Phosphorylation of Nucleolin by a Nucleolar Type NII Protein Kinase†

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ABSTRACT: Nucleolin [C23 or 100 kilodaltons (kDa)] is the major nucleolar phosphorylated protein in exponentially growing Chinese hamster ovary cells. A nucleolar cyclic nucleotide independent protein kinase copurified with nucleolin in a complex which could be dissociated by hydroxyapatite chromatography. The kinase was stimulated by spermine and inhibited by heparin and presented most of the properties of nuclear casein kinase NII. Kinetic analyses showed the apparent K_m value for nucleolin (7×10^{-4} mg/mL) to be lower than those for other casein kinase II substrates such as nuclear protein HMG 14 (0.15 mg/mL), topoisomerase I (0.025 mg/mL), or topoisomerase II (0.04 mg/mL). Similarly, V_{max} values were higher for nucleolin than for other substrates. Nucleolin thus appears to be a natural preferential substrate of nucleolar casein kinase NII. The kinase phosphorylated nucleolin in vitro at serine residues in a 29-kDa CNBr fragment located near the amino terminus of the molecule. The enzyme labeled typical casein kinase II sites. These sites were found predominantly in two highly acidic tryptic fragments designated A (residues 21–49) and C (residues 180–221) which contained serines having at least two acidic residues on their carboxyl-terminal sides. These results demonstrate the existence in the nucleolus of a type of NII protein kinase that uses a protein involved in ribosome assembly as preferential substrate.

Nucleolin [also called C23 or 100 kilodaltons (kDa)[1]] is the major nucleolar phosphoprotein in exponentially growing cells and is believed to play a key role in ribosome biogenesis. It has been found associated with nucleolar chromatin (Olson & Thompson, 1983), nascent preribosomal RNA, and preribosomes (Bouche et al., 1984; Herrera & Olson, 1986). Several experimental approaches have established a direct relationship between the amount of nucleolin in the nucleolus and the rate of preribosomal RNA synthesis (Bouche et al., 1984). Nucleolin also presents properties which suggest that it is involved in the structural organization of the nucleolus (Escande et al., 1985).

Nucleolin is subject to several postsynthetic modifications including methylation (Lischwe et al., 1982) and phosphorylation (Olson et al., 1975; Rao et al., 1982; Bourbon et al., 1983). Correlations have been established between phosphorylation of the protein and the rate of ribosome biogenesis (Ballal et al., 1975) as well as the process of its maturation into defined subfragments (Bourbon et al., 1983; Suzuki et al., 1985). This maturation process was suggested to directly control the transcription of ribosomal genes (Bouche et al., 1984). Similarly, in *Physarum polycephalum*, a 70-kDa phosphorylated protein appears to regulate the transcription of ribosomal genes (Kuehn et al., 1979).

So far, limited information is available concerning the phosphorylation of nuclear proteins by specific cyclic nucleotide independent protein kinases. Two classes of these (NI and NII) which use casein as substrate have been described (Hathaway & Traugh, 1982). In each class, specific substrates have been characterized: poly(A) polymerase is phosphorylated by kinase NI (Stetler et al., 1984); high mobility group 14 (HMG 14) (Walton et al., 1985), regulatory subunit of the cAMP-dependent kinase (Hemmings et al., 1982), topoisomerase I (Durban et al., 1985), topoisomerase II (Ackerman et al., 1985), and a subunit of RNA polymerase I (Duceman et al., 1981) are phosphorylated by kinase NII.

Earlier studies indicated that nucleolin is phosphorylated by a cyclic nucleotide independent nucleolar protein kinase with properties similar to the NII kinase (Olson et al., 1978). More recently, nucleolin was shown to be the major substrate of casein kinase II in concanavalin A stimulated lymphocytes (Geahlin & Harrison, 1984), in mouse embryos, and in Krebs II mouse ascites tumor cells (Schneider et al., 1986). In all of these studies, the casein kinase II activity, as well as the level of phosphorylation of nucleolin, was correlated with the rate of proliferation of the tissue. These results suggest that the phosphorylation of nucleolin is physiologically significant and plays a regulatory role.

The current investigation was undertaken to further characterize the nucleolar protein kinase associated with nucleolin and the sites which it phosphorylates on its major substrate. In the present study, we have purified from isolated nucleoli a protein kinase NII that phosphorylates nucleolin in vitro at typical casein kinase substrate sites. Phosphorylated serines have been localized within highly acidic regions of nucleolin. This NII type kinase copurifies with nucleolin but can be dissociated by hydroxyapatite chromatography.

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[1] Abbreviations: SDS, sodium dodecyl sulfate; TCA, trichloroacetic acid; HPLC, high-performance liquid chromatography; CHO, Chinese hamster ovary; rRNA, ribosomal RNA; rDNA, DNA containing the gene for ribosomal RNA; kDa, kilodalton(s); Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; DTT, dithiothreitol; PTH, phenylthiohydantoin; PAGE, polyacrylamide gel electrophoresis.

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Materials and Methods

Cell Culture and Fractionation. Chinese hamster ovary cells were grown in monolayer culture in Falcon flasks (Caboche & Bachellerie, 1977). Cells were harvested and fractionated into nuclei and nucleoli (Zalta et al., 1971). Nucleoli from Novikoff hepatoma ascites cells were prepared as previously described (Rao et al., 1982).

Purification of Nucleolar Protein Kinase. Nucleoli (5×10^9) were extracted by low salt (10 mM Tris/10 mM NaCl, pH 8.0). The resulting S2 extract (16 mg of protein) was applied to a heparin–Sepharose column (5 mL) equilibrated with 50 mM Tris-HCl (pH 7.9), 5 mM $MgCl_2$, 0.1 mM EDTA, and 1 mM dithiothreitol. The column was washed with 8 bed volumes of the same buffer containing a $(NH_4)_2SO_4$ gradient (200–800 mM). Fractions containing the peak of protein kinase activity from the heparin–Sepharose column were pooled and applied to a hydroxyapatite–Ultrogel column (1 mL). The column was washed with 20 mL of 10 mM sodium phosphate buffer (pH 7) containing 10% glycerol. Elution was carried out with a 10 mM–1 M sodium phosphate gradient. A final purification step utilized centrifugation in a 10–30% glycerol gradient containing 50 mM Tris-HCl (pH 7.9), 5 mM $MgCl_2$, 0.3 M KCl, and 1 mM DTT. Centrifugation was done in a Beckman SW 41 rotor for 15 h at 40 000 rpm.

Protein Analysis. Proteins of different fractions were separated by electrophoresis on 10–16% polyacrylamide slab gels as previously described (Caizergues-Ferrer et al., 1980). Wet gels were first exposed for autoradiography using Kodak X-OMAT MA or AR autoprocess film and then silver stained (Oakley et al., 1980).

Enzyme Assays and in Vitro Labeling. Reactions (60 μL) contained 10 mM Tris-HCl (pH 7.5), 10 mM β-mercaptoethanol, 5 mM $MgCl_2$, 0.1 mM EDTA, 10 μg/mL leupeptin (Sigma), 0.1 mM [γ-^{32}P]ATP (1500 cpm/pmol), and the indicated amounts of enzyme and substrate. All incubations were performed in triplicate for 10 min at 30 °C. Reactions were stopped by addition of 15% trichloroacetic acid and filtration onto nitrocellulose filters. After the filters were washed, they were counted in a scintillation counter. One unit of activity is the amount of enzyme which catalyzed the incorporation of 1 nmol of phosphate into casein at 10 mg/mL in 1 min at 30 °C. When nucleolin was used as a substrate, it was prepared under native conditions using hydroxyapatite chromatography similar to the method described under Purification of Nucleolar Protein Kinase. In this case, column fractions which contain essentially only nucleolin by electrophoretic analyses were taken for the assays. Prior to the assay, the preparations were heated at 55 °C for 10 min to remove any contaminating kinase activity. Topoisomerase I (0.5–2 mg/mL) used for kinase assays was obtained from Promega Biotec, Madison, WI. Casein (0.1–1 mg/mL) was obtained from Sigma, St. Louis, MO. The kinase was the fraction eluting from the hydroxyapatite column at 0.2 M phosphate. The in vitro labeling was carried out as described above. The labeled material was TCA precipitated and then redissolved appropriately for gel analysis or CNBr digestion.

CNBr and Tryptic Phosphopeptide Mapping. Purified nucleolin ^{32}P labeled in vitro was subjected to electrophoresis on a polyacrylamide gel. The slice of acrylamide containing labeled protein was treated with CNBr (350 mg/mL) in 88% HCOOH for 2 h at 37 °C (Pepinsky et al., 1983). Ammonium bicarbonate (50 mM) containing 0.1% β-mercaptoethanol was then added prior to lyophilization. After four cycles of washing and lyophilization, peptides were eluted from the gel slice by 0.1% SDS in ammonium bicarbonate for 15 h at 37 °C and fractionated by polyacrylamide slab gel electrophoresis. For tryptic digests, nucleolin was labeled in vitro by incubating the kinase fraction with a hydroxyapatite fraction containing only nucleolin. The labeled nucleolin (approximately 25 000 cpm) was TCA precipitated and digested with trypsin (5 μg in 200 μL of 50 mM ammonium bicarbonate, pH 8.0) for 2 h at 37 °C. For determination of molecular masses of ^{32}P-labeled tryptic fragments, the gel system of Burr and Burr (1983) was used.

Amino Acid and Sequence Analysis. In vitro ^{32}P-labeled nucleolin was purified as described above. The gel slice containing nucleolin was treated for 15 h at 37 °C with trypsin (1/100 w/w) in 50 mM ammonium bicarbonate, pH 8.3. The supernatant was lyophilized, after which the phosphopeptides were hydrolyzed in 6 N HCl for 15 h at 110 °C. Aliquots of hydrolysates were electrophoresed at pH 3.5 (5% acetic acid/0.5% pyridine) in the presence of phosphoserine, phosphothreonine, and phosphotyrosine as markers. The amino acid composition of the 29-kDa CNBr fragment was determined on a Beckman 119CL amino acid analyzer. For sequencing studies, rat nucleolin was digested with CNBr and fractionated by reverse-phase HPLC as previously described (Lapeyre et al., 1986). Protein sequencing was done on an Applied Biosystems 470A sequencer using the 03RPTH program. PTH-amino acids were identified with a Waters HPLC directly interfaced with the sequencer using an Applied Biosystems dedicated PTH column (0.2 mm). Phosphorylated amino acids were located by analyses of ^{32}P radioactivity released at each cycle. Since the yield of phosphate removed from the filter is low, the positions of the phosphoamino acids were confirmed by using the technique of Wang et al. (1986).

Results

Purification of Nucleolin-Associated Protein Kinase. A low ionic strength extract of isolated nucleoli highly enriched in nucleolin and in the associated protein kinase was fractionated by chromatography on a heparin–Sepharose column (Figure 1). To detect protein kinase activity, each fraction was incubated with [γ-^{32}P]ATP in the presence of casein as exogenous substrate. The kinase activity was eluted from the column between 0.35 and 0.45 M ammonium sulfate, while nucleolin was eluted between 0.4 and 0.5 M as detected by silver staining (Figure 1B). Since protein kinase and nucleolin overlapped extensively, the same experiment was carried out in the absence of casein; the kinase preferentially transferred phosphate to nucleolin but also to two other proteins of 130 and 20 kDa (Figure 1C). Although protein B23, another nucleolar phosphoprotein (Mamrack et al., 1977), is present in some of the kinase-containing fractions, it is not labeled under these conditions.

The fraction (0.35–0.45 M ammonium sulfate) was further chromatographed on a hydroxyapatite column. The casein kinase activity was detected with casein as exogenous substrate in two fractions (Figure 2) eluting at 0.2 M (fractions 14–19) and 0.3 M sodium phosphate (fractions 20–25). The protein content of the various fractions was analyzed by SDS–PAGE. The material which eluted at 0.2 M sodium phosphate (fractions 14–17) contained small quantities of peptides with molecular masses between 25 and 42 kDa plus high molecular weight material near the top of the gel (Figure 2B); the most prominent band was the one at 42 kDa. The fraction which eluted at 0.3 M sodium phosphate (fractions 20–23) contained predominantly nucleolin and protein B23 (Figure 2B). The specific activity of the enzyme was 100 times higher in the 0.2 M fraction than in the nucleolin (0.3 M) fraction. With casein

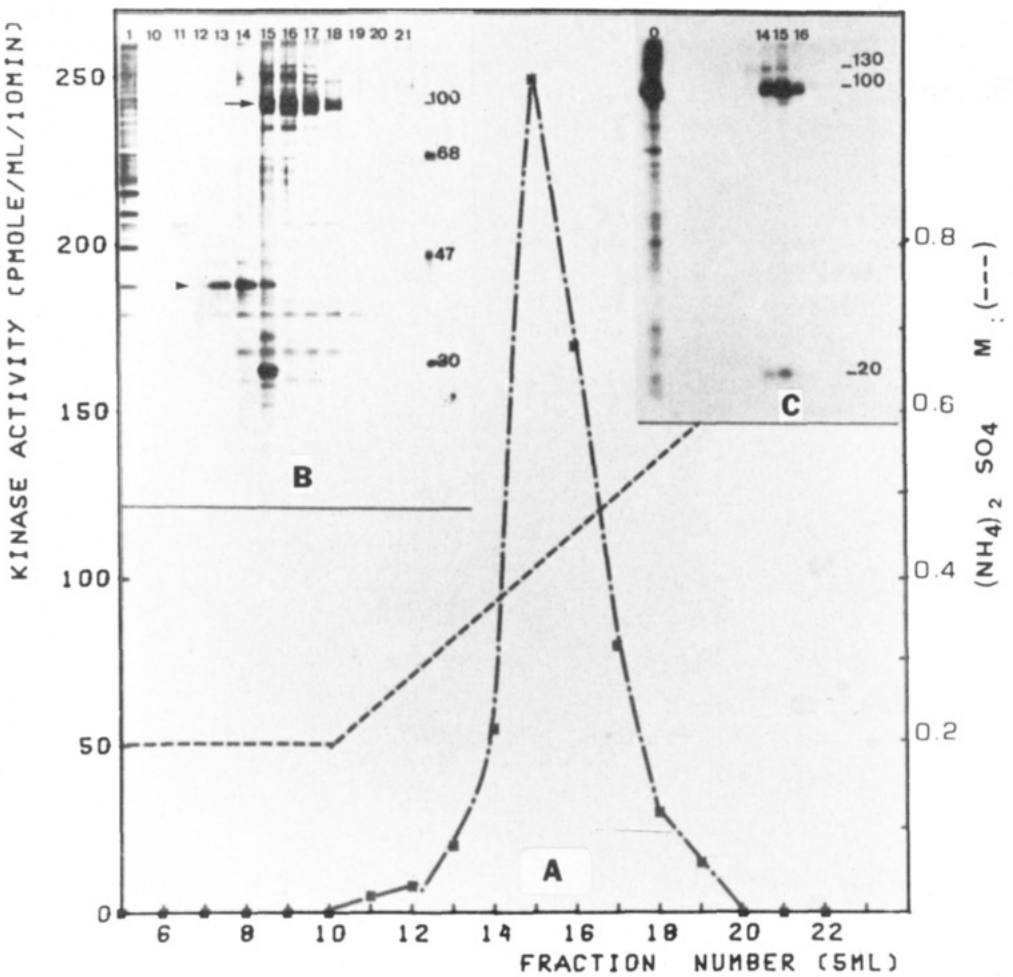

FIGURE 1: Heparin–Sepharose chromatography of nucleolar protein kinase. Nucleolar extract was applied in 0.2 M ammonium sulfate. Ten microliters of each fraction (5 mL) was assayed for casein kinase activity as described under Materials and Methods (A). Aliquots from column fractions (B and C) were analyzed by polyacrylamide gel electrophoresis. The wet gel was subjected to autoradiography for 4 h (C) and then silver stained (B). Fractions 14–17 containing the protein kinase were pooled for further fractionation. Approximate molecular masses are indicated on the right of insets. The positions of nucleolin and protein B23 are indicated by the arrow and arrowhead, respectively.

as phosphate acceptor, apparently 50% of the enzyme was recovered in each peak. However, the 0.3 M peak contained nucleolin that was shown to be the preferential substrate for the kinase (see Table II). To determine the amount of the kinase in the two peaks, assays were carried out in which nucleolin was used as a substrate and was added to the 0.2 M fraction at the same concentration that was endogenously present in the 0.3 M fraction. The exogenous nucleolin added to the 0.2 M fraction was preheated to destroy any contaminating kinase activity. Ten times more phosphate was incorporated into the 0.2 M fraction than into the 0.3 M fraction. This experiment established that 90% of the kinase activity toward nucleolin was present in the fraction which eluted at 0.2 M sodium phosphate. Samples of the 0.3 M fraction incubated with [γ-^{32}P]ATP in the absence of exogenous kinase were analyzed by gel electrophoresis (Figure 2C). Among proteins visible on the gel, the enzyme transferred phosphate only to nucleolin. A summary of the purification steps of the nucleolin-associated kinase is shown in Table I.

Characteristics of the Nucleolar Kinase. The enzyme was optimally active at magnesium concentrations between 5 and 10 mM (not shown). Enzymatic activity was not stimulated by the addition of calcium, although concentrations higher than 2 mM completely inhibited activity, as did zinc (5 mM) or heparin (50% inhibition at 0.3 μg/mL). Activity was not affected by addition of either dibutyryl cyclic AMP (1 mM) or cyclic GMP (1 mM). Spermine was a potent activator (50% stimulation at 1 mM). The extent of maximum stimulation was dependent on the protein used as phosphate acceptor (3-fold for nucleolin, 10-fold for casein). These properties, i.e., stimulation by spermine, inhibition by heparin and calcium, and no effect of cyclic AMP, strongly suggested that the kinase was casein kinase type NII (Hathaway & Traugh, 1982).

Table I: Purification of Nucleolar Kinase NII[a]

step	protein (mg)	units	sp act. (units/mg of protein)
S_2	16.8	290	16
heparin–Sepharose	4	354	88
hydroxyapatite	0.24	180	750

[a] One unit is defined as that amount of enzyme which catalyzes the incorporation of 1 nmol/min of inorganic phosphate from [γ-^{32}P]ATP into dephosphorylated casein at 30 °C. The 0.2 M peak (first peak) from the hydroxyapatite column (Figure 2) was used for calculating the specific activity.

This classification was further supported by the characteristics of the protein. In sedimentation experiments using glycerol gradients, a value of 7 ± 0.5 S was obtained (data not shown). This is relatively close to the value of 7.5 ± 0.3 S determined for casein kinase II by Hathaway and Traugh (1979). Furthermore, the presence of polypeptides of molecular masses of 25, 40, and 42 kDa in the high specific activity fraction (Figure 2B) suggests a subunit structure

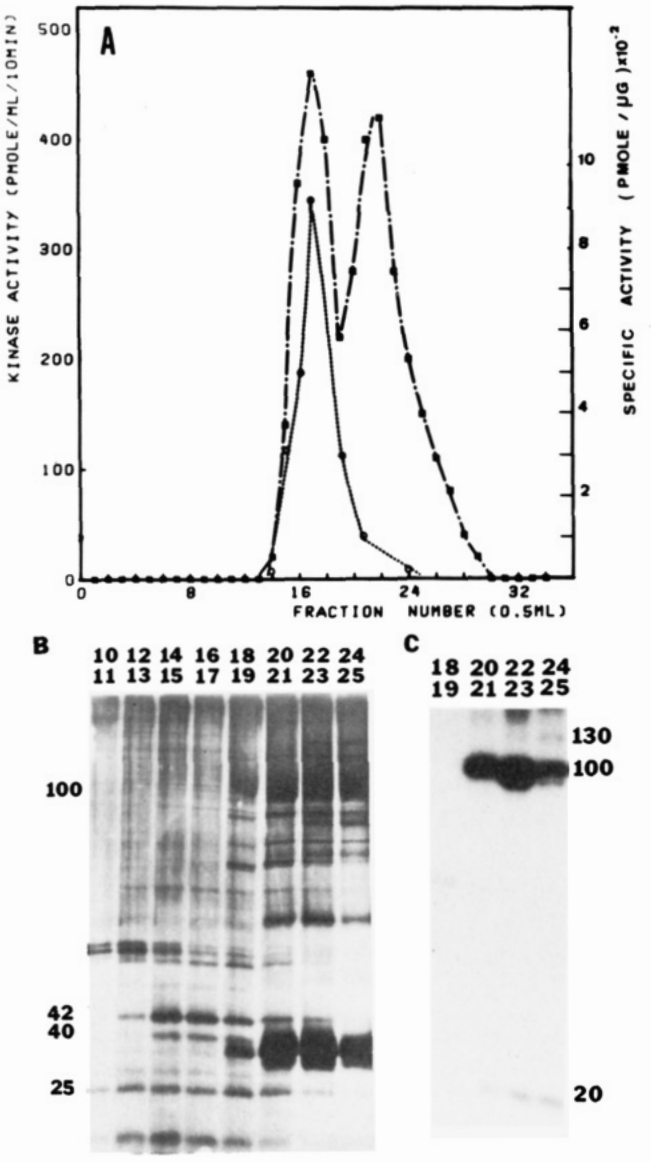

FIGURE 2: Hydroxyapatite chromatography of nucleolar protein kinase. Fractions 14–17 from the heparin–Sepharose column were directly applied as a hydroxyapatite–Ultrogel column. (A) Proteins were eluted by a 10 mM–0.6 M sodium phosphate gradient in the presence of 10% glycerol and 10 µg/mL leupeptin. Ten microliters of each fraction was assayed for casein kinase activity (■). The specific activity (●) of each fraction (picomoles of ^{32}P incorporated per milligram of protein) was determined. Half of each aliquot was analyzed by electrophoresis as described in the legend of Figure 1 (panel B, silver staining of the gel; panel C, autoradiogram). Approximate molecular masses in kilodaltons are indicated. The 25-, 40-, and 42-kDa bands correspond to the putatiave subunits of the kinase.

similar to that found in casein kinase II from rabbit reticulocytes (Hathaway & Traugh, 1979) or rat liver (Inoue et al., 1984; Qi et al., 1986).

Protein Acceptor Specificity. As was suggested by results presented in Figures 1 and 2, the type NII kinase was the major protein kinase in isolated nucleoli. In other experiments using histone H1 as substrate, casein kinase I, cyclic AMP dependent protein kinase, or protein kinase C activities were not detected (data not shown) in the extracts. This kinase possesses a high substrate preference for nucleolin but also transfers phosphate in vitro to a 130-kDa protein which is also labeled in vivo (data not shown). The 130-kDa protein appears to be identical with pp 135 previously isolated by Pfeifle et al. (1986) and shown to have many characteristics in common with nucleolin. In addition, a polypeptide of approximate

Table II: Apparent K_m and V_{max} Values for Nucleolar Kinase II

substrate	K_m	V_{max}	V_{max}/K_m
nucleolin[a]	7×10^{-4}	1.2	1714
topoisomerase I[a]	0.025	0.95	38
topoisomerase I[b]	0.03	0.7	23
topoisomerase II[c]	0.04	3.3	82
HMG 14[d]	0.15	0.17	1.1
casein[e]	0.65	0.46	0.7
casein[a]	1.0	0.25	0.25

[a] Conditions were as described under Materials and Methods. Assays containing various substrate concentrations (0–1 mg/mL) were initiated by the addition of [^{32}P]ATP. Values were deduced from Lineweaver–Burk plots, corresponding to three different experiments. [b] Values for topoisomerase I as substrate for Namalwa cell kinase NII from Mills et al. (1982). [c] Values for topoisomerase II as substrate for casein kinase II (both enzymes from *Drosophila melanogaster*) from Ackerman et al. (1985). [d] Values for HMG 14 as substrate for rabbit reticulocyte casein kinase II. [e] Values for native casein phosphorylated by rabbit reticulocyte casein kinase II from Hathaway and Traugh (1982).

molecular mass 20 kDa was labeled. This is possibly the β-subunit of the kinase which appears to migrate anomalously (20 kDa vs 25 kDa) on the gradient gels (10–16%) used for the autoradiography experiments. No other nucleolar protein was used as substrate by the purified kinase when it was added to nucleoli in which endogenous kinase had been heat inactivated.

The affinities of the enzyme for nucleolin, topoisomerase I, and casein were determined. The K_m of 7×10^{-4} mg/mL was respectively 36- and 1400-fold lower than values for topoisomerase I and casein (Table II). The values for these two substrates were similar to those reported by other authors using total nuclear kinase NII as enzyme. Furthermore, the V_{max} was high with nucleolin than with the other substrates. Using the term V_{max}/K_m as a measure of kinetic specificity, nucleolin appears to be the more specific substrate of nucleolar kinase NII (Table II). There was essentially no incorporation when histones H1 and H2A were used as substrates (data not shown).

Identification of the Phosphorylation Sites. To characterize the residues phosphorylated in vitro, ^{32}P-labeled nucleolin was hydrolyzed with 6 N HCl for 2 h at 105 °C. Phosphoamino acids were then separated by electrophoresis at pH 3.5. Only serine was labeled, and neither phosphothreonine nor phosphotyrosine was detected even when the in vitro labeling was carried out in the presence of spermine as activator (data not shown). This was confirmed by treatment of the protein with 0.33 M KOH overnight at 37 °C; under these conditions, no phosphoamino acids were detected, indicating that phosphotyrosine was not present.

It was previously shown that the in vitro labeled phosphorylation sites reside within the NH_2-terminal half of rat nucleolin (Rao et al., 1982). To further localize these sites, ^{32}P-labeled proteins were cleaved by CNBr. The majority of the label was recovered in a peptide with an apparent molecular mass of 29 kDa, with lesser and somewhat variable amounts incorporated into a 33-kDa fragment (Figure 3A). In addition, extracts were prepared from mouse and rat as well as CHO cell nucleoli and subjected to in vitro phosphorylation. Nucleolin was isolated and cleaved with CNBr, and the fragments were analyzed as above. The labeled cleavage products were essentially identical in all three cell types (Figure 3B).

To locate these fragments in the sequence, it was necessary to isolate them and do partial sequencing on them. This was done on Novikoff hepatoma rat nucleolin since sufficient quantities could only be obtained from that source. The CNBr

Table III: Sequence of 29- and 33-kDa CNBr Fragments[a]

	major sequence		minor sequence	
cycle	residue	yield (pmol)	residue	yield (pmol)
1	Ser	114	Ala	140
2	Glu	262	Pro	75
3	Asp	224	Pro	96
4	Glu	185	Pro	93
5	Asp	221	Lys	59
6	Asp	214	Glu	80
7	Ser	111	Val	38
8	Ser	135	Glu	67
9	Gly	72	Glu	62

[a] Approximately 400 pmol of the unphosphorylated mixture of the 29- and 33-kDa CNBr fragments of rat nucleolin was applied to the gas-phase sequencer using the on-line system. The yields of PTH-amino acids are uncorrected. For comparison, the published sequences of segments of CHO nucleolin (Lapeyre et al., 1987) are Ala-Pro-Pro-Pro-Lys-Glu-Val-Glu-Glu- and Ser-Glu-Glu-Glu-Asp-Asp-Ser-Ser-Gly beginning at residues 17 and 33, respectively.

digest of nucleolin was fractionated by HPLC as previously described (Lapeyre et al., 1986). The 29-kDa fragment and smaller quantities of a 33-kDa fragment (Figure 3C) eluted relatively early on the gradient [see Figure 2 of Lapeyre et al. (1986)]. It was also possible to phosphorylate this preparation to obtain ^{32}P incorporation into both fragments (Figure 3C). This HPLC-purified material was subjected to Edman degradation, and the predominant sequence was found to begin at residue 33 of the protein (Table III). The rat sequence was found to be identical with the CHO sequence with the exception of residue 35 (a Glu exchanged for an Asp). A minor sequence was also found which began at residue 17 (Table III). Thus, the minor sequence (33 kDa) is derived from an amino-terminal extension of the 29-kDa fragment as a result of partial cleavage by CNBr (Figure 4). By molecular mass and amino acid composition (not shown), the 29-kDa fragment extends to residue 284 in CHO nucleolin (Lapeyre, 1987).

To more precisely locate the phosphorylation sites, ^{32}P-labeled nucleolin was subjected to tryptic digestion and mapping. It was previously shown that virtually all of the phosphorylation sites in nucleolin are contained in highly acidic tryptic peptides (Mamrack et al., 1979; Rao et al., 1982). The locations of these regions are now known from the cDNA-derived protein sequence in CHO nucleolin (Lapeyre et al., 1986). Because the highly acidic peptides do not run well on standard thin-layer or paper chromatography electrophoresis systems, the fragments were separated on the basis of molecular mass using the Burr and Burr (1983) gel system. After autoradiography, two tryptic phosphopeptide bands of apparent molecular masses 3300 and 4600 Da were seen (not shown). The 3.3-kDa peptide corresponds to acidic region A (Figure 4) which begins at residue 22 and ends at residue 49, thereby confirming the results of sequence analyses. The 4.6-kDa peptide corresponds to the molecular mass of the C region (residues 180–221) or the D region (residues 236–271). The D region of nucleolin does not contain serine or threonine and therefore cannot be phosphorylated by this enzyme. It some preparations, traces of radioactivity were seen in a 3.9-kDa band possibly corresponding to the B region (Figure 4), but this was always a minor component. Thus, only two of the highly acidic regions appear to be significantly phosphorylated by the nucleolin-associated kinase under the conditions employed.

To locate the phosphorylated serines near the N-terminal end (A region) of the 29-kDa fragment, the ^{32}P-labeled preparation was subjected to six cycles of Edman degradation, and the radioactivity released at each cycle was monitored.

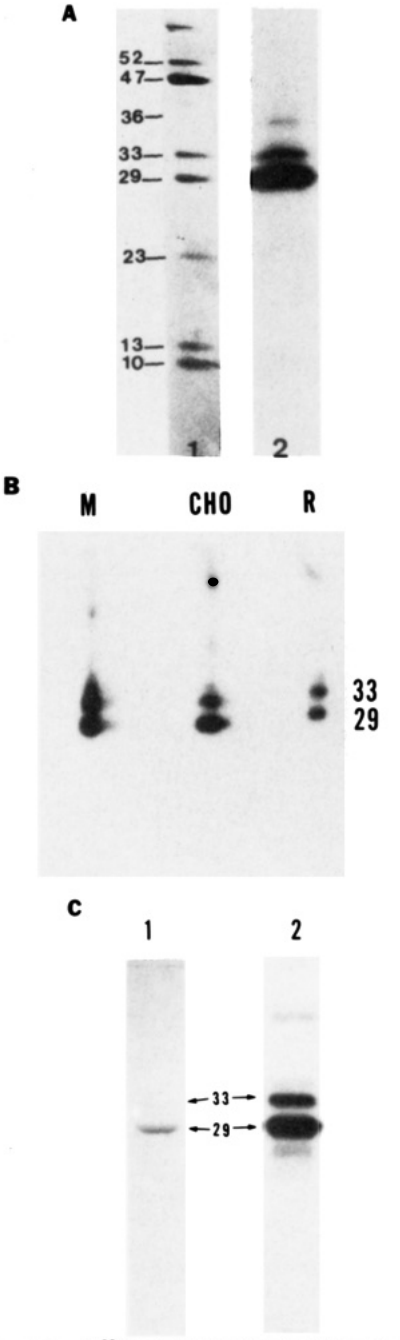

FIGURE 3: Analysis of ^{32}P-labeled CNBr peptides of nucleolin by polyacrylamide gel electrophoresis. In vitro labeled nucleolin was digested with CNBr as described under Materials and Methods. (A) Analysis of CNBr peptides labeled in vitro. Lane 1, Coomassie blue staining; lane 2, autoradiogram of phosphorylated peptides. (B) Species comparison of labeling of CNBr fragments of nucleolin. Heparin–Sepharose fractions containing kinase and nucleolin (see Figure 1) from mouse (M), Chinese hamster ovary cell (CHO), or rat (R) nucleoli were subjected to labeling in vitro and analyzed as in panel A, lane 2. The 33- and 29-kDa fragments are indicated. (C) Analyses of HPLC-purified 29-kDa CNBr fragment from rat nucleolin. The CNBr digest was subjected to purification as described under Materials and Methods and analyzed by polyacrylamide gel electrophoresis as in panels A and B. Lane 1, Coomassie blue stained material. Minor quantities of the 33-kDa fragment were visible. Lane 2, autoradiograph of gel as in lane 1 after HPLC-purified material was subjected to phosphorylation with purified nucleolar kinase.

Figure 5 indicates that radioactivity was released predominantly from cycle 1 which corresponds to serine-33 in the nucleolin sequence. Radioactivity in cycles 2–6 appears to be carryover since none of these positions contains serine or threonine. Although serines are present at positions 39 and

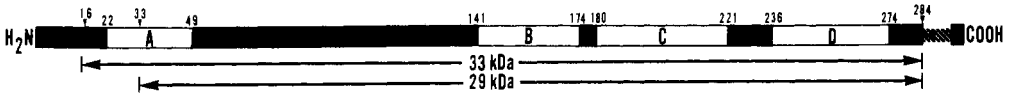

FIGURE 4: Location of acidic regions and CNBr fragments in the amino-terminal third of nucleolin. The map is based on the cDNA-deduced protein sequence of CHO cell nucleolin; the rat nucleolin sequence is not significantly different. Clear parts of the diagram with capital letters indicate the highly acidic tryptic fragments. The C-terminal two-thirds of the molecule is represented by the short cross-hatched area. Numbers directly above the map denote the positions of the lysine residues which define the boundaries of the acidic regions. The phosphorylated CNBr fragments (29 and 33 kDa) were placed in the sequence by Edman degradation (see Table III). Numbers above the arrowheads designate positions of methionine residues cleaved to produce the major CNBr fragments (33 and 29 kDa) represented by the solid lines below the map.

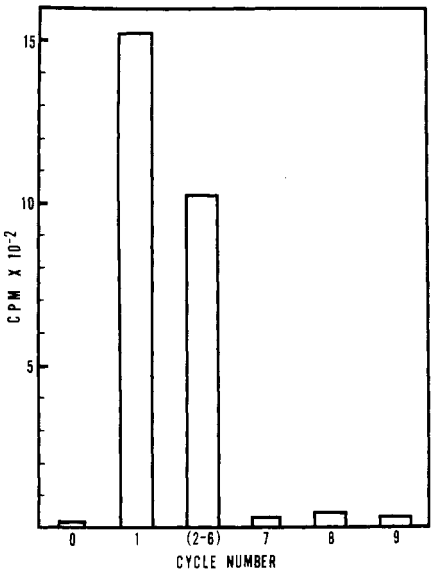

FIGURE 5: Location of the phosphorylated residue in the amino-terminal end of the 29-kDa CNBr fragment. Approximately 400 pmol of in vitro labeled 29-kDa fragment was applied to the gas-phase sequencer. Release of ^{32}P radioactivity at each cycle of Edman degradation was measured by scintillation counting. The cpm in cycles 2–6 were combined since no serines were present. Actual cpm for cycles 2, 3, 4, 5, and 6 are 628, 284, 209, 66, and 44, respectively. The N-terminal sequence of the unlabeled 29-kDa fragment is Ser-Glu-Asp-Glu-Asp-Asp-Ser-Ser-Gly.

40, significant quantities of radioactivity were not released in the corresponding cycles. Because of the relatively low yield of ^{32}P released (approximately 1%), the position of the labeled serine was checked by using the method described by Wang et al. (1986) in which a portion of the filter disk was analyzed after each cycle of Edman degradation. The protein on the filter aliquots was removed with 70% formic acid and subjected to SDS gel electrophoresis followed by autoradiography. The results of this experiment (not shown) were essentially the same as above; i.e., significant reduction in radioactivity was found only at cycle 1. Therefore, in the A region of the protein, only the serine at position 33 appears to be labeled under these conditions.

The sites in the other in vitro phosphorylated acidic region C have been previously identified after labeling under similar conditions (Mamrack et al., 1979). Therefore, no attempt was made to determine the sites labeled. There are two serine residues in the CHO cell nucleolin C region sequence: positions 187 and 209. Both of the equivalent residues in rat nucleolin appear to be phosphorylated, and it is likely that they are phosphorylated in the CHO cell protein. The sites phosphorylated in nucleolin by this enzyme are summarized in Table IV.

Table IV: Recognition Sequences and Phosphorylation Sites for Casein Kinase II

substrate	phosphorylated sequence	ref
dephosphorylated		
S_1 casein	Glu^{63}-Ser(P)-Ile-Ser(P)-Ser(P)-Glu-Glu-Ile	*b*
S_2 casein	His^{6}-Val-Ser(P)-Ser(P)-Ser(P)-Glu-Glu-Ser	*b*
HMG 14	Glu^{85}-Ser-Pro-Ala-Ser(P)-Asp-Glu-Ala-Glu-Glu	*c*
nucleolin		
rat	Asp^{29}-Glu-Glu-Met-Ser(P)-Glu-Asp-Glu-Asp	*d*
CHO	Asp^{29}-Glu-Glu-Met-Ser(P)-Glu-Glu-Glu-Asp	*e*
rat and CHO	Ala^{183}-Ala-Pro-Ala-Ser(P)-Glu-Asp-Glu-Asp-Glu	*e, f*
rat	Asp-Ser-Gln-Glu-Ser(P)-Glu-Glu-Glu-Asp	*f*
CHO	Glu^{205}-Glu-Asp-Asp-Ser(P)-Glu-Glu-Glu-GluGlu	*d*
protein B23	Glu-Asp-Ala-Glu-Ser(P)-Glu-Asp-Glu-Asp-Glu	*g*

[a] Underlined residues indicate similarities in positions of acidic residues on the carboxyl sides of serines typical of casein kinase II sites. The serines phosphorylated by the nucleolar kinase are contained in two tryptic peptides (residues 22–49 and 180–221) of nucleolin. Numbers indicate the position of the amino acid in the proteins. In the case of nucleolin, numbers correspond to the CHO protein since the complete sequence of the rat protein is not known. In CHO nucleolin, there are two serines between residues 180 and 221; it was not possible from these studies to determine whether either one or both were phosphorylated. [b] Hathaway & Traugh (1982). [c] Walton et al. (1985). [d] This paper. [e] Lapeyre et al. (1986). [f] Mamrack et al. (1979). [g] Chan et al. (1986).

Discussion

In the present study, we have characterized a nucleolar kinase that preferentially phosphorylates nucleolin compared to other substrates tested. The properties of this kinase allow it to be classified as a casein kinase NII. The enzyme is cyclic AMP independent, inhibited by heparin, and stimulated by spermine with casein as substrate. The subunit structure of the enzyme appears to be similar to the structure of casein kinase NII (Hathaway & Traugh, 1979); i.e., three major peptides, having molecular masses of 25, 40, and 42 kDa which could correspond to α, α', and β subunits, are present in the kinase-enriched fraction. However, there is now evidence that these subunits are derived from a single polypeptide (Delpech et al., 1986).

It has recently been shown that the major nuclear product of phosphorylation by casein kinase II in rapidly proliferating tissues is nucleolin (Geahlen & Harrison, 1984; Schneider et al., 1986). These studies confirm this and suggest that the kinase is closely associated with its substrate in the nucleolus. The simplest explanation of the high affinity of the enzyme for nucleolin is the presence of multiple highly acidic regions (Mamrack et al., 1979; Lapeyre et al., 1987). Since the enzyme appears to have a high affinity for acidic regions, as judged by its recognition of sites rich in aspartic and glutamic acids as well as its inhibition by heparin, the presence of four highly acidic regions in nucleolin (Figure 4) would be expected to enhance this affinity. On the other hand, it is conceivable that the structure of the nucleolar enzyme differs from that of kinases which phosphorylate other nuclear substrates; i.e., different enzymes have their own preferred substrates. The existence of at least two different NII kinases has been shown (Qi et al., 1986), although further work is needed to show identity or nonidentity of this nucleolar kinase with nucleoplasmic or chromatin-associated enzymes.

The purified nucleolar kinase behaves for the most part like a typical casein NII kinase in its recognition of acidic sequences in substrates. However, the kinase did not appear to phosphorylate all of the potential casein kinase type sites. Casein kinase II generally phosphorylates serine or threonine residues which carry at least two aspartic or glutamic acid residues on their C-terminal sides (Hathaway & Traugh, 1982). The nucleolar enzyme labeled these kinds of sites in the A and C acidic regions (residues 33, 187, and 209) but did not phosphorylate to a significant extent serines-143 and -156 which are flanked by acidic residues in the B region. It is also interesting to note that two other serine residues (39 and 40) which are separated from two glutamic acid residues by a glycine were not phosphorylated. This is presumably because the enzyme prefers acidic residues to be immediately adjacent to the serines, although in some substrates this is not an absolute requirement (Marin et al., 1986). The final potential site is serine-27 in the CHO protein which is a methionine in the rat protein (M. O. J. Olson, unpublished results). It was not possible to obtain enough of the CHO 33-kDa fragment to determine whether the serine is phosphorylated in that species. Another observation was that although protein B23 contains typical casein kinase NII sites (Chan et al., 1986), it was not labeled by partially purified enzyme preparations. Additional studies will be necessary to determine whether all of the potential NII types of sites are actually phosphorylated in vivo.

Several pieces of evidence suggest that phosphorylation of nucleolin contributes to its function. Nucleolin has been shown to be involved in rDNA transcription in vitro (Bouche et al., 1984) by acting as an inhibitor of pre-rRNA synthesis when its proteolysis is inhibited. In the absence of the protease inhibitor, maturation of phosphorylated nucleolin into defined peptides occurs, and transcription of rDNA is allowed. It has recently been shown that testosterone and polyamines stimulate the phosphorylation of nucleolar proteins in rat ventral prostate and increase degradation of nucleolin to a 59-kDa peptide (Suzuki et al., 1985). Furthermore, several correlations have been established between the increase in intracellular levels of polyamines and stimulation of pre-rRNA synthesis (Tabor & Tabor, 1984). Ornithine decarboxylase plays a key role in polyamine metabolism and has been shown to act directly on rDNA transcription in *Xenopus laevis* (Atmar et al., 1980; Russell, 1983). From these results and from the data presented in this paper, we propose the following model of regulation of rDNA transcription: in its unphosphorylated form, nucleolin acts as an inhibitor of rDNA transcription. As a result of some stimuli (e.g., androgens), an increase in the synthesis of ornithine decarboxylase occurs followed by an increase in polyamine concentration. This could result in an activation of nucleolar kinase II and in an increase of nucleolin phosphorylation. Maturation of the phosphorylated nucleolin would then allow rDNA transcription either at the initiation or at the elongation level. Furthermore, activation of nucleolar kinase II would also result in the phosphorylation of RNA polymerase I (Duceman et al., 1981) and in the activation of the enzyme. Evidence has also been presented that topoisomerase (whose activity may be regulated by phosphorylation) is involved in control of rDNA transcription (Bonven et al., 1985). Thus, nucleolar kinase II may play a key role in the regulation of rDNA transcription and ribosome assembly.

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Registry No. Casein kinase, 52660-18-1; topoisomerase, 80449-01-0.

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Structure of Cutinase Gene, cDNA, and the Derived Amino Acid Sequence from Phytopathogenic Fungi†

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ABSTRACT: Cutinase is an extracellular fungal enzyme that allows pathogenic fungi to penetrate through the cuticular barrier into the host plant during the initial stages of the fungal infection. mRNA isolated from glucose-grown *Colletotrichum capsici*, induced to produce cutinase by the addition of cutin hydrolysate, was used to prepare cDNA which was cloned in the expression vector λ gt11. The primary structure of the cutinase from *C. capsici* was deduced from the nucleotide sequence of the cloned cutinase cDNA. Amino acid sequences of two tryptic peptides isolated from cutinase produced by *C. capsici* completely matched with two segments of the amino acid sequence deduced from the nucleotide sequence, strongly suggesting that the cloned cDNA was authentic cutinase cDNA. The cDNA clone was used as a probe to screen *C. capsici* and *Colletotrichum gloeosporioides* genomic libraries constructed in Charon 35 and EMBL 3, respectively. The nucleotide sequences of the cutinase structural genes from *C. capsici* and *C. gloeosporioides* were also determined. S1 mapping was used to reveal the transcriptional start sites and polyadenylation site of the primary transcript from *C. capsici*. The primary sequences and gene structure of the enzymes from the *Colletotrichum* species were compared with the primary structure and gene structure of a cutinase from *Fusarium solani* f. sp. *pisi*. A comparison of the deduced primary structures of the enzymes showed that residues involved in the catalytic triad and disulfide cross-linking of cutinase are strongly conserved. Yet, only 43% of the residues are conserved between all three enzymes. A comparison of the structure of the three genes revealed the location of the single intron has been conserved. The transcriptional start site of the *C. capsici* gene was centered on the sequence TCCAGACCA, the core of which (CAGAC) is found repeated after 21 nucleotides. The same core sequence, repeated after 11 nucleotides, was also identified in the 5′ nontranslated regions of the *C. gloeosporioides* and *F. solani* genes.

Aerial plant organs are protected by a cuticle that is composed of an insoluble polymeric structural component called cutin and associated soluble lipids collectively called waxes (Kolattukudy, 1980a). Cutin is a polyester composed of hydroxy and hydroxyepoxy fatty acids (Kolattukudy, 1980b, 1981). Germinating fungal spores of phytopathogens secrete cutinase to assist in the penetration through the cuticular barrier, and inhibition of this enzyme prevents infection through the intact cuticular barrier (Kolattukudy, 1985). Cutinase is induced in the spores of virulent pathogenic fungi soon after contact with cutin (Woloshuk & Kolattukudy, 1986). Evidence was presented that the unique cutin monomers released by the small amounts of cutinase present on the spores are the true inducers. Although such monomers were shown to cause a large and rapid increase in the level of cutinase transcripts, the mechanism of regulation of expression of this gene has not been elucidated.

An understanding of how hydroxy fatty acids regulate cutinase gene expression may lead to the design of novel methods

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