Reorganization of Alpha-Actinin and Vinculin Induced by a Phorbol Ester in Living Cells

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Abstract. We have used fluorescent analogue cytochemistry, image intensification, and digital image processing to examine the redistribution of alpha-actinin and vinculin in living cultured African green monkey kidney (BSC-1) cells treated with the phorbol ester 12-O-tetradecanoylphorbol-13-acetate (TPA). Before treatment, microinjected alpha-actinin shows characteristic distribution along stress fibers and at adhesion plaques; vinculin is localized predominantly at adhesion plaques. Soon after the addition of TPA, highly dynamic membrane ruffles begin to form. These incorporate a large amount of alpha-actinin but little vinculin. Alpha-actinin is subsequently depleted, more or less uniformly, from stress fibers. Disrupted stress fibers often fragment into aggregates and move into the perinuclear region. Careful analyses of fluorescence intensity distribution indicate that alpha-actinin is depleted more rapidly from adhesion plaques than from stress fibers. Furthermore, the depletion of alpha-actinin from adhesion plaques is also faster than either the depletion of vinculin or the disappearance of focal contacts. These observations indicate that TPA may initiate disruption of stress fibers by interfering with a link between alpha-actinin and vinculin, causing alpha-actinin to be preferentially depleted from adhesion plaques.

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Materials and Methods

Preparation of Fluorescent Analogues

Smooth muscle alpha-actinin was isolated from frozen chicken gizzards (Pell-Freez Biologicals, Rogers, AR) according to Feramisco and Burridge (15) with the following modification: after elution from a DE-52 column, fractions containing alpha-actinin were pooled and fractionated with a hydroxypatite column (DNA-grade, Bio-Gel HTP, Bio-Rad Laboratories, Richmond, CA) before further fractionation with a Sepharose 6B-Cl column (Sigma Chemical Co., St. Louis, MO). Purified alpha-actinin was concentrated by precipitation with solid ammonium sulfate, dialyzed exhaustively against 2 mM Pipes, 0.02% NaN3, pH 7.0, and stored in liquid nitrogen at 13–15 mg/ml.

Smooth muscle vinculin was isolated from frozen chicken gizzards according to Evans et al. (13). Purified vinculin was concentrated and dialyzed as for alpha-actinin but was stored on ice at 4–5 mg/ml.

Labeling of alpha-actinin and vinculin with tetramethylrhodamine was performed by dissolving iodacetamidotetramethyl rhodamine (IATR) (Research Organics, Cleveland, OH, or Molecular Probes, Inc., Junction City, OR) in 200 mM potassium borate buffer, pH 9.0. The solution was clarified in a Beckman type 42.2 Ti rotor at 100,000 g for 20 min to remove undissolved dye aggregates. Stored alpha-actinin or vinculin was mixed 1:1 (vol/vol) with a solution of rhodamine isothiocyanate-labeled (Research Organics) ovalbumin was prepared for bound tetramethylrhodamine. The vinculin conjugate had a final concentration of 1.3 to 10 mg/ml and a final dye to protein molar ratio of 0.50 to 1.9 to 2.2 estimated using a molar extinction coefficient of 23,000 at 555 nm and 495 nm for bound tetramethylrhodamine. The vinculin conjugate had a final concentration of 1.3 to 10 mg/ml and a final dye to protein molar ratio of 0.50 to 0.75. The range of concentrations and labeling ratios did not affect our results.

Vinculin was labeled with fluorescein isothiocyanate (FITC) (Research Organics) by dissolving the dye in 200 mM potassium borate buffer, pH 9.0, and mixing 1:1 (vol/vol) with purified vinculin at an initial dye to protein molar ratio of 20. The solution was incubated at 0°C for 4 h, applied to a 1 x 15 cm column of Sephacryl G-25 (Sigma Chemical Co.), and eluted with 2 mM Tris-Cl, 0.1 mM dithiothreitol, pH 8.5. Fluorescent fractions in the void volume were pooled, concentrated with a Centricon-30 device (Amicon Corp., Danvers, MA), and dialyzed against injection buffer containing 1 mM Pipes, 0.1 mM dithiothreitol, pH 6.95. The alpha-actinin conjugate had a final concentration of 5 mg/ml and a final dye to protein subunit (100,000 dalton) molar ratio of 1.9 to 2.2 estimated using a molar extinction coefficient of 23,000 at 555 nm for bound tetramethylrhodamine. The vinculin conjugate had a final concentration of 1.3 to 10 mg/ml and a final dye to protein molar ratio of 0.50 to 0.75. The range of concentrations and labeling ratios did not affect our results.

All conjugates were stored for up to a month on ice and were clarified at 100,000 g for 20 min before microinjection. The purity of the proteins and the absence of unbound dye were determined by SDS gel electrophoresis. The average fluorescence signal was calculated for each cell by dividing the integrated intensity by the number of pixels. Accumulation of molecules at adhesion plaques that were identifiable in corresponding IRM images through changes in response to TPA were apparently affected by the absence of adhesion plaques in a given cell. Thus any measured change in fluorescence signal represents a change over a constant area. Areas to be measured were delimited using a graphics tablet. The average fluorescence intensity in the delimited area was determined by dividing the integrated intensity by the number of pixels. Accumulation of molecules at adhesion plaques was expressed in terms of the ratio between the average fluorescence in an adhesion plaque to that in the surrounding cytoplasm. Thus any photobleaching during observation was normalized and values from different cells could be directly compared. The boundary of each adhesion plaque was determined based on the image before treatment. The same set of boundaries were used in a given cell at each subsequent time point. Thus any measured change in fluorescence signal represents a change over a constant area. Only adhesion plaques that were identifiable in corresponding IRM images through the course of measurement (~95% of all adhesion plaques in a given cell) were analyzed. Essentially the same technique was used to determine the ratio of average adhesion plaque fluorescence intensity to average associated stress fiber fluorescence intensity.

Image Recording and Processing

Images were detected using a Dage-MTI (Michigan City, IN) ISIT image intensifier coupled to an image processing system comprised of a DEC PDP 11/73 microcomputer, a 330-megabyte hard disk, image processing boards (three frame buffers, arithmetic logic unit, and analog processor; Imaging Technology Inc., Woburn, MA), and a graphics tablet (GTCO, Rockville, MD). The hardware was assembled by G. W. Hannaway and Associates (Boulder, CO); the image processing software was developed by G. W. Hannaway and Associates and by the authors.

During an experiment, raw images from were fed from the image intensifier into a hard disk, which averaged 128 successive frames, and stored the average image on the hard disk. Image averaging dramatically improved signal-to-noise ratio and yield high-quality images even at extremely low levels of illumination. The gain and high-voltage settings on the image intensifier were kept constant, and the gamma correction circuitry was disabled for all experiments. The image intensifier has a linearly better than 95% over the range of operation.

For photography, an image of the dark-current was subtracted from each fluorescence image to remove background. Images were photographed from a video monitor on Tri-X film which was then developed in Diafine. For quantitative analysis, images were recalled and the background subtracted. Areas to be measured were delimited using a graphics tablet. The average fluorescence intensity in the delimited area was determined by dividing the integrated intensity by the number of pixels. Accumulation of molecules at adhesion plaques was expressed in terms of the ratio between the average fluorescence in an adhesion plaque to that in the surrounding cytoplasm. Thus any photobleaching during observation was normalized and values from different cells could be directly compared. The boundary of each adhesion plaque was determined based on the image before treatment. The same set of boundaries were used in a given cell at each subsequent time point. Thus any measured change in fluorescence signal represents a change over a constant area. Only adhesion plaques that were identifiable in corresponding IRM images through the course of measurement (~95% of all adhesion plaques in a given cell) were analyzed. Essentially the same technique was used to determine the ratio of average adhesion plaque fluorescence intensity to average associated stress fiber fluorescence intensity.

Results

Within 3 h after microinjection into BSC-1 cells (an epithelial cell line from African green monkey kidney), IATR alpha-actinin showed characteristic distribution along the length of stress fibers and at adhesion plaques, as described previously (14, 20). Microinjected IATR vinculin and FITC vinculin became incorporated primarily at adhesion plaques, also as described previously (5, 16). We detected no difference in the pattern of incorporation between the IATR and FITC conjugates of vinculin.

The time course and specific details of morphological changes in response to TPA were apparently affected by the size of the cell. To reduce the range of variation, only cells between 50 and 100 μm in diameter will be considered here. The responses were in general similar to those described...
previously (3, 11, 26, 30). The somewhat slower rate of change may be related to the lower temperature used here. None of the effects described below for TPA were observed with DMSO, 4-alpha-PDD, or medium alone. We have also processed cells microinjected with IATR alpha-actinin or IATR vinculin for indirect immunofluorescence using fluorescein-conjugated secondary antibodies. There invariably was a high degree of correlation, indicating that the microinjected conjugates were reliable indicators of the distribution of endogenous counterparts, at least at this level of resolution.

**Formation of Membrane Ruffles and Disintegration of Stress Fibers**

The most immediate change in BSC-1 cells after treatment with TPA was a dramatic increase in membranous ruffle-like structures (hereafter referred to as ruffles), which contained a large amount of injected IATR alpha-actinin (Figs. 1d and 2b, asterisks). These ruffles were dynamic, transient structures. Once formed, they could change shape, move laterally, or disappear within 2–10 min (Figs. 1 and 2). Since microinjected tetramethylrhodamine isothiocyanate-labeled ovalbumin did not concentrate in TPA-induced ruffles (not shown), the high fluorescence intensity of IATR alpha-actinin was probably not a result of high accessible volume. In addition, microinjected IATR vinculin entered ruffles to a much smaller extent than did IATR alpha-actinin. The difference between alpha-actinin and vinculin was clear in cells co-injected with IATR alpha-actinin and FITC vinculin (see Fig. 7).

Stress fibers lost alpha-actinin across their entire length

![Figure 1. Time-lapse sequence of an IATR alpha-actinin-injected BSC-1 cell which is subsequently treated with 100 ng/ml TPA at t = 0.](image)

Fluorescence images are shown in upper photographs and the corresponding IRM images shown in lower photographs. Alpha-actinin appears to deplete rapidly from most adhesion plaques within 20 min (a, c, e, and g; arrows) and more slowly from associated stress fibers (a and c; arrowheads). Most adhesion plaques can be identified in the IRM images long after the depletion of alpha-actinin. Depletion of alpha-actinin from stress fibers appears to be uniform along the length. The stress fibers later move into the perinuclear region and form a network-like structure with alpha-actinin enriched at the foci (f; arrowhead). Some of the stress fibers fragment into small bundles. A bright fluorescent ruffle forms (d; asterisk) and subsequently disappears. Minutes after TPA treatment are indicated in the lower right corner of each fluorescence micrograph. Bar, 10 μm.
Figure 2. Time-lapse sequence of an IATR alpha-actinin-injected BSC-I cell which is subsequently treated with 100 ng/ml TPA at t = 0. Several ring-shaped ruffles appear within 11 min after treatment (b; asterisk) and disappear by 20 min (c). Smaller ruffles later appear and disappear around the periphery (d, e, h, and i; arrowheads). At 51 min (f), peripheral stress fibers begin to move towards the nucleus. Over the next 30 min, these fibers become incorporated into a perinuclear network-like structure, with alpha-actinin enriched at the foci (j; white arrow). Some fibers become curved during the movement (k; black arrow). By 120 min (l) the cell has assumed a highly irregular morphology. Alpha-actinin aggregates and contorted fibers litter the cytoplasm. Minutes after TPA treatment are indicated in the upper right corner of each micrograph. Bar, 10 μm.

(Fig. 1 c, arrowheads) and frequently became fragmented into short bundles and aggregates (Figs. 1 and 2). In no case was depletion observed to proceed from one end of a fiber toward the other, or from the center of a fiber toward either end. In addition, there did not seem to be any direct temporal or spatial relationship between the formation of ruffles and the dissolution or reorganization of stress fibers.

Disintegrating stress fibers often moved into the perinuclear region (Figs. 1 and 2). Sometimes the fibers became curved as if they were pulled at a specific point in a direction...
perpendicular to the axis of the fiber (Fig. 2k). In 20–30% of cells observed, a perinuclear network-like structure can be detected with alpha-actinin enriched at the foci (Fig. 1j, arrowhead, and Fig. 2j, white arrow). These networks eventually dispersed or disintegrated into random aggregates.

We have stained IATR alpha-actinin-injected cells with fluorescein–phalloidin to examine the distribution of actin filaments. Except for the more punctate appearance of alpha-actinin along stress fibers, there was a high degree of correlation between the rhodamine alpha-actinin and fluorescein–phalloidin images in both untreated and treated cells (Fig. 3). This suggests that the distribution of alpha-actinin can also be used as a close indicator of the distribution of actin filaments.

**Alpha-actinin Is Depleted More Rapidly from Adhesion Plaques Than from Stress Fibers**

In most cells examined, TPA appeared to induce a more rapid depletion of alpha-actinin from adhesion plaques than from stress fibers. For example, in the cell shown in Fig. 1a, adhesion plaques represented the most conspicuous structures before treatment, but diminished rapidly after the addition of TPA (Fig. 1c, arrows). A clear, high magnification view of this process is shown in Fig. 4, where alpha-actinin disappeared preferentially from adhesion plaques during the first 20 min of TPA treatment. Most focal contacts in the corresponding IRM image remained detectable long after alpha-actinin fluorescence had disappeared; they slowly lost their distinct shapes and darkness over a relatively long period of time (Figs. 1 and 4).

To quantify the relative loss of alpha-actinin from adhesion plaques and from stress fibers, we used digital image analysis to determine the ratio between the average fluorescence intensity of adhesion plaques (AP) and the average fluorescence intensity of associated stress fibers (SF). In untreated cells, the ratio AP/SF should be equal to or greater than one, as alpha-actinin is localized in both stress fibers and adhesion plaques but is enriched in adhesion plaques. If alpha-actinin is preferentially depleted from adhesion plaques after TPA treatment, then this ratio should decrease to a value less than one. This indeed was the case, as shown in Fig. 5. The ratio for control cells, on the other hand, remained larger than one over the same period of observation.
Figure 6. Time-lapse sequence of an IATR vinculin-injected BSC-1 cell which is subsequently treated with 100 ng/ml TPA at t = 0. Fluorescence images are shown in upper photographs and the corresponding IRM images shown in lower photographs. Vinculin fluorescence is diminished but still present in most adhesion plaques 60 min after treatment (a and d; arrows, upper photographs). Corresponding focal contacts also persist in the IRM image (a and d; arrows, lower photographs). Minutes after TPA treatment are indicated in the lower left corner of each fluorescence micrograph. Bar, 10 μm.
Alpha-actinin Is Depleted More Rapidly Than Vinculin from Adhesion Plaques

Since the dark focal contacts in IRM images remained detectable even long after IATR alpha-actinin fluorescence had disappeared from the corresponding adhesion plaques, we asked whether vinculin might remain in adhesion plaques longer than alpha-actinin. From Figs. 1 and 6, it appeared that microinjected IATR vinculin dissociated much more slowly from adhesion plaques than did alpha-actinin. Even after 60 min, all of the original adhesion plaques can still be identified in the fluorescent vinculin image. Staining of the cell in Fig. 6 with fluorescein-phalloidin indicated that most stress fibers indeed had been disrupted (not shown). In all cells injected with IATR vinculin, vinculin was present in adhesion plaques as long as they can be detected in the corresponding IRM images.

To quantify the loss of alpha-actinin or vinculin fluorescence from adhesion plaques, we measured the ratio of average adhesion plaque fluorescence (AP) to average adjacent cytoplasmic fluorescence (AC). If an adhesion plaque can be seen in a fluorescence image, the ratio AP/AC will be greater than one. If the adhesion plaque subsequently disappears this ratio will approach one. As shown in Table I, the mean ratio AP/AC for alpha-actinin decreased significantly after 40 min of TPA treatment. The mean ratio AP/AC for IATR vinculin, on the other hand, decreased only slightly within 60 min. The mean AP/AC for control cells decreased to some extent at 20 min, but remained constant thereafter. This decrease may be related to perturbations of the cells during the addition of solutions to the culture medium.

To demonstrate directly that alpha-actinin is depleted more rapidly than vinculin from adhesion plaques, we simultaneously microinjected IATR alpha-actinin and FITC vinculin into BSC-1 cells (Fig. 7). Before treatment, there was good correspondence between the two proteins at adhesion plaques. By 40 min after treatment, however, rhodamine fluorescence in most adhesion plaques has diminished considerably, while the corresponding fluorescein image showed little change.

Discussion

Although morphological alterations of cells treated with TPA have been studied previously by the use of phase-contrast, immunofluorescence, and electron microscopy (3, 11, 26, 30), the sequence of alteration remains uncertain. Fluorescent analogue cytochemistry allows us to follow dynamic processes directly in living cells and reach a more definite understanding of the structural transformation process. The present study provides important information about how stress fibers disintegrate after TPA treatment, how the dissolution of stress fibers is related to the formation of ruffles and actin-contain-

Table I. Ratio of Average Fluorescence Intensities at Adhesion Plaques and in Adjacent Cytoplasm

<table>
<thead>
<tr>
<th>Time after addition of TPA or control solution</th>
<th>0</th>
<th>20 min</th>
<th>40 min</th>
<th>60 min</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Alpha-actinin</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TPA-treated cells</td>
<td>1.46 ± 0.16</td>
<td>1.24 ± 0.19</td>
<td>1.02 ± 0.13</td>
<td>—</td>
</tr>
<tr>
<td>Control cells</td>
<td>1.50 ± 0.14</td>
<td>1.29 ± 0.10</td>
<td>1.33 ± 0.15</td>
<td>1.29 ± 0.14</td>
</tr>
<tr>
<td><strong>Vinculin</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TPA-treated cells</td>
<td>1.48 ± 0.18</td>
<td>1.37 ± 0.16</td>
<td>1.29 ± 0.16</td>
<td>1.20 ± 0.18</td>
</tr>
<tr>
<td>Control cells</td>
<td>1.42 ± 0.14</td>
<td>1.29 ± 0.09</td>
<td>1.34 ± 0.16</td>
<td>1.34 ± 0.17</td>
</tr>
</tbody>
</table>

Cells were microinjected with IATR alpha-actinin or IATR vinculin and incubated for at least 3 h. TPA dissolved in DMSO or an equal volume of control solution, which consisted of either medium, DMSO, or 4-alpha-PDD in DMSO, was then added at t = 0 min. Fluorescence intensities were then measured and ratios calculated at specified time points as described in Materials and Methods.

* Numbers indicate that data were collected from 110 adhesion plaques in 5 cells.

Figure 7. A BSC-1 cell coinjected with IATR alpha-actinin (a and c) and FITC vinculin (b and d). Fluorescence images are recorded before (a and b) and 40 min after (c and d) treatment with 100 ng/ml TPA. Alpha-actinin is significantly depleted from most adhesion plaques by this time (a and c; arrows); vinculin distribution remains relatively unchanged (b and d; arrows). A large ruffle forms which contains a large amount of alpha-actinin (c; arrowhead) but much less vinculin (d; arrowhead). Bar, 10 μm.
ing aggregates, and how the integrity of stress fibers is related to the association with adhesion plaques.

The structures induced most immediately by TPA are the highly dynamic ruffles, which, unlike ruffles in untreated cells, can appear close to stress fibers. However, although previous investigators have noted a strong coincidence between increased membrane ruffling and loss of stress fibers (2, 4, 24, 29, 34), we have never detected a direct temporal relationship between the disruption of a specific stress fiber and the appearance of ruffles in its vicinity. Thus it is unlikely that ruffles directly induce the breakdown of stress fibers.

TPA, however, may induce breakdown of stress fibers through disruption of adhesion plaques. The possible role of adhesion plaques and vinculin in the maintenance of the integrity of stress fibers has long been a subject of speculation (19). On the one hand, vinculin appears to co-localize with the transforming kinase, pp60^c-src, in cells infected with the Rous sarcoma virus (27), and is one of the primary components phosphorylated during viral transformation as well as during treatments of TPA and growth factors (12, 31, 37). The subsequent disruption of stress fibers suggests that vinculin and adhesion plaques may play a crucial role in their stability. On the other hand, the extent of vinculin phosphorylation seems limited in transformed cells (31), and increases in vinculin phosphorylation alone appears insufficient to cause dissolution of stress fibers (28).

Our results indicate that both adhesion plaques and associated vinculin persist even after most stress fibers have become disrupted. Therefore, it is unlikely that the disintegration of stress fibers is caused by the depletion of vinculin molecules or the detachment of substrate contact. Conversely, neither the maintenance of substrate contact nor the association of vinculin with adhesion plaques appears to be directly dependent on the association with stress fibers or the presence of alpha-actinin.

However, our data indicate that alpha-actinin at adhesion plaques may play an important role in the stability of stress fibers. The depletion of alpha-actinin from adhesion plaques represents one of the earliest events following TPA treatment. This was then followed by the dissociation of alpha-actinin (and presumably, other components, including actin) along the length of stress fibers as well as fragmentation of stress fibers. Many fibers subsequently move into the perinuclear region. Eventually the stress fibers disperse or disintegrate into numerous actin- and alpha-actinin–containing bundles and aggregates. Thus it is possible that the dissociation of alpha-actinin from adhesion plaques causes stress fibers to dissociate from adhesion plaques and subsequently disintegrate.

It will be very important to determine the mechanism involved in the rapid depletion of alpha-actinin from adhesion plaques. At adhesion plaques, vinculin is localized closer to the plasma membrane than alpha-actinin (7). TPA may interfere with a link between alpha-actinin and vinculin, causing alpha-actinin, but not vinculin, to dissociate from adhesion plaques. Alternatively, alpha-actinin may be depleted from adhesion plaques through competitive binding with ruffles, or through an alteration of the cytoplasmic ionic environment (10, 23).

Our results contrast with the recent report by Herman and Pledger (18) that vinculin redistribution is the primary event in microfilament reorganization induced by the platelet-derived growth factor. Although there is no clear explanation at present, the difference may reflect some subtle but important differences between the disruptions induced by various agents. For example, the different kinases activated by TPA and platelet-derived growth factor may have different effects on vinculin. In addition, the formation of ruffles is much more pronounced in cells treated with TPA than with platelet-derived growth factor (18, 30). Future applications of fluorescent analogue cytochemistry to cells treated with growth factors and cells infected with transforming viruses should shed light on the similarities as well as differences among these related processes.

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Note Added in Proof: Drs. Takahashi, Junker, and Heine at the National Cancer Institute informed us, through personal communication, of their observation that stress fibers in the epidermal cell line JB6 disappear more rapidly than vinculin plaques after TPA treatment.

References

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