

Mutation of the RGD sequence does not affect plasma membrane association and growth inhibitory effects of elevated IGFBP-2 in vivo

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Abstract Using insulin-like growth factor-binding protein-2 (IGFBP-2) transgenic mice (D mice) as a model of elevated IGFBP-2 expression, which is often found in unphysiological conditions, we found association of IGFBP-2 to purified plasma membranes of many organs. To determine whether the RGD (Arg-Gly-Asp) motif of IGFBP-2 mediates cell surface binding in vivo, we mutated the RGD motif of IGFBP-2 into an RGE (Arg-Gly-Glu) sequence and produced transgenic mice (E mice) which express elevated amounts of mutated IGFBP-2. Our data demonstrate that in vivo IGFBP-2 cell surface association is not dependent on the RGD motif and that mutation of this sequence does not alter growth inhibitory effects of IGFBP-2. © 2002 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.

Key words: Insulin-like growth factor-binding protein-2; Overexpression; Transgenic mouse; Cell surface association; RGD sequence

1. Introduction

The insulin-like growth factor-binding proteins (IGFBPs) present in different biological fluids can also be found in association with cell surfaces which they can bind via heparin-binding domains [1] or RGD motifs [2]. The presence of these binding sites for the insulin-like growth factors (IGFs) on the cell surface or in the extracellular matrix clearly points to a role for the IGFBPs in controlling IGF-I bioavailability in the pericellular space, e.g. by regulating receptor access of IGF-I [3]. As a consequence of cell surface association reduced affinity for the IGFs has been described for IGFBP-2 [4], -3 [5,6] and -5 [7]. In addition, IGF-independent actions of IGFBPs are implicated with the feature of membrane association [8–10]. IGFBP-2 possesses a putative heparin-binding domain (HBD) [11], possibly similar to that present in IGFBP-3 and -5 [12,13]. Additionally, an RGD motif exists in IGFBP-2 which is also present in IGFBP-1 and is involved in binding to $\alpha 5 \beta 1$ integrin [8]. IGFBP-2 cell surface association has been reported in a number of tissues and cells [4,14–16]. However, the relative contributions of the HBD or the

RGD domain for membrane association are unknown. A function of the RGD motif present in IGFBP-2 in vivo remains to be established. To identify target tissues of IGFBP-2, we asked if IGFBP-2 binds to plasma membranes from different tissues in IGFBP-2 transgenic mice (D mice). The potential relevance of the RGD motif for cell surface binding was characterized in a transgenic mouse model (E mice) overexpressing mutant IGFBP-2 which lacks the RGD motif.

2. Materials and methods

2.1. Generation of two point mutations leading to an amino acid exchange

The wild-type mouse IGFBP-2 containing expression vector CMV-int-IGFBP-2 [17] was mutated by overlap extension PCR. Two partially overlapping PCR products were generated by use of the mouse IGFBP-2 cDNA sequence and primer 1s (C CAC CAT CCG CGG AGA ACC CGA GTG C) and primer 1as (CTC TCT AAC AGA AGC AAG G) as well as primer 2s (GAC GCT ACG CTG CTA TCC CAA) and primer 2as (GCA CTC GGG TTC TCC GCG GAT GGT GG). The resulting PCR product was *KpnI/PstI*-digested and cloned into the *KpnI/PstI*-digested purified expression vector backbone void of the cDNA sequence. The generated expression vector coded for mouse IGFBP-2 containing an RGE instead of an RGD sequence (Fig. 1A). In parallel, a second point mutation was introduced to generate a restriction site which allowed for simplified screening of the mutation via *SacII* digestion. The mutation was verified by sequence analysis. The mutated IGFBP-2 differs by only one methylene group compared to wild-type IGFBP-2, while the net charge is identical in both proteins.

2.2. Generation of transgenic mice

Transgenic mice were generated by microinjection of the 3.2-kb *SpeI/XhoI* fragment from the expression vector pCMV-int-mIGFBP-2 into pronuclei of fertilized eggs from superovulated donors (B6D2F1 \times B6D2F1). The injected embryos were transferred to pseudopregnant female mice (CD-[®]). Genomic DNA from tail tips of 3-week-old offspring was isolated using the Puregene genomic DNA purification system (Biozym, Hess. Oldendorf, Germany). Transgenic offspring of two founder mice were produced by backcrossing with C57BL/6 mice and identified by PCR analysis (CMV-specific sense primer: 5'-GTG TAC GGT GGG AGG TC-3'; IGFBP-2-specific antisense primer: 5'-TCG GCA GCA TGT TGG CTT GT-3') according to standard protocols from 100 ng of genomic DNA from tail tips.

2.3. Serum measurements of IGF-I, -II and IGFBP-2

Radioimmunoassays were performed as described before [17].

2.4. Analysis of body and organ growth in E mice

Mice were weighed to the nearest 0.1 g. Transgenic E mice and littermate controls of different ages were anesthetized with ether and

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Munich, Germany). Separated proteins were transferred to a nitrocellulose membrane (Millipore, Eschborn, Germany). To check equal loading, the blots were routinely stained in 0.2% Ponceau red/3% acetic acid. The blots were blocked with 1% fish gelatin and incubated with [125 I]IGF-II (10^6 cpm per blot). Binding proteins were visualized and quantified on Phosphor-Imager Storm (Molecular Dynamics, Krefeld, Germany). All incubations and washing steps were performed at 4°C.

3. Results and discussion

IGFBP-2 transgenic mice [17] were used in the present study as a model of elevated IGFBP-2 expression found in many unphysiological conditions [18] and to identify target tissues binding IGFBP-2 on their cell surface. To unequivocally answer the question if the RGD sequence affects cellular distribution of IGFBP-2 in vivo we generated IGFBP-2 transgenic mice which express high levels of mutant IGFBP-2 lacking the RGD motif.

3.1. Presence of the mutation in RNA transcripts

Elevated IGFBP-2 mRNA levels and the mutation were verified in the E mice by reverse transcription PCR from total RNA isolated from muscle, kidney, heart and mesentery and by digestion IGFBP-2-specific PCR products using the *Sac*II restriction enzyme. In cDNA derived from E mice *Sac*II digestion resulted in partial hydrolysis of the 329-bp PCR product to two fragments of 273 bp and 56 bp, whereas in D mice the PCR product was not digested (Fig. 1B). Thus the mutation was verified by analytical digestion using a restriction enzyme in addition to sequence determination.

3.2. Serum concentrations of IGFBP-2, IGF-I and IGF-II

Serum concentrations of IGFBP-2 were significantly ($P < 0.05$) increased by about 30% in E mice (795 ± 172 ng/ml) compared to non-transgenic littermates (606 ± 127 ng/ml). D mice had much higher serum IGFBP-2 concentrations (1021 ± 320 ng/ml) [17] compared to E mice. The differences of serum IGFBP-2 concentrations in the different transgenic lines might be explained by different transgene expression due

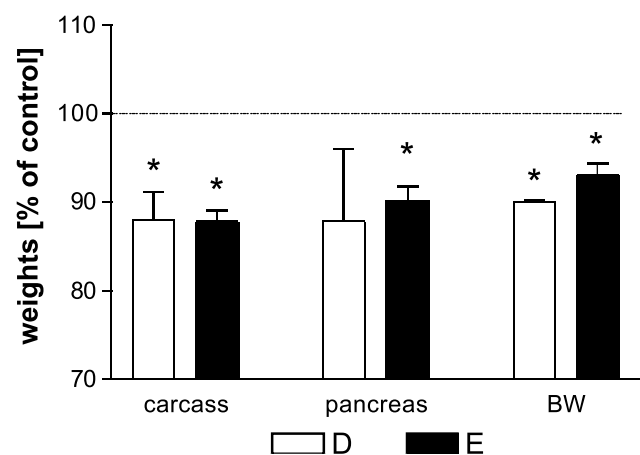


Fig. 2. Weight reductions in E mice. Weights were recorded from animals of different ages and expressed in percent of their respective sex- and age-matched non-transgenic littermates. The weights of carcass and pancreas were divided by the respective body weights and thus represent relative weights ($n = 12$; $*P < 0.05$). For direct comparison the weight reductions present in D mice as shown previously [19] have been included in the figure.

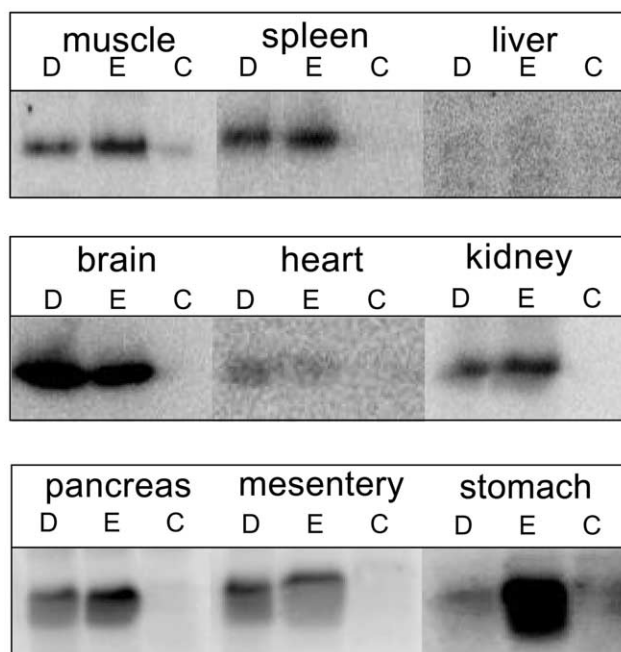


Fig. 3. Transgene expression in different tissues from D and E mice and non-transgenic controls (C). Tissue lysates were prepared as described in Section 2 and the presence of IGFBPs was analyzed by Western ligand blotting using [125 I]IGF-II.

to different genomic integration sites of the expression vectors. Total serum levels of IGF-I and IGF-II in E mice (377 ± 58 ng/ml and 28 ± 4 ng/ml) were similar compared to non-transgenic littermates (407 ± 40 ng/ml and 26 ± 2.5 ng/ml).

3.3. Effects on body and organ weights

E mice were weighed at different ages between 4 and 9 months and compared with sex- and age-matched non-transgenic littermates (Fig. 2). Body weight was significantly ($P < 0.001$) reduced by 7% in the E mice. For carcass and pancreas the reductions of relative weights amounted to 13% ($P < 0.001$) and 10% ($P < 0.001$), respectively, compared to controls. The weight reductions were similar in E mice and in D mice described earlier [17]. The carcass displayed the strongest weight reduction in both transgenic groups, followed by pancreas and body weight. Therefore, E mice had a similar overall phenotype compared to D mice although both transgenic lines had different serum IGFBP-2 levels. This finding suggests that a relatively small increase in serum IGFBP-2 levels is sufficient for a limited growth inhibitory effect or that tissue levels of IGFBP-2 are the more relevant factor for growth inhibition by elevated IGFBP-2. The latter possibility is in line with the revised somatomedin hypothesis which points to important local effects of the IGF system for organ growth [19].

3.4. Tissue expression

Transgene expression of RGE-IGFBP-2 was analyzed by Western ligand blotting in different organs and compared with transgene expression of RGD-IGFBP-2 transgenic mice. In both transgenic groups, a strong band at 32 kDa was found in muscle, spleen, kidneys, brain, heart, pancreas, mesentery and stomach. No obvious differences in tissue expression were observed between both transgenic groups (Fig.

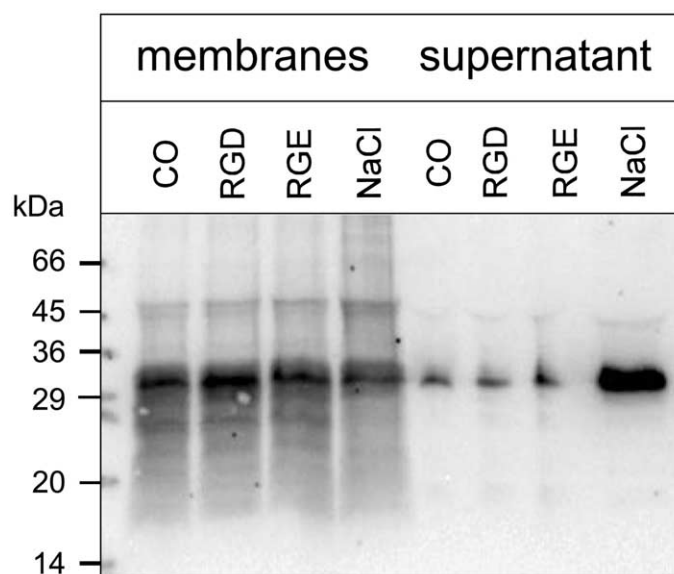


Fig. 4. IGFBP-2 is dissociated by high concentrations of NaCl but not by synthetic RGD- or RGE-containing peptides. Purified plasma membranes from D mice were incubated with or without different agents to demonstrate specificity of cell surface association and the absence of soluble cytosolic contamination.

3). IGFBP-2 protein could be detected in liver tissue from both IGFBP-2 transgenic groups only after long exposure of the membrane. In tissues from non-transgenic littermates only a relatively faint band of similar molecular weight was present. It can thus be concluded that the strong signals present in both transgenic groups are due to transgenic IGFBP-2 and not to endogenous IGFBP-2 expression. Therefore, the RGD sequence is not necessary for binding of IGFs. This conclusion has been drawn before from structural studies of IGFBP-1 [20].

3.5. Membrane association of IGFBP-2

Having demonstrated transgene expression in different tissues from both transgenic groups we investigated by Western ligand blotting if IGFBP-2 is associated with plasma membranes isolated from the respective tissues. First we aimed to demonstrate the specificity of IGFBP-2 binding to the plasma membranes. Thus, we tested the effects of different agents on membrane association of IGFBP-2. Synthetic peptides (GRGDSP or GRGDESP) had no effects on binding of IGFBP-2 to purified membranes (Fig. 4). By contrast, NaCl at high concentration was able to dissociate IGFBP-2 molecules from their binding sites and dissociated IGFBP-2 was found in the supernatant, demonstrating the absence of cytosolic contamination and the specificity of cell surface association. However, the use of RGD-containing peptides might produce biased results since the three-dimensional structure of the RGD sequence is dependent on the flanking sequences [21,22] and may be different in peptides or proteins. We thus investigated the cell surface association of mutated IGFBP-2 lacking the RGD motif. Under non-reducing conditions at 32 kDa a band was present in membrane preparations from all tissues in both transgenic groups which was able to bind [125 I]IGF-II (Fig. 5). We conclude that the strong signals present in E and D mice are due to transgenic IGFBP-2. Since both wild-type and mutant IGFBP-2 was cell surface-associated we conclude that the RGD motif is not necessary for cell surface association in the tissues examined. However, condi-

tional generation of high-affinity integrin-binding sites by either enhanced integrin gene expression [23] or conformational changes [24] has been described. It thus cannot be excluded by the present study that IGFBP-2 under certain, e.g. malignant, conditions could make use of its RGD domain and activate integrin-dependent signal cascades as demonstrated for IGFBP-1 [8], while in the absence of the RGD domain integrin-dependent signal cascades might remain silent.

In mouse IGFBP-2 a putative HBD is resident on the C-terminal domain (amino acids 195–200; PKKLRP). This pu-

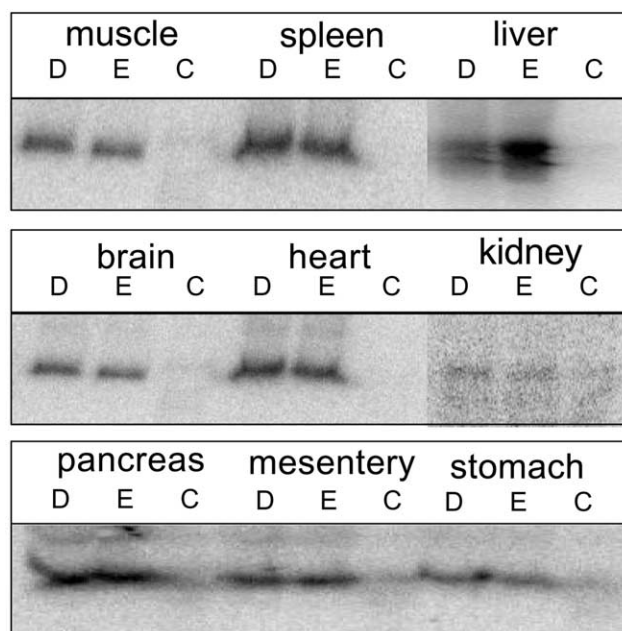


Fig. 5. Association of IGFBP-2 to purified plasma membranes from different tissues of D and E mice and non-transgenic controls (C). Plasma membranes were isolated from various tissues as described in Section 2 and the presence of IGFBPs was analyzed by Western ligand blotting using [125 I]IGF-II.

tative sequence motif was discussed as being involved in cell surface association in several species [11]. The findings that this sequence motif is absent in zebrafish and IGFBP-2 is not cell surface-associated in that species gives at least some evidence for an involvement of the HBD with membrane association [11]. In vitro it has been shown that IGFBP-2 binds to heparin or the extracellular matrix [25] and cell surface proteoglycans have been characterized and shown to bind IGFBP-2 in olfactory bulbs from the rat brain [4]. Since proteoglycans are widely distributed [26] and we demonstrate the presence of IGFBP-2 on the cell surface in all tissues investigated, it might be assumed that IGFBP-2 binds to tissues in a ubiquitous manner. Cell surface association might result in high local tissue levels of IGFBP-2 but not in adequate serum concentrations. The finding that local IGFBP-2 concentrations and weight reductions were similar in both transgenic groups while the serum concentrations were different indicates a more important role of local versus systemic IGFBP-2.

In conclusion, our data clearly demonstrate that IGFBP-2 binds to the cell surface in many tissues and that the RGD motif present in IGFBP-2 is not necessary for cell surface association or growth inhibitory effects of IGFBP-2 in transgenic mice. Our results suggest an important role of IGFBP-2 in the pericellular compartment.

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