Targeting of Frog Prodermorphin to the Regulated Secretory Pathway by Fusion to Proenkephalin

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Abstract. We have investigated the sorting and processing of the amphibian precursor prepro-dermorphin in mammalian cells. Dermorphin, a D-alanine-containing peptide with potent opioid activity, has been isolated from the skin of the frog Phyllomedusa sauvagei. The maturation of this peptide from the precursor involves several posttranslational steps. Recombinant vaccinia viruses were used to infect AtT-20, PC12, and HeLa cells to study the sorting and processing of prepro-dermorphin. While this precursor was not processed in any of the examined cell lines, AtT-20 cells were able to process ~40% of a chimeric precursor consisting of the first 241 amino acids of prepro-enkephalin fused to a carboxy-terminal part of pro-dermorphin. By immunogold-EM, we could show that the chimeric protein, but not pro-dermorphin, was sorted to dense-core secretion granules. The processing products could be released upon stimulation by 8-Br-cAMP. We conclude that the pro-enkephalin part of the fusion protein contains the information for targeting to the regulated pathway of secretion, while this sorting information is missing in pro-dermorphin. This indicates that sorting mechanisms may differ between amphibian and mammalian cells.

It is well established that secretion of peptides and proteins from cells can be either regulated or constitutive (1). In the former case, products are stored at high concentration in specialized vesicles which accumulate in the cytoplasm until exocytosis is triggered by external stimuli. Conversely, in the constitutive pathway newly synthesized secretory products are transported to the plasma membrane and released independent of extracellular signals. While the constitutive pathway is present in all cells, the regulated secretion pathway is restricted to certain secretory cells. In such cells, special sorting mechanisms must exist which direct a set of secretory products to this alternative route. The precursors of secretory peptides are frequently directed to the regulated pathway where processing to the mature products takes place. This sorting into the regulated pathway also functions with heterologous precursors. It has been demonstrated that in mouse pituitary AtT-20 cells transfected with cDNAs encoding mammalian prohormones the precursors are stored in secretory vesicles and proteolytically converted to mature hormones (2, 4, 8, 16, 19, 26, 30). On the other hand, cells lacking the regulated secretory pathway like fibroblasts secrete intact prohormones.

In this paper we describe similar experiments using the cDNA coding for a precursor of peptides present in the skin of Phyllomedusa sauvagei, a South American tree frog (23). The end products liberated from this precursor, the heptapeptides dermorphin and deltorphin, have high affinity and selectivity for μ- and δ-opiate receptors, respectively (15, 17). This biological activity is dependent on the presence of D-alanine or D-methionine in the second position. By cDNA cloning, it has been shown that normal codons for these amino acids are present at the relevant positions, suggesting the existence of a posttranslational epimerization of the configuration of certain residues (23, 24).

From the sequence of these cloned cDNAs, it is evident that the processing of the precursors for dermorphins and deltorphins must be rather complex (see Fig. 1 A). Besides cleavage at pairs of basic amino acids, three additional reactions must take place. These are cleavage of the carboxy-terminal Glu-Ala dipeptide, formation of the terminal amide, and epimerization of the second amino acid of the end product from the L- to the D-isomer. The temporal order of these processing reactions is unknown.

The precursors of skin peptides also have to be sorted to specialized granules where processing takes place (5, 7). One might assume that sorting of skin peptides occurs by a mechanism similar to the regulated pathway of secretion. To answer the question whether these amphibian precursors would also be sorted and processed in mammalian cells, we have expressed the cDNA for one of the dermorphin precursors in mammalian cells. Our results show that in these cells, the major part of prodermorphin accumulates intracellularly and is degraded while the rest is secreted constitutively without any detectable processing. Conversely, a hybrid proen-
Materials and Methods

Plasmid Constructions

Standard recombinant DNA procedures were used for the construction of the following plasmids.

**p7.5K131-derm.** The 663-bp fragment of clone D-1/2 (23) was inserted into the Small site of the vaccinia virus expression vector p7.5K-131 after the tandem early/late 7.5 K promoter. The vector p7.5K-131 contains the 7.5 K promoter of the virus fused to the polylinker of pEMBL-131, which was ligated between the ClaI and EcoRI sites within the viral thymidine kinase gene and inserted into the plasmid pUC8.

**P7.5K-131-enk-derm.** The 825-bp BamHI-HindIII fragment of the plasmid pRPE2 (36), which contains the rat prepro-enkephalin cDNA, was treated to Klein-Polymerase to fill up the HindIII site. The 700-bp BamHI-EcoRI fragment of p7.5K-131-derm was partially digested by AluI. This yielded a 262-bp AluI-EcoRI fragment which contains the carboxy-terminal part of the prodermorphin cDNA. This was ligated to the 825-bp fragment encoding the amino-terminal part of prepro-enkephalin. After St-Nuclease treatment it was inserted into the Small site of the vector p7.5K-131 after the 7.5 K promoter.

All vector constructions were confirmed by restriction endonuclease mapping and in case of p7.5K131-enk-derm also by nucleotide sequencing.

Cell Lines and Culture Conditions

HeLa cells, RK-13 (rabbit kidney) cells, and human 143 TK-cells were grown in MEM, containing 10% FCS. AFT-20 cells were grown in DME supplemented with 0.35% glucose and 10% heat-inactivated FCS; PC12 cells in DME, supplemented with 0.35% glucose and 10% heat-inactivated FCS; PC12 cells in DME, containing 10% horse serum and 5% FCS.

Preparation of Recombinant Vaccinia Viruses

Recombinant vaccinia viruses (vv) were prepared using the temperature-sensitive mutant ts (6) essentially according to the procedure of Kieny et al. (14). Stocks for vv:7.5K-derm and vv:7.5K-enk-derm were prepared by infecting RK-13 cells. After 2 d at 37°C, cells and medium were harvested and centrifuged (30 min, 10,000 rpm, 4°C). The pellet was washed in PBS, centrifuged again, and then suspended in 1 mM Tris-HCl, pH 9.0, and sonicated. The nuclei were removed by centrifugation (2,000 rpm, 5 min). The virus was collected from the supernatant by centrifugation at 15,000 rpm through a 36% sucrose cushion for 60 min. The pelleted virus was resuspended in PBS, homogenized briefly, and stored at −20°C.

Infection of Cells with Vaccinia Virus

Before infection, the virus was sonicated for 30 s, diluted in 250 μl PBS (for a 33-mm dish); cells were washed in PBS and virus was added. The inoculum was removed after incubation for 1 h at room temperature and replaced with medium. Poly peptides synthesized in cells infected with the different recombinant viruses were analyzed by pulse labeling, immunoprecipitation, and Western blots.

Production of Antibodies

All peptides were obtained from Neo Systems (Strasbourg). [L-Ala-2]dermorphin-Gly-Glu-Ala (decapetide) was coupled to BSA using glutaraldehyde (3). [L-Ala-2]dermorphin and dermorphin were coupled to BSA via the SH group of a cysteine residue introduced at the carboxy termini of the deamidated peptides, using maleimidobenzoyl-N-hydroxysuccinimide ester as crosslinker agent (3). Antisera against the peptides were raised in rabbits (female, New Zealand). After initial subcutaneous injections at multiple sites of 100 μg of the peptide-protein complex emulsified in complete Freund's adjuvant, animals were twice boosted at 4-wk intervals using equivalent amounts of complexes in incomplete Freund's adjuvant. Animals were bled 15 d after the last injection. Antisera against dermorphin (63 III B), [L-Ala-2]dermorphin (3A), and [L-Ala-2]decapetide (58 IV a) were obtained.

Radioimmunoassays

Decapetide RIA: The tracer was prepared by iodination of the decapetide with iodine-125 in the presence of chloramine-T (12). The moniodinated compound was purified by HPLC. The RIA was performed essentially as described previously for met-enkephalin (22) with a final dilution of the decapetide-antiserum (58 IVa) of 1/240,000. Under these conditions the sensitivity of the assay (corresponding to a representative value which can be distinguished from maximum binding with 95% confidence) was 4 fmol decapetide/tube with an IC50 of 40 fmol decapetide/tube. The cross-reactivity of decapetide-Lys-Lys and decapetide-Lys, expressed on a molar basis, was <0.01%, while that of [D-Ala-2]-decapetide was 100%.

The RIA for met-enkephalin, performed as described before (22), had a sensitivity of 1 fmol and an IC50 of 12 fmol.

Pulse-Chase Experiments

Confluent cells (33-mm dishes) were infected with the recombinant vaccinia viruses as before. After incubation for 6 h at 37°C, the cells were washed with PBS and incubated in methionine-free MEM (Gibco) which was replaced after 1 h by fresh medium containing 50 μCi of [L-35S]methionine (Amersham). After labeling for 30 min, cells were washed twice with medium, containing a tenfold excess of methionine (chase medium) and incubated for the indicated periods at 37°C in this medium. Finally, media were collected and after addition of 1 mM PMSF they were stored at −20°C. The cells were washed twice in ice-cold PBS, harvested in RIPA-buffer (10 mM Tris HCI pH 7.6, 150 mM NaCl, 1% SDS, 1% NaODC, 1% Triton-X-100, 1 mM PMSF) and lysed by vortexing. Cell extracts were centrifuged (15,000 rpm, 15 min, 4°C) and the supernatants were stored at −20°C. For the experiment with leupetin, the inhibitor (100 μg/ml) was added to the medium one hour before the radioactive amino acid was added.

Immunoprecipitation of Pro-dermorphin and Pro-enkephalin-dermorphin and Western Blots

Immunoprecipitation with anti-[L-Ala-2]dermorphin antibody 3A at a dilution of 1:200 was performed in RIPA buffer. After incubation overnight at 4°C, Protein-A-Sepharose beads (Pharmacia Fine Chemicals, Piscataway, NJ) were added to RIPA buffer. The beads were washed twice with PBS, centrifuged again, and then suspended in 1 mM Tris-HCl, pH 9.0, and sonicated. The nuclei were removed by centrifugation (2,000 rpm, 5 min). The virus was collected from the supernatant by centrifugation at 15,000 rpm through a 36% sucrose cushion for 60 min. The pelleted virus was resuspended in PBS, homogenized briefly, and stored at −20°C.

HPLC Analysis of Peptides

Cells were infected with recombinant vv as before. After 16 h at 37°C, the cells were washed twice with PBS, harvested in 5N acetic acid, 1 mM PMSF, and lyzed by freezing/thawing and brief sonication on ice. Cell debris was removed by centrifugation at 15,000 rpm, the supernatant was lyophilized, dissolved in 0.1% trifluoroacetic acid (TFA) and applied to a C8 column (2 × 220 mm, Brownlee) for HPLC. Peptides were separated by an initial 10 min isocratic elution in 0.1% TFA containing 7% acetonitrile, followed by a linear gradient of 7–45% acetonitrile in 1% TFA, at a flow rate of 200 μl/min. 1-min fractions were collected, lyophilized, and analyzed by RIA for the decapetide, [L-Ala-2]dermorphin, derrmorphin and met-enkephalin.

Peptides secreted from AFT-20 cells were analyzed as follows. Confluent cells were infected with recombinant vv at an m.o.i. of 1. After incubation for 16 h the cells were washed twice with serum-free DME, containing 0.07% BSA, and incubated for 1 h in the same medium, either in the absence or in the presence of 5 mM 8-Br-cAMP. Media were collected, and 1 mM PMSF was added and lyophilized. An aliquot was directly assayed by RIA, the rest was applied to a C8 column, and the fractions were assayed by RIAs as described above.

Abbreviations used in this paper: VV, vaccinia virus; m.o.i., multiplicity of infection.

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Digestion with Trypsin and Carboxypeptidase B

Cells were harvested in 5 N acetic acid and lysed as described above. Cell debris was removed by centrifugation. The supernatant was adjusted to 0.1 M NaCl and then 100 mM Tris-HCl, pH 8.4, 2 mM CaCl₂, boiled for 10 min and incubated with trypsin (50 μg/ml) for 2 h at 37°C. After boiling for 10 min, carboxypeptidase-B (0.1 μg/ml) was added and the incubation was continued for an additional hour.

Immunogold-electron microscopy

AtT-20 cells were infected with vv:wt, vv:7.5K-derm or vv:7.5K-enk-derm at an m.o.i. of 1 for 16 h. The cells were removed from the monolayer with 20 μg/ml of proteinase K on ice, fixed in 8% para-formaldehyde (in 200 mM Hepes, pH 7.4), cryo-sectioned, and labeled with antibodies against [L-Ala-2]-dermorphin (3A) as described by Griffiths et al. (11). For quantification, random micrographs were taken at a primary magnification of 28,000 of the granules, irrespective of labeling. The number of gold particles per area of granule profile was estimated by point counting as described (10).

Results

Expression of the Prepro-dermorphin cDNA in Mammalian Cells

We decided to use recombinant vaccinia virus (vv) for the expression of prepro-dermorphin in animal cells. This virus system has the advantage that the foreign protein is expressed in high yields and that many different cell lines can be infected. The cDNA was inserted after the 7.5K promoter into the vaccinia virus vector 7.5K-131 and recombinant vaccinia virus (vv:7.5K-derm) was prepared.

In a first set of experiments three different cell lines were infected with recombinant vaccinia virus. These were HeLa cells which only secrete proteins via the constitutive pathway, as well as AtT-20 and PC12 cells, two neuroendocrine cell lines where regulated and constitutive pathways coexist. Confluent cells were infected with vv:7.5K-derm for 16 h, then labeled for 1 h with L-[3H]tyrosine and subsequently incubated for 2 h in the presence of unlabeled tyrosine. Extracts from cells and media were boiled and then analyzed by SDS-PAGE. Pro-dermorphin with an M₉ of 29 kD could be detected in cells as well as in media (data not shown). It was shown previously that in vitro translation of prepro-dermorphin cDNA yielded a polyepitope with an M₉ of 31 kD. This was cleaved to pro-dermorphin (M₉, 29 kD) in the presence of dog pancreas microsomes (our unpublished experiments).

For further characterization of the dermorphin precursor, cell extracts and media were digested with trypsin and carboxypeptidase B to liberate the processing intermediate [L-Ala-2]dermorphin-Gly-Glu-Ala, henceforth called the decapetide. After immunoprecipitation, the radioactive decapetide was identified by HPLC. The product comigrated with the [D-Ala-2]decapetide, while no label could be detected in the [D-Ala-2]decapetide region (data not shown).

To analyze the secretion kinetics of pro-dermorphin in HeLa and AtT-20 cells, these were infected for 6 h with recombinant vaccinia virus. Cells were then labeled for 30 min with L-[35S]-methionine and subsequently incubated in the presence of excess unlabeled methionine for the indicated periods of time. Extracts from cells and media were immunoprecipitated and then analyzed by SDS-PAGE. In HeLa as well as in AtT-20 cells (see Fig. 2, A and B), less than one third of prodermorphin is secreted during a 4-h chase period, while the rest is retained in the cells and degraded slowly. This degradation is retarded in the presence of leupeptin (see Fig. 2 C), an inhibitor of lysosomal proteases.

Figure 1. Schematic representation of prepro-dermorphin and the chimeric precursor prepro-enkephalin-dermorphin. In A, the postulated processing of pro-dermorphin is shown. The following reactions must take place to liberate dermorphin from its precursor: (1) cleavage at and removal of pairs of basic amino acids; (2) removal of the carboxy-terminal Glu-Ala dipeptide; (3) formation of the carboxy-terminal amide; and (4) epimerization of the alanine from the L to the D-isomer. B presents the fusion protein consisting of amino acids 1-241 of rat preproenkephalin and residues 124-197 of propro-dermorphin. DM, dermorphin; DL, deltorphin; M, met-enkephalin; O, octapeptide; L, leu-enkephalin.
To determine the extent of processing of pro-dermorphin in the various cell lines, we used RIAs. Polyclonal antibodies were prepared against dermorphin, [L-Ala-2]dermorphin, and the decapeptide. Confluent cells (HeLa, PC12 and AtT-20) were infected for 16 h with recombinant vaccinia virus, media were collected, and cells were subsequently lysed by treatment with 5 N acetic acid. Aliquots of cell extracts and media were then analyzed by RIAs. Neither the decapeptide nor smaller products could be detected in significant amounts, indicating that prodermorphin is not processed in any of these cells. In the case of HeLa cells this was to be expected as these do not have a regulated pathway of secretion. Moreover, for PC12 cells, which have a regulated pathway of secretion, it has been reported that these cells are not able to process foreign hormone-precursors (26). However, the fact that the mouse anterior pituitary cell line AtT-20 did not process this amphibian precursor was quite unexpected.

Expression of the Prepro-enkephalin-dermorphin cDNA in Mammalian Cells

The fact that this amphibian precursor is not processed in AtT-20 cells could be due to the following reasons. (a) Prodermorphin is not recognized by the endoprotease(s) involved in processing at the pairs of basic amino acids; and (b) in these cells, the precursor was not directed to the regulated pathway of secretion and thus did not reach the compartment where the processing enzymes are active.

Figure 2. Pulse-chase experiments. HeLa (A) and AtT-20 cells (B) were infected with vv.7.5K-derm for 6 h, labeled for 30 min with L-[35S]methionine and chased for the times indicated. Cells and medium were treated with anti-[L-Ala-2]-dermorphin antiserum 3A and immune complexes were resolved by SDS-PAGE on 15% gels. (C) Same experiment as in A, but in the presence of 100 µg/ml of leupeptin. The amount of radioactive pro-dermorphin present after the pulse (30P) is set at 100% and fractions thereof present in cells and medium after the chase periods are given below each figure. Arrows indicate the position of pro-dermorphin migrating with an M, of 29 kD.
decapetide, dermorphin, and [L-Ala-2]dermorphin. The results obtained with cell extracts are shown in Fig. 4 and Table I.

In Table I, the amount of decapetide and met-enkephalin present after and before digestion with trypsin and carboxypeptidase B are compared. These values correspond to the total amount of precursor and the extent of processing, respectively. In case of the fusion protein, >40% of the immunoreactivity present in the cell extract is already in the decapetide form before digestion. The corresponding value for prodermorphin was only 5%, which corroborates the above mentioned findings that this amphibian precursor is not processed to any significant extent in AtT20 cells. Immunoreactive material was identified as decapetide and met-enkephalin by HPLC (see Fig. 4). These results demonstrate that the processing enzymes of AtT20 cells can liberate the decapetide from prodermorphin. On the other hand, using the respective antibodies, no evidence for the presence of [L-Ala-2]dermorphin or dermorphin could be obtained (data not shown). This indicates that in AtT20 cells further processing of the decapetide, i.e., removal of the carboxy-terminal Glu-Ala dipeptide and conversion of the L- to the D-alanine, does not take place.

As controls, HeLa cells and PC12 cells were also infected with the same recombinant vaccinia virus. In these cells, no evidence for the processing of the pro-enkephalin-dermorphin fusion protein could be obtained (data not shown).

The above experiments indicate that in AtT20 cells the proenkephalin-prodermorphin fusion protein, but not prodermorphin itself, can enter the regulated pathway of secretion. It is known that processing in this pathway mainly takes place after acidification of immature secretory vesicles (20, 21). That this route is also taken by the chimeric precursor is supported by the fact that its processing is abolished in cells treated with 10 mM ammonium chloride (data not shown).

**Regulated Secretion of the Processing Products**

The previous experiments indicate that the hybrid precursor is processed in AtT20 cells with high efficiency. We also...
wanted to check whether the processing products could be released from their site of storage by secretagogues. Cells were infected with recombinant vaccinia virus for 16 h. Subsequently, 8-bromo-cAMP was added and the amount of peptides released after 1 h was measured. As shown in Fig. 5, in the presence of 8-bromo-cAMP the amount of met-enkephalin and decapetide in the medium increased five-to-sixfold. This confirms that in AtT20 cells, the proenkephalin-prodermorphin fusion protein enters the regulated pathway of secretion and that the processing products are stored and released upon suitable stimulation.

**Pulse-Chase Experiments**

The different fate of prodormorphin as opposed to the proenkephalin-prodermorphin hybrid protein was investigated further by pulse-chase experiments. AtT20 cells were infected with vv:7.5K-enk-derm for 6 h, pulse-labeled with L-[35S]methionine for 30 min and then incubated in the presence of excess unlabeled methionine for different periods of time. As shown in Fig. 6, the fusion protein and its N-glycosylated form are processed rather quickly in AtT20 cells. In contrast, HeLa cells constitutively secrete the chimeric precursor in the unprocessed form (data not shown). As shown earlier, pro-dormorphin turns over much more slowly during the chase period (see Fig. 2, A and B).

**Pro-enkephalin-dormorphin, But Not Pro-dormorphin Is Sorted to Dense-core Secretion Granules**

The above results clearly indicate that prodormorphin and the proenkephalin-prodermorphin hybrid protein enter different subcellular compartments. To investigate this further, we employed immunogold-EM using a polyclonal peptide antibody against [L-Ala-2]dormorphin on thawed cryosections. This antibody also recognizes prodormorphin as well as the pro-enkephalin-prodermorphin fusion protein (see Fig. 3). Using this technique the proenkephalin-prodermorphin fusion protein could be detected in significant amounts in dense-core secretion granules (see Fig. 7). Conversely, no significant labeling of these granules could be detected in AtT20 cells producing prodormorphin. A quantitation of these results is shown in Table II.

**Discussion**

The present work addresses two questions, namely whether sorting and processing mechanisms were conserved during the evolution of animal organisms. Sorting into the regulated pathway of secretion is present in specialized animal cells (1). The process has only been studied in some detail in mammalian endocrine cells, and current evidence indicates that it is neither cell nor species specific. After transfection of the cDNAs for several mammalian prohormones into different neuroendocrine cells mature hormones were generated, stored, and released upon addition of secretagogues (2, 4, 8, 16, 19, 26, 28, 30). The molecular mechanism involved in directing certain molecules to the regulated pathway of secretion is currently not known. As no conserved sequences are present, e.g., among the different peptide precursors, other factors must be involved. The condensation-sorting hypothesis proposes that aggregation of certain secretory prod-
Figure 7. Immunogold-EM. A–C show immunolabeled cryosections of ACT-20 cells infected with vv:7.5K-enk-derm. A and B show details of the secretion granules at the periphery of the cells (P, plasma membrane; V, vaccinia virus). The large arrowheads indicate labeled granules, while the small arrowheads indicate granules that have no labeling associated with them. In C, a region of the Golgi complex (G) is seen which has low but specific labeling over the stack (arrows); a labeled secretion granule is also evident (large arrowhead). Bars, 100 nm.
ucts is a crucial step in this sorting process (1, 13, 34). Some experimental evidence for this appealing theory has been presented (9, 25).

Dermal glands of amphibia may represent a special case of regulated secretion. In case of *Xenopus laevis*, these glands are depleted by adrenergic stimulation. Their regeneration within about 3 wk proceeds via the accumulation of secretory cells which subsequently fuse to form a syncytium filled with ellipsoid granules (5, 7). It is currently not known whether this is also true for the dermal glands of *Ph. sauvagei*.

The results presented in this paper demonstrate that in AtT-20 cells, the precursor of the frog skin peptides dermorphin and deltorphin is not sorted into the regulated pathway of secretion. As shown by pulse-chase experiments, prodermorphin is largely retained in these cells and slowly degraded. It is currently not known in which subcellular compartment this degradation takes place. The fact that leupeptin does partly inhibit the intracellular degradation of pro-dermorphin indicates that lysosomal enzymes may be involved in this process. About one third of unprocessed prodermorphin is released constitutively. In a similar study, it was recently shown that one of two somatostatin precursors from anglerfish islets were also secreted constitutively in transfected AtT-20 cells (27).

Conversely, a hybrid protein containing the first 241 amino acids of prepro-enkephalin fused to the carboxy-terminal part of prodermorphin is sorted and processed in these cells. The secretion of the processing products decapeptide and met-endorphin was stimulated by the addition of 8-Br- cAMP. Using immunogold-EM, this fusion protein, but not pro-endorphin, could in fact be detected in dense-core granules. This indicates that the structural determinants for entry into the regulated pathway may differ between amphibian and mammalian cells. In line with earlier findings with chimeric polypeptides (18, 27, 29), these experiments suggest that segregation into the regulated pathway of secretion is an active process which depends on the presence of certain "sorting domains."

We were also interested to study the extent of processing of prodermorphin in mammalian cells. Processing of precursors from organisms as diverse as yeast and mammals appear to have common features. This is exemplified by the fact that two yeast-processing enzymes, the KEX-2 endoprotease and the KEX-1 carboxypeptidase, both function in mammalian cells (32, 33). Using the proenkephalin-dermorphin fusion protein, the extent of processing in AtT-20 cells could be assessed. Only the cleavage at pairs of basic amino acids and their removal from newly exposed carboxyl ends was observed. No further processing of the decapeptide occurred, indicating that the enzyme that cleaves a dipeptide from the carboxyl end and, in particular, the hypothetical enzyme which converts the second amino acid of the end product from the L- to the D-isomer are apparently not present in AtT-20 cells.

Our results show that besides common features present in the secretory pathway of all animal cells, more specific and restricted mechanisms operate in the sorting of precursors to the regulated pathway and their subsequent processing.

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### Table II. Quantitation of Cryo-section Labeling

|                        | Number of micrographs | Gold/μm² granules |
|------------------------|-----------------------|-------------------|
| vv:7.5K-enk-derm       | 12                    | 104.6 ± 25 SEM    |
| vv:7.5K-derm           | 12                    | 13.5 ± 5 SEM      |

AtT-20 cells were infected with vv:7.5K-enk-derm or vv:7.5K-derm for 16 h. The cells were fixed, cryo-sectioned, and labeled with the antiserum anti-[L-Ala-2]dermorphin 3A. Random micrographs were taken and the number of gold particles per area of granule profile was estimated by point counting as described previously (10).

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