Expression of I_2^{PP2A} , an inhibitor of protein phosphatase 2A, induces c-Jun and AP-1 activity

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Transient expression of I_2^{PP2A} , a potent inhibitor of protein phosphatase 2A (PP2A), in HEK-293 cells increased the concentration and DNA binding of the proto-oncogene c-Jun. In contrast, expression of the catalytic subunit of PP2A ($PP2A_C$) markedly decreased the concentration and DNA binding of c-Jun. Expression of I_2^{PP2A} also increased the transcriptional activity of activator protein-1, and this effect was diminished in a dose-dependent manner by expression of $PP2A_C$. Densitometric analysis following Western blotting of extracts with antibodies specific for phospho-Ser⁶³ and Ser⁷³ suggests that the effects of

I_2^{PP2A} and $PP2A_C$ expression might be mediated, in part, by changes in the phosphorylation of c-Jun at Ser⁶³. The results indicate that I_2^{PP2A} elicits effects that are consistent with it acting as an inhibitor of PP2A in intact cells, and suggest that PP2A might exhibit site selectivity with respect to c-Jun phosphorylation.

Key words: cell signalling, HEK-293 cells, protein kinase, protein phosphorylation.

INTRODUCTION

Protein phosphatase 2A (PP2A) is a major mammalian protein serine/threonine phosphatase that regulates diverse cellular processes [1–4]. Physiological targets of this phosphatase include cell-surface receptors and ion channels, protein kinases involved in mitogenic signalling and the cell cycle, key regulatory enzymes and proteins involved in metabolism, and numerous transcription factors [1–4]. Of the transcription factors, the proto-oncogene c-Jun might be a direct and/or indirect substrate for PP2A. Evidence supporting this conclusion includes the observation that incubation of cells with okadaic acid, a potent cell-permeable inhibitor of the phosphatase [5], increases the concentration, phosphorylation and DNA binding of the proto-oncogene, as well as the transcriptional activity of the activator protein-1 (AP-1) transcription-factor complex, of which c-Jun is a major component [6,7]. Moreover, relative to the other major mammalian protein serine/threonine phosphatases, i.e. PP1, PP2B and PP2C [1–4], purified preparations of PP2A preferentially dephosphorylate c-Jun isolated from okadaic-acid-treated cells [6,7]. Expression of the simian virus 40 (SV40) small t antigen, which inhibits PP2A by either displacing or replacing its regulatory B subunit [8], also increases the concentration and phosphorylation of c-Jun, as well as AP-1 transcriptional activity [9,10].

Recently, we identified two potent PP2A-specific inhibitor proteins that were tentatively designated as I_1^{PP2A} and I_2^{PP2A} [11–13]. Molecular cloning studies [12] revealed that I_1^{PP2A} was equivalent to PHAP-I, a proliferation-associated protein of previously undetermined function that exhibited a calculated molecular mass of 28 500 Da and an apparent M_r of 32 000, as estimated by SDS/PAGE [14,15]. Other studies [13] showed that I_2^{PP2A} was equivalent to the acute undifferentiated leukaemia-

associated SET protein [16]; also termed PHAP-II [15] and TAF-1 β [17]), also of previously unknown function. This protein exhibits a calculated molecular mass of 30 800 Da, and an apparent M_r of 39 000, as estimated by SDS/PAGE. Subcellular localization studies indicate that I_1^{PP2A} might be present largely in the cytoplasm [15,18] or nucleus [14], apparently depending on cell type. In contrast, I_2^{PP2A} is located largely in the nuclei of all cell types examined to date [15,19]. Both I_1^{PP2A} and I_2^{PP2A} are non-competitive inhibitors that act on purified preparations of trimeric PP2A₁, dimeric PP2A₂ and the isolated catalytic C subunit of the phosphatase [11]. Thus the inhibitor proteins appear to act, at least in part, by binding to the C subunit of the phosphatase. To date, however, no evidence has been provided that I_1^{PP2A} and/or I_2^{PP2A} might act as inhibitors of PP2A in intact cells.

In this study, we have determined the effects of I_2^{PP2A} expression on the concentration and DNA binding of c-Jun, as well as the transcriptional activity of AP-1. The evidence presented in this paper is the first to indicate that I_2^{PP2A} elicits effects that are consistent with it acting as an inhibitor of PP2A in intact cells, and suggest that the phosphatase might exhibit site selectivity with respect to c-Jun phosphorylation. Because I_2^{PP2A} is highly expressed in Wilm's tumour cells [20], the findings reported here raise the possibility that c-Jun and AP-1 might also be elevated in these cells.

EXPERIMENTAL

Materials

HEK-293 cells were obtained from the American Tissue Culture Collection (Rockville, MD, U.S.A.). Donor horse serum and Basal Medium Eagles (BME) were from Atlanta Biologicals (Norcross, GA, U.S.A.). LipofectAMINE Plus was from Gibco

Abbreviations used: AP-1, activator protein-1; BME, Basal Medium Eagle's; CAT, chloramphenicol acetyltransferase; HA, haemagglutinin; MEM, minimal essential medium; PP(1/2A/2B/2C), protein phosphatases 1, 2A, 2B and 2C respectively; SV40, simian virus 40.

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BRL (Gaithersburg, MD, U.S.A.). pCMV/PP2A, which encodes a haemagglutinin (HA)-tagged form of human PP2A_{Cx} [21] was generously given by Dr. René Bernards (The Netherlands Cancer Institute, Amsterdam, The Netherlands). Rabbit antibody Ab-1, which recognizes c-Jun, was from Oncogene Science Research (Uniondale, NY, U.S.A.). Monoclonal antibody 16B12 was from Berkeley Antibody Company (Richmond, CA, U.S.A.), and recombinant human c-Jun, rabbit antibody raised against PP2A_{Cx}, pSV-βGal and the Chloramphenicol Acetyltransferase (CAT) Enzyme Assay System were obtained from Promega (Madison, WI, U.S.A.). The AP-1 gel-shift oligonucleotide [5'-CGCTTGATGACTCAGCCGGAA-3' (its mutant form containing a CA → TG substitution in the AP-1 binding-site motif)], and antibodies raised against Sp1 and the p65 subunit of nuclear factor κB, were from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, U.S.A.). ³²P_i (6000 Ci/mmol) and D-threo-[dichloroacetyl-1,2-¹⁴C]chloramphenicol (50–60 mCi/mmol) were from New England Nuclear (Boston, MA, U.S.A.). c-Jun (Ser⁶³) II and (Ser⁷³) antibodies, which specifically recognize c-Jun after phosphorylation of its activating Ser⁶³ and Ser⁷³ residues [22–27], were from New England Biolabs (Beverly, MA, U.S.A.). Details of other materials are given elsewhere [11–13,28].

Generation of I₂^{PP2A} cDNA

Human kidney mRNA, and 5' (GGGGTACCACCATGTGCGCGCCGGCGGCCAAAG) and 3' (CGGGATCCTTAGTCATCTTCTCCTTCATCCTCC) primers containing *Kpn*I and *Bam*HI restriction sites (underlined) were used to generate I₂^{PP2A} cDNA by reverse transcriptase-PCR, as described previously [13], except that the annealing step of the PCR was performed at 60 °C, and the product was subsequently cloned into pcDNA3.1(+). The fidelity of the construct was confirmed by automated DNA sequencing.

Generation of pAbI₂

Affinity-purified antibody to I₂^{PP2A}, pAbI₂, was generated in rabbits at Quality Controlled Biochemicals (Hopkinton, MA, U.S.A.), and was raised against a synthetic peptide corresponding to residues 3–18 of I₂^{PP2A} [16].

Cell transfection and extract preparation

Unless indicated otherwise, cells were seeded at $\approx 2 \times 10^5$ cells/well in six-well plates, and transfected with effector constructs using 2 μl of Plus solution and 5 μg of LipofectAMINE/1 μg of DNA. Control and mock transfections were performed with empty vectors and LipofectAMINE alone respectively. After growth for 30 h in BME with Earl's salts and L-glutamine supplemented with 10 % (v/v) donor horse serum containing 1 % (v/v) penicillin and streptomycin, serum was withdrawn and the cells were allowed to grow for a further 18 h. Transfection efficiencies were determined using *in situ* staining of cells transfected with pSV-βGal. Under the standard conditions employed, about 40 % of the cells were transfected. ³²P-labelling of cells was performed for 3 h in phosphate- and serum-free minimal essential medium (MEM), supplemented with ³²P_i (1 mCi/ml) after incubation in serum-free BME for 14 h, and then for 1 h in phosphate- and serum-free MEM.

Nuclear extracts were prepared as described previously [29], except that buffers contained phosphatase inhibitors (50 mM NaF/0.5 mM sodium orthovanadate/20 mM β-glycerophosphate/10 mM sodium pyrophosphate) and protease inhibitors (1 mM PMSF/1 mM benzamide). Western blotting was performed using the Enhanced Chemiluminescence ECLTM kit

(Amersham Pharmacia Biotech, Piscataway, NJ, U.S.A.). Control experiments were performed with preimmune serum. SDS/PAGE was performed in mini-slab gels [10 % (v/v) acrylamide] [30], and protein concentration was determined by the method of Bradford [31]. Nuclear extracts were routinely subjected to Western blotting with antibodies against c-Jun, the nuclear transcription factor Sp1 and the cytoplasmic p65 subunit of nuclear factor κB. These analyses demonstrated that the nuclear preparations were essentially free of cytoplasmic contamination (results not shown).

Electrophoretic mobility-shift and CAT assays

Nuclear extracts (3 μg) from HEK-293 cells transfected with pcDNA3.1(+), pcDNA3.1(+)/I₂^{PP2A} or pCMV/PP2A were incubated at room temperature with ³²P-labelled, double-stranded oligonucleotide 5'-CGCTTGATGACTCAGCCGGAA-3' (specific radioactivity $\approx 1 \times 10^9$ c.p.m./μg) containing the consensus AP-1-specific TRE sequence (highlighted in bold) in a final vol. of 20 μl. After 20 min, 5 μl of sample buffer was added [32], and the mixtures were subjected to non-denaturing PAGE (5 % acrylamide). The gel was dried and exposed to X-ray film. CAT activity was measured using the CAT Enzyme Assay System, after extracts were prepared according to the manufacturer's recommendations (Promega).

RESULTS

Effect of I₂^{PP2A} on c-Jun concentration

Initial experiments to test whether expression of I₂^{PP2A} causes effects that are consistent with it acting as an inhibitor of PP2A in intact cells revealed that, relative to controls transfected with the empty vector, cells transfected with pcDNA3.1(+)/I₂^{PP2A} exhibited a marked general increase in the phosphorylation of nuclear proteins (results not shown). To investigate the effect of I₂^{PP2A} expression on c-Jun as a potential specific marker for intracellular PP2A activity, cells were transfected with pcDNA3.1(+), pcDNA3.1(+)/I₂^{PP2A} or pCMV/PP2A. Nuclear extracts were then prepared, and their c-Jun content was examined by Western blotting with Ab-1 antibody. Relative to extracts from control cells transfected with the vector alone, extracts from cells transfected with pcDNA3.1(+)/I₂^{PP2A} exhibited an increase in c-Jun concentration (Figure 1A). In contrast, extracts from cells transfected with pCMV/PP2A showed a marked decrease in their concentration of c-Jun relative to controls (Figure 1A). Densitometric analyses of Western blots revealed that the concentration of c-Jun increased by about 1.5–2.5-fold following expression of I₂^{PP2A}. In contrast, relative to controls, extracts from cells transfected with pCMV/PP2A exhibited an approx. 3–5-fold decrease in their concentration of c-Jun. Expression of ectopic I₂^{PP2A} and HA-PP2A_{Cx} was verified by Western blotting of the nuclear extracts with pAbI₂ (Figure 1B) and rabbit antibody raised against PP2A_{Cx} (Figure 1C) respectively. On the basis of densitometric analysis, relative to the endogenous protein there was an approx. 2–3-fold increase in the amount of I₂^{PP2A} detected by Western blotting in extracts of cells expressing the inhibitor protein. Similar analysis indicated that HA-PP2A_{Cx} was expressed to about 3-fold-higher levels, relative to the endogenous phosphatase.

Effect of I₂^{PP2A} on c-Jun phosphorylation

The appearance of c-Jun as a ladder of different mobilities from extracts of control and I₂^{PP2A}-expressing HEK-293 cells as determined by Western blotting with Ab-1 (Figure 1A) suggested

Figure 1 Expression of I₂^{PP2A} increases c-Jun concentration

(A) Nuclear extracts (10 µg) from cells transfected with pcDNA3.1(+) (lane 1), pcDNA3.1(+)/I₂^{PP2A} (lane 2) and pCMV/PP2A (lane 3) were subjected to Western blotting with Ab-1 antibody. (B) The extracts from cells transfected with pcDNA3.1(+) (lane 1) and pcDNA3.1(+)/I₂^{PP2A} (lane 2) were subjected to Western blotting with pAbI₂. The arrow denotes the position of I₂^{PP2A}. (C) Extracts from cells transfected with pcDNA3.1(+) (lane 1) and pCMV/PP2A (lane 2) were subjected to Western blotting with rabbit antibody raised against PP2A_c. The arrows denote the positions of endogenous and HA-tagged PP2A_c, as shown. The extracts from cells transfected with pcDNA3.1(+) (lane 1), pcDNA3.1(+)/I₂^{PP2A} (lane 2) and pCMV/PP2A (lane 3) were subjected to Western blotting with phospho-specific c-Jun (Ser⁶³) II (D) and c-Jun (Ser⁷³) (E) antibodies. The positions of c-Jun in (A), (D) and (E) are indicated by the square bracket. Similar results were obtained from four separate experiments.

that the transactivating factor might be highly phosphorylated in these cells. Furthermore, because this ladder was considerably diminished in extracts of cells expressing HA-PP2A_c (Figure 1A), even when the extracts were normalized for c-Jun relative to extracts from control or I₂^{PP2A} expressing cells (not shown), the results suggested that the transactivating factor might have been dephosphorylated in these cells. Consistent with these ideas,

Figure 2 Effect of I₂^{PP2A} and PP2A_c expression on AP-1 DNA binding

(A) Nuclear extracts (3 µg) from cells transfected with pcDNA3.1(+) (lane 1), pcDNA3.1(+)/I₂^{PP2A} (lane 2) and pCMV/PP2A (lane 3) were incubated with 30 pg of ³²P-labelled AP-1 probe (specific radioactivity approx. 1 × 10⁹ c.p.m./µg) and subjected to electrophoretic mobility-shift analysis, as described in the Experimental section. An autoradiogram of the dried gel is shown. (B) Nuclear extracts from cells transfected with pcDNA3.1(+)/I₂^{PP2A} were incubated with the ³²P-labelled AP-1 probe (as described above) in the absence (lane 1) or presence of a 50-fold (lane 2) or 100-fold (lane 3) molar excess of unlabelled wild-type AP-1 probe. In lane 4, mutant probe was included at a 100 × molar excess. The mixtures were subjected to electrophoretic mobility shift analysis, and the gel was dried. An autoradiogram of the gel is shown. (C) Nuclear extracts from I₂^{PP2A}-expressing cells were incubated in the absence (lane 1) or presence (lane 2) of Ab-1 for 60 min at room temperature, and then with the ³²P-labelled probe described above. The mixtures were subjected to polyacrylamide gel electrophoresis; an autoradiogram of the dried gel is shown. Similar results to those shown in (A–C) were obtained in three separate experiments.

Western blotting with phospho-specific (Ser⁶³) II antibody showed that c-Jun was phosphorylated at Ser⁶³ in nuclear extracts of control cells (Figure 1D, lane 1). In addition, relative to these extracts, Western blotting of extracts from cells expressing I₂^{PP2A} showed that the phosphorylation of c-Jun at Ser⁶³ was increased (Figure 1D, lane 2). Furthermore, phosphorylation of c-Jun at Ser⁶³ was markedly decreased following expression of HA-PP2A_c (Figure 1D). Densitometric analysis revealed that expression of I₂^{PP2A} had increased c-Jun phosphorylation at Ser⁶³ by about 2.5-fold. In contrast, c-Jun phosphorylated at Ser⁶³ could not be detected using 10 µg of extract from cells expressing HA-PP2A_c (Figure 1D). However, at 3-fold-higher concentrations of the extracts, c-Jun phosphorylated at Ser⁶³ was detectable after performing Western blotting with phospho-specific (Ser⁶³) II antibody (results not shown). Densitometric analysis of these extracts revealed that, relative to controls, HA-PP2A_c expression decreased c-Jun phosphorylation at Ser⁶³ by approx. 4–5-fold.

Western blotting with phospho-specific (Ser⁷³) antibody showed that c-Jun was phosphorylated at Ser⁷³ in nuclear extracts of control cells (Figure 1E). In addition, relative to these extracts, Western blotting of extracts from cells expressing I₂^{PP2A} and HA-PP2A_c showed that the phosphorylation of c-Jun at Ser⁷³ was increased and decreased respectively (Figure 1E). Densitometric analysis revealed that expression of I₂^{PP2A} had increased c-Jun phosphorylation at Ser⁷³ by about 1.5–2.5-fold, similar to the increase in c-Jun concentration. In addition, HA-PP2A_c decreased this phosphorylation by approx. 3-fold, also similar to

Figure 3 Effect of I_2^{PP2A} and PP2A_c on AP-1 transcriptional activity

Cells were transfected with —73 Col/CAT (●) and —63 Col/CAT (○) (each at 4 μ g) with the indicated concentration of pcDNA3.1(+)/ I_2^{PP2A} . CAT activity was determined as described in the Experimental section using whole-cell extracts. The extracts were subjected to Western blotting with pAbI₂, and the expression of the inhibitor protein was determined by densitometric analysis relative to extracts from control cells transfected with the vector alone (▲). All results shown in (A) represent the mean values from two separate experiments with an average S.D. of ± 130 c.p.m. Cells were also transfected with —73 Col/CAT (1 μ g) and pcDNA3.1(+)/ I_2^{PP2A} (1 μ g) in the presence of the indicated concentration of pCMV/PP2A. CAT activity was then measured using the extracts. The mean values from two separate experiments are shown in (B). CAT activity at 100% is equivalent to the activity from extracts of cells transfected with —73 Col/CAT and pcDNA3.1(+). The DNA concentration used in each transfection was normalized with pcDNA3.1(+), and extracts were normalized for protein concentration. (C) Extracts from cells either transfected with pcDNA3.1(+)/ I_2^{PP2A} or co-transfected with pCMV/PP2A were subjected to Western blotting with pAbI₂ (upper panel) or monoclonal antibody 16B12 (lower panel).

the decrease in c-Jun concentration. Transfection of cells with twice the standard amount of pCMV/PP2A increased the level of HA-PP2A_c expression by about 1.8-fold, as determined by Western blotting of nuclear extracts with monoclonal antibody 16B12 (cf. Figure 3). However, no further decrease was detectable in either the concentration of c-Jun or in its phosphorylation at Ser⁷³ relative to control cells transfected with the vector alone (results not shown).

Regulation of AP-1 DNA binding

The effects of I_2^{PP2A} and HA-PP2A_c expression on the binding of c-Jun to DNA were subsequently examined. A radiolabelled probe that specifically binds the Jun component of Jun homodimers and Jun/Fos heterodimers was used in these experiments to measure the effect on DNA binding. Expression of I_2^{PP2A} increased DNA binding relative to controls (Figure 2A). Furthermore, relative to controls, AP-1 binding to DNA was substantially diminished in extracts from cells expressing PP2A_c (Figure 2A). PhosphoImager analysis revealed that expression of

I_2^{PP2A} and HA-PP2A_c had increased or decreased DNA binding by about 2-fold or 40-fold respectively, relative to controls. Binding to the radiolabelled DNA probe was specific, since it was completely inhibited by the presence of a 50-fold molar excess of unlabelled wild-type oligonucleotide (Figure 2B). In contrast, up to a 100 $\times$ molar excess of a mutant oligonucleotide failed to eliminate DNA binding (Figure 2B). Incubation of the mixtures with Ab-1, which recognizes a sequence in the DNA-binding domain of c-Jun, markedly reduced reactivity with the wild-type oligonucleotide, demonstrating that c-Jun was the major component of the DNA-binding complexes detected in this study (Figure 2C). Because the increase in DNA binding was similar to that of c-Jun concentration following I_2^{PP2A} expression, the results suggest that there was little, if any, change in the specific activity of DNA binding of c-Jun. In contrast, because the decrease in DNA binding was about 13-fold higher than the decrease in c-Jun concentration, the results indicate a significant decrease in the specific activity of DNA binding following HA-PP2A_c expression, consistent with the notion that c-Jun phosphorylation at regulatory sites was affected. Whether

members of the Jun and Fos families other than c-Jun are also present in the AP-1–DNA-binding complex remains to be determined.

Regulation of AP-1 transcriptional activity

To determine the effect on AP-1 transcriptional activity, cells were transfected in the absence or presence of increasing pcDNA3.1(+)/I₂^{PP2A} concentrations with either an AP-1 reporter plasmid containing a single AP-1 recognition site upstream of the CAT gene (–73 Col/CAT) or a mutant plasmid lacking the AP-1 site (–63 Col/CAT) [28].

Relative to controls transfected with –73 Col/CAT, CAT activity was enhanced by up to 5-fold in cells co-transfected with –73 Col/CAT and pcDNA3.1(+)/I₂^{PP2A} (Figure 3A). In contrast, little or no effect on CAT activity was detected in –63 Col/CAT-transfected cells following transfection with up to 4 µg of pcDNA3.1(+)/I₂^{PP2A} (Figure 3A). Western blotting with pAbI₂ confirmed that the amount of I₂^{PP2A} expressed depended on the concentration of pcDNA3.1(+)/I₂^{PP2A} employed in the transfections (Figure 3A).

We have previously shown that, at a fixed concentration of I₂^{PP2A}, inhibition of purified preparations of PP2A is inversely dependent on the concentration of the C subunit of the phosphatase [11]. Thus, if the effects of I₂^{PP2A} expression on AP-1 transcriptional activity were due to PP2A inhibition, these effects might be expected to be diminished in a dose-dependent manner following co-expression of HA-PP2A_C. Consistent with these ideas, the results presented in Figure 3(B) show that co-expression of HA-PP2A_C prevented the I₂^{PP2A}-stimulated increase in AP-1 transcriptional activity in a dose-dependent manner. Expression of HA-PP2A_C by itself reduced endogenous AP-1 activity by about 3-fold relative to control transfections with the vector alone (results not shown). Western blotting showed that co-expression of HA-PP2A_C had little or no effect on the concentrations of either endogenous or ectopic I₂^{PP2A}, and vice versa (Figure 3C).

DISCUSSION

The results in the present study are the first to demonstrate that I₂^{PP2A} produces effects that are consistent with it acting as an inhibitor of PP2A in intact cells. First, transient expression of I₂^{PP2A} caused a marked increase in c-Jun concentration (Figure 1A). In contrast, HA-PP2A_C expression markedly decreased it relative to controls (Figure 1A). Secondly, co-incidentally with the increase in c-Jun concentration, expression of I₂^{PP2A} enhanced the binding of the transactivating factor to the AP-1 DNA probe, as shown by electrophoretic mobility-shift assays (Figure 2A) and analysis of the DNA-binding complex with c-Jun Ab-1 antibody (Figure 2C). In contrast, HA-PP2A_C expression markedly decreased the specific activity of this binding relative to controls (Figure 2A). Thirdly, expression of I₂^{PP2A} stimulated AP-1 transcriptional activity, whereas expression of HA-PP2A_C decreased this effect (Figure 3) as well as that of endogenous AP-1 transcriptional activity (results not shown). Evidence does exist that alterations in the transcription of the c-Jun gene and the stability of the transactivating factor might be a consequence of changes in its phosphorylation [7,33–35]. However, further studies are needed to determine how I₂^{PP2A} and HA-PP2A_C expression affect c-Jun phosphorylation. Together with previous observations [33–35], our results suggest that some of the effects of HA-PP2A_C expression might be mediated, in part, by changes in the proportion of c-Jun phosphorylated at Ser⁶³. This possibility is suggested by the results of densitometric analysis

following Western blotting of extracts from HA-PP2A_C-expressing cells with antibody raised against c-Jun (Figure 1A) and phospho-specific (Ser63) II antibody (Figure 1D). These analyses indicated that the proportion of c-Jun that was phosphorylated at Ser⁶³ was markedly diminished following expression of HA-PP2A_C. Moreover, because there was no apparent change in the proportion of c-Jun phosphorylated at Ser⁷³, as determined by similar analyses, it appears to be unlikely that the effect of HA-PP2A_C expression reported here is mediated by alterations in phosphorylation at this residue. In this regard, it is interesting to note that, relative to Ser⁷³, significantly larger alterations in the phosphorylation state of c-Jun at Ser⁶³ occur in response to a variety of extracellular stimuli [36–42]. Thus it is possible that PP2A might play an important role in controlling (either directly and/or indirectly) the site specificity of c-Jun phosphorylation at Ser⁶³ and Ser⁷³ in response to extracellular stimuli. The larger effects of HA-PP2A_C relative to I₂^{PP2A} expression on c-Jun concentration (Figure 1A), DNA binding (Figure 2A), phosphorylation (Figure 1D) and AP-1 transcriptional activity (Figure 3) suggest that the transactivating factor, and hence AP-1, might already be highly active in HEK-293 cells.

The results in the present study indicate that PP2A and I₂^{PP2A} act, respectively, as suppressor and activator of c-Jun and AP-1-directed gene expression. These results appear to be at variance with a study by Alberts et al. [43], who reported that transient expression of PP2A_C with c-Jun potentiated serum-stimulated activation of an AP-1-regulated promoter. The reason for this discrepancy is uncertain, but might be related to the cell type employed and/or the effects of serum, of which Alberts et al. reported in [43] and which we have not investigated in the present study. In this regard, it is pertinent that I₂^{PP2A} is analogous to several inhibitors of PP2A, including okadaic acid [6,7] and the small t antigen of SV40 [9,10], that have also been shown to induce AP-1 activity. However, unlike okadaic acid and the small t antigen of SV40, I₂^{PP2A} is endogenous to the cell and is highly expressed during early development [16] and in Wilms' tumour formation, although not in renal carcinoma, adult polycystic kidney disease or in transitional cell carcinoma [20]. The observations presented in the present report suggest that c-Jun concentration and DNA binding, as well as AP-1 transcriptional activity, might be elevated early in development and in Wilms' tumour. Studies to examine the latter possibility are under way in this laboratory.

One surprising finding of the present study is that, relative to the endogenous nuclear phosphatase, HA-PP2A_C was expressed to about 3-fold higher levels (Figure 1C) in an active form, as evidenced by the effects of its expression on c-Jun concentration (Figure 1A), phosphorylation (Figures 1D and 1E), DNA binding (Figure 2) and AP-1 activity (Figure 3), and by further recent studies [21]. This expression of HA-PP2A_C is significantly higher than that previously obtained either in extracts of stably transfected rat fibroblasts or transiently in COS cells [44]. The reasons for these discrepancies might well be related to the cell type and/or differences in the expression vectors or transfection methods employed. In the present study, we used the recently described pCMV/PP2A [21] and the LipofectAMINE reagent for transfections. The expression vector we used employs the cytomegalovirus promoter for a high-level expression of cloned genes. This promoter is considered more efficient than the SV40 promoter (e.g. see [45]) contained in vectors used previously for expression of the PP2A_C gene [44]. We estimate that the transfection efficiency is about 40% using LipofectAMINE as a reagent (results not shown), which is significantly higher than that obtained using the calcium phosphate method employed in previous studies [44]. Baharians and Schonthal [46] recently

described a potentially novel autoregulatory mechanism by which PP2A_C synthesis might be controlled in stably transfected NIH-3T3 fibroblasts. The findings described in the present paper suggest that this mechanism might not be operating as efficiently in transiently transfected HEK-293 cells. Alternatively, HEK-293 cells might have an altogether different mechanism for controlling PP2A_C synthesis.

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