Steady State Dynamics of Intermediate Filament Networks

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Abstract. We have conducted experiments to examine the dynamic exchange between subunit and polymer of vimentin intermediate filaments (IF) at steady state through the use of rhodamine-labeled vimentin in fluorescence recovery after photobleaching (FRAP) analysis. The rhodamine–vimentin incorporated into the endogenous vimentin IF network after microinjection into fibroblasts and could be visualized with a cooled charge-coupled device (CCD) camera and digital imaging fluorescence microscopy. Bar shaped regions were bleached in the fluorescent IF network using a beam from an argon ion laser and the cells were monitored at various times after bleaching to assess recovery of fluorescence in the bleached zones. We determined that bleached vimentin fibers can recover their fluorescence over relatively short time periods. Vimentin fibers in living cells also can exhibit significant movements, but the recovery of fluorescence was not dependent upon movement of fibers. Fluorescence recovery within individual fibers did not exhibit any marked polarity and was most consistent with a steady state exchange of vimentin subunits along the lengths of IF.

Intermediate filaments (IF) are generally considered to be the most stable of the cytoskeletal components. Even so, consideration of their properties supports the view that IF are dynamic. In this regard, the ability of IF networks to undergo transitory changes in response to different stimuli is well documented. For example, fibroblasts reorganize their vimentin-IF networks from a juxtanuclear “cap” to an extended cytoplasmic network during cell spreading and shape formation (Goldman et al., 1986). Dramatic organizational changes in IF networks also are seen in response to different stimuli such as adipose conversion of cultured cells (Franke et al., 1987) or mitogenic stimulation of lymphocytes (Paulin-Levasseur and Brown, 1987). In addition, IF exhibit dynamic properties during mitosis of numerous cell types (Aubin et al., 1980; Franke et al., 1982; Horwitz et al., 1981; Lane et al., 1982; Jones et al., 1985; Rosevear et al., 1990). In BHK-21 cells, these properties include the depolymerization of the IF network to form amorphous aggregates during metaphase and the eventual reformation of polymerized, cytoplasmic IF at the conclusion of mitosis (Rosevear et al., 1990). Likewise, another member of the IF protein family, the nuclear lamins, exhibits cell cycle–dependent dynamics and becomes disassembled during mitosis or meiosis (Gerace and Blobel, 1980; Dessev and Goldman, 1988). Although the molecular mechanisms governing these processes remain unclear, they can be divided into three categories: (a) the molecular requirements for IF assembly; (b) the site(s) of assembly; and (c) the temporal sequence of events leading to assembly.

Transfection studies have been used with great success to determine the domains of IF proteins that are essential for assembly in vivo. The effect on IF assembly of mutant IF proteins lacking portions of their nonhelical amino or carboxyl domains has been addressed using this technique (Albers and Fuchs, 1987, 1989; Gill et al., 1990; Lu and Lane, 1990; Raats et al., 1990; Wong and Cleveland, 1990). While transfection of mutated IF genes is a powerful technique for mapping the functional domains of IF proteins, its temporal resolution is low with respect to determining IF assembly mechanisms. This makes it less suitable for studying posttranslationally regulated IF dynamics.

Studies of the site(s) of assembly have suggested subunit incorporation into IF either in juxtanuclear organization centers or throughout the cytoplasm. Data from transfection experiments (Albers and Fuchs, 1987; Chin and Liem, 1989; Sarria et al., 1990) and microinjection of vimentin (Vikstrom et al., 1989) support the initiation of IF assembly in the juxtanuclear region. On the other hand, studies of newly synthesized IF after keratin mRNA injection into fibroblasts (Kreis et al., 1983) as well as microinjection of keratin (Miller et al., 1991) have demonstrated that keratin assembly does not necessarily require a juxtanuclear organizing center. That vimentin and desmin assembly can also proceed without the aid of a juxtanuclear organizing center has also been supported by transfection studies (Ngai et al., 1990; Sarria et al., 1990; Raats et al., 1990). Some of the dis-

1. Abbreviations used in this paper: IF, intermediate filaments.

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crepencies concerning the nature of the sites of IF assembly may have resulted from attempts to compare steady state versus non-steady state experiments. Microinjection and most transient transfections may provide the cell with a sizable pool of unpolymerized IF proteins and the events seen may not be typical of the assembly of IF in interphase cells at steady state (Ngai et al., 1990; Steinert and Liem, 1990).

To investigate the sequence of events leading to assembly, biochemical analysis has shown that some nascent vimentin molecules associate with IF during translation (Isaacs et al., 1989). This suggests that IF can polymerize cotranslationally (Isaacs et al., 1989). Pulse-chase experiments indicate that newly synthesized vimentin may also enter a soluble pool before all (Blikstad and Lazarides, 1983) or a portion of the newly synthesized vimentin (Soeller et al., 1985) incorporates into the insoluble cytoskeleton. At this point, it has not been clearly established whether after entering a soluble pool of newly synthesized protein, vimentin becomes stably incorporated into polymer or whether there is an equilibrium between soluble and polymer forms of vimentin. Clarifying this issue through the use of analytical techniques with greater temporal and spatial resolution is central to determining the molecular mechanisms underlying the distribution and composition of IF.

We hope to resolve some of these issues by examining the dynamic properties of IF at steady state using the technique of FRAP (Petersen et al., 1986). FRAP analysis allows steady state systems to be analyzed with high temporal resolution and has proven to be extremely valuable for studying the steady state dynamics of both microtubules (for examples, see Wadsworth and Salmon, 1986; Gorbsky et al., 1988; Sammak and Borisy, 1988) and microfilaments (Kreis et al., 1982; Wang, 1985). In this report we use this technique to demonstrate that there is an equilibrium between subunit and polymer in the IF systems of living cells.

Materials and Methods

Cell Culture

3T3 cells were grown at 37°C in DME containing 10% calf serum, penicillin, and streptomycin. For microinjection, cells were plated onto etched grid coverslips (Belco Biotechnology, Vineland, NJ) which had been cleaned with RBS-35 (Pierce Chemical Co., Rockford, IL). The coverslips were attached with a 1:1:1 mixture of Vaseline, beeswax, and lanolin to a 35-mm dish in which an 18-mm hole had been drilled (Lii et al., 1990).

Preparation of Xrhodamine-labeled Vimentin

Vimentin was purified from bovine lens and polymerized into IF as described elsewhere (Vikstrom et al., 1989, 1991). IF were collected by centrifugation at 100,000 g for 30 min at 4°C. The resulting pellet was solubilized in disassembly buffer (8 M urea, 5 mM sodium phosphate, pH 7.2, 0.2% 2-mercaptoethanol, 1 mM PMSF) by stirring for 30 min at room temperature. The sample was then dialyzed overnight against room temperature versus subunit buffer (5 mM sodium phosphate, pH 7.4, 0.2% 2-mercaptoethanol, 0.2 mM PMSF). The protein concentration was determined by the method of Bradford (1976) using BSA as a standard and then it was adjusted to 2.0-2.5 mg/ml. A 40:1 molar excess of 5-(and-6)-carboxy-X-rhodamine versus subunit buffer (5 mM sodium phosphate, pH 7.4, 0.2% 2-mercaptoethanol, 0.2 mM PMSF). The protein concentration was determined by the method of Bradford (1976) using BSA as a standard and then it was adjusted to 2.0-2.5 mg/ml. A 40:1 molar excess of 5-(and-6)-carboxy-X-rhodamine for the microinjection experiments.

To begin the first cycle of disassembly/reassembly the pellet of xrhodamine-labeled IF was solubilized in disassembly buffer and passed over a Sephadex G-25 column which had been equilibrated in subunit buffer. Sodium chloride was added to a final concentration of 0.17 M and the preparation was dialyzed overnight at room temperature versus PBS containing 0.2% 2-mercaptoethanol and 0.2 mM PMSF (Zackroff and Goldman, 1979). The in vitro disassembly/reassembly conditions included both gel filtration chromatography over Sephadex G-25 and dialysis to completely remove unconjugated xrhodamine from the preparation. After a second cycle of disassembly/reassembly the xrhodamine vimentin sample was frozen dropwise in liquid N2 and stored at -70°C. A typical vimentin preparation contained 0.5 mole xrhodamine per mole of vimentin, calculated using an extinction coefficient of 5.3 x 10^4 M^-1 cm^-1 for xrhodamine in 5 mM sodium phosphate and a molecular weight of 55,000 for vimentin.

Microinjection

Xrhodamine-labeled vimentin was prepared for microinjection as described for biotinylated vimentin (Vikstrom et al., 1989, 1991). Volumes were adjusted at all steps to yield final samples with concentrations of 2.0-3.0 mg/ml xrhodamine-vimentin in 5 mM sodium phosphate, pH 8.5, 0.05% 2-mercaptoethanol. Microinjections were performed on an inverted microscope (Nikon Inc., Melville, NY) with the aid of a micromanipulator (The Leitz Co., Overland Park, KS) (Lim et al., 1989). After microinjection, the cells were returned to the incubator for 5-13 h to allow full incorporation of xrhodamine-labeled vimentin into the endogenous vimentin networks. At this time, the culture medium was changed to phenol red-free DME containing 10% calf serum and antibiotics.

Photobleaching

The photobleaching apparatus was set up essentially as described by Lim et al. (1989) following the general procedure of Petersen et al. (1986). Microinjected cells were located on the etched grid and the image of the fluorescent IF network was focused using a SIT TV camera. Since excessive illumination of fluorescent cytoskeletal components such as microtubules can be deleterious (Vigers et al., 1988), neutral density filters were placed in front of the mercury arc lamp to attenuate the light and to minimize photodamage to the cells. Cells were bleached with a bar of green light (514 nm) by 200 ms of irradiation from an argon ion laser operating at 200 mW. These conditions result in laser exposures that are eightfold less than those reported to damage microtubules in vitro (Lim et al., 1990). Fluorescence and phase contrast images of the cells were taken before and after photobleaching using a cooled charge-coupled device (CCD) camera (Photometrics Ltd., Tucson, AZ) and the resulting digitized images were stored on a computer disk and archived on an optical disk using a WORM drive (Model 3363, IBM Corp., Danbury, CT) (Lim et al., 1990).

Image Analysis

Images were first flat-fielded using the Photometrics system and further analysis was carried out with the Image-I system (Universal Imaging Corp., West Chester, PA). To adjust for sample fading during image acquisition the brightness of the prebleach image of each cell was measured and this value was used to normalize the brightness level of subsequent images of that cell. Digitized pixel values were read along each fiber being measured. The maximum pixel value across the width of the fiber was recorded both in the bleached and unbleached regions of the individual xrhodamine-vimentin-containing fibers. The average pixel intensity of the background fluorescence of the coverslip was subtracted from these values and the resulting relative fluorescence intensities were plotted.

Immunofluorescence

Cells were rinsed rapidly with PHEM (60 mM Pipes, 25 mM Hepes, 10 mM EGTA, 2 mM MgCl2, pH 6.95) at 37°C, and then lysed for 30 s in 0.15% Triton X-100 in PHEM (Schiwa and van Blerkom, 1981). After lysis the cells were fixed for 15 min at room temperature in 5 mM EGS (ethylene glycol bis [succinic acid N-hydroxy succinimide ester]) (Sigma Chemical Co., St. Louis, MO) in PHEM (Goldsky et al., 1988). After fixation the coverslips were rinsed with PBS and stored overnight at 4°C. Fixed cells
on coverslips were absorbed with 1% BSA in PBS for 30 min at 37°C and then incubated with a rabbit polyclonal antibody directed against vimentin (Yang et al., 1985) for 30 min at 37°C. After their incubation, the coverslips were washed with PBS (4–5 changes over 15 min) and then incubated with a fluorescein-labeled goat anti-rabbit antibody (Kirkegard & Parry Labs, Inc., Gaithersburg, MD) for 30 min at 37°C. The coverslips were washed as described above and then examined using the CCD camera and narrow band fluorescein and rhodamine filters.

**Results**

**Xrhodamine–Vimentin Forms IF in vitro**

Twice-cycled xrhodamine vimentin IF (see Materials and Methods) were examined by negative stain EM and only samples which contained smooth walled 10-nm diameter IF (Fig. 1 a) were frozen and stored at −70°C for use in subsequent experiments. When examined by SDS-PAGE, the resulting preparation contained one major polypeptide as detected by Coomassie-blue staining, which was fluorescent when unstained gels were examined under UV illumination (Fig. 1 b).

**Microinjected Xrhodamine–Vimentin Is Incorporated into the Vimentin-IF Network of 3T3 Cells**

For xrhodamine–vimentin to be useful in photobleaching studies, it must be incorporated into the endogenous vimentin IF network after microinjection into cultured cells. In addition, the fluorescent vimentin networks must not be damaged under the conditions used to photobleach a bar in this network. To determine if we had achieved these conditions, xrhodamine–vimentin was microinjected into 3T3 cells growing on “locator” coverslips and their positions were noted. Injected cells were incubated for 5–13 h and then examined by fluorescence microscopy using the cooled CCD camera. A bar was bleached in the fluorescent IF network and the cell was lysed and fixed within 2 min of photobleaching (see Materials and Methods). The fixed preparation was processed for indirect immunofluorescence with an antibody directed against vimentin and a fluorescein-conjugated secondary antibody. In fixed-stained cells, xrhodamine–vimentin colocalized with endogenous vimentin. Furthermore, endogenous vimentin appeared intact in regions where the rhodamine fluorescence had been bleached (compare Fig. 2, A and B, and C and D). In addition, over the course of these experiments we did not observe any dissolution of the fluorescent IF network such as had been described for photo damaged fluorescent microtubule networks (Vigers et al., 1988). These data indicate that rhodamine–vimentin is incorporated into the cytoplasmic IF network and that photobleaching does not cause obvious breaks or damage in the vimentin fibers. We refer to these structures as fibers since the limit of resolution for the light microscope does not allow individual IF to be distinguished from small bundles of IF.

**Photobleached Vimentin Networks Recover Their Fluorescence**

The ability of bleached zones to recover their fluorescence was assessed. After microinjection with xrhodamine–vimentin, 3T3 cells were allowed to incorporate the injected protein into their endogenous vimentin IF networks. Flat, peripheral regions of microinjected cells were selected and reference images were taken and stored on a computer. A bar was then bleached in the vimentin IF network and images of the cell were taken at 1–10 min intervals to monitor recovery in the bleached zone (Fig. 3). In some cases fluorescent fibers were seen crossing the bleached zone within 5 min, and by 13–14 min after photobleaching many of the bleached fibers had recovered a significant proportion of their fluorescence (Fig. 3 C).
Figure 2. A 3T3 cell microinjected with xrhodamine-vimentin and photobleached 8 h later. The cell was lysed and fixed 2 min after photobleaching. (A) Direct fluorescence of the xrhodamine-vimentin pattern. (B) Indirect immunofluorescence using an antibody against vimentin and a fluorescein-conjugated secondary antibody to show continuity of the vimentin fibers running through the bleached zone. (C and D) Higher magnification of the region indicated in A and B. Bar, 5 μm.

Figure 3. Recovery of fluorescence in xrhodamine-vimentin networks in living cells after photobleaching. A 3T3 cell was microinjected with xrhodamine-vimentin and 13 h later was photobleached with a 200 ms pulse from a 200 mW argon ion laser beam. (A) Cell before photobleaching; (B) image of the cell after 0.5 min; and (C) 13.5 min after photobleaching. Bar, 5 μm.
Stationary Fibers Recover Their Fluorescence

In some cases fluorescent fibers moved during the recovery period such that the remnants of the bleached bar did not remain straight. To clearly distinguish between fluorescence recovery because of subunit exchange or because of movements of fibers we carried out two experiments. First, we bleached multiple parallel bars in the fluorescent network to provide additional landmarks in assessing the movement of fibers. In addition, we looked for fluorescence recovery in fibers which remained stationary during the time we monitored the cell. When stationary fibers were monitored after photobleaching, fluorescence recovery was observed. In addition, the position of the bleached bars in the cells remained the same. In the example shown in Fig. 4, the bleached zones were clearly apparent 0.5 min after photobleaching. By 6.5 min, the bleached zones no longer had distinct margins and fluorescent fibers could be seen crossing them (Fig. 4). Many bleached fibers that remained stationary (e.g., see the fiber indicated by arrows in Fig. 4, B–F) regained fluorescence during the time that the cell was observed with no apparent translocation of the bleached zones along the fibers.

Fluorescence Recovery Exhibits No Detectable Polarity

To further assess the nature of fluorescence recovery in pho-
Figure 5. Fluorescence intensity measurements along photobleached fibers during the initial stages of recovery. The arrow marks the center of the bleached zones. The times after photobleaching are indicated. (a) 0.5 (△), min, 13.5 (□) min, (b) 0.5 (△), 2.5 (□) and 12.5 (▲) min.

To confirm this observation, we then monitored fluorescence recovery in fibers that had been bleached longitudinally. In this manner we were able to produce an effectively wider bleached zone without changing the photobleaching parameters. When longitudinally bleached fibers were monitored over time, fluorescence returned to the fibers throughout their length (Fig. 6). As in the previous experiment there was no apparent polarity to the recovery.

Discussion

Microinjection of labeled IF proteins has been used to determine the location of sites where subunits incorporate into IF (Vikstrom et al., 1989; Mittal et al., 1989). In this study we combine microinjection with FRAP to determine exchange of vimentin subunits in vivo under conditions which approximate steady state. We report that vimentin IF are dynamic in steady state situations, and therefore, they appear to exchange subunits with a pool of depolymerized or disassembled vimentin.

Xrhodamine-Vimentin Functions as a Probe for Examining the Vimentin Networks of Living Cells

Central to using fluorescently labeled IF proteins as probes for IF dynamics is the premise that once microinjected into living cells they will function as a tracer for the behavior of the entire IF network. Therefore, care must be taken to ensure that the fluorescent protein subunits can become incorporated into IF and that once incorporated, they behave normally. The primary in vitro functional assay for fluorescent IF protein is its ability to polymerize into IF (Ip and Fellows, 1990). We have found that vimentin conjugated with 0.5 mole xrhodamine per mole protein retains its ability to assemble into morphologically normal IF in vitro. In addition, the in vivo pattern of xrhodamine vimentin seen after microinjection appears to reflect the entire IF network (see Fig. 2). The excellent correspondence between xrhodamine-vimentin and the total IF network seen 5-10 h after microinjection argues against the possibility that the microinjected protein only polymerizes into new IF. More vigorous proof of the incorporation of xrhodamine-vimentin into IF in vivo would require the localization of xrhodamine subunits at the electron microscope level. Using immuno-EM techniques, biotinylated vimentin has been localized to IF in microinjected cells (Vikstrom et al., 1991). However, similar localization of xrhodamine-vimentin would require probes that are not yet available, such as anti-rhodamine antibodies.

Fluorescence Recovery in Bleached Zones Indicates Subunit Exchange

Xrhodamine does not recover its fluorescence after bleaching (Jacobson et al., 1983), so recovery of fluorescence in the bleached zone is because of the incorporation of unbleached vimentin into that region. There is evidence for a small soluble pool of tetrameric vimentin subunits at the electron microscope level. Using immuno-EM techniques, biotinylated vimentin has been localized to IF in microinjected cells (Vikstrom et al., 1991). However, similar localization of xrhodamine-vimentin would require probes that are not yet available, such as anti-rhodamine antibodies.
Figure 6. Recovery of fluorescence in a 3T3 cell with a longitudinal bleach zone. 12 h after the cell was microinjected with rhodamine-vimentin, the aster beam was oriented longitudinal to a prominent fiber. The fiber was photobleached and the cell was then monitored at 15-min intervals for >45 min; images of the living cell were taken at the times indicated. (A) Rhodamine-vimentin network in the living cell before photobleaching, and after (B) 0.5, (C) 15, (D) 30, and (E) 45 min after photobleaching. Bar, 5 μm.

Possible Mechanisms of Fluorescence Recovery

We do not detect any polarity in the recovery of fluorescence in bleached vimentin fibers nor do we see migration of the bleached zones towards the cell periphery. The former possibility would be expected if the incorporation of subunits into steady state IF only occurred in the juxtanuclear region, as was suggested by our previous microinjection experiments (Vikstrom et al., 1989). Furthermore, migration of the bleached zones towards the cell periphery would occur if IF exhibited treadmilling, as would be expected from the model of vectorial assembly. This model suggests that the nucleation of IF occurs at the nuclear surface and assembly progresses towards the cell surface (Georgatos and Blobel, 1987).

Based on these observations and the probability that IF are apolar structures (Geisler et al., 1985; Steven, 1990) it seems likely that vimentin-IF at steady state exchange subunits along their length (Fig. 7). This hypothesis is consistent with biophysical analyses of IF structure. For example, STEM analysis of the mass per unit length of individual IF indicates polymorphisms in the number of protein chains per cross-sectional area (Steven et al., 1982; Engel et al., 1985) and nuclear magnetic resonance (NMR) studies show that IF are highly flexible, loosely packed polymers (Mack et al., 1988). Taken together, these data suggest that IF are more like a partially disordered braid, rather than a crystalline lattice, therefore subunits would be expected to dissociate from polymer throughout the filament. The exchange of subunits along the length of IF also is supported by the microinjection of biotinylated keratin which is incorporated into tonofilaments throughout the cytoplasm, possibly along the lengths of filaments (Miller et al., 1991). In addition, transfection experiments by Ngai et al. (Ngai et al., 1990) demonstrate that newly synthesized vimentin subunits may incorporate into IF along their length. These studies are limited to determining the incorporation of subunits into IF, while our FRAP data suggest that the disassociation of vimentin subunits also may occur along the lengths of IF. A qualification should be noted, as FRAP analyses are of insufficient resolution to distinguish between events occurring within individual IF or small bundles of IF. The vimentin fibers detected by light microscopy may actually contain numerous IF of undetermined length. Therefore, multiple "ends" of individual IF could exist throughout the length of the fluorescent fibers and consequently an exchange mechanism limited to the ends of IF cannot be ruled out. However, if the biophysical picture of IF as a disordered braid is correct, subunit vacancies and ends exist within single IF, thus obviating a sharp distinction between exchange at ends and along the length of IF.

Subunit Exchange as a Mechanism to Alter the Composition of an IF Network

The exchange of subunits between a polymer and a soluble...
In addition, subunit exchange may provide a mechanism to alter the protein composition of an IF system without depolymerizing the existing network. During development, the transition from vimentin to different IF proteins seen in differentiating muscle cells, glial cells and neurons may occur through a process of subunit exchange (Bennett et al., 1979; Bignami et al., 1982; Cochard and Paulin, 1984; Tapp et al., 1981; Tokuyasu et al., 1984). It is tempting to speculate that in these instances vimentin-IF provide the initial “IF framework” into which different IF proteins exchange. It should be emphasized that at present the significance of these changes in IF composition remains unclear. However, the two-way exchange of vimentin between polymer and a cytoplasmic pool suggests another level at which IF organization is regulated; the size and composition of the subunit pool.

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