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Induction of morphological change by tyrosine kinase inhibitors in Rous sarcoma virus-transformed rat kidney cells

Kazuo Umezawa, Kuro Tanaka, Takashi Hori, Satoko Abe, Ryuichi Sekizawa and Masaya Imoto

Department of Applied Chemistry, Faculty of Science and Technology, Keio University, 3-14-1 Hiyoshi, Kohoku-ku, Yokohama 223, Japan

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Erbstatin and methyl 2,5-dihydroxycinnamate, related tyrosine kinase inhibitors, induced a morphological change in temperature-sensitive Rous sarcoma virus-transformed rat kidney (RSV^{ts}-NRK) that brought the cells close to the morphology of their normal counterpart. Erbstatin did not change the morphology of normal or Kirsten sarcoma virus-transformed rat kidney cells. Erbstatin also inhibited morphological transformation of RSV^{ts}-NRK cells induced by a shifting in temperature. Actin stress fibres were observed only in normal cells and not in transformed cells. Erbstatin induced stress fibre organization in transformed cells. Erbstatin and methyl 2,5-dihydroxycinnamate increased fibronectin gene expression in RSV-transformed cells. Thus, tyrosine kinase inhibitors induced normal phenotypes specifically in v-src-expressing cells.

Tyrosine kinase; Erbstatin; Src; Cytoskeleton; Morphology; Fibronectin

1. INTRODUCTION

The src oncogene product is a phosphoprotein, $p60^{src}$, with tyrosine kinase activity [1]. Genetic analysis has shown that the tyrosine kinase activity of $p60^{src}$ plays an essential role in the process of transformation [2]. Membrane association of $p60^{src}$ may also be important for transformation [3], and more recently, the active src gene product was shown to be associated with the Triton X-100-resistant cytoskeletal structure in chicken fibroblasts, while non-transforming src proteins including $p60^{c-src}$ were only found in soluble fractions [5]. Therefore, rather direct contribution of the src protein to the cellular cytoskeleton and morphology has been suggested.

Erbstatin has been isolated from *Streptomyces* as an inhibitor of tyrosine kinase [6]. It inhibited epidermal growth factor (EGF) receptor- and v-src product-associated tyrosine kinase in cell culture [7]. Recently, it was shown to delay the EGF-induced DNA synthesis in quiescent normal rat kidney cells without showing irreversible toxicity [8]. Methyl 2,5-dihydroxycinnamate, a stable analogue of erbstatin, showed more prominent inhibition of the S-phase induction [8]. In the present study we employed these tyrosine kinase inhibitors to examine the role of v-src tyrosine kinase in the expression of cellular morphology, cytoskeleton and fibronectin.

Correspondence address: K. Umezawa, Department of Applied Chemistry, Faculty of Science and Technology, Keio University, 3-14-1 Hiyoshi, Kohoku-ku, Yokohama 223, Japan

2. MATERIALS AND METHODS

2.1. *Materials*

Erbstatin [6], methyl 2,5-dihydroxycinnamate [8], and 5'-*O*-methylerbstatin [9] shown in Fig. 1 were prepared as described before. Rat kidney cells transformed by a temperature-sensitive mutant of Rous sarcoma virus (RSV^{ts}-NRK cells) were kindly supplied by Dr M. Yoshida, University of Tokyo [10]; and the temperature-sensitive Kirsten sarcoma virus-infected normal rat kidney cells (K-ras^{ts}-NRK cells) by Dr T.Y. Shih, NIH, Bethesda [11]. A human fibronectin cDNA clone was isolated from the cDNA library of human breast carcinoma HS578T cells [12]; and a β-actin gene fragment, pRβAc3'ut [13], was kindly supplied by Dr K. Tokunaga, Chiba Cancer Center Research Institute.

2.2. *Methods*

2.2.1. Cell culture

RSV^{ts}-NRK cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% calf serum and antibiotics at 33°C. The cells were inoculated at 2×10^4/well with 1 ml medium in a 12-well plate, and incubated 1 or 2 days at 33 or 39°C. Then, chemicals were added and after the indicated times cells were photographed under a phase-contrast microscope. When erbstatin was added, the medium was changed to DMEM containing 2% calf serum.

2.2.2. Fluorescence staining of actin filament

Cells grown on cover glasses were fixed with 3.5% paraformaldehyde in PBS for 20 min at room temperature. After a brief rinse in PBS, fixed cells were exposed to acetone at −20°C for 5 min. The cover glasses were washed with Ca^{2+}, Mg^{2+}-free PBS and incubated with rhodamine-conjugated phalloidin at 37°C for 20 min. Cells thus treated were sealed in glycerin buffer and examined by fluorescence microscopy (Axiovert: Zeiss).

2.2.3. Northern blotting

Total RNA was extracted from sub-confluent monolayers by the guanidine hydrochloride and cesium chloride method [14]. About 100

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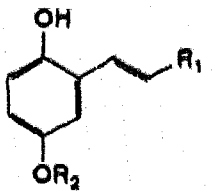

	R_1	R_2
erbstatin	NHCHO	H
methyl 2,5-dihydroxy-cinnamate	COOMe	H
5'-O-methylerbstatin	NHCHO	Me

Fig. 1. Erbstatin analogues.

μg of RNA was extracted from the cells in a 150-mm plate. Transfer of RNA from electrophoresis gels containing formaldehyde to nitrocellulose membranes and hybridization to the fibronectin or β-actin probe were carried out as described by T. Maniatis et al. [15].

3. RESULTS

RSV^{ts}-NRK cells are small and triangular at the permissive temperature (33°C), as shown in Fig. 2A. Incubation with 0.6 μg/ml of erbstatin for 8 h changed the morphology to the round and flattened shape shown in Fig. 2B. This morphological change began 4 h after the chemical addition and lasted for about 15 h, after which cells resumed their transformed morphology. Therefore, the effect of erbstatin is apparently reversible. The morphological change was observed with erbstatin at 0.3–3 μg/ml, while at 10 μg/ml the inhibitor appeared to be toxic, as evidenced by cellular shrinkage. Methyl 2,5-dihydroxycinnamate at 0.3–3 μg/ml also induced the same morphological change as erbstatin (data not shown). The altered morphology by tyrosine kinase inhibitors was not exactly the same as the normal morphology of RSV^{ts}-NRK cells at the non-permissive temperature (39°C, Fig. 3A), however, cells exposed to either inhibitor showed a flattened shape.

5'-*O*-Methylerbstatin, which does not inhibit tyrosine kinase even at 30 μg/ml, did not induce the

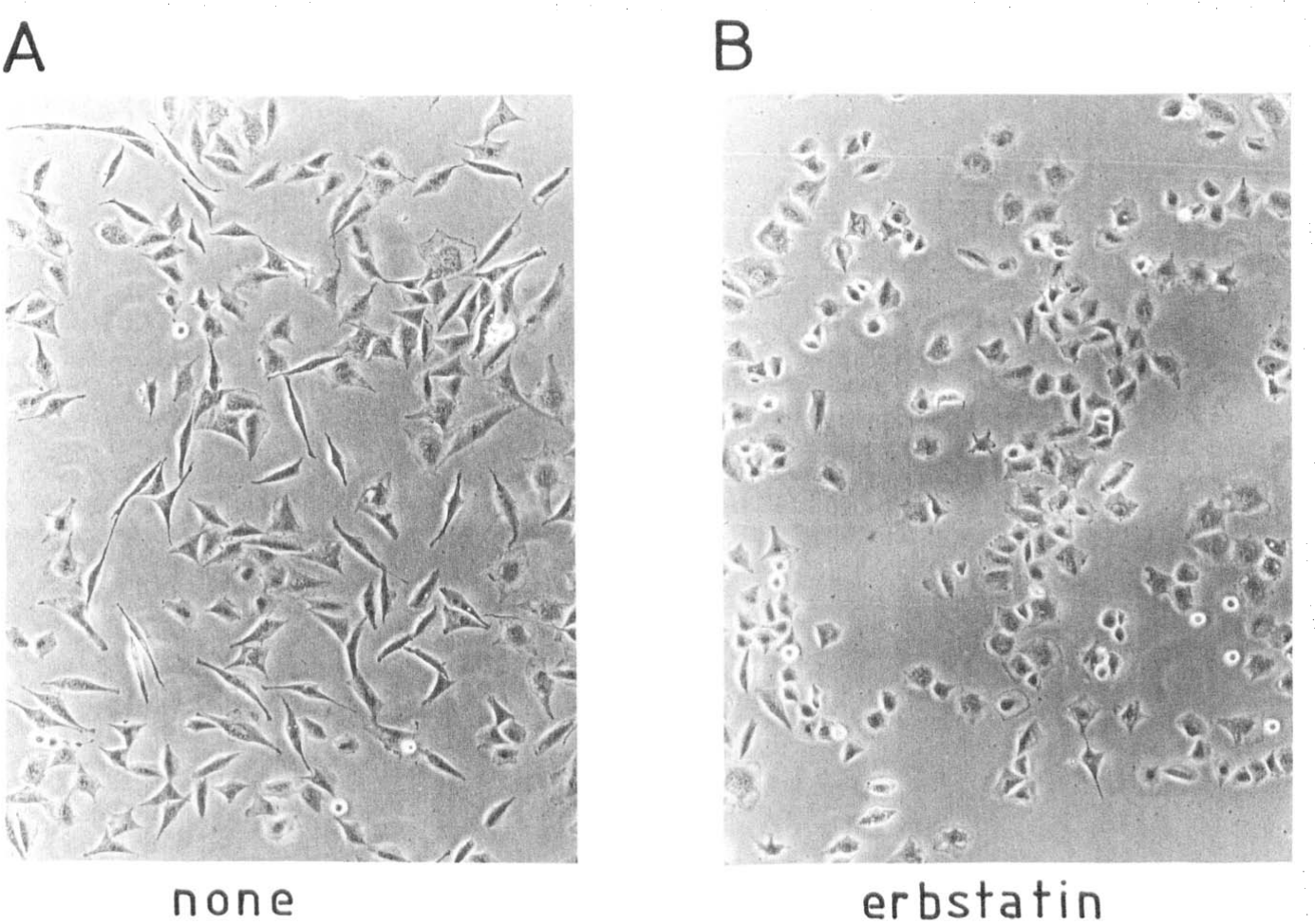

Fig. 2. Induction of morphological change by erbstatin in RSV^{ts}-NRK cells. RSV^{ts}-NRK cells were incubated at 33°C for 8 h without (A) or with (B) 0.6 μg/ml of erbstatin and photographed under a phase-contrast microscope.

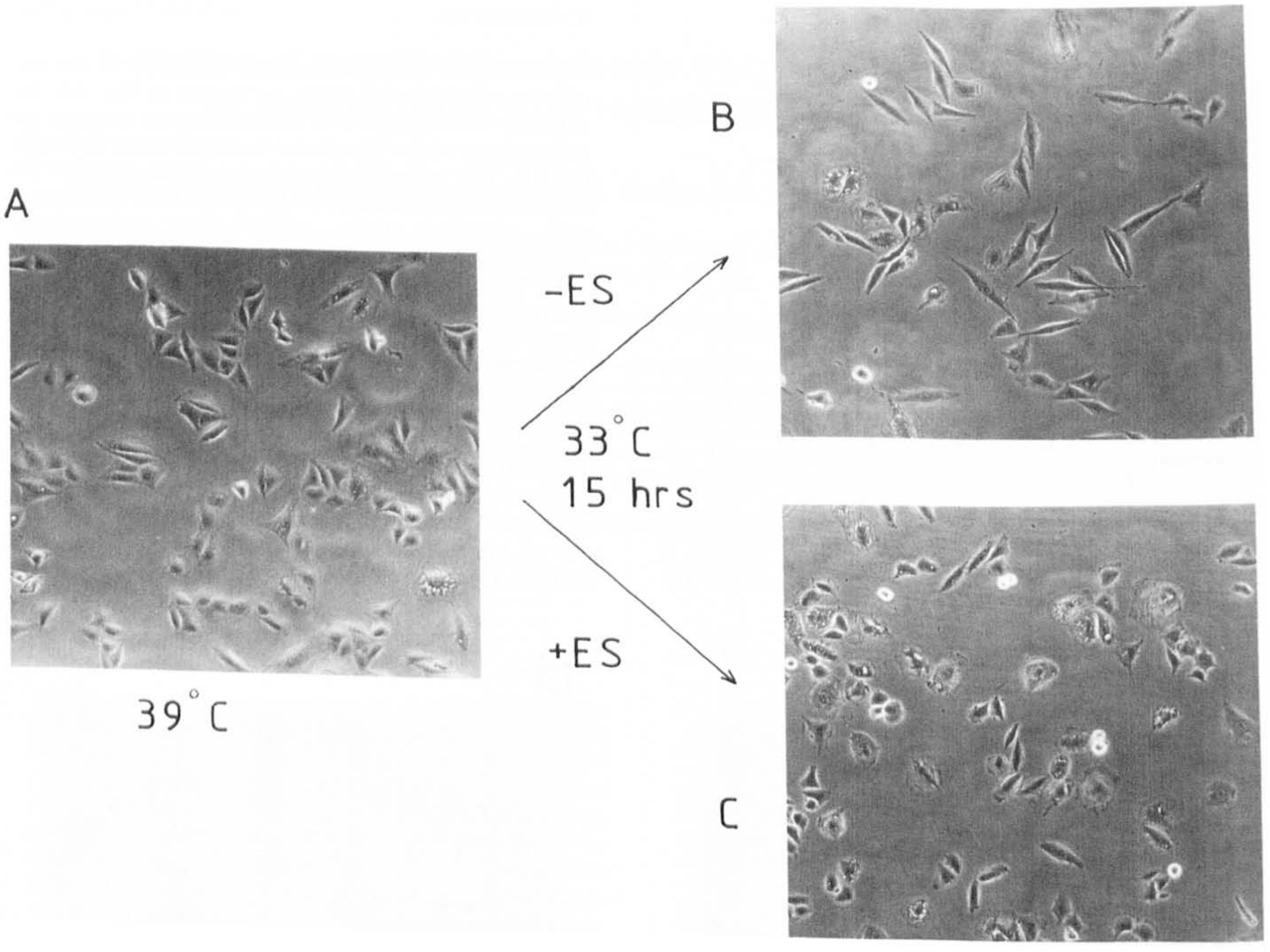

Fig. 3. Inhibition of morphological transformation by erbstatin in RSV^{ts}-NRK cells. Cells were incubated at 39°C for 2 days (A) and then transferred to a 33°C incubator without (B) or with (C) 1 μg/ml erbstatin and incubated for a further 15 h.

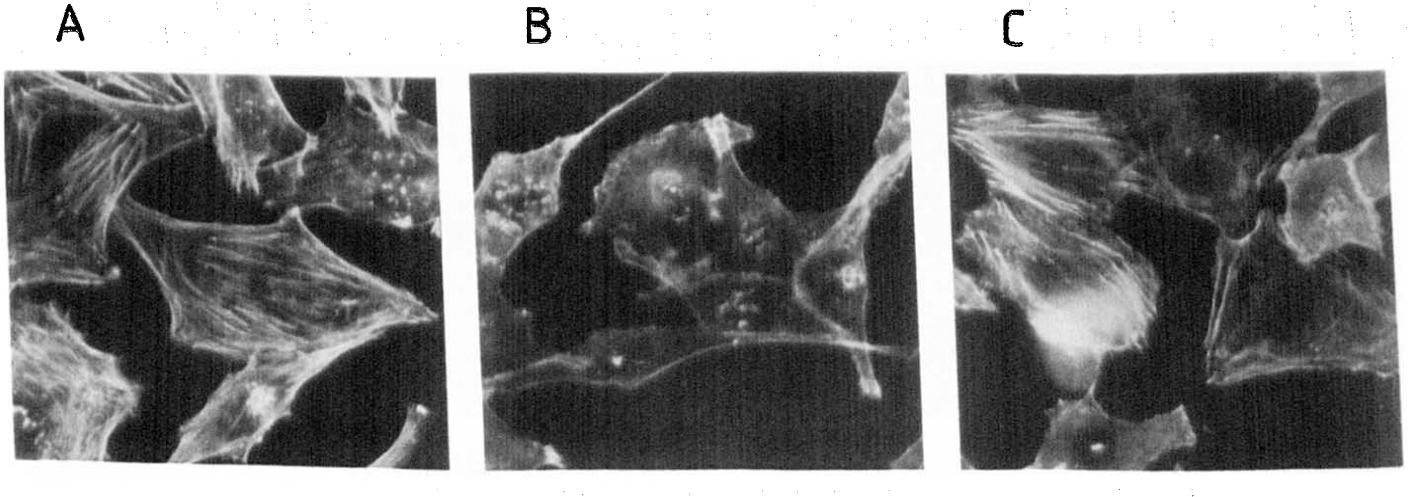

Fig. 4. Induction of actin stress fibre organization by erbstatin in RSV^{ts}-NRK cells. Cells were incubated for 8 h at 39°C (A) or at 33°C without (B) or with 0.6 μg/ml of erbstatin (C).

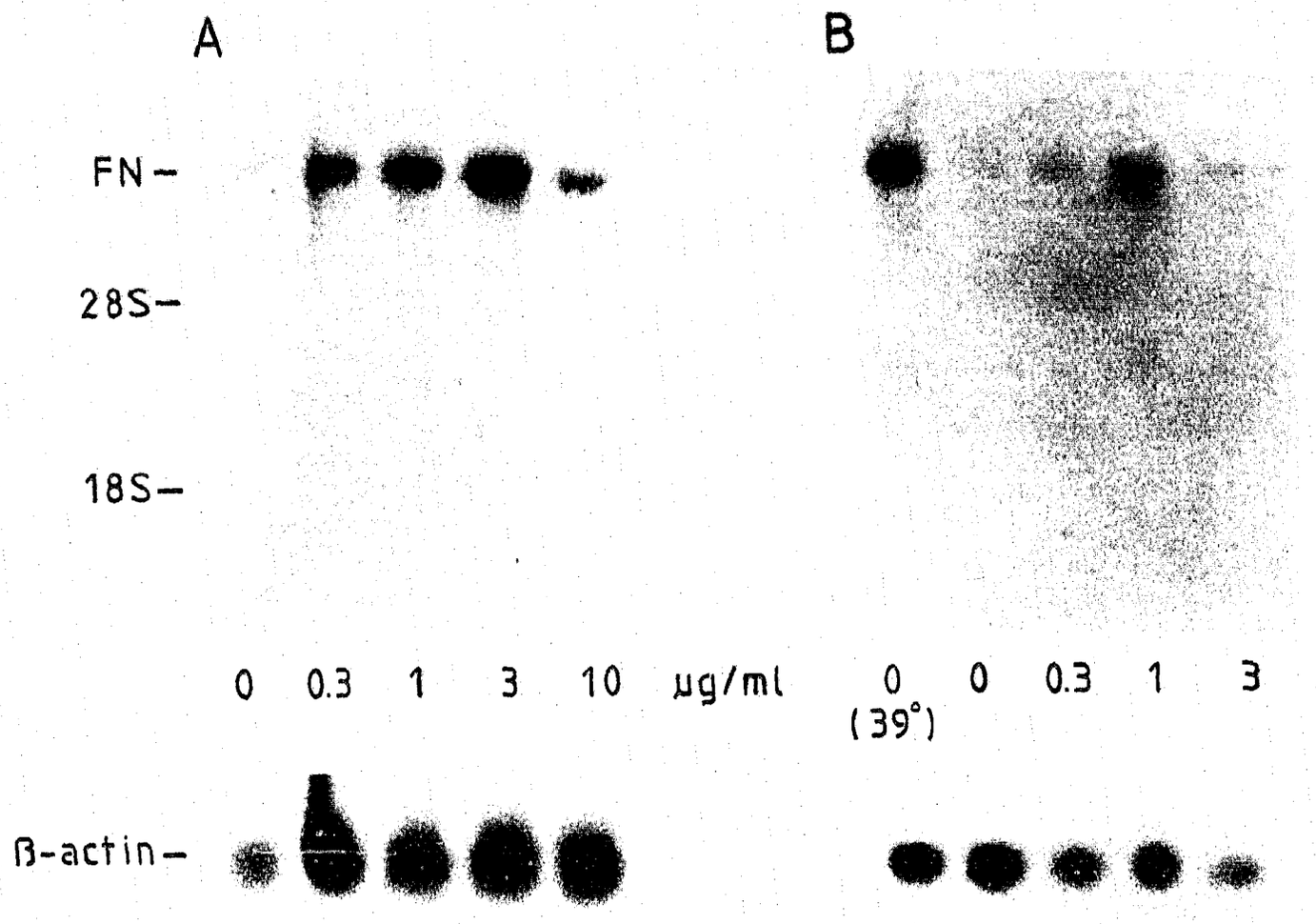

Fig. 5. Enhancement of fibronectin gene expression by erbstatin in RSV^{ts}-NRK cells. Cells were incubated at 33°C for 15 h with erbstatin (A) or methyl 2,5-dihydroxycinnamate (B). Then, total RNA was extracted, and an aliquot (10 µg) was electrophoresed and hybridized with the fibronectin or β-actin probe. RNA was also extracted from cells cultured at 39°C. The results represent two similar experiments.

morphological change. Erbstatin at 1 µg/ml did not change the morphology of RSV^{ts}-NRK cells cultured at the non-permissive temperature, and it also had no effect on the morphology of temperature-sensitive Kirsten sarcoma virus-infected rat kidney cells at the permissive temperature. Thus, the morphological effect was observed specifically for tyrosine kinase inhibitors, and in src-expressing cells.

By shifting of the temperature from 39°C to 33°C, cells can be transformed. The morphological transformation was observed within 15 h as shown in Fig. 4B. Addition of 1 µg/ml of erbstatin inhibited this transformation, and cells remained flattened, as shown in Fig. 4C. Methyl 2,5-dihydroxycinnamate also inhibited the transformation, while 5′-*O*-methylerbstatin did not (data not shown).

Actin stress fibre organization was almost completely lost in the transformed state in RSV^{ts}-NRK cells, while it was clearly seen in the normal cell state, as shown in Fig. 5A and B. Addition of 1 µg/ml erbstatin induced stress fibre organization in the cells at 33°C, as shown in Fig. 5C, although it was not as extended as in Fig. 5A. 5′-*O*-methylerbstatin again did not induce the cytoskeletal organization.

Fibronectin gene expression is usually less active in transformed cells than in normal cells, and this was previously shown to be the case for RSV^{ts}-NRK cells [16]. As shown in Fig. 6A and B, erbstatin and methyl 2,5-dihydroxycinnamate increased the level of fibronectin mRNA in transformed RSV^{ts}-NRK cells, while they did not change β-actin mRNA expression markedly.

4. DISCUSSION

Two related tyrosine kinase inhibitors induced the morphological and cytoskeletal change but their inactive analogue did not. Also, erbstatin showed no effect on normal or ras-transformed cells. Therefore, the p60^{src} tyrosine kinase activity is considered to be involved in the mechanism of cytoskeletal organization.

Erbstatin inhibits EGF receptor tyrosine kinase with an IC_{50} of about 0.2 μg/ml in vitro, and inhibits growth of RSV^{ts}-NRK cells at about 5 μg/ml either at 33°C or 39°C. Therefore, erbstatin induces normal phenotypes at lower concentrations than those showing cytotoxicity. On the other hand, a higher concentration (12.5 μg/ml) was required to inhibit autophosphorylation of the src protein (data not shown). However, autophosphorylation may not reflect essential tyrosine kinase activities of the src protein.

The morphological change induced by erbstatin was almost complete within 4 h, which is slightly faster than the change induced by the temperature shift. This may be because it takes time to inactivate $p60^{src}$ at the non-permissive temperature. The morphological effect of erbstatin was reversible even when the chemical was present in the medium, probably because of inactivation of erbstatin. In fact, the inhibitor has been shown to be unstable in serum [17].

Erbstatin did not induce normal phenotypes in wild RSV-NRK or RSV-NIH3T3 cells. Possibly, the $p60^{src}$ tyrosine kinase activity would be much higher in these cells, and the inhibitors could inhibit it only partially. We have also reported that oxanosine induces normal phenotypes in K-ras^{ts}-NRK cells, but not in K-ras-NRK cells [17].

Tyrosine kinase inhibitors induced other normal phenotypes as well. They increased the level of fibronectin mRNA and adhesion plaques (S. Abe, unpublished results). Thus, $p60^{src}$ tyrosine kinase activity was shown by the use of specific inhibitors to be involved in the expression of transformed phenotypes.

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