The Kangaroo Cation-independent Mannose 6-Phosphate Receptor Binds Insulin-like Growth Factor II with Low Affinity*

(Received for publication, February 11, 1999, and in revised form, June 10, 1999)

Catherine A. Yandell†‡§§, Andrew J. Dunbar‡‡§§, John F. Wheldrake¶¶, and Zee Upton‡‡§§

From the ††Cooperative Research Centre for Tissue Growth and Repair, P. O. Box 10065, Adelaide B.C., South Australia, Australia, 5000, ‡‡CSIRO Human Nutrition, P. O. Box 10641, Adelaide B.C., South Australia, Australia, 5000, and §§School of Biological Sciences, Flinders University of South Australia, GPO 2100, Adelaide, South Australia, Australia, 501

The mammalian cation-independent mannose 6-phosphate receptor (CI-MPR) binds mannose 6-phosphate-bearing glycoproteins and insulin-like growth factor (IGF)-II. However, the CI-MPR from the opossum has been reported to bind bovine IGF-II with low affinity (Dahms, N. M., Bzycki-Wessell, M. A., Ramanujam, K. S., and Seetharam, B. (1993) Endocrinology 133, 440–446). This may reflect the use of a heterologous ligand, or it may represent the intrinsic binding affinity of this receptor. To examine the binding of IGF-II to a marsupial CI-MPR in a homologous system, we have previously purified kangaroo IGF-II (Yandell, C. A., Francis, G. L., Wheldrake, J. F., and Upton, Z. (1998) J. Endocrinol. 156, 195–204), and we now report the purification and characterization of the CI-MPR from kangaroo liver. The interaction of the kangaroo CI-MPR with IGF-II has been examined by ligand blotting, radioreceptor assay, and real-time biomolecular interaction analysis. Using both a heterologous and homologous approach, we have demonstrated that the kangaroo CI-MPR has a lower binding affinity for IGF-II than its eutherian (placental mammal) counterparts. Furthermore, real-time biomolecular interaction analysis revealed that the kangaroo CI-MPR has a higher affinity for kangaroo IGF-II than for human IGF-II. The cDNA sequence of the kangaroo CI-MPR indicates that there is considerable divergence in the area corresponding to the IGF-II binding site of the eutherian receptor. Thus, the acquisition of a high-affinity binding site for regulating IGF-II appears to be a recent event specific to the eutherian lineage.

The cation-independent mannose 6-phosphate receptor (CI-MPR),1 is a multifunctional protein that binds proteins bearing mannose 6-phosphate moieties, as well as insulin-like growth factor (IGF)-II. IGF-II is a polypeptide mitogen related to insulin that is believed to be particularly important during placental and embryonic development (3–6). The binding sites for IGF-II and mannose 6-phosphate-bearing ligands have been shown to be distinct (7–9).

Whereas the role of the CI-MPR in lysosomal enzyme sorting and transport has been largely elucidated (for a review, see Ref. 10), the physiological role of IGF-II binding to this receptor remains unresolved and somewhat controversial. Understanding the functions of the CI-MPR in the IGF system is complicated by the presence of two other receptors, the type 1 IGF and the insulin receptors, which also bind IGF-II. Indeed, many of the effects attributed to IGF-II are mediated by the CI-MPR has been difficult to prove, and much of the evidence has been contradictory. Okamoto et al. (17–19) and Nishimoto et al. (20) have suggested that the receptor binds to the guanylate nucleotide-binding protein, G_{i-2}, by means of a specific motif within the receptor’s cytoplasmic domain. However, others have not been able to demonstrate interactions between G proteins and the receptor upon stimulation by IGF-II (21, 22). Indeed, many studies have not supported a model in which the CI-MPR acts as a signaling protein (23–25).

Whereas the CI-MPR has been highly conserved in mammalian species, purification and characterization of the CI-MPR from the chicken and frog revealed that this receptor is unable to bind IGF-II (26–28). In addition, there is no evidence for an IGF-II-specific receptor in any other non-mammalian species examined thus far. This suggests that the CI-MPR acquired an IGF-II binding site after the separation of aves from mammals. Interestingly, the CI-MPR from a marsupial, the American opossum, does contain a binding site for IGF-II, albeit with an apparent 75-fold lower affinity for bovine IGF-II than the bovine receptor (1). It is not known whether the reported lower binding affinity is due to the use of a heterologous assay and possible amino acid differences between opossum and bovine IGF-II, or whether it indeed reflects the true binding affinity of this receptor. This information is important for determining where in evolution the CI-MPR acquired the ability to bind IGF-II. This knowledge, in turn, may lead to a greater understanding of the physiological role of this receptor in IGF-II action.

To examine the marsupial CI-MPR in a homologous system, we have previously purified kangaroo IGF-II (2), and we now report the purification and characterization of the kangaroo CI-MPR. The interaction of the kangaroo CI-MPR with IGF-II has been examined by radioreceptor assay, Western ligand blotting, and real-time biomolecular interaction analysis using both homologous and heterologous ligands. Furthermore, we have cloned the kangaroo CI-MPR cDNA sequence for the region proposed to be the IGF-II binding site on the mammalian receptor and have compared the sequences.

* The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1794 solely to indicate this fact.

† To whom correspondence should be addressed. Present address: Hanson Centre for Cancer Research, PO Box 14, Rundle Mall, Adelaide, South Australia, Australia, 5000. Tel.: 61 8 82223720; Fax: 61 8 82324092; E-mail: catherine.yandell@imvs.sa.gov.au.

‡‡ The abbreviations used are: CI-MPR, cation-independent mannose 6-phosphate receptor; IGF, insulin-like growth factor; hIGF, human insulin-like growth factor; rIGF, recombinant human insulin-like growth factor; kIGF, kangaroo insulin-like growth factor; RT-PCR, reverse transcription-polymerase chain reaction; SDS-PAGE, SDS-polyacrylamide gel electrophoresis; HBS, 10 mM HEPES, pH 7.4, 150 mM NaCl, 3.4 mM EDTA, and 0.005% surfactant P-20; PCR, polymerase chain reaction.

1 The abbreviation used are: CI-MPR, cation-independent mannose 6-phosphate receptor; IGF, insulin-like growth factor; hIGF, human insulin-like growth factor; rIGF, recombinant human insulin-like growth factor; kIGF, kangaroo insulin-like growth factor; RT-PCR, reverse transcription-polymerase chain reaction; SDS-PAGE, SDS-polyacrylamide gel electrophoresis; HBS, 10 mM HEPES, pH 7.4, 150 mM NaCl, 3.4 mM EDTA, and 0.005% surfactant P-20; PCR, polymerase chain reaction.
**EXPERIMENTAL PROCEDURES**

**Materials**—Mannose-6-phosphate, aprotinin, vinyl sulfone, human γ-globulin, and 4-chloro-1-naphthol were purchased from Sigma/Alrich. Sepharose 6B was purchased from Amersham Pharmacia Biotech, whereas the *Hansenula holstii* (NRRL Y-2154) phosphomannan was a generous gift from Dr. M. E. Slodki (United States Department of Agriculture, National Center for Agricultural Utilization Research, Peoria, IL). IS-401 and rhIGF-II were supplied by GroPop Pty Ltd (Adelaide, Australia), and kIGF-II was purified as described previously (2). Kangaroo livers were obtained from wild western gray kangaroos (*Macropus fuliginosus*), which were professionally culled at Peterborough, South Australia, Australia, and immediately placed on solid CO2 until stored at −80 °C. Bovine livers from freshly slaughtered cattle were obtained from Agpro Abattoir (Gepps Cross, Australia) and snap-frozen in liquid nitrogen before being stored at −80 °C. Radish peroxidase-conjugated antibody was purchased from DAKO (Botany, Australia). The lysosomal enzyme 4-sulfatase was recombinantly produced in Chinese hamster ovary cells (30) and was generously provided by Prof. J. J. Hopwood (Department of Chemical Pathology, Women and Children’s Hospital, Adelaide, Australia). 4-Sulfatase was iodinated using the lactoperoxidase method (31) to a specific activity of 1.42 × 108 Ci/mmol. The Bradford Protein Assay Reagent was purchased from Bio-Rad.

**Restriction enzymes (NsiI and EcoRI), pGEM-7zf vector, MJ109 competent cells (subcloning efficiency), Wizard PCR DNA Purification System, Wizard Plus SV Miniprep Purification System, T4 DNA ligase, and M13 lacZ forward and reverse primers were purchased from Promega (Madison, WI). The RNeasy Mini Kit was purchased from Qiagen (Clifton Hill, Australia), whereas the ELONGASE™ enzyme mix, 10 mM dNTP Mix, oligo(dT), and Superscript II RT were purchased from Life Technologies. The specific oligonucleotide primers detailed below were synthesized by Life Technologies, Inc. The Expand™ High Fidelity PCR System was obtained from Roche Molecular Biochemicals, and the Thermosequenase sequencing kit was obtained from Amersham Pharmacia Biotech.

**Preparation of the Phosphomannan Affinity Column**—H. holstii phosphomannan was hydrolyzed into core and small oligosaccharide fragments by mild acid treatment as described previously (32). The phosphomannan core (1.4 g) was then coupled to vinyl sulfoacteivated Sepharose 6B (50 ml) (33), and the gel was transferred to a glass column 1 cm in diameter and 5 cm in length. The CI-MPR was isolated from kangaroo and bovine liver by phosphomannan-Sepharose affinity chromatography essentially as described by Dahms et al. (1), except that MnCl2 was omitted from the Buffer D: 50 mM Na2CO3, 150 mM NaCl, 0.5 mM Na2EDTA, 1.0 mM Triton X-100, 5 mM sodium glycerophosphate, pH 7.0) and the homogenate. The material from the phosphomannan affinity column was dialyzed against water and then lyophilized. The samples were concentrated to one-tenth of the original volume by resuspending the lyophilized material in 0.25 mM HEPES, pH 7.0.

**Gel Electrophoresis and Western Ligand Blotting**—Purified receptor was concentrated as described above before electrophoresis. Samples and standards were boiled for 15 min in the presence of 2% SDS and applied to 6% or 8% SDS-polyacrylamide gels. Gels were run as described previously (34) and either stained with Coomassie Blue or transferred to nitrocellulose membranes (35). The membranes were probed with either 125I-labeled KIGF-II or 125I-labeled rhIGF-II in the presence or absence of rhIGF-II (1 μg) as indicated in the figure legends. Membranes were also probed with 125I-labeled 4-sulfatase in the presence or absence of 10 mM mannose 6-phosphate. Radiolabeled bands were visualized by Phosphor Imaging (ImageQuant, Molecular Dynamics). Immuno blotting was performed using a polyclonal anti-rat IGF-II receptor antibody (C6) (36) at a dilution of 1:3000. Immunoreactive protein bands were visualized by reaction with a 1.100 dilution of goat anti-rabbit peroxidase-conjugated secondary antibody and 4-chloro-1-naphthol as detailed by the manufacturer.

**IGF-II Receptor Assay**—Binding of 125I-labeled rhIGF-II to the purified CI-MPR was determined as described by Scott and Baxter (36).

**Real-Time Biomolecular Interaction Analysis**—All experiments were performed on a BIAcore 2000 system (Pharmacia Biosensor AB) using HBS buffer at 25 °C. Purified kIGF-II and rhIGF-II were coupled to the dextran-modified gold surface of a CMS sensor chip by amine coupling as described in the BIAcore systems manual. Briefly, the dextran surface of the chip was activated with N-hydroxysuccinimide/N-ethyl-N′-(3-diethylaminopropyl) carbodiimide (40 μl) followed by the addition of a 3 mM IGF-II solution in 0.1 M CH3COONa, pH 4.6 (30 μl). The remaining activated groups were blocked with ethanolamine (40 μl). Concentrations of 390 and 380 resonance units were generated for hIGF-II and kIGF-II, respectively.

Before data collection, several methods of surface regeneration after ligand binding were evaluated. It was found that washes with 1 M NaCl/0.1 M HCl (30 μl) could remove the bound protein and also preserve the binding capacity of the biosensor surface. Before analysis, receptor preparations were dialyzed against HBS buffer, and protein concentrations were determined by the method of Bradford (37) using Bio-Rad reagent and human γ-globulin as the standard.

Different concentrations (20 μl) of either bovine or kangaroo CI-MPR were injected over the chip at a flow rate of 10 μl/min. A non-protein, blocked surface (flow cell 1) served as a blank, and sensorgrams from this flow cell were subtracted from all others. To investigate whether rebinding was apparent during the dissociation phase, HBS buffer containing a 10-fold excess of rhIGF-II (140 μl) was injected during the dissociation phase using the COINJECT command.

**Protein Estimation**—Membrane and receptor concentrations were determined as outlined above. Human and kangaroo IGF-II concentrations were determined by reverse-phase high performance liquid chromatography as described previously (38) using a wavelength of 214 nm and correlation coefficients of 31.701 and 31.69 g liter−1 cm−1, respectively.

**Reverse Transcription-Polymerase Chain Reaction and Sequence Analysis**—The cDNA sequences for the putative IGF-II binding region and the G protein recognition site were obtained using mRNA extracted from kangaroo liver, followed by reverse transcription-polymerase chain reaction (RT-PCR). Total kangaroo liver RNA was extracted using the RNeasy Mini Kit, and first-strand cDNA was synthesized using 0.5 μg of oligo(dT) primer and 200 units of Superscript II reverse transcriptase enzyme in a total volume of 20 μl (according to the manufacturer’s protocols).

**Primer Design and Amplification**—Amplification of the putative IGF-II binding region was performed using two degenerate oligonucleotide primers that were based on the cDNA sequence of the human CI-MPR. The primer pair 5′-ATCAATGTCGCAA-3′ (IGF-1) and 5′-CGTCCAGGAGA-3′ (IGF-2) amplify a 732-base pair fragment spanning repeats 10 and 11. The PCR was carried out in a total volume of 20 μl containing 60 mM Tris-SO4 (pH 9.1), 18 mM (NH4)2SO4, 1.5 mM MgSO4, 0.2 mM dNTP, 0.5 unit of Expand™ High Fidelity PCR enzyme mix (Roche Molecular Biochemicals), 100 ng of oligonucleotide primers, and 4.5 μl of cDNA template. After denaturation at 94 °C for 3 min, the PCR reaction proceeded for 40 cycles of 1 min at 94 °C (denaturation), 1 min at 50 °C (annealing), and 1 min at 72 °C (extension). After electrophoresis and ethidium bromide staining, the correct sized fragment was excised from the agarose gel, purified using Wizard PCR DNA Purification System, and subcloned into the vector (pGEM-7).

**Amplification**—The putative G protein recognition site was performed using two fully degenerate oligonucleotide primers deduced from the amino acid sequences from the chicken (39), human (40), bovine (41), and mouse (42) CI-MPRs. This primer pair was based on the amino acid sequences of Gin2855-Glu2586-Asn2581-Glu2565-His2539 (GP1) and Phe2474-His2470-Asp2466-Asp2461-Ser2459 (GP2) (numbering is based on the human sequence). Amplification using primer set GP1 and GP2 generates a 315-base pair fragment. The PCR was carried out in a total volume of 50 μl containing 60 mM Tris-SO4 (pH 9.1), 18 mM (NH4)2SO4, 1.5 mM MgSO4, 0.2 mM dNTP, 0.5 unit of Expand™ High Fidelity PCR enzyme mix, 100 ng of oligonucleotide primers, and 4.5 μl of cDNA template. After denaturation at 94 °C for 3 min, the PCR reaction proceeded for 40 cycles of 1 min at 94 °C (denaturation), 1 min at 50 °C (annealing), and 1 min at 72 °C (extension). After electrophoresis and ethidium bromide staining, the correct sized fragment was excised from the agarose gel, purified using Wizard PCR DNA Purification System, and subcloned into the vector (pGEM-7).

Cloned PCR products were sequenced in both directions using a radiolabeled terminator cycle sequencing kit, Thermosequenase (Amersham Pharmacia Biotech). To verify the sequence, two independent clones were sequenced.

**RESULTS**

**Purification**—Phosphomannan-Sepharose was used to affinity purify the CI-MPR from membrane extracts of kangaroo and bovine livers. The Triton X-100 solubilized liver membranes were passed over the phosphomannan-Sepharose column, and the protein retained after washing with Buffer D was
eluted with 4 mM mannose 6-phosphate. Peak fractions were pooled, concentrated, and analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) (Fig. 1a). The purified protein migrated as two forms, one with a molecular mass similar to that predicted for the CI-MPR, and a second approximately 50 kDa larger than expected. A faint band at approximately 60 kDa was also observed that most likely represents a small amount of cation-dependent mannose 6-phosphate receptor that was able to bind to the column in the absence of Mn$^{2+}$. Incubation of the purified receptor under reducing conditions before SDS-PAGE inhibited the formation of the slower-migrating, high molecular mass protein, and only one band at approximately 250 kDa, along with the 60-kDa band, was present (data not shown). The purified protein was subjected to SDS-PAGE under nonreducing conditions, transferred to nitrocellulose membrane, and probed with a polyclonal antibody raised against the rat CI-MPR. Both the higher molecular mass proteins reacted with the anti-rat CI-MPR antibody (Fig. 1b), whereas the smaller 60-kDa protein did not.

**Western Ligand Blotting**—The bovine and kangaroo CI-MPRs were subjected to SDS-PAGE, transferred to nitrocellulose membrane, and probed with either $^{125}$I-labeled rhIGF-II or $^{125}$I-labeled kIGF-II (Fig. 2, a and b). Both the kangaroo and bovine receptors specifically bound $^{125}$I-labeled IGF-II, and this binding could be displaced by the addition of excess unlabeled rhIGF-II (Fig. 2, a and b), but not IGF-I (data not shown). However, the kangaroo CI-MPR bound IGF-II with an apparent lower affinity than the bovine CI-MPR. Moreover, the low affinity of the kangaroo receptor for IGF-II did not appear to be dependent upon whether the heterologous or homologous ligand was used as the probe. On the other hand, when probed with $^{125}$I-labeled 4-sulfatase, both the kangaroo and bovine CI-MPRs bound this lysosomal enzyme with equal apparent affinities (Fig. 2c). Again, this binding was specific because the radioligand was displaced by the addition of 10 mM mannose 6-phosphate. Both species of the receptor (i.e., both bands above 220 kDa) were able to bind IGF-II and 4-sulfatase.

**IGF-II Receptor Assay**—Radioreceptor binding assays confirmed the lower binding affinity of the kangaroo CI-MPR for IGF-II. The specific binding of hIGF-II to the kangaroo and bovine CI-MPRs was proportional to the amount of receptor added (Fig. 3, a and b). However, greater amounts of kangaroo receptor than bovine receptor were required to achieve a similar level of specific binding. For example, at the highest concentrations tested, 34 and $2460$ ng for the bovine and kangaroo CI-MPRs, respectively, specific binding was determined to be 52% and 34% of the radioligand added. Similarly, in an assay measuring the ability of unlabeled rhIGF-II to compete with $^{125}$I-labeled rhIGF-II for binding to the CI-MPR, specific binding of radiolabeled rhIGF-II to the kangaroo receptor (100 ng/tube) in the absence of competing unlabeled hIGF-II was only 5% of the total counts added (Fig. 4). However, specific
binding of the bovine receptor (20 ng/tube) in the absence of added unlabeled rhIGF-II was approximately 80% of the total counts added (Fig. 4), with half-maximal inhibition of radiolabeled rhIGF-II binding to the bovine receptor observed with the addition of 0.6 nM rhIGF-II. Thus, this assay was not useful for analysis of the relative binding affinities of these receptors because of the large discrepancy in the amounts of protein required to obtain a competitive binding curve. Additionally, competitive binding curves were not performed using 125I-labeled kIGF-II because of the low specific activity of this radioligand. Characterization of the interaction between IGF-II and the kangaroo CI-MPR was therefore further investigated by real-time biomolecular interaction analysis.

Real-Time Biomolecular Interaction Analysis—Preliminary experiments examining the binding of kangaroo CI-MPR to IGF-II using real-time biomolecular interaction indicated that the increase in resonance signals when analyzing different concentrations of receptor was dose-dependent (data not shown). However, the dissociation of both the bovine and kangaroo receptors appeared to be very slow. Therefore, we examined the possibility that the receptors were rebinding during the dissociation phase, thus giving a misleading indication of the dissociation rate. Co-injection of excess rhIGF-II immediately after injection of the receptor indicated that significant rebinding was occurring, and all subsequent analysis was performed using a co-injection of excess rhIGF-II. Analysis of the sensorgrams indicated that whereas the association rates of the bovine and kangaroo CI-MPRs are similar, the lower affinity of the kangaroo CI-MPR is due to the fast dissociation of this receptor from IGF-II (Fig. 5). Furthermore, the dissociation of the kangaroo CI-MPR appears to be faster when binding to hIGF-II than kIGF-II, whereas the dissociation rate of the bovine CI-MPR is very similar for both IGF-II proteins.

RT-PCR and Sequence Analysis—A primer pair designed to span the proposed IGF-II binding region was used for the RT-PCR amplification of mRNA isolated from kangaroo liver. A product of approximately 750 base pairs was isolated, cloned, and sequenced (Fig. 6a). The deduced amino acid sequence of the proposed IGF-II binding site, residues 1508–1575, revealed that there is a 60% identity between the kangaroo and bovine CI-MPRs (41) and a 49% identity between the kangaroo and chicken receptors (39) (Fig. 6b). Of the 27 amino acids that differ between the bovine and kangaroo receptors, 22 are found in the N-terminal region of repeat 11 (1533–1575).

The cDNA sequence of the putative G protein recognition site was obtained using RT-PCR, and a PCR product of approximately 500 base pairs was detected. Analysis of the amino acid sequence deduced from the cDNA sequence and comparison with the sequences reported for bovine (41), chicken (39), and human CI-MPRs (40) revealed interesting differences between the species (Fig. 7). Seven of the 14 amino acids comprising the putative G protein binding site differ between the kangaroo and bovine CI-MPRs, whereas 6 of the 14 differ between the kangaroo and chicken sequences. However, 4 of the 14 differ between kangaroo and human CI-MPRs, 3 of the 14 differ between human and bovine CI-MPRs, and 1 of the 14 differs between mouse and human receptors.
DISCUSSION

The CI-MPR from the opossum has previously been shown to bind bovine IGF-II with a 75-fold lower apparent affinity than the bovine receptor (1). The opossum study was performed using a heterologous ligand, and it was not known whether amino acid differences between opossum and bovine IGF-II or amino acid differences in the IGF-II binding region of the receptor itself accounted for this difference in affinity. Therefore, we used a homologous approach to examine the interactions of IGF-II with a marsupial CI-MPR. Kangaroo and bovine CI-MPRs were purified from liver using phosphomannan affinity chromatography. The purified proteins from both the kangaroo and bovine liver preparations migrated on a SDS-polyacrylamide gel as two high molecular mass forms, both of which reacted with the anti-rat CI-MPR antiserum. The larger of these two bands could be prevented from forming by the addition of reducing agents. Hence, the formation of the larger of the CI-MPR species under non-reducing conditions may be due to inter-domain disulfide bonds that result in the protein becoming more rigid, thus causing the protein to have decreased mobility when subjected to SDS-PAGE. Alternatively, the larger of the two bands could be the result of dimerization.

Western ligand blotting demonstrated that the kangaroo CI-MPR was able to specifically bind IGF-II, albeit with a lower apparent affinity than the bovine receptor. The binding of IGF-II to the kangaroo CI-MPR was not dependent on whether

FIG. 5. Real-time biomolecular analysis sensorgrams of kangaroo and bovine CI-MPR (62 nM) binding to (a) hIGF-II and (b) kangaroo IGF-II. Excess human IGF-II was injected during the dissociation phase to inhibit rebinding as outlined under “Experimental Procedures.”

FIG. 6. a, the cDNA and deduced amino acid sequence of the kangaroo CI-MPR amino acid residues 1405–1642. The primer sequences used for the RT-PCR amplification of the cDNA are underlined. b, comparison of human CI-MPR amino acid residues 1488–1610 with aligned sequences of bovine, mouse, chicken, and kangaroo CI-MPRs. The proposed IGF-II binding region is boxed. The arrow indicates the junction of repeats 10 and 11 in the alignment we used (40). Sequences are those reported by Oshima et al. (40) [human], Szebenyi and Rotwein (42) [mouse], Lobel et al. (41) [bovine], and Zhou et al. (39) [chicken], whereas the sequence for the kangaroo is deduced from the present study. Dashed lines represent a sequence identical to the human CI-MPR. Periods were used to frameshift proteins for maximal alignment between sequences. Numbering is shown according to the human sequence.
IGF-II Binding to the Kangaroo CI-MPR

Codon repeats 9–11 led to the observation that mutation of the Ile residue at position 1572 to Thr abolishes IGF-II binding (43). It was suggested that substitution of Ile with Thr may cause a structural distortion in this region, thus inactivating the IGF-II binding site (43). Interestingly, examination of the cDNA sequence of the chicken receptor, which does not bind IGF-II, revealed striking divergence from the mammalian receptors in repeat 11 (39). Repeat 11 of the chicken CI-MPR is the least conserved region of the entire receptor, with only 15–22% identity between the chicken and mammalian receptors in residues 1532–1575. In this region of the kangaroo CI-MPR, there is 60% identity between the kangaroo and bovine CI-MPRs (41) and 49% identity between the kangaroo and chicken receptors (39). In contrast, there is approximately 90% identity between eutherian CI-MPR sequences in this region. The Ile at position 1572 has been conserved in the kangaroo CI-MPR and in all other mammalian receptors sequenced thus far. However, of the 14 residues surrounding Ile 1572, which are also highly conserved in eutherian species, 6 are different in the kangaroo receptor. Indeed, this region of the kangaroo receptor shares more identity with the chicken CI-MPR sequence than the eutherian sequences. This supports the suggestion that the secondary structure or conformational stability of this region is important for the binding of IGF-II.

More recently, it has been demonstrated that a second region within the extracellular domain interacts with repeat 11 to form the high-affinity IGF-II binding site of the human CI-MPR (48). Whereas repeat 11 contains the minimal requirements for IGF-II binding (43, 47), a second region within repeat 12 or 13 is required for full binding (48). Indeed, receptors containing a deletion of the 43-residue fibronectin type II repeat in repeat 13 exhibited low IGF-II binding relative to the wild-type receptor. Whereas the fibronectin type II repeat in repeat 13 is not part of the primary IGF-II binding site, it acts as an affinity-enhancing domain. Because stabilization of IGF-II binding appears to involve the fibronectin type II repeat, further analysis of the sequence divergence in this region of the kangaroo receptor may reveal a further explanation for the rapid dissociation rate of this receptor from IGF-II as demonstrated by the real-time biomolecular interaction analysis.

It has been proposed that the CI-MPR mediates the mitogenic actions of IGF-II through specific activation of a heterodimeric G protein, G_{1a} (17, 49, 50). A synthetic peptide representing residues 2410–2423 of the cytoplasmic domain of the receptor was also shown to initiate G_{1a} binding in a manner similar to the G-coupled receptor (17, 19). These results suggested that the G protein recognition site on the CI-MPR resides in the cytoplasmic domain of the receptor. Although these findings have been contradicted in more recent studies (21, 22), it was interesting to note that this region of the chicken receptor is also highly divergent (39). Therefore, we were interested in establishing whether this region of the kangaroo receptor was similarly divergent. Sequence analysis of this region of the CI-MPR from a number of species, along with that we report here for the kangaroo, has revealed that this 14-amino acid motif is not well conserved. Moreover, within the 14-amino acid sequence, there is as much divergence between non-eutherian (which bind IGF-II with little or no affinity) and eutherian (which bind IGF-II) CI-MPRs as there is between the eutherian CI-MPRs themselves. Therefore, if this 14-amino acid region is indeed important for G protein recognition, it seems unlikely, given this evidence, to be correlated with the ability to bind IGF-II.

The presence of a low-affinity binding site for IGF-II on the CI-MPR in metatherian species (Ref. 1 and this study) suggests that the ability to bind IGF-II is a recent functional acquisition by the receptor, which preceded the separation of metatherian
from eutherian mammals. However, the high-affinity binding site for IGF-II appears to be restricted to eutherian CI-MPRs. Whereas separate genes for IGF-I and IGF-II first appear in evolution with the appearance of Chondrichthyes (51), no explanation for the recent evolution of a second IGF receptor that specifically binds IGF-II has been elucidated. In light of the convincing research that has been undertaken in eutherians pointing to a major role for IGF-II in fetal development and placental growth (52), it is tempting to speculate that the emergence of a second IGF receptor in eutherians may be related to the marked changes in the nature of the feto-maternal interactions that have evolved in this group of vertebrates. Whereas many of the components that regulate IGF-II are present in non-mammalian species, the finding that the high-affinity binding site for IGF-II on the CI-MPR is limited to eutherians suggests that the regulation or possibly the function of IGF-II may be different in non-eutherians. Clearly, additional studies are required to elucidate the role of the CI-MPR and IGF-II during marsupial and eutherian development.

Acknowledgments—We gratefully acknowledge Dr. Morey Slodki for generously providing H. holstii phosphomannan, Prof. John Hopwood for the recombinant 4-sulfatase, and Dr. Carolyn Scott for the rabbit anti-CD CI-MPR antibody. We also thank Doug Evans for giving up his time to help us collect kangaroo livers, Darryl Lantheis (Agpro Abattoir) for providing the bovine liver, and Graham Hobba for advice on the use of the BlaCcore (Department of Biochemistry, University of Adelaide, South Australia, Australia) for allowing us to use the BlaCcore and to Drs. Carolyn Scott, Nancy Dahms, and Vicky Avery for helpful discussions.

REFERENCES
1. Dahms, N. M., Bryzicky-Wessell, M. A., Ramanujam, K. S., and Seetharam, B. (1993) Endocrinology 133, 440–446
2. Yandell, C. A., Francis, G. L., Wheldrake, J. F., and Upton, Z. (1998) J. Biol. Chem. 273, 19155–19160
3. Dahms, N. M., Brzycki-Wessell, M. A., Ramanujam, K. S., and Seetharam, B. (1993) Endocrinology 133, 440–446
4. Walther, C. A., Francis, G. L., Wheldrake, J. F., and Upton, Z. (1998) J. Biol. Chem. 273, 19155–19160
5. Dahms, N. M., Brzycki-Wessell, M. A., Ramanujam, K. S., and Seetharam, B. (1993) Endocrinology 133, 440–446
6. Dahms, N. M., Brzycki-Wessell, M. A., Ramanujam, K. S., and Seetharam, B. (1993) Endocrinology 133, 440–446
7. Roth, R. A., Stover, C., Hari, J., Morgan, D. O., Smith, M. C., Sara, V., and Fried, V. A. (1987) Biochem. Biophys. Res. Commun. 149, 490–496
8. 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
9. Tait, J. F., Weinman, S. A., and Bradshaw, R. A. (1991) J. Biol. Chem. 266, 11086–11092
10. Corvol, M., and Rechler, M. M. (1984) Biochem. Biophys. Res. Commun. 135, 1410–1415
11. Rechler, M. M. (1984) Biochem. Biophys. Res. Commun. 135, 1351–1357
12. Zick, Y., Sasaki, N., Rees-Jones, R. W., Grumberger, G., Nisley, S. P., and Rechler, M. M. (1987) Biochem. Biophys. Res. Commun. 149, 490–496
13. Okamoto, T., Katada, T., Murayama, Y., Ui, M., Ogata, E., and Nishimoto, I. (1990) Cell 62, 709–717
14. Okamoto, T., Katada, T., Murayama, Y., Ui, M., Ogata, E., and Nishimoto, I. (1990) Cell 62, 709–717
15. Polychronakos, C., Gwyda, H. J., Janhyth, U., and Posner, B. I. (1990) Endocrinology 127, 1681–1686
16. Polychronakos, C., Gwyda, H. J., Janhyth, U., and Posner, B. I. (1990) Endocrinology 127, 1681–1686
17. Okamoto, T., Katada, T., Murayama, Y., Ui, M., Ogata, E., and Nishimoto, I. (1990) Cell 62, 709–717
18. Okamoto, T., Katada, T., Murayama, Y., Ui, M., Ogata, E., and Nishimoto, I. (1990) J. Biol. Chem. 265, 16395–16392
19. Okamoto, T., and Nishimoto, I. (1990) Proc. Natl. Acad. Sci. U. S. A. 88, 8020–8023
20. Nishimoto, I., Ogata, E., and Okamoto, T. (1991) J. Biol. Chem. 266, 12747–12751
21. Braulke, T., Korner, C., Rosorius, O., and Nurnberg, B. (1994) The Insulin-like Growth Factors and Their Regulatory Proteins (Baxter, R. C., Gluckman, P. D., and Rosenfeld, R. G., eds) pp. 117–129. Elsevier Science, New York
22. Korner, C., Nurnberg, B., Udude, M., and Braulke, T. (1996) J. Biol. Chem. 270, 287–295
23. Braulke, T., Tipper, S., Neher, E., and von Figura, K. (1989) EMBO J. 8, 681–686