Protein Kinase CK1α Regulates mRNA Binding by Heterogeneous Nuclear Ribonucleoprotein C in Response to Physiologic Levels of Hydrogen Peroxide*  

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At low concentrations, hydrogen peroxide (H\textsubscript{2}O\textsubscript{2}) is a positive endogenous regulator of mammalian cell proliferation and survival; however, the signal transduction pathways involved in these processes are poorly understood. In primary human endothelial cells, low concentrations of H\textsubscript{2}O\textsubscript{2} stimulated the rapid phosphorylation of the acidic C-terminal domain (ACD) of heterogeneous nuclear ribonucleoprotein C (hnRNP-C), a nuclear restricted pre-mRNA-binding protein, at Ser\textsuperscript{240} and at Ser\textsuperscript{Ser225–Ser228}. A kinase activity was identified in mouse liver that phosphorylates the ACD of hnRNP-C at Ser\textsuperscript{240} and at two sites at Ser\textsuperscript{Ser225–Ser228}. The kinase was purified and identified by tandem mass spectrometry as protein kinase CK1α (formerly casein kinase 1α). Protein kinase CK1α immunoprecipitated from primary human endothelial cell nuclei also phosphorylated the ACD of hnRNP-C at these positions. Pretreatment of endothelial cells with the protein kinase CK1-specific inhibitor IC261 prevented the H\textsubscript{2}O\textsubscript{2}-stimulated phosphorylation of hnRNP-C. Utilizing phosphoserine-mimicking Ser-to-Glu point mutations, the effects of phosphorylation on hnRNP-C function were investigated by quantitative equilibrium fluorescence RNA binding analyses. Wild-type hnRNP-C1 and hnRNP-C1 modified at the basal sites of phosphorylation (S247E and S286E) both avidly bound RNA with similar binding constants. In contrast, hnRNP-C1 that was also modified at the CK1α phosphorylation sites exhibited a 14–500-fold decrease in binding affinity, demonstrating that CK1α-mediated phosphorylation modulates the mRNA binding ability of hnRNP-C.

At low concentrations, hydrogen peroxide (H\textsubscript{2}O\textsubscript{2}) functions as an endogenous positive modulator of mammalian cell proliferation and cell survival (reviewed in Refs. 1–3). Application of low concentrations (<10 \textmu m) of H\textsubscript{2}O\textsubscript{2} to cultured mammalian cells stimulates cell growth and enhances survival, whereas the application of catalase inhibits proliferation and stimulates cell death. In mammalian systems, H\textsubscript{2}O\textsubscript{2} is generated by plasma membrane NADPH oxidases in response to growth factor stimulation (4, 5). Many of the downstream effects of growth factors are in fact inhibited by treatment of the cells with catalase. In contrast to the effects of low concentrations of H\textsubscript{2}O\textsubscript{2}, the application of higher concentrations (>20 \textmu m) of H\textsubscript{2}O\textsubscript{2} to cultured cells results in oxidative stress and generally inhibits cell proliferation and stimulates cell death. Although several stress-responsive pathways have been implicated in the response of mammalian cells to oxidative stress, the biochemical mechanisms by which low concentrations of H\textsubscript{2}O\textsubscript{2} stimulate cell proliferation and enhance survival are poorly understood (6).

Previously, a functional proteomic analysis demonstrated that low concentrations of H\textsubscript{2}O\textsubscript{2} stimulate the rapid phosphorylation of heterogeneous nuclear ribonucleoprotein (hnRNP)\textsuperscript{1} C1/C2 in human endothelial cells (7), hnRNP-C1/C2 is a nuclear pre-mRNA-binding protein that appears to regulate pre-mRNA processing (8, 9). Deletion of the gene for hnRNP-C1/C2 in the mouse is lethal, with developmental arrest at the egg cylinder stage (10). Murine stem cells lacking hnRNP-C1/C2 are viable, but show impaired survival and decreased rates of proliferation and differentiation. Interestingly, heterogeneous expression of the gene for hnRNP-C1/C2 in yeast cells, which normally lack this gene, is also lethal (11). In this latter case, it appears that heterologously expressed hnRNP-C1/C2 translocates to the yeast nucleus and binds mRNA. However, yeast cells appear to lack the ability to stimulate the release of hnRNP-C1/C2 from the mRNA, resulting in inhibition of mRNA export from the nucleus.

Structurally, hnRNP-C1/C2 isolated from HeLa cells is a heterotetramer (C1\textsubscript{4}C2\textsubscript{4}) in which C1 and C2 are splice variants of the same gene, differing by the presence of an additional 13 amino acids in C2 (12, 13). Each hnRNP-C subunit has four functional domains. There is an N-terminal RNA recognition motif (residues 8–87), followed by a basic high affinity RNA-binding domain (residues 140–179), which may be responsible for much of the affinity of the protein for RNA (14, 15). Next is a leucine zipper (residues 180–207), which mediates subunit interactions in the heterotetramer (16). Finally, there is an acidic C-terminal domain (ACD; residues 208–290), which is the major site of phosphorylation in the protein (17). Previous tandem mass spectrometry phosphate mapping studies utilizing endogenously phosphorylated protein from human endothelial cells revealed that, under basal conditions, hnRNP-C1/C2 is phosphorylated at Ser\textsuperscript{247} and Ser\textsuperscript{286} (17). Low

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1 The abbreviations used are: hnRNP, heterogeneous nuclear ribonucleoprotein; ACD, acidic C-terminal domain; GST, glutathione S-transferase; pDTT, dithiothreitol; HUVEC, human umbilical vein endothelial cell; IEF, isoelectric focusing; CHAPS, 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonic acid; HPLC, high pressure liquid chromatography.
concentrations of H₂O₂ stimulate phosphorylation at Ser²⁴⁰ and at a series of 4 contiguous serine residues at positions 225–228.

Previous studies have implicated protein kinase CK2 (formerly casein kinase 2) as phosphorylating Ser²⁴⁰, one of the basal sites of phosphorylation (17). However, the kinase responsible for the H₂O₂-stimulated phosphorylation has been unclear, as has the effect of this phosphorylation on the function of hnRNP-C. Here, we report that protein kinase CK1α (formerly casein kinase 1α), a kinase implicated in regulating cell cycle progression and pre-mRNA processing (18–20), mediates the H₂O₂-stimulated phosphorylation of hnRNP-C. Furthermore, we show that this CK1α-mediated phosphorylation modulates the RNA binding activity of hnRNP-C.

**Experimental Procedures**

**Expression and Purification of hnRNP-C ACDs**—A DNA segment containing residues 217–293 of hnRNP-C1 was amplified from a nearly full-length clone of hnRNP-C1 (Invitrogen) using the PCR. The 1.2-kb fragment was ligated into the BamHI site of the expression vector pGEX-2T (Amersham Biosciences). Casein kinase CK1α was expressed in Escherichia coli BL21 cells and purified as described previously (7). The kinase was then affinity-purified using a glutathione-Sepharose 4B column (Amersham Biosciences). The purified CK1α was incubated with 5 μM ATP and 5 μM MgCl₂ for 30 min at 37 °C and then centrifuged to remove ATP. The kinase was then used to phosphorylate the ACDs in vitro.

**Endothelial Cell Culture**—Human umbilical vein endothelial cells (HUVECs) were purchased from Cambrex and cultured as described previously (7). HUVECs were grown in 10-cm dishes at passage 3 in M199 medium (Cambrex) containing 10% fetal calf serum, 100 μg/ml heparin (Sigma), and 50 μg/ml endothelial cell growth supplement (Sigma). After reaching confluence, confluent cultures were treated with 5 μM H₂O₂, 100 μM 5,6-dichloro-2'-deoxyuridine (IC261, Calbiochem) for 3 h prior to H₂O₂ stimulation. After treatment, the cells were washed twice with ice-cold phosphate-buffered saline; harvested by scraping in 10 mM Tris, 140 mM NaCl, and 1 mM EDTA, pH 8.0; and pelleted by spinning at 500 × g for 15 min. The nuclear pellets were resuspended in 10 mM Hepes, 750 μM spermidine, 150 μM spermine, 50 μM NaF, 1 mM sodium orthovanadate, 2 mM EDTA, 5 mM DTT, and 1× AKH-200; incubated on ice for 10 min; and then centrifuged at 16,000 × g for 5 min. The supernatant was removed, and the nuclear pellet was washed once with 0.8 μl of Buffer A.

**Immunoprecipitation of Protein Kinase CK1α**—HUVEC nuclei were lysed by application of 0.5 ml of 10 mM Tris and 1× NaCl, pH 7.4, with 1 mM EDTA for 20 min at 4 °C. The nuclear extracts were incubated with 40 μl of agarose-conjugated anti-CK1α (Roche Applied Science) at 4 °C for 2 h, followed by one wash with 1 ml of 10 mM Tris, pH 7.5. The final washed immunocomplexes were then incubated with 5 ml of polymerase chain reaction (PCR) buffer containing 5 mM MgCl₂, 10 μM NaNO₃, and 0.2 mM ADP and centrifuged at 4000 × g for 5 min. The resulting supernatant was then applied to a Bio-Spin 6 chromatography column (Bio-Rad) equilibrated with IEF sample buffer, followed by electrophoresis on a 4–20% polyacrylamide gel. After exposure, film was developed using an Eastman Kodak X-Omat processor.

**Protein Kinase CK1α Regulation of hnRNP-C**

In *In Vitro* Phosphorylation Assays—Samples were assayed for hnRNP-C kinase activity by incubating aliquots with 5 μg of purified recombinant ACD-2E or ACD-3E in 10 mM Tris, pH 7.5, containing 5 mM DTT, 5 mM MgCl₂, 100 μM ATP, and 5 μCi of [γ-³²P]ATP (PerkinElmer Life Sciences) at 37 °C in a reaction volume of 50 μl. Reactions were quenched after 1 h by the addition of SDS-PAGE sample buffer containing 5% (w/v) acrylamide (Bio-Rad) containing 1× phosphatase inhibitor mixture (one tablet/50 ml) using a food processor. All subsequent steps were at 4 °C. Protein phosphorylation was measured by autoradiography of the phosphorylated products. The phosphorylated bands were cut out of the autoradiographs, and the proteins were eluted by incubating the resin for 16 h at 4 °C with 5 ml of 50 mM Tris, 5 mM EDTA, and 2 mM DTT, pH 7.4. The sample was then diluted to 100 μl with centrifugation at 3000 × g with a Centricon YM-3 centrifugal concentrating device (Amicon, Inc.). The sample was then submitted to SDS-PAGE, and the entire sample lane was analyzed by tandem mass spectrometry following tryptic in-gel digestion (see below).

In *In Vivo* Phosphorylation Assays—Samples were assayed for hnRNP-C kinase activity by incubating aliquots with 5 μg of purified recombinant ACD-2E or ACD-3E in 10 mM Tris, pH 7.5, containing 5 mM DTT, 5 mM MgCl₂, 100 μM ATP, and 5 μCi of [γ-³²P]ATP (PerkinElmer Life Sciences) at 37 °C in a reaction volume of 50 μl. Reactions were quenched after 1 h by the addition of SDS-PAGE sample buffer containing 5% (w/v) acrylamide (Bio-Rad) containing 1× phosphatase inhibitor mixture (one tablet/50 ml) using a food processor. All subsequent steps were at 4 °C. Protein phosphorylation was measured by autoradiography of the phosphorylated products.
Tandem Mass Spectrometry—Gel portions were subjected to tryptic in-gel digestion by liquid chromatography-tandem mass spectrometry analysis of the extracted peptides as described previously (17). Specifically, excised gel portions were cut into ∼1-mm³ pieces. Gel pieces were washed and dehydrated with acetonitrile for 10 min, followed by removal of acetonitrile. Pieces were then completely dried in a SpeedVac. Rehydration of the gel pieces was with 50 mM ammonium bicarbonate solution containing 12.5 ng/µl modified sequencing grade trypsin (Promega) at 4 °C. After 45 min, the excess trypsin solution was removed and replaced with 50 mM ammonium bicarbonate solution to just cover the gel pieces. The samples were then placed overnight in a 37 °C room. Peptides were later extracted by removing the ammonium bicarbonate solution, followed by two washes, for 20 min each, with a solution containing 50% acetonitrile and 5% formic acid. The extracts were then dried in a SpeedVac (~1 h). The samples were stored at 4 °C until analyzed.

On the day of analysis, the samples were reconstituted in 5 µl of HPLC solvent A (5% acetonitrile, 0.005% heptfluorobutyric acid, and 0.4% acetic acid). A nanoscale reverse-phase HPLC capillary column was created by packing 5-µm C₁₈ spherical silica beads into a fused silica capillary (75-µm inner diameter × 12-cm length) with a flame-dried tip. After equilibrating the column, each sample was pressure-loaded offline onto the column. The column was then reattached to the HPLC system. A gradient was formed, and peptides were eluted with increasing concentrations of HPLC solvent B (95% acetonitrile, 0.005% heptfluorobutyric acid, and 0.4% acetic acid).

As the peptides were eluted, they were subjected to electrospray ionization, at which time they entered into either an LCQ DECA ion trap mass spectrometer or an LTQ linear ion trap mass spectrometer (ThermoFinnigan, San Jose, CA). Eluting peptides were detected, isolated, and fragmented to produce a tandem mass spectrum of specific fragment ions for each peptide. Sites of phosphorylation were determined by matching the protein sequence with the acquired fragmentation pattern using the software program Sequest (ThermoFinnigan) by allowing for a modification comprising an additional 80 Da on Ser, Thr, or Tyr.

Purification of Full-length hnRNP-C Tetramers—A DNA segment containing full-length hnRNP-C1 (residues 1–293) (10) was amplified from a nearly full-length clone of hnRNP-C1 using the following primers: 5'-TTCATGGATCCGGATCCTGTGTCGAAAGCTTTTGGG-3' and 5'-AGCTCGAGTCAGCGGACAGTCAAGAGTCCTGTG-3'. The PCR product was inserted into the BamHI sites of pET-3b (Novagen). Automated dyeodeoxynucleotide site-directed mutagenesis kit to generate the S247E,S286E double mutant containing Ser-to-Glu mutations at the two sites shown to be phosphorylated endogenously in endothelial cells in response to low levels of H₂O₂ (17).

RESULTS

Purification of an hnRNP-C Kinase from Mouse Liver—The purified recombinant hnRNP-C ACD was used as a substrate to identify hnRNP-C kinase activities in mouse liver extracts. Previously, the purified ACD was shown to be phosphorylated by protein kinase CK2 at the position corresponding to Ser²⁴⁷, but was not phosphorylated by multiple other kinases, including protein kinase A and multiple isoforms of protein kinase C. A double mutant containing Ser-to-Glu mutations at the two basal sites of phosphorylation (S247E,S286E, ACD-2E) was utilized to identify kinases capable of phosphorylating the H₂O₂-stimulated sites of phosphorylation. Such an activity was identified in mouse liver extracts, and the kinase was isolated using red-agarose affinity chromatography, ion exchange chromatography, and an affinity column consisting of the GST-ACD-2E fusion protein immobilized on glutathione-Sepharose (Fig. 1A). This kinase activity readily phosphorylated the ACD-2E protein and also the ACD-3E protein, although to a lesser extent. The ACD-3E protein contained a third mutation (S240E) at one of the H₂O₂-stimulated sites of phosphorylation.

Tandem mass spectral analyses demonstrated that the kinase phosphorylated ACD-2E at the position corresponding to Ser²⁴⁰ in full-length hnRNP-C and phosphorylated twice the stretch of 4 contiguous Ser residues corresponding to Ser²⁴⁵–Ser²⁴⁸ (Fig. 1, B and C). The high susceptibility of this second phosphophosphate to H₂PO₄ loss during acquisition of collision-induced dissociation spectra (24) prevented precise localization of these two phosphates. However, the two phosphates were observed at Ser²⁴⁵–Ser²⁴⁸. Thus, hnRNP-C kinase isolated from mouse liver phosphorylates hnRNP-C at precisely the same locations as shown to be phosphorylated endogenously in endothelial cells in response to low levels of H₂O₂ (17).
Protein Kinase CK1α Is an hnRNP-C Kinase—The partially purified protein kinase from mouse liver was subjected to liquid chromatography-tandem mass spectrometry to identify all of the proteins present. The only protein kinase identified was murine protein kinase CK1α (Fig. 2). The kinase was identified by the presence of two tryptic peptides, YASINAHLGIEQSR and ILQGGVGIPHIR. The analysis also revealed the presence of murine centaurin-α2 (data not shown), consistent with the previous observation that CK1α co-purifies with members of the centaurin-α family of proteins (25).

Protein kinase CK1α family members are most frequently noted to phosphorylate Ser or Thr located 3–4 residues downstream of a phospho-Ser or phospho-Thr residue (26). Although a single Asp or Glu residue at this upstream position is often insufficient to direct CK1 phosphorylation, CK1α is also able to phosphorylate Ser residues 3 residues downstream of a stretch of multiple acidic residues without prior phosphorylation (27, 28). In hnRNPC-1, the 3 consecutive Glu residues at positions 221–223 likely direct the initial CK1α-mediated phosphorylation at Ser225. The resulting phospho-Ser225 then likely directs a second CK1α phosphorylation at Ser228. It has also been recently reported that a cluster of negatively charged residues 6–13 residues downstream of a Ser residue may direct CK1 phosphorylation at that site (29).

Protein Kinase CK1α Regulation of hnRNP-C

FIG. 1. Purification of an hnRNP-C kinase from mouse liver. A, an hnRNP-C kinase activity from mouse liver purified by red-agarose and anion exchange chromatography and immobilized on GST-ACD-2E-conjugated glutathione-Sepharose was assayed by 32P autoradiography. Lane 1, the purified wild-type ACD phosphorylated in vitro with protein kinase CK2; lanes 2–4, the purified kinase from mouse liver assayed with no added substrate or with the addition of purified ACD-2E or purified ACD-3E, respectively; lanes 5–7, negative controls lacking the purified kinase. The arrow indicates the phosphorylated ACD. The arrowhead indicates the phosphorylated GST-ACD-2E fusion protein used in the final affinity chromatography step. B, the tandem mass spectrum of a +2 charged m/z 1411.3 peptide from the ACD-2E substrate showing an additional 80 Da (consistent with phosphorylation) on the Ser residue corresponding to Ser240 in full-length hnRNP-C1 is presented. The intense signal at m/z 1362.5 is consistent with loss of a 98-Da H3PO4 moiety from the doubly charged parent ion. Note that the Glu residue at position 10 in the peptide (corresponding to position 247 in full-length hnRNP-C) was mutated from Ser in this construct. C, the tandem mass spectrum of a +2 charged m/z 1336.7 peptide from the ACD-2E substrate showing an additional 160 Da (consistent with double phosphorylation) on the stretch of 4 contiguous Ser residues corresponding to Ser225–Ser228 in full-length hnRNP-C1 is presented. The intense signals at m/z 1288.0 and 1239.1 are consistent with loss of one and two 98-Da H3PO4 moieties, respectively, from the doubly charged parent ion. Note that the inset of the spectrum is scaled by x/25. Doubly (+2) charged fragment ions are indicated by a superscript 2.
H$_3$PO$_4$ during the acquisition of the collision-induced dissociation spectra. Thus, CK1$\alpha$ immunopurified from endothelial nuclei phosphorylates hnRNP-C at the precise locations where phosphorylation is stimulated by low levels of H$_2$O$_2$ in cultured endothelial cells (17). The immunopurified kinase activity from HUVEC nuclei was not altered by prior treatment of the cells with low concentrations of H$_2$O$_2$ (data not shown), suggesting that the kinase is purified in the fully active form.

**Inhibition of Protein Kinase CK1$\alpha$ Prevents H$_2$O$_2$-stimulated Phosphorylation of hnRNP-C**—To assess the role of protein kinase CK1$\alpha$ in phosphorylating hnRNP-C in cells, the effects of a CK1 family-specific inhibitor (IC261) (30) were assessed. As shown previously (7, 17), hnRNP-C was present in confluent endothelial cells predominantly in the biphosphorylated form (pI 5.05) and, to a lesser extent, in the triphosphorylated form (pI 5.00). Treatment of the cells with 5 $\mu$M H$_2$O$_2$ for 20 min enhanced the formation of hyperphosphorylated forms containing four to five phosphates/subunit at pI 4.90–4.95 (Fig. 4). However, in the presence of the CK1 inhibitor, the addition of H$_2$O$_2$ had no effect on the phosphorylation pattern of endogenous hnRNP-C1/C2. In addition, even in the absence of added H$_2$O$_2$, there was a clear reduction in the triphosphorylated form of the protein in the presence of the CK1 inhibitor. This effect was very similar to that observed previously by treating endothelial cells with catalase (7), suggesting the presence of a tonic H$_2$O$_2$-stimulated CK1$\alpha$ activity in the nuclei of confluent endothelial cells. As IC261 shows specificity for all CK1 family members (30), phosphorylation of hnRNP-C by other CK1 family members in addition to CK1$\alpha$ cannot be ruled out. However, the purification of CK1$\alpha$ using an hnRNP-C ACD affinity column described here and the previously reported localization of CK1$\alpha$ at sites of pre-mRNA processing (18) strongly implicate CK1$\alpha$ as the kinase mediating the H$_2$O$_2$-stimulated phosphorylation of hnRNP-C. It is important to note that whereas CK1$\alpha$ appears to localize to nuclear speckles, hnRNP-C is present diffusely throughout the nucleoplasm without specific localization to nuclear speckles (31, 32).

**Protein Kinase CK1$\alpha$ Phosphorylation Sites in hnRNP-C Are Evolutionarily Conserved**—To gain insight into the evolutionary conservation of the CK1$\alpha$ phosphorylation sites in hnRNP-C1/C2, the protein sequences from several vertebrate species were aligned using ClustalW (Fig. 5). All of the phosphorylation sites in hnRNP-C are highly conserved. The basal site of phosphorylation at Ser$^{286}$ is invariant, being present in fish, amphibians, and mammals. The CK2 basal phosphorylation site at Ser$^{247}$ is nearly invariant, substituted with Tyr in fish. The CK1$\alpha$ phosphorylation site at Ser$^{240}$ is also nearly invariant. Interestingly, this site contains a Glu residue in frogs, perhaps indicating constitutive phosphoserine effects at this position in this species. The CK1$\alpha$ phosphorylation sites at Ser$^{225}$–Ser$^{228}$ are also highly conserved. Whereas Ser$^{225}$ is invariant, Ser$^{226}$ and Ser$^{228}$ are highly conserved, being present in all of the mammals and amphibians. In contrast, Ser$^{227}$ is the least conserved Ser residue in this region, present only in humans and rabbits. Based on these variable degrees of conservation and on the known tendency of CK1$\alpha$ to phosphorylate Ser residues that have acidic or phosphorylated residues at the n-3 position, it can be concluded that, in humans, Ser$^{225}$ and Ser$^{228}$ are the most likely sites of phosphorylation in this contiguous stretch of 4 Ser residues. The high degree of conservation of the CK1$\alpha$ phosphorylation sites among the different vertebrate species suggests that CK1$\alpha$-mediated phosphorylation of hnRNP-C1/C2 constitutes a signaling pathway widespread among vertebrate species.

**Protein Kinase CK1$\alpha$ Modulates the RNA Binding Activity of hnRNP-C**—To assess the effects of ACD phosphorylation on hnRNP-C function, full-length hnRNP-C tetramers were overexpressed in E. coli and purified to homogeneity using anion exchange, cation exchange, and size exclusion chromatography (Fig. 6A). The presence of phospho-Ser residues was mimicked by formation of Ser-to-Glu point mutations. Previously, mutating a Ser residue to Glu has been shown to mimic the effects of phosphorylation in several proteins, including MEK-1 (mitogen-activated protein kinase/extracellular signal-regulated kinase-1) (33) and protein kinase D (34). The three proteins purified were wild-type hnRNP-C1, hnRNP-C1 with S247E and S286E mutations (2E-hnRNP-C1) to mimic basal phosphorylation, and hnRNP-C1 with S247E, S286E, S240E, S226E, S225E, and S228E point mutations (5E-hnRNP-C1) to mimic the effects of protein kinase CK1$\alpha$ phosphorylation. Upon size exclusion chromatography, all three proteins exhibited an aberrantly high apparent molecular mass of $\sim$360 kDa (data not shown), in good agreement with previous observations with both recombinant hnRNP-C1 tetramers and hnRNP-C tetramers isolated from cells (23). The mRNA binding affinities of
these three purified proteins were assessed by monitoring the enhanced fluorescence of ethenoadenosine-modified poly(A)-labeled RNA as described previously (23). Data were fit by non-linear regression using a previously described model for the binding of large ligands to a homogeneous lattice (22). This model incorporates both an intrinsic affinity constant ($K$) and a cooperativity parameter ($\omega$), with the overall affinity represented by the product ($K_\omega$) and thus an overall dissociation constant represented by $1/K_\omega$. Wild-type hnRNP-C1 tetramers avidly bound the RNA with high affinity and marked positive cooperativity (Fig. 6B and Table I), with binding constants in good agreement with previously reported values (23). The 2E-hnRNP-C1 mutant showed RNA binding constants similar to those shown by the wild-type protein. Thus, the biphosphorylated form of hnRNP-C1/C2, which is the predominant form in mammalian cells, appears to bind mRNA with high affinity and substantial positive cooperativity. In contrast, the 5E-hnRNP-C1 mutant displayed markedly reduced RNA binding affinity. Because of the inability of this protein to saturate the RNA, the binding curve could not be fit with absolute certainty, but a range for the binding and cooperativity constants could be ascertained (Table I). The intrinsic affinity constant for the 5E-hnRNP-C1 mutant was decreased by 10–100-fold compared with those for the wild-type hnRNP-C1 and 2E-hnRNP-C1 proteins. In addition, the cooperativity constant was diminished by 1.4–5-fold. Thus, the overall binding affinity of the 5E-hnRNP-C1 mutant was decreased by 14–500-fold compared with those of...
the wild-type and 2E-hnRNP-C1 proteins. This result indicates that the CK1α-mediated phosphorylation of hnRNP-C1/C2 greatly reduces the mRNA binding affinity of the protein, thus modulating its function.

**DISCUSSION**

Low concentrations of H$_2$O$_2$ serve as a positive regulator of mammalian cell growth and survival (1–3); however, the mechanisms underlying this phenomenon are unclear (6). A previous functional proteomic analysis revealed that primary human endothelial cells respond to low levels of H$_2$O$_2$ by the rapid and reversible phosphorylation of hnRNP-C1/C2, a nuclear restricted pre-mRNA-binding protein (7). Interestingly, the phosphorylation status of this protein is correlated with cell cycle progression, with higher levels of phosphorylation during mitosis compared with interphase (35). The protein is important for normal mammalian cell growth and differentiation, as gene deletion in the mouse is lethal, with the embryos failing to develop beyond the egg cylinder stage (10). In addition, cultured stem cells from these embryos show impaired survival and slowed rates of proliferation and differentiation. Although hnRNP-C1/C2 is clearly involved in pre-mRNA metabolism in the nucleus, the specific function of this protein has remained unclear. It has been proposed that this protein binds most (if not all) mRNA transcripts in the nucleus (23). However, hnRNP-C1/C2 is predominantly restricted to the nucleus with a nuclear retention sequence (36). Thus, this protein must dissociate from processed mRNA transcripts to allow for their export to the cytosol. In fact, heterologous expression of hnRNP-C in yeast is lethal, apparently because the protein enters the nucleus and prevents the export of mRNA transcripts (11). Yeast cells appear to lack the mechanisms to enable the release of hnRNP-C1/C2 from mRNA.

**FIG. 4.** Inhibition of CK1α prevents H$_2$O$_2$-stimulated phosphorylation of hnRNP-C1/C2. Shown are two-dimensional immunoblots for hnRNP-C1/C2 from HUVEC nuclei. In the resting state, the protein was present predominantly in the biphosphorylated form (pI 5.05) and, to a lesser extent, in the triphosphorylated form (pI 5.00). Treatment with 5 μM H$_2$O$_2$ for 20 min stimulated the formation of hyperphosphorylated forms at pI 4.90–4.95. Pretreatment of the cells with 100 μM IC261, a CK1-specific inhibitor, prevented the H$_2$O$_2$-stimulated formation of hyperphosphorylated forms of hnRNP-C and also decreased the amount of the triphosphorylated protein.

**FIG. 5.** Evolutionary conservation of the phosphorylation sites in hnRNP-C. The ACDs from several vertebrate species were aligned using ClustalW. The NCBI Protein Database accession numbers for the sequences utilized were AAH03394 (human), AAC61695 (rabbit), BAB31934 (mouse), XP_214160 (rat), AAH71084 (Xenopus laevis), and AAQ97793 (zebrafish). Invariant residues are in **boldface**. Phosphorylation sites are boxed.

**FIG. 6.** CK1α phosphorylation of hnRNP-C modulates RNA binding. Full-length hnRNP-C1 tetramers were overexpressed in E. coli, purified to homogeneity, and utilized in quantitative equilibrium RNA binding studies. A, representative Coomassie Blue-stained SDS-polyacrylamide gel showing the purification of wild-type hnRNP-C1. Lane 1, molecular mass markers shown in kilodaltons; lane 2, supernatant; lane 3, anion exchange chromatography; lane 4, cation exchange chromatography; lane 5, size exclusion chromatography. B, plot of the enhanced fluorescence of ethenoadenosine-modified poly(A) (1 μM nucleotides) upon binding wild-type hnRNP-C1 (●), 2E-hnRNP-C1 (○), or 5E-hnRNP-C1 (□). The **solid lines** indicate simulations using constants derived from nonlinear regression analysis: K = 1 × 10$^5$ M$^{-1}$ and ω = 500 for wild-type hnRNP-C1 and 2E-hnRNP-C1, and K = 7.5 × 10$^5$ M$^{-1}$ and ω = 210 for 5E-hnRNP-C1.
The plots in Fig. 6B were fit by nonlinear regression using the McGhee-von Hippel model for binding large ligands to a homogeneous lattice (22), which encompasses an intrinsic affinity constant ($K_i$) as well as a cooperativity constant ($\omega$).

To date, the only post-translational modification that has been shown to occur on endogenous hnRNP-C in cells is the phosphorylation of the ACD (17). Whereas the protein is basally phosphorylated at Ser$^{237}$ by protein kinase CK2 and at Ser$^{236}$ by an as yet unidentified kinase, H$_2$O$_2$ stimulates phosphorylation at Ser$^{240}$ and Ser$^{225}$–Ser$^{238}$ by protein kinase CK1$\delta$. The equilibrium RNA binding studies presented here indicate that the CK1$\delta$-mediated phosphorylation of hnRNP-C modulates the mRNA binding function of the protein, as would be required during mRNA processing and mRNA export. Studies in general agreement with a previous study in which incubation of hepatoma cells with H$_2$O$_2$ was shown to increase the mRNA binding constants for hnRNP-C phosphorylation site mutants (38–40), this mechanism appears to apply to CK1$\delta$ autophosphorylation sites (38–40), this mechanism appears to apply to CK1$\delta$ autophosphorylation sites (38–40), this mechanism appears to apply to CK1$\delta$ autophosphorylation sites (38–40), this mechanism appears to apply to CK1$\delta$ autophosphorylation sites (38–40), this mechanism appears to apply to CK1$\delta$ autophosphorylation sites (38–40), this mechanism appears to apply to CK1$\delta$ autophosphorylation sites (38–40), this mechanism appears to apply to CK1$\delta$ autophosphorylation sites (38–40), this mechanism appears to apply to CK1$\delta$ autophosphorylation sites (38–40), this mechanism appears to apply to CK1$\delta$ autophosphorylation sites (38–40), this mechanism appears to apply to CK1$\delta$ 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