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Src-Dependent Syk Activation Controls CD69-Mediated Signaling and Function on Human NK Cells[1]

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CD69 C-type lectin receptor represents a functional triggering molecule on activated NK cells, capable of directing their natural killing function. The receptor-proximal signaling pathways activated by CD69 cross-linking and involved in CD69-mediated cytotoxic activity are still poorly understood. Here we show that CD69 engagement leads to the rapid and selective activation of the tyrosine kinase Syk, but not of the closely related member of the same family, ZAP70, in IL-2-activated human NK cells. Our results indicate the requirement for Src family kinases in the CD69-triggered activation of Syk and suggest a role for Lck in this event. We also demonstrate that Syk and Src family tyrosine kinases control the CD69-triggered tyrosine phosphorylation and activation of phospholipase Cγ2 and the Rho family-specific exchange factor Vav1 and are responsible for CD69-triggered cytotoxicity of activated NK cells. The same CD69-activated signaling pathways are also observed in an RBL transfectant clone, constitutively expressing the receptor. These data demonstrate for the first time that the CD69 receptor functionally couples to the activation of Src family tyrosine kinases, which, by inducing Syk activation, initiate downstream signaling pathways and regulate CD69-triggered functions on human NK cells. *The Journal of Immunology,* **2002, 169: 68–74.**

Natural killer cell interaction with target cells relies upon a vast array of triggering and inhibitory receptors, mainly belonging to Ig and C-type lectin superfamilies, whose ultimate balance dictates the development of cytotoxic activity. Besides activating receptors expressed on their endogenous state, such as NKp46, NKp30, NKG2-D, MHC-I-specific CD94/NKG2-C, and triggering counterparts of killer inhibitory receptors (1–3), activation of human NK cells induces the de novo expression of other triggering receptors, such as CD69, NKp44, CD40 ligand, and CD80/CD86 counterstructures, which endow activated NK cells with new recognition capability (3–7). The ligands for the receptors responsible for natural cytotoxic activity remain mostly unidentified yet.

The signaling pathways activated by triggering receptors on NK cells and responsible for NK cytotoxicity have not yet been satisfactorily clarified. Although belonging to different molecular families, many activating NK receptors associate with conserved tyrosine-based sequence-containing subunits, such as TCR ζ, FcϵRI γ, DAP-12, and DAP-10, which allow the SH2-dependent recruitment and activation of intracellular effectors in a protein tyrosine kinase (PTK)[4]-dependent fashion (8). Thus, receptor-mediated activation of PTK has been acknowledged as a critical step in the development of cytotoxic activity, and, in particular, the central role of Syk has been shown (8–10). The possible contribution of PTK belonging to other families, such as Src, remains mostly unexplored. Among the downstream events, the crucial involvement of phospholipase Cγ (PLCγ)-activated calcium influx and the Vav-Rac-extracellular signal-regulated kinase (ERK) pathway in cytotoxic activity and degranulation has been reported (11–17).

CD69 belongs to the family of C-type lectin receptors whose genes are clustered in the NK gene complex (18–21) and bears strong similarity to the closely related NK receptors CD94 and Ly49A (22, 23). Although constitutively expressed on few cell types, CD69 is rapidly acquired following in vitro activation of T, B, and NK cells, neutrophils, and eosinophils (24, 25).

CD69 cross-linking induces the cytotoxic activity and costimulates cytokine production of activated NK cells and selected T cell clones, thus representing a putative receptor for target cells of activated cytotoxic lymphocytes (4, 5, 24, 26, 27).

The signal transduction pathways activated by CD69 engagement and responsible for its functional ability are poorly understood. Although CD69 cross-linking has been reported to induce intracellular signaling events in different cell types (5, 24), no clear information is available on the receptor-proximal mechanisms that endow CD69 with signaling ability. Moreover, no recognizable sequences for connection to intracellular effectors are present in the CD69 intracellular domain or has association with accessory chains been reported to date. In particular, no evidence on its ability to activate PTK-dependent pathways has been provided.

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[4] Abbreviations used in this paper: PTK, protein tyrosine kinase; DAG, diacylglycerol; ERK, extracellular signal-regulated kinase; GAM, goat anti-mouse IgG F(ab′)$_2$; ITAM, immunoreceptor tyrosine-based activation motif; Lck-KI, kinase-inactive Lck; MBP, myelin basic protein; PI, phosphoinositide-specific; PI4,5PIP$_2$, phosphatidylinositol 4,5-bisphosphate; PLC, phospholipase C; PP2, 4-amino-5-(4-chlorophenyl)-7-(*t*-butyl)-pyrazolo[3,4-d]pyrimidine; pTyr, phosphotyrosine.

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The aim of our work was to investigate the involvement of PTK-dependent signaling pathways in the CD69-mediated cytotoxic activity of human NK cells. Our results demonstrate that CD69 engagement specifically induces the activation of Syk and disclose the essential involvement of members of the Src family in this event. In an effort to identify downstream effectors for CD69-activated PTKs, we show that CD69 cross-linking initiates two important pathways involved in the development of cytotoxicity, namely the activation of PLCγ and Vav1. Our results also indicate that Src- and Syk-dependent pathways control CD69-triggered cytotoxic activity. The same signaling events are also triggered by CD69 stimulation in an RBL transfectant clone constitutively expressing the human CD69 receptor.

Materials and Methods

Cell lines and Abs

The murine FcγR$^+$ P815 mastocytoma cell line was used for redirected cytotoxicity assay. The RBL-2H3 clone transfected with human CD69 cDNA R8#6 has been previously described (5).

The following mouse mAbs were used: anti-CD3 (Leu 4), anti-CD16 (Leu 11c), anti-CD56 (Leu 19), and anti-CD69 (Leu 23), all from BD Biosciences (Franklin Lakes, NJ); anti-CD69 (clone TP1/8 (28) and clone 227 (4)) provided by Dr. F. Sanchez-Madrid (Hospital de La Princesa, Madrid, Spain) and Prof. A. Moretta (University of Genoa, Genoa, Italy), respectively; anti-CD16 (B73.1) and anti-CD56 (C218) provided by Dr. G. Trinchieri (Schering-Plough, Dardilly, France), and Prof. A. Moretta, respectively; anti-FcϵRI α-chain (BC4) was kindly provided by Dr. R. Siraganian (National Institutes of Health, Bethesda, MD). Goat anti-mouse IgG F(ab′)$_2$ (GAM) was from Cappel Laboratories (ICN Biomedicals, Opera, Milan, Italy); FITC-conjugated GAM was purchased from Zymed (South S. Francisco, CA); rabbit anti-ERK, anti-Syk, and anti-PLCγ2 antisera, and anti-Syk and anti-Lck mAb were obtained from Santa Cruz Biotechnology (Santa Cruz, CA); anti-Vav1 and anti-ZAP70 mAb were purchased from Upstate Biotechnology (Lake Placid, NY).

NK cell preparation and immunofluorescence analysis

Polyclonal NK cell cultures were obtained by coculturing nylon nonadherent PBMC with irradiated RPMI 8866 cells for 9–10 days at 37°C in a humidified 5% CO_2 atmosphere as previously described (5); NK cell cultures (>90% CD16$^+$, CD56$^+$, CD3$^-$, as assessed by immunofluorescence and cytofluorometric analysis) were treated with human rIL-2 (EuroCetus, Amsterdam, The Netherlands; 500 U/ml) for 8 h at 37°C to induce CD69 expression and then were kept overnight in RPMI 1640 plus 0.1% FCS. At the end of incubation, cells were >95% CD69$^+$, as evaluated by FACS analysis.

For immunofluorescence staining, cells were incubated with saturating concentrations of specific mAbs, either directly conjugated to fluorochromes or revealed by a second incubation with FITC-conjugated GAM, and analyzed on a FACSCalibur (BD Biosciences). Fluorescence intensity is expressed in arbitrary units on a logarithmic scale.

Cell stimulation, lysis, immunoprecipitation, and Western blot analysis

IL-2-activated human NK cells or R8#6 transfectants were incubated with primary mAb for 30 min on ice, then stimulated with GAM (1.5 μg/10^6 cells) for different time periods at 37°C. In some experiments cells were pretreated with either piceatannol (Biomol, Plymouth Meeting, PA) or 4-amino-5-(4-chlorophenyl)-7-(*t*-butyl)pyrazolo[3,4-d]pyrimidine (PP2) (Calbiochem, Darmstadt, Germany) for 20 min at 37°C and kept in the presence of inhibitors during the assay. After stimulation cells were lysed and immunoprecipitated as previously described (29); immunocomplexes were resolved by SDS-PAGE and transferred to Immobilon-P nitrocellulose membranes (Millipore, Bedford MA). After blocking nonspecific reactivity, filters were probed with specific Abs diluted in TBS-T (20 mM Tris-HCl (pH 7.5), 150 mM NaCl, and 0.05% Tween 20). After extensive washing, immunoreactivity was detected using an ECL kit (Amersham Pharmacia Biotech Italia, Cologno Monzese, Milan, Italy).

In vitro kinase assay

Immunoprecipitates were processed for kinase assay as previously described (29). Kinase reaction was performed at 30°C in 25 μl kinase assay buffer plus 10 μCi [γ-^{32}P]ATP (4500 Ci/mmol; Amersham), 1 μM ATP, and 5 μg myelin basic protein (MBP; Sigma-Aldrich, Italy) (when indicated). Samples were separated by SDS-PAGE, transferred to nitrocellulose membranes, exposed to autoradiography, and then subjected to Western blot analysis.

Vaccinia virus infection

WR strain and recombinant vaccinia viruses encoding wild-type or a kinase-inactive Lck mutant (lysine to arginine mutation at position 273) were provided by Dr. P. J. Leibson (Department of Immunology, Mayo Clinic and Foundation, Rochester, MN). Viruses were amplified, semipurified, and titrated using standard techniques (30). Semipurified vaccinia viruses were used to infect human NK cells for 1 h in serum-free medium at a multiplicity of infection of 20:1. Cells were then incubated for an additional 4 h in RPMI containing 0.1% BSA and 25 mM HEPES, as previously described (31). After infection, dead cells were removed by Ficoll-Hypaque density gradient centrifugation for 15 min at 1400 rpm. The stimulation was performed as described above.

Cytotoxicity assay

The ^{51}Cr release assay was used to measure redirected cytotoxic activity against P815 target cells in the presence of saturating amounts of different mAbs, as previously described (5, 29). IL-2-activated NK cell cultures, used as effector cells, were preincubated with PP2, piceatannol, or diluent (DMSO) for 30 min, then tested for cytotoxicity. Inhibitors were present throughout the assay.

Phosphoinositide-specific (PI)-PLC activity assay

After stimulation, IL-2-activated NK cells were resuspended in 50 mM Tris-HCl, pH 7.4, containing protease inhibitors and lysed by sonication, and protein concentrations were determined using the Bio-Rad protein assay (Bio-Rad, Hercules, CA). PI-PLC activity in cell lysates was quantitated by the release of diacylglycerol (DAG) from radiolabeled phosphatidylinositol 4,5-bisphosphate (PI4,5PIP$_2$; DuPont-New England Nuclear, Boston, MA)-containing vesicles and is expressed as picomoles of DAG per milligram of protein, as previously described (32).

Results

CD69 engagement induces the selective activation of Syk, but not ZAP70, kinase in human NK cells

Several immunoreceptor tyrosine-based activation motif (ITAM)-coupled activating receptors have been reported to direct the activation of Syk/ZAP70 family PTKs on NK cells (8, 10, 11). To investigate whether CD69 receptor could induce the activation of these enzymes, highly purified IL-2-stimulated human NK cells were triggered by anti-CD69 mAb plus GAM treatment, and the tyrosine phosphorylation status of Syk and ZAP70 was evaluated after immunoprecipitation with specific Abs. As shown by anti-phosphotyrosine (anti-pTyr) immunoblot, CD69 engagement induces the rapid and transient tyrosine phosphorylation of Syk compared with untreated sample (Fig. 1*A*, *upper panel*). Strikingly, under the same conditions, no increased tyrosine phosphorylation of ZAP70 protein above the basal level was found (Fig. 1*B*, *upper panel*). However, CD16 stimulation potently induced the tyrosine phosphorylation of both Syk and ZAP70, as previously reported (11, 29), while treatment with an isotype-matched control mAb did not affect the phosphorylation level of either kinase. Western blots of the same membranes with anti-Syk and anti-ZAP70 mAb (Fig. 1, *A* and *B*, *lower panel*, respectively) confirmed the equivalent loading of all lanes.

Syk enzymatic activation was then directly assessed by in vitro kinase assay on specific immunoprecipitates. As shown in Fig. 2*A* (*upper panel*), CD69 engagement on NK cells induced the significant augmentation of Syk activity on the exogenous substrate MBP compared with the untreated or control mAb-treated sample. CD69-triggered activation of Syk (evaluated as in vitro autophosphorylation) was also observed in the CD69$^+$ RBL transfectant cell clone R8#6 (Fig. 2*B*, *upper panel*), in which the ability of

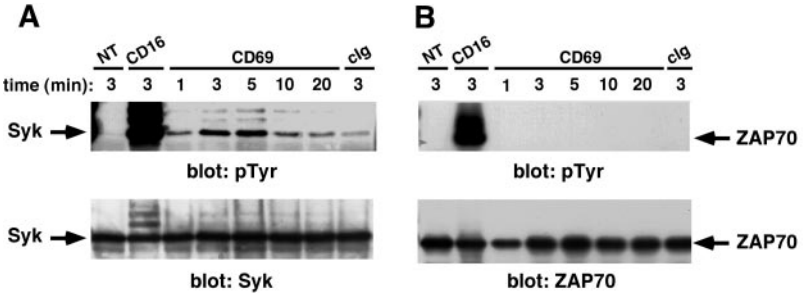

FIGURE 1. CD69 engagement induces Syk, but not ZAP70, activation in human NK cells. IL-2-activated NK cells (50×10^6/sample) were left untreated (NT) or were stimulated with saturating doses of different mAb plus GAM at 37°C for the indicated time periods. Cell lysates were immunoprecipitated with anti-Syk (*A*) or anti-ZAP70 (*B*) mAb, electrophoresed under reducing conditions, blotted with anti-pTyr mAb (*upper panel*), and, after stripping off bound Ab, reprobed with anti-Syk and anti-ZAP70 mAb, respectively (*lower panel*). The data shown are representative of one of four independent experiments.

CD69 to trigger signaling and functional events has been previously assessed (5). Syk activation through CD16 and FcεRI receptor triggering was also shown in NK and RBL transfectants, respectively, as a positive control (29, 33). Western blot analysis showed a CD69-mediated increase in tyrosine phosphorylation of Syk (Fig. 2, *A* and *B*, *middle panel*) and normalization with the specific anti-Syk Abs (Fig. 2, *A* and *B*, *lower panel*) in both cell types.

Collectively taken, this set of data demonstrates that CD69 engagement induces the selective activation of Syk, but not ZAP70, in human NK cells; moreover, the ability of CD69 to activate Syk kinase is also evident in a heterologous cell system, where CD69 is constitutively expressed.

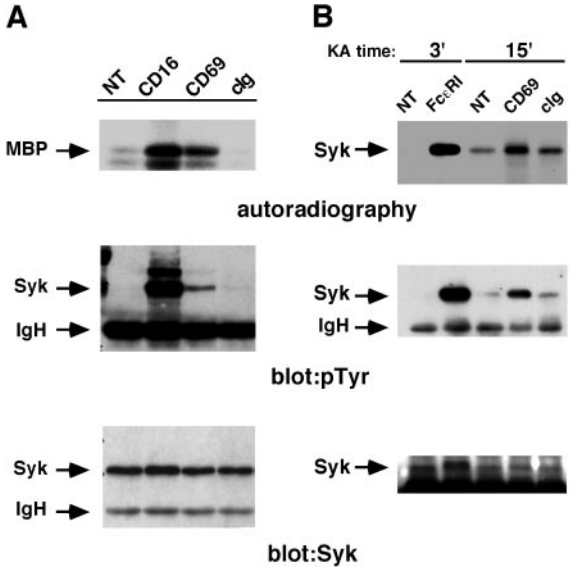

FIGURE 2. CD69 engagement induces Syk activation in human NK cells and in CD69$^+$ RBL transfectants. *A*, IL-2-activated NK cells (20×10^6/sample) were left untreated (NT) or were stimulated with saturating doses of different mAb plus GAM for 3 min at 37°C, and cell lysates were immunoprecipitated with anti-Syk mAb. Syk immunoprecipitates were subjected to in vitro kinase assay in the presence of MBP. Then, samples were separated on 12.5% (MBP) or 7.5% (Syk immunoprecipitates) SDS-PAGE, transferred to nitrocellulose membranes, and either subjected to autoradiography (*upper panel*) or immunoblotted with anti-pTyr mAb (*middle panel*) and, after stripping off bound Ab, with anti-Syk Ab (*lower panel*) as indicated. *B*, CD69$^+$ R8#6 transfectants (10×10^6 cells/sample) were left untreated (NT) or were stimulated with saturating doses of different mAb plus GAM for 3 min at 37°C, and cell lysates were immunoprecipitated with anti-Syk Ab. Syk immunoprecipitates were subjected to in vitro kinase assay (KA) for 3 or 15 min as indicated. After separation by SDS-PAGE, the gel was transferred to membrane, subjected to autoradiography (*upper panel*), then blotted with anti-pTyr mAb (*middle panel*) and, after stripping off bound Ab, reprobed with anti-Syk Ab (*lower panel*). IgH indicates the reactivity of the heavy chain of the Ab used in the immunoprecipitation. Results are representative of one of three independent experiments.

CD69-triggered activation of Syk is mediated by Lck

The PTK-dependent activation of Syk by immunoreceptors may depend on autophosphorylation or be under the control of Src family kinases (33–35); no definitive information exists on the upstream events involved in Syk activation upon engagement of NK-activating receptors. To address the role of Src family kinases in CD69-triggered activation of Syk, we initially used the Src-selective pharmacological inhibitor PP2 (36). The experiment reported in Fig. 3 shows that pretreatment of IL-2-activated NK cells with PP2 completely abrogated the CD69-triggered tyrosine phosphorylation of Syk; conversely, the Syk/ZAP70-specific inhibitor piceatannol (37) did not affect the CD69-induced tyrosine phosphorylation of Syk under the same experimental conditions. It is worth noting that the ability of piceatannol to substantially suppress the Syk-dependent tyrosine phosphorylation of different substrates was assessed in the same samples (data not shown and Figs. 6 and 8). However, a slight inhibitory effect of piceatannol on CD69-triggered tyrosine phosphorylation of Syk could be occasionally appreciated in some donors. This result thus indicates that Src family PTK activity is an essential requisite for the CD69-mediated tyrosine phosphorylation of Syk.

Among the Src family members, Lck has been shown to be associated with NK receptors of the C-type lectin family (38, 39) and with CD16, where its crucial involvement in the early steps of signaling has been reported (40–44). To directly analyze the role

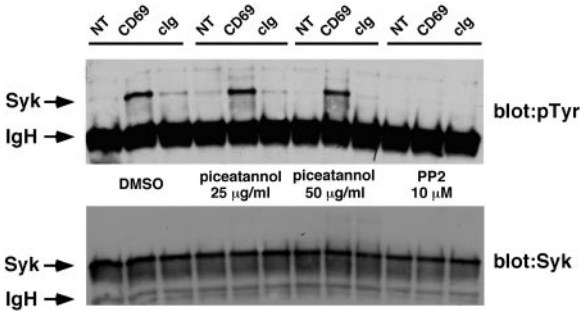

FIGURE 3. CD69-triggered phosphorylation of Syk depends on Src family kinases. IL-2-activated NK cells (25×10^6/sample) were preincubated with piceatannol, PP2, or diluent (DMSO) for 20 min at 37°C, then left untreated (NT) or stimulated with saturating doses of anti-CD69 or control mAb plus GAM for 3 min at 37°C, and cell lysates were immunoprecipitated with anti-Syk mAb. Immunoprecipitates were separated on SDS-PAGE, transferred to nitrocellulose membranes, and subjected to Western blot with anti-pTyr mAb (*upper panel*) and, after stripping off bound Ab, with anti-Syk mAb (*lower panel*) as indicated. IgH indicates the reactivity of the heavy chain of the Ab used in the immunoprecipitation. Results are representative of one of three independent experiments.

of Lck in CD69-initiated signaling, the wild-type or kinase-inactive form of Lck kinase was overexpressed in human IL-2-activated NK cells by means of recombinant vaccinia virus vector infection. As shown in Fig. 4*A*, the overexpression of wild-type Lck (Lck) greatly augmented the CD69-induced tyrosine phosphorylation of Syk, while the kinase-inactive enzyme (Lck-KI) did not affect Syk phosphorylation levels compared with those in samples infected with the empty viral vector (WR). It is worth noting that although overexpression of wild-type Lck augmented the basal level of phosphorylated Syk (40), the net level induced by CD69 engagement was nevertheless >3-fold higher than that observed in empty virus- or Lck-KI-infected samples, as indicated by densitometric analysis (data not shown). The Western blot of whole cell lysates from the same cell samples showed the overexpression levels of Lck and the comparable levels of Syk protein in the different samples (Fig. 4*B*).

These results show that PTKs belonging to the Src family control the CD69-triggered activation of Syk on human NK cells and strongly suggest a role for Lck in this event.

CD69 induces the PTK-dependent activation of PLCγ

PTK activation by NK triggering receptors initiates many signaling pathways through the regulation of different enzymes and adaptor proteins; in particular, PTK-dependent activation of PLCγ leads to the generation of inositol 1,4,5-trisphosphate and DAG second messengers, which are involved in the development of cytotoxic activity (8, 11, 45). To investigate whether CD69 engagement could trigger PLCγ tyrosine phosphorylation and activation, PLCγ2 was immunoprecipitated from CD69-stimulated IL-2-activated NK (Fig. 5*A*) or $CD69^+$ R8#6 transfectant (Fig. 5*B*) cell lysates and immunoblotted with anti-pTyr mAb. CD69 cross-linking specifically induced the tyrosine phosphorylation of PLCγ2 in both cell types, while no tyrosine phosphorylation above basal levels was induced in samples treated with control mAb. CD16- and FcεRI-induced PLCγ phosphorylation in human NK or R8#6 transfectant, respectively, is shown in the same figure and was previously described (11, 46). Fig. 5*C* shows that CD69 stimulation rapidly induces augmentation of the corresponding PI-PLC enzymatic activity in human NK cell lysates at levels comparable to those observed after CD16 stimulation.

We then addressed the relative roles of Syk and Src PTK in CD69-mediated PLC activation. The results shown in Fig. 6 indicate that pretreatment of human NK cells with the Syk- or Src-selective pharmacological inhibitor, either alone or in combination, substantially inhibited CD69-stimulated PLC enzymatic activity (Fig. 6*B*) and PLCγ2 tyrosine phosphorylation (Fig. 6*A*).

Together, these data demonstrate that both Syk and Src PTKs control the CD69-mediated activation of PLCγ in human NK cells.

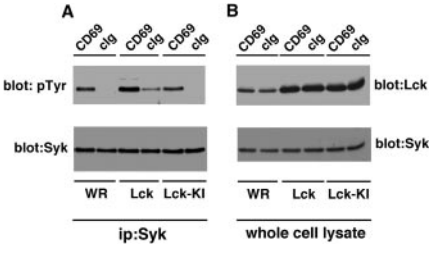

FIGURE 4. CD69-triggered Syk phosphorylation depends on Lck. IL-2-activated NK cells were infected with empty (WR), wild-type-expressing (Lck), or Lck-KI-expressing vaccinia viruses, and then stimulated (20×10^6/sample) with anti-CD69 or control mAb (cIg) plus GAM for 3 min at 37°C. *A*, Cell lysates were immunoprecipitated with anti-Syk mAb, separated on SDS-PAGE, transferred to nitrocellulose membranes, and subjected to Western blot with anti-pTyr mAb (*upper panel*) and, after stripping off bound Ab, with anti-Syk mAb (*lower panel*) as indicated. *B*, Whole cell lysate were normalized for protein content, and aliquots were blotted with anti-Lck (*upper panel*) and anti-Syk mAb (*lower panel*). Results are representative of one of two independent experiments.

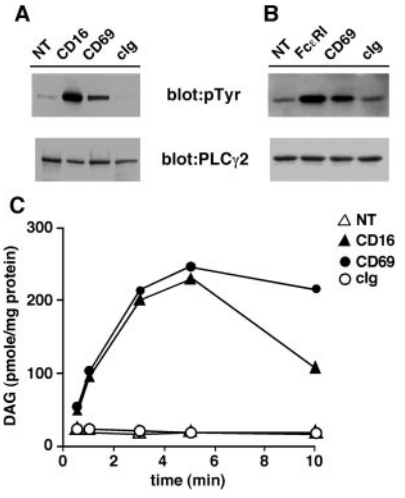

FIGURE 5. CD69 engagement induces PLCγ2 activation in human NK cells and $CD69^+$ transfectants. *A* and *B*, IL-2-activated NK cells (50×10^6 cells/sample; *A*) or $CD69^+$ R8#6 transfectants (20×10^6 cells/sample; *B*) were left untreated (NT) or were stimulated with saturating doses of different mAb plus GAM for 3 min at 37°C. Cell lysates were immunoprecipitated with polyclonal anti-PLCγ2 Ab, electrophoresed under reducing conditions, blotted with anti-pTyr mAb (*upper panel*), and then, after stripping off bound Ab, reprobed with polyclonal anti-PLCγ2 Ab (*lower panel*). Results are representative of one of three independent experiments. *C*, IL-2-activated NK cells (10×10^6/sample) were left untreated (NT) or were stimulated with saturating doses of different mAb plus GAM for different time periods at 37°C. PI-PLC activity was assessed on cell lysates by measuring DAG release from radioactive $PI4,5PIP_2$-containing lipid vesicles. Results are representative of one of three independent experiments.

Together, these data demonstrate that both Syk and Src PTKs control the CD69-mediated activation of PLCγ in human NK cells.

CD69 engagement induces the PTK-dependent activation of Vav

We have previously reported the ability of CD69 engagement of inducing ERK activation (5), a crucial intermediate in the development of NK cell cytotoxicity (12–14); the existence of a Vav/Rac/ERK pathway leading to natural killing has been recently described in NK cells (15). As the GDP/GTP exchange factor for Rac, Vav, is regulated in a PTK-dependent manner (47), we analyzed whether CD69 engagement could induce the tyrosine phosphorylation of Vav. The results reported in Fig. 7 show that CD69 engagement led to the rapid tyrosine phosphorylation of Vav1 in both human NK cells (Fig. 7*A*) and R8#6 transfectant clone (Fig. 7*B*). The enhancement of phosphorylation was not observed after treatment with an irrelevant mAb (control Ig), used as a negative control. We then analyzed the roles of Syk and Src kinases in CD69-induced Vav phosphorylation, and we found that pretreatment of NK cells with Syk- and Src-specific pharmacological inhibitors, alone or in combination, almost completely abrogated the CD69-induced tyrosine phosphorylation of Vav1 (Fig. 8).

In summary, these results demonstrate that both Syk and Src family PTKs are responsible for CD69-triggered tyrosine phosphorylation of Vav1, the GTP/GDP exchange factor for Rac, in human NK cells.

Role of Syk and Src family PTK in CD69-triggered NK cytotoxicity

We then sought to determine whether Syk and Src family PTK-dependent pathways were involved in CD69-triggered cytotoxicity

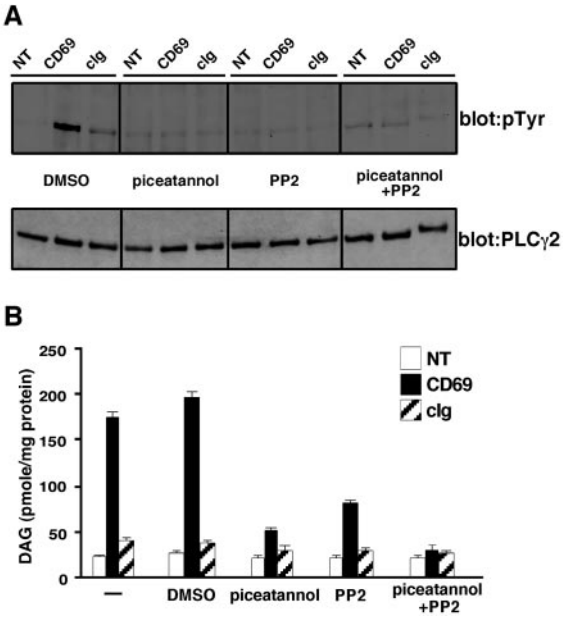

FIGURE 6. CD69-triggered activation of PLCγ depends on Syk and Src family kinases. IL-2-activated NK cells were pretreated with vehicle (DMSO), piceatannol (35 μg/ml), PP2 (10 μM), or both inhibitors and stimulated with saturating doses of anti-CD69 or control (cIg) mAb plus GAM for 3 min at 37°C. *A*, Cell lysates (50×10^6/sample) were immunoprecipitated with polyclonal anti-PLCγ2 Ab, electrophoresed under reducing conditions, blotted with anti-pTyr mAb (*upper panel*), and then, after stripping off bound Ab, reprobed with polyclonal anti-PLCγ2 Ab (*lower panel*). *B*, PI-PLC activity was assessed on cell lysates (10×10^6/sample) by measuring DAG release from radioactive PI4,5PIP_2-containing lipid vesicles. Results are representative of one of three independent experiments.

of human NK cells. To this purpose, IL-2-activated polyclonal NK cell cultures were assayed in a redirected cytotoxicity assay against P815 FcγR^+ target cells in the presence of Syk- and Src-specific inhibitors. The experiments reported in Fig. 9 show that treatment with PP2 (Fig. 9*A*) abrogated CD69-dependent cytotoxicity, and piceatannol (Fig. 9*B*) almost completely impaired it. The cytolytic activity against P815 cells in the absence of Ab or in the presence of control mAb was imputable to the IL-2-dependent activation of the NK effectors, as previously reported (5). These data thus show the essential involvement of both Syk and Src PTK in the CD69-triggered cytolytic function.

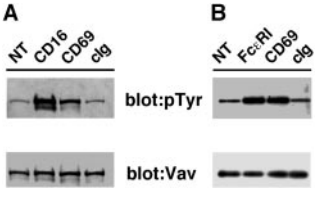

FIGURE 7. CD69 engagement induces the tyrosine phosphorylation of Vav1 in human NK cells and CD69$^+$ transfectants. IL-2-activated NK cells (40×10^6 cells/sample; *A*) or CD69$^+$ R8#6 transfectants (15×10^6 cells/sample; *B*) were left untreated (NT) or were stimulated with saturating doses of different mAb plus GAM for 2 min at 37°C. Cell lysates were immunoprecipitated with anti-Vav mAb, electrophoresed under reducing conditions, blotted with anti-pTyr mAb (*upper panel*), and, after stripping off bound Ab, reprobed with anti-Vav mAb (*lower panel*). Results are representative of one of four independent experiments.

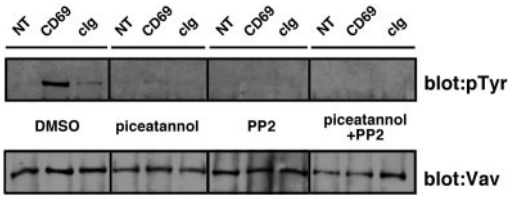

FIGURE 8. CD69-triggered activation of Vav1 depends on Syk and Src family kinases. IL-2-activated NK cells were pretreated with vehicle (DMSO), piceatannol (35 μg/ml), PP2 (10 μM), or both inhibitors and stimulated with saturating doses of different mAb plus GAM for 3 min at 37°C. Cell lysates (40×10^6/sample) were immunoprecipitated with anti-Vav1 mAb, electrophoresed under reducing conditions, blotted with anti-pTyr mAb (*upper panel*), and then, after stripping off bound Ab, reprobed with anti-Vav1 mAb (*lower panel*). Results are representative of one of four independent experiments.

Discussion

In the present work we describe for the first time the ability of the C-type lectin receptor CD69 to initiate PTK-dependent signaling pathways in human IL-2-activated NK cells. Our results clearly show that CD69 engagement induces the selective activation of Syk, but not ZAP70, kinase. We provide evidence that this event is under the control of Src family members and suggest that Lck can be responsible for it. We also show that Syk and Src family PTK regulate the development of the CD69-induced cytolytic function of human NK cells and identify PLCγ2 and Vav1 as downstream effectors of CD69-activated PTK-dependent pathways. Comparable results have been obtained in human NK cells by cross-linking the receptor with two different mAbs, as the identity of CD69 ligand is still unknown. The same signaling pathways are observed in a heterologous transfectant cell clone, constitutively expressing human CD69 receptor.

NK/target interaction is regulated by multiple activating receptors that contact mostly unidentified counterstructures on target cell membrane, and whose expression pattern depends on the NK cell activation state. CD69 is a hemopoietic C-type lectin receptor, whose gene is located in the NK gene complex, clustered with the genes for other membrane receptors that regulate NK cell functions, such as CD94/NKG2 heterodimers, NKG2-D, and NKR-P1 (18–21). CD69 is rapidly induced on NK and other hemopoietic cells in response to cytokines or other activating stimuli (24, 25) and has been shown to induce cytotoxic activity and costimulate cytokine production of activated NK cells and T cell clones (4, 5, 26, 27). It thus represents one of the receptors that endow activated NK cells with new recognition capability in the context of natural killing activity (3–7).

The signaling pathways regulating CD69-mediated functions are mostly unknown; we have recently described the ability of CD69 of inducing ERK activation (5), and the rise of intracellular calcium levels and the generation of arachidonic acid have been reported in different cell types (24, 26, 48). Here we present evidence on the selective and rapid activation of Syk, but not ZAP70, PTK in IL-2-activated human NK cells by CD69 engagement.

Syk/ZAP70 family PTKs are functionally coupled to several immunoreceptors and critically contribute to the signaling and functional ability of Ag and Fc receptors on T, B, and NK lymphocytes, myeloid cells, and platelets (33–35). Syk and ZAP70 are highly homologous; nevertheless, they do not seem to fulfill redundant roles. Recent evidence have shown that Syk, but not ZAP70, activity is crucially involved in the signaling pathways activated by the triggering receptors responsible for natural killing of human NK cells (9, 10), although the mechanisms upstream of Syk activation have not yet been clarified.

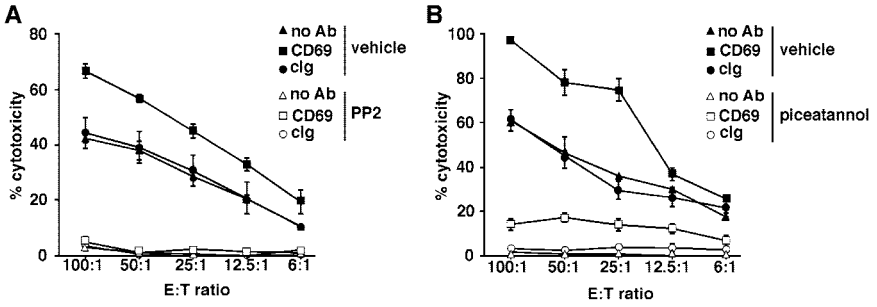

FIGURE 9. Syk- and Src-specific inhibitors abrogate CD69-dependent cytotoxicity. IL-2-activated NK cells were pretreated with vehicle (DMSO; both panels, closed symbols), PP2 (5 μM), or piceatannol (25 μg/ml; both panels, open symbols) for 30 min at 37°C, then tested in a redirected cytotoxicity assay against P815 target cells in the presence of anti-CD69 (squares) or control (circles) mAb or no mAb (triangles). Inhibitors were present throughout the assay (3 h). Results are representative of one of four independent experiments.

Here we show that CD69-triggered activation of Syk essentially relies upon the catalytic activity of Src family PTKs and propose Lck as the main enzyme responsible for the regulation of Syk. However, it cannot be ruled out that a low grade autophosphorylation of Syk may also play a role. Syk activation has been shown to involve several mechanisms, including a conformational change due to ITAM binding, autophosphorylation, and phosphorylation by other kinases (33–35). The relative importance of such mechanisms may be dictated by the modalities regulating Syk recruitment to different receptors. The lack of evidence for CD69-associated ITAM-containing subunits could offer an explanation for the complete dependence of Syk activation on Src family PTKs.

How CD69 receptor is coupled to Src family PTKs is presently unknown. CD69 is endowed with a brief intracellular tail that does not display consensus motifs for protein-protein interactions (19–21). Interestingly, we could evidentiate a kinase activity physically associated to CD69 receptor, which is specifically induced by receptor engagement on RBL transfectants (unpublished observations); the molecular identity of such activity(ies) and the modalities regulating its association with the receptor are unknown and are presently under investigation. The possibility that CD69 could localize in a raft-like compartment, where some members of the Src family have been shown to partition (49), is presently under investigation. Indeed, polarization of glycosphyngolipid-rich areas on NK cell membrane has been recently reported to occur after conjugation with sensitive targets, and the polarization of Lck and Syk in the area facing target cell has been observed (50–52).

PTK activation by NK triggering receptors initiates many signaling pathways by means of several enzymes and adaptor proteins that are involved in the development of cytotoxic activity (8, 11). Here we describe for the first time the ability of CD69 receptor to induce the activation of PLCγ2 and of the exchange factor for Rho family GTPases, Vav1; moreover, our results show that CD69-induced activation of PLC and Vav lies downstream of Syk and Src family PTK.

PI-PLC enzymatic activation is an important requisite for the development of NK cytotoxicity, as shown by the impairment of NK killing in PLCγ2 knockout mice (53); indeed, several receptors able to activate NK cell cytotoxic activity have been shown to induce PLCγ activation (8, 10, 11). PI-PLC activity products lead to the augmentation of the intracellular Ca^{2+} concentration, and can also contribute to activation of the ERK pathway (45, 54). Although CD69 engagement has been reported to induce the augmentation of cytoplasmic Ca^{2+} levels in several cell types (24, 26, 48), the contribution of PLC-activated pathways had not been directly addressed to date.

Tyrosine-phosphorylated Vav1 promotes the exchange of Rac GTPase to its active GTP-bound state (47). Vav1 has been previously shown to undergo tyrosine phosphorylation in NK cells upon contact with sensitive target cells or CD16 stimulation (16, 17), and the importance of the Vav1-Rac-ERK pathway in the development of NK cytotoxic activity has been previously shown (15–17). As Vav1-dependent pathways have been also reported to affect calcium metabolism (47), it is possible that Vav1 activation could participate in CD69-triggered PLCγ2 and ERK activation.

Finally, the functional relevance of Syk and Src family PTK-activated pathways is shown by the effects of the selective pharmacological inhibitors on CD69-triggered cytolytic function, thus providing the first evidence on the role of Src PTKs in the development of natural cytotoxicity.

The signaling pathways we have described may also play a role in the development of natural killing initiated by other activating receptors on NK cells. Moreover, as CD69 expression is rapidly induced on several leukocyte subpopulations, where it triggers biologically relevant functions, the signal transduction pathways described herein could be relevant in the understanding of the mechanisms regulating CD69-triggered functions in different hemopoietic cell types.

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