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CD63 Associates with Transmembrane 4 Superfamily Members, CD9 and CD81, and with β1 Integrins in Human Melanoma

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CD63 belongs to the Transmembrane 4 superfamily (TM4SF) of membrane proteins whose functions are largely unknown. Previous results have suggested that CD63 may play an important role in the regulation of melanoma progression. To explore the role of CD63 in melanoma we have examined its association with other molecules by immunoprecipitation of CD63 from detergent induced lysates of melanoma cells. These results are the first to demonstrate an association between CD63 and two other TM4SF members, CD9 and CD81 in 2 human melanoma cell lines. We were also able to identify an association between CD9 and CD63 with β1 integrins in melanoma. The results suggest that CD63 is capable of forming multicomponent complexes with TM4SF members and β1 integrins on the surface of melanoma. These findings provide further insights into the function of CD63.

CD63 is a heterogeneous membrane-bound glycoprotein which belongs to the newly identified transmembrane 4 superfamily (TM4SF) of cell surface proteins (1, 2). Members of this family are characterised by the presence of four highly conserved transmembrane domains, suggesting that these proteins may perform closely related functions. TM4SF members have been shown to play roles in signal transduction pathways (3–7) and to regulate cell activation (8, 9), development (10), proliferation (11), motility (12, 13) and adhesion (14) in a number of cell types. TM4SF members have also been shown to form non-covalent associations with each other and other molecules such as those involved in signal transduction (15–19) and adhesion (20–23). These results have suggested that TM4SF members are capable of existing in large multicomponent complexes, and that they may serve as receptor-associated ion channels or act to transduce of signals across the plasma membrane (2).

Until recently, relatively little was known about the function of CD63. CD63 is known to be ubiquitously expressed on the surface of most cultured melanoma cell lines (24) and its mRNA is detected in many cell types (25). Its expression in human tissue is, however, limited to glandular tissues (24, 26), macrophages (27) and the lysosomal membranes of platelets and vascular endothelial cells. In the latter it is translocated to the cell surface upon activation (28, 29). Interestingly, CD63 is strongly expressed on the cell surface in the primary stages of melanoma, but is weaker, or absent in the more malignant stages and in normal melanocytes (27, 30). This has led to speculation that CD63 may limit the progression of melanoma. Studies on CD63-transfected 3T3 cells have shown that CD63 suppresses their growth in nude mice (31). These results were extended in our previous studies by showing that transfection of CD63 into human melanoma cells suppressed their growth and metastasis in nude mice whilst having no effect on the growth of melanoma cells *in vitro* (32).

In the present study we have examined the molecules that associate with CD63 on the surface of melanoma. We employed immunoprecipitation techniques of mild detergent lysates to identify associated proteins and confirmed their identity by re-precipitation and western blotting. The

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Abbreviations: TM4SF, Transmembrane 4 superfamily; MAb, monoclonal antibody; PBS, phosphate-buffered saline.

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results indicate that CD63 is associated with other TM4SF family members, CD9 and CD81, and β1 integrins in melanoma.

MATERIALS AND METHODS

Cell lines and antibodies. The MEL-FC cell line was isolated from the subcutaneous melanoma metastasis of a 72 year old male by our laboratory, and the MV3 melanoma cell line (a gift of Dr. G.N.P. van Muijen) was isolated from a lymph node metastasis as previously described (33). These cell lines were routinely cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% FCS.

The LS62 monoclonal antibody (MAb) against CD63 (24) was kindly supplied by Dr. M. Jerry (Calgary, Canada). Mab13 against β1 and polyclonal anti-β1 were generous gifts of Dr. Ken Yamada (NIH, Bethesda, MD). MAbs FMC56, 5A6, and 1D4.5 were gifts from Dr. H. Zola (Flinders Medical Centre, Bedford Park, South Australia), Dr. S. Levy (Department of Medicine, Stanford University, CA), and Dr. L. Ashman (Adelaide, South Australia) respectively. HB57 was obtained from the American Type Culture Collection (ATCC, Rockville, MA.). Non-immune mouse serum was purchased from Vet Services (Australia).

Flow cytometry. Cells were washed in PBS and incubated in saturating concentrations of MAbs at 4°C for 30 min. Cells were washed twice in PBS and incubated in FITC-conjugated sheep anti-mouse immunoglobulin (1:100, Silenus, Dandenong, Australia) for 30 min at 4°C. After washing twice cells were fixed with 1% formaldehyde in PBS. Stained cells were analysed on a Becton Dickinson FACScan (Mountain View, CA).

Cell radioiodination and immunoprecipitation. Cells were harvested with trypsin-EDTA, allowed to recover for 1h in DMEM and then washed 3 times in PBS before radioiodination. Cells were surface labelled with 1mCi $Na^{125}I$ by a modified lactoperoxidase method utilising glucose oxidase for 15 min at room temperature (33). The cells were then washed 3 times with PBS prior to detergent lysis and immunoprecipitation studies. Cells were lysed for 30 min at 4°C in lysis buffer (10mM Tris-HCl pH 7.4, 140mM NaCl, 0.5mM $CaCl_2$, 0.5mM $MgCl_2$, 0.02% NaN_3, 1mM phenylmethylsulfonyl fluoride (Sigma-Aldrich, St Louis, MO), 0.1mg/ml aprotinin (Sigma)) containing either 1% CHAPS (Boehringer Mannheim, W. Germany), 1% NP-40 (Sigma), 1% Brij 96 (Sigma) or 60mM octylglucoside (Amresco, Solon, Ohio). Insoluble material was removed by centrifugation at 10,000rpm, 4°C for 10 min, and the lysate precleared twice with irrelevant MAb 1D4.5 or rabbit anti mouse immunoglobulins (RAM) (DAKO, Carpentaria, CA) coupled to CnBr activated Sepharose beads (Pharmacia Biotech, Uppsala, Sweden) for 2 hours at 4°C. Cells lysates were then incubated overnight with specific antibodies directly coupled to CnBr-activated Sepharose beads or added to the lysates together with RAM beads. Where samples were subjected to re-precipitation, proteins were eluted from the beads at low pH in 2.25ml elution buffer (50mM glycine-HCl pH 2.5, 0.15M NaCl, 0.1% Triton X-100) and neutralised in 250μl 2M Tris pH 8.0. The eluate was passed through a PD-10 desalting column (Pharmacia) and eluted in 1% Triton X-100 lysis buffer. The lysate was then subjected to a second round of immunoprecipitation. The beads were washed 4 times in lysis buffer, eluted with Laemmli sample buffer by boiling (9), and then resolved by SDS-PAGE. Gels were fixed, stained, dried and labelled protein bands visualised by autoradiography.

Western blotting. For western blotting, cells were lysed with 1% CHAPS and immunoprecipitated as before. Associated proteins were eluted from the beads in 1% NP-40 lysis buffer for 1 hour at room temperature. The eluate was mixed with an equal volume of non-reducing Laemmli sample buffer, boiled for 5 minutes and resolved by SDS-PAGE. Proteins were transferred to nitrocellulose at 40V for 1 hour in transfer buffer (25mM Tris, 192mM glycine and 20% methanol) according to an established method(34). The membrane was blocked for 1 hour at room temperature in TTBS (20mM Tris pH 7.5, 500mM NaCl, 0.05% Tween-20) containing 5% skimmed dried milk. The membrane was then incubated with MAb (1ug/ml) in TTBS + 1% skim milk powder for 2 hours at room temperature, washed twice in TTBS, then incubated for 1 hour in horseradish peroxidase-conjugated (HRP) goat anti-mouse immunoglobulins (BioRad, Hercules, CA). After 3 washes the HRP complexes were detected by the Renaissance chemiluminesence system (DuPont NEN, Boston, MA).

RESULTS

Melanoma cell lines MV3 and MEL-FC were examined for surface expression of TM4SF members, CD63, CD9 and CD81 (TAPA-1) by flow cytometry. As shown in Fig. 1 both cell lines expressed CD63 but at lower levels than CD9 or CD81. Other TM4SF members CD53 and CD37 were not detected (data not shown).

MV3 and MEL-FC melanoma cells were surface radioiodinated and solubilised in the detergents NP-40, CHAPS, or octylglucoside, and immunoprecipitated with MAbs against CD63 and CD9 or an irrelevant control (Fig. 2). In the lysates induced by NP-40 and octylglucoside the LS62 MAb against CD63 precipitated a variably glycosylated 40–60kd smear, characteristic of the CD63 antigen. Similarly, FMC56 MAb against CD9 precipitated a single 27kd band typical of the CD9 antigen. The results were consistent for both the MV3 and MEL-FC cell lines.

When cells were lysed in the milder detergent, CHAPS, a number of other protein bands

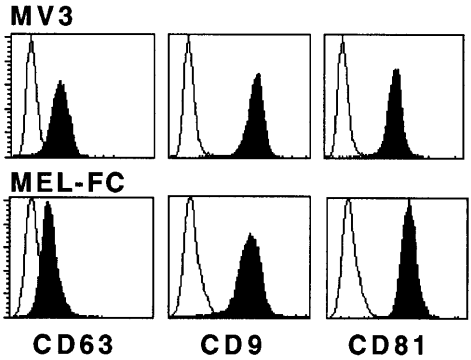

FIG. 1. Expression of TM4SF members on melanoma cell lines. MV3 and MEL-FC cells were stained with MAbs LS62 (CD63), FMC56 (CD9), 5A6 (CD81), represented by the black profiles, or HB57 (irrelevant control anti-human μ chain) represented by the white profiles, and examined by flow cytometry.

co-precipitated with CD63. In MV3 the MAb LS62 precipitated the 40–60kd CD63 smear, and a number of other prominent bands at estimated sizes of 150, 130, 90, and 27kd. Similarly bands of approximate sizes 130, 27, and 24kd were precipitated from MEL-FC. The 27kd protein that co-precipitated with CD63 in both cell lines had the same electrophoretic mobility as the CD9 antigen. In MEL-FC the CD9 MAb (FMC56) precipitated the 27kd CD9 band, along with bands at 100, 66 and 24kD. The band of approximately 24kd in both the CD9 and CD63 precipitates of MEL-FC was consistent with the size of CD81 under reducing conditions. Lysis of the cells in 1% Brij 96 revealed similar immunoprecipitation profiles compared to the CHAPS lysates for both cell lines (data not shown).

To confirm the suggested associations between CD63 and other TM4SF proteins we utilised immunoprecipitation followed by western blot analysis. To minimise interference of immunoglobulin cross-reactivity on the blots we eluted the beads with a more stringent detergent (NP-40) which

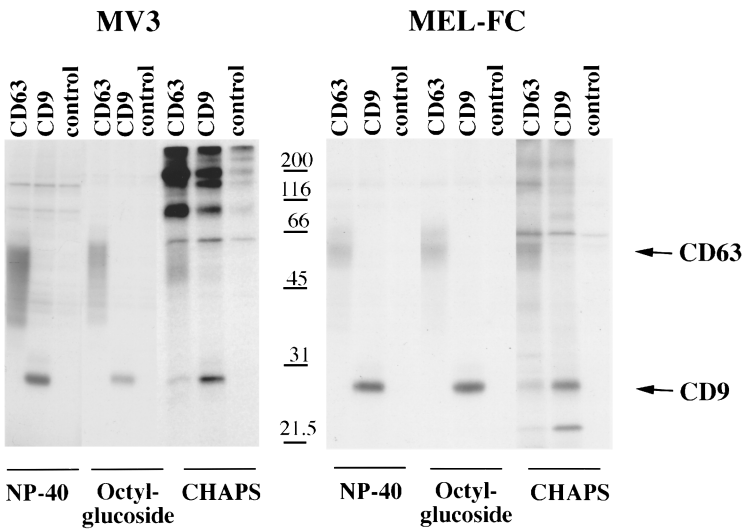

FIG. 2. Protein associations of CD63 in MV3 and MEL-FC. Cells were surface radioiodinated, lysed in NP-40, octylglucoside or CHAPS and immunoprecipitated with MAbs LS62 (CD63), FMC56 (CD9) or normal mouse serum (IgG control) coupled to RAM beads as described in the methods. Proteins were resolved by 12% SDS-PAGE under reducing conditions.

recovered the associated proteins but not the antibodies bound to the beads. First we examined CD63 and CD81 immunoprecipitates from MEL-FC for the presence of CD9 (Fig. 3a). Using this technique CD9 was detected in association with CD63 and CD81 but not in the control IgG immunoprecipitate. The CD81/CD9 association was confirmed by reciprocal blotting of a CD9 immunoprecipitate with 5A6 MAb against CD81 (data not shown). We could not confirm the presence of CD81 in the immunoprecipitate of CD63 by this procedure. In a reciprocal experiment we tried to identify CD63 in the immunoprecipitates of CD9 and CD81 but the diffuse nature of the CD63 band makes it difficult to detect in low quantities above the background signal.

To further examine the molecules associated with CD63 an alternative method based on re-precipitation was employed for the analysis of MV3 cells. MV3 cells were surface radioiodinated and lysed in 1% Brij 96 lysis buffer prior to immunoprecipitation with MAbs against CD63, CD9 or control MAbs. Associated proteins from the CD63 immunoprecipitate were removed from the beads by acid elution and subjected to a second round of immunoprecipitation by MAbs against CD81, CD9 and the β1 integrin subunit (Fig. 3b). This study confirmed that CD63 is associated with CD9, CD81 and β1 integrins in MV3.

The association between CD9 and β1 in MV3 was further confirmed by first immunoprecipitating with MAbs against CD63 or CD9 followed by western blotting for β1 (Fig. 3c). β1 integrins were associated with CD63 by re-precipitation analysis (Fig. 3b) but not in the β1 western blot of CD63 immunoprecipitates (Fig. 3c). This suggests that only a small proportion of β1 molecules may be associated with CD63. The association of the β1 integrin in mild detergent lysates of MV3 with CD63 and CD9 is consistent with the β1 integrin being the unidentified 130 kD protein observed in the preliminary experiments (Fig. 2). The unidentified 150kD band is likely to be the corresponding α chain of the co-precipitated β1 integrin subunit.

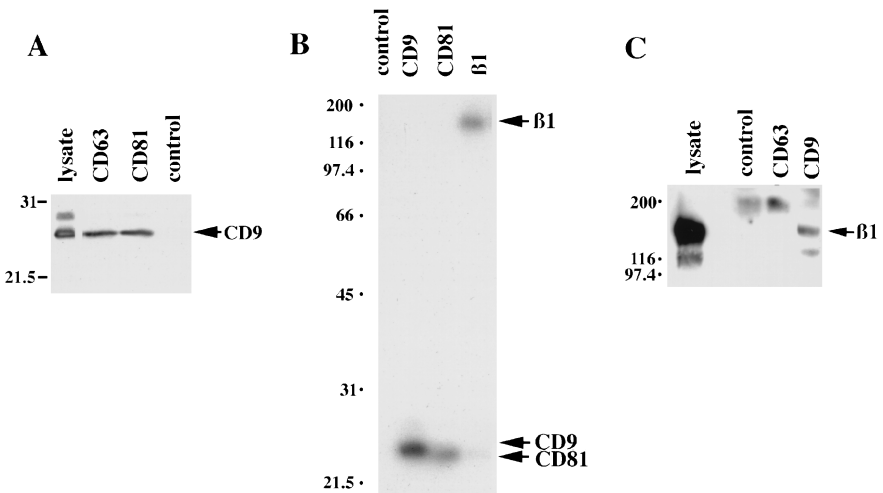

FIG. 3. (A) Association of CD63 and CD81 with CD9 by western blot analysis. MEL-FC cells were lysed in 1% CHAPS and immunoprecipitated with MAbs LS62 (CD63), 5A6 (CD81), or normal mouse serum (IgG control). Proteins were eluted from beads in 1% NP-40 lysis buffer and resolved by 12.5% SDS-PAGE under non-reducing conditions. Proteins were transferred to nitrocellulose and probed with MAb against CD9 (FMC56) as described in the methods. (B) Association of CD63 with CD9, CD81 and β1 integrins by reprecipitation analysis. MV3 cells were lysed in 1% Brij 96 lysis buffer and immunoprecipitated ME491-directly coupled beads against CD63. Proteins were eluted from the beads and re-precipitated with MAbs FMC56 (CD9), 5A6 (CD81), Mab13 (β1), or 1D4.5 (control) as described in the methods. Proteins were resolved by 10% SDS-PAGE under reducing conditions. (C) Association of β1 with CD9 in MV3 by western blot analysis. MV3 cells were lysed in 1% CHAPS and immunoprecipitated with normal mouse serum (IgG control), LS62 (CD63), or FMC56 (CD9). Proteins were resolved by 10% SDS-PAGE under non-reducing conditions, transferred to nitrocellulose and probed with polyclonal anti-β1 Ab as described in the methods.

DISCUSSION

CD63, like other TM4SF family members, has a structure consisting of 4 transmembrane regions and relatively short amino and carboxy termini in the cytoplasm. It therefore appeared highly likely that CD63 may form associations with other molecules in the membrane or cytoplasm of the cell (35). The present studies show that when appropriate non-ionic detergents were used to solubilize melanoma cell membranes, MAbs against CD63 immunoprecipitated several other molecules in association with CD63 on the cell surface. Western blot and re-precipitation techniques indicated that 2 of these were the TM4SF members, CD9 and CD81. Another associated protein of molecular weight 130kd was shown to be the β1 integrin subunit, and an unidentified 150kd band is likely to be its corresponding α chain.

To our knowledge these results are the first to demonstrate an association of CD63 with CD9 and CD81 in melanoma and support the idea that CD63, CD9 and CD81 may form a complex in the surface membrane of melanoma cells. This would be analogous to that reported for other TM4SF members CD81, CD82, CD53 and CD37 in human B cells (15). CD81, originally referred to as TAPA-1 (target of an anti-proliferative antibody) (11), is highly expressed in melanoma (8). In B cells CD81 is associated with other molecules thought to be involved in signal transduction such as CD19, CD21 and Leu-13 (5). CD81 has also been shown in B cells to associate with DR antigens (36). In T cells it is associated with CD4, CD8 and CD82 (17). The formation of these complexes is consistent with the view that the TM4SF molecules in general are involved in signal transduction. Others have suggested that TM4SF complexes resemble ion channels and are involved in ion fluxes in the cells (2). Whether CD63 has similar roles in melanoma cells is yet to be shown.

Our findings that β1 integrins are associated with CD63 and CD9 is consistent with reports by others in various cells. CD9 has been shown to form associations with GPIIb-IIIa complex in platelets (23), β1 in human pre-B cell and megakaryocytic cell lines (21), and α3β1 in monkey kidney cell lines (22). It has been hypothesized that these associations may be important in cell-cell adhesion, proliferation and migration. CD9 has been shown to both enhance (13) and suppress tumor cell motility (12), and it was suggested that it may exert these effects by participating in the transduction of integrin-dependent signals. Recently CD63 was found to be associated with $\alpha^3\beta^1$ and $\alpha^6\beta^1$ integrins in human fibrosarcoma and erythroleukemia cell lines (20). Our results confirm the association observed between CD63 and β1 integrins and extend these to human melanoma cells. During melanoma progression, integrins have been shown to play major roles in the acquisition of invasive capacity of cancer cells. With particular regard to the β1 subfamily of integrins, the $\alpha^3\beta^1$ integrin has been related to the invasiveness of melanoma and has been suggested to contribute to tumorigenicity or metastatic cell motility (7, 37). Conceivably the physical association of β1 integrins and TM4SF could contribute to integrin-mediated cell interactions. However, the functional significance of the association between TM4SF and β1 integrins at this moment is only speculative and needs further clarification. These results provide greater insight into the function of CD63 and provide a basis for further investigation of their function in human melanoma.

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