

Activation of type-1 protein phosphatase by cdc2 kinase

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Purified cdc2 or cdc2 obtained from HeLa cells in association with p13^{suc1} activate inactive type-1 protein phosphatase (PP1) (catalytic subunit + inhibitor-2 complex, purified from skeletal muscle). Likewise in the case of PP1 activation by F_A/GSK3, activation by cdc2 is accompanied by phosphorylation of inhibitor-2 (I2) and free I2 can be phosphorylated as well. Correlation between PP1 activation and I2 phosphorylation is suggested by the fact that both activation and phosphorylation (a) increase in parallel during incubation with cdc2, (b) decrease in parallel upon subsequent cdc2 inhibition by EDTA, and (c) are inhibited by the cdc2 inhibitor 5,6-dichlorobenzimidazole riboside. cdc2 also phosphorylates the catalytic subunit of PP1, whether in the complex with I2 or as free molecule. The activation of PP1 by cdc2 and by F_A/GSK3 is compared.

Protein phosphatase; Protein kinase; Phosphorylation; Cell cycle

1. INTRODUCTION

Protein phosphatase of type-1 (PP1) is the most abundant serine/threonine phosphatase in most cells (see [1] for review) and is regulated by hormones and growth factors [2–5] and during the cell cycle [6–8]. The PP1 catalytic subunit exists in various active (E_a) and inactive (E_i) conformations [9] and is purified either as a free catalytic subunit or bound to the regulatory subunits inhibitor 2 (I2) and G [1,10,11]. Regulation of PP1 is primarily through phosphorylation of its regulatory subunits. In fact the kinase F_A/GSK3 and casein kinase II activate the cytosolic PP1 by phosphorylating the regulatory subunit I2 [9,12,13]; an insulin-stimulated kinase ([14] identical to the 90K S6 kinase) activates PP1 by phosphorylating the G-subunit in muscle glycogen particles, and also protein kinase A inhibits PP1 through the G-subunit (reviewed in [1]). Additionally, PP1 may be regulated by tyrosine phosphorylation of the catalytic subunit by the kinases v-src or c-src [15,16] and v-abl [17]. Tyrosine phosphorylation occurs in the

C-terminal, trypsin-sensitive region of the catalytic subunit and may inhibit the activity [15,16] or facilitate the inactivation by I2 in diluted conditions [17].

cdc2 is the catalytic subunit of the kinase complex that is activated at both the mitotic and meiotic M phase in eukaryotic cells. The activation of cdc2 at the M phase is controlled by reversible multiple phosphorylations and association with cyclins (see [18–20] for review). Dephosphorylation of cdc2 is thought to be the final event to activate the kinase and involves the tyrosine phosphatase cdc25 [21] as well as okadaic acid-sensitive serine/threonine phosphatases, such as PP1 and protein phosphatase-2A [8]. A number of substrates are supposedly phosphorylated by the activated cdc2 and these, in turn, may lead to the major M-phase events such as chromosome condensation, nuclear envelope breakdown and cytoskeletal reorganization. Although few such substrates have been found so far (reviewed in [22]), their identification and the assessment of their role 'in vivo' is of primary importance for the understanding of the M-phase events. Here we report that PP1 might be one of such substrates since cdc2 purified from HeLa cells phosphorylates and activates PP1.

2. MATERIALS AND METHODS

2.1. Materials

ATP, BSA, PMSF, benzamidine, DTT, TPCK, DRB, HEPES and molecular weight markers were purchased from Sigma. [³²P]P_i was from DuPont. Hyperfilms-MP were from Amersham. The chemicals for electrophoresis were from Bio-Rad. Triton X-100 and leupeptin were from Boehringer. Protein A-Sepharose was from Pierce. The Opti Phase II scintillant was from LKB.

2.2. Cell culture and extracts

HeLa cells were grown on 150-mm tissue culture plates in DMEM

Abbreviations: TPCK, *N*-tosyl-L-phenylalanine chloromethyl ketone; PMSF, phenylmethylsulfonyl fluoride; 2-ME, 2-mercaptoethanol; DTT, dithiothreitol; DRB, 5,6-dichlorobenzimidazole riboside; HEPES, *N*-[2-hydroxyethyl]piperazine-*N'*-[ethanesulfonic acid]; BSA, bovine serum albumin; SDS, sodium dodecyl sulfate; DMEM, Dulbecco's Modified Eagle Medium; PP1, protein phosphatase of type-1; E_a, active catalytic subunit of PP1; E_i, inactive catalytic subunit of PP1; E_i · I2, inactive cytosolic PP1 complex; I2, inhibitor-2 of PP1; F_A/GSK3, protein kinase that activates PP1, also called glycogen synthase kinase-3; cdc2, cyclin-dependent serine kinase that is activated at the G2/M transition of cell cycle.

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supplemented with 1 mM sodium pyruvate, 1 mM non-essential amino acids, 10% foetal calf serum, 100 units/ml penicillin and 100 µg/ml streptomycin at 37°C with 5% CO₂. After washing twice with 5 ml cold PBS, the cells were scraped off with a rubber policeman in the presence of 10 mM HEPES, pH 7.4, 0.1 mM EDTA, 10 mM NaCl, 0.004% benzamidine, 0.004% PMSF, 0.004% TPCK, 4 µg/ml leupeptin, 30 mM 2-ME, broken with 20 strokes in a Dounce homogenizer at 4°C and centrifuged at 23,000 × *g* for 90 min. The supernatant thus obtained, defined as cytosolic fraction, was quickly frozen and stored at -70°C for subsequent assay.

2.3. Enzyme and protein purifications

Inactive cytosolic PP1 E_i · I2 complex [23], cytosolic free E_i [24], I2 [12] and phosphorylase *b* were purified from rabbit skeletal muscle [9,23]. cdc2 purified from HeLa cells [25], p13^{cas} covalently bound to Sepharose (p13-beads, [26]) and anti-human cdc2 peptide antiserum G6 [27] were a gift from Dr. G. Draetta (EMBL, Heidelberg, Germany). F_A/GSK3 was purified from rabbit skeletal muscle up to the Blue Sepharose chromatography [28]. Muscle phosphorylase kinase was a gift from Dr. L.M.G. Heilmeyer (Ruhruniversität-Bochum, Germany). Rabbit muscle phosphorylase *b* was used to produce ³²P-labeled phosphorylase *a* [29]. [³²P]ATP (2000 Ci/mmol) was prepared using inorganic [³²P]phosphate (as described in [29]). Protein was determined by the method of Bradford [30], using BSA as a standard.

2.4. p13^{cas}-bead precipitates and immunoprecipitates

40 µl p13-beads [26] resuspended in 1 vol. of PBS was used to bind cdc2 from HeLa cell cytosolic fractions during 30 min rotation at 4°C. Alternatively cdc2 was immunoprecipitated from 1.8 mg of HeLa cell cytosolic fractions with 5 µl of anti-human cdc2 peptide antiserum G6 [27] during 4 h incubation at 4°C, followed by the addition of 40 µl Protein A-Sepharose resuspended in 1 vol. PBS and 30 min rotation at 4°C. The pellets were washed three times in 50 mM Tris, pH 7.5, 5 mM EDTA, 0.4 M NaCl, 0.1% Triton X-100, 0.002% benzamidine, 0.002% PMSF and 15 mM 2-ME, and used as source of active cdc2 kinase.

2.5. PP1 activation and assays

Inactive PP1 E_i · I2 complex (0.1–0.2 µg) was activated by cdc2 or F_A/GSK3 [32] during incubation for 2 min, unless otherwise stated, at 30°C in the presence of 0.5 mM ATP (Fig. 1) or 0.05 mM ATP (Figs. 3 and 4) and 1 mM Mg²⁺, in 25 µl PP1 assay buffer [9]. PP1 was assayed by the release of ³²P_i from [³²P]phosphorylase *a* (1–2 × 10⁵ cpm/nmol) in 50 µl final volume [9,23] and the ³²P_i released was counted in a β scintillation counter using the Opti Phase II scintillant.

2.6. Phosphorylation of I2 and PP1

For the phosphorylation of free I2 and PP1 shown in Fig. 2, 25 mM Tris, pH 7.5, 80 µM ATP, 6 mM Mg²⁺, 0.8 mM DTT, 30 mM NaF, 5–10 × 10³ cpm/pmol [³²P]ATP, I2, PP1 and protein kinases as specifically indicated were incubated in 20–25 µl at 30°C. For the PP1 phosphorylation shown in Figs. 3 and 4, 25 mM imidazole, pH 7.5, 50 µM ATP, 1 mM Mg²⁺, 15 mM 2-ME, 80–120 × 10³ cpm/pmol [³²P]ATP, PP1 and protein kinases as specifically indicated were incubated in 125 µl at 30°C. At the indicated time-points 25 µl aliquots were mixed with 1 vol. of Laemmli buffer, boiled for 3 min and subjected to 10% SDS-PAGE [31] and autoradiography by exposure to Hyperfilm-MP at -70°C with intensifying screen. To measure the phosphorylation of I2 or of the PP1 catalytic subunit (38 kDa protein) the corresponding protein bands were excised from the gel, hydrated, mixed with Opti Phase II scintillant and counted in a β scintillation counter. The stoichiometry of the phosphorylation was calculated assuming phosphorylation on one site and the molecular weight used for I2 was 22,835, as deduced by protein sequence [1].

3. RESULTS AND DISCUSSION

cdc2 kinase precipitated from HeLa cells extract in

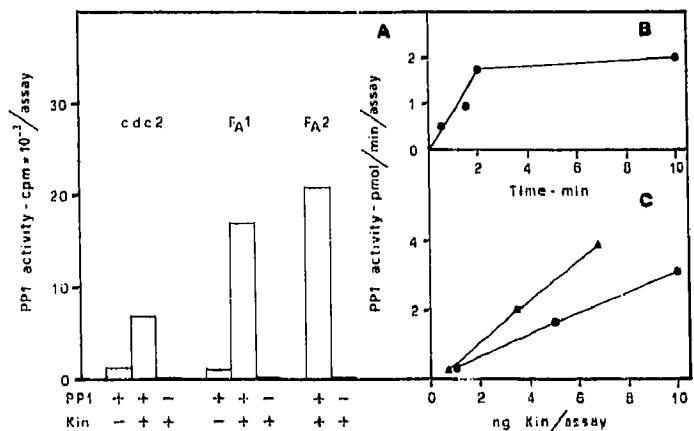


Fig. 1. (A) Activation of the inactive PP1 E_i · I2 complex by cdc2 or by two different F_A/GSK3 preparations (F_A1 and F_A2). 200 ng of inactive PP1 was incubated with 100 ng purified cdc2 or 67 ng F_A1 or 86 ng F_A2, for 2 min at 30°C in the presence of 0.5 mM ATP and 1 mM Mg²⁺, followed by 3-min phosphatase assay, as described in section 2. Incubations containing PP1 or kinase only were run in parallel. (B) 1 µg of PP1 was incubated with 25 ng cdc2 as in (A), in a final volume of 125 µl. At each time point a 25 µl aliquot was taken for a 3-min PP1 assay (●). The PP1 activity generated by the activation was calculated after subtracting the cpm of parallel incubations that contained PP1 or kinase only. PP1 activity is given as pmol of P_i liberated from the substrate phosphorylase *a* per min during the 3-min PP1 assay. (C) The indicated amounts of cdc2 (●) or F_A2 (▲) were incubated with 200 ng PP1 for 2 min, under the conditions indicated in (A), followed by a 3-min phosphatase assay. PP1 activity was calculated as in (B).

association with p13-beads was found to activate exogenous inactive PP1 (E_i · I2 complex). Since such cdc2 preparation still contains contaminants that might be responsible for the activation, the experiments were repeated with cdc2 purified from HeLa cells [25] (in this preparation cdc2 and cyclins accounted for over 90% of the protein detected on silver-stained SDS gel electrophoresis). In Fig. 1A the activation of PP1 by purified cdc2 is shown and compared with the activation by two preparations of F_A/GSK3. Activation by cdc2 was linear in time for the first few minutes (Fig. 1B) (similar to what was described in the case of F_A/GSK3 [9,32]) and with respect to the amount of protein used, within the protein concentrations tested (Fig. 1C). Fig. 1C also shows the linearity of PP1 activation by F_A2. From the data of Fig. 1B and C the specific activity of cdc2 as PP1 activator was found to be 170 U/mg at 2 min incubation (calculated after subtracting the cpm due to PP1 or kinase alone from the cpm obtained in the presence of both PP1 and kinase), while F_A2 had specific activity of 340 U/mg. PP1 activation by cdc2 was lost in the presence of the cdc2 inhibitor 5,6-dichlorobenzimidazole riboside (DRB) (not shown).

The results described above indicate that cdc2 shares with F_A/GSK3 the ability to activate PP1. PP1 activation by F_A/GSK3 involves phosphorylation of I2 in the PP1 complex [9,10,12,13] and conversion of the cata-

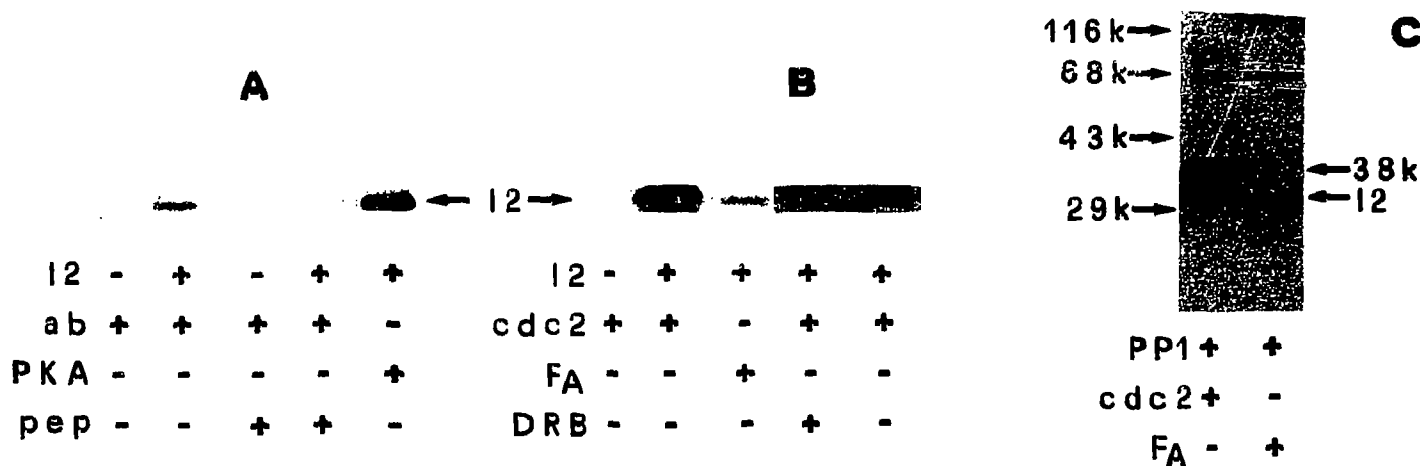


Fig. 2. (A) Phosphorylation of I2 (1 μ g) by immunoprecipitated cdc2. For each incubation cdc2 was precipitated from 1.8 mg of HeLa cell cytosolic fraction with anti-human cdc2 peptide antiserum (ab), in the absence or in the presence of the peptide (pep) used as immunogen, and incubated in the presence of [32 P]ATP for 30 min at 30°C with or without I2. I2 phosphorylated by 50 ng protein kinase A (PKA) was used as I2 marker. (B) Phosphorylation of I2 (2 μ g) by purified cdc2 (20 ng) or FA/GSK3 (14 ng). I2 was incubated in the presence of [32 P]ATP for 60 min at 30°C with or without kinase. One incubation contained also 500 μ M of the cdc2 kinase inhibitor DRB. (C) Phosphorylation of PP1 (38 kDa) by cdc2 or FA/GSK3. 1 μ g of PP1 was incubated with 50 ng of cdc2 or 68 ng of FA/GSK3 for 30 min at 30°C, in the presence of [32 P]ATP. Standard proteins: 116 k, β -galactosidase; 68 k, BSA; 43 k, ovalbumin; 29 k, carbonic anhydrase. Incubation conditions are further described in section 2.

lytic subunit from inactive (E_i) to active form (E_a) [9]. During activation I2 phosphorylation in the PP1 complex by FA/GSK3 is just measurable [13], and this may be explained by dephosphorylation of I2 by the activated complex [23]. However, also phosphorylation of free I2 by FA/GSK3 is very low (up to 0.3 mol/mol in [13]). In order to test whether also PP1 activation by cdc2 involved I2 phosphorylation it was first tested whether I2 was a substrate for cdc2. cdc2 immunoprecipitated from HeLa cell extracts with anti-cdc2 antibodies phosphorylated free I2. Phosphorylation did not occur in a control immunoprecipitate in which the peptide used as immunogen was added to prevent formation of the cdc2-antibody complex (Fig. 2A). Purified cdc2 phosphorylated free I2 at higher degree. Phosphorylation by cdc2 was also higher than phosphorylation by a comparable amount of FA/GSK3 (Fig. 2B) and did not occur in the presence of the cdc2 kinase inhibitor DRB (Fig. 2B). cdc2 phosphorylated I2 also in the PP1 E_i · I2 complex (Fig. 2C) and phosphorylation was inhibited by DRB (not shown). Surprisingly cdc2 phosphorylated also the 38 kDa catalytic subunit of PP1 (Fig. 2C). In a separate experiment it was found that cdc2 phosphorylated also the free active E_a form (purified from muscle cytosol) without affecting its activity and phosphorylation was in the trypsin-sensitive C-terminal region, since the proteolyzed active form of E_a was no longer phosphorylated (not shown). On the other hand FA/GSK3 phosphorylated I2 only (Fig. 2C; see also below and Fig. 4A).

The correlations between PP1 activation and phosphorylation by cdc2 and FA/GSK3 were investigated in two parallel incubations, one to assay PP1 activity and the other to measure I2 phosphorylation in the presence

of [32 P]ATP. The stoichiometry of I2 phosphorylation was calculated assuming phosphorylation on one site. Fig. 3 shows that the activation of PP1 by cdc2 was accompanied by parallel increase in I2 phosphorylation and both events were faster in the first few minutes of reaction but continued to increase throughout the incubation time. A comparable amount of FA/GSK3 induced higher PP1 activation but lower I2 phosphorylation (Fig. 3; compare also with ref. [13]). The two kinases together had additive effects on PP1 activation and I2 phosphorylation, at least in the first few minutes of reaction. The differences between cdc2 and FA/GSK3 were confirmed upon prolonged incubation (Fig. 4A). Both PP1 activation and I2 phosphorylation by cdc2 increased in parallel for up to 2 h and PP1 activation reached values comparable to that obtained with FA/GSK3. On the other hand activation by FA/GSK3 reached its maximum by 20 min, during which time I2 phosphorylation was constantly low (this is in agreement with previous data; see [9,13] for the FA/GSK3 mechanism). In all these experiments inactive PP1 was present in excess and the activity generated by cdc2 in 2 h represented approximately 1% of the potential PP1 activity in each assay, based on the activity that would be assayed after activation by trypsin and Mn^{2+} [9,23]. Fig. 4A shows also that the PP1 catalytic subunit (38 kDa protein) is phosphorylated by cdc2 (but not by FA/GSK3) in a reaction that is almost linear with time for up to 2 h.

Further indication of the involvement of I2 phosphorylation in PP1 activation by cdc2 came from studying the reversibility of the activation. In the experiment shown in Fig. 4B PP1 was activated by cdc2 for 10 min, then the kinase was inhibited by EDTA and the incuba-

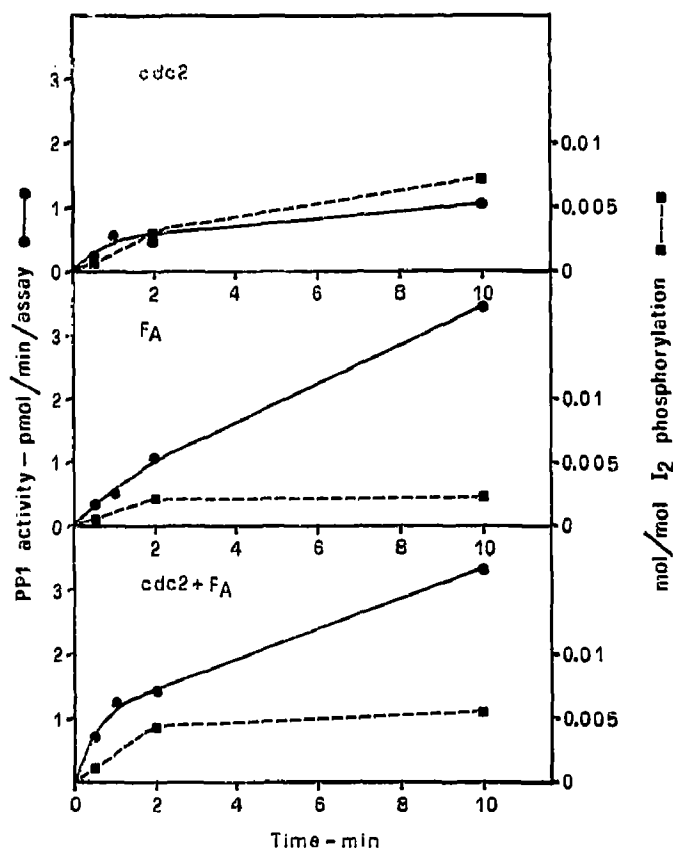


Fig. 3. For activity assays 0.6 μ g of inactive PP1 (E_1 ·I2 complex) was incubated at 30°C with 30 ng cdc2 or with 41 ng F_A /GSK3 or with the same amounts of both kinases in 150 μ l 20 mM imidazole, pH 7.5, 0.05 mM ATP and 1 mM Mg^{2+} . At each time point 25 μ l were taken to assay PP1 activity. For I2 phosphorylation the incubation was as above but contained in addition [32 P]ATP. At each time point 25 μ l were taken for subsequent electrophoresis and autoradiography. The phosphorylated protein bands corresponding to I2 were excised from the gel for radioactivity count and calculations of mol/mol phosphorylation, as described in section 2.

tion proceeded for the subsequent 110 min. Following kinase inhibition, PP1 activation and I2 phosphorylation returned almost to the basal values within 10–20 min. Also PP1 activation by F_A /GSK3 was reversed by EDTA with a similar time pattern (Fig. 3B and [9]), but in this case I2 dephosphorylation and PP1 inactivation were complete.

Altogether the data shown in Figs. 3 and 4 suggest the involvement of I2 phosphorylation in PP1 activation by cdc2. Furthermore, in I2 the P-Y-H sequence immediately downstream the Thr-72 phosphorylated by F_A /GSK3 during PP1 activation bears some resemblance to the S(T)-P-X-Z sequence recognized by cdc2 (where X is a polar amino acid and Z is generally a basic amino acid [22]), suggesting the possibility that also PP1 activation by cdc2 involves phosphorylation of Thr-72. On the other hand the present experiments indicate also differences in PP1 activation and I2 phosphorylation by

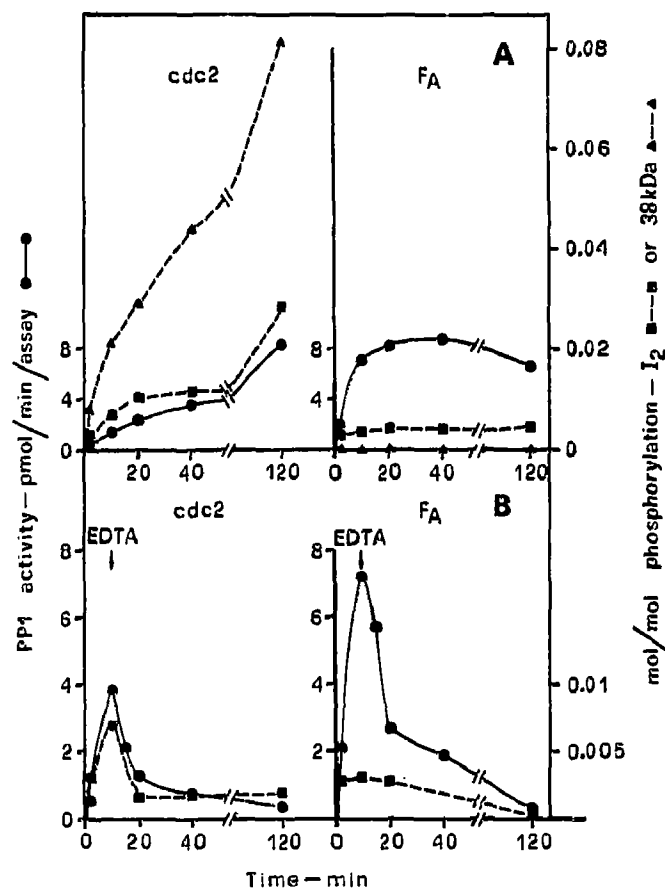


Fig. 4. (A) Incubations for PP1 activation and phosphorylation were as in Fig. 3. In this experiment the mol/mol phosphorylation was calculated for both PP1 catalytic subunit (38 kDa protein) and I2, in the way described in Fig. 3 and in section 2. (B) Incubation conditions were as in (A). After taking the 10 min aliquot, EDTA was added to 10 mM final concentration.

the two kinases. Consequently for a better understanding of the mechanism by which cdc2 activates PP1 the I2 site(s) phosphorylated by cdc2 need to be investigated. For what concerns phosphorylation of the 38 kDa catalytic subunit, it is less likely that this is responsible by itself for PP1 activation since (i) it occurs only when PP1 is activated by cdc2 and not by F_A /GSK3, (ii) it is less correlated in time with enzyme activation, (iii) it takes place also in the case of E_{38} , a 38 kDa form that is already active, and removal of the phosphorylation site by proteolysis does not affect activity. However, such phosphorylation might have additional roles in PP1 regulation that need to be studied. Of equal importance will be to assess if PP1 activation and phosphorylation by cdc2 may take place also 'in vivo'.

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