of the amyloid precursor protein. The reactivity of the peptides to this protein was increased by β-elimination or treatment with heparinase 2. In addition to the 65,000-dalton protein, some of these antisera also react with a doublet of 105,000 and 110,000 daltons in brain and PC12 supernatant. Because these proteins can be degraded with heparinase 2 to a 65,000-dalton protein, it is possible that they are partially glycosylated forms of the same core protein.
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Yeast *KEX2* Endopeptidase Correctly Cleaves a Neuroendocrine Prohormone in Mammalian Cells

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Mammalian cell lines (BSC-40, NG108-15, and GH_4C_1) that cannot process the murine neuroendocrine peptide precursor prepro-opiomelanocortin (mPOMC) when its synthesis is directed by a vaccinia virus vector were coinfected with a second recombinant vaccinia virus carrying the yeast *KEX2* gene, which encodes an endopeptidase that cleaves at pairs of basic amino acid residues. mPOMC was cleaved intracellularly to a set of product peptides normally found in vivo, including mature γ-lipotropin and β-endorphin$_{1-31}$. In GH_4C_1 cells (a rat pituitary line), product peptides were incorporated into stored secretory granules. These results suggest that the inability of any particular cell line to process a prohormone precursor is due to the absence of a suitable endogenous processing enzyme.

PREPRO-OPIOMELANOCORTIN (POMC) is one of the most thoroughly characterized polyprotein precursors (*1*). The processing of POMC is complex and varies in different cell types (*2*). Each cryptic peptide in POMC is flanked by a pair of basic amino acid residues, namely, -Lys-Arg-, -Arg-Arg-, -Arg-Lys-, -Lys-Lys- (Fig. 1), a motif found in essentially all prohormones (*1*). Liberation of the bioactive segments is a two-step process (*3*): (i) the precursor is cleaved at the carboxyl side of the pair of basic residues by a trypsin-like endopeptidase; and (ii) the doublet of basic residues exposed on the carboxyl-terminal end is removed by an enzyme similar to carboxypeptidase B.

The biochemical basis for the tissue specificity of processing reactions is not understood. Several factors may be involved, including: (i) selective expression of distinct processing enzymes, (ii) differential compartmentation of either one or more proteases or the precursor, and (iii) modulation of cleavage-site accessibility by differential modification of the precursor. In order to distinguish among these possibilities, identification of one or more endopeptidases responsible for the initial cleavages in prohormone processing is essential. Several classes of proteases (*3–9*) have been proposed as candidates for the true prohormone endoprotease. A physiologically relevant role has not been established for any of these enzymes in vivo, however. In contrast, the enzyme required for excision of a yeast peptide hormone (α-factor mating pheromone) from its precursor has been unequivocally identified [for review, see (*10*)]. The *KEX2* gene of *Saccharomyces cerevisiae* (baker's yeast) encodes a membrane-bound endopeptidase specific for cleaving on the carboxyl side of pairs of basic residues that contain arginine (-Lys-Arg- and -Arg-Arg-) (*11*). The *KEX2* enzyme is an unusual serine protease (*12*) that is strictly Ca^{2+}-dependent and has a neutral *p*H optimum (*11–13*).

We describe here the identification of cell lines and the establishment of conditions that permitted the detection of proper processing in vivo of a mammalian prohormone by the precursor-cleaving enzyme encoded by the yeast *KEX2* gene.

Cell lines were infected with recombinant vaccinia viruses expressing the cDNAs for prepro-enkephalin (*14*) or mouse POMC (mPOMC) (*15*). The fate of these precursors was observed by radiolabeling and electrophoretic, chromatographic, and immunological analysis. Three cell lines (NG108-15, GH_4C_1, and BSC-40 cells) (*16*) were unable to release bioactive peptides from these precursors (*14, 15a*), (Fig. 2).

To determine whether the *KEX2* gene could produce a functional membrane-bound enzyme in tissue culture cells, we inserted the *KEX2* gene into a vaccinia virus expression vector by the marker transfer method (*14, 17*). When a variety of mammalian cell lines were infected with this construction (VV:*KEX2*), a large increase in an activity capable of cleaving a specific fluorogenic peptide substrate was observed (Table 1). Depending on the cell type, the specific activity in cells infected with VV:*KEX2* was 16 to 33 times as great as that in mock-infected cells or cells infected by control viruses (VV:WT and VV:mPOMC) (Table

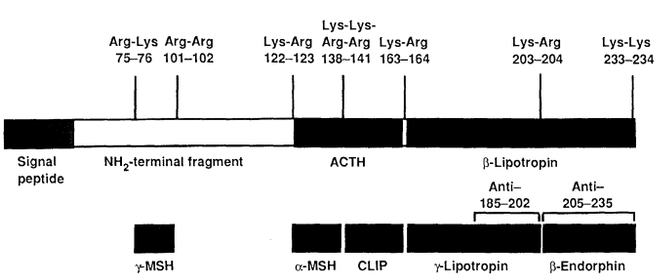

Fig. 1. Structure and processing of the mouse polyprotein precursor, POMC. Mouse POMC (mPOMC) is a 235-residue polypeptide that is processed by cleavage at the indicated pairs of basic amino acid residues into distinct sets of peptide hormones in the anterior and neurointermediate lobes of the pituitary (*1, 2*). Numbering of amino acids in mPOMC is according to that predicted from the cDNA sequence (*30*), beginning with the initiator methionine of the signal peptide. The two specific antisera used in this study are directed against the regions indicated by the brackets. Antiserum 185–202 is selective for γ-LPH (but will also recognize β-LPH and intact mPOMC); similarly, antiserum 205–235 is selective for β-END (but will also recognize β-LPH and intact mPOMC). ACTH, adrenocorticotropic hormone; γ-MSH, γ-melanocyte–stimulating hormone; α-MSH, α-melanocyte–stimulating hormone; CLIP, corticotropin-like intermediate lobe peptide.

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1). This activity was apparently Ca^{2+}-dependent (Table 1) and displayed the same spectrum of sensitivity toward protease inhibitors [resistant to phenylmethylsulfonyl fluoride, tosyl-lysine-chloromethyl ketone, and tosyl-phenylalanine-chloromethyl ketone but inactivated by Ala-Lys-Arg-chloromethylketone (*18*)] as has been documented for the *KEX2* enzyme isolated from yeast (*11–13*). Furthermore, cell-associated activity appeared to be membrane bound, because the majority was found in the particulate fraction (Table 1). Thus, the *KEX2* enzyme produced in mammalian cells had properties similar to those of the *KEX2* protease found in yeast cells. In contrast to yeast where all *KEX2* activity remains intracellular even when the enzyme is overproduced 200- to 500-fold (*12*), a significant fraction (25 to 50%) of the total *KEX2* activity was released from the mammalian cells into the culture medium (Table 1). This extracellular activity was not sedimentable (200,000*g* for 2 hours), suggesting that the catalytic domain had become separated from the sequences required for its membrane retention (*12*).

A reversed-phase high-performance liquid chromatographic (HPLC) separation system was developed which, in combination with the use of specific antibodies, was able to resolve all of the bioactive peptides generated by POMC processing (*19*) (Fig. 2). The peptides produced by AtT-20 cells were used as standards for authentic mPOMC cleavage in vivo, because AtT-20 cells (derived from a mouse anterior pituitary tumor) produce endogenously high levels of mPOMC and cleave the prohormone in a pattern similar to that observed in intact anterior lobe corticotrophs (*20*). Because maturation of adrenocorticotropic hormone (ACTH) and the amino-terminal fragment of POMC (Fig. 1) involves other posttranslational events [glycosylation, phosphorylation, and sulfation (*1*)] that complicate analysis, we focused on the carboxyl-terminal cleavage products of mPOMC to determine if the *KEX2* protease was able to process correctly mPOMC synthesized simultaneously in the same cells.

Each of the "maturation deficient" cell lines (NG108-15, BSC-40, and GH_4C_1) was infected with either VV:mPOMC alone or coinfected with VV:mPOMC and VV:*KEX2*. After 20 hours, the culture medium was removed, and the washed cells were extracted with acetic acid. The peptides in the cell extracts were then resolved by HPLC and analyzed by radioimmunoassay (Fig. 2). In BSC-40 cells (identical results were obtained in NG108-15 cells) infected with VV:mPOMC, a prominent peak of immunoreactivity was present that comigrated with the authentic mPOMC produced in AtT-20 cells (Fig. 2). A similar major peak was found in GH_4C_1 cells infected with VV:mPOMC, although several minor peaks of immunoreactivity, corresponding to γ-lipotropin (γ-LPH, 39 and 41 min), β-endorphin (β-END, 48 min), and β-lipotropin (β-LPH, 55 min), were also detected (Fig. 2).

In contrast, in cells coinfected with VV:*KEX2* and VV:mPOMC, the steady-state level of intact mPOMC present in the cells was significantly reduced and several new prominent mPOMC-derived peptides were observed (Fig. 2). When cells separately infected with VV:mPOMC alone and VV:*KEX2* alone were mixed before extraction with acetic acid, the peptide profile obtained was identical to that in cells infected with VV:mPOMC alone, demonstrating that the proteolytic cleavages observed had not occurred artifactually during cell extraction.

In all three cell lines (data for NG108-15 not shown), a prominent doublet was found at the position of γ-LPH (Fig. 2) and a major peak eluted at 48 min, corresponding to the position of mature β-END_{1-31} (Fig.

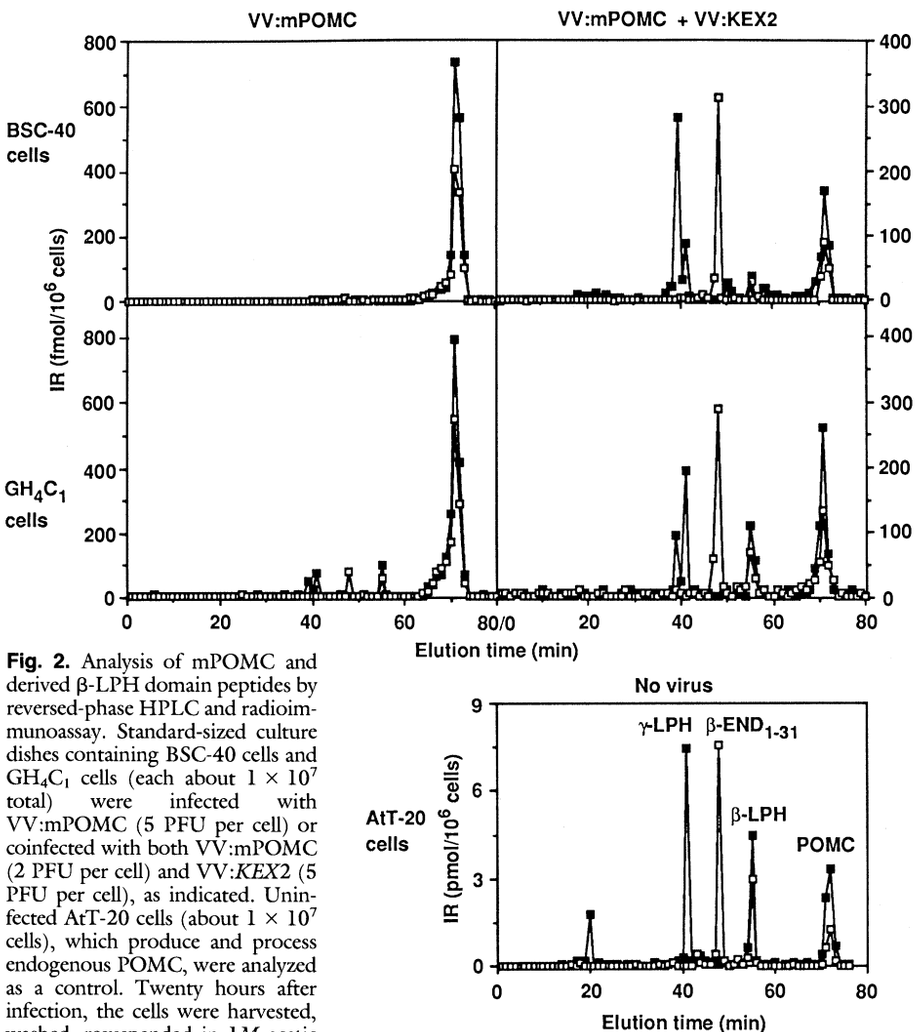

Fig. 2. Analysis of mPOMC and derived β-LPH domain peptides by reversed-phase HPLC and radioimmunoassay. Standard-sized culture dishes containing BSC-40 cells and GH_4C_1 cells (each about 1×10^7 total) were infected with VV:mPOMC (5 PFU per cell) or coinfected with both VV:mPOMC (2 PFU per cell) and VV:*KEX2* (5 PFU per cell), as indicated. Uninfected AtT-20 cells (about 1×10^7 cells), which produce and process endogenous POMC, were analyzed as a control. Twenty hours after infection, the cells were harvested, washed, resuspended in 1*M* acetic acid, 10 m*M* HCl (*p*H 1.9) containing 1 m*M* PMSF and lysed by brief sonication on ice. Insoluble debris was removed by centrifugation, and the extracted peptides were lyophilized and redissolved in 1 ml of 12.8% acetonitrile containing 0.1% trifluoroacetic acid. This material was applied to a C4 reversed-phase column (Vydac 214TP54) and eluted with a linear gradient of acetonitrile from 12.8 to 39.2% over 80 min at a flow rate of 1 ml/min. The content of cross-reacting material in 25 μl of each fraction (one fraction = 1 min) was determined by radioimmunoassay with specific antibodies, 185–202 (filled squares) and 205–235 (open squares) (Fig. 1). BSC-40 cells infected with VV:mPOMC alone produced 1.8 and 1.2 pmol of total cross-reacting material per 10^6 cells against each antiserum, respectively; GH_4C_1 cells infected with VV:mPOMC produced 2.5 and 2.3 pmol of total cross-reacting material per 10^6 cells against each antiserum, respectively. In the same cells coinfected with VV:*KEX2* and VV:mPOMC at a somewhat lower multiplicity of infection, the total amount of cross-reacting material produced was only slightly reduced (30 to 40%). The elution times (in minutes) for authentic mPOMC peptides in the HPLC system used were: γ-LPH, 41; β-END_{1-31}, 48; β-END_{1-26}, 52; β-END_{1-27}, 55; β-LPH, 55; and mPOMC, 71–73 (*31*).

2). In GH_4C_1 cells, but not in the other two lines, a significant amount of immunoreactivity eluted at 55 min, corresponding to the position of β-LPH (Fig. 2).

The peptide that eluted at 55 min had an apparent mobility upon electrophoresis in an 18% polyacrylamide gel corresponding to a molecular weight of 8200, cross-reacted with antisera that recognize both γ-LPH and β-END, and comigrated with authentic β-LPH derived from AtT-20 cells, which confirmed its identity as β-LPH. The peptide that eluted at 48 min was subjected to cation-exchange chromatography and yielded a peak of β-END cross-reacting material that coeluted with synthetic $\beta\text{-END}_{1-31}$ (Fig. 3A), confirming its identity as $\beta\text{-END}_{1-31}$. The γ-LPH–related material that eluted at 41 min, in a position identical to mature γ-LPH from AtT-20 cells (Fig. 2), was also subjected to cation-exchange chromatography and coeluted with authentic γ-LPH from AtT-20 cells (Fig. 3B). In contrast, the γ-LPH–related material that eluted at 39 min on the reversed-phase column (Fig. 2) was significantly retarded on cation-exchange chromatography, compared to mature γ-LPH (Fig. 3B). The stronger retention of this species suggested that it represented γ-LPH from which one or both of the carboxyl-terminal basic residues had not yet been removed after the liberation of this peptide from mPOMC by *KEX2* action.

To test this possibility, this same material was treated with carboxypeptidase B before cation-exchange chromatography. Under the incubation conditions used, the peptide was quantitatively converted to a species that comigrated with mature γ-LPH (Fig. 3B). If briefer incubation times were used, most of the material was converted to a species that eluted from the cation-exchange column at an intermediate position (29 min). The two-step shift in mobility upon digestion with carboxypeptidase B supports the conclusion that this peptide represents γ-LPH from which the carboxyl-terminal Lys-Arg residues have not yet been removed. These results, and the fact that mature $\beta\text{-END}_{1-31}$ was also produced, define precisely the location of one cleavage of mPOMC by the *KEX2* enzyme in mammalian cells: specifically, at the carboxyl side of $Lys^{203}Arg^{204}$. These findings also indicate that the *KEX2* enzyme does not cleave at the carboxyl side of Lys-Lys doublets in vivo, in agreement with the substrate specificity of the enzyme both in vitro (*11–13, 21*) and in yeast (*10, 11, 22*).

Clearly, however, one or more other factors influence the preferential cleavage of one potential processing site versus another in different cell types and even within the same cell type. For example, in BSC-40 cells, there was efficient cleavage at both Lys-Arg sites in the carboxyl-terminal region of mPOMC, resulting in apparently complete conversion of the β-LPH segment into γ-LPH and β-END. In contrast, in GH_4C_1 cells, a significant amount of uncleaved β-LPH was observed (Fig. 2). Thus, despite the presence of a similar level of *KEX2* activity in both cell types (Table 1), the cleavage pattern found in BSC-40 cells mimics that observed in the neurointermediate lobe of the pituitary, whereas the pattern seen in GH_4C_1 cells is more reminiscent of the products found in the anterior lobe (*2*).

In BSC-40 cells, the majority of the cell-associated γ-LPH retained the carboxyl-terminal Lys-Arg residues exposed by *KEX2* enzyme cleavage. In GH_4C_1 cells, this species was present, but was a minor fraction of the γ-LPH produced. This intermediate is not normally observed in AtT-20 cells (Fig. 2). Furthermore, only mature γ-LPH is produced in "maturation proficient" Rin m5F cells (derived from a rat insulinoma) upon infection with VV:mPOMC (*14, 15a*). Hence, accumulation of the form of γ-LPH that retains the carboxyl-terminal Lys-Arg residues is not due to perturbation of normal processing events by vaccinia virus infection. Inefficient removal of the carboxyl-terminal basic residues in the maturation deficient cell lines could be due to an insufficiency of the appropriate carboxypeptidase activity.

In yeast, prepro-α-factor is synthesized, processed, and secreted via a constitutive secretory pathway (*23*); however, the intracellular compartment in yeast in which the *KEX2* enzyme resides has not been definitively established. The successful processing of mPOMC by the *KEX2* enzyme in BSC-40 cells demonstrates that a mammalian constitutive secretory pathway can accommodate the proteolytic processing machinery required for prohormone maturation.

Endocrine cells typically utilize a regulated secretory pathway in which processed peptides are stored in specialized secretory granules, which can be released in response to certain secretagogues (*24*). Immunochemistry and electron microscopy indicate that in endocrine cells the trans-Golgi network and associated nascent secretory vesicles are the compartments in which prohormone processing begins (*25*). To determine whether the peptides generated from

Table 1. Expression of *KEX2* enzyme activity in mammalian cells. Cultures (*28*) of the indicated mammalian cell lines (about 1×10^7 cells total) were either mock-infected or infected with wild-type or recombinant vaccinia viruses (5 PFU/cell). After 24 hours, the medium was removed from each culture and stored at −70°C after addition of glycerol to a final concentration of 10%. The enzymic activity in the culture fluid represents the "secreted" activity. The cells were washed once in phosphate-buffered saline containing 10 m*M* EDTA and harvested in 1 m*M* EDTA, 10 m*M* Hepes (*p*H 7.5). Cells were broken by multiple passages in a Dounce homogenizer, and this crude extract was clarified by centrifugation at 10,000*g* for 20 min. Greater than 80% of the total activity of the crude extract was recovered in the clarified supernatant solution, and this fraction was designated the "cell-associated activity." The clarified lysate was then subjected to centrifugation at 100,000*g* for 60 min; the enzymic activity in the resulting pellet (particulate fraction) represents the portion of the cell-associated activity that was "membrane-bound." The pellet fraction was resuspended in 50 m*M* Hepes (*p*H 7.5), 1 m*M* EDTA, and stored at −70°C prior to assay. Recovery of activity in the combined pellet and supernatant fractions after high-speed centrifugation was at least 95%. *KEX2* protease activity was measured spectrofluorimetrically, as described elsewhere (*11, 12*), and 0.5 m*M* phenylmethylsulfonyl fluoride and 0.1 m*M* tosyl-phenylalanine-chloromethyl ketone [which do not inhibit the *KEX2* enzyme (*11, 12*)] were included to prevent proteolytic degradation of the *KEX2* enzyme in the extracts and to reduce the nonspecific hydrolysis of the fluorogenic peptide substrate. One unit of activity is defined as the hydrolysis of 1 pmol of substrate per minute. Protein concentration was determined by the method of Markwell *et al.* (*29*). Numbers in parentheses represent the ratio of activity measured in the presence of 1 m*M* $CaCl_2$ to that measured in the presence of 25 m*M* EDTA (*p*H 7). ND, not determined. Total activity is secreted activity plus cell-associated activity. The results of two independent experiments are given (values presented are single determinations).

Condition	Experiment	Cell type infected			
		BSC-40	GH_4C_1	NG108-15	Ltk^-
		Cell-associated activity (units per milligram of protein)			
Mock-infected	1	29 (0.4)	26 (0.7)	ND	34 (0.5)
Infected with:					
VV:WT	1	19 (0.4)	30 (0.6)	46 (0.8)	7 (0.2)
	2	29 (0.7)	4 (0.7)	ND	1 (0.4)
VV:POMC	1	23 (0.5)	50 (1.0)	ND	6 (0.2)
VV:*KEX2*	1	587 (8.8)	864 (15.0)	741 (8.9)	380 (7.5)
	2	540 (7.6)	208 (3.1)	ND	488 (9.0)
		Percentage membrane-bound (of cell-associated activity)			
		86	ND	85	88
		Percentage secreted (of total activity)			
		54	33	36	58

mPOMC by *KEX2* enzyme cleavage were sorted into the regulated pathway present in GH_4C_1 cells, we used the secretagogues, forskolin (an adenylate cyclase activator) and phorbol 12-myristate 13-acetate (a protein kinase C activator), to stimulate release of secretory granules (*26*). Increase in the amount of prolactin released by such treatment provided an internal control for the function of the regulated secretory pathway in GH_4C_1 cells. When produced in GH_4C_1 cells, mPOMC was targeted to the regulated pathway, and prolactin delivery to the regulated pathway was not affected (Fig. 4). Similarly, when active *KEX2* enzyme was also present, both prolactin and at least one mPOMC-derived peptide (β-END) were still sorted into the regulated pathway (Fig. 4). This result suggests that *KEX2* protease can act at the correct stage in a regulated secretory pathway to permit sorting of the mature product peptides into storage granules.

Introduction of a single heterologous gene for a known prohormone-cleaving enzyme (the yeast *KEX2* gene) into mammalian cell lines otherwise incapable of causing maturation of prohormones restored their ability to process a polyprotein precursor (mPOMC) to a proper set of product peptides. This reconstitution of prohormone maturation indicates: (i) that yeast *KEX2* protease was produced and was active inside the mammalian cells, as well as in vitro (Table 1); (ii) that the *KEX2* enzyme was translocated into mammalian secretory systems and was able to recognize and cleave at correct processing sites in a native mammalian precursor protein in its natural environment, despite reported differences between the yeast and mammalian secretory pathways (*27*); and, (iii) that precursor cleavage by the *KEX2* enzyme apparently occurred at a stage in the secretory pathway that did not interfere with the delivery of mature bioactive peptides into regulated secretory granules. These results all support the view that the *KEX2* processing endopeptidase is a genuine prototype of its mammalian counterpart. In contrast, the cDNA for a mammalian kallikrein expressed in the same cell lines produced a functional enzyme, but led to no detectable cleavage of mPOMC (*15a*).

Our results also suggest that the primary defect that makes these cell lines incompetent to process prohormones is the absence of the endogenous prohormone-cleaving endoprotease. Therefore, this system might be used to screen cDNA libraries from appropriate tissues in similar complementation assays as a means to identify the gene or genes encoding the true prohormone endopeptidase of animal cells.

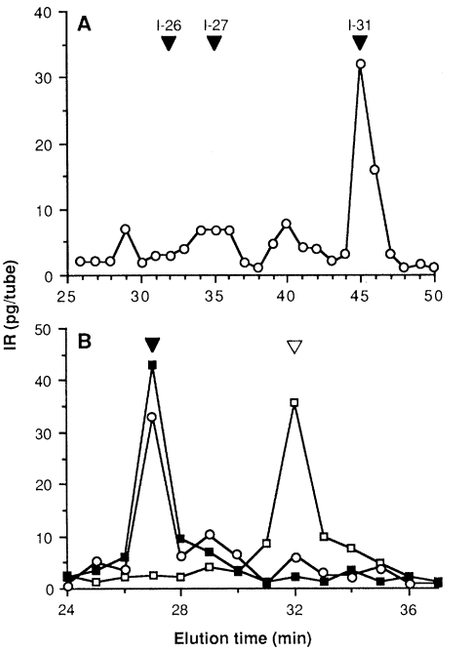

Fig. 3. (**A**) Identification of β-END_{1-31} by cation-exchange HPLC. A sample (100 pg) of the peptide from BSC-40 cells coinfected with VV:mPOMC and VV:*KEX2* that eluted at 48 min from the reversed-phase column (Fig. 2) and cross-reacted with the β-END–specific antiserum (205–235) was applied to a cation-exchange column (NEST WCX 1850-02) in 25% acetonitrile containing 10 m*M* ammonium acetate (*p*H 4.5) and eluted with a linear gradient from 10 to 300 m*M* ammonium acetate (*p*H 4.5) in 25% acetonitrile over 50 min at a flow rate of 0.5 ml/min. After drying, the content of cross-reacting material in each fraction (1 min) was measured by radioimmunoassay with antiserum 205–235. Arrows mark the elution positions (in minutes) of synthetic β-END peptides run as standards: β-END_{1-26}, 32; β-END_{1-27}, 35; and β-END_{1-31}, 45. Identical results were obtained for the peptide from GH_4C_1 cells coinfected with VV:mPOMC and VV:*KEX2* that eluted at 48 min on the reversed-phase column (Fig. 2). (**B**) Identification of γ-LPH–related peptides by cation-exchange HPLC. Samples (100 pg) of the peptides from BSC-40 cells coinfected with VV:mPOMC and VV:*KEX2* that eluted at 39 and 41 min from the reversed-phase column (Fig. 2) and cross-reacted with the γ-LPH–specific antiserum (185–202) were analyzed by cation-exchange chromatography and radioimmunoassay, as described above, except that the gradient was from 10 m*M* to 500 m*M* ammonium acetate (*p*H 4.5). Material from 41-min peak (filled squares); the filled arrow marks the elution position of mature γ-LPH from AtT20 cells. Material from 39-min peak (open squares). Material from 39-min peak treated by digestion with carboxypeptidase B (Sigma, 2 μg/ml) in 50 m*M* tris-HCl (*p*H 8), 2 m*M* $CaCl_2$, bovine albumin (8 mg/ml) for 30 min at 37°C (open circles); the open arrow marks the elution position of an identical sample in which the carboxypeptidase B was heat-inactivated immediately after addition. Results were the same for the peptides in the 39- and 41-min peaks resolved by reversed-phase HPLC from the GH_4C_1 cells (Fig. 2).

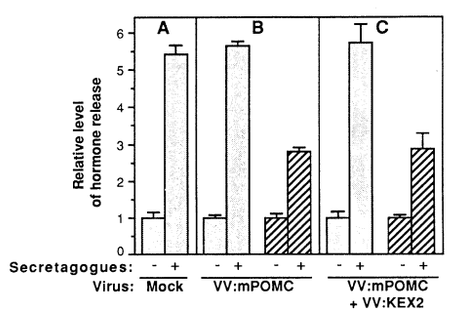

Fig. 4. Stimulated secretion of prolactin and mPOMC-related peptides from GH_4C_1 cells infected by recombinant vaccinia viruses. Separate cultures of GH_4C_1 cells were either mock-infected (**A**), infected with VV:mPOMC alone (**B**), or coinfected with both VV:mPOMC and VV:*KEX2* (**C**) at the multiplicities of infection described (Fig. 2). After 16 hours, the culture fluid was removed from each culture and replaced with fresh medium containing bovine serum albumin (0.07%) in either the absence (−) or the presence (+) of a mixture of forskolin (Calbiochem, 50 μ*M* final concentration) and phorbol 12-myristate 13-acetate (Sigma, 100 n*M* final concentration) to stimulate the release of secretory granules. After 1 hour, the medium was withdrawn from each culture, and the amount in the medium of both prolactin (stippled bars) and mPOMC-related material [either intact mPOMC or mature β-END (*32*)] (hatched bars) was measured by radioimmunoassay (*33*). All infections were performed in triplicate, and samples of the medium from each of the three replicate cultures were assayed for cross-reacting material in duplicate. Hence, the value given for each condition is the average of six independent determinations and was normalized, in each case, to the amount of cross-reacting material released by the unstimulated cells during the 1-hour incubation (which was given an arbitrary value of 1). Error bars represent standard deviation of the mean.

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15. Plasmid pMSKU16 (*30*) containing the cDNA for mPOMC was digested partially with Stu I and completely with Pst I to produce a fragment containing the entire mPOMC coding sequence preceded by 15 bp of its 5′ untranslated region. After incubation with T4 DNA polymerase to generate flush ends, the fragment was inserted by blunt-end ligation with T4 DNA ligase into the polylinker region (multiple cloning site) of the vector, pVV3, that was digested with Bam HI and Bgl II and converted to flush ends. This vector, pVV3, contains the vaccinia virus thymidine kinase (*tk*) gene interrupted by the constitutive vaccinia virus promoter, VV 7.5K, placed immediately proximal to the multiple cloning site (*14*). The resulting construct, pVV:mPOMC, was introduced by DNA-mediated transformation into mouse *tk*-deficient L cells that were infected with wild-type vaccinia virus (VV:WT), strain WR [0.05 plaque-forming units (PFU)/cell]. The mPOMC sequences were transferred into the vaccinia virus genome by homologous recombination. The recombinant virus, VV:mPOMC, was identified by plaque-filter hybridization techniques, and a stock of this recombinant was propagated as before (*14*).
15a. B. A. Thorne and G. Thomas, in preparation.
16. NG108-15 cells are a hybrid line (derived from a fusion of rat C6 glioma and mouse N18 neuroblastoma cells) and produce a low level of endogenous enkephalins [K. M. Brass and S. R. Childers, *J. Neurosci.* **3**, 1713 (1983)]. GH_4C_1 cells are a line of rat pituitary somato- or lactotrophs and secrete the large protein hormones, prolactin and growth hormone, via a well-characterized, regulated secretory pathway [A. H. Tashjian, F. C. Bancroft, L. Levire, *J. Cell Biol.* **47**, 61 (1970); J. G. Scammell, T. G. Burrage, P. S. Dannies, *Endocrinology* **119**, 1543 (1986)]. BSC-40 cells are a line of kidney epithelial cells from African green monkey that do not produce any endogenous prohormones and secrete other proteins via a constitutive secretory pathway (*15a*).
17. A 3.3-kb DNA fragment containing the entire yeast *KEX2* gene (*12*) was inserted into the Bam HI site of pVV3 and used to generate a recombinant vaccinia virus, VV:*KEX2*, as described above (*15*).
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28. Cell lines were grown at 37°C in an atmosphere of 5% CO_2 in the following media: AtT-20 cells, Dulbecco's modified Eagle's medium (DMEM) with 10% fetal calf serum (HyClone); NG108-15 cells, DMEM with 10% serum and 100 μ*M* hypoxanthine, 0.4 μ*M* aminopterin, and 16 μ*M* thymidine; GH_4C_1 cells, Ham's F-10 (Gibco) with 10% serum; and BSC-40 cells and L*tk*$^-$ cells (*14*).
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31. In all the cell lines examined here, the only form of unmatured mPOMC found intracellularly was proopiomelanocortin, indicating that the NH_2-terminal hydrophobic leader (pre-sequence) has been removed from the primary translation product presumably by the action of signal peptidase during translocation of mPOMC into the lumen of the endoplasmic reticulum.
32. Analysis (by HPLC and radioimmunoassay) revealed that the cross-reacting material secreted into the medium (either with or without secretagogue stimulation) by cells infected with VV:mPOMC was almost exclusively uncleaved mPOMC, whereas the majority of the cross-reacting material released by cells coinfected with VV:mPOMC and VV:*KEX2* was processed peptides.
33. Antiserum 205–235 (Fig. 1) was used to measure mPOMC-related material. The prolactin radioimmunoassay utilized ^{125}I-labeled rat prolactin (New England Nuclear), synthetic rat prolactin (Peninsula Labs), and specific antibodies against prolactin (gift of P. Albert).
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(Perspective, continued from page 145)

structures like Fig. 1C, which are at present only conjectural.

In summary, gramicidin A appears to be a structure whose polymorphism may underlie a dynamic mechanism of ion transfer. Although much more remains to be learned about gramicidin function, particularly regarding details of mechanism and the origins of ion transport specificity, the crystallographic studies finally provide a structural basis for definitive spectroscopic studies and computational simulations.

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