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Association of Spectrin With Desmin Intermediate Filaments

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The association of erythrocyte spectrin with desmin filaments was investigated using two in vitro assays. The ability of spectrin to promote the interaction of desmin filaments with membranes was investigated by electron microscopy of desmin filament-erythrocyte inside-out vesicle preparations. Desmin filaments bound to erythrocyte inside-out vesicles in a spectrin-dependent manner, demonstrating that spectrin is capable of mediating the association of desmin filaments with plasma membranes. A quantitative sedimentation assay was used to demonstrate the direct association of spectrin with desmin filaments in vitro. When increasing concentrations of spectrin were incubated with desmin filaments, spectrin cosedimented with desmin filaments in a concentration-dependent manner. At near saturation the spectrin:desmin molar ratio in the sedimented complex was 1:230. Our results suggest that, in addition to its well characterized associations with actin, spectrin functions to mediate the association of intermediate filaments with plasma membranes. It might be that nonerythrocyte spectrins share erythrocyte spectrin's ability to bind to intermediate filaments and function in nonerythroid cells to promote the interaction of intermediate filaments with actin filaments and/or the plasma membrane.

Key words: spectrin, spectrin binding, desmin filaments, intermediate filaments, intermediate filament binding protein

There have been numerous reports during the past 3 years on the occurrence of spectrinlike proteins in a variety of nonerythroid cells [1-3]. Many of the nonerythrocyte spectrins were shown by immunofluorescent microscopy to be within the cell cortex and/or associated with the plasma membrane, and it has been suggested that these spectrins function, as in red cells, to anchor actin filaments to the cell membrane. However, no direct evidence for this has been forthcoming. There is some indirect evidence that spectrin also associates with other elements of the cytoskeleton in nonerythroid cells, which are not present in the mature mammalian erythrocyte. Granger and Lazarides [4] have shown that in chicken erythrocytes vimentin inter-

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mediate filaments connect the nucleus with the cell membrane. After nonionic detergent extraction of these nucleated erythrocytes, the vimentin filaments remain attached to the membrane skeleton, which, as in mammalian erythrocytes, is a spectrin-actin network. Mangeat and Burridge [5] have also demonstrated indirectly an association of vimentin filaments with nonerythrocyte spectrin. When they injected antibodies to fodrin into MDCK cells and fibroblasts, a condensation of the spectrin around the nucleus and a distortion of the vimentin filaments resulted. The microfilaments and microtubules were not affected, suggesting a specific disruption of the association of nonerythrocyte spectrin with the intermediate filaments. Also Lehto and Virtanen [6] have reported on a 230 kD protein that associates with the vimentin filaments of lens cells and is antigenically similar to the α-subunit of erythrocyte spectrin.

The purpose of this investigation was to determine if spectrin binds to intermediate filaments. We show that desmin filaments bind erythrocyte spectrin in a concentration-dependent manner and can mediate their association with erythrocyte plasma membranes. These results suggest a possible role of nonerythrocyte spectrins in mediating the interaction of intermediate filaments with plasma membranes and other elements of the cytoskeleton.

MATERIALS AND METHODS

Purification of Spectrin

Spectrin was purified as described by Cohen and Foley [7]. Fresh or recently outdated human whole blood was washed in phosphate-buffered saline and 0.5 mM EGTA, pH 8.0, and the washed cells were lysed in 20 volumes of lysis buffer (5 mM Na phosphate, 0.5 mM EGTA, pH 8.0). The ghosts were washed with lysis buffer until they were free of all hemoglobin. Spectrin was extracted by incubation of the ghosts in 0.1 mM EGTA, pH 8.5, 10 μg/ml PMSF, and 5 μg/ml pepstatin for 30 min at 37°C. The crude spectrin extract was separated from the membranes by centrifugation at 150,000g for 45 min. The supernatant, which also contained some actin and protein 4.1, was adjusted to 5 mM Na phosphate, 100 mM KCl, pH 7.6, 0.06 mM PMSF, and 0.02% Na azide and was chromatographed on a Sepharose-4B gel filtration column. Pure spectrin was eluted in the included volume. Fractions containing pure spectrin were pooled, concentrated to 1 mg/ml, and dialyzed over night against 5 mM Na phosphate, pH 7.6, and 1 mM DTT prior to use.

Purification of Desmin

Desmin was isolated from chicken gizzard as described by Geisler and Weber [8]. Fresh chicken gizzards (40 gm) were stripped of all nonmuscle tissue and minced and homogenized in 40 mM imidazole-HCl, pH 6.9, 0.6 M KCl, 1 mM EGTA, 1 mM 2-mercaptoethanol, and 0.5% Triton X-100. The homogenate was stirred on ice for 30 min and then centrifuged for 10 min in a Sorval GSA rotor at 10,000 rpm. Pellets were washed twice in the same buffer without Triton and then were resuspended in 600 ml of 10 mM imidazole-HCl, pH 6.9, 0.6 m KI, 1 mM EGTA, 0.5% Triton X-100, 0.5 mM DTT, and 0.2 mM ATP. The suspension was stirred for 6 hr and then centrifuged for 15 min at 10,000 rpm in Sorval GSA rotor. The pellets were extracted twice more with the same buffer and then suspended in 150 ml of 8 M urea-50 mM Tris HCl, pH 7.4, 5 mM $MgCl_2$, 5 mM DTT. The desmin was extracted by incubation for 2 hr at 37°C. The crude desmin extract was centrifuged at 10,000 rpm

in a Sorval GSA rotor to remove unsolubilized material, and the supernatant was loaded onto a DEAE 52 (Whatman) anion exchange column equilibrated in the desmin extraction buffer. Pure desmin was eluted with a 0–100 mM NaCl gradient and was dialyzed extensively against 10 mM Tris-acetate, pH 8.5, and 1 mM DTT. Desmin was induced to polymerize into filaments by adjusting to 150 mM KCl, pH 7.6, or by dialysis against 5 mM Na phosphate, pH 7.6, 150 mM KCl, and 1 mM DTT over night.

Erythrocyte Inside-Out Vesicle Binding Assay

Erythrocyte inside-out vesicles (IOVs) were prepared by incubation of erythrocyte ghost membranes in 30 volumes of 0.3 mM Na phosphate, pH 7.6, at 37°C for 30 min. This results in the depletion of spectrin and actin and the formation of vesicles that are morphologically inside out. The IOVs can be reconstituted with spectrin by incubation of the IOVs and spectrin in 120 mM KCl and 5 mM Na phosphate, pH 7.6, at 4°C for 1 hr as described by Cohen and Foley [9].

Desmin filaments (100 μg/ml) were incubated with IOVs (200 μg/ml) and IOVs that had been reconstituted with spectrin in 5 mM Na phosphate, pH 7.6, 150 mM KCl, 2 mM $MgCl_2$, and 1.0 mM DTT for 1 hr at 22°C. A drop of each suspension was applied to a carbon-coated copper electron microscope grid and negatively stained with 1% uranyl acetate. The grids were then examined in a JEOL S-100 transmission electron microscope.

In Solution Sedimentation Assay

The in solution sedimentation assay used to measure spectrin binding to desmin filaments was performed as previously described for spectrin binding to F-actin [7,10]. Increasing concentrations of ^{125}I-Bolton-Hunter-labeled spectrin were incubated with 500 μg/ml desmin filaments in 5 mM Na phosphate, pH 7.6, 120 mM KCl, 2 mM $MgCl_2$, and 1 mg/ml BSA for 1 hr at 37°C. Aliquots of the incubation mix were then centrifuged in a 42.2 Ti rotor for 15 min at 20,000 rpm to sediment the desmin filaments and bound spectrin. Control mixes, which contained ^{125}I-spectrin but no desmin, were incubated and centrifuged along with the sample which contained desmin filaments. The amount of ^{125}I-spectrin that sedimented with the desmin filaments and the amount of spectrin that sedimented in the absence of desmin were measured by its disappearance from the supernatant. Spectrin was incubated at 37°C for 30 min prior to the experiment to convert any tetramers to dimers.

RESULTS

Our first approach to test the hypothesis that spectrin associates with intermediate filaments was to determine whether spectrin could promote the binding of intermediate filaments to red cell inside-out vesicles.

Desmin was purified to greater than 95% purity as judged by SDS-gel electrophoresis (Fig. 1A) and polymerized into native 10-nm filaments after incubation in physiologic buffer (Fig. 1B). When polymerized desmin filaments were incubated with red cell inside-out vesicles that are nearly devoid of endogenous spectrin (as well as actin), few or no associations of filaments with the vesicles could be detected (Fig. 2A,B). However, if the inside-out vesicles had been previously reconstituted with purified red cell spectrin dimers, numerous associations between desmin filaments

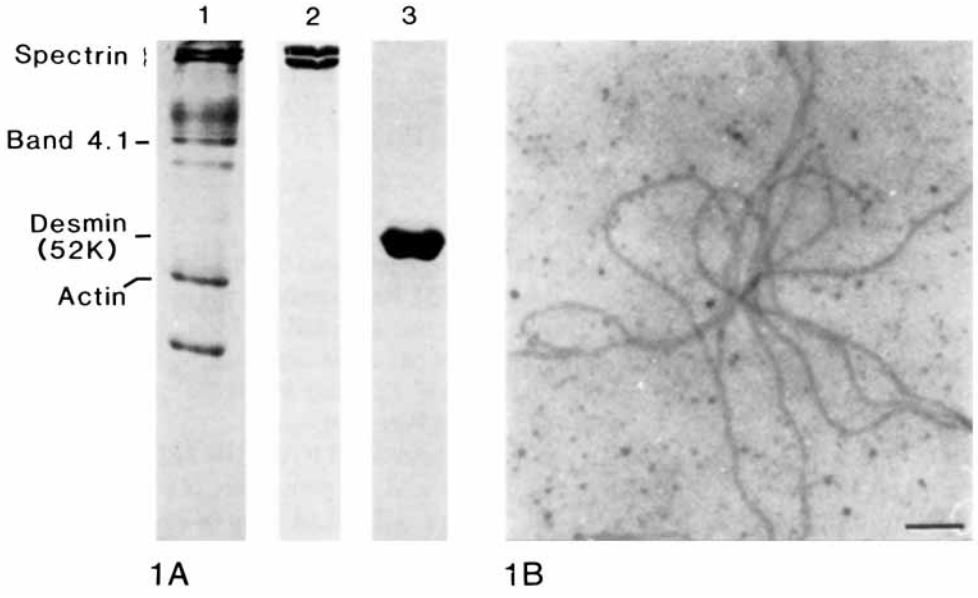

Fig. 1. A) SDS-polyacrylamide gel. Lane 1, human erythrocyte ghost membranes; lane 2, purified human erythrocyte spectrin; lane 3, purified chicken gizzard desmin. B) desmin filaments polymerized by dialysis against 10 mM Tris acetate, pH 7.6, 150 mM KCl, and 0.5 mM DTT and negatively stained with uranyl acetate. Bar = 0.3 μm.

and vesicles were observed (Fig. 2C,D). Aggregated complexes of the spectrin-containing inside-out vesicles connected by a network of desmin filaments were also seen but were never seen with vesicles lacking spectrin.

Although the above results provide a qualitative demonstration of the association of spectrin with desmin filaments, the possibility that other red cell membrane components in addition to spectrin were required for binding cannot be ruled out. To determine whether spectrin alone could form direct associations with intermediate filaments, we used a sedimentation binding assay. Figure 3 shows that incubating purified spectrin dimers in the presence of filamentous desmin results in an enhanced sedimentation of spectrin, presumably because of its binding to desmin. Figure 3 shows that the binding of spectrin to desmin filaments approaches saturation at a spectrin concentration of 250 μg/ml in the presence of 500 μg/ml desmin. Control experiments using ^{125}I-desmin demonstrated that under the conditions of incubation and centrifugation 90% of the desmin was sedimented. Calculation of the molar ratio of spectrin:desmin in the sedimented complex at the highest value of spectrin bound from Figure 3 gives a value of 1:230. Similar results have been obtained using vimentin intermediate filaments, which appear to bind to spectrin with a higher affinity and exhibit spectrin binding that saturates at a molar ratio of 1:16 [11].

This low molar ratio of bound spectrin to desmin suggested the possibility that spectrin was binding to an intermediate filament-associated protein or some other component not detected on our SDS gels. The recent demonstration of an association between ankyrin and vimentin [12,13] gave us reason to consider the possibility that either our spectrin or desmin preparations were contaminated with ankyrin or ankyrin

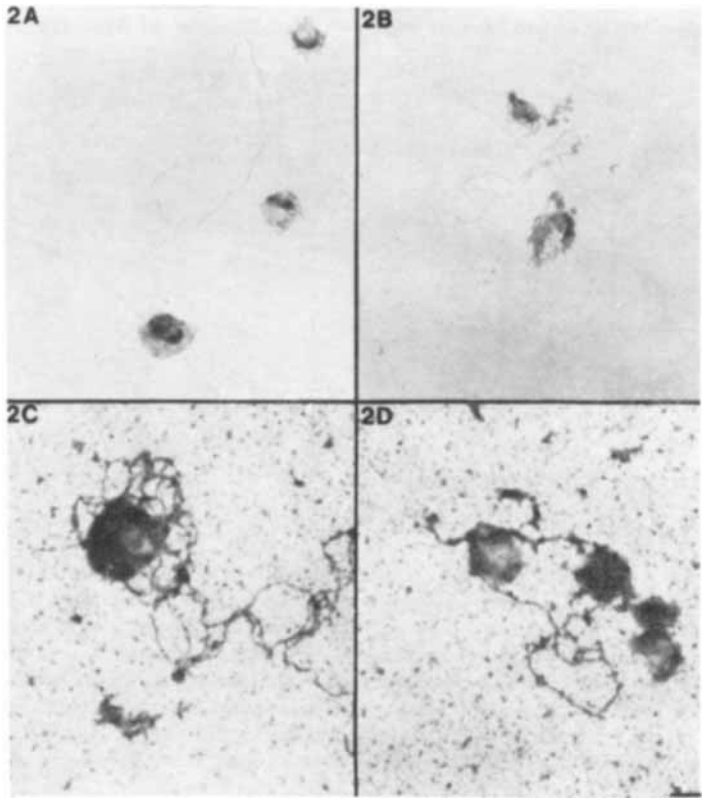

Fig. 2. Erythrocyte inside-out vesicle binding assay. A,B) spectrin-depleted erythrocyte inside-out vesicles (200 μg/ml) were incubated with desmin filaments (100 μg/ml) in 5 mM Na phosphate, pH 7.6, 150 mM KCl, 2 mM $MgCl_2$, and 1 mM DTT for 1 hr at 22°C. C,D) spectrin-reconstituted erythrocyte inside-out vesicles were incubated with desmin filaments as in A and B except that the vesicles were reconstituted with spectrin prior to incubation. Bar = 0.3 μm.

analogues at a level below detection on Coomassie blue-stained gels. Examination of our spectrin and desmin on electroblots of heavily loaded SDS gels using antibodies to red cell ankyrin revealed no cross-reacting components (not shown). However, this negative finding must be reviewed with the knowledge that, although ankyrin analogues have been detected with this antibody in many cells and tissues, crude homogenates of chicken gizzard, the source of our desmin, have shown no cross-reactivity [V. Bennett, personal communication]. Thus the negative finding could be due either to a lack of ankyrinlike proteins in gizzard or to a simple lack of cross-reactivity between erythrocyte and gizzard ankyrin.

DISCUSSION

We have shown that spectrin can bind in an apparently saturable fashion to desmin intermediate filaments. We have obtained similar results with vimentin intermediate filaments as well [11]. Our results provide the first direct evidence to support earlier reports that spectrin can bind to intermediate filaments [4–6]. Although the

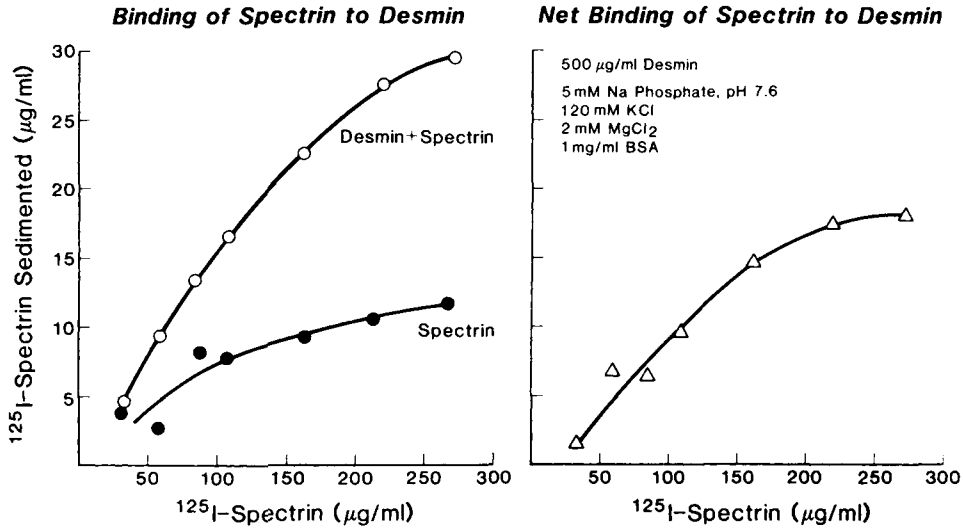

Fig. 3. In solution sedimentation assay. Desmin filaments (500 μg/ml) were incubated with the indicated concentration of ^{125}I-spectrin in 5 mM Na phosphate, pH 7.6, 120 mM KCl, 2 mM $MgCl_2$, 1 mg/ml bovine serum albumin, and 1.0 mM DTT for 1 hr at 22°C. Aliquots of each sample were centrifuged for 15 min at 20,000 rpm in Beckman 42.2 Ti rotor to sediment the desmin filaments and bound spectrin. The amount of spectrin sedimented was measured by its disappearance from the supernatant. Similar concentrations of ^{125}I-spectrin were incubated and centrifuged alone to determine the amount of spectrin that sediments in the absence of desmin filaments. Net binding of spectrin to desmin filaments was calculated by subtracting the amount of spectrin that sedimented in the absence of desmin from the amount of spectrin that sedimented with desmin filaments.

binding of spectrin to intermediate filaments appeared to be saturable, the molar ratio of spectin:desmin was low, and consequently the nature of the binding remains to be clarified. We did attempt to examine spectrin-desmin and spectrin-vimentin complexes by low angle-rotary shadow electron microscopy, but we observed only a small number of associations. It is possible that the 70% glycerol in which the samples were mixed prior to shadowing caused the complexes to dissociate or that the shearing action during spraying had a disruptive effect on the complexes.

One possible explanation for the low molar ratio of spectrin:desmin at or near saturation is that spectrin was binding not to desmin molecules but to some other protein associated and copurified with the intermediate filaments. Although our desmin preparation appeared to be greater than 95% pure on SDS gels, it is nonetheless possible that small amounts of contaminating proteins were present. There have been several reports of proteins that copurify with or become associated with desmin intermediate filaments, and these have been called *intermediate filament-associated proteins* (IFAP) [14,15]. One of these, synemin, can be purified to homogeneity [14]. Experiments to determine the effect of specific IFAPs in spectrin-intermediate filament binding are in progress.

It has recently been reported by Georgatos and colleagues [12,13] that ankyrin (band 2.1) binds to the intermediate filament protein vimentin. Our results demonstrate a second association between an intermediate filament protein, desmin, and an erythrocyte membrane skeleton protein, spectrin. The association between ankyrin

and vimentin appears to be of a different nature than that of spectrin with desmin filaments reported here or with vimentin [11]. It was reported [12] that only the unpolymerized form of vimentin, probably a tetramer, bound to ankyrin on IOVs. Our results, in fact, corroborate those of Georgatos and Marchesi [12], in that we detected almost no binding of intermediate filaments to IOVs that were not reconstituted with spectrin (but did contain ankyrin), implying that there is little or no association of filamentous desmin with ankyrin. We have not investigated the association of spectrin with unpolymerized desmin; however, Georgatos and Marchesi [12] showed that IOVs reconstituted with spectrin did not bind more unpolymerized vimentin than unreconstituted IOVs (which contain ankyrin but no spectrin).

Another apparent difference between the binding of spectrin and ankyrin to intermediate filaments is that ankyrin binds to the amino-terminal head domain of the vimentin monomer and prevents the formation of filaments [13], whereas all the observed contacts between desmin filaments and spectrin-reconstituted IOVs were due to multiple lateral associations of the filaments with the membranes. Thus the nature of the ankyrin-vimentin association apparently precludes an ankyrin contaminant in our desmin preparation accounting for our results. An ankyrin contaminant, assuming that it is bound only to the ends of filaments, as was shown by Georgatos et al [13], would have resulted in end-on associations of the filaments with either the IOVs or IOVs reconstituted with spectrin. This was not observed.

Our results also show that spectrin can promote the binding of desmin intermediate filaments to human erythrocyte membrane inside-out vesicles. Similar results have been obtained with vimentin [R.C. Langley and C.M. Cohen, in preparation]. This function in human erythrocyte spectrin might not be an irrelevant one. Although the mature erythrocyte does not contain intermediate filaments, nucleated erythroid precursors do. Thus the intermediate filament binding capability of spectrin might play some role during erythroid development. In addition, avian erythrocytes that are nucleated and do contain intermediate filaments have a spectrin-actin band 4.1 membrane skeleton that is similar to that of the human red cell [4], and the attachment of intermediate filaments to the membrane has been documented [4]. Although there are clearly important immunologic [16] and possibly functional distinctions between avian and human red cell spectrin, they might share the capacity to promote intermediate filament binding to the cell membranes.

Another important instance when spectrin may interact with intermediate filaments occurs in skeletal muscle. In muscle, desmin intermediate filaments have been described primarily in two orientations relative to the muscle fiber: transverse and longitudinal. Transverse intermediate filaments appear to connect Z lines of adjacent myofibrils and might also connect Z lines to the sarcolemma or muscle plasma membrane [17,18]. Intermediate filaments, which run longitudinally within the myofibril, appear to connect successive Z lines within the myofibril [17,18]. The localization of spectrin within the myofibril suggests that it serves as an anchorage site for the intermediate filaments. Nelson and Lazarides [19] have shown that spectrin is localized at the Z line and M band and also appears to be distributed along the sarcolemma in a ringlike pattern corresponding to the cross-striations of Z lines. They have suggested that spectrin in these regions interacts with the intermediate filaments of the transverse fibers providing a linkage between the sarcolemma and the Z discs and providing structural integrity to the myofibril during contraction and relaxation. Our results showing an in vitro association between spectrin and desmin provide support for this hypothesis.

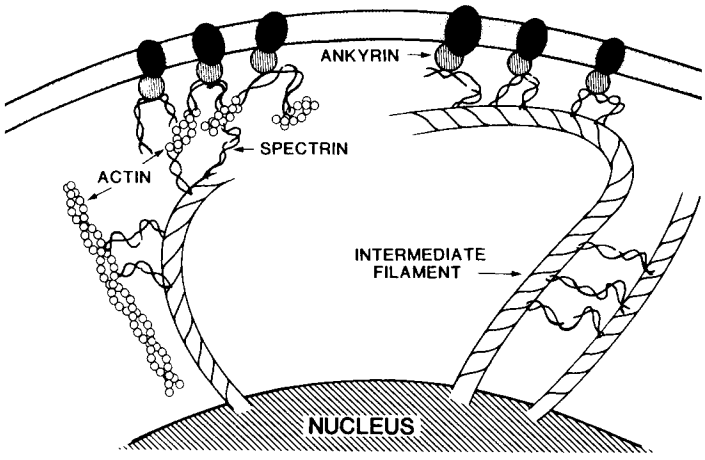

Fig. 4. Model illustrating some of the possible associations of spectrin in a eukaryotic cell. Shown are the following associations: 1) spectrin association with integral membrane protein via ankyrin or ankyrinlike proteins, 2) spectrin binding to F-actin and spectrin-mediated association of F-actin with the plasma membrane, 3) spectrin binding to intermediate filaments and spectrin mediated cross-linking of intermediate filaments, 4) spectrin-mediated cross-linking of actin filaments to intermediate filaments (this association has not as yet been demonstrated but should be possible), 5) spectrin-mediated anchorage of intermediate filaments to the plasma membrane. Note that the cross-linking of various components by spectrin requires that spectrin be in a bivalent or tetrameric form.

The association between spectrin and intermediate filaments might also be regulated during development as the species of spectrin subunits and intermediate filaments changes. For example, during myogenesis the intermediate filament composition of striated muscle changes from a mixture of vimentin and desmin to all desmin at maturity [20,21]. During the same time period, the subunit composition of skeletal muscle spectrin changes from $\alpha\gamma$ to $\alpha\beta$ [22]. In light of our findings, it would clearly be of interest to determine whether the different forms of spectrin interact differently with the two types of intermediate filaments and perhaps have different functions within the myofibrillar cytoskeleton.

The function of spectrin in the erythrocyte has been well characterized [23,24]. Spectrin has multiple protein binding domains, which enables it to cross-link short actin filaments to form the two-dimensional spectrin-actin membrane skeleton and mediate its connection with the plasma membrane. In nonerythroid cells in which spectrinlike proteins have been identified and in some cases purified, the function is not clear. A number of reports have shown that nonerythrocyte spectrins are concentrated at the cytoplasmic aspect of the plasma membrane; others have reported a more diffuse distribution in the cell [1,5,22,25]. In brain, at least some of the spectrin might have a function similar to red cell spectrin, because brain contains membrane-associated ankyrin, and brain spectrin can bind actin in a band 4.1-stimulated manner [26,27]. Thus brain and other nonerythroid spectrins might function in mediating the association of actin filaments with membranes [1,2,28].

The demonstration that spectrin binds to intermediate filaments as well as actin filaments indicates that spectrin is clearly a multifunctional protein in nonerythroid cells, as it is in erythroid cells. Figure 4 shows some of the possible associations that

might be mediated by spectrin given its multifunctional capacity. Our results support the idea that spectrin promotes the association of intermediate filaments as well as actin filaments with plasma membranes. Also, in that spectrin forms bivalent tetramers and higher-order oligomers and is capable of binding both actin and intermediate filaments, it might be able to cross-link these different types of filaments to one another and therefore play a complex and important role in the organization of eukaryotic cytoskeletons.

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