Targeting of the zymogen-granule protein syncollin in AR42J and AtT-20 cells

Alois HODEL and J. Michael EDWARDSON¹

Department of Pharmacology, University of Cambridge, Tennis Court Road, Cambridge CB2 1QJ, U.K.

Syncollin is a 13-kDa protein associated with the membranes of pancreatic zymogen granules. Here we determine the *in situ* localization of syncollin in pancreatic acinar cells from adult and neonatal rats, and study the targeting of green fluorescent protein-(GFP-) and His₆-tagged syncollin chimaeras in model exocrine and endocrine secretory cells. Immunocytochemical analysis of the distribution of syncollin in fully differentiated and neonatal acinar cells revealed a granular pattern that corresponded with that of the zymogen-granule markers synaptobrevin 2 and amylase. In fully differentiated acinar cells syncollin-positive vesicles were detected in the apical region of the cells, whereas in neonatal acinar cells they were found clustered near the cell nucleus. Both GFP- and His₆-tagged syncollin entered the secretory pathway when transiently expressed in AR42J or AtT-20 cells. Syncollin-GFP was found predominantly in amylase-

positive granules in AR42J cells and in adrenocorticotrophic hormone- (ACTH-) positive granules in AtT-20 cells. Syncollin-GFP was also present in the Golgi complex in AR42J cells. Syncollin-His₆ became localized in ACTH-containing granules in the neuritic processes of AtT-20 cells. In AR42J cells syncollin-His₆ did not co-localize with amylase, but was detected in acidic vesicles. These results show that the exocrine protein syncollin contains intrinsic cell-type-independent targeting information that is retained in both exocrine and endocrine cells after fusion to the GFP tag. In contrast, His₆-tagged syncollin is efficiently targeted to secretory granules only in AtT-20 cells and not in AR42J cells.

Key words: endocrine, exocrine, intracellular targeting, pancreas.

INTRODUCTION

Secretory proteins enter an intracellular route that leads either directly to the cell surface (constitutive secretory pathway), or to specialized secretory storage vesicles that release their contents upon appropriate stimulation (regulated secretory pathway). In pancreatic acinar cells, segregation of proteins into constitutive and regulated pathways takes place in the *trans*-Golgi network (TGN) and in immature secretory granules [1]. The precise mechanism that mediates efficient sorting of regulated secretory proteins into zymogen granules has not been elucidated, although various factors, such as binding to sorting receptors, assembly into higher-order complexes and subsequent retention in immature secretory granules, as well as low pH and elevated calcium concentrations, are likely to be important [1].

Whether or not sorting mechanisms are cell-type-specific remains a matter of debate. For example, the exocrine granule proteins trypsinogen, parotid proline-rich protein and salivary amylase are packaged into secretory vesicles in neuroendocrine AtT-20 cells [2,3]. Conversely, the endocrine hormone gastrin is routed into the regulated pathway in the exocrine cell line AR42J [4], and in transgenic mice neuroendocrine chromogranin B is stored in pancreatic zymogen granules [5]. These findings suggest that cell-type-independent mechanisms operate to target proteins into secretory granules and that sorting signals are conserved between cell types. Evidence against such a generalization includes the exclusion of the major zymogen-granule membrane protein GP-2 from secretory granules in AtT-20 cells, although it is sorted to secretory granules in AR42J cells [6]. Hence sorting of secretory-granule proteins can be cell-type-specific, presumably through specific packaging mechanisms that involve hetero-

typic binding to cell-type-specific proteins, lipids or proteoglycans in the TGN or in immature secretory granules [1].

Syncollin was identified originally as a novel protein present on the membranes of pancreatic zymogen granules [7]. Recently, it has also been found in epithelial cells of the duodenum [8]; its expression here is increased in response to feeding, suggesting a role for syncollin in the secretion of digestive enzymes. Syncollin was isolated through its ability to bind to syntaxin *in vitro* [7]. However, it is now known to be tightly associated with the luminal surface of the zymogen-granule membrane [9], a result that calls into question the physiological significance of its interaction with syntaxin. Here we present further evidence that syncollin is a zymogen-granule-associated protein, and describe the subcellular localization of heterologously expressed recombinant syncollin chimaeras in both exocrine pancreas-derived AR42J cells and endocrine AtT-20 cells.

MATERIALS AND METHODS

Antibodies and other reagents

Polyclonal anti-syncollin antibody 'B' was generated by immunizing rabbits with a recombinant glutathione S-transferase-syncollin fusion protein. Monoclonal anti-syncollin antibody 87.1 was generated by immunizing mice with the same fusion protein. Monoclonal anti-synaptobrevin 2 (clone 69.1) was kindly provided by R. Jahn (Max Planck Institute for Biophysical Chemistry, Göttingen, Germany). Monoclonal antibodies against GM130 and TGN38 were generous gifts of P. Luzio (University of Cambridge, Cambridge, U.K.). Polyclonal anti-human amylase and polyclonal anti-human adrenocorticotrophic hormone (ACTH) were from Sigma (Poole, Dorset, U.K.).

Abbreviations used: ACTH, adrenocorticotrophic hormone; GFP, green fluorescent protein; TGN, *trans*-Golgi network; TRITC, tetramethylrhodamine isothiocyanate.

¹ To whom correspondence should be addressed (e-mail jme1000@cus.cam.ac.uk).

Monoclonal anti-His₆ antibody was from Invitrogen (Groningen, The Netherlands). FITC- and tetramethylrhodamine isothiocyanate- (TRITC-) coupled anti-rabbit secondary antibodies were from Calbiochem-Novabiochem (Nottingham, U.K.). Cy3-coupled secondary anti-mouse antibody was from Sigma. Horseradish peroxidase-coupled secondary antibodies were from Bio-Rad (Hemel Hempstead, Herts., U.K.). LysoTRACKER was from Molecular Probes (Leiden, The Netherlands). All other chemicals and reagents were from Sigma.

Subcellular fractionation and immunoblotting

Pancreatic subcellular fractions were prepared from Wistar rats as described previously [7]. Equal amounts of protein from each fraction (10 µg per lane) were separated by SDS/PAGE and transferred electrophoretically to nitrocellulose (Schleicher & Schuell, Dassel, Germany). Blots were probed with anti-syncollin 'B' (diluted 1:2000), anti-synaptobrevin 2 (1:1000) and anti-amylase (1:2000) antibodies, and immunoreactive bands were visualized using horseradish peroxidase-coupled secondary antibodies (1:1000) and enhanced chemiluminescence (Pierce and Warriner, Chester, U.K.).

Isolation of rat pancreatic acinar cells

Differentiated acinar cells were isolated by collagenase digestion of rat pancreas. Isolation medium (120 mM NaCl, 5 mM KCl, 2 mM CaCl₂, 1 mM MgCl₂, 1 mM NaH₂PO₄, 1 mM NaHCO₃, 25 mM Hepes/NaOH, 2 mg/ml glucose, 0.7 mg/ml BSA, 0.3 mg/ml soya bean trypsin inhibitor and basal Eagle's medium amino acids including 2 mM glutamine, pH 7.2) containing 1000 units of collagenase (5 ml) was injected into a 5–8-week-old pancreas. After a 10-min incubation at 37 °C the tissue was dispersed in isolation medium, filtered through a 200-µm-pore mesh, layered over isolation medium containing 4% BSA and spun at 15 g for 3 min. The pellet was washed twice, resuspended in isolation medium, and cells were left to settle on to poly-lysine-coated coverslips for 30 min.

Neonatal pancreatic acinar cells were isolated from the splenic ends of pancreases from 8-day-old rats. Pancreases were cut into 1-mm³ pieces and incubated in isolation medium (containing collagenase) for 45 min at 37 °C. Cells were dissociated by pipetting, filtered through a 200-µm-pore mesh, washed twice in pre-warmed cell-culture medium and dispensed into culture dishes containing poly-lysine-coated coverslips.

Cell culturing and transfection

AR42J and AtT-20 cells were grown on poly-lysine-coated coverslips to 60–80% confluence at 37 °C in Dulbecco's modified Eagle's medium containing 10% (v/v) fetal bovine serum, 100 units/ml penicillin and 100 µg/ml streptomycin in the presence of 5% CO₂. The syncollin cDNA sequence, including the signal sequence [9], was sub-cloned into the pEGFP-N1 expression vector (Clontech, Basingstoke, Hants., U.K.); syncollin-His₆ was subcloned into the vector pCIN4 [10]. Transient transfections were carried out using FuGENE 6 (Roche Diagnostics, Lewes, East Sussex, U.K.) according to the manufacturer's instructions. Dexamethasone (100 nM) was added to AR42J cells immediately after transfection. Both AR42J and AtT-20 cells were analysed 24 h post-transfection.

Immunocytochemistry and confocal microscopy

Cells were fixed in 2% (w/v) paraformaldehyde in a buffer containing 0.1 M Pipes/NaOH, 1 mM EGTA and 1 mM MgSO₄, pH 6.9–7.0, at room temperature for 50 min (AR42J, AtT-20, neonatal acinar cells), or in 4% (w/v) paraformaldehyde in the same buffer for 30 min (differentiated acinar cells). Samples were washed once in PHEM buffer (60 mM Pipes/NaOH, 25 mM Hepes, 10 mM EGTA and 2 mM MgSO₄, pH 6.9–7.0), once in 120 mM phosphate buffer and once in PBS, and incubated for 30 min in PBS containing 0.2% (w/v) fish gelatin (PF buffer). Cells were permeabilized in PHEM buffer containing 0.05% (w/v) saponin [0.1% (w/v) Triton X-100 for differentiated acinar cells] for 15 min, washed once in PF buffer and incubated with primary antibodies in PF buffer for 1 h at room temperature. Antibody dilutions were 1:200 except for anti-syncollin 'B' (1:100), anti-amylase (1:100) and anti-His₆ (1:500). Samples were washed twice in PF buffer and incubated with secondary antibodies coupled to FITC (1:250), TRITC (1:100) or Cy3 (1:500). Cells were washed once each in PBS, 120 mM phosphate buffer and 5 mM phosphate buffer, before being mounted in VectaShield (Vector Labs, Burlingame, CA, U.S.A.) on glass slides.

Specimens were analysed on an inverted Leica TCS-NT confocal laser scanning microscope (Leica, Deerfield, IL, U.S.A.). The images were collected with the appropriate filters: green fluorescent protein (GFP) and FITC were excited using the 488-nm line of a krypton/argon laser and imaged with a 515–540-nm band-pass filter. Cy3 and TRITC were excited with the 568-nm line and imaged with a long-pass 590-nm filter. Images were processed and annotated using Adobe Photoshop 5.0 (Mountain View, CA, U.S.A.) and Corel Draw 8 (Ottawa, Canada).

Figure 1 Distribution of syncollin in pancreatic subcellular fractions

Subcellular fractions were prepared from adult rat pancreas. Equal amounts of protein were separated by SDS/PAGE and electrophoretically transferred on to nitrocellulose. Proteins were probed using polyclonal anti-syncollin antibody 'B' (A), a monoclonal anti-synaptobrevin 2 antibody (B) or a polyclonal anti-amylase antibody (C), and visualized using horseradish peroxidase-coupled secondary antibodies and enhanced chemiluminescence. ZG, zymogen granule; ZGM, zymogen-granule membrane; PM, plasma membrane; cyt, cytosol. For (A), the positions of molecular-mass markers are indicated on the right.

Figure 2 Localization of syncollin, synaptobrevin 2 and amylase in pancreatic acinar cells

Syncollin was visualized using polyclonal antibody 'B' and a TRITC-coupled secondary antibody (A). Synaptobrevin 2 was detected using a monoclonal antibody and a Cy3-coupled secondary antibody (B). Amylase was visualized using a polyclonal antibody and an FITC-coupled secondary antibody (C). Specimens were analysed by confocal microscopy. N, nucleus; ZG, zymogen granules. Scale bar, 10 μ m.

Figure 3 Localization of syncollin and amylase in 8-day-old neonatal pancreatic acinar cells

Neonatal cells were grown in culture for 48 h before processing for immunocytochemistry. Syncollin was visualized using monoclonal antibody 87.1 and a Cy3-coupled secondary antibody (A). Amylase was visualized using a polyclonal antibody and an FITC-coupled secondary antibody (B). Syncollin- and amylase-positive structures were co-localized (arrows) and clustered towards one side of the nucleus (box). The broken line indicates the cell perimeter. N, nucleus. Scale bar, 10 μ m.

Figure 5 Co-localization of syncollin-GFP with GM130 and TGN38

AR42J cells were transiently transfected with a plasmid encoding syncollin-GFP and fixed 24 h later. GM130 and TGN38 were visualized using monoclonal antibodies and a Cy3-coupled secondary antibody. Cells contain perinuclear structures containing both syncollin-GFP (A) and GM130 (B), and syncollin-GFP (C) and TGN38 (D). Note that in both cases the syncollin-GFP labels many additional punctate structures. The broken lines indicate the cell perimeters. N, nuclei. Scale bar, 10 μ m.

Figure 4 Co-localization of syncollin-GFP and amylase in secretory granules of AR42J cells

AR42J cells were transiently transfected with a plasmid encoding syncollin-GFP and fixed 24 h later. Amylase was visualized using a polyclonal antibody and a TRITC-coupled secondary antibody. Arrows indicate punctate structures containing both syncollin-GFP (A) and amylase (B). Arrowheads indicate structures containing amylase but not syncollin-GFP. The broken line indicates the cell perimeter. Scale bar, 10 μ m.

RESULTS

Immunoblot analysis of subcellular fractions using a specific polyclonal antibody raised against a syncollin peptide revealed previously that syncollin is enriched in zymogen-granule membranes [7] along with synaptobrevin 2, an established zymogen-granule membrane marker [11,12]. Unfortunately this antibody did not reliably detect syncollin in immunocytochemical experiments. In the present study, two new antibodies, polyclonal

Figure 6 Comparison of the distribution of syncollin-His₆, amylase and LysoTRACKER in AR42J cells

AR42J cells were transiently transfected with a plasmid encoding syncollin-His₆ and fixed 24 h later. Syncollin-His₆ was visualized using a monoclonal anti-His₆ antibody and a Cy3-coupled secondary antibody (A and E). Amylase was visualized using a polyclonal antibody and an FITC-coupled secondary antibody (B and D). (C and F) Cells were incubated for 2 h in 1 μ M LysoTRACKER before fixation. (A and B) Comparison of the staining for syncollin-His₆ and amylase. (C and D) Comparison of the staining for LysoTRACKER and amylase. (E and F) Comparison of the staining for syncollin-His₆ and LysoTRACKER. Arrows indicate vesicular structures containing both syncollin-His₆ (E) and LysoTRACKER (F). The broken lines indicate the cell perimeters. Scale bar, 10 μ m.

antibody 'B' and monoclonal antibody 87.1, were used to detect syncollin *in situ*. The specificity of antibody 87.1 has been demonstrated previously [9]. As shown in Figure 1, polyclonal anti-syncollin antibody 'B' also detects a single band of apparent molecular mass 16 kDa on immunoblots of zymogen granules and granule membranes. As expected, both syncollin and synaptobrevin 2 were highly enriched in the granule membrane fraction, confirming that syncollin is associated with the granule membrane. In contrast, the protein amylase, known to be in the granule content, was enriched in the total granule fraction and gave a much weaker signal with granule membranes.

Anti-syncollin antibody 'B' was used to examine the distribution *in situ* of syncollin in pancreatic acinar cells. As shown in Figure 2(A), syncollin staining was punctate and concentrated at one pole of the cell, consistent with the labelling of zymogen granules, which are known to have a diameter of 0.5–1.0 μ m [13]. Synaptobrevin 2 and amylase both gave staining patterns that were identical to that for syncollin (Figures 2B and 2C), clearly

Figure 7 Co-localization of syncollin-GFP and ACTH in secretory granules of AtT-20 cells

AtT-20 cells were transiently transfected with a plasmid encoding syncollin-GFP and fixed 24 h later. (A and C) Syncollin-GFP labelling. ACTH was visualized using a polyclonal anti-ACTH antibody and a TRITC-coupled secondary antibody (B and D). Arrows indicate punctate structures in neurites containing both syncollin-GFP (C) and ACTH (D). (C and D) Higher-magnification views of the boxed areas in (A) and (B). The broken lines indicate the cell perimeters. Scale bars, 10 μ m.

demonstrating that syncollin is a zymogen-granule-associated protein. Immunocytochemical analysis of acinar cells from 8-day-old neonatal rats [14], using antibody 87.1, also showed a co-localization of syncollin with amylase (Figure 3). Punctate staining with both antibodies was found to be clustered in a perinuclear region. This perinuclear distribution of granules is a known manifestation of cellular polarization [14]; hence, the granular localization of syncollin in acinar cells is established at an early stage of post-natal development (i.e. before the cells are fully polarized).

Intracellular targeting of syncollin was investigated in AR42J cells. Dexamethasone differentiation of these cells is known to produce a model exocrine cell line which does not express syncollin [15]. Cells were transiently transfected with an expression vector encoding full-length syncollin fused at the C-terminus to a red-shifted variant of GFP (syncollin-GFP). Transfected cells were analysed by immunocytochemistry and confocal microscopy 24 h after transfection. A punctate fluorescence was seen throughout the cytoplasm (Figure 4A). At least half of the syncollin-GFP-containing structures were also positive for amylase (Figure 4B), a known marker for secretory granules in this cell type [16]. Thus a significant fraction of the transiently expressed syncollin-GFP was sorted into secretory granules. In addition, many AR42J cells that had been transfected with syncollin-GFP also showed clear perinuclear labelling (Figure 5). This labelling overlapped with staining for the *cis*-Golgi marker GM130 [17] and the TGN marker TGN38 [18], indicating that the syncollin chimera was present throughout the Golgi stack.

To assess the influence on sorting of the relatively large GFP tag, full-length syncollin fused at the C-terminus to a much

Figure 8 Co-localization of syncollin-His₆ and ACTH in secretory granules of AtT-20 cells

AtT-20 cells were transiently transfected with a plasmid encoding syncollin-His₆ and fixed 24 h later. Syncollin-His₆ was visualized using a monoclonal anti-His₆ antibody and a Cy3-coupled secondary antibody (**A** and **C**). ACTH was visualized using a polyclonal anti-ACTH antibody and an FITC-coupled secondary antibody (**B** and **D**). Arrows indicate punctate structures in neurites containing both syncollin-His₆ (**A** and **C**) and ACTH (**B** and **D**). Arrowheads indicate structures containing syncollin-His₆ (**A**) but not ACTH (**B**). The broken lines indicate the cell perimeters. Scale bar, 10 μ m.

smaller tag (His₆) was subcloned into an appropriate eukaryotic expression vector. AR42J cells were transiently transfected with this vector and examined 24 h later by immunocytochemistry and confocal microscopy. Syncollin-His₆ immunoreactivity was found associated with vesicular structures of varying size that were distributed throughout the cytoplasm (Figure 6A), albeit often concentrated near the nucleus. In contrast to labelling with syncollin-GFP, syncollin-His₆ staining did not coincide with staining for amylase (Figure 6B). To investigate the possibility that syncollin-His₆ had entered the endosomal-lysosomal system, transfected AR42J cells were co-stained with LysoTRACKER, a marker for acidic organelles [19]. LysoTRACKER staining did not coincide with staining for amylase (Figures 6C and 6D), but did overlap partially with syncollin-His₆ staining (Figures 6E and 6F), indicating that syncollin-His₆ had indeed entered endosomes and/or lysosomes.

AtT-20 cells are derived from the mouse anterior pituitary and are known to process correctly and store endogenous as well as exogenous secretory proteins [20–22]. This cell line was used to study the sorting and localization of syncollin in an endocrine environment. Syncollin-GFP (Figure 7) and syncollin-His₆ (Figure 8) were detected in punctate structures in transiently transfected AtT-20 cells. Some of them were found in the cell body, but the majority were present in neuritic processes, where ACTH-containing granules are known to be located predominantly [23]. When transfected cells were co-stained with an anti-ACTH antibody, strong immunoreactivity was seen to coincide extensively with labelling by syncollin-GFP and syncollin-His₆. In addition, syncollin-His₆ was also present in ACTH-negative

vesicles along neurites and within the cell body (Figure 8A). These results indicate that both syncollin chimaeras were faithfully translocated into the secretory pathway in AtT-20 cells, and targeted to secretory granules.

DISCUSSION

In this study we have extended our initial characterization of syncollin [7] by describing its subcellular localization *in situ*. We have also studied the localization of heterologously expressed recombinant syncollin in an exocrine (AR42J) and an endocrine (AtT-20) cell line. AR42J cells are capable of sorting exogenous zymogen-granule proteins to secretory vesicles, as illustrated by the observation that GP-2 was targeted to amylase-positive granules in stably transfected cells [6]. Here we show that GFP-tagged syncollin is also packaged into amylase-positive granules in transfected AR42J cells. Syncollin-GFP was also present in the Golgi apparatus, as judged by its co-localization with both GM130 and TGN38. This result indicates that syncollin-GFP was properly routed through Golgi stacks, where it accumulated before being packaged into granules. Only about half of the amylase-positive granules contained detectable levels of syncollin-GFP. Similar results have been reported previously: for example, GFP fusion proteins of chromogranin B and neuropeptide Y expressed in PC12 cells [24] and preproinsulin in INS-1 β cells [25] were also partially targeted to secretory granules. An effect of the GFP tag on protein folding or complex formation could account for these low sorting efficiencies. Alternatively, the secretory granules might turn over too slowly to permit efficient packaging of syncollin-GFP into the total granule population over the 24-h time course of a typical experiment. In any case, the targeting of syncollin-GFP in AR42J cells appears efficient enough for it to be used in the future as a tool for studying the nature of the sorting signal(s) and for monitoring secretory-granule dynamics in real time.

When syncollin-His₆ was expressed in AR42J cells it was found in amylase-negative vesicles of varying size scattered throughout the cytoplasm. A fraction of syncollin-His₆-containing vesicles co-localized with acidic organelles, suggesting partial sorting of syncollin-His₆ to endosomes or lysosomes. In stressed cells (e.g. cells that overexpress foreign proteins), endoplasmic-reticulum vesicles have been observed pinching off and maturing into lysosome-like autophagosomes [26]. Induction of autophagy in syncollin-His₆-transfected cells may have resulted in the accumulation of syncollin-His₆ in vesicles that gradually acquired lysosomal markers [26–28]. As a result, they would have become more acidic and thus able to accumulate the LysoTRACKER dye. Alternatively, syncollin-His₆ may have passed through the Golgi complex and immature secretory granules *en route* to endosomes and lysosomes, a pathway utilized by lysosomal pro-enzymes in exocrine cells [29]. In fact, illegitimate lysosomal localization was observed for GP-2 in Rin5F cells [6], and for both GFP-tagged vesicular stomatitis virus glycoprotein [30] and a fusion protein lacking its glycoposphatidylinositol anchor in COS cells [31,32], suggesting that partial lysosomal sorting is a common phenomenon in cells overexpressing foreign proteins.

AtT-20 cells have been used previously to explore the targeting of several exocrine secretory-granule proteins. The majority of these (e.g. pancreatic trypsinogen [2], salivary amylase and parotid proline-rich proteoglycans [3], and peptidylglycine α -amidating mono-oxygenase [33]) were found to be packaged into secretory granules. The exception was GP-2, which was not sorted into granules, presumably because it uses an exocrine-

specific sorting mechanism [3,6,16]. In AtT-20 cells, syncollin-His₆ and syncollin-GFP were both targeted to ACTH-containing granules in neuritic processes. Hence, syncollin can be faithfully sorted to endocrine secretory granules. It has been reported that the zymogen-granule-content proteins chymotrypsinogen and trypsinogen bind to regulated but not constitutive secretory proteins in AtT-20 cells and hence become packaged into granules [34]. Such a sorting mechanism may also be utilized by the syncollin chimera. However, even proteins that are normally secreted constitutively can be sorted to secretory granules in AtT-20 cells [22,35,36], suggesting that proteins lacking specific sorting signals may enter secretory granules by virtue of a bulk-flow mechanism. Because syncollin is known to oligomerize *in situ* [9], it seems more likely that syncollin-His₆ and syncollin-GFP are packaged into immature secretory granules through homotypic assembly and subsequent retention during granule maturation [1].

Syncollin-His₆-positive but ACTH-negative vesicles were found in the cell bodies of transfected AtT-20 cells. The size of these vesicles was uniform and similar in size to ACTH-positive granules, suggesting that they also represent secretory granules. Conceivably, the high expression of syncollin-His₆ may have caused the biogenesis of granules that became packed with syncollin-His₆ but not with ACTH. In support of this idea, ACTH-containing granules were found in the cell body when the ACTH precursor protein pro-opiomelanocortin was over-expressed in Neuro-2a cells [37]. Alternatively, a novel type of granule may have been produced, as was seen when multimeric von Willebrand factor pro-polypeptide [38] or fibronectin [39] were expressed in AtT-20 cells. This seems less likely because these proteins, in contrast to syncollin-His₆, were excluded from ACTH-containing granules.

Syncollin is not normally expressed in either AR42J cells [15] or AtT-20 cells (A. Hodel and J. M. Edwardson, unpublished work). Nevertheless, syncollin-GFP and syncollin-His₆ are both successfully targeted to ACTH-positive granules in AtT-20 cells, and syncollin-GFP is delivered to amylase-positive granules in AR42J cells. These data argue strongly in favour of a general, cell-type independent, sorting mechanism for syncollin.

We thank Reinhard Jahn and Paul Luzio for providing antibodies, and Paul Thomas and Manuel Campos-Toimil for sharing their acinar cells with us. This work was supported by project grant 053024 from the Wellcome Trust.

REFERENCES

- Arvan, P. and Castle, D. (1998) Sorting and storage during secretory granule biogenesis: looking backward and looking forward. *Biochem. J.* **332**, 593–610
- Burgess, T. L., Craik, C. S. and Kelly, R. B. (1985) The exocrine protein trypsinogen is targeted into the secretory granules of an endocrine cell line: studies by gene transfer. *J. Cell Biol.* **101**, 639–645
- Castle, A. M., Huang, A. Y. and Castle, J. D. (1997) Passive sorting in maturing granules of AtT-20 cells: the entry and exit of salivary amylase and proline-rich protein. *J. Cell Biol.* **138**, 45–54
- Dickinson, C. J., Takeuchi, T., Guo, Y. J., Stadler, B. T. and Yamada, T. (1993) Expression and processing of prohormones in nonendocrine cells. *Am. J. Physiol.* **264**, G553–G560
- Natori, S., King, A., Hellwig, A., Weiss, U., Iguchi, H., Tsuchiya, B., Kameya, T., Takayanagi, R., Nawata, H. and Huttner, W. B. (1998) Chromogranin B (secretogranin I), a neuroendocrine-regulated secretory protein, is sorted to exocrine secretory granules in transgenic mice. *EMBO J.* **17**, 3277–3289
- Hoops, T. C., Ivanov, I., Cui, Z., Colomer-Gould, V. and Rindler, M. J. (1993) Incorporation of the pancreatic membrane protein GP-2 into secretory granules in exocrine but not endocrine cells. *J. Biol. Chem.* **268**, 25694–25705
- Edwardson, J. M., An, S. and Jahn, R. (1997) The secretory granule protein syncollin binds to syntaxin in a Ca²⁺-sensitive manner. *Cell* **90**, 325–333
- Tan, S. and Hooi, S. C. (2000) Syncollin is differentially expressed in rat proximal small intestine and regulated by feeding behaviour. *Am. J. Physiol.* **278**, G308–G320
- An, S. J., Hansen, N. J., Hodel, A., Jahn, R. and Edwardson, J. M. (2000) Analysis of the association of syncollin with the membrane of the pancreatic zymogen granule. *J. Biol. Chem.* **275**, 11306–11311
- Rees, S., Coote, J., Stables, J., Goodson, S., Harris, S. and Lee, M. G. (1996) Bicistronic vector for the creation of stable mammalian cells that predisposes all antibiotic-resistant cells to express recombinant protein. *BioTechniques* **20**, 102–110
- Gaisano, H. Y., Sheu, L., Foskett, J. K. and Trimble, W. S. (1994) Tetanus toxin light chain cleaves a vesicle-associated membrane protein (VAMP) isoform 2 in rat pancreatic zymogen granules and inhibits enzyme secretion. *J. Biol. Chem.* **269**, 17062–17066
- Hansen, N. J., Antonin, W. and Edwardson, J. M. (1999) Identification of SNAREs involved in regulated exocytosis in the pancreatic acinar cell. *J. Biol. Chem.* **274**, 22871–22876
- Ermak, T. H. and Rothman, S. S. (1981) Zymogen granules of pancreas decrease in size in response to feeding. *Cell Tissue Res.* **214**, 51–66
- Anderson, K. L. and McNiven, M. A. (1995) Vesicle dynamics during regulated secretion in a novel pancreatic acinar cell in vitro model. *Eur. J. Cell Biol.* **66**, 25–38
- Gaisano, H. Y., Huang, X., Sheu, L., Ghai, M., Newgard, C. B., Trinh, K. Y. and Trimble, W. S. (1999) Snare protein expression and adenoviral transfection of amphiphrin AR42J. *Biochem. Biophys. Res. Commun.* **260**, 781–784
- Colomer, V., Lal, K., Hoops, T. C. and Rindler, M. J. (1994) Exocrine granule-specific packaging signals are present in the polypeptide moiety of the pancreatic granule membrane protein GP-2 and in amylase: implications for protein targeting to secretory granules. *EMBO J.* **13**, 3711–3719
- Nakamura, N., Rabouille, C., Watson, R., Nilsson, T., Hui, N., Slusarewicz, P., Kreis, T. E. and Warren, G. (1995) Characterization of a cis-Golgi matrix protein. *J. Cell Biol.* **131**, 1715–1726
- Luzio, J. P., Brake, B., Banting, G., Howell, K. E., Braghetta, P. and Stanley, K. K. (1990) Identification, sequencing and expression of an integral membrane-protein of the trans-Golgi network (TGN38). *Biochem. J.* **270**, 97–102
- Guasch, R. M., Guerri, C. and O'Connor, J. E. (1993) Flow cytometry analysis of concanavalin A binding to isolated Golgi fractions from rat liver. *Exp. Cell Res.* **207**, 136–141
- Moore, H. P., Walker, M. D., Lee, F. and Kelly, R. B. (1983) Expressing a human proinsulin cDNA in a mouse ACTH-secreting cell. Intracellular storage, proteolytic processing, and secretion on stimulation. *Cell* **35**, 531–538
- Moore, H. P. and Kelly, R. B. (1985) Secretory protein targeting in a pituitary cell line: differential transport of foreign secretory proteins to distinct secretory pathways. *J. Cell Biol.* **101**, 1773–1781
- Schmidt, W. K. and Moore, H.-P. H. (1994) Synthesis and targeting of insulin-like growth factor-I to the hormone storage granules in an endocrine cell line. *J. Biol. Chem.* **269**, 27115–27124
- Rivas, R. R. and Moore, H.-P. H. (1989) Spatial segregation of the regulated and constitutive secretory pathways. *J. Cell Biol.* **109**, 51–60
- Lang, T., Wacker, I., Steyer, J., Kaether, C., Wunderlich, I., Soldati, T., Gerdes, H. H. and Almers, W. (1997) Ca²⁺-triggered peptide secretion in single cells imaged with fluorescent protein and evanescent-wave microscopy. *Neuron* **18**, 857–863
- Pouli, A. E., Kennedy, H. J., Schofield, J. G. and Rutter, G. A. (1998) Insulin targeting to the regulated secretory pathway after fusion with green fluorescent protein and firefly luciferase. *Biochem. J.* **331**, 669–675
- Dunn, W. A. (1990) Studies on the mechanism of autophagy: maturation of the autophagic vacuole. *J. Cell Biol.* **110**, 1935–1945
- Tooze, J., Hollinshead, M., Ludwig, T., Howell, K. E., Hofflack, B. and Kern, H. F. (1990) In exocrine pancreas, the basolateral endocytic pathway converges with the autophagic pathway immediately after the early endosome. *J. Cell Biol.* **111**, 329–345
- Rabouille, C., Strous, G. J., Crapo, J. D., Geuze, H. G. and Slot, J. W. (1993) The differential degradation of two cytosolic proteins as a tool to monitor autophagy in hepatocytes by immunocytochemistry. *J. Cell Biol.* **120**, 897–908
- Klumperman, J., Kuliawat, R., Griffith, J. M., Geuze, H. J. and Arvan, P. (1998) Mannose 6-phosphate receptors are sorted from immature secretory granules via adaptor protein AP-1, clathrin and syntaxin 6-positive vesicles. *J. Cell Biol.* **141**, 359–371
- Hirschberg, K., Miller, C. M., Ellenberg, J., Presley, J. F., Siggia, E. D., Phair, R. D. and Lippincott-Schwartz, J. (1998) Kinetic analysis of secretory protein traffic and characterization of Golgi to plasma membrane transport intermediates in living cells. *J. Cell Biol.* **143**, 1485–1503
- Moran, P. and Caras, I. W. (1992) Proteins containing an uncleaved signal for glycosylphosphatidylinositol membrane anchor attachment are retained in a post-ER compartment. *J. Cell Biol.* **119**, 763–772

- 32 Field, M. C., Moran, P., Li, W., Keller, G.-A. and Caras, I. W. (1994) Retention and degradation of proteins containing an uncleaved glycosylphosphatidylinositol signal. *J. Biol. Chem.* **269**, 10830–10837
- 33 Milgram, S. L., Johnson, R. C. and Mains, R. E. (1992) Expression of individual forms of peptidylglycine α -amidating monooxygenase in AtT-20 cells: endoproteolytic processing and routing to secretory granules. *J. Cell Biol.* **117**, 717–728
- 34 Gorr, S. U., Hamilton, J. W. and Cohn, D. V. (1992) Regulated, but not constitutive, secretory proteins bind porcine chymotrypsinogen. *J. Biol. Chem.* **267**, 21595–21600
- 35 Fennewald, S. M., Hamilton, R. L. and Gordon, J. I. (1988) Expression of human preproapoA1 and pre(deltapro)apoA1 in a murine pituitary cell line (AtT-20). *J. Biol. Chem.* **263**, 15568–15577
- 36 Castle, A. M., Huang, A. Y. and Castle, J. D. (1998) Immunoglobulin-derived peptides enter the regulated secretory pathway in AtT-20 cells. *FEBS Lett.* **439**, 341–345
- 37 Cool, D. R., Fenger, M., Snell, C. R. and Loh, Y. P. (1995) Identification of the sorting signal motif within pro-opiomelanocortin for the regulated secretory pathway. *J. Biol. Chem.* **270**, 8723–8729
- 38 Wagner, D. D., Saffari, S., Bonfanti, R., Sadler, J. E., Cramer, E. M., Chapman, B. and Mayadas, T. N. (1991) Induction of specific storage organelles by von Willebrand factor propolypeptide. *Cell* **64**, 403–413
- 39 Castle, A. M., Schwarzbauer, J. E., Wright, R. L. and Castle, J. D. (1995) Differential targeting of recombinant fibronectins in AtT-20 cells based on their efficiency of aggregation. *J. Cell Sci.* **108**, 3827–3837

Received 23 March 2000/12 June 2000; accepted 5 July 2000