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Cutting Edge: NKp80 Uses an Atypical Hemi-ITAM To Trigger NK Cytotoxicity

Kevin M. Dennehy,*† Sascha N. Klimosch,‡ and Alexander Steinle*‡

The human NK cell receptor NKp80 stimulates cytotoxicity upon engagement of its genetically linked ligand AICL. However, the mechanisms underlying NKp80-mediated signaling are unknown. In this study, we dissected NKp80 signaling using the NK cell line NK92MI. We demonstrated that NKp80, but not NKp80 mutated at tyrosine 7 (NKp80/Y7F), is tyrosine phosphorylated. Accordingly, NKp80/Y7F, but not NKp80/Y30F or NKp80/Y37F, failed to induce cytotoxicity. NKp80 phosphopeptides comprising the hemi-ITAM–like sequence surrounding tyrosine 7 bound Lck- and Syk-family kinases; accordingly, cross-linking of NKp80, but not NKp80/Y7F, induced Syk phosphorylation. Moreover, inhibition of Syk kinase, but not ZAP-70 kinase, impaired cytotoxic responses through NKp80. Atypical residues in the hemi-ITAM–like motif of NKp80 cause an altered stoichiometry of phosphorylation but did not substantially affect NK cytotoxicity. Altogether, these results show that NKp80 uses an atypical hemi-ITAM and Syk kinase to trigger cellular cytotoxicity. The Journal of Immunology, 2011, 186: 657–661.

Responses of NK cells are governed by various activating and inhibitory receptors, as well as by the net signals emanating from their interplay (1, 2). Although inhibitory receptors mostly rely on recruiting phosphatases SHP-1 and SHP-2 via ITIMs to relay their signals, signaling via activating NK cell receptors (NKRs) is more diverse (1, 2); many activating receptors of NK cells, such as CD16, NKp46, NKp44, and NKp30, signal through associated ITAM-bearing adaptors (i.e., CD3ζ, FcRγ, and DAP12) (1, 2). Upon ITAM phosphorylation by Src-family kinases, Syk-family kinases ZAP-70 and/or Syk are recruited and initiate downstream signals promoting cytotoxicity and cytokine production (1, 2). Activating signals are also transduced by the adaptor DAP10 upon NKG2D engagement via recruitment of PI3K and a Grb2-Vav intermediate (1, 2). In contrast, signaling by the human NKR NKp80 remained unknown. Like NKG2D, NKp80 is an activating homodimeric C-type lectin-like receptor encoded in the natural killer gene complex (3). NKp80 binds to the genetically linked activation-induced C-type lectin (AICL) and, thereby, stimulates NK cytotoxicity against malignant myeloid cells, promotes a mutual cross-talk between NK cells and monocytes, but also augments responses of effector memory CD8 T cells (4, 5).

To elucidate NKp80 signaling, we scrutinized potential signaling motifs of NKp80 and defined an atypical hemi-ITAM that, by activating the Syk-kinase pathway, triggers NKp80-mediated cytotoxicity. Hemi-ITAMs were defined in Syk-recruiting receptors of myeloid cells (6–8), but there are no such receptors known for NK cells.

Materials and Methods

Reagents, constructs, and transductants

NKp80 mAb 5D12 was described previously (5). mAbs specific for NKp30 (R&D Systems), phosphotyrosine (PY100-PO; BD Biosciences), and Syk (4d10) and polyclonal Abs specific for Lck (Santa Cruz Biotechnology), ZAP-70, ERK, and phosphoT202/Y404)ERK (Cell Signaling) were purchased. Biotinylated peptides (NKp80: MQDERYMTLNVQSK-bio, pNKp80: MQDEEypYITLNVNKRK-bio) were provided by S. Stevanovic; R406 (9) was provided by Rigel Pharmaceuticals; and U0126, Ly294002, and Pp2 were from Calbiochem. NKp80 with a C-terminal FLAG-tag (5) was cloned into pmXSP (10), and kinase-defective ZAP-70 (11) and Syk (12) were cloned into pCEF5SIGZ (11). Retroviruses were generated in phoenix-ampho cells for transduction of NK92MI cells (13). Statistical analyses were performed using the Student t test.

Cellular and biochemical assays

NK92MI cells were stimulated with 0.1 mM Na3VO4 for the indicated times at 37°C before lysis (1% Nonidet P-40, 140 mM NaCl, 5 mM EDTA, 5 mM iodoacetamide, 5 mM NaF, 1 mM Na3VO4). For NKp80 stimulation, cells were pretreated on ice with mAb 5D12; after the addition of goat anti-mouse IgG (Jackson Laboratories), they were heated to 37°C for the indicated times before lysis and subsequent immunoprecipitation. For precipitation, 1–30 μM biotinylated peptide was immobilized on streptavidin-Sepharose beads (Invitrogen) and incubated with lysates of Jurkat E6.1 (11), WT8 (8), or NK92MI cells at 4°C for 2 h. After SDS-PAGE, membranes were probed with relevant Abs. NK92MI transductants were tested for cytotoxicity against malignant myeloid cells, as well as by the net signals emanating from their interplay (1, 2). Although inhibitory receptors mostly rely on recruiting phosphatases SHP-1 and SHP-2 via ITIMs to relay their signals, signaling via activating NK cell receptors (NKRs) is more diverse (1, 2); many activating receptors of NK cells, such as CD16, NKp46, NKp44, and NKp30, signal through associated ITAM-bearing adaptors (i.e., CD3ζ, FcRγ, and DAP12) (1, 2). Upon ITAM phosphorylation by Src-family kinases, Syk-family kinases ZAP-70 and/or Syk are recruited and initiate downstream signals promoting cytotoxicity and cytokine production (1, 2). Activating signals are also transduced by the adaptor DAP10 upon NKG2D engagement via recruitment of PI3K and a Grb2-Vav intermediate (1, 2). In contrast, signaling by the human NKR NKp80 remained unknown. Like NKG2D, NKp80 is an activating homodimeric C-type lectin-like receptor encoded in the natural killer gene complex (3). NKp80 binds to the genetically linked activation-induced C-type lectin (AICL) and, thereby, stimulates NK cytotoxicity against malignant myeloid cells, promotes a mutual cross-talk between NK cells and monocytes, but also augments responses of effector memory CD8 T cells (4, 5).

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Results and Discussion

Tyrosine 7 of NKp80 is phosphorylated and required for NK cytotoxicity

NKp80 is an activating receptor of human NK cells and some T cells (3–5); however, in contrast to many other NKRs, the molecular mechanisms of signal transduction by NKp80 are completely unknown. To dissect NKp80 signaling, we sought a suitable NK cell line for reconstitution experiments. NK92 cells have been used extensively to dissect signaling pathways involved in the activation of NK cytotoxicity (14). NK92MI is a derivative of NK92 cells stably transfected with the IL-2 gene (13) and does not express NKp80 at significant levels (Fig. 1B). When NKp80 cDNA was introduced into NK92MI cells, NKp80 was readily detectable at the cell surface (Fig. 1B). We performed redirected lysis experiments to assess functionality of ectopically expressed NKp80. P815 cells loaded with an NKp80-specific Ab were markedly lysed by NK92MI-NKp80 cells but not by the parental NK92MI cells (Fig. 1C).

Activating and inhibitory receptors of NK cells mediate their function through tyrosine phosphorylation of associated activating or inhibitory motifs. Given that NKp80 contains three tyrosine residues in its cytoplasmic tail (Fig. 1A), we determined whether these are required for cytotoxicity. We expressed FLAG-tagged NKp80 and NKp80 mutants Y7F, Y30F, and Y37F in NK92MI cells (Fig. 1B) and used these lines for redirected lysis of P815 cells loaded with an NKp80-specific Ab. Although NKp80, NKp80/Y30F, and NKp80/Y37F transductants readily lysed P815 cells with comparable efficiency, NKp80/Y7F transductants showed no significant cytolysis, similar to parental NK92MI cells (Fig. 1C). Hence, tyrosine 7 is required for the cytotoxic function of NKp80.

To determine whether NKp80 is tyrosine phosphorylated, we stimulated NK92MI transductants with pervanadate, a potent inhibitor of tyrosine phosphatases, because NKp80 cross-linking with available Abs induced only low levels of phosphorylation. Subsequently, NKp80 was immunoprecipitated and probed with phosphoryrosine-specific or FLAG-tag–specific Ab. NKp80, but not NKp80/Y7F, was tyrosine phosphorylated, suggesting that tyrosine 7 is the only or initial site of tyrosine phosphorylation in NKp80 (Fig. 2A).

NKp80 signals through a hemi-ITAM–like motif

The sequence surrounding tyrosine 7 in NKp80 shows remarkable similarity to the recently described hemi-ITAM (6, 7). Notably, 8 of the first 11 aa in NKp80 are identical to those in C-type lectin-like protein 2 (CLEC-2), a receptor for which hemi-ITAM–mediated signaling is well established (Fig. 1A) (6, 15). Aspartate 5 and glycine 6 of CLEC-2 are reportedly critical for hemi-ITAM signaling (6), but they are critical for NKp80 signaling.
altered in NKp80 to glutamate and arginine, respectively, suggesting that there may be functional differences between the motifs in CLEC-2 and NKp80. To directly compare signaling through NKp80 with hemi-ITAM–mediated signaling, we generated a double-point mutant, termed NKp80/DG, in which the first seven residues are identical to those in CLEC-2 (Fig. 1A). This mutant was expressed on NK92MI cells at levels comparable to NKp80 (Fig. 1B) and analyzed in functional assays. Tyrosine phosphorylation of NKp80/DG occurred earlier and at greater stoichiometry compared with that of NKp80 (Fig. 2A). Despite these differences in NKp80 phosphorylation, downstream signaling, indicated by phosphorylation of ERK, was efficiently induced through NKp80 and NKp80/DG, as well as through NKp80/Y30F and NKp80/Y37F, but not through NKp80/Y7F (Fig. 2B). In line with this, NKp80/DG triggered redirected cytotoxicity of NK92MI cells only slightly more efficiently than did NKp80 (Fig. 2C). These data demonstrated that deviations in the aminoterminal NKp80 signaling motif from a described consensus hemi-ITAM sequence (6) did not affect the capacity to induce NK cytotoxicity, despite a markedly lower level of phosphorylation.

In addition to cytotoxicity as a functional readout, we determined how NKp80 affects production of IFN-γ by NK92MI cells (Fig. 2D). NK92MI-NKp80 or NK92MI-NKp80/DG cells were stimulated with plate-bound mAb, and IFN-γ production was measured by ELISA (Fig. 2D). Stimulation of both cell lines through NKp30 resulted in high and comparable levels of IFN-γ secretion. Stimulation by NKp80–specific mAb also induced marked IFN-γ production by NK92MI-NKp80/DG cells but not NK92MI-NKp80 cells (Fig. 2D). Thus, although cytotoxicity is induced through NKp80 and consensus hemi-ITAM–containing NKp80 receptors with comparable efficiency, the capacity to induce secretion of IFN-γ by NK92MI cells is strongly impaired by the alterations in the NKp80 hemi-ITAM–like motif.

NKp80 signaling involves Src and Syk kinases and activates the common cytotoxicity pathway

Hemi-ITAM–containing receptors recruit and signal through Src-family and Syk-family kinases (6, 7, 15). Therefore, we used peptide pull-down experiments to determine whether the N-terminal NKp80 sequence can interact with these kinases. Using a biotinylated peptide comprising the aminoterminal NKp80 sequence from a described consensus hemi-ITAM sequence (6) did not affect the capacity to induce NK cytotoxicity, despite a markedly lower level of phosphorylation.
To determine whether Src and Syk kinases are required for NKp80-mediated cytotoxicity, we performed redirected cytotoxicity assays in the presence of the Src-family kinase inhibitor PP2 and the Syk kinase inhibitor R406 (9) (Fig. 3B, 3C). NKp80-mediated cytotoxic responses were abolished in the presence of PP2, suggesting that Src-family kinases are involved in NKp80 signaling. Similarly, cytotoxic responses were inhibited by R406 in a dose-dependent manner (Fig. 3C). An ~2-fold inhibition was observed using 0.25 μM R406, well in agreement with the EC50 reported for Syk inhibition in THP-1 macrophages (9). Consistent with this, half-maximal inhibition of cytotoxicity induced through NKp80/DG, which contains the hemi-ITAM of CLEC-2 that efficiently activates Syk (15), was also observed using 0.25 μM R406 (data not shown). These results indicated that NKp80 mediates cytotoxicity through Syk kinase. A common cytotoxicity-signaling pathway was described, incorporating Src-family kinases, PI3K, and the MAPK pathway (14). Thus, we used well-characterized inhibitors to determine whether NKp80 addresses this pathway (Fig. 3D). Inhibition of PI3K and, to a lesser extent, inhibition of MAPK diminished cytotoxicity, indicating that NKp80 activates this pathway.

To address whether the Syk pathway is also used upon AICL-mediated ligation of NKp80, we assessed degranulation of primary NK cells stimulated with C1R-AICL in the presence of the Syk inhibitor R406 (Fig. 3E). Similarly to blockade with anti-NKp80 mAb 5D12, the addition of R406 inhibited NK degranulation in a dose-dependent manner (Fig. 3E), suggesting that NKp80 also couples to the Syk pathway in primary NK cells for cytolysis of AICL-expressing cells.

NKp80 mediates cytotoxicity through Syk kinase

We next determined whether Syk kinase is activated in the NKp80-signaling pathway. First, we compared binding of Syk to phosphopeptides of NKp80 and CLEC-2, which is NKp80-signaling pathway. First, we compared binding of NKp80 mediates cytotoxicity through Syk kinase. A common cytotoxicity-signaling pathway was described, incorporating Src-family kinases, PI3K, and the MAPK pathway (14). Thus, we used well-characterized inhibitors to determine whether NKp80 addresses this pathway (Fig. 3D). Inhibition of PI3K and, to a lesser extent, inhibition of MAPK diminished cytotoxicity, indicating that NKp80 activates this pathway.

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NKp80 mediates cytotoxicity through Syk kinase

We next determined whether Syk kinase is activated in the NKp80-signaling pathway. First, we compared binding of Syk to phosphopeptides of NKp80 and CLEC-2, which is known to directly recruit Syk (Fig. 4A) (15). Therefore, we used lysates of WT8 cells that express Syk but not ZAP-70. Binding of Syk was comparable for both phosphopeptides at concentrations between 1 and 10 μM, suggesting that the interaction between Syk and NKp80 is physiologically relevant. However, although Syk readily coprecipitated with NKp80/DG, we failed to coprecipitate detectable amounts of Syk with wild-type NKp80 from lysates of pervanadate-treated NK92MI transductants (data not shown), presumably as a result of the markedly lower phosphorylation levels of NKp80 in this setting (Fig. 2A). Alternatively, we assayed for Syk phosphorylation as a consequence of NKp80 triggering, because Syk kinase is phosphorylated and activated upon recruitment (16): NKp80 on NK92MI-NKp80 or -NKp80/Y7F cells was cross-linked, and Syk was precipitated from lysates and probed with a phosphotyrosine-specific Ab. Ligation of NKp80, but not NKp80/Y7F, readily induced phosphorylation of Syk (Fig. 4B). Thus, Syk kinase is part of the NKp80-signaling pathway, and phosphorylation of Syk requires integrity of the Y7-containing motif of NKp80.

To complement the biochemical approaches above with more functional data, we used dominant-negative kinase defective (KD) Syk and ZAP-70 variants (11, 12). Expression of KD Syk in NK92MI-NKp80 cells severely reduced cytotoxic responses (Fig. 4C). By contrast, expression of KD ZAP-70 had little or no effect on NKp80-mediated cytotoxicity, but it impaired cytotoxic responses through NKp30, which couples to ZAP-70 (Fig. 4D). Altogether, these data strongly suggested that cytotoxicity triggered via NKp80 is mediated through a hemi-ITAM and Syk kinase.

In NKp80, two residues immediately N-terminal to tyrosine 7 are distinct from the described consensus hemi-ITAM sequence (6). Because mutation of these residues in CLEC-2 abolishes signaling through this receptor (6), an impact on the function of NKp80 was anticipated. Unexpectedly, such sequence alterations did not substantially affect NKp80-mediated cytotoxic responses, but they resulted in relatively low levels of NKp80 phosphorylation. It is known that substrate specificity of protein tyrosine kinases is determined by acidic and hydrophobic residues N-terminal of the relevant tyrosine (17). We found that Syk bound comparably well to NKp80 and CLEC-2 phosphopeptides, indicating that the residues preceding tyrosine 7 in these receptors do not directly impact Syk recruitment. Instead, arginine at position 6 in NKp80 may lead to less effective phosphorylation by Src kinases, resulting in less efficient downstream signaling and impaired cytokine secretion. It is not clear why signaling through NKp80 is weakened through an atypical hemi-ITAM sequence. However, it is conceivable that weaker proximal signaling may allow for a shorter dwell time of NK cells on target cells, resulting in more efficient cytotoxic responses, as it was proposed for CD8 T cells with low-affinity TCR (18). This remains to be tested for the NKp80–AICL interaction in a relevant setting.

Taken together, our data demonstrated that the activating receptor NKp80 induces cytotoxicity by using an atypical hemi-ITAM and the Syk-kinase pathway.

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Disclosures

A.S. filed a patent application on NKp80.

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