Identification of kex2-related Proteases in Chromaffin Granules by Partial Amino Acid Sequence Analysis*

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We have characterized glycoprotein H (GpH) from bovine adrenal medullary chromaffin granules. Two-dimensional gel electrophoresis was used to purify GpH from an insoluble fraction obtained following extraction of chromaffin granule membranes with lithium diiodosalicylate. The GpH material was recovered from two-dimensional gel spots by concentration and recovery on a one-dimensional gel followed by electroblotting to a poly(vinylidene difluoride) membrane. This material was subjected to in situ tryptic digestion. The released peptides were purified by microbore high performance liquid chromatography and sequenced. The peptide sequences revealed extensive similarity to the mammalian kex2/subtilisin-related proteases (PC2 and PC3) which have been characterized recently by molecular cloning and sequence analysis (Smeekens, S. P., and Steiner, D. F. (1990) J. Biol. Chem. 265, 2997-3000; Smeekens, S. P., Avruch, A. S., Lamendola, J., Chan, S. J., and Steiner, D. F. (1991) Proc. Natl. Acad. Sci. U. S. A. 88, 340-344). The sequence similarity involved in regions that contain residues equivalent to the aspartic acid and histidine residues which are involved in the active site of the subtilisin family of serine proteases. The sequence data revealed the presence of tryptic peptides derived from both PC2 and PC3. NH2-terminal sequence analysis of GpH gave two sequences which were aligned with residues 110-121 of PC2 and PC3. It is likely that these sequences represent the mature form of PC2 and PC3 in chromaffin granules. These forms would be generated by cleavage at a site which is conserved in mammalian kex2-related enzymes and which would result in the release of approximately 80-residue pro-peptides. It was concluded that the spot identified as GpH by two-dimensional gel electrophoresis contains the bovine counterparts of both PC2 and PC3. The direct identification of these components in chromaffin granules supports their role in the processing of protein precursors.

Most peptide hormones, neuropeptides, and other biologically active peptides are synthesized as part of larger precursor molecules. Conversion to active peptides requires packaging into secretory granules and proteolytic processing (1, 2). Cleavage at paired basic amino acids has been shown to be important to the processing of a number of prohormones, including proopiomelanocortin and proopressin (3, 4).

In the yeast Saccharomyces cerevisiae, the maturation of two proproteins, pro-a-mating factor and pro-killer toxin, requires processing at dibasic amino acid residues. The endoprotease involved has been mapped to the KEX2 locus (5). Subsequent characterization indicated that the enzyme is a Ca2+-dependent serine protease which is homologous to bacterial subtilisins (6, 7). This enzyme also converted proinsulin to insulin when this prohormone was expressed in yeast (8). Furthermore, co-expression of the kex2 gene product and proopiomelanocortin in various eukaryotic cell lines using the vaccinia expression system resulted in correct processing (9). Two Ca2+-dependent proteolytic activities have been partially purified from secretory granules from a rat-transplantable insulinoma and shown to process proinsulin (10) and to convert proalbumin to albumin (11). Recently, Smeekens and Steiner (12) identified a human insulinoma cDNA (PC2) that encodes a protein which is homologous to the yeast kex2 protease and the related bacterial subtilisin enzymes. A second related and putative prohormone convertase (PC3) has been identified and cloned from the mouse AtT20 anterior pituitary cell line (13).

Bovine proenkephalin contains seven copies of enkephalin peptides which are bordered by dibasic residues (Lys-Arg, Lys-Lys, and Arg-Arg). Relatively large amounts of enkephalin-containing peptides which are intermediates in the processing of this precursor are present in chromaffin granules, the secretory vesicles of the adrenal medulla (14). Endoproteolytic activities which can cleave proenkephalin peptides at basic residues have been identified in chromaffin granules (15-17). Carboxypeptidase H, which cleaves COOH-terminal basic amino acids from enkephalin-containing peptides is known to be present in the membrane and soluble fractions of chromaffin granules (18).

Chromaffin granules are relatively easy to purify in good yields and share a number of functions in common with both endocrine secretory granules and neurotransmitter-storing vesicles (for a recent review, please see Refs. 19 and 20). The chromaffin granule membrane contains a number of glycoproteins which are located on the matrix side of the membrane. The major ones, dopamine β-hydroxylase and glycoprotein components II-V and H, J, and K are readily identified by two-dimensional gel electrophoresis in combination with lectin blotting (19, 21). Glycoproteins J and K have been shown to react with antisera which recognize carboxypeptidase H (22).

We have separated chromaffin granule membranes into insoluble and soluble fractions by extraction with lithium diiodosalicylate (LDIS). This removes ~75% of the membrane proteins. The remaining fraction (LDIS-insoluble) is considerably enriched with respect to glycoproteins III, H, J, and...
and K (23). Glycoprotein III has been characterized by molecular cloning and is the bovine counterpart of rat Sertoli cell-sulfated glycoprotein 2, ram clusterin, and human serum protein-40,40 (24). Glycoproteins J and K were isolated from the LDL-insoluble fraction by electroblotting from two-dimensional gels and characterized by protein sequence analysis. This revealed that GpJ is an NH₂-terminally extended form of GpK and that both are molecular forms of carboxypeptidase H (23). We were interested in the possibility that GpH may also have a role in prohormone processing. In the present study, glycoprotein H has been characterized using similar methodology and the sequencing of peptides generated by in situ digestion of glycoprotein H with trypsin. The sequence data resulted in the identification of bovine homologues of the kex2-related proteases PC2 and PC3 (12, 13). These products may have a role in the processing of proenkephalin.

EXPERIMENTAL PROCEDURES

Preparation of Chromaffin Granules and Extraction of Chromaffin Granule Membranes with LDLIS—Procedures for the preparation of chromaffin granules, membranes, and extraction with LDLIS were as described in Ref. 23. The LDL-insoluble fraction obtained from 10 mg of membrane protein was redisolved in 1 ml of 1% SDS and stored (−20 °C) prior to use. In some experiments, chromaffin granules were lysed and the membranes were washed in 5 mM Tris sucrose containing 0.5 mM phenylmethylsulfonyl fluoride, 100 mM dithiodipropionate, 1 mg/ml pepstatin A, 1 mg/ml leupeptin, and 5 mM EDTA.

Two-dimensional Gel Electrophoresis and Electroblotting to PVDF Membranes—Prior to two-dimensional gel electrophoresis, aliquots (150 μl) of the LDL-insoluble fraction (solubilized in 1% SDS) were precipitated by ethanol at −20 °C overnight. Samples were run on two-dimensional mini-gels as described in Ref. 23. For NH₂-terminal sequence analysis, proteins separated by twodimensional gel electrophoresis were electroblotted to PVDF membranes (Immoblizer Transfer, 0.45 μm, Millipore) as in Ref. 25. Protein bands were detected by staining with 0.1% Ponceau S in 1% acetic acid. Following destaining with water, the membrane was dried and stored (−20 °C) until sequence analysis.

To prepare GpH for in situ proteolytic digestion experiments, two-dimensional gels were run and stained with Comassie Blue. The GpH bands were cut out, washed with several changes of water, and stored overnight at 4 °C. Gel pieces from 18 two-dimensional gels were cut into 1-mm pieces and distributed evenly into two sample wells of a new mini-gel (8 × 10 cm) which was cast using 1.5-mm spacers and a 5-well comb ( Hoeffer). A longer stacking gel (2 cm) which was cast using 1.5-mm acrylamide gel; the gel pieces were overlaid with 0.125 M Tris-HCl, pH 6.8, 0.1% SDS, 20% glycerol, 0.3% 2-mercaptoethanol, 0.06% bromphenol blue. After 20 min, the gels were run at 50 V until the dye reached the stacking gel when the voltage was increased to 150 V until the dye reached the bottom of the gel. The gel was then soaked in 10 mM CAPS, 10% methanol, pH 11, buffer for 5 min and electroblotted (90 V for 35 min) to a PVDF membrane (25). The membrane was rinsed several times with water and stained for 1 min with 0.1% Ponceau S in 1% acetic acid. The membrane was then destained with water, and the GpH bands were cut out and processed for in situ digestion.

In Situ Digestion of Glycoprotein H with Trypsin and Separation of Peptides by Microbore HPLC—The two electroblotted bands of GpH (estimated to be 5–10 μg of protein) were combined and treated with polyvinylpyrrolidone, average Mr = 40,000 to prevent adsorption of trypsin (26). Excess Mr = 40,000 polyvinylpyrrolidone was removed by extensive washing with water (6 changes). The PVDF membrane was then cut into small pieces (1 × 1 mm) and placed in a 1.5-ml polypropylene tube. The pieces were covered with 50 μl of 500 mM Tris-HCl, pH 8.5, acetonitrile, 95:5, v/v, and the protein was digested by the addition of trypsin (Boehringer Mannheim, sequencing grade, 0.5 μg in 5 μl of 0.01% trifluoroacetic acid) and incubation at 37 °C overnight. After digestion, the supernatant was transferred into a second tube, and the membrane pieces were washed once with 50 μl of distilled water. The wash was combined with the digestion mixture in the second tube and acidified by the addition of 10 μl of 10% trifluoroacetic acid. Trypsin cleavage peptides were separated by microbore HPLC using a 140A solvent delivery system and a 1000 s diode array detector (Applied Biosystems). The solvent system consisted of solvent A, 0.1% trifluoroacetic acid in water (purified using a Milli Q system, Millipore), and solvent B, 0.085% trifluoroacetic acid in acetonitrile/water, 80:20 (v/v). An aliquot (50 μl) of the digest was injected onto the column (Aquanat RP300, 0.2 × 22 cm) equilibrated with 90% solvent A, 10% solvent B at a flow rate of 200 μl/min. After 30 s, an additional 50 μl was injected, and, 30 s later, a 45-min gradient from 10–65% solvent B at 200 μl/min was started. Peptides were detected by absorbance at 214 nm and collected manually into 1.5-ml polypropylene tubes. Samples were stored at −20 °C until sequence analysis.

Protein Sequence Analysis—Sequence analysis was performed using a gas-phase Sequencer (model 470A) equipped with an on-line phenylthiohydantoin amino acid analyzer (120A) with chemicals and the program (03RPTF) supplied by the manufacturer (Applied Biosystems). Peptides were applied to glass fiber discs which had been coated with Polybrene and pretreated by two cycles of Edman degradation as recommended by the manufacturer. GpH samples electroblotted to PVDF membranes were sequenced by positioning membrane pieces in the upper cartridge block of the Sequencer above a Polybrene-treated glass fiber disc.

Preparation of Antisera against Glycoprotein H and Immunoblotting—Glycoprotein H was purified from the LDL-insoluble fraction by two-dimensional gel electrophoresis and electroelution (27). The recovered material (10–15 μg of protein) was emulsified with Freund’s complete adjuvant and used to immunize a rabbit by multiple subcutaneous injections. After 5–6 weeks, the rabbit was boosted with approximately 10 μg of electroeluted GpH emulsified in incomplete Freund’s adjuvant. After 7 days, the rabbit was bleed from the marginal ear vein, and the resulting serum was used for Western blotting at a dilution of 1:200. Procedures for electroblotting to nitrocellulose membranes and immunochemical detection have been described previously (28).

RESULTS AND DISCUSSION

Protein sequence analysis is an important technique in current biological research. In combination with high resolving one- and two-dimensional gel electrophoresis techniques, it is often possible to obtain 10–20 residues of sequence data with 10–20 pmol of protein (25, 29). Further developments have involved the digestion of electroblotted protein bands with proteolytic enzymes in situ and subsequent sequence analysis of the resulting peptides following purification by microbore HPLC (26). In this way, proteins obtained from two-dimensional gels can be identified directly by partial sequence analysis (30). Isolation of organelles such as secretory granules enables this methodology to be applied directly to identify components present at only relatively low levels in cell extracts. In the present work, we have used this approach to characterize glycoprotein H from the LDL-insoluble fraction of chromaffin granule membranes.

Chromaffin granule membrane components known as glycoproteins III, H, J, and K (19, 21, 23) are easily visualized by Comassie Blue staining following analysis of the LDL-insoluble fraction by two-dimensional gel electrophoresis (Fig. 1). Glycoprotein H runs as a discrete spot (Mr = 66,000). Its position was identified with reference to lectin binding experiments (data not shown). GpH was purified by two-dimensional gel electrophoresis from the LDL-insoluble fraction. The GpH material (purified from 18 gels, equivalent to the LDL-insoluble fraction obtained from 27 mg of membrane protein) was concentrated and recovered by one-dimensional gel electrophoresis, electroblotted to a PVDF membrane, and stained with Ponceau S. The amount of protein recovered was estimated to be 5–10 μg. This material was digested with trypsin, and the soluble peptides were separated by microbore HPLC (Fig. 2).

Seven peptides were sequenced giving a total of 67 residues (Fig. 3). The recovery of phenylthiohydantoin amino acid derivatives corresponded to 10–30 pmol of peptide material.
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Proteins H, J, K, and III have been labeled.

Extensive sequence similarity was observed between these sequences and regions of the mammalian kex2-related proteases PC2 and PC3 (Fig. 3). In PC3, all of the regions corresponding to the GPH tryptic peptide sequences are preceded by basic amino acids further supporting the similarity between GPH peptides and the kex2-related proteases. Importantly, two of the peptides, T26 and T12 corresponded to the GPH spot (designated GPH-a and GPH-b) are equivalent to sequences found in both PC2 and PC3 commencing at residue 110 (Fig. 4). It is likely that the unidentified residues at positions 3 and 4 in the GPH-a and -b sequences represent Arg residues. Arginine derivatives, unlike those of lysine, are difficult to detect at this level of sequence analysis. Both of these sites are occupied by basic residues in PC2 and PC3.

The low yield of aspartic acid at positions 3 and 4 in the GPH-a and -b sequences represent Arg residues. Arginine derivatives, unlike those of lysine, are difficult to detect at this level of sequence analysis. Both of these sites are occupied by basic residues in PC2 and PC3.

To examine this possibility further, the NH2-terminal se- quence of GPH was determined following two-dimensional gel electrophoresis and blotting to a PVDF membrane. The results obtained from 12 cycles of automated Edman degradation are shown in Table I. The results are consistent with the presence of two sequences in approximately equal amounts. The low yield of aspartic acid at positions 4, 5, and 9 is due to the poor extraction of the anilinothiazolinone deriviative of this amino acid from PVDF membranes. Careful inspection of the sequence data obtained from cycles 5 to 12 indicated the presence of a sequence Asp-Ser-Ala-Leu-Asp-Leu-Phe-Asn which comprises the NH2-terminal 8 residues of peptide T30 (Fig. 3). Subtraction of this sequence from residues identified in cycles 5 to 12 gave the sequence Ile-Asn-Glu-Ile-Asp-Ile-Asn-Val, which is similar to residues 114-121 of PC2. This enabled the NH2-terminal sequence data to be interpreted. It was apparent that the two sequences obtained from the GPH spot (designated GPH-a and GPH-b) are equivalent to sequences found in both PC2 and PC3 commencing at residue 110 (Fig. 4). It is likely that the unidentified residues at positions 3 and 4 in the GPH-a and -b sequences represent Arg residues. Arginine derivatives, unlike those of lysine, are difficult to detect at this level of sequence analysis. Both of these sites are occupied by basic residues in PC2 and PC3.

2 D. J. Hakes, N. P. Birch, E. Mezey, and J. E. Dixon, manuscript submitted for publication.
kex2-related Proteases in Chromaffin Granules

Samples of the LDIS-insoluble fraction were subjected to two-dimensional gel electrophoresis followed by electroblotting to PVDF membranes. A total of 6 GpH spots, detected by Pronéou S staining, were combined for automated sequence analysis. The discovery of phenylthiohydantoins in the sequence runs were calculated relative to standards.

Table I

<table>
<thead>
<tr>
<th>Cycle</th>
<th>Residue identified (pmol)</th>
</tr>
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<tr>
<td>1</td>
<td>Gly (10), Ser (5)</td>
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<tr>
<td>2</td>
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<tr>
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<tr>
<td>10</td>
<td>Ile (4), Leu (5)</td>
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<tr>
<td>11</td>
<td>Asn (5), Phe (7)</td>
</tr>
<tr>
<td>12</td>
<td>Val (4), Asn (4)</td>
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GpH-a
PC2 (110-121) G Y L D I N E I D I N V
PC2 (110-121) M Q K
GpH-b
PC3 (110-121) S V L D S A L D L F N

which is consistent with the recovery of peptide T30 from tryptic digests.

It is clear from the sequence data of tryptic peptide and the NH2-terminal sequence of electroblotted GpH that this spot in two-dimensional gels contains bovine homologues of both PC2 and PC3. The direct identification of kex2-related enzymes in chromaffin granules is important in a number of respects. Mammalian kex2-related enzymes have been identified and characterized by molecular cloning techniques, but the protein products have not been previously isolated. It has also not been established whether mammalian kex2 proteases undergo proteolytic processing. Our NH2-terminal sequence data indicate that the bovine counterparts of PC2 and PC3 found in adrenal medullary secretory granules commence at a position equivalent to residue 110. This requires cleavage at a site preceded by the sequence Arg-Ser-Lys-Arg- and Arg-Lys-Arg-Lys- in PC3 and PC2, respectively. This would result in the loss of an approximately 80-residue propeptide. It is of interest that other workers have predicted this site to be involved in the proteolytic maturation of kex2 enzymes based on a sequence comparison of recently cloned rat pancreatic enzymes and prosubtilisin. Assuming that the bovine proteins are the same length as the mouse, human, and rat products, processing at this site would be expected to generate 644 (PC3)- and 629 (PC2)-residue proteins. In this respect, it is difficult to reconcile both proteins having the same mobility on two-dimensional gel electrophoresis. A possible explanation is differences in glycosylation. PC2 contains three consensus sequences for N-linked carbohydrate attachment sites, while only two are present in PC3 (12, 13).

It appears that the NH2-terminal sequences we have determined represent the major forms of kex2 enzymes present in chromaffin granules. A characteristic and reproducible pattern of glycoproteins is obtained following analysis of chromaffin granule membranes by two-dimensional gel electrophoresis in combination with lectin binding (19, 23). Glycoprotein H is a major lectin binding component, and no other major spots were obvious candidates for precursor or further processed forms of the kex2 enzymes. To investigate this further, antisera were prepared against the glycoprotein H spot isolated from two-dimensional gels and used to characterize GpH-related material in chromaffin granule membranes by Western blotting. The major species detected in chromaffin granule membranes following one- or two-dimensional gel electrophoresis corresponds to a 66-kDa protein (Fig. 5). A minor 74-kDa component is also detected although the relative amounts of these products are not dependent on whether or not chromaffin granules are lysed in the presence of protease inhibitors. The mobility of the major immunoreactive spot on two-dimensional gels corresponds exactly to the GpH material characterized from the LDIS-insoluble fraction. It is very likely that the minor 74-kDa species corresponds to the precursor form of one or both of the kex2 enzymes. While this component can also be visualized on Coomassie Blue-stained two-dimensional gels of the LDIS-insoluble fraction (Fig. 1), the levels are too low to permit direct sequence analysis. Biosynthetic experiments using isolated cell systems will be required to determine whether the kex2 enzymes are processed prior to, or after, packaging into secretory granules as the half-lives of chromaffin granules have been estimated to be several days in vivo (31). In preliminary experiments, it appears that most of the GpH material is associated with membranes rather than the soluble contents of chromaffin granules. Neither PC2 nor PC3 contains obvious membrane-spanning sequences (12, 13). Thus, it appears that bovine kex2 enzymes can be added to the growing list of chromaffin granule components for which the basis of their membrane association is unknown.

The expression of PC2 and PC3 appears to be restricted to neural and endocrine cell types (13) which is consistent with the presence of bovine counterparts in adrenal medullary

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**Table 1**

**NH2 terminal sequence analysis of glycoprotein H**

Samples of the LDIS-insoluble fraction were subjected to two-dimensional gel electrophoresis followed by electroblotting to PVDF membranes. A total of 6 GpH spots, detected by Pronéou S staining, were combined for automated sequence analysis. The discovery of phenylthiohydantoins in the sequence runs were calculated relative to standards.

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**Fig. 4.** Alignment of the NH2-terminal sequence data obtained for GpH with regions of PC2 and PC3. NH2-terminal sequence analysis of GpH revealed two sequences in approximately equal amounts (Table I). The two sequences have been denoted GpH-a and GpH-b. The portion underlined in the GpH-b sequence is identical with NH2-terminal 8 residues of the tryptic peptide GpH T30 (Fig. 3), (unidentified residue). The GpH-a and -b sequences have been aligned with residues 110-121 of the kex2 proteases PC2 and PC3 (12, 13).

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**Fig. 5.** Analysis of GpH-related material in chromaffin granule membranes by one- and two-dimensional gel electrophoresis and immunoblotting. Samples (10-15 µg of protein) of chromaffin granule membranes prepared in the usual manner (a) or by lysing chromaffin granules and washing the membranes in the presence of protease inhibitors, 0.5 mM phenylmethylsulfonyl fluoride, 100 mM dithiodipyridine, 1 mg/ml pepstatin A, 1 mg/ml leupeptin, and 5 mM EDTA (b) were run on a 10% SDS-polyacrylamide gel. c, a sample of chromaffin granule membranes (10-15 µg of protein) run on a two-dimensional gel as described for Fig. 1. Following electrophoresis, proteins were electroblotted to nitrocellulose membranes and reacted with an anti-rabbit GpH serum and peroxidase-conjugated anti-rabbit IgG. Diaminobenzidine was used as the substrate for color development.
chromaffin granules. It is apparent from sequence analysis that the LDIS-insoluble fraction contains approximately equal amounts of PC2 and PC3, and it will be interesting to see if this is reflected by mRNA levels in the adrenal medulla. The reason that the majority of tryptic peptides for which sequence data were obtained were derived from PC3 probably reflects the fact that recovery of peptides following in situ digestion is a selective process greatly affected by the properties of individual peptides.

Mammalian kex2/subtilisin-like proteases appear to represent cellular precursor processing enzymes. Two Ca\(^{2+}\)-dependent endoproteolytic activities (designated types I and II) from transplacental rat insulinoma secretory granules with specificity for Arg-Arg and Lys-Arg cleavage sites are required for the processing of proinsulin (10). It is tempting to speculate that the kex2-related enzymes in chromaffin granules may be involved in the processing of proenkephalin. Chromaffin granules contain a number of enkephalin-containing peptides which are intermediates in the processing of proenkephalin (14). Major intermediates correspond to 27-, 20.5-, and 16.5-kDa enkephalin-containing peptides (28). The processing in chromaffin granules is slow, although it is possible to observe the cleavage of a 27-kDa intermediate using Western blotting and an antiserum which recognizes all the major intermediates of proenkephalin (32). It will be necessary to demonstrate that the kex2 enzymes in chromaffin granules are enzymatically active and to assess their roles in the processing of proenkephalin.

Our characterization of the chromaffin granule membrane component known as Gph has led to the identification of bovine counterparts of two kex2-related proteases, PC2 and PC3. This study provides an important link with the sequencing of proenkephalin. Intermediates of proenkephalin (32). It will be necessary to observe the cleavage of a 27-kDa intermediate using Western blotting and an antiserum that recognizes all the major intermediates of proenkephalin (32). It will be necessary to demonstrate that the kex2 enzymes in chromaffin granules are enzymatically active and to assess their roles in the processing of proenkephalin.

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