Journal of Cellular Biochemistry 80:37–45 (2000)

GTP-Dependent Permeabilized Neutrophil Secretion Requires a Freely Diffusible Cytosolic Protein

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Abstract Guanosine triphosphate (GTP) has been implicated in the regulation of Ca^{2+}-mediated secretion from neutrophils. We further examined the role of GTP in neutrophil secretion using streptolysin O permeabilized cells. We found that, in the presence of GTP, 1.0 μM free Ca^{2+} causes maximum secretion—equivalent to that achieved with 100 μM free Ca^{2+}—whereas GTPγS inhibits Ca^{2+}-stimulated secretion. Interestingly, GTP by itself stimulates secretion. These results indicate the existence of a GTP-regulated mechanism of secretion in neutrophils that requires GTP hydrolysis to stimulate secretion in the presence and absence of Ca^{2+}. The stimulatory effect of GTP is only observed when GTP is present during permeabilization. Addition of GTP after permeabilization, when the cytosolic contents have leaked out from cells, gives no stimulatory response, implying that the GTP-dependent secretory apparatus requires at least one cytosolic protein. GTP-dependent secretion can be reconstituted with crude HL-60 and bovine liver cytosol. The reconstituting activity binds to GTP-agarose, suggesting that the cytosolic factor is a GTP-binding protein or forms a complex with a GTP-binding protein. However, it is not a member of the rho or rac families of GTPases. By gel filtration chromatography, the secretion-reconstituting activity eluted at 870 and 200 kDa, but in the presence of GTP, eluted at 120 kDa, indicating that it is part of a high-molecular-weight complex that dissociates in the presence of GTP. Retention of adenosine diphosphate-ribosylation factor (ARF) in permeabilized cells and insensitivity of the cytosolic reconstituting activity to brefeldin A led to our speculation that ARF6 may be the GTPase involved in GTP-dependent secretion, and that activity from a BFA-insensitive ARF6 guanine nucleotide exchange factor reconstitutes secretion. J. Cell. Biochem. 80:37–45, 2000.† © 2000 Wiley-Liss, Inc.

Key words: GTP; neutrophil secretion; cytosolic protein

The study of vesicular transport between the endoplasmic reticulum and golgi compartments resulted in the identification of the soluble and membrane proteins NSF-SNAP (N-ethylmaleimide sensitive factor-soluble NSF attachment protein) and SNAREs (SNAP receptors). These proteins mediate vesicle coat formation, budding, and vectorial fusion between target and vesicle membranes [Rothman, 1994]. This system of vesicular transport, however, does not distinguish between constitutive and regulated secretion. For example, in neuroendocrine cells, PEPs (priming for exocytosis proteins) [Hay and Martin, 1992] and a 145-kDa protein [Walent et al., 1992], which are not part of the NSF-SNAP–SNARE complex, are required for secretion. Although neutrophils share certain mechanisms of secretion with neuroendocrine cells, neutrophils also have distinct mechanisms. This is evident in our finding that, unlike chromaffin cells where cytosolic proteins are required for Ca^{2+}-dependent secretion [Sarafian et al., 1987], the Ca^{2+}-dependent mechanism of secretion in neutrophils is mostly, if not entirely, membrane-bound [Rosales and Ernst, 1997]. Moreover, although vesicle-associated membrane protein (VAMP)-2, syntaxin 4, and SCAMP are expressed in neutrophils, other secretory proteins present in neuronal cells, such as VAMP-1, syntaxin 1, SNAP-25 (synaptic

Grant sponsor: National Institutes of Health; Grant number: HL56001; Grant sponsor: University of California Tobacco Related Diseases Research Program; Grant number: RT-066; Grant sponsor: Arthritis Foundation.

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Received 21 April 2000; Accepted 5 June 2000

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This article published online in Wiley InterScience, September xx, 2000.

vesicle-associated protein-25 kDa), synaptophysin, and cellubrevin, have not been detected in neutrophils [Brumell et al., 1995].

Investigations on intracellular membrane fusion and secretion in other systems has established an essential role for low-molecular-weight guanosine triphosphatases (GTPases) [Fischer von Mollard et al., 1991, Fischer von Mollard et al., 1994; Lenhard et al., 1992; Norman et al., 1996; Oberhauser et al., 1992; Padfield et al., 1992; Rothman, 1994]. For example, in neuroendocrine cells, rho regulates phosphatidylinositol 4-phosphate (PIP) 5-kinase [Chong et al., 1994], a regulator of PIP_2 that is involved in secretion; and in mast cells, rac and rho were found to be important in secretion. In neutrophils, the low-molecular-weight GTPases rac and adenosine diphosphate (ADP)-ribosylation factor (ARF) have been implicated as regulators of superoxide production [Abo et al., 1992; Heyworth, 1994; Rotrosen et al., 1992] and PLD activity, respectively [Cockcroft et al., 1994; Lambeth et al., 1995]. Although there have been reports that GTP [Smolen and Sandborg, 1990] and a GTP-binding protein [Gomperts, 1990] may regulate secretion in neutrophils, no specific GTPase that mediates the effects of GTP in secretion has been identified.

In this study, we present evidence for the existence of a GTP-dependent mechanism of secretion in neutrophils that requires one or more cytoplasmic proteins that are leaked after streptolysin O permeabilization. Using a GTP-dependent secretion reconstitution system, we found that the cytosolic secretion-reconstituting factor binds to GTP-agarose. While this suggests that the cytosolic factor is a GTP-binding protein, it is also possible that it forms a complex with a GTP-binding protein. By Superose-12 gel filtration chromatography, we found that it exists as a macromolecular complex. Additional evidence indicates that the essential secretion-reconstituting factor is not a member of the rac or rho families of small GTPases, but may be a guanine nucleotide exchange factor for ARF6.

MATERIALS AND METHODS

Materials

GTP-agarose and brefeldin A were from Sigma (St Louis, MO); GTP and GTPγS were from Boehringer Mannheim (Indianapolis, IN); botulinum C3 exotoxin was from List Biological Laboratories Inc. (Campbell, CA); bovine tissues were from Pelfreez Inc. (Brown Deer, WI); rhoGDI was a kind gift from Dr. Gary Bokoch.

Neutrophil Permeabilization, Stimulation, and Reconstitution Experiments

Neutrophils were prepared and permeabilized as described previously [Rosales and Ernst, 1997]. Briefly, 2×10^6 cells/ml were permeabilized with 0.05 i.u./ml streptolysin O in permeabilization buffer (50 mM Hepes, pH 7.0, with 100 mM KCl, 20 mM NaCl, 1 mM EGTA, and 0.1% dextrose) in the presence or absence of GTP and GTPγS. Nucleotide stimulation was continued for an additional 15 min at 37°C after a 10-min permeabilization at 37°C. Ca^{2+} buffers and Ca^{2+} stimulation were performed as described previously [Rosales and Ernst, 1997].

For reconstitution of GTP-dependent secretion, a wash step was added after permeabilization of cells to remove the remaining cytosolic contents. Permeabilized and washed neutrophils were then resuspended in permeabilization buffer containing HL-60 or tissue cytosol and incubated for 2 min at 37°C before adding 300 μM GTP and allowing secretion to proceed for 15 min at 37°C.

Pretreatment of cytosol was done as follows: Botulinum C3 exoenzyme was added at 1 μg/ml in the presence of 1 mM NAD at 37°C for 15 min, rhoGDI was added at 10 μM at 37°C for 5 min, and brefeldin A was added at 5 μg/ml for 30 min.

HL-60 Cell Culture and Cytosol Preparation

HL-60 cells were cultured in RPMI-1640 supplemented with 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine, 50 μg streptomycin/ml, and 50 i.u. penicillin/ml. Cells were seeded at 2×10^5/ml and maintained at $<2 \times 10^6$/ml. They were then harvested and lysed at 8×10^8/ml by sonication in permeabilization buffer containing 2 mM diisopropylfluorophosphate. Cytosol was obtained after two consecutive centrifugations at 30,000*g* for 15 min (Beckman J2-21 centrifuge, JA-14 rotor) and 100,000*g* for 60 min (Beckman L5-50B ultracentrifuge, 50 Ti rotor). The $>$10 kDa fraction of the cytosol was obtained by filtration using centricon-10 (Amicon Inc., Beverly, MA).

Preparation of Tissue Cytosol and Partial Purification of the GTP-Dependent Secretion Reconstituting Factor

Bovine tissues (liver, spleen, or brain) were rinsed with four volumes (w/v) of 0.15 M NaCl and homogenized in an equal volume (w/v) of permeabilization buffer containing 1 mM dithiothreitol, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, 10 μg/ml aprotinin, 1 mM benzamidine, 2 μM pepstatin, and 10 nM E-64. The tissue homogenates were centrifuged at 22,000*g* for 20 min. The resulting supernatants were centrifuged at 100,000*g* for 60 min. Proteins from liver cytosol were fractionated by successive ammonium sulfate (pH = 7.5) precipitation at saturations of 16%, 35%, 55%, and 80%. Precipitated protein fractions were collected by centrifugation at 22,000*g* for 15 min, dissolved in permeabilization buffer, and dialyzed against the same buffer. The dialyzed protein solutions were centrifuged at 100,000*g* for 60 min and the supernatants aliquoted and stored at −85°C.

GTP-agarose affinity chromatography of the active sample from the 55% ammonium sulfate precipitate was performed according to the method of Volpp, et al. [1988]. Briefly, 70 mg of the sample was incubated with 5 ml GTP-agarose at 4°C for 1 h. The mixture was loaded into a column and washed with 50 ml wash buffer (10 mM piperazinediethanesulfonic acid, pH 7.3, containing 100 mM KCl, 3 mM NaCl, and 3.5 mM $MgCl_2$). Bound proteins were eluted with wash buffer containing 150 mM NaCl and 1 mM GTP. Fractions (5 ml) were pooled, concentrated to 1 ml using centricon-10 and washed with permeabilization buffer. GTP-dependent secretion reconstituting activity was tested using 20-μl aliquots of the concentrated fractions.

Dissolved samples (2.5 mg) of the 55% ammonium sulfate precipitate in 200 μl volume were also separated in a 25 ml Superose 12 gel filtration column (Pharmacia Biotech, Uppsala, Sweden), attached to an FPLC, at a flow rate of 0.5 ml/min in the presence and absence of GTP. Molecular weight standards used were blue dextran (200 kDa), rabbit immunoglobulin G (150 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), soybean trypsin inhibitor (21.5 kDa), lysozyme (14.4 kDa), and vitamin B_{12} (1.355 kDa).

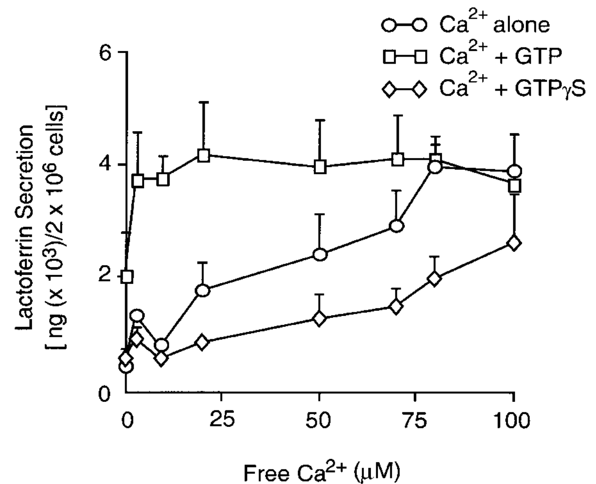

Fig. 1. Effects of guanine nucleotides on lactoferrin secretion of Ca^{2+}-stimulated cells. Streptolysin O-bound neutrophils were resuspended in permeabilization buffer alone or in buffer containing 300 μM GTP or 100 μM GTPγS. Permeabilized neutrophils were then stimulated with the indicated concentrations of Ca^{2+}. Buffer designated as containing 0 μM Ca^{2+} contained 1.0 mM EGTA. Values are means ± SEM of duplicate determinations from three experiments.

Miscellaneous Procedures

Quantitation of the extent of permeabilization and secretion of lactoferrin into the extracellular medium was performed as described previously [Rosales and Ernst, 1997].

RESULTS

Effects of Guanine Nucleotides on Ca^{2+}-Stimulated Secretions

A role for GTP in neutrophil secretion has previously been implicated [Cockcroft, 1991; Cockcroft, 1992; Lu and Grinstein 1990; Smolen and Sandborg, 1990]. In this study, we investigated the regulatory function of GTP in secretion by examining its effect on the biphasic secretory response of neutrophils to increasing concentrations of Ca^{2+} [Rosales and Ernst, 1997]. We found that when GTP was present during permeabilization (in buffer containing 1.0 mM EGTA), lactoferrin secretion was observed even when no Ca^{2+} was added (Fig. 1). Addition of Ca^{2+} stimulated secretion. Indeed, GTP together with 1.0 μM Ca^{2+} caused secretion of lactoferrin equivalent to that achieved with 100 μM Ca^{2+} alone. In other words, GTP by itself stimulates some secretion, but when used together with a low concentration of Ca^{2+}, causes a maximum response. In contrast, 100

μM GTPγS decreased Ca^{2+}-dependent secretion (Fig. 1). At higher $[Ca^{2+}]_f$ concentrations (i.e., 100 μM), a diminishing effect of GTP was evident. Together, these findings indicate that hydrolysis of GTP by one or more GTPase(s) is responsible for stimulation of secretion in the presence or absence of Ca^{2+}. Because a concentration-dependent response to GTP in the range of 10–500 μM was optimal at 300 μM (data not shown), this concentration was used for subsequent studies.

GTP-Dependent Secretion

Our results demonstrate that a GTP-regulated mechanism exists in secretion by permeabilized neutrophils, and that this system may interact with the high-affinity Ca^{2+} mechanism. However, the stimulatory effect of GTP was only observed when GTP was added during permeabilization (Fig. 2). Addition of GTP after permeabilization gave no stimulatory response.

To determine whether the lack of response to GTP added after permeabilization was due to leakage of an essential cytosolic factor, we tested the ability of isolated cytosol to reconstitute GTP-dependent secretion. Because we were unable to prepare neutrophil cytosol free of contaminating lactoferrin, we sought reconstituting activity in cytosol from other sources. We found that crude cytosol from the promyelocytic cell line, HL-60, and cytosol from several bovine tissues were able to reconstitute GTP-dependent secretion (liver > spleen > brain) from permeabilized neutrophils (Fig. 3). Cytosol from undifferentiated and differentiated HL-60 cells reconstitutes secretion to a similar extent (data not shown). Using HL-60 cytosol, we found that the cytosolic factor that reconstitutes GTP-dependent secretion has a molecular weight >10 kDa (Fig. 3). This cytosolic fraction can reconstitute GTP-dependent secretion to a greater extent than the crude cytosol, indicating partial purification. The cytosolic factor is also heat and trypsin sensitive (data not shown), implying that it is a protein.

To partially purify the GTP-dependent neutrophil secretion-reconstituting factor in larger quantities, we fractionated proteins from liver cytosol by ammonium sulfate precipitation. We found the greatest activity in the 35–55% precipitate (data not shown). Activity was also found to bind reversibly to GTP-agarose (Fig. 4). For the purpose of analyzing results of pu-

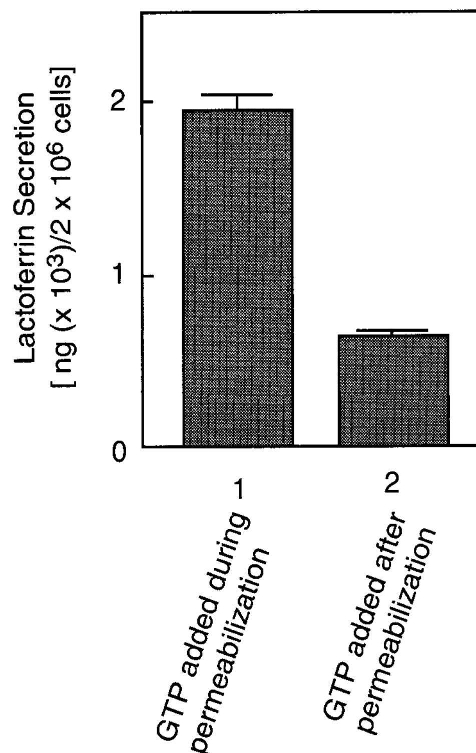

Fig. 2. GTP treatment during or after permeabilization. Neutrophils were initially incubated with streptolysin O at 4°C, washed in cold buffer to remove unbound streptolysin O, then resuspended in prewarmed (37°C) permeabilization buffer with (left bar) or without (right bar) 300 μM GTP. After 10 min incubation at 37°C, 300 μM GTP was added to cells that previously lacked it, and incubation was continued for an additional 15 min. Values are means ± SEM of duplicate determinations from three experiments.

rification procedures, we designated 1 unit of activity as that which causes secretion of 1 ng lactoferrin from 2×10^6 permeabilized cells. Because we occasionally observed some GTP-independent activity in crude cytosol and the ammonium sulfate fraction, we calculated the specific activity of the GTP-dependent secretion-reconstituting factor(s) as follows: (secretion in the presence of 300 μM GTP − secretion in the absence of added GTP)/μg protein. In Fig. 4, the specific activity of the ammonium sulfate fraction prior to loading into the GTP-agarose column was 1.5 units/μg protein while maximum specific activity from the column was 100.5 units/μg protein, indicating a 67-fold purification. We also observed a dose-response to increasing concentrations of the eluate from the column with lactoferrin secretion starting to plateau at an eluate concentration of 120 μg/ml or 10 μl (Fig. 5).

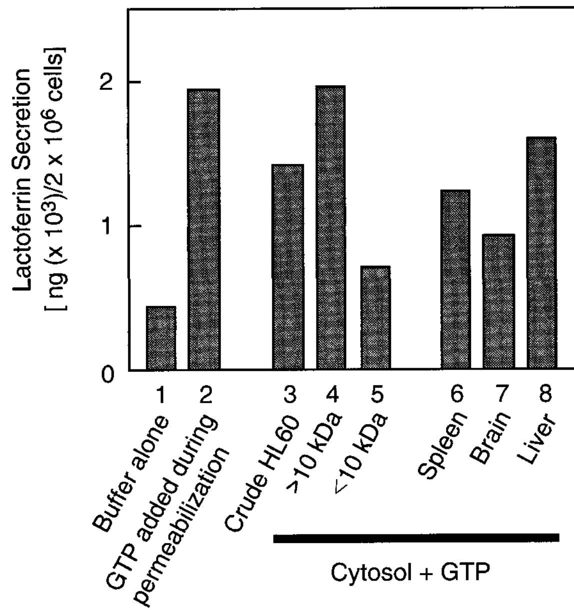

Fig. 3. Reconstitution of GTP-dependent secretion by HL-60 and bovine tissue cytosol. Cytosol was prepared as described in Materials and Methods. Cells were washed after permeabilization to remove the remaining endogenous cytosolic contents then resuspended in prewarmed cytosol (5 mg/ml). After incubation at 37°C for 2 min, 300 μM GTP was added, and incubation was continued for another 15 min. Data are means of duplicate determinations of a representative experiment (n = 3).

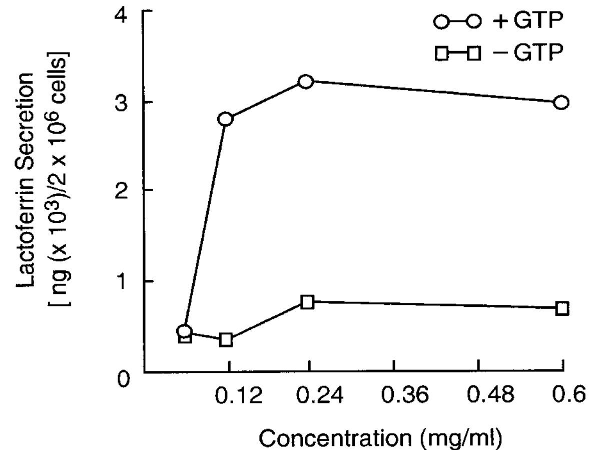

Fig. 5. Dose-response to increasing concentrations of the GTP-agarose eluate. Fraction #12 (elution fraction #2) from the GTP-agarose column was collected, concentrated, and analyzed for GTP-dependent secretion-reconstitution activity as described in Materials and Methods. Data are means of duplicate determinations of a representative experiment (n = 3).

Because members of the rho, rac, and ARF families of GTPases have been implicated in vesicular trafficking and secretory processes, we examined the possibility that at least one of these GTPases regulate GTP-dependent secretion in permeabilized neutrophils. We found that HL-60 cytosol pretreated with botulinum C3 exotoxin reconstitutes GTP-mediated secretion to the same extent as untreated cytosol (Fig. 6), suggesting that the cytosolic secretion-reconstituting factor is not rho or rac. Further evidence that the reconstituting activity in cytosol is not attributable to rac was obtained by the finding that, on immunoblots, rac was undetectable in leaked cytosol but was quantitatively retained in the streptolysin O permeabilized cells (data not shown). In contrast, approximately 40% of rhoA was detectable in the leaked cytosol (data not shown). However, rhoGDI (GDP dissociation inhibitor for rhos and racs) up to 10 μM had no effect on the secretion-reconstituting activity of HL-60 cytosol (Fig. 6). This provides further evidence that the cytosolic factor is not a member of the rho or rac family of GTPases. Using an anti-

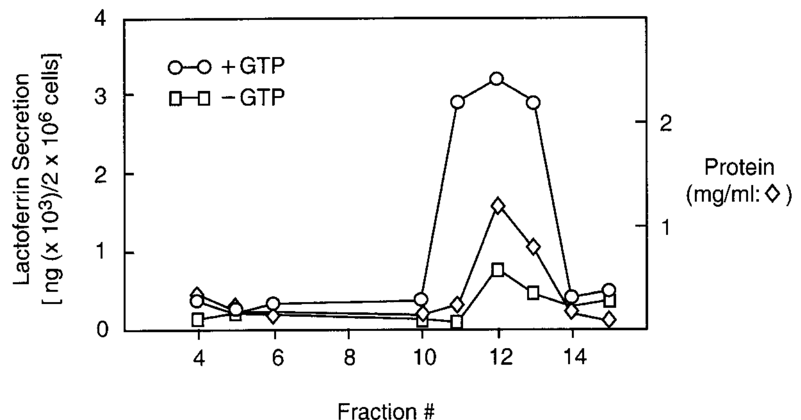

Fig. 4. Elution profile of the GTP-dependent secretion-reconstituting factor from a GTP-agarose column. GTP-agarose affinity chromatography was performed as described in Materials and Methods (fractions 1–10, wash; fractions 11–15, elution). Data are means of duplicate determinations of a representative experiment (n = 3).

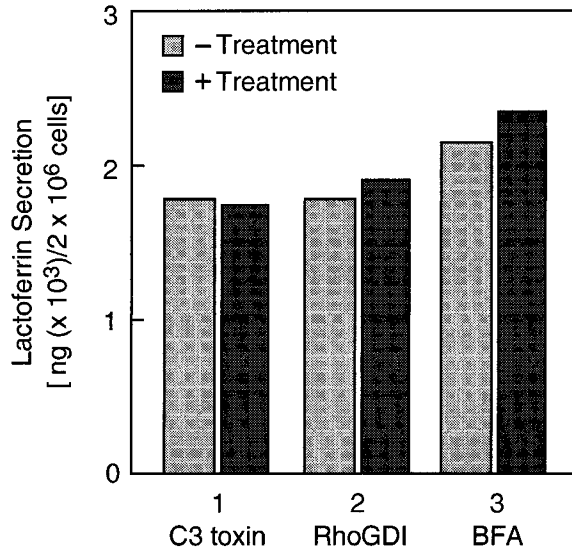

Fig. 6. Effect of botulinum C3 exotoxin, rhoGDI, and brefeldin A on the GTP-dependent secretion-reconstituting activity of HL-60 cytosol. Pretreatment of the cytosol with toxin, rhoGDI, and brefeldin A was performed as described in Materials and Methods. Treated cytosol preparations were tested for GTP-dependent secretion-reconstituting activity on washed permeabilized neutrophils. Values are means of duplicate determinations of a representative experiment (n = 4).

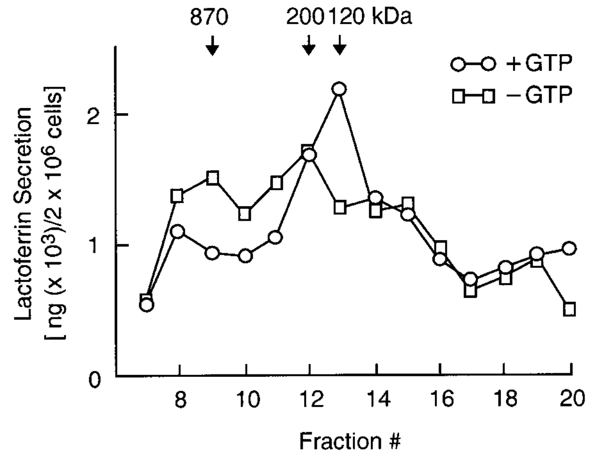

Fig. 7. Elution profile of the GTP-dependent secretion-reconstituting activity from a Superose-12 gel filtration column. Superose-12 gel filtration chromatography was performed as described in Materials and Methods. Data are means of duplicate determinations of a representative experiment (n = 3).

body that is broadly reactive to members of the ARF family, it was determined by Western blot that ARF was also retained in permeabilized cells (data not shown). Treatment of HL-60 cytosol with brefeldin A did not affect its secretion-reconstituting activity (Fig. 6). Brefeldin A (up to 50 μg/ml) also did not inhibit secretion from intact neutrophils stimulated with f-met-leu-phe (data not shown).

To further characterize the cytosolic factor that reconstitutes GTP-dependent secretion, the 55% ammonium sulfate precipitate was analyzed by gel filtration chromatography. Secretion-reconstituting activity eluted from a Superose 12 column in two peaks at fractions corresponding to 870 and 200 kDa (Fig. 7). However, when the sample was pretreated with GTP and separated through the column in a GTP-containing buffer, a single peak of activity eluted in a fraction corresponding to 120 kDa. This suggests dissociation of the secretion-reconstituting factor from the higher-molecular-weight structures.

DISCUSSION

It is now known that guanine nucleotides play a role in neutrophil secretion. Smolen and Sandborg [1990] reported that GTP enhances and GTPγS inhibits Ca^{2+}-induced secretion from electroporated neutrophils. We noted similar findings from streptolysin O permeabilized neutrophils, indicating that hydrolysis of GTP by a GTPase is essential for GTP-dependent secretion in these cells. However, in this study, we also found that GTP alone stimulates secretion. Maximum secretion obtained with GTP in the presence of 1.0 μM $[Ca^{2+}]_f$ may be a result of either an additive effect of GTP- and high-affinity Ca^{2+}-dependent secretion or convergence of the two pathways. Further work will be required to determine which of these is correct.

Unlike previous reports, our technique of adding GTP during permeabilization and adding Ca^{2+} after permeabilization allows one to distinguish secretion induced by these two stimuli. For example, in the novel finding by Nusse et al. [1998] on the existence of a high- and low-affinity Ca^{2+}-induced secretion from individual neutrophils, it cannot be ruled out that GTP did not contribute to stimulation of secretion, as GTP is most likely retained in patch-clamped cells. In the current study, we found that in contrast to Ca^{2+}-mediated secretion where maximum response occurs even when cytosolic contents have leaked out from cells [Rosales and Ernst, 1997], GTP-dependent secretion requires a cytosolic factor(s) as indicated by the loss of secretion competence of cells when GTP is added after

permeabilization. It is likely that when GTP is present during permeabilization, it activates the relevant GTPase and either causes its translocation to a membrane compartment or activates a downstream effector before it leaks from cells. When GTP is absent during permeabilization, either the GTPase involved or an upstream activator or downstream effector is lost by leakage from the permeabilized cells.

While reconstitution of GTP-mediated regulation of PLC [Thomas et al., 1991] and PLD [Cockcroft et al., 1994] have been demonstrated in permeabilized HL-60 cells, we present here the first report of a GTP-dependent secretion-reconstitution system in permeabilized neutrophils using a cytosol preparation. Previous studies have implicated the small GTP-binding proteins, particularly, the rab/rab-like proteins [Fischer von Mollard et al., 1991; Fischer von Mollard et al., 1994; Oberhauser et al., 1992; Padfield et al., 1992] and ARF [Lenhard et al., 1992; Rothman, 1994], in membrane fusion and secretion. However, in neutrophils, the GTP-binding protein(s) responsible for GTP-dependent secretion has not been identified. In the present study, binding of the neutrophil secretion-reconstituting factor to GTP-agarose suggests that it is either a GTP-binding protein or associates with a GTP-binding protein to form a complex.

We have examined the possibility that one of the members of the small GTPases, rho, rac, and ARF, may mediate GTP-dependent secretion in permeabilized neutrophils. Our findings indicate that the secretion-reconstituting factor is not rho or rac because botulinum C3 exotoxin, which inactivates these GTPases [Bowman et al., 1993], has no effect on the secretion-reconstituting activity of the cytosol. This conclusion is supported by the absence of rac in the leaked cytosol of permeabilized cells. Although rho is present in the leaked cytosol, rhoGDI has no effect on the reconstituting activity of the cytosol, further suggesting that rho or rac is not the relevant cytosolic factor involved in reconstituting GTP-dependent secretion. However, it is possible that the important cytoplasmic protein is an upstream effector of a GTPase such as a guanine nucleotide exchange factor (GEF) complexed to a GTP-binding protein. Furthermore, it is possible that the protein of interest may be a GTPase accessory protein, like p190 rhoGAP [Foster et al., 1994], which can be a GTP-binding protein itself. It is also conceivable that the cytosolic secretion-reconstituting factor is a GTP-binding GTPase effector protein that, when added to permeabilized neutrophils, exerts its action towards a membrane-bound retained GTPase. Among the small GTPases, ARF6 has been found to be membrane-bound both in the active (plasma membrane) and inactive (granule membrane) forms [Galas et al., 1997; Caumont et al., 2000]. The currently known GEFs for ARF6, EFA6 (exchange factor for ARF6) [Franco et al., 1999], and ARNO (ARF nucleotide binding site opener [Caumont et al., 2000] are insensitive to brefeldin A. Recent findings have also implicated that ARF6 has a role in secretory exocytosis from chromaffin cells [Galas et al., 1997; Caumont et al., 2000]. These findings, together with our observations that ARF is retained in permeabilized neutrophils and that the cytosolic secretion reconstituting activity is unresponsive to brefeldin A, led us to speculate that ARF6 may be the GTPase involved in GTP-dependent secretion, and that an ARF6 GEF may be the cytosolic factor that reconstitutes secretion.

Further characterization of the GTP-dependent secretion-reconstituting factor shows that it exists as part of an 870-kDa and a 200-kDa macromolecular complex. However, in the presence of GTP, the relevant cytoplasmic factor dissociates from the complex and reveals a molecular weight of 120 kDa. Interestingly, this corresponds to the size of a dimeric form of ARNO whose coiled-coil sequence at its N-terminal allows dimerization [Chardin et al., 1996] as well as potential interaction with other protein molecules.

In summary, we show evidence of a GTP-dependent mechanism of secretion from neutrophils that requires a cytosolic protein(s). We developed a GTP-dependent secretion-reconstitution system in permeabilized cells and found that the cytosolic secretion-reconstituting factor does not belong to the rho or rac family of GTPases. However, it is possible that ARF6 is the GTPase that participates in GTP-dependent secretion, and that a cytosolic ARF6 GEF that assumes a molecular size of 120 kDa reconstitutes secretion from neutrophils. Further characterization of this cytosolic factor will aid in our understanding of the molecular basis of GTP-dependent secretion from neutrophils.

ACKNOWLEDGMENTS

This work was performed during the tenure of an Established Investigator award from the American Heart Association to I.D.E.

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