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Characterization of Insulin-Like Growth Factor Binding Protein-1 Kinases From Human Hepatoma Cells

D.P. Ankrapp, J.I. Jones, and D.R. Clemmons

Department of Medicine, School of Medicine, University of North Carolina, Chapel Hill, North Carolina 27599

Abstract The phosphorylation of insulin-like growth factor binding protein-I (IGFBP-1) alters its binding affinity for insulin-like growth factor I (IGF-I) and thus regulates the bioavailability of IGF-I for binding to the IGF-I receptor. The kinase(s) responsible for the phosphorylation of IGFBP-1 has not been identified. This study was designed to characterize the IGFBP-1 kinase activity in HepG2 human hepatoma cells, a cell line that secretes IGFBP-1 primarily as phosphorylated isoforms. IGFBP-1 kinase activity was partially purified from detergent extracts of the cells by phosphocellulose chromatography and gel filtration. Two kinases of approximate M_r 150,000 (peak I kinase) and M_r 50,000 (peak II kinase) were identified. Each kinase phosphorylated IGFBP-1 at serine residues that were phosphorylated by intact HepG2 cells. The kinases were distinct based on their differential sensitivity to inhibition by heparin (IC_{50} = 2.5 and 16.5 μg/ml, peak I and II kinase, respectively) and inhibition by the isoquinoline sulfonamide CKI-7 (IC_{50} = 50 μM and 100 μM, peak I and II kinase, respectively). In addition, a tenfold molar excess of nonradioactive GTP relative to [gamma-^{32}P]ATP lowered the incorporation of ^{32}P into IGFBP-1 by 80% when the reaction was catalyzed by the peak I kinase, whereas GTP had no effect on the reaction catalyzed by the peak II kinase. In the presence of polylysine, IGFBP-1 was radiolabeled by the partially purified kinase activity when [gamma-^{32}P]GTP served as the phosphate donor indicating the presence of casein kinase II activity. Furthermore, IGFBP-1 was phosphorylated by purified casein kinase I and casein kinase II at sites phosphorylated by the peak I and peak II kinases. Our data suggest that at least two kinases could be responsible for the phosphorylation of IGFBP-1 in intact HepG2 cells and that the kinases are related to the casein kinase family of protein kinases.

Key words: phosphorylation, protein kinase, growth factor, casein kinase, human hepatoma cells, IGFBP-1

The insulin-like growth factor binding proteins (IGFBPs) bind to insulin-like growth factors I and II (IGF-I, -II), but not insulin, with high affinity [Baxter and Martin, 1989]. IGFBPs are present in extracellular fluids and are secreted by many cell types in culture. One of the functions of the IGFBPs is to modulate the biological activity of the IGFs [Jones and Clemmons, 1995]. In particular, IGFBP-1 has been shown to potentiate or inhibit IGF-1–stimulated DNA synthesis [Elgin et al., 1987; Busby et al., 1988a; Koistinen et al., 1990; Kratz et al., 1992; Figueroa et al., 1993; Liu et al., 1991], to inhibit amino acid transport [Ritvos et al., 1988], and in vivo to potentiate IGF-I–stimulated wound healing [Jyung et al., 1994]. The ability of IGFBP-1 to potentiate or inhibit the biologic actions of IGF-I is at least partially due to the phosphorylation of IGFBP-1 [Jones et al., 1991; Frost and Tseng, 1991]. Phosphorylated IGFBP-1 attenuates, whereas nonphosphorylated IGFBP-1 potentiates, the biological activity of IGF-I [Busby et al., 1988a; Jyung et al., 1994]. The underlying mechanism for the biological difference between phosphorylated and nonphosphorylated IGFBP-1 may be attributable to the sixfold higher binding affinity of phosphorylated IGFBP-1 for IGF-I [Jones et al., 1991].

Phosphorylation of IGFBP-1 occurs exclusively on serine residues [Frost and Tseng, 1991; Jones et al., 1993], and this posttranslational modification is an intracellular event [Jones et al., 1991]. To date, the kinase(s) responsible for the phosphorylation of IGFBP-1 has not been identified. In the work described here, we have partially purified two kinases from a human liver carcinoma cell line that secretes IGFBP-1 primarily as four phosphorylated isoforms [Jones et al., 1991]. Each partially puri-

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Address reprint requests to D.P. Ankrapp, Department of Medicine, School of Medicine, University of North Carolina, Chapel Hill, NC 27599.

fied kinase phosphorylated IGFBP-1 at sites similar or identical to sites that were phosphorylated in intact cells. The kinases appear to be distinct based on differences in their apparent molecular weight, their differential sensitivity to inhibition by heparin and CKI-7, and differences in their utilization of GTP in the phosphotransferase reaction.

MATERIALS

HepG2 human hepatoma cells were obtained from the American Type Culture Collection (Rockville, MD). [gamma-^{32}P]ATP (3,000 Ci/mmol) and [gamma-^{32}P]GTP (3,000 Ci/mmol) were obtained from Amersham (Arlington, IL). Ortho [^{32}P]phosphate (8,000 Ci/mmol) was purchased from DuPont–New England Nuclear (Wilmington, DE). Phosphoserine, phosphothreonine, phosphotyrosine, dephosphorylated alpha-casein, phosvitin, heparin (M_r 12–24 kd, 180 USP units/mg), poly-I-lysine (M_r 35,000), ATP, GTP, rabbit antimouse IgG alkaline phosphatase conjugate, phenylmethylsulfonyl fluoride (PMSF), leupeptin, protein A-sepharose CL-4B, Sephacryl S-200, and the gel filtration molecular weight standards were purchased from Sigma Chemical Co (St. Louis, MO) CKI-7 was purchased from Seikagaku (Rockville, MD) and was dissolved in DMSO at a concentration of 100 mM and then kept at 4°C. Triton X-100 was purchased from Pierce Chemical Co. (Rockford, IL), Phenyl-sepharose CL-4B from Pharmacia LKB Biotechnology Inc. (Piscataway, NJ), and Centricon 10 microconcentrators from Amicon (Beverly, MA). Nitrocellulose membrane (0.05 μm pore size) was purchased from Schleicher and Schuell (Keene, NH). Endoproteinase Asp-N and the monoclonal antibody to the α-subunit of casein kinase II (clone 1AD9) were purchased from Boehringer-Mannheim (Indianapolis, IN). Casein kinase I and II were purchased from Promega (Madison, WI). Thin-layer cellulose plates (20 × 20 cm) were from E.M. Sciences (Gibbstown, NJ). The Hunter Thin-layer Peptide Mapping Electrophoresis Apparatus (model HTLE-7000) was purchased from C.B.S. Scientific (Del Mar, CA). Phosphocelulose (P11) and 3MM chromatography paper were purchased from Whatman (Maidstone, U.K.). Tissue culture plates were purchased from Falcon LabWare Division, Becton-Dickinson (Lincoln Park, NJ). Protein concentrations were determined using the Bio-Rad Coomassie Blue Dye Binding reagent with bovine serum albumin as the standard. All other chemicals were from Fisher Scientific (Pittsburgh, PA) or Sigma Chemical Co.

METHODS

Tissue Culture

HepG2 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) fetal calf serum. The cells were passaged at a 1:4 split ratio once weekly. Transfected CHO cells carrying an expression plasmid with the cDNA for human IGFBP-1 (CHOBP1-D6) were maintained as previously described [Jones et al., 1991]. Transfected CHO cell lines carrying expression plasmids with the cDNA for IGFBP-1 in which Ser^{101}, Ser^{169}, or both serines were substituted for alanine ([Ala^{101}]IGFBP-1, [Ala^{169}]IGFBP-1, or [$Ala^{101,169}$]IGFBP-1) were developed and maintained in this laboratory using previously described methods [Jones et al., 1993]. The mutations were made at serine residues in IGFBP-1 that we have shown to be phosphorylated by the CHOBP1-D6 cells [Jones et al., 1993].

Partial Purification of Kinase Activity

HepG2 cells were grown to confluence in 10 cm dishes. The cells were rinsed twice with 5 ml of ice-cold Dulbecco's-buffered saline solution (DBSS), and the cultures were transferred to 4°C. All subsequent procedures were performed at 4°C. One milliliter of lysis buffer (100 mM HEPES, pH 7.5, 100 mM NaCl, 0.5% (v/v) Triton X-100, 1 mM DTT, 10 mM EDTA, 1 mM EGTA, 1 mM PMSF, 10 μg/ml leupeptin) was added to each dish, and after 30 min the lysate was collected and centrifuged (12,000*g*, 10 min), and the supernatant was added to phosphocellulose (10 mg of lysate protein/milliliter packed volume of resin) equilibrated with buffer A (100 mM HEPES, pH 7.5, 100 mM NaCl). After mixing for 60 min, the phosphocellulose was collected by centrifugation (500*g*, 5 min) and the supernatant discarded. The resin was washed twice with five bed volumes of buffer A, and bound protein was eluted with five bed volumes of 100 mM HEPES, pH 7.5, 1 M NaCl, 10 mM $MgCl_2$, 1 mM DTT, and 10% (v/v) glycerol. The eluate was concentrated to 0.2 ml by centrifugation using Centricon 10 microconcentrators. The retained material was diluted twelvefold with 100 mM HEPES, pH 7.5, 10 mM $MgCl_2$, 1 mM DTT, and 10% (v/v) glycerol, and the concentration step was repeated once. The final protein

concentration of the phosphocellulose-purified kinase preparation was 0.5–2 mg/ml as determined by the Bio-Rad (Hercules, CA) Coomassie Dye Binding Protein Assay using bovine serum albumin as the standard. This kinase preparation was used in many of the characterization studies described below. For some experiments, the kinase preparation was further purified by Sephacryl S-200 gel filtration chromatography. It was eluted at 10 ml/h using 100 mM HEPES, pH 7.5, 200 mM NaCl, 10 mM $MgCl_2$, 1 mM DTT, and 10% (v/v) glycerol. Aliquots (10 μl) of selected fractions (0.9 ml) were assayed for casein kinase activity as described below. Fractions containing kinase activity were pooled and concentrated. The phosphocellulose-purified and the gel filtration–purified kinase preparations were stored at −80°C.

Purification of IGFBP-1

For use in the kinase assays, human recombinant IGFBP-1 was purified from conditioned medium obtained from confluent cultures of CHOBP1-D6 cells as previously described [Jones et al., 1993]. For some experiments, the IGFBP-1 from CHOBP1 conditioned medium was partially purified only by phenyl-sepharose chromatography and then used as the substrate in the kinase reaction. The concentration of the purified or partially purified IGFBP-1 was determined by radioimmunoassay [Busby et al., 1988b].

Protein Kinase Assays

The standard incubation mixture contained 100 mM HEPES, pH 7.5, 100 mM NaCl, 10 mM $MgCl_2$, 1 mM DTT, 100 μM [gamma-^{32}P]ATP (500–5,000 cpm/pmole), partially purified kinase (0.1–0.25 mg/ml), and either IGFBP-1 (0.2–0.5 mg/ml), alpha-casein (0.5–1 mg/ml), phosvitin (0.5 mg/ml), or no added substrate. Final assay volumes were 20–25 μl. In some experiments, heparin (2.5–25 μg/ml), CKI-7 (10–200 μM), or GTP (100 μM) was included in the reaction mixture, and the ATP concentration for each of these experiments was 10 μM. Where indicated, 100 μM [gamma-^{32}P]GTP (1,000–2,500 cpm/pmole) was substituted for ATP as the nucleotide substrate, and polylysine (0.1–10 μM) was included in the reaction mixture. The assays were routinely performed at 30°C for 30 min except for assays of the gel filtration fractions for which the incubation time was 90 min. Assays were terminated by adding 6.5 μl (20 μl assay volume) or 8.5 μl (25 μl assay volume) of 4× Laemmli buffer (200 mM Tris-HCl, pH 6.8, 8% (v/v) SDS, 400 mM DTT, 40% (v/v) glycerol) to each sample and then placing the samples in boiling water for 10 min. Proteins were separated on 12.5% SDS-PAGE gels, and the radiolabeled IGFBP-1, casein, or phosvitin was localized on dried gels by autoradiography using Kodak X-OMAT film. The radiolabeled protein was excised from the gels, and the radioactivity in the gel pieces was quantitated by liquid scintillation counting. Alternatively, the dried gels were exposed to a phosphor screen, and the extent of radiolabeling of IGFBP-1 or casein was analyzed and quantitated using a Phosphor Imager (Molecular Dynamics (Sunnyvale, CA)). For the assay of the gel filtration fractions, the autoradiographic intensities of the phosphocasein bands were quantitated by scanning densitometry (model GS 300; Hoefer Scientific Instruments (San Francisco, CA)). For assays using pure casein kinase I and II, the same reaction conditions that were used for IGFBP-1 and casein phosphorylation were utilized except that 5 units of enzyme were added to each tube.

For two-dimensional phosphopeptide mapping of the in vitro phosphorylated IGFBP-1, the kinase reaction was performed as described above for 90 min and then terminated by diluting the sample with 1 ml of 25 mM HEPES, pH 7.5, 100 mM NaCl, 10 mM EDTA, 1 mM ATP, and 1% (v/v) Triton X-100. Rabbit polyclonal antiserum (0.025 ml) against human IGFBP-1 was added, and the binding protein was immunoprecipitated as described below. This antiserum was prepared as previously described [Busby et al., 1988b]. It specifically binds human IGFBP-1 and has $<0.2\%$ cross-reactivity for other forms of IGFBPs.

Western Blot Analysis of the Partially Purified Kinase Fractions Using Anti-Casein Kinase II Antibody

Equal volume aliquots of peak I and peak II containing approximately the same amount of IGFBP1 kinase activity were subjected to SDS-PAGE (10% gel), and the separated proteins were electrophoretically transferred onto an Immobilon membrane. The membrane was incubated in TBS (50 mM Tris-HCl, pH 7.4, 200 nM NaCl) containing 3% (w/v) bovine serum albumin (BSA) for 1 h, washed with TBS containing 0.1% (v/v) Nonidet P-40 and 0.05% (v/v) Triton X-100, and then incubated overnight

(12–16 h) at 4°C with an antibody against the alpha subunit of casein kinase II (1 μg/ml in TBS containing 1% 9 (w/v) BSA). The membrane was washed and incubated (3 h) with rabbit antimouse IgG conjugated to alkaline phosphatase (1:1,000 dilution in TBS containing 1% BSA), and the detectible proteins were visualized using 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium substrate.

Radioactive Labeling of IGFBP-1 From HepG2 Cells and CHO Cells

Near-confluent HepG2 cells or transfected CHO cells in a 10 cm dish were incubated (18–24 h) in 5 ml of phosphate- and serum-free Eagle's minimal essential medium supplemented with the nonessential amino acids, 0.5% (w/v) BSA, and 100 μCi/ml of ortho[^{32}P]phosphate. Aliquots (0.9 ml) of the conditioned media were stored in siliconized tubes at −80°C.

Immunoprecipitation of Radiolabeled IGFBP-1 and Phosphopeptide Mapping

Aliquots (0.9 ml) of radiolabeled HepG2 conditioned medium each received 0.1 ml of 10× concentrated immunoprecipitation buffer (1× buffer = 25 mM HEPES, pH 7, 0.1 M NaCl, 10 mM EDTA, 1% (v/v) Triton X-100) and 0.025 ml of rabbit polyclonal antiserum against human IGFBP-1. After an overnight (18–24 h) incubation at 4°C, 10 mg of protein A-sepharose was added to each sample, and the incubation was continued at 4°C for 2 h. The antibody–protein A complex was collected by centrifugation (12,000*g*, 2 min) and washed twice with 1× immunoprecipitation buffer followed by one wash with 50 mM Tris-HCl, pH 6.8. For both the in vivo and in vitro phosphorylated IGFBP-1, the immunoprecipitated proteins were separated on 12.5% SDS-PAGE gels under reducing conditions and electroblotted (75 min at 1 mA/cm^2 gel) onto nitrocellulose membranes. The band of radiolabeled IGFBP-1 was localized by autoradiography and excised from the membrane. The binding protein was proteolytically cleaved on the membrane strip [Luo et al., 1990] by endoproteinase Asp-N (1 μg/ml) in 50 mM $NaHCO_3$, pH 7.8 (0.2 ml), for 18–24 h at 37°C. Two-dimensional mapping of the phosphopeptides was carried out on thin-layer cellulose plates by the method of Boyle et al. [1991]. In the first dimension, electrophoresis was performed at 15 mA constant current for 45 min at room temperature at pH 1.9 with formic acid/acetic acid/H_2O (5:16:179). In the second dimension, ascending chromatography was performed with 1-butanol/pyridine/acetic acid/H_2O (75:50:15:60). The radiolabeled peptides were visualized by autoradiography. Two-dimensional phosphoamino acid analysis was performed essentially as described by Boyle et al. (1991).

RESULTS

Partial Purification of IGFBP-1 Kinase Activity From HepG2 Cells By Phosphocellulose Chromatography

A HepG2 cell lysate was adsorbed onto and eluted from phosphocellulose. The phosphocellulose-purified protein was incubated with [gamma-^{32}P]ATP in the absence and presence of IGFBP-1, alpha-casein, or phosvitin, and the proteins were separated by SDS-polyacrylamide gel electrophoresis in the presence of 100 mM dithiothreitol. Following autoradiography (Fig. 1), a phosphorylated protein of approximate M_r 34,000 was detected in the lane containing IGFBP-1 (lane 3), and a phosphoprotein of approximate M_r 30,000 was present in the lane containing alpha-casein (lane 2). In addition, phosvitin was phosphorylated by the partially purified kinase activity (not shown). In contrast, there was no IGFBP-1 kinase activity in the fraction of the cell lysate that did not bind to phosphocellulose. This result indicates that the phosphocellulose-purified protein preparation contained kinase activity that was capable of phosphorylating IGFBP-1 as well as the known casein kinase substrates alpha-casein and phosvitin. Kinase activity was not detected in the lysates prior to purification by phosphocellulose chromatography possibly due to the presence of kinase inhibitors and/or serine/threonine phosphatases.

Phosphopeptide and Phosphoamino Acid Analysis

The two-dimensional phosphopeptide map of IGFBP-1 phosphorylated by intact HepG2 cells was compared with the map of IGFBP-1 phosphorylated in vitro by the phosphocellulose-purified kinase in order to determine if the sites of phosphorylation were similar. Four phosphopeptides were generated by the proteolytic digestion of [^{32}P]-labeled IGFBP-1 secreted by HepG2 cells (Fig. 2A). Each of these phosphopeptides migrated to positions that were similar to those

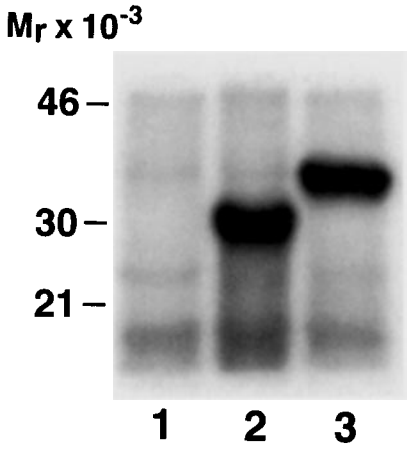


Fig. 1. Phosphorylation of IGFBP-1 and alpha-casein by the phosphocellulose-purified kinase preparation from detergent-solubilized HepG2 cells. Partially purified kinase (0.1 mg/ml) was incubated in the absence (*lane 1*) or presence of 0.25 mg/ml alpha-casein (*lane 2*) or IGFBP-1 (*lane 3*). The reaction was terminated after 30 min by the addition of Laemmli sample buffer, and the proteins were separated by SDS-PAGE. Figure shows an autoradiograph of the dried gel.

of four peptides (Fig. 2B) that were generated when IGFBP-1 was phosphorylated in vitro and digested with the same enzyme. This suggests that the kinase activity in the phosphocellulose-purified protein preparation phosphorylates IGFBP-1 in vitro at all of the sites phosphorylated by the kinase(s) in intact HepG2 cells. An additional phosphopeptide (Fig. 2B, peptide Y) was detected in the digest of IGFBP-1 phosphorylated by the phosphocellulose-purified kinase, suggesting that an additional site was phosphorylated in vitro. Phosphoamino acid analysis of peptide Y indicated that this peptide was phosphorylated only on threonine (not shown). In contrast phosphoamino acid analysis of IGFBP-1 secreted by HepG-2 cells demonstrated phosphorylation exclusively of serine residues (not shown).

CHO cells transfected with a plasmid containing the cDNA for human IGFBP-1 express multiple phosphorylated isoforms of the binding protein [Jones et al., 1991]. Radiosequence analysis has shown that IGFBP-1 secreted by CHO cells is phosphorylated at Ser^{101}, Ser^{119}, and Ser^{169} [Jones et al., 1993]. Of the phosphopeptides generated by the Asp-N digest of [^{32}P]-labeled IGFBP-1 from CHO cells (Fig. 3A), three phosphopeptides comigrated with peptides #1, #2, and #3 in the map of IGFBP-1 phosphorylated by HepG2 cells. This suggests that IGFBP-1 is phosphorylated by HepG2 cells at the same sites phosphorylated by CHO cells.

To assign each of the known serine phosphorylation sites in IGFBP-1 to the appropriate phosphopeptide spots generated by the Asp-N digest of IGFBP-1, we compared the two-dimensional phosphopeptide map of wild-type IGFBP-1 (Fig. 3A) with the maps of the phosphorylated IGFBP-1 analogs (Fig. 3B–D) in which each known site of serine phosphorylation had been substituted with alanine. The peptide map of [Ala^{101}]IGFBP-1 (Fig. 3B) showed a phosphopeptide pattern similar to the map of wild-type IGFBP-1, but there was a significant reduction in the radioactivity associated with peptide #1. Likewise, the phosphopeptide map of [Ala^{169}]-IGFBP-1 (Fig. 3C) showed a reduction in the radioactivity of peptide #3. The phosphopeptide map of [$Ala^{101,169}$]IGFBP-1 (Fig. 3D) showed a significant loss of radioactivity associated with both peptide #1 and peptide #3. These results suggest that peptide #1 contained the Ser^{101} site, peptide #2 contained the Ser^{119} site, and peptide #3 contained the Ser^{169} site (Table I). The residual phosphorylation in each of the peptides that contained a specific serine to alanine mutation might be explained by the presence of serine and/or threonine residues that become phosphorylated in the absence of the primary site. A spot comigrating with peptide Y was detected in the maps of both wild-type IGFBP-1 and the [Ala^{169}]IGFBP-1 after phosphorylation by CHO cells (Fig. 3A,C). The intensity of this spot increased with the amount of proteolytic enzyme used to digest the ^{32}P-labeled IGFBP-1, suggesting that its appearance resulted from the known minor activity of endoproteinase Asp-N to cleave at glutamyl residues. Phosphoamino acid analysis of this peptide detected phosphorylation exclusively on serine residues (not shown), supporting the conclusion that this spot represents one of the serines known to be phosphorylated by intact CHO cells rather than the threonine that is phosphorylated in vitro and contained in peptide Y (Fig. 2B). Since the mutation of Ser^{101} to alanine appears to prevent phosphorylation of this peptide (Fig. 3B,D), this spot most likely represents Ser^{101}.

IGFBP-1 is phosphorylated in vitro by casein kinase II [Frost and Tseng, 1991]; however, the sites of phosphorylation are not known. Since IGFBP-1 from transfected CHO cells is phosphorylated at serines located within consensus sequences recognized by both the casein kinase I and casein kinase II family of protein kinases,

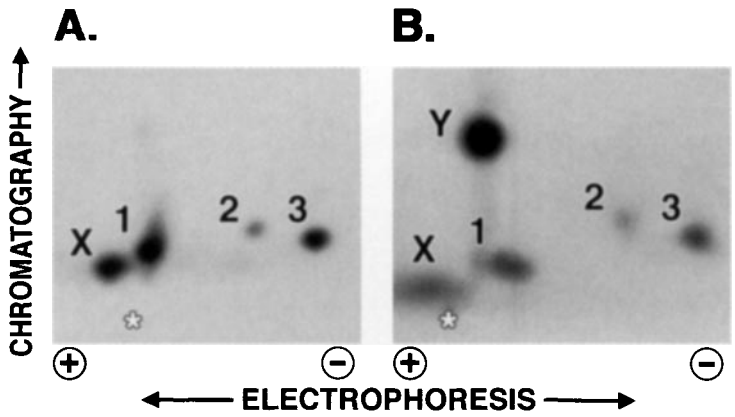


Fig. 2. Two-dimensional phosphopeptide maps of [^{32}P]-labeled IGFBP-1 immunoprecipitated from (**A**) the medium conditioned by HepG2 cells and (**B**) the reaction mixture catalyzed by the phosphocellulose-purified kinase. The immunoprecipitated binding protein preparations were subjected to phosphopeptide analysis as described in Methods. The sample origins are identified by *asterisks*. Peptides are identified for reference in the text.

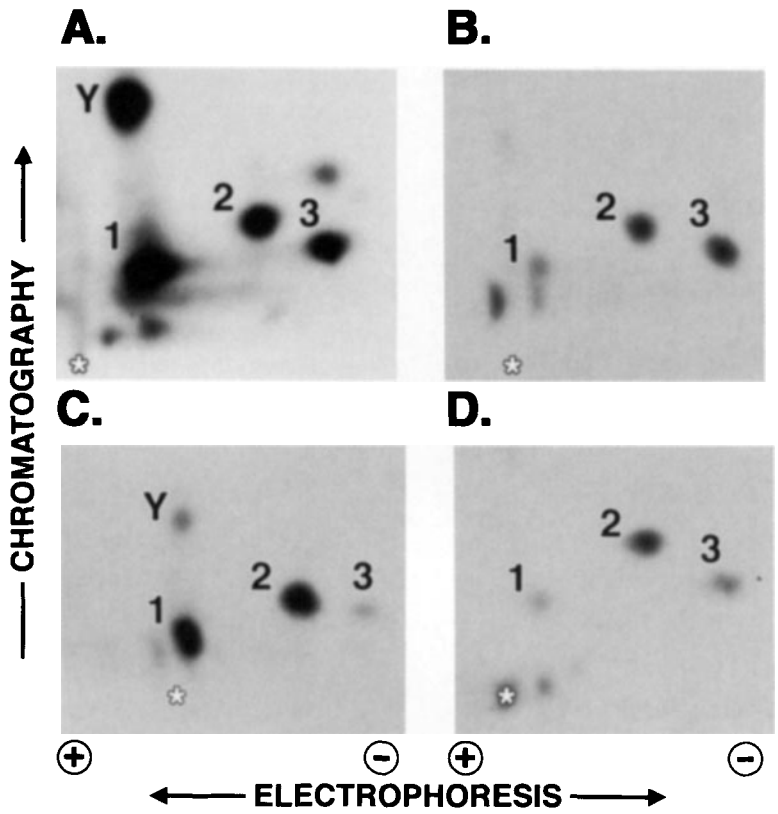


Fig. 3. Two-dimensional phosphopeptide maps of [^{32}P]-labeled IGFBP-1 immunoprecipitated from the conditioned medium of transfected CHO cells expressing (**A**) wild-type IGFBP-1, (**B**) [Ala^{101}], (**C**) [Ala^{169}], or (**D**) [Ala^{101,169}]IGFBP-1. The immunoprecipitated binding protein preparations were subjected to phosphopeptide analysis as described in Methods. The sample origins are identified by *asterisks*. Peptides are identified as in Fig. 2.

TABLE I. Amino Acid Sequences of the [^{32}P]-Labeled Endoproteinase Asp-N Peptides in IGFBP-1 Secreted by CHO Cells Incubated With Ortho[^{32}P]phosphate*

Peptide #1	^{84}DASAPHAAEAGSPESPE**S**^{101} TEITEEELL^{110}
Peptide #2	^{111}DNFHLMAP**S**^{119}EE^{121}
Peptide #3	^{134}DGSKALHVTNIKKWKEPCRIEL-YRVVESLAKAQET **S**^{169}GEE ^{173}IS-KFYLPNCNKGFYHSRQCETSM^{196}

*The sites of Asp-N cleavage were predicted based on the known amino acid sequence of IGFBP-1 [Lee et al., 1988], and the locations of the phosphorylated serine residues (bold) were previously determined by radiosequence analysis [Jones et al., 1993].

we determined whether these kinases phosphorylated IGFBP-1 in vitro at sites that were phosphorylated by kinases in the intact CHO and HepG2 cells. Casein kinase I (Fig. 4A) phosphorylated IGFBP-1 at two sites (peptides #2 and #3) that were phosphorylated by the kinases present in intact HepG2 cells and CHO cells. Likewise, casein kinase II (Fig. 4B) phosphorylated IGFBP-1 at two sites (peptides #1 and #3) that were phosphorylated by HepG2 and CHO cells. The majority of the radioactivity incorporated into IGFBP-1 by either casein kinase I or casein kinase II resided in a peptide that comigrated with peptide Y of IGFBP-1 phosphorylated in vitro by the partially purified kinase activity from HepG2 cells and with peptide Y of IGFBP-1 phosphorylated by intact CHO cells. Phosphoamino acid analysis of the peptides that were generated using either casein kinase I or II showed phosphorylation exclusively on serine residues (data not shown). By comparison, peptide Y was phosphorylated on serine by intact CHO cells but exclusively on threonine by the partially purified kinase activity.

Characterization of the IGFBP-1 Kinase Activity

Since purified casein kinase II phosphorylates IGFBP-1, we determined whether the kinase activity isolated from HepG2 cells could belong to the casein kinase II family of protein kinases. A property unique to the casein kinase II family is the ability to use GTP as the nucleotide substrate [Pinna, 1990]. Thus, we tested whether GTP could substitute for ATP as the phosphate donor in the phosphorylation of IGFBP-1 by the phosphocellulose-purified kinase preparation. IGFBP-1 was incubated with the kinase preparation in the presence of either [gamma-^{32}P]ATP or [gamma-^{32}P]GTP at a nucleotide concentration of 100 μM (Fig. 5A). Incorporation of radioactivity into IGFBP-1 occurred only when [gamma-^{32}P]ATP (lanes 3 and 4) but not [gamma-^{32}P]GTP (lanes 7 and 8) served as the phosphate donor. In contrast, incorporation of radioactivity into alpha-casein was only 2.5-fold greater with ATP relative to GTP as the phosphate donor (data not shown). The phosphorylation of casein with GTP indicated the presence of casein kinase II activity in the kinase preparation. The presence of casein kinase II protein in the partially purified kinase preparation was confirmed by Western blot analysis using a monoclonal antibody against the alpha subunit of casein kinase II (see Fig. 9, lane 1). Since phosphorylation of calmodulin by casein kinase II requires the presence of polylysine or other polybasic effectors [Meggio et al., 1992], we tested whether polylysine could facilitate the phosphorylation of IGFBP-1 by the Hep G2 kinase preparation. To ensure that the radiolabeling of IGFBP-1 was limited to casein kinase II activity, [gamma-^{32}P]GTP (100 μM) was used as the nucleotide substrate. In the absence of polylysine (Fig. 5B, lane 1) there was no significant phosphorylation of IGFBP-1. However, phosphorylation of IGFBP-1 was enhanced approximately sevenfold (Fig. 5B, lane 2), 12.5-fold (Fig. 5B, lane 3), and threefold (Fig. 5B, lane 4) in the presence of 0.1 μM, 1 μM, and 10 μM polylysine, respectively. These results indicate that polylysine is required for the phosphorylation of IGFBP-1 by the casein kinase II activity in the phosphocellulose-purified kinase preparation. The decreased stimulation of phosphorylation by 10 μM vs. lower concentrations of polylysine has also been observed for the polylysine-dependent phosphorylation of the low density lipoprotein receptor [Kishimoto et al., 1987] and calmodulin peptide [Meggio et al., 1992] by purified casein kinase II.

The phosphocellulose-purified kinase activity from HepG2 cells was resolved into two peaks of kinase activity by gel filtration chromatography (Fig. 6). The peak I kinase had an apparent M_r 175,000, whereas the peak II kinase had an apparent M_r 50,000. Each peak of kinase activity phosphorylated IGFBP-1 at sites phosphorylated by intact HepG2 cells. The endoproteinase Asp-N peptide map of IGFBP-1 phosphorylated by the peak I kinase showed five phosphopeptides (Fig. 7A), including the phosphopeptides

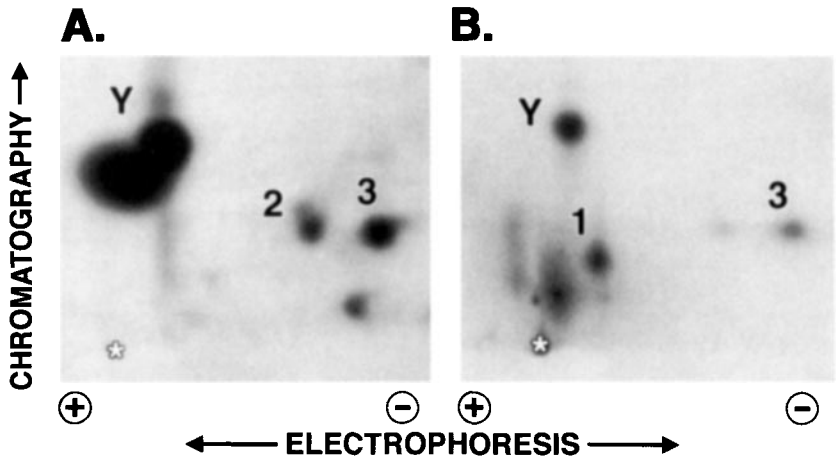


Fig. 4. Two-dimensional phosphopeptide maps of [^{32}P]-labeled IGFBP-1 phosphorylated by purified preparations of (**A**) casein kinase I or (**B**) casein kinase II. The radiolabeled binding protein was immunoprecipitated from the reaction mixture and subjected to phosphopeptide analysis as described in Methods. Peptides are identified as in Fig. 2. The sample origins are identified by asterisks.

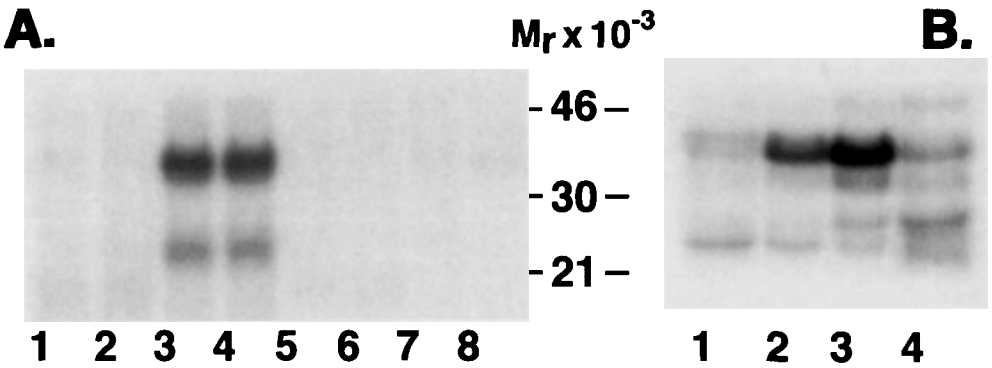


Fig. 5. **A:** Phosphorylation of IGFBP-1 by the phosphocellulose-purified kinase activity with 100 μM [gamma-^{32}P]ATP (*lanes 1–4*) or 100 μM [gamma-^{32}P]GTP (*lanes 5–8*) as the phosphate donor. The phosphorylation reaction was performed for 30 min with the kinase preparation (0.1 mg/ml) in the absence (lanes 1, 2, 5, 6) or presence (lanes 3, 4, 7, 8) of IGFBP-1 (0.2 mg/ml). The reactions were terminated by the addition of Laemmli sample buffer, and the proteins were separated by 12.5% SDS-PAGE. An autoradiogram of the dried gel is shown. Nucleotide specific activities: ATP = 574 cpm/pmole; GTP = 700 cpm/pmole. **B:** Phosphorylation of IGFBP-1 by the phosphocellulose-purified kinase activity with 100 μM [gamma-^{32}P]GTP in the absence (*lane 1*) and presence of polylysine at 0.1 μM (*lane 2*), 1 μM (*lane 3*), and 10 μM (*lane 4*). The assay was performed as described in A. An autoradiogram of the dried gel is shown.

containing Ser^{101} (peptide #1), Ser^{119} (peptide #2), and Ser^{169} (peptide #3). Likewise, the phosphopeptides containing Ser^{101} and Ser^{169} were present in the Asp-N peptide map of IGFBP-1 phosphorylated by the peak II kinase (Fig. 7B). For each kinase activity, the majority of the radioactivity incorporated into IGFBP-1 was at a site (peptide Y) containing exclusively phosphothreonine that was not phosphorylated by intact HepG2 or CHO cells.

The peak I kinase was more sensitive than the peak II kinase to the inhibitory effect of heparin (Fig. 8A) (IC_{50} = 2.5 μg/ml and 16.5 μg/ml for the peak I and peak II kinases, respectively). Likewise, the peak I kinase was more sensitive to the inhibitory effect of the casein kinase–specific inhibitor, CKI-7 (Fig. 8B) (IC_{50} = 50 μM and 100 μM for the peak I and peak II kinases, respectively). In the presence of a tenfold molar excess of unlabeled GTP relative to [gamma-^{32}P]ATP, the amount of radioactivity incorporated into IGFBP-1 by the peak I kinase was decreased by 80% (Fig. 8C, lane 3), whereas the radiolabeling of IGFBP-1 by the peak II kinase was not changed (Fig. 8, lane 6). The decrease in the incorporation of ^{32}P into IGFBP-1 with [gamma-^{32}P]ATP in the presence of excess unlabeled GTP suggested casein kinase II activity in

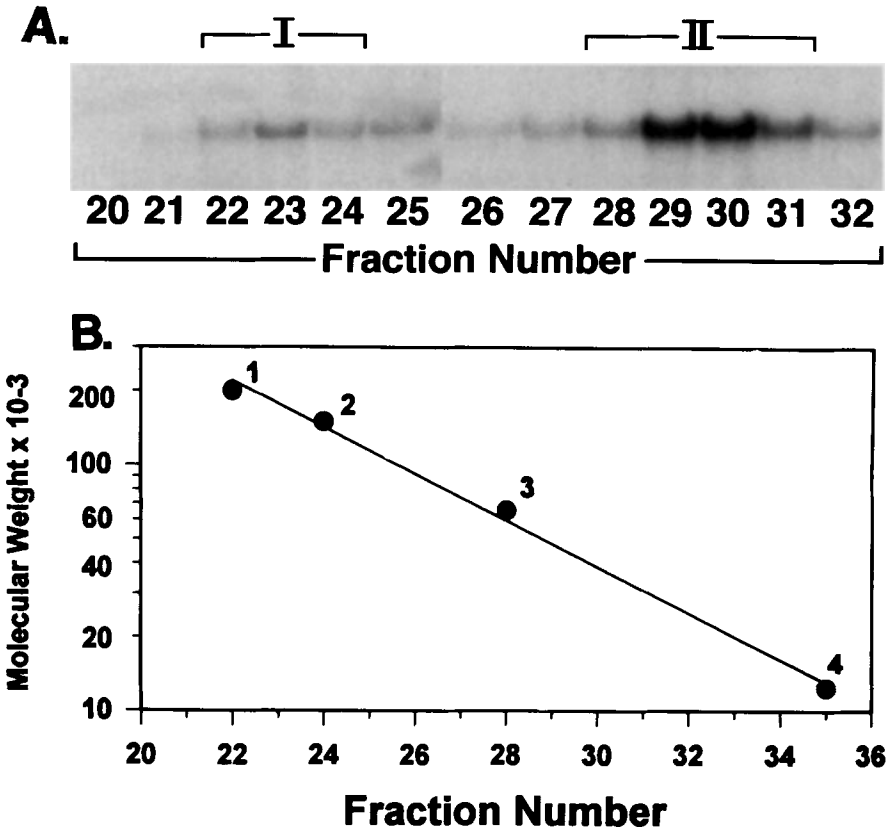


Fig. 6. **A:** Resolution of the phosphocellulose-purified kinase activity from detergent-solubilized HepG2 cells into multiple peaks of kinase activity by Sephacryl S-200 gel filtration. Ten microliters of each fraction was incubated with alpha-casein (1 mg/ml) for 90 min, and the reaction was terminated by the addition of Laemmli sample buffer. Proteins were separated by 12.5% SDS-PAGE. An autoradiogram of the dried gel is shown. Routinely, fractions 21–23 (*peak I*) and fractions 28–31 (*peak II*) were separately pooled for subsequent characterization studies. **B:** Gel filtration calibration curve: 1) alpha-amylase (200 kd); 2) yeast alcohol dehydrogenase (150 kd); 3) bovine serum albumin (66 kd); and 4) horse heart cytochrome C (12.4 kd).

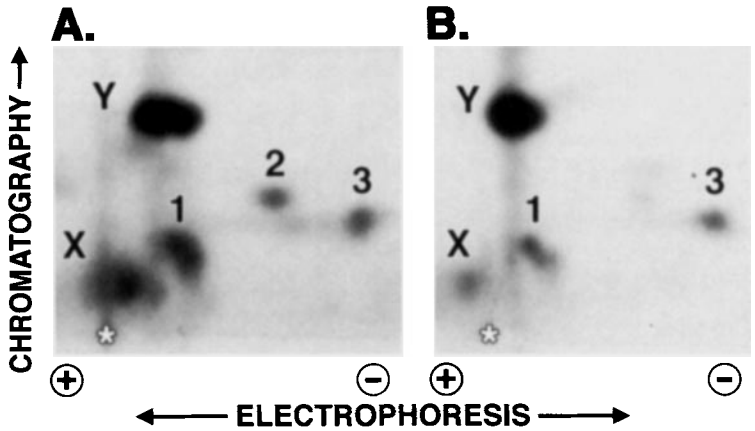
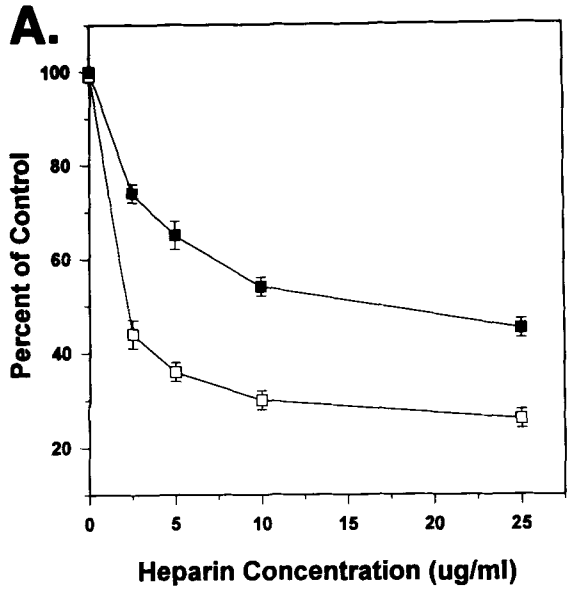
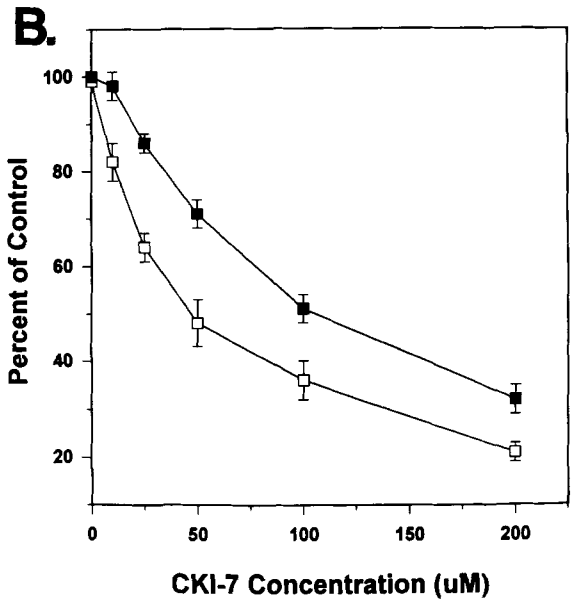
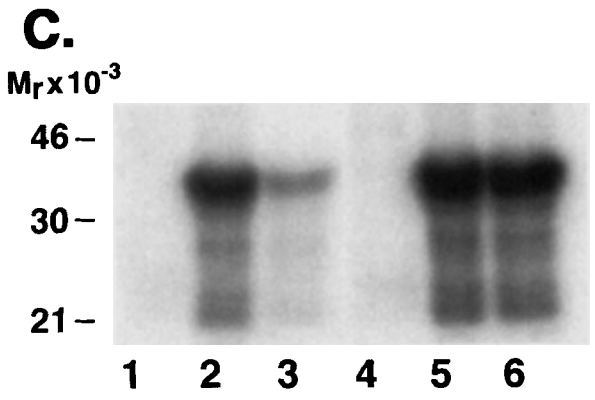


Fig. 7. Two-dimensional phosphopeptide maps of [^{32}P]-labeled IGFBP-1 phosphorylated by the (**A**) peak I kinase and (**B**) peak II kinase. For each reaction, IGFBP-1 was immunoprecipitated from the assay mixture and subjected to phosphopeptide analysis as described in Methods. Peptides are identified as in Fig. 2. The sample origins are identified by *asterisks*.







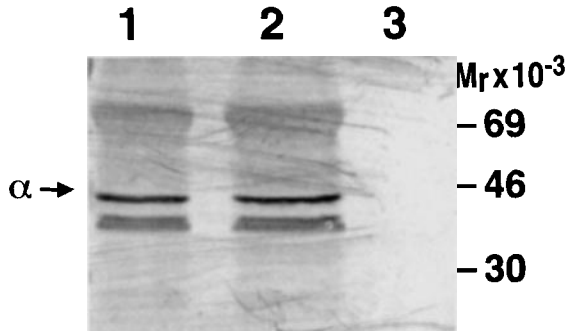


Fig. 9. Anti-casein kinase II immunoblot analysis of Hep G2 kinase preparations. Equal aliquots of the phosphocellulose-purified (*lane 1*) and the peak I (*lane 2*) and peak II (*lane 3*). Sephacryl S-200 purified kinase preparations were resolved by 10% SDS-PAGE, transferred to PVDF membranes, and probed with a monoclonal antibody to the α subunit of casein kinase II as described in Methods. The location in the blots of the α subunit is shown by the *arrow*. Casein kinase II was detected in the phosphocellulose-purified kinase preparation and peak I of the Sephacryl S-200 purified fraction but not in peak II.

the peak I kinase fraction. Immunoblotting showed that the peak I kinase fraction contained detectable casein kinase II alpha subunit (Fig. 9, lane 2), whereas casein kinase II was not detectable in the peak II kinase fraction (Fig. 9, lane 3). Taken together, these results indicate that the two peaks of IGFBP-1 kinase activity are distinct and that each is biochemically related to the casein kinases, with the peak I activity being closely related to CK-II.

DISCUSSION

The principle finding in these studies is that detergent-solubilized extracts of HepG2 human hepatoma cells contain at least two serine/threonine kinases that phosphorylate IGFBP-1. The peak I (M_r 175,000) and the peak II (M_r 50,000) IGFBP-1 kinase activities appeared to

Fig. 8. Effect of heparin (**A**) and CKI-7 (**B**) on the phosphorylation of IGFBP-1 (0.2 mg/ml) by the peak I (□) and peak II (■) kinase activities. The assay was for 30 min with 0.1 mg/ml protein of each kinase and was terminated by the addition of Laemmli sample buffer. Proteins were separated by 12.5% SDS-PAGE. [^{32}P]-labeled IGFBP-1 was localized on the gel by autoradiography. The radiolabeled binding protein was excised from the gels, and radioactivity in the gel pieces was quantitated by liquid scintillation counting. **C:** Phosphorylation of IGFBP-1 with 10 μM [gamma-^{32}P]ATP as the phosphate donor in the presence of 100 μM GTP. The assay was for 30 min with 0.1 mg/ml protein of peak I (*lanes 1–3*) and peak II (*lanes 3–5*) kinase activities without (lanes 1, 4) and with (lanes 2, 3, 5, 6) IGFBP-1 (0.2 mg/ml) in the absence (lanes 1, 2, 4, 5) and presence (lanes 3, 6) of GTP. Proteins were separated by 12.5% SDS-PAGE. An autoradiogram of the dried gel is shown.

be distinct. There was a 6.5-fold difference between the kinases with respect to the IC_{50} of heparin, and there was a twofold difference with respect to the IC_{50} of CKI-7. Furthermore, radiolabeling of IGFBP-1 by the peak I kinase with [gamma-^{32}P]ATP was reduced by 80% in the presence of excess nonradioactive GTP, while the activity of the peak II kinase was not affected by GTP. Finally, the two peaks of IGFBP-1 kinase activity phosphorylated IGFBP-1 at three identical sites (peptides #1, #3, and Y), but the peak I kinase also phosphorylated a distinct site (peptide #2). These findings support our conclusions that at least two distinct kinases are responsible for the phosphorylation of IGFBP-1 in vitro, but it is not known whether the intracellular phosphorylation of IGFBP-1 in intact cells is due to the activity of one or both enzymes.

We have assigned the partially purified IGFBP-1 kinases to the ubiquitous family of casein kinases based on the following observations. First, the IGFBP-1 kinase activities in the HepG2 cell extracts were partially purified by phosphocellulose chromatography. Casein kinases I and II in rat liver cytosol [Itarte et al., 1981; Singh and Huang, 1985] and in rabbit reticulocyte lysate [Hathaway and Traugh, 1979] bind to phosphocellulose, and this separates them from cAMP-dependent protein kinase, protein kinase C, and other serine/threonine kinases. Second, both IGFBP-1 and alpha-casein were radiolabeled by the phosphocellulose-purified kinase activity when [gamma-^{32}P]GTP served as the phosphate donor. The casein kinase II family of protein kinases are the only known kinases that utilize GTP in the phosphotransferase reaction [Pinna, 1990]. The presence of casein kinase II protein in the partially purified kinase preparation was confirmed by Western blot analysis. In addition, several substrates for casein kinase II are phosphorylated only in the presence of polylysine, and polybasic effectors may be important regulators of casein kinase II activity in vivo [Meggio et al., 1992]. Finally, both the peak I and peak II kinase activities were inhibited by the isoquinoline sulfonamide CKI-7 at concentrations (50–100 μM) reported to inhibit casein kinases [Chijiwa et al., 1989; Zhai et al., 1992].

Indirect evidence for designating the HepG2-derived IGFBP-1 kinases as casein kinases is based on the phosphorylation sites in IGFBP-1. Although peptides #1, #2, and #3 from the IGFBP-1 phosphorylated in vitro and in vivo were not radiosequenced, the migration of the three peptides was similar to the migration of phosphopeptides generated by the endoproteinase Asp-N digest of wild-type, [Ala^{101}]; [Ala^{169}]; and [Ala^{101,169}]IGFBP-1 from CHO cells. This suggests that Ser^{101} (peptide #1), Ser^{119} (peptide #2), and Ser^{169} (peptide #3) are the residues phosphorylated in vitro and by the kinase(s) in intact HepG2 cells. These phosphoserines are located within acidic amino acid–rich sequences (Table I) that are potential sites of recognition by members of the casein kinase family [Kennelly and Krebs, 1991]. This was confirmed by showing that purified casein kinase I phosphorylated IGFBP-1 at Ser^{119} (peptide #2) and Ser^{169} (peptide #3), and purified casein kinase II phosphorylated IGFBP-1 at Ser^{101} (peptide #1) and Ser^{169} (peptide #3). It has been reported [Frost and Tseng, 1991; Koistinen et al., 1993] that protein kinase A (PKA) can phosphorylate IGFBP-1 in vitro. However, PKA is not likely to be a physiologic IGFBP-1 kinase since none of the serines phosphorylated in vivo are potential PKA phosphorylation sites. Furthermore, neither the non-phosphocellulose-bound fraction of the Hep G2 cell lysate (which should contain PKA) nor purified catalytic subunit of PKA phosphorylated IGFBP-1 (D. Ankrapp and J. Jones, unpublished observations).

The peak I kinase fraction from gel filtration has properties similar to casein kinase II. A tenfold molar excess of nonradioactive GTP relative to [gamma-^{32}P]ATP decreased by 80% the amount of radioactivity incorporated into IGFBP-1 by the peak I kinase. Additionally, IGFBP-1 is phosphorylated by the phosphocellulose-purified kinase using [gamma-^{32}P] GTP as the phosphate donor, and the alpha subunit of casein kinase II is immunologically detectable in peak I. These findings suggest that the peak I IGFBP-1 kinase activity is closely related to casein kinase II. The apparent molecular weight of the peak I kinase (M_r 175,000) is higher than the M_r 130,000 reported for the canonical heterotetrameric casein kinase II purified from liver [Pinna, 1990]. However, a kinase with a higher molecular weight estimate (e.g., 500,000 daltons) that phosphorylates the low density lipoprotein receptor was shown to share many biochemical properties with casein kinase II and to use GTP in the phosphotransferase reaction in particular [Kishimoto et al., 1987]. Our demonstration that the peak I kinase phosphorylated IGFBP-1 at all of the sites phosphorylated by

casein kinase II lends further support for the presence of casein kinase II activity in the peak I fraction. However, the peak I kinase also phosphorylated IGFBP-1 at a site (peptide #2) that was not phosphorylated by casein kinase II. Because of the impurity of the peak I kinase preparation we cannot rule out the presence of multiple IGFBP-1 kinases including a kinase that phosphorylates the peptide #2 site.

The phosphorylation of IGFBP-1 by purified casein kinase I suggested that one of the partially purified kinases from HepG2 cells might be related to casein kinase I. The peak II kinase had an apparent molecular weight (M_r 50,000) within the range reported (M_r 35,000–50,000) for the various isoforms of casein kinase I [Rowles et al., 1991]. In addition, a tenfold molar excess of GTP relative to [gamma-^{32}P]ATP had no effect on the ability of the peak II kinase to incorporate radioactivity into IGFBP-1. This suggests that the peak II kinase uses only ATP in the phosphotransferase reaction, a property of the casein kinase I family. However, the peak II kinase phosphorylated IGFBP-1 at a site (peptide #1 containing Ser^{101}) not phosphorylated by a purified preparation of casein kinase I from rat liver. Furthermore, the Y peptide was exclusively phosphorylated on threonine residues by the partially purified kinase preparation, whereas purified casein kinase I phosphorylated this peptide exclusively on serine. This observation suggests that either the peak II kinase is distinct from the commercially available casein kinase I preparation or that multiple kinases are in peak II including kinases that phosphorylate the peptide #1 site and peptide Y.

The majority of the radioactivity incorporated into IGFBP-1 phosphorylated by the partially purified IGFBP-1 kinases occurred at a threonine residue (peptide Y) that was not phosphorylated in intact HepG2 cells. The observation of a site phosphorylated in vitro but not in vivo could be due to the presence of a serine/threonine phosphatase in the cells that selectively dephosphorylates this site or to an intracellular inhibitor that prevents phosphorylation at that site. Alternatively, the kinases extracted from the HepG2 cells could preferentially phosphorylate IGFBP-1 at a threonine residue that is not accessible to the kinases in the intact cell. Phosphorylation of the type II IGF receptor from rat hepatoma cells [Corvera et al., 1988] and elongation initiation factor II-beta from rabbit reticulocyte [Welsh et al., 1994] by purified casein kinase II occurs at sites found to be phosphorylated in vivo and also at sites found to be phosphorylated only in vitro.

IGFBP-1 is present as multiple phosphorylated isoforms in extracellular fluids [Busby et al., 1988a; Koistinen et al., 1993; Westwood et al., 1994] and in the cell culture media from multiple cell types [Jones et al., 1991; Frost and Tseng, 1991]. Under normal physiological conditions, IGFBP-1 is produced by the liver and, during pregnancy, the endometrium [Baxter and Martin, 1989]. Phosphorylation of IGFBP-1 affects its binding affinity for IGF-1, which may modulate the bioactivity of IGF-1 in the pericellular environment [Jones et al., 1991]. Understanding the biological regulation of the IGFBP-1 kinases may be important in determining the physiological relevance of IGFBP-1 phosphorylation.

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