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Biochemical Pharmacology 65 (2003) 1317–1328
Biochemical Pharmacology
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Activation of the coactivator four-and-a-half-LIM-only protein FHL2 and the c-*fos* promoter through inhibition of protein phosphatase 2A

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Received 27 November 2002; accepted 21 January 2003

Abstract

Previous studies have demonstrated that the serine/threonine protein phosphatase 2A (PP2A) can modulate the transcriptional activity of several sequence-specific DNA-binding proteins. However, less is known about the effect of PP2A on the activities of general transcription factors and transcriptional coregulators. Here we describe that the activity of a general coactivator, the four-and-a-half-LIM-only protein 2 (FHL2), is regulated in a PP2A-dependent manner. Specific inhibition of PP2A by simian virus 40 (SV40) small t-antigen (st-ag) stimulated the intrinsic transcriptional activity of FHL2 more than 10-fold, while a st-ag mutant unable to bind PP2A had no effect. Overexpression of the B56 subunits α, β, and γ1 of PP2A impaired the induction of FHL2 by st-ag. FHL2 functioned as a coactivator for CREB-mediated transcription, and inactivation of PP2A further increased FHL2-induced CREB-directed transcription. Overexpression of FHL2 readily enhanced the transcription of the *luciferase* reporter gene driven by the c-*fos* promoter, and inhibition of PP2A further stimulated FHL2-induced transactivation of this promoter. These results suggest that dephosphorylation of the general coactivator FHL2 may represent a novel mechanism by which PP2A modulates the transcription of FHL2-responsive genes.

Keywords: FHL2; SV40 small t-antigen; c-*fos*; CREB; NIH 3T3

1. Introduction

Expression of eukaryotic protein-encoding genes requires the concerted action of many proteins that can be divided in three functional classes: RNA polymerase II and the general transcription factors TFIIs, sequence-specific DNA-binding activators and repressors, and coactivators and corepressors including the chromatin-remodeling and modifying enzymes (reviewed in [1]). The activity of several transcription factors is regulated by reversible phosphorylation events [2,3]. While phosphorylation of transcription factors by protein kinases has been extensively studied, less attention has been paid to the role of protein phosphatases in the regulation of the activity of transcription factors. The serine/threonine PP2A is a heterotrimeric enzyme that consists of the catalytic subunit C, the regulatory subunit A, and one of a diverse array of regulatory subunits (B) (reviewed in [4]). PP2A has been demonstrated to be involved in the regulation of the transcriptional activities of CREB [5,6], AP-1 [7,8], NFκB [9,10], HOX11 [11], STAT3 [12], Sp1 [13], Zfhep [14], and retinoid X receptor [15]. Furthermore, PP2A inhibits the activity of E2F by dephosphorylation of p107 [16]. Phosphorylation and dephosphorylation events seem to contribute to the maximal transcriptional activity of general transcription factors and coregulators, but the involvement of PP2A awaits to be identified [17–22].

In many studies, the criterion used to link PP2A involvement to the regulation of transcriptional activity was the application of the PP2A inhibitor OA isolated from marine

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Abbreviations: CRE, cAMP response element; CREB, cAMP response element-binding protein; FAP, *fos* AP-1 site; FHL2, four-and-a-half-LIM-only protein 2; OA, okadaic acid; PP2A, protein phosphatase 2A; SIE, *sis*-inducible element; st-ag, small t-antigen; SV40, simian virus 40.

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doi:10.1016/S0006-2952(03)00071-6

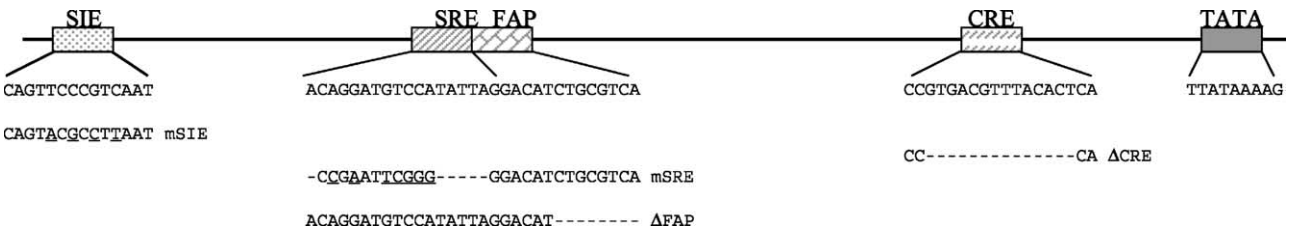


Fig. 1. Schematic presentation of the human c-*fos* promoter spanning nucleotides −361 to +13 (with +1 the start site of transcription). The relative positions of the *sis*-inducible element (SIE), serum response element (SRE), Fos-AP-1 site (FAP), cAMP responsive element (CRE), and TATA motif are indicated. The wild-type sequences of these motifs are shown in the upper line, while the lower line represents the introduced mutations that were tested in this study. Mutated bases are underlined, while deleted bases are shown as a stippled line.

dinoflagellates. OA has been shown to bind directly to the catalytic subunit of PP2A [23]. The use of OA as a specific PP2A inhibitor has, however, some drawbacks. OA also inhibits protein phosphatase (PP1), albeit with an approximately 100-fold lower potency. PP4 was found to be almost as sensitive to OA as PP2A, while PP5 had a comparable sensitivity to OA as PP1 [24]. Moreover, OA penetrates the cell membrane rapidly, but accumulates slowly. This finding and the fact that the efflux of OA varies considerably between different cell lines makes it difficult to control the actual intracellular concentration of the compound *in vivo* [25]. Thus, the inhibitory effect assigned to PP2A might actually have resulted from OA inhibition of other protein phosphatases. Alternatives for OA are the cellular inhibitors of PP2A, I_1^{PP2A} and I_2^{PP2A}. However, in the presence of near-physiological concentrations of Mn^{2+}, I_1^{PP2A} and I_2^{PP2A} stimulate the activity of PP1, which may hamper the interpretation of the results observed with these cellular inhibitors [26]. The st-ag is also being used as a valuable tool in the research for PP2A-dependent processes in mammalian cells. This viral protein and PP2A's regulatory B subunits bind in a mutually exclusive manner to a region of the A subunit which is distinct from the region that binds the C subunit [27]. The interaction of PP2A with st-ag alters the substrate specificity of PP2A and inhibits its serine/threonine phosphatase activity [28]. Thus, st-ag appears to be a specific inhibitor for PP2A-mediated serine/threonine phosphorylation of its substrates and makes this protein suitable for studying PP2A-dependent processes.

Previous studies with OA and st-ag have demonstrated a PP2A-dependent involvement in the regulation of the promoter of the proto-oncogene c-*fos* [7,15,29]. Stimulation of the mitogen-activated protein (MAP) kinase pathways MEK/ERK (MAPK-ERK-kinases/extracellular signal-regulated kinase) and JNK (Jun NH_2-terminal kinase), and the calmodulin-dependent protein kinase IV pathway through specific inhibition of PP2A may be one way by which the activity of the c-*fos* promoter is controlled [7,30–33]. Indeed, both the MAP kinase pathway and the calmodulin-dependent protein kinase IV pathway have been shown to activate c-*fos* expression (reviewed in [34]) and enhance the transcriptional activity of the transcription factors such as CREB [6,33]. The st-ag also regulates the transcriptional activity of STAT3 [12], AP-1 [7], and Sp1 [13], all of which can bind the c-*fos* promoter (Fig. 1, reviewed in [35] and in [36]). Coregulators and chromatin structure add to the complexity of transcriptional regulation of the c-*fos* promoter. The c-*fos* promoter binding proteins AP-1, CREB, NFκB, STAT and Sp1 can all interact with CBP/p300 *in vivo* [20], while signal-induced phosphorylation and acetylation of histone H3 contribute to the regulation of this promoter [37]. Recently, a novel family of coregulators for CREB has been identified. The members of this family, referred to as four-and-a-half-LIM-only proteins (FHL), possess intrinsic transcriptional activities and can stimulate CREB-mediated transcription. While a prerequisite for transcriptional activation of CREB is phosphorylation of serine residue 133 [38,39], FHL proteins induce CREB-mediated transcription in a phosphoserine-133-independent fashion [40]. Stimulation of the Rho signaling pathway induces nuclear translocation of FHL2 and subsequent activation of FHL2-responsive genes. However, no changes in the phosphorylation pattern of FHL2 after stimulation have been reported so far [41].

Here we describe that specific inhibition of PP2A by st-ag stimulated the intrinsic transcriptional activity of FHL2 and potentiated transactivation of the c-*fos* promoter by FHL2. Furthermore, inhibition of PP2A enhanced FHL2-induced transcription mediated by CREB. These observations suggest that a PP2A-dependent signaling pathway may regulate gene expression of FHL2-responsive genes.

2. Materials and methods

2.1. Cells

NIH 3T3 cells (ATCC CRL 1658, American Type Culture Collection) were maintained as described before [42]. All cells were used between passages 132 and 143.

2.2. Chemicals

Forskolin was purchased from Sigma. Sonicated salmon sperm DNA was from Amersham Pharmacia. Newborn calf serum was from BioWhittaker Inc. OA was obtained from Alexis Corporation.

2.3. Plasmids

The st-ag expression vectors pCMV5st, pCMV5#mt3, and the empty vector pCMV5 were kindly provided by Dr. E. Sontag [30]. The plasmid pG5E1bLuc was a kind gift of Dr. R. Davis [43]. The expression vector encoding the DNA-binding domain of GAL4 (residues 1-147) was a kind gift of Dr. R.A. Maurer [44]. Expression plasmids for GAL4 fusion proteins pGAL4-CREB, pGAL4-CREB S133A, pGAL4-VP16, pGAL4-p65, and pGAL4-Elk were gratefully donated by Dr. R.A. Maurer [44], Dr. M. Green [45], Dr. J.A. Didonato [46], Dr. S. Khan [47], and Dr. T. Johansen [48]. The GAL4-p53 expression plasmid was purchased from Clontech Laboratories. The plasmids $pfos_{(-361/+13)}$-LUC, $pfos_{(-361/+13)}$mSIE-LUC, $pfos_{(-361/+13)}\Delta$CRE-LUC, $pfos_{(-361/+13)}\Delta$SRE-LUC, and $pfos_{(-361/+13)}\Delta$FAP-LUC were generated by subcloning the *Hin*dIII-*Bgl*II fragment of the previously described plasmids pfosCAT, pfosCATmSIE, pfosCATΔCRE, pfosCATΔSRE, and pfosCATΔFAP [49], respectively, in the corresponding sites of the promoter-less reporter plasmid pO-Luc [50]. Plasmids pCMXGal-FHL2 and pCMX-Flag-FHL2 were generously provided by Dr. Schüle [51], while expression plasmids for the B56α, B56β, and B56γ1 regulatory subunits of PP2A were a kind gift of Dr. D.M. Virshup [52]. The murine FHL2 EST clone (Image

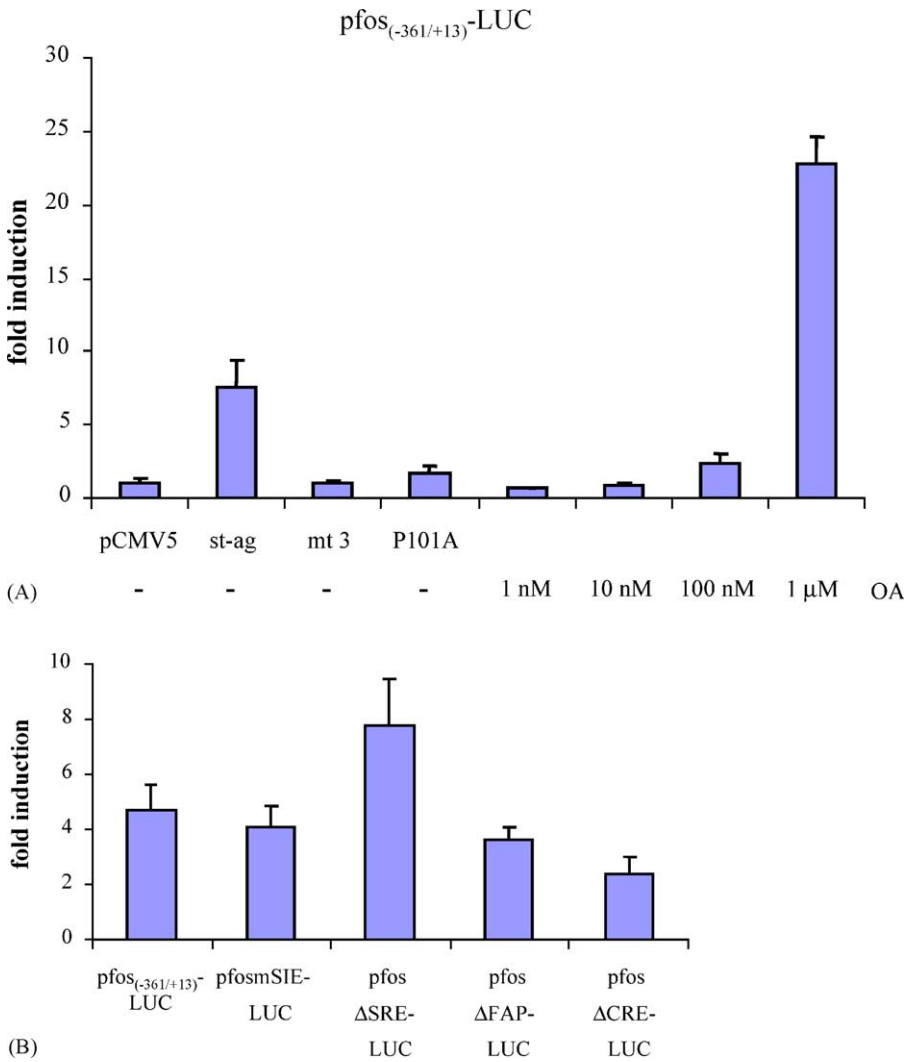


Fig. 2. Inhibition of PP2A in NIH 3T3 cells stimulates the c-*fos* promoter activity through the CREB-binding motif. (A) Cells were transiently transfected with 1 μg of a c-*fos* promoter driven luciferase reporter plasmid and with either 1 μg of expression vector for wild-type small t-ag expression vector (st-ag), for the C-terminal truncated mutant 3 [30], for the PP2A deficient-binding mutant P101A [55], or empty vector pCMV5. The c-*fos* promoter sequences span from nucleotide −361 to +13 with +1 as the transcription start site. The luciferase activity in the presence of the empty expression vector pCMV5 was arbitrarily set as 1, while the luciferase activity in the presence of small t-ag are shown as fold induction. Okadaic acid (OA) treatment was for 3 hr with increasing concentrations as shown in the figure. The luciferase activity in the absence of OA was arbitrarily set as 1. The results represent the average ± SD of three independent parallels. Similar results were obtained in separate experiments. (B) The influence of deletions in the SIE, SRE, FAP, and CRE motifs on PP2A-mediated transactivation of the c-*fos* promoter was tested. The mutations in the distinct responsive elements are shown in Fig. 1. A representative experiments is shown. The results (±SD) are the average of three independent parallels.

Clone 3325842) was obtained from UK HGMP Resource Centre.

2.4. Site-directed mutagenesis

Site-directed mutagenesis was performed with the QuickChange™ Site-Directed Mutagenesis Kit from Stratagene, according to the instructions of the manufacturer. Oligonucleotides were ordered from Eurogentec. The st-ag mutant Pro101 → Ala (st-ag P101A) was obtained by site-directed mutagenesis using the complementary primers (only one primer is shown): 5′-CTG CAA ACA ATG GGC TGA GTG CGC AAA G-3′. The mutation was verified by cycle sequencing using the Big Dye sequencing kit (Perkin-Elmer Tropix). Sequencing reactions were analyzed on an ABI377 Prism Sequencer (Perkin-Elmer).

2.5. Transfections and luciferase activity

For transient transfections, 2×10^5 NIH 3T3 cells were seeded per well in a 6-well culture dish and transfected 24 hr later as described previously [42]. Cells were serum-starved (0.2%) for 18 hr before they were harvested. The amount of total DNA in each transfection mix was kept constant (5 μg per well) by adding appropriate control vector DNA and salmon sperm DNA. All plasmids were purified using the Qiagen plasmid purification kit (Qiagen GmbH). Different plasmid DNA preparations were tested. Luciferase activity was determined in 20 μL lysate using the Luciferase assay system kit (Perkin-Elmer Tropix) and a Luminoscan RT (Labsystems). Each experiment was performed several times with three independent parallels to ensure reproducibility of the observations. The variation between the parallels ranged between 1 and 38%, and was less than 20% in more than 75% of the transfections. Co-transfections with a β-galactosidase reporter plasmid were avoided because expression of st-antigen influenced the β-galactosidase values. Moreover, it is our experience that correction for protein concentrations in each cell lysate had negligible effect on the results (our unpublished results).

2.6. Total RNA isolation and reverse transcriptase-polymerase chain reaction (RT-PCR)

Total RNA of NIH 3T3 cells was isolated and reversed transcribed into cDNA as previously described [53]. FHL2 cDNA was amplified using the primers 5′-ATG ACT GAA TTC TTT GAC TGC CAC CAC TGC-3′ and 5′-TCA AAT AAG CTT TCC ACA GTC AGG ACA GAG-3′. The PCR cycling conditions were 40 times 94° for 30 s; 62° for 1 min, and 68° for 1 min. PCR product was analyzed by agarose gel electrophoresis.

2.7. Western blotting

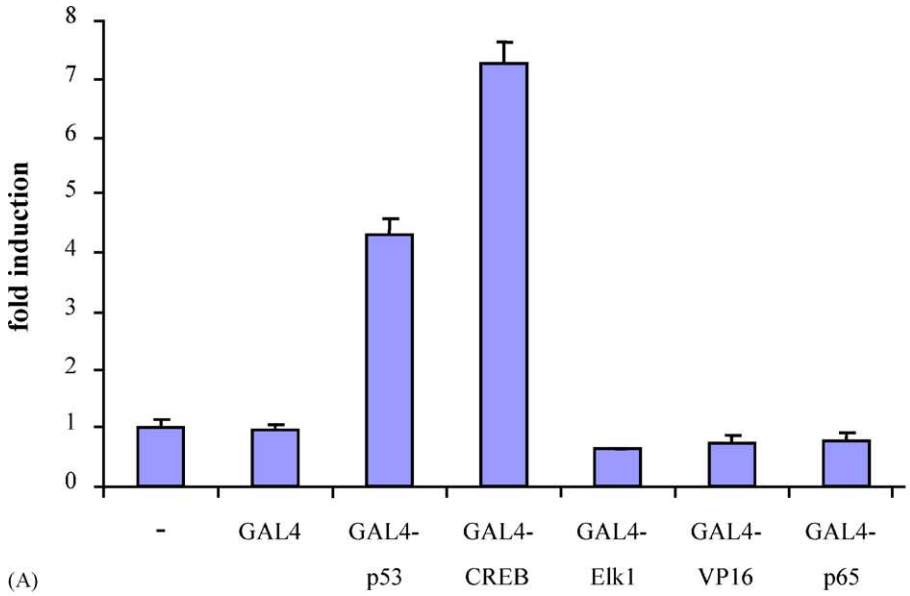
Western blotting was performed as previously described [42]. Phospho-CREB and CREB-specific antibodies were purchased from New England Biolabs (catalogue no. 919 and 912, respectively). GAL4 antibodies were from Santa Cruz Biotechnology (catalogue no. sc-577). Densitometry quantitation of the hybridization signals was performed with a Lumi-Imager F1™ using LumiAnalyst™ Software.

3. Results

3.1. Activation of the c-fos promoter after inhibition of PP2A by st-ag is mediated through the CRE binding motif

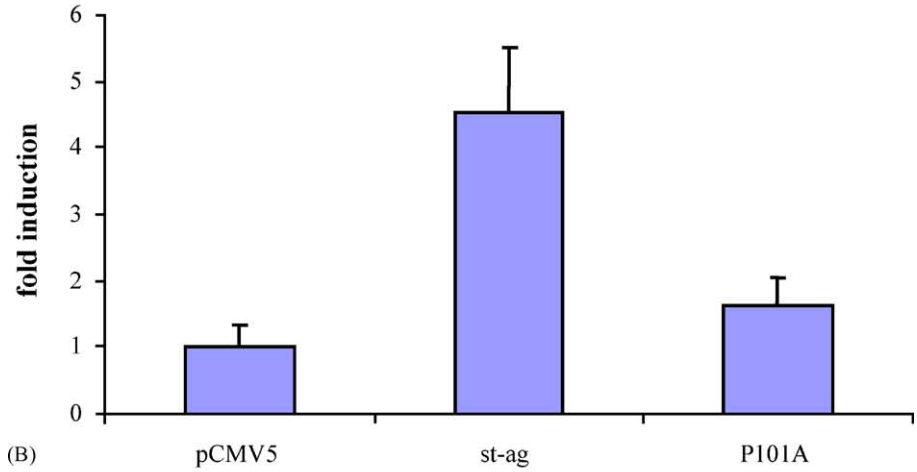
Several lines of evidence indicate a PP2A-dependent mechanism for the regulation of promoter activity of the proto-oncogene c-*fos* [7,15,29]. However, the exact mechanisms by which PP2A affects the c-*fos* promoter activity remains unknown. First, we established whether PP2A binding to st-ag was required for transactivation of

Fig. 3. Inhibition of PP2A stimulates CREB-mediated transcription. (A) NIH 3T3 cells were co-transfected with 1 μg of the GAL4-luciferase reporter plasmid GAL4-E1b-Luc and pCMV5 or the st-ag expression vector. Co-transfection was performed with expression plasmids encoding a fusion protein of the GAL4 DNA-binding domain (amino acid residues 1-147) and one of the following transcription factors: p53, CREB, Elk1, VP16, and RelA/p65, respectively. The (−) indicates that no GAL4 DNA-binding domain expression plasmid was added, while GAL4 represents the DNA-binding motif of GAL4 without any fusion partner. The luciferase activity in the presence of pCMV5 was set as 1 and the activity in the presence of st-ag is shown as fold induction. The results represent the average of three independent parallels (±SD) and similar results were obtained in separate experiments. (B) Induction of CREB-mediated transcription by st-ag requires binding to PP2A. Transfections and calculations of luciferase activities are described as in (A). P101 is the non-PP2A binding st-ag mutant. The results are the average ± SD of three independent parallels and similar results were obtained in separate transfection experiments. (C) NIH 3T3 cells were co-transfected with GAL4-CREB, or GAL4-FHL2 expression plasmids, respectively and with either empty vector (pCMV5) or a vector encoding wild-type st-ag. Expression levels of the GAL4 fusion proteins were monitored by using anti-GAL4-specific antibodies (upper panel). Lane 1: cells co-transfected with GAL4-CREB expression plasmid and empty vector pCMV5; lane 2: cells co-transfected with expression plasmids for GAL4-CREB and st-ag; lane 3: cells co-transfected with GAL4-FHL2 expression plasmid and pCMV5; lane 4: cells co-transfected with expression plasmids for GAL4-FHL2 and st-ag. To correct for variations during the Western blotting, membranes were stripped and rehybridized with antibodies against CREB (lower panel). The intensity of the hybridization signals was determined by densitometry. The numbers on the bottom represent the ratio of the relative levels of GAL4-fusion protein:endogenous CREB. The relative protein level of GAL4-CREB, GAL4-FHL2, respectively, in the absence of st-ag was arbitrarily set as 1. The results of a representative experiment is shown and similar results were obtained in two other experiments.

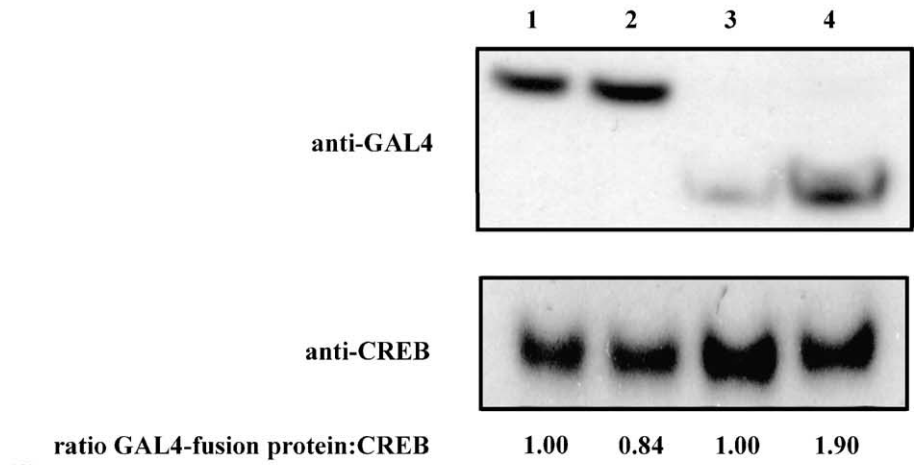


(A)

pGAL4-CREB



(B)



(C)

this promoter. For this purpose, NIH 3T3 cells were co-transfected with a luciferase reporter plasmid under control of wild-type or mutated c-*fos* promoter and either empty expression vector or a plasmid encoding wild-type SV40 st-ag, st-ag mutant 3 or mutant P101A. The mutant 3 lacks the carboxy-terminal amino acid residues 102-172, while mutant P101A has a proline residue replaced by alanine. Both mutant proteins have been shown to be expressed with comparable levels as wild-type st-ag, but the mutations almost completely abolished the ability of the mutants to bind PP2A and to inhibit its protein phosphatase activity [30–32,54,55]. Transient expression of full-length st-ag increased the activity of the wild-type c-*fos* promoter on average about 5-fold (range 3–7.5-fold in 10 separate transfection studies). No activation was observed with the st-ag mutants that failed to interact with PP2A. Induction of the c-*fos* promoter already occurred at OA concentrations that inhibit PP2A but not PP1 (Fig. 2A). Next, we examined which c-*fos* promoter sequences could mediate the PP2A-induced activation. Mutations in the SIE, SRE, FAP, or CRE motifs did not affect the basal promoter activity (results not shown). However, the CREB-binding motif is required for the induction of the c-*fos* promoter upon inhibition of PP2A because deletion of this element reduced transactivation by st-ag on average by 41% (22–51%, N = 3; Fig. 2B). Deletion of the serum responsive element (SRE) increased the induction of the promoter when PP2A was inhibited probably because it released autorepression of the c-*fos* promoter by this site [56].

3.2. CREB-mediated transcription is stimulated by wild-type st-ag, but not by a non-PP2A binding mutant

Because activation of the c-*fos* promoter by PP2A-binding st-ag partially required the CRE binding motif, we examined whether inhibition of PP2A modulated CREB-mediated transcription. For this purpose, the GAL4 system with GAL4-CREB fusion protein and the G5-E1b-LUC reporter plasmid was used. Expression of the *luciferase* gene is under the control of the adenovirus E1b minimal promoter fused to five binding motifs for GAL4 [43]. Dephosphorylation of p53 by PP2A has been shown to be impaired by st-ag [57], so pGAL4-p53 was therefore included as a control to confirm that st-ag could enhance GAL4-p53-mediated transcription in our experimental set up. Co-expression of wild-type st-ag enhanced the transcriptional activity of GAL4-CREB 4–19-fold (10 independent transfection experiments) compared with the activity observed in the absence of st-ag (Fig. 3A). The transcriptional potential of GAL4-p53 augmented 4.3-fold in the presence of st-ag. Increased transcription of the *luciferase* reporter gene was mediated by the transactivation domains of the tested transcription factors because st-ag had no effect on GAL4-directed transcription, nor could st-ag alone stimulate luciferase activity of the G5-E1b-LUC plasmid (Fig. 3A). The effect of st-ag was transcription factor specific because the activities of the GAL4-Elk-1, GAL4-VP16, GAL4-RelA/p65 (Fig. 3A), and the GAL4-oestrogen receptor [58] fusion proteins were not effected by co-expression of st-ag. The non-PP2A binding st-ag mutant P101A was unable to stimulate transcription mediated by GAL4-CREB (Fig. 3B). These data suggest the PP2A-dependency of the activation of CREB.

To rule out that st-ag enhanced GAL4-CREB-mediated transcription resulted from the increased concentration of the GAL4-fusion proteins, we compared the levels of GAL4-CREB in lysates of cells expressing st-ag with cell extracts not expressing st-ag. Western blot analyses revealed no obvious increase in the levels of the GAL4-CREB when st-ag was expressed (Fig. 3C). This suggests that increased transcription of CREB-directed transcription by inhibition of PP2A by st-ag is not merely the result of increased levels of these transcription factors.

3.3. PP2A-mediated modulation of CREB-directed transcription is independent of Ser-133

A prerequisite for transcriptional activation of CREB is the phosphorylation of Ser-133 (reviewed in [38,39]).

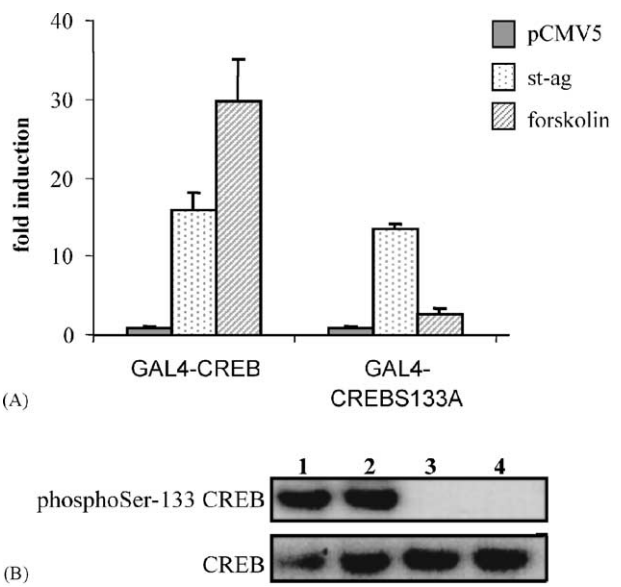


Fig. 4. Activation of CREB-mediated transcription by inhibition of PP2A is independent of phosphorylation of Ser-133. (A) NIH 3T3 cells were co-transfected with empty (pCMV5) or st-ag expression vector, and with a GAL4-fusion protein-encoding plasmid. The fusion partner of GAL4 was wild-type CREB or CREB Ser-133Ala mutant. Stimulation with 10 μM forskolin was for 3 hr. Luciferase activity was determined and presented as described in the legend of Fig. 2. (B) Inhibition of PP2A by st-ag does not alter the Ser-133 phosphorylation pattern of CREB. NIH 3T3 cells were co-transfected with GAL4-CREB expression plasmid and empty (pCMV5) or st-ag expression vector. Phosphoserine-133 levels were determined by phosphoCREB-specific antibodies. The protein levels of GAL4-phospho-CREB are shown in the upper panel, while the lower panel represents endogenous CREB as determined with anti-CREB antibodies. Lane 1: cell transfected with pCMV5 and GAL4-CREB; lane 2: cells transfected with st-ag and GAL4-CREB expression vectors; lane 3: cells transfected with pCMV5; lane 4: mock transfected cells.

Sustained phosphorylation through inhibition of PP2A may thus account for enhanced GAL4-CREB-mediated transcription. To investigate the role of Ser-133 in the st-ag-induced transactivation by CREB, we used the nonphosphorylable CREB mutant in which Ser-133 was substituted by Ala [59]. The transcriptional activity of wild-type GAL4-CREB, but not the GAL4-CREB S133A mutant, was strongly induced by the adenylyl cyclase activator forskolin. (Fig. 4A). Surprisingly, st-ag-activated GAL4-CREB S133A-mediated transcription equally well as wild-type GAL4-CREB (Fig. 4A). This indicated that the S133A mutation strongly impaired activation of CREB through the classical cAMP-dependent protein kinase (PKA) pathway, but had no effect on PP2A-dependent regulation of CREB. Ser-133-independent activation of CREB-mediated transcription by st-ag was further tested by comparing the Ser-133 phosphorylation pattern in NIH 3T3 cells lacking or transiently expressing st-ag. Western blotting with phosphoSer-133 CREB-specific antibodies revealed no quantitative changes in the phosphorylation pattern of CREB when st-ag was co-expressed (Fig. 4B). These observations underscore the findings that induction of CREB-mediated transcription after PP2A inactivation can be uncoupled of Ser-133 in NIH 3T3 cells.

3.4. Inhibition of PP2A increases the intrinsic transcriptional activity of the coactivator FHL2

The family of FHL has recently been shown to bind and stimulate CREB-mediated transcription in a phosphoSer-133-independent manner. Moreover, the FHL proteins possess intrinsic transcriptional activities [40]. Our finding that phosphoSer-133 was dispensable for PP2A-dependent activation of CREB-mediated transcription, suggested to

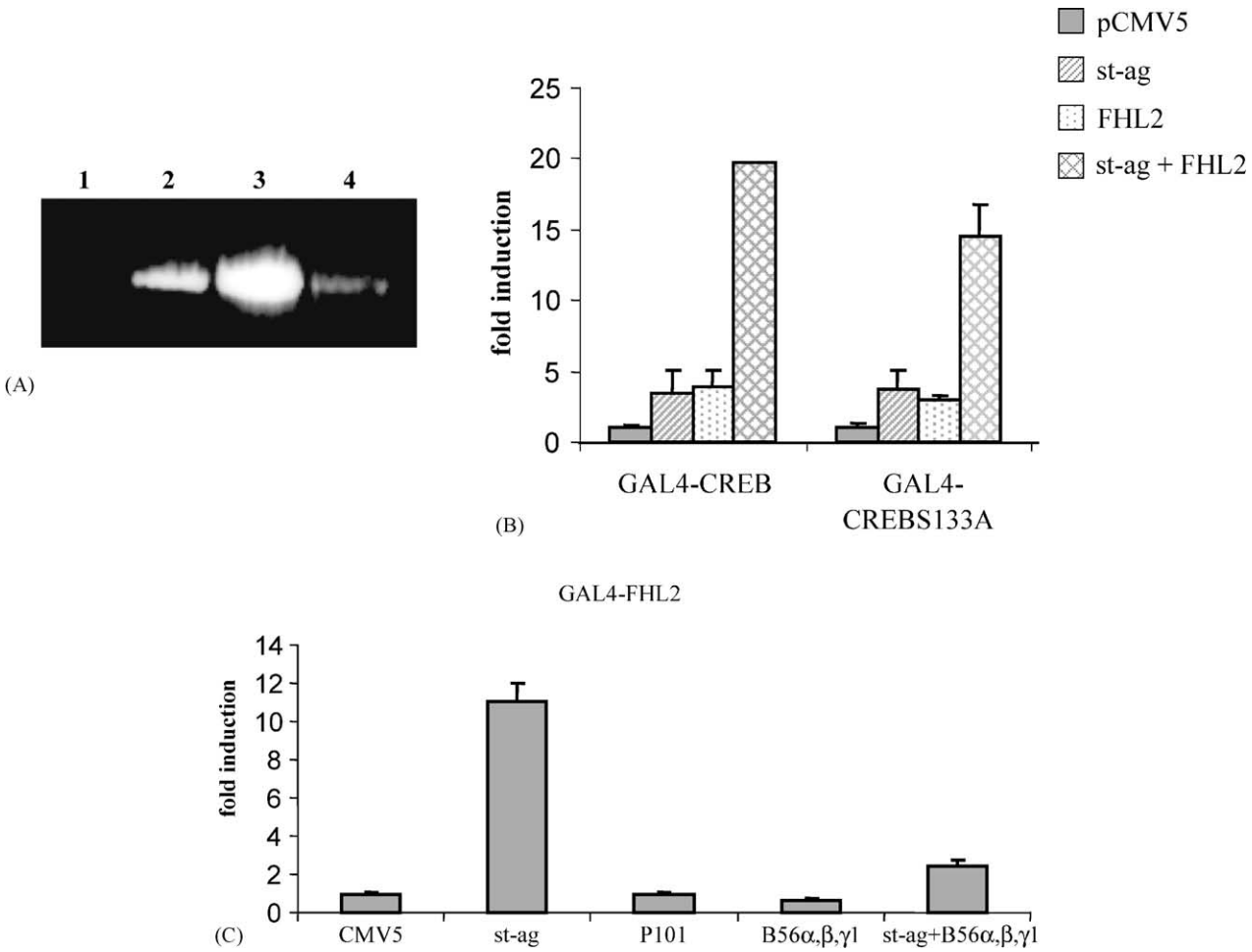


Fig. 5. FHL2-induced activation of CREB-mediated transcription is enhanced by inhibition of PP2A. (A) FHL2 transcripts can be detected in NIH 3T3 cells by RT-PCR. Total RNA was reversed transcribed and the cDNA was amplified by specific primers for FHL2. PCR products were analyzed by agarose gel electrophoresis. Lane 1: H_2O control; lane 2: 25 pg FHL2 EST clone DNA; lane 3: 100 pg FHL2 EST clone DNA; lane 4: cDNA of NIH 3T3 cells. (B) NIH 3T3 cells were co-transfected with expression plasmids for GAL4-CREB or GAL4-CREBS133A and with pCMV5 or st-ag or/and FHL2 expression plasmids. (C) Inhibition of PP2A stimulates the intrinsic transcriptional activity of FHL2. GAL4-FHL2-mediated transcription was monitored in the absence (pCMV5) or presence of st-ag. Simultaneous overexpression of the B56α,β and γ1 subunits of PP2A strongly reduced st-ag-induced activation of FHL2. Luciferase activity was determined and presented as described in the legend of Fig. 2. The results are the average ± SD of three independent parallels and similar results were obtained in separate experiments.

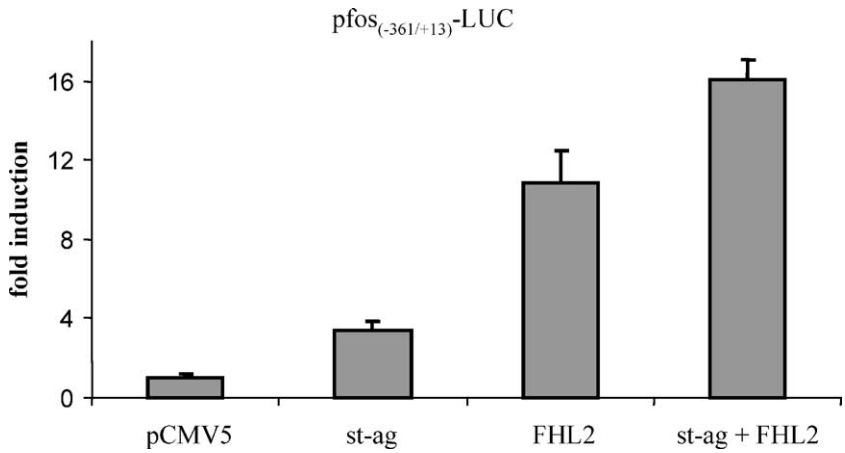


Fig. 6. Inhibition of PP2A superinduces FHL2-stimulated c-*fos* promoter activity. Transcription of the *luciferase* gene driven by the c-*fos* promoter (nucleotides −361 to +13) was measured in the presence of st-ag or/and FHL2. The results are the average ± SD of three independent parallels and similar results were obtained in separate experiments.

us that FHL proteins could be involved in mediating PP2A-dependent activation of CREB-directed transcription. Fimia *et al*. have demonstrated that FHL2, FHL3, and ACT stimulated CREB-mediated transcription. As ACT is exclusively expressed in testis, we decided to focus on FHL2 as a putative mediator for PP2A-dependent regulation of CREB. RT-PCR ensured that FHL2 was expressed in NIH 3T3 cells (Fig. 5A). Subsequently, we tested the effect of FHL2 on CREB-induced transcription. Overexpression of FHL2 stimulated CREB-directed expression, while co-expression of st-ag further enhanced the transcriptional potential of CREB in the presence of FHL2. Transcription directed by GAL4-CREBS133A was also stimulated when FHL2 was overexpressed, and an additional stimulatory effect was measured in the presence of st-ag (Fig. 5B). To test whether PP2A could be involved in regulating the intrinsic transcriptional activity of FHL2, transient transfection studies with a GAL4-FHL2 fusion protein in the presence or absence of st-ag were performed. As depicted in Fig. 5C, st-ag strongly stimulated the intrinsic transcriptional potential of FHL2 (6–11-fold; five independent experiments). The st-ag P101A mutant, unable to bind PP2A, could not augment the transcriptional activity of FHL2. The implication of PP2A in the modulation of FHL2's transcriptional activity was further confirmed by overexpression of the B56α, B56β, and B56γ1 subunits of PP2A. When present in excess, the B56 regulatory subunits of PP2A can displace st-ag from the AC-st-ag complex [60]. Simultaneous overexpression of the B56 subunits almost completely abrogated st-ag-induced activation of GAL4-FHL2-mediated transcription (Fig. 5C). These results support a PP2A-dependent mechanism for the regulation of FHL2. Enhanced FHL2-mediated transcription in the presence of st-ag was probably not due to increased GAL4-FHL2 protein levels as Western blot analysis detected only a marginal increase (1.3–1.9-fold in three independent experiments) in GAL4-FHL2 protein levels when st-ag was co-expressed (Fig. 3C).

3.5. st-ag potentiates FHL2-induced activation of the c-fos promoter

We and others have shown that PP2A can modulate the activity of the c-*fos* promoter (Fig. 2A [7,15,29]). Moreover, FHL2 is a coactivator for the transcription factors CREB (Fig. 5B and [40]) which can bind the c-*fos* promoter (Fig. 1). This prompted us to investigate whether FHL2 could influence the c-*fos* promoter activity. Overexpression of FHL2 stimulated the promoter 5–10-fold (four independent experiments). Inhibition of PP2A by st-ag further enhanced the activity of the c-*fos* promoter (16-fold, Fig. 6). These results suggest that the general coactivator FHL2 can be involved in the transcriptional activity of the c-*fos* promoter and that the PP2A-dependency of this regulation may be exerted through FHL2.

4. Discussion

Previous studies have demonstrated that PP2A can alter the activity of the c-*fos* promoter by a dual mechanism: dephosphorylation of sequence-specific transcription factors, and inhibition of signal transduction pathways that converge either directly or indirectly through interaction with other signaling pathways, to transcription factors that can bind the c-*fos* promoter [5,7,30–34]. The present study describes a third mechanism by which PP2A may control the c-*fos* promoter activity. We found that PP2A modulates the transcriptional potential of the general coactivator FHL2, which in turn affects CREB-mediated transcription and c-*fos* promoter activity. This model is based upon the following experimental evidences. Specific inhibition of PP2A by expression of SV40 wild-type st-ag increased c-*fos* activity on average 5-fold in transiently transfected NIH 3T3 cells. Expression of non-PP2A binding st-ag mutants did not stimulate this promoter (Fig. 2A). This is in agreement with the findings of Mullane *et al*., who

demonstrated that polyomavirus wild-type st-ag, but not a mutant deficient in PP2A binding (*ins*AL107), stimulated the c-*fos* promoter 5–6-fold in NIH 3T3 cells [61]. We were then able to identify that the CRE-binding motif was involved in mediating PP2A-dependent induction of the c-*fos* promoter in NIH 3T3 cells. Frost *et al*. found that transient co-expression of st-ag in combination with either c-Raf-1, MEK1 or ERK1 in REF52 fibroblasts, resulted in increased activity of a human c-*fos* promoter fragment containing the first 711 base-pairs upstream of the transcription start site [7]. This region includes the SIE, the serum response element (SRE) and the *fos*-AP-1 site (FAP), located at around −300, and the cAMP responsive element (CRE) at −50 (Fig. 1). However, a SV40 st-ag-mediated repression of the human c-*fos* promoter, spanning nucleotides −710 to +42 relative to the start site of transcription, has been observed in CV-1P, HeLa and A31 cells. The authors found that the region from −710 to −363 did not contain sequences responsible for repression by st-ag, while the region from −225 to −61 did [62]. The use of different cell lines may account for these discrepancies. Schüchner *et al*. reported that mouse polyomavirus st-ag activated the cyclin A promoter about 10-fold in Swiss 3T3 cells. This promoter also possesses a CRE motif [64]. Mutation of the CRE reduced the induction of the cyclin A promoter by st-ag with ca. 50%. The authors also showed that mutation in the PP2A binding region of st-ag (*ins*107AL) strongly impaired transactivation of the cyclin A promoter [63]. This is in agreement with our own findings. Using the same c-*fos* promoter fragment, Manfroid *et al*. found that mutation in SRE, but not in SIE or FAP, strongly reduced induction by OA in the rat pituitary tumour cell line GH3B6. The authors did not test the possible contribution of the CRE binding site [65]. The cell line and distinct experimental conditions to inhibit PP2A (50 nM OA for 18 hr) may account for the differences with our results. Obviously, the regulation of the c-*fos* promoter is very complex. Low concentrations of OA not only inhibit PP2A, but also PP4 [24]. A role for PP4 in regulation of the c-*fos* promoter activity has, however, not been addressed so far. Although stimulation of GAL4-CREB-mediated transcription by st-ag was higher than transactivation of the c-*fos* promoter by st-ag, deletion of the CRE did not completely abolish transactivation by st-ag, indicating that additional promoter sequences can mediate the effect of st-ag. Co-expression of st-ag and FHL2 resulted in an enhanced activity of the c-*fos* promoter. This may suggest that both proteins, in addition to affecting each others potentials, may influence the activity of other proteins binding to the c-*fos* promoter.

Our promoter deletion studies together with reports that PP2A has clearly been identified as the phosphatase for CREB [5], all strongly point to CREB as mediator for the induction of the c-*fos* promoter through inhibition of PP2A by st-ag. On the other hand, several reports suggest that PP1 could be responsible for the dephosphorylation of CREB. Dephosphorylation of CREB at Ser-133 was completely reversed by 1 μM OA in PC12 cells, while no effect was obtained with 1 nM OA. Using GAL4-CREB fusion protein, these authors found that 100 nM OA enhanced the transcriptional activity of CREB 2–3-fold [66]. These relatively high concentrations of OA suggest a role for PP1. Furthermore, Montminy and co-workers presented convincing evidence that PP1, and not PP2A, dephosphorylated CREB in NIH 3T3 and PC12 cells [66,67]. Moreover, both this group and we observed that st-ag expression in NIH 3T3 cells had no effect on the Ser-133 phosphorylation status of CREB ([67], this study). Overexpression of PP1, but not PP2A, in GH3B6 cells repressed the c-*fos* promoter [65]. These contradictory results may be explained by assuming that PP2A does not regulate the activity of CREB by direct dephosphorylation, but by controlling the activity of another protein such as a coregulator. Because PP2A-dependent regulation of CREB-mediated transcription could be uncoupled from phosphorylation of Ser-133, we investigated whether FHL2 could be a target for PP2A. We were able to demonstrate that FHL2 was a coactivator for CREB, and that inhibition of PP2A not only stimulated the intrinsic transcriptional activity of FHL2, but also enhanced FHL2-induced CREB-mediated transcription. FHL2 was recently reported to stimulate CREB-mediated transcription in a phosphoSer-133-independent manner [40]. Overexpression of FHL2 in NIH 3T3 cells stimulated the c-*fos* promoter activity 5–10-fold (Fig. 5). The group of Sassone-Corsi reported only a 2-fold increase when FHL2 was overexpressed in COS cells. The use of different cell types may explain this discrepancy. Alternatively, we used the c-*fos* promoter fragment spanning nucleotides −361 to +13, while Sassone-Corsi and co-workers used a fragment encompassing nucleotides −220 to +40. However, both fragments contain the CREB-binding site, but we monitored promoter activity with the *luciferase* reporter gene, while they applied the *chloramphenicol acetyltransferase* reporter gene.

Co-expression of st-ag increased very moderately the GAL4-FHL2 levels, while the intrinsic transcriptional activity of this fusion protein was strongly enhanced (6–11-fold). No effect of st-ag on the GAL4-CREB levels was observed. Transcription of the GAL4-CREB coding sequences is directed by the SV40 promoter, while transcription of the GAL4-FHL2 coding sequences is under control of the immediate early CMV promoter. Both promoters are not transactivated by st-ag [68,69]. However, the effect of st-ag on the latter was not tested in NIH 3T3 cells.

In conclusion, our studies support a role for FHL2 in the PP2A-dependent regulation of transcription. The exact mechanism by which PP2A regulates the activity of FHL2 remains to be established. PP2A may either directly dephosphorylate phosphoacceptor sites on FHL2, or the enzyme may inhibit a protein kinase that phosphorylates

FHL2. Further studies are needed to reveal which residues can be reversibly phosphorylated by a PP2A-dependent signaling pathway. FHL2 has also been described as a coactivator for the androgen receptor [51], while in a recent report, FHL2 was identified as a genuine interaction partner of WT1 able to modulate the transcriptional potentials of this transcription factor [70]. FHL2 was also shown to interact and augment the transcriptional repression of the promyelocytic zinc finger protein, a sequence-specific DNA-binding repressor involved in the control of cellular proliferation [71]. PP2A may thus, through regulating the transcriptional activity of FHL2, modulate the expression of genes that utilize one or several of these transcription factors. Aberrant expression of PP2A may affect the activity of FHL2 and therefore disturb the expression of a large spectrum of genes, which may explain a possible role for PP2A in tumorogenesis and other pathological conditions [4,24,72].

Acknowledgments

The authors thank Drs. R.J. Davis, J.A. Didonato, M. Green, T. Johansen, S. Khan, R.A. Maurer, E. Sontag and D.M. Virshup for the kind gift of various plasmids. This work was supported by grants from the Norwegian Cancer Society (DNK Project A01037), the Norwegian Research Council (NFR Project 135823/310), and the Erna and Olav Aakre Foundation. One of us (B.J.) is supported by the Norwegian Cancer Society.

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