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2B4 Is Constitutively Associated with Linker for the Activation of T Cells in Glycolipid-Enriched Microdomains: Properties Required for 2B4 Lytic Function

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2B4 is a receptor belonging to the Ig superfamily and is found on all murine NK cells as well as a small subset of T cells. Previous studies have found that cross-linking of the 2B4 receptor results in both increased cytotoxicity and IFN-γ secretion. We have discovered that 2B4 from transfected NK and T cell lines, as well as from primary murine cells, coimmunoprecipitates with the phosphoprotein linker for the activation of T cells (LAT), which is essential for TCR-mediated signaling. This association is independent of both 2B4 phosphorylation and the cytoplasmic tail of 2B4. We have found that, along with LAT, 2B4 is constitutively located in glycolipid-enriched microdomains of the plasma membrane. In fact, 2B4 appears to associate with LAT only when it localizes to glycolipid-enriched microdomains. This localization of 2B4 occurs due to a CxC cysteine motif found in the transmembrane region, as determined by mutagenesis studies. 2B4-mediated cytotoxicity is defective in the absence of LAT, indicating that LAT is a required intermediate for 2B4 signal transduction. However, we have also shown that LAT association alone is not sufficient for maximal 2B4 activation. The Journal of Immunology, 2002, 169: 55–62.

Natural killer cells are large granular lymphocytes that mediate killing of tumors and virally infected cells in a non-MHC-restricted manner (1). Lysis of target cells is regulated by both positive and negative signaling cell surface receptors. Negative, or inhibitory, signaling receptors have been well characterized in the murine system and include members of the C-type lectin superfamily represented by the Ly49 and CD94/NKG2 receptors (1). Upon binding self MHC ligands, these receptors transmit inhibitory signals that prevent lysis of the MHC-bearing target cells. Recently, stimulatory members of the Ly49 and NKG2 families have also been identified. The positive, or activating, murine NK cell receptors for non-MHC ligands are less well defined but are thought to include NKG2D, NK1.1, MAR1, and 2B4 (2–5). Upon ligand binding, activating NK cell receptors initiate signaling pathways that lead to increased cytotoxicity of NK-sensitive targets. However, the molecules and mechanisms involved in this activation pathway are poorly understood, although recent evidence indicates that 2B4 uses p38 and extracellular signal-regulated kinase/mitogen-activated protein kinase signaling pathways (Ref. 6 and J. Mooney and J. Schatzle, unpublished results).

2B4 is expressed on the surface of all murine NK cells, all dendritic epidermal γδ T cells, and the subset of αβ T cells that exhibit non-MHC-restricted killing after culture with IL-2 (4, 7). 2B4 belongs to the CD2 subfamily of the Ig superfamily (8) and is a receptor for CD48 (9). The murine 2B4 gene encodes two distinct polypeptides, as a result of alternative splicing, that differ solely in their cytoplasmic regions (8). However, only one isoform has been described for human 2B4 (10). The longer murine isoform, 2B4L, contains seven potential phosphorylation sites, five of which are unique to 2B4L (8). Several of these sites resemble immunoreceptor tyrosine-based switch motifs found in the cytoplasmic region of a fellow CD2 subfamily member, signaling lymphocytic activation molecule (11). This motif has been named a “switch” motif because it appears to control binding of a Src homology 2 (SH2)3 domain-containing protein tyrosine phosphatase vs an SH2 domain-containing inositol phosphatase through association with signaling lymphocytic activation molecule-associated protein (SAP). The shorter isoform, 2B4S, contains only one of these immunoreceptor tyrosine-based switch motif sites, suggesting a possible difference in signaling function (8). The phosphorylated 2B4L isoform has been shown to associate with SH2 domain-containing protein tyrosine phosphatase 2, whereas both isoforms associate with the defective X-linked lymphoproliferative disease gene product, SAP (S. Stepp and J. Schatzle, unpublished observations). Studies in transfected rat NK cell lines suggest distinct functions for the two isoforms (12). 2B4L appears to act as an inhibitory receptor, while 2B4S provides stimulatory functions (12). However, the molecular mechanisms of signal transduction by 2B4 have yet to be determined.

2B4 shows homology to CD48 and CD2 (7, 8), both of which are signaling molecules resident in specialized subdomains of the plasma membrane that contain a unique lipid composition of sphingolipids, cholesterol, and GPI-anchored proteins (13, 14). These glycolipid-enriched microdomains (GEM) are nonionic detergent insoluble, which allows them to be separated from all other cellular components via density centrifugation. Studies in T cells have led to the discovery that many signaling molecules are present in these GEM fractions (15), suggesting that GEMs may concentrate signaling components, thereby inducing more efficient

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pathway activation. Linker for the activation of T cells (LAT) is a 36- to 38-kDa phosphoprotein resident within the GEM fractions of T cells (16, 17). Palmitoylation of LAT appears to target this transmembrane protein to GEMs (18). LAT is a critical signaling molecule that couples proximal TCR-mediated activation events with downstream activation of phospholipase C-\(\gamma\), Vav, SLP-76, and other signaling molecules (16, 17). Less is known about the signaling pathways of NK cell activation, but there is some evidence to suggest that they may bear resemblance to T cell pathways. In particular, LAT has not only recently been found to be essential for T cell activation (17) but is also implicated in the FeR activation pathway in NK cells (19). Recent studies demonstrate the importance of GEMs in NK cell activation (20, 21). Because 2B4 is an activating receptor found in NK and T cells, we examined whether it localizes in GEMs and associates with GEM resident signaling molecules.

Studies using human NK cells have demonstrated an association between 2B4 and LAT (6, 22). Our studies in mice confirm this, but in addition we show that LAT associates with both the long and short isoforms of 2B4 in two transfected cell lines: RNK-16 (NK cells) and Jurkat (T cells). Our data also reveal that neither phosphorylation nor the cytoplasmic tail of 2B4 is necessary for its association with LAT. Furthermore, we show that 2B4 is resident in GEM fractions, where it associates with LAT. In fact, mutation of a CxC cysteine motif in the 2B4 transmembrane region impairs not only 2B4 localization to GEM fractions but also its association with LAT. Redirected lysis assays demonstrate that the 2B4 signaling pathway is defective in the absence of LAT, suggesting an important role for LAT in 2B4-mediated cytotoxicity. Even though LAT is required for 2B4 function, we have also shown that its association with 2B4 is not sufficient for maximal 2B4 activation to occur.

Materials and Methods

Cells and tissue culture

293 T cells were grown in DMEM medium supplemented with 10% FCS, 2 mM t-glutamine, 100 U/ml penicillin, and 100 \(\mu\)g/ml streptomycin. All other cells and cell lines were grown in RPMI 1640 medium supplemented with 10% FCS, 2 mM t-glutamine, 100 U/ml penicillin, and 100 \(\mu\)g/ml streptomycin. A total of 1000 U/ml IL-2 or 1 ng/ml IL-12 and 100 ng/ml IL-18 were provided for the lymphokine-activated killer (LAK) cultures as described (23). Transfected cell lines were selected for growth in complete RPMI 1640 or DMEM supplemented with 1 ng/ml G418.

Mice

C57BL/6 mice were obtained from The Jackson Laboratory (Bar Harbor, ME) and bred and maintained in a conventional colony at the University of Texas Southwestern Medical Center (Dallas, TX). C57BL/6 SCID and 129 mice were obtained from The Jackson Laboratory and LAT^{-/-} mice were obtained from P. Love and L. Samelson (National Institutes of Health, Bethesda, MD). Both were maintained in a specific pathogen-free colony at the University of Texas Southwestern Medical Center. Mice used were 2–4 mo of age.

Antibodies

Anti-phosphotyrosine mAb and anti-Lck mAb were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-LAT mAb was obtained from Upstate Biotechnology (Lake Placid, NY). Anti-H2B4 mAb (C1.7) was obtained from Immunotech (Westbrook, ME). Purified, FITC-conjugated, and PE-conjugated anti-m2B4 mAb, anti-RT1A, and anti-LFA-1 mAb were obtained from BD PharMingen (San Diego, CA). All HRP-conjugated secondary Abs were obtained from Amersham Pharmacia Biotech (Piscataway, NJ). Polyclonal rabbit antiserum was prepared to an Ig G protein of 2B4 (S. Stepp, W. Lai, and J. Schatzle, unpublished results).

Flow cytometry

Surface expression of 2B4 on Jurkat cells, 293T cells, and LAKs was examined by staining with FITC-conjugated or PE-conjugated anti-2B4 mAb. For staining, cells were washed and resuspended in PBS supplemented with 2% FCS. Cells were incubated with mAb for 15 min at 4°C. A total of 10,000 events were analyzed using a FACScan flow cytometer (BD Biosciences, San Jose, CA).

Immunoprecipitation and Western blot analysis

Surface proteins were biotinylated using the EZ Linker Sulfo-NHS-LC Biotinylation reagent system from Pierce (Rockford, IL) according to the manufacturer’s instructions. Where indicated, cells were treated with 0.03% hydrogen peroxide and 100 \(\mu\)m orthovanadate (pervanadate) for 10 min at 37°C as previously described (12). Approximately 1 \(\times\) 10^6 cells were harvested for each condition and washed in PBS; cell pellets were then lysed at 4°C in lysis buffer (50 mM HEPES (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1 mM MgCl_2, and 10% glycerol) containing 1% Brij-35. Immunoprecipitation and Western blot analysis were performed as previously described (12). Blots were developed with the SuperSignal chemiluminescence kit from Pierce.

Vector constructs, mutagenesis, transfections, and infections

2B4S or 2B4TMC->A cDNA was subcloned into the MigR1 retroviral vector as described (24). 293T cells were transfected using Effectene kit from Qiagen (Valencia, CA) following the manufacturer’s protocol. LAKs were infected with MigR1:2B4S or MigR1:2B4TMC->A retroviruses by spinfection on d1 as described (25), with the following change: LAKs were incubated at 32°C for 24 h immediately after spinfection. cDNAs corresponding to each of the 2B4 isoforms or various mutant forms of 2B4 were subcloned into the pEMCVoEN vector as previously described (12). Mutagenesis was performed using the Altered Sites Mutagenesis kit from Promega (Madison, WI). All constructs were sequenced for confirmation before transfection. Transfections and establishment of stable cell lines were performed as previously described (12).

Sucrose density gradient centrifugation and GEM isolation

For each condition, \(\sim\)1 \(\times\) 10^8 cells were harvested, washed in PBS, and lysed in lysis buffer with 1% Brij-35 as described above. Lysates were adjusted to 4 ml of 40% sucrose using a 60% sucrose stock dissolved in lysis buffer without glycerol. The cell lysate was then placed on the bottom of 14 \(\times\) 89 mm ultraclear centrifuge tubes from Beckman Coulter (Fullerton, CA). Sequential 2-ml fractions (30, 20, 10, and 5% sucrose in lysis buffer) were overlaid on top of the cell lysate. Samples were centrifuged at 39,000 rpm for 18 h at 4°C in a SW41 rotor. After centrifugation, 1-ml fractions were collected from the top of the tube. Aliquots of each fraction were then subjected to immunoblotting or immunoprecipitation followed by immunoblotting as described above.

Cytotoxicity assays

Specific lysis of targets was determined by using a standard 4-h ^51Cr release assay as previously described (23). Redirected lysis assays using P815 FcR+ targets were performed as previously described (12).

Results

Because murine 2B4 is expressed as two distinct isoforms (8), we examined potential differences between these isoforms using a rat NK cell line, RNK-16, which has been transfected separately with each murine 2B4 isoform (12). Previous studies using these transfectants demonstrate that the 2B4 isoforms not only are structurally distinct but also have different functions. In Fig. 1A, perivandate-treated RNK-16/2B4L transfectants were lysed and immunoprecipitated with anti-m2B4. Immunoprecipitates were resolved by SDS-PAGE and immunoblotted with anti-phosphotyrosine Abs. This process revealed a 66-kDa phosphoprotein corresponding to the molecular mass of 2B4L. In addition, a 38-kDa phosphoprotein coimmunoprecipitated with 2B4L. Fig. 1B demonstrates that the 38-kDa phosphoprotein immunoprecipitated by anti-m2B4 is indeed LAT. Immunoprecipitation with anti-RT1A, a mAb against a rat class I molecule (Fig. 1B), and isotype control Ab (data not shown) failed to coimmunoprecipitate the LAT protein, thereby serving as negative controls.

To determine whether LAT associates with both 2B4 isoforms, lysates from untransfected RNK-16 cells or from RNK-16 transfectants expressing either 2B4S or 2B4L were immunoprecipitated...
with m2B4 mAb followed by blotting with anti-LAT. All 2B4 transfectants were assayed for 2B4 expression via FACS analysis, as previously described (12). Fig. 2A reveals that LAT associates with both the short and long 2B4 isoforms (Fig. 2A, lanes 2 and 3, respectively). Because 2B4 is also expressed in a subset of T cells, we wondered whether this association would exist in a 2B4-transfected Jurkat T cell line. Blotting of whole cell Jurkat lysates with anti-LAT in Fig. 2B, lane 1, demonstrates the migration of the LAT protein. No LAT protein is detected from m2B4 immunoprecipitates of untransfected Jurkat cells (Fig. 2B, lane 2). However, the anti-LAT blots of m2B4 immunoprecipitates from Jurkat transfectants in Fig. 2B, lanes 3 and 4, show that LAT associates with both 2B4S and 2B4L, respectively. Therefore, the LAT-2B4 association occurs in both T and NK cell lines expressing exogenous murine 2B4. To demonstrate the association between LAT and endogenous 2B4 in primary murine cells, murine LAKs were immunoprecipitated with either anti-m2B4 or anti-LFA-1 mAb followed by blotting with anti-LAT. As shown in Fig. 2C, LAT coimmunoprecipitates with 2B4, but not LFA-1, another surface protein found on NK cells, illustrating the specific association between LAT and 2B4 from murine NK cells.

Because LAT coimmunoprecipitated with 2B4 in the absence of pervanadate treatment (Fig. 2), we tested whether 2B4 phosphorylation was necessary for this association. A mutant 2B4L construct (2B4LMY) was generated by site-directed mutagenesis in which all cytoplasmic tyrosines were mutated to phenylalanines. This mutant form of 2B4 exhibited no phosphorylation in the presence of pervanadate (data not shown). RNK-16 transfectants expressing this 2B4LMY mutant, as well as those expressing wild-type 2B4L, were immunoprecipitated with anti-m2B4 followed by immunoblotting with anti-LAT. These mutations do not prevent the association of LAT with 2B4 (Fig. 3A, compare lanes 1 and 2). RNK-16 transfectants expressing a mutant 2B4S protein (2B4SMY) in which all cytoplasmic tyrosines were mutated to phenylalanines were also used in this assay. Again, 2B4 associates with LAT (data not shown), confirming that 2B4 phosphorylation is not required for this interaction to occur. Although LAT associates with both isoforms of 2B4 independent of 2B4 phosphorylation status, the nature of this association is unknown. The 2B4 isoforms have shared cytoplasmic domains as well as domains unique to each isoform. To examine which regions are required for LAT association, RNK-16 cells were transfected with a previously described mutant 2B4 construct.

**FIGURE 1.** LAT coimmunoprecipitates with anti-2B4 mAb in RNK-16-2B4L transfectants. A, RNK-16-2B4L transfectants were incubated with pervanadate, lysed, and subjected to immunoprecipitation with anti-m2B4 mAb. Immunoprecipitates were resolved by SDS-PAGE under nonreducing conditions and immunoblotted with anti-phosphotyrosine. A 66-kDa phosphoprotein representing 2B4 and a 36- to 38-kDa phosphoprotein coimmunoprecipitated. B, RNK-16-2B4L transfectants were immunoprecipitated with either anti-m2B4 or anti-RT1A mAb and immunoblotted with anti-LAT mAb. A 36- to 38-kDa band representing LAT coimmunoprecipitated with anti-m2B4 but not anti-RT1A mAb.

**FIGURE 2.** 2B4 associates with LAT in transfected cell lines and in primary murine NK cells. A, Untransfected or transfected RNK-16 cells were lysed and subjected to immunoprecipitation with anti-m2B4 mAb. Immunoprecipitates were resolved by SDS-PAGE under nonreducing conditions and immunoblotted with anti-LAT mAb. Lane 1, Untransfected RNK-16 cells. Lane 2, RNK-16-2B4S transfectants. Lane 3, RNK-16-2B4L transfectants. B, Untransfected or transfected Jurkat cells were treated as described in A, Lane 1. Whole cell lysates from untransfected Jurkat cells were immunoblotted with anti-LAT mAb. Lane 2, Immunoprecipitates from untransfected Jurkat cells. Lane 3, Immunoprecipitates from Jurkat-2B4S transfectants. Lane 4, Immunoprecipitates from Jurkat-2B4L transfectants. C, Lysates from murine LAK stimulated with IL-12 and IL-18 were subjected to immunoprecipitation with either anti-m2B4 or anti-LFA-1 mAb. Immunoprecipitates were resolved by SDS-PAGE under nonreducing conditions and immunoblotted with anti-LAT mAb.

**FIGURE 3.** Neither phosphorylation of 2B4 nor its cytoplasmic tail is required for LAT association in RNK-16 transfectants, but both are required for maximal activation. A, RNK-16 cells transfected with 2B4L or 2B4LMY (mutant with Y → F) were lysed and subjected to immunoprecipitation with anti-m2B4 mAb. Immunoprecipitates were resolved by SDS-PAGE under nonreducing conditions and immunoblotted with anti-LAT mAb. Lane 1, RNK-16-2B4L transfectants. Lane 2, RNK-16-2B4LMY transfectants. B, RNK-16 cells transfected with 2B4S or 2B4D1 (mutant with no cytoplasmic tail) were treated as described in A, Lane 1. RNK-16-2B4S transfectants. Lane 2, RNK-16-2B4D1 transfectants. C, RNK-16 transfectants, as indicated, were used as effectors in a redirected lysis assay against FcR γ P815 targets at an E:T ratio of 100:1. As shown on the right, 10 μg/ml anti-m2B4 mAb was added to effectors before addition of targets, where indicated.
LAT IS REQUIRED FOR 2B4 LYTIC FUNCTION

(2B4D2) truncated at aa 301, leaving only regions shared by the two isoforms (12). LAT was still found to associate with this mutant 2B4 protein, indicating that this association does not require any domains unique to either 2B4 isoform (data not shown). To determine whether the shared domains of the 2B4 cytoplasmic tail were necessary for LAT association, RNK-16 cells were transfected with 2B4D1, a mutant form of 2B4 with the cytoplasmic tail removed by a truncation at aa 247 (12). Anti-LAT blots of m2B4 immunoprecipitates from RNK-16 cells expressing 2B4S or 2B4D1 revealed the presence of LAT (Fig. 3B, compare lanes 1 and 2), indicating that the cytoplasmic tail of 2B4 is not necessary for LAT association. It should be noted in Fig. 3B, lane 2, that the mutant 2B4 seems to elicit a stronger association with LAT than the wild-type 2B4. However, this apparent increased association is probably due to the higher expression of 2B4 in the RNK-16-2B4D1 transfectants, as determined by FACS analysis (data not shown). To test the function of 2B4 proteins with mutated cytoplasmic domains, a redirected lysis assay was performed using 2B4S, 2B4D1, and wild-type 2B4S RNK-16 transfectants as effectors. When murine 2B4 mAb was added to P815 FcR+ targets, all three effectors (expressing similar levels of surface 2B4, as determined by FACS analysis) demonstrate an increase in cytotoxicity, although the two transfectants expressing mutant forms of 2B4 exhibit a decrease relative to the wild-type 2B4 transfectants (Fig. 3C). Therefore, the tyrosine motifs and the cytoplasmic tail of 2B4 are required for maximal activation. Because these mutant forms of 2B4 still associate with LAT, this association alone is not sufficient for maximum function of 2B4.

LAT is exclusively localized to GEMs found in the plasma membrane (18). Many signaling intermediates appear to accumulate in these regions, suggesting the importance of GEMs to cellular activation functions (15). To determine whether the 2B4-LAT association occurs within GEMs, lysates from RNK-16 transfectants were separated by density gradient centrifugation. GEMs are detergent-insoluble, remaining intact during lysis with nonionic detergents (26). Because of their buoyancy, GEMs rise near the top of a density gradient, whereas detergent-soluble proteins remain at the bottom. Individual fractions of the gradient were collected and blotted with anti-LAT Abs. Because LAT is found exclusively within GEMs (18), immunoblotting with anti-LAT mAb demonstrates the location of GEM fractions within the gradient (Fig. 4A).

To determine whether the LAT-2B4 association occurs within GEMs, fractions from the same lysates were subjected to immunoprecipitation with anti-m2B4 followed by blotting with anti-LAT. Fig. 4B shows that LAT and 2B4 coimmunoprecipitate in GEM fractions 7 and 8, suggesting that the LAT-2B4 interaction occurs within GEMs. Similar results were observed for Jurkat transfectants as well as primary NK cells (see Fig. 7 and data not shown).

The human homolog of 2B4 has recently been described and is expressed in the human NK cell line YT (25). To determine whether 2B4 and LAT colocalize in an untransfected cell line, lysates from YT cells were separated by density gradient centrifugation followed by immunoblotting of individual gradient fractions. The anti-LAT immunoblot in Fig. 5A shows that GEMs are located in fractions 7–9. Immunoblotting with anti-h2B4 indicates that 2B4 is also constitutively located within GEM fractions from YT cells (Fig. 5B). Furthermore, anti-LAT blots of h2B4 immunoprecipitates from YT whole cell lysates demonstrate 2B4 and LAT association (data not shown). Thus, it appears that 2B4 and LAT colocalize to GEMs within a variety of cell types, including human NK and T cell lines and murine primary cells.

Proteins can be targeted to GEMs in one of three ways: GPI linkage, acylation, or noncovalent association with other resident proteins (27). Palmitoylation at two cysteines within the transmembrane region of LAT is required to target LAT to GEMs (18). This palmitoylation site, Cys-Val-Arg-Cys, is similar to aa 244–247 in the transmembrane region of 2B4 (Cys-Phe-Cys-Val) (7, 18), indicating that 2B4 may also be targeted to GEMs by this mechanism. In fact, a similar CxC motif is present in the cytoplasmic tails of CD4 and CD8α, where it mediates association with LAT (28). However, using radiolabeled palmitate, we were unable to demonstrate palmitoylation of 2B4 (data not shown). To determine the importance of the CxC cysteine motif in GEM localization, Jurkat cells were transfected with either wild-type 2B4 (J.2B4S) or with a 2B4 construct in which the cysteines of the CxC motif were mutated to alanines (J.2B4TMC>A). Transfectants expressing similar expression levels of 2B4 were selected for further study (Fig. 6A, C and D). To determine whether 2B4 is resident in GEMs of Jurkat cells, and to compare the localization of wild-type and mutated 2B4 as well, we used 2B4 mAb to immunoprecipitate biotinylated surface proteins from density gradient fractions of J.2B4S and J.2B4TMC>A lysates. These immunoprecipitates were then blotted with streptavidin-HRP. Fig. 6A shows the location of wild-type 2B4 to exist predominantly within GEM fractions 7–9. As an additional confirmation that these buoyant fractions contain GEMs, we immunoblotted the fractions from the same

FIGURE 4. LAT associates with 2B4 in GEM fractions from RNK-16 transfectants. A, RNK-16-2B4L transfectants were lysed and subjected to density gradient centrifugation. Fractions were then collected, resolved by SDS-PAGE under nonreducing conditions, and immunoblotted with anti-LAT mAb. GEM fractions correspond to fractions 6–9. B, RNK-16-2B4L transfectants were lysed and subjected to density gradient centrifugation. Fractions were collected and immunoprecipitated with anti-m2B4 mAb. Immunoprecipitates were resolved by SDS-PAGE under nonreducing conditions and immunoblotted with anti-LAT mAb.

FIGURE 5. 2B4 and LAT are both resident in GEM fractions from YT human NK cells. YT cells were lysed and subjected to density gradient centrifugation. Fractions were collected and resolved by SDS-PAGE under nonreducing conditions. A, Fractions were immunoblotted with anti-LAT mAb. B, Fractions were immunoblotted with anti-h2B4 mAb.
J.2B4S lysates with Lck, a protein known to be disproportionately represented in GEMs (29). Lck was predominantly located in fractions 7–10 (Fig. 6B), indicating that the fractions containing the majority of wild-type 2B4 are indeed GEM fractions. Fig. 6A also suggests that the majority of the 2B4 protein normally found in the GEM fractions is shifted to the soluble fractions when the CxC motif is mutated. To more directly compare the relative localization to GEMs of the wild-type and mutant forms of 2B4, we chose to immunoblot fractionated lysates with polyclonal m2B4 Ab rather than first immunoprecipitate with anti-m2B4 mAb. This allows a direct comparison of the GEM localization of the two proteins to be made. Despite having similar expression levels (Fig. 6, C and D), the presence of mutant 2B4 is much reduced in GEM fractions compared with wild-type 2B4 (Fig. 6E), suggesting that the mutation in J.2B4TMC>A shifts the majority of 2B4 away from GEM fractions. These results demonstrate that the CxC motif found in the transmembrane region of 2B4 is important for GEM localization.

Because mutation of the CxC motif produced a shift in the localization of 2B4 away from GEM fractions, we were interested to determine whether 2B4-LAT association would also be affected by this mutation. Therefore, fractionated lysates from Jurkat transfectants, J.2B4S and J.2B4TMC>A, were immunoprecipitated with anti-m2B4. Blotting of the immunoprecipitates with anti-LAT mAb indicates that LAT has a much-reduced association with mutated 2B4S from J.2B4TMC>A when compared with wild-type 2B4S (Fig. 7). Furthermore, this association is not seen in any of the detergent-soluble fractions, indicating that 2B4 localization to GEM fractions is important to the 2B4-LAT interaction.
In an attempt to determine the functional significance of the localization of 2B4 to GEMs, we planned to use LAKs retrovirally transduced with either 2B4 or 2B4TMC>A constructs as effectors in a redirected lysis assay. To obtain the viral supernatants needed to perform the transduction, we first transfected 293T cells with bicistronic retroviral constructs containing both 2B4 and green fluorescent protein (GFP) open reading frames linked by an internal ribosome entry site. This allows us to assess transduction efficiency and sort transduced cells by flow cytometry. As seen in Fig. 8A, both 2B4 and 2B4TMC>A proteins are expressed on the surface of transfected 293T cells (2B4 PE+GFP+). Splenocytes from 129 mice, which lack the allele of 2B4 recognized by murine 2B4 mAb, were then used to generate the LAK cultures. However, extracellular staining of these LAKs after retroviral transduction demonstrates that only the wild-type 2B4 protein reaches the cell surface (Fig. 8B). Additionally, we were unable to obtain RNK-16 transfectants exhibiting surface expression of 2B4TMC>A (data not shown). These data suggest that the CxC mutation found in 2B4TMC>A negatively affects the capacity of 2B4 protein to reach the cell surface of LAKs. Although it is unclear why 2B4TMC>A can be expressed on the surface of 293T and Jurkat cells (Figs. 8A and 6C), this may be due to an abnormality in membrane localization and composition, as is seen in some tumor cell lines (30).

We next tested whether LAT was required as an intermediate in the 2B4 signaling pathway. To do this, we examined 2B4 function in NK cells cultured from LAT−/− mice (a kind gift from L. Samelson and P. Love, National Institutes of Health). It has previously been shown that LAT−/− mice have functional NK cells (31). To demonstrate that LAT−/− NK cells are capable of lysing susceptible targets, a chromium release assay was performed in which LAKs from C57BL/6 wild-type mice or LAT−/− mice were tested against chromium-labeled NK-sensitive targets, YAC-1. Both NK effectors efficiently lyse YAC-1 targets (data not shown), confirming that natural cytotoxicity is not impaired by the absence of LAT, as previously described (31). Redirected lysis assays were then performed to determine whether 2B4-mediated lysis was disrupted in LAKs generated from LAT−/− mice. Because LAT−/− mice lack T cells, SCID mice were chosen as an appropriate wild-type control. mAb to m2B4 were added to FcR− P815 target cells. As shown in Fig. 9A, Abs to m2B4 stimulate lysis of P815 targets at the indicated E:T ratios. As shown on the right, 10 μg/ml anti-m2B4 mAb was added to effectors before addition of targets, where indicated. Results are representative of three independent experiments.

Discussion

2B4 is a cell surface receptor found on all murine NK cells, as well as a subset of T cells (4, 7). The ligand for 2B4 in both humans and mice is CD48 (9), a molecule expressed on all cells of the hematopoietic lineage. It is still unclear exactly how the 2B4-CD48 interaction regulates NK and T cell function.
interaction affects cellular function, although evidence suggests that the interaction stimulates human NK cell cytotoxicity and murine T cell proliferation (32–34). Murine 2B4 is expressed as two isoforms differing in both structure and function (8, 12). Single-cell RT-PCR analysis of murine NK cells indicates that both coexist within a single cell (L. Hays and J. Schatzle, unpublished observations), although the relative expression levels and functional dominance of the two isoforms within a single cell remain unresolved.

These studies and others (6, 22) have determined that 2B4 associates with LAT, an important intermediate in both T cell and NK cell activation (17, 20). Furthermore, we have seen this association in both NK and T cell lines, as well as in primary murine NK cells. Because LAT is localized to GEMs (18), 2B4 membrane localization was also examined. GEMs are enriched in sphingolipids, cholesterol, and GPI-anchored proteins (35). All of these components have higher melting temperatures than glycerolipids due to the saturation of their fatty acyl chains. Lipids with saturated acyl chains can tightly pack together in a more ordered state due to favorable interactions among the acyl chains. This property creates clustering of sphingolipids, cholesterol, and GPI-anchored or fatty-acylated proteins in specialized areas of the membrane (GEMs) that are relatively detergent-insoluble. It is known that many signaling molecules important to both NK and T cell activation accumulate in GEMs (17, 19). Some of these molecules are GPI-anchored, some are palmitoylated, and some simply associate with other resident proteins. Clustering of signaling pathway intermediates results in amplification of signal transduction cascades, allowing a cell to more efficiently respond to an activation event. Therefore, GEM structures appear to be important to cellular activation (15, 18, 36). Unlike many signaling molecules, which are recruited to GEMs during activation, we have discovered that 2B4 is resident within GEMs. Residence within such an environment may allow 2B4 to respond more quickly to a ligand-induced interaction than a recruited receptor could respond. Through its localization to GEMs, 2B4 also has an increased likelihood of impacting the pathways of other GEM signaling receptors, either resident or recruited. In fact, recent studies in human NK cells suggest a coreceptor role for 2B4, in which 2B4 communicates with the NKp46 receptor (37). Therefore, GEM localization of 2B4 may impact the receptor pathways with which 2B4 can communicate.

We have determined that the 2B4-LAT interaction occurs within GEMs, which is not surprising given that both molecules reside there. Targeting of LAT to GEMs is dependent upon palmitoylation of a CxC motif (18). We discovered that a similar CxC motif found in the transmembrane region of 2B4 is also involved in targeting 2B4 to GEMs. Mutagenesis studies have shown that this CxC motif is also necessary to retain LAT association. However, unlike with LAT, we were unable to demonstrate palmitoylation of 2B4 (data not shown). Therefore, the mechanism by which this motif functions in 2B4 localization remains unclear.

Although Jurkat cells transfected with 2B4TMC> A clearly demonstrate a reduced capacity to reside in GEMs and associate with LAT, a small percentage of 2B4 protein shows residual association with GEMs. It is possible that 2B4 has other motifs or domains that aid in the localization to GEMs. Therefore, mutation of the CxC motif may only shift the balance away from GEM localization rather than abrogate it. Any remaining 2B4 present in GEMs may be free to associate with LAT.

Although we were unable to test the functional significance of the CxC mutation because of the inability of 2B4TMC> A to reach the cell surface in NK cells, we can conclude that LAT association alone is not sufficient for 2B4 function. Both mutation of cytoplasmic tyrosines (2B4SMY) and truncation of the cytoplasmic tail (2B4D1) lead to significant reductions in 2B4-mediated cytotoxicity. This is probably due to the fact that both mutations prevent the association of 2B4 with SAP (J. Schatzle, unpublished results), which has been shown to be essential to 2B4 function in human NK cells (38). However, the residual cytotoxicity seen with these mutations may be due to the remaining association with LAT.

Recent evidence, in fact, suggests that LAT is involved in 2B4 signaling (6, 22). When the 2B4 pathway in human NK cells is stimulated, LAT is phosphorylated and LAT substrates phospholipase C-y1 and Grb2 are recruited to the 2B4 signaling complex. Therefore, we examined the function of 2B4 in the absence of LAT using NK cells from LAT−/− mice. We found that redirected lysis triggered by cross-linking 2B4 is inhibited when LAT is absent, although natural cytotoxicity is still intact. We conclude that, under these circumstances, LAT is a required intermediate in the 2B4 signaling pathway. Future studies will focus on the establishment of 2B4 function in T cells and the further characterization of the molecules involved in the 2B4 signaling pathway in both NK and T cells.

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References


