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Flat reversion by okadaic acid of *raf* and *ret*-II transformants

(protein phosphatase inhibitor/tumor promoter/fibronectin)

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ABSTRACT **Okadaic acid is a non-phorbol 12-myristate 13-acetate (PMA)-type tumor promoter on mouse skin and known to be a potent inhibitor of serine/threonine protein phosphatases. Contrary to expectation from its tumor-promoting activity, okadaic acid was shown to have a potential to revert the phenotype of cells transformed by *raf* and *ret*-II to that of normal cells. Two to 3 days after addition of 8 ng of okadaic acid per ml to the culture medium, *raf* and *ret*-II transformants changed to flat cells and gained contact inhibition. The amount of fibronectin, which was decreased in malignant transformed cells, was increased in the flat revertants. Moreover, okadaic acid caused a dose-dependent loss of ability to grow in soft agar. The morphology of the cells reverted to malignant phenotype within 1 week after removal of okadaic acid. The levels of mRNA and protein of activated c-*raf* in flat revertants were similar to those in parental transformed cells. The level of mRNA of *ret*-II was also not changed by flat reversion. No induction of flat reversion was observed with okadaic acid tetramethyl ether, an inactive compound, or a phorbol ester, PMA. As okadaic acid is a potent inhibitor of protein phosphatases 1 and 2A, the possibility that these phosphatases are involved in signal transduction from the *raf* and *ret*-II oncogenes is suggested.**

Okadaic acid is a polyether compound of a C_{38} fatty acid and has been implicated as one of the causative agents of diarrhetic shellfish poisoning (1). It was found recently to be a potent tumor promoter in a two-stage carcinogenesis experiment on mouse skin (2). In contrast to phorbol 12-myristate 13-acetate (PMA)-type tumor promoters, okadaic acid does not either activate protein kinase C or bind to the receptor of phorbol esters (3). Thus, okadaic acid is classified in the group of non-PMA type tumor promoters, which includes palytoxin and thapsigargin (4). Its cellular mediator is unknown. Okadaic acid has been shown to be a strong inhibitor of serine/threonine protein phosphatases 1 and 2A (5, 6) and to induce hyperphosphorylation of a proteolytic fragment of nucleolin, N-60, in fibroblasts (7).

Cellular signal transduction from the membrane to the nucleus is mediated by protein phosphorylation catalyzed by protein kinases, including oncogene products. Serine/threonine kinases such as *raf*-encoded protein are involved in signal transduction, and the involvement of protein phosphatases in regulation of the phosphorylation states of various key proteins is strongly suspected. To clarify the role of protein phosphatases in cell transformation, we examined the effect of okadaic acid on NIH 3T3 transfectants expressing normal or activated c-*raf* mRNA, and we found that okadaic acid induced reversion to flat cells of activated c-*raf* transformants. Okadaic acid also induced reversion of the *ret*-II transformant to flat cells. *ret*-II is an activated form of the *ret* protooncogene predicted to encode the receptor-type tyrosine kinase by a rearrangement. In this study we examined the effects of okadaic acid on morphological changes, oncogene expression, and the phosphorylation state of normal *raf*-encoded protein in various transformants.

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MATERIALS AND METHODS

Chemicals. PMA was purchased from CCR (Eden Prairie, MN). Okadaic acid was isolated from a black sponge (1) and okadaic acid tetramethyl ether was synthesized as described (2, 3). These compounds were dissolved at a concentration of 8 ng/μl in dimethyl sulfoxide before use.

Cells. NIH 3T3 cells and a secondary transformant of NIH 3T3 cells with activated human c-Ha-*ras* (a1-1) were provided by M. Wigler (Cold Spring Harbor Laboratory). Other secondary transformants of NIH 3T3 cells transfected with rat and human activated *raf* (IQ7-2 and 2C-1, respectively), human activated N-*ras* (Nh-1), rat activated Ki-*ras* (Kr-6), and human *ret*-II (SIC-2) were obtained as described (8–12). An NIH 3T3 transfectant of normal rat c-*raf* cDNA ligated to the metallothionein promoter-I (EV-R3) was isolated (13). Each transfectant or transformant was cloned by limiting dilution just before use. Point mutations of activated *ras* oncogenes are described in Table 1.

Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Nissui Pharmaceutical, Tokyo) supplemented with 10% calf serum (GIBCO). Cell morphology was examined by phase-contrast microscopy.

Treatment of Cells with Okadaic Acid. Cells ($2–3 \times 10^5$) were plated in 10-cm Petri dishes and cultured overnight in DMEM containing 10% calf serum. Then the medium was changed to that containing okadaic acid at a concentration of 2–8 ng/ml. Two or 3 days after the addition of okadaic acid, the cell morphology was examined. Control cultures were treated with okadaic acid tetramethyl ether following the same protocol. For examination of the reversibility of morphological changes of cells, cells were cultured with 8 ng of okadaic acid per ml for 7–10 days, and then the medium was changed to that without okadaic acid. Cell morphology was examined at 7–14 days after removal of okadaic acid. The culture medium was renewed every 3 days during this experiment.

Cell Growth. Cells (5×10^4) were plated in several 6-cm Petri dishes, and the next day, cells were counted, and the medium in each plate was changed to that with or without 8 ng of okadaic acid per ml. On days 1, 2, 3, 4, and 6 after addition of okadaic acid, cells in triplicate plates were counted. For examination of the growth in soft agar, a total of 10^3 cells suspended in DMEM containing 0.3% Bacto agar

Abbreviation: PMA, phorbol 12-myristate 13-acetate.

(Difco) was seeded on basal agar containing DMEM and 0.5% Bacto agar. The basal and top agar both contained 0, 4, or 8 ng of okadaic acid per ml or 8 ng of okadaic acid tetramethyl ether per ml. Numbers of visible colonies of more than four or five cells were counted at 2 weeks after plating.

Northern Blot Analysis. Total RNAs were extracted from cells by the acid guanidinium thiocyanate/phenol/chloroform method (14). Total RNAs were separated in 0.9% agarose by electrophoresis and transferred to a nylon filter as described (15). Hybridization was carried out at 42°C in a solution containing 50% formamide, 0.65 M NaCl, 0.1 M sodium Pipes (pH 6.8), 5× Denhardt's solution (Denhardt's solution = 0.02% bovine serum albumin/0.02% Ficoll/0.02% polyvinylpyrrolidone), 0.1% sodium dodecyl sulfate (SDS), 5 mM EDTA, 10% dextran sulfate, salmon sperm DNA (100 μg/ml), and ^{32}P-labeled probes. An *Xho* I–*Bst*EII fragment of v-*raf* (11) and an *Eco*RI–*Nco* I fragment of *ret*-II cDNA (16) were used as probes. Filters were washed four times for 20 min each with washing solution containing 2× SSC (1× SSC = 0.15 M NaCl/0.015 M sodium citrate, pH 7.0), 0.2% sodium pyrophosphate, and 0.1% SDS at 50°C.

Western Blot Analysis. Cells were harvested and lysed in lysis buffer containing 1% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, 10% glycerol, 140 mM NaCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, and 20 mM Tris·HCl (pH 8.0) and centrifuged at 100 × *g* for 5 min, and the supernatant was collected.

Aliquots of 25 μg of each sample were applied to 8% SDS polyacrylamide gels. After polyacrylamide gel electrophoresis (PAGE), blotting of proteins from the gel onto a Durapore filter (Millipore) was carried out in a semidry blotting apparatus (Atto) for 2 hr at 2 mA/cm^2. The filters were then treated with blocking solution consisting of 5% skim milk and 1% bovine serum albumin in distilled water for 30 min. The filters were incubated overnight with the first antibody (αsp63) diluted 1:200 with Tris/saline (150 mM NaCl/10 mM Tris·HCl, pH 7.4) containing 1% gelatin and 10% goat serum (IBL, Gunma, Japan). αsp63 was rabbit antibody for the C-terminal 12 amino acids of c-*raf* (17). Then the filters were washed twice with washing buffer (Tris/saline with 0.1% Tween 20) and treated with the second antibody [biotinylated goat anti-rabbit IgG (heavy and light chain specific) (Vector Laboratories)] for 2 hr at room temperature. They were then washed twice with washing buffer, incubated with avidin-biotin complex (Vector Laboratories) for 30 min, washed four times with washing buffer, and stained with 0.05% diaminobenzidine/0.05% H_2O_2 in 50 mM Tris·HCl (pH 7.4).

Immunofluorescence Microscopy. Cells were cultured with or without okadaic acid (8 ng/ml) for 2 days on chamber slides (Nunc) and then fixed as follows. The chamber slides were washed twice with phosphate-buffered saline (PBS), and the frame of each chamber was removed. The cells on the slide were fixed in 0.1 M phosphate buffer (pH 7.4) containing 4% paraformaldehyde for 1 hr at 4°C, then washed successively with 0.1 M phosphate buffer, 70% ethanol, 95% ethanol, 90% ethanol, and PBS, and dried at 4°C. For immunofluorescence staining, the cells on the slides were incubated with PBS containing 1% bovine serum albumin (fraction V, Sigma) and 5% normal goat serum for 15 min at room temperature to mask nonspecific binding sites. The slides were drained and incubated with a rabbit antibody against human fibronectin (Cappel Laboratories, diluted 1:100) or a rabbit antibody against human tenascin (gift from Teruyo Sakakura, Tsukuba Life Science Center, RIKEN, diluted 1:100). After incubation for 30 min at room temperature, the glass slides were washed three times with cold PBS. They were then incubated with fluorescein isothiocyanate-conjugated goat antibody against rabbit IgG (Tago, diluted 1:200) for 30 min at room temperature. After washing the slides with cold PBS, the slides were counterstained with 0.01% Evans blue (Merck) and mounted in 90% nonfluorescent glycerol (Nakarai Chemicals, Kyoto) in PBS. Immunofluorescence was examined in an inverted fluorescence light microscope (18).

RESULTS

Changes of Cell Morphology. On treatment with 5–8 ng of okadaic acid per ml, transformants of activated human c-*raf* (2C-1) and *ret*-II (SIC-2) changed in morphology within 48 hr, becoming flat and showing clear contact inhibition (Fig. 1). The morphological change was dose-dependent: cell flattening increased and the frequency of criss-cross piling up decreased with increase in okadaic acid concentration in the range of 2–8 ng/ml, and at a concentration of 8 ng/ml, almost all cells became flat and showed strict contact inhibition within 48 hr. IQ7-2, a transformant of activated rat c-*raf*, showed flat morphology by okadaic acid, but the contact inhibition was not as strict as in 2C-1 or in SIC-2. However, with the transformant of human Ha-*ras*, a1-1, the change in cell morphology was only moderate; the cells showed some extent of flattening with piling up (Fig. 1). The N-*ras* transformant, Nh-1, and Ki-*ras* transformant, Kr-6, showed less

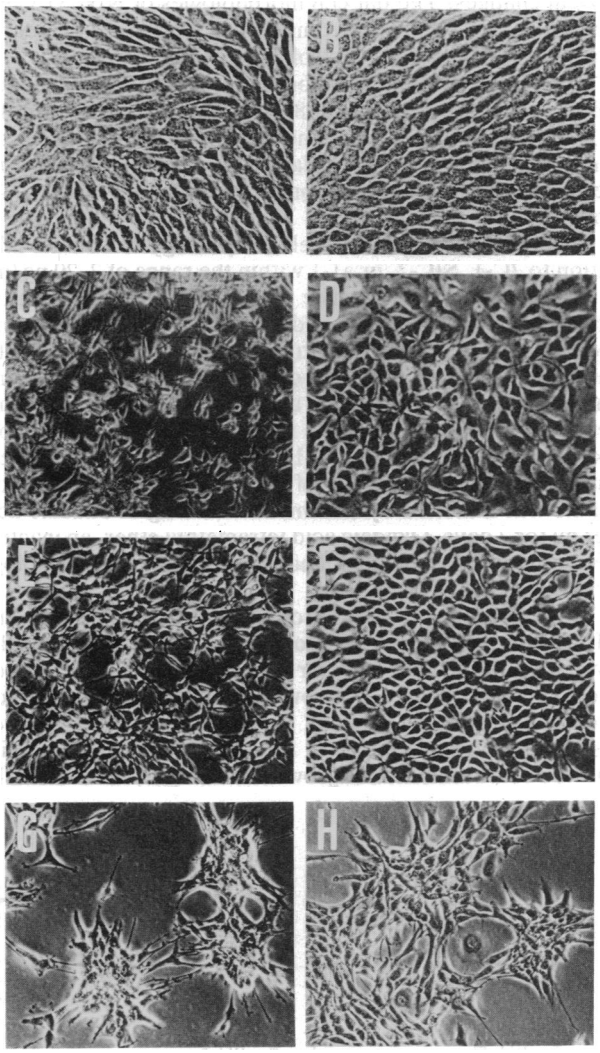

FIG. 1. Morphological changes of transformants treated with okadaic acid. NIH 3T3 transformants were cultured in the presence of 8 ng of okadaic acid per ml for 2 days. The cell morphologies of NIH 3T3 (*A* and *B*), 2C-1 (human *raf*) (*C* and *D*), SIC-2 (*ret*-II) (*E* and *F*), and a1-1 (Ha-*ras*) (*G* and *H*) cultured with okadaic acid (*B*, *D*, *F*, and *H*) or without okadaic acid (*A*, *C*, *E*, and *G*) are shown. (×70.)

Table 1. Changes in morphology and growth rate of transformants treated with okadaic acid

Clone	Exogenous DNA: Oncogene	Species	Mutation	Flat reversion
NIH 3T3	None			−*
EV-R3	c-*raf* cDNA	Rat	None	−*
IQ7-2	c-*raf*	Rat	5′ truncated	+
2C-1	c-*raf*	Human	5′ truncated	++
SIC-2	*ret*-II	Human	5′ truncated	++
a1-1	Ha-*ras*	Human	$Gly^{12} \rightarrow Val^{12}$	±
Nh-1	N-*ras*	Human	$Gly^{13} \rightarrow Arg^{13}$	−
Kr-6	Ki-*ras*	Rat	$Gly^{12} \rightarrow Cys^{12}$	−

Cells were cultured with 8 ng of okadaic acid per ml for 2–3 days and morphological changes were then examined. ++, Flattened with strict contact inhibition; +, flattened with moderate contact inhibition; ±, flattened to some extent with piling up; −, no obvious change.
*Originally flat cells.

Table 2. Colony formation on soft agar in the presence of okadaic acid

Cells	No. of colonies per plate: OA, ng/ml 0	4	8	OA-TE, 8 ng/ml
2C-1	231, 262	34, 23	0, 0	174, 212
SIC-2	522, 591	65, 101	0, 3	577, 614
a1-1	325, 333	262, 277	190, 158	349, 366
Kr-6	288, 273	237, 212	131, 171	290, 286
NIH 3T3	0, 0	0, 0	0, 0	0, 0

Samples of 10^3 cells were plated in soft agar and visible colonies were counted 14 days after plating. Each value indicates the number of colonies on each plate. OA, okadaic acid; OA-TE, okadaic acid tetramethyl ether.

morphological change on treatment with okadaic acid (Table 1). At a higher concentration of 20 ng of okadaic acid per ml, the 2C-1 and SIC-2 cells as well as other transformants and NIH 3T3 cells died within a week without showing morphological changes. The flat cell morphologies of NIH 3T3 cells and the normal rat c-*raf* transfectant were not affected by concentrations of 2–8 ng of okadaic acid per ml.

The 2C-1 and SIC-2 transformants retained their flat morphologies in the presence of 8 ng of okadaic acid per ml for >1 month. However, on removal of okadaic acid from the culture medium after 7–10 days, their flat morphology reverted to the original transformed morphology within 7–10 days.

PMA did not induce flat cell morphology or contact inhibition to 2C-1, SIC-2, or a1-1 within the range of 2–20 ng/ml.

Effect on Cell Growth. The effects of okadaic acid on the growth of NIH 3T3 cells and *raf* and *ret*-II transformants were examined (Fig. 2). Treatment with okadaic acid slightly inhibited growth of NIH 3T3 cells after 4 days of treatment. In the absence of okadaic acid, the SIC-2 transformant replicates at almost the same rate as NIH 3T3 cells without okadaic acid, whereas the 2C-1 transformant replicates at less than half this rate. Growth of the 2C-1 and SIC-2 transformants was inhibited by treatment with 8 ng of okadaic acid per ml for 2 days. Okadaic acid tetramethyl ether, an inactive derivative of okadaic acid, had no effect on the morphology or growth rate of these cells.

In the presence of 8 ng of okadaic acid per ml, 2C-1 and SIC-2 lost their ability to grow in soft agar. Interestingly, even 4 ng of okadaic acid per ml, which had little effect on their cell morphology or growth, markedly inhibited their growth in soft agar (Table 2). Kr-6 and a1-1, which showed less morphological change in the presence of okadaic acid, showed some inhibition of growth in soft agar, but even in the presence of 8 ng of okadaic acid per ml, the number of viable colonies was more than half that without okadaic acid.

Effect on Extracellular Matrix Protein Expression. Three kinds of extracellular matrix proteins, fibronectin, laminin, and tenascin, were examined by immunofluorescence microscopy. The amount of fibronectin, which decreases in several transformants (ref. 19 for review), was also reduced markedly in 2C-1 and SIC-2. After treatment with 8 ng of okadaic acid per ml for 2 days, the increase in the amount of fibronectin was clearest in SIC-2, the intensity of fluorescence being the same as that of NIH 3T3 cells (Fig. 3). The increase in the amount of fibronectin was less marked in 2C-1. In a1-1 transformants, expression of fibronectin was not markedly decreased compared with NIH 3T3 and was not increased even after addition of okadaic acid. Okadaic acid tetramethyl ether induced no apparent changes in the amount of fibronectin.

The expression levels of laminin and tenascin in transformants of 2C-1 and SIC-2 were almost the same as those in NIH 3T3 and were apparently not affected by treatment with okadaic acid.

Effect on *raf* and *ret*-II Gene Expression. Expression of c-*raf* in the flat revertant of 2C-1 induced by 8 ng of okadaic acid per ml for 3 days was the same as that before treatment with okadaic acid (Fig. 4). Since 2C-1 expresses 3.2-kilobase (kb) mRNA of activated human c-*raf*, which is the same size as NIH 3T3 mouse c-*raf* mRNA, IQ7-2 was also used for analysis of mRNA expression. The probe used for analysis of the *raf* gene expression recognizes normal c-*raf* transcripts (3.2 kb) and activated c-*raf* gene transcripts (3.0 kb). As shown in Fig. 4, neither the expression level of activated c-*raf* nor that of normal c-*raf* in IQ7-2 was affected by okadaic acid. Expression of the *ret*-II gene in SIC-2 was also not affected by the addition of okadaic acid (Fig. 4). Expression of the *ret* protooncogene in NIH 3T3 cells is very low and could not be detected under the conditions used in this experiment, as reported previously (8). Thus the mRNAs detected in the

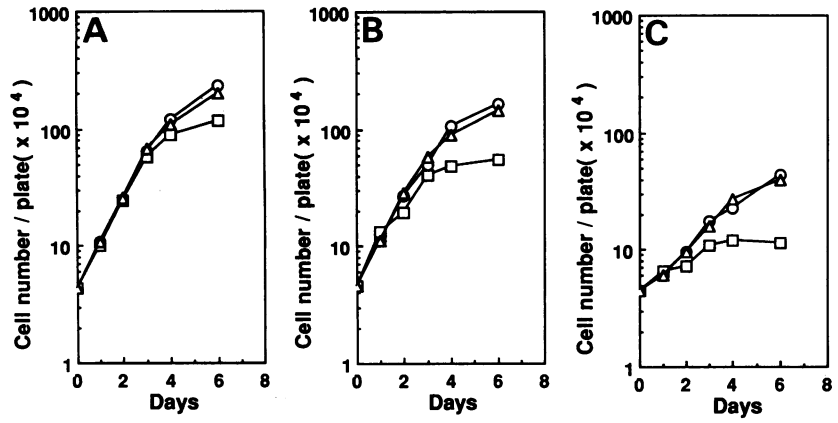

FIG. 2. Effect of okadaic acid on cell growth. Cells were cultured in 6-cm Petri dishes at a starting population of 5×10^4 cells per dish. Okadaic acid or its tetramethyl ether was added 24 hr after the plating at a concentration of 8 ng/ml. Each point indicates the average of cell numbers in three dishes. NIH 3T3 (*A*), SIC-2 (*B*), and 2C-1 (*C*) were cultured with okadaic acid (□), with okadaic acid tetramethyl ether (△), or without treatment (○).

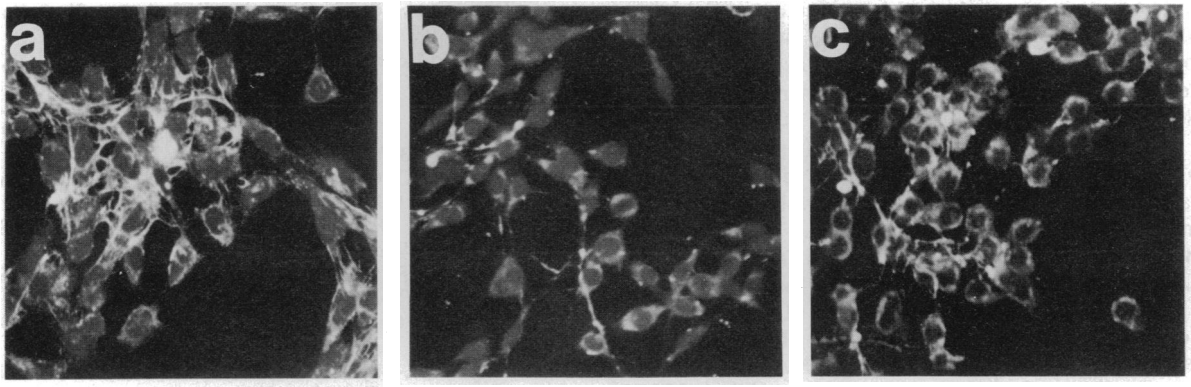

FIG. 3. Immunofluorescence staining of fibronectin after treatment with okadaic acid. NIH 3T3 cells (*a*), SIC-2 (*b*), and SIC-2 cultured 2 days with okadaic acid (8 ng/ml) (*c*).

SIC-2 transformant were the transcripts of *ret*-II. Further, no effect of okadaic acid on the copy number of *ret* oncogene was confirmed by Southern blot analysis (data not shown).

Effect on *raf* Product. To detect changes in the amount of oncogene product or its phosphorylation state in two c-*raf* transformants, we performed Western blot analysis using polyclonal antibody αsp63. Normal *raf*-encoded protein usually migrates as a doublet at a position corresponding to about 70 kDa on SDS/PAGE. The upper band corresponds to the phosphorylated product and the lower band corresponds to the nonphosphorylated product (20). These two bands were detected in NIH 3T3, 2C-1, IQ7-2, and SIC-2. No difference in the amount of normal *raf* protein before and after treatment with okadaic acid was observed in these cells (Fig. 5). Also, no apparent change existed in the ratio of the phosphorylated and nonphosphorylated *raf* products on addition of okadaic acid. In 2C-1 and IQ7-2, a third band for activated *raf* products with an expected molecular mass of 66 kDa and 68 kDa, respectively, was detected. These bands of activated *raf* products were single and we have no information about the state of phosphorylation of these activated *raf* proteins, but their amounts in these transformants were not markedly changed by okadaic acid treatment (Fig. 5).

DISCUSSION

Our results revealed that okadaic acid has activity to induce the normal phenotype of cell morphology, with contact inhibition and anchorage dependency in *ret*-II and activated *raf* transformants. We also found that expression of fibronectin by these cells was increased to the level of normal NIH 3T3 cells by treatment with okadaic acid. These changes did not seem to be associated with genetic changes, because they were reversible, and we detected no change in copy number of the activated oncogene. Okadaic acid inhibited the growth of the transformants more than that of NIH 3T3 cells. These findings indicate that okadaic acid reverses cellular transformation by inhibiting some key process(es) that triggers a series of changes in cellular transformation, such as morphological changes, suppression of contact inhibition, and fibronectin expression.

The fact that *raf* and *ret*-II transformants were more susceptible to okadaic acid than transformants of *ras* family genes indicates the existence of specificity in some oncogenes for these reversions. It is possible that signals from *raf* and *ret*-II are mediated by the same pathway that is blocked by okadaic acid, but those from *ras* families are by way of different pathways.

Another possible explanation is that these differences in susceptibility to okadaic acid of various transformants are due to differences in the degree of malignancy of these transformants. Both *raf* transformants, 2C-1 and IQ7-2, and *ret*-II transformants have relatively long doubling times, which are comparable to NIH 3T3, whereas a1-1, Nh-1, and Kr-6 grow more rapidly than NIH 3T3 cells. Possibly only slow-growing, less malignant transformants can be reverted to flat cells on treatment with okadaic acid. The expression level of activated c-*raf* is much higher in IQ7-2 than in 2C-1, as shown by Northern and Western blot analyses (Figs. 4 and 5). This may be why flat reversion was more obvious in 2C-1 than in IQ7-2. These results indicate that suppression of transformation by okadaic acid is rather common but that complete reversion to flat cells can be achieved only with cells that are in low-grade of malignancy.

Okadaic acid is a strong inhibitor of protein phosphatases 1 and 2A (5, 6); its IC_{50} values for phosphatases 1 and 2A are 500 nM and 1 nM, respectively, *in vitro* (21). In our experi-

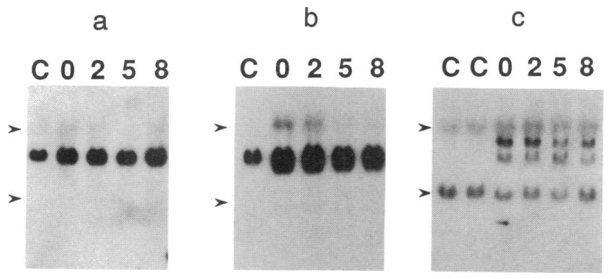

FIG. 4. Northern blot analysis of *raf* and *ret*-II transformants treated with okadaic acid. Expression of *raf* oncogene was examined in 2C-1 (*a*) and IQ7-2 (*b*) and that of *ret* oncogene was examined in SIC-2 (*c*) after culture with or without okadaic acid. Untreated NIH 3T3 cells were used as controls (C) and each number indicates the concentration of okadaic acid in ng/ml. Arrowheads indicate positions of ribosomal 28S and 18S RNA bands.

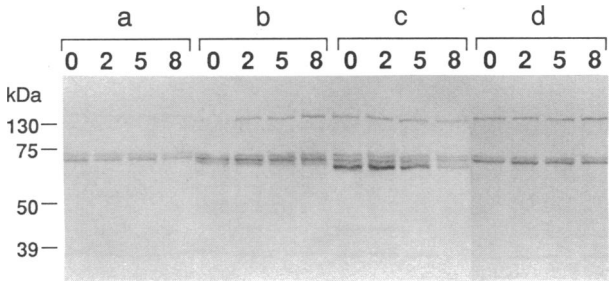

FIG. 5. Western blot analysis of *raf* transformants after treatment with okadaic acid. NIH 3T3 cells (*a*), 2C-1 (*b*), IQ7-2 (*c*), and SIC-2 (*d*) were treated with various amounts of okadaic acid and *raf* protein was stained using αsp63 polyclonal antibody. Numbers above the lanes indicate the concentrations of okadaic acid (ng/ml). Two bands with molecular masses of about 70 kDa are seen in *a*–*d*, and an additional lower molecular mass band is seen in *b* and *c*.

ments, its optimum concentration for induction of flat reversion was about 10 nM. Flat reversion is, therefore, considered to be due to its inhibition of protein phosphatases, which may result in hyperphosphorylation of some proteins. In fibroblasts, N-60, a proteolytic fragment of nucleolin, was found to show the clearest change in phosphorylation by okadaic acid (7). N-60 has been found to be a nuclear–cytoplasmic shuttle protein (22), but involvement of this protein in flat reversion is not yet known.

Skin tumors induced by 7,12-dimethylbenz[*a*]anthracene (DMBA) and okadaic acid were recently found to be associated with mutation in the Ha-*ras* codon 61 (23). The same mutation in Ha-*ras* has frequently been observed in skin tumors induced by DMBA plus PMA or PMA alone (23–25). Thus, mechanisms of the tumor-promoting activity of PMA and okadaic acid seem to be similar. On the contrary, the effects of okadaic acid *in vitro* are different from those of PMA, which enhance the cell growth rate and promote transformed phenotypes of cells (26, 27). The mode of action of okadaic acid in tumor promotion *in vivo* may be different in some ways from that *in vitro*, or it may show different effects in different tissues, such as skin epithelial cells and fibroblasts.

We showed here that a phosphatase inhibitor can revert malignant phenotype of transformants at low concentration. This suggests that phosphatase 1 or 2A is involved in a critical step in cellular transformation. There are reports that protein phosphatase 1 is involved in dephosphorylation of S6 peptide of ribosomes and in sister chromatid segregation in *Saccharomyces pombe* (28) and that protein phosphatase 2A is involved in dephosphorylation of S6 kinase II and microtubule-associated protein 2 protein kinase (29). The activity of RNA polymerase II in *Saccharomyces cerevisiae* is also regulated by protein phosphatase 1 or 2A, and a phosphatase mutant revealed a transcriptional defect (30). These findings suggest multifunctional and critical roles of protein phosphatases in normal and malignant cell growth. Very possibly a phosphatase(s) adroitly regulates the processes of interconversion of normal and malignant cells.

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