Identification of glyceraldehyde-3-phosphate dehydrogenase as a Ca^{2+}-dependent fusogen in human neutrophil cytosol

Ronald J. Hessler,* R. Alexander Blackwood,† Thomas G. Brock,* Joseph W. Francis,*‡ Donna M. Harsh,† and James E. Smolen*§⇑

*Department of Pediatrics, Division of Hematology-Oncology, University of Michigan, Ann Arbor; †Department of Pediatrics, Division of Infectious Diseases, University of Michigan, Ann Arbor; ‡Department of Science and Mathematics, Cedarville College, Cedarville, Ohio; §Department of Pathology, University of Michigan, Ann Arbor; and ⇑Department of Pediatrics, Leukocyte Biology Section, Baylor College of Medicine, Houston, Texas

Abstract: The membrane fusion events observed during neutrophil degranulation are important aspects of the immunoregulatory system. In an attempt to understand the regulation of granule-plasma membrane fusion, we have begun characterizing human neutrophil cytosol for fusion activity, finding that 50% of the fusogenic activity could be attributed to members of the annexin family of proteins. The major non-annexin fusion activity (25% of the total cytosolic activity) was enriched by ion exchange chromatography after depletion of annexins by Ca^{2+}-dependent phospholipid affinity chromatography. The fusion activity co-purified with a 10,14-kDa dimer identified as leukocyte L1 (which was non-fusogenic), along with an approximately 36-kDa protein. This protein was identified as glyceraldehyde-3-phosphate dehydrogenase (GAPDH) by amino-terminal sequencing, and the fusion activity was verified using commercially available GAPDH. GAPDH may play an important role in degranulation because it is as potent as annexin I on a mass basis and may constitute up to 25% of the total cytosolic fusion activity of the neutrophil. *J. Leukoc. Biol.* 63: 331–336; 1998.

Key Words: *fusion · liposome · degranulation*

INTRODUCTION

Neutrophil degranulation is an important aspect of the immune system's inflammatory response. Invagination of the plasma membrane to engulf foreign particulates, followed by fusion of these phagosomes with specific and azurophil granules containing bactericidal compounds and hydrolytic enzymes, are well-known processes [1]. Although we have considerable knowledge about neutrophil degranulation, the mechanism(s) and regulation of the membrane-membrane interactions are not well understood. It has been observed that the annexins, a class of Ca^{2+}-dependent phospholipid binding proteins [2], can promote fusion of both phospholipid vesicles and neutrophil specific granules [3, 4]. Therefore, they have been implicated as potential mediators of the membrane fusion events observed during degranulation.

There are several non-annexin proteins that have been shown to mediate membrane fusion in systems analogous to neutrophils. The release of neurotransmitters at synaptic junction sites is a membrane-membrane fusion event similar to degranulation. This process in neurons is mediated by proteins such as SNAP-25, VAMP, and NSF [5, 6]. These proteins have been shown to effect neurotransmitter release on stimulation with Ca^{2+}, which can reach the micromolar range locally [7].

Hypothesizing that neutrophil degranulation may be mediated at least in part by one or more non-annexin fusogens, we depleted human neutrophil cytosol of annexins, finding that considerable fusion activity remained. Subsequent isolation, partial purification, and analysis demonstrated that a significant portion of the fusogenic activity was due to glyceraldehyde-3-phosphate dehydrogenase (GAPDH). These results were confirmed by Western blot, enzymatic assay, and fusion using commercial GAPDH. We believe that native GAPDH may play an important role in neutrophil degranulation.

MATERIALS AND METHODS

Reagents

Phosphatidylethanolamine (PE), phosphatidic acid (PA), *N*-[7-nitrobenz-2-oxa-1,3-diazol-4-yl]-PE (NBD-PE), and rhodamine-PE (Rh-PE) were purchased from Avanti Polar lipids (Birmingham, AL). *p*-Xylene-bis-pyridinium bromide (DPX) and 8-aminonaphthalene-1,3,6, trisulfonic acid disodium salt (ANTS) were purchased from Molecular Probes (Eugene, OR). Mouse anti-GAPDH was purchased from Biogenesis, Sandown, NH. Protein-A Sepharose CL-4B, chicken muscle GAPDH (95% pure protein, with $<$0.01% 3-phosphoglyceric phospho-

Abbreviations: GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PE, phosphatidyl ethanolamine; PA, phosphatidic acid; NBD-PE, *N*-[7-nitrobenz-2-oxa-1,3-diazol-4-yl]-PE; DPX, *p*-xylene-bis-pyridinium bromide; ANTS, 8-aminonaphthalene-1,3,6 trisulfonic acid disodium salt; HEPES, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; DFP, diisopropyl fluorophosphate; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; RBC, red blood cells; PBS, phosphate-buffered saline; PS, phosphatidylserine.

Correspondence: James E. Smolen, Department of Pediatrics, Children's Nutrition Research Center, 1100 Bates, Room 6014, Baylor College of Medicine, Houston, TX 77030-2600. E-mail: jsmolen@bcm.tmc.edu

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kinase and <0.1% triosephosphate isomerase), ethylenediaminetetraacetate, ethyleneglycol-aminoethylether-*N,N,N,N*-tetraacetic acid (EGTA), NaCl, KCl, *N*-2-hydroxyethylpiperazine-*N′*-2-ethanesulfonic acid (HEPES), dithiothreitol, $MgCl_2$, molecular weight standards, TEMED, DEAE Sephacel, and diisopropyl fluorophosphate (DFP) were obtained from Sigma. All reagents for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) were obtained from Bio-Rad (Richmond, CA). Leupeptin and aprotinin were obtained from Boehringer Mannheim. Phenylmethylsulfonyl-fluoride was purchased from United States Biochemical Co. (Cleveland, OH).

Cells

Neutrophils were isolated from the fresh venous blood of healthy donors by use of the method of Boyum [8]. Briefly, acid-citrate-dextrose anticoagulated venous blood was separated on a Ficoll-Hypaque cushion, followed by dextran sedimentation of red blood cells (RBC). Residual RBC were removed by hypotonic lysis. The resulting neutrophils were washed and suspended in buffer A (100 mM KCl, 50 mM HEPES, 1 mM EGTA, pH 7.0) at 1×10^8/mL. After treatment with DFP (2 mM), the neutrophils were disrupted by nitrogen cavitation [9] and the nuclei removed by low-speed centrifugation in a Beckman J6 centrifuge (1000 *g* for 10 min at 4°C), followed by removal of granules and plasma membrane by ultracentrifugation (158,000 *g* for 30 min at 4°C).

HL-60 cells were also used for the final protein isolation and purification step due to high cell yields and the presence of the fusogenic activity in these cells. Cells were grown in RPMI 1640 supplemented with 10% fetal calf serum and 1% penicillin-streptomycin. After washing of the undifferentiated cells with phosphate-buffered saline (PBS), cell pellets were treated with DFP, cavitated, and clarified as outlined for blood.

Fusogen purification

Annexins were removed from cytosol by exhaustive affinity chromatography using a phospholipid column (3 PS:1 PE) similar to that used for annexin purification in the presence of 1 mM Ca^{2+}, as previously described [10]. The pooled flow-through was dialyzed into 10 mM sodium phosphate, pH 7.0, and subjected to DEAE Sephacel anion-exchange chromatography. The subsequent flow-through was dialyzed into 50 mM MES, 0.1 mM EGTA, pH 5.5, and subjected to FPLC (MonoS HR 10/10 cation exchange, Pharmacia, 17-1557-01). Elution was at 1 mL/min with 0–1.0 M NaCl concentration gradient executed over 50 min (see Fig. 2). Collected eluate fractions (1 mL) were dialyzed back into Buffer A for experimentation.

Fusion assay

Large unilamellar liposomes (LUV) composed of PA/PE (1:3) were prepared by the reverse-phase evaporation method of Duzgunes et al. [10, 11]. For lipid mixing assays, PA:PE (1:3) LUV labeled with 1% NBD-PE and 1% Rh-PE were formed in buffer A by reverse-phase evaporation. At these concentrations of NBD-PE and Rh-PE, the distance between NBD and Rh was sufficiently close that Rh quenched NBD fluorescence. Labeled and unlabeled LUV (1:9, 180 μg total phospholipid) were combined in a cuvette (final volume 1 mL) and fusion initiated with the addition of increasing [Ca^{2+}]. Fusion between a labeled and an unlabeled LUV resulted in an increase in the distance between NBD and Rh and a decrease in quenching. The resulting increase in NBD fluorescence was detected by a SLM 8000 spectrofluorometer (excitation, 450 nm; emission, 530 nm).

Content mixing fusion was assayed with ANTS/DPX as described by Wilschut et al. [12].

Miscellaneous procedures

Western blots were performed by the method of Gillespie [13]. Protein sequencing was carried out by the University of Michigan Carbohydrate Facility using acid hydrolysis followed by high-performance liquid chromatography. GAPDH enzyme assays were performed according to Heinz and Freimuller [14].

RESULTS

The annexins have been implicated as potential fusogens mediating the fusion between neutrophil granules and plasma membrane fusion [4]. However, non-annexin fusogens have been shown to be important in other cell systems and in fact may work in conjunction with the annexins. To determine the potential role of non-annexin fusogenic activity, we took advantage of the characteristic affinity of annexins for phospholipid vesicles [10]. Neutrophil cytosol was depleted of annexins by Ca^{2+}-dependent affinity chromatography using a phosphatidylserine (PS)/PE liposome column. We found that, following depletion of annexins by exhaustive affinity chromatography, the column flow-through retained approximately half of total cytosol fusion activity (**Fig. 1**). Annexin depletion was confirmed by Western blot analysis using an antibody to the annexin consensus sequence (not shown).

The flow-through from the liposome affinity column was subjected to anion-exchange chromatography on DEAE Sepharose. Although 50% of total protein bound to the DEAE column, virtually all of the fusion activity was found in the flow-through (Fig. 1). The flow-through from the anion-exchange column was bound to an FPLC cation exchange column and the remaining proteins were eluted with a 0–1.0 M NaCl gradient. This eluate was, according to the elution program, divided into five fractions designated peak 1 (corresponding to 0.15–0.25 M NaCl elution), peak 2 (0.25–0.35 M NaCl), peak 3 (0.35–0.50 M NaCl), peak 4 (0.50–1.00 M NaCl), and flow-through (**Fig. 2**). FPLC peak 2 contained most of the fusion activity present in the initial affinity column flow-through, but only one-sixth of the proteins. Because of this potent fusion activity, samples were tested for trace amounts of annexin by Western blotting and were found to be free of annexin (data not shown).

Figure 3 shows by SDS-PAGE that stepwise chromatography removed most contaminating proteins from the fusogenic fraction. Lanes 1, 2, 3, and 6 show the purification achieved in cytosol, affinity column flow-through, anion exchange column flow-through, and FPLC peak 2, respectively (other lanes show proteins removed in anion column flow-through, FPLC flow-through, and FPLC peaks 1, 3, and 4). It can be seen that the

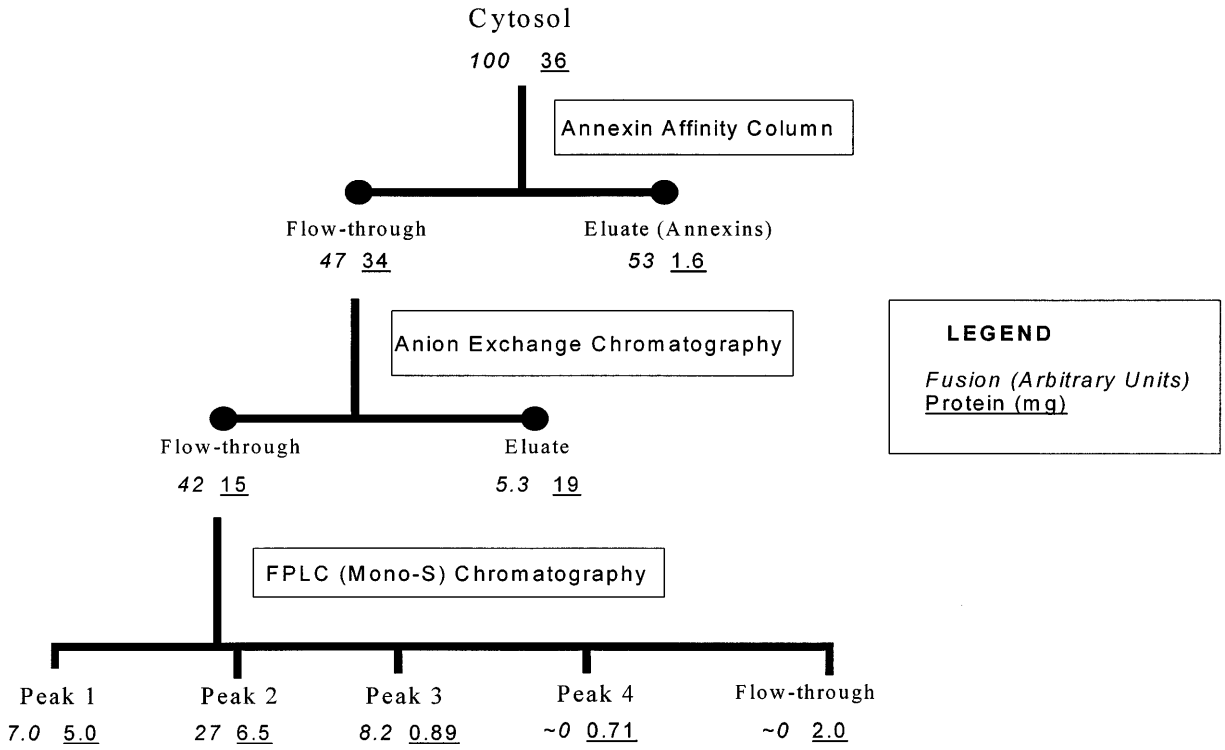

Fig. 1. Fractionation scheme for concentrating non-annexin fusogen. Protein (mg), shown underlined, and fusion activity (arbitrary units), shown in italics, of successive neutrophil cytosol preparatory fractions indicate that fusion activity was concentrated in FPLC peak 2 after 85% of total protein was removed. These results were compiled from five separate experiments.

vast majority of protein in the fusogenic peak 2 (lane 6) was in a low-molecular-weight range (about 10–15 kDa), whereas higher-molecular-weight bands were faint. These prevalent bands were believed to be the subunits of reduced leukocyte L1 protein, a 30-kDa multimer that is believed to constitute about 45% of total neutrophil cytosolic protein [15]. Identity was confirmed by reduced and non-reduced Western blots using antibodies to L1 (not shown).

Further purification of the fusion activity was attempted by use of size-selective membranes. Activity was retained by a 30-kDa cut-off filter, partially passed through a 50-kDa, and completely passed through a 100-kDa filter (Amicon 4208,

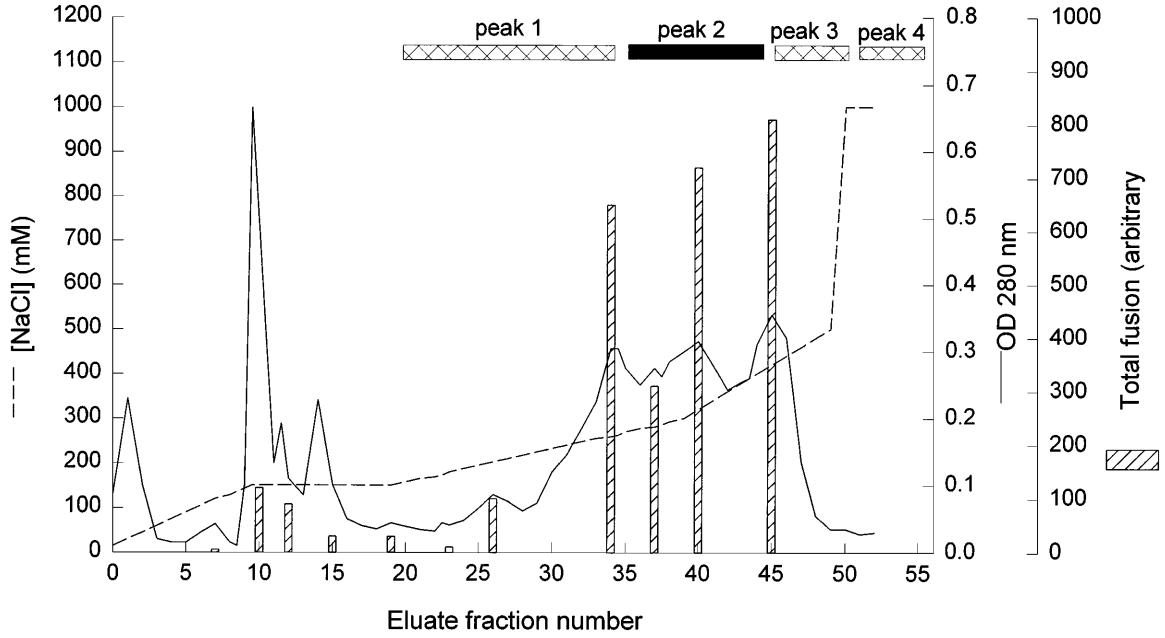

Fig. 2. Mono-S elution profile and fusion activity. Proteins were eluted from the Mono-S column using a NaCl gradient (0–1.0 M, at 1 mL/min). Samples were pooled, designated peaks 1, 2, 3, and 4, and flow-through. These fractions were assayed for fusion activity using NBD-Rh fusion assay with PA/PE liposomes (results are from a single representative experiment).

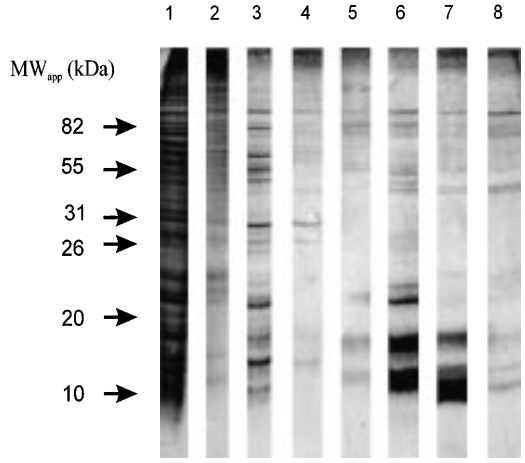

Fig. 3. SDS-PAGE of the purification of non-annexin fusogen. Silver-stained gel (14%) shows reduction of protein contaminants in relevant fractions. Lane 1, cytosol; lane 2, flow-through from phospholipid affinity column; lane 3, flow-through from DEAE anion exchange column; lane 4, flow-through from FPLC MonoS ion exchange column; lane 5, peak 1 (0.15–0.25 M NaCl) from FPLC; lane 6, peak 2 (0.25–0.35 M NaCl) from FPLC; lane 7, peak 3 (0.35–0.50 M NaCl) from FPLC; lane 8, peak 4 (0.50–1.00 M NaCl) from FPLC.

4224, and 4211, respectively; not shown). Thus the estimated molecular mass of the protein was between 30 and 100 kDa. Because L1 was a primary component of our preparation, we examined purified L1 isolated from human blood and authentic lyophilized L1 (generously donated by Kenneth Miyasaki and Nancy Hogg) [15, 16] for fusion activity. Both forms of authentic protein lacked any appreciable activity in our fusion assay (data not shown). We also attempted to immunodeplete the fusion activity from our preparation by use of MAC 387 (a commercial antibody against L1) and an antibody against L1 provided by Nancy Hogg. Although significant amounts of L1 were removed, the fusion activity was unaffected (not shown), indicating that L1 was not responsible for membrane fusion in our system.

By comparing multiple preparations from neutrophils and HL-60 cells, we found that fusogenic activity appeared to correlate with the presence of a 36-kDa band that often appeared in conjunction with L1. This band was purified from HL-60 cells, excised, and subjected to acid hydrolysis and sequencing. Peptide sequencing yielded a 100% homology match to human liver GAPDH. Identity was confirmed by Western blot using monoclonal antibody to GAPDH. As can be seen in **Figure 4**, a 36-kDa band persisted in fusogenic samples throughout the preparation. Lanes 1 and 2 contained authentic human GAPDH (positive control) and bovine serum albumin (negative control), respectively. Lane 3 contained crude cytosol, lanes 4 and 5 contained affinity column flow-through and eluate, lanes 6 and 7 contained anion-exchange column flow-through and eluate, lane 8 contained FPLC cation-exchange column flow-through, and lanes 9–12 contained FPLC peaks 1, 2, 3, and 4, respectively. FPLC peak 2 (lane 10) contained the majority of the GAPDH in the FPLC start material.

Assays of GAPDH enzymatic activity showed that the active protein was enriched throughout the preparation as protein was removed in column chromatography eluates. In particular, it was found that GAPDH segregated almost completely to the

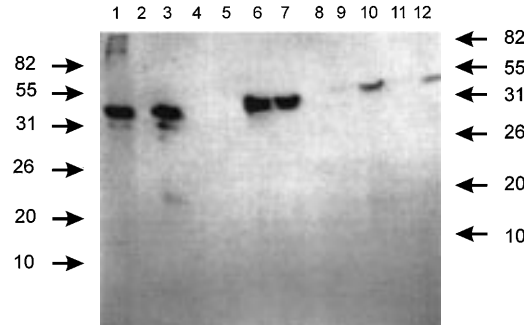

Fig. 4. Western blot analysis of GAPDH. Fractions containing the non-annexin fusogen were subjected to Western blot analysis using antibody against GAPDH. It can be seen that GAPDH segregated predominantly to Mono S Peak 2. Lane 1, GAPDH positive control; lane 2, albumin-negative control; lane 3, cytosol; lane 4, phospholipid affinity column flow-through; lane 5, phospholipid affinity column eluate; lane 6, DEAE anion exchange column flow-through; lane 7, DEAE anion exchange column eluate; lane 8, FPLC flow-through; lane 9, FPLC eluate peak 1 (0.15–0.25 M NaCl); lane 10, FPLC eluate peak 2 (0.25–0.35 M NaCl); lane 11, FPLC eluate peak 3 (0.35–0.50 M NaCl); lane 12, FPLC eluate peak 4 (0.50–1.00 M NaCl).

flow-throughs of the affinity- and anion-exchange columns. Unfortunately, enzyme (but not fusion) activity was lost after FPLC on the cation-exchange column. Thus, the enzyme assays provided additional evidence that GAPDH was concentrated in fractions with the highest fusion activity, at least until the final preparatory step.

We confirmed that commercial GAPDH from chicken muscle (but not from human erythrocytes, data not shown) promotes Ca^{2+}- and dose-dependent fusion of PA/PE liposomes with unlabeled complex liposomes (designed to mimic neutrophil membrane composition; **Fig. 5**) [17]. We observed that 1 unit (14 μg) of GAPDH was capable of inducing up to 45% of

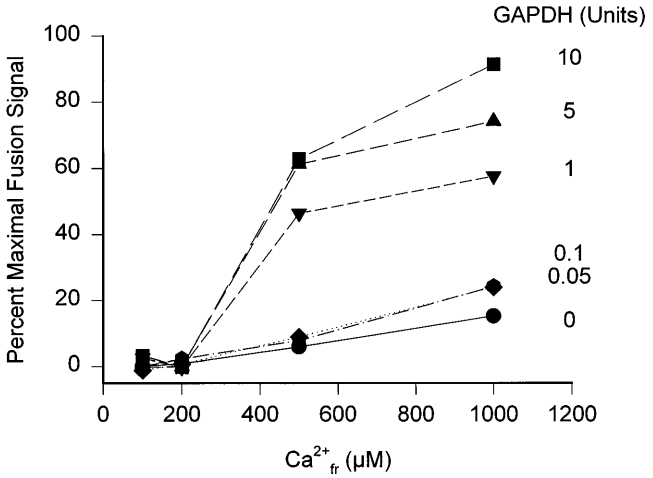

Fig. 5. Ca^{2+}- and dose-dependent fusion of commercial GAPDH. Standard fusion experiments were conducted using PA/PE (1:3) liposomes dually labeled with NBD-PE and Rh-PE along with unlabeled complex liposomes (designed to mimic neutrophil membrane composition) [17]. The liposomes were preincubated for 1 min with the indicated concentration of commercial (chicken muscle) GAPDH. Fusion was initiated with the addition of Ca^{2+} to the indicated level and was monitored for 90 s on an SLM spectrofluorometer (excitation, 450 nm; emission, 530 nm). The data shown are from a single representative experiment.

maximal fusion at 500 μM free Ca^{2+}. Higher concentrations of GAPDH or Ca^{2+} produced more fusion signal.

We also wished to compare our native fusogen to both annexin I and commercial GAPDH with respect to fusion activity. As shown in **Figure 6**, 1 unit (14 μg) of commercial GAPDH had very potent fusion activity. In addition, our semi-purified samples containing native GAPDH (derived from peak 2) were also highly fusogenic. As little as 4 μg (shown above to contain approximately 90% L1) could induce fusion equivalent to that of 4 μg human placenta annexin I (Fig. 6). Thus we propose that native neutrophil GAPDH in our hands may have up to 10 times the fusion activity of annexin I on a mass basis.

DISCUSSION

We have outlined here the identification of a 36-kDa protein partially purified from human neutrophils that can induce fusion of unilamellar liposomes. This protein, identified by sequencing as GAPDH, co-enriched with leukocyte L1 protein (which was not a fusogen). We confirmed prior findings [18–21] that GAPDH has potent fusion activity. We have also compared commercial as well as human neutrophil GAPDH fusion activity to annexin I, finding that GAPDH was more fusogenic on a mass basis. Because our non-annexin fusogen comprised at least 25% of the total cytosolic activity, we speculate that GAPDH may be an important contributor to the degranulation process.

This conclusion must be tempered with two considerations. The first is that some contaminant in the commercial GAPDH preparation might be responsible for the observed fusion. Sigma reports that this preparation is 95% pure protein, with $<0.01\%$ 3-phosphoglyceric phosphokinase and $<0.1\%$ triosephosphate isomerase. These contamination levels are not only very low but to our knowledge neither of these enzymes has been reported to possess fusion activity. Also, it is unlikely that the many preparations of GAPDH that have been reported with fusion activity [18–21] would all possess the same putative fusogenic contaminant. The second consideration is that GAPDH, like other glycolytic enzymes, is usually found both free in the cytosol and bound to cytoskeleton [22–24]. We do not know how much of the enzyme is free in neutrophils nor indeed whether the bound enzyme has any fusogenic activity. Hence, the impact of this distributed state of GAPDH on our findings is unclear.

The annexins are a class of Ca^{2+}-dependent phospholipid binding proteins found in neutrophils and other secretory cells. Annexin I has high affinity for both PS and PA [10], whereas GAPDH does not bind to PS. In fact, this difference was exploited in the isolation of GAPDH. Extremely small amounts of PA are found in resting neutrophils. However, after activation of the cells, membrane phosphatidylcholine is hydrolyzed to PA by phospholipase D [25], resulting in transient elevated levels of intracellular PA. It is hypothesized that granule-plasma membrane apposition and fusion may in part be mediated by the annexins through the newly generated PA [10, 26]. GAPDH, which has no appreciable affinity for PS, appears to be a potent endogenous fusogen that may also mediate granule-plasma membrane fusion through PA.

Multiple potential interactions between GAPDH and membrane phospholipids have been previously reported. Sidorowicz et al. [27] found that interaction of rabbit muscle GAPDH with negatively charged phospholipids decreases enzymatic activity, and that nicotinamide adenine dinucleotide could protect GAPDH from inactivation by membrane phosphatidylinositol. Glaser et al. [18] proposed that a GAPDH isoform isolated from rabbit brain cytosol could mediate Ca^{2+}-independent fusion of complex liposomes constructed to resemble a biological membrane (PC 27%, PS 6%, cholesterol 40%, and plasmenylethanolamine 27%). This fusion was believed to be due to lipid phase changes of plasmenylethanolamine mediated by GAPDH that could not be achieved with phosphatidylethanolamine [19]. Although Ca^{2+} was not required for catalysis of membrane fusion in their system, it modulated the activity of the isoform examined. In addition, isoforms that had dehydrogenase activity were distinguished from isoforms that catalyzed membrane fusion activity. This could be related to our observation of an apparent loss of enzymatic activity in the final FPLC fractions, which retained fusion activity (data not shown).

Direct interactions between GAPDH and lipids were investigated by Vinals et al. [20]. These investigators induced optimal fusion of liposomes (9 PC:1 PA) with commercial GAPDH by lowering pH to 5.0, demonstrating that increasing negative charge on phospholipid vesicles also increased fusion potential in the presence of GAPDH. However, Morero et al. [21] demonstrated Ca^{2+}-independent fusion of 10% PA liposomes mediated by commercial GAPDH, questioning whether GAPDH fusogenicity was due solely to its affinity for acidic phospholipids. We also detected low levels of Ca^{2+}-independent aggregation and fusion activities, but this accounted for only a small

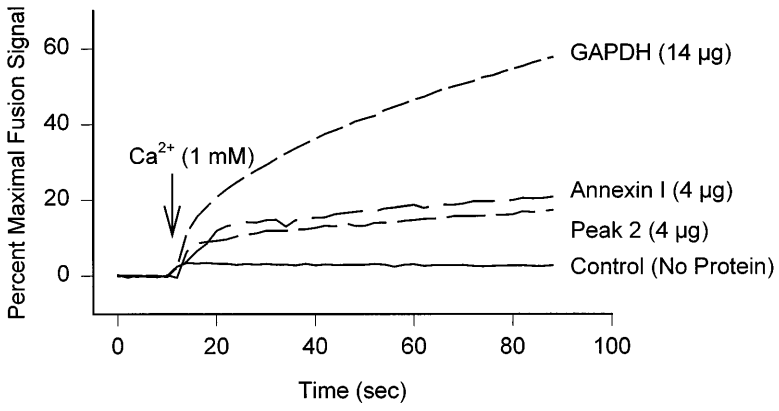

Fig. 6. Comparative fusion activity between commercial GAPDH, peak 2 and annexin I. Standard fusion experiments were performed as described in the legend to Figure 4, except that a fixed $[Ca^{2+}]$ of 1 mM was used and actual fluorimeter traces are shown. The data compare the fusion potential of equivalent masses of peak 2 (partially purified endogenous GAPDH) and annexin I. A larger mass of commercial GAPDH is shown for comparison. The data are from a single representative experiment.

fraction of that achievable with Ca^{2+} (not shown). We have also shown that GAPDH in our fusion system apparently has a potential for Ca^{2+}-mediated fusion that is at least equivalent to annexin I. Robbins et al. [28] recently discovered in late endosomes that a single amino acid substitution in GAPDH caused a marked difference in endocytosis of liquid phase markers. Thus, it can be seen in an unrelated system that GAPDH appears to serve in a membrane-membrane fusion capacity.

Our data suggest a possible role for GAPDH in the neutrophil degranulation process. Although fusogenic samples were not purified to a single protein, our experimental data and related research by other laboratories clearly implicate GAPDH as a membrane fusion mediator. Further study may reveal this protein to be an as yet unexplored factor of the degranulation process.

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