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ISOLATION AND CHARACTERIZATION OF INSULIN RECEPTORS FROM RAT KIDNEY GLOMERULI AND TUBULES

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SUMMARY: In order to directly compare the structural characteristics of renal glomerular and tubular insulin receptors, the purified isolated nephron subunits were extracted with 1% Triton X-102, fractionated by DEAE-Sephacel ion exchange column chromatography and the fractions containing insulin binding proteins were identified by the precipitation of ^{125}I-insulin-protein complexes with polyethylene glycol (PEG). The fractions containing insulin binding proteins were pooled, incubated with ^{125}I-insulin and covalently cross-linked with disuccinimidyl suberate, followed by chromatography of the cross-linked samples on Sepharose CL-6B. From both glomeruli and tubules, three ^{125}I-insulin-binding complexes with molecular weights of 560KDa, 220KDa and 95KDa were found. SDS-PAGE of these complexes from glomeruli and tubules under both reducing and nonreducing conditions gave similar patterns of ^{125}I-insulin-crosslinked components, with the exception of the polypeptide pattern from the 560KDa peak fraction which was markedly different between glomeruli and tubules with the former giving major labeled components at 170 and 68KDa while the latter showed labeled components of 125KDa and > 250KDa. Glomerular and tubular insulin receptors, therefore, display similar subunit composition under reducing conditions, but differ in the non-reduced state, suggesting that these complexes may differ in the extent and/or nature of disulfide bonding.

Specific receptors for insulin in isolated renal glomeruli and tubules were initially identified by ourselves (1-3) and others (4-6), and are of interest because of the significant amounts of this hormone to which the kidney is continually exposed via the routes of glomerular circulation and tubular reabsorption, and the possible role which insulin may play in renal metabolism and physiology. Although renal glomerular and tubular insulin receptors have characteristics that resemble such receptors in other tissues, there is evidence that the tubular receptors have a lower average affinity and higher capacity for binding ^{125}I-insulin than the glomerular receptors (1-3). The basis for these differences in affinity and capacity between glomerular

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and tubular insulin receptors is unclear, but the high-affinity insulin receptors in tubules have recently been reported to be almost completely localized to the basolateral membranes, with few receptors present on the brush border surface (7). We have recently confirmed and extended these results by demonstrating that although the renal tubular brush border lacks significant amounts of a high-affinity classical insulin receptor, it possesses a low affinity, broadly-specific, high capacity acceptor site for insulin which may mediate reabsorption of the hormone from the tubular lumen (Meezan, et al., submitted for publication). In order to elucidate the possible structural basis for the differences between low- and high-affinity glomerular and tubular insulin receptors, we have isolated and characterized the insulin receptor populations in glomeruli and tubules isolated concommitantly from the same kidneys using a non-enzymatic, magnetic iron oxide technique (8).

MATERIALS AND METHODS

Materials-Receptor grade ^{125}I-insulin (2,200 Ci/mmol) was purchased from New England Nuclear Corporation; Triton X-102, bovine serum albumin (BSA), gamma globulin, polyethylene glycol (PEG) and molecular weight marker proteins for SDS-PAGE were from Sigma Chemical Co.; disuccinimidyl suberate was from Pierce Chemical Co.; DEAE-Sephacel, Sepharose CL-6B and molecular weight markers for gel filtration chromatography were from Pharmacia Fine Chemicals; reagents for SDS-PAGE were from Bio-Rad Laboratories; nylon sieves (86 μm) were from Kressilk Products Inc., Monterey Park, California; Amicon concentrator was from Amicon Corp., Lexington, Mass; Spectrapor dialysis membrane tubing #2 (nominal cutoff range = 12,000-14,000) was from Fisher Scientific Co.; GF/C filtration membranes were from Whatman; Cronex X-ray films were from E.I. Dupont Co.; and Kodak GBX X-ray film developer kits were from Eastman Kodak Co. Other chemicals used were reagent grade.

Solubilization and isolation of renal glomerular and tubular insulin-binding proteins-Purified preparations of rat renal glomeruli and tubules were isolated as described in detail previously (3,8). Insulin-binding proteins were solubilized and fractionated using a procedure applied by us to the isolation of insulin receptors from other tissues (9-11). In brief, glomeruli (1 g wet weight) and tubules (6 g wet weight) were solubilized in 1% Triton X-102 and the resulting supernatant extracts were chromatographed on DEAE-Sephacel as described previously (11). To identify the fractions containing insulin receptors, ^{125}I-insulin binding was carried out essentially according to the polyethylene glycol precipitation procedure described by Cuatrecasas (12).

Characterization of ^{125}I-insulin-receptor complexes and their subunits-The insulin receptor-containing pooled Peak II samples from the DEAE-Sephacel ion exchange chromatography were dialyzed overnight at 4°C, incubated with ^{125}I-insulin and the insulin-receptor complexes were covalently cross-linked by the addition of disuccinimidyl suberate according to the procedure of Pilch and Czech (13), as used previously by us (11). The crosslinked, concentrated samples were dialyzed overnight at 4°C and were chromatographed on a Sepharose CL-6B column exactly as described previously (11). Radioactive protein peak fractions were separately pooled, and the pooled samples were dialyzed and subjected to SDS-PAGE electrophoresis as previously reported (11).

Specificity of insulin binding to rat glomerular and tubular insulin receptors- Glomeruli or tubules used directly in binding studies were resuspended in Buffer A composed of 1% Triton X-100 in 0.25 M sucrose, 5 mM Tris-HEPES, pH 7.5, 2.5 mM EDTA, 5 mM N-ethylmaleimide and 100 mM KCl. Following a 60 min incubation at 4°C, the samples were centrifuged at 35,000 g for 15 min to remove iron oxide particles and insoluble basement membrane material, and the solubilized material recovered in the supernatant solution was used in binding studies. Solubilized renal tissue was incubated in plastic microfuge tubes containing the following substances in a final volume of 354 µl: glomerular or tubular tissue extract (40-80 µg protein/sample), [^{125}I]insulin (0.5 ng/ml, 0.05 µCi/sample), plus or minus unlabelled insulin, in buffer A. Samples incubated with 40 µg/ml unlabelled insulin were used to assess non-specific binding and specific binding was calculated after subtraction of non-specific binding values. The reaction was stopped by addition of 667 µl of 25% polyethylene glycol and 667 µl of 0.1% human gamma globulin. The samples were placed in an ice bucket for 30 min and duplicate aliquots of 600 µl were removed, placed in plastic microfuge tubes containing 0.5 ml of a mixture of dinonylphthalate:dibutylphthalate (1:5, v:v) and centrifuged at 12,000 g for 3 min. An aliquot of the supernatant solution was counted to measure unbound [^{125}I]insulin and the pellet, containing bound radioligand, was counted after removal of the supernatant and underlying oil layer by aspiration. The tip of the plastic microfuge tube was cut off with a razor blade and counted in a gamma counter to minimize the contribution of [^{125}I]insulin binding to the tube.

In cross-linking studies utilizing intact renal membranes, plasma membranes were partially purified from the glomeruli or tubules by sonication and centrifugation essentially as described previously by us for cerebral microvessels (14). The membranes were then cross-linked with ^{125}I-insulin, solubilized in SDS with or without dithiothreitol and subjected to electrophoresis on a 7.5-15% gradient polyacrylamide gel followed by autoradiography to visualize the ^{125}I-insulin-receptor complexes (14).

RESULTS: Solubilization of glomerular and tubular fractions in 1% Triton X-102, followed by fractionation of the detergent-soluble components by DEAE-Sephacel ion exchange chromatography yielded similar elution profiles of proteins and insulin-binding components for both glomerular (Fig. 1A) and tubular (Fig. 1B) samples. In both preparations, the insulin-binding components (receptors) were found in the peak II fractions eluted with 0.6 M NaCl. About 8-fold purification was achieved for the insulin receptors of both types of tissue by DEAE-Sephacel ion exchange chromatography.

The insulin receptor-containing Peak II fractions were separately pooled, cross-linked with ^{125}I-insulin and subjected to Sepharose CL-6B gel filtration chromatography for the separation and characterization of ^{125}I-insulin-receptor complexes. From both glomeruli (Fig. 2A) and tubules (Fig. 2B), three radiolabeled protein peaks corresponding to molecular weights of 560KDa (Peak A), 220KDa (Peak B) and 95KDa (Peak C) were found. Among the three ^{125}I-insulin-labeled complexes found in both glomeruli and tubules, the 95KDa complex was present in the largest amounts. The radioactive peaks eluting at a volume of > 150 ml represented free ^{125}I-insulin and ^{125}I-insulin degradation products and were discarded. The fractions of each radiolabeled protein peak

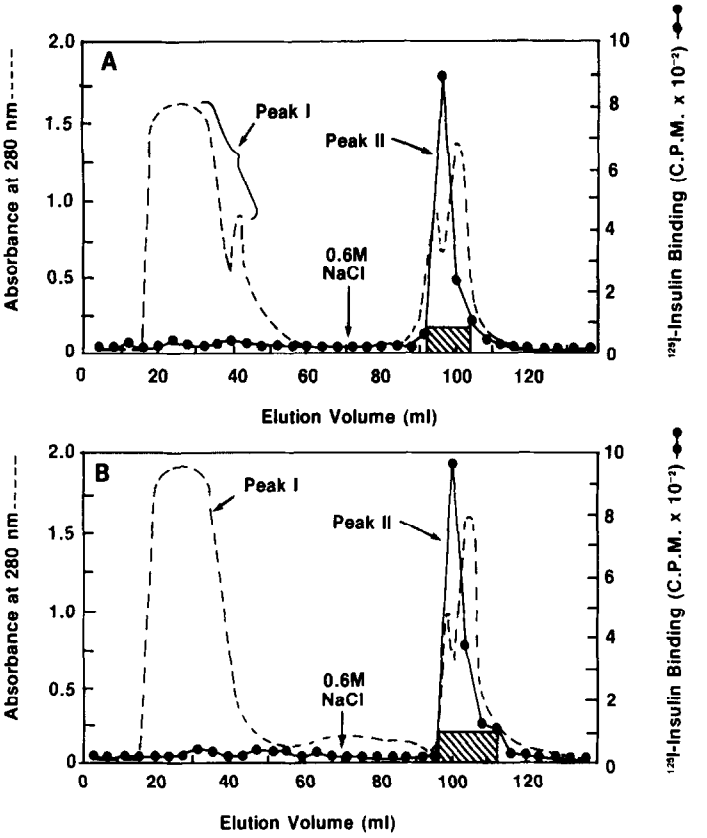

Fig. 1. Fractionation of Triton X-102-solubilized glomerular and tubular membrane proteins on DEAE-Sephacel ion exchange chromatography. Isolated renal glomeruli (A) or tubules (B) were solubilized in detergent and applied to a DEAE-Sephacel ion exchange column (1.6 x 15 cm). The column was washed with 70 ml of starting buffer (50 mM sodium phosphate, pH 7.6, containing 0.05% Triton X-102), followed by 70 ml of starting buffer containing 0.6 M NaCl. The flow rate was 8 ml/h and 4 ml fractions were collected and monitored at 280 nm, as well as for insulin binding activity.

in Fig. 2 (hatched bars) were separately pooled, dialyzed, and concentrated for the characterization of ^{125}I-insulin-binding subunits by SDS-PAGE.

After electrophoresis, the gels were stained with Coomassie brilliant blue dye (Figs. 3 and 4, Panel 1), then dried and autoradiography was performed. Gels run in the presence of DTT (Figs. 3 and 4, Panel I) revealed distinct differences in the polypeptide profiles of Peaks A, B and C from glomerular (Fig. 3) and tubular (Fig. 4) samples. These differences within each tissue fraction represent the separation of different polypeptide components on the Sepharose CL-6B column. The differences between the polypeptide pattern of comparable fractions from the glomerular and tubular samples are representative of the distinctive protein composition of each of these nephron subunits. Despite the polypeptide diversity of these tissue fractions, autoradiograms of the gels show a limited and distinct pattern of

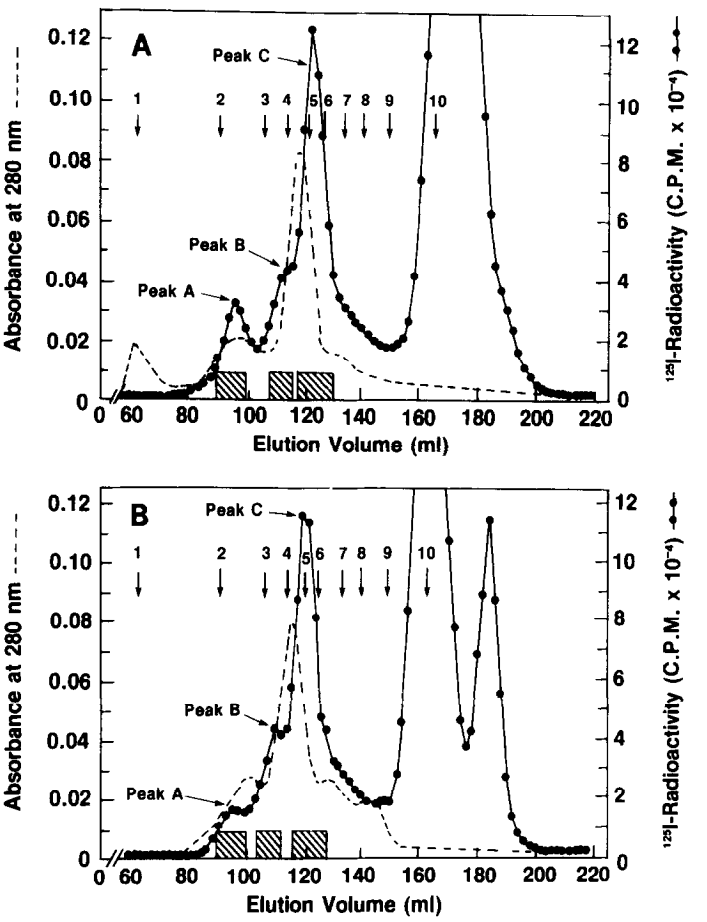

Fig. 2. Fractionation of cross-linked ^{125}I-insulin-protein complexes on Sepharose CL-6B. Peak II samples from the DEAE-Sephacel ion exchange chromatography column (Fig. 1) were pooled, dialyzed against Krebs-Ringer phosphate buffer and incubated with 5 µCi ^{125}I-insulin. The cross-linker dissuccinimidyl suberate was added to covalently couple ^{125}I-insulin to proteins and the samples were subsequently dialyzed against column buffer (50 mM sodium phosphate, pH 7.6, 0.1 M NaCl, 0.05% Triton X-102). The samples were applied to Sepharose CL-6B columns (1.6 x 95 cm) which had been pre-calibrated with molecular weight markers (1, Blue dextran; 2, thyroglobulin; 3, ferritin; 4, catalase; 5, aldolase; 6, BSA; 7, ovalbumin; 8, chymotrypsinogen; 9, RNase-A; 10, insulin). The flow rate was 4 ml/h and 2 ml fractions were collected, absorbancy was monitored at 280 nm and radioactivity was counted in a gamma counter for the presence of [^{125}I]-insulin.

^{125}I-labeled components which are strikingly similar for each fraction between glomeruli and tubules (Figs. 3 and 4, Panel II).

All three peaks depicted in Fig. 2 from the glomerular (Fig. 3, Panel II) and tubular (Fig. 4, Panel II) samples contain a prominent band of [^{125}I]-insulin cross-linked protein-complex of 55KDa. Furthermore, for the 220KDa and 95KDa complexes of both tissues (lanes B and C), this represents the only protein species intensively labelled. The 560KDa complex of glomerular origin

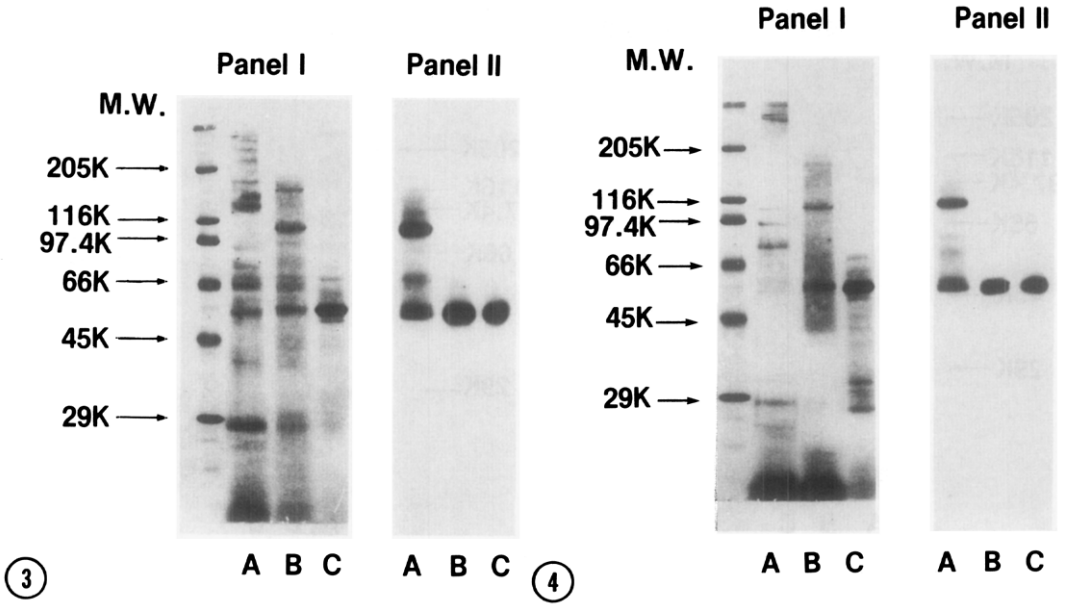

Fig. 3. Characterization of covalently cross-linked ^{125}I-insulin-protein complexes from glomerular tissue by SDS-PAGE. Peaks A, B and C from the Sepharose CL-6B column described in Fig. 2A were concentrated, dialyzed and 1% SDS plus 40 mM DTT were added to 100 µl aliquots, which were then applied to a 7.5-15% polyacrylamide slab gel. Following electrophoresis, the gel was stained with Coomassie brilliant blue (Panel I) and autoradiography was performed with the dried gel (Panel II). The positions of molecular weight markers (myosin, β-galactosidase, phosphorylase b, BSA, ovalbumin and carbonic anhydrase) are indicated.

Fig. 4. Characterization of covalently cross-linked ^{125}I-insulin-protein complexes from tubular tissue by SDS-PAGE. Peaks A, B and C from the Sepharose CL-6B column described in Fig. 2B, containing detergent-solubilized tubular proteins cross-linked with ^{125}I-insulin, were studied on SDS-PAGE as described in Fig. 3. Panel I, Coomassie brilliant blue stain; Panel II, autoradiography.

(Fig. 3, Panel II, lane A), on the other hand, also contains a major binding component of 115KDa, as well as several minor components (130KDa, 120KDa and 68KDa). By comparison, the tubular 560KDa complex (Fig. 4, Panel II, lane A) contains a very similar array of ^{125}I-labelled components, the most prominent being 115KDa and 55KDa. There is a weakly labelled band of 75KDa which is not labelled in the glomerular sample (Fig. 3, Panel II, lane A).

These samples were concomittantly studied by gel electrophoresis run in the absence of DTT to further characterize their ^{125}I-insulin-binding components (Figs. 5 and 6). The Coomassie brilliant blue stain of the glomerular fractions (Fig. 5, Panel I) reveals a much different pattern than that observed in the presence of DTT (Fig. 3), especially for the 560KDa species (lane A), suggesting that disulfide-linked protein complexes are present in this fraction. The tubular fractions (Fig. 6, Panel I) gave a unique pattern in the absence of DTT, quite different than that observed in the presence of

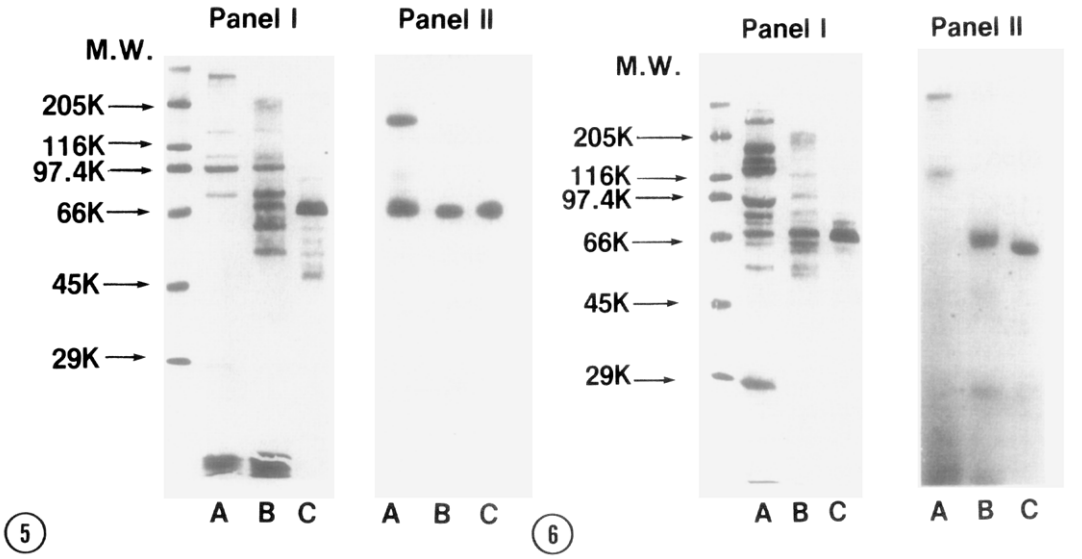

Fig. 5. Characterization of covalently cross-linked ^{125}I-insulin-protein complexes from glomerular tissue by SDS-PAGE in the absence of DTT. Detergent-solubilized glomerular proteins cross-linked with ^{125}I-insulin were collected in Peaks, A, B and C (Fig. 2A) and were subjected to SDS-PAGE in the absence of DTT as described in Fig. 3. Panel I, Coomassie brilliant blue stain; Panel II, autoradiography.

Fig. 6. Characterization of covalently cross-linked ^{125}I-insulin-protein complexes from tubular tissue by SDS-PAGE in the absence of DTT. Detergent-solubilized tubular proteins cross-linked with ^{125}I-insulin were collected in Peaks A, B and C (Fig. 2B) and were subjected to SDS-PAGE in the absence of DTT as described in Fig. 4. Panel I, Coomassie brilliant blue stain; Panel II, autoradiography.

DTT (Fig. 4, Panel I). The 560KDa species, in particular, showed numerous high molecular weight protein bands, some of which are absent in the presence of DTT (Fig. 4). The autoradiogram of glomerular tissue fractions run in the absence of DTT (Fig. 5, Panel II) shows that all three fractions contain a 68KDa ^{125}I-insulin-binding species, while the 560KDa fraction (lane A) also contains a prominently labelled band of 170KDa. In contrast, the tubular tissue fractions (Fig. 6, Panel II) show three distinct patterns of labelled material. The 560KDa species (lane A) contains labelled bands of 125KDa and > 250KDa, both of which differ from the glomerular material presented in Fig. 5. The 220KDa and 95KDa species (lanes B and C) show a labeled band of 70KDa and 66KDa, respectively. It is also interesting to note that of all the fractions tested, only the tubular 560KDa fraction run in the absence of DTT fails to show prominent labelling of a 66-70KDa species.

In order to facilitate comparison of renal glomerular and tubular insulin receptors with each other and with insulin receptors from other tissues, additional binding and cross-linking studies were conducted with renal

Table I

Insulin binding by rat renal glomeruli and tubules

Tissue	Unlabelled Insulin	[^{125}I]Insulin Bound (pg)
Glomeruli	-	0.90 ± 0.05
	+	0.20 ± 0.02
Tubules	-	1.29 ± 0.07
	+	0.15 ± 0.08

Rat renal tissues were solubilized in buffer containing 1% Triton X-100 and equivalent amounts of solubilized protein (42 µg protein per sample) were incubated for 90 min at 22°C in the presence of [^{125}I]insulin (0.05 µCi/sample) with or without the addition of unlabelled insulin (40 µg/ml). Data represent the mean ± S.E. for triplicate samples.

glomerular and tubular membrane proteins which had not been partially purified by size-exclusion and ion exchange chromatography. In order to evaluate the impact of detergent-solubilization on renal tissue insulin binding specificity, renal glomeruli and tubules were solubilized in 1% Triton X-100 and [^{125}I]insulin binding studies were conducted in the presence of this detergent (Table I). Interestingly, both renal tissues displayed specific insulin binding activity, with the relative amount of hormone binding per mg of protein in both tissues being comparable or greater to that seen in preparations of intact glomeruli and tubules (1-3). Glomerular tissue solubilized in 1% Triton X-100 showed approximately 30% less [^{125}I]insulin binding capacity per mg protein than tubular tissue treated in the same manner (Table I), as was shown previously for the intact nephron fractions (1-3).

In order to examine insulin receptor size in tissue that is not solubilized in detergent and is minimally subjected to proteolysis by endogenous enzymes, renal glomerular and tubular membranes were isolated and incubated with [^{125}I]insulin in buffer that did not contain any detergent. Covalent cross-linking of [^{125}I]insulin to its receptor on renal glomerular and tubular membranes was performed as described previously (11). In the absence of unlabelled insulin, both renal glomerular and tubular membranes were weakly, but specifically, labelled with [^{125}I]insulin (Fig. 7). The apparent molecular weight of the insulin binding subunits of the insulin receptors in the presence of 40 mM dithiothreitol were approximately Mr = 130,000 for both tissues, and samples run in the presence of 4 mM or 0 mM dithiothreitol showed more prominent receptor complexes of Mr > 230,000. These results (Fig. 7) suggest that the original renal tissue preparations used throughout this study contain insulin receptors which behave in covalent cross-linking studies precisely the same way that insulin receptors from other tissue sources behave (11,13-16), whereas partially purified, detergent-

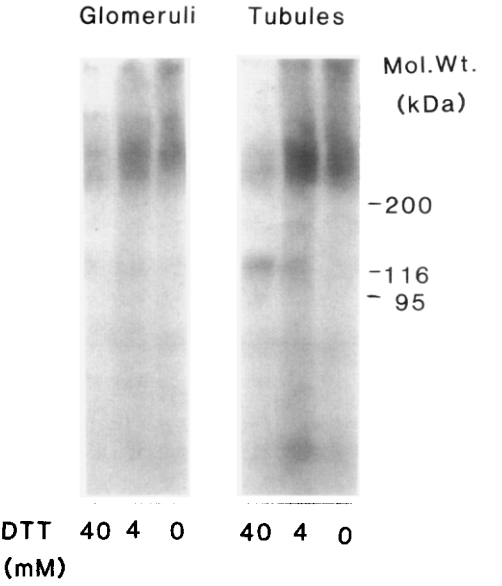

Fig. 7. Characterization of covalently cross-linked ^{125}I-insulin-protein complexes from intact glomerular and tubular tissue by SDS-PAGE in the presence and absence of DTT. Renal membranes were isolated, incubated with 0.5 μCi ^{125}I-insulin in the absence of unlabelled insulin and ^{125}I-insulin-receptor complexes were cross-linked and subjected to SDS-PAGE in the presence of 0, 4 and 40 mM DTT.

soluble renal insulin receptors contain an insulin-binding α-subunit that is somewhat smaller in molecular size (Figs. 3-6). Nevertheless, the molecular size of the α-subunits of the insulin receptor isolated from glomerular and tubular renal fractions are identical.

DISCUSSION: The molecular structure of the insulin receptor has been the subject of intensive study resulting in the characterization of the receptor as a membrane glycoprotein composed of two α-subunits of Mr 115,000-135,000 and two β-subunits of Mr 87,000-95,000 (15). The subunits are linked by disulfide bonds and can exist in multiple redox states (16,17), including a number of high molecular weight forms (18). Additional putative receptor subunits of various molecular weights including 85,65 and 40KDa have also been reported to be associated with the insulin receptor (17,19-22). Some of these lower molecular weight polypeptide components may represent fragments of α- or β-subunits produced by proteolytic cleavage, since the β-subunit in particular is extremely sensitive to such cleavage, producing a β_1-fragment which remains disulfide-linked to the receptor complex and a free β_2-fragment (15).

The present results on the isolation and characterization of the renal glomerular and tubular insulin receptors reveal a composite picture of the subunit composition of these receptors in two kidney subfractions which is complicated by the multiple cell types present in the glomerulus and the

asymmetric distribution of high-affinity hormone binding sites in the renal tubule. Nevertheless, there is a remarkable similarity and a surprising simplicity in the polypeptide patterns of ^{125}I-insulin-crosslinked components in the two nephron subunits. Interestingly, on gel filtration chromatography of the crosslinked ^{125}I-insulin-binding fraction, the bulk of the ^{125}I-insulin was found not with a component in the high-molecular weight range associated with an intact insulin receptor (Mr ~ 440,000), but rather with a fairly low molecular weight fraction (Mr = 95,000) (Fig. 2). Smaller amounts of ^{125}I-insulin were associated with fractions corresponding to proteins or protein complexes of Mr = 560,000 and Mr = 220,000, the former being large enough to include a complete high-affinity insulin receptor (Fig. 2).

Gel electrophoresis of ^{125}I-insulin-labelled fractions from the gel filtration columns revealed a strong similarity in the low molecular weight protein species that are found in the 560, 220, and 95 KDa fractions from both glomerular (Figs. 3, 5) and tubular (Figs. 4, 6) samples. Both samples contain an insulin binding component (55 KDa) in the presence of DTT. While the identity of this 55 KDa protein is not known, it is possible that this component could represent a fragment of the insulin receptor produced by the action of endogenous protease activity. This interpretation of the results is supported by the subsequent demonstration that cross-linking studies conducted with intact renal membrane fractions failed to reveal such a 55 kDa labelled species (Fig. 7). Alternatively the 55 kDa component could constitute a low molecular weight, low affinity binding protein which may be responsible for the low-affinity, high-capacity ^{125}I-insulin binding sites present in renal tissue. However, the results described above in Fig. 7 fail to reveal the presence of such a component in renal membranes which had not been previously solubilized in detergent and partially purified by DEAE-Sephacel ion exchange chromatography. Hence it seems most likely that the 55 kDa component (Figs. 3 and 4, Panel II) and the 66 kDa component (Figs. 5 and 6, Panel II) represent proteolytic fragments of an insulin receptor complex which is found in all three of the radiolabelled peaks separated by Sepharose CL-6B chromatography (Fig. 2). In contrast, the electrophoretic mobility of the high molecular weight ^{125}I-insulin-receptor complex (Fig. 2, Peak A), is consistent with the known electrophoretic mobility of the high-affinity insulin receptor α-subunit when evaluated on dodecyl sulfate polyacrylamide gels (Figs. 3-6, Panel II, lane A). This electrophoretic mobility and the fact that ^{125}I-insulin could be successfully cross-linked to these renal fractions is consistent with the conclusion that a genuine high-affinity insulin receptor is present in these glomerular and tubular extracts, but definitive proof of this conclusion requires either immunological or sequencing data.

While the Coomassie brilliant blue profiles of glomerular vs. tubular proteins presented in Figs. 3-6 effectively demonstrate that these two renal subfractions contain distinctive profiles of detergent soluble proteins, indicating that tissue cross-contamination is minimal, the more intriguing observation is that the autoradiograms of glomerular vs. tubular fractions, derived from the 560KDa peak of the gel filtration column (Fig. 2), show similar ^{125}I-labeled subunit profiles in the presence of dithiothreitol (Figs. 3 and 4, Panel II, Lane A) but distinctly different patterns in the absence of the reducing agent (Figs. 5 and 6 Panel II, Lane A). When fully reduced, both glomerular and tubular samples contain a distinct ^{125}I-labeled component, 115 KDa, which is consistent with the α-subunit of the insulin receptor (Figs. 3 and 4, Panel II, Lane A). In the nonreduced state, however, the glomerular sample has a prominently labeled band at 170 KDa as well as another distinct band at 68 KDa (Fig. 5, Panel II, Lane A), while the tubular sample contains a distinct high molecular weight component at > 250 KDa and another component at 115 KDa, with no labeled component present in the region of 68 KDa (Fig. 6, Panel II, Lane A). The latter component is not only prominent in the glomerular sample derived from the 560 KDa gel filtration fraction, but is also present in both the glomerular and tubular samples derived from the 220 KDa and 95 KDa gel peaks. The distinct difference in the multimeric subunit structure of the nonreduced insulin receptor in glomeruli and tubules provides a possible structural basis for the affinity differences between these two nephron fractions reported previously by us (1-3). Whether this structural difference is significant in terms of the possible physiological roles of high-affinity and low-affinity receptor sites for insulin in renal glomeruli and tubules will require further investigation. Nevertheless the current study is the first direct comparison of the insulin receptor in isolated glomeruli and tubules and reveals a unique duality of hormone binding components within a single organ.

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