Synthetic Peptide GRGDS Induces Dissociation of Alpha-Actinin and Vinculin from the Sites of Focal Contacts

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Abstract. The synthetic peptide Gly-Arg-Gly-Asp-Ser (GRGDS) mimics the cellular binding site of many adhesive proteins in the extracellular matrix and causes rounding and detachment of spread cells. We have studied whether its binding affects the associations of two major components, alpha-actinin and vinculin, at the adhesion plaque. Living 3T3 cells were microinjected with fluorescently labeled alpha-actinin and/or vinculin and observed using video microscopy before and after the addition of 50 µg/ml GRGDS. As soon as 5 min after treatment, fluorescent alpha-actinin and vinculin became dissociated simultaneously from the sites of many focal contacts. The proteins either moved away as discrete structures or dispersed from adhesion plaques. As a result, the enrichment of alpha-actinin and vinculin at these focal contacts was no longer detected. The focal contacts then faded away slowly without showing detectable movement. These data suggest that the binding state of integrin has a transmembrane effect on the distribution of cytoskeletal components. The dissociation of alpha-actinin and vinculin from adhesion plaques may in turn weaken the contacts and result in rounding and detachment of cells.

THE interactions between the cell surface and the extracellular matrix (ECM)[1] affect many fundamental properties of the cell, such as shape, locomotion, growth, and differentiation (for reviews see Ruoslahti and Pierschbacher, 1987; Burridge, 1986; Buck and Horwitz, 1987). However, it is not clear how the effects of these interactions are transmitted across the plasma membrane.

One possible way for the ECM to exert its effect is through the cytoskeleton. There is accumulating evidence that the ECM may interact with the microfilament system. For example, the distribution of fibronectin bundles on the surface of cultured fibroblasts correlates closely with the distribution of intracellular actin filament bundles (stress fibers; Hynes and Destree, 1978; Singer, 1979). In addition, focal contacts, where cultured cells make an extremely close adhesion with the substrate, contain not only ECM receptors, but also actin, alpha-actinin, vinculin, and talin (Burridge, 1986; Kelly et al., 1987). Consistent biochemical observations indicating direct associations of fibronectin receptors and talin (Horwitz et al., 1986) have also been reported. Thus an attractive hypothesis is that the binding of the ECM may cause changes in their receptors, which in turn affect the organization of membrane-associated cytoskeletal components.

One approach to this question is to manipulate the association of the cell with the ECM and examine the effects on the distribution of structural components, such as alpha-actinin and vinculin, that are normally enriched at the cytoplasmic side of focal contacts (referred to as the adhesion plaque in this article). Recent studies indicate that the cellular association of many ECM proteins can be disrupted with a synthetic peptide, Gly-Arg-Gly-Asp-Ser(GRGDS), which mimics a cellular binding domain of these proteins (Ruoslahti and Pierschbacher, 1987; Yamada and Kennedy, 1987). After a brief treatment of cultured fibroblasts with this peptide, the colocalization of GRGDS-binding receptors, referred to as integrins, with extracellular fibronectin fibers becomes progressively lost (Chen et al., 1986). However, little is known about the organization of cytoskeletal proteins in the adhesion plaques during this process; specifically, whether they remain associated with focal contacts and with each other.

In this study, we examined the organization of alpha-actinin and vinculin at adhesion plaques after a brief treatment of GRGDS. To study directly the sequence of events in living cells, we have microinjected cells with functional fluorescent analogues of vinculin and alpha-actinin. The cells were then observed before and after the addition of GRGDS using fluorescence and interference reflection microscopy (IRM). Our data indicated that the peptide induces a rapid, simultaneous dissociation of alpha-actinin and vinculin from the sites of focal contacts well before the disappearance of the contacts themselves.

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1. *Abbreviations used in this paper:* ECM, extracellular matrix; IRM, interference reflection microscopy.

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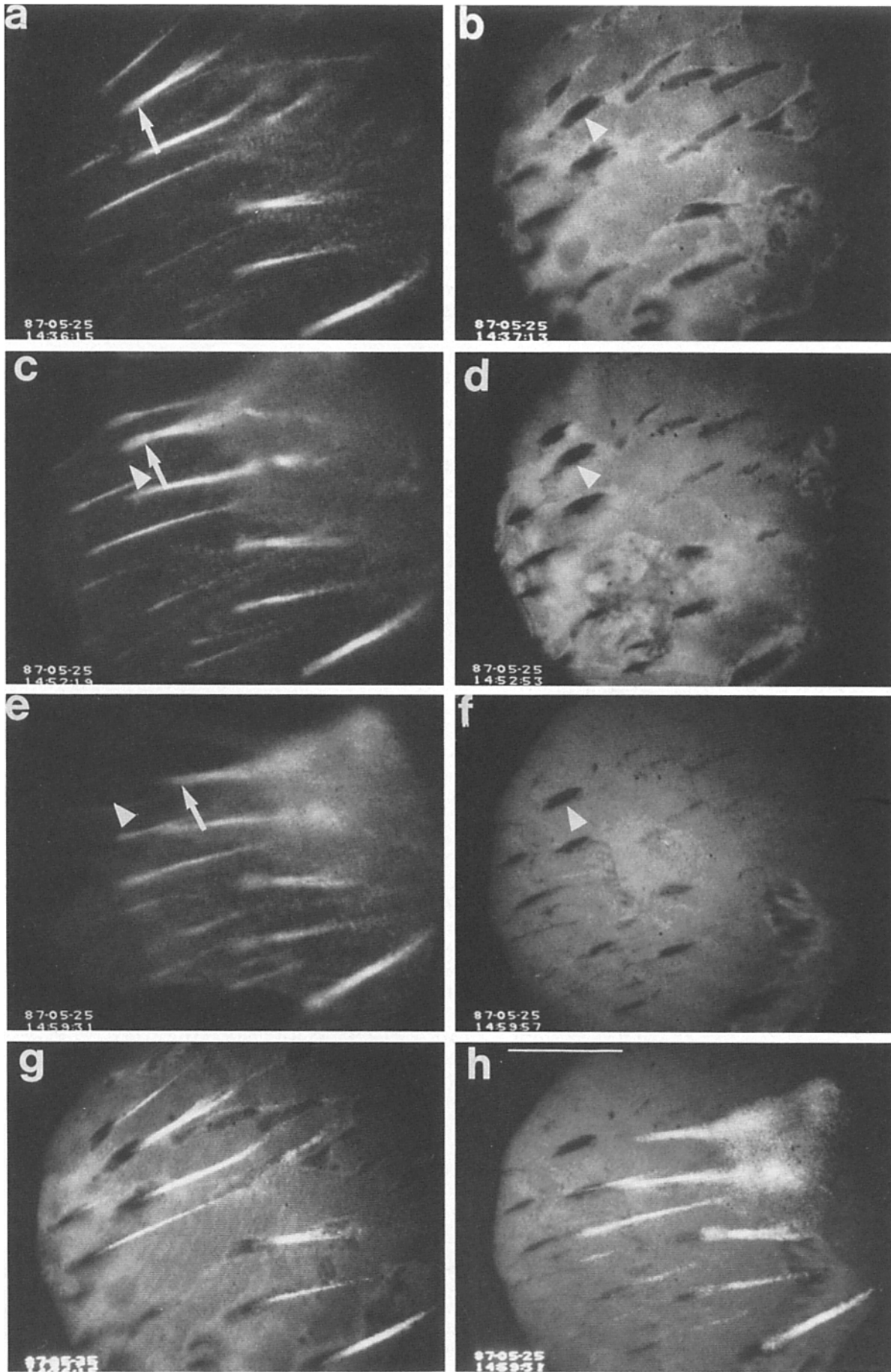
Materials and Methods

Cell Culture and Protein Preparation

Swiss 3T3 cells (American Type Culture Collection, Rockville, MD) were grown in DME (KC Biological Inc., Lenexa, KS) supplemented with 10% calf serum (Colorado Serum Co., Denver, CO), 50 U/ml penicillin, 50 μg/ml streptomycin, and 2 mM L-glutamine. Cells were plated onto injection dishes (Wang, 1984) 2–3 d before experiments.

Alpha-actinin and vinculin were purified from turkey gizzards as described previously (Meigs and Wang, 1986). Alpha-actinin was labeled with tetramethylrhodamine iodoacetamide or with 5-iodoacetamidofluorescein (Molecular Probes, Inc., Junction City, OR; Meigs and Wang, 1986). The conjugate had a final concentration of 5.0 mg/ml and an estimated labeling ratio of 0.7–1.3 fluorophore per 100,000-D polypeptide.

Vinculin was labeled with TRITC (10% on celite; Research Organics, Inc., Cleveland, OH) by mixing equal volumes of vinculin (1.0 mg) and 200 mM potassium borate buffer, pH 9.0, and then adding 1.2 mg of TRITC on celite. The mixture was stirred at 0°C for 2 h and clarified in a rotor (model 42.2 Ti; Beckman Instruments, Inc., Palo Alto, CA) at 100,000 g for 20 min to remove celite. An equal volume of 100 mM lysine in borate buffer was added to the mixture to quench the reaction. The mixture was incubated on ice for 2 h before it was applied to a 0.7 × 15-cm column of Bio-Beads SM-2 (Bio-Rad Laboratories, Richmond, CA) and eluted with 2 mM Tris, pH 8.5. Fluorescent fractions were pooled and concentrated in a Centricon-30 (Amicon Corp., Danvers, MA). The protein solution was then dialyzed against 2 mM Pipes, 0.1 mM dithiothreitol (DTT), pH 6.95. The protein conjugate had a final concentration of 4.0–5.0 mg/ml and a final dye to protein molar ratio of 0.6–1.5 using a molar extinction coefficient of 55,000 at 555 nm for bound tetramethylrhodamine.

Microinjection and Microscopy

Cells were cultured on the microscope stage and microinjected as described previously (Wang, 1984). Fluorescence microscopy, IRM, and digital image processing were performed as in previous studies (Meigs and Wang, 1986). A Zeiss Neofluar 63× objective (NA 1.25) was used for all experiments.

Application of Synthetic Peptides to Living and Permeabilized Cells

All synthetic peptides were obtained from Peninsula Laboratories, Inc. (Belmont, CA) and stored lyophilized at −20°C. Before applying the peptides to microinjected living cells, fluorescence and IRM images were recorded. Culture medium was then removed and replaced with medium containing 50 μg/ml of resuspended peptide without moving the culture dish from its initial position on the microscope stage. Subsequent images were recorded at various time intervals depending upon how quickly the cell responded to treatment.

Experiments involving permeabilized cells and isolated membranes were performed by first microinjecting living cells with fluorescent alpha-actinin or vinculin. Cells were then permeabilized by incubating in a buffer of 50 mM 2-(N-morpholino)ethane sulfonic acid (MES), 3 mM EGTA, 5 mM MgCl₂, 0.5% Triton X-100, pH 6.0 for 2 min (Avnur et al., 1983). Isolated membranes were prepared after the ZnCl₂ method of Avnur et al. (1983). Addition of peptides was performed as described for living cells with the exception of resuspending the peptides in the membrane isolation buffer or the permeabilization buffer without Triton.

Results

Fluorescent analogue cytochemistry (Wang et al., 1982) was used to examine the distribution of alpha-actinin and vinculin in living cells before and after the treatment of GRGDS. As in previous studies (Meigs and Wang, 1986), the fluorescent analogues became incorporated into adhesion plaques within 1 h of microinjection into 3T3 cells. Before treatment with GRGDS, there was a close correlation between the distribution of focal contacts, as revealed by IRM, and the distributions of alpha-actinin and vinculin (e.g., Fig. 1, *a* and *b*; see Fig. 3, *a* and *b*). In addition, most adhesion plaques showed no detectable translocation over a period of at least 20 min.

The addition of GRGDS caused rounding and eventual detachment of the cell from the substrate. At a concentration of 50 μg/ml, GRGDS induced a gradual response and allowed intermediate steps to be observed. Complete rounding generally occurred between 20 and 30 min but varied from cell to cell.

We were first interested in the possible changes of alpha-actinin near the sites of focal contacts before cell rounding took place. Two major types of response were observed. In the first case, alpha-actinin at adhesion plaques moved toward the center of the cell as discrete structures (Fig. 1, *a*, *c* and *e*; see also Fig. 5). As a result, alpha-actinin–containing structures appeared separated from the corresponding focal contacts (Fig. 1, *g* and *h*; see also Fig. 5). The distance between the two can reach 3–5 μm within 10 min of treatment. The focal contacts faded gradually. However, many remained detectable until the cell rounded up (Figs. 1 *f* and 2 *f*).

In a second type of response, alpha-actinin dispersed away from adhesion plaques (Fig. 2, *a*, *c* and *e*), while focal contacts persisted longer (Fig. 2, *b*, *d*, and *f*). As in the first type of response, the affected focal contacts lost their characteristic enrichment of alpha-actinin and faded gradually. By following the distribution of alpha-actinin in 152 adhesion plaques located near the periphery of 14 cells, we found 63 showing the movement (41%), 24 showing the dispersion (16%), 34 showing both responses simultaneously (22%), and 31 showing no apparent change (21%).

We have also examined the distribution of vinculin after the addition of GRGDS. Vinculin, like alpha-actinin, either moved away from the sites of focal contacts as discrete structures (Fig. 3; see also Figs. 4 and 5) or dispersed from adhesion plaques (Fig. 5). By following 387 vinculin-labeled peripheral adhesion plaques in 18 cells, we found 141 showing movement (36%), 58 showing dispersion (15%), 74 showing both responses (19%), and 114 showing no apparent change (29%).

Direct correlation of the two proteins was performed with

Figure 1. GRGDS-induced movement of alpha-actinin–containing structures. Fluorescein-labeled alpha-actinin was microinjected into 3T3 cells where it became localized in stress fibers and adhesion plaques. Fluorescence images were recorded before (*a*), and 1 (*c*) and 8 (*e*) min after the addition of 50 μg/ml GRGDS. Corresponding IRM images (*b*, *d*, and *f*) were taken within 1 min of recording the alpha-actinin images. As the cell starts to respond (*c*), alpha-actinin–containing structures (*arrows*) move toward the center of the cell, while the corresponding focal contacts remain virtually unchanged (*b* and *d*, *arrowheads*). As alpha-actinin continues to move away from the cell edge (*e*, *arrow*), focal contacts remain stationary but appear to fade gradually (*f*, *arrowhead*). The location of the focal contact is indicated on alpha-actinin images (*c* and *e*, *arrowheads*). To facilitate the visualization of the movement of alpha-actinin relative to focal contacts, a superimposed image of *a* and *b* is shown in *g* and a superimposed image of *e* and *f* is shown in *h*. Bar, 10 μm.
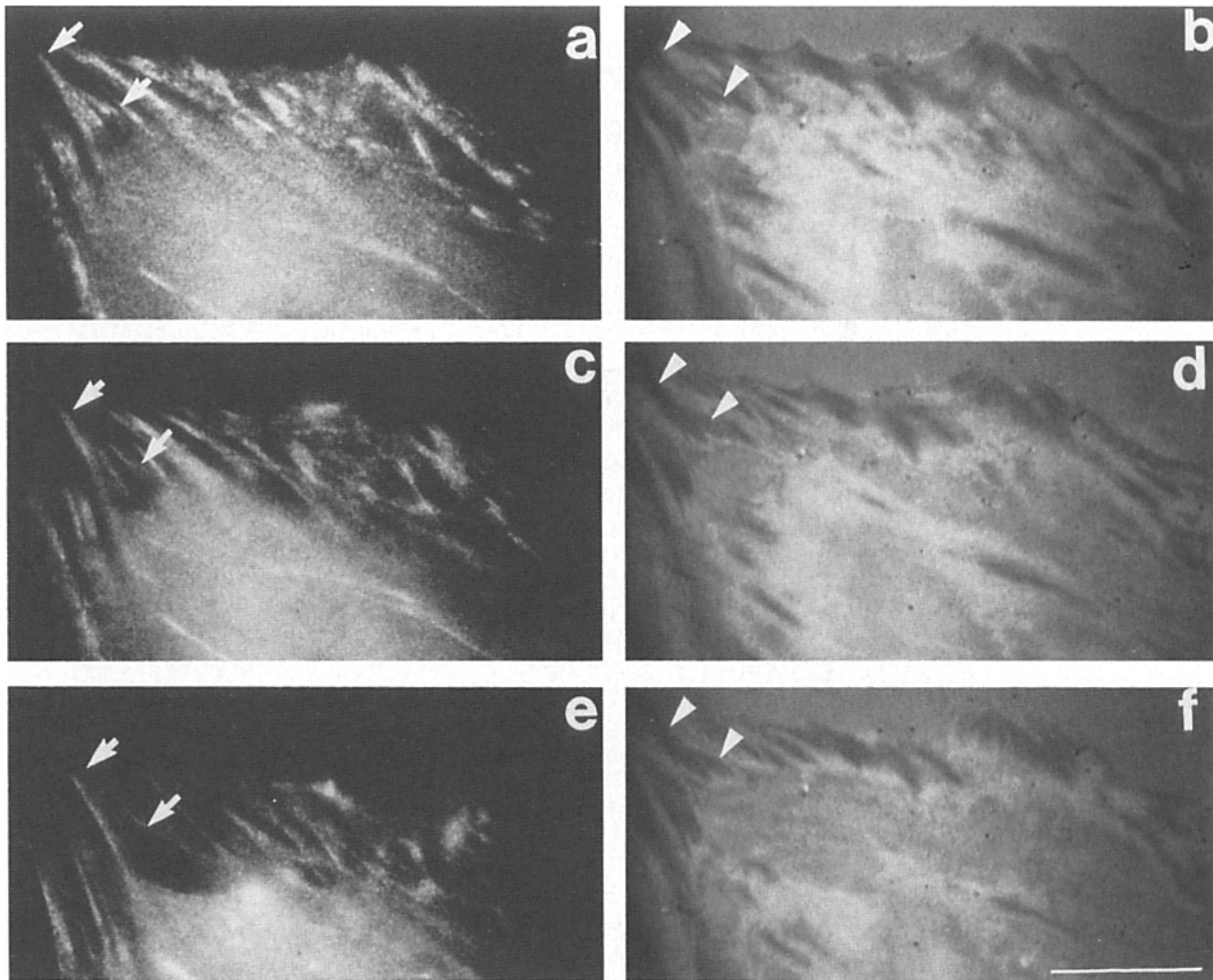
Figure 2. GRGDS-induced dispersion of alpha-actinin from adhesion plaques. Fluorescein-labeled alpha-actinin was microinjected into 3T3 cells where it became localized in adhesion plaques. Fluorescence images were recorded before (*a*), and 6 (*c*) and 20 (*e*) min after the addition of 50 μg/ml GRGDS. Corresponding IRM images (*b*, *d*, and *f*) were taken within 1 min of recording the alpha-actinin images. Alpha-actinin gradually disappears from the adhesion plaques (*arrows*). Concentration of alpha-actinin at adhesion plaques is no longer detected at the last time point, while the IRM patterns undergo little changes (*arrowheads*). Bar, 10 μm.

cells injected sequentially with fluorescein-labeled alpha-actinin and rhodamine-labeled vinculin. As shown in Fig. 4, both proteins moved simultaneously away from their original sites after GRGDS treatment. Fig. 5 shows both simultaneous movement and simultaneous dispersion of the two proteins from adhesion plaques. These results suggested that vinculin and alpha-actinin molecules probably dissociated from focal contacts as a complex.

To determine whether similar responses can be reproduced in a model system, we applied the peptide to permeabilized cells and isolated membranes, which were prepared without disturbing the focal contacts and membrane-associated vinculin and alpha-actinin (Avnur et al., 1983). As shown in Fig. 6, no change in alpha-actinin or vinculin organization was observed in permeabilized cells after treatment with GRGDS, indicating that intact cells or extractable components were required for the responses. An identical result was obtained with isolated membranes.

Control experiments were performed with related pep-

tides, Gly-Arg-Gly-Asp and Gly-Arg-Gly-Glu-Ser-Pro, which have a much weaker effect compared to GRGDS (Yamada and Kennedy, 1987). Little effect on alpha-actinin and vinculin was detected over the first 20 min when GRGDS usually produced dramatic effects (Fig. 7). With prolonged exposure, slow reorganizations qualitatively similar to those induced by GRGDS were observed. However, detachment of cells did not occur over several hours.

Discussion

The ability of GRGDS to induce rounding and detachment of cultured cells has been described previously (Chen et al., 1986). The mechanism probably involves a competitive binding against a wide spectrum of ECM proteins, including fibronectin, laminin, vitronectin, and type I collagen (Yamada and Kennedy, 1987) for their integrin receptors. The perturbation of the binding of these proteins eventually leads to the detachment of the cell from the substrate.
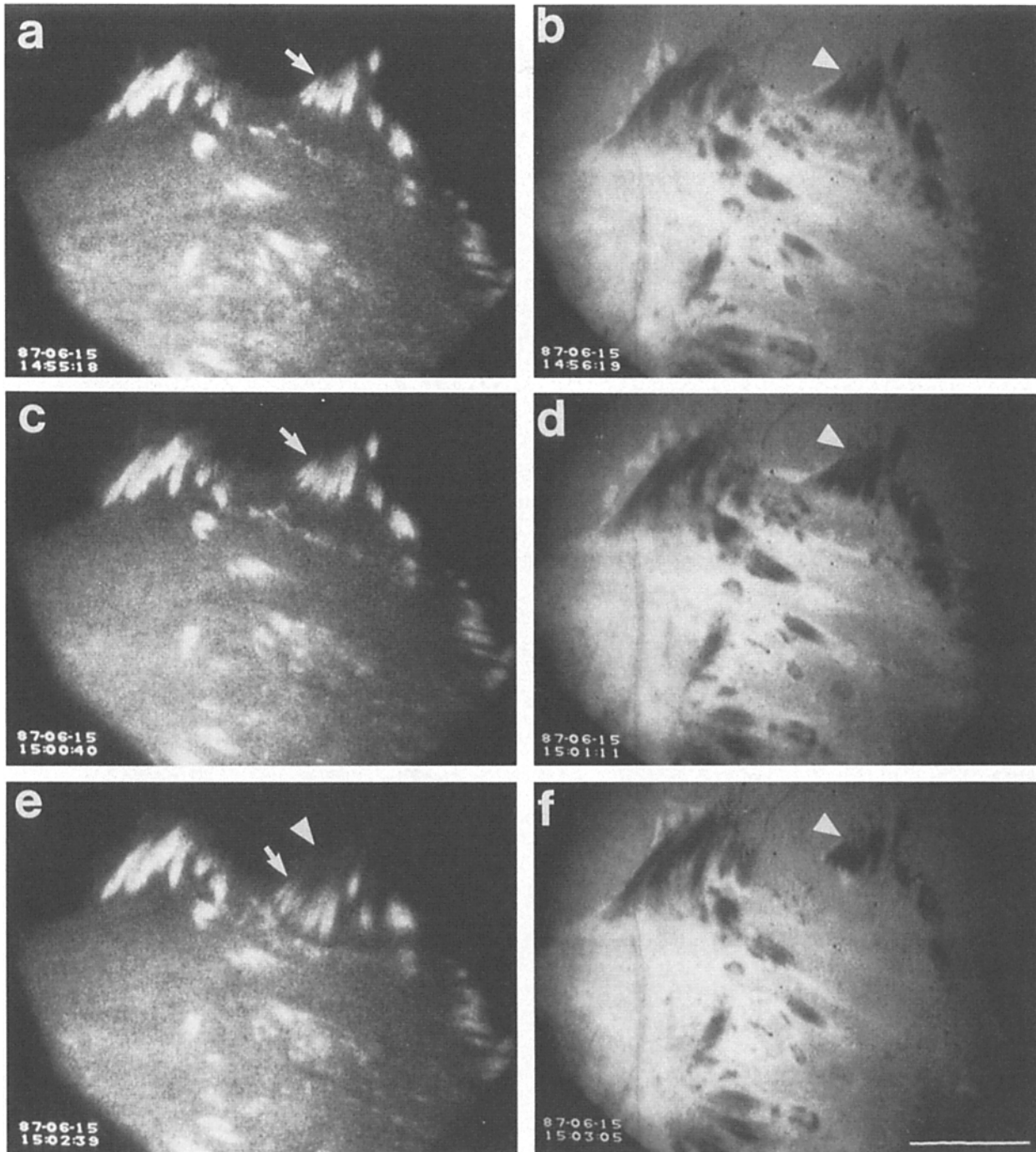
Figure 3. GRGDS-induced movement of vinculin plaques. Rhodamine-labeled vinculin was microinjected into 3T3 cells where it became localized in the adhesion plaques. Fluorescence images were recorded before (*a*), and 3 (*c*) and 5 (*e*) min after the addition of 50 μg/ml GRGDS. Corresponding IRM images (*b*, *d*, and *f*) were taken within 1 min of recording the vinculin images. The fluorescent vinculin, which remains in discrete plaques (*arrows*), moves away from the edge toward the center of the cell, while the focal contacts, as shown in IRM images, remain stationary (*arrowheads* in *b*, *d*, and *f*). The position of focal contact is also indicated on the vinculin image (*e*, *arrowhead*). Bar, 10 μm.

The present results indicate that one of the early responses induced by GRGDS is a depletion of alpha-actinin and vinculin from the sites of focal contacts. This was then followed by a gradual disappearance of the contact structures. Al-though we have observed two different patterns of dissocia-tion, dispersion and movement as discrete structures, a com-mon mechanism may be involved and the pattern may be determined by the local environment of the adhesion plaque.
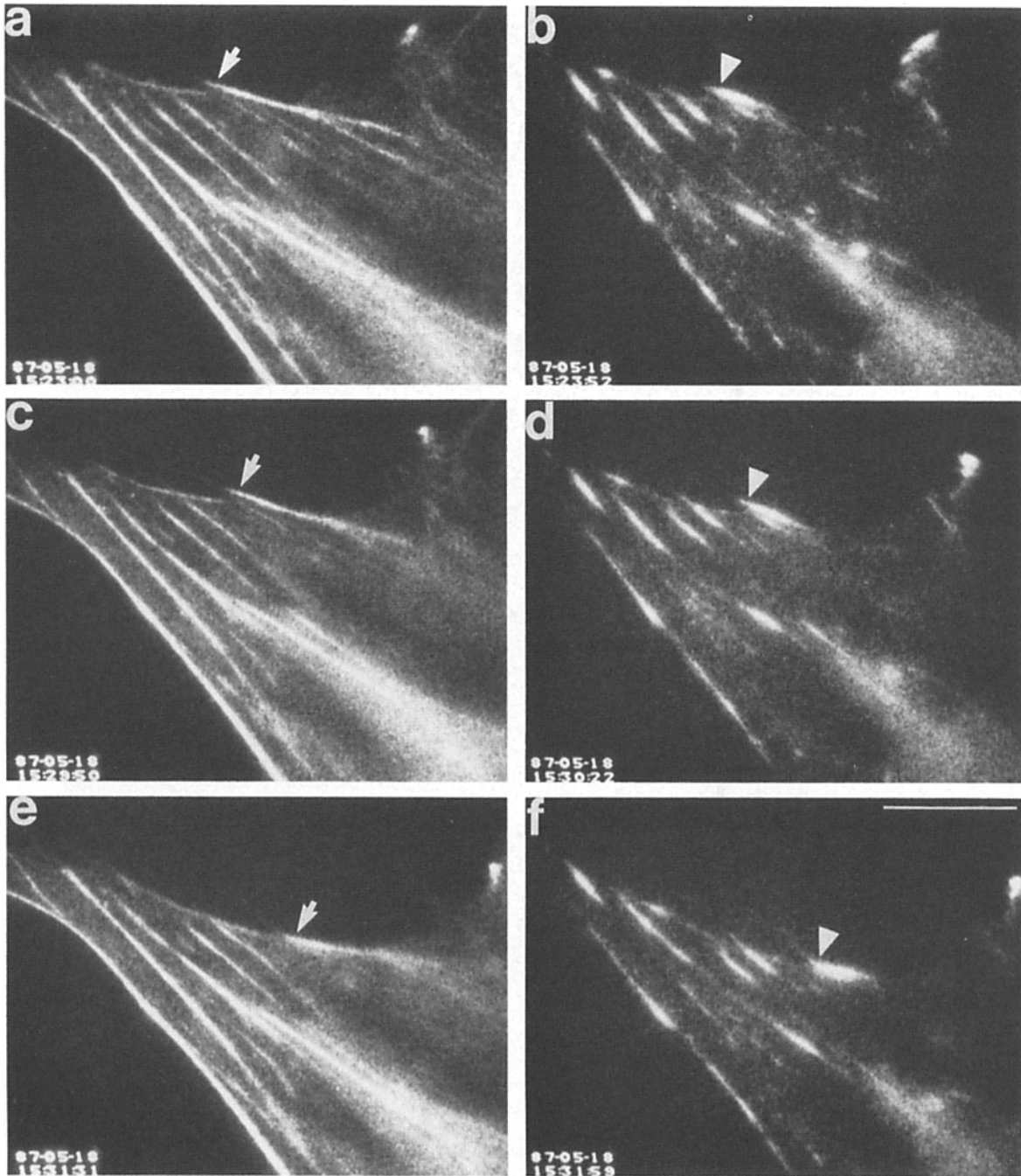
Figure 4. Simultaneous movement of vinculin- and alpha-actinin–containing structures after the addition of GRGDS. Fluorescein-labeled alpha-actinin and rhodamine-labeled vinculin were microinjected into 3T3 cells where they became colocalized in adhesion plaques as identified by IRM (not shown). Images of alpha-actinin images were recorded before (*a*), and 1 (*c*) and 3 (*e*) min after the addition of 50 μg/ml GRGDS. Corresponding vinculin images (*b*, *d*, and *f*) were taken within 1 min of recording the alpha-actinin images. Alpha-actinin (*arrows*) and vinculin (*arrowheads*) move together away from their original positions. Bar, 10 μm.

For example, local tension or contractile forces may cause detached structures to move toward the same direction as an aggregate.

Similar dissociations of alpha-actinin or vinculin have been observed under other conditions. For example, when cells were treated with a tumor-promoting phorbol ester, alpha-actinin and stress fibers became dissociated from the focal contacts (Meigs and Wang, 1986). However, unlike the present case, vinculin stayed with the residual focal contacts for a longer period of time. In another experiment, Herman and Pledger (1985) observed that platelet-derived growth factor induced a rapid depletion of vinculin from adhesion plaques, whereas talin, also a component of the adhesion plaque, was unaffected.
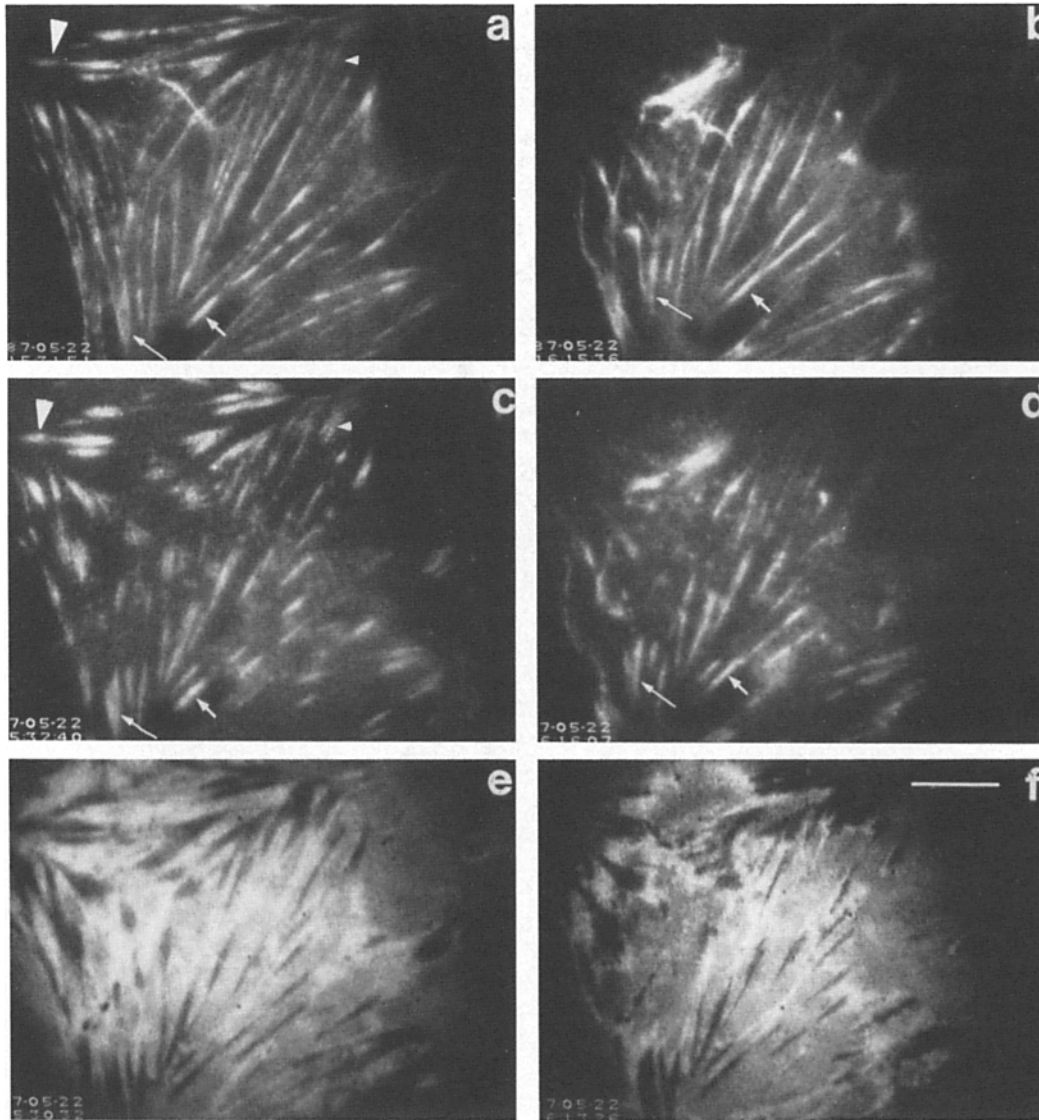
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Figure 5. Simultaneous movement and dispersion of vinculin and alpha-actinin after the addition of GRGDS. Rhodamine-labeled vinculin and fluorescein-labeled alpha-actinin were microinjected sequentially into 3T3 cells where they became colocalized in adhesion plaques as identified by IRM (*e* and *f*). Images of alpha-actinin were recorded before (*a*) and 40 min after (*b*) the addition of 50 µg/ml GRGDS. Corresponding vinculin (*c* and *d*) and IRM images (*e* and *f*) were also recorded. Vinculin and alpha-actinin either move simultaneously (*arrows*) or disperse simultaneously (*arrowheads*) away from adhesion plaques. Changes in IRM images are much less pronounced. Bar, 10 µm.

Based on the present observations, one may conclude that, by binding to the extracellular domain of integrins, GRGDS causes the dissociation of cytoskeletal structures from surface molecules that are directly responsible for creating the IRM pattern. Furthermore, our negative results with permeabilized cells and cell models indicate that the process requires soluble factors and/or structural integrity of the membrane or cytoplasm. Two possibilities may be considered regarding the mechanism of the dissociation. First, the disruption of the binding of integrins to ECM may induce a transmembrane conformational change in the cytoplasmic portion of the integrin molecule, causing alpha-actinin and vinculin to dissociate from the membrane. This may involve a change in the state of phosphorylation of integrin, which

has a potential target site for the tyrosine-specific kinases in the cytoplasmic domain (Hirst et al., 1986; Tamkun et al., 1986). Second, GRGDS may cause integrin to dissociate from ECM and become mobile on the plasma membrane. The molecule may then move away from focal contacts as a complex with vinculin and alpha-actinin. This mechanism appears consistent with the progressive loss of integrin-fibronectin association at the cell periphery after GRGDS treatment (Chen et al., 1986).

At least in the present case and in the experiment with phorbol esters (Meigs and Wang, 1986), the dissociation of cytoskeletal components was followed by a gradual disappearance of focal contacts. Therefore, it is possible that cytoskeletal elements, while responding to the interactions of
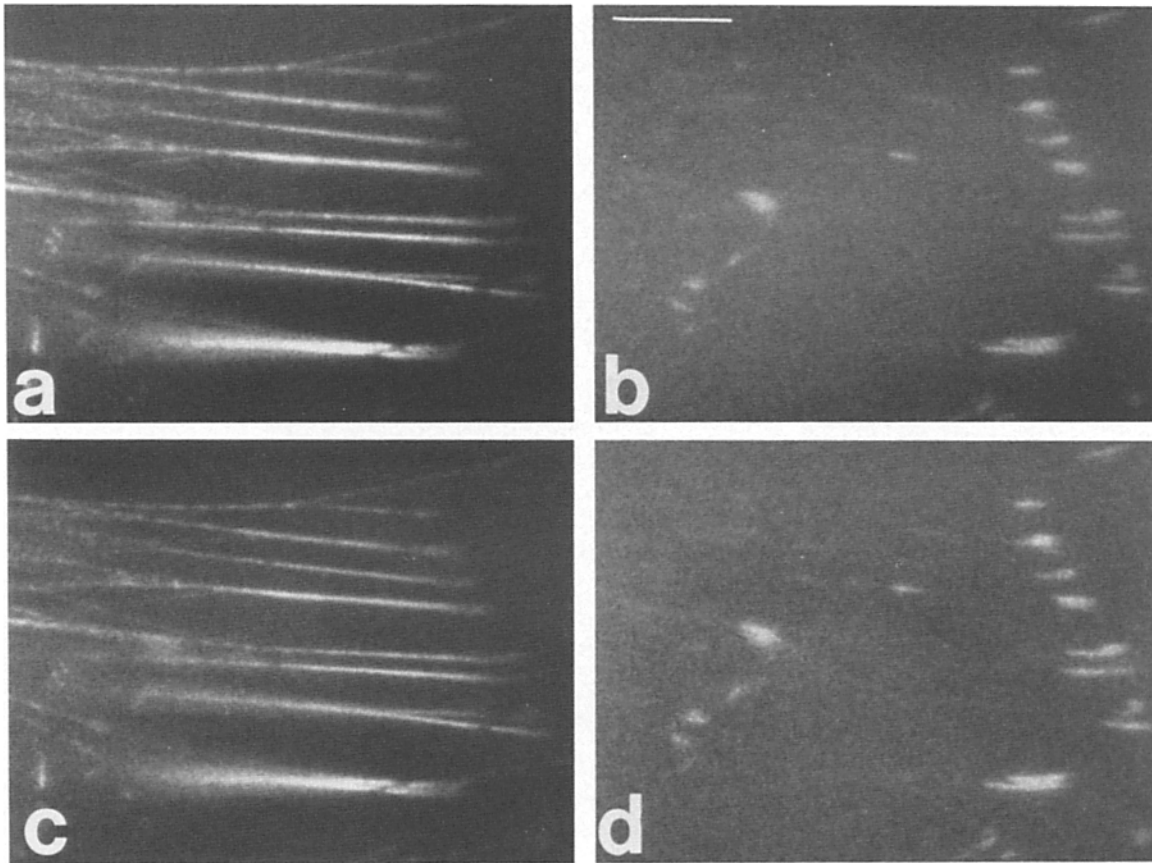
Figure 6. Lack of response of alpha-actinin and vinculin to GRGDS in permeabilized cells. 3T3 cells were microinjected with fluorescein-labeled alpha-actinin and rhodamine-labeled vinculin and permeabilized as described in Materials and Methods. Fluorescence images of alpha-actinin (*a* and *c*) and vinculin (*b* and *d*) were recorded before (*a* and *b*) and 20 min after (*c* and *d*) the addition of GRGDS. No change in fluorescence images is detected during the 20-min period of treatment. Bar, 10 μm.
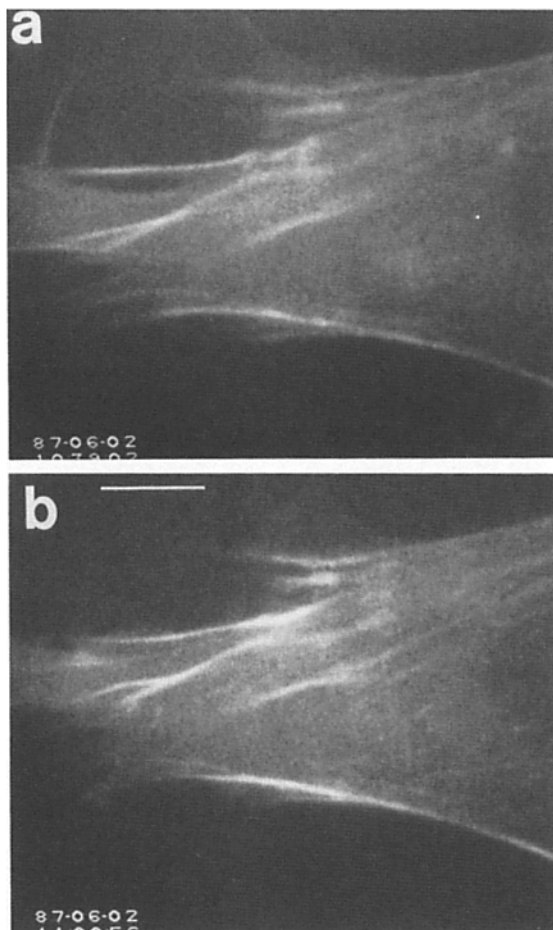
surface receptors with ECM, may at the same time play an important role in the stability of the interactions between the surface and the extracellular substrate. This is consistent with the observations that microinjections of proteins that disrupt actin structures induce rounding of the cell (Fuchtbauer et al., 1983; Cooper et al., 1987). A similar conclusion may be drawn regarding the role of integrin in focal contacts. Since focal contacts were detected well after the application of GRGDS peptides, the association between integrins and ECM may not be directly responsible for the appearance of the contact structures as detected by IRM. However, either through interactions with other surface molecules or through interactions with the cytoskeleton as discussed above, integrin may nevertheless play an important role in the stability of focal contacts.

Figure 7. Lack of response of alpha-actinin distribution to the control peptide Gly-Arg-Gly-Glu-Ser-Pro. Fluorescence images of fluorescein-labeled alpha-actinin were recorded before (*a*) and 20 min after (*b*) applying the peptide. Stress fibers show minor changes in arrangement, common in living cells, but responses as seen in GRGDS treatment are not observed. Bar, 10 μm.

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