Site Specificity in Vimentin-Membrane Interactions: Intermediate Filament Subunits Associate with the Plasma Membrane via Their Head Domains

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ABSTRACT Fragments of vimentin, generated by chemical or enzymatic cleavages, were analyzed for their capacity to bind to human inverted erythrocyte membrane vesicles. Only peptides comprising the amino-terminal head domain of vimentin molecules were competent in associating with the membranes. In vitro studies also demonstrated that isolated ankyrin (the major vimentin acceptor site on the membrane) binds to an oligomeric species of vimentin and prevents the formation of characteristic 10-nm filaments. These data, taken together with the observation that the NH2-terminal end of vimentin is implicated in the polymerization process (Traub, P., and C. Vorgias, J. Cell Sci., 1983, 63:43-67), imply that intermediate filaments may contact the membrane in an end-on fashion, using the exposed head domains of their terminal subunits.

Vimentin binds to inverted human erythrocyte membrane vesicles, or inside-out membrane vesicles (IOVs)1 through a high-affinity association with ankyrin, one of the major components of the membrane skeleton (1). The characteristics of the association suggest that ankyrin may serve as an attachment site for intermediate filaments through an end-on association mechanism. Vimentin possesses a tripartite molecular substructure composed of a head piece, a helical middle domain, and a tail region (2, 3). It undergoes polymerization via lateral associations of individual monomers followed by an elongation of short protofilaments, to form extended 10-nm filaments (4). The amino-terminal head domains are implicated in this latter step (5).

The studies described below show that vimentin molecules bind to membranes through their amino-terminal domains, and, as it could be expected, the binding of ankyrin to vimentin oligomers inhibits the formation of higher polymers in vitro.

Abbreviations used in this paper: IOVs, inside-out membrane vesicles; NTCB, 2-nitro-5-thiocyanobenzoic acid; PMSF, phenylmethylsulfonyl fluoride.

MATERIALS AND METHODS

Chemicals: a-Chymotrypsin was purchased from Worthington Biochemical Corp. (Freehold, NJ) trypsin from Miles Laboratories, Inc. (Elkhart, IN). 2-Nitro-5-thiocyanobenzoic acid (NTCB) was synthesized according to the method of Degani and Patchornic (6).

Purification of Vimentin: In a typical experiment, 50 calf lenses (44-50 g) were homogenized in phosphate-buffered saline (PBS) containing 2 mM MgCl2, 5 mM 2-mercaptoethanol and 1 mM phenylmethyisulfonyl fluoride (PMSF) (pH 7.4) at 4°C. After centrifugation in a JAI4 Beckman rotor at 14,000 rpm for 40 min, the pellet was extracted for 15 min with 0.6 M KCl, 50 mM Tris-HCl, 2 mM MgCl2, 5 mM 2-mercaptoethanol, 1 mM PMSF, 1 mM NaN3, and 0.5% Triton X-100 (pH 7.4) on ice; it was then Dounce-homogenized (12 strokes) and spun for 45 min. This procedure was repeated three times. The final pellet was washed with 150 mM KCl, 5 mM Tris-HCl, 5 mM 2-mercaptoethanol, 1 mM PMSF, and 1 mM NaN3 (pH 7.4), and then extracted with 7 M urea, 2 mM Tris-HCl, 10 mM EDTA, 5 mM 2-mercaptoethanol, 1 mM PMSF (pH 7.4) (3 h at 23°C or 8 h at 4°C with 6 M urea). The extract was high-speed centrifuged (100,000 g for 30 min) after dilution (1:1) with double-distilled water, and the supernate was collected. Urea was removed by dialysis against 2 mM Tris-HCl, 5 mM PMSF, and 1 mM NaN3 (pH 7.4), and then extracted with 7 M urea, 2 mM Tris-HCl, 10 mM EDTA, 5 mM 2-mercaptoethanol, 1 mM PMSF (pH 7.4) (3 h at 23°C or 8 h at 4°C with 6 M urea). The extract was high-speed centrifuged (100,000 g for 30 min) after dilution (1:1) with double-distilled water, and the supernate was collected. Urea was removed by dialysis against 2 mM Tris-HCl, 1 mM EDTA, 5 mM 2-mercaptoethanol, 0.2 mM PMSF (pH 7.4), and vimentin was precipitated with ammonium sulfate at 23°C saturation. The precipitate was resuspended in 6 M urea, 5 mM Tris-HCl, 1 mM EDTA, 2 mM 2-mercaptoethanol, 0.1 mM PMSF (pH 7.6), and dialyzed against the same buffer. After adjustment of the protein concentration to 1 mg/ml, this material was combined batchwise with DEAE-cellulose (DE52) equilibrated in the same buffer, shaken for 30 min at 4°C, and
extensively washed with 2 liters of the low-salt buffer. The resin was poured into a column, washed with another 2 bed volumes of buffer, and finally eluted with a 0-300-mM NaCl gradient. Vimentin-containing fractions were identified by SDS PAGE, pooled, dialyzed for 2-3 days against 3 mM NaPO₄, 0.1 mM EDTA, 2 mM 2-mercaptoethanol, and 0.1 mM PMSF (pH 7.4), and the protein concentration was adjusted to 0.1-0.2 mg/ml. The preparation was kept in 1-ml aliquots at 0°C and remained stable over the course of at least 1 mo. Upon addition of salt (to isotonic) characteristic 10-nm filaments were formed in vitro. (The purification and radiolabeling of vimentin are shown in Fig. 8.)

Purification of Ankyrin: Ankyrin was isolated from human erythrocyte ghosts as previously described (7).

Chymotryptic Cleavages of Vimentin: Digestion of purified vimentin was performed at 23°C with a-chymotrypsin at an enzyme to substrate ratio of 1:250 for 1, 15, and 30 min or at 4°C for 2 min. Digestion was stopped by the addition of PMSF to 1.3 mM and cooling on ice. Digests remained stable for at least 1 wk.

Chemical Cleavage of Vimentin: Purified vimentin (200 µg/ml) was made 6 M in urea by the addition of solid urea, and then dialyzed against 2-mercaptoethanol (pH 7.4). Urea was then added to 2 mM and the preparation was incubated for 40 min at room temperature. After that, the pH was raised to 9 by the addition of Tris base and the reaction mixture was incubated at 37°C for 4 and 8 h. The reaction was stopped by the addition of 2-mercaptoethanol to 10 mM and samples were subsequently dialyzed extensively against 3 mM NaPO₄, 0.1 mM PMSF, 2 mM 2-mercaptoethanol (pH 7.4).

Radiolabeling Procedures: Purified vimentin (at 90-100 µg/ml), or NTCB- cleaved vimentin (220 µg/ml) were dialyzed against 50 mM NaPO₄ (pH 8.1) and then reacted with low-specific activity (500 Ci/mmol) Bolton-Hunter reagent-125I. Reaction was allowed to proceed for 30 min at 0°C after which the proteins were extensively dialyzed against 3 mM NaPO₄, 0.1 mM EDTA, 1 mM PMSF (pH 7.4). Specific activities varied between 50 and 70,000 cpm/µg. Protein-bound radioactivity was estimated to 99.1% after precipitation with trichloroacetic acid (10%) at 0°C, in the presence of 3% bovine serum albumin (BSA) (carrier protein).

Immunoprecipitation: 125I-Vimentin (33,000 cpm/µg) was incubated with 17 µl of isolated ankyrin (30 min in 20 mM KCl, 5 mM Tris, 0.5 mM PMSF (pH 7.4) and then reacted with low-specific activity (500 Ci/mmol) Bolton-Hunter reagent-125I. Reaction was allowed to proceed for 30 min at 0°C after which the proteins were extensively dialyzed against 3 mM NaPO₄, 0.1 mM EDTA, 1 mM PMSF (pH 7.4). Specific activities varied between 50 and 70,000 cpm/µg. Protein-bound radioactivity was estimated to 99.1% after precipitation with trichloroacetic acid (10%) at 0°C, in the presence of 3% bovine serum albumin (BSA) (carrier protein).

Sedimentation Assays: 125I-Vimentin (10,000 cpm/µg) was incubated with 125I-ankyrin (300,000 cpm/µg) for 25 min at 30 min in an airfuge. Then, increasing amounts of ankyrin were incubated for 30 min with vimentin in 50 mM KCl, 5 mM Tris, 0.1 mM PMSF (pH 7.4) at 23°C (or alternatively in PBS/10). After this incubation, the mixture was centrifuged through a 150-µl cushion of 1 M KCl and then reacted with 0.1 mg/ml BSA. After that, 3 µl of anti-ankyrin antiserum or nonimmune serum were added, and the proteins were incubated for an additional 30 min at 23°C. 20 µl of a Staphylococcus aureus suspension were then pipetted and, after 15 min, the samples were centrifuged (5 min in a minifuge). Pellets were washed five times with 100 mM KCl, 10 mM Tris, 0.1 mM PMSF (pH 7.4) containing 0.06% Triton X-100. The combined supernates and the pellets were then counted in a gamma counter.

Electrophoresis: One- and two-dimensional electrophoresis was performed as previously described (8, 9).

RESULTS

Vimentin Binds to IOVs through its Head Domain

Treatment of vimentin with NTCB is known to cleave the polypeptide chain at a single cysteine residue, 137 amino acids from the COOH-terminal, and to separate the molecule into two segments. One (C-I) contains the amino-terminal head domain and part of the middle domain and the second (C-II) is composed of the tail region and the remainder of the middle segment (2, 3). When the two NTCB fragments of vimentin are incubated with IOVs under physiological conditions, only one, the C-I fragment binds selectively to the surfaces of the IOVs (Fig. 1). The specificity of the binding of this 37,000-mol-wt fragment to IOVs is demonstrated by showing that unlabeled intact vimentin molecules were able to quantitatively displace both labeled intact vimentin and the labeled C-I peptide (Fig. 2). The data shown in Fig. 2 show that unlabeled intact vimentin molecules are able to displace labeled vimentin molecules to a greater extent than the labeled C-I peptides, as would be expected from the difference in molar concentrations of the two peptides. (The molar ratio between C-I and uncleaved vimentin in this particular digest was estimated to 1.52:1). It is worth noting that some products of endogenous degradation (probably lacking the head domain and migrating between uncleaved vimentin and C-I or between Cl and CII) also partition with the supernate.

Since the C-I peptide derived from vimentin is composed of both the head domain of the molecule and part of the middle segment, further analysis was carried out using limited chymotryptic digestion. Brief incubations of intact vimentin with low concentrations of chymotrypsin at room temperature produced a series of peptides with molecular weights of 55,000, 50,000, 47,000, and 45,000. This characteristic "stair-
Figure 2. Displacement of $^{125}$-vimentin NTCB fragments bound to IOVs by unlabeled, intact vimentin. 20 μg of IOVs were incubated with a standard amount of NTCB fragments of $^{125}$-vimentin (1.1 μg) and 0 μg (2), 1.5 μg (3), 3.5 μg (4), and 5.25 μg (5) of unlabeled, intact vimentin in PBS (pH 7.4), and in a final volume of 100 μl. Sample 1 contained only IOVs. All five samples were processed as in Fig. 1. (Panel A) 1-5 (left), pellets; 1-5 (right), supernates (Coomassie-stained gel). (Panel B) Corresponding autoradiogram. Arrowheads denote (from top to bottom) the positions of intact $^{125}$-vimentin, $C_o$, and $C_i$.

Figure 3. Binding of chymotryptic fragments of vimentin to IOVs. 15 μg of purified vimentin were digested at room temperature for various times with α-chymotrypsin. The reaction was stopped by the addition of PMSF to a final concentration of 1.5–2.0 mM. The digests were incubated with IOVs in PBS, 0.5 mM PMSF for 90 min at 23°C, processed as in Fig. 1, analyzed by SDS PAGE, and stained by Coomassie Blue. (Panel A) (1) 55 μg of IOVs incubated with a blank sample (no vimentin; α-chymotrypsin inhibited by PMSF); (2 and 7) 55 μg of IOVs incubated with a 30-min vimentin digest; (3 and 6) 55 μg of IOVs incubated with a 10-min digest; (4 and 5) 55 μg of IOVs incubated with 15 μg of undigested vimentin. 1–4 correspond to the membrane pellets and 5–7 to the supernates. The position of band 6 is indicated by a dash. Arrows point to the two major vimentin peptides with molecular weights of 45,000 and 22,000 (10% gel). (Panel B) (1) 20 μg of IOVs incubated with a blank sample as in A. (2 and 5) 20 μg of IOVs and 10 μg of a 30-s digest; (3 and 6) 20 μg of IOVs and a 1-min digest; (4 and 7) 20 μg of vimentin and a 2-min digest. 1-4 correspond to the membrane pellets and 5-7 to the supernates (7.5–15% gradient gel). Arrows indicate the major early fragments with apparent molecular weights of 50,000, 47,000, and 45,000. Band 6 is indicated by dash.
Case pattern of vimentin degradation results from a gradual cleavage of the vimentin molecule from the basic amino-terminal end as indicated by two-dimensional gel electrophoresis (not shown; see reference 12). More extensive degradation of vimentin produced mainly a 45,000-mol-wt peptide, analogous to desmin's 40,000-mol-wt middle piece (2, 4), then cleaved into a 22,000-mol-wt broad band analogous to the 21,000- and 18,000-mol-wt subfragments of desmin derived from the middle segment. After appropriate inhibition of residual chymotryptic activity, the different digest fractions were analyzed for their capacity to bind to IOVs. Of the digests analyzed, no fragment showed appreciable capacity to bind to IOVs (Fig. 3), a result consistent with the idea that the active site of vimentin is contained within the protease-sensitive amino-terminal domain and not in the protease-resistant middle piece.

**Ankyrin Inhibits the Assembly of Intermediate Filaments In Vitro**

Vimentin and ankyrin form a specific complex in vitro that can be demonstrated by sucrose gradient centrifugation (Fig. 4). Vimentin alone at 100 μg/ml sediments predominantly as a 6–7S species under physiologic salt and pH conditions. The addition of excess ankyrin causes a significant shift in the migrating peak (Fig. 4). Incubation of vimentin preparations with threefold excess ankyrin also causes a significant decrease in the amount of pelleted (filamentous) vimentin that is formed under these conditions.

The association between ankyrin and vimentin was also analyzed using a radioimmunoassay with anti-ankyrin antibodies. The data in Fig. 5 show that the association between vimentin and ankyrin was concentration-dependent and saturable, and corresponded roughly to a 1:1 molar stoichiometry, assuming a tetrameric organization for the vimentin oligomer (4).

Based on the results of the sedimentation experiments and the fact that the head domain of the vimentin molecule seemed to bind specifically to IOVs, we considered it likely that ankyrin might affect the capacity of vimentin molecules to polymerize into intermediate filaments by binding to the head domains of the protofilaments and thereby blocking the arginine-rich regions that are thought to be involved in the assembly process (5). This question was approached by two independent methods. Vimentin was assembled in vitro in the presence of increasing amounts of ankyrin, and the relative amounts of polymer that were sedimented were correlated with the ankyrin-vimentin ratio in the original reaction mixture. The data in Fig. 6 show that there is a decrease in the relative amount of vimentin species greater than 54S as a function of increasing ankyrin concentrations. In contrast, if vimentin polymers were preassembled and then incubated with increasing amounts of ankyrin for the same time intervals, such a decrease was not detected (Table I). These results are further supported by the fact that only small amounts of ankyrin co-pelleted with prepolymerized vimentin under the same conditions (not shown).
The capacity of ankyrin to inhibit the polymerization of vimentin into intermediate filaments was demonstrated most strikingly by analyzing preparations of ankyrin and vimentin by negative staining and electron microscopy (Fig. 7). Characteristic 10-nm filaments were readily demonstrated by incubation of vimentin alone (Fig. 7A), whereas no recognizable filament forms were seen when ankyrin and vimentin were incubated together before negative staining (Fig. 7, B and C). Instead, short rod-like forms were seen scattered throughout the fields. The rod-like forms had the same approximate diameter of vimentin filaments (10–11 nm); this is consistent with the idea that lateral association of protofilament units did occur, but the elongation of protofilaments was inhibited by the presence of ankyrin.

The purification and radiolabeling of vimentin are shown in Fig. 8.

**DISCUSSION**

The results described here and in the preceding paper (1) provide new insight into how and where intermediate filaments may attach to plasma membranes. Intermediate filaments composed of vimentin are able to bind to IOVs prepared from human erythrocytes, and they appear to do so by associating to an attachment protein, previously identified as ankyrin, which also links other components of the cytoskel-

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**Table I**

| Concentration of vimentin (μg/ml) | Ankyrin/Vimentin* | Percent pelletable vimentin |
|----------------------------------|-------------------|----------------------------|
| 60                               | 0                 | 8.3                        |
| 60                               | 0.05              | 9.4                        |
| 60                               | 0.35              | 9.3                        |
| 100                              | 0                 | 20                         |
| 100                              | 0.05              | 18.9                       |
| 100                              | 0.35              | 16.4                       |
| 200                              | 0                 | 38                         |
| 200                              | 0.35              | 40                         |

* Vimentin was preassembled in isotonic KCl buffer for 1 h at 23°C and then co-incubated with isolated ankyrin for additional 40 min. Samples were processed as described in Materials and Methods.

* In molar terms.

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**Table**

**Effect of Ankyrin on the Stability of Pre-assembled Vimentin Polymers under In Vitro Conditions**

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**Figure 7** The effect of ankyrin on vimentin polymerization as detected by negative staining and electron microscopy. (A) Vimentin alone (300 μg/ml). (B) Vimentin (300 μg/ml) and ankyrin at a molar ratio of 4:1. (C) Vimentin and ankyrin at a molar ratio of 2:1. × 42,000.
vivo, it seems logical to hypothesize that membrane-bound vimentin does not provide nucleating centers for intermediate filament assembly, since the ankyrin-vimentin association would be expected to inhibit filament growth at the binding ends. Thus, centers for nucleation of vimentin filaments that are attached to membranes should be located elsewhere inside cells, possibly at the nuclear envelope. It follows that native vimentin intermediate filaments contain distinct nucleation sites and membrane attachment sites, such filaments also assume a distinct polarity within the cell. If only the ends of vimentin filaments are capable of binding to membrane-bound ankyrin sites, a limited number of such sites will be required to attach the number of vimentin filaments usually found inside cells, and this prediction agrees with earlier morphological studies in which it has been demonstrated that in certain vimentin-rich cells such as avian erythrocytes, the intermediate filaments associate at a relatively few foci along the plasma membrane rather than being diffusely distributed over the entire cell surface (13).

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