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Reduction of the disulfide bond of chromogranin B (secretogranin I) in the *trans*-Golgi network causes its missorting to the constitutive secretory pathway

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The role of the single, highly conserved disulfide bond in chromogranin B (secretogranin I) on the sorting of this regulated secretory protein to secretory granules was investigated in the neuroendocrine cell line PC12. Treatment of PC12 cells wtih dithiothreitol (DTT), a membrane permeable thiol reducing agent known to prevent disulfide bond formation in intact cells, resulted in the secretion of newly synthesized chromogranin B, but only slightly decreased the intracellular storage of newly synthesized secretogranin II, a regulated secretory protein devoid of cysteines. The secretion of newly synthesized chromogranin B in the presence of DTT occurred with similar kinetics to those of a heparan sulfate proteoglycan, a known marker of the constitutive secretory pathway in PC12 cells. Analysis of the various secretory vesicles derived from the *trans*-Golgi network (TGN) indicated that DTT treatment diverted newly synthesized chromogranin B to constitutive secretory vesicles, whereas the packaging of secretogranin II into immature secretory granules was unaffected by the reducing agent. The chromogranin B molecules diverted to constitutive secretory vesicles, in contrast to those stored in secretory granules, were found to contain free sulfhydryl residues. The effect of DTT on chromogranin B occurred in the TGN rather than in the endoplasmic reticulum. We conclude that the sorting of CgB in the TGN to secretory granules is dependent upon the integrity of its single disulfide bond.

Key words: chromogranin B/disulfide bond/secretion/*trans*-Golgi network

Introduction

The biogenesis of immature secretory granules (ISGs) and constitutive secretory vesicles (CSVs) from the *trans*-Golgi network (TGN) includes the sorting of regulated secretory proteins from constitutive secretory proteins (Burgess and Kelly, 1987). Morphological and biochemical data indicate that the selective aggregation of regulated secretory proteins in the TGN is a key step in this sorting process (Tooze *et al.*, 1993). Studies on chromogranin B (CgB; secretogranin I) and secretogranin II (SgII), two members of the granin (chromogranin/secretogranin) family (Rosa *et al.*, 1985b; Huttner *et al.*, 1991b), have shown that the selective aggregation of these regulated secretory proteins in the TGN is triggered by the lumenal milieu of this compartment, specifically an acidic pH and an elevated calcium concentration (Chanat and Huttner, 1991). This low pH/calcium-induced aggregation is thought to involve structural features common to CgB and SgII, in particular an abundance of acidic residues and a secondary structure alternating between helix and turns (Gerdes *et al.*, 1989).

Despite many common structural features (Huttner *et al.*, 1991b), CgB differs from SgII in that it contains two cysteines close to its amino-terminus (Benedum *et al.*, 1987) whereas SgII lacks cysteine (Gerdes *et al.*, 1989). These two cysteines form an intramolecular disulfide bond, resulting in the formation of a 20 amino acid loop in the polypeptide (Benedum *et al.*, 1987). Interestingly, this loop is highly conserved between CgB and chromogranin A (CgA) (Benedum *et al.*, 1986, 1987; Iacangelo *et al.*, 1986), another member of the granin family (Rosa *et al.*, 1985b; Huttner *et al.*, 1991b). For both CgA and CgB, this domain is encoded by a separate exon, exon 3 (Pohl *et al.*, 1990; Iacangelo *et al.*, 1991; Wu *et al.*, 1991), which is not present in the SgII gene (Schimmel *et al.*, 1992). Furthermore, this domain is highly conserved in CgA and CgB from various species (Simon and Aunis, 1989; Pohl *et al.*, 1990). This is in contrast to the sequence of most of the remainder of either polypeptide, which shows substantially less conservation across species. This high degree of conservation suggests that the disulfide-bonded loop is somehow important for CgA and CgB. We have been interested in the possibility that this loop might have a role in the sorting of these proteins to secretory granules.

Expression of mutated proteins has been a powerful approach to investigate the mechanism of protein sorting in eukaryotic cells. However, regulated secretory proteins are sorted to secretory granules as aggregates (Tooze *et al.*, 1993). Thus, if the disulfide-bonded loop is required for the sorting of CgB, mutation of this structure would not, however, result in missorting if the mutated transfected CgB is still able to co-aggregate with endogenous, normal CgB. It would therefore be desirable to be able to interfere with the disulfide bond in the entire population of CgB molecules that aggregate and are to be sorted.

Several recent studies have shown that it is possible to perturb, in living cells, the formation of disulfide bonds in proteins synthesized in the rough endoplasmic reticulum (ER) by the addition of thiol reducing agents (Alberini *et al.*, 1990; Braakman *et al.*, 1992a,b). In the present study, we have exploited this possibility to investigate a potential role of the disulfide-bonded loop in the sorting of CgB to secretory granules. We used PC12 cells, in which secretory protein sorting in the TGN has been well characterized (Tooze and Huttner, 1990). These cells express a heparan sulfate proteoglycan (hsPG), which has been shown to be a marker for the constitutive secretory pathway (Tooze and Huttner,

1990), and CgB and SgII (but undetectable amounts of CgA), which are very efficiently sorted to, and stored in, secretory granules (Lee and Huttner, 1983; Gerdes *et al.*, 1989; Tooze and Huttner, 1990). Using sulfation to selectively label these marker proteins in the TGN (Kimura *et al.*, 1984; Baeuerle and Huttner, 1987; Rosa *et al.*, 1992), the segregation of the two granins from the hsPG and the packaging of these proteins into ISGs and CSVs, respectively, can easily be monitored (Tooze and Huttner, 1990). Finally, and of particular relevance for the present study, the presence of SgII, which because of its lack of cysteines cannot be directly affected by thiol reducing agents, provides a convenient internal control for any indirect effects of the reducing agent on the regulated secretory pathway of these cells.

Results

DTT treatment of PC12 cells causes the selective release of newly synthesized CgB

To study the effect of thiol reducing agents on the secretion of the hsPG, a marker of the constitutive secretory pathway (Tooze and Huttner, 1990), and CgB and SgII, two markers of the regulated secretory pathway (Rosa *et al.*, 1985b; Huttner *et al.*, 1991a), these molecules were pulse-labelled in the TGN with [^{35}S]sulfate and chased for 60 min in the absence or presence of dithiothreitol (DTT). The medium and cells were analysed by two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) to separate the hsPG and CgB from each other. This revealed two effects of the DTT treatment. First, the amount of [^{35}S]sulfate-labelled molecules recovered at the end of the chase was markedly decreased by DTT (Figure 1, compare top and bottom panels; see Figure 2A for quantification of the hsPG). This reduction was largely due to an inhibition of [^{35}S]sulfate incorporation into these molecules since it was also observed at the end of the 5 min pulse (data not shown). The reduction of [^{35}S]sulfate incorporation differed in its sensitivity to increasing DTT concentrations, and in its extent, for the hsPG and CgB/SgII (data not shown), raising the possibility that it, at least in part, reflected a differential sensitivity to DTT of the various sulfotransferases which catalyse the sulfation of these molecules.

Secondly, and more importantly, DTT treatment exerted a differential effect on the intracellular storage of CgB and SgII. In contrast to the control condition, in which the vast majority (~90%) of both CgB and SgII were found intracellularly at the end of the 60 min chase (Figure 1, top left), the majority of CgB was found in the medium in the presence of DTT (Figure 1, bottom right, arrowhead). Interestingly, in the case of SgII, the majority of the protein

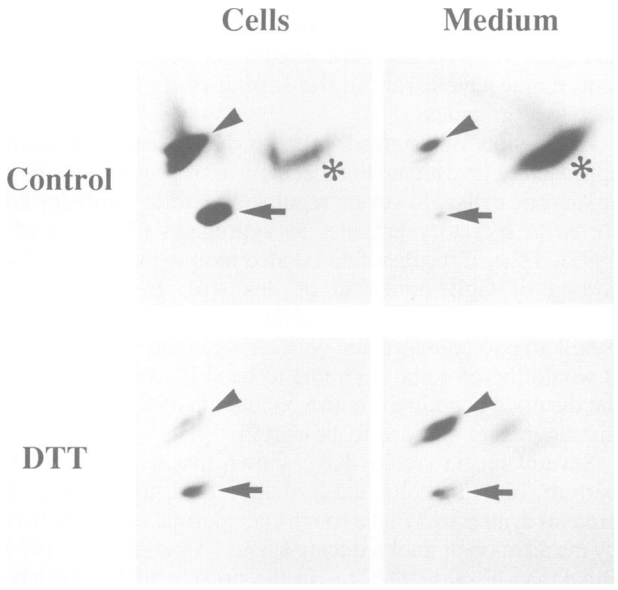

Fig. 1. The majority of newly synthesized CgB, but not SgII, is released upon DTT treatment of PC12 cells. PC12 cells were preincubated for 2.5 min, pulse-labelled for 5 min with [^{35}S]sulfate and chased for 60 min, all in either the absence (Control) or the presence (DTT) of 5 mM DTT. The medium and the heat-stable protein fraction of the cells were analysed by 2D-PAGE and fluorography. Arrowheads: CgB; arrows: SgII; asterisks, hsPG.

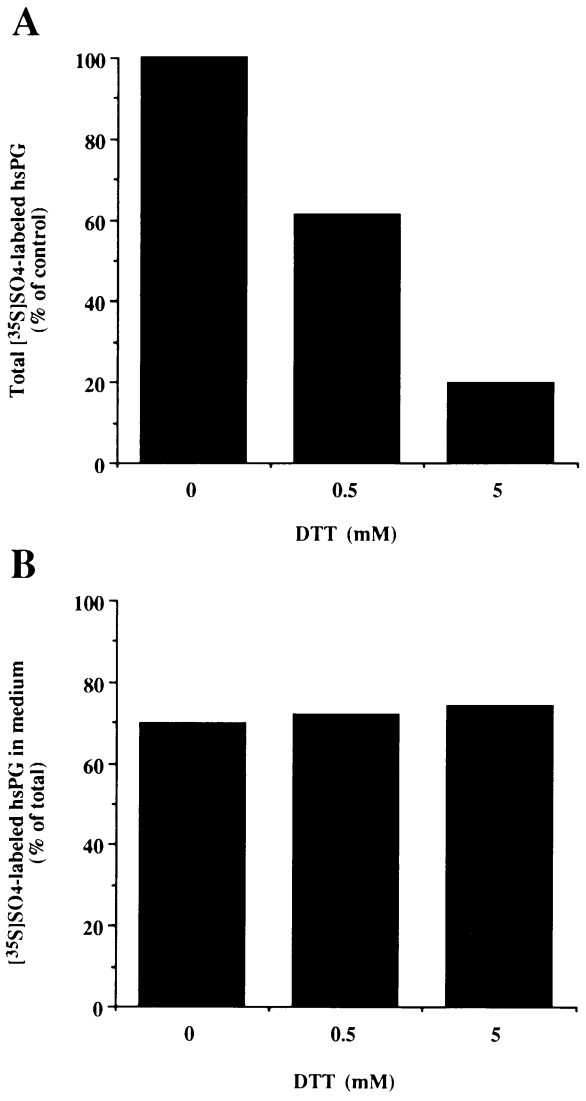

Fig. 2. DTT does not affect the constitutive release of the hsPG from PC12 cells. PC12 cells were preincubated for 2.5 min, pulse-labelled for 5 min with [^{35}S]sulfate and chased for 60 min, all in either the absence or the presence of the indicated concentrations of DTT. The medium and the heat-stable protein fraction of the cells were analysed by 2D-PAGE followed by fluorography, and the hsPG was quantified. (**A**) Total (sum of cells plus medium) [^{35}S]sulfate-labelled hsPG. The labelled hsPG found in the presence of DTT is expressed as percent of that found in the absence of DTT (control). (**B**) [^{35}S]sulfate-labelled hsPG in the medium. For each condition, the labelled hsPG in the medium is expressed as percent of total.

was still found intracellularly in the presence of DTT (Figure 1, bottom left, arrow), showing that the reducing agent preferentially affected CgB (for the quantification of this differential effect, see Figures 3, 4 and 7).

As to the constitutive secretory pathway, ~70% of the total [^{35}S]sulfate-labelled hsPG was found in soluble form in the medium after 60 min of chase in the absence of DTT (Figure 2B). The same proporton of [^{35}S]sulfate-labelled hsPG was found in the medium when the pulse-labelling and the chase had been performed in the presence of 0.5 mM or 5 mM DTT. Thus, treatment of PC12 cells with the reducing agent had no detectable effect on the constitutive secretory pathway.

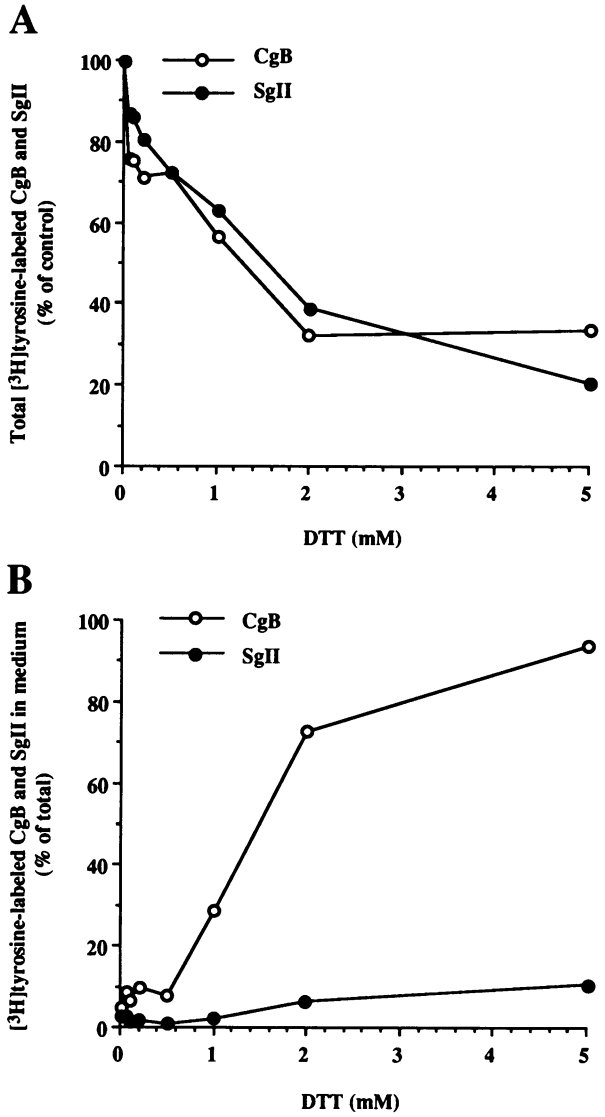

Fig. 3. Effect of different concentrations of DTT on the release of newly synthesized CgB from PC12 cells. PC12 cells were preincubated for 5 min, pulse-labelled for 5 min with [^{3}H]tyrosine and chased for 60 min, all in the absence or the presence of the indicated concentrations of DTT. The medium and the heat-stable protein fraction of the cells were analysed by SDS–PAGE followed by fluorography, and the [^{3}H]tyrosine-labelled CgB (open circles) and SgII (closed circles) were quantified. (**A**) Total (sum of cells plus medium) [^{3}H]tyrosine-labelled CgB and SgII. The total amount of labelled CgB and SgII found in the presence of DTT is expressed as percent of that found in the absence of DTT (control). (**B**) [^{3}H]tyrosine-labelled CgB and SgII in the medium. For each condition, the labelled CgB and SgII in the medium is expressed as percent of total.

Figure 3 shows the effects of various concentrations of DTT on the synthesis (panel A) and the secretion (panel B) of CgB and SgII. PC12 cells were preincubated for 5 min with DTT, pulse-labelled for 2 min with [^{3}H]tyrosine, and chased for 60 min. Quantification of the sum of the [^{3}H]tyrosine-labelled CgB and SgII recovered in the medium and the cells at the end of the chase (Figure 3A) revealed that the amount of both labelled proteins decreased similarly in the presenc of increasing concentrations of the reducing agent, with a maximum decrease by 60–70% at 2–5 mM DTT. This decrease appeared to be largely due to an effect of DTT on the synthesis of these proteins, because (i) a similar decrease was found for actin, a cytoplasmic protein, and (ii) an ~40% decrease was also observed when CgB was quantified immediately at the end of the [^{3}H]tyrosine pulse in the presence of 5 mM DTT (data not shown).

While the effect of DTT on the [^{3}H]tyrosine labelling of CgB and SgII was very similar (Figure 3A), its effect on the secretion of the newly synthesized proteins was highly selective for CgB (Figure 3B). Approximately 90% of the total [^{3}H]tyrosine-labelled CgB, but only ~10% of SgII, were found in the medium at the end of the 60 min chase in the presence of 5 mM DTT. The selective effect of the reducing agent on the secretion of newly synthesized CgB was already detectable at 1 mM DTT and was nearly maximal at 2 mM DTT.

DTT treatment results in the diverson of newly synthesized CgB to the constitutive pathway of secretion

In PC12 cells, both CgB and SgII are normally sorted with high efficiency to the regulated pathway of secretion (Gerdes *et al.*, 1989; Tooze and Huttner, 1990). The observation that DTT treatment resulted in the selective secretion of newly synthesized CgB could therefore have been due to one of the following reasons. First, CgB and SgII may have been packaged into separate secretory granules, with the

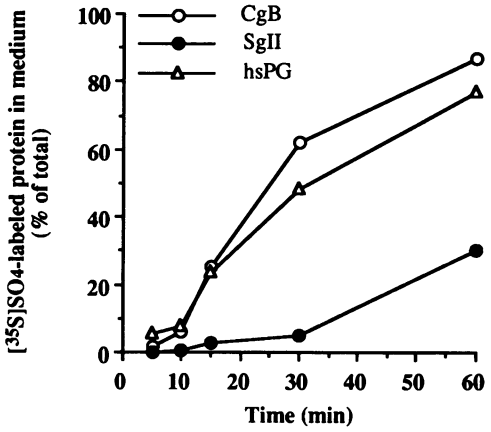

Fig. 4. The kinetics of the DTT-induced release of CgB are similar to those of the constitutively secreted hsPG. PC12 cells were preincubated for 5 min, pulse-labelled for 5 min with [^{35}S]sulfate and chased for the indicated times, all in the presence of 5 mM DTT. The cells and the medium collected at the end of the various chase periods were analysed by SDS–PAGE and fluorography, followed by quantification of CgB (open circles), SgII (closed circles) and the hsPG (open triangles) by densitometric scanning as described in Materials and methods. For each marker, the amount in the medium is expressed as percent of total (sum of cells plus medium).

CgB-containing granules having a shorter period of storage in the presence of DTT than the SgII-containing granules. Secondly, DTT treatment might have perturbed the sorting of CgB, but not that of SgII, by diverting the former to the constitutive secretory pathway. To investigate these possibilities, we studied the kinetics of release of CgB, and characterized the post-TGN vesicles containing CgB, in the presence of DTT.

Figure 4 shows the comparison of the kinetics of secretion of CgB, SgII and the hsPG in the presence of 5 mM DTT after pulse-labelling of these molecules in the TGN with [^{35}S]sulfate. The secretion of SgII was very low during the first 30 min of chase (~5%) and increased significantly only from 30 to 60 min of chase. In contrast, the secretion of both CgB and the hsPG increased rapidly after a 10 min lag period and resulted in the release of ~90% of the CgB and ~80% of the hsPG into the medium after 60 min of chase. Moreover, the secretion of CgB and the hsPG followed very similar kinetics. These observations suggested that DTT treatment caused the diversion of newly synthesized CgB to the constitutive secretory pathway.

To obtain further evidence for this, we investigated

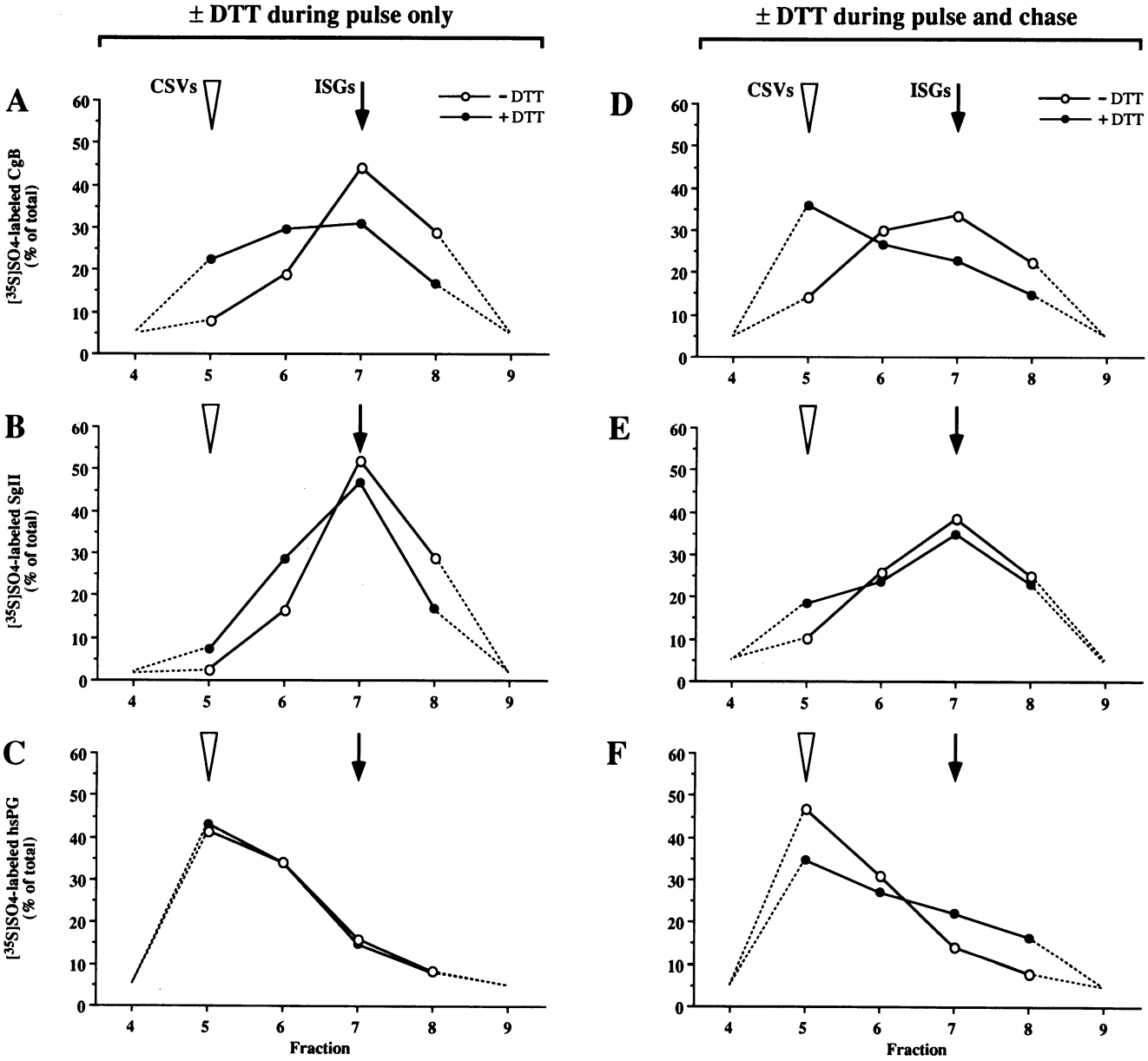

Fig. 5. DTT induces the missorting of CgB to CSVs. (**A–C**) PC12 cells were preincubated for 2.5 min and pulse-labelled for 5 min with [^{35}S]sulfate in the absence (−DTT, open circles) or presence (+DTT, closed circles) of 5 mM DTT. They were then chased for 15 min in the absence of DTT. (**D–F**) PC12 cells were preincubated for 2.5 min, pulse-labelled for 5 min with [^{35}S]sulfate and chased for 15 min, all in the absence (−DTT, open circles) or presence (+DTT, closed circles) of 5 mM DTT. (A–F) The PNS prepared from the four sets of cells was subjected to velocity sucrose gradient centrifugation, and the post-TGN vesicle-containing fractions were pooled and subjected to a second, equilibrium sucrose gradient centrifugation. An aliquot of fractions 5–8 from each second gradient was analysed by 2D-PAGE and fluorography. CgB, SgII and hsPG were quantified, and the [^{35}S]sulfate-labelled CgB (panels A and D), SgII (panels B and E) and hsPG (panels C and F) in each fraction is expressed as percent of the total recovered in fractions 5–8. SDS–PAGE and fluorography carried out in parallel with other aliquots of each fraction of the second gradients revealed that the amount of these proteins in fractions 4 and 9 was less than 5% of the total (dashed part of the curves); these fractions were therefore not analysed by 2D-PAGE. The open arrowheads and the arrows indicate the peak of the CSVs and ISGs, respectively.

whether in the presence of DTT, newly synthesized CgB was packaged into CSVs. CgB, SgII and the hsPG, pulse-labelled in the TGN with [^{35}S]sulfate, were chased for 15 min. During such a chase, these molecules are known to be packaged into ISGs and CSVs, which can be separated from the TGN and from each other by sequential velocity and equilibrium sucrose gradient centrifugation (Tooze and Huttner, 1990). Confirming previous observations (Tooze and Huttner, 1990), SgII and the hsPG showed a different distribution across the equilibrium gradient, with SgII peaking in fraction 7 indicative of the position of ISGs (Figure 5B, E, open circles), and the hsPG peaking in fraction 5 indicative of the position of CSVs (Figure 5C and F, open circles), which are known to exhibit a lower buoyant density in sucrose than ISGs (Tooze and Huttner, 1990; Tooze *et al.*, 1991; Régnier-Vigouroux *et al.*, 1991). This distribution of SgII and the hsPG was not significantly altered when 5 mM DTT was present during the [^{35}S]sulfate pulse only (Figure 5B and C, closed circles) or during the [^{35}S]sulfate pulse and the 15 min chase (Figure 5E and F, closed circles). CgB, in the absence of DTT, co-distributed with SgII (Figure 5A and D, open circles), consistent with it being packaged into ISGs. However, when DTT was present during the [^{35}S]sulfate pulse, the distribution of CgB across the equilibrium gradient differed from that observed in the absence of DTT by being shifted towards the position of CSVs (Figure 5A, closed circles). Moreover, when DTT was present not only during the [^{35}S]sulfate pulse but also during the chase, the distribution of CgB across the equilibrium gradient became indistinguishable from that of the hsPG, peaking in fraction 5 (Figure 5D, closed circles, compare with Figure 5F, closed circles). This indicated that the newly synthesized CgB that had been labelled in, and chased from, the TGN in the presence of DTT had been packaged into CSVs.

The effect of DTT that results in the missorting of CgB occurs in the TGN

As shown in Figures 1 and 5, a DTT treatment starting very shortly (2.5 min) before the pulse-labelling with [^{35}S]-sulfate, which is known to label proteins selectively in the TGN (Kimura *et al.*, 1984; Baeuerle and Huttner, 1987; Rosa *et al.*, 1992), was sufficient to cause the diversion of newly synthesized CgB to the constitutive pathway of secretion. In addition, removal of DTT at the end of the [^{35}S]sulfate pulse decreased the degree of missorting of CgB to CSVs as compared with that seen when the presence of DTT was continued into the chase (compare Figure 5A with Figure 5D). These observations suggested that the effect of DTT that resulted in the missorting of CgB occurred in the TGN. To investigate this possibility further, we first determined the kinetics of arrival of newly synthesized CgB in the TGN (Figure 6), and then used this information to vary the beginning of the DTT treatment with respect to the localization of the pulse-labelled CgB within the secretory pathway (Figures 7 and 8).

PC12 cells were pulse-labelled for 2 min with [^{3}H]tyrosine and chased for various times, in either the absence or presence of 5 mM DTT. The newly synthesized rough ER form of CgB in PC12 cells had an electrophoretic mobility corresponding to an M_r of 97 000 (Figure 6, left, arrowhead). After 4 min of chase, this form was converted to more slowly migrating forms with an M_r of 113 000–105 000 (Lee and Huttner, 1983; Rosa *et al.*, 1985b) (Figure 6, left, bracket). This reduction in electrophoretic mobility is due to post-translational modifications, notably sialylation, occurring in the *trans*-Golgi (Huttner *et al.*, 1991a; Rosa *et al.*, 1992). In line with this, the M_r 113 000–105 000 forms corresponded to mature CgB which, in control conditions, is stored in secretory granules. In control cells, the M_r 113 000 form was predominant (Figure 6, left) whereas in DTT-treated cells, the M_r 113 000 and 105 000 forms were present in approximately equal proportion (Figure 6, right). [The latter was the case for both the CgB molecules diverted to CSVs and those stored in secretory granules (data not shown), and thus the change in proportion between the M_r 113 000 and 105 000 forms upon DTT treatment is unlikely to be causally related to the missorting of CgB.] In control cells, conversion of newly synthesized CgB to the mature form, i.e. its arrival in the *trans*-Golgi, occurred with a $t_{1/2}$ of ~7 min and was virtually complete by 10 min of chase (Figure 6, middle, open circles). In DTT-treated cells,

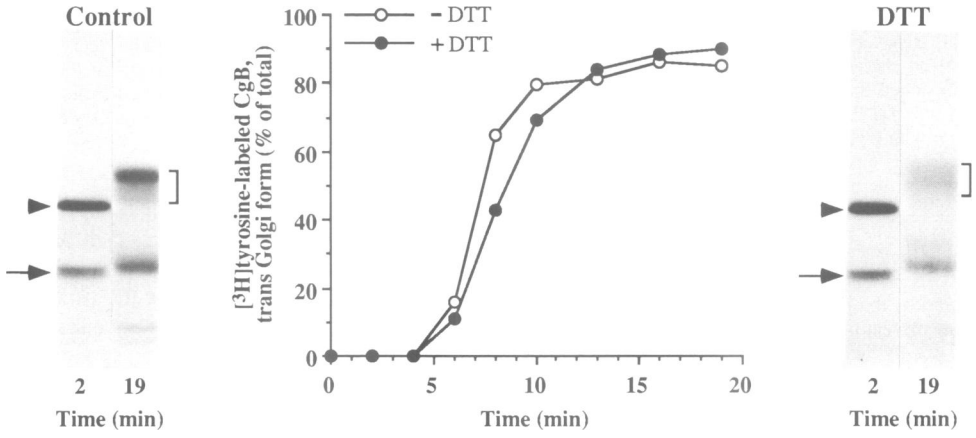

Fig. 6. Time course of arrival of newly synthesized CgB in the *trans*-Golgi. PC12 cells were preincubated for 1 min, pulse-labelled for 2 min with [^{3}H]tyrosine and chased for the indicated times, all in the absence (control, open circles) or presence (DTT, closed circles) of 5 mM DTT. At the end of the various chase periods, a heat-stable protein fraction was prepared from the cells and analysed by SDS–PAGE and fluorography (shown on the left and right for 2 and 19 min of chase), followed by quantification of the rough ER form (arrowheads) and the *trans*-Golgi forms (brackets) of CgB. The arrows indicate SgII. Middle panel: the *trans*-Golgi forms of CgB are expressed as percent of total (sum of rough ER and *trans*-Golgi forms of CgB).

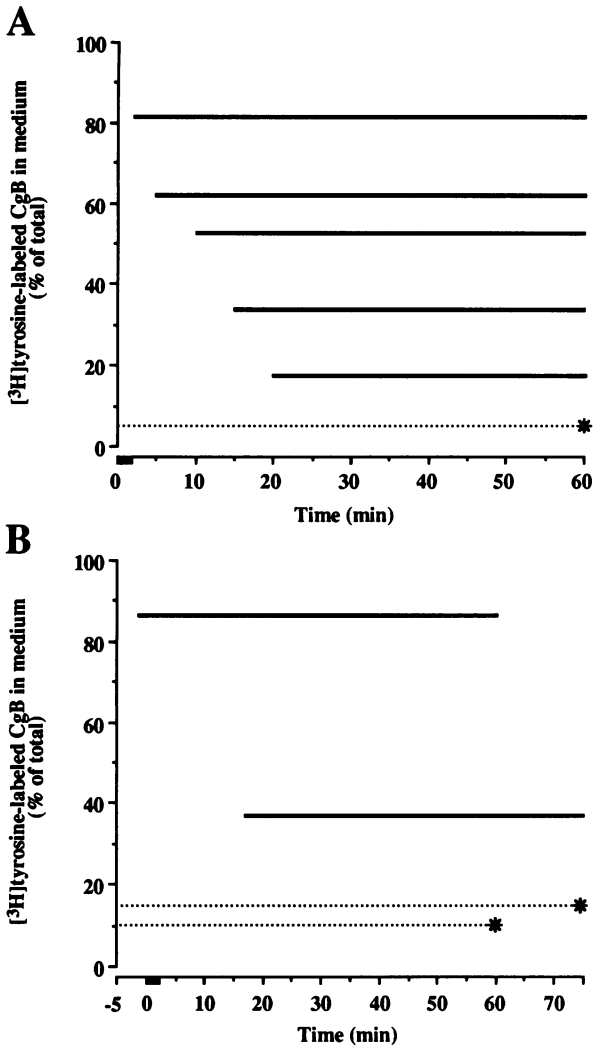

Fig. 7. The effect of DTT takes place before sorting of CgB into secretory granules. (**A**) PC12 cells were pulse-labelled for 2 min with [^{3}H]tyrosine (thick bar on abscissa). The control dish was then chased until 60 min in the absence of DTT (dotted line with asterisk). The other dishes received fresh medium containing 5 mM DTT either at the beginning of the chase (2 min) or at various time points thereafter (5, 10, 15 and 20 min), and were incubated until 60 min, as indicated by the length of the solid lines. The beginning of the solid lines corresponds to the time point of addition of DTT. (**B**) PC12 cells were preincubated for 1 min, pulse-labelled for 2 min with [^{3}H]tyrosine (thick bar on abscissa) and chased until 60 min, all either in the absence (control, lower dotted line with asterisk) or the presence (upper solid line) of 5 mM DTT. Alternatively, PC12 cells were pulse-labelled for 2 min with [^{3}H]tyrosine and chased until 75 min either without DTT (control, upper dotted line with asterisk), or with 5 mM DTT being added to the medium at 15 min followed by incubation until 75 min as indicated by the beginning and end of the lower solid line. (A and B) The medium collected at the end of the chase and the heat-stable protein fraction prepared from the cells were analysed by SDS−PAGE and fluorography, followed by quantification of CgB. For each condition, the [^{3}H]tyrosine-labelled CgB in the medium is expressed as percent of total (sum of cells plus medium). These values are indicated by the height of the dotted (control) and solid (DTT) lines with respect to the ordinate.

this conversion occurred with a $t_{1/2}$ of ~8 min and was virtually complete by 13 min of chase (Figure 6, middle, closed circles). Thus, DTT treatment of PC12 cells had little effect on the kinetics of transport of newly synthesized CgB to the *trans*-Golgi.

When PC12 cells were pulse-labelled for 2 min with

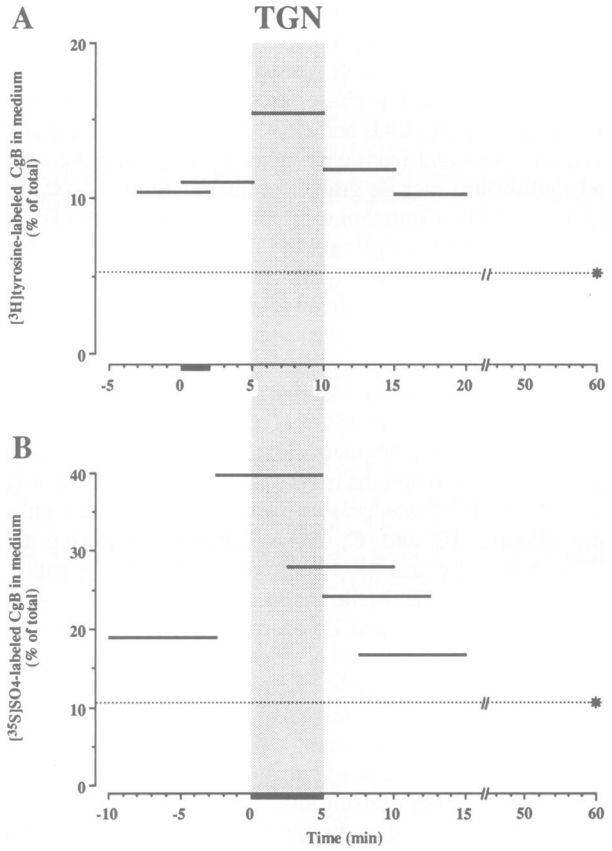

Fig. 8. The effect of DTT on the sorting of CgB takes place in the TGN. (**A**) PC12 cells were pulse-labelled for 2 min with [^{3}H]tyrosine (thick bar on abscissa) and chased until 60 min, either without DTT (control, dotted line with asterisk) or with 5 mM DTT being present for a 5 min period (−3 to 2 min; 0−5 min; 5−10 min; 10−15 min; 15−20 min) as indicated by the length of the solid lines. The beginning of the solid lines corresponds to the time point of addition of DTT, the end to the removal of DTT. (**B**) PC12 cells were pulse-labelled for 5 min with [^{35}S]sulfate (thick bar on abscissa) and chased until 60 min, either without DTT (control, dotted line with asterisk) or with 5 mM DTT being present for a 7.5 min period (−10 to −2.5 min; −2.5 to 5 min; 0−7.5 min; 2.5−10 min; 5−12.5 min; 7.5−15 min) as indicated by the length of the solid lines. The beginning of the solid lines corresponds to the time point of addition of DTT, the end to the removal of DTT. (A and B) The chase medium collected at 60 min and the heat-stable protein fraction prepared from the cells were analysed by SDS−PAGE ([^{3}H]tyrosine) or 2D-PAGE ([^{35}S]sulfate) followed by fluorography, and CgB was quantified. For each condition, the labelled CgB in the medium is expressed as percent of total (sum of cells plus medium). These values are indicated by the height of the dotted (control) and solid (DTT) lines with respect to the ordinate.

[^{3}H]tyrosine and chased until 60 min, the greatest amount of constitutively secreted CgB (>80%) was observed when 5 mM DTT was added at the beginning of the chase (Figure 7A, top solid line). The later the time point of addition of DTT, the lower was the constitutive secretion of CgB (Figure 7A). The constitutive secretion of CgB was almost back to control levels when DTT was added at 20 min (Figure 7A, bottom solid line), i.e. at a time at which newly synthesized CgB had presumably passed through the TGN (compare with Figure 6).

The decrease in constitutive secretion of CgB seen at later time points of DTT addition was not due to the shorter DTT treatments. When DTT was added at 15 min and the chase continued until 75 min, i.e. for 60 min in the presence of

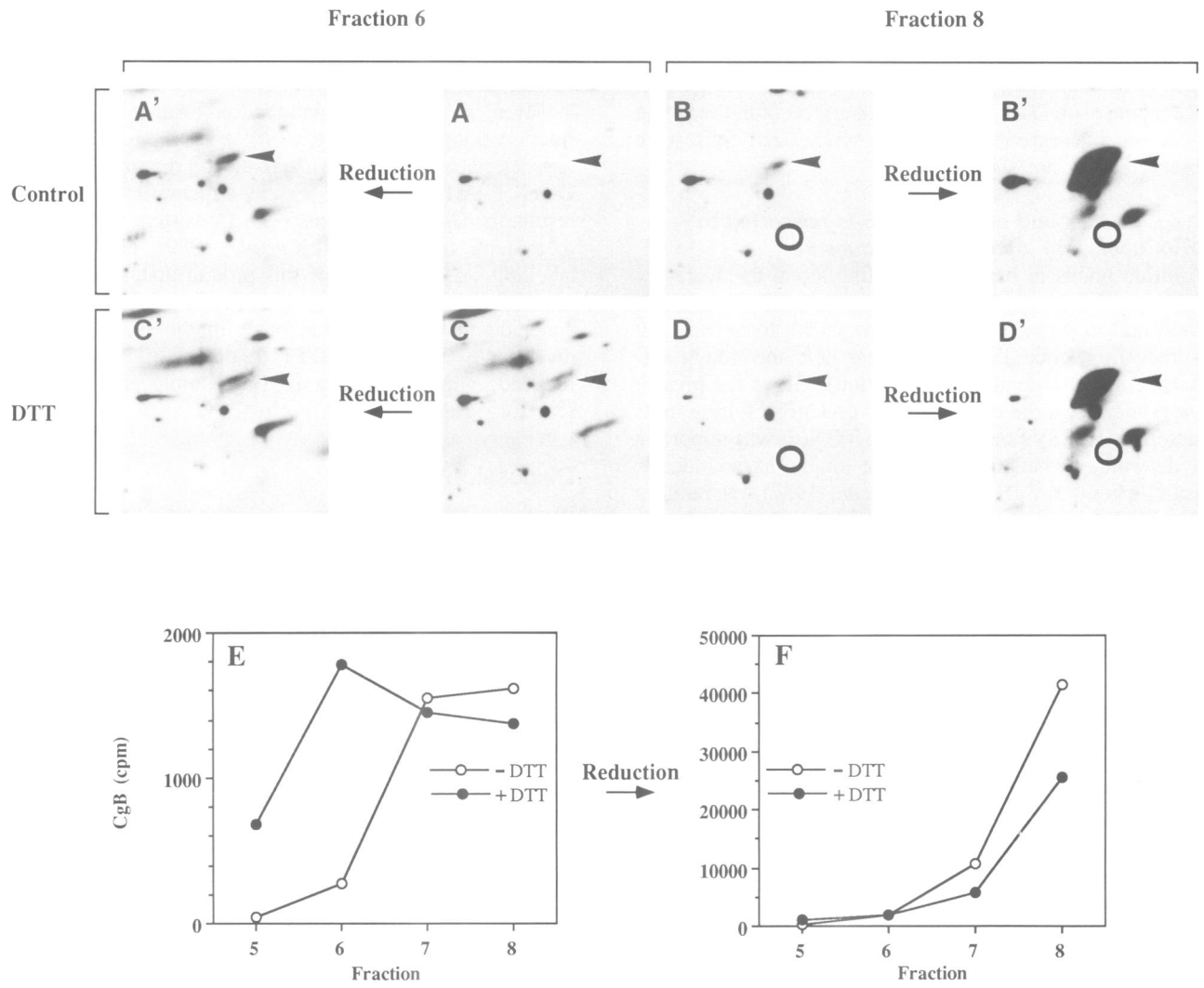

Fig. 9. The disulfide bond in the CgB molecules which have been diverted to CSVs upon DTT treatment of PC12 cells is reduced. PC12 cells were incubated for 22.5 min in the absence (Control) or presence (DTT) of 5 mM DTT. The PNS prepared from the two sets of cells was subjected to velocity sucrose gradient centrifugation, and the post-TGN vesicle-containing fractions were pooled and subjected to a second, equilibrium sucrose gradient centrifugation. Proteins contained in fractions 5–8 were either labelled with [^{3}H]NEM before *in vitro* reduction, or subjected to *in vitro* reduction with 5 mM DTT followed by [^{3}H]NEM labelling. Samples were analysed by 2D-PAGE followed by fluorography. (**A–D** and **A′–D′**) fluorograms of [^{3}H]NEM-labelled proteins in fraction 6 (CSVs) and fraction 8 (mature secretory granules) before (A–D) and after (A′–D′) *in vitro* reduction. Only the area of the gel surrounding CgB is shown. Arrowheads: CgB; circles in B, B′, D and D′: location of SgII as revealed by Coomassie Blue staining. (**E** and **F**) Quantification of the [^{3}H]NEM-labelled CgB in fractions 5–8 from control (open circles) and DTT-treated (closed circles) cells, before (E) and after (F) *in vitro* reduction. Note the difference in the scales of the ordinates.

DTT, the amount of constitutively secreted CgB was much less (~35%, Figure 7B, lower solid line) than the >80% observed when the 60 min DTT treatment started 1 min before the pulse (Figure 7B, upper solid line) or with the beginning of the chase (Figure 7A, top solid line). Furthermore, it was similar to that observed when DTT was added at 15 min and the chase stopped at 60 min (Figure 7A, second solid line from bottom). Under the conditions of DTT treatment shown in Figure 7, only a small proportion of the newly synthesized SgII (at most ~20%) was found in the medium (data not shown), which is consistent with the results shown in Figure 3. It is interesting to note, however, that this small proportion of SgII behaved similarly to CgB in the various conditions of DTT treatment.

To obtain further evidence that the effect of DTT on CgB which resulted in its missorting occurred in the TGN, we performed pulse–chase experiments, using either [^{3}H]-tyrosine (2 min pulse) or [^{35}S]sulfate (5 min pulse), in which the cells were exposed to 5 mM DTT only for short (5 min and 7.5 min, respectively) periods. With both types of labelling, we observed that the peak of sensitivity of newly synthesized CgB to DTT resulting in missorting occurred during a short window of time. This showed that the effect of DTT not only was rapidly reversible but also took place at a specific site in the intracellular transport of the newly synthesized CgB. In the case of the pulse–chase using [^{3}H]tyrosine, the peak of sensitivity to DTT occurred between 5 and 10 min (i.e. between 3 and 8 min of chase, Figure 8A). Since DTT was found to inhibit [^{3}H]tyrosine incorporation into CgB after 3 min, but not 1 min, of preincubation (data not shown), it is reasonable to assume a lag period of ~2 min for DTT to begin acting in the secretory pathway. Thus, the time window of the peak of sensitivity of [^{3}H]tyrosine-labelled CgB to DTT correlated well with the arrival of the bulk of newly synthesized CgB in the TGN (Figure 6). In the case of the pulse–chase using

[^{35}S]sulfate, the peak of sensitivity occurred during the pulse-labelling (Figure 8B), i.e. when the newly synthesized CgB was in the TGN. In these experiments using short incubations with DTT, no significant effects of the reducing agent on the secretion of newly synthesized SgII were observed (data not shown).

The disulfide bond of CgB which is missorted to CSVs upon DTT treatment is reduced

Disulfide bonds in proteins travelling along the secretory pathway are typically formed in the rough ER (Freedman, 1989) and in some cases have been shown to be resistant to reduction once the protein has left the rough ER (A.Helenius, personal communication). Given the present observations that the effect of DTT on CgB resulting in its missorting to CSVs occurred in the TGN, it was important to determine whether or not the single intramolecular disulfide bond in CgB (Benedum *et al.*, 1987) was reduced upon DTT treatment of PC12 cells. Unlabelled PC12 cells were incubated in the absence or presence of 5 mM DTT for 22.5 min, a time period corresponding to the sum of the 2.5 min preincubation, 5 min pulse and 15 min chase used in the experiment shown in Figure 5 in which newly synthesized CgB had been diverted to CSVs (Figure 5D). The unlabelled cells were then homogenized and the postnuclear supernatant (PNS) subjected, in the absence of DTT, to sequential velocity and equilibrium sucrose gradient centrifugation to obtain fractions enriched in CSVs (fraction 6) and secretory granules (fraction 8). [Note that because a larger sample volume was loaded onto the equilibrium gradient, fraction 6 rather than fraction 5 contained the peak of CSVs (see Materials and methods and legend to Figure 9).] An aliquot of the proteins of fractions 6 and 8 was then labelled with the alkylating agent *N*-ethyl maleimide (NEM) to detect the proteins that contained free sulfhydryl residues either in the control condition or upon *in vivo* treatment of the PC12 cells with DTT (Figure 9, top, A–D). To measure the total thiol-containing proteins, irrespective of the redox state of the cysteines, another aliquot of the proteins of fractions 6 and 8 was first reduced with DTT *in vitro* and then labelled with [^{3}H]NEM (Figure 9, top, A′–D′).

The CSV-containing fraction 6 from control cells did not contain any detectable amount of [^{3}H]NEM-labelled CgB (Figure 9A, arrowhead). Upon reduction with DTT *in vitro*, a small amount of [^{3}H]NEM-labelled CgB, constituted predominantly by the M_r 113 000 form of CgB (Lee and Huttner, 1983; Rosa *et al.*, 1985b, 1992), was observed (Figure 9A′). Thus, fraction 6 from control cells contained a small amount of non-reduced CgB. This CgB was most likely not present in CSVs but, given the abundance of granular CgB (see below), represented the small amount of secretory granules contaminating fraction 6. In contrast to fraction 6 from control cells, fraction 6 from DTT-treated cells contained, without *in vitro* reduction, a significant amount of [^{3}H]NEM-labelled CgB (Figure 9C). Upon *in vitro* reduction with DTT, only a small increase in the amount of [^{3}H]NEM-labelled CgB in this fraction was observed (Figure 9C′), which could be accounted for by the CgB in secretory granules contaminating this fraction. Analysis of the secretory granule-containing fractions 8 from both control and DTT-treated cells after *in vitro* reduction revealed the presence of a large amount of [^{3}H]NEM-labelled CgB (Figure 9B′ and D′). This reflected the abundance of CgB in secretory granules, which was also observed by Coomassie Blue staining (not shown). For both control and DTT-treated cells, virtually all of this CgB, however, was obtained in non-reduced form after subcellular fractionation since only very little [^{3}H]NEM-labelled CgB was detectable without *in vitro* reduction (Figure 9B and D). Panels E and F of Figure 9 show a quantification of these results for CgB in fractions 5–8. Consistent with the lack of cysteine in SgII (Gerdes *et al.*, 1989), no [^{3}H]NEM-labelling was observed for this protein under any experimental condition (Figure 9, circles in B, B′, D and D′). We conclude that the disulfide bond in the CgB molecules diverted to CSVs upon DTT treatment of PC12 cells is reduced, whereas that in the CgB molecules stored in secretory granules is not.

Discussion

We have shown that DTT treatment of PC12 cells results in the missorting of CgB to the constitutive pathway of secretion. In contrast, DTT treatment had virtually no effect on the sorting of SgII, which like CgB is a regulated secretory protein but unlike CgB lacks cysteines. These results suggest that the effect of DTT on the sorting of CgB reflects a direct action on the CgB molecule rather than an indirect effect on the sorting machinery. Consistent with DTT exerting an effect on the CgB molecule, the single intramolecular disulfide bond in those CgB molecules which had been diverted to CSVs was found to be reduced. We conclude that the missorting of CgB is due to the reduction of the disulfide bond, as will be further discussed below.

For DTT-induced missorting to occur, DTT had to be present at the time when the pulse-labelled CgB was passing through the TGN. No significant missorting of CgB was observed when DTT was removed before the arrival of CgB in the TGN, or when DTT was added after the pulse-labelled CgB had left the TGN. These observations have several implications. First, since DTT has been shown to reduce disulfide bonds in the rough ER, the lack of missorting upon DTT removal prior to the arrival of CgB in the TGN implies that the effect of DTT on CgB was rapidly reversible. This in turn implies that the disulfide bond in CgB can form after its exit from the rough ER and just before sorting, e.g. in the *trans*-Golgi. Second, since in the absence of DTT the disulfide bond in CgB is likely to form in the rough ER, the ability of DTT to induce missorting when added concomitantly with the arrival of CgB in the TGN implies that the disulfide bond can be reduced in the TGN. We do not know whether the disulfide bond formation in the presumably thiol oxidizing milieu of the TGN, or its disruption in this compartment upon DTT addition, occur spontaneously or are catalysed by an enzyme with protein disulfide isomerase activity. Such enzymes are present not only in the ER but may well be present in the *trans*-Golgi since it has been found that certain KDEL-containing proteins can be stoichiometrically galactosylated and thus must transiently be present in the *trans*-Golgi (Peter *et al.*, 1992).

The rapid reversibility of the effect of DTT on CgB may explain, at least in part, why the extent of missorting of [^{35}S]sulfate-labelled CgB was greater when the reducing agent was present during both pulse and chase (~90% missorting) than when it was present during the [^{35}S]sulfate pulse only (~40% missorting). Since the exit of [^{35}S]-

sulfate-labelled proteins from the TGN occurs with a $t_{1/2}$ of ~5 min (Tooze and Huttner, 1990), the disulfide bond may have re-formed in some CgB molecules, upon DTT removal, before their exit from the TGN.

In contrast to the CgB molecules present in CSVs from DTT-treated cells, the CgB molecules stored in secretory granules from DTT-treated cells were not recovered with free sulfhydryl residues after subcellular fractionation. One possible explanation for the latter finding is that DTT treatment did not result in the reduction of the disulfide bond in those CgB molecules that had been packaged into secretory granules prior to exposure of the cells to the reducing agent. In this context, it is worth noting that certain disulfide bonds in the influenza virus haemagglutinin are resistant to disruption by DTT once the polypeptide has undergone oligomerization (Tatu *et al.*, 1993). Alternatively, it is possible that DTT treatment did reduce the disulfide bond of CgB in secretory granules, but that the disulfide bond re-formed during subcellular fractionation which was performed in the absence of additional DTT. Whatever the explanation, both imply that the conformation of CgB in secretory granules must favor either the stability or the re-formation of the disulfide bond in CgB, compared to the CgB that had been diverted to CSVs.

Two principal possibilities can be envisaged as to the mechanism by which the DTT-induced reduction of the disulfide bond of CgB in the TGN results in missorting. First, the disulfide bond may be required for CgB to adopt an aggregation-competent structure. However, in preliminary experiments in which TGN vesicles prepared from DTT-treated cells were perforated by saponin in aggregative buffer (Chanat and Huttner, 1991) containing DTT, we observed quantitative aggregation of CgB, as in the control condition (data not shown). This is consistent with the notion that the same structural features of CgB and SgII (acidic amino acids, secondary structure) are likely to be involved in the low pH-/calcium-induced aggregation (Gerdes *et al.*, 1989). These are encoded by exon 4 of the CgB gene, which corresponds to ~90% of the CgB polypeptide (Pohl *et al.*, 1990), and by exon 2 of the SgII gene, which corresponds to 100% of the SgII polypeptide (Schimmel *et al.*, 1992). A second principal possibility is that the disulfide bond of CgB may be required for the interaction of CgB with components of the TGN membrane during the sorting process. This interaction has been proposed to involve a membrane-associated form of CgB which exists not only in secretory granules (Pimplikar and Huttner, 1992) but also in the TGN (E.Chanat and W.B.Huttner, unpublished observations). It will be important to investigate whether DTT treatment affects the interaction of soluble CgB with membrane-associated CgB.

Besides the effect of DTT on the sorting of CgB, we observed few effects of the reducing agent on the intracellular transport of the marker proteins used in the present study. First, although we observed a rapid loss of some (~30%) of the newly synthesized CgB which was presumably due to degradation in the ER, the rate of transport of the remainder of the newly synthesized CgB from the rough ER to the TGN was almost as fast in the presence of DTT as in its absence. Clearly, DTT did not prevent exit of this protein from the rough ER, as has previously been observed for membrane proteins which contain multiple disulfide bonds and which undergo oligomerization in the rough ER (Braakman *et al.*, 1992a). Secondly, the constitutive secretion of the hsPG was not affected by DTT and occurred with similar kinetics in the presence of DTT to those observed previously in the absence of the reducing agent (Tooze and Huttner, 1990). Finally, although the storage of SgII in secretory granules was slightly decreased in the presence of DTT, the sorting of SgII to ISGs in itself was not significantly affected by the reducing agent.

The fact that SgII, which lacks cysteines, is sorted to secretory granules implies that disulfide bonds are not an obligatory requirement for the sorting of every regulated secretory protein. However, the question arises whether disulfide bonds have a general role in the sorting of those regulated secretory proteins which contain this structural motif. For two other regulated secretory proteins, proopiomelanocortin (POMC) and von Willebrand factor (vWF), the role of disulfide bonds in the sorting to secretory granules has been investigated using site-directed mutagenesis. In the case of POMC, which contains two intramolecular disulfide bonds, the interference with both disulfide bonds inhibited sorting whereas the interference with one disulfide bond, or the deletion of the portion containing the four cysteines, did not (Roy *et al.*, 1991). In the case of vWF, which multimerizes via the formation of intermolecular disulfide bonds (Wagner, 1990), a point mutation which prevented this multimerization did not inhibit sorting (Mayadas and Wagner, 1992). However, in both cases, the cells which expressed the mutated proteins also produced, aggregated and packaged into secretory granules, endogenous regulated secretory proteins whose disulfide bonds were not manipulated. These studies therefore do not strictly exclude a role for the disulfide bonds in the sorting of these proteins if the transfected, mutated proteins co-aggregated with the endogenous, normal regulated secretory proteins. Perhaps DTT treatment *in vivo* would be an alternative approach to investigate this issue. On a more general note, DTT treatment *in vivo* may be a valuable tool to study the post-ER traffic of proteins to their various intracellular destinations.

Materials and methods

Cell culture and metabolic labelling

PC12 cells were grown in DMEM supplemented with 10% horse serum and 5% fetal calf serum as previously described (Tooze and Huttner, 1990). To label CgB and SgII in the rough ER, PC12 cells (3.5 cm dishes) were preincubated for 30 min in tyrosine-free DMEM (DMEM without tyrosine and with 10% of the normal concentration of phenylalanine) and then pulse-labelled for either 2 or 5 min with fresh tyrosine-free DMEM containing 50 μCi/ml L-[2,3,5,6-^{3}H]tyrosine (Amersham). To label CgB and SgII in the TGN, PC12 cells (3.5 cm dishes or 15 cm dishes when subcellular fractionation was performed) were preincubated for 30 min with sulfate-free DMEM (DMEM containing $MgCl_2$ instead of $MgSO_4$ and 10% of the normal concentration of methionine and cysteine) and then pulse-labelled for 5 min with fresh sulfate-free DMEM containing 1 mCi/ml carrier-free [^{35}S]sulfate (Amersham). To chase the sulfate or the tyrosine label, PC12 cells were incubated in regular DMEM.

DTT treatment of PC12 cells and analysis of secretion

DTT treatment. DTT was added to the medium from a 20-fold concentrated stock in H_2O. The standard final DTT concentration used in most experiments was 5 mM. In some experiments, lower final DTT concentrations were used as indicated in the figures. Controls received an equivalent amount of H_2O. The time point of DTT addition with respect to preincubation, pulse-labelling and chase, and the length of incubation varied between experiments and are indicated in the figure legends. The mode of addition of DTT was as follows. At the beginning of preincubation, the DTT stock

solution was added directly to the dish. At the beginning of pulse-labelling, the medium was replaced with DTT-containing labelling medium. During pulse-labelling, the DTT stock solution was added directly to the dish. At the beginning and during chase, medium was replaced with DTT-containing chase medium (except for Figure 7B, see legend). In the experiments in which the DTT treatment did not extend until the end of the chase, the DTT treatment was terminated by replacing the DTT-containing medium with the appropriate DTT-free medium.

Analysis of secretion. At the end of the chase, dishes were placed on ice to stop intracellular transport. The medium was collected, and cleared by centrifugation, and proteins were precipitated with trichloroacetic acid (TCA) (10% final). In some experiments, the medium was first supplemented with Tween 20 (0.3% final) and EDTA (5 mM final) followed by the preparation of a heat-stable protein fraction (see below) which was then TCA-precipitated. Cells were washed three times with ice-cold TBSS (25 mM Tris−HCl pH 7.4, 4.5 mM KCl, 137 mM NaCl, 0.7 M Na_2HPO_4, 1.6 mM Na_2SO_4) containing 0.5 mM PMSF and lysed in the dish (3.5 cm) in 1 ml of TNTE (20 mM Tris−HCl pH 7.4, 150 mM NaCl, 0.3% Tween 20, 5 mM EDTA) supplemented with 0.5 mM PMSF. The cell lysate (or the supplemented medium) was boiled for 5 min, and the heat-stable protein fraction was obtained by centrifugation for 15 min at 14 000 r.p.m. at 4°C in an Eppendorf centrifuge followed by TCA precipitation of the supernatant. TCA-precipitated proteins were analysed by SDS−PAGE or 2D-PAGE followed by fluorography as described by Lee and Huttner (1983) and Rosa *et al.* (1985a).

Subcellular fractionation of PC12 cells

The effect of DTT on the exit of CgB, SgII and the hsPG from the TGN to post-TGN vesicles was analysed by pulse−chase labelling in conjunction with subcellular fractionation using velocity and equilibrium sucrose gradient centrifugation as previously described (Tooze and Huttner, 1990, 1992). Briefly, to monitor the transport of secretory proteins from the TGN to ISGs and CSVs, PC12 cells (15 cm dishes) were pulse-labelled for 5 min with [^{35}S]sulfate and chased for 15 min, with 5 mM DTT being present as indicated in the figure legends. At the end of the 15 min chase, a PNS was prepared from the cells and subjected to velocity sucrose gradient centrifugation. Fractions 2−4 near the top of the gradient, which contain the peak of post-TGN vesicles, were pooled, loaded on a second sucrose gradient and centrifuged to equilibrium to separate ISGs and CSVs from each other. Aliquots (100 μl) of each fraction (1 ml) from the second gradient were analysed by SDS−PAGE and fluorography. The remainder of fractions 5−8, which contained the bulk of the [^{35}S]sulfate-labelled hsPG, CgB and SgII, was precipitated with TCA and the proteins were analysed by 2D-PAGE and fluorography.

NEM labelling

PC12 cells (15 cm dishes) were incubated for 22.5 min, corresponding to a 2.5 min preincubation followed by a 5 min pulse and a 15 min chase, in the absence or presence of 5 mM DTT. At the end of the incubation, the medium was removed, and a PNS prepared from the cells was subjected to subcellular fractionation by velocity and equilibrium sucrose gradient centrifugation as described in the previous section. Fractions 5−8 were each slowly diluted with an equal volume of 10 mM HEPES−KOH pH 7.2 to decrease the sucrose concentration, and the organelles contained in these fractions were pelleted at 130 000 *g* for 30 min at 4°C. Pellets were resuspended in 200 μl of 10 mM HEPES−KOH pH 7.7, and vesicles were lysed by three cycles of freezing and thawing. Aliquots (100 μl) of each lysate were incubated in the absence or presence of 5 mM DTT for 10 min at room temperature. After precipitation of proteins at −20°C using 80% acetone to remove DTT, proteins were resuspended in 175 μl of 10 mM HEPES−KOH pH 7.7, supplemented with 5 μl of pentane containing 5 μCi of [^{3}H]NEM (NEN), and incubated for 15 min at 0°C. The reaction was stopped by addition of 20 μl of 100 mM DTT on ice. Proteins were precipitated by acetone and analysed by 2D-PAGE and fluorography.

Quantifications

The radioactivity present in [^{3}H]NEM-labelled CgB and in [^{35}S]sulfate-labelled CgB, SgII and the hsPG contained in 2D-gel pieces was quantified by liquid scintillation counting after pronase digestion (Friederich *et al.*, 1988). In some experiments performed in the presence of 5 mM DTT, [^{35}S]sulfate-labelled CgB, SgII and the hsPG were quantified by densitometric scanning (Pharmacia/LKB ultroscan XL) of fluorograms of one-dimensional gels. Because in the presence of 5 mM DTT, the total [^{35}S]sulfate radioactivity present in the molecular weight area containing CgB was found to be mostly due to [^{35}S]sulfate-labelled CgB (see Figure 1 bottom panels), the absorbance values obtained for the CgB area of the one-dimensional gel were taken to represent [^{35}S]sulfate-labelled CgB. To obtain absorbance values for the [^{35}S]sulfate-labelled hsPG from one-dimensional gels, the region of the fluorograms corresponding to the top area of the gel, which also contains the hsPG (Tooze and Huttner, 1990), was scanned. Quantification of [^{3}H]tyrosine-labelled CgB and SgII was performed by densitometric scanning of the fluorograms.

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