Tenacious binding of lipids to vimentin during its isolation and purification from Ehrlich ascites tumor cells

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Vimentin enriched in cytoskeletal frameworks by Triton X-100 extraction of Ehrlich ascites tumor cells and purified from a low ionic strength extract of the cell residues by $(NH_4)_2SO_4$ precipitation and DEAE-Sepharose and ssDNA-cellulose chromatography in the presence of 6 M urea was highly contaminated with lipids. Thin-layer chromatography of a chloroform-methanol extract of the purified protein revealed, besides small amounts of phospholipids, the presence of large quantities of neutral lipids.

Intermediate filament *Vimentin* *Triton cytoskeleton* *Lipid* *(Ehrlich ascites tumor cell)*

1. INTRODUCTION

The isolation of intermediate filament (IF) subunit proteins usually employs, in a first step, extraction of cultured cells or tissues with non-ionic detergents in the presence of high concentrations of mono- or divalent cations [1–10]. The enrichment of intermediate filaments in the resulting detergent-resistant residual cell structures or cytoskeletal frameworks is mainly due to their high insolubility under these ionic conditions. During removal of most of the membrane lipids and substantial fractions of cytoplasmic materials, the filaments collapse onto the nucleus from where they can be released either with urea or, in the case of non-epithelial IF proteins, with low ionic strength buffers containing EDTA [5,11,12].

For the recovery of the mesenchyme-specific IF protein vimentin from such low ionic strength extracts, we routinely employed an $(NH_4)_2SO_4$ precipitation step [11,12]. Although a high concentration of Triton X-100 had been used for the initial extraction of, for instance, cultured Ehrlich ascites tumor (EAT) cells, the precipitated protein still contained substantial amounts of lipids [11]. Approx. 50% of the isolated vimentin accumulated in the meniscus fraction upon KBr density gradient equilibrium centrifugation. Here, we present evidence that the transfer of vimentin to the top of KBr gradients is not due to its indirect interaction with other lipid-associated proteins but is the result of its strong affinity particularly for neutral lipids.

2. MATERIALS AND METHODS

2.1. *Preparation of vimentin from EAT cells*

EAT cells were grown in minimum essential medium supplemented with 5% heat-inactivated calf serum to a density of 1.4×10^6 cells/ml [13]. The extraction of 400 g frozen cells with 10 mM Tris-HCl, pH 7.6, 1 mM EGTA, 4 mM $MgCl_2$, 6 mM 2-mercaptoethanol, 0.5% Triton X-100, the release of vimentin from the resulting cytoskeletal frameworks with 10 mM Tris-HCl, pH 7.6, 1 mM EGTA, 6 mM 2-mercaptoethanol and $(NH_4)_2SO_4$ fractionation of the solubilized protein were carried out as described [11,12]. The $(NH_4)_2SO_4$ precipitate was dissolved in 10 mM Tris-HCl, pH 7.6, 3 mM EDTA, 6 mM 2-mercaptoethanol, 6 M urea and dialysed against the same buffer overnight. The protein solution was applied to a 27 × 3 cm DEAE-Sepharose CL-6B column (Pharmacia, Uppsala) [11] and bound material was

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eluted with a 2 l linear 0–400 mM KCl gradient in Tris/EDTA/urea buffer. Vimentin-containing fractions were combined, dialysed against Tris/EDTA/urea buffer and passed through a 42 × 2 cm ssDNA-cellulose column [12]. Vimentin was eluted with a 2 l linear 0–300 mM KCl gradient in Tris/EDTA/urea buffer. After removal of urea by extensive dialysis against distilled H_2O, the vimentin solution was lyophilized. The yield was 350 mg protein.

2.2. *Extraction of vimentin with chloroform-methanol*

The dry powder was extracted 5 times with 35-ml portions of chloroform-methanol (2:1, v/v) by sonication at room temperature. Interval centrifugation was at 20000 × g_{av} for 5 min. The delipidated protein was dried in vacuo, dissolved in Tris/EDTA/urea buffer, dialysed against distilled H_2O, lyophilized and again extracted with 3 35-ml portions of chloroform-methanol (2:1). Following evaporation of the combined chloroform-methanol extracts to dryness in a rotary evaporator, the residue was dissolved in 20 ml chloroform. The solution was washed with 30 ml of 0.1 M KCl according to Folch et al. [14] and evaporated to dryness. Finally, the slightly yellow lipid residue was taken up in 1 ml chloroform and kept under nitrogen at 0°C.

2.3. *Thin-layer chromatography*

Phospholipid analysis was carried out on 10 × 20 cm precoated (silica gel 60) HPTLC plates (Merck, Darmstadt) in the solvent system chloroform:methanol:glacial acetic acid:water (52.5:50:4:1) [15], neutral lipid analysis on 20 × 20 cm precoated silica gel TLC plates (Merck) in the tandem system benzene:2-propanol:ethyl acetate:glacial acetic acid (72.5:3.5:22:2) and carbon tetrachloride:benzene (30:70) [16]. The lipid bands were made visible by exposure of the thin-layer plates to iodine vapor. Lipid standards used for lipid identification were purchased from Sigma (St. Louis, MO).

2.4. *Other methods*

SDS-polyacrylamide gradient slab gel electrophoresis of chromatography fractions and purified vimentin was performed as in [13]. For KBr density gradient equilibrium centrifugation, a small fraction of the $(NH_4)_2SO_4$-precipitated vimentin was dissolved in 500 μl of 10 mM Tris-HCl, pH 7.6, 1 mM EGTA, 6 mM 2-mercaptoethanol by sonication and mixed with 12 ml of a solution obtained by dissolving 46.7 g KBr in 100 ml Tris/EGTA buffer; centrifugation was at 35000 rpm and 2°C for 65 h in the SW 40 Ti rotor of a Beckman L2 65B centrifuge.

3. RESULTS AND DISCUSSION

When crude vimentin obtained from Triton X-100-resistant residual cell structures by extraction with low ionic strength buffer and fractionation of the extract with $(NH_4)_2SO_4$ was subjected to KBr density gradient equilibrium centrifugation, 52.7% of the filament protein was concentrated in a thin film floating on top of the KBr gradient; the remainder banded at a density of ϱ = 1.300 g/cm^3 (fig.1). Since extraction of the $(NH_4)_2SO_4$-precipitated vimentin with chloroform-methanol (2:1) prevented its accumulation in the meniscus fraction (not shown), it had to be assumed that the original, non-delipidated material was carried to the top of the KBr density gradient by lipids. Indeed, TLC analysis of a chloroform-methanol (2:1) extract of crude vimentin revealed the presence of substantial amounts of lipids (not shown).

To demonstrate that the accumulation of vimentin in the meniscus fraction was not due to its interaction with lipid-carrying membrane proteins that co-fractionated with vimentin, the filament protein was purified by DEAE-Sepharose CL-6B [11] and ssDNA-cellulose chromatography [12] in the presence of 6 M urea. The electrophoresis pattern of fig.2 shows that all contaminating proteins had been removed. When this purified material was extracted with chloroform-methanol (2:1) and the extract subjected to TLC, substantial quantities of lipids could still be detected. While fig.3 shows the phospholipid pattern, fig.4 presents the profile of neutral lipids. It is evident from both chromatograms that vimentin preferentially associated with neutral lipids. Using standard lipids for comparison, mainly phosphatidylcholine, sphingomyelin and phosphatidylethanolamine besides smaller amounts of phosphatidylserine and phosphatidylinositol could definitely be identified among the phospholipids. The

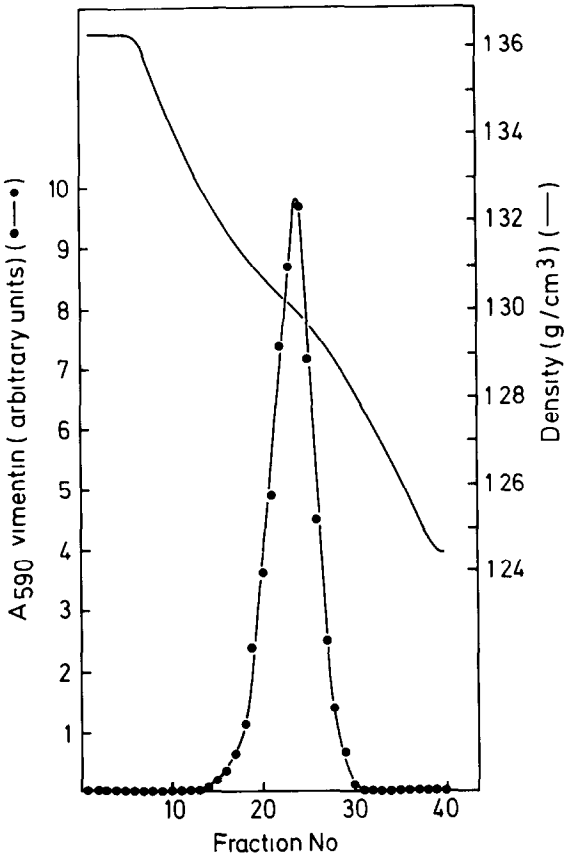

Fig.1. KBr density gradient equilibrium centrifugation of vimentin released from Triton cytoskeletons of EAT cells with low ionic strength buffer and concentrated with $(NH_4)_2SO_4$ at 25% saturation. 52.7% of the total amount of vimentin accumulated in the meniscus fraction of the density gradient (not shown).

neutral lipid fraction consisted of mono-, di- and triglycerides and, very surprisingly, unusually large quantities of cholesterol and cholesterol fatty acid esters. Some of the neutral lipids could not be identified but from the differential kinetics of their staining with iodine vapor it might be deduced that particularly the strongly staining, fast moving lipids are cholesterol derivatives. There were at most only traces of Triton X-100 in the lipid extract. We wish to emphasize that the heavy loading of vimentin with lipids is probably artifactual. Very likely, the filament protein came into contact with lipids only after these had been solubilized by Triton X-100 during the preparation of Triton

Fig.2. SDS-polyacrylamide gradient slab gel electrophoresis of vimentin after purification by DEAE-Sepharose and ssDNA-cellulose chromatography in the presence of 6 M urea and before delipidation with chloroform-methanol.

cytoskeletons. It cannot be excluded, however, that due to its high binding potential, also in vivo a minor but distinct fraction of vimentin is complexed by lipids (see below).

It should also be noted here that, on the basis of the present findings, we undertook a detailed study on the association of non-epithelial IF proteins with individual lipid classes and mixtures thereof. The results of this investigation indeed showed that all these proteins have high affinities for various types of lipids. In addition, their interactions with octyl-Sepharose 4B were found to be extremely resistant to denaturing reagents (to be published). Of course, these results also had practical consequences. Since the biochemical characterization of IF proteins in terms of their affinities for other cellular constituents might be considerably hampered by lipid contamination, we routinely include a delipidation step in the purification of IF subunit proteins [10,11,17,18].

Considering that EAT cells were extensively extracted with Triton X-100 and the vimentin released from the resulting cell residues was purified to homogeneity in the presence of 6 M urea, it is astonishing how much lipid material was still attached to the filament protein. Obviously, vimentin is capable of forming tight association products with hydrophobic molecules, a property which is probably of considerable physiological relevance. In this context, it is noteworthy that in electron microscopy intermediate filaments are often seen to be in close contact with intracellular membrane systems such as the plasma membrane, the endo- and sarcoplasmic reticulum, the nuclear envelope, the outer mitochondrial membrane and a series of cytoplasmic vesicular structures (review [19]). It is conceivable that these associations are brought about by direct interaction of hydrophobic domains, notably the α-helical rod domains, of IF

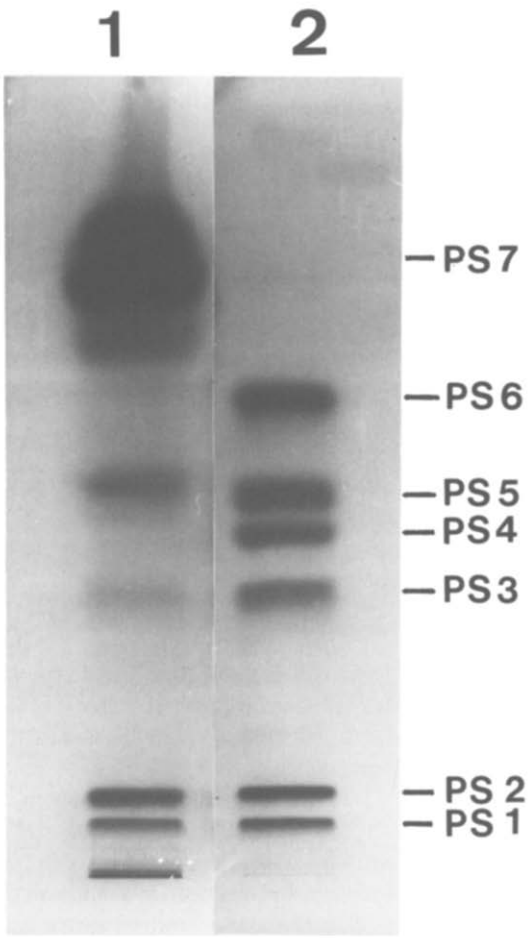

Fig.3. Phospholipid analysis of a chloroform-methanol (2:1) extract of vimentin purified by DEAE-Sepharose and ssDNA-cellulose chromatography in the presence of 6 M urea. For TLC of 250 μl extract (from 100 mg vimentin) on 10 × 20 cm precoated silica gel HPTLC plates, the solvent system chloroform:methanol:glacial acetic acid:water (52.5:50:4:1) was used. Lanes: (1) vimentin extract, the heavily staining bands at the top of the chromatogram represent neutral lipids; (2) phospholipid standards (PS1, sphingomyelin; PS2, phosphatidylcholine; PS3, phosphatidylserine; PS4, phosphatidylinositol; PS5, phosphatidylethanolamine; PS6, phosphatidic acid; PS7, phosphatidylglycerol). All lipid standards were obtained from natural sources and contained, therefore, unsaturated fatty acids; 100 μg of each reference compound was used. Lipid bands were made visible with iodine vapor.

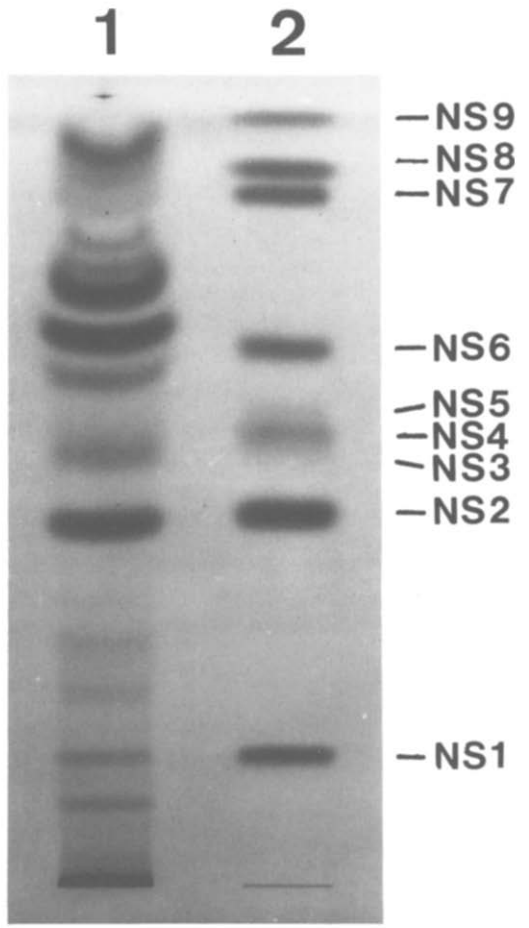

Fig.4. Neutral lipid analysis of a chloroform-methanol (2:1) extract of vimentin purified by DEAE-Sepharose and ssDNA-cellulose chromatography in the presence of 6 M urea. For TLC of 125 μl extract (from 50 mg vimentin) on 20 × 20 cm precoated silica gel TLC plates, the solvent systems benzene:2-propanol:ethyl acetate:glacial acetic acid (72.5:3.5:22:2) and carbon tetrachloride:benzene (30:70) were used in tandem. Lanes: (1) vimentin extract, the distortion and retardation of the bands at the top of the profile are due to the presence of unknown, chloroform-soluble, fast-moving compounds which are not stainable with iodine; phospholipids stayed at the origin; (2) neutral lipid standards (NS1, 80 μg 1-monooleoylglycerol; NS2, 100 μg cholesterol; NS3, 250 μg palmitic acid; NS4, 150 μg 1,2-dipalmitoylglycerol; NS5, 12 μg 1,2-dioleoylglycerol; NS6, 70 μg 1,3-dioleoylglycerol; NS7, 80 μg trioleoylglycerol; NS8, 100 μg cholesteryl butyrate; NS9, 50 μg cholesteryl oleate). The thin layer plates were stained with iodine vapor.

proteins with the lipid bilayer of the various membranes. Such a relationship has been suggested by Ramaekers et al. [20] to exist between lens membranes and vimentin newly synthesized in a reticulocyte cell-free system. However, these associations seem to be different from those interactions occurring between vimentin (filaments) and the plasma membrane of erythrocytes; they are mediated by the peripheral membrane protein ankyrin [21,22].

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