DAP12-mediated Signal Transduction in Natural Killer Cells

A DOMINANT ROLE FOR THE Syk PROTEIN-TYROSINE KINASE

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The murine Ly49 family contains nine genes in two subgroups: the inhibitory receptors (Ly49A, B, C, E, F, G2, and I) and the noninhibitory receptors (Ly49D and H). Unlike their inhibitory counterparts, Ly49D and H do not contain immunoreceptor tyrosine-based inhibitory motifs but associate with a recently described coreceptor, DAP12, to transmit positive signals to natural killer (NK) cells. DAP12 is also expressed in myeloid cells, but the receptors coupled to it there are unknown. Here we document the signaling pathways of the Ly49D/DAP12 complex in NK cells. We show that ligation of Ly49D results in 1) tyrosine phosphorylation of several substrates, including phospholipase Cγ1, Cbl, and p44/42 mitogen-activated protein kinase, and 2) calcium mobilization. Moreover, we demonstrate that although human DAP12 reportedly binds the SH2 domains of both Syk and Zap-70, ligation of Ly49D leads to activation of Syk but not Zap-70. Consistent with this observation, Ly49D/DAP12-mediated calcium mobilization is blocked by dominant negative Syk but not by catalytically inactive Zap-70. These data demonstrate the dependence of DAP12-coupled receptors on Syk and suggest that the outcome of Ly49D/DAP12 engagement will be regulated by Cbl and culminate in the activation of transcription factors.

Natural killer cells play a central role in cell-mediated immunity by virtue of their ability to recognize and lyse tumor or virus-infected cells and to regulate the T-helper cell repertoire through the production of interferon γ (1). Although the receptor(s) involved in the positive recognition of targets by natural killer (NK) cells are still unknown, a large number of human major histocompatibility complex class I-binding killer cell inhibitory receptors (KIRs) have now been reported (2, 3). Human KIRs are Ig superfamily receptors expressed primarily on NK cells and a small subset of T cells. When bound by major histocompatibility complex class I ligands on target cells, KIRs transmit a negative signal to the effector cell, causing the activation program to be aborted. KIRs possess two or three extracellular Ig-like domains and contain at least one intracellular immunoreceptor tyrosine-based inhibitory motif (ITIM), defined as (IV/XXXVXII) (2–4). KIR ligation results in the phosphorylation of the tyrosine residues of the ITIM. Tyrosine-phosphorylated ITIMs then serve as docking sites for recruitment of the Src homology 2 (SH2) domain-containing protein-tyrosine phosphatase SHP-1, resulting in the dephosphorylation of cellular substrates and the termination of positive signaling events (4–6). Consistent with this model, mutation of ITIMs within KIRs or expression of dominant negative isoforms of SHP-1 blocks the inhibitory function of these receptors (4).

Murine NK cells express the Ly49 family of major histocompatibility complex class I-binding inhibitory receptors (reviewed in Ref. 7). Although these type II lectin-like receptors differ dramatically from the Ig superfamily human KIRs, they also contain cytoplasmic ITIMs. Biochemical evidence from our laboratory, as well as others, has demonstrated that inhibitory Ly49 molecules are tyrosine-phosphorylated following ligation and that their phospho-ITIMs recruit SHP-1 (8–10). As expected, Ly49 inhibitory function is severely compromised, but notably not absent, in SHP-1-deficient viable motheaten mice (9). The existence of KIRs in NK cells, together with the discovery of multiple families of ITIM-containing Ig superfamily receptors in the B cell (leukocyte immunoglobulin-like receptors (LIR), paired immunoglobulin-like receptors (PIR)p91), and leukocyte-associated immunoglobulin-like receptor (LIAR) and myeloid lineage (immunoglobulin-like transcripts (ILT), PIR)p91, LIAR, and monocyte/macrophage immunoglobulin-like receptor (MIR)), suggests that these receptors likely play a critical role in the regulation of multiple facets of the immune system (11–18).

Concurrent with the description of multiple families of inhibitory receptors are reports of family members lacking the characteristic ITIM and instead containing charged residues within their putative transmembrane domains. This receptor configuration is reminiscent of the T cell receptor (TCR), the B cell receptor, and Fc receptors, all of which lack intrinsic cat-

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alytic activity yet deliver activation signals via receptor-associated chains. In fact Ly49D, which lacks an ITIM and contains an arginine residue in its transmembrane domain, delivers an activation signal to murine NK cells, resulting in reverse anti-body-dependent cellular cytoxicity (8, 19). Noninhibitory iso-forms of the human KIRs have also been demonstrated and termed killer cell activitory receptors (KARs) (20–22). In addition, the PIR and LIR families contain receptors with charged residues in their transmembrane domains that lack the ITIM consensus (11, 14). No positive signaling capabilities, however, have been demonstrated for PIR or LIR receptors. Despite the potential importance of these activating receptors in the B cell, NK cell, and myeloid cell lineages, little is known regarding the biochemical basis of noninhibitory Ig family receptor function. Moretta and co-workers (23) have reported that ligation of noninhibitory KIRs on human NK cells leads to the activation of Lck and to tyrosine phosphorylation of the TCRγ chain. However, we have recently described the physical and functional association of a 16-kDa tyrosine-phosphorylated polypeptide (pp16) with Ly49D in mouse NK cells (24). In addition, we have shown that pp16 is the murine homolog of a protein associated with noninhibitory KIRs, DAP12 (25). DAP12 is a 12-kDa transmembrane protein similar to TCRγ and the FcεRIγ chain that contains an aspartic acid residue in its transmembrane domain and a single cytoplasmic, immunoreceptor tyrosine-based activation motif (ITAM). Consistent with the expression of putative positive signaling receptors in various lineages, DAP12 expression is detected not only in NK cells but also in monocytes (24, 26). In this report, we have examined signal transduction by the DAP12-associated receptor Ly49D. We have identified several proteins that become tyrosine-phosphorylated following Ly49D/DAP12 stimulation. In addition, we show that although NK cells express both Syk-family protein-tyrosine kinases Zap-70 and Syk, Zap-70 is only weakly activated following Ly49D ligation, whereas Syk becomes heavily phosphorylated and activated. Moreover, dominant negative Syk efficiently blocks mobilization of intracellular calcium by Ly49D, whereas catalytically inactive Zap-70 does not. These data demonstrate a critical role for Syk but not Zap-70 in receptors coupled to DAP12.

EXPERIMENTAL PROCEDURES

Antibodies—The monoclonal antibodies (mAbs) 4D11 (rat IgG2a) for Ly49G2 and 4E5 (rat IgG2a) for Ly49D were developed in this laboratory (27). A rabbit antisera to rat IgG (Zymed Laboratories Inc., San Francisco, CA) was used to cross-link the rat mAb. Rabbit polyclonal antibodies recognizing Cbl and Syk were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). In some cases, anti-Syk blotting was done with an anti-Syk antibody generated against a GST fusion protein containing the 35-kDa carboxyl-terminal kinase domain of murine Syk or with anti-Syk provided by Dr. Joseph Bolen (DNAX, Palo Alto, CA). Phospho-specific and Pan-p42/44 mitogen-activated protein (MAP) kinase antibodies were from New England Biolabs (Beverly, MA). Biotinylated 4G10 and monoclonal anti-phosphotyrosine Cy1 (FLC-cy1) antibodies were purchased from Upstate Biotechnologies Inc. Anti-Zap-70 was the gift of Dr. Ronald Wang (NIA, National Institutes of Health, Baltimore, MD). Rabbit anti-DAP12 was generated in our laboratory using 4E5 immunoprecipitates as an immunogen. Tumor Cell Lines—The rat NK cell line, RNK-16, was maintained in RPMI 1640 medium containing 10% fetal bovine serum, 2 mM glutamine, 100 units/ml penicillin, 100 µg/ml streptomycin, and 5.5 × 10−5 M 2-mercaptoethanol. 293T cells were maintained in DMEM containing 10% fetal bovine serum, glutamine, and antibiotics. Plasmids and Transfections—The Ly49D cDNA was obtained by polymerase chain reaction amplification from cDNA prepared from 7-day interleukin-2-activated lymphokine activated killer cells as described.4 RNK-16 cells were transfected as described previously (9). Briefly, RNK-16 cells were electrotransfected with 20 µg of Scrl-linearized BSrκEn plasmid using a BTX electroporator (San Diego, CA). After electroporation, cells were cultured overnight and then plated into 96-well plates at 1 × 105 cells/well, in medium containing 1 mg/ml active G418 (Boehringer Mannheim). Transfectants were subcloned and characterized by flow cytometry (referred to as RNK-D).

The murine cDNA for DAP12 was identified by searching an expressed sequence tag data base using the NCBI Blast Program. The query sequence was the human cDNA DAP12 sequence reported by Lanier et al. (26). The homologous murine expressed sequence tag (mEST accession number AA085965) was purchased from ATCC (Rockville, MD) and sequenced using the T7 Sequenase Version 2.0 DNA sequencing kit (United States Biochemical, Cleveland, OH). For transfection of 293T cells, the Ly49D and Ly49D[4E5]+ mCDAs (24) were digested with NotI, blunted using the Klenow fragment of DNA polymerase I, and then subcloned into the pEF expression vector (28) (the gift of Dr. Gary Koretzky, University of Iowa, Iowa City, IA) under the control of the elongation factor promoter. The murine FceRI cDNA in the pSVL vector (29, 30) was the gift of Dr. J. P. Kinet (Harvard University, Boston, MA). The Myc-tagged Syk cDNA was prepared from the original Syk cDNA construct (31) by annealing it to oligonucleotides containing the sense and antisense sequences of the Syk myc epitope along with a stop codon (ATT) and SalI and NotI restriction sites. The resulting PCR product and cDNA was cloned into the HindIII/NotI sites of the pcR-CMV expression vector (Invitrogen, San Diego, CA). The day before transfection, 3 × 105 293T cells were plated into each well of a 6-well plate in complete medium. After culture overnight, cells were transfected with 0.5 µg each of the indicated combinations of the Ly49D, DAP12, and Syk expression constructs using the FuGene 6 transfection reagent as directed by the manufacturer (Boehringer Mannheim). Expression of Ly49D was monitored by flow cytometry. Stimulation, Immunoprecipitation, Electrophoresis, and Blotting—Cells were stimulated with 1 µM pervanadate for 15–20 min at 37 °C as described (24). Stimulation with rat mAb to the Ly49 receptors, or control mAb, was accomplished by the addition of 1 µg/106 cells in 1.0 ml of prewarmed medium, followed by the addition of 1 µg/106 cells of rabbit anti-rat antibody 20 s later. The cells were then incubated at 37 °C for 1 min unless otherwise noted. After stimulation, the cells were pelleted, the supernatant was removed, and the cells were lysed in lysis buffer (1% Triton X-100, 50 mM Tris, pH 7, 300 mM NaCl, 2 mM EDTA, 0.4 mM NaVO₃, aprotinin, leupeptin, and phenylmethylsulfonyl fluoride) (24). Lysates were clarified by centrifugation and immunoprecipitated with antibody bound to protein G-agarose (Life Technologies, Inc.) for 60 min at 4 °C. Immunoprecipitates were washed complexes were washed with wash buffer (1% Triton X-100, 50 mM Tris, pH 7.5, 300 mM NaCl, 2 mM EDTA), and proteins were eluted in nonreducing or reducing Laemli buffer and separated by SDS-PAGE as noted. Proteins were transferred to Immobilon-P (Millipore, Bedford, MA), and the blots were blocked in 5% bovine serum albumin. Biotinylated 4G10 (Upstate Biotechnologies, Lake Placid, NY) followed by streptavidin-horseradish peroxidase was used to detect phospho-proteins. Blots were developed using ECL (Amersham Pharmacia Biotech) and exposed to Kodak XAR film (24).

Cell lysis and immunoblotsting with phosphospecific Erk antibodies were done as directed by the manufacturer (New England Biolabs). Immunoblotting with anti-Syk and anti-PLCγ1 was done using 5% milk for blocking. After incubation with primary antibodies in 5% milk and extensive washing, bound antibody was detected by using either horseradish peroxidase-conjugated goat anti-rabbit antibody (Boehringer Mannheim) or goat anti-mouse Ig (Amersham Pharmacia Biotech) and ECL (Amersham Pharmacia Biotech). Where noted, filters were stripped for 30 min at 50 °C in 2% SDS, 100 mM 2-mercaptoethanol, 62.5 mM Tris-HCl, pH 6.7. After extensive washing, filters were reblocked and subsequently reprobed.

GST Fusion Binding Assays—GST fusion proteins were produced and purified by using established protocols. The GST fusion construct of the Syk SH2 domains was derived from the pNTXSyk plasmid (32) by digestion with KpnI to remove coding sequences for the carboxyl-terminal region of Syk. The resulting fragments were blunt-ended with T4 polymerase and ligated to the unphosphorylated EcoRI linker (CG-GAATTCCG). The vector/insert fusion-terminal fragment was gel purified and ligated. An 800-base pair Xhol/EcoRI fragment was then excised from this plasmid and subcloned into the Xhol/EcoRI site of pGEX-2T (Amersham Pharmacia Biotech). The fusion protein of the

2 P. Gosselin, L. H. Mason, J. Willette-Brown, J. R. Ortaldo, D. W. McVicar, and S. K. Anderson, submitted for publication.
3 J. R. Ortaldo, A. T. Mason, R. Winkler-Pickett, A. Raziuddin, W. J. Murphy, and L. H. Mason, submitted for publication.
4 M. C. Nakamura, P. A. Linnemeyer, E. C. Niemi, L. H. Mason, J. R. Ortaldo, J. C. Ryan, and W. E. Seaman, submitted for publication.
SH2 domain of Chk will be described elsewhere. For binding assays, 2 μg of fusion protein was bound to glutathione Sepharose 4B beads (Amersham Pharmacia Biotech) at 4 °C for 1 h. Cells were stimulated as described and lysed in lysis buffer containing 10% glycerol. Glutathione beads were pelleted, unbound fusion proteins removed, and cleared lysates were added for 1–2 h at 4 °C. After washing with wash buffer, the bound proteins were eluted with SDS sample buffer.

Elution of DAP12 from Ly49D immunoprecipitations was done after immunoprecipitation of 40 × 10^6 RNK-D cells as described above. Washed immunoprecipitates were incubated for 30 min at 4 °C in 50 μl of modified radioimmune precipitation buffer (150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 10 mM Tris, pH 7) containing phosphatase inhibitors and protease inhibitors as described above. After pelleting the beads, the supernatant was removed and diluted to 3 ml in lysis buffer containing 1% Brij 96 in place of Triton X-100. One ml of this was then added to each of the prebound GST fusion proteins and incubated for 1–2 h at 4 °C. After washing in 0.2% Triton X-100 wash buffer, the bound proteins were eluted with SDS sample buffer, resolved by SDS-PAGE, and blotted with anti-phosphotyrosine as described above.

**Immunoprecipitations** — Immunoprecipitations were done after stimulation, lysis, and immunoprecipitation as described above. Immunoprecipitates were washed twice in wash buffer, twice in lithium buffer (0.5M LiCl, 50 mM Tris, pH 7.5, 0.1% Triton X-100), and then twice in water. Immunoprecipitates were then resuspended in 50 μl of kinase buffer (20 mM Tris, pH 7.5, 10 mM MnCl2, 1 μM ATP) supplemented with 10 μCi of [γ-32P]ATP (3000 Ci/mM, NEN Life Science Products). One μg of the cytoplasmic domain of erythrocyte band 3 (the gift of Dr. Ronald Wange, NIA) was added to each reaction as an exogenous substrate. Reactions were carried out for 10 min at room temperature and then stopped by the addition of 50 μl of 2× SDS sample buffer and boiling. Reaction products were resolved on 8% SDS-PAGE gels and detected by autoradiography.

**Vaccinia Infections** — The vaccinia constructs pSC65 and Syk-T (33, 34) were the gifts of Dr. Deborah Burshtyn (NIAID, Rockville, MD). The WR and Myc-Zap-70(KH7) vaccinia were the gifts of Dr. Paul Leibson (Mayo Clinic, Rochester, MN) (35). Vaccinia virus stocks were maintained and propagated as described (4). For vaccinia infections, cells were suspended in DMEM with 2% fetal bovine serum at a concentration (10^6 cells/ml). Infection was with a multiplicity of infection of 10–20 for 4 h at 37 °C. Cells were then washed and loaded with calcium dyes as described below. Expression of vaccinia encoded proteins was confirmed by Western blotting using an antisera that recognizes both Syk and Zap-70.

**Calcium Flux** — Analysis of the changes in intracellular Ca^{2+} concentration ([Ca^{2+}]_i) was carried out using a FACSort flow cytometer (Becton Dickinson, Mountain View, CA) and the calcium sensitive fluoro chrome Fluo-3 (Molecular Probes, Eugene, OR). Briefly, cells (2 × 10^6/ml) were incubated at 25 °C in Dulbecco’s phosphate-buffered saline without Ca^{2+} or Mg^{2+} containing 20 μg/ml Fluo-3. After 30 min, cells were washed in Dulbecco’s phosphate-buffered saline and held at room temperature in the dark until analysis. The [Ca^{2+}]_i was monitored with the loaded cells (40 μl) diluted to 500 μl with Dulbecco’s phosphate-buffered saline with Ca^{2+} and Mg^{2+}, glucose, and sodium pyruvate at 37 °C. Cells were kept at 37 °C during analysis. Baseline data were collected for 20–30 s, and then cells were stimulated with primary (10 μg/ml) mAb followed 20–25 s later by rabbit anti-rat antibody (10 μg/ml). Data were analyzed using the MultiTime kinetic experiment analysis software (Phoenix Flow Systems, San Diego CA) and are expressed as the percentage of responding cells relative to unstimulated baseline measurements.

**RESULTS**

**Mouse Ly49D Couples to DAP12 in RNK-16 Cells** — In order to study the proximal signal transduction of DAP12 in NK cells, we expressed murine Ly49D, a receptor known to physically and functionally couple to DAP12, in the rat NK cell line RNK-16. Clones were grown under selection in G418-containing medium and screened for Ly49D expression using the mAb 4E5. Positive RNK-16 cells express rat Ly49 molecules (36); however, they did not express any molecules that react with anti-Ly49D mAb 4E5 (Fig. 1A). Transfection of RNK-16 with the mouse Ly49D cDNA yielded a number of clones that stably express Ly49D as detected by 4E5 staining. Due to its high level of stable expression of Ly49D, clone D2.38 (referred to as RNK-D) was selected for further analysis of DAP12 signaling.

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We and others have previously demonstrated the co-immunoprecipitation of DAP12 and Ly49D in mouse NK cells and transfectants (25). Therefore, we next determined whether mouse Ly49D was able to physically couple to rat DAP12 in RNK-D. Ly49D was immunoprecipitated from RNK-D cells before or after nonspecific stimulation with pervanadate or receptor cross-linking using anti-Ly49D mAb (Fig. 1B). Phosphoproteins within the receptor complexes were resolved using nonreducing SDS-PAGE and immunoblotted with anti-phosphotyrosine. These data demonstrate that, similar to mouse NK cells, cross-linking of Ly49D in RNK-D results in co-immunoprecipitation of phosphotyrosine-containing polypeptides of a mass consistent with that of DAP12 homodimers (Fig. 1B).
To confirm the identity of the Ly49D co-immunoprecipitating peptides in RNK-D, we injected rabbits with Ly49D immunoprecipitates from mouse NK cells. We determined the specificity of this antibody by using it to immunoblot Ly49D receptor complexes that had been immunoprecipitated from transfected 293T cells or RNK-D. First, 293T cells were transfected with combinations of Ly49s and signal transduction chains, Ly49D was immunoprecipitated, and the complexes were immunoblotted with our rabbit antiserum (Fig. 2A). This analysis demonstrated that our antibody detected a 12-kDa protein in Ly49D immunoprecipitates only after transfection with both Ly49D and DAP12. Ly49D with Arg54 mutated to Leu (Ly49DR54L) failed to associate with DAP12, confirming the specificity of this interaction. Similarly, an inhibitory Ly49, Ly49G2, failed to associate with DAP12 (Fig. 2A, fifth lane). Our anti-DAP12 antibody did not react with FcεRIg that was directly immunoprecipitated (data was not shown). Therefore, DAP12 is specifically detected by our anti-DAP12 antibody and fails to associate with Ly49DR54L or Ly49G2 (Fig. 2A and data not shown). We next used this antibody to analyze Ly49D receptor complexes in RNK-D that had been stimulated with pervanadate (Fig. 2B). Immunoprecipitation with anti-Ly49D from lysates of RNK-D (bottom panels), but not RNK-16 (top panels), followed by nonreduced immunoblotting with anti-DAP12 clearly showed the presence of DAP12 homodimers (Fig. 2B). Furthermore, the bands detected by our antiserum co-migrated with the phosphotyrosyl-containing bands characteristically seen in Ly49D immunoprecipitates (Fig. 2B, right panels). The electrophoretic shift of nonreduced DAP12 homodimers from an apparent mass of 24–32 kDa in response to stimulation is consistent with the potent tyrosine phosphorylation of DAP12 under these conditions (Fig. 2B) (24). Together, these data demonstrate the presence of DAP12 in the Ly49D complexes of RNK-D.

Ly49D Cross-linking Induces Tyrosine Phosphorylation of Multiple Cellular Proteins—Having established the linkage between Ly49D and DAP12 in RNK-16, we examined postnuclear lysates for the presence of tyrosine phosphoproteins following Ly49D cross-linking. When RNK-D cells were stimulated with anti-Ly49D mAb and a cross-linking antibody and lysed and post-nuclear lysates were analyzed using anti-phosphotyrosine, we detected a marked increase in the phosphotyrosine content of several cellular substrates (Fig. 3A, arrows). Affected substrates had apparent masses of approximately 180, 130, 110, 97, 75, and 70 kDa. Stimulation of RNK-D cells with F(ab)2 fragments of anti-Ly49D alone were also capable of eliciting increases in cellular phosphotyrosine content (data not shown). We next immunoprecipitated specific proteins from stimulated RNK-D cells and performed anti-phosphotyrosine immunoblotting to determine whether they might be phosphorylated in response to Ly49D ligation. This approach demonstrated that Cbl was potently tyrosine-phosphorylated following Ly49D cross-linking (Fig. 3B). Tyrosine-phosphorylated Cbl co-migrates with the heavily phosphorylated 110-kDa band seen in the whole cell lysates, suggesting that Cbl is one of the most prominent tyrosine phosphoproteins in the DAP12 pathway (compare Fig. 3, A and B). The Cbl homologue in Caenorhabditis elegans, Sli-1, is a negative regulator of the Ras pathway in these organisms (37). Therefore, we asked whether Ly49D/DAP12 engagement would result in phosphorylation of
the p44/42 MAP kinases (Erk1/Erk2). RNK-D cells were stimulated with either anti-Ly49G2 (αLy49G2, 1 μg/10^6 cells) (middle lane) or anti-Ly49D (αLy49D, 1 μg/10^6 cells) (right lane) followed by cross-linking with rabbit anti-rat Ig (1 μg/10^6 cells) cross-linker (XL) or with cross-linker alone (left lane) and lysed, and whole cell lysates were resolved by SDS-PAGE and immunoblotted with anti-phosphotyrosine. B, Ly49D-induced phosphorylation of Cbl. RNK-D (10^7 cells/lane) were stimulated with anti-Ly49G2 (αLy49G2, 1 μg/10^6 cells) (left lane) or anti-Ly49D (αLy49D, 1 μg/10^6 cells) (middle and right lanes) followed by cross-linking with rabbit anti-rat Ig (XL, 1 μg/10^6 cells), lysed, and immunoprecipitated with anti-Cbl (left and middle lanes) or control antibody (right lane). Immunoprecipitates were resolved by SDS-PAGE and immunoblotted with anti-phosphotyrosine (top). Filters were stripped with SDS and then reprobed with anti-Cbl (bottom). C, Ly49D/DAP12-mediated phosphorylation of p44/42 MAP kinases. RNK-D cells were stimulated with anti-Ly49D (αLy49D, 1 μg/10^6 cells) and cross-linker or anti-Ly49G2 (αLy49G2, 1 μg/10^6 cells) and cross-linker for the indicated times and lysed, and phospho-p44/42 MAP kinase (p44/42 MAPK) was detected in whole cell lysates (100 μg/lane) by SDS-PAGE followed by immunoblotting with phospho-p44/42 MAP kinase specific antibodies (top). Filters were stripped with SDS and reprobed with pan anti-p44/42 MAP kinase to confirm equal loading (bottom).

**FIG. 3.** Ly49D-induced tyrosine phosphorylation in RNK-D. A, whole cell lysates. 10^6 RNK-D cells were stimulated with either anti-Ly49G2 (αLy49G2, 1 μg/10^6 cells) (middle lane) or anti-Ly49D (αLy49D, 1 μg/10^6 cells) (right lane) followed by cross-linking with rabbit anti-rat Ig (1 μg/10^6 cells) cross-linker (XL) or with cross-linker alone (left lane) and lysed, and whole cell lysates were resolved by SDS-PAGE and immunoblotted with anti-phosphotyrosine. B, Ly49D-induced phosphorylation of Cbl. RNK-D (10^7 cells/lane) were stimulated with anti-Ly49G2 (αLy49G2, 1 μg/10^6 cells) (left lane) or anti-Ly49D (αLy49D, 1 μg/10^6 cells) (middle and right lanes) followed by cross-linking with rabbit anti-rat Ig (XL, 1 μg/10^6 cells), lysed, and immunoprecipitated with anti-Cbl (left and middle lanes) or control antibody (right lane). Immunoprecipitates were resolved by SDS-PAGE and immunoblotted with anti-phosphotyrosine (top). Filters were stripped with SDS and then reprobed with anti-Cbl (bottom). C, Ly49D/DAP12-mediated phosphorylation of p44/42 MAP kinases. RNK-D cells were stimulated with anti-Ly49D (αLy49D, 1 μg/10^6 cells) and cross-linker or anti-Ly49G2 (αLy49G2, 1 μg/10^6 cells) and cross-linker for the indicated times and lysed, and phospho-p44/42 MAP kinase (p44/42 MAPK) was detected in whole cell lysates (100 μg/lane) by SDS-PAGE followed by immunoblotting with phospho-p44/42 MAP kinase specific antibodies (top). Filters were stripped with SDS and reprobed with pan anti-p44/42 MAP kinase to confirm equal loading (bottom).

**FIG. 4.** Ly49D-induced tyrosine phosphorylation of PLCγ1 and calcium mobilization. A, tyrosine phosphorylation of PLCγ1. RNK-D cells (10^7/point) were stimulated as in Fig. 3B, and lysates were immunoprecipitated with anti-PLCγ1 (left and middle lanes) or control antibody (right lane). Immunoprecipitates were resolved by SDS-PAGE and immunoblotted with anti-phosphotyrosine (top). Filters were stripped with SDS and then reprobed with anti-PLCγ1 (bottom). B, Ly49D-induced calcium mobilization. RNK-D were loaded with the calcium-sensitive dye Fluo-3 as described under “Experimental Procedures” and then stimulated with anti-Ly49G2 (10 μg/ml) (solid trace) or anti-Ly49D (10 μg/ml) (dashed trace) at the first event arrow. 30 s later (event arrow 2), the antibodies were cross-linked using rabbit anti-rat Ig (10 μg/ml). Intracellular calcium was monitored using a flow cytometer and is expressed as the percentage of cells responding to the stimulus over time.

bllotting demonstrated tyrosine phosphorylation of PLCγ1, suggesting that this protein represents the 130-kDa band seen in whole cell lysates (Fig. 4A). We have previously reported that cross-linking Ly49D in the murine mastocytoma P815 results in DAP12 phosphorylation and calcium mobilization (24). However, calcium mobilization by a DAP12-coupled receptor has never been demonstrated in murine NK cells. Therefore, we assessed the effects of Ly49D cross-linking on [Ca^{2+}]_i of RNK-D. These data demonstrate that cross-linking of Ly49D results in a vigorous increase in [Ca^{2+}]_i in 70–80% of the RNK-D cells (Fig. 4B). Importantly, addition of antibody against Ly49G2 with cross-linker had no effect on [Ca^{2+}].
phosphorylated 28–32-kDa complex. The specificity of this interaction is demonstrated by the lack of 28–32-kDa complex binding to an unrelated tyrosine kinase SH2 domain (Chk-SH2). Although the 28–32-kDa complex exhibited the properties of DAP12 homodimers, these proteins may be different from the Ly49D associated polypeptides. To demonstrate that these proteins were associated with Ly49D, we stimulated RNK-D cells with pervanadate and immunoprecipitated the Ly49D receptor complex. After washing, the proteins within the complex were eluted using SDS and then diluted and passed over various GST fusion proteins. This study demonstrated that DAP12 eluted from the Ly49D receptor immunoprecipitations was bound by the Syk SH2 domains but not by GST alone or by the unrelated SH2 domain (Fig. 5B).

The binding of DAP12 to the Syk SH2 domain suggests that Syk may be activated following Ly49D ligation. Many laboratories have shown that the state of activation of Syk closely parallels its tyrosine phosphorylation (39–42), so we stimulated RNK-D cells with anti-Ly49D, immunoprecipitated Syk, and blotted with anti-phosphotyrosine. These data clearly show that F(ab)2 fragments of anti-Ly49D are capable of inducing tyrosine phosphorylation of Syk in RNK-D cells (Fig. 6A). Kinetic experiments confirmed that 1 min of stimulation resulted in peak phosphorylation of Syk in these cells (data not shown).

Although no role for Zap-70 in NK lytic activity has been established, NK cells express this kinase (35, 43). Therefore, we immunoprecipitated Zap-70 and Syk sequentially from RNK-D lysates after stimulation with anti-Ly49D. Immunoblotting of these immunoprecipitates demonstrated marked phosphorylation of Syk with only a minimal increase in Zap-70 tyrosine phosphorylation (Fig. 6B). To more directly assay the activation status of Syk and Zap-70, we stimulated RNK-D with anti-Ly49D, immunoprecipitated Syk or Zap-70, and, after washing, subjected these immunoprecipitates to in vitro kinase assays using 32P-labeled ATP and the cytoplasmic domain of the erythrocyte band 3 protein as an exogenous substrate. Although basal levels of kinase activity was detected in both Syk and Zap-70 immunoprecipitations, Ly49D/DAP12 engagement caused an increase only in Syk catalytic activity. These findings, together with our phosphotyrosine data, confirm that engagement of Ly49D/DAP12 leads to strong activation of Syk but only weak activation of Zap-70 (Fig. 6C).

To further define the role of Syk and/or Zap-70 in DAP12-mediated signal transduction, we used the vaccinia expression system to transiently express Syk lacking a catalytic domain (Syk-T) or catalytically inactive Myc-tagged Zap-70 (Myc-Zap-70K369R). Previous studies in NK cells have shown catalytically inactive kinases to be nearly as effective as truncated enzymes in the suppression of NK cell function (35). An anti-Syk antibody that cross-reacts with Zap-70 was used to confirm the expression of Syk-T and Zap-70K369R (Fig. 7B). The WR’ strain or vaccinia carrying the empty expression vector, pSC65, was used as controls and we assayed Ly49D-mediated calcium mobilization as described above. These data show that Ly49D-mediated increases in [Ca2+]i, are completely intact after infection with control vaccinia or Myc-Zap-70K369R (Fig. 7A). Infection with Syk-T, however, almost completely blocked the Ly49D/DAP12-mediated increases in [Ca2+]i. Thus, Syk is essential to the Ly49D/DAP12 signaling pathway. Moreover, Western blotting demonstrated levels of Zap-70 in RNK-D comparable to those seen in Jurkat T cells, indicating that the failure of Ly49D/DAP12 to activate Zap-70 is not due to a lack of this enzyme (data not shown).

Using this approach, we have also determined that F(ab)2 fragments of anti-Ly49D are sufficient to cause increases in [Ca2+]i, whereas heat-aggregated rabbit and/or rat immunoglobulin does not (data not shown). These findings rule out the possible involvement of RNK-16 Fc receptor in the response to Ly49D cross-linking. Several additional clones of RNK-16 that express Ly49D were tested with similar results (data not shown).

**Fig. 5.** Ly49D-activated DAP12 binds the Syk SH2 domains. A. anti-Ly49D-induced binding of phosphoproteins to the Syk SH2 domains. RNK-D (10^7/lane) were stimulated with cross-linked anti-Ly49D (αLy49D) or anti-Ly49G2 (αLy49G2) as in Fig. 3B. Lysates were then incubated with GST fusion proteins of the Chk SH2 domain or the Syk SH2 domains. After washing, proteins bound to the GST fusion proteins were eluted with SDS sample buffer, resolved by nonreducing SDS-PAGE, immunoblotted, and probed with anti-phosphotyrosine. B. DAP12 eluted from the Ly49D complex binds Syk SH2 domains. RNK-D cells were stimulated with pervanadate, lysed, and immunoprecipitated with anti-Ly49D. After washing, bound proteins were eluted with 0.1% SDS, diluted, and incubated with the indicated fusion proteins (1.3 x 10^7 cell equivalents/lane). After a second round of washing, bound proteins were eluted in SDS sample buffer, resolved by nonreducing SDS-PAGE, immunoblotted, and probed with anti-phosphotyrosine.

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**A.**

- **Stimulation:** αLy49G, αLy49D
- **GST:** Chk-SH2, Syk-SH2, GST-SH2
- **kDa:** 19, 21, 27, 31
- **I.B. Phosphotyrosine**

**B.**

- **GST:** GST, Syk-sgk, Chk-sgk, Chk-sgk
- **kDa:** 30
- **I.B. Phosphotyrosine**

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**DAP12 Signal Transduction in NK Cells**

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Here, for the first time, we report details of the DAP12 signal transduction pathway. We demonstrate the ability of the DAP12-coupled receptor, Ly49D, to induce tyrosine phosphorylation of multiple substrates, including PLC\(\gamma_1\), c-Cbl, p44/42 Erk, and Syk in RNK-D cells. Moreover, we demonstrate the preferential use of Syk versus Zap-70 by this DAP12-coupled receptor, and we show a requirement for Syk but not Zap-70 in Ly49D-mediated calcium mobilization in these cells.

DISCUSSION

The strong dependence of Ly49D on Syk activation was intriguing. Murine NK cells, including RNK-16, express both Syk and Zap-70. In fact, direct immunoblotting of Zap-70 showed that RNK-D have as much Zap-70 as Jurkat T cells (data not shown). Fc receptor cross-linking in human NK cells results in the activation of both Syk and Zap-70 (35). Moreover, phosphopeptides derived from the human DAP-12 ITAM bind both Syk and Zap-70, implying that both would be activated by receptor engagement (26). In fact, peptide sequence analysis...
demonstrates that the DAP12 ITAM is more similar to the membrane distal ITAM of TCRζ, a chain best known for mediating the activation of Zap-70, than to the ITAMs of FcεRIγ, TCRε, or TCRζ.\(^5\)

The preferential use of Syk by the Ly49D/DAP12 complex is, however, consistent with the requirement for multiple ITAMs within a receptor chain for efficient activation of Zap-70. Whereas Syk catalytic activity is enhanced following binding of phosphopeptides containing a single ITAM, the activity of Zap-70 is not (44, 45). Zap-70 activation requires the binding of a phosphopeptide containing multiple ITAMs, suggesting that in addition to occupancy of its SH2 domains, this kinase may require clustering and/or intermolecular phosphorylation for complete activation (46, 47). The signal transduction pathways used by the murine Fc receptor are consistent with this model. Although murine NK cells express both TCR and FcεRIγ, their Fc receptors utilize FcεRI homodimers exclusively (48). Consequently, antibody-dependent cellular cytotoxicity mediated by these Fc receptors is fully intact in mice lacking Zap-70 (49).

Our finding that DAP12, a polypeptide containing only one ITAM per molecule, efficiently activates Syk but not Zap-70 adds further support to this model of differential utilization of Syk-family kinases.

The utilization of Syk versus Zap-70 by the Ly49D/DAP12 complex fits with the expression of DAP12 in cells other than T cells and NK cells (24, 26). Monocytes do not express Zap-70 but do express DAP12 and Syk (26, 50). The receptors to which DAP12 couples in these cells are currently unknown, but several groups of Ig superfamily receptors with homology to the KIRs have been described in the monocyte lineage. Both the ILT family in the humans and the PIR family in mice include putatively positive signaling receptors (14, 18). These proteins lack the inhibitory domains of KIR-like receptors, and they contain charged residues in their transmembrane domains. The possibility that these receptors physically and/or functionally couple to DAP12 is currently under investigation. Regardless of the receptors coupled to DAP12 in monocytes, our data suggest that Syk may be critical to their function.

Cbl functions as a multivalent adaptor molecule in a variety of immune receptor systems, where it interacts with proteins such as Grb-2, the p85 regulatory subunit of phosphatidylinositol 3-kinase (PI3K), and various protein-tyrosine kinases (42, 51–58). Simulation of CD16 in human NK cells results in potent tyrosine phosphorylation of Cbl and the generation of Cbl/p85 PI3K complexes, implying that Cbl may be involved in CD16-mediated cytolytic activity (51, 59). In addition, interleukin-2, a potent activator of NK cytolytic activity, induces the tyrosine phosphorylation of Cbl and the SH2/3 containing adaptor Crk-L (55). Exactly how Cbl, Grb-2, and/or Crk-L complexes control the cytolytic events of NK cells is unknown. A likely pathway, however, is via the control of PI3K activity. PI3K enhances calcium mobilization by producing ligand for pleckstrin homology domains of protein-tyrosine kinases of the Tec-family (60).

In NK cells, strong, sustained calcium mobilization is required for optimal granule exocytosis and cytotoxicity (43). Therefore, Ly49D-induced phosphorylation of Cbl may represent a means of controlling PI3K activation, calcium mobilization, and ultimately Ly49D-mediated cytotoxicity.

c-Cbl may also exert its effects at the level of the Syk family kinases. In mast cells, Cbl reduces receptor-mediated activation of Syk (42). Overexpression of wild type Cbl, but not the 70Z/3 mutant, effectively reduces both receptor-mediated Syk tyrosine phosphorylation and catalytic activity, resulting in decreased FcεRI-mediated serotonin release. These observations suggest that Cbl may function in NK cells to attenuate Syk activity. It is worth noting, however, that Syk activation by the Ly49D/DAP12 complex is vigorous even in the presence of extensive phosphorylation of Cbl. Regardless, the possibility that Cbl may negatively regulate the signal transduction cascade of DAP12-mediated receptors is currently under investigation.

In human NK cells, there is a great deal of evidence documenting the outcome of the ligation of TCRζ and/or FcεRIγ-coupled receptors. The best characterized of these receptors is the low affinity receptor for IgG (FcγRIIA, CD16). Ligation of CD16 leads to tyrosine phosphorylation of multiple cellular substrates, including Zap-70, PLCγ, Lck, Cbl, and the TCRζ chain (34, 51, 61, 62). In addition, this activation pathway leads to significant increases in [Ca\(^{2+}\)], a response critical for granule exocytosis and CD16-mediated killing (43). Like CD16, Ly49D coupled through DAP12 is capable of delivering signals that result in target cell lysis, suggesting that the outcome of a TCRζ-mediated pathway and that of a DAP12-mediated pathway are similar.

The data presented here demonstrate that Ly49D/DAP12 signaling has some similarities with signals delivered through TCRζ- and/or FcRIγ-coupled receptors. However, Ly49D, Ly49H, and their functional homologues human KAR and CD94/NKG2C are the only receptors known to use DAP12 (25, 26, 63). So why would Ly49D and noninhibitory KIRs choose to use DAP12 over other chains? One possibility is that a DAP12-mediated response will be qualitatively different from that of TCRζ- or FcRIγ-coupled receptors. To date, only two functional outcomes have been associated with Ly49D ligation: cytotoxicity via reverse antibody-dependent cellular cytotoxicity, and apoptosis. Both of these activities can be mediated by CD16 as well (19). In addition to cytotoxicity and apoptosis, CD16 ligation leads to transcription and translation of cytokine genes, including interferon-γ and granulocyte-macrophage colony-stimulating factor (1). The stimulation of these cytokines through DAP12-coupled Ly49D or KAR has not yet been examined. More definition of the outcomes of DAP12-coupled receptors will help in the elucidation of functional differences between this pathway, TCRζ- or FcRIγ-mediated pathways, and others. The expression of DAP12 in myeloid cells, a population that does not express Ly49 or KAR, indicates that other DAP12-coupled receptors will be identified.

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\(^5\) D. W. McVicar, unpublished observations.
DAP12-mediated Signal Transduction in Natural Killer Cells: A DOMINANT ROLE FOR THE Syk PROTEIN-TYROSINE KINASE
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