Inhibition of c-Ras Reactivates Thyroglobulin Synthesis in Middle-T Transformed Thyroid Cells

Bruno Di Jeso, Antonio Feliciello, Francesco Pacifico, Antonio Iannelli, Franco D’Armiento, Domenico Liguori, Paola Giuliano, Giuseppe Ferulano, and Enrico V. Avvedimento


Abstract

Differentiated thyroid cells expressing polyoma Middle-T became transformed and tumorigenic when injected into syngenic animals. The expression of thyroglobulin was greatly reduced and no longer responsive to thyrotropin (TSH) and to cAMP. Inhibition of endogenous c-ras by the expression of two transdominant negative mutant H-ras genes, Asn17 and Leu61-Ser186, reactivated thyroglobulin synthesis. Reactivation of thyroglobulin synthesis by c-ras inhibition was not observed in the absence of TSH. These findings indicate that MT elicits dedifferentiation of thyroid cells by activating endogenous c-ras and that c-ras interferes with TSH or cAMP signaling.

Introduction

Cellular transformation interferes with the expression of differentiation genes in several cell types (1–4). The thyroid transformed phenotype is almost invariably associated with the loss of expression of differentiation markers. The dedifferentiated phenotype appears to be independent on the type of oncogene expressed. Viral oncogenes ras, bas, src, mos, raf, and MT1 from polyomavirus inhibit, to various extents, the expression of the thyroid-specific genes (5–7).

Polyoma MT associates with several cellular proteins in transformed cells. These include several members of the src family of tyrosine-kinases (pp62c-yes, pp60c-src, pp59c-fyn) (Refs. 8–10), PI-3' kinase (11), and recently shc (12). It is now known that all the aforementioned proteins are involved in a common signal transduction pathway or whether several distinct pathways are used by MT to elicit transformation and dedifferentiation. There are several indications that p21-Ras is the central point where tyrosine-kinase receptors converge to transduce their signals. In the last 2 years, molecular adaptors have been found to link membrane tyrosine-kinase receptors to v-Ras and v-Raf (13). Revertants of Ras-transformed mouse cells are resistant to transformation by pp60v-src (14). Since MT mutants, which fail to associate or stimulate pp60 src, are transformation deficient (15), it is possible that MT, as pp60 src, requires p21 Ras activity to cause dedifferentiation and transformation. To test this hypothesis, we have made use of two transdominant negative mutant ras genes, Asn17 and Leu61-Ser186, and we have determined whether they affect Tg expression in MT-transformed thyroid cells. Here we report that MT expression in thyroid cells inhibits Tg expression and that inactivation of endogenous ras reverses this inhibition. These results indicate that MT requires an active cellular ras to cause dedifferentiation of thyroid cells.

Results and Discussion

Polyoma MT Dedifferentiates Thyroid Cells and Inhibits Tg Expression. Differentiated thyroid cells (FRTL-5 cell line) were cotransfected with a plasmid containing polyoma MT under the control of the virus early promoter (16) and the neomycin resistance gene driven by the long terminal repeats of the Rous sarcoma virus. We first selected for neomycin-resistant cells and eventually screened for TSH-independent clones. From 80 to 90% of neomycin-resistant clones (neo+) were also expressing polyoma MT sequences (data not shown). These clones expressing MT were also TSH-independent for growth. A pool of about 100 clones expressing MT antigen (L5-MT cells) was further analyzed and characterized. L5-MT cells became tumorigenic when injected in syngenic rates (Table 1). Tumors appeared at the site of inoculation after a latency period of 3–4 weeks, reached a diameter of 1–3 cm, and then remained stable. The tumors were excised and subjected to histochemical analysis. The structural organization was peculiar; follicularlike structures, resembling thyroid follicles, were evident (Fig. 1A, arrows). The cells were stained by anti-cytokeratin and anti-thyroglobulin antibodies (Fig. 1, B and C, respectively). The tumors closely resembled papillary carcinomas of the thyroid.

The expression of thyroid differentiation genes was analyzed in MT-expressing cells. mRNA levels of both Tg and TSH receptor decreased ~10-fold compared to untransformed cells (Fig. 2). We also determined the Tg protein levels in L5 and L5-MT cells. In MT cells, the decreased expression of Tg at the protein level closely paralleled the decreased expression of Tg mRNA (reduction to ~10% relative to L5 cells), (Fig. 3). Interestingly, cell lines established from the tumors induced by MT cells revealed a synthesis of Tg comparable to that of MT cells (Fig. 3). The iodide carrier activity was also profoundly reduced (Table 2). The residual iodide carrier activity displayed the same $K_m$ of the normal carrier (Table 2).

The residual Tg synthesis did not increase following TSH stimulation. This was not caused by the reduction of TSH receptor molecules in L5-MT cells, since stimulation of adenyl cyclase by cholera toxin, pertussis toxin, or for-
kolin did not induce Tg expression (Fig. 4 and data not shown). Consistent with these data, we did not find any difference in the cAMP steady-state levels in MT cells compared to the differentiated controls in cells either starved in 0.2% BSA or grown in the 6H mixture (which includes TSH) (Table 3). However, in the presence of the 6H mixture, the cAMP levels were increased both in L5neo+ and in MT cells. These data indicate that in MT cells the TSHR, albeit reduced, was functional. Also, Tg promoters fusions (pTgCAT) were not efficiently transcribed in L5-MT cells and were not stimulated by cAMP (17). These data indicate that in thyroid cells, MT interferes downstream with cAMP in the transduction of signals from the membrane to the nucleus.

Inhibition of Endogenous c-Ras Restores Tg Synthesis in MT-transformed Thyroid Cells. To dissect the MT-activated pathway leading to dedifferentiation of thyroid cells, we have used two transdominant negative mutants of the ras gene, Asn17 and Leu61–Ser186. Asn17 interferes with the GDP/GTP exchange reaction stimulated by guanine nucleotide-releasing factors. This mutant, in which Ser17 has been changed to Asn, inhibits the proliferation of NIH 3T3 cells (18) and neuronal differentiation in PC12 cells (19). The growth-inhibitory effect of Ha-Ras Asn17 can be bypassed by expression of Raf or Mos oncogene, both of which function downstream from cellular Ras proteins (20). Leu61–Ser186 has a serine at position 186, replacing a cysteine. This alteration prevents proper membrane attachment and consequently renders the protein nonfunctional (21). Furthermore, the mutant protein has a change at position 61, where leucine is substituted for glutamine, resulting in a loss of GTPase activity and an affinity for the GTPase-activating protein that is 50-fold higher than that of wild-type p21-ras (22, 23). This mutant has been proposed to inhibit the function of p21-ras by competition for its cellular targets (22, 24).

We transfected L5-MT cells with vectors expressing Asn17 and Leu61–Ser186 under the control of the RSV promoter. We measured Tg synthesis 36 h after transfection by immunoprecipitation of [35S]-methionine-labeled proteins. Fig. 5, A and B, shows that Tg synthesis was greatly stimulated in LT-MT cells transfected with Asn17 and Leu61–Ser186, as compared to control cells transfected with the neomycin resistance gene under the control of the RSV promoter (NEO). We also transfected L5-MT cells with other DNA constructs and did not find any change in Tg synthesis. It has been shown that Tg gene expression is in part constitutive and in part regulated by TSH (25). To test which part of Tg expression was inhibited by MT, we measured Tg synthesis in Asn17-transfected cells in the presence and in the absence of the 6H mixture (which includes TSH). Fig. 5C shows that Tg synthesis was stimulated by Asn17 only in the presence of the 6H mixture. This finding indicates that c-Ras inactivation restores TSH sensitivity in polyoma-transformed thyroid cells and suggests that c-Ras mediates MT inhibition of thyroglobulin expression. It should be noted that, in MT cells, the TSHR is functional, albeit reduced (Table 3).

It is well known that transformation interferes with the expression of differentiation genes (1–4). Recently, however, progress has been made on the mechanisms by which oncogenes inhibit the expression of differentiation genes. For example, LT inhibits terminal differentiation of myoblasts by binding to the product of the retinoblastoma susceptibility gene (26) and c-myc by down-regulating the expression of the myoD family gene (27). The focus of this study was to identify the step(s) mediating the inhibition of

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**Table 1** Tumorigenicity of FRTL-5 cells transfected by polyoma middle-T oncogene

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Tumor incidence</th>
<th>Latency period</th>
<th>Histological type of tumor</th>
</tr>
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<tbody>
<tr>
<td>L5neo+</td>
<td>0/9</td>
<td></td>
<td>Differentiated carcinoma</td>
</tr>
<tr>
<td>L5-MT</td>
<td>27/30</td>
<td>1–4 weeks</td>
<td>Differentiated carcinoma</td>
</tr>
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* Number of animals with tumor/number of animals injected with $2 \times 10^6$ cells/animal.

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![Fig 1](image-url) Morphology and immunocytochemistry of the tumors induced by MT. A, Hematoxylin and eosin (x 400) section shows solid masses of cells with nuclear atypia and small follicles (arrows); B, anti-cytokeratin (x 106); C, anti-thyroglobulin (x 250).
Fig. 1. TG protein levels in L5neo+, L5-MT cells, and in cell lines established from tumors. Normal and transformed cells were grown in medium containing 5% calf serum and the 6H mixture. Ten μg total RNA were loaded for each lane. The same filter was sequentially hybridized with TG and GADPH (A) or TG and GADPH (B). The expression of TG was decreased also in another thyroid cell line transformed with MT, the P3-13 cells, although to a lesser extent (7).

Fig. 2. TG and TSHR mRNA expression in L5neo+ and L5-MT cells. Northern blot analysis of TG (A), TSHR (B), and glyceraldehyde-3-phosphate dehydrogenase (GADPH; A and B) mRNAs in L5neo+ (LS) and L5-MT. Normal and transformed cells were grown in medium containing 5% calf serum and the 6H mixture. Ten μg of total RNA were loaded for each lane. The same filter was sequentially hybridized with TG and GADPH (A) or TSHR and GADPH (B). The expression of TG was decreased also in another thyroid cell line transformed with MT, the P3-13 cells, although to a lesser extent (7).

Table 2. Iodine uptake in L5neo+ and L5-MT cells

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Vmax (μmol/μg DNA/min)</th>
<th>Km (μmol/μg DNA)</th>
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<tr>
<td>L5neo+</td>
<td>12.7 ± 1.7</td>
<td>50 ± 0.7</td>
</tr>
<tr>
<td>L5-MT</td>
<td>3.6 ± 0.6</td>
<td>50 ± 0.8</td>
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Tg expression elicited by MT. The data reported above indicate that Ras p21 is an essential step in the inhibition of Tg synthesis by MT. We have extensively characterized the mechanism by which Ki-Ras dedifferentiates thyroid cells (28, 29). One of the early events stimulated by Ki-Ras is the down regulation of nuclear protein kinase A mediated by protein kinase C leading to the down-regulation of the cAMP-induced thyroid genes. This effect cannot be overcome by cAMP (29). Stimulation of cellular Ras by MT may mimic the effects caused by oncogenic Ki-Ras, i.e., down-regulation of the nuclear PKA and inhibition of the transcription of thyroglobulin gene (5, 28, 29). We have evidence indicating that also in differentiated FRTL-5 cells the inhibition of cRas leads to stimulation of Tg expression induced by TSH (5). This suggests that in normal differentiated thyroid cells, c-Ras inhibits and cAMP activates the differentiation of thyroid cells. The interplay between these signals may regulate the coordinate expression of thyroid-specific genes.

As to the pathway connecting MT to p21-Ras, the recent finding that MT binds and stimulates tyrosine phosphorylation of shc protein provides an important clue (12). It is possible that MT brings p60src close to shc, which becomes

* B. Di Jeso et al., manuscript in preparation.
tyrosine phosphorylated. In this configuration, the complex binds Grb2 and possibly stimulates p21-Ras via the mammalian guanine nucleotide-releasing factor Sos (13). The reposition of the GRF to the membrane, close to p21, has been proposed as mechanism for p21 activation. MT is also located on the membrane and could facilitate the formation of a multiprotein complex leading to the activation of p21 Ras.

Materials and Methods

Materials. Guanidine thiocyanate was obtained from Fluka. α[32P]dGTP, α[32P]dATP (800 Ci/mmol), and [35S]methionine (1000 Ci/mmol) were from Dupont NEN Research Laboratories (Boston, MA). Restriction endonucleases were purchased from Boehringer (Indianapolis, IN). The random priming labeling reagents were from Amershamb. All antibodies for the immunocytochemical analysis were from Dako (Glostrup, Denmark).

Cell Lines. FRTL-5 cells are a cloned line of thyroid differentiated cells (30). FRTL-5 neo+ (L5) are FRTL-5 cells transfected with RSVneo; FRTL-5 PyMT (L5-MT) are FRTL-5 cells cotransfected with RSVneo and p53.A6.6, a plasmid expressing the middle-T antigen of polyomavirus (16).

Cell Culture. L5 and L5-MT were cultured in Coon's modified Ham's F-12 medium supplemented with 5% calf serum (GIBCO, Grand Island, NY), a six-hormone (6H) mixture (1 μg/ml insulin, 1 milliunit/ml TSH, 10 ng/ml glucyl-histidyl-lysin, 5 μg/ml human transferrin, 10 nm cortisone, 10 ng/ml somatostatin), and 50 μg/ml G418. In some experiments, cells were starved for 4–5 days in medium containing 0.2% BSA but lacking serum and hormones.

Transfection of FRTL-5 Cells and Selection of Stable Transformants. FRTL-5 cells were plated 48 h before transfection at a density of 0.5 × 10^4 cells/100-mm diameter dish. Calcium phosphate precipitates of plasmid DNA were made with a 20:1 molar ratio of relevant plasmid to RSVneo. Precipitate DNAs were added to the culture medium, and 4 h later, the cells were washed twice with 10 ml of serum-free medium before replacement with medium containing 5% calf serum and the 6H mixture. After an additional 30 h the medium was replaced with selection medium containing 50 μg/ml G418. After approximately 14 days, about 100 colonies of neo+ cells were apparent.

Establishment of Cell Lines from Tumors. Tumors were excised from the animals, minced, and washed three times with PBS. After an incubation of 3 h at 37°C in PBS containing 20 units/ml trypsin (ICN Pharmaceuticals), the suspension was allowed to settle for 2 min; then the supernatants were collected, washed in complete medium, and plated in complete medium plus 5% calf serum, the 6H mixture, and 50 μg/ml G418.

Immunocytochemical Analysis. The biopsy specimens were fixed in 10% formalin and stained with hematoxylin and eosin. The following mAb were used for the immunocytochemical staining with a peroxidase anti-peroxidase reaction: anti-cytokeratin, marker for epithelial cells; and anti-thyroglobulin, marker for thyroid cells. For negative control staining, anti-leukocyte common antigen and antivimentin were used.

Protein Labeling and Immunoprecipitation. Cells were labeled with [35S]-methionine (1000 Ci/mmol; 30 μCi/ml medium) for 3 h in methionine-free medium plus 5% calf serum and the 6H mixture. Medium was aspirated, and cells were lysed as described previously (31). Immunoprecipitation and SDS-PAGE were carried out as described previously (31).

Hybridization Probes. The Tg cDNA probe was a 680-bp EcoRI-PstI fragment corresponding to the 3’ end of the rat Tg cDNA (32). The TSHR probe was the purified insert from the clone T8A6B and represent residues 54 to 2780 of the nucleotide sequence reported for the rat FRTL-5 TSHR (33).

RNA Isolation and Northern Blot Analysis. RNA isolation and Northern blot analysis were carried out as described previously (7).

Other Procedures. Iodine uptake measurements were carried out as reported previously (7). The protein determinations were performed using the Bio-Rad protein assay reagent.

Acknowledgments

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References

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