Reversible inhibition of a thyroid-specific trans-acting factor by Ras

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Exposure of rat thyroid cells for 1 week to a temperature-sensitive variant of Kirsten murine sarcoma virus (KiMSV) Ras inactivated the thyroglobulin promoter (pTg). Cellular dedifferentiation was paralleled by the loss of the thyroid-specific trans-acting factor, TgTFl, which binds to pTg. When Ras was denatured by shifting cells to 39°C, TgTFl binding and pTg function recovered rapidly without the synthesis of new protein. TgTFl could be reactivated in vitro by treating nuclear extracts with protein kinase A. After 4 weeks of exposure to the oncogene, denaturation of Ras no longer restored TgTFl binding or reactivated pTg. Incubation of nuclear extracts with protein kinase A likewise did not reactivate TgTFl. Cells chronically exposed to Ras did, however, yield redifferentiated clones after treatment with 5-azaactidine. We suggest that Ras induces dedifferentiation in two sequential steps: (1) Ras reduces PKA activity; TgTFl (or an auxiliary protein) becomes dephosphorylated, and binding to pTg is abolished. (2) The effects of Ras become imprinted by methylation, possibly of the TgTFl gene.

[Key Words: Ras; dedifferentiation; thyroid-specific trans-acting factor]

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The expression of transforming oncogenes can inhibit the establishment and the maintenance of cellular differentiation [Fiszman and Fuchs 1975; Beug et al. 1982; Schmidt et al. 1985; Dmitrowsky et al. 1986; Prochownik and Kukowska 1986; Olson et al. 1987; Freytag 1988]. In cell lineages where cellular proliferation and expression of tissue-specific functions are mutually exclusive, oncogene expression might abort differentiation by providing a constitutive mitogenic signal. In the best-studied case, however, oncogenic transformation of myoblasts by activated ras or fos interferes directly with the expression of MyoD, the gene that induces terminal differentiation. MyoD expressed from a retroviral promoter overrides ras, reduces the rate of proliferation, and reestablishes the differentiation program [Lassar et al. 1989].

Unlike terminally differentiating cell lineages, proliferating thyroid cells express tissue-specific markers. Cell growth and differentiation are linked by their dependence on cAMP [Ambesi-Impiombato et al. 1980; Weiss et al. 1984; Hen et al. 1989]. Thyroid cells transformed with a variety of activated oncogenes, for example, v-ras, v-src, v-raf, and v-mos, become cAMP independent for growth. At the same time, the thyroid differentiation markers are suppressed; transformed cells do not express thyroglobulin, thyrotrypsin receptor, and the iodide carrier [Fusco et al. 1987].

To identify the mechanism by which v-ras inhibits thyroglobulin expression, we have used an established and differentiated rat thyroid cell line [TL]. TL was transformed with Kirsten murine sarcoma virus [KiMSV]-ts carrying a temperature-sensitive v-ras allele [line Ats]. Although the transformed phenotype [e.g., anchorage independence] of the Ats cells was temperature sensitive, Ats cells were dedifferentiated at both permissive (33°C) and nonpermissive (39°C) temperatures [Colletta et al. 1983]. Ats cells carrying a fusion between the thyroglobulin promoter and a gene encoding resistance to the neomycin analog, G418, [pTg-NEO] were sensitive to the drug. Treatment of these cells at 39°C with a 5-azaacytidine, a DNA demethylating agent, generated a cell line [Ats-aza] that expressed thyroglobulin. Ats-aza cells could be stably propagated at 39°C in the presence of G418 [Avvedimento et al. 1989].

The activity of pTg is associated with the presence of a thyroid-specific trans-acting factor, TgTFl, which binds to the -60 region of the promoter [Musti et al. 1987; Civitareale et al. 1989; Ursini et al. 1989]. TgTFl was absent in Ats cells, either at 33°C or 39°C, and reappeared in the Ats-aza line accompanied by G418 resistance and thyroglobulin expression [Avvedimento et al. 1988, 1989].

In this manuscript we report that dedifferentiation and the loss of TgTFl-binding activity occur rapidly after exposure of thyroid cells to active Ras. In Ats-aza cells grown for 1 week at 33°C, both processes are reversible. TgTFl binding can be restored in nuclear extracts from Ras-exposed cells by treatment with protein kinase
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A (PKA). Longer exposure to active Ras irreversibly dedifferentiates thyroid cells. After 4 weeks at 33°C, TgTFl activity cannot be restored by denaturing Ras or by treating nuclear extracts with PKA. We suggest that Ras inhibits thyroglobulin transcription in two sequential steps: (1) Ras reduces PKA activity; TgTFl (or an auxiliary protein) becomes dephosphorylated and binding to pTg is abolished. (2) Later, the effects of Ras become imprinted, and active Ras is no longer required to block differentiation. This second step, which is reversed by 5-azacytidine, may involve inactivation of the TgTFl gene by DNA methylation.

Results

Reversible inactivation of TgTFl trans-acting factor in KiMSV-ts-transformed cells

We have used the thyroid-derived Ats-aza line, which bears a temperature-sensitive v-ras allele, to explore early steps in Ras-induced dedifferentiation. When an Ats-aza culture is shifted from 39°C to 33°C, Ras is activated, and dedifferentiation, as exemplified by the loss of thyroglobulin expression, begins. In the experiment described in Figure 1A, we measured the formation of complexes between the thyroid-specific trans-activating factor (TgTFl) and labeled oligonucleotide derived from the –60 region of the thyroglobulin promoter [oligo(C)]. TgTFl-binding activity disappeared almost entirely from the nuclei of Ats-aza cells within 1 week after Ras activation [lanes 1 and 2]. Unlike cells exposed to Ras for long periods [e.g., Ats], at this stage the loss of TgTFl activity was reversible. Inactivation of Ras by shift-up of the culture to 39°C for 3 days fully restored TgTFl binding [lane 3]. In contrast, Ats-aza cells maintained at 33°C for 4 weeks failed to regain TgTFl activity when shifted back to 39°C [lanes 4 and 5]. Nuclear extracts of TL cultures grown at 33°C or 39°C contained high TgTFl levels (Fig. 1B). A shift up from 33°C to 39°C for 1 week likewise did not stimulate TgTFl activity in TL nuclear extracts [data not shown]. As control, an oligonucleotide spanning the –150 region of pTg [oligo(A)], which binds a non-tissue-specific factor, was retarded by all Ats-aza cell extracts tested (Fig. 1C).

The activity of TgTFl paralleled the expression of the pTg-NEO fusion present in the Ats-aza cells. Growth of Ats-aza cultures at 33°C for 1 week followed by a return to 39°C, did not reduce the titer of G418-resistant colonies. After 4 weeks at 33°C, however, no resistant clones could be recovered after temperature shift-up (Avvedimento et al. 1989).

The loss of TgTFl activity and of G418-resistant revertants, spontaneous G418 resistance did not result from pTg reactivation.

Figure 1. Reversible and irreversible inactivation of TgTFl in Ats-aza cells. Nuclear extracts were prepared and used for gel retardation assays of labeled oligonucleotides as described in Materials and methods. Oligo(C) derives from the –60 region of pTg and binds TgTFl. Oligo(A) derives from the –150 region of pTg and binds a non-tissue-specific factor[s]. (A) Oligo(C) binding activity in extracts from Ats-aza cells maintained at 39°C [lane 1], shifted to 33°C for 1 week [lane 2], and returned to 39°C for 3 days [lane 3]. Ats-aza cells shifted to 33°C for 4 weeks [lane 4], and then returned to 39°C for 3 days [lane 5]. (B) Oligo(C) binding by extracts from TL cells grown at 39°C [lane 1], TL cells grown at 33°C for 1 week [lane 2]. Ats cells grown at 39°C [lane 3]. (C) Oligo(A) binding by extracts from cells described in A.

Figure 2. Transcription from pTg parallels TgTFl-binding activity. RNase A mapping of pTg-NEO RNA in Ats-aza cells. A specific antisense RNA probe for the pTg-NEO transcript was prepared and assayed as described previously [Avvedimento et al. 1989]. Total RNA was extracted from TL, Ats, and Ats-aza cells [lanes 1–7, 9]. All lines carry a pTg-NEO fusion. A total of 30 µg of RNA from each cell extract was assayed. The arrows indicate the expected protected bands of correctly initiated pTg-NEO RNA (360 nucleotides) and of rat glyceraldehyde-3-phosphate dehydrogenase (GPDH) (70 nucleotides), used as an internal control. [Lanes 1 and 9] Ats cells; [lane 2] TL cells; [lane 3] Ats-aza cells grown at 39°C; [lane 4] Ats-aza cells grown at 33°C for 4 days and then shifted to 39°C for 3 hr [lane 5]; [lane 6] Ats-aza cells grown at 33°C for 10 days and then returned to 39°C for 1 day [lane 7]; [lane 8] RNA from a rare, spontaneous G418-resistant Ats clone. Unlike 5-azacytidine-induced revertants, spontaneous G418 resistance did not result from pTg reactivation.
failed to transcribe a pTg-NEO fusion (lanes 1 and 9). A TL line carrying a pTg-NEO fusion and an Ats-aza culture maintained at 33°C both expressed the fusion (lanes 2 and 3). Shifting Ats-aza cells to 33°C for 4 days reduced pTg-NEO transcription; transcription was stimulated by returning the cells to 39°C for 3 hr (lanes 4 and 5). After 10 days growth at 33°C, pTg-NEO transcripts were absent from Ats-aza cells (lane 6). Shifting the cells to 39°C for 24 hr restored the levels of pTg-NEO RNA to those of the original Ats-aza culture (lane 7).

**TgTF1 reactivation does not require de novo protein synthesis**

Detectable loss of TgTF1 activity in Ats-aza cells required a minimum of 1 week at 33°C (data not shown). However, the degree of TgTF1 inactivation was dependent on the condition of cell culture. TgTF1 from older-passage cells or from cultures close to confluency was more resistant to v-ras [Fig. 3A, lane 2; see Fig. 5B, lane 2] than TgTF1 isolated from early-passage cells or subconfluent cultures [Fig. 4A, lane 2]. In contrast to TgTF1 inactivation, restoration of TgTF1 binding occurred very rapidly after a return to 39°C. Some recovery of TgTF1 activity was detectable after 1 hr of incubation at 39°C, and recovery to 20–50% of Ats-aza levels was reached at 3 hr [Fig. 3A].

The rapid reappearance of TgTF1 activity at 39°C suggests that TgTF1 protein is present in cells newly transformed by v-ras but is unable to bind to pTg. Consistent with this prediction, the reactivation reaction did not require de novo protein synthesis. Ats-aza cells were incubated for 1 week at 33°C and then shifted to 39°C for 12 hr in the absence or presence of cycloheximide [Fig. 4A, lanes 3 and 4, respectively]. TgTF1 reactivation occurred rapidly without new protein synthesis. We conclude that TgTF1 converts to an inactive form during a week of exposure to Ras. The process is reversed rapidly when Ras is denatured.

Loss of TgTF1-binding activity after 2 weeks exposure of Ats-aza cells to v-ras could still be reversed by storing the culture to 39°C. However, the recovery of TgTF1 activity was partially sensitive to cycloheximide [Fig. 4B, cf. lanes 3 and 4]. After 3 weeks of exposure to v-ras, the loss of TgTF1 binding was essentially irreversible [Fig. 4C].

**Reactivation of TgTF1 in Ras-transformed cells by PKA**

These data suggest that TgTF1 or some associated protein must be modified for activity, and that v-ras affects this modification. A likely candidate for the modification is phosphorylation. Therefore, we assayed TgTF1-binding activity in TL nuclear extracts after treatment with acid phosphatase. As shown in Figure 5A, TgTF1 binding was exquisitely phosphatase sensitive [cf. lanes 1 and 2]. TgTF1 binding could be restored in the treated extracts by phosphorylation with PKA [Fig. 5A, lane 3]. The role of PKA in thyroid cell differentiation will be discussed in detail elsewhere [A.M. Musti, V.E. Avvedimento, M. Ueffing, and M.E. Gottesman, in prep.].

These results indicate that phosphorylation of a protein in thyroid cell nuclear extracts is necessary for the binding of TgTF1 to pTg. To test the possibility that Ras might inhibit the phosphorylation reaction, we prepared a nuclear extract from Ats-aza cells grown for 1 week at 33°C. Treatment of the extract with activated bovine PKA restored pTg-binding activity [Fig. 5B, cf. lanes 2 and 3]. The activity of the PKA-treated extracts was comparable to that of extracts from Ats-aza cells incubated at 33°C for 1 week and returned to 39°C for 1 day [Fig. 5B, lane 4]. Oligo(C)-binding by nuclear extracts from cultures returned to 39°C was completely sensitive to treatment with phosphatase [Fig. 5B, lane 5].

In contrast to our results with Ats-aza cells grown at 33°C for 1 week, inactive nuclear extracts from Ats-aza cells exposed to Ras for 4 weeks were unresponsive to PKA. Similarly, oligo(C)-binding activity in nuclear extracts from Ats cells was not restored by PKA treatment (data not shown). Presumably, nuclei from these cells contain no TgTF1 protein. Recent experiments indicate that the TgTF1 gene is not transcribed in the Ats line [R. Di Lauro, pers. comm.]. PKA treatment likewise did not

**Figure 3. Kinetics of TgTF1 reactivation.** [A] Shown are oligo(C) complexes formed by nuclear extracts from Ats-aza cells grown at 39°C [lane 1]. Ats-aza cells shifted to 33°C for 1 week [lane 2], and returned to 39°C for 3 hr [lane 3], 3 hr [lane 4], or 1 day [lane 5]. [B] As in A, except that complexes with oligo(A) are shown.
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Figure 5. Reactivation of TgTFI in vitro with PKA. (A) Oligo(C) binding by nuclear extracts from TL cells. (Lane 1) Untreated extract, (lane 2) TL nuclear extract treated with potato acid phosphatases (0.6–0.8 units, Sigma type III or type X, for 5 min at room temperature); (lane 3) the extract of lane 2 was brought to 1 mM NaF and sodium vanadate and then incubated with PKA holoenzyme (60 μg/ml and 0.5 mM cAMP for 10 min at room temperature. (B) Oligo(C) binding by Ats-aza nuclear extracts. (Lane 1) Ats-aza cells grown at 39°C, (lane 2) Ats-aza cells shifted from 39°C to 33°C for 1 week and then incubated with PKA holoenzyme and 0.5 mM cAMP for 10 min at room temperature; (lane 4) Ats-aza cells grown at 39°C for 1 week and then shifted to 33°C for 1 day; (lane 5) the extract of lane 4 treated with phosphatase as described in Fig. 5A. (C) Oligo(C) binding by extracts from Ats-aza cells incubated at 33°C for 1 week (lane 1). These extracts were treated with 60 μg/ml PKA [lane 2] 600 μg/ml PKA [lane 3], 60 μg/ml PKC [lane 4], or 60 μg/ml PKC and PS/DO lipid cofactors as described in Materials and methods. PKA from bovine heart (Sigma) was preactivated with cAMP as described in Materials and methods.

These results indicate that Ras inhibits TgTFI activity consequent to the dephosphorylation of some nuclear protein, perhaps TgTFI itself. The inhibition was reversed in vivo by denaturing Ras and in vitro by treatment of the nuclear extract with PKA. How might the activation of Ras result in the dephosphorylation of the responsible nuclear protein? We considered the possibility that Ras lowered cellular cAMP levels by inhibiting adenylate cyclase. Without the kinase activity of PKA to reverse nuclear phosphatases, TgTFI [or an associated protein] would become unmodified. In this model, dedifferentiation by Ras should be blocked by providing cAMP. This was not the case. Ats-aza cultures were shifted to 33°C for 1 week and then incubated for an additional week at 33°C in the presence or absence of dibutyryl cAMP. As control, cells incubated without dibutyryl cAMP were returned to 39°C for the final 2 days of the experiment. Whereas Ras denaturation restored TgTFI binding, dibutyryl cAMP did not [data not shown]. The target(s) of Ras must thus lie distal to adenylate cyclase.

That dibutyryl cAMP can replace endogenously generated cAMP was shown by the following experiment. The adenylate cyclase activity of TL cells depends on thyroid-stimulating hormone (TSH). As expected, cells starved for TSH lose TgTFI activity. Dibutyryl cAMP added to the medium maintained TgTFI activity in the absence of TSH (A.M. Musti, M. Ueffing, V.E. Avvedimento, and M.E Gottesman, in prep.).

PKA activity in Ras-transformed cells

These results are consistent with the idea that the expression of Ras reduced cellular PKA activity. We measured the phosphorylation of a PKA-specific peptide substrate in cytosolic extracts from transformed and nontransformed thyroid cells [Table 1]. Our experiments show that Ats or Ats-aza cells grown at 33°C contained significantly less cAMP-dependent phosphorylating activity than cells grown at 39°C. As control, TL cells grown at 33°C or at 39°C showed similar PKA levels. We note, however, that the reduction in PKA levels in Ats-aza cells grown at 33°C for 1 week was ~50%, whereas the reduction in TgTFI activity was considerably greater.

Discussion

DNA methylation follows gene inactivation

The differentiation program of thyroid cells is suppressed by transformation with KiMSV carrying an activated v-ras. The suppression is imprinted; a rat thyroid cell line transformed with a temperature-sensitive v-ras allele did not differentiate when the v-ras product was thermally inactivated. Although the transformed thyroid cells lost anchorage independence and the capacity to form foci when shifted to 39°C, they did not regain the ability the express thyroglobulin, to incorporate iodide, or to bind TSH [Colletta et al. 1983]. Treatment

Table 1. PKA activity is reduced in Ras-transformed thyroid cells

| Cell line | PKA [units/μg] |
|-----------|----------------|
|           | 33°C | 39°C |
| TL        | 29.5 (±5.0) | 34.0 (±6.0) |
| Ats       | 6.7 (±2.0)  | 17.0 (±2.7) |
| Ats-aza   | 19.5 (±3.0) | 39.1 (±3.1) |

PKA phosphorylation by whole-cell extracts of the specific synthetic substrate, Kemptide, was determined as described in Materials and methods. Where indicated, cells grown at 39°C were shifted to 33°C for 1 week. The values shown above represent the mean of at least three experiments. The concentration of the cellular proteins was 4–10 μg/ml, within the linear range of enzymatic activity.
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with 5-azacytidine readily yielded clones [Ats-aza] expressing thyroglobulin [Avvedimento et al. 1989] and TSH receptor (T. Akamizu, M. Saji, S. Ikuyama, and L. Kohn, unpubl.). The reactivation of the differentiation markers was accompanied by the reappearance of TgTFl, which binds pTg at –60 from the transcription start site [Avvedimento et al. 1989].

In this paper, we describe early steps in dedifferentiation that follow Ras reactivation in Ats-aza cells. The activity of the thyroglobulin promoter was lost after 1 week of exposure of Ats-aza cells to Ras. The loss was paralleled by the disappearance from nuclear extracts of TgTFl-binding activity. At this stage, both events were reversible by thermal inactivation of Ras. New protein synthesis was not required.

Two weeks after Ras reactivation, dedifferentiation could still be reversed by returning the Ats-aza culture to 39°C. However, the restoration of TgTFl binding was now partially sensitive to cycloheximide.

In contrast, in cells exposed to the Ras oncogene for 4 weeks or longer, Ras denaturation did not restore TgTFl-binding activity or pTg expression [this work, Avvedimento et al. 1989]. To obtain clones expressing pTg, chronically exposed cells must be treated with DNA demethylating agents. By analogy to the processes of globin gene switching [Enver et al. 1988] and suppression of HPRT during X chromosome inactivation [Lock et al. 1987], we propose that methylation and inactivation of the TgTFl gene follows the inhibition of the binding activity of the TgTFl protein. Possibly TgTFl, like other cell-specific trans-activators, is positively autoregulating [Thayer et al. 1988]. In the absence of active TgTFl, the quiescent TgTFl gene becomes methylated. The inhibition of the gene product by Ras is thus imprinted by an epigenetic DNA modification.

Mechanism of TgTFl inactivation by Ras

In cells briefly exposed to Ras, TgTFl-binding activity could be restored in vitro by treatment of nuclear extracts with PKA. PKC was ineffective. These results indicate that Ras interferes with the phosphorylation by PKA of TgTFl or an accessory protein. Our finding of reduced cAMP-dependent kinase activity in Ras-transformed cells supports the idea that Ras mediates dedifferentiation in thyroid cells by inhibiting PKA.

The central role of PKA in thyroid differentiation is also suggested by our recent experiments in TL cells. TL cells grown in the absence of TSH had lowered cAMP levels and, consequently, reduced PKA activity. These cells rapidly lost TgTFl binding activity. With short periods of TSH starvation, TgTFl binding-activity was restored by adding TSH or by increasing cAMP levels. Reminiscent of cells briefly exposed to Ras, new protein synthesis was not required to reactivate TgTFl. Furthermore, treatment of TL nuclear extracts with acid phosphatase greatly reduced TgTFl-binding activity; binding was restored by phosphorylation with PKA [see Fig. 5A], but not PKC [A.M. Musti, M. Ueffing, V.E. Avvedimento, and M.E. Gottesman, in prep.].

That phosphorylation regulates the activity of nuclear trans-acting transcription factors is well documented. Transcription of adenovirus early functions, c-fos, and various other genes depends on a set of CREB phosphoproteins that binds to promoter region sequences (CRE). The transcriptional activity of the CREB proteins as well as their binding affinity is stimulated in vivo and in vitro by phosphorylation with PKA [Sassone-Corsi et al. 1988, Merino et al. 1989; but see Montminy and Bilezikjian 1987].

We do not know whether TgTFl or an associated protein is the substrate for PKA. We note that oligo(dC), to which TgTFl binds, does not carry a CRE sequence, arguing against a role for CREB in reactivation by PKA. Another model for reactivation is suggested by the properties of trans-acting factor NF-κB (Ghosh and Baltimore 1990). Nuclear factor NF-κB is found in an inactive cytoplasmic complex with an inhibitory protein. Phosphorylation of the inhibitor with PKC or PKA allows active NF-κB to enter the nucleus. In the thyroid system, however, inactive TgTFl is present in the nucleus, rather than the cytoplasm, and its activation is PKA specific. Furthermore, nuclear extracts from Ras-exposed cells treated with deoxycholate did not reveal cryptic TgTFl activity [data not shown]. Similarly, addition of nuclear or cytosolic extracts from Ras-transformed cells to nuclear extracts from TL or Ats-aza cells did not inhibit TgTFl-binding activity [data not shown].

The action of Ras in thyroid cells has analogies to its role in blocking differentiation in myoblasts. In both cases, the activity of a tissue-specific trans-acting factor was reduced after transformation. Unlike the thyroid system, however, activated Ras inhibits the synthesis rather than the activity of MyoD [Lassar et al. 1989].

PKA activity in Ras-transformed cells

We have found reduced PKA activity in thyroid cells transformed with Ras. The induction by cAMP of c-fos is also defective in Ras-transformed thyroid lines [Colletta et al. 1987]. Nuclear extracts from Ats or Ats-aza cells grown at 33°C show no binding of CRE to the CRE element in the c-fos promoter. Cells grown at 39°C yield extracts with normal CRE binding activity [S. Obici, M.E. Gottesman, and V.E. Avvedimento, unpubl.]. Although injection of active Ras stimulates phosphodiesterase activity in Xenopus oocytes [Sadler et al. 1990], no results linking PKA to Ras have been reported. In recent experiments we have noted that active Ras injected into Xenopus oocytes lowered PKA levels and induced rapid maturation. Simultaneous injection of PKA prevented early maturation [S. Fisch, S. Obici, M.E. Gottesman, and V.E. Avvedimento, in prep.; I. Daar and G. Van de Woude, pers. comm.].

Activated ras has been shown to stimulate PKC in Xenopus oocytes [Lacal et al. 1987] and in the PC12 rat pheochromocytoma line [Lacal et al. 1990, Thomson et al. 1990]. Conversely, cells overproducing PKC are more susceptible to transformation by an H-ras oncogene [Hsiao et al. 1989]. Since the PKC and PKA pathways are frequently antagonistic [Mechta et al. 1989], it is entirely possible that the inhibition of PKA by Ras may be mediated through stimulation of PKC. Supporting this notion is our recent finding that PKC overexpression in
TL or Ats-aza cells mimics Ras transformation [M. Ueffing, S. Obici, A.M. Musti, M.G. Gottesman, and V.E. Avvedimento, unpubl.]. Conversely, inhibition of PKC restores TgTF1 binding in cells exposed to Ras [M. Bonapace, E. Benusiglio, M.E. Gottesman, and V.E. Avvedimento, unpubl.]. How the increase in PKC and the down-regulation of PKA activities by Ras are related to its induction of cell proliferation is a subject of lively interest in our laboratory.

Materials and methods

Cell lines

The rat thyroid line, TL (previously referred to as FRTL), grows in chemically defined medium [Ambesi-Impiombato et al. 1980]. It was transformed with a KMSV bearing a temperature-sensitive v-ras allele (Shih et al. 1970), yielding clone Ats [Colletta et al. 1983]. Ats cells were transfected with a pTg-NEO fusion, and G418-resistant derivatives [Ats-aza] clones were isolated after selection with 5-azacytidine [Avvedimento et al. 1989].

RNA analysis

RNA was isolated from cells by the quanidiunum thiocyanate–acid phenol procedure [Chirgwin et al. 1979]. RNase protection assays with antisense RNA probes synthesized with SP6 RNA polymerase employed standard protocols [Melton et al. 1984]. The Tg-NEO probe was described previously [Avvedimento et al. 1989].

DNA-binding assays

Assays for TgTF1 binding used oligo(C), a 25-bp oligonucleotide derived from pTg, which includes the high-affinity TgTF1–binding site at −60 relative to the transcription start site derived from pTg, which includes the high-affinity TgTF1–binding site at −60 relative to the transcription start site. The oligo(C) is 5'‐GCCCACGCCCCAGTCAAGTGTTFCT. The TgTFl core-binding site is underlined. Oligo(C*) is identical to oligo(C), except for a 3'-end substitution in which C is replaced by T.

As control, oligo(A) [5'-GATTACTCAAGTATTCTTAAC-3'] derived from the −150 region of pTg was used. Oligo(A) binds a non-tissue-specific nuclear factor(s) [Civitareale et al. 1989; Ursini et al. 1989]. The sequence of oligo(C) is 5'-GCCACGCCCCAGTCAAGTGTTFCT. The TgTF1 core-binding site is underlined. Oligo(C*) is identical to oligo(C), except for a mutant core, CAAGTATT.

As control, oligo(A) [5'-GATTACTCAAGTATTCTTAAC-3'] derived from the −150 region of pTg was used. Oligo(A) binds a non-tissue-specific nuclear factor(s) [Civitareale et al. 1989; Ursini et al. 1989]. The DNA oligonucleotides were end-labeled with T4 polynucleotide kinase [Maniatis et al. 1982], and 50 fmol were incubated for 15 min at room temperature with 5 μg of nuclear proteins [Wu 1985] in the presence of 2 μg/ml of poly(dI‐dC), 5 mM spermidine in 10% glycerol, 10 mM HEPES (pH 7.9), 50 mM NaCl, and 0.1 mM PMSF in a final volume of 20 μl. After incubation, the reaction mixture was brought to 5 mM DTT. The suspended cells were passed 20 times through 1-ml syringes (needle size #23) to obtain cytosolic extract. Assays [final volume 100 μl] were performed at 30°C for 10 min in a solution containing 100 μM [32P]ATP (125–150 cpm/pmol) 5 mM MgAc, 15 μM Kemptide [Sigma], 10 mM Tris-HCl (pH 7.4), 250 μM 3-isobutyl-1-methyIxanthine, 5 mM DTT, 2.5 mM NaF, and 40–100 μg of cellular proteins. Where indicated, cAMP was added at 5 μM. Kemptide phosphorylation was monitored by spotting 50 μl of incubation mixture on phosphocellulose filters [Whatman, P81] and washing with 75 mM phosphoric acid as described [Clegg et al. 1987]. At concentrations of extracts exceeding 200 μg/ml the reaction was no longer linear. The units of PKA represent picomoles of [32P]phosphate transferred to the peptide substrate per 10 min of incubation in the presence of cAMP.

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