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Decreased Retinoylation in NIH 3T3 Cells Transformed with Activated Ha-*ras*

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Retinoylation (retinoic acid acylation) is a post-translation modification of proteins occurring in a variety of mammalian cell lines and *in vivo*. To gain further knowledge of the role of retinoylation we studied it in NIH 3T3 cells and NIH 3T3 cells transformed by an activated Ha-*ras* oncogene (NIH Ha-*ras*-3T3 cells). In serum-free medium retinoic acid (RA) inhibited growth of NIH 3T3 cells but did not inhibit growth of NIH Ha-*ras*-3T3 cells. After incubation with [^{3}H]RA, the level of retinoylated protein in NIH 3T3 cells was about 1.5-fold greater than in NIH Ha-*ras*-3T3 cells. On one-dimensional polyacrylamide gel electrophoresis, both the rate and the extent of retinoylation were greater in NIH 3T3 cells. We detected about 40 retinoylated proteins in NIH 3T3 cells by two-dimensional polyacrylamide gel electrophoresis. Only about 15 proteins were retinoylated, but at reduced levels, in NIH Ha-*ras*-3T3 cells. These results suggest that the activated *ras* oncogene inhibits retinoylation. This inhibition may in turn be related to the loss of other RA responses of NIH 3T3 cells, including growth inhibition, retinoic acid catabolism, down-regulation of fibronectin biosynthesis, and induction of tissue-type transglutaminase, which are not seen to the same extent in NIH Ha-*ras*-3T3 cells.

In NIH 3T3 cells retinoic acid (RA) induces tissue-type transglutaminase (TGase)(1), decreases the levels of fibronectin, RARα and RARγ (2), increases the level of RARβ (2), inhibits growth (3), and is rapidly catabolized to more polar metabolites (3). Transformation of NIH 3T3 cells with an activated Ha-*ras* oncogene negates these effects (1-3). The mechanism by which *ras* interferes with these RA actions is unclear.

The mechanism for the effects of RA in a variety of cell types involves the RA nuclear receptors (RARs and RXRs) (4-9). These receptors are members of the steroid/thyroid nuclear receptor multigene family (8) and have specific high affinity binding sites for RA and some of its metabolites. It is generally accepted that one or more of the nuclear receptors mediate the biological activity of RA. These receptors directly activate or repress transcription of their target genes by binding to specific DNA sequences. However, a correlation is not always seen between the biological potency of a retinoid and its affinity to an RAR (10-13).

We have reported on RA acylation (retinoylation) of proteins in mammalian cells (14). In HL60 cells retinoylation is dependent on the initial concentration of RA in a saturable manner (15). Furthermore, the dose-response curves for RA-induced differentiation and for retinoylation are similar. These results suggested that retinoylation may be involved in the RA-induced differentiation of HL60 cells.

In this study, we examined the relationship between retinoylation in mouse NIH 3T3 cells and NIH 3T3 cells transfected with an activated Ha-*ras* oncogene. We show a correlation between the biological potency of RA in inhibiting cell growth and retinoylation.

MATERIALS AND METHODS

Cells and cell culture. NIH 3T3 cells were obtained from the American Type Culture Collection (Rockville, MD). Activated Ha-*ras*-transfected cells were generated as described (1). All comparative studies were done with matched pair clones, *i.e.*, cells were transfected with vector plus/minus the activated *ras* construct. In this study, cells transfected with vector minus activated *ras* are designated NIH 3T3 and cells transformed with activated *ras* are designated NIH Ha-*ras*-3T3. Cells were maintained in DMEM containing

Abbreviations used: RA, all-*trans* retinoic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazinethane sulfonic acid; PBS, phosphate-buffered saline (1.5 mM KH_2PO_4, 8.1 mM Na_2HPO_4, 136.9 mM NaCl, pH 7.2); SDS, sodium dodecyl sulfate: RAR, retinoic acid receptor; TGase, transglutaminase; EDTA, ethylenediaminetetraacetic acid; PAGE, polyacrylamide gel electrophoresis; 1D-PAGE, one-dimensional polyacrylamide gel electrophoresis; 2D-PAGE, two-dimensional polyacrylamide gel electrophoresis; DMEM, Dulbecco's modified Eagle medium.

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10% bovine calf serum. Cell cultures were grown at 37° C in a humidified atmosphere of 5% CO_2 in air and subcultured every week. Cells were removed from the surface of the tissue culture flask with Trypsin-EDTA (0.05% trypsin and 0.53 mM EDTA in Hanks' balanced salt solution without Ca or Mg, (GIBCO)). We estimated cell number of the stock cultures on an electronic particle counter (Coulter Electronics, Hialeah, FL) and viability by trypan blue dye exclusion.

Cell proliferation. Cells (2×10^4) were suspended in DMEM containing 10% bovine calf serum and cultured for one day at 37° C in a humidified atmosphere of 5% CO_2 in air. After changing from serum-containing DMEM medium to serum-free DMEM medium (DMEM containing 5 μg insulin and 5 μg transferrin/ml and 10 mM 4-(2-hydroxyethyl)-1-piperazinethane sulfonic acid, pH 7.3), various concentrations of RA were added to the cultures. Cells were incubated for 2-3 days and then viable cell number was estimated using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide as described (16, 17).

Retinoylation. NIH 3T3 cells and NIH Ha-*ras*-3T3 cells from exponentially growing cultures were used for all experiments. The serum-containing DMEM medium was replaced with the serum-free DMEM medium. RA (Sigma, St. Louis, MO) and [11,12^{-3}H]RA (50 Ci/mmol, Du Pont-New England Nuclear, Boston, MA) were dissolved in absolute ethanol and added to the growth medium. The final concentration of ethanol was no higher than 0.1%.

NIH 3T3 cells and NIH Ha-*ras*-3T3 cells were grown for various times with 100 nM [^{3}H]RA. Cells were removed from the growth surface with a rubber policeman, harvested by centrifugation (200 $\times$ g, 5 min), and washed extensively with PBS. The cell pellet was then extracted by the Bligh-Dyer procedure (18) and centrifuged at 10,000 $\times$ g for 5 min in a microcentrifuge. This extraction was repeated about five times until there was $<$300 cpm/ml in the supernatant fraction. The delipidated pellet was then dried in a centrifugal vacuum device (Savant, Farmingdale, NY). Part of the pellet was dissolved in 10% (w/v) SDS solution, and radioactivity was measured on a liquid scintillation spectrometer. Proteins were measured with the Bicinchoninic acid (BCA) Protein Assay Reagent (Pierce, Rockford, IL) with bovine serum albumin as the standard. Both cell lines contained about 130 μg protein/1 $\times$ 10^6 cells.

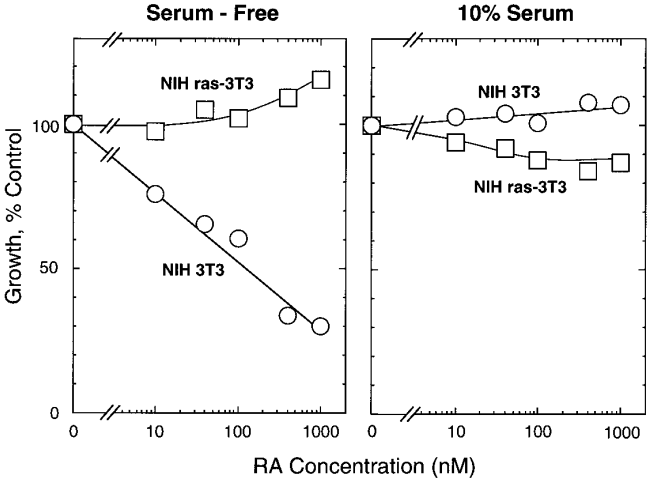

FIG. 1. Comparison of the effects of RA on growth of NIH 3T3 cells and NIH Ha-*ras*3T3 cells. NIH 3T3 cells (○) and NIH Ha-*ras*-3T3 cells (□) were grown with various concentrations of RA in serum-free medium (*left panel*) or medium containing 10% bovine calf serum (*right panel*). Growth was measured at 48 h as described under Materials and Methods. Each *point* is the mean of at least four measurements. The SE of each point was ⩽8% of the mean.

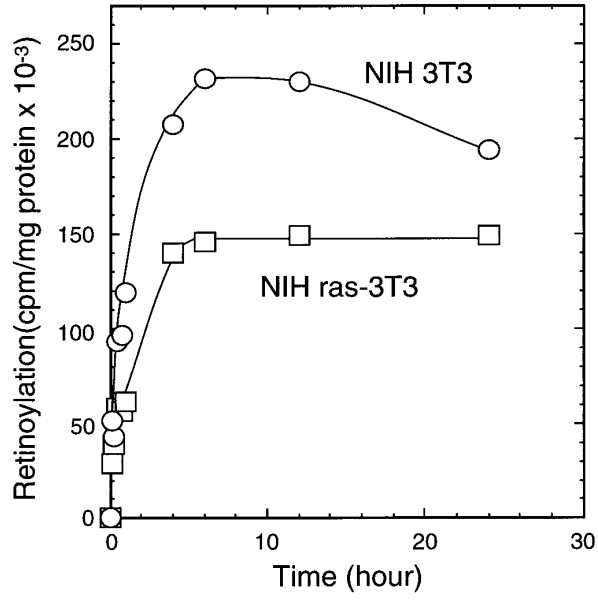

FIG. 2. Changes in the levels of retinoylated protein during growth of NIH 3T3 cells and NIH Ha-*ras*-3T3 cells. NIH 3T3 cells (○) and NIH Ha-*ras*-3T3 cells (□) were grown in serum-free medium in the presence of 100 nM [^{3}H]RA. Cells were scraped, harvested by centrifugation, washed with PBS, and extracted by the Bligh-Dyer procedure as described under Materials and Methods. The residue was dissolved in 10% SDS and radioactivity measured in a liquid scintillation spectrometer.

1D- and 2D-PAGE. The dried delipidated residue, prepared as described above, was dissolved in either SDS sample buffer containing 2.5% 2-mercaptoethanol for 1D-PAGE or in isoelectric focusing buffer (9.5 M urea, 2% NP-40, and 2% ampholytes (pH 3.5-10, Pharmacia LKB) for 2D-PAGE. 1D-PAGE was carried out according to Laemmli (19). Samples were heated at 100° C for 5 min in the sample solution and applied to 10-20% gradient polyacrylamide slab gels (Integrated Separation System). 2D-PAGE was according to O'Farrell(20). First dimension isoelectric focusing gels contained 2% ampholytes (pH 3.5-10). Second dimension gels were 10-20% gradient polyacrylamide gels (Integrated Separation System). Gels were fixed, stained with Coomassie Brilliant Blue R-250, and prepared for fluorography with ENTENSIFY (Du Pont-New England Nuclear) according to the manufacturer's instructions. The dried gels were exposed to Kodak XAR-5 film at <-120°C. Molecular weight markers were from GIBCO-BRL.

Proteinase K treatment. The delipidated pellet prepared as described above was dissolved in 1% (w/v) SDS. The reaction mixture (100 μl), containing 40 mM Tris-HC1, pH 7.5, 2 mM EDTA, and 0.4 mg of proteinase K (Sigma), was incubated at 37°C for 1 h. Bovine serum albumin (final concentration 50 to 100 μg/ml) and trichloroacetic acid (final concentration of 10% (w/v)) were added. The reaction mixture was chilled in an ice water bath and then centrifuged in a microcentrifuge at 10,000 $\times$ g for 5 min. Radioactivity in both the supernatant and the precipitate fractions was determined in a liquid scintillation spectrometer.

RESULTS

Effects of RA on growth of NIH 3T3 and NIH Ha-ras-3T3 cells. RA did not inhibit the growth of either NIH 3T3 cells or NIH Ha-*ras*-3T3 cells in me-

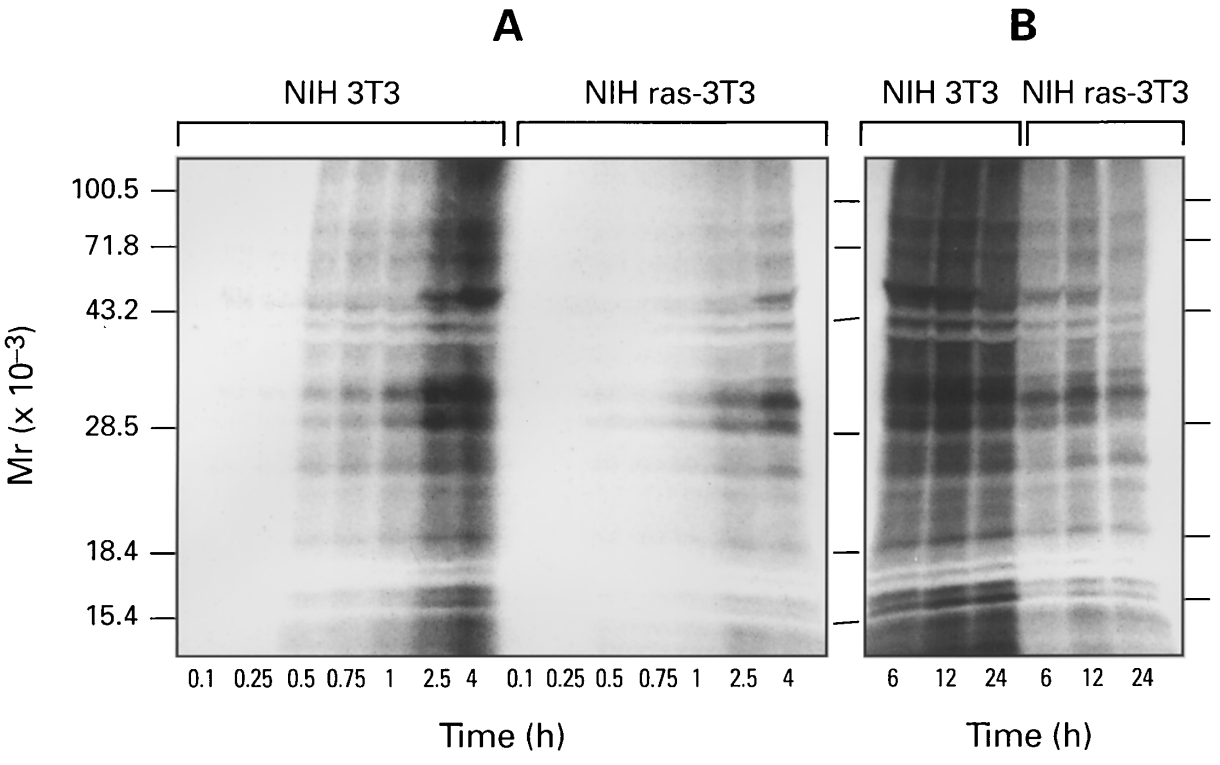

FIG. 3. Kinetics of retinoylation in NIH 3T3 and NIH Ha-*ras*-3T3 cells. Cells were grown in serum-free medium with 100 nM [^{3}H]RA. At the indicated times, cells were harvested and extracted by the Bligh-Dyer procedure. Part of each residue, containing 80 μg protein, was subjected to 1D-PAGE. The gels were prepared for fluorography and exposed to the film for 170 days. *A* shows the fluorogram of one gel containing samples from both cell lines grown with [^{3}H]RA for $\leq$4 h. *B* shows the fluorogram of a different gel containing samples from both cell lines grown with [^{3}H]RA for $\geq$6 h. The samples used in *A* and *B* were from one experiment.

dium containing 10% bovine calf serum (Fig. 1). However, in serum-free medium, RA inhibited the growth of NIH 3T3 cells (50% growth inhibition at 100 nM RA) but did not inhibit the growth of NIH Ha-*ras*-3T3 cells (Fig. 1).

Retinoylation in NIH 3T3 and NIH Ha-ras-3T3 cells. During growth of cells in serum-free medium containing 100 nM [^{3}H]RA, there was a time-dependent increase in the incorporation of RA into a form that was not extracted by the Bligh-Dyer procedure (Fig. 2). Digestion of the Bligh-Dyer residues, from cells exposed to RA for 24 h, with proteinase K converted 94% of the NIH 3T3 bound radioactivity and 91% of the NIH Ha-*ras*-3T3 bound radioactivity from an acid-insoluble form to an acid-soluble form. This result showed that RA (or a metabolite) was covalently bound to protein.

Both the rate and the extent of retinoylation were greater in NIH 3T3 cells than in NIH Ha-*ras*-3T3 cells (Fig. 2). In both cell lines the maximum level of retinoylation occurred at 6 h. At times between 1 and 6 h retinoylation was 1.5- to 2-fold greater in NIH 3T3 cells than in NIH Ha-*ras*-3T3 cells (Fig. 2). After 6 h, the retinoylation gradually decreased in NIH 3T3 cells while it remained constant in NIH Ha-*ras*-3T3 cells.

1D-PAGE analysis of retinoylated proteins at various times. The differences in the rate and the extent of retinoylation between NIH 3T3 cells and NIH Ha-*ras*-3T3 cells (Fig. 2) prompted an examination by 1D-PAGE of the retinoylated proteins in both cell lines.

As shown in Fig. 3 the patterns of retinoylated proteins were similar in both cell lines. A primary difference was the more rapid retinoylation of NIH 3T3 proteins compared with NIH Ha-*ras*-3T3 proteins. The patterns of Coomassie Brilliant Blue R-250-stained proteins were similar in all lanes, suggesting that differences in rates of retinoylation were not simply a reflection of variations in the intracellular concentrations of substrate proteins.

2D-PAGE analysis. Retinoylated proteins in NIH 3T3 cells and NIH Ha-*ras*-3T3 cells were analyzed further by 2D-PAGE. We detected at least 40 retinoylated proteins in NIH 3T3 cells (Fig. 4, *composite,* open and solid symbols). In contrast, the only proteins detected in NIH Ha-*ras*-3T3 cells were *proteins 1-7* accounting for 15 retinoylated proteins (Fig. 4, *composite,* solid and shaded symbols). The proteins in groups 1-6 (solid symbols) were retinoylated in both cell lines. However, labeling of *proteins 1-6* were very low in NIH Ha-*ras*-3T3

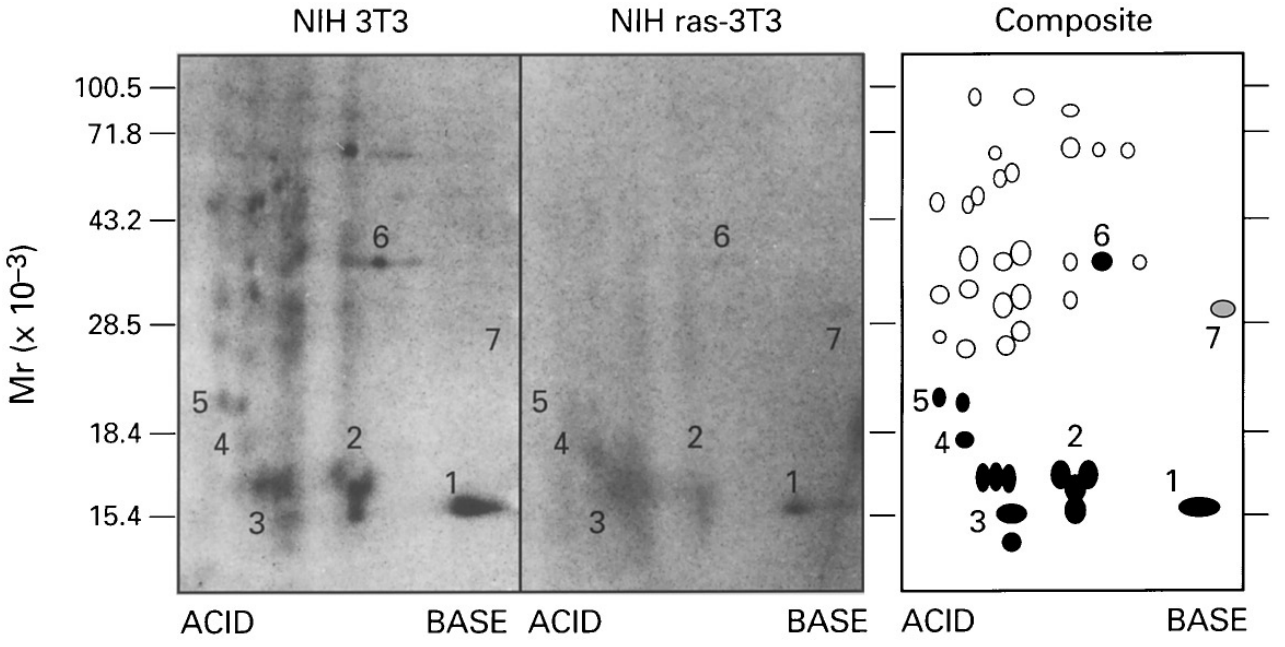

FIG. 4. 2D-PAGE of retinoylated proteins in NIH 3T3 and NIH Ha-*ras*-3T3 cells. Cells were grown in serum-free medium for 26 h in the presence of 100 nM [^{3}H]RA. Cells were scraped and harvested by centrifugation. Cell pellets were washed with PBS and extracted by the Bligh-Dyer procedure. The residues were dissolved in isoelectric focusing buffer (pH 3.5-10) and portions analyzed by 2D-PAGE and fluorography. We applied 264 μg of NIH 3T3 cell protein containing 4.5×10^4 cpm and 264 μg of NIH Ha-*ras*-3T3 cell protein containing 2.6×10^4 cpm to each first-dimension isoelectric focusing gel. The second-dimension electrophoresis was done on the same gel. Exposure to the film was for 170 days. The composite identifies the retinoylated proteins. Open symbols are retinoylated proteins seen only in NIH 3T3 cells. Solid symbols are retinoylated proteins seen in both cell lines. The shaded symbol is a retinoylated protein (*protein 7*) seen only in NIH Ha-*ras*-3T3 cells.

cells compared with NIH 3T3 cells. *Protein 7* was the only protein retinoylated by NIH Ha-*ras*-3T3 cells and not by NIH 3T3 cells. The 2D-PAGE patterns of Coomassie Brilliant Blue R-250-stained proteins were similar in both cell lines, suggesting that differences in retinoylation did not simply reflect variations in the intracellular concentrations of substrate proteins.

DISCUSSION

This study establishes that the RA growth-inhibited cell line NIH 3T3 (Fig. 1) shows a higher amount of protein retinoylation (Figs. 2-4) than the nonresponsive NIH Ha-*ras*-3T3 cell line. Retinoylation is widespread and occurs in a variety of intact cells (15, 21-24) and *in vivo* (25). The 2D-PAGE pattern of retinoylation in NIH 3T3 cells (Fig. 4) was unique among other cell lines. An emerging pattern is that different cell-types may retinoylate different proteins. It is tempting to speculate that retinoylation may be partially responsible for the pleiotropy that is characteristic of retinoid action.

RA is essential for embryogenesis and the maintenance of growth and differentiation in the adult (26). The RA nuclear receptors mediate many of its actions on cells. To aid in determining whether RA exerts its effects by retinoylation we studied this phenomenon in mouse NIH 3T3 cells and NIH Ha-*ras*-3T3 cells.

The greater level of retinoylation in NIH 3T3 cells compared with NIH Ha-*ras*-3T3 cells (Figs. 2, 3, and 4) correlated with the greater response of NIH 3T3 cells to RA (1-3). This difference may reflect a difference in the levels of the enzymes that activate and transfer RA to protein (27) because we saw no marked variation in the Coomassie Brilliant Blue staining intensity of individual proteins from the two cell lines separated by either 1D- or 2D-PAGE.

The transfection of activated Ha-*ras* oncogene into NIH 3T3 cells diminishes various RA effects including the induction of TGase (1), RA catabolism (3), down-regulation of fibronectin and RARα biosynthesis (2), and, as shown in this study, growth inhibition of the cells (Fig. 1) and retinoylation (Figs. 2-4). These results raise the possibility that signal transduction by RA interacts with a Ha-*ras*-activated pathway(s). Ha-*ras* is a member of the family of GTP-binding proteins involved in transmembrane signaling (28). The mature *ras* p21 protein is at the inner surface of the plasma membrane and is a substrate for myristoylation, palmitoylation, and isoprenylation (29).

We do not know whether Ha-*ras* is involved in RA action directly. Various reports show opposite effects

of RA and *ras* on regulating the expression of different genes (30-33), suggesting that there is an interaction between the signal transduction pathways mediated by RA and *ras*. It is of interest that retinoylated *proteins 5* (Fig. 4) have migrations similar to those of *ras* (34). The retinoylation of *ras* may explain how RA and *ras* interact. There is speculation that *ras* requires reversible association with membranes. Palmitoylation, myristoylation, and isoprenylation are active in this process. Retinoylation, like palmitoylation, is a reversible process (15). Therefore, retinoylation also could play a role in the dynamic localization of *ras* proteins. We presently are determining whether *ras* is retinoylated.

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