Brief Definitive Report

Physical and Functional Association of p56^{lck} with FcγRIIIA (CD16) in Natural Killer Cells

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Summary

The transmembrane receptor for immunoglobulin G immune complexes on natural killer (NK) cells and macrophages, FcγRIIIA (CD16), mediates cellular activation through a tyrosine kinase–dependent pathway. We show that FcγRIII crosslinking results in activation of the *src*-related kinase p56^{lck} in NK cells and demonstrate a physical association of p56^{lck} with FcγRIIIA in immunoprecipitates from NK cells obtained using anti-FcγRIII antibodies or immune complexes. Our studies show that the ζ chain, the signal transducing subunit of FcγRIIIA and of T cell receptor, associates with p56^{lck} and, in NK cells, is a substrate for this kinase. Such direct association of p56^{lck} with the ζ subunit was confirmed by demonstrating the interaction in heterologous cells transfected with cDNA expressing p56^{lck} and ζ. Our findings demonstrate both functional and physical association of p56^{lck} with FcγRIIIA, through direct interaction of the kinase with the ζ and/or the γ signal transducer subunits of the receptor. These data suggest a possible mechanism by which activation via FcγRIIIA occurs.

The receptor for the Fc fragment of IgG (FcγRIIIA) (CD16) binds IgG immune complexes with low affinity and mediates the antibody-dependent cytotoxicity of NK cells (1). This receptor is a multimeric complex composed of three functionally and biochemically distinct proteins: IIIAα, a 254-amino acid transmembrane glycoprotein containing the extracellular ligand binding domain, and IIIAγ and IIIAζ, membrane-spanning subunits responsible for both assembly and signal transduction (1). The γ and ζ chains are members of a family of homologous proteins present as homo- or heterodimers, first described as subunits of the high affinity Fc receptor for IgE, FcεRI, and of the TCR–CD3 complex (2). Ligand binding and crosslinking of FcγRIII induce NK cell activation with the release of intracytoplasmic granules and upregulation of genes encoding surface activation molecules and cytokines relevant to NK cell biology and functions (3, 4). The early biochemical events induced in NK cells upon engagement of FcγRIII include tyrosine phosphorylation of intracellular substrates (ζ and γ chains, phospholipase C [PLC]-γ1 and PLC-γ2, phosphatidylinositol-3 [PI-3] kinase), hydrolysis of membrane phosphoinositides (PIP_2), increased intracellular calcium concentration ($[Ca^{2+}]_i$), and activation of PI-3 kinase (5–7, and Kanakaraj, P., and B. Perussia, manuscript in preparation). The observation that treatment of NK cells with tyrosine kinase inhibitors blocks both FcγRIII-induced hydrolysis of membrane PIP_2 and subsequent increase in $[Ca^{2+}]_i$ (5) and later activation events (8) has indicated the involvement of a tyrosine kinase(s) in initiating and/or mediating FcγRIII-induced signal transduction events. No intrinsic enzymatic activities have been described for FcγRIII that could account for its ability to activate cells upon crosslinking. Results from experiments with chimeric molecules containing ζ or γ cytoplasmic domains linked with extracellular domains of heterologous molecules support the hypothesis that a nonreceptor kinase(s) associates with FcγRIII possibly via the γ or ζ subunits (9–13). In cells expressing these chimeric molecules, stimulation of the extracellular domains results in signal transduction. We set out to determine how FcγRIII stimulates protein tyrosine phosphorylation in NK cells by testing the hypothesis that FcγRIII interacts directly with protein tyrosine kinases in these cells.

Materials and Methods

NK Cell Preparations and Stimulation. PBL, obtained by density gradient centrifugation of venous peripheral blood from healthy donors, were cultured with 30-Gy irradiated RPMI-8866 B lymphoblastoid cells (14). NK cells were purified from the 10-d cocultures by negative selection after sensitization with anti-CD3 (OKT3), anti-CD5 (B36.1), and anti-CD14 (B52.1) mAb and indirect antiglobulin rosetting (14). The purity of each preparation (>95% $CD16^+/CD56^+/CD3^-$ cells) was confirmed in indirect immunofluorescence (flow cytometry) using a panel of mAb.

Transfections. COS cells were cultured in modified Eagle's medium containing 10% FCS. Mouse *fyn* cDNA (15) (from R.

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Perlmutter, University of Washington, Seattle, WA), human *yes* cDNA (16) (from T. Yamamoto, Tokyo University, Tokyo, Japan, and J. Sukegawa, Rockefeller University, New York), and human *lck* cDNA (17) (from T. Mak, Ontario Cancer Institute, Toronto, Canada) were cloned into the pCEXV-3 vector. DNA (15 μg each DNA/60-mm dish) was transfected into COS cells using the calcium-phosphate method (18) in the presence of 100 μM chloroquine. The IIIA/ζ construct contained the extracellular region of FcγRIIIA and the transmembrane and cytoplasmic regions of human ζ chain (19). Cells were analyzed 2 d after transfection.

Monoclonal and Polyclonal Antibodies and IgG. The mAb 3G8 (IgG1, anti-CD16) and B159.5 (IgG1, anti-CD56) have been previously described (5, 14); anti-*yes* (20) and anti-p56^{lck} mAb (21) were from T. Yamamoto and J. Sukegawa, and Y. Koga (Kyushu University, Fukoaka, Japan), respectively. Polyclonal rabbit antisera to *fyn* and *yes* were purchased from Upstate Biotechnology Inc. (Lake Placid, NY) and provided by T. Yamamoto and J. Sukegawa, respectively. The anti-p56^{lck} polyclonal serum was produced in rabbits immunized with a synthetic peptide corresponding to amino acids 39-64 of the murine p56^{lck} sequence (5). Goat anti-rabbit and sheep anti-mouse Ig antibodies conjugated to horse radish peroxidase (HRP) were from Amersham Corp. (Arlington Heights, IL). The goat anti-mouse Ig (GaMIg) used for precipitation was produced in our laboratory. IgG from the rabbit anti-ζ and anti-γ chain sera (prepared at Sloan- Kettering) were purified on a protein A-Sepharose column. Human IgG (Cohn fraction IV; Sigma Chemical Co., St. Louis, MO), and their F(ab')$_2$ fragments prepared by pepsin digestion, were heat aggregated (63°C, 30 min).

Immunoprecipitation and Western Blotting. The indicated numbers of the different cell types were lysed in: (*a*) 1% digitonin, 150 mM NaCl, 20 mM Tris, pH 8, 1 mM PMSF, 10 μg/ml aprotinin, and 10 μg/ml leupeptin; (*b*) 2% NP-40, 150 mM NaCl, 20 mM Tris, 2 mM PMSF, and 25 μg/ml each aprotinin, leupeptin, antipain; or (*c*) 3% NP-40, 50 mM Tris, pH 8, 150 mM NaCl, 50 mM NaF, 10 μM molibrate, 0.2 mM Na vanadate, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 2.5 μg/ml antipain, and 0.1 mM PMSF, as indicated. Postnuclear supernatants were precleared (30 min–15 h) with: (*a*) CNBr-activated/quenched Sepharose (Pharmacia Fine Chemicals, Uppsala, Sweden); (*b*) protein G–Sepharose to which GaMIg had been immobilized using dimethylpimelidate; or (*c*) protein A–Sepharose, as indicated. Immunoprecipitations (2 h, 4°C) were performed with the indicated antibodies or with immune complexes and the appropriate controls, as indicated. The immunoprecipitates were washed five to six times with the respective lysis buffer, Sepharose-bound complexes were eluted into sample buffer containing 2% SDS and 1% 2-ME, as indicated. Postnuclear cell lysates or immunoprecipitates were separated in reducing SDS-PAGE, transferred to Immobilon-P sheet or nitrocellulose membrane and subjected to Western immunoblotting (5) using the indicated antibodies. Filters were developed using a goat anti-rabbit or a sheep anti-mouse Ig antibody conjugated to HRP and enhanced chemiluminescence (ECL) (Amersham Corp.), or ^{125}I-labeled anti-p56^{lck} mAb.

In Vitro Kinase Assays. *src*-related kinases were immunoprecipitated from postnuclear supernatants of NK cells solubilized in 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 150 mM NaCl, 10 mM Tris, 5 mM EDTA, or from the NP-40 eluate of FcγRIII immunoprecipitated from NK cells lysed in 1% digitonin, using protein A–Sepharose (rabbit polyclonal antisera) or protein A–Sepharose coated with anti–mouse Ig (anti-*src* mAb). Precipitates were washed twice with lysis buffer and once with 100 mM NaCl, 10 mM Tris, pH 7.5, 5 mM $MnCl_2$. The kinase reaction was performed (15 min, 20°C) in 30-μl assay buffer in the presence of 1 μM ATP and 10 μCi γ-[^{32}P]ATP (sp act 4,500 Ci/mmol, ICN Biomedicals, Costa Mesa, CA) without or with 1 μg enolase (5, 22). The proteins were electrophoresed in reducing SDS-PAGE, as indicated.

Results and Discussion

Expression of *src*-related kinases was analyzed in homogeneous NK cell populations obtained from short-term (10 d) cocultures of PBL with irradiated RPMI-8866 B lymphoblastoid cells (14). These NK cell preparations are >95% homogeneous and have phenotypic and functional properties identical to those of freshly isolated NK cells except that they express late activation antigens and are more readily activated (3–6, 14). These NK cells expressed several *src*-related tyrosine kinases, including p53^{lyn} and p56^{lyn}, p56^{lck}, p60^{fyn}, and p62^{yes}, as measured by kinase autophosphorylation in immune-complex protein kinase assays (Fig. 1 *A*). Upon stimulation of FcγRIII with the anti-FcγRIII mAb 3G8, we detected a rapid activation of at least one of the *src*-related kinases, p56^{lck}, as analyzed by in vitro kinase assay on p56^{lck} immunoprecipitates isolated from cells after receptor stimulation (Fig. 1 *B*). Increased p56^{lck} autophosphorylation and phosphorylation of the exogenous substrate enolase were detected as early as 10 s after receptor stimulation. These results are consistent with those we previously reported using CD3$^-$

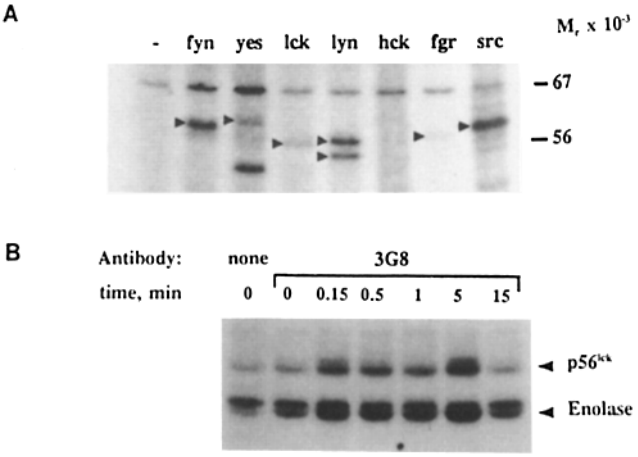

Figure 1. Expression and activation of p56^{lck} in NK cells. (*A*) The indicated *src*-related kinases (►) were immunoprecipitated from postnuclear supernatants of NK cells (10 × 10^6 cells/sample) lysed in 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 150 mM NaCl, 10 mM Tris, 5 mM EDTA using protein A–Sepharose (for rabbit polyclonal antisera) or protein A–Sepharose coated with anti–mouse Ig (anti-*src* mAb). The products of in vitro kinase assays were analyzed in reducing 7.5% SDS-PAGE. (*B*) NK cells (5 × 10^6/ml RPMI 1640 containing 10% fetal bovine serum) were incubated for the indicated times with anti-CD16 mAb 3G8 (5 μg/ml). After incubation and lysis in 1% NP-40, p56^{lck} was precipitated from the postnuclear supernatants. Kinase assay was performed after addition of 1 μg enolase and the product of the kinase assay was analyzed in reducing 7.5% SDS-PAGE. No increased phosphorylation of p56^{lck} or enolase was detected in p56^{lck} immunoprecipitates from NK cells stimulated with anti-CD56 mAb B159.5 used as control (data not shown).

Jurkat cells expressing transfected FcγRIIIAα chain in association with endogenous ζ (5), and indicate that $p56^{kk}$ is functionally associated with FcγRIII in primary NK cells.

To determine how $p56^{kk}$ is stimulated upon FcγIII crosslinking, we precipitated the receptor from digitonin lysates of NK cells and assayed tyrosine kinase activity in the immunoprecipitates. Tyrosine kinase activity was coprecipitated with FcγRIII and resulted in the phosphorylation of the ζ chain subunit. Phosphorylated ζ chain was preferentially observed within the FcγRIII immunoprecipitates when reprecipitated with anti-$p56^{kk}$ or anti-ζ antibodies (Fig. 2 *A*). These data clearly indicate that ζ is a substrate for $p56^{kk}$-dependent tyrosine phosphorylation and strongly suggest that $p56^{kk}$ coprecipitates with FcγRIII.

To determine directly whether $p56^{kk}$ and FcγRIII are physically associated, anti-$p56^{kk}$ immunoblotting was performed on immunoprecipitates isolated from NK cells using FcγRIII ligands. NK cells were solubilized in 1% digitonin to preserve the association of FcγRIIIA subunits. $p56^{kk}$ was specifically detected in immunoprecipitates isolated with either antireceptor antibody (3G8) (Fig. 2 *B*, *a*) or the natural ligand immune complexes (heat-aggregated IgG) (Fig. 2 *B*, *b*). Aggregates lacking Fc did not yield $p56^{kk}$ complexes, and isotype-matched anti-CD56 antibodies yielded significantly lower amounts of them. Western blot analysis with an anti-CD16 rabbit polyclonal antibody confirmed that both FcγRIIIAα and ζ chain are present in the 3G8 and the aggregated IgG, but not in the $F(ab')_2$ precipitates (data not shown). The stoichiometry of the FcγRIII-$p56^{kk}$ association have been reported between TCR-ζ and *fyn* in T cells (22) and may reflect the instability of receptor subunits upon detergent extraction. Increased $p56^{kk}$-FcγRIII association could not be demonstrated upon receptor crosslinking (data not shown).

To directly assess which FcγRIII subunit is responsible for the association with $p56^{kk}$, anti-$p56^{kk}$ immunoblotting experiments were performed on immunoprecipitates isolated with anti-ζ polyclonal antisera. NK cells were lysed in 2% NP-40 to reduce possible nonspecific precipitation of $p56^{kk}$. Using a large number of NK cells and a sensitive detection system (ECL) a small fraction of total cellular $p56^{kk}$ was detected in the anti-ζ precipitates (Fig. 2 *B*, *c*; compare anti-$p56^{kk}$ precipitates with anti-ζ). In addition, a phosphoprotein with molecular weight similar to phospho-ζ (~21 kD) was detected in the respective $p56^{kk}$ immunoprecipitates isolated from digitonin- and, to a lesser extent, NP-40–solubilized NK cells, as analyzed by in vitro kinase assays (data not shown).

To confirm that $p56^{kk}$ associates with ζ and to determine whether this association is direct or is, in part, mediated by additional proteins, experiments were performed using COS cells cotransfected with various *src* family-related kinase cDNA (mouse *fyn*, human *yes*, and human *lck*) and a cDNA encoding a chimeric protein composed of the extracellular region of FcγRIIIAα and the transmembrane and cytoplasmic regions of human ζ (IIIA/ζ). Transfected cells were lysed in

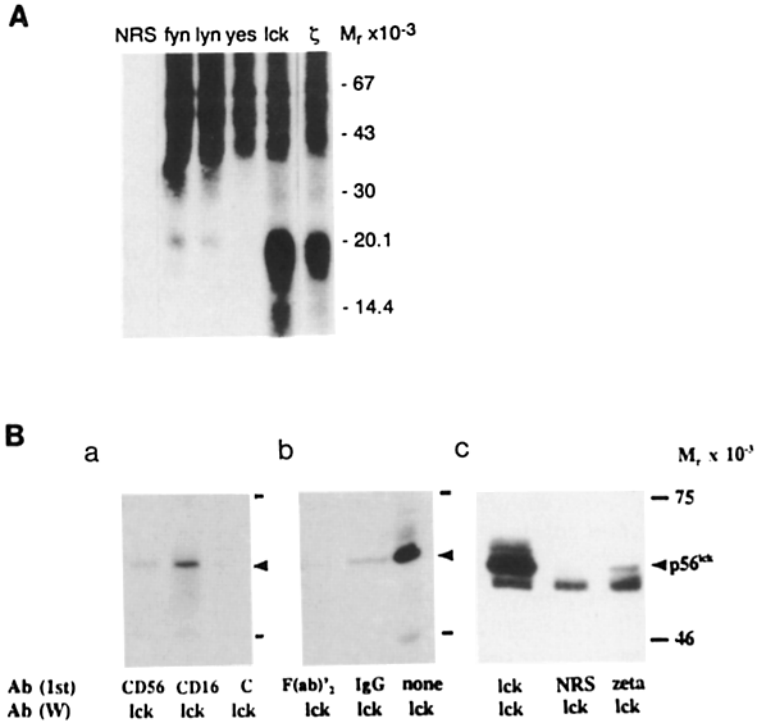

Figure 2. Association of $p56^{kk}$ with FcγRIIIA in NK cells. (*A*) FcγRIII was precipitated from NK cells (10×10^6 cells per precipitation) lysed in 1% digitonin, 150 mM NaCl, 20 mM Tris, pH 8, 1 mM PMSF, 10 μg/ml aprotinin, 10 μg/ml leupeptin using anti-CD16 mAb 3G8, and in vitro kinase assay was performed on the immunoprecipitate. Kinase products were eluted from the beads (1% NP-40, 1 h) and the indicated proteins were precipitated using specific antibodies or normal rabbit serum as control. Immunoprecipitates were analyzed in reducing 13% SDS-PAGE. (*B*) In *a*, postnuclear supernatants from NK cells (50×10^6 per precipitation), lysed as in A, were precleared and precipitated [*Ab (1st)*] with anti-CD16 mAb 3G8 or anti-CD56 mAb B159.5 coupled to goat anti-mouse Ig protein G–Sepharose. Goat anti-mouse Ig protein G–Sepharose was used as control (*C*). Immune complexes were washed and proteins analyzed in 7.5% reducing SDS-PAGE and Western blotting [*Ab (W)*] using anti-$p56^{kk}$ and ^{125}I-labeled goat anti-rabbit IgG. In *b*, postnuclear supernatants from NK cells (35×10^6 cells per precipitation), lysed in digitonin buffer as in *a*, were precleared and precipitated with heat-aggregated IgG-Sepharose or $F(ab')_2$-Sepharose (control) for 5 h. Complexes were washed with lysis buffer and proteins analyzed on 7.5% reducing SDS-PAGE followed by Western blotting using anti-$p56^{kk}$ mAb, HRP-sheep anti-mouse Ig, and ECL. (*None*, lysate from ~10^6 cell equivalents, no precipitation). In *c*, postnuclear supernatants from NK cells (30×10^6 cells/precipitation), lysed in 2% NP-40, were precleared and incubated with rabbit anti-ζ, anti-$p56^{kk}$, nonimmune serum) followed by protein A–Sepharose precipitation. Beads were washed with lysis buffer and analyzed in 7.5% reducing SDS-PAGE followed by Western blotting for $p56^{kk}$ as in *b*. The lower bands in *c* represent rabbit IgG used for precipitation.

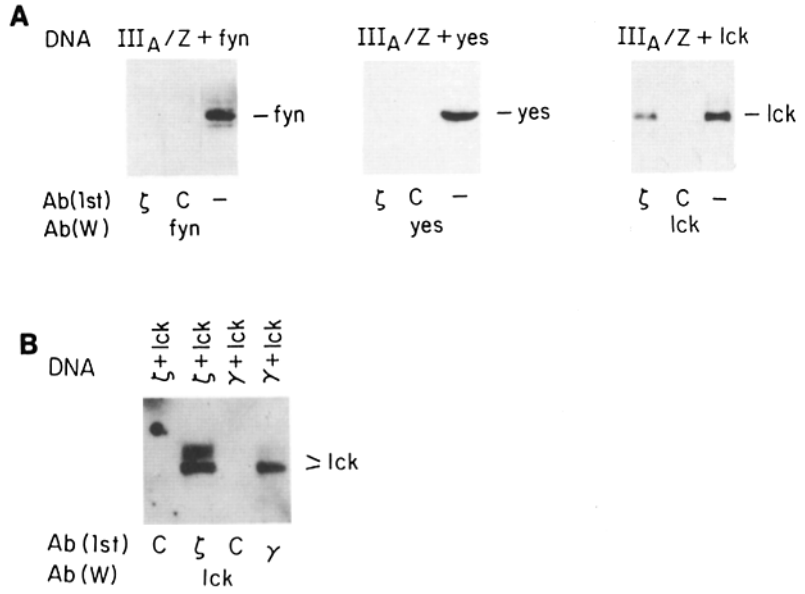

Figure 3. Association of p56^{lk} with γ and ζ chains. 2 d after transfection with the cDNA indicated at the top, COS cells were solubilized in lysis buffer (3% NP-40, 50 mM Tris pH 8, 150 mM NaCl, 50 mM NaF, 10 mM molibrate, 0.2 mM Na vanadate, 1 μg/ml aprotinin, 10 μg/ml leupeptin, 2.5 μg/ml antipain, 0.1 mM PMSF). Cell lysates were precleared with Sepharose, incubated with the indicated Ab [*Ab (1st)*] coupled-Sepharose for 2 h and washed with lysis buffer five times. Antibodies against γ and ζ chains (19) and control antibodies were purified by protein A–Sepharose and directly coupled to CNBr-activated Sepharose. Sepharose-bound complexes were eluted into sample buffer containing 2% SDS and 1% 2-ME, separated in reducing 8% SDS-PAGE, and transferred to Immobilon-P sheet or nitrocellulose membrane. Anti-*fyn*, -*yes*, and -p56^{lk} antibodies were used for detection in Western blotting, as indicated. Filters were developed using a goat anti-rabbit or a sheep anti-mouse Ig antibody conjugated to HRP and ECL (*A*) or ^{125}I-labeled anti-p56^{lk} mAb (*B*).

3% NP-40, immunoprecipitates were collected using either anti-ζ antibody-coupled Sepharose or control antibody–Sepharose and subjected to immunoblotting with the respective anti-*src*–related kinase antibody. Coprecipitation of IIIA/ζ and p56^{lk}, but not of *fyn*, *yes* (Fig. 3 *A*) or *src* (data not shown), was detected. Similar experiments in COS cells cotransfected with p56^{lk} and γ cDNAs revealed association of these two proteins, although to levels lower than those observed with ζ (Fig. 3 *B*).

Our results indicate that the *src*-related kinase p56^{lk} associates both functionally and physically with the FcγRIIIA complex on NK cells. This association appears to be mediated in part via the ζ chain. The results of ζ/γ/p56^{lk} cotransfection experiments in COS cells prove that p56^{lk} and either ζ or γ subunits can associate via direct interaction. Although the molecular basis of the association remains to be determined, it is likely to depend, in part, on the antigen receptor homology 1 motifs (ARH1) of ζ/γ, which are conserved sequences ([Asp or Glu]-X$_7$-[Asp or Glu]-Tyr-X$_3$-Leu-X$_7$-Tyr-X$_2$-[Leu or Ile]) found in many receptor signal transducing chains, including TCR ζ, η, γ, δ, and ε, FcεRI β and γ chains, B cell antigen receptor chains Ig-α (mb1) and Ig-β (B29), and human FcγRIIA (23, 24). Evidence to support the contention that these sequences mediate coupling of receptors to signalling pathways has been provided for the B cell antigen receptor chains Ig-α and Ig-β (25). Differential binding patterns of the ARH1 regions in these proteins for cytoplasmic effectors were observed, indicating that the presence of an ARH1 motif is insufficient for binding cytoplasmic effector molecules but that additional chain-specific residues determine binding specificity and a single motif can bind more than one effector molecule (26). Our preliminary data indicate that the p56^{lk}–ζ interaction depends on the presence of ARH1 motifs in ζ, and deletion of one or more of them results in a proportionally decreased association (data not shown). This may also explain, in part, the detection of lower levels of p56^{lk} associated with γ chain (a single ARH1 motif) as compared to ζ (three ARH1 motifs). The p56^{lk} domain involved in this interaction has not been defined. It is likely to differ from that involved in the interaction between p56^{lk} and CD4, shown to depend on the NH_2-terminal sequence of this molecule (26–28), because no sequence homology is found between the ARH1 motif and CD4.

Functional interaction between p56^{lk} and the ζ subunit is supported by observations in T cells. Elegant studies using p56^{lk} deficient cell lines (which endogenously express *fyn*) strongly support a role for p56^{lk} in signal transduction via the TCR and in cell-mediated cytotoxic responses (29, 30). Cytotoxic functions are restored upon reexpression of p56^{lk} and, most interestingly in regard to NK cells, appear independent of CD4 or CD8 engagement (29, 30). Our cotransfection experiments in COS cells demonstrate a direct interaction of p56^{lk} and ζ/γ. However, additional proteins may be necessary to mediate optimal association or disassociation of these two molecules in primary cells and the situation in NK cells may be analogous to that observed in T cell lines. A 70-kD protein (ZAP-70) has been observed to associate with ζ in the Jurkat T cell line upon TCR/CD3 stimulation (31–33). Proteins of similar size are rapidly phosphorylated upon engagement of the B cell antigen receptor complex (p72^{syk}), the FcεRI complex (34, 35), and FcγRIII in NK cells (5, and our unpublished data). Although the role of these 70–72-kD proteins/kinases is unknown, they may function to stabilize the primary interaction of ARH1 containing subunits with *src*-related protein tyrosine kinases.

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