Vol. 196, No. 1, 1993 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
October 15, 1993 Pages 320-327

V-SRC-INDUCED TRANSFORMATION IS INHIBITED BY OKADAIC ACID[1]

Ruchika W. Gupta[#], Cecil K. Joseph[2] and David A. Foster[#3]

[#]Institute for Biomolecular Structure and Function and Department of Biological Sciences, The Hunter College of The City University of New York, New York 10021

Received August 31, 1993

The tumor promoter okadaic acid is a potent inhibitor of the serine/threonine protein phosphatases 1 and 2A. Addition of okadaic acid to v-Src-transformed BALB/c 3T3 cells reverted them to a flat morphology, increased fibronectin levels in the extracellular matrix, reduced saturation density, and inhibited the formation of colonies in soft agar. The ability of v-Src-transformed cells to proliferate in low serum was also inhibited by okadaic acid. These data implicate serine/threonine phosphatases in v-Src-induced transformation.

Rous sarcoma virus encodes a protein-tyrosine kinase, v-Src, which transforms cells in culture and induces tumors in animals (1). The molecular events leading from the protein-tyrosine kinase activity of v-Src to cellular transformation are poorly understood; however it has been pointed out that transformation by v-Src leads to an increase in the phosphorylation state of several proteins on serine and threonine residues as well as on tyrosine (2-4). Due to the specificity of v-Src for tyrosine residues, the increase in serine/threonine phosphorylation must arise from either the activation of serine/threonine-specific protein kinases or the inhibition of phosphatases specific for serine and threonine residues.

[1] This investigation was supported by grants from the National Institutes of Health (CA46677) and the Council for Tobacco Research (3075) to DAF, and a Research Centers in Minority Institutions (RCMI) award from the Division of Research Resources, National Institutes of Health (RRO-3037-03) to Hunter College.

[2] Present address: Memorial Sloan Kettering Cancer Center, 1275 York Ave., New York, New York 10021.

[3] To whom correspondence should be addressed: Inst. for Biomolecular Structure and Function and Dept. of Biological Sciences, The Hunter College of The City University of New York, 695 Park Avenue, New York, NY 10021.

Abbreviations: DMF, dimethylformamide; OA, okadaic acid; PP1, protein phosphatase 1; PP2A, protein phosphatase 2A; SRD, Schmidt-Rupin D.

0006-291X/93 $4.00

The serine/threonine specific protein phosphatases, which are localized in the cytosol and nucleus, have been classified into two groups, type 1 and type 2, depending upon their ability to dephosphorylate the B-subunit of phosphorylase kinase, their sensitivity to two heat- and acid-stable proteins termed inhibitor 1 and inhibitor 2, and their requirement for divalent cations (see 5 for review). PP2A , one of four major serine/threonine phosphatases characterized to date, may play a significant role in the transformation of cells by v-Src. The regulatory and catalytic subunits of PP2A have been identified as p63 and p36 respectively, two cellular proteins known to associate with the polyoma middle and small T antigens, and the SV40 small T antigen (6). The middle T antigen of polyoma virus interacts with c-Src (7) and enhances its tyrosine kinase activity (8-9). In cells transformed with polyoma middle T, antisera against c-Src was found to immunoprecipitate p36, the catalytic subunit of PP2A (6), suggesting that PP2A may play a role in the intracellular signals generated by v-Src.

It has previously been shown that OA, a potent inhibitor of PP1 and PP2A (10), can act as a tumor promoter (11). However, it has also been reported that OA reverts the transformation of cells by an activated Raf-1 (12). We previously demonstrated that v-Src activates both Raf-1-dependent and Raf-1-independent signalling pathways (13-14). We also demonstrated that v-Src-induced transformation was inhibited by a dominant negative Raf-1 mutant (15). Since OA has been reported to revert transformation induced by v-Raf (12), we examined the effect of OA upon v-Src-transformed Balb/c 3T3 fibroblasts. We report here that treatment with OA inhibits several transformation-related phenotypes induced by v-Src.

MATERIALS AND METHODS

Cells and Culture Conditions
SRD and LA90 transformed BALB/c 3T3 cells (kindly provided by J. Brugge) were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% calf serum. LA90 cells are non-transformed at 39.5°C and transformed at 35°C (16, 17). Cell morphology was examined by phase contrast microscopy. The rate of cell proliferation was determined by seeding $3x10^5$ SRD cells per 35mm dish in DMEM containing 20% or 1% calf serum and the appropriate drug. The next day (day 1), the medium was changed, and fresh drugs were added on days 1 and 3.

Materials
OA was obtained from Calbiochem and diluted in DMF. Fluoromount-G and fluorescein-labelled goat-anti-mouse IgM were from Southern Biotechnology. All other reagents were obtained from Sigma.

Colony Formation in Soft Agar
$1x10^3$ SRD cells were suspended in DMEM containing 0.35% Bacto agar and seeded onto basal agar containing 0.6% Bacto agar in DMEM. The basal and top agar both contained the indicated concentration of drug. Cells were incubated at 37°C and fed with 1mL of fresh DMEM containing 10% calf serum and drugs at the appropriate concentration every third day. After 10 days, viable colonies were stained overnight with 1mL of 40% p-iodonitrotetrazolium violet (INT) and visible colonies were counted the next day.

Immunofluorescence Microscopy
LA90 cells were grown in Lab-Tek (Nunc) culture chambers as described previously

(12) with the following modifications. The cells were fixed in 3.7% paraformaldehyde for one hour and blocked with 1% bovine serum albumin (BSA) in phosphate-buffered saline (PBS) for 15 minutes at room temperature to block non-specific binding sites. They were then incubated for 30 minutes at room temperature with monoclonal anti-cellular fibronectin (Sigma, diluted 1:200), washed three times in PBS and incubated with fluorescein-labelled goat-anti-mouse IgM (Southern Biotechnology, diluted 1:100) for 30 minutes at room temperature. After washing in cold PBS, the slides were mounted in flouromount-G and immunofluorescence was examined under an inverted fluorescence light microscope.

RESULTS

OA Reverts v-Src-Transformed Cells to a Flat, Non-Transformed Morphology

BALB/c 3T3 fibroblasts transformed with the SRD strain of Rous sarcoma virus were incubated for 20 hours alone, with 20 nM OA, or with 0.1% DMF, the solvent for OA. Untreated cells and cells treated with DMF showed a high degree of piling-up and loss of contact-inhibition, as well as a refractile, rounded morphology characteristic of transformed cells (Fig. 1A and 1B). Incubation with OA, however, resulted in the appearance of a flat, non-transformed morphology similar to that of the parental BALB/c 3T3 cell line (Fig. 1C and 1D). Removal of OA from the SRD

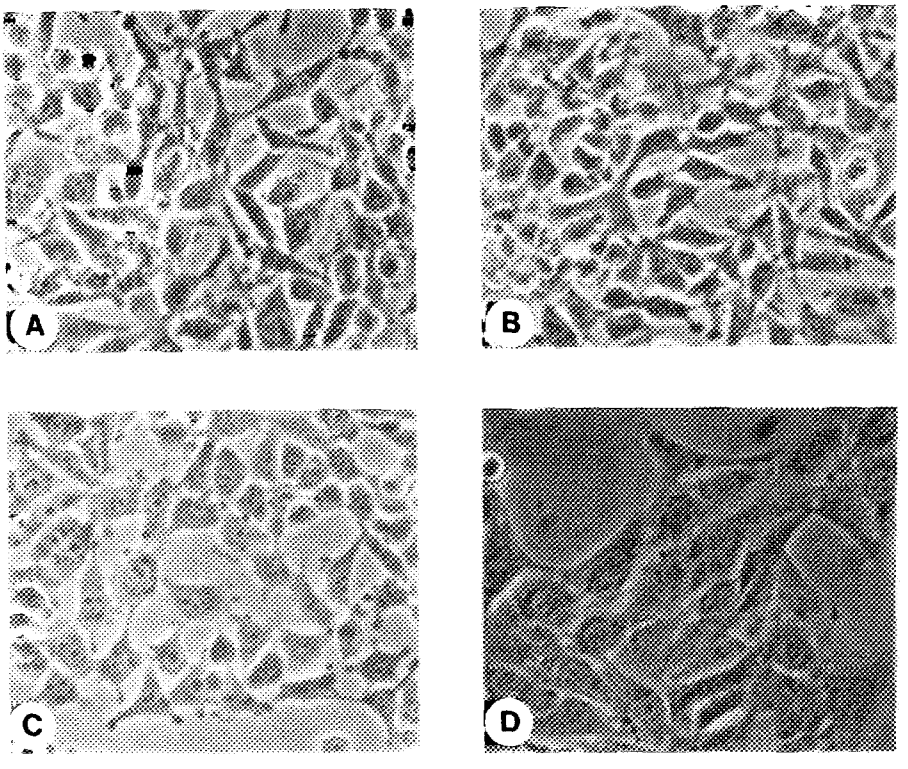

Fig. 1. OA reverts v-Src-transformed cells to a flat, non-transformed morphology. SRD (A-C) and BALB (D) cells were cultured alone (A and D), with 0.1% DMF (B) or 20nM OA in 0.1% DMF (C). After 20 hours, the cells were photographed under a phase contrast microscope (200x magnification).

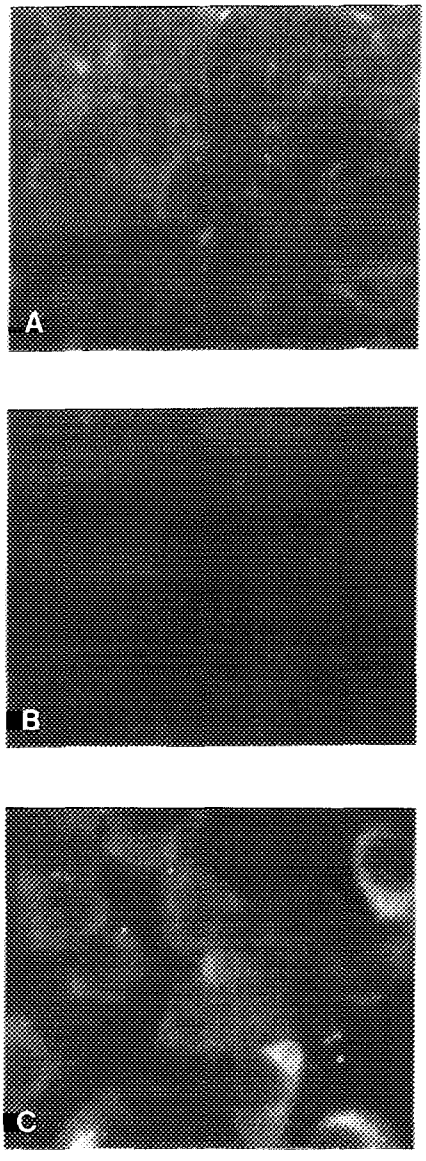

Fig. 2. OA increases the level of fibronectin in v-Src-transformed cells. LA90 cells were incubated for 2 days at 39.5°C (A) or 35°C (B and C) in the absence (A and B) or presence (C) of 20nM OA. Fibronectin was detected as described under "Materials and Methods" and cells were examined at 400x magnification with a fluorescence microscope.

cells resulted in the re-appearance of the transformed morphology within one day (data not shown). These data suggest that OA inhibits the morphological changes that accompany v-Src-induced transformation.

OA Increases Fibronectin Levels in v-Src-Transformed Cells

Fibronectin is an extracellular matrix protein that plays a central role in cell adhesion, and its level in transformed cells is often low with respect to that in normal cells (see

18 for review). We therefore examined the effect of OA upon the level of fibronectin in v-Src-transformed cells. To avoid artifacts arising from variability between different cell lines in the secretion of the extracellular matrix, we used BALB/c 3T3 cells transformed with LA90, a temperature-sensitive strain of Rous sarcoma virus (described by 16 and 17). LA90 transformed BALB/c 3T3 cells were grown at either the permissive (35°C) or non-permissive (39.5°C) temperature for v-Src. LA90 transformed cells cultured at the permissive temperature express less fibronectin than do those grown at the non-permissive temperature (Fig. 2A and 2B). Incubation with OA, however, resulted in an increase in the level of fibronectin in LA90 cells maintained at the permissive temperature (Fig. 2B and 2C). These data further suggest that OA inhibits transformation-related phenotypes induced by v-Src.

OA Inhibits the Ability of v-Src-Transformed Cells to Reach High Saturation Densities and to Grow Under Low-Serum Conditions

Transformed cells often overcome the density-dependent growth arrest that is characteristic of normal cells and grow to high saturation densities (19). The effect of OA upon the saturation density of v-Src-transformed cells was therefore examined. SRD cells were cultured in growth medium supplemented with 20% serum either alone, or in the presence of DMF or OA. Untreated and DMF treated SRD cells grew rapidly and reached a high cell density that was maintained for two days (Fig. 3A). In contrast, SRD cells incubated with OA had a slower rate of proliferation and reached a lower saturation density that was similar to that observed

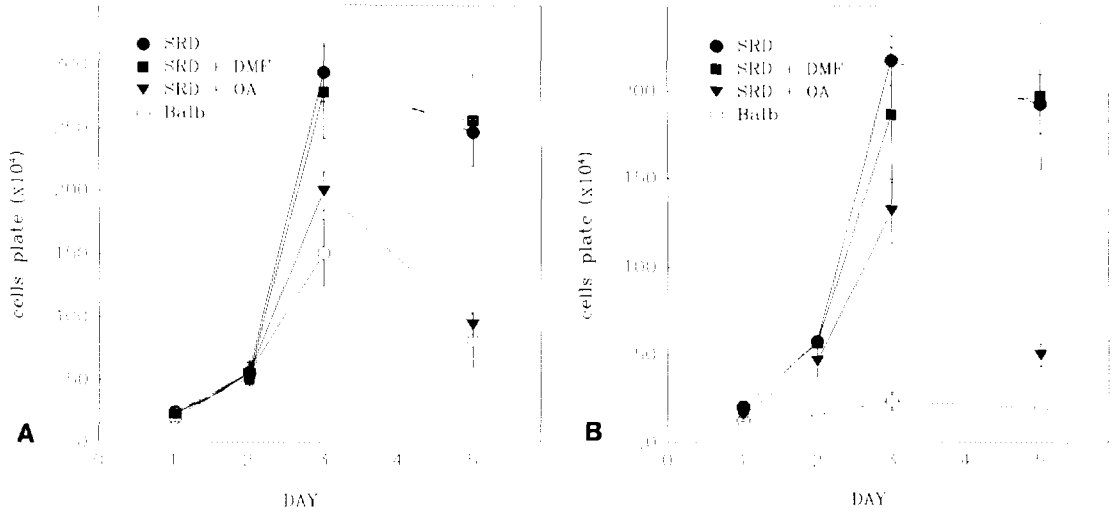

Fig. 3. OA inhibits the ability of v-Src-transformed cells to reach high saturation densities and to grow in low serum.

$3x10^5$ SRD (filled symbols) or BALB/c 3T3 (open circle) cells were seeded per 35mm tissue culture dish and incubated with DMEM supplemented with 20% (A) or 1% (B) calf serum. Cells were either untreated (circles), or incubated with 0.1% DMF (squares) or 20nM OA (triangles). On the indicated days, cells were trypsinized and counted with a hemocytometer. Values are the means of triplicate samples for SRD cells, and of duplicate samples for BALB/c 3T3 cells.

for the parental BALB/c 3T3 cells. Furthermore, both the parental BALB/c 3T3 cells and SRD cells incubated with OA declined in number after reaching maximal cell density on the third day. Thus, the presence OA caused SRD cells to behave like the non-transformed parental BALB/c 3T3 cells with regard to growth rate and saturation density.

Transformed cells also often have a reduced requirement for serum growth factors (20). We therefore examined the effect of OA upon the growth of v-Src-transformed cells under low serum conditions. SRD cells were cultured in growth medium supplemented with 1% serum either alone, with 0.1% DMF or with 20nM OA. Untreated and DMF treated SRD cells grew rapidly and reached a high cell density by the third day (Fig. 3B). In contrast, the parental BALB/c 3T3 cells were completely growth-arrested under these conditions. SRD cells incubated with OA were still able to proliferate under low serum conditions, but with a slower growth rate and lower saturation density than untreated SRD cells or SRD cells incubated with DMF. This decrease in the growth rate and saturation density can not be explained by an inability of the cells to proliferate in the presence of OA since cells could be grown in the presence of OA for at least 10 days (data not shown). Furthermore, removal of OA from the growth medium resulted in the reappearance of the transformed morphology (data not shown). Thus, OA inhibits the ability of v-Src-transformed cells to overcome density-dependent growth arrest and inhibits their ability to grow under low serum conditions.

OA Inhibits Anchorage-Independent Growth by v-Src-Transformed Cells

Perhaps the most stringent transformation-related phenotype in culture is the ability to grow in suspension. Normal fibroblasts must attach to a solid matrix in order to

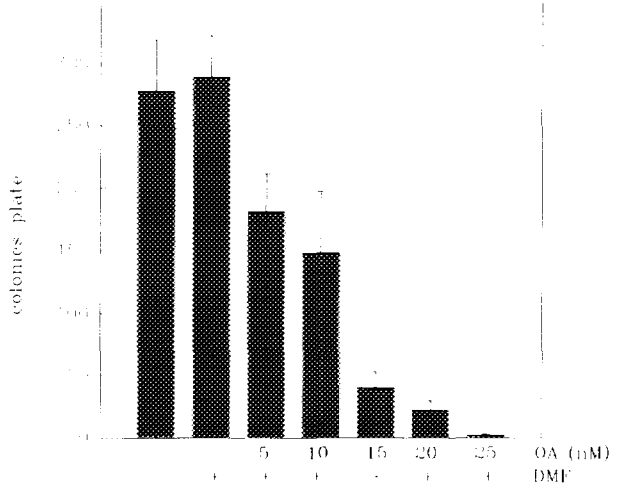

Fig. 4. **OA inhibits anchorage-independent growth by v-Src-transformed cells.** $1x10^3$ SRD cells were suspended in Bacto-agar as described under "Materials and Methods" with the indicated concentration of OA and/or 0.1% DMF. Colonies were counted after 11 days. Values are the mean of at least 4 independent samples.

proliferate. In contrast, transformed fibroblasts can proliferate in suspension without attachment to a solid surface (21). We therefore examined the effect of OA upon the ability of v-Src-transformed cells to grow in semi-solid medium. SRD cells were suspended in soft agar alone, with DMF, or with OA. There was no difference between the number of colonies formed by the untreated cells and cells incubated with DMF (Fig. 4). Treatment with OA, however, led to a concentration-dependent decrease in colony formation by SRD cells (Fig. 4). Thus, OA also inhibits anchorage-independent growth induced by v-Src-transformed cells. The effect of OA on v-Src-induced colony formation or the other transformation-related parameters is not likely due to a direct effect of OA upon v-Src since OA had no effect upon either v-Src protein levels or the phosphorylation state of v-Src upon treatment with OA (data not shown).

DISCUSSION

Transformation of cells by v-Src is the result of a constitutively activated protein-tyrosine kinase (1). The protein-tyrosine kinase activity of v-Src initiates multiple intracellular signalling mechanisms including the activation of protein kinases that phosphorylate proteins on serine and threonine (13, 17). Two serine/threonine kinases, protein kinase C and Raf-1, have been shown to be required for v-Src-induced intracellular signals (13, 17, 22). The regulation of intracellular signals mediated by serine/threonine kinases also involves dephosphorylation by phosphatases specific for serine and threonine. We report here that OA, an inhibitor of PP1 and PP2A, reverts v-Src-transformed fibroblasts to a flat morphology, increases the level of fibronectin in the extracellular matrix, inhibits their ability to overcome density-dependent growth arrest and to grow in low serum, and blocks colony formation in soft agar. Taken together, these data suggest that OA reverses v-Src-induced transformation.

While our results demonstrate that OA can block the appearance of several transformation-related phenotypes in cells expressing v-Src, OA has also been shown to be a powerful tumor promoter in two-stage carcinogenesis experiments (11). Unlike other tumor promoters, such as phorbol esters which activate and then down-regulate protein kinase C, OA inhibits serine/threonine phosphatase activity (10). This suggests that serine/threonine phosphatases may promote or suppress tumor formation depending upon the cell type or transforming agent. PP1 and PP2A, which are inhibited by OA exhibit extreme evolutionary conservation (5). Therefore, these phosphatases must play critical roles in the cell. The effect of OA on v-Src-induced transformation suggests that PP1 and/or PP2A are important mediators of intracellular signals activated by protein-tyrosine kinases.

It has been demonstrated that OA also blocks transformation by an activated Raf-1 (12). Since OA prevents transformation by both v-Src and activated Raf-1, PP1 and PP2A may be acting on a signalling intermediate that is common to both v-

Src- and Raf-1- mediated intracellular signals. Whether the same OA-sensitive phosphatase is required for transformation by v-Src and activated Raf-1, and whether this phosphatase is required in a common intracellular signalling mechanism activated by these oncogenic stimuli remains to be determined.

Acknowledgments: We thank Marcello Curto and Qun Zang for comments on the manuscript, and Dr. William Cohen for use of his flourescence microscope. This investigation was supported by grants from the National Institutes of Health (CA46677), The Professional Staff Congress of the City University of New York (661151), and the Council for Tobacco Research (3075) to DAF, and a Research Centers in Minority Institutions (RCMI) award from the Division of Research Resources, National Institutes of Health (RRO-3037-03) to Hunter College.

REFERENCES

1. Jove, R. and Hanafusa, H. (1987) Ann. Rev. Cell Biol. **3**:31-56.
2. Cooper, J.A. and Hunter, T. (1981) Mol. Cell Biol. **1**:165-178.
3. Decker, S. (1981) Proc. Natl. Acad. Sci. USA **78**:4112-4115.
4. Sefton, B.M., Hunter, T., Beemon, K., and Eckhart, W. (1980) Cell **20**, 807-816.
5. Cohen, P. (1989) Annu. Rev. Biochem. **58**:453-508.
6. Pallas, D.C., Sharick, L.K., Martin, B.L., Jaspers, S., Miller, T.B., Brautigan, D.L., Roberts, T.M. (1990) Cell **60**:167-176.
7. Courtneidge, S.A., and Smith, A.E. (1983) Nature **303**:435-439.
8. Bolen, J.B., Thiele, C.J., Israel, M.A., Yonemoto, W., Lipsich, L.A., and Brugge, J.S. (1988) Cell **38**:767-777.
9. Courtneidge, S.A. (1985) EMBO J. **4**:14771-1477.
10. Bialojan, C., and Takai (1988) Biochem. J. **256**:283-290.
11. Suganuma, M., Fujiki, H., Suguri, H., Yoshizawa, S., Hirota, M., Nakayasu, M., Ojika, M., Wekamatsu, K., Yamado, K., and Sugimura, T. (1988) Proc. Natl. Acad. Sci. USA **85**:1768-1771.
12. Sakai, R., Ikeda, I., Kitani, H., Fujiki, H., Takaku, F., Rapp, U., Sugimura, T., and Nagao, M. (1989) Proc. Natl. Acad. Sci. USA **86**:9946-9950.
13. Qureshi, S.A., Rim, M., Bruder, J., Kolch, W., Rapp, U., Sukhatme, V.P., and Foster, D.A. (1991b) J. Biol. Chem. **266**:20594-20597.
14. Qureshi, S.A., Alexandropoulos, K., Joseph, C.K., Rim, M., Bruder, J., Rapp, U.R., and Foster, D.A. (1992) J. Biol. Chem. **267**:17635-17639.
15. Qureshi, S.A., Joseph, C.K., Hendrickson, M., Song, J., Gupta, R., Bruder, J., Rapp, U., and Foster, D.A. (1993) Biochem. Biophys. Res. Comm. **192**:969-975.
16. Gray, G.M. and Macara, I.G. (1988) J. Biol. Chem. **263**:10714-10719.
17. Qureshi, S.A., Joseph, C.K., Rim, M., Maroney, A., and Foster, D.A. (1991a) Oncogene **6**:995-999.
18. Ruoslahti, E. (1988) Ann. Rev. Biochem. **57**:375-413.
19. Holley, R.W. and Kiernan, J.A. (1968) Proc. Natl. Acad. Sci. USA **60**:300-304.
20. Dulbecco, R. (1970) Nature **227**:802-806.
21. Macpherson, I. and Montagnier, L. (1964) Virology **23**:291-294.
22. Spangler, R., Joseph, C.K., Qureshi, S.A., Berg, K.L., and Foster, D.A. (1989) Proc. Natl. Acad. Sci. USA **86**:7017-7021.