INVOLVEMENT OF THE N-TERMINAL POLYPEPTIDE OF VIMENTIN IN THE FORMATION OF INTERMEDIATE FILAMENTS

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SUMMARY

The potential to form intermediate filaments of a 54 × 10^3 molecular weight (M_r) polypeptide derived from vimentin by cleavage by the intermediate filament-specific, Ca^{2+}-activated proteinase was investigated. Under physiological conditions of assembly, the breakdown product did not form intermediate filaments. Electron microscopy revealed short, rod-like structures similar to those described by Geisler et al. for a 38 × 10^3 M_r, α-helical core particle derived from desmin. Since the specific, Ca^{2+}-activated proteinase degrades vimentin preferentially from its N terminus, this result suggests the involvement of the basic, N-terminal polypeptide of vimentin in the assembly of intermediate filaments. This was supported by the observation that arginine inhibits the formation of intermediate filaments from intact vimentin. Whereas lysine had very little effect on the assembly process, guanidinium hydrochloride was effective at the same concentration as arginine. On the basis of these findings, an affinity chromatography method for the identification and isolation of intermediate filament subunit proteins was developed. Beside vimentin, desmin, the 68 × 10^3 M_r, neurofilament triplet protein, the glial fibrillary acidic protein and cytokeratins also bound to arginine methylester Sepharose 4B in a salt-stable manner and could be eluted with arginine. The 145 × 10^3 M_r, neurofilament triplet protein exhibited reduced binding activity, whereas the 210 × 10^3 M_r, subunit did not bind to the affinity matrix. Among the degradation products of vimentin produced by the specific, Ca^{2+}-activated proteinase, only those with molecular weights higher than 40 × 10^3 bound to arginine methylester Sepharose 4B. The same applied to the high molecular weight degradation products of desmin with a proteinase-resistant 37 × 10^3 M_r, polypeptide as the major component. The results suggest that arginine residues of the non-α-helical, N-terminal polypeptides of intermediate filament subunit proteins play an important role in filament assembly.

INTRODUCTION

The observation that the subunit proteins of all five major classes of intermediate filaments constitute, regardless of their different molecular sizes and antigenic and biochemical properties, the same type of long protein tubes with an electron-microscopically defined diameter of 8–10 nm suggests that they possess common structural features (for reviews on intermediate filaments, see Anderton, 1981; Lazarides, 1982; Osborn et al. 1982). One such feature is their high α-helix content. In order to account for the α-type X-ray diffraction pattern of intermediate filaments, the α-helical regions of their subunit proteins are thought to be arranged in coiled-coils (Day & Gilbert, 1972; Renner, Mandelkow & Franke, 1980; Renner et al. 1981; Steinert, 1978; Steinert, Idler & Goldman, 1980). Based on the structural analysis of α-helix-enriched core particles obtained by tryptic digestion of intact filaments of the cytokeratin-, desmin- and vimentin-type, a first subunit domain map was constructed.
According to this model, each subunit was supposed to be made up of two equally long $\alpha$-helices of 180 Å length separated by a non-$\alpha$-helical, globular segment and flanked by two non-$\alpha$-helical domains at the amino and carboxy termini. It was further proposed that the filaments themselves consist of repeating three-chain structural units – the so-called protofilament units – in which three subunit chains are aligned in approximate register. Since in this model the non-$\alpha$-helical domains represent the variable parts of the different subunit proteins, they seem to determine the unique physical and chemical properties of the various intermediate filament classes.

However, the determination of the amino acid sequence of desmin and its comparison with the partial sequences of other intermediate filament proteins (Geisler, Kaufmann & Weber, 1982; Geisler & Weber, 1982) confirmed only the basic concept of this model. It had to be modified in many respects; for instance, regarding the number and sizes of the $\alpha$-helical and non-$\alpha$-helical domains and their positioning along the polypeptide chain. The amino acid sequence of desmin dictates the formation of three $\alpha$-helical domains. The two shorter helices on the N-terminal side plus their short, non-$\alpha$-helical spacer sequence are approximately equal in length to the longer $\alpha$-helix on the C-terminal side. The three $\alpha$-helices together with their spacer regions constitute a $38 \times 10^3$ molecular weight ($M_r$) central piece which in the electron microscope appears as a rod-like structure some 500 Å in length (Geisler et al. 1982). It can be prepared by proteolytic cleavage of the N- and C-terminal, non-$\alpha$-helical domains of the filament protein, which are $7.5 \times 10^3 M_r$ and $5.5 \times 10^3 M_r$, respectively. Moreover, the amino acid sequence analysis of desmin has shown that the $7.5 \times 10^3 M_r$, N-terminal polypeptide is rather basic, due to the presence of 10 randomly distributed arginine residues and the absence of acidic amino acids (Geisler et al. 1982). The high susceptibility of the N-terminal headpiece to proteolytic degradation was ascribed to the presence of several strong $\beta$-turns. Finally, recent results of chemical cross-linking experiments performed on the isolated desmin rod suggested that the architecture of the intermediate filaments themselves is not based on triple-stranded coiled-coils but on dimers of normal, double-stranded coiled-coils (Geisler & Weber, 1982).

From the inability of the rod-like central piece of desmin to form intermediate filaments and from results of the amino acid sequence analysis of $\alpha$-keratins of sheep wool (Weber & Geisler, 1982), it was concluded that one or both of the non-$\alpha$-helical, terminal domains are involved in the assembly of protofilaments into intermediate filaments. While in the case of wool filaments the assembly process is made irreversible due to the formation of disulphide bonds between the cysteine residues of the non-$\alpha$-helical, terminal polypeptides and the $\alpha$-helical central piece, in the case of desmin and possibly also of most of the other cytoplasmic filament subunit proteins, the high arginine content of the non-$\alpha$-helical, N-terminal polypeptide appears to stabilize the interaction of protofilaments in the filament assembly process (Weber & Geisler, 1982).

In the course of the large-scale isolation and purification of the intermediate filament-specific, Ca$^{2+}$-activated proteinase from Ehrlich ascites tumour cells, we
isolated from the same cell homogenate a $54 \times 10^3\ M_r$ proteolytic breakdown product of vimentin. Its formation is the result of the action of the $Ca^{2+}$-activated proteinase, which degrades vimentin from its N terminus (our unpublished results). We used this breakdown product to investigate whether the removal of a $4 \times 10^3\ M_r$ polypeptide from the basic, non-$\alpha$-helical N terminus of vimentin has an influence on the capability of vimentin to form intermediate filaments. Here we show that the $54 \times 10^3\ M_r$ polypeptide does not form filaments under physiological conditions of assembly and we present some indirect evidence that arginine residues of the non-$\alpha$-helical, N-terminal polypeptides of intermediate filament subunit proteins may play an important role in filament assembly.

**MATERIALS AND METHODS**

**Materials**

Arginine methylster hydrochloride was obtained from Serva (Heidelberg, FRG), CNBr-activated Sepharose 4B and DEAE-Sepharose CL-6B from Pharmacia (Uppsala, Sweden), CF-11 cellulose from Whatman (Maidstone, England), and arginine, agarose and calf thymus DNA from Sigma (St Louis, Mo., U.S.A.). Single-stranded (ss)DNA-cellulose was prepared as described recently (Nelson & Traub, 1982a) and arginine methylster Sepharose 4B as described by Unkeless, Dans, Kellerman & Reich (1974), except that $0.1\ M\ NaHCO_3$ (pH 8.9) was used as coupling buffer. Vimentin (Nelson & Traub, 1982b; Nelson, Vorgias & Traub, 1982) and the intermediate filament-specific, $Ca^{2+}$-activated proteinase (Nelson & Traub, 1982c) were isolated from Ehrlich ascites tumour (EAT) cells according to the protocols published previously. Desmin was isolated from porcine stomach smooth muscle (Vorgias & Traub, unpublished data), glial fibrillary acidic protein from bovine brain white matter (Vorgias & Traub, unpublished data) and cytokeratins from newborn rat skin (Traub, unpublished data) using ssDNA-cellulose and arginine methylster Sepharose 4B affinity chromatography. The preparation of purified neurofilament triplet proteins from porcine spinal cord followed the procedure described by Delacourte et al. (1980) as modified by Geisler & Weber (1981a). The monoclonal antibody $\alpha$-IFA was a gift from Dr A. J. Dowding and Dr B. H. Anderton (St George's Hospital Medical School, London, England). All other chemicals were of reagent grade and purchased from Merck AG (Darmstadt, FRG).

**Cell culture**

Ehrlich ascites tumour cells were grown in suspension culture using minimum essential medium supplemented with 5% foetal calf serum (FCS) or 5% heat-inactivated, normal calf serum as described previously (Egberts, Hackett & Traub, 1976). Adenovirus-transformed marmoset skin fibroblasts (HF-TAV cells) were propagated in monolayer culture in RPMI 1640 medium and baby hamster kidney (BHK-21) cells also in monolayer culture in Dulbecco's modified Eagle's medium. Both media were supplemented with 5% FCS. EAT cells were harvested at a density of $1.5 \times 10^6$ cells/ml, HF-TAV and BHK-21 cells when they had reached confluency.

**Isolation of the $54 \times 10^3\ M_r$ degradation product of vimentin**

The $54 \times 10^3\ M_r$ proteolytic degradation product of vimentin was isolated in the course of the large-scale purification of the intermediate filament-specific, $Ca^{2+}$-activated proteinase from EAT cells. It was separated from intact vimentin and slightly smaller degradation products by essentially following the procedure described previously for the purification of vimentin (Nelson & Traub, 1982b). Briefly, 500 g frozen EAT cells were homogenized in 10 mm-Tris-acetate (pH 7.6), 1 mm-EGTA, 6 mm-mercaptoethanol (buffer A) in a Waring Blender. Insoluble material was removed by centrifugation at 13,000 g, for 10 min and then at 160,000 g, for 5 h in the presence of 1 mm-Mg$^{2+}$. The supernatant was adjusted to 23% (NH$_4$)$_2$SO$_4$ saturation and, after stirring for 1 h, centrifuged at 30,000 g, for 10 min. The pellet contained vimentin and its proteolytic degradation
products, the supernatant was further processed for enzyme purification. The pellet of the 160,000 g, centrifugation contained ribosomes and a considerable amount of vimentin and its high molecular weight degradation products. It was resuspended in buffer A and the solution was adjusted to 1.5 mM-Mg²⁺. Samples were underlayered with 30% (w/w) sucrose in buffer A containing 1.5 mM-Mg²⁺ and centrifuged at 160,000 g for 15 h. The supernatant was subjected to (NH₄)₂SO₄ fractionation as described above and the precipitate was combined with the major fraction of vimentin. The further purification of vimentin and its 54 × 10^6 M, breakdown product included delipidation with chloroform/methanol (2:1), DEAE-Sepharose CL-6B chromatography and ssDNA-cellulose affinity chromatography, both in the presence of 6 M-urea, exactly as described previously (Nelson & Traub, 1982b; Nelson et al. 1982). The 54 × 10^6 M, polypeptide required a higher KCl concentration for its elution from DEAE-Sepharose CL-6B and a lower KCl concentration for its elution from ssDNA-cellulose. The final yield was 75 mg.

Arginine methylester Sepharose 4B affinity chromatography

A sample containing 2 mg filament protein in 2 ml 10 mM-Tris-acetate (or sodium phosphate) (pH 7-6), 3 mM-EDTA, 6 mM-2-mercaptoethanol (buffer B), was applied to a 10 cm × 1 cm arginine methylester Sepharose 4B column previously equilibrated with buffer B. Fractions (2 ml) were collected at a flow rate of 25 ml/h. The column was washed with 40 ml buffer B, then with 20 ml buffer B containing 0.5 M-KCl and again with 10 ml buffer B. For further protein elution, a 100 ml linear 0 to 0.5 M-arginine hydrochloride or a 0 to 1 M-guanidinium hydrochloride gradient in buffer B was used. The salt concentration of each fraction was determined by conductivity measurement with a Digitalmeter Digi 610 (Wissenschaftlich-Technische Werkstatten, Weilheim, FRG). A portion (50 µl) of each fraction was mixed with 10 µl of a solution containing 45 ml 10% sodium dodecyl sulphate (SDS), 12.5 ml 2-mercaptoethanol, 50 mg Bromophenol Blue and 25 µl of each sample was used for polyacrylamide gradient slab gel electrophoretic analysis in the presence of SDS. The neurofilament triplet proteins, the glial fibrillary acidic protein and the cytokeratins were applied to the affinity column in buffer B containing 8 M-urea. The glial fibrillary acidic protein and the cytokeratins were circulated over the column overnight, resulting in a dilution of urea to 1 M. Thereafter, the chromatography was continued as described above.

Extraction of HF-TAV and BHK-21 cells and isolation of intermediate filament proteins

A sample (5 g) of frozen cells of each cell line was extracted three times with 12 ml portions of buffer A containing 0.5% Triton X-100 in tightly fitting Dounce homogenizers. Interval centrifugation was at 30,000 g, for 5 min. The supernatants were adjusted to 3 mM-spermidine and, after standing for 30 min, centrifuged at 30,000 g, for 10 min. The pellets were resuspended in 6 ml distilled water in Dounce homogenizers; 0.7 ml 2.5 M-HCl was added to each suspension. After shaking for 2 h, insoluble material was removed by centrifugation at 30,000 g, for 5 min and solubilized protein was precipitated from the supernatants with 70 ml acetone. It was dissolved in 5 ml 10 mM-Tris-acetate (pH 7-6), 3 mM-EDTA, 6 mM-2-mercaptoethanol, 6 M-urea (buffer C) by brief sonication. The solutions were adjusted to pH 7-6 with 1M-KOH. The nuclear pellets were processed exactly as the spermidine precipitates. For filament protein isolation, the solutions were applied to 10 cm × 1 cm arginine methylester Sepharose 4B columns previously equilibrated with buffer C, and chromatographed as described above. The flow-through and 0.5 M-KCl washed fractions were combined. Adsorbed protein was eluted with buffer C containing 1M-KCl. Equivalent volumes of each protein solution before and after affinity chromatography were mixed with 2 vol. distilled water and 3 vol. 40% trichloroacetic acid to achieve a final concentration of 20%. After standing for 2 h at room temperature, the precipitates were collected by centrifugation at 2000 g, for 10 min, washed with 20% trichloroacetic acid and acetone, respectively, and dissolved in sample buffer for polyacrylamide gradient slab gel electrophoretic analysis in the presence of SDS.

Assembly of intermediate filaments and electron microscopy

Purified vimentin was incubated at a concentration of 200 µg/ml for 30 min at 37 °C in 10 mM-Tris-acetate (pH 7-6), 150 mM-KCl, 6 mM-2-mercaptoethanol in the absence and the presence of
varying amounts of arginine, lysine, guanidinium hydrochloride and degradation products of vimentin, respectively. When the reaction mixtures were supplemented with basic amino acids, the ionic strength was kept constant at that of a 150 mM-KCl solution. The $54 \times 10^3 M_r$ proteolytic breakdown product of vimentin was treated in the same way, but with the omission of any additives. The reaction products were negatively stained as described by Moore, Huxley & De Rosier (1970) and viewed in a Zeiss EM9 electron microscope.

Polyacrylamide gel electrophoresis

The kinetics of the degradation of vimentin and the $54 \times 10^3 M_r$ polypeptide, as well as the distribution of filament proteins and their proteolytic degradation products during column chromatography, were followed by polyacrylamide gradient slab gel electrophoresis in the presence of SDS as described previously (Egbertsef et al. 1976). The gels were stained with Coomassie Brilliant Blue, destained with 10% acetic acid and scanned at a wavelength of 590 nm in a Gilford 2400S spectrophotometer.

RESULTS

The Ca$^{2+}$-activated proteinase specific for intermediate filament proteins is normally isolated from cultured cells or tissues in the presence of EGTA, mainly in order to prevent its autodigestion, which is induced by Ca$^{2+}$ (Nelson & Traub, 1981, 1982c). Under these conditions, vimentin, if present, should be stable. However, when we prepared the Ca$^{2+}$-activated proteinase from a large amount of suspension-grown Ehrlich ascites tumour cells, we found a substantial part of the vimentin to be partially degraded. We isolated the degradation products, besides intact vimentin, to study their structural and biochemical properties. For their isolation, we employed a combination of (NH$_4$)$_2$SO$_4$ fractionation, DEAE-Sepharose chromatography and affinity chromatography on ssDNA-cellulose (data not shown). We concentrated our efforts mainly on the purification of a $54 \times 10^3 M_r$ degradation product.

To show that the $54 \times 10^3 M_r$ polypeptide is indeed derived from vimentin, it was digested with the intermediate filament-specific, Ca$^{2+}$-activated proteinase and the peptide composition of the digest was compared with that of a digest of intact vimentin, employing one-dimensional polyacrylamide gradient slab gel electrophoresis in the presence of SDS. As depicted in Fig. 1, the $54 \times 10^3 M_r$ breakdown product and vimentin were degraded with the formation of the same set of stable polypeptides.

Since the Ca$^{2+}$-activated proteinase degrades vimentin from its N terminus (our unpublished results), the $54 \times 10^3 M_r$ polypeptide should lack a considerable number of the basic amino acid residues originally present in the non-$\alpha$-helical, N-terminal polypeptide of vimentin, provided that the analytical data obtained with desmin also apply to vimentin (Geisler & Weber, 1981b; Geisler et al. 1982). If so, the $54 \times 10^3 M_r$ polypeptide should be somewhat more acidic than vimentin and elute from DEAE-Sepharose slightly behind vimentin. This was actually observed when a mixture of vimentin and the $54 \times 10^3 M_r$ polypeptide was chromatographed on the anion exchanger in 6 M-urea (Fig. 2). Because a steep KCl gradient was used for protein elution, only incomplete separation of both proteins was achieved.

Another characteristic property of vimentin is its affinity for ssDNA (Traub, Nelson, Kühn & Vorgias, 1983). In the presence of 6 M-urea, it can be purified by affinity chromatography on ssDNA-cellulose (Nelson et al. 1982). Limited digestion
Fig. 1. Kinetics of the degradation of vimentin (A) and its $54 \times 10^3 M_r$ breakdown product (B) by the intermediate filament-specific, $Ca^{2+}$-activated proteinase. For each time point, 40 $\mu$g vimentin or $54 \times 10^3 M_r$ breakdown product was digested with 1.5 $\mu$g of $Ca^{2+}$-activated proteinase in 200 $\mu$l of 10 mM-Tris-acetate (pH 7.6), 150 mM-KCl, 0.4 mM-Ca$^{2+}$, 6 mM-2-mercaptoethanol as reaction buffer. The samples were incubated at 37 $^\circ$C for: lanes 1, 0 min; 2, 15 s; 3, 30 s; 4, 1 min; 5, 2 min; 6, 3 min; 7, 5 min; 8, 10 min; 9, 15 min; 10, 30 min. After incubation, the reaction mixtures were processed for polyacrylamide gel electrophoretic analysis as described previously (Nelson & Traub, 1982a,c).
by the Ca\(^{2+}\)-activated proteinase, however, causes total loss of the nucleic acid-binding activity due to loss of the basic, N-terminal polypeptide (our unpublished results; see also Nelson & Traub, 1982a). It was of interest, therefore, to determine the effect of only partial removal of the N terminus on the DNA-binding capacity of vimentin. A mixture of intact vimentin and the 54 × 10^3 M, polypeptide was chromatographed in 6 M-urea on ssDNA-cellulose. As illustrated in Fig. 3, the proteolytic breakdown product eluted at a considerably lower KCl concentration (25 mM-KCl) than vimentin (80 mM-KCl).

The major goal of this investigation was to obtain information on the function of the basic, non-\(\alpha\)-helical N terminus of vimentin in the assembly of intermediate filaments. Vimentin and the 54 × 10^3 M, breakdown product, respectively, were incubated at 37°C in the presence of 150 mM-KCl; i.e., under conditions that are optimal for filament assembly. Whereas the vimentin solution stayed clear, the 54 × 10^3 M, polypeptide precipitated during incubation. Electron-microscopic examination of both incubation mixtures revealed the formation of intermediate filaments from vimentin (Fig. 4A), whereas in the case of the 54 × 10^3 M, breakdown product only irregular, dense aggregates consisting of short, rod-like particles were detected with the occasional indication of the formation of short, filament-like
Fig. 3. Single-stranded (ss)DNA-cellulose co-chromatography of vimentin and its $54 \times 10^3 M_r$ breakdown product; 2.5 mg vimentin and 2 mg of $54 \times 10^3 M_r$ degradation product was applied to a 10 cm $\times$ 1 cm ssDNA-cellulose column equilibrated with buffer C. The chromatogram was developed with a 100 ml linear 0 to 300 mM-KCl gradient in buffer C. For gradient analysis by polyacrylamide gel electrophoresis, see Materials and Methods. P58 (O—O), vimentin; P54 (●—●), degradation product.

aggregates (Fig. 4B, C). In addition, we polymerized vimentin in the presence of the $54 \times 10^3 M_r$ breakdown product at protein ratios of 1:1 and 1:2, respectively. There was no significant interference of the degradation product with the formation of filaments from intact vimentin. In both cases, the filaments had the same appearance as those of the control, with respect to both length and shape (data not shown). The inability of the proteolytic degradation product to form filaments under normal assembly conditions strongly indicates that the N-terminal polypeptide of vimentin is essential for filament assembly.

This raised the question as to whether the incompetence to form intermediate filaments is due to the lack of some of the N-terminal basic amino acid residues or of the $4 \times 10^3 M_r$ polypeptide as a whole. If the basic amino acid residues play a role in filament assembly, the polymerization should be prevented in the presence of free amino acids as competitive inhibitors. To solve this problem, vimentin was incubated under physiological ionic conditions in the presence of increasing concentrations of arginine and the incubation mixtures were examined by electron microscopy. As shown in Fig. 5B, arginine at a concentration of 60–90 mM inhibited the assembly of 10 nm filaments. At 150 mM-arginine, rod-like structures, probably protofilaments,
Fig. 4. An attempt to form intermediate filaments from vimentin (A) and its $54 \times 10^3 M$, breakdown product (B); 20 µg vimentin and $54 \times 10^3 M$, breakdown product, respectively, were incubated at 37°C for 30 min in 100 µl 10 mM-Tris-acetate (pH 7.6), 150 mM-KCl, 6 mM-2-mercaptoethanol. The filaments were negatively stained on carbon-coated grids and viewed in a Zeiss EM9 electron microscope. ×38,000.
Fig. 5. An attempt to form intermediate filaments from vimentin in the presence of arginine (a), lysine (c) and guanidinium hydrochloride (d); 20 μg portions of vimentin were incubated at 37°C for 30 min in 100 μl 10 mM-Tris-acetate (pH 7.6), 6 mM-2-mercaptoethanol containing 30, 60, 90, 120 and 150 mM-arginine, lysine and guanidinium hydrochloride, respectively. The conductivity of each solution was adjusted with KCl to that of a 150 mM-KCl solution. The reaction products were negatively stained on carbon-coated grids and viewed in a Zeiss EM9 electron microscope. ×30,000. A. Control filaments assembled in the presence of 150 mM-KCl alone. In b, c and d, only the reaction products obtained in the presence of 90 mM-arginine hydrochloride, lysine hydrochloride and guanidinium hydrochloride, respectively, are presented.
N terminus of vimentin and filament assembly

were observed (data not shown). This was paralleled by precipitation of vimentin in the incubation mixture. The assembly of intermediate filaments proceeded normally at lower arginine concentrations, whereas at higher concentrations the production of non-specific aggregates was intensified. Compared with arginine, lysine did not exert a significant effect on the formation of intermediate filaments, except that at high amino acid concentrations the filaments were somewhat shortened and less smooth in appearance (Fig. 5c). Guanidinium hydrochloride at a concentration of 60–90 mM, however, interfered considerably with filament assembly (Fig. 5d). It induced the formation of a network of electron-dense ribbons of varying diameter. With increasing guanidinium hydrochloride concentration, the network became extremely compact and this was accompanied by precipitation of the filament protein in the incubation mixture.

These data suggest that arginine residues of the non-α-helical, N-terminal polypeptide of vimentin are essential for filament assembly and that they specifically interact with a partial structure different from the N terminus of a second molecule of vimentin. On the basis of this assumption, it should be possible to bind vimentin to arginine or derivatives of arginine covalently coupled to a solid matrix. To test this possibility, vimentin was passed over an arginine methylester Sepharose 4B column. As shown in Fig. 6, vimentin bound quantitatively to the affinity matrix and it could not be eluted even with 0.5 M-KCl. However, it was desorbed by 320 mM-arginine when a 0 to 0.5 M-arginine gradient was applied to the column. Moreover, as illustrated in Fig. 7, 0.5 M-lysine eluted, although continuously, very little vimentin from the column. Most of the bound vimentin was recovered with 0.5 M-arginine. In contrast to this, when a 0 to 1 M-guanidinium hydrochloride gradient was used for protein

Fig. 6. Affinity chromatography of vimentin on arginine methylester Sepharose 4B; 2 mg vimentin was applied to a 10 cm × 1 cm arginine methylester Sepharose 4B column equilibrated with buffer B. The column was washed with buffer B, buffer B containing 0.5 M-KCl (position 1) and buffer B (position 2). In position 3, a 100 ml linear 0 to 0.5 M-arginine hydrochloride gradient in buffer B was started. For gradient analysis by polyacrylamide gradient gel electrophoresis, see Materials and Methods.
Fig. 7. Effect of lysine on the binding of vimentin to arginine methylester Sepharose 4B; 2 mg vimentin was applied to a 10 cm X 1 cm arginine methylester Sepharose 4B column equilibrated with buffer C. The column was washed with buffer B (position 1), buffer B containing 0.5 M-KCl (position 2), buffer B (position 3), buffer B containing 0.5 M-lysine hydrochloride (position 4), buffer B (position 5) and buffer B containing 0.5 M arginine hydrochloride (position 6). For gradient analysis by polyacrylamide gradient gel electrophoresis, see Materials and Methods.

In this context, the affinity-chromatographic behaviour of the degradation products of vimentin produced by the intermediate filament-specific, Ca$^{2+}$-activated proteinase was of special interest. Vimentin was digested with the proteinase for 1 min and 15 min, respectively, and the breakdown products were applied to an arginine methylester Sepharose 4B column. The column was washed with 0.5 M-KCl and, thereafter, a 0 to 0.5 M-arginine gradient was employed for further protein elution. From Fig. 8, in which the protein compositions of the KCl washes and the arginine
eluates are presented, it can be seen that the breakdown products with molecular weights between $27 \times 10^3$ and $20 \times 10^3$ were washed out with 0.5 M-KCl, whereas those with molecular weights higher than $40 \times 10^3$ were only eluted with arginine. The effective arginine concentration was the same as that for the elution of intact

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**Fig. 8.** Affinity chromatography on arginine methyl ester Sepharose 4B of the digestion products of vimentin produced by the intermediate filament-specific, Ca$^{2+}$-activated proteinase; 2 mg portions of vimentin were digested with the specific, Ca$^{2+}$-activated proteinase as described in the legend to Fig. 1 at 37 °C for 1 min and 15 min, respectively. The reactions were stopped with EDTA and the digests were chromatographed on a 10 cm × 1 cm arginine methyl ester Sepharose 4B column as outlined in the legend to Fig. 6. The figure shows the protein compositions of the peak fractions of the various column eluates. M, molecular weight standards: a, myosin ($M_r = 205000$); b, β-galactosidase ($M_r = 116000$); c, phosphorylase a ($M_r = 92500$); d, bovine serum albumin ($M_r = 68000$); e, ovalbumin ($M_r = 45000$); f, carbonic anhydrase ($M_r = 31500$); g, myoglobin ($M_r = 17800$). Lanes 1, fraction immediately in front of the flow-through of the 1 min digest; 2, 0.5 M-KCl wash of the 1 min digest; 3, arginine gradient eluate of the 1 min digest; 4, flow-through of the 15 min digest; 5, 0.5 M-KCl wash of the 15 min digest; 6, arginine gradient eluate of the 15 min digest. The position of vimentin is indicated by an arrow.
Fig. 9. The effect on the formation of intermediate filaments from intact vimentin of a 12 × 10^3 M, basic polypeptide derived from vimentin by Ca^{2+}-dependent proteolysis. Vimentin (20 μg/100 μl reaction mixture) was incubated at 37°C for 45 min in the presence of 150 mM-KCl and a 12 × 10^3 M, polypeptide cleaved from the N terminus of vimentin by the intermediate filament-specific, Ca^{2+}-activated proteinase. The ratio of vimentin to the 12 × 10^3 M, polypeptide is shown on the right in the polyacrylamide gel electrophoresis profile. A. Control filaments obtained in the absence of the 12 × 10^3 M, polypeptide; B, aggregates obtained in the presence of the 12 × 10^3 M, polypeptide. The filaments were negatively stained and viewed in a Zeiss EM9 electron microscope.

vimentin: 320 nm. Fig. 8 also shows that during a short digestion of vimentin a small, 12 × 10^3 M, polypeptide was formed, which upon discontinuous elution with 0.5 mM-KCl was washed out just in front of the major protein peak. It is a basic polypeptide because it binds to ssDNA-cellulose in the presence of 6 M-urea and because it does not bind to DEAE-Sepharose at low ionic strength (data not shown). When vimentin was digested for a longer period of time, the 12 × 10^3 M, polypeptide was degraded. However, a slightly larger breakdown product (14.5 × 10^3 M,) was formed, which appeared in the flow-through during arginine methylester Sepharose 4B affinity chromatography; it did not bind to ssDNA-cellulose in the presence of 6 M-urea.

We have isolated the basic, 12 × 10^3 M, polypeptide of a vimentin digest from the flow-through fraction from DEAE-Sepharose chromatography (data not shown) and studied its effect on the assembly of intermediate filaments from intact vimentin. At higher concentrations of the polypeptide, we observed the disappearance of normal 10 nm filaments and the formation of electron-dense ribbons of varying diameter (Fig. 9). The appearance of these ribbons was similar to that observed in the presence of guanidinium hydrochloride (Fig. 5D). With increasing concentration of the basic polypeptide, there was a continuous change from tube-like 10 nm filaments to electron-dense ribbons.
Fig. 10 depicts the results of the arginine methylester Sepharose 4B affinity chromatography of the digestion products of desmin produced by the specific, Ca$^{2+}$-activated proteinase. Polypeptides with molecular weights down to 37 x 10$^3$ bound efficiently to the affinity matrix. However, even the greatest part of the smaller degradation products with molecular weights of 25 x 10$^3$ and 21 x 10$^3$ could be released only with arginine. These polypeptides are the main constituents of the second group of desmin-derived breakdown products, which is separated from the first group by a gap of 12 x 10$^3$ daltons (Nelson & Traub, unpublished results). Intact desmin was eluted with 320 mM-arginine (data not shown).

It was also interesting to find out whether other intermediate filament subunit
proteins have the same high affinity for arginine methylester as vimentin and desmin. Thus, also neurofilament proteins from porcine spinal cord, glial fibrillary acidic protein from bovine brain white matter and cytokeratins from newborn rat skin were subjected to affinity chromatography on arginine methylester Sepharose 4B. With the exception of the $145 \times 10^3$ and $210 \times 10^3 M_r$, neurofilament triplet proteins (Fig. 11a, c), all intermediate filament subunit proteins were retained by the affinity matrix in a salt-stable manner and were eluted, on average, with an arginine concentration close to $300 \text{mM}$ (data only shown for the neurofilament triplet proteins, Fig. 11). For the elution of the cytokeratins, however, an arginine concentration of $400-450 \text{mM}$

![Affinity Chromatography Diagram](attachment:affinity_chromatography_diagram.png)

Fig. 11. Affinity chromatography of neurofilament triplet proteins (NFP) on arginine methylester Sepharose 4B; 2 mg of each neurofilament triplet protein were dissolved in 2 ml buffer C and applied to a 10 cm × 1 cm arginine methylester Sepharose 4B column. The column was washed with buffer B (position 1), buffer B containing 0.5 M-KCl (position 2) and buffer B (position 3). In position 4, a 100 ml linear 0 to 0.5 M-arginine hydrochloride gradient in buffer B was applied to the column. For gradient analysis by polyacrylamide gradient gel electrophoresis, see Materials and Methods. A, NFP 68; B, NFP 145; C, NFP 210.
was needed. Owing to the strong tendency of the cytokeratins to aggregate even in the presence of urea, we had some difficulties in adsorbing these proteins to the affinity matrix. We managed to overcome this obstacle by dissolving the cytokeratins in 8 M-urea and circulating them on the arginine methylester Sepharose 4B column that had been equilibrated with a low ionic strength buffer (buffer B) in the absence of urea. In time, mixing and diffusion caused a slow reduction of the urea concentration of the applied protein solution to 1 M. When complete equilibration had been achieved, the chromatography was continued as normal. In addition, in the case of glial fibrillary acidic protein and the cytokeratins, a fraction of the proteins was so tightly bound that it could not be eluted with 0.5 M-arginine; it was recovered from the column with 6 M-urea, 1 M-KCl.

Finally, we explored the possibility of whether the binding of intermediate filament subunit proteins to an arginine affinity matrix could be exploited for their identification, isolation and purification from cellular extracts. As examples, we chose adenovirus-transformed marmoset skin fibroblasts (HF-TAV) and baby hamster kidney (BHK-21) cells. The cells were extracted with Triton X-100 at low ionic strength and the homogenates were separated into nuclear and supernatant fractions. Intermediate filament proteins were precipitated from the postnuclear supernatants, together with nucleic acids, with 3 mM-spermidine. The precipitates as well as the nuclear fractions were extracted with 0.25 M-HCl. Solubilized protein was
precipitated with acetone and dissolved in a low ionic strength buffer containing 6M-urea. Figs 12 (HF-TAV cells) and 13A (BHK-21 cells) show that when the nuclear and supernatant-derived protein fractions were passed over an arginine methylester Sepharose 4B column, the intermediate filament proteins (vimentin in the case of HF-TAV cells; vimentin plus desmin in the case of BHK-21 cells) almost quantitatively adsorbed to the affinity column in a salt-resistant manner. They could

Fig. 12. Isolation by arginine methylester Sepharose 4B affinity chromatography of vimentin from a nuclear and postnuclear supernatant fraction of HF-TAV cells. Cells were extracted with Triton X-100 in buffer A and the homogenate was separated into a nuclear and postnuclear supernatant fraction. Vimentin was precipitated from the supernatant with 3 mM-spermidine. The nuclear fraction and the spermidine precipitate were extracted with 0.25 M-HCl. Solubilized protein was precipitated with acetone, dissolved in buffer B and chromatographed on a 10 cm x 1 cm arginine methylester Sepharose 4B column as described in Materials and Methods. The various protein fractions were analysed by polyacrylamide gel electrophoresis in the presence of SDS. Lanes 1, total nuclear protein; 2, flow-through plus 0.5 M-KCl wash of total nuclear protein; 3, urea/KCl eluate of bound total nuclear protein; 4, spermidine-precipitated supernatant protein; 5, flow-through plus 0.5 M-KCl wash of spermidine-precipitated supernatant protein; 6, urea/KCl eluate of bound spermidine-precipitated supernatant protein. For gel electrophoresis, equivalent amounts of each protein fraction were used. The arrows indicate the position of vimentin.
be eluted in a highly enriched form with urea at high ionic strength. It is evident from the electrophoresis profiles of the various protein eluates that the high molecular weight degradation products of the filament proteins also were quantitatively retained by arginine methylester Sepharose 4B. Immunoblotting with the monoclonal antibody α-IFA (Pruss et al. 1981) of the BHK-21 cell-derived proteins (Fig. 13B) indicates that only very little filament protein passed the affinity column. Vimentin and desmin extracted from BHK-21 cells were further characterized by their chromatographic behaviour in affinity chromatography on ssDNA-cellulose and by their characteristic peptide patterns produced by the intermediate filament-specific, Ca²⁺-activated proteinase (data not shown).

**DISCUSSION**

We have shown previously that the intermediate filament-specific, Ca²⁺-activated, neutral thiol proteinase digests vimentin and desmin from their N termini in a step-wise manner and that eventually a number of high molecular weight breakdown products are formed, which are resistant to further degradation (our unpublished results). However, the formation of the first intermediate products in this degradation cascade cannot be controlled *in vitro*, so that only very low amounts of these polypeptides can be isolated, which are not sufficient for a detailed biochemical analysis. In the course of the large-scale isolation of the intermediate filament-specific, Ca²⁺-activated proteinase from Ehrlich ascites tumour cells, we obtained a relatively large amount of a 54 × 10³ Mᵣ polypeptide, which was identified as being derived from vimentin by its peptide pattern produced by the Ca²⁺-activated proteinase (Fig. 1). Like the somewhat smaller degradation products of vimentin that are resistant to further proteolysis, this polypeptide was not able to form intermediate filaments under physiological conditions of assembly. Only occasionally was the formation of short fibrils from small, rod-like structures indicated. This was similar to the situation described by Geisler et al. (1982), who tried to assemble filaments from desmin-derived, rod-like core particles of molecular weight 38 × 10³ and 83% α-helix content.

The first polypeptides of the degradation cascade are arranged in a typical staircase in two-dimensional polyacrylamide gel electrophoresis (see, e.g., Nelson et al. 1982; Ochs, McConkey & Guard, 1981), indicating that with continuing degradation they become more acidic. Obviously, small basic polypeptides are cleaved from vimentin during its proteolysis. This is also indicated by the higher salt concentration required for the elution of the 54 × 10³ Mᵣ polypeptide from DEAE-Sepharose (Fig. 2) and the lower salt concentration for its elution from ssDNA-cellulose (Fig. 3) in the presence of 6 M-urea, always in comparison with the corresponding properties of intact vimentin. Furthermore, Geisler et al. (1982) have recently demonstrated that the 7.5 × 10³ Mᵣ, non-α-helical, N-terminal polypeptide of desmin is rather basic. It contains 10 arginine residues in nearly random distribution, but no lysine, histidine or acidic amino acids; it is highly susceptible to proteolytic degradation. On the basis of the close structural similarity between desmin and vimentin (Geisler & Weber, 1981b),
Fig. 13
it is likely that vimentin also possesses, in its basic N-terminal polypeptide, exclusively arginine residues as positively charged structural elements. This would explain our recent finding that extensive modification of vimentin with the lysine-specific reagent pyridoxal-5'-phosphate does not have any influence on the RNA-binding activity of vimentin at low ionic strength (Traub & Nelson, 1982). Provided that the interaction of vimentin with nucleic acids through its positively charged, N-terminal polypeptide is a merely electrostatic one, this result suggests that the N terminus of vimentin is rich in basic amino acids other than lysine.

With this background information, and as already suggested by Geisler & Weber (1982), our experimental results indicate that the basic, N-terminal polypeptide of desmin and vimentin plays an essential role in the process of filament assembly. Because of their high, local concentration, arginine residues appear to be the active principle. This contention is supported by the strong inhibitory effect of free arginine on the formation of intermediate filaments under normal assembly conditions. Since lysine has very little effect on filament assembly, the function of the arginine residues does not seem to be merely electrostatic. Conformation of this supposition comes from affinity chromatography experiments performed with arginine covalently coupled to agarose. Vimentin could be quantitatively eluted in a sharp peak from such an affinity column with 6 M-urea at low ionic strength (10 mM-Tris-acetate, pH 7.6, 3 mM-EDTA; data not shown). Apparently, hydrogen bonds are, at least in part, responsible for the strong salt-resistant binding of arginine residues to the filament protein. However, a configurational change of vimentin with distortion of the arginine binding site under denaturing conditions could explain this observation as well. Nevertheless, ionic bonds also seem to mediate the binding of arginine residues to vimentin, since at high ionic strength the complexes are dissociated at a much lower urea concentration (0.8 M-urea) than at low ionic strength (6 M-urea).

At low salt concentration, urea does not elute vimentin from arginine methylester Sepharose 4B. This is due to the fact that, because of esterification of the carboxyl group of arginine with methanol, the positive net charge of arginine methylester is higher than that of arginine, particularly when the α-amino group of arginine is incorporated into an iso urea group during reaction with cyanogen bromide-activated Sepharose or agarose. Since at physiological pH vimentin is negatively charged (pI = 5.3), it can still bind to arginine methylester Sepharose 4B through ionic bonds.

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*Fig. 13.* Gel electrophoresis (A) and immunoblotting (B) of intermediate filament proteins isolated from a nuclear and postnuclear supernatant fraction of BHK-21 cells by arginine methylester Sepharose 4B affinity chromatography. Cells were extracted and processed exactly as briefly outlined for HF-TAV cells in the legend to Fig. 12. Lanes 1 and 9, vimentin and actin; 2, supernatant protein after spermidine precipitation; 3, total nuclear protein; 4, flow-through plus 0.5 M-KCl wash of total nuclear protein; 5, urea/KCl eluate of bound total nuclear protein; 6, spermidine-precipitated supernatant protein; 7, flow-through plus 0.5 M-KCl wash of spermidine-precipitated supernatant protein; 8, urea/KCl eluate of bound spermidine-precipitated supernatant protein. For immunoblotting (B), the monoclonal antibody α-IFA was used, which defines a common antigenic determinant shared by all intermediate-filament proteins (Pruss et al. 1981). The positions of vimentin and desmin are indicated by arrows.
in the presence of 6 M-urea. Therefore, arginine methylester Sepharose 4B should be
preferred as an affinity matrix; it can be charged with filament protein in the presence
of urea, thus allowing full advantage to be taken of the dissociating and solubilizing
effect of urea on protein aggregates.

This has been exploited for the isolation of intermediate filament proteins and their
high molecular weight degradation products from adenovirus-transformed marmoset
skin fibroblasts (HF-TAV) and baby hamster kidney (BHK-21) cells. HF-TAV cells
have been chosen because their extracts often contain relatively large amounts of
proteolytic degradation products of vimentin, BHK-21 cells because they contain
vimentin and desmin (Gard, Bell & Lazarides, 1979). Even in the presence of Triton
X-100 (data not shown) and histones, respectively, vimentin and desmin and their
high molecular weight degradation products bind to arginine methylester Sepharose
4B. The latter observation is noticeable because vimentin also has high affinity for
arginine-rich histones (Traub, unpublished results). However, the concentration of
histones in the nuclear extracts of HF-TAV and BHK-21 cells was probably too low
for efficient competition with the affinity matrix for the intermediate filament proteins
and their proteolytic degradation products. Thus, the arginine affinity matrix proved
to be an excellent tool for the identification and enrichment of vimentin and desmin
in and from cellular extracts, respectively. We have successfully used it for the purif-
ication of vimentin from in vitro cultured cells, of desmin from porcine stomach
smooth muscle and of cytokeratins from newborn rat skin (our unpublished results).

The intermediate filament-specific, Ca^{2+}-activated proteinase degrades vimentin
and desmin with the production of two major groups of breakdown products with
molecular weights between 56 \times 10^3 and 40 \times 10^3, and 27 \times 10^3 and 20 \times 10^3, respec-
tively, in the case of vimentin and between 52 \times 10^3 and 37 \times 10^3, and 25 \times 10^3
and 21 \times 10^3, respectively, in the case of desmin (Nelson & Traub, unpublished
results). Among these digestion products, only those of higher molecular weight bind
to arginine methylester Sepharose 4B, indicating that the high affinity binding site for
arginine residues is lost during further proteolytic cleavage of the high molecular
weight breakdown products. We do not regard the apparently tight binding of the
lower molecular weight degradation products derived from desmin (Fig. 10) as the
result of a genuine affinity interaction. We rather assume that, even at high ionic
strength, the \( \alpha \)-helical segments of these polypeptides interact, on the basis of a coiled-
coil relationship, with the \( \alpha \)-helical domains of the major 37 \times 10^3 M, breakdown
product, which itself is tightly bound to the arginine residues of the affinity matrix.
There are three reasons for this assumption. Firstly, the smaller degradation products
are very slowly, but significantly, eluted from arginine methylester Sepharose 4B with
0.5 M-KCl, in contrast to the 37 \times 10^3 M, polypeptide, which can be desorbed only
with arginine or salt under denaturing conditions (data not shown). Secondly, we
noticed that at 0°C and high ionic strength desmin exhibits a much stronger tendency
to aggregate spontaneously than, for instance, vimentin. This very often leads to
precipitation of desmin, whereas vimentin responds to sudden increases in the salt
concentration with slow formation of filaments. Thirdly, in the case of desmin, the
quantities of high molecular weight degradation products bound to the affinity matrix
are much higher than in the case of vimentin; this increases the probability of coiled-coil formation with the lower molecular weight degradation products.

We assume that the $37 \times 10^3 M_r$ polypeptide, which is derived from desmin by $Ca^{2+}$-dependent proteolysis and still exhibits high affinity for arginine methylester, is very similar or identical to the $38 \times 10^3 M_r$ polypeptide described by Geisler et al. (1982); this polypeptide comprises the entire $\alpha$-helical domain with two intervening, non-$\alpha$-helical segments (Geisler & Weber, 1982). Therefore, the binding site for arginine must reside in the central piece of the filament protein. Since $\alpha$-helical domains are usually very resistant to proteolytic cleavage (Geisler et al. 1982; Geisler & Weber, 1982; Steinert et al. 1980), it is tempting to speculate that the loss of the arginine-binding activity during $Ca^{2+}$-dependent proteolysis of the high molecular weight breakdown products of vimentin and desmin is due to degradation of one or both of the central, non-$\alpha$-helical polypeptide segments (Geisler & Weber, 1982).

In the past, many studies have revealed the close morphological and structural similarity of the different intermediate filament classes (for reviews, see Anderton, 1981; Lazarides, 1982; Osborn et al. 1982). Here, we describe another property that is shared by all subunit proteins that are competent in forming morphologically normal intermediate filaments in vitro. In addition to vimentin and desmin, also the $68 \times 10^3 M_r$ neurofilament triplet protein, the glial fibrillary acidic protein and the cytokeratins exhibit strong affinity for arginine methylester. Their binding to the affinity matrix is resistant to high concentrations of salt and the proteins can be eluted with arginine. This observation suggests that, by analogy to vimentin and desmin, all subunit proteins that are capable of assembling into intermediate filaments possess an arginine-rich, N-terminal polypeptide, which, in the course of filament assembly, interacts with a central domain of a neighbouring or successive protein molecule. The $145 \times 10^3 M_r$ neurofilament triplet protein has a reduced affinity for arginine methylester, whereas the $210 \times 10^3 M_r$ neurofilament protein is completely incapable of binding to the affinity matrix. Whether this is related to the increasing degree of phosphorylation of these proteins (Jones & Williams, 1982; Julien & Mushynski, 1982) remains to be determined.

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N terminus of vimentin and filament assembly


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