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Stable Expression, Secretion, and Characterization of Active Human Renin in Mammalian Cells

JON A. NORMAN, OPHELIA HADJILAMBRIS, ROSEANN BASKA, DARU Y. SHARP, and RAMESH KUMAR

Departments of Cardiovascular Biochemistry (J.A.N., O.H., R.B.) and Molecular Biology (D.Y.S., R.K.), Bristol-Myers Squibb Pharmaceutical Research Institute, Princeton, New Jersey 08543-4000

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SUMMARY

Human renin is synthesized as a 406-amino acid preprorenin protein that is processed by a signal peptidase during secretion, to release prorenin as a 386-amino acid zymogen. The 46-amino acid "pro" domain is removed by a renin-processing enzyme, to produce enzymatically active renin, by cleavage at an Arg-Leu bond. The effects of the renin-processing enzyme can be mimicked by trypsin activation, where high concentrations of trypsin are incubated with prorenin for brief periods of time, followed by excess trypsin inhibitor to minimize secondary proteolytic processing by trypsin. In order to study the role of the pro segment in the secretion, folding, and activity of human renin, we engineered a construct where the pro domain from the preprorenin cDNA was deleted. This construct was introduced into mammalian cells and its expression was assayed in transient and stable systems. In COS-1 cells transfected with the prerenin expression vector pREN3, active renin was secreted with a specific activity of 1360 μg of angiotensin I/min/mg, compared with trypsin-activated prorenin, which has a specific activity of 818 μg of angiotensin I/min/mg. The active renin secreted in this system had a significantly reduced potency for the renin inhibitor SQ 32,970. These results demonstrate that the pro segment is dispensable for the folding and secretion of renin. A permanent cell line expressing the active form of renin was obtained by cotransfection of NRP cells with pREN3 and pHyg. A colony designated B/1 was identified, subcloned, and shown to secrete active renin (110 pg of renin/10^6 cells) optimally when maintained in both G418 and hygromycin.

Renin, an aspartic protease, is an important first enzyme in the renin-angiotensin cascade controlling blood pressure and electrolyte balance. It is uniquely specific for its only known substrate, angiotensinogen, and releases the amino-terminal decapeptide AI from this circulating glycoprotein (1). Renin is synthesized primarily by the juxtaglomerular cells of the kidney and is secreted mainly in an inactive form or zymogen (2). It has long been assumed that inactive renin is the prorenin predicted to exist from the report of Imai *et al.* (3), who first cloned and sequenced the cDNA encoding preprorenin. Recently, inactive renin has been purified and partially sequenced by Higashimori *et al.* (4). They confirmed that inactive renin is prorenin, as predicted by Imai *et al.* (3).

The gene encoding human renin spans about 12 kb and consists of 10 exons and 9 introns (5, 6). The renin mRNA is approximately 1.5 kb long, and the predicted preprorenin translation product is a 406-amino acid polypeptide (3). This protein contains a 20-amino acid signal sequence or "pre" region that is required for the translocation of the protein into the lumen of the rough endoplasmic reticulum for assignment to the secretion pathway. The signal sequence is followed by a 46-amino acid "pro" domain that prevents renin from being catalytically active until it is removed by a renin-processing enzyme. The active form of renin is 340 amino acids in length. The renin-processing enzyme may be a membrane-associated thiol protease (7) that removes the pro region at the time of secretion, and its activity can be mimicked by trypsin treatment *in vitro* (8). It is not yet clear whether the pro domain is required for the correct folding or secretion of renin. In the case of subtilisin E, a bacterial serine protease, the pro domain of preprosubtilisin has been shown to be necessary for the correct folding and subsequent activity of subtilisin (9).

In order to produce large amounts of active renin without trypsin treatment and to determine the role of the pro segment in secretion and folding, a modification of the renin cDNA was made. The coding sequences of the signal peptide and the active renin were fused, thus deleting the pro domain of preprorenin. This modified construct was inserted into an expression vector and used for transient transfection of COS-1 cells and for the establishment of a stable cell line expressing renin. Active renin secreted from these transfected cells is biochemically identical to trypsin-activated prorenin but has a higher estimated specific activity and a reduced affinity for the renin inhibitor SQ 32,970. These results indicate that the pro domain of preprorenin is not necessary for the secretion or folding of renin.

ABBREVIATIONS: AI, angiotensin I; FBS, fetal bovine serum; bp, base pair(s); kb, kilobase(s); DMEM, Dulbecco's modified Eagle medium.

Experimental Procedures

Materials. Restriction enzymes were purchased from either BRL (Gaithersburg, MD) or New England Biolabs (Beverly, MA). Trypsin, soybean trypsin inhibitor, and hygromycin B were obtained from Sigma Chemical Co. (St. Louis, MO). Lipofectin reagent was obtained from BRL (Gaithersburg, MD). Geneticin (G418 sulfate) and DMEM were purchased from GIBCO (Grand Island, NY). Preprorenin cDNA inserted into plasmid pIBI76 was generously provided by Dr. John Chirgwin (University of Texas, San Antonio).

Expression plasmid construction. All plasmids were constructed using recombinant DNA techniques and guidelines described by Sambrook *et al.* (10). The cDNA encoding all but the first 10 nucleotides of the 5′ untranslated region was removed from pIBI76 by cutting with *Xba*I and *Bam*HI restriction enzymes and was inserted into the same sites in the expression vector pREX2. This expression plasmid, pREN2, contains the preprorenin coding sequences under the transcriptional control of an avian retroviral long terminal repeat. The pREN2 plasmid also contains an SV40 origin of replication, making it suitable for COS cell expression. To construct pREN3 encoding prerenin, four different DNA fragments were ligated (Fig. 1). These fragments were derived as follows: sequences corresponding to the 5′ untranslated region, including the restriction enzyme sites *Sal*I, *Not*I, and *Xba*I, all of the 20 amino acids of the pre region, and the first two amino acids, Thr-Met, of renin were synthesized by polymerase chain reaction, using two synthetic deoxyoligonucleotide primers of 47 (3′) and 49 (5′) nucleotides, according to the method of Saiki *et al.* (11). The amplification of this segment of pREN2 resulted in a 116-bp fragment that was digested with *Sal*I and *Nco*I restriction enzymes to produce a 104-bp fragment. A segment of the renin cDNA corresponding to amino acids 3–20 and flanked by *Nco*I and *Sca*I restriction sites was produced by annealing two oligonucleotides of 53 and 49 nucleotides. The DNA sequence encoding amino acid 21 through the termination codon (TGA) of the 3′ untranslated region was obtained by cleavage of pREN2 with *Sca*I and *Bam*HI. The expression vector fragment was obtained by cleavage of pREX2 with *Sal*I and *Bam*HI. These four components were purified by gel electrophoresis, and a four-fragment ligation reaction was set up to assemble the complete prerenin expression plasmid.

Cell culture. COS-1 cells were maintained in DMEM supplemented with 10% FBS. NRP cells (mouse fibroblasts transformed by polyoma virus) were also maintained in DMEM supplemented with 10% FBS.

Transfection protocols. COS-1 cells were plated out in 25-cm^2 flasks, at a density of 10^6 cells/flask, and grown to 80% confluency (3×10^6 cells) before transfection was carried out with 20 μg of DNA using lipofectin (BRL, Gaithersburg, MD), to mediate the uptake of DNA, according to the procedure of Felgner *et al.* (12). After the overnight transfection step, the cells were maintained in DMEM plus 10% FBS, and the medium was sampled for renin secretion assay every 24 hr for the next 10 days. A permanent cell line secreting active renin was obtained by co-transfection of NRP cells with pREN3 and pHyg (vector containing the selectable marker hygromycin phosphotransferase), using electroporation. After 24 hr, the transfected cells were selected in medium containing 150 μg/ml hygromycin B. Resistant clones were picked after 14 days, grown to confluency in 60-mm Petri dishes, and assayed for renin production. The positive clone B/1 was subsequently subcloned and assayed again for renin production.

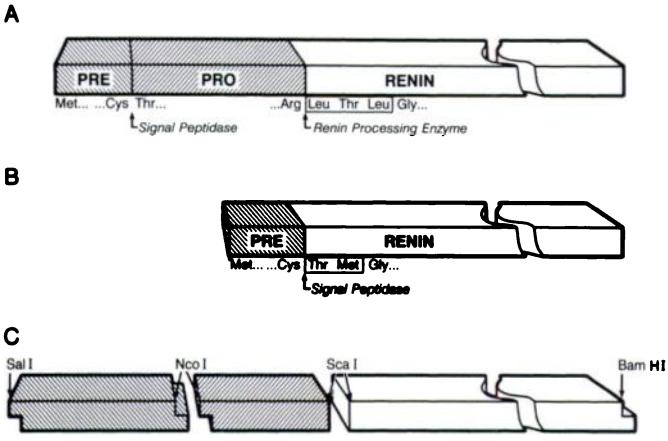

Fig. 1. Schematic representation of the targeted deletion of the pro domain from preprorenin. A, Sites of proteolytic processing by signal peptidase and the renin-processing enzyme in preprorenin; B, amino acid changes made in the amino terminus of renin in the prerenin protein; C, three components of the prerenin cDNA construct described in Experimental Procedures.

Southern blot analysis. High molecular weight genomic DNA was prepared from cells by protease digestion and organic extractions, followed by ethanol precipitation. DNA was quantitated, and 10 μg of each sample were subjected to digestion with *Eco*RI. The digests were electrophoretically resolved in a 0.7% agarose gel, transferred to nitrocellulose, and hybridized to a renin-specific probe. The probe was prepared by digesting pREN3 with *Sca*I and *Bam*HI, followed by labeling with [^{32}P]dCTP by random priming, using a Pharmacia oligolabelling kit. Hybridization was carried out at 42° in a buffer containing 40% formamide, and the blot was washed at high stringency in aqueous buffers. The autoradiogram was scanned on an LKB densitometer. The density values, expressed as area under the peak, for the three bands representing the endogenous gene were normalized. The two bands representing the transfected DNA were averaged and compared in each of the four subclones tested.

Renin assay. Prorenin was activated with trypsin, at a final concentration of 0.1 mg/ml, for 15 min at room temperature, according to a modification of the procedure of Atlas *et al.* (8). This activation step was stopped by the addition of excess soybean trypsin inhibitor at a final concentration of 2 mg/ml.

Quantities of active renin were measured by a sandwich immunoassay procedure that utilized two monoclonal antibodies directed toward different epitopes on renin (13). In this procedure, culture medium from transfected cells was diluted as necessary and incubated with the primary antibodies (which were covalently linked to magnetic beads) in a total volume of 250 μl, for 60 min at room temperature, in 0.2 M imidazole buffer, pH 7.4. Imidazole wash buffer was added and magnetic bars were inserted before the supernatant was decanted. The ^{125}I-labeled second antibodies were added in 50 mM Tris, pH 7.4, containing 10% horse serum, for 3 hr at room temperature before washing and decanting as described above. The pellets were counted in a γ counter and the concentration of renin was derived from a standard curve. The assay components described above were available as a kit from Diagnostics Pasteur (Paris, France).

Renin activity was measured by the production of AI, using human angiotensinogen as a substrate. Twenty-five microliters of test sample were incubated with substrate in TES buffer (containing phenylmethylsulfonyl fluoride) at 37°, in a total volume of 250 μl, for 30 min. Concentrations of AI were measured using a radioimmunoassay kit produced by Biotecx Laboratories (Friendswood, TX).

Results

Construction of prerenin expression plasmids. The strategy for the construction of prerenin is shown in Fig. 1. The Cys-Thr scissile bond for signal peptidase was retained in the new construct at the junction between the signal sequence and renin, because little is known about the subsite requirements for signal peptidase. Methionine was introduced at the second position as a conservative substitution for leucine. This allowed a novel *Nco*I restriction enzyme recognition site to be introduced into the construct, to facilitate future modifications. This construct would encode an active form of renin with a Thr-Met substituted for Leu-Thr-Leu at the amino terminus. This recombinant form of active renin would be one amino

acid shorter than native renin, but these modifications were predicted to have no effect on renin activity. The amino terminus is considered to be distal to the active site of renin and is not believed to play any role in the catalytic mechanism of this aspartic protease (14). The approach taken to engineer the deletion of the nucleic acid sequence encoding the pro domain of preprorenin is shown in Fig. 1C. The *SalI* to *ScaI* region was made in two parts using polymerase chain reaction and direct chemical synthesis of the oligonucleotides. After the four-component ligation reaction, *Escherichia coli* transformants were selected that had a 1200-bp *SalI-BamHI* insert. A plasmid containing the correct insert was designated pREN3, verified by partial sequencing, and used in the subsequent transfection experiments.

Transient expression of active renin. COS-1 cells grown to 80% confluency (3×10^6 cells) were transfected with either pREN2 (encoding preprorenin) or pREN3 (encoding prerenin), using the lipofectin procedure. In several experiments, lipofectin transfection consistently gave higher levels of renin expression than the $Ca(PO_4)_2$ or DEAE/dextran methods (data not shown). Fig. 2 shows the levels of renin secreted into the medium over a 12-day period after transfection. The levels of prorenin were consistently 3–4 times higher than those of active renin over this extended period. The higher level of prorenin secretion by COS-1 cells was not due to a higher level of transfection efficiency by pREN2 versus pREN3. In a separate experiment, the transfection efficiency was shown to be the same for both plasmids (data not shown). The transient expression and secretion of both prorenin and active renin for more than 12 days suggest that the renin mRNA may have a very long half-life. Additionally, no detectable renin was evident in nontransfected cells or mock-transfected cells (Table 1) and the accumulation of prorenin or active renin was reduced by 50% in transfected cells grown in the absence of FBS. Therefore, the presence of FBS in the culture medium appears to confer stability to the secreted renin. In subsequent experiments, COS-1 cells were washed and lysed, to determine whether renin was accumulating within the cells rather than being secreted. In these experiments, approximately 30% of either prorenin or active renin was found in the cells, and the remaining prorenin or renin was secreted into the medium (data not shown). Additionally, Western blot analysis with

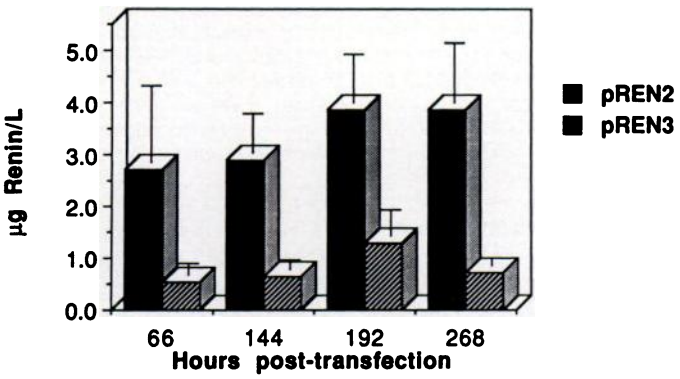

Fig. 2. Transient expression of renin in COS-1 cells by transfection with pREN2 and pREN3. COS-1 cells were grown to 80% confluency in 25-cm^2 flasks before transfection with 20 μg of pREN2 or pREN3, as described in Experimental Procedures. Culture medium was changed at the indicated days, and renin content and activity were measured. Each point represents the mean ± standard error of three experiments.

TABLE 1

Expression of recombinant human prerenin and preprorenin in COS-1 cells

Culture medium was assayed 114 hr after transfection with the indicated expression plasmid. Trypsin treatment was performed as indicated, with 0.1 mg/ml trypsin for 15 min at 20°, followed by the addition of 2 mg/ml soybean trypsin inhibitor.

Expression plasmid	Trypsin treatment	Active renin concentration	Renin activity	Specific activity
		ng/ml	*ng of AI/min/ml*	*μg of AI/min/mg*
None ($n = 3$)[a]	−	0	0	ND[b]
	+	0	0	ND
pREN2 (preprorenin)	−	0.07	0.13	2810 ± 770
($n = 5$)	+	2.9	2.38	818 ± 67
pREN3 (prerenin)	−	0.65	0.84	1360 ± 140
($n = 4$)	+	0.77	0.67	870 ± 60

[a] *n*, number of experiments.
[b] ND, not determined.

antisera raised to one of the epitopes on renin revealed that most of the renin synthesized by the transfected cells was present in the culture medium, indicating that most of the renin or prorenin is secreted and does not accumulate intracellularly in an unfolded state (data not shown). These results suggest that the signal peptide from both prerenin and preprorenin was removed with equal efficiency. Prorenin consistently appeared to be synthesized and secreted more readily than active renin. Renin activity in the culture medium was measured at several time points after transfection, including the 114-hr time point shown in Table 1. Because the active renin concentration and the activity of renin were determined, it was possible to estimate the specific activities of each form of secreted recombinant renin. The prorenin secreted from the COS-1 cells transfected with pREN2 had an artificially high specific activity, due to the stringency of the sandwich assay for active renin (11). The second monoclonal antibody in the assay was directed toward an epitope at the active site of renin. Consequently, this assay did not recognize prorenin. Because there was a 42-fold increase in the concentration of active renin after trypsin treatment and only an 18-fold increase in enzyme activity after trypsin treatment, the sandwich assay was more stringent than the enzyme assay for detecting active renin. This is probably due to the pro domain existing in a state of equilibrium with the active site that permits limited catalysis but restricts the binding of the active site-directed antibody in the sandwich assay. After trypsin treatment, the secreted prorenin had a specific activity of 818 ± 67 μg of AI/min/mg. This value is in excellent agreement with the specific activity of trypsin-treated native human renin, which was 900 μg of AI/min/mg when assayed under identical reaction conditions (pH 7.0, 37°, 0.5 μM human angiotensinogen). The active renin secreted by the COS-1 cells transfected with pREN3 had a specific activity of 1360 ± 140 μg of AI/min/mg, which is significantly higher than trypsin-treated prorenin ($p < 0.0085$; Student *t* test). This result indicates that the trypsin treatment probably produces secondary proteolysis of renin, such that the specific activity is lower than expected for endogenous renin that has been processed under physiological conditions. It is interesting that trypsin treatment of active renin produces a specific activity that is indistinguishable from that obtained after trypsin treatment of prorenin. This result confirms that trypsin treatment has some secondary effect on renin that results in a lower specific activity.

Stable cell line secreting active renin. A cell line secret-

ing active renin was established by co-transfecting NRP cells with pREN3 and pHyg DNA, using electroporation. Electroporated cells were plated out at varying dilutions and grown in the presence of 150 μg/ml hygromycin B. After 14 days, several colonies were identified and isolated for subsequent renin assay. Clone B1 was found to secrete active renin at the level of 757 pg/ml (Table 2). Cells from this colony were maintained for several weeks before subcloning by limiting dilution. Surviving subclones were identified and isolated for renin secretion as shown in Table 2. Subclones B1–10 and B1–11 had the highest levels of renin secretion, with values of 745 and 736 pg/ml, respectively. The various subclones had different levels of renin secretion, suggesting that multiple copies of the prerenin cDNA inserted into the genome of the NRP cells were rearranging. To address this question, Southern blot analysis was carried out on selected subclones that expressed varying amounts of active renin. Genomic DNA was isolated from untransfected NRP cells, colony B1 cells, and subclones B1.2, B1.9, B1.10, and B1.11. Ten micrograms of *Eco*RI-digested DNA from each of these cell lines were run on an agarose gel and blotted onto nitrocellulose for subsequent hybridization with ^{32}P-labeled prerenin cDNA. The resulting blot is shown in Fig. 3. The 2.2-kb and 0.7-kb bands were present in the transfected cell lines but not in the untransfected NRP cells. These two bands were expected from the *Eco*RI restriction sites present in the expression plasmid. The intensity of these bands correlated with the levels of renin expression in these subclones (Table 2). Comparative evaluation of the densitometric scan of this blot showed relative values of 1, 1.5, 2.7, and 5.2 for the transfected bands in subclones B1.2, B1.9, B1.10, and B1.11, respectively. This indicated that multiple copies of the prerenin cDNA inserted into the genome rearranged by either amplification or deletion of the renin-coding sequences during propagation in culture. The three bands at the top of the gel that were seen in every lane containing the digested genomic DNAs were due to the cross-hybridization of the human probe to the murine genomic sequences encoding endogenous renin. The intensity of these bands in different lanes was comparable, which is consistent with the equivalent amount of DNA applied to each lane in the gel.

Fig. 4 illustrates the optimal growth conditions for subclone B1.10, when grown for more than 2 weeks in different selection

TABLE 2

Selection of stable cell lines secreting active renin

Clone B-1 was isolated from NRP1 cells co-transfected with pREN3 and pHyg, followed by selection in medium containing 150 μg/ml hygromycin B. The culture medium of B-1 and the 12 subclones of B-1 were assayed for active renin concentration, as described in Experimental Procedures.

Clone/subclone	Active renin concentration
	pg of renin/ml
B1	757
B1-1	40
B1-2	118
B1-3	43
B1-4	66
B1-5	28
B1-6	67
B1-7	0
B1-8	5
B1-9	181
B1-10	745
B1-11	736
B1-12	1

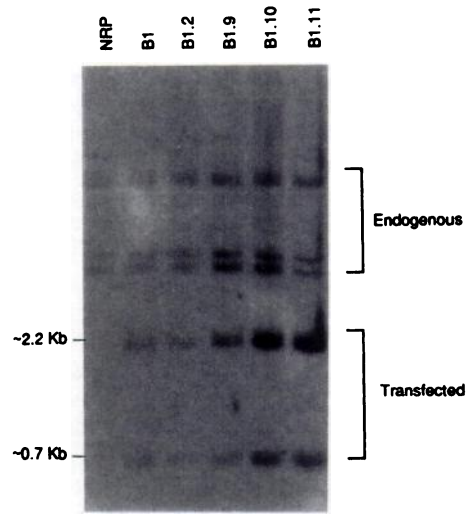

Fig. 3. Southern blot analysis of NRP cells stably transfected with pREN2. Genomic DNA was extracted from nontransfected NRP cells (*NRP*) and the indicated clone or subclone described in Table 2. DNA was digested with *Eco*RI and separated on a 0.8% agarose gel before transfer to nitrocellulose filters for probing with ^{32}P-labeled prerenin DNA, as described in Experimental Procedures.

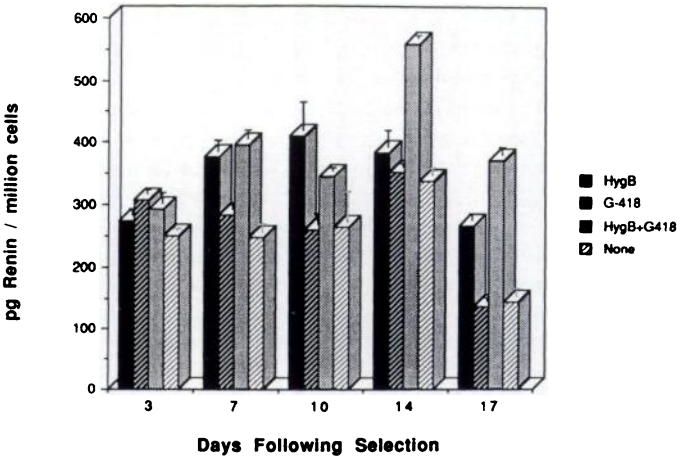

Fig. 4. Optimal growth conditions for subclone B1.10. Twelve 25-cm^2 flasks were each seeded with 10^6 cells/flask and grown in DMEM plus 10% FBS. Twenty-four hours later, the medium was removed and groups of three flasks were replaced with the indicated medium. Where indicated, hygromycin B (*HygB*) and G418 were present in the medium at 150 μg/ml and 500 μg/ml, respectively. On the indicated day, 500 μl of culture medium were removed for determination of the quantity of renin secreted. Cells were then removed after treatment with 0.25% trypsin/EDTA and counted using a hemocytometer. Cells were then reseeded at a density of 9×10^5 cells/flask, and the aforementioned process was repeated at the next time point. Values shown represent the mean ± standard error of three experiments.

conditions. Optimal production of active renin was evident when the cells were maintained in both G418 (500 μg/ml) and hygromycin B (150 μg/ml).

Pharmacological characterization of recombinant human renin. SQ 32,970 is a tripeptidic thiazole inhibitor of human renin (15). This inhibitor was used to demonstrate that the recombinant renin produced in this study is pharmacologically identical to native renin. Fig. 5 shows the inhibition of trypsin-activated prorenin secreted by COS-1 cells after transfection with pREN2. The IC_{50} value obtained for this preparation of recombinant renin is 25 nM, which is essentially iden-

tical to that obtained from trypsin-activated native renin (22 nM). Active renin secreted from COS-1 cells transfected with pREN3 or secreted from the B1.10 subclone was also inhibited by SQ 32,970 in a concentration-dependent manner (Figure 5). However, SQ 32,970 was significantly less potent (IC_{50} = 68 nM) as an inhibitor of active renin ($p < 0.01$, Student t test). Trypsin activation of active renin secreted by subclone B1.10 had the effect of decreasing the IC_{50} value (51 nM), but this was not statistically significant.

Discussion

An expression plasmid was prepared for transient and stable expression of a secreted form of active renin. This construct differs from preprorenin in that the 46-amino acid pro segment has been deleted. Therefore, the activation step mimicked by trypsin is not necessary to obtain enzymatically active renin. The pro domain was not necessary for secretion of renin, although higher levels of expression were consistently observed in COS-1 cells transfected with a plasmid encoding preprorenin. This suggested that the pro domain influences the expression of renin but not the secretion. Transfected COS-1 cells did not accumulate intracellular renin to a greater extent when transfected with pREN3, when compared with pREN2 (data not shown). Recently Harrison *et al.* (16) have shown that a cDNA construct with the domain encoding active renin joined to an immunoglobulin signal sequence was capable of secreting active renin after transfection of a myeloma cell line. This suggests that the pro domain is not required for secretion in myeloma cells where an immunoglobulin signal sequence is utilized.

Renin belongs to the family of aspartic proteases that are characterized by an Asp-Thr-Gly motif in each of the two domains of the enzyme (17). There are five known aspartic proteases in humans, pepsinogen A, pepsinogen C, renin, cathepsin D, and cathepsin E. These enzymes perform very diverse physiological functions but have many common structural features, as seen from the predicted protein sequences of each of these enzymes (18–21). These five proteases have a signal sequence varying from 15 to 20 amino acids, followed by a pro domain of approximately 45 amino acids and the active form of the enzyme, which is approximately 340 amino acids.

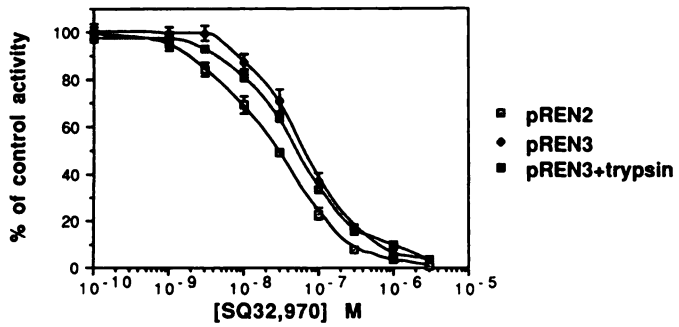

Fig. 5. Inhibition of different forms of recombinant human renin by SQ 32,970. Culture media from cells transfected with the indicated plasmid were assayed for renin activity, as described in Experimental Procedures. SQ 32,970 was dissolved in 100% dimethyl sulfoxide and diluted to the indicated concentration, such that the final dimethyl sulfoxide concentration was 1%, which had no effect on renin activity. Trypsin treatment of active renin was identical to that for prorenin, as described in Experimental Procedures. Prorenin was obtained from COS-1 cells transfected with pREN2. Active renin was obtained from NRP cells permanently transfected with pREN3.

These enzymes show a high degree of sequence homology, in addition to the invariant Asp-Thr-Gly sequence that appears twice in the polypeptide sequence and forms the active site of these enzymes (6). Pepsinogen A and pepsinogen C are secreted as inactive zymogens from chief cells into the stomach, where they undergo acid activation and autoproteolysis. Renin is secreted as an inactive zymogen from juxtaglomerular cells and chorion cells and requires a specific proteolytic activation to remove the pro domain. Cathepsins D and E are intracellular proteases that undergo an undefined activation step within the cell (22). It is not clear what the molecular determinants are that target the human aspartic proteases for secretion or intracellular locations. The signal sequence for each of these five proteases obeys von Heijne's (23) rules for the basic requirements of a mammalian signal sequence. A minimal signal sequence should have at least 15 amino acids with a charged amino terminus (n region), a hydrophobic core (h region) of at least six hydrophobic residues permitting no more than one intervening Ser, Gly, Thr, or Pro, and a charged carboxyl terminus (c region) containing at least five residues. Although Bird *et al.* (24) have proposed that an h region of only five hydrophobic amino acids is necessary for a functional signal sequence and the overall hydrophobicity of this region is more important, it is evident that all five human aspartic proteases have a functional signal sequence to permit the translocation of the newly synthesized proteins from the polysomes of the rough endoplasmic reticulum to the lumen of this organelle. After the translocation of the aspartic proteases into the lumen of the rough endoplasmic reticulum, glycosylation of specific arginine residues can occur. However, the pattern of glycosylation for this group of aspartic proteases does not clearly explain why prorenin and the pepsinogens are secreted and the cathepsins remain intracellular. Neither pepsinogen A or C is *N*-glycosylated or possesses potential sites of *N*-glycosylation, but they are secreted. Prorenin and cathepsin E are *N*-glycosylated, but only prorenin is secreted. Therefore, some unknown molecular determinant or combination of determinants must govern the assignment of prorenin as a secretory protein. This study has demonstrated that the pro domain is not necessary for the secretion of renin. However, only one secretion pathway within the cell may be utilized by the prerenin protein, because increasing evidence suggests that prorenin is secreted via a constitutive pathway and active renin is secreted by a regulated pathway (25). This was recently demonstrated in a mouse clonal cell line isolated from kidneys of transgenic mice expressing a renin-promotor/SV40 T antigen fusion transgene (26). In this cell line, 95% of the secreted renin is prorenin, even though active and inactive renin exist in equal proportions within the cell. When these cells are stimulated with 8-Br-cAMP, only active renin secretion is significantly increased, thus indicating that active renin is secreted by a regulated pathway. The NRP subclone B1.10 or the pREN3 construct in another cell line may be useful for characterizing this regulated pathway, because only active renin is produced by this cell line.

The pro domain of renin does not appear to be necessary for the correct folding of renin, which is in agreement with the original suggestion of Harrison *et al.* (16). They demonstrated that active renin could be secreted from myeloma cells transfected with a DNA construct encoding an immunoglobulin signal sequence adjacent to the active renin coding sequence. Their results show that active renin is secreted by these cells

but do not indicate the specific activity of the active renin. The demonstration in this study that active renin has a higher specific activity than trypsin-activated prorenin is more indicative that the pro domain does not play a role in the correct folding of renin. Additionally, unusually large quantities of renin did not appear in the Western blot experiments performed with transfected cell pellets and culture medium (data not shown). This suggests that the renin translational product is correctly folded and detected by the sandwich assay and enzyme assay, because the Western blot would detect large amounts of incorrectly folded protein, which were not seen. These results are not necessarily expected, because the bacterial serine protease subtilisin E has been shown to require its pro domain for correct folding of the enzyme (9). Subtilisin E is derived from a preprosubtilisin translational product encoding a 29-amino acid signal sequence that is necessary for secretion (pre domain), followed by a 77-amino acid prodomain that precedes the active region consisting of 275 amino acids. Ikemura *et al.* (9) showed that DNA constructs having either the subtilisin E signal sequence or the Omp A signal sequence were capable of directing prosubtilisin E to the periplasmic space in a correctly folded active conformation. However, when the Omp A signal sequence was joined directly to the active sequence of subtilisin E, the correct translational product was delivered to the periplasmic space, but in a totally inactive conformation. This inactive mature Subtilisin E could not be refolded correctly unless exogenous pro sequence was added to the renaturing buffer (27). These results clearly demonstrate that the pro domain of preprosubtilisin E is necessary for the correct folding and subsequent activity of Subtilisin E. This is not the case for renin and this is the first demonstration that the pro domain is not required for correct folding of an aspartic protease. It will be interesting to examine this property with other members of the aspartic protease class.

The estimated specific activity of secreted active renin was found to be significantly higher than that of trypsin-activated prorenin. This is not surprising, considering that trypsin is a nonspecific serine protease that will cleave after arginine residues in all proteins. Recently, Vlahos *et al.* (28) have shown that recombinant human prorenin secreted from a transfected human 293 cell line is cleaved at a secondary site, Arg^{53}-Leu^{54}, after trypsin activation. There are seven other arginine residues in human renin that are potential cleavage sites for trypsin, so it would not be surprising that these sites are also attacked by trypsin, but the digestion products might not be found after purification. The higher IC_{50} value observed for active renin is a surprising but reproducible result. The trypsin activation step must alter the active site such that it has a higher affinity for SQ 32,970 than observed with active renin. SQ 32,970 is a competitive inhibitor designed to interact directly with the active site of the enzyme (15). Therefore, secondary proteolysis by trypsin at some position near the active site might enhance the affinity of the inhibitor for the enzyme in a minor but significant manner. Trypsin may have a different cleavage pattern with prorenin versus active renin, such that it may not be possible to mimic the effects of trypsin activation of prorenin by subjecting active renin to trypsin treatment.

One of the objectives in establishing a stable cell line expressing active renin was to develop a source of renin for purification and crystallization studies. As shown in this report, the amount of renin produced by our best cell line is less than 1 ng/ml. The prohibitive cost and effort of preparing crystallizable amounts of renin from this source compelled us to look for alternative means of expression. Our attempts using inducible bacterial secretion vectors were unsuccessful. However, high level expression using the baculovirus system has been achieved. Preliminary results indicate that this system may be most suited for the production of large quantities of renin for structural and biochemical studies.

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Send reprint requests to: Jon A. Norman, Department of Cardiovascular Biochemistry, Bristol-Myers Squibb Pharmaceutical Research Institute, P.O. Box 4000, Princeton, NJ 08543-4000.