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Recruitment of Pleckstrin and Phosphoinositide 3-Kinase γ into the Cell Membranes, and Their Association with Gβγ After Activation of NK Cells with Chemokines

Ala Al-Aoukaty, Bent Rolstad, and Azzam A. Maghazachi

The role of phosphoinositide 3 kinases (PI 3-K) in chemokine-induced NK cell chemotaxis was investigated. Pretreatment of NK cells with wortmannin inhibits the in vitro chemotaxis of NK cells induced by lymphotactin, monocyte-chemoattractant protein-1, RANTES, IFN-inducible protein-10, or stromal-derived factor-1α. Introduction of inhibitory Abs to PI 3-Kγ but not to PI 3-Kα into streptolysin O-permeabilized NK cells also inhibits chemokine-induced NK cell chemotaxis. Biochemical analysis showed that within 2–3 min of activating NK cells, pleckstrin is recruited into NK cell membranes, whereas PI 3-Kγ associates with these membranes 5 min after stimulation with RANTES. Recruited PI 3-Kγ generates phosophatidylinositol 3,4,5 trisphosphate, an activity that is inhibited upon pretreatment of NK cells with wortmannin. Further analysis showed that a ternary complex containing the βγ dimer of G protein, pleckstrin, and PI 3-Kγ is formed in NK cell membranes after activation with RANTES. The recruitment of pleckstrin and PI 3-Kγ into NK cell membranes is only partially inhibited by pertussis toxin, suggesting that the majority of these molecules form a complex with pertussis toxin-insensitive G proteins. Our results may have application for the migration of NK cells toward the sites of inflammation. The Journal of Immunology, 1999, 162: 3249–3255.
protein-protein interactions. For example, the PH domains of the β adrenergic receptor kinase (G-protein-coupled receptor kinase 2) bind the βγ dimer of PT-sensitive G proteins (15). Although Gβγ activates PI 3-K, resulting in the activation of the mitogen-activated protein kinase pathway (16), or Jun kinase (17), the binding of PI 3-K to Gβγ has been disputed (18). Pleckstrin, the major substrate for protein kinase C in platelets, is composed of two PH domains (N-terminal and C-terminal) separated by 150 amino acids. Activation of platelets with thrombin results in the phosphorylation of pleckstrin by protein kinase C. Phosphorylated pleckstrin in turn inhibits PI 3-K activity as a result of activating GPCR (19). This inhibition was reversed upon the addition of purified Gβγ, suggesting that phosphorylated pleckstrin may interact with the βγ dimer. However, a correlation of coupling and/or function between Gβγ, pleckstrin, and PI 3-K has not been reported.

In this study, we explored the possibility that there is an interaction among these components as a result of activating NK cells with chemokines.

Materials and Methods

Culture medium

Culture medium consisted of RPMI 1640 supplemented with 10% human AB serum (Ulleval Hospital, Oslo, Norway), 10 U/ml penicillin, 100 μg/ml streptomycin, 1 mM L-glutamine, 1% nonessential amino acids (all from Life Technologies, Paisley, U.K.), and 5 × 10⁻⁶ M 2-ME (Sigma, St. Louis, MO). AIM-V medium was from Life Technologies.

Preparation of NK cell membranes

IL-2-activated NK cells were prepared by adherence to plastic flasks, as previously described (20, 21). The majority of these cells (more than 85%) show the CD16⁺ CD56⁻ CD3⁻ phenotype, as determined by flow-cytometric analysis. Before performing the biochemical assays described in this work, these cells were incubated overnight in a serum-free (AIM-V) medium. This was necessary since serum has been reported to induce the recruitment of PH-containing proteins into the plasma membranes of various cell types (14). NK cell membranes were prepared by incubating the cells in a lysis buffer containing 10 mM HEPES, pH 7.5, 3 mM MgCl₂, 40 μg/ml PMSF, 10 μg/ml leupeptin, 2 μg/ml pepstatin A, and 2 μg/ml aprotinin. After homogenization and sonication, the mixtures were centrifuged at 1000 × g for 10 min. The supernatants were transferred into Beckman tubes; ultracentrifuged in a buffer containing 10 mM HEPES, 3 mM MgCl₂, 10 mM EDTA, and 200 μg/ml PMSF, 1% Triton-X, and 0.5% Nonidet P-40) overnight at 4°C, centrifuged, and washed three times. The pellets were suspended in electrophoresis sample buffers before running on SDS-PAGE. Immunoblotting was performed by running the samples on SDS-PAGE and then electrotransferred into polyvinylidene difluoride (PVDF) membranes, blocked with 5% skim milk in TBS buffer for 2 h, washed, and incubated with 1/1000 of the primary Abs, and 1/500 dilution of the secondary Abs. Development was done by using either horseradish peroxidase-color development reagents (Bio-Rad) or enhanced chemiluminescence (ECL) reagents (Amersham, Arlington Heights, IL) according to the manufacturer’s specifications.

Pretreatment with PT or wortmannin

Cells were incubated overnight in AIM-V medium. They were either left intact or were treated overnight with 100 ng/ml activated PT (Sigma). These cells were harvested and membranes were prepared as described above. Pretreatment with wortmannin (Sigma) was done by incubating NK cells (1 × 10⁶) with various concentrations of this metabolite at 37°C for 30 min, or with the appropriate concentration of DMSO, washed, and examined as above.

Phosphatidylinositol kinase assay

NK cells were either left intact or pretreated with 100 nM of wortmannin. These cells were incubated with 100 pg/ml RANTES for 5 min, and membranes were prepared from these cells. The membranes were immunoprecipitated with anti-PI 3-Kγ overnight at 4°C in the presence of protein A/G agarose. The immunocomplex was washed once with ice-cold PBS and once with a buffer containing 0.5 M LiCl, 100 mM Tris-HCl, pH 7.5, and 1 mM sodium orthovanadate. It was then washed once with distilled water, and once with a washing buffer containing 20 mM Tris-HCl, pH 7.5, 0.5 mM EDTA, and 100 mM NaCl. The immunocomplex was suspended in a 50 μl of washing buffer, and was added to 0.5 μl of P(32)P, 20 mg/ml (Sigma). This mixture was incubated for 10 min at 25°C with 10 μCi/sample of [γ-32P]ATP (Amersham). MgCl₂ (20 mM) was then added to this mixture, which was incubated for additional 15 min. The reaction was stopped by the addition of 150 μl of chloroform/methanol/1.16 N HCl, with the volume ratio of 100:200:2. The organic phase was separated by the addition of 100 μl chloroform. The lipid in the organic phase was separated on TLC aluminum silica gel 60 precoated sheets (Merck, Darmstadt, Germany). TLC plates were developed and resolved in chloroform/methanol/ammonium hydroxide/distilled water, with the volume ratio of 124:114:15:21. Radioactive PIP products were visualized by autoradiography.

Statistical analysis

Significant values were determined by using the two-tailed Student’s t test.

Results

PI 3-Kγ controls chemokine-induced NK cell chemotaxis

PI 3-K has been shown to play an important role in NK cell activity upon perturbation of FcR (23, 24). In addition, wortmannin, a fungal inhibitor of PI 3-K activity, inhibits RANTES-induced T cell chemotaxis (25). To investigate the role of PI 3-K in chemokine-induced NK cell chemotaxis, we pretreated NK cells with different concentrations of wortmannin. Fig. 1 shows that the C chemokine lymphotactin (Ltn), the CC chemokines MCP-1 and RANTES, or the CX3 chemokines IP-10 and SDF-1α induced the in vitro chemotaxis of NK cells (p < 0.005, as compared with cells migrating toward culture medium only). Low concentration of wortmannin (10 pM) did not inhibit chemokine-induced NK cell chemotaxis. However, 1 nM of wortmannin inhibited the chemotactic activity induced by Ltn, MCP-1, or IP-10 (p < 0.02, as compared with the migration of cells not treated with wortmannin), whereas higher concentrations (10, 100, and 1000 nM) inhibited RANTES and SDF-1α (p < 0.05), as well as Ltn-, MCP-1-, and
IP-10-induced NK cell chemotaxis. None of the concentrations of wortmannin used affected the viability of NK cells, as determined by trypan blue exclusion test (not shown). To establish which PI 3-K isotype is involved in the chemokine activity, we introduced Abs to PI 3-K into SLO-permeabilized cells. Results in the upper panel of Fig. 2 show that affinity-purified goat anti-PI 3-K inhibited Ltn, MCP-1, RANTES, IP-10, or SDF-1α induction of NK cell chemotaxis (p < 0.001, p < 0.003, p < 0.004, p < 0.02, and p < 0.001, respectively, as compared with the migration of cells treated with goat IgG). Neither goat IgG, nor Ab to PI 3-K in NK cell chemotaxis (not shown). About 10–15% of the cells migrated within the 2-h incubation period. Mean ± SD of three to six experiments.

These results suggest that PI 3-K plays an important role in the chemotaxis of NK cells. To biochemically address the nature of PI 3-K effect, we investigated its recruitment to the membranes after stimulating NK cells with RANTES. Fig. 3A shows that PI 3-K is not present in NK cell membranes. However, it is recruited into these membranes upon stimulation of these cells with RANTES, as determined by immunoblotting NK cell membranes with a specific Ab to the catalytic p110γ subunit (lane 2). No such recruitment was observed in the absence of stimulation with RANTES (lane 1 in Fig. 3A). To examine the association of PI 3-K with Gβγ, NK cells were activated with culture medium or with RANTES for 5 min. Membranes were prepared from these cells, immunoprecipitated overnight with anti-PI 3-K, and then immunoblotted with Ab to the common β subunit of G protein. The results in lane 1 of Fig. 3B show that in the absence of stimulation with RANTES, anti-Gβγ picked up a 37-kDa band representing Gβγ in NK cell membranes. Importantly, an association between PI 3-K and βγ subunit of G proteins occurred after stimulation with RANTES (lane 2 in Fig. 3B). Taken together, the results clearly show that chemokine activation of GPCR results in the recruitment of PI 3-K into NK cell membranes, and its association with the Gβγ dimer.

Chemoynes induce the recruitment of pleckstrin into NK cell membranes

After incubation in serum-free medium for 18 h, NK cells were activated for 5 min with culture medium, Ltn, MCP-1, RANTES, IP-10, or SDF-1α. Membranes were prepared from these cells, and were examined for the presence of pleckstrin. Fig. 4A shows that all five chemokines induced the recruitment of pleckstrin into NK cell membranes (lanes 2–6). To investigate whether chemokines recruit pleckstrin from the cytosol, NK cells were treated with either culture medium or RANTES. The results show that pleckstrin is abundant in NK cell lysates, but not in NK cell membranes (lane 1 in the left and right panels of Fig. 4B). However, it is recruited into the membranes after stimulating the cells for 5 min with RANTES (lane 2 in the left and right panels of Fig. 4B), suggesting that stimulation with chemokines facilitates the distribution of this protein from the cytosol into the membranes. A physical association between pleckstrin and Gβγ dimer was then examined. NK cells were stimulated with culture medium or with RANTES, and membranes were prepared from these cells, immunoprecipitated with anti-pleckstrin-PH, and then immunoblotted with anti-G β Ab (left panel in Fig. 4C). Reciprocally, the membranes were immunoprecipitated with anti-G β, and then immunoblotted with anti-pleckstrin-PH (right panel in Fig. 4C). Only after stimulation with RANTES (lane 2 in both panels of Fig. 4C) and not with culture medium (lane 1 in both panels), an association occurred between pleckstrin and Gβγ.
beta dimer of PT-resistant G proteins forms a complex with pleckstrin and PI 3-Ky in NK cell membranes

To demonstrate the nature of G proteins involved in the recruitment of pleckstrin and PI 3-Ky, NK cells were either left untreated or pretreated with 100 ng/ml of PT. Results in Fig. 5, A and B, show that RANTES induced the recruitment of pleckstrin and PI 3-Ky, respectively, as compared with culture medium-treated cells (lane 3 versus lane 1). However, there was only a partial inhibition of the recruitment of pleckstrin (lane 2 in Fig. 5A) or PI 3-Ky (lane 2 in Fig. 5B) upon pretreatment of NK cells with 100 ng/ml of PT.

Higher concentrations of PT affected the viability of the cells (data not shown).

Recruitment of pleckstrin or PI 3-Ky into NK cell membranes is resistant to wortmannin treatment

To investigate whether PI 3-Ky plays a role in the recruitment of pleckstrin into NK cell membranes, NK cells were pretreated with culture medium or wortmannin, washed, and then stimulated with RANTES. In lane 1 of Fig. 5C, a faint band representing pleckstrin was present in NK cell membranes in the absence of stimulation. The existence of such a band was variable in cells generated from different donors. More important, treatment with RANTES resulted in the recruitment of pleckstrin into NK cell membrane (lane 3 in Fig. 5C). Concentration of wortmannin (100 nM), which inhibited RANTES-induced NK cell chemotaxis (Fig. 1), did not inhibit the recruitment of pleckstrin into NK cell membranes (lane 2 in Fig. 5C).

Similarly, pretreatment of NK cells with wortmannin failed to inhibit the association of PI 3-Ky with NK cell membranes upon stimulation with RANTES. Fig. 6A shows that incubation of NK cells for 5 min with RANTES, and not with culture medium resulted in the association of PI 3-Ky with NK cell membranes (left versus right lanes; similar to the results obtained in Fig. 2A). Pretreatment of NK cells with 100 nM wortmannin before incubation with RANTES did not inhibit the association of PI 3-Ky with NK cell membranes (Fig. 6A, middle versus right lane). To correlate the inhibitory effect of wortmannin on chemokine-induced NK cell chemotaxis with the recruitment of PI 3-Ky into NK cell membranes, we examined the ability of membrane-associated PI 3-Ky to generate phospholipids. After stimulation with RANTES, NK cell membranes were immunoprecipitated with Ab to PI 3-Ky overnight. This immune complex was mixed with PI(4,5)P2 in the presence of [32P]ATP. PI 3-Ky activity and the generation of PI(3,4,5)P3 were only seen after stimulation with RANTES and not with culture medium (Fig. 6B, right and left lanes, respectively). The ability of PI 3-Ky immunoprecipitated from NK cell membranes after stimulation with RANTES to generate PI(3,4,5)P3 was inhibited upon prior pretreatment of NK cells with 100 nM of wortmannin (Fig. 6B, middle versus right lane).

RANTES induces the formation of a ternary complex in NK cell membranes

To examine the association of these molecules in NK cell membranes, NK cells were stimulated with RANTES for 1–20 min, and membranes were prepared from these cells, immunoprecipitated with anti-PI 3-Ky, and then immunoblotted with Abs to either PI 3Ky (Fig. 7A) or pleckstrin-PH (Fig. 7B). An association between these two molecules occurred after 3 min (B). The same association was observed when NK cell membranes were immunoprecipitated with anti-pleckstrin first, and then immunoblotted with anti-PI 3-Ky (C). However, the majority of the band detected after 3 min may be due to pleckstrin since there was only a low recruitment of PI 3-Ky at this time (A). A strong association between
pleckstrin and PI 3-Ky occurred 5 min after stimulation with RANTES (B). This coincided with the robust recruitment of PI 3-Ky into NK cell membranes at this time point (A). The association between these two molecules was also apparent 10 min after stimulation. Both pleckstrin and PI 3-Ky almost disappeared from this complex 20 min after stimulation with RANTES (B and C), indicating a transient recruitment of these molecules after stimulation with RANTES. Time kinetic association between pleckstrin and Gβγ was also examined. After RANTES stimulation of NK cells for various times, membranes from these cells were immunoprecipitated with anti-pleckstrin-PH and then immunoblotted with anti-Gβγ. Fig. 7D shows that an association between these molecules occurred after 2 min and was strong after 3 and 5 min of stimulation. However, after 10 min, most of the Gβγ dimer dissociated from pleckstrin.

Discussion

Chemotactic cytokines (chemokines) are proinflammatory mediators implicit for the recruitment of various cell types into the inflammatory sites (reviewed in Refs. 2 and 5). Depending on the presence and the arrangement of the first cysteine residues in the N-terminal region, chemokines are divided into four subfamilies: CXC (α), CC (β), C (γ), and CX₃C (δ). The CC chemokine MIP-1α, MCP-1, RANTES, macrophage-derived chemokine, MIP-3α, or MIP-3β (20, 26–30); the C chemokine lymphotactin (7, 31, 32); the CXC chemokine IP-10, or SDF-1α (7, 27, 33); and the CX₃C chemokine fractalkine (30, 34) have been shown to induce the chemotaxis of NK cells. Furthermore, MIP-1α recruits NK cells toward the livers of CMV-infected mice, which resulted in increased inflammation and decreased susceptibility to infection with this virus (3).

Receptors for CC chemokines are coupled to the heterotrimeric G proteins, which are composed of three subunits, α, β, and γ. The presence and identity of the G proteins in human and rat NK cell membranes have been investigated. It was reported that these membranes expressed Gα, Gα, Gγ, Gγ, Gγ, and G13. These G proteins play vital roles as early transducers of various biological functions in NK cells, such as chemotaxis (reviewed in Ref. 6). However, the downstream signaling molecules important for the chemotaxis of NK cells are not known. It has been observed that wortmannin, a fungal inhibitor of PI-3K, inhibits RANTES-induced T cell chemotaxis (25). Because wortmannin has effects other than inhibition of PI-3-K, it is not clear whether this enzyme is involved in mediating chemokine-induced cellular chemotaxis. In this study, we showed that wortmannin as well as inhibitory Abs to PI 3-Ky but not PI 3-Kα inhibit C, CC, and CXC chemokine-induced NK cell chemotaxis, suggesting that PI 3-KI plays an important role in chemokine activation of NK cells.

In addition, we showed that PI 3-Ky is recruited into NK cell membranes within 5 min after stimulation of these cells with the CC chemokine RANTES. At almost the same time, an association between PI 3-Ky and the βγ dimer of G proteins occurred. In addition, we observed that pleckstrin is recruited from the cytosol into NK cell membranes within 2–3 min after stimulating NK cells with C, CC, or CXC chemokines. The recruitment of pleckstrin was insensitive to wortmannin pretreatment, suggesting that PI 3-Ky products such as P(3,4,5)P3 or P(3,4,5)P3 are not important for the recruitment of pleckstrin into NK cell membranes shortly after activating GPCR. These results contrast the recruitment of PLCγ-PH into the cell membranes upon stimulation with growth factors, which occurs as a result of binding the PLCγ-PH to phospholipids, which facilitates its association with the cell membrane. This activity was sensitive to wortmannin pretreatment (13). Therefore, there may be multiple mechanisms by which PH domain-containing proteins are recruited into the membranes, depending on the pathway utilized by the ligands.
In addition, we observed that wortmannin did not inhibit the recruitment of PI 3-Ky into NK cell membranes. To correlate the finding that wortmannin inhibits NK cell chemotaxis with the recruitment of this kinase into the membranes of these cells, we observed that wortmannin inhibited the activity of this kinase. Hence, the generation of PI(3,4,5)P3 (Fig. 6) or PI(3,4)P2 (data not shown) was abrogated upon pretreatment of NK cells with this metabolite. These results suggest that during activation with RANTES, PI 3-Ky is recruited into NK cell membranes, placing it in close proximity to its phospholipid substrates, resulting in the phosphorylation of various phosphatidylinositol lipids.

Interestingly, pretreatment of NK cells with PT only partially inhibited the recruitment of either pleckstrin or PI 3-Ky into their membranes, suggesting that the majority of pleckstrin or PI 3-Ky is associated, presumably reassociating with the α subunit in the membranes, whereas the PI 3-Ky mediates NK cell chemotaxis by phosphorylating phospholipids such as phosphatidylinositol 4-phosphate (PI(4)P) or phosphatidylinositol 4,5 bisphosphate (PI(4,5)P2), which are important for this process. These results may shed some light on the biochemical events utilized by chemokines to induce the polarization and the extravasation of NK cells into the sites of virus infection, or tumor growth (3, 4).

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FIGURE 7. Formation of a ternary complex in NK cell membranes after stimulation with RANTES. NK cells were incubated with 100 pg/ml RANTES for 1–20 min. Membranes of these cells were immunoprecipitated with anti-PI 3-Kγ, and then immunoblotted with either anti-PI 3-Kγ (A) or anti-pleckstrin-PH (B). Reciprocally, NK cell membranes were im-
munoprecipitated with anti-pleckstrin-PH, and then immunoblotted with anti-PI 3-Kγ (C). In D, NK cells were treated as above, except that the membranes were immunoprecipitated with anti-pleckstrin-PH, and then immunoblotted with anti-βγ."
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