Modulation by Retinyl Acetate of Microfilament Bundle Formation in C3H/10T1/2 Cells

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Retinyl acetate has been previously shown to inhibit carcinogen-induced neoplastic transformation in 10T1/2 cells and to accentuate many aspects of the nontransformed phenotype. Scanning electron microscopy of logarithmic phase 10T1/2 cells treated for 3 days with 0.3 µg/ml retinyl acetate revealed that this treatment caused extensive flattening of cells to the plastic substrate. In contrast the tumor promoter tetradecanoyl phorbol acetate, which antagonizes the antineoplastic activity of retinyl acetate, caused cell rounding and completely inhibited the action of retinyl acetate on cell morphology. During this same time course, the formation of microfilament bundles was also found to be modulated by retinyl acetate. Transmission electron micrographs of unsectioned peripheral regions of flattened cells showed that while the unit density of microfilament bundles was not influenced, the thickness of bundles, particularly those with a diameter of 100 nm or more, was increased by retinyl acetate. Tetradecanoyl phorbol acetate had little effect on microfilament bundle diameters but did partially antagonize the action of retinyl acetate. To determine if this increase was associated with an increase in total actin/cell, total cell proteins, and proteins not extractable by glycerol-triton extraction, were subjected to sodium dodecylsulfate/ polyacrylamide gel electrophoresis. It was found that while total cellular actin was not increased by retinyl acetate, the proportion of nonextractable actin (which includes microfilament bundles) increased from 65% to 88% of total actin. This increase was not inhibited by inhibitors of protein or RNA synthesis. These studies again demonstrate that retinyl acetate accentuates the nontransformed phenotype of 10T1/2 cells; it is hypothesized that these actions are related to the antineoplastic activity of retinoids.

Key words: retinoids, microfilaments, tumor promoters, actin, cell spreading

Numerous reports have demonstrated that cultured fibroblasts transformed by chemical, physical, or viral carcinogens are less adhesive to the culture substratum and assume a less polar and more rounded morphology [1]. This has been attributed

to the disorganization of the cytoskeletal network of microtubules and microfilaments [2,3]. Although it has been shown that cell shape and adhesiveness to the substratum are related to growth control [4,5], a causal relationship between cytoskeletal disorganization and neoplastic transformation has not been established. Agents such as fibronectin and cAMP, which temporarily normalize the growth and morphology of transformed fibroblasts [6,7], also induce reorganization of the cytoskeleton in these cells. Thus, a clear association exists between the integrity of the cytoskeleton and the malignant phenotype.

Vitamin A and certain of its natural and synthetic analogues (retinoids) inhibit chemically induced oncogenesis in various rodent epithelial tissues [8] and the neoplastic transformation in C3H/10T1/2 mouse embryo fibroblasts [9]. High dietary and serum vitamin A levels have also been associated with a reduced risk of cancer in humans [10,11]. Although the precise mechanism of this inhibitory effect is unknown, considerable evidence has accumulated demonstrating a possible role for vitamin A in protein glycosylation [12]. In a preliminary study, we reported that retinyl acetate (RAC) causes nearly 90% of the C3H/10T1/2 fibroblasts in growing culture to assume the very flat morphology usually seen in confluent, contact-inhibited cultures [13]. RAC also induces these cells to adhere more avidly to [14], and spread more extensively over [13], the culture substratum, without any effect on their growth rate [15]. Similar effects of other vitamin A analogues on the morphology and adhesion of other normal and transformed cell lines have been reported [16].

Because the microfilament bundles (MFB) of the cytoskeleton have been shown to originate at the sites of cell adhesion to the substratum [17], we examined the possibility that the effects of retinoids on adhesion and spreading also altered the formation of MFB. Analysis by light and transmission electron microscopy revealed that RAC significantly enhanced MFB formation independent of de novo RNA and protein synthesis. These results demonstrate that RAC, by increasing the degree of polymerization of actin into MFB, is producing an effect opposite to that caused by neoplastic transformation, and is thus, as we have previously stated [18], accentuating the normal phenotype. Whether this effect on MFB is a primary action, is induced by plasma membrane protein glycosylation patterns as implied by the work of De Luca [12], or is a consequence of regulation at the gene level is not known. A preliminary report of these studies has been made [13].

METHODS
Cell Culture Conditions and Treatments

The C3H/10T1/2 (10T1/2) mouse embryo fibroblast cell line was cultured, as previously described [19], in Eagle's basal medium (BME, Grand Island Biological Company, Grand Island, NY) with 5% fetal calf serum (Rehatuin, Reheis Chemical Company, Division of Armour Pharmaceutical Company, Phoeniz, AZ) and 25 μg/ml Gentamicin Sulfate (Schering Corporation, Kenilworth, NJ). Retinyl acetate (RAC; Sigma Chemical Company, St Louis, MO) was stored in air-tight containers in liquid N_2, prepared just prior to use, and administered in 25 μl of acetone/5 ml medium. The phorbol ester tumor promoter 12-O-tetradecanoylphorbol-13-acetate (TPA; C.C.R.,Inc, Eden Prairie, MN) was stored at $-20°C$ in light- and air-tight containers and administered in 25 μl of acetone. Control cultures were treated with 25 μl of acetone, which was nontoxic. Experimental cultures were seeded with 5×10^3 to 10^4

cells/60-mm dish and treated with RAC, TPA, or both for 3 to 4 days beginning 1 day after seeding.

Analysis of Morphological Types

Gross morphological cell types were established on the basis of the degree of flattening of the cell to the tissue culture substrate. Both treated and control cells grown on cover slips were fixed in 2.5% glutaraldehyde and postfixed in 1% osmium tetroxide. The cells were dehydrated in graded percentages of ethanol, critical-point dried in CO_2, sputter-coated with gold and examined in a ETEC autoscanner microscope operated at 25 kV. A cell was considered "flat" if the entire periphery was spread out away from the nucleus and over the substrate. If no more than 50% of the peripheral membrane was asymetrically retracted, the cell was considered "semiflat." These cells typically take the shape of a half-moon. If areas of contact with the substrate alternate with retracted sections of the peripheral membrane, giving the cell a many-sided appearance, it was considered a "raised polygonal" cell. "Spindle"-shaped cells were elongated and fibroblastlike in appearance. These morphological types observed by scanning electron microscopy of control cultures is shown in Figure 1. One hundred cells in each treatment group were analyzed. Statistically significant differences (P < .05) were determined by the Z-test statistic for comparisons between percentages.

Determination of MFB Density and Diameter

Cells grown on glass coverslips were extracted with 1% Triton X-100 in 4 M glycerol and stained with Coomassie blue R-250 according to the procedure of Pena [20]. This technique reveals MFB as efficiently as immunofluorescent techniques. In flat and semiflat cells the number of microfilament bundles oriented roughly parallel to the long axis of the cell, which includes the vast majority of MFB in 10T1/2 cells, were counted by light microscopy (LM) at four equally spaced points along the entire length of the cell. The distance between the plasma membranes on opposite sides of the cell at these points was also measured and divided into the number of MFB counted. This yielded a density value which was expressed as the number of MFB/100 μm, thus eliminating possible artifacts due to effects on cell size.

The diameter of MFB was determined by transmission electron microscopy of cells grown on carbon/Formvar-coated gold grids. The cells were fixed in cold 2.5% glutaraldehyde in phosphate buffered 0.9% NaCl for at least 1 hr at 4°C and postfixed in 1% osmium tetroxide for 30 min. These cells were then dehydrated in graded concentrations of ethanol and critical-point dried in CO_2. The whole cell mounts were examined in a Siemens 101 microscope operating at 80 kV. Thin, peripheral areas of cells were examined and photographed at 5,000× magnification. Because of the thinness of these peripheral cytoplasmic regions, no sectioning was required. MFB density and diameters were measured directly from about 30 photomicrographic negatives of individual cells of each group, randomly selected from twice repeated experiments.

Measurements of both the light and electron micrographic images were performed by projecting photographic images onto the touch-sensitive pad of a digitizer connected to a Hewlett-Packard desktop computer programmed to compute length and statistical error. Densities of MFBs were then readily calculated.
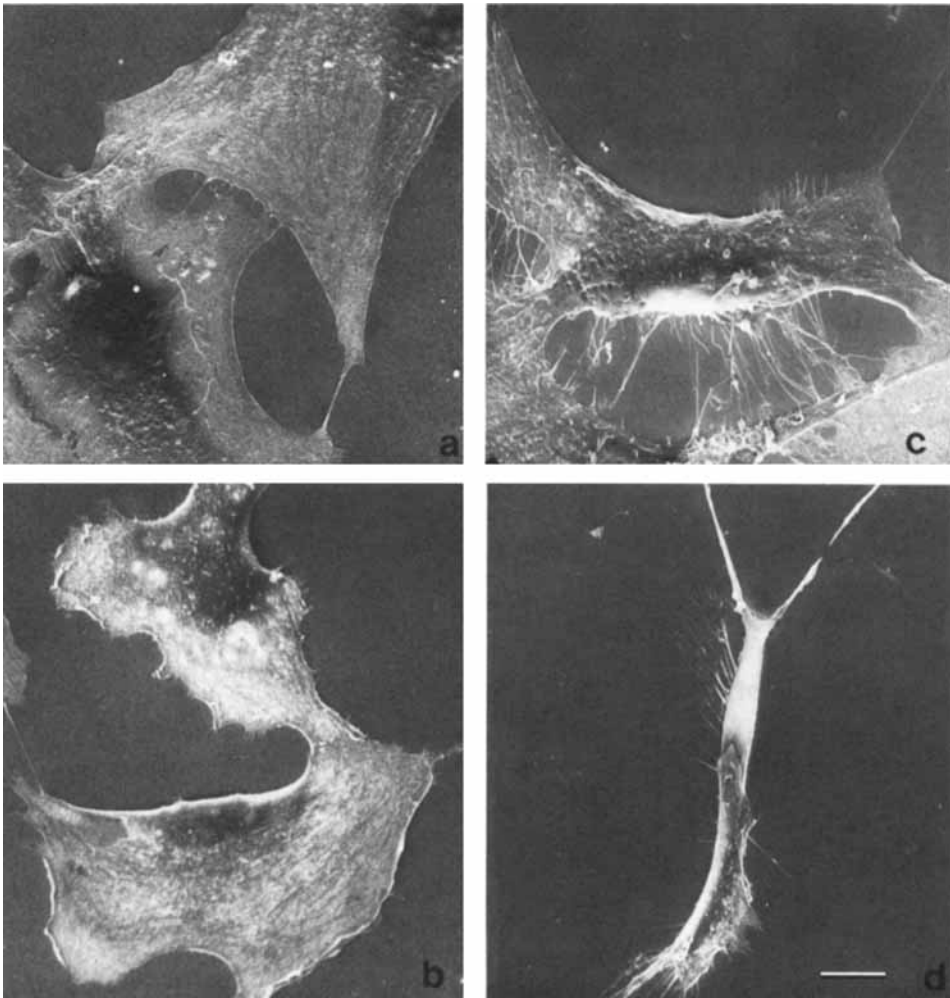
Fig. 1. Scanning electron micrographs of the 4 morphological types of 10T1/2 cells given in Table I: (a) flat cell, (b) semiflat cell, (c) raised polygonal cell, and (d) a spindle-shaped cell. The morphological criteria are given in the text. Bar = 10 μM.

Quantitation of Actin

Total cellular actin and actin resistant to glycerol/triton (G/T) extraction were determined by sodium dodecylsulfate-polyacrylamide (SDS) gel electrophoresis [21]. Whole cells, or G/T-resistant material, were solubilized in electrophoresis buffer and applied to a 9% gel in amounts representing equal numbers of cells. Proteins in the gel were stained with Coomassie blue R250 and quantitated by densitometric scanning. Rabbit muscle actin was run simultaneously. Relative actin content was determined from the areas under the densitometric scan of the protein band comigrating with the rabbit muscle actin.

TABLE I. Effect of RAC and TPA on Cell Morphology*

Morphology[a]	Acetone control (0.5%)	RAC (0.3 μg/ml)	TPA (0.1 μg/ml)	RAC and TPA
Flat	48	83[b]	41	37
Semiflat	28	16	23	28
Raised polygonal	17	1	28	29
Spindle	7	0	8	6

*Logarithmic phase cells were treated for 3 days with the stated drug concentrations, then fixed and examined by scanning electron microscopy. One hundred cells/treatment group were examined and allocated according to morphological types.
[a]See Materials and Methods for description of morphologies.
[b]Significantly different from control, $P < .05$.

RESULTS

In a preliminary report, we demonstrated that RAC caused growing 10T1/2 cells to flatten extensively over the culture substratum [13]. We have confirmed and extended this observation in the present study (Table I). RAC, 0.3 μg/ml, caused a significant increase in the percentage of cells with a flat morphology (48% to 83%) and a compensatory decrease in the percentage of less-flattened cells. In contrast, cells treated with 0.1 μg/ml TPA were significantly less flattened than controls, as indicated by the increase from 17% to 28% in the proportion of raised polygonal cells. Cells treated simultaneously with RAC and TPA were not significantly different from cells treated with TPA alone. Thus, RAC and TPA had opposite effects upon the morphology of 10T1/2 cells when administered separately. However, at the concentrations tested, RAC was not able to reverse the effects of TPA on cell morphology.

Since cell morphology appears to be related to the organization of the cytoskeleton, we examined the effect of RAC on the organization of MFB in flat and semiflat 10T1/2 cells which constitute up to 99% of the population of treated cells. In RAC-treated cells, the density of Coomassie blue-stained MFB resistant to G/T extraction as detected by light microscopy (LM) was 2.5-fold higher than in control cells, 52.8 vs 20.9 MFB/100 μm, respectively (Table II). Thus, the significant enhancement of cell flattening following RAC treatment was accompanied by a dramatic increase in MFB formation, as observed in the LM. This effect on MFB formation cannot simply be a consequence of cell spreading, since when spreading of logarithmic cultures was induced by decreasing the serum content of the medium to 2.5%, spreading was induced that was comparable to that seen after exposure to 0.1 μg/ml/RAC, but without the major increase in MFB density observed after RAC treatment (Table III). It is clear from these data that spreading induced by serum reduction itself causes an increase in MFB density, but of a much smaller magnitude from that caused by RAC, and that a clear dose-response relationship exists between RAC concentration and MFB density as detected by LM.

We next attempted to confirm these observations with the transmission electron microscope (TEM). We found that, whereas the density of MFB in RAC-treated cells

TABLE II. Effect of RAC and TPA on MFB Density

	MFB density (No./100 μm)	
Treatment	Light microscope	Transmission electron microscope
Control	20.9 ± 1.8	41 ± 12
RAC	52.8 ± 4.2[a]	43 ± 18
TPA	19.4 ± 2.6	55 ± 1
RAC and TPA	24.6 ± 2.1	44 ± 15

Cells were treated as in Table I and the MFB density assessed by light microscopy after Triton X extraction and Coomassie blue staining, or by scanning electron microscopy after critical-point drying as described in the text. Analyses were performed on about 30 cells/treatment group.
[a]Significantly different from control, P < .05.

TABLE III. Changes in MFB Density With Cell Spreading Induced by Either Retinyl Acetate (RAC) or by Decreasing Concentrations of Serum*

	Cell area (μM^2)	MFB 100 μm
RAC		
Concentration (μg/ml)		
0	2,930 ± 250	20.9 ± 1.8
0.03	3,870 ± 305	35.4 ± 4.2
0.1	4,850 ± 270	49.2 ± 3.4
0.3	6,410 ± 320	52.8 ± 4.2
Serum		
concentration (%)		
10	2,560 ± 250	17.2 ± 1.5
5	2,930 ± 250	20.9 ± 1.8
2.5	5,430 ± 350	25.5 ± 2.3

*Measurements of cell area and MFB density were performed as described in the text on logarithmic phase cultures of 10T1/2 cells. Cultures receiving RAC were in medium containing 5% serum. Results stated are means ± standard deviation.

as measured by TEM was comparable to that measured with the LM—43 vs 52.8 MFB/100 μ—control cells had twice the MFB density as previously seen with the LM—41 vs 20.9 MFB/100 μm, respectively. Consequently, we could detect no significant difference between control and RAC-treated MFB densities when determined by TEM (Table II).

Since the LM and TEM have vastly different resolving powers, we investigated the possibility that RAC may actually be increasing the size of the MFB, thus making them more visible in the LM and resulting in an apparent increase in density. Typical electron micrographs of control and treated cells are shown in Figure 2. From measurements of diameters of about 150 MFB/treatment group, three size classes of MFB were found (Fig. 3.). In control cells, class I was composed of MFB of <80 nm diameter and contained 26% of the MFB; class II contained MFB of 80 to 120 nm and constituted 44% of the total MFB, while 17% of the MFB were >120nm in diameter and composed class III. RAC caused a dramatic increase in the percentage of MFB in class III from 17% to 46% of total. Class I MFB were reduced by nearly two-thirds to 8% of total MFB, while class II MFB were unaffected. We thus conclude
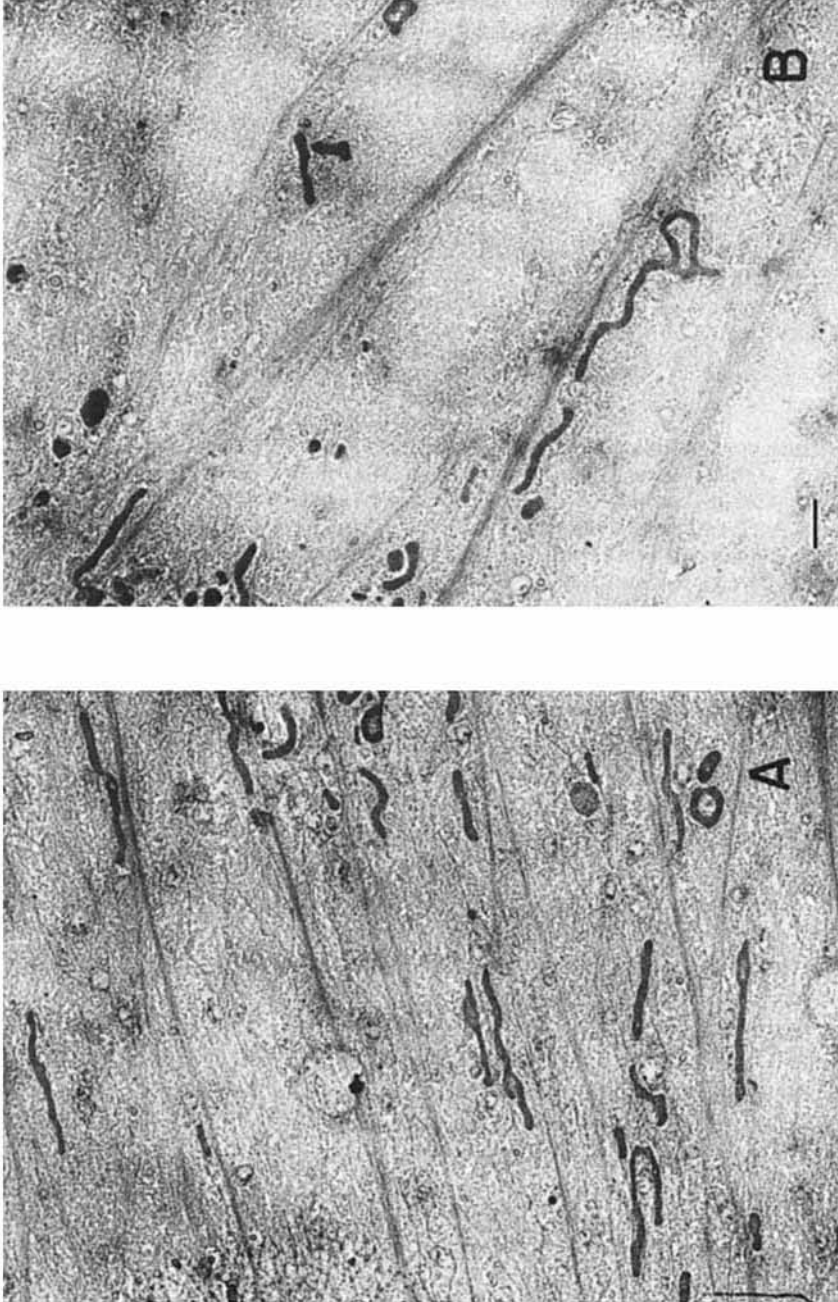
Fig. 2. Transmission electron micrographs of critical-point-dried whole 10T1/2 cells showing MFB in the peripheral cytoplasmic regions. Dark rodlike objects are whole mitochondria. A. Control treated with acetone, 0.5%. B. Cell treated with retinyl acetate, 0.3 µg/ml. Treatments were commenced 3 days before fixation, Bar = 1µM.
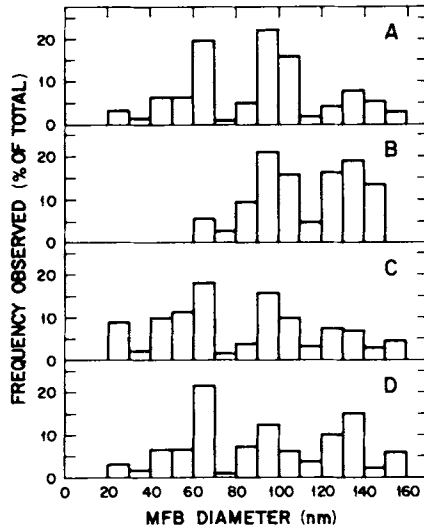
Fig. 3.   Modulation of MFB diameters by retinyl acetate and TPA in 10T1/2 cells. Cultures were seeded at a density of 5 × 10³ cells/60-mm Petri dish and after 24 hr treated with the following drugs or combinations: A. Acetone control 0.5%. B. Retinyl acetate 0.3μg/ml. C. TPA 0.1μg/ml. D. Retinyl acetate 0.3μg/ml + TPA 0.1μg/ml. After 3 days treatment cultures were processed for TEM analysis. Results represent determinations of MFB diameters made from direct measurements of TEM micrographs.

that the 2.5-fold increase in MFB density detected by LM in RAC-treated cells was due to a dramatic shift in the distribution of MFB toward bundles of larger diameter which were only then detectable by LM.

The density of MFB in cells treated with TPA alone or with TPA plus RAC was not significantly different from control cells in either LM or TEM preparations (Table II). The distribution of MFB diameters in TPA-treated cells was not significantly different from control cells in all three size classes. Cells treated with TPA plus RAC showed a significant increase in class III MFB to 33% of total compared to 17% in control cells. This was accomplished by a compensatory decrease in class II MFB to 26%. Class I MFB were unaffected. Thus, the antagonism of TPA and retinoids which has been described for transformation in vivo and in vitro, is also evident in their effects on the MFB of 10T1/2 cells.

These results suggest that RAC is causing either (1) a coalescence of small MFB into larger MFB or (2) is enhancing actin synthesis, thus making more actin available for microfilament polymerization. We therefore determined the effect of RAC on total cellular and glycerol/triton-resistant actin in the presence of inhibitors of protein (cycloheximide) and RNA (actinomycin D) synthesis (Table IV). RAC induced a 23% increase in the percentage of glycerol/triton-resistant actin without any effect on total cellular actin. Neither inhibition of protein synthesis by cycloheximide, nor of RNA synthesis by actinomycin D for 36 hr, had any effect upon this increase. From these results we concluded that RAC enhances the polymerization of actin into microfilaments and the coalescence of these microfilaments into MFB. Since this is accomplished without de novo protein and RNA synthesis, it appears to represent the result of effects upon the cellular mechanisms controlling actin polymerization and MFB formation.

TABLE IV. Effect of RAC on Resistance of Actin to Glycerol-Triton Extraction*

	Relative actin content (%)	
Treatment	G-T resistant	Whole cell
Acetone control 0.5%	64.7	100.0
RAC 0.3 μg/ml	87.8	102.1
RAC + cycloheximide 0.1 μg/ml	84.6	97.4
RAC + actinomycin D 2 ng/ml	86.3	98.2

*Logarithmic phase cells were treated for 36 hr with the stated drugs, then analyzed for actin content. One group was first extracted with glycerol/Triton X-100 (G/T) to remove nonpolymerized actin, the other was dissolved without pretreatment and assayed for actin content by SDS-polyacrylamide gel electrophoresis.

DISCUSSION

We had previously reported that RAC induced a profound increase in the substrate adhesion and flattening of 10T1/2 fibroblasts [13,14]. In this report we have extended these observations by examining effects on the cytoskeleton, since it appeared that the above effects could cause, or be caused by, cytoskeletal alterations. Initial LM observations indicated that RAC caused a 2.5-fold increase in the density of MFB in 10T 1/2 cells (Table II). We were unable to confirm this observation using TEM because of a major increase in the numbers of MFB detectable by this technique in control cells, but not in RAC-treated cells, which had similar MFB densities regardless of the method of detection used. However, further analysis revealed that RAC treatment increased the diameter of MFB; thus, the discrepancy between data obtained by LM and TEM is almost certainly due to the limited resolution of LM, which failed to detect what we have called class I MFBs in control cells (Fig. 2). Similar discrepancies between LM and TEM observations of MFB have been reported and have emphasized the importance of TEM in validating LM observations [22]. Since RAC enhanced MFB formation in the absence of RNA and protein synthesis (Table IV), we conclude that RAC has profound effects upon the cellular mechanisms controlling actin polymerization and the cross-linking of microfilaments into bundles.

RAC and TPA have been shown to have opposite effects on the growth and morphology of fibroblasts [13,23] and to be mutually antagonistic to the transformation of 10T1/2 cells by methylcholanthrene [24]. TPA-treated cells were less flattened than control cells (Table I) but had a similar MFB densities (Table II) as measured by LM and TEM. Simultaneous RAC treatment did not prevent the action of TPA on cell spreading, while the effects of RAC on MFB aggregation were antagonized by TPA. Thus, for these drug concentrations, TPA exerts a dominant effect over RAC. This correlates well with the dominant action of TPA's inducing the transformation of RAC-treated initiated cells [24]. TPA has been reported to cause loss of MFB in chick embryo fibroblasts as detected by LM using fluorescently tagged actin antibodies [2]; however, it is possible that MFB were present but not detectable by this method, since these same authors have shown that SV40-transformed 3T3 cells contain MFB detectable by TEM that are only poorly resolvable by immunofluorescence [22]. Retinoids have previously been shown to antagonize the actions of TPA on binding of growth factors [25], induction of ornithine decarboxylase [26], and some of the alterations in glycoprotein composition of the plasma membrane [27].

The effects of RAC on the MFB of 10T1/2 cells suggests some interesting possibilities for the role of vitamin A in the physiology of cells other than in the eye and the inhibition of neoplastic transformation in these cells. Since MFB originate at the sites of cell-substrate adhesion [17], and are composed of cross-linked microfilaments [28], our results suggest that RAC may be influencing (1) the size of the adhesion site, (2) the number of nucleation points for microfilament formation within the adhesion site, or (3) the cross-linking of microfilaments by α-actinin to form MFB. We have previously shown that RAC increases the adhesion of 10T1/2 cells to a plastic substrate [14], which could also be accomplished by an increase in the size and/or the number of adhesion sites. Our current results are thus compatable with the concept that effects of RAC on adhesion, spreading, and MFB formation are due to effects on the adhesion site. These effects could be mediated by the proposed direct role of retinoids as intermediates in the glycosylation of membrane components [12] or could result from direct modulation of gene expression [29]. Whether these effects are involved in the inhibitory effects of RAC on neoplastic transformation is unclear.

ACKNOWLEDGMENTS

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