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Zinc Bound to the Killer Cell-Inhibitory Receptor Modulates the Negative Signal in Human NK Cells

Sumati Rajagopalan and Eric O. Long

The lysis of target cells by human NK cells is inhibited by several kinds of receptors with varying specificities for the MHC class I molecules of target cells. The requirements for complete inhibition of NK cytotoxicity appear to be complex and not well defined. The HLA-C-specific members of the killer cell-inhibitory receptor (KIR) family, carrying two Ig domains (KIR2D), are unusual among Ig superfamily members in their ability to bind zinc. A role for the zinc-binding site in KIR-mediated inhibition was demonstrated in this study using a functional reconstitution system in NK cells. Replacement of six histidines by alanine residues in putative zinc binding sites of a KIR2D ablated zinc binding and markedly impaired its inhibitory function, but left intact its ability to bind HLA-C and to transduce a positive signal through an immunoreceptor tyrosine-based activation motif grafted onto its cytoplasmic tail. Thus, zinc modulates specifically the negative signal transmitted by this KIR molecule. Mutation of an exposed amino-terminal zinc-binding motif alone was sufficient to impair the inhibitory function of KIR. The data suggest that complete inhibition of HLA-C-specific NK cells requires a zinc-dependent protein-protein interaction via the amino-terminal end of KIR2D.


A n inhibitory signal is transmitted to NK cells upon engagement of specific receptors that recognize MHC class I ligands on the target cell (1, 2). Human NK cells express at least two different types of inhibitory receptors that are involved in recognition of HLA class I allotypes on target cells (3). One kind of human NK inhibitory receptor is a family of type I transmembrane glycoproteins of the Ig superfamily, including the p58 and p70 molecules called killer cell-inhibitory receptors (KIR).2 Specific recognition of different HLA-C allotypes is mediated by p58 members (2) of the KIR family with two Ig domains (KIR2D) and cytoplasmic tails that contain immunoreceptor tyrosine-based inhibition motifs (ITIM) (4, 5). The second type of NK inhibitory receptor belongs to the C-type lectin family (6). It consists of the type II glycoprotein CD94 covalently associated with structurally heterogeneous members of the type II NKG2 receptor family (7–10). The CD94/NKG2 receptors exhibit specificity for the nonclassical class I molecule HLA-E (11, 12). Most NK clones express multiple inhibitory receptors on their cell surface. The contribution of each of these receptors to the final inhibition of lysis observed in cellular assays is often difficult to assess. At present, it is still unclear whether these diverse inhibitory receptors act independently of each other, whether signals from several receptors are integrated, or whether additional molecules are involved to achieve proper inhibition of NK cells.

KIR2D have the intriguing and unique property, among all Ig superfamily members, of binding zinc, as shown with KIR2DL1 and KIR2DL3 (13). KIR2DL1 is specific for HLA-C allotypes that carry amino acids asparagine at position 77 and lysine at position 80, such as HLA-Cw4, whereas KIR2DL3 recognizes HLA-C with serine at 77 and asparagine at 80, such as HLA-Cw3 (4, 14–16). Potential zinc binding sites are present in the first Ig domain of KIR2D molecules, including the amino-terminal end, which corresponds to the canonical Zn-binding motif HEXXH (17). In addition to zinc binding, a role for zinc in the KIR-mediated inhibition of NK killing was demonstrated using NK clones (13). KIR-mediated inhibition of target cell lysis was prevented by the zinc chelator 1,10-phenanthroline (13). However, as 1,10-phenanthroline is membrane permeable, such cytolytic assays did not distinguish between a requirement for extracellular zinc, possibly bound to KIR, and an intracellular role for zinc bound to other molecules involved in the negative signaling pathway.

Zinc coordination in proteins can serve a structural role by stabilizing a folded conformation, or a catalytic role as part of the active site of enzymes. Zinc can also promote protein-protein interactions when it is coordinated by amino acid residues on two different molecules. From the study of several zinc-binding proteins that have been crystallized, it appears that zinc is typically coordinated by histidine, cysteine, and less commonly, aspartate and glutamate residues, with histidine being the most frequent ligand in zinc active sites (18). By analogy with known zinc-mediated receptor-ligand interactions, such as HLA-DR with the superantigen staphylococcal enterotoxin A (19), and prolactin receptor with growth hormone (20), the zinc-binding property of KIR2D suggested that zinc may be required for KIR binding to HLA-C. However, binding of a soluble KIR2DL1-Fc fusion protein to HLA-C was unaffected by chelation of zinc (C. Winter and S.R., unpublished observations). Furthermore, soluble KIR2DL1 produced in Escherichia coli could be refolded in the absence of zinc and was still competent to bind soluble HLA-C (21). Thus, a requirement for zinc in the direct interaction between KIR and HLA-C was ruled out.

At least four possible roles for zinc in KIR function can be envisaged. First, zinc may be required for the activity or the inhibition of intracellular proteins that control the negative signaling
pathway. Zinc is a necessary structural component of many trans- 
cription factors and enzymes such as proteases (18, 22). Second, 
zinc could confer a conformation to KIR that is necessary for 
the delivery of a signal through the cytoplasmic tail. It is still un- 
known to what extent signaling through KIR occurs via receptor ag- 
gregation or via a conformational change. Third, zinc may promote 
oligomerization of KIR at the cell surface, which may be a nec- 
esary early step in the negative signaling cascade. Dimerization of 
proteins such as HIV tat and human growth hormone is zinc de- 
pendent (23, 24). Fourth, zinc might mediate intramolecular inter-
actions between KIR and another protein that would strengthen the 
inhibitory signal. To distinguish between these different possibil-
ities, mutational analysis of the putative zinc binding sites of KIR 
inhibitory signal. To distinguish between these different possibil-
ities, mutational analysis of the putative zinc binding sites of KIR 
and functional reconstitution experiments in NK cells were con-
ducted. The results definitively established a role for the zinc-bind-
ing site on KIR in the inhibitory function of this receptor. The data 
support a model whereby zinc bound to KIR promotes a protein-
protein interaction.

Materials and Methods

Cells and Abs

The human NK cell line NK92 (a gift of H. G. Klingemann, The Terry 
Fox Laboratory, Vancouver, Canada) (25) was maintained in Myel-
ocult H5100 medium (Stem Cell Technologies, Vancouver, Canada) 
and supplemented with 100 U/ml hIL-2 (gift from Hoffmann-La Roche, 
Nutley, NJ). The HLA class I transfectants 221-Cw3 and 221-Cw4 of the 
B lymphoblastoid cell line 721.221 were provided by J. Gumperz 
and P. Parham (Stanford University, Stanford, CA). The RMA-S-Cw4 
transfectant and its loading with peptide QYDDAVYKL have been de-
scribed (26). The following Abs were used: F4/326 (IgG2a) (a gift from 
S. Y. Yang, Memorial Sloan-Kettering Cancer Center, New York, 
NY) (27); the anti-KIR2D mAb HP3E4 (IgM) (a gift from M. Lopez-
Botet, Hospital de la Princesa, Madrid, Spain) (28); and the anti-
KIR2DL1 mAb EB6 (IgG2a) from Immunotech (Westbrook, ME). 
The zinc chelators polyhistidine (m.w. 5,000 –15,000) and 1,10-phenan-
trene were obtained from Sigma (St. Louis, MO).

cDNA and construction of mutant and chimeric constructs

The mutants H1, H2, and H1,2,3 were generated by site-directed mutagenis-
es of the cDNA cl-42 (29), encoding the KIR2DL1 molecule KIR-42, 
using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, 
CA). Mutant H1 was engineered by replacing the histidines at positions 1 
and 5 of KIR-42 with alanine using oligonucleotide 5′-GGGGGCTGGCC 
CAGCTGAGGAGTGCACAGAAGAACCT-3′ and its complement. Mu-
ant H2 was engineered by replacing the histidines at positions 36 and 40 
of KIR-42 with alanine using oligonucleotide 5′-GTCTAGTGTGGAAC 
CTTCTTCTGGCCGAGAGGGG-3′ and its complement. Mutant 
H1,2,3 was generated by sequentially mutating the histidines described 
in H1 and H2 and additionally mutating a third pair of histidines at positions 
55 and 56 using oligonucleotides 5′-CTCATGGGAAGAGCCGTAG 
GGGTTCTC-3′ and its complement. Mutations were confirmed by se-
quencing, and the mutants H1, H2, and H1,2,3 were cloned into the plas-
mid pcSC66 and used to generate recombinant vaccinia viruses, as described 
(30).

The chimeric KIR-42-ITAM and H1,2,3-ITAM receptors were pro-
duced by ligation of fragments encoding the extracellular and transmem-
brane regions of KIR-42 and H1,2,3 with the ITAM-containing cyto-
plasmic region of the γ-chain of FcERI. The cytoplasmic tail of the 
FcERI-γ was amplified from a cDNA encoding FcERI-γ (a gift from M.-H. Jouvin, 
Botet, Hospital de la Princesa, Madrid, Spain) (28); and the anti-
KIR2DL1 mAb EB6 (IgG2a) from Immunotech (Westbrook, ME). The 
zinc chelators polyhistidine (m.w. 5,000 –15,000) and 1,10-phenan-
trene were obtained from Sigma (St. Louis, MO).

Soluble KIR-Fc fusion proteins and direct binding assays

The construction of soluble KIR-42-Fc fusion protein of KIR fused to the 
Fc portion of human IgG1 has been described (4). The sequences encoding 
the extracellular portions of the mutant KIR-42 molecules generated in 
this study, namely H1, H2, and H1,2,3, were amplified by PCR. For H1 and H1,2,3, 
the forward primer used was 5′-CAGGGGGGCTAGGCGGCT 
GAGGGACTGCCAGAAAACCT-3′ containing an Nhel site, and the re-
verse primer used was 5′-GAGGCTTCCCGAGGTGCTCATGAGCTG 
AGTGGGTTGATT-3′ upstream of the transmembrane and containing a 
BamHI site. For H2, the forward primer was 5′-CAGGGGGGCTAGGCG 
GCTAGGCGGACTCCAGAAAACCT-3′, and the reverse primer was the 
same as that used for H1 and H1,2,3. These PCR-generated Nhel-BamHI 
fragments were cloned into the expression vector CD51ing (a gift from B. 
Seed, MGH Cancer Center, Charlestown, MA). COS-7 cells were trans-
fected with these cDNAs, and supernatants were harvested and affinity 
purified on protein A-Sepharose, as previously described (4). Protein con-
centrations were determined using the Micro bicinchoninic acid assay 
(Pierce, Rockford, IL). The HLA-Cw4-transfected and untransfected 
721.221 cells were incubated for 30 min at 4°C with 40 μg/ml of the 
purified KIR-Fc fusion proteins KIR-42-Fc, H1-Fc, H2-Fc, and H1,2,3-Fc. 
The cells were then washed and incubated with Fc-specific, FITC-conju-
gated goat anti-human IgG (Jackson ImmunoResearch, West Grove, 
PA) for 30 min at 4°C. Fluorescence of 5000 cells was analyzed by flow 
cytometry on a FACScan (Becton Dickinson, Mountain View, CA).

6Zn blot assay

The Fc portion of human IgG1 (control), wild-type KIR-42-Fc, and mutant 
H1,2,3-Fc fusion proteins (5 μg and 10 μg of each) were dried down onto 
nitrocellulose filters. Filters were incubated with metal-binding buffer 
(MBB: 100 mM Tris-HCl, pH 6.8, 50 mM NaCl) for 30 min at room 
temperature. The blot was incubated in 50 μCi of 65ZnCl2 (3,31 Ci/g) in 
7.5 ml MBB for 1 h at room temperature. After three washes in MBB, the 
filter was subjected to autoradiography.

Peptide-binding assay for stabilization of HLA-C surface 
expression

RMA-S transfectants were plated at 2.5 × 103 cells/well in a 48-well plate in 
a final volume of 0.5 ml. Cells were incubated for 18 h at 25°C. Peptide 
(100 μM final concentration) was added at the onset of culture, and again 
16 h later. Cells were harvested, washed with PBS containing 2% FCS, 
and divided into two aliquots. One aliquot was used to measure the surface 
levels of class I molecules on peptide-loaded RMA-S transfectants, as de-
scribed previously (26). The second aliquot was used for targets in the 
cytotoxicity assay described below.

Vaccinia virus infections and cytotoxicity assays

Purified viruses encoding KIR-42, H1, H2, H1,2,3, KIR-42-ITAM, and 
H1,2,3-ITAM were used to infect the human cell line NK92, as described 
(4). Lysis of B cell targets by human NK92 cells was measured in a 4-h 
51Cr release assay, as described (4). The RMA-S-Cw4 target cells were 
incubated with peptide, as described above, and labeled overnight at 25°C
incubated at 37°C for 3 h, and 51 Cr release was measured as previously described (31). mAbs and zinc chelators were added at the onset of the assay. The recombinant vaccinia virus expression system in NK cells, the membrane-nonpermeable zinc chelator polyhistidine was used. The membrane-permeable zinc chelator 1,10-phenanthroline concentrations (data not shown).

**Results**

**Polyhistidine interferes with the inhibition of NK cells mediated by KIR-42**

To test whether the requirement for zinc in the KIR-mediated inhibition of NK cells reflects a need for zinc inside or outside the cells, the membrane-nonpermeable zinc chelator polyhistidine was used. The recombinant vaccinia virus expression system in NK cells was used to study the effect of zinc on KIR2DL1, the well-defined HLA-Cw4-specific KIR2D encoded by cDNA cl-42 (referred to in this study as KIR-42; see Fig. 1). KIR-42 was expressed in the cell line NK92 and tested for its ability to inhibit lysis of HLA-Cw4 targets in the presence of polyhistidine (Fig. 2). There was no significant effect of polyhistidine on the lysis of 51Cr-loaded targets in the absence of effector NK cells. Similar results were obtained at an E:T ratio of 6 and in an independent experiment. Even though zinc is not required for the binding of KIR-42 to HLA-C (21), the six histidine to alanine substitutions that obtained with thermolysin, a protein known to bind zinc via the HEXXH motif (data not shown). In contrast, the mutant KIR-42-Fc bound zinc (Fig. 3A, center), wild-type KIR-42-Fc bound zinc (Fig. 3A, left), and HH (amino acids 55–56), were replaced to produce mutant KIR-42-Fc were bound to nitrocellulose filters using a slot-blot apparatus. Filters were equilibrated in MBB and probed with 50 μCi of 65ZnCl₂, washed, and subjected to autoradiography. B, the human B cell line 721.221 transfected with HLA-Cw4 was incubated with 40 μg/ml of purified Fc portion of human IgG (control), KIR-42-Fc, and H1,2,3-Fc fusion proteins. Cells were washed, incubated with FITC-conjugated goat anti-human IgG, and analyzed by flow cytometry. C, Titration of KIR-42-Fc and H1,2,3-Fc binding to HLA-Cw4. Binding and flow cytometry were performed as indicated in B, except that several concentrations of KIR-42-Fc (circles) and H1,2,3-Fc (squares) were tested. MFI, mean fluorescence intensity.

**Histidine residues in KIR necessary for zinc binding are not required for KIR binding to HLA-C**

To directly test whether zinc bound to KIR is necessary for inhibition of NK cells, several histidine residues that were potential zinc binding sites on KIR were replaced by alanine. A total of six histidines in the first Ig domain, included in the three histidine pairs HEGVH (amino acids 1–5), HFLLH (amino acids 36–40), and H1 (amino acids 55–56), were replaced to produce mutant H1,2,3 (Fig. 1). Soluble forms of wild-type KIR-42 and of mutant H1,2,3 were produced as fusion proteins with the Fc portion of human IgG1 to assess their zinc- and HLA-C-binding potential. Binding of 65Zn to proteins attached on nitrocellulose filters was measured in a zinc-blot assay (Fig. 3A). While there was no zinc binding by the control Fc protein (Fig. 3A, left), wild-type KIR-42-Fc bound zinc (Fig. 3A, center) to an extent comparable with that obtained with thermolysin, a protein known to bind zinc via the HEXXH motif (data not shown). In contrast, the mutant H1,2,3-Fc protein exhibited greatly reduced zinc binding (Fig. 3A, right). Competition studies indicated that metal binding was specific. Zn2⁺ and, to a lesser extent, Ni2⁺ competed for the binding of 65Zn, whereas Ca2⁺ and Mg2⁺ did not, even at 10-fold higher concentrations (data not shown).

Even though zinc is not required for the binding of KIR-42 to HLA-C (21), the six histidine to alanine substitutions could have...
disrupted the HLA-C binding site on KIR-42 or a conformation of KIR-42 compatible with binding to HLA-C. To test this possibility, binding of the soluble KIR fusion proteins to HLA-Cw4 expressed on transfected cells was measured (Fig. 3B). The H1,2,3-Fc mutant bound to HLA-Cw4 as well as KIR-42-Fc, even at suboptimal concentrations (Fig. 3C). Thus, the histidine residues that control zinc binding to KIR are not required for KIR binding to HLA-C.

Impaired inhibitory function of KIR-42 lacking the zinc binding site

To test whether mutation of the zinc-binding motifs had an effect on the inhibitory potential of KIR, KIR-42 and H1,2,3 on NK92 cells infected with 15 and 10 PFU/cell of the respective recombinant vaccinia viruses. B. Specific lysis of the B cell line .221 and its transfectants .221-Cw3 and .221-Cw4 by NK92 infected with either Vac-KIR-42 or Vac-H1,2,3. Lysis was determined in a 4-h $^{31}$Cr release assay at an E:T ratio of 3. Similar results were obtained at an E:T ratio of 6 and in three other experiments.

FIGURE 4. Impaired inhibitory function of the zinc-binding mutant H1,2,3. A. Surface expression of KIR-42 and H1,2,3 on NK92 cells infected with 15 and 10 PFU/cell of the respective recombinant vaccinia viruses. B. Specific lysis of the B cell line .221 and its transfectants .221-Cw3 and .221-Cw4 by NK92 infected with either Vac-KIR-42 or Vac-H1,2,3. Lysis was determined in a 4-h $^{31}$Cr release assay at an E:T ratio of 3. Similar results were obtained at an E:T ratio of 6 and in three other experiments.

Inhibited function of KIR-42 lacking the zinc binding site

To test whether mutation of the zinc-binding motifs had an effect on the inhibitory potential of KIR, KIR-42 and the mutant H1,2,3 were expressed by recombinant vaccinia viruses in NK cells (Fig. 4A). NK92, an NK cell line that lacks endogenous KIR-42 expression (32), was used to express these recombinant receptors and was tested for its ability to kill 721.221 target cells transfected with either HLA-Cw4 (ligand for KIR-42) or HLA-Cw3 (an allotype recognized by other KIR2D receptors). As expected, KIR-42 provided specificity for HLA-Cw4 on target cells, resulting in inhibition of lysis (Fig. 4B). In contrast, expression of the mutant H1,2,3 resulted in a partial inhibition of lysis of target cells bearing HLA-Cw4. The residual inhibition mediated by mutant H1,2,3, similar to that observed after chelating zinc (Fig. 2) (13), was observed reproducibly and is not due to lower expression of H1,2,3 (Fig. 4A). This residual inhibition was not reversed to complete lysis by the addition of the zinc chelator polyhistidine (data not shown). These data suggest that zinc, although not absolutely required, contributes to the inhibition of NK cells by KIR-42.

Mutation of the zinc binding site on KIR-42 may have compromised its ability to transmit a signal through the cytoplasmic tail upon ligand binding. To test whether the mutation caused a generalized inability to transduce positive or negative signals, the ITIM-containing cytoplasmic tails of mutant H1,2,3 and wild-type KIR-42 were replaced by the ITAM-containing tail of the $g$-chain of the FcERI receptor (Fig. 1). To detect an activation signal in NK cells, it was necessary to use target cells that are not normally lysed by NK92 cells, such as the mouse TAP-deficient cell line RMA-S (26). Direct binding of soluble KIR2D to transfected RMA-S cells expressing HLA-C allotypes is dependent on the addition of specific exogenous peptides (26). Thus, RMA-Cw4 cells loaded with the specific peptide QYDDAVYKL were tested for their ability to activate the lytic function of NK92 cells expressing KIR-42, H1,2,3, and the ITAM-containing forms of these receptors (Fig. 5A). NK92 cells, either uninfected or infected with recombinant vaccinia viruses expressing any one of these receptors, were unable to bind empty HLA-Cw4 molecules and lyse RMA-Cw4 cells (Fig. 5B). Upon peptide-induced stabilization of surface

FIGURE 5. Mutant H1,2,3 is not impaired in its ability to transduce a signal. A. Surface HLA-Cw4 expression on RMA-Cw4 transfectants. Cell surface stabilization of HLA-Cw4 on RMA-Cw4 cells in the absence (left panel) or the presence (right panel) of specific peptide QYDDAVYKL (100 $\mu$M). Cells were incubated at 25°C for 18 h and stained with the HLA-C-specific mAb F4/326. B. Specific lysis of the RMA-Cw4 cell line without peptide (left panel) or loaded with peptide (right panel) by NK92 cells infected with recombinant vaccinia viruses encoding KIR-42 (45 PFU/cell), H1,2,3 (25 PFU/cell), KIR-42-ITAM (100 PFU/cell), and H1,2,3-ITAM (100 PFU/cell). Cell surface expression of these receptors at the onset of the cytotoxicity assay is shown on the left as mean fluorescence intensity (MFI). Lysis was determined in a 3-h $^{31}$Cr release assay at an E:T ratio of 5. Similar results were obtained at an E:T ratio of 10 and in an independent experiment.
HLA-Cw4 molecules, activation of the lytic function of NK92 cells was observed after infection with vaccinia viruses encoding KIR-42-ITAM and H1,2,3-ITAM, but not KIR-42 and H1,2,3 (Fig. 5B). Despite a lower level of surface expression as compared with that of KIR-42-ITAM, the mutant H1,2,3-ITAM was equally competent to activate NK92 cells. Thus, removal of zinc-binding site(s) on KIR-42 expressed on NK cells affected neither ligand binding nor the ability to transduce an activation signal. These results point to a specific role for the zinc-binding histidines in the inhibitory function of KIR-42.

The N-terminal zinc-binding motif of KIR-42 is required for receptor function

During the course of this work, structural data on KIR-42 became available (33). The position of several histidine residues in the three-dimensional structure of KIR-42, including those at residues 36–40 (HFLLH) and 55–56 (HH), was not favorable to zinc binding. In contrast, the first five amino acids of KIR-42, which correspond to the zinc-binding motif HEXXH, are not visible in the crystal structure and may be disordered (33). Furthermore, the amino-terminal end of KIR-42 lies at an edge of the molecule, in a position seemingly well suited for intermolecular interactions. To test whether this N-terminal HEGVH motif is indeed necessary for KIR function, mutant H1 (HEGVH→AEGVA) was generated (Fig. 1). Mutant H2 (HFLLH→AFLLA) was generated to compare mutant H1 with a KIR-42 molecule that had a very similar mutation, but at a position not favored structurally to bind zinc. Soluble forms of mutants H1 and H2 were produced to evaluate binding to HLA-C. As expected, given the normal binding of mutant H1,2,3-Fc (Fig. 3), both H1-Fc and H2-Fc bound to HLA-Cw4 expressed on transfected .221-Cw4 cells (Fig. 6A). Mutants H1 and H2 were expressed in NK92 cells using the vaccinia virus expression system. Whereas mutant H2 retained the inhibitory function upon specific recognition of HLA-Cw4, mutant H1 was greatly impaired in the inhibitory function, despite a similar surface expression level (Fig. 6B). Thus, the histidines of the prototypic zinc-binding motif HEGVH at the N terminus of KIR-42 contribute to the inhibitory function of KIR-42.

Discussion

The decision by NK cells to kill or spare target cells results from the balance of multiple molecular interactions between NK receptors and their ligands on target cells that can either activate or inhibit cytolytic function. A large number of different receptors on NK cells can induce cytotoxicity upon cross-linking (3). The ligands of several of these activating receptors are still unknown. Inhibition of NK cytotoxicity occurs upon recognition of HLA class I molecules on target cells by different types of receptors. As each NK cell expresses several receptors with inhibitory potential and with different HLA class I specificities, the contribution of each one to the inhibition of target cell lysis is often unclear. An additional level of complexity was revealed in this study, in that complete inhibition of NK cells specific for HLA-C requires extracellular zinc bound to KIR2D via histidine residues in the first Ig domain.

A role for zinc bound to KIR2D molecules was deduced from mutagenesis of the KIR-42 zinc binding site. Both the H1,2,3 and the H1 mutants showed impaired inhibitory potential compared with wild-type KIR-42. Despite the substitution of six histidine residues by alanines, the zinc-binding-deficient mutant H1,2,3 was fully competent to bind HLA-C and to transmit an activation signal through an ITAM-containing artificial cytoplasmic tail upon recognition of HLA-C molecules on target cells. In agreement with this, earlier work had shown that folding of a soluble H1-2,3-Fc and binding to a soluble form of HLA-C occurred in the absence of zinc (21). Therefore, the zinc dependence of the inhibitory function of KIR lies downstream of ligand binding.

Given that proper folding and ligand binding of KIR-42, and the ability of KIR-42 to deliver an activation signal through an ITAM-containing cytoplasmic tail upon binding to HLA-C on target cells, are zinc-independent properties, the specific effect of zinc is best explained by a zinc-mediated interaction of KIR-42 with proteins that contribute to the negative signal. Zinc bound to KIR-42 may either mediate a protein interaction directly, or it may induce a conformation of KIR2D that is necessary for a protein interaction. By analogy with nerve growth factor (34), it is also possible that a N-terminal zinc-dependent conformation prevents an interaction...
with another protein. Several possibilities that could facilitate inhibition of NK cells through a zinc-mediated interaction with KIR-42 can be considered. First, receptors that activate NK cytotoxicity may interact with KIR to facilitate their own inhibition. Second, KIR-42 may interact with itself. Third, other proteins may interact with KIR-42 to strengthen the inhibitory signal.

Coligation of KIR with activating receptors appears to be necessary for inhibition (35), presumably because the required activity of the KIR-associated tyrosine phosphatase SHP-1 (5) needs to be localized near the activation signal. It is still unknown whether and how KIR interacts with receptors that activate NK cells. Zinc may facilitate such an interaction. However, many structurally unrelated proteins, such as the Ig-like CD16 and the lectin-like CD69, can trigger NK cells (3). It is therefore unlikely that zinc could mediate an interaction between KIR2D and each of these different activating receptors, unless it mediates an interaction with a common intermediate.

Dimerization or multimerization of KIR2D may be a requirement for an effective negative signal, as it often is for signal transduction mediated by other receptors (36). However, zinc-dependent oligomerization was not observed in studies of soluble rKIR-42 produced in E. coli. Such soluble KIR-42 remained monomeric in the presence or the absence of zinc, as judged by size exclusion (21). It remains possible that such a zinc-mediated KIR dimerization occurs at the surface of NK cells, but only after KIR binding to HLA-C to avoid constitutive dimerization.

The third hypothesis proposes that zinc mediates the interaction of KIR2D with another protein. Such a protein could be a coreceptor for KIR2D that provides an accessory function in the inhibition of NK cells, a ligand for KIR on target cells that enhances KIR function, or a protein that interferes with KIR function by binding to KIR in the absence of zinc. Preliminary biochemical experiments have failed to date to reveal a zinc-dependent protein interaction with KIR2D. A low affinity interaction, or a transient interaction dependent on target cell recognition by NK cells, could explain the lack of detection.

The protein interaction proposed by this hypothesis would be distinct from the KIR-42 dimerization or heterodimerization suggested by the three-dimensional structure of KIR-42 (33). The high resolution x-ray structure of KIR-42 revealed that this molecule belongs to the hemopoietic receptor family that includes cytokine and growth hormone receptors. All of the members of this receptor family form either homodimers (e.g., growth hormone and erythropoietin receptors) or heterodimers (e.g., IL receptors) upon ligand binding. A similar dimerization of KIR2D upon binding to HLA-C on target cells would involve contacts along one face of the second Ig domain (33). The HEGVH zinc-binding motif on KIR2D that contributes to inhibition of NK cytotoxicity is located away from this hypothetical dimerization site, and at some distance from the HLA-C binding site (Fig. 7). Thus, a protein interacting with the N-terminal end of KIR2D via zinc would not interfere with HLA-C binding or receptor dimerization. The first five HEGVH amino acids of KIR-42 were not visible in the crystal structure, indicating they may be disordered (33). Conceivably, these amino acids could gain an ordered structure upon zinc binding or upon a zinc-mediated interaction with a protein, as has been observed or postulated for other proteins that bind zinc at their N terminus, such as HIV integrase (37) and nerve growth factor (34).

The main conclusion from this study is that tight negative regulation of NK cytotoxicity by HLA-C-specific KIR, which is essential to avoid autoimmune destruction, is dependent on zinc. This requirement was observed for the inhibition of the NK cell line NK92 and of normal NK clones (13). This complex inhibitory regulation is a unique example, among receptors of the Ig superfamily, of a signal-transduction pathway that is modulated by zinc.

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