Phosphorylation of Casein Kinase II†

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ABSTRACT: Casein kinase II from rabbit reticulocytes is a tetramer with an $\alpha,\alpha'\beta_2$ or $\alpha_2\beta_2$ structure; the α subunits contain the catalytic activity, and the β subunits are regulatory in nature [Traugh, J. A., Lin, W. J., Takada-Axelrod, F., & Tuazon, P. T. (1990) *Adv. Second Messenger Phosphoprotein Res. 24*, 224–229]. When casein kinase II is isolated from rabbit reticulocytes by a rapid two-step purification of the enzyme, both the α and β subunits are phosphorylated to a significant extent. In vitro, purified casein kinase II undergoes autophosphorylation on the β subunit. In the presence of polylysine and polyarginine, phosphorylation of the β subunits is inhibited, and the α subunits (α and α') become autophosphorylated. The effectiveness of polylysine coincides with the molecular weight. With basic proteins, including a number of histones and protamine, autophosphorylation of both subunits is observed. With histones, autophosphorylation of each subunit can be greater than that observed with the autophosphorylated enzyme alone or with a basic polypeptide. Thus, the potential exists for modulatory proteins to alter the autophosphorylation state of casein kinase II. Taken together, the data suggest that phosphorylation of the α subunit of casein kinase II in vivo may be due to an unidentified protein kinase or due to autophosphorylation. In the latter instance, casein kinase II could be transiently associated with specific intracellular compounds, such as basic proteins, with a resultant stimulation of autophosphorylation.

Casein kinase II has been identified in all eukaryotes examined and is found in the nucleus, cytoplasm, and mitochondria and associated with the cell membrane (Hathaway & Traugh, 1982). Casein kinase II has been shown to modify a number of substrates in vivo and in vitro by recognizing a serine (or threonine) followed by acidic residues [see Tuazon and Traugh (1991) for a review]. The protein kinase has an $\alpha_2\beta_2$ or an $\alpha,\alpha'\beta_2$ structure, where the α subunits contain the catalytic activity and the β subunits are regulatory in nature (Tuazon & Traugh, 1991; Traugh et al., 1990; Lin et al., 1991). Unlike many protein kinases, the purified enzyme is active and is regulated directly by acidic and basic physiological compounds. Polyamines have been shown to stimulate activity (Cochet et al., 1980; Hathaway & Traugh, 1984b; Meggio et al., 1987), while 2,3-bisphosphoglycerate, heparin, and other sulfated glycosaminoglycans inhibit the protein kinase activity (Feige et al., 1980; Hathaway & Traugh, 1984a; Hathaway et al., 1980; Tuazon & Traugh, 1991). Basic polypeptides, such as polylysine and polyarginine, also have a stimulatory effect, while acidic polypeptides are inhibitory (Criss et al., 1978; Meggio et al., 1984, 1987). The α subunit from *Drosophila*, which has been cloned and expressed in *Escherichia coli*, contains the catalytic activity, is autophosphorylated, and is directly modulated by acidic compounds which inhibit the activity of the holoenzyme (Traugh et al., 1990; Lin et al., 1991). Upon comparison of the α subunit with the holoenzyme, the β subunit appears to be required for optimal protein kinase activity, to mediate the stimulation observed via basic compounds, and to be involved in recognition and/or binding to specific substrates (Traugh et al., 1990; Lin et al., 1991).

The mode of regulation of casein kinase II activity in vivo has been enigmatic. In cultured cells, growth-promoting compounds and insulin have been shown to stimulate casein kinase II activity from 30 to 600% within minutes (Ackerman & Osheroff, 1988; Carroll & Marshak, 1989; Sommercorn & Krebs, 1987; Sommercorn et al., 1987); a second stimulation, observed after a duration of hours, appears to be due to production of new enzyme [see Tuazon and Traugh (1991) for a discussion]. Casein kinase II has been shown to be phosphorylated on the β subunit in human carcinoma A-431 cells in vivo (Ackerman et al., 1990). In the studies described herein, we have shown that both the α and the β subunits of casein kinase II are phosphorylated in rabbit reticulocytes.

Purified casein kinase II is autophosphorylated on the β subunit (Christmann & Dahmus, 1981; Dahmus, 1981; Hathaway & Traugh, 1979; Tao et al., 1980). Addition of polylysine has been shown to inhibit autophosphorylation of the β subunit and stimulate autophosphorylation of the α subunit (Meggio & Pinna, 1984). Although polyamines also stimulate casein kinase II, they do not stimulate autophosphorylation of the α subunit (Meggio & Pinna, 1984). In these studies, we have examined the effects of basic polypeptides and proteins on autophosphorylation of casein kinase II and compared the results with those obtained in vivo.

Experimental Procedures

Materials. Poly(L-lysine) (M_r 3800–22 400, hydrobromide, and M_r 36 500, hydrochloride), poly(L-arginine) (M_r 40 000, hydrochloride), protamine sulfate, mixed histone (type II AS), and histone 1 (type IIIS) were from Sigma. Histones 3/4 (HA, arginine-rich) were obtained from Cooper Biomedical. Okadaic acid was from Moana BioProducts.

Purification of Casein Kinase II. Casein kinase II was purified by chromatography on DEAE-cellulose and phosphocellulose (with the sample loaded at 0.3 M NaCl), as described by Hathaway and Traugh (1979), followed by chromatography on a hydroxylapatite column (2.5 × 10 cm) equilibrated in buffer A (20 mM Tris-HCl, pH 7.8, 400 mM NaCl, 1 mM EDTA, 1 mM EGTA, and NaN_3). Following application of the sample (approximately 200 mL), the column was washed sequentially with 150 mL of buffer A, with 150

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mL of buffer A minus NaCl, and with 150 mL of buffer B (25 mM KH_2PO_4/K_2HPO_4, pH 7.2, 1 mM EDTA, and 0.02% NaN_3); casein kinase II eluted at 350–400 mM with a 300-mL gradient from 25 to 500 mM KH_2PO_4/K_2HPO_4 in buffer B; 6-mL fractions were collected. Fractions (10 mL) were dialyzed against buffer C (20 mM Tris-HCl, pH 7.5, 1 mM EGTA, 1 mM EDTA, and 0.02% NaN_3) and then chromatographed by FPLC on a Mono Q HR5/5 column equilibrated in buffer C. Casein kinase II eluted at 550 mM NaCl in buffer C using a gradient from 0.1 to 1.1 M NaCl. The enzyme, purified to apparent homogeneity, was stored at 4 °C. Casein kinase II was assayed at pH 7.5 with dephosphorylated casein as described by Hathaway et al. (1979).

Autophosphorylation of Casein Kinase II. Autophosphorylation of casein kinase II was carried out in 0.070-mL reaction mixtures containing 50 mM Tris-HCl, pH 7.5, 140 mM KCl, 10 mM $MgCl_2$, 0.14 mM [γ-^{32}P]ATP, and casein kinase II, in the presence and absence of basic polypeptides or proteins. Incubation was at 30 °C for 15 min. The reactions were terminated by addition of 100 mM ATP followed by gel electrophoresis sample buffer containing sodium dodecyl sulfate and analyzed by electrophoresis in 12.5% polyacrylamide gels followed by autoradiography (Hathaway et al., 1979). ^{32}P incorporated into each subunit was determined from densitometric scans of the autoradiograms or by excising the protein and liquid scintillation counting.

Phosphorylation of Casein Kinase II in Reticulocytes. Rabbit reticulocytes were incubated for 2.5 h with [^{32}P]P_i, as described by Morley and Traugh (1989). The cells were washed and lysed in the presence of phosphatase and protease inhibitors; the postribosomal supernate was immediately chromatographed on a small phosphocellulose column (2.5 × 1.5 cm) equilibrated in buffer D (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.1 mM EDTA, 1 mM dithiothreitol, 20 mM β-glycerophosphate, and 0.5 mM phenylmethanesulfonyl fluoride). The column was washed with 75 mL of buffer D, and protein was eluted in a single step with 1 M NaCl in the same buffer; 0.5-mL fractions were collected. Immediately following chromatography, the peak fractions were pooled, diluted to 150 mM salt, and applied to a Mono Q HR 5/5 column equilibrated in buffer D; casein kinase II eluted at 550 mM with a gradient from 0.1 to 1.1 M NaCl in buffer D. The enzyme was identified by assaying the column fractions with casein and [γ-^{32}P]ATP. The individual subunits were visualized following gel electrophoresis on 10% polyacrylamide gels by staining with Coomassie blue and autoradiography. The identity of the visualized protein was confirmed by immunoblotting of the β subunit with antibody prepared in rabbit to a synthetic peptide corresponding to amino acids 172–186 of the β subunit of casein kinase II (Tuazon & Traugh, 1991), cross-linked to keyhole limpet hemocyanin, and injected into rabbit. The Coomassie-stained β subunit was excised and rerun on 12.5% polyacrylamide gels; the protein was electroblotted onto Immobilon-P, subjected to antibody overnight, and visualized with perioxidase-conjugated sheep anti-rabbit IgG.

Results

Phosphorylation of Casein Kinase II in Reticulocytes. Rabbit reticulocytes were incubated in the presence of [^{32}P]P_i for 2.5 h. Following cell lysis, the postribosomal supernate was chromatographed on phosphocellulose, step-eluted, and purified further by FPLC on Mono Q. The fractions were assayed for casein kinase activity and for radiolabeled protein by gel electrophoresis and autoradiography. The casein kinase activity eluted at 550 mM KCl, the same elution position as purified casein kinase II. The peak fractions contained only proteins corresponding to casein kinase II. Two of the bands, M_r 43 000 and 39 000, were equivalent to those identified previously as the α and α' subunits of casein kinase II from reticulocytes; the other migrated at M_r 26 000, equivalent to that of the β subunit (Figure 1). To confirm that the phosphoproteins observed in these fractions were due to casein kinase II, the Coomassie-stained β-subunit was excised and subjected to gel electrophoresis, and the immunoblot was incubated with antibody prepared in rabbit against a synthetic peptide prepared to a 15 amino acid sequence of the β subunit. The antibody was specific for the β subunit of casein kinase II and reacted with the phosphoprotein (M_r 26 000), confirming the identify of the β subunit (Figure 1C). This indicates that the three subunits identified by Coomassie staining and autoradiography corresponded to the α,α' and β subunits of casein kinase II. The reticulocytes used in these studies were from a pool of seven animals; the amount of α was greater than that of α' since some of the animals were $\alpha_2\beta_2$, while others were $\alpha,\alpha'\beta_2$.

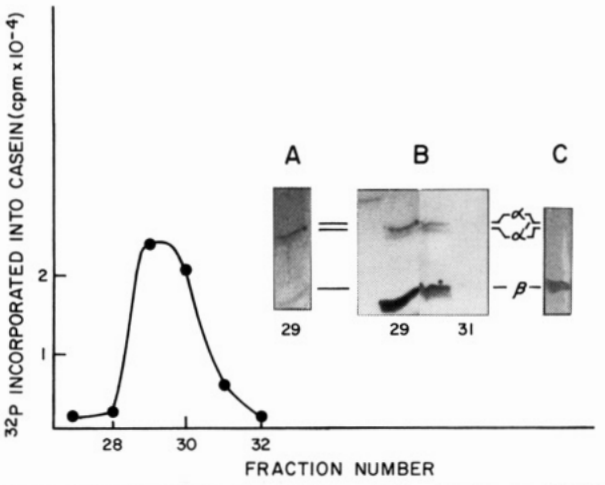

FIGURE 1: Phosphorylation of casein kinase II in reticulocytes. Rabbit reticulocytes were incubated with [^{32}P]P_i and lysed, and casein kinase II was isolated by chromatography on phosphocellulose and FPLC on Mono Q as described under Experimental Procedures. The activity for casein kinase II following elution from Mono Q is shown. The fractions were examined by gel electrophoresis followed by autoradiography. (A) Coomassie blue stain of fraction 29; (B) autoradiogram of the peak fractions from Mono Q; (C) immunoblot of the protein migrating at the position of β subunit of casein kinase II with antibody prepared to a synthetic peptide of casein kinase II.

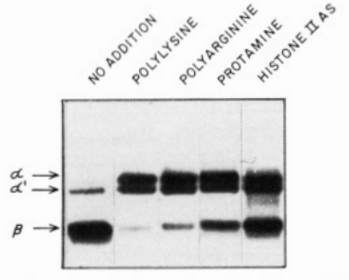

FIGURE 2: Autophosphorylation of casein kinase II in the presence of basic polypeptides. Casein kinase II (280 units) was autophosphorylated with [γ-^{32}P]ATP (2000 dpm/pmol) in the absence or presence of 1 μg of the synthetic polypeptides polylysine (M_r 36 500) or polyarginine (M_r 40 000) or the natural proteins protamine and mixed histone. The reactions were analyzed by gel electrophoresis followed by autoradiography. The autoradiogram is shown.

Effects of Polylysine on Autophosphorylation. Casein kinase II, purified to apparent homogeneity, was autophosphorylated on the β subunit; little or no phosphorylation of the α subunit was observed (Figure 2). When casein kinase

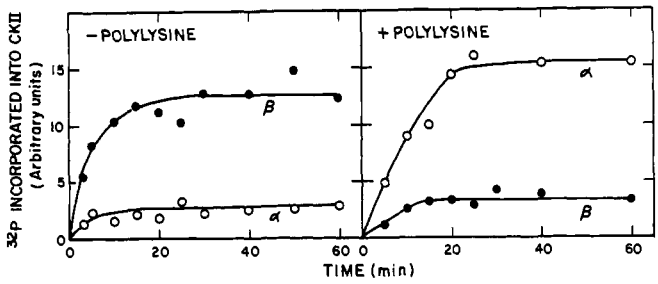

FIGURE 3: Time course of autophosphorylation of casein kinase II. Autophosphorylation of casein kinase II (80 units) with [γ-^{32}P]ATP (500 dpm/pmol) was carried out in the presence or absence of 1 μg/assay of polylysine (M_r 36 500) for the times indicated. Following gel electrophoresis, radioactivity was determined by scanning the autoradiograms and has been expressed in arbitrary units.

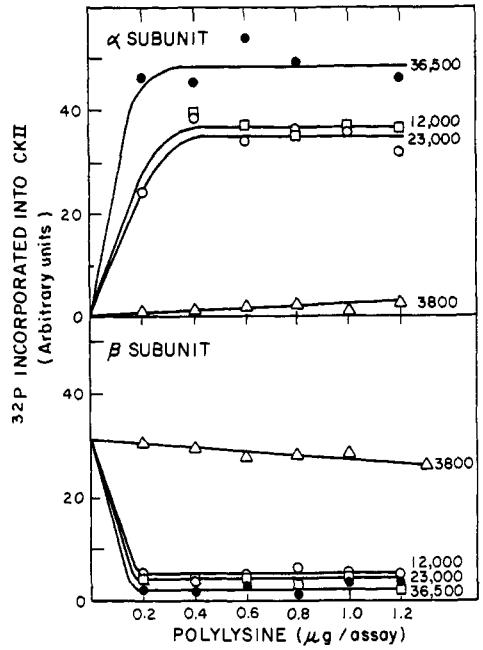

FIGURE 4: Effects of the molecular weight of polylysine on autophosphorylation of casein kinase II. The autophosphorylation reaction mixtures utilized 80 units of casein kinase II and [γ-^{32}P]ATP (500 dpm/pmol) and contained the indicated amounts of polylysine, M_r 36 500, 23 000, 12 000, or 3800. ^{32}P incorporation into the α and β subunits of casein kinase II was analyzed, after 20 min of incubation, by gel electrophoresis and quantified by densitometric scanning of the autoradiograms.

II was incubated with the basic polypeptide polylysine, autophosphorylation of the β subunit was inhibited, and the α and α' subunits were highly modified. The amount of phosphate incorporated into the α subunits was simular to that incorporated into the β subunits in the absence of polylysine. As shown in Figure 3, autophosphorylation of the β subunit was complete after 20 min of incubation with [γ-^{32}P]ATP. A similar time course of autophosphorylation for the α and α' subunits was observed in the presence of polylysine. The effect of the length of the polylysine polymer on autophosphorylation of casein kinase II was examined. As shown in Figure 4, autophosphorylation of the α and α' subunits was optimal with polylysine, M_r 36 500. At 0.2 μg/assay (4 μg/mL), phosphorylation was maximal, and phosphorylation of the β subunits was totally inhibited. The I_{50} for inhibition of phosphorylation of the β subunits and the A_{50} for phosphorylation of the α subunits were 10 and 30 ng/assay, respectively (0.2 and 0.6 μg/mL). With polylysine, M_r 23 000 and 12 000, maximal incorporation into the α subunits was around 80% of that observed with the higher molecular weight polypeptide. Polylysine, M_r 3800, did not stimulate autophosphorylation

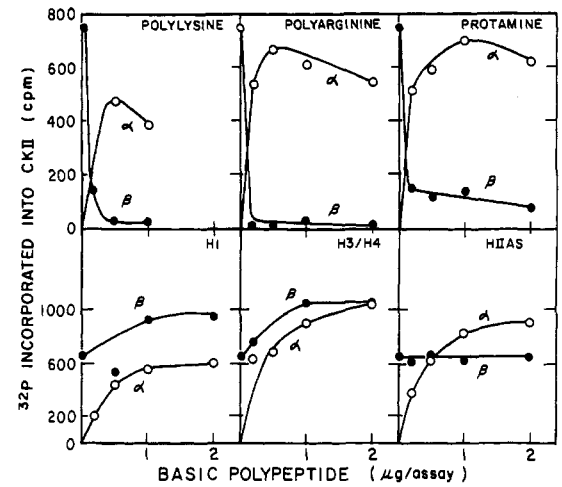

FIGURE 5: Autophosphorylation of casein kinase II with different basic polypeptides. Casein kinase II (80 units) was autophosphorylated under standard conditions with [γ-^{32}P]ATP (1000 dpm/pmol) in the presence of increasing amounts (0–2 μg) of different basic polypeptides or proteins for 20 min and analyzed by gel electrophoresis and autoradiography. Radiolabeled bands corresponding to the α and β subunits were excised and counted in a scintillation counter.

of the α subunits to any extent, and little inhibition of autophosphorylation of the β subunits was observed even at high concentrations of polylysine (1.2 μg/assay; 24 μg/mL).

Effects of Other Basic Polypeptides and Proteins on Autophosphorylation. The autophosphorylation pattern of casein kinase II was examined in the presence of another basic polypeptide, polyarginine (M_r 40 000), and basic proteins, including protamine and mixed histone (type II AS). As shown in Figure 2, all three compounds stimulated autophosphorylation of the α subunits to at least the same extent as polylysine (M_r 36 500), when added at a concentration of 1 μg/assay (20 μg/mL). No difference in autophosphorylation between α and α' was observed. However, differences were observed in the effect of the compounds on autophosphorylation of the β subunits. Polyarginine almost completely inhibited phosphorylation of the β subunits, with data similar to those observed with polylysine. However, some phosphorylation of the β subunits was detected with protamine, and significant phosphorylation was observed with mixed histone. Phosphoamino acid analysis showed that in all instances the β subunit was phosphorylated only on serine, whereas autophosphorylation of the α subunits was on serine, with some phosphothreonine observed also (data not shown).

Incorporation of phosphate into casein kinase II was investigated further with increasing concentrations of the basic compounds (Figure 5). With polyarginine, optimal stimulation of phosphorylation of α and inhibition of β were observed at $\leq$0.2 μg/assay (4 μg/mL). Higher levels of protamine (about 1 μg/assay) were required to achieve optimal phosphorylation of the α subunits; inhibition of the β subunits was 80% at 0.2 μg of protamine/assay, but the residual autophosphorylation was difficult to inhibit further. With the histones, optimal stimulation was observed at 1–2 μg/assay (20–40 μg/mL). No inhibition of phosphorylation of the β subunits was identified at any concentration; with histone 1 and a mixture of histones 3 and 4, autophosphorylation of the β subunits was stimulated 1.4–1.6-fold, while mixed histone did not affect autophosphorylation of the β subunits to any extent. All three compounds stimulated autophosphorylation of the α subunits with histone 3/4 > mixed histone > histone 1.

The ratio of autophosphorylation of the α and β subunits with casein kinase II alone was less than 0.1; with polylysine

(M_r 36 500), the α/β ratio was around 50. With protamine, the ratio was 5, while histone 1, histones 3/4, and mixed histone had a ratio between 0.7 and 1.4. With all three types of histone, incorporation of phosphate into the β subunits was greater than with the enzyme alone, and incorporation into the α,α' subunits was greater than in the presence of polylysine. Thus, more than twice as much phosphate was incorporated into the holoenzyme in the presence of histone than under the other conditions of phosphorylation.

Discussion

Phosphorylation of casein kinase II was examined in reticulocytes incubated with [^{32}P]P_i following a rapid, two-step purification of the enzyme. In the highly purified enzyme, both the α and β subunits were shown to be phosphorylated, with the majority of the phosphate in the β subunits. Previously, Ackerman et al. (1990) showed that the β subunits were phosphorylated in human carcinoma A-431 cells incubated with epidermal growth factor; little phosphorylation of the α subunits was detected. In the studies described herein, all manipulations following cell disruption were rapidly carried out in the presence of phosphatase and protease inhibitors, which may explain the differences between the results.

With purified casein kinase II, only autophosphorylation of the β subunits was observed; up to 1 mol of phosphate was incorporated into the β subunits, with little phosphorylation of the α subunits detected (Hathaway & Traugh, 1979). Autophosphorylation of the α subunits was observed in the presence of polylysine under conditions where autophosphorylation of the β subunit was inhibited (Meggio et al., 1984), although in earlier experiments, with higher concentrations of polylysine, no phosphorylation of the α subunits was observed (Meggio et al., 1983). In these experiments, we examined in detail the requirements for autophosphorylation of both the α and the β subunits. The basic polypeptides, polylysine and polyarginine, stimulated autophosphorylation of α and α' and inhibited autophosphorylation of the β subunits. The degree of effectiveness of the basic homopolymer was dependent on length; the higher the molecular weight, the greater the degree of stimulation of phosphorylation of the α subunits. Polylysine has been shown to form complexes with the purified monomer (Traugh et al., 1990) and to cause dissociation of the filamentous form (Mamrack, 1989).

The histones, which have an overall basic charge, but do not contain extensive basic sequences, stimulated autophosphorylation of the α subunits, but had differential effects on autophosphorylation of the β subunits. Histone 1 and a mixture of histones 3 and 4 stimulated autophosphorylation of both the α and β subunits; the amount of phosphate incorporated into the protein kinase was up to twice that observed with the enzyme incubated alone or with polylysine or polyarginine. The amount of histone required to effect autophosphorylation was 10–15-fold higher than that observed for polylysine.

From sequence analysis, the β subunit has been shown to have a considerable degree of polarity, with the amino terminus containing a higher portion of acidic residues and the carboxyl terminus enhanced in basic residues (Jakobi et al., 1989; Saxena et al., 1987). In studies with the purified α subunit, cloned and expressed in *E. coli*, it has been determined that the β subunit is regulatory in nature and stimulates the protein kinase activity, mediates the stimulatory effects of basic compounds, and is important in selectivity of substrates (Lin et al., 1991; Traugh et al., 1990). Thus, basic proteins/polypeptides could bind to the amino-terminal domain of the β subunit, altering the structure of the protein kinase so that alternative autophosphorylation sites are available.

Casein kinase II appears to be ubiquitous in all eukaryotes and is found associated with all subcellular components. The protein kinase can be tightly associated with numerous substrates and is often a contaminant of otherwise "pure" proteins. In this regard, casein kinase II could be associated with basic proteins inside the cell, including histones and/or other regulatory molecules, as well as with the membrane. It should be noted that the polyamines, although having a stimulatory effect on casein kinase II (Hathaway & Traugh, 1984b), do not stimulate autophosphorylation of the α subunit (Meggio & Pinna, 1984; data not shown).

The studies described herein support the conclusion that basic polypeptides and proteins can act at the level of the protein kinase as shown by changes in autophosphorylation of the α and the β subunits. These compounds appear to alter the structure of the protein kinase so that the α subunits now become substrates. The role of differential autophosphorylation of casein kinase II in regulation of activity is currently unknown. Difficulties abound in analyzing the effects in vitro, in that the modulatory molecules required for changes in the autophosphorylation patterns also have effects on individual substrates. For instance, polylysine has been reported to stimulate phosphorylation of some cellular proteins by casein kinase II (Criss et al., 1978), and basic polypeptides or proteins are required for phosphorylation of specific proteins, including calmodulin (Meggio et al., 1987; Nakajo et al., 1988; Sacks et al., 1988) and elongation factor 1 (Palen et al., 1991). In vitro, neither protein can be phosphorylated to any extent alone, by casein kinase II but significant phosphorylation is observed in the presence of these basic compounds.

Thus, we have shown that both the α and β subunits of casein kinase II are phosphorylated in reticulocytes incubated with [^{32}P]P_i and that basic compounds can differentially affect the autophosphorylation pattern of purified casein kinase II in vitro. Over a number of years, we have analyzed whether casein kinase II could be phosphorylated by other protein kinases. To date, these studies have been negative. Recently, the β subunit, but not the α subunits, has been reported to be modified in vitro by cdc 2 kinase (Mulner-Lorillon et al., 1990). Phosphorylation of the α subunits observed in reticulocytes is due either to autophosphorylation or to an unidentified protein kinase.

Acknowledgments

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Registry No. Casein kinase II, 88232-11-5; polylysine, homopolymer, 25104-18-1; polyarginine, homopolymer, 25212-18-4; polylysine, SRU, 38000-06-5; polyarginine, SRU, 24937-47-1.

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Interaction of Assembly Protein AP-2 and Its Isolated Subunits with Clathrin†

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ABSTRACT: The clathrin assembly protein complex AP-2 is a multimeric subunit complex consisting of two 100–115-kDa subunits known as α and β and 50- and 16-kDa subunits. The subunits have been dissociated and separated by ion-exchange chromatography in 7.5 M urea. Fractions highly enriched in either the α or β subunit were obtained. The α fraction interacted with clathrin as evidenced by its ability to bind to preassembled clathrin cages. It also reacted with dissociated clathrin trimers under conditions that favor assembly of coat structures, but did not yield discrete clathrin polygonal lattices. The enriched β fraction (containing small amounts of α) reacted with clathrin to yield intact coats with the incorporation of approximately equivalent amounts of α and β subunits into the polymerized species; excess free β subunit was unreactive. The AP-2 complex was also completely dissociated in a highly denaturing solvent, 6 M Gdn·HCl, and the constituent subunits of 100–115, 50, and 16 kDa were separated by gel filtration. In a coassembly assay with clathrin, the clathrin polymerizing activity was exclusively associated with the 100-kDa subunit fraction with stoichiometric incorporation of both α and β subunits of 100 kDa into the polymerized coats, and with no requirement for 50- or 16-kDa subunits. These observations demonstrate that the assembly activity of the complex is associated with the α and β subunits and suggest that both subunits, through independent interactions with clathrin, are required for expression of complete lattice assembly activity.

Clathrin coated vesicles are involved in a variety of cellular processes, the best characterized among them being receptor-mediated endocytosis (Brodsky, 1988; Goldstein et al., 1985). In addition to clathrin, the principal component, the coat of in vitro isolated coated vesicles consists of several other proteins known as assembly, associated, or adaptor proteins,

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