Disruption of disulfide bonds exhibits differential effects on trafficking of regulated secretory proteins

SVEN-ULRIK GORR,1 XUE FEN HUANG,2 DARRIN J. COWLEY,3 REGINA KULIAWAT,2 AND PETER ARVAN2

1Department of Biological and Biophysical Sciences, University of Louisville Health Sciences Center, Louisville, Kentucky 40292; and 2Division of Endocrinology and Department of Developmental and Molecular Biology, Albert Einstein College of Medicine, Bronx, New York 10461

Gorr, Sven-Ulrik, Xue Fen Huang, Darrin J. Cowley, Regina Kuliawat, and Peter Arvan. Disruption of disulfide bonds exhibits differential effects on trafficking of regulated secretory proteins. Am. J. Physiol. 277 (Cell Physiol. 46): C121–C131, 1999.—For several secretory proteins, it has been hypothesized that disulfide-bonded loop structures are required for sorting to secretory granules. To explore this hypothesis, we employed dithiothreitol (DTT) treatment in live pancreatic islets, as well as in PC-12 and GH4C1 cells. In islets, disulfide reduction in the distal secretory pathway did not increase constitutive or constitutive-like secretion of proinsulin (or insulin). In PC-12 cells, DTT treatment caused a dramatic increase in unstimulated secretion of newly synthesized chromogranin B (CgB), presumably as a consequence of reducing the single conserved chromogranin disulfide bond (E. Chanat, U. Weiss, W. B. Huttner, and S. A. Tooze. EMBO J. 12: 2159–2168, 1993). However, in GH4C1 cells that also synthesize CgB endogenously, DTT treatment reduced newly synthesized prolactin and blocked its export, whereas newly synthesized CgB was routed normally to secretory granules. Moreover, on transient expression in GH4C1 cells, CgA and a CgA mutant lacking the conserved disulfide bond showed comparable multimeric aggregation properties and targeting to secretory granules, as measured by stimulated secretion assays. Thus the conformational perturbation of regulated secretory proteins caused by disulfide disruption leads to consequences in protein trafficking that are both protein and cell type dependent.

proinsulin; insulin; chromogranin

CONCEPTS CONTINUE TO EVOLVE concerning the storage of secretory granule proteins in the regulated exocytotic pathway of endocrine cells. A decade or more ago, the sole view was that regulated secretory proteins required specific sorting signals for entry into forming storage granules (i.e., active sorting), whereas soluble secretory proteins lacking this specific sorting information entered the constitutive secretory pathway by default (10, 50, 51). More recently, however, a sorting-by-retention model has been suggested in which several soluble proteins that are not stored in secretory granules can nevertheless enter immature granules, only to be later sorted out during the granule maturation process (13, 39, 43). Thus, whereas regulated secretory proteins tend to be retained (i.e., stored via passive sorting) during granule maturation (41), some fraction of nonretained proteins may be released via the constitutive-like secretory pathway (3).

Although many secretory proteins contain disulfide bonds, the evidence for a role of disulfide bonds in Golgi/post-Golgi sorting remains limited, and the requirement for a disulfide loop in the sorting of regulated secretory proteins (25, 40), separate from a role in overall protein folding, has been questioned (2, 58). To further examine the potential role of disulfide bonds in the sorting of regulated secretory proteins, we set out to investigate the events that follow dithiothreitol (DTT)-mediated reduction of proinsulin (which contains three disulfide bonds) in β-cells of live pancreatic islets and compared them to the effects of DTT treatment on CgB (which contains one disulfide bond) in live PC-12 and GH4C1 cells. Interestingly, on treatment with DTT, neither reduced proinsulin nor insulin exhibits increased release via constitutive or constitutive-like secretory pathways. By contrast, in PC-12 cells, unstimulated secretion of newly synthesized CgB is signifi-
GH4C1 cells, neither the absence nor presence of the mediated reduction, and, in transiently transfected made CgB is not significantly increased by DTT-ing in the distal secretory pathway. That may influence the final outcome of protein targeting in the distal secretory pathway.

**METHODS**

Materials. Collagenase was from Worthington (Freehold, N J); Hypaque (sodium diatrizoate), human serum albumin, BSA, soybean trypsin inhibitor, DTT, iodoacetamide (IAA), and phorbol 12-myristate 13-acetate (PMA) were from Sigma (St. Louis, MO); BAY K 8644 was from Calbiochem-Novabiochem (La Jolla, CA); calf serum was from GIBCO (Long Island, NY); gelding equine serum and FCS were from HyClone (Logan, UT); antibiotic-antimycotic solutions were from either Gibco or Sigma; [35S]methionine/oysteine (Express) was from NEN (New Bedford, MA); [3H]leucine was from ICN; [14C]leucine was from Amersham; DMEM, both deficient and complete, were either from Sigma or from Life Technologies (Grand Island, NY), while mouse epidermal growth factor (EGF) and Lipofectamine were from Life Technologies. Rabbit antisera to the amino terminus and carboxy terminus of CPE were graciously provided by Dr. L. Fricker (Albert Einstein College of Medicine, Bronx, NY), while the antiserum to bovine CgA was generously provided by Dr. D. V. Cohn (Univ. of Louisville, Louisville, KY).

Isolation and pulse-chase studies of mouse pancreatic islets. Islets from CD-1 mice were isolated by pancreatic ductal perfusion with collagenase, flotation on a Hypaque gradient, picking of individual islets, and recovery overnight in DMEM containing 10% calf serum plus 1% penicillin-streptomycin. Islets were washed twice with methionine-free, cysteine-free DMEM, before pulse labeling, for times ranging up to 12 min at 37°C in the same deficient medium that contained ~300 µCi of [35S]methionine plus cysteine. Labeling of islets in batch was performed as described in Ref. 34. When appropriate, labeled proinsulin accumulation in the TGN was accomplished with chase incubation of 2 h at 19.5°C. When appropriate, labeled proinsulin accumulation in the immature secretory granules was accomplished by a 105-min chase at 37°C. Appropriately chased islets were divided into equal numbers for different treatment conditions. Preliminary experiments confirmed that, with the numbers of islets used (800 per experiment), equally divided aliquots contained roughly equal amounts of total protein. The islet aliquots were then either mock treated or incubated in chase medium containing DTT at a final concentration of 20 mM. Unlike lower concentrations of DTT necessary for protein reduction in the endoplasmic reticulum, this concentration is required to achieve adequate reduction of proinsulin in the TGN of pancreatic islets (35). For islets in which labeled proinsulin was accumulated in the TGN, the DTT treatment was performed first for 10 min at 19.5°C, and the islets were then further warmed for 10 min to 37°C before continuation of the experiment (the warm-up was included because intracellular DTT-mediated reduction is less effective at 19.5°C). For islets in which labeled proinsulin chased to immature secretory granules, the DTT treatment was simply performed for 10 min at 37°C.

After the transient treatment, washout of DTT and subsequent chase were performed as described in the figure legends. All labeling and chase media also contained 0.5 mg/ml human serum albumin as well as 0.005% soybean trypsin inhibitor. For the purposes of this report, chase medium collected under "unstimulated" conditions was simply complete DMEM, which contains 5.5 mM glucose. In some cases, samples were stimulated with a β-cell secretagogue cocktail that included 22 mM glucose, 1 mM tolbutamide, 1 mM IBMX, and 100 nM PMA. Stimulated secretion for 1 h from isolated mouse islets generally achieves 15–20% of total insulin measured by RIA.

At the conclusion of all chase incubations, labeled islets were rapidly washed in ice-cold Tris-buffered saline containing 50 mM IAA and were lysed by bath sonication in the same buffer including 1% Triton X-100, 25 mM IAA, and an anti-protease cocktail of aprotonin (1 µM/ml), leupeptin (0.1 mM), pepstatin (10 µM), EDTA (5 mM), and disopropyl fluorophosphate (1 mM). The islet lysate was spun briefly in a microcentrifuge to remove debris, and proportionate volumes of the clarified lysate and secretions were analyzed by SDS-PAGE.

PC-12 cell culture and pulse-chase experiments. PC-12 cells (American Type Culture Collection, Manassas, VA) were cultured in DMEM supplemented with 10% FCS and penicillin/streptomycin in a humidified atmosphere at 37°C and 5% CO2. For each experiment one-half of the cells from near-confluent 75-cm2 flasks were plated in six-well cluster plates. The cells were maintained in culture for an additional 1–2 days before experiments.

For secretion experiments, PC-12 cells were preincubated for at least 45 min with labeling medium (0.3 mM CaCl2, 3.82 mM KCl, 0.62 mM MgCl2, 227 mM NaCl, 1.1 g/l glucose, 1.0 mM sodium pyruvate, 2.05 mM glutamine, 15 mM HEPES, pH 7.4). The preincubation medium was discarded and the cells were labeled with 1 ml per well of labeling medium containing 100 µCi [3H]leucine or 8 or 50 µCi of [14C]leucine for 5 min at 37°C. The cells were briefly rinsed and then chased for 1 h at 37°C in complete DMEM to which had been added 5 mM DTT from a freshly prepared 20× stock solution or an equivalent volume of water.

At the conclusion of the chase, 10 mM N-ethylmaleimide (NEM) was added to the collected chase media, which were then spun briefly in a microcentrifuge to remove debris. The cells were briefly rinsed in a buffer containing 20 mM NEM and were lysed by freeze-thawing in solubilization buffer (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 5 mM EDTA, 0.3% Tween 20, 1 mM phenylmethylsulfonyl fluoride) to which 10 mM NEM was then added; these extracts were then spun briefly in a microcentrifuge to remove cellular debris. In some experiments, to make conditions for protein recovery identical for all samples, radiolabeled secretion was subsequently mixed with unlabeled cell extracts from a control plate that had been incubated in parallel, while radiolabeled cell extracts were mixed with unlabeled medium from the control plate. In experiments in which the mixing was not performed, the media samples were adjusted to 5 mM EDTA and 0.3% Tween 20 before further analysis. At this time, radiolabeled cell extracts and medium samples were heated for 30 min at 100°C, followed by centrifugation for 20 min at 16,000 g. The heat-stable supernatant fractions were mixed with TCA (10% final concentration), incubated 20 min on ice, and spun for 20 min at 16,000 g. In all cases, visible pellets were obtained without the addition of carrier protein. The TCA precipitates were finally washed with 80% acetone before resuspension in solubilization buffer.
For secretion experiments, the cells were plated at a concentration of 10^5 cells/well in six-well cluster plates and were hormone treated for 4 days. The cells were rinsed with Krebs-Ringer-HEPES (KRH) buffer and preincubated in fresh KRH buffer for 75 min at 37°C. The cells were then labeled for 5 min in 1 µl/well of KRH containing 100 µCi [3H]leucine (or, in some cases, 250 µCi [3H]orpine). The labeling media were discarded and the cells were briefly rinsed and then chased for 1 h at 37°C in KRH containing 2 mM leucine to which had been added 5 mM DTT from a freshly prepared 20× stock solution or an equivalent volume of water. For analysis of stimulated secretion following the first 60-min chase period (see Fig. 8B), the cells were rinsed with KRH containing 2 mM leucine and then chased for 30 min in fresh KRH with leucine plus either 50 mM NaCl, 100 mM 4-k-phorbol 12,13-didecanoate, or 1 µl ethanol (unstimulated) or 50 mM KCl, 100 mM PMA and 1 µM BAY K 8644 (stimulated). The chase media were collected and centrifuged to remove cellular debris. The media and cell samples were mixed with unlabeled samples and prepared for SDS-PAGE as described for PC-12 cells (see above). In some experiments, NEM was omitted from the samples.

For experiments in which prolactin secretion was analyzed, 10^5 cells per well were hormone treated. The cells were preincubated in KRH for 45 min and then labeled and chased as described above. The labeled medium and cell samples were mixed with unlabeled samples and treated with NEM as described for PC-12 cells above; however, samples were not heated before TCA precipitation. One-half of each sample was analyzed by nonreducing SDS-PAGE on 15% polyacrylamide gels and the other half was analyzed by reducing SDS-PAGE in the presence of 20 mM DTT.

Transient expression of CgA in GH4C1 or PC-12 cells. The wild-type CgA (wt-CgA) cDNA (1) was subcloned into pcDNA3 (Invitrogen, San Diego CA). A mutant form of CgA (''CgA-CC'') lacking the amino-terminal disulfide bond was prepared by PCR-based mutagenesis, using separate primers (5'-'catgtgctcctttgagagtgtgtgcagatgcagctgatggacttcat-3' and 5'-'tacgcgttcctcccccgcgcagctgaggtggagtcggtcgtt) designed to mutate the cysteine residues at positions 17 and 38, respectively, to serine residues. Primers for the 5'- and 3'-untranslated regions were also designed. A new Kpn I restriction site was incorporated in the coding sequence between the two mutation sites. The amino- and carboxy-terminal PCR products were cloned into pcDNA3 and joined in frame at the new Kpn I site. Incorporation of the Kpn I site necessitated two conservative changes at Pro293Gly and Ser307Thr. The identity of the mutated CgA was confirmed by DNA sequencing.

GH4C1 or PC-12 cells were plated at a density of 5 × 10^4 cells/cm² in six-well plates in complete medium (described above). Two days later, the cells were transfected using 8 µl of Lipofectamine with 1 µg DNA/well (GH4C1 cells) or 8 µl of Perfect Lipid (Invitrogen) with 2 µg DNA/well (PC-12 cells). The medium was changed the following day, and stimulated secretion (in response to a secretagogue cocktail described above for GH4C1 cells or 2 mM BaCl₂ for PC-12 cells) was assayed on the second day after transfection. The medium containing secreted CgA was heated at 100°C for 10 min and centrifuged to remove denatured proteins.

For GH4C1 cells, 200 µl of the heat-stable supernatant (containing CgA) were then assayed quantitatively by dot immunoblotting essentially as previously described for secretogranin II (SgII) and prolactin (26). Control samples consisted of the heat-stable supernatant from media bathing cells that had been transfected with pcDNA3 not containing the CgA insert. The samples were applied to nitrocellulose membranes in a 96-well dot-blot apparatus (Bio-Rad Laboratories, Hercules CA). These membranes were washed in 20 mM Tris-HCl, pH 7.4, 500 mM NaCl, 0.05% Tween 20 (TTBS). For PC-12 cells, the medium was washed with TTBS followed by 20 mM HEPES, pH 7.4, and 20 mM CaCl₂ plus 100 mM MES, pH 6.0, followed by centrifugation for 20 min at 16,000 g. Pellets and supernatants were analyzed by SDS-PAGE and immunoblotting with anti-CgA. The in vitro aggregation of CgA shown in Fig. 8 actually represents the difference value between the percent sedimentation at pH 6.0 minus background values at pH 7.4.

SDS-PAGE and fluorography. Islet samples were analyzed by 15% acrylamide SDS-PAGE plus urea using a Tricine buffer system (61). Reduced and oxidized mouse proinsulins were run as standards on all gels. Insulin gels were fixed initially in 20% TCA without alcohol, then in 12.5% TCA plus 50% methanol, then incubated briefly with water, and finally incubated with 1 M sodium salicylate for 20 min. Dried gels were exposed to XAR film at −70°C. Films of media samples were typically exposed four- or fivefold longer than those of the companion islet lysates. PC-12 and GH4C1 samples were analyzed by conventional SDS-PAGE (44) under reducing or nonreducing conditions as indicated. Fluorography and densitometry were performed as described in Ref. 26. GH4C1 samples were also analyzed by direct quantitation of radioactive gel slices as described in Ref. 27.

Western blotting for CPE. PC-12 and GH4C1 cells were lysed by sonication in the following buffer: 100 mM NaCl, 1% Triton X-100, 0.2% sodium deoxycholate, 0.1% SDS, 10 mM EDTA, 0.7 mM DTT, 10 mM IAA, and 25 mM Tris, pH 7.4. An anti-protease cocktail yielding final concentrations of aprotilin (1 µM), leupeptin (0.1 mM), pepstatin (10 mM), EDTA (5 mM), diisopropylfluorophosphate (1 mM), and IAA (1 mM) was added to the lysates. The lysates were then normalized for cellular DNA using bis-benzimide fluorescence according to the protocol provided by Hoefer Scientific (San Francisco, CA). Lysates equivalent to 16 µg cellular DNA were analyzed in each lane. Cell lysates were electrotransferred to nitrocellulose and blotted with rabbit sera prepared against the amino terminus and carboxy terminus of CPE (a mixture of "carboxy-terminus bleed 5" and "amino-terminus bleed 3" was graciously provided by Dr. L. Fricke, Albert Einstein College of Medicine, Bronx, NY). A goat anti-rabbit IgG...
coupled to peroxidase was used as a secondary antibody, with
enhanced chemiluminescence detection.

Calculations and statistical analysis. The relative secre-
tion of PC-12 and GH4C1 proteins was calculated as described
in Ref. 26. The data were analyzed by two-tailed Student's
\( t \)-test. A value of \( P < 0.05 \) was considered statistically
significant.

RESULTS

Effects of in vivo disulfide reduction of proinsulin and
insulin in islet \( \beta \)-cells. The conserved disulfide loop in
CgB (which behaves as a regulated secretory protein)
has been proposed to function as a sorting signal at the
TGN for entry into forming secretory granules (25, 40).
The amino-terminal disulfide bond was originally found
to be an essential part of this signal, since disulfide
disruption using DTT treatment in live PC-12 cells
causes newly synthesized CgB to exhibit enhanced
unstimulated release rather than intracellular storage,
suggesting its missorting from the regulated to the
constitutive secretory pathway (16). Disulfide disrup-
tion of a similar amino-terminal disulfide-bonded loop
in POMC reportedly produces an analogous phenotype
in Neuro-2a cells (19); thus we have been interested in
exploiting disulfide disruption to explore the general
significance of disulfide bond integrity in regulated
secretory protein sorting.

As proinsulin advances along the secretory pathway
in pancreatic \( \beta \)-cells, hexamerization causes proinsulin
disulfide bonds to be increasingly buried and less
accessible to membrane-permeant reducing agents, so
that disulfide disruption in vivo can no longer be
obtained with monothiol reducing agents like 2-mercap-
toethanol (70) or with DTT at doses \(< 5 \text{ mM} \) (35).
Nevertheless, brief exposure to DTT at 20 mM can still
result in significant intracellular reduction of luminal
protein disulfides within the TGN or secretory granules
of pancreatic islets (35). To determine whether DTT
treatment also affects proinsulin trafficking, we first
pulse labeled isolated mouse pancreatic islets with
\( ^{35} \text{S}-\text{amino acids for 10 min followed by accumu-
lation of labeled prohormone and/or hormone from islets
of media by nonreducing SDS-PAGE (not shown) re-
duced insulin chains as a standard; Ox Pro, position of an oxidized
proteins. All samples were analyzed without prior immunoprecipita-
tion. Ins, position of an oxidized insulin standard; Red Ins, position of
proteins. All samples were analyzed without prior immunoprecipita-
tion. Ins, position of an oxidized insulin standard; Red Ins, position of
proinsulin (Fig. 1C). After washout
of DTT, subsequent unstimulated and stimulated chase
periods were collected. Interestingly, the unstimulated
chase period contained only modest labeled proinsulin
secretion that was clearly not stimulated by prior DTT
treatment (Fig. 1D). Furthermore, stimulated secre-
tion of labeled prohormone and/or hormone from islets
briefly treated with DTT was also less than that
observed from control islets (Fig. 1E). Accompanying
the decrease in labeled proinsulin and/or insulin secre-
tion from DTT-treated islets was an increase in the
labeled proinsulin that remained intracellular at the
end of the experiment (Fig. 1F). Importantly, analysis
of media by nonreducing SDS-PAGE (not shown) re-
vealed that only the residual oxidized forms of proinsu-
lin and insulin were secreted from islets treated with
DTT, suggesting that proinsulin molecules with dis-
rupted disulfide bonds could not advance from the TGN
into any secretory pathway. In addition, DTT treat-
ment led to obvious inhibition of endoproteolytic conver-
sion to labeled insulin (Fig. 1, E and F).

To exclude a general toxic effect of the brief islet
exposure to 20 mM DTT, it was important to examine
proinsulin and insulin that had already entered inma-
ture granules of the regulated secretory pathway. For
this purpose, we modified our protocol such that pancreatic
islets were chased to enrich for labeling of imma-

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1 We have previously reported that, because a fresh isolation of
pancreatic islets is performed for each experiment, there is variabil-
ity in the degree of reduction of the contents of post-Golgi compart-
ments in different preparations of islets, which are clusters of
1,000–5,000 cells. Incomplete reduction is commonly observed in
preparations enriched in larger islets, which may diminish the
degree of DTT penetration during incubation with the reducing agent
(35).
Disulfide disruption and regulated secretion

In mature β-granules (see METHODS), the compartment in which endoproteolytic conversion of proinsulin to insulin takes place (34, 41). The islets were then briefly exposed to DTT or were mock treated (see METHODS). It was clear that DTT penetrated the granules and structurally altered proinsulin as well as insulin, because there was a dramatic decrease in the recovery of both labeled intracellular proteins on nonreducing SDS-PAGE (Fig. 2A). This evidently reflected a structural perturbation rather than proteolysis, because, in separate experiments, DTT-treated islets boiled in the presence of 2% SDS and 25 mM DTT yielded recovery of reduced labeled proinsulin and insulin that was comparable with control samples (Fig. 2B). Thus DTT does not alter the amount of insulin or proinsulin in the immature granules but instead alters the structure of these proteins so as to affect their mobility on nonreducing gels. [This kind of structural perturbation induced by in vivo reduction, detected by differential underrecovery on nonreducing SDS-PAGE, has been reported previously for other proteins (22, 36, 38, 54, 59).]

Importantly, however, after in vivo reduction of labeled proinsulin and insulin within immature granules, the unstimulated and stimulated secretions did not differ from those of control (mock-treated) islets (Fig. 3). Essentially identical results were obtained on reduction of insulin after chase to mature secretory granules (chase time = 5 h at 37°C); indeed, by reducing SDS-PAGE followed by fluorography/scanning densitometry, stimulated insulin secretion from islets that had been briefly treated with 20 mM DTT averaged 18.3%, whereas stimulated insulin secretion from control islets averaged 18.4% (n = 4 experiments). Together, these data suggest that the effects of DTT on labeled proinsulin secretion were not a general effect of the DTT treatment on the viability or responsiveness of the islets. Rather, disruption of proinsulin disulfides in the TGN resulted in structural alterations that inhibited its advance into the post-Golgi secretory compartments comprising the constitutive, constitutive-like, and stimulated secretion pathways. Although there were also major structural sequelae for disruption of proinsulin or insulin disulfides within secretory granules (Fig. 2), because these proteins were already contained within the regulated secretory pathway, there was no demonstrable diminution of the fraction of labeled intracellular hormone that underwent stimulus-dependent exocytosis (Fig. 3).

In vivo disruption of CgB disulfide in PC-12 and GH3 cells. Because newly synthesized proinsulin did not exhibit increased unstimulated release in response to DTT treatment (Fig. 1), we decided to reexamine the effect of DTT-mediated disulfide reduction on the intracellular routing of newly synthesized CgB in endocrine cells. For this, we deferred DTT treatment or mock treatment of newly synthesized proteins until immediately after radiolabeled amino acid incorporation, because DTT inclusion during the pulse labeling can substantially inhibit the synthesis of CgB and other secretory proteins (16). After a 5-min pulse labeling of PC-12 cells with radioactive leucine, the cells were chased for an additional 60 min in the presence or absence of 5 mM DTT. The heat-stable radioactive proteins recovered from media or cell lysates (containing CgB) were analyzed by reducing SDS-PAGE and fluorography (14, 16, 24). The identity of CgB was independently confirmed by immunoprecipitation (not shown) with a specific antiserum (kindly provided by

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**Fig. 2.** Disulfide reduction of proinsulin and insulin within immature β-granules in vivo. A: preparation of islets was pulse labeled and chased for 105 min at 37°C before a brief mock treatment or DTT treatment as described in text. Islet lysates analyzed by nonreducing SDS-PAGE without prior immunoprecipitation, immediately after treatment period, are shown. Note apparently diminished recovery of both proinsulin and insulin in islets exposed to DTT. B: in this experiment, islet lysates were resolved by reducing SDS-PAGE. Ins, position of an oxidized insulin standard; Red Ins, position of reduced insulin chains as a standard; Ox Pro, position of an oxidized proinsulin standard; Red Pro, position of a reduced proinsulin standard. Data shown are representative of 3 experiments.

**Fig. 3.** Effect of DTT treatment on subsequent unstimulated and stimulated secretion from immature secretory granules. Isolated mouse islets were pulse chased and then mock treated or treated with DTT (A). Islets were then further chased in absence of DTT without secretagogue (B) and with secretagogue (C), each for 45 min. Secretions were analyzed by reducing SDS-PAGE and fluorography, without prior immunoprecipitation. Note that in vivo DTT treatment did not increase secretion of labeled proteins. Unstimulated secretion (B) may include exocytosis of immature granules but is also enriched in products of constitutive-like secretory pathway (4, 41), whereas secretagogue stimulation (C) leads to direct exocytosis of granules. Positions of reduced proinsulin and reduced insulin chains are shown. A radiolabeled band in middle of C is of uncertain identity but might be incompletely reduced insulin; intensity of this band is equal between the 2 samples. Red Ins, position of reduced insulin chains as a standard; Red Pro, position of a reduced proinsulin standard.
Dr. J. Scammell, Univ. of South Alabama). In agreement with earlier results (16), the presence of DTT during the chase resulted in enhanced unstimulated release of newly synthesized CgB from PC-12 cells, which was increased approximately sixfold over that released from control PC-12 cells (Fig. 4, left).

The foregoing data confirmed a major difference in the handling of reduced proinsulin in pancreatic islets and reduced CgB in PC-12 cells. To determine whether these results reflected a general aspect of sorting in regulated secretory cells, we extended our studies to include examination of GH4C1 cells that secrete prolactin along with CgB via the regulated secretory pathway (26, 33, 60). Using the same protocol as that employed for PC-12 cells, we first examined the fate of newly synthesized prolactin. As shown by nonreducing SDS-PAGE in Fig. 5, labeled prolactin was fully reduced by the DTT treatment. Moreover, an abnormally small fraction of newly synthesized prolactin was exported from these cells over a 60-min chase (see legend to Fig. 5). This dramatic perturbation of prolactin trafficking in GH4C1 cells clearly indicated DTT penetration and disulfide disruption within the secretory pathway. Remarkably, however, in GH4C1 cells, there was no significant increase in the unstimulated secretion of newly synthesized CgB after DTT treatment (Fig. 4, right).

Quite unlike results in PC-12 cells (16), the inability of DTT treatment to enhance the unstimulated secretion of newly synthesized CgB in GH4C1 cells might be explained by an inability of reduced CgB to exit the endoplasmic reticulum in these cells, making the protein unavailable to the distal secretory pathway. If newly synthesized CgB were to fail to escape the endoplasmic reticulum in GH4C1 cells, this would lead to its inability to acquire Golgi-type carbohydrate modifications. However, an SDS-PAGE assay based on the upward mobility shift of Golgi-modified CgB (15) strongly suggested that CgB did indeed exit the endoplasmic reticulum in DTT-treated GH4C1 cells (Fig. 6A). Furthermore, after DTT treatment, newly synthesized CgB still exhibited regulated exocytosis in response to secretagogue stimulation of GH4C1 cells (Fig. 6B). Thus, with the use of identical methodology in PC-12 and GH4C1 cells, the data do not support the hypothesis that the disulfide loop in CgB is important for its targeting to secretory granules in GH4C1 cells.

Disulfide disruption does not affect the targeting of CgA in GH4C1 cells. We next wished to test the role of the amino-terminal disulfide bond in chromogranin targeting using a means independent of DTT treatment. For this, we chose CgA, which also is targeted to...
secretory granules and exhibits homotypic calcium/low-pH-dependent assembly properties (31, 73) and contains the highly conserved disulfide-bonded loop structure (6) that has been described as essential for sorting to secretory granules in PC-12 cells (67). We transiently expressed wt-CgA or CgA in which both cysteine-17 and cysteine-38 (which form the amino-terminal disulfide loop) were converted to serine by site-directed mutagenesis (CgA-CC; see METHODS), thereby abolishing the sole granin disulfide bond. It is important to note that the expression of a disulfide-deficient mutant of CgB has recently been shown to be targeted normally to secretory granules in PC-12 cells, presumably because the disulfide-deficient CgB can homotypically associate with wild-type endogenous CgB (40). In addition, PC-12 cells express endogenous CgA. Indeed, when we overexpressed CgA-CC in PC-12 cells, it also exhibited a clear stimulus-dependent secretion (similar to that observed for expressed wt-CgA, Fig. 7), quite possibly because the disulfide-deficient CgA-CC mutant can homotypically associate with endogenous wt-CgA. However, GH4C1 cells do not express endogenous CgA, effectively eliminating homotypic interactions between transfected and endogenous CgA molecules. [Note also that, while CgA may interact with CgB under highly acidic conditions not comparable with those found in the TGN (73, 74), our previously published (and unpublished) results have suggested that heterotypic CgA-CgB interac-
tions are in fact quite limited (31).] We therefore compared the degree of stimulation of wt-CgA or CgA-CC secretion induced by secretagogue treatment of GH4C1 cells at steady state (a measure of granule targeting and storage efficiency). The data in Fig. 8A clearly demonstrate that the ability of secretagogue addition to elicit a stimulated secretion of CgA from GH4C1 cells is independent of the presence of its amino-terminal disulfide bond.

Because multimeric aggregation under conditions of mildly acidic pH and high calcium have been suggested to be an essential feature for the efficient sorting and storage of secretory granule proteins (14, 17), it seemed important to consider the significance of the granin disulfide bond in this context. Notably, DTT treatment does not affect the calcium/low-pH-induced aggregation of CgB or SgII from PC-12 cells (15), and similar results have been obtained for CgB and SgII from GH4C1 cells (25a). In addition, calcium/low-pH-induced multimeric aggregation of CgA-CC in vitro was not significantly different from that of wt-CgA (Fig. 8B) or significantly different from that previously reported for wt-CgA (31). Thus all the available data, taken together, indicate that, independent of the amino-terminal granin disulfide bond, other structural information is employed for the calcium/low-pH-induced granin aggregation, which is likely to contribute significantly to granin targeting to secretory granules in GH4C1 cells.

CPE levels are not very different in PC-12 and GH4C1 cells. One possible difference in chromogranin routing in PC-12 vs. GH4C1 cells could be in the binding of chromogranins to a putative sorting receptor that could interact with the amino-terminal disulfide bond. Thus far, the only common component that has been postulated to function as a neuroendocrine-specific receptor for the entry of secretory proteins into forming granules (21) or in secretory protein retention within maturing granules (57) [including in cells synthesizing growth hormone or prolactin (63)] is CPE. It therefore was of interest to determine whether there might be important differences in the levels of CPE between GH4C1 and PC-12 cells. As measured by immunoblotting (see METHODS), total immunoreactive CPE appeared comparably abundant in these cells (Fig. 9). These data are consistent with the idea that CPE per se does not directly relate to the mechanism of chromogranin sorting to the regulated secretory pathway (20, 52, 53) and cannot explain differences in sorting between PC-12 and GH4C1 cells after disruption of the chromogranin disulfide bond.

DISCUSSION

We have endeavored to explore the general significance of disulfide disruption on the fate of luminal
proteins in the distal secretory pathway of several regulated secretory cell types. Although there are several distinct models of protein sorting to secretory granules (3, 66), one idea common to all models is that sorting depends on conformational features in the secretory proteins. In particular, recent evidence has implicated disulfide-enclosed loop structures in the sorting of several regulated secretory proteins (15, 16, 25, 28, 40, 65, 67).

The results presented in this report raise new issues that must be considered when trying to interpret how disulfide disruption in newly synthesized secretory proteins may affect their targeting. Proinsulin contains three highly conserved disulfide bonds, including the A6-A11 bond that is known to be highly inaccessible to solvent, based on the insulin crystal structure (5, 7). Thus global structural rearrangement is expected to accompany reduction of the three disulfide bonds in proinsulin or insulin. Remarkably, our results in pancreatic β-cells seem to suggest that DTT treatment does not alter the fraction of labeled insulin that undergoes regulated exocytosis, because that fraction of molecules has already gained entrance to the regulated secretory pathway before exposure to the reducing agent (Fig. 3). (These findings also eliminate the idea that transient DTT exposure produces a toxic effect that blocks subsequent regulated exocytosis.) By contrast, when proinsulin is accumulated in the TGN of islet β-cells (Fig. 1) or shortly after prolactin synthesis in GH4C1 cells (Fig. 5), disulfide disruption renders these molecules unable to advance into either unstimulated or stimulated secretory pathways. Thiol-dependent conformational perturbation of secretory proteins within the endoplasmic reticulum is well established (8, 9, 36–38, 47, 48, 59); however, to our knowledge, such an effect has not been previously reported for labeled proteins that have already reached the TGN (Fig. 1). Indeed, these observations contrast with a previous conclusion that protein retention mediated by disulfide reduction (to free thiols) does not take place in or beyond the Golgi (70). Thus the mechanism of thiol-mediated retention of proteins in the Golgi complex appears to be protein dependent and quite possibly does not involve endoplasmic reticulum molecular chaperones (37). Moreover, recent studies have established that luminal proteins may be retained within the Golgi complex, even in the absence of disulfide disruption (32, 46, 62).

Unlike proinsulin or prolactin, reduction of the single conserved disulfide bond does not impair chromogranin transport through the endoplasmic reticulum and Golgi complex (Fig. 6A). Indeed, we have confirmed that, in PC-12 cells, unstimulated secretion of newly synthesized CgB is greatly augmented as a result of DTT treatment (Fig. 4). In GH4C1 cells, dramatic perturbation of newly synthesized prolactin clearly indicated DTT penetration and disulfide disruption within the secretory pathway (Fig. 5); however, DTT treatment did not lead to any significant rerouting of CgB in these cells (Figs. 4 and 6B). Consistent with these findings, CgA mutated to lack the homologous amino-terminal disulfide bond also exhibits normal stimulated secretion when transiently expressed in GH4C1 cells (Fig. 8). Importantly, although CgA-CC targeting to secretory granules in PC-12 cells (Fig. 7) can be explained by homotypic interactions with endogenous CgA that “rescue” the mutant [analogous to that reported for the rescue of disulfide-deficient CgB by endogenous CgB (40)], the same result in GH4C1 cells cannot be explained by interactions with endogenous CgA (because it is not expressed) but can be explained by homotypic multimeric aggregation of the transfected mutant CgA molecules (Fig. 8 and see below). We have used two entirely independent approaches, and neither has provided any evidence to support the idea that chromogranin sorting or for differences in sorting (which is gaining increasing acceptance) that cell-type-specific protein interactions play important roles in the targeting of regulated secretory proteins (13, 18, 64). However, interactions with CPE are unlikely to account for chromogranin sorting or for differences in sorting between PC-12 and GH4C1 cells [and this agrees with recent reports (20, 52, 53)], as levels of CPE by immunoblotting were comparable between the two cell types (Fig. 9).

There is a growing consensus that multimeric aggregation in the luminal ionic environment of the TGN and immature granules is a critical step in the sorting of numerous regulated secretory proteins (3, 68). For example, this appears to be of major importance not
We therefore hypothesize that multimeric aggregation of CgA containing or lacking the disulfide bond (Fig. 8). The targeting of SgII to secretory granules cannot be explained by rescue interactions with CgB, since these two granins apparently do not form heterotypic complexes (15, 16), although it is known that SgII independently exhibits calcium/low-pH-induced multimeric aggregation properties (24). Thus, in both PC-12 and GH4C1 cells, molecular interactions employing structural features over and above the disulfide-bonded loop are likely to be involved in granin targeting to secretory granules.

With these ideas in mind, it seemed reasonable to examine the multimeric aggregation properties of disulfide-deficient granins (which might potentially serve as a starting point for further investigations of granin sorting differences in PC-12 vs. GH4C1 cells). Notably, reduction of the disulfide bond in CgB does not affect its properties of calcium/low-pH-induced multimeric aggregation (15). Also, we do not find a significant difference in the calcium/low-pH-induced multimeric aggregation of CgA containing or lacking the disulfide bond (Fig. 8). We therefore hypothesize that multimeric aggregation behavior is likely to account largely for the disulfide-independent granin targeting to secretory granules in GH4C1 cells (Figs. 4, 6, 8). We intend to test this hypothesis by expressing in PC-12 and GH4C1 cells a truncated, nonaggregating form of CgA (31) that, nevertheless, contains the amino-terminal disulfide-bonded loop.

In conclusion, protein trafficking in regulated secretory cells appears to be a complex process involving multiple potential interactions among different secretory proteins, including potential interactions between secretory proteins and the surrounding membranes that form both the constitutive and the regulated secretory pathways (3). Thus, even with experiments that may demonstrate that a given region of a model protein is necessary and sufficient for trafficking to granules within the context of a single cell type, no one model protein or cell line can be trusted to faithfully replicate and to be fully representative of all aspects of post-Golgi trafficking among the great variety of neuroendocrine secretory cells.

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Address for reprint requests and other correspondence: P. Arvan, Div. of Endocrinology and Department of Development and Molecular Biology, Albert Einstein College of Medicine, Golding Bldg., Rm 501, 1300 Morris Park Ave., Bronx, NY 10461 (E-mail: arvan@ aecom.yu.edu).

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