The Propeptide of Preprosomatostatin Mediates Intracellular Transport and Secretion of α-Globin from Mammalian Cells

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Abstract. We have investigated the role of the somatostatin propeptide in mediating intracellular transport and sorting to the regulated secretory pathway. Using a retroviral expression vector, two fusion proteins were expressed in rat pituitary (GH₃) cells: a control protein consisting of the β-lactamase signal peptide fused to chimpanzee α-globin (142 amino acids); and a chimera of the somatostatin signal peptide and proregion (82 amino acids) fused to α-globin. Control globin was translocated into the endoplasmic reticulum as determined by accurate cleavage of its signal peptide; however, α-globin was not secreted but was rapidly and quantitatively degraded intracellularly with a $t_{1/2}$ of 4–5 min. Globin degradation was insensitive to chloroquine, a drug which inhibits lysosomal proteases, but was inhibited at 16°C suggesting proteolysis occurred during transport to the cis-Golgi apparatus. In contrast to the control globin, ~30% of the somatostatin propeptide–globin fusion protein was transported to the distal elements of the Golgi apparatus where it was endoproteolytically processed. Processing of the chimera occurred in an acidic intracellular compartment since cleavage was inhibited by 25 μM chloroquine. 60% of the transported chimera was cleaved at the Arg-Lys processing site in native prosomatostatin · yielding "mature" α-globin. Most significantly, ~50% of processed α-globin was sorted to the regulated pathway and secreted in response to 8-Br-cAMP. We conclude that the somatostatin propeptide mediated transport of α-globin from the endoplasmic reticulum to the trans-Golgi network by protecting molecules from degradation and in addition, facilitated packaging of α-globin into vesicles whose secretion was stimulated by cAMP.

MOST neuropeptides and small peptide hormones are synthesized as part of larger inactive polyprotein precursors in which the hormone sequence is flanked by pairs of basic amino acids (9). To generate a bioactive molecule, the precursors undergo one or several posttranslational modifications including glycosylation, proteolysis, phosphorylation, amidation, and acetylation (9). These processing events occur in different organelles during intracellular transport and therefore peptide hormone precursors are useful models to study movement through the secretory pathway. Recent observations demonstrate that several sorting and processing events occur in the distal elements of the Golgi apparatus/trans-Golgi network (TGN)[1] and in maturing secretory granules (12, 29, 30, 44). In particular, endoproteolytic cleavage at paired basic residues is initiated in acidic, clathrin-coated vesicles which bud from the TGN and the resulting mature hormone is stored in secretory granules (30, 31, 44).

Peptide hormone–producing cells characteristically concentrate and store their secretory product in electron-dense secretory granules (32). Upon stimulation by an extracellular signal, these granules fuse, through a calcium-dependent process, with the plasma membrane releasing their contents into the external milieu. This type of secretion is designated "regulated" or "stimulated" (see references 4, 14, and 33 for recent reviews). Hormone-secreting cells also undergo basal or "constitutive" secretion whereby nonhormone secretory proteins and plasma membrane proteins are neither concentrated nor stored and are transported in vesicles which continuously fuse with the plasma membrane in a calcium-independent manner (4, 14). Since hormone-secreting cells undergo basal secretion, a mechanism must exist that discriminates between molecules destined for the regulated or constitutive pathways.

The molecular signals that target a polypeptide hormone to the regulated secretory pathway are poorly understood, although morphological evidence has implicated selective aggregation or precipitation, initiated in the TGN, in the sorting process (44). Current evidence suggests that in the absence of a specific topogenic signal, e.g., for retention in the ER (27, 34) or Golgi apparatus (19) or for sorting to lysosomes (16), secretion through the constitutive pathway oc-

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1. *Abbreviations used in this paper:* TCR, T-cell receptor; TFA, trifluoroacetic acid; TGN, trans-Golgi network.

curs by default (14, 36). Expression of heterologous precursors in different endocrine cell lines results in proteolytic cleavage to the mature hormone and, in some cases, targeting to the regulated secretory pathway; e.g., preproinsulin (26), pretrypsinogen (5), preproenkephalin (43), preprorenin (10), prepproneuropeptide Y (8), and preprosomatostatin (39, 41). Since this diverse group of proteins, which lack amino acid sequence homology, can be sorted to the regulated secretory pathway, it is likely that a common structural feature rather than a primary sequence is involved in targeting. However, the identity of putative sorting signals has been elusive.

We are studying the processing of anglerfish prosomatostatin (proSRIF) in order to identify structural domains within peptide hormone precursors which might function in targeting to the regulated secretory pathway (41, 46). Somatostatin is a 14–amino acid peptide hormone that is synthesized as part of a larger precursor, preproSRIF (13); it consists of a 25–amino acid signal peptide followed by a proregion of 82 residues. The mature hormone is located at the carboxyl terminus of the propeptide and is preceded by a single pair of basic amino acids: ArgLys. These residues are a subset of a hexapeptide domain (AlaProArgGluArgLys) which is conserved in all proSRIF species. Apart from endoproteolytic cleavage, the precursor undergoes no other posttranslational modifications.

We recently demonstrated (41) that growth hormone–producing cells (GH₃) efficiently cleave proSRIF and sort the mature hormone into the regulated secretory pathway. We previously postulated that the SRIF propeptide might function in mediating intracellular transport (46). To test this hypothesis directly, we used a retroviral vector, pLJ (15), to express a fusion protein (PRO-GLO) consisting of the SRIF signal peptide and proregion and chimpanzee α-globin in pituitary GH₃ cells. The α-globin polypeptide was chosen as a reporter group for these studies since (a) it is a cytoplasmic protein and is unlikely to possess intrinsic sorting information; (b) previous work demonstrated that attachment of a bacterial signal peptide to the NH₂ terminus of α-globin resulted in its translocation into the ER in vitro and in vivo (17, 40); and (c) expression of an ovalbumin signal peptide–α-globin fusion protein in Xenopus oocytes led to efficient segregation of α-globin into the ER but not to its secretion (42). Here we show that a signal peptide–globin fusion protein (SIG–GLO) was translocated into the ER in vivo, but in the absence of the SRIF propeptide it was rapidly degraded with a half-life of 4–5 min. In contrast, PRO-GLO was transported from the ER to the TGN where it underwent proteolytic cleavage yielding mature α-globin which was secreted into the medium.

Materials and Methods

Materials

The following plasmids were used: pLJ, a recombinant retrovirus expression vector (15), a gift of Dr. R. Mulligan, The Whitehead Institute, Boston, MA; pSP125E encoding the β-lactamase signal peptide fused to chimpanzee α-globin (40), a gift of Dr. V. R. Lingappa, University of California, San Francisco, CA; and pLaS1 (13), containing a 462-bp cDNA encoding anglerfish preproSRIF I to which 5′ and 3′ Eco RI–Bam HI linkers were added (41). Rabbit anti–human hemoglobin antiserum was purchased from Cappel Laboratories (Cochranville, PA).

Construction of Recombinant Virus Encoding SIG-GLO and PRO-GLO

Standard recombinant DNA procedures (21) were followed to generate the hybrid molecules.

pLJSIG-GLO Construction. A Bam HI fragment encoding the β-lactamase signal peptide–α-globin (SIG-GLO) was generated from pSP125E by the addition of Bam HI linkers at a blunt-ended Bgl III site; this fragment was ligated directly into the Bam HI site of pLJ to generate pLJSIG-GLO.

pLJPRO-GLO Construction. pLaS1 was digested with Eco RI to generate a 462-bp fragment encoding preproSRIF (41, 46). This fragment was ligated into the Eco RI site of a pBR322 derivative in which all four Nae I sites were deleted by digestion with Nae I and Bal I generating plasmid pDWS18 (Fig. 1 A). Plasmid pSP125E was digested with Nco I, filled-in using the Klenow fragment of DNA polymerase I, 12-mer Bam HI linkers were added, and following digestion with Bam HI and blunt-ending with Klenow, the DNA fragment encoding α-globin was ligated into pDWS18 which was partially digested with Nae I. This generated a hybrid gene encoding the preproSRIF signal peptide and proregion in frame with α-globin at the 3′ end; four amino acids (AlaAspProArg) were introduced (double lines) by this construction. Plasmid pPRO-GLO was digested with Bam HI and the appropriate DNA fragment ligated into the unique Bam HI site of pLJ to generate pLJPRO-GLO.

Production of Recombinant Retrovirus Expressing α-Globin-related Molecules

Plasmid DNAs were amplified in *Escherichia coli* strain MC1000, grown in the presence of 40 μg/ml of kanamycin and plasmid DNA prepared. Infectious virus particles containing globin-related RNA transcripts were generated by transfecting Psi-2 cells (22) with plasmid DNA.

Cell Culture

Cells were grown at 37°C in an atmosphere of 7.5% CO₂. GH₃ cells were grown in Ham's F10 medium supplemented with 15% equine serum, 2.5% fetal bovine serum (41). Psi-2 cells were grown in DME supplemented with 10% fetal bovine serum, 2 mM glutamine, 25 U/ml penicillin, and 25 mg/ml streptomycin.

Infection of Target GH₃ Cells

Medium from semiconfluent G418 resistant Psi-2 cells was filtered (41) and a dish with 10⁶ GH₃ cells was incubated for 2 h with 1 ml of the filtrate containing 10 μg of polybrene. The cells were incubated with complete Ham's F10 containing 1 mg/ml G418 as previously described (41). Single G418 resistant cells were subcultured by limiting dilution in a 96-well plate and 10 clonal lines were grown to mass culture.

Biosynthetic Labeling of Cells

60-mm dishes were seeded with 2 × 10⁶ cells, 24–72 h later the cells were washed with PBS and pulse-labeled with 1 ml of labeling medium supplemented with 2 mM glutamine, 250 μCi/ml [³⁵S]methionine exactly as previously described (41). For chase incubations, the medium also contained 10 μl/ml of antiserum (41). After the labeling and chase periods, the medium was removed, centrifuged, and stored on ice or at −20°C until further treated. Cells were harvested (41), the suspension centrifuged, and the pellet lysed by vortexing in lysis buffer. Nuclei were removed by centrifugation (41) and the postnuclear supernatants were stored on ice or at −20°C until treated with antisera.

Immunoprecipitation

To determine the intracellular levels of globin-related polypeptides, postnuclear supernatants were adjusted to 1% SDS and incubated at room temperature for 10 min. 10 vol of buffer A (41) were added followed by addition of 10 μl of rabbit anti–human hemoglobin antiserum. Samples were incubated at 4°C for 12–24 h with constant mixing (41). To assay for secreted polypeptides, the medium was adjusted to buffer A conditions by addition of 1/3 vol of a 4× buffer A solution. Samples were incubated with constant mixing at 4°C for 12–24 h followed by incubation with protein A–Sepharose at 4°C for 3 h (41). Immune complexes were isolated by centrifugation, washed twice with buffer B containing 1 mg/ml BSA (41), twice with PBS,
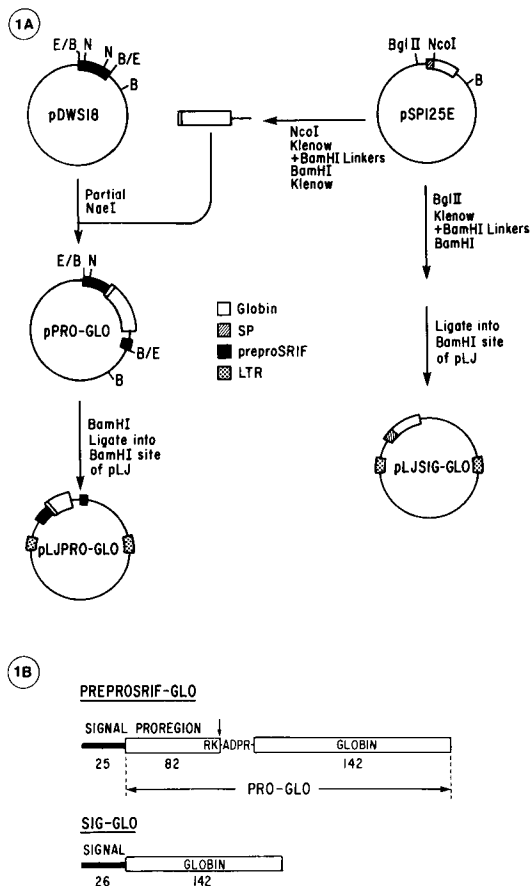
Figure 1. Construction of SIG-GLO and PRO-GLO chimeras. (*A*) pLJSIG-GLO Construction. A Bam HI fragment encoding the β-lactamase signal peptide–α-globin fusion (SIG-GLO) was generated from pSP125E by addition of Bam HI linkers at a blunt-ended Bgl II site (see Materials and Methods). This fragment was ligated directly into the Bam HI site of pLJ to generate pLJSIG-GLO. pLJPRO-GLO Construction. Plasmid pDWS18 encoding anglerfish preproSRIF I was partially digested with Nae I and a blunt-ended fragment encoding chimpanzee α-globin, derived from pSP125E, was ligated into the 3′ Nae I site of preproSRIF cDNA to generate plasmid pPRO-GLO (Materials and Methods). The Bam HI fragment of pPRO-GLO was ligated into the Bam HI site of the retroviral expression vector pLJ. *B*, Bam HI; *E*, Eco RI; *N*, Nae I; *P*, Pvu II; *SP*, β-lactamase signal peptide; *LTR*, long terminal repeat of pLJ (15). (*B*) PREPRO-GLO consists of the anglerfish preproSRIF I 25–amino acid signal peptide (*black box*); 82 residue propeptide, Ala (*A*), the first residue of mature SRIF; three residues derived from the cloning strategy AspProArg (*DPR*); and 142 residues of chimpanzee α-globin. The arrow indicates the single set of paired basic amino acids ArgLys (*RK*) in native proSRIF which are the site of endoproteolytic cleavage to yield mature SRIF-14. SIG-GLO is the identical fusion used by Simon et al. (40) and consists of the β-lactamase signal peptide (26 amino acids, *black box*) fused to chimpanzee α-globin.

and used immediately or stored at −20°C until analyzed by HPLC or SDS-PAGE.

Automated Microsequencing of Globin-related Products

Automated Edman degradation was performed using a Beckman Instru-

ments Inc. (Fullerton, CA) 890C spinning cup sequencer (41). GH₃ cells were radiolabeled with [³⁵S]methionine and the medium or cells treated with antihemoglobin antisera as outlined above. The protein A–Sepharose beads were incubated with 50 μl of 2 M acetic acid and 8 M urea for 30 min at 50°C. The supernatant was removed and the pellet washed twice with 50 μl of the same solution. A SEP-PAK (Waters Associates, Inc., Milford, MA) was prepared by washing with 5 ml of 80% CH₃CN in 0.1% trifluoroacetic acid (TFA) followed by 5 ml of 0.1% TFA. To maximize binding, the combined supernatants were loaded on the SEP-PAK three times which was then washed with 5 ml of 0.1% TFA. Bound material was eluted by three washes with 1 ml of 60% CH₃CN in 0.1% TFA and three washes with 1 ml of 80% CH₃CN in 0.1% TFA. 25 μl of each fraction was counted. The first two 60% CH₃CN washes containing 90% of the radioactivity were combined and loaded directly into the sequencer.

Results
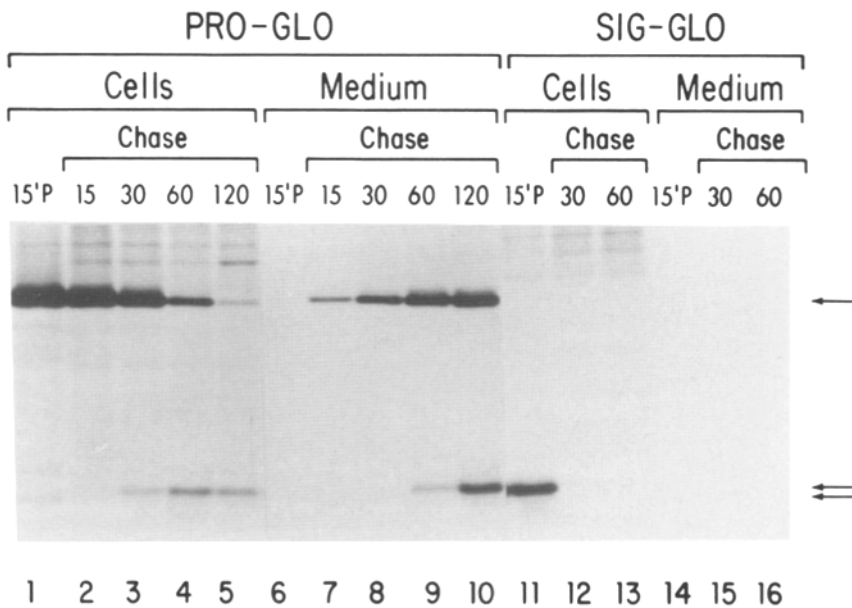
Expression of Globin-related Proteins in GH₃ Cells

To test the hypothesis that the SRIF propeptide mediates intracellular transport, we expressed two chimeric proteins in GH₃ cells: PRO-GLO, a fusion of the SRIF preproregion and chimpanzee α-globin; and a control precursor, SIG-GLO, consisting of the β-lactamase signal peptide fused to α-globin (Fig. 1 *B*). The rationale for these experiments was to determine if the propeptide could facilitate transport of a normally cytoplasmic protein through the secretory pathway. In addition, our aim was to determine if PRO-GLO would be proteolytically processed at the proSRIF ArgLys processing site and if the resulting globin polypeptide would be targeted to the regulated or constitutive secretory pathway.

The starting cDNAs for these constructions were plasmids pSP125E encoding the β-lactamase signal peptide fused to chimpanzee α-globin (40), and pDWS18 which contained anglerfish preproSRIF I cDNA (13) cloned into the Eco RI site of a pBR322 derivative in which all four Nae I sites were deleted (Fig. 1). After digestion, the appropriate DNA fragments were ligated into the Bam HI site of the retroviral expression vector pLJ (15) and were designated pLJSIG-GLO and pLJPRO-GLO, respectively. Plasmid pLJSIG-GLO encoded the β-lactamase signal peptide (26 amino acids) fused to α-globin (40). Plasmid pLJPRO-GLO encoded the preproSRIF I signal peptide, 82 residue proregion, the first amino acid of mature SRIF (Ala), three residues (AspProArg) resulting from the cloning and the complete chimpanzee α-globin chain sequence (Fig. 1, PrePROSRIF-GLO). We used the expression vector pLJ since the recombinant virions can infect rat pituitary GH₃ cells and these cells efficiently cleave native preproSRIF to the mature hormone (41).

### Generation of Stable GH₃ Cell Lines Expressing Globin-related Proteins

Psi-2 cells were transfected with pLJSIG-GLO and pLJPRO-GLO DNAs and grown in the presence of G418; subsequent virus production and target cell (GH₃) infection were exactly as previously described (41). 10 G418 resistant clonal lines expressing SIG-GLO (GH₃SIG-GLO) and PRO-GLO (GH₃PRO-GLO) were generated and one clonal line expressing each chimera was selected for analysis. GH₃SIG-GLO.9 and GH₃PRO-GLO.9 cells were pulse-labeled for 15 min with [³⁵S]methionine and chased in complete media for times up to 2 h. The cells and medium were treated with anti–human hemoglobin antiserum and the immune com-

Figure 2. Expression of SIG-GLO and PRO-GLO in GH₃ cells. PRO-GLO expression: GH₃PRO-GLO cells were pulse labeled for 15 min with [³⁵S]methionine and chased for the indicated times. Cells and medium were treated with antihemoglobin antiserum and immune complexes resolved by SDS-PAGE on 12% gels. Lanes *1–5,* intracellular material; lanes *6–10,* secreted products. Lanes *1* and *6,* 15-min pulse; lanes *2* and *7,* 15-min chase; lanes *3* and *8,* 30-min chase; lanes *4* and *9,* 60-min chase; lanes *5* and *10,* 120-min chase. (*Top arrow*) Migration of in vitro synthesized proSRIF–α-globin (M_r 25,000); (*middle arrow*) migration position of globin endoproteolytically excised from PRO-GLO. SIG-GLO expression: GH₃SIG-GLO cells were pulse labeled for 15 min with [³⁵S]methionine and chased for 30 and 60 min. Cells and medium treated as in PRO-GLO expression. (Lanes *11–13*) Intracellular material; (lanes *14–16*) secreted material. (Lanes *11* and *14*) 15-min pulse; (lanes *12* and *15*) 30-min chase; (lanes *13* and *16*) 60-min chase. (*Bottom arrow*) Migration of mature α-globin.

plexes resolved by SDS-PAGE (Fig. 2). During the 15-min pulse of GH₃SIG-GLO cells, a major globin–immunoreactive polypeptide having the same electrophoretic mobility as α-globin was evident (Fig. 2, lane *11, bottom arrow*). After 30 min of chase, this polypeptide had completely disappeared from the cell lysate (Fig. 2, lane *12*) but no globin-related material was recovered in the medium (Fig. 2, lanes *14–16*).

We hypothesized that SIG-GLO was transported to lysosomes and degraded. To test this idea, GH₃SIG-GLO cells were incubated with several concentrations of chloroquine
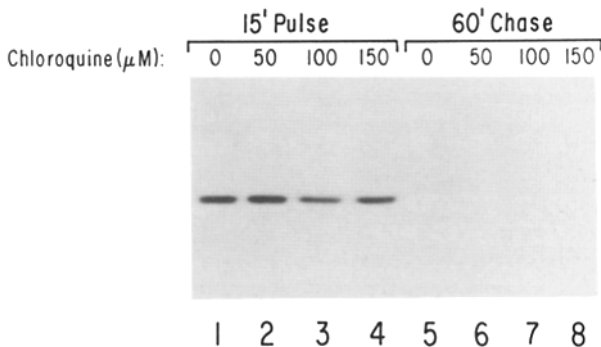

Figure 3. Effect of chloroquine on SIG-GLO turnover. GH₃SIG-GLO cells were pulse labeled for 15 min with [³⁵S]methionine in the absence or presence of the indicated concentrations of chloroquine and chased for 60 min in the absence and presence of chloroquine. At each time point, the cell lysates and medium (not shown) were treated with antihemoglobin antisera and the immunoprecipitates resolved by SDS-PAGE. (Lanes *1* and *5*) No chloroquine; (lanes *2* and *6*) 50 μM chloroquine; (lanes *3* and *7*) 100 μM chloroquine; (lanes *4* and *8*) 150 μM chloroquine.

(Fig. 3), a weak base that neutralizes the intralysosomal acidic pH, thereby inactivating lysosomal hydrolases (23, 28). At each chloroquine concentration (up to 150 μM), α-globin was degraded intracellularly and globin secretion was not observed (data not shown). This result suggested that the rapid turnover of the α-globin molecules was not dependent on or occurring in an acidic compartment. The intracellular half-life of SIG-GLO was then determined by pulse-labeling GH₃SIG-GLO cells with [³⁵S]methionine for 15 min and chasing for times up to 40 min; at each time point cells were lysed and the globin–immunoreactive products analyzed by SDS-PAGE (Fig. 4). By 5 min of chase, >60% of α-globin had disappeared and by 40 min of chase globin was undetectable in the lysate; at no time point was globin detected in the medium. However, when cells were chased for 40 min at 16°C to prevent exit from the ER, 80–90% of the pulse-labeled α-globin was still present intracellularly. Based on the rapid kinetics of the α-globin disappearance, the temperature sensitivity, and the chloroquine data, it seemed unlikely that globin was transported to lysosomes for degradation.

The preceding data confirmed that a signal peptide was sufficient to effect translocation of globin into the ER in vivo but not for globin secretion. To determine if a propeptide could mediate secretion, GH₃PRO-GLO cells were pulse-labeled for 15 min with [³⁵S]methionine and chased for up to 2 h (Fig. 2). During the 15-min pulse, a prominent globin–immunoreactive polypeptide migrating with the predicted molecular weight for PRO-GLO lacking a signal peptide (M_r 25,000) was detected intracellularly (Fig. 2, lane *1, top arrow*). A second intracellular, globin-specific polypeptide (M_r 15,000) was detected between 15 and 30 min of chase (*middle arrow*; Fig. 2, lanes *2* and *3*). The appearance of this
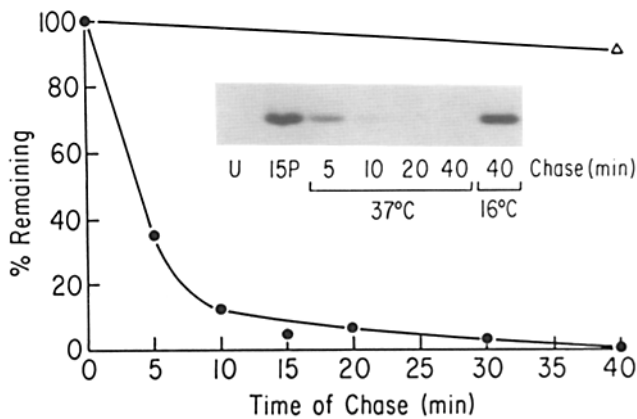
Figure 4. Kinetics of SIG-GLO turnover. GH₃SIG-GLO cells were pulse labeled with [³⁵S]methionine for 15 min (*15P*) and chased for times up to 40 min at 37°C or at 16°C for 40 min. At each time point cells were lysed and the intracellular material treated with antihemoglobin antisera and the immunoprecipitates resolved by SDS-PAGE (*inset*). The autoradiographs were scanned using an LKB Instruments, Inc. (Gaithersburg, MD) ultrascan laser densitometer. (Lane *U*) Uninfected control GH₃ cell lysate treated with antihemoglobin antiserum. (●) Percent α-globin remaining in cells incubated at 37°C; (△) percent α-globin from cells incubated at 16°C.

molecule, which had an electrophoretic mobility slightly slower than authentic α-globin (Fig. 2, *bottom arrow*), was consistent with PRO-GLO being proteolytically processed at the single ArgLys site in proSRIF generating "mature" α-globin: α-globin with four additional NH₂-terminal amino acids (see Fig. 1). Cleavage following the ArgLys was subsequently confirmed by partial NH₂-terminal sequencing (see below and Fig. 6). With prolonged chase times, the level of M_r 15,000 globin increased with a concomitant reduction of the PRO-GLO polypeptide (Fig. 2, lanes 2–5) suggesting a precursor product relationship. Some unprocessed M_r 25,000 PRO-GLO was rapidly secreted (Fig. 2, lanes 7–10) but most significantly, processed mature M_r 15,000 globin was also secreted into the medium (Fig. 2, lanes 8–10, and Fig. 5).

PRO-GLO processing efficiency and the kinetics of α-globin secretion were determined by excising globin-specific polypeptides from gels similar to that shown in Fig. 2 and determining the radioactivity (Fig. 5). With increasing chase times, PRO-GLO decreased with a concomitant increase in the level of intracellular mature α-globin (Fig. 5 *A*) which remained constant from 90 to 180 min of chase. Processing of PRO-GLO to mature globin was detected between 15–30 min of chase and some α-globin was secreted into the medium following a lag of ∼30 min (Fig. 5 *B*). It is noteworthy that in these cells, the kinetics of PRO-GLO cleavage to α-globin were identical to those of native proSRIF processing to the mature hormone (41), suggesting that the chimera and native precursors were transported through the same intracellular compartments at similar rates. About 30% of the total pulse-labeled globin–immunoreactive material could be recovered intracellularly and in the medium at 120 min of chase. We presume that the initially synthesized PRO-GLO which was not recovered after 15 min of chase was degraded intracellularly in the same compartment as SIG-GLO. Since
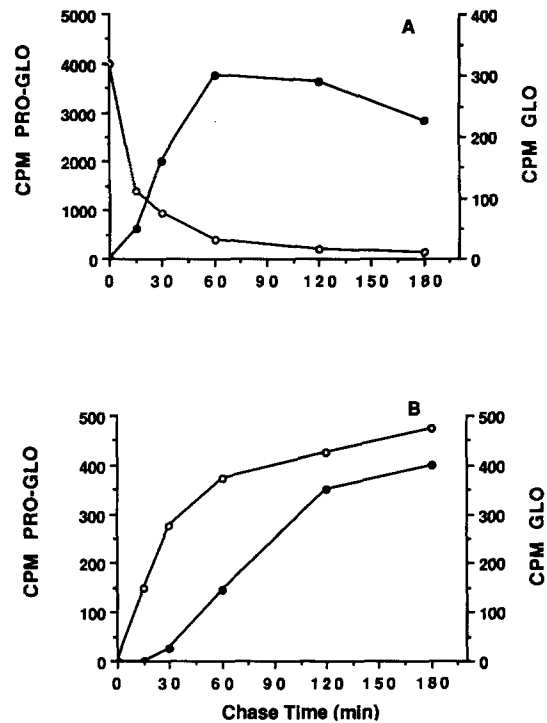

Figure 5. Kinetics of PRO-GLO processing and secretion. GH₃PRO-GLO cells were subjected to a pulse-chase protocol as outlined in Fig 2. The cell lysates and media were treated with antihemoglobin antisera and the immunoprecipitates resolved by SDS-PAGE. The gel bands corresponding to globin-specific polypeptides were excised and the radioactivity in the solubilized gel pieces determined. Note: PRO-GLO has three methionine residues whereas mature α-globin contains two methionines; data, which are the average from two separate experiments, are not corrected for differences in methionine content. (*A*) Intracellular material; (*B*) secreted polypeptides. (○) PRO-GLO; (●) mature α-globin.

PRO-GLO contains three methionine residues whereas mature α-globin has two methionines, we estimate that ∼60–65% of the transported PRO-GLO, i.e., that present after the 15-min chase, was processed to mature α-globin at 2 h of chase. Of this processed globin, ∼45% was stored intracellularly with a half-life >4 h.

Pro-SRIF-GLO Is Processed to Mature α-Globin

Approximately 60% of the transported PRO-GLO was processed to mature α-globin. Partial NH₂-terminal sequencing was performed to determine if proteolytic cleavage had occurred at the predicted processing sites: on the carboxyl side of lysine in the hexapeptide domain in PRO-GLO and the junction of the signal peptide and α-globin in SIG-GLO (Fig. 6). Chimpanzee α-globin has methionine residues at positions 1 and 33. Consistent with correct signal peptide cleavage, methionine residues were detected at positions 1 and 33 in the α-globin polypeptide from GH₃SIG-GLO cells. PRO-GLO contains four additional NH₂-terminal amino acids (AlaAspProArg; Fig. 1). Therefore, if the prohormone processing enzyme(s) recognized the endoproteolytic processing site of PRO-GLO (ArgLys), a methionine residue should be present at position 5. The sequence data demonstrated that this was the case (Fig. 6). Thus, despite the pres-
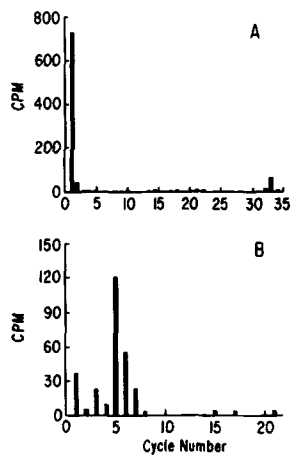
Figure 6. Partial NH₂-terminal amino acid sequencing of processed α-globin polypeptides. (*A*) GH₃SIG-GLO cells were labeled with [³⁵S]methionine for 60 min, lysed, and the cell lysate treated with antihemoglobin antiserum. The immunoprecipitates were purified on a SEP-PAK (see Materials and Methods) and subjected to 34 cycles of automated Edman degradation. (*B*) GH₃PRO-GLO cells were radiolabeled for 60 min, chased for 60 min, and then chased for an additional 60 min in the presence of 5 mM 8-Br-cAMP to stimulate globin secretion (see Fig. 8). The chase medium was treated with antihemoglobin antiserum, the immunoprecipitates separated on a SEP-PAK and applied directly to the sequencer, and subjected to 21 cycles of automated Edman degradation. The radioactivity in each cycle was determined by liquid scintillation counting.

ence of a 142–amino acid foreign protein at the carboxyl terminus of the propeptide, the chimera was recognized by the processing enzymes nearly as efficiently as native proSRIF (70%) and cleaved at the native processing site. The data also indicated that a low percentage of PRO-GLO molecules were cleaved at a single arginine located in the linker region between proSRIF and α-globin (Fig. 1). It is possible that we may have generated a cryptic cleavage site in PRO-GLO for a distinct monobasic-processing enzyme thought to be present in secretory granules (38). In ∼1/3 of precursors cleaved at single basic residues, proline immediately precedes or follows the basic amino acid. Consistent with this hypothesis, ProArg was present in the PRO-GLO linker peptide.

In endocrine cells proteolytic processing and packaging of the mature hormone into secretory granules requires acidification of the TGN compartment (1, 7, 31). To demonstrate that PRO-GLO processing occurred in an acidic intracellular compartment and to exclude the possibility of extracellular proteolysis, GH₃PRO-GLO cells were incubated in the presence of increasing concentrations of chloroquine. At 25 μM chloroquine PRO-GLO processing was inhibited by ∼95% and at higher concentrations proteolysis was quantitatively inhibited resulting in secretion of only unprocessed precursor (Fig. 7). In the absence of proteolytic processing at 25 and 50 μM chloroquine, enhanced secretion of unprocessed PRO-GLO was seen (Fig. 7, lanes 7 and 8). At higher chloroquine concentrations secretion was diminished (Fig. 7, lane 9) most likely due to cytotoxic effects of the drug. These results contrast with those of SIG-GLO and further demonstrate that the two precursors were transported to distinct cellular compartments.

Characterization of the Intracellular Pool of PRO-GLO and α-Globin

Approximately 50% of processed mature globin was stored intracellularly with a half-life >6 h. It was therefore of interest to determine if globin was targeted to the regulated secretory pathway (Fig. 8). Cells were pulse labeled for 15

min and chased for 2 h, the medium was then changed and the cells incubated for an additional 2 h in the absence (Fig. 8, lanes 4 and 8) or presence of the secretagogue, 8-Br-cAMP (Fig. 8, lanes 5 and 9). During the 120-min chase in the absence of 8-Br-cAMP, unprocessed PRO-GLO and mature globin were secreted (Fig. 8, lane 6) but a significant level of both molecules was still present intracellularly at 240 min of chase (Fig. 8, lanes 3 and 4). However, in the presence of 8-Br-cAMP (cf. Fig. 8, lanes 5 and 9), 90% of both mature globin and PRO-GLO were secreted and virtually no globin-related material remained intracellular (Fig. 8, lane 5). We conclude that the release of stored globin and PRO-GLO in response to 8-Br-cAMP indicated targeting to the regulated pathway.

Discussion

A characteristic morphological feature of many endocrine cells is the possession of dense core secretory granules in which the mature hormone is stored in a semicrystalline form. Upon stimulation, these granules fuse with the plasma membrane releasing their contents into the circulation. In general, there is little sequence homology among a diverse array of proteins targeted to secretory granules, thus, it is unlikely that a primary consensus sequence is involved in targeting. Recent evidence, using chimeric proteins, implies targeting to the regulated secretory pathway may be an "active" sorting process that contrasts with constitutive secretion which probably occurs by bulk flow (4, 33, 47). For exam-
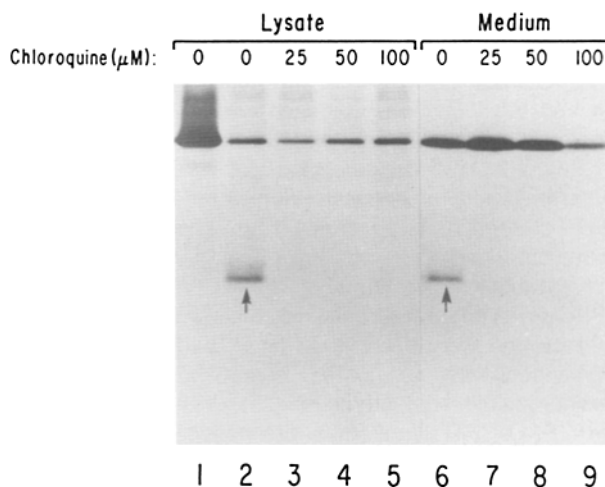
Figure 7. Effect of chloroquine on PRO-GLO processing. GH₃PRO-GLO cells were pulse labeled with [³⁵S]methionine for 15 min and chased for 120 min in the absence and presence of chloroquine. After 120 min of chase, the cell lysates and media were treated with antihemoglobin antiserum and the products resolved by SDS-PAGE. (Lane *1*) Cell lysate, 15-min pulse in the absence of chloroquine; (lanes *2-9*) 120-min chase, cell lysates and secreted material; (lane *2*) cell lysate in the absence of chloroquine; (lane *6*) medium, absence of chloroquine; (lanes *3* and *7*) cell lysates and medium, respectively, in the presence of 25 μM choloroquine; (lanes *4* and *8*) cell lysate and medium, respectively, 50 μM chloroquine; (lanes *5* and *9*) cell lysate and medium, respectively, 100 μM chloroquine. Arrows in lanes 2 and 6 indicate "mature" α-globin. Note the enhanced levels of PRO-GLO secretion in the presence of 25 and 50 μM chloroquine (lanes *7* and *8*).
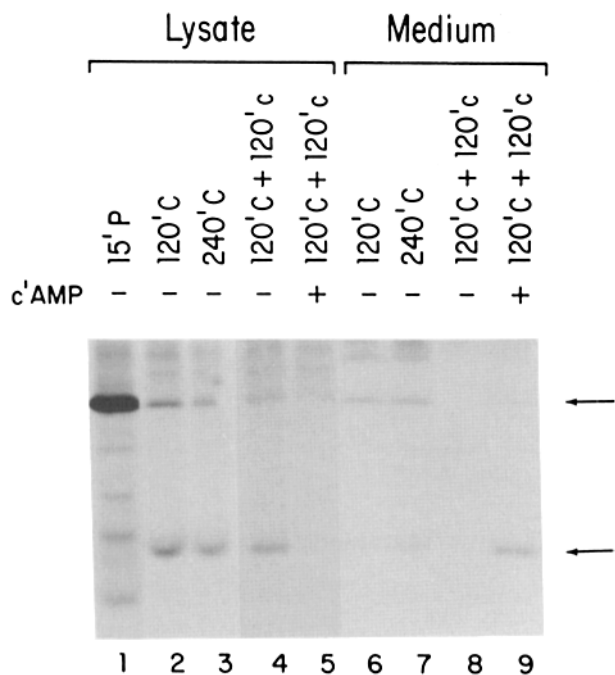
Figure 8. Secretagogue-mediated secretion of intracellular PRO-GLO and α-globin. GH₃PRO-GLO cells were pulse-labeled (P) with [³⁵S]methionine for 15 min (lane 1) and chased (C) for 2 (lanes 2 and 6) or 4 h (lanes 3 and 7). After 120 min of chase, the medium was removed and the cells incubated for an additional 120 min in the absence (lanes 4 and 8) or presence (lanes 5 and 9) of 5 mM 8-Br-cAMP. The cells and medium from each sample were then treated with antihemoglobin antisera and the globin-immuno-reactive material resolved by SDS-PAGE. (Top arrow) Migration of proSRIF-α-globin (M_r 25,000); (bottom arrow) α-globin endo-proteolytically cleaved from PRO-GLO.

ple, a fusion between growth hormone and a truncated form of a constitutively secreted viral glycoprotein (vesicular stomatitis virus glycoprotein [VSV-G]) resulted in transport to the regulated pathway (24). Although this result suggests that a positive signal in growth hormone confers a "dominant" sorting phenotype on the truncated VSV-G protein, the sorting sequence was not identified.

We previously showed that heterologous cells process proSRIF to mature SRIF and package it into vesicles which enter the regulated pathway (41). The targeting information could be contained in a structural feature of the proregion, the mature sequence, or the entire prohormone. Computer predictions suggest that proSRIF from several species contains conserved domains (2) which we hypothesize might be important for targeting to the regulated pathway. To investigate this possibility, we expressed two chimeric molecules in GH₃ cells: PRO-GLO and SIG-GLO. Both chimeras were translocated into the ER as judged by accurate cleavage of the signal peptide. While SIG-GLO rapidly turned over intracellularly and was not secreted, 30% of PRO-GLO traversed the entire secretory pathway. The majority (60%) of this PRO-GLO was cleaved to α-globin and was targeted to the regulated pathway (45%).

In the Absence of a Propeptide α-Globin Is Rapidly Degraded

The choice of α-globin for use in these experiments was

based on previous in vitro (17) and in vivo (40, 42) studies which indicate that α-globin is competent for translocation across the ER membrane when fused to a signal peptide; furthermore, no degradation of the in vitro–translocated α-globin was observed (17). The rapid disappearance of α-globin ($t_{1/2}$ = 4–5 min) after cleavage of the signal peptide demonstrated that globin alone did not contain sufficient sorting information to mediate secretion. The site of α-globin degradation was not determined but its rapid turnover indicated degradation shortly after completion of protein synthesis. The lack of inhibition by chloroquine, a drug that inactivates lysosomal enzymes, suggested that degradation did not occur in a lysosomal compartment. Since globin degradation was inhibited between 16 and 20°C, a temperature which prevents exit from the ER (37), the site of degradation may be between the ER and "early" Golgi regions. However at present, we cannot exclude the possibility that the degradation process is temperature sensitive; i.e., at low temperatures the proteases are inhibited and/or the substrates are less accessible. Thus, additional experiments are therefore necessary to identify the compartment where proteolysis occurs.

The rapid degradation of globin might be related to its inherent instability in the absence of β-globin or heme, as in certain thalassemias. It is also possible that α-globin was incompletely translocated into the lumen of the ER and degraded via the ubiquitin system; this possibility seems unlikely given its efficient translocation in Xenopus oocytes (40, 42) and in cell-free systems (17). The aforementioned studies failed to observe α-globin secretion from Xenopus oocytes; since these experiments used the same or a similar signal peptide–globin construction as was used here, most probably the resulting globin molecules were degraded intracellularly.

Our data are similar to those reported by Lippincott-Schwartz et al. (18) and Chen et al. (6) who demonstrated degradation of incompletely assembled T cell receptor (TCR) α- or Δ-subunits in the ER. The kinetics of globin degradation were significantly faster than for the TCR α- and Δ-subunits; we did not observe a lag period of 20 min before degradation and the half-life of globin was 4–5 min compared to ∼50 min for TCR α-chains. These results suggest the presence of enzymes which recognize and rapidly degrade "aberrant" proteins. This function may be distinct from any role BiP, the heavy chain binding protein (3), may have in controlling export of proteins from the ER. Our data and those on TCR assembly (6, 18) suggest that the ER or an "early" Golgi compartment has multiple proofreading mechanisms which regulate the flow of proteins through the secretory apparatus.

The SRIF Proregion–mediated Transport of α-Globin to the Regulated Pathway

Our results are consistent with the suggestion that the cellular sorting machinery recognizes precursor molecules rather than mature hormones (45), in this case a domain or structure in proSRIF. In contrast to the proinsulin C-peptide (35), the presence of the SRIF propeptide was essential for targeting globin to the regulated pathway. We speculate that the propeptide may enhance folding or oligomerization of the chimera which could be necessary for efficient exit from the ER as observed for other proteins (11). Alternatively, the

propeptide could facilitate efficient transport by preventing nonspecific interactions which might retard movement in the bulk phase (36). Once the precursor reached the TGN, sorting could result from intrinsic chemical properties of the molecules rather than a sorting signal per se. For example, during passage through the acidic environment of the post-TGN, a precursor with an acidic pI value might precipitate or aggregate as it reached its isoelectric point. Precipitation, which may require the presence of a suitable cofactor such as a metal ion (e.g., proinsulin and zinc), would result in a highly condensed nucleus of protein leading to the formation of immature secretory granules (30, 44, 45). In this context, the SRIF proregion confers an acidic pI on both the native molecule and α-globin. Mature SRIF and α-globin have predicted pIs of 9.15 and 9.78, respectively; while proSRIF and PRO-GLO have predicted pIs of 5.15 and 6.49, respectively. The propeptide of SRIF therefore may function to initiate precipitation and storage of the prohormone which is subsequently cleaved by processing enzymes activated in the acidic environment of forming secretory granules.

It might be argued that since only a fraction ($\sim$30%) of PRO-GLO was transported from the ER, that the secreted globin was transported to granules by default in these cells, or that the SRIF propeptide may have facilitated nonspecific associations with endogenous secretory proteins, thereby leading to "passive" sorting into the regulated pathway. The obvious candidate for such a protein is growth hormone which is by far the most abundant polypeptide synthesized by and secreted from GH$_3$ cells. However, we recently demonstrated (41) that, in these cells, only 5% of newly synthesized growth hormone is stored intracellularly in a cAMP-responsive compartment, whereas 95% is secreted constitutively. Consequently, if PRO-GLO were "sticking" to growth hormone, for example, only $\sim$5% would be expected to enter the regulated pathway. In fact our data demonstrate that $\sim$45% of the processed PRO-GLO is stored intracellularly; i.e., significantly more efficiently than endogenous growth hormone. This figure is consistent with the observation (44) that 55% of processed native proSRIF was sorted to the regulated pathway and secreted in response to 8-Br-cAMP. Approximately 90% of the stored intracellular material was secreted in response to cAMP; equivalent to a fourfold stimulation over the basal level of secretion. This observation is consistent with storage of globin in mature secretory granules. We conclude that the propeptide-mediated targeting to the regulated pathway and facilitated packaging of globin into a cAMP-stimulatable compartment. Morphologic studies are currently in progress to determine the precise cellular localization of these molecules.

Proteolytic Processing of PRO-GLO

Similar to observations for several native prohormones (7, 25, 29, 31) proteolytic processing of PRO-GLO required an acidic environment; cleavage was quantitatively inhibited even at low concentrations (25 μM) of chloroquine. High concentrations of chloroquine may be toxic to some cells, however, at the concentrations used in these experiments (25–100 μM) we observed no inhibition of total protein synthesis. Thus, although chloroquine was reported to not affect processing of proopiomelanocortin in pituitary AtT-20 cells (20), we observed processing inhibition at all chloroquine concentrations and the concomitant enhanced secretion of

unprocessed PRO-GLO. Similar results were observed for native proSRIF (41a). We interpret our results to suggest that PRO-GLO was transported to the distal elements of the Golgi apparatus/TGN and packaged into acid vesicles where proteolytic processing was initiated.

PRO-GLO was accurately processed indicating that the native propeptide cleavage site (ArgLys) was recognized by the paired basic processing enzyme(s). Thus, even though chimpanzee α-globin possesses a single pair of basic amino acids (Lys$_{61}$ and Lys$_{62}$), most probably these were not cleaved since no smaller forms of globin-related peptides were detected. Accurate processing demonstrated the conformational dominance of the propeptide and in particular the highly conserved hexapeptide region. This is noteworthy since α-globin is 10 times the size of mature SRIF and nearly twice the size of the proregion itself; thus, it is unlikely that the chimera and native precursor would be structurally related.

In conclusion, our results have identified two novel functions for a propeptide: partial protection of nascent precursors from degradative enzymes and targeting to the regulated secretory pathway. The SRIF proregion overcame the efficient ER/pre-Golgi "proofing" mechanisms and facilitated transport of α-globin to the distal elements of the Golgi apparatus. The proregion may have afforded protection from proteolysis by allowing the molecule to assume a conformation which (a) is not recognized as "abnormal" and therefore is not a substrate for proteolysis; and (b) mediated rapid ER to Golgi transport thus separating the substrate from the degradative enzyme; these two mechanisms probably act in concert.

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