The unique proline-rich domain of parotid proline-rich proteins functions in secretory sorting

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SUMMARY

When expressed in pituitary AtT-20 cells, parotid proline-rich proteins enter the regulated pathway. Because the short N-terminal domain of a basic proline-rich protein is necessary for efficient export from the ER, it has not been possible to evaluate the role of this polypeptide segment as a sorting signal for regulated secretion. We now show that addition of the six-amino acid propeptide of proparathyroid hormone to the proline-rich protein, and especially to a deletion mutant lacking the N-terminal domain, dramatically accelerates intracellular transport of these polypeptides. Under these conditions the chimeric deletion mutant is stored as effectively as the full-length protein in dense core granules. The propeptide does not function as a sorting signal in AtT-20 cells as it does not reroute a constitutively secreted reporter protein to the regulated pathway. During transit, the propeptide is cleaved from the chimeric polypeptides such that the original structures of the full-length and the deletion mutant proline-rich proteins are reestablished. We have also found that the percentage stimulated secretion of the proline-rich proteins increases incrementally (almost twofold) as their level of expression is elevated. The increase reflects an enrichment of these polypeptides in the granule pool and its incremental nature suggests that sorting of proline-rich proteins involves an aggregation-based process. Because we can now rule out contributions to sorting by both N- and C-terminal segments of the proline-rich protein, we deduce that the unique proline-rich domain is responsible for storage. Thus at least some of the determinants of sorting for regulated secretion are protein-specific rather than universal.

Key words: Proline-rich protein, Protein sorting, Regulated secretion, AtT-20 cell

INTRODUCTION

In endocrine and exocrine secretory cells, storage granules are formed from a subset of the proteins that are transported through the secretory pathway. Molecular sorting is required to determine which proteins are retained in these granules for stimulus-dependent exocytosis. Several studies have implicated the function of specific cellular machinery and interactions among secretory proteins, especially selective aggregation (Chanat and Huttner, 1991; Kuliawat and Arvan, 1994; Colomer et al., 1994), in these sorting events (reviewed by Arvan and Castle, 1992; Bauerfeind and Huttner, 1993). Transfection of cultured cells with cDNAs encoding secretory proteins from different cell types has shown that several exogenous proteins are sorted as efficiently as the endogenous secretory products (e.g. Moore and Kelly, 1985; Burgess et al., 1985; Moore et al., 1983; Dickerson et al., 1987), suggesting that targeting for regulated secretion might involve a common mechanism. However, other proteins are not sorted as efficiently as endogenous products (e.g. Fennewald et al., 1988; Castle et al., 1992; Schmidt and Moore, 1994; Reaves et al., 1990; Stoller and Shields, 1989), and recent work has suggested that sorting processes may differ among types of cells and types of secretory products (Colomer et al., 1994). Thus the sorting machinery may be more diverse than originally anticipated.

Several efforts have been made to identify structural determinants within secretory proteins that may function in sorting. Among endocrine and exocrine proteins, structural regions have been identified that are either necessary or sufficient for targeting to granules (Stoller and Shields, 1989; Chu et al., 1990; Cool et al., 1995; Arrandale and Dannies, 1994; Castle et al., 1992). Many, but not all, of these regions are located at or near the N termini of the polypeptides. No bona fide consensus structural signal has been identified, although common structural motifs involved in sorting have been postulated (Kizer and Tropsha, 1991; Gorr and Darling, 1995). However, there is limited insight regarding how these domains function in sorting.

We have been examining the mechanisms of sorting of salivary proline-rich proteins (PRPs). PRPs are elongate proteins lacking disulfide bonds and they have an unusually simple structure consisting of three domains: a short N-terminal segment, a central proline-rich domain of repeating cassettes, and a short C-terminal segment. They are efficiently targeted to secretion granules in parotid acinar cells (Arvan and
Castle, 1986; Blair et al., 1991), and when expressed from cloned cDNAs in a pituitary cell line, AtT-20, they are stored in modest amounts in the dense core granules (Castle et al., 1992; Castle and Castle, 1993). Deletion of the N-terminal thirteen amino acid segment (transition region) from a basic PRP eliminated storage of the protein but also resulted in its inefficient transport from the ER. Because the ability to detect regulated secretion of biosynthetically labeled proteins is compromised by slow ER export, we were not able to determine whether the transition region was truly needed for storage in granules or whether the deletion mutant (PRPΔT) would be stored if its ER export was efficient. Consequently, we have sought ways to restore more rapid ER export of PRPΔT so as to allow examination of its storage in dense core granules.

We now report that rapid export of PRPΔT can be achieved by attaching the hexapeptide prosequence of parathyroid hormone (PROPTH) to its N terminus. We selected PROPTH for this approach for several reasons. First, PROPTH is normally removed rapidly and efficiently from PTH during intracellular transport (Habener et al., 1979; Hellerman et al., 1984) and we hoped that similar processing might occur from the chimera, thereby generating PRPΔT. Second, PROPTH is thought to function in translocation into the ER but does not affect post-ER transport through the secretory pathway in GH4 cells (Wieren et al., 1988). Third, PROPTH is short and its strongly basic and hydrophilic character differs from the amphipathic nature of hydrophobic helical motifs that have been postulated to function in post-Golgi sorting for regulated secretion (Kizer and Tropsha, 1991; Gorr and Darling, 1995). Finally, the PTH signal and propeptide have been used in chimeras previously (Blair et al., 1986; Blair et al., 1991), and when expressed from both chase media and cell lysates were quantitated and the percentage of secretagogue and then chased for an additional 3 hours in the absence (basal secretion) or the presence (stimulated secretion) of 5 mM 8-Br-cAMP. Radiolabeled PRP- or ACTH-related polypeptides in the medium and cell lysates were quantitated and the level of expression was calculated as the total amount of radiolabeled polypeptide synthesized during the labeling (sum of radiolabeled polypeptide from the medium and cell lysate). The data were corrected for the number of prolines in each molecule and total trichloroacetic acid-precipitable counts in each sample. The level of expression was measured following a 15 hour labeling period with [3H]proline. Radiolabeled PRP-related polypeptides in the medium and cell lysates were quantitated and the level of expression was calculated as the total amount of radiolabeled PRP-related polypeptide synthesized during the labeling (sum of radiolabeled polypeptide from the medium and cell lysate). The data were corrected for the number of prolines in each molecule and total trichloroacetic acid-precipitable counts in each sample.

Materials and Methods

Antibodies
Anti-ACTH (adrenocorticotropic hormone) antiserum and the polyclonal antibody against PRPs were characterized previously (Blair et al., 1991; Castle and Castle, 1993). The polyclonal antibody against the vesicular stomatitis protein G (Indiana strain) was a gift from Dr. Robert Wagner (University of Virginia).

Construction of vectors
PROPTH-PRPΔT and PROPTH-PRP
The signal and pro sequences of preproPTH were synthesized by the polymerase chain reaction (PCR) with a HindIII restriction site included at the 5' end and an EcoRI site at the 3' end. The prepro fragment was cloned into HindIII and EcoRI sites of pBluescript (Stratagene, Inc., CA). Fragments encoding PRP and PRPΔT lacking the signal sequence were synthesized by PCR with an EcoRI site at the 5' end and cloned into pBluescript containing the prepro sequence of PTH. The extra bases resulting from inclusion of the EcoRI site were deleted by oligonucleotide-directed mutagenesis according to the instructions from Bio-Rad (Muta-Gene kit, Bio-Rad Laboratories, Richmond, CA). Mutated clones were screened by limited DNA sequencing, and the selected constructs were sequenced in entirety to confirm their structure. For expression in eukaryotic cells, the cDNA fusion constructs were excised from pBluescript and cloned into the HindIII/BamHI site of the pBhR1100 expression vector (provided by Dr Tim Reudelhuber, Montreal Cancer Research Institute, Quebec) which is driven by the Rous sarcoma virus long terminal repeat (Chu et al., 1990).

HISPTH-PRPΔT
HISPTH-PRP was obtained by site-directed mutagenesis of a restriction fragment containing the signal and propeptide sequences of preproPTH and 93 base pairs of PRPΔT. The sequence of the entire mutagenized fragment was confirmed by sequencing. The mutagenized fragment was then attached to the remainder of PRPΔT using standard cloning techniques.

tG and PROPTH-tG
The cDNA for tG was a gift from Dr. J. Rose and was cloned into the pLEN expression vector (Neufeld et al., 1987). The signal and propeptide sequences of preproPTH with added 5’ SalI and 3’ HindIII sites were synthesized by PCR, inserted into pBluescript, and the primary structure was confirmed by DNA sequencing. tG cDNA lacking nucleotides encoding a signal sequence was also synthesized by PCR, and was inserted into the plasmid containing the signal and propeptide sequence of preproPTH. The amino acid sequence across the junction of PTH propeptide and tG was KRSK/LKTS where the underlined amino acids result from HindIII. The final construct was cloned into the pLEN expression vector.

Cell culture and transfection
Mouse pituitary AtT-20 D16v cells were cultured as described previously (Castle et al., 1992). DNA transfections were carried out by calcium phosphate precipitation. Selection of stable transfectants and screening for expression of polypeptides by western blotting were also performed as described (Castle et al., 1992).

Metabolic labeling and immunoprecipitation
Cells expressing PRP, PROPTH-PRP, PROPTH-PRPΔT or HISPTH-PRPΔT were plated in 24-well plates with 1x10^5 cells/well. After 48-72 hours in culture, cells were labeled for 15 hours with 0.4 mCi/ml [3H]proline (Amersham Corp.) and chased as specified in individual experiments. The cells and medium containing secreted proteins were harvested and radiolabeled PRP-related polypeptides or ACTH were immunoprecipitated and quantitated by SDS-PAGE and scintillation counting as previously described (Castle et al., 1992).

Level of expression was measured following a 15 hour labeling period with [3H]proline. Radiolabeled PRP-related polypeptides in the medium and cell lysates were quantitated and the level of expression was calculated as the total amount of radiolabeled PRP-related polypeptide synthesized during the labeling (sum of radiolabeled polypeptide from the medium and cell lysate). The data were corrected for the number of prolines in each molecule and total trichloroacetic acid-precipitable counts in each sample. The level of expression of ACTH-related peptides was estimated from the same experiment as the sum of radiolabeled proopiomelanocortin, ACTH biosynthetic intermediate and ACTH. The data were corrected for the number of prolines in each molecule and total trichloroacetic acid-precipitable counts in each sample.

To measure the percentage stimulated secretion, duplicate wells of cells were labeled for 15 hours, chased first for 6 hours in the absence of secretagogue and then chased for an additional 3 hours in the absence (basal secretion) or the presence (stimulated secretion) of 5 mM 8-Br-cAMP. Radiolabeled PRP- or ACTH-related polypeptides from both chase media and cell lysates were quantitated and the per-
percentage stimulated secretion was calculated as the difference between stimulated and basal secretion of radiolabeled polypeptides, expressed as the percentage of total (chases + cell extract).

Cells expressing tG and PROPTH-tG were plated in 35 mm dishes at a density of 3×10^5 cells per dish. After 48-72 hours, the cells were labeled for 1 hour with 0.25 mM/ml EXPRESS 35S-labeled (ICN Radiochemicals, Irvine, CA) in minimal essential medium (Gibco BRL, Gaithersburg, MD) supplemented with 15% dialyzed Nuserum, 20 mM Hepes, 4 mM glutamine or for 15 hours with 0.15 mM/ml EXPRESS 35S-labeled in the same medium supplemented with 2.5 μg/ml of methionine and 4 μg/ml of cystine. Chases were carried out in the same medium containing excess cold methionine and cystine plus Trasylol as proteinase inhibitor (50 kallikrein units/ml).

**Determination of ER exit rates**

To estimate ER exit rates, cells were labeled with [3H]proline for 15 hours and chased for different lengths of time. At each timepoint, medium and cells were harvested, immunoprecipitated with anti-PRP antibody and digested with Endoglycosidase H (Endo H) and analyzed as described previously (Castle et al., 1992). Endo H-sensitive and Endo H-resistant bands were quantitated by densitometric scanning. Half-times of exit from the ER were obtained from log-linear plots of the fraction of each polypeptide that was Endo H-sensitive versus time.

**Quantitation of stimulated secretion using western blotting**

AtT-20 cells expressing PROPTH-PRP and PROPTH-PRPΔT were plated at equal cell densities in duplicate dishes. After 2-3 days, cell monolayers were washed thoroughly and incubated in serum-free medium with or without 5 mM 8-Br-cAMP for 3 hours. Cells were lysed and assayed for protein (BCA protein assay; Pierce, Rockford, IL) and DNA content (West et al., 1985). Aliquots of the medium were analyzed by SDS-PAGE followed by western blotting with the anti-PRP antibody. Bound antibody was detected with 125I-conjugated secondary antibody and the amount quantitated by phosphoimager analysis using ImageQuant software (Molecular Dynamics). The values were normalized to DNA content, and the net stimulated secretion was calculated as the amount of PRP secreted in the presence minus the amount secreted in the absence of 8-Br-cAMP.

**Automated microsequencing of PROPTH-PRPΔT**

AtT-20 cells expressing PROPTH-PRPΔT were biosynthetically labeled for 20 hours with 1 mCi/ml [3H]proline and labeled PROPTH-PRPΔT polypeptides were immunoprecipitated. The immunoprecipitates were heated for 15 minutes at 55°C in SDS sample buffer (Laemmli, 1970) containing 5 mM dithiothreitol and then treated with 10 mM iodoacetamide for 2 hours at 20°C in the dark. Samples were resolved on 12.5% tube gels which were sliced into 1 mm slices and treated with 10 mM iodoacetamide for 2 hours at 20°C in the dark. Samples were resolved on 12.5% tube gels which were sliced into 1 mm slices and eluted with 5 mM ammonium acetate. Bovine serum albumin (0.5 mM) was added as a carrier and the eluted material was exchanged into 0.01% SDS and less than 0.38 mM Tris using a Centricon microconcentrator (Amicon, Beverly MA). This preparation was loaded into 0.01% SDS and less than 0.38 mM Tris using a Centricon microconcentrator (Amicon, Beverly MA). This preparation was loaded into 0.01% SDS and less than 0.38 mM Tris using a Centricon microconcentrator (Amicon, Beverly MA). This preparation was loaded into 0.01% SDS and less than 0.38 mM Tris using a Centricon microconcentrator (Amicon, Beverly MA). This preparation was loaded into 0.01% SDS and less than 0.38 mM Tris using a Centricon microconcentrator (Amicon, Beverly MA). This preparation was loaded into 0.01% SDS and less than 0.38 mM Tris using a Centricon microconcentrator (Amicon, Beverly MA). This preparation was loaded into 0.01% SDS and less than 0.38 mM Tris using a Centricon microconcentrator (Amicon, Beverly MA). This preparation was loaded into 0.01% SDS and less than 0.38 mM Tris using a Centricon microconcentrator (Amicon, Beverly MA). This preparation was loaded into 0.01% SDS and less than 0.38 mM Tris using a Centricon microconcentrator (Amicon, Beverly MA). This preparation was loaded into 0.01% SDS and less than 0.38 mM Tris using a Centricon microconcentrator (Amicon, Beverly MA).

**Rates of export from the ER were estimated by following the kinetics of the loss of sensitivity to Endo H digestion (Castle et al., 1992).** As shown by the values of t1/2ER in Table 1, the presence of PROPTH causes a dramatic acceleration in transport of PRP. PROPTH-PRPΔT exited the ER with a t1/2ER of 1.3-1.4 hours in contrast to >7 hours for PRPΔT (Table 1). Similarly, PROPTH-PRP exhibited a t1/2ER of 1.1 hours. The independence of the export rate on level of expression for both PROPTH-PRPΔT and PROPTH-PRP also contrasts with the results obtained for PRP, for which t1/2ER was ~6 hours for a 33 of PRP are deleted in the PRPΔT portion of the construct (Castle et al., 1992). PROPTH-PRP, encodes the same PTH segment attached to the N terminus (residue 17) of full-length PRP. Full-length PRP containing its native signal sequence and transition domain was included in some of the studies (Castle et al., 1992). PROPTH-PRPΔT and PROPTH-PRP cDNAs were transfected into AtT-20 cells and stable cell lines were created. We selected cell lines with different levels of expression of PROPTH-PRPΔT, PROPTH-PRP and PRP. The level of expression of all PRP-related polypeptides varied over a 100-fold range (Tables 1, 2 and 3) but in all cases was less than 20% of the level of endogenous ACTH-related peptides. Notably, the level of expression of ACTH-related peptides was nearly the same in all the clones examined (Table 1, legend).

**Acceleration of ER export by PROPTH**

Rates of export from the ER were estimated by following the kinetics of the loss of sensitivity to Endo H digestion (Castle et al., 1992). As shown by the values of t1/2ER in Table 1, the presence of PROPTH causes a dramatic acceleration in transport of PRP. PROPTH-PRPΔT exited the ER with a t1/2ER of 1.3-1.4 hours in contrast to >7 hours for PRPΔT (Table 1). Similarly, PROPTH-PRP exhibited a t1/2ER of 1.1 hours. The independence of the export rate on level of expression for both PROPTH-PRPΔT and PROPTH-PRP also contrasts with the results obtained for PRP, for which t1/2ER was ~6 hours for a
low expressing clone and increased to a plateau of 1.8 hours for progressively higher expressing clones (Table 1 above; Castle et al., 1992). Thus the presence of the propeptide bypasses the concentration-dependent step during transport from the ER, which is normally mediated by the transition domain of PRP.

**Secretion and storage of PROPTH-PRPΔT and PROPTH-PRP**

As the PROPTH chimeras were transported from the ER with rapid and essentially identical kinetics, it was now possible to evaluate whether the presence of the transition region of PRP was truly necessary for targeting to storage granules. We used stimulation of secretion of radiolabeled cells by a secretagogue to assess whether the chimeric polypeptides were routed to the regulated pathway. Secretion of both chimeric polypeptides was stimulated with 8-Br-cAMP, indicative of their presence in secretory granules. The percentage of total radiolabeled PROPTH-PRPΔT and PROPTH-PRP undergoing stimulated secretion is shown in Table 2 and will be referred to as the percentage stimulated secretion throughout this work. The results show that the percentage stimulated secretion of PROPTH-PRPΔT and PROPTH-PRP is the same (10-20%) for comparable levels of expression. Therefore, in this context, the transition region appears unnecessary.

For both chimeric polypeptides, we also observed that the percentage stimulated secretion exhibited a single-step increase with increased level of expression. For several different clones expressing low levels of PROPTH-PRPΔT or PROPTH-PRP (representative examples are clones 20 and 39, respectively, in Table 2) the percentage stimulated secretion was ~10%. At higher levels of expression (e.g. clones 10 and 8 in Table 2), the percentage stimulated secretion increased to ~20% but did not rise significantly beyond this level with yet higher levels of expression (e.g. clones 43 and 4 in Table 2). To confirm that the differences in stimulus-dependent secretion of the chimeric polypeptides do not reflect variations in intrinsic storage capacity or secretory response, we measured 8-Br-cAMP-stimulated secretion of radiolabeled ACTH-related peptides in untransfected AtT-20 cells and selected clones expressing PROPTH-PRPΔT. As shown in Table 3, the percentage stimulated secretion of ACTH-related peptides was essentially the same in all cell populations.

The studies of stimulated secretion suggested that low and high expressing clones contain different fractions of PROPTH-PRPΔT and PROPTH-PRP in the stimulus-releasable (granule) pool. These differences should be reflected in the fraction of PROPTH-PRPΔT and PROPTH-PRP in the post-Golgi (Endo H-resistant) pool. We compared the percentage of cell-associated Endo H-resistant PROPTH-PRPΔT and PROPTH-PRP at different times of chase following a long-term labeling for high and low expressing clones (Fig. 2). For each clone this value decreases rapidly during the first hour probably reflecting release of PROPTH-PRPΔT and PROPTH-PRP via the constitutive pathway and reaches a plateau with longer times of chase. At all timepoints, the percentage of cell-associated Endo H-resistant PROPTH-PRPΔT and PROPTH-PRP is higher (by ~10% on average) in a high expressing clone than in a low expressing clone.

Our results using radiolabeled polypeptides imply that the level of expression has a significant influence on the distribution of PROPTH-PRP and PROPTH-PRPΔT among the major

### Table 1. Kinetics of exit PRP-related polypeptides from the ER

<table>
<thead>
<tr>
<th>Construct</th>
<th>Clone</th>
<th>LOE&lt;sup&gt;PRP&lt;/sup&gt;</th>
<th>t&lt;sub&gt;1/2&lt;/sub&gt;&lt;sup&gt;ER&lt;/sup&gt; (hours)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PROPTH-PRPΔT</td>
<td>20</td>
<td>3.3</td>
<td>1.3</td>
</tr>
<tr>
<td>PROPTH-PRPΔT</td>
<td>43</td>
<td>66.0</td>
<td>1.4</td>
</tr>
<tr>
<td>PRPΔT</td>
<td>26</td>
<td>2.9</td>
<td>7.0</td>
</tr>
<tr>
<td>PRPΔT</td>
<td>6</td>
<td>12.0</td>
<td>9.7</td>
</tr>
<tr>
<td>PROPTH-PRP</td>
<td>39</td>
<td>1.0</td>
<td>1.1</td>
</tr>
<tr>
<td>PROPTH-PRP</td>
<td>4</td>
<td>60.0</td>
<td>1.1</td>
</tr>
<tr>
<td>PRP</td>
<td>A2</td>
<td>2.9</td>
<td>6.0*</td>
</tr>
<tr>
<td>PRP</td>
<td>A1</td>
<td>21.5</td>
<td>2.5*</td>
</tr>
<tr>
<td>PRP</td>
<td>B9</td>
<td>59.4</td>
<td>1.8</td>
</tr>
<tr>
<td>PRP</td>
<td>B35</td>
<td>97.5</td>
<td>1.8</td>
</tr>
</tbody>
</table>

LOE<sup>PRP</sup> represents level of expression of PRP-related polypeptides corrected to total trichloroacetic acid-precipitable counts. It was determined as described in Materials and Methods. The values shown were normalized to the lowest expressing clone, PROPTH-PRP 39. The level of expression of ACTH-related polypeptides determined from the same experiments had an average value of 506±20 (s.e.m.).

<sup>*</sup>t<sub>1/2</sub><sup>ER</sup> is the halftime of exit of PRP-related polypeptides from the ER. It was determined as described in Materials and Methods.

<sup>†</sup>Values of t<sub>1/2</sub><sup>ER</sup> were taken from Table 3 of Castle et al. (1992).

### Table 2. 8-Br-cAMP-dependent secretion of PRP-related polypeptides

<table>
<thead>
<tr>
<th>Construct</th>
<th>Clone</th>
<th>LOE&lt;sup&gt;PRP&lt;/sup&gt;</th>
<th>% Stim. sec.</th>
</tr>
</thead>
<tbody>
<tr>
<td>PROPTH-PRPΔT</td>
<td>20</td>
<td>3.3</td>
<td>10.3±1.2</td>
</tr>
<tr>
<td>PROPTH-PRPΔT</td>
<td>10</td>
<td>13.2</td>
<td>18.3±1.3</td>
</tr>
<tr>
<td>PROPTH-PRPΔT</td>
<td>43</td>
<td>66.0</td>
<td>21.0±2.1</td>
</tr>
<tr>
<td>PROPTH-PRP</td>
<td>39</td>
<td>1.0</td>
<td>9.4±1.1</td>
</tr>
<tr>
<td>PROPTH-PRP</td>
<td>8</td>
<td>10.0</td>
<td>24.6±1.6</td>
</tr>
<tr>
<td>PROPTH-PRP</td>
<td>4</td>
<td>60.0</td>
<td>18.2±1.1</td>
</tr>
<tr>
<td>PRP</td>
<td>A2</td>
<td>2.9</td>
<td>6.1±0.5*</td>
</tr>
<tr>
<td>PRP</td>
<td>A1</td>
<td>21.5</td>
<td>5.2±0.8*</td>
</tr>
<tr>
<td>PRP</td>
<td>B9</td>
<td>59.4</td>
<td>11.0±2 †</td>
</tr>
<tr>
<td>PRP</td>
<td>B35</td>
<td>97.5</td>
<td>9.0 ‡</td>
</tr>
</tbody>
</table>

LOE<sup>PRP</sup> represents level of expression of PRP-related polypeptides normalized to total trichloroacetic acid precipitable cpm. It was determined as described in Materials and Methods.

% Stim. sec. represents the difference between stimulated (+ 8Br-cAMP) and basal (~8-Br-cAMP) secretion of radiolabeled PRP-related polypeptides expressed as percentage of total (chases + cell extract). Detailed description of the experiment is presented in Materials and Methods. Data are presented as the mean of four experiments ± s.e.m.

<sup>‡</sup>Data taken from Castle et al. (1992).

<sup>†</sup>Mean of two experiments ± deviation from the mean.

<sup>‡</sup>Result of a single experiment.

### Table 3. 8-Br-cAMP-dependent secretion of ACTH

<table>
<thead>
<tr>
<th>Construct</th>
<th>Clone</th>
<th>LOE&lt;sup&gt;PROPTH-PRPΔT&lt;/sup&gt;</th>
<th>% Stim. sec.</th>
</tr>
</thead>
<tbody>
<tr>
<td>PROPTH-PRPΔT</td>
<td>20</td>
<td>3.3</td>
<td>49</td>
</tr>
<tr>
<td>PROPTH-PRPΔT</td>
<td>43</td>
<td>66.0</td>
<td>47</td>
</tr>
<tr>
<td>AtT-20</td>
<td>NA</td>
<td>49</td>
<td></td>
</tr>
</tbody>
</table>

LOE<sup>PROPTH-PRPΔT</sup> refers to the level of expression of PROPTH-PRPΔT in each clone.

% Stim. sec. of ACTH was calculated as described for PRP-related polypeptides (Materials and Methods).

NA, not applicable.
intracellular pools, the granules and the ER. Because steady-state labeling enriches for the slowest turnover pools of protein, to a first approximation, the granule and ER pools may be represented by Endo H-resistant and Endo H-sensitive PRPs, respectively. Using the clones expressing PROPTH-PRPΔT as examples, at steady state the fraction of Endo H-resistant PROPTH-PRPΔT is 0.62 in clone 43 (high level of expression) and only 0.40 in clone 20 (low level of expression) (Fig. 2). Based on these fractions and the difference in level of expression, it is possible to predict that the granule pool in clone 43 should be 31-fold higher (0.62/0.4×20) than in clone 20 while the ER pool (calculated on basis of Endo H-sensitive PROPTH-PRPΔT) should be only 13-fold higher (0.38/0.6×20) in clone 43 than in clone 20. We have confirmed the predicted increase in the granule pool experimentally by comparing the stimulated discharge of PROPTH-PRPΔT by clones 43 and 20 using quantitative western blotting (see Table 4). The results indicate a 33-fold higher stimulated secretion of PROPTH-PRPΔT by clone 43. Applying the same analysis to PROPTH-PRP clones 39 and 4, the predicted fold difference in stimulated secretion of 88 (0.53/0.36×60) is in good agreement with the experimental value of 83 (see Table 5).

Secrecion and storage of full-length PRP

Our previous study showed that the percentage stimulated secretion of full-length PRP did not increase when levels of expression of PRP varied over the range corresponding to 0.6-4% of ACTH (Table 2, this work; Castle et al., 1992). In view of the increased percentage stimulated secretion of the PROPTH chimera observed at higher levels of expression, we have now measured 8-Br-cAMP-stimulated secretion of full-length PRP in clones expressing it at yet higher levels. Interestingly, an approximately twofold increase in the percentage stimulated secretion was observed when the level of expression of PRP was increased (Table 2). Yet, as in the case of PROPTH-PRPΔT and PROPTH-PRP, no further significant rise in the percentage stimulated secretion occurred at still higher levels of expression (Table 2). Thus the secretion of full-length PRP shows the same trend as that of the chimeric polypeptides. But overall, the percentage stimulated secretion is lower at all levels of expression.

The role of the PTH propeptide

The presence of the chimeric polypeptides in regulated secretion has lead us to examine in more detail the potential role(s) of PROPTH in sorting of PRPs. We considered two possibilities. First, we examined whether storage might relate to cleavage of the propeptide, reflecting an interaction with the processing enzyme (Journet et al., 1993). Second, we examined whether the propeptide might serve as a sorting signal.

Proteolytic processing of the PTH propeptide

The kinetics of processing of native proPTH to PTH are very rapid and are consistent with proteolytic cleavage occurring within or just after exiting the Golgi complex (Habener et al., 1979; Hellerman et al., 1984). If processing of PROPTH is occurring in the chimeric polypeptides, then the propeptide should be absent in secreted polypeptides. For cell-associated polypeptides, the propeptide should be present in Endo H-sensitive forms (ER forms) and absent in Endo H-resistant forms (Golgi/post-Golgi forms). Using cells expressing PROPTH-PRPΔT and PRPΔT, we compared the electrophoretic mobilities of secreted and cell-associated [3H]proline-labeled polypeptides. Fig. 3A shows that secreted PROPTH-PRPΔT (PROΔT in Fig. 3A) and PRPΔT (ΔT in Fig. 3A) have identical apparent Mr values suggesting that the propeptide has been removed from PROPTH-PRPΔT. The cell-associated Endo H sensitive PROPTH-PRPΔT has higher apparent Mr than the Endo H sensitive PRPΔT, indicating that the propeptide is still attached. The cell-associated Endo H resistant PROPTH-PRPΔT has an identical Mr as the secreted PRPΔT indicating the absence of the propeptide in the post-Golgi pool of PROPTH-PRPΔT. No band of higher Mr was ever observed in samples of secreted or cell-associated Endo H-resistant PROPTH-PRPΔT, suggesting that the propeptide is cleaved off from the majority of transported PROPTH-PRPΔT, whether or not it is stored in the granules. This is illustrated by the identical mobility of PROPTH-PRPΔT released in the absence and presence of 8-Br-cAMP (Fig. 3B). Together, these results imply that the propeptide stored in the granules of cells expressing PROPTH-PRPΔT corresponds to PRPΔT, the deletion mutant that was judged to be defective in storage and transport previously (Castle et al., 1992). Analysis of secreted and cell-associated PROPTH-PRP yielded similar results (not shown).

To confirm that the propeptide was cleaved at the predicted processing site (the carboxyl side of arginine-1 of the hexapeptide prosequence; Fig. 1), we performed limited N-terminal sequencing of secreted radiolabeled PROPTH-PRPΔT. Using [3H]proline for biosynthetic labeling, radioactivity was antici-
pated in the 5th and 6th residues from the N terminus (Fig. 1; Castle et al., 1992). As seen in Fig. 3C, these expectations were confirmed, indicating correct cleavage of the PTH propeptide.

**Mutagenesis of the processing site in PROPTH-PRPΔ**

Having established correct cleavage of PROPTH during intracellular transport, we sought to test the effect of preventing the cleavage of the chimeras by mutagenesis of the processing site. The PTH propeptide of chimeric PROPTH-PRPΔ and PROPTH-PRP may be cleaved by PC3 or the more ubiquitous furin (Smeekens et al., 1991; reviewed by Steiner et al., 1992; Seidah et al., 1993). In order to alter amino acid residues commonly recognized by these enzymes and at the same time retain the same positive charge of the PTH propeptide, the lysines at positions -2 and -6 were changed to histidines (Fig. 1) by in vitro mutagenesis of PROPTH-PRPΔ. The resulting construct (HISPTH-PRPΔ) was transfected into AtT-20 cells and stable lines with different levels of expression were selected.

To demonstrate that the propeptide is not cleaved, we examined the fate of HISPTH-PROPTH after labeling with [3H]histidine. As shown in Fig. 4, both secreted and cell-associated HISPTH-PROPTH were labeled with [3H]histidine. Since PROPTH contains no histidine residues, this indicates that the propeptide was not removed. Further, the ratio of [3H]histidine:[3H]proline labeling for secreted HISPTH-PROPTH is the same as that for cell-associated HISPTH-PROPTHΔ (Fig. 4), consistent with no cleavage.

Using cells expressing HISPTH-PROPTH, we tested whether secretion of HISPTH-PROPTH was stimulated with 8-Br-cAMP. Results presented in Table 5 show that the secretion of HISPTH-PROPTH was stimulated with 8-Br-cAMP; however, the percentage stimulated secretion was slightly lower than observed for PROPTH-PRPΔ for all levels of expression examined (Table 2). As in the case of PROPTH-PRPΔ, PROPTH-PRP and PROPTH-PRPΔ, there was an approximately twofold jump in the percentage of stimulated secretion of HISPTH-PROPTH as the level of expression increased. Thus removal of the propeptide is not essential for storage in granules.

**Effect of PROPTH on the storage of truncated VSVG**

To address the second possibility that PROPTH carries independent sorting information, we fused it to the truncated G protein of vesicular stomatitis virus (tG), which lacks its N-terminal cytoplasmic and transmembrane segment (Fig. 1). By itself, tG is secreted constitutively in AtT-20 cells (Moore and Kelly, 1985), but it is rerouted to the regulated pathway when fused to growth hormone (Moore and Kelly, 1986). Both tG and the PROPTH-tG chimera (Fig. 1) were transfected into AtT-

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**Table 4. Western blotting of stimulated secretion of PROPTH-PRPΔ and PROPTH-PRP**

<table>
<thead>
<tr>
<th>Construct</th>
<th>Clone</th>
<th>LOE</th>
<th>HISPTH-PRPΔ</th>
<th>Stim. sec.</th>
</tr>
</thead>
<tbody>
<tr>
<td>PROPTH-PRPΔ</td>
<td>43</td>
<td>39</td>
<td>100,000</td>
<td>1,370</td>
</tr>
<tr>
<td>PROPTH-PRP</td>
<td>20</td>
<td>39</td>
<td>130,000</td>
<td>3,600</td>
</tr>
</tbody>
</table>

Net stimulated secretion represents the difference in secretion of PROPTH-PRPΔ or PROPTH-PRP in the presence and absence of 5 mM 8-Br-cAMP. The data are from a sample experiment performed as described in Materials and Methods. The values presented in arbitrary units (AU) are taken from phosphomager analysis and normalized for DNA content.

Stimulated secretion ratio is: (net stimulated secretion PROPTH-PRPΔ 43)/(net stimulated secretion PROPTH-PRPΔ 20) and (net stimulated secretion PROPTH-PRP 4)/net stimulated secretion PROPTH-PRP 39). This is the fold difference in stimulated secretion between high and low expressing clones.

Mean stimulated secretion ratio is an average for three separate experiments ± s.e.m.

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**Table 5. 8-Br-cAMP-dependent secretion of HISPTH-PRPΔ**

<table>
<thead>
<tr>
<th>Construct</th>
<th>Clone</th>
<th>LOE</th>
<th>HISPTH-PRPΔ</th>
<th>Stim. sec.</th>
</tr>
</thead>
<tbody>
<tr>
<td>HISPTH-PRPΔ</td>
<td>16</td>
<td>5.4</td>
<td>7±1</td>
<td></td>
</tr>
<tr>
<td>HISPTH-PRP</td>
<td>15</td>
<td>21.5</td>
<td>14±2</td>
<td></td>
</tr>
<tr>
<td>HISPTH-PRPΔ</td>
<td>10</td>
<td>86.0</td>
<td>11±2</td>
<td></td>
</tr>
</tbody>
</table>

LOE HISPTH-PRPΔ and % Stim. sec. were determined as described in Table 2. The mean ± s.e.m. of three experiments are presented.
20 cells and stable cell lines secreting each construct were created.

The glycosylation pattern of PROPTH-tG suggests that it correctly traverses the intracellular transport pathway. As is the case for tG (see Fig. 5A; Fig. 2, Moore and Kelly, 1985), the predominant intracellular form of PROPTH-tG is Endo H-sensitive (Fig. 5A) indicating its presence in the ER. A minor fraction of both cell-associated tG and PROPTH-tG is Endo H-resistant (not visible at the exposure shown) and represents the post-Golgi pool. The small amount of intracellular Endo H-resistant tG and PROPTH-tG is consistent with the absence of both polypeptides in secretory granules. In contrast to the intracellular polypeptides, secreted tG and PROPTH-tG are entirely resistant to Endo H digestion (Fig. 5B).

We examined the secretion in the presence and absence of 8-Br-cAMP to determine whether PROPTH-tG is rerouted to the regulated secretory pathway. The effect of secretagogue was examined following a 1 hour pulse with Expre35S35S label and a 5 hour chase. As shown in Fig. 6, no 8-Br-cAMP-dependent stimulation of PROPTH-tG was observed. The same result was obtained with tG (Fig. 6) as was expected based on previous findings (Moore and Kelly, 1985). These results obtained with a 1 hour labeling were confirmed with cells that were labeled for 15 hours to approach steady state (data not shown). Our findings suggest that PROPTH does not contain independent sorting information for the regulated pathway.

**DISCUSSION**

In this study we have achieved rapid intracellular transport of a deletion mutant, PRPAT, and of full-length PRP by attaching to them the propeptide of PTH. Using this approach we have gained new insight about the intracellular transport and sorting of these unusual secretory proteins. Our findings argue against the presence of a universal sorting signal for regulated secretory proteins and are consistent with the notion that protein-specific interactions are important.

**Intracellular transport**

The relatively slow ER export of full-length PRP suggested the presence of an ER retention mechanism for PRPs (Castle et al., 1992). Since polypeptides lacking either the transition domain (PRPΔT) or the C-terminal domain (PRPΔC) also exit the ER slowly (Castle et al., 1992), this ER retention most likely involves the proline-rich repeats. Proline-rich domains are known to function in protein-protein interactions (Williamson, 1994), and PRPs may bind to resident ER protein(s) via their proline-rich repeats. For full-length PRP, ER retention decreases with increasing concentration (level of expression) of PRP (Castle et al., 1992). Based on this observation, we have inferred that self-association of PRPs competes with retention in the ER. This self-association appears to be mediated by the transition region as its deletion causes extensive retention of PRPΔT regardless of concentration (Castle et al., 1992).

Replacement of the transition region with the short and basic PTH propeptide causes rapid ER export that is independent of level of expression, suggesting that the ER retention mechanism has been bypassed. The same results are obtained when the PTH propeptide and the transition regions are both present (PROPTH-PRP), suggesting that the propeptide has a dominant effect. As pointed out by Mains et al. (1995) the PTH propeptide exhibits a striking sequence similarity to the propeptides of PAM and of albumin, both of which are thought to accelerate the rate of secretion of the respective polypep-
tides, most likely as a consequence of enhanced ER export (Mains et al., 1995; McCracken and Kruse, 1989). Together with the finding that the effect of the PAM propeptide is transferable to another protein, PC2 (Mains et al., 1995), these results are suggestive of a specialized mechanism involving recognition of the propeptide sequence. However, deletion of the propeptide from PTH does not alter its own rate of transport (Wiren et al., 1988) suggesting that not all proteins are affected by the presence of the propeptide. Clearly, further studies will be required to elucidate the mode of action of these propeptides.

Using both electrophoretic analysis and N-terminal microsequencing, we have demonstrated that the propeptide is cleaved from the chimeric PROPTH-PRPΔT before secretion. Further, processing was observed regardless of the pathway of secretion, suggesting that cleavage occurred before or concomitantly with sorting. Therefore, the post-Golgi and secreted forms of PROPTH-PRPΔT and PROPTH-PRP have identical primary structures to the original PRPΔT and PRP, validating the strategy of propeptide attachment as a bypass for slow ER export.

**Sorting and storage of PRP-related polypeptides**

It is clear that by accelerating ER export, PROPTH enhances the ability to detect granule storage of PRP and PRPΔT. We regard the possibility that PROPTH contains sorting information for the regulated secretory pathway as unlikely for two reasons. First, deletion of the propeptide from the precursor of PTH does not affect the pathway of secretion of PTH in GH4 cells (Wiren et al., 1988). Second, when the PTH propeptide was fused to a reporter protein, tG, the chimeric polypeptide was not targeted to the regulated pathway of AtT-20 cells (Fig. 6, this work). We also considered the possibility that the propeptide might affect sorting through its interaction with the proteolytic processing machinery. An intact cleavage site has been shown to be necessary for the storage of von Willebrand factor in Weibel-Palade bodies, and it has been proposed that the processing enzyme may function in sorting either by facilitating local concentration and aggregation of propeptide-containing polypeptides or by serving as a 'receptor' at sites of sorting (Journet et al., 1993). However, we have shown that disruption of PTH propeptide processing by mutagenesis of the cleavage site did not abrogate regulated secretion. While this would appear to rule out a major role of the processing enzymes in targeting of the propeptide-containing chimeras, we can’t discount the possibility that processing enzymes might affect the degree of storage because the percentage of 8-Br-cAMP dependent secretion of HISPTH-PRPΔT was slightly lower than that of PROPTH-PRPΔT.

Because the major role of PROPTH is to accelerate ER export of PRPs rather than to serve as a sorting signal, we believe that sorting of PRPs for stimulated secretion must rely on structural characteristics of the PRP itself. While we previously regarded the transition domain of PRP as necessary for storage in secretory granules (Castle et al., 1992), the present findings argue convincingly that this is not the case. Rather the transition domain mainly facilitates efficient transport of the native protein from the ER. Thus sorting of PRP for regulated secretion must depend on one of the two remaining domains, either the central proline-rich repeats or the C-terminal segment. Our earlier study ruled out any effect of the C-terminal segment on sorting, so by elimination, we are left with the proline-rich repeats as being responsible. Since this domain is unique in primary and probably higher order structure among regulated secretory proteins, any interactions that it mediates are likely to be specific to the PRP family.

Strikingly, regulated secretion of PRP-related polypeptides shows a dependence on level of expression. An incremental increase in the percentage stimulated secretion is observed when expression is increased beyond a certain level. While both ER and storage granule pools of PRP-related polypeptides are elevated when the level of expression is increased, our data clearly indicate that the storage pool is preferentially enriched (Table 4). In contrast, the fractional storage of the endogenous hormone ACTH was unaffected by the expression of PRP-related polypeptides. Consequently, PRP-related polypeptides either are stored in increased amounts in the ACTH-containing granules or are segregated into a second storage compartment with the same regulation.

Because the incremental increase in storage was observed for the full-length PRP as well as for the chimeric polypeptides, the presence of PROPTH is not necessary for this effect. We propose that the jump in storage is driven by the increased steady state concentration at sites of secretory granule formation and reflects homo- or heterotypic aggregation involving PRP-related polypeptides. The nonlinear dependence on concentration (level of expression) is consistent with a cooperative process which is catalyzed once a threshold concentration is reached. The jump in storage for PROPTH-PRPΔT and PROPTH-PRP occurs at a 5- to 10-fold lower expression level than for full-length PRP, suggesting that PROPTH may promote somewhat different interactions than occur for full-length PRP. In any case, the concentration-dependent storage of PRPs is best explained as an aggregation process, and it may reflect the same type of associations that cause PRPs to concentrate visibly within the content of parotid acinar granules (Kousvelari et al., 1982).

In conclusion, our studies put in question the existence of a universal sorting signal for regulated secretion as has been postulated previously (Kizer and Tropsha, 1991; Gorr and Darling, 1995). On the other hand, we wish to emphasize that the fractional storage that we have observed for PRPs and that others have observed for several nonendocrine proteins (e.g. Fennewald et al., 1988; Castle et al., 1992; Schmidt and Moore, 1994; Reaves et al., 1990; Stoller and Shields, 1989; Castle et al., 1995) and free glycosaminoglycan chains (Matsuuchi and Kelly, 1991) is typically well below the extent of storage achieved by the endogenous hormone, ACTH. Thus we believe that it will be important in future studies to evaluate sorting for regulated secretion on the basis of the amount of polypeptide stored. Cell- or protein-specific sorting signals (Cool et al., 1995; Arrandale and Dännies, 1994; Stoller and Shields, 1989) or highly efficient aggregation and retention mechanisms (Kuliyawat and Arvan, 1994) may be superimposed on a bulk flow pathway leading to forming granules, and proteins that are either absent or poorly represented in regulated secretion may be progressively excluded from the maturing storage compartment.

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