p34cdc2 Is Physically Associated with and Phosphorylated by a cdc2-specific Tyrosine Kinase

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Abstract

The mammalian homologue of the yeast cdc2 gene encodes a 34-kilodalton serine/threonine kinase that is a subunit of M phase-promoting factor. Recent studies have shown that p34cdc2 is also a major tyrosine-phosphorylated protein in HeLa cells and that its phosphorysitory content is cell cycle regulated and related to its kinase activity. Here, we show that cdc2 is physically associated with and phosphorylated in vitro by a highly specific tyrosine kinase. Tyrosine phosphorylation of cdc2 in vitro occurs at tyrosine 15, the same site that is phosphorylated in vivo. The association between the two kinases takes place in the cytosolic compartment and involves cyclin B-associated cdc2. Evidence is presented that a substantial fraction of cytosolic cdc2 is hypophosphorylated, whereas nuclear cdc2 is hyperphosphorylated. Finally, we show that the tyrosine kinase associated with cdc2 may be a 67-kilodalton protein and is distinct from src, abl, fms, and other previously reported tyrosine kinases.

Introduction

Genetic studies in yeast have identified a number of genes that are necessary for completion of a normal cell cycle (reviewed in Ref. 1). Among these genes, cdc2 has been of great interest because its 34-kilodalton serine/threonine kinase product is required both at "Start" and at G2-M in Schizosaccharomyces pombe (2). cdc2 is highly conserved evolutionarily, and its homologue has been found in every eukaryotic organism in which it has been sought, including man. In Xenopus oocytes, cdc2 has been shown to be the catalytic subunit of M phase-promoting factor, a protein complex consisting of p34cdc2 and p45 cyclin, which is required for the initiation of meiosis (3, 4). In human HeLa cells, the amount of p34cdc2 is relatively consistent throughout the cell cycle, although its histone H1 kinase activity is greatly modulated, rising from low levels in G1-S to a peak in G2-M (5, 6). Phosphorylation of p34cdc2 on tyrosine is also cell cycle regulated in both yeast and human cells and inversely related to p34cdc2 activity; in fact, tyrosine dephosphorylation of cdc2 is a prerequisite for its activation (5, 7–9). Therefore, a more complete understanding of the cell cycle requires identification of a number of positive and negative regulators of p34cdc2 kinase activity.

Results

In an attempt to identify the critical tyrosine kinase involved in regulating cdc2, we have lysed murine myeloid cells and immunoprecipitated cdc2 under mild conditions designed to preserve protein complexes prior to in vitro kinase reactions. We found that multiple proteins were phosphorylated after immunoprecipitation with an antiserum, 88-2, directed against the COOH-terminal cdc2 peptide DNQIKKM (Figure 1A, Lane 1), but that phosphorylation of p23, p34cdc2, p50, p60, p95, and p140 was specifically competed by an excess of the peptide immunogen incubated with the antibody before immunoprecipitation (Fig. 1A, Lane 2); preclearing the lysates with normal rabbit serum before immunoprecipitation with the carboxyl-terminal antiserum had no effect on phosphorylation of p34cdc2 (data not shown). Since cdc2 is not reported to autophosphorylate efficiently, these results suggested that a second protein kinase was present in the immunoprecipitate.

We performed both KOH hydrolysis of SDS3 gels containing in vitro phosphorylated cdc2 immunoprecipitates and phosphoamino acid analysis of proteins eluted from gel slices to determine which amino acids were phosphate acceptors. Fig. 18 shows autoradiography of immunoprecipitated cdc2 before (Lane 1) and after (Lane 2) 1 m KOH treatment. Only three phosphoproteins, p95, p60, and p34cdc2, were relatively stable to alkaline hydrolysis, suggesting that they contained phosphotyrosine or phosphothreonine. Phosphoamino acid analysis of in vitro phosphorylated cdc2 eluted from gels not treated with KOH (Fig. 1C) showed that cdc2 contained mostly phosphotyrosine, with lesser amounts of both phosphoserine and phosphothreonine, whereas both p60 and p95 contained phosphothreonine and phosphoserine, but no phosphotyrosine.

We next attempted to assay the activity of the immunoprecipitated tyrosine kinase using several exogenous tyrosine kinase substrates, including, poly(Glu-Tyr), and a 20-aminoc acid peptide derived from the human cdc2 sequence and containing tyrosine 15, the site of tyrosine phosphorylation in vivo (9). Under conditions where tyrosine phosphorylation of p34cdc2 occurred readily, none of the exogenous substrates was phosphorylated (data not shown).

Received 4/4/91.

1 This work was supported with funds from the Department of Health and Human Services under Contract NO1-CO-74102 with Program Resources, Inc./DynCorp and under Contract NO1-CO-74101 with ABL. The contents of this publication do not necessarily reflect the views or policies of the Department of Health and Human Services, nor does mention of trade names, commercial products, or organizations imply endorsement by the U.S. Government.

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3 The abbreviations used are: SDS, sodium dodecyl sulfate; HPLC, high performance liquid chromatography; BSA, bovine serum albumin; IL-3, interleukin 3.
not shown). This result suggested that the tyrosine phosphorylation of p34<sup>cdk2</sup> was not the result of nonspecific trapping in the immunoprecipitates of a highly active and promiscuous tyrosine kinase.

In order to further ensure that the presence of the tyrosine kinase in cdk2 immune precipitates was not an artifact, two additional cdk2 antipeptide antisera were used. SP304, kindly provided by Richard S. Paules, is directed against the conserved 16-amino acid sequence EGVPSTAIRRESLKLK (10), or PSTAIR region, as it is commonly called. David Beach graciously provided an additional antibody directed against the same COOH-terminal sequence as 88-2. Fig. 2A shows the results of using antibodies specific for different epitopes to immunoprecipitate p34<sup>cdk2</sup> and associated proteins before in vitro kinase reactions. We found that while both COOH-terminal peptide antisera (Fig. 2A, Lanes 2 and 3) coprecipitated the tyrosine kinase activity with p34<sup>cdk2</sup>, the SP304 PSTAIR antiserum did not (Fig. 2A, Lane 1). SP304 antiserum did, however, reprecipitate cdc2 which had previously been precipitated with 88-2 and phosphorylated in vitro (Fig. 2A, Lane 4). Therefore, the reason that cdc2 was not phosphorylated in the SP304 immunoprecipitation could not be because SP304 did not react with cdc2. We considered it possible that, because the PSTAIR region is only 27 amino acids COOH-terminal to tyrosine 15, binding of the PSTAIR antibody might sterically block the in vitro phosphorylation. This was not the case, however, because addition of an excess of PSTAIR antiserum to a COOH-terminal cdc2 immunoprecipitate was not capable of inhibiting the in vitro tyrosine phosphorylation of cdc2. An alternative explanation of this result is that the tyrosine kinase is associated with cdc2 through the conserved PSTAIR sequence also recognized by SP304.

We further examined the specificity of the reaction by reprecipitating both the protein complexes immunoprecipitated with carboxyl terminal and PSTAIR antisera after in vitro kinase reactions with a monoclonal antibody specific for phosphotyrosine (Fig. 2B) Lanes 1–4 show in vitro phosphorylations after immunoprecipitation with COOH-terminal and SP304 PSTAIR antisera performed with and without peptide competition. We again found that p34<sup>cdk2</sup> was only phosphorylated in the COOH-terminal immunoprecipitation, although similar amounts of p34<sup>cdk2</sup> protein were present in SP304 immunoprecipitates as detected by Western blot (data not shown). When duplicate in vitro reactions were denatured, reduced, and reprecipitated with a monoclonal antibody to phosphotyrosine (Fig. 2B, Lanes 5–8), we found that no phosphotyrosine-containing proteins were detected in SP304 immunoprecipitations, whereas both p34<sup>cdk2</sup> and p100 were detected in carboxyl-terminal immunoprecipitations performed in the absence of the peptide competitor. These results strongly suggest that the tyrosine phosphorylation of cdc2 in vitro is due to the activity of a highly specific tyrosine kinase that is complexed with cdc2, possibly through the PSTAIR region.
Fig. 2. Specificity of in vitro tyrosine phosphorylation. A, autoradiography of in vitro kinase reactions after immunoprecipitation with amino-terminal peptide antiserum SP304 (Lane 1). COOH-terminal peptide antiserum from D. Beach (Lane 2), COOH-terminal peptide antiserum 88-2 (Lane 3), and reprecipitation with SP304 of cdc2 phosphorylated in 88-2 immune complex kinase reaction (Lane 4). B, immunoprecipitations performed with COOH-terminal or PSTAIR antisera with and without competition were phosphorylated in vitro. Duplicate samples were then denatured with boiling SDS and reprecipitated with a monoclonal antibody to phosphotyrosine (UBI).

If the tyrosine kinase present in the carboxyl-terminal cdc2 immunoprecipitations were the same tyrosine kinase that inactivates cdc2 in vivo, then it would be expected to phosphorylate cdc2 on tyrosine 15, the only site of tyrosine phosphorylation in vivo (9). To determine which tyrosine was phosphorylated during the in vitro reaction, we used a synthetic peptide corresponding to amino acids 7-26 of S. pombe p34cdc2, which contains the tyrosine phosphorylated in vivo. Fig. 3A shows the peptide sequence and protease cleavage sites. When this peptide was phosphorylated in vitro by immunoprecipitated pp60^v-src^, we found that only tyrosine was phosphorylated, as demonstrated by the phosphoamino acid analysis performed on the peptide (Fig. 3B). Since the peptide contains two tyrosine residues, we wished to know whether both or only one of them was phosphorylated. To determine this, we digested the peptide with V8 protease, which cleaves at the COOH-terminal side of the glutamic acid, three amino acids NH2-terminal to tyrosine 15 (Fig. 3A). The radioactive product of the V8 digestion was then subjected to Edman degradation and scintillation counting to determine with which of the two tyrosine residues the radioactivity was associated (Fig. 3C). We found that the radioactivity was released on the third cycle of degradation, corresponding to tyrosine 15.

Having found that the synthetic peptide was phosphorylated by src only on tyrosine 15, we next performed two-dimensional peptide mapping on digests of both the src-phosphorylated peptide and cdc2 phosphorylated in vitro in the complex precipitated by 88-2 antiserum (Fig. 3, D-I). We found that the radioactive product generated by trypsin digestion of the synthetic peptide comigrated with the major trypsin product from in vitro phosphorylated cdc2 (Fig. 3, D-F). Since the predicted trypsin digestion product contains both tyrosine 15 and tyrosine 19, we performed a further digestion with thermolysin, which cleaves between the two tyrosines. Double digestion of the synthetic peptide generated a radioactive product containing only tyrosine 15, which comigrated with the major radioactive cdc2 double digestion product (Fig. 3, G-I). Phosphoamino acid analysis of the cdc2 digestion product eluted from the thin layer plate confirmed that it contained only phosphotyrosine (data not shown). These results establish that the tyrosine kinase associated with immunoprecipitated cdc2 phosphorylates the same tyrosine residue that is phosphorylated in vivo and that has previously been shown to inhibit cdc2 activity (5, 8, 9). No other site of tyrosine phosphorylation was detected, further suggesting the specific nature of the reaction.

cdc2 has been reported to be present in both cytoplasmic and nuclear fractions by immunofluorescence (11, 12), and we therefore wished to determine where the interaction of cdc2 and the tyrosine kinase takes place. Using our standard lysis conditions, nuclei remain microscopically intact, and chromatin is not solubilized; however, exposure to the detergent could have released most nuclear cdc2 into the cytosol. To examine the proteins associated with cdc2 in cytoplasmic and nuclear compartments, lysates from unlabelled cells and cells
metabolically labeled with $[^35]S$-methionine or with $^{32}P$ was separated into nuclear and cytosolic fractions and immune precipitated with 88-2 antisera in the presence or absence of the peptide competitor. The unlabeled immunoprecipitates were then subjected to the in vitro kinase reaction and compared with the in vivo labeled immunoprecipitates (Fig. 4). cdc2 immunoprecipitated from the cytosol (Fig. 4, Lane 2) was phosphorylated in vitro, whereas cdc2 immunoprecipitated from the nuclear fraction was not (Fig. 4, Lane 4). This result was not due to loss of all of the cdc2 from the nuclear fraction, since similar amounts of cdc2 were found in the cytosolic (Fig. 4, Lane 10) and nuclear (Fig. 4, Lane 12) fractions from cells labeled with $[^35]S$-methionine. The slower electrophoretic mobility of the nuclear cdc2 (Fig. 4, Lane 12) compared with cytoplasmic cdc2 (Fig. 4, Lane 10) is probably due to differences in phosphate content, since cdc2 immunoprecipitated from cytoplasmic (Fig. 4, Lane 6) and nuclear (Fig. 4, Lane 8) fractions of cells labeled with orthophosphate reflect the same difference in mobility and since in vitro phosphorylation of cdc2 immunoprecipitated from cytosol (Fig. 4, Lane 2) produces cdc2 with similar mobility to nuclear cdc2 (Fig. 4, Lanes 8 and 12). It is also noteworthy that the cdc2 that is
phosphorylated in the in vitro reaction is associated with cyclin B, as seen in Fig. 4, Lanes 2, 6, and 10. In other experiments (data not shown), we have found that performing the in vitro kinase reaction on cdc2/cyclin complexes immunoprecipitated with a cyclin B antiserum, kindly given to us by Jonathan Pinas, produces a pattern of phosphorylation identical to that obtained with the COOH-terminal antiserum. These observations are in agreement with the results of Solomon et al. (13), who reported that cyclin-associated cdc2 is the form that is phosphorylated on tyrosine in vivo. Given this, it is noteworthy that both we (data not shown) and others (14) have found that cdc2 precipitated with PSTAIRE antiserum is not associated with cyclin, and, as shown in Fig. 2, PSTAIRe-precipitated cdc2 is not phosphorylated during the in vitro reaction. The results of our fractionation experiments are consistent with the suggestion that the interaction of cdc2 with the tyrosine kinase takes place in the cytosol and also show that the phosphorylation state of cdc2 is correlated with, and may regulate, its subcellular distribution.

As a first approach toward identifying which of the coprecipitated proteins is the tyrosine kinase, we have used the photoaffinity probe 8-azido-ATP, which specifically cross-links to proteins containing ATP binding sites. When cdc2 immunoprecipitates were labeled with the probe, p34cdc2 and p67 were specifically labeled and competed by the 88-2 carboxyl-terminal peptide, suggesting that p67 could be the cdc2 tyrosine kinase (Fig. 5). In addition, other experiments involving reprecipitations of in vitro phosphorylated cdc2 immunoprecipitates with antiserum to various tyrosine kinases indicated that the cdc2 tyrosine kinase is not src, abl, fer, fes, fms, or yes (data not shown).

**Discussion**

We have shown in this paper that a tyrosine kinase that phosphorylates p34cdc2 on tyrosine 15 can be isolated in COOH-terminal cdc2 peptide immunoprecipitates and assayed by in vitro kinase reactions. The tyrosine kinase present in the cdc2 immunocomplexes is specific for tyrosine 15, which is the only tyrosine residue in cdc2 that is phosphorylated in vivo. Furthermore, our work shows that only cdc2 that is associated with cyclin can be tyrosine phosphorylated in the immunoprecipitate and that the tyrosine kinase associates with cdc2/cyclin in a cytosolic compartment. These results from our in vitro studies replicate what has been found in vivo (13) and in Xenopus egg extracts (15).

Our findings indicate that the cdc2-regulatory tyrosine kinase forms a relatively stable complex with cdc2/cyclin that can be assayed and potentially purified. In fact, we have recently been able to partially purify the cdc2/cyclin tyrosine kinase complex by HPLC (data not shown). Given this, it can fairly be asked, why in vitro tyrosine phosphorylation of cdc2 not been observed by other groups investigating p34cdc2 regulation? In partial
answer to that question, we have found that replacing the Nonidet P-40 in the lysis buffer with Triton X-100, or a number of other detergents, strongly inhibits the in vitro reaction and that the presence of BSA in the lysis buffer stabilizes the complex. An additional constraint on the reaction is that it absolutely requires Mn2+. Finally, we have tested a number of different mammalian cell lines and tissues and have found wide variation in the amount of tyrosine phosphorylation observed in the in vitro reaction, presumably due to differences in the stability of the cdc2/cyclin/tyrosine kinase complex; HeLa cells, for instance, are not a particularly good source, whereas the murine 32D cell line and the human neuroblastoma cell line BE2 both exhibit extensive tyrosine phosphorylation of cdc2 in vitro.

Recently, it has been shown that p107-reg., the negative regulator of p34cdc2 activity in yeast, has both serine and tyrosine kinase activity in vitro, although its sequence is more similar to other serine/threonine kinases than to tyrosine kinases (16). Due to its inhibitory effect on p34cdc2 activity in vivo and its in vitro kinase activity, it has been suggested that p107-reg. may directly phosphorylate cdc2 on tyrosine 15 (16). However, there are two good reasons to be skeptical of this hypothesis: first, in previous studies, it has been shown that p34cdc2 is phosphorylated on tyrosine in yeast wee1-1 mutants (17); and second, no physical association was demonstrated between cdc2 and p107-reg. Indeed, purified cdc2 was not phosphorylated by p107-reg. in vitro (16). More recently, a yeast wee1-related 66-kilodalton kinase, termed mik1, has been cloned (18), but in this case, too, cdc2 is apparently tyrosine phosphorylated in mik1-1 mutant strains, although wee1-1 mik1-1 double mutation results in lethal mitosis and loss of tyrosine phosphorylation of p34cdc2. These results do not suggest that either mik1 or wee1 is directly responsible for the tyrosine phosphorylation of cdc2. The current model for the regulation of cdc2 involves its association with cyclins A and B and its reversible covalent modification by phosphorylation on threonine residues 14 and 167 and tyrosine residue 15 (7, 9; reviewed in Ref. 1). At present, the identity of the protein kinases directly responsible for regulating cdc2 are unknown; however, our finding that cdc2 is physically associated with its regulatory tyrosine kinase provides a relatively clear route to characterization and cloning of the enzyme. Such experiments are currently under way.

The detection of hyperphosphorylated and presumably inactive cdc2 in the nucleus and hypophosphorylated cdc2 in the cytoplasm, in association with the tyrosine kinase that phosphorylates it, suggests that tyrosine phosphorylation may regulate not only the activity of cdc2 but its association with other proteins and its subcellular distribution. In accord with this hypothesis, we have found that hyperphosphorylated cdc2 can be isolated in complex with proteins distinct from those associated with hypophosphorylated cdc2, and we are currently investigating whether tyrosine phosphorylation may directly affect the ability of cdc2 to interact with other proteins in vitro.

Materials and Methods

Tissue Culture. IL-3-dependent murine myeloid 32D cells were grown in RPMI containing 10% fetal calf serum supplemented with 20% WEHI-conditioned media to provide IL-3.

Metabolic Labeling. Metabolic labeling was carried out essentially as described (19, 20). Briefly, cells were washed three times in either phosphate-free or methionine-free RPMI and resuspended at a density of 1 × 10⁷ cells/ml. The labeling medium was supplemented with 1000 units/ml of dialyzed recombinant human IL-3 and 5% fetal calf serum that had been dialyzed against either phosphate-free or methionine-free RPMI. Labeling was initiated by the addition of 0.5 mCi/ml of either [35S]methionine or [32P]P, and proceeded for 3–4 h.

Cell Lysis and Immunoprecipitation. The cells (1 × 10⁷) were lysed 30 min at 4°C in 1 ml of phosphate-buffered saline containing 1% Nonidet P-40, 1 mM phenylmethyisulfonyl fluoride, 1 μg/ml leupeptin, and 1% BSA for the in vitro kinase assays (buffer 1) or in buffer 1 supplemented with 5 mM NaF, 1 mM orthovanadate, and 5 mM pyrophosphate for precipitations of metabolically labeled cells. The lysates were centrifuged for 20 min (12,000 × g), and supernatants were immune precipitated for 2 h with antisera (1:1000 dilution) or with antisera + peptide competitor (1 μg). Immune precipitations were washed three times with buffer 1 and three times with buffer 1 without BSA (buffer 2).

Kinase Reactions and Gel Electrophoresis. Immune complexes were performed in 20 μl of kinase buffer (20 mM Tris HCl-10 mM MnCl₂, pH 7.5) containing 30–50 μCi [γ-32P]ATP (3000 Ci/mmol) for 15 min at 37°C. Immune complexes were analyzed by single dimension 10% SDS-polyacrylamide gel electrophoresis and autoradiography.

Cell Fractionation. The cells were lysed 10 min with buffer 1, and nuclei were pelleted by 10 min centrifugation at 3000 × g. The nuclei were resuspended to the original volume in buffer 1 and disrupted by sonication. Both the cytosolic and nuclear fractions were clarified by centrifugation prior to immunoprecipitation. Cell lysis and fractionation were monitored by Hoffman modulation contrast microscopy.

Phosphoamino Acid Analysis and Peptide Mapping. Phosphoamino acid analysis was performed as described (19). The synthetic peptide IEKIGETGCVYKAKKLS was purchased from Multiple Peptide Systems (San Diego, CA) and further purified by reversed phase HPLC, and the sequence of the purified peptide was confirmed as described (21). The purified peptide was phosphorylated by immunoprecipitated pp60±ac and repurified by thin layer electrophoresis as described (9). The phosphorylated peptide was scraped from the thin layer plate and subjected to phosphoamino acid analysis as described (19). A portion of the phosphorylated peptide was digested with V8 protease and purified by reversed phase HPLC, and the single radioactive peak was subjected to Edman degradation and scintillation counting.

Protein digestions with trypsin and thermolysin and two-dimensional peptide analysis of in vitro phosphorylated cdc2 and src-phosphorylated, synthetic cdc2 peptide, were performed essentially as described (9).

Phototaffinity Labeling. Unlabeled cells were lysed and immunoprecipitated as described. After immunoprecipitation, 20 μCi of gamma [γ-32P]8-azido-ATP (ICN; 8.7

* C. Norbury, unpublished observation.
Cl/mmol) were added to each sample in 20 μl of 10 mM MnCl$_2$-20 mM Tris (pH 7.5) and illuminated with UV light for 5 s through the bottom of the microfuge tube on a Hoefer Transilluminator. The labeled proteins were separated by 10% SDS-gel electrophoresis, and autoradiography was performed.

Acknowledgments
We thank John Groffen and Ira O. Daar for helpful discussions and R. Heath Coats for technical assistance. We also thank Stephen Oroszlan for the use of his laboratory to perform the sequence analyses.

References
12. Bailly, E., Doree, M., Nurse, P., and Borens, M. P34 cdc2 is located in both nucleus and cytoplasm; part is centrosomally associated at G2/M and enters vesicles at anaphase. EMBO J., 8: 3985–3995, 1989.