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RasGRP1 is required for human NK cell function[1]

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Abstract

Cross-linking of NK activating receptors activates PLC-γ and subsequently induces DAG and Ca^{2+} as second messengers of signal transduction. Previous studies reported that Ras guanyl nucleotide-releasing protein (RasGRP) 1, which is activated by DAG and Ca^{2+}, is crucial for T cell receptor-mediated Ras-ERK activation. We now report that RasGRP1, which can also be detected in human NK cells, plays an essential role in NK cell effector functions. To examine the role of RasGRP1 in NK cell functions, the expression of RasGRP1 was suppressed using RNAi. Knockdown of RasGRP1 significantly blocked ITAM-dependent cytokine production as well as NK cytotoxicity. Biochemically, RasGRP1-knockdown NK cells showed markedly decreased ability to activate Ras, ERK and JNK. Activation of the Ras-MAPK pathway was independently shown to be indispensable for NK cell effector functions via the use of specific pharmacological inhibitors. Our results reveal that RasGRP1 is required for the activation of the Ras-MAPK pathway leading to NK cell effector functions. Moreover, our data suggest that RasGRP1 might act as an important bridge between PLC-γ activation and NK cell effector functions via the Ras-MAPK pathway.

Introduction

Natural killer (NK) cells are effector lymphocytes that eliminate tumor cells or virus-infected cells (1). NK cells also have the ability to produce immunoregulatory cytokines, such as interferon-γ (IFN-γ), granulocyte-macrophage colony-stimulating factor (GM-CSF), and tumor necrosis factor-α (TNF-α) (2). These NK cell effector functions are regulated by multiple activating and inhibitory NK cell receptors (3,4). Whereas inhibitory NK cell receptors transmit

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Authorship

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inhibitory signals to the downstream signaling molecules through their cytoplasmic ITIM, activating NK cell receptors signal through ITAM-containing adaptors, such as CD3ζ, FcRγ, and DAP12, or the cytoplasmic Tyr-Ile-Asn-Met (YINM) motif-containing adaptor DAP10 (5). Upon engagement of activating receptors, such as natural cytotoxicity receptors (NCRs) and NKG2D, tyrosine phosphorylation in the ITAM or YINM motif of adaptor molecules is mediated by Src family kinases, leading to the subsequent activation of a variety of downstream signaling molecules, including the Vav family, PI3K, and phospholipase C-γ (PLC-γ). Finally, transmitted signals result in activation of mitogen-activated protein kinases (MAPKs), which are crucial for cytolytic granule release and cytokine generation (6,7). Among these molecules propagating activation signals, PLC-γ plays an essential role in the signal transduction leading to NK cell cytotoxicity and cytokine production (8-10). PLC-γ, which hydrolyzes membrane phosphatidylinositols into inositol 1,4,5-triphosphate (IP_3) and diacylglycerol (DAG) (11, 12), modulates the intracellular calcium ion concentration (Ca^{2+}) and activates downstream-signaling cascades for NK cell effector functions.

Ras guanyl nucleotide-releasing protein 1 (RasGRP1) is a member of the RasGRP family that contain a DAG-binding C1 domain, a Ras exchange motif, calcium-binding EF-hands, and a guanine-nucleotide exchange factor (GEF) domain (13). Following PLC-γ activation, RasGRP1 is activated by DAG and Ca^{2+} leading to activation of the Ras family via guanine-nucleotide exchange with dissociation of GDP from Ras and association with GTP (14,15). RasGRP1 plays an essential role in T cell receptor (TCR) signaling. Thymocytes isolated from RasGRP1$^{-/-}$ mice display a defect in Ras-mediated extracellular signal-regulated protein kinase (ERK) activation in response to TCR stimulation, and a block in the double-positive stage of thymocyte development (16). In addition, RasGRP1 plays an important role in IgE-mediated signal transduction and mast cell function. IgE-mediated degranulation is impaired in mast cells of RasGRP1$^{-/-}$ mice, and these mice display severely defective IgE-evoked systemic anaphylaxis (17).

Despite the evidence that RasGRP1 plays a critical role in TCR-mediated and IgE-mediated signaling, whether it functions in NK cell receptor signaling has not been examined. Because activation of Ras has been shown to be involved in NK cell-mediated cytotoxicity and IFN-γ production (6,18,19), we examined if RasGRP1 plays a role in NK cell activation and identified an essential role in effector functions and receptor-mediated Ras-MAPK activation.

Materials and Methods

Cell culture and reagents

Human primary NK cells were isolated from the umbilical cord blood (UCB) using the human NK Cell Isolation Kit (Miltenyi). The isolated primary NK cell populations which were >95% CD56^{+}CD3^{-} were cultured in Myelocult H5100 (StemCell Technologies) supplemented with IL-15 (10 ng/ml). The UCB was collected from umbilical veins after neonatal delivery, with informed consent from the pregnant mothers and following the guidance of the local institutional review board (IRB). To prepare hematopoietic stem cell (HSC)-derived mature NK cells, CD34^{+} HSCs were isolated from UCB using the CD34 MicroBead Kit (Miltenyi). CD34^{+} HSCs were differentiated into NK cell precursors by incubating the cells in Myelocult H5100 supplemented with SCF (30 ng/ml) and Flt3 ligand (50 ng/ml) for 14 days. NK cell precursors were differentiated into mature NK cells by stimulation with IL-15 (30 ng/ml) for an additional 14 days. Mature NK cells (>95% CD56^{+}CD3^{-} cells) were maintained in Myelocult H5100 with 10 ng/ml IL-15 and used for the functional assays. The human NK cell line NK-92 (American Type Culture Collection, ATCC) was cultured in alpha MEM (Gibco), supplemented with 20% heat-inactivated FBS (Hyclone), 2 mM $_{L}$-glutamine and IL-15 (10 ng/ml). The K562 cell line (ATCC) was cultured in RPMI 1640 (Gibco) supplemented with 10% heat-inactivated FBS (Hyclone). Recombinant human SCF, Flt3 ligand, IL-12, and IL-15 were

purchased from PeproTech. FTI-277, PD98059, SP600125 and SB203580 were purchased from Calbiochem.

Lentiviral infection and siRNA nucleofection

For lentiviral infection of NK-92 cells, the pLKO.1-non-target shRNA control vector (SHC002) and pLKO.1-RasGRP1 shRNA vector (TRCN0000048268) were purchased from Sigma. Lentiviruses were produced using a third-generation packaging system (pMDLg/pRRE, pRSV-Rev and pMD2.G) in HEK293T cells. The lentivirus-containing supernatants were cleared by centrifugation at 3000 rpm for 5 min at 4 °C, passed through a 0.45 μm filter, and concentrated by ultracentrifugation at 50,000 *g* for 90 min at 4 °C. Upon infection, shRNA-expressing NK-92 cells were selected with puromycin (2 μg/ml) for 3 weeks before functional analysis. Nucleofections of NK cell precursors and mature NK cells were performed using the Amaxa Human CD34 Cell Nucleofector™ Kit (program U-08), and the non-target control siRNA and RasGRP1 siRNA SMARTpool were purchased from Dharmacon.

NK cell functional assays

Cytotoxicity was examined using a standard 4-h ^{51}Cr-release assay. ^{51}Cr-labeled target K562 cells (3×10^5 cells/well) and serial dilutions of NK cells were plated in triplicate. The ^{51}Cr released into the supernatant was measured using a γ-counter. The percentage of specific lysis was calculated using the formula: (experimental release - spontaneous release)/(maximum release - spontaneous release)×100. To evaluate cytokine secretion, NK cells were stimulated in duplicate for 16 h with plate-bound antibodies (10 μg/ml), IL-12 (20 ng/ml), or PMA/ionomycin (1 or 2 ng/ml, 0.1 or 0.2 μg/ml). The secretion of IFN-γ (Assay Designs), TNF-α, and GM-CSF (R&D Systems) into the supernatant was measured by ELISA.

Real-time PCR

Total RNA was extracted using TRIZOL (Invitrogen) and reverse transcribed into cDNA using M-MLV reverse transcriptase (Promega) with random primers (Takara Bio). Real-time PCR was performed using a Dice TP 800 Thermal Cycler and the SYBR Premix Ex Tag (Takara Bio). The data were normalized to the amount of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) transcript. The primer sequences were: 5′-ggctcaaggagacaagttcg-3′ and 5′-gaagtcggtgcactctccata-3′ for RasGRP1, 5′-gtccaacgcaaagcaataca-3′ and 5′-ctcttcgacctcgaaacagc-3′ for IFN-γ, 5′-gtcctcttcaagggccaag-3′ and 5′-tagtcgggccgattgatct-3′ for TNF-α, 5′-catgatggccagccactac-3′ and 5′-taatctgggttgcacaggaa-3′ for GM-CSF, and 5′-cagcctcaagatcatcagca-3′ and 5′-gtcttctgggtggcagtgat-3′ for GAPDH.

Western blotting

Cells were washed twice with ice-cold PBS and lysed in RIPA buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.25% SDS, 1% NP-40, and 1 mM EDTA, supplemented with a protease inhibitor cocktail tablet and a phosphatase inhibitor cocktail tablet from Roche). The cell lysates were resolved in 8 or 12% SDS-PAGE gels and transferred to a PVDF membrane (Millipore). The membrane was probed with antibodies specific to the following molecules: p-PLC-γ1^{Tyr783}, p-Src^{Tyr416}, Src, p-ERK^{Thr202,Tyr204}, ERK, p-JNK^{Thr183,Tyr185}, JNK, p-p38^{Thr180,Tyr182} and p38 (Cell Signaling); RasGRP1, PLC-γ1 and β-actin (Santa Cruz); and GAPDH (Assay Designs). After incubation with peroxidase-conjugated anti-rabbit or anti-mouse IgG (Jackson ImmunoResearch), the signals were detected using SuperSignal West Pico Chemiluminescent Substrate (Pierce) or Immobilon Western Chemiluminescent HRP Substrate (Millipore).

Ras activation

NK-92 cells (1×10^7 cells) were stimulated with K562 cells (1×10^6 cells) or plate-bound anti-NKp30 mAb (10 μg/ml) for 5 min. Ras activation was assessed with the Ras Activation kit (Assay Designs), according to the manufacturer's instructions. In brief, stimulated NK cells were lysed in the Lysis/Binding/Wash buffer. The lysates were incubated with 80 mg of GST-Raf1-RBD on immobilized glutathione resin for 1 h. The resin was then washed and boiled in 2× SDS sample buffer, and GTP-bound pan-Ras was detected by western blotting with anti-pan-Ras antibody.

Statistical analyses

Comparisons were analyzed for statistical significance by the Student's *t* test using Microsoft Excel software. A p value < 0.05 was considered as significant.

Results

Knockdown of RasGRP1 inhibits NK cell cytolytic activity

To determine the potential role of RasGRP1 in NK cells, we first investigated whether RasGRP1 is expressed in human NK cells. RasGRP1 protein was detected in NK-92 and primary NK cells (Fig. 1A). Additionally, RasGRP1 was expressed in mature NK cells (mNK), but not in NK cell precurors (pNK) (Fig. 1B). Next, we examined whether RasGRP1 functions in NK cytotoxicity. To investigate the cytolytic activity of NK-92 cells in which expression of RasGRP1 was silenced (Fig. 1C), we measured lysis of the NK target, K562 cells (a human erythromyeloblastoid leukemia cell line). RasGRP1 shRNA-expressing NK-92 cells exhibited reduced capacity to kill target K562 cells (Fig. 1D). RasGRP1 siRNA-nucleofected mature NK cells (Fig. 1E) also displayed diminished cytolytic activity compared with control siRNA-treated mature NK cells (Fig. 1F). Thus, RasGRP1 is expressed in human NK cells and functions in NK cytotoxicity, leading us to hypothesize that RasGRP1 might be involved in the K562-evoked NK receptor signaling pathway that leads to expression of cytolytic activity.

RasGRP1 is essential for NCR-ITAM-dependent cytokine production

After engagement of NK activating receptors that associate with ITAM-containing adaptors, NK cells produce proinflammatory cytokines, such as IFN-γ, TNF-α, and GM-CSF (7,20,21), in addition to exhibiting cytotoxicity. To address the impact of RasGRP1 on ITAM-dependent cytokine production, we analyzed NKp30-mediated cytokine generation in control shRNA- and RasGRP1 shRNA-expressing NK-92 cells. NKp30-mediated secretion of IFN-γ, TNF-α, and GM-CSF was significantly reduced in RasGRP1 shRNA-expressing NK-92 cells (Fig. 2A), and transcription of these cytokines was also decreased (Fig. 2B). After cross-linking of NK activating receptors, ITAM-dependent signaling cascades occur including activation of PLC-γ (5,6). As it has been reported that RasGRP1 is directly activated by PLC-γ-induced DAG and Ca^{2+} (13,14,22), we evaluated the effect of PMA (as a DAG analog) and ionomycin (as a calcium ionophore) on cytokine production in RasGRP1 shRNA-expressing NK-92 cells. PMA/ionomycin-induced cytokine generation was markedly reduced in RasGRP1 shRNA-expressing NK-92 cells compared with control shRNA-expressing NK-92 cells (Fig. 2A, 2B). IFN-γ production in response to IL-12, a major stimulatory cytokine of IFN-γ, was normal in RasGRP1 shRNA-expressing NK-92 cells, but IL-12 did not induce GM-CSF or TNF-α production in NK-92 cells (Fig. 2A, 2B). Similar to RasGRP1 shRNA-expressing NK-92 cells, cytokine production was impaired after stimulation through NCRs (NKp30, NKp44 and NKp46) and by PMA/ionomycin, but not by stimulation of IL-12 receptors in RasGRP1 siRNA-nucleofected mature NK cells (Fig. 2C). Thus, these results indicate that RasGRP1 is essential for ITAM-dependent cytokine production in NK cells.

RasGRP1 is required for Ras activation in NK cells

Our data suggested that impaired intracellular signaling events might be accountable for the defective NK cell effector functions in RasGRP1-knockdown NK cells. In addition, other studies have demonstrated that DAG and Ca^{2+}-bound RasGRP1 play a critical role in the activation of Ras protein (14,15). Before evaluating the effect of RasGRP1-knockdown on Ras activation, we investigated whether knockdown of RasGRP1 affects activation of PLC-γ, the upstream signaling molecule of RasGRP1. NKp30-mediated activation of PLC-γ1 was normal in RasGRP1 shRNA-expressing NK-92 cells (Fig. 3A). Moreover, knockdown of RasGRP1 did not affect Src phosphorylation, an important kinase for promoting ITAM-mediated proximal signaling events. Next, we examined the Ras activity in RasGRP1-knockdown NK-92 cells using a GST protein fused to the Ras-binding domain (RBD) of Raf1 (GST-Raf1-RBD). The amount of GTP-bound Ras precipitated by GST-Raf1-RBD was reduced in RasGRP1 shRNA-expressing NK-92 cells after incubation with K562 or NKp30 stimulation (Fig. 3B). As shown in Figures 1 and 2, RasGRP1-knockdown NK cells displayed defective effector functions. To examine if Ras activation is important for NK cell cytotoxicity and cytokine production, we measured NK cell effector functions in NK-92 cells treated with the Ras inhibitor FTI-277. NK cell cytolytic activity was decreased by treatment with FTI-277 in a dose-dependent manner (Fig. 3 C). In addition, NKp30-mediated and PMA/ionomycin-induced production of IFN-γ, TNF-α, and GM-CSF was markedly reduced in FTI-277-treated NK-92 cells (Fig. 3D). However, treatment with FTI-277 did not affect IL-12-induced IFN-γ secretion similar to knockdown of RasGRP1. Overall, these data suggest that RasGRP1 regulates Ras activation, and that Ras activation is important for NK cell cytotoxicity and ITAM-dependent cytokine production.

RasGRP1 regulates ERK and JNK activation in NK cells

RasGRP1 plays an important role in TCR-mediated Ras-ERK activation, and Ras protein is an essential factor in the regulation of the MAPK pathway (23,24). Additionally, MAPK activation is crucial for cytolytic granule release and cytokine generation in NK cells (6,7). Therefore, we investigated whether reduced Ras activity by RasGRP1-knockdown affects the MAPK pathway responsible for NK cell effector functions. To investigate the impact of RasGRP1 on MAPK activation, control shRNA- and RasGRP1 shRNA-expressing NK-92 cells were stimulated with K562, plate-bound anti-NKp30 or PMA/ionomycin. After incubation with K562 target cells, RasGRP1 shRNA-expressing NK-92 cells displayed reduced phosphorylation of ERK and JNK compared with control shRNA-expressing NK-92 cells (Fig. 4A). Similarly, NKp30-mediated and PMA/ionomycin-induced activation of ERK and JNK were decreased in RasGRP1-knockdown NK-92 cells (Fig. 4B, 4C). However, p38 phosphorylation was not reduced in RasGRP1 shRNA-expressing NK-92 cells in response to K562, anti-NKp30 or PMA/ionomycin, indicating that p38 activity is not affected by RasGRP1. Next, we confirmed the role of MAPKs in NK cell effector functions using specific pharmacological inhibitors of MAPKs. Use of the JNK inhibitor SP600125 substantially reduced lysis of K562 target cells, and both the ERK inhibitor PD98059 and the p38 inhibitor SB203580 slightly decreased cytolytic activity (Fig. 4D). Additionally, SP600125 significantly blocked NKp30-mediated IFN-γ production, and PD98059 weakly diminished NKp30-mediated IFN-γ production, but SB203580 did not affect NKp30-mediated IFN-γ production (Fig. 4E). Collectively, these results indicated that RasGRP1 is required for the activation of the ERK and JNK pathways in NK cells, and we conclude that the impairment of Ras-mediated ERK and JNK activation by RasGRP1 RNAi is a probable cause for the defect in cytotoxicity and cytokine production in RasGRP1-knockdown NK cells.

Discussion

NK cells are large granular lymphocytes of innate immunity that contribute to the killing of tumor or virus-infected cells (1). Additionally, NK cells produce important inflammatory cytokines, such as IFN-γ, TNF-α, and GM-CSF (2). These effector functions of NK cells are controlled by the coordinated balance between activating and inhibitory receptors (4). The inhibitory NK receptors suppress cellular responses via ITIMs present in their cytoplasmic tails. Upon stimulation of the inhibitory receptors, phosphorylated ITIMs bind to the SH2 domains of protein tyrosine phosphatases, such as SHP-1 and SHP-2, which dephosphorylate a number of tyrosine-phosphorylated signaling proteins (25,26). The activating NK cell receptors associate with adaptor molecules that signal through their cytoplasmic signaling motifs, such as ITAMs, which initiates both cytotoxicity and cytokine production, or the YINM motif, which triggers only cytotoxicity. The NCR family that recognizes unidentified ligands on tumor cells contains three defined members (NKp46, NKp44 and NKp30). NKp44 associates with ITAM-containing DAP12, whereas NKp30 and NKp46 recruit ITAM-bearing CD3ζ and FcRγ as adaptor molecules (5). NKG2D is an activating receptor that recognizes ULBP-1/2/3 (27) and MIC-A/B (28) on the surface of target cells. In mice, NKG2D recruits both ITAM-bearing KARAP/DAP12 and YINM motif-bearing DAP10. In contrast to mice, NKG2D in humans only associates with DAP10. Upon engagement of NK activating receptors including NCRs and NKG2D, tyrosine residues in ITAM or YINM motif are phosphorylated by the recruited Src family kinases (29). Phosphorylated ITAM recruits Syk protein family members, which subsequently activate PLC-γ, whereas the phosphorylated YINM motif directly recruits Grb2-mediated PLC-γ independent of Syk. Activated PLC-γ hydrolyzes membrane phosphatidylinositols into IP_3 and DAG for downstream signaling events. PKC-θ has been reported to be a putative signaling protein downstream of PLC-γ in NK cells (30). However, PKC-θ-deficient NK cells displayed a defect only in ITAM-dependent IFN-γ secretion, whereas PLC-γ is required for both NK cytotoxicity and cytokine production (8). In addition, several signaling molecules, such as PDK1, SLP76, and Vav1, are also involved in PKC-θ activation (31-33). Despite the fact that PLC-γ is essential for NK cell effector functions, a major downstream signaling protein of PLC-γ, especially DAG and Ca^{2+}-binding protein, has not been well-defined.

In the present study, we stimulated NK cells with K562 cells, engagement of NCRs, or PMA/ionomycin treatment to induce NK cell effector functions. K562 cells expressed two NKG2D ligands, MICA/B and ULBP2, and treatment with the NKG2D blocking mAb markedly reduced NK cytotoxicity against K562 (34). Thus, the incubation with K562 cells could trigger NKG2D-DAP10-mediated signaling events and cytotoxicity in NK cells. Upon engagement of NCR, the ITAM-mediated signaling pathway is activated, and NK cell effector functions are evoked. Both stimulation with K562 and cross-linking of NCRs activate PLC-γ and produce DAG and IP_3-mediated Ca^{2+} influx (5,6). PMA is a DAG analog that binds to and activates a DAG target protein. Ionomycin is a calcium ionophore that transports Ca^{2+} across biological membranes and raises the intracellular Ca^{2+} level. Therefore, treatment with PMA/ionomycin mimics the induction of DAG and Ca^{2+}, which are produced by receptor-mediated PLC-γ activation, and activate DAG and Ca^{2+} target signaling proteins that regulate the production of inflammatory cytokines from NK cells (21,35). In this paper, we demonstrate that RasGRP1 might be a major downstream signaling molecule of PLC-γ in NK cells.

RasGRP1 has the ability to bind to and activate Ras family proteins via a Ras exchange motif, a guanine-nucleotide exchange factor domain (GEF), and is an important regulator in the control of the Ras-mediated signaling pathway. At the beginning of this study, we hypothesized that RasGRP1 plays a role in the NK receptor signaling pathway because it is essential for TCR-mediated and IgE-dependent signaling pathways (16,17), and it is also expressed in human NK cells. We observed that knockdown of RasGRP1 inhibited NK cell effector

functions, including cytotoxicity and ITAM-dependent cytokine production. Similar to RasGRP1-deficient thymocytes, RasGRP1-knockdown NK cells displayed an impaired activation of the Ras-MAPK pathway, suggesting that RasGRP1 connect PLC-γ activation to the Ras-MAPK pathway in NK cells (Fig. 5). MAPKs are known as essential kinases of NK cell effector functions (6,7). It has also been reported that treatment with the Ras inhibitor FTI-277 significantly reduces ITAM-dependent IFN-γ production in mouse NK cells (19), and a Ras-ERK pathway has been implicated in ADCC (18). However, Wei et al. reported that the spontaneous cytotoxicity is independent of Ras-mediated signaling despite the detection of Ras activation followed by target ligation (36). We treated NK-92 cells with FTI-277 for 30 min before triggering NK cell effector functions, but Wei et al. treated with FTI-277 for 24 h prior to stimulation. It is possible that this difference in pretreatment time might lead to disparate cytotoxicity results. Nevertheless, we confirmed that Ras activation is important for cytotoxicity and cytokine production using a pharmacological specific inhibitor of Ras and knockdown of RasGRP1, a Ras activator. However, further analysis is required to elucidate which Ras member (N-Ras, K-Ras, and/or H-Ras) is activated by RasGRP1 in NK cells.

As shown in Figure 2, knockdown of RasGRP1 did not affect IL-12-induced IFN-γ production. This result was expected because the IL-12 receptor signals mainly through STAT4, and not through Ras proteins (37). Similarly, IL-12-mediated IFN-γ secretion was normal in FTI-277-treated NK-92 cells (Fig. 3). This result also showed that treatment with FTI-277 had targeted activity and did not globally affect cellular activity or survivial of NK-92 cells.

In conclusion, we identified RasGRP1 as an essential regulator of NK cell cytotoxicity and cytokine production. Knockdown of RasGRP1 inhibited activation of Ras, ERK, and JNK, and consequently blocked NK cell effector functions. In addition, our results indicate that RasGRP1 might connect PLC-γ activation to NK cell effector functions via the Ras-MAPK pathway.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Abbreviations used in this paper

pNK	NK cell precursor
mNK	mature NK cell
RasGRP1	Ras guanyl nucleotide-releasing protein 1
PLC-γ	phospholipase C-gamma
DAG	diacylglycerol
RNAi	RNA interference
NCR	natural cytotoxicity receptor
RBD	Ras-binding domain
GEF	guanine-nucleotide exchange factor
ADCC	antibody dependent cell-mediated cytotoxicity
UCB	umbilical cord blood

References

1. Trinchieri G. Biology of natural killer cells. Adv Immunol 1989;47:187–376. [PubMed: 2683611]

2. Perussia B. The Cytokine Profile of Resting and Activated NK Cells. Methods 1996;9:370–378. [PubMed: 8812690]
3. Yokoyama WM, Plougastel BF. Immune functions encoded by the natural killer gene complex. Nat Rev Immunol 2003;3:304–316. [PubMed: 12669021]
4. Lanier LL. NK cell recognition. Annu Rev Immunol 2005;23:225–274. [PubMed: 15771571]
5. Tassi I, Klesney-Tait J, Colonna M. Dissecting natural killer cell activation pathways through analysis of genetic mutations in human and mouse. Immunol Rev 2006;214:92–105. [PubMed: 17100878]
6. Vivier E, Nunes JA, Vely F. Natural killer cell signaling pathways. Science 2004;306:1517–1519. [PubMed: 15567854]
7. Guo H, Samarakoon A, Vanhaesebroeck B, Malarkannan S. The p110 delta of PI3K plays a critical role in NK cell terminal maturation and cytokine/chemokine generation. J Exp Med 2008;205:2419–2435. [PubMed: 18809712]
8. Caraux A, Kim N, Bell SE, Zompi S, Ranson T, Lesjean-Pottier S, Garcia-Ojeda ME, Turner M, Colucci F. Phospholipase C-gamma2 is essential for NK cell cytotoxicity and innate immunity to malignant and virally infected cells. Blood 2006;107:994–1002. [PubMed: 16204312]
9. Tassi I, Presti R, Kim S, Yokoyama WM, Gilfillan S, Colonna M. Phospholipase C-gamma 2 is a critical signaling mediator for murine NK cell activating receptors. J Immunol 2005;175:749–754. [PubMed: 16002670]
10. Upshaw JL, Schoon RA, Dick CJ, Billadeau DD, Leibson PJ. The isoforms of phospholipase C-gamma are differentially used by distinct human NK activating receptors. J Immunol 2005;175:213–218. [PubMed: 15972651]
11. Wilde JI, Watson SP. Regulation of phospholipase C gamma isoforms in haematopoietic cells: why one, not the other? Cell Signal 2001;13:691–701. [PubMed: 11602179]
12. Berridge MJ, Lipp P, Bootman MD. The versatility and universality of calcium signalling. Nat Rev Mol Cell Biol 2000;1:11–21. [PubMed: 11413485]
13. Ebinu JO, Bottorff DA, Chan EY, Stang SL, Dunn RJ, Stone JC. RasGRP, a Ras guanyl nucleotide-releasing protein with calcium- and diacylglycerol-binding motifs. Science 1998;280:1082–1086. [PubMed: 9582122]
14. Bivona TG, Perez De Castro I, Ahearn IM, Grana TM, Chiu VK, Lockyer PJ, Cullen PJ, Pellicer A, Cox AD, Philips MR. Phospholipase Cgamma activates Ras on the Golgi apparatus by means of RasGRP1. Nature 2003;424:694–698. [PubMed: 12845332]
15. Roose J, Weiss A. T cells: getting a GRP on Ras. Nat Immunol 2000;1:275–276. [PubMed: 11017094]
16. Dower NA, Stang SL, Bottorff DA, Ebinu JO, Dickie P, Ostergaard HL, Stone JC. RasGRP is essential for mouse thymocyte differentiation and TCR signaling. Nat Immunol 2000;1:317–321. [PubMed: 11017103]
17. Liu Y, Zhu M, Nishida K, Hirano T, Zhang W. An essential role for RasGRP1 in mast cell function and IgE-mediated allergic response. J Exp Med 2007;204:93–103. [PubMed: 17190838]
18. Perussia B. Signaling for cytotoxicity. Nat Immunol 2000;1:372–374. [PubMed: 11062493]
19. Tassi I, Cella M, Gilfillan S, Turnbull I, Diacovo TG, Penninger JM, Colonna M. p110gamma and p110delta phosphoinositide 3-kinase signaling pathways synergize to control development and functions of murine NK cells. Immunity 2007;27:214–227. [PubMed: 17723215]
20. El Costa H, Casemayou A, Aguerre-Girr M, Rabot M, Berrebi A, Parant O, Clouet-Delannoy M, Lombardelli L, Jabrane-Ferrat N, Rukavina D, Bensussan A, Piccinni MP, Le Bouteiller P, Tabiasco J. Critical and differential roles of NKp46- and NKp30-activating receptors expressed by uterine NK cells in early pregnancy. J Immunol 2008;181:3009–3017. [PubMed: 18713971]
21. Hara H, Ishihara C, Takeuchi A, Xue L, Morris SW, Penninger JM, Yoshida H, Saito T. Cell type-specific regulation of ITAM-mediated NF-kappaB activation by the adaptors, CARMA1 and CARD9. J Immunol 2008;181:918–930. [PubMed: 18606643]
22. Di Fiore PP. Signal transduction: life on Mars, cellularly speaking. Nature 2003;424:624–625. [PubMed: 12904771]
23. Stone JC. Regulation of Ras in lymphocytes: get a GRP. Biochem Soc Trans 2006;34:858–861. [PubMed: 17052215]

24. Mor A, Philips MR. Compartmentalized Ras/MAPK signaling. Annu Rev Immunol 2006;24:771–800. [PubMed: 16551266]
25. Burshtyn DN, Long EO. Regulation through inhibitory receptors: Lessons from natural killer cells. Trends Cell Biol 1997;7:473–479. [PubMed: 17709010]
26. Stebbins CC, Watzl C, Billadeau DD, Leibson PJ, Burshtyn DN, Long EO. Vav1 dephosphorylation by the tyrosine phosphatase SHP-1 as a mechanism for inhibition of cellular cytotoxicity. Mol Cell Biol 2003;23:6291–6299. [PubMed: 12917349]
27. Cosman D, Mullberg J, Sutherland CL, Chin W, Armitage R, Fanslow W, Kubin M, Chalupny NJ. ULBPs, novel MHC class I-related molecules, bind to CMV glycoprotein UL16 and stimulate NK cytotoxicity through the NKG2D receptor. Immunity 2001;14:123–133. [PubMed: 11239445]
28. Groh V, Bahram S, Bauer S, Herman A, Beauchamp M, Spies T. Cell stress-regulated human major histocompatibility complex class I gene expressed in gastrointestinal epithelium. Proc Natl Acad Sci U S A 1996;93:12445–12450. [PubMed: 8901601]
29. Gilfillan S, Ho EL, Cella M, Yokoyama WM, Colonna M. NKG2D recruits two distinct adapters to trigger NK cell activation and costimulation. Nat Immunol 2002;3:1150–1155. [PubMed: 12426564]
30. Tassi I, Cella M, Presti R, Colucci A, Gilfillan S, Littman DR, Colonna M. NK cell-activating receptors require PKC-theta for sustained signaling, transcriptional activation, and IFN-gamma secretion. Blood 2008;112:4109–4116. [PubMed: 18784374]
31. Toker A, Newton AC. Cellular signaling: pivoting around PDK-1. Cell 2000;103:185–188. [PubMed: 11057891]
32. Dienz O, Moller A, Strecker A, Stephan N, Krammer PH, Droge W, Schmitz ML. Src homology 2 domain-containing leukocyte phosphoprotein of 76 kDa and phospholipase C gamma 1 are required for NF-kappa B activation and lipid raft recruitment of protein kinase C theta induced by T cell costimulation. J Immunol 2003;170:365–372. [PubMed: 12496421]
33. Villalba M, Bi K, Hu J, Altman Y, Bushway P, Reits E, Neefjes J, Baier G, Abraham RT, Altman A. Translocation of PKC[theta] in T cells is mediated by a nonconventional, PI3-K- and Vav-dependent pathway, but does not absolutely require phospholipase C. J Cell Biol 2002;157:253–263. [PubMed: 11956228]
34. Li C, Ge B, Nicotra M, Stern JN, Kopcow HD, Chen X, Strominger JL. JNK MAP kinase activation is required for MTOC and granule polarization in NKG2D-mediated NK cell cytotoxicity. Proc Natl Acad Sci U S A 2008;105:3017–3022. [PubMed: 18287025]
35. Roncagalli R, Taylor JE, Zhang S, Shi X, Chen R, Cruz-Munoz ME, Yin L, Latour S, Veillette A. Negative regulation of natural killer cell function by EAT-2, a SAP-related adaptor. Nat Immunol 2005;6:1002–1010. [PubMed: 16127454]
36. Wei S, Gilvary DL, Corliss BC, Sebti S, Sun J, Straus DB, Leibson PJ, Trapani JA, Hamilton AD, Weber MJ, Djeu JY. Direct tumor lysis by NK cells uses a Ras-independent mitogen-activated protein kinase signal pathway. J Immunol 2000;165:3811–3819. [PubMed: 11034387]
37. Morinobu A, Gadina M, Strober W, Visconti R, Fornace A, Montagna C, Feldman GM, Nishikomori R, O'Shea JJ. STAT4 serine phosphorylation is critical for IL-12-induced IFN-gamma production but not for cell proliferation. Proc Natl Acad Sci U S A 2002;99:12281–12286. [PubMed: 12213961]

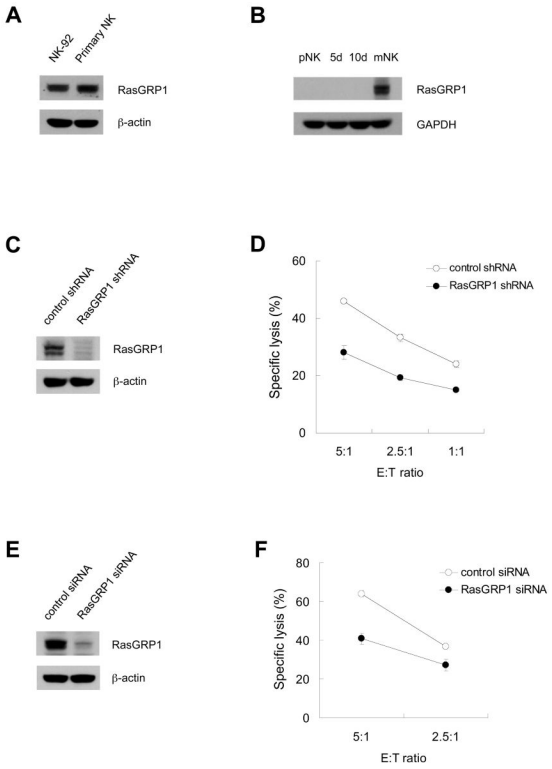

Figure 1. RasGRP1 in NK cell cytotoxicity

(A) Expression of RasGRP1 in NK-92 and human primary NK cells. NK cell lysates were analyzed by western blotting with anti-RasGRP1 antibody. (B) Expression of RasGRP1 in NK cell precursors and mature NK cells. NK cell precursors were stimulated with IL-15 (30 ng/ml) for 0, 5, and 10 days, and mature NK cells were harvested. Expression of RasGRP1 was analyzed by western blotting. (C) Cell lysates of control shRNA- and RasGRP1 shRNA-expressing NK-92 cells were analyzed by western blotting with anti-RasGRP1 antibody. (D) Control shRNA- and RasGRP1 shRNA-expressing NK-92 cells were incubated with ^{51}Cr-labeled K562 cells at the indicated effector/target (E:T) ratios. (E) Cell lysates of control siRNA- and RasGRP1 siRNA-nucleofected mature NK cells were analyzed by western blotting with anti-RasGRP1 antibody. (F) Control siRNA- and RasGRP1 siRNA-nucleofected mature NK cells were incubated with ^{51}Cr-labeled K562 cells at the indicated E:T ratios. The data are representative of two independent experiments, and the error bars represent the standard deviation (s.d.) of triplicates.

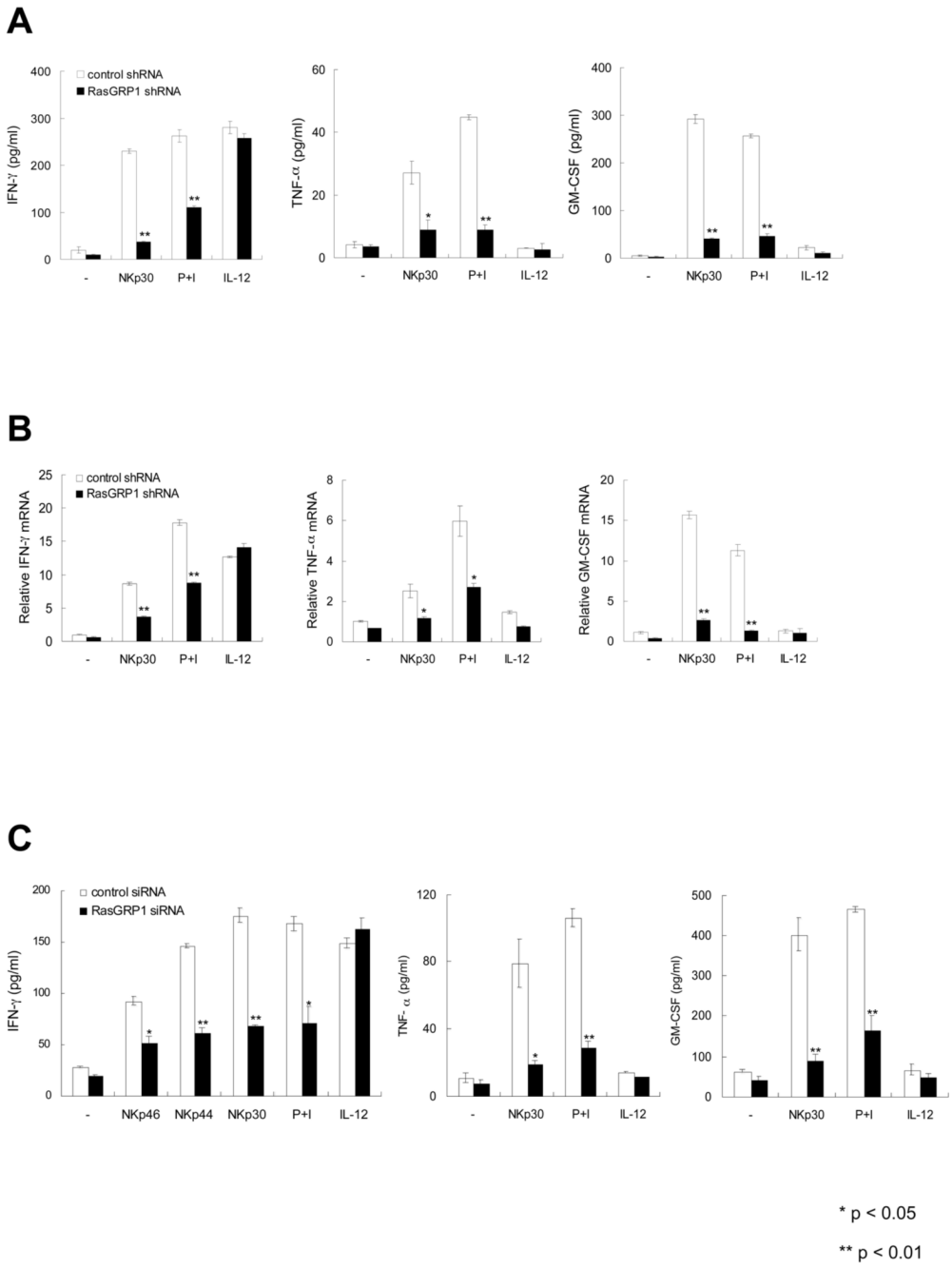

Figure 2. Impaired ITAM-dependent cytokine production in RasGRP1-knockdown NK cells

(A) Control shRNA- and RasGRP1 shRNA-expressing NK-92 cells were stimulated with plate-bound antibodies to NKp30 (10 μg/ml), or were stimulated with PMA (1 ng/ml)/ionomycin (0.1 μg/ml) or IL-12 (20 ng/ml) in the culture medium. After 16 h, the cytokines (IFN-γ, TNF-α, and GM-CSF) released into the supernatant were measured by ELISA. * $p < 0.05$, ** $p < 0.01$. (B) shRNA-transduced NK-92 cells were stimulated with anti-NKp30 mAb (10 μg/ml), PMA (1 ng/ml)/ionomycin (0.1 μg/ml), or IL-12 (20 ng/ml) for 4 h, and the mRNA expression of the cytokines was analyzed by real-time PCR. * $p < 0.05$, ** $p < 0.01$. (C) Control siRNA- and RasGRP1 siRNA-nucleofected mature NK cells were stimulated with plate-bound antibodies to NCRs (10 μg/ml) or were stimulated with PMA (2 ng/ml)/ionomycin (0.2 μg/ml)

or IL-12 (20 ng/ml) in the culture medium. After 16 h, the cytokines released into the supernatant were quantified by ELISA. * $p < 0.05$, ** $p < 0.01$. The data are representative of three independent experiments, and the error bars represent the s.d. of duplicates.

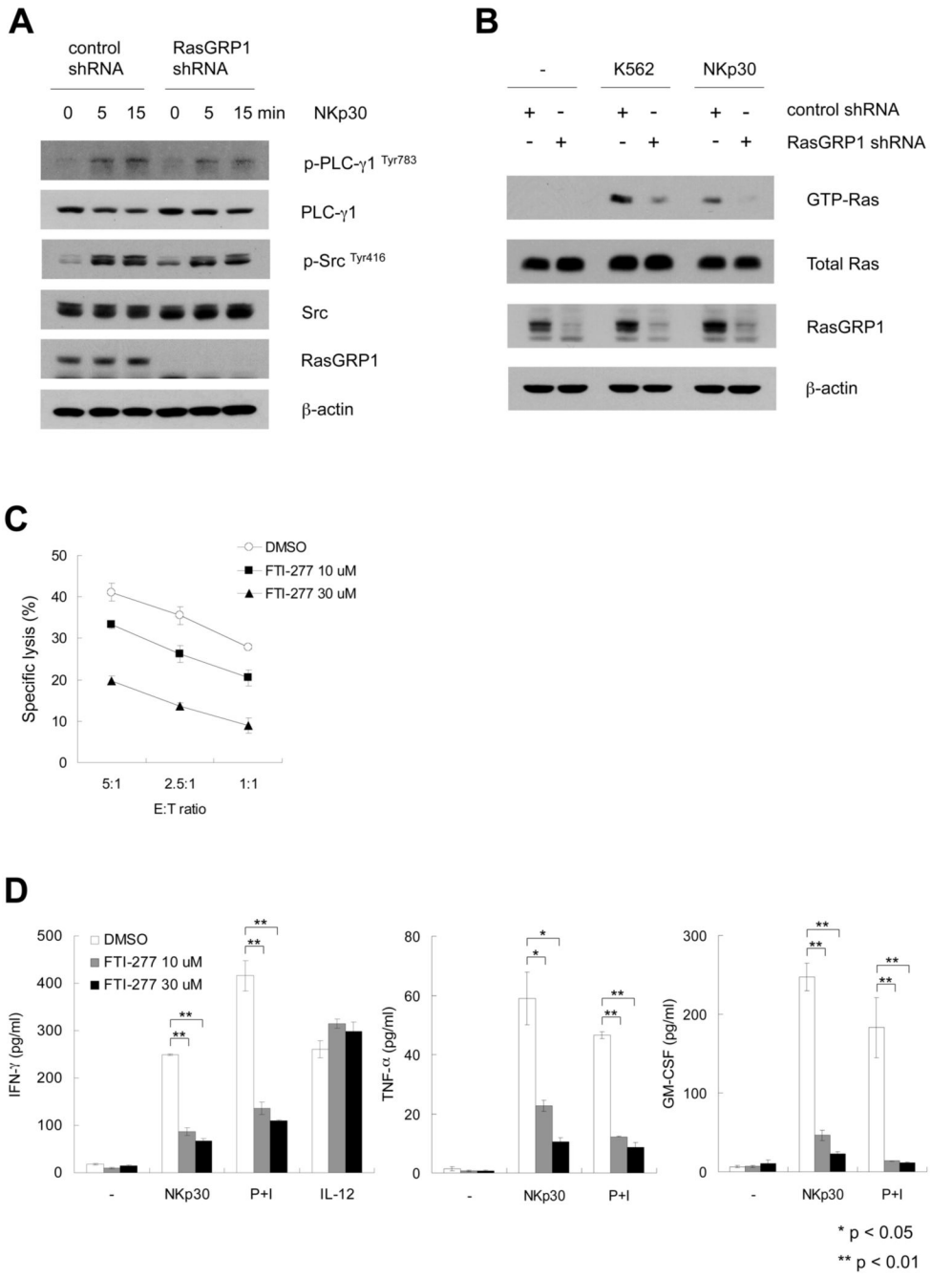

Figure 3. Reduced Ras activation in RasGRP1-knockdown NK cells

(A) Control shRNA- and RasGRP1 shRNA-expressing NK-92 cells were stimulated with plate-bound antibodies to NKp30 for the indicated times. Phosphorylation of PLC-γ1 and Src was detected by western blotting. (B) Control shRNA- and RasGRP1 shRNA-expressing NK-92 cells were pelleted with K562 cells or were stimulated with plate-bound antibodies to NKp30 for 5 min. Cell lysates were precipitated with GST-Raf-RBD, followed by western blotting with anti-pan-Ras antibody. (C) NK-92 cells were pretreated with FTI-277 (10 or 30 μM) for 30 min followed by incubation with ^{51}Cr-labeled K562 cells at the indicated E:T ratios. (D) IFN-γ, TNF-α, and GM-CSF were measured by ELISA using the supernatant obtained from FTI-277 (10 or 30 μM)-pretreated NK-92 cells that were subsequently stimulated by NKp30

(10 μg/ml) cross-linking or PMA (1 ng/ml)/ionomycin (0.1 μg/ml). The data are representative of two independent experiments, and the error bars represent the s.d. of triplicates (C) or duplicates (D).

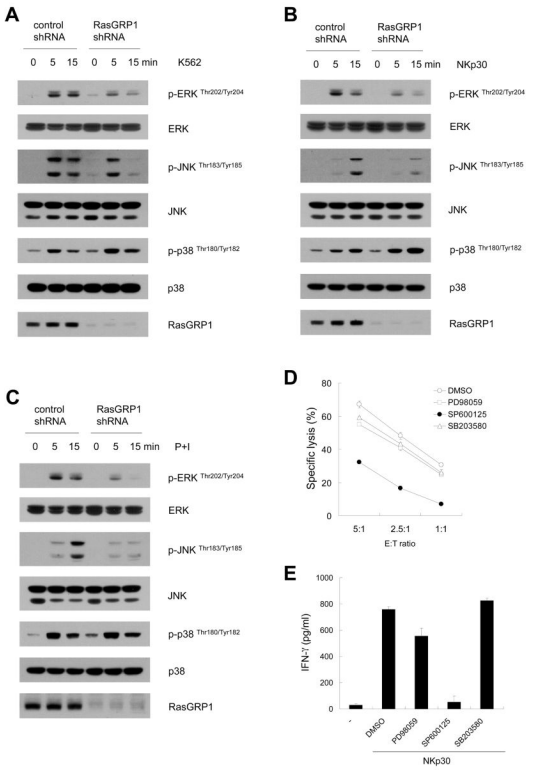

Figure 4. Impaired activation of ERK and JNK in RasGRP1-knockdown NK cells

Control shRNA- and RasGRP1 shRNA-expressing NK-92 cells (1×10^6 cells/sample) were stimulated with (A) K562 (1×10^5 cells/sample), (B) plate-bound antibodies to NKp30 (10 μg/ml), or (C) PMA (1 ng/ml)/ionomycin (0.1 μg/ml) for the indicated times. Phosphorylation of ERK, JNK and p38 was detected by western blotting. (D) Mature NK cells were pretreated with DMSO, the ERK inhibitor (PD98059; 10 μM), the JNK inhibitor (SP600125; 10 μM), or the p38 inhibitor (SB203580; 10 μM) for 30 min, followed by incubation with ^{51}Cr-labeled K562 cells at the indicated E:T ratios. (E) Mature NK cells were treated with DMSO, PD98059 (10 μM), SP600125 (10 μM) or SB203580 (10 μM) for 30 min before stimulation. Pretreated mature NK cells were stimulated with plate-bound antibodies to NKp30 (10 μg/ml). Secreted IFN-γ was quantified by ELISA. The data are representative of three independent experiments, and the error bars represent the s.d. of triplicates (D) or duplicates (E).

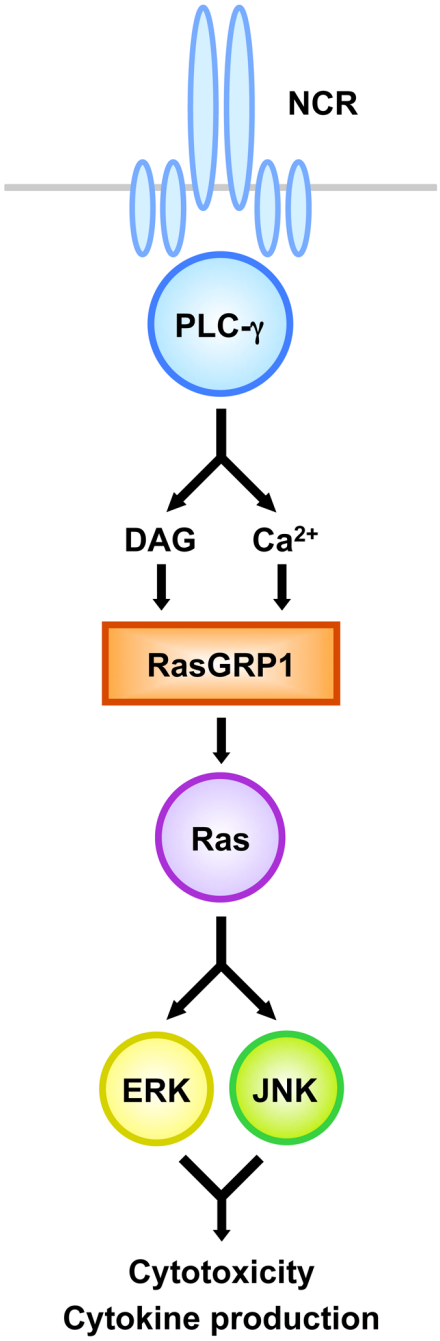

Figure 5. Proposed model of RasGRP1 in NK cell receptor signaling

Upon cross-linking of NK activating receptors, PLC-γ is activated and induces DAG and IP_3-dependent Ca^{2+}. DAG and Ca^{2+}-bound RasGRP1 interacts with and activates Ras, which regulates activation of ERK and JNK. Finally, activated ERK and JNK evoke NK cytotoxicity and cytokine production.