ADP-ribosylation of the ras-related, GTP-binding Protein RhoA Inhibits Lymphocyte-mediated Cytotoxicity* (Received for publication, February 24, 1992)

Paul Langé, Lamia Guizani, Isabelle Vitté-Mony, Rodica Stancou, Olivier Dorsuelj, Gérard Gacoin, and Jacques Berthoglio†

From the Institut National de la Santé et de la Recherche Médicale (INSERM), Unité 333, Institut Gustave Roussy, 94805 Villejuif, and INSERM Unité 257, Institut Cochin de Génétique Moleculaire, 75014 Paris, France

The Rho proteins are identified as a subgroup of the Ras superfamily of low molecular weight GTP-binding proteins. We have studied the expression of these proteins in human cytotoxic natural killer cells and found that RhoA is the most abundantly expressed member of the Rho family. The Rho proteins are specific substrates for ADP-ribosylation catalyzed by the C3 exoenzyme from Clostridium botulinum. We report here that introduction of recombinant C3 in electroporemediated normal killer cells or in cytotoxic T lymphocytes resulted in a dose-dependent inhibition of their cytolytic function. Furthermore, a single substrate is efficiently ADP-ribosylated by C3 in extracts from cytotoxic cells. Biochemical analysis indicates that this substrate is RhoA, and subcellular fractionation experiments demonstrate that it is essentially present in the cytosol of the cells. Western blot analysis, however, revealed that a small proportion of the Rho protein can be found associated with the cell membrane as well as with the cytotoxic granules. These results indicate that the low molecular weight GTP-binding protein RhoA is present in cytotoxic lymphocytes and plays a critical role in cell-mediated cytotoxicity.

Lympohocyte-mediated cytotoxicity is an essential function of the immune system which mediates elimination of virus-infected and of tumor cells. Two types of cytotoxic effector lymphocytes are known: (a) the CD8-positive cytotoxic T lymphocytes (CTL), which express a polymorphic T cell receptor and recognize target cell antigens in the context of MHC class I, and (b) natural killer (NK) cells, which do not possess a defined receptor and bind to their targets through adhesion molecules. Target cell recognition by cytotoxic cells triggers transmembrane signaling pathways that result in exocytosis of cytolytic granules (1). This study was undertaken to investigate the possible role of the rho gene products in the mechanisms of cytotoxicity. The rho and rac gene products belong to the p21^superfamily of GTP-binding proteins (2, 3), whose cellular functions are just being discovered. Recent work has contributed to implicate several low molecular weight (LMW) GTP-binding proteins in intracellular traffic of vesicles, exocytosis and cytoskeleton organization in yeast and various mammalian cell types (4-8). Little information is however available about the cellular functions of ras-related G-proteins in lymphocytes. Recently, p21^superfamily has been shown to be activated during T cell receptor-mediated signal transduction (9). In addition, expression of the mRNA for Rac2, a hemopoietic restricted LMW G-protein of the Rho subfamily is increased during mitogenic activation of human T lymphocytes (10). Recent investigations performed in neutrophils suggest that the Rac1 and Rac2 proteins may participate in the formation of the NADPH oxidase complex, whereas a 24-kDa G-protein related to Rho may control cell motility (11-13). GTP-binding proteins of the Rho subfamily are the only reported substrates for the C3 exoenzyme, an ADP-ribosyl transferase produced by Clostridium botulinum, which results in alteration of the G-protein function (14-16). We have used this unique tool to investigate the function of the Rho proteins in cytotoxic lymphocytes. We report here that lymphocyte-mediated cytotoxicity can be blocked by introducing the C3 exoenzyme into intact permeabilized effector cells, suggesting that cytotoxicity is one of the cellular functions that is somehow controlled by the LMW GTP-binding protein RhoA.

EXPERIMENTAL PROCEDURES

Cell Cultures and Cytotoxicity—All cultures were performed in RPMI 1640 medium supplemented with 10% fetal calf serum, glutamine, and antibiotics. The YT line is a transformed, NK-like cell line established from a leukemic patient (17). The K562 and Daudi cell lines were used as targets for NK cell cytotoxicity. NK cells were purified from normal blood donor peripheral lymphocytes (PBL) using a multistep negative selection procedure. Briefly, non-adherent PBL were depleted of the majority of T cells by rosetting with sheep red blood cells at 29°C for 1 h (18). Enriched NK cell preparations were recovered from the interface of a Ficoll-Hypaque gradient and further purified by incubating with a mixture of monoclonal antibodies against T and B cell antigens (including anti-CD3, CD4, CD5, and -MHC class II). After two washes, cells were incubated with anti-mouse Ig-coated magnetic beads and passed over a magnet. The purity of the negatively selected population was assessed by flow cytometry, and cells were activated or expanded in the presence of human recombinant interleukin-2 (IL-2) at 100 units/ml in complete culture medium. IL-2-dependent NK cell lines established in this way were used after 3-6 weeks of growth. A cytotoxic T cell (CTL) line was generated in one-way secondary mixed lymphocyte culture between peripheral blood lymphocytes from a normal donor and the HLA-incompatible, Epstein-Barr virus-transformed B cell line 2145 in the presence of IL-2 and recombinant interleukin-4 (50 ng/ml). The resulting allospecific CTL line did not kill unrelated Epstein-Barr virus-transformed B cells or NK targets. Cytotoxicity was assayed in a conventional 4-h 51Cr release assay at multiple effector:target cell ratios against the appropriate target cells, and data converted into lytic units (LU) as previously described (19).

Northern Blot Analysis—Total cellular RNA was extracted using a modified guanidinium procedure (20). RNA (10-15 µg) were elec-
trophoresed through 1% agarose in the presence of formaldehyde and transferred to nylon filters. cDNA probes for rhoA, rhoB, and rhoC (21) were kindly provided by B. Olofsson (INSERM Unité 248, Paris). Obtention of the rac2 cDNA and probe have been described previously (10). The probes were labeled with [32P]dCTP by random-primed synthesis and hybridized at 65 °C in standard solution. The same filter was used throughout, with dehybridization by boiling in 0.1% SDS in between different probes. Films were exposed for 1-3 days.

**Electropermeabilization**—Cells were electroporated using a Celect apparatus (Eurogentec, Belgium) generating a decaying exponential waveform double electric pulse (800 V, 100 microfarads for 1.7 ms, and then 200 V, 1500 microfarads for 66 ms) with highly purified recombinant C3 exoenzyme (kindly provided by P. Bosquet, Pasteur Institute, Paris). Cells were allowed to recover membrane integrity for 1 h at 37 °C in culture medium, and viability was assessed by trypan blue exclusion.

**ADP-ribosylation**—*In vitro* ADP-ribosylation was performed essentially as described (22). Briefly, cell lysates were prepared by freeze-thawing in 20 mM Hepes (pH 8), and the post-nuclear fraction (10 μg of proteins) was ADP-ribosylated using 50 ng/ml recombinant C3 and 5.10^8 cpm [32P]NAD (30 Ci/mmol, Du Pont-New England Nucl.) in ADP-ribosylation buffer (20 mM Hepes, pH 8, 1 mM MgCl₂, 1 mM AMP, and 15 mM thymidine). After 30 min at 37 °C, the reaction was stopped by addition of an equal volume of 2× sample buffer for SDS-PAGE or two-dimensional gel analysis. For two-dimensional gels, samples were first separated by isoelectric focusing (ampholine range 3.5-10) and then by SDS-PAGE on 15% acrylamide gel according to standard procedures. After drying, gels were autoradiographed for 3-4 days.

**Subcellular Fractionation**—10 cells were disrupted in a Dounce homogenizer in relaxation buffer (100 mM KC1, 3 mM NaCl, 3.5 mM MgCl₂, 1 mM ATP, and 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin as protease inhibitors) and briefly cleared from debris by centrifugation (5 min, 12,000 rpm). The supernatant was then centrifuged for 2 h in a Beckman SW 41 rotor at 35,000 rpm on a discontinuous gradient consisting of 40, 50, and 60% sucrose in GTP-binding buffer (23). Plasma membranes were recovered at the top of the gradient, and cytoxic granules, containing perforin as identified by Western blotting, sedimented at the 40-50% sucrose interface. These particulate fractions were washed and recovered by ultracentrifugation, and the cytoxic fraction was dialogized against 20 mM Hepes buffer, pH 8.

**GTP-binding and Western Blot Analysis**—Proteins (50 μg) from total cell lysates or from subcellular fractions were separated by SDS-PAGE and electroblotted onto nitrocellulose (0.1-μm pore size, Schleicher & Schuell, Dassel, Germany). For GTP-binding analysis, the blot was washed for 30 min in GTP-binding buffer (50 mM Tris, pH 7.5, 1 mM EGTA, 5 mM MgCl₂, 0.5% Tween 20), then incubated for 1 h with 10 μCi/ml [3H]GTP (3000 Ci/mmol, Amersham, U. K.) in the same buffer. After 3 washes, the blot was allowed to dry and autoradiographed for 16 h. Western blot analysis was performed on the same blot, using antibodies to the Rho proteins raised in rabbits by immunization with a synthetic peptide corresponding to residues 124-146 of the human RhoA amino acid sequence coupled to keyhole limpet hemocyanin. Antibodies were affinity-purified on a peptide-CNBr-Sepharose column, and specifically recognize the three Rho proteins A, B, and C in Western blot analysis (to be described elsewhere). Following saturation for 3 h in 5% nonfat dry milk in PBS, 1% Tween 20, nitrocellulose membranes were incubated in sequential washes with affinity purified anti-Rho antibodies and peroxidase-labeled swine antirabbit Ig. The blots were developed using an enhanced chemiluminescence detection system (ECL, Amersham) and the film were exposed for 2-5 min.

**RESULTS AND DISCUSSION**

We have observed a high expression of mRNA for rhoA in normal, human cytoxic natural killer (NK) cells grown in IL-2-supplemented medium as well as in the transformed NK-like cell line YT (Fig. 1). In contrast, rac2 is expressed at a low level, and we have so far been unable to detect mRNA for rhoB and rhoC by conventional Northern blot hybridization (not shown), suggesting that these proteins, if present at all, are not abundantly expressed.

To test for a possible involvement of these ras-related proteins in the cytotoxic function, we took advantage of the specificity of Clostridium botulinum C3 exoenzyme, which selectively ADP-ribosylates the Rho proteins at an asparagine residue (Asn-41) located within or close to the putative effector domain of the molecule (24, 25). As C3 does not penetrate the lymphocyte membrane spontaneously, we introduced it inside the cells, using an electropermeation technique under controlled conditions where over 75% of the cells are able to functionally recover from the electric shock. When NK cell lines, which are cytolytic for the K562 and Daudi targets, are treated in this way in the presence of various concentrations of recombinant C3, a dramatic decrease in their cytotoxicity is observed (Fig. 2, a and b) as compared with untreated or sham controls. This inhibition, in several experiments using different NK effector cell lines, averaged 60-70% at the highest dose of C3 tested (4 μg/ml). The effects of C3 are dose-dependent and non-lethal. Morphological changes, however, occurred, and the cells assumed a round and regular shape (not shown). Similar inhibition of cytotoxicity was observed using an allogeneic cytotoxic T cell line generated against Epstein-Barr virus-transformed B cells (Fig. 2c). Because the amount of C3 which actually penetrates the cells cannot be controlled, its in vivo effect was assessed indirectly, by measuring substrate which remained available for in vitro [32P]ADP-ribosylation by C3 in extracts of C3-treated or control NK cells. Fig. 2d evidences a dose-dependent reduction in substrate availability which correlates with functional inhibition.

The mammalian rho subfamily is comprised of at least five identified gene products, including the two Rac proteins and the three Rho proteins (25). We thus attempted to identify which of these potential C3 substrates were present in NK cells. Fig. 3 shows a representative two-dimensional gel analysis of [32P]ADP-ribosylated protein extracts from NK cells and from the YT cell line. A single 32P-labeled protein spot could be identified (even when the film was heavily overexposed, not shown), migrating between pH 5.8 and 6.2 in the first dimension and with an apparent molecular mass of 22 kDa. This pattern of migration is clearly different from that of the Rac proteins (pl 7.5 and 8.5) (26) and identifies this protein as being most probably RhoA (pl 6.2, based on its amino acid sequence, as opposed to 6.8 for RhoC and 5.3 for...
The RhoA Protein Is Involved in Cell-mediated Cytotoxicity

was assayed in a conventional, their specific targets, the 2145 B-cell line, were used. Cytotoxicity expressed in lytic units (LU) per 10^6 effector cells (26). LU at effector:target cell ratios against the appropriate target cells, and data concentrations. c, same as table material was counted and expressed as cpm/5 l.

Ribosyl transferase. NAD was performed on the post-nuclear fractions obtained from control or C3-treated cell lysates as described under "Experimental Procedures." Incorporation of [^32P] into trichloroacetic acid-precipitable material was counted and expressed as cpm/5 µg proteins.

FIG. 2. Inhibition of lymphocyte cytotoxicity by C3 ADP-ribosyltransferase. a and b, lytic activity of human NK cell lines against K562 (a) and Daudi (b) targets, either untreated (CTRL) or following electroporation in the absence (SHAM) or in the presence of C. botulinum recombinant C3 exoenzyme at the indicated concentrations. c, same as a and b, except that allogeneic CTLs and their specific targets, the 2145 B-cell line, were used. Cytotoxicity was assayed in a conventional, 4-h [^51Cr] release assay at multiple effector:target cell ratios against the appropriate target cells, and data expressed in lytic units (LU) per 10^6 effector cells (26). LU at 25% specific lysis are shown for experiments using Daudi (b) or 2145 targets (c), as cytotoxicity of cells treated with 4 µg/ml C3 never reached 30% with these two targets, even at the highest (30:1) effector to target ratio (at this ratio, untreated cells gave over 65% specific lysis). Standard error of the mean was less than 10% for all measurements (data not shown). c, reduction in the amount of substrate available for ADP-ribosylation in cell extracts prepared following treatment of NK cells with C3. In vitro ADP-ribosylation with [^32P]NAD was performed on the post-nuclear fractions obtained from control or C3-treated cell lysates as described under "Experimental Procedures." Incorporation of [^32P] into trichloroacetic acid-precipitable material was counted and expressed as cpm/5 µg proteins.

Fig. 3. Two-dimensional SDS-PAGE analysis of proteins ADP-ribosylated by C3 in cell lysates from an IL-2-dependent NK cell line (a) and from YT cells (b). Cell lysates were prepared by freeze-thawing in 20 mM Hepes (pH 8), and the post-nuclear fraction (0.1 µg of proteins) was ADP-ribosylated using 50 ng/ml recombinant C3 and 5 × 10^7 cpm[^32P]NAD (30 Ci/mmol) for 30 min at 37°C. The reaction mixture was solubilized in sample buffer, separated by IEF (ampholine range 3.5-10) and then by SDS-PAGE on 15% acrylamide gel. After drying, gels were autoradiographed for 3-4 days.

Rho). This result is in agreement with Northern blot analysis, which revealed a predominant expression of the rhoA mRNA.

Knowing the intracellular localization of the RhoA protein in NK cells would clearly be of importance to further understand its role in cytotoxicity. YT cells and normal IL-2-dependent NK cells were fractionated on discontinuous sucrose gradient to prepare cytosol, membranes, and a fraction containing cytolitic granules as identified by Western blot using anti-perforin antibodies (not shown). Gel analysis of these fractions following [^32P]ADP-ribosylation clearly demonstrated that the C3 substrate was essentially found in the cytosolic fraction (Fig. 4A, lane 2), whereas no signal was detected in the plasma membrane fractions. In some experiments, a faint band was observed in the granule fraction, indicating that a small proportion of cellular RhoA protein may be associated with the cytotoxic granules. Western blot analysis using anti-Rho antibodies (Fig. 4B) confirmed that the majority of the 22-kDa immunoreactive protein was localized in the cytosol (lane 2). However, a small amount of the protein is clearly identified in the membrane fraction, and some also appear to be present in the granules (lanes 3 and 4). This subcellular distribution is clearly different from that of the majority of the LMW G-proteins (20-25 kDa) which, for the most part are localized in the plasma membrane and granule fractions as assessed by binding of [a[^32P]GTP on electroblotted proteins (Fig. 4C). The reason why ADP-ribosylation by C3 failed to consistently identify RhoA in the particulate fractions (membranes or granules) is not yet established. However, in addition to the apparently higher sensitivity of the Western blot technique, it is possible that, when Rho is associated with the membranes, its effector domain is engaged in molecular interactions which block access of the ADP-ribosyl transferase to its target residue Asn-41.

Thus our data demonstrate that a C. botulinum C3 substrate, identified as the GTP-binding protein RhoA, is critical for the function of cytotoxic lymphocytes. The current concept of cell-mediated cytotoxicity divides this function in two major steps: 1) transmembrane signaling triggered by cell surface interactions between effectors and targets and 2) exocytosis of cytotoxic granules. Although NK cells and specific CTLs may use a somewhat overlapping set of membrane molecules for target recognition, including CD8 and adhesion molecules such as CD2 and LFA1, major differences exist between NK cells and CTLs, inasmuch as the former do not use a polymorphic, antigen-specific receptor. This presumably translates into differences in the initial steps of activation.

Fig. 4. Subcellular localization of RhoA in NK cells. IL-2-dependent NK cells (10^6 cells) were homogenized in relaxation buffer and fractionated on a discontinuous sucrose gradient to prepare four fractions for analysis: lane 1, total cell extract; lane 2, cytosol; lane 3, plasma membranes; lane 4, cytoplasmic granules. These fractions were analyzed using three different parameters. A, ADP-ribosylation by C3; 10 µg of proteins from each fractions were ADP-ribosylated as in Fig. 3 and analyzed by SDS-PAGE (15% acrylamide). B, Western blot analysis using anti-Rho antibodies; 50 µg of proteins from the same fractions were separated by SDS-PAGE and electroblotted onto nitrocellulose. The membrane was incubated with rabbit anti-Rho antibodies followed by swine anti-rabbit Ig conjugated to peroxidase. The reaction was developed by enhanced chemiluminescence and exposed for 3 min to an Amersham high performance autoradiography film. For technical reasons, in this particular experiment the total cell extract could not be dialyzed against the appropriate buffers, resulting in a much lower level of detection of RhoA, which is not visible in A' or B1 on the exposures shown. C, GTP-binding analysis; prior to Western blot analysis, the membrane shown in B was washed for 30 min in GTP-binding buffer, then incubated for 1 h with 10 µCi/ml [α[^32P]GTP (3000 Ci/mmol). After 3 washes, the blot was allowed to dry and autoradiographed for 16 h.
pathways. Our finding that both types of cytotoxicity are equally inhibited by C3 suggests that RhoA is more likely implicated distal to the events of target recognition and transmembrane signaling. Thus, RhoA could be directly involved in the regulated exocytosis of cytotoxic granules. Alternatively, RhoA could play in NK cells a role similar to that ascribed to RhoC which, in other cell systems, has been found to control microfilament organization (5, 22, 27). ADP-ribosylation by C3 may induce disruption of the cytoskeletal network, thus preventing intracellular redistribution of granules and exocytosis. Consistent with this hypothesis, C3 treatment is accompanied by rounding up of the cells and by inhibition of tubulin polarization which normally occurs in NK cells at the point of contact with their targets (data not shown).

Whether other LMW G-proteins are associated with the granules of cytolytic lymphocytes and which functions they perform remain to be investigated. For instance, several ras-related GTP-binding proteins, including Rap1, were found to be associated with cytochrome b and specific granules in neutrophils (23, 28). However, unique to the system we have studied is the necessity to degranulate in a polarized fashion so that granular components are delivered into the contact zone between cytotoxic and target cells. This requires a tight control of cytoskeletal microfilaments, and we hypothesize that this is one of the possible mechanisms whereby RhoA may control the function of cytolytic lymphocytes.

Acknowledgments—We thank P. Boquet for kindly providing recombinant C3 exoenzyme, B. Olofsson for rho cDNA probes and comments on the manuscript, and H. Wakasugi for the YT line. We also thank F. Farace and G. Ferr for help and advice in growing NK cell lines and F. Trostien and J. P. Levillain for peptide synthesis.

REFERENCES