rabbit IgG and polyclonal antibody to PKCβII were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA), and anti-PLCγ antibody was from Upstate Biotechnology, Inc. (Lake Placid, NY). Other mAbs were as follows: anti-CD3, OKT3 (American Type Cell Culture Collection, Rockville, MD), anti-CD29 (Becton Dickinson), anti-CD25 (Dr. C. Rudder, Dana Farber Cancer Institute); anti-CD71, DF1513 (Sigma); anti-CD98, DB122; anti-MHC class I, W6/32. Rhodamine-conjugated goat anti-mouse secondary antibodies (for immunofluorescence staining) were from BIOSOURCE (Camarillo, CA). Goat anti-mouse IgG antibody (for cell surface antibody anti-cross-linking) was from Roche Molecular Biochemicals. For most experiments involving reimmunoprecipitation or Western blotting, mAbs used for initial immunoprecipitation were covalently conjugated to CNBr-activated Sepharose 4B beads.

**Immunoprecipitation and Reimmunoprecipitation**—Cells were surface-labeled with Na251 (PerkinElmer Life Sciences) using lactoperoxidase by an established protocol, or cells were 32P-labeled by growth in sodium phosphate deficient medium supplemented with [32P]orthophosphate (PerkinElmer Life Sciences) for 3–6 h. Experiments involving PMCA stimulation, cells were treated with 100 nM PMA for 20–30 min at 37 °C prior to lysis. Cells were lysed in immunoprecipitation buffer (1% Brij 96 or 1% Brij 99, 25 mM HEPES, pH 7.4, 150 mM NaCl, 5 mM MgCl2, 2 mM phenylmethylsulfonyl fluoride, 20 mg/ml aprotinin, and 10 mg/ml leupeptin) for 1 h at 4 °C. For 32P-labeled, immunoprecipitation buffer was supplemented with phosphatase inhibitors (1 mM sodium orthovanadate, 1 mM NaF, and 10 mM β-glycerophosphate). Immunoprecipitations and reimmunoprecipitations were then carried out as described (10, 44). Immune complexes collected on beads were then washed three times with immunoprecipitation buffer and analyzed by SDS-PAGE under nonreducing conditions, and radiolabeled proteins were visualized by autoradiography.

**Immunofluorescence**—For Western blot analysis, immunoprecipitated samples were subjected to SDS-PAGE under reducing conditions and then electrophoretically transferred to nitrocellulose membrane. After blocking with 5% nonfat milk in PBS-Tween 20 buffer at room temperature for 1 h, nitrocellulose membranes were sequentially blotted at room temperature for 1 h with specific antibody and then horseradish peroxidase-conjugated goat anti-mouse IgG. Each step was followed by three 5-min washes with PBS-Tween 20 buffer. Membranes were then developed using chemiluminescence (Renaisance; PerkinElmer Life Sciences).

**RESULTS**

**PKC Forms Complexes with Specific Tetraspanin Proteins**—During our studies of tetraspanin protein association with intracellular PI 4-K (16, 17), we noticed that another enzyme, PKC, showed an even stronger tetraspanin association. From a series of K562 erythroleukemia cell transfectants, the tetraspanin protein CD81 was immunoprecipitated, and then PKCβII (a conventional PKC isozyme) was detected by immunoblotting, provided that the K562 cells had been activated with PMA (Fig. 1A, compare upper and lower panels). Immunoprecipitates of CD98 (right lanes) yielded no associated PKC. To confirm results in Fig. 1B, PKCα was immunoprecipitated from lysates of Jurkat T cells, and immunoblotted to detect PKCα (another conventional PKC isozyme). As indicated, the TM4SF proteins CD9, CD81, and CD82 each showed a clear association with PKCα (Fig. 1B, upper panel). In contrast, PKCα was not present in immunoprecipitates of A15 (another TM4SF protein), CD71 (transferrin receptor), CD98 (another prominent transmembrane protein), or MHC class 1. Cell surface levels of A15, CD71, CD98, and MHC class 1 were each greater than that of CD9, and were comparable with CD81 and CD82 (not shown). Association of PKCα with TM4SF proteins was not observed unless Jurkat cells were pretreated with PMA (Fig. 1B, upper panel). PMA had no effect on the levels of TM4SF proteins (CD9, CD81, CD82, and A15) or control proteins (CD71, CD98, and MHC class I) directly immunoprecipitated using appropriate mAbs (not shown). The TM4SF protein CD151 also co-precipitated with PKC (see below). Compared with total cell lysate samples in Fig. 1, A and B, comparable levels of PKC were detected in CD9 and CD81 immunoprecipitates derived from 20–30-fold more cell equivalents. Thus, >3–5% of the total PKC may be associated with each of these TM4SF proteins.

To confirm results in Fig. 1B, PKCα was immunoprecipitated from lysate of Jurkat T cells that had been PMA-treated and 32P-labeled. Reimmunoprecipitations were then carried out showing that CD9, CD53, CD81, and CD82 were associated with PKCα (Fig. 1A, lanes a–d). In control experiments, a non-tetraspanin protein (MHC-1) was not recovered from PKCα immunoprecipitates (lane e) although it could be directly immunoprecipitated (lane h). Furthermore, no tetraspanins or other proteins were recovered from immunoprecipitations of PI 3-K (lanes f–j) or PKA (not shown).

Because PKC and tetraspanins may both associate with integrins, we considered that TM4SF-PKC associations are mediated through integrins. In 1% Brij 96 conditions, CD81 in K562 cells associates readily with α9, and α9 integrins but hardly at all with any α2, α3, or α4 integrins (12). However, native K562 cells (containing only endogenous α9β1), and transfected cells expressing abundant levels of α2, α3, α4, or α6 integrins

---

2. B. Mannion, F. Berditchevsky, J. Bodorova, and M. E. Hemler, unpublished data.
Likewise, TM4SF-PKC association of PKCα cross-linking of the CD3-T cell receptor on 125I-surface-labeled Jurkat T cells triggered association of PKCα on the surface of T cells (47). Antibody/second antibody-induced antibody cross-linking of the CD3-T cell receptor complex on no TM4SF-integrin complexes were present (not shown). Jurkat cells under detergent conditions (1% Brij 96) in which lane b few other unknown cell surface proteins (Fig. 2, lane b). In contrast, antibody/second antibody cross-linking of MHC class I, CD81, and CD98 (all abundant on Jurkat T cells) failed to stimulate PKCα association with CD81 protein complexes (lanes a and c–e). In another control experiment, CD81 and associated proteins were not associated with PKA following CD3 antibody cross-linking (lane f). The identity of CD81 was verified in Fig. 2B. After antibody cross-linking of CD3 on Jurkat cells, a PKCα immunoprecipitation was carried out, and from the resulting protein complex, CD81 could be reimmunoprecipitated (Fig. 2B, lane i). In contrast, α3β1, integrin, CD98, and MHC-1 proteins could not be reimmunoprecipitated (lanes h, j, and k). Likewise, CD81 could not be reimmunoprecipitated from a PI 3-K immunoprecipitate after CD3 antibody cross-linking of Jurkat cells (lane l). A direct immunoprecipitation of CD81 and associated proteins is shown in lane g. The pattern of surface-labeled proteins looks remarkably similar to that immunoprecipitated with an anti-PKCα antibody (lane b), thus providing further evidence for the presence of PKCα in CD81 complexes.

From human peripheral blood mononuclear cells stimulated with either PMA or anti-CD3 antibody cross-linking, PKCα again formed complexes with TM4SF proteins (not shown). Another agent that stimulates PKC translocation (bryostatin 1 (48)) also induced association of PKC with TM4SF proteins (not shown). In other experiments, PKC inhibitors (chelerythrine D, Go6976, calphostin C, and staurosporine) did not inhibit PMA-induced association of PKC with TM4SF proteins (data not shown). Thus, although PKC translocation is required for TM4SF protein association, PKC activity is not required.

**TM4SF-PKC Interactions Are Stabilized by Covalent Cross-linking**—To characterize TM4SF-PKC interactions further, intact K562 cells were treated with dithiobis(succinimidyl propanoate) (DSP), a homobifunctional cross-linking agent with a span of 12 Å. Without cross-linker, and under relatively mild detergent conditions, immunoprecipitation of CD81 from PMA-stimulated K562 cells yielded associated PKCβII that was readily visualized by Western blotting (Fig. 3A, lane a), in
PKC immunoprecipitations under any conditions (PKC of intact cells with DSP cross-linker, association with A the Brij 96 lysis buffer (Fig. 3)

In a second experiment (Fig. 3B), another TM4SF protein (CD151) maintained association with PKCβII antibody. B, intact K562 cells were treated with or without PMA and with or without DSP as indicated. Cells were lysed in 1% Brij 96 plus 0.2% SDS, and then CD71 (transferrin receptor) and CD151 (TM4SF protein) were immunoprecipitated, and PKCβII was immunoblotted.

PKM, a form lacking the N-terminal C1 and C2 regulatory domains (49). The addition of calcium and the omission of the protease inhibitor leupeptin caused the partial conversion of intact PKCβII in K562 cells (Fig. 4A, lane a) into two closely migrating fragments of ~50 kDa characteristic of PKM (lane g). No PKM was observed even upon overexposure of lane g, such that the PKC intensity was comparable with that in lanes a–d (not shown). Also, no PKC was co-precipitated with CD81 in the absence of PMA treatment (lane e) or with control Ig (lanes f and h). In conclusion, the PKM form of PKCβII, lacking N-terminal regulatory domains, does not associate with CD81. In addition, we failed to recover PKCε, PKCζ, or PKCμ from TM4SF immunoprecipitates, despite testing multiple cell lines and 4–6 different TM4SF proteins for each isozyme (Fig. 4B).

TM4SF Specificity—As seen in Fig. 1, PKCα interacted with four different TM4SF proteins (CD9, CD53, CD81, and CD82) but not another TM4SF protein (A15/Talla1). Showing a similar specificity, PKCβII in K562 cells interacted again with CD9 but not A15 (Fig. 5). With A15 being negative for PKC association, an opportunity for a chimeric mapping approach was provided. So far, nearly all TM4SF associations and functions have mapped to the large extracellular loop (42, 50–52). However, the A15-lel34.CD9 chimera (A15 with the large extracellular loop and flanking transmembrane domains of CD9) failed to interact with PKCβII in K562 cells (Fig. 5). Thus, the CD9 large extracellular loop and flanking transmembrane domains are not sufficient for PKC association. Because the short inner loop of A15 (RGSPW sequence) is quite distinct from most other known tetraspanins (53), we hypothesized that it could selectively prevent PKC association. However, replacement of the short inner loop QESQC of CD9 with the loop RGSPW from A15 (CD9-il.A15) did not abolish PKC interaction. Conversely, replacement of the A15 RGSPW with the QESQC from CD9 (A15-il,CD9) did not confer PKC interaction. Therefore, the unusual structure of the A15 inner loop appears to be required for interaction with PKCβII.
PKC Isoforms Are Induced to Form Complexes with Specific Integrins—Because TM4SF proteins associate with specific integrins (see Introduction) and with activated PKC, we predicted that TM4SF proteins may link activated PKC to integrins. In this regard, antibodies to either CD81 (from HT1080 cells) or PKCα (from activated HT1080 cells) co-immunoprecipitated similar surface-labeled proteins (Fig. 6A, lanes b and c) that precisely comigrate with α3β1 integrin (lane a). Upon longer exposure of lanes b and c (see lanes k and l), a small amount of surface-labeled CD81 was also obvious in both CD81 and PKCα immunoprecipitations. Notably, integrin-like proteins were not obtained using anti-PKA, anti-PLCγ, or anti-PI 3-K mAbs (lanes d–j) and were not seen if HT1080 cells were not stimulated with PMA (lanes g–j). Upon long exposure (lane m), immunoprecipitation of another conventional PKC (PKCγ) also yielded integrin-like protein, whereas control PLCγ (lane n) did not. As seen previously (12), PMA pretreatment was not required for CD81-integrin association (lanes b and k). Anti-PKC, anti-PKA, anti-PI 3-K, and anti-PLCγ mAbs immunoprecipitated comparable amounts of appropriate target proteins from 35S-labeled cells (not shown). Considering that the immunoprecipitations are from 125I-labeled HT1080 cell total lysate, the patterns of proteins associated with CD81 and activated PKC are remarkably simple. Thus, few other cell surface proteins may be present in PKCα-CD81-α3β1 complexes under these conditions. To identify specific β integrin co-immunoprecipitated with PKCα, complexes derived from 125I-labeled HT1080 cells were dissociated and subjected to reimmunoprecipitation (Fig. 6B). As indicated, anti-integrin antibodies yield specific integrin β1, α3, and α6 subunits (lanes f, h, and j) but not α2 or α5 subunits (lanes g and i). Each of these subunits was expressed in HT1080 cells at moderate to high levels as seen by direct immunoprecipitation (lanes a–e) and by flow cytometry (not shown). Complexes obtained using anti-PI 3-K failed to yield reprecipitated integrin subunits (lanes k–o).

To confirm co-immunoprecipitation of specific integrins with PKCs, we analyzed another conventional isoform (PKCβII) and another cell line (K562). From cell surface 125I-labeled K562-α3 and K562-α6 Brij 96 lysates, anti-PKCβII antibody co-immunoprecipitated abundant integrins (Fig. 7A, lanes g and j). However, little if any integrin was co-precipitated from K562-α2, K562-α5, or K562 mock transfectants containing substantial endogenous α2, α5 (lanes f, h, and i). Labeled proteins of ~120 kDa in these latter lanes do not resemble heterodimeric integrins and appear to be background proteins. All integrins tested were well expressed in their respective K562 transfectants (lanes a–e). No integrins were detected from normal rabbit IgG control immunoprecipitations (lanes k–o) or from

**Fig. 5.** TM4SF domains needed for PKC association. K562 cells expressing CD9/A15 chimeras were treated with PMA, lysed in 1% Brij 96, and immunoprecipitated (I.P.) using mAb to CD9 (first three panels) or A15 (last two panels) or control antibodies (to CD98 or MHC-1). After SDS-PAGE, proteins were blotted using anti-PKCβII antibody. By flow cytometry, CD9, A15, and chimeras were at comparable levels (210–290 mean fluorescence intensity units), compared with negative control antibody (30–40 mean fluorescence intensity units). Direct immunoprecipitation of CD9, A15, and chimeras also yielded comparable protein levels (not shown).

**Fig. 6.** PKC association with integrins. A, HT1080 cells were treated with or without PMA (100 nM at 37 °C, 20 min), surface-labeled with 125I, and then lysed in 1% Brij 96, mAbs to the indicated proteins were used for immunoprecipitations. B, HT1080 cell lysates (prepared as in A) were immunoprecipitated (I.P.) (lanes a–e), using mAbs TS2/16, A2-IIE10, A3-IVA5, A5-PJ32, and A6-ELE, respectively. Also, protein complexes initially precipitated (from PMA-treated cells) using anti-PKCα or PI3-K antibodies were dissociated and then reimmunoprecipitated using mAb to the indicated integrin subunits (lanes f–o).

PKCβII immunoprecipitations from cells not treated with PMA (not shown).

Whereas Figs. 6 and 7A show integrins co-precipitated with PKCα and PKCβII, the reciprocal result (PKC co-precipitated with specific integrins) is shown in Fig. 7B. Integrin α2, α4, and α6 immunoprecipitations, from Brij 96 lysates of K562-α2 and K562-α6 cells, respectively, yielded prominent PKCβII proteins as detected by Western blotting (lower panel, +PMA). In contrast, no PKCβII was detected in α2, α4, or α6 immunoprecipitations (Fig. 7B, lower panel), although those proteins are well
represents the respective K562 transfectants (Fig. 7A, lanes a–c). Furthermore, in the absence of PMA treatment of K562 cells, no PKCβII was detected from integrin immunoprecipitations, although it was present in the whole cell lysate (Fig. 7B, upper panel). While PMA greatly stimulated PKCα and PKCβII association with integrins, PMA had no effect on the solubilization and direct immunoprecipitation of any of the integrins tested. Besides PMA, brystatin 1 also induced PKC-βII association, consistent with its ability to induce PKC-TM4SF association (as mentioned above). As seen for TM4SF association (see above), PKC inhibitors did not prevent integrin association (not shown). Thus again, PKC translocation was required for integrin association, but PKC catalytic activity was not needed.

As seen by immunofluorescent staining of HT1080 cells spread on fibronectin (Fig. 8), localization of integrin α3 and TM4SF protein CD81 to lamellipodia was substantially increased following PMA treatment. Additionally, PKCα was translocated from the cytoplasm to lamellipodia, whereas PI 3-K distribution was relatively unaffected upon PMA treatment. These results are again consistent with PKC-CD81-α3 integrin complex formation.

Specificity for Intracellular PKC Determined by Integrin Extracellular Domain—Specificity for TM4SF-integrin association resides within the ectodomains of both TM4SF proteins and integrins (42). Thus, if PKC is linked to integrins via a TM4SF linker protein, an extracellular integrin site should be needed for recruitment of intracellular PKC. In this regard, deletion of the cytoplasmic tail of α3 (K562-X3C0 transfectant, Fig. 9A, lane c) or exchange of the α3 transmembrane and tail regions with those from α6 (K562-X3TC5 transfectant, lane d) did not diminish association with PKCβII, relative to that seen for wild type α3 integrin (K562-α3, lane b). Conversely, an α2 integrin bearing an α3 tail (K562-X2C3) failed to associate with PKCβII (lane e). Also, anti-PKCBII antibody did not co-precipitate integrin from cells bearing predominately α6β1 (mock-transfected K562 cells, lane f), and none of the K562 transfectants yielded integrins with normal rabbit IgG control antibody (lanes g–h). Thus, the integrin α3 chain extracellular domain determines specificity for interaction of an intracellular protein (PKC) with integrin. These results are consistent with PKC-integrin association requiring a transmembrane linker protein such as a tetraspan. Similar to results seen in Fig. 6a, anti-PKCBII antibody co-immunoprecipitation of α3 integrin was remarkably devoid of other surface-labeled proteins (Fig. 9A, lanes b–d).

Potential Relevance of PKC Recruitment to α3, α6 Integrins—Cytoplasmic domains from integrin α3A and α3A but not α2, α4, or α5 are phosphorylated by a mechanism that involves activated PKC (29, 31, 54). Furthermore, α3A phosphorylation may regulate integrin-dependent cell motility, signaling, and cytoskeletal organization (31). If TM4SF proteins are required to link PKC to integrin, then an integrin unable to associate with TM4SF proteins should not be phosphorylated even if the correct α tail is present. Consistent with this prediction, the α3 tail present within an X2C3 integrin chimera was not phosphorylated in PMA-treated K562-X2C3 cells (Fig. 9B, lane i), although the α3 tail was phosphorylated in K562-α3 cells (lane k).
Triton X-100, and then immunoprecipitated using the relevant mAb to stabilize by covalent cross-linking. The covalent cross-linking complexes (lanes a–h) or control rabbit Ig (lanes g–k). Co-precipitated proteins (lanes b–d) align with control αβIII (directly immunoprecipitated, lane a). As indicated by flow cytometry, the X3C0, X3TC5, X2C3, and αl proteins were all expressed at comparable levels (not shown). B, K562 transfectants, with or without PMA stimulation, were labeled with 32P, lysed in 1% Triton X-100, and then immunoprecipitated using the relevant mAb to integrin α2 or α5 or control mAb to CD98.

Also, no phosphorylation was observed if the α3 tail was deleted (lane m) or if the tail and transmembrane regions were replaced with those of α5 (lane o). In addition, no phosphorylation was observed in CD98 control immunoprecipitations or if PMA treatment was omitted (lanes a–h).

DISCUSSION

TM4SF-PKC Association—Activation and translocation of PKC promoted a relatively robust association of conventional PKC isoforms with multiple TM4SF proteins. TM4SF-PKC complexes were seen in multiple cell lines (including both adherent and nonadherent cells), were seen for several different tetraspanins (CD9, CD53, CD81, CD82, and CD151), and were promoted by multiple PKC activating stimuli (phorbol ester, brystatin 1, or CD3-T cell receptor triggering). Analysis of TM4SF-PKC complexes in Jurkat T cells suggests a reasonable stoichiometry. Although each TM4SF protein might only associate with ~3–5% of the total PKC, the presence of about five or more different TM4SF proteins in a given cell type would engage a substantially greater fraction of the total PKC. Four kinds of biochemical evidence support the presence of TM4SF-PKC complexes. (i) Immunoprecipitation of TM4SF proteins yielded PKC; (ii) immunoprecipitation of PKC yielded multiple TM4SF proteins; (iii) immunoprecipitation of either TM4SF protein (i.e. CD81) or PKC yielded essentially identical protein complexes (e.g. Fig. 2); and (iv) TM4SF-PKC complexes were stabilized by covalent cross-linking. The covalent cross-linking results provide perhaps the most compelling evidence for TM4SF-PKC association. The span of the cross-linking agent (12 Å) is such that only highly proximal interactions would be captured. Furthermore, the membrane-permeable cross-linking agent was added to intact cells and thus captured the native complexes before exposure to any detergents. Finally, the cross-linked complexes were solubilized using relatively stringent detergent conditions, such that uncross-linked proteins are largely removed.

The occurrence of TM4SF-PKC complexes was specific, with respect to both TM4SF proteins and PKC. Considering the tendency of TM4SF proteins to associate with each other, it was reassuring to find that at least one prominently expressed TM4SF protein (A15/Talla1) did not associate with PKC in Jurkat cells. Among PKC isozymes, conventional PKCs α, βII, and γ associated with TM4SF proteins, whereas representative other PKC types (ε, ζ, and μ) did not. Likewise, PKM (PKCβII lacking regulatory domains (49)) failed to associate. Thus, unique features within the regulatory regions (e.g. C1 or C2 domains) of conventional PKCs are critical for TM4SF association. The C1 and C2 regulatory domains interact with membrane diacylglycerol and phosphatidylserine, respectively, and play key roles in PKC activation and translocation (19). We now suggest that C1 and/or C2 domains of PKC also could directly interact with TM4SF proteins, thus facilitating membrane targeting of conventional PKC isoforms. Alternatively, the C1 and/or C2 domains could act indirectly. They may only be required insofar as they bring activated PKC into proximity with the membrane, while other PKC domains then associate with TM4SF proteins. While the C2 domain of PKC looks promising, it remains to be demonstrated which particular PKC regulatory domains are especially important for enabling and/or mediating TM4SF protein interactions.

Thus, it remains to be demonstrated which particular PKC regulatory domains are especially important for enabling and/or mediating TM4SF protein interactions.

Compared with tetraspanin interactions with other intracellular signaling proteins (PI 4-kinase, phosphatase, and GTP binding proteins) the PKC interactions described here are quite distinct and perhaps more robust. These other interactions have not been demonstrated using covalent cross-linking. Furthermore, tetraspanin association with PI 4-K was observed, not in Brij 96, but in the less stringent Brij 99 conditions. Also, whereas tetraspanin-PKC associations are induced, association with PI 4-K is constitutive (8, 16). Finally, PI 4-K associates well with TM4SF protein A15 but not with CD82 and CD53 (17), whereas PKC associates well with CD82 and CD53, but not with A15. Thus, TM4SF proteins may have distinct sites for recruitment of these two key signaling enzymes (PI 4-K and PKC). The CD53 and CD63 tetraspanins may associate with membrane diacylglycerol and phosphatidylserine, respectively, and play key roles in PKC activation and translocation (19). We now suggest that C1 and/or C2 domains of PKC also could play key roles in PKC activation and translocation (19). We now suggest that C1 and/or C2 domains of PKC also could
due to the rather nonstringent conditions utilized, this association is probably part of a large complex.

**Tetraspanins Link PKC to Integrins (PKC-TM4SF-Integrin Model)—**Reciprocal co-immunoprecipitation experiments showed that specific integrins (α3β1, α5β1) form complexes with conventional PKCs. Our evidence suggests that tetraspanin proteins provide a key linker function between PKC and integrins. First, we found PKC, tetraspanins, and integrins all within the same complexes. For example, in many experiments (e.g., Fig. 6A and not shown), antibodies to PKC and tetraspanins both yielded the same pattern of co-immunoprecipitated integrins. Also, all experiments showing PKC-integrin complexes were carried out under conditions (1% Brij 99, 1% Brij 96) in which TM4SF-integrin complexes are maintained (12, 44). Second, immunofluorescence staining revealed a similar localization pattern for α3β1 integrin, CD81, and activated PKCα at the periphery of spread cells. Third, only tetraspanin proteins have been shown (by covalent cross-linking) to have high proximity to both PKC (as shown here) and relevant integrins (42). Fourth, linkage through tetraspanin proteins explains how an extracellular integrin site could determine specificity for an associated intracellular enzyme such as PKC. Importantly, it is the extracellular domains of both integrin α chains and TM4SF proteins that determine specificity for TM4SF-integrin association (8, 10, 44), whereas it is intracellular and/or transmembrane domains of tetraspanin proteins that most likely determine tetraspanin-PKC association. In this regard, the TM4SF protein CD151 may similarly link the integrin α3β1 extracellular domain to another intracellular enzyme, PI-4-K (8). Fifth, those integrins (e.g., α3β1 and α6β1) that associate strongly with tetraspanins were seen in association with PKC, whereas integrins not well associated with tetraspanins (α2β1, and α5β1) did not associate with PKC. Perhaps most importantly, experiments with chimeric integrins showed that α chain mutants lacking capability for tetraspanin association also lost PKC association, whereas α chain tail and transmembrane mutants that retain tetraspanin association also retained PKC association. Together, these results strongly support a PKC-TM4SF-integrin arrangement.

An alternative model would involve integrins providing a link between PKC and tetraspanins (PKC-integrin-TM4SF model). However, this model does not explain how integrin association with PKC, an intracellular enzyme, would be specified by the extracellular domain of the integrin α chain. Likewise, the model does not account for the formation of PKC-TM4SF complexes in the absence of associated integrins (e.g., as seen in K562 cells). Finally, we have not yet observed PKC-integrin complexes in the absence of TM4SF proteins. In another report describing PKCα-β1 integrin complexes (28), the potential presence of TM4SF proteins was not addressed. At present, we prefer a model (PKC-TM4SF-integrin) in which, upon activation and translocation, PKC is brought into direct association with either TM4SF proteins or preexisting TM4SF-integrin complexes in cellular membranes.

**Do Other Proteins Contribute to the Linking of PKC to Integrins?—**Because integrins, PKCα, and tetraspanin proteins have all been found in organized lipid microdomains (58–60), we considered that our PKC-TM4SF-integrin complexes may occur in the context of large, incompletely solubilized membrane aggregates. However, in direct co-immunoprecipitation experiments (such as shown in Figs. 2, 5A, and 8A), antibodies to PKC and/or tetraspanins yielded (especially in HT1080 and K562 cells) a remarkably clean pattern of 125I-labeled surface-labeled proteins. In some experiments, we observed no prominent surface-labeled proteins, aside from surface-labeled integrin, and a low amount of labeled TM4SF protein.

In 1% Brij 96 lysate conditions, the majority of tetraspanin complexes appeared in the dense fractions of sucrose gradients, indicating that associations with other proteins (including α9 integrin) typically do not depend on a low density lipid microdomain (60). Furthermore, in 1% Brij 96, the majority of CD81 and CD9 complexes were included well within Sepharose 6B gel filtration columns, again indicating that they are well solubilized and of a reasonable size (<2 × 10^6 Da). Even in the less stringent (i.e. less hydrophobic) Brij 99 detergent, tetraspanin-integrin complexes appeared to be well solubilized and of reasonable size (60). These prior results, coupled with our co-immunoprecipitation and biochemical cross-linking results, suggest that few other proteins, besides tetraspanins, may be needed to facilitate PKC-integrin complex formation.

PKC may interact with a number of substrate proteins (20) and other intracellular proteins termed receptors for activated C kinase (RACKs) (61). However, none of these are transmembrane proteins. A protein associating with PKCβ2I, termed RACK1, may bind to the integrin β1 cytoplasmic domain. Furthermore, association of integrins with intact RACK1 was promoted upon stimulation with PMA (62). However, both the RACK1 integrin specificity (β1 and β2 cytoplasmic domains) and the PKC specificity (restricted to PKCβ) are distinct from the specificities seen here for integrin-TM4SF-PKC complexes. PKCα may also interact with syndecan-4, a transmembrane proteoglycan regulating localization of PKC to focal adhesions (21). However, syndecan complexes may be distinct, since TM4SF proteins are not usually in focal adhesions (63).

**Functional Role of PKC-TM4SF-integrin Complexes—**The α3 and α6 integrins undergo serine phosphorylation, dependent on both activated PKC and an unidentified serine kinase (31). Mutation of the α3 phosphorylation site caused alterations in cell morphology, α3 integrin distribution and signaling, actin distribution, and cell migration (31). Our evidence suggests that TM4SF proteins may play a role during PKC-dependent integrin phosphorylation. First, as indicated above, TM4SF proteins are closely associated with both integrins and activated PKC. Second, only those integrins (α3 and α6 integrins) able to associate with TM4SF proteins become phosphorylated. In the most informative example, the chimeric X2C3 integrin failed to associate with TM4SF proteins and was not phosphorylated, although it contained the α3 cytoplasmic tail phosphorylation site and was well expressed at the cell surface. Third, the same agents that promoted integrin α3 or α6 phosphorylation (PMA, bryostatin 1) also promoted activated PKC-TM4SF complex formation.

The formation of integrin-TM4SF-PKC complexes may allow PKC localization to be closely coordinated with cell adhesion involving particular integrins. Thereby PKC may become optimally positioned to regulate a host of downstream events involving cytoskeletal organization and signaling. For example, PKC regulates the interaction of cellular membranes with several cytoskeletal proteins (including the myristoylated alanine-rich C kinase substrate protein) that are also PKC substrates (20, 64). Notably, the myristoylated alanine-rich C kinase substrate protein also colocalizes with TM4SF proteins at the periphery of spread cells (63). Integrin-TM4SF-PKC complexes may be particularly important during cell migration, since TM4SF proteins (13–15), TM4SF-integrin complexes (8, 65), PKC (66), and PKC-integrin complexes (28) have each been linked to cell migration.

The role of PKC in the context of integrin-TM4SF-PKC complexes is distinct from previously described PKC-dependent modulation of integrin adhesion (23, 24), cell spreading (26), and focal adhesion formation (67). For example, some integrins (e.g., α1β2, α6β1, and α2β1) that show PKC-dependent adhesion

**Integrin-TM4SF-PKC Complexes**
and/or spreading functions are not those typically found in integrin-TM4SF-PKC complexes under moderately stringent detergent conditions. In addition, PKC-dependent triggering of cell adhesion, for example through CD28 on T cells (68), can occur in the absence of integrin-TM4SF-PKC complex formation (not shown). Also, in contrast to cell migration, cell adhesion is typically not regulated by TM4SF proteins (8, 44).

In conclusion, studies of integrin signaling can now be expanded to include not only integrin cytoplasmic domains but also membrane-proximal α chain extracellular domains. These latter domains provide specificity for the formation of integrin-TM4SF-PKC signaling complexes. Results shown here contribute to an emerging paradigm whereby association and activity of intracellular signaling enzymes can be determined through integrin extracellular domains. Also, for the first time we have demonstrated a close and possibly direct association of PKC with a class of widely expressed transmembrane proteins (TM4SF proteins) that probably play a role in PKC activation, translocation, subcellular distribution, and signaling.

REFERENCES

1. Clark, E. A., and Brugge, J. S. (1995) Science 268, 233–239
2. Hynes, R. O. (1996) Dev. Biol. 180, 402–412
3. Miyamoto, S., Teramoto, H., Coss, O. A., Gutkind, J. S., Burbello, P. D., Akiyama, S. K., and Yamada, K. M. (1995) J. Cell Biol. 131, 791–805
4. Wary, R. K., Mainiero, F., Isakoff, S. J., Marcantoni, E. E., and Giancotti, F. G. (1996) Cell 87, 733–743
5. Zhang, Z. H., Vuori, K., Reed, J. C., and Ruoslahti, E. (1995) Proc. Natl. Acad. Sci. U.S.A. 92, 6161–6165
6. Huhuala, P., Humphries, M. J., McCarthy, J. B., Tremble, P. M., Werb, Z., and Damasky, C. H. (1995) J. Cell Biol. 129, 867–879
7. Liu, S., Calderwood, D. A., and Ginsberg, M. H. (2000) J. Biol. Chem. 275, 2715–2765
8. Rabinstein, E., Le Naour, F., Billard, M., Prenant, M., and Boucheix, C. (1994) Eur. J. Immunol. 24, 3005–3013
9. Berditchevski, F., Bazzoni, G., and Hemler, M. E. (1995) J. Biol. Chem. 270, 17784–17790
10. Takagi, S., Fujuhara, K., Imai, T., Takahashi, T., Kobayashi, T., and Mekada, E. (2000) J. Biol. Chem. 275, 18284–18290
11. Amiot, M. (1995) in Leukocyte Typing V-White Cell Differentiation Antigens (Schlossman, S. F., Boumsell, L., Gilks, W., Harlan, J. M., Kishimoto, T., Morimoto, C., Ritz, J., Shaw, S., Silverstein, R., Springer, T., Todd, T. F., and Todd, R. F., ed.) 37, 506–558. Oxford University Press, Oxford
12. Fujukida, K., Furuse, M., Imai, T., Nishimura, M., Takagi, S., Hinuma, Y., and Yoshie, O. (1992) J. Virol. 66, 1394–1401
13. Shattil, S. J., and Brass, L. F. (1987) J. Biol. Chem. 262, 7974–7984
14. Armstrong, A. (1994) Biochem. Biophys. Res. Commun. 204, 262–272
15. Bergelson, J. M., St. John, N. F., Kawaguchi, S., Pasqualini, R., Berditchevski, F., Hemler, M. E., and Finberg, R. W. (1994) Cell Adhes. Commun. 2, 453–464
16. Weitzman, J. B., Pasqualini, R., Takada, Y., and Hemler, M. E. (1993) J. Biol. Chem. 268, 8651–8657
17. Hober, M., Huang, C., Takada, Y., Schwarz, L., Strominger, J. L., and Clabby, M. L. (1987) J. Biol. Chem. 262, 11478–11485
18. Pujadas, C., Teixido, J., Bazzoni, G., and Hemler, M. E. (1996) Biochem. J. 313, 899–908
19. Lee, R. T., Berditchevski, F., Cheng, G. C., and Hemler, M. E. (1995) Circ. Res. 76, 209–214
20. Hemler, M. E., Ware, C. F., and Strominger, J. L. (1983) Immunol. Today 13, 244–249
21. Hemler, M. E., Sánchez-Madrid, F., Flotte, T. J., Kremsy, A. M., Burkoff, S. J., Bhan, A. K., Springer, T. A., and Strominger, J. L. (1984) J. Immunol. 132, 1031–1038
22. Amiot, M. (1995) in Leukocyte Typing V-White Cell Differentiation Antigens (Schlossman, S. F., Boumsell, L., Gilks, W., Harlan, J. M., Kishimoto, T., Morimoto, C., Ritz, J., Shaw, S., Silverstein, R., Springer, T., Todd, T. F., and Todd, R. F., ed.) 37, 506–558. Oxford University Press, Oxford
23. Takagi, S., Fujuhara, K., Imai, T., Takahashi, T., Kobayashi, T., and Mekada, E. (2000) J. Biol. Chem. 275, 18284–18290
24. Shimizu, Y., Van Seventer, G. A., Horgan, K., and Shaw, S. (1992) J. Biol. Chem. 277, 2974–2984
25. Doms, R. W., and Kornfeld, S. (1989) Science 243, 15–21
26. Weitzman, J. B., Pasqualini, R., Takada, Y., and Hemler, M. E. (1993) J. Biol. Chem. 268, 8651–8657
27. Chun, J.-S., and Jacobson, B. S. (1993) Mol. Biol. Cell 4, 129–145
28. Weitzman, J. B., Pasqualini, R., Takada, Y., and Hemler, M. E. (1993) J. Biol. Chem. 268, 8651–8657
29. Shaw, L. M., Messier, J. M., and Mercurio, A. M. (1990) J. Cell Biol. 110, 2167–2174
30. Hugger, F., Kuikman, I., Noteboom, E., and Sonnenberg, A. (1993) J. Biol. Chem. 268, 18427–18430
31. Zhang, X. A., Bontrager A. L., Stipp, C. S., Kraeft, S.-K., Bazzoni, G., Chen, L. B., and Hemler, M. E. (2001) Mol. Biol. Cell 12, 351–365
32. Dumont, J. A., and Bitonni, A. J. (1994) Biochem. Biophys. Res. Commun. 204, 262–272
33. Bergelson, J. M., St. John, N. F., Kawaguchi, S., Pasqualini, R., Berditchevski, F., Hemler, M. E., and Finberg, R. W. (1994) Cell Adhes. Commun. 2, 453–464
34. Weitzman, J. B., Pasqualini, R., Takada, Y., and Hemler, M. E. (1993) J. Biol. Chem. 268, 8651–8657