Identification of Soluble N-Ethylmaleimide-Sensitive Factor Attachment Protein Receptor Exocytotic Machinery in Human Plasma Cells: SNAP-23 Is Essential for Antibody Secretion

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Identification of Soluble N-Ethylmaleimide-Sensitive Factor Attachment Protein Receptor Exocytotic Machinery in Human Plasma Cells: SNAP-23 Is Essential for Antibody Secretion

Elena Reales, Francisco Mora-López, Verónica Rivas, Antonio García-Poley, José A. Brieva, and Antonio Campos-Caro

Plasma cells (PC) are B-lymphocytes terminally differentiated in a postmitotic state, with the unique purpose of manufacturing and exporting Igs. Despite the importance of this process in the survival of vertebrates, no studies have been made to understand the molecular events that regulate Ig exocytosis by PC. The present study explores the possible presence of the soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) system in human PC, and examines its functional role in Ig secretion. Syntaxin-2, Syntaxin-3, Syntaxin-4, vesicle-associated membrane protein (VAMP)-2, VAMP-3, and synaptosome-associated protein (SNAP)-23 could be readily detected in normal human PC obtained from intestinal lamina propria and blood, as well as in human PC lines. Because SNAP-23 plays a central role in SNAREs complex formation, it was chosen to examine possible functional implications of the SNARE system in PC Ig secretion. When recombinant SNAP-23 fusion protein was introduced into the cells, a complete abolishment of Ig production was observed in the culture supernatants of PC lines, as well as in those of normal PC. These results provide insights, for the first time, into the molecular machinery of constitutive vesicular trafficking in human PC Ig secretion and present evidence indicating that at least SNAP-23 is essential for Ab production. The Journal of Immunology, 2005, 175: 6686–6693.

The humoral response is one of the most important strategies of the immune system against foreign invaders. Plasma cells (PC) are considered the final stage of the B lymphocyte differentiation process and, as such, they show features that reveal their full specialization in the synthesis and secretion of the Abs or Igs, the effective weapons of humoral immune response. PC Ab production has been classically considered a paradigm of cell protein secretion, in which newly synthesized Ig is processed by the Golgi, packaged into small vesicles, and immediately secreted with little, if any, intracellular storage (1–7). Nevertheless, despite the importance of Ab secretion, the ultimate mechanism by which this process is accomplished by PC has not been yet elucidated. This process necessarily requires the fusion of two lipid bilayers (8), one from the Ig-containing vesicles and the other belonging to the external plasma membrane. Cell membranes fusion and, in particular, the fusion between secretory vesicles and the plasma membrane for exocytosis, must be specific to maintain organelle identity, cell function and viability, and requires a molecular mechanism to juxtapose and fuse the two membranes involved. This process was first described in cell systems of regulated exocytosis from yeast to neurons (9), where fusion of a secretory vesicle with its target membrane is mediated by a set of proteins called SNAREs (N-ethylmaleimide-sensitive factor attachment protein receptor) (10). Subsequently, SNAREs have been implicated in a wider range of membrane-to-membrane recognition and fusion processes. The minimal fusion components of the SNARE complex consists of three protein families associated in a 1:1:1 stoichiometry. Two of these protein families, previously designated as t-SNAREs (later named Q-SNAREs, after a conserved Glutamine (Q) at central position), are associated with the target membrane. The proteins of one of these families, the Syntaxin family, are integral membrane proteins, whereas those of the other, the SNAP-25 family (synaptosome-associated protein of molecular mass 25 kDa), are peripherally attached to the target membrane by means of palmitoylated cysteine residues. The third SNAP protein family is located at the vesicle membrane, and was initially termed v-SNAREs (now R-SNAREs, after a conserved Arginine (R) at central position). Members of this protein family have been named Synaptobrevins or VAMPs (vesicle-associated membrane proteins) and are integral membrane proteins. The association of the three proteins forms a very stable parallel four-helical bundle, to which Syntaxin and VAMP contribute one α-helix each and SNAP-25 contributes two α-helices (11). It has been proposed that this heterotrimeric interaction occurs between particular components of these family proteins, and this fact confers the specificity for the fusion between appropriate membranes “in vivo” (10). It is clear that particular SNAREs specifically localize to distinct subcellular compartments. Although the critical role of SNAREs in cell secretion has been mainly described in neurons and neuroendocrine cells, recent evidences have implicated these proteins in the secretory pathways in a broad range of cellular systems including hematopoietic cells (12) such as platelets, neutrophils, eosinophils, and mast cells. Accordingly, as the molecular mechanism controlling Ab secretion remains unknown, it might be hypothesized that SNARE proteins could play a role in PC Ig exocytosis.
The present study explores this possibility in human normal PC obtained from intestinal lamina propria (LP) and peripheral blood (PB), and in human PC lines. All of these PCs show a very high rate of Ig secretion. The results revealed that all of these cells expressed a similar pattern of SNARE components, including Syntaxin-2, Syntaxin-3, Syntaxin-4, VAMP-2, VAMP-3, and SNAP-23. These molecules were expressed by human PC at both mRNA and protein levels. In contrast, Syntaxin-1, VAMP-1, and SNAP-25 were not detected. Additional experiments demonstrated that the functional blockade of SNAP-23 provoked a marked inhibition of PC Ig-secretion. Taken together, present data demonstrate for the first time that human PCs express a particular set of SNARE components, and that SNAP-23 is essential for PC Ig-secretion.

Materials and Methods

Reagents

Synthetic oligonucleotides and glutathione-Sepharose 4B beads were purchased from Sigma-Genosys. The following Abs were obtained from the suppliers indicated: against VAMP-2 (Stressogen Biotechnologies), against SNAP-23 (Synaptic Systems), against SNAP-25 (Santa Cruz Biotechnology), against Syntaxin-1, Syntaxin-3, VAMP-3, and β-actin (Abcam), against Syntaxin-2 (Calbiochem), and against Syntaxin-4, Calnexin, and CD138 (BD Biosciences); FITC-conjugated Abs against human IgA, IgG, and IgE for fluorescence microscopy (DakoCytomation); rhodamine-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch Laboratories); Alexa488-conjugated rabbit anti-mouse IgG (Molecular Probes); alkaline phosphatase-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch Laboratories); rabbit anti-mouse IgG (Jackson ImmunoResearch Laboratories); and alkaline phosphatase-conjugated secondary Abs for Western blots (Sigma-Aldrich).

Tissue samples and cell lines

The human cell lines U266 and IM-9 were purchased from the European Collection of Cell Cultures and maintained in RPMI 1640 supplemented with 10% heat-inactivated FBS, 100 IU/ml penicillin, and 100 μg/ml streptomycin (Invitrogen Life Technologies).

Human PC were obtained from colon LP tissue (LP-PC) of surgically resected specimens, and purified as described previously (13). They were also obtained from PB samples (PB-PC) of tetanus toxoid-vaccinated healthy volunteers and purified as described previously (14). This study was approved by the Institutional Review Board (Comision Etica, Hospital Universitario, Puerta del Mar, Cádiz, Spain). Human samples were obtained with informed consent according to the Declaration of Helsinki.

RT-PCR and RNase protection assays (RPA)

To perform PCR experiments, total RNA was purified from human PCs (5 × 10⁸–10⁹ LP-PC and 5 × 10⁶–10⁷ PB-PC) and cell lines U266 and IM-9 (5–10⁶ cells) using the Strataprep total RNA extraction kit (Stratagene). All RNA obtained was reverse transcribed using pdN6 (random hexamers) and AMV-RT (Amersham Biosciences). Then, cDNAs were amplified with specific primer pairs for each SNARE tested. PCR conditions were 94°C (30 s), 58°C (30 s), 72°C (45 s), 30 cycles, performed in a volume of 20 μl, and 15 μl of each reaction were analyzed on 1.5% agarose gels. Primer nucleotide sequences and product sizes are detailed in Table I.

To perform RPA, total RNA from cell lines (10⁷ cells) was obtained following standard protocols (15). For each different SNARE transcript tested, a fragment of cDNA, corresponding to sequence coding for protein was subcloned into pSP68 vector (Roche), and the corresponding antisense strands riboprobes were labeled [α-³²P]CTP (300 Ci/mmol) (Amersham Biosciences) using SP6 or T7 RNA polymerases and purified with Sephadex G-25 spin columns (Roche). The corresponding riboprobes (Table II) were hybridized with total RNA at 45°C for 16 h and digested according to RPA-kit instructions (Roche). The protected fragments were resolved on a 6% denaturing PAGE, which was dried and exposed to autoradiographic films for 24 h at −70°C with an amplification screen.

Western blotting

Cell lines U266 and IM-9 grown in T-25 flasks were washed twice with PBS buffer, centrifuged, and cell pellets (5 × 10⁶–10⁷ cells) were resuspended in ice-cold homogenization buffer (0.25 M sucrose, 1 mM EDTA, 20 mM HEPES-KOH [pH 7.4], 1 mM magnesium acetate, 1 mM PMSF, 1 μg/ml pepstatin A, 1 μg/ml leupeptin). The cells were frozen/thawed several times until >90% of cells were positive by trypan blue staining. Nuclei and unbroken cells were removed by centrifuging the lysates at 1000 x g for 10 min. The protein concentration in the postnuclear fraction was determined by the BCA kit (Pierce), and 60 μg of protein were resolved on a 10% SDS-PAGE and transferred to polyvinylidene fluoride membranes (Immobilon-P; Millipore). Blots were conducted as described (16). Briefly, polyvinylidene fluoride membranes were blocked for 1 h at 4°C with 5% dry nonfat milk/PBS and incubated overnight at 4°C with the primary Abs in the same blocking buffer. After three 15-min washes in TBST (25 mM Tris-HCl [pH 8], 500 mM NaCI, 25 mM KCl, 0.05% w/v Tween 20) and one wash with PBS, the membranes were incubated with secondary Abs in 5% dry nonfat milk/PBS for 1 h.

<table>
<thead>
<tr>
<th>Table I. Primer sets used for PCR</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>SNARE Transcripit</strong></td>
</tr>
<tr>
<td>Syntaxin-1A</td>
</tr>
<tr>
<td>Syntaxin-1B</td>
</tr>
<tr>
<td>Syntaxin-2</td>
</tr>
<tr>
<td>Syntaxin-3</td>
</tr>
<tr>
<td>Syntaxin-4</td>
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<tr>
<td>VAMP-2</td>
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<tr>
<td>VAMP-3</td>
</tr>
</tbody>
</table>

<sup>a</sup> The primers were designed to detect the possible different alternative splicing. S, Sense primer; AS, antisense primer; nt, nucleotides; bp, base pairs; a.n., accession numbers.

<sup>b</sup> For primer location numbering, the adenine from the initial coding ATG is considered number 1.
Table II. Probes used for RPA

<table>
<thead>
<tr>
<th>SNARE Transcript</th>
<th>Probe Size (b)</th>
<th>Protection Size (b) (expected)</th>
<th>Location on GenBank Transcript</th>
<th>GenBank a.n</th>
</tr>
</thead>
<tbody>
<tr>
<td>Syntaxin-1A</td>
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<td>317</td>
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<td>L37792</td>
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<td>258</td>
<td>479–737</td>
<td>BC047496</td>
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<td>Syntaxin-3</td>
<td>261</td>
<td>213</td>
<td>1–250</td>
<td>BT006666</td>
</tr>
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<td>Syntaxin-4</td>
<td>329</td>
<td>268</td>
<td>86–354</td>
<td>X85784</td>
</tr>
<tr>
<td>SNAP-23</td>
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<td>189</td>
<td>1–222</td>
<td>Y09567</td>
</tr>
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<td>229</td>
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<td>VAMP-3</td>
<td>268</td>
<td>213</td>
<td>93–303</td>
<td>U64520</td>
</tr>
</tbody>
</table>

* The probes include the protection sizes plus subcloning-derived sequences. nt, Nucleotides; b, bases; a.n., accession numbers.

** For location numbering, the adenine from the initial coding ATG is considered number 1.

at room temperature for 1 h, washed with TBST, and the specific proteins were detected by the appropriate secondary alkaline phosphatase-conjugated Abs and a chromogenic reaction nitroblue tetrazolium 5-bromo-4-chloro-3-indolylphosphate (Bio-Rad).

Expression and purification of GST fusion proteins
The constructs for native SNAP-23 protein (211 aas) and deleted SNAP-23 proteins (SNAP-23del1–203, lacking the last eight C-terminal residues; and SNAP-23del1–150, lacking the last 61 C-terminal residues) were generated by PCR from cDNA using specific oligonucleotide primers. For deleted protein mutants, antisense oligonucleotides encoding the corresponding deletion with a stop codon were designed. Then cDNA were cloned into the pGEX-3X vector (Amersham Biosciences), transformed into Escherichia coli BL21-DE3 strain, verified by automated DNA sequence analysis, and screened for colonies expressing GST-recombinant fusion proteins. Expression and purification of GST-recombinant proteins were performed as described elsewhere (17).

In vitro binding assays
U266 cells (105 cells) were homogenized in 3 ml of 150 mM NaCl, 50 mM Tris-HCl (pH 8), containing 1 mM PMSF, 1 μg/ml pepstatin A, and 1 μg/ml leupeptin, and disrupted by repeated freeze-thaw. Triton X-100 (1% v/v) was added to the homogenate, and the mixture was incubated at 4°C for 1 h in constant agitation. Mixture was clarified by centrifugation at 12,000 rpm for 10 min in a microfuge and buffer (20 mmol/L glutathione, 100 mmol/L Tris-HCl (pH 8.0), containing 1 mM PMSF, 1 μg/ml pepstatin A, and 1 μg/ml leupeptin), and 1 ml of the supernatant was used for binding to GST and GST fusion proteins. Glutathione-Sepharose 4B beads were added to the supernatant and incubated with gentle mixing for 3 h at 4°C. Beads were then sedimented, washed 5 times in ice-cold PBS, with gentle mixing, and proteins bound to matrix were eluted with 50 μl of elution buffer (20 mmol/L glutathione, 100 mmol/L Tris-HCl (pH 8.0), 120 mmol/L NaCl) for 30 min with gentle mixing. Eluates were separated from the beads by centrifugation at 12,000 rpm for 10 min in a microfuge and processed for SDS-PAGE and Western blotting (18).

Flow cytometry
Flow cytometry was performed using a BD Biosciences FACSCalibur flow cytometer (BD Biosciences). Fluorescent channels were set at logarithmic gain. Ten thousand events were acquired for each sample. A 530/30 band-pass filter was used for FL-1 fluorescence, and a 585/42 band-pass filter was used for FL-2 fluorescence. FITC was measured in the F1 channel, and the propidium iodine was measured in the F2 channel. Data were analyzed using CellQuest software (BD Biosciences).

Immunofluorescence (IF) confocal microscopy
Cell suspensions were fixed with 4% paraformaldehyde in PBS for 20 min at 4°C, washed three times with PBS, and permeabilized in PBS-S (PBS containing 0.2% saponin). After washing three times with PBS-S, antisera anti-SNAP23, anti-SNAP25, anti-Calnexin or anti-CD138 were added in PBS-BF (PBS containing 1% BSA and 5% FBS) for 1 h at room temperature. After washing three times with PBS-S, cells were incubated with the corresponding fluorescein isothiocyanate-conjugated secondary Ab for 1 h at room temperature. For direct IF detection of IgA, IgG, and IgE, three additional washes with PBS-S and incubation with FITC-conjugated rabbit anti-human IgA, IgG, or IgE for 1 h at room temperature were performed. After washing three times with PBS-S, the IF localization was performed using a laser confocal imaging system (TCS-SL; Leica Microsystems).

Protein transfections and ELISA
To introduce the recombinant proteins into the cells, we used the protein transfection kit Pro-Ject (Pierce), following the manufacturer’s directions. Briefly, the cell lines or the cells obtained from LP collagenase digestion or from PB Ficoll gradients were cultured at 105 cells/well in a 96-well plate. The reagent (2.5 μl/well) was mixed with 10 μl of RPMI 1640 and with 2 μg of purified recombinant protein and incubated for 10 min at room temperature. The mixes were added to the wells containing the cells and incubated at 37°C. Then an equal volume of prewarmed RPMI 1640 with 20% FBS was added to each well and incubated as described above. After the time indicated for each experiment, cell-free supernatants were collected, and IgG for IM-9 and PB-PC, IgE for U266, or IgA for LP-PC was tested by ELISA technique in microtiter plates, as reported previously (19).

Results
Human normal PC and PC lines exhibit a particular set of SNARE transcripts
The initial aim of this study was to determine the SNARE components expressed by human PC. To this end, the presence of transcripts of SNARE components described in other cell systems as implicated in the last steps of the exocytotic pathway (20) was examined in human PC by RT-PCR. For this, cDNA was synthesized from normal PC purified from colon LP (LP-PC) and PB-PC, and from the human PC lines IM-9 and U266, and the corresponding RT-PCR were performed by using primer pairs suitable for amplifying specific SNARE members. As shown in Fig. 1A, a PCR of 30 cycles revealed the occurrence of transcripts for SNAP-23, VAMP-2, VAMP-3, Syntaxin-2, Syntaxin-3, and Syntaxin-4, in both normal PC and PC lines. Neither Syntaxin-1 (-1A or -1B) nor SNAP-25 were detected in these cells. These results indicated that normal as well as tumoral PC exhibited a rather similar pattern of SNARE mRNA. In addition, VAMP-1 transcripts could be observed as a weak band and only in normal PC. To confirm these results, RPA technique was performed. The low number of PC obtained from normal tissues did not allow normal PC to be examined by this procedure. For this reason, these studies (and others like Western blotting) were only performed in PC lines. Fig. 1B shows that RPA experiments corroborated the results obtained previously by RT-PCR (Fig. 1A). The most abundant transcripts observed corresponded to SNAP-23, VAMP-2, and VAMP-3. Curiously, the protection of the VAMP-3 transcripts showed two bands of similar intensities, the expected band and another slightly lower. However, a single protection fragment was observed by using a different probe (data not shown), and a single robust amplification was observed by RT-PCR (Fig. 1A). RPA for Syntaxin-4 also revealed two bands, but in this case different amplification transcripts were also detected in RT-PCR experiments (Fig. 1A), and this was probably due to an alternative splicing. Syntaxin-1, VAMP-1, and SNAP-25 were not detected, despite testing with two different probes (data not shown).

A similar pattern of SNARE proteins is detected in human PC lines
Considering that the expression of transcripts was indicative of the possible protein expression, the presence of protein components corresponding to previously detected SNARE transcripts was examined by Western blotting. Postnuclear fraction lysates of U266 and IM-9 human PC lines were used for these experiments, and, as can be seen (Fig. 2), these cells expressed several SNARE proteins including SNAP-23, Syntaxin-2, Syntaxin-3, Syntaxin-4, VAMP-2, and VAMP-3. It should be noted that there was a doublet in the SNAP-23 Western blot. Whether this represented partial
proteolysis, alternative splicing, or post-translational modification has not been explored. For both Syntaxin-2 and Syntaxin-4, a band slightly smaller than that obtained for brain lysates was observed. Neither Syntaxin-1 nor SNAP-25 (Fig. 2) protein was present in these cells. No specific Ab for VAMP-1 protein was available at the time of performing the study, and, accordingly, its presence was not examined. Nevertheless, the results obtained with the RT-PCR and RPA suggested that this protein was probably not expressed in human PC. Collectively, these experiments indicated that human PC lines (and probably normal PC) exhibited a broad set of SNARE proteins, showing a pattern of SNARE expression similar to that observed in mRNA analysis above.

**IF localization of SNAP-23 within human PC**

As mentioned above, the SNARE core complex necessary for membrane fusion requires a member of the Syntaxins, VAMPs, and SNAP-25 families. The present study shows that human PC expressed several Syntaxins and VAMPs members, but only one SNAP-25 family component, the SNAP-23 protein, a reason that makes this molecule an ideal target for functional studies in the present system. In addition, it has been established in other cell systems that SNAP-23 plays a central role in the SNARE complex formation, where it acts as a bridge by binding the other two SNAREs partners (21). Therefore, indirect IF and confocal microscopy were used to examine the presence and the appropriate subcellular distribution of the SNAP-23 protein in normal and PC lines. Fig. 3, a, d, and g, shows the intracytoplasmic labeling for the corresponding IgG, IgE, and IgA isotypes for IM-9. U266, and LP-PC, respectively. The SNAP-23 labeling was clearly detectable in the PC lines IM-9 and U266 (Fig. 3, b and e), as well as in normal human PC (Fig. 3h). No labeling at all was observed when anti-SNAP-25 Ab was used (Fig. 3k), as expected considering the results obtained by Western blotting using the same Ab (Fig. 2). To test the possibility that the peripheral staining observed for SNAP-23 could be due to ER staining around the inner face of plasma membrane, the labeling with anti-Calnexin Ab, to identify the ER, and with anti-CD138 (Syndecam) Ab, to label the external plasma membrane, were explored. The labeling patterns for ER (Fig. 3m) and CD138 (Fig. 3p) show that the SNAP-23 staining (Fig. 3, n and p) was predominant in the plasma membrane. In all of the cases, staining was observed almost exclusively on the PC external boundary, indicating predominant localization at the cell membrane, although low levels of cytosolic staining could be detected.

**SNAP-23 is involved in human PC Ig secretion**

The predominant location of SNAP-23 on the cell surface of human PC prompted us to investigate whether this SNARE protein...
could play a role in Ig secretion. Due to the difficulties found for cDNA transfection into human PC, we decided to use a protein carrier reagent to introduce exogenous SNAP-23 fusion proteins into these cells, to study the putative functional role of this SNARE component in PC Ig exocytosis. To establish the conditions under which efficient protein transfer occurred, 2\(\mu\)g of an irrelevant FITC-labeled mAb was introduced by this technique into cell lines, and, after 6, 15, and 24 h of cultures, the FITC-label content, as well as the viability of cells tested by propidium iodide staining, were determined by flow cytometry and fluorescence microscopy (data not shown). FITC labeling was readily observed in \(\sim 80\%\) of the transfected cells (Fig. 4A), and at least 24 h after protein transfection FITC-labeled cells remained viable because they did not show increased propidium iodide staining (data not shown). Because the FITC-Ab molecular mass (\(\sim 150\) kDa) was higher than that of the fusion proteins used in this study (\(\sim 50–60\) kDa), at least similar efficiency should be expected. Another important question was to know how much protein could be introduced into the cells by this method, without disturbing the secretion process in an unspecific way. To test this point, different quantities of GST protein were introduced into U266 cells, and the IgE secreted by the cells was measured in the culture supernatant. As can be seen in Fig. 4B (dotted line), cell IgE secretion was not affected by the transfection of GST in a range of 0.5–2.5\(\mu\)g. Accordingly, a concentration of 2\(\mu\)g of protein was used in the subsequent experiments. C, U266 cell lysates were used for in vitro protein interactions as we have described in Materials and Methods. Specific binding of the Syntaxin-3 to the bacterially expressed GST-SNAP-23 fusion protein was demonstrated. Supernatants (Triton X-100 clarified extracts) were applied on Western blots as positive control to identify the corresponding band to Syntaxin-3.
To directly test a role of SNAP-23 in Ig secretion by human PC, recombinant GST, GST-SNAP-23, and GST-SNAP-23delC8 (C-terminal 8-aa deletion) fusion proteins were introduced into the cells using the described protein transfection technique, and their effect was assessed by measuring the subsequent Ig secretion by these cells, including U266 and IM-9 cell lines, and normal PC obtained from colon LP (LP-PC) and PB (PB-PC). As shown in Fig. 5A, the transfection reagent alone induced a small inhibition of Ig secretion and, for this reason, the results obtained were normalized to this value. The addition of cycloheximide drastically reduced Ig secretion in all of the cases, as expected. Introduction of GST alone did not seem to exert any effect. In contrast, the introduction of GST-SNAP-23 as well as GST-SNAP-23delC8 markedly inhibited the secretion of the Ig newly synthesized by PC cell lines and normal PC (Fig. 5A). The inhibitory effect of the fusion proteins was a concentration-dependent phenomenon, as shown for GST-SNAP-23 in Fig. 4 (continuous line). Fig. 5B shows a Western blotting corresponding to the cell pellets of one representative experiment presented in Fig. 5A, showing that similar quantities of the delivered recombinant fusion protein were observed, and that the intrinsic native SNAP-23 served as an internal control of protein charge. Finally, the introduction of a largely truncated fusion protein (SNAP-23delC61, a C-terminal 61-aa deletion) into the U266 cell line (Fig. 6) did not affect Ig secretion, a result similar to that obtained with GST alone, and clearly different from those observed by the introduction of GST-SNAP-23delC8 and GST-SNAP-23. Identical results were observed when normal human PC were used in similar experiments (data not shown).

Discussion

Our understanding of the PC biology has increased substantially in recent years. Thus, the central role of the transcription factors BLIMP-1 and XBP-1 in the development of the terminal PC differentiation program is now well established (22, 23). However, less is known about the mechanisms that ultimately control the main function of the PC, i.e., the secretion of Ig. The aim of the present study was to identify the molecules implicated in this process. In this regard, the hypothesis of this work was that human PC Ig secretion is similar to that found for this activity in certain cell types, in that it depends on extensive membrane fusion phenomena, a process where the SNARE components play a key role (24, 25). Present results show that human normal PC, as well as PC lines, express a similar set of SNARE components. Specifically, Syntaxin-2, Syntaxin-3, and Syntaxin-4 of the Syntaxin family, VAMP-2 and VAMP-3 members of the v-SNARE family, and SNAP-23 were readily detected by RT-PCR, RPA, and immunoblotting of whole cell extracts from these cells, including U266 and IM-9 cell lines, and normal PC obtained from colon LP (LP-PC) and PB (PB-PC). As shown in Fig. 5A, the transfection reagent alone induced a small inhibition of Ig secretion and, for this reason, the results obtained were normalized to this value. The addition of cycloheximide drastically reduced Ig secretion in all of the cases, as expected. Introduction of GST alone did not seem to exert any effect. In contrast, the introduction of GST-SNAP-23 as well as GST-SNAP-23delC8 markedly inhibited the secretion of the Ig newly synthesized by PC cell lines and normal PC (Fig. 5A). The inhibitory effect of the fusion proteins was a concentration-dependent phenomenon, as shown for GST-SNAP-23 in Fig. 4 (continuous line). Fig. 5B shows a Western blotting corresponding to the cell pellets of one representative experiment presented in Fig. 5A, showing that similar quantities of the delivered recombinant fusion protein were observed, and that the intrinsic native SNAP-23 served as an internal control of protein charge. Finally, the introduction of a largely truncated fusion protein (SNAP-23delC61, a C-terminal 61-aa deletion) into the U266 cell line (Fig. 6) did not affect Ig secretion, a result similar to that obtained with GST alone, and clearly different from those observed by the introduction of GST-SNAP-23delC8 and GST-SNAP-23. Identical results were observed when normal human PC were used in similar experiments (data not shown).

Discussion

Our understanding of the PC biology has increased substantially in recent years. Thus, the central role of the transcription factors BLIMP-1 and XBP-1 in the development of the terminal PC differentiation program is now well established (22, 23). However, less is known about the mechanisms that ultimately control the main function of the PC, i.e., the secretion of Ig. The aim of the present study was to identify the molecules implicated in this process. In this regard, the hypothesis of this work was that human PC Ig secretion is similar to that found for this activity in certain cell types, in that it depends on extensive membrane fusion phenomena, a process where the SNARE components play a key role (24, 25). Present results show that human normal PC, as well as PC lines, express a similar set of SNARE components. Specifically, Syntaxin-2, Syntaxin-3, and Syntaxin-4 of the Syntaxin family, VAMP-2 and VAMP-3 members of the v-SNARE family, and SNAP-23 were readily detected by RT-PCR in all the cases, and by RT-PCR, RPA, and immunoblotting of whole cell extracts from PC lines. This finding supports the view that the SNARE system might be mediating Ig secretion, because many of the components observed in human PC have been implicated in the final stages of exocytosis in other cellular systems (26, 27). Thus, Syntaxin-2 is expressed in several tissues and is thought to participate in secretion (28) and other different processes, such as cytokinesis (29) or morphogenesis (30, 31). Likewise, Syntaxin-3 and Syntaxin-4 have been involved in exocytotic processes in different cell types, including GLUT4 translocation in adipocytes (32), and platelets (33) and neutrophils granule secretion (34, 35). The Western blot analysis of human PC extracts in the present study reveals that Syntaxin-2, Syntaxin-3, and Syntaxin-4 exhibit a high level of expression in these cells. In addition, the band sizes

![FIGURE 5. Effect of GST-SNAP-23 introduction on human PC Ig secretion. A, U266 and IM-9 PC lines as well as human normal LP-PC and PB-PC were cultured for 15 h in the absence (CELLS) and in the presence of cycloheximide (CELLS-CHX), Pro-Jec reagent (CONTROL), Pro-Ject reagent plus GST-SNAP-23, Pro-Ject reagent plus GST-SNAP-23delC8, and Pro-Ject reagent plus GST. In all cases we used 2 μg protein/10⁵ cells/well. Ig secreted to the supernatant was determined by ELISA. Data were normalized to the Ig secreted by cells plus Pro-Ject reagent alone (CONTROL). Results represent the mean and the SEM of at least three experiments. B, Western blot of one representative experiment showing the protein transfected in the same cultures was as follow: 21.1 (4.8) of IgE for U266, 107.7 (46) of IgG for IM-9, 136.5 (75) of IgA for LP-PC, and 18.3 (7) of IgG for PB-PC. Western blot analysis of human PC extracts in the present study was to identify the molecules implicated in this process. In this regard, the hypothesis of this work was that human PC Ig secretion is similar to that found for this activity in certain cell types, in that it depends on extensive membrane fusion phenomena, a process where the SNARE components play a key role (24, 25). Present results show that human normal PC, as well as PC lines, express a similar set of SNARE components. Specifically, Syntaxin-2, Syntaxin-3, and Syntaxin-4 of the Syntaxin family, VAMP-2 and VAMP-3 members of the v-SNARE family, and SNAP-23 were readily detected by RT-PCR in all the cases, and by RT-PCR, RPA, and immunoblotting of whole cell extracts from PC lines. This finding supports the view that the SNARE system might be mediating Ig secretion, because many of the components observed in human PC have been implicated in the final stages of exocytosis in other cellular systems (26, 27). Thus, Syntaxin-2 is expressed in several tissues and is thought to participate in secretion (28) and other different processes, such as cytokinesis (29) or morphogenesis (30, 31). Likewise, Syntaxin-3 and Syntaxin-4 have been involved in exocytotic processes in different cell types, including GLUT4 translocation in adipocytes (32), and platelets (33) and neutrophils granule secretion (34, 35). The Western blot analysis of human PC extracts in the present study reveals that Syntaxin-2, Syntaxin-3, and Syntaxin-4 exhibit a high level of expression in these cells. In addition, the band sizes.
for Syntaxin-2 and Syntaxin-4 are slightly smaller than those obtained with human brain extracts, suggesting a protein expression with pre- and/or post-transductional modification. VAMP-2 is broadly expressed in brain tissue and participates in the neuronal secretion, unlike that which occurs with VAMP-3, which is not restricted to this organ and has been implicated preferentially in recycling processes (36, 37).

Syntaxin-1 and SNAP-25 were not detected in human PC, and VAMP-1 appeared also to be lacking in human PC lines. These data are consistent with the observation that the expression of these molecules is more restricted to neuronal and neuroendocrine tissues (38–40). However, the presence of Syntaxin-1 and VAMP-1 has been documented in other non-neuronal cells as neutrophils and osteoblasts (41–43), and the expression of SNAP-25 has been demonstrated in gastric parietal cells and eosinophils (44, 45). The RT-PCR results presented here in normal PC, but not in PC lines (Fig. 1A), show amplification for VAMP-1 transcripts. Accordingly, VAMP-1 might be expressed by human normal PC, a fact that could not be confirmed at the protein level, because a suitable Ab is not available yet.

The third protein family required for SNARE core complex formation, the SNAP-25 family, only contains two possible isoforms implicated in the latter stages of exocytosis, the SNAP-25 and the SNAP-23 members. As mentioned above, the first isoform is considered to be expressed more specifically in neuronal and neuroendocrine tissues, unlike SNAP-23, which represents a counterpart of SNAP-25 expressed in certain non-neuronal cells (21). Present data obtained by RT-PCR, RPA, Western blotting, and IF show that all human PC express SNAP-23, but not SNAP-25, and confocal studies confirm its predominant cell membrane location, a place where this protein, and also SNAP-25, plays a clear role in secretion in other cell systems (46, 47). The fact that human PC express several Syntaxin and VAMP components, but just one SNAP-25 member, the SNAP-23, makes this molecule an ideal target for functional studies aimed at determining whether Ig secretion is mediated by the SNARE system.

The functional involvement of the SNARE complex in PC Ig secretion was first suggested by the inhibitory effect observed with N-ethylmaleimide treatment (data not shown). To check this assumption in a more direct way, experiments were conducted to block SNAP-23 protein activity in human PC, by introducing recombinant fusion protein GST-SNAP-23 and GST-SNAP-23delC8 into these cell populations. Both constructs strongly inhibited Ig secretion by human normal PC and PC lines, reaching a level of reduction similar to that observed with cycloheximide treatment. This effect was specific because GST alone did not exert any effect. In addition, the introduction in human PC of anti-SNAP-23, but not an irrelevant antiserum, also provoked a marked reduction of the subsequent Ig secretion (data not shown). The inhibitory effect of GST-SNAP-23del1–203 on PC Ig secretion is consistent with the results observed in 3T3-L1 adipocytes infected with recombinant adenoviruses carrying SNAP-23delC8 (48). The explanation of how GST-SNAP-23delC8 inhibits cell secretion remains unknown, but it has been suggested that a competition could exist between the deleted protein and the endogenous SNAP-23, resulting in either the formation of unstable SNARE complexes of GST-SNAP-23delC8 with the other SNARE partners (49, 50), or the prevention of endogenous SNAP-23 from forming a SNARE complex (51). The finding that undeleted GST-SNAP-23 also produces an identical inhibition of PC Ig secretion indicates that additional events might explain the present results. One of the mechanisms by which both inhibitory fusion proteins exerted this activity appears to be their capacity to associate with partner SNARE components in the complex. Thus, it has been demonstrated that the SNAP-23 N-terminal region is essential for the binding of the Syntaxin partner, whereas both N-terminal as well as C-terminal regions are needed for interacting with the VAMP partner (52). Indeed, when we addressed in vitro binding assays of GST-SNAP-23 proteins with U266 cell extracts, we observed that the three constructs have the capability to bind Syntaxin-3 (data not shown). The explanation could be that the constructs can interact equally well with the Syntaxins but not with VAMP partners. These results may explain why the introduction of GST-SNAP-23delC61 did not inhibit Ig secretion (Fig. 6). GST-SNAP-23delC61 lacks one of the two central α-helices comprising the domain that mediates this interaction with the other SNARE partners. In addition, it is well known that fusion proteins generated in bacteria, like those used in the present study, lack post-translational modifications that are required for the proper activity of many proteins. In fact, at least two post-translational modifications of SNAP-23 have been described as being essential for membrane fusion. The first modification is the phosphorylation of SNAP-23 mediated by kinases as SNAK kinase (53, 54) and protein kinase C (54), a process that has been implicated in the regulation of SNARE-complex formation. The other modification is the palmitoylation of cysteine residues clustered in the segment in between the two helical domains forming the core complex. Recent reports suggest that these palmitic residues allow the insertion of SNAP-23 (and SNAP-25) into membranes, a phenomenon that is essential for membrane fusion (55, 56). Moreover, early palmitoylation of SNAP-25 seems to be inherent to an intact secretory pathway (57). Therefore, bacterially produced SNAP-23 fusion proteins could be capable of associating to the corresponding SNARE partners (Fig. 4C), but could fail to form a productive SNARE complex (Fig. 6). In accordance, SNAP-23 fusion proteins probably act as inhibitory peptides that compete with normal endogenous SNAP-23 partners for the SNARE complex (58) and provoke the subsequent blockade of the Ig secretion pathway. Whatever the explanation, present data indicate that SNAP-23 is involved in human PC Ig secretion. Further work will be required to identify additional components of the SNARE complex that participate in this process, although preliminary data obtained from in vitro binding assays suggest that Syntaxin-3 appears to be one of them (Fig. 4C).

In summary, the present study demonstrates that human normal PC and PC lines share the expression of a wide and similar set of SNARE components, mainly involved in exocytic phenomena, that includes Syntaxin-2, -3, and -4, VAMP-2 and -3, and SNAP-23. Moreover, the introduction into PC of recombinant SNAP-23 proteins capable of interfering with endogenous SNAP-23 leads to the prevention of Ig secretion. Taken together, these observations support the view that the SNARE system, and specifically SNAP-23, plays a critical role in the Ig secretion by human PC.

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**Disclosures**

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**References**


