Study of the Antigenic Determinants of Human Renin

Geneviève Evin, William D. Carlson, Mark Handschumacher, Jiří Novotný, Gary R. Matsueda, Edgar Haber, Jacob Bouhnik, François-Xavier Galen, Joël Ménard, and Pierre Corvol

SUMMARY The primary structure of human renin, recently established from the complementary DNA sequence of its messenger RNA, shows a strong homology to other aspartyl proteases. This homology has permitted the construction of a model of the three-dimensional structure of renin based on the crystallographically determined structures of three aspartyl proteases: penicillopepsin, endothiapepsin, and rhizopuspepsin. Using an algorithm in which a spherical probe approximating the size of the antibody-binding domain (1-nm radius) was allowed to contact the surface of the renin model, we predicted 12 to 15 peptides to be immunogenic epitopes. We synthesized peptides corresponding to three different regions of the model: Cys-Gly-Ser-Asp-Pro-Gln-His-Tyr-Glu-Gly-amide (C-180-188), Tyr-Leu-Leu-Cys-Glu-Asp-Gly-Cys-Leu-Ala-Leu-amide (Y-215-224; disulfide bond between cysteines) and Tyr-Gly-Ser-Ser-Thr-Leu-Cys-Glu-Asp-Gly-Cys-Leu-Ala-Leu-amide (Y-211-224; disulfide bond between cysteines), and Cys-Tyr-Ser-Ser-Leu-Cys-Asp-Gly (C-290-296-G; disulfide bond between cysteines). All four peptides were tested for their binding to 11 polyclonal and 7 monoclonal antibodies raised against pure human renin, in both a solution assay and an enzyme-linked immunosorbent assay. Peptides Y-215-224 and Y-211-224 bound to all 11 polyclonal antibodies in the solution assay, and peptide Y-211-224 bound to eight of them in the enzyme-linked immunosorbent assay. Therefore, region 211-224 can be identified as a major epitope of the human renin molecule. Peptide C-180-188 bound to four polyclonal antibodies in the solution assay and to three in the enzyme-linked immunosorbent assay, whereas peptide C-290-296-G bound to only three antibodies in the enzyme-linked immunosorbent assay and none in the solution assay. None of the four peptides bound to any of the seven available monoclonal antibodies in either assay. Thus, we have successfully identified two renin epitopes (C-180-188 and Y-211-224) by means of a new computer algorithm. (Hypertension 8 [Suppl II]: II-72-II-77, 1986)

KEY WORDS • human renin • antigenic determinants • renin epitopes • renin antibodies • epitope prediction • renin three-dimensional model

The renin-angiotensin system is involved in the regulation of blood pressure, renal function, and electrolyte balance, and in several forms of hypertension as well. At an early stage in the system, renin, an aspartyl protease synthesized in the kidney, cleaves its specific substrate angiotensinogen to release angiotensin I. Angiotensin I is then converted into a potent vasoconstrictor octapeptide, angiotensin II, by the action of a carboxydipeptidase, the angiotensin converting enzyme. Both polyclonal and monoclonal antibodies to human renin have been raised and found to be useful in the study of the physiological role of renin. To date, however, nothing is known about the target sites of these antibodies.

The primary structure of human renin has recently been established from the complementary DNA sequence of human renin messenger RNA. An alignment of the human renin sequence with those of other aspartyl proteases shows a striking homology and has allowed models of its three-dimensional structure to be constructed. The model used in this study is based on the crystallographically determined structures of three aspartyl proteases: penicillopepsin, endothiapep-
The coupling reactions were carried out with a fourfold excess of protected amino acid and either dicyclohexyl carbodiimide or benzotriazolyl-oxy-tris(dimethylamino)phosphonium hexafluorophosphate (BOP). The last reagent is especially useful for incorporating glutamine and histidine and for other difficult couplings. The protected peptidyl-resin was treated with anhydrous hydrogen fluoride containing 10% anisole for 1 hour at 4°C. After removal of the hydrogen fluoride, the residue was extracted with diethyl ether, then with 1 M acetic acid (or 50% acetic acid in the case of Y-215-224), and the aqueous solution was lyophilized. Cyclizations of peptides Y-215-224 and Y-211-224 were performed by air oxidation of the crude lyophilizate in 0.1 M ammonium acetate (pH 8.7) for 24 hours, at a concentration of 0.5 mg of peptide per milliliter. Peptides were purified by gel filtration on Sephadex G-25, followed by ion-exchange chromatography. They were characterized by amino acid analysis, reverse-phase high-performance liquid chromatography, and thin-layer chromatography.

Binding of [125I]-Labeled Peptides to Antirenin Antibodies

The preparation of 7 monoclonal and 11 polyclonal antibodies against pure human renin has been described previously. Peptide C-180-188 was radioiodinated by the chloramine-T method of Greenwood et al. For radioiodination of the three other peptides, lodogen was utilized, as described by Fraker and Speck. Unincorporated [125I] was separated from the peptide with a C-18 Sep-pak cartridge (Waters-Millipore, Milford, MA, USA). The peptide was retained on the reverse phase and eluted with 40% acetonitrile in distilled water. A tracer amount of peptide (5000 cpm) was incubated overnight with 200 μl of pure, or 1:10 diluted, anti-human renin polyclonal (or monoclonal) antibody at 4°C, in 500 μl (final volume) of 0.1 M phosphate buffer (pH 7.5). The bound fraction of the peptide was precipitated with 1 ml of 20% (wt/vol) polyethylene glycol (molecular weight, 8000; Sigma, St. Louis, MO, USA), in the presence of 1 mg of bovine gamma globulin (Sigma), and was counted after centrifugation. Nonspecific binding was 6 to 8%.

Enzyme-Linked Immunosorbent Assay of Synthetic Peptides with Antirenin Antibodies

Polyvinyl chloride, 96-well microtiter plates (Falcon, Becton Dickinson, Oxnard, CA, USA) were coated with 50 μl of peptide solution (5-10 mg/ml in 0.1 M

\[
\text{C-180-188: CYS-GLY-SER-ASP-PRO-GLN-HIS-TYR-GLU-GLY-AMIDE}
\]

\[
\]

\[
\]

\[
\text{C-290-296-G: CYS-TYR-SER-lys-lys-lys-lys-CYS-GLY}
\]

**Figure 1.** Sequences of synthetic peptides studied as renin epitopes. The numbering refers to that of the primary structure of human renin. Residues that do not exist in the native structure of human renin are underlined. C = cysteine; Y = tyrosine; G = glycine.
tris(hydroxymethyl)aminomethane buffer, pH 9.6). After extensive washing, 225 μl/well of 1% (10 mg/ml) bovine serum albumin (radioimmunooassay grade, Sigma) in phosphate-buffered saline, pH 7.4, was added in order to block all the nonspecific binding sites. The plate was incubated overnight at room temperature. It was extensively washed with phosphate-buffered saline and 50 μl/well of anti-human renin antibody (1:500 dilution of pure antiserum in 1% bovine serum albumin and phosphate-buffered saline). After 30 minutes of incubation at 37°C with agitation, the plates were washed again, and 50 μl/well of alkaline phosphatase-labeled anti-rabbit (or anti-mouse) gamma globulin (Sigma) was added. After 30 minutes of incubation at 37°C, the plates were washed and p-nitrophenylphosphate was added. The colorimetric reaction was read at 405 nm on a Titertek Multiskan Photometer (Flow Laboratories, Merkenheim, Federal Republic of Germany) after 1 to 2 hours of incubation. A background value was obtained from wells that were not coated with the peptide. The peptides were tested with normal rabbit (or mouse) serum as a negative control. A ratio to control of 2.5 or more was interpreted as significant binding of antibody to peptide.

**Results**

Some 12 to 15 peptides have been predicted to be potential epitopes of human renin because of their location on the surface of a three-dimensional model of the molecule and their accessibility to a 1-nm spherical probe, which approximates the size of the antibody-binding domain. Among these peptides, three corresponding to different regions of the model were selected for initial study (Figure 2) because of their particular secondary and tertiary structures. They have been numbered C-180-188, Y-215-224, and C-290-296-G, in reference to the numbering of the human renin sequence. Two of the three are cyclic disulfide-bridged peptides: Y-215-224 corresponds to a natural loop that includes a disulfide bond present in the renin structure; in contrast, we intentionally added a disulfide bond to peptide C-290-296-G in order to constrain its secondary structure so that it would adopt a conformation closely resembling the one it adopts in the native renin molecule. For this purpose, serine 289 was exchanged for a cysteine linked to cysteine 296, thereby creating a disulfide bond not present in the native structure of human renin. In the native structure, cysteine 296 is linked by a disulfide to the cysteine of another part of the sequence (residue 259). Peptide 215-224 was also extended at its N-terminal with a tyrosine in order to allow radiiodination (Y-215-224). Unlike these two peptides, peptide 180-188 has a linear structure in native renin. No disulfide was introduced into this peptide; its three-dimensional structure was left unconstrained. However, for immunization purposes, peptide 180-188 was extended by a cysteine at its N-terminal to allow selective anchoring to a carrier protein by the cysteine side chain (C-180-188).

The binding of the peptide to anti-human renin antibodies was tested with 11 polyclonal and 7 monoclonal antibodies. All four peptides were tested with the seven monoclonal antibodies, in both the solution assay and the enzyme-linked immunosorbent assay (ELISA). None of the peptides was found to bind to any of the monoclonal antibodies in either assay (data not shown). Results obtained with the polyclonal antibodies are summarized in Table 1. The solution assay showed that peptide C-180-188 bound to 4 of the 11 polyclonal antibodies and that peptide Y-215-224 bound to all 11, whereas peptide C-290-296-G did not bind to any. The ELISA showed that peptide C-180-188 bound to three of the four antibodies that recognized 125I-labeled peptide in solution. For peptide Y-215-224, none of the antibodies showed significant binding, and peptide C-290-296-G bound to only three of them.

The binding of 125I-labeled Y-215-224 to all antibodies suggests that this peptide corresponds to a major epitope of human renin. The degree of binding was low, however, perhaps because the peptide only partially overlaps the natural epitope. Although a segment of 13 amino acids (210-222) was predicted to be accessible to the large spherical probe, in our initial study we synthesized peptides of no more than 10 residues. Considering the results obtained, we resynthesized the peptide and extended its length by four amino acids at the N-terminal. The results for peptide Y-211-224 are given in Table 2. In the binding assay with the labeled peptide, as much as 50 to 75% of it was bound by the majority of antibodies at a 1:2.5 dilution, and 30 to 65% was bound at a 1:25 dilution. This suggests that the antibodies have a much higher affinity for peptide Y-211-224 than for Y-215-224. Also, the ELISA, which showed no significant binding of peptide Y-215-224 to any of the antibodies, showed significant binding of Y-211-224 to 8 of the 11 polyclonal antibodies.

**Discussion**

The criteria that define the antigenic determinants of a protein are not yet clearly established. The location of the epitopes on the surface of the molecule, the degree to which they are hydrophilic, their specific sequences, and their mobility have all been invoked. The classic approach to the identification of the antigenic determinants of a protein is to synthesize overlapping segments of 10 to 15 amino acids, which represent the entire primary structure of the protein. In the present study, we used a new approach to epitope
by guest on November 4, 2017 http://hyper.ahajournals.org/ Downloaded from

predominant epitope, since it was recognized by all
ported here, the loop 215-224 appears to be a
regions for initial study. Among the three peptides re-
tial antigenic regions. We selected three of these re-
sequence 215-224 at its N-terminal
seem to constitute the whole epitope, however, be-
cotnmon to all these aspartyl proteases. It does not
small loop might belong to a major epitope that is
extension of sequence 215-224 at its N-terminal
cause sequence 215-224 at its N-terminal
by Lys-Glu-Gly in human cathepsin
renin is replaced by Ala-Glu-Gly in human pepsin,
cysteines vary with each enzyme and also among spe-
Hypertension, Vol 8, No 6, June 1986

TABLE 1. Assay of the Binding of Three Synthetic Peptides to 11 Polyclonal Anti-Human Renin Antibodies

<table>
<thead>
<tr>
<th>Peptide</th>
<th>R11</th>
<th>R12</th>
<th>R14</th>
<th>R15</th>
<th>R21</th>
<th>R22</th>
<th>R24</th>
<th>R25</th>
<th>R26</th>
<th>Donc</th>
<th>Bof</th>
</tr>
</thead>
<tbody>
<tr>
<td>C-180-188</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>53</td>
<td>29</td>
<td>51</td>
<td>9</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>ELISA (A/Ao)</td>
<td>1.4</td>
<td>1.7</td>
<td>1.2</td>
<td>1.7</td>
<td>1.2</td>
<td>2.3</td>
<td>10.3</td>
<td>3.9</td>
<td>3.5</td>
<td>1.4</td>
<td>1.5</td>
</tr>
<tr>
<td>Y-215-224</td>
<td>28</td>
<td>21</td>
<td>33</td>
<td>25</td>
<td>27</td>
<td>24</td>
<td>27</td>
<td>27</td>
<td>30</td>
<td>34</td>
<td>26</td>
</tr>
<tr>
<td>ELISA (A/Ao)</td>
<td>1.6</td>
<td>1.7</td>
<td>1.5</td>
<td>1.4</td>
<td>1.9</td>
<td>2.4</td>
<td>1.5</td>
<td>1.3</td>
<td>1.8</td>
<td>1.6</td>
<td>1.5</td>
</tr>
<tr>
<td>C-290-296-G</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ELISA (A/Ao)</td>
<td>1.8</td>
<td>1.5</td>
<td>1.4</td>
<td>1.3</td>
<td>3.2</td>
<td>1.8</td>
<td>1.6</td>
<td>1.9</td>
<td>1.9</td>
<td>2.8</td>
<td>3.0</td>
</tr>
</tbody>
</table>

Results of both the solution assay and the enzyme-linked immunosorbent assay (ELISA) are shown for each peptide. For the solution assay, the percentage of bound tracer represents that amount of 125I-labeled peptide bound by a 1:2.5 dilution of antiserum. For the ELISA, the antibody solution was 1:500, and the results are presented as the ratio of absorbance (A/Ao) due to antibody binding to peptide and antibody binding in the absence of peptide.

TABLE 2. Assay of the Binding of Peptide Y-211-224 to Anti-Human Renin Antibodies

<table>
<thead>
<tr>
<th>Assay</th>
<th>R11</th>
<th>R12</th>
<th>R14</th>
<th>R15</th>
<th>R21</th>
<th>R22</th>
<th>R24</th>
<th>R25</th>
<th>R26</th>
<th>Donc</th>
<th>Bof</th>
</tr>
</thead>
<tbody>
<tr>
<td>1:2.5</td>
<td>74</td>
<td>63</td>
<td>72</td>
<td>65</td>
<td>70</td>
<td>70</td>
<td>57</td>
<td>68</td>
<td>61</td>
<td>65</td>
<td>11</td>
</tr>
<tr>
<td>1:25</td>
<td>65</td>
<td>45</td>
<td>60</td>
<td>52</td>
<td>62</td>
<td>62</td>
<td>24</td>
<td>59</td>
<td>59</td>
<td>50</td>
<td>5</td>
</tr>
<tr>
<td>ELISA (A/Ao)</td>
<td>2.5</td>
<td>4.5</td>
<td>11</td>
<td>3.0</td>
<td>11</td>
<td>8.4</td>
<td>2.0</td>
<td>7.5</td>
<td>2.0</td>
<td>7.0</td>
<td>1.7</td>
</tr>
</tbody>
</table>

Percentages of bound tracer are shown for two dilutions (1:2.5 and 1:25) of pure antiserum in 0.1 M phosphate buffer, pH 7.5. ELISA = enzyme-linked immunosorbent assay; A/Ao = ratio of absorbance due to antibody binding to peptide and antibody binding in the absence of peptide.

 selections: determination of the accessibility of surface regions to a probe approximating the size of the antibody's antigen-binding domain. The computer program predicted the precise residues accessible to an antirenin antibody — identifying some 12 to 15 potential antigenic regions. We selected three of these regions for initial study. Among the three peptides reported here, the loop 215-224 appears to be a predominant epitope, since it was recognized by all antirenin polyclonal antibodies tested. Peptide C-180-188 was also at least partly antigenic.

These results offer confirmation of the proposed structure of human renin, particularly in the region of residues 215-224, and of the presence of a disulfide bond between cysteines 217 and 221. A corresponding pentapeptide cyclic structure exists in most of the aspartyl proteases, particularly in pepsin and cathepsin D; however, the residues included between the two cysteines vary with each enzyme and also among species. In fact, the sequence Glu-Asp-Gly in human renin is replaced by Ala-Glu-Gly in human pepsin, by Lys-Glu-Gly in human cathepsin D, and by Glu-Glu-Gly in mouse submaxillary gland renin. This small loop might belong to a major epitope that is common to all these aspartyl proteases. It does not seem to constitute the whole epitope, however, because extension of sequence 215-224 at its N-terminal by four residues resulted in much greater binding to the antirenin antibodies.

The two assay methods used do give corroborative results, but it must be pointed out that the ELISA was much more sensitive, since a 1:500 dilution of antibody was used in that assay, as opposed to the 1:2.5 or 1:25 dilution used in the solution assay. The low binding observed in the ELISA for peptide Y-215-224 may be explained by a different conformation that the peptide adopts when it is adsorbed on the plate during the course of the assay.

Our results support the hypothesis that the native conformation of the epitope is important to its antigenicity. That a cyclic peptide is well recognized by the antirenin antibodies also supports this hypothesis. In its cyclized form, peptide Y-215-224 would be more restricted in its mobility; it would be constrained to a conformation more closely resembling that which exists in the native renin structure. The disulfide bond in the other cyclic peptide, C-290-296-G, was designed to impose a secondary structure on the molecule that would correspond to the renin model in this particular region. This segment of the sequence protrudes from the surface of the model and is highly accessible, but it is linked to another polypeptide chain segment by the proposed disulfide bond between cysteine 259 and cysteine 296. Thus, it may constitute a discontinuous epitope.
The finding that none of the four synthetic peptides bound to any of the monoclonal antibodies is not surprising. These peptides correspond to only three of the 15 regions predicted to be potential epitopes, and only seven monoclonal antibodies were available; thus, the probability that a peptide would bind to one of the monoclonal antibodies is about one in 10. Furthermore, it has not been determined whether the seven monoclonal antibodies are directed to seven different epitopes. The information we possess allows us to divide them into two groups only: those that inhibit renin enzymatic activity and those that do not. This distinction suggests that the seven antibodies are directed to at least two different epitopes. One must also keep in mind that antibodies raised against a protein are often directed to topographically assembled epitopes formed by residues that are in close proximity in terms of the molecule’s three-dimensional structure but far apart in terms of its primary structure. The peptides reported in this study correspond only to continuous segments of the primary structure, and they may represent only parts of epitopes. Thus, the monoclonal antibodies would have only a slight affinity for these peptides, and binding would not be detected in our experiments.

In conclusion, we have identified two antigenic determinants of human renin on the surface of a three-dimensional model, using a method that allows the prediction of epitopes on the basis of their accessibility to a large spherical probe. The ability to predict the antigenic determinants of a protein from its three-dimensional structure would also be helpful in the development of synthetic vaccines. The use of synthetic peptides of human renin prosegment, recently reported, has allowed the immunological identification of human inactive renin as prorenin. The prediction of the antigenic determinants of human renin constitutes one approach to the generation of site-directed antirenin antibodies and should provide valuable information on the mechanism of renin’s action and on its interactions with angiotensinogen.

Acknowledgments
We are grateful to Josephine De Paolis and Irene Laboulandine for their skillful technical assistance. We acknowledge Thomas J. McVarish for his expert assistance in editing the manuscript.

References
18. Fraker P, Speck J. Protein and cell membrane iodination with a sparingly soluble chloroamide, 1,3,4,6-tetrachloro-3a,6a-diphenylglycoluril. Biochem Biophys Res Commun 1978;80:849–857
Study of the antigenic determinants of human renin.
G Evin, W D Carlson, M Handschumacher, J Novotný, G R Matsueda, E Haber, J Bouhnik, F X Galen, J Ménard and P Corvol