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Isolation of mitotic p34^{cdc2} apoenzyme from human cells

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A simple procedure was devised for isolating from homogenates of mitotic cells the human homolog to the fission yeast *cdc2* gene product The identity of the purified protein was established with anti-p34^{cdc2} antibodies and p13^{suc1}, both specific ligands for p34^{cdc2} Active-site labeling with oxidized [α^{32}P]ATP showed the purified molecule to be an ATP-binding protein Its ability to phosphorylate casein but not histone, and its phosphorylation on tyrosine, detected by anti-phosphotyrosine antibodies, indicates the form of p34^{cdc2} purified is the inactive or apoenzyme form Purified quantities of human p34^{cdc2} should be of considerable value in establishing the mechanism of its activation at mitosis by phosphatases

Mitosis, Phosphotyrosine, p34^{cdc2}, Human

1 INTRODUCTION

The protein kinase encoded by the yeast *cdc2* gene or its homologs in other species plays an essential role in triggering mitotic events [1,2] In the human cell, p34^{cdc2} has been detected in a number of distinct forms at mitosis, both on the basis of size [3,4] or isoelectric point [5,6]. Only a high molecular weight form [3,4], thought to contain both cyclin B [6,7] and p65, a protein phosphatase present as a disulfide-linked dimer at mitosis [8,9], possesses the H1 kinase activity characteristic of M phase-promoting factor (MPF), other forms have only casein kinase activity. Although the several isoforms of human p34 distinguishable by isoelectric focusing [5,6] likely derive from differential phosphorylation at multiple sites [10], the precise relationship of these several forms with the active, H1 kinase complex or holoenzyme is unclear

Activation of the latent H1 kinase activity of p34^{cdc2} is known to occur in several steps. Phosphorylation of p34^{cdc2} during interphase may be required for assembly of the high molecular weight holoenzyme [3], while subsequent dephosphorylation of p34^{cdc2} (particularly on tyrosine) is necessary for activation of histone H1 kinase activity [1,2]. These conclusions derive principally from examination of the phosphorylation state of p34^{cdc2} immunoprecipitated from crude extracts or from gel-filtration column fractions There is evidence, however, that at least in mammalian cells p34^{cdc2} may be partially dephosphorylated during immunoprecipitation [11] Availability of a purified p34^{cdc2} apoenzyme would circumvent this problem by allowing direct determination of p34^{cdc2} kinase activity as a function of controlled alterations in its phosphorylation state This report describes a rapid purification of p34^{cdc2} apoenzyme from human mitotic cell extracts

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2. MATERIALS AND METHODS

2 1 *Cell extracts*

Human D98/AH-2 cells were cultured as previously described [8] Mitotic cells (mitotic index 85–97%) were collected by mechanical shake-off following 16–20 h incubation in 50 ng/ml nocodazole Cell homogenates were prepared as described [8], except that NaF and 2-glycerophosphate were increased to 50 mM and 10 mM, respectively

2 2 *Purification of p34^{cdc2}* apoenzyme

Mitotic extracts were fractionated by $(NH_4)_2SO_4$ precipitation as previously described [8] The 20–60% fraction (approximately 10 mg of protein) was taken up in 2 ml of 10 mM Tris-HCl, 50 mM NaCl, 1 mM EDTA (pH 7 4, 20°C), transferred to 6.4-mm diameter, 12 000–14 000 molecular weight cut-off Spectra/Por membrane tubing (Spectrum Medical Industries) and dialyzed for 18 h against 2 l of the same buffer containing 0 1 mM DTT, 5 mM NaF, 10 mM 2-glycerophosphate, 0 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 0 1 mM benzamidine, 1 μg/ml aprotinin, 1 μg/ml pepstatin A, 0 1 mM $ZnCl_2$, and 1 mM ATP (dialysis buffer) Dialysis was carried out at 4°C, at which temperature the pH of the buffer rose to 8 0, concomitant with the appearance of a precipitate in the dialysis tubing The precipitate was collected by centrifugation at 4°C, washed once with ice-cold dialysis buffer and extracted on ice in 2 ml of 20 mM Bis-Tris, 50 mM NaCl, 4 mM $MgCl_2$, 0 1 mM DTT (pH 6 9) containing 10 mM 2-glycerophosphate, 5 mM NaF, 0 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 0 1 mM $ZnCl_2$ and 1 mM ATP (extraction buffer) The remaining precipitate was removed by centrifugation and discarded The supernatant was applied to a DEAE-Sepharose CL 6B column, and the non-binding fraction was collected, concentrated using a Centricon 10 concentrator (Amicon), and stored at 4°C until

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use The size of the purified apoenzyme was determined by gel-filtration chromatography in 10 mM sodium phosphate, 500 mM NaCl (pH 6 9) on Sepharose CL-6B, using a 50×1 cm column.

2 3 *Gel electrophoresis and immunoblotting*

Gel electrophoresis and immunoblotting were performed as previously described [8] Blots were probed overnight at 4°C with J4 or JP4 ascites, an antibody against the PSTAIR region of p34^{cdc2} [12], anti-phosphotyrosine antibodies [13], anti-p65 antibodies [8] or anti-human cyclin B antibodies [7] Silver staining was according to [14]

2 4 *Protein kinase assay*

Protein kinase assays were carried out as previously described [8], except that some reactions contained 1 mg/ml of dephosphorylated, partially hydrolyzed casein [15] rather than 1 mg/ml of histone H1 as substrate Reactions were initiated by the addition of enzyme and were terminated after 10 min at 37°C by adding 3× electrophoresis sample buffer for analysis by SDS-PAGE and autoradiography

2 5 *p13-Sepharose binding*

Binding of samples to Sepharose CL-4B to which p13 had been coupled was carried out essentially according to [16], except that the samples were applied in dialysis buffer or extraction buffer Following binding, beads were washed exhaustively with 50 mM triethanolamine, 0 9% NaCl, 0 1% SDS, 0 5% Tween 20, 2 mM EDTA (pH 7 4)

3 RESULTS

3 1 *Isolation of a 34-kDa casein kinase*

When isolating protein kinases from mitotic cell extracts, substantial loss of casein kinase activity was noted during dialysis of $(NH_4)_2SO_4$ fractions To discover the source of the loss, the translucent precipitate formed when 20–60% $(NH_4)_2SO_4$ fractions of mitotic cell extracts were dialyzed at pH 8 0 at low ionic strength was extracted at pH 7 0, and the resolubilized portion analyzed by SDS-PAGE and silver staining, a 34-kDa polypeptide (Fig. 1A, lane 1) was prominent DEAE chromatography of the resolubilized fraction produced an essentially homogeneous preparation (lane

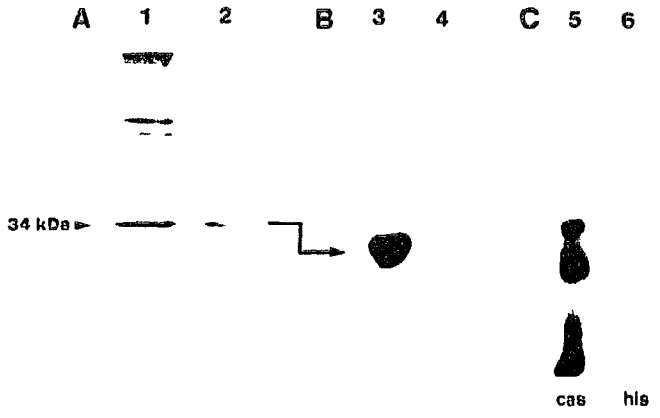

Fig 1 Isolation of a 34-kDa casein kinase A Silver stain of precipitate (lane 1) and DEAE-purified protein (lane 2) Approximately 100 ng of protein were loaded per lane B Active-site labeling of DEAE-purified protein in the absence (lane 3) or presence (lane 4) of 1 mM unlabeled ATP C Casein (lane 5) and histone (lane 6) kinase activity of DEAE-purified protein

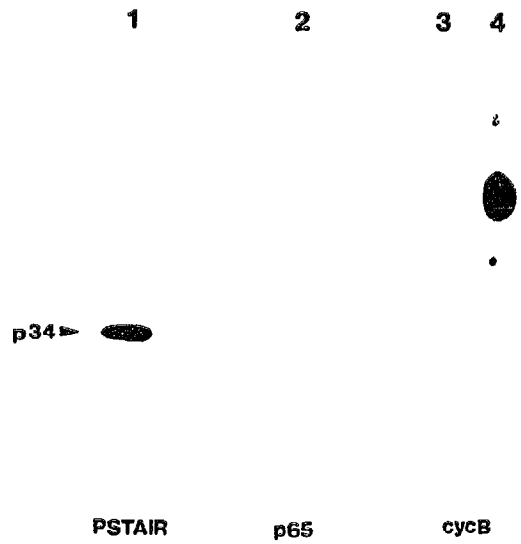

Fig 2 Western blot of the 34-kDa polypeptide and holoenzyme fraction Lanes 1–3, p34 apoenzyme, lane 4, MPF-containing $(NH_4)_2SO_4$ fraction remaining soluble following dialysis at pH 8 0 Antibodies used are indicated below each blot strip

2) which eluted from a gel-filtration column as a single peak of molecular weight 30–40 kDa (data not shown). Active-site labeling with periodate-oxidized [α-^{32}P]ATP [17] revealed the 34-kDa polypeptide to be the only ATP-binding protein present (Fig. 1B, lanes 3 and 4). The preparation readily phosphorylated casein, but did not phosphorylate histone to any appreciable extent (Fig. 1C, lanes 5 and 6), consistent with the substrate specificity reported for the uncomplexed apoenzyme form of human p34^{cdc2} [3,4]

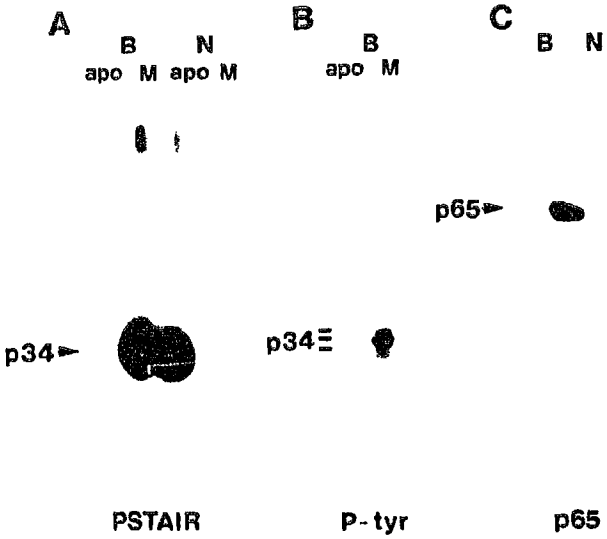

Fig 3 Western blot of protein bound by p13-Sepharose apo, p34^{cdc2} apoenzyme preparation, M MPF-containing $(NH_4)_2SO_4$ fraction remaining soluble at pH 8 0 B, p13-Sepharose-bound protein, N, p13-Sepharose non-binding protein Antibodies used are indicated below each blot section

3 2. *Identification as p34^{cdc2} apoenzyme*

The isolated 34-kDa polypeptide was tested for reactivity with ligands specific for p34^{cdc2}. As shown in Fig 2, lane 1, the 34-kDa polypeptide was recognized on Western blots by antibodies against the highly-conserved PSTAIR sequence of p34^{cdc2} [12], two monoclonal antibodies raised against fission yeast p34^{cdc2} (JP4 and J4, [5]) also recognized the molecule (data not shown). When the preparation was probed with anti-p65 or anti-cyclin B antibodies, no reactivity was detected (lanes 2 and 3), demonstrating the absence of the holoenzyme from the preparation. Lane 4 demonstrates the presence of cyclin B in the fraction remaining soluble after precipitation of the apoenzyme, this fraction also contains p65 (see below)

The product of the yeast *suc1* gene, p13, is able to clear p34^{cdc2} from mitotic extracts when immobilized on Sepharose beads [1,2]. As shown in Fig. 3 (panel A, **apo**), incubation of the purified p34^{cdc2} apoenzyme with p13-coated Sepharose completely removed anti-p34^{cdc2} immunoreactive material. As would be predicted from its lack of histone kinase activity, the p13-bound p34^{cdc2} was phosphorylated on tyrosine as judged by an anti-phosphotyrosine antibody (Panel B, **apo**). In agreement with results obtained in *Xenopus*, phosphotyrosine was found on each component of the p34^{cdc2} triplet [18] p13-Sepharose also quantitatively removed p34^{cdc2} which remained soluble after the apoenzyme had precipitated from the $(NH_4)_2SO_4$ fraction during dialysis (Panel A, M). In contrast, however, this p13-bound p34^{cdc2} contained very little phosphotyrosine (Panel B, M) Consistent with this latter form being part of the active p34^{cdc2} holoenzyme (MPF), p13-Sepharose removed p65 along with p34^{cdc2} (Panel C). Gamma counting of bands excised from blots showed that approximately two-thirds of the p34^{cdc2} present in the 20–60% $(NH_4)_2SO_4$ fraction was complexed in MPF and approximately one-third was apoenzyme

4. DISCUSSION

This report describes a simple procedure for isolating the apoenzyme form of the p34^{cdc2} kinase from human mitotic cells Identity of the purified molecule with p34^{cdc2}, suggested by its protein kinase activity and molecular weight, was confirmed by labeling with specific anti-p34^{cdc2} antibodies and by adsorption on p13-Sepharose While p34^{cdc2} is predicted from its kinase activity and from its nucleotide sequence to bind ATP, this is the first direct demonstration that p34^{cdc2} is an ATP-binding protein The absence of other components of MPF from the purified preparation identifies the form isolated as the apoenzyme That the purified p34^{cdc2} is phosphorylated on tyrosine and lacks H1 kinase activity further distinguishes it from the holoenzyme and identifies it as inactive apoenzyme The ability to separate the human p34^{cdc2} apoenzyme and holoenzyme species, along with the demonstration that the human MPF holoenzyme contains virtually no phosphotyrosine, rectifies earlier observations to coincide with those made in other species [3,5]. Availability of homogeneous p34^{cdc2} apoenzyme will be useful in searching for the physiological activators of the H1 kinase activity of p34^{cdc2} and in evaluating its potential substrates.

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