A Protease Processing Site Is Essential for Prorenin Sorting to the Regulated Secretory Pathway*

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Transfected mouse pituitary AtT-20 cells were used to examine the sorting of human prorenin to dense core secretory granules and the regulated secretory pathway. These cells secrete prorenin constitutively and sort a portion of the prorenin to secretory granules, where it is converted to active renin by proteolytic processing. Pulse-chase labeling of transfected AtT-20 cells demonstrated that regulated secretion of prorenin was prevented by: 1) the mutagenic deletion of the prosegment, 2) the premature proteolytic removal of the prosegment by a Golgi-resident processing protease, or 3) the mutation of the native cleavage site so as to prevent removal of the prosegment. In addition, expression of fusion proteins containing portions of the prorenin prosegment demonstrated that exposure of potential proteolytic cleavage sites was sufficient to confer cleavage-dependent regulated secretion of the corresponding protein. These data implicate the protease cleavage event in the regulated secretion of prorenin and are consistent with the involvement of a subclass of processing proteases in the sorting of certain proteins to secretory granules in AtT-20 cells.

Mammalian cells use several different pathways to secrete proteins (1). All mammalian cells possess the ability to secrete proteins by the constitutive secretory pathway, in which proteins are packaged into low density vesicles and released at a constant rate to the cell surface. Transient time of proteins through the constitutive secretory pathway is on the order of minutes (2). Specialized endocrine and neuroendocrine cells also contain a regulated secretory pathway, in which proteins are packaged and stored in secretory granules for subsequent release in response to physiological stimuli. The transient time of proteins secreted by the regulated secretory pathway is typically in the range of hours (3). Two different models have been proposed to explain the sorting to the regulated secretory pathway. In the first model, peptides to be retained would selectively aggregate in the presence of calcium and an acidic environment, conditions present in immature secretory granules (4, 5). The second model involves a receptor-mediated mechanism in which a structural domain on the sorted protein is recognized by receptors localized in the maturing granule (6, 7). This process could also involve the specific binding of prohormones to immature secretory granule proteins, such as chromogranins, which are themselves sorted to the secretory granules (5, 8, 9). It is possible that aspects of both these models could be operative (1).

In the current studies, the sorting of prorenin to the regulated secretory pathway has been examined. This protein is the precursor to renin, an aspartyl protease that cleaves angiotensinogen to release angiotensin I. The latter is then cleaved to angiotensin II, which plays a critical role in regulation of the cardiovascular system (10). Renin in the circulation comes mainly from the juxtaglomerular cells of the kidney, where it is first synthesized as the precursor prorenin. Prorenin is secreted constitutively. In addition, a portion of the prorenin is sorted to dense core secretory granules, where it is proteolytically cleaved to active renin by removal of a 43-amino acid prosegment. This active renin is stored in dense secretory granules until its release is stimulated by secretagogues such as β-adrenergic agonists (11). Secretory granule sorting of human prorenin has been demonstrated in transfected endocrine cells in culture, including mouse pituitary corticotropic AtT-20 cells, rat somatotrophic GH4 cells, and rat pheochromocytoma PC12 cells (12-14). Protein engineering studies have demonstrated that neither the native signal peptide nor the glycosylation sites are essential for regulated secretion of human prorenin from transfected AtT-20 cells (15). Moreover, it was suggested that the prosegment of prorenin is not necessary for regulated secretion in multiple cell types (14-16). Thus, although these studies indicate that prorenin carries a signal for secretory granule sorting, they have so far failed to identify that signal.

To further define the human prorenin sorting signal, mouse pituitary AtT-20 cells were transfected with expression vectors for either native or mutated human prorenin or fusion proteins containing portions of human prorenin. Pulse-chase experiments were performed to follow labeled proteins in the secretory pathways. Our results demonstrate that the cleavage sites for certain endoproteases are sufficient to direct the sorting/retention of proteins in the regulated secretory pathway of AtT-20 cells.

EXPERIMENTAL PROCEDURES

Recombinant Plasmid Construction—Construction of the expression vectors for native human prorenin (proren, also referred to as pRHR1100) and prosegment-deleted prorenin (pro-Δp)1 have been described previously (15) (Fig. 1). Site-directed mutagenesis of amino

1 The abbreviations used are: pro-Δp, prosegment-deleted prorenin; Pro3-IgG, IgG fusion protein containing the N-terminal 3 amino acids of renin profragment.
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acids in the prosegment of human prorenin was carried out by overlap extension polymerase chain reaction (17). A furin consensus cleavage site (18) at the junction between the prosegment and renin (proren-furin) (Fig. 1) and mutants of the cleavage site (Fig. 1; proren K/A +42 and R/A +43; numbering relative to amino acid 1 of the prosegment) were obtained in this fashion.

Expression vectors for fusion proteins containing portions of human prorenin and the mouse immunoglobulin heavy chain were constructed as follows. A DNA clone done for the heavy chain of mouse immunoglobulin IgG2b (19) was cleaved with XhoI and SadI to release a fragment encoding 221 amino acids from the C-terminus of the protein. The region contains the entire CH2 and CH3 domains and therefore lacks the BIP binding hinge, and intermolecular disulfide bridge regions while maintaining the protein A binding site. Bal-31 nuclease was used to delete the human prorenin cDNA from its 3' end, and the generated fragments were modified by attachment of a BamH1 linker on their 3' ends. Deletions that would result in predicted frame fusions were ligated to the IgG fragment and inserted in the place of the native human prorenin cDNA in the expression vector proren (Fig. 2). Site-directed mutagenesis was carried out by overlap extension polymerase chain reaction. All recombinant plasmid constructions were verified by sequencing of double-stranded DNA.

Cells—Culture—Mice pituitary corticotrophic AtT-20 cells were grown in Dulbecco's modified Eagle's medium supplemented with 5% fetal calf serum and 0.1% streptomycin (Irvine Scientific) in an humidified incubator at 10% CO2, 90% air. AtT-20 cells sort endogenous proopiomelanocortin to secretory granules, process proopiomelanocortin to adrenocorticotropic hormone and other peptides, such as β-endorphin, and release them in response to increases in intracellular cyclic AMP (20).

DNA Transfections and Pulse-Chase Experiments—Purified plasmid DNA (50 μg) was mixed with 107 AtT-20 cells resuspended in serum-free Dulbecco's modified Eagle's medium and electroporated with a single pulse of 300 V/μm, 1000 microfarads. Transfected cells were then transferred to 100-mm plates in complete medium. The following day, cells were transferred to 12-well plates at a concentration of 500,000 cells/well. Twenty-four hours later, parallel wells of transfected cells were depleted of methionine for 1 h in methionine-free Dulbecco's modified Eagle's medium containing 10% dialyzed fetal calf serum. The cells were then labeled for 2 h with 300 μCi of [35S]methionine/Well, washed overnight with complete medium, and subsequently incubated for 3 h in the absence or presence of 10 μM forskolin.

The overnight chase allowed labeled proteins to be targeted to their final destination. Culture supernatants were immunoprecipitated with either protein G-agarose-coupled anti-human prorenin antibody or with protein G-agarose alone (Life Technologies, Inc.) for IgG fusion proteins. Immunoprecipitated proteins were fractionated by SDS-polyacrylamide gel electrophoresis, and gels were subjected to fluorography.

RESULTS

Prorenin Is Sorted to the Regulated Secretory Pathway—Fig. 1 shows the pattern of secretion of native human prorenin from AtT-20 cells transfected with electroporation with a preprorenin expression vector (proren). Transfected cells were labeled for 2 h with radioactive methionine, chased overnight, and then exposed for 3 h with or without 10 μM forskolin, which activates adenylyl cyclase and causes the release of proteins stored in dense core secretory granules. Media were immunoprecipitated with an anti-renin antibody, and proteins were fractionated by SDS-polyacrylamide gel electrophoresis. Prorenin was secreted predominantly in the 2-h pulse period, corresponding to the constitutive secretory pathway. Mature renin was present in the medium in the overnight chase, and its secretion was selectively stimulated by forskolin. Consistent with previous results (12), this experiment demonstrates that human prorenin is sorted to secretory granules since labeled renin still remains in the cell following the overnight chase, suggesting its presence in a storage compartment, and its release can be stimulated by forskolin. It is noteworthy that all of the renin released by forskolin migrates with the expected size of active renin from which the 43-amino acid prosegment has been removed by proteolytic cleavage.

The Prorenin Prosegment Is Necessary for Sorting—To test the importance of the prosegment in secretory granule sorting, AtT-20 cells were transfected with a vector in which the sequence encoding the prosegment of prorenin was deleted (prodel). As shown in Fig. 1, prodel was not present in the medium after the 2-h labeling period, indicating that its secretion was slower than that of prorenin. These results are consistent with a role of the prosegment in the folding of the nascent peptide in the endoplasmic reticulum (21). Pro-del secretion was not stimulated by forskolin, showing that it was not stored in secretory granules and did not enter the regulated secretory pathway. These results suggest that renin prosegment removal inhibits secretory granule targeting and leads in constitutive secretion of the resulting renin protein.

Premature Cleavage of the Prorenin Prosegment Abolishes Sorting to the Regulated Secretory Pathway—To exclude the possibility that the loss of regulated secretion of the pro-del mutant was due to general effects on the conformation of the resulting renin, conditions were devised such that the AtT-20 cells would cleave the prosegment prior to entry of the protein into the trans-Golgi network. To achieve this, AtT-20 cells were transfected with a vector that expresses a mutated prorenin in which the native proprotein cleavage site had been altered to a consensus cleavage sequence for the endoprotease furin (proren-furin) (18). Furin, an ubiquitously distributed member of the subtilisin-like family of processing enzymes called convertases, is localized in the Golgi apparatus (22) and would be expected to remove the prosegment of proren-furin before its sequestration into immature secretory granules. As shown in Fig. 1, renin cleaved from the prorenin-furin precursor was predominant in the 2-h labeling supernatants, suggesting that prosegment cleavage occurred in the constitutive secretory pathway. After the overnight chase, labeled renin was not secreted from the transfected cells in response to forskolin, showing that prosegment cleavage in the Golgi apparatus prevents the sorting of renin to the regulated secretory pathway. Taken together, these results suggest that the presence of the renin prosegment at the trans-Golgi network level is essential for sorting to the regulated secretory pathway.

Mutation of the Prorenin Cleavage Site Abolishes Sorting—To test whether it is the renin prosegment itself or the removal step that is important for sorting, we investigated the role of the native prorenin to renin cleavage site (Lys50-Arg54) in sorting. AtT-20 cells were transfected with expression vectors for human prorenin in which either the lysine residue at position 42 or the arginine residue at position 43 was changed to alanine (Fig. 1; proren K/A +42 and R/A +43). As expected, only prorenin was secreted in the 2-h labeling period and the overnight chase, indicating that both mutations block cleavage at the native site in AtT-20 cells and that secondary processing events (i.e. at two additional Lys-Arg pairs in the prosegment at positions 9–10 and 14–15 or elsewhere in the prorenin molecule) do not occur. In addition, there is no evidence of either retention in the secretory pathway or of stimulation by forskolin for either of the prorenins mutated at the native cleavage site (see 3-h chase). These results strongly suggest that the presence of a cleavage site for a specialized endopro-
Fig. 1. Importance of the prorenin to renin cleavage site in sorting to the regulated secretory pathway. AtT-20 cells were transfected with the expression vectors proren, pro-del, and proren-furin and with the expression vectors for the human prorenin with a mutation at the cleavage site (K/A +42 and R/A +43). Parallel wells of cells were labeled for 2 h with [35S]methionine, chased overnight (O/N), and incubated for 3 h in the absence or presence of 10 μM forskolin. Culture supernatants were immunoprecipitated with an anti-human renin antibody and analyzed by SDS-polyacrylamide gel electrophoresis. Presence and/or increased labeling of a band in the rightmost lane (Forskolin +) is indicative of regulated secretion. Prorenin and renin represent the predicted size of the two proteins based on coelectrophoresis of size markers. Results are representative of those obtained in 3–6 independent experiments.

A Portion of the Renin Prosegment Containing a Dibasic Cleavage Site Can Target a Constitutively Secreted Protein to the Regulated Secretory Pathway—The prorenin prosegment contains two pairs of basic amino acids (Lys-Arg pairs at positions 9–10 and 14–15 in the prosegment) in addition to those at the usual cleavage site (position 42–43). Although in the context of intact prorenin these never serve as cleavage sites (see above), they could become exposed for cleavage by a processing enzyme in the context of a fusion protein. If cleavage sites play a role in secretory granule targeting, exposure of these potential cleavage sites could direct the sorting of a linked protein to the regulated secretory pathway. To test this notion, we transfected AtT-20 cells with vectors containing various prosegment sequences linked to a fragment of the immunoglobulin IgG heavy chain (corresponding to the Fc fragment region), and pulse-chase experiments were performed. As shown in Fig. 2, when the N-terminal 16, 21, or 31 amino acids of the prorenin prosegment, which contain both potential cleavage sites, were linked at their C termini to the IgG fragment, the resulting fusion protein was retained within the secretory pathway after the overnight chase, and its secretion was stimulated by forskolin. Both findings are characteristic of the regulated secretory pathway. By contrast, inclusion of 3, 6, or 11 amino acids of the prorenin prosegment did not lead to either retention of the labeled protein or its regulated secretion. Thus, the ability of the prorenin prosegment to direct a fusion protein to the regulated secretory pathway correlates with the presence of the basic amino acids at positions 9–10 and 14–15. Of note, a shorter exposure of the gels shown in Fig. 2 clearly reveals multiple bands in both the 2-h labeling and the overnight chase supernatants for the fusion proteins with 16, 21, and 31 amino acids of the prorenin prosegment, whereas only the shortest of these forms is secreted in response to forskolin in the subsequent 3-h chase. The generation of these multiple forms suggests that the fusion proteins were cleaved by a processing enzyme. Taken together, these results suggest that the presence of an accessible cleavage site in the prorenin prosegment correlates with the sorting of a linked fusion protein to the regulated secretory pathway in transfected AtT-20 cells.

To confirm the localization of prorenin prosegment-containing fusion proteins in dense core secretory granules, AtT-20 cells transfected with the expression vectors for IgG fusion protein containing either 3 amino acids (Pro16-IgG) or 16 amino acids (Pro3-IgG) of the human renin prosegment were immunoprecipitated with an antibody directed against mouse Fc fragment. Fig. 3A shows representative cells expressing Pro16-IgG. Fc staining was concentrated in the perinuclear area, suggesting localization within the Golgi apparatus. By contrast, cells expressing Pro3-IgG showed staining of both the Golgi apparatus, as well as cell processes with very heavy punctate labeling at the tips of the processes, which is the expected result for a protein localized in secretory granules (Fig. 3B). This result supports the conclusion that the presence of the N-terminal 16 amino acids of human renin prosegment contain sequences capable of inducing the sorting of a constitutively secreted protein (IgG fragment) to secretory granules.

Mutation of the Alternate Cleavage Sites in the Prorenin Prosegment Abolishes Sorting of Prosegment-IgG Fusion Proteins—To test the possibility that the Lys-Arg pairs in the 16-amino acid prosegment participate in the sorting process, we constructed a double mutant for Pro3-IgG in which the two amino acids lys-Arg at positions 9–10 and 14–15 were replaced by Lys-Lys. The replacement of Arg by Lys does not change the predicted secondary structure of the putative sort peptide (data not shown) but should abolish cleavage by potential processing enzymes present in AtT-20 cells (23). As shown in Fig. 4, the protein was secreted during the 2-h pulse period and the overnight chase, but no stimulation by forskolin...
abolishes the sorting of the native prorenin (Fig. 1) and of the (Fig. 2). Third, mutation of the prosegment cleavage site totally is not ordinarily targeted to secretory granules results in the tion of the resulting renin protein (Fig. 1). Second, transfer of a secretory granule targeting and results in constitutive secre-

cleavage in the Golgi apparatus by furin (pro-ren-furin) inhibits regulated secretory pathway. This was shown in several ways.

was observed during the 3-h chase period. Further confirmation of the role of the dibasic residues in secretory granule sorting was obtained by substituting the human renin prosegment peptide with the N-terminal 16 amino acids from the rat renin prosegment, which, although highly conserved, does not contain any pairs of basic amino acids. Pulse-chase experiments clearly demonstrate that the rat prosegment-containing fusion protein was not targeted to the regulated secretory pathway (Fig. 4; rat Pro16-IgG). Taken together, these results strongly indicate that the presence of a functional endopeptidase cleavage site in the prorenin prosegment is essential for regu-

lated secretion of the IgG fusion protein.

**DISCUSSION**

Results from these studies suggest that a functional cleavage site in human prorenin is necessary for prorenin sorting to the regulated secretory pathway. This was shown in several ways. First, renin prosegment removal by engineering (pro-del) or by cleavage in the Golgi apparatus by furin (proren-furin) inhibits secretory granule targeting and results in constitutive secre-

of the resulting renin protein (Fig. 1). Second, transfer of a cleaveable portion of the prorenin prosegment to a protein that is not ordinarily targeted to secretory granules results in the sorting of the fusion protein to the regulated secretory pathway (Fig. 2). Third, mutation of the prosegment cleavage site totally abolishes the sorting of the native prorenin (Fig. 1) and of the fusion protein (Fig. 4). Of interest was the fact that the fusion protein containing the first 16 amino acids of the human prosegment that contains processing sites was sorted (Pro16-IgG), whereas an analogous fragment from rat prorenin that does not contain any dibasic pairs of amino acids was not (Fig. 4). Thus, the data support the hypothesis that retention in the dense core secretory granules may involve interactions of the sorted protein with a processing enzyme recognizing dibasic amino acids. Moreover, cleavage at paired basic amino acids is itself not sufficient for secretory granule sorting since conversion of the native prorenin cleavage site to one recognized by the Golgi-resident protease furin leads to constitutive secretion of the resulting renin, suggesting that secretory granule sorting of prorenin requires interaction with a specific subtype of protease. The identity of the enzyme that cleaves human prorenin and the prorenin prosegment-containing fusion proteins in AtT-20 cells is uncertain. Mouse PC1, a member of the mammalian convertase family responsible for the cleavage of prohormones at pairs of basic amino acids, has been reported to cleave human prorenin with the appropriate site selectivity when the two proteins are co-expressed in somatomammotroph GH4 cells (24). PC1 is expressed in AtT-20 cells, where it is localized to secretory granules (25), and is a good candidate for the prorenin processing enzyme in this cell line.

Interestingly, the protease cleavage sites at positions 9–10 and 14–15 that were shown to direct the sorting of the fusion protein are neither cleaved by protease nor implicated in se-

cretory granule sorting in the context of the native protein. This result suggests that in the context of the native prorenin molecule, the processing enzyme interacts exclusively with the basic pair at position 42–43 and that other paired basic amino acids in the prosegment are masked due to formation of salt bridges with the body of renin (21, 26) or other conformational constraints. This finding also implies that studies using fusion proteins to identify potential sorting peptides should be interpreted with caution.

Our present results seem to be in contradiction with previous reports from several groups (14, 16, 27) and from our own previous data (15) showing that neither the renin prosegment nor the renin-to-prorenin processing event was essential for sorting. However, these differences may be explained by the experimental conditions used. In previous studies, the secretory characteristics of the expressed renin/prorenin were determined by either activity measurements or immunoprecipitation of labeled proteins after a short (2 or 3 h) chase period. Either approach would likely detect secretion of proteins that are only transient in immature granules but have a final des-

ination other than the regulated secretory pathway. Indeed, although it has been proposed that segregation of proteins between the constitutive and regulated pathways occurred in the trans-Golgi network, more recent evidence suggests that both types of proteins are first packaged into immature secretory granules (28, 29). A similar observation was recently re-

ported by Kuliawat and Arvan (30) in a study of insulin secretion from pancreatic islets. With short chase periods, treatment of radiolabeled islets with secretagogue stimulated release of not only unprocessed preproinsulin, but also of precursors of lysosomal enzymes ("constitutive-like secretion"). After a chase period of longer than 8 h, secretagogue only stimulated the release of mature insulin and no longer caused the release of lysosomal enzymes. In the current study, the use of an overnight chase period ensures that labeled proteins have reached their final cellular destination. Under these conditions, there is a direct correlation between the presence of an accessible dibasic amino acid cleavage site and the ability of human prorenin peptides to direct proteins to the regulated secretory pathway.
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In addition to their importance for prorenin, it is likely that protease processing sites may be implicated in the sorting of other proteins whose secretion is regulated. For example, although the use of fusion proteins has suggested that a secretory granule sorting peptide was present in the N-terminal 78 amino acids of the rat somatostatin prosegment (31, 32), more systematic deletion analysis suggests that pro-opiomelanocortin, which contains multiple protease cleavage sites, also contains multiple secretory granule sorting sequences (35).

Association of proteins to processing proteases is likely to be only one of several mechanisms whereby proteins are targeted to dense core secretory granules. For example, growth hormone, prolactin, and chromogranins are sorted to the regulated secretory pathway although none of these are processed by proteolysis. It has been suggested that such proteins may be sorted/retained in secretory granules by a process of selective aggregation (36). Others, such as atrial natriuretic factor, appear to aggregate in the immature secretory granules as a prohormone and are processed upon release of the mature secretory granules (34), systematic deletion analysis suggests that pro-opiomelanocortin, which contains multiple protease cleavage sites, also contains multiple secretory granule sorting sequences (35).

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Fig. 4. Importance of Lys-Arg paired basic amino acids for fusion protein sorting. A23-20 cells were transfected with the expression vectors for native Pro16aa-IgG (Pro16aa-IgG; Fig. 2), the double mutant R/K +10, R/K +15, or a similar IgG fusion protein containing the analogous 16 amino acids of the rat prorenin prosegment. O/N, overnight. Pulse-chase experiments were performed as described under “Experimental Procedures” and in the legend to Fig. 1.