Vimentin’s tail interacts with actin-containing structures in vivo

Robert B. Cary1,*, Michael W. Klymkowsky1,*,†, Robert M. Evans2, Alberto Domingo1,‡, Joseph A. Dent1,§ and Leilah E. Backhus1

1Molecular, Cellular and Developmental Biology, University of Colorado, Boulder, Boulder, Colorado 80309-0347, USA
2Department of Pathology, University of Colorado Health Sciences Center, Denver, Colorado 80262-0216, USA

*These two authors contributed equally to this paper
†Author for correspondence
‡Present address: Department of Biochemistry, School of Medicine, Autonomous University of Madrid, Madrid, Spain
§Present address: Department of Biochemistry, University of Texas Southwestern Medical Center, Dallas, Texas 75235-9038, USA

SUMMARY

The tail domain of the intermediate filament (IF) protein vimentin is unnecessary for IF assembly in vitro. To study the role of vimentin’s tail in vivo, we constructed a plasmid that directs the synthesis of a ‘myc-tagged’ version of the Xenopus vimentin-1 tail domain in bacteria. This polypeptide, mycVimTail, was purified to near homogeneity and injected into cultured Xenopus A6 cells. In these cells the tail polypeptide co-localized with actin even in the presence of cytochalasin. Two myc-tagged control polypeptides argue for the specificity of this interaction. First, a similarly myc-tagged lamin tail domain localizes to the nucleus, indicating that the presence of the myc tag did not itself confer the ability to co-localize with actin (Hennekes and Nigg (1994) J. Cell Sci. 107, 1019-1029). Second, a myc-tagged polypeptide with a molecular mass and net charge at physiological pH (i.e. −4) similar to that of the mycVimTail polypeptide, failed to show any tendency to associate with actin-containing structures, indicating that the interaction between mycVimTail and actin-containing structures was not due to a simple ionic association. Franke (1987; Cell Biol. Int. Rep. 11, 831) noted a similarity in the primary sequence between the tail of the type I keratin DG81A and vimentin. To test whether the DG81A tail interacted with actin-containing structures, we constructed and purified myc-tagged DG81A tail polypeptides. Unexpectedly, these keratin tail polypeptides were largely insoluble under physiological conditions and formed aggregates at the site of injection. While this insolubility made it difficult to determine if they associated with actin-containing structures, it does provide direct evidence that the tails of vimentin and DG81A differ dramatically in their physical properties. Our data suggest that vimentin’s tail domain has a highly extended structure, binds to actin-containing structures and may mediate the interaction between vimentin filaments and microfilaments involved in the control of vimentin filament organization (Hollenbeck et al. (1989) J. Cell Sci. 92, 621; Tint et al. (1991) J. Cell Sci. 98, 375).

Key words: vimentin, actin, tail domain, keratin

INTRODUCTION

Vertebrate intermediate filament proteins (IFPs) all share a similar secondary and tertiary structure and all form ultrastructurally similar filaments (see Aebi et al., 1988; Steinert and Roop, 1988; Parry and Steinert, 1992; van de Klundert et al., 1993). Three distinct structural domains have been recognized: an N-terminal ‘head’, a central α-helical ‘rod’ and a C-terminal ‘tail’. Previous studies have shown that the head and rod domains are important in intermediate filament (IF) assembly (see van de Klundert et al., 1993, for review). Interactions between rod domains drive the formation of α-helical coiled-coil dimers. Dimers associate in an anti-parallel and staggered manner to form tetramers (Geisler et al., 1985, 1992; Potschka et al., 1990; Steinert, 1991; Steinert et al., 1993). Tetramers, in turn, associate into higher order oligomers to form an IF (Ip et al., 1985; Aebi et al., 1988; Steinert, 1991). Isolated head domains of desmin have been shown to form dimers in solution (Saeed and Ip, 1989). The head domain of vimentin binds with high affinity to vimentin’s rod domain (Traub et al., 1992) and a conserved nonapeptide motif in the head has been implicated in the formation of higher order structures (Herrmann et al., 1992; Hofmann and Herrmann, 1992).

In contrast, the role of the tail domains of IFPs are much less clear. Even the location of the tail domain with respect to the IF axis is uncertain. Axial mass measurements of IFs have been interpreted to indicate that the head and tail domains are located at the periphery of the IF (Steven et al., 1983a,b; Engel et al., 1986). In neurofilaments the distal portion of the tail domain clearly projects centrifugally from the filament axis (Hisanaga and Hirokawa, 1989). On the other hand Birkenberger and Ip (1990) reported that an antiserum directed against the C-terminal end of desmin’s tail bound to desmin tetramers but not to desmin filaments, arguing that at least a portion of the tail domain is hidden in the assembled filament.

There have been conflicting reports on the effects of removing or mutating the tail domain on the ability of IFPs to
assemble into IFs. Quinlan et al. (1989) found that deletion of the tail domain inhibited the ability of the IFP glial fibrillary acidic protein (GFAP) to form filaments in vitro. Similarly, Birkenberger and Ip (1990) found that a polypeptide derived from the tail domain of desmin, as well as an antibody directed against this region, were able to perturb desmin filament formation in vitro. Based on the effects of anti-tail antibodies on vimentin filament organization and in vitro binding studies Koulik et al. (1991) suggested that the tail domain of vimentin bound to a region at the end of the rod domain and that this interaction acted to inhibit the lateral association of vimentin filaments. It has also been proposed that interactions between the nuclear lamins and the tail domains of type III IFPs are involved in the nucleation of IF assembly (Georgatos and Blobel, 1987; Georgatos et al., 1987; Djabali et al., 1991; Papa- marcali et al., 1991).

There have also been reports in which removal of the tail domain of vimentin (Shoeman et al., 1990; Eckelt et al., 1992; McCormick et al., 1993), desmin (Kaufmann et al., 1985), and keratins (Wilson et al., 1992) were found to have little if any noticeable effect on the in vitro formation of IFs. McCormick et al. (1993) found that mutations that eliminate the in vitro interaction between the tail and rod domains of vimentin have no apparent effect on the formation or aggregation of vimentin filaments (see below). Tail-deleted IFPs readily integrate into pre-existing IF systems (see Eckelt et al., 1992 and McCormick et al., 1993, and references therein).

In cells with no pre-existing IF system the effects of mutations in the tail domain of IFPs are generally much more dramatic. Deletions in the region of the desmin tail (Raats et al., 1991) lead to failure to form filaments when synthesized in human MCF-7 cells. Lu and Lane (1990) found that tail-less pairs of keratins formed aberrant filament systems in mouse NIH-3T3 cells. Eckelt et al. (1992) found that the removal of the tail domain of Xenopus vimentin lead to aberrant filament network formation in bovine BMGE+H cells (see also Herrmann et al., 1993). On the other hand, Bader et al. (1991) reported that tail-less keratins formed superficially normal looking filament networks in mouse 3T3-L1 cells. In a particularly striking set of observations McCormick et al. (1993) found that a human vimentin protein with its tail removed still formed extended filament networks in human MCF-7 cells, although some abnormalities in the organization of the network were observed.

As this short review indicates, the exact role of the tail domain in IF structure and organization is still unclear. The failure of tail-less IFPs to form extended IF networks in many cells has been interpreted to mean that ‘the establishment of extensive IF arrays in a specific cell type could well involve the coordinate interaction of the tail portions with one or several unknown cellular factors’ (Eckelt et al., 1992). What these cellular factors might be remains unclear. To approach this question we have examined the behavior of the tail domain as an isolated entity. We constructed plasmids that drive the synthesis of N-terminally epitope-tagged forms of the tail domains of Xenopus vimentin-1 and the Xenopus type I epidermal keratin DG81A. The purified tagged tail polypeptides were then studied both in vitro and in vivo. In vivo it appears that the tail domains of vimentin interact with actin-containing structures. The keratin tail, in contrast, behaves quite differently within the cell. These results point to previous unrecognized differences between the tail regions of different IFP types and suggests that the tail domain of vimentin may well mediate interactions between vimentin filaments and microfilaments.

MATERIALS AND METHODS

Construction of myc-tagged vimentin and keratin tail domain plasmids

The tail domain of the Xenopus laevis vimentin-1 (Dent et al., 1992) and the X. laevis type I epidermal keratin DG81A (Jonas et al., 1985) (supplied by Tom Sargent, NIH) were amplified by PCR (Fig. 1). In the case of the vimentin domain, the upstream oligonucleotide included an Nde I site, the sequence encoding the myc-tagging epitope (MEQKLISEEDLN), an EcoRI site, and a sequence complementary to the antisense strand of the 5′ end of the tail region. The downstream oligonucleotide overlapped the 3′ end of the tail domain and contains an XhoI site. The amplification reaction was carried out as follows: 94°C for 3 minutes, 5 cycles of 94°C for 2 minutes, 55°C for 2 minutes, 72°C for 2 minutes followed by two cycles of 94°C for 2 minutes, 72°C for 2 minutes and then 72°C for 8 minutes. The product was restricted with NdeI and XhoI and subcloned into the pSP6.stop plasmid (Dent et al., 1992) to generate pSP6.myc-tagged vimentin tail (pSP6.mycVimTail). This plasmid was then restricted with NdeI and BamHI and the released fragment was subcloned in the pET3a plasmid (Studier et al., 1990) to create pET.mycVimTail. The original pET3a plasmid was a gift of W. Studier (Brookhaven National Laboratory).

pET3a contains a single EcoRI and a single XhoI site; these sites were removed sequentially by cutting the plasmid with the appropriate enzyme, filling the ends with Klenow and re-ligating to form pET+. To create a plasmid encoding the DG81A tail domain we subcloned the vimentin-1 stop coding region from pSP6.vimentin-1.stop (Dent et al., 1992) using the NdeI and BamHI sites into pET+ to form pET+vimentin-1.stop. This plasmid was then restricted with NdeI and XhoI to remove the vimentin-1 sequence and the mycVimTail DNA was subcloned in to form pET+mymcVimTail. The tail domain of the type I keratin DG81A was amplified using oligonucleotide primers similar to those used for vimentin (Fig. 1) following the regime 95°C for 3 minutes, 5 cycles of 95°C for 2 minutes, 37°C for 2 minutes, 72°C for 2 minutes followed by two cycles of 95°C for 2 minutes, 72°C for 2 minutes and then 72°C for 8 minutes. The amplified DG81A DNA was restricted with EcoRI and XhoI and subcloned into the EcoRI/XhoI-digested pET+mymcVimTail plasmid to generate pET+mymcKerTail (Fig. 1B). We also subcloned the original amplified mycVimTail DNA into the pSP6.myc plasmid (Dent et al., 1992) using the NdeI and XhoI sites to create pSP6.mycVimTailmc. Amplified DG81A DNA was restricted with EcoRI and XhoI and subcloned into pSP6.mycVimTailmc to produce pSP6.mycKerTailmc. The mycKerTailmc insert was released from this plasmid by restriction with NdeI and BamHI and subcloned into pET to create pET.mycKerTailmc. The pET plasmids were initially transformed into Escherichia coli JM103 and plasmid DNA was sequenced using the T7 promoter primer and the dideoxynucleotide method (Chen and Seeburg, 1985) (Fig. 1C). The DNA was then transformed into BL21DE3plSlyS cells (Studier et al., 1990) for polypeptide expression.

DNA- and RNA-expression plasmids

Since it is unclear whether the purified mycVimTail polypeptide is in a native configuration when injected into cells, we also constructed DNA- and RNA-expression vectors. pET.mycVimTail was digested with NdeI/BamHI to release the mycVimTail sequence, which was then subcloned into the pSK.tag (Dent et al., 1992) to form pSK.mycVimTail.stop. The mycVimTail.stop insert was liberated
with SalI/BamHI and subcloned into pCsAct.vimentin-1.myc, replacing the vimentin-1.myc sequence. This plasmid was generated from a pCsAct.LacZ plasmid supplied to us by Richard Harland (UC Berkeley). pCsAct contains the X. borealis cytoskeletal actin promoter and the SV40 polyadenylation signal (Cross et al., 1988; Berkeley). pCsAct contains the original sequence TTTGAGCTC TATA and the T7 RNA polymerase insert.

Next, we isolated the NdeI/BamHI mycVimTail fragment from pCsAct.mycVimTail and subcloned it into pT7.vimentin-1.myc, replacing the vimentin-1.myc sequence. The pT7 plasmid is derived from the pSP64T plasmid (supplied by Doug Melton, Harvard University). To construct pT7, the SP6 promoter was removed from pSP64T by digestion with NheI and subcloned into pCskAct.vimentin-1.myc, which contains the SP6 promoter and the SV40 polyadenylation signal (Cross et al., 1988; Berkeley). Subcloning of pCsAct.mycVimTail DNA into the nuclei of A6 cells at a concentration of ~1 mg/ml.

After annealing, the oligos were digested with EcoRI and XbaI and subcloned into either pET.mycVimTail or pET.mycVimTallmyc (b) replacing the VimTail sequence. This produced the pET.mycVimTail plasmid. Amplified DG81A tail DNA was digested with EcoRI and XbaI and subcloned into either pET.mycVimTail or pET.mycVimTallmyc (b) replacing the VimTail sequence. This produced the pET.mycVimTail and pET.mycVimTallmyc plasmids. The resulting pET plasmids were sequenced. The sequence of the mycVimTail, mycKerTail and mycKerTailmyc polypeptides is shown in c. Homology between mycVimTail and mycKerTailmyc polypeptides is noted using a ‘|’ to denote amino acid identity and a ‘:.’ to denote a conservative amino acid difference.

**Myc-tagged control polypeptides**

To test whether the binding of mycVimTail to actin-containing structures was due to the presence of the myc-epitope, we obtained a plasmid encoding an N-terminally myc-tagged lamin tail domain under control of the cytomegalovirus promoter from H. Hennekes and Eric Nigg (Swiss Institute for Experimental Cancer Research, Lausanne, Switzerland) (Hennekes and Nigg, 1994). Quagen purified plasmid DNA was resuspended in distilled water and injected into cells at a concentration of 1.3 mg/ml.

**Fig. 1. Schematic for construction of pET.mycVimTail and pET.mycKerTail.**
was removed by cutting with EcoRI, filling in with Klenow, and religating. The resulting EcoRI-minus plasmid was cut with SacI, blunt-ended with mung bean nuclease and ligated to EcoRI linkers (Promega cat. no. 901028). The EcoRI/XhoI fragment from this plasmid was then ligated into pET*mycVimTail, replacing the VimTail sequence to form pET*myc3xHa. This plasmid was sequenced using the T7 promoter of the pET plasmid. The sequence of the encoded polypeptide was MEOKLISEEALNSHGRRGRIPYYDVDSYDAGPYDYDGHP. The multiple underlined region is the myc-tag sequence and the double-underlined region is the repeated hemagglutinin-derived sequence. The 3' terminus of pET*myc3xHa was then transformed in BL21DE3PlsY3 cells for synthesis of the myc3xHa polypeptide (see below).

**Purification of myc-tagged polypeptides**

For purification of mycVimTail, mycKerTail, and myc3xHa polypeptides, 1 litre cultures of bacteria were grown in LB broth to an OD600 of 0.6-1 and then induced with 0.4 mM isopropyl-β-D-thiogalactopyranoside for 2 hours. The polypeptides were followed through the purification by a dot-blot assay using the anti-myc antibody 9E10 (Evan et al., 1985) (see below). The tail polypeptides do not bind efficiently to nitrocellulose paper but can be fixed using glutaraldehyde. Samples (2 µl) were applied to nitrocellulose paper and dried; the polypeptide was then fixed with 0.5% glutaraldehyde in phosphate-buffered saline (PBS) for 10 minutes. The polypeptide was then blocked for 10 minutes in 2% low fat dried milk (LFDM) in Tris-buffered saline (TBS) and incubated in 10 µg/ml 9E10 antibody for 30 minutes, washed, and incubated in 1:1000 diluted horseradish peroxidase-conjugated anti-mouse immunoglobulin antibody (Bio-Rad) for 30 minutes. Bound antibody was visualized using the diaminobenzidine reaction.

Bacteria were recovered by centrifugation (5,000 × g, 15 minutes); the cell pellet was resuspended in 100 mM Tris-HCl, 2 mM EDTA, pH 8.0, collected by centrifugation and frozen at −70 °C. After thawing, the cell pellet was resuspended in 20 ml 7 M guanidine HCl in 50 mM Tris-HCl, 1 mM EDTA, pH 8.0, and frozen at −70 °C. After thawing, the solution was diluted with 80 ml of 50 mM Tris-HCl, 1 mM EDTA, pH 8.0, and centrifuged at 5,000 × g for 30 minutes. In the case of mycVimTail, mycKerTail and mycKerTail/myc polypeptides the supernatant was recovered, 200 ml of 95% ethanol was added, and the solution was placed at −70 °C for 2 hours or longer. After thawing, the solution was centrifuged at 5,000 × g for 30 minutes; the pellet was solubilized in 10 mM Tris-HCl, 0.2 mM EDTA, pH 8.8, and then centrifuged at 15,000 rpm for 30 minutes in a Sorvall SS-34 rotor. The supernatant was passed through a 0.2 µm syringe filter and then applied to a HQ-5 MonoQ column (Pharmacia) at a rate of 1 ml/minute. The column was eluted with a 30 µl 0 to 130 mM NaCl gradient in 15 mM Tris-HCl, pH 8.8. To recover the purified polypeptides, fractions were combined, precipitated with 2 volumes of 100% ethanol and resuspended in the buffer of choice.

In the case of myc3xHa, following the dilution and centrifugation of the guanidine fraction, 400 ml of 95% ethanol was added to the supernatant and the solution was placed at −70 °C overnight; insoluble material was collected by centrifugation and discarded. A second 400 ml of 95% ethanol was then added to the supernatant, which was again incubated overnight at −70 °C. The insoluble material was then collected, dried down, resuspended in 0.1% aqueous trifluoroacetic acid (TFA) and separated on a 25 cm Vydac 214-TPS46 C4 reversed-phase HPLC column. The column was run at room temperature at a flow rate of 1.5 ml/minute. The sample was applied to the column and run in 0.1% aqueous TFA for 2 minutes. The column was eluted with a linear gradient of 0 to 80% acetonitrile, 0.1% TFA for 8 minutes, 30 to 40% acetonitrile, 0.1% TFA for 10 minutes, and then 40 to 55% acetonitrile, 0.1% TFA for 7 minutes. Fractions were collected, dried under vacuum, and resuspended in distilled water. Samples were assayed for anti-myc reactivity by dot-blot as described above. The major 9E10-reactive fraction was lyophilized and resuspended in distilled water for injection.

The purity of the MonoQ-purified mycVimTail polypeptide was assayed by reverse-phase high pressure liquid chromatography (HPLC) on a 25 cm Vydac 214-TP546 C4 column. The column was run at room temperature with a flow rate of 1.5 ml/minute and samples were applied to the column and run in 0.1% aqueous TFA for 2 minutes. Retained material was eluted with a linear gradient of 0 to 40% acetonitrile, 0.1% TFA for 25 minutes, then 40 to 80% acetonitrile, 0.1% TFA for 5 minutes. The purified mycVimTail was cleaved with 10 mg/ml cyanogen bromide in 70% formic acid for 4 hours. The sample was dried under vacuum and again analyzed by HPLC; under these conditions it produced the two expected cleavage products (data not shown). The purity of the mycVimTail polypeptide was also assayed using polyacrylamide gel electrophoresis (Laemmli, 1980). Because of the presence of only a single aromatic residue, the myc-tagged vimentin and keratin tail polypeptides have little molar absorbance at 280 nm. Protein concentration was estimated using a Coomassie Brilliant Blue binding assay using 2 mg/ml bovine serum albumin as a standard (Pierce).

**Size-exclusion chromatography and cross-linking studies**

The apparent size of the mycVimTail polypeptide was analyzed by size-exclusion chromatography on Sepharose 6-B (1.5 cm × 20 cm column). The column was run in TBS with increasing concentrations of urea or 0.5% SDS. Blue dextran (M, 2 × 109), bovine serum albumin (M, 67,000), lysozyme (M, 14,000) and Phenol Red (M, 354) were used as size markers. For cross-linking studies, MonoQ-purified mycVimTail polypeptide fractions were precipitated with ethanol and resuspended in 50 mM sodium phosphate, 10 mM β-mercaptoethanol buffer (Saeed and Ip, 1989); 50 µl of a 0.1 to 1 mg/ml solution of purified polypeptide was incubated at room temperature (21–23°C) for 30 minutes in the presence of 0.1% glutaraldehyde (EM grade) in the presence or absence of 0.5% SDS. Alternately, purified polypeptide was incubated at room temperature for 60 minutes in 1 mg/ml dimethylsuberimide alone, or in the presence of 2 M urea or 0.5% SDS. The cross-linking reactions were stopped with SDS sample buffer and the samples were electrophoresed on 12.5 or 15% gels and the gel was stained with Coomassie Brilliant Blue.

**Intracellular injection of purified protein and DNA**

*Xenopus* A6 cells (obtained from American Type Culture Collection) were cultured on coverslips in 85% Leibovitz L-15 media supplemented with 10% fetal calf serum and 50 µg/ml gentamycin. Purified polypeptides were resuspended in distilled water (final concentration 1 to 3 mg/ml) and injected into cells using a manually-controlled pressure injection system (Klymkowsky, 1981). The same apparatus was used for the intranuclear injection of pCskAct.mycVimTail DNA or cytoplasmic injection of mycVimTail RNA. In some experiments, injected cells were treated with cytochalasin D (2.5 µg/ml final concentration) or with nocodazole (5 µg/ml final concentration). At times ranging from 5 to 120 minutes after injection, the cells were washed once with PBS, fixed with 3.7% formaldehyde for 10 minutes at room temperature and then extracted with 100% methanol for 1 minute. In some cases, the cells were extracted with PHEM-0.15% Triton X-100 buffer (Schliwa and van Blerkom, 1981) or MES buffer (Algrain et al., 1993) for 15-60 seconds prior to formaldehyde fixation. Fixed cells were washed in TBS and incubated with the anti-myc antibody 9E10, followed by fluorescein-conjugated anti-mouse immunoglobulin G antibody (antiMIg-F). For double labeling, cells were stained with 9E10, fluorescein-conjugated anti-mouse γ-chain specific antibody (antiMIg-γ-F), and a mouse monoclonal anti-actin antibody (IgM) (Amersham - cat no. N.350) and rhodamine-conjugated anti-mouse μ chain-specific antibody (antiMIg-μ-Rd). 9E10 is obtained from the American Type Culture Collection. Cells were examined and photographed using a Zeiss IM35 microscope.
RESULTS

Given the remarkable conservation of vimentin’s tail domain between species (Herrmann et al., 1989) and its apparent non-involvement in vimentin filament assembly (see Eckelt et al., 1992; McCormick et al., 1993) we decided to study the behavior of vimentin’s tail domain as an isolated entity. We constructed plasmids to direct the synthesis of an epitope-tagged form of the *Xenopus* vimentin-1 tail domain in bacteria. A nucleotide sequence encoding the polypeptide sequence MEQKLISEEDLN, which is recognized by the monoclonal antibody 9E10, was attached in-frame to the 5′ end of the sequence encoding the tail domain (Fig. 1). This myc-tagged tail DNA sequence was then subcloned into the bacterial protein expression vector pET3a. The myc-tagged vimentin tail polypeptide (mycVimTail) was purified from cultures of bacteria to near homogeneity as determined by reverse phase HPLC (Fig. 2). Sequencing of the pET.mycVimTail plasmid revealed the expected nucleotide sequence and appropriate stop codon (data not shown). Cleavage of the mycVimTail polypeptide with cyanogen bromide or lysine-specific protease revealed the expected number of cleavage products. The products obtained following cleavage with lysine-specific protease were isolated by HPLC and their amino acid composition compared with that predicted for the mycVimTail polypeptides. For all four predicted lysine-specific protease fragments, the measured amino acid composition corresponded to the predicted amino acid composition of the mycVimTail polypeptide (data not shown). We are therefore confident that the purified mycVimTail polypeptide is identical to the predicted sequence (Fig. 1).

The mycVimTail polypeptide has a calculated molecular mass of 7033 daltons. However, on SDSPAGE gels, it migrates with an apparent molecular mass of ~12 kDa (Fig. 2). A similar sized polypeptide was obtained when the pT7.mycVimTail plasmid was cut with BamHI and used in an in vitro transcription/translation reaction (data not shown). On a Sephacryl S-200 size exclusion column mycVimTail migrated with an apparent size of ~15-20 kDa (Fig. 3). The migration of the mycVimTail polypeptide through a Sephacryl S-200 column was unaffected by the presence of increasing concentrations of urea (up to 8 M) (Fig. 3) or 0.5% SDS (data not shown). In contrast, the position at which lysozyme (14.3 kDa) eluted from the column changed dramatically, it behaved as an increasingly larger structure as the concentration of denaturant increased (Fig. 3). Based on this behavior, it would appear that the mycVimTail polypeptide is in an extended configuration under physiological conditions.

It has been reported that the head domain of desmin exists as a dimer in solution (Saeed and Ip, 1989). Cross-linking studies of the mycVimTail polypeptide failed to reveal any evidence for self-assembly (data not shown). The lack of effects of denaturants on the apparent molecular mass (as determined by size-exclusion chromatography) (Fig. 3) and the absence of evidence for oligomers (as determined by cross-linking studies) indicate that the tail domain exists as a monomer in solution.

Behavior of tail domains in the cell

Based on in vitro data (Georgatos and Blobel, 1987; Georgatos et al., 1987; Djabali et al., 1991; Papamarcaki et al., 1991; Kouklis et al., 1991) the tail domain of vimentin might be expected to interact with both nuclear lamins and vimentin within the living cell. To test this hypothesis we injected *Xenopus* A6 cells with purified mycVimTail polypeptide. A6 cells possess both keratin- and vimentin-type IF networks (Franz et al., 1983; Dent et al., 1989) as well as a lamina Lpl/L3 lamina (Krohne et al., 1984). The mycVimTail polypeptide should be small enough to pass through nuclear pores even in the absence of a specific nuclear transport signal (see Newport and Forbes, 1987) (see below). To our surprise the injected tail domain did not appear to interact with either nuclear lamins or vimentin within the living cell. To test this hypothesis we injected *Xenopus* A6 cells with purifed mycVimTail polypeptide. A6 cells possess both keratin- and vimentin-type IF networks (Franz et al., 1983; Dent et al., 1989) as well as a lamina Lpl/L3 lamina (Krohne et al., 1984). The mycVimTail polypeptide should be small enough to pass through nuclear pores even in the absence of a specific nuclear transport signal (see Newport and Forbes, 1987) (see below). To our surprise the injected tail polypeptide was found to be present in the injected cell’s vimentin filament system (Fig. 4). If the injected cell’s vimentin filament system was ‘collapsed’ by treatment with nocodazole, we again found little if any evidence for an interaction between the injected tail polypeptide and vimentin filaments (data not shown). On the other hand, there was a striking overlap between the distribution of the injected mycVimTail polypeptide and actin (Fig. 4).
mycVimTail-injected cells were treated with cytochalasin D (2.5 µg/ml for 15 minutes), to reorganize their actin filament systems (see Schliwa, 1982), the injected mycVimTail polypeptide remained co-localized with the reorganized actin system (Fig. 5). This type of interaction with actin-containing structures is not a common feature of injected proteins (Stacey and Allfrey, 1977; Klymkowsky, unpublished observation; see below).

To provide an estimate of the strength of the interaction between the injected mycVimTail polypeptide and the cell’s actin system, we extracted injected cells with Triton X-100-containing PHEM or MES buffers. We have found two distinct types of behavior of the mycVimTail polypeptide under these conditions. In some cells the mycVimTail polypeptide remains bound to actin-containing structures, while in others it behaves like a detergent-soluble protein (data not shown). We have not been able to define the variable that leads to these two different behaviors.

The vimentin tail domain appears to be unstable in A6 cells

To test whether mycVimTail polypeptide translated from RNA behaved the same way as the purified mycVimTail polypeptide, we subcloned the mycVimTail sequence into the DNA expression plasmid pCskAct or the RNA synthesis vector pT7. Both of these reagents are highly effective in driving the synthesis of exogenous proteins when introduced into cultured A6 cells (Cary and Klymkowsky, unpublished observation). In four attempts with each, we were unable to detect mycVimTail polypeptide in either pCskAct.mycVimTail or mycVimTail RNA injected cells. This observation suggests that the vimentin tail domain is not stable in A6 cells.

Control myc-tagged polypeptides

To test whether the co-distribution of the injected mycVimTail polypeptide with actin was due simply to the presence of the myc-tagging epitope, we constructed a polypeptide based on the YPYDVPDYA epitope derived from the influenza hemagglutinin protein. The myc-epitope was fused in-frame to the 5′ end of the hemagglutinin sequence to form myc3xHa (see Materials and Methods). The calculated isoelectric point of the myc3xHa polypeptide is 4.22 (as compared to 4.87 for mycVimTail); its net charge at physiological pH (i.e. pH 7.0) is −4 (as compared to a −4 net charge for mycVimTail at pH 7.0) (calculations made using the GCG sequence analysis program) (Table 1). The myc3xHa polypeptide is also similar in size to the mycVimTail polypeptide (6708 versus 7033 daltons). The myc3xHa polypeptide was synthesized in bacteria, purified by HPLC and injected into A6 cells. Myc3xHA exhibited no apparent association with actin-based structures and tended to accumulate in the nucleus of injected cells (Fig. 6), arguing that...

---

**Table 1. Physical parameters of the polypeptides used in this study**

<table>
<thead>
<tr>
<th>Polypeptide</th>
<th>pI</th>
<th>Charge at pH 7.0</th>
<th>Mol. mass</th>
</tr>
</thead>
<tbody>
<tr>
<td>mycVimTail</td>
<td>4.87</td>
<td>−4</td>
<td>7033</td>
</tr>
<tr>
<td>myc3xHa</td>
<td>4.22</td>
<td>−4</td>
<td>6701</td>
</tr>
<tr>
<td>mycKerTail</td>
<td>4.58</td>
<td>−3</td>
<td>6332</td>
</tr>
<tr>
<td>mycKerTailmyc</td>
<td>4.48</td>
<td>−5</td>
<td>8134</td>
</tr>
</tbody>
</table>
the observed interaction between mycVimTail and actin-containing structures is not due to the presence of the myc-epitope or to mycVimTail’s net acidic charge at neutral pH. Similar results were obtained using plasmid DNA to express a myc-tagged form of the lamin A tail domain (data not shown) (see Hennekes and Nigg, 1994).

The DG81A keratin tail behaves differently from vimentin’s tail. Franke (1987) originally reported that the tail domain of the *Xenopus* type I keratin DG81A shared sequence similarity with the tail domain of vimentin. Our own homology searches using the NIH BLAST sequence analysis system failed to identify any significant homology between vimentin and DG81A. We were, therefore, curious to determine whether or not a myc-tagged tail domain of DG81A

Fig. 4. mycVimTail in cells. Shown here are three examples of the distribution of mycVimTail polypeptide following injection into *Xenopus* A6 cells. Cells were fixed for 15–45 minutes following injection and stained first for the injected polypeptide (a,c and e) and then for actin (b,d and f) using immunoglobulin class-specific secondary antibodies. In each example there is a striking co-distribution between the injected mycVimTail polypeptide and the endogenous actin system. mycVimTail is found associated with the ruffling edge of the cell (a,e), with stress fibers (b) and with what appear to be microvilli (e). Bar (a-f), 10 µm.
would behave like the tail domain of vimentin and co-localize with actin in the cell. We constructed a ‘myc-tagged tail’ (mycKerTail) version of the DG81A tail domain (Fig. 1). This polypeptide could be purified to near homogeneity using the mycVimTail protocol (data not shown). Unexpectedly, when we injected the mycKerTail polypeptide into cells we found that needles clogged quickly and precipitated protein accumulated around the needle tip. We take this as evidence that the mycKerTail polypeptide is not soluble under physiological conditions. Nevertheless, cells could be injected and the injected mycKerTail polypeptide was found to form punctate aggregates (Fig. 7a,b). In an attempt to increase the solubility of the keratin tail polypeptide, we constructed a ‘myc-tagged tail-myc’ (mycKerTailmyc) version of the DG81A tail domain. Again, this polypeptide could be purified to near homogeneity using the mycVimTail protocol. The mycKerTailmyc polypeptide was easier to inject, and so presumably more soluble under physiological conditions than mycKerTail. Nevertheless, in the cell it behaved very much like the mycKerTail polypeptide, i.e. it formed aggregates around the injection site (Fig. 7c,d). Aggregates of the keratin tail polypeptides remained following detergent-extraction (Fig. 7e,f) indicating that they are largely insoluble under physiological conditions. Given their insolubility, we were unable to determine whether either mycKerTail or mycKerTailmyc interact with actin-containing structures. Nevertheless, it is clear that the tail domain of the DG81A keratin behaves very differently from the vimentin tail domain.

Fig. 5. Cytochalasin effects on mycVimTail. In these experiments, cells were injected with mycVimTail polypeptide and allowed to recover for 15 minutes. They were then treated with 2 µg/ml cytochalasin D for a further 15 minutes, at which time they were fixed and stained for the injected polypeptide (a,c) and actin (b,d). There is a striking overlap in the distribution of the injected mycVimTail and the endogenous, reorganized actin. Bar (a-d), 10 µm.
DISCUSSION

The tail domain of vimentin appears unnecessary for IF formation (see Eckelt et al., 1992; McCormick et al., 1993, and references therein). This suggests that the tail domain could play a role in the control of IF organization through interactions with other cytoplasmic components. To study the possible functions of the tail domain, we examined its behavior as an isolated entity within the cell. When purified mycVimTail polypeptide was injected into cells it appeared to interact with actin-containing structures (Figs 4, 5). The interaction between the mycVimTail polypeptide and actin-containing structures was not due to the presence of the myc-tag, since control polypeptides containing the identical myc-tag at their N termini, fail to co-localize with actin (Fig. 6) (Hennekes and Nigg, 1994). It is not a non-specific effect of the small size and net negative charge of the mycVimTail polypeptide; the myc3xHa polypeptide is of similar size and charge (Table 1) but does not show any apparent association with actin containing structures (Fig. 6).

The behavior of the vimentin tail domain was quite different from that of the tail domain of the epidermal keratin DG81A (Fig. 7). The two polypeptides do not differ greatly in size, pI or net charge at physiological pH (Table 1), yet the vimentin tail is soluble under physiological conditions, whereas the keratin tail is insoluble. This difference in behavior between vimentin’s and DG81A’s tail presumably reflects physiologically significant differences in tail structure. Whether or not the tail domain of DG81A can interact with actin-containing structures remains unresolved, since its insolubility within the cell obscured any other interactions.

A surprising result of our studies was the apparent absence of interactions between the vimentin tail domain and either nuclear lamins or endogenous vimentin (see Introduction) (Fig. 4). Given that both mycLamTail (Hennekes and Nigg, 1994) and myc3xHa (Fig. 6) accumulate in the nucleus, it is clear that a polypeptide with the size and charge of mycVimTail can enter the nucleus. Why no interaction with the vimentin tail with lamins was observed is not clear. It is possible that the endogenous vimentin tail domain blocks the binding of exogenous tail polypeptide to vimentin. To examine this possibility we constructed plasmids encoding ‘tail-less’ and ‘tail-
truncated' vimentins. In both IF-minus human SW13 clone 2 cells (Sarraia et al., 1990) and in vimentin-minus human MCF-7 cells (supplied by E. Fuchs, University of Chicago) these tail-deleted vimentins failed to form extended filament networks (Cary and Klymkowsky, unpublished observation) (see Eckelt et al., 1992). It therefore remains possible that the vimentin tail domain does bind to vimentin's rod domain as suggested by Kouklis et al. (1991). On the other hand, the observation that this interaction can be disrupted by mutation without apparent effect on vimentin filament formation or behavior (McCormick et al., 1993) makes the significance of this interaction unclear.

The role of the tail in vimentin filament organization

In most vimentin-containing cells, vimentin filaments form an extended network that stretches from the vicinity of the nucleus to the cellular periphery (see Goldman et al., 1986). This extended vimentin filament network appears to be established primarily through interactions with microtubules (Geiger and Singer, 1980; Ball and Singer, 1981; Gyoeva and Gelfand, 1991). Depolymerization of the microtubule system leads to the collapse of the vimentin network into a dense coil near the cell center (Goldman and Knipe, 1972; Croop and Holtzer, 1975; Blose and Chako, 1976; Franke et al., 1978; Hynes and Destree, 1978; Wehland and Willingham, 1983; Blose et al., 1984). This centripetal collapse of the vimentin filament network appears to be mediated by the interaction of IFs with the microfilament system(s) of the cell (Hollenbeck et al., 1989; Tint et al., 1991). Direct interactions between vimentin filaments and microfilaments/microfilament-associated structures have been reported (Knapp et al., 1983; Magneat and Burridge, 1985; Langley and Cohen, 1987; Granger and Lazarides, 1982; Green and Goldman, 1986; Green et al., 1986; Bershadsky et al., 1987). Disruption of normal microfilament organization also leads to the reorganization of IFs (Croop and Holtzer, 1975).

These observations indicate that IFs interact with microfilaments; why then are IFs and microfilaments not co-localized in the cell? The answer would appear to involve the relative strengths of the interactions between IFs, microtubules and microfilaments. The IF-microtubule interaction appears to be dominant, as demonstrated by the co-alignment of the IF and microtubule systems in many cell types. An IF-microfilament interaction is generally apparent only upon the disruption of the microtubule system. In the absence of microtubules, vimentin filaments undergo a microfilament-dependent centripetal collapse (see Hollenbeck et al., 1989; Tint et al., 1991). In addition, the connected nature of the IF itself imposes constraints on the distribution of IFs within the cell. The isolated tail domain of vimentin can not form IFs and does not appear to interact with microtubules, so its distribution in the cell reflects the distribution of its highest affinity partner, i.e. the microfilament system.

Physical properties of the vimentin tail domain

On size-exclusion columns the mycVimTail polypeptide behaves as a much larger structure than its molecular mass would predict (Fig. 3). Since its behavior is not changed by denaturants (Fig. 3), it would appear that the mycVimTail polypeptide has a highly extended configuration in solution. Whether it is also highly extended when it is part of the intact vimentin polypeptide remains to be determined. Solid state NMR measurements (Mack et al., 1988) suggest that the tail and head domains of keratins are not structured, which would be consistent with an extended configuration. STEM measurements of vimentin, vimentin/desmin and keratin filaments (Steven et al., 1983a,b; Engel et al., 1986) suggest that the true diameter of an IF is ~16 nm of which ~8-10 nm is accounted for by the central portion, which is composed primarily of rod...
domains. This would leave 6 to 8 nm of the diameter to be occupied by head and tail domains.

Based on a length of ~0.34 nm/ amino acid residue we calculate the maximum extended length of the vimentin tail domain is ~15 nm. Given STEM mass measurements of intact IFs (see above), it would seem unlikely that the tail is fully extended; it may well fold back upon itself at least once, perhaps twice. This could place the highly conserved end of the tail domain in close proximity to the filament axis and may explain why the extreme C-terminal region of the tail domain of desmin is inaccessible to antibody when assembled into filaments (Birkenberger and Ip, 1990). It could also provide a structural explanation for why mutations in the tail domain in this highly conserved region could effect filament structure (McCormick et al., 1993). In fact, Franke’s (1987) original recognition of conservation in the C-termini of vimentin, desmin, GFAP, and keratin K8 tail domains noted that this sequence motif resembled a ‘loop’ similar to that seen in Ca2+-binding proteins (see also Branden and Tooze, 1991). Such a loop could be involved in the folding of the tail domain (Fig. 8). A folded tail would still project ~5-7 nm from the central axis of the filament and would be well positioned to mediate interactions with microfilaments, either directly or through other actin-binding proteins.

While the tail domain is associated with actin-containing structures in formaldehyde-fixed cells, in detergent-extracted cells we often found that it behaved as a detergent-soluble protein. This would imply that the interaction between the tail domain and microfilaments is of relatively low affinity. This does not mean that within the context of the cell it is not significant. Based on structural studies, it appears that there are 16 IFP subunit dimers per cross-section in an IF (Aebi et al., 1988; Steinert et al., 1993). If the two tails of a vimentin dimer both project outward from the filament axis they could co-operate with one another and with tails elsewhere in the filament to form more stable interactions with microfilaments. A number of such low affinity interactions could mediate the vimentin filament ↔ microfilament interaction (Fig. 8).

We thank Michael Yarus for the use of his FPLC machine; Craig Tuerek for sharing his early results on the behavior of the in vitro synthetic mycVimTail polypeptide; Tom Sargent for the DG81A cDNA; Doug Melton for the pSP64T plasmid; Richard Harland for pCskAct.LacZ; and Danielle Przychodzin for technical assistance in the early stages of the project. Sequence analysis was performed at the NCBI using the BLAST network service; we thank Robin Gutell for introducing us to BLAST and Mark Winney for carrying out the GCG analysis of tail polypeptides. This work was supported by a grant from the National Science Foundation (DCB91-05523) with additional support from the Colorado Chapter of the American Heart Association and the Council for Research and Creative Work of the University of Colorado, Boulder. A half-year sabbatical granted to M.W.K. by the University of Colorado made this work possible. R.B.C. was supported in part by a training grant from the NIH. R.M.E. was supported by NIH grant HL51850; A.D. was supported by a fellowship from the Spanish Ministry of Science; J.A.D. was supported in part by an NSF pre-doctoral fellowship.

REFERENCES


(Received 27 December 1993 - Accepted 15 February 1994)