Renin, a Secretory Glycoprotein, Acquires Phosphomannosyl Residues

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Abstract. Renin is an aspartyl protease which is highly homologous to the lysosomal aspartyl protease cathepsin D. During its biosynthesis, cathepsin D acquires phosphomannosyl residues that enable it to bind to the mannose 6-phosphate (Man-6-P) receptor and to be targeted to lysosomes. The phosphorylation of lysosomal enzymes by UDP-GlcNAc:lysosomal enzyme N-acetylglucosaminylphosphotransferase (phosphotransferase) occurs by recognition of a protein domain that is thought to be present only on lysosomal enzymes. In order to determine whether renin, being structurally similar to cathepsin D, also acquires phosphomannosyl residues, human renin was expressed from cloned DNA in *Xenopus* oocytes and a mouse L cell line and its biosynthesis and posttranslational modifications were characterized. In *Xenopus* oocytes, the majority of the renin remained intracellular and underwent a proteolytic cleavage which removed the propiece. Most of the renin synthesized by oocytes was able to bind to a Man-6-P receptor affinity column (53%, 57%, and 90%, in different experiments), indicating the presence of phosphomannosyl residues. In the L cells, the majority of the renin was secreted but 5–6% of the renin molecules contained phosphomannosyl residues as demonstrated by binding of [^{35}S]methionine-labeled renin to the Man-6-P receptor as well as direct analysis of [2-^{3}H]mannose-labeled oligosaccharides. Although the level of renin phosphorylation differed greatly between the two cell types examined, these results demonstrate that renin is recognized by the phosphotransferase and suggest that renin contains at least part of the lysosomal protein recognition domain.

LYSOSOMAL enzymes, secretory proteins, and plasma membrane proteins are all synthesized in the rough endoplasmic reticulum (ER)[1] and yet have different final destinations in the cell. Several biosynthetic events are shared by these classes of proteins during their transport through the cell. In the ER, the proteins undergo cleavage of the signal peptide, which directs translocation across the membrane, and cotranslational glycosylation of selected asparagine residues. Additionally, the lysosomal enzymes and secretory proteins are completely translocated across the ER membrane and are thus mixed together in the lumen of this organelle. This mixture of proteins is then transported to the Golgi apparatus where they undergo a variety of posttranslational modifications, including processing of oligosaccharide chains. They are then sorted for targeting to the proper destination, e.g., lysosomes, secretory granules, plasma membrane.

The mechanism for the segregation of newly synthesized lysosomal enzymes is best understood. These enzymes contain phosphomannosyl residues that are recognized by specific receptors (mannose 6-phosphate receptor) allowing selective translocation to lysosomes (3, 22). This mannose 6-phosphate (Man-6-P) recognition marker is generated by a two-step reaction (13). First, N-acetylglucosamine-1-phosphate is transferred to selected mannose residues on lysosomal enzymes, giving rise to a phosphodiester intermediate. The N-acetylglucosamine is then removed by a second enzyme to expose the Man-6-P monoester signal. The first reaction is catalyzed by UDP-GlcNAc:lysosomal enzyme N-acetylglucosaminylphosphotransferase (phosphotransferase) (16, 29). This enzyme is able to phosphorylate lysosomal enzymes selectively over nonlysosomal glycoproteins containing similar oligosaccharides by recognition of a protein domain that is common to almost all lysosomal enzymes (26, 28). Therefore, the specific recognition of lysosomal enzymes by the phosphotransferase is the initial and determining step in the generation of the Man-6-P marker and thus eventual targeting to lysosomes by the Man-6-P recognition system.

To learn more about the signals that determine the sorting events, we have initiated a study of the aspartyl protease family of proteins. This family includes cathepsin D, a typical lysosomal enzyme, and renin, a secretory glycoprotein. Hu-

1. *Abbreviations used in this paper:* endo H, endo-β-N-acetylglucosaminidase H; ER, endoplasmic reticulum; Man-6-P, mannose 6-phosphate; phosphotransferase, UDP-GlcNAc:lysosomal enzyme N-acetylglucosaminylphosphotransferase.

man cathepsin D and human renin share 46% identity in amino acid sequence (7) and are believed to have similar three-dimensional structure (32). Cathepsin D is known to be efficiently phosphorylated and targeted to lysosomes in several systems (12, 15, 30). Renin is found primarily in the juxtaglomerular cells of the kidney where it is stored in secretory granules (23). Renin functions to catalyze the first step in the activation pathway of angiotensinogen, and thereby plays a pivotal role in the regulation of blood pressure and extracellular fluid volume (21). There is some evidence that the major action of renin may not occur in the serum but in peripheral tissues, where it is taken up from the serum and acts intracellularly to activate angiotensinogen (1). Although several laboratories have studied the biosynthesis of renin (9, 11, 27), none have determined whether or not this glycoprotein acquires phosphomannosyl residues and, if so, whether these residues have any role in the targeting of renin to its storage granule or in the peripheral uptake of renin from the serum. Indeed, it has been reported that the secretory granules of the juxtaglomerular cell have some properties typical of lysosomes, leading to speculation that they may in fact be modified lysosomes (33). We report here than renin, when expressed from cloned DNA in *Xenopus* oocytes and a mammalian L cell line, is phosphorylated but the extent of this process varies greatly between these two cell types.

Materials and Methods

cDNA Cloning/Plasmids

A human kidney cDNA library was constructed as previously described (7) except that double-stranded cDNAs from 1.2 to 1.8 kb were selected. The library was screened with human renin genomic exons (18). One positive clone was isolated which extended from base 3 of the protein coding region of the human renin cDNA sequence published by Imai et al. (20) through the poly(A) tail. The signal sequence was made complete by fusing a Sau3AI-RsaI restriction fragment (base −32 to 59 of cDNA sequence [20]) isolated from a human renin exon 1 genomic subclone (18) to the renin cDNA correspondingly cut at RsaI (base 59) by partial digestion. The resulting gene-cDNA fusion product was sequenced across the RsaI site to confirm the construction and then subcloned into the expression vector pSP65 (Promega Biotec, Madison, WI).

A cDNA clone for α-lactalbumin in pSP64 was generously provided by Dr. P. K. Qasba (National Cancer Institute, Bethesda, MD) and Dr. V. R. Lingappa (UCSF, San Francisco, CA). A cDNA clone for chicken ovalbumin was generously provided by Dr. A. Colman (University of Warwick, United Kingdom).

Oocyte Expression

RNA transcripts were synthesized in vitro from cloned cDNAs with SP6 polymerase (New England Biolabs, Beverly, MA) and microinjected into stage V-VI *Xenopus laevis* oocytes as previously described (8). Oocytes were then incubated at 19°C in groups of 10–20 with 10 μl/oocyte of modified Barth's saline containing 5% fetal bovine serum (FBS) and 1 mCi/ml [³⁵S]methionine (>1,000 Ci/mmol, Amersham Corp., Arlington Heights, IL). At the termination of the incubation, oocytes and media were collected, rapidly frozen on dry ice, and stored at −20°C.

Pulse Labeling of Cells

L-2234 cells, a murine L929 cell line stably transfected with the human renin gene, were very generously provided by Dr. Peter M. Hobart (Pfizer, Inc., Groton, CT). The isolation of this cell line has been previously described (19). Preliminary studies have demonstrated that the L-2234 cell line contains the 215-kD Man-6-P receptor (data not shown). L-25.336 cells are the parental L929 line from which the L-2234 cells were derived.

L-2234 cells were maintained in α-minimal essential medium (MEM)

containing 10% FBS and 100 μg/ml Geneticin (Gibco, Grand Island, NY). For labeling with [³⁵S]methionine, confluent monolayers in 60-mm dishes were incubated in 2.5 ml of methionine-free MEM that contained 1% FBS, 20 mM Hepes, pH 7, and 100 μCi/ml of [³⁵S]methionine. To initiate the chase, 100 mM methionine in MEM was added to a final concentration of 10 mM.

For labeling with [2-³H]mannose (14 Ci/mmol, ICN, St. Louis, MO), confluent monolayers in 150-mm dishes were rinsed with 20 ml of glucose-free MEM and then incubated for 30 min in 10 ml of glucose/bicarbonate-free MEM containing 20 mM Hepes, pH 7, and 1 mCi of [2-³H]mannose. Cells were then chased for 4 h by removing the radioactive medium and adding 20 ml of complete MEM containing 1% FBS and 20 mM unlabeled mannose.

To harvest, media were collected and clarified by centrifugation. Cells were rinsed and scraped from the dish into ice-cold 0.1 M Tris, 0.15 M NaCl with a rubber policeman, collected by centrifugation at 600 g for 10 min, and frozen in a dry ice-ethanol bath.

Antisera

Rabbit antirenin antiserum, raised vs. pure human renal renin, was generously provided by Dr. V. J. Dzau (Brigham and Women's Hospital, Boston, MA). The antiserum does not cross-react with mouse cathepsin D; immunoprecipitation of mouse L-cell cathepsin D with anti-human cathepsin D antibody revealed proteins of distinct molecular mass from those in antirenin immunoprecipitates (data not shown). Antiserum to α-lactalbumin was provided by Dr. P. K. Qasba. Antiserum to chicken ovalbumin was purchased from Calbiochem-Behring Corp., La Jolla, CA.

A rabbit antiserum (anti-propeptide) was generated against a 17-amino acid synthetic peptide corresponding to residues 17–33 of the putative pro segment of human prorenin (ProSerIleArgGluSerLeuLysGluArgGlyVal-AspMetAlaArgLeu). The peptide was synthesized by solid-phase methods, coupled to keyhole limpet hemocyanin, and injected with adjuvants. The coupled peptide was kindly provided by Dr. Steve Adams (Monsanto Co., St. Louis, MO). Reactivity of the anti-propeptide serum was monitored by the ability to immunoprecipitate renin synthesized in a reticulocyte lysate system (Amersham Corp.) from an in vitro generated renin message.

Immune Precipitations

Frozen, labeled oocytes were homogenized in 40 μl/oocyte of 50 mM imidazole, pH 7, 150 mM NaCl, 5 mM Na β-glycerophosphate, 1% Triton X-100, 0.2 TIU/ml aprotinin (Sigma Chemical Co., St. Louis, MO), 1 mM phenylmethylsulphonyl fluoride (PMSF), and 1:1,000 antiprotease mix (buffer I), and centrifuged in an Eppendorf microfuge (Brinkmann Instruments, Westbury, NY) for 2 min at 4°C to remove insolubles. The antiprotease mix consists of antipain, chymostatin, leupeptin, and pepstatin (each at 2 mg/ml) and aprotinin (10 TIU/ml) in 50% DMSO.

Frozen cell pellets from L-2234 cell labelings were suspended in buffer I and sonicated for three 10-s bursts with a probe sonicator (Bronwill, Rochester, NY). After 30 min on ice, the extracts were centrifuged for 30 min at 40,000 rpm, 4°C in a Ti50 rotor (Beckman Instruments, Inc., Palo Alto, CA) and the pellets were discarded. Medium samples were prepared for immunoprecipitation by addition of one-quarter volume of a detergent solution containing 5% Triton X-100, 0.75 M NaCl, 50 mM EDTA, 0.5 M Tris-HCl, pH 8, and 0.2 TIU/ml aprotinin.

Cell extract and medium samples were incubated with appropriate antisera and immunoprecipitates were isolated as previously described (8). Immunoprecipitated proteins were dissociated from the resin by heating for 5 min at 100°C in SDS gel sample buffer, electrophoresed through a SDS polyacrylamide gel (24) under nonreducing conditions, and fluorographed using ENHANCE (New England Nuclear, Boston, MA). When indicated, the appropriate regions of the dried gel were excised, solubilized with 90% NCS tissue solubilizer (Amersham Corp.), and radioactivity was measured by liquid scintillation counting according to the manufacturer's instructions.

Man-6-P Receptor Affinity Chromatography

Chromatography of oocyte detergent extracts and media samples on Man-6-P receptor columns has been previously described (8). Detergent extracts from L-2234 cell labelings were applied directly to the receptor column. Medium samples from L-2234 cells labeled with [³⁵S]methionine were first dialyzed vs. buffer I without detergent, and Triton X-100 was then added to 0.05% before applying to the column. Medium samples from L-2234 cells labeled with [2-³H]mannose were prepared for chromatography by

addition of $(NH_4)_2SO_4$ to 75% saturation. The precipitate was collected by centrifugation, dissolved in water, and dialyzed extensively at 4°C vs. buffer I without detergent. Triton X-100 was added to 0.05% before applying to the column. Fractions corresponding to the column run-through (RT) and Man-6-P-eluted material were immunoprecipitated with appropriate antisera, as described (8).

Oligosaccharide Analysis

Cell extract and media samples from [2-^{3}H]mannose-labeled L-2234 cells were applied to Man-6-P receptor columns. Renin was isolated by immunoprecipitation from the column run-through and Man-6-P eluates. Glycopeptides were prepared by pronase digestion of SDS-disaggregated immunoprecipitates and applied to concanavalin A (Con A)-Sepharose as previously described (4). The glycopeptides that eluted with 100 mM α-methylmannoside were desalted by chromatography on Sephadex G-25 and then digested with 2 mU of endo-β-N-acetylglucosaminidase H (endo H; Boehringer Mannheim, Indianapolis, IN) in 0.1 ml of 50 mM citrate-phosphate, pH 5.6. A sample of the endo H digest was diluted with 2 mM Tris, pH 8, and analyzed by QAE-Sephadex chromatography (34). Separate samples were subjected to mild acid hydrolysis and/or digestion with Escherichia coli alkaline phosphatase (a generous gift of Dr. M. Schlesinger, Washington University, St. Louis, MO) as previously described (34).

Results

Expression of Renin in Xenopus Oocytes

Oocytes were microinjected with a renin RNA message generated by in vitro transcription of the cloned cDNA inserted behind the bacteriophage SP6 promoter. Injected oocytes were incubated with [^{35}S]methionine over a period of 75 h, and renin present inside oocytes and in the medium was immunoprecipitated with the antirenin antibody (note that incubation past 20 h constitutes a chase in this system [2]). After 5 h of incubation, a M_r 45,000-D protein was immu-

noprecipitated from the oocyte homogenate and was first seen in the medium at 10 h (Fig. 1, lanes 1 and 4, respectively). The majority of the renin, however, remained intracellular and was slowly processed to a M_r 38,000-D protein (Fig. 1); this processed form was not detected in the medium. By 75 h of incubation, <20% of the renin synthesized by oocytes had been secreted and intracellular conversion to the M_r 38,000-D protein was 90% complete (Fig. 1, lanes 9 and 10); distributions were quantified by solubilization and liquid scintillation counting of appropriate regions of the dried gel (data not shown). These immunoprecipitable proteins were not present in antirenin immune precipitates from control, noninjected oocytes (not shown) or in nonimmune antiserum precipitates of renin-injected oocytes (Fig. 1, lane 11).

The M_r 45,000-D form of renin present in oocytes and medium appears to be a form of prorenin as it is also immunoprecipitated by the anti-propeptide antibody (Fig. 1, lanes 12–14). This agrees with the findings of Hirose et al. (17). The M_r 38,000-D protein, however, is not precipitable with this antibody. In addition, trypsin activation (9) of the secreted renin, which is believed to remove the propiece, decreases the apparent molecular mass to that of the intracellular processed form (data not shown). Both the size of the shift in apparent molecular mass and loss of reactivity with the anti-propeptide antibody indicate that the intracellular processing is due, at least in part, to cleavage of the renin propiece.

Studies with expression of the lysosomal aspartyl protease, cathepsin D, in oocytes have shown a similar time course for proteolytic processing which correlated with phosphorylation of mannose residues to generate the Man-6-P
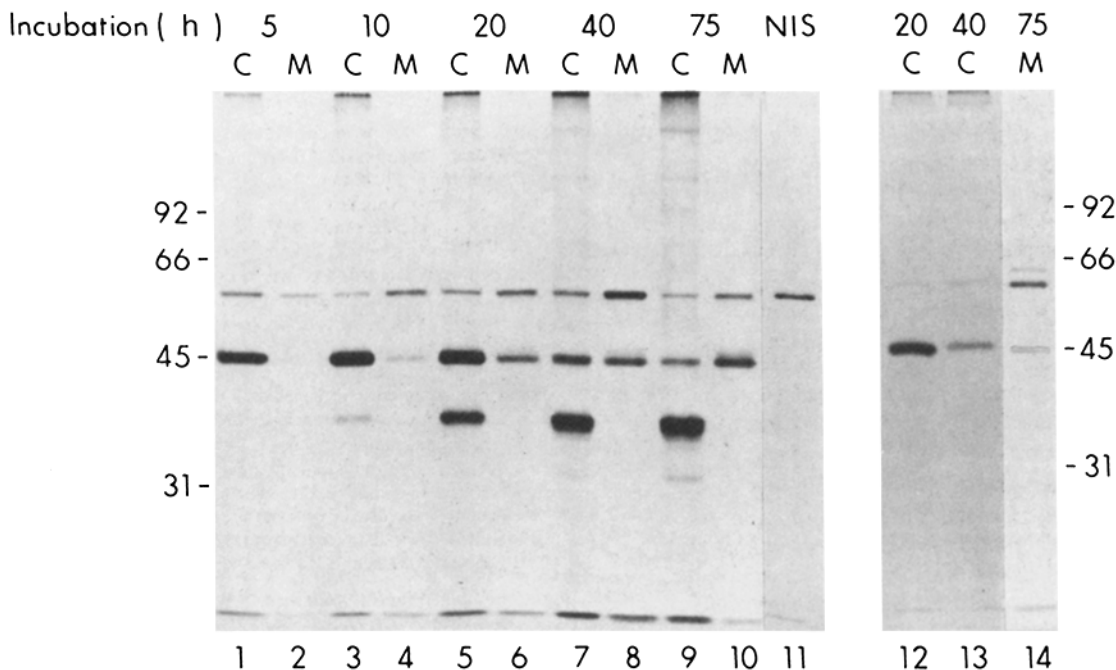

Figure 1. Time course of renin synthesis in Xenopus oocytes. Oocytes were microinjected with renin RNA and then labeled with [^{35}S]methionine for the indicated times. Equivalent samples of an oocyte detergent extract (C) and the medium (M) were then immunoprecipitated with antirenin antiserum (lanes 1–10), nonimmune serum (lane 11) or anti-propeptide antiserum (lanes 12–14) and analyzed by SDS-PAGE (10% gel) and fluorography. The band at M_r 59,000 D is nonspecific as it appears in nonimmune serum control (lane 11; same band present in cell and medium samples). Molecular size standards are in kilodaltons.
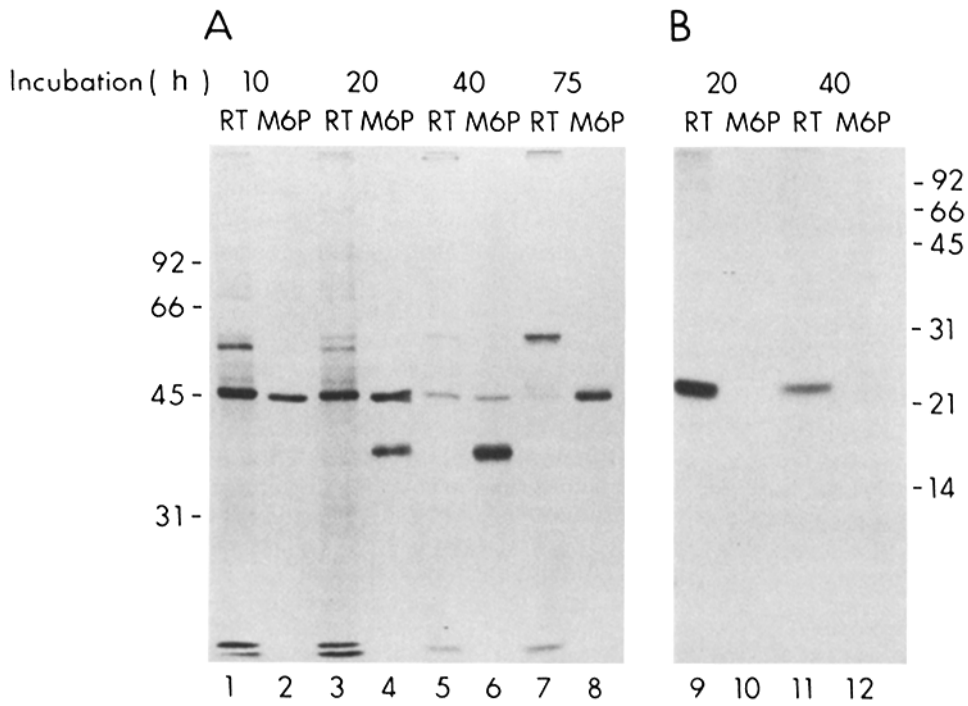
Figure 2. Binding of ^{35}S-labeled proteins produced in oocytes to the Man-6-P receptor. Separate batches of oocytes were injected with (A) a renin message or (B) an α-lactalbumin message. Oocyte homogenates (lanes 1–6 and 9–12) and medium samples (lanes 7 and 8) from the indicated times were applied to Man-6-P receptor affinity columns. The column was washed until no further radioactivity was in the buffer run-through and then eluted sequentially with 2 mM glucose 6-phosphate followed by 5 mM Man-6-P. Fractions corresponding to the column run-through (RT) and Man-6-P eluate (M6P) were immunoprecipitated with (A) antirenin antiserum or (B) antilactalbumin and analyzed by SDS-PAGE (10% gel in A; 15% gel in B) and fluorography. The glucose 6-phosphate-eluted fractions were free of radioactivity and not analyzed further.

recognition marker and targeting to lysosomes (8). Given the close relationship of renin to cathepsin D, we examined whether renin synthesized by oocytes also obtained this marker. [35S]methionine-labeled oocyte homogenate and medium samples were applied to Man-6-P receptor columns, as previously described (8). The radioactive material in the column run-through and the Man-6-P eluate was then immunoprecipitated with antirenin antiserum. As shown in Fig. 2 A, renin present inside oocytes (lanes 1–6) and in the medium (lanes 7 and 8) bound to the receptor column and was specifically eluted with Man-6-P, indicating the presence of the Man-6-P marker. The total extent of receptor binding of the intracellular renin increased from 35% to 91% between 10 and 40 h of incubation (Table I), consistent with the posttranslational nature of phosphorylation (13). Even 80% of the secreted renin bound to the receptor column (Table I), indicating that phosphorylation of renin by the oocyte is very efficient. In fact, in this experiment, renin acquired receptor-binding capability equally as well as cathepsin D (8). In addition, the M_r 38,000-D form of renin was only detected in receptor-bound fractions (Fig. 2 A, lanes 4 and 6). Its appearance lagged behind the phosphorylation event indicating that the proteolytic cleavage occurs after phosphorylation.

The level of renin secretion and phosphorylation varied with oocytes from different animals. In two separate experiments, after 75 h of incubation, about 50% of the renin was secreted and only 15% of this was able to bind to the receptor column (data not shown). The renin that remained intracellular was still highly phosphorylated (84–90%) and underwent proteolytic cleavage. However, because of the greater secretion of nonphosphorylated renin observed in these experiments, the total level of renin phosphorylation (cells and medium material) was only 53–57% vs. >90% in the first experiment described above. The reason for this variability is not understood.

As Xenopus oocytes had not previously been used to examine phosphorylation of lysosomal enzymes, the ability of the phosphotransferase in these cells to distinguish lysosomal from nonlysosomal glycoprotein acceptors was unknown. As a control, in vitro generated RNA for ovalbumin and α-lac-

Table I. Quantification of Renin Phosphorylation in Xenopus Oocytes and L-2234 Cells

Xenopus oocytes Time	Sample	Run-through	Man-6-P eluate	Percent phosphorylated
h		cpm in renin		%
10	Cells	2,145	1,183	35
20	Cells	1,719	3,006	64
40	Cells	323	3,172	91
75	Medium	319	1,260	80
L-2234 cells Chase				
h				
0	Cells	114,580	2,330	2.0
8	Cells	59,580	3,540	5.6*
8	Medium	45,640	2,455	5.1

Appropriate regions of the dried gels shown in Fig. 2 A and Fig. 4 were excised and the radioactivity present was measured by liquid scintillation counting. In the Xenopus oocyte experiment, the counts per minute associated with the M_r 38,000-D form (present in the Man-6-P eluate) were corrected for the loss of three methionines present in the renin propiece (11 methionines in prorenin). Note that in Fig. 4, only one-tenth of the run through samples were applied to the gel.
* If the Con A–Sepharose profile for nonreceptor-bound renin in the medium (Fig. 5 B) and its corresponding SDS gel profile (Fig. 4, lane 5) represents the complete processing of the oligosaccharides of renin, then the higher percentage of high-mannose-type oligosaccharides observed for the nonreceptor-bound renin found intracellularly (Fig. 5 A and Fig. 4, lane 3) suggests that only about one-half of the intracellular renin had passed from the endoplasmic reticulum to the Golgi complex, where it could encounter oligosaccharide processing enzymes. Taking this into account, it is probable that only 50% of this renin had encountered the phosphotransferase, and thus the percentage of intracellular renin which is eventually phosphorylated would actually be closer to 10%.
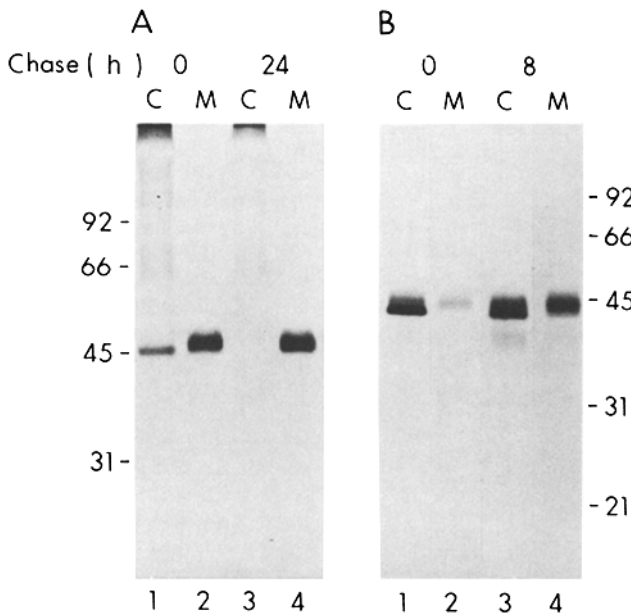
Figure 3. Immunoprecipitation of renin produced in L-2234 cells. Cells were labeled for 4 h with [³⁵S]methionine and chased for 0–24 h, as indicated. Renin present intracellularly (*C*) or in the medium (*M*) was immunoprecipitated with antirenin antiserum. *A* and *B* are from separate experiments.

Figure 4. Binding of ³⁵S-labeled renin from L-2234 cells to Man 6-P-receptor. Cell homogenate (lanes *1–4*) and medium (lanes *5–6*) from the experiment represented in Fig. 3 *B* were processed as described in Fig. 2. Note that only one-tenth of the run-through material was applied to the gel.

talbumin were microinjected into oocytes. Ovalbumin and α-lactalbumin are glycoproteins with no relationship to the aspartyl protease family and are known to be secreted from oocytes (5; E. Perara, P. Quasba, and V. Lingappa, personal communication). Binding of ovalbumin (data not shown) or α-lactalbumin (Fig. 2 *B*) to the Man-6-P receptor column was not detected (one can easily detect 0.5% binding). In addition, *Xenopus* oocytes phosphorylate only ∼2% of their total endogenous glycoproteins (data not shown). Thus, the phosphotransferase enzyme exhibits specificity towards endogenous as well as foreign mammalian glycoproteins.

Phosphorylation of Renin in L-2234 Cells

The efficient phosphorylation of renin seen in *Xenopus* oocytes would lead one to predict that, in mammalian cells, renin would be translocated to a lysosome. However, both Galen et al. (11) and Pinet et al. (27) have shown that renin is efficiently secreted from juxtaglomerular cells which have lost the ability to store renin in secretory granules. It still remained possible that renin was phosphorylated but not retained by the cell, as has been seen for the major excreted protein in transformed cells (31), or that its phosphorylation was inefficient. A mouse L cell line expressing renin (L-2234 cells), established by stable transfer of the renin gene, was used to examine this question. Mouse L cells are particularly suited to a study of phosphorylation as they do not rapidly degrade the Man-6-P marker (10).

L-2234 cells were labeled for 4 h with [³⁵S]methionine and chased in the presence of unlabeled methionine for 0–24 h. Renin present in the cells and medium was then assayed by immunoprecipitation with antirenin antibody. As shown in Fig. 3, renin synthesized by the L-2234 cells ap-
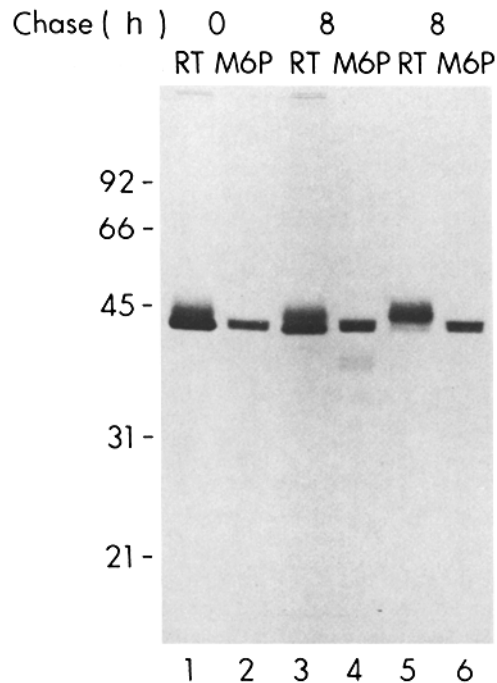
pears intracellularly as an M_r 44,000-D protein.[2] In the medium it is mostly a doublet of M_r 45,000- and 46,000-D proteins with minor species at M_r 44,000 and 47,000 D. All of these species were shown to be prorenin by immunoreactivity with the anti-propeptide antibody (data not shown), in agreement with the findings of Fritz et al. (9).

In the experiment shown in Fig. 3 *A*, 75% of the renin had been secreted at the end of the 4-h labeling period and no renin was detected intracellularly after 24 h of chase. In a similar experiment, shown in Fig. 3 *B*, the L-2234 cells secreted renin at a much slower rate, with only 7% and 40% of the renin synthesized during the pulse secreted at 0 and 8 h of chase, respectively. The more slowly migrating species, characteristic of those previously seen in the medium (Fig. 3 *A*), were now also apparent inside the cells. This slower rate of secretion appeared to correlate with a greater cell confluence. The level of renin expression also increased from 0.08% to 0.6% of newly synthesized proteins as determined by measuring the absolute amount of radioactivity incorporated into renin by solubilizing appropriate regions of the dried gels shown in Fig. 3 (data not shown). In this experiment there was also the intracellular appearance of lower apparent molecular mass forms of renin (M_r 41,000–39,000 D; Fig. 3 *B*, lane *3*).

The renin synthesized by the L-2234 cells, in the experiment represented in Fig. 3 *B* was applied to the Man-6-P

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2. The electrophoretic mobility varied between M_r 44,000 and 46,000 D, as shown in Fig. 3 *B* and 3 *A*, respectively. The mobilities for all renin species, as shown in Fig. 3 *B* and Fig. 4 and as stated in the text, were those most consistently observed.

receptor column. Fig. 4 shows that a significant portion of both the intracellular and secreted renin bound to the column (note that only one-tenth of the renin which ran through the column was applied to the gel). As in the case of the oocytes, the lower molecular mass processed forms of renin were detectable only in the receptor-bound fractions (Fig. 4, lane 4). The percentage of the intracellular renin which bound to the receptor increased from 2.0% to 5.6% over the chase period, and 5.1% of the secreted renin bound to the Man-6-P receptor column (Table I). When the intracellular material from Fig. 3 A (lane 1) was applied to the Man-6-P receptor column, 2.8% of the renin bound to the column. These levels of receptor binding are markedly lower than observed for renin synthesized in oocytes.

Since only ∼0.25% of [^{35}S]methionine-labeled protein in these cells bound to the Man-6-P receptor column, it seemed possible that the apparently inefficient phosphorylation of renin could be due to an overloading of the capacity of the phosphotransferase to phosphorylate the large amount of renin produced by the cell (up to 0.6% of total protein). If this were the case, one might expect there to be competition between renin and lysosomal enzymes for the phosphotransferase, resulting in overall lower levels of phosphorylation and increased secretion of lysosomal enzymes owing to a lack of the Man-6-P marker. To address this question, three experiments were performed. First, the intracellular level for two lysosomal enzymes, β-galactosidase and β-glucuronidase, was determined and found to be similar between the L-2234 cells and the parental L-25.336 line (data not shown), consistent with lysosomal enzyme retention being normal. Second, L-2234 cellular extracts were applied to the Man-6-P receptor column and the extent of receptor binding for the two lysosomal enzymes was determined. 83% of the activity for both enzymes bound to the receptor column (data not shown). For β-glucuronidase, this is even higher than reported by Gabel and Foster (10) for another L cell line in which 60% of intracellular β-glucuronidase was phosphorylated. Third, the targeting of cathepsin D in L-2234 cells was determined in a pulse-chase experiment. The data

revealed that >90% of the cathepsin D remained intracellular (data not shown). These results indicate that the phosphorylation and targeting of lysosomal enzymes are not disturbed by the presence of the large amounts of renin in these cells. In addition, although the level of renin expression was found to vary nearly 10-fold between cell labelings, the percentage of renin that was phosphorylated remained constant. The inefficient phosphorylation of renin is, therefore, a property intrinsic to the mammalian system.

Oligosaccharide Analysis on L-2234 Cell Renin

An analysis of the oligosaccharides of renin was undertaken to compare the phosphorylation of intracellular and secreted renin. L-2234 cells were labeled for 30 min with [2-^{3}H]mannose and chased for 4 h. Cellular extracts and medium samples were applied to the Man-6-P receptor column and renin was immunoprecipitated from the run through and Man-6-P eluates. The immunoprecipitates so obtained were judged >95% pure renin with respect to total radioactivity by comparison with nonimmune precipitates of equivalent fractions. After immunoprecipitation, the renin was digested with pronase, and the resulting glycopeptides were applied to a column of Con A-Sepharose which was eluted sequentially with 10 mM α-methylglucoside and 100 mM α-methylmannoside (Fig. 5). Under these conditions, complex-type asparagine-linked oligosaccharides either pass through the column or are eluted with α-methylglucoside, whereas high-mannose-type oligosaccharides require 100 mM α-methylmannoside for elution (4). 92% of the oligosaccharides of the intracellular receptor-bound renin were of the high-mannose type (Fig. 5 C) vs. 56% of this type in the nonreceptor-bound material (Fig. 5 A).[3] In the medium,

3. The counts per minute shown in Fig. 5 were converted to molar percent oligosaccharides by assuming that the high-mannose-type oligosaccharides contain on average eight mannose residues and the complex-type oligosaccharides contain three mannoses and one fucose residue which could be radioactively labeled.
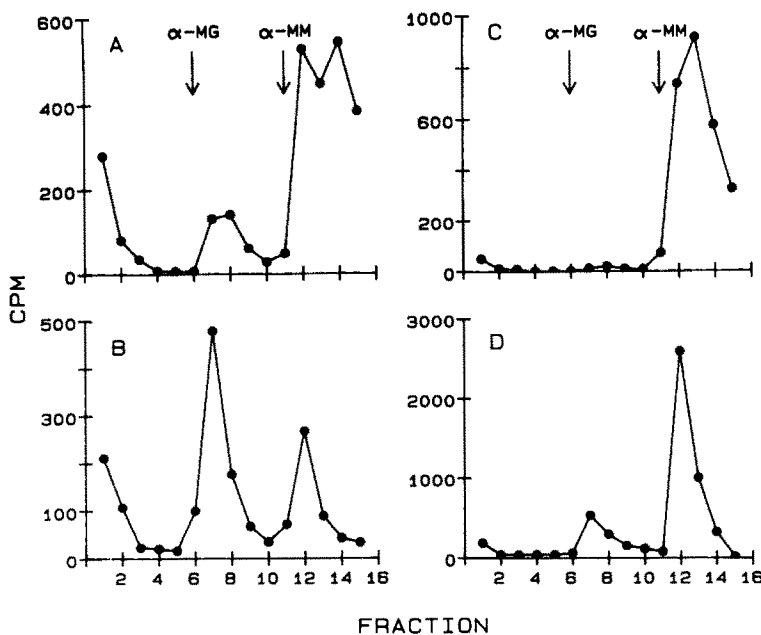

Figure 5. Con A-Sepharose chromatography of L-2234 cell renin glycopeptides. Cells were labeled with [2-^{3}H]mannose for 30 min and chased for 4 h. Cell (*A* and *C*) and medium (*B* and *D*) samples were applied to the Man-6-P receptor column and renin immunoprecipitated from the run-through (*A* and *B*) and Man-6-P eluates (*C* and *D*). Glycopeptides were generated by pronase digestion of the SDS-disaggregated immunoprecipitates and then applied to Con A-Sepharose columns. The columns were eluted with 10 mM α-methylglucoside (α-*MG*) and 100 mM α-methylmannoside (α-*MM*).

the receptor-bound renin contained 60% high-mannose-type oligosaccharides (Fig. 5 D) vs. only 17% in the non-receptor-bound fraction (Fig. 5 B). It is evident from these data that the renin that binds to the receptor is more enriched in high-mannose-type oligosaccharides relative to the renin that does not bind to the receptor. Previous studies have established that only high-mannose-type oligosaccharides are phosphorylated on lysosomal enzymes (25, 34). The higher percentage of this oligosaccharide type in receptor-bound fractions is consistent with the presence of phosphomannosyl residues whose presence is known to block conversion of high-mannose-type oligosaccharides to complex-type oligosaccharides in the Golgi complex (13). In addition, the renin in the secretions that bound to the Man-6-P receptor column contained more complex-type oligosaccharides than its cellular counterpart.

These Con A-Sepharose profiles provide an explanation for the migration pattern of renin in the SDS gel analysis shown in Fig. 4. Thus, the M_r 44,000-D renin would contain high-mannose-type oligosaccharides and the higher molecular mass forms result from varying degrees of oligosaccharide processing to complex-type oligosaccharides. Endo H, which cleaves only high-mannose-type oligosaccharides, caused an increased mobility for only the M_r 44,000-D form (data not shown), thus confirming this relationship. In addition, a partial endo H digest on intracellular renin produced two distinct species of M_r 42,500 and 41,000 D from the M_r 44,000-D high-mannose renin, indicating that both potential asparagine-linked glycosylation sites on renin (20) are utilized (data not shown).

The high-mannose-type glycopeptides from the samples represented in Fig. 5 were treated with endo H to release the oligosaccharides, which were then analyzed for the presence of phosphomannosyl residues by QAE-Sephadex chromatography (34). Under the conditions used, neutral oligosaccharides pass through the resin while oligosaccharides with one, two, three, or four net negative charges bind and are eluted with 20, 70, 100, and 140 mM NaCl, respectively. Of the intracellular renin, 88% of the radioactivity in the nonreceptor-bound fraction behaved as neutral oligosaccharides (Fig. 6 A) whereas 98% of the receptor-bound radioactivity interacted with the resin and eluted at positions characteristic for oligosaccharides with one phosphomonoester (70 mM NaCl) or two phosphomonoesters (140 mM NaCl) (Fig. 6 B). Treatment of this latter material with alkaline phosphatase converted the oligosaccharides to neutral species, confirming the identity of the anionic species as phosphomonoesters (data not shown). Insufficient material was available to identify the nature of the radioactivity eluting at 70 mM NaCl in the nonreceptor-bound renin (Fig. 6 A). This analysis demonstrates that fractionation of renin on the Man-6-P receptor column selectively isolates those molecules that contain phosphorylated oligosaccharides.

The analysis of the secreted renin which bound to the receptor revealed that 95% of the radioactive oligosaccharides bound to QAE-Sephadex (Fig. 6 C) but the elution pattern differed significantly from that seen for the equivalent cell-associated material (Fig. 6 B). These secreted renin oligosaccharides were then subjected to (a) alkaline phosphatase digestion or (b) mild acid hydrolysis (a procedure which will remove the N-acetylglucosamine from phosphodiester residues as well as sialic acid residues) or (c) mild acid hydrolysis followed by alkaline phosphatase digestion,
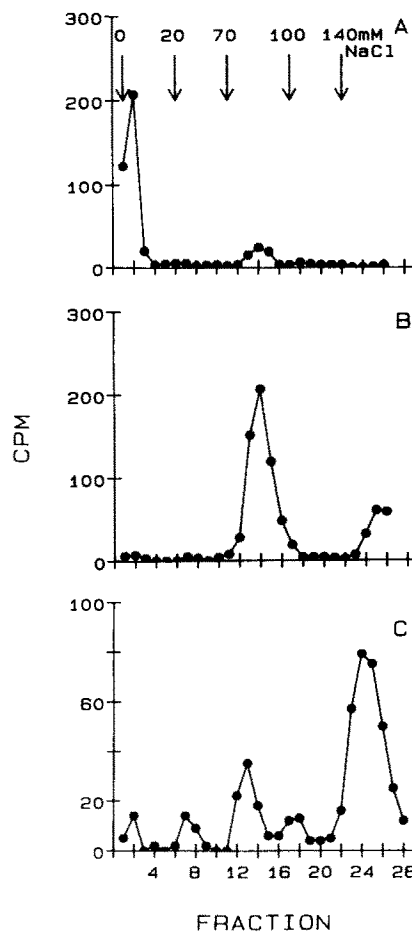

Figure 6. QAE-Sephadex analysis of L-2234 cell renin oligosaccharides. The α-methylmannoside eluted glycopeptides (Fig. 5) were digested with endo H and then applied to analytical QAE-Sephadex columns. (A and B) Cell-associated renin that ran through (A; Fig. 5 A) or bound (B; Fig. 5 C) to the Man-6-P receptor column. (C) Renin from the medium, receptor-bound fraction (Fig. 5 D). 20, 70, 100, and 140 mM NaCl refers to concentration of NaCl in elution buffer.

and then applied to QAE-Sephadex. This analysis allowed identification of the eluted peaks (Fig. 6 C) as oligosaccharides with one phosphodiester (20 mM NaCl), two phosphomonoesters (70 mM NaCl), a hybrid type (35), with one phosphomonoester and one sialic acid (100 mM NaCl), and two phosphomonoesters (140 mM NaCl) (data not shown).

Analysis of the nonrenin, receptor-bound high-mannose-type oligosaccharides on QAE-Sephadex showed a similar difference in the profiles between the intracellular and medium material (data not shown). Therefore, for both renin and the endogenous lysosomal enzymes which are able to bind to the receptor, most of the oligosaccharides on intracellular proteins were of the high-mannose type with one or two phosphomonoesters; for the secreted proteins, only ~60% of the oligosaccharides were of the high-mannose type and of these, oligosaccharides with two phosphomonoesters predominated while neutral, phosphodiesters, and sialylated hybrid species were more prevalent.

Discussion

The data presented in this paper demonstrate that renin, an aspartyl protease which is normally targeted to secretory

granules, acquires Man-6-P residues. However, the extent of renin phosphorylation differed significantly between the two cell types examined. In oocytes, most of the renin synthesized was retained within the cell and 53–90% of the renin molecules were phosphorylated. In the mammalian L-2234 line, greater than 90% of the renin was secreted and only 5–6% of the renin molecules contained the Man-6-P marker.

It is intriguing that the same protein, when expressed in two different cell types, showed strikingly different degrees of phosphorylation. One possible explanation is that there is a difference in the specificity of the phosphotransferase in the two organisms for the protein signal needed for phosphorylation. Given the close evolutionary relationship between renin and the lysosomal aspartyl protease cathepsin D, it is possible that renin contains only part of the protein recognition marker present on cathepsin D, resulting in weak recognition of renin by the mammalian phosphotransferase enzyme. The oocyte phosphotransferase enzyme, although it does not recognize the nonrelated mammalian glycoproteins ovalbumin and α-lactalbumin, phosphorylates renin and cathepsin D almost equally well, and thus does not appear to discriminate between their protein recognition markers. We consider it possible that the amphibian enzyme is less stringent in its requirements for recognition of this protein domain. Precedent for evolution in the ability of phosphotransferase to specifically recognize this domain exists as the phosphotransferase enzyme isolated from rat liver is 50–100-fold better at discriminating between lysosomal and non-lysosomal glycoprotein acceptors than the same enzyme isolated from the soil ameba *Acanthamoeba castellani* (25). Alternatively, the amphibian enzyme may be just like the mammalian enzyme in its specificity, but because the protein substrates move so slowly in the oocyte, the phosphotransferase may have more time to act on the renin substrate than in a mammalian cell and therefore may phosphorylate a higher percentage of it. Many proteins take several hours to pass through the ER and Golgi complex in oocytes whereas the transit time in a mammalian cell is usually less than 1 h (37).

The data presented here indicate that the renin which is phosphorylated has similarities in its processing and targeting to well-studied lysosomal enzymes. The cleavage of the renin propiece, which correlated with acquisition of the Man-6-P marker, is reminiscent of cleavage of lysosomal enzymes after delivery to lysosomal organelles in mammalian cells (12). Studies with cathepsin D expressed in oocytes directly demonstrated that phosphorylated material is translocated to lysosomes (8), suggesting that renin would also be targeted to lysosomes in oocytes. Lower molecular mass, phosphorylated forms of renin also appeared intracellularly in the L cells and these most likely represent renin which has been delivered to lysosomes. However, the renin which is targeted to lysosomes in the L cell appears to be degraded inasmuch as it is not detectable inside the cells after a long chase (Fig. 3 *A*). Although the renin is much more stable in oocytes, the lysosomal organelles in these cells have an unusual enzymatic composition with little proteolytic activity (36).

The oligosaccharide analysis of renin from L-2234 cells also demonstrates similarities between phosphorylated renin and lysosomal enzymes. The cell-associated phosphorylated renin had an oligosaccharide with one phosphomonoester as its predominant species. This is consistent with the findings of Gabel and Foster (10) that cell-associated oligosaccharides with two phosphomonoesters are slowly converted to species with one phosphomonoester. This latter species remains as a stable form. However, the predominant species present on renin and lysosomal enzymes in the medium was an oligosaccharide with two phosphomonoesters. It appears then that the secreted lysosomal enzymes do not encounter the processing phosphatase, and therefore the intracellular site of dephosphorylation must be located after the Golgi apparatus, where lysosomal enzymes are segregated from the secretory pathway (14).

The oligosaccharide analysis demonstrated a major difference between the phosphorylated renin which was secreted vs. that retained by the cell. The intracellular, phosphorylated renin contained essentially all high-mannose-type oligosaccharides with phosphomonoesters. In contrast, phosphorylated renin in the medium contained only 60% high-mannose-type oligosaccharides and of these, 10% were either neutral or contained phosphodiesters. These species do not mediate binding to the Man-6-P receptor (35). Therefore, while both of the oligosaccharides of the cell-associated renin contained phosphomonoesters, the renin in the medium had only one oligosaccharide of this type. A similar pattern of phosphorylation was also observed for lysosomal enzymes in general. In that both cell-associated and medium material bind to the Man-6-P receptor in vitro, the question arises as to why the molecules containing phosphomonoesters are secreted. One possible explanation is that, in vivo, the Man-6-P receptor is limiting. The resultant competition for receptor sites may select for molecules with two or more phosphomonoester-containing oligosaccharides that perhaps bind to the receptor with higher affinity; those with only a single phosphorylated oligosaccharide may bind with lower affinity and are thereby secreted.

The level of renin phosphorylation observed in the L cells, if indicative of that in a juxtaglomerular cell, could not account for quantitative targeting to a secretory granule. Thus the correct targeting of renin to this granule must depend on another, as yet unidentified signal. However, if phosphorylated renin is targeted to lysosomes, renin would then have a true dual localization within a secretory granule-containing cell. Another protein which has been shown to be located in both lysosomes and secretory granules is the thiol protease cathepsin B (6). This dual localization may be due, at least in part, to an incomplete targeting to lysosomes via the Man-6-P pathway. As indicated by this study, this could be effected by the presence of a "weak" protein recognition marker leading to poor binding to the phosphotransferase, and, consequently, inefficient phosphorylation.

The protein domain responsible for the specific recognition of lysosomal enzymes by the phosphotransferase is as yet unidentified. The finding that renin, a related but non-lysosomal glycoprotein, can also be phosphorylated suggests an even further intricacy on the nature of this recognition determinant and in the development of the Man-6-P recognition pathway. The aspartyl protease family (32), which also includes the nonglycoproteins pepsinogen, chymosin, and gastricin and the yeast glycoprotein, proteinase A, is a particularly well suited system for gaining an understanding of the recognition marker. By comparing their known protein

sequences and examining the expression of the phosphorylation marker in appropriate system, the targeting signal may be elucidated.

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