Functional Coupling of NKR-P1 Receptors to Various Heterotrimeric G Proteins in Rat Interleukin-2-activated Natural Killer Cells*

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NKR-P1 molecules constitute a family of type II membrane receptors in natural killer (NK) cells that preferentially activate NK cell killing and release of interferon-γ from these cells. Here, we demonstrate that anti-NKR-P1 enhances GTP binding in rat interleukin-2-activated NK cell membranes; GTP binding to Gi3, Gs, Gq, and G12 increased noticeably in these cell membranes after treatment with anti-NKR-P1. Western blot analysis of membrane proteins prepared from interleukin-2-activated NK cells reveals the presence of Gi1,2, Gi3, Gs, Gq, and G12, but not G13. However, only α3, αs, α11, and αq, but not α11, αo, α12, or α13 subunits when immunoprecipitated with the appropriate anti-G protein antibodies, are associated with NKR-P1 when immunoblotted with anti-NKR-P1. Reciprocally, NKR-P1 immunoprecipitated with anti-NKR-P1 is associated with α3, αs, α11, and αq immunoblotted with anti-G proteins. These results are the first to demonstrate the physical and functional coupling of NKR-P1 to the heterotrimeric G proteins in NK cells.

Natural killer (NK) cells were first discovered by their ability to kill certain tumor cell lines without prior sensitization, but they can also recognize and destroy virally infected cells (1–3). These cells recognize the major histocompatibility complex class I molecules on target cells, resulting in either inhibition or activation of their cytolytic potential (4–7). Target cell recognition by rodent NK cells involves C-type lectin proteins, such as NKR-P1 and Ly 49, that are expressed preferentially on NK cells (8). Three homologous NKR-P1 genes have been identified both in mice and rats and are designated as NKR-P1 (A, B, and C) (9–13). In human NK cells, NKR-P1A has about 46% homology to the rodent NKR-P1 molecules (14). Anti-NKR-P1 monoclonal antibody (3.2.3) reacts with rat NKR-P1 members and induces the production of IP3, the mobilization of intracellular calcium, the secretion of interferon-γ, and the degranulation and cytotoxicity of NK cells (15–17).

Recently, we reported that the heterotrimeric guanine nucleotide-binding (G) proteins play important roles in mediating rat NK cell lysis of allogeneic and tumor target cells (18). The heterotrimeric G proteins are composed of three subunits (α, β, and γ). In its inactive form, the α-subunit binds the guanine nucleotide GDP and exchanges it with GTP upon activation. Both the α-GTP and the βγ-heterodimer transduce regulatory signals from a large number of cell-surface receptors to various intracellular enzymes such as adenyl cyclases, phosphodiestersases, and phospholipases (19, 20). The ability of NKR-P1 to induce various biological activities in NK cells suggests that multiple intracellular signaling pathways may be activated upon ligating NKR-P1. The presence of a number of different G proteins in rat NK cell membranes suggests that some of these may also be involved in the transmission of various signals in NK cells. Since it is not known to what extent signal transmission through NKR-P1 triggering is dependent on G proteins, we have investigated the physical and functional coupling of different heterotrimeric G proteins to NKR-P1 in NK cells.

EXPERIMENTAL PROCEDURES

Animals—Breeding pairs from the rat strains of PVG were bred in our laboratory or were purchased from Harlan Olac Ltd. (Bicester, United Kingdom (UK)).

Reagents—Leupeptin, aprotinin, pepstatin A, phenylmethylsulfonyl fluoride, dithiothreitol, Tris-HCl, HEPES, CHAPS, glyceral, KCl, sodium phosphate, EDTA, EGTA, MgCl2, bovine serum albumin, and GTP were purchased from Sigma. RPMI 1640 medium, PBS, antibiotics, fetal calf serum, 1,2-galactose, nonessential amino acids, and 2-mercaptoethanol solution were from Life Technologies, Inc. (Paisley, Scotland).

Antibodies—Anti-NKR-P1 monoclonal antibody (3.2.3) was a generous gift from Dr. John C. Hiserodt (University of California, Irvine, CA). Anti-Gi3 monoclonal antibody (G4.18) was a gift from Dr. Bruce M. Hall (Liverpool, Australia). Monoclonal mouse OX8 (reacting with rat CD8) was a gift from the Cellular Immunity Unit, Department of Pathology, Oxford University (Oxford, UK). Rabbit polyclonal anti-G protein antibodies AS7 (anti-Gi3,Gq,Go), EC2 (anti-Gi3), GC2 (anti-Gs), RM1 (anti-Gi3), QA (anti-Gi3), GD/1 (anti-Gi3), and GA1 (anti-Gi3) were purchased from NEN Life Science Products (Brussels, Belgium). Anti-Gs was purchased from Gramsch Laboratories (Schwabhausen, Germany). Anti-G12, anti-G13, and goat anti-mouse HRP were from Santa Cruz Biotechnology (Santa Cruz, CA). Goat anti-rabbit HRP-conjugated antibody was from Bio-Rad. Normal rabbit serum (NRS) and rabbit IgG were from Sigma.

Isolation and Culture of NKR-P1-IL-2-activated NK Cells—This was done according to the method described previously (21). Briefly, rat mononuclear splenocytes were obtained by density gradient centrifugation on Lymphoprep for 30 min at 400 × g, 1.077 g/ml (Nycomed Pharma, Oslo, Norway). The cells were washed and were depleted of CD3+ cells using anti-CD3 monoclonal antibody (G4.18) and rabbit complement. Following incubation for 75 min at 37 °C with gentle agitation, the cells were washed several times and incubated with M450 sheep anti-mouse IgG, magnetic Dynabeads (Dynal, Oslo, Norway), precoated with mouse anti-rat NKR-P1 mAb (3.2.3), to positively select...
NKR-P1+ cells, which mark most NK cells. Positively selected NK cells were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum, 2 mM l-glutamine, 1% nonessential amino acids, 10 units/ml penicillin, 100 mg/ml streptomycin, and 5 x 10^5 x 2-mercaptoethanol plus rat recombinant IL-2 equivalent to approximately 100 U/ml IL-2 for 10 days. The cells were washed more than 98% NKR-P1+, CD3−, CD5−, and TCRαβ− cells when examined by flow cytometry.

Membrane Preparation—This procedure was performed according to the described procedure (22). Briefly, IL-2-activated NK cells were harvested after 7–10 days in culture, washed extensively in ice-cold PBS, and centrifuged at 450 x g for 10 min at 4 °C. The cells were suspended in ice-cold lysis buffer containing 10 mM HEPES, pH 7.5, 3 mM EDTA in addition to the enzyme inhibitors (40 μg/ml phenylmethylsulfonyl fluoride, 2 μg/ml pepstatin A, 10 μg/ml leupeptin, and 2 μg/ml aprotinin). After two steps of homogenization and sonication, the mixture was centrifuged at 100,000 x g for 45 min at 4 °C. The supernatants were transferred to Beckman tubes and ultracentrifuged at 150,000 x g for 45 min at 4 °C. The pellets were suspended in a lysis buffer, snap-frozen, and stored at −80 °C.

GTP Binding Assay—GTP binding was measured using a method described by us (23, 24). About 50–100 μg of membrane proteins were incubated in 100 μl buffer containing 20 mM HEPES/NaOH, pH 7.4, 100 mM EDTA, 125 μM MgCl2, and 10 mM γ-[35S]GTP (1000 C/mmoll). The mixture was incubated at 37 °C after addition of the indicated antibodies and protein was terminated by addition of 900 μl of ice-cold 100 mM Tris-HCl, 1% CHAPS, 100 mM NaCl, and 20 μl unlabeled GTP. The mixtures were incubated on ice for 1 h, washed three times with ice-cold PBS plus 0.05% Tween 20, and centrifuged at 14,000 rpm at 4 °C using an Eppendorf centrifuge. The pellets were then suspended in a scintillation mixture and counted in a β counter. Nonspecific binding was determined by the addition of unlabeled GTP.

In addition, an ELISA assay was developed to determine the GTP binding to the α-subunit of G proteins. Nunc ImmunoMaxiSorp 96-well plates (removable wells) were coated with goat anti-rabbit IgG for 2 h at 4 °C. Rabbit antibodies to various G protein α-subtypes, and as a control NRS were added to the plates for additional 2 h. IL-2-activated NK cell membranes stimulated with 10 μg/ml anti-NKR-P1 for 1.5 min at 37 °C were preincubated in a binding buffer containing 10 mM γ-[35S]GTP, and were added to these plates. The plates were left on ice for 2 h and then washed three times with ice-cold PBS plus 0.05% Tween 20. Each well was removed and placed in a scintillation vial filled with liquid scintillation mixture and counted in a β counter. Nonspecific binding was determined by the addition of unlabeled GTP.

To confirm the nature of G protein α-subtypes activated after ligating NKR-P1 with anti-NKR-P1, a method was developed using immunomagnetic beads (Dynabeads) coated with sheep anti-rabbit IgG (Dynal, Oslo, Norway). The beads were incubated for 2 h at 4 °C with rabbit anti-G proteins, rabbit IgG, or NRS in a PBS buffer containing 1% bovine serum albumin. IL-2-activated NK cell membranes were incubated first with 10 μg/ml anti-NKR-P1 for 1.5 min, added to the GTP binding buffer plus γ-[35S]GTP, then mixed with anti-G protein- or NRS-coupled Dynabeads, washed with PBS buffer plus 0.05% Tween 20, suspended in the scintillation mixture and transferred to scintillation vials. All assays were performed in triplicate.

Immunoblot Analysis—Immunoblotting was performed as described (18). Briefly, 100 μg of membrane proteins were suspended in SDS sample buffer and separated by 12% SDS-polyacrylamide gel electrophoresis. The proteins were electrotransferred to PVDF membranes blocked with 5% skim milk in TBS buffer and incubated with primary antibody overnight at room temperature, washed twice with TBS plus 0.05% Tween 20 (TBBS), incubated with HRP-conjugated secondary antibody, washed twice with TBBS, and then developed using HRP development reagents (Bio-Rad).

Immunoprecipitation Assay—Membrane pellets were suspended in a solubilization buffer containing 25 mM sodium phosphate, pH 7.4, 5 mM EDTA, 5 mM EGTA, 200 mM KCl, 25% glycerol, and 25 mM MgCl2, plus 1% CHAPS. They were centrifuged at 100,000 x g, and the supernatants were collected and stored at −80 °C until the time of the assay. The membranes were added to the solubilization buffer plus 0.3% CHAPS and incubated overnight with rabbit antibodies to various α-subtypes of G proteins, rabbit IgG, or anti-NKR-P1 at 4 °C with gentle agitation. The complexes were added with protein A/G-agarose and incubated for additional 4 h. The immunocomplexes were isolated by centrifugation at 14,000 rpm at 4 °C using an Eppendorf centrifuge and washed three times with the solubilization buffer plus 0.3% CHAPS. The pellets were suspended in SDS sample buffer boiled for 5 min, and the agarose beads were removed by spinning the tubes at 2000 rpm for 2 min. The immunoprecipitates were separated by 12% SDS-polyacrylamide gel electrophoresis and immunoblotting using monoclonal anti-NKR-P1 primary antibody and goat anti-mouse IgG HRP-conjugated secondary antibody. Similarly, solubilized membranes were immunoprecipitated with anti-NKR-P1 or mouse IgG, and then immunoblotted with rabbit antiserum to various G protein α-subtypes or with anti-NKR-P1. Goat anti-rabbit-HRP was used as a secondary antibody.

Statistics—Significant values were determined using a two-tailed Student’s t test.
present in NK cell membranes. This binding was specific for anti-G protein antibodies, since no binding was observed with beads coated with normal rabbit serum (data not shown) or rabbit IgG (Fig. 4).

Reciprocally, when IL-2-activated NK cell membranes were

![Fig. 1. Ligation of NKR-P1 molecules results in increased GTP binding to NK cell membranes.](image1)

![Fig. 2. Determination of GTP binding to various G protein α-subtypes by immunoselection methods.](image2)

Coimmunoprecipitation of NKR-P1 with G Protein α-Subtypes—A coimmunoprecipitation assay was utilized to investigate whether there was any direct interaction between NKR-P1 and G proteins. IL-2-activated NK cell membranes were immunoprecipitated with various anti-G protein antibodies or with rabbit IgG (R IgG) as a control and then immunoblotted with anti-NKR-P1. The α-subunits of G protein immunoprecipitated with anti-αi3, anti-αo, anti-αq, or anti-αz, were associated with a 60-kDa band upon immunoblotting with anti-NKR-P1 (Fig. 4), while αi1,2, αo, αq,11, or α13 failed to associate with the 60-kDa band representing NKR-P1. Whereas mouse IgG failed to detect NKR-P1 when NK cell membranes were immunoprecipitated with mouse IgG (M IgG), mouse anti-NKR-P1 was able to detect NKR-P1 immunoprecipitated with anti-NKR-P1 (NKR-P1 in Fig. 4).

Reciprocally, when IL-2-activated NK cell membranes were
immunoprecipitated with anti-NKR-P1, and then immunoblotted with either rabbit IgG as a control or with anti-G protein antibodies, the same G protein α-subunits were shown to associate with NKR-P1. Fig. 5 shows that 40-, 45-, 41-, and 39–45-kDa bands. Representative of three experiments. MWstd., standard molecular weight.

In the present study, we demonstrate that the anti-NKR-P1 mAb 3.2.3, which recognizes certain members of the NKR-P1 family of NK cell receptors, enhances the GTP binding in rat IL-2-activated NK cell membranes. NKR-P1 are 60-kDa homodimeric proteins belonging to the family of transmembrane glycoprotein receptors with lectin domains (16), and were first characterized as activating receptors (15). Although the natural ligand for NKR-P1 is still undefined, 3.2.3 antibody induces redirected lysis (16), transduces signals important for regulating NK cell growth (17), and induces intracellular calcium mobilization (15), phosphoinositide turnover (15), and interferon-γ secretion (17).

Signals are transmitted intracellularly via one of two identified pathways: the tyrosine kinase receptors pathway or the G protein-coupled receptor pathway. The G protein intracellular signaling pathway, being the older one, became specialized and has been conserved for at least the last 1.2 billion years (25). This pathway is important for the activation of various secondary messengers such as phospholipase, in particular phospholipase Cβ (20, 26), and the mitogen-activated protein kinase pathway (27). Recent work has shown that the βγ-dimer binds and activates the phosphatidylinositol 3-kinase γ-isofrom (27, 28). In addition, this dimer binds pleckstrin homology domain (29), suggesting the importance of G proteins in mediating various biological activities inside the cells.

More than 20 α-subunits and at least 5 β- and 10 γ-subunits have been identified so far (30). The α-subunit is divided into four subfamilies. These are (i) αg (stimulatory of adenylyl cyclase), which includes α1γ, α12γ; (ii) αi (inhibitory of adenylyl cyclase), which includes αi1, αi2, αi6, αi11, αi12, αi13, and αi14; (iii) αβ (activator of phospholipases), which includes α0, α11, α14, and α12/α16; and (iv) αq, which includes α1γ, and α13. In its resting state, the α-subunit binds GDP and upon ligation of the receptors, conformational changes occur within the receptor α-subunit initiating the activation of G proteins, resulting in the binding of GTP to the α-subunit and its dissociation from the βγ-dimer (30, 31). Both the α-subunit and the βγ-dimer

FIG. 3. Immunoblot analysis of IL-2-activated NK cell membranes. IL-2-activated NK cell membranes (100–200 μg) were incubated with Dynabeads coated with sheep anti-rabbit/rabbit antibody to various α-subtypes of G protein. The immunocomplexes were collected and immunoblotted with anti-G protein antibodies. α12, α12, α13, α11, α12, and α11, but not α1, are detected, and are represented by 39–45-kDa bands. Representative of three experiments. MWstd., standard molecular weight.

FIG. 4. Physical coupling of NKR-P1 with various G protein α-subtypes. IL-2-activated NK cell membranes were immunoprecipitated with various rabbit anti-G protein antibodies, with anti-NKR-P1, or with rabbit IgG (RIgG in the figure), and then immunoblotted with anti-NKR-P1. The molecular weight marker is indicated at left. The results are representative of two different experiments. IP, immunoprecipitate; IB, immunoblot.

FIG. 5. Various heterotrimeric G proteins are associated with NKR-P1. NK cell membranes suspended in the solubilization buffer were immunoprecipitated overnight with anti-NKR-P1 at 4 °C. Protein A/Ag-agarose was then added to the mixture and incubated for 4 h at 4 °C. The immunocomplexes were suspended in SDS sample buffer, boiled, and run on 12% SDS-polyacrylamide gel electrophoresis, transferred to PVDF membrane, and then immunoblotted with anti-G protein antibodies to α1, α12, α13, α10, α11, α12, α13, and α14 or with rabbit IgG (RIgG) as a control. The molecular weight markers are indicated at left. The results are representative of three different experiments. IP, immunoprecipitate; IB, immunoblot.

FIG. 6. Only NKR-P1, but not an unrelated surface molecule (OX8) is coupled to G proteins in NK cell membranes. A, membranes prepared from NK cells suspended in the solubilization buffer were immunoprecipitated overnight at 4 °C with mouse antibody to OX8, NKR-P1, mouse IgG (MlgG), or rabbit IgG (RIgG). Protein A/Ag-agarose was added to the tubes, incubated for 4 h at 4 °C, and then washed. The immunocomplexes were separated by 12% SDS-PAGE, electrotransferred, and then immunoblotted with anti-NKR-P1. B, similar to A except that the immunocomplexes were immunoblotted with antibody to the common α of G protein instead of anti-NKR-P1. The results are representative of two different experiments.

DISCUSSION

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can then interact with various regulatory effector molecules (30, 31).

Several receptors present on NK cells are coupled to G proteins, which mediate various signals inside these cells. These include: (i) NK cell Fc receptors (32); (ii) receptors present on human NK cells that recognize tumor targets, and are coupled to Gs and Go (22), (iii) receptors present on rat NK cells that recognize tumor or allogeneic target cells, and are coupled to Go and Gq (18), (iv) transforming growth factor-β receptors present on rat NK cells, and are coupled to Gq and Gs (33), (v) the CXC chemokine IL-8 receptors present on human NK cells, and are coupled to Gs and Go (34), (vi) the CXC chemokine IP-10 receptors present on human IL-2-activated NK cells, and are coupled to Gi, Go, and Gq (35), (vii) the CXC chemokine SDF-1 receptors present on human NK cells, and are coupled to Go, Gs, and Gq (36), (viii) the CC chemokines MCP-1 and RANTES receptors present on human NK cells, and are coupled to Go, Gq, and Gz (37), (ix) the C chemokine lymphotactin receptors present on human NK cells, and are coupled to Gi3, Gs, Gq, and Gz (18), (iv) transforming growth factor-β receptors present on rat NK cells that bind the heterotrimeric G proteins, other single transmembrane-domain receptors, and does not belong to the seven-transmembrane-domain receptor that binds G proteins, other single transmembrane-spanning domain receptors, which characteristically bind the heterotrimeric G proteins, other single transmembrane-spanning domain receptors such as transforming growth factor-β receptors (33, 38), or insulin like growth factor-1 receptors (39) also bind G proteins. It is interesting that both transforming growth factor-β type II receptors (40) and NKR-P1 receptors (10, 11) are rich in serine/threonine kinases. Whether these kinases form a motif in the single-transmembrane-spanning domain receptor that binds G proteins is an intriguing possibility that needs to be examined.

In summary, our results are the first to show the functional coupling of NKR-P1, a type II plasma membrane receptor to various heterotrimeric G proteins in NK cell membranes. The promiscuous coupling of four different G proteins in these membranes to NKR-P1 may contribute to our understanding of the diverse biological functions attributed to this family of molecules in NK cells.

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