Antipain-induced Suppression of Oncogene Expression in H-ras-transformed NIH3T3 Cells

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ABSTRACT

Antipain (AP; 50 µg/ml) inhibits transformation of NIH3T3 cells after transfection with an activated H-ras oncogene. To determine whether AP effects on transformation are associated with alterations in oncogene expression, NIH3T3 cells were cotransfected with an activated H-ras oncogene and the selectable marker gene aph, and gene expression was quantified. Fifty percent of geneticin-resistant colonies which were exposed to AP failed to express the transformed phenotype as determined by their inability to grow in soft agar. Northern blot analysis of the transformed and nontransformed colonies revealed that suppression of H-ras transformation by AP was associated with a decrease in expression of the exogenously transfected H-ras gene by approximately 4-fold. Expression of the endogenous oncogene c-myc was decreased by approximately 2.5-fold, to levels seen in untransfected cells. AP-treated colonies that retained the transformed phenotype had levels of oncogene expression that were similar to untreated ras-transformed colonies. Southern blot analysis revealed no effects of AP on incorporation or copy number of the H-ras gene.

INTRODUCTION

Proteases play an important but poorly understood role in the process of cell transformation. Inhibitors of these protein-degrading enzymes represent, nevertheless, a well-studied class of chemopreventive and antineoplastic agents with demonstrable activity in a variety of in vivo or in vitro systems (1, 2).

Previous studies from our laboratory have shown that protease inhibitors, including the tripeptide AP, inhibit transformation of NIH3T3 cells after transfection with an activated H-ras oncogene (3). The molecular mechanisms of this effect, like most of the transformation-suppressing effects of protease inhibitors, are unknown. This question is particularly difficult to address in models involving chemical- or radiation-induced transformation since the molecular events responsible for transformation are themselves not known. When the transforming agent is an activated oncogene, however, it is logical to begin exploration of the mechanisms of transformation inhibition with studies on effects related to oncogene function. Our earlier work suggested that AP did not affect the transfection process itself or interfere with stable incorporation of the ras gene (3).

We hypothesized, therefore, that the ability of AP to suppress oncogenic transformation is associated with its ability to modulate gene expression.

There is evidence that AP modulates gene expression in untransformed cells. Exposure to AP decreases c-myc and serum-stimulated c-fos levels in normal C3H10T1/2 cells which are in the exponential phase of growth (4, 5). This effect is not related to alterations in growth rate or changes in total RNA synthesis or degradation. Exposure to AP does not affect or slightly decreases c-myc levels in cells which have already undergone chemical or radiation transformation, respectively (6).

We have continued our studies concerning the suppression of ras-induced cell transformation by the model protease inhibitor AP with a focus on the expression of the transected ras gene as well as the endogenous c-myc gene. To test the hypothesis that effects of AP on gene expression may be relevant to the functional effects of AP on the process of transformation, NIH3T3 cells were cotransfected with an activated H-ras oncogene and the selectable marker gene aph and were grown in the presence or absence of AP. Northern blot analysis of G418-resistant colonies permitted a direct analysis of gene expression in those cells which had taken up the H-ras oncogene but whose transformation was suppressed by AP treatment as determined by the loss of anchorage-independent growth.

MATERIALS AND METHODS

Cell Culture. NIH3T3 cells were grown in DMEM supplemented with 10% fetal bovine serum, 2 mm l-glutamine, and antibiotics. Cells were subcultured before reaching confluence.

Transfection. NIH3T3 cells were seeded at 5 x 10⁶ cells/25-cm² tissue culture flask on Day 0 into AP-containing or control medium. An AP stock solution (50 mg/ml) was prepared in phosphate-buffered saline (pH 7.2) and stored at -20°C. Media were prepared by adding aliquots of the stock solution to supplemented DMEM. Transfections were performed by the calcium phosphate precipitation method as described by Wigler et al. (7). Plasmids used in these studies included pEG, which contains the 6.6-kilobase fragment of a mutagenically activated H-ras gene isolated from human bladder carcinoma (8); pSV2neo, which contains the neomycin resistance gene aph (9); and pAGT, which contains both an activated human H-ras gene and the neomycin resistance gene aph (gift of Dr. D. Spandidos; 10). Plasmid DNA (0.2 µg) was mixed with NIH3T3 DNA (20 µg) as a carrier and transfected on Day 1. Cultures were fed with fresh medium approximately 18 h after transfection. Cells were split at a 1:3 ratio 48 h after transfection into selection medium containing 0.3 µg/ml geneticin (G418) on Day 2. Medium was changed twice/week thereafter. Colonies were picked on Day 20 and expanded into mass culture (approximately 20 x 10⁶ cells).

Northern and Southern Blot Analysis. DNA and RNA were isolated from cell pellets prepared from subconfluent cultures (approximately 20 x 10⁶ cells). DNA was obtained by phenol extraction as previously described (3). RNA was extracted from pellets using the RNAzol method (Cinna/Biotex, Friendswood, TX). DNA samples (10 µg) were digested with BamHI and separated on 0.8% agarose gels. RNA samples (10 µg) were glyoxalated according to the procedure of Williams and Mason (11) and separated on 1.0% agarose gels. DNA and RNA were transferred onto Zetabind (Cuno, Meriden, CT) membranes overnight and baked for 1.5 h at 80°C. Filters were washed at 65°C for 0.5 h in 0.1 x SSC (0.15 M NaCl and 0.015 M trisodium citrate) and 0.5% sodium dodecyl sulfate to remove the tracking dye front, according to the manufacturer's instructions. Prehybridization of membranes was performed for at least 2 h at 65°C in a solution containing 6x SSC, 0.05 M Na₂HPO₄, 10 x Denhardt's solution (0.2% Ficoll, polyvinylpyrrolidone, and bovine serum albumin), 1.0% glycine, and 1.0 ml of sheared and boiled salmon sperm DNA. DNA probes were prepared.
from pEJ (H-ras); pBS4, the PstI fragment from the third exon of rat c-myc which was a gift from P. Tsichlis; and pHFBA-1, the 2.0-kilobase XhoI fragment of human β actin, obtained from American Type Culture Collection, Rockville, MD. Probes were nick-translated to a specific activity of $10^7$ to $10^8$ cpm/μg using [32P]dCTP (New England Nuclear, Wilmington, DE) according to the procedure of Maniatis et al. (12).

Hybridizations were performed in 6x SSC, 0.08 M NaH2PO4, and 10% dextran sulfate at 65°C overnight. Filters were washed at a final stringency of 0.1x SSC + 0.1% sodium dodecyl sulfate at 65°C and exposed to Kodak XAR-5 film with DuPont Cronex intensifying screens at -70°C for 1-7 days. Signal intensity was determined by densitometry. After hybridization with oncogene probes, RNA filters were stripped in boiling double-distilled water and rehybridized to actin for standardization purposes. Common standards were included on blots to allow interblot comparisons.

Data Analysis. Southern blots were scanned by laser densitometry. Only those bands of 6.6 kilobases (the size of the H-ras insert in the pEJ plasmid) or greater were quantified. Gene copy number was determined relative to that of the normal human fibroblast cell line, MRC-5. Gene expression on Northern blots was quantified by laser densitometry, and values were standardized by actin expression. Differences were analyzed for statistical significance using a one-tailed Student's t test.

Soft Agar Growth. Cells were tested for anchorage-independent growth by plating in soft agar according to the procedure of MacPherson and Montagner (13). Cells (5 x 10^4) were suspended in 2 ml of a 1:3 mixture of supplemented DMEM and agar medium (0.5% Difco agar, 80% DMEM, 10% Gibco tryptose phosphate broth, and 10% fetal bovine serum) and layered onto 7 ml of agar medium. Cells were fed twice/week with appropriate media. Colony growth was scored after approximately 3 weeks.

RESULTS

Effects of Serum. All transfections have routinely been done in medium containing 10% serum. Because serum contains low levels of protease inhibitors, it was important to examine whether changing serum concentrations could alter the effects of AP. As shown in Table 1, transfection efficiencies were independent of serum content of the media, with and without AP exposure. At levels of 0.2% serum, NIH3T3 cell survival was too low to provide usable data.

Antipain Effects on pAGT Transfection. Although AP had no effect on the transfection efficiency of the pSV2neo plasmid, as determined by colony yield after selection with G418 (3), this experiment did not necessarily rule out a specific action of AP on transformation of the pEJ plasmid harboring the ras gene. NIH3T3 cells were, therefore, transfected with pAGT, which contains both an activated human H-ras oncogene and the selectable aph gene. Replicate plates of cells were either treated with G418 to select for neoresistant colonies or allowed to form foci in the absence of selection. As shown in Table 2, the number of colonies and foci seen in the presence and absence of selection, respectively, were similar. These data indicate that the effects of the two genes residing on the same plasmid vector are comparable. However, only the yield of foci was diminished by AP treatment, while the number of G418-resistant colonies actually increased. This experiment clearly shows that the effects of AP on transformation are not related to the stable incorporation of the transfected plasmids but are, instead, specifically associated with mechanisms involving the ras gene itself.

Cotransfection Experiments. In order to study the potential molecular mechanisms by which AP suppresses ras-induced transformation, it was necessary to examine isolated clones of cells that were stably transfected with ras but failed to express the transformed phenotype as a result of AP treatment. Since a very small fraction of the cells in a monolayer of NIH3T3 is actually transfected, data from experiments on the entire treated plate of cells would not be meaningful.

As discussed above, AP has been shown to have no effect on the function of the transfected aph gene which confers resistance to the antibiotic G418 (3). Our strategy, therefore, was to select colonies after cotransfection with pSV2neo and an excess of pEJ in the presence of G418. Surviving colonies represent clones of cells that incorporated the aph gene. Because ras is present in 10-fold excess, most of the colonies should also have taken up the ras gene and, therefore, express the transformed phenotype. Isolated resistant colonies that contained the ras oncogene and that were not transformed by the criteria of anchorage independence could be assumed to be the cells on which AP had exerted its inhibitory effects.

Treatment with AP was previously shown to inhibit H-ras-induced NIH3T3 cell transformation by a maximum of 50%; therefore, half the G418-selected colonies in the cotransfection experiment treated with AP were expected to be transformed. Ten such colonies were isolated after cotransfection with pSV2neo and pEJ. During the transfection experiment cultures were treated with 50 μg/ml AP. In a parallel experiment 5 colonies were isolated that had not been treated with AP. Each isolated colony was grown to mass culture and established as a stable cell line. As shown in Table 3, each of the 5 untreated colonies had the transformed phenotype of soft agar growth. These results are consistent with the frequency of transformation inhibition by AP found earlier (3).

ras Oncogene Incorporation. Southern blot analysis revealed that 2 of the 6 nontransformed lines, 926-44 and 926-54-2, had failed to incorporate the ras oncogene as shown in Fig. 1. However, as shown in Table 3, the other 4 cell lines which were exposed to AP and were negative for transformation had on average a similar number of copies of the transfected EJ gene (4.42 ± 1.60, mean ± SEM) as the 4 positive lines which were exposed to AP (3.87 ± 0.77) and the 5 positive lines which served as untreated controls (4.90 ± 1.17). This result confirms our earlier conclusions based on indirect evidence that the action of AP in inhibiting transformation is not related to effects on transfection or stable incorporation of the exogenous gene. Cell lines 926-44 and 926-54-2 which did not incorporate the ras gene in the cotransfection experiment served as controls for the effects of transfection, colony selection, and

### Table 1

<table>
<thead>
<tr>
<th>% serum AP/control</th>
<th>Foci/μg DNA vehicle control</th>
<th>Foci/μg DNA + AP (50 μg/ml)</th>
<th>Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>994 ± 62*</td>
<td>573 ± 35</td>
<td>0.57</td>
</tr>
<tr>
<td>5</td>
<td>1080 ± 55</td>
<td>595 ± 30</td>
<td>0.55</td>
</tr>
<tr>
<td>1</td>
<td>1090 ± 59</td>
<td>590 ± 54</td>
<td>0.54</td>
</tr>
</tbody>
</table>

* Mean ± SEM (n = 9).

### Table 2

<table>
<thead>
<tr>
<th></th>
<th>Foci/μg DNA</th>
<th>Colonies/μg DNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>205 ± 20</td>
<td>166 ± 17</td>
</tr>
<tr>
<td>Antipain (50 μg/ml)</td>
<td>95 ± 16</td>
<td>302 ± 24</td>
</tr>
<tr>
<td>Ratio (A/C)</td>
<td>0.46</td>
<td>1.8</td>
</tr>
</tbody>
</table>

* Number of transformed foci obtained/μg DNA.
* G418-resistant colonies/μg DNA.
growth of nontransformed cells in the c-myc gene expression studies described below.

ras Oncogene Expression. Fig. 2 presents Northern blot analyses of mRNA levels of the transfected H-ras gene in untreated and AP-exposed cell lines, respectively. The densitometry results are presented in Table 3. H-ras expression was similar in all transformed cell lines whether untreated or AP exposed. In contrast, levels of H-ras mRNA were decreased approximately 4-fold in the AP-treated cell lines whose transformed phenotype was suppressed (P < 0.05; Table 3).

c-myc Oncogene Expression. Fig. 3 presents Northern blot analyses of mRNA levels of the c-myc gene in untreated and AP-exposed cell lines, respectively. As in the case for the transfected ras gene, all transformed cell lines expressed comparable levels of c-myc independently of AP treatment. The level of c-myc expression in nontransformed AP-treated cell lines was approximately 2.5-fold less than in their transformed counterparts (P < 0.10; Table 3). The levels of expression of the endogenous c-myc in cell lines 926-44 and 926-54-2 which did not incorporate the ras gene in the cotransfection experiment were very similar to those seen in H-ras-containing cell lines which were exposed to AP but not transformed (0.644 versus 0.599, respectively). These levels of c-myc were also similar to those in untransformed NIH3T3 cells (0.367). These results suggest that the transfection process itself does not result in altered patterns of c-myc oncogene expression but that transformation by H-ras involves an increase in expression of the endogenous c-myc gene. Furthermore, when such transformation is inhibited by AP, there is a concomitant suppression of c-myc expression to control levels.

DISCUSSION

AP-induced suppression of oncogenic transformation of NIH3T3 cells is associated with alterations in oncogene expression of both the exogenously transfected transforming gene, H-ras, and the endogenous oncogene c-myc. The AP effect on gene expression is seen only in those colonies whose transformation phenotype was inhibited by AP. The reason that approximately half of the transfected cells escape the suppressive effects of AP is still not known. However, the results presented here confirm our earlier findings using focus-forming efficiency of the ras gene and rule out artifactual explanations such as delays in cell division or toxicity. The fact that no decrease in expression was seen in treated cells that escaped the antineoplastic effects of AP lends strong evidence to a mechanistic link between AP-induced suppression of both gene expression and transformation.

The decrease in expression of both H-ras and c-myc is of particular interest, because these oncogenes are considered complementary in the process of transformation of primary cells (14). It is not clear whether the expression of both of these genes must be suppressed in order for AP to exert its transformation inhibitory activity or whether suppression of either one would be sufficient. It may be significant, however, that in each of the inhibited lines examined, expression of both genes was decreased. Further investigation of this issue is clearly warranted. Although the immortalized cell line NIH3T3 is capable of one-step transformation by ras oncogenes, the cells may already contain genetic changes which facilitate conversion to the malignant phenotype upon acquisition of a mutated ras gene. An approximate 2.5-fold enhancement of c-myc expression was found in the transformed cell lines in comparison to NIH3T3 cells or the transfected cell lines 926-44 and 926-54-2, which did not take up the transforming gene. Enhanced c-myc expression has been observed in other systems following ras transformation. Both C3H10T1/2 cells (15) and rat kidney cells (16) express elevated levels of c-myc following ras transfection. In rat kidney cells, enhanced expression of c-myc was attributed to deregulation of the c-myc gene following ras transformation. Although the mechanism by which ras transformation results in enhanced expression of c-myc in NIH3T3 cells is not yet known, the enhanced c-myc expression may be necessary for the maintenance of the transformed phenotype. This idea is supported by the data which show that the suppression of the transformed phenotype which occurs upon exposure to AP is associated with decreases of c-myc levels to those found in NIH3T3 parent cells or transfected control cells. Since AP has been shown to suppress c-myc expression in normal 10T1/2 cells, (6), such an effect in transfected pretransformed NIH3T3 cells may very likely contribute to the transformation inhibitory activity of AP that we observed in this system.

We have previously found that AP appears to be a stage-specific inhibitor of H-ras transformation. Maximal inhibitory activity (approximately 50%) is observed when transfected cells are exposed at the time of subculture (Day 3) and subsequent cell proliferation. Antipain is inactive, however, when applied only before the subculture step or during the postconfluent phase of the experiment. Antipain, therefore, appears to block a proliferation-associated process(es) which is critical in the development of transformation. These processes may involve or require a high level of expression of c-myc and the mutated H-ras gene.

Although the chemopreventive effects of AP have not previously been specifically linked to alterations in oncogene expression, various other chemopreventive agents have been shown to decrease ras and/or myc expression during differentiation of malignant cell lines. The differentiation of human colon carcinoma cells by N-methylformamide is associated with a decrease in c-myc expression (17). 13-O-Tetradecanoylphorbol-12-acetate-induced differentiation of the human neuroblastoma cell line...
activity of ras, resulting in a continuous flow of signal transduction (20). ras transformation has been shown to modulate numerous cellular functions, including those involved in signal transduction, such as protein kinase C activity (21), phosphoinositide turnover (22), and calcium mobilization (23). ras transformation has also been shown to alter cellular responsiveness to a variety of growth factors (24) as well as growth factor production (25). ras-transfected NIH3T3 cells, for example, have been shown to secrete elevated levels of the transforming growth factors α and β (25). Changes in the production of proteases have also been associated with transformation by ras. H-ras-transformed NIH3T3 cells have been shown to exhibit an altered profile of membrane-associated proteases (26) and secrete more plasminogen activator of the nondetermined type in comparison to the parent NIH3T3 cells (27, 28). In those ras-transformed lines which exhibit metastatic potential, type IV collagenase activity is enhanced as well (28). Interestingly, proteases have been shown to activate latent growth factors including transforming growth factor β (29), and growth fac-

line SH-SY5Y, which contains an activated N-ras oncogene and constitutively expresses c-myc, has been correlated with a decrease in c-myc expression (18). The monocytic differentiation of HL60/MRI cells induced by retinoic acid is associated with a 3-fold lower expression of c-myc and a 2-fold lower expression of N-ras (19).

The mechanism by which mutated ras can transform cells is not fully understood. Point mutations in ras, such as the G-T transversion in pEJ, are believed to alter the intrinsic GTPase

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**Fig. 1.** Southern Blot analysis of plasmid pEJ incorporation in transfected cell lines. Individual G418-resistant colonies were picked following cotransfection of H-ras (pEJ) and aph (pSV2 neo) into NIH3T3 cells and grown to mass culture. DNA was isolated by phenol extraction, and samples (10 µg) were digested with BamHI and electrophoresed in 0.8% agarose gels. Samples were transferred to Zetabind, hybridized to a 32P-labeled pEJ probe, and visualized by autoradiography. A, transfected cell lines grown in control medium; B, transfected cell lines grown in the presence of AP (50 µg/ml). T-24 is a human bladder carcinoma cell line containing the mutationally activated H-ras gene.

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**Fig. 2.** Northern analysis of H-ras in transfected cell lines. Individual G418-resistant colonies were picked following cotransfection of H-ras (pEJ) and aph (pSV2 neo) into NIH3T3 cells and grown to mass culture. RNA was isolated by the RNAzol method, and samples (10 µg) were glyoxylated and electrophoresed in 1.0% agarose gels. Samples were transferred to Zetabind, hybridized to a 32P-labeled pEJ probe, and visualized by autoradiography. A, transfected cell lines grown in control medium; B, transfected cell lines grown in the presence of AP (50 µg/ml).
SUPPRESSION OF ONCOGENE EXPRESSION BY ANTIPAIN

Fig. 3. Northern analysis of c-myc in transfected cell lines. Individual G418-resistant colonies were picked following cotransfection of H-ras (pEJ) and aph (psV2neo) into NIH3T3 cells and grown to mass culture. RNA was isolated by the RNAzol method, and samples (10 µg) were glyoxalated and electrophoresed in 1.0% agarose gels. Samples were transferred to Zetabind, hybridized to a 32P-labeled pBS4 probe, and visualized by autoradiography. A, transfected cell lines grown in control medium; B, transfected cell lines grown in the presence of AP (50 µg/ml).


tors, in turn, may also regulate the activity of proteases such as plasminogen activator. The ability of AP to interfere with the cellular function of transformation-induced proteases may be an important mechanism by which it suppresses oncogenic transformation. The regulation of oncogene expression by AP is likely to be indirect, through the modulation of transcriptional or posttranscriptional regulatory mechanisms. Elucidation of the mechanisms by which a protease inhibitor regulates ras and myc expression will provide insight into the chemopreventive action of these compounds.

REFERENCES


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