Basal and Hydrogen Peroxide Stimulated Sites of Phosphorylation in Heterogeneous Nuclear Ribonucleoprotein C1/C2†

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Received September 6, 2002; Revised Manuscript Received December 6, 2002

ABSTRACT: Hydrogen peroxide (H_2O_2) is a recently recognized second messenger, which regulates mammalian cell proliferation and migration. The biochemical mechanisms by which mammalian cells sense and respond to low concentrations of H_2O_2 are poorly understood. Recently, heterogeneous nuclear ribonucleoprotein C1/C2 (hnRNP-C1/C2) was found to be rapidly phosphorylated in response to the application of low concentrations of H_2O_2 to human endothelial cells. Here, using tandem mass spectrometry, four sites of phosphorylation are identified in hnRNP-C1/C2, all of which are in the acidic C-terminal domain of the protein. Under resting conditions, the protein is phosphorylated at S247 and S286. In response to low concentrations of H_2O_2, there is increased phosphorylation at S240 and at one of the four contiguous serine residues from S225−S228. Studies using a recombinant acidic C-terminal domain of hnRNP-C overexpressed in *Escherichia coli* demonstrate that protein kinase CK2 phosphorylates hnRNP-C1/C2 at S247, while protein kinase A and several protein kinase C isoforms fail to phosphorylate the isolated domain. These findings demonstrate that the acidic C-terminal domain of hnRNP-C1/C2 serves as the site for both basal and stimulated phosphorylation, indicating that this domain may play an important role in the regulation of mRNA binding by hnRNP-C1/C2.

Hydrogen peroxide (H_2O_2) is a recently recognized second messenger, which regulates mammalian cell proliferation and migration (reviewed in refs *1*−*3*). The H_2O_2 precursor, superoxide, is generated by mammalian cells by a family of membrane-bound NADPH oxidases, in response to receptor-mediated signaling (reviewed in refs *4* and *5*). The application of low concentrations of H_2O_2 (<10 μM) to mammalian cells in culture results in increased cellular proliferation in a wide variety of cell types (*6*−*10*). In addition, migration and tube formation by endothelial cells in culture are stimulated by these low concentrations of H_2O_2 (*10*, *11*). Overexpression of an NADPH oxidase catalytic subunit has been shown to increase proliferation and induce transformation in NIH3T3 cells (*12*). Likewise, overexpression of catalase was shown to inhibit proliferation of vascular smooth muscle cells (*13*). Although H_2O_2 is freely diffusible, upon application of H_2O_2 to cells in culture a gradient of H_2O_2 is rapidly established across the plasma membrane (*14*, *15*). The presence of this gradient dictates that applied concentrations of H_2O_2 below 10 μM, which are mitogenic to mammalian cells, correlate to intracellular concentrations below 1 μM. The biochemical mechanisms by which mammalian cells sense and respond to such low concentrations of H_2O_2 are poorly understood.

Recently, a functional proteomic analysis demonstrated that heterogeneous nuclear ribonucleoprotein C1/C2 (hnRNP-C1/C2)[1] is rapidly phosphorylated by low concentrations of H_2O_2 in human endothelial cells (*16*). hnRNP-C1/C2 is a nuclear pre-mRNA binding protein that appears to regulate pre-mRNA processing (reviewed in refs *17* and *18*). Deletion of the gene for hnRNP-C1/C2 in the mouse is lethal, with developmental arrest at the egg cylinder stage (*19*). Murine stem cells lacking hnRNP-C1/C2 are viable but show decreased rates of differentiation. Heterologous expression of the gene for hnRNP-C1/C2 in yeast, which normally lack this gene, is also lethal (*20*). In this latter case it appears that the heterologously expressed hnRNP-C1/C2 translocates to the yeast nucleus and binds mRNA. However, yeast appear to lack the biochemical machinery necessary to release hnRNP-C1/C2 from the mRNA, resulting in the inhibition of mRNA export from the nucleus.

Structurally, hnRNP-C1/C2 isolated from HeLa cells is a heterotetramer ($C1_3C2$) in which C1 and C2 are splice variants of the same gene, differing by the presence of an additional 13 amino acids in C2 (*21*, *22*). When bound to RNA, the protein is arranged in 19 S triangular complexes, each of which is composed of three hnRNP-C1/C2 heterotetramers (*23*, *24*). Each hnRNP-C subunit appears to have four functional domains. There is an N-terminal RNA

† This work was supported by NIH Grant HL35716 to T.C. J.R.S. was supported by NIH Grant HL07627.

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[1] Abbreviations: 2D-PAGE, two-dimensional polyacrylamide gel electrophoresis; DTT, dithiothreitol; EGCS, endothelial cell growth supplement; FCS, fetal calf serum; hnRNP, heterogeneous nuclear ribonucleoprotein; HUVEC, human umbilical vein endothelial cell; IEF, isoelectric focusing; LC-MS/MS, liquid chromatography tandem mass spectrometry; NP-40, Nonidet P40.

10.1021/bi0268091 CCC: $25.00
Published on Web 01/15/2003

binding domain (residues 8−87), which has a solution structure consisting of a $\beta\alpha\beta\beta\alpha\beta$ fold in which a four-strand antiparallel β-sheet is tightly packed against two perpendicularly oriented α-helices (*25*). This is followed by a basic high-affinity RNA binding domain (residues 140−179), which is believed to be responsible for much of the affinity of the protein for RNA (*26*). Next is a leucine zipper (residues 180−207), which mediates subunit interactions in the heterotetramer (*27*). Finally, there is an acidic C-terminal domain (residues 208−290). The function of this last domain is not completely clear, although mutational studies indicate that the acid C-terminal domain contributes to tetramer stability (*26*, *27*).

It has been demonstrated that hnRNP-C1/C2 undergoes phosphorylation, not only in vitro but also in chicken MSB cells, in Chinese hamster ovary cells, in human cell lines (HeLa and K562), and in primary human endothelial cells (*16*, *28*−*35*). On the basis of in vitro phosphorylation studies, it has been reported that the presence of mRNA modulates the phosphorylation status of the protein and that phosphorylation of the protein regulates its affinity for mRNA (*36*−*38*). In addition, the phosphorylation level has been reported to be increased during mitosis compared to interphase in HeLa cells (*33*). In confluent human endothelial cells, under resting conditions the hnRNP-C1/C2 subunits are present mostly in the diphosphorylated state with some monophosphorylated and triphosphorylated species present (*16*). After the addition of a low concentration of H_2O_2, there is a rapid increase in the level of phosphorylation, with an increase in the amount of triphosphorylated species and the transient formation of some quatraphosphorylated protein. This transient-increased phosphorylation is followed by dephosphorylation in which the unphosphorylated form of the protein is transiently present.

Previous studies identified protein kinase CK2 (formerly termed casein kinase II) as one kinase that phosphorylates the protein both in vitro and in intact nuclei, and other unidentified kinases have been implicated (*30*, *31*). However, progress toward understanding the role of phosphorylation in hnRNP-C1/C2 function has been hindered by the lack of information concerning the sites of phosphorylation on the protein. Here, using ion-trap tandem mass spectrometry, we identified four sites of phosphorylation in hnRNP-C1/C2 in human endothelial cells. All of the identified sites of phosphorylation are within the acidic C-terminal domain. The basal sites of phosphorylation were found to be S247 and S286, and the H_2O_2 enhanced sites of phosphorylation were found to be S240 and one of the four contiguous serine residues from S225−S228. Furthermore, studies using a recombinant acidic C-terminal domain overexpressed in *Escherichia coli* demonstrate that protein kinase CK2 phosphorylates hnRNP-C1/C2 at S247. These findings suggest that the acidic C-terminal domain of hnRNP-C1/C2 could be a regulatory domain and may play an important role in the regulation of mRNA binding by hnRNP-C1/C2.

EXPERIMENTAL PROCEDURES

Cell Culture. Human umbilical vein endothelial cells (HUVECs) were isolated from fresh human umbilical cords and cultured as described previously (*39*). HUVECs were prepared confluent in 10 cm dishes at passage 3 in medium 199 (Bio Whittaker) containing 20% fetal calf serum (FCS), heparin (100 μg/mL; Sigma), and endothelial cell growth supplement (ECGS, 50 μg/mL; Biomedical Technologies). Twenty-four hours after reaching confluence, the endothelial cells in medium 199 with FCS, heparin, and ECGS were incubated in the absence or presence of 7 μM H_2O_2 for 20 min at 37 °C.

Preparation of Nuclear Extract. After treatment, the cells were washed twice with ice-cold PBS (67 mM phosphate, 150 mM NaCl, pH 7.4) and harvested by scraping in buffer A (10 mM Tris, 140 mM NaCl, 1 mM EDTA, pH 8.0). The cells were pelleted by spinning at 500*g* for 15 min. The cells were resuspended in buffer B [10 mM HEPES, 750 μM spermidine, 150 μM spermine, 20 mM NaF, 1 mM sodium orthovanadate, 2 mM EDTA, 5 mM DTT, complete protease inhibitor (1 tablet/50 mL; Roche), pH 7.9] containing 0.1% Nonidet P40 (Boehringer Mannheim), incubated on ice for 10 min, and then centrifuged at 16000*g* for 5 min. The supernatant was removed, and the crude nuclear pellet was washed once with 0.8 mL of buffer B and, after centrifugation, was resuspended in IEF sample buffer [9 M urea, 65 mM DTT, 1% CHAPS, 0.1% Bio-Lyte 3/10 ampholyte (Bio-Rad), 100 μL/10^6 cells]. The suspension was centrifuged at 16000*g* for 15 min, and the resulting supernatant was then applied to a Bio-Spin 6 chromatography column (Bio-Rad) equilibrated with IEF sample buffer.

Two-Dimensional Electrophoresis. HUVEC nuclear extracts were subjected to isoelectric focusing (IEF) using a Protean IEF cell and 17 cm IPG ready-strips (Bio-Rad), which covered the pH range of 4−7. Upon completion of the IEF, the IPG ready-strips were incubated first with equilibration buffer (375 mM Tris, 6 M urea, 2% SDS, 20% glycerol, pH 8.8) containing 130 mM DTT for 10 min and then with equilibration buffer containing 135 mM iodoacetamide for 10 min. The strips were then placed on 7.5% polyacrylamide gels and electrophoresed with a constant current of 20 mA/gel. The gels were stained with Coomassie (Bio-Rad).

Tandem Mass Spectrometry. The portion of the gel containing a protein spot of interest was excised using a razor blade, cutting as close to the spot as possible. The Coomassie-stained gel portions were destained and subjected to tryptic in-gel digestion followed by LC-MS/MS analysis of the extracted peptides as described previously (*40*−*42*). Specifically, excised gel portions were cut into approximately 1 mm^3 pieces. Gel pieces were washed and dehydrated with acetonitrile for 10 min followed by removal of acetonitrile. Pieces were then completely dried in a speed-vac. 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. Samples were then placed in a 37 °C room overnight. 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 speed-vac (∼1 h). The samples were then stored at 4 °C until analysis.

On the day of analysis the samples were reconstituted in 5 μL of HPLC solvent A (5% acetonitrile, 0.005% heptafluorobutyric acid, 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 $\times$ 12 cm length) with a flame-drawn tip. After equilibrating the column, each sample was pressure-loaded off-line onto the column. The column was then reattached to the HPLC system. A gradient was formed, and peptides were eluted with increasing concentrations of solvent B (95% acetonitrile, 0.005% heptafluorobutyric acid, 0.4% acetic acid).

As the peptides eluted, they were subjected to electrospray ionization, at which time they entered into an LCQ DECA 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 in hnRNP-C1 were determined by matching the protein sequence (*19*) with the acquired fragmentation pattern using the software program Sequest (ThermoFinnigan, San Jose, CA), by allowing for a modification comprising an additional 80 Da on Ser, Thr, or Tyr. An observed peptide mass was considered to match a theoretical value if the absolute mass difference was $\leq$1.5 Da. The mass accuracy of the instrument employed is 1 Da.

Plasmid Construction. A DNA segment containing residues 217−293 of hnRNP-C1 was amplified from a nearly full-length clone of hnRNP-C1 (Invitrogen) using the polymerase chain reaction. This amplified fragment was then subcloned into the *Bam*HI and *Eco*RI sites of pGEX-2T (Pharmacia). Automated dideoxynucleotide sequencing of the construct verified the authenticity of the insert. The predicted protein product consists of the acidic C-terminal domain of hnRNP-C fused with glutathione *S*-transferase (GST). The amino acid sequence of the final protein product after cleavage from GST with thrombin consists of the 79-residue sequence GSNDKSEEEQSSSSVKKDETNVKMESEGGADDSAEEGDLLDDDDNEDRGDDQLELIKDDEKEAEEGEDDRDSANGEDDS, which consists of hnRNP-C1 residues N217−S293 preceded by "GS" and has a calculated isoelectric point of 3.7 and molecular mass of 8.62 kDa.

Bacterial Expression and Purification of the hnRNP-C Acidic C-Terminal Domain. E. coli BL21 bacteria (Novagen) transformed with the pGEX-hnRNP-C fusion construct were grown to an OD_{600} of 0.6 at 37 °C and then induced with 0.1 mM isopropyl β-D-thiogalactopyranoside for 1−2 h. The cells were pelleted at 4000*g* for 30 min. The cells were then suspended in PBS (30 mL per 1 L culture) containing 5 mM DTT, 0.1% Nonidet P40 (Boehringer Mannheim), and complete protease inhibitor (1 tablet/50 mL). The cells were lysed by sonication, and the homogenate was centrifuged at 3000*g* for 30 min. Glutathione−Sepharose 4B (Amersham-Pharmacia) was added to the resulting supernatant (0.75 mL of resin per 1 L culture). The supernatant was then agitated on a rocker for 20 min and then centrifuged at 500*g* for 5 min. The resulting supernatant was removed, and the resin was placed in a chromatography column and washed with 2 bed volumes of TBS (5 mM Tris, 125 mM NaCl, pH 7.5) containing 5 mM DTT. The resin was then incubated as a 50% slurry with thrombin (80 units/0.75 mL of resin) at 25 °C overnight. All subsequent steps were performed at 4 °C. The resin was washed with 1 bed volume of TBS, and the eluate was concentrated to 0.5 mL with a Centriprep YM-3 concentrator (Amicon, 3000 Da MWCO) at 3000*g*. The sample was then applied to a Superdex 200 HR 10/30 size-exclusion chromatography column (10 $\times$ 300 mm; Amersham-Pharmacia) equilibrated in TBS containing 5 mM $MgCl_2$ at a flow rate of 0.4 mL/min using a Biologic HR chromatography system (Bio-Rad).

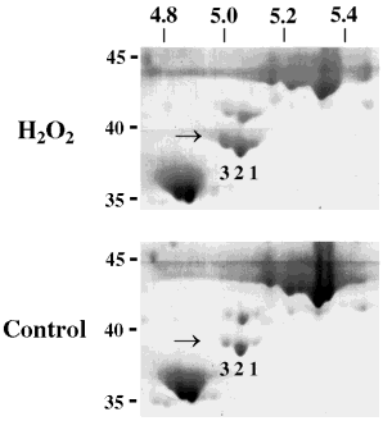


FIGURE 1: Two-dimensional electrophoresis of HUVEC nuclear extract. The soluble nuclear extract from 10^7 HUVECs was subjected to 2D PAGE, first with isoelectric focusing over the pH range of 4−7, followed by SDS−PAGE on a 7.5% gel. Cells were treated with (top) or without (bottom) 7 μM H_2O_2 for 20 min. Displayed are gel portions encompassing the pH range of 4.8−5.4. The arrow indicates hnRNP-C1. The numbers indicate the predicted number of phosphates per subunit (*16*).

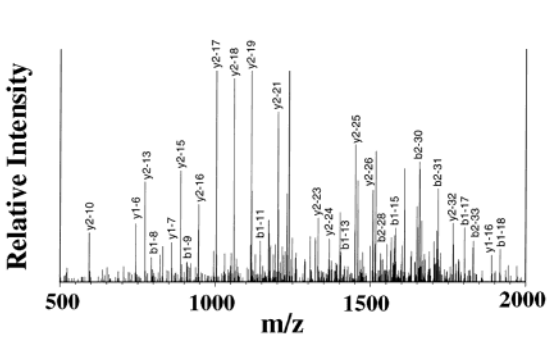
In Vitro Phosphorylation Studies. The recombinant purified hnRNP-C1/C2 acidic C-terminal domain (20 μg) was incubated with the following recombinant protein kinases: protein kinase CK2 (Roche), protein kinase A (Calbiochem), and protein kinase C isoforms α, β1, γ, ϵ, and ζ (Calbiochem). Each incubation contained 0.5 unit of the respective kinase, defining a unit as the amount of kinase required to transfer 1 nmol of phosphate from ATP to substrate per minute as indicated by the supplier. Incubations were performed in TBS containing 2 mM DTT, 5 mM $MgCl_2$, 100 μM ATP, and 20 μCi [γ-^{32}P]ATP at 37 °C in a reaction volume of 125 μL. Incubations with all protein kinase C isoforms contained 10 μM diolein and 20 μg/mL phosphatidylserine. Incubations with protein kinase C isoforms α, β1, and γ also contained 200 μM $CaCl_2$. Reactions were quenched after 1 h by the addition of SDS−PAGE sample buffer containing β-mercaptoethanol (Bio-Rad). Samples were then subjected to reducing SDS−PAGE on a 12% polyacrylamide gel, followed by Coomassie staining and autoradiography. After exposure, film was developed using a Kodak M35A X-Omat processor.

To identify the site(s) of phosphorylation by protein kinase CK2, 0.1 mg of purified recombinant hnRNP-C acidic C-terminal domain was incubated with or without 2 units of protein kinase CK2 in TBS containing 2 mM DTT, 5 mM $MgCl_2$, and 150 μM ATP at 37 °C in a reaction volume of 200 μL. Reactions were quenched after 2 h by the addition of SDS−PAGE sample buffer containing β-mercaptoethanol. Samples were then subjected to reducing SDS−PAGE on a 20% polyacrylamide gel. After SDS−PAGE, the gel was stained with Coomassie. The gel bands were then excised and subjected to LC-MS/MS analysis as described above.

Miscellaneous Methods. H_2O_2 concentrations of stock solutions were determined using an ϵ of 81 M^{-1} at 230 nm (*43*). UV−visible absorption spectra were recorded on a Cary 50 Bio UV−visible spectrophotometer. The purity of the

A.

	Seq	b#	b1	b2	y1	y2	y#
	*M	1	148.2	74.6	-	-	34
	E	2	277.3	139.2	3661.5	**1831.3**	33
	S	3	364.4	182.7	3532.4	**1766.7**	32
	E	4	493.5	247.3	3445.3	**1723.2**	31
	G	5	550.6	275.8	3316.2	**1658.6**	30
	G	6	607.6	304.3	3259.1	**1630.1**	29
	A	7	678.7	339.8	3202.1	1601.5	28
	D	8	**793.8**	397.4	3131.0	**1566.0**	27
	D	9	**908.9**	454.9	3015.9	**1508.5**	26
Phospho-S		10	1075.9	538.5	2900.8	**1450.9**	25
S247	A	11	**1147.0**	574.0	2733.7	**1367.4**	24
	E	12	1276.1	638.6	2662.7	**1331.8**	23
	E	13	**1405.2**	703.1	2533.6	1267.3	22
	G	14	1462.3	731.7	2404.4	**1202.7**	21
	D	15	**1577.4**	789.2	2347.4	**1174.2**	20
	L	16	1690.5	845.8	2232.3	**1116.7**	19
	L	17	**1803.7**	902.4	2119.1	**1060.1**	18
	D	18	**1918.8**	959.9	2006.0	**1003.5**	17
	D	19	2033.9	1017.4	**1890.9**	**945.9**	16
	D	20	2149.0	1075.0	1775.8	**888.4**	15
	D	21	2264.1	1132.5	**1660.7**	830.9	14
	N	22	2378.2	1189.6	1545.6	**773.3**	13
	E	23	2507.3	1254.1	1431.5	716.3	12
	D	24	2622.4	1311.7	1302.4	651.7	11
	R	25	2778.6	1389.8	1187.3	**594.2**	10
	G	26	2835.6	1418.3	1031.1	516.1	9
	D	27	2950.7	1475.9	974.1	487.5	8
	D	28	3065.8	**1533.4**	**859.0**	430.0	7
	Q	29	3193.9	1597.5	**743.9**	372.5	6
	L	30	3307.1	**1654.0**	615.8	308.4	5
	E	31	3436.2	**1718.6**	502.6	251.8	4
	L	32	3549.4	**1775.2**	373.5	187.3	3
	I	33	3662.5	**1831.8**	260.3	130.7	2
	K	34	-	-	147.2	74.1	1



B.

	Seq	b#	b1	b2	y1	y2	y#
	G	1	58.1	29.5	-	-	31
	D	2	173.1	87.1	3463.3	1732.1	30
	D	3	288.2	144.6	3348.2	**1674.6**	29
	Q	4	416.4	208.7	3233.1	1617.1	28
	L	5	**529.5**	265.3	3105.0	**1553.0**	27
	E	6	658.6	329.8	2991.8	1496.4	26
	L	7	**771.8**	386.4	2862.7	**1431.8**	25
	I	8	**885.0**	443.0	2749.5	**1375.3**	24
	K	9	1013.1	507.1	2636.4	**1318.7**	23
	D	10	1128.2	564.6	2508.2	1254.6	22
	D	11	**1243.3**	622.2	2393.1	**1197.1**	21
	E	12	**1372.4**	686.7	2278.0	**1139.5**	20
	K	13	**1500.6**	750.8	2148.9	1075.0	19
	E	14	1629.7	**815.4**	2020.7	1010.9	18
	A	15	1700.8	**850.9**	1891.6	**946.3**	17
	E	16	1829.9	**915.5**	1820.5	910.8	16
	E	17	1959.0	980.0	1691.4	846.2	15
	G	18	2016.1	1008.5	**1562.3**	781.7	14
	E	19	2145.2	**1073.1**	**1505.3**	**753.1**	13
	D	20	2260.3	**1130.6**	**1376.1**	688.6	12
	D	21	2375.4	**1188.2**	**1261.0**	631.0	11
	R	22	2531.6	**1266.3**	**1146.0**	573.5	10
	D	23	2646.6	**1323.8**	989.8	495.4	9
Phospho-S		24	2813.7	**1407.4**	**874.7**	437.8	8
S286	A	25	2884.8	1442.9	**707.6**	354.3	7
	N	26	2998.9	**1500.0**	**636.5**	318.8	6
	G	27	3056.0	1528.5	522.4	261.7	5
	E	28	3185.1	1593.0	465.4	233.2	4
	D	29	3300.2	1650.6	336.3	168.6	3
	D	30	3415.2	1708.1	221.2	111.1	2
	S	31	-	-	106.1	53.5	1

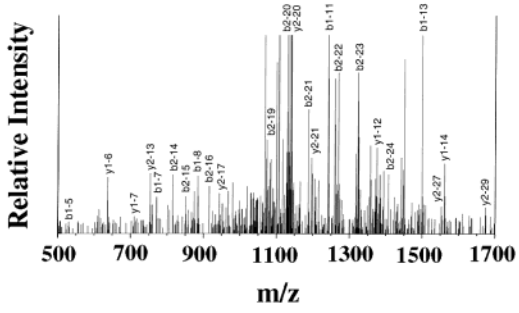


FIGURE 2: Identification of basal sites of phosphorylation in hnRNP-C1/C2. The lower hnRNP-C spot (hnRNP-C1) with an apparent p*I* of 5.05 (labeled 2 in Figure 1) was subjected to tryptic in-gel digestion followed by LC-MS/MS analysis of the extracted peptides. Phosphates were found to be present on S247 (A**)** and on S286 (B). Sites of phosphorylation are indicated by Phospho-S and artifactual methionine oxidation by *M. Listed are the theoretical mass to charge ratios for all possible fragmentation products of the tryptic phosphopeptides from either the N-terminus (b) or the C-terminus (y) and considering both singly charged (b1, y1) and doubly charged (b2, y2) ions. The underlined and bolded numbers indicate the fragmentation products that were observed, some of which are labeled in the corresponding mass spectra.

recombinant acidic C-terminal domain was assessed by SDS−polyacrylamide gel electrophoresis using an XCell Surelock electrophoresis cell (Novex) and 12% precast gels. Chemicals not otherwise specified were obtained from Sigma.

RESULTS

Identification of Sites of Phosphorylation in hnRNP-C1/C2. Previous studies employing 2D immunoblots revealed that, in resting HUVECs, the hnRNP-C1/C2 subunits are present mostly in the diphosphorylated state with some monophosphorylated and triphosphorylated species present (*16*). After the addition of a low concentration of H_2O_2, there was a rapid increase in the level of phosphorylation with an increase in the amount of triphosphorylated species and the formation of some quatraphosphorylated protein. This effect can also be discerned on the Coomassie-stained 2D gels of HUVEC nuclear extracts in Figure 1. The arrow indicates hnRNP-C1, and the numbers indicate the predicted number of phosphates per subunit based on previous studies (*16*). Note that the level of the triphosphorylated species (labeled "3") is increased 20 min after the addition of 7 μM H_2O_2. The amount of monophosphorylated species (labeled "1") is also modestly increased; as shown previously, the H_2O_2-stimulated phosphorylation, which occurs at 10−20 min, is followed immediately by dephosphorylation, which is maximal at 60 min. The lower abundance quatraphosphorylated species previously detected on immunoblots is present after H_2O_2 treatment but is more difficult to discern on the Coomassie-stained gels.

The three labeled hnRNP-C1 spots in Figure 1 were subjected to LC-MS/MS analysis to determine the sites of

A.

	Seq	b#	b1	b2	y1	y2	y#
	*M	1	148.2	74.6	-	-	38
	E	2	277.3	139.2	4229.0	2115.0	37
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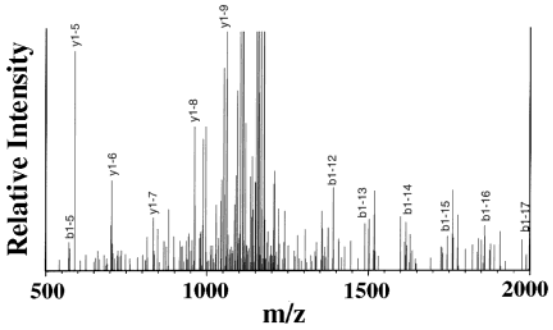
S240	E	4	573.5	287.3	3932.8	**1966.9**	35
	G	5	630.6	315.8	3803.7	**1902.3**	34
	G	6	687.6	344.3	3746.6	1873.8	33
	A	7	758.7	379.8	3689.5	**1845.3**	32
	D	8	873.8	437.4	3618.5	1809.7	31
	D	9	**988.9**	494.9	3503.4	**1752.2**	30
Phospho-S		10	1155.9	578.5	3388.3	**1694.6**	29
S247	A	11	1227.0	614.0	3221.2	**1611.1**	28
	E	12	1356.1	678.6	3150.1	**1575.6**	27
	E	13	1485.2	743.1	3021.0	**1511.0**	26
	G	14	1542.3	771.7	2891.9	**1446.5**	25
	D	15	1657.4	**829.2**	2834.9	1417.9	24
	L	16	1770.5	885.8	2719.8	**1360.4**	23
	L	17	1883.7	942.4	2606.6	**1303.8**	22
	D	18	**1998.8**	999.9	2493.4	**1247.2**	21
	D	19	2113.9	**1057.4**	2378.4	**1189.7**	20
	D	20	2229.0	1115.0	2263.3	**1132.1**	19
	D	21	2344.1	1172.5	2148.2	**1074.6**	18
	N	22	2458.2	**1229.6**	2033.1	**1017.0**	17
	E	23	2587.3	**1294.1**	**1919.0**	960.0	16
	D	24	2702.4	**1351.7**	1789.9	895.4	15
	R	25	2858.6	1429.8	1674.8	**837.9**	14
	G	26	2915.6	1458.3	**1518.6**	759.8	13
	D	27	3030.7	**1515.9**	1461.5	731.3	12
	D	28	3145.8	**1573.4**	**1346.5**	673.7	11
	Q	29	3273.9	**1637.5**	**1231.4**	616.2	10
	L	30	3387.1	**1694.0**	1103.2	552.1	9
	E	31	3516.2	1758.6	990.1	495.5	8
	L	32	3629.4	**1815.2**	861.0	431.0	7
	I	33	3742.5	**1871.8**	747.8	374.4	6
	K	34	3870.7	**1935.8**	634.6	317.8	5
	D	35	3985.8	**1993.4**	506.5	253.7	4
	D	36	4100.9	2050.9	391.4	196.2	3
	E	37	4230.0	2115.5	276.3	138.6	2
	K	38	-	-	147.2	74.1	1

B.

	Seq	b#	b1	y1	y#
	N	1	115.1	-	21
	D	2	230.2	2335.3	20
	K	3	358.4	2220.2	19
	S	4	445.4	2092.1	18
	E	5	**574.6**	2005.0	17
	E	6	**703.7**	1875.9	16
	E	7	**832.8**	1746.8	15
	Q	8	**960.9**	**1617.6**	14
	S	9	-----	1489.5	13
Phospho-	S	10	-----	------	12
S225-S228	S	11	-----	------	11
	S	12	**1389.2**	------	10
	V	13	**1488.4**	**1061.2**	9
	K	14	**1616.5**	**962.1**	8
	K	15	**1744.7**	**833.9**	7
	D	16	**1859.8**	**705.7**	6
	E	17	**1988.9**	**590.6**	5
	T	18	2090.0	461.5	4
	N	19	2204.1	360.4	3
	V	20	2303.3	246.3	2
	K	21	-	147.2	1



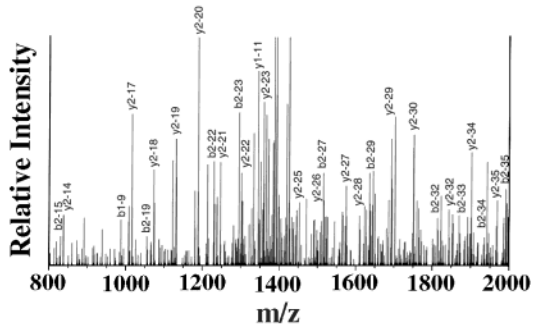


FIGURE 3: Identification of H_2O_2-stimulated sites of phosphorylation in hnRNP-C1/C2. The lower hnRNP-C spot (hnRNP-C1) with an apparent p*I* of 5.00 (labeled 3 in Figure 1) was subjected to tryptic in-gel digestion followed by LC-MS/MS analysis of the extracted peptides. In addition to phosphorylations (Phospho-S) at positions S247 and S286, phosphates were also found to be present at S240 (A**)** and at one of the four contiguous serine residues from S225−S228 indicated by S (B). Artifactual methionine oxidation is indicated by *M. Listed are the theoretical mass to charge ratios for all possible fragmentation products of the tryptic phosphopeptides from either the N-terminus (b) or the C-terminus (y), considering singly charged (b1, y1) and/or doubly charged (b2, y2) ions. The underlined and bolded numbers indicate the fragmentation products that were observed, some of which are labeled in the corresponding mass spectra.

phosphorylation. Two phosphorylation sites (S247 and S286) were identified in all three spots (Figure 2). Thus these two sites are the basal sites of phosphorylation in hnRNP-C1/C2. The monophosphorylated protein present in spot 1 appears to be heterogeneous, containing a single phosphate located either at S247 or at S286. The analysis of the triphosphorylated species (spot 3) also revealed two sites of phosphorylation, which were not found in spots 1 and 2 (Figure 3). One was at S240, and the other was at one of the four contiguous serine residues from S225−S228. The data did not allow for precise localization of this fourth phosphorylation site. Thus the triphosphorylated protein contains phosphates at the two basal positions (S247 and S286), as well as a third phosphate, which may be located either at S240 or at S225−S228. Presumably, the quatraphosphorylated protein described previously (*16*) is phosphorylated at all four positions. The low abundance of the quatraphosphorylated form precluded direct LC-MS/MS analysis on this species. Additional sites of phosphorylation cannot be completely ruled out.

These results indicate that, in confluent human endothelial cells, hnRNP-C1/C2 is phosphorylated basally at two positions, S247 and S286, and that the presence of a low concentration of H_2O_2 stimulates two additional phosphor-

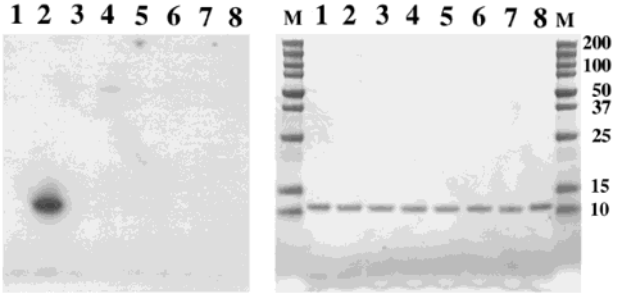


FIGURE 4: In vitro phosphorylation of the isolated acidic C-terminal domain of hnRNP-C. The purified recombinant hnRNP-C acidic C-terminal domain was incubated with [γ-^{32}P]ATP in the presence of various recombinant protein kinases and subjected to SDS−PAGE, followed by autoradiography (left) and Coomassie staining (right). Lanes: 1, no kinase present; 2, protein kinase CK2; 3, protein kinase A; 4, protein kinase Cα; 5, protein kinase Cβ1; 6, protein kinase Cγ; 7, protein kinase Cϵ; 8, protein kinase Cζ; M, molecular weight markers.

ylations, one at S240 and one at S225−S228. All fours sites of phosphorylation, basal and H_2O_2-stimulated, are within the acidic C-terminal domain, suggesting that this domain may play a key role in regulating the binding of mRNA by hnRNP-C1/C2.

In Vitro Phosphorylation of the hnRNP-C Acidic C-Terminal Domain. To help to elucidate the kinases responsible for phosphorylating the acidic C-terminal domain, the isolated domain, consisting of residues 217−293 of hnRNP-C1, was overexpressed as a recombinant GST fusion protein in *E. coli*. After isolation on glutathione−Sepharose and cleavage with thrombin, the isolated acidic C-terminal domain was further purified by size-exclusion chromatography. On SDS−PAGE, the purified protein migrates as a single band with an apparent molecular mass of ~11 kDa (Figure 4). The sequence of the protein was completely verified by LC-MS/MS (not shown).

To gain insight into the protein kinases responsible for phosphorylating the hnRNP-C acidic C-terminal domain, several recombinant protein kinases were evaluated for their ability to phosphorylate the purified recombinant domain (Figure 4). It was observed that protein kinase CK2 readily phosphorylates the acidic C-terminal domain, while protein kinase A and several protein kinase C isoforms do not phosphorylate the domain. To determine the protein kinase CK2 mediated site(s) of phosphorylation in the recombinant acidic C-terminal domain, the phosphorylated recombinant protein was subjected to tryptic in-gel digestion, followed by LC-MS/MS analysis of the extracted tryptic peptides. In the absence of protein kinase CK2, no phosphorylation sites were observed (not shown). In the presence of protein kinase CK2, one site of phosphorylation was identified, which corresponds to S247 in the full-length hnRNP-C1 sequence (Figure 5). This Ser residue does in fact lie within a protein kinase CK2 consensus sequence, having an acidic residue at the $n + 3$ position along with several other acidic residues in the region from $n - 2$ to $n + 5$ (*44*). Together, these studies indicate that, in human endothelial cells, hnRNP-C1/C2 is basally phosphorylated at S247 by protein kinase CK2 and at S286 by an as yet unidentified kinase and that H_2O_2 stimulates phosphorylation at S240 and at S225−S228 by one or more unidentified kinases.

	Seq	b#	b1	b2	y1	y2	y#
	*M	1	148.2	74.6	-	-	34
	E	2	277.3	139.2	3661.5	**1831.3**	33
	S	3	364.4	182.7	3532.4	**1766.7**	32
	E	4	493.5	247.3	3445.3	1723.2	31
	G	5	550.6	275.8	3316.2	**1658.6**	30
	G	6	607.6	304.3	3259.1	**1630.1**	29
	A	7	678.7	339.8	3202.1	**1601.5**	28
	D	8	**793.8**	397.4	3131.0	**1566.0**	27
	D	9	**908.9**	454.9	3015.9	**1508.5**	26
Phospho-S		10	1075.9	538.5	2900.8	**1450.9**	25
	A	11	**1147.0**	574.0	2733.7	**1367.4**	24
	E	12	1276.1	638.6	2662.7	**1331.8**	23
	E	13	**1405.2**	703.1	2533.6	1267.3	22
	G	14	**1462.3**	731.7	2404.4	**1202.7**	21
	D	15	**1577.4**	789.2	2347.4	**1174.2**	20
	L	16	1690.5	845.8	2232.3	**1116.7**	19
	L	17	**1803.7**	902.4	2119.1	**1060.1**	18
	D	18	**1918.8**	959.9	2006.0	**1003.5**	17
	D	19	2033.9	1017.4	**1890.9**	**945.9**	16
	D	20	2149.0	1075.0	**1775.8**	**888.4**	15
	D	21	2264.1	1132.5	**1660.7**	**830.9**	14
	N	22	2378.2	1189.6	**1545.6**	**773.3**	13
	E	23	2507.3	1254.1	1431.5	716.3	12
	D	24	2622.4	1311.7	1302.4	651.7	11
	R	25	2778.6	1389.8	1187.3	594.2	10
	G	26	2835.6	1418.3	**1031.1**	516.1	9
	D	27	2950.7	**1475.9**	974.1	487.5	8
	D	28	3065.8	**1533.4**	**859.0**	430.0	7
	Q	29	3193.9	1597.5	**743.9**	372.5	6
	L	30	3307.1	1654.0	615.8	308.4	5
	E	31	3436.2	**1718.6**	502.6	251.8	4
	L	32	3549.4	**1775.2**	373.5	187.3	3
	I	33	3662.5	**1831.8**	260.3	130.7	2
	K	34	-	-	147.2	74.1	1

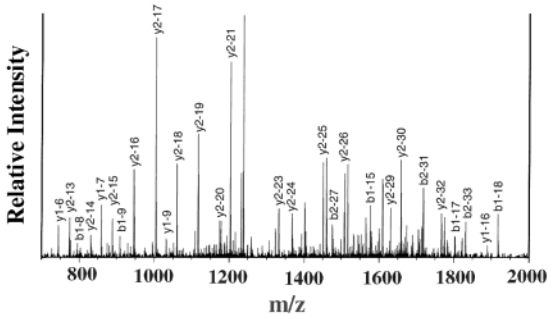


FIGURE 5: Identification of the protein kinase CK2 site of phosphorylation. The recombinant acidic C-terminal domain of hnRNP-C, phosphorylated in vitro with recombinant protein kinase CK2, was subjected to tryptic in-gel digestion followed by LC-MS/MS analysis of the extracted peptides. A single phosphorylated serine (+80 Da) was identified at the position corresponding to S247 in full-length hnRNP-C1, indicated by Phospho-S. Artifactual methionine oxidation is indicated by *M. Listed are the theoretical mass to charge ratios for all possible fragmentation products of the tryptic phosphopeptide from either the N-terminus (b) or the C-terminus (y), considering both singly charged (b1, y1) and doubly charged (b2, y2) ions. The underlined and bolded numbers indicate the fragmentation products that were observed, some of which are labeled in the corresponding mass spectrum.

DISCUSSION

H_2O_2 is a recently recognized diffusable second messenger playing a role in proliferation and migration in mammalian cells (reviewed in refs *1*−*3*). Although low concentrations of H_2O_2 (<10 μM) stimulate proliferation in a wide variety of cells types (*6*−*10*), understanding of the biochemical mechanism by which cells sense low concentrations of H_2O_2 has been severely limited due to a lack of information concerning the signaling pathways involved. A recent functional proteomic analysis identified hnRNP-C1/C2 as a

protein that is rapidly phosphorylated upon application of low concentrations of H_2O_2 to human endothelial cells (*16*).

hnRNP-C1/C2 is a nuclear restricted pre-mRNA binding protein (reviewed in refs *17* and *18*). Its precise function is not entirely clear. It appears to play an important role in pre-mRNA processing and may be considered as a mRNA chaperonin, maintaining long stretches of mRNA in an unfolded single-stranded state to allow for more efficient pre-mRNA processing. It has been proposed that hnRNP-C1/C2 may bind to and completely saturate all newly synthesized pre-mRNA transcripts (*45*). However, this view may not fully explain the protein's apparent specificity for particular mRNA sequences or the significant variation in hnRNP-C1/C2 protein levels among different cell types (*46*−*47*). Unlike other members of the hnRNP family, which readily cycle between the nucleus and the cytoplasm, hnRNP-C1/C2 is predominantly restricted to the nucleus with a nuclear retention sequence (*48*). Thus, hnRNP-C1/C2 must be removed from the mRNA prior to export from the nucleus. The manner in which the binding of mRNA by hnRNP-C1/C2 is regulated is not known.

Currently, the only posttranslational modification known to occur on hnRNP-C1/C2 is phosphorylation. hnRNP-C1/C2 has been demonstrated to be phosphorylated in vitro in nuclear extracts, in chicken MSB cells, in Chinese hamster ovary cells, in human cell lines (HeLa and K562), and in primary human endothelial cells (*16*, *28*−*35*). A series of studies employing in vitro phosphorylation indicate that the presence of mRNA may alter the phosphorylation status of the protein and that phosphorylation of the protein may regulate its affinity for mRNA (*36*−*38*). Recently, a 2D PAGE analysis of nuclear extract from human endothelial cells revealed that each hnRNP-C1/C2 subunit is predominantly biphosphorylated under resting/basal conditions and that low concentrations of H_2O_2 stimulate a rapid increase in the amount of tri- and quatraphosphorylated subunits (*16*). The phosphorylation site determinations reported here indicate that all four of these sites of phosphorylation are within the acid C-terminal domain of the protein. These results suggest that the acidic C-terminal domain may be functioning as a regulatory domain, regulating the binding of mRNA by hnRNP-C.

Previous studies have indicated that hnRNP-C1/C2 is phosphorylated by protein kinase CK2 (both in vitro and in intact nuclei) and by at least one other Ser/Thr kinase (*30*, *31*). On the basis of the in vitro phosphorylation studies reported here, it is clear that protein kinase CK2 phosphorylates the protein at S247. The kinases responsible for the other three phosphorylation sites remain unknown at this time. The target serine residues are not within readily identifiable consensus sequences, and the recombinant domain was not phosphorylated by protein kinase A or by several protein kinase C isoforms. There have been several reports of protein kinase activities that copurify with the hnRNP complex (*49*−*52*). It may be that there are protein kinases specific to the hnRNP particle that phosphorylate the hnRNP-C acidic C-terminal domain. Identification of the remaining hnRNP-C kinases, their mechanisms of activation, and the structural and functional alterations resulting from their phosphorylating activity should allow for a much better understanding of the mechanisms by which mRNA binding by hnRNP-C1/C2 is regulated.

ACKNOWLEDGMENT

We acknowledge the Taplin Biological Mass Spectrometry Facility in the Department of Cell Biology at Harvard Medical School under the direction of Dr. Steven Gygi for acquisition of LC-MS/MS spectra and also Kay Case, Vannessa Davis, and Deanna Lamont for excellent technical assistance in HUVEC isolation and culture.

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BI0268091