Co-expression of several human syntaxin genes in neutrophils and differentiating HL-60 cells: variant isoforms and detection of syntaxin 1

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Abstract: Syntaxins are major components of vesicle trafficking and their pattern of expression depends on the cell type. Using reverse transcriptase-polymerase chain reaction (RT-PCR), cloning, and sequencing techniques, we have found that human neutrophils and neutrophil-differentiated HL-60 cells co-express syntaxins 1A, 3, 4, 5, 6, 7, 9, 11, and 16. These genes are also expressed in human peripheral blood lymphocytes and SH-SY5Y neuroblastoma cells, which, unlike neutrophils, also expressed syntaxin 10. We have identified two isoforms of syntaxin 3. Syntaxin 3A, similar to the previously reported syntaxin 3, and the novel isoform syntaxin 3B, which is identical to syntaxin 3A but lacks 37 amino acid residues at the carboxy-terminal region. Syntaxin 1 was mainly located to neutrophil granule membranes by confocal microscopy and by immunoblotting of subcellular fractions. These data indicate that syntaxin 1 cannot be considered specific to neural tissues. The level of expression of syntaxins 3, 4, 6, and 11 was increased during neutrophil differentiation of HL-60 cells, whereas that of syntaxins 1A, 5, 9, and 16 was unchanged. Syntaxin 7 was not expressed in undifferentiated HL-60 cells, but its expression was induced on neutrophil differentiation. The expression of several syntaxin genes in human neutrophils could be related to the high secretory capacity of these cells as well as to the presence of different cytoplasmic granules with distinct exocytic capabilities. *J. Leukoc. Biol.* 65: 397–406; 1999.

Key Words: *SNARE · cytoplasmic granules · reverse transcriptase-polymerase chain reaction · polymorphonuclear leukocytes · lymphocytes · neuroblastoma*

INTRODUCTION

Human neutrophils constitute the first line of defense against infection and play a major role in inflammation. Exocytosis of the distinct granules present in human neutrophils plays a critical role in neutrophil biology and seems to regulate a number of important functions of these cells in both inflammation and infection, such as adhesion, extravasation, generation of reactive oxygen metabolites, and release of lytic enzymes. Furthermore, certain cytoplasmic granules constitute a reservoir of plasma membrane proteins that are mobilized to the cell surface on cell activation [1–5]. These mobilizable granules, named specific or secondary granules and gelatinase-containing tertiary granules, are readily exocytosed on neutrophil activation, whereas azurophilic granules are hardly mobilized [1, 6–8]. However, little is known about the underlying mechanisms that regulate the exocytic process in these cells.

In contrast, a great number of proteins have been identified in the neuronal tissue involved in the docking and fusion of synaptic vesicles with the plasma membrane [9–11]. This has led to the postulation of the SNARE hypothesis [9–11], which constitutes an attractive model for vesicle-membrane fusion in eukaryotic cells. According to the SNARE hypothesis, docking and fusion of vesicles with the plasma membrane seems to be modulated by the specific interaction of vesicle proteins (v-SNAREs) with target plasma membrane-located proteins (t-SNAREs). Both v- and t-SNAREs seem to be required on opposing membranes for membrane fusion [12, 13]. The prototype of v-SNARE is the synaptic vesicle membrane protein synaptobrevin, also named vesicle-associated membrane protein (VAMP). The prototypes of t-SNAREs are syntaxin and the 25-kDa synaptosome-associated protein (SNAP-25). SNAP-25 is mainly detected in neuronal tissues [14], but its presence in non-neuronal cells has also been recently

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Abbreviations: DMSO, dimethyl sulfoxide; EST, expressed sequence tag; RT-PCR, reverse transcriptase-polymerase chain reaction; SNARE, soluble *N*-ethylmaleimide-sensitive factor (NSF) attachment protein (SNAP) receptor; SNAP-25 and SNAP-23, 25- and 23-kDa synaptosome-associated protein; VAMP, vesicle-associated membrane protein; PBS, phosphate-buffered saline; PMSF, phenylmethylsulfonyl fluoride; SDS, sodium dodecyl sulfate; TBS, Tris-buffered saline.

The nucleotide sequence data reported in this work have the GenBank/EMBL accession numbers AJ000541 (syntaxin 4 precursor), AJ002076 (syntaxin 3A), and AJ002077 (syntaxin 3B).

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reported [15, 16]. Another recently reported protein named SNAP-23 [17], showing two isoforms A and B [18], seems to fulfill the role of SNAP-25 in non-neuronal mammalian cells. Syntaxins constitute a family of receptors for transport vesicles [19], and it has been suggested that each target membrane may be identified by a specific member of the syntaxin family [19]. Seven syntaxins have been previously identified and characterized, namely 1A, 1B, 2, 3, 4, 5, and 6 [19, 20]. Syntaxins are 255- to 301-amino acid-long proteins that contain α-helical coiled-coil regions, likely to be involved in protein-protein interactions, and also a region of highly hydrophobic amino acids at the extreme carboxyl terminus, which can serve as a membrane anchor. The fact that syntaxins 1A and 1B are only present in the nervous system and related tissues, as well as the diverse syntaxin distribution in distinct tissues [19–22], suggests that each cell type expresses a different set of syntaxins, and this could reflect peculiarities in cell-specific vesicle-membrane fusion processes. In addition, the existence of 10 new syntaxins has been postulated, numbered consecutively from syntaxin 7 to syntaxin 16, by sequence homology between well-known syntaxins and partial DNA sequences from EST database searches [23]. Some of these postulated new syntaxins have been cloned very recently, such as syntaxin 7 [24, 25], syntaxin 10 [26], syntaxin 11 [27], syntaxin 13 [28], and syntaxin 16 [29].

In this study we have demonstrated, using a PCR-based cloning and sequencing approach, that human neutrophils as well as neutrophil-differentiated HL-60 cells express a wide number of syntaxins, namely syntaxins 1A, 3, 4, 5, 6, 7, 9, 11, and 16. We have also identified and cloned two isoforms of syntaxin 3, namely syntaxin 3A (previous syntaxin 3) and the novel isoform syntaxin 3B. Furthermore, the presence of syntaxin 1 in human neutrophils was further assessed by immunoblotting and confocal microscopy studies using the monoclonal antibody HPC-1, able to recognize the distinct isoforms of syntaxin 1 [30, 31]. Thus, both cloning and immunological analyses demonstrate the expression of syntaxin 1 in human neutrophils, and thereby indicate that expression of syntaxin 1 is not exclusive of neural tissues.

MATERIALS AND METHODS

Cell culture and leukocyte isolation

The human promyelocytic leukemia HL-60 cell line was grown in RPMI-1640 medium supplemented with 10% (v/v) heat-inactivated fetal calf serum, 2 mM glutamine, 100 units/mL penicillin, and 24 μg/mL gentamicin at 37°C in a humidified atmosphere of air/CO_2 (19/1). Neutrophil differentiation of HL-60 cells was induced by adding 1.3% (v/v) dimethyl sulfoxide (DMSO) for the times indicated in the respective figure as previously described [32]. The human neuroblastoma SH-SY5Y cell line was grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) heat-inactivated fetal calf serum, 2 mM glutamine, 100 units/mL penicillin, and 24 μg/mL gentamicin at 37°C in a humidified atmosphere of air/CO_2 (19/1).

Neutrophils were obtained from fresh human peripheral blood by dextran sedimentation and centrifugation on Ficoll-Hypaque (Pharmacia LKB Biotechnology, Uppsala, Sweden), followed by hypotonic lysis of residual erythrocytes as previously described [33]. The gradient centrifugation was repeated once more and the final cell preparation contained more than 99% neutrophils as assessed by Wright-Giemsa staining. Cell viability was evaluated by trypan blue dye exclusion and was always higher than 98%. Isolation of peripheral blood lymphocytes was performed as previously described [34]. The band corresponding to mononuclear cells, obtained after Ficoll-Hypaque centrifugation, was washed twice with phosphate-buffered saline (PBS) and the cell suspension was added to Petri dishes to allow cells to adhere. Monocytes were depleted by culture dish adherence. After overnight incubation at 37°C, the nonadherent cells (lymphocytes) were washed with PBS and collected. Lymphocyte preparations were typically 67–73% $CD3^+$, 25–28% $CD19^+$, and $<0.4\%$ $CD14^+$.

Subcellular fractionation

Soluble and membrane fractions from resting human neutrophils were prepared as previously described [16]. Neutrophils were resuspended in 50 mM Tris-HCl, pH 7.5, containing 2 mM phenylmethylsulfonyl fluoride (PMSF), and then disrupted by freeze-thaw. Homogenate was centrifuged at 1200 rpm (Sorvall T 6000D) for 10 min and the supernatant, representing the postnuclear extract (E), was saved. Total membranes were prepared by centrifugation of the postnuclear extract at 45,000 rpm in a TLA rotor (Optima TL Ultracentrifuge, Beckman Instruments, Palo Alto, CA) for 90 min at 4°C. Pellets were resuspended in 50 mM Tris-HCl, pH 7.5, containing 2 mM PMSF. To prepare subcellular fractions, resting neutrophils (about 5×10^8 cells) were fractionated by rate zonal sedimentation in sucrose gradients as previously described [35, 36]. Then, fractions enriched in cytosol, plasma membrane, and cytoplasmic granules, assessed by using specific marker proteins [35, 36], were saved. The cytoplasmic granule fraction was a combination of the different granule fractions obtained after the sucrose gradient centrifugation and contained altogether the distinct types of intracellular granules present in neutrophils without resolving each other. Membranes from subcellular fractions enriched in plasma membrane and in cytoplasmic granules were obtained by diluting the corresponding subcellular fractions with 50 mM Tris-HCl, pH 8.0, 100 mM NaCl, and centrifugation in a 30-type rotor (Beckman Instruments) at 29,000 rpm for 90 min at 4°C as previously described [35, 36]. Then membrane pellets were resuspended in 50 mM Tris-HCl, pH 7.5, containing 2 mM PMSF.

Western blot analysis

Proteins were separated through sodium dodecyl sulfate (SDS)-15% polyacrylamide gels under reducing conditions, transferred to nitrocellulose filters, and subjected to immunological detection as previously described [16, 37]. After blocking for 3 h at room temperature in 2% (w/v) powdered defatted milk in Tris-buffered saline (TBS) buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl), blots were incubated overnight with HPC-1 monoclonal anti-syntaxin 1 antibody (Sigma Chemical, St. Louis, MO; and kindly provided by Dr. Juan Blasi, Barcelona, Spain) at a dilution of 1:2000 in TBS buffer containing 0.05% Tween 20. Antibody reactivity was monitored with biotinylated anti-mouse IgG (diluted 1:1000 in TBS buffer), followed by streptavidin-horseradish peroxidase conjugate (diluted 1:1000 in TBS buffer), with the use of an enhanced chemiluminescent detection system (Amersham, Buckinghamshire, UK).

Confocal laser scanning microscopy

Confocal microscopy was performed essentially as previously described [8]. Resting cells were resuspended (10^7 cells/mL) in HEPES/glucose buffer (150 mM NaCl, 5 mM KCl, 10 mM HEPES, 1.2 mM $MgCl_2$, 1.3 mM $CaCl_2$, 5.5 mM glucose, pH 7.5); an aliquot (40 μL) of the cell suspension was placed on slides and incubated at 37°C for 5 min. Cells were washed with PHEM buffer (60 mM PIPES, 25 mM HEPES, 10 mM EGTA, 3 mM $MgCl_2$, pH 7), and fixed in 4% paraformaldehyde in PHEM for 10 min. After extensive washing with PBS containing 0.1 M glycine, cells were permeabilized for 5 min with −20°C acetone, and then washed again with PBS. Cells were blocked with 5% normal goat serum, 5% fetal calf serum, and 1% bovine serum albumin in PBS for 1 h at room temperature. Incubation with HPC-1 anti-syntaxin 1 monoclonal antibody (diluted 1:1000 in PBS containing 1% bovine serum albumin) was for 1 h at 37°C in a humidified chamber. After washing with PBS, cells were incubated with fluorescein isothiocyanate-conjugated anti-mouse antibody (diluted 1:200 in PBS containing 1% bovine serum albumin) for 45 min in a humidified chamber at room temperature in the dark. Slides were then washed extensively with PBS and mounted in the aqueous medium Crystal/Mount (BIOMEDA Corp., Foster City, CA). Negative controls were routinely prepared by omitting the primary antibody or by using an irrelevant antibody, showing no

fluorescence staining of the samples. Fluorescence was visualized with a ZEISS LSM 310 laser scan confocal microscope.

RT-PCR

Total RNA was extracted and purified from the different cell types used in this work according to the protocol of Chomczynski and Sacchi [38] or using the Trizol reagent (GIBCO-BRL, Grand Island, NY) following the manufacturer's instructions. RNA preparations were carefully checked by gel electrophoresis and found to be free of DNA contamination. Total RNA (5 μg), primed with oligo-dT, was reverse-transcribed into cDNA with 30 units of AMV reverse transcriptase from Promega (Madison, WI) according to the manufacturer's instructions in a final volume of 20 μL. The mixture was incubated at 37°C for 2 h and stored at −20°C until use. The PCR mixture (50 μL) contained the template cDNA (1–2 μL), 20 pmol of the corresponding primers, 0.2 mM dNTPs, 1.5–2.5 mM $MgCl_2$, and 5 units of EcoTaq DNA polymerase derived from *Thermus aquaticus* (ECOGEN, Barcelona, Spain). PCR reactions were performed in a GeneAmp PCR System model 9600 (Perkin-Elmer, Norwalk, CT). The primers used are listed in **Table 1**, where the nucleotide numbers indicate the primer location in the corresponding sequences of human or rat origin obtained from the GenBank/EMBL database.

Primers were designed with the PCgene programs for DNA analysis from Intelligenetics (Mountain View, CA). The conditions for PCR amplification of cDNA prepared from the different cell types were as follows: one cycle at 95°C for 5 min as an initial denaturation step, then denaturation at 95°C for 30 s, annealing for 30 s, and extension at 72°C for 90 s (30–35 cycles), followed by further incubation for 15 min at 72°C (one cycle). The annealing step was carried out at 50°C (syntaxin 7 and syntaxin 16), 55°C (syntaxin 1A, syntaxin 4, syntaxin 9, and syntaxin 10), 57°C (syntaxin 3 and syntaxin 11), 62°C (syntaxin 6), 65°C (syntaxin 2), and 69°C (syntaxin 5 and syntaxin 13).

In the case of HL-60 cells, we also used a semiquantitative RT-PCR analysis to assess mRNA expression as previously described [39] and using the β-actin gene (GenBank/EMBL accession number: X00351) as an internal control. The sense and anti-sense primers for β-actin cDNA amplification were 5′-CTGTCTGGCGGCACCACCAT-3′ and 5′-GCAACTAAGTCATAGTCCGC-3′. This primer pair amplifies a 254-bp fragment (nucleotides: 936–1189). For the RT reaction, total RNA (5 μg) from untreated and DMSO-treated HL-60 cells was primed with oligo-dT and reverse-transcribed into cDNA with 30 units of AMV RT (Promega) in a 20-μL volume. A 50-μL PCR mixture contained 2 μL of the RT reaction and the reagents used were as described above. The PCR conditions, to analyze syntaxin gene expression during neutrophil differentiation of HL-60 cells, were the same as above except for the following changes for annealing temperature, Mg^{2+} concentration, and number of cycles: syntaxin 1A (55°C, 1.5 mM Mg^{2+}, 29 cycles), syntaxin 3 (60°C, 1.5 mM Mg^{2+}, 27 cycles), syntaxin 4 (60°C, 1.5 mM Mg^{2+}, 27 cycles), syntaxin 5 (69°C, 1.5 mM Mg^{2+}, 27 cycles), syntaxin 6 (62°C, 1.5 mM Mg^{2+}, 27 cycles), syntaxin 7 (57°C, 1.5 mM Mg^{2+}, 27 cycles), syntaxin 9 (69°C, 1.5 mM Mg^{2+}, 26 cycles), syntaxin 11 (69°C, 1 mM Mg^{2+}, 27 cycles), syntaxin 16 (66°C, 1.5 mM Mg^{2+}, 27 cycles), β-actin (60°C, 1.5 mM Mg^{2+}, 24 cycles). These experimental conditions were shown to be at the linear phase of amplification for each gene. An aliquot of the PCR reaction was analyzed on a 2% agarose gel (syntaxins 1A, 3, 4, 5, 6, and 13) or 3% agarose gel (syntaxins 7, 9, 10, 11, and 16) in 1 × TAE (40 mM Tris-acetate, 1 mM EDTA, pH 8.0), and checked for the expected PCR products.

cDNA cloning and sequencing

The PCR products were directly cloned into the pCR™2.1 vector using the TA cloning kit (Invitrogen, San Diego, CA) following the manufacturer's instructions. DNA sequencing was performed by thermal cycle sequencing using Cy5™AutoCycle™ sequencing kit (Pharmacia) and by automated laser fluorescent DNA sequencing (Pharmacia) as well as using a PE Applied Biosystems 377 DNA Sequencer (Perkin-Elmer). DNA sequencing was performed on both strands from at least 10 independent cDNA clones for each gene.

TABLE 1.

syntaxin 1A (accession number: L37792)	
(forward; nt 133–152):	5′-TGACAAGATCGCAGAGAACG-3′
(reverse; nt 593–612):	5′-TTGATGATCTCACTGTGCCG-3′
syntaxin 2A (accession number: L20823)	
(forward; nt 77–99):	5′-AGAAGGACCACTTCATGGATGCC-3′
(reverse; nt 860–879):	5′-ACGCAATCATTTGCCAACCG-3′
syntaxin 3 (accession number: U32315)	
set 1	
(forward; nt 221–244):	5′-GATCTCAGAACATGTAGAGGAGGC-3′
(reverse; nt 924–944):	5′-ATTCAGCCCAACGGAAAGTCC-3′
set 2	
(forward; nt 221–244):	5′-GATCTCAGAACATGTAGAGGAGGC-3′
(reverse; nt 924–947):	5′-TTAATTCAGCCCAACGGAAAGTCC-3′
set 3	
(forward; nt 78–101):	5′-ATGAAGGACCGTCTGGAGCAGCTG-3′
(reverse; nt 924–947):	5′-TTAATTCAGCCCAACGGAAAGTCC-3′
syntaxin 4 (accession number: X85784)	
set 1	
(forward; nt 1–20):	5′-ATGCGCGACAGGACCCACGA-3′
(reverse; nt 477–496):	5′-CAGCATTGGTGATCTTCAGC-3′
set 2	
(forward; nt 128–146):	5′-ACAAGGTCCGGACAATTCG-3′
(reverse; nt 872–894):	5′-TTATCCAACCACTGTGACGCCAA-3′
set 3	
(forward; nt 128–146):	5′-ACAAGGTCCGGACAATTCG-3′
(reverse; nt 477–496):	5′-CAGCATTGGTGATCTTCAGC-3′
syntaxin 5 (accession number: U26648)	
(forward; nt 107–130);	5′-TAAGCCAGCTTTGCGTGCTGTCCG-3′
(reverse; nt 812–835):	5′-TTGAGGATCTCTGAATGGGCGGCC-3′
syntaxin 6 (accession number: U56815)	
(forward; nt 206–230):	5′-TGCCCAGGGATTGTTCCAGAGATGG-3′
(reverse; nt 856–880):	5′-AGGACCGCGAAGAGGATGGCTATGG-3′
syntaxin 7 (accession number: D60600)	
(forward; nt 147–169):	5′-TCAACCATGTCTTACACTCCAGG-3′
(reverse; nt 335–359):	5′-GTACTTATCTGTTTCTTTGGCAAGC-3′
syntaxin 9 (accession number: AA150357)	
(forward; nt 9–32):	5′-GAACAGGAAGCAAAGTACCAGGCC-3′
(reverse; nt 333–357):	5′-TTCATCACAATAGAGTGGAAATCCG-3′
syntaxin 10 (accession number: W24393)	
(forward; nt 64–88):	5′-GTTGGAGAAGAGCTGGACGAGCAGG-3′
(reverse; nt 315–339):	5′-AAGATGAGAACGAGGAGAAGCACCC-3′
syntaxin 11 (accession number: AA227632)	
(forward; nt 157–176):	5′-TTTGGTGCGTCCTTCCCAGC-3′
(reverse; nt 386–406):	5′-TCTTCTTGCAGATGGCGGTGC-3′
syntaxin 13 (accession number: AA167677)	
(forward; nt 21–45):	5′-GCTCCTCGTCATGTCATACGGTCCC-3′
(reverse; nt 581–602):	5′-TCAGCTCAACTGCCGAATGCCG-3′
syntaxin 16 (accession number: AA100145)	
(forward; nt 43–64):	5′-GGGCGATGATTGTAGAACAGGG-3′
(reverse; nt 234–255):	5′-TGCCACTTATCGAGACTTCACG-3′

RESULTS

Syntaxin expression in human neutrophils

We have used RT-PCR to analyze the expression of the distinct syntaxins in human neutrophils, using appropriate sets of primers for the corresponding human (syntaxins 1A, 3, 4, and 5) and rat (syntaxins 2 and 6) genes. Two isoforms of syntaxin 1, named syntaxin 1A and syntaxin 1B, specific for neuronal tissues have been identified in rat [19, 22, 31]. They share 84% amino acid identity. Syntaxin 1A from human brain has also been cloned and sequenced [40]. This human syntaxin 1A shows a 98% amino acid sequence identity with the rat syntaxin 1A homolog [40]. Thus, we used primers for human syntaxin 1A to examine expression of syntaxin 1 in human neutrophils. We found expression of syntaxins 1A, 3, 4, 5, and 6, as assessed by detection of the appropriate PCR bands with the expected sizes for the syntaxins 1A, 3, 4, 5, and 6 amplified fragments (**Fig. 1A**), and by subsequent cloning and sequencing of these amplified fragments (data not shown). In the case of syntaxin 4, we detected two amplified PCR fragments (Fig. 1A), correspond-

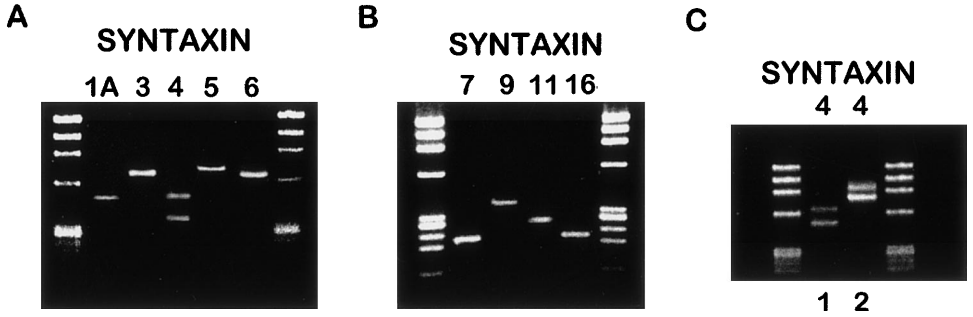

Fig. 1. PCR of cDNAs from human neutrophils using primers for several syntaxins. Total RNA was extracted from resting cells, reverse-transcribed into cDNA, and followed by amplification by cycle repeating denaturation, annealing, and primer extension as described in Materials and Methods. Primer sequences and conditions for cDNA amplification are detailed in Materials and Methods. (A) PCR products of cDNA amplification using specific primers for syntaxin 1A, syntaxin 3, syntaxin 4, syntaxin 5, and syntaxin 6. The sets of primers 1 and 3, detailed in Materials and Methods, were used for RT-PCR amplification of syntaxins 3 and 4, respectively. (B) PCR products of cDNA amplification using specific primers for syntaxin 7, syntaxin 9, syntaxin 11, and syntaxin 16. (C) PCR products of cDNA amplifications using the sets of primers 1 and 2 for syntaxin 4 detailed in Materials and Methods. Standards (φX174 DNA digested with *Hae*III) were run at both sides of each gel.

ing the lower band to the previously sequenced syntaxin 4 [15]. We failed to detect expression of syntaxin 2 under the experimental conditions used. However, this lack of syntaxin 2 detection could be due to the rat origin of the primers used.

The existence of several new syntaxins has been postulated by Bock and Scheller [23], ranging from syntaxin 7 to syntaxin 16, based on the homology between well-characterized syntaxins and several related EST sequences. Some of these postulated new syntaxins, derived from DNA partial sequences of EST databases, are of human origin (syntaxins 7, 9, 10, 11, 12, 13, 14, and 16), but others are of mouse origin (syntaxins 8 and 15). Thus, we designed primers for the EST sequences corresponding to the postulated syntaxins 7, 9, 10, 11, 13, and 16 derived from human EST sequences, and analyzed the expression of these new syntaxins in human neutrophils by RT-PCR. The sequences available in the EST database for both syntaxins 12 and 14 were very short, not suitable for primer design and subsequent analysis of the respective amplified fragments, precluding their appropriate detection by RT-PCR. As shown in Figure 1B, we detected bands with the expected sizes for the amplified fragments of syntaxins 7, 9, 11, and 16, which were then cloned and sequenced. Analysis of the cloned nucleotide sequences (data not shown) revealed that these PCR fragments corresponded to syntaxins 7, 9, 11, and 16. However, we were not able to detect any amplified fragment with the expected size for syntaxins 10 and 13 under the experimental conditions used. These results indicate that human neutrophils express the postulated new syntaxins 7, 9, 11, and 16.

Cloning of two isoforms of syntaxin 3 and of a syntaxin 4 precursor

We used different sets of PCR primers with wide overlapping regions designed from the corresponding genes (see Materials and Methods) in order to sequence the complete encoding regions of syntaxins 3 and 4. Following this approach, we have cloned and sequenced two variant isoforms of syntaxin 3, termed syntaxin 3A and syntaxin 3B (**Fig. 2**). The complete nucleotide sequence of syntaxin 3A contained an open reading frame of 870 nucleotides coding for a protein composed of 289 amino acids with a deduced molecular mass of about 33.2 kDa. Syntaxin 3A from human neutrophils showed a strong homology with that of rat origin previously reported [19], with 75 differences in nucleotide sequence (91.4% identity) and only six changes in the amino acid sequence (97.9% identity; Fig. 2). In addition, using the set 2 of primers for syntaxin 3 (Materials and Methods), we were able to clone and partially sequence a novel syntaxin 3 isoform, termed syntaxin 3B, which shows a total identity to syntaxin 3A between the nucleotides 145–675 and 787–870, and a gap between the nucleotides 676–786 of the syntaxin 3A coding sequence. Thus, syntaxin 3B is identical to syntaxin 3A but lacks 37 amino acid residues, from amino acid 226 to amino acid 262 (Fig. 2). Syntaxin 3A has one potential target sequence of phosphorylation by cAMP/cGMP-dependent protein kinase in Thr-161 and four potential sites of phosphorylation by protein kinase C at the residues Ser-124, Thr-156, Ser-207, and Thr-250, this last one being absent in syntaxin 3B. The syntaxin 3A protein also contains two potential amino-myristoylation sites at Gly residues 180 and 283, which are preserved in syntaxin 3B, as well as an epimorphin family signature between amino acid residues 197–235. Mouse syntaxin 3 has been reported to show four different isoforms with differences either in the carboxyl- or the amino-terminal region [41] but the novel human syntaxin 3B described here (Fig. 2) does not correspond to any of the previously reported mouse syntaxin 3 isoforms.

The two-band PCR pattern obtained for syntaxin 4 (Fig. 1A) was always observed using the three different pairs of primers described in Materials and Methods (Fig. 1, A and C). The upper and lower amplified fragments, obtained from sets 1 and 2 of primers for syntaxin 4 (see Materials and Methods) shown in Figure 1C, were cloned in the pCR2.1 vector using the TA cloning system and sequenced. The sequence of the lower band corresponded exactly to the previously reported sequence for human syntaxin 4 from skeletal muscle [15]. The sequence of the upper band corresponded to that of syntaxin 4, but contained an insertion of 136 nucleotides (**Fig. 3**). This insert had several stop codons and contained the highly conserved sequences for RNA splicing in higher eukaryotes, namely GT at the 5′ splice site (donor site) and AG at the 3′ splice site (acceptor site; Fig. 3), which are required for intron removal. The complete sequence of the larger band rendered a complete sequence of 1030 nucleotides showing an intron sequence of

Fig. 2. Amino acid sequences of syntaxin 3A and syntaxin 3B from human neutrophils, and homology with rat syntaxin 3. The complete amino acid sequence of human syntaxin 3A (Hum-SYNT3A) is compared with the partial amino acid sequence of human syntaxin 3B (Hum-SYNT3B), showing the lack of 37 amino acids between residues 226–262. The deduced amino acid sequences of syntaxins 3A and 3B were aligned with that of rat syntaxin 3 (Rat-SYNT3). The bold amino acid residues indicate differences between human and rat amino acid sequences.

136 nucleotides between the coding regions 1–232 and 369–1030.

Syntaxin expression in neutrophil-differentiated HL-60 cells

To further assess the presence of the above-described syntaxins in human neutrophils, we examined the expression of these neutrophil-expressed syntaxins in the human promyelocytic HL-60 cell line. This is a homogeneous human cell line that can be differentiated toward the neutrophil lineage and it is used as a cell culture model to study neutrophil biology [32]. We found that the pattern of expression of syntaxin genes shown in peripheral blood mature neutrophils (Fig. 1) was identical to that found in DMSO-differentiated HL-60 cells (**Fig. 4**), which were used as a cell culture model for neutrophils [32]. It has been previously shown that treatment of HL-60 cells with DMSO for 4 days leads to their differentiation toward cells displaying many neutrophil functions and characteristics [32]. As shown in Figure 4, all the syntaxin genes expressed in human neutrophils were also detected by RT-PCR in neutrophil-differentiated HL-60 cells (see Figs. 1 and 4). The identification of each syntaxin was further assessed by cloning and subsequent sequencing of each amplified fragment. Cloning and sequencing of these syntaxin genes (syntaxins 1A, 3A, 3B, 4, 5, 6, 7, 9, 11, and 16) expressed in neutrophil-differentiated HL-60 cells revealed the same sequences as their corresponding counterparts from peripheral blood neutrophils, further confirming their expression in human neutrophils. In addition, following the same approach as that used in peripheral blood human neutrophils, we also cloned and sequenced the above-described syntaxin 3B as well as the syntaxin 4 precursor in neutrophil-differentiated HL-60 cells.

We also determined the level of expression of different syntaxins during DMSO-induced differentiation of the human promyelocytic leukemia HL-60 cell line toward the neutrophil lineage by semiquantitative RT-PCR. This RT-PCR analysis revealed the presence of relatively high levels of syntaxins 3, 4, 5, 9, and 16, as well as lower levels of syntaxins 1A, 6, and 11 in undifferentiated HL-60 cells (Fig. 4). Syntaxin 7 was not expressed in undifferentiated HL-60 cells (Fig. 4). The level of expression of syntaxin 6 was highly increased after 1-day treatment of HL-60 cells with DMSO (Fig. 4), the time required to commit HL-60 cells toward the neutrophil lineage [42]. Syntaxin 7 was expressed after a 2-day treatment of HL-60 cells with DMSO (Fig. 4). These data indicate that the expression of syntaxin 7 is under a tight regulatory control during neutrophil differentiation. Also, the expression of syntaxins 3, 4, and 11 was increased during neutrophil differentiation, whereas the level of expression of syntaxins 1A, 5, 9, and 16 was not modified along the DMSO-induced differentiation process of HL-60 cells (Fig. 4). It is interesting to note that the precursor form of syntaxin 4 observed in human neutrophils (Figs. 1 and 3) was not detected in undifferentiated HL-60 cells, but was accumulated after DMSO treatment (Fig. 4). This result corrobo-

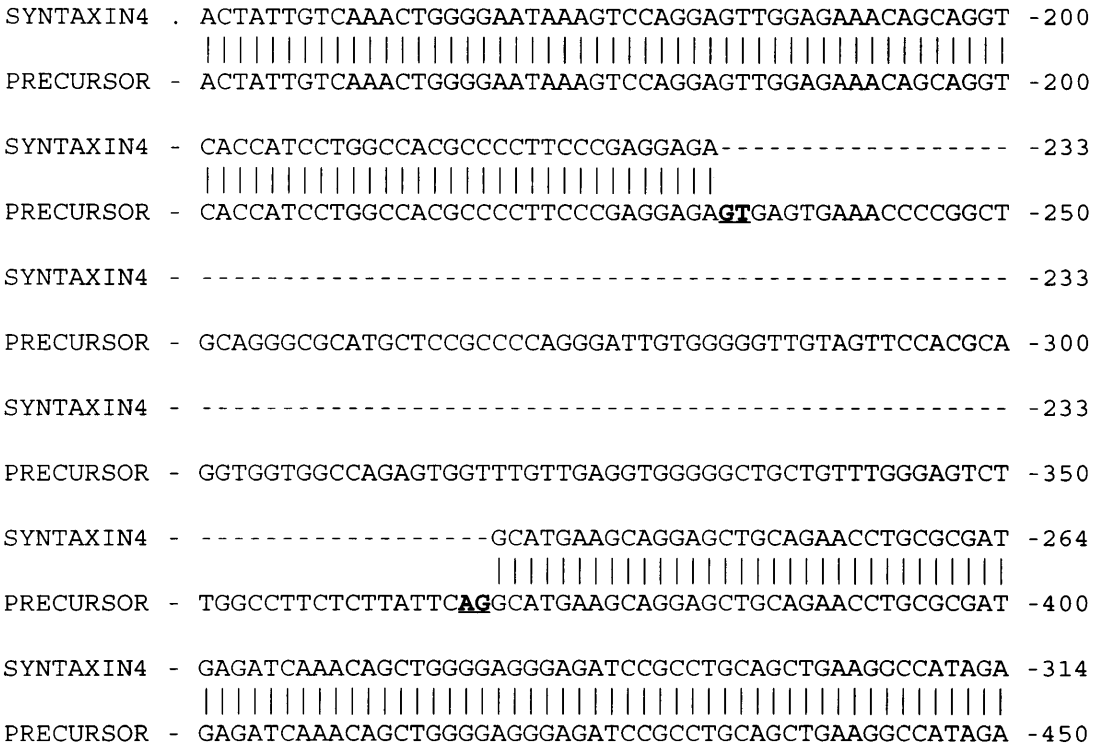

Fig. 3. Nucleotide sequence of human neutrophil syntaxin 4 containing an intron sequence. Partial sequences of the DNA strands corresponding to the mature mRNA (SYNTAXIN4) and to the mRNA precursor (PRECURSOR) sequences of syntaxin 4 from human neutrophils are shown. The mRNA precursor of syntaxin 4 contains an intron sequence of 136 nucleotides as indicated. The highly conserved GT and AG nucleotides at the beginning and at the end of the intron are underlined and shown in bold.

rates that obtained from human neutrophils and suggests that a regulatory mechanism at the level of RNA splicing could constitute a rate-limiting step in the modulation of the levels of syntaxin 4 during neutrophil development.

Comparative expression of syntaxins in neutrophils, lymphocytes, and SH-SY5Y neuroblastoma cells

We have compared the patterns of expression of syntaxins in human neutrophils, lymphocytes, and SH-SY5Y neuroblastoma cells using the same experimental conditions described in Materials and Methods for all the cell types (**Fig. 5**). Because the number of cycles used in these studies was higher than that used in Figure 4, we have also included the data obtained from undifferentiated and neutrophil-differentiated HL-60 cells, to further examine the expression of some syntaxins that are either weakly or scarcely expressed (syntaxins 3B, 6, and 7) in undifferentiated HL-60 cells when semiquantitative RT-PCR conditions were used (Fig. 4). Syntaxin 2 has not yet been cloned in human tissues and we were unable to detect any amplification PCR product of the expected size for syntaxin 2 using the primers of rat origin. We also failed to detect expression of syntaxin 13 under the experimental conditions used. However, this lack of expression in the cell types tested, together with the rat origin of the syntaxin 2 primers, does not permit us to conclude whether these two syntaxins are expressed in neutrophils. As shown in Figure 5, we have shown that SH-SY5Y neuroblastoma cells express, in general, higher levels of syntaxins than neutrophils. Human peripheral blood lymphocytes also express all the syntaxins found in human neutrophils. In addition, both SH-SY5Y cells and lymphocytes express significant levels of syntaxin 10, which is not detected in human neutrophils (Fig. 5). Syntaxins 1A and 4 are expressed in neutrophils at much lower levels than in the other cell types assayed (Fig. 5). The two-band PCR pattern for syntaxin 4 was also observed in lymphocytes and SH-SY5Y cells (Fig. 5), even though the relative proportion of the syntaxin 4 precursor (upper band) compared to the fully processed syntaxin 4 (lower band) seems to be higher in human neutrophils, and is very low in SH-SY5Y cells. We have also been able to detect a two-band PCR pattern for syntaxin 3 in undifferentiated and differentiated HL-60 as well as in SH-SY5Y cells (Fig. 5). This lower band of the two-band pattern for syntaxin 3 was also present but hardly visible in gel, in human neutrophils, due to its much lower content in this cell type (Fig. 5). Cloning and sequencing analyses indicated that these two bands corresponded to the above-mentioned syntaxin 3A (upper band) and syntaxin 3B (lower band; Fig. 2). The data shown in Figure 5 indicate that syntaxin 3B is expressed at a much lower level than syntaxin 3A in the different cell types assayed (Fig. 5). Under the experimental conditions used in Figure 5, we could observe detectable levels of syntaxin 6 expression in undifferentiated HL-60 cells that were increased on DMSO treatment, whereas no expression of syntaxin 7 was detected in undifferentiated HL-60 cells. These results further indicate that untreated HL-60 cells do not express syntaxin 7 and that its expression is induced during HL-60 differentiation toward neutrophils.

Fig. 4. Expression of human syntaxin genes during DMSO-induced differentiation of HL-60 cells toward the neutrophil lineage. The expression of syntaxin 1A, syntaxin 3 (set 1 of primers), syntaxin 4 (set 3 of primers), syntaxin 5, syntaxin 6, syntaxin 7, syntaxin 9, syntaxin 11, and syntaxin 16 was assessed by a semiquantitative RT-PCR analysis. Total RNA was purified from untreated HL-60 cells as well as from HL-60 cells treated with 1.3% (v/v) DMSO for the indicated times, and subjected to semiquantitative RT-PCR analysis with oligonucleotide primers specific for each syntaxin gene as described in Materials and Methods. PCR amplification of β-actin was used as an internal control. After 29 cycles (syntaxin 1A), 27 cycles (syntaxin 3, syntaxin 4, syntaxin 5, syntaxin 6, syntaxin 7, syntaxin 11, and syntaxin 16), 26 cycles (syntaxin 9), and 24 cycles (β-actin), shown to be at the linear phase of amplification, the PCR products were electrophoresed onto 2 or 3% agarose gels and stained with ethidium bromide.

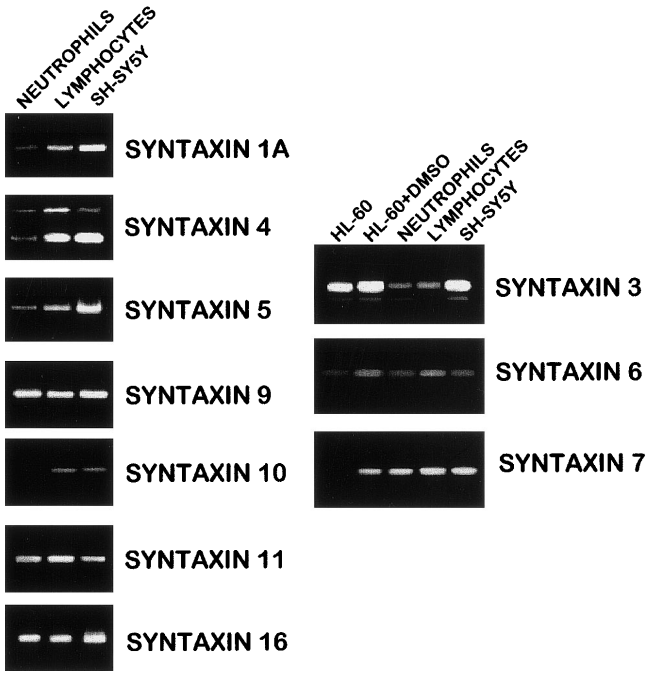

Fig. 5. Expression of several human syntaxin genes in neutrophils, lymphocytes, and SH-SY5Y neuroblastoma cells. Total RNA was extracted from cells, reverse-transcribed into cDNA, and followed by amplification by cycle repeating denaturation, annealing, and primer extension as described in Materials and Methods. Primer sequences and conditions for cDNA amplification are detailed in Materials and Methods. The expression of some syntaxins (syntaxins 3, 6, and 7) in undifferentiated HL-60 cells (HL-60) and in HL-60 cells treated for 4 days with DMSO (HL-60+DMSO) is also included. The number of cycles used for PCR amplification of syntaxin 3 was increased up to 40. The two-band PCR patterns obtained for syntaxin 4 and syntaxin 3 correspond to the amplification of the normal syntaxin 4 (lower band) and the syntaxin 4 precursor described in Figures 1 and 3 (upper band), as well as to syntaxin 3A (upper band) and syntaxin 3B (lower band).

Identification and intracellular localization of syntaxin 1 in human neutrophils

As shown in Figures 1A, 4, and 5, we detected an amplified PCR product, with the expected size, in human neutrophils and HL-60 cells using specific primers for syntaxin 1A. After subsequent cloning and sequencing of this PCR amplified product, we found that this PCR band corresponded to syntaxin 1A. These results indicate that syntaxin 1, particularly syntaxin 1A, previously thought to be specific for neuronal tissues [19, 22, 31], is expressed in human neutrophils and HL-60 cells. In addition, we found that syntaxin 1A was also expressed in human peripheral blood lymphocytes and in the SH-SY5Y neuroblastoma cell line (Fig. 5).

The presence of syntaxin 1 in human neutrophils was further assessed by immunoblotting and confocal microscopy techniques. To examine the presence of syntaxin 1 in human neutrophils, we carried out immunoblotting analysis from neutrophil extracts as well as from cytosol and membrane fractions. We used the monoclonal antibody HPC-1 against syntaxin 1A/1B, which has been widely used to immunolocalize syntaxin 1 [22, 30, 31, 43, 44]. This HPC-1 monoclonal antibody recognized only an intense band, which corresponded to a protein with an apparent molecular mass of about 35 kDa, similar to that of syntaxin 1 (**Fig. 6A**). In fact, the HPC-1 monoclonal antibody recognized an identical band of about 35 kDa in homogenates of both rat brain and human neutrophils (data not shown), which further assessed the specificity of the immunodetected band. The protein recognized by the HPC-1 antibody in human neutrophils was membrane-bound (Fig. 6A). Immunoblotting analysis of distinct subcellular fractions prepared from resting human neutrophils indicated that this syntaxin 1 immunoreactive protein band was mainly located in the fraction enriched in the membranes prepared from cytoplasmic granules, with a minor location in plasma membrane, and was absent from cytosol (Fig. 6B). The granule membranes were prepared from the whole neutrophil granule population, without resolving the distinct intracellular granules present in human neutrophils. Immunofluorescence confocal microscopy for syntaxin 1, using the HPC-1 monoclonal antibody, showed a granular pattern in resting human neutrophils (**Fig. 7**). This further indicates a granule localization for this protein.

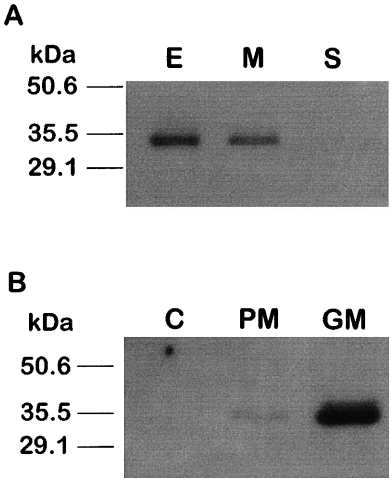

Fig. 6. Immunodetection and subcellular distribution of syntaxin 1 in resting human neutrophils. (A) Membrane association of syntaxin 1. Postnuclear extract (E) from resting human neutrophils was prepared as described in Materials and Methods. Then the total membrane fraction (M) as well as the soluble fraction (S), including cytosolic and soluble proteins, were obtained as described in Materials and Methods. Proteins from postnuclear extract (60 μg), membrane (30 μg), and soluble (60 μg) fractions were run on a SDS-15% polyacrylamide gel and analyzed by immunoblotting using the anti-syntaxin-1 HPC-1 monoclonal antibody. (B) Granule membrane localization of syntaxin 1. Equal amounts of cytosolic (C), plasma membrane (PM), and granule membrane (GM) proteins (60 μg), prepared from resting human neutrophils as described in Materials and Methods, were run on a SDS-15% polyacrylamide gel and analyzed by immunoblotting using the anti-syntaxin-1 HPC-1 monoclonal antibody. Molecular masses (kDa) of protein markers are indicated on the left.

DISCUSSION

This study is the first report analyzing the pattern of expression of syntaxins in human neutrophils, a non-neuronal cell type where exocytosis of cytoplasmic granules plays a key role in its physiology. In order to assess the neutrophil origin of the cloned genes herein reported, we used both a highly purified human peripheral blood neutrophil preparation and the homogeneous human promyelocytic leukemia HL-60 cell line, which can be differentiated toward neutrophils and is widely used as a cell culture model for the study of different aspects of human neutrophil biology [32, 45, 46]. HL-60 cells are committed to differentiate toward the neutrophil lineage after 1-day DMSO treatment [42], and are fully differentiated toward cells sharing many mature neutrophil features after a 4-day DMSO treatment [32]. We have found here an identical pattern of expression of syntaxin genes in both human peripheral blood mature neutrophils and HL-60 cells differentiated toward neutrophils with DMSO. From these data, we conclude that human neutrophils, and neutrophil-differentiated HL-60 cells, express syntaxins 1A, 3A, 3B, 4, 5, and 6, as well as the postulated new syntaxins 7, 9, 11, and 16, as assessed from RT-PCR, cloning, and sequencing analyses. Previous reports indicated that human neutrophils contained several SNARE proteins, namely: syntaxin 4 [47], VAMP-2 [47], and SNAP-25 [16], based on the use of specific antibodies; and SNAP-23 A and B [18], based on cloning and sequencing approaches. Taking these data together, it can be envisaged that human neutrophils contain all the components, v-SNAREs and t-SNAREs, required by the SNARE hypothesis. The presence of Rap1 and Rap2 proteins in human neutrophils, putatively involved in the regulation of exocytosis, has also been reported [37, 48]. The putative presence of distinct combinations of these docking-fusion proteins in the different types of cytoplasmic granules present in human neutrophils could explain the independent mobilization of these granules during neutrophil activation.

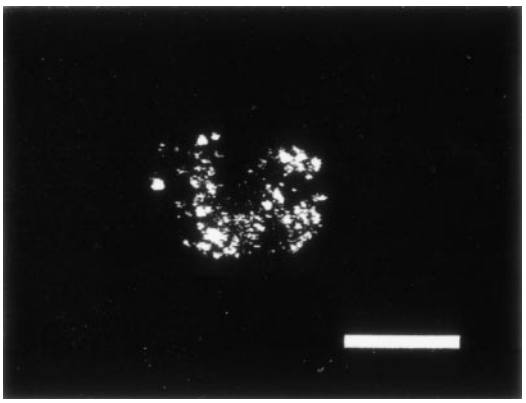

Fig. 7. Localization of human syntaxin 1 in resting neutrophils by confocal laser scanning microscopy. Cells were permeabilized and incubated with anti-syntaxin 1 HPC-1 monoclonal antibody as described in Materials and Methods. One cell is shown. Bar, 8 μm. Data shown are representative of three separate experiments.

Syntaxin 3 was found to be expressed as two isoforms, named 3A and 3B. This novel human syntaxin 3B isoform is identical to syntaxin 3A, but lacks 37 amino acid residues, and it is expressed at much lower levels than syntaxin 3A. It is interesting to note the detection by RT-PCR of a syntaxin 4 precursor in human neutrophils that results from an incomplete RNA splicing. This syntaxin 4 precursor was accumulated on DMSO-induced HL-60 differentiation. The accumulation of an RNA splicing intermediate could suggest the putative existence of a regulatory mechanism at the level of RNA splicing, acting as a rate-limiting step in modulating the levels of syntaxin 4 during neutrophil development and in mature neutrophils. This RNA splicing intermediate was also found in human lymphocytes and SH-SY5Y neuroblastoma cells. Thus, the observation of a syntaxin 4 splicing intermediate with an intron of 136 nucleotides might reflect a limiting step in the processing of this gene RNA, perhaps related to the large structure of the spliceosome complex [49, 50] and the small size of this intron.

To compare neutrophil syntaxin expression with that found in other human cell types, we examined comparatively syntaxin expression in human neutrophils, lymphocytes, and SH-SY5Y neuroblastoma cells. We have found that both peripheral blood lymphocytes and SH-SY5Y cells expressed all the syntaxins detected in human neutrophils. In addition, syntaxin 10 was expressed in lymphocytes and neuroblastoma cells, but not in human neutrophils. This suggests that syntaxin 10 is not expressed in human neutrophils. A recent report showed the presence of syntaxin 1 in SH-SY5Y neuroblastoma cells with the use of Western blot analysis [44] but other syntaxins were not analyzed [44]. Thus, this study constitutes the first evidence for syntaxin expression in human lymphocytes and human SH-SY5Y neuroblastoma cells, and demonstrates that these cell types express syntaxins 1A, 3A, 3B, 4, 5, 6, 7, 9, 10, 11,

and 16. The ubiquitous expression of these syntaxins suggests their involvement in pathways that are common to all cell types. However, it could be envisaged that some syntaxins may play distinct roles in different cell types. Human neutrophils are terminal nonproliferating cells that have a reduced capacity of macromolecule biosynthesis [51] but manifest a prominent secretory capacity [1, 5]. Accordingly, the cytoplasm of these short-lived, highly specialized phagocytes is full of cytoplasmic granules, many of them readily exocytosed on cell activation [1, 5]. Functional and morphological changes coupled to the conversion of proliferating cells toward terminal non-proliferating, phagocytic, and secretory cells are observed during neutrophil maturation in bone marrow, and HL-60 neutrophil differentiation partially mimics some of these changes [32, 45, 46]. The fact that expression of some syntaxins is increased during HL-60 neutrophil differentiation suggests a more specialized role for these syntaxins. The induction of syntaxin 7 during HL-60 differentiation toward the neutrophil lineage is of particular interest. This syntaxin has been recently cloned [24, 25] and found to be associated with the early endosome compartment [25]. We have found that syntaxin 7 is not expressed in undifferentiated HL-60 cells, but its expression is induced during the early phases of DMSO-induced HL-60 differentiation toward the neutrophil lineage. These data indicate that expression of syntaxin 7 is highly regulated during neutrophil differentiation and, thereby, suggest a putative role for syntaxin 7 in some specialized functions of mature neutrophils. In addition, the low level of syntaxin 6 expression detected in undifferentiated HL-60 cells is increased on neutrophil differentiation. Bock et al. [20, 52] found that syntaxin 6 is widely expressed and localizes in the region of the Golgi apparatus. Therefore, it seems to play a role in a universal biological process. However, it could also be speculated that syntaxin 6 could play an additional and more specialized role in human neutrophils because its expression is increased during neutrophil differentiation of HL-60 cells. Similar more specialized roles occurring in mature neutrophils could be putatively assigned to syntaxins 3, 4, and 11, which are also induced during neutrophil differentiation of HL-60 cells. In this regard, we have previously found that expression of SNAP-23 is also increased during DMSO-induced differentiation of HL-60 cells toward neutrophils [18].

A major finding in this report is the identification of syntaxin 1 in human neutrophils by both genetic and immunological approaches. RT-PCR analysis, followed by cloning and sequencing, allowed identification of syntaxin 1A in human neutrophils and HL-60 cells. This gene was also expressed in human lymphocytes and SH-SY5Y neuroblastoma cells. Thus, the data herein reported indicate that syntaxin 1A is not exclusive of neuronal tissues as previously reported [19, 22, 31]. In addition, we report evidence for the identification of syntaxin 1 in human neutrophils using the monoclonal antibody HPC-1, which has been broadly used to immunolocalize syntaxin 1 [22, 30, 31, 43, 44]. Immunoblotting of subcellular fractions and immunofluorescence confocal microscopy studies reveal that syntaxin 1 is mainly located in the membranes of cytoplasmic granules in human resting neutrophils, with a minor location in plasma membrane. This HPC-1 monoclonal antibody is able to recognize the previously two reported isoforms of syntaxin 1, named 1A and 1B [30, 31]. Jagadish et al. have recently reported a novel isoform of syntaxin 1, termed 1C, which is also recognized by the HPC-1 monoclonal antibody [43]. Unlike previous syntaxins 1A and 1B, which were previously reported to be specific for neuronal tissues, syntaxin 1C is expressed in several human tissues [43]. Syntaxins 1A and 1B consist of 288 amino acid residues, whereas syntaxin 1C contains 260 residues [43]. Due to the close molecular masses of the syntaxin 1 isoforms, it is possible that HPC-1 monoclonal antibody recognizes other syntaxin 1 isoforms, in addition to the cloned syntaxin 1A, in human neutrophils. The main granule localization of syntaxin 1 in human neutrophils differs from the plasma membrane location previously reported for this syntaxin in neuronal tissues [19, 22, 31], consistent with its proposed role as t-SNARE in nerve terminals [53]. Although syntaxin 1 has normally been described as a t-SNARE, some studies report its association with synaptic vesicles and with chromaffin granules [21, 54, 55].

The expression of a wide number of proteins involved in vesicle-membrane fusion processes in neutrophils could be related to their high secretory capacity and to the presence of distinct neutrophil cytoplasmic granules with different exocytic capabilities. However, the roles of the distinct syntaxin family members in human neutrophils remain to be elucidated.

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