Tyrosine Phosphorylation of a Human Killer Inhibitory Receptor Recruits Protein Tyrosine Phosphatase 1C

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Summary

Natural killer (NK) cells express killer inhibitory receptors that mediate negative regulation of NK cell cytotoxicity upon binding to MHC class I molecules on target cells. Unrelated inhibitory receptors on B cells have recently been shown to function through recruitment of phosphotyrosine phosphatase 1C (PTP-1C). Here, we show that a human killer inhibitory receptor specific for HLA-C also recruits PTP-1C after phosphorylation induced either by the pharmacological agent phenylarsine oxide or by conjugation with target cells. This recruitment is mediated by the binding of specific cytoplasmic phosphotyrosine-containing sequences to PTP-1C. These results implicate PTP-1C as a cytosolic component of the negative signaling pathway through NK cell inhibitory receptors.

Cytotoxicity by NK cells is controlled by an interplay of signals from both stimulatory and inhibitory cell surface receptors (1). Activating receptors trigger NK cell-mediated cytotoxicity upon binding to specific target cell ligands. Killer inhibitory receptors (KIRs)[1] engage with MHC class I molecules on target cells, blocking the functional capacity of the stimulatory receptors. When target cells lose or reduce expression of MHC class I molecules as a consequence of transformation or viral infections, the balance between activation and inhibition is altered, resulting in the release of target-directed NK cell cytotoxicity.

Human KIRs have recently been identified by mAbs and cDNA cloning as transmembrane glycoproteins of the Ig superfamily that recognize the distinct polymorphic determinants of HLA-B and -C molecules (2–7). Structurally, the extracellular region of these receptors is composed of two (HLA-C receptors) or three (HLA-B receptors) Ig superfamily domains. The cytoplasmic region includes a motif characterized by two tyrosine-x-x-leucine (YXXL) pairs spaced by 26 amino acids. A subgroup of KIRs lack this motif because of a premature truncation of the cytoplasmic tail and function as activatory rather than inhibitory receptors (8).

Recent observations in B cells have suggested a potential role for these cytoplasmic tyrosines in the function of KIRs. The B cell-surface receptors FcγRIIB1 and CD22 are characterized by cytoplasmic tyrosine-containing sequences, termed immune receptor tyrosine-based inhibitory motifs (ITIM) (9–12), which bind the SH2 domain(s) of protein tyrosine phosphatase-1C (PTP-1C, also known as SHPTP-1, SHP, or HCP; 13–16) upon phosphorylation (9, 17). Coligation of FcgRIIB1 with the B cell antigen receptor (BCR) induces ITIM tyrosine phosphorylation, recruitment of PTP-1C, and subsequently inhibits the capacity of the BCR to activate phospholipase C and to mobilize calcium (9). Similarly, CD22, which is weakly associated with BCR, becomes tyrosine phosphorylated and recruits PTP-1C upon BCR cross-linking (17). Separating CD22 from BCR with anti-CD22 antibody can substantially enhance proliferative responses induced by BCR cross-linking (17). The ITIM motifs in CD22 and FcγRIIB1 are surprisingly similar to the YXXL motifs of the human KIRs. A further parallel with CD22 and FcγRIIB1 is the observation that ligation of inhibitory receptors by MHC class I molecules on target cells inhibits the calcium mobilization in NK cells induced by susceptible target cells (18).

These structural and functional parallels between the human KIRs and the CD22 and FcγRIIB1 receptors have suggested a role for a phosphatase in KIR function (5, 19). Therefore, we investigated whether the KIRs also bind PTP-1C upon tyrosine phosphorylation. The results indicate that a human p58 KIR specific for HLA-C indeed binds PTP-1C upon phosphorylation of either tyrosine residue.

Materials and Methods

Cell Lines and Antibodies. The NK cell clone 5F12 was derived from human peripheral blood and cultured as described (20).

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[1] *Abbreviations used in this paper:* BCR, B cell antigen receptor; FSC, forward scatter; HRP, horseradish peroxidase; ITIM, immune receptor tyrosine-based inhibitory motifs; KIR, killer inhibitory receptors; PAO, phenylarsine oxide; PTP-1C, phosphotyrosine phosphatase 1C.

721.221 is a class I–deficient, human EBV-transformed lymphoblastoid cell line (21), and the HLA-C transfectants of 721.221 have been described (22). Cell-surface expression of class I alleles was periodically tested by FACS® analysis (Becton Dickinson & Co., Mountain View, CA) using the W6/32 mAb (mouse IgG_{2a} anti–HLA-A, -B, and -C; American Type Culture Collection, Rockville, MD) and the F4/326 mAb (mouse IgG_1 anti-HLA-C; kindly provided by Soo Young Yang, Sloan-Kettering Institute, New York, NY; 23). Anti-p58 KIR monoclonal antibody HP-3E4 (mouse IgM) has been previously described (4). The anti–mouse IgM mAb b-7-6 (rat IgG_1, kindly provided by Dr. John Cambier, National Jewish Center, Denver, CO) was conjugated to cyanogen bromide–activated Sepharose 4B beads (Pharmacia Biotech Inc., Piscataway, NJ) according to the manufacturer's instructions. Anti–PTP-1C antibody (rabbit polyclonal) and horseradish peroxidase (HRP)-conjugated antiphosphotyrosine mAb (4G10; mouse IgG_{2b}) were purchased from Upstate Biotechnology Inc. (Lake Placid, NY). Monoclonal anti-PTP-1C (mouse IgG_1) was from Transduction Laboratories (Lexington, KY) and was detected with rabbit anti–mouse IgG-HRP from Dako Corp. (Carpinteria, CA). The antiphosphotyrosine antibody PY20 (Transduction Laboratories) was conjugated to cyanogen bromide–activated Sepharose 4B beads (Pharmacia).

Peptides. The tyrosine-phosphorylated (p) and unphosphorylated p58 peptide sequences were EVTYAQLNH (YAQL), EVT(p)YAQLNH (pYAQL), IVYTELPNAE (YTEL), and IV(p)YTELPNAE (pYTEL). The control phosphopeptide sequence was (p)YMAPYDNY (amino acids 739–746 from the PDGF receptor β; 24). Peptides were synthesized with a carboxy-terminal amide by Quality Controlled Biochemicals, Inc. (Hopkinton, MA) and purified by reversed-phase (rp) HPLC. Their purity was >97%, as assessed by rpHPLC, mass spectrometry, and ^{31}P-NMR. Peptides were coupled to AminoLink Plus coupling gel (1 mg/1 ml packed gel; Pierce Chemical Co., Rockford, IL) overnight at 4°C and pH 7.2, according to the manufacturer's instructions. Coupling efficiency from the supernatant was determined by monitoring rpHPLC column elution at OD_{220}. Peptide beads (~15 μg/precipitation) were used immediately after coupling.

Cell-surface ^{125}I-Labeling. Cells were first labeled with a water-soluble Bolton-Hunter reagent (36 μg/ml Sulfo-SHPP; Pierce) according to the method of Portolés et al. (25) and subsequently with $Na^{125}I$ (0.5 mCi/50 million cells; Amersham, Arlington Heights, IL) using Iodogen (Pierce), according to the manufacturer's instructions.

Cytosolic Calcium Measurement of NK Cells Conjugated with Tumor Target Cells. The NK cell clone 5F12 was loaded with Indo-1 AM (Sigma Immunochemicals, St. Louis, MO), as described (26). Cells were mixed at a 2:1 ratio with 721.221 cells or with HLA-Cw4, -Cw5 transfectants of 721.221, centrifuged 1 min at 400 *g*, incubated 2 min at 37°C, resuspended, and analyzed on a Coulter Elite Flow cytofluorimeter (Coulter Electronics Inc., Hialeah, FL) to detect Ca^{2+} fluxes and conjugate formation, as described (26). In some experiments, target cells were preincubated for 30 min at 4°C with the anti–HLA-C antibody F4/326 or the anti–pan MHC class I antibody W6/32 or the anti-CD19 antibody (clone HD37; Boehringer Mannheim, Indianapolis, IN) before mixing. Only live (based on forward scatter [FSC] criteria) and indo-1–loaded cells (based on 405-nM vs. 525-nM emission spectra) were included in the analysis. In this way, the unloaded target cells were only visible when conjugated with a loaded NK cell. Because conjugates have a higher FSC than single cells, we could directly correlate mean cytosolic Ca^{2+} concentrations within the conjugated NK cell populations.

Metabolic Labeling with $[^{35}S]Cysteine/Methionine$. Cells were washed twice with PBS and resuspended at 4 million/ml in cysteine/methionine–free DME (Sigma) containing 10% FCS (TBS-dialyzed) and 1,000 U/ml rIL-2. $[^{35}S]$methionine/cysteine (Amersham) was added at 0.1 μCi/ml, and cells were sealed in a 75-cm² culture flask and incubated at 37°C for 4 h. Cells were washed twice before subsequent lysis.

Cell Stimulations. For phenylarsine oxide (PAO) treatment, 30 million NK cells/ml were unstimulated (–) or stimulated (+) for 2 min with 100 μM PAO (1 μl of 100 mM stock in DMSO) in a 37°C shaking heat block. An aliquot of 1 million cells was lysed in 4× reducing Laemmli sample buffer (27) and immediately frozen in liquid nitrogen, while the remainder was rapidly centrifuged, lysed in 1% Triton X-100 buffer, and subjected to immunoprecipitation (see below). This PAO treatment regimen has been shown to produce optimal global tyrosine phosphorylation in T and B cells (28–30). In conjugation experiments, 50 million NK cell clones (5F12) and/or 25 million target cells were mixed in 2 ml of IMDM medium at 37°C in a 14-ml round-bottom tube and immediately centrifuged at 2200 rpm for 5 s. Pelleted cells were incubated at 37°C for 2 min, mixed with 10 ml ice-cold PBS containing 200 μM sodium orthovanadate, pelleted for 2 min at 1,600 rpm, lysed in 1% Triton X-100 buffer, and subjected to immunoprecipitation (see below).

Protein Precipitations with Antibodies and Peptides. Cells were lysed on ice in 1% Triton X-100 (Surfact-Amps; Pierce), 10 mM Tris, pH 7.4, 150 mM NaCl, 2 mM Na orthovanadate, 1 mM Pefabloc SC (Boehringer-Mannheim), 0.4 mM EDTA, 10 mM NaF, and 1 μg/ml each of leupeptin, aprotinin, and soybean trypsin inhibitor (all from Sigma). Lysates were cleared by centrifugation at 12,000 rpm and precipitated with antibodies or gel-coupled peptides at 4°C for 2–4 h. HP-3E4 (1–3 μl of ascites/sample) was immunoprecipitated with b-7-6–coupled Sepharose, and W6/32 (100 μl culture supernatant/sample) was precipitated with protein A–Sepharose (Pharmacia). All precipitates were washed five to six times with lysis buffer and resuspended in reducing Laemmli sample buffer (27).

Immunoblotting. Precipitated samples were boiled for 5 min and separated on discontinuous SDS-PAGE according to the method of Laemmli (27). Proteins were electrophoretically transferred to nitrocellulose (Hybond-C Extra; Amersham) and blocked with 5% BSA. Membranes were probed with either HRP-coupled 4G10 (375 ng/ml) or initially with anti–PTP-1C and secondarily with HRP-coupled protein G or rabbit anti–mouse IgG-HRP. Immunoblotted proteins were visualized by chemiluminescence using the ECL detection reagents (Amersham). Membranes were stripped of antibody by incubating for 30 min at 50°C in 100 mM 2-ME, 2% SDS, 62.5 mM Tris, pH 6.7 according to a protocol supplied by Amersham. Stripped blots were reprobed after blocking. ^{35}S-labeled samples were subsequently autoradiographed from dried membranes.

PTP-1C Phosphatase Assay. The src family tyrosine kinase–derived phosphorylation site peptide RRLIEDAEYAARG (Santa Cruz Biotechnology) was ^{32}P-phosphorylated by incubating for 3 h with fyn kinase immunoprecipitates in the presence of $[^{32}P]ATP$, and was purified on P81 paper (Whatman, Clifton, NJ), eluted with 100 mM NH_4HCO_3, and lyophilized as described by Matthews et al. (16). PTP-1C phosphatase was purified from lysates of the human NK cell clone, NK3.3, with pYAQL tyrosine phosphopeptide-coupled beads, eluted with 50 mM *p*-nitrophenyl phosphate, and washed and concentrated in a Centricon-30 (Amicon, Inc., Beverly, MA). Dephosphorylation of the ^{32}P-peptide substrate by purified PTP-1C was assayed in the presence of

p58-derived phosphopeptides for 30 min at 30°C (within the linear range of peptide dephosphorylation), as described by Struelí et al. (31). About 50 ng of ^{32}P-peptide labeled with ∼55,000 cpm was added to each 50-μl sample of assay mixture.

Results

Inhibition of Cytotoxicity of NK Cell Clones by Class I Ligands is Paralleled by Inhibition of Calcium Mobilization. The human NK cell clone 5F12 expresses the p58 KIR recognized by the HP-3E4 mAb, which has previously been shown to mediate NK cell inhibition by a group of HLA-C molecules on target cells that share Asn^{77}-Lys^{80} (2). Indeed, in cytotoxicity assays, lysis of class I–deficient B cell line 721.221 by 5F12 was inhibited by transfection of HLA-Cw4, -Cw5, and -Cw6, whereas other HLA-C alleles did not affect cytotoxicity (data not shown).

To assess whether the inhibition of NK cell cytotoxicity was paralleled by changes in calcium mobilization in this clone, 5F12 was loaded with a calcium-reactive dye, indo-1, and conjugated with sensitive or resistant target cells. Cytosolic calcium levels were measured in the NK cells that formed conjugates. Interaction with sensitive target cells substantially elevated the mean cytosolic calcium levels in the conjugated NK cell population (Fig. 1). Conjugation with HLA-C–transfected resistant target cells, however, resulted in only a minimal increase of cytosolic calcium in the NK cells during the same time course. Interestingly, masking of HLA-C in these resistant target cells with antibodies resulted in increased calcium mobilization in the NK cells (Fig. 1), while the isotype-matched, anti-CD19 antibody had no effect in control experiments (data not shown). Therefore, the NK cell calcium mobilization response is negatively regulated by the interaction of inhibitory receptor with HLA-C molecules expressed on resistant target cells.

PTP-1C Associates with Tyrosine-phosphorylated p58 KIR. Since in B cells, CD22 and FcγRIIB1 inhibitory receptors downregulate calcium mobilization by phosphorylation of ITIMs and binding with PTP-1C, we tested whether p58 KIR could be tyrosine phosphorylated in NK cells, and whether the phosphorylated KIR could bind PTP-1C. In initial experiments, we induced tyrosine phosphorylation in human NK cells by a pharmacologic agent, PAO, a potent phosphotyrosine phosphatase inhibitor that has been shown to induce substantial protein tyrosine phosphorylation in treated T and B lymphocytes (28–30). Aliquots of the NK cell clone 5F12 were either untreated or treated with 100 μM PAO for 2 min, then lysed and immunoprecipitated with anti-p58 or anti–HLA class I antibodies. Whole-cell lysates and immunoprecipitates were separated by SDS-PAGE and analyzed by sequential immunoblotting using antiphosphotyrosine and anti–PTP-1C antibodies.

Antiphosphotyrosine immunoblotting revealed substantial increases in tyrosine phosphorylation of many proteins in whole-cell lysates of PAO-treated NK cells, as compared to the untreated cells (Fig. 2 *A, left panels*). PAO treatment also dramatically increased the intensity and number of tyrosine phosphorylated species in p58 immunoprecipitations. The major tyrosine phosphoprotein in this immunoprecipitation directly overlapped with the p58 protein (Fig. 2 *B*), indicating that the p58 KIR is inducibly tyrosine phosphorylated by PAO treatment. Additional p58-associated tyrosine phosphoprotein bands were detected with apparent molecular masses of 63, 71, 74, 78, and 105 kD. In control MHC class I immunoprecipitations, a single tyrosine phosphoprotein of ∼55 kD that was unaffected by PAO stimulation was detected.

Anti–PTP-1C immunoblotting detected equal amounts of a protein with the expected molecular mass of 65 kD in lysates from both untreated and PAO-treated cells (Fig. 2 *A,*
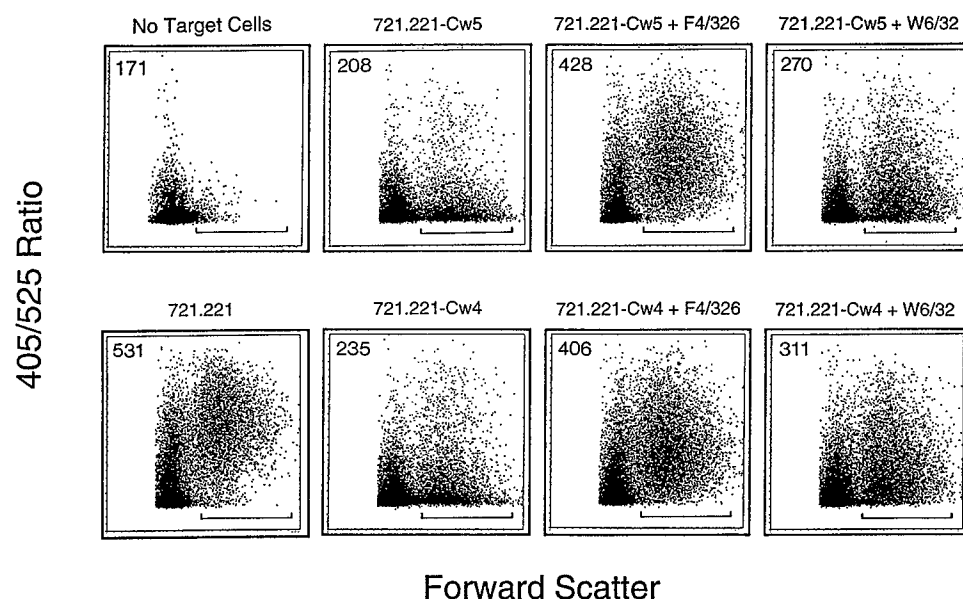

No Target Cells   721.221-Cw5   721.221-Cw5 + F4/326   721.221-Cw5 + W6/32

171   208   428   270

405/525 Ratio

721.221   721.221-Cw4   721.221-Cw4 + F4/326   721.221-Cw4 + W6/32

531   235   406   311

Forward Scatter

Figure 1. NK cell calcium mobilization is inhibited by MHC class I recognition on cytotoxicity-resistant target cells. The NK cell clone 5F12 was loaded with indo-1 and centrifuged with the indicated target cells before flow cytometric analysis. Some target cells were preincubated for 30 min at 4°C with the anti–HLA-C antibody F4/326 or the anti–pan MHC class I antibody W6/32 before mixing. NK cells that are directly conjugated with targets exhibit distinct shift in FSC (indicated by brackets). The 405/525 emission ratio directly correlates with the intracellular Ca^{2+} concentration of the individual indo-1-loaded NK cells. The mean fluorescence of the conjugated cell population is indicated in the upper left corner of each panel. Each panel represents individual cell recordings accumulated during a 4-min period. This profile was maintained for at least 15 min (data not shown).

A

B

Figure 2. Phenylarsine oxide treatment of NK cells induces tyrosine phosphorylation of p58 receptor and association of PTP-1C. (*A*) The NK cell clone 5F12 was unstimulated (-) or stimulated (+) for 2 min with 100 μM PAO in a 37°C shaking block. An aliquot of 1 million cells was immediately lysed in Laemmli buffer, while the remainder was lysed in 1% Triton X-100 buffer and immunoprecipitated with W6/32 (*MHC*) or anti-p58 (*p58*) antibodies. A control was performed in which anti-p58 was identically precipitated from lysis buffer lacking cells (no cells), to rule out immunoblotting reactivity with the heavy chains of the immunoprecipitating antibody. Lysates and immunoprecipitates were separated by SDS-PAGE, transferred to nitrocellulose, immunoblotted with antiphosphotyrosine antibody (4G10-HRP; *left panel*), stripped, and secondarily immunoblotted with anti–PTP-1C antibody (polyclonal; *right panel*). (*B*) The NK cell clone 5F12 was surface ^{125}I-labeled, lysed in 1% Triton X-100, and immunoprecipitated with either anti–MHC class I antibody W6/32 and protein A–Sepharose (MHC), or with the anti-p58 antibody and anti–mouse IgM (p58; HP-3E4 and b-7-6), or with the anti–mouse IgM alone (anti-IgM; b-7-6). Immunoprecipitates were separated on SDS-PAGE, the gel was dried, and labeled proteins were detected by autoradiography.

right panels). Interestingly, in the p58 immunoprecipitate from PAO-treated cells, a substantial amount of PTP-1C was detected in association with the p58 KIR, while only minimal anti–PTP-1C reactivity was observed in immuno-

precipitates from untreated cells. PTP-1C was not detected in anti–MHC class I immunoprecipitates from either untreated or PAO-treated cells. In conclusion, these results indicate that PAO induces substantial tyrosine phosphory-

Figure 3. PTP-1C associates with tyrosine-phosphorylated p58 receptor in NK cells conjugated with resistant target cells. The NK cell line 5F12 was conjugated with target cells (721.221, sensitive target and HLA-Cw4–transfected 721.221, resistant target) by centrifugation as detailed in Materials and Methods. Lysates of conjugated cells or identically treated NK cells or target cells were precleared (b-7-6), immunoprecipitated with anti-p58 antibody (HP-3E4 and b-7-6), and secondarily immunoprecipitated with antiphosphotyrosine antibody (PY20). Immunoprecipitates and NK cell lysate (1 million cells lysed in sample buffer) were separated on SDS-PAGE and probed first with antiphosphotyrosine antibody (4G10-HRP) and secondarily with anti–PTP-1C (monoclonal and goat anti-mouse IgG-HRP). The migration of p58 and PTP-1C are marked with arrows.

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lation of p58 and association of PTP-1C with p58 KIR. Moreover, PAO induces the phosphorylation of and/or association of several additional tyrosine phosphoproteins that coprecipitate with the p58 KIR.

PTP-1C Is Recruited to p58 KIR upon NK Cell Conjugation with Target Cells. Since PAO treatment is a nonphysiological stimulus of tyrosine phosphorylation, we also tested whether PTP-1C could inducibly associate with p58 KIR upon receptor ligation that occurs during the conjugation of our characterized NK cell clone 5F12 with target cells. As shown in Fig. 3, significant tyrosine phosphorylation is induced upon conjugation of the NK cells with either sensitive or resistant target cells, as has been shown previously by Kaufman et al. (18). Immunoprecipitation of p58 KIR from lysates of both cell conjugates revealed that a protein

migrating at 58 kD was tyrosine phosphorylated and that numerous additional tyrosine phosphorylated proteins became associated with the receptor. Immunoblotting with anti–PTP-1C demonstrated that the phosphatase indeed becomes associated with p58 KIR upon conjugation of NK cells with resistant targets. To a lesser extent, PTP-1C also associated with p58 KIR upon conjugation with sensitive target cells, which was also observed in several additional experiments. Nevertheless, PTP-1C associated more efficiently with p58 KIR upon NK cell interaction with resistant target cells.

Both Tyrosine Residues in the Cytoplasmic Domain of p58 KIR Bind and Activate PTP-1C upon Phosphorylation. Since the cytoplasmic domain of p58 KIR has two YXXL motifs, the role of phosphorylation of each of these motifs on PTP-1C
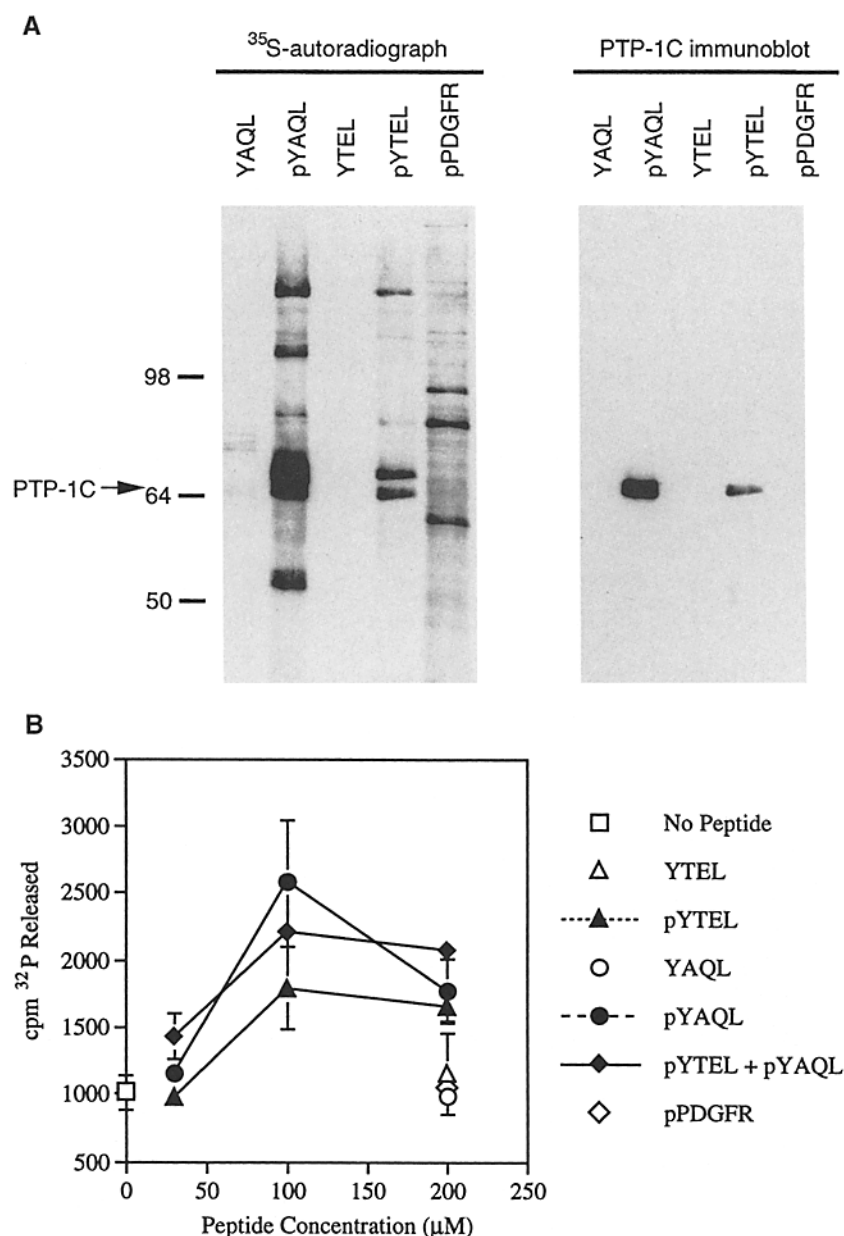
Figure 4. Tyrosine phosphopeptides bind several proteins from NK cell lysates, including PTP-1C, and enhance PTP-1C phosphatase activity. (*A*) The NK cell clone 5F12 was ^{35}S-metabolically labeled and lysed in 1% Triton X-100 buffer (25 million cells/sample lysed in 0.5 ml). The lysates were cleared by centrifugation and precipitated with peptides coupled to AminoLink gel. Precipitates were separated by SDS-PAGE and transferred to nitrocellulose. The membrane was immunoblotted with anti–PTP-1C (polyclonal; *right panel*) and subsequently dried and autoradiographed (*left panel*). (*B*) Enzymatic activity of PTP-1C was assayed by measuring the radioactivity released from a [^{32}P]phosphotyrosine peptide substrate in the presence or absence of the p58-derived peptides and phosphotyrosine peptides, as detailed in Materials and Methods. The cpm of ^{32}P-O$_4$ released are averages with range bracketed from duplicate samples, as measured by scintillation counting and are from a representative of three experiments. The PDGF receptor tyrosine phosphopeptide (pPDGFR) encompassed residues 739–746. The combination samples (pYTEL + pYAQL) were treated with the indicated concentration of each phosphopeptide.

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binding was examined with synthetic tyrosine phospho-peptides. Peptides containing sequences surrounding the p58 KIR tyrosine residues were coupled to AminoLink Plus coupling gel and used as affinity matrices to bind proteins from Triton X-100 lysates of ^{35}S-metabolically labeled NK cell clone 5F12. Few, if any, proteins bound to un-phosphorylated tyrosine–containing peptides, but phos-phorylated versions of the same peptides bound several distinct protein species (Fig. 4 A, *left panel*). The pYAQL-containing phosphopeptide bound at least nine proteins of 54, 65, 69, 81, 83, 102, 108, 120, and 131 kD while the pYTEL-containing phosphopeptide bound a subset of the same proteins of 65, 69, 81, 108, 120, and 131 kD. Alternatively, a control tyrosine phosphopeptide from the cyto-plasmic domain of the PDGF receptor-β exhibited different binding specificity and adsorbed three major proteins (60, 81, and 90 kD). The 65-kD protein band that was bound by both of the p58 phosphopeptides corresponded to the predicted molecular mass for PTP-1C. Subsequent immunoblotting identified this band as PTP-1C (Fig. 4 A, *right panel*). Nevertheless, PTP-1C routinely bound more to the pYAQL peptide than to the pYTEL peptide and was less susceptible to elution by 50 mM p-nitrophenylphos-phate when associated with the pYAQL peptide (data not shown). Thus, both tyrosine residues in the cytoplasmic domain of p58 have the capacity to bind PTP-1C and additional proteins exhibit distinct binding capacities towards the phosphopeptides.

Both of the p58-derived tyrosine phosphopeptides elevated enzymatic activity of PTP-1C in a concentration-dependent manner, as presented in Fig. 4 B. The pYAQL phosphopeptide was more potent in enhancing phosphatase activity than pYTEL, which parallels the PTP-1C–binding capacity of the phosphopeptides. The combination of both phosphopeptides was at best additive. Unphosphorylated peptides or the PDGF receptor–derived tyrosine phospho-peptide did not stimulate phosphatase activity.

Discussion

These observations provide advances in our understanding of the mechanism of inhibitory receptor function in human NK cells. It has been previously shown that inhibi-tion of the inositol phosphate turnover and calcium mobili-zation response are critical components of the downregula-tion of NK cell cytotoxicity by class I molecules (18). Our results parallel these observations and imply a role for PTP-

1C as a cytosolic mediator of KIR function. Upon tyrosine phosphorylation, the p58 KIR binds to PTP-1C via two separate ITIM motifs, which may interact with one or both of the SH2 domains of PTP-1C. Recruitment of PTP-1C is paralleled by activation, as suggested by the stimulation of tyrosine phosphatase activity of PTP-1C in vitro (Fig. 4 B) by p58-derived phosphotyrosine peptides.

Tyrosine phosphorylation of p58 KIR and association of PTP-1C occurs in response to pharmacological stimulation with PAO, as well as to physiological conjugation of NK cells with resistant target cells. In our experiments, p58 KIR is also tyrosine phosphorylated when NK cells are con-jugated with sensitive target cells. This is probably caused by a profound global tyrosine phosphorylation that is stim-ulated in NK cells that interact with sensitive target cells (18). Still, PTP-1C is more efficiently recruited when the receptor is specifically engaged by the inhibitory ligand. It is also likely that PTP-1C is only one of the components of the inhibitory cascade. Further characterization of the addi-tional proteins that bind p58 KIR (Fig. 3) and KIR phos-phopeptides (Fig. 4 A) is underway.

The apparent role for PTP-1C in the inhibitory receptor mechanism is strikingly similar to the mechanism by which the FcγRIIB1 and CD22 receptors negatively regulate BCR function (9, 17). A critical aspect of the mechanism of these inhibitory receptors is their direct association with the BCR, thereby bringing the phosphatase directly to the antigen receptor and negatively regulating calcium mobili-zation through the antigen receptor. FcγRIIB1 can be re-cruited to the BCR by bound antigens with associated se-rum IgG (9, 11, 12), whereas CD22 is believed to be weakly associated with the BCR (17, 32). It is not known whether KIRs are associated with an activating receptor(s) and whether this association is required to induce negative signaling. Previous experiments with bystander cells have shown that NK cell cytotoxicity against class I–deficient target cells is not affected by the presence of class I–positive bystanding cells (22). This lack of bystander effect suggests that the KIRs generate a negative signaling directed at the interface of effector–target cell interaction rather than a global cell anergy. Thus, colocalization of KIRs with stimulatory re-ceptors may be required to mediate cross-talk inhibition. Further studies are required to characterize the human NK cell stimulatory receptors, determine whether they associ-ate with inhibitory receptors, and establish how they are regulated through PTP-1C recruitment.

References

1. Yokoyama, W.M. 1993. Recognition structures on natural killer cells. *Curr. Opin. Immunol.* 5:67–73.
2. Moretta, A., M. Vitale, C. Bottino, A.M. Orengo, L. Morelli, R. Augugliaro, M. Barbaresi, E. Ciccone, and L. Moretta. 1993. p58 molecules as putative receptors for major histocompatibility complex (MHC) class I molecules in human natural killer (NK) cells. Anti-p58 antibodies reconstitute lysis of MHC class I–protected cells in NK clones displaying different specificities. *J. Exp. Med.* 178:597–604.
3. Litwin, V., J. Gumperz, P. Parham, J.H. Phillips, and L.L. Lanier. 1994. NKB1: a natural killer cell receptor involved in the recognition of polymorphic HLA-B molecules. *J. Exp. Med.* 180:537–543.
4. Melero, I., A. Salmeron, M.A. Balboa, J. Aramburu, and M. Lopez-Botet. 1994. Tyrosine kinase–dependent activation of human NK cell functions upon stimulation through a 58-kDa surface antigen selectively expressed on discrete subsets of NK cells and T lymphocytes. *J. Immunol.* 152:1662–1673.
5. Colonna, M., and J. Samaridis. 1995. Cloning of immunoglobulin-superfamily members associated with HLA-C and HLA-B recognition by human natural killer cells. *Science (Wash. DC).* 268:405–408.
6. Wagtmann, N., R. Biassoni, C. Cantoni, S. Verdiani, M.S. Malnati, M. Vitale, C. Bottino, L. Moretta, A. Moretta and E.O. Long. 1995. Molecular clones of the p58 NK cell receptor reveal immunoglobulin-related molecules with diversity in both the extra- and intracellular domains. *Immunity.* 2:439–449.
7. D'Andrea, A., C. Chang, K. Franz-Bacon, T. McClanahan, J.H. Phillips and L.L. Lanier. 1995. Molecular cloning of NKB1, a natural killer cell receptor for HLA-B allotypes. *J. Immunol.* 155:2306–2310.
8. Moretta, A., S. Sivori, M. Vitale, D. Pende, L. Morelli, R. Augugliaro, C. Bottino, and L. Moretta. 1995. Existence of both inhibitory (p58) and activatory (p50) receptors for HLA-C molecules in human natural killer cells. *J. Exp. Med.* 182:875–884.
9. D'Ambrosio, D., K.L. Hippen, S.A. Minskoff, I. Mellman, G. Pani, K.A. Simonovitch and J.C. Cambier. 1995. Recruitment and activation of PTP1C in negative regulation of antigen receptor signaling by FcγRIIB1. *Science (Wash. DC).* 268:293–297.
10. Thomas, M.L. 1995. Of ITAMs and ITAMs: turning on and off the B cell antigen receptor. *J. Exp. Med.* 181:1953–1956.
11. Amigorena, S., C. Bonnerot, J.R. Drake, D. Choquet, W. Hunziker, J.G. Guillet, P. Webster, C. Sautes, I. Mellman, and W.H. Fridman. 1992. Cytoplasmic domain heterogeneity and functions of IgG Fc receptors in B lymphocytes. *Science (Wash. DC).* 256:1808–1812.
12. Muta, T., T. Kurosaki, Z. Misulovin, M. Sanchez, M.C. Nussenzweig, and J.V. Ravetch. 1994. A 13-amino-acid motif in the cytoplasmic domain of Fc gamma RIIB modulates B-cell receptor signalling. *Nature (Lond.).* 368:70–73.
13. Shen, S.-H., L. Bastien, B.I. Posner, and P. Chrétien. 1991. A protein-tyrosine phosphatase with sequence similarity to the SH2 domain of the protein-tyrosine kinases. *Nature (Lond.).* 352:736–739.
14. Yi, T., J.L. Cleveland, and J.N. Ihle. 1992. Protein tyrosine phosphatase containing SH2 domains: characterization, preferential expression in hemopoietic cells, and localization to human chromosome 12p12-p13. *Mol. Cell. Biol.* 12:836–846.
15. Plutzky, J., B.G. Neel, and R.D. Rosenberg. 1992. Isolation of a src homology 2–containing tyrosine phosphatase. *Proc. Natl. Acad. Sci. USA.* 89:1123–1127.
16. Matthews, R.J., D.B. Bowne, E. Flores, and M.L. Thomas. 1992. Characterization of hematopoietic intracellular protein tyrosine phosphatase: description of a phosphatase containing an SH2 domain and another enriched in proline-, glutamic acid-, serine-, and threonine-rich sequences. *Mol. Cell Biol.* 12:2396–2405.
17. Doody, G.M., L.B. Justement, C.C. Delibrias, R.J. Matthews, J. Lin, M.L. Thomas, and D.T. Fearon. 1995. A role in B cell activation for CD22 and the protein tyrosine phosphatase SHP. *Science (Wash. DC).* 269:242–244.
18. Kaufman, D.S., R.A. Schoon, M.J. Robertson, and P.J. Liebson. 1995. Inhibition of selective signaling events in natural killer cells recognizing major histocompatibility complex class I. *Proc. Natl. Acad. Sci. USA.* 92:6484–6488.
19. Yokoyama, W.M. 1995. Right-side-up and up-side-down NK-cell receptors. *Curr. Biol.* 5:982–985.
20. Cella, M., A. Longo, G.B. Ferrara, J.L. Strominger, and M. Colonna. 1994. NK3-specific natural killer cells are selectively inhibited by Bw4-positive HLA alleles with isoleucine 80. *J. Exp. Med.* 180:1235–1242.
21. Shimizu, Y., and R. De Mars. 1989. Production of human cells expressing individual transferred HLA-A,-B,-C genes using an HLA-A,-B,-C null human cell line. *J. Immunol.* 142:3320–3328.
22. Colonna, M., G. Borsellino, M. Falco, G.B. Ferrara, and J.L. Strominger. 1993. HLA-C is the inhibitory ligand that determines dominant resistance to lysis by NK1- and NK2-specific natural killer cells. *Proc. Natl. Acad. Sci. USA.* 90:12000–12004.
23. Mizuno, S., S.H. Kang, H.W. Lee, S.A. Trapani, B. Dupont, and S.Y. Young. 1989. Isolation and expression of a cDNA clone encoding HLA-Cw6: unique characteristics of HLA-C encoded gene products. *Immunogenetics.* 29:323–330.
24. Gronwald, R.G., F.J. Grant, B.A. Haldeman, C.E. Hart, P.J. O'Hara, F.S. Hagen, R. Ross, D.F. Bowen-Pope, and M.J. Murray. 1988. Cloning and expression of a cDNA coding for the human platelet-derived growth factor receptor: evidence for more than one receptor class. *Proc. Natl. Acad. Sci. USA.* 85:3435–3439.
25. Portoles, P., J.M. Rojo, and C.A. Janeway, Jr. 1990. A simple

method for the radioactive iodination of CD4 molecules. *J. Immunol. Methods.* 29:105–109.

26. Valitutti, S., M. Dessing, K. Aktories, H. Gallati, and A. Lanzavecchia. 1995. Sustained signaling leading to T cell activation results from prolonged T cell receptor occupancy. Role of T cell actin cytoskeleton. *J. Exp. Med.* 181:577–584.

27. Laemmli, U.K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage. *Nature (Lond.).* 227:680–685.

28. Garcia-Morales, P., Y. Minami, E. Luong, R.D. Klausner, and L.E. Samelson. 1990. Tyrosine phosphorylation in T cells is regulated by phosphatase activity: studies with phenylarsine oxide. *Proc. Natl. Acad. Sci. USA.* 87:9255–9259.

29. Oetken, C., M. von-Willebrand, A. Marie-Cardine, T. Pessa-Morikawa, A. Stahls, S. Fisher, and T. Mustelin. 1994. Induction of hyperphosphorylation and activation of the

p56[l]ck protein tyrosine kinase by phenylarsine oxide, a phosphotyrosine phosphatase inhibitor. *Mol. Immunol.* 31:1295–1302.

30. Campbell, K.S., W.D. Bedzyk, and J.C. Cambier. 1995. Manipulation of B cell antigen receptor tyrosine phosphorylation using aluminum fluoride and sodium orthovanadate. *Molec. Immunol.* 32:1283–1294.

31. Strueli, M., N.X. Krueger, A.Y.M. Tsai, and H. Saito. 1989. A family of receptor-linked protein tyrosine phosphatases in humans and *Drosophila. Proc. Natl. Acad. Sci. USA.* 86:8698–8702.

32. Leprince, C., K.E. Draves, R.L. Geahlen, J.A. Ledbetter, and E.A. Clark. 1993. CD22 associates with the human surface IgM-B-cell antigen receptor complex. *Proc. Natl. Acad. Sci. USA.* 90:3236–3240.