Intermediate filaments (IFs) are present in the cytoplasm and nucleoplasm of almost all mammalian cells. IF proteins constitute a large family of proteins and usually have distinct tissue distributions (for review, see Fuchs and Weber, 1994). All IF proteins have a tripartite structure, consisting of N-terminal head and C-terminal tail domains flanking a protease-resistant, $\alpha$-helical central rod domain with a heptad repeat of hydrophobic amino acids (for review, see Parry and Steinert, 1995). The first step for filament formation is dimerization, mediated by the rod domains through coiled-coil interactions. The head and tail domains are also involved in filament formation. Amino-terminally deleted IF proteins lose their ability to self-assemble, whereas carboxyl-terminally truncated proteins may form normal looking IF networks, abnormally closely packed IF networks, or amorphous non-filament aggregates depending on the protein and the site of truncation. However, nearly all the head-truncated IF proteins, as well as some tail-truncated mutants can be incorporated into the IF network (reviewed by Heins and Aebi, 1995).

Recent studies have shown that IFs play crucial roles in maintaining the structural integrity of skin and muscle cells. These conclusions are based on mutations or deletions of keratins that cause blistering diseases (for review, see McLean and Lane, 1995) and on the deletion of desmin in mice, which resulted in the disorganization of myofibrils in the most actively contracting muscles (Li et al., 1996; Milner et al., 1996). Neurofilament deficient animals have axons with smaller diameter (Ohara et al., 1993; Eyer and Peterson, 1994; Zhu et al., 1997). In mice, the animals lacking neurofilaments exhibit no overt phenotype (Zhu et al., 1997), although the quail mutant quivers (Ohara et al., 1993). These

In order to study the dynamic behavior of intermediate filament networks in living cells, we have prepared constructs fusing green fluorescent protein to intermediate filament proteins. Vimentin fused to green fluorescent protein labeled the endogenous intermediate filament network. We generated stable SW13 and NIH3T3 cell lines that express an enhanced green fluorescent protein fused to the N-terminus of full-length vimentin. We were able to observe the dynamic behavior of the intermediate filament network in these cells for periods as long as 4 hours (images acquired every 2 minutes). In both cell lines, the vimentin network constantly moves in a wavy manner. In the NIH3T3 cells, we observed extension of individual vimentin filaments at the edge of the cell. This movement is dependent on microtubules, since the addition of nocodazole stopped the extension of the intermediate filaments. Injection of anti-IFA causes the redistribution or ‘collapse’ of intermediate filaments. We injected anti-IFA antibodies into NIH3T3 cells stably expressing green fluorescent protein fused to vimentin and found that individual intermediate filaments move slowly towards the perinuclear area without obvious disassembly. These results demonstrate that individual intermediate filaments are translocated during the collapse, rather than undergoing disassembly-induced redistribution. Injections of tubulin antibodies disrupt the interactions between intermediate filaments and stable microtubules and cause the collapse of the vimentin network showing that these interactions play an important role in keeping the intermediate filament network extended. The nocodazole inhibition of intermediate filament extension and the anti-IFA microinjection experiments are consistent with a model in which intermediate filaments exhibit an extended distribution when tethered to microtubules, but are translocated to the perinuclear area when these connections are severed.

Key words: Intermediate filament, Vimentin, GFP, Microtubule, Cytoskeleton

INTRODUCTION

Intermediate filaments (IFs) are present in the cytoplasm and nucleoplasm of almost all mammalian cells. IF proteins constitute a large family of proteins and usually have distinct tissue distributions (for review, see Fuchs and Weber, 1994). All IF proteins have a tripartite structure, consisting of N-terminal head and C-terminal tail domains flanking a protease-resistant, $\alpha$-helical central rod domain with a heptad repeat of hydrophobic amino acids (for review, see Parry and Steinert, 1995). The first step for filament formation is dimerization, mediated by the rod domains through coiled-coil interactions. The head and tail domains are also involved in filament formation. Amino-terminally deleted IF proteins lose their ability to self-assemble, whereas carboxyl-terminally truncated proteins may form normal looking IF networks, abnormally closely packed IF networks, or amorphous non-filament aggregates depending on the protein and the site of truncation. However, nearly all the head-truncated IF proteins, as well as some tail-truncated mutants...
studies have therefore not completely clarified all the potential functions of IFs.

To study these potential functions of IFs, it would be useful to be able to visualize these structures in living cells. In previous studies using fluorescently labeled IF proteins, a number of phenomena regarding the dynamics of subunit exchange and filament assembly and disassembly have been described (Angelides et al., 1989; Okabe et al., 1993; Vikstrom et al., 1992). However, the movements of IFs over long periods of time could not be observed due to the limitations of the available fluorescent reagents (Mittal et al., 1989), or were only observed over a period of about one hour (Vikstrom et al., 1992). In this study, we describe strategies for the preparation of chimeras of green fluorescent protein (GFP) (Chalfie et al., 1994) with IFs. These GFP/IF chimeras have enabled us to observe the movements of the IF network in living cells. For example, we observed vimentin filaments extending toward the edge of a cell, which is dependent on the presence of microtubules and we have been able to study the mechanism of ‘collapse’ of IFs, when cells are infected with the anti-IFA antibody (Klymkowsky, 1981).

**MATERIALS AND METHODS**

**DNA constructs**

EGFP-vimN56: The amino-terminal fusion protein EGFP-vimN56 was prepared by inserting the Smal-BamHI fragment of pRSVi-vimentin (Ching and Liem, 1993), which contains amino acids 56-465 of rat vimentin and the SV40 poly(A) signal, into pEGFP-C1 (Clontech Laboratories Inc., Palo Alto, CA), which was doubly digested by restriction enzymes Smal and BamHI.

EGFP-vimFL: The amino-terminal fusion protein EGFP-vimFL was prepared by inserting the BstBI-BamHI fragment of pRSVi-vimentin, which contains amino acids 1-465 of rat vimentin and the SV40 poly(A) signal, into the BamHI site of pEGFP-C1.

S65T-gfapN30: The amino-terminal fusion protein S65T-gfapN30 was prepared by inserting the KpnI-BamHI fragment of pRSVi-gfap (Chen and Liem, 1994), which contains amino acids 30-430 of rat GFAP and the SV40 poly(A) signal, into pS65T-C1 which was doubly digested by restriction enzymes KpnI and BamHI.

S65T-gfapFL: The amino-terminal fusion protein S65T-gfapFL was prepared by inserting the EcoRI fragment of pRSVi-gfap, which contains amino acids 1-430 of rat GFAP, into the BamHI site of pS65T-C1. In order to do this, the EcoRI sites of the EcoRI fragment of pRSVi-gfap were changed to BamHI sites through linker ligation.

pCEN-env and pLEN-env: These constructs were prepared by first ligating the Eco47III-BglII fragment of pEGFP-C1 which contains the EGFP sequence and the BamHI-HindIII fragment of pRSVi-vim into the EcoRI and HindIII sites of pGEM7Zf (-) (Promega Corp., Madison, WI) after the Eco47III site of the Eco47III-BglII fragment of pEGFP-C1 was changed to an EcoRI site. A fragment encoding GFP-vimentin can be cut out by EcoRI digestion from this plasmid (pGEM-env). pCEN-env was prepared by inserting the EcoRI fragment of pGEM-env into the EcoRI cloning site of XhoI whereas pCEN contains an internal ribosomal entry site (Ghattas et al., 1991) a neo resistance gene after the EcoRI cloning site and a CMV promoter before the EcoRI site. Therefore, EGFP-vimFL and the neo resistance gene product will be produced from the same transcript. pLEN-env was prepared by inserting the EcoRI fragment of pGEM-env into the EcoRI cloning site of the eukaryotic expressing vector pLEN (Brieseswitz et al., 1993).

**Antibodies**

The monoclonal anti-vimentin (clone V9) was obtained from Sigma Chemical Company, St Louis, MO, USA. It was used at a dilution of 1:100. The monoclonal anti-vimentin (clone vim 13.2) was also obtained from Sigma and used at a dilution of 1:200. The rabbit polyclonal antibody to GFAP has been described previously (Wang et al., 1984) and was used at a dilution of 1:200. Goat anti-mouse secondary antibody conjugated with fluorescein isothiocyanate (FITC, used at a dilution of 1:200) and tetramethyl rhodamine isothiocyanate (TRITC) labeled goat anti-rabbit IgG (used at a dilution of 1:400) were purchased from Cappel Laboratories, Durham, NC. Goat anti-rabbit IgG secondary antibody conjugated with Cy5 was used at a dilution of 1:100 (from Jackson ImmunoResearch Laboratories Inc., West Grove, PA).

**Cell culture**

Human adrenal carcinoma SW13 cl. 2 Vim-, cl. 1 Vim+ and MFT16 cells (Sarria et al., 1990; Holwell et al., 1997) were generously provided by Dr Robert Evans and grown in DMEM/F12 medium (Gibco BRL, Gaithersburg, MD) supplemented with 5%, 10% and 10% fetal bovine serum, respectively. NIH3T3 cells were grown in DMEM medium supplemented with 10% calf serum. All the cells were grown at 37°C in a humidified atmosphere of 5% CO₂.

**DNA transfections and indirect immunofluorescence staining**

Procedures for DNA transfections were essentially the same as described previously (Ho et al., 1995). Briefly, 36 hours before transfection, cells were plated to 6 cm culture dishes which contained two sterile coverslips. About 10 μg of DNA were introduced into cells by calcium phosphate-mediated transfection. After transfection, cells were incubated overnight, then treated with DMEM/F12 medium containing 15% dimethyl sulfoxide at room temperature for 2 minutes (SW13 cells) or 30 minutes (MFT16 and NIH3T3 cells). Cells were processed 48 hours after transient transfection. Procedures of indirect immunofluorescence staining were described previously (Ho et al., 1995).

To establish the stable SW13 vim+ cell lines, 36 hours after the cells were plated on a 10 cm culture dish, about 30 μg of EGFP-vimFL and 1 μg pGKneo were introduced into cells by calcium phosphate-mediated transfection. After transfection, cells were incubated overnight, then treated with DMEM/F12 medium containing 15% dimethyl sulfoxide at room temperature for 2 minutes. About 48 hours later, cells were trypsinized and plated on ten 10 cm culture dishes in normal culture medium and incubated overnight. The next day, the medium was changed to selection medium with 500 μg/ml G418 (Gibco BRL, Gaithersburg, MD). About 10 days later, colonies were trypsinized and plated on ten 10 cm culture dishes. After the cells reached subconfluency, they were trypsinized and subjected to FACS (fluorescence activated cell sorting, described below) to sort cells expressing higher levels of the GFP. The sorted cells were kept in medium containing 300 μg/ml G418.

For establishing stable NIH3T3 lines, cells were plated to a 10 cm culture dish 36 hours before transfection. 30 μg of pLEN-env and 1 μg pCEN-env were introduced into cells by calcium phosphate-mediated transfection. The cells were incubated overnight and treated with DMEM/F12 medium containing 15% DMSO at room temperature for 30 seconds. About 48 hours later, cells were trypsinized and plated to twenty 10 cm culture dishes in normal culture medium and incubated overnight. The medium was changed to selection medium containing 1,000 μg/ml G418. 14 days later, individual colonies were picked and kept in medium containing 300 μg/ml G418. Positive clones were determined by observing the green fluorescence.

To determine the relative amounts of GFP-vimentin and vimentin in the stable NIH3T3 cell lines, the cells were washed with phosphate-buffered saline and then lysed with 10 mM Tris-HCl (pH 7.5) containing 1% SDS. The lysates were boiled for 10 minutes, centrifuged at 13,000 g for 5 minutes, separated on SDS-PAGE and electrotransferred to a nitrocellulose filter. Following incubation with
Fluorescence activated cell sorting (FACS)
Cells for FACS were trypsinized, washed in def-PBS two times and filtered through a nylon mesh. The final concentration was about one million cells in 1 ml def-PBS. FACS was performed using a FACSTAR Plus cytometer (Becton Dickinson, Franklin Lakes, NJ) equipped with an Innova argon ion laser (Coherent Inc., Santa Clara, CA). The excitation wavelength was tuned to 488 nm. A 530±30 nm bandpass filter was used for detection of the green fluorescence. The flow rate was about 3,000 cells/second with a droplet frequency of 30,000 droplets per second.

Time-lapse recordings
Cells for recording were plated out on 35 mm dishes containing 25 mm round coverslips at a density so that they became confluent two days later. The cells were cultured in normal medium as described above for one day. The next day the medium was changed to DMEM medium supplemented with 10% calf serum, 300 nM tocopherol and 4.8 mM N-acetyl-cysteine (from Sigma, St Louis, MO) to reduce photodamage. On the third day, the confluent monolayer was wounded (Gurland and Gundersen, 1995) and incubated in medium for at least 3 hours. The cells at the edge of the wound polarize and begin migrating into the wound. This provides a uniform population of cells with IFs clearly detectable in the front of the cell (Gurland and Gundersen, 1995). The coverslips were assembled in a Sykes-Moore Chambers (Bellco Glass Inc., Vineland, NJ) filled with GFP recording medium: Hanks' balanced salt solution (without Phenol Red) supplemented with 1x MEM essential amino acid solution, 1x MEM non-essential amino acids solution (both from Gibco), penicillin and streptomycin (10,000 units/ml), 5% calf serum, and 4.8 mM N-acetyl-cysteine. Vitamins were left out of the GFP recording medium to reduce autofluorescence of the medium. The recording was performed with a MicroMax cooled CCD camera containing a Kodak KAF1400 chip (Princeton Instruments Inc, Trenton, NJ), an Optiphot-2 fluorescence microscope (Nikon, Tokyo, Japan) equipped with a custom-made warm stage to hold the Sykes-Moore Chamber, an Uniblitz D122 shutter (from Vincent Associates, Rochester, NY) and a Pentium computer with 128 Mbyte RAM running Metamorph v. 2.5 (from Universal Imaging Corp, West Chester, PA). HiQ fluorescein filter cube (Nikon, Tokyo, Japan) was used for GFP fluorescence and a rhodamine G2B filter cube (Nikon) was used to detect the injected marker protein, rhodamine-transferrin. The exposure time for GFP fluorescence was 150 milliseconds for NIH3T3 cells and 250 milliseconds for SW13 cells. An ND2 filter was inserted to reduce the intensity of the fluorescence light source. Images were acquired every one, two or two and a half minutes for NIH3T3 cells and every four minutes for SW13 cells. The total recording times were 1-4 hours. The time exposure for filaments at the edge of a cell was 1,000 milliseconds.

Nocodazole treatment and life history plots
Approximately 20 images were taken of intermediate filaments at the edge of the cell before perfusion to establish their behavior before nocodazole treatment. The chamber was perfused with approximately 5 ml of 20 µM nocodazole over 5 minutes. The image was refocused during the perfusion in order to obtain as many usable images as possible. The life history plots of intermediate filaments were obtained in the following manner. Using Metamorph v.2.5 and the Track Points dropin accessory for Metamorph, the end of each filament was traced over the course of the recording. Track Points determines the pixel location of each point and calculates the distance between them. The rate of movement may also be calculated. The pixels are converted to microns using the calibration function in Metamorph v.2.5. The beginning of each trace was set to 0 and the distance each filament moved was plotted over time using Sigma Plot.

Microinjections
Wounded 3T3 monolayers were prepared as described above. Three or more hours after wounding, cells were pressure microinjected with a mixture of anti-IFA antibodies and rhodamine-conjugated transferrin using back-loaded glass capillaries and a Narishige micromanipulator (Narishige, Greenvale, NY). The coverslips were then assembled in Sykes-Moore Chambers (Bellco Glass Inc., Vineland NJ) filled with GFP recording medium. It took ~35 minutes to assemble the chamber and locate a cell for recording. To minimize the collapse of IFs before recording, the above procedures were done at room temperature. The warm stage was then turned on and recording was begun. Time lapsed recording was performed as described above.

RESULTS
GFP/IF proteins label the vimentin network in cultured cells
Previous studies have shown that amino-terminal deletion mutants of IF proteins can coassemble with the endogenous vimentin network. Therefore, we fused GFP to the amino terminus of IF proteins in the hope that the GFP/IF fusion protein would still coassemble with the endogenous IF network without disrupting it and thereby label the IF network (see Fig. 1 for a diagram of the constructs). We initially used the S65T variant of GFP (Heim et al., 1995), but later switched to EGFP (Cormack et al., 1996), when it became commercially available. EGFP has an F to L mutation at amino acid 64 in addition to the S to T mutation at amino acid 65 and codon usage is optimized for expression in human cells. As a result, EGFP is reported to be considerably brighter than S65T-GFP in mammalian cells.

The amino-terminal fusion proteins EGFP-vimN56 and EGFP-vimFL, as well as S65T-vimN56 showed diffuse and punctate staining patterns in SW13 vim- cells (Fig. 2A, see also summary in Fig. 1), but coassembled with vimentin into filamentous networks in SW13 vim+ cells (Fig. 2B). However, qualitative differences could be observed. For example, EGFP-vimFL labels very fine filaments of the vimentin network (Fig. 2B), whereas EGFP-vimN56 usually labels only thicker filaments and at the same time shows a diffuse staining pattern (see summary in Fig. 1). These results suggest that EGFP-vimFL associates better with the endogenous vimentin network than EGFP-vimN56. In order to determine whether other IF proteins assembled in the same manner as S65T-vimN56, we also fused S65T GFP to GFAP. The constructs are illustrated in Fig. 1 and include S65T fused to amino-terminal truncated (S65T-gfapN30), as well as full-length GFAP protein (S65T-gfapFL). Both S65T-gfapN30 and S65T-gfapFL labeled the vimentin network at least as strongly as the S65T/vimentin fusion proteins (summarized in Fig. 1). None of these N-terminal fusion proteins formed aggregates, except that EGFP-vimFL formed multiple small dots in the transfected SW13 vim- cells (Fig. 2A).

These results show that these GFP/IF constructs label the endogenous IFs in living cells. None of the GFP/IF fusion
proteins self-assembled in SW13 vim- cells. To test whether these proteins can self-assemble into filaments in other cells, we transfected them into the MFT16 fibroblastic cell line, which was derived from vimentin knock-out mice (Holwell et al., 1997). EGFP-vimFL forms multiple small dots in about half of the transfected cells (Fig. 2C), whereas in the other half of the transfected cell population, EGFP-vimFL forms some linear structures resembling short filaments (Fig. 2D). However, no filamentous network was found in the MFT16 cells expressing EGFP-vimFL. In MFT16 cells, S65T-gfapFL also only showed diffuse staining pattern (not shown). Therefore, none of the full-length fusion proteins can self-assemble into a filamentous network.

### Stable cell lines expressing GFP-vim fusion proteins

We prepared stable cell lines in SW13 vim- cells and NIH3T3 cells, expressing the GFP/vim fusion proteins. The vimentin network detected by GFP fluorescence looked essentially the same as that detected by immunofluorescent staining (Fig. 2C). With EGFP-vimFL in SW13 cells, we were able to record up to 50 useful images. After 50 frames, the fusion protein became too weak to reveal finer filaments (data not shown). Interestingly, when EGFP-vimFL was expressed in NIH3T3 cells, we were able to record 120 frames (up to 4 hours) without any significant photobleaching. In these experiments, the filaments in the last images were nearly as bright as in the first image. For some unidentified reason, the GFP fusion protein in NIH3T3 cells is more resistant to photobleaching than in SW13 cells. Under these circumstances, the limiting factor for the duration of the experiment was not the brightness of the fluorescence, but rather the energy and patience of the researcher.

To determine the relative amount of GFP-vimentin fusion protein in the stable NIH3T3 cells, we performed western blots on cell extracts with an anti-vimentin antibody and observed bands representing both the endogenous vimentin and the GFP-vimentin. From scans of the autoradiograms, we determined that the ratio of GFP-vimentin to vimentin is 1:5, indicating that the levels of the GFP/vim fusion protein are relatively low compared to the endogenous vimentin.

The IFs in both the SW13 vim- cells and the NIH-3T3 cells were not static, since many filaments exhibited a wavy movement (Fig. 3, for NIH3T3 cells). Several filaments at the cell edge were seen to extend and move (Fig. 4). The arrows in Fig. 3A-C point to a filament that is changing in shape and orientation. Fig. 3D and E are color overlays of Fig. 3A-C with...
Green fluorescent intermediate filaments each color depicting the progression of movement. When the original 75 planes of the recording are played back as a video clip, many of the filaments showed obvious wavy movements. We did not observe any of the IFs disappear due to disassembly. Similar wavy movements were observed in SW13 cells expressing EGFP-vimFL. However, some heterogeneity of the movements was observed. In stable SW13 vim+ cells and in non-polarized stable NIH3T3 cells, essentially all the filaments were wavy, whereas in polarized stable NIH3T3 cells, the filaments waved less and at certain periods did not move at all. Non-moving filaments are easily distinguished in the colored images (Fig. 3D,E).

**IF dynamics at the edge of the cell**

We examined the leading edges of cells and looked for IFs with observable ends. Fig. 4A-D show examples of the movements of filament ends at the edge of the cell. Fig. 4E-G show color overlay images of two sequential frames of the time lapse recording. The first time point of each series is represented in green, the second time point in red. The yellow represents areas where there was fluorescence in both time points. The arrows in Fig. 4F point to two red filaments, which were absent in the previous frame and are examples of extending filaments. Also visible at the edge of the cell are smaller fragments of vimentin IFs. One such fragment is clearly seen moving along the edge of the cell shown in Fig. 4E (arrowheads). In addition to extensions of IFs at the edge of the cell, lateral movements of individual IFs are also visible (Fig. 4G, double arrow). The observations portrayed in Figs 3 and 4 therefore clearly demonstrate that IFs are dynamic structures.

To determine whether the extension of filaments is dependent on the presence of microtubules, we perfused 20 µM nocodazole while recording IFs and analyzed the recordings by preparing life history plots of the ends of filaments immediately before and after the nocodazole treatments. If
MTs were necessary for IF extension, we expected to see an inhibition of extension within ~5-10 minutes, since we previously found that 5-10 minutes of 20 μM nocodazole treatment was sufficient to depolymerize virtually all of the MTs at the edge of cells (A. Mikhailov and G. G. Gundersen, unpublished). In every case we examined (n=12 filaments, in 4 cells), filament ends did not extend after the nocodazole treatment. Five of the filament ends were extending at the time of nocodazole addition and in four of these cases, the filament ends stopped extending with 5-10 minutes (see Fig. 5A,B). In the remaining case, the filament end began collapsing towards the cell center. The other 7 filament ends were paused (i.e. neither extending or collapsing) at the time of nocodazole treatment and these filaments either began collapsing (5 out of 7) (see Fig. 5C) or remained unchanged (2 of 7) after nocodazole treatment.

The life history plots in Fig. 5 show that IF extension and nocodazole-induced IF collapse occurred linearly for several minutes. This allowed us to calculate a rate of each of these processes. Interestingly, the rates of the two processes were different. Extension occurred at a rate of 0.61±0.33 μm/minute (n=8), whereas IF collapse occurred at a rate of 0.24±0.08 μm/minute (n=9). These different rates are consistent with the idea that the processes involved in extending IFs are distinct from those involved in collapsing IFs.

**IF dynamics after microinjection of anti-IFA antibodies**

Previous studies have shown that injections of anti-IFA antibodies will cause the vimentin network to collapse to a perinuclear location (Gurland and Gundersen, 1995; Klymkowsky, 1981). To look at this effect in real time, we injected anti-IFA antibodies into the stable 3T3 cells and recorded the injected cells for a period of 4 hours. As can be seen in Figs 6 and 7, the filament network collapses to a perinuclear area as intact filaments. Fig. 6A,B and C were taken shortly after microinjection and show respectively the phase contrast of the cells, the coinjected rhodamine-conjugated transferrin (as a marker for injection) and the green fluorescent vimentin network. Fig. 6D-F show the collapse...
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process (images were taken every 2 minutes, only selected panels are shown). Fig. 6G is the phase contrast image of the cell taken at the end of the recording, showing that the edge of the cell did not shrink during the collapse of the vimentin network. Fig. 6H shows the immunostaining of microtubules, demonstrating that the microtubules are not depolymerized by anti-IFA antibodies.

Fig. 7 shows parts of the same recording at higher magnification. We can see that the IFs do not disassemble and reassemble near the nucleus, but rather seemed to be ‘pushed back’ to the perinuclear area. The arrowhead points to an individual collapsing filament, which demonstrates that IFs move to the juxtanuclear area as intact filaments. Fig. 8 is a control study for the antibody injection. As can be observed, in a cell injected with non-specific human IgG, the IF network slowly extended during the recording.

DISCUSSION

The GFP/IF fusion proteins label the vimentin network, but do not self-assemble

The proteins with GFP fused to the N terminus of IF proteins did not self-assemble into filamentous networks, but incorporated into the endogenous vimentin network without
disrupting this network. Furthermore, the IF arrays labeled by the GFP/IF chimeras were similar to the normal anti-vimentin immunofluorescence patterns. These constructs are therefore useful to observe IFs in living cells. We also found that full-length vimentin fused at its N terminus to GFP (EGFP-vimFL) coassembled better with vimentin than EGFP-vimN56, indicating that the first 55 amino acids of vimentin may be important for coassembly of GFP/vimentin with IFs. In transfected SW13 vim− cells, we occasionally saw these GFP fusion proteins in a filamentous pattern. Since SW13 vim− cells are known to have a low percentage of revertants, we were not sure if GFP/vimentin can occasionally self-assemble. To solve this problem, we used the MFT16 cell line (Holwell et al., 1997), which was derived from the vimentin knockout mice and confirmed that GFP/vimentin fusion proteins are not able to self-assemble into filamentous networks.

The N-terminal fusion proteins did not affect the endogenous IF network

There is always a concern when tagging a protein that the endogenous network will be disturbed by the tag. Other studies from our laboratory have shown that when GFP is fused to the carboxyl terminus of IF proteins, the GFP/IF act as dominant negative disrupters, collapsing and aggregating the endogenous

**Fig. 6.** Anti-IFA antibody injection (low magnification). Anti-IFA antibody was injected into stable NIH3T3 transfectants expressing EGFP-vimFL at a wound edge and the cell was recorded as described in the Materials and Methods section. Images were taken at 2 minute intervals. (A) A phase contrast picture of the first image which was taken about 35 minutes after microinjection. (B) The rhodamine channel of the first image showing the coinjected rhodamine-conjugated transferrin which was used as a marker for the injected cell. (C-F) The vimentin network collapses to the perinuclear area. (G) Phase contrast of the last image which demonstrates that the cell itself did not collapse. After recording, the cells were fixed with methanol at −20°C for 10 minutes and stained with monoclonal mouse anti-β-tubulin antibody 3F3. As revealed in H, the microtubule network did not collapse either. Bar, 10 μm.
vimentin network (C.-L. Ho, D. Sun and R. K. H. Liem, unpublished). In contrast, when GFP was fused to the N termini of IF proteins, the staining patterns of the IF networks coincided with the staining of the GFP. The GFP/IF proteins did not change the structure of the endogenous vimentin network. The level of GFP-vimentin in the stable cell lines is a fifth of the endogenous vimentin, which may be sufficiently low that it does not overwhelm or disrupt the endogenous vimentin network. However, for lack of a functional assay of IF function, we can only be certain that the vimentin network labeled by GFP/vimentin looks normal. We need to bear this caveat in mind when interpreting the data from the cells expressing GFP/IF chimeras. However, this new tool has given us the opportunity to look at phenomena that we have not been able to observe before in living cells.

**Novel observations from the green fluorescent IF network**

**Normal movements of IFs in vivo**

In cells that express the GFP/vimentin chimera, we observed the movements of the IFs. These movements appear wavy, consistent with the curved morphology of IFs under the EM. Two previous reports have described the movements of fluorescently labeled IFs in living cells. Mittal et al. (1989)
microinjected rhodamine-conjugated desmin into cells and followed the IF network from prophase to the completion of cytokinesis. They took seven images in a span of about 45 minutes and also monitored the IF network intermittently in interphase cells for 20 to 30 minutes. Most of the interphase filaments remained stationary. We believe that the reason they did not observe the wavy movements of the IF network is due to the limitation of the technique available at that time. In our experience, we have to play back at least 15 consecutive images, acquired in 2 minute intervals to detect the wavy movements convincingly. With the EGFP fusion proteins expressed in NIH3T3 cells and with the sensitivity of a cooled CCD camera, we can take 120 images at intervals of 2 minutes. In another study, Vikstrom et al. (1992) injected cells with rhodamine-labeled vimentin and reported movements of vimentin filaments. However, these movements were not described in detail, since the focus of these studies was on the recovery of fluorescence after photobleaching. Furthermore, the filaments were only followed for one hour. Our results show that over a period of four hours, the vimentin filaments move in a wavy manner without any catastrophic depolymerization. In NIH3T3 cells, at the wound edge, we sometimes observed a subset of IFs that did not undergo the wavy movements. Usually these filaments tend to be rather straight, implying that

Fig. 8. Control (human IgG) injection. Human IgG along with rhodamine-conjugated transferrin were injected into stable NIH3T3 transfectants expressing EGFP-vimFL at a wound edge and the cells were recorded as described in Materials and Methods. The images were taken at 2 minute intervals. (A) Phase contrast picture of the first image which was taken about 35 minutes after microinjection. (B) The rhodamine channel of the first image showing the coinjected rhodamine-conjugated transferrin which was used as a marker for the injected cell. (C-G) The vimentin network actually extends in this cell. (H) Phase contrast of the last image. Bar, 10 μm.
they might be associated with other straight filaments such as microtubules or microfilaments. These non-wavy filaments may represent IFs that are tethered to other components in the cell. The mechanism of the wavy movement of the IFs is not clear. They may depend on actin filaments, or they may be a manifestation of the relative movements of the cell membrane and the nuclear membrane, since vimentin has been shown to be attached to both the nuclear and the cell membranes (Georgatos and Blobel, 1987). A final possibility is that the movements may reflect Brownian movements of the IFs.

Extensions of IFs at the cell edge is dependent on microtubules

Over the course of a recording, we observed the movements of individual ends of IFs at the leading edge of 3T3 cells. Whether these filament ends represent individual IFs or bundles of IFs is an important question that we cannot resolve at this time. However, these filaments behaved as a unit, since we did not observe fraying of the ends, or extension or collapse of just part of the fluorescence associated with the filaments. Thus, these filament ends represent individual IFs or bundles of IFs that extend or collapse in concert. The addition of nocodazole stopped the ongoing extension of the IFs with a lag of 5-10 minutes. During this time, virtually all MTs at the leading edge of fibroblasts are depolymerized (A. Mikhailov and G. G. Gundersen, unpublished), suggesting that the extension of IFs at the cell edge depends on MTs. This MT dependence of the IF extension makes it unlikely that this IF extension represents IF polymer elongation, since this process would be independent of MTs. Also, we observed the movement of IF fragments, which cannot be attributed to polymer elongation, since the fragments stayed about the same size and IFs are not able to treadmill.

One question raised by these results is what molecule(s) may be involved in the movement of IFs on MTs. Plectin has been shown to cross-bridge IFs with microtubules (Svitkina et al., 1993). However, the plectin cross-links between IFs and microtubules do not show a preference for detyrosinated or Glu-tubulin (Svitkina et al., 1996). Recent evidence has shown that microinjection of antibodies specific for detyrosinated tubulin cause the vimentin network to collapse, whereas microinjections of antibodies specific for tyrosinated tubulin do not affect the vimentin network (Gurland and Gundersen, 1995). Thus, it appears to be unlikely that plectin is responsible for the movements of IFs on MTs. Instead, a more likely scenario is that a kinesin-related motor moves IFs from the cell center outwards along MTs. Consistent with this interpretation is the observation that microinjected antibody to kinesin head domain of a nearby molecule, since the assembly of IFs and MTs. It is tantalizing to assign the effects of the anti-IFA antibody to its ability to block the KLEGEE motif or adjacent regions, implying that the binding site for the MT-IF linker protein may reside here. However, it should be noted that an antibody against the tail domain may mask a region in the head domain of a nearby molecule, since the assembly geometry of IFs is quite complex (Parry and Steinert, 1995).

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