Direct Extracellular Contact between Integrin \( \alpha_3\beta_1 \) and TM4SF Protein CD151*

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Previously we established that the \( \alpha_3\beta_1 \) integrin shows stable, specific, and stoichiometric association with the TM4SF (tetraspannin) protein CD151. Here we used a membrane impermeable cross-linking agent to show a direct association between extracellular domains of \( \alpha_3\beta_1 \) and CD151. The \( \alpha_3\beta_1 \)-CD151 association site was then mapped using chimeric \( \alpha_3/\alpha_3 \) integrins and CD151/NAG2 TM4SF proteins. Complex formation required an extracellular \( \alpha_3 \) site (amino acids (aa) 570–705) not previously known to be involved in specific integrin contacts with other proteins and a region (aa 186–217) within the large extracellular loop of CD151. Notably, the anti-CD151 monoclonal antibody TS151r binding epitope, previously implicated in integrin contacts with other proteins and a region (aa 186–217). Finally, we demonstrated that both NH\(_2\)- and COOH-terminal domains of CD151 are located on the inside of the plasma membrane, thus confirming a long suspected model of TM4SF protein topology.

The integrin family of adhesion receptors controls a variety of biological events, including cell migration, proliferation, survival, and differentiation. Integrins span the plasma membrane and link extracellular matrix proteins, as well as cellular ligands, to the cytoskeleton and associated signaling enzymes (1–5). Electron microscopy studies suggest that integrin \( \alpha \beta \) heterodimers contain a large globular head (comprised of NH\(_2\)-terminal domains) on two elongated stalks that may extend into the membrane (6, 7). Ligand and divalent cation binding sites have been largely mapped to the NH\(_2\)-terminal (large globular head) regions of integrins (8). Also, integrin cytoplasmic tails have been suggested to associate with various intracellular molecules, including cytoskeletal proteins, chaperone proteins, and signaling enzymes (3, 9). However at present, few if any direct interactions have been described for the extracellular stalk-like region of integrins.

Particular integrins may engage in lateral interactions with a variety of other transmembrane proteins, including members of the transmembrane-4 superfamily (TM4SF proteins). The TM4SF proteins (also called tetraspannins) contain two extracellular loops (of 20–27 and 75–130 amino acids) and four putative hydrophobic transmembrane domains. The TM4SF proteins may play key roles in the regulation of cellular proliferation, fusion, development, motility, tumor cell growth, metastasis, and in vitro angiogenesis (10–16). Various integrins, including \( \alpha_3\beta_1 \), \( \alpha_6\beta_1 \), \( \alpha_5\beta_1 \), \( \alpha_1\beta_1 \), \( \alpha_5\beta_2 \), \( \alpha_6\beta_2 \), and \( \alpha_6\beta_4 \), may associate with one or more TM4SF proteins, including CD9, CD53, CD63, CD81, CD82, CD151, and NAG-2 (9). Besides integrins, TM4SF proteins also have been suggested to associate with each other (17–19), as well as with Ig superfamily proteins CD2, CD4, CD8, CD19, L1, MHC I, and MHC II; proteoglycans CD44 and syndecan-1; and other proteins CD20, CD21, and \( \gamma \)-glutamyl transpeptidase (20–27). Among this plethora of proposed interactions, little is known about which proteins directly associate with integrin subunits and which proteins are indirectly recruited into complexes with integrins.

Many suggested integrin–TM4SF protein associations are based on co-immunoprecipitation results from cells lysed in detergents such as CHAPS, Brij 99, Brij 58, and octyl glucoside. However, in lysates prepared using detergents that are more hydrophobic (e.g. Brij 96, Triton X-100), integrin–TM4SF interactions appear to be much more restricted. For example, in 1% Triton X-100 cell lysates, \( \alpha_3\beta_1 \) did not associate with any TM4SF protein except for CD151, and CD151 did not associate with any other integrin except \( \alpha_3\beta_1 \) (28). Also in contrast to other integrin–TM4SF associations, \( \alpha_3\beta_1 \)-CD151 association occurred at an unusually high stoichiometry (at least 90% of \( \alpha_3\beta_1 \) was associated), was relatively resistant to the effects of denaturing detergents, and occurred in the apparent absence of any other cell surface proteins (28). The \( \alpha_3\beta_1 \)-CD151 complex was also one of the few integrin–TM4SF protein complexes not disrupted by digitonin (29). In addition, a novel CD151 epitope has been defined (using mAb TS151r) and shown to be quantitatively diminished following \( \alpha_3\beta_1 \) overexpression (29).

The \( \alpha_3\beta_1 \)-CD151 complex may contribute to cell signaling and cell motility. For example, the CD151 protein serves to link \( \alpha_3\beta_1 \) to the signaling molecule, phosphatidylinositol 4-kinase (28). Also, antibodies to CD151 inhibited \( \alpha_3\beta_1 \)-dependent motility of neutrophils (28), and antibodies to both CD151 and \( \alpha_3\beta_1 \) similarly inhibited the motility of endothelial cells (30). Possibly, \( \alpha_3\beta_1 \)-CD151–phosphatidylinositol 4-kinase complexes could serve as a functional unit to support cell migration. Here we have investigated the biochemical basis for \( \alpha_3\beta_1 \)-CD151 complex formation. We provide evidence that the association between CD151 and \( \alpha_3\beta_1 \) is direct and may involve a site in the extracellular “stalk” region of \( \alpha_3 \), interacting with...
a site within the large extracellular loop of CD151. Also, we have provided perhaps the first experimental demonstration that the NH₂ and COOH termini of a TM4SF protein (in this case CD151) are indeed located intracellularly.

**EXPERIMENTAL PROCEDURES**

*Cell Lines and Antibodies—* HT1080, A431, and COS7 cells were maintained in Dulbecco’s modified Eagle’s medium EM supplemented with 10% fetal bovine serum and antibiotics. K562 cells were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum and antibiotics. K562 cells transfected with full-length, wild-type α₅ (K562-α₅-WT) and α₅ (K562-WT) were described previously (31, 32). The following monoclonal antibodies were used in this study: anti-integrin α₂, A2-HE10 (33); anti-integrin α₅, A3-IVA5 and A3-IF5 (31); anti-integrin α₆, AE6-ELE (34); anti-integrin β₁, TS2/16 (35); anti-CD151, 5C11 (28), 11B1 (36), and TS151r (29); anti-CD81, 6/7 (37); anti-CD9, DU-ALL (Sigma); anti-CD-HA (Berkeley Antibody Co., Richmond, CA); anti-vinculin (Sigma); and negative control antibodies, 1B7 (38) and J2A2 (39). Unless otherwise indicated, mAb 5C11 was used for all CD151 immunoprecipitations, and mAb 11B1 was used for all CD151 immunoblots. Polyclonal antibodies against caveolin (Transduction Laboratories, Lexington, KY), the hemagglutinin “HA” tag (Berkeley Antibody Company), and the cytoplasmic domain of integrin α₅A (40) were also utilized in this study. Rabbit polyclonal anti-CD151 was raised against a 15-amino acid peptide (MEGFENKAKTS-GTVC) very similar to the amino-terminal sequence of mouse and human CD151. The peptide was coupled to carrier protein (keyhole limpet hemocyanin) using m-maleimidobenzoyl-N-hydroxysulfosuccinimidyl ester (Pierce) as described previously (41). A rabbit was immunized four times at 2-week intervals and then serum was collected, purified on a column of peptide conjugated to Thiopropyl-Sepharose 6B (Amersham Pharmacia Biotech), and concentrated to 1.4 mg/ml. Pre-purified immune serum was purified using protein A-Sepharose.

**Immunoprecipitation—** Cells were lysed for 1 h in immunoprecipitation buffer (150 mM NaCl, 5 mM MgCl₂, and 25 mM HEPES, pH 7.5) supplemented with 1 mM phenylmethylsulfonyl fluoride, 20 μg/ml aprotinin, 10 μg/ml leupeptin, 2 mM NaF, 0.1 mM Na₃VO₄, and 1% Triton X-100 detergent, unless otherwise indicated. Insoluble material was cleared from lysates by centrifugation at 12,000 rpm for 15 min. To eliminate nonspecific binding material, lysates were then incubated for 1 h with protein G-Sepharose (Amersham Pharmacia Biotech) or protein A-Sepharose alone, or prebound with 187.1 antibody. For immunoprecipitation lysates were incubated with specific antibodies pre-bound to protein G-Sepharose for either 1 h or overnight at 4 °C. In some cases, anti-CD81 or anti-CD151 monoclonal antibodies directly conjugated to CNBr-activated Sepharose (Amersham Pharmacia Biotech) were used. Immune complexes were washed four times in the appropriate lysis buffer and solubilized in either nonreducing or reducing (100 mM dithiothreitol) sample buffer prior to SDS-PAGE.

For analysis of surface epitopes by immunoprecipitation, COS7 transfected with plasmid, washed twice in PBS supplemented with 1% bovine serum albumin, and 0.02% sodium azide (assay buffer), and the respective antibodies were added in assay buffer. Cells were incubated for 1 h at 4 °C, washed three times, and cells were lysed with 1% Triton X-100. Lysates were clarified as above and immune complexes were captured by addition of protein A-Sepharose (keyhole limpet hemocyanin) using m-maleimidobenzoyl-N-hydroxysulfosuccinimidyl ester (Pierce) as described previously (41). A rabbit was immunized four times at 2-week intervals and then serum was collected, purified on a column of peptide conjugated to Thiopropyl-Sepharose 6B (Amersham Pharmacia Biotech), and concentrated to 1.4 mg/ml. Pre-purified immune serum was purified using protein A-Sepharose.

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out and then complexes were disrupted by treatment with 0.1 M DTSSP, a membrane-impermeable cross-linker (Pierce) for 30 min at room temperature prior to quenching with 40 mM Tris, pH 8.0, for 15 min. Cells were then lysed in 1% Brij 96 supplemented with 0.2% SDS, and proteins were immunoprecipitated (IP) as described under “Experimental Procedures,” using the indicated antibodies, including anti-α3 mAb A3-1V6 and anti-CD151 mAb 5C11. Antibody–antigen complexes were dissociated in 0.1 M Tris, pH 8.0, and then further disrupted by incubation with lysis buffer containing 0.5% SDS for 30 min at 4 °C. The dissociated protein solution was centrifuged at 12,000 rpm to remove any remaining debris and then re-immunoprecipitation was carried out using anti-CD151 antibody 5C11 directly conjugated to CNBr-activated Sepharose. These immune complexes were washed four times in lysis buffer supplemented with 0.5% SDS and resolved by reducing SDS-PAGE, to disrupt thiol bonds in the chemical cross-linker. Immunoblots were carried out using polyclonal antibodies to α3 light chain (upper panel) or anti-CD151 mAb 11B1 (lower panel) and were developed using chemiluminescence.

experiments, both primary and secondary antibody incubations were carried out in the presence or absence of 0.5% saponin. Immunofluorescence—COS7 cell transfectants were grown overnight on acid-washed coverslips, washed in warm PBS, and fixed in fresh, 4% paraformaldehyde for 15 min. Cells were either left nonpermeabilized or were permeabilized for 4 min with 0.5% Triton X-100, prior to blocking coverslips for 45 min at 37 °C with PBS supplemented with 10% goat serum. Cells were stained with primary antibodies in 10% blocking coverslips for 45 min at 37 °C with PBS supplemented with 10% goat serum. Cells were either left nonpermeabilized or permeabilized with 0.5% SDS for 30 min at 4 °C. The dissociated protein solution was centrifuged at 12,000 rpm to remove any remaining debris and then re-immunoprecipitation was carried out using anti-CD151 antibody 5C11 directly conjugated to CNBr-activated Sepharose. These immune complexes were washed four times in lysis buffer supplemented with 0.5% SDS and resolved by reducing SDS-PAGE, to disrupt thiol bonds in the chemical cross-linker. Immunoblots were carried out using polyclonal antibodies to α3 light chain (upper panel) or anti-CD151 mAb 11B1 (lower panel) and were developed using chemiluminescence.

Chemical cross-linking of CD151 to integrin αβ3. Intact HT1080 cells were left untreated (−) or cross-linked (+) with 2 mM DTSSP, a membrane-impermeable cross-linker (Pierce) for 30 min at room temperature prior to quenching with 40 mM Tris, pH 8.0, for 15 min. Cells were then lysed in 1% Brij 96 supplemented with 0.2% SDS, and proteins were immunoprecipitated (IP) as described under “Experimental Procedures,” using the indicated antibodies, including anti-α3 mAb A3-1V6 and anti-CD151 mAb 5C11. Antibody–antigen complexes were dissociated in 0.1 M Tris, pH 8.0, and then further disrupted by incubation with lysis buffer containing 0.5% SDS for 30 min at 4 °C. The dissociated protein solution was centrifuged at 12,000 rpm to remove any remaining debris and then re-immunoprecipitation was carried out using anti-CD151 antibody 5C11 directly conjugated to CNBr-activated Sepharose. These immune complexes were washed four times in lysis buffer supplemented with 0.5% SDS and resolved by reducing SDS-PAGE, to disrupt thiol bonds in the chemical cross-linker. Immunoblots were carried out using polyclonal antibodies to α3 light chain (upper panel) or anti-CD151 mAb 11B1 (lower panel) and were developed using chemiluminescence.

RESULTS

Direct Association of αβ3 with CD151—It was shown previously that association of CD151 with the integrin αβ3 is highly stoichiometric, stable, and specific. To determine whether these proteins might be directly associated, we carried out chemical cross-linking of intact HT1080 cells, using a membrane-impermeable reagent (DTSSP; 3′,3′-dithiobis(sulfosuccinimidyl propionate)) with a 12-A spacer arm. Initially, anti-α3, anti-CD151, or control immunoprecipitations were carried out and then complexes were disrupted by treatment with 0.1 M glycine, pH 2.7, and 0.5% SDS. Finally, from the resolubilized material, CD151 was re-immunoprecipitated. As expected from uncross-linked cells, CD151 was isolated from complexes originally immunoprecipitated using anti-α3 or CD151 antibodies (Fig. 1, lanes c and d). However, no integrin α3 subunit remained associated with the CD151. In contrast, from cells treated with DTSSP, re-isolated CD151 remained in association with the integrin α3 subunit (lanes g and h). Thus, cross-linking stabilized αβ3 association with CD151 and made it resistant to harsh, dissociating conditions.

In negative control experiments (using anti-α3 or no primary antibody), we failed to re-immunoprecipitate CD151 or α3 integrin (Fig. 1, lanes a, b, e, and f) even though α3 is highly expressed on HT1080 cells (28). In another control experiment, we immunoprecipitated CD81 from DTSSP-cross-linked cells lysed under stringent detergent conditions (1% Triton X-100), but we failed to co-immunoprecipitate β1 integrin (data not shown). However, under the same conditions, we readily co-immunoprecipitated β1 integrin with CD151 (not shown).

Recent evidence has suggested that caveolin-1 could co-immunoprecipitate with integrins, including αβ3, in Triton X-100 cell lysates (46, 47). Since the majority of αβ3 is associated with CD151 in 1% Triton X-100 lysates (28), we considered that caveolin-1 may also be present in αβ3–CD151 complexes. Caveolin-1 was clearly present in the lysates of A431 cells (lane a). However upon immunoprecipitation of either α3β1 or CD151 from A431 cells, we failed to observe co-immunoprecipitation of caveolin-1 (Fig. 2, lower panel, lanes c and h). Under the same conditions, we did readily observe β1 integrin co-immunoprecipitated with CD151 (upper panel, lane h). Also, caveolin-1 was not co-immunoprecipitated with the integrins α3β1 and α3β2 (lower panel, lanes c and e) and was not obtained using antibodies to TM4SF proteins, CD9 and CD81 (lanes f and g) or control IgG (lane b). In a separate experiment, we failed to detect caveolin in association with CD151 or integrins in 1% Triton X-100 lysates from HT1080 cells (data not shown). Together these data suggest a direct association between α3β1 and CD151 that is independent of caveolin.

Analysis of α3 Ectodomain Chimeras—To determine which α3 extracellular domains are needed for CD151 association, we produced chimeric proteins in which extracellular regions of α3 were swapped with regions from α2, a structurally similar integrin subunit (Fig. 3). Chimeric and wild-type integrins were stably transfected into K562 cells, and multiple subclones of each chimeric integrin transfected were tested for capability to co-immunoprecipitate with CD151. Under reducing condi-
sponding regions of α3 are replaced by the corresponding regions of α6. This chimeric subunit was shown previously to retain CD151 association (28). Numbers (569, 705, 934, 959) represent the first a3 residue adjacent to the a3 sequence, or the last a3 residue adjacent to the a6 sequence.

In another experiment, we utilized transiently transfected K562 cells and again observed that CD151 immunoprecipitates from any K562 transfectant associated with mature α3 (displaying an αL fragment), association with immature α3 (not yet cleaved) was also observed (Fig. 7B, compare top and middle panels, lanes b–i). In all experiments, HA-tagged wild-type and chimeric CD151 were well expressed (Fig. 7, A and B, bottom panels). The occurrence of two forms of these proteins is due to variable glycosylation (data not shown) of one or more of the two glycosylation sites present in the large loop of NAG2 (45).

An anti-CD151 mAb, TS151r, was shown previously to bind to a CD151 site that was masked by the presence of α3 integrin (29). Notably, the TS151r antibody did not bind to the C(185)-N-C(218) mutant on transfected COS7 cells (Fig. 8), but did bind to wild-type CD151 or CD151 mutant in an adjacent region (C(217)-N). Structural integrity of mutant CD151 proteins was maintained as each was comparable with wild-type CD151 with respect to reactivity with the anti-CD151 mAb 5C11. Neither anti-CD151 antibody (5C11, TS151r) bound to wild-type NAG2 protein.

TM4SF Protein Topology—The putative membrane topology of TM4SF proteins (e.g. Fig. 9) is based primarily on hydrophobicity plots. Also, the extracellular location of the large loop is supported by epitope mapping (48, 49) and the presence of sites that undergo N-glycosylation. However, it has yet to be demonstrated that the proposed intracellular amino- and carboxy-terminal domains are indeed intracellular. To gain insights into the topology of CD151, we utilized antibodies against the carboxyl-terminal CD151 HA tag in cell surface binding studies. In a flow cytometry experiment, anti-HA antibodies (α-HA(C′)) failed to recognize COS7 cells stably transfected with HA-tagged CD151 (CD151-HA), unless the cells were first permeabilized with saponin (Fig. 10A). In contrast, unpermeabilized COS7 cells transfected with CD151-WT and CD151-HA were both recognized by an antibody to the extracellular domain of CD151 (α-CD151(EC)). In control experiments, the α-HA(C′) antibody failed to recognize cells transfected with wild-type CD151 (lacking an HA tag), or with vector alone, regardless of saponin permeabilization.

To extend our CD151 carboxyl tail analysis, we also stained COS7 transfectants on coverslips, with or without Triton X-100 permeabilization. As summarized in Table I, monoclonal and polyclonal antibodies against the carboxyl-terminal HA tag (anti-HA(C′), mAb; anti-HA(C′), pAb) each stained CD151-HA

Analysis of CD151 Chimeras—The CD151 molecule contains two putative extracellular domains that could be involved in extracellular contact with α5β1. To ascertain which region might be critical, we prepared and analyzed chimeric CD151 molecules (Fig. 6), in which we incorporated portions of another TM4SF protein, called NAG-2 (45). Swaps were engineered within the third putative transmembrane domain (TM3) or within the second extracellular domain (EC2). HA-tagged mutant and wild-type proteins were transiently expressed in HT1080 cells and immunoprecipitated using anti-HA antibody. Immunoprecipitations were carried out under stringent conditions (1% Triton X-100) such that CD151, but not NAG2, would associate with α5β1 integrin. As indicated, immunoprecipitation of CD151-HA and chimeric N-C(105)-HA each yielded co-immunoprecipitation of the integrin β1 and α3 chains (Fig. 7, A and B, upper panels, lanes b and e). In contrast, NAG2-HA and C(104)-N-HA proteins showed no integrin co-immunoprecipitation (Fig. 7, A and B, upper panels, lanes c and d). These data indicate that for α5β1 integrin association, the small extracellular loop of CD151 (EC1) is not essential, whereas the large loop (EC2) may be required.

In another experiment, co-immunoprecipitation of integrin β1 and α3 chains was seen for CD151 and the C(217)-N mutant, but not for NAG2 or the C(185)-N mutant (Fig. 7, A and B, upper panels, lanes g–j). These results implicate CD151 residues 186–217 as being critical for α3 integrin association. To confirm this, the CD151 aa 186–217 region was replaced by the analogous region from NAG2. Indeed this mutant (C(185)-N-C(218)) lost association with the integrin (Fig. 7A, upper panel, lane o), while association was again maintained for wild-type CD151 (lane l) and for the C(217)-N mutant (lane n). In all cases in which wild-type or mutant CD151 associated with mature α3 (displaying αL fragment), association with immature α3 (not yet cleaved) was also observed (Fig. 7B, compare top and middle panels, lanes b–i). In all experiments, HA-tagged wild-type and chimeric CD151 were well expressed (Fig. 7, A and B, bottom panels). The occurrence of two forms of these proteins is due to variable glycosylation (data not shown) of one or more of the two glycosylation sites present in the large loop of NAG2 (45).
transfected COS7 cells that had been permeabilized. However, these antibodies failed to stain cells that had not been permeabilized, or that had been permeabilized, but not transfected with CD151-HA. A mAb to the extracellular domain (EC) of CD151 strongly stained both permeabilized and unpermeabilized cells. Together with the data in Fig. 10A, these results strongly indicate that the carboxyl terminus of CD151 is intracellular and does not extend into the extracellular environment.

To address whether the amino terminus of CD151 is also intracellular, we prepared polyclonal antibodies to a peptide representing the first 15 amino acids of CD151 (Fig. 9) and tested this reagent (a-CD151(N9)) in an immunoprecipitation/Western blotting procedure (Fig. 10B). Selective immunoprecipitation, of only cell surface molecules, was carried out by pretreating stable COS7 transfectants with antibodies at 4 °C in the presence of sodium azide to prevent internalization. Then unbound antibodies were removed, cells were lysed, and immune complexes were collected. Under these conditions, antibody to the CD151 NH2 terminus, exposed only to cell surface CD151, failed to immunoprecipitate any CD151 (lane d). In contrast, the same antibody immunoprecipitated ample CD151 from total COS7-CD151 cell lysate (lane l). In a control experiment, cell surface CD151 was recognized by an antibody to the extracellular region of CD151 (lane h). In other control experiments, no CD151 was obtained using rabbit preimmune serum (lanes a, c, i, and k) or mouse control Ig (lanes e and g), and no CD151 was obtained from COS7 cells transfected with vector alone (lanes b, d, f, and j).

As indicated in Table I, our rabbit antibody to the CD151 NH2 terminus also stained COS7-CD151 transfectants that

Fig. 4. Association of stably transfected α3 chimeras with CD151. A, K562 cells stably transfected with either vector alone, wild-type α3, wild-type α6, or three separate subclones each of α6/α3-VIII and α6/α3-V chimeras were lysed in 1% Triton X-100 and immunoprecipitated (IP) with antibodies to CD81 or CD151. Immune complexes or whole lysates were resolved by SDS-PAGE under reducing conditions and subjected to immunoblotting with polyclonal antibody to the α3,α6 cytoplasmic tail. Chimeric and wild-type α subunits showed variable extents of maturation, but consistently high levels of precursor α3 (≈160,000 kDa). Thus we chose to analyze precursor α3 (α3pre), instead of the mature, cleaved light chain that was analyzed in Figs. 1, 5, and 7B. B, immune complexes prepared using anti-CD151 (5C11) or anti-CD81 antibodies were blotted with antibody to CD81 (upper panel) and CD151 (11B1, lower panel).

Fig. 5. Association of transiently transfected α3/α6 chimeras with CD151. K562 cells transiently transfected with either vector alone, wild-type α3, wild-type α6, or α3/α6-V, α3/α6-VI, and α3/α6-VIII chimeras were lysed in 1% Triton X-100. Either whole cell lysates (upper panel) or anti-CD151 mAb 5C11 immunoprecipitates (lower panels) were resolved by SDS-PAGE under reducing conditions and subjected to immunoblotting with polyclonal antibody to α3 (upper panels) or CD151 mAb 11B1 (lower panel). Both precursor (α3pre, ≈160,000 kDa, not cleaved) and mature (α3L, ≈30,000 light chain) proteins were detected in the upper panels. Note, in whole cell lysates, more wild-type α3 was recovered compared with mutants, due to the use of a possibly more potent promoter (pFneo compared with cytomegalovirus).

Fig. 6. Chimeric CD151/NAG2 molecules. A, schematic representations of HA-tagged, wild-type CD151, wild-type NAG-2, and chimeric CD151/NAG-2 molecules are shown. The proposed TM and EC domains are indicated. Mutant numbers refer to either the last or first CD151 residue adjacent to downstream or upstream NAG2 sequence, respectively. For example, in the C(185)-N-C(218) chimera, CD151 aa 186–217 have been replaced with the corresponding residues from NAG2.
had been fixed and then permeabilized, but failed to stain COS7 cells that were either mock-transfected or not permeabilized. In control experiments, COS7 transfectants were not stained by rabbit preimmune serum or by FITC-anti-rabbit secondary antibody alone. As a positive control for permeabilization (Table I), the cytoskeletal protein vinculin was stained only when cells were permeabilized. Together, the data in Fig. 10B and Table I strongly suggest that the CD151 NH₂-terminal epitopes recognized by α-CD151(N') antibodies are intracellular, rather than extracellular.

**DISCUSSION**

**Direct Association of α₃β₁ Extracellular Domain with CD151**—Here we have established that protein complex formation between integrin α₃β₁ and TM4SF protein CD151 is direct, not dependent on caveolin, and likely involves a lateral interaction between the extracellular domains of each protein. Evidence for direct contact was obtained through covalent cross-linking using the bivalent agent DTSSP. With a 12-Å spacer arm, that reagent typically only links proteins that are in direct contact. This result is consistent with our previous findings that α₃β₁ can stably associate with CD151 in the absence of any other surface-labeled proteins (28). Also, cross-linking was highly specific, as α₃β₁ did not cross-link with another TM4SF protein (CD81), and CD151 did not cross-link with another integrin (α₂β₁). Previous cross-linking experiments did provide some evidence for α₃β₁–CD81 and other integrin–TM4SF complexes, but the results were much less obvious than seen here, and the presence of multiple components in the complexes complicated interpretation of the results (18).

We considered that a membrane-associated protein such as caveolin-1 could contribute to α₃β₁–CD151 association. However, under conditions that allow strong association between α₃β₁ and CD151 we saw no evidence for any caveolin-1 association. Thus, while caveolin-1 may indeed associate with various integrins on different cell types (46, 47), it is not needed to stabilize CD151–α₃β₁ association.
Multiple lines of evidence indicate that extracellular domains are critical for αβ₁−CD151 association. First, cross-linking was achieved using a membrane-impermeable reagent (DTSSP) that only links extracellular domains. Second, αδ/α3 chimeras were used to map CD151 association to a site (aa 569–705) within the extracellular domain of α2. The use of αδ/α3 chimeras takes advantage of fact that even though αδ and α3 have somewhat similar amino acid sequences (~37%), and although both associate with CD151 under nonstringent conditions, the αδβ₁ integrin does not associate with CD151 under stringent (i.e. Triton X-100) conditions (28). Our conclusions regarding the importance of CD151 and αδ extracellular domains are consistent with previous studies showing that neither the transmembrane nor cytoplasmic tail of α2 was needed for CD151 protein association (28). We conclude that extracellular domains clearly provide specificity and likely sites for direct interaction. Nonetheless, it is possible that hydrophobic transmembrane domains may also make a necessary contribution, even though these domains are not sufficient to stabilize a strong and specific CD151−αδβ₁ interaction when key extracellular sites are mutated.

Thus far, integrin contacts with other proteins have largely been mapped to N-terminal “globular head” regions of the α and β chains (8) and to cytoplasmic tail regions (3, 9). Notably, no specific protein-protein interactions have been reported previously for the membrane proximal stalk-like region of αδβ₁ or any other integrin. Now we demonstrate that CD151 interaction requires an integrin αδ site (aa 569–705) that occurs within the membrane proximal stalk-like region that is predicted by structural models derived from electron microscopy of purified integrins (6, 7). The current study has focused on the very robust αδβ₁−CD151 interaction. In future studies it will be interesting to determine whether the same integrin α chain region (aa 569–705) mediates the observed lateral interactions of αδβ₁ with other TM4SF proteins (9) and with non-TM4SF proteins such as CD147/EMMPRIN (50).

In an earlier study, mutations D346E and D408E within the putative divalent cation binding regions of the αδ integrin chain caused diminished association of αδβ₁ with TM4SF protein CD81 (51). However, comparable mutations within αδ divalent cation sites did not disrupt association with TM4SF proteins.2 We suspect that requirements for strong αδβ₁−TM4SF associations may differ considerably from the weaker αβ₁−TM4SF interactions observed previously. For example, αδβ₁−TM4SF interactions were observed in Brij 99, a

2 A. Chen and M. E. Hemler, unpublished data.
less stringent (i.e., less hydrophobic) detergent, but were abolished in more stringent (i.e., more hydrophobic) detergents such as Triton X-100 and Brij 96. Furthermore, αβ3 could not be cross-linked to TM4SF proteins (not shown), suggesting that αβ3–TM4SF interactions may be indirect.

Previously, we observed that αβ3 appearance was accompanied by CD151 on every cell and tissue type that we examined (28). Here we extend that correlation as we show that not only mature αβ3, but also the uncleaved biosynthetic precursor form of α5 associates with CD151. In fact, in all five cases (Figs. 5 and 7) in which mature wild-type or mutant α5 associated with wild-type or mutant CD151, the immature αβ3 was also found to associate. Is it possible that those few precursor α5 chimeras that did not mature failed to do so because they lacked CD151 association? Indeed our results support a hypothesis (still needing to be further tested) in which CD151 association, occurring early in biosynthesis, might actually be required for α5 integrin maturation and cell surface expression.

Elsewhere it was shown that the TM4SF protein CD9 could associate with a precursor form of the integrin β3 chain, with no apparent involvement of integrin chains (49). However the CD9–β3 interaction is readily lost in stringent detergent conditions (i.e. 1% Nonidet P-40; not shown) or in the presence of digitonin (29). Thus it appears to be quite distinct from the CD151–αβ3 interaction described here and possibly may be less direct. Furthermore, we have preliminary evidence that a single chain truncated form of CD151/NAG2 chimeras were cross-linked to TM4SF proteins (not shown), suggesting that even in the absence of the integrin αβ3 chain, with less direct. Furthermore, we have preliminary evidence that a single chain truncated form of CD151 may associate with CD151, even in the absence of the integrin β3 chain.3

Structural Features of CD151—CD151/NAG2 chimeras were used to map αβ3 integrin association to a region (aa 186–217) within the COOH-terminal portion of the large extracellular loop (Fig. 6). The use of CD151/NAG2 chimeras takes advantage of the fact that NAG2 fails to associate with αβ3 under stringent detergent conditions. However, under less stringent detergent conditions (e.g. Brij 96, Brij 99) NAG2 and every other TM4SF protein that we have tested do associate with αβ3 and other integrins. It remains to be determined whether this same region in the COOH-terminal large loop of CD151 will be involved in its weaker interactions with many other integrins (16). Also it will be interesting to determine for other TM4SF proteins whether the COOH-terminal ends of their large loops are involved in their relatively weaker integrin interactions. In this regard, the relatively nonstringent CD9 interaction with mature β3 integrin was mapped to a region containing the large loop of CD9 plus the fourth transmembrane domain (49).

The standard topological model for TM4SF proteins (Fig. 9) assumes that there are four transmembrane domains, and two extracellular loops, flanked by short intracellular NH2- and COOH-terminal domains. Previous studies of N-glycosylation sites and mAb epitope mapping have shown definitively that the putative large loop of TM4SF proteins must have an extracellular orientation (48, 49). However, the intracellular orientation of the NH2- and COOH-terminal regions had not been demonstrated explicitly. Here, in the process of expressing and analyzing CD151, we have determined that antibodies to an NH2-terminal peptide and to a COOH-terminal HA tag did not react with CD151 from intact cells, but did recognize CD151 from cells that had been lysed or permeabilized. These results establish that both the NH2- and COOH termini of the molecule are indeed highly likely to be oriented extracellularly, consistent with the assumed topological model (Fig. 9).

In summary, we have uncovered a novel α5 integrin site, within the membrane proximal stalk region, that is involved in direct lateral association with the TM4SF protein CD151. Also we have established that integrin interaction requires a relatively small region within the large extracellular loop of CD151. Finally, we have obtained evidence that strongly supports the predicted TM4SF protein topology by demonstrating that both NH2- and COOH termini are indeed intracellular. These results begin to provide the biochemical details needed to understand how a TM4SF protein such as CD151 may form functionally relevant complexes with the αβ3 integrin.

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