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Engagement of the natural killer cell IgG Fc receptor results in tyrosine phosphorylation of the ζ chain

(tyrosine kinase/signal transduction/T-cell antigen receptor/large granular lymphocytes)

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ABSTRACT **The ζ chain has emerged to be a key subunit of the T-cell antigen receptor with central roles not only in intracellular assembly of the multimeric receptor but also in mediating signal transduction events. This subunit is present in natural killer (NK) cells that lack the other subunits of the T-cell antigen receptor. In NK cells, the ζ chain appears to be associated with the NK Fc receptor [type 3 receptor for the Fc portion of IgG (FcγRIII or CD16)] and may be necessary for efficient cell surface expression of this receptor complex. In T cells, the ζ chain is a prominent substrate that becomes phosphorylated on tyrosine residues after occupancy of the TCR; ζ chain phosphorylation was in fact the first evidence that the TCR was coupled to a protein-tyrosine kinase as well as to inositol phospholipid hydrolysis. To determine if FcγRIII is coupled to a protein-tyrosine kinase in a manner analogous to the T-cell antigen receptor, we investigated ligand-dependent ζ-chain phosphorylation in NK cells. We observed that activation of NK cells with an anti-FcγRIII monoclonal antibody induced tyrosine phosphorylation of the ζ chain whereas other activating stimuli, such as the combination of phorbol ester and ionomycin or a lymphokine, interleukin 2, did not result in phosphorylation of this protein. Perturbation of FcγRIII by the more physiological stimulus, incubation of NK cells with antibody-coated target cells, also induced ζ-chain phosphorylation. Previous data have indicated that the NK-cell FcγRIII is coupled to inositol phospholipid hydrolysis. This present finding that FcγRIII is coupled to a protein-tyrosine kinase illustrates that there are significant similarities in the signaling pathways activated by FcγRIII in NK cells and the T-cell antigen receptor in T cells; the ζ chain is a common element that may serve as a coupling protein for both of these receptors.**

Natural killer (NK) cells are cytolytic effector lymphocytes that mediate major histocompatibility complex-independent killing of tumor- or virally infected-targets and are morphologically identifiable as large granular lymphocytes (1, 2). In addition, NK cells are capable of carrying out antibody-dependent cellular cytotoxicity, by virtue of the occupancy of their Fc receptors [type 3 receptor for the Fc portion of IgG (FcγRIII or CD16)] with an antibody directed against targets (2, 3). The molecular basis of recognition of target cells in the absence of antibody and the mechanisms by which induction of cytotoxicity is coupled to this recognition is poorly understood. Although the principal receptor(s) responsible for recognition of target cells by NK cells have yet to be delineated, it is clear that NK cells do not express on their cell surfaces either the T-cell antigen receptor (TCR) rearranged gene products or CD3 proteins. Anderson *et al.* (4) have recently shown that the ζ chain is expressed in $CD3^-$ NK cells. Previous studies in T cells have indicated that expression of the ζ chain at the plasma membrane requires association with other proteins (for review, see ref. 5). Lanier *et al.* (6) demonstrated that the ζ chain is associated with the FcγRIII in NK cells and is required for plasma membrane expression of this receptor. This role in surface expression may be analogous to the role played by the ζ chain in the multisubunit TCR where it appears to be uniquely required for conferring competency for transport of the receptor from the Golgi apparatus to the cell surface (5).

The TCR is comprised of polypeptides encoded by rearranging genes associated with the so-called invariant chains, which include the CD3 proteins (γ, δ, ε) and the ζ polypeptide (7–13). The proteins encoded by rearranging genes are responsible for recognition of ligand, whereas the invariant chains appear to be important not only for optimal surface expression of the TCR but also for mediating signal transduction. The ζ subunit is a critical component in this process. Indeed mutant T cells that lack the normal complement of receptors containing ζ homodimers and ζ–η heterodimers have impaired signaling capability (10, 14, 15). More recently, the importance of the ζ chain in receptor-mediated signaling has been demonstrated by expressing truncated mutants of the ζ chain; although surface expression of the TCR is normal in these cells, antigen-mediated signaling is dramatically impaired (16). Another feature of the ζ chain is that it is a substrate of the protein-tyrosine kinase (PTK) that is activated after ligand binding to the TCR (17–22); in murine T-cell hybridomas and in human peripheral blood T cells, the ζ chain undergoes multiple seemingly cooperative phosphorylations of its tyrosine residues upon perturbation of the TCR. The functional significance of ζ-chain phosphorylation in signal transduction has not been elucidated. Studies of TCR-dependent tyrosine phosphorylated substrates indicate that ζ-chain phosphorylation is not the earliest detectable phosphorylated species induced by receptor occupancy (23).

Because of the presence of the ζ chain in NK cells in association with FcγRIII, we sought to determine if this receptor, like the TCR, is coupled to a PTK. Specifically, we asked if the ζ chain serves as a substrate of a receptor-coupled PTK in NK cells. We observed that occupancy of the NK FCγRIII either by anti-receptor monoclonal antibody (mAb) or by antibody-coated target cells resulted in phosphorylation of the ζ chain. In contrast, activation of the cells with phorbol esters and ionomycin or interleukin 2 (IL-2) failed to induce this modification. This finding is consistent with a signaling pathway in which the FcγRIII is coupled to a nonreceptor PTK for which the ζ chain may serve as a substrate. Previous studies have indicated that perturbation of the FcγRIII results in inositol phospholipid metabolism (24). Thus it appears that the NK FcγRIII is strikingly similar to the TCR

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Abbreviations: mAb, monoclonal antibody; TCR, T cell receptor for antigen; PTK, protein tyrosine kinase; NK, natural killer; FcγRIII, type 3 receptor for the Fc portion of IgG also designated CD16; IL-2, interleukin 2.

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in that both receptors couple to at least two kinase pathways (PTK and protein kinase C) and the ζ chain may potentially subserve an analogous signal transducing role in both cell types.

MATERIALS AND METHODS

Reagents and Antibodies. Anti-CD16 mAb (3G8), anti-CD2 mAb (9.6), and anti-CD3 mAb (OKT3) were prepared as ascites fluid and were used at a final immunoglobulin concentration of ≈30 μg/ml. The anti-ζ peptide antiserum 387 was provided by David Orloff, National Institute of Child Health and Human Development (25). Affinity-purified anti-phosphotyrosine antibodies were provided by Lawrence Samelson, National Institute of Child Health and Human Development. The antibody used to coat tumor cells was a rabbit polyclonal antiserum prepared against Raji cells and was used at a dilution of 1:250. In separate experiments, this concentration was shown to be optimal for inducing cytolysis of Raji cells by large granular lymphocytes (data not shown).

Isolation of NK Cells and Cell Lines. NK cells and T lymphocytes were obtained from buffy coats of blood donated by normal volunteers, as described (26). Briefly, mononuclear cells were separated on a Ficoll/Hypaque gradient and were passed sequentially over plastic and nylon to remove adherent cells. The nonadherent cells were then fractionated on a seven-step discontinuous Percoll gradient ranging from 40 to 60%. NK cells were collected from the low-density fractions whereas T cells were collected from the high-density bottom fractions. NK cells were further depleted of contaminating T cells by rosette formation with sheep erythrocytes, as described. The resulting NK cells typically were found to be 80–90% large granular lymphocytes by histochemical staining and morphological analysis. These NK cell preparations were noted to be 70–75% $CD11b^+$, ≈82% $CD56^+$, ≈72% $CD16^+$, <2% $LeuM3^+$, and ≈5% $CD3^+$, as determined by flow cytometry. The T cells that contaminated the NK cell populations were demonstrated to be <1% $CD16^+$.

Phosphorylation Assay, Immunoprecipitation, and Electrophoresis. Isolated NK and T cells were washed three times with phosphate-free RPMI 1640 medium, resuspended in the same medium with 10% (vol/vol) dialyzed fetal calf serum at 1×10^7 cells per ml, and incubated with [^{32}P]orthophosphate (ICN) (1–2 mCi/ml; 1 Ci = 37 GBq) for 2 hr at 37°C. The cells (≈10^7 cells per condition) were then stimulated as indicated for 1 hr at 37°C. After stimulation, the cells were washed three times with ice-cold phosphate-buffered saline containing 1 mM EDTA, 10 mM sodium fluoride, and 0.4 mM sodium orthovanadate to inhibit phosphatases and lysed in 0.5% Triton X-100 containing 300 mM NaCl, 50 mM Tris·HCl (pH 7.6), 2 mM EDTA, 1 mM sodium orthovanadate, 10 mM sodium fluoride, leupeptin (10 μg/ml), aprotinin (10 μg/ml), and 2.5 μM *p*-nitrophenyl *p'*-guanidinobenzoate (lysis buffer). The lysates were immunoprecipitated with anti-ζ peptide antiserum or control antiserum bound to staphylococcal protein A-agarose. After washing and eluting the beads in nonreducing sample buffer, the samples were subjected to SDS/polyacrylamide gel electrophoresis (13% gel) in tubes. The tubes were then equilibrated in 0.5% dithiothreitol, overlaid on 13% slab gels, and electrophoresed in the second dimension. The gels were fixed, dried, and exposed to film for 2–5 days as indicated.

Anti-Phosphotyrosine Immunoblot Analysis. Approximately 1×10^7 isolated NK cells were stimulated or not with the anti-FcγRIII mAb for 1 hr at 37°C as indicated, washed with phosphate-buffered saline containing phosphatase inhibitors, lysed, immunoprecipitated with anti-ζ antibodies, electrophoresed on a 13% polyacrylamide gel containing SDS, transferred to nitrocellulose, and immunoblotted with affinity-purified anti-phosphotyrosine antibodies, as described (17–23). To normalize for the amount of ζ chain immunoprecipitated, the filter was sequentially probed with an anti-ζ peptide antiserum. Analysis of total cellular tyrosine phosphorylated substrates was assessed as described (23). Briefly, 1×10^7 NK cells, unstimulated or stimulated for 15 min at 37°C, were washed, lysed, electrophoresed on a 10.5% polyacrylamide gel containing SDS, transferred to nitrocellulose, and immunoblotted with anti-phosphotyrosine antibodies.

RESULTS AND DISCUSSION

Ligand binding to the TCR or engagement of other receptors that transmit a mitogenic signal in T cells results in the tyrosine phosphorylation of the ζ chain, presumably through the activation of a TCR-coupled PTK. Based on these observations in T cells, we hypothesized that engagement of a receptor that activates NK cells might also induce tyrosine phosphorylation of the ζ chain and thus would imply a role for a receptor-coupled tyrosine kinase in these cells. Activating receptors in NK cells include the FcγRIII, the IL-2 receptor (27), and an as-yet-undetermined receptor or group of receptors responsible for non-major-histocompatibility-complex-dependent binding and cytolysis of targets recognized by NK cells. In addition, pharmacologic agents such as phorbol esters can activate NK cells (27). To assess ligand-dependent phosphorylation of the ζ chain, isolated NK cells were loaded with [^{32}P]orthophosphate to label cellular ATP pools and stimulated as indicated. Phosphorylated ζ chain was detected by immunoprecipitation and then two-dimensional electrophoresis. Monomeric ζ chain has a molecular weight of 16,000. It exists in T cells primarily as a disulfide-linked homodimer and so migrates with a molecular weight of 32,000 prior to reduction. In two-dimensional nonreduced–reduced gels, the ζ chain is, therefore, visualized below the diagonal as a M_r 16,000 species that can be extrapolated to a molecular weight of 32,000 on the diagonal. However, in activated T cells, the tyrosine-phosphorylated form of the ζ chain migrates aberrantly with a molecular weight of 21,000 under reducing conditions. Under nonreducing conditions, the migration of phosphorylated ζ chain is not aberrant and so migrates with a molecular weight of 32,000. In two-dimensional gels, phosphorylated ζ chain is visualized as a M_r 21,000 reduced protein that can be extrapolated to a molecular weight of 32,000 on the diagonal. In NK cells, ζ-chain expression is more complicated; it exists as two forms: a ζ–ζ homodimer and a ζ–p12 heterodimer (4). In NK cells that were stimulated with an anti-FcγRIII mAb, enhanced phosphorylation of the ζ chain was evident (arrow, Fig. 1*b*). This species migrated with a molecular weight of ≈21,000 after reduction. By extrapolation, it is evident that this species was derived from a M_r 32,000 nonreduced polypeptide and thus is consistent with representing the homodimeric form of ζ. Anderson *et al.* (28) have reported preferential physical association of homodimeric ζ with FcγRIII. The finding that the phosphorylated form of the ζ chain seen after activation of NK by FcγRIII was the homodimeric form of the ζ chain suggests that this species of ζ chain is functionally as well as physically associated with this receptor. The inability to detect phosphorylation of ζ–p12, however, may be a reflection of the sensitivity of the assay or the inability of the antipeptide antiserum to efficiently immunoprecipitate phosphorylated heterodimer. The migration of phosphorylated ζ chain was confirmed by showing that the species seen in anti-CD16 mAb-stimulated NK cells comigrated with the form of phosphorylated ζ chain seen in anti-CD3-stimulated T cells (data not shown). The finding that anti-CD16 mAb induced ζ-chain phosphorylation in NK cells was confirmed using five donors. In contrast to NK cells that had been

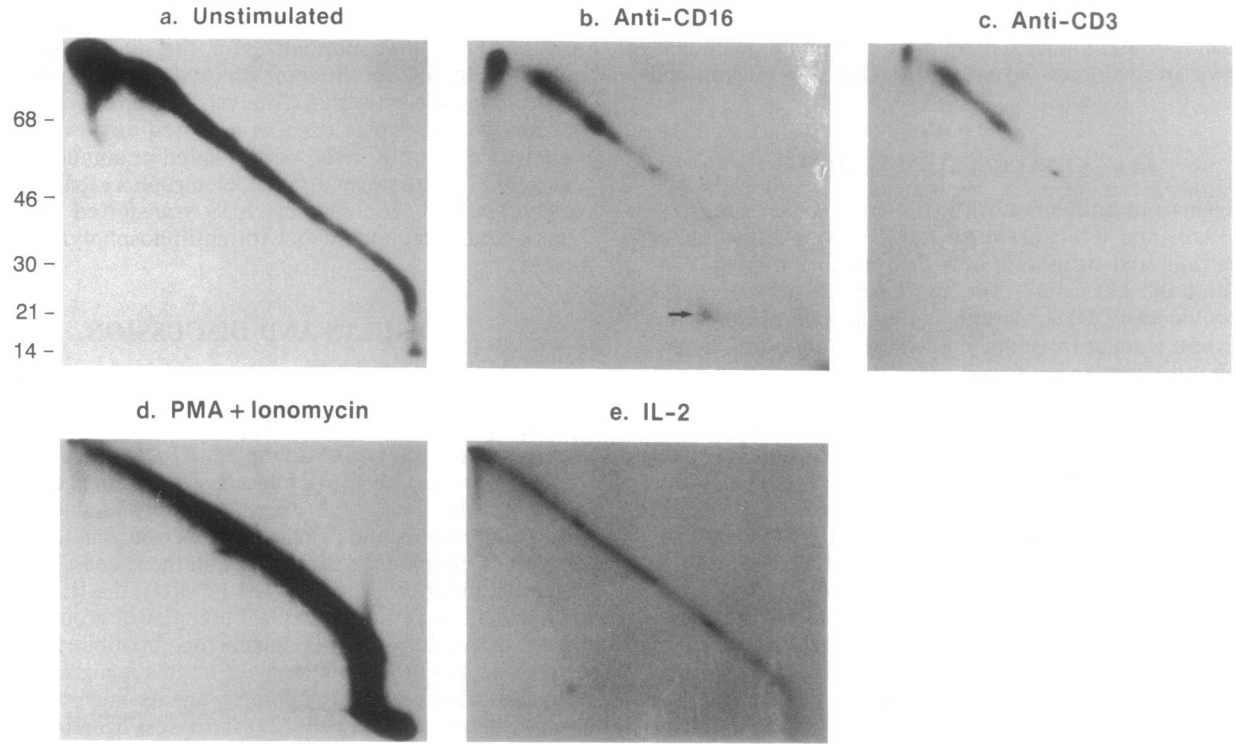

FIG. 1. Anti-FcγRII antibody induced ζ-chain phosphorylation. Fifteen million isolated NK cells were labeled with [^{32}P]orthophosphate (*a*) and then stimulated with anti-FcγRIII antibody (*b*), anti-CD3 mAb (*c*), phorbol 12-myristate 13-acetate (PMA; 50 ng/ml) and ionomycin (1 μM) (*d*), or IL-2 (1000 units/ml) (*e*). The cells were washed, lysed, immunoprecipitated with anti-ζ antibody, and subjected to two-dimensional nonreducing–reducing electrophoresis in 13% polyacrylamide gels. The gels were then dried and autoradiography was performed for 4 days at −70°F. Labeled NK cells used in *a–c* were from one donor and cells used in *d* and *e* were from another donor; however, comparable levels of anti-CD16-induced ζ chain were evident in cells from both donors (not shown). The arrow indicates phosphorylated ζ chain migrating below the diagonal as discussed in detail in the text.

activated by anti-FcγRIII mAb, isolated NK cells that were not further stimulated showed little basal phosphorylation of the ζ chain (Fig. 1*a*). Although the basal level of ζ phosphorylation is not apparent in this exposure, upon longer exposures, basal phosphorylation of the ζ chain was evident (data not shown). In one donor the basal level of ζ-chain phosphorylation was higher than others relative to the level of ζ-chain phosphorylation induced by anti-FcγRIII mAb. Several factors could potentially contribute to this finding including individual variation or perturbing the cells in the process of purification. Indeed, T cells isolated from peripheral blood have substantial levels of constitutive ζ-chain phosphorylation immediately after purification that diminishes upon culturing the cells (21). Since isolated human NK cells were utilized in this study, one potential artifact was that the phosphorylation seen might be due to contaminating T cells. We controlled for the presence of T cells by incubating the NK fraction of cells with a stimulatory anti-CD3 mAb (Fig. 1*c*). We observed that even under conditions that result in optimal phosphorylation of the ζ chain in T cells, ζ-chain phosphorylation due to activation of T cells maximally accounted for <10% of the signal generated by anti-FcγRIII antibodies (as assessed by two-dimensional densitometric scanning). A further potential confounding factor is that T cells may express the CD16 molecule under certain circumstances (29, 30). However, we excluded this possibility by analyzing the T-cell expression of CD16 by using flow cytometry and finding that the T cells used in this study were negative for this marker. Thus these data indicate that the phosphorylation seen was not due to T-cell contamination but was rather a measure of ζ-chain phosphorylation in NK cells. In contrast to stimulation of cells by FcγRIII, other activating stimuli in NK cells such as phorbol 12-myristate 13-acetate, ionomycin, and IL-2 failed to induce ζ-chain phosphorylation (Fig. 1 *d* and *e*). A below-the-diagonal species is noted in IL-2-stimulated NK cells; however, this does not correspond to the expected nonreduced molecular weight of phosphorylated ζ chains. We believe that this species is unrelated to the ζ chain as phosphorylated ζ chain is also not detected in IL-2-activated NK cells by anti-phosphotyrosine immunoblot analysis (data not shown). The nonstimulating anti-CD2 mAb (9.6) was also used as a control antibody that bound to NK cells but this also did not result in enhanced ζ-chain phosphorylation (data not shown).

The finding that phorbol esters did not stimulate ζ-chain phosphorylation suggested that the phosphorylation observed was not due to the serine/threonine kinase protein kinase C. Previous studies in T cells have demonstrated that the phosphate incorporated into the ζ chain after cell activation is solely on tyrosine residues, as determined by anti-phosphotyrosine immunoblot analysis and phospho amino acid analysis (17, 18). To assess whether tyrosine phosphorylation of the ζ chain occurred in NK cells stimulated with FcγRIII, we utilized anti-phosphotyrosine immunoblot analysis. We observed that stimulation of NK cells with anti-FcγRIII mAb resulted in a >10-fold increase in tyrosine phosphorylation of the ζ subunit (Fig. 2*a*). This estimate of phosphorylation was even greater if corrected for amount of ζ protein present (Fig. 2*b*), though the slight reduction in total ζ-chain expression in activated NK cells was not a consistent finding. In contrast, anti-CD2 mAb (9.6), anti-CD3 mAb, or IL-2 did not induce detectable increase in ζ-chain phosphorylation (data not shown). The experiment shown does not exclude the possibility that residues other than tyrosine are also phosphorylated, though based on previous studies (17, 18) this is not likely. If perturbation of FcγRIII activates a PTK, one would predict that a number of substrates might be evident after perturbation of this receptor. As can be seen in

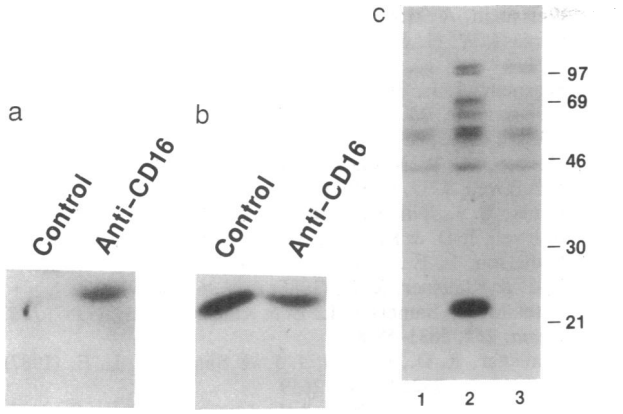

FIG. 2. Immunoblot analysis of ζ chains in NK cells depicting anti-phosphotyrosine immunoblot analysis of stimulated NK cells (*a*), immunoblot analysis with anti-ζ antibodies (*b*), and immunoblot analysis of whole cell lysates (*c*). (*a*) NK cells (10^7 cells per lane) were used unstimulated or were stimulated with anti-FcγRIII mAb for 1 hr at 37°C. The cells were washed with phosphate-buffered saline containing phosphatase inhibitors, lysed, immunoprecipitated with an anti-ζ peptide serum (387), and electrophoresed on a 13% polyacrylamide gel containing SDS. The gel was transferred to nitrocellulose and immunoblotted with affinity-purified anti-phosphotyrosine antibodies. The filter was exposed to film for 6 days. (*b*) The quantity of ζ chain precipitated was determined by immunoblot analysis of the samples with anti-ζ antibody. The filter was exposed to film for 2 days. (*c*) NK cells, unstimulated (lane 1) or stimulated with anti-CD16 mab (lane 2) or with a control mAb (lane 3) were washed, lysed, and analyzed by immunoblotting with anti-phosphotyrosine antibodies.

Fig. 2*c*, stimulation of NK cells with anti-CD16 mAb results in enhanced phosphorylation of a variety of substrates in addition to the M_r 21,000 protein that corresponds to phosphorylated ζ chain. A control mAb did not have this effect nor did an anti-CD11b mAb (data not shown).

To determine the physiologic relevance of the effect of anti-receptor antibodies, we investigated if ζ-chain phosphorylation also occurred as a consequence of recognition by NK cells of antibody-coated targets, since the phenomenon of antibody-dependent cellular cytotoxicity is also mediated by the NK FcγRIII. Incubation of NK cells with Raji cells in the absence of an antibody directed against surface molecules of these cells, resulted in little ζ-chain phosphorylation (Fig. 3*a*). In contrast, engagement of FcγRIII on NK cells by stimulating with antibody-coated targets induced an increase in the ζ-chain phosphorylation (Fig. 3*b*). In T cells, anti-receptor antibodies appear to be a more potent inducer of ζ-chain phosphorylation relative to the physiologic ligand, antigen (19), and it is possible that the anti-FcγRIII mAb was a more potent stimulus of ζ-chain phosphorylation relative to antibody-coated targets as well. However, more detailed dose–response and time-course experiments need to be performed to establish this point with certainty. Because of the difficulties in isolating large numbers of NK cells from donors, these issues were not addressed in the present study. No phosphorylated ζ chain was detected if orthophosphate-labeled NK cells were stimulated with antibody-coated Raji cells and were immunoprecipitated with a control antiserum (Fig. 3*c*). The lysis of some tumor targets by NK cells does not require antibody though the recognition structures are not well characterized. To determine if ζ-chain phosphorylation occurred as a consequence of recognition of tumor cells, isolated NK cells were also incubated with K562 cells, a typical NK target, and this failed to induce ζ-chain phosphorylation (data not shown). To exclude the possibility that the phosphorylation seen was due to ζ-chain phosphorylation in the target cells, Raji cells and K562 cells were assayed for ζ chain by immunoblot analysis. The target cells were found to lack ζ chains by this technique (data not shown).

In the present study, we provide evidence that in NK cells the ζ chain is phosphorylated on tyrosine residues after perturbation of FcγRIII, whether by an anti-receptor antibody or by engagement of the receptor by its physiologic ligand, immunoglobulin bound to a target. The data suggest that FcγRIII couples to a nonreceptor PTK in a manner similar to the TCR.

Recent studies in T cells indicate that at least two PTKs are candidates for the TCR-coupled PTK, the lck and fyn PTKs (29–33), and the latter has been shown to be specifically coprecipitated with the TCR. It will be important to determine what kinase is associated with FcγRIII in NK cells. In this regard it is of interest that NK cells have been shown to express at least two PTKs encoded by the *lck* and *fgr* genes (34, 41). Activation of NK cells by targets independent of antibody did not induce ζ-chain phosphorylation. This indicates that the recognition of targets in the absence of antibody and the biochemical pathways involved in lysis by this mechanism are distinct from the pathways activated by FcγRIII. This contrasts with signaling in T cells in which many surface molecules that activate these cells result in ζ-chain phosphorylation and suggests a convergence of signaling mechanisms. Interestingly, although it has been shown that ligand binding to the IL-2 receptor results in tyrosine phosphorylation of a number of substrates in T cells and NK cells (35, 36), the ζ chain appears not to be a substrate of the putative tyrosine kinase activated by this receptor. This implies that this pathway too is divergent from those involved

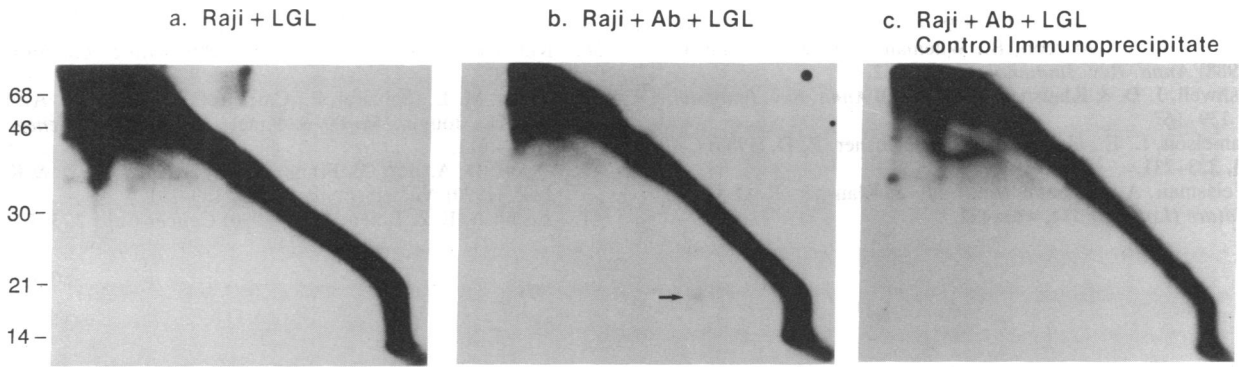

FIG. 3. Assessment of ζ-chain phosphorylation in NK cells activated by incubation with antibody-coated target cells. Isolated NK cells were labeled with [^{32}P]orthophosphate (10^7 cells per condition) were incubated with 10^7 Raji cells (*a*) or Raji cells that had been precoated with antibody for 1 hr at 37°C (*b*). Phosphorylated ζ chain was detected as described above. No phosphorylated ζ chain was detected if labeled NK cells were stimulated with antibody-coated Raji cells but immunoprecipitated with a control antiserum (*c*). The arrow indicates phosphorylated ζ chain. LGL, large granular lymphocytes.

in FcγRIII signaling. Perturbation of FcγRIII has been shown to induce inositol phospholipid metabolism (24) and thus, in turn, may lead to activation of protein kinase C. Like the TCR, the NK FcγRIII may couple to two kinase pathways: protein kinase C and a PTK. Recent studies have indicated the importance of the ζ chain both as a homodimer (ζ–ζ) and as a heterodimer (ζ–η) in coupling the TCR to both of these pathways. It remains to be determined if a species of ζ-linked η chain exists in NK cells and if it is responsible for coupling the FcγRIII to inositol phospholipid metabolism. Also proof of the importance of the ζ chain in coupling FcγRIII to kinase pathways must await the generation of NK cells bearing mutated receptors. The finding that the ζ chain undergoes tyrosine phosphorylation in these cells, as it does in T cells, supports the hypothesis that these phosphorylation events may play a role in lymphocyte function. However, at this time the nature of this function has yet to be determined.

The invariant chains of the TCR also function in the transport of the multimeric receptor complex to the cell surface. Recent studies indicate that FcγRIII may utilize the ζ chain for transport of the receptor to the plasma membrane (6); it appears that the principles that govern the assembly of this putative multimeric receptor complex may be the same as that of the TCR. Thus the ζ chain seems to have highly analogous roles in both T cells and NK cells, since the ζ chain is not only involved in signaling but also in assembly of the multimeric receptor. One of the subunits of the Fc ε receptor on mast cells, the γ subunit, is homologous to the ζ chain (37). As the γ subunit present in NK cells allows surface expression of FcγRIII and can pair with the ζ chain, it might be that the low molecular weight species of the ζ chain seen in NK cells represents ζ–γ (38–40). The functional significance of this species of the ζ chain is unclear at the present time.

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