The disulfide-bonded loop of chromogranin B mediates membrane binding and directs sorting from the *trans*-Golgi network to secretory granules

Michael M.Glombik, Andreas Krömer¹,
Thorsten Salm, Wieland B.Huttner and
Hans-Hermann Gerdes²

Department of Neurobiology, University of Heidelberg,
Im Neuenheimer Feld 364, D-69120 Heidelberg, Germany

¹Present address: Institut für Klinische Pharmakologie Bobenheim,
Richard-Wagner-Straße 20, D-67269 Grünstadt, Germany

²Corresponding author
e-mail: hhgerdes@sun0.urz.uni-heidelberg.de

M.M.Glombik and A.Krömer contributed equally to this work

The disulfide-bonded loop of chromogranin B (CgB), a regulated secretory protein with widespread distribution in neuroendocrine cells, is known to be essential for the sorting of CgB from the *trans*-Golgi network (TGN) to immature secretory granules. Here we show that this loop, when fused to the constitutively secreted protein α_1 -antitrypsin (AT), is sufficient to direct the fusion protein to secretory granules. Importantly, the sorting efficiency of the AT reporter protein bearing two loops (E2/3–AT–E2/3) is much higher compared with that of AT with a single disulfide-bonded loop. In contrast to endogenous CgB, E2/3–AT–E2/3 does not undergo Ca^{2+} /pH-dependent aggregation in the TGN. Furthermore, the disulfide-bonded loop of CgB mediates membrane binding in the TGN and does so with 5-fold higher efficiency if two loops are present on the reporter protein. The latter finding supports the concept that under physiological conditions, aggregates of CgB are the sorted units of cargo which have multiple loops on their surface leading to high membrane binding and sorting efficiency of CgB in the TGN.
Keywords: chromogranin B/regulated secretory pathway/secretory granule/sorting signal/*trans*-Golgi network

Introduction

During the biogenesis at the *trans*-Golgi network (TGN) of immature secretory granules (ISGs) and constitutive secretory vesicles (CVs), regulated secretory proteins are segregated from constitutive secretory proteins (Burgess and Kelly, 1987; Tooze and Huttner, 1990). Constitutive secretory proteins exit the TGN in CVs which continuously fuse with the plasma membrane (PM) to release their content. Regulated secretory proteins are sorted into ISGs which undergo maturation. During maturation, a subset of proteins is removed from ISGs in a process termed constitutive-like secretion (Arvan and Castle, 1998). The mature secretory granule is the storage compartment of regulated secretory proteins which are released from the cell after stimulation.

Morphological (Tooze *et al.*, 1987, 1989) and biochem-

ical (Gerdes *et al.*, 1989; Chanut and Huttner, 1991; Huttner *et al.*, 1995) evidence suggests that a key step in the sorting of regulated secretory proteins at the level of the TGN is their selective aggregation. For chromogranin B (CgB) and secretogranin II (SgII) this aggregation depends on millimolar Ca^{2+} concentrations and on a mildly acidic pH, conditions which are present in the lumen of the TGN. Ca^{2+} /pH-dependent aggregation of proteins is thought to be mediated by a structural feature composed of numerous acidic amino acids distributed over large parts of the polypeptide chain (Gerdes *et al.*, 1989). Whether other motifs of regulated secretory proteins, comprised by short stretches of amino acids and often referred to as sorting signals, are also involved in sorting at the level of the TGN by a mechanism distinct from aggregation, is poorly understood.

In the past, amino acid sequences of regulated secretory proteins have been found to affect storage in secretory granules of these proteins as determined by morphological analyses and stimulation-evoked secretion experiments (Stoller and Shields, 1989; Sevarino and Stork, 1991; Castle *et al.*, 1992; Chevrier *et al.*, 1993; Tam *et al.*, 1993; Cool *et al.*, 1995; Brechler *et al.*, 1996; Varlamov and Fricker, 1996). However, it has not been shown whether the sequences identified were involved in protein sorting at the level of the TGN or in protein retention at the level of the ISG (Thiele *et al.*, 1997). Furthermore, it is not known whether the identified sequences exert their effect on sorting via aggregation and/or via binding to membrane components in the TGN.

The first indication for the existence of a specific sorting signal functioning at the level of the TGN was obtained for CgB, a regulated secretory protein of the granin family of proteins with widespread distribution in neuroendocrine cells. Near the N-terminus, CgB contains a disulfide-bonded loop which is highly conserved across species, and between CgB and chromogranin A (CgA) (Huttner *et al.*, 1995), another member of the granin family. This loop motif is encoded by a separate exon, exon 3, for both CgB and CgA. Two lines of evidence show that the loop structure is necessary for sorting of CgB to the regulated pathway of protein secretion. First, reductive cleavage of the disulfide bond led to constitutive secretion of CgB (Chanat *et al.*, 1993) but did not interfere with its aggregative properties in the TGN (Chanat *et al.*, 1994). Secondly, vaccinia-based expression of a loop-deleted form of CgB, i.e. expression of the mutant in the absence of endogenous granin synthesis by infection with a recombinant virus, revealed that the 22 amino acids comprising the loop are necessary for sorting of CgB from the TGN to ISGs (Kroemer *et al.*, 1998). The finding that the loop-deleted mutant of CgB is sorted to ISGs in the presence but not in the absence of full-length CgB highlighted the function of aggregative processes at the level of the TGN during sorting to the regulated pathway.

Fig. 1. Schematic representation of reporter constructs. **(A)** N-terminal aa 1–43 of mature rat CgB. Grey box, sequence encoded by exon 3. The disulfide bond in this sequence is indicated by a bracket. The two double arrows indicate the sequence tested in reporter constructs (E2/3, E3). Note that E3 refers to aa 10–12 encoded by the end of exon 2 and to aa 13–43 encoded by exon 3. Numbers indicate position of amino acids in mature rat CgB. **(B)** Different recombinant proteins tested in this study. The constitutive reporter molecule AT was fused to the C-terminus of full length rat CgB (CgB–AT). E2/3 and E3 were inserted both at the N-terminus of AT (E2/3–AT and E3–AT, respectively). E2/3 was fused to the C-terminus of AT (AT–E2/3). E2/3 or E3 were inserted at the N- and C-terminus of AT (E2/3–AT–E2/3 and E3–AT–E3, respectively).

Here we analyse whether the disulfide-bonded loop of CgB is sufficient to direct secretory proteins into ISGs and whether it promotes aggregation and/or membrane binding in the TGN. We generated fusion proteins of the loop motif and α_1 -antitrypsin, a constitutive secretory protein, and analysed their membrane binding, sorting and storage after transient transfection in the neuroendocrine cell line PC12.

Results

Expression of recombinant proteins

CgB of different species shows a striking conservation of amino acids (aa) 1–43, of which aa 1–12 are encoded by the 3' end of exon 2, and aa 13–43 by exon 3 (Pohl *et al.*, 1990; Huttner *et al.*, 1995). To analyse whether the N-terminal 43 aa of CgB (Figure 1A) contain sufficient sorting information to direct a constitutive protein to the regulated pathway of protein secretion, we fused this

Fig. 2. Secretion kinetics of recombinant proteins. PC12 cells transiently transfected with AT, E3–AT, E2/3–AT–E2/3 or CgB–AT were pulse-labelled with [35 S]sulfate for 5 min. Equal aliquots of cell lysates and media collected at the end of the indicated chase times were analysed, either after immunoprecipitation (for recombinant proteins) or directly (endogenous SgII), by SDS–PAGE and phosphorimaging. Secretion values for each time point were calculated as percent of total (sum of cell lysate and medium). For AT, E3–AT, CgB–AT and SgII single experiments and for E2/3–AT–E2/3 the mean of two experiments are shown. The broken line indicates the endpoint of constitutive protein secretion.

particular region to the constitutive secretory protein α_1 -antitrypsin. Two series of reporter constructs were produced, one employing the complete N-terminal sequence 1–43 of rat CgB referred to as E2/3, and one with aa 10–42 referred to as E3, the latter almost entirely encoded by exon 3. E2/3 and E3 contain 22 aa which form a disulfide-bonded loop (Figure 1A; Benedum *et al.*, 1987).

We used a modified α_1 -antitrypsin (AT) containing a tyrosine sulfation site at the C-terminus (Leitinger *et al.*, 1994). AT was shown to be efficiently secreted by PC12 cells (Kroemer *et al.*, 1998). The tyrosine sulfation site allows [35 S]sulfate labelling of the reporter fusion proteins specifically in the TGN, the compartment in which the initial segregation of regulated and constitutively secretory proteins takes place (Kelly, 1985; Tooze and Huttner, 1990). CgB sequences containing the disulfide-bonded loop, i.e. E2/3 or E3, were inserted three amino acids downstream of the signal peptidase cleavage site of AT and/or were fused to the C-terminus of AT (Figure 1B, see Materials and methods). Chimeric molecules with one loop, either at the N- or C-terminus, or with two loops, one at each terminus, were generated. Two controls, AT without rat CgB sequences and full-length CgB fused to the N-terminus of AT, were employed.

Secretion kinetics of recombinant proteins

We analysed the secretion of recombinant proteins in PC12 cells over a time period of 3 h. Transiently transfected cells were pulse-labelled for 5 min with [35 S]sulfate. At the end of various chase times the labelled recombinant protein was quantitated in cell lysates and media after immunoprecipitation with an antiserum against AT followed by SDS–PAGE.

During the first hour of chase the recombinant proteins were rapidly secreted (Figure 2). After 90 min of chase secretion reached a plateau (Figure 2, broken line). Notably, the plateau for each recombinant protein was at a different level indicating a different degree of secretion. Endogenous SgII, which is known to be sorted almost

Fig. 3. Storage efficiency (S_{eff}) of recombinant proteins after transient transfection. **(A)** PC12 cells transfected with the recombinant proteins (each on a separate dish) were pulse-labelled for 5 min with [^{35}S]sulfate and chased for 90 min. Equal aliquots of cell lysates and media collected at the end of the chase were analysed for recombinant proteins by immunoprecipitation and SDS-PAGE followed by phosphorimaging. Signals for the indicated recombinant proteins in cell lysates (C) and media (M) of a single experiment are shown. The mol. wt of recombinant proteins (kDa) is indicated. **(B)** The graph shows the S_{eff} of individual recombinant proteins calculated from several experiments. Error bars show standard deviation. Number (n) of experiments: AT, $n = 9$; CgB-AT, $n = 7$; E2/3-AT, $n = 10$; AT-E2/3, $n = 12$; E3-AT, $n = 6$; E2/3-AT-E2/3, $n = 17$; E3-AT-E3, $n = 8$.

exclusively into ISGs upon exit from the TGN (Tooze and Huttner, 1990), exhibited only little secretion during the first 90 min. Notably, the $t_{1/2}$ of secretion of all recombinant proteins was ~ 20 min. This value is very similar to the $t_{1/2} \sim 17$ min observed for AT after transient expression in Cos-7 cells (Leitinger *et al.*, 1994) and is characteristic of constitutive protein secretion. Most likely two main components contribute to the secretion profile of the recombinant proteins; the portion released into the medium during the first 90 min reflects constitutive secretion in CVs, whereas the portion stored intracellularly beyond this time point implies storage in secretory granules.

AT containing two loop motifs is significantly retained in PC12 cells

Next, we quantitated the amount of all pulse-labelled recombinant proteins retained in PC12 cells after 90 min of chase. The relative amount stored intracellularly at this timepoint is referred to as storage efficiency (S_{eff}). All recombinant proteins were detected as single bands of the expected mol. wt in cell lysates and in the corresponding media (Figure 3A). The total amounts of [^{35}S]sulfate-labelled proteins with and without chase were compared and were found to be slightly increased after chase (not shown). This finding has been observed previously (Baeuerle and Huttner, 1987) and shows that no degradation of [^{35}S]sulfate-labelled proteins occurred during the chase.

The basal level of storage in this system was determined by expression of AT alone. To determine the S_{eff} of full-length CgB in this system, it was fused to AT (CgB-AT). For AT and CgB-AT, S_{eff} of $7.9\% \pm 2.3$ SD and $40.3\% \pm 6$ SD, respectively, were determined (Figure 3B). The reduction in cellular storage of transfected CgB-AT compared with endogenous CgB ($S_{\text{eff}} \sim 90\%$; Chanut *et al.*,

1993), or exogenous human CgB ($S_{\text{eff}} \sim 70\%$; Kroemer *et al.*, 1998) most likely reflects steric hindrance in sorting of the fusion protein due to its AT moiety. When the E2/3 domain was present either at the N-terminus (E2/3-AT) or at the C-terminus (AT-E2/3) of AT, the S_{eff} was found to be $14.2\% \pm 3.1$ SD and $12.9\% \pm 3.3$ SD, respectively (Figure 3B). Likewise, for E3, when inserted at the N-terminus of AT (E3-AT), a similarly low storage efficiency was obtained ($S_{\text{eff}} 12.5\% \pm 2.6$ SD; Figure 3B). Importantly, Student's t -test analysis revealed that the S_{eff} values of all recombinant proteins with one loop, though not as high as CgB-AT ($S_{\text{eff}} \sim 40\%$), were significantly greater than that of AT ($p \leq 0.005$).

A more striking result emerged from the analysis of recombinant AT containing two disulfide-bonded loops per molecule, one located at the N-terminus and a second at the C-terminus. In the case of E2/3-AT-E2/3, the S_{eff} of $33.3\% \pm 3.9$ SD (Figure 3B) was significantly higher than that of AT fusion proteins with only one disulfide-bonded loop. Notably, E2/3-AT-E2/3 was stored to almost the same level as CgB-AT. The shorter fusion protein E3-AT-E3 was stored as high as E2/3-AT-E2/3 ($S_{\text{eff}} = 35.5\% \pm 3.9$ SD; Figure 3B). This result clearly shows that amino acids 10–42 encoding the disulfide-bonded loop of CgB are sufficient to confer intracellular storage to AT when present at both ends of the reporter molecule.

AT containing two disulfide-bonded loops is efficiently sorted to secretory granules

The high S_{eff} values of E2/3-AT-E2/3 and E3-AT-E3 suggested sorting of these proteins to the regulated pathway of protein secretion. This was investigated in three ways. First, we characterized the type of vesicle in which E2/3-AT-E2/3 left the TGN using a 5 min [^{35}S]sulfate pulse followed by a 12 min chase. At this time point [^{35}S]sulfate-labelled secretory proteins have left the TGN in secretory

Fig. 4. E2/3-AT-E2/3 is sorted to immature secretory granules upon exit from the TGN. PC12 cells transfected with AT (**A**), E2/3-AT (**B**) or E2/3-AT-E2/3 (**C**) were pulse-labelled with [35 S]sulfate for 5 min and chased for 12 min. Postnuclear supernatants were prepared and subjected to sequential velocity and equilibrium sucrose gradient centrifugation. Equal aliquots of gradient fractions were analysed, either after immunoprecipitation (AT, E2/3-AT, E2/3-AT-E2/3) or directly (hsPG, SgII), by SDS-PAGE and phosphorimaging. The graphs show the quantitation of the indicated [35 S]sulfate-labelled proteins across the equilibrium sucrose gradient (fraction 1, top). The arrows indicate the peak of CVs and ISGs.

vesicles. CVs and ISGs were separated by sucrose gradient centrifugation as described previously (Tooze and Huttner, 1990). In addition to E2/3-AT-E2/3, AT and E2/3-AT, both with a low S_{eff} (Figure 3B), were analysed in parallel. On the equilibrium gradient AT comigrated with heparan sulfate proteoglycan (hsPG), a marker for constitutive vesicles in PC12 cells (CV, Figure 4A). This result is consistent with the low S_{eff} obtained for AT (Figure 3B) and confirms its secretion by the constitutive pathway. As expected from its slightly higher S_{eff} , E2/3-AT largely comigrated with CVs (Figure 4B) and only a shoulder

Fig. 5. Double immunogold electron microscopy showing colocalization of E2/3-AT-E2/3 and endogenous CgB in dense-cored secretory granules. Ultrathin cryosections prepared from PC12 cells transfected with E2/3-AT-E2/3 were double immunolabelled for E2/3-AT-E2/3 (large arrowhead) and endogenous CgB (small arrow). The bar corresponds to 300 nm.

was present at the position of ISGs. In contrast, most of E2/3-AT-E2/3 comigrated with endogenous SgII, a marker protein for ISGs, whereas a minor portion comigrated with the marker for CVs, endogenous hsPG (Figure 4C). These data clearly show that the majority of E2/3-AT-E2/3 had entered the ISGs, whereas a minor portion exited the TGN in CVs. Determination of the sorting efficiency of E2/3-AT-E2/3 (see Materials and methods) revealed that at the level of the TGN ~60% of E2/3-AT-E2/3 is sorted to ISGs. This sorting value is significantly higher than the storage value determined at the level of mature secretory granules after 90 min of chase (Figure 3B).

Secondly, we performed double immunogold-electron-microscopy on PC12 cells transiently transfected with E2/3-AT-E2/3. Two first antibodies were used, one against transfected AT and another specific for endogenous CgB. As shown in Figure 5, a specific labelling of E2/3-AT-E2/3 (large gold arrowhead) and CgB (small gold arrow) was obtained for dense-cored granules. Many granules showed colocalization of both proteins. When cells transiently transfected with AT were probed with an antibody against AT, no immunolabelling of dense core granules was detected (data not shown). The latter observation is consistent with the low storage efficiency of AT and its comigration with CVs on equilibrium gradients.

Thirdly, we tested for storage of recombinant AT containing two loops in secretory granules by performing depolarization-induced secretion experiments. Therefore PC12 cells, transfected with either E2/3-AT-E2/3 or E3-AT-E3, were [35 S]sulfate pulse-labelled for 5 min followed by a chase of 90 min. Subsequently, the cells were stimulated by depolarization in a medium containing

Fig. 6. Depolarization-induced, calcium-dependent secretion of E2/3-AT-E2/3 and E3-AT-E3. PC12 cells transfected with E2/3-AT-E2/3 (top) or E3-AT-E3 (bottom) were pulse-labelled for 10 min with [³⁵S]sulfate and chased for 90 min. Cells were then incubated for 10 min as indicated. Equal aliquots of cells and media were analysed either directly for endogenous SgII, or by immunoprecipitation for transfected proteins, followed by SDS-PAGE and phosphorimaging. The graphs show the quantitation of released [³⁵S]sulfate-labelled proteins during the 10 min incubation. Each value is expressed as percentage of total (sum of cells plus medium). Error bars show standard deviation of three independent experiments.

55 mM potassium. The relative amount of [³⁵S]sulfate-labelled proteins released into the medium after depolarization as compared with the cellular amount prior to stimulation, was quantified. As depicted in Figure 6, exogenous E2/3-AT-E2/3 and E3-AT-E3 were released to $11.7\% \pm 3.7$ SD and $10.6\% \pm 3.1$ SD, respectively. Simultaneously, endogenous SgII was released to similar amounts, i.e. $12.8\% \pm 6.1$ SD from cells transfected with E2/3-AT-E2/3 and $12.0\% \pm 3.1$ SD from cells transfected with E3-AT-E3. Under control conditions (either 5 mM KCl/2.2 mM CaCl₂ or 55 mM KCl/10 mM MgCl₂/no CaCl₂), secretion of all three proteins was significantly lower. Consistent with the low S_{eff} , a stimulated release of AT was not detected under the same experimental conditions (not shown). Taken together, these data show that E2/3-AT-E2/3 and E3-AT-E3 were sorted to ISGs upon exit from the TGN and stored in secretory granules which undergo a calcium-dependent stimulated secretion.

Disulfide bond formation is necessary for efficient sorting of E2/3-AT-E2/3

Previously it was shown that reductive cleavage of the disulfide bond stabilizing the loop in CgB upon dithiothreitol (DTT)-treatment *in vivo* leads to missorting of CgB (Chanat *et al.*, 1993). At the same time endogenous SgII, lacking cysteines, is efficiently sorted to secretory

Fig. 7. Reductive cleavage of the disulfide-bonded loop induces missorting of E2/3-AT-E2/3. PC12 cells transfected with E2/3-AT-E2/3 or AT were pulse labelled in the absence or presence of 2 mM DTT. After 90 min of chase, equal aliquots of cell lysates and media were analysed either directly for SgII or by immuno-precipitation for recombinant proteins, followed by SDS-PAGE and phosphorimaging. S_{eff} was calculated for the proteins indicated from three independent experiments, except for AT in the presence of 2 mM DTT, for which the standard deviation of a duplicate experiment is shown. Error bars, standard deviation.

granules. We used this approach to analyse whether disulfide bond formation is important for the sorting of E2/3-AT-E2/3. The S_{eff} of E2/3-AT-E2/3 was determined after pulse-chase labelling with [³⁵S]sulfate in the absence and presence of 2 mM DTT. As a control, AT of transfected PC12 cells was analysed in parallel. Figure 7 shows that storage of E2/3-AT-E2/3 dropped significantly from $33.3\% \pm 3.9$ SD in the absence of DTT to $7.0\% \pm 1.6$ SD in the presence of DTT. Importantly, this value is very similar to that obtained for AT ($S_{\text{eff}} = 7.9\% \pm 2.3$ SD in the absence of DTT; $S_{\text{eff}} = 9.4\% \pm 0.1$ SD in the presence of DTT). This S_{eff} reflects the background level of the constitutive reporter (see also Figure 3). In contrast, endogenous SgII with a S_{eff} of $91.7\% \pm 3.6$ SD in the absence of DTT was still stored efficiently in DTT-treated transfected PC12 cells ($S_{\text{eff}} = 68.8\% \pm 2.9$ SD, Figure 7), as found previously by Chanat *et al.* (1993) for DTT-treated non-transfected PC12 cells. This indicates that the sorting machinery for the regulated pathway of secretion was still functioning in the presence of DTT. Therefore the lack of storage of E2/3-AT-E2/3 indicates that disulfide bond formation is necessary for sorting of E2/3-AT-E2/3 to the regulated pathway of protein secretion.

The loop structure of CgB is stabilized by an intramolecular disulfide bond (Benedum *et al.*, 1987). Because E2/3-AT-E2/3 contains the loop at both termini, we analysed whether intermolecular disulfide bonds were formed between E2/3-AT-E2/3 molecules leading to covalently linked oligomers. These oligomers may mimic aggregates which could hypothetically result in their sorting to ISGs. To investigate this possibility we labelled E2/3-AT-E2/3, and as a control AT, which lacks cysteine-containing loops, in the TGN by a 5 min [³⁵S]sulfate pulse. Subsequently the mol. wts of the [³⁵S]-labelled forms were analysed by SDS-PAGE under reducing and non-reducing conditions. Figure 8 shows that the mobility of neither AT nor E2/3-AT-E2/3 is affected by the presence or absence of β -mercaptoethanol. Note that AT and E2/

Fig. 8. E2/3-AT-E2/3 molecules do not form intermolecular disulfide bonds. PC12 cells transfected with AT or E2/3-AT-E2/3 were pulse-labelled for 5 min with [35 S]sulfate. Cell lysates were analysed for recombinant proteins by immunoprecipitation followed by SDS-PAGE in the absence (-) or presence (+) of β -mercaptoethanol (β -ME), and phosphoimaging. Note the same mobility of AT or E2/3-AT-E2/3 under reducing and non-reducing conditions. In addition to the higher mol. wt form of labelled AT and E2/3-AT-E2/3 (arrowheads), which is the only form when pulse-labelling is followed by a chase (Figure 3A), a lower mol. wt form is visible for each protein (asterisks). This form reflects a different degree of sialylation.

3-AT-E2/3 were detected as doublets of lower (Figure 8, asterisk) and higher (Figure 8, arrowhead) mol. wt bands. In each case the lower band reflects incomplete sialylation (Leitinger *et al.*, 1994) and is no longer seen after 5 min (Leitinger *et al.*, 1994) or 90 min of chase (Figure 3). In conclusion, no intermolecular disulfide bonds had been formed between E2/3-AT-E2/3 molecules during biosynthesis and secretory transport to the TGN. Formation of 'aberrant' intramolecular disulfide bonds, i.e. between N- and C-terminal cysteines, we regard as most unlikely because E2/3-AT-E2/3 underwent [35 S]sulfation in the TGN, showing that it passed the quality control mechanisms for misfolded proteins of the endoplasmic reticulum.

The disulfide-bonded loop does not promote Ca^{2+} /low pH aggregation

CgB has been shown to aggregate selectively in the presence of millimolar Ca^{2+} and mildly acidic pH, conditions mimicking the luminal milieu of the TGN (Chanat and Huttner, 1991). From this it has been proposed that selective aggregation of CgB in the TGN is an important step for its sorting to ISGs (Chanat and Huttner, 1991). To test whether the disulfide-bonded loop of CgB is involved in Ca^{2+} /pH-mediated aggregation, we analysed in isolated TGN vesicles (Chanat and Huttner, 1991) the aggregative properties of AT bearing two loops. PC12 cells transfected with E2/3-AT-E2/3 were pulse-labelled for 5 min with [35 S]sulfate. After subcellular fractionation TGN vesicle preparations were perforated with 0.1%

Fig. 9. E2/3-AT-E2/3 does not undergo Ca^{2+} /pH-dependent aggregation. PC12 cells transfected with E2/3-AT-E2/3 were pulse-labelled for 5 min with [35 S]sulfate. TGN vesicles prepared by velocity gradient centrifugation were perforated in a 0.1% saponin buffer, either under non-aggregative conditions (NA, absence of Ca^{2+} at pH 7.4), or under aggregative conditions (A, presence of 10 mM Ca^{2+} at pH 6.4.), and separated into supernatant (S) and pellet (P). Supernatants and pellets were divided into two equal aliquots. One aliquot of each fraction was subjected to immunoprecipitation for E2/3-AT-E2/3, the other for endogenous CgB. Immunoprecipitates were analysed by SDS-PAGE and phosphoimaging. Arrowhead, full-length endogenous CgB; brackets, degradation products of endogenous CgB; arrow, E2/3-AT-E2/3.

saponin under aggregative (10 mM Ca^{2+} at pH 6.4) or non-aggregative conditions (absence of Ca^{2+} at pH 7.4). They were sedimented and supernatants and pellets were analysed for recombinant E2/3-AT-E2/3 and endogenous CgB. Under aggregative conditions 94.9% of full-length CgB and its fragments were obtained in the pellet, whereas under non-aggregative conditions endogenous CgB appeared almost completely in the supernatant and only 4.9% were found in the pellet (Figure 9). However, E2/3-AT-E2/3 was only detectable in the supernatant even in the presence of Ca^{2+} /pH 6.4 (Figure 9). Thus, the disulfide-bonded loop does not confer the Ca^{2+} /pH-dependent aggregative properties to the reporter and probably functions independently of Ca^{2+} and mildly acidic pH.

The disulfide-bonded loop of CgB promotes binding to the TGN membrane of E2/3-AT-E2/3

To investigate whether E2/3-AT-E2/3 interacts with the membrane of the TGN we exploited sulfation to specifically label proteins in this compartment. A post-nuclear supernatant was prepared from transfected PC12 cells directly after pulse-labelling for 5 min with [35 S]sulfate. At this time point no labelled proteins have exited the TGN (Tooze and Huttner, 1990). After opening of TGN vesicles by treatment with 0.03% saponin (Chanat and Huttner, 1991) followed by sonication, membranes were pelleted by centrifugation and the amount of [35 S]sulfate-labelled E2/3-AT-E2/3 in the membrane fraction compared with the supernatant was determined and is referred to as binding efficiency. In parallel, membrane binding of transfected AT and E2/3-AT was analysed. The presence

Fig. 10. E2/3-AT-E2/3 binds more efficiently to TGN membranes than E2/3-AT. PC12 cells, transfected with E2/3-AT-E2/3, E2/3-AT or AT, were pulse-labelled for 5 min with [35 S]sulfate. The post-nuclear supernatants were sonicated in the presence of saponin and subjected to centrifugation at 100 000 g for 45 min. Equal aliquots of the membrane-pellets and supernatants were analysed, either directly for CgB, or by immunoprecipitation for the recombinant proteins, followed by SDS-PAGE and phosphorimaging. The graph shows a quantitation of the transfected proteins (black bars) together with the corresponding endogenous CgB (grey bars) of the same cell culture dishes. Error bars show SEM of six independent experiments.

of 0.03% saponin in our assay did not lead to a reduction in membrane binding (not shown). Figure 10 shows the quantitation of membrane-bound recombinant proteins. AT was virtually unbound to membranes of the TGN ($0.1\% \pm 0.03$ SD). This result also implies that secretory proteins were not trapped in sealed TGN vesicles under our experimental conditions. For E2/3-AT a significant but small fraction ($1.1\% \pm 0.08$ SD) was found in the membrane pellet. However, E2/3-AT-E2/3 was bound ~5-fold more efficiently to membranes of the TGN ($5.0\% \pm 0.9$ SD). In all experiments endogenous CgB, for which a membrane-bound form has been described (Pimplikar and Huttner, 1990), was found at ~5% in the membrane fraction.

Newly exocytosed E2/3-AT-E2/3 is bound to the PM after depolarization-induced release of secretory granules

To analyse whether the observed membrane binding in the TGN of E2/3-AT-E2/3 occurs in the regulated secretory pathway we performed a cell-surface staining after stimulation of regulated protein secretion. The same approach was used to identify a membrane-bound form of newly exocytosed CgB (Pimplikar and Huttner, 1992). PC12 cells, transfected with E2/3-AT-E2/3 or AT, were depolarized with 55 mM potassium for 7 min and subsequently probed with an antibody against AT at 4°C prior to paraformaldehyde fixation. A clustered immunostaining of E2/3-AT-E2/3 was detected at the cell surface (Figure 11C). No surface staining was detectable in the absence of a stimulus (Figure 11A) or when calcium was replaced by magnesium during depolarization (Figure 11B). In the case of AT, no surface staining was detectable even after

a stimulus in the presence of calcium (Figure 11D). This shows that under stimulation conditions no nonspecific immunostaining occurred. Under all experimental conditions endogenous CgB, which was analysed with a monoclonal antibody in parallel, showed the same result as E2/3-AT-E2/3 (not shown). These data show that the membrane binding of the disulfide-bonded loop, observed biochemically in the TGN, persists throughout the regulated pathway of protein secretion even at the PM shortly after exocytosis of secretory granules.

Discussion

Loop-mediated sorting of recombinant proteins to secretory granules

In this report we show that the disulfide-bonded loop of CgB contains transferable sorting information to direct a constitutive secretory protein to the regulated pathway of protein secretion. This sorting signal acts at the level of the TGN and was confined to aa 10–42 of CgB. The missorting of E2/3-AT-E2/3 upon reductive cleavage of the disulfide bonds demonstrates that not only the amino acid sequence of the loop but also its three-dimensional conformation stabilized by the disulfide bond is essential for its function as a sorting signal. Similarly, missorting of endogenous CgB in PC12 cells was observed upon DTT treatment (Chanat *et al.*, 1993).

Non-aggregative properties of the disulfide-bonded loop

Our data show that the disulfide-bonded loop mediates membrane binding, observed at the level of the TGN, by a mechanism distinct from the Ca^{2+} /pH-dependent aggregation. This is in agreement with the concept proposed previously (Gerdes *et al.*, 1989) that the highly negatively charged region of CgB encoded by exon 4 is the structure responsible for Ca^{2+} /pH-dependent aggregation.

The inability of E2/3-AT-E2/3 to form Ca^{2+} /pH-dependent aggregates may explain the observation that the sorting efficiency of E2/3-AT-E2/3 to ISGs ($S_{\text{eff}} \sim 60\%$ after 12 min of chase as determined by gradient analysis; Figure 4) was significantly higher than the storage efficiency (33% as determined after 90 min of chase; Figure 3B). This result suggests that constitutive-like secretion may have occurred during maturation of ISGs. This pathway was originally proposed by Arvan and Castle (1992) and is thought to remove secretory proteins which stay soluble during ongoing condensation in the maturation process. If constitutive-like vesicles budded from ISGs, they seem to have fused directly (and not via the endosome) with the PM, because the secretion kinetics of E2/3-AT-E2/3 are rapid and very similar to that of AT (Figure 2). The fact that E2/3-AT-E2/3 is only partially removed by constitutive-like secretion may be explained at least in part by the membrane binding of E2/3-AT-E2/3. Whether in addition loop-loop interactions, as observed *in vitro* (Corti *et al.*, 1997; Thiele and Huttner, 1998), play a role in retention of E2/3-AT-E2/3 in ISGs remains to be investigated. As an alternative explanation for the difference between the sorting and the storage efficiency of E2/3-AT-E2/3, stimulus-independent exocytosis of secretory granules may have occurred.

Fig. 11. Membrane binding of E2/3-AT-E2/3 after depolarization-induced exocytosis of secretory granules. PC12 cells transfected with E2/3-AT-E2/3 were incubated in non-stimulating media either 5K/Ca medium (A) or 55K/Mg medium (B) or stimulated for exocytosis 55K/Ca medium (C). As a control, cells transfected with AT were stimulated in parallel with 55K/Ca medium (D). Thereafter cells were probed for surface-staining of E2/3-AT-E2/3 or AT with an antiserum against AT and analysed by confocal microscopy. Single sections midway up the cell are shown. Scale bar, 5 μ m.

The disulfide-bonded loop binds to the membrane in the TGN

Interestingly, membrane-binding was 5-fold higher for E2/3-AT-E2/3 compared with E2/3-AT, i.e. duplication of the number of loops per AT molecule had a more than additive effect on membrane binding. This result is consistent with the concept that both loops of E2/3-AT-E2/3 bind directly and synergistically to the membrane resulting in more membrane-bound E2/3-AT-E2/3 than E2/3-AT. Alternatively, the higher signal of E2/3-AT-E2/3 in the membrane pellet might not result from single molecules of E2/3-AT-E2/3 that are directly bound to the membrane but from chains of E2/3-AT-E2/3 molecules, with one or both ends attached to the membrane in the TGN. E2/3-AT-E2/3 chains may have formed by loop-loop interactions which are known to promote dimerization of CgA *in vitro* (Thiele and Huttner, 1998). However, we did not detect oligomeric forms of E2/3-AT-E2/3 by sedimentation of cell lysates on sucrose gradients (not shown). If chains existed *in vivo* (high concentration of E2/3-AT-E2/3 at pH 6.4) they presumably would be unstable under our experimental conditions (high protein dilution at pH 7.3). Thus, our membrane binding assay most likely detects E2/3-AT-E2/3 molecules directly bound to components of the membrane. In addition to membrane binding in the TGN, membrane-bound E2/3-AT-E2/3 was detected at the PM after exocytosis of SGs.

The latter observation suggests that the loop is bound to membrane components in the TGN which give rise to ISG membrane and points to a role of the loop-mediated membrane binding in the sorting of E2/3-AT-E2/3 to the regulated pathway of protein secretion.

Which components in the membrane of the TGN interact with the disulfide-bonded loop? Carboxypeptidase E (CPE) was reported to function as a sorting receptor for the regulated pathway of protein secretion, in particular for proopiomelanocortin (POMC), proinsulin and proenkephalin (Cool *et al.*, 1997). Two lines of evidence argue against CPE as the binding partner for the disulfide-bonded loop of CgB in the TGN. First, in Neuro2A cells expressing CPE-antisense RNA, CgA with a disulfide-bonded loop highly homologous to that of CgB was not missorted to constitutive vesicles, whereas proinsulin and proenkephalin exhibited an increased basal release (Normant and Loh, 1998). Secondly, despite the absence of CPE in exocrine tissue, CgB expressed in transgenic mice is sorted into amylase containing exocrine granules (Natori *et al.*, 1998). Thus the binding site for the disulfide-bonded loop of CgB remains to be identified.

Membrane binding versus sorting in the TGN

We found that ~60% of E2/3-AT-E2/3 was sorted into ISGs but only 5% was recovered in the membrane fraction. Two possible reasons may account for the apparent discrep-

any. First, it is conceivable that the percentage of membrane-bound E2/3-AT-E2/3 in living cells is higher than detected in our assay. Directly membrane-bound E2/3-AT-E2/3 may have been lost due to the mechanical stress during the isolation procedure. In addition, a loss of membrane-linked E2/3-AT-E2/3 chains may have occurred upon dilution due to a similarly modest homodimerization affinity as obtained for full-length CgA ($K_D = 1.6 \mu\text{M}$) or CgA₁₋₆₀ ($K_D = 85 \text{ nM}$). Secondly, a contribution to the high sorting value of E2/3-AT-E2/3 may result from the direct interaction of E2/3-AT-E2/3 to loops of CgB aggregates and not to the membrane. However, binding to endogenous cargo is not supported by our data, showing that E2/3-AT-E2/3 did not sediment together with aggregates of CgB *in vitro*. We therefore favour the explanation that the different extents of sorting and membrane binding of E2/3-AT-E2/3 as determined in our assays is due to a loss of the membrane-bound fusion protein during the analysis.

Sorting efficiency of one versus two disulfide-bonded loops

Our results demonstrate that sorting to ISGs of AT bearing two loops is much more efficient than AT with only one loop. How can this be explained? Several lines of evidence indicate that the Ca^{2+} /pH-mediated aggregation is an essential step in chromogranin sorting to the regulated pathway of secretion (Huttner *et al.*, 1995). Protein aggregates have been detected in the TGN by electron microscopy (Tooze *et al.*, 1987). Furthermore, aggregates of CgB and SgII were found biochemically in perforated TGN vesicle preparations (Chanat and Huttner, 1991). The importance of aggregate formation for sorting of regulated secretory proteins was demonstrated by preventing aggregation upon neutralization of the pH in the TGN with NH_4Cl *in vivo* (Gerdes *et al.*, 1989) and by missorting loopless CgB in the absence of endogenous granins (Kroemer *et al.*, 1998). Therefore, under physiological conditions most likely not single molecules but aggregates of regulated secretory proteins exit the TGN into ISGs. Our working hypothesis is that aggregates represent the sorting units and have exposed many sorting signals at their surface. According to this model we suggest that, in addition to segregation of regulated from constitutive secretory proteins and concentration of the former, aggregation in the TGN has a third function which is multimerization of sorting signals for the regulated pathway of protein secretion. To generate a sorting unit carrying multiple sorting signals with a non-aggregative reporter, we expressed AT with two disulfide-bonded loops. The finding that this recombinant protein was sorted and stored much more efficiently than AT bearing only one loop is in agreement with our model that multiple signals are necessary for efficient sorting to ISGs (Figure 12). Furthermore, the finding that E3-AT-E3 and E2/3-AT-E2/3 were stored with nearly the maximal efficiency possible in our reporter system, suggests that two sorting signals on a reporter suffice to mimic sorting of an aggregate (Figure 12). Evidence in favour of such a model is provided by the higher binding efficiency to TGN membranes of E2/3-AT-E2/3 as compared with E2/3-AT.

In line with our observation, it was demonstrated for apical targeting in Madin-Darby canine kidney cells

Fig. 12. Sorting model based on membrane interaction of the disulfide-bonded loop in the TGN. (A) Fusion proteins consisting of AT and the disulfide-bonded loop do not undergo Ca^{2+} /pH-mediated aggregation in the TGN. Single molecules are the sorting units. Efficient loop-mediated membrane-binding in the TGN occurs only when more than one loop is fused to AT. (B) CgB undergoes Ca^{2+} /pH-mediated aggregation in the TGN. The formed CgB aggregates are the sorting units to ISGs which have exposed multiple loops at their surface. More than one loop interacts with the membrane in the TGN leading to high binding efficiency.

that the doubly *N*-glycosylated form of growth hormone showed increased apical delivery compared with the monoglycosylated form (Scheiffele *et al.*, 1995). Increased binding affinity as the consequence of multiple binding sites is implicated in sorting of lysosomal hydrolases to lysosomes (Tong *et al.*, 1989). In particular, it has been shown that the presence of multiple mannose-6-phosphate residues per molecule greatly increased the binding affinity to the large mannose-6-phosphate receptor. Notably, a 100- to 1000-fold increase in binding affinity was detected with a bivalent ligand compared with a monovalent ligand (Tong *et al.*, 1989).

Comparison of the disulfide-bonded loop with structures of other regulated secretory proteins implicated in sorting

The signal identified in CgB, i.e. aa 10–42, is in fairly good agreement with the hypothesis of Gorr and Darling (1995). They postulated an N-terminal hydrophobic sorting signal between residues 9 (± 4) and 26 (± 6) in many regulated secretory proteins. However, comparison of the results obtained for E2/3-AT-E2/3 and E3-AT-E3, i.e. similar storage efficiency but different distance of the signal to the N-terminus, clearly shows that the spacing of the disulfide-bonded loop to the N-terminus does not affect its function as a sorting signal.

In the past, a 13 aa sequence in the N-terminus of POMC was described to contain sorting information for the regulated pathway of secretion (Cool and Peng Loh, 1994). It was reported that this sequence is necessary for sorting of POMC by transfection of deletion mutants in Neuro2A cells (Cool and Peng Loh, 1994). The same group found that aa 1–26 of POMC is sufficient to direct the constitutive reporter chloramphenicolacetyltransferase (CAT) to the regulated pathway of protein secretion (Tam

et al., 1993) when it is fused in a single copy to the reporter. Storage in the regulated pathway of secretion was analysed at the level of secretory granules by immunofluorescence analysis and depolarization-induced stimulated release experiments. However, storage efficiencies of the transfected proteins by pulse-chase analysis were not determined. Although not discussed by Cool and Peng Loh (1994), it is interesting in the context of this study that immediately after its synthesis CAT oligomerizes into trimers which cannot be dissociated by 1 M thiocyanate (Shaw and Leslie, 1991). Therefore, in line with our model multiple sorting signals per sorting unit may have been generated by oligomerization of the CAT-fusion proteins.

Materials and methods

Chemicals and antibodies

Sodium butyrate was purchased from Merck (Germany), radiochemicals from Amersham Buchler (UK) and cell culture reagents from Gibco-BRL, Gaithersburg, MD. A polyclonal antibody (Rosa *et al.*, 1985) was used for detection of rat CgB. A polyclonal anti-human α_1 -antitrypsin antibody was kindly provided by Dr J.L.Brown (Denver, USA) and bought as IgG fraction from Sigma (Germany).

Expression vector cloning

For cloning of fusion proteins, the cDNA of α_1 -antitrypsin containing the tyrosine sulfation site of rat cholecystokinin precursor (AT) (Leitinger *et al.*, 1994) was subcloned into pBluescript II KS (Stratagene). Rat CgB cDNA (Forss-Petter *et al.*, 1989) was used in PCRs as a template to generate cDNA fragments encoding amino acid sequences with the disulfide-bonded loop. The loop-encoding sequences were verified by DNA sequencing. The different fragments (E2/3, E3; Figure 1) were inserted either at the 5'- or 3'-end of the AT cDNA. All insertions into the 5' end were cloned using the unique *Bam*HI restriction site. For cloning at the 3' end a *Bam*HI restriction site was introduced by PCR using a T7 primer (forward) and the oligonucleotide GCGGGATCCG-ATGGGTATTCGTAGTC as reverse primer. For subsequent insertion, *Bam*HI and *Bgl*III restriction sites were introduced by PCR at the 5'- and 3'-ends of the N-terminal loop sequences to be inserted. Therefore, the following forward (forw) and reverse (rev) primers (restriction sites: underlined) were used:

rCgBforw, E2/3forw: GCGGGATCCCGCTCCAGTGGATAACAG;
rCgBrev: GCGAGATCTTCGCCCCGCTGGCTGAAC;
E2/3rev: GCGAGATCTTTCCTCAGGACTTGCC;
E3forw: CTCCTTAGGGGATCCCGAAGAAATGGTGACTCGCTGC;
E3rev: CTCCTAAGGATCCGTTCTCAGGACTTGCCGCACTC.

For insertion into the 3' *Bam*HI restriction site of AT in addition to the *Bgl*III restriction site (underlined), a stop codon (italics) was introduced by PCR at the 3' end of the sequence coding for the disulfide-bonded loop. For PCR a T7 forward primer and the following oligonucleotide as a reverse primer was used:

E2/3rev,stop: GCGAGATCTTACTTCTCAGGACTTGCC.

For cloning of the cDNA constructs, which encode AT fused with an N- and C-terminal disulfide-bonded loop (E2/3-AT-E2/3, E3-AT-E3), the following cloning strategy was used. Because of the additional 3' *Bam*HI restriction site introduced in AT by PCR, it was necessary to split AT by restriction with *Hind*III-*Eco*RV into a 5' and a 3' half. Then, the sequence encoding for the disulfide-bonded loop was inserted in each half of AT separately. For subsequent cloning of E2/3-AT-E2/3 and E3-AT-E3 the 5' restriction fragment encoding the N-terminal disulfide-bonded loop and the N-terminal part of AT was excised with *Hind*III-*Eco*RV and inserted into the *Hind*III-*Eco*RV opened pBluescript II KS Vektor containing the sequence encoding the C-terminal half of AT and the C-terminal disulfide-bonded loop. To obtain the expression vector constructs used for transfection, the final constructs were excised by *Hind*III-*Xba*I from pBluescript II KS and subcloned into pRc/CMV.

Cell culture and transfections

PC12 cells (rat pheochromocytoma cells, clone 251) were grown in DMEM supplemented with 10% horse serum and 5% fetal calf serum (growth medium) as previously described (Tooze and Huttner, 1990) and transfected by electroporation as published (Kroemer *et al.*, 1998). Except for the stimulated release experiments, growth medium was

replaced by growth medium containing 10 mM sodium butyrate 17.5 h before pulse-labelling.

Metabolic labelling

PC12 cells were pulse-labelled in the TGN with [³⁵S]sulfate as previously described (Kroemer *et al.*, 1998). To determine storage efficiencies, chase times of 90 min, and for subcellular fractionation, chase times of 12 min were used.

Pulse-chase labelling in the presence of DTT was basically carried out as described previously (Chanat *et al.*, 1993). In brief, 2.5 min before the pulse DTT was added to a final concentration of 2 mM and was present throughout the 5 min pulse and during the first 30 min of chase. After 30 min the chase medium was replaced by DTT-free chase medium and cells were incubated for an additional 60 min. Chase media with and without DTT of the same dish were pooled and processed simultaneously with the corresponding cell lysates for analysis.

Subcellular fractionation

The exit of sulfate-labelled proteins from the TGN into CVs and ISGs was followed by sequential velocity and equilibrium sucrose gradients of post-nuclear supernatants according to standard procedures (Tooze and Huttner, 1990; Kroemer *et al.*, 1998). Aliquots of 0.8 ml of fractions 2-4 from the top of the velocity gradients were pooled and analysed by equilibrium gradient centrifugation.

Determination of the sorting efficiency in the TGN

E2/3-AT-E2/3 exits the TGN in both CVs and ISGs as defined by gradient profiles of hsPG and SgII, respectively. The portion of E2/3-AT-E2/3 exiting the TGN in CVs and ISGs was determined from a best fit between the distribution curve across the equilibrium gradient of E2/3-AT-E2/3 and that of an arbitrary (hsPG+SgII)-composite calculated as follows: for each protein (hsPG, SgII, E2/3-AT-E2/3) the values giving the distribution across the equilibrium gradient were normalized such that the peak had the same arbitrary value. For each fraction, the values of the hsPG (CVs) and SgII (ISGs) were then multiplied by the factors *x* and *y*, respectively. The factors *x* and *y* have been chosen such that the sum of the two products (*x*hsPG + *y*SgII) yielded a distribution across the equilibrium gradient that fitted best to that of E2/3-AT-E2/3. The percentage of E2/3-AT-E2/3 in CVs and ISGs was then determined according to %CV = (*x*/*x* + *y*)100 and %ISG = (*y*/*x* + *y*)100, respectively. %ISG is referred to as sorting efficiency.

Analysis of radioactively labelled proteins

Immunoprecipitations with polyclonal antisera against AT or rat CgB were carried out as described (Kroemer *et al.*, 1998). For direct analysis of granins and hsPG, equal aliquots of various samples were processed for SDS-PAGE and analysed by phosphoimaging.

Analysis of the aggregative state of AT and E2/3-AT-E2/3 in the TGN

Transfected PC12 cells were pulse-labelled for 5 min with 3 mCi/ml [³⁵S]sulfate. Isolation of TGN membrane vesicles was performed as described previously (Chanat and Huttner, 1991). TGN membrane vesicles were concentrated by centrifugation at 130 000 *g* for 20 min. Pellets were resuspended in 300 μ l buffer A (non-aggregative milieu: 10 mM MES-NaOH pH 7.4, 30 mM KCl, 5 mM EDTA, 1% Triton X-100, 1.2 mM leupeptin) or buffer B (aggregative milieu: 10 mM MES-NaOH pH 6.4, 10 mM CaCl₂, 1% Triton X-100, 1.2 mM leupeptin). After 30 min incubation on ice, samples were centrifuged at 130 000 *g* for 20 min. Pellets were resuspended in 300 μ l buffer A or B as before. Samples in buffer A were adjusted to 5 mM EDTA. Aliquots of each sample were adjusted to 100 mM NaCl, diluted with 1 vol. cell dilution buffer, split into two aliquots and analysed by immunoprecipitation with antisera against AT or CgB and SDS-PAGE.

Analysis of membrane binding in the TGN

PC12 cells were transfected and labelled for 5 min with [³⁵S]sulfate as described. Post nuclear supernatants were prepared as described, using strip-buffer (10 mM HEPES buffer pH 7.3, 100 mM NaCl) and incubated in 0.03% saponin (Sigma S-4521) for 15 min at 4°C with gentle shaking. Thereafter the samples were sonicated at 4°C (three intervals of 10 s) followed by centrifugation for 45 min at 100 000 *g*. The supernatants were removed for analysis and the pellets were washed once in strip-buffer. Pellets, supernatants and wash buffer were analysed, either directly for endogenous CgB, or by immunoprecipitation for transfected proteins with an antiserum against AT, followed by SDS-PAGE and phosphoimaging.

Depolarization-induced release

Three days after transfection PC12 cells were labelled with [³⁵S]sulfate for the indicated time. After a 90 min chase time cells were washed twice with either 5K/Ca-medium (127 mM NaCl, 5 mM KCl, 2.2 mM CaCl₂, 0.33 mM Na₂HPO₄, 0.44 mM KH₂PO₄, 4.2 mM NaHCO₃, 5.6 mM glucose, 10 mM HEPES–NaOH pH 7.4) or 5K/Mg-medium for depolarization of cells in the absence of calcium (5K/Ca-medium in which 2.2 mM CaCl₂ was replaced by 10 mM MgCl₂). Thereafter cells were incubated for 15 min in 5K/Ca-medium, 55K/Ca-medium (5K/Ca-medium in which 127 mM NaCl and 5 mM KCl were replaced by 77 mM NaCl and 55 mM KCl) or 55K/Mg-medium (5K/Mg-medium in which 127 mM NaCl and 5 mM KCl were replaced by 77 mM NaCl and 55 mM KCl). At the end of the stimulation, the dishes were placed on ice, the release medium was collected, and the cells and medium were analysed as described under immunoprecipitation.

Cell surface staining of E2/3–AT–E2/3

The same release and control media as described for the depolarization-induced release were used here. PC12 cells transfected with E2/3–AT–E2/3 were washed twice with 5K/Ca-medium, incubated for 7 min in control media (5K/Ca-medium or 55K/Mg-medium), or release medium (55K/Ca-medium), washed twice with ice-cold phosphate-buffered saline (PBS) supplemented to remove non-specifically-bound secretory proteins. Immunostaining was performed with an antiserum against AT (Sigma) diluted 1:1000 in PBS containing 0.2% gelatine for 30 min on ice. Cells were then fixed with 3% paraformaldehyde on ice for 5 min and subsequently at room temperature for 20 min. After quenching with 50 mM NH₄Cl the cells were stained with the secondary antibody coupled to rhodamin (Dianova) and analysed by confocal microscopy.

Immunogold electronmicroscopy

Ultrathin cryosections were prepared as described (Aaku-Saraste *et al.*, 1996). Antibodies against AT (Dr J.L.Brown) were diluted 1:60 and polyclonal antibodies against endogenous rat CgB were used undiluted.

Acknowledgements

We thank Dr Martin Spieß for providing the C-terminally modified cDNA of α_1 -antitrypsin, Dr Jerry L.Brown for providing the antiserum against α_1 -antitrypsin and Drs Frederico Calegari and Patrizia Rosa for antiserum against rat chromogranin B. We are grateful to Denis Corbeil and Jeremy Garwood for critical comments on the manuscript. M.G. was supported by the Graduiertenkolleg 'Molekulare und Zelluläre Neurobiologie'. H.-H.G. and W.B.H. are recipients of grants from the Deutsche Forschungsgemeinschaft (SFB 317/C7 and C2).

References

- Aaku-Saraste, E., Hellwig, A. and Huttner, W.B. (1996) Loss of occludin and functional tight junctions, but not ZO-1, during neural tube closure—remodeling of the neuroepithelium prior to neurogenesis. *Dev. Biol.*, **180**, 664–679.
- Arvan, P. and Castle, D. (1992) Protein sorting and secretion granule formation in regulated secretory cells. *Trends Cell Biol.*, **2**, 327–331.
- Arvan, P. and Castle, D. (1998) Sorting and storage during secretory granule biogenesis: looking forward and looking backward. *Biochem. J.*, **332**, 593–610.
- Baeuerle, P.A. and Huttner, W.B. (1987) Tyrosine sulfation is a trans-Golgi-specific protein modification. *J. Cell Biol.*, **105**, 2655–2664.
- Benedum, U.M. *et al.* (1987) The primary structure of human secretogranin I (chromogranin B): comparison with chromogranin A reveals homologous terminal domains and a large intervening variable region. *EMBO J.*, **6**, 1203–1211.
- Brechler, V., Chu, W.N., Baxter, J.D., Thibault, G. and Reudelhuber, T.L. (1996) A protease processing site is essential for prorenin sorting to the regulated secretory pathway. *J. Biol. Chem.*, **271**, 20636–20640.
- Burgess, T.L. and Kelly, R.B. (1987) Constitutive and regulated secretion of proteins. *Ann. Rev. Cell Biol.*, **3**, 243–293.
- Castle, A.M., Stahl, L.E. and Castle, J.D. (1992) A 13-amino acid N-terminal domain of a basic proline-rich protein is necessary for storage in secretory granules and facilitates exit from the endoplasmic reticulum. *J. Biol. Chem.*, **267**, 13093–13100.
- Chanat, E. and Huttner, W.B. (1991) Milieu-induced, selective aggregation of regulated secretory proteins in the *trans*-Golgi network. *J. Cell Biol.*, **115**, 1505–1519.
- Chanat, E., Weiß, U. and Huttner, W.B. (1994) The disulfide bond in chromogranin B, which is essential for its sorting to secretory granules, is not required for its aggregation in the *trans*-Golgi network. *FEBS Lett.*, **351**, 225–230.
- Chanat, E., Weiß, U., Huttner, W.B. and Tooze, S.A. (1993) Reduction of the disulfide bond of chromogranin B (secretogranin I) in the *trans*-Golgi network causes its missorting to the constitutive secretory pathway. *EMBO J.*, **12**, 2159–2168.
- Chevrier, D., Fournier, H., Nault, C., Zollinger, M., Crine, P. and Boileau, G. (1993) Targeting of pro-opiomelanocortin to the regulated secretory pathway may involve cooperation between different protein domains. *Mol. Cell. Endocrinol.*, **94**, 213–221.
- Cool, D.R. and Peng Loh, Y. (1994) Identification of a sorting signal for the regulated secretory pathway at the N-terminus of pro-opiomelanocortin. *Biochimie*, **76**, 265–270.
- 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.
- Cool, D.R., Normant, E., Shen, F.-S., Chen, H.-C., Pannell, L., Zhang, Y. and Loh, Y.P. (1997) Carboxypeptidase E is a regulated secretory pathway sorting receptor: Genetic obliteration leads to endocrine disorders in *Cpe^{fat}* mice. *Cell*, **88**, 73–83.
- Corti, A., Sanchez Perez, L., Gasparri, A., Curnis, F., Longhi, R., Brandazza, A., Siccardi, A.G. and Sidoli, A. (1997) Production and structure characterisation of recombinant chromogranin A N-terminal fragments (vasostatins). *Eur. J. Biochem.*, **248**, 692–699.
- Forss-Petter, S., Danielson, P., Battenberg, E., Bloom, F. and Sutcliffe, G.J. (1989) Nucleotide sequence and cellular distribution of rat chromogranin B (secretogranin I) mRNA in the neuroendocrine system. *J. Mol. Neurosci.*, **1**, 63–75.
- Gerdes, H.-H., Rosa, P., Phillips, E., Baeuerle, P.A., Frank, R., Argos, P. and Huttner, W.B. (1989) The primary structure of human secretogranin II, a widespread tyrosine-sulfated secretory granule protein that exhibits low pH- and calcium-induced aggregation. *J. Biol. Chem.*, **264**, 12009–12015.
- Gorr, S.U. and Darling, D.S. (1995) An N-terminal hydrophobic peak is the sorting signal of regulated secretory proteins. *FEBS Lett.*, **361**, 8–12.
- Huttner, W.B. *et al.* (1995) Biogenesis of neurosecretory vesicles. *Cold Spring Harbor Symp. Quant. Biol.*, **60**, 315–327.
- Kelly, R.B. (1985) Pathways of protein secretion in eukaryotes. *Science*, **230**, 25–32.
- Kroemer, A., Glombik, M.M., Huttner, W.B. and Gerdes, H.-H. (1998) Essential role of the disulfide-bonded loop of chromogranin B for sorting to secretory granules is revealed by expression of a deletion mutant in the absence of endogenous granin synthesis. *J. Cell Biol.*, **140**, 1331–1346.
- Leitinger, B., Brown, J.L. and Spiess, M. (1994) Tagging secretory and membrane proteins with a tyrosine sulfation site. *J. Biol. Chem.*, **269**, 8115–8121.
- Natori, S. *et al.* (1998) Chromogranin B (secretogranin I), a neuroendocrine-regulated secretory protein, is sorted to exocrine secretory granules in transgenic mice. *EMBO J.*, **17**, 3277–3289.
- Normant, E. and Loh, Y.P. (1998) Depletion of carboxypeptidase E, a regulated secretory pathway sorting receptor, causes misrouting and constitutive secretion of proinsulin and proenkephalin, but not chromogranin A. *Endocrinology*, **139**, 2137–2145.
- Pimplikar, S.W. and Huttner, W.B. (1990) Interaction of chromogranin B (secretogranin I) with secretory granule membranes in PC12 cells. *J. Cell. Biochem. Suppl.*, **14C**, H211.
- Pimplikar, S.W. and Huttner, W.B. (1992) Chromogranin B (secretogranin I), a secretory protein of the regulated pathway, is also present in a tightly membrane-associated form in PC12 cells. *J. Biol. Chem.*, **267**, 4110–4118.
- Pohl, T.M., Phillips, E., Song, K., Gerdes, H.-H., Huttner, W.B. and Rütter, U. (1990) The organisation of the mouse chromogranin B (secretogranin I) gene. *FEBS Lett.*, **262**, 219–224.
- Rosa, P., Hille, A., Lee, R.W.H., Zanini, A., De Camilli, P. and Huttner, W.B. (1985) Secretogranins I and II: two tyrosine-sulfated secretory proteins common to a variety of cells secreting peptides by the regulated pathway. *J. Cell Biol.*, **101**, 1999–2011.
- Scheiffele, P., Peränen, J. and Simons, K. (1995) N-glycans as apical sorting signals in epithelial cells. *Nature*, **378**, 96–98.
- Sevarino, K.A. and Stork, P. (1991) Multiple preprosomatostatin sorting signals mediate secretion via discrete cAMP- and tetradecanoylphorbolacetate-responsive pathways. *J. Biol. Chem.*, **266**, 18507–18513.

- Shaw,W.V. and Leslie,A.G.W. (1991) Chloramphenicol acetyltransferase. *Annu. Rev. Biophys Chem.*, **20**, 363–386.
- Stoller,T.J. and Shields,D. (1989) The propeptide of presomatostatin mediates intracellular transport and secretion of α -globin from mammalian cells. *J. Cell Biol.*, **108**, 1647–1655.
- Tam,W.W.H., Andreasson,K.I. and Peng Loh,Y. (1993) The amino-terminal sequence of pro-opiomelanocortin directs intracellular targeting to the regulated secretory pathway. *Eur. J. Cell Biol.*, **62**, 294–306.
- Thiele,C. and Huttner,W.B. (1998) The disulfide-bonded loop of chromogranins, which is essential for sorting to secretory granules, mediates homodimerization. *J. Biol. Chem.*, **273**, 1223–1231.
- Thiele,C., Gerdes,H.-H. and Huttner,W.B. (1997) Puzzling receptors. *Curr. Biol.*, **7**, R496–R500.
- Tong,P.Y., Gregory,W. and Kornfeld,S. (1989) Ligand interactions of the cation-independent mannose 6-phosphate receptor. *J. Biol. Chem.*, **264**, 7962–7969.
- Tooze,S.A. and Huttner,W.B. (1990) Cell-free protein sorting to the regulated and constitutive secretory pathways. *Cell*, **60**, 837–847.
- Tooze,J., Tooze,S.A. and Fuller,S.D. (1987) Sorting of progeny coronavirus from condensed secretory proteins at the exit from the *trans*-Golgi network of AtT20 cells. *J. Cell Biol.*, **105**, 1215–1226.
- Tooze,J., Kern,H.F., Fuller,S.D. and Howell,K.E. (1989) Condensation-sorting events in the rough endoplasmic reticulum of exocrine pancreatic cells. *J. Cell Biol.*, **109**, 35–50.
- Varlamov,O. and Fricker,L.D. (1996) The C-terminal region of carboxypeptidase E involved in membrane binding is distinct from the region involved with intracellular routing. *J. Biol. Chem.*, **271**, 6077–6083.

Received October 1, 1998; revised and accepted December 22, 1998