Use of Transcriptional-Enhancer Elements Responsive to Ras, Protein Kinase A and Protein Kinase C to Evaluate Inhibitors of Specific Signal Transduction Pathways

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To evaluate *ras*-mediated signal transduction, we constructed a transient transfection assay system that measures chloramphenicol acetyl transferase activity expressed under the transcriptional-enhancer elements responsive to *ras*, protein kinase C, and protein kinase A in NIH3T3 cells. Characterization of the assay system with several known activators and inhibitors of signal transduction pathways proved that our system could reliably evaluate agents that affect individual pathways. Pirarubicin ((2''*R*)-4'-tetrahydropyranyladriamycin, THP), which has recently been found able to reverse *ras*-transformed cells, appeared to selectively inhibit the *ras* signal transduction pathway.

Keywords *ras*; anthracycline; transfection; signal transduction pathway; transcriptional enhancer element

Mammalian *ras* proteins (p21^{ras} or Ras) are believed to be involved in signal-transduction mechanisms and to play an important role in the control of cell proliferation and differentiation.¹⁾ Point mutations of *ras* oncogenes at positions 12, 13, 59 or 61 in their coding sequences lead to the constitutive activation of the proteins and are implicated in the transformation of many types of malignant tumors in humans.¹⁾ Inhibitors of Ras-mediated signal transduction pathways may therefore be effective as antitumor agents. We recently found that some of the anthracycline antitumor antibiotics that are used clinically, such as doxorubicin and pirarubicin, but not aclarubicin, selectively reversed transformed phenotypes of *ras*, but not *src*, oncogene-expressed cells *in vitro*,²⁾ and inhibited the growth of solid tumors of K-*ras*-NIH3T3 cells transplanted into nude mice.³⁾ However, the molecular mechanisms underlying this selective effect on *ras*-transformed cells remain unknown.

To examine the capacity of pirarubicin to inhibit *ras* stimulated signal transduction in *ras*-transformed cells, we employed systems consisting of cells expressing a chloramphenicol acetyl transferase (CAT) reporter gene that was placed under the transcriptional control of a *ras* responsive element (RRE). The RRE has been shown to be activated by an oncogenic form of *ras*.⁴⁾ Control plasmids containing the same reporter gene under the transcriptional control of the cyclic AMP response element (CRE) and the TPA response element (TRE) were also constructed. TRE has been shown to be activated by both TPA and *ras*.⁵⁾ In this study, we first characterized these transcriptional elements for their ability to respond to stimulators and inhibitors of signal transduction pathways and then examined the effects of anthracycline antibiotics on the transcriptional activation of these response elements.

MATERIALS AND METHODS

Materials Pirarubicin and aclarubicin were gifts from Dr. T. Takeuchi, Institute of Microbial Chemistry

(Tokyo, Japan). Lovastatin, in the inactive lactone form, was provided by Dr. T. Kobayashi, Sankyo Co., Ltd. (Tokyo, Japan). It was converted to the active form as described elsewhere.⁶⁾ H-7 and H-89 were purchased from Seikagaku Kogyo Co., Ltd. (Tokyo, Japan), chelerythrine and 12-*O*-tetradecanoylphorbol-13-acetate (TPA) were from LC Laboratories (MA, U.S.A.), and forskolin and 3-isobutyl-1-methylxanthine (IBMX) were from Sigma Chemical Co. (MO, U.S.A.).

Plasmids pH1,⁷⁾ a v-Ha-*ras* expression plasmid including Ha-MuSV whole genome, was provided by JCRB (Japanese Cancer Research Resources Bank). pGC1, a CAT expression plasmid, contains a 1440 bp mouse β -globin major gene promoter region⁸⁾ (–1414 to +26 with respect to the RNA cap site) upstream of the CAT gene (Eco RI-Bam HI fragment from pSV2CAT).⁹⁾ pRSV-SEAP¹⁰⁾ was a gift from Dr. B. R. Cullen and a plasmid containing a β -galactosidase gene driven by the RSV-LTR (pRSV- β gal)¹¹⁾ was the gift of Dr. S. Yamagoe, National Institute of Health, Japan.

Construction of Reporter Plasmids Plasmid pGC3 was constructed by replacing the 1292 bp 5' region of the promoter (–1414 to –123) of pGC1 with an Xho I linker. Double-stranded oligonucleotides including each of the *ras*-responsive enhancer element (RRE: AGGA-TATGACTCTTTGG corresponding to –117 to –101 of *ras* responsible NVL3 U3-region),^{4a)} the TPA response element (TRE: CGAGGAAGTGACTAACTGACCG)⁵⁾ and the cAMP response element (CRE: ATGGCCGTCATACTGTGACGT)¹²⁾ were synthesized. All had Xho I and Sal I cutting ends upstream and downstream, respectively. These oligonucleotides were multimerized, and cloned into the Xho I site in pGC3 (Fig. 1). The repeating number of response elements in each pGC3 construct was confirmed by the dideoxynucleotide sequencing method.¹³⁾

Cell Culture and Transient Transfection The cell lines used were NIH3T3 and its v-Ha-*ras* oncogene transformed derivative.¹⁴⁾ Cells were cultured in Dulbecco's modified Eagle medium (DMEM) containing 10% calf

serum (GIBCO) at 37°C in a humidified atmosphere containing 5% CO₂.

For transfection experiments, cells were seeded at 6×10^5 to 8×10^5 cells per 10-cm-diameter dish or 2.5×10^5 to 5×10^5 cells per 6-cm-diameter dish and 24 h later the cells were transfected with DNA using the procedure of Chen and Okayama.¹⁵ A total of 20 µg DNA was added to each 10-cm-diameter dish. As an internal control, pRSV-βgal was included in the DNA precipitates.

In experiments comparing the expression of reporter plasmids in NIH3T3 and v-Ha-ras NIH3T3, NIH3T3 (5×10^5) or v-Ha-ras NIH3T3 (3×10^5) cells were plated on a 6-cm-diameter dish 1 d prior to transfection. The cells were transfected with 8 µg reporter and 2 µg pRSV-βgal plasmids. The cells were then incubated with the DNA co-precipitates for 5 to 6 h, washed with phosphate buffered-saline (PBS), treated with fresh medium containing 10% fetal bovine serum and incubated for 48 h.

For transient expression of *ras*, the expression plasmid pH1 was used with pRSV-SEAP as a control. In co-transfection experiments, NIH3T3 cells (2.5×10^5) were plated on a 6-cm-diameter dish 1 d prior to transfection. To each dish was added 3.3 µg reporter, 3.3 µg pH1 and 1.3 µg pRSV-βgal. After incubation for 5 to 7 h, the calcium phosphate-DNA coprecipitates were removed by washing the cells with PBS. Fresh medium containing 10% calf serum was then added to the dish and the cells were incubated for an additional 40 h.

For assays involving treatment of transfected cells with stimulators or inhibitors of signal transduction pathways, NIH3T3 cells (6.5×10^5) were plated on a 10-cm-diameter dish, transfected with a mixture of 8 µg reporter plasmid, 8 µg expression plasmid and 4 µg pRSV-βgal, incubated for 16 to 20 h in fresh medium containing 10% calf serum, trypsinized and transferred to 12 or 24-well culture plates. After 3 to 5 h incubation in fresh medium containing 10% calf serum, a stimulant [TPA (100 ng/ml), or a combination of forskolin and IBMX (20 and 500 µM, respectively)] or blank was added, followed by incubation for 24 h. The inhibitors to be studied were added 1 h before the addition of stimulant.

Quantitation of CAT Protein Cells were lysed by repeated freezing/thawing or using a lysis buffer (MOPS, 20 mM; NaCl, 10 mM; MgCl₂, 1.5 mM; Triton X-100, 1%). The CAT protein content of the cell lysates was assayed using an enzyme-linked immunoabsorbent assay (ELISA) with a CAT assay kit (Boehringer Mannheim Biochemica) and quantified using a standard curve constructed during each experiment with purified CAT supplied with the kit.

β-Galactosidase activity was measured as described previously.¹⁶ The protein content of the cell extracts was determined using the method of Bradford.¹⁷

RESULTS AND DISCUSSION

As shown in Fig. 1, each reporter construct contained four repeats of each of the synthetic elements, RRE, CRE, and TRE. These multimerized response elements were inserted upstream of the minimal promoter of mouse major β-globin gene containing CAAT and TATA

Fig. 1. Structures of Transcriptional Response Element-CAT Reporter Plasmids

Plasmids were constructed as described in Materials and Methods. Ras response element (RRE)-CAT contains four tandem copies of RRE inserted upstream of the minimal mouse major β-globin gene promoter attached to the CAT reporter gene. CRE and TRE stand for cAMP response element and TPA response element, respectively. The underlined bases represent the consensus sequences for transcriptional regulation by each response element.

Fig. 2. Enhanced Expression of RRE-CAT in Ha-ras Transformed NIH3T3 Cells

NIH3T3 cells and v-Ha-ras-transformed NIH3T3 cells were transiently transfected with CAT reporter plasmids pGC3 or pGC3-4RRE. 48 h after transfection, cell lysates were made and the CAT content was determined. To measure the relative efficiency of transfection and to normalize separate experiments, pRSV-βgal was co-transfected with the CAT constructs. Data are the means of the CAT contents, normalized to the β-gal activity of each of three transfected plates. The levels of β-gal in each plate were similar in all the experiments (data not shown). The relative ratios of CAT expression in Ha-ras-NIH3T3 cells compared with that in NIH3T3 cells are indicated as multiples of the degree of stimulation. □, NIH3T3; ▨, Ha-ras-NIH3T3.

sequences. A gene encoding CAT reporter gene, followed by an SV40 t intron and a poly(A) addition signal, was placed downstream from these sequences.

Owen *et al.*⁴ have shown that the expression of *ras* oncogene induces an increase in transcription of the NVL-3 gene and that an RRE is involved in this *ras* responsiveness. To test the ability of our multimerized RRE construct to confer *ras* responsiveness, we transfected the recombinant reporter plasmid pGC3-4RRE into NIH3T3 cells and v-Ha-ras-transformed NIH3T3 cells. Transfection of pGC3-4RRE into Ha-ras-NIH3T3 cells resulted in a 5-fold increase in CAT compared with NIH3T3 cells (Fig. 2). The control vector pGC3, however, did not

Fig. 3. Stimulation of RRE-CAT Expression by Co-transfected Oncogenic *ras*

NIH3T3 cells were transiently transfected with one of the CAT reporter plasmids: pGC3, pGC3-4RRE, pGC3-4CRE, or pGC3-4TRE. Each plasmid was co-transfected either with a v-Ha-*ras* expression plasmid (pH1) or a control plasmid (pRSV-SEAP). pRSV- β gal was used as the internal control plasmid and CAT was determined 40 h later, as described in the legend to Fig. 2. Relative ratios of CAT expression in each pair of cells, one containing *ras* oncogene and the other not, are indicated as multiples of the degree of stimulation. ▨, +*ras*; □, control.

induce CAT gene expression in Ha-*ras*-NIH3T3 cells. These results confirmed that the RRE is capable of conferring a *ras*-enhanced increase in the expression of the CAT gene.

To further confirm the *ras*-responsiveness and specificity of RRE, we next transfected four CAT reporter plasmids, pGC3, pGC3-4RRE, pGC3-4CRE, and pGC3-4TRE individually into NIH3T3 cells in combination with pH1, a v-Ha-*ras* expression plasmid which showed transforming activity after transfection of NIH3T3 cells, or with a control vector (pRSV-SEAP) lacking the v-Ha-*ras* sequence. Co-transfection of NIH3T3 cells with the v-Ha-*ras* expression vector pH1 in combination with pGC3-4RRE resulted in more than a 30-fold increase in CAT compared with basal (control) CAT (Fig. 3). Co-transfection of pH1 with pGC3-4TRE resulted in a moderate increase in CAT, but co-transfection with pGC3-4CRE or pGC3 did not increase CAT, as shown in the figure. The results suggest that v-Ha-*ras* activates the target gene expression *via* RRE- and TRE-transcriptional control elements in the transient expression assay systems.

We next tested whether TPA (a PKC activator) or a combination of forskolin and IBMX (abbreviated to F/I), an activator of adenylate cyclase and an inhibitor of phosphodiesterase, respectively, would induce the expression of CAT reporter gene downstream of RRE, CRE, or TRE. NIH3T3 cells were transiently transfected with each plasmid and treated with the inducers for 24 h. As expected, CAT was induced approximately 14-fold by F/I from CRE, and 7-fold by TPA from TRE (Fig. 4). In contrast, it was not induced by either F/I or TPA from RRE. It seems, therefore, that RRE, CRE, and TRE can be used as selective transcriptional enhancer elements for *ras*, protein kinase A (PKA), and protein kinase C (PKC) signal transduction pathways, respectively.

We then tested four well characterized inhibitors of the individual signal transduction pathways for their possible effects. Lovastatin, an inhibitor of hydroxymethylglutaryl coenzyme A reductase, blocks processing of the *ras* precursor protein to its mature form and inhibits mammalian cell growth *in vitro*.¹⁸⁾ H-89, an isoquinoline

Fig. 4. Effects of TPA and F/I on the RRE, CRE, and TRE Transcriptional Elements

NIH3T3 cells were transfected with one of the CAT reporter plasmids pGC3, pGC3-4RRE, pGC3-4CRE, or pGC3-4TRE with the internal control plasmid pRSV- β gal. 20 h after transfection, the cells were replated to smaller dishes and cultured for 24 h. Cells were then either untreated or stimulated with forskolin/IBMX (20 μ M/500 μ M) or TPA (100 ng/ml) for 24 h. Responses of each CAT construct to the inducers are expressed as multiples of the degree induction; induction in unstimulated cells is defined as 1.0. □, none; ■, forskolin/IBMX; ▨, TPA.

TABLE I. Effects of Inhibitors on Transcriptional Activation of the RRE, CRE, and TRE Response Elements

Inhibitor	Percentage inhibition			
	RRE + <i>ras</i>	CRE + F/I	TRE + TPA	TRE + <i>ras</i>
Lovastatin (5 μ M)	59	1.7	0	32
H-89 (10 μ M)	0	45	8.1	0
H-7 (20 μ M)	0	23	60	N.D.
Chelerythrine (5 μ M)	0	0	78	41

NIH3T3 cells were transfected with each CAT reporter plasmid either with (RRE + *ras*, TRE + *ras*) or without Ha-*ras* expression plasmid pH1. 20 h after transfection, cells were trypsinized and replated into smaller dishes. Inhibitors were added at the concentrations indicated, and 1 h later induction with F/I or TPA was initiated and continued for 24 h, as described in the legend to Fig. 4. Values represent the averages of three dishes and the S.D. was <10%. Percentage inhibition = $[1 - (\text{inhibitor-treated cells}/\text{untreated cells})] \times 100$. N.D. = not determined.

sulfonamide, was recently synthesized as a specific inhibitor of PKA¹⁹⁾ and its selectivity has been confirmed.²⁰⁾ H-7 is an inhibitor of both PKC and PKA.²¹⁾ Chelerythrine is reported to selectively inhibit PKC.²²⁾ These inhibitors were expected to interfere with one or more of the signal transduction pathways described above.

As summarized in Table I, lovastatin selectively inhibited the expression of *ras*-induced transcription of RRE- and TRE-CAT but inhibited neither the F/I-stimulated expression of CRE-CAT nor the TPA-stimulated expression of TRE-CAT. H-89 inhibited only the expression of CRE-CAT, without affecting other combinations of transcriptional activation. H-7 strongly inhibited the expression of TPA-induced TRE-CAT and, to a lesser extent, F/I-induced expression of CRE-CAT. Chelerythrine inhibited both the TPA- and *ras*-induced expression of TRE-CAT but not F/I-induced CRE-CAT or *ras*-induced RRE-CAT. CRE thus appears to be

Fig. 5. Effect of Pirarubicin and Aclarubicin on the Transcriptional Activation of RRE-, CRE-, and TRE-CAT

Experiments were performed in triplicate as described in the legends to Fig. 4 and Table I. (—) Indicates experiments in the absence of each stimulator.

activated only *via* the PKA signaling pathway while TRE is activated by signals involving PKC and *ras*, but not PKA, which is consistent with previous studies.²³⁾ These results suggest that the three reporter plasmids we constructed provide a reliable assay system for evaluating or identifying inhibitors of specific signal transduction pathways involving either *ras*, PKA or PKC in NIH3T3 cells.

We found recently that anthracycline antitumor antibiotics could be grouped into two types, one (doxorubicin and pirarubicin, *etc.*) capable of reversing transformed cell phenotypes of *ras* oncogene-expressed cells, and the other (aclarubicin, for example) which does not.²⁾ We therefore examined the capacity of these anthracyclines to inhibit the Ras-stimulated signal transduction in the system described above. Pirarubicin, at concentrations effective in reversing transformed phenotypes of *ras*-transformed cells,²⁾ significantly inhibited *ras*-induced expression of RRE-CAT as shown in Fig. 5. In contrast, aclarubicin which is unable to reverse *ras* transformed cells, stimulated RRE-CAT expression. The two anti-tumor drugs, however, enhanced F/I-induced expression of CRE-CAT, while showing almost no effect on TPA-induced expression of TRE-CAT. Similar results were obtained in the absence of inducers for CRE and TRE (data not shown). The selective inhibition by pirarubicin of *ras* induced transcription of RRE-CAT was reproducibly observed. Because the protein content of p21^{ras} and its membrane association is not affected by the drug treatment (unpublished results), the target point appears to be a step downstream of the signaling pathway from activated Ras. Thus, the target site of pirarubicin is located somewhere on the signal transduction pathway downstream from activated Ras.

Ras proteins have been implicated as transducers of

PKC-dependent²⁴⁾ and PKC-independent intracellular signals.²⁵⁾ Our results suggest that the Ras signal transduction for RRE is distinct from the TPA/PKC pathway, because TPA or inhibitors of PKC neither stimulate nor inhibit RRE-CAT transcription, confirming a previous suggestion.^{4b)} Pirarubicin, therefore, may act on an unidentified component distinct from the pathway involving PKC. RRE has recently been shown to contain two partially overlapping binding sites, one for AP-1 related nuclear factor, probably *c-jun*, and the other for transcription factors of the *ets*-family.²⁶⁾ To further define the target of pirarubicin, an investigation of the binding ability of these transcription factors to the RRE might provide useful information.

Stimulation of transcription of CRE by pirarubicin, and RRE and CRE by aclarubicin was unexpected. However, this observation is very interesting because it has been reported that the typical anthracycline antibiotic, adriamycin, stimulates proliferation of human and murine cells at subtoxic concentrations.²⁷⁾ The biochemical mechanism, however, remains unexplained. It is possible that the enhanced transcription we observed may contribute to the stimulation of the growth of some cells.

In this study we conducted a transient transfection assay using CAT as a reporter gene. To characterize known compounds, and to screen new agents that affect specific signal transduction pathways, it is desirable to establish and use permanent cell lines containing individual DNA response elements linked to a common reporter gene. We are currently isolating such cell lines.

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