Association of Killer Cell Ig-like Receptor (KIR) with an Adaptor Protein Shc

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ABSTRACT

Background: Cytotoxic function of killer cells is inhibited by specific recognition of class I MHC molecules on target cells by inhibitory killer Ig-like receptors (KIR) expressed on NK cells and some cytotoxic T cells. The inhibitory effect of KIR is accomplished by recruitment of SH2-containing protein tyrosine phosphatase (SHP) to the phosphotyrosine residues in the cytoplasmic tail. **Methods:** By *in vitro* coprecipitation experiments and transfection analysis, we investigated the association of KIR with an adaptor protein Shc in Jurkat T cells. **Results:** The cytoplasmic tail of KIR appeared to associate with an adaptor protein Shc in Jurkat T cell lysates. Similar *in vitro* experiments showed that phosphorylated KIR cytoplasmic tail bound SHP-1 and Shc in Jurkat T cell lysates. The association of KIR with Shc was further confirmed by transfection analysis in 293T cells. Interestingly, however, Shc appeared to be replaced by SHP-2 upon engagement of KIR in 293T cells. **Conclusion:** Our data indicate that KIR associate with an adaptor protein Shc in Jurkat T cells, and suggest that KIR might have an additional role which is mediated by this adaptor protein. **(Immune Network 2006;6(2):67-75)**

Key Words: KIR, Shc, SHP-1, SHP-2, cell activation, cell proliferation, inhibitory signal transduction

Introduction

NK cells and some T cells express members of a multigenic family of killer cell Ig-like receptors (KIRs)[3] that recognize polymorphic class I MHC molecules on target cells (reviewed in refs. 1-4). Both natural cytotoxicity and antibody dependent cell cytotoxicity of NK cells, and CD3/TCR dependent cytotoxicity of T cells are inhibited by the coengagement of KIRs and activating receptors such as FcR and TCR (5-8). Recent studies have suggested that KIR plays a role in the survival of memory-phenotype T cells (9,10) and in the inhibition of T cell activation induced cell death (AICD) (9,11,12). However, the exact mechanism of the AICD inhibition mediated by KIR has not been revealed yet. Inhibitory KIRs (KIR hereafter) are type I transmembrane glycoproteins belonging to the Ig superfamily and consist of either two (for p58 KIR and KIR103: KIR2D family) or three (for p70 KIR: KIR3D family) extracellular Ig-related domains, a transmembrane part and a cytoplasmic tail (13-16). The extracellular domain of KIR specifically recognizes particular class I MHC molecules expressed on target cells (17-20). The cytoplasmic tail of KIR contains either one (for KIR103) or two (for p58 and p70 KIR) immunoreceptor tyrosine-based inhibition motifs (ITIMs) that function in triggering an inhibitory signal transduction that, in turn, prevents killer cell-mediated cytotoxicity (21-24). The ITIM, which has the consensus sequence of (I/V) xYxx (L/V), was originally found in the cytoplasmic tail of FcγRIIB (25,26). FcγRIIB has also been shown to transmit the inhibitory signals in B cell activation by recruitment of SH2-containing inositol phosphatase (SHIP) upon tyrosine phosphorylation of the ITIM (25,27,28). The consensus sequence of ITIM is similar but clearly distinguishable from the immunoreceptor tyrosine-based activation motif (ITAM) found in the signaling molecules associated with several lymphocyte antigen receptors (29,30).

Upon engagement of KIR, tyrosine residues in

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ITIMs are phosphorylated by a src-family protein tyrosine kinase (like p56 Lck), and in turn a SH2-containing protein tyrosine phosphatase (SHP), SHP-1, is recruited to the phosphotyrosyl residues (21-24,31). It has also been shown that another SHP, SHP-2 (31, 32), and the p85α regulatory subunit of phosphatidylinositol (PI) 3-kinase (32) are recruited to the phosphotyrosyl residues of KIR. The functional importance of SHP-1 in the KIR-mediated inhibition of NK cell activation is further supported by the observation that the overexpression of a dominant negative form of SHP-1 reverses the inhibitory effect of KIR (21,22). It is thought that the inhibitory effect of KIR results from the dephosphorylation of phospho-tyrosine residues in signaling molecules involved in the activating signal transduction pathway by SHP (SHP-1 or SHP-2) recruited to the KIR. For FcR-mediated ADCC of NK cells, Binstadt et al. (21) reported that coengagement of FcR and KIR inhibited the phosphorylation of FcR-associated signaling molecules including TCRζ, ZAP-70 and PLC-γ. Similarly, when ζ, ZAP-70, and Lck were coexpressed with SHP-1 in Sf21 insect cells, the levels of tyrosine phosphorylation in these molecules decreased substantially (33). It has also been demonstrated that class I MHC recognition by KIR blocks the formation of a complex between tyrosine-phosphorylated pp36 (LAT) and PLC-γ in human NK cells (34). And Binstadt et al. have demonstrated that the SH2-containing leukocyte protein 76 (SLP-76) may be a specific target for dephosphorylation by SHP-1 in T cells and NK cells (35). These results suggest that SHP recruited to KIR may dephosphorylate those signaling molecules in NK cells and T cells.

Shc is an adaptor protein composed of a unique N-terminal phospho-tyrosine binding domain (PTB), a glycine/proline-rich collagen homology domain (CH) and a C-terminal SH2 domain (36-38). Shc is ubiquitously expressed in three isoforms of 46, 52, and 66 kDa, but only the 46 and 52 kDa forms are present in hematopoietic cells (36). Shc has been implicated in Ras activation triggered by a number of receptors, including growth factor receptors, antigen receptors and cytokine receptors (reviewed in refs. 39,40). The SH2 and PTB domains of Shc have been shown to interact with the tyrosine phosphorylated receptors. Shc is tyrosine phosphorylated upon stimulation of the receptors and subsequently it interacts with Grb2 through the CH domain. The Shc : Grb2 complex, in turn, associates with the GTP/GDP exchange factor, SOS, and finally leads to Ras activation. Shc can also activate the Akt pathway via Shc-Grb2-Gab2-PI3K (phosphatidylinositol 3-kinase) in B cells (41).

In this report, by *in vitro* coprecipitation experiments and transfection analyses, we showed that KIR associates with an adaptor protein Shc in resting cells. We further demonstrated that Shc is replaced by SHP upon engagement of KIR by antibody.

Materials and Methods

Cell lines. The human T cell lymphoma cell line, Jurkat was obtained from the American Type Culture Collection (ATCC, Rockville, MD) and maintained in culture with RPMI 1640 medium (Flow Laboratories, Rockville, MD) supplemented with 10% (V/V) Fetal Bovine Serum (FBS, Sigma, St. Louis, MO), 1% L-glutamine and 1% antibiotic solution. Human embryonic kidney epithelial cells expressing SV 40 large T antigen (293T) were obtained from the ATCC and cultured in Dulbecco's modified Eagle medium (DMEM)(Flow Laboratories, Rockville, MD) supplemented with 10% FBS 1% L-glutamine and 1% antibiotic solution.

Antibodies and other reagents. Anti-phospho tyrosine antibody (PY20), anti-SHP-1, anti-SHP-2, anti-Grb2 and anti-Shc antibodies were purchased from Transduction Laboratories (Lexington, KY). Fluorescein isothiocyanate (FITC) conjugated anti-mouse IgG and Texas-red conjugated anti-rabbit IgG were obtained from Vector Laboratories (Burlingamme, CA), and PE-conjugated anti-mouse IgG and anti-CD8 antibodies were from Beckton Dickinson (San Jose, CA). Dithiobissuccinimidyl propionate (DSP) was purchased from Pierce (Rockford, IL). Ni-NTA resin was obtained from Qiagen (Santa Clarita, CA), and protein A-agarose and protein G-agarose were from Boeringer Manheim (Indianapolis, IN). cDNA of a p70 KIR (NKB1) was a kind gift from Dr. Lanier (DNAX Research Inst., Palo Alto, CA). Polyclonal antibodies to the cytoplasmic tail of KIR were produced in mice using the bacterially expressed His-tag fusion protein of KIR (His-CytKIR; refs. 42,43). Briefly, the His-CytKIR protein was further purified on SDS polyacrylamide gels and the protein band was excised with a scalpel. The gel slices were homogenized and injected into mice intraperitoneally. A booster injection was given 2 weeks after the primary immunization. Another two booster injections were given every two weeks, and the animals were bled 2 weeks later.

His-tag and CD8 fusion constructs of KIR cytoplasmic tail, and other DNA constructs. The His-tag fusion protein construct of a p70 KIR (NKB1; ref. 14) cytoplasmic tail (pHis-CytKIR) was described previously (42,43), and the generation of plasmid containing the extracellular and transmembrane portion of CD8α, pCD 8T, was also described previously (44). A CD8 fusion construct of KIR cytoplasmic tail was generated by subcloning the cytoplasmic tail of NKB1 into the pCD8T. Briefly, the cytoplasmic tail of NKB1 was amplified by PCR with primers 5'-GGGGAATTCC

ATCTCTGGTGCTCCAACAAAAAAAAT-3' and 5'-GGGGGATCCTCATGGGCAGGAGACAACTTT GG-3'. The amplified DNAs were cloned into EcoRI and BamHI sites of pBluscript (Strategene, La Jolla, CA) and verified by nucleotide sequencing. Then the KIR cytoplasmic fragment was inserted in frame into the EcoRI sites of pCD8T. The resulting chimeric receptor (CD8-KIR) was excised from the plasmid and ligated into SalI and BamHI sites of the eucaryotic expression vector, pHβAPr-1-neo (45).

Metabolic labeling of Jurkat T cells/Preparation of cell lysates. Jurkat cells (5×10^6 cells) were washed with 10 ml of methionine-free RPMI 1640 and preincubated for 1 hr in 10 ml of methionine-free RPMI 1640 supplemented with 10% heat-inactivated dialyzed FBS. Cells were then incubated in the presence of 100μ Ci/ml L-[^{35}S]methionine at 37°C for 2 hr. After labeling, the cells were washed two times with cold PBS, and lysed in 0.5 ml of cold lysis buffer containing 20 mM Na-Phosphate (pH 7.5), 0.15 M NaCl, 1% Triton X-100, 1 mM PMSF, 5μg/ml leupeptin, 5μg/ml pepstatin, 0.02% NaN₃, and 1 mM iodoacetamide. Cell lysates were cleared by centrifugation at 13,000 rpm for 20 min, and the supernatant was used for coprecipitation and immunoblot analysis. Non-labeled Jurkat cell lysates were also similarly prepared.

Coprecipitation and Western blotting. The His-tag fusion protein of KIR cytoplasmic tail (His-CytKIR) was added to [^{35}S]-labeled Jurkat cell lysates in the presence and absence of a chemical cross-linker (DSP, a final concentration of 2 mM), and incubated for 1 hr at 4°C. The reaction mixtures were precipitated with Ni-NTA resin, as previously described (43). The precipitates were separated on 12% SDS polyacrylamide gels, and the gels were then fixed, dried, and autoradiographed. For Western blot analysis, His-CytKIR was mixed with Jurkat cell lysates in the presence of DSP, and incubated for 1 hr at room temperature. The reaction mixtures were precipitated with Ni-NTA resin, and washed three times. Precipitates were resolved by SDS-PAGE using 12% acrylamide gels, and proteins were transferred onto polyvinylidene difluoride (PVDF) membranes (Immobilon-P; Millipore, Marlborough, MA). Western blot analysis was performed with anti-Shc antibody, anti-Grb2 antibody, and anti-phosphotyrosine antibody, as previously described (43).

In vitro phosphorylation of His-CytKIR. Jurkat cell lysates and the His-CytKIR protein were mixed and incubated in a kinase reaction buffer, as previously described (43). After *in vitro* phosphorylation, the His-Cyt KIR was precipitated with Ni-NTA resin. The precipitates were resolved on SDS PAGE gels, and the gels were blotted onto PVDF membranes and analyzed with anti-Grb2, anti-Shc and anti-phosphotyrosine

antibodies.

Immunofluorescence staining and Confocal Microscopy. Cells transfected with the pCD8T or pCD8-KIR cDNA were grown on sterile glass coverslides precoated with 1% gelatin overnight prior to antibody staining. 36 hr after transfection, cells were fixed with ice-cold methanol and aceton for 10 min each and incubated with 3% H₂O₂ for 30 min at room temperature. The coverslides were washed three times with PBS and incubated with blocking solution (5% BSA, 3% normal horse serum, 0.3% Tritonx-100 in PBS) for 3 hr. The cells were simultaneously stained by incubating them with anti CD8 antibody ascites (1 : 100) followed by a goat antiserum against mouse IgG coupled to FITC and rabbit anti-Shc antibody followed by a goat antiserum against rabbit IgG coupled to Texas-red at 37°C for 2~3 hr and then washed 4 times in PBS. After 3 additional washes in PBS, the slides were mounted in mounting solution. Cells were viewed by confocal microscope (Lica PCSMT, Heidelberg, Germany) using the 100× objective lens.

Immunoprecipitation and Western blotting. 293T cells transfected with CD8T or CD8-KIR were stimulated with OKT8 ascites (1 : 200 dilution). After 30 min stimulation, the cells were rinsed twice with ice-cold PBS and lysed in a lysis buffer (10 mM Tris, pH 7.5, 1% Triton X-100, 150 mM NaCl, 10 mM Na₃VO₄, 1 mM NaF, 1 mM Na pyrophosphate, 5 mM EDTA, 50μg/ml leupeptin, 1 U/ml aprotinin, 1 mM pepstatin, 1 mM PMSF). Samples were incubated for 1hr at 4°C and centrifuged (13,000 rpm) for 20 min at 4°C. For immunoprecipitation, the lysates from 5×10^6 cells in 700μl were precleared by mixing for 3 hr at 4°C with protein A agarose. The precleared lysates were then incubated with anti-Shc antibody on a rotating shaker for 1 hr at 4°C, and protein A agarose beads were added and further incubated overnight at 4°C. The precipitates were washed 4 times with wash buffer (cell lysis buffer with salt concentration increased by 200% and detergent concentration decreased by 50%) and twice with 10 mM Tris (pH 7.5) containing 50 mM NaCl and 0.1% triton X-100. After SDS PAGE, the proteins were transferred onto PVDF membrane, and blotted with anti-His-CytKIR antibodies. The membrane was reprobed with anti-SHP-2 antibody. A reciprocal immunoprecipitation experiment was also carried out with anti-His-CytKIR antibodies followed by immunoblotting with anti-Shc antibody and subsequently with anti-SHP-2 antibody.

Results

The cytoplasmic tail of KIR associates with Shc, Grb2, TCR-ζ and a protein of 36 kDa in Jurkat T cell lysates. Coaggregation of an activating receptor, such as TCR or FcR, and KIR is required for the inhibition of NK
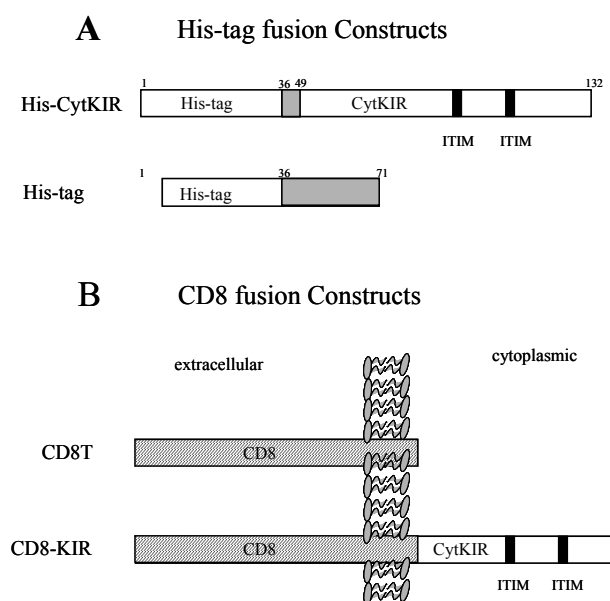
A His-tag fusion Constructs

His-CytKIR
```
      1              36 49                              132
      |  His-tag  |  |    CytKIR    |  ■  ■  |
                                      ITIM ITIM
```

His-tag
```
      1                    36        71
      |  His-tag  |                |
```

B CD8 fusion Constructs

extracellular   cytoplasmic

CD8T
```
|  CD8  |
```

CD8-KIR
```
|  CD8  |          | CytKIR  ■  ■ |
                              ITIM ITIM
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Figure 1. His-tag and CD8 fusion protein constructs. (A) The His-CytKIR construct encodes a His-tag fusion protein of the cytoplasmic tail of a p70 KIR (NKB1). The protein coding region of the cytoplasmic tail of KIR was subcloned into an *E. coli* expression vector pRSETA. The His-tag construct (pRSETA itself) encodes a control His-tag fusion protein which lacks the cytoplasmic tail of KIR. (B) The CD8 fusion protein constructs. The generation of plasmid containing the extracellular and transmembrane portion of CD8α, pCD8T, was described previously (44). A CD8 fusion construct of KIR was generated by subcloning the cytoplasmic tail of KIR into eucaryotic expression vector, pH βAPr-1 (45).

or T cell activation (46,47), implying that the inhibitory effect mediated by KIR may occur in the immediate vicinity of the activating receptor. We first asked whether the cytoplasmic tail of KIR interacts with signaling molecules that are also associated with activating receptors, such as TCR, BCR and FcR. To identify proteins associated with the cytoplasmic tail of KIR, we generated a His-tag fusion protein of the cytoplasmic tail (His-CytKIR, Fig. 1A) and used this fusion protein to co-precipitate proteins from metabolically-labeled Jurkat T cells. Ni-NTA resin was used to coprecipitate proteins interacting with the His-CytKIR. In Jurkat cell lysates, the cytoplasmic tail of KIR coprecipitated the proteins of 46 kDa, 36 kDa, 23 kDa and 16 kDa (Fig. 2A, lane 2). The coprecipitates appeared as higher Mr (50∼100 kDa) complexes under nonreducing conditions (Fig. 2A, lane 3), suggesting that the four proteins are really cross-linked to His-CytKIR by DSP, a disulfide containing cross-linker. A control His-tag protein expressed from the pRSETA expression plasmid *per se* did not result in any detectable proteins coprecipitated in the

cell lysates (Fig. 2A, lane 1). The protein bands were also detectable when the precipitates were obtained without a chemical cross-linking procedure (Fig. 2C for the 46 kDa protein; data for other proteins not shown), though less prominent than with chemical cross-linking. These results suggest that the cytoplasmic tail of KIR associates specifically with the 46 kDa, 36 kDa, 23 kDa and 16 kDa proteins in Jurkat cell lysates.

Previously, we identified that the 16 kDa protein is TCRζ by immunoblotting experiments with antibodies against TCRα,β, CD3ε andζ (43). Interestingly, we found that the remaining coprecipitated proteins of 46, 36 and 23 kDa have molecular weights consistent with Shc, pp36 (LAT) and Grb2, respectively (36,48,49), that have been implicated to function in and downstream of TCR signal transduction. To identify the 46 kDa and 23 kDa proteins, we performed immunoblotting experiments with commercially available anti-Shc and anti-Grb2 antibodies. The results shown in Fig. 2B clearly indicate that the 46 kDa and 23 kDa proteins are Shc and Grb2, respectively. Mammalian Shc exists in three isoforms of 46 kDa, 52 kDa and 66 kDa, but only the former
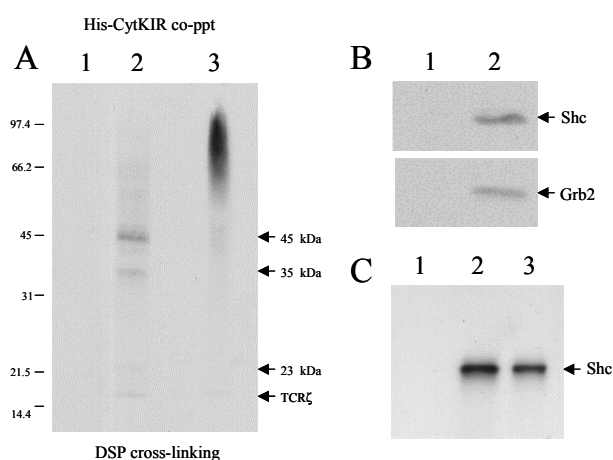
His-CytKIR co-ppt

DSP cross-linking

Figure 2. The His-CytKIR associates with Shc, Grb2, TCRζ, and a protein of 35 kDa in Jurkat T cell lysates. (A) His-CytKIR was mixed with [^{35}S] labeled Jurkat cell lysates and cross-linked with DSP. The His-CytKIR was precipitated with Ni-NTA resin, and the precipitates were resolved by SDS-PAGE under reducing (lane 2) and nonreducing (lane 3) conditions. (B) The 46 kDa and 23 kDa proteins are Shc and Grb2, respectively. The His-CytKIR and Jurkat cell lysates were mixed and incubated for 1 hr at 4°C. The His-CytKIR was precipitated with Ni-NTA resin, and the precipitates were resolved on SDS gels. The protein bands were detected with anti-Shc (upper pannel) and anti-Grb2 (lower pannel) antibodies. (C) Similar experiments with (B) were performed with (lane 2) and without (lane 3) chemical cross-linking. The precipitates were detected with anti-Shc antibody. Lane 1 in each figure shows the precipitates obtained by using the His-tag control protein in the reaction mixtures.

two are expressed by hematopoietic cells (36). Our

results show that only the 46 kDa form of Shc associates with His-CytKIR in Jurkat cell lysates.

Phosphorylated KIR cytoplasmic tail binds SHP-1 and Shc in Jurkat T cell lysates. To investigate the effect of tyrosine phosphorylation on the molecular association between KIR and the signaling molecules, we carried out similar His-tag pull-down experiments. Metabolically-labeled Jurkat cell lysates were mixed with His-CytKIR in a kinase reaction buffer and incubated at
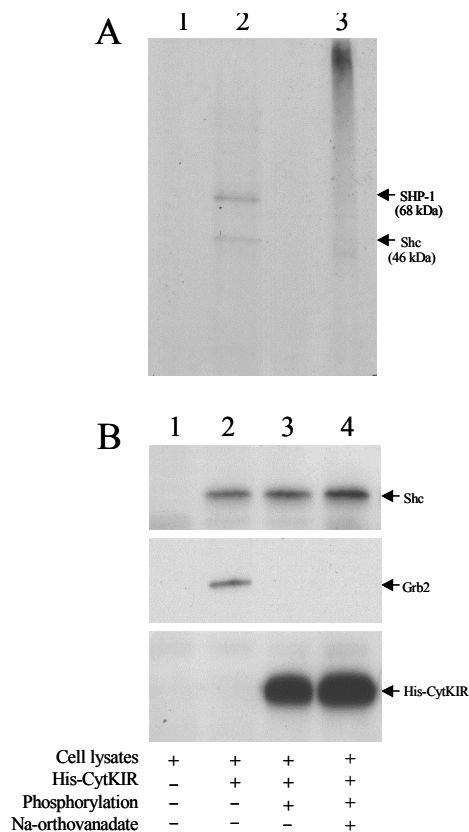

Figure 3. Tyrosine phosphorylated His-CytKIR associates with SHP-1 and Shc in Jurkat T cell lysates. (A) His-CytKIR was mixed with [^{35}S] labeled Jurkat cell lysates in a Kinase reaction buffer and incubated at 37°C. The reaction mixtures were cross-linked with DSP, and the His-CytKIR was precipitated with Ni-NTA resin. The precipitates were resolved by SDS-PAGE under reducing (lane 2) and nonreducing (lane 3) conditions. Lane 1 shows the precipitates obtained by using the His-tag control protein in the reaction mixtures. (B) Effects of tyrosine phosphorylation of KIR on the Shc and Grb2 binding. Same amounts of Jurkat cell lysates (500 μg) and the His-CytKIR protein (200 μg) were mixed in a kinase reaction buffer (total volume of 700 μl) and cross-linked with DSP. *In vitro* phosphorylation reactions were performed in the presence (lane 4) and absence (lane 3) of sodium orthovanadate, a protein tyrosine phosphatase inhibitor (58). Then the His-CytKIR was precipitated with Ni-NTA resin and the precipitates were detected with anti-Shc antibody (top), anti Grb2 antibody (middle), and anti- phosphotyrosine antibody (bottom).

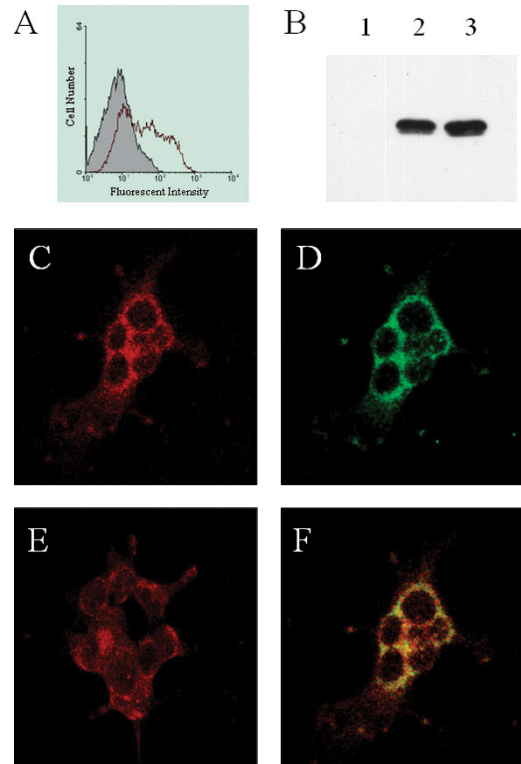37°C as previously described (42,43). The reaction

Figure 4. Expression of CD8-KIR on 293T cells and the colocalization of CD8-KIR and Shc in transfected cells. 293T cells were transiently transfected with the CD8T and CD8-KIR constructs, respectively. (A) A histogram shows the surface expression level of transfected CD8-KIR as determined by FACS staining with PE-conjugated anti-CD8 antibody, where shaded histogram indicates staining with PE-conjugated anti-mouse IgG. (B) Expression level of transfected CD8-KIR protein was detected by immunoblotting with polyclonal anti-His-CytKIR antibodies. Extracts from 5×10^5 cells were loaded. Lane 1 is 293T cells transfected with CD8T; lane 2 is 293T cells transfected with CD8-KIR; lane 3 is 293T cells transfected with CD8-KIR and stimulated with PMA/Ionomycin. (C-F) Colocalization of Shc and CD8-KIR by confocal microscopy in transfected 293T cells. Cells were double-labeled with anti-Shc antibody (red, detected with Texas red-conjugated goat anti-rabbit IgG) and with mouse antiserum to CD8 (green, detected with FITC-conjugated goat anti-mouse Ig). Fluorescent confocal images were obtained for Shc expression (red) in transfected (C) and untransfected (E) cells, and CD8-KIR expression (green, D). The two images were then superimposed (F) to show regions expressing both Shc and CD8-KIR (yellow).

mixtures were chemically cross-linked and precipitated with Ni-NTA resin. The precipitates were separated on a SDS polyacrylamide gel and the protein bands were visualized by autoradiography. Unlike the unphosphorylated His-CytKIR (Fig. 2A), the phosphorylated His-CytKIR was associated with the 68 kDa and 46 kDa proteins (Fig. 3A), which turned out to be SHP-1 and Shc, respectively, by Western blot experiments (data not shown). These results are consistent with previous reports showing that the pho-

sphorylated KIR recruits SHP-1 (21-24,31), and that the synthetic peptide representing the phosphorylated form of the ITIM of FcγRIIB binds Shc (50). Previous studies have also shown that Shc associates with tyrosine phosphorylated TCRζ and Grb2 (51) in T cells, both of which also appeared to associate with KIR in this report. However, in contrast to TCRζ (43) and Grb2 (Fig. 3B, middle), Shc appeared to associate with both phosphorylated (Fig. 3B, top, lane 3) and non-phosphorylated His-CytKIR (lane 2). As well, Shc binding was most prominent among the proteins associating with His-CytKIR (Fig. 2A). These findings suggest that Shc directly associates with His-CytKIR, not through the TCRζ or Grb2. *Interaction of KIR and Shc in transfected cells.* To verify the association of KIR and Shc in cells, a CD8 fusion protein construct of KIR cytoplasmic tail (CD8-KIR, Fig. 1B) was transfected into 293T cells. FACS staining with a monoclonal antibody to CD8 (OKT8) and Western blot analysis with polyclonal antibodies to the His-CytKIR protein showed that CD8-KIR was expressed on the surface of 293T cells (Fig. 4A, B, respectively). We next investigated whether the CD8-KIR expressed on 293T cells interacts with Shc. The transfected cells were double stained with anti-Shc antibody and OKT8, and then observed on a confocal microscope. Most of the transfected cells abundantly expressed CD8-KIR, and the CD8-KIR appeared to be localized predominantly on the cell surface (Fig. 4D). In 293T cells, Shc protein was found in the cytoplasm as well as around the plasma membrane (Fig. 4E). However, in 293T cells transfected with the CD8-KIR expression vector, most of Shc appeared to be localized around the plasma membrane (Fig. 4C). Double staining of the transfected cells clearly showed that CD8-KIR and Shc were co-localized on the membrane (Fig. 4F), suggesting that the two proteins associate in the mammalian cells.

The binding interaction between the CD8-KIR and Shc in transfected cells was confirmed by coimmunoprecipitation analysis. Shc was immunoprecipitated with anti-Shc antibody, and the precipitates were resolved on a SDS polyacrylamide gel. The protein bands were transferred onto PVDF membrane, and sequentially blotted with anti-His-CytKIR antibody and anti-SHP-2 antibody, respectively. Fig. 5A shows that CD8-KIR coprecipitated with Shc (Fig. 5A, top, lane 3). Interestingly, however, the association was almost blocked when the CD8-KIR was cross-linked by OKT8 antibody prior to the anti-Shc immunoprecipitation (lane 4). On the other hand, SHP-2 binding was only observed when the transfectants were treated with OKT8 antibody cross-linking (Fig. 5A, bottom, lane 4). The weak intensity of the SHP-2 band may result from the fact that most of the phosphory-
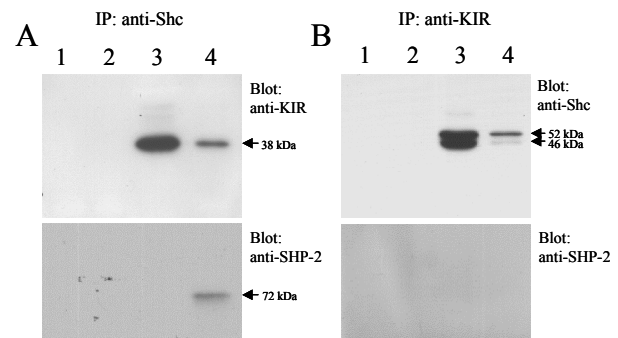

Figure 5. Association of CD8-KIR and Shc in 293T cells transiently transfected with CD8-KIR. CD8-KIR associates Shc in resting 293T cells, but it associates SHP-2 instead of Shc in cells treated with anti-CD8 antibody. (A) Shc was immunoprecipitated with anti-Shc antibody and blotted with anti-His-CytKIR antibody (top), and reprobed with anti-SHP-2 antibody (bottom). (B) Shc was immunoprecipitated with anti-Shc antibody and blotted with anti-His-CytKIR antibody (top), and reprobed with anti-SHP-2 antibody (bottom). Lanes in (A, B): lane 1, 293T cells transfected with CD8T; lane 2, 293T cells transfected with CD8T and treated with anti-CD8 antibody; lane 3, 293T cells transfected with CD8-KIR; lane 4, 293T cells transfected with CD8-KIR and treated with anti-CD8 antibody.

lated KIR could not precipitate with Shc. Immunoprecipitation with anti-His-CytKIR antibodies and immunoblotting experiments with anti-Shc and anti-SHP-2 antibodies resulted in similar results (Fig. 5B). Shc coprecipitated with CD8-KIR and the association was greatly reduced by OKT8 antibody cross-linking (Fig. 5B, top). Both the 46 kDa and 52 kDa forms of Shc appeared to associate with the CD8-KIR in transfected cells. However, SHP-2 binding was not detected in this case (Fig. 5B, bottom), probably due to the antibody blocking effect. Taken together, these results indicate that CD8-KIR constitutively interacts with Shc in 293T cells but the Shc is replaced by SHP-2 upon engagement of CD8-KIR.

Discussion

By *in vitro* coprecipitation experiments, we demonstrated that the cytoplasmic tail of KIR associates with proteins of 46 kDa, 36 kDa, 23 kDa, and 16 kDa in Jurkat T cell lysates. Previously, we identified that the 16 kDa protein is TCRζ and demonstrated that the association between KIR and TCRζ is hindered by the tyrosine phosphorylation of KIR, but is independent of the tyrosine phosphorylation of the TCRζ-chain (43). In this report, we identified that the 46 kDa and 23 kDa proteins are Shc and Grb2, respectively, by immunoblotting experiments. Like the case of TCRζ, tyrosine phosphorylation of the KIR cytoplasmic tail abolished the association with Grb2 and the 36 kDa protein in Jurkat cell lysates. However, the interaction between KIR and Shc ap-

peared to be unaffected *in vitro*. The relatively weak band intensity of Grb2 (Fig. 2A) and previous reports, where Grb2 appears to associate with Shc, TCRζ, and LAT (51), suggest that Grb2 may be coprecipitated with other proteins. We also demonstrated that tyrosine phosphorylated KIR associates with SHP-1 and Shc in Jurkat T cell lysates, suggesting that the molecular interaction between KIR and the signaling molecules identified in this study are differently modulated by the phosphorylation status of KIR.

To investigate the molecular interaction between KIR and Shc in cells, we transfected the CD8-KIR expression vector in 293T cells. Double staining of the transfectants with anti-Shc antibody and OKT8 antibody revealed that CD8-KIR and Shc are colocalized around the plasma membrane, and immunoprecipitation analysis confirmed that CD8-KIR binds Shc in transfected cells. In contrast to the in vitro data, however, KIR interacts with Shc in a phosphorylation-dependent manner in cells. Shc binds to CD8-KIR in resting cells, but dissociates in OKT8 treated-cells. Upon engagement of CD8-KIR with OKT8 antibody, KIR appears to associate with SHP-2 instead of Shc, suggesting that the phosphorylation of tyrosine residues in the KIR cytoplasmic tail hinders Shc binding and induces SHP-2 (maybe SHP-1 in NK and T cells) binding in 293T cells where no SHP-1 is expressed (our unpublished observation). The *in vitro* association of Shc with the phosphorylated KIR may be caused by either incomplete phosphorylation of KIR or excess use of KIR for the *in vitro* coprecipitation experiments. Also, the association between KIR and Grb2 observed in *in vitro* experiments was not detected in transfection analysis. This result suggests that Grb2 may be coprecipitated with TCRζ or the 36 kDa protein which are not expressed in 293T cells but are present in Jurkat T cells.

Shc is composed of three different domains, a phospho-tyrosine binding domain (PTB), collagen homolog domain (CH), and SH2 domain (36-38). Our data demonstrate that unphosphorylated KIR binds to Shc, but the KIR : Shc complex dissociates upon tyrosine phosphorylation of KIR, suggesting that Shc may bind around the ITIMs of KIR. This is quite distinct from the common binding modes of the SH2 and PTB domains, which bind only with phosphorylated tyrosine sites. As well, the CH domain is known to bind with Grb2 via phosphorylated tyrosine residue. Therefore, the binding mode between KIR and Shc seems to be unique. Interestingly, we have shown that the His-CytKIR binds only the 46 kDa form of Shc in Jurkat cell lysates, while the CD8-KIR and endogenous KIR bind the 46 kDa and 52 kDa forms of Shc in 293T cells and in NK and

T cells, respectively. The 46 kDa form is distinct from the 52 kDa form only at the N-terminus, where about 50 amino acid residues are truncated in the 46 kDa form by an alternative translation mechanism (36). Also, the 66 kDa form of Shc has an additional CH domain at the N-terminus of the 52 kDa form (39). Taken together, the KIR binding sites on the Shc molecule seems to be located at the N-terminal region.

We demonstrated that the cytoplasmic tail of KIR constitutively associates with Shc *in vitro* as well as in cells. It would be interesting to investigate the functional implications of this observation. Shc proteins recruited by a variety of activated receptors mediate the activation of the Ras signaling pathway through the complex formation of Grb2 : SOS (39,51,52). We investigated whether Shc bound to KIR would also turn on the Ras signaling pathway. However, no direct evidence suggesting that the Ras signaling pathway is activated by overexpressing CD8-KIR in 293T cells was obtained (data not shown). CD8-KIR : Shc association was evident, but Shc : Grb2 association was not observed in the transfected cells. Furthermore, no MAP kinase activation was observed in the transfectants even after the engagement of CD8-KIR with OKT8 antibody. Recent studies have implied that Shc might send another signal distinct from Ras activation for an anti-apototic pathway (53), for mitogenesis of fibroblast and Drosophila cells (54,55), and for cellular transformation (56). Interestingly, it has been demonstrated that human cytotoxic T cells that express KIR represent oligoclonally or monoclonally-expanded cell populations (57). In addition, KIR expressed on T cells has been shown to inhibit the activation-induced cell death of T cells in a ligation-independent manner by blocking FasL induction upon stimulation (9,12). Furthermore, KIR expressing clones demonstrated greater forward scatter values by FACS analysis and larger cell sizes by image analysis (12). These observations suggest that KIR might constitutively transmit a certain signal that affects the cell size and the growth pattern of Jurkat T cells.

Acknowledgements

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