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IGF-II receptors in luminal and basolateral membranes isolated from pars convoluta and pars recta of rabbit proximal tubule

Christian Jacobsen [a,*], Henrik Jessen [a], Allan Flyvbjerg [b]

[a] *Department of Medical Biochemistry, University of Aarhus, Ole Worms Allé, Bldg. 170, DK-8000 Aarhus C, Denmark*
[b] *Institute of Experimental Clinical Research and Medical Department M, Aarhus Kommunehospital, DK-8000 Aarhus C, Denmark*

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Abstract

The binding of ^{125}I-labeled insulin-like growth factor-II (^{125}I-IGF-II) to luminal and basolateral membrane vesicles isolated from pars convoluta and the straight part (pars recta) of rabbit proximal tubule was investigated. Analyses of the binding data by use of the general stoichiometric binding equation revealed, that in all preparations IGF-II was bound to one high-affinity binding site and other sites with lower affinities. The specificity of the high-affinity ^{125}I-IGF-II binding to the membrane vesicles assessed by displacement by unlabeled IGF-II, IGF-I and insulin showed that IGF-I displaced ^{125}I-IGF-II in the range 22.5–47.9 nM (IC_{50}) whereas insulin did not effect ^{125}I-IGF-II binding at all. β-Galactosidase inhibited the ^{125}I-IGF-II binding with half-maximal inhibition of 20–30 nM β-galactosidase. D-Mannose 6-phosphate increased the binding of ^{125}I-IGF-II and reversed the inhibitory effect of β-galactosidase. Analyses of ^{125}I-IGF-II binding curves in the presence of β-galactosidase or D-mannose 6-phosphate demonstrated that none of these compounds changed the binding affinity of ^{125}I-IGF-II for the membrane vesicles. The IGF-II/M6P receptor content in the luminal membranes was in the range 0.21–0.34 pmol IGF-II/M6P receptor per mg protein and very low compared to 2.27–2.86 pmol IGF-II/M6P receptor per mg protein in basolateral membranes.

Keywords: Insulin-like growth factor II; Receptor; Proximal tubule membrane; (Rabbit)

1. Introduction

The IGF-II receptor and the cation-independent mannose 6-phosphate receptor are the same protein (the IGF-II/M6P receptor) [1]. The cation-independent mannose 6-phosphate receptor plays a role in transport of soluble glycoproteins synthesized in the endoplasmic reticulum [2] whereas in the membrane the IGF-II/M6P receptor function is to internalize exogenous lysosomal enzymes and IGF-II [2,3]. In contrast to M6P lysosomal enzymes impair the IGF-II binding, and IGF-II inhibits lysosomal enzyme binding [1,4–6]. However, the molecular mechanism of this effect is still a controversial question [5,7]. Another aspect, which is still disputed, is whether the receptor mediates signal transduction or only functions in the transport of lysosomal enzymes, IGF-II, and other ligands [2]. It has been suggested that IGF-II/M6P receptor has two distinct signaling functions that positively or negatively regulate the activity of GTP-binding protein ($G\alpha_i$) in response to IGF-II or M6P [8,9]. Likewise, a possible role in signal transduction by increasing inositol triphosphate also has been reported for renal proximal tubular basolateral membranes from mongrel dogs [10].

The majority of cellular IGF-II/M6P receptor has been reported to be localized intracellularly whereas only a smaller proportion of the total receptor pool is at the cell surface [3]. Examination of the latter by vesicle studies showed that the IGF-II/M6P receptors are distributed symmetrically in luminal and basolateral membranes from the proximal tubule of mongrel dog kidney. By contrast, similar experiments revealed that the IGF-I receptors are located primarily at the basolateral cell membrane [11]. Likewise, we have previously reported a 10-fold higher binding capacity for IGF-I to basolateral than to luminal membrane vesicles isolated from either the convoluted part

Abbreviations: IGF-II, insulin-like growth factor-II; IGFBP, IGF-binding proteins; M6P, D-mannose 6-phosphate; IGF-II/M6P, insulin-like growth factor II/D-mannose 6-phosphate.

* Corresponding author. E-mail: cj@biokemi.aau.dk. Fax: +45 86131160.

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(pars convoluta) or the straight part (pars recta) of rabbit proximal tubule [12]. These findings correspond to results obtained by electron microscope autoradiography of isolated, perfused proximal tubules after basolateral exposure [12]. However, recent studies on rat renal cells have indicated that the IGF-II/M6P receptor is located primarily in the pars convoluta and mainly in components of the vacuolar system in the apical cytoplasm of the proximal convoluted tubule cells [13]. Evidently, there seems to exist a discrepancy in the observations of the renal IGF-II/M6P receptor distribution in luminal and basolateral membranes. In addition, the segmental localization of the receptors in the proximal tubule needs to be further examined since it is rather puzzling that the IGF-II/M6P receptors should be confined only to the pars convoluta and not the pars recta. Thus, the purpose of the present study was to examine the distribution of IGF-II binding and IGF-II/M6P receptors in luminal and basolateral membranes from the pars convoluta and pars recta of rabbit proximal tubule. The presence of IGF-II/M6P receptors was verified by studying the displacements of ^{125}I-IGF-II with unlabelled IGF-II, IGF-I and insulin. Furthermore, the interactions of M6P and β-galactosidase with the ^{125}I-IGF-II binding to the receptor in membrane associated environments were investigated.

2. Material and methods

2.1. Materials

(3-[^{125}I]Iodotyrosyl)insulin-like growth factor-II (^{125}I-IGF-II) with a specific activity of 2000 Ci/mmol (purchased from Amersham International, Amersham, Bucks, UK) was used in all experiments. Unlabeled biosynthetic IGF-I and IGF-II were purchased from Bachem Bioscience, Bubendorf, Switzerland, unlabeled insulin was obtained from Novo-Nordisk A/S, Bagsværd, Denmark. β-Galactosidase (EC 3.2.1.23) isolated from bovine testes with activity of 3.0 U/mg (one unit will hydrolyze 1.0 μmol of *o*-nitrophenyl-β-D-galactoside to *o*-nitrophenol and D-galactose per min at pH 4.4 at 25° C) and D-mannose 6-phosphate were obtained from Sigma, St. Louis, MO, USA.

2.2. Preparation of luminal and basolateral membrane vesicles from pars convoluta and pars recta of rabbit proximal tubule

Adult male and female rabbits weighing approx. 3–4 kg were killed by a blow to the neck, exsanguinated and the kidneys were excised as previously described [14,15]. Luminal and basolateral membrane vesicles were isolated from pars convoluta (cortex corticis) and from pars recta (outer stripe of outer medulla) of the proximal tubule as previously described [15]. Briefly, the outer cortical tissue was obtained by taking slices < 0.4 mm thick from the surface of the kidney. Strips of outer medulla approx. 1 mm thick (representing predominantly pars recta) were dissected from outer stripe of outer medulla. The tissue was homogenized and luminal and basolateral vesicles were prepared by differential centrifugation and by Mg^{2+} precipitation analogous with Ca^{2+} precipitation as described in [15]. The purity of the membrane vesicle preparations was examined by electron microscopy and by measuring specific activities of various enzyme markers [14]. The activities of the enzyme markers in the luminal membrane vesicle fractions were enriched as compared with the corresponding homogenates by the following factors ($n = 15$): 12.4 ± 1.8-fold (alkaline phosphatase), 31.5 ± 2.7-fold (leucine aminopeptidase), and 9.4 ± 2.4-fold (maltase). Average enrichments in specific activity of the basolateral marker, Na^+/K^+-stimulated ATPase, and that of the mitochondrial marker, succinate dehydrogenase, were in all cases < 0.4 and < 0.04, respectively. The amount of protein was determined as described by Lowry et al. [16] with modification as described by Peterson [17] and using bovine serum albumin as a standard. All solutions used in this study were sterilized before use.

2.3. Binding studies

Membrane vesicles prepared from the four regions, i.e., luminal and basolateral membrane vesicles from pars convoluta and pars recta, respectively, were tested for radiolabeled IGF-II binding. The protocol for binding experiments were essentially as described previously [12]. Binding experiments were performed in duplicate in 1.5 ml polypropylene microfuge tubes. The buffer system was a HBS buffer consisting of 140 mM NaCl, 5 mM KCl, 5.5 mM $CaCl_2$, 10 mM Hepes, 10 mM glucose, 1% BSA, 0.01% bacitracin, and unless otherwise stated pH 7.1. The incubation mixture consisted of 50 μl membrane suspension, 425 μl of buffer solution with unlabeled peptide or other competitive or non-competitive agent or buffer alone, and 25 μl of ^{125}I-IGF-II (approx. 10^{-11} M, 20 000–30 000 cpm). The final protein concentration in basolateral and in luminal membrane suspension were 0.1 and 1 mg/ml, respectively. Following 20 h incubation at 4° C membrane bound, radiolabeled peptide was separated from free peptide by centrifugation ($7500 \times g$, 5 min) in a Beckman Microfuge B. The supernatant was aspirated and the tube and the pellet were gently washed with ice-cold binding buffer (0.5 ml). The tip of the tube with the pellet was cut off and bound and free fractions were counted in a LKB, Wallac γ-counter. The specific binding was calculated as the difference between the amount of ^{125}I-IGF-II bound in the absence (total binding) and the presence of 1 μM unlabeled IGF-II (non-specific binding). The degradation of the tracer in the incubation medium was routinely checked at the end of the incubation period by precipitation with 15% (wt/vol) TCA. Degradation of ^{125}I-IGF-II

was 11% (luminal convoluta), 8% (luminal recta), 5% (basolateral convoluta) and 5% (basolateral recta) after incubation of the membranes 20 h at 4° C. The optimal conditions for ^{125}I-IGF-II binding to luminal and basolateral membrane vesicles occurred after incubation for 20 h at pH 7.1 at 4° C. Accordingly all binding studies were conducted at 20 h at pH 7.1 at 4° C.

2.4. Binding parameters

The general stoichiometric binding equation may be formulated as:

$$r = \frac{K_1 c + 2K_1K_2c^2 + ... + NK_1K_2...K_N c^N}{1 + K_1 c + K_1K_2c^2 + ... + K_1K_2...K_N c^N} \quad (1)$$

where r = average number of moles of ligand bound per mol of protein ($(\text{ligand})_{\text{bound}}/(\text{Pr})_{\text{total}}$); c = equilibrium concentration of ligand ([ligand]); N = the maximum value of r, i.e., when $c \rightarrow \infty$. K_j = stoichiometric binding constant for step j as described by Adair and Klotz (see Ref. [18]). The equation can be transformed into:

$$r = \sum_{j=1}^{N} \frac{cK_j}{1 + cK_j} \quad (2)$$

Simplifying Eq. (2) to only two terms, the equation is equivalent to a two sites binding model:

$$\text{IGF-II}_{\text{bound}} = \frac{n_1(\text{Pr})_{\text{total}}K_1[\text{IGF-II}]}{1 + K_1[\text{IGF-II}]} + \frac{n_2(\text{Pr})_{\text{total}}K_2[\text{IGF-II}]}{1 + K_2[\text{IGF-II}]} \quad (3)$$

Equivalent to:

$$\text{IGF-II}_{\text{bound}} = \frac{B_{\text{max.1}}[\text{IGF-II}]}{K_{\text{diss.1}} + [\text{IGF-II}]} + \frac{B_{\text{max.2}}[\text{IGF-II}]}{K_{\text{diss.2}} + [\text{IGF-II}]} \quad (4)$$

$K_{\text{diss.}}$ = stoichiometric dissociation constant. The equation is sometimes named a two terms Scatchard. In this case $B_{\text{max.1}}$ and $B_{\text{max.2}}$ denote the concentrations of binding sites. In case of $N = 1$ equivalent to a one site binding model the above equation is further simplified:

$$\text{IGF-II}_{\text{bound}} = \frac{B_{\text{max}}[\text{IGF-II}]}{K_{\text{diss}} + [\text{IGF-II}]} \quad (5)$$

The Eqs. (4) and (5) were fitted to the data by finding the parameters, i.e., B_{max}, $K_{\text{diss.}}$ (Eq. 5) or $B_{\text{max.1}}$, $K_{\text{diss.1}}$, $B_{\text{max.2}}$, $K_{\text{diss.2}}$ (Eq. (4) that cause the equations to best fit the data using nonlinear regression. Iterations were continued until minimum of squares of the residuals were reached. The norm represents the closeness of the fit of the iteration. Numerically, it is the square root of the sum of squares of the residuals.

2.5. Competitive binding parameters

$$\text{IGF-II}_{\text{bound}} = \frac{A - D}{1 + ([X]/\text{IC}_{50})^B} + D \quad (6)$$

Eq. (6) is a four parameter logistic function describing a sigmoid curve. X is the concentration of unlabeled displacer. The parameters A is the maximal radioligand binding, i.e., when X is zero; D is the minimal radioligand binding, i.e., $X \rightarrow \infty$. B is the slope, and IC_{50} refers to the inflection point and thus to the concentration of unlabeled compound displacing 50% of the radiolabeled binding. A, B, D, and IC_{50} were estimated by non-linear curve fitting.

The IC_{50} values estimated from Eq. (6) were used for calculating the K_I, the dissociation constant of displacer by using the Cheng-Prusoff equation [19].

$$\text{IC}_{50} = K_I(1 + [\text{IGF-II}^*]/K_d) \quad (7)$$

IC_{50} is the concentration of the unlabeled compound that displaces 50% of radiolabeled ligand binding. [IGF-II*] is the concentration of the radiolabeled compound. K_d is the dissociation constant of radiolabeled compound.

3. Results

3.1. IGF-II binding to luminal and basolateral membrane vesicles

Luminal and basolateral membrane vesicles from pars convoluta and pars recta of rabbit proximal tubules were tested for IGF-II binding. Fig. 1 displays binding of IGF-II at a fixed membrane protein concentration. Unless otherwise stated the data are representative of at least three experiments, each performed in duplicate on separate occasions. The binding data obtained by luminal and basolateral membrane vesicles from pars recta are illustrated in a direct (inset) and a Scatchard plot. The binding data were analyzed by the two terms stoichiometric binding equation by using least-squares non-linear regression to a one site (Eq. (5)) or a two-sites binding model (Eq. (4)) [18]. Table 1 summarizes the binding parameters obtained by the approximations and the norm value, which represents the closeness of the fit of the iterations. The plots reveal that IGF-II binds to more than one site since a one site model should result in a straight line in the Scatchard plot. This is supported by the direct binding curves (inset) showing that no saturation phenomenon were obtained in any of the cases. It is seen from the Table that $K_{\text{diss.1}}$ parameters are within a range from 1 to 1.6 nM. As expected the norm values shown in Table 1 were essentially lower for a two-sites model compared to a one-site model. The curves seen in Fig. 1 have been drawn only on the basis of the parameters estimated by the two-sites binding model (Eq.

Table 1
IGF-II binding to luminal and basolateral membrane vesicles

	$B_{max.1}$	$K_{diss.1}$	$B_{max.2}$	$K_{diss.2}$	Norm (Eq. (5))	Norm (Eq. (4))
Vesicles from:						
Pars convoluta luminal	0.21	1.14	58.3	1185	0.356	0.244
Pars recta luminal	0.34	1.64	175	5748	0.471	0.260
Pars convoluta basolateral	2.86	1.63	166	1001	3.55	1.92
Pars recta basolateral	2.27	1.00	55	154	2.83	1.04

Binding parameters, $B_{max.}$ (pmol/mg) and $K_{diss.}$ (nM), were obtained by using nonlinear regression of Eqs. (5) and (4) to the experimental ^{125}I-IGF-II binding data.

(4)). It should be emphasized that the binding parameters, $B_{max.1}$, $K_{diss.1}$, $B_{max.2}$ and $K_{diss.2}$ are a set of constants causing the stoichiometric binding equation to fit the experimental data using nonlinear regression [18,20]. The dotted lines in Scatchard plot illustrate the high-affinity binding to the membranes and were drawn using the constants, $K_{diss.1}$ and $B_{max.1}$ given in Table 1. If $B_{max.1}$ corresponds to the high-affinity IGF-II/M6P receptor site concentration, as supported by the experiments described in the following, it means that luminal and basolateral membranes from pars recta contains 0.34 pmol and 2.27 pmol IGF-II/M6P receptor per mg protein, respectively. The corresponding values for luminal and basolateral membranes from pars convoluta were 0.21 pmol/mg and 2.86 pmol/mg.

3.2. Effects of IGF-I and insulin on IGF-II binding to luminal and basolateral membrane vesicles

In a series of experiments, we examined the specificity of the ^{125}I-IGF-II binding by measuring the displacement of radiolabeled IGF-II bound to luminal and basolateral membrane vesicles at increasing concentrations of unlabeled IGF-II, IGF-I or insulin in order to differentiate between the binding of IGF-II to IGF-II/M6P receptor and to other proteins. The results shown in Fig. 2 are from pars recta. The maximal ^{125}I-IGF-II binding was set to 100%. The IC_{50} values for IGF-II and IGF-I are summarized in Table 2. The IC_{50} values by homologous displacement by IGF-II were in the range 0.88–1.37 nM. By homologous displacement, the $K_I = K_d$, and since the tracer ^{125}I-IGF-II concentration is known (0.01 nM), this gives by insertion in Eq. (7) a $K_d = 1.0$ nM. This value is close to the mean value of the high-affinity constant estimated by a two terms stoichiometric binding equation. Furthermore, Fig. 2 and Table 2 show IC_{50} values of IGF-I in the range 22.5–47.9 nM with a mean value of 31.3 nM. By contrast, insulin even at a high concentration of 1 μM did not affect the ^{125}I-IGF-II receptor interaction. The figures clearly demonstrate that the ^{125}I-IGF-II is bound with high affinity to IGF-II/M6P receptors in both luminal and basolateral membranes as IGF-I displaced ^{125}I-IGF-II, but insulin did not effect ^{125}I-IGF-II binding at all.

3.3. Effects of β-galactosidase and D-mannose 6-phosphate on ^{125}I-IGF-II binding to IGF-II / M6P receptors

We examined the inhibitory effect of β-galactosidase on the ^{125}I-IGF-II binding to membrane vesicles by addition of increasing concentrations of β-galactosidase to the incubation mixture. In Fig. 3, the inset shows the data obtained by basolateral membrane vesicles from pars recta, but virtually similar patterns were obtained by the other membrane preparations (not shown). Binding of ^{125}I-IGF-II is expressed as a percentage of stock input radioactivity (25 000 cpm; concentration 27 pM), giving a total binding of 18% and a binding of 4.5% in the presence of 100 nM IGF-II. The concentration of β-galactosidase which under these conditions was required for half-maximal inhibition was determined from the curve as 20.5 nM. Likewise, half-maximal inhibition was found to be 20–30 nM β-galactosidase for the other membrane preparations (not shown). We found no differences in the results obtained by diverse batches of commercial available β-galactosidase from bovine testes. The inhibitory effect of β-galactosidase on ^{125}I-IGF-II binding was subsequently studied

Table 2
Specificity of ^{125}I-IGF-II binding

	IGF-II; IC_{50} (nM)	IGF-I; IC_{50} (nM)	Insulin	Norm (Eq. (6))
Vesicles from:				
Pars convoluta luminal	0.88	31.1	no dis.	10.3 ; 7.26
Pars recta luminal	0.98	47.9	no dis.	6.88; 12.5
Pars convoluta basolateral	1.37	23.7	no dis.	12.9 ; 7.55
Pars recta basolateral	0.90	22.5	no dis.	10.3 ; 8.74

The specificity of the binding to membrane vesicles was assessed by displacement by unlabeled IGF-II, IGF-I and insulin.
no dis., no displacement.

more closely. To clarify probable changes in high-affinity binding a number of ^{125}I-IGF-II binding isotherms in the presence of inhibiting concentrations of β-galactosidase

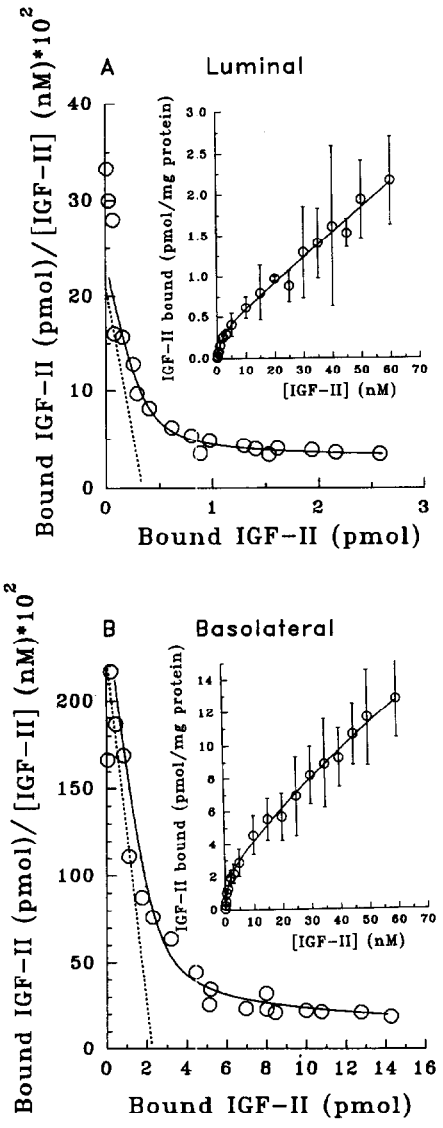

Fig. 1. Concentration dependence of IGF-II binding to vesicles isolated from luminal and basolateral membranes from pars recta of rabbit proximal tubule, depicted as Scatchard plot or as direct plots in insets. The incubation mixtures consisted of 50 μl membrane suspension, 425 μl of buffer solution with unlabeled peptide or other competitive or non-competitive agent or buffer, and 25 μl of ^{125}I-IGF-II (approx. 10^{-11} M, 20000–30000 cpm). Final protein concentrations were 0.1 mg/ml (basolateral) and 1 mg/ml (luminal) membrane suspension. After 20 h incubation at 4° C membrane bound, radiolabeled peptide was separated from free peptide by centrifugation. The specific binding was calculated as the difference between the amount of IGF-II bound in the absence (total binding) and the presence of 1 μM unlabeled IGF-II (non-specific binding). The lines through the points were drawn by approximating the binding data by using least-squares non-linear regression to a two terms stoichiometric binding equation (Eq. (4)). The data comprise of least three set of experiments, each perform in duplicate on separate occasions. Mean values ± S.D., are given. (A) Luminal membrane vesicles from pars recta; (B) basolateral membrane vesicles from pars recta.

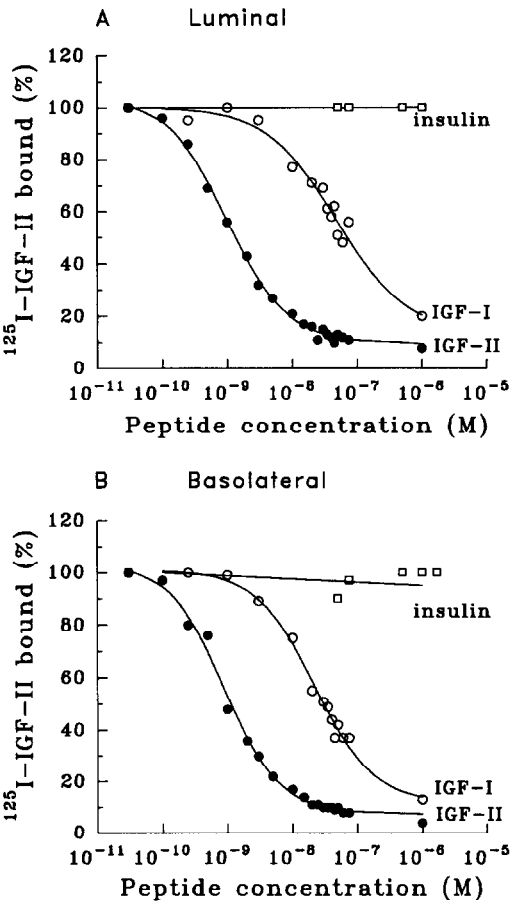

Fig. 2. Displacement of (3-[^{125}I]iodotyrosyl)IGF-II bound to luminal and basolateral membrane vesicles by increasing concentrations of unlabeled IGF-II, IGF-I and insulin. The maximal ^{125}I-IGF-II binding was set to 100%. Values are means of three set of experiments. (A) Luminal membrane vesicles from pars recta, (B) basolateral membrane vesicles from pars recta.

were generated. Fig. 3 shows binding of ^{125}I-IGF-II in the presence of 0 nM, 30 nM and 100 nM β-galactosidase to basolateral membrane vesicles from pars recta. Non-linear curve fitting of the data to Eq. (6), gave IC_{50} values of 1.57 nM, 1.80 nM and 2.30 nM, respectively. In another set of experiments with basolateral membrane vesicles from pars convoluta curve fitting revealed values 1.31 nM, 1.25 nM and 1.29 nM, respectively (not shown). All in all we did not observe any significant change in dissociation constant with increasing β-galactosidase.

The influence of M6P on the ^{125}I-IGF-II binding to IGF-II/M6P receptor was examined and a representative experiment is shown in Fig. 4. The maximal binding of a standard preparation was in this case 17% of added ^{125}I-IGF-II. By increasing concentrations of M6P the binding reached a level of 30% at approx. 1 mM M6P (upper curve). Half-maximal activation was calculated to 72 μM D-mannose 6-phosphate. The combined influence of β-galactosidase and D-mannose 6-phosphate on the IGF-II binding was investigated and shown in the lower curve. In

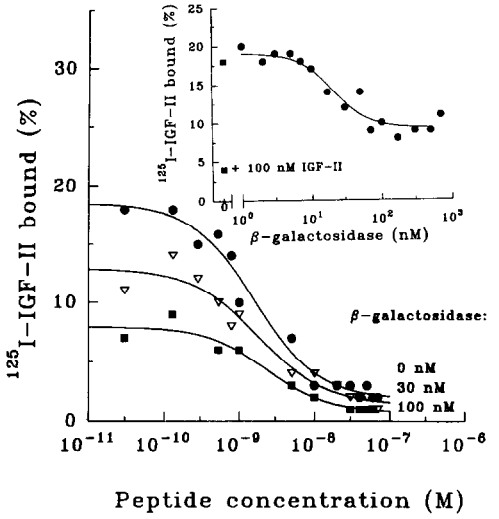

Fig. 3. Inhibitory effect of β-galactosidase on the ^{125}I-IGF-II binding to basolateral membrane vesicles. Binding of ^{125}I-IGF-II is expressed as percentage of input radioactivity (25000 cpm; concentration 27 pM) at three separate β-galactosidase concentrations: 0 nM (●), 30 nM (▽) and 100 nM (■) by increasing concentrations of unlabeled IGF-II or by increasing concentrations of β-galactosidase as shown in inset.

these cases 100 nM β-galactosidase were included in all samples. The figure shows that the inhibition of ^{125}I-IGF-II binding by β-galactosidase was reversed to approximately the starting value by M6P and completely abolished by a M6P concentration of 0.1 M.

The increase in ^{125}I-IGF-II binding to the receptor in the presence of 0 mM, 0.1 mM and 5 mM D-mannose 6-phosphate are shown in Fig. 5. The binding data were fitted to Eq. (6), and IC_{50} values calculated. All preparations showed a significant stimulation in ^{125}I-IGF-II binding. In the presence of 5 mM M6P the binding was increased from 20% to 30% for basolateral membrane preparations. The obtained IC_{50} values were virtually identical. This indicates that the M6P stimulation effect did not

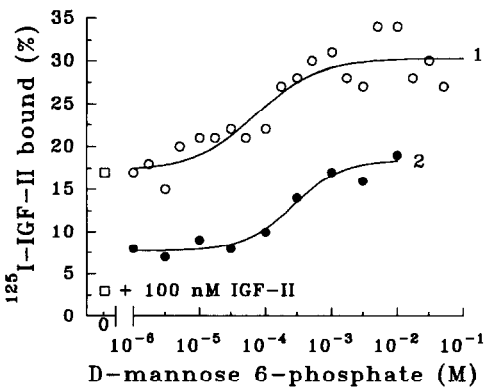

Fig. 4. Activation effect of M6P on ^{125}I-IGF-II binding to basolateral membrane vesicles. Representative experiments showing binding of ^{125}I-IGF-II is expressed as percentage of radioactivity (curve 1). Reversal of the inhibitory effect of β-galactosidase with M6P. In these experiments 100 nM β-galactosidase was included in all samples (curve 2).

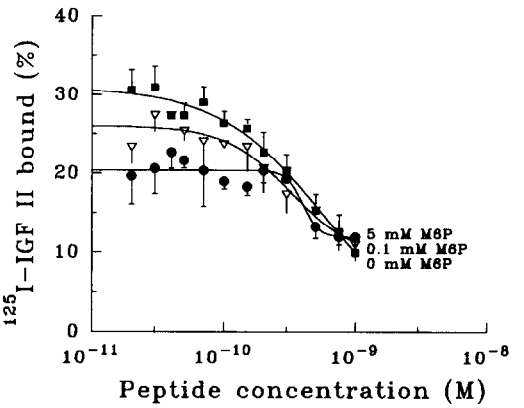

Fig. 5. ^{125}I-IGF-II binding in the presence of D-mannose 6-phosphate. Binding curves in the presence of 0 mM, 0.1 mM, and 5 mM M6P by increasing concentrations of unlabeled IGF-II are shown. The binding data are fitted to Eq. (6), the four parameter logistic function.

influence the affinity of IGF-II for the IGF-II/M6P receptor.

4. Discussion

Hammerman and Rogers observed an asymmetrical distribution of IGF-I receptors in isolated basolateral and brush-border membranes from mongrel dog kidney with localization being primarily on the basolateral side, whereas they found a symmetrical binding of ^{125}I-IGF-II in both types of membranes [11]. A recent study on rat renal cells claims that IGF-II/M6P receptors are located predominantly in pars convoluta and mainly in components of the vacuolar system in the apical cytoplasm of the proximal convoluted tubule cells [13]. Thus, the purpose of the present study was to examine the distribution of IGF-II binding and IGF-II/M6P receptors in luminal and basolateral membranes from pars convoluta and pars recta of rabbit proximal tubule. The binding curves illustrated in Fig. 1 and the corresponding values given in Table 1 revealed that a saturation level of IGF-II was never obtained. Consequently, it seems reasonable to conclude that IGF-II binds to more than one binding site. This is in accordance with our previous results which demonstrated that both luminal and basolateral membranes possess IGF-I receptors which, in addition to IGF-I, competitively bind IGF-II [12]. As mentioned previously, the binding parameters, $B_{max.1}$, $B_{max.2}$, $K_{diss.1}$ and $K_{diss.2}$ are one set of constants that causes the stoichiometric binding equation to fit the experimental data [18,20]. Expressed in Scatchard equation, $B_{max.1}$ and $B_{max.2}$ denote the concentrations of the first and following binding sites, respectively, and $K_{diss.1}$ is the high-affinity dissociation constant. Furthermore, it is evident from Fig. 1 and 2 that $K_{diss.1}$ represents the high-affinity IGF-II binding to IGF-II/M6P receptor as the relative potencies of IGF-II, IGF-I and insulin are in accordance with the characteristic of the affinities of these

ligands [1,21,22]. However, the binding patterns cannot definitely exclude the possibility that part of IGF-II is bound to IGF-binding proteins (IGFBPs), as IGFBPs (IGFBP-1, IGFBP-3, IGFBP-4) are characterized by binding IGF-I and IGF-II with almost same affinity or IGF-II with higher affinity than IGF-I (IGFBP-2, IGFBP-5, IGFBP-6) and do not bind insulin at all [23,24]. It should be noticed that the $K_{diss.1}$ dissociation constant can be determined from either the direct plot or calculated from competition curves cf. Fig. 2, as the latter primarily represent the high-affinity binding. On the basis of $B_{max.1}$ we conclude that the IGF-II/M6P receptor is asymmetrically distributed with 2.27 pmol/mg in basolateral membranes compared to 0.34 pmol/mg in luminal membranes from pars recta. Approximately same values of $B_{max.1}$ was found for luminal membranes from pars convoluta (data not shown) indicating that the IGF-II/M6P receptor content in luminal membranes of rabbit proximal tubule in either cases is very low, almost negligible. The low content of IGF-II/M6P receptors in both luminal and basolateral membranes could explain why immunolabeling and autoradiographic techniques were not able to detect any IGF-II/M6P receptors in rat renal membranes, but only in the vacuolar system in the apical cytoplasm [13]. This is further consistent with the observation that although many cell types have a distinctive pattern of cell distribution of IGF-II/M6P receptors the majority of these receptors are reported to be localized intracellularly and only a minor part at the cell surface [3,25].

The IGF-II/M6P receptor in membrane associated environments was further examined as to the mannose 6-phosphate recognition site using D-mannose 6-phosphate and β-galactosidase, an acid hydrolyse with phosphomannosyl residues which serve as ligands for binding to the receptor [2,3]. We found that very low concentrations of β-galactosidase (20 nM for half-maximal inhibition of IGF-II binding) inhibited the ^{125}I-IGF-II binding. Thus, β-galactosidase inhibits binding of ^{125}I-IGF-II to the IGF-II/M6P by binding to the receptor with high affinity. We found the same concentration of β-galactosidase required for half-maximal inhibition as observed by binding of highly purified β-galactosidase to pure IGF-II/M6P receptor [4,5,26]. The ^{125}I-IGF-II binding was studied at three fixed β-galactosidase concentrations in order to reveal changes in ^{125}I-IGF-II binding affinities. Our data did not reflect any correlation between dissociation constant and inhibiting concentrations of β-galactosidase (Fig. 3). Likewise, we did not find any changes in affinity of ^{125}I-IGF-II for the receptor irrespective of M6P concentration as shown by the stimulation experiments (Fig. 5). This may support the assumption that the M6P sites and the IGF-II site do not interact directly. By contrast, Kiess et al. [5] detected by Scatchard analysis of IGF-II binding to pure IGF-II/M6P receptors isolated from rat placenta in the presence and absence of β-galactosidase that β-galactosidase decreased the binding affinity for IGF-II (K_d 0.26 nM versus 1.0 nM in the presence of 57 nM β-galactosidase). It has been reported that the IGF-II/M6P receptor monomer has one binding site for β-galactosidase and two binding sites for M6P [26]. The binding sites of M6P has been further localized to domains 1-3 and 7–11 of the extracytoplasmic region [27]. The inhibition by β-galactosidase could be explained by assuming that β-galactosidase binds to one (or both?) of the two M6P recognition sites on the receptor. When β-galactosidase with its phosphomannosyl residue is bound to the M6P binding sites, the binding of IGF-II may be impeded by sterical hindrance of the β-galactosidase molecule or alternatively by conformational changes of the receptor on β-galactosidase binding. The exact IGF-II binding site of the IGF-II/M6P receptor still has to be defined, but it has been suggested that the IGF-II binds to a sequence localized close to the domain 13 which is near to the transmembrane spanning region. An explanation of the M6P effect could be that M6P displaces other compounds carrying a mannose 6-phosphate residue including β-galactosidase at the M6P receptor binding sites as demonstrated here. Fig. 4 shows that although M6P reversed the inhibitory effect of β-galactosidase it did not reverse it completely. The ^{125}I-IGF-II binding did not reach the level obtained in absence of β-galactosidase in the incubation medium. So M6P reverses the inhibitory effect of β-galactosidase to various extent dependent on the receptor preparation procedure, a phenomenon also observed by others [5]. Thus, the interactions between β-galactosidase and M6P still remains unclear and probably other approaches have to be used.

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References

[1] MacDonald, R.G., Pfeffer, S.R., Coussens, L., Tepper, M.A., Brocklebank, C.M., Mole, J.E., Anderson, J.K., Chen, E., Czech, M.P. and Ullrich, A. (1988) Science 239, 1134–1137.
[2] Dahms, N.M., Lobel, P. and Kornfeld, S. (1989) J. Biol. Chem. 264, 12115–12118.
[3] Kornfeld, S. (1992) Annu. Rev. Biochem. 61, 307–330.
[4] Kiess, W., Thomas, C.L., Greenstein, L.A., Lee, L., Sklar, M.M., Rechler, M.M., Sahagian, G.G. and Nissley, S.P. (1989) J. Biol. Chem. 264, 4710–4714.

[5] Kiess, W., Thomas, C.L., Sklar, M.M. and Nissley, S.P. (1990) Eur. J. Biochem. 190, 71–77.
[6] Distler, J.J., Guo, J.F., Jourdian, G.W., Srivastava, O.P. and Hindsgaul, O. (1991) J. Biol. Chem. 266, 21687–21692.
[7] Kiess, W., Greenstein, L.A., White, R.M., Lee, L., Rechler, M.M. and Nissley, S.P. (1987) Proc. Natl. Acad. Sci. USA 84, 7720–7724.
[8] Murayama, Y., Okamoto, T., Ogata, E., Asano, T., Iiri, T., Katada, T., Ui, M., Grubb, J.H., Sly, W.S. and Nishimoto, I. (1990) J. Biol. Chem. 265, 17456–17462.
[9] Nishimoto, I., Ogata, E. and Okamoto, T. (1991) J. Biol. Chem. 266, 12747–12751.
[10] Rogers, S.A. and Hammerman, M.R. (1989) J. Biol. Chem. 264, 4273–4276.
[11] Hammerman, M.R. and Rogers, S. (1987) Am. J. Physiol. 253, F841–F847.
[12] Flyvbjerg, A., Nielsen, S., Sheikh, M.I., Jacobsen, C., Ørskov, H. and Christensen, E.I. (1993) Am. J. Physiol. 265, F624–F633.
[13] Cui, S., Flyvbjerg, A., Nielsen, S., Kiess, W. and Christensen, E.I. (1993) Kidney Int. 43, 796–807.
[14] Sheikh, M.I., Kragh Hansen, U., Jørgensen, K.E. and Røigaard Petersen, H. (1982) Biochem. J. 208, 377–382.
[15] Sheikh, M.I. and Møller, J.V. (1987) in Biochemical Toxicology: A Practical Approach (Snell, K. and Mullock, K., eds.), pp. 153–182, IRL Press, Oxford.
[16] Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275.
[17] Peterson, G.L. (1977) Anal. Biochem. 83, 346–356.
[18] Brodersen, R., Honoré, B., Pedersen, A.O. and Klotz, I.M. (1988) Trends. Pharmacol. Sci. 9, 252–257.
[19] Wiener, H.L. and Reith, M.E. (1992) Anal. Biochem. 207, 58–62.
[20] Pedersen, A.O. and Brodersen, R. (1988) J. Biol. Chem. 263, 10236–10239.
[21] Kiess, W., Blickenstaff, G.D., Sklar, M.M., Thomas, C.L., Nissley, S.P. and Sahagian, G.G. (1988) J. Biol. Chem. 263, 9339–9344.
[22] Kiess, W., Blickenstaff, G.D., Sklar, M.M., Thomas, C.L., Nissley, S.P. and Sahagian, G.G. (1988) J. Biol. Chem. 263, 9339–9344.
[23] Forbes, B., Szabo, L., Baxter, R.C., Ballard, F.J. and Wallace, J.C. (1988) Biochem. Biophys. Res. Commun. 157, 196–202.
[24] Shimasaki, S., Shimonaka, M., Zhang, H.-P. and Ling, N. (1991) in Modern Concepts of Insulin-like Growth Factors (Spencer, E.M., ed.), pp. 343–358, Elsevier, New York.
[25] Chin, E., Michels, K. and Bondy, C.A. (1994) J. Clin. Endocrinol. Metab. 78, 156–164.
[26] Tong, P.Y., Gregory, W. and Kornfeld, S. (1989) J. Biol. Chem. 264, 7962–7969.
[27] Westlund, B., Dahms, N.M. and Kornfeld, S. (1991) J. Biol. Chem. 266, 23233–23239.