CD16-mediated p21^{ras} Activation Is Associated with Shc and p36 Tyrosine Phosphorylation and Their Binding with Grb2 in Human Natural Killer Cells

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Summary

The Src homology (SH) 2/SH3 domain–containing protein Grb2 and the oncoprotein Shc have been implicated in a highly conserved mechanism that regulates p21^{ras} activation. We investigated the involvement of these adaptor proteins in the signaling pathway induced by CD16 or interleukin (IL) 2R triggering in human natural killer (NK) cells. Both p46 and p52 forms of Shc were rapidly and transiently tyrosine phosphorylated upon CD16 or IL-2 stimulation with different kinetics. Shc immunoprecipitates from lysates of CD16- or IL-2–stimulated NK cells contained Grb2 and an unidentified 145-kD tyrosine phosphoprotein. Grb2 immunoprecipitates from anti-CD16–stimulated NK cells contained not only Shc, but also a 36-kD tyrosine phosphoprotein (p36). The interaction between Grb2 and Shc or p36 occurred via the Grb2SH2 domain as indicated by in vitro binding assays using a bacteriologically synthesized glutathione S-transferase–Grb2SH2 fusion protein. We also present evidence that p21^{ras} is activated by CD16 and IL-2R cross-linking. Accumulation of guanosine triphosphate–bound Ras was detected within 1 minute and occurred with kinetics similar to inductive protein tyrosine phosphorylation and Grb2 association of Shc and p36 adaptor proteins.

N K cells are a CD3$^-$CD16$^+$CD56$^+$ subset of lymphocytes that play a key role in nonadaptive immune response against microbial infections and tumor cell growth. They lyse a variety of target cells by direct cell-mediated cytotoxicity or in an Ab-dependent manner without prior sensitization, secrete several cytokines, and proliferate in response to IL-2 (1).

CD16, a major surface structure on NK cells capable of triggering their functional program, is the low-affinity receptor for the Fc fragment of IgG (FcγRIIIA) (2, 3). It exists as an oligomeric complex composed of one Fc-binding α chain associated with homo- or heterodimers of the ζ family proteins such as CD3ζ and the γ subunit of the high-affinity IgE receptor, FcεRI. FcγRIIIA occupancy by either immunocomplexes or specific mAb initiates a cascade of biochemical intracellular signals including protein tyrosine phosphorylation (4–9), phospholipase activation (6, 7, 10), inositol triphosphate generation, and intracellular Ca^{2+} mobilization (11).

Engagement of multimeric antigen receptor complexes on T and B lymphocytes and a number of cytokine receptors induce activation of the p21^{ras} signaling pathway (12). The conversion of Ras from its inactive GDP-bound state

to its active GTP-bound state is regulated by the opposite effects of proteins that stimulate the intrinsic rate of GTP hydrolysis (guanosine triphosphatase–activating proteins) and proteins that regulate the exchange rate of bound GDP for cytosolic GTP (guanine nucleotide exchange factors) (13). One of the exchange proteins involved in Ras activation by a number of receptors is Sos, a homologue of the Drosophila son of sevenless gene product (14), which is recruited to the cell membrane by the adaptor protein Grb2/Sem-5. Grb2, a 23-kD protein, comprises two Src homology (SH)[1] 3 domains that bind the COOH-terminal proline-rich domain of Sos and a SH2 domain that interacts with tyrosine phosphoproteins (15, 16). Shc is one of several substrates of protein tyrosine kinases (PTKs) interacting with Grb2. Shc exists in three isoforms of 46, 52, and 66 kD (only the former two are expressed by hemopoietic cells) and is composed of a single SH2 domain, a glycine/proline–rich collagen homology domain, and a unique NH$_2$-terminal domain (designated N) (17). It functions as a

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[1]Abbreviations used in this paper: GAM, goat anti–mouse IgG; GST, glutathione S-transferase; PTK, protein tyrosine kinase; SH, Src homology.

second adaptor molecule in Ras signaling via a number of receptors either endowed with tyrosine kinase activity (18–20) or capable of activating nonreceptor tyrosine kinase(s) (21–27). Phosphorylated Shc interacts with Grb2, which, in turn, interacts with Sos. In T and B cells, antigen receptor–mediated Ras activation is associated with tyrosine phosphorylation of Shc (21–24). However, a 36-kD membrane-bound phosphoprotein has also been proposed as an adaptor that links TCR-activated PTKs to Grb2–Sos complex (22, 28).

No information is currently available on the Ras signaling pathway in NK cells activated through CD16 ligation or other stimuli including IL-2, which activates Ras and induces a rapid formation of Shc–Grb2–Sos complexes in T cells (25, 26).

Here we investigated whether the p21^{ras} pathway is activated in human NK cells upon CD16 or IL-2 stimulation. In particular, we were interested in studying whether Grb2 complexes might be involved in Ras activation by examining the phosphotyrosine proteins associated with Grb2 after CD16 or IL-2R triggering. Our results indicate that activation of human NK cells through CD16 or by IL-2 results in a rapid accumulation of GTP-bound p21^{ras}. CD16-mediated Ras activation is accompanied by Grb2 association with both Shc and p36 tyrosine phosphoproteins, whereas only phosphorylated Shc forms a stable complex with Grb2 upon IL-2 stimulation.

Materials and Methods

Antibodies and Reagents. The following mouse mAbs were used. Anti-CD3 (Leu4), anti-CD16 (Leu11c), and anti-CD56 (Leu19) were purchased from Becton Dickinson & Co. (San Jose, CA). Anti-CD16 (B73.1) mAb was kindly provided by Dr. G. Trinchieri (Wistar Institute, Philadelphia, PA). Anti-CD56 (C218) mAb was generously provided by Dr. A. Moretta (University of Genoa, Genoa, Italy). Goat F(ab')$_2$ anti–mouse IgG Ab (GAM) was purchased from Cappel Laboratories (Cooper Biomedical Inc., Malvern, PA). Affinity-purified rabbit anti-Shc antiserum used in immunoprecipitation and anti-Grb2 mAb used in Western blotting were obtained from Transduction Laboratories (Lexington, KY). Anti-Shc mAb was kindly provided by Dr. P. G. Pelicci (University of Perugia, Perugia, Italy). The polyclonal antiserum against Grb2 used in immunoprecipitation was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). The antiphosphotyrosine (anti-pTyr) mAb 4G10 was purchased from Upstate Biotechnology, Inc. (Lake Placid, NY). Y13-259 (anti-Ras) mAb was a kind gift of Dr. S.A. Aaronson (The Mount Sinai Medical Center, New York). Human rIL-2 was provided by EuroCetus B.V. (Amsterdam, The Netherlands). Bacteria expressing either the glutathione S-transferase (GST)–Grb2SH2 fusion protein or GST alone were kindly provided by Dr. P.G. Pelicci.

Preparation of Human NK Cells. NK cell cultures were obtained by coculturing nylon nonadherent PMBC (4 × 10^5/ml) with irradiated (3,000 rad) RPMI 8866 cells (10^5/ml) for 10 d at 37°C in a humidified 5% CO$_2$ atmosphere as previously described (29). On day 10, the cell population was routinely 80–90% CD56$^+$CD16$^+$CD3$^-$, as assessed by cytofluorimetric analysis. When purity was <90%, contaminating T cells were further eliminated by panning or by rigorous immunomagnetic negative

selection with anti-CD5 mAb. The resulting NK cell population was >90% pure.

Cell Stimulation and Lysate Preparation. Highly purified cultured human NK cells (5 × 10^7/tube) were incubated with saturating concentrations of anti-CD16 (B73.1) or anti-CD56 (C218) mAb for 20 min on ice. After washing off unbound antibody, cells were resuspended in prewarmed RPMI medium, and GAM F(ab')$_2$ was added at a concentration of 2 μg/10^6 cells; IL-2 stimulation was performed by incubating the cells with 500 IU rIL-2 in 0.5 ml. Cells were lysed (lysis buffer containing 1% vol/vol Triton X-100, 50 mM Tris-HCl [pH 8], 150 mM NaCl, 5 mM EGTA [pH 8], 1.5 mM MgCl$_2$, 10% glycerol, 100 μg/ml PMSF, 1 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin, 200 μg/ml Na$_3$VO$_4$, and 50 mM NaF [pH 8]). Lysates were cleared of debris by centrifugation at 14,000 *g* for 20 min, and the supernatants were used for immunoprecipitation and affinity-binding experiments.

Immunoprecipitation and Immunoblotting. Cell lysates were immunoprecipitated with appropriate concentrations of polyclonal anti-Shc or anti-Grb2 antibody and protein A–Sepharose beads for 2 h at 4°C. The beads were washed four times in a mixture of 0.1% vol/vol NP-40, 50 mM Tris HCl (pH 8), 150 mM NaCl, 1 mM EDTA (pH 8), 200 μg/ml Na$_3$VO$_4$, separated on SDS-PAGE gels, and transferred to nitrocellulose filters. After blocking nonspecific reactivity, filters were probed with specific antibodies diluted in TBS-T (20 mM Tris HCl pH 7.8, 150 mM NaCl, 0.02% Tween 20) containing 1% BSA. After extensive washing, immunoreactivity was detected using an enhanced chemiluminescence kit (Amersham International plc, Amersham, UK). In some experiments, blots were stripped with 62.5 mM Tris-HCl (pH 6.8), 2% SDS, and 100 mM β-ME at 50°C for 30 min and reprobed.

In Vitro Binding Studies Using GST–Grb2SH2 Fusion Protein. Recombinant GST or GST–Grb2SH2 fusion protein was purified onto glutathione Sepharose 4B (Pharmacia LKB, Uppsala, Sweden) and used for binding assays. NK cell lysates (30 × 10^6/ sample) were incubated for 2 h at 4°C with an appropriate concentration of either GST or GST fusion protein bound to glutathione Sepharose 4B. Protein complexes were washed eight times in ice-cold lysis buffer, eluted, denatured by heating at 95°C for 3 min in reducing Laemmli buffer, and subjected to SDS-PAGE and Western Blot analysis.

p21^{ras} Activation. p21^{ras} was immunoprecipitated with Y13-259 mAb from cells in which guanine nucleotides were biosynthetically labeled with [^{32}P]orthophosphate as described (30). Ras-bound labeled guanine nucleotides were eluted, separated by TLC, and then quantitated by direct scanning for β radiation using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA). Results are expressed as the percentage of p21^{ras} proteins bound to GTP relative to total guanine nucleotides complexed to the protein.

Results

Shc Proteins Are Tyrosine Phosphorylated upon CD16 and IL-2R Stimulation in Human NK Cells. CD16 cross-linking results in the tyrosine phosphorylation of several cellular substrates (4–9). To investigate whether Shc is phosphorylated on tyrosine residues upon NK cell stimulation, cultured human NK cells were stimulated with rIL-2 or saturating concentrations of anti-CD16 (B73.1) or anti-CD56 (C218) mAb and cross-linked with GAM F(ab')$_2$ fragments for the indicated times at 37°C. Shc proteins

were immunoprecipitated from the cell lysates with a poly-clonal anti-Shc antiserum, and Shc immunoprecipitates were analyzed for the presence of tyrosine phosphoproteins by immunoblotting with an antiphosphotyrosine (anti-pTyr) mAb. Marked tyrosine phosphorylation of both the 46- and 52-kD isoforms of Shc was detected after CD16 cross-linking (Fig. 1 B). CD16-mediated Shc phosphorylation was rapid, with a peak at 1 min and a decline at 5 min. IL-2 also stimulated a rapid tyrosine phosphorylation of both p46 and p52 isoforms of Shc, but unlike CD16, phosphorylation was still observed 10 min after stimulation. No changes in the phosphorylation status of Shc were observed in NK cells treated with anti-CD56 mAb used as control. After stripping off bound antibodies, the same membrane was incubated with anti-Shc mAb to verify that an equivalent amount of Shc proteins was present in all lanes. Samples stimulated with anti-CD16 mAb or IL-2 showed a small shift of both Shc isoforms to a lower electrophoretic mobility due to induced phosphorylation (Fig. 1 D).
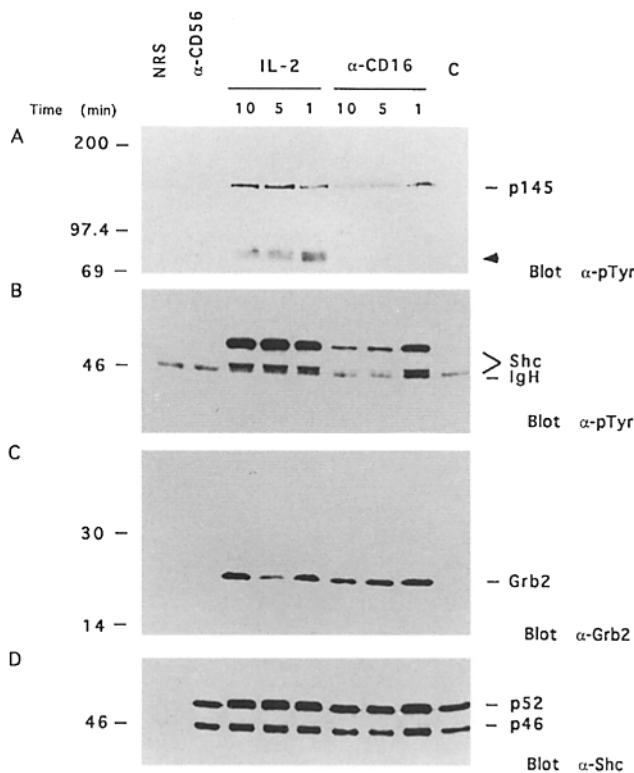

Figure 1. CD16 and IL-2R stimulation induce the tyrosine phosphorylation of Shc and its association with Grb2 and a 145-kD tyrosine phosphoprotein: time course. Cultured NK cells were stimulated with control medium or appropriate doses of rIL-2, anti-CD16 (B73.1), or anti-CD56 (C218) mAb for the indicated times at 37°C. Cell lysates (50 × 10⁶ cells/sample) were subjected to immunoprecipitation with polyclonal anti-Shc or preimmune serum (NRS). Resulting protein complexes were resolved by 10% SDS-PAGE, transferred to nitrocellulose, and immunoblotted with either anti-pTyr (A and B), anti-Grb2 (C), or anti-Shc (D) antibodies. All panels are from the same experiment and from different regions of the same blot. In A, the arrowhead indicates the IL-2Rβ chain. IgH denotes the heavy chain of the precipitating Ab. Sizes are indicated in kilodaltons. These results are representative of one out of three independent experiments.

Interestingly, an additional unidentified tyrosine-phosphorylated protein of ~145 kD consistently coimmuno-precipitated with Shc from CD16- or IL-2–stimulated NK cells lysates; tyrosine phosphorylation of Shc and p145 occurred with the same kinetics (Fig. 1 A). The band migrating at ~70 kD in the immunoprecipitates from IL-2–activated NK cell lysates likely corresponds to IL-2Rβ chain, as previously reported (26); the background band migrating at 46 kD is the IgH chain of the antibody used for immunoprecipitation.

In light of the evidence showing that the SH2 domain–containing adaptor protein Grb2 binds tyrosine-phosphorylated Shc (15, 18), we then investigated whether this complex is also formed in activated NK cells. CD16 or IL-2R triggering of NK cells resulted in Grb2–Shc association, as shown by immunoblotting of Shc precipitates with an anti-Grb2 antibody (Fig. 1 C).

These observations indicate that NK cell stimulation through CD16 and IL-2R induces tyrosine phosphorylation of Shc and its association with Grb2.

A 36-kD Tyrosine Phosphoprotein Coimmunoprecipitates with Grb2 in CD16-stimulated NK Cells. Immunoprecipitation of Grb2 from cell lysates of untreated, anti-CD16-, or IL-2–stimulated NK cells was performed to visualize the interactions between Grb2 and molecules that become tyrosine phosphorylated after NK cell activation. Associated molecules were separated by SDS-PAGE, blotted on nitrocellulose filters, and probed with anti-pTyr mAb. As shown in the upper portion of Fig. 2, Grb2 coimmunoprecipitates a strongly tyrosine-phosphorylated protein of ~36 kD in lysates of CD16- but not IL-2–stimulated NK cells. Tyrosine phosphorylation of the 36-kD protein and its association with Grb2 occurred very rapidly, peaking at 1 min and decreasing significantly at 5 min after CD16 ligation. As expected, Grb2 immunoprecipitates of stimulated NK cells also contained the 46- and 52-kD isoforms of phosphorylated Shc as identified by anti-Shc immunoblotting (data not shown). The faint band migrating at ~46 kD, observed in the unstimulated sample, was not reactive with anti-Shc antibody, suggesting that it was the IgH of the immunoprecipitating antibody. The anti-CD56–treated sample is comparable to the untreated sample (not shown). Immunoblot of Grb2 immunoprecipitates with an anti-Grb2 antibody shows that an equivalent amount of Grb2 was present in all lanes (Fig. 2, *bottom*).

To establish whether the p36 phosphoprotein detected in the Grb2 complex was directly bound to Grb2, we attempted to immunodeplete Shc and determine whether p36 was still associated with Grb2 under these conditions (Fig. 3). After complete depletion of Shc from cell lysates, the intensity of tyrosine-phosphorylated p36 protein in anti-Grb2 immunoprecipitates was unaffected, suggesting that p36 directly interacts with Grb2.

The SH2 Domain of Grb2 Complexes with p36 or Shc Tyrosine Phosphoproteins. The Grb2 molecule binds to several phosphotyrosine-containing proteins via the SH2 domain (15, 16, 18). Moreover, a 36-kD tyrosine-phosphorylated protein has been reported to complex with the Grb2SH2
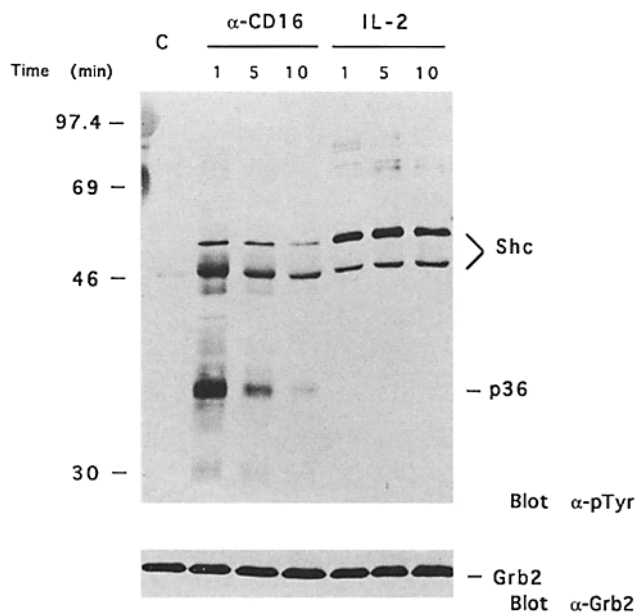
Figure 2. Analysis of phosphotyrosine proteins in the immunoprecipitates of Grb2 after CD16 or IL-2R stimulation. Lysates from 50×10^6 cultured NK cells stimulated with control medium or appropriate concentrations of rIL-2 or anti-CD16 (B73.1) mAb for the indicated times at 37°C were immunoprecipitated with polyclonal anti-Grb2 antibody. Resulting protein complexes were resolved by 10% SDS-PAGE, transferred to nitrocellulose, and immunoblotted with either anti-pTyr (*upper panel*) or anti-Grb2 (*lower panel*). The panels are from the same experiment and from different regions of the same blot. Shc bands were identified by reprobing the blot with anti-Shc antibody (data not shown). Sizes are indicated in kilodaltons. These results are representative of one out of three independent experiments.

Figure 3. 36-kD tyrosine-phosphorylated protein interacts directly with Grb2. Cultured NK cells were stimulated with control medium or appropriate doses of rIL-2 and anti-CD16 (B73.1) mAb for 2 min at 37°C. Cell lysates (50×10^6 cells/sample) were, when indicated, depleted of Shc proteins by two subsequent cycles of immunoprecipitation with anti-Shc antibody and immunoprecipitated with anti-Grb2 antibody or normal rabbit serum (*NRS*). Immunoprecipitates were subjected to 8% SDS-PAGE, transferred to nitrocellulose, and blotted with anti-pTyr antibody. IgH denotes the heavy chain of the precipitating Ab. Sizes are indicated in kilodaltons. These results are representative of one out of two independent experiments.

domain in TCR-activated T cells (22, 28). To test whether the interaction of p36 and Shc phosphoproteins with Grb2 is mediated through the SH2 domain, lysates from untreated, CD56-, CD16- or IL-2–stimulated NK cells were incubated with a bacterially expressed Grb2SH2 fusion protein immobilized on glutathione–agarose beads, and the bound phosphotyrosine proteins were analyzed. The results, shown in Fig. 4, indicate that the SH2 domain of Grb2 in vitro binds both tyrosine-phosphorylated Shc and p36 in CD16-stimulated NK cells and only phosphorylated Shc in IL-2–stimulated NK cells. The control GST alone failed to precipitate any tyrosine-phosphorylated proteins.

These results indicate that the association between tyrosine-phosphorylated p36 or Shc and the adaptor molecule Grb2 occurs via the interaction with the SH2 domain of Grb2.

CD16 and IL-2R Triggering Activate p21^{ras} in Human NK Cells. Grb2 complexes containing a p36 phosphoprotein or phosphorylated Shc have been implicated in the activation of the p21^{ras} guanine nucleotide-binding cycle in TCR- (28) or IL-2–stimulated human T cells (25, 26). To determine whether p21^{ras} is activated upon CD16 or IL-2R triggering in NK cells, p21^{ras}/GTP loading experiments were performed. NK cells were exposed to anti-CD16 and anti-CD56 mAbs, IL-2, or the phorbol ester PMA, which has been shown to activate Ras in a PKC-dependent man-
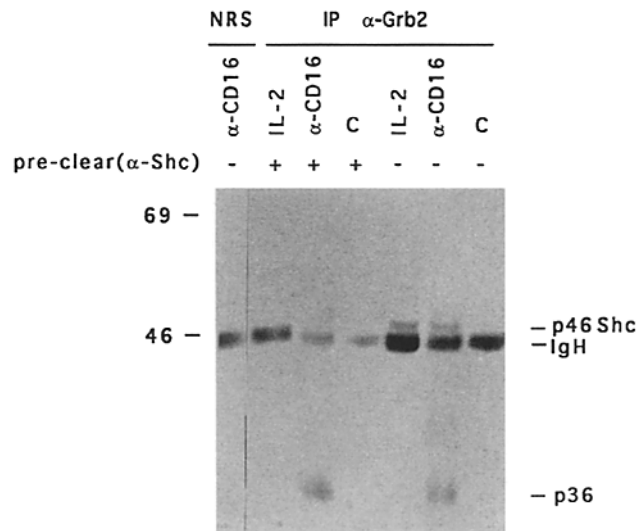
ner in T lymphocytes (30). [^{32}P]Orthophosphate-loaded cells were lysed and p21^{ras} was immunoprecipitated. The proportion of p21^{ras}-bound GTP, expressed as the ratio between GTP and GTP plus GDP on p21^{ras}, was evaluated by emission scanning of TLC-resolved guanine nucleotides eluted from p21^{ras} immunoprecipitates. Fig. 5 shows that
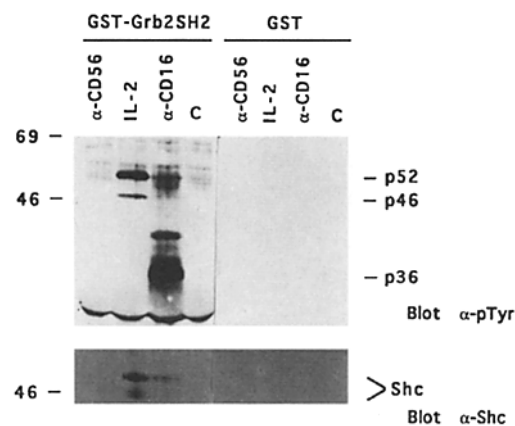

Figure 4. In vitro association of the 36-kD and Shc tyrosine phosphoproteins with the SH2 domain of Grb2. Cultured NK cells were stimulated with control medium or appropriate doses of IL-2, anti-CD16 (B73.1), or anti-CD56 (C218) mAbs for 2 min at 37°C. Cell lysates (30×10^6 cells/sample) were incubated with GST alone or GST–Grb2SH2 fusion protein bound to glutathione–agarose beads. The resulting protein complexes were resolved by 8% SDS-PAGE and immunoblotted with anti-pTyr antibody (*upper panel*). The blot was stripped and reprobed with anti-Shc antibody (*bottom panel*). Sizes are indicated in kilodaltons. These results are representative of one out of four independent experiments.
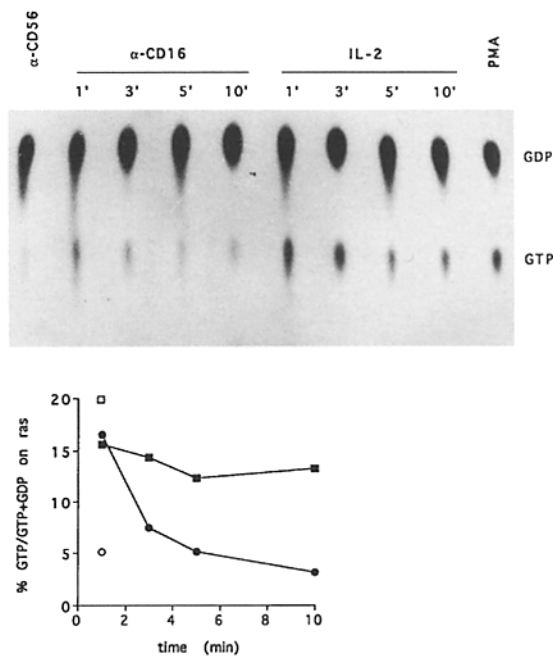
Figure 5. Kinetics of p21^{ras} activation by triggering via CD16 and IL-2R. TLC of the nucleotides eluted from p21^{ras} immunoprecipitates of [^{32}P]orthophosphate-labeled NK cells (*upper panel*). Cells were stimulated for the indicated times with appropriate doses of anti-CD56 (C218) and anti-CD16 (B73.1) mAbs, rIL-2, or PMA (100 ng/ml) before lysis and immunoprecipitation of p21^{ras} with Y13-259 mAb as described (30). The position at which GTP and GTP standards run is indicated. The lower panel shows the data derived from quantitation by direct scanning for β radiation of the same experiment. C218 (*open circles*), B73.1 (*solid circles*), PMA (*open squares*), IL-2 (*solid squares*). These results are representative of one out of three independent experiments.

CD16 triggering results in a threefold increase in the proportion of GTP-bound Ras (from 5 to 17%), an increase similar to that observed after IL-2 stimulation. PMA also resulted in accumulation of GTP-bound Ras, suggesting that p21^{ras} can be activated in NK cells via a PKC-dependent pathway.

The time course of CD16- or IL-2–mediated p21^{ras} activation strictly paralleled the induction of Shc and p36 tyrosine phosphorylation and Grb2-containing complex formation; upon CD16 cross-linking, Ras activation peaked at 1 min and returned to basal levels within 5 min, whereas it persisted until 10 min after IL-2R stimulation. The anti-CD56 control mAb–treated sample shows a p21^{ras} GTP/GDP+GTP ratio comparable to the untreated sample (not shown).

Discussion

Many recent studies have indicated the SH2/SH3 domain–containing protein Grb2 in the coupling of PTK activation with the Ras signaling pathway in mammalian cells (15). In this study, we investigated the possible role of Grb2 in CD16- and IL-2R–mediated activation of p21^{ras} in human NK cells. Our findings show that CD16 ligation results in a rapid tyrosine phosphorylation of both p46 and

p52 isoforms of the Shc protein. IL-2R stimulation also induces Shc phosphorylation in NK cells, as previously described for T cells (25, 26). IL-2–induced tyrosine phosphorylation of Shc occurs at levels comparable to those observed upon CD16 ligation, although it is more persistent, remaining at about the same levels 10 min after cytokine stimulation.

Shc tyrosine phosphorylation correlates with the formation of complexes containing Grb2 and an unidentified tyrosine-phosphorylated protein of 145 kD. Phosphoproteins of a similar molecular mass (pp145) have been reported to associate with Shc in T, B, and myeloid cells in response to several stimuli (21, 24, 27). The binding domain for pp145 (designated PTB domain) has recently been mapped in the NH$_2$ terminus of the Shc protein (31).

In contrast to the rapid Shc phosphorylation observed in CD16-stimulated NK cells, TCR stimulation by itself induces only low levels of Shc tyrosine phosphorylation and Grb2 association, and CD4 coengagement is required to potentiate Shc phosphorylation (21). Similarly, BCR-induced Shc tyrosine phosphorylation is markedly enhanced by CD19 receptor coligation in B cells (32). The molecular basis of this phenomenon is still poorly understood. It is possible that CD4 and CD19, which are physically and functionally coupled to Src-family PTKs (33–35), might augment Shc tyrosine phosphorylation elicited by the antigen receptors by recruiting Src-family kinases in their proximity. Several lines of evidence indicate that Shc may be a good substrate for Src-family PTKs: first, p56^{lck} is detected in Shc immunoprecipitates after CD4 cross-linking (36) and phosphorylates Shc fusion protein in vitro (37); second, Src SH3 domain binds to the proline-rich domain of Shc (38); and finally, Shc is constitutively phosphorylated on tyrosine residues in cells expressing v-Src or v-Fps and forms a complex with Grb2 (39). It is conceivable, therefore, that the lack of requirement for coreceptor engagement in the stimulation of Shc phosphorylation upon CD16 and IL-2R ligation may be attributable to the constitutive coupling of these receptors to Lck (40, 41).

In addition to Shc, we detected a major 36-kD tyrosine-phosphorylated protein interacting with Grb2 upon CD16 stimulation. The interaction between p36 and Grb2 occurred very rapidly after CD16 stimulation with a kinetics similar to that of Grb2–Shc association. p36–Grb2 complex formation appears to be receptor specific, as it was not detected in IL-2–stimulated NK cells. Furthermore, interaction between Grb2 and p36 or Shc phosphoproteins is mediated through the SH2 domain of Grb2 and is likely induced by Shc and p36 phosphorylation. As individual SH2 domains interact with only one binding pattern, our results suggest that two populations of Grb2 exist in CD16-stimulated NK cells, one that is complexed with Shc and the other with p36.

Recent reports (22, 28) describe a membrane-bound 36-kD phosphoprotein that binds to the SH2 domain of Grb2 in TCR-stimulated T cells that appears to be identical to the 36-kD phosphoprotein described here. p36 is one of the major PTK substrates in T cells and may also associate

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with the SH2 domain of PLC-γ (22, 42). No evidence of p36 tyrosine phosphorylation or association with Grb2 has been found in B lymphocytes upon BCR stimulation (23, 24, 32). In view of the possible common origin of T and NK cells (43, 44), it would be of interest to investigate whether p36 expression or its tyrosine phosphorylation and consequent Grb2 association is restricted to these two lineages.

These results also show that both CD16 ligation and IL-2R stimulation induce a rapid accumulation of $p21^{ras}$–GTP complexes in human NK cells. The kinetics of Ras activation strictly parallel that of Shc and p36 phosphorylation and Grb2 association observed upon CD16 and IL-2R triggering. The role of Grb2 in $p21^{ras}$ activation is further supported by the finding of Grb2-Sos complex in NK cells (data not shown).

Taken together, our results indicate that two different mechanisms might couple CD16 to the Ras pathway in NK cells: phosphorylation of Shc, which has been implicated in both receptor tyrosine kinase– and non–receptor tyrosine kinase–induced Ras activation (18–20, 25, 26), and p36, which has been proposed to be involved in TCR-mediated $p21^{ras}$ activation (22, 28). It is possible that Shc and p36 represent additive complementary pathways that couple CD16 to $p21^{ras}$. On the other hand, one of these pathways could be dominant and the other redundant. The existence of more than one Grb2-Sos complex potentially involved in $p21^{ras}$–GTP formation has been described for several growth factor receptors, including EGFR and insulin receptor. For these receptors, Shc has been found to be the major molecular connection between the receptor and the Grb2-Sos-$p21^{ras}$ signaling cascade (45, 46). To better

understand the relative role of Shc–Grb2–Sos and p36–Grb2–Sos complexes in mediating the CD16-induced increase in cellular $p21^{ras}$–GTP, a further characterization of p36 is required.

The function of $p21^{ras}$ in NK cells remains unclear. Cross-linking of both CD16 and IL-2R delivers intracellular signals that trigger the functional program of NK cells. Stimulation of NK cells through CD16 or by IL-2 promotes expression of genes encoding for cytokines such as IFN-γ, TNF-α, GM-CSF, or activation markers including IL-2Rα chain and CD69 (11, 47, 48). In addition, IL-2 is the major cytokine capable of sustaining NK cell growth (1).

The role of $p21^{ras}$ in downstream biologic responses to ligand has been addressed in several systems. Several lines of evidence suggest that $p21^{ras}$ acts ubiquitously in signal transduction pathways leading to cellular proliferation and differentiation (49, 50). The transmission of signals from $p21^{ras}$ to the nucleus involves the activation of mitogen-activated protein kinases as the extracellular signal–regulated (ERKs) and Jun kinases, which have several transcriptional factors as main targets (51).

In T lymphocytes, a role of $p21^{ras}$ in the regulation of IL-2 gene and CD69 expression has been demonstrated (52, 53). Ras regulation of other cytokine genes has also been proposed (54). Preliminary results indicate that CD16 ligation induces activation of p42 and p44 ERK in human NK cells (Gismondi, A., and A. Santoni, unpublished observations). It is conceivable, therefore, that $p21^{ras}$ activation induced upon IL-2R and CD16 stimulation on NK cells plays a major role in the control of their proliferation and functions.

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