Identification and Purification of a Kidney Membrane Protein Which Specifically Binds the Amino-terminal Domain of Native Parathyroid Hormone*

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Nitrocellulose blots of bovine kidney membrane proteins were prepared from denaturing polyacrylamide gels. Strips of the blots were incubated with parathyroid hormone (PTH), washed, and then incubated with antisera against the hormone. Exposure to horseradish peroxidase-linked second antibody led to staining of a 51-kDa protein. No staining was observed in blots not incubated with PTH. Fragments 35–84 and 19–84 of PTH reacted strongly with the antisera, but did not lead to staining of the 51-kDa protein on the blots. Staining was visible, but greatly reduced, when fragment 9–84 was used. Oxidation of the native hormone at positions 8 and 18 led to reductions in staining of the band which were quantitatively similar to the reductions in biological activity induced by such oxidations. These properties suggested that the 51-kDa protein recognizes the amino-terminal portions of PTH, which is the segment of the molecule required for its biological activities. Several micrograms of the 51-kDa protein were purified to homogeneity by selective extraction from the membranes with detergent and by elution from multiple two-dimensional gels. The purified protein retained its PTH-dependent staining and specificity. This protein may be a PTH receptor or a fragment of a PTH receptor from kidney.

Parathyroid hormone is the principal regulator of blood calcium and phosphate levels in mammals. The hormone acts on the kidney to regulate phosphate and calcium transport and the hydroxylation reactions which activate vitamin D in the kidney. In bone, PTH stimulates mineral resorption, which leads to increased calcium and phosphate in the blood. Many, if not all, of the actions of this hormone are believed to be mediated by cAMP, and bone and kidney cells contain a PTH-activated adenyl cyclase (1). The major secreted form of PTH is an 84-residue, single-chain polypeptide. However, the known physiological actions of this hormone can be expressed by its amino-terminal fragment, PTH(1–34) (2, 3).

Putative receptors for the amino-terminal PTH fragment have been identified in kidney, bone, and human fibroblasts using photoaffinity labeling and cross-linking techniques. Coltrera et al. (4) identified a protein of about 70 kDa in kidney membranes, and later this protein was also described in bone cells and fibroblasts (5). Draper et al. (6) described a 90-kDa species in canine renal membranes. Brennan and Levine (7) used biotinylated PTH to identify a 70-kDa protein on Western blots of kidney membranes. More recently, Wright et al. (8) described both a 90-kDa species and a 25-kDa protein in addition to the 70-kDa form, and Nissenson et al. (9) reported cross-linking of PTH to an 80-kDa protein, a 70-kDa form, and a 51-kDa species. The relationship of these various proteins to one another is not known, but it has been suggested that some are proteolytic fragments of the others (8).

Earlier, we described (10) a method for detection of PTH-binding proteins on nitrocellulose blots using antisera against PTH to detect bound hormone. We now describe the use of this methodology in detection and purification of a 51-kDa protein which requires the amino-terminal domain of native PTH for binding. The specificity of binding of PTH-related peptides is in agreement with the suggestion that it is also a receptor (or receptor fragment) for this hormone.

MATERIALS AND METHODS

Parathyroid Hormone—Native PTH was purified from defatted bovine parathyroid tissue (Sigma) by methods described in the literature (3, 12). The oxidized forms which contaminate most preparations of hormone were removed by HPLC (13). Fragment 1–34 of bovine PTH was obtained from Bachem.

Antiserum—One rabbit antiserum (RSL) was provided by Dr. Bernard Roos (American Lake Veteran’s Administration Medical Center, Tacoma, WA). Two antisera (RSL and RS1) were raised in our laboratory by immunization of rabbits with bovine PTH emulsified in Freund’s adjuvant according to standard procedures. All the antisera were of high titer for PTH (normally used at 1:20,000 or higher dilutions) and were specific for the peptide sequences carboxyl-terminal to residue 34 (i.e., none showed cross-reactivity with PTH-(1–34)-peptide at the dilutions used in our experiments.)

Fragments and Oxidized Forms of PTH—PTH selectively oxidized at methionine 8, methionine 18, and at both positions was prepared as described in our earlier work (13, 14). Fragment 9–84 of PTH was prepared by CNBr cleavage of fragment 1–84 oxidized at position 18. Fragment 19–84 was prepared by CNBr cleavage of native PTH. Fragments 35–54 and 55–84 were prepared by cathepsin D digestion of PTH (15, 16). All the peptides were purified by HPLC and have been chemically identified by amino acid analysis.

Preparation and Handling of Nitrocellulose Blots from Polyacrylamide Gels—Two sizes of polyacrylamide gels were used: the standard gels were 16 × 13 cm, and minigels were 6 × 8 cm. The amount of membrane protein applied to the normal gels ranged between 500 and 1000 μg and between 200 and 400 μg for the minigels. All electrophoresis was conducted with denaturing gels (15). Proteins were transferred to nitrocellulose paper electrophoretically in Tris/glycine buffer with 20% methanol. One well of the gels was usually utilized for molecular mass markers. Normally, the nitrocellulose blots were cut into strips about 7 mm in width (or corresponding to the width of the individual wells) for staining. The strip containing the molecular mass markers and one additional strip were stained with Amido Black. The remaining strips were blocked with bovine serum albumin.
prior to immunostaining. The blots, or individual strips, could be stored in the freezer for months without loss of activity.

Immunostaining—Originally, PTH was preincubated with the nitrocellulose strips, and this step was followed by washing and incubation with anti-PTH antisera. Later, to save time, the antisera and hormone or appropriate hormone fragment or derivative were added simultaneously so that the strips were exposed to the hormone-receptor complex in the first step of the assay. Both methods gave similar results. Incubation of the strips with the hormone or hormone/antisera mixtures was conducted overnight in the cold. The strips were then washed with several changes of phosphate-buffered saline containing 0.5% Tween; and subsequently, appropriately diluted second antibody linked to horseresoridin peroxidase was added. After 2 h at room temperature, the strips were washed again and then added to freshly prepared substrate solution (0.0035% o-dianisidine in 0.02 n Tris-HCl at pH 7.4 and 0.01% hydrogen peroxide) for development. The timing of the various steps and the dilutions of the antisera varied somewhat with different antisera. Control experiments were run in the absence of PTH and/or the first antibody.

Characterization of PTH Antiserum on Dot Blots—The various antisera against PTH were compared for amount and specificity of antibodies against the hormone by the following method. Dot blots (Bio-Rad) were prepared with serial dilutions of PTH or fragments of the hormone. These ranged from 100 ng to 10 pg of hormone. The blots were then incubated with serial dilutions of first antibody. Following washing and application of a second antibody (horseresoridin peroxidase) the strips were washed once more with the subdeveloped with the substrate. The dots could then be quantitatively scanned with a densitometer to provide a rapid, sensitive, and quantitative estimate of the relative strength and specificities of the antisera.

Starting Material for Receptor Purification—Beef kidneys were obtained fresh at slaughter and transported to the laboratory on ice. The outer cortex was removed with a scalpel, and a 50% homogenate was prepared in 0.25 M sucrose, 0.01 M Tris (pH 7.4), 1 mM EDTA (STE buffer) by three 20-s bursts in a Sorvall Omni-Mixer. This homogenate was then further disrupted by three passes in a Teflon-glass homogenizer. Partially purified plasma membranes were then prepared as described earlier (17).

Selective Extraction of the 51-kDa Protein—Earlier work (18) had shown that PTH-binding proteins could be extracted from purified bovine kidney membranes with 1% Triton X-100. However, the efficiency of extraction was a function of the protein concentration. We employed this observation to remove selectively proteins including the PTH receptor from the crude membrane preparation. The membrane pellet was first resuspended in STE buffer, to a protein concentration of 50 mg/ml, and the membranes were extracted with 1% Triton X-100 in the cold overnight. The extract was then centrifuged at 10,000 × g for 1.5 h. A large amount of protein was extracted by this method, but little of the 51-kDa protein was removed. The pellet was then resuspended in STE buffer to a final protein concentration of 10 mg/ml and again extracted overnight with 1% Triton X-100. This extract was again centrifuged as described above. The PTH-binding protein was present in the supernatant, and the pellet was discarded. The detergent was removed from this extract by treatment with Bio-Beads as described earlier (18). After keeping overnight in the cold, this solution turned cloudy, and it was centrifuged again at 150,000 × g for 2 h. The 51-kDa protein was found to be primarily localized in the pellet obtained in this step. This pellet was then taken up into the same buffer for two-dimensional electrophoresis.

Purification of the 51-kDa Protein on Two-Dimensional Gels—The dissolved pellet from the extraction (650 μg of protein/gel) was loaded on 4.0% polyacrylamide tube gels (5 × 120 mm) for isoelectric focusing according to standard procedures. Isoelectric focusing was for 8000–9000 V-h. Separation in the second dimension was exactly as described previously (20). The gels were lightly stained with Coomassie Blue, and the 51-kDa protein was identified on each gel by its characteristic position with regard to other proteins in the gel. This spot was then cut from each two-dimensional gel. The purified protein from this gel was denatured and added to a 200-μl aliquot of 0.2% o-dianisidine in a 800-μl reaction mixture using an ISCO electroforetic concentrator. The amount of protein recovered was estimated by silver staining as described by Merrill and Pratt (21). The amount of pure receptor obtained was estimated to be 5–25 μg/kg of tissue using bovine serum albumin as a standard. However, this is an approximation because of the variable extent of silver staining observed with different proteins.

Detection of a PTH-binding Protein on Nitrocellulose Blots—The theory for the use of antibodies against PTH to detect receptors on nitrocellulose blots is outlined in Fig. 1. The assay is based on the concept that carboxy-terminal segments of PTH are not involved in binding of the hormone to its receptors. Thus, receptor-bound native PTH will have a portion of the peptide chain free for interaction with antibodies. A similar approach to membrane binding of PTH, using iodinated antibodies, was described earlier by McIntosh and Hesch (22).

Fig. 2 shows early results obtained with this assay using two different polyclonal antisera against PTH. These antisera were specific for regions of the PTH-peptide beyond residue 34 and showed no cross-reactivity with fragment 1–34 at the dilutions used. Thus, they meet the criteria outlined in Fig. 1. Preliminary studies had indicated that the putative receptor band has a size between 40 and 70 kDa (in agreement with earlier photoaffinity labeling studies); and therefore, the experiments were conducted only with the sections of the blots which included proteins of this size. As shown in Fig. 2, a membrane protein with a mass of about 51 kDa was detected by both antisera in the presence of PTH. Staining of the protein required relatively high concentrations of PTH; the band could occasionally be detected with 10−15 M hormone, but was only consistently observed at higher concentrations. This band did not stain when either the hormone or the first antibody was omitted or when normal rabbit sera were used.

![Fig. 1. Theoretical basis for detection of PTH-binding proteins on nitrocellulose blots by use of antibodies against carboxy-terminal segments of PTH. Ab, antibody; HRP, horseresoridin peroxidase; R, receptor.](image)

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![Fig. 2. Detection of a PTH-binding protein in bovine kidney membranes by use of the method described in Fig. 1 using two different antisera against PTH. Membrane proteins were separated on denaturing polyacrylamide gels and transferred to nitrocellulose paper, and the region of the blot comprising the mass range between approximately 40 and 70 kDa was cut out. These strips were first blocked with bovine serum albumin and then incubated with 1:100 dilutions of RSL (left) and RSR (right) in the presence of 10−7 M PTH (lanes B and F) and the absence of the hormone (lanes A and E) at 4 °C overnight. The strips were then washed, incubated with enzyme-linked second antibody, and developed with substrate as outlined under "Materials and Methods." Lane C shows the staining in the absence of the first antibody, and lane D shows the mass markers.**

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Both antisera stained some proteins in the absence of PTH, but this nonspecific staining was not altered significantly by PTH. Although the band near 80 kDa appears to be more heavily stained with the RSL antiserum, this was not reproducible. The nonspecific staining was due to the first antibody and apparently reflects the cross-reactivity of the rabbit immunoglobulins with a variety of bovine kidney membrane proteins.

Another aspect of the method apparent in Fig. 2 is the higher background staining on the blots in the presence of PTH. This is due to the nonspecific binding of the hormone to the paper. The extent of this background staining varied between experiments. Thus, each study of PTH binding by this method represents a compromise between the intensity of the staining of the band which could be observed and the background staining levels. Dark staining of the 51-kDa protein could be obtained with less extensive washing of the blots, but this led to significantly increased background (e.g. see Fig. 5). On the other hand, background staining could be reduced to a great extent with more extensive washing, but this led to reduced staining of the 51-kDa protein. For these reasons, it is only possible to compare the intensity of staining within experiments, where the conditions are identical for all samples.

Specificity of PTH Binding to the 51-kDa Protein—It was difficult to examine the specificity of PTH binding with the antisera described for Fig. 2. In one case (RSL), the staining of the band was unacceptably light; and with the other antiserum (RSR), a second band which stained heavily but was unrelated to PTH ran very close to the 51-kDa protein and sometimes was not completely resolved. Although it could be shown that neither fragment 1–34 nor 35–84 of the hormone led to staining, intermediate levels of staining produced by other PTH-derived peptides were more difficult to demonstrate in reproducible fashion. Therefore, additional antisera were examined for their potential use in specificity studies.

One antiserum (RS1) was found to be acceptable for this purpose. With the exception of a protein at 54 kDa, this antiserum gave relatively little staining of the bovine kidney membrane proteins in the absence of PTH. As indicated in Fig. 3, the staining of the 51-kDa protein could be demonstrated by use of this antiserum under a variety of experimental conditions. Staining was obtained when the blots were incubated with the hormone first, followed by washing and subsequent exposure to antibody or by direct incubation with the hormone/antibody mixture. The protein was never stained in the absence of PTH.

An additional finding illustrated in Fig. 3 is that the length of time of exposure of the blots to the RS1 antiserum led to more intense staining of the 54-kDa species. Under these conditions, the presence of PTH decreased the staining of this band. Also, antibodies against the 54-kDa protein were not found in the preimmune serum (see Fig. 5). These observations cannot be interpreted presently, and additional examination of their significance is currently underway.

The sensitivity and specificity of the RS1 antiserum for different forms of PTH are illustrated in Fig. 4. As indicated, this antiserum showed similar sensitivity to the intact hormone, fragment 19–84, and the fully oxidized form of PTH. Fragment 35–84 also showed strong cross-reactivity with this antiserum (data not shown). Staining was inversely proportional to the log of the peptide amount for these peptides over a range of 100 to 0.1 ng. Thus, this antiserum has the desired properties of cross-reactivity with carboxyl-terminal segments of PTH, and the extent of reactivity was apparently not altered by such modifications as deletion of amino-terminal segments or oxidation of methionines in the amino-terminal portions of the chain.

Specificity studies for staining of the 51-kDa protein with different forms of PTH using the RS1 antiserum are shown in Fig. 5. PTH-(1–34)-peptide did not lead to staining of the 51-kDa protein, as expected, since it does not react with the antiserum. However, fragment 35–84, a peptide which reacts...
strongly with the antiserum, showed no staining of the 51-kDa protein. Fragment 9–84 did generate some staining of the 51-kDa band, although this was distinctly weaker than that obtained with the intact hormone. Very light staining was produced by fragment 19–84, but was extremely variable. These data indicate that the amino-terminal 19 residues are of importance for the binding of PTH to this protein.

Additional specificity studies of the 51-kDa protein were conducted with forms of PTH specifically oxidized at positions 8, 18, or both. In earlier work, we showed that the form oxidized at position 8 is a full agonist for PTH in the adenyllycyclase assay, with a somewhat lower potency than the native hormone (13, 14). On the other hand, the form oxidized at position 18 is a considerably weaker partial agonist, and the potency of the form oxidized at both positions is reduced even more. The data shown in Fig. 6 indicate that the binding of the oxidized forms of PTH is reduced in a fashion which parallels these biological effects. Whereas the form oxidized at position 8 stains the 51-kDa band nearly as well as the native hormone, the form oxidized at position 8 gives much weaker staining, and the doubly oxidized form even less.

The binding of the oxidized forms of PTH was quantitated by scanning densitometry of the blots shown in Fig. 6. The integrated densities were then converted to the relative amount of binding estimated by the integrated density of the stained bands in Fig. 6. Although the absolute accuracy of these estimates is not high, it is clear that oxidation of the methionines in PTH has the same proportionate effect on PTH binding to the 51-kDa protein on the blots as it has on the biological activity of the hormone.

Light staining of an additional protein of lower mass (about 43 kDa) was also occasionally produced by PTH when the RS1 antiserum was used (Fig. 6). This was not of sufficient intensity to be systematically studied, but it may represent an additional PTH-binding protein of future interest. Evidence for this form is also found in data presented below (Fig. 7).

Oxidation of PTH also slightly altered the staining of the 54-kDa protein (Fig. 6). However, in this case, oxidation enhanced staining, suggesting that the hormone is either competing for the antibodies which bind this protein or binding this protein directly. Again, interpretation of the results related to the 54-kDa protein is not yet possible.

Identification of PTH-binding Proteins on Two-dimensional Gels—We examined whether the 51-kDa protein can be detected on two-dimensional blots of the membrane proteins. For this study, strips from these blots were cut out which included the molecular mass range of interest and the entire pH range. All proteins on this strip were tested for their ability to bind PTH using the RS1 antiserum. The results are shown in Fig. 7. In the absence of PTH, staining of the 54-kDa protein (and two minor associated forms) was observed, but the 51-kDa protein was not apparent. The isoelectric point of the 54-kDa protein is about 6.5. In the presence of the hormone, the 51-kDa protein was clearly stained; whereas as described above, the staining of the 54-kDa species was somewhat reduced by the hormone. In addition to the 51-kDa protein, however, several additional proteins which bind PTH were apparent on the two-dimensional blots. The major ones consisted of two proteins with masses lower than 51 kDa but which migrated near the 51-kDa protein and a pair with higher isoelectric point (about 6.5) and mass (68 kDa). The
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FIG. 7. Identification of PTH-binding proteins on blots of two-dimensional gels. Left panel, staining of primarily the 54-kDa protein with the RS1 antiserum alone; right panel, effect of PTH (10^{-7} M) on the staining of the 54- and 51-kDa (arrowhead) proteins. Blots were prepared, and a strip containing the 40-70-kDa range was cut out. This strip was developed with the antibodies, and the remainder of the blot was stained with Amido Black.

The smallest of these (43 kDa) may be the low molecular mass species seen faintly in the one-dimensional blots (e.g. Fig. 6). However, the others were not observed in the one-dimensional blots, and they must either be obscured by overlapping bands on the one-dimensional gels or be artifacts generated by the isoelectric focusing step of the two-dimensional gels.

One reasonable artifact which could account for the smaller species is proteolytic breakdown of the 51-kDa forms during the isoelectric focusing step. Since these proteins migrate into the acidic portion of the gels, it is possible that degradation would be accelerated by contaminating lysosomal enzymes activated at the lower pH. This was examined by resuspending the membranes in a buffer containing a mixture of protease inhibitors prior to preparation for electrophoresis. This treatment did not significantly alter the results, however.

Purification of the 51-kDa Protein—The 51-kDa protein is similar in size to a putative PTH receptor fragment described recently by Nissenson et al. (9). Its specificity for binding native hormone is also consistent with the possibility that this protein is related to a PTH receptor in kidney. For these reasons and because it could easily be identified and separated on two-dimensional gels, we undertook its purification. Through use of extraction techniques developed earlier (18) and two-dimensional electrophoresis, we were able to obtain approximately 1-5 µg of this protein by following the steps outlined under "Materials and Methods." A silver-stained two-dimensional gel of the purified protein is shown in Fig. 8. When run in the purified form, the size of this protein is estimated to be 52-53 kDa and its isoelectric point about 5.2. It is clear from the gel that the purified protein contains no significant contaminants.

The PTH-binding properties of the purified protein were examined by use of the method described above. As shown in Fig. 9, staining of the purified protein by the RS1 antiserum was observed in the presence of PTH either when the hormone was preincubated with the blots prior to exposure to antibody or when the antiserum and hormone were coincubated with the blots. No staining was observed in the absence of the hormone. Finally, the specificity of PTH binding to the purified protein was found to be identical to that of the 51-kDa species studied in the membrane preparations. As indicated in Fig. 10, it is clear that this protein requires residues 1-9 of the PTH-peptide for maximum binding, and removal of residues 1-19 almost completely eliminates staining. Furthermore, oxidation of the methionine residues of PTH reduces binding in exactly the same manner as observed in the membrane studies.

FIG. 8. Silver-stained two-dimensional gel of the highly purified PTH-binding protein. Approximately 1 µg of purified protein was utilized.

FIG. 9. Demonstration of PTH binding to the purified 51-kDa protein. Each lane represents about 300 ng of purified protein. Lanes A and B were incubated overnight with PTH (lane A) or buffer (lane B) prior to washing and development with the RS1 antiserum; lanes C and D were incubated overnight with a PTH/antiserum mixture (lane C) or just the RS1 antiserum (lane D) and then developed.

FIG. 10. Specificity of staining the purified 51-kDa protein. Blots were incubated overnight with peptide/antiserum mixtures prior to development. The lanes in the upper panel were exposed to 100 nM peptide, and those in the lower panel to 10 nM peptide. Lane A, no peptide; lane B, PTH oxidized at both methionines; lane C, PTH oxidized at Met-8; lane D, PTH oxidized at Met-18; lane E, native PTH; lane F, fragment 1-34 of PTH; lane G, fragment 35-84 of PTH; lane H, fragment 9-84 of PTH; lane I, fragment 19-84 of PTH.
This work introduces an immunological approach for identification of PTH-binding proteins found in target tissue membranes which does not require antibodies against these proteins. The method is a variation of the antibody sandwich technique and is limited to proteins which bind the hormone in such a fashion as to leave the carboxyl-terminal segments of PTH free for binding to antibodies. For its success, a renatured protein is required since only proteins which bind PTH can be detected. Several examples of renaturation of receptors on nitrocellulose blots are known (23, 24). In addition, Brennan and Levine (7) have recently shown that biotinylated PTH binds putative receptors on nitrocellulose blots. Our results also establish that at least partial renaturation of the 51-kDa protein does occur on the blots.

The identification of the PTH-binding protein by the immunological method could be achieved either by preincubating the blots with PTH, followed by washing and exposure to antibody, or by coincubations of the blots, antibody, and hormone. The time of blotting did not seem to be critical; we obtained similar results either by overnight blotting at low current or by 2-h blotting at higher currents. The backs of the blots did not stain significantly, indicating that most of the protein is bound to the surface facing the gels. Temperature variations during blotting had some effect, and generally better results were obtained at lower temperatures. The method clearly stained the 51-kDa protein with three different antisera against PTH. Although both nonspecific staining and the intensity of staining varied with the different antisera, the 51-kDa protein was always detected with antisera which were known to bind the carboxyl-terminal segments of PTH.

In theory, this method can be used to detect PTH-binding proteins in any tissue, cell type, or cellular fraction, and it may be of value in the study of such problems as the biosynthesis or degradation of receptors, the internalization of receptors, the cellular distribution of receptors, and the role of PTH receptors in various physiological or pharmacological states such as development or aging. One of the advantages of this assay is that it does not require chemically modified forms of the hormone such as are necessary in photoaffinity labeling or biotinylated PTH studies. Also, the fact that the method uses the biologically relevant native form of PTH, PTH-(1–34)-peptide, rather than the commonly used synthetic fragment 1–34, may be an advantage.

A disadvantage of the method is the variability of the staining intensity both of the protein band and of the background. This problem persists even with relatively high hormone concentrations and with the purified protein. It could be due, in part, to variability in the extent of either the renaturation or the affinity of the protein (or both). Although the 51-kDa protein identified by Nissenson et al. (9) showed high affinity for PTH, no conclusions concerning this point are possible from our studies. Many opportunities for hormone dissociation exist during the repeated washing steps and other manipulations of the nitrocellulose strips required by our method, and this precludes any but qualitative estimates of affinity. Also, because of this limitation, this method of assay cannot be used to quantitate absolute amounts of receptor present on a blot.

The specificity of staining of the 51-kDa protein, either as found in membranes or in the purified form, strongly suggests that this protein is associated with the biologic actions of PTH. It is apparent that the first 9 residues of the hormone are required for maximal staining; thus, whereas fragment 9–84 may associate weakly with this protein, its affinity appears to be greatly reduced. This is consistent with literature reports (25, 26) that amino-terminal deleted peptides such as fragment 7–34 do bind to PTH receptors, but only weakly, and they do not initiate a biological response. Although reproducible binding of fragment 19–84 was not observed, some light staining of the band was occasionally seen with this peptide (see Fig. 5). Earlier studies suggested that the amino acids between positions 27 and 34 play a significant role in PTH binding to receptors (27, 28). Thus, weak binding by fragment 19–84 may occur through these residues. Fragment 35–84 produced no staining of the band in any of our experiments.

The effects of oxidation of specific methionine residues in PTH also argue strongly that the 51-kDa protein is a biologically relevant receptor. Thus, oxidation at position 8 (i.e., addition of a single oxygen atom to PTH-(1–84)-peptide) significantly reduces the binding in our assay, whereas oxidation at position 18 has much less effect. Oxidation at both residues nearly eliminates staining. These results are both qualitatively and quantitatively in agreement with the effects of oxidation on the biological action of PTH in activation of renal membrane adenylylcyclase (Table I). At the same time, such oxidation has no impact on binding of antibodies to the hormone, again confirming that the loss of staining is due to reduced interaction of the 51-kDa protein with the hormone derivatives.

Photoaffinity labeling approaches have led to identification of putative PTH receptors with masses of 90, 80, 68–70, 55, 51, and 28 kDa (4–9). From studies with canine renal membranes, Nissenson et al. (9) suggested that the 70- and 51-kDa species may be proteolytic fragments of the 80-kDa form. We examined canine membranes with our method and obtained intense staining of the 51-kDa protein, but none with the 68- or 80-kDa species. However, two-dimensional blots from bovine membranes showed the presence of a 68-kDa species which stained only in the presence of PTH (Fig. 7). Furthermore, we have recently detected a 90-kDa protein with a high affinity for PTH which appears to be quite unstable; at the same time, we have found the 51-kDa protein to be remarkably stable to proteolytic digestion.2 These findings are consistent with the possibility that the 51-kDa protein may indeed be a fragment of a higher molecular mass species which retains its ability to bind the hormone and which accumulates under conditions which lead to degradation of higher molecular mass forms. Whether it is the same protein as that identified by Nissenson et al. (9) remains to be confirmed.

As noted above, during our examination of PTH binding to proteins separated on two-dimensional gels, several additional proteins were observed to bind the hormone (Fig. 7). The pair of proteins with masses near 68 kDa and an isoelectric point of about 6.5 are of particular interest since this is the size range reported for a putative PTH receptor identified by other groups (4–9). It seems possible that these proteins can now also be isolated and their properties examined. The use of our approach for their isolation has the advantage that it is not restricted to chemical conditions which hold the hormone to the blot for its detection, and the isolated species will be the unmodified protein rather than a cross-linked species. Thus, the availability of the immunological assay for PTH binding to such proteins on nitrocellulose blots should facilitate their purification.

Our methods do not allow calculation of the absolute mass of the receptor in the starting tissue or the various fractions, but the fact that the protein has been obtained in a totally purified state does allow an estimate of the minimum number of receptor molecules/kidney cell. We obtained in the range of 5–25 μg of this protein/kg of tissue. Assuming the smaller

1 J. E. Zull, J. Chuang, I. Yike, and R. Laethem, unpublished data.
number is correct and assuming 100% recovery in all the purification steps, a value of 600 receptor molecules/cell results. This is a minimum value; as noted, it does not account for losses during purification, and it assumes all cells in the tissue have PTH receptors. Using similar assumptions and estimated yields of membranes, we also made these calculations from data in the literature. These calculations give values ranging from 1,000 receptor molecules/cell in chicken kidney (29) to 90,000/cell in canine kidney (11).

Although the small amounts of this protein we are able to isolate by present methods are inadequate for classical biochemical studies, they are sufficient to obtain some primary structure information by microsequencing. Thus, the significance of the present report is that it opens the door to obtaining polynucleotide and antibody probes for this protein in cDNA clones. This in turn will lead to the entire primary structure of this protein or the parent protein from which it is derived.

Finally, it is of interest that the RS1 antiserum was found to contain antibodies against a 54-kDa kidney membrane protein, which arose in response to PTH immunization. Also, the staining of this protein appeared to be altered by PTH and some of the fragments we used in our specificity studies. The basis for these results is not presently understood, but one of two conclusions is apparent: either the PTH antibodies are cross-reactive with this protein or PTH immunization has stimulated generation of a second class of antibodies which directly bind this kidney membrane protein. Either conclusion has interesting ramifications.

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