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THE PURIFICATION AND CHARACTERIZATION OF RECOMBINANT HUMAN RENIN EXPRESSED IN THE HUMAN KIDNEY CELL LINE 293

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The cDNA encoding human preprorenin has been introduced into the adenovirus-transformed human kidney cell line 293. The recombinant 293 cells expressed and secreted prorenin; trypsin was used to activate the secreted prorenin to renin in vitro. The recombinant protein was purified to homogeneity by a single affinity chromatographic step. Using synthetic tetradecapeptide, the K_m was 57.1 ± 9.3 uM and the k_{cat} was (7.48 ± 1.57) x 10^3/hr. Activation with trypsin resulted in a secondary cleavage between Arg53 and Leu54 generating a two chain form held together via a disulfide between Cys51 and Cys58. This secondary cleavage did not affect enzyme activity as determined by the ability of renin to degrade a synthetic tetradecapeptide substrate. Our paper demonstrates the potential for producing large quantities of renin from human kidney cells and also suggests that the use of trypsin, which has been widely used to convert prorenin to renin in vitro, causes a secondary cleavage in the renin peptide chain.

Renin is a highly specific aspartyl protease that catalyzes the release of angiotensin I from the plasma protein angiotensinogen. This conversion is the rate limiting step in the cascade by which angiotensin I is ultimately converted to angiotensin II, an octapeptide involved in vascular constriction and aldosterone secretion (1,2).

Renin is secreted as an inactive zymogen (prorenin), which is then converted to the functional protease in the blood stream. Human renin, a glycoprotein of molecular weight 42000 Da, has been purified from a wide variety of sources, including kidney (3,4), plasma (5), and placental chorion (6). Renin has also been purified from other sources such as hog kidney (7), mouse submaxillary gland (8), and rabbit uterus (9). The cleavage specificity for human renin is different from that obtained from other sources. Whereas human renin cleaves a leucyl-valine bond near the amino terminus of angiotensinogen (10), the scissile bond for renin of nonprimate origins is a leucyl-leucine (11).

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Human kidneys are known to secrete prorenin as well as renin, and there is evidence that prorenin is activated by a specific protease *in vivo* (12,13); the conversion of prorenin to renin occurs in the kidney and not in the plasma (14,15). This report describes the expression and purification of recombinant human renin from human kidney 293 cells. Surprisingly, the 293 cells do not activate prorenin to renin during the secretory process but instead secrete only prorenin. Treatment of prorenin with trypsin not only produced active renin but also led to a secondary cleavage site in the renin chain.

MATERIALS AND METHODS

Construction of Expression Vector--Plasmids for the expression of human renin were constructed using the cDNA coding sequence isolated by Faust *et al.* (16). The renin cDNA clone, inserted into the polylinker of pSP65 (Promega Biotech), was excised on a BamHI to HindIII restriction fragment and the 3' protruding ends were made blunt by treatment with the Klenow fragment of *E. coli* polymerase. The plasmid phRN-8 (Figure 1) was constructed by replacing the human protein C coding sequence in pLPC-hd (17) with the blunt ended renin cDNA sequence at the plasmid Bcl.1 site.

Cell Lines, DNA Transfection, and Drug Selection--The adenovirus-transformed human kidney cell line, 293 (American Type Culture Collection CRL 1573), was grown in Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum and was transfected with phRN-8 as described previously (18) by the calcium phosphate method (19). Clonal cell lines resistant to hygromycin B were isolated as described previously (17). These recombinant cell lines were expanded, and the presence of renin activity in the conditioned culture medium was determined by the HPLC analysis described below. RNA was isolated from transfected cells and cytodot analysis was performed as previously described (20) using a ^{32}P-labeled probe to the renin cDNA (HindIII to BamHI restriction fragment).

Renin Assay-- Renin activity was determined by measuring the simultaneous formation of angiotensin I and loss of tetradecapeptide substrate using isocratic reverse phase HPLC on a Dupont Zorbax ODS column (25 cm x 4.6 mm). A solvent system consisting of 0.1% trifluoroacetic acid in 32% CH_3CN was used at a flow rate of 1.5 ml/min. Assay mixtures consisted of 35 nmol renin tetradecapeptide substrate in 100 ul 0.1M sodium acetate pH 6.5. Aliquots (20 ul) of column fractions containing renin were incubated at 37°C with the renin substrate. At various times, 20 ul of the reaction mixture was removed, added to 100 ul HPLC running solvent, and 60 ul of this mixture was then injected onto the Dupont Zorbax ODS column, with effluent monitored at 220nm. Angiotensin I elutes at 4.4 min, while the tetradecapeptide substrate elutes at 6.8 min in the above solvent system.

Activation of Secreted Recombinant Human Prorenin--Serum free culture media from 293 cells (3.6 l) was treated with EDTA (final concentration 5 mM) and N-ethylmaleimide (final concentration 0.5 mM) with stirring at 4°C for one hour. The cell culture media was then concentrated to 270 ml by tangential flow ultrafiltration (Pharmacia/Filtron) using an Omega 8K membrane. TPCK-trypsin (0.75 mg/ml) was added to the media, and activation of the secreted prorenin to renin was allowed to proceed for one hour at 4°C with continuous stirring, at which time the trypsin was inactivated by addition of PMSF to a final concentration of 1mM. The solution containing activated renin was filtered through a Nalgene 0.2 u sterile filter apparatus.

Purification of Recombinant Human Renin--Purification of recombinant human renin using affinity chromatography was similar to the method of McIntyre *et al.* (21) except for the changes described below. The renin inhibitor His-Pro-Phe-His-LeuΨ[CH_2-NH]Val-Ile-His (H-113) (22) was used as the affinity matrix instead of H-77 due to its specificity for human renin, and was covalently coupled to Sepharose as described by McIntyre *et al.* (21). The

activated renin solution was loaded at 0.2 ml/min onto an affinity column (5 x 1.2 cm) previously equilibrated in 0.05 M Tris·HCl buffer, pH 7.4. Fractions (5 ml) were collected, and column effluent was monitored at 280 nm, 0.2 AUFS.

The column was washed successively with 75 ml 0.05 M Tris·HCl (pH 7.4), 75 ml of 0.05 M Tris·HCl (pH 7.4)/1 M NaCl, and then 75 ml of 0.1 M NaOAc (pH 6.0). In all cases, the flow rate was maintained at 0.2 ml/min and 5 ml fractions were collected.

Bound human renin was eluted from the H-113-Sepharose column by applying a decreasing pH gradient of 0.1 M NaOAc from pH 6.0 to 4.3 (65 ml of each buffer), followed by a wash with 100 ml of the pH 4.3 buffer. Fractions containing renin activity (as determined by HPLC) were pooled, adjusted to pH 6.5 with Tris base, and the pool was then concentrated to a volume of 8 ml using an Amicon stirred ultrafiltration cell and a YM10 membrane.

Kinetic Data--The enzyme turnover number (k_{cat}) and Michaelis constant for tetradecapeptide substrate (K_m) were measured in 0.1 M sodium acetate buffer pH 6.0 at 37°C. Assays were performed with 25-125 uM tetradecapeptide substrate in 0.05 ml buffer. Renin (0.02 ug) was added to the reaction mixture, and the reaction was allowed to proceed until 30-60% of the substrate was used, at which time a 20 ul aliqout was withdrawn, added to 40 ul HPLC running solvent (above), and 50 ul of this mixture was then analyzed by HPLC as described above. Controls showed that the levels of angiotensin II produced in the reaction did not inhibit the enzyme.

RESULTS AND DISCUSSION

Although human renin can be purified from human kidney (3,4), the level of enzyme isolated is very low. As an alternative, we have utilized recombinant DNA technology to express human renin in quantities sufficient to carry out detailed protein characterization. Recombinant human renin has previously been expressed in transfected Chinese hamster ovary (CHO) cells (23,24). In this paper, the expression and purification of human renin from the human kidney cell line 293 is described.

Shown in Figure 1 is phRN-8, a vector for the expression of the cDNA for recombinant human renin. The expression vector, utilizing the E1A-responsive BKVP2 enhancer (25), was introduced into the E1A-producing adenovirus-transformed human kidney line 293 by calcium phosphate-mediated transfection, stable recombinant cell lines resistant to hygromycin were isolated, and several individual clones were analyzed for expression from the renin cDNA by cytodot hybridization analysis. For comparative purposes, the nontransfected

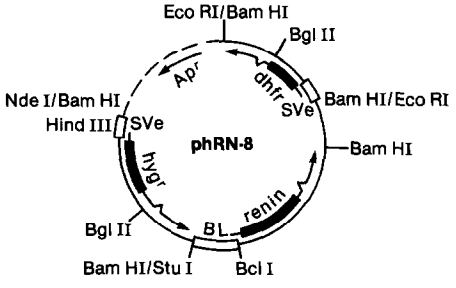

Figure 1. Vector for the expression of the cDNA for human renin. Regulatory regions are indicated by open boxes, protein coding sequences by closed boxes, and mRNA transcripts by arrows.

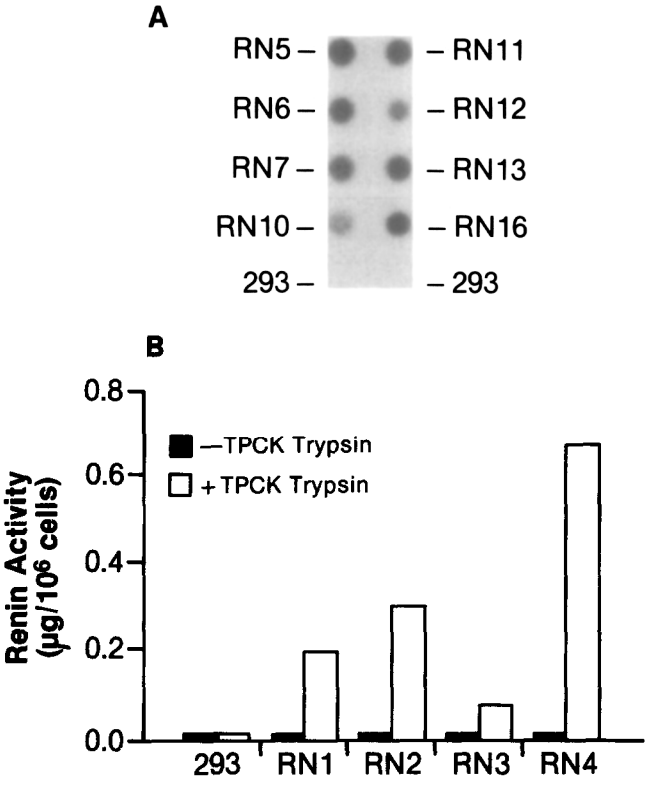

Figure 2. Expression and identification of mRNA for recombinant human renin (A) and secretion of prorenin (B) in human kidney cells. (A) Analysis of total cellular RNA from transfected (RN) and nontransfected 293 cells for renin specific mRNA by a dotblot procedure using a renin-specific ^{32}P-labelled probe. (B) Analysis of conditioned culture media from 293 cells and four stable hyg^r transformants (RN 1-4) for the presence of renin activity before and after treatment with TPCK-trypsin.

parental 293 line also was analyzed. As shown in Figure 2A, specific renin mRNA could be detected in the hygromycin resistant 293 cell transformants but not in the parental cell 293 cell line. Thus, the human kidney cell line does not express the endogenous renin gene at a level detectable by this assay.

To determine if the recombinant 293 cell lines were secreting renin activity, the conditioned culture medium from nine Hyg^r clones were incubated with an angiotensin-containing synthetic tetradecapeptide and the renin-dependent release of angiotensin I was determined by HPLC analysis. In addition, the presence of renin activity in the conditioned culture medium before and after treatment with TPCK-trypsin was determined. As shown in

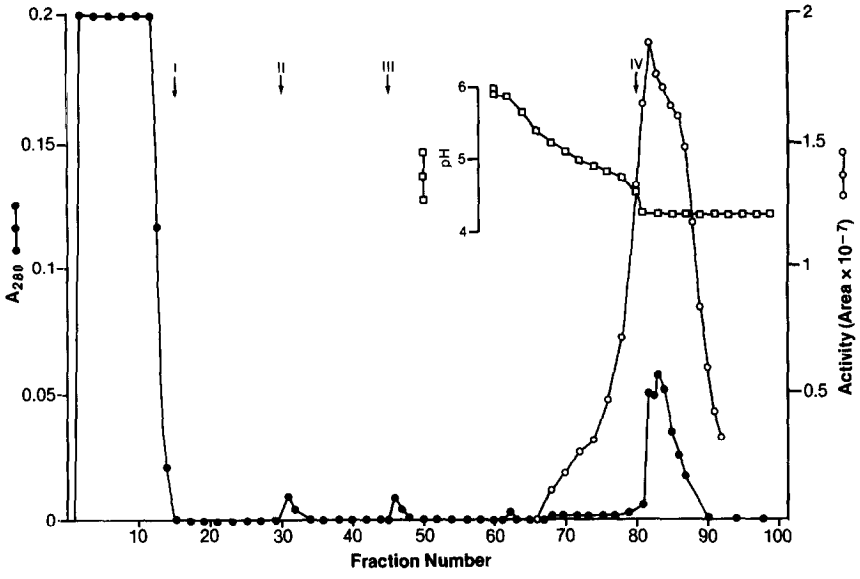

Figure 3. Elution profile of recombinant human renin from an H-113-Sepharose affinity column. Activated renin was applied to the affinity column and washed sequentially with 0.1 M Tris HCl (pH 7.4) (arrow I), 0.1 M Tris HCl (pH 7.4)/1 M NaCl (arrow II), and 0.1 M sodium acetate (pH 6.0)/1 M NaCl (arrow III). Renin was eluted with a pH gradient from pH 6.4 to pH 4.3, followed by an isocratic wash with 0.1 M sodium acetate pH 4.3 (arrow IV). Effluent was monitored at 280 nm for eluted protein (●—●—●). Activity (○—○—○) was determined using HPLC by measuring the area 8under the peak corresponding to angiotensin I as indicated under "Materials and Methods".

Figure 2B, no renin activity could be detected in medium from the parental and recombinant 293 cell lines. However, activity could be detected in the medium from the recombinant cell lines following treatment with trypsin. These data suggested that the recombinant 293 cell lines stably transfected with phRN-8 secreted prorenin and not activated renin. This was confirmed by the biochemical analyses described below. The level of prorenin secreted from the nine hygromycin resistant transformants ranged from 0.2 to 2.0 ug/10^6 cells.

A single-step affinity chromatography method was utilized to purify the active renin from the cell culture medium. The application of a decreasing pH gradient on the affinity column allowed for rapid purification of renin to apparent homogeneity (Figure 3), resulting in an increase in specific activity from 9.68 units/mg in the concentrated filtrate to 1495 units/mg for the purified renin; 1.32 mg renin was obtained, corresponding to a yield of 76% (Table I). The protein, stored at pH 6.5 at 4°C, was stable for several months. The final specific activity of 1495 units/mg is comparable to that observed for CHO-derived recombinant human renin using a similar HPLC assay (23). One unit of renin activity is defined as the amount of renin needed to produce 100 nmol angiotensin I/hour at pH 6.5 and 37°C as measured by HPLC (12).

Table I. Purification of Recombinant Human Renin

	units	mg	U/mg	fold	yield
Activated Filtrate (conc)	2613	269	10	1	100
Final Renin	1973	1.32	1495	154	76

Prior to the affinity chromatography step, the secreted prorenin was activated to renin with trypsin. SDS-polyacrylamide gel electrophoresis (Figure 4) revealed that *in vitro* activation of prorenin to renin using trypsin resulted in an internal cleavage of the renin chain. Under nonreducing conditions, the purified renin migrated as a single band corresponding to a molecular weight of about 41,000 Da which is consistent with the size of human kidney-derived renin (3) (Fig. 4). However, two bands were observed under reducing conditions that corresponded to molecular weights of about 42,000 Da and 32,500 Da, suggesting that the renin chain had been nicked by the *in vitro* trypsin activation.

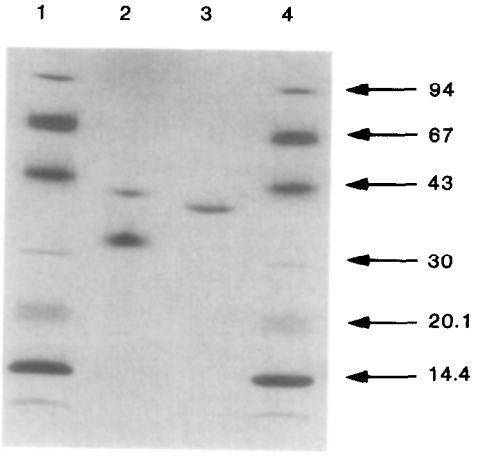

Figure 4. SDS/polyacrylamide gel electrophoresis of purified recombinant human renin. Lanes 1 and 4, standard M_r marker proteins (Pharmacia LMW Calibration Kit): 80 ng each of phosphorylase b (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), trypsin inhibitor (20.1 kDa), and alpha-lactalbumin (14.4 kDa); lane 2, 130 ng renin in the presence of 0.5% (wt:vol) dithiothreitol; lane 3, 130 ng renin.

Table II. Aminoterminal Sequence of Recombinant Human Renin
Sequencing was performed on 200 pmol protein

	Primary Sequence[a]		Secondary Sequence[a]	
Cycle	Amino Acid	Yield(pmol)	Amino Acid	Yield(pmol)
1	Leu	360.9	Leu	b
2	Thr	42.4	Tyr	112.6
3	Leu	198.1	Thr	29.1
4	Gly	72.0	Ala	69.9
5	(Asn)	c	(Cys)	c
6	Thr	35.8	Val	129.8
7	Thr	72.6	Tyr	141.1
8	Ser	16.7	His	42.5
9	Ser	17.7	Lys	148.4
10	Val	109.2	Leu	176.3
11	Ile	67.4	Phe	132.2
12	Leu	122.0	Asp	133.2
13	Thr	30.3	Ala	54.8
14	Asn	28.2	Ser	16.5
15	Tyr	54.2	Asp	43.4
16	Met	71.0	Ser	29.9
17	Asp	42.5	Ser	15.1
18	Thr	14.5	Ser	8.3

Theoretical initial yield = 187.8 pmol (93.9%)
Average repetitive yield = 91.9%[d]

[a]The primary sequence corresponds to residues 1-18 of human renin and the secondary sequence corresponds to residues 54-71 of human renin.

[b]The yield for Leu in cycle 1 accounts for both peptides.

[c]Asn was expected in cycle 5 for the primary peptide while Cys was expected in cycle 5 for the secondary peptide. A blank cycle suggests that Asn5 is glycosylated and that Cys58 is involved in a disulfide linkage.

[d]Average repetitive yield was determined as the mean of the yields for Tyr (cycles 2,7,15), Leu (cycles 3,12), Ala (cycles 4,13) and Val (cycles 6,10).

Aminoterminal sequence analysis showed the presence of two sequences beginning at Asp1 and Leu54, respectively, with both peptides in approximately equimolar amounts (Table II). These data confirm that the purified renin is nicked, with the cleavage site located between Arg53 and Leu54, and that the two chains are held together by a disulfide bond between Cys51 and Cys58. Asparagine5 was not seen in the sequencing, which is consistent with glycosylation at this position (23,26). Two chain renin was fully active (as measured by cleavage of the tetradecapeptide) and was inhibited by pepstatin in a manner analogous to single-chain human renin. A three-dimensional structural model of human renin, based upon the X-ray structure of pepsin, suggests that the loop formed as a result of the disulfide bond between Cys51 and Cys58 is located on the outer surface of the protein and is removed from the substrate binding site. Therefore, cleavage in this region might not be expected to affect enzyme activity. Aside from removal of the propeptide, single-chain human renin does not require further proteolytic cleavage for enzyme activation.

Kinetic analysis determined that the K_m for recombinant human renin for synthetic tetradecapeptide substrate was 57.1 + 9.3 uM while k_{cat} = (7.48 ± 1.57) x 10^3/hr in 0.1 M sodium acetate buffer, pH 6.0. These findings are consistant with a Km of 11.7 uM determined by radioimmunoassay for human renin against tetradecapeptide previously reported (27).

Renin is an attractive target to combat hypertension because it catalyzes the rate-limiting step in the angiotensinogen degradation cascade and because it is a highly specific enzyme. Thus, the development of a specific renin inhibitor could be therapeutically useful to control hypertension. The ability to express human renin at mg/l levels in a human kidney cell will allow for the rapid production of large quantities of purified human renin for enzymatic characterization, crystallographic studies, and the development of therapeutically useful inhibitors.

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