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Identification and Cloning of the SNARE Proteins VAMP-2 and Syntaxin-4 from HL-60 Cells and Human Neutrophils

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Abstract—Degranulation and membrane fusion by neutrophils are essential to host defense. We sought homologues of neuron-specific fusion proteins in human neutrophils and in their precursors, the promyelocytic cell line HL-60. We screened a differentiated HL-60 library and obtained an 848 bp sequence with a 351 bp open reading frame, identical to that published for human VAMP-2 and including 5′ and 3′ untranslated regions. RNA from HL-60 cells during differentiation into the neutrophil lineage was subjected to Northern blot analysis, which revealed a transcript of ~1050 bp at all stages of differentiation. The amount of these transcripts increased approximately threefold during differentiation, a finding confirmed by quantitative RT-PCR. We also detected mRNA for VAMP-2 in human neutrophils and monocytes using RT-PCR. In like fashion, transcripts of syntaxin-4, another fusion protein, were recovered from a neutrophil cDNA library. As with VAMP-2, expression of syntaxin-4 (determined by Northern blots) also increased, but by only 50%, during differentiation of HL-60 cells. These studies demonstrate that neutrophils and their progenitors possess mRNA for the fusion proteins VAMP-2 and syntaxin-4, and that their transcription increases during differentiation, concurrent with the functional maturation of myeloid cells.

KEY WORDS: fusion; VAMP-2; syntaxin-4; synaptobrevin; neutrophil; HL-60 cells.

INTRODUCTION

Exposure of human neutrophils to a variety of particulate and soluble stimuli evokes a series of responses, including chemotaxis, phagocytosis, degranulation, hexose monophosphate shunt stimulation, generation of reactive derivatives of oxygen, release of membrane-bound calcium and reorganization of the cytoskleton (1, 2). The most efficient oxygen-dependent microbicidal system in phagocytes, the myeloperoxidase-H_2O_2-halide system, requires concurrent degranulation and oxidase activation for full potency. Despite its important contribution to microbicidal activity, the least understood of agonist-dependent neutrophil responses is degranulation and the associated fusion events that occur between the granule and plasma or phagosomal membrane. With that in mind, we focused our attention on the annexin and nonannexin elements that regulate fusion in neutrophils.

Synaptosomes, endosomes and Golgi are among the few experimental systems in which *in vitro* fusion has been studied under physiological conditions (3–8). In these systems, several proteins are required at different stages of the docking and fusion process, with various factors often being required transiently during the chore-

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ography. For example, the final fusion steps generally involve a protein that is sensitive to N-ethyl maleimide (designated "NEM-sensitive fusion protein" or NSF), Soluble NSF-Attachment Proteins (SNAP), ATP, acetyl-CoA, vesicle associated membrane protein (VAMP), SNAP-25, syntaxin, synaptotagmin, rabphilin and unc-18. Taken together, these elements represent components of the SNARE (SNAP-Receptor) complex.

Because similar fusion events and requirements have been defined in endosome fractions over a broad range of species and cell types (9–13), an analogous set of factors likely regulates fusion in neutrophils. However, the lack of evidence for NSF-like proteins in neutrophils suggests that neutrophils may exploit unique variations of the neuronal model to achieve the same effect. Indeed, neutrophil appear to lack the entire "neuronal complement" of fusion proteins, as only SNAP-25 (14), syntaxin-1 (15), syntaxin-4 and VAMP-2 (16) have been detected in neutrophils at the protein level. mRNA transcripts for SNAP-23 have been reported in HL-60 cells (17). Recently, several forms of syntaxin were found in neutrophils and HL-60 cells (15) and we have identified annexin I (18) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (19), neither of which is part of the standard neuronal model, as important cytosolic fusion proteins in neutrophils.

Based on these data, we examined expression of VAMP-2 and syntaxin-4 in HL-60 cells and neutrophils. cDNA encoding VAMP-2 was isolated from a differentiated HL-60 library and sequenced, as was a partial sequence for syntaxin-4. Expression of genes encoding VAMP-2 and syntaxin-4 increased during differentiation of HL-60 cells. Circulating neutrophils (cells exhibiting relatively little protein synthesis) and monocytes produced mRNA for both VAMP-2 and syntaxin-4. Taken together, our studies extend findings of previous reports and are the first to show that neutrophils actively produce mRNA for these SNARE proteins.

MATERIALS AND METHODS

Reagents

Agarose (low melting point) and Dulbecco's phosphate buffered saline were obtained from Gibco (Grand Island, NY). RNAsin ribonuclease inhibitor, reverse transcriptase (avian myeloblastosis virus) and EcoRI were purchased from Promega (Madison, WI). [α-^{32}P]-dCTP was purchased from New England Nuclear (Boston, MA). Hybond nylon membranes and Rapid-Hyb buffer were obtained from Amersham (Arlington Heights, IL). Guanidinium thiocyanate was purchased from Fluka (Switzerland). Mono-Poly Resolving Medium was purchased from ICN Biomedicals (Aurora OH). NZCYM agar and NZCYM top agar were purchased from Bio 101, Inc. (Vista, CA). Fast Track mRNA Isolation Kits and pcDNA3.1 vector were purchased from Invitrogen (Carlsbad, CA). Random Priming Labelling Kits, pBlueScript II vector, XL1-Blue, Able C, and Able K were obtained from Stratagene (La Jolla, CA). The PCR MIMIC Construction Kit was purchased from Clontech (Palo Alto, CA). Taq polymerase was purchased from Fisher (Pittsburgh, PA). Human serum albumin was purchased from Alpha Therapeutic Corporation (Los Angeles, CA).

Cells

Venous blood from normal healthy adult donors was collected in 10 U/ml heparin (Elkins-Sinn, Inc., Cherry Hill, NJ). Neutrophils were isolated using Ficoll-Hypaque gradients (Mono-Poly medium, ICN Biomedicals, Aurora, OH) (20). Cells were washed and resuspended in Dulbecco's Phosphate Buffered Saline without Ca^{2+} or Mg^{2+} at 4° and pH 7.4, along with 0.1% human serum albumin. Monocytes were also isolated by this method and routinely subjected to parallel analyses.

HL-60 cells were grown in RPMI 1640 supplemented with 20% fetal calf serum and 1% penicillin–streptomycin. For studies on differentiated cells, 1.25% dimethyl sulfoxide (DMSO) was employed, with samples being taken every day for 4 days.

Libraries

For the majority of this work, a library for differentiated HL-60 cells was employed (21). The mRNA was derived from HL60 cells grown in 1.25% DMSO for 4 days. Corresponding cDNA was cloned into the EcoRI site of lambda ZAP. An undifferentiated HL-60 library in lambda ZAP was the generous gift of Dr. George Dubyak, Case Western Reserve School of Medicine, Cleveland, OH. A neutrophil library in the lambda ZAP Express cloning vector pBK-CMV was generously provided by Drs. Inder Patel and Ashok Amin, Hospital for Joint Diseases, New York University Medical Center, New York, NY. This library was prepared from an atopic patient and contains other cells, such as eosinophils, along with activated neutrophils. A human brain library in lambda ZAP was the generous gift of Dr. A. Zaheer,

Department of Neurology, University of Iowa School of Medicine, Iowa City, IA.

Library Screens

The differentiated HL-60 library (a total of 1×10^6 plaque forming units) was employed to screen for cDNA to VAMP-2. For syntaxin-4, we transformed pBK-CMV in XL-1 Blue cells and screened a total of 10^6 colony-forming units. A radiolabeled probe for VAMP-2 was prepared using the full-length sequence obtained by PCR screening of the total HL-60 library cDNA (1061B4B, *vide infra*). For syntaxin-4, the probe was a cDNA an 820 nt portion of the coding region of human muscle syntaxin-4 (a gift from Dr. M. N. Jagadish, Parkville, Australia) (22). The probes were labeled with [^{32}P] by random priming (Stratagene, La Jolla, CA), incubated with the blots (prehybridized and blocked with Rapid-Hyb Buffer, Amersham) at 65° for 2 h, and then washed at high stringency (0.2X SSC/0.1% SDS). Following autoradiography, several positive clones were identified and used for further studies. We conducted secondary, tertiary, and quaternary screening until we obtained a single positive clone.

Northern Blots

Total RNA was obtained from HL-60 cells (~10^8 total) that were differentiated with 1.2% DMSO for varying periods of time (0 to 4 days) and also from isolated neutrophils and monocytes using RNAzol solution (23). Poly-T-coated beads (Invitrogen, Carlsbad, CA) were sometimes used to isolate mRNA from these samples. For the Northern blots, total RNA (20 μg) or mRNA (4 μg) were subjected to electrophoresis on 1% formaldehyde gels, and then transferred to Hybond nylon membranes (Amersham). Probe cDNA (obtained by PCR screening of the HL-60 library as described under Results) was labeled by random priming, and the blots were hybridized and washed at high stringency as described previously. For a positive control, the coding region of p47^{phox} was also employed (21). As a measure of loading, a sequence of the coding region of GAPDH (nucleotides 2-775, obtained with primers G1 and G2, below) was employed. The developed autoradiographs were scanned using Corel Photopaint and the intensities of bands were quantitated with ImageQuant software. Background subtraction was generally done by "local average," according to the software. These intensity values were then expressed as ratios of VAMP-2/GAPDH, syntaxin-4/GAPDH or p47^{phox}/GAPDH, normalized to Day 0 (VAMP-2 or syntaxin-4) or Day 2 (p47^{phox}). In some cases, intensities of 28S rRNA were used for normalization in place of GAPDH.

Primers for PCR

The primers employed in this work for both PCR and RT-PCR are given in the following. In some cases, minor modifications of the neuronal VAMP-2 sequence were made to avoid hairpins, primer dimers, and primer-pair interactions.

Primer A 5′-AATCTTACCAGTAACAGGAGA-3′ Upper primer corresponding to bp 71–92 of coding region of human VAMP-2 (351 bp total).

Primer B 5′-AGTGCTGAAGTAAACGATGAT-3′ Lower primer corresponding to bp 327–347 of coding region of human VAMP-2 (351 bp total).

Primer C 5′-ATACGAATTCAAACCTCACCAGT-AACAGGAGA-3′ Same as Primer A, except for EcoRI site added.

Primer D 5′-ATCCGAATTCAAGTGCTGAAGTA-AACTATGAT-3′ Same as Primer B, except for EcoRI site added.

Primer E 5′-TTGGGGCTAGTCAGGAAAGAAA G-3′ Lower Primer for the full-length clone designated M40D1. This primer corresponds to bp 665-686 of M40D1 and was used for sequencing.

Primer F 5′-CAGGCCCAGGTGGATGAGGT-3′ Upper Sequencing Primer for M40D1, corresponding to bp 134–153 of M40D1 and 105–124 of the coding region.

Primer G1 5′-GATCGAATTCGCCAGCCGAGCC-ACA TCG-3′ Upper Primer for human GAPDH, corresponding to nucleotides 2 to 21 of the coding region along with an EcoRI site at the 5′ end.

Primer G2 5′-GATCGGTACCGGCAGGTCAGGTC-CAC CACT-3′ Lower Primer for human GAPDH, corresponding to nucleotides 756 to 775 of the coding region along with a Kpn-1 site at the 3′ end of the product.

Primer S1 5′-ATGCGCGACAGGACCCACGAG-CTG-3′ Upper primer corresponding to bp 1–24 of coding region of human syntaxin-4 (894 bp total) (22).

Primer S2 5′-TTATCCAACCACTGTGACGCCAAT-

GAT-3′ Lower primer corresponding to bp 867–894 of coding region of human syntaxin-4 (894 bp total) (22).

RT-PCR

Total RNA was isolated as before from human neutrophils, monocytes, and HL-60 cells that were differentiated with 1.2% DMSO over a 4-day time course. Aliquots of total RNA (1 μg) were prepared and incubated with reverse transcriptase according to manufacturer's instructions (Promega). Antisense primers were Primer D (VAMP-2), Primer S2 (syntaxin-4), or Primer G2 (GAPDH). The conditions of PCR amplification were as follows: initial denaturation at 95° for 3 min, each cycle of PCR including denaturation at 94° for 1 min, annealing at 60° for 2 min, and elongation at 72° for 3 min. Regular PCR was conducted in a similar manner, except that annealing temperature, primers, and template concentrations were routinely varied.

Quantitative RT-PCR

We constructed internal inhibitors according to instructions in the Clontech kit. For VAMP-2, we created elongated versions of Primers C and D that included selected sequences of the MIMIC DNA fragment. PCR using these modified primers and the MIMIC DNA as a template produced an inhibitor fragment of approximately 650 bp. This fragment was then introduced in known quantities into RT-PCR reactions for VAMP-2 in which a 350-bp fragment would ordinarily be produced. Increasing concentrations of the inhibitor decreased production of the 350-bp fragment and increased production of the 650-bp fragment. The intensities of the respective bands, I for inhibitor and N for normal, were measured as a function of inhibitor concentration C. A plot of the log (N/I) vs log (C) was linear and the x-intercept was used to quantitate the nominal amount of VAMP-2 template in the reactions.

Similar protocols were used for GAPDH. In this cases, the inhibitor was constructed to be approximately 350 bp in length and was derived from primers G1 and G2 (GAPDH). This inhibitor competed with a product of greater length, namely, 774 bp for GAPDH.

Sequencing

Sequencing was conducted by Molecular Biology Center for Research core facilities at Baylor College of Medicine.

RESULTS

It has been reported that neutrophils express proteins that are immunochemically related to syntaxin-1 (15), syntaxin-4, and VAMP-2 (16). Because promyelocytic leukemia cell lines are commonly used as cultured models for neutrophils, we wished to examine expression of these molecules in HL-60 cells. In so doing, we also hoped to obtain sequence information concerning the protein in myeloid cells.

Library Screens

VAMP-2

Using primers derived from the sequence of neuronal VAMP-2, we used PCR to screen a differentiated HL-60 cDNA library for related sequences. As a positive control, we used a library for human brain, from which we could reasonably expect to obtain cDNA for neuronal fusion proteins. Primer pairs A/B and C/D, which covered most of the coding region, produced a 278-bp fragment from the pooled cDNA of the neuronal library. Using primer pair C/D, this neuronal fragment was cloned into the EcoRI site of pBluescript. The insert was sequenced and demonstrated to correspond to human VAMP-2. Using primer pair C/D, the pooled cDNA of the differentiated HL-60 library was screened with PCR—again, a 278 bp fragment (designated 1061B4B) was cloned and identified as VAMP-2 by sequencing. Thus, it appeared that the HL-60 library contained cDNA for authentic VAMP-2.

In order to obtain the full length sequence for this cDNA, we screened the library by standard plaque techniques, using 300,000–1,000,000 plaque forming units (pfu). We employed the partial coding sequence 1061B4B as our probe. We obtained a single clone (designated M40D1), 848 bp long, containing an open reading frame with the entire coding region for human VAMP-2, with both 5′- and 3′-untranslated regions. This sequence was identical to that published for the human neuronal protein and contained portions of all five published exons (24).

Syntaxin-4

In a similar manner, we screened several libraries for sequences homologous to syntaxin-4. As a probe, we employed an 820 nt segment of the 5′ coding region of human muscle syntaxin-4, kindly provided by M. N. Jagadish (22). Using high stringency, we were unable

to detect any homologous sequences in HL-60 libraries derived from either differentiated or undifferentiated cells. However, a screen of a human "neutrophil" library (derived from a mixture of activated leukocytes) yielded a single partial sequence. This fragment starts at nt 411 in human muscle syntaxin-4 and contains the full 3′ end of the authentic sequence. The 3′-untranslated region is included, followed by a poly-A sequence.

Northern Blots

In order to determine the expression of these sequences in mRNA, we differentiated HL-60 cells with 1.2% DMSO and withdrew samples at 1-day intervals over a period of 4 days. RNA was isolated from cells at each time point, separated by agarose gel electrophoresis in 1% formaldehyde, and blotted to nylon filters. Filters were probed with radiolabeled 1061B4B, the 278-bp coding sequence for VAMP-2, and subsequently with the 820 nt probe for syntaxin-4. As a positive control, we used the coding sequence of $p47^{phox}$, a component of the phagocyte oxidase system that is expressed during differentiation of HL-60 cells into the neutrophil lineage. Finally, a 774 nt sequence of the coding region of GAPDH was used to assess loading of the blots.

VAMP-2

Using either total RNA or mRNA purified by binding to poly-T-coated beads, we found that the VAMP-2 probe consistently revealed a band at ~1050 bp (not shown). Another band, at ~1.5 kb, was also found on most blots of total and mRNA (not shown). Its abundance paralleled the 1050-bp species and it may represent a precursor or alternatively spliced form. The size of the 1050-bp band is consistent with that of our probe and suggests that most of the sequence of the mRNA is in hand. Northern blot analysis for VAMP-2 using mRNA is shown in the upper part of Fig. 3. Relative loading could be assessed either by blotting for GAPDH or by the staining of the 28S ribosomal RNA bands (as done in this experiment). In either case, the intensities of the bands were measured and expressed as ratios of VAMP-2 to GAPDH or rRNA. In all cases, the ratio observed for HL-60 cells on Day 0 was arbitrarily set at 1.0. As can be seen at the top of Fig. 1, expression of VAMP-2 increased during differentiation of HL-60 cells with DMSO. The ratio of VAMP-2 to 28S rRNA in this experiment increased modestly on Days 1 and 2 and then to threefold or greater on subsequent days.

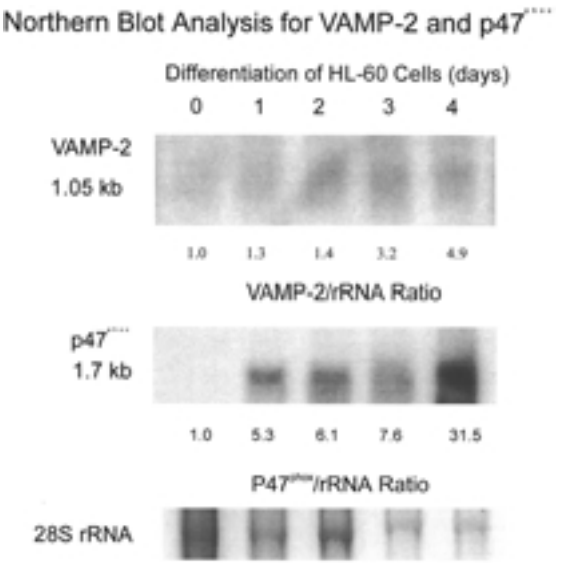

Fig. 1. Northern Blot Analyses for VAMP-2 and $p47^{phox}$. Total RNA from HL-60 cells that were differentiated with 1.2% DMSO was obtained over a period of 4 days. Poly-T-coated beads were used to isolate mRNA from these samples. For the Northern blots, total RNA (20 μg) or mRNA (4 μg) were subjected to electrophoresis on 1% formaldehyde gels. Molecular weight markers were provided by 28S and 18S rRNA. After electrophoresis, the RNA was transferred to nylon filters and hybridized with probes for VAMP-2, $p47^{phox}$, or GAPDH. Total RNA probed for VAMP-2 is shown in the top panel and was exposed for 3 days. The middle panel shows total RNA probed for $p47^{phox}$ and was exposed for 1 day. Ribosomal RNA (28S) bands were scanned and inverted in intensity (lower panel). The intensities of each band were measured with ImageQuant software. The software automatically subtracted background values from areas of the film surrounding each identified band. These values were then expressed as ratios of VAMP-2/rRNA or $p47^{phox}$/rRNA, normalized to Day 0, and are shown on the figure. See Materials and Methods for further details.

We used $p47^{phox}$ as a positive control for a protein that is upregulated during differentiation (21). As expected, the $p47^{phox}$ probe hybridized strongly at 1.7 kb and increased from virtually nothing at Day 0 to a maximum by Day 4. This pattern differed from that for VAMP-2 in that $p47^{phox}$ was virtually undetectable in undifferentiated cells, while some VAMP-2 was observed at all times.

The results from several Northern blots are combined in Fig. 2. VAMP-2 was normalized with GAPDH as well as 28S rRNA. It can be seen that on average, the expression of VAMP-2 by HL-60 cells increased threefold over the 4 days of the experiment. This was observed when expressed as the VAMP-2/rRNA ratio (n = 4). In several experiments, the GAPDH/rRNA ratio also increased during differentiation (not shown).

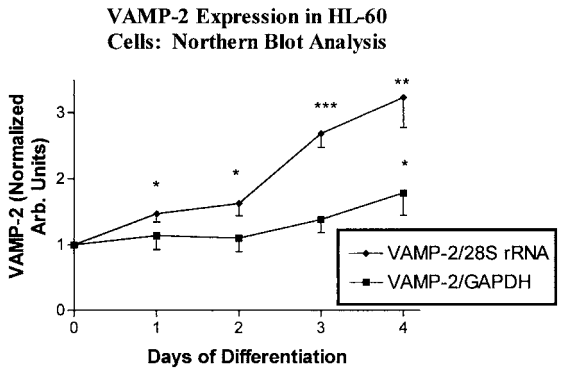

Fig. 2. Compilation of Northern Blot analyses for VAMP-2. Total RNA was obtained from HL-60 cells as described previously and probed for VAMP-2. The blots were stripped and then rehybridized with a probe consisting of the coding region for GAPDH. Autoradiograms were developed and signal intensities were measured as described under Materials and Methods and in the legend to Fig. 1. Intensities of the 28S rRNA bands were also measured. These values were then expressed as ratios of VAMP-2/GAPDH or VAMP-2/28S rRNA, normalized to Day 0 and are shown on the figure. The data shown are the means (±SEM) compiled from five to six separate experiments for VAMP-2/GAPDH and four to five experiments for VAMP-2/28S rRNA. The levels of significance (using a paired *t* test) were calculated for differences from the Day 0 values (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.002$). See Materials and Methods for further details.

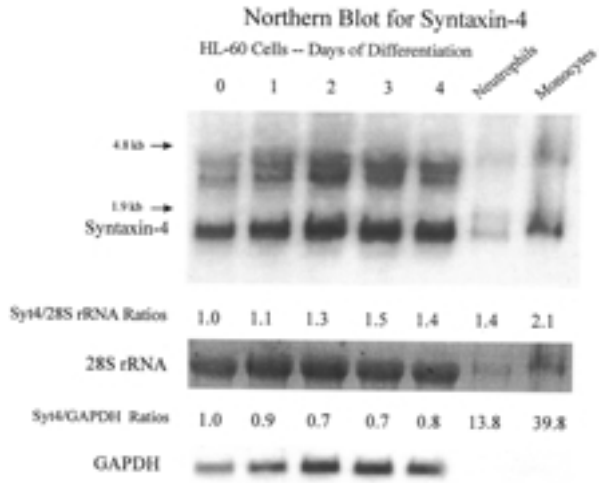

Fig. 3. Northern Blot analysis for Syntaxin-4. Total RNA was obtained from HL-60 cells as described previously. Neutrophils and monocytes were also isolated from human blood and total RNA was extracted. These samples were subjected to gel electrophoresis and Northern blotting for syntaxin-4 (using an 820 nt section of the coding region as a probe). The blots were stripped and then rehybridized with a probe consisting of the coding region for GAPDH. The panels show the Northern blots for syntaxin-4 (top), the inverted 28S rRNA bands (middle), and the Northern blot for GAPDH (bottom). Signal intensities were measured as described under Materials and Methods and in the legend to Fig. 1. These values were then expressed as ratios of syntaxin-4/GAPDH or syntaxin-4/28S rRNA, normalized to Day 0 and are shown on the figure. See Materials and Methods for further details.

As a consequence, the VAMP-2/GAPDH ratio did not increase in a statistically significant manner except on Day 4.

Compared to HL-60 cells, both neutrophils and monocytes had substantially higher expression of VAMP-2. With expression by undifferentiated HL-60 cells being defined as 1.0, the VAMP-2/rRNA ratios were 6.1 ± 5.0 ($n = 3$) for neutrophils and 2.7 ± 1.7 ($n = 3$) for monocytes; VAMP-2/GAPDH ratios were 67 ± 21 ($n = 6$) for neutrophils and 37 ± 21 ($n = 6$) for monocytes.

Syntaxin-4

In a similar manner, we studied the expression of syntaxin-4 during the differentiation of HL-60 cells. Using a probe derived from the coding region of human muscle syntaxin-4, we found three bands to be present during differentiation (Fig. 3). The strongest band was found at ~1.5 kb, a size consistent with the existence of a 894 nt coding region. This band increased ~50% during differentiation (as a ratio with 28S rRNA). The increase in GAPDH during differentiation meant that the syntaxin-4/GAPDH ratio was unchanged. Higher weight transcripts at ~3 kb were also found, suggesting that some transcriptional regulation and processing or alternative splicing was occurring. The 1.5-kb bands were also detected in RNA preparations from neutrophils and monocytes (Fig. 3). The ratios of syntaxin-4/GADPH were substantially higher than for HL-60 cells (defined as 1.0)—14.7 ± 5.6 ($n = 5$) for neutrophils and 14.3 ± 6.7 ($n = 5$) for monocytes. The syntaxin-4/28S rRNA ratios for all three cell types were roughly comparable—0.95 ± 0.34 ($n = 3$) for neutrophils and 1.5 ± 0.4 ($n = 3$) for monocytes.

We performed Northern blot analysis in several separate experiments, the combined results of which are shown in Fig. 4. A pattern similar to that for VAMP-2 (Fig. 2) was found for syntaxin-4. On average, the expression of syntaxin-4 by HL-60 cells, measured as syntaxin-4/28S rRNA, increased by 50% over the 4 days of the experiment (much less than the threefold increase found to VAMP-2). This increase was statistically significant by Day 4. As before, concomitant increases in the expression of GAPDH prevented the ratio of syntaxin-4/GAPDH from achieving a high level of significance.

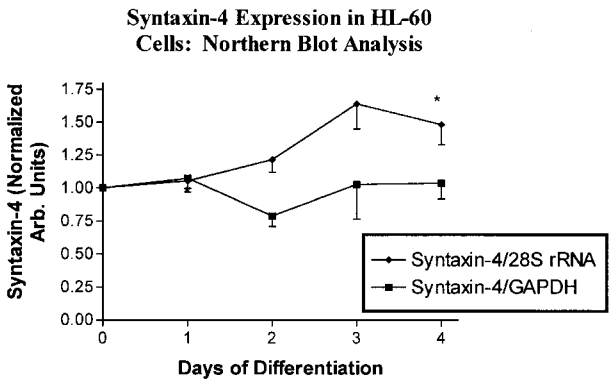

Fig. 4. Compilation of Northern Blot analysis for Syntaxin-4. Total RNA was obtained from HL-60 cells as described previously and probed for syntaxin-4. The blots were stripped and then rehybridized with a probe consisting of the coding region for GAPDH. Autoradiograms were developed and signal intensities were measured as described under Materials and Methods and in the legend to Fig. 1. Intensities of the 28S rRNA bands were also measured. These values were then expressed as ratios of syntaxin-4/GAPDH or syntaxin-4/28S rRNA, normalized to Day 0 and are shown on the figure. The data shown are the means (±SEM) compiled from two to four separate experiments for syntaxin-4/GAPDH and two to three experiments for syntaxin-4/28S rRNA. The levels of significance (using a one-tailed Student t test) were calculated for increases in the ratios above 1.0. For the syntaxin-4/28S rRNA ratio, the level at Day 4 was significantly ($p < 0.05$) greater than that on Day 0. See Materials and Methods for further details.

Quantitative RT-PCR

In order to get an independent measure of the level of mRNA expression for VAMP-2 and syntaxin-4, we performed quantitative RT-PCR. We first determined that our selected primers for VAMP-2 and GAPDH (see Methods) all produced RT-PCR products of the expected size and sequence from the total RNA of HL-60 cells. Using the MIMIC Construction Kit (Clontech), we prepared "internal inhibitors" for VAMP-2 and GAPDH. These internal inhibitors are sequences that have annealing sites for the primers, but are of distinctly different sizes than the authentic PCR products. Hence, in the PCR reaction, increasing amounts of the internal inhibitor will compete with the authentic template for primers, with both products being distinguishable following gel electrophoresis. Careful titration of the PCR reactions with varying amounts of the internal inhibitors permits quantitation of the amount of template in each RT reaction.

The absolute abundances of VAMP-2 and GAPDH RT products are shown in Table 1. The amount of VAMP-2 template in the RT reactions was comparable for HL-60 cells and neutrophils. However, monocytes possessed several-fold more of this template per unit of total RNA. For GAPDH transcripts, high abundances were found for HL-60 cells, with low and comparable quantities measured for neutrophils and monocytes. On a cellular basis, all three cell types had similar amounts of VAMP-2 template. For GAPDH, the amount of template was by far the greatest in HL-60 cells.

RT-PCR confirmed some quantitative aspects of the Northern blot data. First, that the quantity of transcript for VAMP-2 increased during differentiation. The amount of GAPDH transcript also increased, but to a lesser extent (Table 1). These data also demonstrated that monocyte contamination of the neutrophil preparations, generally found to be 5–10%, could not account for the abundance of transcript in the neutrophils.

We also used quantitative RT-PCR as an indepen-

Table 1. mRNA Levels Determined by Quantitative RT-PCR

Cell Type	VAMP-2[a] (per μg Total RNA)	VAMP-2[b] (per 10^6 cells)	GAPDH[a] (per μg Total RNA)	GAPDH[b] (per 10^6 cells)
HL-60 Day 0	2.6 ± 1.7 (6)	40.7 ± 20.7 (4)	23.5 ± 20.3 (6)	485 ± 303 (4)
HL-60 Day 4	11.9 ± 4.6 (5)	92.7 ± 81.7 (4)	32.3 ± 22.9 (5)	408 ± 365 (4)
Neutrophils	8.0 ± 4.2 (5)	15.9 ± 14.9 (3)	1.35 ± 0.50 (5)	1.24 ± 0.84 (3)
Monocytes	24.6 ± 8.4 (5)	25.2 ± 14.4 (3)	0.65 ± 3.0 (5)	0.53 ± 0.18 (3)

Note. Total RNA was extracted from unstimulated HL-60 cells, human neutrophils, and human peripheral blood monocytes. Aliquots of total RNA (1 μg) were used for the reverse transcriptase reaction as detailed under Materials and Methods. Levels of mRNA transcript were estimated by titrating with internal inhibitors, devised using the MIMIC system (Clontech). The data are presented as means ± SEM with number of experiments in parentheses.

[a] Attomoles/μg Total RNA.

[b] Attomoles/10^6 cells.

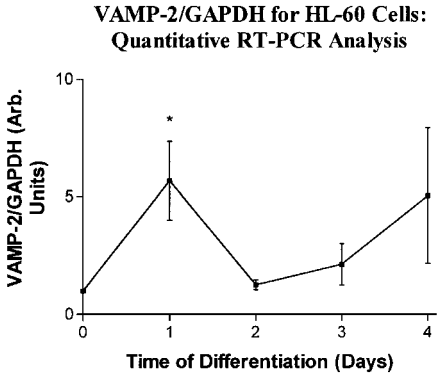

Fig. 5. Quantitative RT-PCR for VAMP-2 and GAPDH. Total RNA was extracted from HL-60 cells that were stimulated with 1.2% DMSO over a 4-day time course from human neutrophils and from human peripheral blood monocytes. Aliquots of total RNA (1 μg) were used for the reverse transcriptase reaction as detailed under Materials and Methods. Levels of mRNA transcript were estimated by titrating with internal inhibitors, devised using the MIMIC system (Clontech). Expression of VAMP-2 was normalized with respect to GAPDH; the ratio at Day 0 was defined as 1.0. Expression at different times are presented as means ±SEM (n = 4). The ratio at Day 1 was significantly different from Day 0 control (*, 1-way ANOVA with Dunnett post-test). The comparable ratios for neutrophils and monocytes were 35 + 27 and 193 ± 129, respectively (n = 5).

dent means to obtain VAMP-2/GAPDH ratios. As can be seen in Fig. 5, the ratio of VAMP-2 to GAPDH increased during the differentiation of HL-60 cells. The increase on Day 1 was statistically greater than the ratio found on Day 1.

DISCUSSION

Degranulation is one of the most important responses of neutrophils in inflammation and bacterial killing. Nonetheless, the prerequisite membrane fusion, involving membrane events employed by a cell engaged in endo- or exocytosis, has been difficult to model in the laboratory and is consequently poorly understood. Several recent advances, such as isolation of endogenous fusogens and development of contents-mixing fusion assays have permitted an investigation of membrane fusion and secretion by human neutrophils (18, 19, 25–28). However, because of the attention paid to the fusion complex associated with neuronal tissues and the apparent ubiquity of many members of this fusion complex in a variety of cell types (11, 13), we sought to determine if such proteins were being produced in HL-60 cells or neutrophils.

VAMP-2

Although previous studies reported immunochemical evidence for VAMP-2 protein in tertiary granules and secretory granules in neutrophils (16), little is known at the molecular level about the expression of VAMP-2 in myeloid cells. Following membrane fusion, VAMP-2 appeared to translocate to the plasma membrane following stimulation. However, this work was limited to assessing immunologically cross-reacting protein in neutrophils. Our studies reported here extend this earlier work by (1) showing that HL-60 cells, as well as resting neutrophils and monocytes, produced mRNA for VAMP-2; (2) that the abundance of VAMP-2 mRNA, assessed by quantiative RT-PCR, was similar in all three cell types when measured on a cellular basis; (3) that the nucleotide, and hence the protein, sequences were identical to those published for human VAMP-2; (4) the mRNA for VAMP-2 was produced in both differentiated and undifferentiated HL-60 cells; and (5) the mRNA for VAMP-2 increased approximately threefold during differentiation.

Screening a library constructed from the cDNA of differentiated HL-60 cells for VAMP-2, we obtained a single clone (termed M40D1). This sequence was 848 bp in length, containing an open reading frame, and was identical (in the coding sequence) to that reported for human VAMP-2, which is encoded in five exons (24). Our 848-bp sequence had 28 bp of 5′-untranslated sequence as well as 427 bp of 3′-untranslated sequence, that was rich in poly-A. Northern blots of total RNA at high stringency revealed a band of hybridization approximately 1050 bp. This length corresponds relatively well to the size of M40D1 and indicates that most of the VAMP-2 mRNA in the HL-60 cells is represented in M40D1. This cDNA covers more of the 3′ end of the published sequence (24) than it does of the 5′ end. Using M40D1 as a frame of reference, exon 1 of the published sequence covers from −919 to 37, with the ORF starting at bp 29. The remaining exons are relatively short. Exon 2 covers bp 5–158, exon 3 covers 129–317, exon 4 covers 290–368, and exon 5 covers 336–485. Except for the overlap regions between the exons, which contain sequence variability at the splicing sites, the published sequence is identical to that of M40D1 over the common 485-bp region.

The fidelity of the 3′ region of M40D1 can be illuminated by a published sequence of rat VAMP-2 (29) that is 2071 bp long. This sequence has 86% identity in the common overlap region and is almost completely

identical from bp 21 to 600. Between 600 and 848, the two sequences deviate substantially. Hence, M40D1 has virtual identity to published sequences, both within the coding regions and over mesial stretches of the 3′-untranslated end.

Our Northern blots using total RNA or isolated mRNA often showed an additional band at ~1.5 kb, suggesting that alternative splicing or processing is taking place at the mRNA level. Indeed, it appears that the abundance of mRNA increased three- to fourfold during the differentiation of HL-60 cells as measured by several different criteria. This was confirmed by Northern blot analysis (VAMP-2/28S rRNA ratios) and quantitative RT-PCR. It should be noted that the tedious nature of quantitative RT-PCR precluded the routine use of the technique.

A somewhat different trend was observed with $p47^{phox}$, our positive control. As expected (21), $p47^{phox}$ mRNA was virtually undetectable in undifferentiated HL-60 cells (in spite of the high loading of this lane) and increased rapidly during differentiation (Fig. 1). Hence, it appeared that VAMP-2 mRNA increased modestly during differentiation, while $p47^{phox}$ increased dramatically. Nevertheless, these increases in mRNA occur at a time when HL-60 cells become more competent with respect to both superoxide generation and degranulation. It is likely that enhanced transcription of VAMP-2 helps support degranulation in the differentiated cells.

We are also the first to measure of absolute abundances of mRNA, using quantitative RT-PCR, in these three cell types. On a cellular basis, HL-60 cells, neutrophils, and monocytes all had comparable amounts of transcript (Table 1). In contrast, HL-60 cells had far more transcript for GAPDH than did monocytes or neutrophils (Table 1). One possible explanation is that HL-60 cells in culture are actively undergoing proliferation and differentiation, requiring higher expression of housekeeping genes. Furthermore, neutrophils are notoriously deficient in both RNA (30) and protein (31, 32) synthesis, consistent with the relatively low levels found in Table 1. Indeed, we had previously noted that HL-60 cells were a particularly abundant source of GAPDH protein when we were isolating it as an endogenous fusogen (33).

The RT-PCR product from the RNA of human neutrophils had a sequence identical to that of HL-60 cells (not shown). This was gratifying since differentiated HL-60 cells are not identical to neutrophils, the two cells types differing in many details of biochemistry, cell biology, and function (34–36). Whereas the array of proteins actively synthesized by neutrophils is quite limited, our data from RT-PCR indicate that VAMP-2 is a member of this highly selective group of proteins. Such speculations are consistent with the possibility that VAMP-2 is an essential component of the degranulation apparatus that must be expressed in greater abundance as the cells become more competent in degranulation.

Syntaxin-4

Syntaxin-4 was originally detected in neutrophils by means of immunoblotting (16). Recently, Martin-Martin *et al.* used RT-PCR to detect expression of syntaxins 1A, 3, 4, 5, 6, 7, 9, 11, and 16 in neutrophils and HL-60 cells (15). They also reported that expression of syntaxins 3, 4, 6, and 11 increased during differentiation of HL-60 cells. Our studies extend this work by showing that (1) monocytes, in addition to HL-60 cells and neutrophils, express mRNA highly homologous to syntaxin-4 when examined by Northern blot; (2) Northern blot analysis confirms that a ~1.5 kb transcript, consistent in size with the 894 nt coding region of syntaxin-4, is increased during differentiation of HL-60 cells; (3) additional sequence information has been provided by screening a human neutrophil library.

As shown in Figs. 3 and 4, differentiation increased expression of syntaxin-4 approximately 50% in HL-60 cells. These findings are consistent with the possibility that enhanced synthesis of syntaxin-4 is, like VAMP-2, necessary for increased degranulation in mature HL-60 cells. However, this increase is far more modest than that for VAMP-2. It is possible that syntaxin-4 is normally produced in surplus even in undifferentiated cells. These data are also comparable to the observations of Martin-Martin *et al.* (15), although this group did not quantitate their findings. Our findings of comparable monocyte expression, but considerably lower neutrophil expression, were also observed by Martin-Martin *et al.* (15). However, because 28S rRNA or GAPDH are relatively scarce in neutrophils, the ratios of syntaxin-4/28S rRNA or syntaxin-4/GAPDH were comparable to those of monocytes (Fig. 3).

We obtained sequence information complementary to that of Martin-Martin *et al.* (15), who reported only the coding region. Our partial sequence, derived from a human neutrophil library, starts at nt 411, continues into the 3′-untranslated region and is followed by a poly-A sequence. This fragment begins downstream of a reported splice point containing an intron of 136 nt (15), that we consequently could not detect. Combining

our sequence information with that of Martin-Martin *et al.* (15) and including the 136 nt intron, we would expect a length of almost 1.3 kb. The 5′-untranslated region is unknown, but is likely to be no more than a few hundred base pairs. Hence, the putative transcript is consistent with the ~1.5 kb band that we observed in Fig. 5. It should be noted that longer transcripts were also observed in differentiating HL-60 cells (Fig. 3), suggesting additional processing of mRNA. It is also important to note that the neutrophil library did contain contaminants from other cell types (see Materials and Methods). Hence, it is possible that this complementary sequence information is not attributable to neutrophils.

Speculations—A Nontraditional Fusion Apparatus for Neutrophils?

It has recently been reported tha the full set of SNARE proteins is not necessary for docking and fusion of membranes. Rather, only VAMP-2 syntaxin-1A, and SNAP-25 are sufficient for docking and low-rate fusion (37–39). It is likely that such low-rate fusion could be greatly accelerated by other cofactors, such as phospholipases or annexins. These latter proteins are abundant in neutrophils and are activated following cell stimulation.

There is increasing evidence that the fusion proteins operating in cells of the myeloid lineage are substantially different from those in neurons. For example, VAMP-2, syntaxin-4, and secretory carrier membrane protein (SCAMP) were detected in neutrophils by Western blot (16). These investigators also reported that they were unable to find the classical neuronal components syntaxin-1, VAMP-1, SNAP-25, synaptophysin, and cellubrevin. Another research group did find SNAP-25 protein, but it was on the "wrong" vesicle (granule membrane rather than plasma membrane) (14). Human neutrophils and HL-60 cells have been reported to have two forms of SNAP-23 (17), based on molecular studies. SNAP-23 is non-neuronal and these researchers found that mRNA for this protein increased during differentiation of HL-60 cells. Interestingly, those components that have been detected in neutrophils and HL-60 cells, namely, VAMP-2, multiple syntaxins, and SNAP-23 (25), are homologues of the SNAREpin set (37).

It is possible that many cell types feature subsets of the neuronal system, along with other components, some of which might be unique. Neutrophils, for example, might have only VAMP-2 on the granule membranes as a direct representative on the neuronal complement. Myeloid cells have multiple forms of syntaxin (15) corresponding to SNAREpin syntaxin-1, although syntaxin-1 itself is found on the wrong (granule) membrane. The other neuronal components of the SNARE complex appear to be absent. The roles of these missing components might be played by other proteins known to induce fusion in neutrophils. For example, we have demonstrated that annexin I (18) and GAPDH (19) are cytosolic fusogens in neutrophils. We have also shown that phospholipase D, in conjunction with annexin I, is capable of promoting the aggregation of granules and artificial membranes (27). Finally, phospholipase A_2 that is activated in stimulated neutrophils (40–44) is exquisitely sensitive in promoting fusion (26). We expect that cells of the granulocytic lineage will have a set of fusion proteins that has both commonality with some elements of the neuronal complement, as well as uniqueness.

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