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Cytoplasmic and Nuclear Distribution of Casein Kinase II: Characterization of the Enzyme Uptake by Bovine Adrenocortical Nuclear Preparation†

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ABSTRACT: Casein kinase II (CK II) is a ubiquitous protein kinase that has been found in both nuclear and soluble subcellular fractions and whose precise cellular functions and mechanisms of control remain to be clarified. Using immunocytochemical localization, it was observed that the intracellular distribution of CK II exhibited a striking shift toward an increased nuclear concentration during active proliferation of bovine adrenocortical cells in primary culture. The interaction of CK II with purified adrenocortical cell nuclear preparation was thus examined in vitro. CK II was found to rapidly associate with nuclei in a temperature-dependent and saturable process, resulting in a tight binding of the kinase to nuclear components, as shown by various extraction procedures. This association resulted in a concentration of the kinase in the nuclear preparation about 100-fold that in the medium and exhibited two types of binding sites with K_a of 10^9 and 10^7 M^{-1}, respectively. The nuclear CK II uptake was dependent upon the presence of ATP and was stimulated by a kinase activator such as spermine, although the enzyme activity did not appear to be required for the process. These observations would be in line with a pore-mediated, energy-dependent nuclear uptake of the kinase. Since a number of potential nuclear CK II targets have been reported, including the oncoprotein myc, it is suggested that the nuclear translocation of the kinase as characterized in vitro may have a biological significance in living cell, especially in the control of nuclear activities related to cell proliferation and the mechanism of action of growth factors.

Casein kinase II (CK II) is an ubiquitous enzyme belonging to the threonine–serine protein kinase family (Edelman et al., 1987; Meggio et al., 1984). It was also named casein kinase G (CKG) due to its ability to use GTP as well as ATP as phosphoryl donor (Cochet et al., 1980; Cochet & Chambaz, 1983b), phosvitin kinase, glycogen synthase 5 kinase, casein kinase TS, PC 0.7 kinase, troponin T kinase, and protein kinase NII (Edelman et al., 1987). The enzyme has been purified from various tissues and shown to be an oligomeric protein made of two different subunits, with an $\alpha_2\beta_2$ stoichiometry

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(Cochet & Chambaz, 1983a). The α subunit has been shown to bear the catalytic site whereas the β subunit is the target of the kinase self-phosphorylation and appeared to be required for optimal enzymatic activity (Cochet & Chambaz, 1983a; Feige et al., 1983). The α subunit cDNA has been obtained from *Drosophila* (Saxena et al., 1987) and human origin (Meisner et al., 1989), and it was suggested that the protein may represent a heterogenous product (α, α') of two different genes (Meisner et al., 1989). The bovine β subunit has recently been sequenced (Takio et al., 1987). CK II has been isolated either from soluble tissue fractions or from nuclear extracts, in which it was termed N II kinase (Hathaway & Traugh, 1982; Delpech et al., 1986). CK II and N II kinase exhibit mostly identical properties, although subtle differences have been suggested (Baydoun et al., 1986).

The biological functions and mechanisms of control of CK II in the living cell remain to be fully understood. A number of potential substrates have been characterized, mostly following in vitro studies. They include enzymes of glycogen and lipid metabolism, as well as cytoskeleton components and factors involved in the protein synthesis machinery (Edelman et al., 1987; Hathaway & Traugh, 1982; Gonzatti-Haces & Traugh, 1982). On the other hand, reported potential nuclear targets include HMG proteins (Walton & Gill, 1983), RNA polymerase II (Stetler & Rose, 1983; Lee et al., 1984), and topoisomerase II (Ackerman et al., 1988). An additional interest was raised by reports showing that components of the DNA transcription machinery such as nucleolin (Caizergues-Ferrer et al., 1987) and, more recently, a number of oncoproteins such as myc (Luscher et al., 1989) could be regulated by CK II phosphorylation. Sequence homology study between various protein kinases and the nuclear factor 1 (NF-1) suggested that CK II may belong to a large family of nuclear factors involved in the regulation of DNA expression (Mannermaa & Oikarinen, 1989). Immunolocalization of CK II has clearly shown a nuclear and more specifically a nucleolar concentration of the enzyme (Pfaff & Anderer, 1988; Belenguer et al., 1989), in line with a proposed role of the kinase in ribosomal RNA gene transcription process (Caizergues-Ferrer et al., 1987; Belenguer et al., 1989).

Possible major nuclear functions for CK II may also be suggested by the fact that the enzyme activity was reported to increase in actively growing tissues (Pêrez et al., 1988) and to be stimulated under cell exposure to serum and several growth factors (Carroll & Marshak, 1989; Sommercorn et al., 1987; Klarlund & Czech, 1988) as well as during cell differentiation (Sommercorn & Krebs, 1987) and embryogenesis (Schneider et al., 1986). We have previously suggested that CK II may represent a target for the intracellular actions of polyamines, whose presence is an absolute requirement for active cell growth (Mamont et al., 1980).

The present study was initiated by the observation that the nuclear versus cytosolic distribution of CK II in bovine adrenocortical cells in culture was clearly modified according to the cell growth status. An assay system was designed to examine the interaction of bovine CK II with purified adrenocortical cell nuclear preparations in vitro. It was observed that CK II associated very efficiently with nuclei, resulting in a tight binding to nuclear components. The kinetic parameters of this nuclear uptake of CK II were characterized. It was shown that the process was temperature dependent and was activated in the presence of ATP and polyamines. These observations strongly suggest that the nuclear uptake of CK II, as observed in vitro, may be of biological significance in the intranuclear import of CK II in the intact cell and subsequently in the regulation of the enzyme distribution between the cytoplasmic and the nuclear compartments.

Experimental Procedures

Chemicals. [γ-^{32}P]Adenosine triphosphate (3000 Ci/mmol) was from Amersham (U.K.) and [^{125}I]Na was purchased from the CEA (Paris, France). Nucleotides, proteins, spermine (tetrahydrochloride), glucose 6-phosphate, amiloride, quercetin, 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (DRB),[1] leupeptin, Triton X-100, and EDTA were from Sigma Chemicals (St. Louis, MO), while sucrose was purchased from Merck (Darmstadt, Germany). Collagenase (155 units/mg) was furnished by Millipore Co.; hexokinase and glucose-6-phosphate dehydrogenase were from Boehringer (Mannheim, Germany). Nucleolin and NPT6 were a generous gift from F. Amalric (Toulouse, France), and specific anticasein kinase II antiserum was kindly provided by M. Pfaff (Tubingen, FRG). Lysis buffer was 40 mM Tris-HCl, pH 7.4, containing 0.25 M sucrose, 25 mM KCl, 4 mM $MgCl_2$, 0.1% NPT 6, 0.1 mg/mL collagenase, and 50 μg/mL leupeptin. Nuclear buffer was 10 mM Tris-HCl, pH 7.4, containing 0.25 M sucrose, 2.5 mM $MgCl_2$, and 0.1 mM $CaCl_2$.

Casein Kinase II and Nucleolin Labeling. Casein kinase II was purified to homogeneity from bovine lung tissue following the procedure previously described (Cochet et al., 1983) with slight modifications. The purified enzyme was self-phosphorylated upon incubation with 1 μM [γ-^{32}P]ATP (3000 Ci/mmol) in 10 mM Tris-HCl (pH 7.4) containing 50 mM $MgCl_2$ and 1 mg/mL BSA for 15 min at 25 °C. The reaction was stopped by dilution with 1 volume of Hepes buffer containing 1 mg/mL BSA and 1 M NaCl. Radiolabeled casein kinase II was separated from [γ-^{32}P]ATP by centrifugation of the sample through a Sephadex G-50 column previously equilibrated in Hepes buffer containing 1 mg/mL BSA and 1 M NaCl, according to Penefsky (1977). Self-phosphorylation leads to a stoichiometric ^{32}P labeling of the β subunit (M_r = 27K) of the enzyme together with a substoichiometric incorporation of ^{32}P in the α subunit (M_r = 38K) (Cochet & Chambaz, 1983a). Nucleolin (100 ng) labeling was carried out following incubation with CK II (50 ng) in the presence of 1 μM (3000 Ci/mmol) [γ-^{32}P]ATP. The resulting ^{32}P-labeled nucleolin exhibited a specific radioactivity of 1000 cpm/ng of protein.

Cell Culture. Bovine adrenocortical cells were prepared by successive tryptic digestions of fresh adrenocortical tissue, seeded either in six-well plates (9.6 cm^2 per well) or in 10-cm diameter plates (Becton Dickinson, Oxnard, CA), and grown as previously described (Cochet & Chambaz, 1983b) in Hams F12 medium supplemented with 12.5% horse serum and 2.5% foetal calf serum in an air/CO_2 (95:5) atmosphere.

Immunocytochemical Procedure. For these experiments, cells were grown on glass cover slips. Cells at different states of proliferation were fixed according to Murthy et al. (1986) in 4% paraformaldehyde. The cell layer was then rinsed three times with ice-cold PBS and permeabilized in PBS containing 0.5% Triton X-100 for 5 min at 22 °C, followed by incubation in 100% methanol at −20 °C for 10 min. Rinsed cells were incubated at 4 °C overnight with an antiserum against mouse (L 1210 cells) casein kinase II at a 1/20 or 1/50 dilution, as

[1] Abbreviations: DRB, 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole; EDTA, ethylenediaminetetraacetic acid; BSA, bovine serum albumin; PBS, phosphate-buffered saline; SDS, sodium dodecyl sulfate; AMP-PNP, 5'-adenylyl imidodiphosphate; Tris, tris(hydroxymethyl)-aminomethane.

indicated. Independent experiments showed that the antiserum recognized only the CK II α subunit following SDS–PAGE analysis of the purified kinase and blotting experiment. On the other hand, blots of total adrenocortical cell protein extracts disclosed that, under stringent washing conditions (10 mM Tris-HCl, pH 7.5, containing 150 mM NaCl and 1% Triton X-100), the antibody revealed only the CK II 38K α subunit. After three washes with 0.5% bovine serum albumin in PBS, cells were further incubated for 1 h at 22 °C with fluorescein-conjugated goat anti-rabbit γ-globulin antibody at a 1/250 dilution. Finally, the preparations were extensively washed with PBS, stained with Hoechst 33342 dye (5 μg/mL in PBS), and mounted on glass slides. The preparations were observed in an Axiovert Zeiss microscope equipped with a fluorescence accessory. Quantitative fluorescence assay was carried out by using a video recording and an Hamamatsu analyzer. Control experiments were run with antiserum which was previously incubated with an excess of purified CK II preparation.

Subcellular Preparations and Casein Kinase II Assay. Bovine adrenocortical cell layers were washed with PBS; cells were homogeneized in lysis buffer containing 1 mM dithiothreitol. Nuclei were obtained by centrifugation of the homogenate at 800*g* for 5 min, and the supernatant was cleared by centrifugation at 100000*g* for 30 min to yield the cytosolic fraction. Nuclear proteins were extracted in nuclear buffer containing 2 M NaCl. After centrifugation, the supernatant was diluted in 1 mL of nuclear buffer containing 1% BSA to give a final concentration of 0.1 M NaCl. Cytosolic and nuclear protein extracts were fractionated by ion-exchange chromatography on phosphocellulose (100 μL) previously equilibrated in nuclear buffer. After the column was washed with nuclear buffer containing 0.4 M NaCl, casein kinase II was eluted with 1 M NaCl (300 μL) and 10-μL aliquots of the eluate were used for casein kinase II activity determination. Protein kinase activity assays were performed as previously described, with casein as substrate and ATP as the source of phosphate (Cochet & Chambaz, 1983a).

Nuclei Preparation. All procedures were carried out at 0–4 °C. Bovine adrenocortical cells (4×10^7) were washed three times with cold PBS and homogenized twice for 30 s with a polytron apparatus in lysis buffer. The homogenate was used to prepare nuclear pellet following the procedure of Zalta et al. (1971). The pellet was washed in 4 mL of nuclear buffer and homogenized (30 s) with a polytron. The suspension was centrifuged for 5 min at 900*g*, and the resulting pellet was referred to the nuclear preparation. For some experiments, the method of Chauveau et al. (1956) was also used to obtain a purified nuclear pellet. The CK II uptake results were basically the same with the two types of nuclear preparations, which appeared devoid of detectable contamination when observed under phase contrast microscopy.

In Vitro Assay for Nuclear Association of Casein Kinase II. Samples of the nuclear preparation (10^5 nuclei) were routinely incubated at 30 °C with ^{32}P-labeled casein kinase II (specific activity 1000 cpm/ng of protein) in a final volume of 125 μL of nuclear buffer, for the time indicated. At the end of the incubation, nuclei were washed by sedimentation in 600 μL of nuclear buffer containing 0.05% Triton X-100. Nuclear pellets were then solubilized in Laemmli sample buffer and analyzed by 12% SDS–polyacrylamide gel electrophoresis. The dried gels were autoradiographed, and the amount of CK II associated with nuclei was determined following radioactive counting of the band corresponding to the β subunit of the enzyme.

Protein assay was performed according to the method of Bradford (1976). Glucose-6-phosphate dehydrogenase activity was assayed according to Domagk et al. (1969). Phosphatidylinositol 4-phosphate (5)-kinase was isolated as previously described (Cochet & Chambaz, 1986).

Results

Nuclear and Cytosolic Distribution of Casein Kinase II in Bovine Adrenocortical Cells in Primary Culture. Typically, when fresh adrenocortical cell suspension was seeded at low density (4×10^4/cm^2), proliferation started within 36 h in the presence of serum and stopped as the cell layer reached confluence at day 7 or 8 of culture (Figure 1B). The distribution of CK II in the nuclear and in the cytoplasmic cell compartments was examined during the period of active growth (day 4) and in growth-arrested cells, either at confluence or following a 3-day serum starvation. Localization of casein kinase II in intact cells was first studied by immunodetection of the enzyme, using a rabbit antimouse CK II antiserum. The antiserum exhibited no detectable cross-reaction for other components in either cytoplasmic or nuclear extracts, as examined following SDS–polyacrylamide gel electrophoresis and immunoblotting. Figure 1 shows that, in actively growing cells (a), casein kinase II was mainly detected in the nuclei. By contrast, in nongrowing cells, arrested either at confluence (b) or following serum starvation (c), the enzyme was spread within the cell and no longer concentrated in nuclei. When the anti-CK II antiserum was saturated with purified CK II prior to immunofluorescence study, labeling could no longer be detected (a′, b′, c′). Quantitative evaluation of the fluorescence in the two cell compartments yielded average nuclear over cytoplasmic ratio values of 2.6 ± 0.1 and 1.75 ± 0.15 (n = 5) for growing and arrested cells, respectively. Assay of CK II activity following phosphocellulose chromatography of adrenocortical cell cytosolic and nuclear extracts showed that the total enzyme activity recovered from the extracts remained constant whatever the cell growth status. However, the nuclear over cytosolic CK II activity ratio shifted from 3 ± 0.5 to 1.76 ± 0.3 (n = 3) in actively growing and resting cells, respectively. These observations strongly suggested that a preferential association of the enzyme with the nucleus was concomitant with active proliferation and that the enzyme may be exchanged between nuclear and extranuclear compartments, in a possibly growth-related process. Of particular interest in this context was the possibility of an active translocation of the enzyme toward potential nuclear targets. We thus decided to examine this translocation hypothesis by designing an in vitro assay system using purified nuclear preparations.

Interaction of Casein Kinase II and Purified Nuclei in Vitro. When ^{32}P-labeled casein kinase II was incubated with an adrenocortical cell nuclear preparation at 30 °C, a large part of the radioactivity was rapidly found associated with the nuclear pellet and was not removed following extensive washing. To check whether this nuclear affinity of the enzyme reflected a specific process or might be explained by nonspecific binding to nuclear components, possibly due to nuclei alteration in vitro, the purity of the nuclear preparation was examined by phase contrast microscopy and control experiments were run under identical conditions using either ^{125}I-labeled immunoglobulin G or bovine serum albumin. With both labeled proteins, no detectable radioactivity remained associated with the washed nuclear pellet. Similarly, when the nuclear pellet was incubated with a phosphatidylinositol phosphate kinase preparation (Cochet & Chambaz, 1986), no phosphoinositide kinase activity was found associated with the nuclear material.

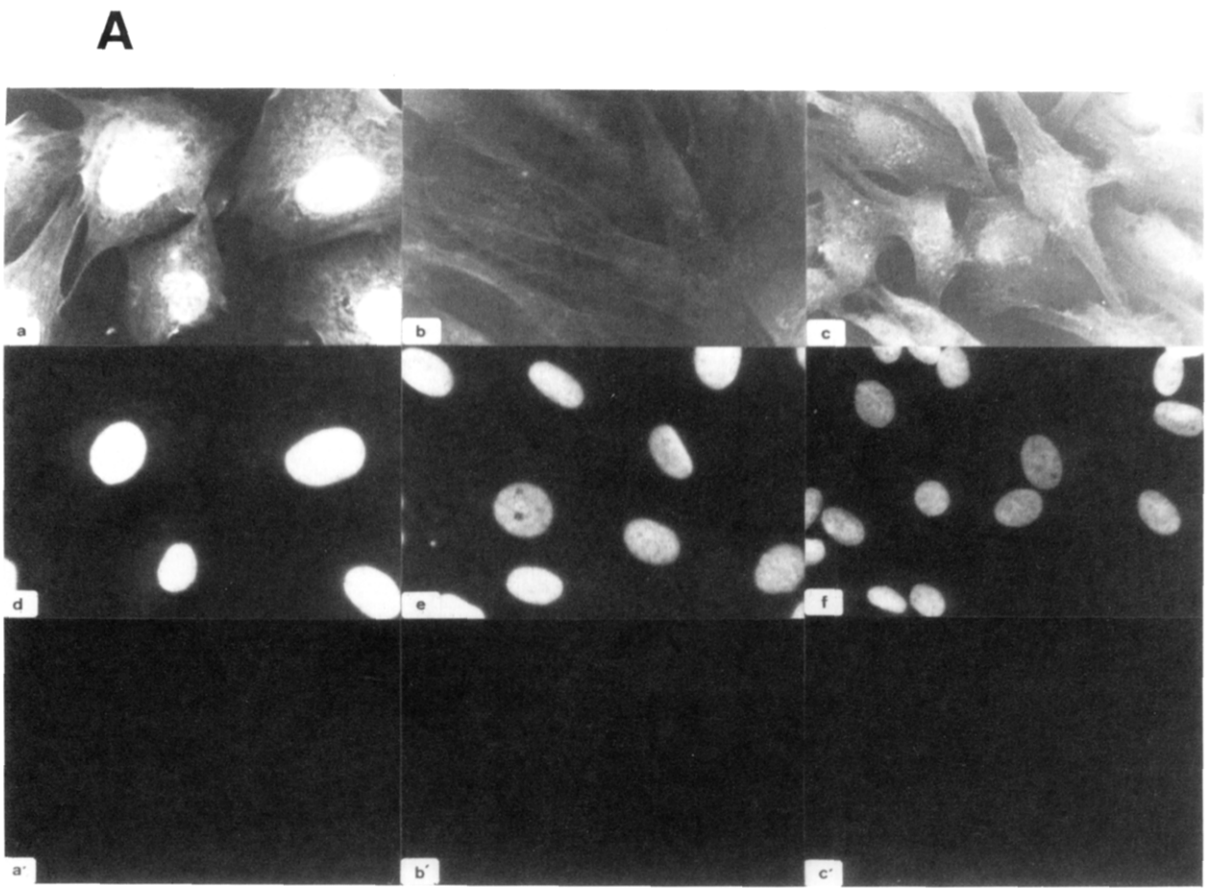

FIGURE 1: Immunocytochemical localization of casein kinase II in bovine adrenocortical cells and cell growth kinetics. (A) Indirect immunofluorescence staining of CK II in actively growing cells (a, a′) and growth-arrested cells, either at confluence (b, b′) or following serum starvation (c, c′); panels a–c show immunofluorescence microscopy of cells using rabbit anti-mouse CK II antiserum (dilution 1/20) as described under Experimental Procedures; panels d–f show the corresponding cell preparations following Hoechst staining of the nuclear material (magnification 1250×). In panels a′–c′, the anti-CK II antiserum was saturated with purified CK II (1 μg) prior to use in immunofluorescence studies. (B) Growth of adrenocortical cells. Cells (3.5×10^5) were seeded in 1.5 mL of growth medium in six-well plates as described under Experimental Procedures. Cell growth was monitored by counting using a hemocytometer. Cells were either maintained in complete serum growth medium for 8 days (B1) or subjected to serum deprivation from day 4 to day 8 (B2). Arrows a–c represent the growth status of the cells when used for immunofluorescence studies.

A well-established cytoplasmic protein (i.e., glucose-6-phosphate dehydrogenase) was also used in additional control experiments. Following incubation of the dehydrogenase (0.28 unit) with adrenocortical nuclear preparations (500000 nuclei), all the enzymatic activity was recovered in the washing buffer, whereas no activity could be detected in the nuclear pellet. On the contrary, when the nuclear protein nucleolin was incubated under identical conditions, a clear nuclear accumulation was observed (see below, Figure 6). These observations showed that our adrenocortical nuclear preparations were capable of selective protein uptake and ruled out a nuclear association of CK II resulting from nonspecific binding to damaged nuclear material. The specificity of the nuclear CK II uptake was further supported by the fact that, when ^{32}P-labeled CK

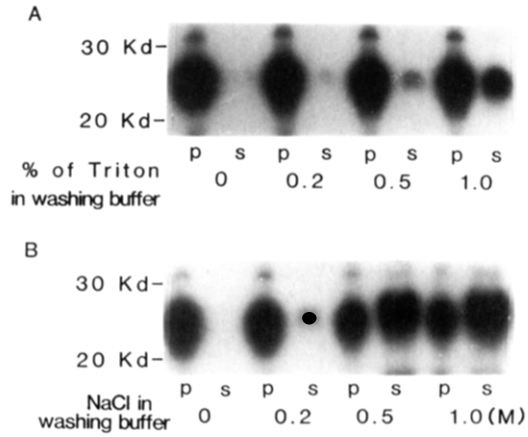

FIGURE 2: Extraction of casein kinase II following association with adrenocortical cell nuclear preparation. Nuclei (4×10^6) were incubated with 1 μg of ^{32}P-labeled casein kinase II for 30 min at 30 °C. After washing, aliquots of 4×10^5 nuclei were sonicated for 5 min in the presence of increasing concentrations of either Triton X-100 (A) or NaCl (B). After centrifugation (100000g, 10 min), the nuclear pellet and the corresponding supernatant were analyzed by 12% SDS–polyacrylamide gel electrophoresis and the 27K β subunit of the enzyme was detected in each fraction following autoradiography of the gel.

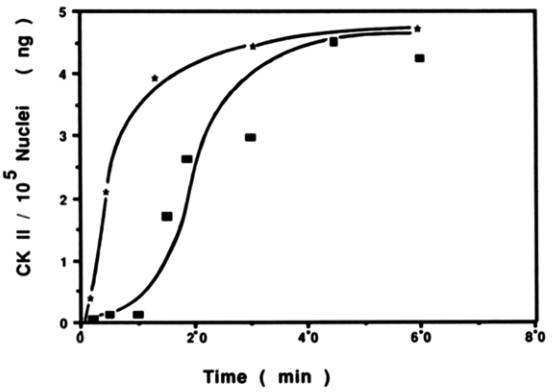

FIGURE 3: Time course of association of casein kinase II to adrenocortical cell nuclear preparation. Samples of nuclei (10^5) were incubated at 30 °C for the indicated times in the presence of 45 ng of ^{32}P-labeled casein kinase II. The nuclear suspension kept at 0 °C was either transferred at 30 °C at the start of the incubation (■) or preincubated for 15 min at 30 °C (*) before addition of the kinase. Thoroughly washed nuclear pellets were counted for their radioactivity content, which was used to calculate the amount of associated CK II. These curves are representative of 3 similar experiments.

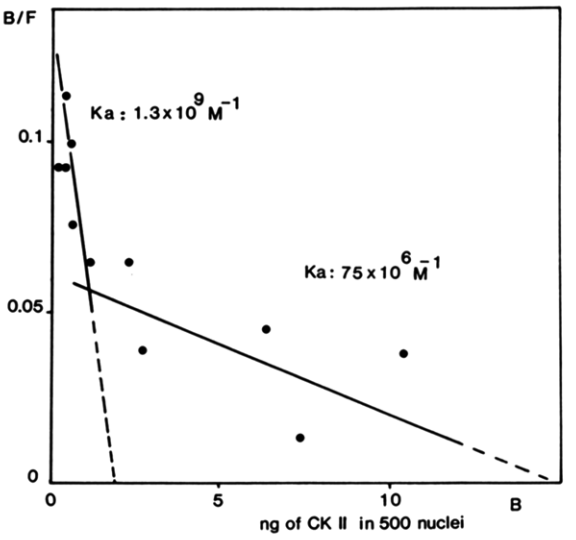

FIGURE 4: Scatchard plot analysis of the interaction of casein kinase II with nuclear preparation. Samples of nuclei (500) were incubated for 50 min at 30 °C in the presence of increasing amounts of ^{32}P-labeled casein kinase. Washed nuclear pellets were counted for radioactivity, and the data were plotted according to Scatchard (1949). These results are representative of 3 similar experiments.

II was preincubated with a specific polyclonal antibody which selectively binds to the CK II α subunit, a marked inhibition of the enzyme association with nuclei was observed (Figure 7) while nonimmune serum in the same condition has no effect. Possible CK II adsorption to nuclear envelope was examined in a series of experiments using enzymatic attack of CK II preloaded nuclear preparations. Neither phospholipases (A_2, C, D) nor trypsin treatments released any detectable CK II previously associated to the nuclear suspension. The tight association of CK II with the nuclear material was further supported by experiments examining the recovery of the nuclei-associated enzyme under various extraction conditions. Figure 2A illustrates the corresponding results following sonication of the CK II loaded nuclear pellet in the presence of various concentrations of Triton X-100: up to 0.5% Triton, most of the kinase remained associated with the pellet while 1% detergent solubilized about 20% of the enzyme. Figure 2B shows that 0.5 M NaCl was required to extract significant amount of CK II. Counting the radioactivity of the excised gel area allowed us to determine that 1 M salt release about 70% of the enzyme. After 2 M NaCl extraction, some radioactivity remained associated with the pellet and required 1% SDS treatment to be recovered. These observations supported the view that, under in vitro conditions, CK II accumulated in the nuclear compartment where it became tightly bound to intranuclear components.

Kinetic Parameters of the Nuclear Association of Casein Kinase II in Vitro. A time course study of ^{32}P-labeled CK II association to an adrenocortical nuclear preparation is illustrated in Figure 3. When the nuclear preparation kept at 0 °C was transferred at 30 °C for the incubation with CK II, the process exhibited a time-dependent sigmoid-shaped curve to reach a plateau within about 50 min of incubation. At this point, the concentration of labeled enzyme in the nuclear pellet was 100–125-fold that in the surrounding medium, thus corresponding to an accumulation in the nuclei, resulting in a marked concentration gradient between the two compartments. Taking into account the specific radioactivity of the labeled kinase, and assuming an average volume of 10^{-12} L per nucleus, it could be calculated that the nuclear translocated enzyme (4.5 ng/5×10^5 nuclei) would reach micromolar concentrations. However, it may be noticed that, under these experimental conditions, we were far from saturation, due to the large number of nuclei used in the incubation (see below). When the nuclei suspension was preincubated at 30 °C for 10 to 15 min, the nuclear uptake started immediately upon addition of the kinase (Figure 3). This showed that the uptake process was highly temperature-dependent and suggested that the sigmoidal kinetic curve (Figure 3) might be explained by the time lag required for the nuclear preparation (total volume 1.3 mL) to reach a permissive temperature. This was confirmed by study of the CK II nuclear accumulation rate which was reduced to 75% at 25 °C and to less than 10% at 0 °C, as compared to that observed at 30 °C, after 1-h incubation.

To further characterize the nuclear interaction of the kinase, saturation experiments were performed using a limited number of nuclei ($\simeq$ 500) incubated with increasing amounts of ^{32}P-labeled CK II at 30 °C, until equilibrium, and the data were treated according to Scatchard (1949). As shown in Figure 4, the corresponding plot exhibited a nonlinear curve, indicating heterogeneous binding sites. The graphical analysis disclosed

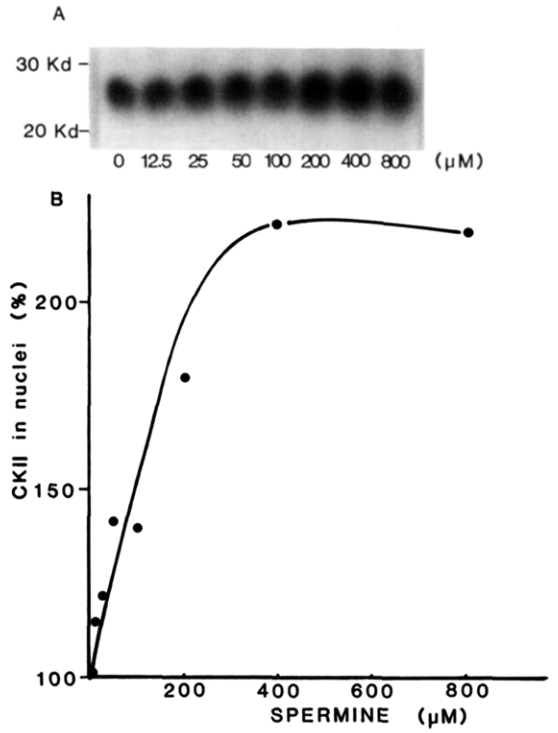

FIGURE 5: Effect of increasing concentrations of spermine on the nuclear uptake of casein kinase II. Samples of nuclei (10^5) were incubated for 30 min at 30 °C with 125 ng of ^{32}P-labeled casein kinase II in the presence of increasing concentrations of spermine. Washed nuclear pellets were analyzed by 12% SDS–polyacrylamide gel electrophoresis, and the nuclear associated enzyme was visualized by autoradiography of the gel (A). The amount of radioactivity was determined by counting of the excised gel area, and the data were plotted as percent of the uptake value in the absence of spermine taken as 100% (B).

at least two binding systems for which apparent association constants K_a of $(1.3 \pm 0.13) \times 10^9$ M^{-1} and $(7.5 \pm 1{,}57) \times 10^7$ M^{-1} ($n = 3$), respectively, could be calculated. The corresponding binding capacities were 25×10^{-6} M (high affinity) and approximately 2×10^{-4} (low affinity).

The energy dependence of the CK II nuclear association was examined by several approaches. Although the presence of hexokinase (together with glucose) depressed by only 35 ± 15% the uptake process, 1 mM ATP was a highly potent activator (265% stimulation ± 10). Moreover, a nonhydrolyzable ATP analogues such as ATP-γ-S or AMP-PNP resulted in a weak inhibition of the enzyme uptake (50 ± 4% and 37 ± 5%, respectively). The data were expressed as percent of uptake value in the absence of addition taken as 100%. These results are representative of three similar experiments and are in line with the fact that the CK II nuclear uptake is an energy (ATP) dependent reaction.

Modulation of Casein Kinase II Nuclear Uptake in Vitro. The requirement for an active, native form of the kinase for its nuclear association was first examined by denaturation experiments. When exposed to 70 °C for 5 min, the enzyme activity was lost by an average of 80% while its nuclear uptake was in parallel inhibited by 60–70%. These observations further support the fact that CK II nuclear association was specific and requires an intact, active conformation of the protein.

Magnesium and the polycations polyamines have been well characterized as activators of CK II activity in vitro (Hathaway & Traugh, 1982; Cochet & Chambaz, 1983b). A 5-fold activation of the process was achieved in the presence of optimal Mg^{2+} concentrations (≃25 mM), similar to those previously reported to maximally support the casein kinase activity

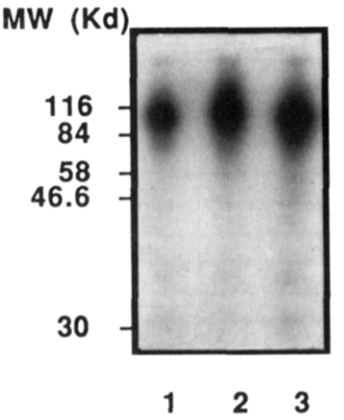

FIGURE 6: Effect of spermine and magnesium on nucleolin nuclear uptake. ^{32}P-Labeled nucleolin was incubated with samples of nuclei (10^5) for 30 min at 30 °C in the absence (1) or in the presence (2) of 0.8 mM spermine or 20 mM $MgCl_2$ (3). Washed nuclear pellets were analyzed by 12% SDS–polyacrylamide gel electrophoresis. The ^{32}P-labeled nucleolin was localized after autoradiography of the gel. The results are representative of 2 similar experiments.

in vitro (Cochet et al., 1981). Among the different naturally occurring polyamines tested, spermine was found the most effective in stimulating CK II nuclear association. Figure 5 shows that a 2-fold stimulation was observed in the presence of submillimolar concentrations of the polycation, similar to those previously shown to optimally activate the enzyme (Cochet & Chambaz, 1983b; Feige et al., 1985). It may be noticed that 400 μM of the polyamine analogue 3,5-diamino-*N*-(aminoiminomethyl)-6-chloropyrazinecarboxamide (amiloride) had no detectable effect on CK II nuclear uptake nor on the enzyme activity.

However, polyamines and Mg^{2+} are known to induce marked changes in chromatin organization (Kaiser et al., 1963). In order to examine whether such structural effects might participate in the increase in CK II nuclear uptake, control experiments were run using the nuclear protein nucleolin. As shown in Figure 6, the nuclear accumulation of ^{32}P-labeled nucleolin (1.5 ng for 3×10^5 nuclei in the presence of 10 ng of nucleolin) was also stimulated in the presence of Mg^{2+} (2.5 ng) or spermine (2.9 ng). This suggests that enzyme activators such as Mg^{2+} and spermine most likely act through chromatin structure modification, leading to increased affinity for the kinase (as well as for other nuclear protein such as nucleolin). On the other hand, flavonoid structures such as quercetin and the adenosine analogue 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (DRB) are both CK II inhibitors by competition with ATP (or GTP) (Hathaway & Traugh, 1982; Cochet et al., 1982; Zandomeni et al., 1986). As illustrated in Figure 7, quercetin, which at 10 μM is a powerful inhibitor of the casein kinase activity, did not exhibit such a clear blocking effect on the enzyme nuclear association. Similarly, DRB (100 μM), which reduced the kinase activity to about 40%, had no detectable effect on its nuclear uptake. Altogether, these results show that there was no clear correlation between the enzymatic activity and its nuclear translocation.

Discussion

Casin kinase II is an ubiquitous protein kinase whose precise cellular functions and mode of control remain to be understood. The high affinity of CK II for nuclear structures in vitro resulted in a very tight association and a concentration of the enzyme in the nuclei. The quantitative aspect of this translocation was remarkable: while the basal CK II concentration could be evaluated to be about 1 μM, saturation experiments

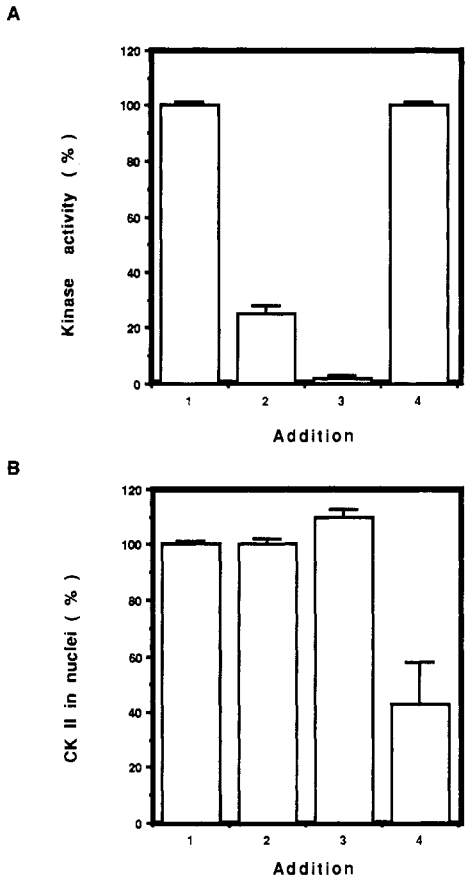

FIGURE 7: Effects of modulators of casein kinase II activity on the nuclear uptake of the enzyme. (A) Casein kinase II activity was assayed, using casein as substrate, in the absence (1) or in the presence of different reagents: (2) DRB, 100 μM; (3) quercetin, 10 μM; (4) anti-CK II antiserum at a 1/10 dilution. Data were calculated with regard to the control value (1) taken as 100%. (B) ^{32}P-Labeled casein kinase II was incubated in the absence (1) or in the presence of different reagents for 10 min at 22 °C prior to the addition of the nuclear preparation, under standard uptake assay conditions. The amount of CK II accumulated in nuclei was compared in each case with the control values taken as 100%. The data are the mean of 3 similar experiments, and error bars indicate standard deviation.

showed that nuclear concentrations reaching 25 μM of translocated enzyme could be obtained. These in vitro experiments are well in agreement with the fact that CK II is, at least in part, a nuclear enzyme in the living cell. The nuclear form has been characterized as an N II kinase by a number of research groups (Hathaway & Traugh, 1982; Delpech et al., 1986), although some subtle differences between CK II and N II kinase have been discussed (Baydoun et al., 1986). A nuclear and specifically a nucleolar accumulation of CK II has been observed by cytoimmunolocalization and biochemical methods (Pfaff & Anderer, 1988; Belenguer et al., 1989; Schneider & Issinger, 1989). However, we never observed a selective nucleolar localization of CK II in the present study. This may be related to the fact that adrenocortical cell represents a highly differentiated model, with a relatively low rate of proliferation.

The fact that CK II concentrates in the nuclear compartment implies that the enzyme must be imported in the nucleus from its cytoplasmic sites of biosynthesis. Nothing is known as yet about a possible nascent form of the kinase bearing peptide signal sequences such as those responsible for the nuclear migration of large proteins through nuclear envelope pores. The seven amino acid lysine-rich signal consensus sequence described in SV40 large T antigen (Lanford et al., 1988; Dang & Lee, 1988) is not found in the recently described CK II structure (Saxena et al., 1987; Meisner et al., 1989). The present observations clearly show that CK II purified from a soluble bovine lung fraction (Cochet et al., 1983) was able to actively penetrate nuclear structure in vitro. The presence of radioactivity associated with the α subunit indicated that the α and β subunits are both translocated into the nuclei. Due to the molecular size of the native, $\alpha_2\beta_2$ tetrameric kinase (140K), a pore-mediated nuclear import process (Richardson et al., 1988; Newmeyer & Forbes, 1988) is likely to be involved. Nuclear pore-mediated protein translocation has been characterized as selectively sensitive to the wheat germ agglutinin (WGA) lectin (Newmeyer & Forbes, 1988). Although this lectin did not influence CK II nuclear transfer in our assay system (not shown), the adrenocortical nuclear uptake of CK II in vitro was clearly temperature- and ATP-dependent as described for this pore-mediated import. Our data clearly demonstrate that CK II tightly bound to nuclear components and that this process involved a heterogeneous population of at least two types of binding sites. Preliminary evidence has been obtained in the laboratory showing that CK II binds to DNA. On the other hand, CK II may bind to nuclear target proteins; this would be in agreement with the observation that an enzyme inhibitor such as heparin, acting as a protein substrate competitor (Feige et al., 1980; Hathaway & Traugh, 1982), inhibits CK II nuclear association. Both DNA and nuclear protein binding affinities would support the hypothesis for a role of CK II in the control of DNA activity. In this context, a particular interest is raised by the stimulatory effect of spermine in our in vitro assay. Polyamines are well-known to greatly influence chromatin structure organization (Tabor & Tabor, 1984) and may thus increase intranuclear CK II binding site affinity and/or accessibility. On the other hand, polyamines are ubiquitous intracellular polycations which are required for normal cell growth (Mamont et al., 1980), and induction of the limiting enzyme of their biosynthesis (i.e., ornithine decarboxylase) is a typical response in growth factor induced cell proliferation (Mamont et al., 1980; Feige et al., 1985). We have previously proposed that polyamines may be examined as intracellular messengers in the regulation of CK II mediated protein phosphorylation, since they can act at the level of the enzyme and at the same time they may counteract endogenous inhibitors and modify the protein substrate conformation (Cochet & Chambaz, 1983b). This hypothesis may be further supported by the present findings, suggesting that intracellular polyamine concentrations may modulate CK II accumulation into the nuclear compartment. Whether the preferential accumulation of CK II in the nuclear compartment observed in the present study in actively growing adrenocortical cells is related to increased intracellular polyamine level remains to be examined.

Considerable strengthening of the hypothesis of major nuclear roles for CK II has been provided in recent years by a number of reports showing that RNA polymerase II (Stetler & Rose, 1983; Lee et al., 1984), chromosomal high mobility group proteins (Walton & Gill, 1983), nucleolin (Caizergues-Ferrer et al., 1987), and several nuclear oncoproteins such as Myc (Luscher et al., 1989) and Myb (Luscher et al., 1990) were substrates of CK II. The CK II α subunit cDNA from *Drosophila* (Saxena et al., 1987) and human genomic DNA (Meisner et al., 1989) have recently been isolated, and the human β subunit has been cloned by two groups. The corresponding deduced protein sequences confirm that the α subunit contains the catalytic site of the enzyme (Meisner et al., 1989). Sequence homology studies suggest that CK II may belong to a superfamily of nuclear proteins including NF-1

and other kinases (Mannermaa & Oikarinen, 1989) as well as the yeast cell division control protein CDC 2 and its homologue CDC 28 (Sommercorn et al., 1987; Takio et al., 1987). Recently, our CK II preparation was shown to phosphorylate the thyroxine receptor on the same site of its amino-terminal domain which is phosphorylated in vivo (Glineur et al., 1989).

Although many questions still remain open as to the biological significance of the present observations, especially concerning the regulation of CK II nuclear concentration and the nature of its specific nuclear targets, the evidence allows to suggest that CK II may be a major kinase in the control of nuclear activities. This will be of particular interest in the control of cell proliferation and/or selective gene expression and in the mechanism of action of growth factors in their target cells. Recent reports showing activation of cellular CK II in response to insulin and epidermal growth factor (Carroll & Marshak, 1989; Sommercorn et al., 1987; Klarlung & Czech, 1988; Ackerman et al., 1990) are in line with this concept. Our observations showing that, in the intact cell, the nuclear over cytosolic ratio of CK II may be dependent upon the growth status also support the hypothesis that the observed in vitro CK II–nuclei interaction may have a biological significance in this regard in living cell.

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Registry No. ATP, 56-65-5; Mg, 7439-95-4; spermine, 71-44-3; casein kinase, 52660-18-1.

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Spectroscopic and Kinetic Characterization of Nonenzymic and Aldose Reductase Mediated Covalent NADP-Glycolaldehyde Adduct Formation†

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ABSTRACT: Reaction of glycolaldehyde with the binary E·NADP complex of bovine kidney aldose reductase (ALR2) produces an enzyme-bound chromophore whose absorbance (λ_{max} 341 nm) and fluorescence (λ^{ex}_{max} 341 nm; λ^{emit}_{max} 421 nm) properties are distinct from those of NADPH or E·NADPH yet are consistent with the proposed covalent adduct structure [1,4-dihydro-4-(1-hydroxy-2-oxoethyl)nicotinamide adenine dinucleotide phosphate]. The kinetics of adduct formation, both in solution and at the enzyme active site, support a mechanism involving rate-determining enolization of glycolaldehyde at high [$NADP^+$] or [E·NADP]. At low [$NADP^+$] or [E·NADP] the reaction is second-order overall, but the ALR2-mediated reaction displays saturation by glycolaldehyde due to competition of the aldehyde (plus hydrate) and enol for E·NADP. Measurement of the pre-steady-state burst of E·adduct formation confirms that glycolaldehyde enol is the reactive species and gives a value of 1.3×10^{-6} for K_{enol} = [enol]/([aldehyde] + [hydrate]), similar to that determined by trapping the enol with I_3^-. At the ALR2 active site, the rate of adduct formation is enhanced 79 000-fold and the adduct is stabilized ≥13 000-fold relative to the reaction with $NADP^+$ in solution. A portion of this enhancement is ascribed to specific interaction of $NADP^+$ with the enzyme since the 3-acetylpyridine analogue, (AP)ADP^+, gives values that are 15-200-fold lower. Additional evidence for strong interaction of ALR2 with both $NADP^+$ and NADPH is reported. Yet, because dissociation of adduct is slow, catalysis of the *overall* adduct formation reaction by ALR2 is ≤67-fold.

Aldose reductase (ALR2;[1] alditol-$NADP^+$ 1-oxidoreductase; E.C. 1.1.1.21), an NADPH-dependent aldehyde reductase that has received much attention as a potential therapeutic target for the prevention or amelioration of diabetic complications [reviewed in Kador et al. (1985)], displays substrate inhibition by a variety of aldehyde substrates (McKercher et al., 1985; Grimshaw et al., 1989). For two- and three-carbon aldoses, inhibition of the initial reaction rate is followed by a time-dependent onset of further inhibition, reminiscent of the substrate inhibition of lactate dehydrogenase by pyruvate (Gutfreund et al., 1968; Burgner et al., 1978). In that case, substrate inhibition arises from the combination of a rapidly reversible component, due to formation of the dead-end E·NAD·pyruvate complex, and a time-dependent component attributed to reaction of pyruvate enol with E·NAD^+ to produce a covalent NAD-pyruvate adduct at the enzyme active site (Everse et al., 1971a; Sugrobova et al., 1972; Burgner & Ray, 1974). Detailed studies of the latter reaction, both on and off the enzyme, have advanced our understanding of the mechanistic basis for the catalytic efficiency of lactate dehydrogenase (Burgner & Ray, 1974, 1978, 1984a,b; Burgner et al., 1978).

In this paper we present a detailed spectroscopic and kinetic study of the nonenzymic and ALR2-mediated reactions of glycolaldehyde with $NADP^+$ and with the 3-acetylpyridine analogue, (AP)ADP^+. UV-visible and fluorescence results

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[1] The nomenclature for aldose reductase (ALR2) was recommended by the First International Workshop on Aldehyde Dehydrogenase and Aldehyde Reductase, held in Bern, Switzerland, on July 12-14, 1982 (Turner & Flynn, 1982). Abbreviations: Na_2EDTA, disodium ethylenediaminetetraacetate; Mops, 3-(*N*-morpholino)propanesulfonic acid; NAD^+, β-nicotinamide adenine dinucleotide; NADH, reduced form of NAD^+; $NADP^+$, β-nicotinamide adenine dinucleotide phosphate; NADPH, reduced form of $NADP^+$; N(Hx)DP^+, β-nicotinamide hypoxanthine dinucleotide phosphate; N(Hx)DPH, reduced form of N(Hx)DP^+; (AP)ADP^+, β-3-acetylpyridine adenine dinucleotide phosphate; (AP)ADPH, reduced form of (AP)ADP^+; (AP)AD^+, β-3-acetylpyridine adenine dinucleotide; NADP-ald or simply "adduct", the covalent adduct [1,4-dihydro-4-(1-hydroxy-2-oxoethyl)nicotinamide adenine dinucleotide phosphate]resulting from reaction of glycolaldehyde with $NADP^+$; E·NADP·ald, the ternary dead-end complex of ALR2 with $NADP^+$ and glycolaldehyde; E·NADP-ald, the *binary* E·adduct complex [similar nomenclature is used for adduct and enzyme complexes containing (AP)ADP^+ and N(Hx)DP^+]. Fluorescence data are given as ${}_{ex}F_{emit}$, with λ_{max} for excitation (λ^{ex}_{max}) and emission (λ^{emit}_{max}) shown as preceding and following subscripts, respectively.

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