The Binding of Vimentin to Human Erythrocyte Membranes: A Model System for the Study of Intermediate Filament–Membrane Interactions

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ABSTRACT We have characterized the association of the intermediate filament protein, vimentin, with the plasma membrane, using radioiodinated lens vimentin and various preparations of human erythrocyte membrane vesicles. Inside-out membrane vesicles (IOVs), depleted of spectrin and actin, bind I^{125}-vimentin in a saturable manner unlike resealed, right-side-out membranes which bind negligible amounts of vimentin in an unsaturable fashion. The binding of vimentin to IOVs is abolished by trypsin or acid treatment of the vesicles. Extraction of protein 4.1 or reconstitution of the membranes with purified spectrin do not basically affect the association. However, removal of ankyrin (band 2.1) significantly lowers the binding. Upon reconstitution of depleted vesicles with purified ankyrin, the vimentin binding function is restored. If ankyrin is added in excess the binding of vimentin to IOVs is quantitatively inhibited, whereas protein 4.1, the cytoplasmic fragment of band 3, band 6, band 4.5 (catalase), or bovine serum albumin do not influence it. Preincubation of the IOVs with a polyclonal anti-ankyrin antibody blocks 90% of the binding. Preimmune sera and antibodies against spectrin, protein 4.1, glycophorin A, and band 3 exhibit no effect. On the basis of these data, we propose that vimentin is able to associate specifically with the erythrocyte membrane skeleton and that ankyrin constitutes its major attachment site.

Eucaryotic cells possess a complex interconnecting network of microfilaments and tubules that seem to be involved in a variety of diverse functions (cell shape, contractility, locomotion, etc.). This network, referred to as the cytoskeleton, organizes the cell interior into three topologically distinct domains. The bulk of the cytoskeleton makes up a matrix which extends throughout the cytoplasm, linking neighboring organelles together and providing some internal order which may serve to coordinate activities of the different components (1).

A special subset of cytoskeletal proteins, some with features quite unlike those that make up the cytoplasmic matrix, are concentrated in the submembranous regions of cells and have come to be known as the membrane skeleton (2–8). In addition to providing structural stability to the overlying lipid bilayer (a function most pronounced in the case of the membrane skeletons of erythrocytes), the membrane skeleton occupies a critical position situated as it is at the interface between the surface membrane and the cell interior, and it is likely that it plays some role in relaying signals from the cell surface to different effector systems located in the cytoplasm or the nucleus.

A third region where cytoskeletal elements are concentrated is the perinuclear region. This especially applied to intermediate filaments and microtubules (1, 9–11). Thus, the interface between the nucleoplasm and the cytoplasm might be considered analogous in some ways to the cell surface membrane skeleton.

The mechanisms of assembly of the macromolecular complexes of each cytoskeletal subdomain also seem to have characteristic features. The subunits of the cytoplasmic matrix cytoskeleton assemble via mass-action–driven polymerization reactions (12). The individual molecules are designed for self-assembly and the processes are regulated by the local ionic milieu, metabolic co-factors, and accessory proteins (13).

However, our understanding of the factors that control the

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assembly of the membrane skeleton and the perinuclear cytoskeleton are less clear, since in both cases filaments must link up with membranes. With respect to intermediate-sized (or 10-nm) filaments, specific contacts have been morphologically identified in specialized regions of the plasma membrane such as the desmosomal plaques (14). Also, in lens and avian erythrocytes there is some evidence in favor of a side-on or end-on attachment of the filaments to unspecialized regions of the plasma membrane (15, 16, 17). Still, the precise molecular mechanism of these interactions remains obscure.

We have found that inverted human erythrocyte membrane vesicles, or inside-out membrane vesicles, (IOVs)[1] is a useful model system to explore the factors that regulate interactions between the intermediate filament subunits and membranes, because these membranes retain binding sites for the intermediate filament protein, vimentin. This protein is normally present in appreciable amounts in developing erythroblasts but not in the mature mammalian erythrocytes (18, 19). The binding of vimentin to IOVs can be studied under in vitro conditions, and the search for the receptors involved has been simplified by the fact that IOVs contain a relatively small number of protein classes.

We found that human erythrocyte IOVs bind vimentin by a specific high-affinity association with ankyrin, one of the proteins that also links spectrin to the inner surface of the membrane.

MATERIALS AND METHODS

Chemicals: Ultrapure urea, ammonium sulfate, and Tris (base) were purchased from Schwarz/Mann (Spring Valley, NY), trypsin from Miles Laboratories Inc. (Elkhart, IN), bovine serum albumin (BSA) (RIA grade) from Sigma Chemical Co. (St. Louis, MO), DEAE-cellulose from Whatman Laboratory Products Inc., (Maidstone, Kent, England), and Bolton-Hunter reagent-^{125}I from Amersham Corp. (Arlington Heights, IL).

Membranes: IOVs were obtained after a 20-min extraction of washed erythrocyte ghosts with 30–40 vol of 0.3 mM $NaPO_4$, 0.1 mM EDTA, and 1 mM phenylmethylsulfonyl fluoride (PMSF) (pH 9.0) at 37°C. Resealed ghosts were prepared by incubating erythrocyte ghosts in 20 vol of phosphate-buffered saline (PBS), 1 mM PMSF for 20 min, at 37°C. Right-side-out vesicles were made as previously described (20) except that the medium also contained 2 mM $MgCl_2$. "Stripping" of the IOVs or partial removal of protein 4.1 was achieved by the method of Hargreaves et al. (21). All preparations were kept in 5 mM $NaPO_4$, 1 mM PMSF on ice for a maximum of 6 d. Human blood was collected from normal donors while bovine blood was obtained from a local slaughterhouse.

Protein Purification: The purification, characterization, and radiolabeling of calf lens vimentin are described in the accompanying report. Vimentin at 100 μg/ml was primarily (84%) migrating as a homogeneous soluble species with an S coefficient of 6–75 in isokinetic sucrose gradients. Protein 4.1 and ankyrin were extracted from IOVs by 1 M KCl and further purified by ion-exchange chromatography (22). Band 4.5 was purified from ghosts obtained after lysis in 2 mM $MgCl_2$, 5 mM $NaPO_4$, subsequent extraction with 0.3 mM $NaPO_4$, 2 mM EDTA, and finally, ion-exchange chromatography (Georgatos, S. D., manuscript in preparation). Partially purified band 6 was prepared after a 20-min extraction of erythrocyte ghosts with 20 vol of PBS, 1 mM PMSF at 0°C. The cytoplasmic 43,000-mol-wt peptide of band 3 was isolated according to the method of Bennet and Stenbuck (23).

Antibodies: The characterization of anti–band 3, anti–glycophorin A, and anti–spectrin are reported elsewhere (24, 25, 26). The characterization of anti-protein 4.1 is to be reported elsewhere (Leto, T., and V. T. Marchesi, manuscript in preparation).

Reconstitution of IOVs: For spectrin reconstitution, 500 μg/ml of IOVs were incubated with 112 μg/ml of column-purified spectrin for 60 min at 4°C in 150 mM KCl, 5 mM Tris-HCl, 0.03 mM PMSF (pH 7.4). The membranes were pelleted and then washed twice with the same media. For ankyrin reconstitution, 500 μg/ml of stripped IOVs were incubated as above with 90 μg/ml of purified ankyrin with the exception that PMSF at 0.1 mM and 2 mM 2-mercaptoethanol were also included. Membranes were washed as previously described. Both preparations were used immediately for binding studies.

Binding Assays: In general, 20 μg of membranes were mixed and purified ^{125}I-vimentin (50,000–70,000 cpm/μg) in 150 mM KCl, 5 mM Tris-HCl, 2 mM $MgCl_2$, 0.03 mM PMSF (pH 7.4), or PBS at a final volume of 100–150 μl. 0.1 mg/ml of BSA was also included to prevent adsorption to the plastic. Vimentin was kept in low salt on ice at 0.1 mg/ml and it was adjusted to the proper salt concentration immediately prior to the assay. The mixture was incubated for 90 min at 23°C and then transferred to hard polyethylene 400-μl tubes and layered over a 150-μl sucrose cushion (4% sucrose [isotonic]). Samples were centrifuged for exactly 12 min in a mini-fuge (15,000 g), frozen in liquid N_2, and the bottoms of the tubes dissected with a razor blade. The pellet containing tips and the rest of the tube were counted in a gamma counter (supernate and pellet).

The pelleting system was prestandardized as follows. Blank samples containing only vimentin or only membranes were processed as described above. After freezing in liquid N_2, the tubes were serially dissected and the pieces counted for radioactivity or analyzed by SDS PAGE. In a different series of pretests, blank samples containing buffer and a trace quantity of bromophenol blue were pelleted, dissected, and analyzed spectrophotometrically at 700 nm. From such assays it was found that only 0.3% of ^{125}I-vimentin was sedimenting in the absence of membranes, >95% of the vesicles were pelleted after a 12-min spin, and no dye had penetrated the sucrose cushion by the end of the run.

Competition Assays: I^{125}-vimentin (10 μg/ml; 50,000 cpm/μg) was mixed with 20 μg of IOVs and increasing amounts of various membrane polypeptides in isotonic salt (pH 7.4) at a final volume of 100 μl. Samples were processed for quantitation as described above in *Binding Assays.*

Blocking by Antibodies: Increasing amounts of specific antibodies up to saturating levels (empirically determined by SDS PAGE) were added to 20 μg of IOVs in PBS, 0.1 mM PMSF. After an incubation period of 60 min at 4°C, equal amounts of ^{125}I-vimentin (12 μg/ml) were added and the system was allowed to equilibrate for another 90 min at 23°C. Bound and nonbound vimentin was quantitated as previously described.

As assays were executed in triplicate, except those shown in Fig. 1 (six independent observations) and in Fig. 7 (duplicates).

Immunoblotting: 10% polyacrylamide SDS gels were transferred electrophoretically to nitrocellulose filters for 24 h. After transfer, the filters were washed in isotonic buffered saline and then incubated for 1 h at 37°C, and then again for 24 h at room temperature, with 3% BSA. Incubation with a 1:100 dilution of anti-ankyrin followed for 8 h at room temperature. After washing (6 changes of buffer), ^{125}I-protein A from *Staphylococceus aureus* was added and the filters were incubated for 4 h. Finally, the nitrocellulose was washed for 6 h with buffer (6 changes) and for another 8 h with 0.01% Triton X-100. Dried filters were exposed at −70°C for 5 d.

Electrophoresis: Regular 10% gels or 5–10% gradient gels were prepared according to the method of Laemmli (27). Two-dimensional gels were made as described by O'Farrell (28).

Protein Determinations: Protein was measured according to Lowry (29) using BSA as a standard.

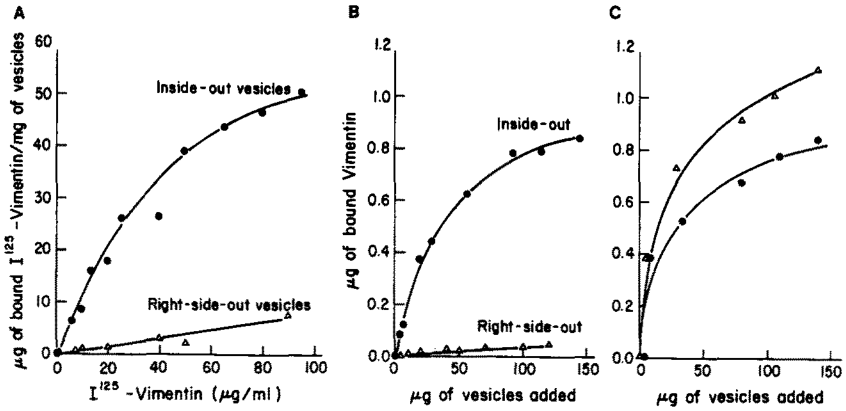
RESULTS

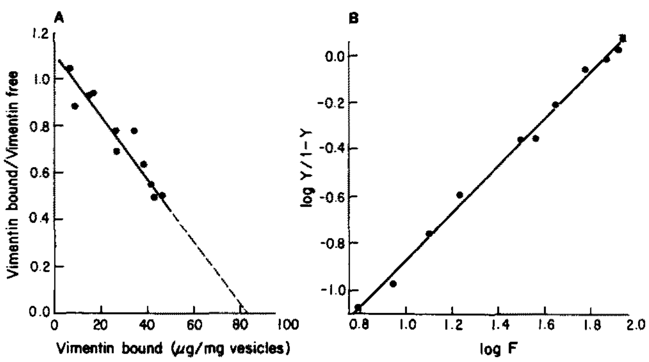
Vimentin Binds Specifically to the Inner Surface of the Human Erythrocyte Membranes

Purified I^{125}-vimentin (see accompanying report) binds to IOVs, prepared from human erythrocyte membranes, in a concentration-dependent, saturable manner. In contrast, the binding of vimentin to either resealed ghost membranes or right-side-out membrane vesicles is nonsaturable and in an order of magnitude less than to IOVs (Fig. 1*A*). When vimentin was incubated with saturating amounts of IOVs, >80% of the intermediate filament protein bound to the membranes (Fig. 1*B*), indicating that the vimentin bound to the erythrocyte IOVs is not a special subset of vimentin molecules. The polymerization state of vimentin played some role in the capacity of vimentin to bind to IOVs. Vimentin prepolymerized before being exposed to IOVs bound to a lesser extent than nonprepolymerized material when assessed under non-equilibrium conditions. Under these conditions, the vimentin

[1] *Abbreviations used in this paper:* IOVs, inside-out membrane vesicles; PMSF, phenylmethylsulfonyl fluoride.

FIGURE 1 (A) Binding of purified ^{125}I-vimentin to IOVs or right-side-out vesicles. For details, see Materials and Methods. (Resealed ghosts gave similar values as the right-side-out vesicles.) (B) The vimentin-binding capacities of IOVs and right-side-out vesicles. The assay volume was 100 μl and the final vimentin concentration 9.8 μg/ml. (C) The effect of vimentin's polymerization state in the binding. ^{125}I-Vimentin at 200 μg/ml was divided in two. One half was kept on ice in 5 mM $NaPO_4$, 0.03 mM PMSF, 2 mM 2-mercaptoethanol (pH 7.5) while the other was induced to polymerize by (a) the addition of salt (final concentrations 1× PBS, 0.03 mM PMSF, 2 mM 2-mercaptoethanol [pH 7.5]) and (b) an incubation of 1 h at 23°C. An aliquot was taken from each preparation and mixed with increasing quantities of IOVs to give a final vimentin concentration of 60 μg/ml. The samples were incubated in phosphate-buffered saline for 10 min at 23°C and then processed as described in Materials and Methods. Δ, nonassembled vimentin; ●, pre-assembled vimentin.





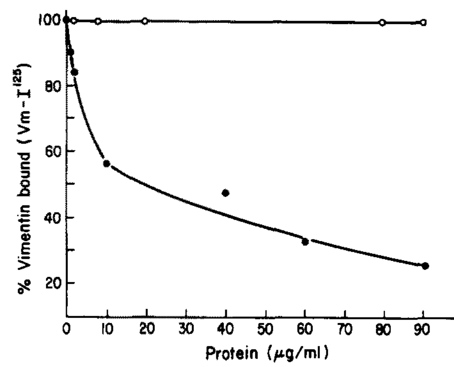


FIGURE 2 (A) Analysis of the binding data presented in Fig. 1A according to Scatchard (43). The nonspecific binding has been subtracted. (B) Analysis of the binding data according to Hill. The Hill coefficient is estimated to be 1.007.

FIGURE 3 Competition between ^{125}I-vimentin, unlabeled vimentin (●), and BSA (○) for binding to IOVs. Standard amounts of ^{125}I-vimentin (final concentration: 10 μg/ml) were added to 20 μg of IOV together with increasing amounts of unlabeled vimentin or BSA. Bound ^{125}I-vimentin was quantitated as above. "100% binding" was assigned to the samples containing only ^{125}I-vimentin and IOVS.

concentration and the quantity of membrane vesicles were exactly the same (Fig. 1C).

An analysis of vimentin binding to IOVs at equilibrium (see below) using the Scatchard plot (Fig. 2A) suggested that vimentin bound to a single class of acceptor sites, present at an approximate concentration of 80 μg/mg of IOVs. Assuming that the smallest vimentin aggregate in solution under the conditions of incubation was a tetramer (30), the K_D was estimated to be 3×10^{-7} M. Plotting the data according to Hill (Fig. 2B) revealed no apparent cooperativity in the binding process (Hill coefficient 1.007). Additional evidence for the specificity and saturability of the binding of vimentin to IOVs was provided by quantitative competition assays, such as the ones shown in Fig. 3. The amount of iodinated vimentin that binds to erythrocyte IOVs can be almost completely blocked by the addition of unlabeled vimentin, with a calculated Ki of approximately 6×10^{-7} M. Comparable amounts of BSA (an acidic protein of approximately the same size as vimentin) were completely ineffective in influencing the binding of vimentin to IOVs, as were other unrelated membrane proteins, described below.

The effects of neutral salt solutions on the binding of vimentin to IOVs is complicated and dependent upon the concentration of vimentin used in the assays. At low vimentin concentration (10 μg/ml), binding to IOVs increased slightly when salt concentrations were raised to ~150 mM, but less protein remained bound with the membranes after salt concentration exceeded 200 mM (Fig. 4A). Magnesium ions did not influence the binding. When the incubations were carried out at higher vimentin concentrations (36 μg/ml), more vimentin was bound to IOVs in the low salt solutions (Fig. 4B) than in higher salt. Since it has been reported that vimentin polymerization does not occur below 20 mM salt (31), this experiment is consistent with the results using prepolymerized vimentin described above. When dilute solutions of vimentin were incubated with membranes, vimentin polymers would not be expected to form because of low protein concentration, and it is likely that binding under that condition reflected the true salt dependency of the vimentin-membrane interaction. When higher concentrations of vimentin were used in the incubation, the protein which should have remained nonpolymerized below 20 mM salt concentrations undoubtedly began to self-associate to a small degree as the salt concentration was raised. The varying degree of binding could be then

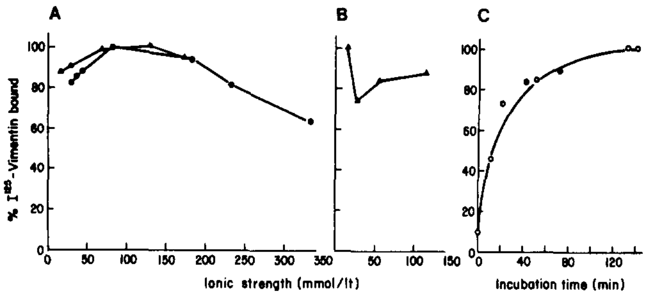


FIGURE 4 The effect of the ionic environment in the binding. (A) ^{125}I-Vimentin (at 10 μg/ml) was reacted with 20 μg of IOV at the indicated ionic strength in either 5 mM $NaPO_4$ and increasing NaCl (●) or 5 mM Tris-HCl, 2 mM $MgCl_2$, and increasing KCl (△) at pH 7.5 and 23°C. The highest binding values were considered "100% binding." (B) ^{125}I-Vimentin (at 36 μg/ml) was assayed in the same way in 5 mM Tris-HCl, 2 mM $MgCl_2$, and increasing KCl at pH 7.5 and 23°C. (C) Time course of the binding. ^{125}I-Vimentin (10 μg/ml) was incubated in isotonic salt (PBS) with 20 μg of IOVs for the indicated time intervals. The time needed to process the samples for counting (12–15 min) was not taken into account.

accounted for by the number of free ends of vimentin polymers that are actually exposed to receptor sites on the IOVs (31).

The association of vimentin to IOVs proceeded relatively slowly at 23°C and equilibrium was approached after a 90-min incubation (Fig. 4C). More prolonged incubations (>4 h) resulted in a decrease in the binding of vimentin to membranes, but this is probably due to proteolytic degradation of the membranes that occurs under these conditions.

Identification of Vimentin Binding Sites

To identify the nature of the binding site or sites on the exposed surfaces of IOVs, vesicles were treated with different proteolytic enzymes or with solvents capable of differentially extracting different classes of proteins from the membranes. Both trypsin and 1 M acetic acid markedly reduced binding of vimentin to the membrane vesicles (Table I). Extraction of IOVs with 1 M KCl, 0.4 M urea at 0°C selectively removed the bulk of protein 4.1 from the membrane while retaining 80% of the ankyrin as has been reported (21). Such vesicles stripped of most of protein 4.1 still retained the capacity to bind vimentin (Fig. 5B). In contrast, when a substantial amount of the ankyrin was stripped from the vesicles by treatment with 1 M KCl, 2.5 M urea at 37°C (14), the capacity of these stripped vesicles to bind vimentin was reduced to 25% of the original activity (Fig. 5B). When purified ankyrin was added back to the urea-stripped vesicles, much of vimentin's original capacity to bind was restored (Fig. 5B). The reconstitution of IOVs, depleted only of spectrin and actin, with purified spectrin did not significantly change the vimentin-binding capacity of vesicles (Fig. 5A). Reconstitution with 36 μg/ml or 112 μg/ml of purified spectrin did not alter the levels of vimentin binding (not shown).

Ankyrin Is a Vimentin Binding Site on IOVs

To further explore the possibility that ankyrin was responsible for the binding of vimentin to IOVs, a series of competition experiments were carried out. A standard amount of vesicles were incubated with ^{125}I-vimentin in the presence of ankyrin, protein 4.1, band 4.5, band 6, or the 43,000-mol-wt peptide derived from band 3 (23). The percentage of bound vimentin was then measured and expressed as a function of the putative competitor concentration.

Consistent with the findings described above, it was observed that a severalfold excess of ankyrin was able to inhibit 80% of the binding of the radiolabeled vimentin (Fig. 6A). This inhibition was of a competitive mode as indicated by the Dixon plots (Fig. 6B). Much less inhibition is found with the other proteins tested (Fig. 6, C–F). The amount of ankyrin needed for quantitative vimentin displacement was greater than expected, because exogenously added material reassociated with the IOVs, leaving only a fraction of it in solution to act as a competitor (not shown). Surprisingly, the 43,000-mol-wt cytoplasmic peptide of band 3 had little effect on the binding (Fig. 6C). This was unexpected since it was thought that an excess of the band 3 cytoplasmic peptide would probably displace some ankyrin from the membranes and would thereby decrease the available vimentin binding sites. However, in separate experiments it was observed that although the 43,000-mol-wt peptide displaces ankyrin from urea-stripped, ankyrin-reconstituted vesicles, that does not

TABLE I
Binding of ^{125}I-Vimentin to Various Preparations of Erythrocyte Membranes

Membranes	Percent binding with respect to control
Human erythrocyte IOVs	100
Bovine erythrocyte IOVs	104
Trypsinized IOVs (human)	17.5
Acetic acid–treated IOVs (human)	3.6
Stored IOVs (human)	45

Bovine IOVs were prepared exactly the same way as the human IOVs. Trypsin digestion of human IOVs was achieved by a brief incubation of 2.9 mg/ml of IOVs with 0.4 μg/ml of trypsin for 10 min at 23°C. The digestion was stopped by PMSF and the membranes were pelleted and washed 3 times with 5 mM $NaPO_4$, 1 mM EDTA, 1 mM PMSF (pH 7.5). Acetic acid treatment involved incubation of IOVs with 1 M acetic acid at 0°C, extensive wash, and brief dialysis against 5 mM $NaPO_4$, 1 mM PMSF (pH 7.5). Stored IOVs were assayed after having been left on ice for 10 d in 5 mM $NaPO_4$, 0.1 mM EDTA, 1 mM PMSF (pH 7.5).

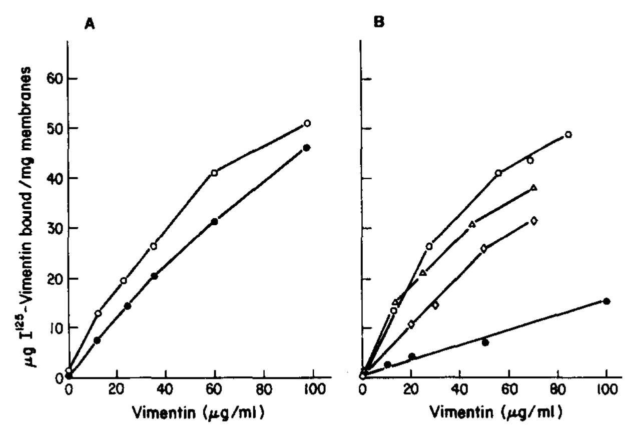


FIGURE 5 (A) Binding of vimentin to spectrin-reconstituted IOVs. O, nontreated IOV; ●, spectrin-reconstituted IOV. (B) Binding of vimentin to various membrane preparations after KCl-urea extraction or ankyrin-reconstitution. △, Protein 4.1–depleted IOV; ●, "stripped" IOV; ◇, ankyrin-reconstituted ("stripped") IOV. For details see Materials and Methods.

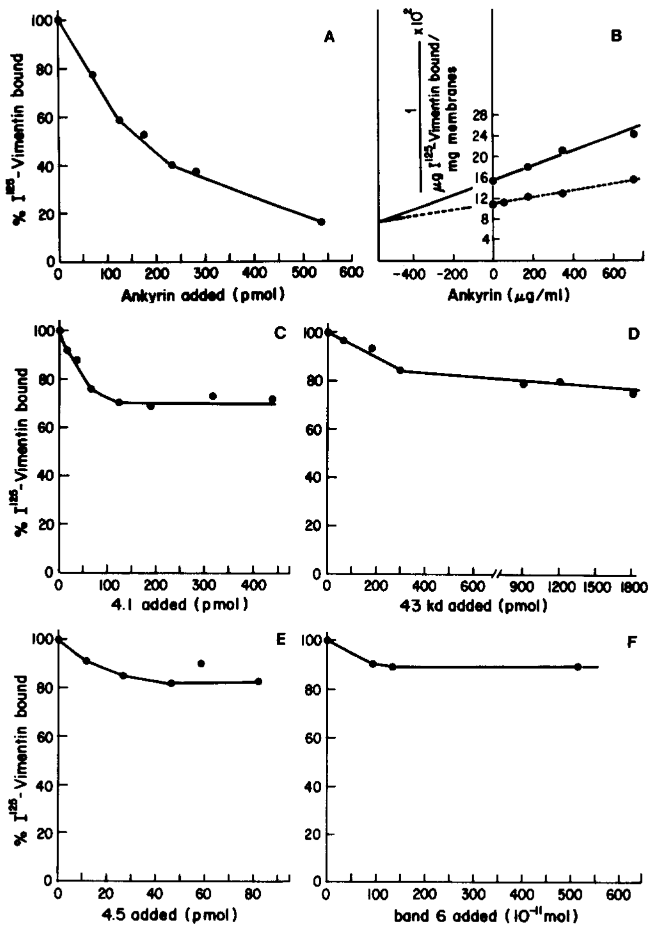


FIGURE 6 Competition assays. ^{125}I-Vimentin and IOVs were assayed in the presence of increasing amounts of exogenously added ankyrin (*A* and *B*); protein 4.1 (*C*); 43,000-mol-wt peptide (from band 3, *D*); band 4.5 (*E*); and band 6 (*F*). For details see Materials and Methods.

happen with the same efficiency in unextracted IOVs (not shown).

Further evidence that ankyrin is the vimentin binding site of IOVs was provided by preincubating IOVs with monospecific antibodies directed against different membrane proteins. Anti-ankyrin antisera completely inhibited the binding of vimentin to IOVs (Fig. 7*A*). Antispectrin antisera had a small effect (Fig. 7*B*), but antisera directed against protein 4.1, glycophorin A, and the 43,000-mol-wt peptide derived from band 3 were completely ineffective (not shown). To ensure that the inhibitory effect of anti-ankyrin was not due to a cross-reaction with vimentin, gels containing IOVs, isolated vimentin, ankyrin, or a crude lens extract (containing vimentin) were immunoblotted and tested for cross-reactivity with the anti-ankyrin antibody. As it could be seen in Fig. 8, even in overexposed autoradiograms such an effect is not detected.

DISCUSSION

Physiological Significance

Although the terminally differentiated erythrocytes of the mammals lack intermediate filaments, the findings described above are likely to be of physiological relevance. The cytoskeleton of immature erythrocytes (up to the orthoblast) is featured by a well-developed network composed largely of vimentin filaments, which is lost after the expulsion of the nucleus (18, 19). Since ankyrin (constitutively present in all erythrocytes) plays a pivotal role in connecting the spectrin-actin meshwork to the membrane while retaining the ability to bind vimentin, it follows that a fundamental regulatory mechanism may operate in vivo to control the selective linkage of membrane skeleton or/and the cytoskeleton to the lipid bilayer. Using various erythrocyte membrane constructs of defined protein composition and sideness and exploiting the sensitive binding assays with purified vimentin, it seems now feasible to study the process of filament attachment on the molecular level (better depicted in the companion paper).

In addition, the main corollaries of this study could be applied to nucleated cells expressing both membrane skeleton components and the full complement of the cyto-skeleton. In fact, the widespread existence of "typical" erythrocyte membrane polypeptides in virtually every tissue examined, taken together with the limited complexity of the erythrocyte skeleton present a paradox whereby filament-membrane interactions occurring in nucleated cells might be better analyzed in a model system that involves anucleate cells.

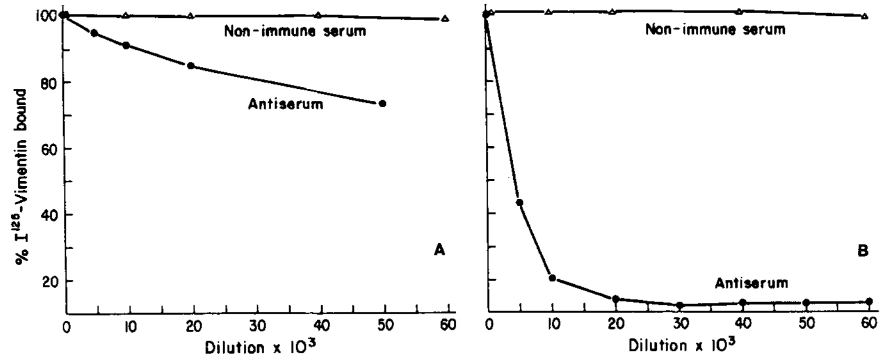
Ankyrin as an Intermediate Filament Attachment Site

The findings presented here show that soluble vimentin purified from lens tissue specifically associates with the inner surface of the human erythrocyte membrane under in vitro conditions. By several criteria we have identified ankyrin (band 2.1) as the vimentin acceptor site on IOVs. Isolated ankyrin competes with the vimentin binding site. If added exogenously, it restores the binding function to membranes that otherwise exhibit minimal binding as a result of KCl-urea "stripping," and it exists in IOVs at approximately the same concentration as the vimentin acceptor (see also reference 21). Antibodies against ankyrin dramatically block the vimentin-membrane association while treatments known to modify ankyrin (trypsin, acetic acid) also reduce the binding of vimentin.

Although these results specifically implicate ankyrin as a vimentin attachment site, it is conceivable that other binding sites may also exist for intermediate filament subunits. As a matter of fact, since our Scatchard analysis was not extended to vimentin concentrations greater than 100 μg/ml (to prevent self-association), the existence of low-affinity sites cannot be excluded. In this regard, it is interesting that protein 4.1 shows a small but measurable ability to compete with the vimentin attachment site (approximately 30% inhibition). Although a direct interaction between vimentin and protein 4.1 could not be documented in vitro (data not shown), it is worth pointing out that a 30% increase in the binding of ^{125}I-vimentin was observed after treating IOVs with affinity-purified anti-protein 4.1.

Vimentin does not seem to interact directly with the spectrin-actin network. IOVs reconstituted with spectrin do not show an increase in binding of vimentin, nor have we been able to detect complexes of vimentin and spectrin or vimentin and actin that can be demonstrated by sedimentation analysis. Furthermore, vimentin does not form a ternary complex when co-incubated with spectrin and protein 4.1 as it happens in the case of actin (Correas, I., and S. D. Georgatos, unpulished observations). Since IOVs reconstituted only with spectrin show appreciable vimentin binding capacity, we would predict that under native conditions the vimentin binding site on ankyrin is not completely masked by spectrin. However, the

FIGURE 7 Blockade of vimentin binding to IOVs by specific antibodies. ^{125}I-vimentin (at 12 μg/ml) was incubated with 20 μg IOVs that had been preincubated with increasing amounts of antispectrin (●) (*A*), anti-ankyrin (●) (*B*), or pooled normal rabbit serum (△). "100% binding" was assigned to samples containing no antibodies.



in vivo situation is complicated by a number of factors, among them the possible presence of spectrin oligomers attached to the inner surface of the membrane and to more than one site, and the possible effects of cross-linking of intermediate filaments by associated proteins (15). For these reasons the precise organization of the vimentin-ankyrin complex in situ cannot be approached only by studies of soluble proteins in vitro. The findings described above differ significantly from previous studies in which it was suggested that a direct association exists between intermediate filaments and the lipid bilayer (16). Both the binding features (saturability, number of acceptor sites, etc.) and the fact that stripped or protease-treated membranes failed to bind vimentin are inconsistent with a simple, nonspecific association with membrane lipids.

It is intriguing that ankyrin-like polypeptides have recently been identified in lens membranes (32), an observation that raises the possibility that a family of ankyrin-like proteins exist that provide attachment sites for intermediate filaments in a wide variety of eucaryotic cells. In fact, recent observations indicate that nonerythroid ankyrin occurs at a threefold excess over the membrane-associated nonerythroid spectrin (33). Thus, even if all spectrin is exclusively bound to the membrane via ankyrin, there is additional ankyrin to be used for other functions. Although we do not have a clear picture of the molecular features of the ankyrin molecule, these findings and other related observations (34) suggest that ankyrin has two functionally distinct domains, one a membrane-linking site and the other a multifunctional domain to which can be attached either spectrin or other components of the cytoskeleton. If the spectrin attachment site and the intermediate filament binding regions are physically close to one another as we suspect, regulatory "switching" phenomena may occur that could be involved with the coupling and uncoupling of membrane receptors with different components of the cytoskeletal system. It is also interesting to consider potential similarities between vimentin-ankyrin interactions and those that have been reported between intermediate filaments (neurofilaments) and microtubule-associated proteins (35). Since a certain degree of functional and structural homology exists between ankyrin and microtubule-associated proteins, the types of interactions between vimentin and ankyrin detected in the erythrocyte may reflect more general mechanisms whereby microtubules, actin filaments, and intermediate filaments form an integrated skeletal unit that regulates the structure and viscoelastic properties of both the membrane and the underlying cytoplasm.

Attachment Pattern

Vimentin filaments could bind to IOVs either by their "free ends" in an end-on fashion, or by a side-on association that could involve associations between multiple segments of the linear polymers. Since the binding of iodinated vimentin to IOVs (via ankyrin) is non-cooperative, and since bound subunits do not seem to serve as filament nucleation sites, we favor an end-on binding mechanism. When measured under nonequilibrium conditions, nonassembled vimentin binds to a larger extent to IOVs than preassembled vimentin, possibly because of the higher concentration of free polymer ends (Fig. 1 *C*). In contrast, with a hypothetical side-on mechanism in which each individual subunit along a polymer should be equally competent to bind, identical binding curves should be obtained regardless of the polymerization state of the vimentin preparation. The data cannot exclude a pattern whereby looping intermediate filaments attach to the membrane via short (proto)filament branches. In support of such a model, it has been observed that some intermediate filaments that connect keratin tonofilaments with desmosomal plaques have both a branching morphology and also have tiny rod-like bridges (36, 37). If the short rod-like bridges are

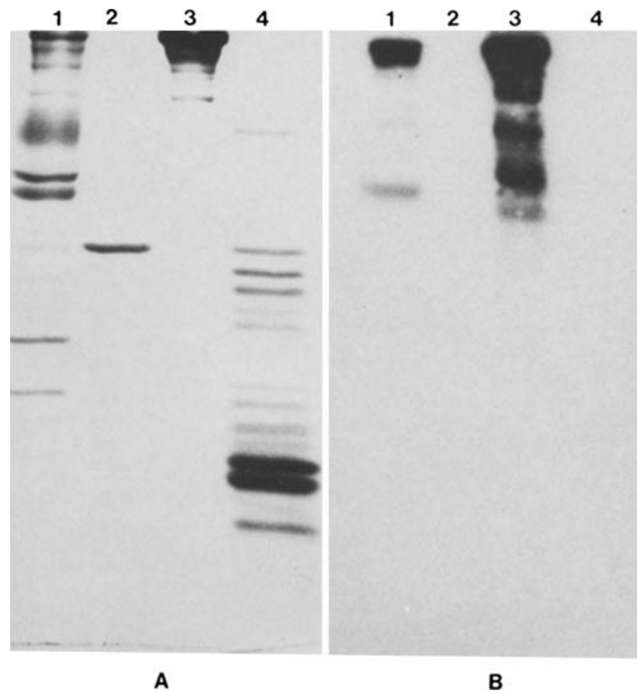


FIGURE 8 Immunoblotting with the anti-ankyrin antibody. Proteins transferred to nitrocellulose sheets and probed as described in Materials and Methods. (*A*) Coomassie-stained gel. (*B*) Autoradiogram of a replica gel. Lane *1*, IOV's; lane *2*, purified lens vimentin; lane *3*, purified erythrocyte ankyrin; lane *4*, KI Triton X-100–insoluble residue of bovine lens. The autoradiogram was exposed for 5 d at −70°C to bring about all possible cross-reacting protein species.

actual attachment points, they might also be oriented in an end-on fashion, even though the overall microscopic picture is one of a combination of end-on and side-on orientations.

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