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Identification of Glycyrrhizin-Binding Protein Kinase as Casein Kinase II and Characterization of Its Associated Phosphate Acceptors in Mouse Liver

Shigeyoshi Harada,* Atsushi Karino,* Yoshihito Shimoyama,*
Fazel Shamsa,† and Kenzo Ohtsuki*,[1]

**Laboratory of Genetical Biochemistry, Kitasato University, School of Allied Health Sciences, Sagamihara 228, Japan; and †Department of Pharmaceutical Chemistry, College of Pharmacy, Tehran University, Tehran, Iran*

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Two forms (G-I and G-II kinases) of casein kinase II (CK-II) in a partially purified CK-II fraction (Mono Q fraction) of mouse liver were separated by means of glycyrrhizin (GL)-affinity column chromatography. Biochemical characterization revealed that these two GL-binding kinases were identical to CK-II. Two phosphate acceptors [p99 (pI 7.0) and p56] copurified with CK-II were identified as ERp99 (Hsp-90 family protein) and calreticulin, respectively. Another protein [p100 (pI 9.0)], which crossreacted with anti-serum against human glucocorticoid receptor (GR), was associated with ERp99. Phosphorylation of p99 [a heterocomplex of p99 (pI 7.0) and p100 (pI 9.0)] and p56 by CK-II *in vitro* was stimulated significantly by low levels (1-3 μM) of GL, but inhibited significantly at doses above 20 μM. However, no effect of GL on autophosphorylation of ERp99 was detected. The data provided here suggest that GL can regulate CK-II-mediated phosphorylation involved in the GL-induced biological effects in mammalian cells.

Glycyryrrhizin (GL) is present in large quantities in the roots and rhizomes of liquorice, *Glycyrrhiza glabra* L., and is composed of a molecule of glycyrrhetinic acid (GA) and two molecules of glucuronic acid. GL is an effective anti-inflammatory agent used in Chinese medicine (1,2) and several of its biological activities have been identified in the human body, i.e. hypolipidemic (3), anti-oxidative (3), anti-viral (4) and IFNγ-inducing (5) activities.

CK-II, a cAMP-, cGMP- and Ca^{2+}/phospholipid-independent serine/threonine nuclear protein kinase (6), is characterized as one of protein kinases responsible for positive and negative regulations of several DNA-binding transcriptional factors [Sp1 (7), Ap1 (8), serum response factor (9), upstream binding factor (10) and G-box binding protein (11)]. Recently, we reported (12) that casein kinase II (CK-II) is one of GL-binding proteins which, when purified from a 1.5 M NaCl nuclear extract of Ehrlished ascites tumor cells (EAT cells), is copurified with effective phosphate acceptors [a heterocomplex of GR like protein (GRP) and ERp99 (Hsp-90 family protein)] for CK-II activity. However, the physiological significance of GL-binding proteins, which function as effective phosphate acceptors for CK-II *in vitro,* in the transcriptional regulation involved in the GL-induced biological effects remains to be elucidated. Therefore, the present study was undertaken to purify a GL-binding protein kinase and its phosphate acceptors, which are involved in the biochemical mechanisms of the GL-induced

[1] Corresponding author. Fax: (81) 427-78-9406.

Abbreviations: CK-II, casein kinase II; DTT, dithiothreitol; ERp99, 99 kDa endoplasmic reticulum protein; HPLC, high performance liquid chromatography; Hsp-90, heat shock protein 90; GL, glycyrrhizin; GR, glucocorticoid receptor; p99, 99 kDa polypeptide; poly-Arg, poly-L-arginine; PMSF, phenylmethylsulfonyl fluoride; SDS-PAGE, sodium dodecyl-sulfate polyacrylamide gel electrophoresis.

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biological effects, from a 1.5 M NaCl extract of mouse liver. Here, we describe (i) purification and characterization of GL-binding protein kinase and its associated proteins, p99 and p56, from the partially purified CK-II (DEAE-cellulose fraction) fraction of mouse liver; and (ii) the effect of GL on phosphorylation of p99 and p56 by CK-II *in vitro*.

MATERIALS AND METHODS

Glycyrrhizin and anti-sera. GL was kindly supplied by Minophagen Pharmaceutical Co., Ltd. (Tokyo). A 10 mM preparation of the compound in 10% dimethylsulphonyl sulfate was diluted with sterilized water. Anti-human GR (antigen: synthetic polypeptide of amino acid sequence 346-367) and mouse monoclonal antibody specific for slime mold Hsp-90 were obtained from Funakoshi Biochemical Laboratory (Tokyo).

Preparation of a 1.5 M NaCl extract from mouse liver. Mouse liver (wet weight about 80 g) was cut to small pieces and suspended in about 400 ml of Buffer A [40 mM Tris-HCl (pH 7.6), 2 mM dithiothreitol (DTT), 2 mM EDTA, 2 mM EGTA, 1 mM phenylmethylsulfonyl fluoride (PMSF) and 10% glycerol] containing 0.2 M NaCl. The suspension was homogenized for 5 min at 4°C using a glass homogenizer. After centrifugation (40,000 ×g for 15 min), the pellet obtained was suspended in about 400 ml of Buffer A containing 1.5 M NaCl, and sonicated for 90 sec at 4°C. After centrifugation (20 min at 105,000 ×g at 4°C), the supernatant obtained was concentrated with solid ammonium sulfate (70% saturation). The resulting precipitates were suspended in about 50 ml of Buffer A containing 0.2 M NaCl and the dialysate was used as a 1.5 M NaCl crude extract.

Partial purification of CK-II. To partially purify CK-II and its phosphate acceptors, the 1.5 M NaCl crude extract (about 1.2 g protein) was applied on a column (2.6 × 25 cm) of DEAE-cellulose, equilibrated previously with Buffer A containing 0.2 M NaCl. Elution was carried out with a linear gradient between 0.2 M and 1.0 M NaCl. CK-II activity was eluted from the column between 0.50 M and 0.65 M NaCl, as reported previously (12). This DEAE-cellulose fraction was used as a partially purified source of CK-II from mouse liver.

Assay for CK-II activity. The reaction mixture (50 μl) comprised 40 mM Tris-HCl (pH 7.6), 2 mM DTT, 3 mM Mn^{2+}, 5 μg of poly-Arg (an activator for CK-II), 10 μg of α-casein (phosphate acceptor), 30 μM [γ-^{32}P]ATP (1,000 cpm/pmol) and the indicated amount of the partially purified or purified CK-II. After incubation for 20 min at 30°C, the ^{32}P-phosphorylated α-casein was determined, as reported previously (12).

SDS-PAGE and autoradiography. To detect ^{32}P-labeled polypeptides in the partially purified and purified CK-II fractions, the fractions were incubated separately for 20 min at 30°C with 30 μM [γ-^{32}P]ATP (1,000 cpm/pmol) in the presence of 3 mM Mn^{2+} and poly-Arg (a CK-II activator, 5 μg). The ^{32}P-labeled polypeptides in the reaction mixtures were detected directly by autoradiography after SDS-PAGE, as reported previously (12,13).

RESULTS

Further purification of CK-II and its phosphate acceptors. CK-II and its phosphate acceptors in a partially purified CK-II fraction were further purified by gel filtration on a Superdex 200pg column (HPLC). CK-II activity was detected between fractions 47 and 59 as a single active peak (Fig. 1A). Several polypeptides phosphorylated by CK-II were detected when the CK-II active fractions (between 47 and 59) were analyzed separately by SDS-PAGE followed by autoradiography after incubation (20 min at 30°C) with 30 μM [γ-^{32}P]ATP in the presence of poly-Arg (5 μg) (Fig. 1B). These CK-II active fractions were divided into two (designated S-I and S-II fractions) on the basis of the following experimental results: (i) autoradiography (Fig. 1B) detected p99 as a main polypeptide phosphorylated by CK-II in fractions 47-51 (S-I fraction), whereas there were several phosphorylated polypeptides (99, 80, 70, 56, 50 and 34 kDa polypeptides) in fractions 52-60 (S-II fraction); and (ii) immunological experiments, with anti-sera against slime mold Hsp-90 (lanes 2 and 5) and human GR (Fig. 2, lanes 3 and 6) after phosphorylation *in vitro*, confirmed that p99 was a main polypeptide phosphorylated by CK-II in the immuno-precipitates of the S-I fraction, whereas at least two phosphorylated polypeptides (p99 and p56) were detected in the immuno-precipitates of the S-II fraction with anti-serum against human GR (lane 6, Fig. 2).

CK-II and its phosphate acceptors (p99 and p56) in the S-II fraction were further purified by means of Mono Q column chromatography (HPLC). CK-II activity was detected at the fractions eluted between 0.45 M and 0.55 M NaCl as a single protein peak (Fig. 3A). Autoradiography (Fig. 3C) detected at least five phosphate acceptors (approximately 99, 70, 56, 50 and 34 kDa) as polypeptides phosphorylated by CK-II in the presence of poly-Arg in the CK-

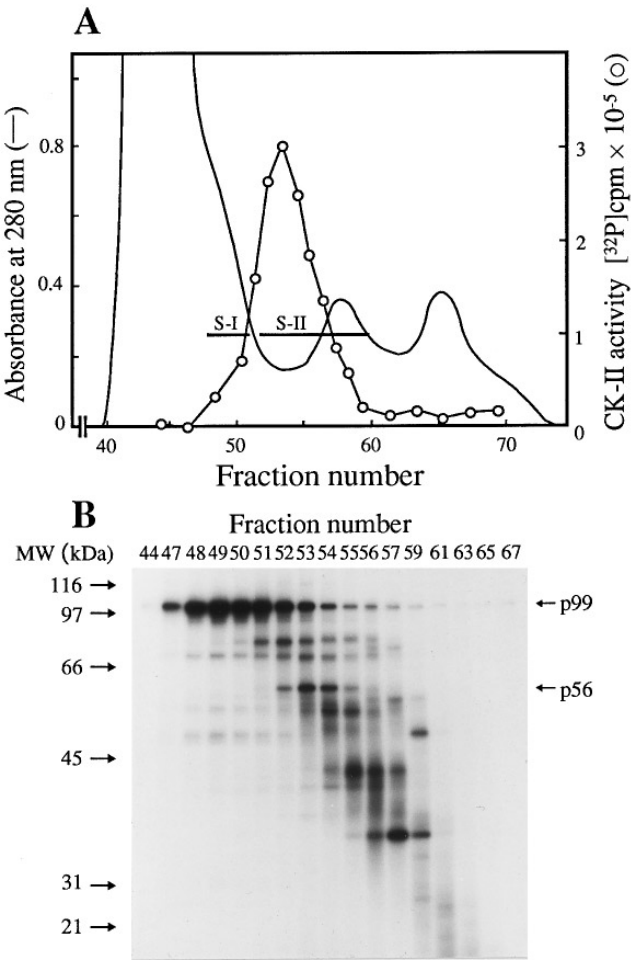

FIG. 1. [A] CK-II and its phosphate acceptors in the DEAE-cellulose fraction (approx. 20 mg protein) were further purified by gel filtration on a Superdex 200pg column (HPLC) in Buffer A containing 1.0 M NaCl. Elution was carried out at a flow rate of 1.0 ml/min, and 1.5 ml fractions were collected. CK-II activity (○-○) in the indicated fractions (10 μl each) was measured using α-casein as a phosphate acceptor. Absorbance at 280 nm (–). [B] The Superdex 200 fractions (10 μl each) between 44 and 67 were separately incubated for 20 min at 30°C with 30 μM [γ-^{32}P]ATP (1,000 cpm/pmol) in the presence of 3 mM Mn^{2+} and poly-Arg (5 μg). The ^{32}P-labeled polypeptides in the reaction mixtures were detected by autoradiography after SDS-PAGE. S-I, fractions 47 through 51; and S-II, fractions 52 through 60.

II peak fractions between 20 and 22. Fractions 20 to 22 contained mainly two polypeptides (p99 and p56), as detected by silver staining (Fig. 3B).

Separation and characterization of GL-binding protein kinases. CK-II and its associated proteins in the Mono Q fraction were further purified by means of GL-affinity column chromatography. Three distinct casein phosphorylating activities (designated G-0, G-I and G-II kinases) were detected when casein was used as a phosphate acceptors *in vitro:* (i) G-0 kinase activity was detected at the break-through fractions; (ii) G-I kinase activity was eluted between 0.10 M and 0.20 M NaCl; and (iii) G-II kinase activity was eluted between 0.45 M and 0.55 M NaCl (Fig. 4A).

To characterize two GL-binding protein kinases (G-I and G-II kinases), the effects of CK-

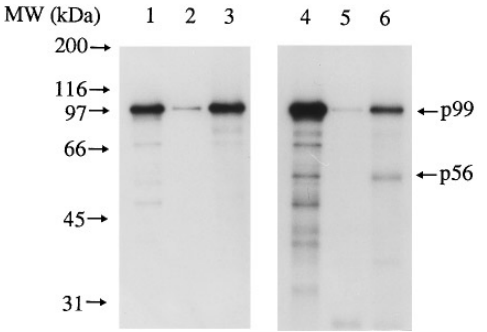

FIG. 2. Immuno-precipitation of the ^{32}P-labeled p99 and p56 in the S-I and S-II fractions with anti-sera against slime mold Hsp-90 and human GR. After incubation (30 min at 30°C) of S-I or S-II fractions (approx. 0.2 μg each) with 30 μM [γ-^{32}P]ATP (1,000 cpm/pmol) in the presence of 3 mM Mn^{2+} and poly-Arg (5 μg) *in vitro,* the ^{32}P-labeled polypeptides in the reaction mixtures were immuno-precipitated with anti-serum against slime mold Hsp-90 or human GR in the presence of protein A-Sepharose. The ^{32}P-labeled polypeptides in the immuno-precipitates were detected by autoradiography after SDS-PAGE. Lane 1, S-I fraction alone; lane 2, S-I fraction immunoprecipitated with anti-Hsp-90; lane 3, S-I fraction immuno-precipitated with anti-GR; lane 4, S-II fraction alone; lane 5, S-II fraction immuno-precipitated with anti-Hsp-90; and lane 6, S-II fraction immuno-precipitated with anti-GR.

II activator (poly-Arg) and CK-II inhibitors (heparin and cold GTP) on phosphorylation of casein or endogenous phosphate acceptors (p99 and p56) by these two kinases were examined. As shown in Fig. 4B, it was found that (i) these two kinases phosphorylated casein (lanes 4 and 9), p99 and p56 *in vitro;* (ii) cold GTP inhibited completely phosphorylation of p99 and p56 by the kinases (lanes 5 and 10); and (iii) phosphorylation of p56 by the kinases was significantly stimulated by poly-Arg (lanes 2 and 7), and inhibited by heparin (lanes 3 and 8). These biochemical characteristics of the G-I and G-II kinases correspond exactly to those reported for CK-IIs from various mouse cells (7). However, it should be noted that no significant inhibition of heparin (0.2 μg/ml) on phosphorylation of p99 by G-I and G-II kinases was observed (Fig. 4B, lanes 3 and 8), although phosphorylation of p56 and casein by CK-II was completely inhibited.

p99 was the major phosphorylated polypeptide in the G-I kinase fraction 9 and p56 was the major phosphorylated polypeptide in the G-II kinase fraction 18 (Fig. 4). As reported previously for EAT cells (12), two-dimensional gel electrophoresis (2DE) confirmed that p99 in the G-II kinase fraction is composed of two distinct polypeptides [99 kDa polypeptide (p99, pI 7.0) and 100 kDa polypeptide (p100, pI 9.0)]. In addition, determination of the partial N-terminal amino acid sequences revealed that p99 (pI 7.0) and p56 was identical to 99 kDa endoplasmic reticulum protein (ERp99) and calreticulin, respectively.

Effect of GL on phosphorylation of p99 and p56 by CK-II in vitro. The dose effect of GL on phosphorylation of p99 and p56 by CK-II in the G-II kinase fraction was examined *in vitro.* It was found that phosphorylation of both p99 and p56 by CK-II was stimulated about 3.3-fold in the presence of 1 μM GL, but was significantly inhibited at doses above 20 μM (Fig. 5). Phosphorylation of p56 by CK-II was sensitive to GL at 30 μM rather than phosphorylation of p99 (Fig. 5).

Since it has been reported that ERp99 has autophosphorylating activity (14), the effect of GL on autophosphorylation of ERp99 (p99, pI 7.0) was examined after CK-II activity in the G-I kinase was inactivated by incubation for 30 min at 55°C. No significant effect of GL even at 100 μM on autophosphorylation of ERp99 was observed (data not shown).

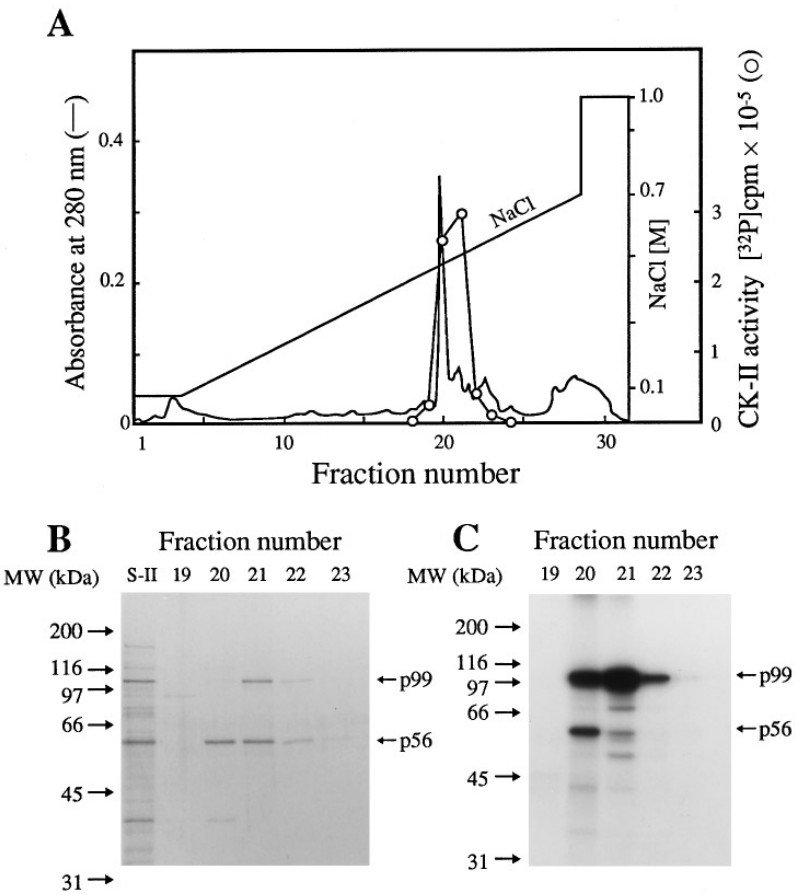

FIG. 3. [A] CK-II and its phosphate acceptors (p99 and p56) and CK-II in the S-II fraction (approx. 3 mg protein) were further purified by Mono Q column chromatography (HPLC). Elution was carried out with a linear gradient between 0.1 M and 0.7 M NaCl at a flow rate of 1.0 ml/min, collecting 1.0 ml fractions. CK-II activity (○-○) in the indicated fractions (10 μl each) was assayed in the presence of poly-Arg (5 μg), using α-casein as a phosphate acceptor. Absorbance at 280 nm (–). [B] Detection of polypeptides in fractions 19 to 23 by silver staining after SDS-PAGE. [C] After incubation with 30 μM [γ-^{32}P]ATP (1,000 cpm/pmol) for 20 min at 30°C in the presence of poly-Arg, the ^{32}P-labeled polypeptides in the Mono Q fractions between 19 and 23 (10 μl each) were detected by SDS-PAGE followed by autoradiography.

DISCUSSION

In the present study, CK-II was identified as a GL-binding protein kinase since (i) two GL-binding protein kinases were purified selectively from the partially purified CK-II fraction by means of GL-affinity column chromatography (Fig. 4); and (ii) the enzymatic characteristics of these two kinases correspond exactly to those reported for CK-IIs from various mammalian cells (6). Moreover, two of the effective phosphate acceptors, p99 and p56, copurified with CK-II activity in the partially purified CK-II (Mono Q) fraction, were identical to ERp99 and calreticulin (Ca^{2+}-binding protein), respectively. In addition, p100 (pI 9.0) was characterized as a GRP, since it crossreacted with anti-serum against human GR (Fig. 2).

We observed that (i) p56 (calreticulin) forms a heterocomplex with p100 (pI 9.0) and p99 (pI 7.0), associated with CK-II activity (Figs. 2 and 4); and (ii) p100 (pI 9.0) in the G-II kinase fraction is also associated with p99 (pI 7.0), p56 and CK-II, as reported previously in EAT

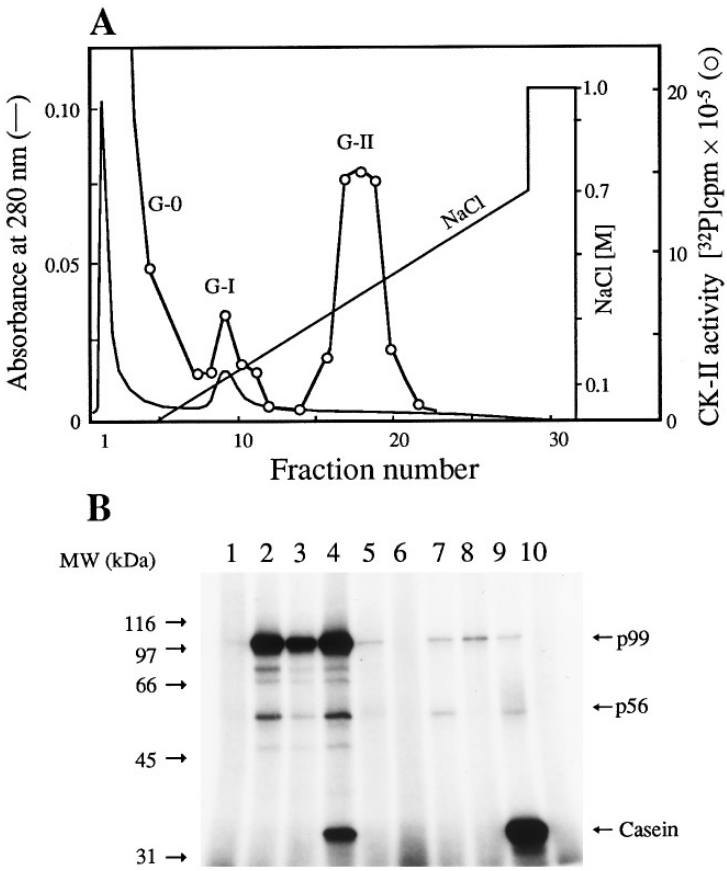

FIG. 4. [A] To separate GL-binding protein kinases in the Mono Q fraction, the fraction (approx. 1.3 mg protein) was applied on a GL-affinity column, previously equilibrated with MES-NaOH buffer (pH 6.8) containing 0.2 M NaCl. Elution was carried out with a linear gradient between 0.0 M and 0.7 M NaCl at a flow rate of 1.0 ml/min, and 1.0 ml fractions were collected. Protein kinase activities (○-○) between fractions 1 and 25 (10 μl each) were assayed using α-casein as a phosphate acceptor. Absorbance at 280 nm (–). [B] To characterize biochemically G-I (lanes 1 through 5) and G-II (lanes 6 through 10) kinases, they (approx. 0.1 μg each) were incubated separately for 20 min at 30°C with 30 μM [γ-^{32}P]ATP (1,000 cpm/pmol) and 3 mM Mn^{2+} in the presence or absence (lanes 1 and 6) of a CK-II activator (poly-Arg, 5 μg) or inhibitors [heparin (0.2 μg) or 50 μM GTP]. These two kinase fractions were incubated separately with 30 μM [γ-^{32}P]ATP in the presence of either heparin (lanes 3 and 8), poly-Arg (lanes 2 and 7) or 50 μM GTP (lanes 5 and 10). After incubation (20 min at 30°C), the ^{32}P-labeled polypeptides in the reaction mixtures were detected by SDS-PAGE followed by autoradiography. Casein was added to the reaction mixtures as an exgenous substrate for these two kinases (lanes 4 and 9).

cells (12). These findings suggest that the partially purified CK-II (Mono Q) fraction contains at least two heterocomplexes [p100 complex (p100, ERp99 and p56) and ERp99 complex (ERp99 and p56)], both of which are associated with CK-II activity since CK-II is tightly bound to ERp99 (15).

Earlier reports have revealed that (i) nuclear receptors (NRs), such as steroid hormone receptors, retinoic acid receptor and GR, are DNA-binding transcriptional factors (16,17); (ii) NRs form a complex with a member of the Hsp-90 family (18); (iii) these receptors have a DNA-binding consensus sequence (KXFFK/RR) and their phosphorylated forms bind specifically to the specific responsive element (GRE) on the enhancer regions of selected genes (19); and (iv) serine/threonine protein kinases, including CK-II, are responsible for phosphorylation

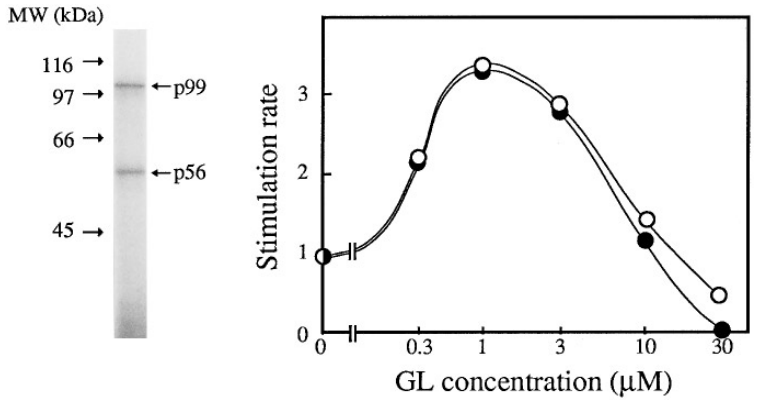

FIG. 5. The effect of GL on the phosphorylation of p99 and p56 by CK-II *in vitro.* The G-II kinase fraction 20 (Fig. 4) was incubated for 15 min at 30°C with 30 μM [γ-^{32}P]ATP (1,000 cpm/pmol), 3 mM Mn^{2+} and poly-Arg (5 μg) in the absence (control or presence of various concentrations of GL. The ^{32}P-labeled p99 and p56 in the reaction mixtures were detected directly by autoradiography after SDS-PAGE. The autoradiogram was scanned with a spectrometer and the stimulation rates were estimated from the averages of the three different experiments under the same conditions (right). Stimulation rate ''1'' represents phosphorylation of p99 (○-○) or p56 (●-●) by CK-II in the absence of GL (left).

of NRs at the cellular level (12). Moreover, recent reports concerning the biological role of calreticulin have demonstrated that (i) this Ca^{2+}-binding protein interacts specifically with the DNA-binding domain of NRs (20); (ii) calreticulin prevents the binding of NRs to the specific DNA binding sites (21); and (iii) the physiological activity of heat shock proteins (Hsps), such as Hsp-90 and Hsp-70, is regulated by their specific phosphorylation by CK-II (12,19). These findings provide indirect evidence to suggest that phosphorylation of both p99 (GRP and ERp99) and p56 (calreticulin) by CK-II in the heterocomplex results in the dissociation of p56 from the heterocomplex as an initial step for activation of GRP. This possibility is supported by our preliminary observations that (i) p56 could be released from the heterocomplex after its full phosphorylation of p56 by CK-II and autophosphorylation of p99 *in vitro;* and (ii) this release was significantly enhanced when phosphorylation of these proteins by CK-II was carried out in the presence of 1-3 μM GL.

Earlier reports concerning GL-induced biological effects have demonstrated that GL has IFNγ-inducing activity (5) and also induces extrathymic T cells in mouse liver (22). Since low levels (1-3 μM) of GL significantly stimulate the CK-II catalyzed *in vitro* phosphorylation of cellular polypeptides, such as GRP and p56, it seems that GL at low levels enhances the CK-II-mediated positive regulation of gene expression in GL-responsive genes, such as the IFNγ gene. However, for complete understanding of the biochemical mechanisms involved in GL-stimulated gene expression, it is necessary to (i) investigate the physiological significance of the CK-II catalyzed phosphorylation of GRP, ERp99 and p56 on the protein-protein interaction between these three phosphate acceptors for CK-II in mammalian cells; (ii) elucidate the physiological role of calreticulin in the CK-II-mediated activation of NRs at the cellular level; and (iii) identify a specific activator for CK-II in the heterocomplex in the T cells activated by GL.

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