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The predicted β12–β13 loop is important for inhibition of PP2Acα by the antitumor drug fostriecin

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Abstract The potential anticancer agent fostriecin (FOS) is a potent inhibitor of the protein Ser/Thr phosphatases PP2A and PP4 and a weaker inhibitor of PP1. Random mutagenesis and automated screening in yeast identified residues in human PP2Acα important for inhibitory FOS binding. A C269S substitution in the predicted β12–β13 loop decreased the FOS sensitivity of intact cells and increased the IC_{50} of PP2Acα by 10-fold in vitro. Changing PP2Acα Cys-269 to phenylalanine, the equivalent residue in PP1, and the Y267G and G270D substitutions caused a similar effect. The results provide information relevant to the design of novel protein Ser/Thr phosphatase inhibitory drugs. © 2001 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

Key words: Fostriecin; Phosphatase; Protein serine/threonine phosphatase 2A; Cancer; Yeast; Drug

1. Introduction

Fostriecin (FOS; NSC339638) is a structurally novel antitumor antibiotic [1,2]. Preclinical studies have revealed potent activity against murine leukemias dependent upon the unsaturated lactone and phosphate ester moieties [3]. Moreover, FOS displays antineoplastic activity against human tumor cells in vitro [4] and it has been evaluated as an anticancer drug in human patients [5]. The antitumor activity of FOS is mediated apparently by interference with reversible protein phosphorylation [6]. It inhibits the activity of protein Ser/Thr phosphatases (PPAs), an important family of enzymes with diverse roles in the control of signal transduction, growth and cell division [7]. Thus, FOS inhibits PP2A (inhibitory concentration $(IC)_{50} \sim 1$ nM) and PP4 ($IC_{50} \sim 3$ nM) potently, and the related PP1 enzyme weakly ($IC_{50} \sim 131$ μM) [8–10] but does not inhibit PP2B [8].

The crystal structure of PP1 [11,12] and molecular modeling [13,14] have predicted residues important for the interaction of PP1 and PP2A with several inhibitors. Mutagenesis of PP1 highlighted the C-terminal β12–β13 loop as a region important for inhibitor binding [15,16]. This flexible portion of the molecule protrudes over the active site and may impede access to substrates when inhibitors are bound [11,12]. It encompasses an amino acid sequence (SAPNYC) that is highly conserved in PP1 and PP2Ac, and downstream residues that diverge between PP1 and PP2Ac and contribute to the differential sensitivity of these enzymes to inhibitors [10,17,18].

The importance of PP1 and PP2Ac C-terminal residues for inhibitor binding was further defined by experiments employing the PP1 catalytic core enzyme and a chimeric protein, CRHM2, consisting of N-terminal PP1 residues and C-terminal residues from PP2Ac [8,10]. These studies indicated that residues C-terminal to the PP1 catalytic core are important for the binding of several protein and toxin inhibitors, including okadaic acid (OA) and microcystin-LR, while PP1 core residues alone mediate the binding of FOS. We extend these observations to reveal that, in the context of the full length PP2Acα molecule, Cys-269 in the predicted β12–β13 loop participates in an interaction with FOS that, while absent from PP1, promotes inhibition of PP2A.

2. Materials and methods

2.1. Media, drugs, strains and plasmids

Rich (YPD and YMI), synthetic (C) and 5-fluoroorotic acid (5-FOA) media were described [19]. OA (Calbiochem) and FOS (DTP-NCI) were suspended to 20 mM in dimethyl sulfoxide (DMSO) and stored at 4°C (OA) or −40°C (FOS). Prior to use FOS was diluted into buffer containing ascorbic acid at 0.1 mM to protect against oxidation. DEY779-1 (**a** *erg6*Δ1::*TRP1 pdr1*Δ1::*LEU2 pdr3*Δ1::hisG *bub3*Δ1 *ura3*, A364a) is a Ura^- recombinant of strain SPY50779[1]. Strains DEY3 [20] and DEY1-Cα [21] are deleted for the chromosomal genes *PPH21*, *PPH22* and *PPH3* encoding yeast PP2Ac and Pph3p. Viability of DEY3 is dependent on plasmid YCpDE8 (*URA3 PPH22*) encoding yeast PP2Ac [20]. Strain DEY1-Cα lacks yeast PP2Ac and is dependent on human PP2Acα expressed at a low level from the *PGK1* promoter of YEpDE-PGK-Cα (*TRP1*) [21]. YEpDE2e is $PPH22^{HA}$ encoding hemagglutinin (HA)-tagged yeast PP2Ac [22] in YEp352. Plasmid YCpDE-ADHU-CHA is the HA-PP2Acα cDNA (*Bam*HI/*Eco*RI fragment) from YCpDE-PGK-CHA [21] expressed from the *ADH1* promoter. Cell number was determined using a hemocytometer.

2.2. Drug toxicity assays with intact yeast cells

Toxicity assays were performed as per Simon et al. [23]. FOS was 3-fold serially diluted in 5% DMSO and aliquots (15 μl) were dispensed in duplicate to a Nunc U96 PP 0.5 ml 96-well plate. DMSO (15 μl) was dispensed in duplicate to control wells. Cells grown in selective (C-ura or C-trp) medium were diluted to 5×10^4 cells per ml and

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Abbreviations: FOS, fostriecin; PP2Ac, catalytic subunit of protein serine/threonine phosphatase 2A; PP1, protein phosphatase 1; OA, okadaic acid; DMSO, dimethyl sulfoxide; 5-FOA, 5-fluoroorotic acid; ORF, open reading frame; HA, hemagglutinin; IC, inhibitory concentration; t_d, population doubling time

[1] NCI yeast anticancer drug screen: http://dtp.nci.nih.gov.

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dispensed (135 μl) into each well. Cultures were incubated at 30°C for 18–24 h until the A_{660} of control cultures was 0.35–0.45 (Bio-Tek Instruments EL340 microplate reader). Relative growth was (A_{660} with drug/A_{660} without drug).

2.3. Mutagenesis of human PP2Acα and selection for functional mutant forms

An 870 bp fragment from YEpDE-PGK-Cα, encoding 653 bp of the PP2Acα 3′ open reading frame (ORF) and 217 bp of vector, was amplified by mutagenic PCR [21]. A 970 bp cDNA encoding human PP2Ac-225-1 [21] was inserted into vector pYPGE2(*TRP1*) and cleaved with *Afl*II/*Bst*EII within the ORF. To produce a library of PP2Acα mutant clones the gapped plasmid and mutant PCR products were introduced into strain DEY3 [20]. Transformants (~55 000) were selected on C-trp medium then replicated to 5-FOA to select for Ura$^-$ recombinants (~10 000) lacking yeast PP2Ac and expressing a functional PP2Acα. Site directed mutagenesis of PP2Acα in plasmid YEpDE-PGK-CHA [21] was performed using QuickChange (Stratagene).

2.4. Identification of functional PP2Acα mutant forms conferring FOS resistance

Naïve strains (1216) were patched onto C agar containing FOS (100 μM), incubated at 30°C for 2 days and scored for growth relative to strain DEY1-Cα. Additional strains (184) were screened by automated, high-throughput toxicity assays in which strains were inoculated (30 μl) initially into duplicate wells of a 96-well plate containing liquid C (270 μl). Saturated cultures were serially diluted to 5×10^4 cells per ml using a MATRIX PlateMATE 96/384 Automated Pippettor robot (OPAL/JENA software) and distributed (135 μl) to two 96-well plates with FOS (15 μl) at 100 μM or 400 μM, or DMSO (15 μl) at 5% in the wells. Duplicate control cultures of DEY1-Cα were included on each plate. Cells were deemed drug resistant if relative growth was 1.8-fold and 1.4-fold higher than that of DEY1-Cα in the presence of 10 μM and 40 μM drug, respectively. In an alternative enrichment approach, colonies functionally expressing PP2Acα were pooled from 5-FOA agar into liquid C. An aliquot (3×10^6 cells) was inoculated into C (3 ml) containing FOS (40 μM) and ascorbic acid (0.1 mM). After 2 days at 30°C a fresh sample of FOS was added to 40 μM. After a further 3 days independent colonies (138) were isolated on YPD and screened for FOS resistance by automated toxicity assay.

2.5. Partial purification of PP2Acα proteins from yeast, and Western blot analysis

PP2Acα proteins were tagged with the HA epitope [21] and inducibly expressed from vector pYES2 in strain INVSC1 (Invitrogen) [21]. HA-tagged proteins were immunoprecipitated from cell extracts (~1 mg) and eluted from beads with HA peptide (Boehringer Mannheim) as described [21,24] except the monoclonal HA.11 (0.4 mg; Covance) was bound to Gammabind Plus Sepharose (1 ml; Amersham Pharmacia Biotech). Tagged proteins were analyzed by Western blotting as described [21] using HA.11 as probe.

2.6. Protein phosphatase activity measurements

Partially purified PP2Acα was eluted from beads and assayed for activity as using malachite green to measure phosphate release from a peptide substrate (RRA(pT)VA) (Promega V2460) as described [21]. Assays (50 μl) were performed in duplicate in a 96-well plate (Costar 3690; Corning). Purified rabbit muscle PP2Ac (V6311) was obtained from Promega.

3. Results

3.1. Fostriecin targets PP2Ac in yeast cells

Fostriecin (FOS) blocks the proliferation of mammalian cells and inhibits the activity of PP2Ac [6,8,25] and PP4 [9] potently. Recently we found that FOS inhibits yeast cell growth, and that the inhibition is exacerbated in the absence of the *BUB3* gene[1]. In an attempt to understand the biological effects of FOS we tested whether PP2Ac is a target in yeast cells. In a toxicity assay [23] FOS inhibited the growth of *bub3*Δ cells potently ($IC_{50} < 1.0$ μM) but its toxic effect was reduced ($IC_{50} \sim 4$ μM) by increased dosage of the yeast PP2Ac gene (Fig. 1A). A similar effect (~2.4-fold increase in IC_{50}) was observed using *BUB3*$^+$ cells (not shown). Moreover, drug resistance was conferred by expression of human PP2Acα from the strong *ADH1* promoter in cells lacking the endogenous yeast enzyme (Fig. 1B). These results indicate that PP2Ac is a target of FOS in yeast.

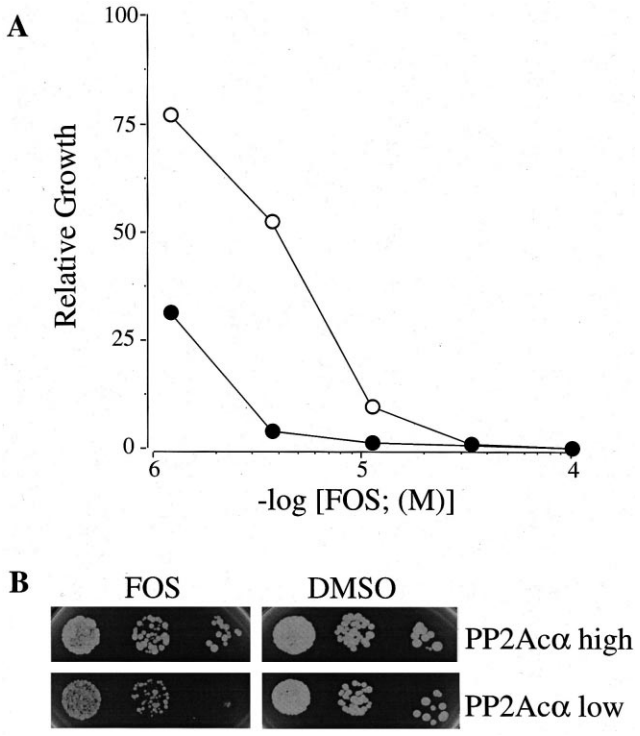

Fig. 1. Increased PP2Ac gene dosage suppresses FOS inhibition of yeast growth. A: Elevated *PPH22* dosage confers FOS resistance. Toxicity was assayed in *bub3*Δ cells (strain DEY779-1) containing plasmid YEpDE2e (*2μ PPH22*, encoding yeast PP2Ac; open circles) or empty vector (filled circles). B: Expression of human PP2Acα from the yeast *ADH1* promoter confers FOS resistance. A plasmid expressing PP2Acα from the strong *ADH1* promoter (PP2Acα high), or empty vector (PP2Acα low) was introduced into strain DEY1-Cα. Cells were grown to saturation in C-ura medium, 10-fold serially diluted, spotted onto YPD agar containing FOS (100 μM) or drug vehicle (DMSO) and incubated at 30°C for 48 h.

3.2. Identification and functional analysis of FOS-resistant forms of PP2Acα in yeast

To identify amino acids important for the inhibition of PP2Ac by FOS, the human PP2Aα cDNA was subjected to random mutagenesis and PP2Acα mutant forms that substituted for yeast PP2Ac were tested for FOS resistance (Section 2). Thus, 1400 strains functionally expressing a PP2Acα protein were patched onto agar containing FOS at 100 μM, or inoculated into liquid medium and tested for FOS resistance at 10 μM and 40 μM by automated screening. This yielded one PP2Acα mutant cDNA (N1) that conferred drug resistance following plasmid rescue and reintroduction into the host strain (inset to Fig. 2). By an alternative strategy, yeast strains (~5000) functionally expressing PP2Acα were pooled and an aliquot was inoculated into medium containing FOS at 40 μM to enrich for drug-resistant cells. After 5 days, independent colonies were tested for FOS resistance at 10 μM and 40 μM by automated screening. By this approach, three

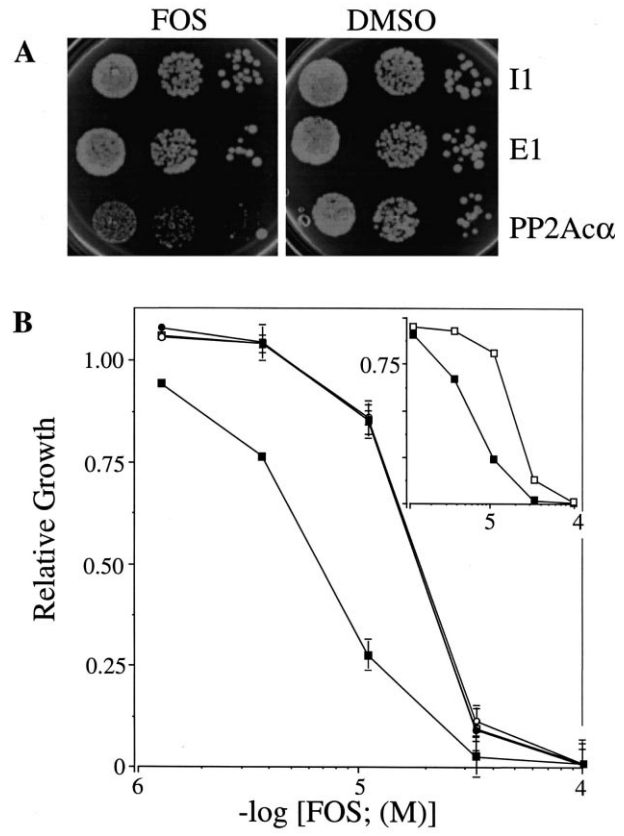

Fig. 2. FOS resistance of yeast cells expressing PP2Acα mutant cDNAs. Cells from a saturated culture and expressing a wild-type (filled squares) or mutant (E1, open squares; E2, open circles; E3, filled circles) form of PP2Acα were assayed for growth in liquid medium containing FOS. Numbers are mean values (±S.D.) from three independent experiments. The inset shows a similar experiment with cells expressing wild-type PP2Acα (filled squares) or a mutant form encoded by cDNA N1 (open squares).

PP2Acα cDNAs (E1, E2 and E3) were isolated that conferred drug resistance reproducibly following plasmid rescue (Fig. 2).

Analysis of cDNAs N1 and E1–E3 revealed a single transversion (t805→a) in each encoding the PP2Acα C269S substitution. Cells functionally expressing PP2Acα C269S exhibited a rate of proliferation (population doubling time (t_d) 102 min at 30°C in YMI) similar to that of cells expressing wild-type PP2Acα (t_d 105 min; strain DEY1Cα) indicating that the mutant protein is largely functional. Remarkably, a substitution at the same position (C269G) was identified during a screen for PP2Acα mutations conferring OA resistance in Chinese hamster ovary (CHO)-K1 cells [25]. Furthermore Cys-269 lies within a C-terminal region of PP2Ac, the predicted β12–β13 loop, that is partly conserved in PP1 [11,12]. This region encompasses a conserved SAPNYC motif and residues immediately downstream (GEFD in PP1 and YRCG in PP2Ac) which contribute to the differential sensitivity of PP1 and PP2Ac to inhibitors [8,10,15–18]. Because FOS is highly specific (~40 000-fold) for the inhibition of PP2Ac relative to PP1 [8], each residue in the PP2Acα YRCG motif (including Cys-269) was changed individually to the equivalent residue in PP1. In addition, phenylalanine was substituted for Tyr-265 in the PP2Acα SAPNYC motif because the equivalent mutation in PP1 confers resistance to OA, calyculin A and tautomycin [15]. The mutant proteins were tested in yeast, for function in vivo and FOS resistance. The PP2Acα Y265F and R268E mutant proteins were non-functional suggesting that they are improperly folded in vivo, and they were not studied further. In contrast, PP2Acα C269F supported a wild-type rate of cell proliferation (t_d 102 min) and conferred resistance to FOS (~4-fold increase in IC_{50}; Fig. 3A). Similarly, the Y267G and G270D forms supported wild-type proliferation (t_d 100 and 92 min, respectively) and caused decreased sensitivity to FOS (1.4-fold increase in IC_{50}) (Fig. 3A) that was observed clearly in cells grown on agar (Fig. 3B). These data support the conclusion that Cys-269 and adjacent residues are important for inhibition of PP2Acα by FOS.

3.3. The PP2Acα C269S substitution confers FOS resistance in vitro

To investigate the role of Cys-269 in drug inhibition of PP2Ac activity, the PP2Acα wild-type and C269S mutant proteins were partially purified from yeast. A similar amount (Fig. 4A) and total activity (see legend to Fig. 4B and C) of enzyme was recovered from yeast expressing the wild-type or mutant form, and each enzyme displayed a similar rate of

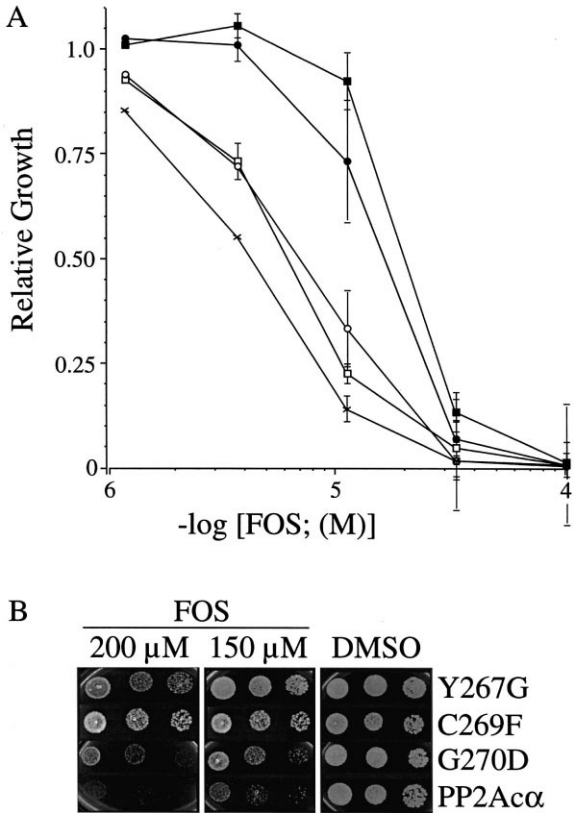

Fig. 3. FOS resistance of cells expressing a PP2Acα form mutated in the predicted β12–β13 loop. A: Toxicity assay of FOS sensitivity. Cells functionally expressing a wild-type (crosses) or mutant (C269F, filled squares; C269S, filled circles; G270D, open circles; Y267G, open squares) PP2Acα were assayed for FOS sensitivity. Numbers are mean values (±S.D.) from three independent experiments. B: Growth on YPD agar. Cells containing a cDNA encoding a wild-type (PP2Acα) or mutant (Y267G, G270D, C269F) PP2Acα were tested for growth in the presence (FOS, 150 μM or 200 μM) or absence (DMSO) of drug as in Fig. 2. Similar results were obtained in three experiments.

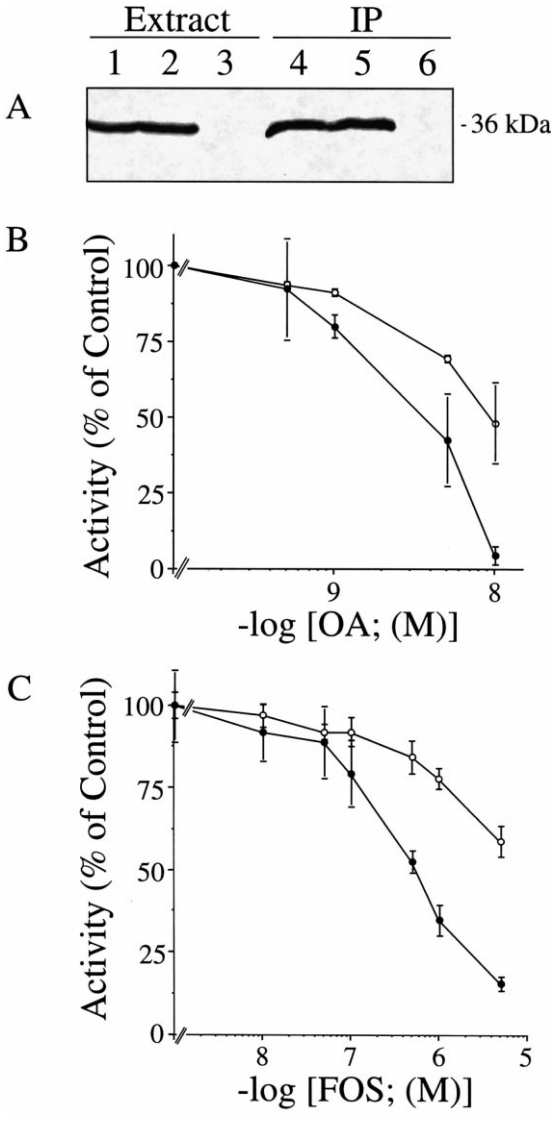

Fig. 4. Analysis of PP2Acα activity in vitro. A: Western blot analysis of partially purified PP2Acα proteins. A plasmid encoding the HA-tagged wild-type or C269S form of PP2Acα (36 kDa), or the empty vector, was introduced into strain INVSC1. Yeast cell extracts (30 μg; extract), and immune complexes (IP) released from beads by boiling, were resolved by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) (10%) prior to Western blot analysis. Lanes 1 and 4, wild-type PP2Acα; lanes 2 and 5, PP2Acα C269S; lanes 3 and 6, no HA-tagged protein (empty vector). Results are representatives of two independent preparations. B/C: Protein phosphatase assays in vitro. The PP2Acα wild-type and C269S mutant enzymes were partially purified from yeast and assayed for activity in the presence of OA (B) or FOS (C). Data are expressed as % of control activity in the absence of drug. B: Numbers are mean values from two independent experiments. Control activity was 39.2 ± 3.4 μU (wild-type; $n = 2$) and 30.1 ± 8.9 μU (C269S; $n = 2$). C: Mean values (± S.D.) from three independent experiments. Control activity was 41.1 ± 4.9 μU, wild-type ($n = 3$) and 40.5 ± 1.4 μU, C269S ($n = 3$). Control activities were corrected for background activity (A, 7.5 ± 1.3 μU, $n = 2$ and B, 6.9 ± 0.6 μU, $n = 3$) present in eluates lacking HA-tagged protein.

phosphopeptide substrate hydrolysis in vitro (not shown). Above a concentration of 0.5 nM, OA inhibited wild-type PP2Acα activity (apparent $IC_{50} \sim 3$ nM) in a dose dependent manner (Fig. 4B). Similarly, FOS inhibited PP2Acα activity (apparent $IC_{50} \sim 500$ nM) in a dose dependent manner (Fig. 4C), but less potently than OA as observed by Walsh et al. [8]. The high apparent IC_{50} of wild-type PP2Acα for OA and FOS in these experiments is likely due to a high concentration of enzyme present in the assay, causing drug titration as described previously [8]. The fold difference between the apparent and reported IC_{50} of wild-type PP2Acα for OA (30-fold) and FOS (> 100-fold) was different. The reason for this is unclear but the effect is inherent to our assay, as under the same conditions the IC_{50} of purified rabbit muscle PP2Ac for OA and FOS was similar to that of the PP2Acα enzyme partially purified from yeast (not shown). Notably, the C269S substitution caused a decrease in the sensitivity of PP2Acα to OA (~3–4-fold increase in IC_{50}) (Fig. 4B) similar to that reported for the C269G substitution [25]. Moreover, consistent with the results obtained with intact cells, the activity of the PP2Acα C269S mutant enzyme was strikingly resistant to inhibition by FOS (apparent $IC_{50} > 5.0$ μM) (Fig. 4B) supporting the conclusion that Cys-269 is important for inhibition of PP2Acα by this drug.

4. Discussion

To identify novel anticancer agents we have been screening for compounds that inhibit yeast cell growth specifically in the context of cancer-like mutations [23]. FOS was identified in this screen due to differential inhibition of *bub3*Δ mutant cells defective for the kinetochore/spindle checkpoint[1]. Towards an understanding of the inhibitory effect of FOS we have investigated whether PP2Ac is a cellular target in yeast. Consistent with this, we demonstrate that increased dosage of PP2Ac enhances the tolerance of yeast cells to FOS, apparently via drug titration [26], and we identify mutations in PP2Acα that confer FOS resistance.

The PP2Acα C269S substitution, located within a region equivalent to the PP1 β12–β13 loop [11,12], increased the FOS resistance of intact cells and the IC_{50} of the partially purified enzyme. Similar to C269G [25], C269S increased the IC_{50} of PP2Acα for OA by 4-fold indicating that Cys-269 is important for FOS and OA binding by PP2Acα. Because it is absent from PP1, Cys-269 must contribute to the 40 000-fold greater sensitivity of PP2Ac to FOS [8]. Accordingly, changing Cys-269 to phenylalanine, the corresponding residue in PP1, increased the FOS resistance of intact cells.

Our results were unexpected because FOS inhibition of PP1 requires residues in the catalytic core, but not in the β12–β13 loop [10]. Thus a PP1 core enzyme, lacking residues downstream of alanine in the SAPNYC motif, and a chimeric enzyme CRHM2, consisting of PP2Ac C-terminal residues fused to PP1 at the SAPNYC motif, exhibit FOS sensitivity characteristic of full length PP1 [8,10]. However, within the context of PP2Acα, Cys-269 contributes to the potent inhibition of activity by FOS because C269S increased the IC_{50} by at least 10-fold. Nevertheless, PP2Acα C269S was inhibited by 40% in the presence of 5 μM FOS, contrasting with PP1 and CHRM2 which are inhibited with an IC_{50} of > 100 μM [8,10]. Indeed our measurements underestimate the inhibition of PP2Acα C269S because the apparent IC_{50} of wild-type PP2Acα for FOS in our assay was > 100-fold higher than that calculated for the purified enzyme diluted to a titration endpoint [8]. The intermediate sensitivity of PP2Acα C269S to FOS, together with results obtained from structural analysis

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Hs PP4c  -VKALCAKAREILVEESNVQRVDSPVTVCGDIHGQFYDLKELFRVGGDVPETNYLFMGDFV   84
Hs PP2Ac -VKSLCEKAKEILTKESNVQEVRCPVTVCGDVHGQFHDLMELFRIGGKSPDTNYLFMGDYV   87
Hs PP1c  -IRGLCLKSREIFLSQPILLELEAPLKICGDIHGQYYDLLRLFEYGGFPPESNYLFLGDYV   94
Bt PP2B  -ALRIITEGASILRQEKNLLDIDAPVTVCGDIHGQFFDLMKLFEVGGSPANTRYLFLGDYV   120

Hs PP4c   DRGFYSVETFLLLLALKVRYPDRITLIRGNHESRQITQVYGFYDECLRKYGSVTVWRYCT   144
Hs PP2Ac  DRGYYSVETVTLLVALKVRYRERITILRGNHESRQITQVYGFYDECLRKYGNANVWKYFT   147
Hs PP1c   DRGKQSLETICLLLAYKIKYPENFFLLRGNHECASINRIYGFYDECKRRYN-IKLWKTFT   153
Bt PP2B   DRGYFSIECVLYLWALKILYPKTLFLLRGNHECRHLTEYFTFKQECKIKYS-ERVYDACM   179

Hs PP4c   EIFDYLSLSAIIDGKIFCVHGGLSPSIQTLDQIRTIDRKQEVPHDGPMCDLLWSDPE-DT   203
Hs PP2Ac  DLFDYLPLTALVDGQIFCLHGGLSPSIDTLDHIRALDRLQEVPHEGPMCDLLWSDPD-DR   206
Hs PP1c   DCFNCLPIAAIVDEKIFCCHGGLSPDLQSMEQIRRIMRPTDVPDQGLLCDLLWSDPDKDV   213
Bt PP2B   DAFDCLPLAALMNQQFLCVHGGLSPEINTLDDIRKLDRFKEPPAYGPMCDILWSDPLEDF   239

Hs PP4c   TG-------WGVSPRGAGYLFGSDVVAQFNAANDIDMICRAHQLVMEGYKWHFN------   250
Hs PP2Ac  GG-------WGISPRGAGYTFGQDISETFNHANGLTLVSRAHQLVMEGYNWCHD------   253
Hs PP1c   QG-------WGENDRGVSFTFGAEVVAKFLHKHDLDLICRAHQVVEDGYEFFAK------   260
Bt PP2B   GNEKTQEHFTHNTVRGCSYFYSYPAVCEFLQHNNLLSILRAHEAQDAGYRMYRKSQTTGF   299

Hs PP4c   ETVLTVWSAPNYCYRCGNVAAILELDEHLQ-280
Hs PP2Ac  RNVVTIFSAPNYCYRCGNQAAIMELDDTLK-283
Hs PP1c   RQLVTLFSAPNYCGEFDNAGAMMSVDETLM-290
Bt PP2B   PSLITIFSAPNYLDVYNNKAAVLKYENNVM-359
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Fig. 5. Candidate FOS binding residues in PP2Ac and PP4. Catalytic core and C-terminal residues of human PP4, PP2Ac and PP1 and bovine PP2B were aligned using CLUSTAL W. Amino acids in bold are conserved in PP2Ac and PP4 (inhibited potently by FOS), but differ from the equivalent residue in PP1 (inhibited weakly) and PP2B (not inhibited). The SAPNY motif in each sequence is underlined. The PP1 β12–β13 loop encompasses residues 268–278.

and mutagenesis of PP1 [8,10,11,16] predict that in addition to Cys-269, residues within the PP2Acα catalytic core are important for inhibitory FOS binding.

Amino acid sequence alignment (Fig. 5) reveals that PP2Ac and PP4 share residues that are absent from PP1 and PP2B and therefore are candidates for mediators of high-affinity FOS binding. We propose that Cys-269 orients FOS optimally in relation to an active site residue(s) that may be unique to PP2Ac and PP4 and which mediates high-affinity FOS binding. The screen employed in this study may not have identified core amino acids involved in FOS binding because it included a selection for PP2Acα function in vivo, and mutation of active site residues can impair catalytic function. However, substituting an alternative residue for PP2Acα Cys-269 may weaken FOS binding by disrupting the structural relationship between the β12–β13 loop and the active site. The increase in FOS resistance caused by the Y267G and G270D substitutions (~1.4-fold increase in the IC_{50} of intact cells) located adjacent to Cys-269 is consistent with this. Interestingly, the unsaturated lactone of FOS resembles the αβ unsaturated *N*-methyldehydroalanine (Mdha) residue in microcystin-LR, the side chain of which forms a covalent linkage with Cys-273 in the PP1 β12–β13 loop [11]. Thus FOS may bind the equivalent Cys-266 residue in PP2Acα. However, the covalent linkage between Mdha and PP1 Cys-273 is not essential for enzyme inhibition [27,28] and our data do not rule out a direct interaction between PP2Acα Cys-269 and FOS.

Fostriecin confers an antitumor effect at concentrations that may inhibit the activity of both PP2Acα and PP1 [4]. An understanding of the molecular interactions occurring between protein Ser/Thr phosphatase inhibitors and their targets may help in the design of novel drugs with increased efficacy and reduced general toxicity.

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References

[1] Stampwala, S.S., Bunge, R.H., Hurley, T.R., Willmer, N.E., Brankiewicz, A.J., Steinman, C.E., Smitka, T.A. and French, J.C. (1983) J. Antibiot. (Tokyo) 36, 1601–1605.
[2] Tunac, J.B., Graham, B.D. and Dobson, W.E. (1983) J. Antibiot. (Tokyo) 36, 1595–1600.
[3] Leopold, W.R., Shillis, J.L., Mertus, A.E., Nelson, J.M., Roberts, B.J. and Jackson, R.C. (1984) Cancer Res. 44, 1928–1932.
[4] Scheithauer, W., Von Hoff, D.D., Clark, G.M., Shillis, J.L. and Elslager, E.F. (1986) Eur. J. Cancer Clin. Oncol. 22, 921–926.
[5] de Jong, R.S., Mulder, N.H., Uges, D.R.A., Sleijfer, D.T.H., Hoppener, F.J.P., Groen, H.J.M., Willemse, P.H.B., van der Graaf, W.T.A. and de Vries, E.G.E. (1999) Br. J. Cancer 79, 882–887.
[6] Roberge, M., Tudan, C., Hung, S.M.F., Harder, K.W., Jirik, F.R. and Anderson, H. (1994) Cancer Res. 54, 6115–6121.
[7] Janssens, V. and Goris, J. (2001) Biochem. J. 353, 417–439.
[8] Walsh, A.H., Cheng, A. and Honkanen, R.E. (1997) FEBS Lett. 416, 230–234.
[9] Hastie, C.J. and Cohen, P.T.W. (1998) FEBS Lett. 431, 357–361.
[10] Connor, J.H., Kleeman, T., Barik, S., Honkanen, R.E. and Shenolikar, S. (1999) J. Biol. Chem. 274, 22366–22372.
[11] Goldberg, J., Huang, H.-B., Kwon, Y.-G., Greengard, P., Nairn, A.C. and Kuriyan, J. (1995) Nature 376, 745–753.
[12] Egloff, M.-P., Cohen, P.T.W., Reinemer, P. and Barford, D. (1995) J. Mol. Biol. 254, 942–959.
[13] Bagu, J.R., Sykes, B.D., Craig, M.M. and Holmes, F.B. (1997) J. Biol. Chem. 272, 5087–5097.
[14] Gauss, C.-M., Sheppeck, J.E., Nairn, A.C. and Chamberlin, R. (1997) Bioorg. Med. Chem. 5, 1751–1773.
[15] Zhang, L., Zhang, Z., Long, F. and Lee, E.Y.C. (1996) Biochemistry 35, 1606–1611.
[16] Huang, H.-B., Horiuchi, A., Goldberg, J., Greengard, P. and Nairn, A.C. (1997) Biochemistry 94, 3530–3535.
[17] Connor, J.H., Quan, H.N., Ramaswamy, N.T., Zhang, L., Barik, S., Zheng, J., Cannon, J.F., Lee, E.Y.C. and Shenolikar, S. (1998) J. Biol. Chem. 273, 27716–27724.
[18] Zhang, Z., Zhao, S., Long, F., Zhang, L., Bai, G., Shima, H., Nagao, M. and Lee, E.Y.C. (1994) J. Biol. Chem. 269, 16997–17000.
[19] Kaiser, C., Michaelis, S. and Mitchell, A. (1994) Methods in Yeast Genetics, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
[20] Evans, D.R.H. (1997) Genetics 145, 227–241.
[21] Evans, D.R.H., Myles, T., Hofsteenge, J. and Hemmings, B.A. (1999) J. Biol. Chem. 274, 24038–24046.

[22] Evans, D.R.H. and Hemmings, B.A. (2000) Mol. Gen. Genet. 264, 425–432.
[23] Simon, J.A., Szankasi, P., Nguyen, D.K., Ludlow, C., Dunstan, H.M., Roberts, C.J., Jensen, E.L., Hartwell, L.H. and Friend, S.H. (2000) Cancer Res. 60, 328–333.
[24] Evans, D.R.H. and Hemmings, B.A. (2000) Genetics 156, 21–29.
[25] Shima, H., Tohda, H., Aonuma, S., Nakayasu, M., DePauli-Roach, A.A., Sugimura, T. and Nagao, M. (1994) Biochemistry 91, 9267–9271.
[26] Cheng, A., Balczon, R., Zuo, Z., Koons, J., Walsh, A.H. and Honkanen, R.E. (1998) Cancer Res. 58, 3611–3619.
[27] Zhang, Z., Zhao, S., Deans-Zirattu, S., Bai, G. and Lee, E.Y.C. (1993) Mol. Cell. Biochem. 128, 113–119.
[28] Moorhead, G., MacKintosh, R.W., Morrice, N., Gallagher, T. and MacKintosh, C. (1994) FEBS Lett. 356, 46–50.