Proc. Natl. Acad. Sci. USA
Vol. 76, No. 8, pp. 3814–3818, August 1979
Biochemistry

In vivo distribution and turnover of fluorescently labeled actin microinjected into human fibroblasts

(cell motility/stress fibers/fluorescence microscopy/reflection contrast microscopy)

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Communicated by A. Frey-Wyssling, May 31, 1979

ABSTRACT **Graessmann's microinjection technique was chosen to introduce fluorescently labeled muscle actin and other proteins into WI-38 human fibroblasts. The injected cells were examined during culture by fluorescence and reflection contrast microscopy. Within 30 min after injection, rhodamine-labeled actin was incorporated into a distinct network of fluorescent filaments, resembling the stress fibers observed by classical immunofluorescence microscopy. Cytochalasin b prevented the formation of these fibers, but colchicine did not. Neighboring fibers often converged into distinct focal points that appeared to be concentrated near the base of the cell. Examination of these fluorescent fibers and focal points by reflection contrast microscopy confirmed their close location to the substratum. After 6 hr of culture, fluorescent actin and the control proteins were segregated into granules located mainly near the cell nucleus. Thus, the injected actin both enters the intrinsic actin pool and participates in an assembly and disassembly of filamentous structures. Segregation into granules traces the natural turnover of this protein within the cell.**

Recent research on nonmuscle cell motility has been directed predominantly toward the isolation and characterization of the proteins likely to be involved and toward the development of immunofluorescence and electron microscopy techniques to localize these components within the cell (cf. ref. 1 for reviews). Such methods, however, have certain limitations, because (*i*) during isolation the complex network of interactions is destroyed and might not be reconstituted to its native form *in vitro*, (*ii*) with the above microscopic techniques artifacts due to fixation might occur, and (*iii*) only insufficient information can be obtained on the dynamic processes of cellular motility.

In order to overcome these limitations specific cytoskeletal structures must be visualized directly within the living cell. Microinjection of fluorescently labeled proteins, as applied by Graessmann and collaborators (2), as well as by other groups (cf. refs. 3–5), appears to be an ideal technique for such purposes. By means of sensitive fluorescence microscopy the behavior of injected tracers can be studied in terms of distribution, turnover, and mutual interactions. Furthermore, a new optical technique—i.e., Leitz' reflection contrast microscopy—offers additional possibilities for characterizing the fluorescent structures in their three-dimensional architecture.

The present communication demonstrates that actin as well as other proteins, after microinjection into tissue culture cells, is, in fact, incorporated into the intrinsic protein pool. Thus, actin enters stress fibers and their focal points at the cell membrane and is finally degraded within cytoplasmic granules.

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MATERIALS AND METHODS

Cells. WI-38 human fibroblasts, a generous gift of G. Thomas (Friedrich Miescher-Institute, Ciba Geigy AG, Basel, Switzerland), were grown on glass coverslips in Dulbecco's modified Eagle's medium containing 10% fetal calf serum and gentamycin at 100 μg/ml. Microinjection of the cells was carried out in phosphate-buffered saline.

Microinjection. The technique was modified from that described by Graessmann *et al.* (2). To this end, microcapillaries were drawn on an M1 Puller (Industrial Science Associates, Ridgewood, NY 11227) from borosilicate glass capillaries (type GC 150 F, Clark Electromedical Instruments, Pangbourne, Reading, England). An inner filament within these needles transferred the solution to be injected, loaded from the rear, to the tip (diameter less than 2 μm). The injected proteins, 1–2 mg/ml, were in either 5 mM sodium carbonate, pH 7.4 (actin) or 5 mM sodium carbonate/100 mM KCl, pH 7.4 (other proteins). The volume of the injected solution has not been accurately determined but is $\approx$5% of the cell volume (2, 3).

Fluorescent Labeling of Proteins. Actin was purified from rabbit psoas muscle by the procedure of Spudich and Watt (6). The F-form, 5 mg/ml, was modified for 3 hr at room temperature with 2 mM tetramethylrhodamine isothiocyanate (from a 100 mM stock solution in dimethyl sulfoxide, isomer R, Becton Dickinson AG, Basel, Switzerland) in buffer A (6) containing 0.6 M KCl/2 mM $MgCl_2$/50 mM sodium borate, pH 8.5. The reaction was quenched with 50 mM NH_4Cl, followed by low speed centrifugation to remove insoluble fluorochrome. The modified F-actin was depolymerized by dialysis as described (6). It was further stripped from any noncovalently bound fluorochrome on a 1 × 50 cm Sephadex G-100 column, equilibrated with buffer A containing 2.5 mM potassium cholate. The eluted actin monomer was concentrated with Aquacide III (Calbiochem, Lucerne, Switzerland) and dialyzed against the injection buffer.

Other proteins were modified with tetramethylrhodamine isothiocyanate under similar conditions. Modification time was chosen to give a fluorochrome-to-protein molar ratio of usually 0.5–1.5. Protein concentration was measured by the Lowry procedure (7) and rhodamine concentration was determined spectrophotometrically by using an extinction coefficient ϵ_{554} of 88 cm^{-1} mM^{-1} (calculated from ref. 8).

Optical Methods. Observation of cells was performed with a Leitz Diavert light microscope equipped with phase contrast, interference contrast, reflection contrast, and epifluorescence optics. A Leitz Ploemopak contained the necessary filter

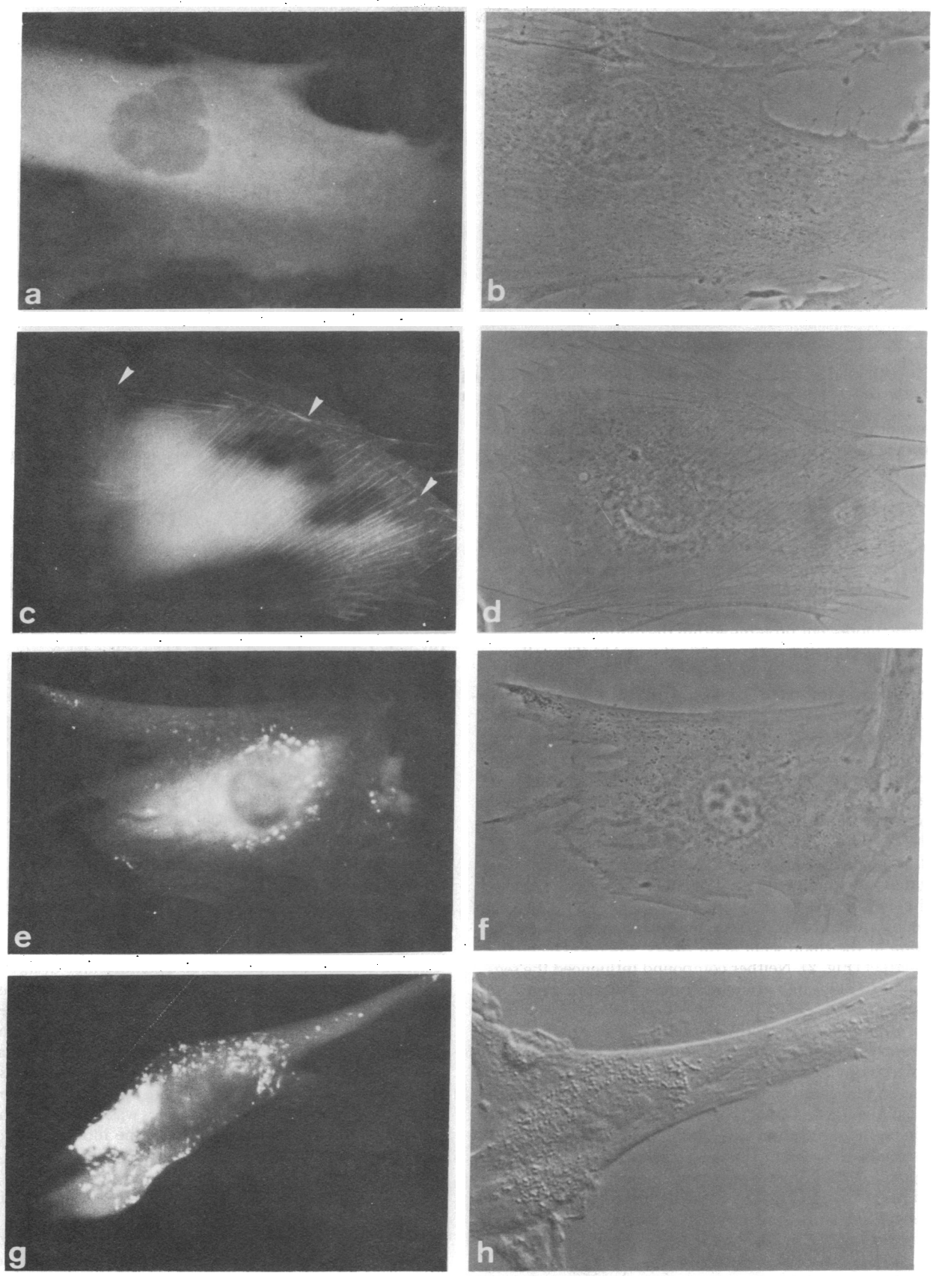

FIG. 1. WI-38 human fibroblasts microinjected with fluorescent proteins and then monitored after different times of culture by fluorescence microscopy and phase contrast or interference contrast microscopy (*h*), respectively. (*a* and *b*) Actin immediately after injection; (*c* and *d*) actin after 1 hr; (*e* and *f*) actin after 6 hr; and (*g* and *h*) bovine serum albumin after 6 hr. The arrows in *c* indicate focal points. (*a* and *b*, × 400; others, ×340.)

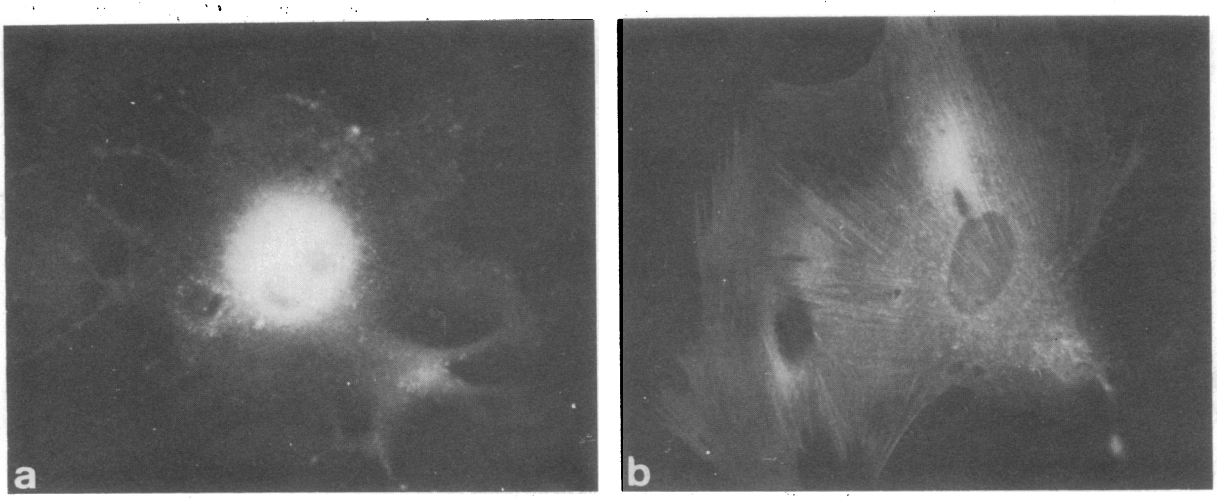

FIG. 2. Effect of cytochalasin b (*a*) and colchicine (*b*) upon the distribution of injected fluorescently labeled actin. The drugs (10 μM) were added to the culture medium immediately after injection of the actin, and the photographs were taken after 2 hr of culture. (×340.)

combinations for both reflection contrast and epifluorescence (N2 for rhodamine) microscopy. Photographs were taken with a Leitz Orthomat Camera on Ilford FP4 and HP5 films.

RESULTS

Muscle actin and several control proteins (e.g., bovine serum albumin, hemoglobin, cytochrome *c*) were modified with tetramethylrhodamine isothiocyanate and injected into WI-38 human fibroblasts. The cells showed a homogeneous distribution of the fluorescent proteins within the cytoplasm immediately after injection (for actin, see Fig. 1 *a* and *b*). The cell nucleus generally showed less intense fluorescence. Furthermore, fluorescence was restricted to injected cells. However, if such cells were viewed after at least 30 min of culture, fluorescent actin, but none of the controls, was incorporated into an array of fibers (Fig. 1*c*). Some of these fibers could also be detected by phase contrast microscopy (Fig. 1*d*) and often extended through the whole cytoplasm. Neighboring fibers converged into distinct focal points (see arrows in Fig. 1*c*) concentrated near the base of the cell (see below). After 6 hr of culture, actin as well as bovine serum albumin and the other controls was segregated into granules located mainly near the cell nucleus (Fig. 1 *e-h*)*.

Cytochalasin b at appropriate concentration prevented the formation of the fluorescent actin fibers, whereas colchicine had no effect (Fig. 2). Neither compound influenced the segregation of proteins into granules or their dislocation into the perinuclear area.

In an attempt to characterize the actin-containing filaments in their three-dimensional organization, Leitz' reflection contrast microscopy was applied. By this technique most of the fluorescent fibers and focal points (Fig. 3 *a* and *e*) could be identified as darkened areas (Fig. 3 *b* and *f*), indicating a close location on the substratum (see *Discussion*). Along the seemingly straight fluorescent fibers (see arrows in Fig. 3*a*), the corresponding dark lines were often characteristically interrupted (see arrows in Fig. 3*b*). In ruffle-like structures shorter fluorescent filaments were enriched, and a close overlap with the dark regions was again observed (Fig. 3 *c* and *d*). In areas of cell–cell contact, actin-containing microspikes were even localized below the neighboring cell edge (see arrows in Fig. 3 *e* and *f*).

DISCUSSION

Our results demonstrate that fluorescently labeled actin, after microinjection into cells maintained in culture, can be visualized by fluorescence microscopy, and its incorporation into cytoskeletal structures can be readily studied. The microinjected cells (*i*) retain a healthy appearance for at least 48 hr of culture, (*ii*) exclude trypan blue when tested immediately after injection as well as during the experiment, and (*iii*) segregate and degrade fluorescent proteins almost completely within less than a day (see also below). Thus, we consider the injected cells to be alive—i.e., to have quickly recovered from microinjection. This belief is supported by the recent observations that injected cells still permit viral gene expression (9) and that injected mRNA undergoes translation (10). The cells were found to carry out mitosis and were still sensitive to enzyme induction (10).

With the injected actin, but not with the control proteins, an impressive network of fluorescent filaments has been visualized (Fig. 1*c* and Fig. 3 *a* and *e*). Thus, during culture the tracer actin† appears to be integrated into structures that resemble the stress fibers known from classical immunofluorescence microscopy (1, 12). They appear to be either new elements or existing fibers in the process of assembly and disassembly. Unspecific association seems unlikely because cytochalasin b, but not colchicine, prevented integration of the fluorescent tracer into any organized structures (Fig. 2 *a* and *b*). The experiments with cytochalasin b, furthermore, indicate that the fluorescent structures are in fact stress fibers and not single F-actin filaments, because the latter are not affected by the drug (1, 13–15). Integration into stress fibers was observed, although the injected tracer was modified by the introduction of tetramethylrhodamine isothiocyanate and was from a different tissue and species. This confirms the previous notion that actin is a structurally and functionally conservative protein (1, 16).

Groups of stress fibers often end at focal points (Fig. 1*c*), organelle-like structures, which we have further analyzed by

* Taylor and Wang (4) have recently injected fluorescently labeled actin into very large cells such as *Physarum* and *Chaos*. For *Physarum* they reported an overall distribution of actin different from that of bovine serum albumin. However, neither a distinct filamentous network with focal points at the membrane nor turnover of structures was reported.

† The injected muscle actin indeed behaves as a tracer for fibroblast actin. If we assume that approximately 5% of the cell volume has been injected (2, 3, 10), the final concentration of the tracer in the cell is 0.1 mg/ml or less (see *Materials and Methods*); actin in fibroblasts, on the other hand, is reported to be 4 mg/ml (11).

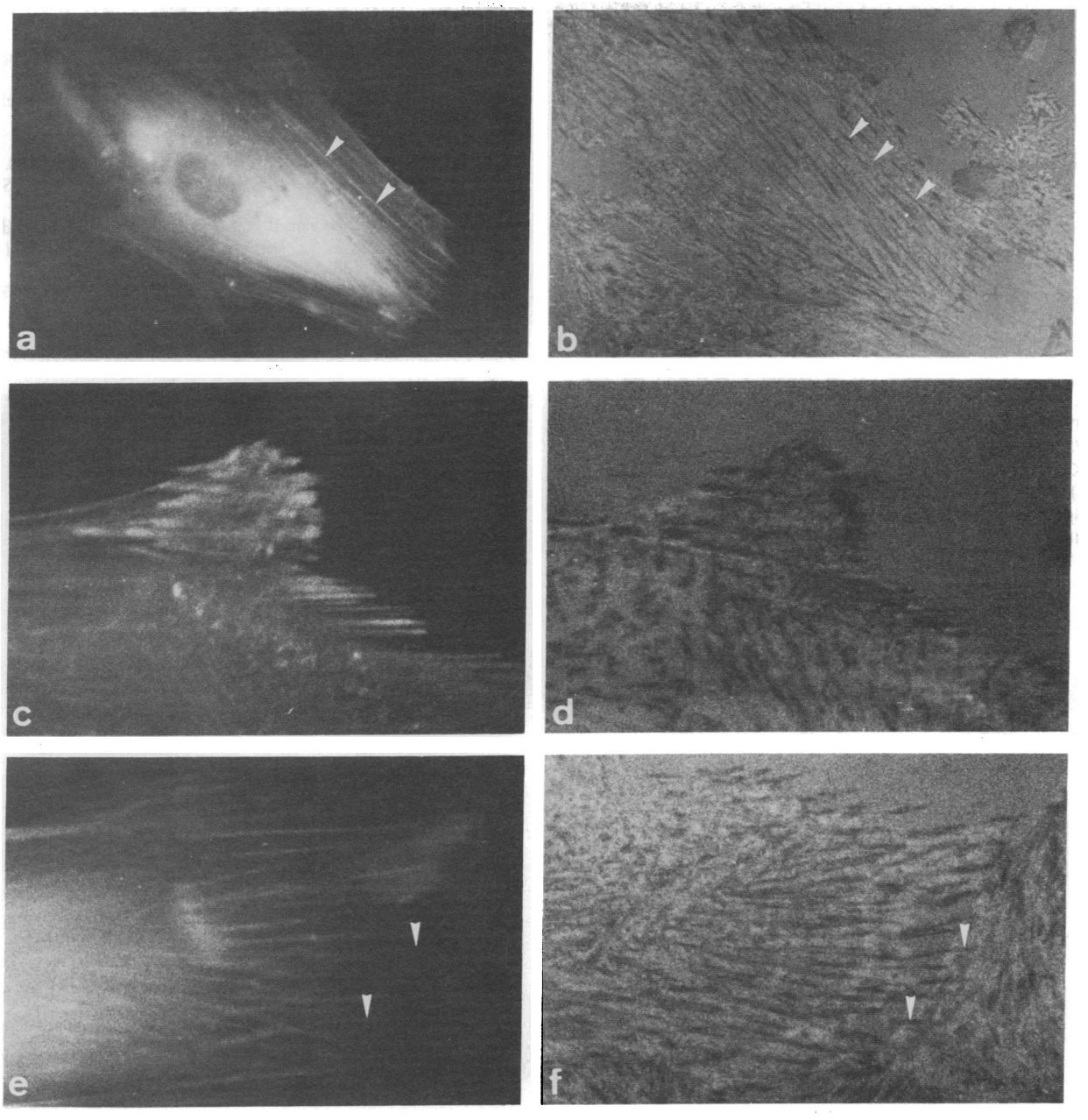

FIG. 3. Fibroblasts injected with fluorescent actin as monitored by both fluorescence and reflection contrast microscopy. (*a* and *b*) Overview on a cell; (*c* and *d*) enlarged ruffle-like structure; (*e* and *f*) enlarged area of a cell-to-cell contact. Arrows in *a* and *b* indicate identical filaments, and arrows in *e* and *f* indicate microspikes underlying neighboring cells. (*a* and *b*, ×340; *c* and *d*, ×900; and *e* and *f*, ×780.)

the recently available technique of Leitz' reflection contrast microscopy. This technique indicates the areas of cellular attachment to the cover slip as dark areas. The image increases in brightness to a maximum at which the distance is $\lambda/4n$ ($\approx$0.1 μm), λ being the wavelength of the incident light and n the refractive index of the medium between cell surface and cover slip (cf. ref. 17). In many areas the actin-containing focal points perfectly overlap with the darkest areas (Fig. 3). Thus, the stress fibers converge to form cusps at the attachment plaques of the cell (cf. refs. 17–19). It could be hypothesized that these focal points might represent the initial organizers of the stress fibers. However, more information—e.g., by continuous visualization of single cells (see below)—will be needed to clarify this point. In ruffle-like structures, actin-containing fibers were found to be enriched (Fig. 3*c*), and thereby the present *in vivo* observations have yielded higher resolution than possible with conventional immunofluorescence microscopy (20).

The injected proteins finally segregated into cytoplasmic granules that moved mainly into a perinuclear position. To what degree the turnover of actin depends on the modification with fluorescent substances remains to be determined. Fluorescence later disappeared, possibly as a consequence of protein degradation. Segregation was not affected by cytochalasin b or by colchicine. Thus, none of the known cytoplasmic filaments (cf. ref. 1) appear to be involved in this process. Stacey and Allfrey (3) have recently analyzed and discussed such autophagic segregation in HeLa cells in detail. It is worth mentioning, however, that protein turnover seems to be faster in the WI-38 fibroblasts in comparison to the HeLa cells.

Further improvements in the culturing techniques of single injected cells directly under the microscope are needed. In combination with the recently introduced video-intensifying system (21), requiring less illumination and capable of continuous monitoring of fluorescent tracers, these improvements

will allow the investigation of regulators of cytoskeletal organization—i.e., proteins that are present at very low concentrations (cf. ref. 22).

We thank Dr. C. Müller, Institute for Molecular Biology, Freie Universität Berlin, for demonstrating the microinjection technique to us; Dr. T. Labhardt, Institute for Zoology, University of Zurich, for suggesting the use of both capillary puller and the particular needles; P. Stocker, Wild-Leitz Zurich, for introducing reflection contrast microscopy to us; J. H. Lanz for excellent technical assistance; and Dr. K. J. Wilson for reading the manuscript. This research was supported by Grant 3.196.-0.77 from the Swiss National Science Foundation.

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