Phosphorylation of C8 and C9 Subunits of the Multicatalytic Proteinase by Casein Kinase II and Identification of the C8 Phosphorylation Sites by Direct Mutagenesis†

José G. Castaño,* Esther Mahillo, Paz Arizti, and Joaquín Arribas‡

Departamento de Bioquímica e Instituto de Investigaciones Biomédicas del Consejo Superior de Investigaciones Científicas, Facultad de Medicina de la UAM, 28029 Madrid, Spain

Received October 25, 1995; Revised Manuscript Received January 23, 1996⊗

ABSTRACT: Two 29 kDa subunits of the multicatalytic proteinase (proteasome) complex, the C8 and C9 components, are phosphorylated in vivo and can be phosphorylated in vitro by casein kinase II (CKII). The major phosphate acceptor is the C8 subunit being phosphorylated in serine, both in vivo and in vitro. The phosphopeptides generated by Glu-C endoprotease digestion from the in vivo 29 kDa labeled subunit and from the in vitro phosphorylation of the recombinant C8 subunit with CKII are identical, suggesting that CKII is likely responsible for the in vivo phosphorylation of the C8 subunit. The in vitro stoichiometry of phosphorylation of the proteasome complex and the recombinant C9 and C8 subunits by CKII is 2−2.5, 0.2, and 2 mol of phosphate per mole, respectively. Several C8 protein constructs allow the location of the CKII phosphorylation sites to be the COOH terminal portion of the protein, and direct mutational analyses show that Ser-243 and Ser-250 are the residues of the C8 subunit phosphorylated by CKII. The in vitro phosphorylation of the proteasome by CKII does not affect its proteolytic activity (on proteins or fluorogenic synthetic peptides), therefore suggesting its involvement in the interaction of the proteasome with other cellular proteins, i.e. in the formation of the 26S complex and/or in the interaction with the nuclear translocation machinery.

The eukaryotic multicatalytic proteinase (MCP)[1] or proteasome is a high-molecular mass proteinase complex composed, at least, of 14 different types of subunits and participates in the degradation of intracellular proteins, by ubiquitin-dependent and -independent pathways (Orlowski, 1990; Goldberg, 1992; Rivett, 1993; Rechsteiner et al., 1993; Hochstrasser, 1995). The MCP polypeptide components separated either by reverse-phase HPLC (Tanaka et al., 1988a,b, 1989) or by two-dimensional polyacrylamide gel electrophoresis (Kleinschmidt et al., 1983; Castaño et al., 1986; Martins de Sa et al., 1986; Tomek et al., 1988; Rivett & Sweeney, 1991; Heinemeyer et al., 1991; Arribas et al., 1994) show up to 20 different polypeptides. Furthermore, changes in proteasomal subunit composition under different physiological conditions (Pal et al., 1988; Klein et al., 1990; Kumatori et al., 1990; Kanayama et al., 1991; Shimbara et al., 1992) and the likely existence of different subpopulations of proteasomes (Falkenburg & Klotzel, 1989; Yang et al., 1992; Hoffman et al., 1992; Brown et al., 1993; Driscoll et al., 1993; Gaczynska et al., 1993) have been reported. All these data suggest that MCP composition, and likely its activity, is under a complex control in vivo. Some of these changes may be due to post-translational modification of MCP subunits, i.e. phosphorylation. Several consensus sequences for tyrosyl and seryl/threonyl kinases are found in the amino acid sequence derived from MCP subunit cDNAs (Tanaka et al., 1992). Previous studies reported the phosphorylation of proteasomal subunits in crude preparations from *Drosophila* culture cells (Haass & Kloetzel, 1989); no phosphorylation of the MCP obtained from hamster kidney cells (Lee et al., 1990) and several MCP subunits became phosphorylated in vitro by a cyclic AMP-dependent protein kinase copurifying with bovine pituitary MCP (Pereira & Wilk, 1990). Recently, the copurification of CKII with human erythrocyte MCP and the in vitro phosphorylation of a 30 kDa MCP subunit was reported (Ludemann et al., 1993).

We describe here that MCP is phosphorylated in vivo and in vitro. CKII is responsible for this phosphorylation in vitro, and the main phosphate acceptor subunit is the C8 component of the MCP complex, both in vivo and in vitro. Furthermore, direct mutagenesis allows us to determine that Ser-243 and Ser-250 in the COOH terminal of the C8 subunit are the amino acid residues phosphorylated by CKII.

MATERIALS AND METHODS

Protein Analysis by SDS−PAGE, 2D Gels, and Immunoblotting. The rat liver MCP was purified and assayed as described (Arribas & Castaño, 1990; Ruiz de Mena et al., 1993). Removal of the copurifying casein kinase II activity was performed by HPLC gel filtration, as described (Arribas & Castaño, 1993). Proteins were analyzed (except where indicated) on 13% SDS−PAGE according to Laemmli

† This work was supported by Comisión Interministerial de Ciencia y Tecnología (SAL94-0685) and Fundación Ramón Areces.

* To whom all correspondence should be addressed at Departamento de Bioquímica e Instituto de Investigaciones Biomédicas del CSIC, Facultad de Medicina de la UAM, 28029 Madrid, Spain. Phone: 34-1-397-5427. Fax: 34-1-585-4587.

‡ Present address: Cell Biology and Genetics program, Memorial Sloan-Kettering Cancer Center, Howard Hughes Medical Institute, 1275 York Avenue, New York, NY 10021.

⊗ Abstract published in *Advance ACS Abstracts,* March 1, 1996.

[1] Abbreviations: SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; EGTA, 1,2-bis(2-aminoethoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid ethylenediamine; PMSF, phenylmethanesulfonyl fluoride; 2D, two-dimensional gel electrophoresis; MCP, multicatalytic proteinase, proteasome; HPLC, high-performance liquid chromatography; CKII, casein kinase II.

0006-2960/96/0435-3782$12.00/0

(1970) or by 2D (O'Farrell et al., 1977) using pH 3.5−10 ampholytes (first dimension, 4 h at 400 V and 1 h at 800 V). After electrophoresis, the gels were either stained with Coomassie blue or transferred to nitrocellulose (BA85, Schleicher and Schuell) as described (Arribas et al., 1991). Nitrocellulose filters were reversibly stained with 0.1% Ponceau red in 1% acetic acid and blocked overnight at 4 °C with blocking buffer: 50 mM Tris-HCl (pH 7.5), 0.5 M NaCl, and 3% (w/v) bovine serum albumin (BSA) for immunoblot analysis. The filters for immunoblotting were incubated with the respective subunit specific antibody at 1/200−1/500 dilution in blocking buffer and processed as described (Arribas et al., 1991); where indicated, the blots were exposed to X-ray film for autoradiography.

Metabolic Labeling of Cultured Cells and Immunoprecipitations. Normal rat kidney cells (ATCC catalog number CRL 1570, NRK-49F) were grown in Dulbecco's modified Eagles medium (DMEM) with 10% fetal calf serum. For labeling, cells grown at 80% confluence in 35 mm plastic petri dishes (Costar) were washed three times with DMEM without phosphate and incubated for 3 h at 37 °C with 1 mL of DMEM without phosphate containing 1 mCi/mL of carrier-free [^{32}P]orthophosphate (ICN). After the labeling period, the cells were cooled on ice (all subsequent steps were performed at 4 °C), washed three times with cold PBS [20 mM NaP_i (pH 7.4) and 68 mM NaCl], and lysed (1 mL per 35 mm dish) in the immunoprecipitation buffer (IP buffer) that contains 50 mM Tris-HCl (pH 7.5), 0.15 M NaCl, 0.5% NP-40, 10 mM EDTA, 5 mM EGTA, 20 mM NaF, 10 mM β-glycero phosphate, 0.1 mM orthovanadate, 0.5 mM PMSF, and 1 μg/mL leupeptin. The labeled cell extracts were centrifuged at 10000*g* for 20 min. Anti-MCP or preimmune sera (Arribas et al., 1991) were precoupled to protein A-Sepharose (Sigma) for immunoprecipitation. The protein A-Sepharose beads containing the coupled antibodies were washed with IP buffer, and then the labeled supernatant was added and incubated for 3 h with rocking. The beads were washed three times with 1 mL of IP buffer, then with IP buffer containing 18.4 g of KCl per 100 mL, and once with distilled water. The proteins were eluted by boiling with SDS/sample buffer and analyzed by SDS−PAGE. For 2D-gel analysis of immunoprecipitates, the eluted proteins were precipitated with 5 volumes of acetone in the cold (1 μg of BSA was added as carrier), centrifuged, washed with cold acetone, dried, and resuspended in the sample buffer of the first dimension of 2D-gel analysis.

In Vitro Phosphorylation of MCP Complex and Recombinant Subunits. The phosphorylation reaction mixture contained in a final volume of 20 μL 25 mM Hepes/NaOH (pH 7.4), 10 mM $MgCl_2$, and 100 μM [γ-^{32}P]ATP (1000 cpm/pmol, from Amersham). For endogenous protein kinase activity of MCP fractions, usually 5 μL of the respective fraction was used. In the assays where the recombinant casein kinase α or α' subunit was used, the reaction mixture contained approximately 0.4 μg of the kinase and 2−5 μg of MCP (CKII-free by HPLC gel filtration, see above) or 0.2−2 μg of the respective purified C8 or C9 recombinant proteins. Reaction mixtures were incubated at 30 °C for 30 min (except where indicated) and stopped by boiling after addition of equal volumes of (2×) SDS/sample buffer (Laemmli, 1970). The products were analyzed on 13% SDS−PAGE, stained with Coomassie blue, destained, dried, and exposed to X-Omat AR X-ray film (Kodak) at −70 °C. For calculation of the stoichiometry of phosphorylation, the corresponding phosphorylated bands were excised from the gel and counted directly by Cerenkov radiation.

Cloning and Bacterial Expression of C8 and CKII α and α' Subunits. The mouse and human C8 subunit cDNAs were cloned by amplification reactions whose mixtures contained in a final volume of 50 μL 20 mM Tris-HCl (pH 7.3), 50 mM KCl, 10 mM $MgCl_2$, 0.25 mM of each deoxynucleotide triphosphate, 0.5 μg of each oligonucleotide primer, 0.1 μg of cDNA from mouse or human liver (Clontech), and 1 unit of Taq DNA polymerase (Cetus-Perkin-Elmer). The 30-cycle amplification profile and the cloning procedures were done as described (Arribas et al., 1994). The oligonucleotide primers were as follows for the 5′ oligonucleotide and the 3′ oligonucleotide, respectively: 5′-TCTAGAATTCATATGAGCTCCATTGGCACTGGG-3′ and 5′-TAGAGCTCGTCGACTTACATATTGTCGTCATCT-3′, derived from the reported rat C8 cDNA sequence (Tanaka et al., 1990). The amplified mouse C8 cDNA was digested with *Eco*RI and *Sal*I for cloning into the Bluescript KS vector (Stratagene), digested with the same restriction enzymes. For expression, the C8 cDNA contained an artificial *Nde*I site in the 5′ oligonucleotide used for the amplification reaction (underlined), so it was excised from the Bluescript plasmid by partial digestion with *Nde*I (the mouse C8 cDNA, like that of the rat, contains an internal *Nde*I site) and *Sal*I and inserted into the pT7-7 plasmid (Tabor & Richardson, 1985), digested with the same restriction enzymes. The corresponding plasmid constructs, named pMC8 (Bluescript) and pTMC8 (pT7-7), respectively, were fully sequenced by the chain-termination method (Sanger et al., 1977). Its deduced amino acid sequence is identical to that of the rat C8 (Tanaka et al., 1990). Similar procedures were followed to express the human C8 subunit from total human liver cDNA (Clontech) and named pHC8 (Bluescript) and pTHC8 (pT7-7). The human CKII α and α' cDNAs were kindly provided by F. J. Lozeman (Lozeman et al., 1990). The α subunit cDNA (1 ng) was amplified in a reaction similar to that described above to create an artificial *Nde*I site (underlined) with the following primers for 5′ oligonucleotide and 3′ oligonucleotide, respectively: 5′-AATCTAGACATATGTCGGGACCCGTGCCAAGC-3′ and 5′-TTCCTAGGTTCGAATGACGACTCGCGGTCGCCG-3′. The amplified product was digested with *Nde*I and *Hin*dIII and subcloned into the pT7-7 vector, digested with the same restriction enzymes. The CKII α' cDNA was digested with *Sma*I and *Pst*I and subcloned into the pT7-7 vector, digested with the same restriction enzymes. The expression plasmids were named pTCKII α and α', respectively.

C8 Protein Constructs and Mutation Analysis of the Phosphorylation Sites. To map the phosphorylation sites by CKII of the C8 subunit, several protein constructs were obtained from the pTHC8 expression construct and generated by partial digestion with *Hin*dIII and religation. The constructs named pTHC8.1, C8.2, and C8.3 resulted from internal *Hin*dIII fragment deletion of C8 cDNA and ligation with the *Hin*dIII site of the pT7-7 vector, as follows: 623, 535, and 284, respectively. The construct pTHC8.6 resulted from the internal in-frame ligation between nucleotide positions 284 and 623 of the HC8 coding sequence, resulting in the deletion of the corresponding internal *Hin*dIII DNA fragments (see schemes in Figure 5).

Point mutation of the putative serine residues of the HC8 subunit was performed by cassette substitution of the *Eco*RV (HC8 cDNA site, 537)-*Sal*I (pT7-7 vector site) fragment of the pTHC8 construct and done by polymerase chain reaction (PCR) amplification with mutant oligos of that fragment, and an *Eco*47III site (not altering the corresponding amino acid sequence) was introduced into the mutant oligos for easy diagnosis of recombinant mutants. The oligonucleotides used were 5′ oligo (corresponding to the unique *Eco*RV site of HC8, underlined) 5′-CCTGCCGTGATATCGTTAAAGAA-GTTGC- 3′ and 3′ oligos, for construct pTHC8 S243A, 5′-GAGCTCGTCGACTTACATATTGTCGTCAT-CTGATTCATCTTCTTCCTTCAGCGCTTCCTT-AGCATATTTCTCTGC-3′ and, for the pTHC8 S243,250A double mutant, 5′-GAGCTCGTCGACTTACATATTGT-CATCATCTGCTTCATCTTCTTCCTTCAGCGCTT-CCTTAGCATATTTCTCTGC-3′. Mutations were confirmed by direct sequencing of the corresponding pTHC8 S243A and pTHC8 S243,250A constructs.

Purification of Recombinant C9, C8, and Casein Kinase II α *and* α′ *Subunits.* Induction of protein expression in bacteria, BL 21 (DE3) cells harboring the corresponding pT plasmid, was done as described (Arribas & Castaño, 1993). All the purifications started from 200 mL of the respective induced bacterial culture and were performed at 4 °C, except where indicated, and proteins analyzed by 13% SDS−PAGE.

The induced bacterial pellets containing the C9 polypeptide were sonicated (three cycles, 30 s power on, 1 min power off) in 20 mL of a buffer containing 20 mM KP_i (pH 6.6), 0.15 M NaCl, 0.1% Triton X-100, 1 mM DTT, and 1 mM PMSF. The pellet, obtained after centrifugation at 10000*g* for 30 min, was rehomogenized in the same buffer as above and centrifuged back under the same conditions. The resulting pellet was extracted with 20 mM KP_i (pH 6.6), 6 M urea, 1 mM DTT, and 1 mM PMSF and centrifuged at 100000*g* for 30 min (Arribas et al., 1994). The supernatant, containing most part of the C9 polypeptide in solution, was applied to a CM-Sepharose (Pharmacia) column (40 mL) equilibrated with 20 mM KP_i (pH 6.6), 1 mM PMSF, and 1 mM DTT. After washing, the bound protein was eluted with a linear gradient up to 0.5 M KCl in the equilibration buffer. Fractions containing the C9 polypeptide (peak elution at 0.2 M KCl) were buffer-exchanged on an Ultracent 10 (10 kDa molecular mass cutoff, from BioRad) with 20 mM Hepes/NaOH (pH 7.4) and 50 mM KCl. The overall yield was 0.5−1 mg of purified recombinant C9 subunit from 200 mL of bacterial cell culture. The C8 (human or mouse) recombinant subunit (identical procedures were followed for the point mutant versions S243A and S243,250A) was purified by sonication of the corresponding bacterial cell pellet (10 cycles, 10 s on, 30 s off) on ice with 50 mL of buffer C [50 mM Tris-HCl (pH 8.0), 1 mM DTT, 50 mM KCl, 5% glycerol, 0.1 mM PMSF, and 1 μg/mL leupeptin] and centrifuged at 10000*g* for 30 min. The supernatant was applied to a 50 mL DEAE-cellulose column (Whatman) equilibrated in buffer C. After washing, proteins were eluted with a linear gradient up to 0.6 M KCl in buffer C. Fractions containing the C8 polypeptide (peak elution at 0.3 M KCl) were pooled and diluted in buffer C without KCl and reapplied to a 5 mL DEAE column (as above) for concentration; elution was by a step of buffer C containing 0.3 M KCl. The concentrated fractions were diluted again with buffer C to 50 mM KCl and injected onto an HPLC DEAE column (at room temperature) equilibrated in buffer A (without $MgCl_2$) and eluted with a linear gradient up to 0.6 M KCl [buffer B without $MgCl_2$; see Ruiz de Mena et al. (1993) for composition of buffers A and B]. The overall yield was 1−2 mg of purified recombinant C8 subunit from 200 mL of induced bacterial cell culture. The production of anti-C8 specific antibodies was performed by injection of the purified recombinant C8 subunit into rabbits, essentially as described (Arribas et al., 1994). Protein constructs HC8.1, C8.2, C8.3, and C8.6 were partially purified as the insoluble protein pellets obtained after two extractions of the corresponding induced bacterial cells with buffer C containing 1% Triton X-100 and 0.5 M KCl and resuspended in 50 mM Tris-HCl (pH 7.5) and 50 mM KCl.

The CKII α and α′ catalytic subunits were purified from the corresponding induced cell pellets by sonication (10 cycles, 10 s on, 30 s off) on ice with 50 mL of buffer D [20 mM Tris-HCl (pH 7.5), 1 mM EDTA, 1 mM DTT, 0.1 mM PMSF, and 1 μg/mL leupeptin] containing 150 mM NaCl and centrifuged at 10000*g* for 30 min. The supernatant, 5-fold diluted with buffer D, was applied to a DEAE-cellulose (DE52, Whatman) column (50 mL) equilibrated in buffer D containing 25 mM NaCl. CKII was eluted with a linear gradient up to 0.6 M NaCl in buffer D. Fractions containing CKII activity (assayed with dephosphorylated β-casein) were pooled, adjusted to 200 mM NaCl by conductivity measurements, and applied to a 40 mL phosphocellulose column (P11, Whatman) equilibrated in buffer D with 200 mM NaCl. CKII was eluted with a linear gradient up to 1.2 M NaCl in buffer D. Fractions containing CKII activity were pooled, diluted to 0.5 M NaCl with buffer D, and applied to a 5 mL heparin/agarose column (Pharmacia) equilibrated in buffer D with 0.5 M NaCl; CKII was eluted by a step of buffer D containing 0.8 M NaCl. The concentrated CKII from the heparin/agarose column was diluted 5-fold with buffer D containing 50% glycerol and stored in aliquots at −70 °C until it was used. The overall yield of the purification was 0.5−0.8 mg of purified α and 0.2−0.5 mg of α′ CKII catalytic subunits per 200 mL of induced bacterial culture, with a specific activity of 80−120 nmol min^{-1} mg^{-1} assayed on β-casein as substrate.

Phosphoamino Acid Analysis and Glu-C Endoprotease Digestion. Proteins phosphorylated either in vitro or in vivo, after separation by 1- or 2D, were excised from the dried gels and hydrolyzed in 6 N HCl and the phosphoamino acids were analyzed by flat bed electrophoresis in the first dimension and thin-layer chromatography (TLC) in the second dimension, as described (Boyle et al., 1991). The TLC plates were exposed to X-Omat AR X-ray films (Kodak) at −70 °C. Glu-C endoprotease (Boehringer) digestion was performed as described by Cleveland et al. (1977).

RESULTS

In Vivo vs in Vitro Phosphorylation of MCP Polypeptides. To analyze the possible in vivo phosphorylation of MCP subunits, we used our anti-MCP polyclonal antibodies (35) for immunoprecipitation of total cell lysates prepared from NRK cells labeled with [^{32}P]orthophosphate. The results from one of those experiments are presented in Figure 1. The anti-MCP polyclonal antibodies specifically immunoprecipitated two phosphorylated polypeptides of 32 and 29

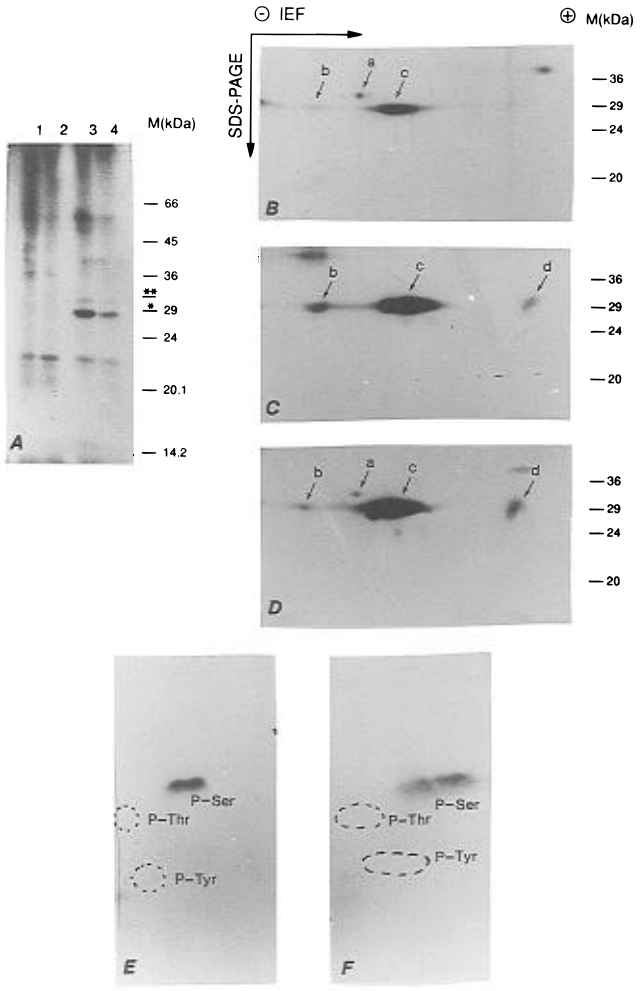

FIGURE 1: In vivo and in vitro phosphorylation of MCP polypeptides. NRK cells were labeled with [^{32}P]orthophosphate and crude extracts prepared and immunoprecipitated with anti-MCP antibodies. Purified rat liver MCP was phosphorylated in vitro by the copurifying CKII holoenzyme. Panel A shows the autoradiogram of a 13% SDS−PAGE of the immunoprecipitates with preimmune (lanes 1 and 2) and immune anti-MCP serum (lanes 3 and 4) showing the specific immunoprecipitation of 29 (*) and 32 kDa (**) labeled MCP polypeptides. Panels B−D show the autoradiogram of 2D-gel analysis of the immunoprecipitate from in vivo-labeled NRK cells (B), the in vitro-phosphorylated MCP (C), and a mixture of both of them (D). Panels E and F show the autoradiogram of the phosphoamino acid analysis from the in vivo and in vitro 29 kDa MCP phosphorylated proteins, respectively. For a complete description of the methods, see Materials and Methods.

kDa (Figure 1A), and two experiments are presented to show that the nonspecific phosphorylated bands vary somewhat from experiment to experiment; this situation was not ameliorated by preclearing the labeled extract with preimmune serum. In vitro studies (data not shown) demonstrate that a CKII activity copurifies with rat liver MCP, similar to the studies reported with the human erythrocyte MCP (Ludemann et al., 1993) being able to phosphorylate a 29 kDa MCP subunit. To compare the in vivo- vs the in vitro-phosphorylated polypeptides, we performed a 2D-gel analysis as shown in Figure 1B−D. By comparison of the in vivo and the in vitro pattern of 2D phosphoproteins, we can conclude that (1) the 32 kDa in vivo-phosphorylated MCP polypeptide (indicated as a) is not observed in the in vitro phosphorylation of MCP by the copurifying CKII; (2) the 29 kDa phosphorylated polypeptides are resolved by 2D analysis into a minor (indicated as b) and a major labeled spot (indicated as c), both of them observed in vivo and in vitro, with almost identical molecular masses and p*I* values, as they comigrate in the mix 2D experiment (Figure 1D); and (3) there is in vitro another very acidic spot in the 29 kDa region (indicated as d) not present in the immunoprecipitate of in vivo-labeled cells and this polypeptide is not always observed in the in vitro phosphorylation of the MCP (see below, Figure 2). A phosphoamino acid analysis of the in vivo- and in vitro-phosphorylated 29 kDa polypeptides shows, in both cases, the presence of phosphoserine (panels E and F of Figure 1, respectively).

Identification of the Phosphorylated MCP Subunits and Stoichiometry of Phosphorylation. With respect to the identification of the 29 kDa MCP phosphorylated subunits, we hypothesized two possible candidates: (1) the C9 component (29 kDa) that by previous 2D analysis and immunoblotting with anti-C9 antibodies was resolved into two isoelectric variants (Arribas et al., 1994) and (2) the C8 subunit (calculated mass of 28.437 kDa) that contains several potential sites of phosphorylation by CKII within its sequence (Tanaka et al., 1990). As a consequence, we proceeded to the bacterial expression and purification of the C8 polypeptide and the purified C8 subunit used to obtain anti-C8 polyclonal antibodies (see Materials and Methods). Then, to test our hypothesis, the experiments presented in Figure 2 were performed. MCP was phosphorylated in vitro by the copurifying CKII and analyzed by 2D. Figure 2A shows the polypeptide pattern stained with Ponceau red after Western transfer, and Figure 2B shows the corresponding autoradiogram (note that in this experiment the spot indicated as d in Figure 1 is not observed). Panels C and D of Figure 2 show the immunoblotting of similar 2D-gel separations with the anti-C9 (C) and anti-C8 (D) specific antibodies, respectively. By alignment of the immunoblots with the autoradiogram, there is a perfect coincidence of the labeled spots indicated as b and c with the anti-C9- and anti-C8-reacting polypeptides, respectively.

To further investigate the phosphorylation of MCP subunits by CKII, we needed to obtain the kinase easily and in high amounts, so we expressed the human cDNAs encoding for the CKII α and α′ catalytic subunits and the recombinant kinases purified. To validate the use of the recombinant catalytic CKII subunits, we confirmed by 2D analysis that the purified recombinant CKII α and α′ subunits phosphorylated the same MCP polypeptides as those observed with the copurifying CKII holoenzyme (data not shown).

If the identification of the MCP polypeptides phosphorylated by CKII was correct, one would predict that the purified recombinant C9 and C8 polypeptides should be substrates of CKII. Direct in vitro phosphorylation experiments showed that the C9 and C8 recombinant proteins were phosphorylated by CKII α and α′ catalytic subunits (no phosphorylation observed without the kinase) and by 2D analysis the phosphorylated recombinant subunits comigrate with the 29 kDa MCP phosphorylated polypeptides indicated as b (C9) and c (C8) in Figures 1 and 2; phosphorylation takes place in serine residues (data not shown). To study the stoichiometry of phosphorylation of the MCP complex and of the purified recombinant C9 and C8 subunits, we performed several experiments with different amounts of the recombinant CKII catalytic subunits and the protein substrates. Figure 3 shows a summary of the stoichiometry of

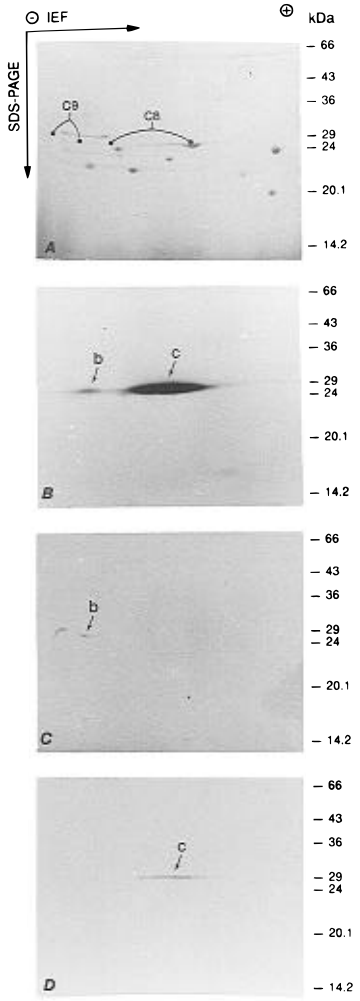

FIGURE 2: 2D-gel analysis of in vitro-phosphorylated MCP and immunoblotting with anti-C9 and -C8 subunit specific antibodies. MCP was phosphorylated in vitro by the copurifying CKII and subjected to 2D-gel analysis. Panel A shows the polypeptide pattern of a 2D-gel blot stained with Ponceau red. We have indicated the regions where the C9 and C8 polypeptides are located. Panel B shows the autoradiogram of a similar 2D-gel blot. Panels C and D show immunoblots with anti-C9 and -C8 specific antibodies of similar 2D-gel analyses, respectively. The alignment of the spots of the immunoblots with the phosphorylated polypeptides was done by X-ray film exposure of the nitrocellulose filters after development with each of the antibodies.

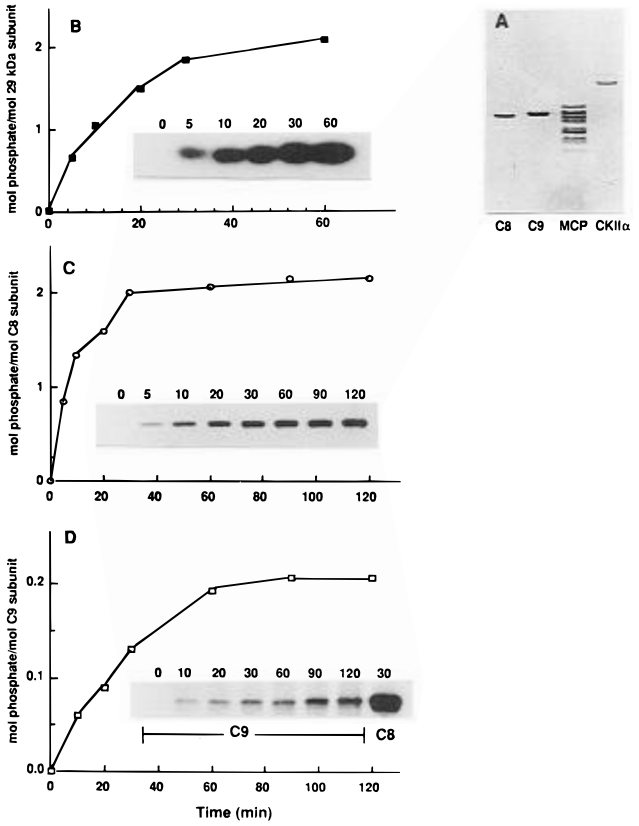

FIGURE 3: Stoichiometry of phosphorylation of purified rat liver MCP and recombinant C8 and C9 subunits by the recombinant CKII α subunit. Panel A shows a 13% SDS−PAGE stained with Coomassie blue of the purified recombinant CKII α (37 kDa), C9 (29 kDa), and C8 (28.5−29 kDa) subunits, together with the rat liver MCP (21−32 kDa) used for the in vitro phosphorylation reactions. Panels B−D show the stoichiometry of phosphorylation for the MCP complex and the C8 and C9 recombinant proteins, respectively. Insets in each panel show the corresponding region of the autoradiograms (incubation times indicated on the top of each lane); in panel D, an extra lane with C8 subunit allows comparison with C9 subunit phosphorylation.

phosphorylation under optimal conditions. Figure 3A shows an SDS−PAGE stained with Coomassie blue of the recombinant proteins and the MCP complex used in these experiments (similar results obtained with the CKII α′ subunit, data not shown). The stoichiometry of phosphorylation of the MCP complex by the CKII α catalytic subunit gives an estimate of 2−2.5 mol of ^{32}P incorporated per mole of 29 kDa subunit (Figure 3B), and similar results were obtained with the copurifying CKII holoenzyme (data not shown). The C8 recombinant subunit gave a maximum stoichiometry of 2 mol of phosphate per mole of C8 subunit (Figure 3C). The C9 recombinant subunit shows a stoichiometry of 0.2 mol of phosphate per mole (Figure 3D). The apparent low stoichiometry of phosphorylation of C9 recombinant subunit (see Figure 3D; the inset shows the C8 phosphorylation in the same autoradiogram for comparison with C9 phosphorylation) could be due to imperfect renaturation of the subunit after urea treatment, but this low stoichiometry is also observed in the in vitro phosphorylation of MCP (even after treatment of the MCP with alkaline phosphatase prior to phosphorylation) and in the in vivo experiments (Figure 1). Direct counting shows, both in vivo and in vitro, that 2−5% of the total radioactivity incorporated in the 29 kDa region corresponds to the phosphorylated C9 polypeptide and 95−98% to the phosphorylated C8 subunit.

The C8 Subunit Is the Main in Vivo-Phosphorylated Polypeptide of the MCP Complex. Experiments presented in Figure 1 show the coincidence in molecular mass and p*I* values of the in vivo and in vitro 29 kDa MCP phosphorylated polypeptides. To further demonstrate that the C8 polypeptide is the main in vivo MCP phosphorylated subunit and to compare the in vivo- vs the in vitro-phosphorylated sites, we took the 29 kDa phosphorylated proteins, from the immunoprecipitates of ^{32}P-labeled cells and from the recombinant C8 protein phosphorylated in vitro by recombinant CKII α subunit, and subjected them to endoGlu-C proteolytic digestion according to Cleveland et al. (1977). Results presented in Figure 4 show that the phosphorylated proteolytic fragments generated from the in vivo-labeled 29 kDa band (mainly derived from the C8-labeled polypeptide, see above) and from the in vitro recombinant C8-phosphorylated polypeptide are identical.

Identification of the Phosphorylation Sites of the C8 Subunit by CKII. The C8 subunit (rat, mouse, and human)

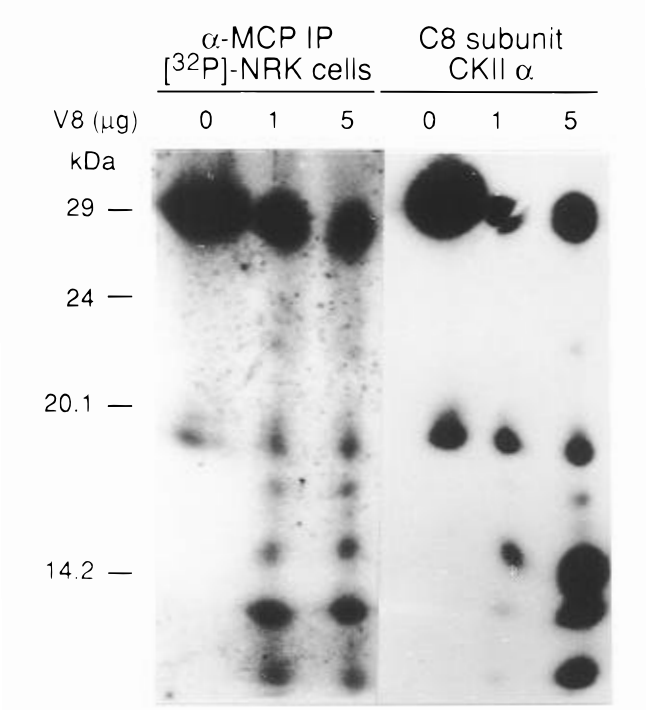

FIGURE 4: EndoGlu-C cleavage of the in vivo-phosphorylated 29 kDa MCP subunit and C8 subunit phosphorylated in vitro by CKII α. Crude extracts from NRK cells labeled with [32P]orthophosphate were immunoprecipitated with anti-MCP antibody, and the purified recombinant mouse C8 subunit was phosphorylated in vitro by recombinant CKII α. The gel pieces containing the phosphorylated proteins were subjected to digestion with Glu-C protease (amounts indicated on the top of each lane), resolved by 18% SDS−PAGE, and exposed to X-ray film for autoradiography as described in Materials and Methods.

contains 21 serine residues, and 4 of them are within consensus sequences for CKII phosphorylation. To approach the mapping of the phosphorylation sites of the C8 subunit, we made several deletion constructs on the pTHC8 expression vector. The human C8 cDNA was more convenient in these studies because it has three internal *Hin*dIII sites (mouse and rat C8 cDNA have only two) and a unique *Eco*RV site (not present in the rat and mouse C8 cDNAs). Figure 5 shows that removal of the COOH terminal portion of the HC8 cDNA (construct HC8.1) completely abolishes the phosphorylation by CKII of the resulting HC8.1 recombinant protein, and in-frame ligation of the coding sequence for this COOH terminal to the smallest HC8 deletion construct, HC8.3, restores the phosphorylation of the resulting protein, HC8.6, by casein kinase II. Two serine residues in the C8 COOH terminal sequence, Ser-243 and Ser-250, are within the consensus sequence for CKII phosphorylation. To prove that those serine residues were the sites of phosphorylation, we generated two C8 full-length mutants (by cassette substitution as described in Materials and Methods): one where Ser-243 was substituted for alanine and another one where both Ser-243 and Ser-250 were substituted for alanine. Figure 6 shows the dose-dependent phosphorylation by CKII of purified recombinant C8, C8 S243A, and C8 S243,250A. Both HC8 and HC8 S243A are phosphorylated by CKII, while the stoichiometry of phosphorylation of HC8 S243A is 1 mol of phosphate per mole of subunit (data not shown), instead of 2. The double mutant HC8 S243,250A is not phosphorylated at all by CKII, clearly demonstrating that Ser-243 and Ser-250 are the phosphorylation sites of the C8 subunit of the proteasome by CKII.

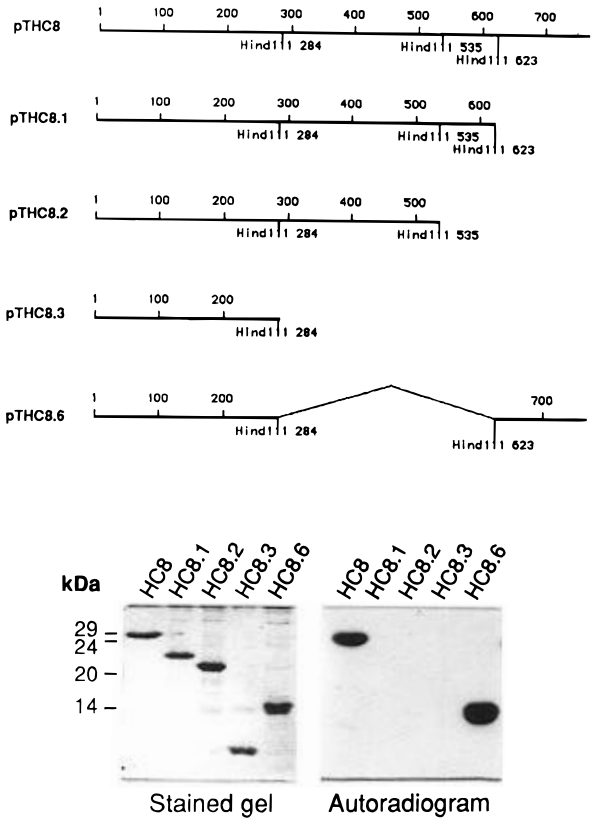

FIGURE 5: Mapping of the C8 phosphorylation sites by CKII. The schemes show the different HC8 constructs generated by deletions of *Hin*dIII fragments (pTHC8.1, C8.2, C8.3) from the original pTHC8 construct and the in-frame ligation that generates the construct pTHC8.6. The two panels presented show the following: on the left, the Coomassie blue-stained gel of the recombinant proteins in a phosphorylation reaction with recombinant CKII α and, on the right, the autoradiogram of the same gel. For experimental details, see Materials and Methods.

DISCUSSION

The present studies show that MCP is phosphorylated in vivo in NRK cells under basal conditions. Similar to the data reported with the human erythrocyte enzyme (Ludemann et al., 1993), a CKII activity copurifies with rat liver MCP complex and phosphorylates two subunits of 29 kDa, now identified as the C8 and C9 components of the MCP. Experiments with the purified human erythrocyte and the *Xenopus laevis* ovaries MCP (Arribas et al., 1991) show the phosphorylation of the human C8 and C9 components by CKII and the phosphorylation of a 29 kDa subunit in the MCP from *X. laevis* (likely to be the C8 component, see below). Both in vivo and in vitro, 95−98% of the radioactive phosphate is incorporated into the C8 subunit and the patterns of phosphopeptides obtained by endoGlu-C protease digestion are identical. These results strongly suggest that CKII is likely to be the enzyme responsible for the phosphorylation of the C8 polypeptide in vivo. By previous experiments of 2D-gel separation of MCP subunits and immunoblotting with antisubunit specific antibodies, we found (Arribas et al., 1994) that the C2 subunit (32 kDa) resolved into one or up to three isoelectric variants, as expected from the results described by Weltman and Etlinger (1992). The 32 kDa phosphorylated protein observed in the 2D analysis of the immunoprecipitates of 32P-labeled cell extracts moves as the most acidic spot of the C2 component of the MCP. CKII is unlikely to be responsible for the C2

Table 1: Comparison of the Sequence of the C8 COOH Terminal Region in Different Species[a]

species	initial amino acid	COOH terminal sequence	final amino acid
human C8	233	REEAEKYAKESLKEEDESDDDNM	255
mouse C8	233	REEAEKYAKESLKEEDESDDDNM	255
rat C8	233	REEAEKYAKESLKEEDESDDDNM	255
X. laevis C8	233	REEAEKYAKESLEEEDDSDDDNM	255
Saccharomyces cerevisiae YC1/PRS1	197	INGDDDQDEDDSDNVMSSDDENA	219

[a] The Ser residues underlined are the potential sites of phosphorylation by CKII in the C8 subunit from different species, on the basis of the mutational analysis of the human C8 subunit. References for the sequences: human (Tamura et al., 1991), rat (Tanaka et al., 1990), mouse (present report), *X. laevis* (Fujii et al., 1993), yeast YC1/PRS1 (Fujiwara et al., 1990).

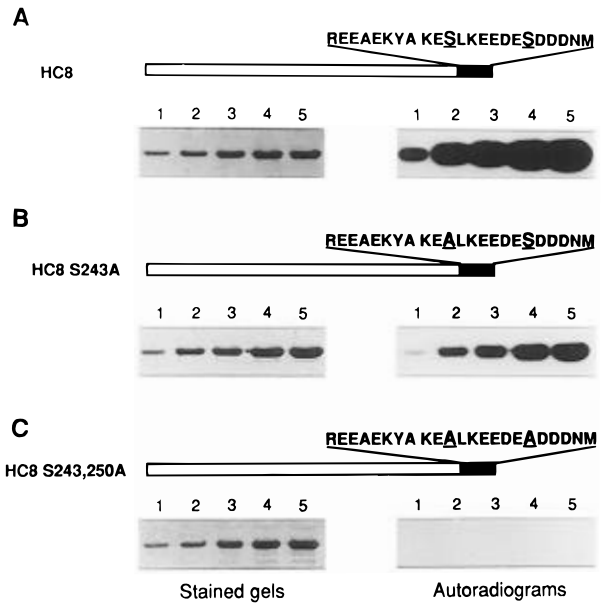

FIGURE 6: Point mutation analysis of the human C8 phosphorylation sites by casein kinase II. Each panel (A−C) shows the amino acid sequence of the COOH terminal of the corresponding C8 full-length recombinant protein. The left side shows the region of the Coomassie blue-stained gels used to analyze the products of phosphorylation reactions with a fixed amount (0.4 μg) of CKII α subunit and different amounts (0.2, 0.4, 0.8, 1, and 2 μg, labeled 1−5, respectively) of the respective recombinant C8 subunit, and on the right are the corresponding autoradiograms. For experimental details, see Materials and Methods.

phosphorylation because the CKII holoenzyme copurifying with the MCP complex and the CKII α and α' catalytic subunits is not able to phosphorylate either the C2 component within the MCP complex (even after treatment of MCP with alkaline phosphatase) or the purified recombinant C2 subunit.

In vitro, the C8 recombinant subunit is a good substrate for CKII with a K_m of 0.5−1 μM and a V_{max} of 60−100 nmol min^{-1} (mg of protein)$^{-1}$ using ATP as donor; these values are similar to those of DNA topoisomerase II (Ackerman et al., 1985), a well-studied substrate of CKII. The maximum stoichiometry of phosphorylation of MCP complex in vitro is around 2.0−2.5 mol of phosphate per mole of 29 kDa subunit, very close to the sum of stoichiometries of the purified recombinant C9 and C8 subunits by CKII, suggesting that the phosphorylation sites of both subunits are equally accessible to the kinase in the MCP complex and in the free recombinant subunits. This point is clearly demonstrated for the C8 subunit because the two phosphorylated serine residues, Ser-243 and Ser-250, are in the COOH terminal of the C8 subunit. The sequence around the Ser residues phosphorylated by CKII in the C8 subunit (Table 1) shows full conservation in the *X. laevis* (Fujii et al., 1993), mouse (present report), rat (Tanaka et al., 1990), and human (Tamura et al., 1991). A similar sequence is also present in the C8 homologue of yeast, YC1/PRS1 (Fujiwara et al., 1990). Furthermore, this C-terminal region of C8 can be predicted as a highly acidic antenna exposed to the outer part of the MCP cylinder, on the basis of the location of the C-terminal in the crystal structure of the proteasome α-type subunit from *Thermosplasma acidophilum* (Lowe et al., 1995).

CK II is a ubiquitous protein serine/threonine kinase found in the nucleus and in the cytoplasm of eukaryotic cells, and many proteins, with a great diversity in cellular distribution and function, are substrates for this kinase (Pinna, 1990; Tuazon & Traugh, 1991). A frequent feature of CKII phosphorylations is that they are apparently "silent", in the sense that they do not cause a significant change in the activity of the proteins after phosphorylation (Pinna, 1990). This seems to be the case for MCP, because the phosphorylation by CKII does not alter significantly its proteolytic activity (either against synthetic fluorogenic peptides or against proteins), in agreement with the observations reported with the human erythrocyte MCP (Ludemann et al., 1993). Also, we detect no change in the peptidase activities of the MCP complex after alkaline phosphatase treatment, and only a modest 2-fold activation of the protease activity toward myelin basic protein was observed. Similarly, phosphorylation or dephosphorylation has no significant effect on the activity of the purified active form of the MCP complex, obtained as described (Arribas et al., 1994). All these data led us to suggest that phosphorylation by CKII in the C8 COOH terminal, a likely outside exposed region of the C8 subunit in the compact cylinder structure of the MCP complex, could participate in the control of MCP interaction with other cellular proteins. Two examples of these interactions could be the formation of the 26S complex (Goldberg & Rock, 1992) and/or the interaction with the nuclear translocation machinery. In the latter case, CKII-phosphorylated sites of the SV40 T-antigen have been reported to be essential for its nuclear targeting (Rihs et al., 1991). Further studies in those directions may clarify the role of CKII phosphorylation in the regulation of the MCP complex.

ACKNOWLEDGMENT

We thank Dr. F. J. Lozeman for providing the human CKII α and α' cDNAs, Dr. J. F. Santaren for help with 2D-gel analysis, C. Gutiérrez for help in handling the rabbits for antibody production, J. Oliva for technical assistance, and A. Fernández for the photographic work of this paper.

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BI952540S