Function of amphiphysin in SRIF receptor targeting

Role of Amphiphysin II in Somatostatin Receptor Trafficking
in Neuroendocrine cells

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ABSTRACT

Amphiphysins are SH3 domain-containing proteins thought to function in clathrin-mediated endocytosis. To investigate the potential role of amphiphysin II in cellular trafficking of G protein-coupled somatostatin (SRIF) receptors, we generated an AtT-20 cell line stably overexpressing amphiphysin IIb, a splice variant that does not bind clathrin. Endocytosis of 125I-[D-Trp\(^8\)]-SRIF was not affected by amphiphysin IIb overexpression. However, the maximal binding capacity (Bmax) of the ligand on intact cells was significantly lower in amphiphysin IIb-overexpressing than in non-transfected cells. This difference was no longer apparent when the experiments were performed on crude cell homogenates, suggesting that amphiphysin IIb overexpression interferes with SRIF receptor targeting to the cell surface and not with receptor synthesis. Accordingly, immunofluorescence experiments demonstrated that, in amphiphysin overexpressing cells, sst\(_{2A}\) and sst\(_5\) receptors were segregated in a juxtanuclear compartment identified as the trans-Golgi network. Amphiphysin IIb overexpression had no effect on CRF-41-stimulated ACTH secretion, suggesting that it is not involved in the regulated secretory pathway. Taken together, these results suggest that amphiphysin II is not necessary for SRIF receptor endocytosis but is critical for its constitutive targeting to the plasma membrane. Therefore, amphiphysin IIb may be an important component of the constitutive secretory pathway.
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Key words: Somatostatin receptors, targeting, endocytosis, ACTH secretion, cytoskeleton, confocal microscopy.
INTRODUCTION

The formation of carrier vesicles is an important aspect of membrane transport along the secretory and endocytic pathways (for review, see (1)). In the past few years, studies on clathrin-mediated receptor endocytosis have led to the identification of novel protein components of the endocytic regulatory machinery. The budding and fission of clathrin-coated vesicles from the plasma membrane, initiated by the recruitment of the adaptor protein-2 (AP-2) and clathrin, were shown to be regulated by several accessory proteins including dynamin, synaptojanin, amphiphysin, Eps15, intersectin, and endophilin (for review, see (2-5)).

The trans-Golgi network (TGN) is a dynamic organelle through which nascent secretory and transmembrane proteins are sorted and packaged into distinct carrier vesicles for transport either to the plasma membrane or to endosomal compartments. Three major pathways out of the TGN have been identified thus far: the regulated secretory pathway, which delivers surface-destined cargo via clathrin-coated vesicles; the constitutive secretory pathway, which mediates sorting from the TGN via non clathrin-coated vesicles; and the clathrin-mediated trafficking route to the endosomal/lysosomal compartments (for review, see (6-9)). Whereas a growing number of proteins have been identified to function in the sorting of proteins towards the endosome/lysosome, such as the adaptor protein-1 (AP-1) and the GGA family (10-17),

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relatively little is known regarding proteins that function in the regulated or constitutive secretory pathways.

Recent studies have suggested that proteins that regulate membrane budding at the cell surface may also be involved in the production of transport vesicles at the TGN. For example, dynamin II has been localized to the TGN of mammalian cells and shown to be required for the formation of transport vesicles (18-23). Accordingly, introduction of the dynamin II mutant (K44A) was found to interfere with the formation of both clathrin-coated and non-clathrin-coated vesicles from the TGN (24,25). Different isoforms of the same accessory proteins could also be involved in clathrin-mediated endocytosis versus production of secretory vesicles. Thus, whereas AP-2, endophilin A1, and synaptojanin are essential for synaptic vesicle endocytosis, AP-1, AP-3 and AP-4, endophilin B1, and synaptojanin-like Inp53p have been implicated in vesicle budding from the TGN (10,26-28).

Amphiphysins belong to the BAR (Bin/Amphiphysin/Rvs) family of proteins, which includes the mammalian bridging-integrators (Bin1, Bin2, Bin3), amphiphysin I and II, and the yeast Rvs161p and Rvs167p (for review, see (29,30)). Amphiphysin I and II act as multifunctional adaptor proteins that cooperate in the recruitment and targeting of other key endocytic proteins. Through direct protein-protein interactions, both isoforms bind their C-terminal SH3 domains to proline-rich sequences of dynamin and synaptojanin (Fig. 1; (31-34)). Amphiphysins I and II have also been reported to interact with AP-2 and endophilin through an SH3 domain-
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independent sequence (31,35-37). The insert region of amphiphysin I and II was reported to interact with clathrin-binding sites, but at sites distinct from the AP-2 and endophilin-binding domains (Fig. 1; (4,29)). Furthermore, the N-terminus of amphiphysin II mediates the dimerization and plasma membrane targeting of this protein (Fig. 1; (38)).

Seven different splice isoforms of amphiphysin II have so far been isolated (Fig. 1; (34,37,39-43). These splice variants have pleiotropic roles, including clathrin-mediated endocytosis, apoptosis, stress signaling processes, and regulation of membrane dynamics, perhaps through interaction with the actin cytoskeleton (for review, see (4,5,30,44-46)). Much of the variability in the splice forms comes from a central region, encoded by exons 12a-12d (Fig. 1; (47)). The latter may convey different binding specificities and functions to the different splice forms. In particular, amphiphysin IIb, which is lacking an important determinant for plasma membrane targeting and does not bind to clathrin, may have functions unrelated to endocytosis. To test this hypothesis, we investigated the effect of amphiphysin IIb overexpression on the trafficking of somatostatin receptors sst\textsubscript{2A} and sst\textsubscript{5} in the pituitary corticotrope cells AtT-20 which express both receptor subtypes endogenously (48,49). Our results demonstrate a role for amphiphysin IIb in the constitutive trafficking of these receptors from the TGN.
Culture and transfection of AtT-20 cells

AtT-20 cells (a mouse ACTH-secreting tumor cell line) were grown and sub-cultured in Dulbecco’s Modified Eagle’s Medium (DMEM) with high glucose (4.5 g/l) supplemented with 10% fetal bovine serum and 10% horse serum in the presence of 100 U/ml penicillin/streptomycin (GibcoBRL, Life Technologies, Burlington, Ont, CAN) as previously described (49). Cell monolayers were grown in T75 cm² flasks, maintained in a humidified atmosphere of 95% O₂ and 5% CO₂ at 37°C and passaged when the monolayer achieved 90% confluence.

An amphiphysin IIb-overexpressing AtT-20 cell line was established according to procedures described previously (50). Briefly, twenty-four hours after plating, semi-confluent AtT-20 cells were transfected with the recombinant pcDNA3 plasmid (5 µg/35-mm dish; Invitrogen, San Diego, Ca, USA) containing a Bam HI Eco RI insert of amphiphysin IIb cDNA using the DAC-30 reagent according to the manufacturer’s recommendation (Eurogentec, Seraing, BEL). After two days, the medium was changed for growth medium containing 0.75 mg/ml geniticin (G418). Surviving colonies were isolated two weeks later and separately cultivated in 24 well plates. Clones were then checked for expression of amphiphysin IIb by immunoblotting and immunofluorescence microscopy using an antibody raised against amphiphysin II (34). The clone
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expressing the highest level of amphiphysin IIb was selected for this study. Cells were maintained in standard growth medium supplemented with 0.5 mg/ml G418. They were plated in 16-mm multi-well dishes for binding and ACTH release experiments at an initial plating density of $10^5$ cells per well.

Immunoblotting analysis

Polyclonal antibodies raised against amphiphysin II have been extensively characterized elsewhere (34,38). For blots, AtT-20 cells were homogenized in 20 mM HEPES-OH, pH 7.4, containing 0.83 mM benzamidine, 0.23 mM phenylmethylsulfonyl fluoride, 0.5 µg/ml aprotinin, and 0.5 µg/ml leupeptin. Insoluble material was removed from the extract by centrifugation for 15 min at 13,000 rpm. Proteins (100 µg/lane) were then separated on a 3-12% gradient SDS-PAGE, transferred to nitrocellulose, and processed for Western blotting analysis using antibodies raised against amphiphysin II. The immunoreactivity was visualized using an enhanced chemoluminescent detection system (Perkin Elmer, Life Sciences, Boston, Ma, USA).

Morphological parameters

To compare the morphological characteristics of amphiphysin IIb (A-IIb)-overexpressing cells with those of wild-type, non-transfected cells (WT), perimeter, area and form factor (defined as $4\pi \times \text{area} \div \text{perimeter}^2$) were determined in 100 cells (50 WT, 50 A-IIb), using a computer-
assisted image analysis system (Biocom, Les Ulis, France). Both WT and amphiphysin IIb-transfected cells were first fixed for 20 min with 4% paraformaldehyde (PFA; Polysciences, Warington, Pa, USA) in 0.1 M phosphate buffer (PB), pH 7.4 and rinsed twice with 0.1 M PB. They were then stained with 0.05% toluidine blue, mounted, and analyzed under a 50 oil immersion objective on a Leitz Diaplan microscope. All calculations and statistical analyses were performed using Excel 5.0 (Microsoft Corp., San Francisco, Ca, USA). Statistical significance was verified using Student’s t-test.

**Preparation of cell homogenates**

Confluent AtT-20 cells were washed and scraped off the culture dishes with ice-cold Tris-buffered saline (pH 7.5). Subsequently, the cells were centrifuged at 15,000 g for 5 min at 4°C in microcentrifuge tubes and resuspended in hypotonic TE buffer (5 mM EDTA and 10 mM Tris-HCl, pH 7.5). Membrane homogenates were then sonicated, recentrifuged at 15,000 g for 30 min at 4°C, and resuspended in the same buffer.

Binding and internalization of $^{125}$I-Tyr$^0$-[D-Trp$^8$]-somatostatin

**Association kinetics**

To investigate somatostatin (SRIF) binding and internalization, WT and amphiphysin IIb-overexpressing AtT-20 cells were equilibrated for 10 min at 37°C in Earle’s buffer (140 mM
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NaCl, 5 mM KCl, 1.8 mM CaCl\(_2\), 0.9 mM MgCl\(_2\) and 25 mM HEPES, pH 7.4) supplemented with 0.1% glucose and 1% Bovine Serum albumin (BSA). The equilibration medium was then replaced by 250 µl of Earle’s buffer containing 0.1 nM \(^{125}\text{I-Tyr}^0\)-[D-Trp\(^8\)]-SRIF (2000 ci/mmol) in the presence of 0.8 mM 1,10-phenanthroline for 5-45 min at 37°C. After various incubation periods, the cells were washed twice with either 0.5 ml of Earle’s buffer, or with 0.5 ml of a hypertonic acid buffer (Earle’s buffer containing 0.2 M acetic acid and 0.5 M NaCl, pH 4) for 3 min to strip off surface-bound radioactivity (but retain intracellularly sequestered SRIF). Cells were then harvested with 1 ml of 0.1 M NaOH and cell-associated radioactivity was counted in a γ-counter. Nonspecific binding, as measured in the presence of 1 µM unlabeled [D-Trp\(^8\)]-SRIF (Neosystem, Lyon, France), represented less than 5% of the total binding.

**Equilibrium binding experiments on whole cells**

After equilibration for 10 min at 37°C in Earle’s buffer, saturation experiments were performed by incubating cells for 30 min at 37°C with increasing concentrations (0.5 to 16 nM) of \(^{125}\text{I-SRIF}\) isotopically diluted with unlabeled [D-Trp\(^8\)]-SRIF in the binding buffer. At the end of the incubation, cells were washed twice with 0.5 ml of equilibration buffer and harvested with 1 ml of 0.1 M NaOH. Nonspecific binding was measured in the presence of 1 µM nonlabeled [D-Trp\(^8\)]-SRIF. Dissociation constant (\(K_d\)) and maximal binding capacity (\(B_{\text{max}}\)) were derived from
Scatchard analysis of the data.

**Equilibrium binding experiments on crude cell homogenates**

Crude cell homogenates (50 µg) were incubated with increasing concentrations (0.5 to 1.6 nM) of $^{125}$I-SRIF for 30 min at 25°C in 250 µl of binding buffer (50 mM Tris-HCl, pH 7.5, 2 mM MgCl$_2$ containing 1% BSA and 0.8 mM 1-10-phenanthroline). Binding experiments were terminated by addition of 3 ml of ice-cold buffer followed by filtration through glass-microfiber filters (GF/C, Whatman, Clifton, Nj, USA) preincubated with 0.5% polyethylenimine. After washing twice with 3 ml of ice-cold buffer, the radioactivity retained on the filter was counted in a $\gamma$-counter. Nonspecific binding was measured in the presence of 1 µM unlabeled [D-Trp$^8$]-SRIF. All binding/internalization data were calculated and plotted using Prism 3.02 (Graph Pad Software) and represent the mean ± standard deviation (S.D.) of n determinations (as indicated in Results).

**Internalization of α-Bodipy Red D-Trp$^8$-SRIF (fluo-SRIF) in AtT-20 cells**

For confocal microscopic tracking of internalized ligand, the pH-insensitive dye Bodipy 576/589 (Molecular Probes, Inc., Eugene, Or, USA) emitting red fluorescence was covalently conjugated to the degradation-resistant SRIF analog D-Trp$^8$-SRIF in the α-position (for more detail, see (51)). AtT-20 cells, grown on 12-mm polylysine-coated glass coverslips in 18 mm Petri dishes
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were equilibrated for 10 min at 37°C in Earle’s buffer containing 1% BSA and 0.1% glucose. They were then incubated for 10 or 40 min in the same buffer with 20 nM fluo-SRIF (kindly provided by Prof. J.P. Vincent), in the presence or absence of 10⁻⁵ M non-fluorescent D-Trp⁸-SRIF. For selective visualization of internalized fluo-SRIF, cells were washed with hypertonic acid buffer (pH 4) for 3 min. Labeled cells were then mounted on glass slides with Aquamount, air-dried, and examined under a Zeiss laser scanning confocal microscope equipped with an Axiovert 100 inverted microscope and an argon-krypton laser. Samples were scanned at 568 wavelength nm excitation. Images were acquired as single transcellular optical sections and averaged over 16 scans/frame and processed using the Carl Zeiss CLSM software 3.1 version. The final composites were adjusted for contrast and brightness using Adobe Photoshop 6.0 software (Adobe, San Jose, Ca, USA) and processed using Deneba’s Canvas 7.0 imaging software (Deneba Software, Miami, Fl, USA) on an Apple Powerbook G3.

Immunodetection of sst2A and sst5 receptors in AtT-20 cells

AtT-20 cells, plated on poly-L-lysine-coated glass coverslips, were fixed for 20 min with 4% PFA in 0.1 M PB, pH 7.4, rinsed twice with 0.1 M Trisma base-buffered saline (TBS), pH 7.4, and pre-incubated for 30 min at room temperature (RT) with a blocking solution consisting of 5% normal goat serum (NGS), 2% BSA and 0.1% Triton X-100 (BDH Inc. Toronto, Ont, CAN) in 0.1 M TBS. Immunostaining was performed by incubating cells overnight at 4°C in TBS
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containing 0.05% Triton X-100 with one of the following rabbit antibodies: 1) 1:1000 dilution of sst5 serum, 2) 1:2000 dilution of sst2A antibody. The specificity of each of these antibodies has been fully established elsewhere (52-56). After three rinses (5 min each) in TBS, cells were incubated for 1 hour at RT with an Alexa 594-conjugated goat anti-rabbit IgG (1:750; Molecular Probes, Eugene, Or, USA). They were then washed twice in TBS and mounted on glass slides with Aquamount for confocal microscopic examination. Images were acquired, stored, and archived as described above.

Double immunofluorescence labeling

To identify the intracellular compartments of somatostatin receptor sequestration, WT and amphiphysin IIb-transfected AtT-20 cells were fixed with 4% PFA for 20 min at RT, washed twice with 0.1 M TBS and pre-incubated for 30 min in the same buffer containing 5% normal goat serum (NGS), 2% BSA and 0.1% Triton X-100. They were then rinsed twice with TBS and incubated in a mixture of primary antibodies in TBS containing 0.5% NGS and 0.05% Triton X-100 overnight at 4°C. The mixture contained the mouse anti-syntaxin 6 antibody (3 µg/ml; Transduction Laboratories, Mississauga, Ont, CAN) and either sst5 (1:1000 of serum) or sst2A (1:2000) antibodies raised in rabbit. After rinsing three times (5 min each) with TBS, bound primary antibodies were revealed by simultaneous incubation with goat anti-mouse Alexa 488-(1:500; Molecular Probes, Eugene, Or, USA) and goat anti-rabbit Alexa 594-conjugated...
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secondary antibodies (1:750; Molecular Probes, Eugene, Or, USA) for 60 min at RT. After washing, the coverslips were mounted on glass slides using Aquamount and viewed with a confocal microscope.

ACTH release studies

WT and amphiphysin IIb-overexpressing AtT-20 cells were plated in 16-mm multi-well culture dishes and allowed to form monolayers for 48 hours prior to experiments. ACTH release was measured on intact and attached cells as previously described (57,58). Each well was washed twice with 1 ml of DMEM supplemented with 0.1% BSA (DMEM/BSA) and then incubated for 1 hour in 1 ml fresh DMEM/BSA at 37°C in a humidified atmosphere of 10% C02 in air. The DMEM/BSA was then decanted and replaced with 500 µl of fresh DMEM/BSA in the absence or presence of corticotrophin-Releasing Factor-41 (CRF-41; Neosystem, Lyon, France) and [D-Trp8]-SRIF, alone or in combination. Zero time samples were taken at this point and the remaining cells were incubated for 2 hours at 37°C in a humidified atmosphere of 10% C02 in air. Incubations were terminated by collecting the DMEM/BSA medium, centrifugating this medium for 30 s at 10,000 g and removing of the supernatant. The ACTH content of the supernatant was measured using a radioimmunoassay kit (Diasorin, Stillwater, Mn, USA). Values were expressed as the mean ± S.E.M. of three determinations performed in duplicate. Calculations and statistical analyses were performed using Prism 3.02 (Graph Pad Software, San
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Diego, Ca, USA). Statistical significance was verified using a repeated Measures ANOVA with a Bonferroni’s Multiple Comparison Test.
RESULTS

Detection of Amphiphysin IIb in AtT-20 Cells

To investigate amphiphysin IIb function, we generated an AtT-20 cell line overexpressing amphiphysin IIb. The expression of amphiphysin IIb was examined by Western blotting on cell homogenates prepared from both WT and amphiphysin IIb-transfected AtT-20 cells using a polyclonal antibody produced against the C-terminal 217 amino acids of amphiphysin II (Fig. 1). In WT cells, this antiserum detected immunoreactive bands at approximately 70-80, 90, and 97 kDa (Fig. 2A, lane 1), which correspond to the molecular weights expected for amphiphysin IIc, IIb and IIa, respectively (Fig. 1; (38)). The presence of a 90 kDa band in WT AtT-20 cells suggests that amphiphysin IIb is endogenously expressed at a low, but detectable level in these cells (Fig. 2A, lane 1). In amphiphysin IIb-transfected AtT-20 cells, the 90 kDa immunoreactive band, corresponding to the amphiphysin IIb isoform, was greatly increased. Quantification of this band revealed that the transfected AtT-20 cells displayed a ~ 40 fold increase in the amphiphysin IIb protein as compared to controls.

Morphological Characterization of AtT-20 Cells Overexpressing Amphiphysin IIb

To determine whether overexpression of amphiphysin IIb induced changes in the phenotype of AtT-20 cells, the perimeter, area and form factor of transfected cells were compared to those of WT cells (Fig. 3). Computer-assisted morphometric analysis revealed that the perimeter of
amphiphysin IIb-transfected cells (204 ± 5.7 µm) was increased by 36% as compared to WT cells (130 ± 4.9 µm ; Fig. 3A). There was also a 41% increase in the surface area of amphiphysin IIb overexpressing cells (656 ± 29 µm²) when compared to WT AtT-20 cells (384 ± 16 µm² ; Fig. 3B). Accordingly, transfected cells (0.21 ± 0.01) displayed significant reductions in form factor compared to WT cells (0.31 ± 0.03 ; Fig. 3C).

Binding and Internalization of Somatostatin in AtT-20 Cells

To determine whether overexpression of amphiphysin IIb affected ligand-induced endocytosis of somatostatin (SRIF) receptors, internalization assays were carried out on WT and amphiphysin IIb-transfected cells. Association kinetics of ¹²⁵I-Tyr⁰-[D-Trp⁸]-SRIF binding were determined on whole cells at 37°C and the proportion of sequestered radioactivity was assessed after hypertonic acid wash of surface bound molecules (Fig. 4, n = 4). ¹²⁵I-SRIF bound specifically to both wild-type (Fig. 4A) and amphiphysin IIb-overexpressing (Fig. 4B) AtT-20 cells in a time-dependent manner and reached a plateau within 20 min. At that time, over 75% of bound radioactivity was resistant to acid/NaCl wash (i.e. was sequestered intracellularly) in both wild-type (77.5 ± 3.2% ; Fig. 4A) and amphiphysin IIb-transfected cells (76.4 ± 2.1% ; Fig. 4B), indicating that overexpression of amphiphysin IIb had no apparent effect on SRIF receptor internalization.

To visualize the intracellular trafficking of internalized SRIF, AtT-20 cells were incubated at
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37°C with 20 nM fluo-SRIF and cell surface labeling was stripped off with hypertonic acid buffer. Following 10 min (Fig. 5A, B) or 40 min (Fig. 5C, D) incubation with the fluorescent ligand, hot spots of internalized fluo-SRIF were observed in the juxtanuclear cytoplasm in both wild-type (Fig. 5A,C) and amphiphysin IIb-transfected (Fig. 5B, D) AtT-20 cells. This labeling was specific in that it was entirely competed for by an excess of nonfluorescent [D-Trp^8]-SRIF (not shown).

Effect of Amphiphysin IIb on the Cell Surface Expression of SRIF Receptors

In order to determine whether the parameters of 125I-SRIF association with SRIF receptors present on the surface of AtT-20 cells were modified by amphiphysin IIb overexpression, saturation experiments were performed for 30 min at 37°C under equilibrium conditions (Fig. 6; n = 3). Incubation of whole AtT-20 cells with increasing doses of 125I-SRIF revealed the presence of specific and saturable 125I-SRIF binding on both WT and amphiphysin IIb-overexpressing cells (Fig. 6A). However, the maximal binding capacity (B_max) was significantly lower in transfected (126.4 ± 12.5 fmol/mg) than in WT AtT-20 cells (334 ± 7.4 fmol/mg; Fig. 6B), whereas the apparent dissociation constant (Kd) was not significantly different between the two cell types (1.30 ± 0.35 nM and 0.98 ± 0.22, respectively). This difference in B_max was no longer apparent when the experiments were performed on crude cell homogenates rather than on intact cells (Fig. 6C). As illustrated by the Scatchard plot in Fig. 6D, the maximal binding
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capacity of $^{125}\text{I}-\text{SRIF}$ to membrane preparations from amphiphysin IIb-transfected (447 ± 76.3 fmol/mg, Kd = 0.26 ± 0.14 nM) and WT (449 ± 7.5 fmol/mg; Kd = 0.31 ± 0.09 nM) AtT-20 cells were identical, suggesting that overexpression of amphiphysin IIb only impaired the number of cell surface SRIF receptors.

Confocal Microscopic Localization of sst$_2^{\text{A}}$ and sst$_5^{\text{R}}$ Receptors

Immunocytochemistry revealed distinct distributional patterns for sst$_2^{\text{A}}$ and sst$_5^{\text{R}}$ receptors in WT and amphiphysin IIb-transfected AtT-20 cells (Fig. 7; n = 5). sst$_2^{\text{A}}$ immunoreactive receptors were exclusively confined to the cell surface in WT AtT-20 cells (Fig. 7A), but were mainly concentrated intracellularly, in a juxtanuclear compartment, in amphiphysin IIb-transfected cells (Fig. 7B). Immunoreactive sst$_5^{\text{R}}$ receptors were observed both at the cell surface and in a prominent cytoplasmic pool in WT cells (Fig. 7C), but were selectively concentrated in a juxtanuclear pool in amphiphysin IIb-transfected cells (Fig. 7D).

To identify the compartment of intracellular sequestration of sst$_2^{\text{A}}$ and sst$_5^{\text{R}}$ receptors in amphiphysin IIb-overexpressing cells, immunohistochemical detection of sst$_2^{\text{A}}$ or sst$_5^{\text{R}}$ receptors was combined with the immunocytochemical localization of the trans-Golgi network (TGN) marker syntaxin-6 (59). Dual immunolabeling studies revealed a complete overlap between sst$_2^{\text{A}}$ (Fig. 8) or sst$_5^{\text{R}}$ (not shown) and syntaxin-6 immunoreactivity, suggesting that both receptors
were sequestered within the TGN in amphiphysin IIb-transfected cells.

Effect of amphiphysin IIb on Stimulated ACTH Secretion

To determine whether the regulated secretory pathway was affected by overexpression of amphiphysin IIb, we assessed the effects of corticotrophin releasing factor (CRF-41) and SRIF on the in vitro release of ACTH from AtT-20 cells. Drug concentrations were chosen so as to induce maximal stimulation (CRF; 100 nM) or inhibition (SRIF; 100 nM) of ACTH secretion (57,58,60). CRF-41 significantly enhanced ACTH secretion in WT AtT-20 cells (235.7 ± 34.2%; Fig. 9). CRF-41 also significantly increased ACTH released from amphiphysin IIb-transfected cells, although not to the same extent as from WT AtT-20 cells (202 ± 20.8%; Fig. 9). However, the difference between WT and transfected cells was not significant, indicating that overexpression of amphiphysin IIb did not affect the regulated secretory pathway.

By contrast, a significant difference between the two cell types was observed in the case of stimulation with SRIF. In WT AtT-20 cells, SRIF markedly inhibited (40%; 143.7 ± 13.5%) CRF-induced ACTH release whereas in amphiphysin IIb-transfected AtT-20 cells, the effect of SRIF on CRF-induced ACTH secretion was considerably less (17.5%; 166.7 ± 18.3%), in keeping with the documented decrease in the density of cell surface SRIF receptors.
DISCUSSION

The present study provides the first evidence for the implication of an amphiphysin isoform, amphiphysin IIb, in the trafficking of G protein-coupled receptors to the plasma membrane of neuroendocrine cells. It also suggests that this amphiphysin isoform is not essential for ligand-induced endocytosis of the same receptors in these cells.

**Amphiphysin IIb overexpression in AtT-20 cells**

In the present study, overexpression of the amphiphysin IIb isoform was used to investigate the potential role of amphiphysin II in budding events at the plasma membrane and the TGN in AtT-20 cells. This cell type was chosen because it had been documented to express five of the six cloned SRIF receptor sub-types (sst1, sst2A, sst2B, sst4 and sst5; (48,49,61)) and to behave as pituitary corticotrophs (57,58,62). Furthermore, these cells were found here by Western blotting to endogenously express amphiphysin IIb, thereby strengthening the biological relevance of this model system for investigating the functional role of this protein. Western blotting experiments showed the presence of other amphiphysin splice variants in AtT-20 cells, in keeping with earlier reports on the presence of amphiphysins in neuroendocrine tissues (42). Western blotting experiments also confirmed that amphiphysin IIb-transfected cells overexpressed this protein isoform. Furthermore, overexpression was selective, as it did not affect the expression levels of
other amphiphysin isoforms present in the same cells.

**Overexpression of amphiphysin IIb affects the size and shape of AtT-20 cells**

Morphometric studies indicated that overexpression of amphiphysin IIb affected both the size and shape of AtT-20 cells, suggesting that the amphiphysin IIb isoform may interact with cytoskeletal proteins important for the control of cell form and size. A growing body of evidence suggests that proteins involved in membrane trafficking such as dynamin, cortactin, and intersectin may interact with the actin cytoskeleton (for reviews, see (4,63-68)). Amphiphysin family members have also been implicated in the dynamics of the cell cytoskeleton. Mutants of the yeast orthologs of amphiphysin, Rvs161p and Rvs167p, exhibit defects in the depolarization of the actin network and actin patches (69-73). Furthermore, Rvs167p was demonstrated by two hybrid assays to interact directly with actin (74) and with the actin binding protein abp1 (75). In *Drosophila*, amphiphysin is localized to actin-rich membrane domains in many cell types and its delocalization in overexpressing mutants results in the mislocalization of F-actin (76,77). In vertebrates, amphiphysins have been shown to affect neuronal actin dynamics and to interact with cdk5, which has been functionally linked to neuronal migration and neurite outgrowth via its action on actin (78,79). It is therefore tempting to postulate that in AtT-20 cells, amphiphysin IIb may affect cytoskeletal organization leading to alteration in cell shape.

*Amphiphysin IIb is not necessary for clathrin-mediated endocytosis of SRIF receptors*
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Overexpression of amphiphysin IIb did not affect the internalization of SRIF receptors in AtT-20 cells, as assessed here by measuring the acid wash-resistant fraction of specifically bound $^{125}$I-D-Trp$^8$-SRIF and by visualizing internalized fluorescent SRIF by confocal microscopy. Similarly, null mutants of an ortholog of amphiphysin II, *Drosophila* amphiphysin, did not affect clathrin-mediated endocytosis at the neuromuscular junction (77,80) or in photoreceptor neurons (76). Likewise also, disruption of the bin/amphiphysin II gene by homologous recombination did not affect synaptic vesicle endocytosis in the mouse or in yeast (81,82).

Yet, several lines of evidence suggest that amphiphysins may be involved in receptor endocytosis, through their interaction with clathrin and/or with other endocytic accessory proteins including dynamin, synaptojanin, AP-2, and endophilin (for review, see (4,5,30,44,45)). Thus, overexpression of the SH3 domain of amphiphysin I was reported to block the uptake of transferrin and of EGF receptors in COS cells (83) and the interaction of amphiphysin II with dynamin was found to be required for the internalization of G protein-coupled AT$_1$A angiotensin receptors in Chinese hamster ovary cells (84). The lack of effect of amphiphysin IIb overexpression on the endocytosis of SRIF receptors in AtT-20 cells therefore suggests that either the interaction of amphiphysin IIb SH3 binding domain with dynamin is not mandatory for receptor endocytosis in AtT-20 cells, or that overexpression of the whole protein does not mimic the effects of overexpression of the SH3 domain of the protein alone. The whole protein, in contrast to the isolated SH3 domain, may not be targeted to endocytic sites on the plasma
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membrane. Indeed, structure-function analyses have revealed a crucial role for the N-terminal domain in the targeting of amphiphysin II to the plasma membrane (38). The lack of a 31-amino acid sequence at the N-terminus of amphiphysin IIb might therefore prevent the recruitment of this isoform to the cell surface during endocytosis, thereby accounting for the lack of inhibitory effect of amphiphysin IIb overexpression on clathrin-mediated endocytosis. Whatever the case may be, the present results suggest that the amphiphysin IIb isoform is not implicated in ligand-induced endocytosis in AtT-20 cells.

Amphiphysin IIb is required for the targeting of SRIF receptors to the plasma membrane

A major finding of the present study was the massive decrease in SRIF binding in whole AtT-20 cells overexpressing the amphiphysin IIb isoform. This decrease in SRIF binding was not due to a reduction in the expression of SRIF receptors since it was no longer apparent when the experiments were carried out on membrane homogenates from the same cells. Therefore, it is best accounted for by impaired trafficking of sst receptors in cells overexpressing amphiphysin IIb. In keeping with this interpretation, both sst_{2A} and sst_{5} SRIF receptor sub-types were found by immunohistochemistry to be sequestered in a juxtanuclear compartment in cells overexpressing amphiphysin IIb. Dual immunolabeling experiments identified this sequestration compartment as the TGN, by virtue of its immunostaining with the TGN marker, syntaxin-6 (85-87). Either of two mechanisms could account for the sequestration of sst_{2A} and sst_{5} receptors within the TGN:
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inhibition of receptor recycling or interference with the targeting of reserve receptors to the plasma membrane. The first possibility appears unlikely since contrary to overexpression of amphiphysin IIb, inhibition of receptor recycling with the ionophore, monensin (25 µM) had no effect on the Bmax of $^{125}$I-SRIF binding in AtT-20 cells (our own unpublished observations). Furthermore, stimulation of amphiphysin IIb-overexpressing cells with [D-Trp$^8$]-SRIF for either 10 or 40 min had no effect on the amount of intra-cellularly sequestered sst$_{2A}$ or sst$_5$ immunoreactive receptors, as would have been expected had amphiphysin overexpression interfered with sst receptor recycling (data not shown). We therefore conclude that overexpression of amphiphysin IIb impairs the constitutive targeting of reserve receptors from the TGN to the plasma membrane. This interpretation is consistent with previous reports showing that interactions of amphiphysin II with nexin 4 and synapsin I regulate vesicular trafficking and exocytosis, respectively (88,89). Dynamin II, which interacts with amphiphysin II (90,91) has also been shown to play a key role in controlling both constitutive and regulated hormone secretion from the Golgi apparatus in AtT-20 cells (62). Thus, overexpression of amphiphysin IIb may block the normal function of dynamin II at the TGN.

It is unclear whether the trafficking of all SRIF receptors, or merely that of sst$_{2A}$ and sst$_5$ subtypes, was affected by amphiphysin overexpression. Indeed, the residual cell surface binding of SRIF could reflect either incomplete blockade of membrane targeting of all SRIF receptor subtypes or selective sparing of the targeting of the other SRIF receptor sub-types (sst$_1$, sst$_{2b}$
and sst₄) expressed in these cells (48,49,61). The latter possibility appears unlikely, however, since sst₂ᴬ and sst₅ receptors are the predominant SRIF receptors expressed by AtT-20 cells (92). Furthermore, the sst₁ and sst₄ subtypes have both a lower affinity than sst₂ᴬ and sst₅ subtypes for D-Trp⁸-SRIF, so that there should have been differences between WT and transfected cells, had these receptors been selectively involved (for review, see (93,94)). Therefore, it is likely that amphiphysin IIb plays a general role in the formation of constitutive transport vesicles at the level of the TGN rather than a restricted role in the targeting of selective receptor subtypes.

The regulated secretory pathway is not affected by amphiphysin IIb overexpression

The effects of amphiphysin IIb overexpression on the membrane targeting of receptor proteins via the constitutive pathway led us to investigate whether proteins trafficking through the secretory pathway would be similarly affected. For this purpose, we compared the CRF-induced release of ACTH, a previously documented measure of secretory activity in the AtT-20 cell line (57,58,62), in WT versus amphiphysin IIb-overexpressing cells. Consistent with earlier reports (95-99), we found that CRF-41 stimulated ACTH release in WT AtT-20 cells and that this CRF-induced release was significantly reduced in the presence of SRIF. Amphiphysin IIb overexpression did not significantly inhibit CRF-41-induced ACTH release, suggesting that amphiphysin IIb is not involved in the trafficking of proteins through the regulated secretory pathway. However, the
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effect of SRIF on CRF-induced ACTH secretion was considerably decreased, in keeping with the demonstrated inhibition of sst$_{2A}$ and sst$_5$ membrane targeting in amphiphysin IIb-overexpressing cells. Indeed, sst$_2$ and sst$_5$ agonists are both known to potently inhibit CRF-41-stimulated ACTH secretion from AtT-20 cells (60).

In conclusion, the present results demonstrate that, in addition to their documented role in endocytosis, amphiphysins are involved in the control of protein trafficking from the TGN in neuroendocrine AtT-20 cells. Furthermore, they suggest a specialization of the diverse amphiphysin isoforms such that whereas certain isoforms (namely here amphiphysin IIb) are involved in protein targeting through the constitutive secretory pathway, others may be implicated in the control of exocytosis through the regulated secretory pathway, or of endocytosis. The mechanisms by which amphiphysin IIb might be controlling receptor recruitment to the plasma membrane remain to be investigated. Recent studies have shown that the trafficking of D1 receptors from the TGN to the plasma membrane requires an intact cytoskeleton (100), suggesting that the interactions proposed here between amphiphysin IIb and cytoskeletal proteins may be involved not only in regulating the cell’s size and shape, but also membrane trafficking events. Further studies will be needed to determine whether amphiphysin IIb regulates cytoskeletal organization and constitutive secretion through the same or separate pathways.
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FIGURE LEGENDS

**Figure 1.** Amphiphysin and the BAR protein family. The domain models of amphiphysin II splice variants, indicated as type IIa, IIb, IIb-1, IIc1, IIc2 and IId, are compared with those of amphiphysin I, SH3P9, and BIN1. BAR, central, SH3 and N-terminal insert domains, as well as nuclear localization signal and amphiphysin I-specific domain, are indicated. The insert domain also contains several specific motifs that interact with proteins of the endocytic regulatory machinery such as endophilin, AP-2, and clathrin. The amphiphysin IIb variant form, overexpressed in AtT-20 cells, is boxed (adapted from (29,38,45)).

**Figure 2.** Identification of endogenously vs. ectopically expressed amphiphysin IIb by Western blotting. Specific immunoreactive bands, corresponding to the splice variants of amphiphysin II (IIa, IIb, IIc), are detected at approximately 70-80, 90 and 97 kDa, respectively, in both wild-type and amphiphysin IIb-transfected AtT-20 cells. Note that the 90 kDa band, corresponding to amphiphysin IIb is markedly stronger in transfected than in WT cells. Data are representative of 3 independent experiments.

**Figure 3.** Effect of amphiphysin IIb overexpression on the morphology of ACTH-secreting AtT-20 cells. Both the perimeter (A) and surface area (B) of amphiphysin II overexpressing cells are higher than those of WT controls. The form factor (C), defined as 4 À x area ÷ perimeter$^2$, is
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accordingly lower in cells overexpressing amphiphysin IIb than in WT. Values are expressed as the mean ± SEM. Levels of significance: *** P < 0.0001; ** P < 0.001.

Figure 4. Association kinetics of $^{125}$I-SRIF binding and internalization in wild-type (A) versus amphiphysin IIb-transfected (B) AtT-20 cells. Cells were incubated with 0.1 nM $^{125}$I-Tyr$^0$-[D-Trp$^8$]-SRIF for 45 min at 37°C. At the indicated times, cells were washed twice with 500 µl of Earle’s buffer (open symbols) or treated with 500 µl of acid-NaCl buffer (pH 4) for 3 min (closed symbols). Hypertonic acid stripping of surface-bound ligand revealed that 77.5 ± 3.2% and 76.4 ± 2.1% of specifically bound $^{125}$I-SRIF was internalized in wild-type and amphiphysin IIb-transfected AtT-20 cells, respectively. The values are expressed as means ± S.D. of four independent experiments carried out in duplicate.

Figure 5. Confocal microscopic images of fluo-SRIF-labeled AtT-20 cells. Single trans-nuclear optical sections scanned after 10 (A, B) or 40 min (C, D) of ligand application followed by hypertonic acid wash. At both time points, the internalized ligand is heavily concentrated in the perinuclear compartment of both wild-type (A, C) and amphiphysin IIb-overexpressing (B, D) AtT-20 cells. This labeling is completely abolished by co-incubation with an excess of nonfluorescent SRIF (data not shown). Scale bar, 15 µm.
Figure 6. Saturation of $^{125}$I-Tyr$^0$-[D-Trp$^8$]-SRIF binding to AtT-20 cells. Experiments were performed on wild-type (closed symbols) and amphiphysin IIb-overexpressing (open symbols) cells for 30 min at 37°C with increasing concentrations of labeled SRIF. A, C: saturation curves; B, D: Scatchard representation of the data. Note that the maximal binding capacity ($B_{\text{max}}$) is markedly higher in WT than in transfected cells when performed on whole cells (A, B), but is similar between the two cell lines when performed on crude membrane homogenates (C, D). The deduced dissociation constant ($K_d$) is, however, similar between WT and transfected cells, in both whole cells (B) and in crude membrane preparations (D). Each point represents the means ± S.D. of three independent experiments carried out in triplicate.

Figure 7. Fluorescence immunolabeling of sst$_{2A}$ and sst$_5$ receptors in AtT-20 cells. In wild-type AtT-20 cells, the sst$_{2A}$ receptor subtype (A) forms a pericellular ring along the cell membrane whereas the sst$_5$ immunoreactivity is also present on the plasma membrane but predominantly within the cytoplasm (C). In amphiphysin IIb-overexpressing cells, both immunoreactive sst$_{2A}$ (B) and sst$_5$ (D) receptors are concentrated in the cytoplasmic core, next to the nucleus. Scale bar, 15 µm.

Figure 8. Dual localization of sst$_{2A}$ and syntaxin-6 immunoreactivity in amphiphysin IIb
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overexpressing AtT-20 cells. The bulk of intracellular sst\(_{2A}\) receptors is concentrated in the juxtanuclear region (A), in a compartment immunopositive for syntaxin-6 (B) and thus corresponding to the TGN/pericentriolar recycling endosome complex. Merged images (C) reveal that sst\(_{2A}\) receptor immunostaining colocalizes perfectly with syntaxin-6 immunoreactivity. Scale bar, 15 µm.

**Figure 9.** Effect of amphiphysin IIb overexpression on CRF-stimulated ACTH secretion. Intact AtT-20 cells are incubated for 2 hours in DMEM/BSA in the absence (closed bars) or in the presence of either 10\(^{-7}\) M CRF alone (open bars) or of CRF in combination with 10\(^{-7}\) M SRIF (hatched bars). Immunoreactive ACTH release is measured with a commercially available radioimmunoassay kit. CRF-41 stimulates ACTH secretion in both wild-type and amphiphysin IIb-transfected AtT-20 cells. No significant difference (ns) is observed between the two cell types. SRIF affects CRF-induced ACTH release in wild-type cells but not in amphiphysin IIb overexpressing AtT-20 cells (ns). All results are expressed as the mean ± SEM of three determinations. The statistical significance of the results compared to the control values is shown as **, P < 0.001.
Role of amphiphysin II in somatostatin receptor trafficking in neuroendocrine cells
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