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Influence of Placental Mannose/N-Acetyl Glucosamine-Binding Proteins on the Interaction of Insulin and Insulin-Like Growth Factors with Their Receptors

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Abstract—Placenta is a source of carbohydrate-binding proteins that function as molecular scavengers, but they could also be involved in interactions that assist in metabolic control. Mannose/N-acetyl-glucosamine (Man/GlcNAc)-binding proteins from placenta were isolated and their reactivity towards placental insulin and insulin-like growth factor receptors (IR and IGF-Rs) was analyzed. The lectins reduced the binding of insulin and IGF-I in a dose-dependent manner, while almost no effect was observed on the binding of IGF-II. The shape of the inhibition curves changed, suggesting altered binding specificity. The presence of sugar could not reverse completely the effect of the lectins, implicating both lectin–sugar and protein–protein conformational recognition. Since biological molecules in our experimental system were those that are in close relation *in vivo*, placental Man/GlcNAc-specific lectins may be regarded as potential allosteric modulators of ligand–receptor interactions in a system of homologous ligands, selectively affecting only binding to tyrosine kinase type receptors (IR and IGF-1R).

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Key words: insulin, insulin-like growth factors, receptors, lectins, placenta

Lectins are carbohydrate-binding proteins that can be found in all living organisms including humans. Mammalian lectins can be present in soluble form or as cell surface constituents. They serve as recognition molecules or they perform intracellular functions. Lectins control synthesis and intracellular trafficking of glycoproteins, participate in cell signaling, and are crucial in the innate immune response [1]. Lectins also contribute to proliferation and metastasis of tumor cells [2]. Animal and plant lectins have different primary structures, but similarity exists at the level of protein folding, in the configuration of the carbohydrate-binding sites [3]. Similar conformational epitopes have thus arisen from unrelated proteins to fulfill similar functional needs.

Plant lectins have been used for a long time to study glycan structures of animal glycoproteins. We have employed plant lectins to characterize placental insulin and insulin-like growth factor (IGF) receptors (IR, IGF-1R, and IGF-2R). Lectins served both to determine the carbohydrate structures of these glycoproteins [4] and the influence of lectins on the ligand–receptor interactions. Certain lectins were shown to be capable of altering the sensitivity and specificity of IR and IGF-Rs [5, 6]. These results stimulated our further research on the possible influence of the placental lectins on the interaction of insulin, IGF-I, and IGF-II with their receptors.

The human placenta is a source of numerous carbohydrate-binding proteins [7]. Two mannose-binding proteins are known to be present: mannose-binding lectin

Abbreviations: CIM6P/IGF-II-R, cation-independent mannose-6-phosphate/IGF-II receptor; Con A, concanavalin A; GlcNAc, N-acetyl-glucosamine; HbA1c, glycosylated (glycated) hemoglobin; HBS, Hepes buffered saline; IDDM, insulin-dependent diabetes mellitus; IGF, insulin-like growth factor; IR, insulin receptor; IGF-1R, type 1 IGF receptor; IGF-2R, type 2 IGF receptor; Man, mannose; MBL, mannose-binding lectin; MMR, macrophage mannose receptor; PBS, phosphate buffered saline; WGA, wheat germ agglutinin.

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(MBL) and macrophage mannose receptor (MMR). They both belong to a family of C-type lectins, binding mannose, N-acetyl-glucosamine, and fucose in a calcium-dependent manner [8, 9]. They function mostly as molecular scavengers, binding pathogenic microorganisms and modified, potentially harmful endogenous glycoproteins. Yet, other novel functions are expected to be found [2]. One of them could be involvement in glycoprotein interactions that may assist in metabolic control of regular physiological processes.

The intention of this study was to isolate mannose/N-acetyl-glucosamine (Man/GlcNAc)-binding proteins from term human placenta, to analyze reactivity of these proteins towards IR and IGF-Rs, and to investigate their possible interference with the ligand–receptor recognition.

MATERIALS AND METHODS

Samples. Human placentas (n = 6) were obtained after normal full-term delivery from healthy mothers (n = 3) and mothers having insulin-dependent diabetes mellitus (IDDM, n = 3), with the approval of the local ethics committee. All of the mothers with diabetes had IDDM class B, without vascular disease, duration 4-7 years, their glycosylated (glycated) hemoglobin (HbA1c) was 5.1-6.2% (measured each month during pregnancy), and they were regularly treated with Actrapid/Insulatard therapy. The two groups of mothers were age (28-33-year-old) and BMI matched (26.4-30.5 kg/m^2).

Homogenization of placental tissue. The tissue was collected in ice-cold 0.1 M phosphate buffered saline (PBS), pH 7.4, and brought to the laboratory within 60 min. The placentas were extensively washed in PBS. Amniotic and chorionic membranes and the large blood vessels were dissected away. The tissue was minced and homogenized in a 0.25 M sucrose solution supplemented with protease inhibitors [10]. After a 10 min centrifugation at 600g the pellet was discarded. The supernatant was centrifuged at 18,000g for 30 min. The resulting supernatant was used for the isolation of placental lectins. The pellet was washed once in 50 mM Hepes buffered saline, pH 7.5 (HBS), resuspended in the same buffer, and used as a source of membrane receptors. Membrane receptors were isolated from both healthy and diabetic placentas, while lectins were isolated only from healthy placentas. The isolates were divided into aliquots and stored frozen at –80°C until used. The protein concentration was determined by the method of Bradford [11].

Isolation of placental lectins. Placental lectins were isolated by affinity chromatography using Man-Sepharose 6B matrix prepared in-house and commercial GlcNAc-Agarose 6B resin (Sigma-Aldrich, USA). Mannose was immobilized on divinyl sulfone-activated Sepharose 6B [12]. Buffers used for the affinity chromatography were those recommended for affinity chromatography with the immobilized plant lectins that share the same carbohydrate specificity (Man-Con A and GlcNAc-WGA).

Thus, the equilibrating (and washing) buffer for the Man-Sepharose column was 50 mM acetate, pH 6.0, supplemented with 1 M NaCl and 1 mM $CaCl_2$. The bound glycoproteins were eluted with 0.3 M Man in the same buffer. The equilibrating buffer for the GlcNAc-Agarose column was 50 mM HBS, and 0.3 M GlcNAc in the same buffer was used for the specific elution. Aliquots of the placental fraction for lectin isolation were re-circulated over the Man-Sepharose and GlcNAc-Agarose matrices (3 ml) packed in columns (2-5 mg proteins/ml packed gel) for 1 h at room temperature, then left overnight at 4°C, and again re-circulated for 1 h at room temperature before elution. Unbound proteins were washed away using equilibrating buffers (10 ml/ml packed gel). Fractions specifically eluted with sugar-containing solutions (10 × 1 ml) were pooled, dialyzed against distilled water, and concentrated by ultrafiltration. The isolated placental lectins were analyzed by electrophoresis and used in ligand-binding assays.

Solubilization of placental membrane receptors. The placental membrane receptors were solubilized by adding Triton X-100 into the membrane suspension at a final concentration of 1% with stirring for 1 h at 4°C. After centrifuging the suspension at 100,000g for 90 min at 4°C, the supernatant containing solubilized membrane proteins was used as a source of I/IGF receptors.

Electrophoresis and immunoblotting. Protein fractions (1 mg/ml) containing isolated placental lectins and a fraction containing solubilized receptors were subjected to SDS-PAGE (6% gel) according to the method of Laemmli [13]. Separated proteins were transferred to nitrocellulose membranes and stained with Ponceau S. Immunoblotting [14] was performed using monoclonal anti-IR (GroPep, Australia), anti-IGF-1R (BioSource, USA), and anti-CIM6P/IGF-II-R (Calbiochem, USA) primary antibodies and horseradish peroxidase-conjugated anti-mouse IgG secondary antibody (BioSource). Receptors were visualized using enhanced chemiluminescence detection reagents (Pierce, Thermo Scientific, USA) followed by autoradiography (Kodak MXB Film and Developing Reagents, France). Densitometric analysis was used to estimate molecular masses of the detected protein bands (ImageMaster™ TotalLab Software; Amersham Biosciences, UK).

Ligand-binding assays. Solubilized receptors (0.1 mg protein in 0.1 ml) were incubated with ^{125}I-labeled ligands ($1 \cdot 10^5$ cpm in 0.1 ml) in test tubes containing 50 mM HBS (0.1 ml) with gelatin (final concentration 0.2% w/v) at 4°C overnight. In competitive ligand-binding assays, the unlabeled ligands were added (0.1 ml, in the concentration range from 27 to 3440 pM in HBS). When effects of the placental lectins were studied, isolated lectin frac-

tions were added (0.1 ml, in the concentration range from 0.1 to 0.8 mg/ml in HBS). To test reversibility of lectin effects on ligand–receptor interactions a hapten sugar was added (0.03 M Man or GlcNAc in HBS, 0.1 ml). The final volume of each incubation mixture was adjusted to 0.5 ml using HBS. Receptor complexes were precipitated by the addition of bovine IgG (0.1 ml, final concentration 0.05% w/v) and ice-cold 20% (w/v) polyethylene glycol in 50 mM PBS, pH 7.4 (1.5 ml). After intensive vortexing, the tubes were centrifuged at 4500*g* for 45 min, supernatants were aspirated, and the radioactivity of precipitates measured in a γ-counter. Nonspecific binding was determined for each incubation combination (a mixture without placental receptors) and subtracted from the specific binding (B/B_o). Each experiment was performed in triplicate. Results obtained for the two groups of placentas were analyzed by Student's *t*-test.

Porcine insulin (Novo, Denmark) and human IGF-I and IGF-II (GroPep) were labeled with ^{125}I by the chloramine T method [15]. The specific activities were approximately 100 μCi/μg. Labeled molecules were used within two weeks.

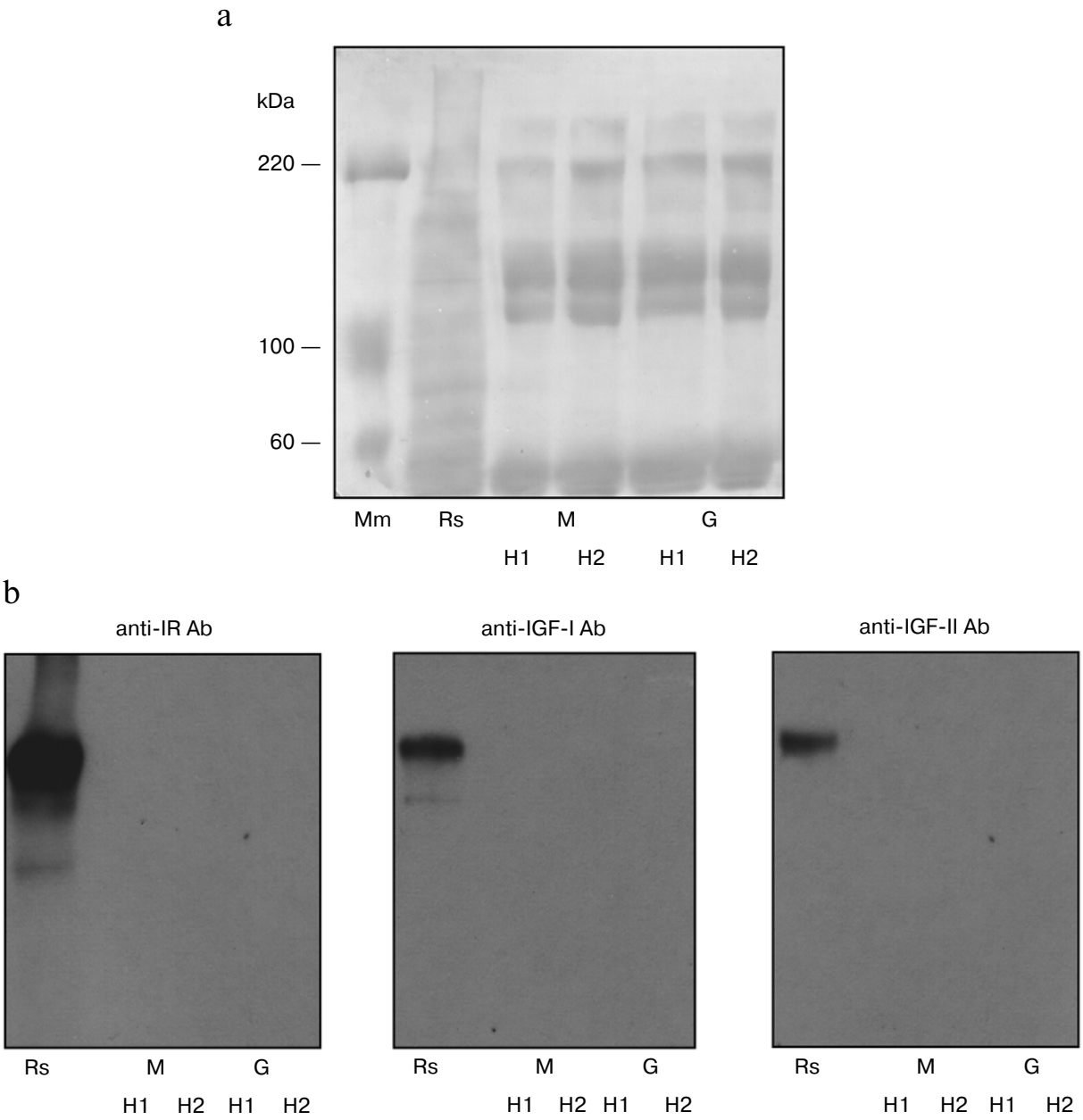

Fig. 1. a) SDS-PAGE/Ponceau S staining and (b) immunoblotting of proteins in solubilized receptor fraction (Rs) and in Man- and GlcNAc-binding fractions (M and G) obtained from healthy placentas (H1 and H2). Molecular mass markers (Mm) are indicated on the left hand-side.

RESULTS AND DISCUSSION

Electrophoretic analysis of placental proteins present in fractions specifically eluted from Man- and GlcNAc-affinity columns demonstrated that the same molecules were isolated by using the two chromatographic matrices (Fig. 1a). Major protein bands were found at approximately 220, 140, 120, and below 40 kDa. A minor band was detected at 280 kDa. The same protein profile was obtained for all healthy placentas (examples for two of them are given in Fig. 1a, together with a protein profile of one solubilized receptor fraction). Immunoblotting (Fig. 1b) demonstrated that IR, IGF-1R, and IGF-2R were present among the solubilized membrane proteins and they were not co-isolated with placental lectins.

MBL is a circulating protein synthesized by hepatocytes [8]. Individual humans differ up to 1000-fold in MBL serum concentration, yet its typical concentration is 1.3 mg/liter [16]. MBL is an acute-phase protein that activates the complement cascade after opsonisation of a pathogen [17]. The role of MBL extends beyond this primary function as it is involved in diseases such as diabetes [18], rheumatoid arthritis [19], and reperfusion injury [20]. A trimeric form is the basic structural unit of MBL, consisting of peptide chains of 25-18 kDa [21]. Structural units form oligomers ranging from dimers to octamers [22], offering a wide interface for the simultaneous recognition of multiple sugars that enables high-avidity binding [23].

MMR is a multidomain monomeric cell surface protein having a mass of approximately 170 kDa [9]. It mediates phagocytosis of pathogens and endocytosis of potentially harmful soluble glycoproteins [24]. Multivalent binding of MMR to several saccharide units is required for high-affinity interaction [25].

According to their estimated masses, the placental glycoproteins isolated in our experiment by affinity chromatography are most likely MBL proteins. Final identification would require an immunochemical detection of MBL and MMR, but the idea of this work was to study the combined effect of all Man/GlcNAc-binding proteins on the interaction of insulin and IGFs with their receptors.

Ligand-binding assays were performed with both Man- and GlcNAc-bound fractions separately. Results of these experiments were not significantly different, and therefore they were used together in an overall statistical analysis of binding and expressed as a mean value and a standard deviation.

The effect of the placental lectins on the maximal binding of insulin, IGF-I, and IGF-II to the corresponding receptors isolated from healthy placentas was first tested. The lectins reduced the maximal binding of insulin and IGF-I in a dose-dependent manner, while almost no effect was observed on the binding of IGF-II (Fig. 2a). Addition of the hapten sugar in the reaction mixture only slightly reduced the effect of the lectins (Fig. 2b). The presence of the lectins (0.2 mg/ml) affected the insulin inhibition curve the most, the IGF-I inhibition curve was less susceptible, whereas the IGF-II curve was almost unchanged (Fig. 3, a-c). Also, the shape of the inhibition curves changed, thus suggesting altered binding specificity. IGF-1R has a tetrameric structure (2α and 2β subunits) displaying over 50% homology to IR [26], whereas IGF-2R is an unrelated, single-chain glycoprotein. Structural characteristics of the three investigated

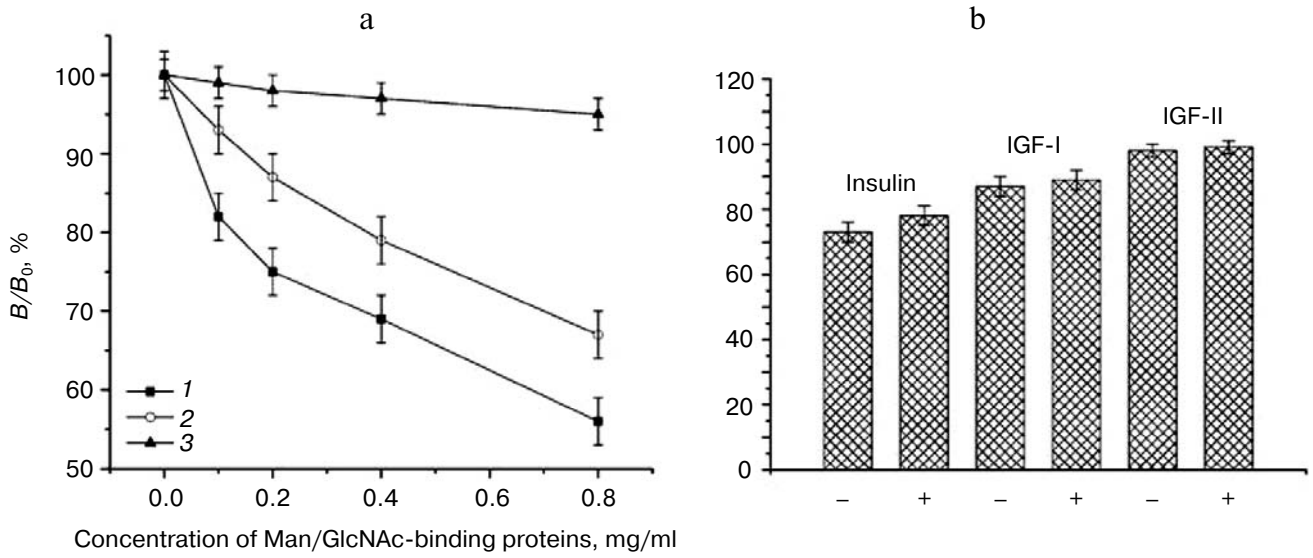

Fig. 2. a) Maximal binding of insulin (*1*), IGF-I (*2*), and IGF-II (*3*) to their receptors in the presence of different concentrations of placental Man/GlcNAc lectins and (b) effect of hapten sugar Man or GlcNAc (+) on maximal binding of ligands to their receptors in incubation mixture with 0.2 mg/ml lectins.

receptors are most likely responsible for their (non)reactivity with the lectins.

The presence of hapten sugar could not reverse, but only moderately reduce, the effect of lectins on ligand–receptor binding. It is known that lectins bind monosaccharides with several orders of magnitude lower affinity than complex structures that form multiple bonds [27]. Even so, glycans of IR and IGF-1R were probably not the only structures involved in interaction with lectins. Although lectins preferably bind carbohydrate groups, peptides that structurally imitate these saccharides can also bind to lectins [28]. The specificity of lectins is thus not limited to saccharide units, but sugar-mimics may be identified by lectin-binding sites as well [29]. Interactions of Man/GlcNAc-binding proteins with IR and IGF-1R can be interpreted both in terms of lectin–sugar and protein–protein conformational recognition.

Posttranslational modifications of proteins can significantly alter their physical and biochemical characteristics, including interactions with other molecules [30]. Diabetes mellitus is a disorder characterized by posttranslational nonenzymatic glycosylation (glycation) of numerous proteins [31]. In women with gestational diabetes mellitus, changes in the distribution of oligosaccharides of glycoconjugates have been reported, and they are related to morphological–functional alteration of the placenta [32]. Insulin and IGF receptors of the human placenta were found to be glycosylated [4] and, in mothers with diabetes, glycation of these receptors could occur. Altered carbohydrate moieties could contribute to differential effect of lectins on ligand–receptor recognition in patients with diabetes, which we intended to investigate. The physiological importance of a single sugar residue was illustrated by Arabkhari et al. [33], who reported that desialylation of IR and IGF-1R quenched the proliferative response of muscle cells to IGF-II.

The same experiments were performed with solubilized receptors isolated from diabetic placentas as with those isolated from healthy placentas. The results were very similar, the effect of lectins being the most pronounced on insulin binding. Quantitative parameters (B/B_o) were not significantly different ($p > 0.05$) between the two groups of samples. In the presence of placental lectins, maximal binding of insulin to its receptor isolated from healthy and diabetic placentas decreased to 75 ± 3 and 72 ± 4%, respectively. No difference, either, was found between the two groups of placentas concerning the electrophoretic mobility and the amount of IR, IGF-1R, and IGF-2R in the cell membranes [34]. If we take into consideration values of HbA1c in mothers with IDDM (5.1-6.2%), it can be assumed that they had stable metabolic glucose control. It seems that their proteins were not subjected to a long-term increased glucose concentration and, therefore, they were not posttranslationally modified by glycation.

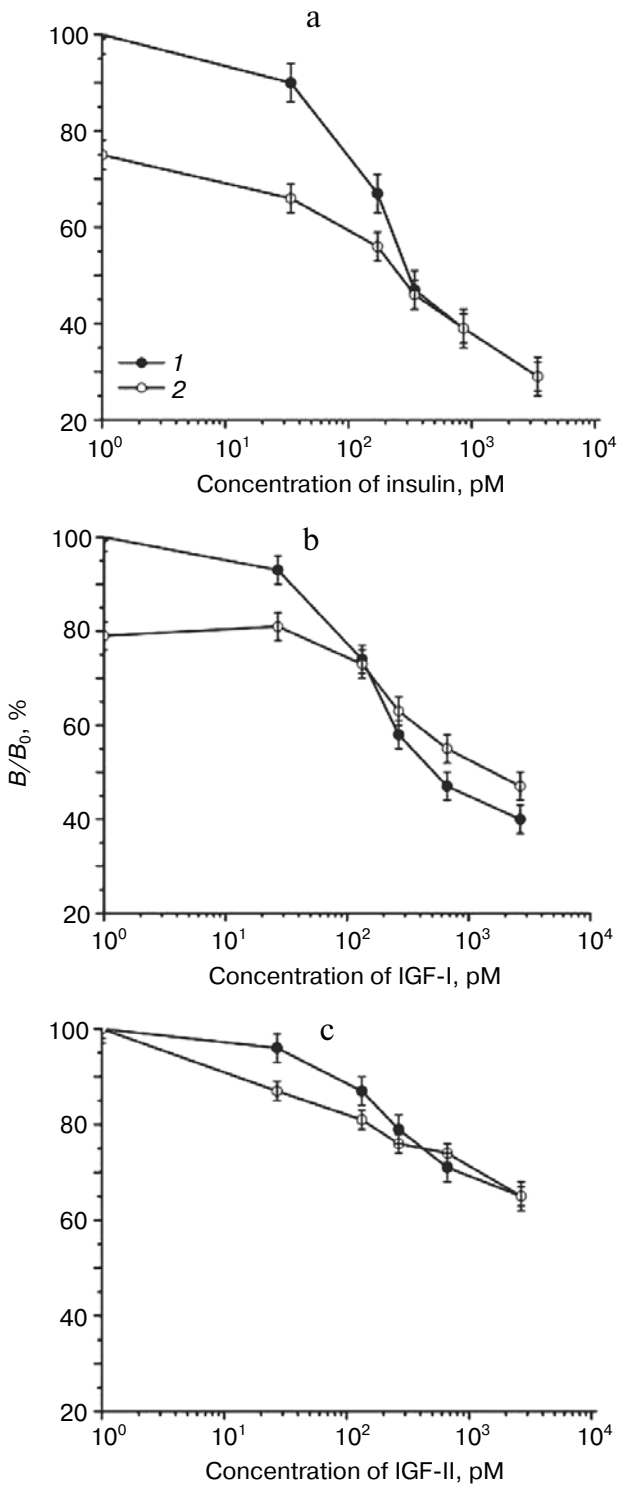

Fig. 3. Ligand-binding assays (inhibition curves) for (a) insulin, (b) IGF-I, and (c) IGF-II in the absence (*1*) and in the presence (*2*) of 0.2 mg/ml Man/GlcNAc lectins.

In conclusion, placental Man/GlcNAc-specific lectins can be regarded as potential allosteric modulators of ligand–receptor interactions in a system of homologous molecules insulin, IGF-I, and IGF-II. The lectins

affected insulin–IR recognition more than IGF-I–IGF-1R recognition, suggesting that some receptor–lectin interactions occurred in the homologous and some in receptor-specific regions. Taking into account that biological molecules in our experimental system were those that are in close relation *in vivo*, the Man/GlcNAc-specific lectins in placenta can be considered as candidates for control factors that are involved in complex physiological mechanisms that underlie metabolic and mitogenic processes triggered by IR and IGF-1R activation.

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