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I (PP-1

I) By Protein 14-3-3

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Phosphorylation Dependent Sequestration Of Protein Phosphatase-1_I (PP-1_I) By Protein 14-3-3

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Abstract.

PP-1 is involved in the control of the meiotic and mitotic cell divisions at the G2 to M transition by dephosphorylating phospho-serine 217 (phospho-serine 287 in *Xenopus*) of Cdc25, accompanied by activation of Cdc25 which would then dephosphorylate and activate Cdk1 . PP-1_I has been proposed to be the form of PP-1 that dephosphorylates and activates Cdc25. Here we show that PP-1_I is sequestered in a phosphorylation dependent manner by protein 14-3-3 involving phosphorylation of serine 71 of I-2 moiety of PP-1_I by C-TAK1. Phosphorylation of serine 71 of the I-2 moiety of PP-1_I by C-TAK1 enhanced the sequestration and inhibition of PP-1_I by Protein 14-3-3. Phosphorylation of serine 71 of the I-2 moiety of PP-1_I caused an increase in the Stability Energy, Binding Energy of the interaction between PP-1_I and Protein 14-3-3 and a significant decrease in the IC₅₀ of the inhibition of PP-1_I by Protein 14-3-3. PP-1_I regulation by C-TAK1 and Protein 14-3-3 is a mechanism for the control of Cdc25 at the G2 to M transition of the cell cycle.

Introduction.

Protein phosphatase-1 (PP-1) was originally identified as an enzyme that dephosphorylates enzymes involved in the control of Glycogen breakdown and synthesis, including Phosphorylase a, Phosphorylase kinase and Glycogen synthase [1-4]. PP-1 was classified based on its preferential dephosphorylation of the subunit of Phosphorylase kinase and its specific inhibition by Inhibitor-1 (I-1) and Inhibitor-2 (I-2) [1-4]. Following the purification to homogeneity of the catalytic subunit of PP-1 (PP-1_C), it was shown to be distinct from Protein phosphatase-2A (PP-2A) [5]. PP-1_C is similar to Phosphatase C-I that was described and purified by Silberman et al. [6]. PP-1 was subsequently shown to be also involved in the control of cell cycle, cell survival and cell death, and other cellular processes [7-22]. PP-1_C does not exist as such in the cell. Instead, it is complexed to other inhibitory and activating proteins, including I-1, I-2, DARPP-32 and CPI-17 which are specific inhibitory proteins [23-38] and Glycogen binding protein (GM, GL,) and Myosin binding protein (MP) [39-42]. GM, GL, and MP have been proposed to be "Targeting Proteins" because they apparently target the catalytic subunit of PP-1, PP-1_C to different cellular compartments where PP-1_C can dephosphorylate its many substrates [43,44]. It has also been argued that PP-1 is regulated by "Protein Targeting Mechanism" (PTM) as opposed to the established regulation of enzymes by activating proteins and inhibitory proteins, "Activating and Inhibitory Mechanism" (AIM) [43,44]. There is no scientific evidence that supports the regulation of PP-1 by the PTM as the PTM implies that the concentration of PP-1_C would be a lot higher than what it is currently estimated to be [45]. Considering that there may be more than 100 different substrates for PP-1_C, under PTM, the concentration of PP-1_C in the cell would have to be at least one hundred times more than what it is currently estimated to be. It must be noted that there is not a single study that has determined the concentration of PP-1_C in the cell with any degree of accuracy. On the other hand, the regulation of PP-1 by AIM follows established mechanism of enzyme regulation: PP-1 is regulated by Activating and Inhibitory Proteins and its concentration in the cell does not need to be proportionally higher in order to account for the dephosphorylation of all of its substrates in the different cellular compartments where its substrates are found [45].

A major proportion of PP-1 in the cell is in the form of PP-1_I which is a complex consisting of PP-1_C and I-2, and other regulatory proteins [46-52]. PP-1_I is the same enzyme as ATP-Mg dependent protein phosphatase [53-57]. For a number of years, it was thought that PP-1_I and ATP-Mg dependent protein phosphatase are two distinct enzymes because the ATP-Mg dependent protein phosphatase was described as a Protein phosphatase consisting of a single protein of apparent molecular mass 70 kDa [55,56] or multiple proteins of apparent molecular masses 62 kDa, 38 kDa, 31 kDa and 30 kDa respectively [57]. It is not clear why the studies of the ATP-Mg dependent protein phosphatase were neglected. In brain tissue, it has been estimated that almost 30% of protein phosphatase activity is in the form of PP-1_I [52]. PP-1_I exists predominantly in an inactive form which can be activated following phosphorylation of I-2 molecule in PP-1_I on threonine 72 by several protein kinases, including GSK-1, PP-1_I-ACK, Cdk5 and Cdk1 [46-52,58-60]. Brain PP-1_I consists of PP-1_C and I-2 which are bound to a number of proteins, including C-TAK1 and Protein 14-3-3 [52]. The inhibition of PP-1_I by Protein 14-3-3 has been demonstrated [52]. However, the effect of Protein 14-3-3 on the activity of reconstituted PP-1_I described by Platholi et al. [46] is most probably substrate directed and not enzyme directed because ³²P-BAD was used as substrate and Protein 14-3-3 is known to bind to BAD phosphorylated by PKA [61]. How Protein 14-3-3 regulates the activity of PP-1_I remains to be established. Examination of the motif surrounding phosphorylation site threonine 72 of I-2 (DEPSPTP) reveals that it is a phosphorylation dependent binding and sequestration site for Protein 14-3-3 [62-67]. Serine 71 of I-2 can be phosphorylated by C-TAK-1 and the motif DEPPSTP in I-2 is very similar to a consensus C-TAK1 phosphorylation motif (RXXpSXP) [62-67]. The motif in I-2 (DEPPSTP) is very similar to DXXpSLP which has been shown to moderately bind Protein 14-3-3 [67]. It is not clear how Protein 14-3-3 sequesters PP-1_I to inhibit PP-1_I.

The role of Protein 14-3-3 in the control of key cellular processes, including the cell cycle, cell survival and cell death is well established [68-81]. Many enzymes and regulatory proteins have been showed to be bound and sequestered in a phosphorylation dependent manner by Protein 14-3-3, including Cdc25, Raf1, and Bad which are involved in the control of the cell cycle, cell survival and cell death respectively [68-81]. In view

of the fact that PP-1_C has been proposed to be involved in the control of Cdc25, Raf1 and Bad [13-15,71,74,75] and PP-1_I is complexed to Protein 14-3-3 and C-TAK1, phosphorylation dependent binding and sequestration of PP-1_I by Protein 14-3-3 suggests that PP-1_I may also be involved in the control of the cell cycle, cell survival and cell death. Here, the sequestration of PP-1_I by Protein 14-3-3 is analyzed. The results indicate that Protein 14-3-3 sequesters PP-1_I in a phosphorylation dependent manner by binding I-2 molecule of PP-1_I at phospho-serine 71 within the phosphorylation motif, DEPPSTP which is part of a phosphorylation recognition motif for C-TAK1 (RXXpSXP or DXXpSLP).

Materials and Methods.

Phosphorylase b, Phosphorylase kinase and PKA were purified from pig skeletal muscle as described in [82,83]. PP-1_I and PP-1_I-ACK were purified brain as described in [51]. C-TAK1 was prepared by inserting the gene coding for C-TAK1 into a Baculovirus Vector as described in [84]. Following infection and growth of infected cells, C-TAK1 was purified from extracts of infected cells by successive chromatographies on DEAE-Sepharose, Phospho-Cellulose and Superdex-200. Protein 14-3-3 was purified from pig brain extracts by successive chromatographies of pig brain extracts on DEAE-Sepharose, Phenyl-Sepharose and Superdex-200 as essentially described by Toker et al. [85].

³²P-labeled Phosphorylase a was prepared by phosphorylation of phosphorylase b with phosphorylase kinase and ³²P-labeled Phosphorylase kinase was prepared by phosphorylation of Phosphorylase kinase with PKA [86]. PP-1_I was assayed for its ability to dephosphorylate ³²P-labeled Phosphorylase a or ³²P-labeled phosphorylase kinase in the presence of PP-1_I-ACK, 1.25 mM MgCl₂ and .125 mM ATP as described in [49].

The full structure of PP-1_I has not been determined from X-ray crystallography because I-2 has very little ordered structure. Only 59 residues of I-2 was observed from the crystal lattice of PP-1_I reconstituted from recombinant PP-1_C and I-2. [87]. The structure of PP-1_I has been rendered from partial crystallographic data, NMR data and SAXS data.

However, these studies have not been verified and did not take into account phosphorylation of serine 71 of I-2 [88]. In the present work, the structure of I-2 was rendered de novo computationally pursuant to Xu and Zhang [89,90]. The structure of Human PP-1_C was also rendered de novo computationally pursuant to Yang et al. [91,92]. The structure of Protein 14-3-3 as determined by Smidova et al. [93] was used in this study.

For the purpose of thermodynamic calculations, the phosphorylation of serine 71 of I-2 moiety in the PP-1_I complex was performed using the Build Model Program of FoldX pursuant to Guerois et al. and Schymkowitz et al. [94,95]. The sequestration and binding of PP-1_I by Protein 14-3-3 was performed by Docking Experiments using Z Dock Program pursuant to Pierce et al. [96]. The structures of PP-1_C, I-2, PP-1_I, and PP-1_I-Protein 14-3-3 were analyzed and visualized by the CCP4 Molecular Graphics Program Version 2.10.11 as described by Mc Nicolas et al [97] and the ZMM Molecular Modeling Program as described by Garden and Zhorov [98]. Determination and calculation of Stability Energy ($\Delta G_{\text{stability energy}}$) of the protein complexes and non-complexed proteins was performed using the Stability Program of FoldX as described by Guerois et al. and Schymkowitz et al. [94,95]. Binding Energy ($\Delta\Delta G_{\text{binding energy}}$) of protein complex was determined and calculated using the Analyze Complex Program of FoldX as described by Guerois et al. and Schymkowitz et al. [94,95]. Binding Energy Difference ($\Delta\Delta\Delta G_{\text{binding energy difference}}$) between phospho-PP-1_I-Protein 14-3-3 complex and dephospho-PP-1_I-Protein 14-3-3 complex was calculated pursuant to Teng et al. and Nishi et al. [99-101] from the equation: $\Delta\Delta\Delta G_{\text{binding energy difference}} = \Delta\Delta G_{\text{binding energy}}$ (binding energy of phospho-PP-1_I-14-3-3 complex) - $\Delta\Delta G_{\text{binding energy}}$ (binding energy of phospho-serine 71-PP-1_I-14-3-3 complex).

Results.

The complete structure of PP-1_I has not been determined from X-ray crystallography because I-2 apparently has very little ordered structure and only 59 residues of rabbit I-2 could be observed from the crystal lattice of PP-1_I reconstituted from recombinant rabbit PP-1_C and I-2 [87]. A PP-1_I structure was rendered from partial crystallographic data,

NMR data and SAXS data. However, these studies have not been verified and phosphorylation of serine 71 was not taken into account in these studies [88]. The structure of Human I-2 which has 90% identity with Rabbit I-2 was rendered de novo pursuant to Xu and Zhang [89,90]. The structure of Human PP-1_C was also rendered pursuant to Yang et al. [91,92]. Through computerized protein docking experiments pursuant to Pierce et al. [96], the structure of PP-1_I consisting minimally of PP-1_C and I-2 could be rendered (Figure 1). The result showed that I-2 interacted with PP-1_C at several sites, including Site A (amino acids 10-22, Site B (amino acids 70-78) and Site C (amino acids 298-300). Site A of PP-1_C (amino acids 10-22) formed contacts with Site X1 (amino acids 48 to 60) and part of Site X2 of I-2 (around amino acids 118, 131 and 150), Site B of PP-1_C (amino acids 70 to 78) formed contacts with parts of Site X2 of I-2 (around amino acid 131), and Site C within PP-1_C interacted with I-2 at two major stretches encompassing amino acids 48 to 60 (Site X1) and amino acids 118 to 160 (Site X2). Site C (amino acids 298 to 300) also formed contact with Site X2 (around amino acid 123).

It has previously been reported that threonine 72 of I-2 moiety of PP-1_I is a phosphorylation site for GSK-3, Cdk1, Cdk5 and PP-1_I-ACK which triggers the activation of PP-1_I [29,46-52]. Adjacent to phospho-threonine 72 is serine 71 which is a phosphorylation site for C-TAK1 as it is part of the phosphorylation recognition site for C-TAK1 (RXXpSTP or DXXpSLP) [62-67] (the actual sequence is DEpSTP). The phosphorylation sites, serine 71 and threonine 72 which are phosphorylated by C-TAK1 and PP-1_I-ACK, GSK-2 and Cdk1 respectively are located at the surface of I-2 (Figure 2A) adjacent to CKII phosphorylation sites (serines 86, 120, 121 and 126) (Figure 2B), Figure 3 shows the ribbon structure and realistic rendering of PP-1_I with its I-2 moiety phosphorylated on serine 71. There was no significant difference in the structure of PP-1_I and phospho-serine 71-I-2-PP-1_I. There was also no significant difference in the Stability Energy and Binding Energy of PP-1_I and phospho-serine 71-I-2-PP-1_I (Table 1) indicating that phospho-serine 71 does not affect the activity of PP-1_I.

It was previously shown that PP-1_I purified from brain is found in a complex consisting of PP-1_C and I-2 and other proteins including Protein 14-3-3 which inhibits the activity of PP-1_I (most probably substrate directed and not enzyme directed) [52]. C-TAK1 can phosphorylate serine 71 of I-2 within the phosphorylation recognition motif DEPPSTP. Figure 3 and Figure 4 summarize the docking experiments between dephospho-PP-1_I, phospho-serine 71-PP-1_I and Protein 14-3-3. Phosphorylation of serine 71 resulted in marked structural change. Computerized thermodynamic calculation shows that phosphorylation of serine 71 increased the Stability Energy ($G_{\text{stability energy}}$) of the trimeric PP-1_C-I-2-Protein 14-3-3 complex from ~893 Kcal/mol to ~918 Kcal/mol and Binding Energy ($G_{\text{binding energy}}$) between PP-1_I and Protein 14-3-3 of the trimeric PP-1_C-I-2-Protein 14-3-3 complex from ~141 Kcal/mol to ~160 Kcal/mol. The Binding Energy Difference ($G_{\text{binding energy difference}}$) between dephospho-PP-1_C-I-2-Protein 14-3-3 complex and PP-1_C-I-2-Protein 14-3-3 complex was calculated to be ~20 Kcal/mol (Table 1). These results are consistent with the conclusion that phosphorylation of serine 71 of I-2 enhances the stability and binding affinity between PP-1_I and Protein 14-3-3.

Figure 5A shows that phosphorylation of serine 71 of I-2 moiety in PP-1_I enhances its inhibition by Protein 14-3-3 by causing a decrease of I_{50} from >300 μM to ~0.03 μM . Phosphorylation of serine 71 of I-2 moiety in PP-1_I did not have a significant effect on the activation of PP-1_I by PP-1_I-ACK (Figure 6).

Discussion.

PP-1_I is a major form of PP-1. PP-1_I represents almost 30% of total PP-1 activity in extracts of brain from freshly killed pigs [49,51,52]. Several studies have implicated PP-1 as an important controller of key steps of the cell cycle, cell survival, cell death [7-22]. However, PP-1_C does not exist by itself in the cell. Instead, it is complexed to a number of Activating and Inhibitory proteins, including Inhibitor-2 (I-2) to form PP-1_I [46-52]. PP-1_I consists minimally of PP-1_C and I-2 and other regulatory proteins, including Protein 14-3-3 and C-TAK1. PP-1_C has been proposed to be a key controller of meiosis and mitosis because (i) inhibiting its activity antagonizes G2 to M transition of the cell

cycle [7-11], (ii) it is regulated by Cdk-1 [58,59], and (iii) it is regulated by Protein 14-3-3 and C-TAK1 [13-15 and present work].

In the present work, structure model analysis and thermodynamic calculations show that PP-1_I can be sequestered by Protein 14-3-3 by a phosphorylation dependent mechanism involving phosphorylation of serine 71 of its I-2 moiety. Phosphorylation of serine 71 of the I-2 moiety of PP-1_I caused an increase in the Stability Energy and Binding Energy of the interaction between PP-1_I and Protein 14-3-3. In vitro enzyme assay of PP-1_I shows that phosphorylation of reconstituted PP-1_I by C-TAK1 significantly enhanced its inhibition by Protein 14-3-3. It was previously shown that PP-1_I purified from brains of freshly killed pigs was complexed to Protein 14-3-3 and C-TAK1 and using ³²P-BAD phosphorylated by PKA as substrate, reconstituted PP-1_I was inhibited by Protein 14-3-3 in vitro [52]. The effect of Protein 14-3-3 on the dephosphorylation of ³²P-BAD by reconstituted PP-1_I described in [52] is most probably substrate directed and not enzyme directed because Protein 14-3-3 is known to bind to phosphorylated BAD and PP-1_I was inhibited with I₅₀ of more than 10 μM when the concentration of ³²P-BAD in the assay was ~5 μM. In the present work, it is shown that phosphorylation of serine 71 by C-TAK1 enhances the inhibition of PP-1_I by Protein 14-3-3 by decreasing the I₅₀ from >300 μM to ~0.03 μM when the concentration of the substrate, ³²P-labeled Phosphorylase kinase was ~5 μM. The results of the present work are consistent with the conclusion that the effects of Protein 14-3-3 on the dephosphorylation of ³²P-labeled Phosphorylase kinase was enzyme directed and not substrate directed.

Phosphorylation of serine 71 of the I-2 moiety of reconstituted PP-1_I by C-TAK1 did not cause any significant inhibition of reconstituted PP-1_I in stark contrast to a report by Platholi et al. [102]. There are major problems in the data presented in the paper by Platholi et al. [102]. The data showing phosphorylation of serine 71 of the I-2 moiety of reconstituted PP-1_I by C-TAK1 has been performed only once and was stolen from Dr H.Y. Lim Tung. The effect of phosphorylation of reconstituted PP-1_I by C-TAK1 on the activity of reconstituted PP-1_I was performed only once and was stolen from Dr H.Y. Lim Tung. The first author of the paper by Platholi et al. [102] was a student working under

the supervision of Dr H.Y. Lim Tung. In the Acknowledgements Section of their paper, Platholi et al. [102] have admitted that they stole the data showing that C-TAK1 phosphorylated serine 71 of I-2 from Dr H.Y. Lim Tung who was never informed that his scientific data was being used without his knowledge and permission. The authors in [102] are lying when they reported that they purified PP-1_I from brains of freshly killed pigs and prepared ³²P-labeled Phosphorylase a. PP-1_I and ³²P-labeled Phosphorylase a described in [102] were purified and prepared respectively by Dr H.Y. Lim Tung. The authors of the paper by Platholi et al. [102] did not and does not know how to purify PP-1_I from brains of freshly killed pigs. The other data in the paper by Platholi et al. [102] showing the so-called effect of PFTAIRE kinase on reconstituted PP-1_I are all fabricated and falsified. PFTAIRE protein kinase is Cyclin dependent protein kinase requiring Cyclin D for full activity [103]. See [104] for a detailed discussion of why the paper by Platholi et al [102] is riddled with data fabrication and falsification.

Serine 71 of I-2 lies within a phosphorylation recognition motif (DEPpSTP) for C-TAK1. C-TAK1 is an enzyme which was first identified for its ability to phosphorylate serine 217 of Cdc25 which forms a binding and sequestration site for Protein 14-3-3 [62,63]. The sequestration of Cdc25 by Protein 14-3-3 has been proposed to be an obligatory control that prevents G2 to M transition of the cell cycle [12-14]. De-sequestration of Cdc25 by Protein 14-3-3 involves a very complex mechanism that remains to be fully elucidated. It has been proposed that a form of PP-1 is involved in the dephosphorylation of serine of Cdc25 [12-14]. The finding that the I-2 moiety of PP-1_I was phosphorylated by C-TAK1 and that phosphorylation of serine 71 of the I-2 moiety of PP-1_I by C-TAK1 enhanced its sequestration and inhibition by Protein 14-3-3 provides a linkage between PP-1_I and the control of G2 to M transition of the cell cycle. It is submitted that PP-1_I is involved in the dephosphorylation of phospho-serine 217 serine (phospho-serine 287 in *Xenopus*) of Cdc25, accompanied by the activation of Cdc25 which would dephosphorylate and activate Cdk1 at the G2 to M transition in mitotic cell division [15]. With respect to meiotic cell division, the activity PP-1_I could be controlled by its specific inhibitors, including I-1 and heat-stable protein inhibitors first identified by Boyer et al. [15,105]. Figure 8 is a working model for the role of PP-1_I in the activation of G2 to M

transition in meiosis and mitosis through dephosphorylation of phospho-serine 217 (phospho-serine 287 in *Xenopus*) of Cdc25.

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	Stability Energy (ΔG) Kcal/mol	Binding Energy ($\Delta\Delta G$) Kcal/mol	Binding Energy Difference ($\Delta\Delta\Delta G$) Kcal/mol
PP-1 _C -Dephospho-I-2	~626	~82	-
PP-1 _C -Phospho-S71-I-2	~625	~82	~0
PP-1 _C - Dephospho-I-2- 14-3-3 complex	~896	~138	-
PP-1 _C -Phospho-S71-I-2- 14-3-3 complex	~1016	~236	~98

Table 1. Thermodynamic calculations for PP-1_C-Dephospho-I-2, PP-1_C-Phospho-S71-I-2, PP-1_C- Dephospho-I-2-14-3-3 complex, and PP-1_C-Phospho-S71-I-2-14-3-3 complex. Phosphorylation of serine 71 of I-2 had no significant effect on Stability Energy and Binding Energy of the PP-1_C-I-2 complex. Phosphorylation of serine 71 of I-2 had significant effect Stability Energy and Binding Energy of the PP-1_C-I-2-14-3-3 complex.

Figure 1. Panel A, Ribbon structure of PP-1_I: The PP-1_C moiety of PP-1_I is shown in blue while the I-2 moiety of PP-1_I is shown in yellow. The C-TAK1 phosphorylation motif, DEPSTP is shown as red spheres; Panel B, Realistic rendering of the structure of PP-1_I: The PP-1_C moiety of PP-1_I is shown in orange while the I-2 moiety of PP-1_I is shown in yellow. The C-TAK1 phosphorylation motif, DEPSTP is shown in red.

Figure 2. Panel A, Ribbon structure of PP-1_I: with its I-2 moiety phosphorylated on serine 71: The PP-1_C moiety of PP-1_I is shown in blue while the I-2 moiety of PP-1_I is shown in yellow. The C-TAK1 phosphorylation motif, DEPpSTP is shown as red spheres; Panel B, Realistic rendering of the structure of PP-1_I: The PP-1_C moiety of PP-1_I is shown in orange while the I-2 moiety of PP-1_I is shown in yellow. The C-TAK1 phosphorylation motif, DEPpSTP is shown in red.

Figure 3. Panel A, Ribbon structure of Dephospho 71-I-2-PP-1_I-Protein 14-3-3 complex. The PP-1_C moiety of PP-1_I is shown in yellow and the I-2 moiety of PP-1_I is shown in grey while Protein 14-3-3 is shown in blue. C-TAK1 phosphorylation site motif (DEPSTP) is shown as red spheres. Panel B, Realistic structural rendering of of Dephospho 71-I-2-PP-1_I-Protein 14-3-3 complex. The PP-1_C moiety of PP-1_I is shown in orange and the I-2 moiety of PP-1_I is shown in yellow while Protein 14-3-3 is shown in grey. C-TAK1 phosphorylation site motif (DEPSTP) is shown in red.

Figure 4. Panel A, Ribbon structure of Dephospho 71-I-2-PP-1_I-Protein 14-3-3 complex. The PP-1_C moiety of PP-1_I is shown in blue and the I-2 moiety of PP-1_I is shown in yellow while Protein 14-3-3 is shown in pink. C-TAK1 phosphorylation site motif (DEPpSTP) is shown as red spheres. Panel B, Realistic structural rendering of of Dephospho 71-I-2-PP-1_I-Protein 14-3-3 complex. The PP-1_C moiety of PP-1_I is shown in orange and the I-2 moiety of PP-1_I is shown in yellow while Protein 14-3-3 is shown in grey. C-TAK1 phosphorylation site motif (DEPpSTP) is shown.

Figure 5. Inhibition of Dephospho-serine 71-I-2-PP-1_I (circles) and Phospho-serine 71-I-2-PP-1_I (squares) by Protein 14-3-3. 40 μ g PP-1_I was phosphorylated by incubation with 4 μ g C-TAK1 in the presence of 5 mM Mg²⁺ and 1.25 mM ATP for 30 minutes at 30⁰ C and then gel-filtered through a Superdex column (1 x 60 cm). The eluates containing phosphorylated PP-1_I were concentrated by vacuum dialysis. Unphosphorylated PP-1_I was treated in a similar fashion except that C-TAK1 was replaced with bovine serum albumin. The stoichiometry of phosphorylation was determined by using ³²P-labeled ATP instead of ATP: it was determined that ~0.83 mole ³²P-phosphate was incorporated per mole of PP-1_I. The activities of dephospho PP-1_I and phospho PP-1_I were assayed by determining the release of ³²P-phosphate from ³²P-Phosphorylase kinase at 10 μ M in the presence of saturating amount of PP-1_I-ACK, 0.125 mM ATP and 1.25 mM Mg²⁺. 100% PP-1_I activity was equivalent to 0.01 unit (the amount of PP-1_I that catalyzed the release of 0.01 nmol Pi/min when the concentration of the substrate was 10 μ M).

A/

B/

C/

Figure 6. Panel A, Specific Activity of Dephospho-serine 71-I-2-PP-1_I (circles) and Phospho-serine 71-I-2-PP-1_I (squares). 40 µg reconstituted PP-1_I was phosphorylated by incubation with 4 µg C-TAK1 in the presence of 5 mM Mg²⁺ and 1.25 mM ATP for 30 minutes at 30⁰ C and then gel-filtered through a Superdex column (1 x 60 cm). The eluates containing phosphorylated PP-1_I were concentrated by vacuum dialysis. Unphosphorylated PP-1_I was treated in a similar fashion except that C-TAK1 was replaced with bovine serum albumin. The stoichiometry of phosphorylation was determined by using ³²P-labeled ATP instead of ATP: it was determined that 0.93 mole ³²P-phosphate was incorporated per mole of PP-1_I. The activities of Dephospho-serine 71-I-2-PP-1_I and Phospho-serine 71-I-2 PP-1_I were assayed by determining the release of ³²P-phosphate from ³²P-Phosphorylase kinase at 10 µM in the presence of saturating amount of PP-1_I-ACK, 0.125 mM ATP and 1.25 mM Mg²⁺. Panel B, Activity of Dephospho-serine 71-I-2-PP-1_I (circles) and Phospho-serine 71-I-2-PP-1_I (squares) at various reaction time (squares). Panel C, Activity of Dephospho-serine 71-I-2-PP-1_I (circles) and Phospho-serine 71-I-2-PP-1_I (squares) at various substrate concentration.

END