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

Gabriele Seethaler,* Michel Chaminade,‡ Reinhard Vlasak,* Maria Ericsson,‖ Gareth Griffiths,‖ Odaly Toffoletto,‡ Jean Rossier,‡ Henk G. Stunnenberg,§ and Günther Kreil*

*Institute of Molecular Biology, Austrian Academy of Sciences, Billrothstr. 11, A-5020 Salzburg, Austria; ‡Laboratoire de Physiologie Nerveuse, Centre National de la Recherche Scientifique, Avenue de la Terrasse, 91190 Gif sur Yvette, France; §Gene Expression Programme; and ‖Cell Biology Programme, European Molecular Biology Laboratory, D-6900 Heidelberg, Germany

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-

kephalin-prodermorphin precursor is sorted into the regulated pathway of secretion and partly processed.

Materials and Methods

Plasmid Constructions

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

p7.5K-131-derm. The 663-bp fragment of clone D-1/2 (23) was inserted into the SmaI 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 BanII-HinfI fragment of the plasmid pRPE2 (36), which contains the rat prepro-enkephalin cDNA, was treated by Klenow-Polymerase to fill up the HinfI 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 S1-Nuclease treatment it was inserted into the SmaI 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 p7.5K-131-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. AtT-20 cells were grown 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)[1] were prepared using the temperature-sensitive mutant ts7 (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, 18,000 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. Polypeptides 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 (decapeptide) 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-succinimide 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

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

B), [L-Ala-2]dermorphin (3A), and [L-Ala-2]decapeptide (58 IV a) were obtained.

Radioimmunoassays

Decapeptide RIA: The tracer was prepared by iodination of the decapeptide with iodine-125 in the presence of chloramine-T (12). The monoiodinated compound was purified by HPLC. The RIA was performed exactly as described previously for met-enkephalin (22) with a final dilution of the decapeptide-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 decapeptide/tube with an IC 50 of 40 fmol decapeptide/tube. The cross-reactivity of decapeptide-Lys-Lys and decapeptide-Lys, expressed on a molar basis, was <0.01%, while that for [D-Ala-2]-decapeptide was 100%.

The RIA for met-enkephalin, performed as described before (22), had a sensitivity of 1 fmol and an IC 50 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 hour 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.HCl pH 7.6, 150 mM NaCl, 0.1% SDS, 1% NaDOC, 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 leupeptin, 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) washed in RIPA buffer were added. The incubation was continued for an additional hour, and beads were pelleted and consecutively washed three times with RIPA buffer and one time with 50 mM Tris-HCl, pH 7.5. The beads were taken up into SDS sample buffer, boiled for 10 min, centrifuged, and the supernatants analyzed by SDS-PAGE on 15% gels followed by fluorography.

For the immunoblots, aliquots of cell extracts and media were fractionated by SDS-PAGE, and transferred to nitrocellulose. This was then incubated with the anti-[L-Ala-2]dermorphin antiserum 3A in a dilution of 1:500 and detected by an anti-rabbit alkaline phosphatase–linked antibody (BRL).

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 lysed 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 55 min of a linear gradient of 7–45% acetonitrile in 0.1% TFA, at a flow rate of 200 μl/min. 1-min fractions were collected, lyophilized, and analyzed by RIAs for the decapeptide, [L-Ala-2]dermorphin, dermorphin and met-enkephalin.

Peptides secreted from AtT-20 cells were analyzed as follows. Confluent cells were infected with recombinant vv at an m.o.i. of 1. After an 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.

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 lyophilized, dissolved in 50 mM Tris-HCl, pH 8.4, 2 mM Ca-chloride, 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-electronmicroscopy

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 quantitation, 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 7.5 K 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 M_r 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 polypeptide with an M_r of 31 kD. This was cleaved to pro-dermorphin (M_r 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 decapeptide. After immunoprecipitation, the radioactive decapeptide was identified by HPLC. The product comigrated with the [L-Ala-2]decapeptide, while no label could be detected in the [D-Ala-2]decapeptide 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.
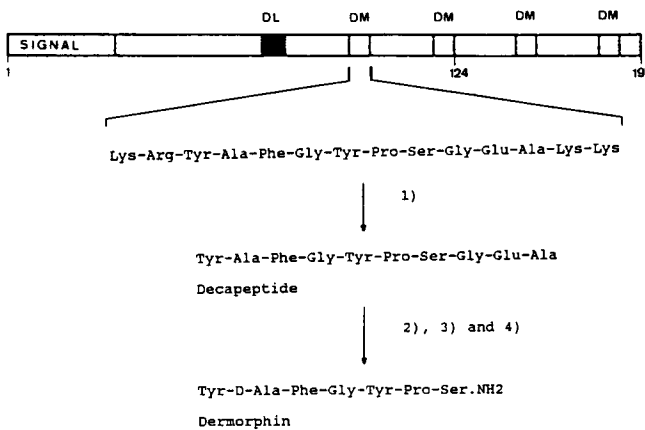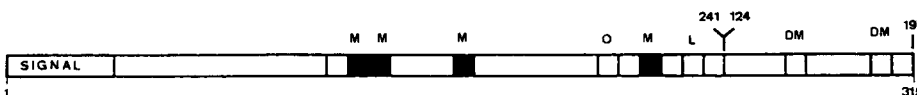
A   PREPRO-DERMORPHIN

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 prepro-dermorphin. DM, dermorphin; DL, deltorphin; M, met-enkephalin; O, octapeptide; L, leu-enkephalin.

B   PREPRO-ENKEPHALIN-DERMORPHIN

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. In AtT-20 cells transfected with the cDNA of human pro-insulin, the precursor is directed to the regulated pathway and processed to insulin (19). Similar results have been obtained for other mammalian hormone precursors (2, 4, 8, 16, 26, 30).

The fact that this amphibian precursor is not processed in AtT-20 cells could be due to the following reasons. (a) Pro-dermorphin is not recognized by the endoprotease(s) in-

volved 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.

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

To discern between these alternatives, a chimeric precursor was constructed which contains the amino-terminal 241 amino acids of prepro-enkephalin and a carboxy-terminal region derived from pro-dermorphin (Fig. 1 B). It has earlier been shown (30) that pro-enkephalin, expressed by recombinant vaccinia virus in AtT-20 cells, is cleaved to met-enkephalin.

The prepro-enkephalin-dermorphin cDNA was inserted after the 7.5 K promoter of vv and recombinant virus was prepared (vv:7.5K-enk-derm). AtT-20 cells were then infected with this virus for 16 h. Cell extracts were analyzed by SDS-PAGE and Western blotting. As shown in Fig. 3, two fusion proteins are present (Fig. 3, lane 4), of which the larger is N-glycosylated in the pro-enkephalin part (31, 35). This is indicated by the fact that in the presence of tunicamycin, this larger product is absent (data not shown). AtT-20 cells infected with this recombinant virus were also analyzed by HPLC and RIAs for the presence of met-enkephalin,
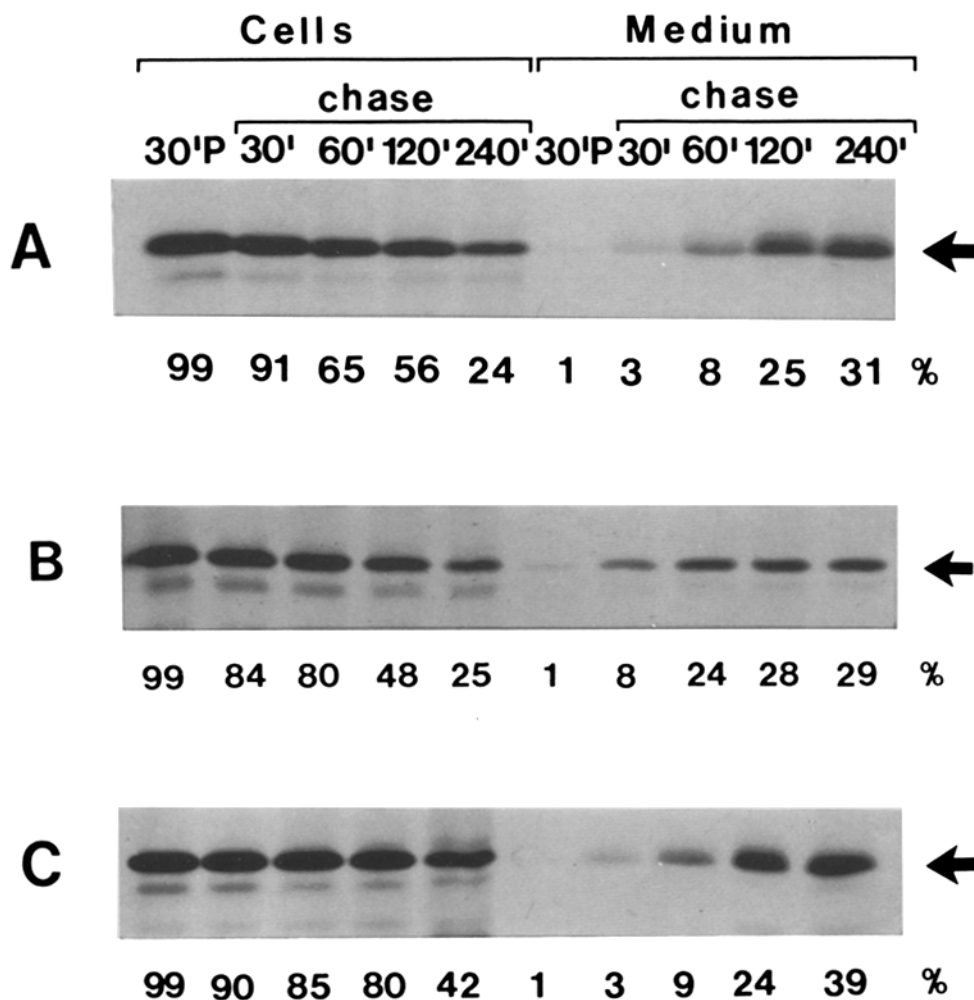

Figure 2. Pulse-chase experiments. HeLa (A) and AtT-20 cells (B) were infected with vv:7.5K-derm for 6 h, labeled for 30 min with L-[³⁵S]methionine and chased for the times indicated. Cells and medium were treated with anti-[L-Ala-2]-dermorphin antiserum 3A and immune complexes were resolved by SDS-PAGE 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 (30'P) 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_r of 29 kD.
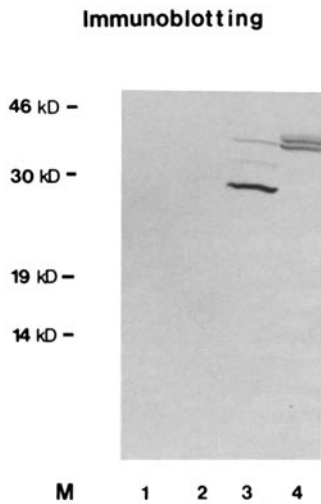
Immunoblotting

46 kD –
30 kD –
19 kD –
14 kD –

M   1   2   3   4

Figure 3. Immunoblotting. AtT-20 cells were either mock infected (lane *1*) or infected with wt virus (lane *2*), vv:7.5K-derm (lane *3*) or vv:7.5K-enk-derm (lane *4*) for 16 h. Cell extracts were fractionated by SDS-PAGE, transferred to nitrocellulose, incubated with the anti-[L-Ala-2]-dermorphin antiserum 3A, and then treated with an antirabbit alkaline phosphatase–linked antibody (BRL). The fusion protein (lane *4*) is partly N-glycosylated. The minor prodermorphin polypeptides with higher molecular weights (lane *3*) probably represent O-glycosylated forms.

Table I. Processing of Pro-enkephalin-dermorphin and Pro-dermorphin

	Decapeptide fmol IR/10⁶ cells Try/CPB		Met-enkephalin fmol IR/10⁶ cells Try/CPB	
	+	−	+	−
vv:7.5K-enk-derm	9,072	3,920 (43%)	44,640	11,780 (26%)
vv:7.5K-derm	24,500	1,240 (5%)		

AtT-20 cells were infected with vv:7.5K-derm or vv:7.5K-enk-derm at an m.o.i. of 5 for 16 h. Cells were extracted by 5 N acetic acid, lyophilized to dryness, and analyzed by RIAs for decapeptide and met-enkephalin either before (−) or after (+) digestion with trypsin and carboxypeptidase B. Each value is the mean of three independent experiments. On serial dilutions the immunoreactive material gave a curve parallel to the standards. The identity of the immunoreactive material was further identified by HPLC (see Fig. 4). The percentage of processing is given in brackets.

decapeptide, 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 decapeptide 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 decapeptide 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 AtT-20 cells. Immunoreactive material was identified as decapeptide and met-enkephalin by HPLC (see Fig. 4).

These results demonstrate that the processing enzymes of AtT-20 cells can liberate the decapeptide from prodermorphin. On the other hand, using the respective antibodies, no evidence for the presence of [L-Ala-2]dermorphin or der-

morphin could be obtained (data not shown). This indicates that in AtT-20 cells further processing of the decapeptide, 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 AtT-20 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 AtT-20 cells with high efficiency. We also
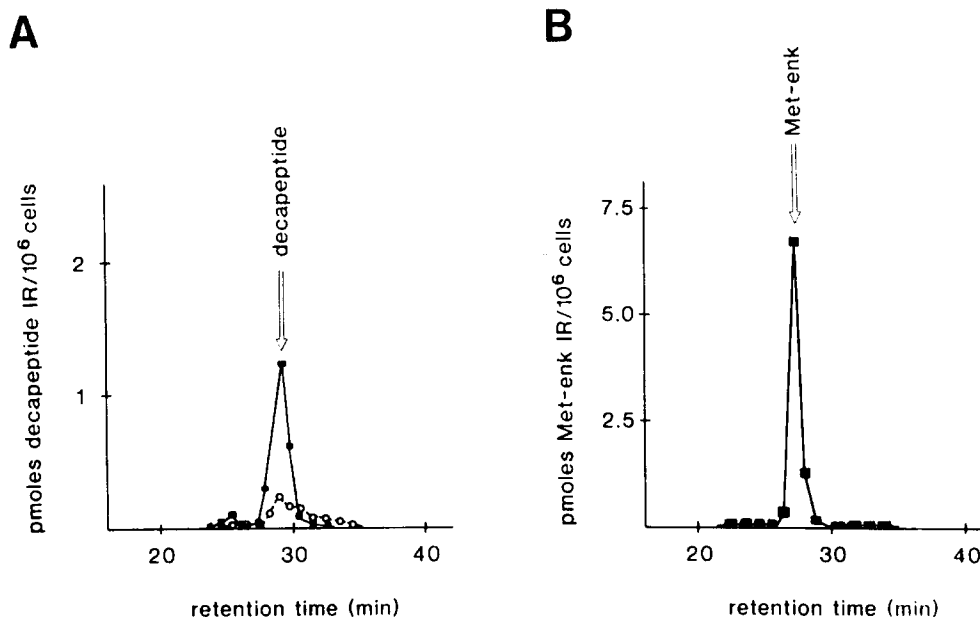
A

B

Figure 4. HPLC analysis of cell extracts followed by RIAs. AtT-20 cells were infected with vv:7.5K-derm (O—O) or vv: 7.5K-enk-derm (■—■) at an m.o.i. of 5 for 16 h. Cells were extracted with 5 N acetic acid, lyophilized, dissolved in 0.1% TFA, and applied to a C8 HPLC column for reversed phase chromatography. Fractions were collected and analyzed by RIAs for decapeptide (*A*) and met-enkephalin (*B*). The arrows indicate the elution times of the standard peptides.

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 decapeptide in the medium increased five- to sixfold. This confirms that in AtT-20 cells, the proenkeph-alin-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 prodermorphin as opposed to the pro-enkephalin-prodermorphin hybrid protein was investigated further by pulse-chase experiments. AtT-20 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 AtT-20 cells. In contrast, HeLa cells constitutively secrete the chimeric precursor in the unprocessed form (data not shown). As shown earlier, pro-dermorphin turns over much more slowly during the chase period (see Fig. 2, A and B).

Pro-enkephalin-dermorphin, but Not Pro-dermorphin Is Sorted to Dense-core Secretion Granules

The above results clearly indicate that prodermorphin 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]dermorphin on thawed cryo-sections. This antibody also recognizes prodermorphin 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
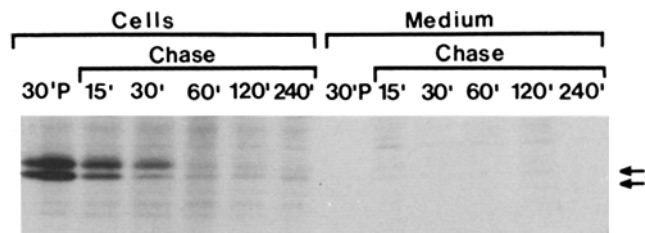

Figure 6. Pulse-chase experiment with the prepro-enkephalin-dermorphin fusion protein. AtT-20 cells were infected with vv: 7.5K-enk-derm for 6 h, pulse labeled for 30 min with L-[³⁵S]methionine and chased for the times indicated. Cells and media were treated with anti-L-dermorphin antiserum 3A, and immune complexes were resolved by SDS-PAGE on 15% gels. Arrows indicate glycosylated pro-enkephalin-dermorphin (M_r 38 kD) and unglycosylated pro-enkephalin-dermorphin (M_r 36 kD).

be detected in AtT-20 cells producing prodermorphin. 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-
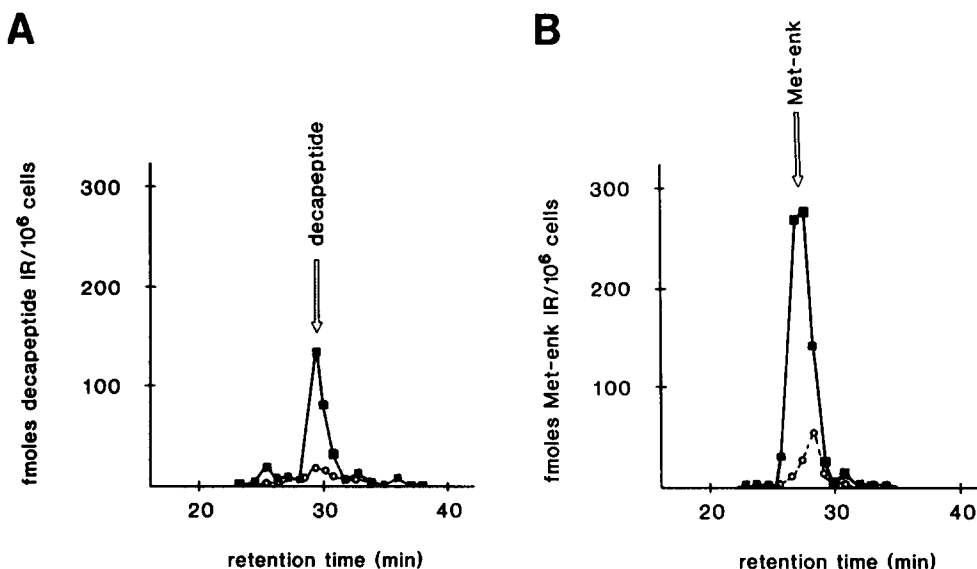

Figure 5. Regulated secretion of the processing products. AtT-20 cells were infected for 16 h with vv:7.5K-enk-derm at an m.o.i. of 1. Medium was removed and the cells were incubated for 1 h in serum-free DMEM containing 0.07% BSA, either in the presence (■—■) or in the absence (○—○) of 5 mM 8-Br-cAMP. Media were collected, lyophilized, dissolved in 0.1% TFA, and applied to a C8 HPLC column for reversed phase chromatography. Fractions were assayed by RIA for decapeptide (A) or met-enkephalin (B). The arrows indicate the elution times of the standard peptides.
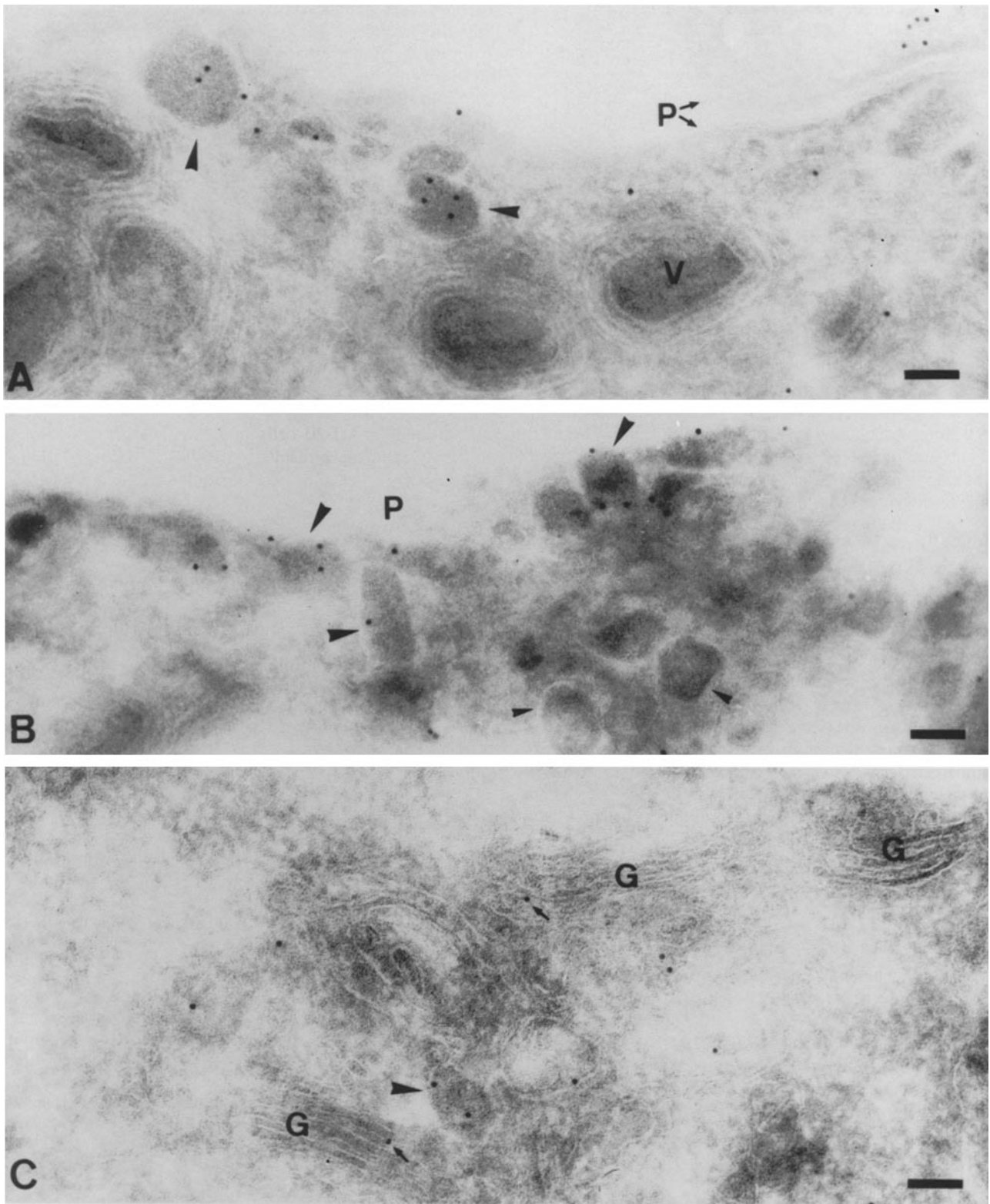
Figure 7. Immunogold-EM. *A–C* show immunolabeled cryosections of AtT-20 cells infected with vv:7.5K-enk-derm. *A* and *B* show details of the secretion granules at the periphery of the cells (*P,* plasma membrane; *V,* vaccinia virus). The large arrowheads indicate labeled granules, while the small 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.

Table II. Quantitation of Cryo-section Labeling

	Number of micrographs	Gold/μm^2 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-derm or vv:7.5K-enk-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).

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 was 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-enkephalin was stimulated by the addition of 8-Br-cAMP. Using immunogold-EM, this fusion protein, but not pro-dermorphin, 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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