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Sequential Involvement of Lck and SHP-1 with MHC-Recognizing Receptors on NK Cells Inhibits FcR-Initiated Tyrosine Kinase Activation

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Summary

Recognition of major histocompatibility (MHC) class I complexes on target cells by killer cell inhibitory receptors (KIR) blocks natural killer (NK) and T cell cytotoxic function. The inhibitory effect of KIR ligation requires the phosphotyrosine-dependent association of KIR with the cytoplasmic SH2-containing protein tyrosine phosphatase SHP-1. Using a somatic genetic model, we first define a requirement for the Src family protein tyrosine kinase (PTK) Lck in mediating KIR tyrosine phosphorylation. We then investigate how KIR ligation interrupts PTK-dependent NK cell activation signals. Specifically, we show that KIR ligation inhibits the Fc receptor (FcR)-induced tyrosine phosphorylation of the FcR-associated ζ signaling chain, the PTK ZAP-70, and phospholipase Cγ. Overexpression of catalytically inactive SHP-1 (acting as a dominant negative) restores the tyrosine phosphorylation of these signaling events and reverses KIR-mediated inhibition of NK cell cytotoxic function. These results suggest sequential roles for Lck and SHP-1 in the inhibition of PTK following MHC recognition by NK cells.

Introduction

The functional response of a cytotoxic lymphocyte to an encounter with a target cell reflects a balance of intracellular signals generated by both activating and inhibitory receptors (Leibson, 1995a; Lanier and Phillips, 1996). Virus-infected cells, tumor cells, and some normal hematopoietic cells may activate natural killer (NK) cells via one of two routes: antibody-dependent cell-mediated cytotoxicity (ADCC) and direct "natural killing" (Trinchieri, 1989). ADCC is initiated by engagement of FcγRIIIA (CD16) on NK cells with the Fc portion of antibodies bound to cell-associated antigens. The receptors initiating natural killing are less well defined, although investigators have implicated roles for certain C-type lectins (Chambers et al., 1989), CD69 (Moretta et al., 1991), CD2, and several other receptors (Lanier and Phillips, 1993). In addition to these activating receptors, NK cells and certain T cells also express killer cell inhibitory receptors (KIR), which recognize specific major histocompatibility (MHC) class I complexes (Long et al., 1996). Engagement of KIR by target cell MHC class I complexes potently inhibits the cytotoxic activity of both NK cells and T cells (Ljunggren and Kärre, 1990; Moretta et al., 1994; Trinchieri, 1994; Yokoyama, 1995; Leibson, 1995a; Mingari et al., 1995; Phillips et al., 1995; Lanier and Phillips, 1996). The present study examines the molecular events underlying KIR-mediated inhibition of NK cell activation.

Activation of NK cells via either ADCC or natural killing depends on early protein tyrosine kinase (PTK) activation (Einspahr et al., 1991; O'Shea et al., 1991, 1992; Ting et al., 1991; Vivier et al., 1991). The PTKs implicated in early NK cell activation include the Src family PTK Lck and both Syk family PTKs, ZAP-70 and Syk (Salcedo et al., 1993; Cone et al., 1993; Pignata et al., 1993; Vivier et al., 1993; Ting et al., 1995). Critical downstream signaling events include the activation of the phospholipase Cγ1 (PLC-γ1) and PLC-γ2, which leads to the production of inositol 1,4,5-trisphosphate (IP_3) and the mobilization of intracellular calcium (Windebank et al., 1988; Ting et al., 1992; Azzoni et al., 1992). Although several of the early activation signals are shared by the two killing pathways, differences do exist, including a specific dependence of ADCC on phosphatidylinositol 3-kinase and of natural killing on protein kinase C (Bonnema et al., 1994).

The nature of the inhibitory signals transduced by KIR is less well defined. The cytoplasmic domains of both p58 and p70 varieties of KIR contain the amino acid sequence $D/E(x)_2YxxL(x)_{26}YxxL$ (Colonna and Samaridis, 1995; Wagtmann et al., 1995; D'Andrea et al., 1995). The similarity of this motif to the immunoreceptor tyrosine-based activation motif (ITAM) suggests that phosphorylation of the tyrosine residues may lead to association with Src homology 2 (SH2) domain–containing proteins. Indeed, cross-linking of KIR results in KIR tyrosine phosphorylation and recruitment of the cytoplasmic SH2-containing protein tyrosine phosphatase, SHP-1 (previously referred to as SHPTP-1, SHP, HCP, and PTP1C) (Burshtyn et al., 1996; Campbell et al., 1996). The phosphotyrosines of the cytoplasmic domain of KIR have recently been shown to be critical for association with SHP-1 and generation of the inhibitory signal (Fry et al., 1996; Olcese et al., 1996).

Although the association of KIR with SHP-1 has been well established, the molecular events both preceding and following this interaction have remained undefined. Here we provide genetic evidence that Lck kinase activity is specifically required for the tyrosine phosphorylation of KIR and its association with SHP-1, whereas Syk family PTKs are not. Additionally, we describe a novel system we have employed to evaluate how SHP-1 recruited to KIR interferes with the PTK-dependent NK

cell activation pathway. Our laboratory has previously demonstrated that KIR engagement inhibits the phosphoinositide generation and intracellular calcium mobilization associated with NK cell activation (Kaufman et al., 1995), suggesting that the inhibitory effect of SHP-1 must occur quite early in the NK cell activation pathway. In this report, we demonstrate that KIR engagement inhibits, via a SHP-1-dependent mechanism, the tyrosine phosphorylation of three critical target proteins of proximal PTKs involved in NK cell activation.

Results

KIR Engagement Blocks FcR-Mediated Killing

The interaction of KIR with their specific MHC class I ligands inhibits NK cell function via an incompletely defined signaling mechanism. We have chosen to study the p70 KIR, which is identified by the monoclonal antibody (MAb) DX9 and whose ligands include the gene products of HLA-Bw4 and related alleles such as B27 and B58 (Litwin et al., 1994; Gumperz et al., 1995). The inhibitory effect of p70 KIR engagement on natural killing is illustrated in Figure 1. C1R is an NK-sensitive, class I–deficient B lymphoblastoid cell line whose class I MHC expression is mostly limited to HLA-Cw4 (Zemmour et al., 1992). As has been previously demonstrated using HLA-B58-transfected C1Rs (Lanier et al., 1995), HLA-B27-transfected C1Rs are resistant to lysis by DX9$^+$ NK cell clones, but remain sensitive to lysis by DX9$^-$ NK cells (Figure 1A). No protection is conferred by the expression of the irrelevant class I ligand, HLA-A3.

The intracellular signaling events involved in natural killing by NK cells are not well characterized, primarily because the use of cellular targets as ligands results in the cross-linking of multiple types of receptors and the target cell proteins complicate biochemical analyses. In contrast, Fc receptor (FcR)-initiated signaling events are well defined and can be readily studied by direct receptor cross-linking. We therefore employed a system that allows us to investigate the effect of KIR engagement on FcR-mediated NK cell activation signals. Previous studies have reported that engagement of p58 KIR inhibits FcR-triggered killing (Vitale et al., 1995). We were therefore interested in determining whether engagement of p70 KIR functionally interferes with FcR-triggered killing. Incubation of the NK-resistant, FcR-bearing P815 target cell with a specific MAb results in binding of the Fc region of the antibody to P815, leaving the antigen recognition domains of the MAb free to bind to receptors on the surface of an NK cell ("reverse ADCC"). In this assay, P815 target cells coated with the anti-FcγRIII MAb 3G8 are killed by CD16$^+$ NK cells (Figure 1B). Coengagement of FcγRIII and KIR on the NK cell by the 3G8 and DX9 antibodies, respectively, completely inhibits FcR-mediated killing of P815 target cells (Figure 1B). The inhibitory effect of KIR engagement on FcR-mediated killing was observed in all of the DX9$^+$ NK cell clones we have tested (14 NK clones in eight separate experiments). No inhibition is observed when DX9$^-$ cells are utilized as effectors. Additionally, an isotype-matched control MAb, B159, which reacts with the CD56 antigen on NK cells, has no effect on FcR-mediated killing, verifying that the inhibitory effect produced by the DX9 MAb

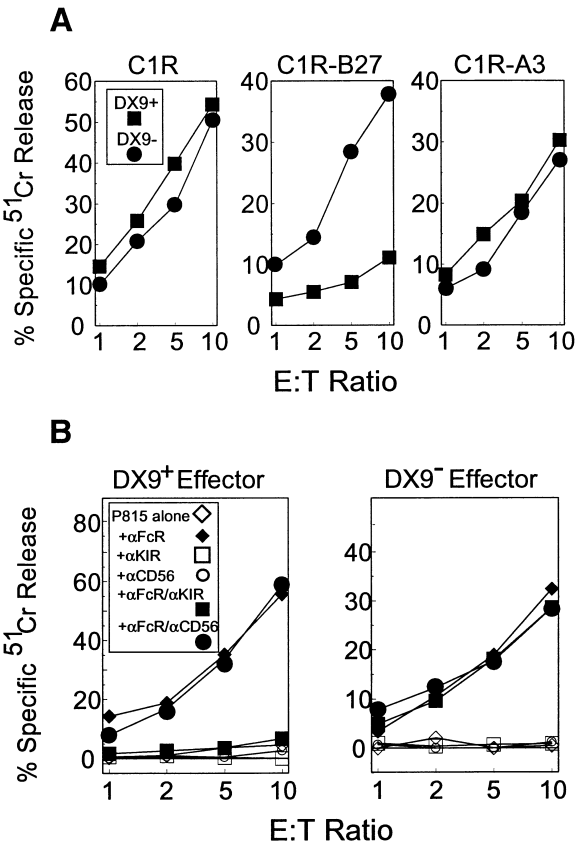

Figure 1. KIR Engagement Blocks Both Natural Killing and ADCC

(A) NK cell clones of the indicated DX9 phenotype were incubated for 4 hr with ^{51}Cr-labeled C1R, HLA-B27-transfected C1R, or HLA-A3-transfected C1R target cells at the indicated effector:target ratios.

(B) DX9$^+$ (left) or DX9$^-$ (right) NK cell clones were incubated with ^{51}Cr-labeled P815 targets for 4 hr in the presence or absence of anti-FcγRIII MAb (3G8), anti-KIR MAb (DX9), or anti-CD56 MAb (B159) alone or with the indicated combinations. Each percentage is the mean of triplicate wells.

is specific for its reactivity with p70 KIR (Figure 1B). These results demonstrate that ligation of the p70 KIR on NK cells is sufficient to inhibit not only natural killing but also FcR-mediated killing, providing us with a useful model for studying inhibitory signal transduction by KIR. We next began to explore the molecular events that occur following KIR ligation.

Tyrosine Phosphorylation of KIR and Association with SHP-1

Previous studies have demonstrated that cross-linking of the p58 KIR with the MAb GL183 results in tyrosine phosphorylation of KIR and its association with SHP-1 (Burshtyn et al., 1996). We have obtained similar results with the p70 KIRs recognized by the MAbs DX9 or 5.133. Cross-linking of the p70 KIR alone or in combination with FcγRIII results in the tyrosine phosphorylation of KIR, whereas cross-linking of only FcγRIII, the adhesion molecule LFA-1, or CD56 does not result in KIR tyrosine phosphorylation (Figure 2; data not shown). Reprobing of the membrane with SHP-1 antiserum demonstrates

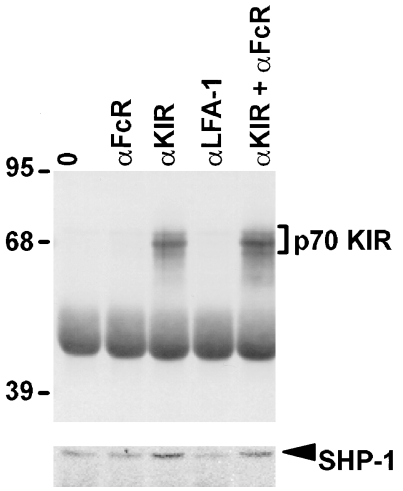

Figure 2. Tyrosine Phosphorylation of KIR and Recruitment of SHP-1

$DX9^+$, 5.133^+ NK cell clones (10×10^6 per sample) were incubated at 37°C for 1 min with cross-linked anti-FcR MAb, anti-KIR MAb (5.133), or anti-LFA-1 MAb alone or with the indicated combination. KIR were immunoprecipitated from cell lysates with rabbit antiserum specific for the cytoplasmic region of KIR, resolved by SDS–PAGE, transferred to membrane, and immunoblotted with anti-phosphotyrosine MAb (upper panel) and reprobed with SHP-1-specific antiserum (lower panel). By densitometry, the ratio of SHP-1 present relative to unstimulated cells is 1.2, 2.1, 0.6, and 2.0 for lanes 2–5, respectively. The prominent band around 50 kDa is the heavy chain of the immunoprecipitating serum. These results are representative of eight experiments with seven independent NK cell clones.

a basal level of association between SHP-1 and KIR in NK clones that is increased 2-fold following KIR cross-linking (Figure 2).

The presence of the modified ITAM in the cytoplasmic region of KIR suggests that a Src family PTK may be responsible for tyrosine phosphorylation of KIR (Chan et al., 1994). In fact, the PTK Lyn, which is not expressed by NK cells, is able to tyrosine phosphorylate KIR when both are expressed in fibroblasts (Burshtyn et al., 1996). To begin to identify the PTK responsible for KIR phosphorylation in lymphocytes, we used a vaccinia virus system to overexpress two Src family PTKs found in NK cells, Lck and Fyn. Relative to infection with control WR vaccinia virus, overexpression of Lck resulted in a 2-fold enhancement in the degree of KIR tyrosine phosphorylation after receptor cross-linking, whereas Fyn overexpression was indistinguishable from the control by densitometry (Figure 3). Additionally, this enhanced phosphorylation of KIR was associated with an increased level of SHP-1 recruitment (data not shown). The degrees of Lck and Fyn overexpression obtained via vaccinia infection of NK clones (relative to control WR virus) were evaluated by in vitro kinase assays against the exogenous substrate enolase and were 4- and 9-fold, respectively (data not shown).

Lck Is Required for Tyrosine Phosphorylation of KIR

Although data presented in Figure 3 suggest that Lck phosphorylates KIR, formal demonstration that Lck is

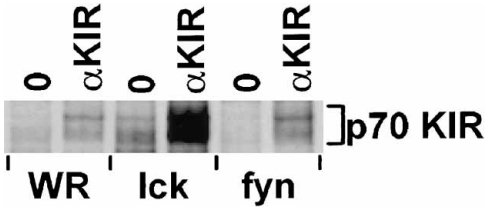

Figure 3. Lck Overexpression in NK Cells Enhances KIR Tyrosine Phosphorylation

$DX9^+$, 5.133^+ NK cell clones (10×10^6 per sample) were infected for 3 hr at a multiplicity of infection of 20 with WR strain vaccinia virus or recombinant vaccinia encoding the indicated PTK. Cells were stimulated as described in Figure 2, and KIR were immunoprecipitated with anti-KIR MAb (DX9) coupled to agarose and analyzed for phosphotyrosine as described in Figure 2.

required for KIR tyrosine phosphorylation requires a genetic approach. NK cells have a limited life span in culture and are thus not candidates for standard genetic manipulation. We therefore developed a system to analyze KIR phosphorylation in Jurkat T cells. The JCaM1 cell line is an Lck-deficient derivative of Jurkat (Strauss and Weiss, 1992). Additionally, we have isolated and characterized a ZAP-70-deficient subclone of the Syk-deficient Jurkat E6-1 cell line (Fargnoli et al., 1995; B. W., unpublished data). Important for these studies, each of the three sublines contained an equivalent amount of SHP-1 protein as measured by Western blotting of whole-cell extracts (data not shown). Recombinant vaccinia viruses expressing the p70 KIR encoded by NKAT3 and recognized by the MAbs DX9 and 5.133 were used to infect each of the three Jurkat lines. Equivalent surface expression of KIR in each of the cell lines was confirmed by flow cytometry (data not shown). As in NK cells, cross-linking of KIR alone or in combination with the multisubunit immune recognition receptor resulted in tyrosine phosphorylation of KIR and association with SHP-1 in the parental Jurkat line as well as the ZAP-70-deficient Jurkat subclone (Figure 4A). However, no tyrosine phosphorylation of KIR was observed in the Lck-deficient JCaM1 cell line (Figure 4A). These results have been confirmed with an independently derived Lck-deficient Jurkat subclone characterized in our laboratories and also with a distinct KIR, the p58 molecule encoded by NKAT1 (Colonna and Samaridis, 1995; data not shown). Reexpression of Lck in JCaM1 resulted in restoration of cross-linking-inducible KIR tyrosine phosphorylation, whereas overexpression of Fyn or kinase-dead Lck (R273) did not (Figure 4B). Vaccinia virus–driven expression of Lck or Lck (R273) in JCaM1 restored Lck protein expression to levels approximating that of the parental Jurkat line, and Lck expression in JCaM1 restored Lck kinase activity to levels approximating those in the parental Jurkat line (data not shown). Overexpression of Fyn (relative to control WR infection) in JCaM1 resulted in a 3-fold increase in Fyn kinase activity against the exogenous substrate enolase (data not shown). Taken together, the above results demonstrate that the catalytic activity of Lck is required for KIR tyrosine phosphorylation.

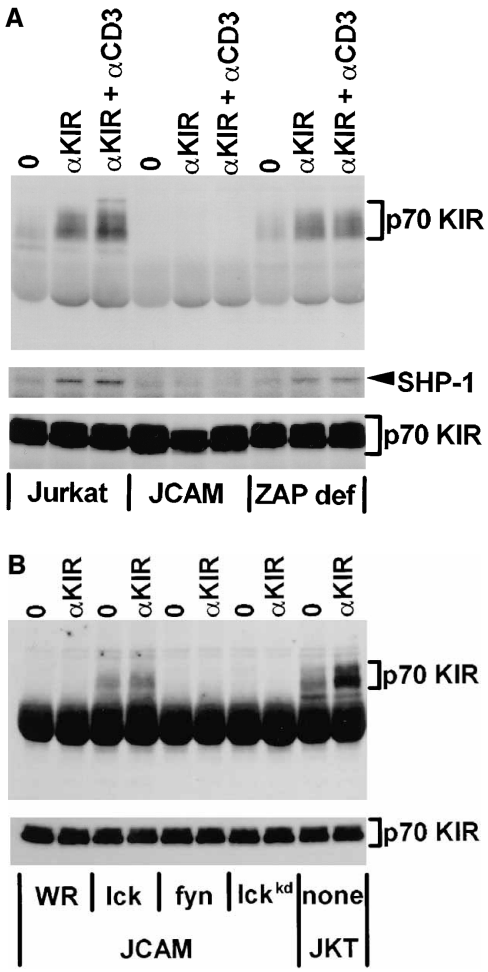

Figure 4. Lck Is Required for KIR Tyrosine Phosphorylation

Jurkat T cells, the Lck-deficient Jurkat line JCaM1, and the ZAP-70-deficient Jurkat line (10×10^6 per sample) were infected with recombinant vaccinia virus encoding NKAT3 (p70 KIR) for 4 hr at a multiplicity of infection of 10. Where indicated, cells were coinfected with vaccinia expressing the indicated PTK for 3 hr at a multiplicity of infection of 20. Cells were stimulated with anti-KIR MAb (5.133) and anti-CD3 MAb as indicated. KIR were immunoprecipitated as described in Figure 2, resolved by SDS–PAGE, and transferred to membrane.

(A) The membrane was probed with anti-phosphotyrosine MAb (upper panel), anti-SHP-1 serum (middle panel), and the immunoprecipitating serum (lower panel).

(B) The membrane was probed with anti-phosphotyrosine MAb (upper panel) and the immunoprecipitating serum (lower panel). The kinase-dead Lck mutant (R273) is indicated (lck^{kd}).

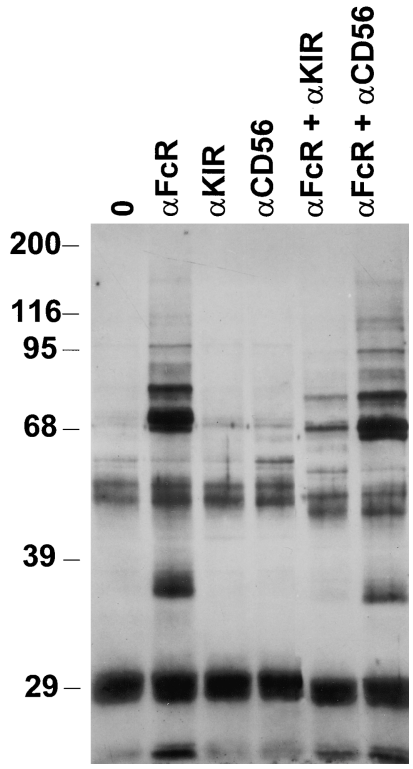

Figure 5. KIR Engagement Inhibits Specific FcR-Triggered Tyrosine Phosphorylation Events

$DX9^+$ NK cells (10×10^6 per sample) were incubated at 37°C for 1 min with cross-linked anti-FcR MAb, anti-KIR MAb (DX9), or anti-CD56 MAb alone or with the indicated combinations. Tyrosine phosphorylated proteins were immunoprecipitated from cell lysates, resolved by SDS–PAGE, transferred to a membrane, and immunoblotted with anti-phosphotyrosine MAb. The light chain of the immunoprecipitating antibody migrates at 29 kDa.

KIR Engagement Inhibits PTK-Dependent Activation Signals

We next were interested in defining how SHP-1 recruited to KIR interferes with NK cell activation signals. FcR-initiated NK cell–mediated killing depends on the rapid activation of specific PTKs and the subsequent tyrosine phosphorylation of specific cellular substrates (Einspahr et al., 1991; O'Shea et al., 1991, 1992; Ting et al., 1991; Vivier et al., 1991). We first evaluated whether KIR ligation inhibits these tyrosine phosphorylation events. $DX9^+$ NK cells were incubated with anti-FcR alone or in combination with DX9 MAb. Following cross-linking of the receptors for 1 min, the cells were lysed and phosphotyrosine-containing proteins were immunoprecipitated. Proteins were resolved by SDS–polyacrylamide gel electrophoresis (SDS–PAGE), transferred to Immobilon-P membranes, and analyzed by Western blotting with the anti-phosphotyrosine MAb, 4G10. Coligation of FcR and KIR inhibits tyrosine phosphorylation of many but not all of the substrates phosphorylated by stimulation of the FcR alone (Figure 5, contrast lanes 2 and 5). The specificity of this effect is demonstrated by the fact that cross-linking of the FcR with CD56 did not inhibit FcR-induced tyrosine phosphorylation (Figure 5, lane 6). Because the tyrosine phosphorylation status of only certain substrates was affected by KIR engagement, we next evaluated the effect of KIR engagement on the tyrosine phosphorylation of key signaling molecules in the FcR-initiated signal transduction cascade.

Signal transduction through FcγRIII is mediated by the noncovalently associated ζ signaling chain (Leibson, 1995b), and FcγRIII ligation results in the tyrosine phosphorylation of ζ (O'Shea et al., 1991; Vivier et al., 1991). We therefore next investigated whether tyrosine phosphorylation of ζ following FcR ligation is modified by coligation of KIR. $DX9^+$ NK cells were stimulated as described above, followed by immunoprecipitation of ζ

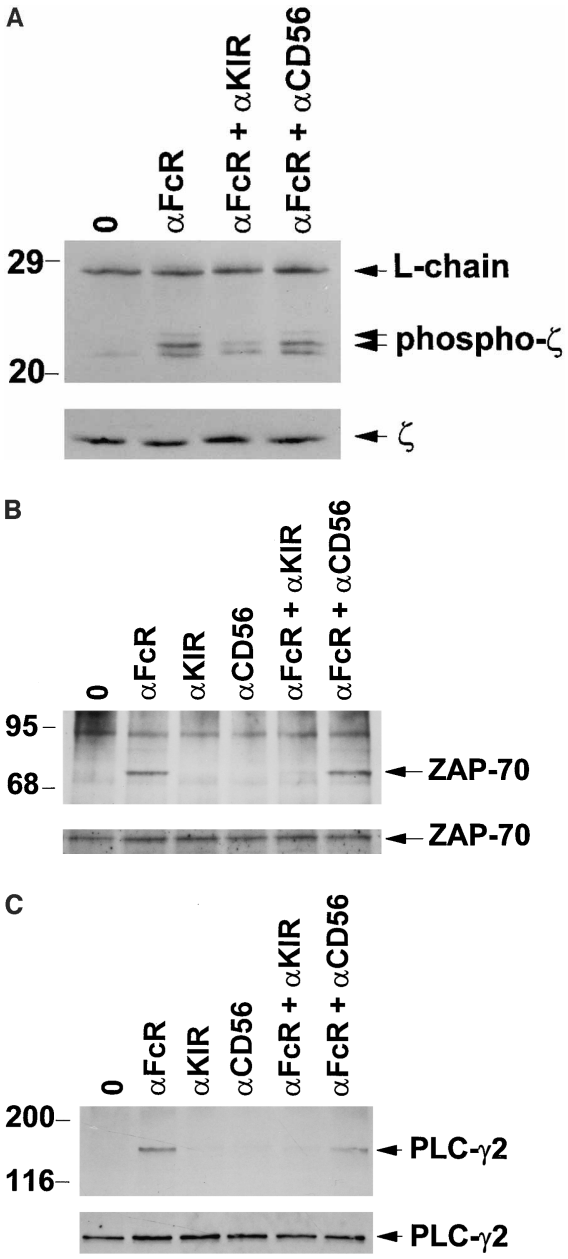

Figure 6. KIR Engagement Decreases the FcR-Triggered Tyrosine Phosphorylation of ζ, ZAP-70, and PLC-γ2

$DX9^+$ NK cells (10×10^6 per sample) were stimulated with the indicated cross-linked MAb as described in Figure 5 after being coinfected with vaccinia expressing the indicated PTK for 3 hr at a multiplicity of infection of 20. Cell lysates were subjected to immunoprecipitation with anti-ζ serum (A), anti-ZAP-70 serum (B), or anti-PLC-γ2 serum (C). Immunoprecipitated proteins were resolved by SDS–PAGE and immunoblotted with anti-phosphotyrosine MAb (upper panels). In the lower panels, the same membranes were reprobed with the immunoprecipitating antiserum. For (A), The p21 and p23 isoforms of phospho-ζ are indicated with arrows; L-chain, the light chain of the immunoprecipitating antiserum.

from cell lysates. FcR-triggered tyrosine phosphorylation of ζ results in the appearance of distinct bands of decreased electrophoretic mobility (p21 and p23) (Figure 6A, lane 2). Coligation of the FcR and KIR decreased formation of both forms of phospho-ζ (70% reduction by densitometry), whereas the isotype-matched control MAb did not (Figure 6A, lanes 3 and 4).

Tyrosine phosphorylation of ζ results in the recruitment of the PTK ZAP-70 and activation of ZAP-70 by tyrosine phosphorylation (Vivier et al., 1993; Chan et al., 1994, 1995; Ting et al., 1995). We therefore investigated whether the KIR-mediated decrease in FcR-triggered ζ phosphorylation we observed was accompanied by an inhibition of ZAP-70 phosphorylation. We found that coligation of the FcR and KIR markedly inhibited the FcR-induced tyrosine phosphorylation of ZAP-70, whereas the isotype-matched control MAb did not (Figure 6B).

Our group has previously demonstrated that KIR engagement blocks the generation of phosphoinositides and mobilization of intracellular calcium associated with NK cell activation (Kaufman et al., 1995). These signaling events are preceded by the activation of PLC-γ isoforms via tyrosine phosphorylation (Azzoni et al., 1992; Ting et al., 1992). PLC-γ hydrolyzes membrane phosphatidylinositol (4,5)bisphosphate into IP_3 and diacylglycerol. IP_3 then mediates the mobilization of intracellular calcium. Consistent with our previous results studying natural killing (Kaufman et al., 1995), we observed that KIR ligation inhibits FcR-induced inositol phosphate generation and calcium mobilization (data not shown). We were therefore interested in determining whether KIR engagement blocks the tyrosine phosphorylation of PLC-γ isoforms. Coligation of the FcR and KIR decreases the level of PLC-γ1 and PLC-γ2 tyrosine phosphorylation relative to FcR stimulation alone (Figure 6C; see Figures 9C and 9D), suggesting that KIR-mediated inhibition of inositol phosphate release and calcium mobilization is due to decreased activation of PLC-γ isoforms. Thus, we have demonstrated that ligation of KIR inhibits FcR-induced tyrosine phosphorylation of three critical signaling molecules: ζ, ZAP-70, and PLC-γ.

Dominant-Negative SHP-1 Reverses the KIR-Mediated Inhibition of PTK-Dependent Signals

The importance of SHP-1 for KIR-mediated inhibition is supported by the finding that overexpression of a catalytically inactive SHP-1 can reverse the inhibitory effect of KIR engagement on NK cell cytolytic activity (Burshtyn et al., 1996). Consistent with this previous report, we found that vaccinia virus–driven overexpression of the catalytically inactive SHP-1-C453S in $DX9^+$ clones restores lysis of targets expressing Bw4-related alleles (data not shown). We extended these findings to evaluate a potential role for SHP-1 in KIR-mediated inhibition of ADCC. Using our reverse ADCC assay, we found that overexpression of SHP-1-C453S significantly reverses the inhibitory effect of KIR coligation on FcR-mediated killing of P815 targets (Figure 7). Importantly, overexpression of an equivalent amount of wild-type SHP-1 did not reverse KIR-mediated inhibition, demonstrating that SHP-1-C453S is not simply interfering with the association of the KIR ITAM with another SH2 domain–containing protein, but rather that SHP-1 catalytic activity is specifically required for inhibitory signaling by KIR. Equivalent levels of overexpression of SHP-1

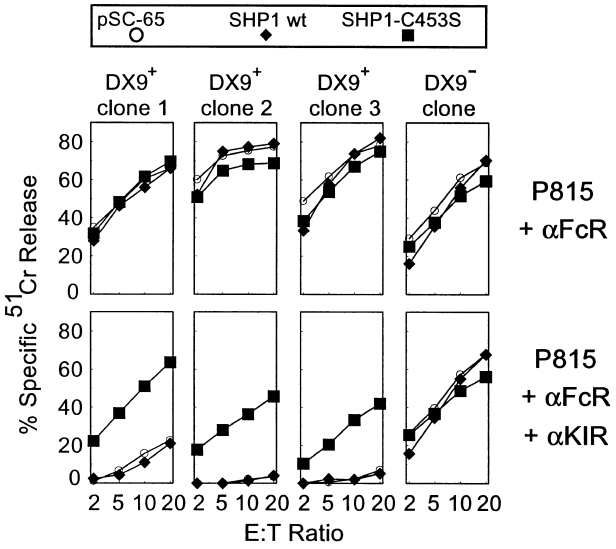

Figure 7. Overexpression of Catalytically Inactive SHP-1 Reverses KIR-Mediated Inhibition of ADCC

NK cells were infected for 4 hr at a multiplicity of infection of 20 with either control recombinant vaccinia viruses (containing the empty vector pSC-65) or recombinant vaccinia encoding either wild-type SHP-1 or catalytically inactive SHP-1-C453S. ^{51}Cr-labeled P815 cells in the presence of anti-FcR MAb (3G8) alone (top) or in combination with anti-KIR MAb (DX9) (bottom) were used as targets. Results are shown for three DX9$^+$ NK cell clones (first three panels) and one DX9$^-$ NK cell clone (far right).

and SHP-1-C453S were confirmed by Western blotting of whole-cell extracts (data not shown).

Although SHP-1-C453S clearly reversed the KIR-mediated inhibition of natural killing and ADCC by NK cells, the point at which KIR-associated SHP-1 interrupts the FcR-triggered signal transduction cascade remained undefined. We therefore investigated whether the KIR-mediated inhibition of tyrosine phosphorylation of the substrates examined could be attributed to the catalytic activity of SHP-1. Vaccinia virus–driven overexpression of SHP-1-C453S, but not wild-type SHP-1, reversed the KIR-mediated inhibition of FcR-induced tyrosine phosphorylation evaluated both at the level of whole-cell extracts (Figure 8) and for each of the specific substrates analyzed, i.e., ζ (Figure 9A), ZAP-70 (Figure 9B), PLC-γ1 (Figure 9C), and PLC-γ2 (Figure 9D). These results demonstrate that KIR ligation interrupts early PTK-dependent NK cell activation signals via a SHP-1-dependent mechanism.

Discussion

Engagement of KIR by MHC class I complexes on potential target cells inhibits NK cell effector function. Here we explore the molecular mechanisms of KIR-mediated inhibition of NK cell activation. Specifically, we provide genetic evidence that Lck is required for the initial tyrosine phosphorylation of KIR. Furthermore, we show that the catalytic activity of the recruited SHP-1 is required for the KIR-mediated inhibition of the tyrosine phosphorylation of three key substrates mediating NK cell activation: ζ, ZAP-70, and PLC-γ. Taken together, our results provide significant and novel evidence in support of a sequential model of KIR function involving Lck-dependent phosphorylation of KIR, recruitment of SHP-1, and SHP-1-mediated interruption of PTK-dependent activation signals.

We have previously demonstrated that interaction of an NK cell with a MHC class I–bearing target can result in the tyrosine phosphorylation of selected substrates in the NK cell (Kaufman et al., 1995), supporting the fact that expression of MHC class I by a target cell does not globally block NK cell recognition of that target. However, the interaction of an NK cell with a target cell engages a wide variety of NK cell surface molecules, including but not limited to various activating receptors, KIR, and adhesion molecules, each of which may trigger

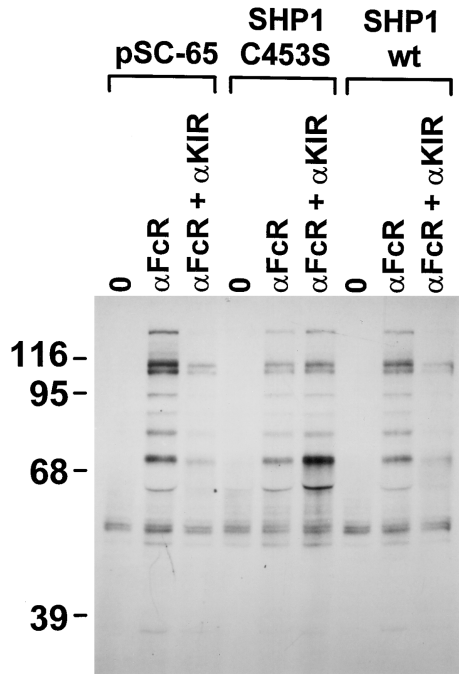

Figure 8. Overexpression of Catalytically Inactive SHP-1 Reverses KIR-Mediated Inhibition of FcR-Triggered Tyrosine Phosphorylation

DX9$^+$ NK cell clones (10^6 per sample) infected for 2 hr at a multiplicity of infection of 10 with either control recombinant vaccinia viruses (containing the empty vector pSC-65), or recombinant vaccinia encoding either wild-type SHP-1 or catalytically inactive SHP-1-C453S were stimulated as described in Figure 5. Whole cell extracts were resolved by SDS–PAGE, transferred to membrane, and probed with anti-phosphotyrosine MAb.

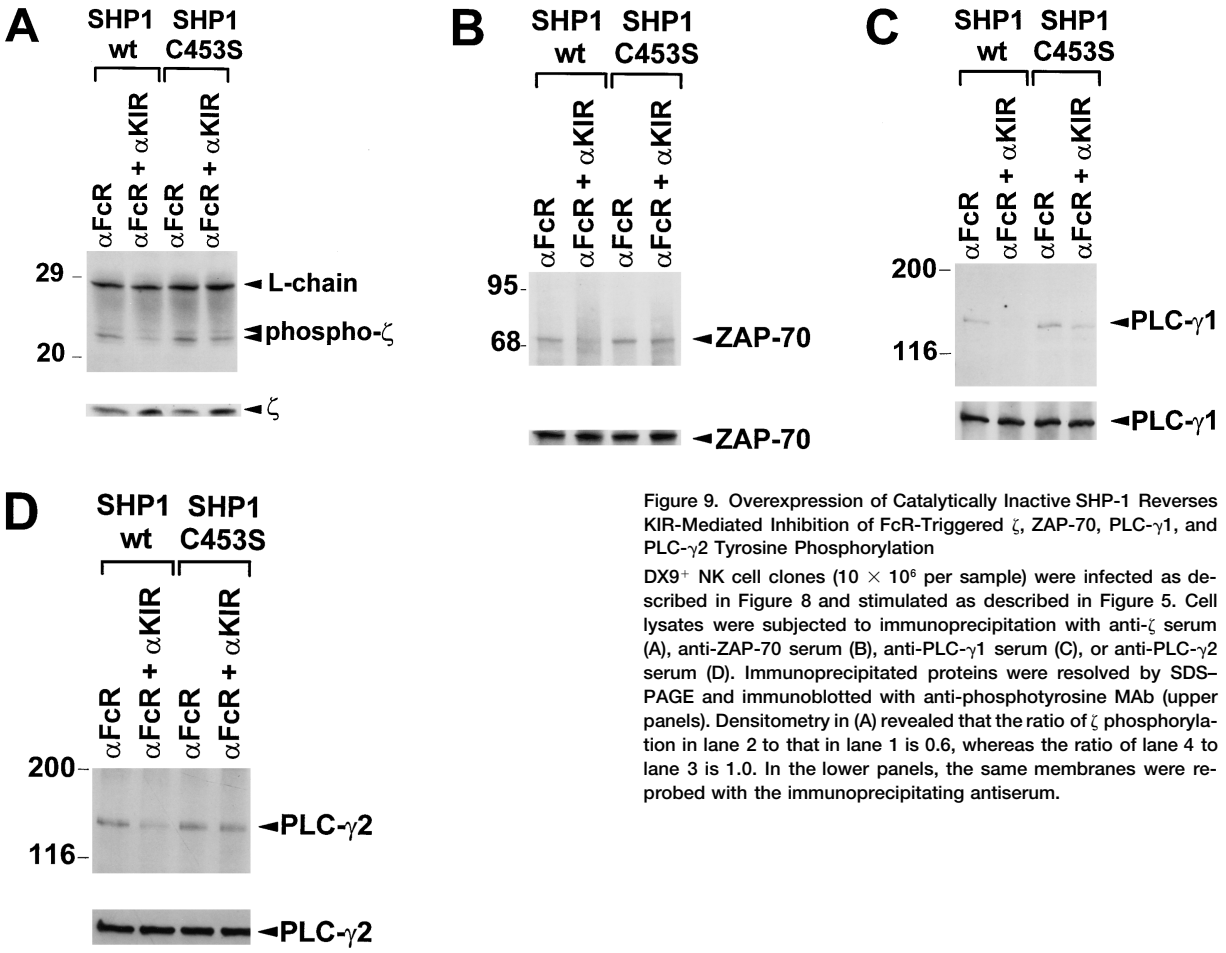

Figure 9. Overexpression of Catalytically Inactive SHP-1 Reverses KIR-Mediated Inhibition of FcR-Triggered ζ, ZAP-70, PLC-γ1, and PLC-γ2 Tyrosine Phosphorylation

$DX9^+$ NK cell clones (10×10^6 per sample) were infected as described in Figure 8 and stimulated as described in Figure 5. Cell lysates were subjected to immunoprecipitation with anti-ζ serum (A), anti-ZAP-70 serum (B), anti-PLC-γ1 serum (C), or anti-PLC-γ2 serum (D). Immunoprecipitated proteins were resolved by SDS–PAGE and immunoblotted with anti-phosphotyrosine MAb (upper panels). Densitometry in (A) revealed that the ratio of ζ phosphorylation in lane 2 to that in lane 1 is 0.6, whereas the ratio of lane 4 to lane 3 is 1.0. In the lower panels, the same membranes were reprobed with the immunoprecipitating antiserum.

several intracellular biochemical pathways. Therefore, to study KIR signaling more directly, we developed the system presented in this study which, utilizes MAbs to ensure ligation of only specific NK cell receptors. This model has allowed us to demonstrate that the KIR-mediated inhibitory signal modulates a subset of intracellular PTK-dependent activation events.

Our demonstration that Lck is required for KIR tyrosine phosphorylation raises several mechanistic possibilities. Since ITAMs are known to serve as substrates for Src family PTKs (Chan et al., 1994; Weiss and Littman, 1994), Lck may directly phosphorylate KIR. Alternatively, we cannot formally rule out the possibility that Lck-dependent KIR phosphorylation occurs via an intermediate signaling molecule, although we have provided evidence that Syk and ZAP-70 are not necessary for KIR phosphorylation (Figure 4A) and that overexpression of Fyn does not increase KIR phosphorylation (Figures 3 and 4B). Since cross-linking KIR alone (without coligation of a triggering receptor) results in KIR phosphorylation, our results raise the additional possibility that KIR may associate with and activate Lck. Coimmunoprecipitation of Lck with p58 KIR has been previously reported (Bottino et al., 1994), but the nature and significance of this interaction remain undefined.

Although we have not formally identified substrates of SHP-1, our data provide critical information regarding what those substrates might be. The SHP-1-dependent inhibition that we report could be produced by several conceivable mechanisms. The simplest interpretation is that ζ serves as a direct substrate of SHP-1. Because tyrosine phosphorylation of ZAP-70 is thought to occur following the association of ZAP-70 with phospho-ζ (Vivier et al., 1993; Chan et al., 1994; Ting et al., 1995), the KIR-mediated inhibition of ζ tyrosine phosphorylation that we observe would also be expected to prevent ZAP-70 phosphorylation. Although this type of mechanism could account for KIR-mediated inhibition of FcR-initiated NK cell activation and T cell activation, ζ has not been implicated as a signal transducer during natural cytotoxicity. Therefore, this mechanism would require that an as yet unidentified ITAM-containing molecule homologous to ζ is required for natural cytotoxicity. Alternatively, based on the reported association of SHP-1 with ZAP-70 (Plas et al., 1996), one could argue that SHP-1 down-regulates ZAP-70 kinase activity. However, this change in ZAP-70 catalytic activity alone would not account for the decreased tyrosine phosphorylation of ζ. Finally, the decreased tyrosine phosphorylation of ζ could reflect decreased catalytic activity of the PTK mediating ζ phosphorylation. Tyrosine phosphorylation of ζ is likely due to a Src family PTK such as Lck or Fyn (Chan et al., 1994; Weiss and Littman, 1994; Ting et al., 1995; van Oers et al., 1996). Regulation of the catalytic

activity of Lck is complex, involving an activating autophosphorylation site (Abraham and Veilette, 1990) as well as a carboxy-terminal inhibitory tyrosine phosphorylation site, which serves as a substrate for the PTK Csk and the tyrosine phosphatase CD45 (Chan et al., 1994). Thus, SHP-1-dependent modulation of the catalytic activity of Lck could conceivably be effected via several mechanisms. Note that such a mechanism would place Lck in the dual role of being required for KIR phosphorylation and serving as a substrate for the SHP-1 recruited to the phosphorylated KIR.

KIR ligation also inhibits activation of certain T cells following T cell antigen receptor engagement (Phillips et al., 1995; D'Andrea et al., 1996). ζ, ZAP-70, and PLC-γ play well-established roles in T cell receptor signaling (Chan et al., 1994). Therefore, one may speculate that the SHP-1-dependent inhibitory signaling mechanism of KIR that we have defined in NK cells may be shared by other lymphocytes. The ability of T cells to recognize MHC class I via both the T cell antigen receptor and KIR may play an important role in the modulation of an on-going immune response (Lanier and Phillips, 1996). Particularly intriguing in this regard is the similarity between the KIR-mediated modulation of ζ phosphorylation that we report and that induced by T cell recognition of altered peptide ligands (Sloan-Lancaster et al., 1994; Madrenas et al., 1995).

Our findings support a model of inhibitory receptor function involving Lck-dependent phosphorylation of KIR, recruitment of SHP-1, and SHP-1-mediated blockade of proximal PTK-dependent NK cell activation signals. Inhibitory receptors have been described in each of the three lymphocyte lineages, but significant differences exist between the proposed models of inhibitory signaling in each case. SHP-1 that has been recruited to the B cell inhibitory receptor FcγRIIB may act on the B cell coreceptor CD19 (J. C. Cambier, submitted). Alternatively, FcγRIIB may modulate calcium influx by recruiting another SH2-containing signaling element, SHIP (an SH2-containing inositol phosphatase) (Ono et al., 1996). In contrast with these mechanisms, our data suggest that MHC class I–recognizing KIR negatively regulate cellular activation by directly targeting the earliest detectable signaling events. This novel and potent means of inhibition is likely to be critical for protecting normal MHC class I–bearing cells from immune destruction by the host's own cytotoxic lymphocytes.

Experimental Procedures

Reagents, Cells, and Antibodies

Unless otherwise indicated, all chemicals were from Sigma Chemical Co. (St. Louis, MO). Human NK cells were cloned and passaged as previously described (Windebank et al., 1988). The P815 murine mastocytoma cell line, the Jurkat and JCaM1 T cell lines, and the anti-CD3-producing hybridoma OKT3 (murine IgG2a) were obtained from American Type Culture Collection (Rockville, MD). The ZAP-70-deficient Jurkat cell line was recently isolated and characterized (B. W., unpublished data). The HLA class I–deficient C1R cell line and its HLA-transfected derivatives were supplied by Peter Creswell (Yale University). The HLA class I–deficient 721.221 cell line and its HLA-transfected sublines were provided by Peter Parham (Stanford University). Anti-FcγRIII MAb 3G8 (Perussia and Trinchieri, 1984) and the anti-CD56 MAb B159 (murine IgG1) were supplied by Bice Perrusia (Thomas Jefferson University). The anti-LFA-1 MAb MHM23 (murine IgG1) was provided by Timothy Springer (Harvard Medical School). Anti-p70 KIR MAb DX9 (murine IgG1) (Litwin et al., 1994) and the anti-p70 KIR MAb 5.133 (murine IgG1) (Döhring et al., 1996) have recently been described. ζ-Specific antiserum was obtained by immunization of rabbits with the synthetic peptide CRRRGKGHDGLYQG (Orloff et al., 1989) conjugated to keyhole limpet hemocyanin. Antiserum to the KIR cytoplasmic domain was obtained by immunization of rabbits with the synthetic peptide TQRKITRPSQRPKTPPTD conjugated to keyhole limpet hemocyanin. The rabbit antisera specific for Lck, PLC-γ1, PLC-γ2, and ZAP-70 have been described previously (Ting et al., 1992, 1995). Rabbit antiserum specific for SHP-1 and the antiphosphotyrosine MAb 4G10 were purchased from Upstate Biotechnology (Lake Placid, NY). Rabbit antiserum specific for Fyn was purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

Cell Phenotype Analysis

$DX9^+$ NK cell clones were identified by incubating 10^6 cells with 1 μg of DX9 MAb or a control IgG1 myeloma protein (Organon Teknika, West Chester, PA) for 20 min at 4°C. After washing, the cells were incubated for 20 min at 4°C with fluorescein-conjugated goat anti–mouse IgG (Becton Dickinson, Mountain View, CA). Washed cells were then fixed in 1 ml of 1% paraformaldehyde in PBS, and 10,000 cells were analyzed by flow cytometry on the FacScan (Becton Dickinson). The DX9 phenotype status of NK cell clones was stable over the duration of their life in culture. Additionally, all $DX9^+$ NK cell clones also reacted with the MAb 5.133.

Vaccinia Viruses

NKAT1 and NKAT3 cDNAs were excised from the pCRIII vectors (Colonna and Samaridis, 1995) with EcoRI, and Klenow fragments were utilized to generate blunt ends. Blunt-ended fragments were inserted into the SmaI cloning site of the vector pSC11 and introduced into WR strain vaccinia virus via homologous recombination (Ting et al., 1995). Recombinant vaccinia viruses encoding wild-type and catalytically inactive HCP-C453S as well as the pSC-65 vector control have been described (Burshtyn et al., 1996). Generation of recombinant vaccinia viruses encoding the Src family PTKs and viral propagation were performed as described (Ting et al., 1995). Cells (2×10^6 per milliliter) were infected at the indicated multiplicity of infection for 1 hr in serum-free RPMI 1640 at 37°C and then for the remainder of the infection time at 10^6 cells per milliliter in RPMI 1640 supplemented with 10% bovine calf serum.

Cytotoxicity Assays, Inositol Phosphate Release, and Calcium Mobilization

The ^{51}Cr-release assays measuring direct NK cell–mediated cytotoxicity or reverse ADCC, measurement of inositol phosphate release, and analysis of calcium mobilization were performed as previously described (Windebank et al., 1988).

Cell Stimulation, In Vitro Kinase Assays, and Immunoblot Analysis

Cells (10×10^6 per sample for immunoprecipitation or 10^6 per sample for whole-cell extracts) were incubated at 4°C for 3 min with anti-FcR MAb (3G8 [10 μg/ml]), anti-p70 KIR MAb (DX9 [10 μg/ml] or 5.133 [5 μl of 1:3200 ascites per 100 μl sample]), anti-CD56 MAb (B159; 10 μg/ml), or anti-LFA-1 MAb (MHM23; 10 μg/ml). Washed cells were then mixed with goat anti–mouse IgG $F(ab')_2$ fragments (Organon Teknika) and incubated at 37°C for 1 min. Following stimulation, cells were lysed in buffer containing 20 mM Tris–HCl, 40 mM NaCl, 5 mM EDTA, 50 mM NaF, 30 mM $Na_4P_2O_7$, 0.1% bovine serum albumin, 1 mM Na_3VO_4, 1 mM PMSF, 5 μg/ml aprotinin, 10 μg/ml leupeptin, and 1% Triton X-100 (pH 7.4). Insoluble material was removed by centrifugation for 5 min at 15,000 × g. Whole cell extracts were resolved by SDS–PAGE. Alternatively, cell lysates were subjected to immunoprecipitation for 1–2 hr with rabbit antiserum bound to protein A–Sepharose beads, with the anti-phosphotyrosine MAb 4G10 coupled to agarose (Upstate Biotechnology) or with DX9 coupled to agarose. In vitro kinase assays were performed as described previously (Ting et al., 1995). Proteins were eluted from

washed immunoprecipitates into 40 μl of SDS sample buffer, resolved by SDS–PAGE, and transferred electrophoretically to Immobilon-P membranes (Millipore, Bedford, MA). Tyrosine-phosphorylated proteins were detected with the 4G10 MAb, followed by sheep anti–mouse IgG coupled to horseradish peroxidase (Amersham, Buckinghamshire, England). KIR, ZAP-70, Lck, Fyn, PLC-γ1, PLC-γ2, and ζ were analyzed with specific rabbit antisera and detected with protein A–horseradish peroxidase and the ECL detection system from Amersham. ^{32}P-labeled proteins were detected by autoradiography. Densitometry was performed using AMBIS software (Ambis, Inc., San Diego, CA).

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