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Phosphorylation of the catalytic subunit of type-1 protein phosphatase by the v-abl tyrosine kinase

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The catalytic subunit of type-1 protein phosphatase (PP1) was phosphorylated by the tyrosine kinase v-abl as follows: (i) cytosolic PP1 was phosphorylated more (0.73 mol/mol) than PP1 obtained from the glycogen particles (0.076 mol/mol), while free catalytic subunit isolated in the active or inactive form from cytosolic PP1 was phosphorylated even less and catalytic subunit complexed with inhibitor-2 was not phosphorylated; (ii) phosphorylation stoichiometry was dependent on the concentration of PP1 and 3 h incubation at 30°C was required for maximal phosphorylation; (iii) phosphorylation was on a tyrosine residue located in the C-terminal region of PP1 which is lost during proteolysis; (iv) phosphorylation did not affect enzyme activity but allowed conversion from the active to the inactive form upon incubation with inhibitor-2 of a PP1 form that in its dephospho-form did not convert.

Protein phosphatase; Protein kinase; Tyrosine kinase; Phosphorylation

1. INTRODUCTION

Phosphatases that dephosphorylate protein at serine/threonine residues (PP) are involved in metabolic regulations, such as activation of glycogen and fatty acid synthesis and regulation of contractile proteins (see [1] for review), as well as in the regulation of cell proliferation (see [2] for review). PP of type-1 (PP1), the most abundant PP in many cells and tissues [1], is activated by hormones and growth factors (e.g. insulin [3–5] and EGF [6]) and during the cell cycle [2,7]. PP1 is regulated through covalent modifications of its regulatory subunits: inhibitor-2 (I2) in the cytosol is a target for the kinases F_A/GSK3 and casein kinase II [8–11] that activate PP1; the G-subunit in the glycogen particles is a target for an insulin-stimulated kinase [12] that activates PP1, and for protein kinase A, through which adrenaline inhibits PP1 (reviewed in [1]). The PP1 catalytic subunit exists in various active and inactive conformations [8] that may be obtained as free catalytic subunits or bound to the regulatory subunits. Some years ago it was reported that PP1 might be also regulated by direct phosphorylation of the catalytic subunit by the tyrosine kinases v-src or c-src [13]. Phosphorylation, which was in a trypsin-sensitive region of PP1, was up to 0.34 mol/mol and induced a parallel decrease in enzyme activity [13,14]. Preliminary data also suggested that the v-abl tyrosine kinase was able to phosphorylate the PP1 catalytic subunit [14,15]. Here we report that cytosolic PP1 purified as active catalytic subunit is indeed a substrate for the v-abl tyrosine kinase and that phosphorylation facilitates its inactivation by I2 in diluted conditions.

Abbreviations: TPCK, *N*-tosyl-L-phenylalanine chloromethyl ketone; PMSF, phenylmethylsulfonylfluoride; BSA, bovine serum albumin; SDS, sodium dodecylsulfate; PP, protein serine/threonine phosphatase assayed here as phosphorylase phosphatase; PP1, protein phosphatase of type-1; E, catalytic subunit of PP1; E_a, active E; E_a^{cl}, cytosolic E_a purified by a procedure that includes ethanol precipitation; E_a^G, catalytic subunit of PP1 purified from the glycogen particles; E_i, inactive E isolated from the 70 kDa E_i–I2 cytosolic complex; E_a^{Mn}, obtained from E_i following activation by Mn^{2+}, E_a^{FA}, E_a isolated from the 70 kDa cytosolic complex that had been activated by the kinase F_A/GSK3; I2, inhibitor-2 of PP1; F_A/GSK3, protein kinase that activates PP1, also called glycogen synthase kinase-3.

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2. MATERIALS AND METHODS

2.1. *Materials*

TPCK-treated trypsin, soybean trypsin inhibitor, ATP, BSA, PMSF, benzamidine, TPCK, poly(Glu/Tyr) (4:1), Freund adjuvants phosphotyrosine, bovine IgG, molecular weight markers, peroxidase conjugated anti-guinea pig IgG, bovine IgG and Keyhole Limpets hemocyanin were purchased from Sigma. [^{32}P]Pi was from Du Pont. Peroxidase conjugated anti-rabbit IgG, Hyperfilms-MP and Hyperfilms-ECL and ECL were from Amersham. Affi-Gel 10 and the chemicals for electrophoresis and transblot were from Bio-Rad. The FPLC system and the SI column were from Pharmacia. Opti Phase II scintillant was from LKB.

2.2. *Enzyme assays*

PP was assayed by the release of ^{32}Pi from [^{32}P]phosphorylase *a* (2–4 × 10^5 cpm/pmol) with or without trypsin treatment (20 μg/ml trypsin for 5 min at 30°C followed by 120 μg/ml soybean trypsin inhibitor) in the presence or not of 0.5 mM $MnCl_2$ [8], in 50 μl final volume [5,8].

The tyrosine kinase activity of v-abl was assayed with the substrate poly(Glu/Tyr) (4:1) co-polymer at 2 mg/ml, final concentration, in the presence of 0.5 mM ATP, [^{32}P]ATP (100–200 cpm/pmol), 20 mM imidazole, pH 7.5, 3% glycerol, 50 mM NaCl, 12 mM $MgCl_2$, 4 mM $MnCl_2$, 0.2 mM EGTA, 0.05% Triton X-100, 0.2 mM orthovanadate. After 10 min at 30°C the reaction mixture was applied to Whatman P-81 chromatography paper. After 4 washes in 10 mM H_3PO_4, P-81 paper was dryed and counted in Opti Phase II scintillant.

2.3. *Enzyme and protein purifications*

Inactive 70 kDa cytosolic PP1 [16], cytosolic E_a^{ct} [17], glycogen particle E_a^G [18], I2 and phosphorylase *b* were purified from rabbit skeletal muscle [8]. E_i, E_a^{Mn} and E_a^{FA} were isolated by SI ion exchange chromatography at pH 5.0 on FPLC from cytosolic 70 kDa PP1, as previously described [8]. The trypsin-treatment to produce the 33 kDa form from the various 37 kDa E_a was performed at 20 μg/ml trypsin for 10 min at 30°C, followed by 120 μg/ml soybean trypsin inhibitor [8]. Muscle phosphorylase kinase was a gift from Dr. L.M.G. Heilmeyer (Ruhuniversität-Bochum, Germany). Rabbit muscle phosphorylase *b* was used to produce ^{32}P-labeled phosphorylase *a* [18]. [^{32}P]ATP (2000 Ci/mmol) was prepared using inorganic [^{32}P]phosphate as described [18]. v-abl was isolated [19] from *E. coli* expressing recombinant v-abl [20], concentrated on Amicon Centricon, and stored at −70°C in the presence of 50% glycerol. The final preparation had a spec. act. of 15.3 nmol/mg using poly(Glu/Tyr) (4:1) as substrate and was not contaminated by serine/threonine kinase since neither phosphoserine nor phosphothreonine were detected in myelin basic protein phosphorylated by v-abl (E.V.-M., S. Lapi, G. Gaudino and P.M. Comoglio, in preparation). Protein was determined by the method of Bradford [21], using BSA as standard.

2.4. *Phosphorylation of PP1*

The incubation (50 μl) contained 0.1 mM ATP, 12 mM $MgCl_2$, 4 mM $MnCl_2$, 0.2 mM EGTA, 0.1 mM sodium orthovanadate, 50 mM NaCl and 4–5 × 10^3 cpm/pmol [^{32}P]ATP, the amount of v-abl that would incorporate 46 pmoles of ^{32}P into poly(Glu/Tyr) (4:1) per min at 30°C (see above) and PP1 as specifically indicated. After 8 h at 30°C, unless indicated otherwise, the reaction was stopped by the addition of 8 μl 0.1% deoxycholate and 9 μl 50% trichloroacetic acid. After 10 min on ice the samples were centrifuged at 4°C for 5 min in a Microfuge E, the pellet was resuspended in 12 μl electrophoresis sample buffer for subsequent electrophoresis [22] and autoradiography on Hyperfilms-MP.

2.5. *Peptide synthesis and production of anti-PP1 antibodies*

A 16 amino acid-long peptide reproducing the sequence from residue 294–309 of PP1α [23] was carried out essentially according to Merrifield [24] with an Applied Biosystems model 430 Automatic Peptide Synthetizer. The synthetic peptide was cleaved from the solid support and side chain protective groups were removed by treatment with anhydrous hydrofluoric acid [25]. 1.5 mg of crude peptide bound to 1.5 mg of Keyhole Limpet Hemocyanin, as previously described [26], was mixed with complete Freund adjuvant and injected into a rabbit [26]. Anti-PP1 catalytic subunit antibodies were detected by both ELISA and Western blot using PP1 as antigen.

2.6. *Production of anti-phosphotyrosine antibodies*

4.8 mg of phosphotyrosine bound to 4.6 mg bovine IgG was injected into 3 guinea pigs as previously described [26]. Detection of positivity in sera was done by ELISA using phosphotyrosine bound to ovalbumine as antigen [26]. Antibodies were purified by affinity chromatography on a column prepared with 1.5 ml Affi-Gel 10 and 5 mg phosphotyrosine [26].

2.7. *Immunoblotting*

After electrophoresis [22] on a 10% polyacrylamide gel in a Mini-Protean Bio-Rad apparatus and Western blot onto nitrocellulose [27], PP1 catalytic subunit was detected with 5 μl of anti-PP1 serum, and proteins phosphorylated on tyrosine were detected with 2–3 μg of affinity purified anti-phosphotyrosine IgG. Peroxidase-conjugated anti-IgG, ECL reagents and Hyperfilms-ECL were used for immunodetection.

3. RESULTS AND DISCUSSION

To investigate whether recombinant v-abl tyrosine kinase phosphorylates the catalytic subunit of PP1 we used the active catalytic subunits E_a^{ct} obtained from cytosol [17] or E_a^G from glycogen particles [18], or inactive cytosolic 70 kDa complex (consisting of inactive catalytic subunit E_i and I2 [16]). We found that v-abl phosphorylated E_a^{ct} (Figs. 1c and 2A) more efficiently than E_a^G (Figs. 1a and 2A), while E_i bound to I2 in the 70 kDa complex purified from cytosol (Fig. 1e) or E_a^{ct} in a complex reconstituted from E_a^{ct} and I2 (not shown) was not phosphorylated. Failure to phosphorylate the 70 kDa E–I2, either as purified or as reconstituted complex, may be due to the association with I2 that prevents access to the phosphorylation site (as suggested also for src phosphorylation [13]) or, alternatively, to the enzyme conformation (see below).

The stoichiometry of v-abl phosphorylation depended on the concentration of E_a^{ct} (Fig. 2A). Up to 0.73 mol/mol Pi was introduced into E_a^{ct} at 18 nM while E_a^G was phosphorylated only up to 0.076 mol/mol. Phosphorylation of E_a^{ct} was relatively slow and reached its maximum in about 3 h (Fig. 2B). This is similar to that reported for v-src or c-src phosphorylation of cytosolic E_a [13,14], although in the latter case phosphorylation was only up to 0.34 mol/mol [13]. The fact that high stoichiometry was obtained only at high E_a dilution might be explained by assuming a high affinity between v-abl kinase and E_a, with the consequence that dissociation of the enzyme–product complex is obtained only at high dilution. This mechanism or, alternatively, the presence of some inhibitory component in the PP1 preparation, would explain also the marked inhibition of v-abl autophosphorylation that we detected at the highest E_a^{ct} or E_a^G concentrations (not shown).

Limited tryptic proteolysis reduces the SDS-electrophoresis size of cytosolic E_a forms [16] and of E_a^G [18] from 37 kDa to 33 kDa. The 33 kDa E_a obtained from both E_a^{ct} and E_a^G could no longer be phosphorylated by v-abl (Fig. 1b and d), indicating that the phosphorylation site was located in the ≈4 kDa fragment removed by proteolysis. Based on the size of the proteolyzed E_a and assuming that trypsin removes about 30 residues from the C-terminus [1], we raised antibodies to a 16-amino acid peptide reproducing the sequence of PP1α from residue 294–309 [23]. These antibodies recognized the 37 kDa (Fig. 1g) but not the 33 kDa proteolyzed E_a^{ct} (Fig. 1h) confirming that the ≈4 kDa fragment removed by trypsin was from the C-terminus. Phosphorylation was confirmed to be on tyrosine since anti-phosphotyrosine antibodies recognized the v-abl-phosphorylated E_a^{ct} in Western blot (Fig. 1l), whereas they did not

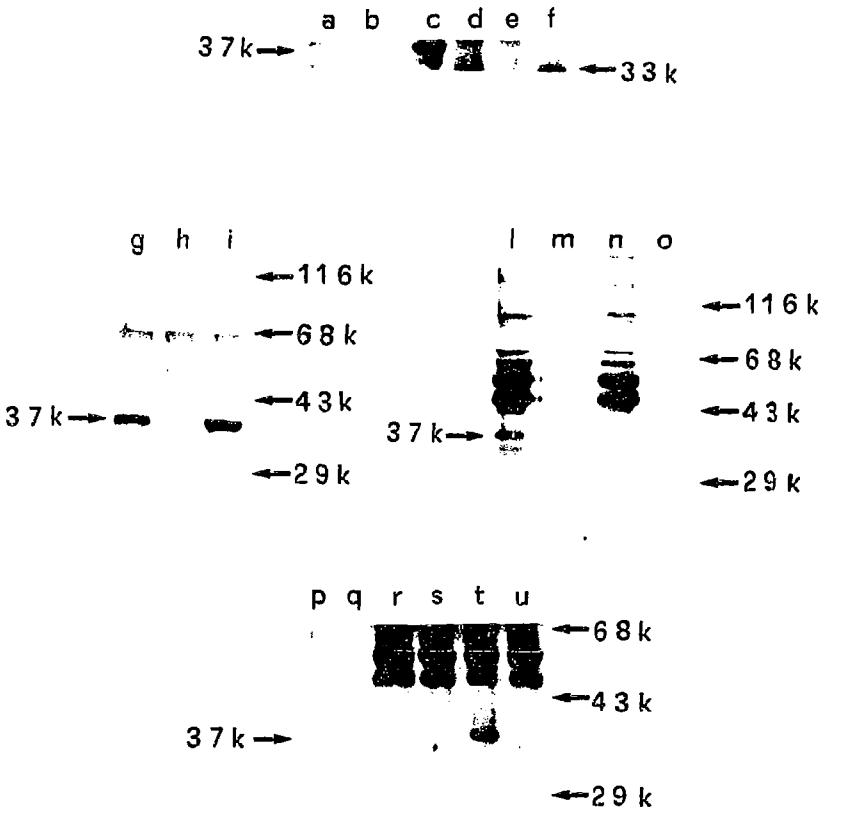

Fig. 1. Phosphorylation of various PP1 forms by recombinant v-abl tyrosine kinase. (a) 1 μg of catalytic subunit from glycogen particles (E_a^G) in native 37 kDa, (b) proteolyzed 33 kDa form of E_a^G, (c) 1 μg of cytosolic catalytic subunit (E_a^{ct}) in native 37 kDa (d) proteolyzed 33 kDa form of E_a^{ct}, (e) 1.6 μg cytosolic 70 kDa PP1 (37 kDa catalytic subunit E_i–inhibitor 2 complex) and (f) buffer only, were phosphorylated with v-abl tyrosine kinase in the presence of [^{32}P]ATP for 8 h at 30°C and subjected to SDS-electrophoresis on a 10% polyacrylamide gel followed by autoradiography. The procedure to obtain the proteolyzed 33 kDa form is described in section 2. (d) and (f) show also the upper part of a heavily phosphorylated 30 kDa band present in the v-abl preparation, whose degree of phosphorylation varies in the presence of the various PP1 samples.
Detection of PP1 catalytic subunit on Western blot with anti-PP1 peptide antibodies. (g) 125 ng of E_a^{ct} in native 37 kDa; (h) proteolyzed 33 kDa form; (i) 200 ng of 37 kDa E_a^G.
Detection of PP1 catalytic subunit on Western blot with anti-phosphotyrosine antibodies. (l,m) 125 ng, (t) 280 ng of 37 kDa E_a^{ct}, (n) 500 ng of proteolyzed 33 kDa E_a^{ct}, (o) 850 ng of 37 kDa E_a^G, (p,r) 280 ng of E_i (inactive catalytic subunit), (s) 280 ng of E_a^{Mn} (E_i activated by Mn^{2+}) and (q,u) 280 ng of E_a^{FA} (E_a isolated from 70 kDa PP1 complex after activation by F_A/GSK3), phosphorylated (l,n,r–u) or not (m,o,p,q) with v-abl in the presence of unlabeled ATP for 8 h at 30°C. The ≈45 kDa and ≈55 kDa bands in lanes l,n, r–u are the 2 autophosphorylated v-abl products. Standard proteins: 116 kDa, β-galactosidase; 68 kDa, BSA; 43 kDa, ovalbumin; 35 kDa, PP1 catalytic subunit.

recognize unphosphorylated E_a^{ct} (Fig. 1m), indicating the absence of endogenous phospho-tyrosine. Also the proteolyzed 33 kDa E_a^{ct} was not recognized by the anti-phosphotyrosine antibodies (Fig. 1n), thus confirming that the phosphorylation site was in the trypsin-sensitive region.

Low phosphorylation of E_a^G was neither due to phosphorylation site occupancy, since anti-phosphotyrosine antibodies did not recognize E_a^G in Western blot (Fig. 1o), nor to absence of the trypsin-sensitive region, since E_a^G was recognized by the anti-PP1 antibodies (Fig. 1i). The possibility that some G bound to E_a might prevent phosphorylation, similar to that described for I2 (see [13–15] and above), was also discarded since E_a^G had been obtained by a purification procedure that removes G [18], and in fact no G was detected in the E_a^G preparation by anti-G antibodies [26] in Western blot (not shown). Consequently the results seem to indicate that the different level of phosphorylation between E_a^{ct} and E_a^G may be due to different conformations or possibly to different isozymes (see also [8]).

To further investigate the effect of E conformation on phosphorylation by v-abl we tested various E forms, all of them isolated from the 70 kDa cytosolic PP1 by SI chromatography on FPLC [8]. E_i was obtained directly from the inactive 70 kDa PP1, E_a^{Mn} was obtained by activating E_i with Mn^{2+} and E_a^{FA} was obtained from 70 kDa PP1 that had been activated by F_A/GSK3. None of these E forms were recognized by anti-phosphotyrosine antibodies before incubating with v-abl (see e.g. E_i and E_a^{FA} in Fig. 1p and q). After v-abl phosphorylation still none of these forms was recognized by the antibodies (Fig. 1r,s and u) in an experiment where E_a^{ct} was phosphorylated, as expected (Fig. 1t). Phosphorylation

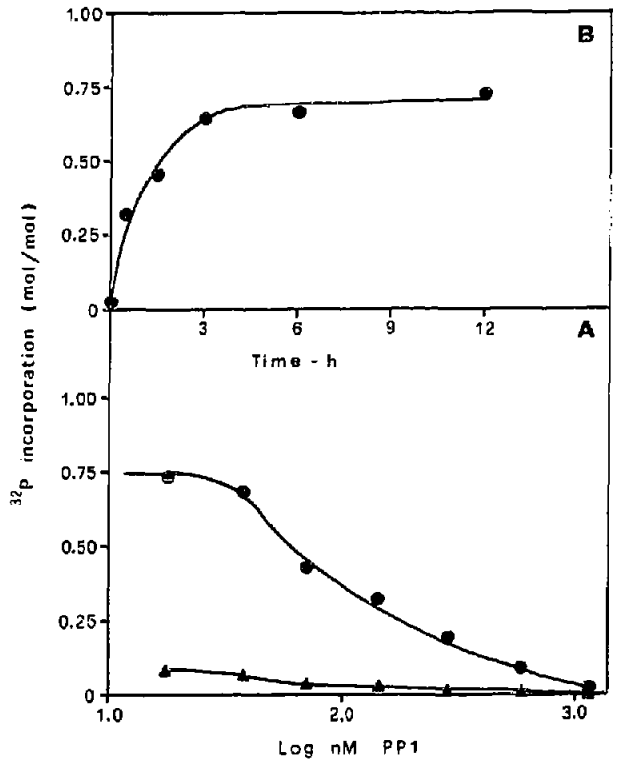

Fig. 2. Phosphorylation of the 37 kDa E_a^{et} (●) or E_a^{G} (▲) by the v-abl tyrosine kinase. (A) PP1 was phosphorylated for 8 h at 30°C in the presence of [^{32}P]ATP at the indicated final concentrations and then subjected to SDS-electrophoresis and autoradiography. The 37 kDa protein bands were excised from the gel, mixed with scintillant and counted. (B) E_a^{et} (18 nM, final concentration) was phosphorylated at 30°C for the time indicated as described in (A). Stoichiometry was calculated assuming phosphorylation at one site.

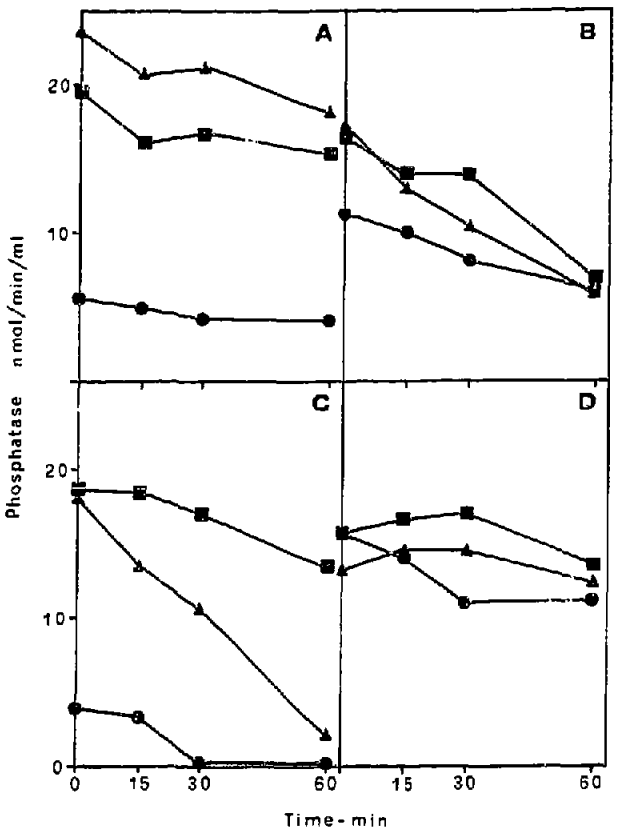

Fig. 3. Conversion of the 37 kDa E_a^{et} from active to inactive form. E_a^{et} phosphorylated (C and D) or not (A and B) with v-abl in the presence of unlabeled ATP was incubated at the final concentration of 36 nM with (A and C) or without (B and D) 350 nM inhibitor-2. At each time point aliquots were taken from the incubation mixture to assay PP1 for basal activity (●) or following activation with trypsin (▲) or trypsin and Mn^{2+} (■).

of E_a^{FA} was also attempted at various Ea concentrations in the presence of [^{32}P]ATP and the highest stoichiometry obtained was 0.05 mol/mol at 18 nM E_a (compare with Fig. 2A).

Phosphorylation did not affect the activity of E_a^{et} or E_a^{G} towards phosphorylase *a* in up to 8 h incubation at 30°C (not shown). In this respect v-abl phosphorylation was different from src phosphorylation, which was accompanied by a parallel decrease in enzyme activity [13–15]. We have no explanation for such a discrepancy, since the phosphorylation site seems to be the same as well as the substrate for PP1 assay. However, we found that phosphorylation by v-abl influenced the interaction of E_a^{et} with I2. When E_a^{et} was incubated in diluted conditions (36 nM) with 10-fold excess I2 the activity was inhibited by approximately 75% (compare basal activity and activity after trypsin at time 0 in Fig. 3A). We have shown previously [8,16] that trypsin-treatment removes I2 allowing the assay of E_a. Upon incubation in the presence of I2, E_a slowly converts to the inactive E_i conformation, which needs Mn^{2+} for activation. Conversion is revealed by the loss of reactivation with trypsin alone (activity is recovered with trypsin and Mn^{2+}). We found that when we used E_a^{et} it did not convert to E_i, as indicated by the fact that all the activity was recovered by trypsin-treatment (Fig. 3A) even after 2 h incubation. However, the same E_a^{et} preparation converted to E_i within 1 h when E_a^{et} had been previously phosporylated with v-abl (Fig. 3C), as indicated by the recovery of the activity with trypsin and Mn^{2+}. Incubation involved some degree of irreversible loss, ranging from 20–30% to over 50% in the case of free E_a, although free E_a became more stable after v-abl phosphorylation. The slight activation of isolated E_a^{et} by trypsin (Fig. 3B and D) is common and increases upon enzyme storage [18]. In the literature there are conflicting reports of the E_a forms that are able to convert to E_i upon incubation with I2 [1,8–10,16,17]. We confirmed that conversion could be obtained with E_a^{FA} or E_a^{G} under the same conditions as in Fig. 3 (further described in [8,18]) but not in the case of E_a^{et}, although the 75% enzyme inhibition indicated that binding to I2 did take place. The fact that the E_a^{et} to E_i conversion became possible after phosphorylation by v-abl may indicate that phosphorylation induces a conformational change that favours conversion.

Altogether our results indicate an inverse relationship

between E_a phosphorylation and inactivation in diluted conditions. The cytosolic E_a forms and E_a^G, that are readily inactivated, are not phosphorylated to a significant degree, and this is not due to the presence of endogenous phosphotyrosine. On the other hand the E_a^{et} form that is phosphorylated, does not convert readily to E_i until it has been phosphorylated. E_a^{et} and E_a^G might be different conformations or even different isozymes, whereas the difference among the cytosolic E_a forms is more likely due to different conformations. One possibility is that early removal of I2 by ethanol during the purification induces or keeps Ea^{et} in a conformation that is different from the conformation of E that has been associated with I2 throughout the purification, and is finally obtained as E_i in the 70 kDa complex. It is not known at present whether PP1 is a physiological substrate for v-abl or src kinase or other tyrosine kinases. The almost stoichiometric phosphorylation, as well as the preference for one specific E_a form, may be in favour of such an hypothesis. The absence of any effect on PP1 activity following v-abl phosphorylation may be due to the use of an unsuitable substrate, or, alternatively, the function of such phosphorylation might be in regulating the association with other molecules rather than on the activity. If such phosphorylation were taking place in vivo it would allow the introduction of a control by tyrosine kinases into pathways that are regulated by serine/threonine phosphorylation. Hence the physiological relevance of such a mechanism needs to be tested in cells expressing abl or src kinase or possibly other kinases of these same families.

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