Inhibition of H-ras Oncogene Transformation of NIH3T3 Cells by Protease Inhibitors

Seymour J. Garte, Diane D. Currie, and Walter Troll

New York University Medical Center, Institute of Environmental Medicine, New York, New York 10016

ABSTRACT

The protease inhibitors antipain, leupeptin, a,-antitrypsin, and e-aminocaproic acid were found to inhibit transformation of NIH3T3 cells after transfection with an activated H-ras oncogene. Antipain was inactive if present only during the first 2 days of the gene transfer protocol or only during the final 10 days of the experiment. However, the full effect was observed when antipain was added at the subculture step on day 3 and during the subsequent cell proliferation. If cells were not subcultured, the yield of the foci per µg of DNA was sharply reduced and addition of antipain did not further suppress the transformation rate. Subculture of NIH3T3 cells 3 days after transfection at lower cell densities resulted in higher transformation efficiency. The results suggest that transformation of NIH3T3 cells by a single mutated oncogene may involve multiple stages including cell proliferation and that part of this process is susceptible to inhibition by protease inhibitors.

INTRODUCTION

The transformation of NIH3T3 cells by DNA-mediated gene transfer, or transfection, has been an important tool in the identification and characterization of viral and cellular transforming oncogenes (1–6). The mechanism by which expression of a single mutated ras gene transforms NIH3T3 fibroblasts is not known. Transformation of primary cells requires expression of at least two active oncoproteins (7), a finding that is consistent with the multistage nature of carcinogenesis in vivo (8).

Proteases have been shown to specifically modify DNA expression (9) and are involved selectively in DNA amplification (10). Support for an active role of proteases in carcinogenesis has come from multiple observations that protease inhibitors suppress transformation in vivo and carcinogenesis in several animal models (11–18). Two laboratories have shown that ras transformation is enhanced by the tumor promoter TPA (19, 20). Treatment of various cells and tissues with the tumor promoter induces proteases, including plasminogen activator (21). The promoting activity of TPA is inhibited by protease inhibitors in vivo and in vitro (see Ref. 9 for review).

We now report that ras transformation of NIH3T3 cells is suppressed by four protease inhibitors, antipain, e-aminocaproic acid, leupeptin, and a,-antitrypsin. The results raise new questions regarding the mechanism of ras oncogene-induced NIH3T3 cell transformation.

MATERIALS AND METHODS

NIH3T3 cells were routinely grown and subcultured before confluence in DMEM supplemented with 10% fetal bovine serum. The pEJ (22) and pSVneo plasmids were provided by Dr. A. Pellicer and DNA was purified according to standard methods (23). The transfection assay was performed as described previously (24) using 20 µg NIH3T3 DNA/plate as a carrier for plasmid DNA. The standard protocol for the assay involved transfection of 5 x 10⁶ cells with a calcium phosphate coprecipitate of plasmid and carrier DNA at day 1. The cells were fed 18 h later (day 2) and were subcultured at 1:3 on day 3. Thereafter cells were fed twice a week. Plates were randomized and coded after subculture.

Protease inhibitors were added as concentrated solutions in sterile DMEM, Dulbecco’s modified Eagle’s medium. The NIH3T3 focus assay provides a useful quantitative tool for analysis of the biochemical events associated with ras oncogene-induced cellular transformation (Fig. 1). A representative dose response for focus formation by pEJ is illustrated in Fig. 2. As shown in Table 1, the number of transformed foci induced by transfection with the H-ras oncogene in NIH3T3 cells was reproducibly and significantly decreased to about 50% of control by addition of protease inhibitors to the media. The concentration dependence of antipain (Fig. 3) indicates a maximal effect at 50 µg/ml of ~50% inhibition, with no further effect at concentrations up to 250 µg/ml. Both e-aminocaproic acid and a1-antitrypsin showed concentration-dependent inhibition of ras-induced transformation (Table 1).

In order to determine whether a general effect of the inhibitors on the incorporation or expression of exogenous genes in the gene transfer assay was responsible for the inhibition of ras transformation, we repeated these experiments using the neomycin resistance gene pSVneo and scored G418 (geneticin)-resistant colonies. As seen in Fig. 3, there was no significant depression in the number of G418-resistant colonies by either 50 or 100 µg/ml antipain. Similar results were obtained using a1-antitrypsin (data not shown). The results on inhibition of H-ras transformation by antipain cannot therefore be explained by effects on the cellular uptake or stable genomic integration of the gene. This conclusion is strengthened by experiments in which antipain was added to the medium during various periods of the 19-day transformation protocol (Table 1). When antipain was present only during the first 2 days (before the 1:3 subculture step), no effect on transforming efficiency was seen. On
the other hand, when antipain was present only after the subculture step at day 3, the complete inhibitory effect was seen. It appears that the early stage of this phase is susceptible to the effects of antipain since addition of the inhibitor starting on day 9 had no effect on transformation. This suggests that antipain is unable to reverse the transformed phenotype once it has been established. In order to further test the ability of antipain to revert H-ras-transformed cells to a normal phenotype the T110 line of NIH3T3 cells previously transformed by transfection with the H-ras oncogene (25) were treated at low seeding density with antipain (50 μg/ml) for two weeks. No morphological evidence of reversion to the normal phenotype was seen.

The period of antipain efficacy in inhibition of NIH3T3 cell transformation corresponds to a period of cell proliferation after the 1:3 subculture step at day 3. Neither antipain or α1-antitrypsin had any effect on the viability or proliferation of NIH3T3 cells (Fig. 4). The possibility that protease inhibitor action involves the growth stage following subculture was tested further. When cells were not subcultured 3 days after transfection, the yield of foci was dramatically reduced compared to that seen following the normal protocol of 1:3 subculture, and antipain exerted no inhibition of transformation (Table 2). These data support the idea that protease inhibitors act during cell proliferation and only block transformation of that fraction of cells which require division in order to express the transformed phenotype.

The observation that subculture and concomitant cell division increased transformation efficiency after transfection of NIH3T3 cells with a single active ras oncogene resembles effects described by Kennedy and others of X-ray and chemically induced transformation in other cell systems (26). We extended this observation by comparing the transformation efficiency of the ras gene in NIH3T3 cells subcultured at different densities. As seen in Table 2, splitting cells at day 3 to lower cell densities yielded increased numbers of foci per μg of DNA.

**DISCUSSION**

The NIH3T3 line of established mouse fibroblasts is exquisitely sensitive to transformation by the ras family and other...
interference by protease inhibitors with the uptake or expression of the exogenous gene during transfection: (a) the transfection efficiency of another, nontransforming gene was not affected by antipain; (b) antipain present only 4 h before, during, and 48 h after transfection did not inhibit transformation. In contrast, when antipain was present during the period of cell proliferation following subculture, focus formation was reproducibly suppressed. Since subculture involves use of trypsin and antipain is added directly after replating of trypsinized cells, it is formally possible that some residual trypsin activity is responsible for enhanced transformation, and this is blocked by the inhibitors. This seems unlikely since cells are exposed to minimal levels of trypsin for only 3 min, and any residual trypsin activity should be halted by the protease inhibitors present in serum. This question will be definitively resolved when other protease inhibitors including non-trypsin inhibitors are tested in this assay and by alternative methods of subculture. Nevertheless, whether cell proliferation or some more specific stimulus is responsible for protease inhibitor-sensitive transformation, it is apparent that the entire process may require several steps.

The unexpected finding of a maximal inhibition at 50% of the normal transformation efficiency of H-ras by antipain suggests the possibility that there are two populations of potential transformed cells. It may be hypothesized that some fraction of the cells which have taken up and expressed the activated ras gene requires no further mechanistic steps to proceed to transformation and focus formation, or that if other steps are required, they are not affected by protease inhibitors. Another fraction of ras-transfected cells apparently does require the involvement of other events (which our data indicate occur during cell proliferation) for complete transformation and one or more of these events are protease inhibitor sensitive.

The 50% maximum inhibition value would suggest that one-half of the transfected cells are in the "complete transformation" pathway. The experiments on subculture show that indeed elimination of the cell proliferation phase following subculture results in a 50% reduction of foci per flask scored (Table 2). However, when the data are corrected for transformation efficiency per µg DNA transfected the decrease is closer to only 20% of the control (1:3 subculture) value. This apparent inconsistency may be explained by the fact that from 1 to 2 rounds of cell division occur between transfection of the subconfluent cultures on day 1 and the subculture step 48 h later. This will result in a 2- to 4-fold expansion of the transfected cells. When these cells are trypsinized, mixed, and reseeded, the ultimate number of foci in the scored flasks will depend on the degree of cell division between transfection and subculture. If an average of ~ 1.5 cell divisions are assumed to occur during this time, then in order to compare transfection efficiencies in subcultured versus nonsubcultured cells (in which such division will not yield new foci, since the cells are never reseeded) the latter value must be corrected by a factor of 3 (2 x 1.5). If this is done with the data in Table 2, it can be seen that the actual number of originally transformed cells in the nonsubculture experiment is one-half that of the subcultured cells. The value of 1.5 cell divisions is a reasonable approximation based on growth curves (data not shown).

Protease inhibitors, such as α2-macroglobulin, α1-protease inhibitor, and α1-trypsin inhibitor, are major constituents in human plasma amounting to about 10% of the total proteins. The lack of α1-trypsin inhibitor formation in certain families leads to an early onset of emphysema (29). Some plasma protease inhibitors control the functions of biologically impor-
Protease inhibitors and ras transformation

tant processes such as thrombin, plasminogen activator, plasmin, and complement activation (29). These and other pro-
teases have been shown to contribute to the metastatic potential of cancer cells (30). Protease inhibitors capable of inhibiting
collagenase or of blocking the protease cascade which leads to
of cancer cells (30). Protease inhibitors capable of inhibiting
song of thrombin, plasminogen activator, plasmin
tant processes such as thrombin, plasminogen activator, plas
sion of lower levels of myc RNA in anti-
pain-treated fibroblasts (9) may be relevant to the functional
ction of metastatic pro-
tant processes such as thrombin, plasminogen activator, plas

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