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Biochemistry

Characterization of the PP2Aα gene mutation in okadaic acid-resistant variants of CHO-K1 cells

(protein serine/threonine phosphatase)

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ABSTRACT **Okadaic acid (OA)-resistant variants of Chinese hamster ovary cells, clones CHO/OAR6-6 and CHO/OAR2-3, were isolated from a CHO-K1 culture. These variant cells were 17- to 26-fold more resistant to OA than the parental cells. The phosphorylase phosphatase activity of the variant cell extracts was 2- to 4-fold more resistant to OA than that of the parental cells in the presence of inhibitor 2, a specific inhibitor of type 1 protein serine/threonine phosphatase (PP1). Nucleotide sequencing of PP2Aα (an isotype of PP2A catalytic subunit) cDNA demonstrated that both variants have a T → G transversion at the first base of codon 269 (805 nt), which results in substitution of glycine for cysteine. We expressed in COS-1 cells a mutant PP2Aα tagged with the influenza hemagglutinin epitope. The recombinant mutant PP2Aα protein immunoprecipitated with an anti-influenza hemagglutinin antibody was more resistant than the wild type to OA, their IC_{50} values being 0.65 nM and 0.15 nM, and their IC_{80} values being 4.0 nM and 0.45 nM, respectively. The cysteine at residue 269 present only in highly OA-sensitive protein serine/threonine phosphatase catalytic subunit isozymes, PP2Aα, PP2Aβ, and PPX, is suggested to be involved in the binding of OA. CHO/OAR6-6 and CHO/OAR2-3 cells also overexpressed the P-glycoprotein, and the efflux of OA was more rapid. It is suggested that the PP2Aα mutation in cooperation with a high level of P-glycoprotein makes the CHO-K1 variants highly resistant to OA.**

Protein serine/threonine phosphatases (PPs) have been known to play important roles in signal transduction. PPs have been classified into four major classes: type 1 (PP1), type 2A (PP2A), type 2B (PP2B), and type 2C (PP2C) (1). Okadaic acid (OA), a fatty acid polyether, inhibits these PPs to different extents (2). It inhibits the PP2A form most strongly, causing complete inhibition at 1 nM (1), while it inhibits the PP1 enzyme moderately, with an IC_{50} of about 50 nM (3), PP2B is even less sensitive, and PP2C is not inhibited (1). In addition to these four major PPs, PPX was recently found to be ubiquitously expressed in various tissues and to be as sensitive to OA as PP2A (4).

OA induces various biological effects *in vivo*, such as diarrhea, due to prolonged smooth muscle contraction (5) and promotion of skin and stomach carcinogenesis in experimental animals (6, 7). Unlike inhibitors 1 and 2, which specifically inhibit the PP1 catalytic subunit (1), OA is unique in inhibiting both major cellular PPs, PP2A and PP1. In addition, OA easily penetrates into cells; therefore OA has been used for analysis of the functions of PPs.

OA at concentration of <10 nM, which mainly inhibit PP2A and PPX, has various effects on mammalian cells *in vitro*, causing flattening of NIH 3T3 cell transformants that bear various activated oncogenes (8), inhibition and promotion of cell transformations by chemical carcinogens (9, 10), diphtheria toxin-resistant mutations (DT^r) (11), sister chromatid exchanges (SCEs) (12), and aneuploidy (10). Inhibition by low concentrations of OA of Rb protein phosphorylation might be involved in inhibition of transformation (13, 14). Although various genetic alterations have been induced in mammalian cells as described above, there is no evidence that OA directly interact with DNA, and OA showed no mutagenicity in *Salmonella typhimurium* (11). These results indicate that inhibition of PP2A and/or PPX leads to genetic alterations. However, it is still unclear whether the effects of OA on cells are due to inhibition of PPs or modulation of other proteins.

To clarify the roles of PPs involved in cell physiology, including genetic instability and malignant transformation, in this study we isolated OA-resistant variants from a CHO-K1 cell culture and examined which isoenzyme of PP was mutated.

MATERIALS AND METHODS

Cells and Culture Medium. A Chinese hamster ovary cell line, CHO-K1, was grown in culture as monolayers in RPMI 1640 medium supplemented with 7% fetal bovine serum, 2 mM L-glutamine, and 50 μg of kanamycin per ml. CHO-K1 cells were cultured in the presence of 8 ng of OA per ml and the OA concentration was increased gradually up to 320 ng/ml (data not shown). Two resistant variants were isolated and their clones were designated as CHO/OAR2-3 and CHO/OAR6-6.

Cloning of Reverse Transcription PCR Product and DNA Sequencing. The primers were designed based on the rat PP2Aα (α isotype of PP2A catalytic subunit) sequence, as follows: Pa-1, 5′-ATGGACGAGAAGTTGTTCAC-3′; Pa-2, 5′-GACCACCATGTAGACAGAAG-3′; Pb-1, 5′-CAGTTACACTGCTTGTAGCT-3′; Pb-2, 5′-CAGGAAGTAGTCTGGGGTAC-3′; Pb-3, 5′-GAGCAGACAGATCACACAAG-3′. Total RNAs from CHO-K1 cells were prepared by acid guanidinium thiocyanate/phenol/chloroform extraction (15) and cDNA was synthesized as described (11). The cDNA was amplified by the PCR using a GeneAmp kit and cloned using a TA cloning system (Invitrogen). Sequencing was performed by the dideoxynucleotide chain-termination method using Sequenase Version 2 (United States Biochemical) according to the manufacturer's protocol.

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Abbreviations: OA, okadaic acid; PP, protein serine/threonine phosphatase; PP2Aα, α isotype of PP2A catalytic subunit; HA, influenza hemagglutinin; SSCP, single-strand conformation polymorphism; DT^r, diphtheria toxin resistant; SCE, sister chromatid exchange.
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PCR Single-Strand Conformation Polymorphism (PCR-SSCP) Analysis. PCR-SSCP analysis was performed basically by a reported method (16). The primers used were Pc-1, 5′-GATATAACTGGTGCCATGAC-3′ and Pc-2, ATCAAGTTCCATGATTGCAG. The cDNA (2 μl) or genomic DNA (100 ng) was amplified by PCR with 0.4 μM of Pc-1 and Pc-2 primers, 0.1 unit of *Taq* polymerase, 0.2 mM of each dNTP, and 0.2 ml of [α-^{32}P]dCTP (370 MBq/ml, 110 TBq/mmol, Amersham) in 5 μl of reaction mixture. Forty cycles of the reaction were performed at 94°C, 58°C, and 72°C for 0.5, 0.5, and 1 min, respectively. The products were applied to 6% polyacrylamide gel containing 5% glycerol and separated by electrophoresis at 20°C.

Phosphatase Assay. Cells were homogenized in 10 volumes of extraction buffer [10 mM Tris·HCl (pH 7.4) containing 100 μM phenylmethylsulfonyl fluoride, 10 μg of aprotinin per ml, 5 μg of leupeptin per ml, 5 μM pepstatin, 1 mM benzamidin, and 10 mM EDTA] and centrifuged at 15,000 × *g* for 10 min at 4°C. The supernatant, which contained most of the PP2A, was used as an enzyme source. PP2A phosphatase assay was carried out as described (17) using 300-fold diluted cell extract in the presence of 1 μg of recombinant inhibitor 2 per ml (18). One unit of activity was defined as that releasing 1 μmol of phosphate from the ^{32}P-labeled phosphorylase *a* in 1 min under the defined conditions.

Plasmid Construction. The rat PP2Aα cDNA plasmid that we reported previously (19) was used. Gene mutations were introduced with an oligonucleotide-directed *in vitro* mutagenesis system (Amersham). A primer Pm-1 (5′-TTGGTTACCACCACGATAGCA-3′) was used for the construction of plasmid PP2Aα269G, which has a mutation in codon 269 (cysteine to glycine). For preparation of the construct for influenza hemagglutinin (HA) epitope-tagged PP2Aα protein, two-step PCR was performed sequentially as described by Wadzinski *et al.* (20) using wild-type and mutated plasmids as templates. For the first PCR, primers HA-1 (5′-ATGTACCCATACGATGTTCCAGATTACGCTGACGAGAAGTTGTTCACC-3′) and HA-2 (5′-CTAGAATTCTTACAGGAAGTAGTCTGGGG-3′) were used. For the second PCR, primers HA-2 and HA-3 (5′-ATACTGCAGATGTACCCATACGATGTTCC-3′) were used for the first PCR products. The final PCR products of PP2Aα and PP2Aα269G were digested with *Pst* I and *Eco*RI, purified by phenol/chloroform extraction, and ligated to the *Pst* I–*Eco*RI fragment of pks(+)/CMV (21). These plasmids were named CMV/HA-2Aα and CMV/HA-2Aα269G, respectively.

Transfection. COS-1 cells were transfected essentially by the method of Chen and Okayama (22). For determination of expression efficiency, cell homogenates were prepared by resuspending the cells in 20 volumes of extraction buffer and sonicating for 30 sec on ice. The particulate and soluble fractions obtained by centrifugation at 5000 × *g* for 10 min at 4°C were then subjected to Western blot analysis.

Purification of HA-Tagged PP2Aα. A volume of 200 μl of the soluble fraction of the transfected cells was mixed with protein A-Sepharose (40 μl) and incubated for 1 hr at 4°C. The supernatant obtained by centrifugation at 5000 × *g* for 10 min at 4°C was incubated overnight at 4°C with 2 μl of an anti-HA antibody (12CA5, Berkeley Antibody, Richmond, CA) and for 1 hr at 4°C with protein A-Sepharose (40 μl). The beads were washed with Nonidet P-40 (NP-40) lysis buffer [50 mM Tris·HCl (pH 7.4) containing 150 mM NaCl, 10 μg of aprotinin per ml, 5 μg of leupeptin per ml, 5 μM pepstatin, 1 mM benzamidin, 10 mM EDTA, and 0.5% NP-40] three times, then washed twice with extraction buffer, and finally divided into two portions. One was used for Western blotting and the other was suspended in 300 μl of reaction buffer for phosphatase assay.

Western Blot Analysis. Western blot was performed as described (23) using rabbit antiserum against the PP2A catalytic subunit (anti-PP2AC) (24) or the anti-HA antibody (20) with ^{125}I-labeled protein A (Amersham).

RESULTS

Isolation of OA-Resistant Variant Clones from the CHO-K1 Cell Line. Two OA-resistant variant clones of Chinese hamster cells, CHO/OAR2-3 and CHO/OAR6-6, were isolated from the CHO-K1 cell line. The IC_{50} of OA for colony formation of the parental CHO-K1 cells was 13 nM and those for OAR2-3 and OAR6-6 were 26- and 17-fold higher, respectively (data not shown).

Sensitivity of Phosphatase to OA *in Vitro*. There was no difference in the phosphorylase phosphatase activities of crude cell extracts of the parental cells and the two variants in terms of amounts of protein. The phosphatase activity in the extracts of both variants was more resistant to OA than that of the parental cells, although the extent was small at OA concentrations between 0.5 nM and 2 nM. OA at concentrations of >10 nM inhibited the enzyme activities in the extracts of the parental cells and of the two variants with similar efficiencies (data not shown). These results suggested that the OA resistance of the variants was due to mutation in PP2A or PPX. To confirm the OA resistance of PP2A or PPX, we measured the phosphatase activity in the presence of recombinant inhibitor 2 (1 μg/ml), a specific inhibitor of PP1. The phosphatase activity of the extracts from parental and variant cells was reduced by approximately half, but the extracts of both variants were clearly more resistant to OA than that of the parental cells (Fig. 1). The IC_{50} and IC_{80} values of the two variants were 2- and 4-fold higher, respectively. These results showed the presence of an OA-resistant mutation on PP2A or PPX in these variants.

Detection of PP2Aα Gene Mutation by Sequencing of Reverse Transcription PCR Fragments. Since the nucleotide sequence of the coding region of the hamster PP2Aα was not reported, we performed PCR cloning for the hamster PP2Aα cDNA using primers based on rat PP2Aα cDNA sequences (19). Two overlapping cDNA clones were obtained from parental CHO-K1 cells. The 5′ half (fragment a) and the 3′ half (fragment b) of the wild PP2Aα cDNA fragment were amplified using two paired primers, Pa-1 and Pa-2 (fragment a) and Pb-1 and Pb-2 (fragment b), and subcloned (Fig. 2). The nucleotide sequence of hamster PP2Aα corresponding to the region from 67 nt to 885 nt of rat PP2Aα coding region was

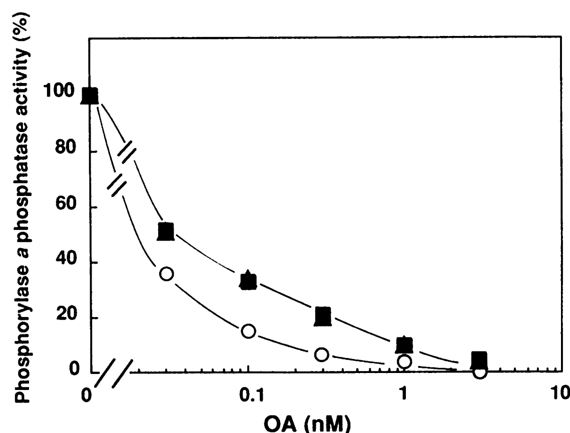

FIG. 1. OA resistance of phosphatase activity *in vitro*. Crude cell extracts were prepared from parental CHO-K1 cells (○) and OA-resistant variant cells, clones OAR2-3 (▲) and OAR6-6 (■), and used as enzyme sources. The enzyme reaction was performed in the presence of 1 μg of inhibitor 2 per ml with phosphorylase *a* as substrate and various concentrations of OA. Each point is the mean value from two experiments.

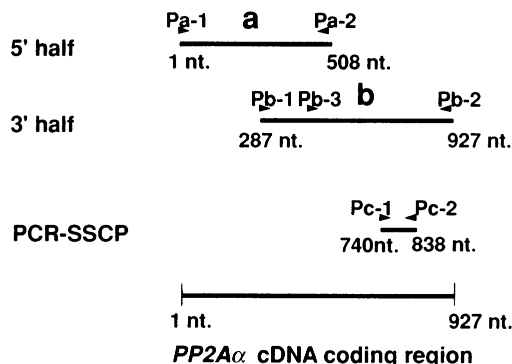

FIG. 2. Primer design for analysis of the mutation in hamster *PP2Aα*. First and second lines, design for sequencing hamster PP2Aα cDNA. PP2Aα cDNA was amplified as two fragments (a and b) using primers Pa-1 and Pa-2 (fragment a) and Pb-1 and Pb-2 (fragment b). The products were cloned. Four independent clones of each fragment were sequenced. For sequencing, primers Pa-1, Pa-2, Pb-1, Pb-2, and Pb-3 were used. Third line, design for PCR-SSCP analysis. Primers Pc-1 and Pc-2 were used for PCR of cDNA and genomic DNA. All of the primer sequences were based on rat PP2Aα cDNA sequences.

determined. The amino acid and nucleotide sequences from codon 23 to codon 295 of *PP2Aα* showed 100% and 97.6% identities, respectively, with those of the rat and hamster. The PP2Aα cDNA sequence of a variant (CHO/OAR6-6) was determined by the same method. The first base of codon 269 (805 nt) was mutated from T to G (Fig. 3*A*). All four cDNA clones examined had the same mutation and no wild-type sequence was detected. The mutation resulted in the substitution of glycine for cysteine. No other mutations were found in the region from 67 nt to 885 nt. The other variant, CHO/OAR2-3 cells, also had the same mutation (Fig. 3*A*).

We used cDNA PCR-SSCP analysis to determine whether the mutant and wild-type alleles are expressed in these variants (Fig. 3*B*, lanes 1–3). Both variants showed only shifted bands, suggesting that the wild-type allele was not expressed in the variants. Direct sequencing analysis also supported this (data not shown). Next we analyzed PP2Aα genomic DNA by PCR-SSCP analysis. We used Pc-1 and Pc-2 primers, corresponding sequences of which are located in the exon 6 of the human PP2Aα gene (25). The PCR-SSCP analysis of genomic DNA revealed that both variants contained the wild-type allele as well as the mutated allele (Fig. 3*B*, lanes 4–6). These data suggest that this gene mutation is heterozygous and functionally hemizygous in the variants. Two other bands observed commonly in the genomic DNA samples (Fig. 3*B*, lanes 4–6) might be due to the presence of a pseudogene.

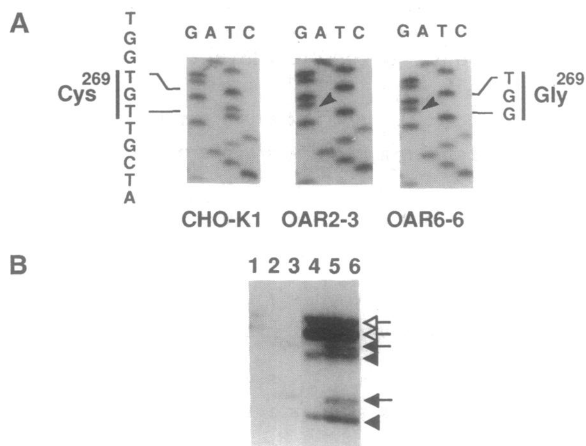

FIG. 3. Detection of *PP2Aα* mutation in OAR2-3 and OAR6-6. (*A*) Nucleotide sequencing of PP2Aα cDNA from CHO-K1 and its variants. cDNA clones from OAR2-3 and OAR6-6 variants showed a T → G transversion at the first letter of codon 269, as indicated by arrowheads. (*B*) PCR-SSCP analysis of the PP2Aα gene (740–838 nt). Lanes 1–3, cDNA samples from CHO-K1 cells and variants, OAR2-3 and OAR6-6, respectively; lanes 4–6, genomic DNA samples from CHO-K1 and the variants OAR2-3 and OAR6-6, respectively. Bands of wild (◁–) and mutated (◀–) alleles were detected. Two bands that were not detected in the cDNA SSCP analysis (◀) may be those of a pseudogene.

Expression of HA-Tagged PP2Aα Gene in COS-1 Cells. High-level expression of HA-tagged wild and mutated PP2Aα proteins was detected by Western blotting using anti-HA antibody, 48 hr after transfection into COS-1 cells of CMV/HA-2Aα or CMV/HA-2Aα269G. Most of the recombinant proteins were found to be inactive in the particulate fraction, and only a small amount was active in the soluble fraction, as previously reported by other investigators (20).

The phosphorylase phosphatase activity of the recombinant enzymes purified from the soluble fraction by immunoprecipitation using anti-HA antibody was examined. The amounts of recombinant wild and mutant enzymes expressed were estimated to be almost the same by Western blotting using anti-HA antibody (Fig. 4) and using anti-PP2AC antibody (data not shown). The phosphorylase phosphatase activities associated with the immunoprecipitates of the wild and mutant PP2A were also similar, being 25 μunits, and 32 μunits, respectively.

The OA sensitivities of recombinant wild and mutant PP2Aα were analyzed using aliquots of these immunoprecipitates prepared from the soluble fractions (Fig. 5*A*). The IC_{50} for the wild PP2Aα was 0.15 nM and that of the mutant PP2Aα269G was 0.65 nM. The IC_{80} for recombinant wild was 0.45 nM and that of the recombinant mutant was 4.0 nM. The mutation at residue 269 conferred severalfold resistance to OA but did not affect the sensitivity to another PP inhibitor, calyculin A (Fig. 5*B*), which shows similar inhibitory effects on PP1 and PP2A.

DISCUSSION

We isolated two OA-resistant variants from a CHO-K1 culture and found that they contained a mutated gene of the

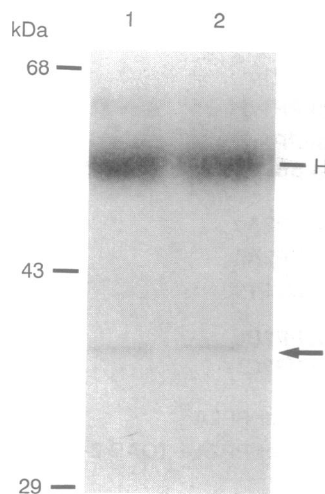

FIG. 4. Detection of the HA-tagged recombinant wild and mutant PP2Aα protein in soluble fractions of COS-1 cells. The immunoprecipitates obtained from soluble fractions of COS-1 cells transfected with the CMV/HA-2Aα and CMV/HA-PP2Aα269G vectors, respectively (lanes 1 and 2), were subjected to Western blot analysis with anti-HA antibody. The heavy chain of rabbit immunoglobulin (H) and the recombinant PP2Aα protein (←) were detected.

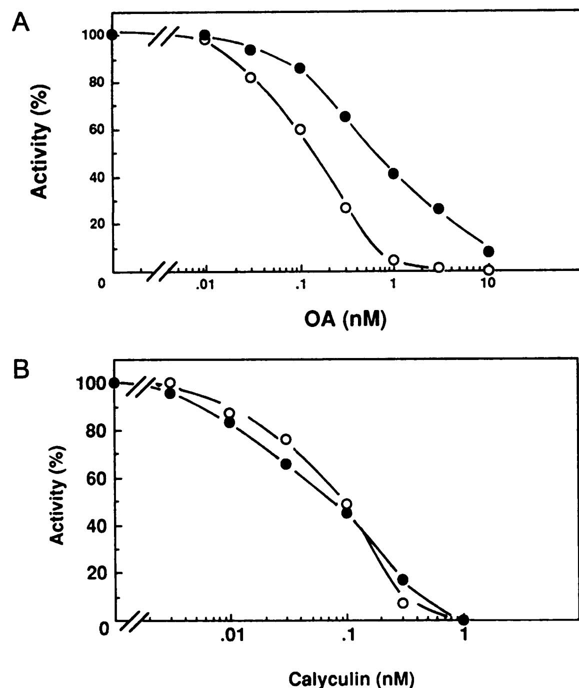

FIG. 5. Sensitivities of the recombinant PP2Aα protein to OA (*A*) and calyculin A (*B*). Aliquots of the immunoprecipitates of HA-2Aα (○) and HA-2Aα269G (●) obtained from soluble fractions (shown in Fig. 4) were subjected to enzyme analysis, using phosphorylase *a* as a substrate. Each point is the mean value from two experiments.

α isoform of PP2A catalytic subunit. Both variants harbor the same mutation, a T → G base substitution at the first base of codon 269, resulting in amino acid substitution of glycine for cysteine. To confirm whether the observed gene mutation confers OA resistance, we constructed a mutant rat PP2Aα cDNA containing the same mutation by site-directed mutagenesis and overexpressed it in COS-1 cells. The phosphatase activity of the recombinant mutant PP2Aα269G was more OA-resistant (4-fold at IC_{50} and 10-fold at IC_{80}) than that of the recombinant wild PP2Aα. These results suggest that the cysteine encoded by codon 269 of PP2Aα plays an important role in interaction with OA.

OA-resistant PPs, PP2Bα and PP2C, have a tyrosine residue at the codon corresponding to 269 of PP2Aα, and all of the PP1 catalytic subunits, PP1α, PP1γ1, and PP1γ2, and PP1δ, which are less sensitive to OA, have phenylalanine, the most OA-sensitive PPs, PP2Aα, PP2Aβ, and PPX, having cysteine (Fig. 6) (3, 4, 26). This observation supports the idea that cysteine encoded at codon 269 may be essential for the high sensitivity to OA. In contrast, the mutation at codon 269 did not affect the sensitivity to calyculin A. Thus, the binding site of PP2Aα for calyculin A may be different from that of OA. Since calyculin A shows an inhibitory potency similar to PP1 and PP2A (27), sequences that are common to both PP catalytic subunits may be involved.

PP2Aα269G (IC_{50} of 0.65 nM) showed an intermediate sensitivity between the recombinant PP2Aα (IC_{50} of 0.15 nM) and recombinant PP1 catalytic subunits, PP1α, PP1γ1, PP1γ2, and PP1δ (IC_{50} of ≈50–120 nM) (3). At present, it is not clear whether only the cysteine 269 confers OA sensitivity. The amino acid sequence from 267 to 270, YRCG, is conserved in all PP2A catalytic subunits and PPX, whereas GEFD is the corresponding residue in all PP1 catalytic subunits (Fig. 6). It is possible that the YRCG sequence plays a key role in high OA sensitivity. Indeed, by replacing the GEFD sequence of PP1α with YRCG, Zang *et al.* (28) in collaboration with us found that the mutant PP1α protein expressed in *Escherichia coli* was 10 times more sensitive to OA than the wild-type recombinant PP1α protein.

The cysteine to glycine mutation at residue 269 does not result in change of the protein level of PP2Aα, because the amount of PP2A catalytic subunit measured by Western blot analysis was similar in the parental CHO-K1 and its variants (data not shown). Furthermore the mutation did not change the phosphorylase phosphatase activity since the activity in the presence of inhibitor 2 was almost the same in the crude extract of the parental and variant cells.

The variants we isolated from a CHO-K1 culture were 17–26 times more resistant to OA than the parental cells in colony formation (data not shown). However, the resistance of PP2Aα269G to OA was much less. To address this discrepancy, we measured the amounts of OA taken up by the cells and found that the [^{3}H]OA level in mutants was about half that in the parental cells and that efflux of [^{3}H]OA was much higher in both variants than in the parental cells. Furthermore, the P-glycoprotein level was elevated in the

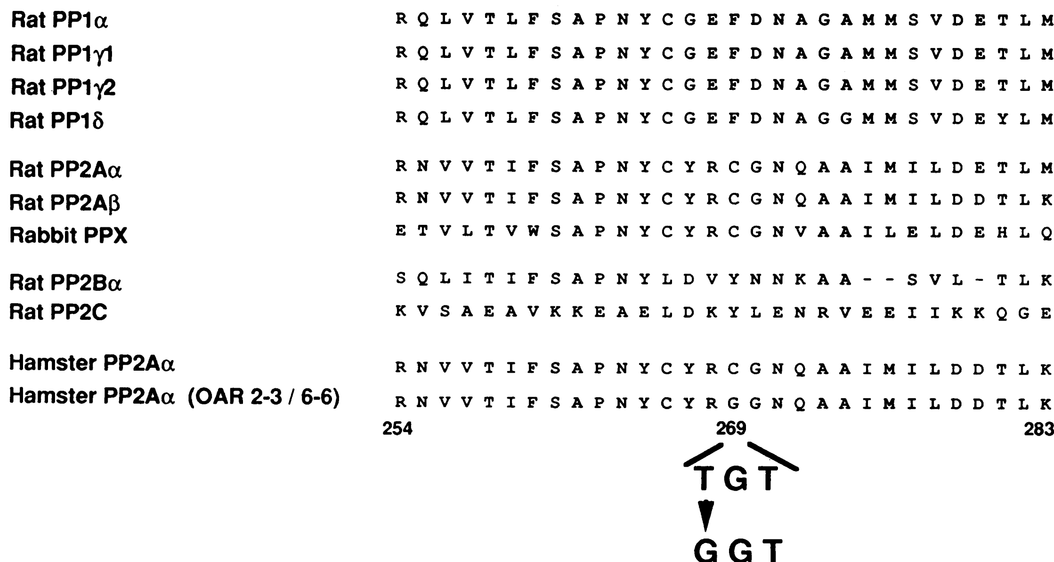

FIG. 6. Comparison of amino acid sequences around codon 269 of *PP2Aα* with various isoenzymes. The reported sequences of protein phosphatases are aligned. The arrow shows the position of mutation.

variants (data not shown). The high efflux may be due to elevated P-glycoprotein and may be partially involved in OA resistance of the variants. One of our purposes in isolating OA-resistant mutant cells was to clarify the role of PP2A in OA-induced genetic changes such as SCEs and DT^r mutation. No SCEs were induced in OAR6-6 cells by 125 nM OA (data not shown), although they were induced in parental CHO-K1 cells, as reported in previous studies with 5–10 nM OA. Since OA is taken up by OAR6-6 cells at half the rate of that of the parental cells (data not shown), the level of OA in OAR6-6 treated with 125 nM OA may be higher than that in the parental cells treated with 10 nM OA. Thus, the PP2Aα mutation might contribute to the absence of induction of SCEs by OA in the variants.

However, the marked efflux of OA in the PP2Aα mutant cells complicates the analysis of the PP2Aα function. It is not clear whether the overexpression of P-glycoprotein is due to the PP2Aα mutation or other mutations. To make this point clear, we tried to express the recombinant mutant PP2Aα protein in mammalian cells, but its level of expression was not high. Development of a method for high-level expression of this protein in mammalian cells should be useful for elucidation of the physiological role of PP2Aα.

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