A 13-Amino Acid N-terminal Domain of a Basic Proline-rich Protein Is Necessary for Storage in Secretory Granules and Facilitates Exit from the Endoplasmic Reticulum*

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We have investigated the role of different domains of a salivary basic proline-rich protein in intracellular transport and sorting of proline-rich proteins to the secretory granules. We have cloned a full-length cDNA of a basic proline-rich protein from the rat parotid and expressed it in AtT-20 cells. It was correctly sorted into secretory granules as shown by EM immunolocalization and by its presence in 8-bromo-cyclic AMP-stimulated secretion. Deletion of the N-terminal thirteen amino acid domain upstream from the proline-rich domain eliminated storage whereas deletion of the C-terminal 20-amino acid domain downstream from the proline-rich domain had no effect. Intracellular transport of full-length and mutant proline-rich proteins was unusually slow due to slow exit from the endoplasmic reticulum. However, the rate of transport increased with increasing level of expression for the full-length protein and the C-terminal deletion mutant. In contrast, the rate of transport of the N-terminal deletion mutant was independent of the level of expression. These results imply that the N-terminal domain is necessary for both storage and efficient intracellular transport. Moreover, interactions (self-aggregation?) that mediate sorting may begin as early as the endoplasmic reticulum.

Secretory proteins reach their extracellular destination by at least two pathways. All cells secrete proteins via vesicles which are rapidly transported from the site of their formation (trans-Golgi (Refs. 1-4) or immature secretory granules (Ref. 5)) to the site of their fusion with the plasma membrane. In specialized endocrine, neural, and exocrine cells many secretory products are stored in granules and released upon appropriate stimulation. In addition, different stored proteins and peptides can be packaged into distinct granules as in the acidophilic cells of the anterior pituitary (6) or the bag cell neurons in Aplysia (7). The coexistence within one cell of several secretory pathways implies that segregation of proteins must occur (8, 9). Some studies have pointed to the importance of selective aggregation or condensation of stored secretory proteins in the formation of granules (10-12). Other work has suggested the role of specific carriers (as in the sorting of lysosomal enzymes) in bringing about the transfer and subsequent concentration of stored proteins (13). However, it is possible that some aspects of both proposals apply to insure that storage occurs in specific membrane carriers.

Transfection studies with foreign cDNAs in cultured cells have shown that (a) sorting mechanisms may be shared among many different cell types (reviewed in Ref. 9) and (b) positive information is necessary for storage (14-16). As no consensus sequence has emerged among the large number of stored proteins, it has been proposed that sorting signals, however they function, may comprise higher order structural features (9, 17).

We have been interested in the mechanisms of sorting and packaging of salivary proline-rich proteins (PRPs)1 (18, 19). PRPs, especially basic PRPs, have certain structural features that seem unusually well suited for attempting to identify domains that might function in secretory sorting. They are elongate, unfolded polypeptides with no cysteine residues (20). They have four easily distinguishable domains consisting of a signal sequence (cotranslationally cleaved), a small N-terminal "transition" region, a proline-rich region consisting of repeated cassettes, and a C-terminal region (21). The proline-rich region comprises more than 70% of the sequence of the mature polypeptide and bears no compositional resemblance to other mammalian polypeptides that undergo secretory storage. Accordingly, putative sorting domains may be restricted to either the transitional or C-terminal domains. Finally, the extended conformation of PRPs suggests that mutation, even deletion of domains may have only minor effects on overall three-dimensional structure.

In this work we focus on the role of the different domains in the intracellular transport and sorting of a rat parotid basic PRP in AtT-20 cells. Using deletion mutants we show that the N-terminal domain may play a role in the storage of bPRP in dense core granules as well as in its efficient exit from the ER.

**EXPERIMENTAL PROCEDURES**

Antibodies—Endorphin antibody JH2 and ACTH antibody Kathy were a kind gift from Dr. Richard Mains (Johns Hopkins University, 1

1 The abbreviations used are: PRP, proline-rich protein; bPRP, basic proline-rich protein, referring specifically to the translation product of the cDNA bPRP23; ER, endoplasmic reticulum; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Endo H, endoglycosidase H; SDS, sodium dodecyl sulfate; 8-Br-cAMP, 8-bromocyclic AMP; POMC, proopiomelanocortin.
Pelvic disease is driven by the Moloney murine sarcoma virus long terminal repeat (pMV7). 100 ng of DNA/bPRP cDNAs were cloned into pLEN/PECE where the EcoRI site is present of canine pancreatic rough microsomes (24) followed by transcription using rabbit reticulocyte lysate (Promega, Madison, WI) in the absence of an upstream promoter. Mutations were confirmed by DNA sequencing. By the lack of hybridization to the oligonucleotide complementary to the deleted domain. Mutations were confirmed by DNA sequencing.

DNA transfections were carried out using a modified calcium phosphate method (25). pMV7-bPRP was used for the experiment 48-60 h later. The labeling was carried out in 5% dialyzed Nusserum, 20 mM HEPES, 1% Triton X-100 and the cell lysates were cleared by centrifugation for 10 min at 5,000 × g.

bPRP in the media and cell lysates was quantitated from immunoprecipitates. Samples were immunoprecipitated with appropriate antisera for 12-24 h at 4°C in radiolabeled immunoprecipitation buffer (20) containing 0.3 mg/ml phenylmethylsulfonyl fluoride, trypsin, and 1 μg/ml leupeptin. The immune complexes were harvested with protein A-Sepharose (Pharmacia LKB Biotechnology Inc.), washed three times with radiolabeled precipitation buffer containing 2 mg/ml BSA and once with phosphate-buffered saline, and resuspended in SDS sample buffer (31). The immunoprecipitates were run on 12.5% SDS-polyacrylamide gels (31), sliced into 1 mm slices, eluted with 5 mM sodium bicarbonate, 0.5 M urea, 0.1% SDS, and bPRP was quantitated by scintillation counting. To assure quantitative recovery, the antisera were used in excess.

For the determination of Endo H sensitivity, samples were immunoprecipitated as above, dissociated from protein A-Sepharose with 0.2% SDS, diluted to 0.05% SDS, 50 mM sodium acetate, pH 5.5, 0.2% Triton X-100 and incubated 20 h at 37°C with 0.05 milliliters/mg Endo H (Genzyme, Boston, MA). The digests were run on 12.5% gels and subjected to fluorography (32), quantitations were done by densitometric scanning of the bands.

**EM Immunolocalization—**Cells growing in 100-mm dishes were fixed for 30 min at room temperature with paraformaldehyde-linseis-peridote (33), scraped and pelleted in a microcentrifuge (11,000 × g for 10 min) and fixed for an additional 2.5 h. The pellets were processed for embedding in LR Gold essentially as described by Berryman and Rodewald (34). Post-embedding immunolabeling of bPRP and ACTH was carried out on thin sections mounted on nickel grids following quenching in 1 M ammonium chloride in Triton-buffered saline, pH 7.4. Affinity-purified anti-PRP antibody (100 μg/ml) and anti-ACTH serum Kathy (1:1000) were diluted in 1% ovalbumin, 1% normal goat serum, 0.05% Tween 20 in Tris-buffered saline, pH 7.4 (binding buffer), and sections were incubated 1 h at room temperature, washed in binding buffer, and immunostained 1 h with 10 nm gold-conjugated goat anti-rabbit IgG (EY Laboratories Inc., San Mateo, CA) diluted in binding buffer. Immunogold particles were quantitated on extended processes containing >10 recognizable dense-core secretory granules in transfected and nontransfected cells. Gold particles were counted on micrographs (magnification, ×25,000) in an outlined area of a process. Areas were estimated using a Zeiss SEM-IPS image processor.

**RESULTS**

**Sequence and Structure of bPRP32—**To obtain a full-length cDNA encoding a basic proline-rich protein, we used synthetic oligonucleotides to screen a parotid XZAP cDNA library. Using restriction mapping and partial sequence information we have identified one cDNA, bPRP23, (out of 10 positive clones) for further analysis and obtained a complete cDNA sequence. The sequence of bPRP23 is highly similar to the known portion of pRP25 (21). It extends from 10 nucleotides 5' of the initiation codon to the poly(A) tail and includes a coding sequence of 223 amino acids with a calculated molecular weight of 23 kDa. The primary sequence (Fig. 1A) contains four easily identifiable domains: the signal sequence (residues 1-16), the transition region (residues 17-33), a proline-rich region composed of one 18 amino acid segment and seven 19 amino acid repeats (residues 34-203), and the C-terminal region (residues 204-233). The cDNA contains a single consensus site for N-glycosylation at Asn-47. Both the primary sequence and the amino acid composition (35% proline, 20% glutamine, 16% glycine, and 8% arginine plus lysine) are characteristic of a basic proline rich protein (21). As discussed by Clements et al. (21) the signal sequence and the proline-rich repeat region are homologous to the same domains of other members of the proline-rich protein family. The transition and C-terminal domains show little primary sequence homology; however, their overall character is similar, with the transition region being acidic and the C-terminal region containing a few aromatic amino acids.

To assess the roles of the transition and C-terminal do-
mains of bPRP in intracellular transport and sorting, two deletion mutants were constructed. The first lacks a portion of the transition region (bPRPΔT), amino acids 21–33. The first four amino acids of the transition region were not deleted (bPRPAC) domains were constructed by oligonucleotide directed mutagenesis.

Western blot analysis of the secretion of clones expressing the full-length clone bPRP23 was derived from the nucleotide sequence. The nucleotide sequence agrees closely with the previously published sequence of pPRP25 (21) with only eight differences. Of those, four do not affect the encoded amino acid and where a different amino acid is predicted, it represents a conservative change. The consensus glycosylation site is underlined. The domains of the proline-rich proteins are indicated to the left of the sequence. The end of the signal sequence was assigned on the basis of the rules developed by von Heijne (35). The arrowheads indicate alternative but less likely sites of signal cleavage. B, schematic structure of bPRP and its mutants. Mutants lacking the transition (bPRPΔT) and the C-terminal (bPRPΔC) domains were constructed by oligonucleotide directed mutagenesis. Amino acids at the junctions of the domains are shown. S, signal sequence; T, transition region; P, proline-rich region; C, C-terminal region.

Expression of bPRP in AtT-20 Cells—The intracellular transport and sorting of bPRP and its mutants was analyzed in the mouse pituitary cell line AtT-20. This cell line has retained the ability to store secretory products and discharge them upon secretagogue stimulation (36, 37), and it has been shown to sort correctly a variety of endocrine hormones as well as the exocrine protein trypsinogen (9).

Western blots of the secretion of clones expressing the full-length bPRP contained a prominent 33,000 band and two minor bands at 31,000 and 33,000 (Fig. 2B). All bands among the pLEN clones. Secretion from the pMV7 clone indicated much lower expression level in this clone.

Expression of bPRP and its mutants. A, Western blot of proteins secreted during a 4-h period from transfected clones carrying pLEN-bPRP (1B2, 2C1, 1A1) and pMV7-bPRP (A2) and nontransfected AtT-20 cells (NT). Note the different intensities of all bands among the pLEN clones. Secretion from the pMV7 clone A2 was collected from 8 times as many cells as for the pLEN clones, indicating much lower expression level in this clone. B, Endo H digestion of 3H-labeled polypeptides translated in vitro in the presence of canine rough pancreatic microsomes from capped mRNA transcribed from bPRP23. The untreated sample contains bands of the same Mr as the secretion from transfected cells. Endo H digestion results in the shift of the 33,000 band to 31,000, indicating glycosylation of the 33,000 species. C, immunoprecipitates of coupled in vitro transcription/translation of full-length and mutant bPRP cDNAs in the presence of microsomes contain two bands. As shown for the full-length bPRP, the upper and lower bands correspond to the glycosylated and unglycosylated species, respectively. The apparent molecular weights of the glycosylated species are indicated in the figure and are 33,000 for the full-length bPRP, 32,000 for bPRPΔT, and 31,500 for bPRPΔC.
pLEN clones (Fig. 2A). To estimate the relative level expression of bPRP, bPRP was immunoprecipitated from extracts of cells labeled to approach steady state (15 h) with [3H]proline. The results presented in Table I show an approximately 5-fold difference in the content of full-length bPRP between the clones transfected with pMV7-bPRP and those transfected with pLEN-bPRP. Clones transfected with the mutant cDNAs in pLEN (bPRPAT-pLEN and bPRPΔC-pLEN) showed similar variation in the content of mutant bPRP (Table I). The content of [3H]proline-labeled ACTH-related species was estimated in some of the same experiments (Table I). It varied at most by a factor of 1.8, and the variation was not correlated to the level of bPRP. The content of ACTH-related species was at least 10 times higher than the content of bPRP. Thus in contrast to the situation when neuropeptide Y was expressed in AtT-20 cells (39), the expression of bPRP is not likely to perturb packaging of ACTH.

**Secretion and Storage of Full-length bPRP**—To address whether bPRP was packaged in secretion granules in AtT-20 cells, we initially examined stimulation of secretion by 8-Br-cAMP. Cells were metabolically labeled with [3H]proline for 15 h in order to (a) achieve sufficient labeling of bPRP and (b) allow for accumulation of labeled secretory proteins in the granule pool. Labeled cells were then chased for two consecutive intervals, and during the second interval 5 mM 8-Br-CAMP was added to discharge the granule contents. The quantitation of bPRP in the secretion and the cell extract for a sample experiment is shown in Fig. 3A. As can be seen, 8-Br-cAMP induced stimulation of bPRP secretion by approximately 1.5-fold. The net amount of bPRP undergoing stimulated discharge represented 6% of total. We observed a comparable (6%) decrease in the amount of bPRP remaining in the cell extract after stimulation. In the same experiment, secretion of ACTH was stimulated approximately 7-fold, with the net amount of ACTH stimulated corresponding to 50% of the total (Fig. 3B). Although the stimulation of secretion of bPRP was smaller than observed for ACTH, it was reproducible in several experiments and in clones with different levels of expression (Table II) indicating that a fraction of bPRP is indeed stored in the granules.

Since the degree of stimulation was relatively low, we wanted to confirm the presence of bPRP in granules by an independent method. Thus we determined the subcellular localization of bPRP and mature ACTH (as the granule marker) in transfected AtT-20 cells by EM immunocytochemistry. Staining with the anti-PRP antibody and anti-ACTH antibody showed clear labeling of dense core granules in both cases (Fig. 4). Quantitation of the bPRP and ACTH labeling in the tips of extended processes showed that the staining was specific (Fig. 4). With the anti-PRP antibody, additional staining was observed within other small vesicles and vacuoles (Fig. 4C). Because we were unable to improve the morphology without significant loss of bPRP antigenicity we could not unambiguously assign the additional stained structures. They could represent components of the ER and Golgi or secretory vesicles carrying bPRP via pathway(s) not sensitive to 8-Br-cAMP.

**Effect of Domain Deletions on Storage and Secretion of bPRP**—To determine if deletion-mutant bPRPs were stored in the secretory granules of transfected cells, 8-Br-cAMP-induced secretion of bPRPΔT and bPRPΔC was compared to that of the full-length bPRP. AtT-20 cells expressing either bPRPΔT or bPRPΔC at approximately the same level were metabolically labeled for 15 h with [3H]proline and subjected to the same chase protocol as described above for bPRP. Table II presents the net percent of mutant bPRP secreted during the second chase in response to 8-Br-cAMP stimulation. Stimulus-dependent secretion of bPRPΔT was comparable to that of full-length bPRP; however, 8-Br-cAMP did not cause an increase in the secretion of bPRPΔC. These results were reproducible among clones expressing different levels of mutant bPRP (data not shown). This intriguing finding suggests that bPRPΔT is not present in the granules (or only to a very low level), which implies that the transition region is necessary for targeting bPRP to granules and/or for retention in granules. To confirm that the lack of stimulus-dependent secretion of bPRPΔT was not simply due to a failure of the transfected cells to respond to secretagouge, stimulation of secretion of ACTH was confirmed for each experiment and was identical to that shown in Fig. 3B.

![Image](3A.png)  

**Fig. 3**. Effect of 8-Br-cAMP on the secretion of bPRP and ACTH for the pMV7-bPRP clone A2. Duplicate wells of cells were labeled for 15 h with [3H]proline and chased first for 6 h (S1), and then for 3 h (S2). Cells (C) were harvested at the end of the experiment. During the second chase 5 mM 8-Br-cAMP was added to one well as indicated. For each condition the radioactively labeled bPRP (expressed in cpm) in the chases and the remaining cell extract was normalized to total labeled bPRP (the sum of cpm of bPRP in the chases and in the cell extract). Radioactively labeled ACTH (the sum of cpm in POMC, the ACTH biosynthetic intermediate and ACTH after each was corrected for proline content) was quantitated in the same way. Data are presented as percent of total cpm of bPRP (A) and ACTH (B). The value for the first chase represents an average from the two wells with the error bars indicating the range.
Fig. 4. EM immunocytochemical localization of bPRP and ACTH in the pLEN-bPRP clone 1A1. A, labeling with the ACTH-specific antibody Kathy gives heavy staining in the secretory granules of a process. Quantitation of the ACTH labeling in cell processes gave 87 ± 13 (S.E.) gold particles/µm². B, labeling with the anti-PRP antibody revealed with gold-conjugated goat anti-rabbit antibody shows clear staining of secretory granules (arrowheads) in the tip of a process. Quantitative evaluation of the staining in cell processes gave 23 ± 3 (S.E.) gold particles/µm² in the transfected clone and 1.5 ± 0.5 (S.E.) gold particles/µm² for nontransfected AtT-20 cells. C, in the cell body staining with the anti-PRP antibody is over large vacuoles (large arrow) and small vesicles (small arrow). Note the lack of nuclear (n) staining. Bar, 0.25 µm.

The amount of cell-associated Endo H-resistant bPRP is another measure that approximates the level of bPRP in granules. Cells expressing full-length bPRP, bPRPΔT, or bPRPΔC were labeled to steady state, chased for 8 h to allow labeled proteins to exit the ER, and secreted and cell-associated bPRPs (full-length and mutant) were analyzed by Endo H digestion. In all cases the secreted bPRP was Endo H-resistant while the majority of cell-associated bPRP was Endo H-sensitive. Densitometric analysis of the cell-associated bPRP indicated that 15% of the full-length bPRP and 33% of bPRPΔC was Endo H-resistant, while at most 4% of bPRPΔT was Endo H-resistant. Positions of the Endo H-resistant (r) and sensitive (s) forms of bPRP are indicated.

The cell-associated full-length bPRP and bPRPΔC was Endo H-resistant (Fig. 5). The same trend was observed at shorter and longer chase times, and an increasing proportion of the full-length bPRP and bPRPΔC became resistant with increased length of chase. These results are consistent with the presence of full-length bPRP and bPRPΔC in post-Golgi compartments, presumably the secretory granules, and the relative lack of accumulation of bPRPΔT therein.

Rate of Exit of the Full-length bPRP from the ER—Comparison of the secreted and cell-associated bPRP following an 8 h chase (Fig. 5) indicates that ~40% of total labeled bPRP is still cell-associated. Furthermore, a large fraction (~85%) of the cell-associated bPRP is still Endo H-sensitive. Taken together, these results suggest an accumulation of bPRP in the ER with a slow exit to subsequent compartments.

The rate of exit of bPRP from the ER was estimated from the kinetics of loss of Endo H sensitivity (Fig. 6). For the clone expressing full-length bPRP depicted in Fig. 5, bPRP exited the ER with a half-time of 6.0 h (Fig. 6, Table III, clone pMV7-bPRP/A2), which is much slower than has been observed for other secretory proteins. Even though bPRP was present in the ER for a long time, it was not degraded; we were able to recover the same total amount of ³H-labeled bPRP for at least 12 h of chase.

Compared the t₁/₂ of loss of Endo H sensitivity among different clones expressing bPRP indicated that the rate of exit from the ER increased with higher level of bPRP expression (t₁/₂ER = 2.5 h for the highest expressing clone, t₁/₂ER = 6.0 h for the lowest expressing clone, Fig. 6, Table III). This surprising observation suggests that bPRP-bPRP interactions may facilitate the exit of bPRP from the ER. In all cases tested, the rate of exit of bPRP from the ER was reiterated in the rate of unstimulated exit from the cell (Table III) as

| Clone       | Secretion of bPRP | Secretion of bPRPΔC | Secretion of bPRPΔT |
|-------------|-------------------|---------------------|---------------------|
| pMV7-bPRP   | 6.1 ± 0.5 (5)     | 5.2 ± 0.8 (4)       | 6.8 ± 0.9 (5)       |
| A2          |                   |                     |                     |
| pLEN-bPRP   | 6.8 ± 0.3 (6)     | 6.8 ± 0.9 (5)       | 0 ± 0.3 (7)         |
| pLEN-bPRPΔC |                   |                     |                     |
| 1A1         |                   |                     |                     |
| pLEN-bPRPΔT |                   |                     |                     |
| 2C1         |                   |                     |                     |
| pLEN-bPRPAC |                   |                     |                     |
| C21         |                   |                     |                     |
| pLEN-bPRPΔT |                   |                     |                     |
| T26         |                   |                     |                     |

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**Table II**

8-Br-cAMP-dependent secretion of full-length and mutant bPRP

Experiments and their analysis were performed exactly as described in Fig. 3. Stimulated secretion represents the difference between percent of total bPRP secreted in the presence and that secreted in the absence of 8-Br-cAMP. Data are expressed as mean ± S.E. Numbers in parentheses indicate the number of experiments.
Kinetics of exit from the ER of full-length bPRP. Transfected cells were labeled for 15 h with \[^{3}H\]proline and chased for varying lengths of time up to 12 h. At each timepoint secretion and cell extracts were harvested, immunoprecipitated with the anti-bPRP antibody, digested with Endo H, and subjected to SDS-PAGE and fluorography. Labeled bPRP was quantitated by densitometry and the data were expressed as percent of total bPRP that was Endo H-sensitive. Linear regression analysis was performed to obtain the lines.

would be expected for a protein which is stored in secretory granules to at most a low level.

Kinetics of Secretion of Mutant bPRPs—We were interested to know whether deletion of the transition or the C-terminal domains of bPRP affected the dependence of ER export rate on the level of bPRP expression. As for the full-length bPRP, the rate of exit of mutant bPRPs from the ER was estimated from the kinetics of loss of Endo H sensitivity. The clones expressing low levels of both bPRP and bPRP AC exhibited similar kinetics of exit from the ER as the full-length clone. At higher expression levels bPRP AC exited the ER more rapidly (decreased \(t_{1/2,\text{ER}}\), Table III) as in the case of the full-length bPRP. Notably, higher expressing clones of bPRP AC behaved differently; there was no increase in the rate of exit from the ER with increased level of expression (Table III). For clone T1, a \(t_{1/2,\text{ER}}\) of approximately 5 h was expected based on its level of expression; yet the measured \(t_{1/2,\text{ER}}\) was 10 h, largely unchanged (or even slightly increased) as compared to the lower expressing clone, T26.\(^3\) These results imply that the transition region of bPRP is important not only for targeting bPRP for storage in granules but also may play a role in promoting its transit through the ER.

**Efficiency of bPRP Storage in Secretory Granules**—As a consequence of the slow rates of exit of full-length and mutant bPRPs from the ER, a fraction of bPRP has not yet reached the trans-Golgi (the presumed site of protein sorting and granule formation) at the time that cells were stimulated with 8-Br-CAMP in the protocol described above. Moreover, this fraction was different for clones with different levels of expression due to the varying rates of exit from the ER. Thus the efficiency of storage may be reflected better by normalizing the amount of 8-Br-cAMP-stimulated bPRP to Endo H-resistant (rather than total) bPRP (Table IV). Notably, the percent of Endo H-resistant bPRP stimulated with 8-Br-CAMP decreased with increased level of expression for the full-length bPRP. The same trend was observed for bPRP AC (not shown). Thus bPRP is stored less efficiently in clones expressing it at higher levels, pointing to a limitation in the packaging of bPRP into the granules.

**DISCUSSION**

Storage of bPRP—We have been using a salivary basic proline-rich protein, bPRP derived from bPRP23 cDNA, as a prototype to identify domains that function in secretory sorting and consequently linear organization of structural domains of bPRP contrasts with the globular configuration of many stored secretory proteins and peptides. These features are potentially quite favorable for analyzing the determinants of sorting provided that bPRP and other proteins are packaged into granules by a common mechanism. When bPRP was expressed in AtT-20 cells, it was found to be present in granules that are released upon stimulation with 8-Br-cAMP. This behavior can be contrasted with that of von Willebrand factor, which is also an unusually elongated molecule. The latter was packaged within separate, 8-Br-cAMP-insensitive granules in AtT-20 cells (40). Thus, in spite of its unusual shape and highly positive charge, bPRP is packaged into the granules in AtT-20 cells by a mechanism which may share similarities with that for the neuroendocrine hormones of AtT-20 cells.

Compared to ACTH and other stored proteins that have been expressed in AtT-20 cells, the rate of bPRP stimulated by 8-Br-cAMP was rather low (i.e. ~6-7% for bPRP versus 50% for ACTH (Fig. 3B), 20% for trypsinogen (41), 35% for insulin (42)). The relatively low storage could

\(^3\)The slow rate of unstimulated secretion of bPRP T was probably not due to inefficient passage into the ER since the signal sequence was cleaved as efficiently for the mutant as for the full-length clone in an in vitro translation (Fig. 2C).

**Table III**

**Kinetics of exit of full-length and mutant bPRP from the ER and the cell**

The experiments for determination of the \(t_{0}\) of exit from the ER (\(t_{0,\text{ER}}\)) were performed as described in Fig. 6. The values of \(t_{0}\) were calculated from log-linear plots of percent of total bPRP that was Endo H-sensitive versus time (as in Fig. 6). For the determination of the \(t_{0}\) of exit from the cell (\(t_{0,\text{Cel1}}\)) the experiments and their analysis were performed similarly except that the Endo H digestions were omitted and the data was expressed as percent of labeled bPRP that was cell-associated. For reference, normalized bPRP content was calculated in relation to the cpm of the lowest expressing clone, pLEN-bPRPAC/C21 (Table I).

| Clone     | pMV7-bPRP | pLEN-bPRP | pLEN-bPRPAC | pLEN-bPRPAC/C21 |
|-----------|-----------|-----------|-------------|-----------------|
|           | A2        | A4        | 1B2         | 2C1             | 1A1             | T26   | T1   | C21  | C18  |
| \(t_{0,\text{ER}}\) (h) | 6.0       | 6.0       | 4.1         | 3.4             | 2.5             | 7.0   | 9.7  | 7.1  | 3.2  |
| \(t_{0,\text{Cel1}}\) (h) | 7.0       | 7.2       | 5.3         | 4.4             | 3.4             | 7.8   | 10.0 | 8.6  | 4.4  |
| Normalized bPRP content | 1.8       | 1.8       | 3           | 6.8             | 15              | 2.3   | 6.8  | 1    | 14.3 |
be due to several factors. First, bPRP may have a "weak" or suboptimal sorting signal in relation to those on other polypeptides that have been examined in AtT-20 cells. According to this interpretation the decreased efficiency of storage of bPRP at higher levels of expression would suggest limiting amounts of sorting machinery. However, this is unlikely, since the sorting of endogenous ACTH was unaffected.

A second contributing factor may be the shape of bPRP as well as its substantial net positive charge. As selective aggregation among secretory proteins is probably an important factor in storage (and possibly sorting) of secretory proteins (10–12), the unusual structure of bPRP may not be conducive to coaggregation with the endogenous secretory products of AtT-20 cells even though the latter include positively-charged as well as negatively-charged components. The situation in AtT-20 cells should be contrasted with that in parotid acinar cells (normal and isoproterenol-treated), where proline-rich proteins are present and very efficiently packaged as a whole family including both acidic and basic species (18). Notably, this emphasizes the possibility that there may be cell-type specific variations among "universal" secretory sorting processes.

A third factor that could contribute to the low level of storage of bPRP in relation to other secretory proteins in AtT-20 cells is the much slower rate of exit of bPRP from the ER. Because the rate of exit of bPRP from the ER ($t_{1/2,ER} = 5$ h for the low expressing clones) approximates the rate of basal release of secretory granules ($t_{1/2} = 7$ h (29)) no appreciable accumulation of bPRP in the granules can occur. In contrast, the rapid exit of proopiomelanocortin from the ER ($t_{1/2} = 0.5$ h) allows for a very effective accumulation of proopiomelanocortin-derived peptides in the secretory granules. If the rate of export from the ER was the only factor governing the extent of storage, then accumulation of bPRP in granules should increase at higher levels of bPRP expression when the rate of export from the ER increases. However, we observed lower, rather than higher, fractional storage with increased bPRP expression, suggesting that there is a limit on how much bPRP can be packaged in the AtT-20 secretory granules.

**Intracellular Transport and Storage of Mutant bPRPs**—One of our most striking findings was that deletion of 13 amino acids from the N-terminal "transition" domain of bPRP resulted in loss of storage in AtT-20 cell granules based on the absence of detectable 8-Br-CAMP-stimulated secretion of bPRPΔT. In contrast, deletion of the C-terminal region had almost no effect on the extent of storage. These observations suggest that the 13-amino acid region alone, within the relatively extended structure of bPRP, may contain a sorting determinant for storage in secretory granules. Examination of this domain in relation to domains of other secretory proteins that have been implicated in secretory sorting (N-terminal region of POMC (16), the proregion of prosomatostatin (15), the minimal transforming region of platelet-deprived growth factor (43) has revealed no primary sequence similarity to the transition domain of bPRP. Furthermore, this domain does not conform to the structural characteristics of a secretory sorting signal that have been predicted using computer modeling (17). Although there may be a tendency to invoke the presence of a universal sorting determinant on the surface of a folded protein in the absence of primary sequence homology, we feel that the identification of a non-homologous signal on a relatively unfolded protein may favor a different situation. That is, there may be a variety of distinct signals that function in the sorting of proteins for storage in secretory granules.

We emphasize that we have not yet established whether the transition domain actually contains a positive sorting signal. It remains possible that the observed low level of bPRP storage is not a result of positive sorting but rather the result of passive retention within the granules. The deletion of the transition domain increases the eccentric nature of bPRP and thereby could eliminate retention in granules. To begin to distinguish among these possibilities, it will be extremely important to determine whether the transition domain is sufficient for targeting non-granule reporter proteins to the storage compartment.

The simultaneous effect of deleting the transition domain on storage in secretory granules and exit from the ER suggests that similar factors play a role in ER exit and storage in secretory granules. Among these factors, a prime candidate is the self-self interaction among bPRPs that has been suggested on the basis of the positive correlation of the rate of exit from the ER and the level of bPRP expression. Such interaction (or aggregation) may contribute to the sorting mechanism. Structurally, it would not be surprising if deletion of the transition domain impaired self-self interaction as it removes all but one negatively charged amino acid from bPRP. The resulting polypeptides either may have too high a net positive charge to undergo effective interaction or may have a reduced capacity to interact with aggregating ions such as calcium. Recent work has pointed to the importance of calcium and pH in mediating the aggregation of neuroendocrine chromogranin/secretogranin in late and post-Golgi compartments (12). It is interesting to note that the transition regions of PRPs identified so far all are acidic (21, 38, 44–46) and thus could be involved in calcium binding.

As a final consideration, it is important to bear in mind that the putative secretory sorting domain that we have identified is not very efficient in the context of the endogenous secretory proteins in pituitary AtT-20 cells. Interactions involving the transition domain of bPRP may enable completion of only part of a hierarchy of interactions comprising the efficient sorting/storage process. Indeed, in salivary acinar cells, PRPs are always present as a family of structurally similar proteins where homologous as well as heterologous associations probably contribute to their efficient intracellular transport and storage in granules. Although our findings and those of others clearly implicate the presence of common secretory sorting mechanisms among different cell types, we suggest that the overall composition of sorting mechanisms may be cell type-specific.

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