Expression of Mutant ELH Prohormones in AtT-20 Cells: The Relationship between Prohormone Processing and Sorting

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Abstract. Posttranslational processing of many proteins is essential to the synthesis of fully functional molecules. The ELH (egg-laying hormone) prohormone is cleaved by endoproteases in a specific order at a variety of basic residue processing sites to produce mature peptides. The prohormone is first cleaved at a unique tetrabasic site liberating two intermediates (amino and carboxy) which are sorted to different classes of dense core vesicles in the bag cell neurons of *Aplysia*. When expressed in AtT-20 cells, the ELH prohormone is also first cleaved at the tetrabasic site. The amino-terminal intermediate is then sorted to the constitutive pathway, and a portion of the carboxy-terminal intermediate is sorted to the regulated pathway. Here, we use mutant constructs of the ELH prohormone expressed in AtT-20 cells to examine the relationship between prohormone processing and consequent sorting. Prohormone which has a dibasic site in place of the tetrabasic site is processed and sorted similarly to wild type. Furthermore, mutant prohormone which lacks the tetrabasic site is processed at an alternative site comprising three basic residues. In these mutant prohormones, mature ELH is still produced and stored in dense core vesicles while aminoterminal products are constitutively secreted. However, deletion of the tetrabasic and tribasic sites results in the rerouting of the amino-terminal intermediate products from the constitutive pathway to the regulated secretory pathway. Thus, in the ELH prohormone, the location of the proteolytic processing events within the secretory pathway and the order of cleavages regulate the sorting of peptide products.

T HE maturation of a prohormone involves many highly regulated cellular and biochemical steps in the secretory pathway. First, the signal sequence of the prohormone is cleaved in the ER (Walter et al., 1984). Then, during vectorial transport through the Golgi complex, further maturation including glycosylation, sulfation, and phosphorylation may occur (Huttner, 1988; Eipper and Mains, 1982). In the late Golgi compartments, possibly the *trans*-Golgi (TG)[1] or TGN, proteolytic processing can occur, as is the case with the ELH prohormone of bag cell neurons (Sossin, et al., 1990*a*), viral proteins (e.g., McCune et al., 1988), growth factors, and receptors (e.g., Yoshimasa et al., 1990). At the TGN, proteins are sorted to one of several destinations: the plasma membrane, lysosomes, the constitutive pathway, or the regulated secretory pathway (Kelly, 1985; Griffiths and Simons, 1986). Prohormone products are sorted to the regulated pathway and are stored in dense core vesicles (DCVs). It is during the maturation of the DCVs that the final processing steps, such as cleavage at dibasic residues (Orci et al., 1987, 1988), removal of the basic residues by a carboxypeptidase, and amidation are thought to occur (for reviews see Docherty and Steiner, 1982; Douglass and Herbert, 1984; Loh et al., 1984; Sossin et al., 1990*a*).

Basic residues, and especially the Lys-Arg dibasic pair, are common proteolytic recognition sites. However, in the course of prohormone maturation, only some of the available basic residue sites are cleaved. Hence, there must be information in the sequence that confers cleavage specificity (Rholam et al., 1986). Biophysical studies using synthetic peptides corresponding to regions surrounding the processing site in prooxytocin have shown that certain secondary structures are important for cleavage. Specifically, a β turn subtype on the amino-terminal side of the dibasic cleavage site and an α helix on the carboxy-terminal side are important structural features for enzyme recognition and utilization of the cleavage site (Paolillo et al., 1992).

Several enzymes involved in the processing of prohormones have been identified and are referred to as prohormone converting enzymes (PCEs) (for reviews see Barr, 1991; Steiner, 1991). Furin, the first mammalian PCE identified due to its homology with the yeast proteolytic prohormone processing enzyme Kex2 (Fuller et al., 1988), is ubiquitous in mammalian cell types (Schalken et al., 1987; Van de Ouweland et al., 1990). The proposed furin consensus sequence, Arg-X-Arg/Lys-Arg (Hosaka et al., 1991)

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1. *Abbreviations used in this paper*: DCV, dense core vesicles; EGH, egg-laying hormone; KLH, keyhole limpet hemocyanin; PCE, prohormone converting enzymes; TG, *trans*-Golgi.

is found more commonly in proviral proteins, progrowth factors, and proreceptors than in prohormones. Other PCEs include PC1/PC3 (Seidah et al., 1991; Smeekens et al., 1991) and PC2 (Seidah et al., 1990; Smeekens and Steiner, 1990). In cellular transfection studies, it was shown that cleavage by PC1/PC3 is specific for the dibasic site between ACTH and β-lipotropin of the proopiomelanocortin precursor, while cleavage by PC2 is more specific for the internal dibasic cleavage in β-lipotropin to produce β-endorphin (Benjannet et al., 1991; Thomas et al., 1991). More PCEs are likely to be characterized, suggesting that particular enzymes may be required for specific cleavages. Furin is thought to be localized in the Golgi complex, because it contains a transmembrane domain; studies also suggest that a furin-like proteolytic activity is present in this compartment. The cellular locations of PC1/PC3 and PC2 have yet to be determined. Since they lack transmembrane domains, it is thought that they are in DCVs. The actual intracellular location and sites of action of these enzymes are of interest and importance in understanding the regulation of prohormone biosynthesis.

The emphases of many studies have been on the identification of structural components of the prohormones and molecular mechanisms involved in sorting proteins to the constitutive or regulated secretory pathways. In this paper, we investigate the role of proteolytic processing in the sorting of prohormone products. The egg-laying hormone (ELH) prohormone encodes several biologically active peptides that regulate the egg-laying behavior in the marine mollusc *Aplysia californica*. The order and cellular location of its processing have been studied intensively (Newcomb et al., 1987; Fisher et al., 1988; Sossin et al., 1990a,b). After the signal sequence cleavage, a unique tetrabasic site is cleaved late in the Golgi apparatus. As a result, amino-terminal (F2) and carboxy-terminal (I3) intermediates are liberated (see Fig. 1). These two intermediates are proposed to be differentially sorted in the TGN: amino-terminal intermediate peptides and the carboxy-terminal product, ELH, are packaged into different DCV classes. Furthermore, the cell modulates the relative levels of the peptides, possibly by shunting some of the amino-terminal intermediate to the lysosomal degradative pathway (Sossin et al., 1990a). Hence, while the bag cell peptides and ELH are initially encoded in an equimolar ratio, the final ratio of recovered peptides is 1 to 5 amino-to carboxy-peptides. The amino-terminal peptide containing DCVs and ELH containing DCVs are also differentially distributed in the different neuronal processes emanating from the bag cells (Sossin et al., 1990b). It has, thus, been hypothesized that the timing and cellular location of the tetrabasic cleavage which liberates the two intermediates from the ELH prohormone is a crucial step in the sorting of bioactive peptides derived from the ELH prohormone.

In pursuit of the molecular mechanisms involved in the differential packaging of the ELH prohormone products, we investigated the processing of the ELH prohormone in the mouse anterior pituitary cell line, AtT-20 (Jung and Scheller, 1991). AtT-20 cells are able to process exogenous prohormones, such as proinsulin, and sort processed products into the regulated secretory pathway (e.g., Moore et al., 1983). Additionally, it has been demonstrated that a regulated secretory protein can redirect a constitutive secretory protein to the DCVs (Moore and Kelly, 1986). In many of these cases,

the prohormone contains only one bioactive peptide. The organization of the ELH prohormone is more complex by comparison; its maturation involves at least nine cleavages. Our previous studies showed that in transfected AtT-20 cells, the ELH prohormone is initially cleaved at the unique tetrabasic site and that the carboxy-terminal intermediate is then cleaved to yield mature ELH, which is stored into DCVs. The amino-terminal intermediate and its products are not detected in DCVs, and thus it appears that the two intermediates are differentially sorted. Hence, processing and sorting of the carboxy-terminal intermediate of the ELH prohormone in AtT-20 cells is similar to that in the bag cells (Jung and Scheller, 1991).

Our next goal was to examine how the specificity and timing of processing governs sorting of peptides derived from the ELH prohormone. To this end, we altered the processing sequence by mutating the unique tetrabasic site. We show in this report that the ELH prohormone lacking the tetrabasic site is cleaved at alternative sites. While processing is slower than that of wild-type prohormone, mature ELH is still produced and stored in DCVs. However, after deleting the tetrabasic site and an additional cleavage site, the amino-terminal products are now sorted to DCVs. Thus, altering the processing sites and location of processing in the secretory pathway changes the sorting of the ELH prohormone peptide products. We conclude that the organization of proteolytic processing sites is a major determinant in the sorting of peptides derived from this prohormone, and that the propensity of mature ELH to be stored in DCVs is dominant over the targeting of the amino-terminal intermediate products to the constitutive pathway in AtT-20 cells.

Materials and Methods

Antibodies

ELH antiserum was raised previously by Fisher et al. Two peptides were synthesized with a cysteine at the carboxy terminus: peptide F3A-C to the first 32 amino acids of the amino terminal intermediate (A-S-V-H-G-K-N-F-A-T-N-R-A-V-J-S-S-S-P-F-V-V-L-S-P-D-D-N-V-V-S-N-S-C) and peptide F3B-C to the first 20 amino acids of fragment F3B (D-Q-D-E-G-N-F-R-R-F-P-T-N-A-V-S-M-S-A-D-C). The peptides were purified by HPLC on a preparative C-18 column (Vydac, Hesperia, CA), eluted with acetonitrile, 0.1% TFA. The peptides then were coupled to keyhole limpet hemocyanin (KLH) via cysteine groups using the heterobifunctional crosslinker sulfo SMCC (Pierce, Rockford, IL) and separated from uncoupled peptide by gel permeation on Sephadex G-50 (Pharmacia Fine Chemicals, Piscataway, NJ) equilibrated in PBS. The two peptides were also coupled to BSA via amino groups of the peptide using sulfo SMCC.

Female New Zealand white rabbits were initially injected with 200 μg of peptide–KLH conjugate in Freund's complete adjuvant (Sigma, St. Louis, MO) with PBS in a final volume of 1 ml. The rabbits were then boosted four times at 20-d intervals with 200 μg of peptide–KLH conjugate in Freund's incomplete adjuvant (Sigma). The final bleeds were taken 9 d after the last boost and serum was obtained by centrifugation of the blood at 4°C and stored at −70°C.

F3A-C and F3B-C antisera were affinity purified by passing 3 mls of serum diluted in 12 mls of PBS over a 1.5 ml column in which 1 mg of peptide was conjugated via cysteine to Affigel 10 (Pierce). The diluted antisera was passed over the column three times or was incubated with the resin for 3 h at 4°C with rotation.

The column was washed as follows: 10 mls of PBS, 10 mls of PBS with 0.3 M NaCl, 10 mls of PBS, 10 mls of PBS with 1.0 M urea, and 10 mls of PBS. The antibody was eluted with 0.1 M glycine at pH 2.5 and 1-ml fractions were neutralized with 1.5 M Tris, pH 8.8 to 7.0–7.5. Fractions were stored with 1 mM sodium azide and 10% glycerol at −20°C. Approximately 0.5 to 1.0 mg of antibody was recovered.

Cell Culture and Transfections

AtT-20 cells were cultured in DME H-21 supplemented with 10% FCS and penicillin–streptomycin at 100 U/ml, 12.5% CO_2, and 37°C. Transfections were performed by calcium phosphate precipitation (Chen and Okayama, 1988). In a typical transfection, a 10-cm plate at 70% confluency was transfected with 50 μg of the PRc/CMV plasmid (Invitrogen, San Diego, CA) containing the ELH cDNA. Stable transfectants were selected with 0.25 mg/ml (active drug) of Geneticin (GIBCO/BRL, Gaithersburg, MD). G418-resistant clones were selected and those expressing ELH were further selected by light level immunocytochemistry using rabbit antisera against mature ELH. Several clones from each construct with different levels of expression were obtained and used for these studies.

In Vitro Mutagenesis and Plasmid Construction

Mutations of the ELH prohormone were produced by oligonucleotide directed mutagenesis (Kunkel, 1985). A cDNA encoding the ELH prohormone (clone U; Mahon et al., 1985) was inserted into the EcoRI site of the KS- plasmid (Stratagene, La Jolla, CA). The deletion of Arg-Arg was produced with the following oligo: 5'ACGGAAAGCCACTCATCTGTCCTA ACGCCT3'. Deletion of the tetrabasic site was made with 5'ACGGAAAGC CACTCAAAACGGTCTGTCCTA3'. The tetra-tri deletion was performed by using the tetra mutant and looping out the tribasic site with 5'CGCTTC TACTCCTTAGCAGGGGAAATGGA. Eight to ten colonies were selected and single-stranded DNA was sequenced (dideoxy; Sanger et al., 1977) to select for the mutated ELH prohormones. The insert was excised at the flanking EcoRI sites and then blunt end ligated into the HindIII site of the PRc/CMV vector.

Light Level Immunocytochemistry

Cells were plated on 8-well Permanox slides (Nunc, Roskilde, Denmark) and grown to 60% confluency. Cells were fixed in 3.5% paraformaldehyde in PBS for 30 min at room temperature. After fixation, cells were washed three times for 15 min in 0.1 M glycine–PBS, and then permeabilized for 5 min in 0.4% Saponin, 5% normal goat serum, 1% BSA, in PBS. Affinity-purified antisera was added at 2–5 μg/ml in the permeabilization buffer and incubated at room temperature for 45 min. After three washes, the cells were incubated for 1 h with goat anti–rabbit conjugated to fluorescein (Chemicon Intl. Inc., Tennecula, CA), diluted 1:100 in permeabilization buffer, and washed three times. The cells were overlaid with Citifluor (Citifluor LTD, London) to inhibit quenching.

Immunogold Labeling of Transfectants

Transfected AtT-20 cells were grown to ~80% confluency in six-well culture plates. Media was aspirated and cells fixed in either 4% paraformaldehyde and 0.2% glutaraldehyde in 0.12 M phosphate buffer, pH 7.2, or in 0.5% glutaraldehyde in 0.12 M phosphate buffer, pH 7.2, for 30 min on ice. After washing with buffer, cells were scraped from the wells and pelleted at 1,000 g for 10 min. The pellets were dehydrated to 50% ethanol at −20°C, stained for 2 h with 2% uranyl acetate in 50% ethanol at 20°C, dehydrated to 100% ethanol, and infiltrated with Lowicryl K4M overnight. Pellets were embedded the following day at −35°C in flat molds using UV polymerization of the resin for 48 h.

Sections (65 nm) of the different transfectants were collected on nickel grids, incubated 10 min on 1% sodium borohydride, 10 min in a solution of 5% normal goat serum, and 1.5% BSA in PBS, and overnight in the primary antisera, either affinity-purified anti-ELH or affinity-purified anti-F3A peptide. The antibodies were used at final concentrations of approximately 10 ng/μl in PBS containing 0.5% normal goat serum and 0.15% BSA. Grids were washed three times in PBS over a 90 min period and transferred to secondary label, either protein A conjugated 10-nm gold colloid (Amersham Corp., Arlington Heights, IL), or goat anti-rabbit conjugated 10 nm gold colloid (Biocell). After a 4-h incubation, grids were washed three times in PBS over 60 min, three times in water over 30 min, and contrasted with uranyl acetate and lead citrate.

Micrographs were taken at 54,800 magnification on a Philips 410 TEM (Philips Electronic Instruments, Inc., Mahwah, NJ).

Quantitation of Immunogold Labeling

Each labeling experiment was repeated at least three times for each transfected prohormone construct. DCVs were defined as relatively circular electron-dense profiles, and their areas were measured using a series of overlays of defined size. For each quantitation, the number of DCVs corresponding to each size overlay was counted and the total area calculated. The total number of gold particles in all DCV areas was divided by this total area to yield the absolute density of immunogold labeling shown in Table I. Golgi areas were defined as stacks or series of electron-lucent cisternae lacking ribosomes, and their areas measured in the same manner as for DCVs. Background, or nonspecific labeling, was measured in the cytoplasm by counting all gold particles not associated with defined Golgi or DCV areas. The absolute densities of immunogold labeling for at least three separate experiments were averaged to yield the values shown in Table I, and the standard deviation calculated.

Pulse Chase Experiments

10-cm dishes of each transfectant at 80% confluency were washed two times with 3 ml of methionine–cysteine deficient media and then prestarved in 2.5-mls deficient media with 2% FCS for 1 h. 0.25 mCi of Expres (New England Nuclear, Boston, MA; >70% [^{35}S]methionine, [^{35}S]cysteine) was added and the cells were incubated for 10 min. Cells were then washed one time with 2 mls of regular media supplemented with 1 mM methionine and cysteine, fed with 2.5 mls of these media, and incubated for the indicated times. At the appropriate time point, these media were collected, and 1 mM PMSF was added while cells were harvested with 1 ml of lysis buffer (50 mM Hepes, pH 7.25, 1% NP-40, 0.15 M NaCl, 2 mM leupeptin, 1 mM PMSF, 2 mM EDTA).

Immunoprecipitations

Cells were incubated in lysis buffer at 4°C for 1 h and then spun for 15 min in a microfuge (Beckman Instruments) at 4°C. The supernatant was diluted with one volume of lysis buffer without NP-40, and 50 μl Sepharose CL-6B slurry (Pharmacia Fine Chemicals) (1:1) was added for preclearing. The cell extracts and media were incubated for 1 h with rotation at 4°C, and then the Sepharose was pelleted by a 1-min spin at 4°C in the microfuge. Supernatants were transferred to new tubes and a 10-μl aliquot of each cell extract was saved for protein determination. BSA was added and samples were incubated with rotation overnight at 4°C. 17 μls of protein A Sepharose (Pharmacia Fine Chemicals) slurry (1:1) was added to each sample and incubated with rotation for 2 h at 4°C. Protein A Sepharose was pelleted by a 10-s spin and the supernatant discarded. The protein A Sepharose pellets were washed once in 50 mM Hepes, pH 7.25, 0.5% NP-40, 0.15 M NaCl, 2 mM EDTA, 2 mg/ml BSA, and then two times in 20 mM Hepes, pH 7.25, 0.3 M NaCl, 1 mM EDTA, BSA 2 mg/ml, and once in 20 mM Hepes, pH 7.25. The pellets were then suspended in 25 μl of gel loading buffer and heated at 100°C for 10 min. After centrifugation, the supernatant was loaded onto a 20% SDS–polyacrylamide gel which contained a separating gel buffer system of pH 9.3 (Hoeffer Scientific Instruments, San Francisco, CA). Gels were run at 15 mAmps, fixed in 25% ethanol, 10% acetic acid, incubated for 30 min in Amplify (Amersham Corp.), dried and then exposed to X-omat film (Eastman Kodak, Rochester, NY) at −70°C.

Quantitation of Storage and Release

The four different transfectants were grown in duplicate 10-cm plates to ~80% confluency. Both sets were treated for pulse chase experiments as described above, and cells were pulsed with 0.25 mCi of [^{35}S]methionine, [^{35}S]cysteine for 10 min. The plates were placed on ice and harvested in 1 ml of lysis buffer. The other set was washed, and 3 mls of chase media were added for 120 min. The samples at each time point were divided into two aliquots. One aliquot was immunoprecipitated with affinity purified ELH antisera and the other with F3A-C antisera. The samples were processed as described above. Quantitation was performed by scanning the autoradiograms with a laser densitometer (Molecular Dynamics, Inc., Sunnyvale, CA) and then by using the volume method for quantitation of the bands.

Radiosequencing

Cells in a 10-cm plate at 80% confluency were pulsed with 0.5 mCi of Expres (New England Nuclear) for 2 h and then chased for 2 h. Immunoprecipitations of either the cell extract or media were carried out as described. After the 20 mM Hepes wash, the protein A Sepharose pellet was resuspended in 30 μls of 1 N HCl for 2 min, spun, and the supernatant collected. This was repeated one time, and the supernatants were then

pooled and sequenced. Analysis was performed on a 470A gas-liquid phase sequencer by Applied Biosystems (Foster City, CA); the fractions from each round of Edman degradation were collected and counted with scintillation fluid.

Results

Antibody Specificity

In our earlier studies, we could not detect the presence of amino-terminal intermediate products with the antibodies available at that time. However, these antibodies are able to detect amino-terminal products in bag cell sections embedded in Lowicryl K4M (Fisher et al., 1988). As the previous antibodies were generated against peptides of 10 amino acids or less, two new antibodies were generated. Antibody F3A-C was generated against the first 30 amino acids of the F3A intermediate (Fig. 1); antibody F3B-C is against the first 20 amino acids of the F3B intermediate (Fig. 1). To test the efficacy of the F3A-C and F3B-C antibodies for light level immunocytochemistry, bag cell sections embedded in Lowicryl K4M (Kreiner et al., 1986) were incubated with anti F3A-C or F3B-C, and visualized with goat anti–rabbit IgG conjugated to fluorescein. Punctate staining was seen in the bag cell body and was blocked with the corresponding peptides. These antibodies immunoprecipitate prohormone and amino-terminal intermediate from bag cells, but immunoprecipitation of the F3A or F3B intermediate products was variable. The ELH antibody was previously characterized and shown to specifically react with mature ELH, as well as prohormone (Fisher et al., 1988).

Construction of the Mutant ELH Prohormones

Earlier experiments suggested that cleavage at the unique tetrabasic site (Arg-Arg-Lys-Arg) was crucial to the differential sorting of the amino- and carboxy-terminal intermediates (Sossin et al., 1990a). Therefore, we chose to mutate this tetrabasic site in two ways. First, we deleted the Arg-Arg pair leaving a Lys-Arg site (Fig. 1), a common proteolytic recognition site. Analysis of the processing of this construct is a way to ascertain the importance of a tetrabasic site cleavage versus a dibasic cleavage site. Second, the entire tetrabasic site was deleted, resulting in a precursor that has no cleavage site at this position. Third, based on results with the tetrabasic site deleted clone (see below), we constructed a tetra–tribasic deleted prohormone in which the tetrabasic site and an amino-terminal tribasic site were both deleted (Fig. 1).

Selection of the ELH Mutant Prohormones Clones

The wild type and mutated ELH prohormone cDNAs were inserted into the PRc/CMV vector and transfected into AtT-20 cells via the calcium phosphate method. With this expression vector, 80% of the G418-resistant clones were immunoreactive with the ELH antisera at the light level. Three clones of each construct which expressed various levels of the transfected ELH were selected. Quantitative Northern analyses (Chomczynski and Sacchi, 1986) were performed and a set of clones was chosen which express the transfected cDNA at levels within threefold of each other. This set was analyzed further, and was designated as follows: EC3 (WT), ELH wild type prohormone; D'36 (DBΔ), prohormone in
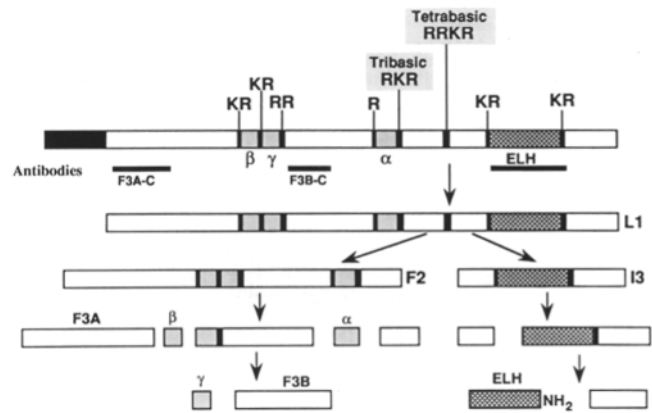
figure 1. The egg laying prohormone structure and processing scheme. The ELH prohormone consists of a signal sequence and several bioactive peptides, the α, b, and γ bag cell peptides, and ELH. The internal cleavage sites are indicated by the vertical solid black bars, and the basic residue cleavage sequence is shown above. After the signal sequence is removed, the first cleavage occurs at a unique tetrabasic site. This partitions the ELH prohormone into the amino- and carboxy-terminal intermediates, labeled F2 and I3, respectively. In the bag cell neurons, these two intermediates are sorted at the TGN to distinct classes of vesicles. The F2 intermediate is further processed into the bag cell peptides and the I3 intermediate into ELH. The tetrabasic and tribasic processing sites that are mutated in this study are shaded.

which the Arg-Arg of the tetrabasic site is deleted leaving a dibasic Lys-Arg; TD6 (TBΔ), prohormone with the tetrabasic site deleted; and T3 (TTΔ), prohormone with the tetrabasic and tribasic sites deleted (Fig. 1).

Expression and Localization of the ELH Prohormone in AtT-20 cells

AtT-20 cells have an elongated morphology and concentrate secretory granules containing peptides derived from the proopiomelanocortin precursor at the tips of the processes. In the transfected cells, ELH immunoreactivity is seen at the tips of the processes, as well as in the perinuclear region of each of the clones (Fig. 2). This staining is blocked with the ELH peptide; untransfected AtT-20 cells are not immunoreactive with the ELH antibody. The four different clones were also incubated with the F3A-C or F3B-C antibodies which react with the amino-terminal region of the ELH precursor. In contrast to the results observed with the ELH antibody, staining of amino-terminal peptides is absent throughout the cells in all of the clones except for the tetra-tribasic-Δ transformants. In the tetra-tribasic-Δ clone, immunoreactivity is observed predominantly in the tips of the processes (Fig. 2 D). Variable levels of immunoreactivity are also observed in ~25% of the tips of processes in the dibasic-Δ clone. The amino-terminal antibody staining is blocked with the corresponding peptide and untransfected AtT-20 cells do not stain. Thus, ELH immunoreactivity is readily expressed in the wild type and all the mutant clones, but amino-terminal products are consistently detected only in the tetra-tribasic-Δ–transfected cells.

Localization to the tips of processes is indicative of proteins that are stored in DCVs, but this is not always the case, since the immunoglobulin light chain also localizes to the
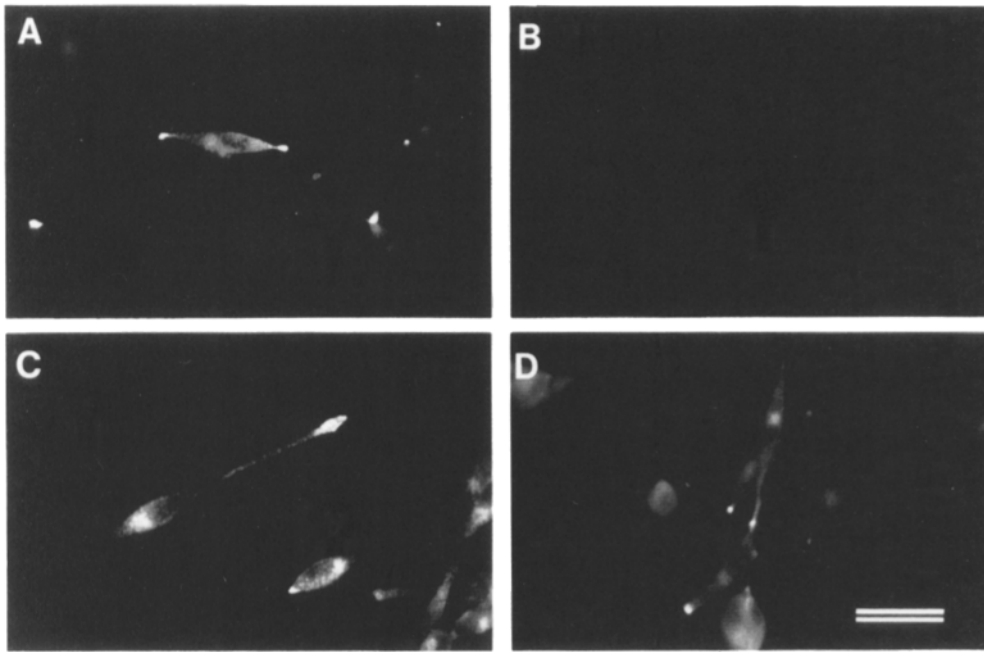
Figure 2. Localization of the ELH precursor proteins in transfected AtT-20 cells. Cells were stained with affinity purified ELH antiserum and F3A-C antiserum and then with goat anti–rabbit antibody conjugated to fluorescein. (*A*) The wild type clone (*WT*) stained with anti-ELH shows staining primarily at the tips of the processes and the poles of the cells. The dibasic-Δ and tetrabasic-Δ also show similar staining. (*B*) The wild type clone stained with anti-F3A-C shows no staining throughout the cell. The dibasic-Δ and tetrabasic-Δ clones also show no staining with this antibody. (*C*) The tetra-tribasic-Δ clone stained with anti-ELH also shows immunoreactivity primarily at the tips of the processes and poles of the cell. (*D*) The tetra-tribasic-Δ clone stained with anti-F3A-C shows prominent staining at the tips of the processes and poles of the cells. Bar, 33 μm.

tips. Thus, we determined the subcellular localization of the ELH and amino-terminal immunoreactivity by immunoelectron microscopy. ELH immunoreactivity was found primarily in dense core vesicles of all four different clones, as opposed to the other compartments within the cell (Fig. 3, Table I). This, along with biochemical data (see below), confirms that mature ELH is sorted into DCVs of the regulated pathway. Furthermore, ACTH and ELH were found to

colocalize in the DCVs of AtT-20 cells transfected with the wild type ELH cDNA (Jung and Scheller, 1991). The transfected cells were also incubated with the amino-terminal intermediate antibodies, F3A-C and F3B-C, and immunoreactivity was observed only in the DCVs of the tetra-tribasic-Δ clone (Table I). These data imply that the amino-terminal intermediate has been redirected to the DCVs in this mutant construct. The absence of staining with the amino-terminal
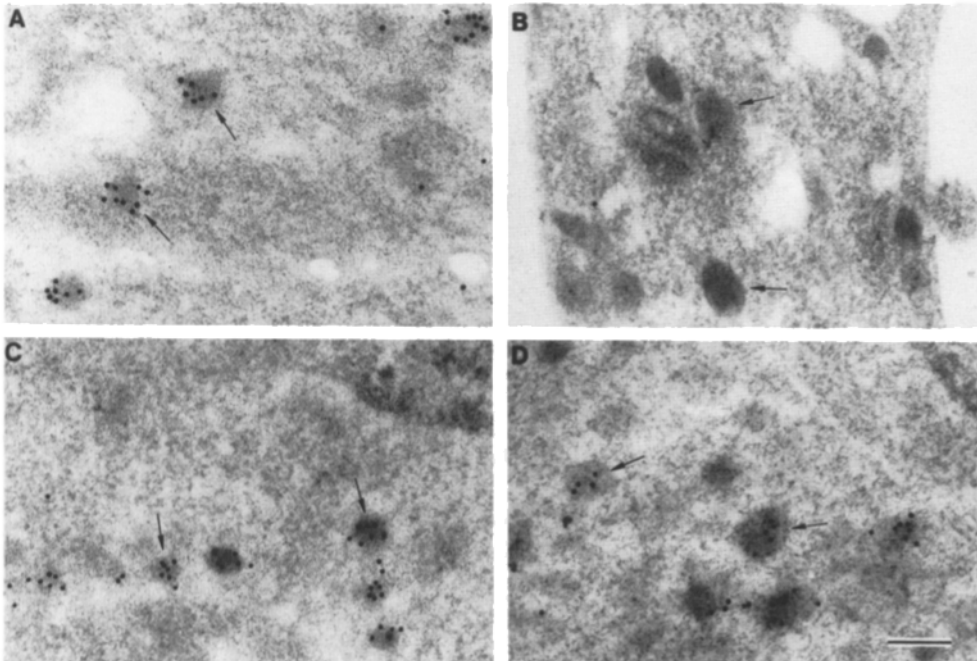
Figure 3. ELH precursor products are localized in dense core vesicles in transfected AtT-20 cells. Sections of each of the four clones were stained with affinity purified antiserum and then with goat anti–rabbit conjugated 10-nm gold colloid. (*A*) In the wild type clone, ELH immunoreactivity is seen primarily in the DCVs. (*B*) In the wild type clone there is no staining in the DCVs with anti-F3A-C. (*C*) ELH immunoreactivity is seen in the DCVs of the tetra-tribasic-Δ clone, and (*D*) immunoreactivity with anti-F3A-C is also seen in the DCVs of this mutant. Bar, 150 nm.

*Table I. Quantitation of Immunogold Labeling of AtT-20 Transfectants**

	WT/ELH‡	DB/ELH	TB/ELH	WT/ELH	WT/F3A-C	WT/F3B-C	TT/ELH	TT/F3A-C	TT/F3B-C
Dense core vesicles									
Mean density	459	460	324	382	10.9	9.59	451	304	218
SEM	9	32	25	19.2	0.7	2.5	57.3	34.4	6.9
Area analyzed (square micrometers)	2.8	9.86	6.21	2.83	0.83	1.18	4.91	5.1	6.35
Golgi apparatus									
Mean density	28.5	32.7	20.7	23.4	29.7	13.9	22.7	11.4	11.1
SEM	2	5.9	1.6	1.8	2.7	0.63	2.3	1.22	0.23
Area analyzed (square micrometers)	34.2	57.9	54.8	17.7	12.7	17.2	16.5	25	18.6
Cytoplasm									
Mean density	1.79	1.87	1.52	1.64	3.19	2.08	1.07	1.37	1.01
SEM	0.49	0.26	0.23	0.16	0.52	0.19	0.13	0.14	0.22
Area analyzed (square micrometers)	177	292	256	119	50.7	75.8	173	209	205

* Quantitation was performed as described in the Materials and Methods. The data is presented as the mean density of gold particles per square microns of surface area for specific organelle.
The form of ELH prohormone transfected into AtT-20 cells/antibody used for staining sections. Wild type ELH (*WT*); dibasic-deleted prohormone (*DB*); tetrabasic-deleted prohormone (*TB*); tetra-tribasic-deleted prohormone (*TT*).
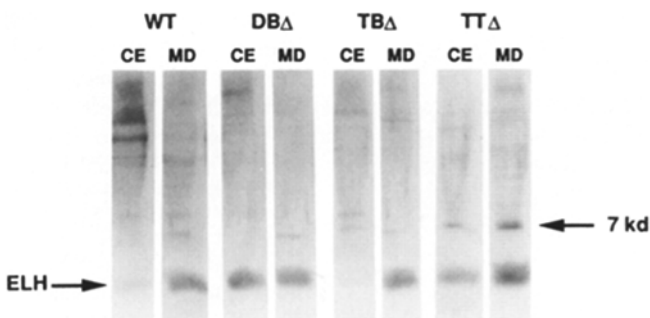
antibodies in the wild type, dibasic-Δ, and tetrabasic-Δ clone, indicates that there is no, or minimal, sorting of the amino-terminal products to the DCVs.

As further biochemical confirmation that ELH was stored in DCVs, the transfected cells were treated with a secretagogue. Each clone was pulsed for 2 h, chased for 3 h, and then treated for 2 h with 5 mM 8-Bromo-cAMP. In the absence of stimulation with secretagogue, ELH is not observed in the media (see below). After this treatment, mature ELH was absent or reduced from the cell extract and present in the media of all the clones. A minor 7-kD ELH–containing band was also isolated from the media of the tetra-tribasic-Δ clone (Fig. 4). This band is most likely the result of alternative cleavage at the arginine amino-terminal to the α bag cell peptide and the Lys-Arg pair carboxy-terminal to ELH (Fig. 1). It is not the same as I3, since the tetrabasic and tribasic sites have been deleted in this mutant. Thus, the wild type

Figure 4. Mature ELH is stored in the regulated secretory pathway. Cells were pulsed for 2 h, chased for 3 h, and then stimulated with 5 mM 8-Br-cAMP for 2 h. After this stimulation, mature ELH was immunoprecipitated from the media (*MD*) of the wild type (*WT*), dibasic-Δ (*DBΔ*), tetrabasic-Δ (*TBΔ*), and the tetra-tribasic-Δ (*TTΔ*) clones; a minor 7-kD ELH– containing product was also isolated from the tetra-tribasic-Δ clone. Mature ELH is not constitutively released into the media (see Fig. 5 *A*). The cell extracts of the wild type, tetrabasic-Δ, and tetra-tribasic-Δ clones, show a lower level of ELH compared to the media.

and all the mutant constructs of the ELH prohormone are processed to yield mature ELH peptide, which is sorted into the regulated secretory pathway. However, as indicated by immunofluorescence and immunoelectronmicroscopy, amino-terminal products derived only from the tetra-tribasic-Δ construct are stored in the DCVs.
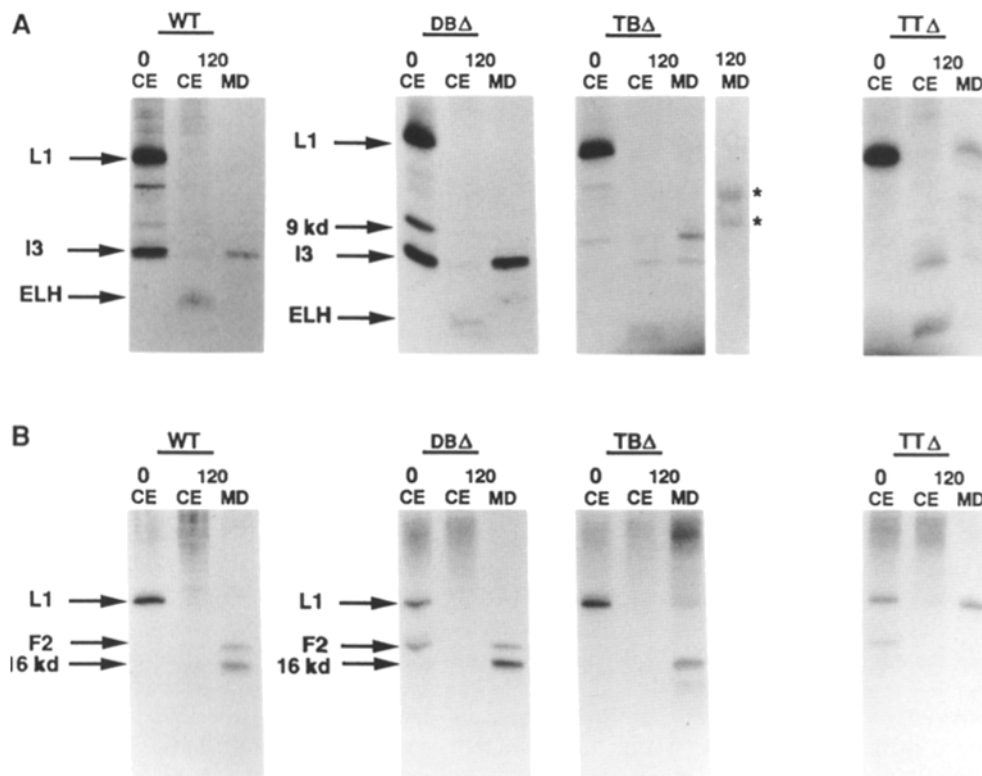
Pathway of Processing

To identify the stored and secreted products, the order of processing, and the site of sorting of the ELH prohormone products, pulse–chase experiments were performed on the ELH-transfected AtT-20 cells. In the first set of experiments, ELH antiserum was used to follow the processing of the prohormone. After a 10-min pulse, prohormone is the predominant product in the wild type clone (Fig. 5 *A*). After a 120-min chase period, ELH is stored in the cell and I3 is secreted into the media. In the dibasic-Δ clone, the prohormone and both a 9-kD intermediate and I3 are present at time 0. At 120 min, the dibasic-Δ clone is very similar to wild type, in that mature ELH is present in the cells and the I3 intermediate is secreted into the media. Prohormone is also initially present at time 0 in the tetrabasic-Δ cells and mature ELH is stored in the cells; however, a 9-kD ELH–containing intermediate is secreted into the media, instead of I3. We also variably observe both a 16- and the 10-kD intermediate (Fig. 5 *A*) secreted into the media from the tetrabasic-Δ clone. To identify the cleavage which produced these two new intermediates, cells were pulsed with [³⁵S]methionine for 2 h and then chased for 2 h; the media was then immunoprecipitated and the products were subjected to radiosequencing. Counts were detected in cycle seven, indicating that the 9-kD protein is produced by cleavage at the tribasic site and based on its size, the 16-kD protein is likely to be produced by cleavage after the γ bag cell peptide (Fig. 1). The processing of the ELH prohormone is also different in the tetra-tribasic-Δ clone. At time 0, prohormone is isolated, as with the other clones, but at 120 min, a minor 7-kD ELH–containing protein and mature ELH are stored in the cells. In further contrast, the prohormone itself is constitutively secreted into the media of the tetra-tribasic-Δ clone (Fig. 5 *A*).

A

WT | DBΔ | TBΔ | TTΔ

Figure 5. Processing and sorting of the wild type and mutant ELH prohormones. (*A*) Immunoprecipitations with anti-ELH. After the 10-min pulse (time *0*) prohormone (*L1*) and the carboxy-terminal intermediate (*I3*) are immunoprecipitated from the wild type cell extract (*CE*). After a 120-min chase period, mature ELH is stored in the cells and I3 is secreted into the media (*MD*). In the dibasic-Δ clone, prohormone, a 9-kD ELH-containing protein, and I3 are immunoprecipitated at time 0. After 120 min, mature ELH is stored in the cells, and I3 is secreted into the media. Prohormone is isolated from the tetrabasic-Δ clone at time 0, and after 120 min, mature ELH is present in the cell extract. In the media at 120 min, a 9-kD ELH-containing protein is secreted and variably, both a 16- and 10-kD ELH containing proteins (*) are secreted into the media (this

sample was run in a urea gel) (Newcomb et al., 1987). For the tetra-tribasic-Δ clone, prohormone is present at time 0. After 120 min, the products in the cell extract are mature ELH and a 7-kD ELH-containing protein. At this same time, prohormone is the primary product secreted into the media. (*B*) Immunoprecipitations with anti-F3A-C. At time 0, all the clones contained the prohormone, and at 120 min the amino-terminal intermediate (F2) is secreted from the dibasic-Δ clone. No apparent products are found from the cell extracts after 120 min of chase. F2 and a 16-kD product are secreted into the media from the wild type and dibasic-Δ clones. Only the 16-kD product is secreted from the tetrabasic-Δ clone, and only prohormone is secreted from the tetra-tribasic-Δ clone.

In a parallel set of experiments, the F3A-C antibody was used to follow the production of the amino-terminal products from the prohormone (Fig. 5 *B*). Again, after a 10-min pulse, prohormone is recovered from the cell extract of all the clones. After a 2 h chase, no amino-terminal products are immunoprecipitated from the cell extract. However, in the media of the wild type and dibasic-Δ clones, the amino-terminal intermediate (F2) is recovered along with a 16-kD protein which is produced by cleavage of F2 at the tribasic site (Fig. 1). These assignments were based on the fact that the F3A-C antibody is directed against the first 32 amino acids of F2. The 16-kD intermediate is the primary product recovered in the media from the tetrabasic-Δ clone and the prohormone was recovered from the media of the tetra-tribasic-Δ clone.

It is notable that no amino-terminal products are immunoprecipitated using the F3A-C and F3B-C antibodies from the tetra-tribasic-Δ clone, despite prominent staining in the DCVs by immunoelectron microscopy. Western blot analysis was also performed on cell extracts of the wild type, dibasic-Δ, tetrabasic-Δ, and tetra-tribasic-Δ clones. However, no amino-terminal products were detected. We reason that one of the following explains the absence of an immunoprecipitated product: (*a*) these antibodies can immunoprecipitate the prohormone and intermediate but are incapable of immunoprecipitating the final product; or (*b*) that F3A or F3B are cleaved further and the labeled methionine is not part of the immunoprecipitated product.

Quantitation of the stored and secreted ELH-containing products was determined for all of the clones. As shown in Table II, all the clones contain a similar amount of mature ELH in the cells after a 2-h chase. However, the dibasic-Δ and tetrabasic-Δ clones constitutively secrete more ELH intermediate(s) than the wild type and tetra-tribasic-Δ clones. It is not clear why there is such a difference. Quantitation of the amount of amino-terminal intermediate or prohormone that was constitutively secreted from the clones showed that the wild type, dibasic-Δ, and tetrabasic-Δ clones secrete at least 66%, while the tetra-tribasic-Δ clone secretes only 37% of the initial level of prohormones into the media. These data imply that sorting of the amino-terminal intermediate of the prohormone is altered in the tetra-tribasic-Δ clone compared to the other clones, and support the storage of amino-terminal products in the DCVs, as shown by the immuno EM data (Table I).

Time Course and Localization of Processing

To identify where the first cleavage occurs, a time course experiment was performed in which the cells were pulsed for 10 min and chased every 5 min up to 20 min. After the 20 min of chase, the prohormone is almost fully cleaved at the unique tetrabasic site in the wild type– and dibasic-Δ–deleted clones. However, ~50% of the prohormone is processed to the 9-kD intermediates in the tetrabasic-Δ clone, and the prohormone remains almost completely unprocessed in the

Table II. Quantitation of the Storage and Release of ELH Prohormone Products from the AtT-20 Transfectants*

	WT CE	WT MD	DB CE	DB MD	TB CE	TB MD	TT CE	TT MD
ELH products								
Percent total	51.68	32.52	35.43	66.75	50.10	60.30	71.43	15.47
SEM	4.05	11.00	5.70	10.62	0.60	1.10	12.90	1.99
Amino-terminal products‡								
Percent total	66.17	–	98.33	–	68	–	37.1	–
SEM	16.9	–	19.22	–	12.89	–	5.26	–

* Transfectants were treated as described in the Materials and Methods. Dta is from the quantitation of several experiments as shown in Fig. 5. ELH-containing products were immunoprecipitated with anti-ELH and amino-terminal products with anti-F3A-C. The values are reported as the percent of total, which is the amount stored in the cell (CE) or secreted in the media (MD), after a 120-min chase period, divided by the total amount isolated at time 0. Wild type prohormone (WT); dibasic-deleted prohormone (DB); Tetrabasic-deleted prohormone (TB); Tetra-tribasic–deleted prohormone (TT).
‡ The percent total of amino-terminal products in the cell extract is not reported as none were detected.

tetra-tribasic-Δ clone (Fig. 6). In the bag cells, EM auto-radiographic experiments demonstrate that the initial tetra-basic cleavage occurs in a late compartment of the Golgi, possibly TGN. In the wild type AtT-20 transfected line, the prohormone was cleaved only at the tetrabasic site and further cleavages were prevented when the clone was treated with the carboxylic ionophore monensin (Jung and Scheller, 1991). This was also the case in bag cells treated with monensin (Sossin et al., 1990a). At 20°C, proteins do not exit from the TGN, and are trapped in this compartment (Saraste and Kuismanen, 1984). To study the site of initial cleavage, all four clones were pulsed for 5 min and then chased for 1 h at 20°C (Fig. 7). When immunoprecipitated with the ELH antibody, the carboxy-terminal intermediate is isolated from wild type and dibasic-Δ clones. The 16.3- and 9-kD intermediates and the prohormone are isolated from the tetrabasic-Δ clone, and only the prohormone is detected in the tetra-tribasic-Δ clone. This indicates that in the wild type, dibasic-Δ, and tetrabasic-Δ cells, the initial cleavage occurs late in the Golgi complex, possibly at the TGN, but in the tetra-tribasic-Δ clone the cleavage occurs after the TGN.

We also believe the TGN is the site of the initial cleavage in the wild type prohormone, based on experiments with

Brefeldin A. In cells treated with Brefeldin A, there is a mixing of the ER and cis-, medial-, and trans-Golgi compartments, but the TGN is not included (Chege and Pfeffer, 1990; Reaves and Banting, 1992). When the wild type clone was pulsed and chased in the presence of Brefeldin A, only prohormone was isolated (Fig. 7). This implies that cleavage at the tetrabasic site was blocked because the prohormone could not enter the TGN. In combination with the immuno-electronmicroscopy and pulse-chase experiments, we interpret these data to mean that in the tetra-tribasic-Δ clone, the entire (unprocessed) prohormone is sorted to DCVs and then processed to release the observed products.

Discussion

Processing of the ELH prohormone in the bag cells of the marine mollusc A. californica includes nine proteolytic cleavages. It is interesting that the processing and sorting machinery of the mammalian AtT-20 cells is able to recognize the structural features of the ELH prohormone, and process it in a similar manner to that of the bag cells. When wild type ELH prohormone is expressed in AtT-20 cells, the tetrabasic site is cleaved in a late Golgi compartment, probably TGN; the amino-terminal and carboxy-terminal intermediates are separated and differentially sorted in a manner similar to the bag cells. A portion of the carboxy-terminal intermediate is
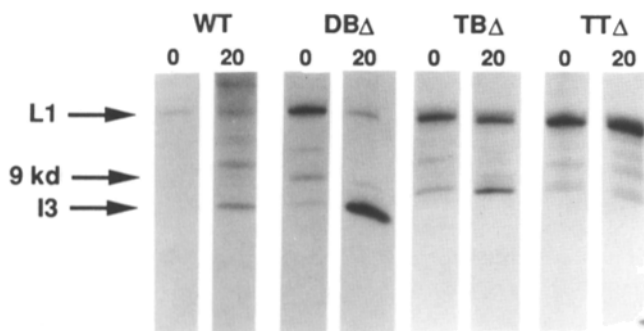

Figure 6. Initial processing of the wild type and mutant prohormones. Clones were pulsed for 5 min and then chased at 5-min intervals. After the pulse (time 0), prohormone is present in all the clones. After 20 min of chase, at least 90% of the prohormone in the wild type and dibasic-Δ clones has been processed to I3 (10-kD protein in WT, time 20 lane, is not ELH specific). However, at 20 min only 50% of the tetrabasic-Δ prohormone has been processed, and virtually none of the tetra-tribasic-Δ prohormone has been processed.
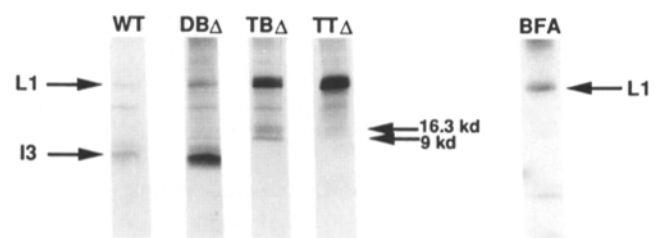

Figure 7. 20°C temperature block. Cells were pulsed for 5 min and then chased for 1 h at 20°C. The cell extracts were immunoprecipitated with anti-ELH and processed as described in the Materials and Methods. The carboxy-terminal intermediate (I3) is the predominant product in the wild type and dibasic-Δ clones. In the tetrabasic-Δ clone, prohormone, 16.3- and 9-kD ELH-containing proteins are isolated: prohormone is the primary product from the tetra-tribasic-Δ clone. In wild type cells treated with BFA (10 μg/ml) for 1 h and then pulsed for 1 h in the presence of BFA, prohormone is the primary product.

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sorted to the regulated pathway and stored in DCVs, whereas most of the amino-terminal intermediate is constitutively secreted into the media. In the bag cells, the amino- and carboxy-terminal intermediates are also sorted differentially but into distinct classes of regulated DCVs, suggesting that these neurons have an additional specialization within the regulated secretory pathway. Furthermore, in the bag cells, a portion of the amino-terminal intermediate is probably degraded intracellularly perhaps by lysosomes. Transfecting mutant forms of the ELH prohormone into AtT-20 cells provides opportunities to examine the biological relevance of the organization of ELH prohormone processing sites, to study how structural features of this prohormone mediate sorting, and to eventually identify the molecular mechanisms governing the differential sorting of the ELH prohormone products.

The primary goal of this study was to understand how the proteolytic processing and the location of processing sites regulate sorting of the amino-terminal and carboxy-terminal intermediates of the ELH prohormone. Our focus was to mutate the unique tetrabasic site, which is the first to be cleaved and allows separation of the two intermediates. A tetrabasic site is not common to prohormones, but is present in some proreceptors. More commonly, the dibasic Lys-Arg pair is a recognition site for proteolytic cleavage. Therefore, we constructed the dibasic-Δ prohormone which contains a Lys-Arg pair instead of the tetrabasic site and a tetrabasic-Δ prohormone which lacks the tetrabasic site. Curiously, the dibasic-Δ prohormone is cleaved at the now dibasic (formerly tetrabasic) site, and the tetrabasic-Δ prohormone is cleaved primarily at an upstream unique tribasic site.
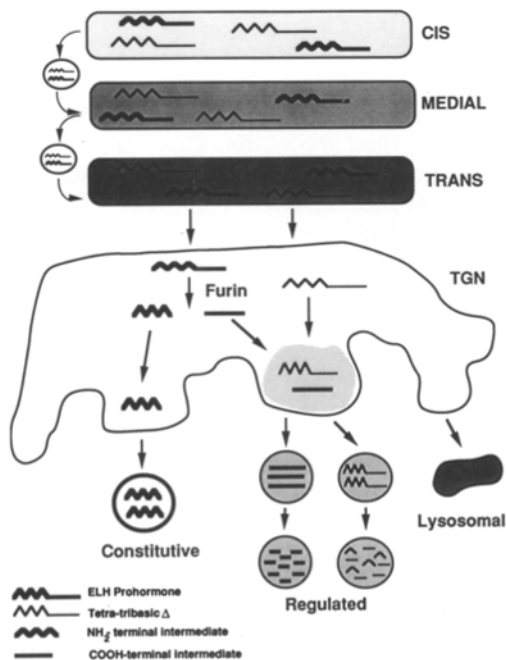
The discovery of furin as a proteolytic enzyme (Van de Ven et al., 1990) involved in cleaving prohormones, viral proteins, and receptors, aided our understanding of the importance of the order of processing and the structure of the ELH prohormone. The first cleavage occurs at the tetrabasic site which fits the proposed consensus sequence for furin (Arg-X-Arg/Lys-Arg). Furthermore, ELH prohormone expressed in oocytes is cleaved at the unique tetrabasic site (Korner et al., 1991). The prevailing thought is that furin is localized in the Golgi (Breshnahan et al., 1990). Thus, it is likely that furin cleaves the ELH prohormone at the tetrabasic site in the Golgi or the TGN. However, the dibasic-Δ prohormone is cleaved at the dibasic site, which is not a furin consensus sequence site. There are several reasons why this may occur. It is possible that another processing enzyme cleaves the dibasic-Δ prohormone with a similar time course, or that there are structural features of the tetrabasic region which favor cleavage of even a dibasic pair by furin. Alternatively, furin may still recognize this as a site because the basic residue histidine is present in the P4 position (position P1 is the carboxy-terminal residue), as opposed to arginine. In prorennin, lysine is present in the P4 position of a cleaved furin site, even though mutational analysis showed that an arginine at P4 is preferred to the lysine (Watanabe et al., 1992). Interestingly, the tetrabasic-Δ prohormone is cleaved at other potential furin consensus sites, though at a slower rate. This suggests that the conformation of the prohormone is not favorable for cleaving these other potential furin sites in the wild type prohormone. Thus, we conclude that, along with the consensus recognition site, there are secondary and tertiary structural features that dictate the initial cleavage at the

tetrabasic site; these structural features are preserved in the dibasic-Δ prohormone.

In the wild type, dibasic-Δ, and tetrabasic-Δ prohormone constructs, the amino-terminal intermediates are primarily constitutively secreted into the media. Consequently, we constructed a new mutant in which the tribasic site was deleted in the tetrabasic deleted prohormone to produce the tetra-tribasic–deleted prohormone. In this case, the sorting of the products is quite different from the other three constructs. The tetra-tribasic-Δ prohormone is not cleaved in the TGN. ELH is still produced, but there is also a minor 7-kD ELH–containing product stored in the DCVs. This indicates that deletion of particular processing sites affects the accessibility of other sites (i.e., those flanking ELH) and reduces the efficiency of mature ELH production. These effects could be mediated in part by the local environment (e.g., pH and ions) of the organelle in which a particular processing enzyme resides. Hence, the conformation of the processing intermediates, and the location and timing of PCE action are crucial to the maturation of prohormone products.

The most interesting result is the presence of amino-terminal immunoreactivity in the DCVs of cells transfected with the tetra-tribasic-Δ prohormone. Thus, by deletion of the two favored potential furin sites, the amino-terminal products are restored to the regulated pathway. There are other cases whereby mutation of furin sites alters the maturation of secreted proteins. For example, a mutation in the furin cleavage site prevents maturation of blood clotting factor IX, resulting in the loss of function (Bentley et al., 1986). However, maturation of the insulin proreceptor appears unaffected by the lack of processing by furin (Yoshimasa et al., 1990). These data on the processing and sorting of the products from mutant ELH prohormones also indicate that the sorting information, whether it is a specific signal or an effect of aggregation, is not dependent on cleavage of the ELH prohormone before sorting. Furthermore, it appears that the propensity for sorting of the ELH product into DCVs is dominant over the targeting of the amino-terminal products to the constitutive pathway. This is in agreement with other workers (Moore and Kelly, 1986).

We are now able to rule out scenarios whereby amino- and carboxy-terminal peptides are copackaged followed by selective degradation in distinct vesicle classes (Sossin et al., 1990b) and propose the following model (Fig. 8). The ELH prohormone is cleaved possibly by furin when it arrives in a late Golgi compartment (TG/TGN). Based on our data, this cleavage is very persistent and is probably defined in part by secondary or tertiary structural features of the prohormone. After this cleavage, the amino- and carboxy-terminal intermediates travel to a sorting area in the TGN. The amino-terminal intermediate is directed to the constitutive pathway; a portion of the carboxy-terminal intermediate is sorted to the regulated pathway, where it is further processed to produce mature ELH which is stored in DCVs. This is the scenario for the wild type, dibasic-Δ, and tetrabasic-Δ ELH prohormones. In contrast, when the tetra-tribasic-Δ prohormone reaches the site of furin, it is not cleaved and passes to the sorting area of the TGN. The unprocessed prohormone is then sorted into the regulated secretory pathway DCVs where mature ELH and amino-terminal products are produced. Some fraction of both the wild type carboxy-terminal intermediate and the tetra-tribasic-Δ prohormone

Figure 8. Processing and sorting model of the ELH and tetra-tribasic ELH prohormones in the secretory pathway of AtT-20 cells. This model depicts the pathway of sorting wild type and tetra-tribasic-Δ ELH prohormones. Both prohormones travel vectorially through the Golgi stacks. Once in the TGN, a processing enzyme, most likely furin, cleaves the wild type prohormone at the unique tetrabasic site. However, the tetra-tribasic prohormone is not cleaved. At this point, the wild type prohormone is separated into the amino- and carboxy-terminal intermediates. The carboxy-terminal intermediate is packaged into DCVs and further processed to mature ELH (Korner et al., 1991). The wild type amino-terminal intermediate is constitutively secreted and is not further processed. The tetra-tribasic-Δ prohormone is packaged into regulated DCVs and further processed to produce mature ELH and amino-terminal products. Both the wild type carboxy-terminal intermediate and the tetra-tribasic-Δ prohormone are also constitutively secreted (not depicted) as the sorting of exogenous prohormones to the regulated pathway in AtT-20 cells is not absolute (Moore et al., 1983).

are constitutively secreted, as the sorting in AtT-20 cells is not absolute (Moore et al., 1983).

Processing can be viewed as a regulatory step in the final sorting of the ELH prohormone products. In the ELH prohormone, the bag cell peptides are encoded in the amino-terminal intermediate and ELH in the carboxy-terminal intermediate. ELH acts as a neuromodulator and a neurohormone, while the bag cell peptides act in an auto-regulatory mode. When the two intermediates are liberated by proteolytic cleavage in the bag cells, they are physically separated, allowing the distinct sorting signals of each intermediate to segregate products to the different classes of DCVs. Thus, the localization of furin, or a related enzyme, in the Golgi/TGN and a furin consensus site (the unique tetrabasic site) in the ELH precursor allow the physiologically active peptides in the two parts of the precursor to be differentially packaged and localized. Cleavage of the intermediates within the DCVs ensures the copackaging of a specific subset of peptides present on the precursor. In this way, different peptides expressed from a single gene can be differentially localized, in order

to serve separate functions which together govern a complex behavior in the animal.

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