Association of vimentin intermediate filaments with the centrosome

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SUMMARY

SW-13 cells that lack cytoplasmic intermediate filaments (IFs) were stably transfected with a human vimentin cDNA expression vector. Isolated subclones displayed two prevalent patterns of vimentin distribution as observed by indirect immuno-localization: (1) cytoplasmic filaments characteristic of a vimentin IF network; and (2) a distinct, juxtanuclear focus with limited filamentous extensions. Comparative analysis of two subclones that uniquely segregated these patterns of vimentin organization indicated that vimentin accumulated as a perinuclear focus in cells that expressed a 4-fold lower level of the protein. The observed variation in cellular organization was not due to detectable differences in vimentin protein modification, as determined by two-dimensional gel analysis. Increasing the amount of vimentin in a low expressing clone by a secondary transfection with human or mouse vimentin cDNA resulted in well-dispersed, cytoplasmic filaments, suggesting that the distinct juxtanuclear organization of vimentin arose due to lower cellular vimentin levels.

Employing anti-γ-tubulin and anti-vimentin antibodies, dual immunofluorescence together with confocal microscopy revealed that the juxtanuclear focus of vimentin was located in the centrosomal region. Electron microscopy showed a spheroidal, filamentous structure with at least some filaments closely associated with the pericentriolar material (PCM). Because vimentin IF organization is at least partially dependent on microtubules, the effects of nocodazole and taxol on perinuclear vimentin foci were examined. Neither drug affected the juxtanuclear localization of foci, although taxol (10 μM, 5 hours) caused a release of pericentriolar γ-tubulin from the nuclear region in 50-60% of the cells. These studies indicate that lower, in vivo, levels of vimentin fail to form extended IFs but rather are organized as a perinuclear aggregate. Moreover, the PCM of the centrosome appears to possess attachment sites for vimentin IFs.

Key words: vimentin, centrosome, intermediate filament

INTRODUCTION

The cytoskeleton of nearly all differentiated eukaryotic cells is a dynamic matrix composed of three major filament classes: microtubules microfilaments and intermediate filaments (IFs). In conjunction with associated proteins, these filaments are considered to establish cellular organization and to perform vital functions in differentiation and development. With regard to organization and function in vivo, the least is known about IF proteins, which share remarkable structural similarities but are highly diverse in their tissue-specific patterns of expression (for reviews, see Klymkowsky et al., 1989; Stewart, 1990; Skalli and Goldman, 1991; Georgatos, 1993). Because most IF subunit proteins are rapidly assembled into insoluble filaments (Blikstad and Lazarides, 1983; Soellner et al., 1985), IFs were assumed to be more static than microfilaments and microtubules. However, recent evidence indicates that IFs are dynamic cellular structures that experience rapid subunit exchange and phosphorylation-mediated assembly and disassembly (for reviews see Skalli and Goldman, 1991; Eriksson et al., 1992; Georgatos, 1993).

Currently, there is no single model for IF assembly in vivo. Previous experiments have demonstrated that IF subunits are incorporated into the cytoskeleton by co-translational and post-translational mechanisms (for review, see Fulton and L'Ecuyer, 1993). Studies involving microinjection of modified IF proteins or introduction of IF genes indicate that a rapid initiation of assembly can occur via two major routes: (1) incorporation into pre-existing, homologous IFs; or (2) random, de novo assembly throughout the cytoplasm in the absence of homotypic IFs. Following microinjection of keratin mRNA into epithelial cells, newly expressed keratin appears to coassemble with endogenous keratin IFs (Franke et al., 1984) while analogous microinjection into nonepithelial cells results in de novo keratin IF assembly at dispersed cytoplasmic sites (Kreis et al., 1983). Microinjected biotinylated keratin protein is also co-localized in a dispersed fashion with the endogenous keratin network of epithelial cells (Miller et al., 1991). In contrast, biotin-labeled vimentin is observed to first associate with endogenous vimentin filaments near the nucleus, with gradual extension towards the more peripheral, cytoplasmic regions (Vikstrom et al., 1989; Miller et al., 1991). Similarly, the induced expression of transfected vimentin cDNA in cell types possessing endogenous vimentin networks results in incorporation of the newly synthesized vimentin into pre-existing filaments that are sometimes localized near the nucleus (Sarria et al., 1990; Ngai et al., 1990; Coleman and Lazarides, 1992). Because the possible modes and sites of IF
assembly might be misinterpreted due to a background of endogenous IFs as well as their regional concentrations within the cell. Sarria et al. (1990) performed transfection studies in human SW-13 cells that lack detectable cytoplasmic IFs. Inductive expression of a vimentin cDNA results in de novo assembly throughout the cytoplasm, irrespective of any pre-existing IFs (Sarria et al., 1990).

The findings that de novo IF assembly occurs throughout the cytoplasm directly contrasts previous microscopic and experimental observations that led to the speculation of vectorial assembly of IFs from specific cellular sites, including the nuclear lamina, nuclear pore complexes and cytoplasmic membrane (Eckert et al., 1982a; Fey et al., 1984; Wang, 1985; Goldman et al., 1985; Katsumata et al., 1987; Georgatos and Blobel, 1987a,b; Albers and Fuchs, 1989; Djabali et al., 1991). Current experimental evidence favors the theory that the ultimate organization of IF cytoarchitecture is governed by the intracellular attachment of IFs to specific IFAPs (intermediate filament-associated proteins; for review, see Foisner and Wiche, 1991). Plectin and related proteins have been proposed to play roles not only in the cross-linking of cytoplasmic IFs but also in IF associations with microtubules and microfilaments, and IF anchoring to the plasma and nuclear membranes (for reviews, see Yang et al., 1990; Foisner and Wiche, 1991). Other IFAPs considered to be involved in IF membrane attachment and organization include the submembranous skeletal proteins ankyrin and spectrin, the epithelial cell surface desmosomal and hemidesmosomal proteins, and the nuclear lamina IF protein, lamin B (for reviews, Green and Jones, 1990; Foisner and Wiche, 1991).

In addition, numerous observations have implicated a perinuclear location near or at the centrosome as a site for IF organization (intermediate filament organizing center, or IFOC; Goldman et al., 1980; Eckert et al., 1982a,b, 1984; Kalnins et al., 1985). Vimentin filaments form juxtanuclear caps in the centrosomal region in response to drugs that depolymerize microtubules, such as colchicine or nocodazole (Goldman et al., 1980; Maro et al., 1984; Traub, 1985). Following microinjection of antibodies against keratin, vimentin, as well as tubulin, a juxtanuclear capping of the respective IFs is observed (Gawlietta et al., 1981; Eckert et al., 1982b; Klymkowsky et al., 1983; Bloše et al., 1984). Using immunological techniques and electron microscopy, several reports have indicated that the purported IFOC for keratin and vimentin IFs is close to, but separate from, the centrosome (Eckert et al., 1984; Maro et al., 1984; Alieva et al., 1992). However, one other report suggested a direct association between IFs and centrioles (Katsumata et al., 1987). It remains ambiguous whether the centrosome, which is known to serve as an organizing center for microtubules (MTOC) (Bornens, 1992; Tucker, 1992; Archer and Solomon, 1994), also influences subcellular IF organization.

In the course of attempting to define better the functional and structural roles of vimentin IFs, we transfected an expression vector encoding human vimentin into the subclone, SW-13/c1.2 vim(−), which is devoid of any known cytoplasmic IFs (Sarria et al., 1990). Here we report the characterization of stable transfomants that display a striking variation in the distribution patterns of vimentin IFs. Unexpectedly, a stable, low level expression of vimentin results in a distinct juxtanuclear organization rather than well-dispersed, cytoplasmonic IFs. We have utilized immunological techniques and electron microscopy to identify a morphological relationship between the centrosome and the perinuclear vimentin focus. Our data suggest that the pericentriolar material (PCM) serves as an attachment site for vimentin IFs.

**MATERIALS AND METHODS**

**Expression vector constructions**

The pGEM3 vector containing a 1.8 kb cDNA insert of human vimentin was obtained from C. Sommers (Sommers et al., 1989). The cDNA insert was excised by digestion with EcoRI and purified. The ends were made flush with Klenow polymerase, and HindIII linkers were added (Sambrook et al., 1989). The human vimentin cDNA was then subcloned into the HindIII site of the expression vector plasmid LK444 (designated LK-HuVi), which placed the cDNA under the control of the human β-actin promoter (Gunning et al., 1987; Trevor, 1990). For expression of mouse vimentin, a 1.8 kb BamHI cDNA insert, encoding the full-length mouse vimentin protein (Sarria et al., 1990), was subcloned into the BamHI site of the LK444 multiple cloning site (Gunning et al., 1987).

**Cell culture and drug treatments**

The subclone SW-13/c1.2 vim(−), which lacks discernable cytoplasmic IFs, was obtained from R. M. Evans (Sarria et al., 1990). Cells were cultivated in Dulbecco’s modified Eagle’s medium supplemented with pyruvate (110 mg/l), glutamine (0.04%), 10% fetal bovine serum and gentamycin sulfate at 30 µg/ml.

For drug treatments, cells were seeded at 6×10⁵ cells in 60-mm dishes and grown for 2 days. Nocodazole (Sigma Chemical Co., St Louis, MO) was kept as a stock solution of 40 mM in DMSO at −20°C and diluted in culture medium just before use. Cells were exposed to a concentration of 10 µM for 18 hours. Taxol (Sigma) was added at a concentration of 10 µM for 5 hours using a stock solution of 10 mM in ethanol.

**DNA transfection**

Stable cell lines expressing human vimentin were obtained by transfecting 10⁶ to 2×10⁶ cells in 10-cm dishes with 20 µg of LK-HuVi DNA by calcium phosphate precipitation (Gorman, 1985). After overnight incubation, the medium was removed and replaced with fresh medium. The next day cells were plated at 1×10⁶ cells in 10-cm dishes and grown in 400 µg/ml G418 for 20 days. Individual colonies were recovered and examined for human vimentin content by indirect immunofluorescence. Stable subclones were subsequently maintained in the presence of 200 µg/ml G418.

For secondary transfection of the HuVi.8 and HuVi.2 clones, 3×10⁵ cells were plated on glass coverslips in 60-mm dishes and transfected with 20 µg of vector DNA by the calcium phosphate precipitation method. Following an overnight incubation, the medium was replaced with fresh medium, and 24 hours later the cells were fixed and immuno-stained for vimentin filament distribution.

**Immunofluorescence and electron microscopy**

For indirect immunofluorescent staining, cells were grown on sterile glass coverslips, rinsed briefly in PBS and then fixed in −20°C methanol for 10 minutes. The coverslips were rinsed in PBS and incubated in PBS containing 1 mg/ml ovalbumin (Sigma) for 5 minutes. Cells were exposed to primary antibody for 30 minutes at 37°C, washed three times in PBS for 5 minutes each and then exposed to secondary antibody for 30 minutes. Following a 10 minute wash in PBS, coverslips were mounted in Immuno Fluore (ICN Biomedicals, Inc., Costa Mesa, CA). For immuno-staining of human vimentin, a mouse monoclonal antibody specific for human vimentin was utilized (V9; Boehringer Mannheim Biochemicals, Indianapolis, IN). Mouse
vimentin was detected, using a rabbit polyclonal anti-vimentin that recognizes only rodent vimentin, by immuno-staining (Sartia et al., 1990); this antibody was tested by immunofluorescent staining on both mouse and human cells and it was determined that it recognizes the vimentin component of mouse but not human cells. The following primary antibodies were used: mouse monoclonal anti-vimentin specific for human vimentin (V9; Boehringer Mannheim Biochemicals, Indianapolis, IN), rabbit polyclonal anti-vimentin specific for mouse vimentin (Sartia et al., 1990), mouse monoclonal anti-β-tubulin (Sigma), and rabbit polyclonal anti-γ-tubulin (Steams et al., 1991). For detection of microtubules, a mouse monoclonal anti-β-tubulin antibody was utilized (Sigma). Anti-γ-tubulin was recognized by a rabbit polyclonal antibody (Steams et al., 1991). Primary antibodies were visualized using the appropriate fluorescently labeled secondary antibodies: FITC-conjugated goat anti-rabbit to mouse IgG, Texas Red-conjugated goat antibody to mouse IgG, and rhodamine-conjugated goat antibody to rabbit IgG (Sigma). Confocal immunofluorescence microscopy was performed on a Zeiss LSM 310 confocal microscope.

The procedure for electron microscopic visualization was essentially as described by Rodionov et al. (1985). For immunogold labeling, cells were grown on sterile coverslips, rinsed with PBS and permeabilized for 30 seconds in Buffer M (50 mM imidazole (Sigma), pH 6.8, 50 mM KCl, 0.5 mM MgCl₂, 0.1 mM EDTA, 1 mM EGTA; 1 mM beta-mercaptoethanol) containing 0.1% Triton X-100 and 4% polyethylene glycol (40,000 mw). Following a brief rinse with the same buffer, the cells were fixed for 15 minutes in 1% glutaraldehyde, washed in PBS and incubated in PBS containing 0.1 M sodium borohydride for 5 minutes. The cells were then incubated for 1 hour in PBS followed by a 2 hour incubation in PBS with 1 mg/ml L-lysine. The cells were exposed to monoclonal mouse anti-human vimentin antisera (V9; Boehringer Mannheim Biochemicals, Indianapolis, IN) for 1 hour at room temperature, washed three times for 10 minutes each with PBS and incubated for 1 hour with a goat anti-mouse IgG conjugated with 5 nm colloidal gold (Amersham, Inc., UK). After washing three times for 20 minutes each with PBS, the cells were post-fixed for 30 minutes in 1 M cacodylate buffer (pH 7.2) containing 1% tannic acid and 3% glutaraldehyde following by exposure to 1% OsO₄ for 1 hour at 4°C. (Cells were fixed for 12 hours before post-osmication in 1% OsO₄.) The fixed cells were embedded in Epon-Araldite after ethanol-propylene oxide dehydration. Ultrathin sections were stained with aqueous uranyl acetate and lead citrate and examined using a Philips 201 Transmission Electron Microscope. For electron microscopy without immunogold labeling, cells were extracted for 10 seconds in Buffer M containing 0.1% Triton X-100 and 4% polyethylene glycol solution. The cells were subsequently processed, omitting exposure to primary and secondary antibodies.

**Protein analysis**

Total protein lysates were prepared according to the method of Oshima (1982). Briefly, cell monolayers were rinsed three times with cold PBS and lysed (dissolved) in 0.1% SDS, 10 mM Tris-HCl, pH 7.2, 3 mM MgCl₂, 0.1 mM CaCl₂, 0.5 mM phenylmethylsulfonyl fluoride, 1 mM N-ethylmaleimide and 0.2 unit/ml Aprotinin (Sigma). DNase I (Gibco/BRL, Gaithersburg, MD) was added to 10 µg/ml, and after approximately 1 minute, SDS was added to 0.5%. The lysate was transferred to 0.1 volume of 0.1 M EDTA, pH 7.2, and heated at 100°C for 2 minutes. After cooling on ice, a 0.1 volume of 10% Nonidet P-40 was added. Triton-insoluble cytoskeletons were prepared by the method of Pasdar and Nelson (1988) as previously described (Trevor and Steben, 1992). Protein concentrations were determined by the BCA method (Pierce, Rockford, IL) and analyzed by one- (Laemmli, 1970) or two-dimensional gel electrophoresis (O’Farrell, 1975).

For immunoblotting, proteins were separated on SDS-polycrylamide (PAGE) gels and transferred electrophoretically to nitrocellulose filters. The filters were processed for protein analysis according to Amersham, Inc. (UK), using the Amersham ECL Western blotting detection system. Human vimentin was recognized using a mouse monoclonal antibody specific for human vimentin (V9; Boehringer Mannheim Biochemicals, Indianapolis, IN). Actin was detected using a mouse monoclonal antibody against β-actin (ICN Immunobiologicals, Costa Mesa, CA). Primary antibodies were visualized using horseradish peroxidase-conjugated goat anti-mouse IgG secondary antibody (Cappel Research Products, Durham, NC) followed by chemiluminescence fluorography. For dual detection of both human vimentin and actin, filters were first exposed to anti-vimentin antibody, processed, and then exposed to the anti-β-actin antibody for the detection of actin protein. The relative amounts of protein were determined by optical scanning of fluorographs using the Ambis Image Acquisition and Analysis System (Ambis Instruments, San Diego, CA).

**RESULTS**

**Differential distribution of human vimentin in stable SW-13 clones**

The human SW-13 subclone utilized in this study, designated SW-13/c1.2 vim(−), has been reported to lack detectable cytoplasmic IFs (Sartia et al., 1990). Preliminary screening by immuno-staining for vimentin indicated that less than 0.5% of the cells possessed a vimentin network. In addition, the cells were negative by immunofluorescence staining for the simple epithelial keratins K8 and K18 (data not shown). Following transfection with the LK-HuVi vector and selection in the antibiotic G418, eight subclones were isolated. Each was examined for human vimentin expression by indirect immunofluorescence staining using a mouse monoclonal antibody specific for human vimentin. Three of the subclones displayed a cytoplasmic, filamentous vimentin pattern in less than 50% of the cells. In three other clonal populations, approximately 40-50% of the cells expressed vimentin protein and showed a more heterogeneous, mixed phenotype with respect to vimentin distribution. Two distinct patterns were obvious. A portion of the cells in each population possessed a filamentous vimentin staining pattern while other cells displayed an unusual juxtanuclear concentration of vimentin protein. The remaining two subclones, designated HuVi.2 and HuVi.8, appeared to segregate these two predominant patterns of vimentin distribution. Fig. 1 shows that the majority HuVi.2 cells possessed dispersed vimentin filaments, typical of a vimentin IF network (Fig. 1A). In contrast, HuVi.8 cells showed distinct perinuclear foci of vimentin with only limited filamentous extensions (Fig. 1B). Approximately 80% of the cells in each clone were positive for vimentin expression and these respective patterns of vimentin distribution.

As at least some of the cells of the untransfected SW-13 parental population (less than 0.5%) displayed vimentin filaments prior to transfection, the possibility existed that the vimentin observed in the isolated clones was derived from the endogenous human gene rather than the LK-HuVi vector. However, in numerous other transfection experiments utilizing this particular SW-13 cell line and the LK444 vector without or including other cDNA inserts, we have never obtained G418-resistant clones that showed endogenous vimentin filaments in greater than 10% of the cells; in total approximately 300 individual clones have been analyzed. In addition, to determine whether the HuVi.8 and HuVi.2 clones expressed...
vimentin RNA encoded by the LK-HuVi vector, the reverse transcription-polymerase chain reaction (RT-PCR) method was utilized (GeneAmp RNA PCR, Perkin Elmer Cetus, Norwalk, CT). This particular vector would express a vimentin mRNA with a small 5′ non-translated sequence (26 bp) derived from the polylinker junction of the vector that would not be present in a vimentin mRNA encoded by the endogenous gene (Gunning et al., 1987). Use of an upstream oligo primer specific for this region and one downstream that recognized vimentin coding sequences resulted in an RT-PCR-generated amplifier that was specific for a vector-encoded vimentin mRNA (data not shown).

**HuVi.8 cells express lower levels of vimentin**

The subclones, HuVi.2 and HuVi.8, provided an opportunity to investigate the unusual, perinuclear accumulation of vimentin observed in at least a portion of the SW-13 cells that stably expressed the LK-HuVi construct. Immunoblotting was performed to examine the amounts of vimentin present in each subclone. Fig. 2 shows that HuVi.8 cells possessed lower levels of the protein relative to HuVi.2 cells. Densitometric scanning and normalization to cellular actin levels indicated that the HuVi.8 cells possessed approximately 4-fold less human vimentin.

The vimentin protein expressed in each subclone was further analyzed by two-dimensional gel electrophoresis in order to determine whether the overall charge content of vimentin expressed by HuVi.8 cells varied from that present in the HuVi.2 subclone. Previous studies have indicated that phosphorylation of vimentin, which increases its acidity, can be a factor in the regulation of vimentin polymerization and subcellular organization (for reviews, see Skalli and Goldman, 1991; Eriksson et al., 1992). Triton X-100-insoluble cytoskeletal proteins prepared from each clone were separated in parallel by two-dimensional gel electrophoresis followed by immunoblotting. As shown in Fig. 3, the vimentin protein expressed in HuVi.8 cells migrated identically to that of HuVi.2 cells relative to actin as well as to the origin of migration. Moreover, when the cytoskeletal extracts were

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**Fig. 1.** Differential distribution of vimentin in stably transfected IF-negative SW-13 subclones. Cells were methanol-fixed and preincubated with a monoclonal antibody against vimentin followed by rhodamine-labeled goat anti-mouse IgG. Hu Vi.2 cells (A); HuVi.8 cells (B). Bar, 10 μm.

**Fig. 2.** Expression levels of vimentin (Vi) in HuVi.2 and HuVi.8 subclones. Cell lysates (50 μg) were separated by SDS-PAGE (12.5% gel), transferred to nitrocellulose and immunoblotted using a mouse monoclonal anti-vimentin antibody. Protein bands were developed using a horseradish peroxidase-labeled anti-mouse antisera followed by chemiluminescence fluorography. For comparison of amounts of protein in the different samples, the same immunoblot was secondarily exposed to a mouse monoclonal antibody against actin (Ac) and similarly developed. Untransfected SW-13 cells (lane 1); HuVi.2 cells (lane 2); HuVi.8 cells (lane 3).

**Fig. 3.** Two-dimensional gel analysis of vimentin protein expressed in HuVi.2 (A) and HuVi.8 (B) cells. Triton X-100-insoluble protein samples (10 μg) were separated in parallel by two-dimensional gel electrophoresis. Human vimentin (V) and actin (A) proteins were detected by chemiluminescence immunoblotting as described in Materials and Methods. Isoelectric focusing (IEF) pH ranged from approximately pH 4 (+) to pH 7 (−). Bars indicate molecular mass standards of the second dimension SDS-PAGE gel (12.5% acrylamide): upper bar, bovine serum albumin (77×10^3 kDa); lower bar, ovalbumin (46.5×10^3 kDa).
mixed and similarly analyzed only one major vimentin species was observed (data not shown).

Secondary transfection of HuVi.8 cells can result in dispersed, cytoplasmic vimentin filaments
Protein analyses indicated that the unique, juxtanuclear accumulation of vimentin observed in HuVi.8 cells was most likely due to a lower level of expression and not the result of vimentin protein modification, such as phosphorylation. An alternative possibility is that this novel aggregation is caused by an unknown mechanism operative in at least some cells of the transfected SW-13 parental population and this phenotype was most obvious in the HuVi.8 subclone. To verify further that the human vimentin collected at a perinuclear site because of

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**Fig. 4.** Cellular distribution of vimentin in HuVi.8 cells following secondary transfection with vimentin expression vectors. Cells were fixed in methanol and stained by indirect immunofluorescence 48 hours after the addition of vector DNAs. (A) HuVi.8 cells following secondary transfection with the LK-HuVi expression vector; (B) double immunofluorescence staining patterns after transfection with the vector encoding mouse vimentin (B and C, D and E). Cells were co-stained with a mouse monoclonal antibody against human vimentin (B and D) and a rabbit polyclonal specific for mouse vimentin (C and E). Primary antibodies were recognized using the appropriate FITC-conjugated (B, D) and rhodamine-labeled (C, E) secondary antibodies. Bars, 10 µm.
reduced amounts of vimentin protein, the cells were transfected a second time with the LK-HuVi vector so as to increase the intracellular concentration of vimentin protein. Immunostaining of HuVi.8 cells 24 hours following the transfection period revealed that approximately 20% of the cells displayed human vimentin filaments that extended throughout the cytoplasm (Fig. 4A).

To examine the fate of the human vimentin in the HuVi.8 cells following the secondary transfection, an LK444 vector encoding mouse vimentin was utilized. The two vimentin antigens were differentiated using antibodies specific for mouse and human vimentin. Following a secondary transfection of HuVi.8 cells, approximately 20% of the cells were positive for mouse vimentin expression by immuno-staining. Of these cells, about 15% exhibited a dispersion of the human vimentin focus into a filamentous form that distributed with mouse vimentin IFs (Fig. 4B,C). The extension of the human filaments was not as obvious as the mouse vimentin most likely because of the relatively low levels of human vimentin present in the cells. In addition, about 5% of the HuVi.8 cells that were positive for mouse vimentin expression showed a co-localization of the mouse vimentin with the original, perinuclear aggregate of the human protein (Fig. 4D,E). Of the cells expressing both mouse and human vimentin, none were observed to retain a focus of human vimentin in the presence of well-dispersed mouse filaments. These results indicate that increased vimentin expression induces a cytoplasmic redistribution of the originally aggregated human vimentin protein.

Fig. 5. Localization of vimentin foci in HuVi.8 cells near the centrosome. HuVi.8 cells (A and B); HuVi.2 cells (C and D). Cells were fixed in methanol and co-stained with a mouse monoclonal against human vimentin (A and C) and a rabbit polyclonal antibody recognizing γ-tubulin (B and D). Primary antibodies were visualized using appropriate secondary antibodies: FITC-labeled goat antiserum recognizing mouse IgG (A and C); rhodamine-conjugated goat antibody to rabbit IgG (B and D). Arrows denote γ-tubulin localization; arrowheads indicate the intense FITC-labeling of the vimentin foci detected by rhodamine optics. Bars, 10 µm.

To examine the fate of the human vimentin in the HuVi.8 cells following the secondary transfection, a LK-HuVi vector encoding mouse vimentin was utilized. The two vimentin antigens were differentiated using antibodies specific for mouse and human vimentin. Following a secondary transfection of HuVi.8 cells, approximately 20% of the cells were positive for mouse vimentin expression by immuno-staining. Of these cells, about 15% exhibited a dispersion of the human vimentin focus into a filamentous form that distributed with mouse vimentin IFs (Fig. 4B,C). The extension of the human filaments was not as obvious as the mouse vimentin most likely because of the relatively low levels of human vimentin present in the cells. In addition, about 5% of the HuVi.8 cells that were positive for mouse vimentin expression showed a co-localization of the mouse vimentin with the original, perinuclear aggregate of the human protein (Fig. 4D,E). Of the cells expressing both mouse and human vimentin, none were observed to retain a focus of human vimentin in the presence of well-dispersed mouse filaments. These results indicate that increased vimentin expression induces a cytoplasmic redistribution of the originally aggregated human vimentin protein.

Secondary transfection of the HuVi.2 cells, which possess filamentous human vimentin, resulted in a co-localization of the mouse protein with the human filament network (data not shown), similar to previous vimentin gene transfection studies (Sarria et al., 1990; Ngai et al., 1990).

Perinuclear vimentin IFs aggregate near the centrosome and infiltrate the PCM

To examine whether the occurrence of juxtanuclear vimentin foci in HuVi.8 cells reflected a random accumulation near the nuclear surface or potentially a more specific perinuclear localization, dual immunofluorescence was performed with antibodies against vimentin and γ-tubulin, a component of the centrosomal PCM (Stearns et al., 1991). Visualization of the centrosome and vimentin focus revealed that the two structures resided in the same juxtanuclear vicinity in greater than 70% of the cells (Fig. 5A,B). A slight escape of the intense FITC-indirect labeling of the vimentin aggregate into the rhodamine detection channel, utilized for indirect secondary antibody detection of γ-tubulin, allowed determination that the most concentrated region of the vimentin aggregate bordered the centrosomal region (arrowhead and arrows, respectively, Fig. 5B). Often, the focus appeared to be localized towards one side of the centrosome. In HuVi.2 cells, the centrosomes appeared perinuclear while the vimentin filaments formed a filamentous cage in the nuclear vicinity and extended throughout the cytoplasm (Fig. 5C,D).

Focal sections obtained by confocal, dual-immunofluores-
Vimentin associates with the centrosome

Confocal dual immunofluorescence microscopy revealed that the most intense staining for each antigen was often observed in the same focal plane (Fig. 6). Employing an interval distance of 0.5 µm to focus on separate planes beginning at the cell surface and moving toward the substratum, anti-γ-tubulin staining was detected over a distance of less than 0.5 µm, within the approximate determined size of the centrosome (Bornens, 1992). Vimentin staining was observed over a distance of 1.5-5 µm, depending on the size of the individual aggregate. Three-dimensional reconstruction of sequential sections revealed that the vimentin aggregate was a rounded, sphere-like structure (data not shown).

Immunogold labeling followed by electron microscopy identified the vimentin aggregate in HuVi.8 cells as a near-spheroidal structure composed of filaments that appeared indistinguishable from typical IFs (Fig. 7A). The aggregated vimentin structure neighbored the centrosomal region, analogous to the position observed by immunofluorescence. In this ultrathin section, only a single centriole was seen. The dense bundle of microtubules observed in the vicinity most likely represents the MTOC (Fig. 7A, arrowheads). Few, if any, vimentin IFs were observed in the MTOC region; moreover, filaments within the focus showed no obvious co-alignment with microtubules. A limited number of immunogold particles appeared to label fragments of cellular material near the centriole (Fig. 7A, inset, arrows), although the halo-like PCM that surrounds the centrioles appeared disrupted. In an attempt to retain the PCM and still remove the amorphous cellular substance that would obscure visualization, cells were only briefly extracted (10 seconds) prior to processing.

Electron microscopy of an ultrathin section of fixed cells prepared by this method is shown in Fig. 7B. The focus (coil) of vimentin IFs was closely situated near the centrosome, in which portions of the two centrioles were obvious, as was the cloud-like halo of the PCM. At higher resolution some of the filaments appeared to directly infiltrate the PCM (Fig. 7B, inset). Again, few microtubules were observed in the vicinity of the vimentin IF focus. In addition, mitochondria and ribosomal material were preserved by this procedure; small, electron-dense, ribosome-like particles appeared throughout, including within the PCM.

Disturbance of microtubule organization does not affect the localization of vimentin foci

Numerous reports have demonstrated a dependency of vimentin organization on microtubules by utilizing drugs known to disturb normal microtubule polymerization (for reviews, Traub, 1985; Klymkowsky et al., 1989; Skalli and Goldman, 1991). In order to examine whether microtubule perturbation might affect the centrosomal localization of the vimentin focus, HuVi.8 cells were exposed to the two different drugs that affect microtubule assembly, nocodazole and taxol.

Fig. 8 (A-D) shows dual immunofluorescence of vimentin and γ-tubulin in both HuVi.8 and HuVi.2 cells after exposure to nocodazole, which induces a disassembly of microtubules. Following an 18 hour treatment of HuVi.8 cells with 10 µM nocodazole, the co-localization of the vimentin focus and the centrosome remained (Fig. 8A,B). Anti-β-tubulin staining demonstrated an absence of microtubules following this lengthy nocodazole treatment (data not shown). HuVi.2 cells...
showed a perinuclear collapse of vimentin IFs toward the centrosomal region (Fig. 8C,D), a characteristic response of the cytoplasmic vimentin IF network following exposure to drugs that disassemble microtubules (Goldman et al., 1980; Maro et al., 1984).

The cells were similarly treated with taxol, which not only
Fig. 8. Effects of nocodazole and taxol on vimentin in HuVi.8 and HuVi.2 cells. Cells were treated with 10 µM nocodazole for 18 hours (A-D) or 10 µM taxol for 5 hours (E-H) prior to methanol fixation and double indirect immuno-staining. Nocodazole-treated HuVi.8 cells (A,B); Huvi.2 cells (C and D). Taxol-treated HuVi.8 cells (E and F); HuVi.2 cells (G and H). Vimentin was detected using a mouse monoclonal antibody recognizing human vimentin followed by FITC-labeled goat antiserum to mouse IgG (A,C,E,G); γ-tubulin was visualized using a rabbit polyclonal antiserum followed by a rhodamine-conjugated goat antibody to rabbit IgG (B,D,F,H). Arrows indicate γ-tubulin localization; arrowheads denote intense FITC-labeled vimentin immuno-staining detected by rhodamine optics. Bar, 10 µm.
induces microtubule polymerization and stabilization but also inactivates the centrosome as an MTOC (De Brabander et al., 1981; Gosti-Testu et al., 1986; Bornens, 1992). In the presence of taxol, the PCM can dissociate from the centrioles and be relocated within the stabilized microtubule arrays (Bornens, 1992). Following exposure of HuVi.8 cells to 10 µM taxol for 5 hours, approximately 50% of the cells showed an obvious dissociation of γ-tubulin (PCM material) from the vimentin focus (Fig. 8E,F). In at least some cells both proteins appeared to remain in the perinuclear region, although at different locations. In other cells, the PCM (as indicated by γ-tubulin staining) appeared in the cytoplasm at some distance from the nucleus, while the vimentin focus appeared to persist near the nucleus. Similarly, taxol-treated HuVi.2 cells showed a relocation of the γ-tubulin staining away from its perinuclear site (Fig. 8G,H).

**Vimentin filaments are observed near the centrosome early after transfection**

De novo vimentin filament assembly has been shown to occur throughout the cytoplasm of IF-negative SW-13 cells induced to express vimentin protein (Sarria et al., 1990). To examine the subsequent organization of polymerizing filaments relative to the centrosome, parental SW-13/c1.2 vim(−) cells were transfected with the LK444-HuVi construct and immuno-stained 24 hours later using antibodies recognizing vimentin and γ-tubulin. Cells with relatively sparse vimentin filaments showed shortened filamentous structures throughout the cell (Fig. 9A). However, careful microscopic examination indicated a distinct focus of vimentin that coincided with the centrosomal region (arrowheads, Fig. 9A and B). In cells that appeared to possess a greater amount of filaments, a knot-like cluster of vimentin staining appeared in the region of the centrosome (arrowheads, Fig. 9C and D).

**DISCUSSION**

In this report we have focused our investigation on one of several subcellular sites that have been purported to serve in the organization of cytoplasmic IFs, the centrosomal region (Goldman et al., 1980; Eckert et al., 1984; Maro et al., 1984; Kalnins et al., 1985; Katsuma et al., 1987; Alieva et al., 1992). Our results indicate that in interphase cells vimentin IFs are not only organized near the centrosome but are also attached
to this organelle, most likely via associations with the peri-centriolar material (PCM) of the centrosome. The original notion of an IF organizing center (IFOC) in close proximity to the centrosome arose from experimental evidence showing that treatment of cells with anti-tubulin drugs or microinjection of antibodies to tubulin, keratin or vimentin can cause a perinuclear collapse of IFs toward this organelle (Goldman et al., 1980; Gawlitza et al., 1981; Eckert et al., 1982b; Klymkowsky et al., 1983; Blose et al., 1984). The centrosome was suggested to serve as a common organizer for IFs, as well as microtubules (MTOC) (Goldman et al., 1980). More recent studies of the MTOC have demonstrated the nucleation of microtubules by the PCM and the essential roles of such PCM proteins as γ-tubulin and pericentrin in the nucleation phenomenon (Bornens, 1992; Tucker, 1992; Archer and Solomon, 1994; and references therein).

In the search for a potential IFOC, both dual immunofluorescence and electron microscopy of interphase cells have indicated the presence of an IF aggregate adjacent to the centrosome from which IFs appear to radiate. However, no direct attachments to the centrosome were observed, and these ‘focal centers’ of IFs were concluded to exist as entities that are separate and distinct from the centrosome (Eckert et al., 1984; Maro et al., 1984; Alieva et al., 1992). In contrast, the electron microscopic visualization of extracted cell cytoskeletons by Katsuma et al. (1987) showed that at least some IFs might be anchored to the centrioles, although a detailed examination of the proposed IF-centriole association was obscured by the extraction procedure.

Our investigations were initiated following the observation that IF-negative SW-13 cells that stably expressed a transfected vimentin cDNA displayed varying intracellular distributions of vimentin filaments. The focal, juxtanuclear accumulation of vimentin in at least some cells appeared to target a distinct subcellular site for IF organization. In one isolated subclone, HuVi.8, the majority of cells possessed this phenotype of vimentin distribution, permitting biochemical and microscopic analyses. Several previous studies have identified unusual perinuclear accumulations of IFs in cultured cells types as well as in cells following experimental transfection of IF cDNAs (Fey et al., 1983; Celis et al., 1984; Blouin et al., 1990; Magin et al., 1990; Kartasova et al., 1993). However, these investigations did not determine the exact causes of such abnormal IF organization or the subcellular sites of localization.

The unusual, focal accumulation of vimentin observed in the HuVi.8 cells was most likely attributable to lower intracellular levels of the protein. When compared with HuVi.2 cells, which displayed characteristic cytoplasmic vimentin IFs, biochemical protein analyses revealed that the HuVi.8 cells possessed a 4-fold lower level of vimentin protein that had not undergone any obvious post-translational modification. A secondary transfection of HuVi.8 cells with the original human vimentin expression vector resulted in cytoplasmic vimentin IFs, indicating that the focal accumulation of vimentin was not simply a cellular characteristic of this particular subclone. Following transient transfection of the mouse vimentin vector, the most prevalent pattern was a dispersion of human vimentin IFs in association with the mouse filaments. This finding demonstrates that the human vimentin component of HuVi.8 cells is capable of forming characteristic, extended filaments in the presence of increased vimentin.

Additionally, a minor population of the HuVi.8 cells that expressed mouse vimentin showed a co-localization of the mouse protein with the human vimentin focus rather than extended filaments. One explanation for the two observed distributions of the mouse/human vimentin components is that the newly expressed mouse vimentin first co-assembles with human vimentin IFs composing the distinct perinuclear accumulation. Assembly would first occur at the perinuclear site where the highest amounts of intracellular vimentin pre-exist. Copolymerized, extended filaments would result in cells expressing higher levels of the mouse protein. This interpretation is consistent with the findings of Sarria et al. (1990). In IF-negative SW-13 cells, cytoplasmic sites of de novo vimentin assembly are seen; in vimentin-positive cells a diffuse perinuclear localization of filaments is initially observed that coincides with the most intense concentration of pre-existing vimentin IFs (Sarria et al., 1990). Similarly, microinjected biotinylated vimentin first shows a diffuse accumulation in the perinuclear region of vimentin-positive cells followed by cytoplasmic extension (Vikstrom et al., 1989; Miller et al., 1991).

Related to these findings is the observation that the IF protein GFAP initiates assembly in the centrosomal region of differentiating astrocytes (Kalnins et al., 1985). As astrocyte precursors possess vimentin IFs, the GFAP may well co-assemble with vimentin filaments that are concentrated at this subcellular site.

In HuVi.8 cells, dual immunofluorescent staining employing anti-vimentin and anti-γ-tubulin antibodies demonstrated a close proximity of the vimentin focus to the centrosome. Electron microscopy extended this finding and further revealed that the vimentin focus was composed of normal-appearing IFs with at least a portion of the filaments in association with the PCM. Most likely, previous attempts by others to identify an IF-centrosome interaction failed because the PCM-associated IFs were obscured by the entangled, dense accumulations of IFs that normally occur in interphase cells.

With respect to cellular IF organization, numerous lines of evidence indicate that the cytoplasmic distribution of IFs depends on microtubules and that microtubules and IFs are physically interconnected. A partial co-alignment of the two cytoskeletal systems is thought to be mediated by IFAPs such as plectin-like proteins and a recently identified 210 kDa protein (Skalli and Goldman, 1991; Foisner and Wiche, 1991; Draberova and Draber, 1993). Two expectations are: that IFs would not only co-align with microtubules as the microtubules emanate from the MTOC but also form attachments at the PCM via microtubule-IFAP interactions. However, electron microscopy of HuVi.8 cells did not reveal any obvious connections between vimentin filaments and microtubules in the region of the PCM or directly at the PCM. The vimentin IFs appeared to associate with the PCM without the involvement of microtubules. Our morphological data suggest that sites of IF attachment to the PCM would comprise a different subdomain of the centrosome than that of the MTOC.

The aggregated conformation of vimentin IFs in HuVi.8 cells may reflect an inability of the relatively low concentrations of vimentin IFs to form the numerous, appropriate contacts with microtubule-IFAPs required for cytoplasmic extension. In cells that possess typical cytoplasmic IFs, such as the HuVi.2 subclone, IF-PCM associations would also occur. Filament dispersion results because the vimentin IFs are
abundant and long enough to develop associations with cytoplasmic microtubules as well as those that emanate from the MTOC. High-resolution electron microscopy of serial sections of the centrosome in HuVi.2 cells may prove useful for identifying vimentin IF-PCM associations in cells with normal-appearing IF networks.

As in a previous study of the association of vimentin IFs and microtubules at the centrosome (Maro et al., 1994), we attempted to alter experimentally the IF-PCM association utilizing drugs known to disturb the normal, polymerized state of microtubules. As is well documented for cytoplasmic vimentin IFs, nocodazole treatment of the HuVi.2 cells caused a perinuclear collapse of vimentin IFs towards the centrosomal region. Long-term nocodazole treatment of HuVi.8 cells did not dissociate the vimentin focus from the centrosomal region, as might be expected if MTOC microtubules were required to maintain an IF-PCM interaction.

Following exposure of HuVi.8 cells to taxanol, an obvious separation of the vimentin aggregate and centrosomal material occurred. In taxol-treated cells, the stabilized network of microtubules is no longer anchored to the centrosome, and the PCM is known to be relocated along the microtubule arrays with a loss of centrosome integrity (De Brabander et al., 1981; Gosti-Testu et al., 1986; Bornens, 1992). Our result showing that the vimentin focus remains at a perinuclear site while the PCM, as determined by γ-tubulin immunofluorescence, can separate from its juxtanuclear location might first suggest that proper microtubule polymerization is required for the association of vimentin IFs with the centrosome. However, as the centrosome itself is disrupted by taxol, essential protein remnants of the centrosome may continue to reside with the juxtanuclear aggregate of vimentin and serve to maintain its subcellular location. Alternatively, the vimentin focus may remain in the vicinity of the nucleus due to additional intracellular interactions, including attachment to the nuclear envelope via such proteins as the nuclear lamin IF protein, lamin B (Georgatos and Blobel, 1987a; Djabali et al., 1991). More detailed electron microscopy is required to examine the potential dual associations of the vimentin focus in HuVi.8 cells with the nuclear envelope and the centrosome in interphase cells.

Preliminary analysis of the fate of the vimentin aggregate during division of HuVi.8 cells indicates that the focus remains associated with at least one of the migrating centrioles (data not shown). Changes in the assembly properties of IFs are known to occur during mitosis in relation to phosphorylation-mediated events (Skalli and Goldman, 1991; Erikkson et al., 1992). However, reports on the morphological organization of vimentin during mitosis are perplexing. In some cases a cage of vimentin IFs is maintained around the mitotic apparatus while in others disassembled aggregates of short filaments are observed (Aubin et al., 1980; Rosevear et al., 1990 and references therein). Possibly, some of these reported discrepancies are due to cell type differences or the result of observations of different stages of mitosis.

During the cell cycle, a variety of proteins are constitutive and facultative components of the centrosome (for review, Kimble and Kuriyama, 1992). Recent studies have identified several filamentous proteins containing coiled-coil α-helical domains, a structural feature of IF proteins, and a number of antibodies have been described that recognize both IFs and centrosomes (Kimble and Kuriyama, 1992; Paul and Quaroni, 1993, and references therein). Such proteins could serve to link vimentin IFs to the PCM by the formation of heterologous coiled-coil structures. Certainly, further experimentation will clarify the protein-protein associations that might govern the attachment of vimentin IFs to the PCM.

With regard to de novo assembly and organization of newly formed vimentin filaments, our examination of SW-13 cells shortly after DNA transfection revealed filamentous fragments of vimentin dispersed throughout the cytoplasm, similar to previous observations (Sarria et al., 1990). In addition, some transfected cells exhibited more extensive cytoplasmic filaments. Our further dual immunofluorescence staining for vimentin and γ-tubulin demonstrated an accumulation of at least some newly synthesized vimentin protein at the centrosome. De novo assembly of vimentin is considered to occur via two pathways with the relative contributions of each varying according to cell type: (1) post-translational assembly from soluble precursors; and (2) co-translational assembly of nascent peptides in association with the cytoskeleton (for reviews, see Fulton and L’Ecuyer, 1993; Georgatos, 1993). In SW-13 cells de novo assembly of a portion of the vimentin could occur co-translationally at the centrosome; our electron micrographs did show ribosome-like particles embedded within the PCM. Alternatively, the centrosomal localization may be attributed to the cytoplasmic diffusion of filamentous vimentin with subsequent binding to specific PCM proteins.

Further clarification of cytoskeletal assembly and intracellular organization is required to better understand how the cytoskeleton influences cellular architecture and biology. Our report implicates the centrosomal PCM as an attachment site for vimentin IFs. More intensive studies are necessary to determine the exact nature of this subcellular association and its potential influence on cellular processes. Together with continued investigations of centrosome activities, unknown aspects of the structural and functional roles of vimentin IFs may well be elucidated.

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Vimentin associates with the centrosome


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