Cutting Edge: Functional Role for Proline-Rich Tyrosine Kinase 2 in NK Cell-Mediated Natural Cytotoxicity

Angela Gismondi, Jordan Jacobelli, Fabrizio Mainiero, Rossella Paolini, Mario Piccoli, Luigi Frati and Angela Santoni

*J Immunol* 2000; 164:2272-2276; doi: 10.4049/jimmunol.164.5.2272

http://www.jimmunol.org/content/164/5/2272

This article cites 23 articles, 18 of which you can access for free at:

http://www.jimmunol.org/content/164/5/2272.full#ref-list-1

Information about subscribing to *The Journal of Immunology* is online at:

http://jimmunol.org/subscription

Submit copyright permission requests at:

http://www.aai.org/About/Publications/JI/copyright.html

Receive free email-alerts when new articles cite this article. Sign up at:

http://jimmunol.org/alerts
Protein tyrosine kinase activation is one of the first biochemical events in the signaling pathway leading to activation of NK cell cytolytic machinery. Here we investigated whether proline-rich tyrosine kinase 2 (Pyk2), the nonreceptor protein tyrosine kinase belonging to the focal adhesion kinase family, could play a role in NK cell-mediated cytotoxicity. Our results demonstrate that binding of NK cells to sensitive target cells or ligation of β2 integrins results in a rapid induction of Pyk2 phosphorylation and activation. By contrast, no detectable Pyk2 tyrosine phosphorylation is found upon CD16 stimulation mediated by either mAb or interaction with Ab-coated P815 cells. A functional role for Pyk2 in natural but not Ab-mediated cytotoxicity was demonstrated by the use of recombinant vaccinia viruses encoding the kinase dead mutant of Pyk2. Finally, we provide evidence that Pyk2 is involved in the β2 integrin-triggered extracellular signal-regulated kinase activation, supporting the hypothesis that Pyk2 plays a role in the natural cytotoxicity by controlling extracellular signal-regulated kinase activation. 

The Journal of Immunology, 2000, 164: 2272–2276.

The proline-rich tyrosine kinase 2 (Pyk2), also known as cell adhesion kinase-β or related adhesion focal tyrosine kinase, is a nonreceptor protein tyrosine kinase (PTK) closely related to p125Fak and is regulated by a variety of extracellular stimuli that elevate intracellular calcium or induce protein kinase C activation (1, 2). Pyk2-related adhesion focal tyrosine kinase/cell adhesion kinase-β is expressed in different cell types including brain cells, fibroblasts, and hemopoietic cells (1–7). In hemopoietic cells, Pyk2 and its alternatively spliced isoform, Pyk2-H, are activated by cytokines, chemokines, and through a number of receptors including multichain immune recognition receptors and integrins (4–8). Pyk2 can interact with several signaling or cytoskeletal molecules such as Src family PTKs, the Grb2 and p130Cas adaptors, paxillin, and the Rho guanine nucleotide exchange factor Graf (4–6). Moreover, recent evidence indicates that in response to different stimuli Pyk2 acts as an upstream activator of the mitogen-activated protein kinase family (1).

NK cells are a CD3−, CD16+, CD56+ lymphocyte subpopulation endowed with the capacity of naturally killing a wide array of target cells. In addition to natural cytotoxicity, NK cells can mediate Ab-dependent cellular cytotoxicity (ADCC) through the low-affinity Fc receptor for IgG, FcyRIII (CD16) (9). The receptor-ligand interactions by which target cells trigger natural cytotoxicity are still poorly defined, although it is becoming increasingly clear that the final outcome of NK cell activity results from a balance between triggering and inhibitory receptors and ligands (10).

Recently, many efforts have been focused to understand the signaling pathways leading to NK cell cytotoxic function, and a crucial role for PTK activation has been demonstrated (10). Ligation of a number of receptors triggering cytotoxicity or NK cell interaction with sensitive target cells results in the activation of both Syk/Zap-70 and Src family PTKs, and a crucial role for Syk in both natural and ADCC activities has been reported (10, 11). Although natural and Ab-dependent cytotoxicity trigger common intracellular signaling events and share downstream targets (12–17), they are also coupled to distinct biochemical pathways. Indeed, Syk is activated by both natural and Ab-dependent cytotoxicity, while Zap-70 is activated only through CD16 (11); in addition, PKC is involved in the regulation of natural killing but not ADCC, and phosphatidylinositols 3-kinase plays a role in CD16-initiated granule exocytosis and killing, but not in natural cytotoxicity (18).

Our previous evidence indicates that human NK cells express the focal adhesion kinase (FAK)-related nonreceptor PTK Pyk2 that is constitutively associated with the cytoskeletal protein paxillin, and engagement of β1 integrins on human NK cells results in tyrosine phosphorylation of both Pyk2 and paxillin (6). We have also reported that upon β1 integrin ligation Pyk2 can bind to Shc and Grb2, suggesting a role for this PTK in the β1 integrin-triggered Ras/mitogen-activated protein kinase cascade (19).
The ability of Pyk2 to form macromolecular complexes potentially capable of regulating cytoskeletal rearrangement and signaling pathways leading to both immediate and later functional responses prompted us to investigate whether Pyk2 could play a role in NK cell-mediated cytotoxic functions.

Materials and Methods

Abs

The following mouse mAbs were used: anti-CD16 (B73.1) was kindly provided by Dr. G. Trinchieri (Wistar Institute, Philadelphia, PA); anti-CD56 (C218) was kindly provided by Dr. A. Moretta (University of Genova, Genoa, Italy); anti-β2 (4B4) integrin subunit was purchased from Coulter Immunology, Hialeah, FL; anti-β2 (TS1/18) was a generous gift by Dr. F. Sanchez-Madrid (La Princesa Hospital, University of Madrid, Madrid, Spain); anti-phosphotyrosine (anti-pTyr) (4G10) was purchased from Upstate Biotechnology (Lake Placid, NY); anti-phospho-Erk (anti-pErk) (E4) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit antiserum against Erk (K23) was purchased from Santa Cruz Biotechnology (Lake Placid, NY); rabbit antiserum against mouse Ig (RAM) was purchased from Zymed Laboratories (San Francisco, CA). Affinity-purified rabbit antiserum against mouse Ig (GAM) were purchased from Cappel Immunology, Hialeah, FL; anti-β2, (TS1/18) was a generous gift by Dr. F. Sanchez-Madrid (La Princesa Hospital, University of Madrid, Madrid, Spain).

Human NK cell preparation

Highly purified (95%) cultured human NK cells were obtained as previously described (6).

Recombinant vaccinia virus generation and infection

cDNAs encoding wild-type Pyk2 and the kinase-dead mutant of Pyk2 (PykM) were kindly provided by Dr. J. Schlessinger (1). The coding sequences were isolated from pRK5 using EcoR1 and subcloned into SalI/Ncol cloning site of pSC-66. The cDNAs within the recombinant pSC-66 vector were then introduced into the WR strain of vaccinia, kindly provided by Jean-Pierre Kinet and Andrew M. Scharenberg (Harvard Medical School, Boston, MA), via homologous recombination (20). Semipurified recombinant vaccinia virus was used to infect human NK cells for 1 h in serum-free medium at a multiplicity of infection of 20:1. The remainder of the infection (4 h) was conducted in RPMI 1640 with 10% FCS. Cellular debris were removed from infected NK cells by Lymphoprep (Nycomed, Oslo, Norway) gradient centrifugation, and viability was >90% before biochemical and functional assays.

Cytotoxicity assay

The K562 human erythroleukemia cell line was used as target for natural cytotoxicity, and the murine mastocytoma cell line FcγR III P815 was used for reverse ADCC. The 51Cr release assay was performed as previously described (21). Lytic units were calculated based on 20% cytotoxicity (9).

[32P]Orthophosphate labeling, cell stimulation, and lysate preparation

NK cells were labeled (2 x 10^7 cells/ml) for 4 h at 37°C with [32P]orthophosphate (0.2 μCi/ml, 4500 Ci/mmol) (Amersham International, Little Chalfont, U.K.) in phosphate-free RPMI 1640 (Life Technologies, Gaithersburg, MD) supplemented with 0.1% phosphate-free FCS. Then, 3 x 10^7 32P-labeled NK cells were incubated with 1.5 x 10^7 targets at 37°C for the indicated times. Incorporated radioactivity was quantified in cell lysates after cold 10% TCA precipitation, and equal amounts of 32P-labeled proteins from each cell lysate were immunoprecipitated with anti-Pyk2 Abs. Binding experiments were also performed using unlabeled NK cells and paraformaldehyde-prefixed K562 target cells (E:T ratio 5:1) as previously reported (15). In experiments involving Ab-mediated cell-surface receptor engagement, NK cells (4 x 10^6 cells/300 μl/tube) incubated with saturating doses of the appropriate mAb for 30 min at 4°C were stimulated for different lengths of time with soluble GAM (1.5 μg/10^6 cells) or GAM coated to polystyrene beads at 37°C (19). Cell lysates, immunoprecipitation, immune complex kinase assay, and immunoblotting analysis were performed as previously described (6).

Results and Discussion

Binding of NK cells to sensitive target cells but not CD16 engagement results in tyrosine phosphorylation of NK cell-derived Pyk2

Upon NK cell binding to target cells, PTK activation is one of the first biochemical events in the signaling pathways leading to the activation of the cytolytic machinery (10). Thus, we investigated whether the nonreceptor PTK, Pyk2 (1, 2), could be phosphorylated upon binding of NK cells to sensitive target cells. To analyze NK cell- but not target cell-derived Pyk2, human NK cells were labeled with [32P]orthophosphate and then incubated with the prototypic NK-sensitive target cell, K562. As shown in Fig. 1A, binding of NK cells to K562 targets resulted in Pyk2 phosphorylation (arrow), which was already evident at 5 min, peaked at 10 min, and declined at 20 min after stimulation. In addition to Pyk2, other proteins migrating at 65–68 kDa were present in the anti-Pyk2 immunoprecipitates, and their phosphorylation was enhanced with the same kinetics of Pyk2. The identity of these proteins is presently unknown, but they likely represent the Pyk2-associated cytoskeletal protein, paxillin, which undergoes phosphorylation upon β2 integrin ligation on NK cells (6). The increase in Pyk2 phosphorylation observed upon [32P]orthophosphate-labeled NK cell binding to K562 targets correlated with increased Pyk2 tyrosine phosphorylation as demonstrated by immunoblotting analysis with anti-pTyr Ab of Pyk2 immunoprecipitates obtained from NK cells stimulated with prefixed K562 targets (Fig. 1B).

We also investigated whether triggering of CD16-mediated cytoxicity could result in Pyk2 activation. 32P-labeled NK cells were allowed to bind to murine P815 mastocytoma cells in the presence of anti-P815 Ab. We found that Pyk2 undergoes phosphorylation 10 min after NK cell binding to unsensitized P815, and no further increase in the levels of Pyk2 phosphorylation is observed upon anti-P815 Ab-mediated triggering of CD16 (Fig. 1A). Stimulation of Pyk2 phosphorylation upon direct NK cell interaction with P815 is not surprising, as these target cells are barely lysed by the NK cell population used in this study (data not shown). No phosphorylated proteins were detected in RAM immunoprecipitates, used as control (data not shown).
To further explore the ability of CD16 engagement to induce Pyk2 phosphorylation, NK cells were treated with mAb directed against CD16, β1 integrin subunit used as positive control, or CD56 used as negative control. As shown in Fig. 2, unlike β1 integrins, ligation of CD16 does not induce any significant tyrosine phosphorylation of Pyk2. Pyk2 phosphorylation was not detected also when CD16 Ag was cross-linked for different time periods (data not shown). Very low levels of Pyk2 tyrosine phosphorylation was observed upon CD16 ligation on NK cells from some donors (data not shown). Overall these results indicate that Pyk2 is phosphorylated by natural but not Ab-mediated cytotoxicity, and suggest that Pyk2 activation is a discriminating event in the signaling pathway leading to natural vs Ab-dependent NK cell cytotoxicity.

Natural cytotoxicity is a function of multiple receptor-ligand interactions between NK and target cells (10). We and others have previously reported that integrins could play an important role in NK cell-mediated cytotoxicity, in that β1 and β2 integrin ligation costimulate NK cytotoxic functions and β2 integrin-mediated triggering of cytotoxicity is observed upon an appropriate redistribution of ICAM-2 on the target cell membrane (21–23). Therefore, we investigated whether β2 integrin engagement on human NK cells could result in stimulation of Pyk2 tyrosine phosphorylation. As shown in Fig. 3, in β2 integrin-stimulated NK cells, Pyk2 tyrosine phosphorylation was already maximal at 1 min and started to decline 10 min after stimulation, as we previously reported for β1 integrins (6). Moreover, tyrosine phosphorylated proteins migrating at 61–68 kDa coprecipitated with Pyk2 upon β2 integrin ligation, as observed following NK cell binding to sensitive target cells (see Fig. 1).

**Induction of Pyk2 PTK activity upon β2 integrin ligation on NK cells or NK cell binding to K562 targets**

To assess whether NK cell stimulation through β2 integrins or binding to K562 targets results in induction of Pyk2 kinase activity, Pyk2 immunoprecipitates from unstimulated, anti-β2 integrin-, or K562 target-stimulated NK cells were analyzed in in vitro kinase assay. As shown in Fig. 4, Pyk2 immunoprecipitates from stimulated cells contained tyrosine kinase activity evaluated as autophosphorylation (Fig. 4, A and B, top) and phosphorylation of an exogenous substrate, poly(Glu-Tyr) (Fig. 4, A and B, middle).

**No phosphorylated proteins were detected in RAM immunoprecipitates used as control (data not shown).**

These results suggest that the intrinsic tyrosine kinase activity of Pyk2 is induced by β2 integrin ligation on NK cells or NK cell binding to K562 targets.

**Pyk2 activation is a crucial event for natural but not Ab-dependent cytotoxicity**

To investigate whether Pyk2 is functionally involved in NK cell-mediated cytotoxicity, NK cells were infected with recombinant vaccinia viruses encoding the wild-type (Pyk2) or the kinase dead mutant of Pyk2 (PykM) shown to prevent Pyk2 enzymatic activity (1), and then assayed for natural cytotoxicity or reverse ADCC.
Overexpression of PykM but not wild-type Pyk2 significantly inhibited natural cytotoxicity without affecting reverse ADCC (Fig. 5). Equal levels of overexpression of the two Pyk2 constructs was demonstrated by Western blot of whole-cell lysates (Fig. 5, right). In addition, enhancement of natural but not CD16-initiated cytotoxicity was observed following overexpression of wild-type Pyk2 in some experiments (data not shown). The ability of PykM to inhibit natural killing suggests that Pyk2 kinase activity is required for the generation of natural cytotoxicity.

Recent evidence indicate that Pyk2 is an upstream activator of Erk cascade (1), and activation of Erk pathway is required for generation of both natural and Ab-dependent cytotoxicity (14–16). Therefore, we investigated whether Pyk2 could regulate β2 integrin-induced activation of Erks. NK cells infected with recombinant vaccinia viruses encoding wild-type Pyk2, PykM, or vaccinia virus alone (WR), were assayed in a 4-h 51Cr release assay against K562 or P815 plus anti-CD16 mAb (reverse ADCC). Data are expressed as the mean ± SD of lytic units/10⁶ cells obtained from three independent experiments. The amounts of overexpressed Pyk2 are shown on the right; NI represents noninfected cells.

FIGURE 5. Pyk2 is involved in natural cytotoxicity but not in ADCC. NK cells, infected for 4 h either with recombinant vaccinia virus encoding wild-type Pyk2, Pyk-M, or vaccinia virus alone (WR), were assayed in a 4-h 51Cr release against K562 or P815 plus anti-CD16 mAb (reverse ADCC). Data are expressed as the mean ± SD of lytic units/10⁶ cells obtained from three independent experiments. The amounts of overexpressed Pyk2 are shown on the right; NI represents noninfected cells.

FIGURE 6. Cross-linking of β2 integrins on human NK cells induces Pyk2-dependent Erk activation. NK cells, infected as indicated in Fig. 5, were left untreated (−) or stimulated with anti-β2 (TS1/18) mAb plus GAM-coated beads for 10 min at 37°C, and Erk activation was examined by Western blot analysis performed on total cell lysates using an anti-phospho Erk mAb. As loading controls, the amounts of Erk protein are shown on the central panel. The amounts of overexpressed Pyk2 are shown on the bottom. Upon β2 stimulation, a 10-fold increase of pErk was observed in WR and Pyk2 and a 5-fold was observed in PykM. These results represent one of three independent experiments.

triggered Ras activation is associated with tyrosine phosphorylation of LAT, which binds to the adaptor Grb2 (24). Moreover LAT tyrosine phosphorylation is rapidly induced following direct NK cell contact with sensitive target cells, and a functional role for LAT in both natural and Ab-dependent cytotoxicity has been demonstrated (17). Thus, one can hypothesize that LAT may be the substrate of different kinases, i.e., Pyk2 vs Syk-family PTKs. At present, it is unclear whether there is any functional interdependence between Pyk2 and Syk upon NK cell interaction with target cells. It has been recently reported that Syk activation is central to the generation of both natural cytotoxicity and ADCC (11) and that Pyk2 activation may occur through Syk-dependent and independent pathways (7). Based on this observation, our data suggest that either Pyk2 activation does not require Syk and Pyk2 may cooperate with this PTK to fully activate natural cytotoxicity or Pyk2 is upstream to Syk.

Acknowledgments

We thank Drs. J. Schlessinger and I. Dikic for kindly providing the anti-Pyk2 Ab and the cDNA coding for Pyk2 and PykM and Dr. A. Serra for collaborating in the preparation of the recombinant vaccinia viruses used in this study. We thank Dina Milana, Anna Maria Bressan, Alessandro Proccaci, Antonio Sabatucci, and Patrizia Biralere for expert technical assistance.

References

2276 CUTTING EDGE


