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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

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Abstract Chromogranin B (secretogranin I), a protein sorted to secretory granules in many endocrine cells and neurons, undergoes selective aggregation during the sorting process in the trans-Golgi network. Reduction of the single, highly conserved intramolecular disulfide bond of chromogranin B by exposure of intact PC12 cells to the thiol reducing agent dithiothreitol has previously been shown to cause its missorting to the constitutive pathway of secretion. Using saponin perforation of membrane vesicles in aggregative buffer mimicking the milieu in the lumen of the trans-Golgi network (pH 6.4, 10 mM calcium), we show here that treatment with dithiothreitol does not prevent the aggregation of chromogranin B in this compartment. This implies that the loop in the chromogranin B polypeptide that is formed by the disulfide bond has a critical role in the membrane recognition of aggregated chromogranin B during secretory granule formation.

Key words: Aggregation; Dithiothreitol; Granin; Proteolysis; Sorting; Trans-Golgi network

1. Introduction

Specialized eukaryotic cells such as endocrine cells and neurons possess two pathways of protein secretion, the constitutive and the regulated secretory pathway [1]. The biogenesis of the vesicles mediating regulated protein secretion, the secretory granules, begins with the formation of immature secretory granules from the trans-Golgi network (TGN) and entails the sorting of the regulated secretory proteins to these vesicles [2]. This sorting process consists of at least two steps. One is the segregation of regulated from constitutive secretory proteins in the lumen of the TGN, a process that is thought to occur via the selective aggregation of the regulated secretory proteins in the TGN [2]. This aggregation presumably reflects the intrinsic property of regulated secretory proteins to become insoluble in the specific conditions of the TGN lumen. In the case of secretogranin II (SgII), a member of the granin (chromogranin/secretogranin) family of secretory proteins which are sorted to secretory granules in a wide variety of endocrine cells and neurons [3,4], the selective aggregation is triggered by the high calcium/low pH milieu characteristic of the TGN [5]. The other step is the enveloping, in the TGN, of the aggregated regulated secretory proteins with the membrane components characteristic of secretory granules [6], a process which must entail a recognition event between the aggregate and the TGN membrane. Little is known about the structural features of regulated secretory proteins that are involved in this recognition.

We recently observed [7] that the single intramolecular disulfide bond in chromogranin B (CgB, previously also called secretogranin I), another member of the granin family [3,4], is of critical importance for its sorting to secretory granules. Opening this disulfide bond by exposing neuroendocrine cells to the thiol reducing agent dithiothreitol (DTT) results in the missorting of CgB to the constitutive secretory pathway [7]. The two cysteine residues forming the disulfide bond flank a highly conserved 20 amino acid-long sequence [8,9], and the disulfide bond presumably stabilizes the loop conformation of this sequence. Given that sorting to secretory granules consists of two steps, aggregation and membrane recognition, it is important to determine whether the missorting of CgB to constitutive secretory vesicles upon reduction of its disulfide bond is due to a lack of aggregation or membrane binding. Here we show that reduction of the disulfide bond of CgB does not block its ability to aggregate in the TGN.

2. Materials and methods

2.1. Cell culture, [^{35}S]sulfate labeling, and DTT treatment in vivo

PC12 cells, a neuroendocrine cell line, were grown and pulse-labeled for 5 min with [^{35}S]sulfate as previously described [7,10]. For [^{35}S]sulfate labeling in the presence of DTT, PC12 cells were preincubated for 2.5 min with 5 mM DTT (added directly to the medium from a 100 mM stock in H_2O) and then pulse-labeled for 5 min with fresh medium containing 5 mM DTT.

2.2. Analysis of CgB and SgII aggregates in the TGN, DTT treatment in vitro

Isolation of TGN membrane vesicles from [^{35}S]sulfate pulse-labeled PC12 cells and their perforation with saponin in either non-aggregative or aggregative milieu was performed essentially as described in detail previously [5]. Isolation of TGN membrane vesicles from PC12 cells treated in vivo with DTT was carried out with 5 mM DTT being present in all solutions. Fractions of the velocity gradient containing [^{35}S]sulfate-labeled TGN membrane vesicles were pooled, slowly diluted with an equal volume of 10 mM HEPES-KOH, pH 7.2, (in the absence or presence of 5 mM DTT) to decrease the sucrose concentration, divided into aliquots (4 each per control and DTT-treated cells), and the [^{35}S]sulfate-labeled TGN vesicles were concentrated by centrifugation at 130,000 × *g* for 45 min. The pellets were resuspended in 100 μl of buffer containing 1.0 mg/ml saponin and either 10 mM MES-NaOH, pH 7.4, 30 mM KCl, 1.2 mM leupeptin (non-aggregative milieu) or 10 mM MES-NaOH, pH 6.4, 10 mM $CaCl_2$, 1.2 mM leupeptin (aggrega-

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Abbreviations: DTT, dithiothreitol; CgB, chromogranin B; SgII, secretogranin II; TGN, trans-Golgi network.

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tive milieu), both in the absence or presence of 5 mM DTT (DTT treatment in vitro). After ≈10 min incubation on ice, samples were centrifuged at 130,000 × *g* for 30 min. Supernatants were adjusted to 10% TCA, and the entire pellets resuspended in 10% TCA. After precipitation, both were centrifuged, and the TCA pellets were washed with ethanol/ether, dissolved in Laemmli sample buffer and boiled. This protocol was chosen to minimize proteolysis after the saponin perforation, and also to allow the analysis of an aliquot of each sample by SDS-PAGE. Proteins in Laemmli sample buffer were precipitated with 80% acetone, re-dissolved in O'Farrell lysis buffer containing 5% NP-40, and analysed by 2D-PAGE followed by fluorography. The radioactivity present in [^{35}S]sulfate-labeled CgB and SgII contained in 2D-gel pieces was quantitated by liquid scintillation counting after pronase digestion.

2.3. NEM labeling

A post-nuclear supernatant prepared from PC12 cells incubated for 22.5 min in the absence or presence of 5 mM DTT was subjected to velocity sucrose gradient centrifugation. Fractions 5 and 6, which have been shown to contain mature secretory granules [11], were pooled, slowly diluted with an equal volume of 10 mM HEPES-KOH, pH 7.2, and centrifuged at 130,000 × *g* for 45 min. The pelleted secretory granules were lysed and the proteins, after reduction with DTT, subjected to labeling with [^{3}H]NEM as described previously [7], followed by 2D-PAGE and fluorography.

3. Results

3.1. Calcium-/low pH-induced aggregation of CgB

We first investigated whether CgB undergoes calcium-/low pH-induced aggregation in the TGN, as we described previously for SgII [5]. For this purpose, we pulse-labeled CgB in the TGN of PC12 cells with [^{35}S]sulfate, isolated a fraction enriched in TGN membrane vesicles, and perforated these vesicles with saponin either at pH 7.4 in the absence of added calcium (non-aggregative milieu), or at pH 6.4 in the presence of 10 mM calcium (aggregative milieu) which is thought to resemble the physiological TGN lumenal milieu [5]. Analysis by 2D-PAGE (used to resolve CgB from a constitutively secreted heparan sulfate proteoglycan [7]) showed that upon perforation in non-aggregative milieu the majority of the labeled CgB was released from the lumen of the TGN into the supernatant, whereas upon perforation in aggregative milieu most of the CgB was recovered in the pellet (Fig. 1), presumably because it was retained in aggregated form within the lumen of the perforated TGN vesicles [5]. This shows that CgB exhibits a similar calcium-/low pH-induced aggregation to SgII.

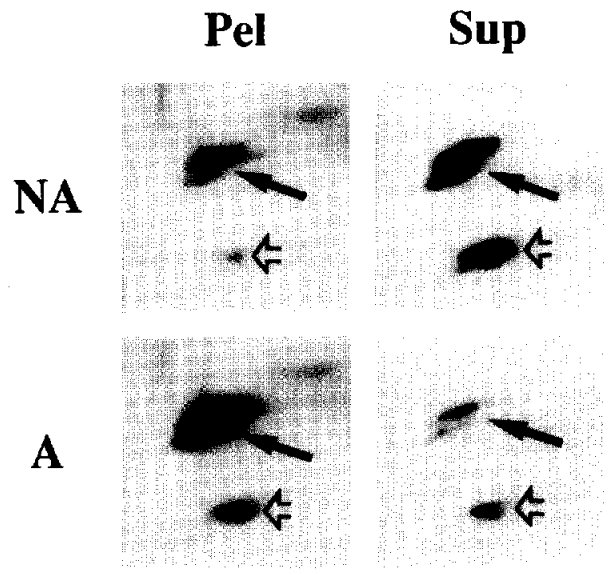

Fig. 1. CgB, like SgII, is recovered in an aggregated form after saponin perforation of TGN membrane vesicles in aggregative milieu. TGN vesicles obtained from [^{35}S]sulfate-labeled PC12 cells were perforated with saponin in non-aggregative (NA) or aggregative (A) milieu, centrifuged, and pellets (Pel) and supernatants (Sup) were analyzed by 2D-PAGE and fluorography. Arrows, CgB; open arrows, SgII.

3.2. DTT treatment does not affect the calcium-/low pH-induced aggregation of CgB in the TGN

These data provided the basis to investigate whether DTT treatment of PC12 cells, which results in the reduction of the intramolecular disulfide bond of CgB and in its missorting to constitutive secretory vesicles [7], affects the calcium-/low pH-induced aggregation of CgB in the TGN, which is thought to

Table 1
The calcium-/low pH-induced aggregation of CgB is unaffected by DTT

Treatment	Saponin perforation	CgB in pellet (% of total)	Total CgB (cpm)	SgII in pellet (% of total)	Total SgII (cpm)
No DTT	non-aggregative	39	2,931	21	1,107
	aggregative	85	2,792	62	888
DTT in vitro	non-aggregative	55	2,385	33	1,213
	aggregative	92	2,108	69	726
DTT in vivo	non-aggregative	21	1,679	21	1,099
	aggregative	80	750	65	603
DTT in vivo plus in vitro	non-aggregative	22	1,671	19	1,081
	aggregative	87	853	72	591

TGN vesicles obtained from [^{35}S]sulfate-labeled PC12 cells were perforated with saponin in a non-aggregative or aggregative milieu, centrifuged, and pellets and supernatants were analyzed by 2D-PAGE. DTT (5 mM) was either absent throughout (No DTT), absent during the labeling of the cells and the isolation of TGN vesicles but added upon saponin perforation (DTT in vitro), present during the labeling of the cells and the isolation of TGN vesicles but not added upon saponin perforation (DTT in vivo), or present during the labeling of the cells, isolation of TGN vesicles and saponin perforation (DTT in vivo plus in vitro). For each condition, the [^{35}S]sulfate-labeled CgB and SgII recovered in the pellet after either non-aggregative or aggregative perforation is expressed as percent of that recovered in the pellet plus supernatant (total).

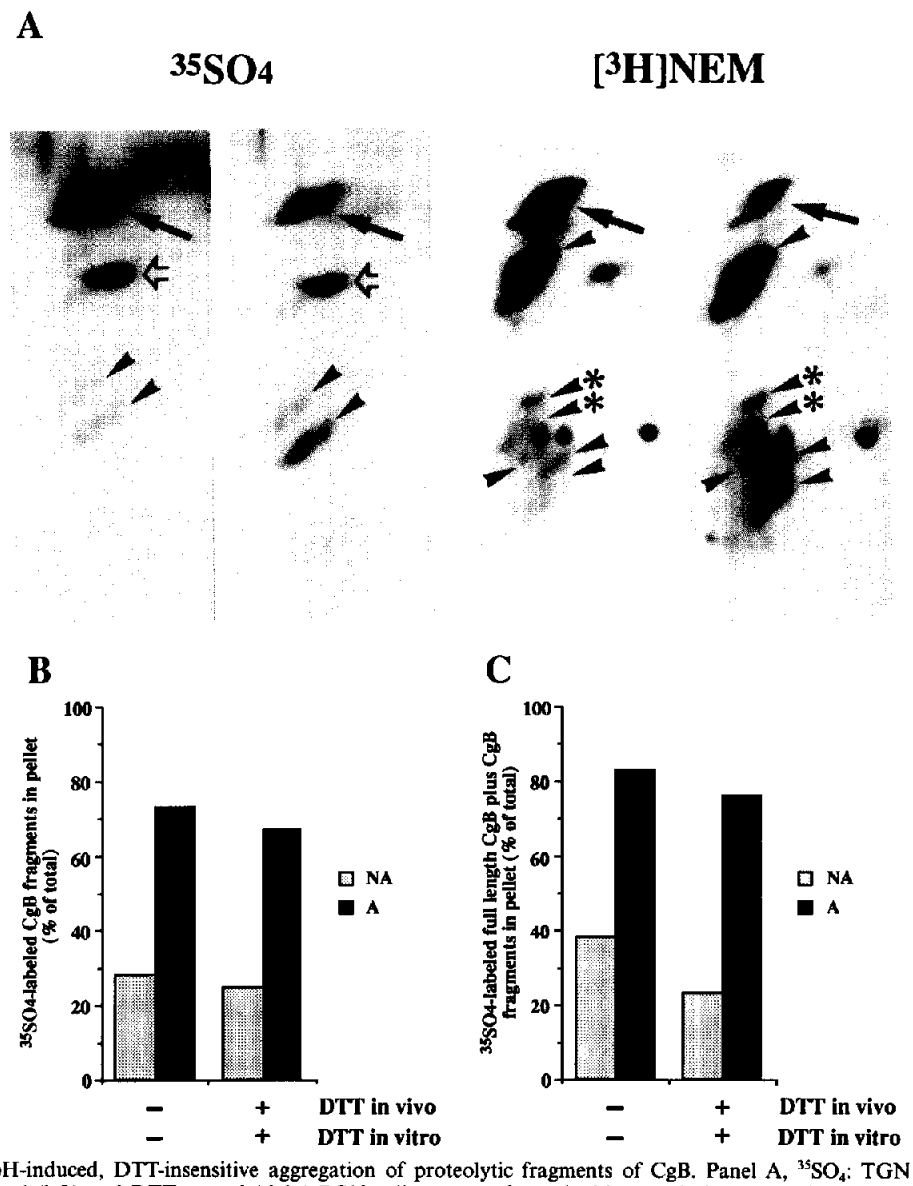

Fig. 2. Calcium-/low pH-induced, DTT-insensitive aggregation of proteolytic fragments of CgB. Panel A, $^{35}SO_4$: TGN vesicles obtained from [^{35}S]sulfate-labeled control (left) and DTT-treated (right) PC12 cells were perforated with saponin in aggregative milieu in the absence (left) and presence (right) of DTT, centrifuged, and pellets were analyzed by 2D-PAGE and fluorography. Arrows: full-length CgB; arrowheads: CgB fragments; open arrows: SgII. Panel A, [^{3}H]NEM: secretory granules obtained from control (left) and DTT-treated (right) PC12 cells were lysed, and the proteins were subjected to disulfide reduction with DTT, labeled with [^{3}H]NEM, and analyzed by 2D-PAGE and fluorography. Arrows: full-length CgB; arrowheads: CgB fragments; the [^{3}H]NEM-labeled fragments corresponding to the [^{35}S]sulfate-labeled fragments shown on the left are indicated by asterisks. Note the absence of labeled SgII. Panels B and C: The [^{35}S]sulfate-labeled full-length CgB and CgB fragments (see panel A) recovered in the pellet and supernatant after the indicated types of DTT treatment and non-aggregative (NA, grey columns) or aggregative (A, black columns) perforation were determined as described in the legend to Table 1. For each condition, either the [^{35}S]sulfate-labeled CgB fragments (B) or the [^{35}S]sulfate-labeled full-length CgB plus CgB fragments (C) recovered in the pellet are expressed as percent of that recovered in pellet plus supernatant (total).

be essential for its efficient sorting to immature secretory granules [2]. The above experimental protocol of saponin perforation was applied to PC12 cells that had been treated with either no DTT, 5 mM DTT during the [^{35}S]sulfate pulse only (DTT in vivo), 5 mM DTT during the saponin perforation of the TGN vesicles only (DTT in vitro), or 5 mM DTT during the [^{35}S]sulfate pulse, subcellular fractionation and saponin perforation of the TGN vesicles (DTT in vivo plus in vitro). As an internal control for any unspecific effects of DTT, we analyzed SgII which (i) lacks cysteine residues [12,13], (ii) has been shown to be sorted, in contrast to CgB, to immature secretory granules not only in the absence but also in the presence of DTT [7], and (iii) would thus be expected to be unaffected by DTT treatment in its calcium-/low pH-induced aggregation in the TGN. Indeed, we observed that for any type of DTT treatment, 60–70% of the labeled SgII was recovered in the pellet upon saponin perforation of the TGN vesicles in aggregative milieu (Table 1). Analysis of the same 2D-gels for [^{35}S]sulfate-labeled CgB revealed that also in the case of this protein, the calcium-/low pH-induced aggregation was unaffected by DTT treatment

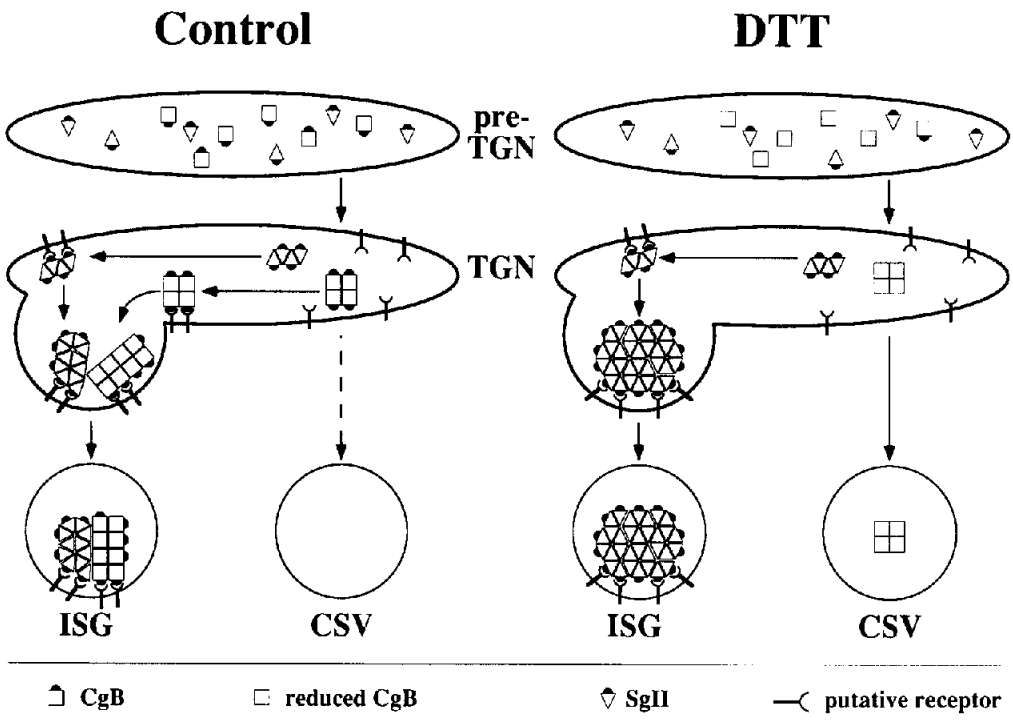

Fig. 3. Model illustrating the sorting of CgB and SgII to immature secretory granules. The filled half-circle on the square and triangular symbols of CgB and SgII, respectively, indicates a loop structure recognized by a putative membrane receptor. In the case of CgB, but not SgII, this loop structure is stabilized by the intramolecular disulfide bond. Aggregation intermediates resulting from homophilic interaction are indicated by tetramers of symbols. ISG, immature secretory granule; CSV, constitutive secretory vesicle.

since, irrespective of the type of DTT treatment used, ≥ 80% of the labeled CgB was recovered in the pellet upon saponin perforation in aggregative milieu (Table 1).

3.3. DTT treatment promotes the proteolytic processing of CgB

The data on CgB described so far concern the full-length, M_r 113,000–105,000 form of this protein found in PC12 cells. In quantitating CgB in the various conditions mentioned above, we noticed that the total amount of [^{35}S]sulfate-labeled full-length CgB recovered in pellet plus supernatant upon saponin perforation of the TGN vesicles in non-aggregative milieu was reduced upon in vivo treatment of PC12 cells with DTT (Table 1). Moreover, the recovery of both [^{35}S]sulfate-labeled CgB and SgII was less upon saponin perforation of the TGN vesicles in aggregative milieu as compared with non-aggregative milieu (Table 1). The aggregative milieu is characterized by parameters (calcium, low pH) known to activate proteolytic processing enzymes which are present in the TGN and which recognize dibasic cleavage sites [14]. Since both CgB and SgII contain multiple dibasic cleavage sites [8,13], it seemed likely that the lower recovery of the full-length forms of these proteins in aggregative milieu, and by analogy their lower recovery in the presence of DTT, reflected increased proteolytic processing in these conditions. As illustrated for CgB in Fig. 2A, this was indeed the case.

To identify the portion of the CgB molecule represented by the two major [^{35}S]sulfate-labeled proteolytic fragments (Fig. 2A), we compared them with those CgB fragments, isolated from secretory granules of PC12 cells, which include the N-terminal, cysteine-containing domain of the molecule [8] and which therefore can be labeled, after thiol reduction, by the alkylating agent *N*-ethyl maleimide (NEM) [7,8]. This comparison (Fig. 2A) indicated that the two major [^{35}S]sulfate-labeled proteolytic fragments of CgB observed in the TGN after DTT treatment contained cysteine residues and thus were derived from the N-terminal half of the molecule. This conclusion is consistent with the observation that these fragments, in contrast to full-length CgB, are not recognized by an anti-peptide antibody directed against the C-terminus of CgB (P. Rosa, personal communication).

3.4. Proteolytic N-terminal fragments of CgB also undergo calcium-/pH-induced, DTT-insensitive aggregation in the TGN

We quantitated these CgB fragments from the same 2D-gels that were used to obtain the data shown in Table 1 (Fig. 2B,C). As with full-length CgB, the majority of the [^{35}S]sulfate-labeled CgB fragments was recovered in the supernatant upon saponin perforation of the TGN vesicles in non-aggregative milieu, whereas the majority was found in the pellet upon perforation in aggregative milieu (Fig. 2B). The distribution of the fragments between pellet and supernatant in these two conditions of perforation was the same for control and DTT-treated cells. Since the processing of CgB to these fragments was already increased when the DTT treatment was confined to the period of [^{35}S]sulfate pulse labeling of the cells (data not shown), and since the saponin-perforated TGN vesicles were incubated for 10 min in the aggregative, i.e. protease activating, milieu before centrifugation, it seems likely that these fragments were generated, at least in part, before rather than after the centrifugation.

This in turn implies that these CgB fragments exhibit the same calcium-/low pH-induced aggregation in the TGN as full-length CgB.

4. Discussion

We have shown that the aggregation of CgB observed upon saponin perforation of the TGN in a high calcium-/low pH buffer (aggregative milieu) is unaffected by exposure of the protein to DTT. The same was found for proteolytic fragments of CgB corresponding to the N-terminal half of CgB. The latter observation is consistent with the domain organization of CgB which suggests that the structural features involved in aggregation (acidic amino acids, secondary structure) are distributed all along the large variable region which comprises ≈ 90% of the polypeptide and which is encoded by the large exon 4 [8,9].

Given that DTT treatment causes the reduction of the disulfide bond in CgB (see Fig. 9 in ref. 7), our previous [7] and present data can be interpreted in at least two ways. First, the reduced CgB is in principal capable of aggregation but, in contrast to the disulfide-bonded CgB in control PC12 cells, was not in an aggregated state in the TGN of DTT-treated cells; rather, it was induced to aggregate upon saponin perforation in aggregative milieu. (We have previously shown [5] that the aggregative milieu is sufficient to induce such an aggregation in the endoplasmic reticulum, in which CgB is not present in an aggregative state under physiological conditions.) This interpretation would imply that the missorting of CgB in DTT-treated cells is due to a DTT-induced alteration in the lumenal milieu of the TGN towards conditions which are less aggregation promoting than the control situation. We do not favor this interpretation because such a change in the lumenal milieu of the TGN should also lead to the missorting of SgII, which undergoes a similar high calcium-/low pH-induced aggregation [5]; this, however, was not the case [7]. Second, the reduced CgB in DTT-treated cells, like the disulfide-bonded CgB in control cells, was in an aggregated state in the TGN; upon saponin perforation in aggregative milieu, this aggregated state was conserved. This interpretation implies that the missorting of CgB in DTT-treated cells is not due to the lack of CgB aggregation but, rather, to the failure of CgB aggregates to become packaged into secretory granules, i.e. to be recognized by the portion of the TGN membrane that gives rise to the nascent immature secretory granule.

If the second interpretation is correct, the CgB missorted to constitutive secretory vesicles in the presence of DTT would be expected to be packaged into these vesicles as an aggregate. Why, then, is the buoyant density of these vesicles in equilibrium sucrose gradients less than that of immature secretory granules [7]? An answer to this question is provided by the model shown in Fig. 3, which is consistent with the data obtained in our previous [7] and present study. In this model, we make the following assumptions. First, the milieu-induced aggregation of CgB and SgII in the TGN is probably not a one-step process in which these proteins are converted, more or less instantaneously, from the soluble form to the final aggregate that is packaged into immature secretory granules, but rather a multi-step process involving aggregation intermediates. Second, the initial aggregation intermediates are likely to be the result of homophilic interactions, i.e. CgB aggregating with CgB and SgII aggregating with SgII. Third, the signal in CgB and SgII (referred to as S-M signal, [15]) which mediates their recognition by a putative membrane receptor in the TGN is a loop structure with a degenerate sequence containing hydroxylamino acids (filled half circles on the square and triangular symbols for CgB and SgII, respectively). In the case of CgB, this degenerate sequence lies between the two cysteine residues [8] and the loop conformation is stabilized by the disulfide bond [7]. Fourth, because of the degenerate nature of the S-M signal, single CgB and SgII molecules exhibit only a low affinity for the putative receptors in the TGN membrane, whereas the aggregation intermediates, being multivalent ligands [16], bind with high affinity to these receptors and induce their clustering. Concerning the latter two assumptions, it is interesting to note that in the case of pro-opiomelanocortin, like with CgB, structural information crucial for its sorting to secretory granules has been shown to be associated with the disulfide-bonded loop near the N-terminus of the molecule [17]. Yet, the sequences of the CgB and pro-opiomelanocortin loops show little homology, and hence recognition of both of these loops by a common membrane receptor could only occur if the recognition process tolerated a substantial degree of sequence degeneracy of the signal.

According to our model, both the disulfide bond-containing and the reduced CgB are induced, by the high calcium/low pH milieu of the TGN, to form the initial homophilic aggregation intermediates. This would explain why CgB aggregates are recovered in both the absence and presence of DTT upon saponin perforation of the TGN in aggregative milieu. However, only the aggregates of CgB containing the disulfide-bonded loop are capable of binding to the receptors in the TGN membrane and thereby becoming incorporated into the nascent immature secretory granules which also contain the SgII aggregates, as well as secretory granule membrane proteins, and which after pinching off from the TGN are characterized by a significantly higher buoyant density than the constitutive secretory vesicles [7,10]. In contrast, the reduced CgB has lost the appropriate loop structure and does not bind, despite its aggregated state, to the receptors in the TGN membrane. Because aggregation is thought to occur, at least initially, by homophilic interaction, the reduced CgB also does not co-aggregate with SgII. Thus, the aggregation intermediates containing reduced CgB leave the TGN by default, i.e. in constitutive secretory vesicles [7]. Perhaps it is the presence of these aggregation intermediates which explains the small increase in the mean buoyant density of these vesicles after DTT treatment (see Fig. 5F in [7]). In contrast to CgB, the S-M signal in SgII, which lacks cysteine residues [12], is not affected by DTT treatment, and hence SgII is efficiently sorted to immature secretory granules also in the presence of DTT [7].

A synthetic peptide corresponding to the CgB sequence between the two cysteine residues has recently been reported to bind to secretory granule membranes in a pH-dependent manner [18]. At first sight, this observation might seem to be consistent with our finding [7] that the disulfide-bonded loop is crucial for sorting. However, it is unclear, at least at present, whether the membrane binding of the synthetic CgB peptide [18] truely reflects the recognition of CgB by a membrane receptor in the TGN involved in sorting and immature secretory granule formation, because (i) binding was performed at pH 5.5 [18], i.e. at the pH of the lumen of secretory granules [19] rather than the TGN, (ii) secretory granule membranes rather than TGN

membranes were used, and (iii) the synthetic peptide lacked the two cysteines [18] and hence the disulfide bond critically required for sorting [7]. Whatever the significance of the membrane binding of the synthetic CgB peptide to secretory granule membranes may be, the previous [7] and present data showing that the disulfide-bonded loop is required for sorting but not aggregation should be helpful in searching for a membrane receptor in the TGN that directs CgB aggregates to immature secretory granules.

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