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8. Analysis of Cytoskeletal Structures by the Microinjection of Fluorescent Probes

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I. INTRODUCTION

The cytoskeleton is composed of three kinds of filamentous structures: actin filaments, microtubules, and intermediate filaments. The former two are known to be involved in a number of crucial functions such as cell locomotion, organelle transport, and mitosis. During the past two decades, a large amount of information has become available on the biochemical and morphological properties of these filamentous structures. It is known that all of them are formed by non-covalent associations of protein subunits and accessory proteins. In addition, actin filaments and microtubules have well-defined polarities, with the two opposite ends showing different rates of assembly. The association of filaments with other structures, such as kinetochores for microtubules and plasma membrane for actin filaments, often involves a uniform filament polarity, indicating that polarity is important for structural organization. Each of the filament systems also has its characteristic distribution in the cell. For example, actin filaments in cultured non-muscle cells often form large bundles (stress fibers), which are associated with the plasma membrane at the ends, at sites where cells make focal contacts with the substrate on the exterior surface (adhesion plaques). On the other hand, microtubules and intermediate filaments appear to emanate from discrete ''organizing centers'' near the nucleus.

The most interesting aspect of cytoskeletal structures in living cells is their high degree of dynamics. Many cytoskeletal structures in non-muscle cells are known to be transient: they assemble in response to stimuli at specific sites, and disassemble when specific functions are completed. Many factors may contribute to the dynamic behavior. For example, both actin and microtubule subunits are known to undergo constant assembly-disassembly reactions at steady state in vitro. The existence of a defined polarity of the filament, coupled to the involvement of nucleotide hydrolysis in structural assembly, confers peculiar patterns to such reactions, such as a preferential assembly or disassembly at the opposite ends [treadmilling; Wegner, 1976], and the manifestation of discrete phases of assembly and disassembly [dynamic instability; Mitchison and Kirschner, 1984]. In addition, the assembly can be modulated by post-translational modifications such as phosphorylation [Suzuki et al., 1978]. The potential of reorganization is further increased by the wide variety of accessory proteins which are capable of severing the filaments into fragments, capping the ends of filaments, inhibiting the polymerization of monomers, nucleating the polymerization reaction, cross-linking filaments into networks or bundles, or inducing movements of filaments [see, e.g., Pollard and Cooper, 1986, for a review of actin-binding proteins]. On the one hand, it is clear that such interactions are critical to account for the versatility of the structures. On the other hand, they also represent a tremendous challenge to cell biologists. Structures are often impossible to isolate in a functional form

to allow biochemical analyses, like those performed with ribosomes or mitochondria. Ultrastructural and immunocytochemical studies are also limited by the tendency of structures to reorganize during fixation, and by the difficulty in determining dynamic reorganizations based on stationary images.

The application of fluorescent probes that bind or incorporate into specific structures represents a unique approach for the study of dynamic structures. Fluorescence techniques have the important advantages of being highly specific and sensitive, yielding excellent signal/noise ratio, and causing minimal disruptions to living cells. These advantages are complemented by the improvement of microinjection techniques, which allows delivery of fluorescent probes into many types of cultured living cells, and by the development of low-light-level detectors and image processing techniques, which allows the detection of extremely low signals from single cells on a real time basis. Furthermore, fluorescence signals can also be analyzed with spectroscopic and photobleaching techniques, and provide information far beyond the resolution of a light microscope.

The purpose of this review is to examine how the microinjection of fluorescent probes has been used to study the dynamics of cytoskeletal proteins in living cells, and what new information has been provided as a result. Closely related experiments, such as microinjection of biotinylated proteins, will also be examined. However, technical aspects will be discussed solely fo the purpose of evaluating existing data and future potentials. The readers are referred to several earlier reviews on various aspects of this technique and on related methods such as the microinjection of antibodies [Taylor and Wang, 1980; Wang et al., 1982a; Kreis and Birchmeier, 1982; Taylor et al., 1984; Jockusch et al., 1985; Simon and Taylor, 1986; Kreis, 1986; Wadsworth and Salmon, 1986a; Wang, 1989].

II. EXPERIMENTAL APPROACHES

In a typical experiment, fluorescent probes are microinjected into living cells. Following the association of the probes with cellular structures, fluorescence images are recorded and analyzed. In the following sections, we will discuss both different fluorescent probes that have been used for microinjection, and different methods for the collection and analysis of data.

A. Fluorescent Probes

1. Fluorescent analogs of cellular components. To date, most probes used for cytoskeletal structures are fluorescently labeled structural components, such as actin, myosin, tubulin, and their accessory proteins (a list of microinjected analogs is shown in Table I). This approach has been referred to as fluorescent analog cytochemistry [Taylor and Wang, 1978; Wang et al., 1982a; Taylor et

TABLE I. Microinjected Fluorescent Analogs of the Cytoskeleton

Protein	Derivatives	Reference
Actin		
	Carboxytetramethylrhodamine succinimidyl ester	Kellogg et al., 1988.
	Fluorescein isothiocyanate	Dome et al., 1988; Hamaguchi and Mabuchi, 1988; Kukulies et al., 1984; McKenna et al., 1985a.
	5-Iodoacetamidofluorescein	Amato et al., 1986, 1983; DeBiasio et al., 1987, 1988; Gawlitta et al., 1980, 1981; Hamaguchi and Mabuchi, 1988; Kukulies et al., 1984; Simon et al., 1988; Stockem et al., 1983; Taylor and Wang, 1978; Taylor et al., 1980; Wang and Taylor, 1979, 1980; Wang et al., 1982b; Wehland and Weber, 1980; Wehland et al., 1980.
	Lissamine rhodamine B sulphonyl chloride	Dome et al., 1988; Kreis, 1986; Kreis et al., 1982; McKenna and Wang, 1986; McKenna et al., 1985a.
	N-(7-dimethylamino-4-methylcoumarinyl) maleimide	Hamaguchi and Mabuchi, 1988.
	Tetramethylrhodamine iodoacetamide	DeBiasio et al., 1988; Dome et al., 1988; Glacy, 1983a, 1983b; Simon et al., 1988; Wang, 1984, 1985.
	Tetramethylrhodamine isothiocyanate	Kreis et al., 1979; Kreis, 1986; Kukulies and Stockem, 1985; Kukulies et al., 1984, 1985; Sanger et al., 1980.
Myosin		
	5-Iodoacetamidofluorescein	DeBiasio et al., 1988; Johnson et al., 1988; McKenna et al., 1989a.
	Lissamine rhodamine B sulfonyl chloride	Glascott et al., 1987.
	Tetramethylrhodamine iodoacetamide	DeBiasio et al., 1988; McKenna et al., 1989a, 1989b.
Myosin light chains		
	Fluorescein isothiocyanate	McKenna et al., 1989a.
	Tetramethylrhodamine iodoacetamide	Mittal et al., 1987a.
Alpha-actinin		
	5-(4,6-Dichlorotriazinyl)aminofluorescein	Kreis, 1986.
	Flourescein isothiocyanate	Mittal et al., 1987a; Sanger et al., 1986a,b, 1987.
	5-Iodoacetamidofluorescein	Simon and Taylor, 1986; Simon et al., 1988; Stickel and Wang, 1988.
	Lissaminerhodamine B sulfonyl chloride	Glascott et al., 1987; Kreis, 1986; Mittal et al., 1987a; Sanger et al., 1984a; Sanger et al., 1986a,b, 1987.
	Lucifer yellow VS	Sanger et al., 1986a.

(continued)

TABLE I. Microinjected Fluorescent Analogs of the Cytoskeleton *(continued)*

Protein	Derivatives	Reference
	Tetramethylrhodamine iodoacetamide	Johnson et al., 1988; McKenna and Wang, 1986; MeKenna et al., 1985b, 1986; Meigs and Wang, 1986; Stickel and Wang, 1987, 1988; Wang, 1986.
	Tetramethylrhodamine isothiocyanate	Feramisco, 1979; Feramisco and Blose, 1980; Hamaguchi and Mabuchi, 1986; Kreis and Birchmeier, 1980; Kreis et al., 1979; Mabuchi et al., 1985.
Filamin		
	5-Iodoacteamidofluorescein	Mittal et al., 1987b.
	Tetramethylrhodamine iodoacetamide	Mittal et al., 1987b; Sanger et al., 1986b.
Gelsolin		
	Lissamine rhodamine B sulfonyl chloride	Cooper et al., 1988.
Metavinculin		
	Tetramethylrhodamine isothiocyanate	Saga et al., 1985.
Tropomyosin		
	Fluorescein isothiocyanate	Dome et al., 1988; Warren et al., 1985.
	5-Iodoacetamidofluorescein	Wehland and Weber, 1980.
	Lissamine rhodamine B sulfonyl chloride	Dome et al., 1988.
Vinculin		
	5-(4,6,-Dichlorotriazinyl)aminofluorescein	Kreis, 1986.
	Fluorescein isothiocyanate	Burridge and Feramisco, 1980; Meigs and Wang, 1986; Saga et al., 1985.
	Lissamine rhodamine B sulfonyl chloride	Glascott et al., 1987; Kreis, 1986.
	Tetramethylrhodamine iodoacetamide	Meigs and Wang, 1986.
	Tetramethylrhodamine isothiocyanate	Stickel and Wang, 1988; Wang, 1986.
Tubulin		
	Bis-caged-carboxyfluorescein	Mitchison, 1989.
	Carboxyfluorescein succinimidyl ester	Vigers et al., 1988.
	Carboxytetramethylrhodamine succinimidyl ester	Kellogg et al., 1988; Schulze and Kirschner, 1988; Vigers et al., 1988.
	Carboxy-X-rhodamine succinimidyl ester	Gorbsky et al., 1988; Gorbsky and Borisy 1989b; Lim et al., 1989; Sammak and Borisy, 1988; Vigers et al., 1988.
	5-(4,6-Dichlorotriazinyl)aminofluorescein	Cassimeris et al., 1988a; Gorbsky et al., 1987; Hamaguchi et al., 1987; Keith, 1987, 1988; Keith et al., 1981; Kellogg et al., 1988;

(continued)

TABLE I. Microinjected Fluorescent Analogs of the Cytoskeleton ***(continued)***

Protein	Derivatives	Reference
		Leslie et al., 1984; Salmon and Wadsworth, 1986; Salmon et al., 1984a,b; Sammak et al., 1987; Saxton and McIntosh, 1987; Saxton et al., 1984; Soltys and Borisy, 1985; Vigers et al., 1988; Wadsworth and Sloboda, 1983; Wadsworth and Salmon, 1986a,b, 1988; Webster et al, 1987.
	Flourescein isothiocyanate	Hamaguchi et al., 1985, 1987.
	Lissamine rhodamine B sulfonyl chloride	Kellogg et al., 1988.
MAPs		
	Iodoacetamidofluorescein	Olmsted et al., 1989; Sherson et al., 1984; Vandenbunder and Borisy, 1986.
	Tetramethylrhodamine iodoacetamide	Vandenbunder and Borisy, 1986.
Desmin		
	Tetramethylrhodamine iodoacetamide	Mittal et al., 1989.

al., 1984; Wang, 1989]. The probes are usually prepared by reacting a limited number of the amino or sulfhydryl groups on the protein molecules with fluorescent reagents, which consist of a fluorophore attached to a reactive group such as isothiocyanate, sulfonyl chloride, or iodoacetamide [Haugland, 1989]. Following microinjection, the conjugates behave as analogs of the endogenous components and often become incorporated into physiological structures. The major advantage in using fluorescent analogues is the stable, direct association of the fluorophore to the protein under study, the potential of direct participation of the probes in structural formation, and the likely low toxicity due to the close similarity of the probe to the endogenous components. However, it is critical to ascertain that the fluorescent analogs have maintained the physiological properties and that they behave in a manner similar to the endogenous components after microinjection.

While well-characterized biochemical properties, such as self-assembly and ATPase activities, can be readily assayed in vitro, there are often interesting components, such as vinculin, whose biochemical functions are not entirely certain. In addition, some proteins, such as actin and tubulin, are likely to interact with a large number of accessory proteins, and it is impossible to be totally sure that all functions have been preserved. Fluorescent labeling may also result in quantitative changes, such as the level of ATPase activities of myosin [Reisler, 1982; Johnson et al., 1988]. It is often difficult to judge how such changes might affect the outcome of the experiments, especially if the physiological roles of the interactions affected are unclear or if the analog

co-assembles with a large excess of endogenous proteins. An example is the decrease in the affinity of actin for profilin following modification of actin at cys-374 [Malm, 1984]. Even if the functions in vitro appear to be intact, it is still possible that the analog may not behave in a fashion identical to its endogenous counterpart following the microinjection. Possible factors affecting the incorporation include accessibility to assembly sites, rates of on-off equilibrium, presence of unoccupied binding sites, induction of adventitious binding by the chemical modification, and involvement of complex interactions in the incorporation process. In addition, fluorescent labeling may also have a profound effect on the rate of protein degradation [Kellogg et al., 1988].

Therefore, equally important is the analysis of the interactions between the analogs and cellular structures. Isolated myofibrils or cell models have been used for characterizing many analogs of the actin filament system [Sanger et al., 1984b,c]. However, associations of structural components with extracted structures may be very different from those with native structures in living cells [Dome et al., 1988; Johnson et al., 1988], due to the requirements of energy or protein factors, disruptions during extraction, or differences in the solution environment. Thus it is usually more informative to observe microinjected cells. Several types of observations have been performed to assess the incorporation and toxic effects of the analogs. First, the distribution of the analog has been compared to that of the endogenous protein, and to that of control proteins such as fluorescent BSA or ovalbumin, within the same cell [Taylor and Wang, 1978; Wang and Taylor, 1979]. The simplest approach to observe endogenous proteins is immunofluorescence, using a fluorophore different from that microinjected. However, immunofluorescence itself may sometimes be affected by problems such as fixation artifacts [Cooper et al., 1988], epitope shielding, or antibody accessibility [midbodies in mitotic cells fail to stain with tubulin antibodies; Saxton et al., 1984]. In addition, the results of immunofluorescence may be misleading if a large amount of the analog, relative to the endogenous counterpart, is microinjected. An equally important experiment is the comparison of structural organization in injected cells with that in uninjected cells. This will also allow the detection of disruptions induced by the analog. Artifacts observed so far include the formation of paracrystals upon the overinjection of actin into some cell lines [Wehland and Weber, 1980], and the formation of abnormally large asters following the overinjection of tubulin [Hamaguchi et al., 1985].

Several other types of assays are also useful. For example, drugs can be used to disrupt the distribution of cytoskeletal structures in microinjected cells, and the response of the analogs compared with that of endogenous components [Wehland and Weber, 1980]. It is also informative to vary the amount of microinjection [Schulze and Kirschner, 1986]; lack of effects would suggest that the process under study is not driven by the microinjected analogs. A

particularly novel way to assay functions is to microinject the analog into mutants lacking the corresponding functional protein [Saxton and McIntosh, 1987]. Rescue of cellular function or morphology would suggest that the analogs are functional.

2. Fluorescent affinity probes. Besides fluorescent analogs, various types of fluorescent affinity probes have been used to label components in living cells. The assay for the binding properties of fluorescent affinity probes is usually easier than that for the functional properties of fluorescent analogs. In addition, since the fluorophore in affinity probes is usually farther separated from the labeled structural components, radiation damage may be reduced [Olmsted et al., 1989]. However, these foreign agents may themselves induce cellular disruptions, especially when used at a high concentration. In addition, experiments examining the dynamic exchange of components may be complicated by the on-off reactions of the probe with the target protein [Wang, 1987].

The best example of affinity probes is fluorescent phalloidin [reviewed by Faulstich et al., 1988]. When microinjected at a low concentration, it labels actin filaments without inducing detectable disruptions [Wehland and Weber, 1981; Hamaguchi and Mabuchi, 1982; Kukulies et al., 1985; Wang, 1987]. However, it causes dramatic changes in cellular morphology and behavior at high concentrations [Wehland et al., 1977]. Fluorescently labeled MAP2, due to its absence (or extremely low concentration) in non-neuronal cells [Vallee et al., 1984], has also been miccroinjected as a probe for microtubules in fibroblasts [Scherson et al., 1984]. Other probes such as fluorescently labeled colcemid [Hiratsuka and Kato, 1987], colchicine [Moll et al., 1982], heavy meromyosin [Sanger, 1975], DNase I [Haugland, 1989], and taxol are also worth consideration for specific purposes.

A related approach is the microinjection of fluorescently labeled antibodies against specific proteins. Two such studies involve the injection of monoclonal antibodies against tubulin [Wehland et al., 1983; Warn et al., 1987]. At low concentrations, the fluorescent antibodies cause no detectable disruptions to 3T3 cells and *Drosophila* embryos, and allow clear observations of microtubules through mitotic cycles. However, disruptions do occur at high concentrations. A second concern in using antibodies is of course the possible limitation in the accessibility of the binding sites. These problems may be minimized by using monoclonal antibodies against specific, nonfunctional domains and by using Fab fragments.

Finally, a new possibility emerges with the design of ''anti-peptides'' [Chaussepied and Morales, 1988]. By analyzing the amino acid sequence of target proteins, it may be possible to design peptides that recognize specific sites based on their charge or hydrophobic properties, and yield small, specific probes while minimizing perturbations to living cells.

B. Detection of Fluorescence Signals

Fluorescence signals from microinjected cells are usually very weak, due to the limited number of probes that can be microinjected without disrupting the cell. This problem cannot be solved by simply increasing the intensity of the excitation light, since the emission is proportional to the excitation intensity only to a certain extent, after which the number of fluorophores at the ground state may become a limiting factor. Intense illumination also induces photobleaching of fluorophores and damage to the structures.

Photodamage is an important concern for fluorescence microscopy of living cells. Detailed studies on the photodamage of actin and tubulin in vitro have been reported recently [Vigers et al., 1988; Simon et al., 1988]. Although photodamage of cells is usually manifested as readily detectable changes in morphology [Vigers et al., 1988], there may also be subtle changes, such as cross-linking of proteins [Leslie et al., 1984], that are difficult to detect by direct observations. However, the extent of damage can be greatly reduced by the presence of reducing agents, and by mixing fluorescent probes with excess unlabeled molecules [Leslie et al., 1984; Vigers et al., 1988]; both conditions are fulfilled in living cells. It also appears that the extent of photodamage is highly dependent on the condition of illumination [Leslie et al., 1984], on the fluorophore used, and on the protein under study. Experimentally, the possibility of photodamage may be ruled out by varying the frequency and duration of illumination (which should not affect the results), by performing immunofluorescence or high-resolution optical and electron microscopy [McKenna et al., 1985a; Saxton et al., 1984], and by testing the response of the illuminated structures to agents that induce structural reorganization.

Photobleaching and photodamage represent the major limitations in earlier studies, where direct photography is used for image recording. Low-light-level video cameras are now used extensively [Spring and Lowy, 1989], allowing intermittent recording of single living cells for several days without detectable disruptive effects [McKenna et al., 1986; Sanger et al., 1986a]. In addition, video signals can be easily processed by digital image processors to improve the signal-to-noise ratio, to enhance the contrast, and to perform quantitative measurements of the fluorescence intensity in specific areas [Arndt-Jovin et al., 1985]. However, in some studies, the signal may remain too weak despite the use of these techniques. One approach to amplify the fluorescence signal is to stain the cell with antibodies against the fluorophores [Gorbsky and Borisy, 1989a]. Although the cells have to be fixed and dynamic processes cannot be followed directly, this method has proven very useful in identifying a small number of incorporated molecules against a high level of unincorporated molecules [Soltys and Borisy, 1985; Amato and Taylor, 1986].

C. Analysis of Fluorescence Signals

What types of information can be obtained from the microinjection of fluorescent probes? Successful incorporation of an analog into physiological structures would indicate that the binding sites are available and accessible. Often this is indicative of a dynamic exchange of components between the soluble and incorporated states. By studying the pattern of fluorescence distribution during incorporation, it is also possible to identify active sites of structural assembly [Soltys and Borisy, 1985]. Furthermore, comparisons can be made between different protein isoforms and between proteolytic domains regarding the ability and pattern of incorporation [McKenna et al., 1985a; Johnson et al., 1988]. Conversely, it may be possible to study the turnover of protein analogs following their incorporation into structures.

The main advantage in studying living cells, of course, is the ability to follow dynamic processes. Both fluorescent analogs and affinity probes have been used extensively for this purpose. Conspicuous structures, such as stress fibers and myofibrils, can be observed directly, and pathways for the assembly or drug-induced disruptions can be determined [e.g., Wang, 1984; Meigs and Wang, 1986]. In addition, it should be possible to study the transport of soluble protein components, by carefully microinjecting fluorescent probes within a localized area and examining the subsequent pattern and rate of dispersal [Cao and Wang, unpublished results].

Photobleaching with laser microbeams, first applied to membrane components [Jacobson et al., 1987], has become a powerful tool for analyzing dynamics at the molecular level. Typically, a laser microbeam at a wavelength absorbed by the fluorophore is used to bleach fluorescence from a small area, 1–5 μm in diameter, without disrupting the integrity of the structures. The recovery of fluorescence then reflects the movement of unbleached molecules into the bleached area. In addition, recovery along discrete structures suggests that structural components undergo an equilibrium between incorporated and soluble states. The bleached spots have been observed either directly or after staining cells with antibodies that recognize unbleached, but not bleached, fluorophores [Sammak et al., 1987]. Light-induced artifacts can be largely ruled out by varying the extent of photobleaching and by comparing the recovery of multiple photobleachings at the same site [Wolf et al., 1980; Salmon et al., 1984b]; neither should affect the outcome of the experiments. In addition, control experiments as discussed above can be performed to rule out light-induced structural damage.

While the half-time and the extent of recovery are useful for qualitative comparisons, quantitative analyses of the recovery kinetics are limited by our knowledge of the way cytoskeletal components move in the cell. The movement may not follow the rule of diffusion. However, equally informative is the

location of the bleached spot and the pattern of recovery, which can yield clues on the occurrence of directional transport and the polarity of structural assembly [e.g., Wang, 1985]. In addition, different patterns, such as multiple lines or spots [Saxton and McIntosh, 1987; McKenna and Wang, 1986] have been used to analyze not only the movement within one specific area but also the relative movement among different regions.

The complementary technique to photobleaching, photoactivation [Ware et al., 1986; Kraft et al., 1988], has recently been applied to cytoskeletal structures [Mitchison, 1989]. Analogs are prepared with "caged" fluorophores, which remain nonfluorescent until activation by a pulse of light. The fate of the bright spot is then followed after photoactivation. The major advantage of this approach is the superb signal to noise ratio: observations are made with a bright spot against a dark background, instead of a faintly fluorescent spot against a bright background as for photobleaching. This greatly facilitates the identification of small fractions of slowly moving molecules, which are extremely difficult to detect with photobleaching techniques [Mitchison, 1989]. In addition, the generation of active fluorophores is likely to be less perturbing to the structures than does photobleaching [Ware et al., 1986; Kraft et al., 1988]. The main drawback of photoactivation is the difficulty in knowing the location or distribution of specific structures. For example, it will be difficult to position the site of activation on a specific fiber, or to measure the movement relative to a particular site. However, this problem may be overcome by microinjecting a mixture of photoactivatable probes and fluorescent probes that absorb or emit at different wavelengths.

Many powerful spectroscopic techniques still await explorations in living cells. For example, the excitation/emission spectra or quantum yield of fluorophores are often affected by the local environment and protein conformation. Good examples are pyrene- and NBD-labeled actins, which show a large increase in fluorescence intensity upon polymerization [Kouyama and Mihashi, 1981; Detmers et al., 1981]. Thus ratio imaging techniques, similar to those used currently for mapping the distributions of intracellular ions [Bright et al., 1989; Tsien, 1989], can be used to map the conformation of proteins in the cell. If fluorophores are rigidly attached to the probe, polarization measurements may also be useful [Axelrod, 1989], at least qualitatively, for studying the assembly of structures from subunits. For example, regions with an increased polarization may be identified before discrete structures become detectable. Resonance energy transfer has also been used to study protein interactions in vitro [Stryer, 1978], as well as the dynamics of lipid molecules and proteins on the membrane of living cells [Uster and Pagano, 1988; Herman, 1989]. If donors and acceptors are located at different sites of the same protein molecule, energy transfer between them (intramolecular) can be used as a highly sensitive tool for detecting conformational changes within living cells.

The application of intermolecular energy transfer will be more difficult, due to the serious dilution of probes upon microinjection. However, the approach may be useful for following the dissociation of macromolecular complexes. If preformed complexes, containing interacting donors and acceptors, are microinjected, subsequent dissociation would be manifested as a loss of resonance energy transfer.

III. MICROINJECTION OF FLUORESCENT PROBES FOR ACTIN FILAMENTS

Fluorescent analogs of various components of the actin filament system have been microinjected into living cells. Valuable insights have been gained by examining interactions between the fluorescent analogs and cellular structures, especially concerning the possible roles of protein isoforms. In addition, important information about the dynamics of lamellipodia, stress fibers, and developing myofibrils has been obtained by studying the reorganization of fluorescently labeled structures, using both direct observations and photobleaching techniques.

A. Association of Fluorescently Labeled Actin and Accessory Proteins With Cellular Structures

1. Incorporation of microinjected actin analogs. The earliest microinjection of a fluorescent analog of the cytoskeletal components was performed with actin labeled with 5-iodoacetamidofluorescein at cys-374 [Taylor and Wang, 1978]. Successful incorporation subsequently was obtained with actin labeled by a variety of fluorescent reagents (Table I). Assays performed on some of the conjugates indicated normal polymerizability and activation of the myosin Mg-ATPase [Wang and Taylor, 1980].

Following microinjection, fluorescent analogs of actin incorporate, within 30 min, into structures in amoeba [Taylor and Wang, 1978], acellular slime molds [Taylor and Wang, 1978], sea urchin eggs [Wang and Taylor, 1979], macrophages [Amato et al., 1983], muscle cells [Glacy, 1983a], and fibroblasts [Kreis et al., 1979]. The distribution of the analog is sensitive to cytochalasin B, as expected for cytoplasmic actin filaments [Kreis et al., 1979; Wehland and Weber, 1980]. At steady state, the distribution of labeled structures is very similar to that revealed by fluorescent phalloidin staining or immunofluorescence [Kreis et aal., 1982]. In addition, there is a diffusely distributed signal throughout the cytoplasm, which probably represents a combination of filament networks and soluble molecules. The latter have also been suggested based on the large mobile fraction detected by photobleaching recovery techniques [Kreis et al., 1982; Wang et al., 1982b]. Thus, it is likely that the polymerization of actin analogs is regulated by cytoplasmic factors.

In fibroblasts, microinjected actin analogs are first incorporated into lamellipodia, and then into adhesion plaques and stress fibers. A steady state is reached within 20 min [Glacy, 1983b]. Under the light microscope, the incorporation takes place simultaneously along the entire length of stress fibers [Glacy, 1983b]. However, when immunoelectron microscopy is used to detect the labeled actin, periodic sites of incorporation can be observed along the stress fiber 5 min after microinjection [Amato and Taylor, 1986]. Such sites may correlate with the periodic localization of alpha actinin or other accessory proteins along stress fibers.

The incorporation of fluorescent actin has also been examined in muscle cells. An early study yielded the unexpected results that the analog is incorporated into the I-bands (where endogenous actin filaments are located) and the M-lines (in the middle of the A-bands, where myosin filaments are located), but not into the Z-lines (where the ends of actin filaments are anchored), of myofibrils in cardiac myocytes [Glacy, 1983b]. However, more recent studies indicate that fluorescent actin incorporates into the I-bands and the Z-lines, but not the M-lines [McKenna et al., 1985a; Dome et al., 1988]. These discrepancies may be related to the metabolic state of the cell or to the handling of actin before microinjection [McKenna et al., 1985a].

The microinjection approach has been used to compare the incorporation properties of actin isoforms [McKenna et al., 1985a]. Brain actin and skeletal muscle actin, labeled with different fluorophores, were co-injected into muscle and non-muscle cells. At the resolution of a light microscope, no difference was detected between the isoforms along myofibrils in cardiac myocytes or stress fibers in fibroblasts, suggesting that the binding sites on these structures cannot differentiate between the isoforms. It was suggested that differential localization of isoforms, as observed with immunofluorescence in adult muscle tissues [Lubit and Schwartz, 1980; Pardo et al., 1983], may be achieved through differential synthesis, stabilization, or degradation. However, it is also possible that subtle differences between isoforms may exist between submicroscopic domains [Otey et al., 1988] or in some specific structures such as lamellipodia [DeNofrio et al., 1989].

2. Incorporation of microinjected myosin analogs. Myosins from skeletal or smooth muscles have been fluorescently labeled and microinjected into muscle and non-muscle cells [Johnson et al., 1988; DeBiasio et al., 1988; McKenna et al., 1989a,b]. Labeled smooth muscle myosin was used as an analog for non-muscle myosin since the two share similar biochemical properties [Scholey et al., 1982]. However, McKenna et al. [1989b] reported a partial inhibition of the ATP-induced conformational change [Suzuki et al., 1978], upon fluorescent labeling of the smooth muscle myosin.

Following microinjection into myotubes, skeletal muscle myosin disperses over a period of 1/2 to 3 h and becomes incorporated into A-bands [Johnson et

al., 1988]. This is significant since skeletal muscle myosin is relatively insoluble under physiological ionic conditions. Once incorporation reaches a steady state, the exchange occurs very slowly as indicated by a very slow recovery of fluorescence after photobleaching [Johnson et al., 1988]. Thus, the association of the myosin analog with myofibrils probably represents addition to existing unoccupied binding sites, rather than an on-off reaction of the myosin molecules. Johnson et al. also microinjected different proteolytic fragments of myosin. Incorporation was observed ith the light meromyosin portion but not with heavy meromyosin, indicating that actin-myosin interactions are probably not required.

Both labeled skeletal and smooth muscle myosins have been microinjected into fibroblasts [DeBiasio et al., 1988; McKenna et al., 1989a,b]. Both analogs were incorporated into periodically arranged beads along stress fibers, indistinguishable from those shown by immunofluorescence [McKenna et al., 1989a,b]. In addition, a large number of elongated beads, either poorly organized or organized as fine linear arrays or networks, can be discerned in thin, spread regions of the cell [DeBiasio et al., 1988; McKenna et al., 1989a,b]. Judging from their length, $\sim$0.7 μm, the beads probably represent single myosin filaments.

Each myosin molecule is known to consist of two heavy chains and four light chains. These polypeptides appear to associate stably in vitro and undergo only very slow exchange [Burke and Sivaramakrishnan, 1981]. However, when fluorescently labeled light chains of skeletal muscle myosin were microinjected, the analogs became colocalized with A-bands of myofibrils in myotubes and with stress fibers in epithelial cells within 3–5 h [Mittal et al., 1987a]. In dividing cells, the fluorescent light chains became concentrated in the equatorial region, as expected with myosin molecules. Similar observations were made when labeled regulatory (20 kD) light chain of smooth muscle myosin was microinjected into 3T3 cells [McKenna et al., 1989a]. The incorporation into stress fibers in this case reached a steady state within 1 h [McKenna et al., 1989b]. Thus, although it is difficult to demonstrate that the light chain analogs have associated correctly with heavy chains, these results strongly suggest an ability of myosin molecules, even different isoforms, to exchange their light chains in vivo. In addition, since the phosphorylation of light chains plays an important role in the regulation of smooth and non-muscle myosins, the results raise the interesting possibility that one phosphorylated light chain may be able to activate multiple myosin molecules [McKenna et al., 1989b]. Another interesting question is whether muscle and non-muscle myosin heavy chains are known to locate in different regions [Fallon and Nachmias, 1980].

3. Incorporation of microinjected actin-binding proteins. A number of actin binding proteins have been fluorescently labeled and microinjected into cultured muscle and non-muscle cells. The distribution of alpha actinin analogs in fibroblasts mimics that of endogenous alpha actinin as detected

by immunofluorescence [Feramisco, 1979; Feramisco and Blose, 1980]. Since the distribution of alpha actinin closely parallels that of actin filaments but shows a lower diffuse signal, alpha actinin analogs have been used as an alternative probe for actin filaments. In addition, the clarity of the punctate alpha actinin structures along stress fibers has greatly facilitated the observation of contractile events [Kreis and Birchmeier, 1980; Wang, 1986; Sanger et al., 1986b].

When microinjected into muscle cells [Kreis and Birchmeier, 1980; Sanger et al., 1984a], analogs of alpha actinin were incorporated within 30 min into Z-lines of myofibrils. As for myosin analogs, fluorescent alpha actinin probably binds to myofibrils through the association with unoccupied binding sites rather than the on-off equilibrium. Once associated with Z-lines, the rate of exchange is low as shown by photobleaching recovery techniques [McKenna et al., 1985b].

Non-muscle alpha actinin, unlike muscle alpha actinins, cross-links actin filaments in a calcium-sensitive manner [Burridge and Feramisco, 1981]. However, alpha actinin analogs prepared from smooth muscles are able to associate with non-muscle structures [Feramisco, 1979]. In addition, when a mixture of muscle and non-muscle alpha-actinins was microinjected into myotubes, both types of alpha actinins incorporated into Z-lines with an apparently equal efficiency [Sanger et al., 1986a]. Therefore, these experiments fail to identify a physiological role of the calcium sensitivity of the non-muscle alpha actinin in resting cells. However, it is still possible that the different isoforms may behave differently in some Ca-activated events.

Filamin, another actin filament cross-linking protein, has been fluorescently labeled without affecting its cross-linking activity [Mittal et al., 1987b]. Following microinjection into epithelial cells (PtK2) and fibroblasts (3T3), the filamin analog became associated with stress fibers in a punctate pattern. In muscle cells, fluorescent filamin is associated with the Z-line and its precursors, consistent with immunofluorescence observations [Gomer and Lazarides, 1981].

Gizzard tropomyosin has been derivatized with iodoacetamidofluorescein and microinjected into epithelial cells and fibroblasts [Wehland and Weber, 1980]. The initial study indicates that the fluorescent analog is associated with stress fibers but not with lamellipodia. However, Warren et al. [1985], using a combination of immunofluorescence and fluorescent analog cytochemistry, demonstrated that tropomyosin is present in the lamellipodia during early stages of spreading, and becomes depleted from the area as the cell spreads out and assembles large stress fibers. Thus it appears that the absence of tropomyosin in the lamellipodia of well spread cells may not represent an inherent property of the lamellipodia. One possible explanation is that tropomyosin associates tightly with actin filaments and undergoes only slow exchanges among the filaments. If actin turns over rapidly in the lamellipodia, there may not be enough time for tropomyosin molecules to redistribute from existing filaments in the stress fibers and bind to newly assembled actin subunits in the lamellipodia.

Isoforms of tropomyosin differ in length and in their affinity for actin filaments [Fine et al., 1973]. Dome et al. [1988] compared the incorporation of fluorescently labeled brain (~30 kD) and gizzard (~42 kD) tropomyosin isoforms into contractile structures in muscle and non-muscle cells. In PtK epithelial cells, both brain and gizzard isoforms incorporate into stress fibers. However, when injected into skeletal myotubes, only the gizzard tropomyosin became incorporated into myofibrils. The simplest explanation is that brain tropomyosin, with its lower affinity for actin filaments, is unable to compete with the endogenous skeletal muscle tropomyosin [Dome et al., 1988]. This also implies that non-muscle tropomyosin may undergo a more rapid exchange among different structures than does muscle tropomyosin.

Microinjection of fluorescently labeled vinculin was performed soon after the identification of the protein [Burridge and Feramisco, 1980]. Although it was uncertain how much its biochemical properties were affected by the fluorescent labeling, the analog did maintain the ability to associate with adhesion plaques. Microinjection has been used to compare the localization of vinculin and meta-vinculin, which is similar to vinculin but is present only in muscle tissues [Feramisco et al., 1982; Siliciano and Craig, 1982; Saga et al., 1985]. A mixture of vinculin and meta-vinculin analogs, labeled with different fluorophores, was microinjected into myotubes and fibroblasts [Saga et al., 1985]. Both analogs became incorporated into periodic, linear structures in myotubes, and into adhesion plaques in fibroblasts, indicating that cellular binding sites cannot discriminate vinculin from meta-vinculin. However, it is still unclear whether or not endogenous vinculin and meta-vinculin assume identical distributions in muscle cells. Furthermore, since vinculin can bind to adhesion plaques through at least two domains [Bendori et al., 1989], it is possible that vinculin and meta-vinculin may be incorporated through different interactions.

Gelsolin is a calcium-activated protein that caps and severs existing actin filaments and nucleates the assembly of new filaments [Yin and Stossel, 1980]. In resting cells, gelsolin is probably inactive, since microinjection of intact gelsolin induces no detectable effect but injection of a calcium-independent fragment causes dramatic disruptions to actin filaments [Cooper et al., 1987]. In order to determine the localization of gelsolin in resting cells, Cooper et al. microinjected a fluorescent analog into fibroblasts [Cooper et al., 1988]. The analog assumed a diffuse distribution without a detectable association with stress fibers, contrary to what was seen by immunofluorescence. However, association of the analog with stress fibers became visible after fixation of microinjected cells. Although alternative explanations are possible, Cooper et al. suggested that gelsolin probably exists predominantly in the soluble form, and makes only transient associations with stress fibers. Chemical fixation may render the association irreversible and create the bright staining of stress

fibers. This explanation appears consistent with the photobleaching recovery data, which show gelsolin to be close to 100% mobile, and with the ease of extraction of endogenous gelsolin molecules as shown in the same study.

B. Dynamics of Actin and Myosin as Studied by Microinjected Fluorescent Probes

1. Dynamics of actin in lower eukaryotic cells. Microinjection of fluorescent probes has been applied to study the dynamics of actin-containing structures in *Amoeba proteus* [Taylor et al., 1980; Gawlitta et al., 1980, 1981], and *Physarum polycephalum* [Kukulies and Stockem, 1985; Kukulies et al., 1984, 1985; Brix et al., 1987]. Formation of discrete actin-containing structures was observed during phagocytosis [Stockem et al., 1983], lectin-induced capping [Taylor et al., 1980], and polyamine-induced cleavage [Gawlitta et al., 1981]. The active motility of such organisms makes them attractive targets for examining the organization of actin and myosin. However, detailed analysis has been limited by the irregular shape and the large thickness of the cell. The application of new cell flattening techniques [Fukui et al., 1986], confocal microscopy [Brakenhoff et al., 1989], and ratio imaging [for correcting variations due to the pathlength; Bright et al., 1989] should greatly facilitate the analysis of images from these organisms.

The dynamics of actin and alpha actinin have also been examined in sea urchin eggs [Wang and Taylor, 1979; Hamaguchi and Mabuchi, 1986, 1988]. No distinct structures were detectable in unfertilized eggs with microinjected analogs. However, within 5 min of fertilization, a high concentration of actin and alpha actinin was observed in the membrane cortex and in the fertilization cone, corresponding to the polymerization of actin and elongation of microvilli reported previously [Begg and Rebhun, 1979]. However, results of photobleaching recovery indicate that actin subunits undergo very rapid on-off reactions along the cortex [Hamaguchi and Mabuchi, 1988]. The intensity of cortical fluorescence decreases 5–6 min after fertilization [Wang and Taylor, 1979; Hamaguchi and Mabuchi, 1986, 1988], but appears to increase again before the first mitosis [Wang and Taylor, 1979]. During mitosis, injected actin can be detected within the mitotic spindle, but this is likely due to a large accessible volume in the spindle since injected ovalbumin shows a similar distribution. During cytokinesis, the contractile ring, which is known to contain bundles of actin [Schroeder, 1981], cannot be detected with the fluorescent actin, indicating that although actin filaments are organized, the concentration may not be much higher than that in other regions of the cell. Injection of fluorescent alpha actinin similarly revealed no clear concentration but only a slight thickening of the cortex on the equatorial plane [Mabuchi et al., 1985]. However, the contractile ring has been detected with injected alpha actinin in dividing PtK epithelial cells, which maintain a spread morphology during cytokinesis, facilitating the detection of fine structures [Sanger et al., 1987].

2. Dynamics of stress fibers and developing myofibrils. Much attention has been paid to the stress fibers and adhesion plaques in cultued higher animal cells. Wehland and Weber [1981] microinjected fluorescent phalloidin into rat mammary cells and detected changes in the pattern of stress fibers over time. Direct time-lapse observations of stress fibers were subsequently made by following microinjected actin analogs [Wang, 1984]. Formation of new stress fibers appears to originate at discrete sites near the leading and trailing edges of the cell. The nascent fibers subsequently extend toward the nucleus. As the cell moves and changes shape, stress fibers undergo concomitant changes such as reorientation and fragmentation. Elongation of stress fibers is achieved by the formation of new segments near the edge and subsequent merging with existing fibers, while the distal ends of existing fibers (which are associated with adhesion plaques) remain stationary [Wang, 1984].

Reorganization of stress fibers at the molecular level was examined by photobleaching techniques [Kreis et al., 1982; Wang, 1987]. Fluorescence recovery of stress fibers labeled with either fluorescent actin or alpha actinin shows a half-time of ~10 min, independent of the length of the bleached segment [Kreis et al., 1982; McKenna et al., 1985b]. It was originally proposed that this may reflect an on-off equilibrium of actin subunits and accessory proteins along the length of stress fibers [Kreis et al., 1982]. However, a similar half-time was subsequently obtained with the photobleaching of fluorescent phalloidin, which associates tightly with actin filaments but not monomers [Wang, 1987]. Thus it is possible that an exchange of filamentous actin along stress fibers may be responsible for the fluorescence recovery, and that actin filaments may be mobile in living cells. A second mode of reorganization, which has been detected in stress fibers near the trailing edge, is the centripetal translocation of actin and alpha-actinin along stress fibers. The movement occurs at a rate of 0.2–0.3 μm/min [McKenna and Wang, 1986], and may be due to either an active flux or a passive stretching.

The organization of stress fibers can be disrupted under various conditions, for example, during the initiation of mitosis [Sanger et al., 1987], during oncogenic transformation [Vasiliev, 1985], or following treatments with various agents such as metabolic inhibitors and phorbol esters [Rifkin et al., 1979; Bershadsky et al., 1980]. Microinjection of fluorescent analogs has been used as a powerful tool to determine the pathways of the destruction and recovery of stress fibers [Wehland et al., 1980; Sanger et al., 1980; Meigs and Wang, 1986; Wang, 1986; Sanger et al., 1987; Glascott et al., 1987; DeBiasio et al., 1987; Stickel and Wang, 1988]. There are clearly multiple pathways for the disruption. For example, while phorbol esters and a synthetic peptide that disrupts the association of extracellular matrices induce a dissociation of stress fibers from adhesion plaques [Meigs and Wang, 1986; Stickel and Wang, 1988], metabolic inhibitors induce contractions toward the adhesion sites [Wang, 1986].

The mobility of proteins may also be profoundly affected. For example, although substrate adhesion structures in transformed cells are similar to adhesion plaques in many ways, studies with photobleaching recovery indicate that alpha actinin exchanges at a much faster rate at the adhesion structure in transformed cells than in normal cells [Stickel and Wang, 1987].

Fluorescent analog cytochemistry also has been performed on developing muscle cells to examine the mechanism of myofibrillogenesis. Arrays of punctate structures, which are precursors of Z-lines and are labeled with fluorescent alpha actinin, can be detected near the periphery of developing myotubes and cardiac myocytes [Sanger et al., 1984a; Sanger et al., 1986a]. Sanger et al. [1986a, 1986b] reported that the distance between neighboring punctate structures in developing myotubes increases from 0.9–1.3 μm to 1.6–2.3 μm over 24 h, as the punctate structures develop into Z-lines. They proposed that the increase in distance is a result of direct elongation of nascent sarcomeres. However, similar experiments by McKenna et al. [1986] failed to reveal such an elongation. Instead, adjoining punctate structures appear to coalesce to form fewer, larger punctate structures with a longer distance, while the distance between groups of coalescing punctate structures gradually becomes more regular and approaches the length of sarcomeres in mature myofibrils (~2 μm). Thus the apparent increase in the distance between Z-line precursors may be achieved through coalescence. However, it is difficult to rule out direct growth based on negative results. It would be informative to follow directly the extension of myofibrils at their ends. If direct elongation takes place as proposed by Sanger et al. (1986a), the ends should move by a distance equal to as much as 70% of the starting myofibril length over 24 h.

3. Dynamics of lamellipodia. Lamellipodia, or "ruffles," are the most motile area in cultured fibroblasts and epithelial cells. Previous studies have demonstrated the presence of a high concentration of actin filaments and small filament bundles in this area [Small, 1981]. However, it is not clear how the filaments are related to the motile activity.

In fibroblasts, actin analogs are incorporated into the lamellipodium within 5 min of microinjection [Glacy, 1983b], indicating that actin subunits may undergo a rapid turnover. The pattern of turnover has been studied by bleaching a small spot on the lamellipodia of cells microinjected with a fluorescent actin analog [Wang, 1985]. The bleached spot moves toward the nucleus at a constant rate of ~0.8 μm/min, suggesting that there is a constant flux of actin subunits in the lamellipodia. Confirming results were subsequently obtained with video enhanced DIC optics in 3T3 cell and neuronal growth cones [Forscher and Smith, 1988; Fisher et al., 1988]. The flux of actin subunits is probably generated by a constant assembly of actin at the filament-membrane interface and a disassembly at the opposite end. This process may play a critical role in cytoplasmic protrusion.

Equally important is the distribution of myosin in the lamellipodium region. Early immunofluorescence observations indicated that myosin may be absent from this area. However, observation of injected myosin analogs indicated that myosin is absent only during the earliest stage of lamellipodia formation, but can be detected in diffuse or bead forms after the initial protrusion stops [DeBiasio et al., 1988]. In a recent study with myosin analogs, McKenna et al. [1989b] demonstrated that bead structures in the lamellipodium area appear as a result of de novo assembly, rather than forward movement of existing structures. The assembly of new beads, which probably correspond to myosin filaments, is coupled to a backward movement of the beads at an average rate of 0.18 μm/min and an organization of the beads into linear arrays. The de novo assembly of beads near lamellipodia is likely to be coupled to a disassembly in other regions of the cell, thus myosin molecules may undergo a constant cycle between the backward moving, filamentous form and the forward diffusing, unassembled form. Although the role of myosin in cytoplasmic protrusion has been questioned [De Lozanne and Spudich, 1987; Knecht and Loomis, 1987], it is possible that the flux of myosin may play an important role in the determination of cell polarity. Disruption of cell polarity has indeed been observed following the microinjection of myosin antibodies [Honer et al., 1988].

IV. MICROINJECTION OF PROBES FOR MICROTUBULES

Compared to actin filaments, the larger size and smaller number of microtubules greatly facilitate their detection and analysis in living cells. However, due to the high susceptibility of tubulin to denaturation, fluorescently labeled tubulin was not successfully prepared until 1981 [Keith et al.]. The first analog was prepared with dichlorotriazinyl fluorescein (DTAF), which reacts primarily with amino groups at a neutral pH. conjugates with other reagents, including succinimidyl esters of carboxyfluorescein and carboxyrhodamine, were subsequently reported (Table I). Assays of these labeled preparations indicate a more-or-less normal polymerization behavior, although some appear to have a relatively low labeling stoichiometry and may not show clear differences from unlabeled controls. However, microinjection of different analogs has so far yielded relatively consistent results in both incorporation behavior and patterns of reorganization, as discussed in the following sections. The availability of rhodamine-labeled tubulin marks a significant advancement since earlier studies with fluorescein conjugates have been limited by photobleaching, photodamage, and interference from autofluorescence.

A. Association of Fluorescently Labeled Tubulin and Microtubule-Associated Proteins With Cellular Structures

Fluorescent tubulins become readily incorporated into fibrous structures after microinjection, showing a distribution very similar to that of tubulin immu-

nofluorescence [Saxton et al., 1984]. The fine linear structures in interphase higher animal cells probably represent single microtubules. The distribution of microinjected tubulin is sensitive to agents such as colcemid, nocodazole, and taxol, as expected for cytoplasmic microtubules [Keith et al., 1981; Wadsworth and Sloboda, 1983; Salmon et al., 1984b; Saxton et al., 1984].

Of great interest are the initial sites of incorporation, which most likely correspond to sites of active assembly of tubulin dimers. Observations of living cells soon after microinjection often give an impression that the incorporation takes place along the entire length of microtubules [Hamaguchi et al., 1985]. However, due to the presence of high concentrations of unincorporated molecules, detailed analysis is possible only after extraction of injected cells. Using immunofluorescence against fluorescein to amplify the signal, Soltys and Borisy [1985] identified discrete segments of labeled microtubules near the centrosomes and at the distal, "plus" ends of existing microtubules in interphase fibroblasts 7–14 min after microinjection. Schulze and Kirschner [1986] subsequently made similar observations with biotin-labeled tubulin and demonstrated that the incorporation near centrosomes represents de novo assembly rather than addition of tubulin to the "minus" ends of existing microtubules.

In metaphase cells, assembly also takes place near the centrosomes [Mitchison et al., 1986]. Like interphase microtubules, the kinetochore microtubules incorporate labeled subunits at the plus end [associated with kinetochores, Schulze and Kirschner, 1986], indicating that the association with kinetochores does not inhibit the assembly of microtubules. Alternatively, it is possible that microtubules may undergo constant on-off reactions with the kinetochore [Cassimeris et al., 1988a], and the incorporation of subunits may occur prior to the association with kinetochores.

Microinjection has also been used to study the possible differences between tubulin isoforms and the effects of post-translational modifications. Hamaguchi et al. [1985] reported that fluorescent analogs prepared from brain and sperm flagella tubulin showed a similar distribution in the mitotic spindle of sand dollar eggs, although it was difficult to rule out subtle differences at the resolution of a light microscope. The effects of tubulin tyrosination on incorporation have been studied by microinjecting fluorescently labeled, detyrosinated tubulin into human fibroblasts [Webster et al., 1987]. The analog was rapidly incorporated into microtubules, indistinguishable from the incorporation of tyrosinated tubulin, and then became tyrosinated over 1 h. In a converse experiment biotinylated tubulin was microinjected into human retinoblastoma cells, which contain discrete populations of tyrosinated-nonacetylated and detyrosinated-acetylated microtubules [Schulze et al., 1987]. The tubulin analog was preferentially incorporated into tyrosinated-nonacetylated and detyrosinated-acetylated microtubules [Schulze et al., 1987]. The tubulin analog was preferentially incorporated into tyrosinated-nonacetylated microtubules over a period of 1–2 h, indicating that tubulins in detyrosinated-acetylated microtubules turn over

more slowly. Similar observations were made with the neuron-like PC12 cells [Lim et al., 1989]. The turnover of tubulin appears slower along the axon, where acetylated tubulins are preferentially localized, than at the growth cone. However, this correlation does not hold in many other cell types such as chick fibroblasts [Schulze et al., 1987], which often contain detyrosinated-acetylated microtubules that undergo a rapid turnover. Therefore, stable microtubules may simply have a tendency to accumulate certain species of tubulins, but post-translational modification itself appears insufficient to affect the stability of microtubules.

In addition to tubulin analogs, functional analogs of microtubule-associated proteins (MAPs), including MAP2 and MAP4, have been prepared [Scherson et al., 1984; Vandenbunder and Borisy, 1986; Olmsted et al., 1989]. Since MAP2 has been found predominantly in neuronal cells [Vallee et al., 1984], fluorescent MAP2 should be viewed as an affinity probe, rather than an analog of endogenous proteins, when injected into non-neuronal cells. However, following microinjection into fibroblasts and epithelial cells, both MAP2 and MAP4 became rapidly associated with microtubules [Scherson et al., 1984; Olmsted et al., 1989]. Despite the effects of MAPs in stabilizing microtubules in vitro [Murphy et al., 1977], microinjection of even a significant amount of these analogs does not appear to affect the cold or drug-induced reorganization of microtubules, or the time course of mitosis [Vandenbunder and Borisy, 1986; Olmsted et al., 1989].

B. Dynamics of Microtubules as Studied by Microinjected Analogs

Microinjected tubulin analogs remain associated with microtubules for many hours and through multiple cell cycles [Keith et al., 1981; Wadsworth and Sloboda, 1983], thus providing a powerful means for following changes in microtubule distribution. For cells that are difficult to fix, microinjection of fluorescent tubulins has provided useful details about how microtubules reorganize through the cell cycle [Hamaguchi et al., 1985; Kellogg et al., 1988].

1. Dynamics of microtubules in interphase cells. Studies have been performed to determine the rate of the incorporation of tubulin analogs into microtubules in interphase cells. For higher animal cells, the half-time is estimated to be between 10 and 30 min [Saxton et al., 1984; Schulze and Kirschner, 1987]. Schulze and Kirschner [1986] demonstrated that the incorporation into the plus ends (discussed above) of microtubules follows a steady time course, with a rate independent of the concentration of the tubulin analog microinjected. Thus the incorporation probably reflects the normal turnover of microtubules, rather than polymerization driven by the increase in tubulin concentration following microinjection. The average rate of growth, 3–5 μm/min, is fast enough to allow a microtubule to reach from the centrosome to the cell periphery in 5 min. Therefore, in order to maintain a constant total length of microtubules in

a cell at steady state, such a fast assembly must be balanced by an equally rapid disassembly.

More detailed information on the dynamics of microtubules was obtained by examining the behavior of individual microtubules. It appears that the incorporation of tubulin analogs into microtubules occurs asynchronously; in some cells there are even "quasi-stable" microtubules that do not bind microinjected tubulin over an extended period of time [Schulze and Kirschner, 1986, 1987]. These results were extended recently by the direct observations of individual microtubules labeled with rhodamine tubulin analogs [Sammak and Borisy, 1988; Schulze and Kirschner, 1988]. Not only do microtubules show a wide range of growth rates, varying both over time and among different microtubules, individual microtubules also show distinct phases of growth, stability, and shortening. Each microtubule spends on the average 50% of the time growing, 32% of the time shortening, and 18% of the time resting [Sammak and Borisy, 1988]. As expected from the frequency, shortening is more rapid than growth, but rarely results in a complete disassembly of microtubules. Instead, after shortening for ~5 μm, a microtubule is as likely to grow as to continue to shorten. These observations, although differing in quantitative details, are similar to those made with uninjected cells using video-enhanced DIC optics [Cassimeris et al., 1988b]. Thus it is unlikely that the rapid shortening is due to the microinjection of tubulin analogs or photodamage. The manifestation of discrete phases of microtubule assembly and disassembly closely parallels the dynamic instability of microtubules as observed in vitro [Mitchison and Kirschner, 1984; Walker et al., 1988].

The results of these elegant studies are complemented by photobleaching studies. In unfertilized sea urchin eggs microinjected with DTAF tubulin, where no microtubules are detectable, the kinetics of fluorescence recovery indicate a single mobile species with an apparent diffusion coefficient of 5×10^{-8} cm^2/s [Salmon et al., 1984a]. This value is close to what one would expect if tubulin moves freely as dimers. In interphase PtK and BSC cells, there is a similar rapid recovery, reflecting the existence of a pool of unpolymerized tubulin dimers [Saxton et al., 1984; Salmon and Wadsworth, 1986]. However, a large portion of fluorescence recovers with a half-time of 4–5 min. This presumably represents exchange of tubulin dimers on and off polymers.

The mechanism of recovery was further studied by analyzing the location of the bleached spot and the pattern of recovery. No longitudinal movement of the bleached spot was detected with microtubules containing either fluorescent tubulin or MAP2 [Saxton et al., 1984; Sammak et al., 1987; Scherson et al., 1984]. In addition, when a wide band was bleached across a group of microtubules, the recovery occurred asynchronously, such that the bleached area gradually contained an increasing number of completely recovered microtubules [Sammak et al., 1987]. The probability of recovery increases with a

decreasing distance between the bleached area and the area of the cell. Taken together, these observations suggest that the recovery is a rapid, asynchronous process occurring predominantly at the distal end of the microtubule, consistent with a model involving repeated incomplete depolymerization and repolymerization as suggested by the incorporation experiments. On the other hand, "treadmilling" of tubulin along microtubules, which involves a continuous assembly of tubulin subunits at the distal end, coupled to a centripetal movement of tubulin dimers along microtubules and disassembly at the centrosomes [Margolis and Wilson, 1981], does not appear to play a major role in the turnover of interphase microtubules.

The method of fluorescence recovery after photobleaching was also used to study axonal transport in neuron-like PC12 cells [Keith, 1987]. The cells were microinjected with DTAF tubulin and subsequently photobleached with an unattenuated mercury arc lamp at 450–490 nm for 5 min. The bleached spot split into two spots moving at different rates. The rate of the slower component is similar to that of neurite growth. However, the main concern is whether the prolonged bleaching, which has been found to cause severe damage to microtubules in vitro [Leslie et al., 1984], induces artifacts. Recent studies indicate that quite different results can be obtained depending on the condition of photobleaching [Keith, 1988; Lim et al., 1989]. The correlation between the transport of microtubules and neurite growth therefore awaits further study.

2. Dynamics of microtubules in mitotic cells. Microtubules in prophase and metaphase mitotic spindles appear to be on the average much more dynamic than those in interphase cells [Salmon et al., 1984b; Saxton et al., 1984; Olmsted et al., 1989]. Incorporation of tubulin analogs into the centrosomal region occurs with a half-time of 5–40 s. Studies with photobleaching recovery of fluorescent tubulin similarly yielded a very short half time, on the order of 10–20 s in cultured higher animal cells and sea urchin eggs. It was originally proposed that such a rapid exchange of subunits may be a result of tubulin dimers undergoing association-dissociation all along the length of microtubules [Inoue and Ritter, 1975; Salmon et al., 1984b]. However, since subsequent incorporation experiments with biotinylated tubulin revealed a clear segregation of labeled segments from unlabeled segments [Mitchison et al., 1986], an end-dependent process is more likely.

The increase in exchange when cells enter prophase may be due to a decrease in the average length of microtubules. This will be coupled to an increase in the number of ends and in the net rate of turnover. In addition, if microtubules undergo cycles of incomplete disassembly-reassembly at the plus ends as in interphase cells, a shorter average length will decrease the average distance of tubulin dimers from the plus ends and increase the population of tubulin dimers undergoing active turnover. However, stimulation of turnover could also be induced by increases in the rate constants of tubulin association and dissocia-

tion. Although there is no definitive evidence for such modulation, dramatic effects on the rate of tubulin turnover have been observed following treatment of cells with metabolic inhibitors [Wadsworth and Salmon, 1988]. Heterogeneous dynamics have also been reported for kinetochore, astral, and interzonal microtubules within the same dividing cell [Mitchison et al., 1986; Saxton and McIntosh, 1987]. Furthermore, fluorescent MAPs also show different rates of on-off reactions along microtubules, both between microtubules in interphase and in mitotic cells, and among different subpopulations of microtubules within the same mitotic cell [Olmsted et al., 1989]. These observations are consistent with the existence of a mechanism that can modulate the stability of individual microtubules.

The incorporation of tubulin analogs into the plus ends of kinetochore microtubules appears much slower than the incorporation into non-kinetochore microtubules or into microtubules in interphase cells [Mitchison et al., 1986; Schulze and Kirschner, 1986], possibly due to the association of microtubules with the kinetochore. However, recent photobleaching studies by Gorbsky and Borisy [1989b] indicated that about 50% of microtubules that associate with kinetochores recover rapidly, with a half-time ~70 s. Although there is no definitive explanation, this apparent discrepancy may be explained if kinetochores can release at least a fraction of the associated microtubules and catch microtubules that undergo rapid tubulin exchange. Thus the mechanism responsible for short-term (~10 s) incorporation at the kinetochore end may be different from that for the long-term (several minutes) exchange along the length of kinetochore microtubules.

Of particular importance is the possible involvement of microtubule treadmilling in the movement of chromosomes. Early experiments with UV microbeams indicate a poleward movement of UV-marked microtubules (shown as areas of reduced birefringence) along metaphase microtubules [Forer, 1965]. This was later cited as a support for the treadmilling model. Treadmilling also is implied by the apparently slow but constant assembly of tubulin at kinetochore during metaphase [Mitchison et al., 1986]. However, photobleaching techniques, which should show a transport of bleached spots toward the poles if treadmilling occurs, have failed to yield definitive evidence. For example, although Hamaguchi et al. [1987] reported indications that spots bleached in the metaphase spindle move poleward, in most studies such movement is undetectable [Salmon et al., 1984b; Saxton et al., 1984; Wadsworth and Salmon, 1986b; Cassimeris et al., 1988a; Gorbsky and Borisy, 1989b]. In addition, the recovery of fluorescence follows an exponential time course, at a rate independent of the size of the bleached spot [Wadsworth and Salmon, 1986b], inconsistent with a flux being the mechanism of recovery. However, Wadsworth and Salmon cautioned that it is difficult to rule out the treadmilling of a subpopulation of microtubules, such as the kinetochore microtubules. The major

problem with the photobleaching studies is the difficulty in identifying the bleached spots over an extended period of time due to the recovery of fluorescence. Recent application of a tubulin analog labeled with a photoactivatable probe, indeed, has yielded the first clear evidence of poleward movement [Mitchison, 1989]. The rate, 0.5–0.6 μm/min, is close to the previous estimation based on the rate of incorporation [Mitchison et al., 1986]. However, the percentage of microtubules undergoing such movement appears low, and decreases continuously during observation.

During anaphase, disassembly of microtubules appears to occur at the kinetochore, as suggested by the loss of previously labeled segments [Mitchison et al., 1986]. More definitive evidence for the disassembly at kinetochores is provided by photobleaching studies, which show little or no movement of bleached spots relative to the pole while the distance between the spots and the chromosomes decreases [Gorbsky et al., 1987, 1988; Gorbsky and Borisy, 1989b]. These observations suggest an important role of microtubule disassembly at the kinetochore in chromosomal movement, but are clearly inconsistent with treadmilling being the primary mechanism. Available data also indicate a considerably slower exchange of tubulin dimers during anaphase than during metaphase. Incorporation of tubulin analog appears to occur at a much slower rate than during metaphase [Wadsworth and Sloboda, 1983; Mitchison et al., 1986]. Bleached spots on kinetochore microtubules also recover much more slowly [Gorbsky and Borisy, 1989b].

Experiments have been performed to study the role of interzonal microtubules, those located between the separating chromosomes, in the elongation of spindles during late anaphase and telophase [Saxton and McIntosh, 1987]. Following photobleaching, the recovery is slower near the equator than near the chromosomes. However, the equatorial region later accumulates a higher level of fluorescence than in surrounding regions. When a series of bars perpendicular to the spindle axis were bleached in the interzonal region, the two groups of bleached lines on the opposite sides of the equator moved apart from each other, but the lines within each group maintained their distances. These results suggest a limited on-off reaction of tubulin dimers near the equator, where the plus ends of many microtubules are located. However, microtubules in the equatorial region probably undergo a net assembly at the plus ends while sliding apart from each other toward the poles.

V. MICROINJECTION OF PROBES FOR INTERMEDIATE FILAMENTS

Among the three cytoskeletal systems, intermediate filaments are the least soluble in vitro and show the least reorganization in vivo. Nevertheless, the filaments must accommodate to such processes as cell spreading, locomo-

tion, and division. The dynamics of intermediate filaments represent an important unknown topic in cell biology.

Only recently have analogs of intermediate filament proteins, including biotinylated vimentin and rhodamine-labeled desmin, been microinjected into living cells [Vikstrom et al., 1989; Mittal et al., 1989]. In vitro, both analogs can form filaments indistinguishable from normal intermediate filaments. Microinjection was performed with a carrier solution of low ionic strength and high pH, in order to maintain solubility. The incorporation of injected vimentin and desmin into cellular structures occurs much more slowly than does injected actin or tubulin, on the order of several hours. However, at steady state, both analogs become colocalized with the endogenous vimentin (but not cytokeratin), and, upon exposing cells to nocodazole or acrylamide, form aggregates as expected for endogenous vimentin filaments [Mittal et al., 1989]. The distribution of fluorescent desmin during cell division also is similar to that revealed by immunofluorescence of vimentin.

These observations are significant since they indicate that, despite the low solubility of vimentin and desmin in the cytoplasm, the analogs can disperse from the site of microinjection and find sites of incorporation. Questions therefore arise as to whether or not endogenous intermediate filaments and the small, but identifiable [Soellner et al., 1985], pool of soluble components undergo any dynamic reorganization. Equally revealing is the pattern of incorporation. The microinjected vimentin appears to move as small aggregates into the perinuclear area before incorporating into the filament network [Vikstrom et al., 1989], suggesting that the perinuclear area may contain factors that facilitate the dissolution of vimentin aggregates. In addition, at steady state, vimentin may undergo a continuous assembly in perinuclear area and diassembly near the periphery of the cell. The observations also suggest that there may be a force-generating mechanism responsible for the directional movement of the vimentin aggregates. These possibilities are difficult to address with biotinylated vimentin, but should be quite approachable with fluorescent analogs and photobleaching techniques.

VI. CONCLUSIONS AND PROSPECTUS

So far the strategy in using the fluorescent probes falls into two main categories: either examining the association of microinjected fluorescent probes with cellular structures, or following the movement of the probe itself or associated structures. The results discussed above illustrate that even such relatively simple approaches can yield a tremendous amount of information which is difficult to obtain otherwise. However, it is also important to remember the basic assumptions of the approach and the complicated, unpredictable nature of living cells. For example, it is difficult to be totally sure that the microinjected

analogs are incorporated correctly at a molecular level, that *all* biochemical functions of the analogs are maintained in injected cells, and that the observed processes are unaffected by the microinjection or the observation. At the quantitative level, it is even more difficult to make sure that the measured values, such as diffusion coefficients or assembly rates, represent true physiological values. Therefore, whenever possible, it is important to seek support of the results by applying other techniques. Successful examples include the use of video-enhanced DIC optics to confirm the flux of actin subunits at the lamellipodia and the dynamic instability of microtubules. Such parallel experiments will continue to play an important role in the application of fluorescent probes.

The versatility of fluorescence techniques has been discussed in other chapters of this volume. Many powerful methods, such as polarization, resonance energy transfer, and spectroscopy, remain to be explored. Theoretically, it should be quite feasible to apply these techniques and delineate molecular interactions in living cells. Unlike conventional spectroscopy or flow cytometry, microscopic analyses of single cells will reveal not only what happens in the cell, but also where. If the molecule assumes several discrete conformations, it should also be possible to map the distribution of these different states and to follow the changes.

Fluorescent structural probes can also be combined with fluorescent indicators of cellular parameters. For example, many fluorescent indicators are now available for determining the cellular ionic environment such as Ca, pH, Na, or membrane potential [Tsien, 1989; Haugland, 1989]. A combination of these indicators and cytoskeletal probes should reveal correlations between structural dynamics and ionic conditions [Waggoner et al., 1989]. Furthermore, cytoskeletal probes can be applied in conjunction with caged compounds, to allow observations of structural reorganizations in response to stimuli at specific sites. Finally, the application of confocal microscopy should greatly increase the resolution of fluorescence images, and allow the observation of three-dimensional structures such as cells within matrices or tissues. A combination of these powerful techniques will turn living cells into a biochemical laboratory for testing various hypotheses, rather than simply beautiful objects to watch.

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