Generation of Monoclonal Antibodies to Integrin-associated Proteins

EVIDENCE THAT $\alpha^3\beta_1$ COMPLEXES WITH EMMPRIN/BASIGIN/OX47/M6*

(Received for publication, March 24, 1997, and in revised form, August 27, 1997)

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The $\alpha^3\beta_1$ integrin forms complexes with other cell-surface proteins, including transmembrane-4 superfamily (TM4SF) proteins (e.g. CD9, CD53, CD63, CD81, and CD82). To identify additional cell-surface proteins associated with $\alpha^3\beta_1$ integrin, a monoclonal antibody selection protocol was developed. Mice were immunized with integrin $\alpha^3\beta_1$-containing complexes isolated from HT1080 fibrosarcoma cells, and then 712 hybridoma clones were produced, and 95 secreted antibodies that recognized the HT1080 cell surface. Among these, 12 antibodies directly recognizing integrin $\alpha^3$ or $\beta_1$ subunits were eliminated. Of the remaining 83, 16 co-immunoprecipitated proteins that resembled integrins under non-stringent detergent conditions. These 16 included 15 monoclonal antibodies recognizing EMMPRIN/basigin/OX-47/M6, a 45–55-kDa transmembrane protein with two immunoglobulin domains. The EMMPRIN protein associated with $\alpha^3\beta_1$ and $\alpha^3\beta_2$, but not $\alpha^2\beta_1$ or $\alpha^3\beta_3$, as shown by reciprocal immunoprecipitation experiments. Also, association with $\alpha^3\beta_1$ was confirmed by cell-surface cross-linking and immunofluorescence co-localization experiments. Importantly, EMMPRIN-$\alpha^3\beta_1$ complexes appear not to contain TM4SF proteins, suggesting that they are distinct from TM4SF protein-$\alpha^3\beta_1$ complexes.

It is well established that ligand binding and cell-surface clustering of integrins can lead to the assembly of large multi-component intracellular signaling complexes (1–4). More recently, integrins have also been found to associate with other cell-surface molecules. For example, the CD47/IAP molecule associates with integrin $\alpha^3\beta_3$ (5, 6), and glycosylphosphatidylinositol-linked receptors such as CD87/uPAR, CD16b/FcγRIIB, and CD14 show functionally relevant interactions with $\beta_1$ (7) and $\beta_3$ (8, 9) integrins. In addition, proteins from the transpersion or transmembrane-4 superfamily (TM4SF), including CD9, CD53, CD63, CD81, and CD82, interact with several integrins, including $\alpha^3\beta_2$, $\alpha^3\beta_1$, $\alpha^3\beta_3$, and $\alpha^6\beta_1$ (10–20). Some of these interactions may regulate integrin-dependent cell adhesion, spreading, and migration (21–23). Importantly, these transmembrane complexes may also contain intracellular signaling proteins thus suggesting that the complexes may play an important role in adhesion-dependent signaling (24).

To identify and characterize additional cell-surface proteins that might form complexes with integrins, we designed a systematic approach based on a three-step monoclonal antibody screening protocol. After immunizing mice with integrin complexes, we screened for mAbs to integrin-associated proteins by (i) flow cytometry to select mAbs reactive with the cell surface, (ii) flow cytometry to eliminate anti-integrin mAbs, and (iii) high and low stringency immunoprecipitation. By applying this approach we have discovered novel interactions between two integrins ($\alpha^3\beta_1$ and $\alpha^6\beta_1$) and the EMMPRIN/basigin/OX47/M6 protein. The latter molecule is a widely distributed cell-surface protein with two immunoglobulin-like domains (25–28) that may play a role in regulating metalloproteinase production (29).

EXPERIMENTAL PROCEDURES

Cell Lines—The human fibrosarcoma cell line HT1080 was maintained in DMEM medium supplemented with 10% fetal calf serum (FCS), CHO and transfected CHO-$\alpha^3\beta_2$, CHO-$\beta_3$, CHO-$\alpha^3\beta_2$ cells were prepared as described previously (30, 31) and maintained in α-minus minimum essential medium supplemented with 1 mg/ml G418. CHO-EMMPRIN was developed by stably transfecting CHO cells with EMMPRIN cDNA (32) that had been cloned into the pZeoSV expression vector (INVITROGEN Co.).

Antibodies—Anti-integrin mAbs utilized were anti-$\alpha^3$, A2-HI6 (33); anti-$\alpha^3$, A3-X8, A3-IVA5, and A3-HIF5 (34); anti-$\alpha^3$, A5-PUJ2 (35); anti-$\alpha^3$, A6-ELE (36); anti-$\beta_1$, TS2/16 (37); and A-1A5 (38). Anti-integrin $\alpha^6$ mAb, A2-VIIc6, was selected following immunization of RBF/DnJ mice with human lung carcinoma cells in a previously described hybridoma fusion (39). Other mAbs were anti-CD61, M38 (40), anti-MHC class I, W6/32 (41), and negative control mAb P3 (42). Rabbit polyclonal antisera against peptides representing the cytoplasmic domains of integrin $\alpha^3$ (43) and $\beta_1$ (44) subunits were previously described.

Immunoprecipitation—HT1080 cells were surface-labeled with NHS-LC-biotin (Pierce) or Na125I according to established protocols and lysed in immunoprecipitation buffer (1% Brij 96, 25 mM HEPES, pH 7.5, 150 mM NaCl, 5 mM MgCl2, 2 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 10 μg/ml leupeptin) for 1 h at 4 °C. Immune complexes were collected onto Sepharose 4B beads (Pharmacia, Uppsala, Sweden) that were pre-bound with mAb, followed by four washes with the immunoprecipitation buffer. For immunoprecipitation under stringent conditions, the immunoprecipitation buffer was supplemented with 0.2% SDS. Immune complexes were eluted from beads with Laemmli sample buffer and resolved by 10% SDS-PAGE under non-reduced conditions. Proteins were detected using OXAR films (Eastman Kodak Co.) for 1–20 days at −70 °C. Biotin-labeled proteins were trans-
ferred to nitrocellulose membranes and visualized with peroxydase-conjugated ExtrAvidin (Sigma) using Renaissance Chemiluminescent Reagents (NEN Life Science Products). In some experiments 1% Brij 96 in lysis and washing buffers was substituted by 1% octyl glucoside.

Re-immunoprecipitation experiments were performed as described earlier (11). Briefly, protein complexes were immunopurified using anti-EMMPRIN (8G6) or anti-integrin mAb-conjugated Sepharose 4B from nonstringent (without 0.2% SDS) Brij 96 lysates of surface-biotinylated HT1080 cells. After five washes, the protein complexes were dissociated for 30 min at 4 °C with Brij 96 buffer containing 0.2% SDS. The eluates were subsequently reprecipitated with mAbs directly coupled to Sepharose 4B. In some experiments 1% Brij 96 was utilized (Fig. 1, lanes a–e). A control antibody yielded only a few background proteins (lanes f and l). Integrin co-immunoprecipitation experiments from several other cell lines (data not shown) yielded cell-surface proteins with sizes similar to those co-immunoprecipitated in Fig. 1.

Production and Initial Characterization of mAb to α\(\beta_1\)-associated Proteins—To identify additional integrin-associated cell-surface proteins (such as those seen in Fig. 1, lane h), a strategy for systematic selection of mAbs was developed. We focused on proteins complexed with α\(\beta_1\), because of the apparent abundance of diverse cell-surface proteins associated with that integrin. Beads coated with antibody A3-IVA5 were used to purify α\(\beta_1\)-containing protein complexes from the HT1080 human fibrosarcoma cell line, and mice were immunized with integrin complexes directly immobilized on the beads. Next, 712 hybridoma clones were prepared, and then mAbs were screened by a three-step protocol. In step 1, flow cytometry was utilized to show that 95 of the 712 hybridoma clones secreted antibodies that recognize cell-surface structures on HT1080 cells. To eliminate antibodies directly recognizing human α\(3\) or β1 subunits (step 2), mAb clones were next tested for staining of CHO, CHO-α\(\beta_1\), CHO-β1, or CHO-α\(3\)β1 transfectants. Of the 95 mAbs from step 1, 12 stained α\(3\) or β1 transfectants, but not untransfected CHO cells, and thus are likely to be anti-α\(3\) or β1 integrin antibodies. The remaining 83 antibodies (after steps 1 and 2) are candidates to recognize cell-surface structures distinct from but potentially associated with the α\(3\)β1 integrin. An example of these is hybridoma clone 8G6, selected in step 1 because it stained the surface of HT1080 cells (Fig. 2, top right). The 8G6 mAb was retained after step 2, because it did not show preferential binding to a CHO-α\(\beta_1\) transfectant (Fig. 2, bottom right) compared with CHO cells (Fig. 2, middle right). The presence of human α\(3\) integrin on HT1080 cells and CHO-α\(3\)β1 transfectants, but not CHO cells, is confirmed in the left panels of Fig. 2.

![Fig. 1. Immunoprecipitation of integrin-associated proteins from HT1080 cells.](image-url)
In step 3, non-stringent immunoprecipitation experiments were carried out to determine which mAbs might co-precipitate integrin-like proteins. Of the 83 mAbs tested, 16 mAbs co-immunoprecipitated integrin-like proteins of 120–150 kDa, together with several other proteins. Examples of these are mAb 5C11 (Fig. 3, lane d) and mAb 8G6 (lane j). In contrast, mAb 7C3 (lane f) and 8D8 (lane h) are examples of the 67 mAbs that did not co-precipitate detectable integrin-like proteins. The results of mAb screening are summarized in Table I.

Identification of αβ₁-associated Proteins—The 5C11 mAb precipitated fewer total proteins under stringent conditions (Fig. 3, lane c) compared with non-stringent conditions (Fig. 3, lane d). A protein of 27–29 kDa was seen in lane c, but the presence of several other proteins made it difficult to determine which protein was directly recognized by mAb 5C11. The associated protein defined by this antibody will be more fully described elsewhere.

Under non-stringent conditions, multiple biotin-labeled proteins were precipitated by mAb 8G6 (Fig. 3, lane j). In contrast, only 45–55-kDa protein bands were seen using 8G6 under stringent conditions (Fig. 3, lane i). Notably, under stringent detergent conditions, 14 other mAbs (all selected as in Table I) co-precipitated 45–55-kDa proteins nearly identical to that obtained using 8G6. To characterize the 45–55-kDa protein(s), mAbs recognizing EMMPRIN were selected and characterized further. M Abs recognizing EMMPRIN (45–55 kD) 15
2. mAbs recognizing another protein(s) 1

![Flow cytometry screening of mAbs produced against αβ₁ complexes.](image1)

![Examples of mAbs that co-immunoprecipitate integrin-like heterodimers.](image2)

![The mAb 8G6 recognizes EMMPRIN.](image3)
further, we utilized 8G6-coated Sepharose beads to purify ~400 pmol of protein from ~800 g of human placenta. Purified material, visualized by silver staining, is shown in Fig. 4A, lanes 3 and 4). Amino-terminal amino acid analysis revealed a perfect match, at 22 of 24 positions (Fig. 4B), between the purified protein and the amino terminus of the human EMMPRIN/M6 antigen (25, 28). Confirming conclusively that mAb 8G6 recognizes human EMMPRIN, CHO cells transfected with EMMPRIN cDNA gained the 8G6 epitope as detected by flow cytometry (Fig. 4C). Three additional mAbs (6G6, 7E7, and 4F10) that recognize the 45–55-kDa protein also showed strong reactivity toward the EMMPRIN-transfected CHO cells (not shown).

Association of EMMPRIN with Integrins—To confirm that EMMPRIN is indeed associated with α3β1 integrins, and to determine which other integrins might be associated, we carried out three sets of experiments. First, by Western blotting we detected β1 integrins within an EMMPRIN immune complex (Fig. 5A, lane b) but not in a MHC class I complex (Fig. 5A, lane a). Notably, anti-β1 antibodies reacted with both immature (pre-β1) and mature forms of β1 in the whole cell lysate (Fig. 5A, lane c), but only mature β1 was associated with EMMPRIN (Fig. 5A, lane b). Second, in re-immunoprecipitation experiments, both the α3β1 and α6β1 integrins but not α2β1 or α5β1 could be recovered from mAb 8G6 immunoprecipitates of EMMPRIN (Fig. 5B, lanes a–d). Third, mAb 8G6 re-immunoprecipitated EMMPRIN only from α3 and α6 but not α2 or α5 immunoprecipitates (Fig. 5C, lanes a–d). Notably, the size of the EMMPRIN associated with integrins (Fig. 5C) appears a little smaller than the total EMMPRIN as seen in Figs. 3 and 4A. Possibly, a smaller form of EMMPRIN could selectively associate with integrins. Alternatively, excess glycosylation in the larger forms of EMMPRIN could block access of tyrosines to iodination (used in Fig. 5), while not blocking availability for silver staining (used in Fig. 4A) or access of amino groups to biotin (used in Fig. 3).

Integrin-EMMPRIN Complexes on the Cell Surface—To establish whether integrin-EMMPRIN complexes may be present on the cell surface, intact HT1080 cells were treated with the cleavable cross-linker dithiobis(succinimidyl propionate). The mAb 8G6 was then used to immunoprecipitate EMMPRIN under stringent conditions to disrupt non-covalent interactions with integrins, and precipitated proteins were subsequently analyzed under reducing conditions to disrupt covalent cross-linker bonds. As indicated, without cross-linker mAb 8G6 precipitated only EMMPRIN (Fig. 6, lane b), but in the presence of 1 mM dithiobis(succinimidyl propionate), additional proteins resembling integrins were present (lane d). These proteins co-migrate with α3β1 (lane e) but likely contain α5β1, as well as α3β1. Besides α3β1, a small amount of 45–55-kDa protein co-migrating with EMMPRIN was also present in the α3 immunoprecipitate prepared from the cells treated with cross-linker (lane e). In contrast, proteins resembling EMMPRIN are not typically seen in stringent α3 immunoprecipitations in the absence of cross-linking (e.g. see Fig. 1, lane h).

Co-localization of α3β1, Integrin and EMMPRIN in Cell-Cell Contacts—In HT1080 cells grown on coverslips for 24–72 h, EMMPRIN and α3β1 were found to strongly co-localize at cell-cell contact sites, as seen by immunofluorescent double staining (Fig. 7, A and B). In control experiments, mAb to MHC class I (Fig. 7C) and integrin α2β1 (Fig. 7D) showed even staining of cell surfaces, and some intracellular staining, but no appreciable staining of cell-cell contact sites. When HT1080 cells are plated on laminin substrates, the α3β1, α6β1, and TM4SF proteins localize into focal complexes (24), a type of complex that contains phosphotyrosine and is assembled at the periphery of spread cells (45). However, EMMPRIN does not appear to localize in focal complexes. In HT1080 cells plated on a laminin-5-containing ECM substrate, focal complexes (at the cell periphery) were prominently stained with anti-phosphotyrosine mAb (Fig. 8B). In marked contrast, double staining with mAb 8G6 showed that EMMPRIN was evenly distributed on the cell surface, with elevated levels at cell-cell contacts, but was essentially absent from focal complexes (Fig. 8A).

EMMPRIN-α3β1 and CD81-α3β1 Complexes Are Distinct—Given the difference in their cellular localization (focal com-
plexes versus cell-cell contact sites), we hypothesized that EMMPRIN-α3β1 and TM4SF-α3β1 complexes may be distinct. To examine this issue further, we carried out immunoprecipitation experiments to determine whether CD81, one of the most predominant TM4SF proteins on HT1080 cells, was present in α3β1-EMMPRIN complexes. As indicated, anti-CD81 mAb precipitated an abundance of the 22-kDa CD81 molecule, together with proteins co-migrating with α3β1, but no proteins resembling EMMPRIN (Fig. 9, lane b). Conversely, anti-EMMPRIN mAb 8G6 precipitated an abundance of the 45–55-kDa EMMPRIN protein and proteins resembling α3β1, but no proteins co-migrating with CD81 (lane c). From proteins precipitated using anti-EMMPRIN mAb 8G6, re-immunoprecipitation experiments clearly showed the α3β1 integrin (Fig. 9, lane d) but no detectable CD81 (lane e). Thus, CD81 does not appear to associate with EMMPRIN. Non-stringent precipitation with an anti-α3 mAb yielded a range of proteins (Fig. 9, lane a), but those corresponding to the 22-kDa CD81 protein and the 44–55-kDa EMMPRIN doublet were not very prominent.
Proteins Complexing with $\alpha^3\beta_1$ Integrin

Identification of Associated Proteins—It is becoming increasingly apparent that full understanding of integrin functional activities may require understanding of integrin associations with other cell-surface molecules (see Introduction). Here we describe a novel approach for identifying cell-surface proteins that associate with integrins. Our screening protocol is based on well established techniques (flow cytometry and immunoprecipitation) and requires only 3–4 days to identify mAbs that recognize integrin-associated proteins. Our protocol has two important advantages over alternative approaches used previously (46). First, selected mAbs recognize proteins that interact with intact integrin molecules. Thus we may avoid adventitious interactions that arise upon un-natural presentation of fragments. Second, use of immunoprecipitation early in screening provides a reliable assessment of protein-protein interactions. Furthermore, cross-linking subsequent to immunoprecipitation provides an early control for the authenticity of putative associations in the context of intact cells.

In a previous study, we immunized mice with intact cells from a mammary epithelial cell line, screened all of the antibodies by immunoprecipitation, and thus identified a novel association between integrins and the TM4SF protein CD63 (10). Our current immunization and antibody selection procedure is designed to generate fewer irrelevant antibodies upon initial immunization, and it requires many fewer time-consuming immunoprecipitations. Furthermore, it seems likely that this approach could be easily applied to identify proteins associated with other integrins or with other cell-surface proteins (e.g., see the accompanying paper (46)).

Integrin-TM4SF Associations—Various co-immunoprecipitation experiments have shown previously that $\alpha^{\text{th}}\beta_3$, $\alpha^6\beta_1$, and $\alpha^\beta_1$ integrins associate with several TM4SF proteins, including CD9, CD53, CD63, CD81, and CD82 (10–20). As mentioned here, mouse immunization with $\alpha^3\beta_1$ complexes yielded mAb 5C11. As will be fully described elsewhere, the 5C11 mAb recognizes the TM4SF protein PETA-3/SFA-1. This result confirms and extends the concept that nearly all TM4SF proteins may associate with certain integrins.

Integrin Association with EMMPRIN—Perhaps the more novel finding is that specific integrins ($\alpha^3\beta_1$ and $\alpha^6\beta_1$) can associate with EMMPRIN (25), a widely distributed 44–45-kDa cell-surface protein with two immunoglobulin domains. Integrin/EMMPRIN association was established by means of reciprocal immunoprecipitation experiments, and $\alpha^3\beta_1$/EMMPRIN association was confirmed by cell-surface cross-linking and immunofluorescence co-localization experiments.

The human EMMPRIN (25) protein is named for its extra-cellular matrix metalloproteinase induction (29) and is identical to the M6 leukocyte activation antigen (28). Also, EMMPRIN is highly homologous to a rat molecule named Ox-47 (27) or CEs9 (47), the mouse basigin (26) or gp42 (48) molecule, and the chicken HT7 (49) or neurothelin (50) molecule. Previous analyses have shown that EMMPRIN size variation results from glycosylation heterogeneity (28). Interestingly, by screening a cDNA expression library with polyclonal sera raised against partially purified integrin $\beta_1$ subunit (51), Alturda and co-workers (48) cloned the mouse EMMPRIN/gp42 molecule. Although this earlier study lacks firm biochemical evidence for the EMMPRIN-integrin interactions, to some extent it foreshadows our current findings.

From co-immunoprecipitation studies we estimate that at least 2–5% of the total $\alpha^3\beta_1$ and $\alpha^6\beta_1$ integrin may be associated with EMMPRIN. However, this may be an underestimation due to the technical limitations of immunoprecipitation. Indeed, a much larger proportion of both $\alpha^3\beta_1$ and EMMPRIN appears to be co-localized by immunofluorescence and thus at least are in proximity, if not directly associated.

The EMMPRIN/basigin/HT7/OX-47/CE9/neurothelin molecule has been proposed to be a blood-brain barrier molecule (49), but knock out mice lacking this gene have not yet displayed alterations in blood-brain barrier function (52). Also, EMMPRIN has been suggested to be involved in cell-cell interactions. For example, a mAb against the chicken HT7/neurothelin protein caused a perturbation in cell contact-dependent maturation of glial cells in vitro (53). Indeed, our immunofluorescent studies showed that EMMPRIN co-localizes with $\alpha^3\beta_1$ integrin in cell-cell contacts and therefore may potentially interact with one another in a receptor-ligand fashion. In this regard, several transmembrane proteins in the immunoglobulin superfamily act as ligands for various integrins. For example, VCAM-1 binds to $\alpha^4\beta_1$ and $\alpha^6\beta_1$ (54); CD31/PECAM-1 binds to the $\alpha^6\beta_1$ integrin (55); and ICAM-1, ICAM-2 and ICAM-3 bind to $\alpha^4\beta_1$ (56). However, expression of moderate to high levels of EMMPRIN in CHO cells did not result in increased adhesion to $\alpha^3$-positive cells (not shown). Also, EMMPRIN-integrin co-precipitation was not diminished in the presence of EDTA, an agent known to inhibit nearly all integrin-ligand interactions (not shown). Furthermore, unlike EMMPRIN, no established integrin immunoglobulin-like ligands (VCAM-1, CD31, and ICAM) have ever been shown to co-precipitate with soluble integrins in detergent lysates. Therefore, it is possible that $\alpha^3\beta_1$ and $\alpha^6\beta_1$ associate with EMMPRIN in lateral fashion, similar to interactions of $\beta_3$ integrins with CD47 (5, 6), another IgSF protein.

Several lines of evidence suggest that integrin-TM4SF and integrin-EMMPRIN complexes may represent two separate entities on the cell surface. First, immunofluorescence studies clearly showed that EMMPRIN is distributed in regions of cell-cell contact and diffusely throughout the membrane but not in focal complexes. In contrast, TM4SF proteins such as CD63 and CD81 can readily localize to focal complexes at the periphery of a spread cell, in a pattern that looks quite distinct from EMMPRIN (24). Second, $\alpha^3$ integrin was readily re-precipitated from an EMMPRIN immunoprecipitation, but the most prominent TM4SF protein on HT1080 cells, CD81, could not be detected. Finally, neither EMMPRIN itself nor EMMPRIN-integrin complexes appear to associate with phosphatidylinositol 4-kinase activity (not shown). In contrast, phosphatidylinositol 4-kinase activity is readily detected in association with several TM4 proteins and TM4-integrin complexes (24).

The functional relevance of integrin-EMMPRIN association is unclear. The preferential association of EMMPRIN with mature $\beta_1$ integrins suggests that the interaction may not play a role during integrin chain association or biosynthetic processing. So far we have found that functions of mature integrins such as cell-cell adhesion or adhesion to extracellular matrix were not altered by either ectopic EMMPRIN expression or anti-EMMPRIN antibodies (not shown). Also, preliminary evidence suggests that $\alpha^3\beta_1$-EMMPRIN association is not regulated by cell adhesion, since the stoichiometry of the $\alpha^3\beta_1$-EMMPRIN interaction was comparable on K562 suspension cells and on HT1080 adherent cells. However, it is perhaps more than a coincidence that both EMMPRIN (29) and $\alpha^3\beta_1$ (57, 58) may help to regulate matrix metalloproteinase production. Future studies will be aimed at determining the extent to which the physical association between EMMPRIN and $\alpha^3\beta_1$ may play a role in adhesion-induced matrix metalloproteinase production.
In conclusion, here we have demonstrated a new strategy for characterization of cell-surface proteins that form complexes with integrins. By using this approach we have discovered that integrins (αβ3 and αβ6) not only associate with TM4SF proteins but also with EMMPRIN, a member of the Ig superfamily. This represents the first clear demonstration that the EMMPRIN/basigin/OX47/M6 molecule can associate with other proteins.

Acknowledgments—We thank Bryan P. Toole (Tufts University) for EMMPRIN cDNA and Osamu Yoshie (Shionogi Institute, Osaka, Japan) for anti-TM4SF antibodies. We also thank John McDonald (Mayo Clinic, Scottsdale, AZ) and Richard Hynes (MIT) for rabbit polyclonal antibodies to integrin tails.

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