Coexpression of both α and β Subunits Is Required for Assembly of Regulated Casein Kinase II†

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ABSTRACT: Casein kinase II is an ubiquitous serine–threonine kinase whose functional significance and regulation in the living cell are not clearly understood. The native enzyme has an oligomeric structure made of two different (α and β) subunits with an $\alpha_2\beta_2$ stoichiometry. To facilitate the study of the structure–activity relationship of the kinase, we have expressed its isolated subunits in a baculovirus-directed insect cell expression system. The resulting isolated recombinant α subunit exhibited a protein kinase catalytic activity, in agreement with previous observations [Cochet, C., & Chambaz, E. M. (1983) *J. Biol. Chem. 258*, 1403–1406]. Coinfection of insect cells with recombinant viruses encoding the two kinase subunits resulted in the biosynthesis of a functional enzyme. Active recombinant oligomeric kinase was purified to near homogeneity with a yield of about 5 mg of enzymatic protein per liter, showing that, in coinfected host cells, synthesis was followed, at least in part, by recombination of the two subunits with an $\alpha_2\beta_2$ stoichiometry. The catalytic properties of the recombinant enzyme appeared highly similar to those previously observed for casein kinase II purified from bovine tissue. Access to the isolated subunits and to their $\alpha_2\beta_2$ association disclosed that the β subunit is required for optimal catalytic activity of the kinase. In addition, the β subunit is suggested to play an essential role in the regulated activity of the native casein kinase II. This is clearly illustrated by the observation of the effect of spermine which requires the presence of the β subunit to stimulate the kinase catalytic activity which is borne by the α subunit. Production of the oligomeric enzyme by coinfected insect cells should provide a powerful experimental system to study structural modifications that may be responsible for the regulation of casein kinase II functions, in living cells.

Casein kinase II is a ubiquitous serine–threonine kinase which is found in both the cytosol and the nucleus of eukaryotic cells (Tuazon & Traugh, 1991; Pinna, 1990; Edelman et al., 1987; Krebs et al., 1988). The enzyme phosphorylates in vivo and in vitro a broad spectrum of nuclear and cytosolic protein substrates, some of which play a critical role in regulating cellular growth and metabolic activities (Tuazon & Traugh, 1991; Pinna, 1990; Edelman et al., 1987; Krebs et al., 1988). Although the existence of casein kinase II has been known for a number of years, its functional significance and regulation in the intact cell are incompletely defined. The development of a specific peptide substrate for the enzyme (Kuenzel & Krebs, 1985) has been useful for estimating changes in kinase activity in response to several cellular stimuli. Casein kinase II was found transiently stimulated following treatment with insulin (Sommercorn et al., 1987; Klarlund & Czech, 1988), insulin-like growth factor I (Klarlund & Czech, 1988), and epidermal growth factor (EGF) (Sommercorn et al., 1987; Ackerman & Osheroff, 1989). Similar activation occurred following serum stimulation (Carrol & Marshak, 1989), and the enzyme was observed to accumulate in nuclei of actively growing cells (Filhol et al., 1990a). Recent evidence indicates that hormonal stimulation of the kinase coincides with an increase in its phosphorylation state (Ackerman et al., 1990).

Casein kinase II from most sources exists as a heterotetramer composed of two dissimilar subunits; i.e., an α subunit of 35–44 kDa and a β subunit of 24–29 kDa which associate to form a native $\alpha_2\beta_2$ structure (Hathaway & Traugh, 1979; Dahmus, 1981). The α polypeptide has been identified as the catalytic subunit by independent methods (Hathaway et al., 1981; Feige et al., 1983; Cochet & Chambaz, 1983a; Meggio & Pinna, 1984) and is homologous with the catalytic domain of the yeast cell division cycle protein kinase CDC 28 (Takio et al., 1987). Cloning of its cDNA has revealed that the protein may represent a heterogeneous product of two different (α, α′) genes (Lozeman et al., 1990; Maridor et al., 1991). The sequence of the β subunit, determined directly from the protein purified from beef lung (Takio et al., 1987) and derived from its cDNA for the *Drosophila* enzyme (Saxena et al., 1987), shows no homology with any other known protein.

The enzyme exhibits several distinctive properties which can be used for its characterization; it can utilize either ATP or GTP as the nucleotide triphosphate donor to phosphorylate serine or threonine residues in protein substrates. The β subunit becomes phosphorylated in an intramolecular autophosphorylation reaction (Hathaway & Traugh, 1979; Dahmus, 1981; Meggio & Pinna, 1984; Glover et al., 1983). Casein kinase II is selectively inhibited by heparin (Hathaway & Traugh, 1982; Feige et al., 1980), and the enzyme can be activated by naturally occurring polyamines (Cochet & Chambaz, 1983b; Feige et al., 1985).

Although the $\alpha_2\beta_2$ oligomeric structure is well established (Glover et al., 1983; Cochet et al., 1983; Hathaway & Traugh, 1979; Dahmus, 1981), the respective roles of the two different subunits in the kinase activity and its regulation remain to be understood. It has been shown that the β subunit is required for optimal catalytic activity (Cochet & Chambaz, 1983a); however, its possible regulatory function and the significance of its autophosphorylation are unknown. Biochemical studies with the purified enzyme have shown that the tetrameric structure of casein kinase II can be dissociated only upon rather

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drastic treatments such as SDS or urea/formamide (Cochet & Chambaz, 1983a). This is a limitation for the isolation of the enzyme subunits under their native forms.

Another approach would be the expression of both the casein kinase II α and β subunits in a host cell system. In order to circumvent the denaturation–renaturation process frequently encountered when using expression in bacteria, we turned to the use of the baculovirus system, for its potential to yield large amounts of functionally active proteins from eukaryotic host cells (Summers & Smith, 1987).

The present report describes the baculovirus-directed production of the isolated casein kinase II subunits. We show that coinfection of insect cells with two viruses containing the genes encoding the two casein kinase II subunits leads to the expression of a functional recombinant holoenzyme. Purification and study of the properties of the recombinant casein kinase II products disclosed that the β subunit is required for optimal kinase activity and plays a regulatory role in some enzyme functions such as its regulation by polyamines.

Experimental Procedures

Materials. [γ-^{32}P]ATP (3000 Ci/mmol) was purchased from Amersham (U.K.) and [^{125}I]NaI was from the CEA (Paris, France). Nucleotides, proteins, spermine (tetrahydrochloride), 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (DRB),[1] leupeptin, Triton X-100, and EDTA were from Sigma Chemicals (St. Louis, MO). Grace's insect cell culture medium was obtained from Gibco. Topoisomerase II was a generous gift from Dr. Annette Larsen (Villejuif, France).

The peptide Arg-Arg-Arg-Glu-Glu-Glu-Thr-Glu-Glu-Glu, which is referred to in the article as the peptide substrate for casein kinase II, was purchased from Peninsula Laboratories Inc. (Belmont, CA).

Antiserum prepared against *Drosophila* casein kinase II (Dahmus et al., 1984; Padmanabha & Glover, 1987) was kindly provided by Dr. C. V. C. Glover (University of Georgia, Athens, GA).

Cells and Viruses. Spodoptera frugiperda (Sf9) cells were maintained in supplemented Grace's medium (Summers & Smith, 1987). For protein expression, Sf9 cells were infected with virus at a multiplicity of infection of 5–10 plaque-forming units. To produce viral stocks, cells were infected at a multiplicity of infection of 0.1–1 plaque-forming units.

DNA Constructs and Isolation of Recombinant Baculoviruses. Full-length *Drosophila melanogaster* casein kinase II α and casein kinase II β cDNAs (Saxena et al., 1987) (Dm92 and Dm98, respectively), independently inserted into the expression plasmid pEV55 (Miller et al., 1986) to yield pEV55 Dmα and pEV55 Dmβ plasmids, respectively, were a kind gift from Dr. C. V. C. Glover (University of Georgia). Transfer of the casein kinase II subunit cDNAs into the *Autographa californica* nuclear polyhedrosis virus (AcNPV) genome was accomplished by homologous recombination following cotransfection of either pEV55 Dmα or pEV55 Dmβ and wild-type AcNPV DNA into Sf9 insect cells. Limiting dilution was used for three rounds of visual screening, and dot-blot hybridization with the α or the β subunit cDNA (Summers & Smith, 1987) was used to identify and isolate the corresponding recombinant viruses designated EV55 Dmα and EV55 Dmβ.

Purification of Recombinant Casein Kinase II. Sf9 cells (1×10^6 cells/mL) were infected with either EV55 Dmα or EV55 Dmβ viruses or were coinfected with both viruses at a multiplicity of infection of 5–10 and cultured in 9- or 15-cm plates. At 3–4 days postinfection, the cells were harvested and homogenized in 2–5 mL of homogenization buffer: 15 mM Tris-HCl, pH 8.2, containing 5 mM KCl, 1 M NaCl, 0.5 mM $MgCl_2$, 50 μM EDTA, 2 mM dithiothreitol, 0.05% Triton X-100, 350 mM sucrose, 10 mM sodium metabisulfite, 10 μM molybdate, 100 μM sodium orthovanadate, 50 mM sodium fluoride, 10 μg/mL leupeptin, and 1 mM phenylmethanesulfonyl fluoride. The cell lysate was sonicated for 3 min and centrifuged at 100000g for 30 min. The soluble extracts were diluted to give a final concentration of 0.2 M NaCl and applied into a phosphocellulose column previously equilibrated with 10 mM Tris-HCl, pH 7.5/1 mM dithiothreitol/1% glycerol/0.1% Triton X-100 (buffer A) and recycled three times through the column. A 0.2–1.5 M linear NaCl gradient in buffer A was applied. Aliquots of collected fractions were used for casein kinase II activity and for Western blot assays. Gel filtration was performed using AcA34 Ultrogel column (0.9 × 29 cm) equilibrated in buffer A containing 1 M NaCl. Aldolase and bovine serum albumin were used as protein calibration standards.

Casein Kinase II Assays. Casein kinase II activity was assayed with slight modifications, as previously described (Ackerman & Osheroff, 1989). The reaction mixture contained (unless otherwise indicated) 50 mM Tris-HCl, pH 8.2, 50 mM NaCl, 25 mM KCl, 100 μM $NaVO_4$, 20 mM $MgCl_2$, 10 μM [γ-^{32}P]ATP [(1–3) × 10^4 cpm/pmol], 1 mM synthetic peptide substrate, and 3 μL of casein kinase II fraction in a final volume of 18 μL. Reactions were incubated for 10–20 min at 25 °C and stopped by addition of 60 μL of 4% trichloroacetic acid and 200 μg of casein as a carrier. The reaction mixture was centrifuged for 20 min at 10000g, and ^{32}P incorporation into the peptide substrate was determined by spotting the supernatant onto phosphocellulose paper disks (Whatman P-81, 4 cm^2). The disks were washed four times in cold 75 mM phosphoric acid and dried, and their radioactivity was measured. When indicated, casein was used as substrate in the reaction mixture in a final volume of 80 μL (Cochet et al., 1981).

Immunoblotting. Samples were run on 12% SDS–polyacrylamide gels, and proteins were electrotransferred onto a nitrocellulose filter for 2 h (500 mA). Casein kinase II subunits (α and β) were detected using an antiserum prepared against *Drosophila* casein kinase II (Dahmus et al., 1984; Padmanabha & Glover, 1987). Briefly, the nitrocellulose filters were treated for 2 h in 10 mM Tris-HCl, pH 7.4, containing 50 mM NaCl, 0.05% Tween-20 (TBS), and 3% BSA at 22 °C. The filters were then incubated with 5 mL of a 1/200 dilution of the casein kinase II antiserum in TBS containing 1% BSA for 2 h at 22 °C or overnight at 4 °C. The filters were washed with TBS and incubated with ^{125}I-labeled protein A at 22 °C for 1 h. Filters were washed, dried, and exposed to X-ray film for autoradiography.

Results

Expression and Coexpression of the Casein Kinase II Subunits. To express the α and β casein kinase II subunits, the pEV55 Dmα and pEV55 Dmβ plasmids, respectively (C. V. C. Glover, personal communication), were used to prepare the corresponding recombinant baculoviruses. These viruses (referred to as EV55 Dmα and EV55 Dmβ) were expected to encode the nonfused α and β polypeptides, respectively. Initially, expression of the casein kinase II subunits was analyzed by [^{35}S]methionine labeling of Sf9 cells infected with EV55 Dmα or EV55 Dmβ. SDS–polyacrylamide gel elec-

[1] Abbreviations: DRB, 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole; MOI, multiplicity of infection; pfu, plaque-forming unit; CK II, casein kinase II; SDS, sodium dodecyl sulfate; AcNPV, *Autographa californica* nuclear polyhedrosis virus.

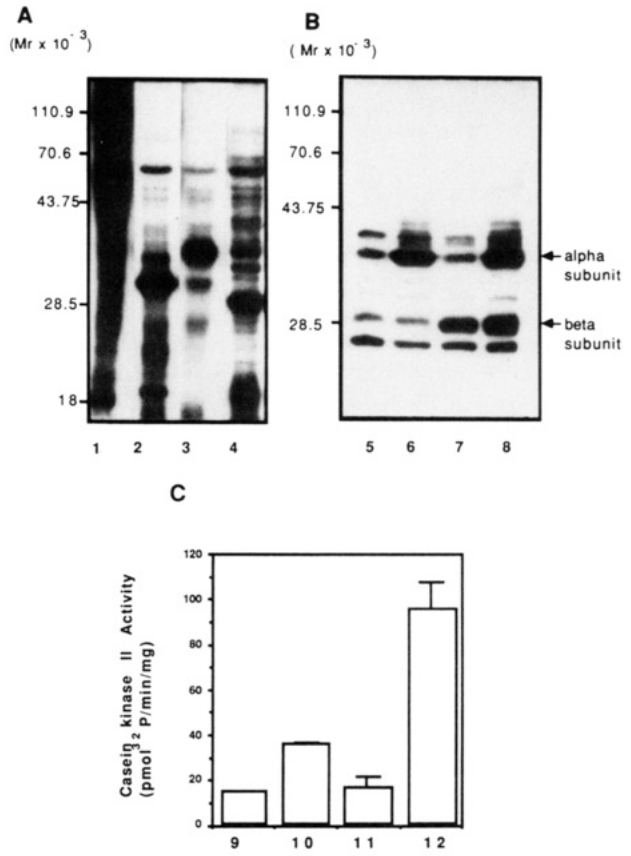

FIGURE 1: Detection of casein kinase II in Sf9 cells. (A) At 3 days postinfection, Sf9 cells (0.2×10^6) uninfected with virus (lane 1), infected with AcNPV (lane 2), infected with EV55 Dmα (lanes 3) or infected with EV55 Dmβ (lane 4) were metabolically labeled with [^{35}S]methionine for 5 h. The cells were solubilized with Laemmli sample buffer, and the proteins (40% of total) were resolved by SDS–PAGE (12%). An autoradiogram of the dried gel is shown with molecular weight markers indicated at the left. (B) Uninfected (lane 5), EV55 Dmα infected (lane 6), EV55 Dmβ infected (lne 7), or EV55 Dmα and EV55 Dmβ coinfected (lane 8) Sf9 cells (2×10^7) were solubilized at 3 days postinfection, sonicated, and centrifuged at 100000g. The supernatant (1% of total) was resolved by SDS–PAGE (12%) and electrotransferred onto nitrocellulose. The nitrocellulose was blotted with antiserum prepared against *Drosophila* casein kinase II (Dahmus et al., 1984). Bound antibodies were detected with ^{125}I-labeled protein A. An autoradiogram of the nitrocellulose is shown with molecular weight markers indicated at the left. (C) Supernatants (0.2% of total) corresponding to the experiment described in panel B were analyzed for casein kinase II activity using the specific peptide substrate in the kinase assays: uninfected (lane 9), EV55 Dmα infected (lane 10), EV55 Dmβ (lane 11), or EV55 Dmα and EV55 Dmβ coinfected (lane 12) Sf9 cells.

trophoresis of total cell protein extracts followed by autoradiography revealed a labeled protein with an estimated molecular weight of 36000 in EV55 Dmα infected cells (Figure 1A, lane 3) and a protein of 28000 in EV55 Dmβ infected cells (Figure 1A, lane 4). These proteins were not detectable in uninfected Sf9 cells (Figure 1A, lane 1) or wild-type virus (AcNPV) infected Sf9 cells (Figure 1A, lane 2). A strong band at approximately 30000 molecular weight was present in AcNPV-infected Sf9 cells (Figure 1A, lane 2) in agreement with the expected high expression of the polyhedrin protein (Summers & Smith, 1987).

Expression of the casein kinase II subunits in Sf9 cells was further assessed by immunoblotting characterization using *D. melanogaster* casein kinase II rabbit polyclonal antibodies (Dahmus et al., 1984; Padmanabha & Glover, 1987). An immunoblot of a total cellular protein extract revealed an immunoreactive band at M_r 36000 in EV55 Dmα infected Sf9 cells (Figure 1B, lane 6) whereas an immunoreactive protein of M_r 28000 was detected in EV55 Dmβ infected cells (Figure 1B, lane 7). These two proteins were also expressed in Sf9 cells following coinfection with EV55 Dmα and EV55 Dmβ (Figure 1B, lane 8). Both proteins were detected, although at low level, in uninfected Sf9 cell extracts (Figure 1B, lane 5).

The molecular weights of 36000 and 28000 are in agreement with the predicted molecular weights of 39833 and 24700 on the basis of the expected amino acid composition of the α and β *Drosophila* casein kinase II subunits. Moreover, purified *Drosophila* casein kinase II is composed of a 37-kDa α and a 28-kDa β subunit, which are also in agreement with the observed molecular weights. These results indicate that the two casein kinase II subunits were correctly expressed in Sf9 cells independently infected or coinfected with the recombinant baculoviruses EV55 Dmα and EV55 Dmβ.

Casein Kinase II Activity of the Baculovirus-Directed Expression Products. Further characterization of casein kinase II subunit expression was performed by examination of casein kinase II activity in the cellular extracts of Sf9 cells. Casein kinase II activity was assayed in the extracts from uninfected, EV55 Dmα infected, EV55 Dmβ infected, or EV55 Dmα and EV55 Dmβ coinfected Sf9 cells (Figure 1C). The results show that casein kinase II was present in uninfected Sf9 cells (Figure 1B, lane 5, and Figure 1C, lane 9); following infection with EV55 Dmα, the expression of the α subunit led to a 2.5-fold increase of casein kinase II activity in the host cell extract (Figure 1C, lane 10). Expression of the β protein alone had no detectable effect on the cell extract casein kinase II activity (Figure 1C, lane 11). By contrast, coexpression of both the α and the β casein kinase II subunits resulted in a 6-fold increase in casein kinase II activity, as compared to control cells (Figure 1C, lane 12).

Since the casein kinase II catalytic site is borne by the α subunit, expression of α protein at variable levels in the host cells could explain these observations. The kinase activity was therefore expressed with regard to the amount of α subunit, as determined by immunoblot scanning (Figure 1B, lanes 6 and 8). This yielded values of casein kinase II activity 2.5-fold higher in Sf9 cell extracts expressing both subunits than in the cells expressing the α subunit only. This strongly suggested that the β peptide contributed to the overall casein kinase II activity and that this may be due, at least in part, to synthesis of a recombinant active holoenzyme, made of both the α and β peptides, by the coinfected Sf9 cells. To examine whether such an α–β recombination could occur without the active contribution of the host cells, supernatants containing independently expressed α and β subunits were mixed in vitro in various proportions. No evidence of an α–β subunit association was obtained as judged by assay of casein kinase II activity and gradient centrifugation analysis (Cochet & Chambaz, 1983a). Further attempts using 6 M guanidine denaturation of the mixture followed by slow renaturation under dialysis were unsuccessful in yielding any detectable α–β association (data not shown).

These observations strongly suggested that coinfected Sf9 cells most likely performed the biosynthesis of the α and β casein kinase II subunits and, at least in part, were able to process and assemble the two peptides in a catalytically active recombination, whose stoichiometry required further characterization (see below).

To optimize the coexpression of both the α and β casein kinase II subunits, coinfection of Sf9 cells was carried out with EV55 Dmα and EV55 Dmβ viruses at different multiplicities of infection. At day 4 postinfection, the cells were homo-

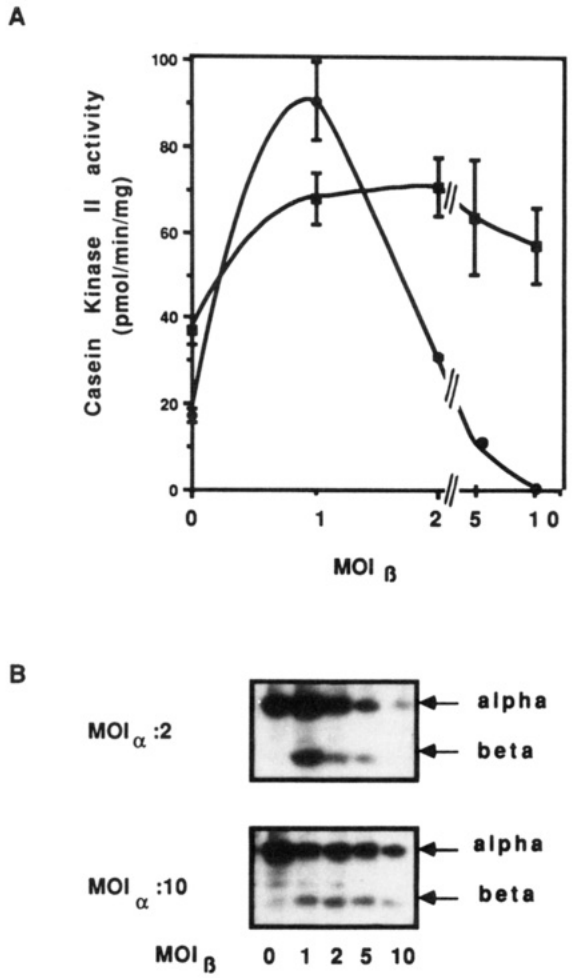

FIGURE 2: Casein kinase II activity of Sf9 cell extracts after infection with both EV55 Dmα and EV55 Dmβ viruses at different multiplicities of infection. At day 4 postinfection, Sf9 cells (10^6) infected with EV55 Dmα (virus) at a MOI of either 10 (■) or 2 (●) and with increasing EV55 Dmβ virus (MOI from 1 to 10) were homogenized, and aliquots of cell extracts were assayed for casein kinase II activity (panel A). Aliquots were also analyzed by Western blotting (panel B), as in Figure 1.

genized and the corresponding extracts were assayed for casein kinase II activity. As illustrated in Figure 2, when the EV55 Dmα MOI was increased from 2 to 10, casein kinase II activity recovered from host cells was increased by 2-fold. When coinfection with various MOI of EV55 Dmβ was carried out with the EV55 Dmα MOI being kept constant (either at 2 or 10), a rather complex picture emerged with regard to the resulting casein kinase II activity expressed by the host cells. At a high EV55 Dmα MOI, expression of the β subunits led to an increase of casein kinase II activity to reach a plateau for a β/α MOI ratio between 0.1 and 0.5. However, at a EV55 Dmα MOI of 2, the introduction of EV55 and Dmβ strikingly increased expression of casein kinase II activity in a rather narrow range (β/α MOI ratio around 0.5) whereas higher β MOI resulted in inhibition of casein kinase II expression. Further study will be required to examine possible cross-regulations in the subunit expression. For the present work, coinfection conditions yielding optimal expression of casein kinase II activity were thereafter employed (i.e., same MOI, between 5 and 10, for both viruses) for further experiments.

Purification and Molecular Characterization of Active Recombinant Casein Kinase II. Aliquots of Sf9 cell extracts, following EV55 Dmα and EV55 Dmβ coinfection (under conditions as defined above), were loaded onto a phosphocellulose column which was developed with a 0.2–1.2 M linear NaCl gradient. Figure 3 (panel A) shows that the endogenous casein kinase II activity from uninfected Sf9 cell extract eluted at 0.9 M NaCl (fractions 8–9) as previously observed for native casein kinase II from other tissues (Cochet et al., 1981, 1983a).

Electrophoretic analysis of the protein contents of the active fractions did not disclose any detectable polypeptide with an M_r corresponding to the α or β subunits in the extract from uninfected cells (Figure 3A, inset). The casein kinase II activity from coinfected Sf9 cells was dramatically higher (about 40-fold) and eluted at the same position as the endogenous kinase. Moreover, the SDS–PAGE analysis of the active fractions and Coomassie blue staining disclosed two polypeptides exhibiting the expected M_r of the α and β casein kinase II subunits. Active fractions from both control and coinfected cell extracts were concentrated and applied to a AcA34 Ultrogel column equilibrated in buffer A containing 1 M NaCl. As illustrated in Figure 3B, casein kinase II activity from uninfected cells was eluted as a symetrical peak with an apparent molecular size of 140 kDa. The presence of α and β polypeptides was not detectable after SDS–PAGE analysis of the corresponding fractions (Figure 3B, inset). Casein kinase II activity from coinfected Sf9 cells eluted at the same position, and SDS–PAGE analysis of the active fractions showed two polypeptides exhibiting the molecular sizes of the α and β subunits (Figure 3B, inset). Western blot analysis of the same fractions using an anti-casein kinase II antiserum was in agreement with the fact that those polypeptides were casein kinase II subunits.

These observations clearly showed that coinfection of Sf9 cells with the two recombinant viruses led to the expression of an oligomeric form of recombinant casein kinase II. The fact that in vitro combination of extracts from separately infected cells did not allow an efficient reassociation of the subunits of the enzyme after cell lysis (see above) suggests that the subunits assembly takes place in the host cells, following biosynthesis of the peptides. The isolated recombinant casein kinase II exhibited a similar pattern on SDS–PAGE analysis, as casein kinase II purified from bovine lung (Cochet et al., 1983). Using bovine serum albumin and bovine casein kinase II as quantitative internal standards, it could be estimated that 1 L of coinfected Sf9 cell culture yielded about 5 mg of purified recombinant casein kinase II. By Coomassie blue and silver staining, the recombinant casein kinase II appeared to be more than 90% pure. Using the peptide substrate, the specific activity of the recombinant enzyme was estimated to be 40 nmol/(min·mg of protein) whereas that of purified endogenous insect casein kinase II was 800 nmol/(min·mg of protein). This indicates that the recombinant casein kinase II exhibited a specific activity of about 5% that of the purified endogenous enzyme.

As shown in Table I, coinfection led to the expression of a high level of recombinant casein kinase II, which represent about 10% of the total proteins in the Sf9 cell extracts. In line with this marked enrichment, purified casein kinase II was obtained with a purification factor of 7, while the endogenous casein kinase II has to be purified by about 6400-fold to reach a similar degree of homogeneity.

Enzymatic Characterization of Purified Recombinant Casein Kinase II. Study of the enzymatic properties of the purified baculovirus-directed recombinant casein kinase II was carried out using the purified preparation obtained following phosphocellulose chromatography of EV55 Dmα and EV55 Dmβ coinfected Sf9 cell extracts (Figure 4). The divalent metal ion requirement to support casein kinase II activity was

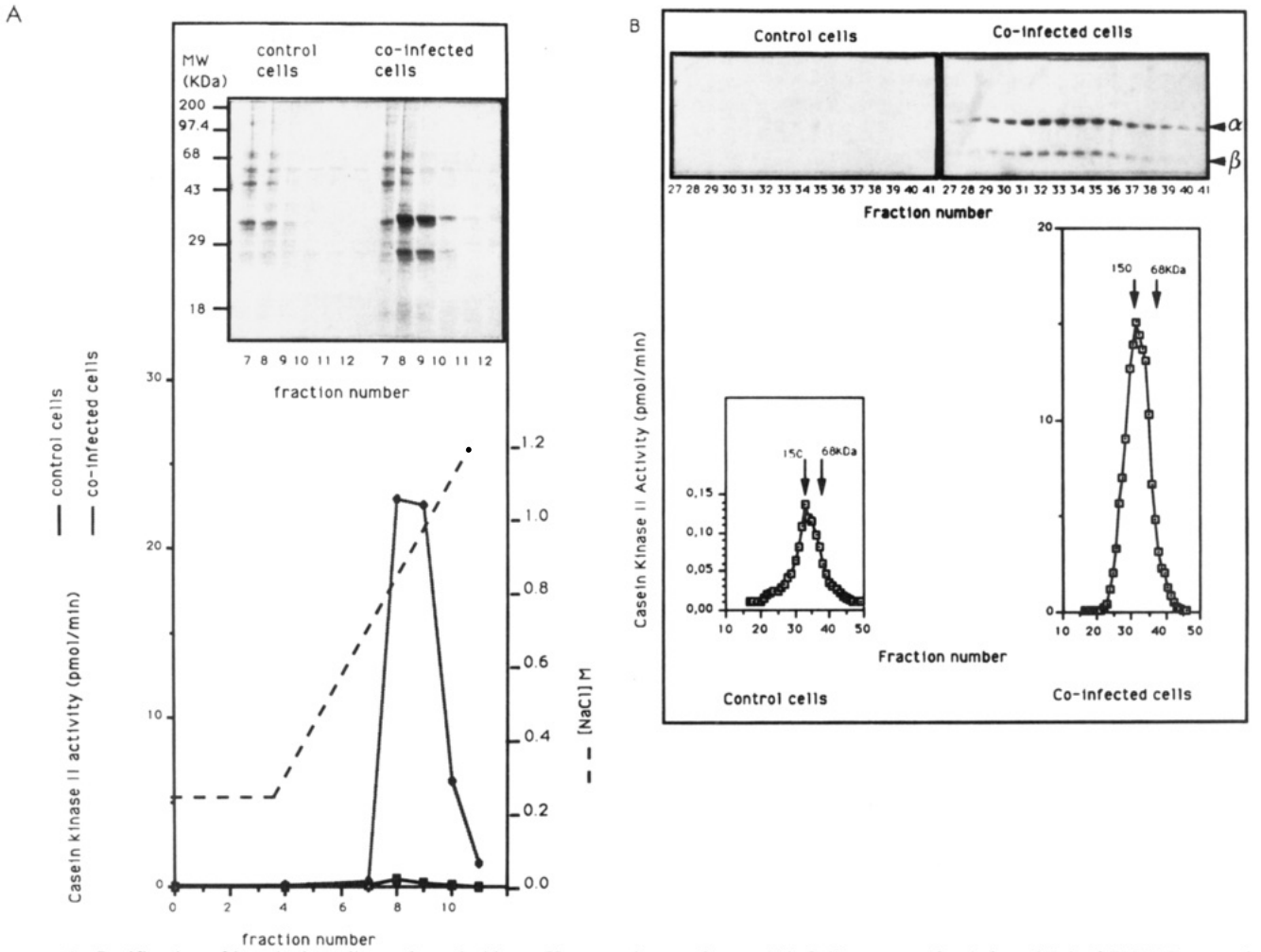

FIGURE 3: Purification of baculovirus-directed casein kinase II expression products. (A) Cell extracts of uninfected (■) of EV55 Dmα and EV55 Dmβ coinfected Sf9 cells (●) (1×10^8) at 3 days postinfection was lysed and centrifuged at 100000g. The supernatant was diluted to give a final concentration of 0.2 M NaCl and applied to a phosphocellulose column equilibrated and eluted as described under Experimental Procedures. Samples (2.5 μL) were assayed for casein kinase II activity. Fractions (20 μL) 7–12 were collected and analyzed by SDS–PAGE (12%), and proteins were detected by Coomassie blue staining (inset). (B) Phosphocellulose chromatography fractions 8 and 9 from control and coinfected cells were pooled, concentrated, and loaded onto Ultrogel AcA34 column (11 mL) and eluted as described under Experimental Procedures. Collected samples (2.5 μL) were assayed for casein kinase II activity. Fractions 27–41 (20 μL) were analyzed by 12% SDS–PAGE, and proteins were detected with Coomassie blue staining (inset). Arrows indicate the position of bovine serum albumin (68 kDa) and aldolase (158 kDa) used as calibration standards in the filtration chromatographic step.

Table I: Purification of Endogenous and Recombinant Monomeric and Oligomeric Forms of Casein Kinase II from Sf9 Cell Extracts

step	protein (μg)	sp act. (nmol/(min·mg of protein))	purification factor
Endogenous Casein Kinase II from Uninfected Sf9 Cell Extracts			
100000g supernatant	3000	0.125	1
phosphocellulose	6.4	38	300
filtration	0.25	800	6400
Recombinant Oligomeric Casein Kinase II from Coinfected Sf9 Cell Extracts			
100000g supernatant	3000	5.1	1
phosphocellulose	500	18	3.5
filtration	208	36	7
Recombinant α Subunit of Casein Kinase II from Sf9 Cell Extracts			
100000g supernatant	3000	0.7	1
phosphocellulose	1000	2.0	2.8

first analyzed. A maximal (about 10-fold) stimulation of the kinase activity was seen with optimal Mg^{2+} concentrations of 20–40 mM (panel A). Spermine at millimolar concentrations stimulated the enzyme activity by an average of 6-fold (panel B). The use of ATP or GTP as phosphate donors (panel C) showed that the recombinant casein kinase II could use GTP as well as ATP. Because heparin is a well-established selective inhibitor of casein kinase II activity (Cochet & Chambaz, 1983b), it was important to determine whether heparin inhibited the baculovirus-expressed casein kinase II. Heparin inhibited 50% of the casein kinase II activity at 0.03 μg/mL and 90% of the enzyme activity at 0.2 μg/mL (panel D). Another reported inhibitor of casein kinase II activity, DRB (Zandomeni et al., 1986), was examined. As shown in Figure 4 (panel E), inhibition of the enzyme was obtained at 50 μM DRB. These data indicate that the baculovirus-directed recombinant casein kinase II enzymatic activity exhibited all of the typical traits of its native counterpart purified from mammalian sources (Tuazon & Traugh, 1991; Pinna, 1990; Edelman et al., 1987).

Contribution of the α and β Subunits in the Modulation of Casein Kinase II Activity. We have previously reported that when the tetrameric structure of native casein kinase II was dissociated in urea/formamide, the catalytic activity was recovered with the α subunit (Cochet & Chambaz, 1983a). This was confirmed by the present data, showing that the α subunit expressed in insect cells is catalytically active. The access to a recombinant oligomeric casein kinase II as well as to its recombinant isolated subunits allowed us to examine

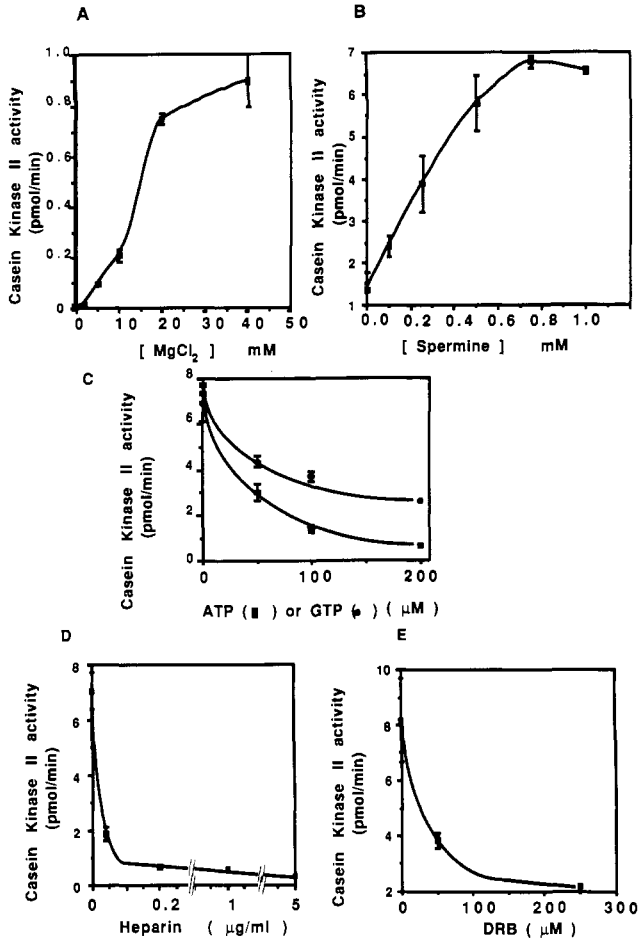

FIGURE 4: Characterization of recombinant casein kinase II enzymatic properties. An aliquot (5 μL) of casein kinase II, expressed by coinfection of Sf9 cells with EV55 Dmα and EV55 Dmβ viruses and fractionated on a phosphocellulose column was incubated for 10 min at 30 °C with 10 mM Tris-HCl, pH 7.4/0.1 mg/mL BSA/7.5 mg/mL casein/10 μM [γ-^{32}P]ATP (6 × 10^3 cpm/pmol) and the indicated concentrations of magnesium (panel A), spermine (panel B), unlabeled ATP or GTP (panel C), heparin (panel D), or DRB (5,6-dichloro-1-β-D-ribofuranosylbenzimidazole) (panel E), in a reaction volume of 80 μL. Mg^{2+} concentrations were 2 mM (panel B) and 20 mM (panels C, D, E). The incorporation of ^{32}P into casein was measured as described (Cochet et al., 1981). Each point is the mean of duplicate points, and similar results were obtained in two separate experiments.

the contribution of each subunit to the overall kinase activity and its regulation. The α subunit expressed in EV55 Dmα infected Sf9 cells was purified to near homogeneity (90%) on a phosphocellulose column from which it eluted at 0.8 M NaCl. As shown in Table I, this α subunit preparation exhibited a specific activity of 2 nmol of ^{32}P incorporated per minute per milligram of protein. As compared to this value, the recombinant $\alpha_2\beta_2$ enzyme showed a specific activity about 20-fold higher (Table I): this clearly indicates that the β subunit is required for optimal kinase activity of the oligomeric enzyme. In addition, we examined the effect of spermine on the activity of oligomeric and monomeric recombinant forms of the enzyme. This activity was assayed using two different substrates, i.e., casein and topoisomerase II (Figure 5). Phosphorylation of both substrates by the oligomeric form of the recombinant enzyme was stimulated 3–5-fold in the presence of submillimolar concentrations of spermine. By contrast, spermine at the same concentrations was without any effect on the kinase activity of the isolated α subunit. Similarly, high magnesium concentrations required for optimal activity of the oligomeric enzyme had no effect on the α subunit alone. This strongly indicates that although the casein

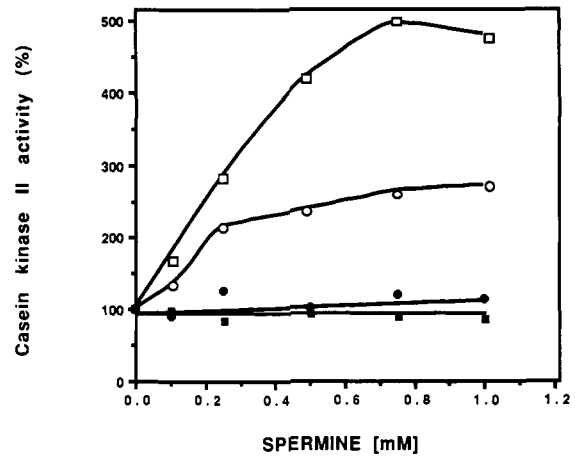

FIGURE 5: Differential effect of spermine on oligomeric and monomeric casein kinase II activity. Casein kinase II activity from insect cells either coinfected with EV55 Dmα and EV55 Dmβ viruses (□, ○) or infected with EV55 Dmα alone (■, ●) was purified by phosphocellulose chromatography. The active fractions were assayed for protein kinase activity with either casein (□, ■) or topoisomerase II (○, ●) as substrate in the presence of 2 mM magnesium and increasing amounts of spermine. The incorporation of ^{32}P into each substrate was measured, and the data were plotted as the percent of the ^{32}P incorporation value in the absence of spermine taken as 100% for both oligomeric and monomeric activities.

kinase II α subunit bears the catalytic site, the β subunit plays an important role in the regulation of the oligomeric casein kinase II activity.

Discussion

To facilitate the biochemical analysis of the structure–activity relationship and the study of the biochemical significance of the ubiquitous casein kinase II, we have expressed the isolated subunits of the enzyme in the baculovirus-directed insect cell expression system (Summers & Smith, 1987).

Insect cells infected with the casein kinase II α or β subunits encoding viruses produced large quantities of the corresponding expected proteins. The proteins accumulated over 3 days postinfection and remained stable during this time, being then easily isolated because most cell protein synthesis was turned off at this stage of infection. Using a peptide substrate, we found that the isolated α subunit expressed in this system was catalytically active, confirming initial observations demonstrating that this subunit contains the catalytic sites of the enzyme (Cochet & Chambaz, 1983a; Feige et al., 1983; Traugh et al., 1990; Lin et al., 1991; Hu & Rubin, 1990). Coinfection of insect cells with viruses encoding for the two subunits led to the simultaneous expression of the two corresponding polypeptides with a concomitant increase in the recovery of casein kinase II activity. Because the β subunit appears to be required for optimal kinase activity (Cochet & Chambaz, 1983a), this result suggests functional oligomerization of the enzyme in the coinfected cells. Filtration analysis of coinfected cell extracts confirmed that these cells express an $\alpha_2\beta_2$ heteromeric form of the enzyme. Coinfected cell extract was used to purify the $\alpha_2\beta_2$ oligomeric enzyme to near homogeneity. This purification provided approximately 5 mg of oligomeric casein kinase II per liter of coinfected cell culture. The recombinant enzyme was activated in the presence of high magnesium concentrations, could use ATP as well as GTP as phosphate donor, was strongly inhibited by heparin and DRB, and was stimulated by polyamines (Tuazon & Traugh, 1991; Pinna, 1990; Edelman et al., 1987). The enzymatic properties of the recombinant enzyme are thus highly similar to those previously described for casein kinase II purified from mam-

malian tissues (Cochet et al., 1983; Zandomeni et al., 1986; Tuazon & Traugh, 1991). The β subunit, which by itself was devoid of any kinase activity, increased the activity of the catalytic α subunit. Contribution of the β subunit appeared to be essential for optimal activity of the oligomeric $\alpha_2\beta_2$ enzyme. This was evident from the differential effect of spermine on activity of oligomeric and monomeric casein kinase II. Polyamines are potent activators in vitro of casein kinase II activity (Feige et al., 1985; Hathaway & Traugh, 1984), of the nuclear uptake of the enzyme (Filhol et al., 1990a), and of its DNA binding activity (Filhol et al., 1990b). Using both casein and a well-known physiological substrate such as topoisomerase II (Ackerman et al., 1988), we found that the oligomeric recombinant enzyme was strongly sensitive to spermine. By contrast, this polyamine at the same concentrations was without effect on the isolated α subunit. This observation suggests that polyamines stimulate casein kinase II activity by interacting at least in part with the β subunit. This is in agreement with a recent report describing the catalytic properties of the α subunit of casein kinase II expressed in *Escherichia coli* (Traugh et al., 1990; Hu & Rubin, 1990; Lin et al., 1991).

Interestingly, calculation of the specific activity of the isolated recombinant enzyme disclosed that it was about 5% that of the endogenous Sf9 casein kinase II. These results suggest that the posttranslational processing and assembly of the α and the β casein kinase II subunits may be only partially performed in the coinfected insect cells. Attempts to reconstitute a fully active casein kinase II by in vitro recombination of the α and β recombinant subunits were unsuccessful, even following a denaturation–renaturation cycle. This observation is in contrast with that from Grankowski et al. (1991), who reported that recombinant α and β casein kinase II subunits produced by *E. coli* could reconstitute in vitro an optimal casein kinase II activity, upon simple mixing in vitro. The $\alpha_2\beta_2$ structure expressed in coinfected Sf9 cells thus most likely results from processing and combination during biosynthesis of α and β subunits in the host cells. The low specific activity of the recombinant $\alpha_2\beta_2$ casein kinase II obtained in the present study remains to be understood. It may be explained by a lack in some posttranslational process, such as phosphorylation, as observed in the case of baculovirus-directed synthesis of S6 kinase (Vik et al., 1990). Casein kinase II phosphorylation on its β subunit has been correlated with increased casein kinase II activity in EGF-stimulated cells (Ackerman et al., 1990); however, the recombinant casein kinase II isolated in the present study was able to self-phosphorylate (not shown), and we have no evidence that additional phosphorylation is required for optimal kinase activity.

Because coinfection of Sf9 cells with recombinant baculoviruses allows in vivo assembly of native enzyme, this provides an experimental system for studying both in vivo and in vitro modifications that may prove to be essential for expression of full catalytic activity of casein kinase II.

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REFERENCES

Ackerman, P., & Osheroff, N. (1989) *J. Biol. Chem. 264*, 11958–11965.

Ackerman, P., Glover, C. V. C., & Osheroff, N. (1988) *J. Biol. Chem. 263*, 12653–12660.

Ackerman, P., Glover, C. V. C., & Osheroff, N. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 821–825.

Carroll, D., & Marshak, D. R. (1989) *J. Biol. Chem. 264*, 7345–7348.

Cochet, C., & Chambaz, E. M. (1983a) *J. Biol. Chem. 258*, 1403–1406.

Cochet, C., & Chambaz, E. M. (1983b) *Mol. Cell. Endocrinol. 30*, 247–266.

Cochet, C., Job, D., Pirollet, F., & Chambaz, E. M. (1981) *Biochim. Biophys. Acta 658*, 191–201.

Cochet, C., Feige, J. J., & Chambaz, E. M. (1983) *Biochim. Biophys. Acta 743*, 1–12.

Dahmus, M. E. (1981) *J. Biol. Chem. 256*, 3319–3325.

Dahmus, G. K., Glover, C. V. C., Brutlag, D., & Dhamus, M. E. (1984) *J. Biol. Chem. 259*, 9001–9006.

Edelman, A. M., Blumenthal, D. K., & Krebs, E. G. (1987) *Annu. Rev. Biochem. 56*, 567–613.

Feige, J. J., Pirollet, F., Cochet, C., & Chambaz, E. M. (1980) *FEBS Lett. 121*, 139–142.

Feige, J. J., Cochet, C., Pirollet, F., & Chambaz, E. M. (1983) *Biochemistry 22*, 1452–1459.

Feige, J. J., Cochet, C., & Chambaz, E. M. (1985) in *Recent Progress in Polyamine Research* (Selmeci, L., Brosnan, M. E., & Seiler, N., Eds.) Vol. 13, pp 181–190, Akademiai Kiado, Budapest.

Filhol, O., Cochet, C., & Chambaz, E. M. (1990a) *Biochemistry 29*, 9928–9936.

Filhol, O., Cochet, C., & Chambaz, E. M. (1990b) *Biochem. Biophys. Res. Commun. 173*, 862–871.

Glover, C. V. C., Shelton, E. R., & Brutlag, D. L. (1983) *J. Biol. Chem. 258*, 3258–3265.

Grankowski, N., Boldyreff, B., & Issinger, O. G. (1991) *Eur. J. Biochem. 198*, 25–30.

Hathaway, G. M., & Traugh, J. A. (1979) *J. Biol. Chem. 254*, 762–768.

Hathaway, G. M., & Traugh, J. A. (1982) in *Current Topics in Cellular Regulation* (Stadtman, E., & Horecker, B., Eds.) Vol. 21, pp 101–127, Academic Press, New York.

Hathaway, G. M., & Traugh, J. A. (1984) *J. Biol. Chem. 259*, 7011–7015.

Hathaway, G. M., Zoller, M. J., & Traugh, J. A. (1981) *J. Biol. Chem. 256*, 11442–11446.

Hu, E., & Rubin, C. S. (1990) *J. Biol. Chem. 265*, 20609–20615.

Klarlund, J. K., & Czech, M. P. (1988) *J. Biol. Chem. 263*, 15872–15875.

Krebs, E. G., Eisenman, R. N., Kuenzel, E. A., Litchfield, D. W., Lozeman, F. J., Lüscher, B., & Sommercorn, J. (1988) *Cold Spring Harbor Symp. Quant. Biol. 53*, 77–84.

Kuenzel, E. A., & Krebs, E. G. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 737–741.

Lin, W. J., Tuazon, P. T., & Traugh, J. A. (1991) *J. Biol. Chem. 266*, 5664–5669.

Lozeman, F. J., Litchfield, D. W., Piening, C., Takio, K., Walsh, K. A., & Krebs, E. G. (1990) *Biochemistry 29*, 8436–8447.

Maridor, G., Park, W., Krek, W., & Nigg, E. A. (1991) *J. Biol. Chem. 266*, 2362–2368.

Meggio, F., & Pinna, L. A. (1984) *Eur. J. Biochem. 145*, 593–599.

Miller, D. W., Safer, P., & Miller, L. K. (1986) in *Genetic Engineering* (Setlow, J. K., & Hollander, A., Eds.) Vol. 8, pp 277-298, Plenum Publishing Corp., New York.
Padmanabha, R., & Glover, C. V. C. (1987) *J. Biol. Chem. 262*, 1829-1835.
Pinna, L. A. (1990) *Biochim. Biophys. Acta 1054*, 267-284.
Saxena, A., Padmanabha, R., & Glover, C. V. C. (1987) *Mol. Cell. Biol. 7*, 3409-3417.
Sommercorn, J., Mulligan, J. A., Lozeman, F. J., & Krebs, E. G. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 8834-8838.
Summers, M. D., & Smith, G. E. (1987) *Bulletin No. 1555*, Texas Agricultural Experiment Station, College Station, TX.
Takio, K., Kuenzel, E. A., Walsh, K. A., & Krebs, E. G. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 4851-4855.
Traugh, J. A., Lin, W. J., Takada-Axelrod, F., & Tuazon, P. T. (1990) in *Advances in Second Messenger and Phosphoprotein Research. Vol. 24: The Biology and Medicine of Signal Transduction* (Nishizuka, Y., Endo, M., & Tanaka, C., Eds.) Raven Press, New York.
Tuazon, P. T., & Traugh, J. A. (1991) in *Advances in Second Messenger and Phosphoprotein Research* (Greengard, P., & Robinson, G. A., Eds.) Vol. 23, pp 124-164, Raven Press, New York.
Vik, T. A., Sweet, L. J., & Erickson, R. L. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 2685-2689.
Zandomeni, R., Zandomeni, M. C., Shugar, D., & Weinmann, R. (1986) *J. Biol. Chem. 261*, 3414-3419.

Stability and Sequence-Specific DNA Binding of Activation-Labile Mutants of the Human Glucocorticoid Receptor†

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ABSTRACT: The stability and DNA-binding properties of activation-labile (*act*[1]) human glucocorticoid receptors (hGRs) from the glucocorticoid-resistant mutant 3R7.6TG.4 were investigated. These receptors are able to bind reversibly associating ligands with normal affinity and specificity, but become unstable during attempted activation to the DNA binding form [Harmon et al. (1984) *J. Steroid Biochem. 21*, 227-236]. Affinity labeling and immunochemical analysis demonstrated that *act*[1] receptors are not preferentially proteolyzed during attempted activation. In addition, analysis of binding to calf thymus DNA showed that after loss of ligand, *act*[1] receptors retain the ability to bind to DNA nonspecifically. A 370 bp MMTV promoter fragment containing multiple GREs and an upstream 342 bp fragment lacking GRE sequences were used to assess the binding of *act*[1] hGR to specific DNA sequences. Immunoadsorption of hGR-DNA complexes after incubation with ^{32}P-end-labeled fragments showed that both normal and *act*[1] hGR bound selectively to the GRE-containing fragment in an activation-dependent manner. Binding of both normal and *act*[1] hGRs could be blocked with a synthetic oligonucleotide containing a perfect palindromic GRE, but not with an oligonucleotide in which the GRE was replaced by an ERE. Analogous results were obtained for normal and *act*[1] hGR activated in the absence of ligand, or after incubation with the glucocorticoid antagonist RU 38486. These results suggest that sequence-specific binding of the hGR does not require the presence of bound ligand and suggest a role for the ligand in trans-activation of hormonally responsive genes.

The ability of steroid hormone receptors to modulate the expression of hormonally responsive genes is strictly dependent upon the presence of bound ligand, and activation of the steroid-receptor complex to a form capable of sequence-specific DNA binding (Evans, 1988; Beato, 1989; Carson-Jurica et al., 1990). Analysis of proteolytic receptor fragments and site-directed mutagenesis have mapped the ligand binding domain of the glucocorticoid receptor (GR)[1] to the carboxyl-terminal one-third of the steroid binding protein (Carlstedt-Duke et al., 1987; Giguere et al., 1986; Danielsen et al., 1987; Rusconi et al., 1987). Deletion of the entire GR ligand binding domain, or of an internal segment postulated to be responsible for the interaction of the GR with hsp90, results in a receptor protein with constitutive DNA binding and trans-activating activity (Danielsen et al., 1987; Godowski et al., 1987; Hollenberg et al., 1987; Pratt et al., 1988). Conversely, coupling of the GR ligand binding domain to GAL4 (Webster et al., 1988; Hollenberg & Evans, 1988) or to the adenovirus E1A protein (Picard et al., 1988; Becker et al., 1989) results in hormone-regulated expression of the activities of the chimeric proteins. Thus, the ligand binding domain appears to directly or indirectly participate in the repression of the DNA binding and trans-activating activities of the receptor.

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[1] Abbreviations: *act*[1], activation-labile; DM, dexamethasone 21-mesylate; ERE, estrogen response element; GR, glucocorticoid receptor; GRE, glucocorticoid response element; HEPES, *N*-(2-hydroxyethyl)-piperazine-*N*'-2-ethanesulfonic acid; hsp90, 90-kDa heat shock protein; LTR, long-terminal repeat; MMTV, mouse mammary tumor virus; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; TA, triamcinolone acetonide.