Human Neutrophil Annexin I Promotes Granule Aggregation and Modulates Ca^{2+}-dependent Membrane Fusion

Joseph W. Francis, Kenneth J. Balazovich, James E. Smolen, David I. Margolis, and Laurence A. Boxer
Division of Hematology/Oncology, Department of Pediatrics, The University of Michigan School of Medicine, Ann Arbor, Michigan 48109

Abstract

The mechanism and cofactor requirements of exocytotic membrane fusion in neutrophils are unknown. Cytosolic proteins have been implicated in membrane fusion events. We assessed neutrophil cytosol for the presence of fusogenic proteins using a liposome fusion assay (lipid mixing). A fusogenic 36-kD protein containing amino acid sequence homology with human annexin I was purified from the cytosol of human neutrophils. This protein also shared functional characteristics with annexin I: it associated with and promoted lipid mixing of liposomes in a Ca^{2+}-dependent manner at micromolar Ca^{2+} concentrations. The 36-kD protein required diacylglycerol to promote true fusion (contents mixing) at the same Ca^{2+} concentrations used for lipid mixing. The 36-kD protein exhibited a biphasic dose–response curve, by both promoting and inhibiting Ca^{2+}-dependent lipid-mixing between liposomes and a plasma membrane fraction. The 36-kD protein also promoted Ca^{2+}-dependent increases in aggregation of a specific granule fraction, as measured by a turbidity increase. Antiannexin I antibodies depleted the 36-kD protein from the cytosol by > 70% and diminished its ability to promote lipid mixing. Antiannexin I antibodies also decreased by > 75% the ability of neutrophil cytosol to promote Ca^{2+}-dependent aggregation of the specific granules. These data suggest that annexin I may be involved in aggregation and fusion events in neutrophils. (*J. Clin. Invest.* 1992. 90:537–544.) Key words: leukocytes • protein • purification • liposomes • lipid mixing

Introduction

Neutrophils participate in host defense by releasing antimicrobial proteins from intracellular granules into phagosomes. Membrane fusion plays an important role in the degranulation process by creating a pathway by which granule contents have access to phagosomes or to the extracellular milieu. Although much is known about the conditions required for neutrophil activation, relatively little is known about the cofactors and mechanisms that promote fusion between granules and the plasma membrane. Several cofactors may participate in exocytotic membrane fusion in neutrophils. One likely candidate is Ca^{2+}, since it promotes fusion of artificial membranes and its concentration is elevated near forming phagolysosomes in activated neutrophils (1, 2). However, we have recently shown that Ca^{2+} by itself does not promote fusion between neutrophil granule and plasma membrane fractions using a cell-free fusion assay (3). Therefore other components must be required to bring about phagolysosomal formation.

Cytosolic proteins are postulated to participate in intracellular fusion events. In studies involving fusion of Golgi vesicles, fusogenic proteins were identified and isolated from the cytosol (4, 5). Cytosolic fusion proteins that may be involved in exocytosis have also been identified and have been implicated in neurotransmitter release (6), exocytosis in chromaffin cells (7), and the constitutive exocytotic pathway of yeast (8). Two potential fusogenic proteins that have been isolated from neutrophil cytosol, annexin III (9) and VII (10), share the properties of binding to phospholipid membranes in a Ca^{2+}-dependent manner and contain a conserved sequence of 17 amino acids (11). Previous studies have shown that annexins aggregate neutrophil granules and promote fusion between liposomes and granules only at millimolar concentrations of Ca^{2+} (10). Thus, proteins that promote Ca^{2+}-dependent fusion of natural membranes at lower (< 1 mM) or physiological Ca^{2+} concentrations have not been identified in the neutrophil cytosol.

In this study, we isolated an abundant 36-kD cytosolic protein from human neutrophils that enhances both Ca^{2+}-dependent aggregation and fusion of liposomes and neutrophil membrane fractions at micromolar concentrations of Ca^{2+} (> 30 μM). Amino acid sequence analysis identified this protein as annexin I.

Methods

Materials. Phosphatidylethanolamine (PE),[1] phosphatidic acid (PA), and phosphatidylserine were purchased from Avanti Polar Lipids, Inc. (Birmingham, AL) and the Sigma Chemical Co. (St. Louis, MO). 1,2-dioctanoyl-*sn*-glycerol (DiC_8) was purchased from Avanti Polar Lipids, Inc. *p*-Xylene-*bis*-pyridinium bromide (DPX), 8-aminonaphthalene-1,3,6, trisulfonic acid disodium salt (ANTS), and octadecyl rhodamine (R18) were purchased from Molecular Probes, Inc. (Eugene, OR). Protein-A Sepharose CL-4B, EDTA, ethyleneglycol-aminoethyl-ether-*N,N,N,N*-tetraacetic acid (EGTA), NaCl, KCl, Hepes, DTT, $MgCl_2$, molecular weight standards, TEMED (*N,N,N,N*-tetramethylethylenediamine), sucrose, anti-sheep IgG, and DEAE Sephacel were obtained from Sigma Chemical Co. All reagents for SDS-PAGE were obtained from Bio-Rad Laboratories (Richmond, CA). Leupeptin and aprotinin were obtained from Boehringer Mannheim, Indianapolis, IN. PMSF was purchased from United States Biochemical Corp. (Cleveland, OH). Antilipocortin III and antiannexin consensus sequence

Address correspondence to Dr. Joseph W. Francis, Department of Pediatrics, Room 7500 MSRB I, Box 0684, University of Michigan, Ann Arbor, MI 48109.

Received for publication 2 July 1991 and in revised form 2 March 1992.

J. Clin. Invest.

0021-9738/92/08/0537/08 $2.00
Volume 90, August 1992, 537–544

1. *Abbreviations used in this paper:* ANTS, 8-aminonaphthalene-1,3,6 trisulfonic acid disodium salt; DiC_8, 1,2-dioctanoyl-*sn*-glycerol; DPX, *p*-xylene-bis-pyridinium bromide; PA, phosphatidic acid; PE, phosphatidylethanolamine; R18, octadecyl rhodamine.

antibodies were the generous gift of Dr. J. R. Dedman (Dept. of Physiology and Cell Biology, University of Texas Medical School, Houston). Antiannexin I antibodies were purchased from Zymed Labs, Inc. (South San Francisco, CA).

Neutrophil isolation. Whole blood from healthy adult donors was collected by venipuncture into heparinized syringes. Leukocyte-rich plasma (provided by the American Red Cross, Lansing, MI) was also used as a source of neutrophils. Neutrophils were isolated by density centrifugation on Hypaque-Ficoll gradients (12) followed by dextran sedimentation (13). Residual erythrocytes were removed by hypotonic lysis (13). Neutrophils were washed by centrifugation in PBS.

Isolation of neutrophil membrane fractions. Neutrophil granule and plasma membrane fractions were isolated using nitrogen cavitation and Percoll gradient centrifugation according to a previously published study (3). Fractions were kept on ice before use.

Isolation of fusogenic proteins from neutrophil cytosol. Cytosol was collected from neutrophils disrupted by nitrogen cavitation using a method similar to that described by Jesaitis et al. (14). Neutrophils were suspended in cavitation buffer at pH 7.4 consisting of (mM) 130 KCl, 0.1 $MgCl_2$, 1 EDTA, 1 DTT, 10 Hepes, 1 PMSF and 10 μg/ml each of leupeptin, aprotinin, soybean trypsin inhibitor, and pepstatin. Neutrophils were subjected to nitrogen at 375 psi for 20 minutes at 4°C in a cell disruption chamber (Parr Instrument Co., Moline, IL). The cavitate was collected dropwise and centrifuged first at 1,000 *g* for 5 min to remove intact cells and nuclei and then at 10,000 *g* for 10 min. The resulting supernatant was further clarified by centrifugation at 100,000 *g* for 60 min. The clarified cytosol was dialyzed against 10 mM Hepes, pH 7.4, for 15 h and applied to a 2.5 × 7 cm column packed with DEAE-Sephacel and equilibrated with 10 mM Hepes buffer pH 7.0. Flow-through fractions (2.5 ml) were collected and tested for their ability to promote liposome fusion. Fractions containing fusogenic activity were pooled and concentrated using a YM-10 membrane (Amicon, Beverly, MA).

SDS-PAGE. Fractions from ion-exchange chromatography were pooled, concentrated, mixed with sample buffer (40% glycerol, 20% 2-mercaptoethanol, 8% SDS, 250 mM Tris-HCl, plus bromophenol blue) and heated to 100°C for 5 min. Proteins were separated on 10% polyacrylamide gels, and visualized with 0.1% Coomassie brilliant blue R-250.

Immunoprecipitation. Annexin I was depleted from solution by several rounds of immunoprecipitation using a monoclonal antiannexin I antibody and protein-G Sepharose. Protein fractions containing annexin I were incubated for 1 h at 4°C with 50 μg of antiannexin I antibody. Protein-antibody complexes were incubated with protein-G Sepharose CL-4B for 2 h at 4°C. Immune complexes were removed by centrifugation (10,000 *g*, 30 s) and the supernatants were subjected to SDS-PAGE. The percent depletion of the 36-kD protein from the supernatant fraction was calculated using scanning densitometry.

Western blotting. Western blotting was performed using a modification of the procedure of Towbin et al. (15). Proteins were transferred from electrophoresis gels to nitrocellulose or PVDF membranes at 500 mA for 45 min. Nonspecific protein binding was blocked by treating membranes with a 3% gelatin solution for 1 h at room temperature. Antibody treatment was performed overnight with shaking at 4°C with antiannexin I (1:2000) or antilipocortin III (1:500) antibodies in 1% gelatin. The blots were incubated with ^{125}I-anti-sheep IgG (5×10^5 to 10^6 cpm/ml) or HRP-conjugated anti-mouse IgG for 2 h at room temperature. The blots were washed, dried, and analyzed by autoradiography. Some blots were analyzed by visualizing antibodies using a Bio-Rad Immuno-blot HRP kit (Bio-Rad Laboratories).

Amino acid analysis. Amino acid analysis was performed by the Yale University School of Medicine Protein and Nucleic Acid Chemistry facility. The 36-kD protein identified on electrophoresis gels was transferred to a PVDF membrane and subjected to CNBr cleavage. The resulting peptides were separated by reverse-phase HPLC. The amino acid sequence of selected peptides was carried out on a sequencer (Applied Biosystems, Inc., Foster City, CA) equipped with an on-line HPLC. The obtained sequences were matched with known sequences using the Protein Identification Resource (National Biomedical Research Foundation, Washington, DC) in collaboration with the International Protein Information database (Node, Japan) and the Martinsried Institute for Protein Sequences (Max Planck Institute for Biochemistry, Munich, FRG)

Liposomes. Mixtures of PE/PA (4:1) in chloroform were dried under a steady stream of argon in a partial vacuum for 1 h. The resulting lipid films were hydrated in a solution of (mM) 130 KCl, 5 NaCl, 30 Hepes (KCl–Hepes), and 1 EGTA by sonication first in a water bath for 2 min followed by treatment with a sonication microprobe sonicator (Fisher Scientific Co., Allied Corp., Pittsburgh, PA) for 30 s. This procedure yielded predominantly small, unilammellar vesicles (3). R18 was incorporated into some liposome preparations by adding the R18 to the phospholipid/chloroform mixture; R18 comprised ~ 2% of the total phospholipid concentration of the liposomes. R18 that was not incorporated into vesicles was removed by gel filtration using Sephadex G-75 (Pharmacia Inc., Piscataway, NJ) and a solution of (mM) 130 KCl, 5 NaCl, and 10 Hepes. For the vesicle content-mixing studies, unilammelar liposomes with encapsulated solutes were prepared by drying thin films as described above. The liposomes were rehydrated in the presence of KCl-Hepes buffer (19.5 mM) containing 25 mM ANTS or Hepes buffer containing 117 mM DPX, vortexed, and subjected to five freeze–thaw cycles using liquid N_2 and extruded three times through two 100-nm polycarbonate filters (Nuclepore Corp., Pleasanton, CA) using an Extruder (Lipex Biomembranes, Vancouver, British Columbia, Canada).

Membrane aggregation. Aggregation of neutrophil membrane fractions was assayed using a previously described method with some modification (10). Neutrophil granule and membrane fractions were suspended with continuous stirring in KCl–Hepes at an absorbance equal to 0.1 at 450 nm using a Beckman DU-8 spectrophotometer (Beckman Instruments, Inc.). Aggregation was promoted by addition of cytosol and Ca^{2+}, and changes in absorbance were continuously monitored.

Fusion. Liposome fusion was assessed by both lipid and contents mixing. Lipid mixing was assayed by recording the increase in rhodamine fluorescence due to R18 dilution and resulting relief of self-quenching in fusing membranes. R18-labeled liposomes were stirred with unlabeled liposomes at 37°C (phospholipid = 30 μM). Neutrophil cytosol and calcium were added to the liposomes to promote fusion. Free Ca^{2+} concentrations were determined for the KCl–Hepes buffer by ion-selective electrodes (16). Fusion was expressed as the initial rate of fusion occurring within the first 2 min. 100% fusion is defined as the fluorescence signal that resulted from adding 0.1% Triton X-100 to the labeled membranes. The phospholipid ratio of R18-labeled to unlabeled vesicles or liposomes was 1:4 for all assays.

Contents mixing was assayed by first incorporating DPX and ANTS into separate populations of liposomes (PA/PE) as described above. Unencapsulated DPX and ANTS were removed by G-75 chromatography as described above. Fusion was defined as the decrease in fluorescence as DPX mixed with and quenched ANTS fluorescence. Concentrations of DPX were chosen such that quenching of ANTS could only occur in the concentrated environment of fusing liposomes and not during leakage of liposome contents. Leakage was also measured by assaying the quenching of ANTS fluorescence by free DPX (4.5 mM) as ANTS escaped leaking liposomes. The final phospholipid concentration for all contents-mixing assays was 50 μM. Neutrophil cytosol proteins, calcium, and DiC_8 were used to promote fusion. Rhodamine and ANTS fluorescence were monitored using a spectrofluorometer (650-10S; Perkin-Elmer Corp., Norwalk, CT) equipped with a temperature-regulated cell holder and a magnetic stirring device.

Liposome pelleting. Neutrophil cytosol proteins were tested for their ability to bind to liposomes in a Ca^{2+}-dependent manner. Liposomes were pelleted by centrifugation at 100,000 *g* for 20 min. The pelleted liposomes were resuspended in KCl–Hepes buffer. Use of fluorescently labeled R18 liposomes showed that roughly 80% of the liposomes were recovered by centrifugation. The preselected liposomes were then pelleted at 100,000 *g* for 10 min in the presence of various concentrations of cytosolic proteins and Ca^{2+} (> 30 μM). The pellets

(bound proteins) and supernatants (unbound proteins) were then analyzed by SDS-PAGE.

Results

Isolation of a 36-kD protein that promotes Ca^{2+}-dependent lipid mixing of liposomes. To determine if Ca^{2+}-dependent fusogenic proteins were present in the neutrophil cytosol, we assessed the ability of the neutrophil cytosol to promote Ca^{2+}-dependent lipid mixing of liposomes. Lipid mixing is a measure of the coalescence of membrane phospholipids from two or more vesicles during membrane fusion. Lipid mixing was measured by recording the increase in fluorescence due to the dequenching of R18 as it was diluted in fusing liposome membranes (PE/PA; 4:1). The neutrophil cytosol promoted Ca^{2+}-dependent lipid mixing and also promoted a shift in the Ca^{2+} requirement for this event (Fig. 1). The fusion-enhancing factors were heat- and trypsin-sensitive, suggesting the involvement of proteins (not shown).

Because Ca^{2+}-dependent phospholipid-binding proteins from neutrophil cytosol have been previously isolated from the flow-through fractions from ion-exchange chromatography (9), we analyzed similar fractions to identify and isolate potential fusogenic proteins. Two major peaks of lipid-mixing activity were observed in the cytosol that did not bind to DEAE-Sephacel (flow-through fractions; Fig. 2). The fusogenic factors found in these fractions shifted the Ca^{2+} requirements of lipid mixing from millimolar to micromolar concentrations

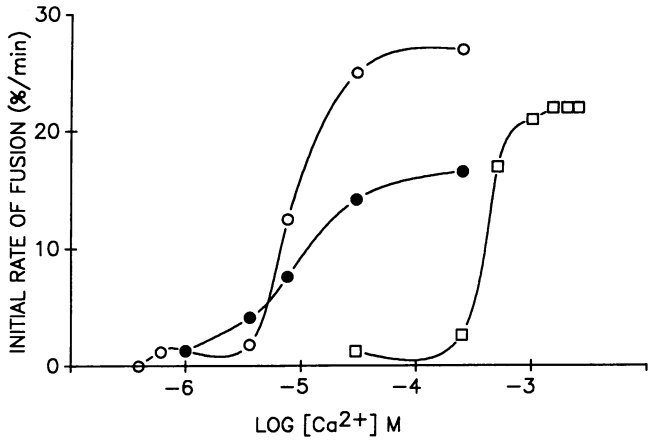

Figure 1. Calcium concentration dependence of liposome fusion as measured by a lipid-mixing assay. Liposomes composed of phosphatidylethanolamine and phosphatidic acid (4:1) were labeled with octadecyl rhodamine (R18) and mixed with unlabeled liposomes of the same phospholipid concentration at 37°C in a KCl-Hepes buffer containing 1 mM EGTA. Fusion was detected as an increase in rhodamine fluorescence as the R18 is diluted in fusing membranes. Ca^{2+} at millimolar concentrations promoted liposome fusion (*squares*). Neutrophil cytosol (10 μg/ml) and a flow-through protein fraction containing the 36-kD protein (5 μg/ml) from ion-exchange chromatography (*closed* and *open circles*, respectively) shifted the Ca^{2+} requirement of liposome fusion to micromolar Ca^{2+} concentrations. The initial rate of fusion is calculated as the rate of fusion (*%/min*) occurring within the first 2 min after the addition of Ca^{2+}. 100% fusion (probe dilution) is derived from the fluorescence signal that occurs after addition of Triton X-100 (0.1% final) to the R18-labeled liposomes. Representative experiment; $n = 4$.

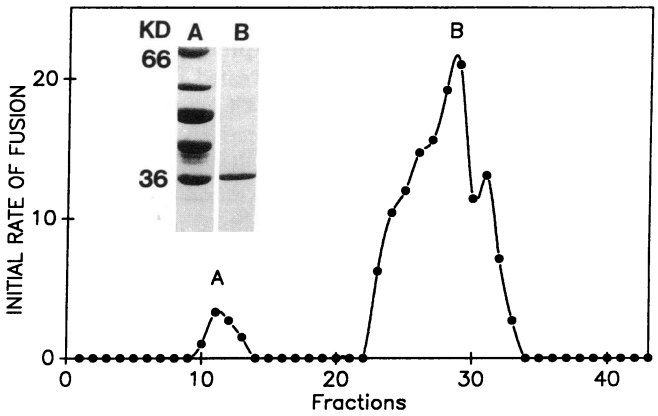

Figure 2. Lipid-mixing activity and SDS-PAGE analysis of neutrophil proteins collected from the run-through fractions of cytosol subjected to ion-exchange chromatography. Neutrophil cytosol (10 ml from 1 × 10^9 cells) was collected as described in methods and applied to an ion-exchange column containing DEAE-sephacel. Individual fractions (2.5 ml) were tested for their ability to support Ca^{2+}-dependent fusion of liposomes. Two major peaks of fusion activity were detected (*A, B*) in the run-through fractions. Fractions corresponding to these two peaks were pooled and analyzed by SDS-PAGE. Proteins were stained with Coomassie blue. Lanes marked A and B correspond to the labeled peaks of fusion activity. Fusion was measured and calculated by the lipid mixing method described in Fig. 1. Representative experiment; $n = 20$.

(Fig. 1). Since protein-mediated detergent or swelling effects could potentially cause relief of R18 self-quenching, the protein fractions were added to R18-labeled liposomes alone (without unlabeled liposomes); no increase in rhodamine fluorescence was detected under these conditions.

SDS-gel electrophoresis of the fractions from the flow-through showed five to seven prominent proteins in the first peak of lipid-mixing activity (Fig. 2; *inset*, lane *A*). The second peak of lipid-mixing activity showed one predominant protein at 36 kD (Fig. 2; *inset*, lane *B*). Some individual fractions from the second DEAE flow-through peak contained purified 36-kD protein as determined by silver staining of the protein on both one and two dimensional electrophoresis gels (not shown). Fractions containing purified 36-kD protein were pooled and used in subsequent assays. Table I shows the protein concentra-

Table I. Protein Concentration and Fusogenic Activity of the 36-kD Protein

Fraction	Protein	Fusion activity	Fold purification
	mg	*U/mg*	
Neutrophils (1 × 10^9)	90	—	—
Cytosol (100,000 *g*)	20.6	30	
36-kD Protein-enriched fraction DEAE-Sephacel	0.08	2,160	72

Cell isolation and protein purification procedures were performed as described in text. One unit of fusion activity was defined as the amount of fusogen that gives 10%/min increase in the initial rate of fusion. Representative data of more than 12 preparations of neutrophil cytosol.

tion and fusogenic activity of the 36-kD protein recovered during purification. The yield of the 36-kD protein based on fusion activity was 28%. Protein analysis revealed that this protein comprised roughly 0.5% (range 0.3–2.4%) of the total protein recovered from the cytosol.

The 36-kD protein binds to phospholipids in a Ca^{2+}-dependent manner. A characteristic of some membrane fusion proteins is their ability to bind to phospholipids in a Ca^{2+}-dependent manner. To determine if the 36-kD protein acted in this manner, we used a liposome-pelleting assay. Results revealed that micromolar concentrations of Ca^{2+} promoted binding of the 36-kD protein to liposomes (Fig. 3). The threshold concentration of Ca^{2+} required to promote binding of the 36-kD protein to liposomes was 30 μM (not shown). Optimal binding occurred at 200 μM Ca^{2+} (Fig. 3). To determine if the PE/PA liposomes pellet with all proteins in a Ca^{2+}-dependent manner or if they can be used to discriminate between Ca^{2+}-dependent and -independent protein binding, we investigated the ability of two other proteins to associate with them. Glyceraldehyde 3-phosphate dehydrogenase bound to liposomes in the absence of Ca^{2+}, whereas BSA was found mostly in the supernatant fraction after pelleting the liposomes (Fig. 3). Some residual BSA was detected in the liposome pellets but Ca^{2+} did not increase its association as observed on the SDS-gels (Fig. 3).

Western blotting and amino acid analysis. Recently, a family of Ca^{2+}-dependent phospholipid-binding proteins called annexins has been shown to promote liposome fusion (17). Annexin III is a 33-kD protein that has been isolated from human neutrophils (9). In this study, Western blotting analysis revealed that antiannexin III antibodies reacted with a 33-kD protein in crude cytosol but did not react with the purified 36-kD protein (Fig. 4). However, the 36-kD protein did react strongly with a monoclonal antibody to annexin I (Fig. 4). We also analyzed the proteins present in peak A (Fig. 2) by immunoblotting; neither antiannexin I or antiannexin III reacted with these proteins, suggesting the presence of other fusogenic proteins in this fraction, which will be the subject of future studies.

Since antibodies to specific annexins often cross-react with other annexins, we identified the 36-kD protein by obtaining its amino acid sequence. The 36-kD protein was subjected to cyanogen bromide and trypsin cleavage, and selected peptides were isolated by HPLC. The sequences of three peptides showed 100% homology with residues 127–140, 167–172, and 213–225 of human annexin I.

Depletion of the 36-kD protein by immunoprecipitation correlated with decreased liposome fusion. Since SDS-PAGE revealed that purified fractions containing the 36-kD protein often contained low concentrations of contaminating proteins as well, we analyzed the involvement of the 36-kD protein in lipid-mixing assays using immunoprecipitation. The 36-kD protein was depleted by several cycles of exposure to antiannexin I antibodies and protein G Sepharose. Analysis of the gels using scanning densitometry revealed that the 36-kD protein was depleted by > 70% after two cycles of immunodepletion (not shown). Fusion activity of this depleted fraction was diminished by roughly the same amount (Fig. 5). After four cycles of immunodepletion the 36-kD protein could not be detected by SDS-PAGE and no fusion activity remained in this fraction (not shown) compared with control samples (without primary antibody), which retained fusion activity.

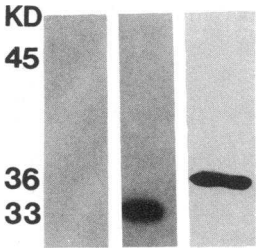

Figure 4. Western blot of the 36-kD protein using an antibody to annexin I or III. Lanes *A* and *B* show the 36-kD protein and cytosol, respectively, after incubation with a sheep antiannexin III antibody (1:500). Lane *C* shows the 36-kD protein after incubation with an antiannexin I antibody (1:2000). Primary antibodies to annexin III and I were detected by ^{125}I-anti-IgG and HRP-conjugated anti-IgG, respectively. Representative experiment; $n = 3$.

The 36-kD protein promotes liposome fusion as measured by a contents-mixing assay. Physiological fusion of membranes involves both mixing of membrane phospholipids and mixing of vesicle contents. Since some potential fusogenic cofactors have been shown to promote lipid mixing but not contents mixing (18), we tested the ability of the 36-kD protein to promote mixing of vesicle contents. Fusion in these assays was defined as a decrease in ANTS fluorescence as it was quenched by DPX. These two components were encapsulated into different liposome populations. The 36-kD protein promoted contents mixing and shifted the Ca^{2+} requirement of fusion to

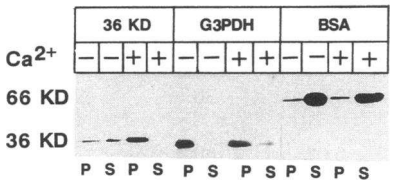

Figure 3. Ca^{2+}-dependent binding of the 36-kD protein to liposomes. The 36-kD bound to liposomes in a Ca^{2+}-dependent manner. Glyceraldehyde-3-phosphate dehydrogenase (*G3PDH*) did not require Ca^{2+} for binding whereas BSA was found mostly in the supernatant. Ca^{2+} concentration was 200 μM. The supernatants (*S*) and pellets (*P*) were analyzed by SDS-PAGE followed by Coomassie blue staining. Representative experiment; $n = 4$.

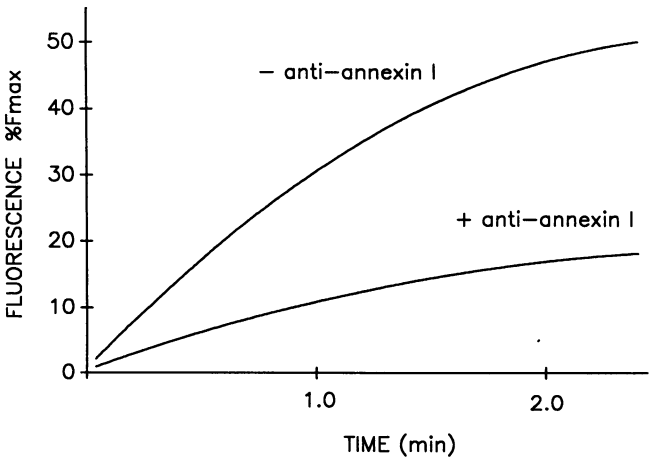

Figure 5. Immunodepletion of the 36-kD protein correlated with decreased liposome fusion. The 36-kD protein was depleted from solution by centrifugation using antiannexin I and protein-G Sepharose. After two cycles of immunodepletion the protein fraction (+ *anti-annexin* I) also showed decreased ability to promote Ca^{2+}-dependent liposome fusion compared with the undepleted fraction (– *anti-annexin* I). Fusion was measured by the lipid-mixing method described in Methods. Representative experiment; $n = 3$.

lower concentrations. However, the threshold concentration (250 μM) and the concentration of Ca^{2+} that promoted contents mixing at 50% of the maximal (800 μM) level were both higher than that required to promote lipid mixing (Fig. 6). Fatty acids and diacylglycerols have been shown to enhance Ca^{2+}-dependent annexin-mediated fusion of liposome membranes (19, 20). In the presence of 25 μM DiC_8 contents mixing promoted by the 36-kD protein of liposomes was shifted to lower Ca^{2+} concentrations (Fig. 6). In control experiments, DiC_8 alone marginally shifted the Ca^{2+} requirements (Fig. 6).

Effect of the 36-kD on aggregation and lipid mixing of neutrophil membrane fractions. Since the 36-kD protein promoted liposome fusion, we investigated whether it affected neutrophil membrane fractions in a similar manner. The initial membrane interactions during fusion involve close apposition of membranes, which is often called aggregation. The 36-kD protein promoted Ca^{2+}-dependent changes in the optical density of suspensions of specific granules (Fig. 7). These changes in optical density have been shown to be due to aggregation of the granules (9). The aggregation promoted by the 36-kD protein was concentration dependent (Fig. 7) and specific for Ca^{2+}, since Mg^{2+} and Mn^{2+} did not promote aggregation (not shown). The 36-kD protein promoted aggregation at high micromolar concentrations of Ca^{2+}, with a threshold at 250 μM and a half-maximal response at 750 μM. Ca^{2+} alone did not promote aggregation of any membrane fraction at any concentration tested (0.001–20 mM).

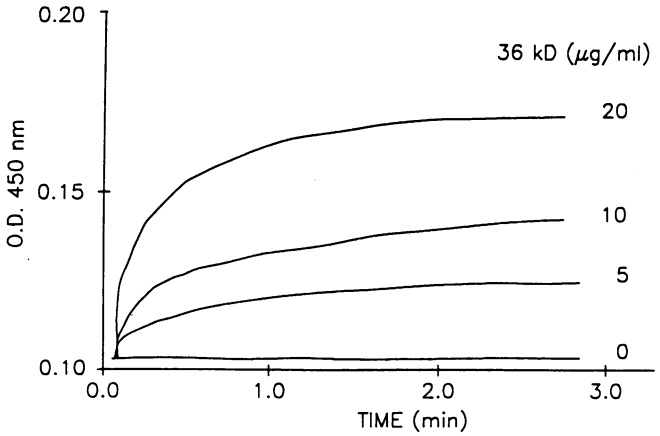

Figure 7. The 36-kD protein promoted Ca^{2+}-dependent aggregation of the specific granule fraction. The specific granule fraction was isolated from Percoll gradients. Aggregation of the granule fraction was indirectly assessed as an increase in turbidity at 450 nm. In the presence of 250 μM Ca^{2+}, the 36-kD protein promoted Ca^{2+}-dependent aggregation in a dose-dependent manner. Representative experiment; $n = 3$.

Because other annexins promote aggregation of specific granule fractions, we determined what proportion of the aggregating activity of crude cytosol could be attributed to annexin I. Fig. 8 shows aggregation of specific granules promoted by cytosol and Ca^{2+}; antiannexin I antibodies significantly diminished the aggregation-promoting activity of crude cytosol by ~ 75%. On the other hand, the 36-kD protein did not promote aggregation of the plasma membrane or primary granule fractions by themselves in mixed assays.

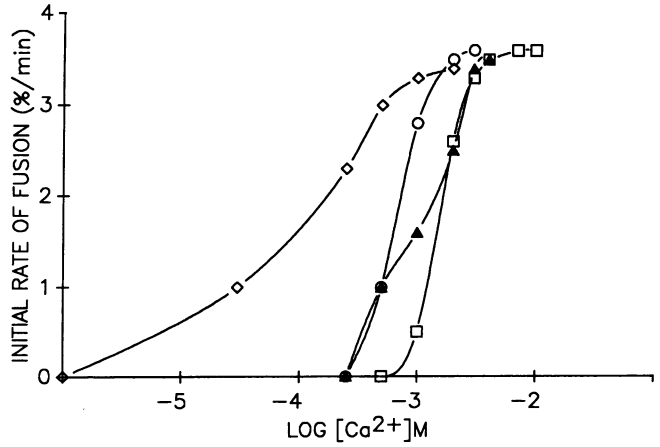

Figure 6. Promotion of contents mixing of liposomes by the 36-kD protein, Ca^{2+}, and diacylglycerol. Contents mixing was detected as the quenching of ANTS by DPX, which were encapsulated in individual liposome populations. The 36-kD protein (2 μg) promoted contents mixing and shifted the Ca^{2+} requirements to lower concentrations (□, Ca^{2+} alone; ○, Ca^{2+} + annexin I). The addition of DiC_8 (25 μM) enhanced the ability of the 36-kD protein to shift the Ca^{2+} concentration (◇). The effect of DiC_8 alone is shown (▲). The initial rate of fusion is calculated from the linear rate of fusion that occurred within the first 2 min after the addition of Ca^{2+}. The maximal rate of fusion (or 100% quenching) was calculated from the fluorescence intensity value obtained after disrupting ANTS-containing liposomes in the presence of 4.5 mM free DPX. Leakage of vesicle contents was monitored by exposing liposomes with encapsulated ANTS to the same experimental conditions in the presence of 4.5 mM free DPX. The initial rate of leakage was calculated and subtracted from the contents mixing initial rate. At low Ca^{2+} levels (10–100 μM) leakage varied from undetectable to 20% of the total rate of quenching. At higher Ca^{2+} levels (> 100 μM) the leakage increased and approached 40% at 2 mM Ca^{2+}. Representative experiment; $n = 3$.

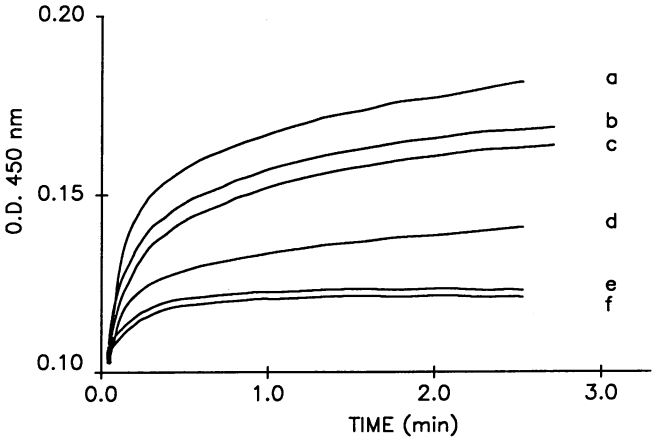

Figure 8. Antiannexin I antibodies decreased cytosol- and Ca^{2+}-dependent aggregation of the specific granule fraction. Cytosol promoted Ca^{2+}-dependent aggregation of the specific granule fraction. Antiannexin-I antibodies decreased the ability of cytosol to promote these changes in concentration dependent manner: (*a*) 0 μg/ml, (*d*) 12.5 μg/ml, (*e*) 25 μg/ml, (*f*) 50 μg/ml. Antiannexin II (25 μg/ml) and antiannexin IV (25 μg/ml) did not significantly diminish aggregation (curves *b* and *c*, respectively). Aggregation was measured as described in Fig. 7. Representative experiment; $n = 3$.

We investigated whether annexin I could promote lipid mixing between isolated granules and plasma membranes. Annexin I did not promote lipid mixing between these membranes; however, it did affect lipid mixing between the plasma membrane fraction and liposomes. We and others have previously shown that plasma membranes fuse with liposomes in a Ca^{2+}-dependent manner (3, 21). The 36-kD protein enhanced Ca^{2+}-dependent lipid mixing between plasma membranes and liposomes at 30 μM Ca^{2+} (Fig. 9), but at higher protein concentrations it inhibited lipid mixing. Bovine serum albumin at equivalent concentrations did not affect lipid mixing (Fig. 9, *closed circles*). The 36-kD protein only enhanced lipid mixing between the plasma membrane fraction and liposomes if it was preincubated with liposomes and Ca^{2+}.

Discussion

Proteins are among the leading candidates in the search for endogenous factors that promote and regulate membrane fusion. Recent evidence has shown that proteins, and possibly a protein complex, guide and promote fusion of intracellular organelle membranes (4, 5, 22). However, fusogenic proteins that promote exocytotic membrane fusion have not been identified. In this report we describe the isolation of a 36-kD fusogenic protein from the cytosol of human neutrophils.

The 36-kD protein is abundant, comprising roughly 0.35–2.4% of the total cytosolic protein. Western blotting and amino acid sequencing revealed that this protein is likely annexin I. Annexin I has previously been shown by Western blotting to be a relatively abundant cytosolic protein in neutrophils, monocytes, and in HL60 cells differentiated along the granulocytic pathway (23). Compared with two annexin proteins previously identified in neutrophil cytosol (III and VII), we show in this study that the 36-kD protein promoted membrane fusion at lower concentrations of Ca^{2+} (micromolar). This observation is consistent with a recent report showing that annexin I and II require lower concentrations of Ca^{2+} to bind to and promote fusion of liposomes compared with other annexins (17). We also observed that membrane interactions involving liposomes required low micromolar Ca^{2+} concentrations (< 100 μM) whereas those involving only native membranes required high micromolar Ca^{2+} (> 250 μM). The requirement of high micromolar Ca^{2+} concentrations could be due to several factors, including the absence of cofactors or the presence of inhibitors of Ca^{2+}-dependent fusion.

We show in this report that the 36-kD protein promoted Ca^{2+}-dependent lipid mixing but not contents mixing when low micromolar concentrations of Ca^{2+} were used. This phenomenon has been previously described as hemi-fusion (24). The 36-kD protein did promote contents mixing at higher Ca^{2+} concentrations (> 250 μM). Upon the addition of DiC_8 to the liposomes, the threshold Ca^{2+} concentration required to promote contents mixing could be lowered to a range similar to that required to promote lipid mixing. This is consistent with recent reports that have shown that annexin-mediated membrane fusion is enhanced by lipid factors (19). For example, annexin VII (synexin) has been shown to have a requirement for fatty acids to promote Ca^{2+}-dependent fusion of native membranes and to promote the mixing of vesicle contents while decreasing contents leakage (19). However, our study may be one of the first reports to show that diacylglycerol can lower the Ca^{2+} requirement of annexin-mediated fusion. This is also consistent with reports showing that diacylglycerols may be cofactors involved in membrane fusion events (20) and is relevant to their possible role in neutrophils, since diacylglycerol levels are increased by exocytotic stimuli (25). A previous study has also shown that annexin VII can promote contents mixing of liposomes in the presence of low micromolar concentrations of Ca^{2+}. However, this reaction also required millimolar concentrations of Mg^{2+}. We found that Mg^{2+} did not lower the Ca^{2+} requirement of contents mixing promoted by the 36-kD protein (not shown).

Even though the 36-kD protein did not promote detectable lipid mixing between isolated granule and plasma membrane fractions, it did modulate lipid mixing between the plasma membrane fraction and liposomes. We showed in a previous report that plasma membranes can fuse with liposomes in a Ca^{2+}-dependent manner (3). In this report, we show that the 36-kD protein both enhanced and inhibited this reaction. Similarly, a recent study has shown that purified annexin I can promote plasma membrane–liposome fusion in a Ca^{2+}-dependent manner at low micromolar Ca^{2+} concentrations (5 μM) (21). An inhibitory activity of annexin I is not reported. Several differences between their study (21) and ours should be noted, (*a*) in their study the source of annexin I is not neutrophil cytosol, (*b*) a dose-response of annexin I fusion activity is not reported, and (*c*) a different liposome phospholipid composition is used. Despite these differences, an interesting general observation can be made from these two studies and our previous study (3), namely that the plasma membrane fraction plays an active role in promoting fusion between plasma membranes and liposomes. For instance, as we have shown previously and in this report, plasma membranes can fuse with liposomes without adding cytosol proteins. The data of Oshry et al. (21) are also consistent with the hypothesis that plasma membrane is involved in promoting fusion, since they show that trypsin treatment of the plasma membrane fraction inhibits its ability to fuse with liposomes. Additionally, we occasionally observed (~ 1 out of 10 plasma membrane preparations) lipid mixing between plasma membranes and liposomes before the addition of Ca^{2+} (unpublished observation). Based on these

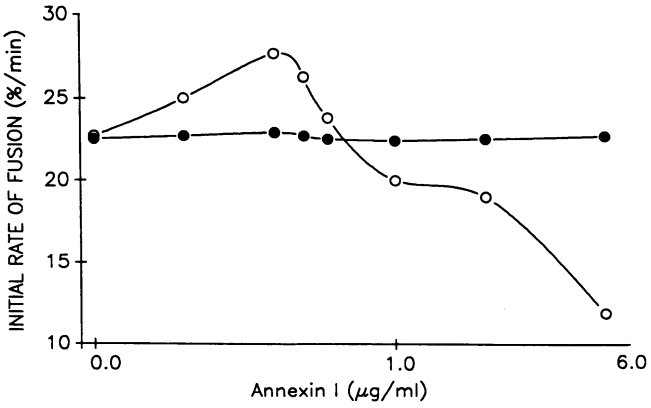

Figure 9. Modulation of Ca^{2+}-dependent fusion between the plasma membrane fraction and liposomes by the 36-kD protein. Ca^{2+} promoted fusion between the plasma membrane fraction and liposomes (30 μM) (initial rate of fusion = 22.5%/min). The 36-kD protein enhanced, as well as inhibited, the rate of fusion in a dose-dependent manner (○). BSA at the same protein concentrations did not affect fusion (●). Fusion was measured as in Fig. 1. $n = 3$.

observations, it could be hypothesized that the plasma membrane contains an integral or up-regulated fusion factor that is trypsin sensitive. It cannot be rule out that this membrane-bound fusogen is annexin I. Our data showing enhancement or inhibition of plasma membrane–liposome fusion would also suggest that this protein or cofactor could be regulated by annexin I.

Another important observation with regard to the different effects of the 36-kD protein on artificial membranes versus natural membranes is that the protein promoted the fusion of liposomes, but only aggregation of granules, under similar conditions. Thus, one interpretation of the aggregation and fusion data presented in this study is that annexin I is a factor that promotes membrane aggregation rather than fusion. The hypothesis that annexin I is a factor that promotes aggregation is based on the following findings: (*a*) the 36-kD protein did not promote detectable lipid mixing between granules and plasma membranes; (*b*) the 36-kD protein enhanced Ca^{2+}-dependent lipid mixing between the plasma membrane fraction and liposomes only within a narrow concentration range; (*c*) at higher concentrations the 36-kD protein was a potent inhibitor of Ca^{2+}-dependent fusion of these fractions; (*d*) it also promoted Ca^{2+}-dependent aggregation of neutrophil-specific granules and liposomes but, in contrast to its effects on fusion, did not inhibit aggregation at elevated concentrations; and (*e*) it required additional cofactors to promote contents mixing at low micromolar concentrations of Ca^{2+}. In addition, the 36-kD protein was only observed to enhance lipid mixing when it was preincubated with liposomes and Ca^{2+}. This observation is consistent with the reported finding that phospholipid increases annexin I affinity for Ca^{2+} and membranes (26).

The hypothesis that the 36-kD protein is an aggregating factor does not invalidate its potential role in the fusion process, since aggregation (or docking) is an important initial step in exocytotic membrane fusion (27). For instance, the calcium that is initially released upon neutrophil activation could cause annexin I to bind to membranes and promote docking of granules with themselves or with the cytoplasmic face of the plasma membrane. A recent report showed that treatment of HL60 cells with A23187, which resulted in a rise in intracellular Ca^{2+} levels, caused annexin I to associate with the membrane-enriched particulate fraction (23). Furthermore, annexin III has been localized near sites associated with phagolysosome formation in neutrophils (28). Although the Ca^{2+} required to promote aggregation in this study is higher than the average level found in the neutrophil cytosol during activation, a recent report showed that elevated concentrations of Ca^{2+} are located near phagolysosomes (2).

Annexins II, III, and VII have been shown to promote Ca^{2+}-dependent aggregation of membranes and the extent of the involvement of these proteins in membrane fusion has been postulated to be limited to these events (29). For example, the ability of annexin VII (synexin) to promote Ca^{2+}-dependent liposome fusion has been shown to be due to its ability to bind to and aggregate liposomes. This interpretation is supported by the finding that annexin VII has been shown to diminish Ca^{2+}-dependent fusion of liposomes of certain phospholipid compositions for which aggregation, as opposed to fusion, is the rate-limiting step (30). With regard to natural membranes, we show in this report that annexin I may promote aggregation of granule membranes to a greater extent than other annexins (Fig. 8).

In constitutive membrane fusion pathways, proteins in a multimeric complex regulate the fusion process (5), each protein presumably performing an independent function that contributes to the overall fusion process. Annexin I could be part of a similar mechanism and contribute to membrane fusion by promoting apposition of membranes. Alternatively annexin I could be a fusogen that requires other cofactors to be present. It should be noted that the three annexins identified in neutrophils each promote aggregation of granules under similar conditions (9, 10). It will be interesting to see if these proteins work synergistically (e.g., such that they reduce Ca^{2+} requirements for fusion or aggregation) or are targeted to specific membranes. Thus, the protein and nonprotein cofactor requirements of annexin-dependent exocytotic membrane aggregation and fusion will be the goals of future investigations.

Acknowledgments

This investigation was supported by Research Grants NIH-AI20065 (L. A. Boxer), NIH-DK32471 (J. E. Smolen), NIH-AI21641 (K. J. Balazovich), and postdoctoral fellowship (J. W. Francis) NIH-HL07517.

References

1. Jacking, M. E. E., D. P. Lew, J. L. Carpentier, K. E. Magnusson, M. Sjogren, and O. Stendahl. 1990. Cytosolic free calcium elevation mediates the phagosome-lysosome fusion during phagocytosis in human neutrophils. *J. Cell Biol.* 110:1555–1564.

2. Sawyer, D. W., J. A. Sullivan, and G. L. Mandell. 1985. Intracellular free calcium localization in neutrophils during phagocytosis. *Science (Wash. DC).* 230:663–666.

3. Francis, J. W., J. E. Smolen, K. J. Balazovich, R. R. Sandborg, and L. A. Boxer. 1990. Calcium-dependent fusion of the plasma membrane fraction from human neutrophils with liposomes. *Biochim. Biophys. Acta.* 1025:1–9.

4. Wattenberg, B. W., and J. E. Rothman. 1986. Multiple cytosolic components promote intra-Golgi protein transport. Resolution of a protein acting at a late stage, prior to membrane fusion. *J. Biol. Chem.* 261:2208–2213.

5. Rothman, J. E., and L. Orci. 1990. Movement of proteins through the Golgi stack: a molecular dissection of vesicular transport. *FASEB (Fed. Am. Soc. Exp. Biol.) J.* 4:1460–1468.

6. Sudhof, T. C., and R. Jahn. 1991. Proteins of synaptic vesicles involved in exocytosis and membrane recycling. *Neuron.* 6:665–677.

7. Drust, D. S., and C. E. Cruetz. 1988. Aggregation of chromaffin granules by calpactin at micromolar levels of calcium. *Nature (Lond.).* 331:88–91.

8. Saliminen, A., and P. J. Novick. 1987. A ras-like protein is required for a post-Golgi event in yeast secretion. *Cell.* 49:527–538.

9. Ernst, J. D., E. Hoye, R. A. Blackwood, and D. Jaye. 1990. Purification and characterization of an abundant cytosolic protein from human neutrophils that promotes Ca^{2+}-dependent aggregation of isolated specific granules. *J. Clin. Invest.* 85:1065–1071.

10. Meers, P., J. D. Ernst, N. Duzgunes, K. L. Hong, J. Fedor, I. M. Goldstein, and D. Papahadjopoulos. 1987. Synexin-like proteins from human polymorphonuclear leukocytes. Identification and characterization of granule-aggregating and membrane-fusing activities. *J. Biol. Chem.* 262:7850–7858.

11. Geisow, M. J., and J. H. Walker. 1986. New proteins involved in cell regulation by Ca^{2+} and phospholipids. *Trends Biochem. Sci.* 11:420–423.

12. Boyum, A. 1968. Isolation of mononuclear cells and granulocytes from human blood. Isolation of mononuclear cells by centrifugation and of granulocytes by combining centrifugation and sedimentation at 1 G. *Scand. J. Clin. Lab. Invest.* 21:77–89.

13. Zurier, R. B., S. Hoffstein, and G. Weissmann. 1973. Cytochalasin B: effect on lysosomal enzyme release from human leukocytes. *Proc. Natl. Acad. Sci. USA.* 70:844–848.

14. Jesaitis, A. J., J. R. Naemura, R. G. Painter, L. A. Sklar, and C. G. Cochrane. 1982. Intracellular localization of *N*-formyl chemotactic receptor and Mg^{2+} dependent ATPase in human granulocytes. *Biochim. Biophys. Acta.* 719:556–568.

15. Towbin, H., T. Staehelin, and J. Gordon. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Natl. Acad. Sci. USA.* 76:4350–4354.

16. Bers, D. M. 1982. A simple method for the accurate determination of free (Ca) in Ca-EGTA solutions. *Am. J. Physiol.* 242:C404–C408.

17. Blackwood, R. A., and J. D. Ernst. 1990. Characterization of Ca^{2+}-dependent phospholipid binding, vesicle aggregation and membrane fusion by annexins. *Biochem. J.* 266:195–200.

18. Burgess, S. W., D. Massenburg, J. Yates, and B. R. Lentz. 1991. Poly(ethylene glycol)-induced lipid mixing but not fusion between synthetic phosphatidylcholine large unilamellar vesicles. *Biochemistry.* 30:4193–4200.

19. Meers, P., K. Hong, and D. Papahadjopoulos. 1988. Free fatty acid enhancement of cation-induced fusion of liposomes: synergism with synexin and other promoters of vesicle aggregation. *Biochemistry.* 27:6784–6794.

20. Siegel, D. P., J. Banschbach, D. Alford, H. Ellens, L. J. Lis, P. J. Quinn, P. Yeagle, and J. Bentz. 1989. Physiological levels of diacylglycerols in phospholipid membranes induce membrane fusion and stabilize inverted phases. *Biochemistry.* 28:3703–3709.

21. Oshry, L., P. Meers, T. Mealy, A. Tauber. 1991. Annexin-mediated membrane fusion of human neutrophil plasma membranes and phospholipid vesicles. *Biochem. Biophys. Acta.* 1066:239–244.

22. Wattenberg, B. W., W. E. Balch, and J. E. Rothman. 1986. A novel prefusion complex formed during protein transport between Golgi cisternae in a cell-free system. *J. Biol. Chem.* 261:2202–2207.

23. William, F., B. Mroczkowski, S. Cohen, and A. S. Kraft. 1988. Differentiation of HL-60 cells is associated with an increase in the 35-kDa protein lipocortin I. *J. Cell. Physiol.* 137:402–410.

24. Song, L., Q. F. Ahkong, D. Georgescauld, and J. A. Lucy. 1991. Membrane fusion without cytoplasmic fusion (hemi-fusion) in erythrocytes that are subjected to electrical breakdown. *Biochem. Biophys. Acta.* 1065:54–62.

25. Agwu, D. E., L. C. McPhail, M. C. Chabot, L. W. Daniel, R. L. Wykle, and C. E. McCall. 1989. Choline-linked phosphoglycerides. A source of phosphatidic acid and diglyceride in stimulated neutrophils. *J. Biol. Chem.* 264:1405–1411.

26. Schlaepfer, D. D., and H. T. Haigler. 1987. Characterization of Ca^{2+}-dependent phospholipid binding and phosphorylation of lipocortin I. *J. Biol. Chem.* 262:6931–6937.

27. Plattner, H. 1989. Regulation of membrane fusion during exocytosis. *Int. Rev. Cytol.* 119:197–286.

28. Ernst, J. D. 1991. Annexin III translocates to the periphagosomal region when neutrophils ingest opsonized yeast. *J. Immunol.* 146:3110–3114.

29. Meers, P., J. Bentz, D. Alford, S. Nir, D. Papahadjopoulos, and K. Hong. 1988. Synexin enhances the aggregation rate but not the fusion rate of liposomes. *Biochemistry.* 27:4430–4439.

30. Hong, K., N. Duzgunes, P. R. Meers, and D. Papahadjopoulos. 1987. Protein modulation of liposome fusion. *In* Cell Fusion. A. E. Sowers, editor. Plenum Publishing Corp., New York. 269–284.