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Immunodetection of insulin-like growth factor binding proteins (IGFBPs) in the sera of different animal species

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Abstract: Knowledge regarding the insulin-like growth factor (IGF) system and the methods for the detection and quantitation of IGF binding proteins (IGFBPs) is greater for the human species than for other animal species. This is mainly because antibodies specifically raised against nonhuman IGFBPs are rarely commercially available. We aimed to investigate which of the antibodies directed against human IGFBPs could be used for the detection of IGFBPs present in other species. We used commercially available antibodies, raised against human IGFBP-1 through IGFBP-4, in order to recognize the corresponding IGFBPs in the sera of different animal species: human, bovine, equine, porcine, ovine, guinea pig, rat, and murine. Serum samples were analyzed by SDS-PAGE followed by western blotting, using 12 different polyclonal antibodies and antisera raised against IGFBPs as primary antibodies. Detection of IGFBP-1, -2, -3, and -4 in the sera of animals was achieved to a different extent depending on the primary anti-IGFBP antibodies used. The results of this article may be considered as an initial guide in the designing of experiments in which the detection of animal IGFBPs is required.

Key words: Western blotting, IGFBPs, human, bovine, equine, porcine, ovine, guinea pig, rat, murine

Introduction

The insulin-like growth factor (IGF) system comprises IGFs, IGF receptors, and IGF binding proteins (IGFBPs). IGFBPs are a family of highly homologous proteins that bind IGFs with high affinity. IGFBPs serve as regulators of IGF bioavailability, although some of them have IGF-independent actions. There are 6 IGFBPs in the serum of adults (1). The human (h) IGFBP-1 has 234 amino acid residues and a deduced molecular mass (Mr) of 25.3 kDa (2). It migrates as a band of 32-34 kDa in reducing sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), as reported by Shimasaki and Ling (3). Both rat (r) and murine (m) IGFBP-1 have 247 residues and are highly homologous to hIGFBP-1 (4,5).

hIGFBP-2 is a 289-residue protein, as determined by Binkert et al. (6), whereas rIGFBP-2 and mIGFBP-2 have 270 and 271 residues, respectively (7,8). Bovine (b) IGFBP-2 is a 283-residue protein, as reported by Bourner et al. (9). According to Yang and Rechler, hIGFBP-2 migrates as a nonglycosylated protein of approximately 34 kDa in PAGE (10).

IGFBP-3 is the most abundantly circulating IGFBP in postnatal animals (11). hIGFBP-3 is composed of 264 residues, and its Mr would be 29 kDa based on its sequence (12). However, its actual Mr ranges from

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40 to 45 kDa, because it is heavily glycosylated (13). On SDS-PAGE gels, hIGFBP-3 is usually found as a characteristic doublet (13). mIGFBP-3 and rIGFBP-3 both have 259 residues (5,14).

hIGFBP-4 contains 237 amino acids and 1 N-linked glycosylation site (15). The deduced Mr of hIGFBP-4 is 26 kDa (16), and it occurs in both N-glycosylated and nonglycosylated forms. Zhou et al. (17) reported that glycosylated IGFBP-4 migrates with an apparent Mr of 28 kDa, whereas the nonglycosylated form migrates as a band of 24 kDa in SDS-PAGE. In adult rats, IGFBP-4 is the second most abundant IGFBP (18). rIGFBP-4 and mIGFBP-4 both have 233 residues (5,18).

The knowledge of the IGF system in humans, its physiological importance, its regulation, and the methods for IGFBP detection and identification is greater than the respective knowledge of the IGF systems of other animal species. IGFBPs play an important role in animal physiology and, therefore, they have been examined to some extent in a variety of species, including pigs, cattle, sheep, horses, and dogs. Quantification of IGFBPs in animals is limited to blotting methods. The problem is a general lack of available anti-IGFBP antibodies that recognize specific animal IGFBPs. Since mouse and rat models are widely used to obtain insight into the functions of the IGF system members in development (19,20), antihuman, antimouse, and antirat IGFBP antibodies are commercially available, but those raised against IGFBPs from other animals (e.g. domestic animals) generally are not.

Determination of individual IGFBPs in specific species thus entails difficulties, so the aim of this study was to compare the applicability of different commercially available anti-hIGFBP antibodies and antisera in the western immunoblot (WIB) of human and animal (bovine, porcine, equine, ovine, guinea pig, rat, and murine) sera.

Materials and methods

Serum samples of healthy humans were obtained from the Institute for the Application of Nuclear Energy (INEP), Belgrade, Serbia, whereas serum samples of healthy animals were provided by Marko Kirovski from the Veterinary Institute of Belgrade, Serbia. After separation from the coagulum, sera were stored at –20 °C until use. The human serum was abbreviated as h, bovine as b, equine as e, porcine as p, ovine as o, guinea pig as g, rat as r, and murine as m. The study was approved by the local ethical committee of the INEP.

Serum samples were analyzed by SDS-PAGE using 4% stacking gel and 12% resolving gel, according to the procedure given by Hossenlopp et al. (21). The total protein concentrations of serum samples (in mg/mL) were as follows: 79.6 (h), 69.1 (b), 64.9 (e), 60.3 (p), 74.1 (o), 61.7 (g), 66.1 (r), and 70.3 (m). Samples were diluted, ranging from 1:10 to 1:100, in PBS buffer (0.05 M sodium phosphate, 0.15 M NaCl; pH 7.5); mixed with an equal volume of reducing sample buffer (0.125 M Tris-HCl, 4% (w/v) SDS, 20% (v/v) glycerol, 10% (v/v) 2-mercaptoethanol, 0.01% (w/v) bromophenol blue; pH 6.8); boiled for 7 min; and then loaded onto the gels (50 μL). Low-range molecular mass markers (Bio-Rad Laboratories, Hercules, CA, USA) were run in parallel. Samples were electrophoresed in a Mini-PROTEAN 3 Cell (Bio-Rad Laboratories) at a constant voltage (150 V) until the bromophenol blue marker reached the bottom of the gel (about 1.5 h). After SDS-PAGE, the proteins were transferred to a nitrocellulose membrane (0.45 μm, Whatman Protran, PerkinElmer Life and Analytical Sciences, Boston, MA, USA). Transfer was carried out at a constant voltage of 25 V for 1 h using a Trans-Blot SD Semi-Dry Transfer Cell (Bio-Rad Laboratories) and a transfer buffer (0.025 M Tris-HCl, 0.19 M glycine, 20% (v/v) methanol; pH 8.3). Electrotransfer was confirmed by reversible staining of the membrane with 5% Ponceau S solution (1 min), and the positions of the mass markers and serum albumin were labeled on the membrane. Nonspecific binding on membranes was prevented by immersing them in TBST buffer (0.01 M Tris-HCl, 0.15 M NaCl, 0.1% (v/v) Tween-20; pH 7.4) containing 5% nonfat dry milk, for 1 h at room temperature, as suggested by Baričević et al. (22). Membranes were shaken in TBST containing 1% nonfat dry milk and the appropriate dilution of the primary (1°) antibody, overnight at 4 °C. In this experiment, 12 different polyclonal antibodies and antisera raised against IGFBPs (native, recombinant, or short peptides) were used as 1° antibodies. The goat polyclonal anti-hIGFBP-1 (Cat. No. sc-6072), anti-

mIGFBP-2 (sc-6002), anti-mIGFBP-3 (sc-6004), and anti-hIGFBP-4 (sc-6005) antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). The affinity-purified goat polyclonal anti-hIGFBP-1 (Cat. No. DSL-R00337), anti-hIGFBP-2 (DSL-R00437), and anti-hIGFBP-3 (DSL-R00536) antibodies were purchased from Diagnostic Systems Laboratories (DSL) Inc. (Webster, TX, USA). Rabbit antisera against hIGFBP-1 (Cat. No. PAP1), hIGFBP-2 (PAQ1), hIGFBP-3 (PAV1), and hIGFBP-4 (PAR1) were obtained from GroPep (Adelaide, Australia). A rabbit polyclonal anti-hIGFBP-2 antibody (Cat. No. A682/R7H) was purchased from Biogenesis (Poole, UK). Different dilutions of 1° antibodies and antisera were tested with the WIB (from 1:50 to 1:10,000).

The membranes were thoroughly washed (10 times for 5 min each with TBST, with constant agitation) and further incubated with the appropriate secondary (2°) antibody for 1 h at room temperature. For the detection of the 1° antibodies bound on the membranes, 2 different horse radish peroxidase (HRP)-conjugated 2° antibodies were used: swine antigoat (SAG) IgG (Biosource, Camarillo, CA, USA) and donkey antirabbit (DAR) IgG (GE Healthcare, Amersham, Little Chalfont, UK). SAG IgG was used in a dilution of 1:10,000, whereas DAR IgG was used in a dilution of 1:7000. After extensive washing of the membranes (10 times for 5 min each with TBST, with constant agitation), IGFBPs were detected using an enhanced chemiluminescence kit (GE Healthcare), and the IGFBP bands were visualized by autoradiography. The X-ray films and autoradiography reagents were purchased from KODAK (Paris, France).

Besides molecular mass markers, the positions of IGFBPs on autoradiograms were confirmed using samples that were known to contain particular IGFBPs. Nonglycosylated recombinant (r)hIGFBP-3 (29 kDa, designated as BP3 on autoradiograms) and rhIGFBP-4 (24 kDa, designated as BP4 on autoradiograms) were obtained from DSL. Amniotic fluid (AF), the source of IGFBP-1, was collected from pregnant women during amniotomy for labor induction (23). Seminal plasma (SP), the source of IGFBP-2, was obtained from healthy men (24). All other chemicals were of reagent grade (Sigma-Aldrich, Steinheim, Germany).

Results

WIB experiments were performed with 12 different primary antibodies and antisera, and the results are arranged in Figures 1-4, each representing the data obtained with antibodies purchased from 1 manufacturer. Only representative WIBs are shown. The positions of mass markers are indicated on the left and those of the IGFBPs on the right.

Figure 1A shows a WIB of the animal sera probed with the goat polyclonal anti-hIGFBP-1 antibody (dilution of 1:1000) obtained from Santa Cruz Biotechnology. IGFBP-1 bands were clearly visible in the samples of bovine, ovine, and guinea pig sera, and faint bands were present in the human, porcine, and murine sera. These bands were in line with the band obtained with AF (protein concentration of 0.02 mg/mL), which was a positive control for IGFBP-1.

Figure 1B shows a WIB of the animal sera probed with the goat polyclonal anti-mIGFBP-2 antibody (dilution of 1:1000) obtained from Santa Cruz Biotechnology. The IGFBP-2 bands were those seen above the position of the 31-kDa mass marker. IGFBP-2 bands were visible in all examined samples and had an apparent size similar to that of the protein detected in the SP sample, which served as a positive control for IGFBP-2. In the human, equine, and porcine sera, 1 band was identified in this region, whereas 2 distinctive bands were seen in bovine, guinea pig, rat, and murine sera. Ovine serum gave a specific pattern of immunoreactive IGFBP-2, having 3 bands.

Figure 1C shows a WIB of the animal sera probed with the goat polyclonal anti-mIGFBP-3 antibody (dilution of 1:1000) obtained from Santa Cruz Biotechnology. According to the literature, the bands of glycosylated IGFBP-3 were those at the level of the 45-kDa marker. IGFBP-3 was detected in all sera, but the 2 separate bands, characteristic of the IGFBP-3 doublet, were not resolved clearly in any sample. However, the IGFBP-3 bands in the lanes of bovine, equine, and ovine sera were broad. It should be noted that the Santa Cruz Biotechnology polyclonal anti-IGFBP-3 antibody does not recognize rhIGFBP-3, since no immunoreactive band was detected in the BP3 lane (Figure 1C).

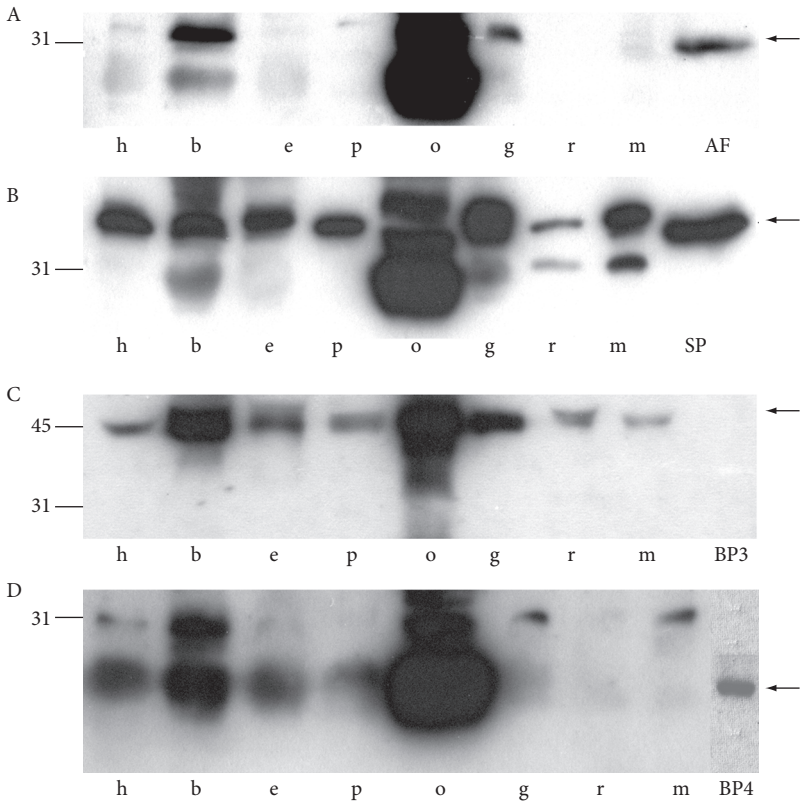

Figure 1. Immunoblots of IGFBP-1 (A), IGFBP-2 (B), IGFBP-3 (C), and IGFBP-4 (D) probed with goat polyclonal antibodies obtained from Santa Cruz Biotechnology (dilution of 1:1000), done following SDS-PAGE. The 2° Ab was HRP-conjugated SAG IgG (1:10,000). Samples were as follows: h (human), b (bovine), e (equine), p (porcine), o (ovine), g (guinea pig), r (rat), and m (murine). Samples were diluted to 1:10 (A, B) or 1:20 (C, D), except the ovine serum, which was diluted to 1:50 (A, B) or 1:100 (C, D). The amount of protein loaded per lane for A and B was 0.4 mg (h), 0.35 mg (b, m), 0.3 mg (e, p, g, r), and 0.07 mg (o); for C and D, it was 0.2 mg (h), 0.17 mg (b, m), 0.15 mg (e, p, g, r), and 0.035 mg (o). AF (0.001 mg protein/lane), SP diluted to 1:20 (0.15 mg protein/lane), rhIGFBP-3 (25 ng/lane), and rhIGFBP-4 (50 ng/lane) were used as biological markers. The positions of mass markers are indicated on the left and those of IGFBP-1, -2, -3, and -4 are indicated on the right.

Figure 1D shows a WIB of the animal sera probed with the goat polyclonal anti-hIGFBP-4 antibody (dilution of 1:1000) obtained from Santa Cruz Biotechnology. Immunoreactive IGFBP-4 bands were detected in all sera, except the rat serum. The 2 separate bands below the 31-kDa mass marker were visible in the lanes of human, bovine, and ovine serum. The upper band at approximately 28 kDa was clearly detectable in the lanes of bovine and ovine serum and faint in the human, guinea pig, and murine sera. The lower band at approximately 24 kDa (indicated by the arrow in Figure 1D) was clearly detectable in the lanes of human, bovine, equine, porcine, and ovine serum. According to the literature, the immunoreactive band of glycosylated IGFBP-4 was that at the level of the 28-kDa marker, whereas the band of nonglycosylated IGFBP-4 was at the level of 24 kDa. Finally, in the control sample (BP4), the antibody recognized a single band that corresponded to nonglycosylated rhIGFBP-4.

Figure 2A shows a WIB of the animal sera probed with the affinity-purified goat polyclonal anti-

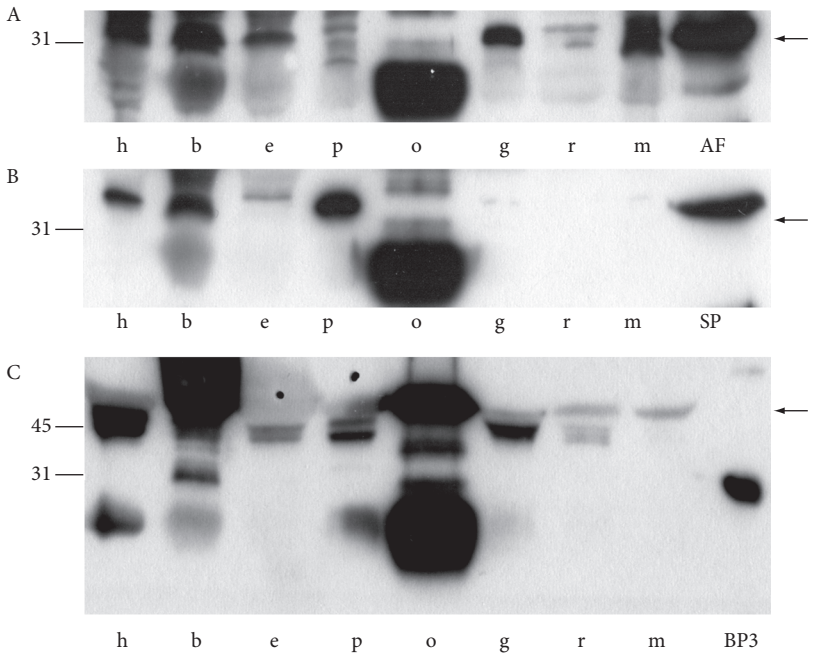

Figure 2. Immunoblots of IGFBP-1 (A), IGFBP-2 (B), and IGFBP-3 (C) probed with goat polyclonal antibodies obtained from DSL (dilution of 1:10,000), done following SDS-PAGE. The 2° Ab was HRP-conjugated SAG IgG (1:10,000). Samples are marked as in Figure 1. Samples were diluted to 1:10 (A, B) or 1:20 (C), and the ovine serum was diluted to 1:100. The amount of protein loaded per lane for A and B was 0.4 mg (h), 0.35 mg (b, m), 0.3 mg (e, p, g, r), and 0.035 mg (o); for C, it was 0.2 mg (h), 0.17 mg (b, m), 0.15 mg (e, p, g, r), and 0.035 mg (o). AF (0.001 mg protein/lane), SP diluted to 1:20 (0.15 mg protein/lane), and rhIGFBP-3 (25 ng/lane) were used as biological markers. The positions of mass markers are indicated on the left and those of IGFBP-1, -2, and -3 are indicated on the right.

hIGFBP-1 antibody (dilution of 1:10,000) obtained from DSL. The immunoreactive IGFBP-1 protein bands were visible in the lanes of human, bovine, equine, guinea pig, and murine serum. Protein bands at the Mr expected for IGFBP-1 band (32-34 kDa) were faint in the lanes of porcine and rat sera, whereas no band at the expected mass was detected in the lane of the ovine serum. There was more than one band in the molecular mass region where IGFBP-1 bands were expected (in parallel with the most intense band from the AF) in the porcine and rat sera.

Figure 2B shows a WIB of the animal sera probed with the affinity-purified goat polyclonal anti-hIGFBP-2 antibody (dilution of 1:10,000) obtained from DSL. The IGFBP-2 band, expected to be at 34 kDa, was clearly visible in the human, bovine, equine, and porcine sera and in the SP sample. However, the anti-hIGFBP-2 antibody obtained from DSL did not recognize the rodent (guinea pig, rat, or murine) IGFBP-2 protein.

Figure 2C shows a WIB of the animal sera probed with the affinity-purified goat polyclonal anti-hIGFBP-3 antibody (dilution of 1:10,000) obtained from DSL. The human, equine, porcine, guinea pig, rat, and most likely bovine sera exhibited the characteristic IGFBP-3 doublet in the region of 46-53 kDa. The doublet in the ovine serum had a smaller Mr, whereas in the murine serum, only one band was visible. Finally, in the control sample (BP3), the antibody recognized a single band with a Mr lower than 31 kDa, which corresponded to nonglycosylated rhIGFBP-3.

Figure 3 shows WIBs of the animal sera probed with the rabbit anti-hIGFBP-2 (dilution of 1:50) and anti-hIGFBP-3 antisera (dilution of 1:100) obtained from GroPep. GroPep anti-IGFBP-2 antiserum failed

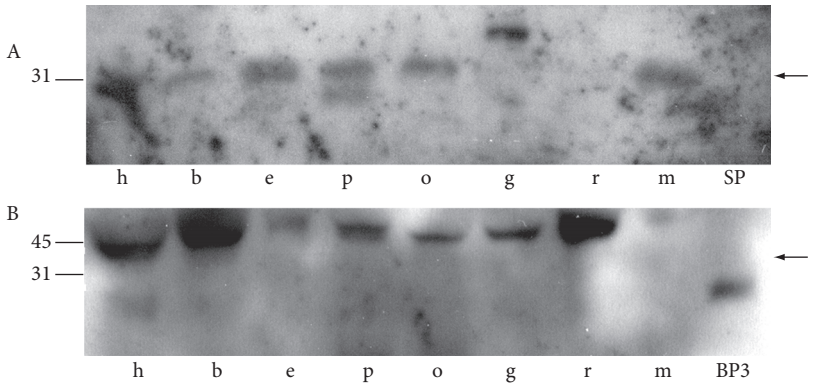

Figure 3. Immunoblots of IGFBP-2 (A) and IGFBP-3 (B) probed with rabbit antisera obtained from GroPep. Anti-IGFBP-2 (dilution of 1:50) and anti-IGFBP-3 (dilution of 1:100) were used following SDS-PAGE. The 2° Ab was HRP-conjugated DAR IgG (1:7000). Samples are marked as in Figure 1. All sera were diluted to 1:10. The amount of protein loaded per lane was 0.4 mg (h, o), 0.35 mg (b, m), and 0.3 mg (e, p, g, r). AF (0.001 mg protein/lane), SP diluted to 1:20 (0.15 mg protein/lane), and rhIGFBP-3 (25 ng/lane) were used as biological markers. The positions of mass markers are indicated on the left and those of IGFBP-2 and -3 are indicated on the right.

to identify IGFBP-2 from the SP (Figure 3A), which was recognized by the respective antibodies from both Santa Cruz Biotechnology and DSL (Figures 1B and 2B). The immunoreactive bands slightly above the position of the 31-kDa mass marker were present in the human, bovine (faint), equine, porcine, ovine, and murine sera (Figure 3A). The immunoreactive band in the lane of guinea pig serum had a greater molecular mass. The anti-hIGFBP-3 antiserum obtained from GroPep recognized a single band (not a doublet) of IGFBP-3, close to 45 kDa, in all of the examined sera, except the murine (Figure 3B). It also recognized rhIGFBP-3 (Figure 3B). In contrast, GroPep anti-hIGFBP-1 and anti-hIGFBP-4 antisera failed to recognize any immunoreactive bands regardless of the antisera and sample dilution or the time of exposure.

Figure 4 shows a WIB of the animal sera probed with the affinity-purified rabbit polyclonal anti-hIGFBP-2 antibody (dilution of 1:1000) obtained from Biogenesis. As shown in Figure 4, the antibody reacted with a protein positioned above the 31-kDa molecular mass marker. The protein band was clearly visible in the human, porcine, and guinea pig sera and was much less intense in the rat and murine sera. The antibody, however, did not recognize IGFBP-2 from SP. The faint bands having high molecular mass, which appeared in the bovine, equine, porcine, and ovine sera, might represent dimeric forms of IGFBP-2 and/or its complexes with certain serum proteins.

The results obtained from the WIB experiments were analyzed in detail and the effectiveness of particular antibodies and antisera for the detection of IGFBPs 1-4 from the examined animal sera are summarized in the Table.

Discussion

Here we describe the use of WIB analysis for testing animal sera for the presence of IGFBP-1, -2, -3, and -4. Most of the available literature on the identification and quantitation of IGFBP-1, -2, and -3 in animal blood is based on radioactive ligand blots. The major advantage of WIB over ligand blotting is that it allows the detection of all immunoreactive species present in a sample, not only the native molecule but also fragments and complexes with other proteins. Since antibodies specifically raised against nonhuman IGFBPs are rarely commercially available, we aimed to investigate which antibodies directed against human IGFBPs could be used to detect IGFBPs present in the circulation of some animal species.

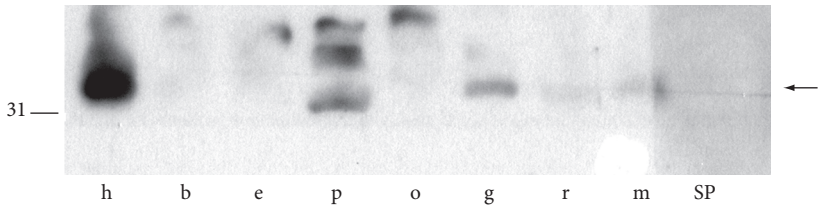

Figure 4. Immunoblot of IGFBP-2 probed with affinity-purified rabbit polyclonal anti-IGFBP-2 antibody obtained from Biogenesis (dilution of 1:1000), done following SDS-PAGE. The 2° Ab was HRP-conjugated DAR IgG (1:7000). Samples are marked as in Figure 1. All samples were diluted to 1:10. The amount of protein loaded per lane was 0.4 mg (h, o), 0.35 mg (b, m), 0.3 mg (e, p, g, r), and 0.15 mg (SP). The positions of mass markers are indicated on the left and that of IGFBP-2 is indicated on the right.

Table. Effectiveness of anti-IGFBP antibodies (Abs) and antisera (As) for the detection of IGFBP-1, -2, -3, and -4 in the sera of different animal species.

	Human	Bovine	Equine	Porcine	Ovine	Guinea pig	Rat	Murine
Santa Cruz anti-IGFBP-1 Ab	○	●		○	●	●		○
Santa Cruz anti-IGFBP-2 Ab	●	●	●	●	●	●	●	●
Santa Cruz anti-IGFBP-3 Ab	●	●	●	●	●	●	●	●
Santa Cruz anti-IGFBP-4 Ab	●	●	●	●	●	●		●
DSL anti-IGFBP-1 Ab	●	●	●	○		●	○	●
DSL anti-IGFBP-2 Ab	●	●	●	●				
DSL anti-IGFBP-3 Ab	●	●	●	●	●	●	●	●
GroPep anti-IGFBP-1 As								
GroPep anti-IGFBP-2 As	●	○	●	●	●			●
GroPep anti-IGFBP-3 As	●	●	○	●	●	●	●	
GroPep anti-IGFBP-4 As								
Biogenesis anti-IGFBP-2 Ab	●			●		●	○	○

● Good recognition ○ Weak recognition

Our experimental protocol was based on the standard immunoblotting procedure, which had originally been set up for human serum by Baričević et al. (22). The procedure was modified where needed to achieve better resolution of protein bands. This was done mostly by altering the dilution of the sample and/or 1° antibody. Applying a reducing instead of nonreducing sample buffer in SDS-PAGE significantly improved the resolution of the WIBs (data not shown).

Although the Santa Cruz Biotechnology anti-IGFBP-1 antibody was recommended for detection of h-, r-, and mIGFBP-1, it effectively detected IGFBP-1 only in bovine, ovine, and guinea pig serum under the experimental conditions explained above. In the lanes of human, porcine, and murine sera, immunoreactive IGFBP-1 bands were faint, so longer exposure of the blotted membranes to autoradiographic film is recommended in order to gain more intense immunoreactive bands. The failure to detect IGFBP-1

from rat serum may have been due to low avidity of the antibody for rat IGFBP-1 or a low IGFBP-1 concentration in the rat serum. The sera with approximately 70 mg protein/mL were diluted to 1:10, which gave a very high protein concentration of 7 mg/mL, bearing in mind that 1 mg/mL is the recommended protein concentration of samples for SDS-PAGE. When sera are diluted to 1:5, the large amount of protein loaded per well puts a strain on the resolving potential of the electrophoretic gel, which may cause substantial distortion of the protein bands. On the other hand, when we diluted the Santa Cruz Biotechnology antibody to 1:500, the resulting immunoblot had increased background stain without the expected improvement in band intensities and sharpness.

The DSL anti-IGFBP-1 antibody displayed selective cross-reactivity toward IGFBP-1 from the different species. Immunoreactive IGFBP-1 bands, which were difficult or impossible to detect using the Santa Cruz Biotechnology anti-IGFBP-1 antibody, were recognized in the lanes of human, equine, porcine, rat, and murine sera. Since the same experimental system was applied with both antibodies (except that the working concentration of the DSL antibody was 0.1 µg/mL, while that of the Santa Cruz Biotechnology antibody was 0.2 µg/mL), it seems that the DSL anti-IGFBP-1 antibody has a higher avidity for IGFBP-1. The Santa Cruz Biotechnology antibody was raised against the C-terminal peptide of IGFBP-1 of human origin (18 amino acids), while the DSL antibody was affinity-purified from a serum IgG fraction of a goat immunized with the full-length, native molecule of IGFBP-1 (234 amino acids), isolated from human amniotic fluid. Therefore, the latter presents a mixture of antibodies directed toward different epitopes on the native IGFBP-1 molecule, increasing its avidity.

IGFBP-1 in human plasma forms a complex with alpha-2-macroglobulin (25). Recently, we found that the majority of circulating IGFBP-1 is not free, but rather is bound to this protein (26). The amount of free IGFBP-1 that is available for interaction with the anti-IGFBP-1 antibody depends on the ability of the complex to dissociate during sample preparation for electrophoresis. Moreover, IGFBP-1 exists in several phosphoisoforms, more or less phosphorylated. Highly phosphorylated IGFBP-1 is the most abundant isoform in human serum, but nonphosphorylated IGFBP-1 and less phosphorylated isoforms exist in amniotic fluid (27). Different antibodies raised against IGFBP-1 may have different avidities toward the different phosphoisoforms.

The polyclonal antibody against IGFBP-2 produced by Santa Cruz Biotechnology was raised against the short peptide located at the C-terminus of murine IGFBP-2 and is recommended for detection of h-, r-, and mIGFBP-2. This was the most successful antibody, as immunoreactive IGFBP-2 bands were detected in all of the sera of the animal species tested. It recognized a doublet band in the lanes of bovine and rodent (guinea pig, rat, and murine) sera. The double immunoreactive IGFBP-2 band was also displayed in the data sheet given by the producer. IGFBP-2 is neither glycosylated nor phosphorylated, and there are no literature data supporting any interaction with other plasma proteins (11). In our experimental system, the DSL anti-IGFBP-2 antibody showed selective cross-reactivity toward IGFBP-2 from the different animal species; for example, it did not recognize rodent IGFBP-2. This antibody was raised against full-length rhIGFBP-2.

IGFBP-3 undergoes posttranslational modification by N-glycosylation. Native IGFBP-3 consists of 2 differently glycosylated isoforms (13), which are usually detected as a characteristic doublet in WIBs. The affinity-purified anti-IGFBP-3 antibody from DSL, produced by immunization of goats with rhIGFBP-3, was the best anti-IGFBP-3 antibody in our tests, as it recognized both of the most abundant forms of IGFBP-3 in several species. The Santa Cruz Biotechnology polyclonal anti-IGFBP-3 antibody was raised against the short peptide located at the C-terminus of murine IGFBP-3 and is recommended for detection of h-, r-, and mIGFBP-3. IGFBP-3 was detected in the sera of all 8 species tested, although this antibody failed to recognize either native isoform of IGFBP-3. A finding that cannot be explained is that 29-kDa nonglycosylated rhIGFBP-3 (produced by DSL) could not be detected using the Santa Cruz Biotechnology anti-IGFBP-3 antibody. This could not be due to a low concentration of rhIGFBP-3 and/or any error in our experimental system, as both the anti-IGFBP-3 antibody obtained from DSL and the antiserum purchased from GroPep recognized this form of IGFBP-3. The latter cross-reacted with

IGFBP-3 from almost all animal species tested, but without clear resolution of the IGFBP-3 isoforms.

The Santa Cruz Biotechnology polyclonal anti-IGFBP-4 antibody was raised against the short peptide located at the C-terminus of human IGFBP-4 and is recommended for detection of h-, r-, and mIGFBP-4. Immunoreactive IGFBP-4 bands were those at approximately 24 kDa and were visible in the lanes of human, bovine, equine, porcine, and ovine serum. A second immunoreactive band, positioned at approximately 28 kDa, probably corresponding to a glycosylated isoform of IGFBP-4 (17), was clearly detectable in bovine and ovine serum and faintly in human, guinea pig, and murine sera.

It is widely known that the binding between an antibody and its antigen is dependent on the affinity constant. The optimal working concentrations (dilutions) of antibodies and antisera were determined empirically for a given set of experimental conditions. For any assay, the optimum dilution is the one that gives the strongest bands with minimum background staining. WIBs obtained using antibodies from Santa Cruz Biotechnology and DSL exhibited generally satisfactory resolutions with a clear background. In contrast, the anti-IGFBP antisera produced by GroPep were of much lower quality. There was substantial background staining on the blots, which complicated the identification of IGFBP bands. The intense background signal was the consequence of the low antisera dilutions, but at higher dilutions, no bands were detected. Furthermore, the GroPep antisera could not be properly dissolved, giving opaque solutions even after applying ultrasound or homogenization techniques.

Taking all this into account, we recommend the use of the goat polyclonal anti-IGFBP-1 antibody produced by DSL for the detection of IGFBP-1 in all tested animal sera, except for ovine serum IGFBP-1. In this case, the goat polyclonal anti-IGFBP-1 antibody from Santa Cruz Biotechnology should be used. The anti-IGFBP-2 antibody produced by Santa Cruz Biotechnology can be used for any of the tested animal sera. Alternatively, the DSL goat polyclonal anti-IGFBP-2 antibody is suitable for the detection of IGFBP-2 in only human, bovine, equine, and porcine sera. Regarding immunodetection of IGFBP-3 in animal sera, we recommend the goat polyclonal antibodies produced by both DSL and Santa Cruz Biotechnology. However, it is important to state that the IGFBP-3 doublet can be detected only by the DSL antibody. Finally, the nonglycosylated form of IGFBP-4 in human, bovine, equine, porcine, and ovine sera can be detected using the Santa Cruz Biotechnology anti-IGFBP-4 antibody. In contrast, rat IGFBP-4 was not detected by any of the antibodies tested in the present study.

The WIB technique presented herein permits the identification of IGFBP-1, -2, -3, and -4 in the sera of certain animals using commercially available antibodies, directed against human IGFBPs. The results of this article may be considered as an initial guide in the designing of experiments in which the detection of animal IGFBPs is required.

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