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The Tetraspan Protein CD82 Is a Resident of MHC Class II Compartments Where It Associates with HLA-DR, -DM, and -DO Molecules

Craig Hammond,* Lisa K. Denzin,*2 Mary Pan,* Janice M. Griffith,‡ Hans J. Geuze,‡ and Peter Cresswell3

In specialized APCs, MHC class II molecules are synthesized in the endoplasmic reticulum and transported through the Golgi apparatus to organelles of the endocytic pathway collectively called MHC class II compartments (MIICs). There, the class II-associated invariant chain is degraded, and peptides derived from internalized Ag bind to empty class II in a reaction that is facilitated by the class II-like molecule HLA-DM. An mAb raised to highly purified, immunoisolated MIICs from human B lymphoblastoid cells recognized CD82, a member of the tetraspan family of integral membrane proteins. Subcellular fractionation, immunofluorescence microscopy, and immunoelectron microscopy showed that CD82 is highly enriched in MIICs, particularly in their internal membranes. Coprecipitation analysis showed that CD82 associates in MIICs with class II, DM, and HLA-DO (an inhibitor of peptide loading that binds DM). Similar experiments showed CD63, another tetraspan protein found in MIICs, also associates with these molecules in the compartment and that CD82 and CD63 associate with each other. Preclearing experiments demonstrated that both CD82 and CD63 form complexes with DM-associated class II and DM-associated DO. The ability of CD82 and CD63 to form complexes with class II, DM, and DO in MIICs suggests that the tetraspan proteins may play an important role in the late stages of MHC class II maturation.


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1 Abbreviations used in this paper: ER, endoplasmic reticulum; MIIC, major histo-compatibility complex class II-enriched compartment; CLIP, class II-associated invariant chain peptide; HB, homogenization buffer; SFM, serum-free medium; PB, permeabilization buffer; CHAPS, 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonate.

The endocytic system of human B cells has recently been described in detail (2). Briefly, six endocytic subcompartments can be distinguished on the basis of morphologic criteria and the presence of marker proteins: two types of early endosomes (EEs), three types of late endosomes (LEs)/lysosomes, and an intermediate type kinetically positioned between EEs and LEs. The majority of intracellular class II molecules are located in the compartments downstream of EEs, collectively called MIICs (for MHC class II-enriched compartments) (2, 3). Typical MIICs are characterized by the presence of internal vesicles and membrane sheets. The multivesicular MIICs probably represent the classical multivesicular bodies or late endosomes in B cells, while MIICs with internal membrane sheets correlate with lysosomes. No exclusive class II-containing organelle has been found, indicating that class II molecules localize to conventional endocytic compartments (2). Loss of the luminal domain of the invariant chain correlates with the transition from multivesicular to multilamellar MIICs and suggests that invariant chain is degraded in this group of organelles (4). Subcellular fractionation and pulse-chase analysis have shown that peptide loading occurs in compartments with characteristics of MIICs and, following loading, class II molecules move from these organelles to the cell surface.

Two other class II-like molecules encoded in the MHC, HLA-DM, and HLA-DO have been localized to MIICs. Experiments in mutant B cell lines and knockout mice have demonstrated that DM is required for efficient Ag presentation by many class II alleles (5–8). In vitro studies have shown that DM facilitates the removal of a fragment of the invariant chain (class II-associated invariant chain peptide (CLIP)) from the peptide binding groove and stabilizes empty class II molecules awaiting antigenic peptide (9–14). A direct low affinity interaction between DM and class II in MIICs has been demonstrated by coprecipitation analysis (10, 15). HLA-DO molecules also bind to DM. However, this interaction is strong and is initiated in the ER, after which the DM/DO complex
is transported to the MIIC (16). HLA-DO inhibits the ability of DM to remove CLIP and facilitate peptide loading (17–19).

To identify new molecules involved in class II processing and presentation, we raised mAbs to immunoisolated MIICs. We report here that one molecule identified by this approach was CD82, a member of the tetraspan (also tetraspanin, transmembrane 4) superfamily of proteins that has previously been found on the surface of B cells, activated T cells, macrophages, and granulocytes (20–22). The tetraspan family consists of at least 18 members with a proposed structure of four transmembrane segments and two extracellular loops of unequal size (reviewed in Refs. 23 and 24). These proteins form large complexes on the cell surface composed of several different tetraspan molecules bound to many different cell surface proteins. CD82 itself associates with the tetraspan proteins CD81, CD63, CD53, CD9, and CD37 as well as CD21, CD19, MHC class I, MHC class II, CD4, and integrins (25–30). At date, no function has been unambiguously assigned to any of the tetraspan proteins.

In this study we demonstrate that CD82 is highly enriched in MIICs of human B lymphoblastoid cell lines. In these compartments, CD82 and CD63, another tetraspan protein previously localized to MIICs, specifically associate with class II, DM, and DO. The results suggest that the late stages of class II maturation may occur in large multicomplexes in MIICs.

Materials and Methods

Cell lines and Abs

The human B lymphoblastoid lines Swei (31), Pala (32), RN (33), and 6H5.DM (34) and the TxB hybrid T2.DM (32) have been previously described. The following Abs used for immunofluorescence microscopy, immunoprecipitation, and immunoblotting have been previously described: L243 (35), R.DRAB (36), HB10 (37), XD5.A11 (38), R.DMB.C (32), and MaP.DMB/C (32). The polyclonal anti-cathepsin D Ab was purchased from Dako (Carpinteria, CA). Abs for immunoelectron microscopy were as follows: HLA-DM (15), HLA-DR (39), human invariant chain C-terminus (40), Lamp-1 (41), CD63 (42), and CD82 (this report). The anti-CD63 mAb H5C6 developed by Dr. J. Thomas August was obtained from the Developmental Studies Hybridoma Bank maintained by the Department of Pharmacology and Molecular Sciences, Johns Hopkins University School of Medicine (Baltimore, MD), and the Department of Biologic Sciences, University of Iowa (Iowa City, IA).

Subcellular fractionation and immunosoliation of MIICs

For each fractionation, 4 × 10^7 Pala cells were pelleted, washed once in homogenization buffer (HB; 250 mM sucrose, 10 mM HEPES, and 1 mM EDTA, pH 7.4), and resuspended in 4.75 ml of HB. Cells were disrupted with two to four passes through a ballbearing homogenizer using a 0.0013 inch gap. Cell breakage was monitored by phase contrast microscopy. Nuclei were pelleted by two successive centrifugation steps at 4 °C for 5 min at 1500 × g in a microfuge. Two milliliters of postnuclear supernatant was mixed with 9 ml of Percoll in HB to give a final Percoll concentration of 27% in Beckman 344059 ultracentrifuge tubes (Palo Alto, CA). The mixture was underlayered with a 0.5-mL isotonic Nycodenz (26%) cushion buffered with 10 mM HEPES to pH 7.4. The gradients were centrifuged in Sorvall TH-641 rotor (Randolph, MA) for 1 h at 17,500 rpm (41,000 × g) at 4°C. MIICs were collected in 0.5 ml from the interface with the cushion bypipette. For further purification, 1 ml of collected MIICs was mixed with Percoll, and the separation was repeated. MIICs purified on sequential gradients was used as a starting material for immunosoliation. The MIIC fraction was first precleared of any residual ER and plasma membrane contaminants with 100 µL of Biomag protein A that was prebound with both a rabbit polyclonal Ab that recognizes the C-terminal 19 amino acids of calnexin (43) and w6/32, which recognizes assembled MHC class I molecules. Binding to Biomag protein A (Perseptive Diagnostics, Cambridge, MA) and subsequent washing of unbound Abs were performed in PBS with 1% IgG-free BSA. Preclearing was performed by rotating for 1 h at 4°C and subsequent removal of the beads on a magnet. Immunosoliation was performed by rotating the precleared MIIC fraction with 100 µL of Biomag protein A bound to MaP.DMB/c, an mAb that recognizes the C-terminus of HLA-DMB, for 3 h at 4°C. Beads were then washed three times for 10 min each time by rotating with HB.

Immunoelectron microscopy

For immunogold labeling, cells were allowed to internalize 5-nm gold particles coated with BSA for 10 min. Cells were then fixed in 2% paraformaldehyde in 0.1 M sodium phosphate buffer at pH 7.4 for 2 h and stored in 1% paraformaldehyde in the same buffer until cryosectioning. Cryosectioning and immunolabeling were performed essentially as described previously (44), except that the cryosections were embedded in a mixture of sucrose and methylocellulose to better visualize membranes (45). Ultrathin cryosections were indirectly single or double immunolabeled with 10- and 15-nm protein A/gold particles (44).

For visualization of immunosoliated MIICs, organelles bound to Bio- mag protein A were pelleted and fixed for 1 h in a buffer containing 1.5% glutaraldehyde, 146 mM sucrose, and 100 mM sodium cacodylate. Cells were then embedded in Epon, sectioned, and counterstained with 3% uranyl acetate and Reynolds lead citrate solutions.

PCR cloning of CD82 and transfection of HeLa cells

The cDNA for CD82 was cloned from a Raji cell cDNA library (32) using Pfu polymerase (Stratagene, La Jolla, CA) according to the manufacturer’s instructions. Primers used for amplification were 5’-GGTACCGGACCATGGGCGTCACCTGTTATCATCAA-3’ and 5’-ATCGATTACGACTTGGGACCTTG-3’. The PCR product was cloned into the TA cloning vector pCR 2.1 (Invitrogen, Carlsbad, CA) and sequenced using an ABI DNA sequencing kit (Applied Biosystems, Foster City, CA). For expression in HeLa cells the cDNA for CD82 was subcloned into the mammalian expression vector PCMFR-PAC (32). HeLa cells were transfected by electroporation in a 0.4-cm gap cuvette using a Bio-Rad gene pulser set at 800 V and 960 µF. Transfectants were selected in medium containing 0.75 µg/ml puromycin and were screened for CD82 expression by immunofluorescence microscopy.

Immunofluorescence microscopy

For staining nonadherent cells, coverslips (12 mm in diameter) were treated with a 1% solution of alcan blue that had been heated to the boiling point. Excess dye was subsequently washed away with water. Cells were washed once in serum-free Iscove’s modified Dulbecco’s medium (SFDM) and probed on coverslips in a 24-well plate at a density of 3 × 10^4 cells/well in 500 µl of SFM. Cells were allowed to adhere for 20 to 30 min. Spreading of the cells was monitored by phase contrast microscopy. Cells were then fixed for 15 min in a freshly prepared solution of 3.7% formaldehyde in SFM. After washing away the fixative with SFM, cells were permeabilized and blocked for 15 min in either PBS or SFM containing 10% bovine serum, 0.05% saponin, 10 mM glycine, and 0.05% sodium azide (permeabilization buffer (PB)). Samples were labeled for 30 min in a moist chamber at room temperature by placing coverslips side down on parafilm in a 20-µl drop of Ab diluted in PB. Cells were washed in the 24-well plate three plates for 5 min each time in 500 µl of PB after incubation in primary Ab or secondary Abs conjugated to FITC or Texas red. After the washes, cells were washed twice in 1 ml water and mounted on glass coverslips in Mowiol 4–88 (Calbiochem-Novabiochem Corp., San Diego, CA). Cells were viewed with a Zeiss axioptil 2 fluorescence microscope (Rockleigh, NJ).

For internalization of anti-CD82 Abs (IgG1) or control mouse IgG, Fab were prepared on an immobilized fcin column (Pierce, Rockford, IL) according to the manufacturer’s directions. Fab were further purified by high performance size exclusion chromatography. In uptake experiments, cells at a concentration of 1 × 10^6/m in 1 ml IMDM containing 5% bovine serum with 10 µg/ml Fab were incubated in SFM. Immediately after incubation, cells were transferred to a 24-well plate three plates for 5 min each time in 500 µl of PB after incubation in primary Ab or secondary Abs conjugated to FITC or Texas red. After the washes, cells were washed twice in 1 ml water and mounted on glass coverslips in Mowiol 4–88 (Calbiochem-Novabiochem Corp., San Diego, CA). Cells were viewed with a Zeiss axioptil 2 fluorescence microscope (Rockleigh, NJ).

Analysis of radiolabeled CD82-associated components

Pala cells (1.5 × 10^7) were labeled with [35S]methionine/cysteine (ICN, Costa Mesa, CA; 2.5 mCi) for 4.5 h and chased in the presence of cold methionine/cysteine for 1.5 h. The cells were fractionated on Percoll gradients, and the dense (MIIC) and light (plasma membrane/ER) fractions were collected. Membranes were solubilized by the addition of 10% (w/v) CHAPS in 0.15 M NaCl/0.01 M Tris, pH 7.4, and PMSF (0.5 mM final concentration) and iodoacetamide (5 mM final concentration) were added. Each extract was applied to an affinity column (1 ml) of 2 mg MaP.CD82 mAb conjugated to Bio-Gel A15 m agarose with a similar precolumn of normal mouse IgG, as previously described for similar affinity purifications (9). After washing with 0.6% CHAPS in 0.15 M NaCl/0.01 M Tris, pH 7.4, CD82-associated proteins were eluted with 1% sodium deoxycholate in 0.15 M NaCl/0.01 M Tris, pH 8.2. Control total glycoproteins were isolated from material that failed to bind to the MaP.CD82 column by the addition
of concanavalin A-Sepharose beads (50 μl/0.75 ml of extract). CD82-associated proteins were ethanol precipitated and dissolved in sample buffer for two-dimensional gels, and concanavalin A-bound proteins were eluted in the same buffer. Two-dimensional gels used a nonequilibrium pH gradient as the first dimension and SDS-PAGE as the second (46). Gels were dried and exposed as previously described (32).

Coimmunoprecipitation

For coprecipitation analysis of MIIC fractions, 4 × 10⁷ Pala cells were fractionated on a Percoll gradient, and the MIIC fraction of the gradient was collected in 600 μl by side puncture. Each immunoprecipitation used one-third of the fraction and was mixed with 400 μl of 2% CHAPS in a pH 7.4 buffer containing 130 mM NaCl, 25 mM HEPES, 1 mM PMSF, and 2% BSA. The organelles were lysed at 4°C for 15 min, and then specific Abs covalently coupled to Bio-Rad A15 M beads or a combination of protein G-Sepharose and Ab were added to the lysate. Immunoprecipitation was conducted by rotating at 4°C for 1.5 h. Lysates were then washed three times in pH 7.4 buffer containing 2% CHAPS, 130 mM NaCl, and 25 mM HEPES. After the final wash, nonreducing sample buffer was added, and the samples were heated to 95°C for 1 min. When Abs used for both immunoprecipitation and immunoblotting were from the same species or when immunoprecipitating Abs were bound to protein G-Sepharose rather than covalently coupled, coprecipitating material was first eluted from the beads with 15 μl of 0.1% Triton X-100 and 0.05% SDS in 10 mM Tris/300 mM NaCl, pH 8.0. Samples were then analyzed by 10% SDS page followed by transfer to an Immobilon (Millipore, Bedford, MA) membrane for immunoblotting. Immunoblots were visualized with Pierce chemiluminescence reagents.

Results

Purification and characterization of MIICs

Partial purification of MIICs for mAb production was performed on Percoll density gradients (Fig. 1A). Fractionation of the human B lymphoblastoid cell line Pala showed MHC class I, which is predominantly in the plasma membrane, and calnexin, a marker for the endoplasmic reticulum, to be in the low density fractions of the gradient. MHC class II molecules (DRαβ) were predominantly located in the same fractions but were also present in a small peak in the high density portion of the gradient (fraction 15). This small peak cofractionated with the major peak of DM and was thus taken to contain MIIC. MIIC were defined throughout this study by the presence of DM, which has previously been shown by us and others to be almost exclusively localized to these compartments at steady state in human B lymphoblastoid cells (47, 48). DM in the lighter portions of the gradient may have been present in lower density MIIC or ER. These lighter fractions were not characterized further.

The high density MIIC from 1.2 × 10⁸ Pala cells were purified on two sequential Percoll gradients followed by immunosolublation on a magnetic matrix coated with mAb MaP.DMB/c, which recognizes the cytosolic tail of DM. Analysis of the immunosolublated MIIC by electron microscopy showed the preparations to contain 200- to 400-nm diameter organelles with abundant internal membranes and very little contamination from other organelles (Fig. 1B).

Identification of CD82 as an MIIC component

BALB/C mice were initially s.c. injected with an MIIC preparation from 4 × 10⁷ Pala cells still associated with the magnetic matrix. They were boosted i.v. before fusion with MIIC that had been eluted from the magnetic substrate with the peptide used to generate mAb. Ab supernatants were screened by immunofluorescence microscopy using Pala cells. Four Abs that gave an MIIC-like staining in Pala cells but not in nonlymphoid cells (HeLa) were chosen for further analysis. Two of these Abs (MaP.DM1 and MaP.DM2) recognized DM, one recognized Ig κ-chain (MaP.IgK), and one (MaP.CD82) recognized a heavily glycosylated protein of 40 to 70 kDa (Fig. 1C).

FIGURE 1. An mAb raised to purified MIIC recognizes CD82. A, Percoll gradient fractionation of the human B lymphoblastoid cell line Pala. Fifteen fractions were collected from the top of density gradient and analyzed by 10% nonreducing SDS-PAGE followed by immunoblotting and visualization of proteins by chemiluminescence. The proteins recognized are listed on the right of the corresponding blot. B, Immunoisolated MIIC from fraction 15 of a Percoll gradient. MIIC were bound to magnetic protein A coated with MaP.DMB/c, which recognizes the cytosolic tail of DM. A representative vesicle is shown surrounded by magnetic protein A. Bar = 0.2 μm. C, Immunoprecipitation with MaP.CD82 from 1 × 10⁶ Pala cells labeled for 4 h with [³⁵S]methionine and cysteine. Immunoprecipitated material was analyzed by 10% nonreducing SDS-PAGE. Molecular weight markers (kilodaltons) are shown on the left. The heavily glycosylated protein was identified by sequencing as CD82.
This protein was purified on an affinity column made from the mAb. Sequencing of a tryptic fragment of the protein identified it as CD82, a member of the tetraspan family of integral membrane proteins that has three extensively sialated N-linked glycans. The lower 35-kDa band was shown to be a core glycosylated precursor of CD82 by pulse-chase analysis and enzymatic digestion of the carbohydrate (data not shown). Western blotting of Percoll gradient fractions showed a peak of CD82 protein in the most dense portion of the gradient, coinciding with the peak of DM (Fig. 1A).

Double-label immunofluorescence studies in Pala cells confirmed that CD82 was highly enriched in MIICs (Fig. 2). CD82 was present in perinuclear vesicles that contained DM, class II, and another member of the tetraspan family, CD63, which has previously been characterized as a marker of MIICs and lysosomes (42). Similar patterns of intracellular CD82 staining were seen in other human B lymphoblastoid lines, including Swei, LOO-1, and Raji. The cDNA for CD82 was cloned by PCR from a Raji cDNA library and expressed in HeLa cells to determine whether any B
cell-specific factors were responsible for the cellular distribution of CD82. Similar to B lymphoblastoid lines, CD82 expressed in HeLa is present on the plasma membrane and in intracellular vesicles that colocalize with the lysosomal hydrolase cathepsin D (Fig. 2, J–L). Apparently, no B cell-specific factors are required for its localization.

Immunoelectron microscopy confirmed the localization of CD82 in MIICs. Immunogold labeling of both 6H5.DM (illustrated in the figures) and RN B cells revealed CD82 labeling at the plasma membrane and in MIICs (Figs. 3 and 4A). Interestingly, the majority of CD82 was found on the internal membranes of the MIICs (Figs. 3 and 4A), similar to the distribution of the lysosomal tetraspan protein CD63 (not illustrated, see Ref. 49). On the other hand, HLA-DM (Fig. 4A) and the lysosomal membrane protein Lamp-1 (Fig. 4B) predominantly localized to the limiting membrane of the MIICs. Multilaminar MIICs showed a higher CD82 labeling in the internal membranes than multivesicular MIICs (Figs. 3 and 4).

Figure 3 shows an irregularly shaped type of MIIC, termed early MIIC to indicate that it represents the most likely entrance site for
biosynthetic class II/invariant chain complexes into the endocytic pathway (2, 50). Typical MIICs are globular, while the shape of early MIICs reflect their formation from the tubulovesicular EEs. After endocytosis for 10 min, 5-nm BSA-gold particles reached the early MIICs but not the later MIIC types (Fig. 3, 2). Early MIICs often showed vesicles in a process of budding into the MIIc to form the internal vesicles (Fig. 3). Early MIICs showed abundant labeling for the C-terminus of the invariant chain and colabeling for HLA-DM (2) and CD82.

To determine whether CD82 can be internalized from the cell surface to the MIIC, purified anti-CD82 Fab were added to Pala cells in culture. The MaP.CD82 Fabs were rapidly internalized to the plasma membrane/ER (A and B) or MIIC (C and D) were elysed in 2% CHAPS and bound to either concanavalin A beads (A and C) to visualize total labeled glycoproteins or to an MaP.CD82 affinity column (B and D). CD82 bound only a small fraction of the total glycoproteins. The predominant, metabolically labeled, CD82-associated proteins eluted from the CD82 column were identified as class II αβ, which are labeled in D and are indicated by arrows in the other panels.

**CD82 specifically associates with proteins in the MIIC**

A characteristic of tetraspan proteins is the formation of large multimolecular complexes (23, 24). We therefore determined whether CD82 associates with resident and itinerant molecules in the MIIC (Fig. 5). Pala cells were labeled with [35S]methionine/cysteine for 4.5 h and were chased for an additional 1.5 h. The cells were fractionated on Percoll gradients, and the most and least dense fractions that contained either MIIC or plasma membrane/ER, respectively, were collected. Fractions were solubilized in 1% CHAPS, and the lysate was run over an MaP.CD82 affinity column. In parallel, total glycoproteins were affinity purified using *Lens culinaris* lectin. Proteins bound to the CD82 column were eluted with the more stringent detergent deoxycholate, which removes all proteins with the exception of CD82 (data not shown), and those bound to the lectin beads were eluted with high pH. Eluted material was analyzed by two-dimensional nonequilibrium pH gradient/SDS-gel electrophoresis and fluorography. The total glycoprotein precipitation from each fraction contained a large number of proteins. In contrast, very few labeled proteins eluted from the CD82 column. In the plasma membrane/ER fractions, spots identifiable as MHC class I heavy chain, β2m, and MHC class II α- and β-chains were prominent, in agreement with previous reports. The MIIC fractions showed only class II α and β and another unidentified spot. Thus, CD82 appeared to quite specifically interact with class II in the MIIC.

Many proteins are difficult to detect by metabolic labeling because of low synthetic rates and large unlabeled cellular pools. Therefore, immunoblotting was used to detect additional proteins that interact with CD82 in the MIIC (Fig. 6A). For these experiments the MIIC-enriched fractions from Percoll gradients (fraction 15) were lysed in 2% CHAPS (similar results were obtained with 1% Brij 58) and immunoprecipitated with agarse beads covalently coupled to MaP.CD82. Control beads were coupled to an isotype-matched Ab, the anti-α-chain Ab, which was found in our immunofluorescence screen and recognizes free Ig light chain in the MIIC (data not shown). In agreement with the labeling results, MHC class II was found to coprecipitate with CD82 (Fig. 6A, lanes 1 and 2). Immunoblotting also showed DM and CD63 to be associated with CD82 (Fig. 6A, lanes 3–6). Beads bound to an anti-CD63 mAb, H5C6, also specifically coprecipitated MHC class II and DM as well as CD82 (Fig. 6B). Similar results were obtained for both CD82 and CD63 coprecipitation using highly purified, immunoisolated MIIC as starting material, thus confirming the subcellular location of the interactions (not shown). Importantly, Lamp 1, a very abundant protein in the MIIC that was easily detected in MIIC lysates, did not coprecipitate with either CD82 or CD63 (Fig. 6, A and B, lanes 7 and 8).

To verify that the interactions were not occurring after cell lysis, mixing experiments were performed using HeLa cells transfected with the class II trans-activator, which express DM but not CD82, and 1.74 cells, which express CD82 but not DM. For comparison, Swei cells were used because, per cell, they express amounts of CD82 and DM similar to those found in the mixed HeLa class II trans-activator/1.74 lysates. Coprecipitation of DM was seen only
have been transfected with cDNA encoding the DM
5 and was determined by coprecipitation from T2.DM cells (Fig. 7, Whether DM could bind to CD82 in the absence of class II and DO
ilar to that found in complexes of known functional significance. The results show that the amount of DM associated with CD82 is sim-
ever, the amount of coprecipitating DM was nearly equal. These somewhat greater than that bound to CD82. In Swei cells, how-
lanes 1–4 associated with class II with that associated with CD82 (Fig. 7, teraction of class II and DM, we compared the amount of DM
previous studies have provided a clear function for the direct in-
how many interactions are broken even by mild detergents. Since it is difficult to determine by coprecipitation because it is not clear
in the Swei cells (Fig. 6C, lane 5) and not in the mixed samples. Thus, the interaction of DM with CD82 exists in cells before lysis. The fraction of total DM and class II bound by CD82 in the cell is difficult to determine by coprecipitation because it is not clear how many interactions are broken even by mild detergents. Since previous studies have provided a clear function for the direct interaction of class II and DM, we compared the amount of DM associated with class II with that associated with CD82 (Fig. 7, lanes 1–4). In Pala cells, the amount of class II-associated DM was somewhat greater than that bound to CD82. In Swei cells, however, the amount of coprecipitating DM was nearly equal. These results show that the amount of DM associated with CD82 is similar to that found in complexes of known functional significance. Whether DM could bind to CD82 in the absence of class II and DO was determined by coprecipitation from T2.DM cells (Fig. 7, lanes 5 and 6). These cells have a chromosomal deletion in the MHC but have been transfected with cDNA encoding the DM α and β subunits. DM was specifically coprecipitated with CD82 from these cells in the absence of the other proteins.

Preclearing experiments were performed to determine the composition of CD82 and CD63 complexes in the MIIC (Fig. 8). Pala cells were lysed in 2% CHAPS, and the lysates were precleared with either a mixture of anti-class II mAbs or a control Ab. Preclearing was repeated until DM coprecipitation with class II was greatly diminished (Fig. 8, lane 6 vs lane 8), then the lysates were immunoprecipitated with either anti-CD82 (lanes 1 and 2) or anti-CD63 (lanes 3 and 4) beads. All coprecipitating material was immunoblotted for DM, the vast majority of which was located in MIIC at steady state. The results show that removing class II-associated DM substantially depleted the pool of DM bound to

FIGURE 6. Proteins associated with CD82 and CD63 in MIIC fractions detected by immunoblotting. A, MIIC fractions (fraction 15) from Percoll gradients were lysed in 2% CHAPS and immunoprecipitated with either MaP.CD82 (lanes 1, 3, and 5) or an isotype-matched control Ab (lanes 2, 4, and 6) covalently bound to agarose beads. Coprecipitating proteins were analyzed by 10% nonreducing SDS-PAGE and immunoblotting with Abs to HLA-DR (rabbit polyclonal antiserum against DR
a 6 ) covalently bound to agarose beads. Coprecipitating proteins were analyzed by 10% nonreducing SDS-PAGE and immunoblotting with Abs to HLA-DR
bmunoprecipitated with MaP.CD82 (anti-CD82; lanes 3 and 4), and CD63 (lanes 5 and 6). Lysate from MIIC was blotted for Lamp 1 (lane 7), which was not detected, even upon overexposure of the blot, by coprecipitation with CD82 (lane 8). B, Similar experiments to those in A, but with beads coupled to the anti-CD63 mAb H5C6. All conditions were the same, except for lanes 5 and 6, which were immunoblotted with MaP.CD82. C, Controls for postlysis association of DM and CD82 were performed by comparing coprecipituation from Swei cells, a normal lymphoblastoid line, to that from a mixture of J74 cells, which contain CD82 but not DM, and HeLa/class II trans-activator, which contain DM but not CD82. Lanes 1 and 2 show immunoblotting of indicated lysates for CD82, while lanes 3 and 4 show blotting for DMβ. Lanes 5 and 6 show coprecipitation with CD82 from the indicated lysates.

FIGURE 7. Comparison of DM association with HLA-DR and CD82 in different cell lines. Pala or Swei cells were lysed in 2% CHAPS and immunoprecipitated with MaP.CD82 (anti-CD82; lanes 1 and 3) or HB10 (anti-HLA-DRβ; lanes 2 and 4). In lanes 5 and 6, lysates from T2.DM cells, which contain CD82 and DM but not class II and DO, were immunoprecipitated with MaP.CD82 or a control Ab. Coprecipitating DMβ was visualized in all lanes by immunoblotting.

FIGURE 8. Characterization of CD82 and CD63 complexes. Lysates from Pala cells (1 × 10^6/lane) were subjected to four successive rounds of preclearing with either a mixture of anti-class II Abs (L243, XD5, HB10) or a control Ab (anti-κ-chain). Precleared lysates were then immunoprecipitated with beads coupled to either MaP.CD82 (anti-CD82; lanes 1 and 2) or H5C6 (anti-CD63; lanes 3 and 4). Material immunoprecipitated in the first (lanes 5 and 6) and fourth (lanes 7 and 8) rounds of preclearing and in subsequent precipitations with MaP.CD82 and H5C6 was analyzed by 10% nonreducing SDS-PAGE and immunoblotting with a polyclonal antiserum against DMβ. The results shown are representative of four independent experiments.
CD82 and CD63. This demonstrates that complexes of the tetraspan proteins, class II, and DM exist in MIICs and also supports the fractionation data (Fig. 6, A and B, lanes 1 and 2) showing that class II in MIICs, localized here by association with DM, can interact with CD82 and CD63.

HLA-DO is a class II-like resident of MIICs that binds DM and has recently been shown to inhibit the DM-catalyzed removal of CLIP releasing DM from endosomes. To determine whether DO associates with CD82 and CD63 in MIICs, coprecipitation analysis from MIIC-enriched Percoll fractions was performed (Fig. 9). As with DM, DO was found to coprecipitate with beads coupled to mAbs recognizing CD82, CD63, and class II, but not a control Ab (lanes 1–4). Preclearing DM-associated DO from cell lysates substantially diminished the amount of DO bound to the tetraspans and class II (lanes 5–10). This shows that much of the DO in tetraspan complexes is DM associated and agrees with our observation that DO binds DM and is highly enriched in intracellular vesicles that are probably MIICs (53) (C. Hammond, H. Geuze, and P. Cresswell, unpublished observations). Our results also suggest MIICs may contain tetraspan protein complexes of different composition. Preclearing experiments demonstrate that class II-associated DM binds both CD63 and CD82. Coprecipitation of DM with CD82 from T2 cells shows that neither class II nor DO is needed for association, which leaves open the possibility that free DM and DM/DO complexes may bind tetraspan proteins in the MIICs of normal B cells. The existence of CD82-CD63-class II complexes at the cell surface, where there is essentially no DM or DO, has been shown by several groups (25, 29, 30). Since a minority of the class II molecules in MIICs are thought to be bound to DM (54), it is likely that CD82 and CD63 bind free class II in these compartments. Given that much of the class II coprecipitated with CD82 from purified MIICs is loaded with peptide, as assessed by stability in SDS, and CD82 does not associate with class II-invariant chain complexes (C. Hammond and P. Cresswell, unpublished observations), CD82 seems to associate with class II molecules nearing the end of their stay in the MIIC.

The domains of CD82 responsible for the interactions described here are unknown. Preventing carbohydrate processing does not affect the interaction of CD82 with either class II (C. Hammond and P. Cresswell, unpublished observations) or class I (27), thus demonstrating that the molecule’s highly modified N-linked glycans are not necessary for these associations. Studies using chimeras between the tetraspan molecule CD9 and CD82 suggest that both the large C-terminal extracellular loop of CD9 and the most C-terminal transmembrane domain may be involved in the interaction between CD9 and β1 integrin (29). For CD82 association, the extracellular and cytosolic regions of CD4 have been implicated, again pointing to the extracellular domain of the tetraspan being important for binding (26).

**FIGURE 9.** Coimmunoprecipitation of DO with CD82, CD63, and class II. MIIC fractions from Percoll gradients were lysed in 2% CHAPS and immunoprecipitated with agarose beads coupled to MaP.CD82 (anti-CD82: lane 1), H5C6 (anti-CD63; lane 2), HB10 (anti-HLA-DR β1 chain; lane 3), or control (anti-k-chain; lane 4). All lanes were then immunoblotted with rabbit polyclonal anti-DOβ antisera. To determine whether bound DO was in a complex with DM, Pala cell lysates (1 × 10⁶ cells/lane) were precleared with a mixture of Abs to DM (MaP.DMB/c, MaP.DM1, and MaP.DM2) or a control Ab (anti-k-chain). After four rounds of preclearing, the lysates were immunoprecipitated with beads coupled to Map.CD82 (lanes 5 and 6), H5C6 (lanes 7 and 8), or HB10 (lanes 9 and 10) and immunoblotted with anti-DOβ antisera. The first (lanes 11 and 12) and fourth (lanes 13 and 14) rounds of preclearing were also blotted. Note that the elution of DO from DM immunoprecipitates with SDS/TX-100 buffer (see Materials and Methods) is not very efficient. All samples were analyzed on 10% nonreducing SDS-PAGE. The arrowhead marks a slower migrating form of DO that disappears upon reduction. The results shown are representative of four independent experiments.

**Discussion**

MIICs represent late endocytic compartments that facilitate class II-mediated Ag presentation. While MIICs share many components, such as lamp proteins, CD63, cathepsins, and Rab proteins, with similar compartments in nonpresenting cells, what makes them specialized is the cohort of proteins involved in the expression of mature, peptide-loaded class II molecules at the cell surface. The best understood of these is DM, which binds class II and facilitates both the removal of CLIP and the loading of antigenic peptide (9–14, 51), and DO, which is tightly associated with DM and has recently been shown to inhibit its activity (17). Also well characterized is the IFN-γ-inducible, cysteine protease cathepsin S, which is highly expressed in APCs and is capable of degrading invariant chain (52). In this study we raised mAbs to highly purified MIICs to identify additional proteins involved in class II-mediated Ag presentation. CD82, a member of the tetraspan family of integral membrane proteins, was found by this approach to be highly enriched in MIICs and bound to class II, HLA-DM, and HLA-DO proteins. These findings identify CD82 as another specialized component of MIICs that is potentially important in the later stages of class II maturation.

Taken together with the tendency of tetraspan proteins to form large multimolecular structures, our findings of CD82 and CD63 association with class II, DM, and DO in MIICs strongly suggest that the late stages of class II maturation occur in the context of tetraspan complexes. Additional tetraspan proteins may also be present in these organelles. CD37, a tetraspan protein found in mature B cells, is highly enriched in intracellular vesicles that are probably MIICs (53) (C. Hammond, H. Geuze, and P. Cresswell, unpublished observations). Our results also suggest MIICs may contain tetraspan protein complexes of several different compositions. Preclearing experiments demonstrate that class II-associated DM binds both CD63 and CD82. Coprecipitation of DM with CD82 from T2 cells shows that neither class II nor DO is needed for association, which leaves open the possibility that free DM and DM/DO complexes may bind tetraspan proteins in the MIICs of normal B cells. The existence of CD82-CD63-class II complexes at the cell surface, where there is essentially no DM or DO, has been shown by several groups (25, 29, 30). Since a minority of the class II molecules in MIICs are thought to be bound to DM (54), it is likely that CD82 and CD63 bind free class II in these compartments. Given that much of the class II coprecipitated with CD82 from purified MIICs is loaded with peptide, as assessed by stability in SDS, and CD82 does not associate with class II-invariant chain complexes (C. Hammond and P. Cresswell, unpublished observations), CD82 seems to associate with class II molecules nearing the end of their stay in the MIIC.

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The pathway by which CD82 arrives in the MIIc is unknown, but the rapid internalization of anti-CD82 Fab suggests that it may reach the compartment after internalization from the cell surface. The C-terminal cytosolic tail of CD82 contains a recently identified clathrin activating protein-2 binding motif (YSKV) that may serve as an internalization signal (55). Expression of CD82 in HeLa cells, which have undetectable levels of endogenous CD82 protein, results in its localization to late endocytic vesicles (Fig. 2, M–O). This result and the observation of a similar distribution in .174 cells, which have deleted the class II region of the MHC, suggest that other components of the class II processing machinery are not necessary for localization of CD82 to the MIIc. Given the rapid internalization of CD82 and its long half-life (>4 h; our unpublished observations), it is possible that CD82 may recycle between the MIIc and the plasma membrane. Further investigation should address whether CD82 travels with class II from the MIIc, as the two molecules are known to associate on the cell surface. Recent evidence showing that activating protein-2 and clathrin bind to lysosomes under certain experimental conditions (56) opens the possibility that the YSKV motif on CD82 may act in the MIIc as well, perhaps facilitating the exit of class II molecules.

Additional possibilities for CD82 function in the MIIc are suggested by the known properties of the tetratrans. CD82 and other tetratrans form large protein networks or membrane microdomains that may limit diffusion of included molecules and facilitate interactions between molecules by keeping them in close proximity (57). In MIIcs, class II and DM are obvious candidates for such a facilitated interaction. Specificity for protein association with different tetratrans networks would presumably come from their particular composition. Work with the tetratrans CD9, which associates with the heparin binding epidermal growth factor-like receptor, has shown that CD9 can enhance ability of the heparin binding epidermal growth factor-like receptor to bind diphtheria toxin and stimulate the growth of neighboring cells by juxtaclapping signaling through the epidermal growth factor receptor (57–59). These studies suggest that tetratrans can facilitate protein interactions outside the plane of the membrane. The differential distribution of CD82 and DM within the MIIc (Fig. 4A) may hint at such an interaction in this case also.

CD82 may also be involved in the recruitment of ancillary molecules to the MIIc. Kinase, GTPase, and phosphatase activities have been found to associate with tetratrans proteins (3, 54, 56), although in many cases these interactions are probably indirect. This scenario is made more attractive by the results of Pierce and co-workers, who have found that signaling through the B cell receptor not only increases the efficiency of class II-mediated Ag presentation in some cases, but also causes an increase in phosphoproteins and GTP binding proteins in the MIIc fractions of Percoll gradients (60). A final possibility for tetratrans function in the MIIc is suggested by the presence of CD63 on regulated secretory vesicles, including Weibel-Palade bodies, cytoplasmic granules of cytotoxic T cells (3), platelet granules, and basophilic granules (61). Platelet α-granules, which are connected to late endocytic compartments, contain the tetratrans proteins CD9, CD63, and CD151 (H. Heijnen and H. Geuze, unpublished observation). Perhaps CD9, CD63, and other tetratrans have a role in regulated secretion and are somehow involved in the recently reported transport and fusion of MIIcs with the plasma membrane (33, 62). During the process, proteins present in the MIIc limiting membrane are inserted into the plasma membrane, whereas the MIIc internal vesicles are released as exosomes (33). Exosomes express peptide-loaded class II molecules and are enriched in tetratrans proteins (H. Geuze, data not shown), in accordance with the abundant labeling of CD82 on the internal membranes of MIIcs shown here.

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References


