Phosphorylation and Functions of Inhibitor-2 Family of Proteins

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ABSTRACT: Protein phosphatase-1 (PP1) is an essential protein Ser/Thr phosphatase that is extraordinarily conserved from yeast to human, and Inhibitor-2 (I-2) is the most ancient of the heat-stable proteins specific for PP1. We identified novel I-2 homologues in *Caenorhabditis elegans* (Ce) and *Xenopus laevis* (Xe) and compared them to the I-2 proteins from *Homo sapiens* (Hs), *Saccharomyces cerevisiae* (GLC8), and *Drosophila melanogaster* (Dm). The Ce I-2 and Dm I-2 showed the highest potency inhibition of rabbit PP1 with IC_{50} near 5 nM compared to Hs I-2 and Xe I-2 with IC_{50} between 10 and 50 nM and GLC8 with >100-fold lower activity. Inhibition of PP1 bound to Nek2 kinase activated the kinase to phosphorylate a C-Nap1 domain substrate. All the species of I-2 except GLC8 activated the Nek2::PP1 to the same extent as microcystin-LR. Only Hs I-2 and Xe I-2, not the I-2 proteins more divergent in sequence, directly activated human Aurora-A kinase. Various species of I-2 have a common PxTP phosphorylation site that showed a wide range of reactivity with GSK3, ERK, or CDC2/cyclinB1 kinases. The Suc1 subunit of CDC2/cyclinB1 enhanced reactivity with I-2, consistent with this being a site of mitotic phosphorylation. The results show species specificity among the I-2 family within the context of conserved PP1 inhibitory activity and variable phosphorylation by Pro-directed kinases.

Protein phosphatase-1 (PP1)[1] is an essential enzyme that dephosphorylates Ser/Thr sites in many proteins and is specifically required for metaphase to anaphase transition in fungi (*1*, *2*) and mammalian cells (*3*). Regulation and distribution of PP1 within cells involves its binding to many different subunits (*4*, *5*), estimated to exceed 150 in number (*6*). Binding of PP1 involves a primary interaction of an R/K-V-x-F motif in the regulatory subunit with sockets for the V and F residues on the backside of PP1, opposite the active site. These details have been visualized in 3D from structures of PP1 complexed with a KVSF-containing peptide (*7*) or the MYPT1 subunit (*8*) plus active site inhibitors (*9*−*11*). The PP1 holoenzymes exhibit narrow substrate specificity and differential regulation, often involving phosphorylation of the regulatory subunits. PP1 operates under an additional layer of regulation from families of inhibitor proteins (*12*), first described 30 years ago (*13*). These are proteins of relatively small size (15−30 kDa) that are mostly hydrophilic and heat-stable, properties common with other "intrinsically unstructured proteins" (*14*) that are flexible and adapt to the shape of their targets. PP1 inhibitor proteins are phosphorylated at multiple sites (*12*). Phosphorylation is required for high potency inhibition by several inhibitors such as I-1, DARPP-32, CPI-17, KEPI, and PHI-1. In the case of CPI-17, NMR demonstrates that phosphorylation produces a conformational change in the protein (*15*). These inhibitors are extremely specific for PP1 compared to other related Ser/Thr phosphatases such as PP2A, PP4, and PP6, even though these enzymes are inhibited with nanomolar potency by the same small molecule toxins binding at their active sites. Furthermore, certain inhibitors have specificity for PP1 holoenzymes where PP1 is already engaged with a regulatory subunit, with perhaps the best example being CPI-17 inhibition of myosin phosphatase (*16*). More recently, PP1 inhibitors have been shown to be multifunctional in that they also bind protein kinases to either inhibit or activate them (*17*, *18*).

Inhibitor-2 is the most ancient PP1 regulatory protein, conserved from yeast to human. Here we compared five species of I-2 in multiple assays. We compare inhibition of PP1 monomer, PP1 complexed to Nek2, and activation of Aurora-A kinase. We demonstrate that the most conserved sequence motif is phosphorylated by multiple Pro-directed kinases, but these show large differences in reactivity with the I-2 family members.

MATERIALS AND METHODS

Reagents. Restriction enzymes and GSK3, CKII, and CDC2/cyclinB1 kinases were purchased from New England BioLabs, and [γ-^{32}P] ATP was obtained from Amersham Biosciences. Oligonucleotides were synthesized by Integrated DNA Technologies. A RT-PCR kit was purchased from Invitrogen and the mRNA purification kit from Amersham Pharmacia Biotech. Aurora-A kinase was expressed in bacteria as described previously (*18*). Recombinant Suc1 protein was provided by Dr. Todd Stukenberg (University of Virginia). Active MAP kinase (ERK2) was provided by

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[1] Abbreviations: I-2, inhibitor-2 of protein phosphatase type-1; PP1, protein phosphatase-1; Ce, *C. elegans*; Xe, *Xenopus laevis*; Dm, *Drosophila melanogaster*; Hs, *Homo sapiens*; I-1, inhibitor-1; PCR, polymerase chain reaction; MCLR, microcystin-LeuArg; MBP, myelin basic protein.

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Drs. J. Smith and D. Lannigan (University of Virginia). A yeast cDNA library was provided by Drs. Nima Mosammaparast and Lucy Pemberton (University of Virginia). The C-terminal domain of C-Nap1 (residues 1979−2442) was cloned by RT-PCR with primers that added a BamH1 site to the 5′-end and a His_6 tag plus Sal I site to the 3′-end. This DNA was cloned into the pMal vector (New England Biolabs) to produce a maltose binding protein (malE) fusion protein with a C-terminal His_6 tag. The recombinant protein was expressed in the BL21 strain of *Escherichia coli* and purified by sequential chromatography on Ni-NTA (Qiagen) and amylose resin (New England Biolabs).

Cloning of cDNAs for Different Species of I-2. The National Center for Biotechnology Information (NCBI) expressed sequence tag database (dbEST) was searched with the human I-2 nucleotide sequence using the TBLASTN algorithm. A cDNA sequence from *Xenopus laevis* (GenBank accession #BI444584) was identified that encoded a portion of a polypeptide with significant sequence identity to human I-2. The *Xenopus* database (//Xenopus.nibb.ac.jp/) was queried using TBLASTN with the cDNA #BI444584 to identify the contig #036101 that contained the full sequence. The sense primer 5′-AGCGGATCCCATATGGCGGCTCAGACCCAGAGG-3′ and antisense primer 5′-GGACTCGAGCGGCCGCTCAGGATGAGCTTCGGTCTGT-3′ were designed from this entire sequence, and the cDNA was amplified by polymerase chain reaction (PCR). The PCR product was subcloned into the BamH1 and Not1 sites of pET28b. By searching the HomoloGene of NCBI, the predicted *C. elegans* I-2 cDNA (GenBank accession #NM_065746) and the cDNA sequences including *S. cerevisiae* GLC8, *D. melanogaster* I-2, and human I-2 were identified. The *C. elegans* ORF clone of I-2 (clone ID: Y32H12A. 4) was purchased from Open Biosystems. The cDNA was amplified by PCR with two primers 5′-GCGGATCCATGGCATCACCCTCCGCTTCT-3′ and 5′-GCGCGGCCGCCTAGTGTTCCATATTTCCACC-3′. A yeast cDNA library was used with specific sense primer 5′-GCGAATTCATGGGAGGTATACTTAAAAAC-3′and antisense primer 5′-GCGCGGCCGCTCATGGTTCTTTTGTCGTACT-3′ for amplifying GLC8 cDNA. A *Drosophila* cDNA library made from *Drosophila* S2 cells was used as template with sense primer 5′-GCGGATCCATGCAGAACAATCCCAGCCCA-3′ and antisense primer 5′-GCGCGGCCGCCTAGTTATTCGATGGCTCCAG-3′ for the cloning of *Drosophila* I-2. The GLC8, Ce I-2, Dm I-2, and Hs I-2 cDNAs were subcloned into the pET30a vector to generate His_6-S-tagged I-2 fusion proteins in *E. coli*.

Mutagenesis of Thr to Ala in Highly Conserved IDEPXTPY Motif. Human I-2 T72 was changed to A72 using the complementary oligonucleotides 5′-ATAGATGAACCAAGCGCTCCTTACCATAGT-3′ and 5′-ACTATGGTAAGGAGCGCTTGGTTCATCTAT-3′; GLC8 T116 was changed to A116 by these two pairs of complementary oligonucleotides 5′-ATAGACGAGCCCAAGGCCCCCTACCAAGGT-3′ and 5′-ACCTTGGTAGGGGGCCTTGGGCTCGTCTAT-3′; *C. elegans* I-2 T79A: 5′-ATTGATGAACCAAAGGCCCCATATCATCAT-3′ and 5′-ATGATGATATGGGGCCTTTGGTTCATCAAT-3′; *D. melanogaster* I-2 T59A: 5′-ATCGATGAGCCCAAAGCGCCGTACAACTAC-3′ and 5′-GTAGTTGTACGGCGCTTTGGGCTCATCGAT-3′; *Xenopus* I-2 T71A: 5′-ATAGATGAACCAAGCGCCCCATATCACAGG-3′ and 5-CCTGTGATATGGGGCGCTTGGTTCATCTAT-3′.

All mutated codons were underlined. QuickChange (Stratagene) mutagenesis reactions were performed on the pET expression constructs.

Bacterial Expression and Purification of His_6-S-Tagged I-2 Proteins. E. coli strain BL21(DE3) plysS was transformed with pET-I-2 bacterial expression vectors and grown overnight at 37 °C in 10 mL of LB medium containing 30 μg/mL of kanamycin, 20 μg/mL of chloramphenicol, and 0.25 mL of 20% glucose. The culture was inoculated into 1.0 L of LB medium including 30 μg/mL of kanamycin and 20 mL of ethanol. The transformed cells were grown to the absorbance of 0.6 at 600 nm at 37 °C, and the expression of I-2 proteins was induced by addition of isopropyl-1-thio-β-D-galactopyranoside into the culture at a final concentration of 1 mM for 6 h at 37 °C. The bacteria were collected by centrifugation at 4500*g* for 10 min, and the cell pellet was suspended by a buffer containing 25 mM MOPS−NaOH, 1.25 mM EDTA, 0.4 mM Pefabloc, 10 μg/mL of lima bean trypsin inhibitor, 0.5 mM TCEP (tris(2-carboxyethyl)phosphine), and 1.25 mg/mL of lysozyme and was stored at −20 °C overnight. On the next day, the pellet was thawed for 10 min in a 37 °C bath, $MgCl_2$ (10 mM final concentration) added, plus DNase1 (0.01 mg/mL final concentration). After incubating at room temperature for 10 min, NaCl (0.8 mM final concentration) and 15 mL of dH_2O were added, and the cells were heated for 10 min in boiling water. The supernatants were collected by centrifugation for 20 min at 20 000*g* and transferred into a 50 mL tube. His_6-tagged I-2 in the extracts was adsorbed to Ni^{2+}-bound chelating sepharose fast flow (Pharmacia) column (1.5 × 5.7 cm) at 4 °C. The column was successively washed with buffer containing 20 mM imidazole/HCl, pH 7.0, 20 mM potassium phosphate, and 0.5 M NaCl. The bound proteins were eluted by buffer with 0.5 M imidazole/HCl, pH 7.0, 20 mM potassium phosphate, and 0.5 M NaCl. The eluted protein was dialyzed against buffer of 25 mM HEPES, pH 7.5, 1 mM DTT, 0.4 mM Pefabloc, 5 mM $MgCl_2$, and 50 mM NaCl, then was stored at −80 °C.

Sequence Alignment and Construction of Phylogenetic Tree. Sequences were aligned by using the multiple alignment algorithm in the MegAlign package (Windows version 3.12e; DNASTAR, Madison, WI) followed by manual optimization. Phylogenetic analyses using distance methods were carried out with the GCG Sequence Analysis Package. The phylogenetic tree is based on the entire amino acid sequence of the members shown. Distance matrices were generated by the Jukes−Cantor correction model. These matrices were analyzed by the UPGMA program to construct a phylogenic tree.

Phosphorylase Phosphatase Assay. PP1 activity was assayed by the release of ^{32}P phosphate from phosphorylase *a* as described by Shenolikar and Ingebritsen (*19*). PP1 purified from rabbit skeletal muscle was incubated with 15 μM phosphorylase *a* in 20 mM MOPS, pH 7.4, 50 mM NaCl, 1 mM $MgCl_2$, 1% (v/v) 2-mercaptoethanol, 1 mM dithiothreitol, 10% glycerol, and 0.1 mg/mL of BSA in a total volume of 40 μL for 15 min at 30 °C. The reaction was terminated by addition of 60 μL of 1% bovine serum albumin and 300 μL of 15% (w/v) trichloroacetic acid and was incubated on ice for 10 min. After centrifugation at 15 000*g*

for 10 min, the supernatant (300 μL) was analyzed for ^{32}P release by liquid scintillation counting.

Kinase Assays. Recombinant GSK3, CKII, ERK2, or CDC2 was incubated with different species of recombinant His_6-S-tagged-I-2 wild type or I-2 (T/A) mutant proteins in reactions containing 25 mM MOPS, pH 7.4, 0.1% 2-mercaptoethanol, 5 mM $MgCl_2$, 50 mM NaCl, 1 mM EGTA, 1 mM Pefabloc, 1 μM MCLR, 10 mM NaF, 1 mM vanadate, 4 mM β-glycerophosphate, 0.1% NP-40, and 100 μM [^{32}P]-ATP (20 μCi). The whole reactions in 50 μL were run at 30 °C for 1 h, then stopped by 2× SDS sample buffer. The samples were resolved by SDS-PAGE, gels stained by Coomassie, and labeled I-2 detected by autoradiography. The ^{32}P-labeled protein bands were cut from gels, and the radioactivity was quantitated with a Beckman model LS-6500 scintillation counter.

For Aurora-A kinase assay, recombinant Aurora-A (300 nM) was incubated with or without species of I-2 proteins for 10 min at 30 °C in the kinase buffer containing 20 mM Tris, pH 7.5, 1 mM $MgCl_2$, 25 mM KCl, 1 mM dithiothreitol, and 40 μg/mL of bovine serum albumin. Reactions were stopped with 2× SDS sample buffer and proteins separated by SDS-PAGE (*18*). Gels were stained with Coomassie blue, dried, and analyzed by Phosphor Imager using Image Quant software (Amersham Biosciences).

For the Nek2 kinase assay, HEK293T cells at 60% confluence in 6 cm dishes were co-transfected with 2 μg of plasmid DNA for HA_3-Nek2 (*20*) and 4 μg plasmid DNA for PP1γ in pcDNA3 using Lipofectamine reagent (Invitrogen). After 36 h, extracts were prepared in 0.4 mL of lysis assay buffer consisting of 50 mM Hepes, pH 7.5, 0.1 M NaCl, 10 mM $MgCl_2$, 0.5 mM $MnCl_2$, 5 mM EGTA, and 0.1% NP-40 with protease inhibitor cocktail (CalBiochem) at 1:100 dilution. Following centrifugation at 13 000 rpm for 10 min, the supernatants were recovered and used for immunoprecipitations with protein A agarose beads (Amersham) preloaded with 12CA5 antibody. After 1 h mixing end-over-end, the beads were recovered by centrifugation, washed once with 1 mL of the lysis assay buffer, and resuspended and dispensed for assay. Beads with the HA_3-Nek2::PP1 complex were incubated alone as control or with 2 μM MCLR or different I-2 before starting reactions with addition of 100 μM [^{32}P]ATP (10 μCi) and 3.5 μg of malE-cNAP1-His_6 as substrates. After 40 min at 30 °C, reactions were stopped with SDS buffer and boiling and products resolved by SDS-PAGE. After Coomassie staining and drying, the band of malE-cNAP1-His_6 protein was excised from the gel and radioactivity was determined by Cherenkov radiation with a Beckman model LC-6500 scintillation counter.

RESULTS

Identification of Novel I-2 Proteins from Various Eukaryotic Species. Phosphatase inhibitor-2 protein from rabbit muscle was sequenced before its cDNA was cloned (*21*), and subsequently, homologues have been identified by DNA sequence in human, rat, *Drosophila*, and *S. cerevisiae* (*22*−*25*). We searched the NCBI EST (expressed sequence tag) database using human I-2 cDNA to identify related proteins in other species. We found a *C. elegans* I-2 homologue (GenBank accession #NM_065746) with an entire open reading frame containing 192 amino acids. This protein had an extra 22 residues at its N-terminus relative to human I-2. Another homologue was identified from *Xenopus laevis* (GenBank accession #BI444584). This sequence contained 85 bp of 5′ untranslated region and was missing a portion of the 3′ coding region. Because this was only a partial clone, we searched the *Xenopus* database (//Xenopus.nibb.ac.jp/) using this cDNA and found the entire sequence of predicted *Xenopus* I-2 encoding a polypeptide of 187 amino acids that was 17 residues shorter than human I-2 at the C-terminus. We also cloned by RT-PCR the I-2 cDNA from *H. sapiens*, *Saccharomyces cerevisiae* (GLC8), and *Drosophila melanogaster*.

The amino acid sequences of these five species of I-2 are aligned in Figure 1A to reveal three regions that are most highly conserved among these proteins. One region is the site (PIKGILKN) at the N-terminus required for high potency inhibition of the PP1 catalytic subunit. This site is identical in Hs I-2 and Xe I-2. In Dm I-2 and Ce I-2, there are two and three residues substituted, respectively, involving the first Ile and the Asn. In the yeast GLC8 protein, this region appears at the N-terminus of the entire protein with the PIK sequence substituted by MG, followed by the GILKN motif. The second conserved region (IDEPx**T**PY) contains a phosphorylation site, and seven of eight residues are identical in all five species. The third conserved sequence region (FExKRKxHY) is in the C-terminal region. The sequence (RKLHY) has been proposed as a secondary PP1 binding site (*26*). In addition to these three sequence motifs, we noted that the C-terminal region of all these I-2 consists of a large proportion of acidic amino acid residues, including several consecutive acidic residues (e.g., DDDEDEE, DEEEDEDEE, EEDDDDDDED, EDDEEE, or DEDEDEDD). The only functions assigned to the C-terminal region of I-2 have been activation of Aurora-A kinase (*18*) and a role in GSK3 reactivation of PP1 in the I-2::PP1 heterodimer (*26*). We constructed a phylogenetic tree to show relationships between I-2 proteins (Figure 1B). This showed the *C. elegans* I-2 as the most primitive metazoan version of I-2, which lies between *Drosophila* and yeast.

Purification of Recombinant I-2 Proteins. The five different species of I-2 were subcloned into pET vectors for expression in bacteria and purified as recombinant heat-stable proteins. Treatment of total bacterial lysates at 100 °C followed by centrifugation resulted in removal of ∼80% of the total protein. The I-2 proteins remained soluble after boiling and were purified from the heat-stable fraction by Ni-agarose affinity chromatography. Analysis of the purified proteins by SDS-PAGE and Coomassie staining showed that these I-2s were essentially homogeneous and all displayed anomalous low mobility on SDS-PAGE (Figure 2). The observed (and expected) sizes in kDa were: GLC8, 45 (*32*); Ce I-2, 33 (*27*); Dm I-2, 35 (*28*); Xe I-2, 32 (*25*); Hs I-2, 39 (*28*). The properties of these I-2 proteins are consistent with them being inherently unstructured or relatively disordered proteins, characteristic of many regulatory proteins involved in cell signaling (*14*).

Inhibition of PP1 by Different Species of I-2. We assayed different species of I-2 for the ability to inhibit the type-1 protein phosphatase monomeric catalytic subunit. PP1 purified from rabbit skeletal muscle dephosphorylates ^{32}P-labeled phosphorylase *a*, and we determined the concentration range

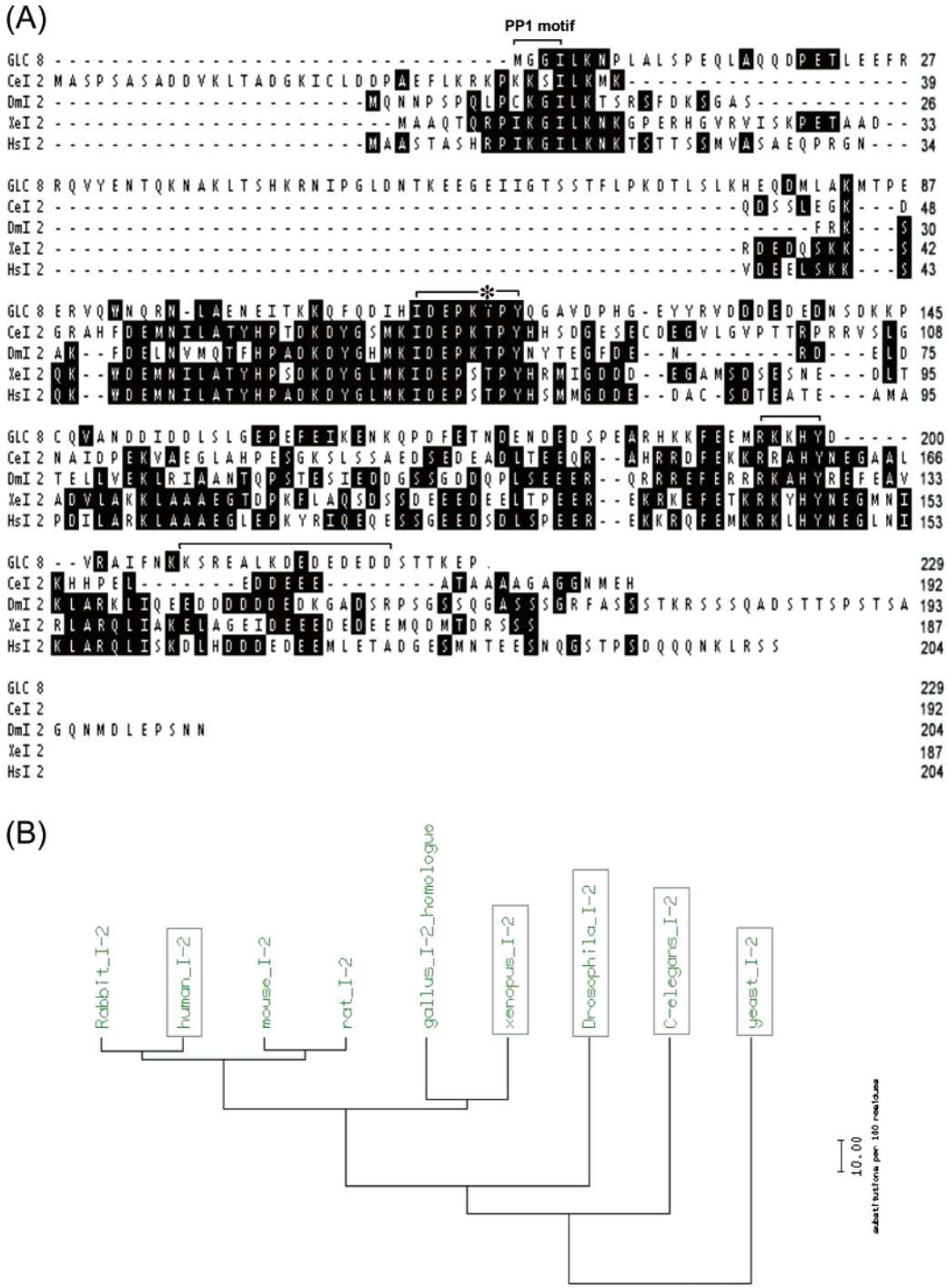

FIGURE 1: (A) Sequence comparison of different species of I-2. The *S. cerevisiae* GLC8, *C. elegans* I-2 (Ce I-2), *D. melanogaster* I-2 (Dm I-2), *Xenopus* I-2 (Xe I-2), and *Homo sapiens* I-2 (Hs I-2) protein sequences in single letter code were aligned. Numbering of the amino acids is from the residue following the initiator Met. The highly conserved IDEPXTPY site in each I-2 protein is indicated and the phosphorylated Thr marked by ∗. Other conserved regions, including the putative PP1 binding motif, are also indicated by brackets. The comparison was created using the program DNASTAR (DNASTAR, Inc. Madison, WI) followed by manual optimization of the alignment, and shading was done using the program BOXshade. (B) Phylogenetic relationship among I-2 genes. The phylogenetic tree is based on I-2 amino acid sequences constructed by UPGMA clustering of Jukes−Cantor distances. The scale bar indicates the scale of sequence divergence. The boxes show I-2 proteins analyzed in this study.

of PP1 in the assay that produced a linear release of ^{32}P (Figure 3A). Using a concentration of PP1 in this range, the dose-dependent inhibition by different species of I-2 proteins was determined (Figure 3B). The concentrations of I-2 proteins were quantitated both by Bradford assay and by Coomassie staining after gel electrophoresis (data not shown). The Ce I-2 and Dm I-2 showed the highest potency with IC_{50} near 5 nM. This shows conservation of potent PP1 inhibition by the novel Ce I-2 protein. By comparison, the two proteins from vertebrate species, Hs I-2 and Xe I-2, had slightly lower activity with IC_{50} between 10 and 50 nM. GLC8 had more than 100-fold lower activity than any of these other I-2s, with IC_{50} of 1 μM. The potency of GLC8 was about the same as that of the truncated protein Hs I-2 (14−197), which has the primary PP1 binding site at the N-terminus deleted. Thus, all these proteins were inhibitors

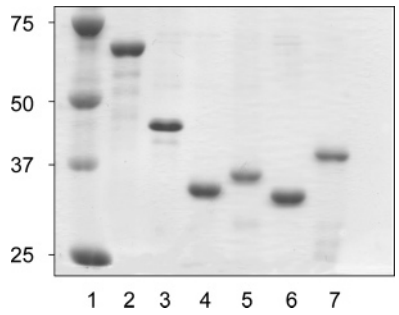

FIGURE 2: Expression of various species of I-2 in *E. coli*. Heat-treated extracts were prepared from transformed *E. coli* cells, with the various tagged I-2s isolated as described in Materials and Methods and proteins analyzed by SDS-PAGE. A Coomassie blue-stained gel is shown. Lane 1, size standard proteins; lane 2, bovine serum albumin; lane 3, GLC8; lane 4, Dm I-2; lane 5, Ce I-2; lane 6, Xe I-2; lane 7, Hs I-2. All I-2 proteins have a His_6-S-tag at the N-terminal except Xe I-2, which has only a His_6 tag. The numbers at the left side indicate relative molecular mass expressed in kilodaltons.

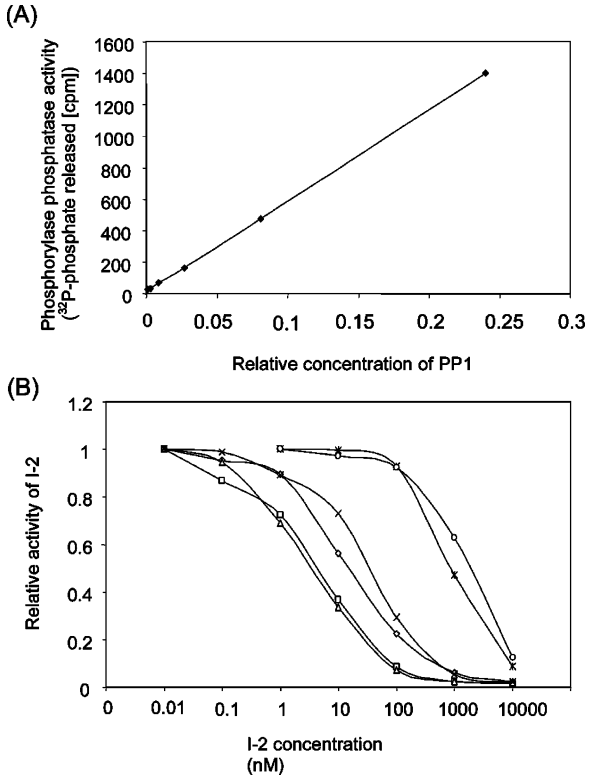

FIGURE 3: (A) Phosphorylase phosphatase activity of rabbit skeletal muscle PP1. ^{32}P-Labeled phosphorylase *a* was incubated with various amounts of PP1 isolated from rabbit skeletal muscle, and release of acid-soluble [^{32}P] phosphate was measured by scintillation counting. Results demonstrate linear response of the assay used to measure inhibitor activity. (B) Effect of I-2 proteins on the phosphorylase phosphatase activity of PP1. ^{32}P-Labeled phosphorylase *a* was incubated with PP1 in the presence of increasing concentrations of recombinant I-2 proteins: GLC8, open circles; human I-2 (14−197) deletion mutant protein, asterisks; Xe I-2, crosses; Hs I-2 diamonds; Dm I-2, squares; Ce I-2, triangles. Results calculated as average of duplicate samples.

of purified PP1 catalytic subunit, and the low activity of GLC8 might be due to sequence variation near the N-terminus.

Inhibition of PP1 Bound to Nek2 Kinase. PP1 exists as a collection of holoenzymes in cells; therefore, we tested inhibitory action of the different species of I-2 using a holoenzyme composed of PP1 bound to Nek2. In this complex, the PP1 is bound to a KVKF motif in the noncatalytic C-terminal region of Nek2. The HA_3-Nek2::PP1 complex was recovered by anti-HA immunoprecipitation from extracts of HEK293T cells that had been co-transfected with plasmids for HA_3-Nek2 and untagged PP1γ. The Nek2 kinase is activated by autophosphorylation but is inactivated by constant PP1 dephosphorylation within the complex. Inhibition of the PP1 in this complex can be indirectly assayed by measuring the increase in Nek2 kinase activity (*20*), using here recombinant C-Nap1 fusion protein as a preferred Nek2 substrate. MCLR was used as positive control to completely inhibit PP1, and it produced a 4-fold increase in Nek2 kinase activity relative to the untreated control where PP1 remained active. In this assay, micromolar concentrations of Ce I-2, Dm I-2, Xe I-2, and Hs I-2 all produced activation of Nek2 about equivalent to the activation seen with MCLR, whereas the yeast I-2 (GLC8) was not effective (Figure 4A). Other assays with lower concentrations of added I-2 showed incomplete activation of the Nek2, compared to the effects of MCLR in the same assay (not shown). We concluded that all these species of I-2 except GLC8 inhibited PP1 that was bound to Nek2, albeit with lower apparent potency relative to PP1C monomer. Inhibition of PP1 in this holoenzyme did not require I-2 interaction with the KVxF site on PP1 because Nek2 already occupied this site on PP1. The added I-2 did not promote dissociation of the HA_3-Nek2::PP1 complex. On the contrary, we found that the I-2 formed ternary complexes, with PP1 binding both Nek2 and I-2 at the same time. Following incubation of the HA_3-Nek2::PP1 holoenzyme with either GLC8 or Hs I-2, the beads were pelleted, washed with assay buffer, and the bound proteins were analyzed by immunoblotting (Figure 4B). Results showed that the PP1 remained bound to Nek2, and furthermore, both GLC8 and Hs I-2 were recovered with the HA_3-Nek2::PP1 complex. We took this as evidence that the GLC8 and Hs I-2 bound to the PP1 already engaged with Nek2. However, the Hs I-2, not the GLC8, inhibited the PP1 to allow activation of the Nek2. The results suggest that the weak inhibitory activity of GLC8 in this assay is not simply due to differences in its primary PP1 binding site at the N-terminus, or even in its ability to associate with PP1, but due to differences in sequence elsewhere in the protein that are critical for inhibiting mammalian PP1.

Aurora Kinase Activation by Various I-2 Proteins. We previously reported (*18*) that Hs I-2 directly stimulated recombinant human Aurora-A kinase activity and co-precipitated with Aurora from cell extracts. Recombinant Aurora-A kinase was purified from *E. coli* and incubated with or without I-2 proteins for 10 min at 30 °C, then kinase activity was measured by adding the substrates MBP and [^{32}P]ATP. In this assay, Hs I-2 produced ~8-fold increase in Aurora-A kinase activity, and Xe I-2 activated Aurora-A kinase ~13-fold compared with control. In contrast, Ce I-2, Dm I-2, and GLC8 only stimulated Aurora-A kinase activity slightly above control, much less effective compared to Hs I-2 and Xe I-2 (Figure 5). We conclude that Hs I-2 and Xe I-2 can bind and activate human Aurora-A kinase. This property is not shared by Ce I-2, Dm I-2, and GLC8, any one of which has only 30% identity to either Hs I-2 or Xe

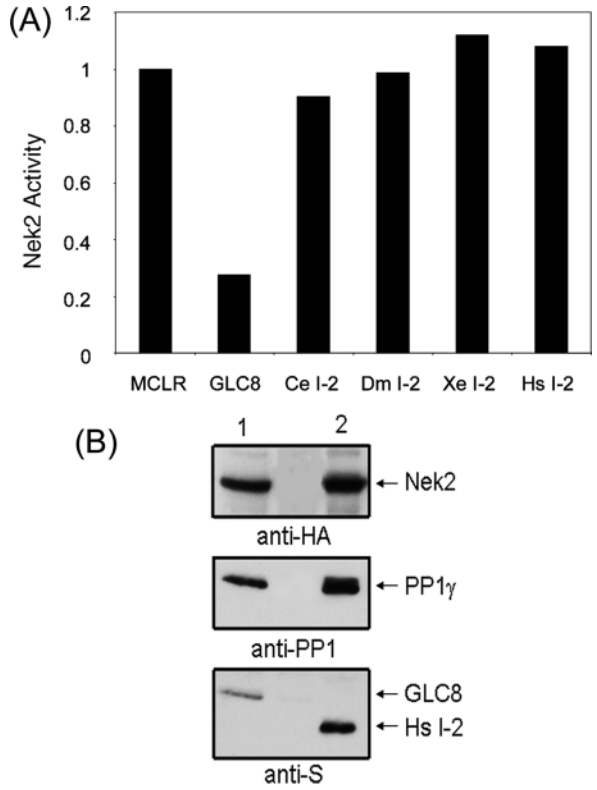

FIGURE 4: (A) Activation of Nek2::PP1 kinase by MCLR and recombinant I-2. Kinase assays were performed as described in Materials and Methods using anti-HA immunoprecipitates from 293T cells transiently transfected with vectors encoding HA_3-Nek2 and untagged PP1γ. Recombinant C-Nap1 domain plus [γ-^{32}P]ATP (0.1 mM) were added as substrates in the kinase reaction. The reaction was started by addition of ATP within 40 min after adding 2 μM MCLR or recombinant I-2 proteins. Results were replicated in two independent experiments, and the average activities are shown. (B) Binding of GLC8 and Hs I-2 to HA_3-Nek2::PP1 heterodimer. Anti-HA immunoprecipitate prepared from 293T cells transiently transfected with vectors encoding HA_3-Nek2 and untagged PP1γ was split and incubated for 1 h with either GLC8 or Hs I-2 (each at 0.2 mM). The beads were collected by centrifugation, washed with 1.0 mL assay buffer and pelleted. Proteins bound to the beads were analyzed by immunoblotting with anti-HA (polyclonal antibody Y-11, Santa Cruz; 1:2000), anti-PP1 (chicken antiserum at 1:10000), and anti-S peptide (monoclonal from Novagen; 1:5000) antibodies, developed with anti-rabbit HRP, anti-mouse HRP, or anti-chicken HRP (all at 1:10000) using enhanced chemiluminescence and exposure of X-ray film that was scanned and processed in Photoshop (*37*).

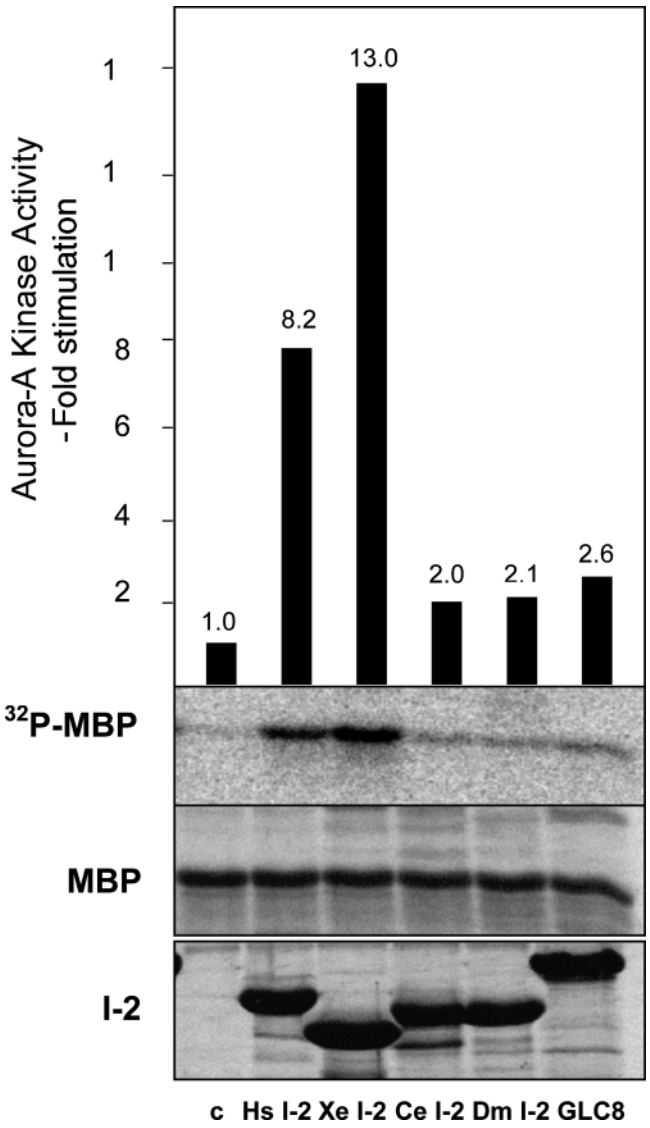

FIGURE 5: Human I-2 and *Xenopus* I-2 stimulate Aurora-A kinase activity. Recombinant Aurora-A was incubated ± added I-2 for 10 min. Assays containing 300 nM Aurora-A were performed for 10 min after addition of substrates myelin basic protein (MBP; Sigma) and 0.1 mM [γ-^{32}P] ATP. Samples were resolved by SDS-PAGE, kinase activity quantitated by PhosphorImager detection of ^{32}P MBP (upper image). Kinase activity was normalized to untreated Aurora-A kinase (leftmost lane), and −fold activation is given as numbers in the histogram in the top panel. The amount of myelin basic protein substrate and I-2 added to assay is shown by scans of the Coomassie stained gel (lower two panels). Results are representative of two independent experiments.

I-2 that are closest to one another in amino acid sequence and 60% identical (Figure 1A,B).

Phosphorylation of Different Species of I-2 by CKII, GSK3, and ERK. The I-2 protein is phosphorylated at multiple Ser residues by purified CKII (*27*). These sites were first identified in the I-2 protein isolated from rabbit muscle (*28*) and also were found in I-2 from human tissue culture cells (*29*). These sites are Ser86 in the SDxE motif and the tandem SerSer at 120−121 in a SSxEE motif (numbering for rabbit I-2 sequence). We reacted various species of I-2 with purified CKII and found that the proteins were all efficiently phosphorylated (Figure 6). Compared to GLC8 (set to 1.0 for normalization), Hs I-2 was phosphorylated about the same and Dm I-2 slightly more, while Xe I-2 and Ce I-2 were phosphorylated about twice as much as GLC8. These results, an average of two independent assays, demonstrate that all of the recombinant I-2 proteins were effective substrates for CKII in the kinase assay and were probably phosphorylated at multiple sites.

Rabbit I-2 can be phosphorylated at Thr72 by GSK3 (*30*−*32*) and ERK (*33*). These kinases phosphorylate I-2 in the PX**T**P site that is the most conserved region of all I-2 proteins (see Figure 1). The I-2 proteins from the five species were reacted with recombinant GSK3 (Figure 7A) or ERK (Figure 7B), and the ^{32}P-labeled I-2 proteins were excised from gels and the radioactivity was quantitated. With GSK3, the Dm I-2 was by far the best substrate, with 60 times more phosphorylation than GLC8 or Ce I-2 or Xe I-2, proteins that were essentially nonreactive under the same conditions (Figure 7A). This observation is all the more impressive when considering the nearly identical reactivity of all the

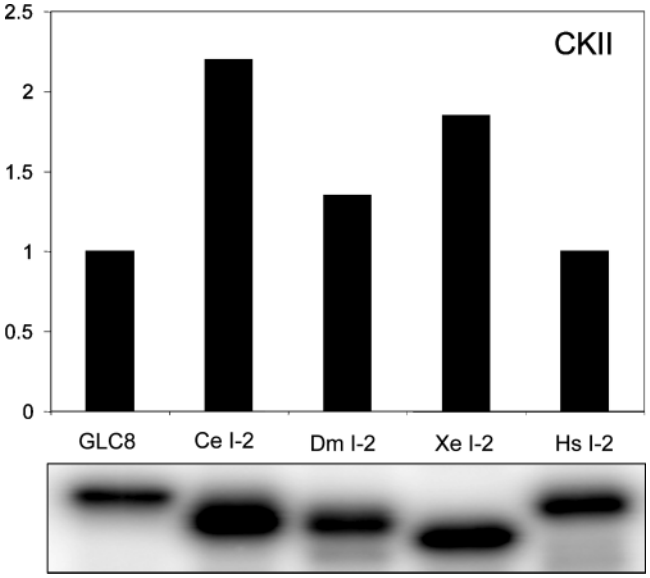

FIGURE 6: Phosphorylation of I-2 proteins by CKII kinase. Different species of I-2 (final concentration 3 μM) were incubated with CKII (500 U) in kinase reaction buffer for 1 h, then stopped by addition of 2× SDS sample buffer. The proteins were resolved by SDS-PAGE and analyzed by autoradiography. Film was scanned and image shown in the bottom panel processed in Photoshop. Protein bands were excised from the gel, radioactivity quantitated with a scintillation counter, and results normalized to GLC8. Data from two independent reactions were averaged to generate the histogram.

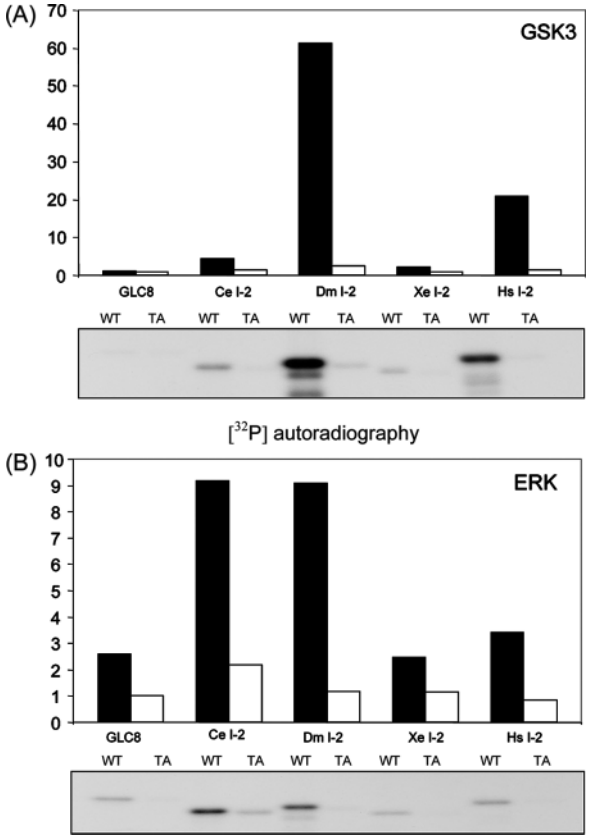

FIGURE 7: Phosphorylation of wild-type I-2 (solid bars) or T/A mutant I-2 proteins (open bars) by GSK3 (panel A) and ERK kinase (panel B). I-2 proteins at a final concentration of 3 μM were reacted with GSK3 (50 U) or ERK (0.3 μg). The kinase reaction was stopped by mixing aliquots of the reaction with 2× SDS sample buffer at 1, 2, and 4 h, and the proteins were resolved by SDS-PAGE. Results at 1 h were analyzed by autoradiography as shown in the bottom panels, and the bands were cut from the gel, quantitated with a scintillation counter, and results normalized to GLC8 (T/A). The relative differences in phosphorylation between different proteins were consistent at different times of assay.

I-2 proteins with CKII (see Figure 6). Hs I-2 was the other family member that was reactive with GSK3 and was phosphorylated about 1/3 as much as Dm I-2 (Figure 7A). As controls, we mutated each of five species of I-2 to change the threonine residue in the PXTP site to alanine. This single residue T to A mutation essentially eliminated phosphorylation, demonstrating this was the only site phosphorylated by GSK3. The results indicate that there must be other sites for recognition by GSK3 besides the consensus motif surrounding the phosphorylation site in these proteins, and there was a substantial difference between species of I-2 for these interactions. Likewise, the reactivity of ERK kinase was different with various species of I-2 and different compared to GSK3 (Figure 7B). In this case, all of the five species of I-2 were phosphorylated at the PXTP site, shown by the difference in radiolabeling of the wild-type (WT) and mutant (TA) proteins (solid and open bars in Figure 7B). Dm I-2 and Ce I-2 were phosphorylated equally and were the best substrates, and there was about 3−4-fold more ^{32}P labeling compared to GLC8, Xe I-2, or Hs I-2. Because at least 8 of 10 residues are identical in the MKIDEPK**T**PY motif defining this site in the five species of I-2, we expected them all to be substrates with both GSK3 and ERK and to be phosphorylated nearly the same. However, it is clear that this phosphorylation site has quite different reactivity with GSK3 and ERK in the context of different I-2 proteins.

The PXTP site in I-2 was phosphorylated by CDC2/cyclinB as previously reported (*29*, *32*), but we found that the reactivity differed between various species of I-2. In this case, Dm I-2 was the best substrate, and Ce I-2, GLC8, and Hs I-2 were phosphorylated to about the same extent, but curiously Xe I-2 was not readily phosphorylated (Figure 8A). Again, the T to A mutants of each I-2 were used as controls to demonstrate phosphorylation in the PXTP site. In this case, the Ce I-2 T/A mutant showed more than double the phosphorylation compare to the other T/A controls. Our interpretation of this result was that Ce I-2 is phosphorylated at another site in addition to the PXTP site. There is a Ser−Pro at residues 3−4 and Pro−Thr at residues 64−65 and 98−99 (Figure 1A). The protein Suc1 (human CKS) binds to the CDC2/cyclinB kinase as a regulatory subunit and enhances reactivity with certain mitotic substrates (*36*). Suc1 addition to reactions increased by 3−4-fold the phosphorylation of all of the various I-2 by CDC2/cyclinB1 (Figure 8B).

DISCUSSION

In this study, we compared phosphorylation and function of five different I-2 proteins from various eukaryotic species, yeast to human. Among this group, are two new proteins that we identified in *C. elegans* and *Xenopus*, whose properties are reported here for the first time. The family of I-2 proteins exhibits considerable sequence variation. GLC8 is the most different, with only 14−17% identity to any of the other proteins. As a group, the I-2s from non-vertebrate species (GLC8, Ce I-2, Dm I-2) have only 30% identity with the I-2s from vertebrate species (Xe I-2 and Hs I-2). Despite this divergence in sequence, all of these I-2 proteins are about

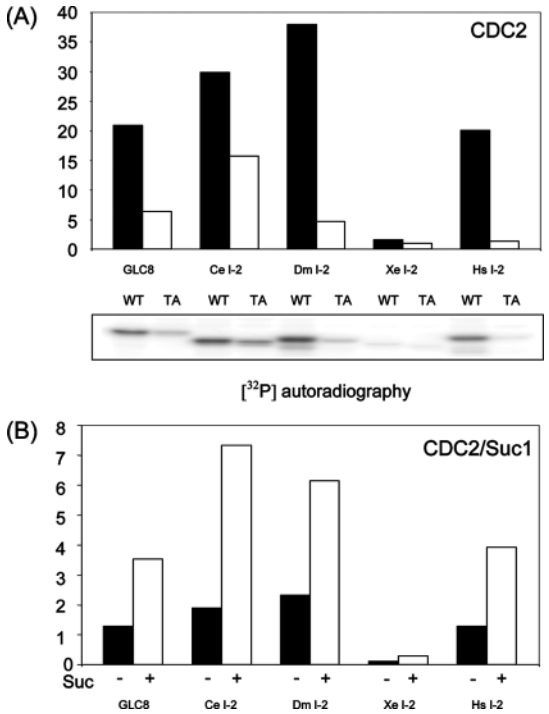

FIGURE 8: Phosphorylation of wild-type I-2 or T/A mutant proteins by CDC2/cyclinB1 or CDC2/cyclinB1 with Suc1. (A) Wild-type I-2 (solid bars) or T/A mutant I-2 (open bars) proteins (3 μM) were mixed with CDC2/cyclinB1 (20 U) and reactions stopped after 1 or 4 h by 2× SDS sample buffer, and the proteins were resolved by SDS-PAGE. Autoradiography results from the 1 h reaction are shown in the bottom panel. The bands were excised, radioactivity was quantitated, and results were normalized to Xe I-2. (B) Reactions with wild-type I-2 and T/A mutant proteins using CDC2/cyclinB1 (20 U) alone (solid bars) or plus recombinant Suc1 (open bars) were done as described above, the results for the wild-type proteins quantified and plotted for comparison.

the same size (187−229 residues) and they are heat stable; that is, they are soluble after boiling, a property probably due to a preponderance of charged and hydrophilic amino acid residues. This amino acid composition is consistent with lower than average binding of SDS, which would account for their relatively low migration during SDS-PAGE. The new *C. elegans* protein (Ce I-2) is the most primitive metazoan I-2, and the phylogenetic tree shows that it and *Drosophila* I-2 (Dm I-2) are well separated from vertebrate I-2. Therefore, it was interesting that Ce I-2 and Dm I-2 displayed the highest potency inhibition of rabbit PP1, better even than the Xe I-2 and Hs I-2 proteins. Inhibition of PP1 is one clearly conserved function of this protein family. The poorest inhibitor of this group is GLC8, and we demonstrate that its potency with PP1 catalytic subunit is the same as that of Hs I-2 deleted of the first 13 residues. The N-terminal sequence IKGI (residues 10−13) in Hs I-2 has been shown by mutagenesis to be necessary for high potency inhibition of the PP1 catalytic subunit (*34*). Binding of I-2 to PP1 was proposed to involve the aliphatic side chains of the Ile residues. GLC8 appears as a natural truncation of I-2, with the first residue aligning with residue 11 of Hs I-2, supporting this model. However, Ce I-2 and Dm I-2 are natural mutants in the first Ile in the IKGI motif but exhibit the highest potency inhibition. We suggest that high potency inhibition does not necessarily depend on the IKGI motif. The sequences necessary for potent and specific inhibition of PP1 are not yet identified, but our results with Ce I-2 impose constraints on their identity.

We demonstrate that I-2 proteins also inhibited PP1 that was engaged in a heterodimer with Nek2 via its KVKF motif. Inhibition of bound PP1 allowed the activation of Nek2 kinase by autophosphorylation, confirmed by addition of MCLR as a positive control in each assay. C-Nap-1 binds to Nek2 and is phosphorylated by Nek2 at multiple sites in the C-terminal domain (*35*), making it an especially good substrate to assay Nek2 activity. Various I-2s were considerably less potent inhibitors of Nek2::PP1 heterodimers, compared to monomeric PP1 catalytic subunit, and required micromolar concentration of I-2 for full activation. In the Nek2 assay, even micromolar GLC8 failed to inhibit PP1. Both GLC8 and Hs I-2 bound to the PP1 tethered to HA_3-Nek2 on beads, demonstrating formation of heterotrimers. In these trimers, GLC8 did not inhibit PP1, whereas the other species of I-2 were inhibitory. This difference in I-2 activity probably is due to sequence variations between GLC8 and Ce I-2 (the closest family relative) and may have to do with the ~50 residue insertion in GLC8 between the N-terminus and the conserved phosphorylation site. The results show that I-2 (other than GLC8) inhibits not just the PP1 monomer but also certain PP1 complexes containing regulatory subunits. This expands the role of I-2 beyond acting as a scavenger to neutralize the PP1 catalytic subunit released from regulatory subunits. Because some PP1 holoenzymes (e.g., myosin phosphatase) are resistant to inhibition by I-2, while others (e.g., Nek2::PP1) are not, this indicates that I-2 can function in cells to regulate the activity of a select subset of the PP1. On the basis of these results, expression of GLC8 in cells would inhibit PP1 monomer but not holoenzymes, so this may prove to be a useful tool for research. Thus, I-2 forms heterotrimeric complexes containing PP1 and a regulatory subunit, and we suspect that the I-2::PP1 heterodimer known as MgATP-dependent phosphatase is derived from such heterotrimers by dissociation of regulatory subunits and rearrangement of the heterodimer.

Only the I-2 from vertebrate species (Xe I-2 and Hs I-2) activated Aurora-A kinase. These I-2 proteins are 60% identical in sequence and are divergent in sequence from all the other I-2s tested. Previous results showed that deletion of the N-terminal 13 residues of I-2 did not impair Aurora-A activation (*18*). Therefore, sequence differences at the N-terminal of the various I-2s cannot account for the lack of Aurora-A activation. This activity reveals the largest functional differences among I-2 family members. Presumably, there are residues conserved between Xe I-2 and Hs I-2 but not in Dm I-2, Ce I-2, or GLC8 that are critical for the binding and activation of Aurora-A kinase, but these remain to be identified.

All I-2 family members have multiple phosphorylation sites for different kinases. CKII effectively phosphorylated all five species of I-2 at multiple sites, showing these recombinant proteins were good substrates in the kinase assay, probably because they are heat-stable and extended or unstructured polypeptides. The incorporation of ^{32}P and the knowledge from direct sequencing that there are three sites of CKII phosphorylation in Hs I-2 (*29*) or rabbit I-2 (*28*) indicate that there are 3−5 sites for CKII phosphorylation in the various I-2 proteins. The CKII sites determined by sequence analysis, namely, Ser86, Ser120, and Ser121,

are not well conserved between species; however, a SSx-[D/E][D/E] motif does appear in the same region of the other I-2 proteins, except GLC8. In contrast to the reactivity of all these I-2 proteins with CKII, the reaction with GSK3 was highly specific for Dm I-2 and Hs I-2, with Dm I-2 by far being the best GSK3 substrate. The phosphorylation of Dm I-2 was at a single site in the PXTP motif, demonstrated by the loss of phosphorylation in the single residue Thr to Ala mutated protein. The IDEPXTPY motif is the single most highly conserved sequence in the entire I-2 family of proteins. Therefore, it was unexpected that phosphorylation of the Thr in this motif was so different in the five I-2 proteins tested, and neither GLC8 nor Ce I-2 nor Xe I-2 was phosphorylated by GSK3 to any significant extent. GSK3 must require other sequence elements distal to the phosphoacceptor for effective phosphorylation of this site, and these elements distinguish Dm I-2 and Hs I-2 from Xe I-2, Ce I-2, and GLC8. Furthermore, this was not seen just with GSK3 because phosphorylation of this same site in the five species of I-2 was distinctively different for ERK2 and CDC2 kinases. Each kinase showed a different pattern of reactivity with the five I-2 proteins, even though they were phosphorylating the same single site within the conserved IDEPxTPY sequence. In this regard the I-2 family offers an opportunity to better understand the basis of substrate specificity for these kinases. The protein Suc1/CKS is a regulatory subunit that binds to the CDC2 kinase subunit and enhances the phosphorylation of I-2 by CDC2/cyclinB1. Because the Suc1/CKS protein binds to CDC2 and selectively enhances reactivity of this kinase with mitotic substrates (*36*), our results support the idea that I-2 is phosphorylated especially during mitosis by CDC2 kinase (*29*, *37*). However, Suc1/CKS did not change the relative specificity of CDC2/cyclinB1 for different species of I-2 proteins, but it increased reactivity about the same extent with each protein. The disparate reactivity of the PxTP site in various species of I-2 with three different kinases shows that even heat-stable proteins that are unstructured and flexible probably interact with different kinases at multiple sites to enhance or reduce their phosphorylation at a common site.

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