Spectrin and Ankyrin-Based Pathways:
Metazoan Inventions for Integrating Cells Into Tissues

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Bennett, Vann, and Anthony J. Baines. Spectrin and Ankyrin-Based Pathways: Metazoan Inventions for Integrating Cells Into Tissues. Physiol Rev 81: 1353–1392, 2001.—The spectrin-based membrane skeleton of the humble mammalian erythrocyte has provided biologists with a set of interacting proteins with diverse roles in organization and survival of cells in metazoan organisms. This review deals with the molecular physiology of spectrin, ankyrin, which links spectrin to the anion exchanger, and two spectrin-associated proteins that promote spectrin interactions with actin: adducin and protein 4.1. The lack of essential functions for these proteins in generic cells grown in culture and the absence of their genes in the yeast genome have, until recently, limited advances in understanding their roles outside of erythrocytes. However, completion of the genomes of simple metazoans and application of homologous recombination in mice now are providing the first glimpses of the full scope of physiological roles for spectrin, ankyrin, and their associated proteins. These functions now include targeting of ion channels and cell adhesion molecules to specialized compartments within the plasma membrane and endoplasmic reticulum of striated muscle and the nervous system, mechanical stabilization at the tissue level based on transcellular protein assemblies, participation in epithelial morphogenesis, and orientation of mitotic spindles in asymmetric cell divisions. These studies, in addition to stretching the erythrocyte paradigm beyond recognition, also are revealing novel cellular pathways essential for metazoan life. Examples are ankyrin-dependent targeting of proteins to excitatory membrane domains in the plasma membrane and the Ca²⁺ homeostasis compartment of the endoplasmic reticulum. Exciting questions for the future relate to the molecular basis for these pathways and their roles in a clinical context, either as the basis for disease or more positively as therapeutic targets.
I. INTRODUCTION

Emergence of metazoans from their unicellular ancestors required solutions to a new set of problems imposed by function of cells in the context of tissues and in motile organisms of immense size compared with individual cells. These requirements of communal life include ability of cells to develop micron-scale spatial organization of cell surfaces and of intracellular compartments to optimize cell-cell interactions and intercellular signaling. In addition, cells incorporated into an actively moving organism must deal with enormous mechanical stresses at the plasma membrane-cytoskeleton interface compared with free-living cells. Understanding the molecular basis for such metazoan adaptations represents an interdisciplinary challenge involving biochemistry, cell biology, physiology, and molecular medicine. This review focuses on the molecular physiology of spectrin and the spectrin-associated proteins ankyrin, adducin, and protein 4.1. These proteins were first discovered as components of the membrane skeleton of human erythrocytes (see below) and are required for survival of erythrocytes in the circulation. The erythrocyte membrane proteins are members of closely related families that are associated with membranes in simple metazoans, including Caenorhabditis elegans and Drosophila melanogaster, and are expressed in most vertebrate tissues. Spectrin, ankyrin, adducin, and protein 4.1 are modular proteins that are not present in their assembled state in the completed Saccharomyces cerevisiae or Arabidopsis thaliana genomes and so far have not appeared in Zea mays genomic sequences. These proteins therefore are likely to have evolved early in evolution of metazoans, following divergence of plants and fungi, and represent candidates for roles in specialized activities of multicellular animals.

Recent discoveries based on studies involving C. elegans and D. melanogaster as well as gene knock-outs in mice will be reviewed that demonstrate functions of spectrin- and ankyrin-based protein assemblies in diverse roles that are all related to multicellular life. Functions that will be described include morphogenesis of epithelial tissues, targeting of ion channels and cell adhesion molecules to specialized regions in myelinated axons, and sorting of Ca\(^{2+}\) homeostasis proteins to the Ca\(^{2+}\) compartment of the endoplasmic reticulum (ER) of striated muscle. The clinical implications of these observations are only beginning to be appreciated and are discussed. Elucidation of physiological roles of spectrin and ankyrin-associated proteins is based on a strong foundation at a molecular level. The review includes current information regarding gene family members, atomic structures, oligomeric state, and protein interactions. The human erythrocyte remains the best understood in terms of its membrane skeleton, and the review begins with a brief summary of this system.

A. Overview of the Erythrocyte Membrane Skeleton

The membrane skeleton of mammalian erythrocytes was first visualized in electron micrographs of detergent-extracted erythrocytes (440). The erythrocyte membrane skeleton is organized as a polygonal network formed by five to seven extended spectrin molecules linked to short actin filaments ~40 nm in length (42, 247, 360) (Fig. 1A). The spectrin-actin network of erythrocytes is coupled to the membrane bilayer primarily by association of spectrin with ankyrin, which in turn is bound to the cytoplasmic domain of the anion exchanger (17, 20, 23, 256, 400, 441). The anion exchanger is associated into dimers (305), which associate with separate sites on the membrane-binding domain of ankyrin to form pseudo-tetramers (53, 281, 332, 437). Anion exchanger dimers also are associated on their cytoplasmic surface with band 4.2 (442). Additional membrane connections are provided at the spectrin-actin junction by a complex between protein 4.1, p55, a member of the MAGUK family, and glycophorin C (170, 266, 267) (Fig. 1B).

Several proteins responsible for capping actin and defining the length of actin filaments as well as stabilizing spectrin-actin complexes have been localized to spectrin-actin junctions by electron microscopy (91, 402). Protein 4.1 stabilizes spectrin-actin complexes (400, 401). Adducin associates with the fast-growing end of actin filaments in a complex that caps the filament and promotes assembly of spectrin (135, 222, 223, 239) (Fig. 1C). A nonmuscle isoform of tropomyosin is associated with the sides of actin filaments (127). Tropomyosin is of the same length as actin filaments visualized in electron micrographs and is a candidate to function as a morphometric ruler defining the length of actin filaments in erythrocyte membranes. Tropomodulin caps the slow-growing end of actin filaments in a ternary complex involving tropomyosin (124–126, 421).

Components of the erythrocyte membrane skeleton have been the subject of recent reviews or papers that include discussions of tropomodulin (125), protein 4.1 (68, 118), protein 4.2 (62), p55 (56), spectrin (164, 410), the anion exchanger, and glycophorin C (4, 381). The contributions of these proteins to mechanical properties of erythrocyte membranes have also been summarized (97, 289).

A major function of the spectrin skeleton in erythrocytes is to provide mechanical support for the membrane bilayer and allow survival of these cells in the circulation. The essential nature of the spectrin-skeleton in red cell biology was first demonstrated in mutant mice with deficiencies in α- and β-spectrin and ankyrin (33, 152). Numerous mutations have subsequently been catalogued in humans with hereditary hemolytic anemias. Defects in lateral associations of the spectrin-actin network result in
abnormally shaped cells in elliptocytosis and poikilocytosis and include loss of spectrin dimer-tetramer interactions (395) and deficiency of protein 4.1 (67, 120, 383). Defects in membrane associations result in loss of unsupported phospholipid bilayer and spherocytosis. Molecular defects include spectrin deficiency from a variety of causes (1, 2, 52, 94, 112). A substantial literature has documented naturally occurring mutations/deficiencies of skeletal proteins resulting in hereditary hemolytic anemias in humans and mice (reviewed in Refs. 87, 166, 396). An emerging area of interest is mutations resulting from targeted gene knock-outs in mice resulting in hemolytic anemias that may foreshadow human disorders. An example is the β-adducin null mouse, which exhibits spherocytosis (143).

II. GENES, PROTEINS, AND PROTEIN INTERACTIONS

Solution of the organization of the spectrin-based membrane skeleton of the human erythrocyte membrane has provided the biochemical equivalent of a high-resolution genetic pathway of interacting membrane structural proteins. The discovery that other tissues express isoforms of ankyrin (18) and spectrin (20, 40, 146, 151, 237, 343) suggested that the erythrocyte membrane skeleton...
had a broad relevance for other cell types. However, although the basic structural principles established in erythrocytes are likely to apply in other tissues, the organization, protein interactions, and functions of spectrin-based structures are considerably more diverse in other cells. Nevertheless, understanding the physiological roles of these proteins begins with their structure and biochemistry. This section focuses on genes, alternatively spliced variants, protein structure, and protein interactions of generally expressed forms of spectrin, and the proteins that interact with spectrin: ankyrin, protein 4.1, and adhucin.

A. Spectrins

Spectrins are extended, flexible molecules ~200–260 nm in length and 3–6 nm across with actin-binding domains at each end (20, 146, 362, 400). Spectrins are comprised of α- and β-subunits, which are both related to α-actinin (43, 105, 319, 387, 408). The α- and β-subunits are associated laterally to form antiparallel heterodimers, and heterodimers are assembled head-to-head to form heterotetramers (Fig. 2, Table 1).

Metazoan spectrins exhibit 50–60% sequence similarity along the length of the predicted polypeptide chains, when compared between Drosophila, C. elegans, and vertebrates, with some regions with 70–80% identity. Candidates for prototypic spectrins, possibly comprised of a single subunit, have also been characterized biochemically and by electron microscopy in Dictyostelium (16) and Acanthamoeba (336). However, sequence information is not yet available for these presumed spectrin ancestors. Spectrin subunits are absent from completed S. cerevisiae and Arabidopsis thaliana genomes, although individual domains of spectrin are represented. Similarly, no spectrin sequence has yet appeared in genome of Zea mays. Polypeptides cross-reacting with spectrin have been reported in higher plants (118, 284, 364) as well as green algae (176) and Chlamydomonas (252). However, these immunoreactive forms of spectrin have not been characterized in terms of primary sequence or visualized by electron microscopy. Definition of the full scope of the spectrin family awaits completion of genome sequencing. However, the available data demonstrate that spectrins are ancient proteins present in their modern form in simple metazoans.

The spectrin repertoire of the completed C. elegans and D. melanogaster genomes includes one α-subunit (105), one β-subunit (41, 159, 292), and one β-H subunit (106, 276, 387). Currently characterized spectrins in humans include two α-subunits (α1, α2) (353, 419), four β-subunits (β1, β2, β3, β4) (24, 187, 261, 286, 310, 372, 427, 428), and a β-H subunit (also referred to as β3) (371) (Table 1). β-Spectrins also include isoforms that have not been characterized at a molecular level. βNM is a β-type subunit identified at neuromuscular junctions based on immunoreactivity (31). βGolgi-Spectrin has been discovered by Beck et al. (9) to be an immunoreactive form of β-spectrin associated with Golgi structures. βGolgi shares epitopes with β1-erythrocyte spectrin, but establishing the relationship of βGolgi spectrin with other β-spectrins will require molecular characterization. Recently, βγ-spectrin has been proposed to function as the Golgi spectrin (372). However, the pattern of expression and cellular localization of βγ-spectrin do not support a general role in Golgi function (310).

Alternative splicing provides additional diversity among α- and β-spectrins. βI, βII, and βIV spectrins are all differentially spliced. βI, βII, and βIV spectrins have COOH-terminal regions that are subject to differential mRNA splicing to generate “short” or “long” COOH-terminal regions (24, 168, 427). The β III polypeptides described to date have a long COOH-terminal region that includes a pleckstrin homology (PH) domain (310, 372). One nomenclature refers to β-spectrin spliceoforms by the order of their discovery (429): short β I is β I σ I, and long β I is β I σ II. However, this system has become confusing as new family members have been discovered: long β IV is β IV σ 1 and short β IV is β IV σ 4, while long β II is β II σ 1 and short β II is β II σ 2. We will refer instead to spliceoforms by molecular weight or in some way to help describe their domain composition.

The long COOH-terminal regions of β-spectrins have a PH domain (see below) linked by an apparently unstructured region of ~100 amino acid residues to the last (partial) triple helical repeat. The polypeptide chain terminates 50–60 residues after the PH domain. Short COOH-terminal isoforms do not contain a PH domain. About halfway through the linker region after the last (partial) triple helical repeat, the sequences diverge and terminate after 22–28 residues. In both β I and β II spectrins, the short COOH-terminal region contains multiple Ser or Thr residues that are potential substrate sites for casein kinase II. In the case of the short β I COOH-terminal region (i.e., in erythrocyte β-spectrin), at least six of these residues are substrates for casein kinase II (163, 323). As described more fully in section III, the PH domain probably represents a major ligand-binding site in β-spectrins. Thus differential splicing modulates both the interactive and regulatory properties of the β-spectrins.

β II is subject to further differential splicing. A splice variant termed ELF1 represents a truncated β-spectrin, consisting of little more than a calponin homology domain (the CHI domain) and the COOH-terminal region of the short β II (287).

Combinatorial association of α-spectrins with various β- and β-H subunits yields α/β and α/β-H heterotetramers with distinct functions and patterns of expression.
Human \(a_1/b_1\)-spectrins were first characterized in mammalian erythrocytes, and also are expressed in striated muscle and a subset of neurons in the central nervous system (227, 300, 345). Avian erythrocytes, in contrast, have \(a_2/b_2\)-spectrin (343). \(a_2/b_3\) and \(a_2/b_4\) represent the major forms of spectrin in nonerythroid vertebrate tissues. Terminal web spectrin comprised of \(a_2\) and a presumed \(\beta\)-H spectrin are localized in apical domains of epithelial tissues such as small intestine, while \(a_2/b_2\)-spectrins are associated with basolateral domains (147).

### 1. Domains

\(\alpha\)-Spectrins contain 22 domains with the following features: domains 1–9 and 11–21 are comprised of triple helical repeats also found in \(\beta\) and \(\beta\)-H spectrins (see below); domain 10 is an SH3 (src homology domain 3) motif; the COOH-terminal domain 22 is related to calmodulin (393) (Figs. 2 and 3). Domain 11 of vertebrate \(\alpha\)-subunits contains a 35-residue extension with the cleavage site for \(\text{Ca}^{2+}\)-activated protease and a calmodulin-binding site (162, 235). *D. melanogaster* spectrin contains...
A predicted calmodulin-binding site at a different position than vertebrates (105).

β-Spectrins contain 19 domains beginning with a highly conserved NH2-terminal actin-binding domain comprised of two adjacent calponin homology domains (6, 46), followed by 17 consecutive triple-helical repeat domains and ending with a COOH-terminal domain which includes a PH domain (Figs. 2 and 3). Ankyrin-binding sites are at a site in the midregion of the tetramer (75, 400) and have been assigned to repeat number 15 of β1-spectrin (210). Repeat 17 is a partial helical repeat that pairs with the COOH terminus of α-spectrins to form a noncovalent triple-helical structure (see below).

β-H spectrins of D. melanogaster (106, 387), C. elegans (276), and humans (371) have NH2-terminal actin-binding domains and COOH-terminal domains closely related to β-spectrins but contain 30 triple-helical repeats instead of 17. β-H spectrins of Drosophila and C. elegans also have an SH3 domain inserted in the fifth triple helical domain. β-H spectrins lack an ankyrin-binding site (276, 371, 387).

A) TRIPLE-HELICAL DOMAINS. Atomic resolution of the structure of triple-helical repeat domains obtained by X-ray crystallography (100, 155, 434) as well as NMR (319) provides a striking confirmation of the structure originally predicted by Speicher and Marchesi (367). Triple helical repeats are comprised of two parallel and one antiparallel α-helices, which are stabilized by interactions between hydrophobic residues spaced in a heptad repeat pattern found in other examples of paired helical structures. Tandem triple helical repeats of spectrin and α-actinin are comprised of antiparallel α-helices connected by an extended α-helix (100, 155) (Fig. 3A). Comparison of hydrodynamic properties of single and multiple domains suggests that serial repeats are flexible and configured such that the average end-end length is reduced compared with values predicted for rigid rods (403). One possible source of flexibility is bending of the α-helix interconnecting domains. A novel mechanism for shortening end-end distances by rearrangement of helices has been proposed by Grum et al. (155) based on alternative structures observed by crystallography. An extended series of triple helical repeats may also exhibit a superhelical twist (155, 274).

Spectrin repeats have recently been demonstrated by atomic force microscopy to reversibly unfold and refold when subjected to forces in the range of 35 pN (347). Spectrin and other proteins (see below) with triple helical domains therefore have the potential to function as molecular springs that can store energy and dampen deformations resulting from mechanical stress. The potential role of this elastic behavior in spectrin function is discussed below.

B) ACTIN-BINDING DOMAIN. The NH2-terminal actin-binding domains of the β-spectrins are comprised of a pair of calponin homology (CH) domains (47). These are similar in sequence to a region of the smooth muscle actin-binding protein calponin; such tandem pairs occur in other proteins that have lateral associations with actin filaments, including dystrophin, utrophin, α-actinin, and fimbrin (Fig. 3F). Related calponin-based actin-binding domains have been recently found in the plakin family of proteins involved in connection of actin filaments with intermediate filaments and microtubules (435, 436), and in cortexillins, which bundle actin filaments (116).

X-ray crystallography has resolved the atomic structures of the NH2-terminal (45) and COOH-terminal (6) CH domains of β-spectrin, and of tandem CH domains of fimbrin (149) and utrophin (291) (Fig. 3B). The tandem pairs of CH domains of fimbrin and utrophin interact with actin, and their binding to actin filaments has been resolved at atomic resolution (149, 161, 208, 291). The β-spectrin CH domains are also likely to interact with actin. The actin binding activity of β-spectrin is restricted to the β-chains (44, 238). A minimal actin-binding fragment of erythrocyte spectrin has been produced by limited trypsin digestion and derives from residues 47–186 (207). These residues represent the NH2-terminal CH domain (known as CH1), which in utrophin and fimbrin is the highest affinity actin binding site (161, 291).

Several considerations suggest that the site of contact with F-actin involves the junction between CH domains, with the first domain providing most of the interactions (6, 161). The second CH domain may contribute by enhancing the affinity for F-actin and/or have a regulatory role (6) (see below). Ironically, the single calponin domain of calponin itself lacks actin-binding activity (145), suggesting the likely possibility that CH domains have additional unresolved functions.
b-Spectrins contain a PH domain located in the COOH-terminal segment, which is deleted in certain alternatively spliced isoforms (see above). These domains extend out from spectrin rods in the midregion of spectrin tetramers and are placed within 10 nm of each other (see Fig. 2). PH domains are 100-residue folding units first resolved in pleckstrin, which is a major protein kinase C substrate in platelets, and subsequently been found in many proteins (340). A unifying feature of proteins with PH domains is a role in signaling and proximity to plasma membranes. Ligands for PH domains may include polyphosphatidylinositol lipids as well as proteins.

The three-dimensional structures of the PH domains of mouse (190, 262) and D. melanogaster β-spectrins (447) reveal similar folds to PH domains of other proteins (340) (Fig. 3C). PH domains include a seven-stranded β-sheet arranged as a β-barrel with a COOH-terminal α-helix. Solution of PH domain structure from other proteins indicates that while the overall folding of PH domains are conserved, variations in loop lengths and composition provide substantial variability in potential interaction surfaces (447). Consistent with structural predictions, binding activities of PH domains of spectrin and other proteins are distinct both with respect to interactions with various phosphatidylinositol lipids and to proteins (340).
d) Calmodulin-related domain. α-Spectrins contain EF-hand motifs located at the NH$_2$ terminus of that are juxtaposed to the actin-binding domain on the adjacent β-subunit. The EF-hand domain of α-spectrin shares structural homology with calmodulin and also exhibits a Ca$^{2+}$-dependent conformational change (392, 393). An important distinction between spectrin and calmodulin is that spectrin only contains the two NH$_2$-terminal EF hands and lacks the two COOH-terminal EF hands present in calmodulin. Intact human erythrocyte spectrin or recombinant α I or α II EF hands bind Ca$^{2+}$ selectively with a stoichiometry corresponding to the number of EF hands but with an unphysiological affinity in the range of hundreds of micromolar (8, 257, 258). However, intact horse spectrin molecules have been reported to bind Ca$^{2+}$ at many sites of micromolar affinity, which presumably are not related to EF hands (414). Fowler and Taylor (123) reported that low micromolar levels of Ca$^{2+}$ influenced human erythrocyte spectrin-actin interactions, although spectrin-actin and spectrin-4.1-actin interactions were described as Ca$^{2+}$-insensitive by Ohanian et al. (307). Clearly much remains to be understood about the significance of Ca$^{2+}$ binding by spectrins.

E) SH$_3$ domain. SH$_3$ domains, initially observed in the Src protein tyrosine kinase, are present in many proteins involved in cell signaling and mediate interactions with proline-rich stretches in a variety of target proteins (321). SH$_3$ domains are inserted in α-spectrins (419) and in invertebrate β-H spectrins (106, 276) (Fig. 2). The structure of the α-spectrin SH$_3$ domain has been resolved at an atomic level by X-ray crystallography (298) and NMR (30, 352) (Fig. 3D). The three-dimensional structure of spectrin SH$_3$ domains is a compact β-barrel and exhibits the same overall fold as other SH$_3$ domains.

2. Subunit interactions

Spectrin heterotetramers are assembled through the following interactions between α- and β-subunits: 1) a lateral and antiparallel association between β-subunits and α-subunits; 2) head-head association between laterally associated heterodimers by linkage between partial triple-helical repeats at the COOH-terminal end of the β-subunits and the NH$_2$-terminal end of α-subunits (see Fig. 2). Structural requirements for lateral association between α- and β-spectrins have been analyzed for erythrocyte (14, 368, 403) and D. melanogaster spectrin (409, 411). The minimal domains required for α-β complexes are the first two triple-helical domains of β-spectrin and the last two triple helical domains of α-spectrin (368). The first two triple helical domains of β-spectrin and last two of α-spectrin are closely related to the four triple helical domains of α-actinin, which also associate laterally in an antiparallel orientation (100). The atomic structure of tandem α-actinin domains reveals a dimer formed stabilized by electrostatic interactions provided by complementary surfaces (100). Association between four triple helical domains of α$_1$- and β$_1$-spectrins is of high affinity with a dissociation constant ($K_D$) of 10 nM (403). Further stabilization of α-β complexes is provided by interaction between the calmodulin-related domain of α-spectrin and the calponin homology domains of β-spectrin (409, 411).

The lateral association of α- and β-spectrin subunits is highly conserved and occurs between D. melanogaster and vertebrate spectrins (43). The high affinity of α- and β-subunits implies that spectrin will not exist as independent α- or β-subunits but is an obligatory heterodimer or tetramer. Reports of β-spectrin unaccompanied by an α-subunit in striated muscle and at neuromuscular junctions (31, 338) suggest the possibility of a heretofore unrecognized α-subunit or an immunoreactive but otherwise highly diverged β-spectrin.

Head-to-head contacts between α- and β-spectrin subunits are believed to occur through contacts resembling pairing between helices in triple helical bundles (89, 211, 221, 366, 395, 433). The NH$_2$ terminus of α-spectrin provides one helical segment, and the COOH-terminal repeat of β-spectrin provides two antiparallel helices, with α-β pairing resulting in a noncovalent triple helical segment. Flanking residues on α-spectrin also contribute to α-β association (55).

Defects in spectrin tetramer formation have been established in erythrocytes of patients with hereditary elliptocytosis (315, 395). Mutations in α- and β-spectrin defined in these patients would be predicted to disrupt helical pairing predicted from biochemical studies (89, 396, 434). These mutations include substitution of prolines, which would be expected to disrupt an α-helix, as well as mutations in residues predicted to provide contacts between helices. Human mutations in the partial helical domain of α-spectrin have been introduced into D. melanogaster α-spectrin and result in a temperature-sensitive phenotype (89) (see below).

3. Spectrin superfamily

Proteins that contain NH$_2$-terminal CH domains, COOH-terminal calmodulin-related domains, and intervening triple helical domains comprise the spectrin superfamily. Currently recognized proteins with these combined features include α-actinins and dystrophins. In addition, trabeculin/macrophin/MACF of vertebrates (236) and Kakapo of D. melanogaster (154) are newly recognized members of the plakin family that have CH domains, 23–29 triple helical domains, and a calmodulin-related domain, as well as domains related to plectin and the plakin family (Fig. 4). The COOH termini of these proteins contain a microtubule-binding domain (206, 236). These proteins thus can interact with actin filaments, cadherins, or integrins through the plakin domain and
microtubules. Spectrin-like repeats also are present in eight to nine tandem copies in unc 73/kalirin/Trio, which also contain a dbl/pleckstrin homology domain (83). Trio/Unc 73 is required for axon pathfinding in *C. elegans* and *D. melanogaster* (259, 375).

Triple-helical repeats of α-actinin are most similar to the first repeats of β-spectrin (repeats 1 and 2) and the last repeats of α-spectrin (domains 20 and 21) (43). α-Actinin thus contains within a single polypeptide the COOH-terminal domain of α-spectrin (calmodulin-related domain) and the NH2-terminal domain of β-spectrin (CH domains and first two triple helical repeats) (Fig. 4). These considerations have led to suggestions that α- and β-spectrins evolved from a homodimeric α-actinin-like precursor polypeptide (41, 319, 387, 408). One possible evolutionary scenario is that α-actinin became elongated through insertion of seven repeats by an unspecified mechanism, followed by duplication events resulting in a giant α-actinin-like protein. Insertion of a transcriptional promoter within a repeat has been proposed to provide the basis for the modern split repeat at the α-β tetramerization site and the origin of separate α- and β-spectrin genes (387, 408).

4. Spectrin-protein interactions

A) **Spectrin-actin interaction.** The site of contact of F-actin with the actin-binding domains of α-actinin (273), fimbrin (161), and utrophin (291) has been mapped to actin subdomains 1 and 2 as well as subdomain 1 of the adjacent actin monomer by image analysis of electron micrographs. These conclusions are supported by genetic mapping of actin contact sites with fimbrin in yeast (175, 177). The fimbrin actin-binding domain induces a confor-
national change in F-actin (161), suggesting the possibility that β-spectrin could also perturb F-actin structure.

Comparison of actin-binding activities of the β-spectrin actin-binding domain alone and with triple-helical repeats suggests that the first triple helical repeat also participates in spectrin-actin complexes (238) (Fig. 1C). These results support a model where β-spectrin actually lies along the actin filament, thus engaging in contacts involving two actin monomers (238). Interestingly, similar studies of dystrophin-actin interactions have concluded that lateral associations occur between actin and dystrophin triple helical domains (in this case repeat number 11) (351).

There is evidence that different β-spectrins exhibit varying modes of interaction with actin. It is interesting to note that a very small β II-spectrin splice variant ELF1 (a “mini-spectrin”) contains a single CH domain that is similar to the CH1 domain of other β-spectrins. Since βγ-spectrin CH1, isolated as a tryptic fragment, binds actin avidly in vitro (207), it is likely that ELF1 is a true actin-binding protein, even though some other proteins with single CH domains do not necessarily bind actin (6). Intriguingly, the CH1 tryptic fragment of β I-spectrin appears to have a higher affinity interaction with actin than might have been expected: it inhibits interaction of whole erythrocyte spectrin with actin with a half-maximal effect at 5 μM, while the intact erythrocyte dimer binds to actin with a KD of 250 μM (309). This indicates a higher affinity interaction than native erythrocyte α I/β I-spectrin dimer. One possibility might be that α I-spectrin has a suppressive effect on the interaction of β1 CH domains with actin. As we note below, the weak interaction of erythrocyte spectrin with actin is enhanced by proteins 4.1 and adducin.

The tandem CH domains in β-spectrins may have additional roles in actin binding. CH1, the NH2-terminal CH domain, probably provides the primary interaction with actin (see Ref. 291). CH2, the COOH-terminal of the pair, binds actin only weakly (46). In utrophin, CH2 provides a specificity for actin subtypes: utrophin binds β-actin (cytoskeletal) with higher affinity than α-actin (sarcomeric). The CH domains of β-spectrin therefore have the potential to target spectrin to particular isoforms of actin, as is the case with utrophin (426). In this context, it is interesting to note that erythrocyte membranes contain only β-actin, rather than the βγ mixture typical of most cytoskeletal systems (333).

B) SPECTRIN-MEMBRANE INTERACTIONS. Spectrins are coupled to membranes by multiple pathways including direct association with membrane-spanning proteins, interaction with phospholipids, and through interactions with ankyrins (see below). Binding of spectrin to ankyrin-independent protein sites has been measured in brain membranes (80, 251, 373, 374). β-Spectrin associates with membranes through two distinct classes of sites. One is regulated by calmodulin and is localized in the NH2-terminal region. The other β-spectrin site is located in the COOH-terminal domain, which includes the PH domain.

Association of the spectrin PH domain with membranes has been demonstrated in living cells using green fluorescent protein-tagged β-spectrin PH domain (415). One class of spectrin-PH domain interactions is likely to involve PI lipids, since the PH domain of β-spectrin associates with sites in brain membranes stripped of peripheral proteins that are blocked by inositol 1,4,5-trisphosphate (IP3) and presumably represent phosphorylated sites (416).

C) SPECTRIN-ION CHANNEL INTERACTIONS. Candidates for spectrin-binding proteins in brain synaptosomes include the NR2 and NR1 subunits of the NMDA receptor (422). Spectrin binds to the NR2B subunit at sites distinct from those of α-actinin-2 and members of the PSD95/SAP90 family. The spectrin-NR2B interactions are inhibited by Ca2+ and fyn-mediated NR2B phosphorylation, but not by Ca2+/calmodulin or by calmodulin kinase II-mediated phosphorylation of NR2B. The spectrin-NR1 interactions are unaffected by Ca2+ but inhibited by Ca2+/calmodulin and by phosphorylation of NR1 by protein kinases A and C. The NR1 subunit thus is a candidate to interact with the Ca2+/calmodulin-regulated site on β-spectrin (80, 374).

Spectrin associates via the α-spectrin SH3 domain with the α-subunit of the amiloride-sensitive Na+ channel, EnNaC (349, 457), and with the Na+/H+ exchanger, NHE2 (59). The amiloride-sensitive Na+ channel/spectrin complexes also contain the protein Apx, which is an apically localized protein identified in Xenopus (457). Association with α-spectrin may be responsible for apical targeting of NHE2, since deletion of the spectrin-binding loop results in basolateral localization of the channel (59). α/βH (DJW) is the most likely form of spectrin to associate with the apically targeted NHE2 and amiloride-sensitive Na+ channels since α/β-spectrin is localized to the basolateral domains of epithelial cells (438).

The cGMP-gated cation channel of rod photoreceptor plasma membranes copurifies with a 240-kDa polypeptide subsequently identified as spectrin based on immunoreactivity (290). Direct linkage between spectrin and the cation channel is likely since antibodies against each protein coimmunoprecipitates the other from relatively pure preparations of the channel. The 240-kDa polypeptide was localized to the inner surface of the rod outer segment plasma membrane and excluded from the disk membranes, as would be expected for an association of this protein with the cGMP-gated cation channel in vivo.

D) SPECTRIN AS A MEMBRANE ADAPTOR FOR CYTOPLASMIC PROTEINS. Cytoplasmic ligands for spectrin include a protein termed HsSH3bp1 that binds to tyrosine kinases and binds to spectrin through association with the α-spectrin SH3 domain (432, 456). HsSH3bp1 is associated with macroinosomes and may couple spectrin to these intracellular
organelles (432). Spectrin PH domains are likely to associate with proteins in addition to their interactions with PI lipids (above). An interesting example is RACK1, a protein kinase C-anchoring protein, which associates selectively with the β-spectrin PH domain and activated protein kinase C to form a ternary complex (348).

A search for annexin VI-binding proteins revealed spectrin as one of ~14 membrane-associated proteins that associate with annexin VI in blot overlays (420). Annexin VI has subsequently been implicated in directing proteolytic degradation of spectrin during receptor-mediated endocytosis (204). The interpretation of these experiments was that annexin VI promoted proteolysis of spectrin, which resulted in relaxation of restraints of budding of a subset of coated pits. Annexin VI, according to this model, utilizes spectrin as an adaptor to direct and possibly activate a protease.

E) SPECTRIN AS A REGULATOR PROTEIN. Spectrin, at nanomolar concentrations, inhibits phospholipase D as well as phospholipases C and A2 in in vitro assays (254, 255). The basis for inhibition was most likely not a direct interaction of spectrin with these enzymes, but rather competition for their phospholipid substrates (255). Spectrin in lymphocytes associates with CD45 (192, 248), which is a member of a family of membrane-spanning glycoproteins with protein-tyrosine phosphatase activity. Spectrin binds to CD45 with a nanomolar affinity and upregulates the tyrosine phosphatase activity of CD45 (248).

B. Ankyrins

Ankyrin in human erythrocytes provides a high-affinity link between the cytoplasmic domain of the anion exchanger and the spectrin/actin network (Fig. 1). The ankyrin family has a general role as an adapter between a variety of integral membrane proteins and the spectrin skeleton. The C. elegans genome contains a single ankyrin gene, (312), whereas the D. melanogaster genome has two ankyrin genes, Dank1 and Dank2 (35, 110). The ankyrin gene family of mammals currently includes three members: ankyrin-R (R for restricted; also termed ankyrin1) (229, 260), first characterized in erythrocytes, (227); ankyrin-B (B for broadly expressed, also termed ankyrin 2) first characterized in brain (313); and ankyrin-G (G for general or giant; also termed ankyrin 3) independently discovered in searches for components of the node of Ranvier (219) and for epithelial ankyrins (93, 327, 385). Ankyrins are expressed in most tissues, and in many cases all three ankyrins are found in the same cell type.

Diseases of humans attributed to ankyrins include hereditary spherocytosis, which can result from decreased expression and/or mutated forms of ankyrin-R (112). In mice, a similar disorder (the nb/nb mutation) results in a nearly complete deficiency of 210-kDa isoforms of ankyrin-R in erythrocytes, neurons, and striated muscle (452) with a phenotype of severe anemia (33) and degeneration of a subset of Purkinje cell neurons accompanied by cerebellar dysfunction (326). Ankyrin mutations in C. elegans result in the unc44 phenotype, which includes abnormal axon guidance and uncoordinated movements (312).

1. Domains

Ankyrins are modular proteins comprised of three domains conserved among family members as well as specialized domains found in alternatively spliced isoforms (see Fig. 5). Conserved domains are an NH2-terminal membrane-binding domain, a 62-kDa spectrin-binding domain, and a 12-kDa death domain. The membrane-binding domains are comprised of 24 copies of a 33-residue repeat known as the ANK repeat that is involved in protein recognition in many types of proteins (34, 359) (Fig. 6). The 24 ANK repeats form 4 subdomains, each comprised of the basic folding unit of 6 repeats (280). The role of six-repeat subdomains as protein binding sites is discussed below. Death domains were first reported in proteins such as Fas and the tumor necrosis factor receptor that participate in apoptosis pathways (394). These domains can associate with related death domains in other proteins. The protein interactions of the ankyrin death domain could involve self-association and/or interactions with other proteins and are not yet resolved. The death domain is followed by a regulatory domain subject to alternative splicing that in the case of ankyrin-R modulates both binding of the anion exchanger and of spectrin (81, 157). A regulatory function for this domain has not been demonstrated for ankyrin-B or ankyrin-G. However, the COOH-terminal domains are the most divergent among ankyrin family members and are likely to have distinct functions that are yet to be defined.

2. Diversity due to alternative splicing

Ankyrin genes are expressed and processed in a complex tissue-dependent and developmentally regulated fashion, giving rise to a large number of isoforms that utilize different combinations of functional domains. For example, alternative splicing generates ankyrin-G isoforms missing all or portions of their membrane-binding domain (93, 178, 219, 327). Alternate exon usage is also used to generate an unusual isoform of ankyrin-R in striated muscle. This isoform lacks both the membrane-binding and spectrin-binding domains and contains only the COOH-terminal 26 kDa plus a hydrophobic stretch (452). The functions of these small ankyrins are likely to involve intracellular as opposed to plasma membrane roles, since these truncated ankyrins are associated with Golgi, lysosomal, and sarcoplasmic reticulum membranes (11, 88, 92, 452).
Ankyrin-B and ankyrin-G spliceoforms also include polypeptides with insertions of up to 2,500 amino acid residues and molecular mass of up to 480 kDa, which are abundantly expressed in axons (49, 219, 224, 225) (see below). The inserted sequences in both genes are placed between the spectrin-binding and death domains (see Fig. 5). Ankyrin-G polypeptides of 270 and 480 kDa contain inserted sequences beginning with a serine/threonine-rich stretch of ~400 residues, that is followed by sequence with similarity to that of the inserted sequence of 440-kDa ankyrin-B. The 270-kDa ankyrin-G lacks a 190-kDa stretch of this sequence resulting from the use of an alternate splice donor site.

Much of the 220-kDa inserted sequence of 440-kDa ankyrin-B has the configuration of an extended random coil based on physical properties of expressed polypeptides (49). Because the alternatively spliced insert of ankyrin-G has a highly polar hydrophilicity profile similar to that of ankyrin-B, it is likely that the 480-kDa ankyrin-G also contains an extended stretch of random coil inserted between its spectrin-binding and death domains. The properties of the inserted sequences suggest a structural model for 440-kDa ankyrin-B and 480-kDa ankyrin-G where the membrane-associated head domain is separated from the death domain by an extended filamentous tail domain encoded by the inserted sequence (see Fig. 5). The length of the tail domain could be up to 0.5 μm if fully extended, which is a distance that, in principle, could be resolved in the light microscope.

Ankyrin isoforms possessing the tail domain are highly localized to axons, whereas isoforms lacking this domain are localized to the neuron cell body (49, 224). Possible functions of the tail domains include axonal targeting motifs, deregulation of ankyrin membrane-binding domains by physical separation from the regulatory domain, and long-range connections between molecules interacting with the death domain/COOH-terminal domains and membrane-binding domains. Given the potential distance spanned by the inserted sequence, these putative ankyrin-binding molecules could be in distinct membrane domains or even different cellular compartments.

3. Ankyrin-binding membrane proteins

A defining feature of the ankyrin family is their ability to interact through the membrane-binding domain with structurally diverse proteins with apparently unrelated primary sequences. Currently identified ion channels/pumps that associate with ankyrin and colocalize in cells include anion exchanger isoforms (AE1, AE2, AE3) (22, 23, 196, 293), the Na\(^+\)-K\(^+\)-ATPase (216, 297, 304), the voltage-dependent Na\(^+\) channel (263, 269), and the Na\(^+\)/Ca\(^{2+}\) exchanger (240). IP\(_3\) receptor and ryanodine receptor Ca\(^{2+}\)-release channels also associate with ankyrins (36, 37, 199). Evidence for an in vivo interaction between ankyrin-B and Ca\(^{2+}\) release channels is based on altered targeting of these proteins in striated muscle of ankyrin-B-deficient mice (397) (see below). Finally, ankyrin also associates with cell adhesion molecules (CAMs) including CD44 (203, 249) and the L1 CAM family (L1/neurofascin/NrCAM/CHL1/NgCAM) (76, 79, 107, 182, 450). Evidence for physiologically important interactions between ankyrins and L1 CAMs include downregulation of L1 in
axons of ankyrin-B (−/−) mice (358), and reduction of Dank2 in cell bodies of neurons of neuroglian mutant flies (35).

The ankyrin membrane-binding domain also interacts with cytoplasmic proteins including tubulin (19, 75, 82) and clathrin (283). Ankyrin associates with microtubules with a relatively low affinity in the micromolar range, and the physiological significance of this interaction remains to be determined. However, ankyrin association with clathrin occurs with a \( K_D \) in the nanomolar range and is specific for the fourth subdomain of ankyrin and the terminal knob domain of clathrin. Evidence that ankyrin-clathrin interactions are functionally important is that microinjection of the fourth subdomain inhibits receptor-mediated endocytosis of low-density lipoprotein (283).

The basis for diversity of binding partners for the ankyrin membrane-binding domains is due to special properties of the ankyrin repeats, a motif which mediates protein recognition for a large number of proteins including the transcription factors GABP-β, NfκB/κB, and SwI 6, p53 binding protein and p16 cyclin inhibitor (reviewed in Ref. 359). Atomic structures for these proteins reveal that ankyrin repeats are folded into a series of antiparallel \( \alpha \)-helices connected by loops that are arranged perpendicular to the helices (Fig. 6). Residues located at the tips of the loops are the most variable between repeats and contain recognition sites for diverse proteins. The four repeat subdomains of ankyrins each have distinct binding properties, and these subdomains also cooperate with each other to generate a further level of diversity (281, 282). An additional feature of ankyrin is that binding interactions can occur simultaneously with different membrane proteins, resulting in multiprotein complexes (281, 282).

Sites of ankyrin interactions have been evaluated for the erythrocyte anion exchanger (AE1) (78, 96, 424), the Na\(^+\)-K\(^+\)-ATPase (197, 453), and the L1 CAM family (181, 450). A general conclusion from these studies is ankyrin is capable of structurally distinct interactions with diverse proteins. This conclusion is reinforced by the fact that ankyrin has independent binding sites for AE1 and L1 CAMs (281, 282). Another conclusion from analysis of ankyrin-binding sites of the Na\(^+\)-K\(^+\)-ATPase (450) and L1

FIG. 6. Schematic view of ankyrin domains and their protein interactions (top) and atomic structure of Ank repeats (bottom). The NH\(_2\)-terminal membrane-binding domain of ankyrins is composed of 24 Ank repeats folded into 6-repeat subdomains. These domains associate with a variety of membrane proteins as well as tubulin and clathrin. The bottom panel shows a stack of six ankyrin repeats in the protein IκB (PDB file 1NFI; Ref. 193). The two images (a and b) show the same structures rotated by 90°. The sites of protein interactions are likely to be the loops of Ank repeats, based on structures of complexes of IκB and several other Ank repeat proteins with their protein partners (see Ref. 359).
CAMs (450) is that ankyrin-binding sites of these proteins share a conformation as a random coil even though they lack obvious sequence similarity. An ankyrin-membrane protein complex has not yet been resolved at an atomic level, but these considerations suggest a model where random coils of at least certain ankyrin-binding proteins associate with loops of ankyrin repeats (see above) (450). Given that ankyrin repeats fold into subdomains, it is likely that loops of ankyrin repeats in different subdomains also could collaborate to create a “binding pocket.”

Cytoplasmic domains of the erythrocyte anion exchanger (5) and neurofascin (450) are both dimers in solution. Considered together with the finding that ankyrin has two binding sites for neurofascin as well as for two sites for the anion exchanger, the existence of dimers implies that these proteins and ankyrin are capable of forming oligomeric complexes. One predicted configuration of the anion exchanger/ankyrin complex at low ratios of ankyrin to the anion exchanger is as a pseudo-dimeric complex. Evidence that such an arrangement occurs in erythrocyte membranes is provided by observations that the anion exchanger behaves as a mixture of dimers and tetramers in native membranes and only as a dimer in ankyrin-deficient membranes (439). One could also imagine that at higher ankyrin-to-membrane protein ratios it would be possible to form linear arrays of dimeric membrane proteins cross-linked by the multivalent ankyrin membrane-binding domain. These complexes could be further immobilized by coupling to the spectrin-based membrane skeleton through the spectrin-binding domain of ankyrin. Ability to form such large immobilized complexes between ankyrin and neurofascin could be important for the assembly of specialized membrane domains such as axon initial segments and nodes of Ranvier where these proteins are localized (see below).

1) L1 CAMS AS GENERAL CORECEPTORS FOR ANKYRINS. The major class of ankyrin-binding proteins in mammalian brain is a group of CAMs in the Ig/FnIII superfamily known as L1 CAMs. These molecules together comprise over 1% of the membrane protein in adult brain tissue and are abundantly expressed on axons in the developing nervous system (reviewed in Ref. 181). The L1 CAM family of cell adhesion molecules in the vertebrate nervous system is comprised of L1, NgCAM, NrCAM, CHL1, and neurofascin, in D. melanogaster by neuroglian (181), and in C. elegans by LAD1 (L. Chen and V. Bennett, unpublished data). L1 CAMs possess variable extracellular domains comprised of six Ig and three to five fibronectin type III domains, along with a relatively conserved cytoplasmic domain (181). Extracellular domains of L1 CAMs participate in homophilic interactions as well as a variety of interactions with soluble proteins and other CAMs (39, 121, 350). Extensive diversity in extracellular interactions of L1 CAM family members is provided by divergence between extracellular domains encoded by different genes as well as multiple alternatively spliced variants of each gene (as many as 50 estimated in the case of neurofascin) (165). Consistent with the diverse extracellular domains, a range of functions has been attributed to the L1 CAM family, including axon fasciculation, axonal guidance, neurite extension, a role in long-term potentiation, synaptogenesis, and myelination (39).

Analysis of LAD-1, the single L1 CAM gene in C. elegans, has provided a global view of the pattern of expression in a metazoan and suggests a general expression at sites of cell-cell contact early in embryonic development and in essentially all cell types (Chen and Bennett, unpublished data). LAD-1 binds to GFP-tagged worm ankyrin (unc44) in cultured cells and colocalizes with ankyrin in most cell types. These findings suggest that LAD-1 is the major receptor for ankyrin (unc44) in the worms. Given that ankyrin is multivalent with respect to neurofascin and the anion exchanger (282), it is possible that ankyrin forms heterocomplexes with LAD-1 and various ion channels (see Fig. 7 for an example of vertebrate L1 CAM/Na\(^{+}\) channel complexes).

An extrapolation from the results in C. elegans is that additional members and/or alternatively spliced forms of the L1 CAM family in vertebrates are expressed in all tissues and very early in development (the two-cell stage), and function as coreceptors for ankyrin. Precise colocalization of L1 CAMs, ankyrin, and voltage-gated Na\(^{+}\) channels has been demonstrated at nodes of Ranvier and axon initial segments in the vertebrate nervous system (77, 228). It will be of interest to determine if other ion channels reported to associate with ankyrin also are colocalized with CAMs and if these have homology to L1 CAMs. Another variation on the theme of ion channels and CAMs may be provided by the Na\(^{+}\)-K\(^{-}\)-ATPase which has an \(\alpha\)-subunit, responsible for ion permeation, associated with a \(\beta\)-subunit which has properties of a CAM (148).

Residues critical for ankyrin-binding activity of L1 CAM cytoplasmic domains are localized in a 26-residue stretch which includes a sequence, EDGSFIGQY, which is highly conserved in all family members from C. elegans to mammals (one exception is CHL1 which has the sequence EDGSFIGAY) (136, 182, 451; Chen and Bennett, unpublished data). Mutations of the FIGQY tyrosine in the cytoplasmic domain of neurofascin (Y81H/A/E) greatly impair neurofascin-ankyrin interactions (451). Mutation of human L1 at the equivalent tyrosine (Y1229H) is responsible for certain cases of mental retardation (212). Mutations F77A and E73Q greatly diminish ankyrin-binding activity, whereas mutation D74N and a triple mutation of D57N/D58N/D62N result in less loss of ankyrin-binding activity. These results provide evidence for a highly specific interaction between ankyrin and L1 CAM members.

The basis for specificity of association between var-
ious L1 CAMs and different ankyrin family members in the context of cells has yet to be determined. However, the ability of neurofascin to participate in high-affinity interactions with both ankyrin-R (282) and ankyrin-B (Chen and Bennett, unpublished data) and the ability of LAD-1 to associate with mammalian ankyrin (Chen and Bennett, unpublished data) suggest that these interactions most likely are not restricted by intrinsic affinity. This raises the interesting question as to how different ankyrins in the same cell are segregated with their respective binding partner and how these interactions are regulated.

B) L1 CAMS AND PHOSPHOTYROSINE-BASED SIGNALING. The association between ankyrin and neurofascin is abolished by phosphorylation of the FIGQY tyrosine, which as noted above is conserved among all L1 CAM family members (136). Phosphorylation of the FIGQY tyrosine inhibits activity of neurofascin in mediating cell adhesion and cell segregation in cultured cells (398). Inhibitory effects of FIGQY phosphorylation on cell-cell interactions of neurofascin were proposed to result from loss of ankyrin-mediated cross-linking of neurofascin (398).

Antibodies specific for the phosphotyrosine form of the FIGQY peptide resolve members of the L1 CAM family at strategic sites in the nervous system of vertebrates including paranodes of nodes of Ranvier, the AChR domain of the neuromuscular junction, and in zones enriched in migrating neurons (S. Jenkins, A. Sen, N. Carmarcy, R. Sealock, and V. Bennett, unpublished data). Phospho-FIGQY tyrosine forms of LAD-1 are localized in the pharynx and in the vulva of *C. elegans* in a complementary pattern to the distribution of ankyrin, indicating the conservation of this phosphorylation as well as the potential for its extraneuronal expression (Chen and Bennett, unpublished data). These considerations suggest that L1 CAMs participate in vivo in a phosphotyrosine-based signaling pathway, with one consequence being loss of ankyrin binding. The extracellular ligand(s), kinase(s), and phosphatase(s) involved in determining the phosphorylation state of L1 CAMs remain to be defined. FIGQY-phosphorylated L1 CAMs, by analogy with SH2 and PTB systems, also may associate with yet to be identified phosphotyrosine-dependent adaptors.
C. Proteins That Promote Spectrin-Actin Interactions

Association of spectrins with F-actin is relatively weak with measured $K_D$ values ranging from 6 $\mu$M for recombinant $\beta_2$-spectrin (238) to 250 $\mu$M for native erythrocyte dimer (309). In fact, spectrin most likely requires accessory proteins in vivo to initiate assembly and to stabilize actin interactions. Advantages of a low-affinity spectrin-actin interaction with the potential for enhancement by accessory proteins include the ability to selectively stabilize spectrin-actin networks in specific locations, as well as providing an additional level of regulation. Protein 4.1 (123, 400, 401) and adducin (23, 134, 135) have been identified in erythrocyte membranes based on their activities in stabilizing spectrin-actin complexes. These proteins have been localized at the spectrin-actin junction in erythrocyte membranes (91), as anticipated from their association with spectrin and actin in vitro.

1. Protein 4.1

A) PROTEIN 4.1 IN ERYTHROCYTES. Protein 4.1 purified from mammalian erythrocytes associates with $\beta$-spectrin at a site in the NH$_2$-terminal region (12, 399, 400), and it also interacts with F-actin (12, 63, 296). Protein 4.1 can promote a spectrin-4.1-F-actin ternary complex (401). In the presence of 4.1, strong spectrin-actin interaction is observed: the association constant for the ternary complex is $1 \times 10^{12}$ $M^{-2}$ (309). The spectrin-4.1 complex also caps the minus end of actin filaments and can sever F-actin filaments (331). Standard preparations of erythrocyte ghosts have minus ends of actin filaments that are capped (330), but it is difficult to be certain of the physiological significance in erythrocytes of the spectrin-4.1 capping activity, since these cells also contain the specific minus end capping protein tropomodulin (reviewed in Ref. 125).

A proportion of protein 4.1 remains membrane-bound after erythrocyte ghosts are extracted with low ionic strength buffers. Several possible membrane associations have been identified including membrane lipids, the anion exchanger band 3, and glycophorin C (e.g., Refs. 3, 137, 138, 169, 195, 266, 267, 320, 341). Protein 4.1 and glycophorin C form a high-affinity ternary complex in vitro with the membrane-associated guanylate kinase (MAGUK) p55 (266). Genetic interactions between human glycophorin C and p55 are indicated by loss of these proteins from membranes of red blood cells from a patient with a 4.1 deficiency (3).

Protein 4.1 interactions in red blood cells are regulated by phosphorylation and by interaction with calmodulin. Protein 4.1 is a substrate for protein kinases A and C, casein kinase II, and an erythrocyte tyrosine kinase. Interestingly, phosphorylation by all these kinases results in downregulation of 4.1 membrane- and/or spectrin-actin binding activities (e.g., Refs. 50, 51, 74, 180, 245, 332, 376). Calmodulin alone (independent of Ca$^{2+}$) can inhibit spectrin-actin and membrane binding (251, 306, 379).

B) MAMMALIAN GENES FOR PROTEIN 4.1 AND DOMAIN STRUCTURES. The prototypical 4.1 from erythrocytes is now known as 4.1R (R for red blood cell) and is encoded by the EPB41 gene. Three other genes encode close human and mouse homologs of 4.1R; these are EPB41L2, EPB41L3, and EPB41L1 (328). The corresponding proteins are 4.1G, 4.1B, and 4.1N. mRNA encoding 4.1R is most abundant in hematopoietic tissue such as bone marrow and fetal liver, but the mRNA is expressed at lower levels elsewhere (328). 4.1G is so named because of its very general expression as determined by multiple tissue Northern blots (317, 328). 4.1B is expressed most prominently in brain (316), and 4.1N also in brain, most particularly in subpopulations of neurons (412). In Drosophila and C. elegans, single 4.1 genes are present. The D. melanogaster 4.1 protein Coracle has been extensively analyzed, as will be described below, and gives clues to the functions of 4.1 proteins in a genetic background not complicated by multiple potentially redundant 4.1 genes.

Products of all four mammalian genes are modular proteins, with three functional domains linked by regions relatively unconserved between them (see Refs. 65, 316). This appears to be a general vertebrate pattern for 4.1 proteins. The two most conserved modules are a domain that binds erythrocyte membranes and MAGUKs; the other is a domain at the COOH terminus. The membrane binding domain is a FERM domain, a “genetically mobile” module found in ezrin, radixin, merlin, and moesin as well as certain tyrosine phosphatases, talin, and a variety of other metazoan and plant proteins (57). The structure of the human 4.1R FERM domain has recently been solved (Fig. 8) and is revealed to have three lobes (160). Each lobe has a fold recognizable in other proteins. Most NH$_2$-terminal is a ubiquitin-like fold; the central region of sequence is a four helix bundle similar to acyl-CoA binding protein; the COOH-terminal lobe is similar to the PH/phosphotyrosine binding protein superfamily fold. Although the three lobes in 4.1R FERM domain appear to be independent folding units, they have extensive contacts and are suggested not to function independently. Interestingly, this structure reveals that individual lobes are regulated by differential mRNA splicing. Conboy et al. (69) and Huang et al. (188) found that a common differential mRNA splicing event is the excision of exons 4 and 5, especially in brain and endothelial cell mRNAs. This splice is predicted to remove most of the first lobe of the FERM domain. Although the functional consequence of loss of the ubiquitin-like domain has not yet been identified, mutational analysis of the D. melanogaster 4.1 pro-
tein Coracle indicates that this eliminates membrane binding activity (418).

The FERM domain is associated with a wide variety of ligand binding activities including membrane proteins (234), membrane lipids (especially acidic lipids and their head groups) (114, 139), calmodulin (306), and 2,3-diphosphoglycerate (295). In cells other than red blood cells, FERM domains interact with numerous transmembrane proteins, including adhesion molecules of the neurexin family, the heparan sulfate proteoglycans CD44 (206) and syndecan (61), and a swelling-induced chloride conductance regulatory protein pCln (380). Neurexins and syndecans are discussed in more detail in section III. The structure of the 4.1R FERM domain and the recent structures of moesin (322) and radixin (158) FERM domains illuminate the nature of the interactions. Band 3 (the anion exchanger AE1) binds at a sequence LEEDY (195) within the most NH2-terminal lobe. A region that binds glycophorin C (and probably NCP neurexins and syndecan 2) is present on the central lobe (307). The binding site for the MAGUK p55 (266) is present in the most COOH-terminal lobe. The major protein binding sites are therefore in separate lobes, so possibly differential splicing gives proteins that can (for example) bind distinct classes of membrane proteins. A mechanism underlying 4.1 binding to a number of small phosphorylated ligands such as inositol phosphates (114) or 2,3-diphosphoglycerate (295) may be indicated by the radixin structure. This contains a binding site for IP3 at the junction of the NH2- and COOH-terminal lobes: this site is composed of several basic residues at the cleft between the two lobes. In 4.1R, a deep cleft between the two lobes is surrounded by basic residues, indicating the possible existence of a ligand-binding site.

The COOH-terminal domain (CTD) is the most diagnostic domain of 4.1 proteins. Whereas the FERM domain is widespread, and the spectrin-actin binding domain not highly conserved, the CTD is preserved among different 4.1 proteins. It is recognizable in D. melanogaster and C. elegans 4.1 homologs, always in association with a FERM domain. The CTD has not yet been found outside metazoan, indicating that like spectrin and ankyrin, 4.1 is an animal protein. The COOH-terminal domain has been reported to bind the nuclear mitotic apparatus protein NuMA and the immunophilin FKBP13 (413). Although a 4.1N-NuMA interaction is indicated to mediate some effects of nerve growth factor on pheochromocytoma (PC12) cells (437), there is no evidence from D. melanogaster genetics of an essential role for 4.1 proteins in neuronal differentiation (119).

The spectrin-actin binding (SAB) domain is well characterized in human 4.1R. A 10-kDa fragment pro-

FIG. 8. Mammalian 4.1 proteins. Left: the four mammalian proteins are known as 4.1R, 4.1G, 4.1N, and 4.1B. They are modular proteins, assembled from common domains linked by unconserved sequences. The common domains are a FERM domain (a protein and lipid binding module common to ezrin, radixin, moesin, and some other proteins, Ref. 57); a domain that binds spectrin and actin (the SAB domain, found only in vertebrate 4.1s so far); and a COOH-terminal domain (CTD) common to all 4.1 proteins. [Modified from Parra et al. (316).] Right: the structure of the FERM domain of moesin (PDB entry 1EF1A; Ref. 322). The 4.1 proteins have FERM domains homologous to the equivalent domains in the ERM proteins. The figure shows the FERM domain of human moesin. Although the sequences of the human 4.1R and moesin proteins are only ~32% identical, they probably fold very similarly. The FERM domain has three lobes. Lobe 1 (the NH2-terminal lobe) is similar to the fold of ubiquitin; the fold of lobe 2 is similar to that of the acyl-CoA binding protein; and the lobe 3 fold is similar to that of the pleckstrin homology domain (from the PDB file 1EF1; Ref. 322).
duced in limited chryomtryptic digests of 4.1 promoted spectrin-actin interaction (71, 72). This is encoded by exons 16 and 17 (98, 99, 179). Exon 17 encodes sequence required for the interaction with spectrin and actin, but high-affinity binding is only achieved if sequence encoded by exon 16 is present too. Because exon 16 expression is upregulated during erythroblast differentiation, high-affinity SAB is effectively a gain of function during erythrocyte differentiation. This indicates that SAB may not be a "core" function of the 4.1 proteins, a suggestion supported by the lack of known SAB activity in D. melanogaster Coracle. 4.1G has close conservation of the 4.1R SAB and in vitro promotes spectrin-actin interactions (144). 4.1B also promotes SAB (316). 4.1N does not have an especially well-conserved SAB domain, which is identical to 4.1R in only 26 of 66 residues.

More than one start codon has been identified in 4.1R mRNAs. Erythrocyte 4.1 is predominantly an 80-kDa form. The corresponding mRNA has a 17 nucleotide exon in the 5′-region (exon 2) excised and translation starts at a codon in a 210 nucleotide exon (exon 4) (188). If the 17 nucleotide exon is expressed, translation starts within this exon yielding 135-kDa isoforms typical of nonerythroid 4.1R proteins. The extra NH₂-terminal sequence contains a calmodulin-binding site (209, 230). So far, only one translation initiation codon has been identified in 4.1B, 4.1G, and 4.1N, equivalent to the one that yields the 135-kDa form of 4.1R. However, as with 4.1R, these other proteins are encoded by mRNAs that are extensively spliced.

c) CORACLE, THE 4.1 PROTEIN IN D. MELANOGASTER. Fehon et al. (119) have found a FERM domain protein in D. melanogaster they named Coracle. Coracle is distinguished as a 4.1 protein by the presence of a conserved 4.1 CTD, although there is little sequence evidence of a functional SAB. Mutation of the Coracle gene resulted in defects in dorsal closure; homozygotes are embryonic lethal. Coracle does not obviously colocalize with either spectrin or ankyrin in fly epithelial cells. Together with the lack of an obvious SAB sequence, this indicates that coracle is not a SAB protein. However, the sequence relationship of the FERM and CTD regions of Coracle with mammalian 4.1 proteins provides a useful guide to their core functions.

Coracle is selectively localized at pleated septate junctions of ectodermally derived epithelial cells. Smooth septate junctions of, for example, the midgut do not express coracle. This contrasts again with the very wide distribution of spectrin and actin in the fly. It is also clear that there is no Coracle in fly nuclei. Nonerythroid 4.1 proteins of mammals have been found in nuclei, in association with NuMA (271) and spliceosomes (84). Nuclear import requires sequence encoded by exon 16 of mammalian 4.1 (138), which is not present in Coracle. Because exon 16 encodes both a nuclear localization sequence and part of the SAB domain, nuclear functions, like SAB, probably evolved with vertebrates and are not conserved among 4.1 proteins.

Coracle is required for correct functioning of at least two D. melanogaster transmembrane proteins, the epidermal growth factor (EGF) receptor (119) and the cell adhesion molecule neurexin IV (418). Coracle mutations suppress a hypermorphic allele of the EGF receptor el-lipse, indicating that full expression of EGF receptor activity requires Coracle. Coracle deficiency results in partial loss of adult cuticular structures. A possible mechanism for this phenotype is that EGF receptor function is compromised in flies with Coracle deficiency, resulting in failure of cell proliferation in imaginal epithelia (226).

Neurexin and Coracle depend on each other for correct localization at the septate junction, and physically interact since they communoprecipitate from fly extracts (7, 418). The coracle FERM domain is both necessary and sufficient for correct localization of neurexin (418). The mechanism underlying this is unclear but may require the MAGUK disks lost (DLT; Ref. 27). DLT is essential for cell polarity and is required for correct localization of neurexin IV at septate junctions. By analogy with the erythrocyte 4.1/p55/glycophorin C complex, it seems possible that a ternary coracle/DLT/neurexin IV complex may form at septate junctions, stabilizing the incorporation the CAM at the junction, but at the time of writing, no published account has tested the existence of such a complex. Correct incorporation of neurexin in these junctions seems to be essential for creation of the transepithelial barrier at the septate junction. In this context a recently described interaction of mammalian 4.1R with the tight junction protein ZO-2 (270) may indicate a general role for 4.1 proteins in barrier functions in vertebrates as well as flies.

d) FUNCTIONS OF MAMMALIAN 4.1R REVEALED BY GENETICS. Deficiency or functional inactivation of erythrocyte 4.1R has been linked for some years with the disease hereditary elliptocytosis (HE). Surprisingly, in view of the widespread expression of 4.1R mRNA, no other tissue in these patients has been reported to be affected, even in patients suffering total loss of 4.1R from their red blood cells. For a more detailed analysis of 4.1R mutations in HE, readers are referred to comprehensive reviews in this specialty, e.g., Reference 87. Characterization of numerous mutations in the 4.1R gene has revealed the importance of the various domains in erythrocyte function. Several mutations have been reported within the SAB domain and confirm that spectrin-4.1-actin interaction is essential for normal erythrocyte function. Loss of one or more exons encoding this domain, or mutation of a critical lysine residue in this domain, gives markedly reduced mechanical stability and deformability (66, 70, 95, 253, 265). These mutations are also typically associated with some spectrin loss from the membrane. These cases of HE are associated with anemia, in contrast to cases where the
mutations are in the CTD. A very mild phenotype has been reported in a homozygous HE family in which there are mutations in the CTD (294). In these patients, 4.1R assembles correctly in the membrane skeleton, indicating this domain is not required for any of the classical interactions of 4.1 at the membrane. The relationship of the mutations to the phenotype remains elusive.

So far, the only mouse 4.1 gene to be knocked out is 4.1R (361). Unlike coracle deficiency, loss of 4.1R is not lethal. Indeed, the phenotype is surprisingly mild. However, functional redundancy among the products of the four 4.1 genes could mask potential defects. As would be expected, 4.1R-deficient mice suffer hemolytic anemia. The membranes of erythrocytes are misshapen and frequently fragmented. There is a loss of ~30% of spectrin from the membranes, complete loss of p55, and >80% loss of glycophorin C, i.e., 4.1 is required for stable accumulation of the latter two proteins in erythrocyte membranes. However, platelet function is generally normal, and (at a gross level) the mice are generally unaffected. Nevertheless, subtler defects are manifest in the nervous system. 4.1R null mice exhibit deficits in learning, coordination, balance, and movement (413). The mechanism underlying these defects is unclear, but they fit with the normal pattern of expression of 4.1R mRNA, especially in the dentate gyrus and cerebellum.

Like Coracle and neurexin IV, glycophorin and 4.1 have an interdependent relationship for accumulation at the membrane. Leach elliptocytosis results from mutations in the glycophorin C gene (384) and is associated with loss of both 4.1R and p55 from the erythrocyte membrane (3).

2. Adducin

Adducin (from the Latin adducere, to draw together) forms ternary complexes between spectrin and actin and promotes association of spectrin with actin filaments (21, 135). Although adducin associates directly with the sides (285, 382) as well as fast-growing ends of actin filaments (223), adducin complexes with the combination of spectrin and actin at 5- to 20-fold lower concentrations than with actin alone (239). Adducin exhibits the highest affinity for complexes between spectrin and the fast-growing ends of actin filaments, which form with an association constant of 15 nM adducin (239). The relative activities of adducin for actin filament ends and sides in the presence and absence of spectrin suggest that the preferred role of adducin in cells is to form a complex with the fast-growing ends of actin filaments that recruits spectrin and prevents addition or loss of actin subunits (Fig. 9). Adducin thus is an actin-capping protein that recruits other proteins to actin filament ends and could represent a new class of assembly factor with the function of integrating actin into other cell structures.

Adducin interactions with spectrin and actin are mediated by a COOH-terminal basic stretch of residues with homology to the MARCKS family (myristoylated alanine rich C kinase substrate; Refs. 29, 298) (Fig. 9). The adducin MARCKS-related domain also contains the site of phosphorylation by protein kinase C at the RTPS-serine (268, 269) as well as for binding of Ca$^{2+}$/calmodulin (135, 268). Adducin activities involving spectrin and actin are all inhibited by Ca$^{2+}$/calmodulin (135, 223) and by phosphorylation by protein kinase C (269). Antibodies specific for RTPS-serine phosphorylated adducin reveal that adducin is an in vivo substrate for phorbol ester-activated protein kinases that presumably include members of the protein kinase C family (269). Gelsolin competes for adducin recruitment of spectrin to the fast-growing ends of actin filaments (298) and may provide yet another means of regulating adducin activity.

Adducin has been identified by Kaibuchi and colleagues (213) as a substrate for the Rho-dependent protein kinase at a site distinct from protein kinase C. Experiments with mutated forms of adducin lacking the Rho-phosphorylation site suggest that Rho-dependent phosphorylation of adducin plays an essential role in promoting cell motility (130).

A) ADDUCIN DOMAINS, SUBUNITS, AND QUATERNARY STRUCTURE. Adducin in humans and rodents includes three closely related subunits termed α, β, and γ (102, 134, 201). Adducin subunits all contain an NH$_2$-terminal globular domain that participates in subunit interactions, a neck domain that also promotes formation of oligomers, an unstructured tail domain, and finally a COOH-terminal basic stretch of residues with homology stretch containing the spectrin/actin interaction site as well as targets for calmodulin and protein kinase C (Fig. 9). Erythrocyte adducin is comprised of α/β-subunits in a 1:1 ratio, and in other cells include α/γ as well as α/β combinations of subunits (102, 134). Hydrodynamic and cross-linking experiments with erythrocyte and brain adducins (134, 189, 200) support a physical model of adducin as a mixture of dimers and tetramers comprised of ball and chain-shaped subunits associated via their globular domains with tail domains extending into solution (Fig. 9). Adducin is represented by single genes in C. elegans and D. melanogaster (see below) and presumably exists as either homodimer or homotetramer in these organisms.

Sites of interactions between adducin subunits include an oligomerization region within the neck domain (298), as well as undefined sites in the globular head domains (189). Mechanism(s) operate in vertebrate adducins that somehow ensure assembly of adducin heteromers with 1:1 ratios of α- and β-subunits or α- and γ-subunits. One possibility is that one subunit is synthesized in limiting amounts and monomeric subunits in excess of available partners are degraded. However, recombinant
adducin constructs can form homodimers, suggesting additional aspects of regulation.

All of the spectrin/actin association activities of the adducin are reproduced with recombinant polypeptides containing the neck, tail, and MARCKS domains (298). Tail/MARCKS and neck/tail domains are inactive. These results and oligomerization activity of the neck domain (see above) suggest that the active form is a parallel dimer with dimerization provided by the neck domain and actin contacts provided by the MARCKS domain (298).

The role of adducin globular domains beyond promoting subunit interactions currently is a mystery. As noted above, the neck and tail domains of adducin are necessary and sufficient for all activities involving spectrin and actin (298). The issue of undiscovered roles for the head domain is particularly relevant in view of the high degree of conservation of this domain and the existence of alternatively spliced variants of adducin lacking the COOH-terminal MARCKS-related domain (see below). A possible clue to the head domain function is a similarity in primary sequence to an E. coli enzyme, aldolphosphofructohydrolase, which requires zinc for catalytic activity. Histidines involved in zinc coordination are conserved in adducin, although an aspartic acid residue required for catalytic activity is missing. These considerations suggest that adducin lacks enzymatic activity but may require zinc for function of the globular domain. A corollary of a requirement for zinc is that adducin may have been at least partially inactive in previous biochemical experiments using adducin isolated with the metal chelator EDTA (134).

B) GENES AND ALTERNATIVELY SPliced VARIANTS. Adducins, like spectrins, are, so far at least, found only in metazoans, although one of the adducin domains is expressed in
E. coli (see above). The C. elegans and D. melanogaster genomes each contains a single adducin gene. Adducin in D. melanogaster was discovered as a transposon-induced mutation in oocyte development termed huli-tai shao or hts (too-little nursing) (423, 444, 445). Adducin of vertebrates is encoded by three closely related genes termed α-, β-, and γ-adducins (102, 201). α-Adducin is expressed in most tissues, while β-adducin has a more restricted pattern of expression limited to brain and hematopoietic cells (201). γ-Adducin is a likely companion for α-adducin in cells lacking the β-subunit (102).

Alternative splicing is a general feature of metazoan adducins. The D. melanogaster adducin gene encodes four alternatively spliced forms, including one with a basic COOH-terminal domain, which is expressed in somatic cells (423). Other D. melanogaster adducin transcripts in germ line cells lack the MARCKS-related domain and have distinct 3′-UTR regions resulting in selective targeting of mRNAs within oocytes and nurse cells (423). The C. elegans adducin gene encodes two transcripts, one containing a MARCKS-related domain and the other with a distinct sequence (S. Moorly and V. Bennett, unpublished data). In vertebrates, α- (241), β- (389, 377) and γ-adducins (60) also include variant transcripts lacking the COOH-terminal MARCKS-related domains. Other alternatively spliced versions of adducin subunits have predicted internal deletions within the tail as well as the head domain.

III. PHYSIOLOGICAL FUNCTIONS

A. Overview

Hypotheses for roles of spectrin and ankyrin-related genes have been generated through the classic maneuvers of determining cellular localization and interacting proteins. Manipulation of gene expression in animal models recently has provided critical tests of these hypotheses and provided new functions as well. The purpose of this section is to briefly summarize proposals for function based on localization and protein neighbors, and then to critically evaluate these ideas in the physiological context of animals.

Initial efforts to explore functions of spectrin in cultured fibroblasts by microinjection of spectrin antibodies resulted in aggregation and collapse of intermediate filaments in cells that otherwise were normal (264). These findings suggested that spectrin was not likely to be required for survival of fibroblasts in culture, and apparently was not essential for fundamental metabolic processes. Subsequently, spectrin and ankyrin were discovered to be localized at basolateral domains of a variety of epithelial tissues (103, 104, 302). These morphological observations were accompanied by Nelson’s discovery (304) that ankyrin associated with the Na⁺-K⁺-ATPase, which is well known to be localized at basolateral domains of many epithelial tissues. Nelson and colleagues (277) subsequently discovered that expression of E-cadherin in fibroblasts promoted cell-cell contact and recruited spectrin to sites of cell-cell contact. These investigators also characterized a complex of spectrin, cadherin, and ankyrin that formed during assembly of cultured Madin-Darby canine kidney (MDCK) cells into epithelial sheets (301). Moreover, overexpression of ankyrin-binding and actin-binding domains of β-spectrin resulted in highly abnormal cells lacking polarized distribution of the Na⁺-K⁺-ATPase (92, 186). Taken together, these and other observations have led to a widely accepted view that spectrin and ankyrin have a role either in the initial formation of epithelial cell polarity immediately following cadherin-based cell adhesion, or in maintenance of this polarity (438).

Spectrin and ankyrin have also been implicated in intracellular roles involving the Golgi (11, 88). The concept of a Golgi-based spectrin skeleton is based on the observation of immunoreactive forms of β-spectrin (9, 372) and ankyrin (10, 93, 327) associated with Golgi membranes. The finding that lysosomal membranes contain a 120-kDa ankyrin-G spliced form suggests other organelles also utilize ankyrin and/or spectrin (178). Moreover, spectrin is associated with dynactin, an organelle-based microtubule motor, in cells overexpressing ARP2 (176). Evidence for function of spectrin in post-Golgi targeting is based on disruption of polarized delivery of the Na⁺-K⁺-ATPase in cultured MDCK cells overexpressing of the NH₂-terminal portion of β-spectrin (92).

Spectrin has been proposed to participate in axonal transport based on the observation by Willard and colleagues (237) that spectrin (named fodrin by this group) was present in multiple classes of axonally transported proteins and organelles. Spectrin also has been suggested to participate in tethering synaptic vesicles within nerve terminals (363). This hypothesis is based on association of spectrin with synaptic vesicle proteins synapsin (365) and MUNC 13 (354) and observations of filament-shaped structures the size of spectrin associated with synapses (156).

D. melanogaster and C. elegans with their simplified and completely characterized genomes provide model systems to test function in the context of living animals. The spectrin repertoire of both organisms is comprised of three genes encoding one α, one generally expressed β (β-G), and one β-H subunit (Table 1). The pattern of expression of spectrins in these organisms closely parallels their behavior in vertebrates. β-G spectrin of C. elegans is localized at sites of cell-cell contact beginning at the two-cell embryo and continuing in adult epithelial tissues (292). β-G spectrin in adult C. elegans, as in vertebrates, is most highly expressed in the nervous system.
in axons as well as cell bodies and is localized in muscle cells at sites of contact with the extracellular matrix (292). β-H spectrin of \textit{D. melanogaster} is localized in apical domains of epithelial cells and is in an equivalent location to \(\beta_{\text{TW}}\) of chickens (85, 108, 147, 388).

Several observations, based on consequences of disruption of expression of spectrin genes in \textit{C. elegans} and \textit{Drosophila}, suggest that spectrin, although essential for survival beyond the embryonic stage, actually is not required for initial development of epithelial polarity, Golgi function, axonal transport, or accumulation of synaptic vesicles at presynaptic structures. Normal development of epithelial polarity and normal progression of morphogenesis as well as embryonic viability occurs in \textit{C. elegans} in the absence of \(\beta\)-G spectrin (159, 292). Moreover, \(\beta\)-G spectrin was not required for neuronal migration or axon guidance of dorsal axons, for assembly of synaptic vesicles at nerve endings, or for polarized secretion of collagen. In addition, the plasma membranes of epithelial cells, muscle, and neurons were normal in appearance in electron micrographs, with no evidence for a generalized loss of membrane integrity. Equivalent phenotypes were observed with deficiency of \(\beta\)-G spectrin as a consequence of either injection of double-stranded RNA (292) or a null allele of \textit{unc-70} (159). Normal epithelial polarity in \(\alpha\)-spectrin null mutants also has been observed in \textit{D. melanogaster} larvae (89, 232). Interpretation of these findings was complicated by the possible contributions of maternal spectrin (232, 325). However, the principal period of spectrin expression in both organisms occurs during gastrulation, and spectrin synthesized at this time is most likely not of maternal origin. Moreover, double-stranded RNA injection in \textit{C. elegans} reduces levels of \(\beta\)-G spectrin to \(<\)2% of normal in early embryos.

In summary, direct evaluation of spectrin function in \textit{C. elegans} and \textit{D. melanogaster} has raised serious doubts regarding the previously hypothesized roles in maintenance of plasma membrane integrity, establishing cell polarity, Golgi-related roles, axonal transport, and a structural role in the synapse. Nevertheless, all is not well for spectrin function, as well as its associated proteins in animal models is just beginning, and these results are summarized below.

### B. Stabilization of Cell Surface Membranes at Sites of Cell-Cell Contacts

A unifying role for \(\alpha/\beta\)-G spectrin emerging from results with \textit{D. melanogaster} and \textit{C. elegans} is the stabilization of cell-cell and cell-matrix attachments after these structures have formed. The requirement for \(\alpha/\beta\)-G spectrin becomes most apparent in the larval stage when the animals leave the supportive environment of the egg case and presumably generate mechanical stress on their tissues during movement. Evidence for this idea is that defects in muscle and nervous system are reduced in \textit{unc-54} worms that exhibit reduced movement due to a myosin mutation (159). Moreover, \textit{D. melanogaster} \(\alpha\)-spectrin null larvae that had successfully completed embryonic morphogenesis and established normal cell polarity subsequently exhibited loss of cell-cell contact and cell-substratum contacts (232).

Conditional mutations have allowed evaluation of spectrin function in the developing \textit{Drosophila} ovary, which is surrounded by a follicular epithelium. This epithelial layer is subjected to stress during oogenesis, especially when yolk formation in the oocyte requires an increase in egg volume. \(\alpha\)-Spectrin has been eliminated selectively in follicular cells through site-specific recombination by the FLP/FRT system (231) and in both follicular cells and germ cells through rescue of \(\alpha\)-spectrin null animals with a temperature-sensitive mutation (89). In either case, loss of \(\alpha\)-spectrin resulted in disruption of the follicular cell monolayer and changes in cell shape (89, 231). The absence of \(\alpha\)-spectrin would be expected to disrupt both laterally associated \(\alpha/\beta\)-G as well as apical \(\alpha/\beta\)-H complexes. Observations of \(\beta\)-H-deficient eggs suggest that the loss of \(\alpha/\beta\)-G is responsible for disruption of the monolayer while loss of \(\alpha/\beta\)-H causes changes in cell shape (446) (see below). \(\beta\)-Spectrin mutations in \textit{D. melanogaster} have recently been characterized as embryonic lethal, precluding a direct evaluation of \(\alpha/\beta\) contribution to follicular monolayer integrity (106). However, it will be of interest in future work to target \(\beta\)-spectrin deletion to follicular cells using the FLP/FRT technique.

Activity of \(\alpha/\beta\) and \(\alpha/\beta\)-H spectrin in stabilizing cell-cell contacts and in achieving normal columnar epithelial cell shape requires formation of tetramers. The best evidence for spectrin tetramers as a functional unit is that either a deletion or a point mutation of \(\alpha\)-spectrin that abolish head-head \(\alpha/\beta\) interactions also prevent rescue of \(\alpha\)-spectrin null \textit{D. melanogaster} (89). The requirement for spectrin tetramers suggests that cross-linking of actin filaments is an important aspect of spectrin function and, moreover, suggests an essential role for a spectrin-actin network as resolved in erythrocytes. Coupling of cell adhesion molecules to a spectrin lattice could be anticipated to promote their interaction with molecules on adjacent cells through restricted diffusion and through formation of closely spaced complexes linked to dual sites on spectrin tetramers, thereby increasing the effective local concentration. Also, mechanical coupling of a large number of adhesion molecules on both cytoplasmic surfaces at sites of cell-cell contact into a transcellular...
C. Cell Sheet Morphogenesis

Creation of tissues and early embryos from ensembles of cells requires mechanisms for coordinating forces generated in individual cells to produce morphogenesis. For example, the developmental processes of embryonic elongation of *C. elegans*, dorsal closure in *Drosophila*, formation of enclosed epithelial tissues in metazoans, and neurulation in vertebrates all involve transcellular transmission of forces resulting from constriction of apically associated actin filaments. β-H spectrin has been implicated in these processes based on the phenotypes of β-H mutations in *C. elegans* (276) and in *D. melanogaster* (388, 446).

β-H spectrin mutants in *C. elegans*, termed sma-1 for their small size, are defective in embryonic elongation (276). Normally, the epidermal actin filaments reorganize soon after ventral enclosure to form an array oriented around the circumference of the embryo. Contraction of these actin fibers is accompanied by elongation of the embryo. β-H mutant worms elongate at only 20% of the rate of wild type and attain only half the final length (276). Moreover, these mutants also exhibit defective morphogenesis of the pharynx and excretory cell. β-H mutant worms, although mishaped, are viable.

β-H localization and the phenotype of β-H mutations in *D. melanogaster* provide strong evidence for a role in epithelial morphogenesis. β-H spectrin is expressed in most epithelial tissues and is restricted to apical cell domains where it is present associated with zonula adherens as well as the brush border (231, 386, 388, 446). Mutations of β-H result in extensive larval lethality as well as defective eye morphology, wing morphogenesis, and tracheal defects resulting in loss of hemolymph (388). In addition, follicular epithelial cells lining egg chambers do not undergo apical constriction but instead remain cuboidal (446).

Adherens junctions are abnormal in β-H mutant follicular epithelial cells (446). This observation combined with lack of shape change in β-H deficient epithelial cells suggests that β-H spectrin physically interacts with adherens junction components and couples these to the actomyosin contractile system. Initial targeting of β-H spectrin to apical domains requires α-spectrin, while targeting of α-spectrin requires β-H spectrin (231, 446). The "receptor" for α/β-H spectrin thus requires both subunits. The identity of molecular partners for β-H spectrin remains to be determined. DE cadherins or associated proteins are candidates for such an activity based on colocalization (388).

D. Assembly of Voltage-Gated Na⁺ Channel-Rich Domains in Excitable Cells

Clustering of voltage-gated Na⁺ channels (NaCh) at axon initial segments, nodes of Ranvier, and postsynaptic folds of the neuromuscular junction is vital for generating sufficient local current to overwhelm membrane capacitance and resistance and to initiate sufficient depolarization for effective signaling. Several clues point to a role of ankyrin in NaCh clustering. Ankyrin and the NaCh copurify and associate in vitro as well as in cell models (263, 369). Moreover, 480/270-kDa isoforms of ankyrin-G are localized at axon initial segments and nodes of Ranvier (219), sites where the existence of high density of NaCh has been well documented (48, 404, 430). As yet undefined ankyrin-G spliced forms also colocalize with NaCh in postsynaptic folds of mammalian neuromuscular junctions (122, 220, 431). Ankyrin-G also is associated with NaCh clusters in the dystrophic mouse in regions lacking myelination (86), as well as clusters of NaCh induced in cultured retinal ganglion neurons (205). β II spectrin, in contrast to ankyrin-G, occurs along the length of axons, does not exhibit increased density at nodes of Ranvier (390) or axon initial segments (449), and thus is not likely to play a role in NaCh localization. However, β IV spectrin is colocalized with 480/270-kDa ankyrin-G at nodal and initial axon segments in the central and peripheral nervous system (24). Ankyrin-G and β IV spectrin thus define specialized domains within a more general spectrin-actin network.

Evidence that ankyrin is required for localization of both Na⁺ channels and L1 CAM at axon initial segments is based on targeted knock-out of ankyrin-G expression in the postnatal cerebellum of mice (453). Mutant mice lacking cerebellar 480/270-kDa ankyrin-G developed characteristic cerebellar defects with symptoms of abnormal gait and tremor, and reduced locomotion that became more severe with increasing age. Electrophysiological data from analysis of cerebellar slices demonstrate that Purkinje cell neurons of mutant mice were unable to fire normal action potentials (453). Na⁺ channels were not concentrated at initial segments of granule cells from mutant mice (453). Neurofascin and NrCAM also exhib-
laid loss of restriction to axon initial segments of Purkinje neurons of ankyrin-G mutant mice (453). It will be of interest to determine if ankyrin-G also is required for targeting of β IV spectrin to axon initial segments.

Similarities between nodes of Ranvier and other sites of Na channel concentration support the prediction that ankyrin-G also is required for restriction of Na channels at these domains. Nodes of Ranvier, the neuromuscular junction, and axon initial segments are each highly specialized, suggesting the likely possibility that Na channel/ankyrin-G assemblies will include additional proteins that perform specific functions adapted to each cell domain. Syntrophins, for example, associate with Na channels and are concentrated at the neuromuscular junction (140, 357). The β-subunits of Na channels with their extracellular Ig domains may also be candidates to mediate important domain-specific interactions as well as provide a linkage with ankyrin (263).

Ankyrin-G could form heterocomplexes between Na channels and neurofascin in vivo by directly binding to both proteins, since the ankyrin membrane-binding domain has distinct binding sites for the Na channel, located on subdomains 3 and 4 (370), and for neurofascin, located on subdomains 2 and 3 as well as 3 and 4 (282) (Fig. 7). Possible physiological roles for such Na channel/adhesion molecule/ankyrin complexes include directing extracellular interactions with adjacent cells to sites of channel clusters. Another role of co-clustering of Na channels with neurofascin/NrCAM could be related to the fact that L1 CAM family members are palmitoylated at a conserved cysteine site in their membrane-spanning segments (342). A high density of neurofascin-linked palmitoyl groups (likely to be in the range of 1,000 molecules/μm²) could perturb the local organization of phospholipids. Potential consequences could be recruitment of palmitoylated or lipid-modified signaling proteins and an increased membrane viscosity. It is pertinent in this regard that evidence of a diffusion barrier for glycosphatidylinositol (GPI)-linked proteins and membrane lipids at axon initial segments has been reported, suggesting some difference in the lipid domain at this site (425).

Ankyrin-G, neurofascin, and the voltage-dependent Na channel cosegregate into axon microdomains early in the morphogenesis of the node of Ranvier (228). The 480-kDa ankyrin-G and 440-kDa ankyrin-B are coexpressed in premylinated dorsal root ganglion axons at early stages of axonal growth during embryonic life. However, at the onset of myelination in the sciatic nerve, 440-kDa ankyrin-B disappears and 480/270-kDa ankyrin-G redistributes from a continuous distribution along premyelinated axons to localized patches adjacent to the ends of myelin-associated glycoprotein (MAG)-staining processes of myelinating Schwann cells. All foci of ankyrin-G also contain neurofascin, NrCAM, and the voltage-dependent Na channel. However, neurofascin and NrCAM clusters precede ankyrin-G and are candidates to either recruit or stabilize ankyrin-G at these sites (228). Further observations at later stages of sciatic nerve development suggest pairs of ankyrin/neurofascin/NrCAM/Na channel clusters fuse to form the mature node of Ranvier (228). Interestingly, similar clusters of Na channels have been reported in remyelinating peripheral nerve (111), which may represent a reversion to an earlier developmental stage of the axon as a response to injury.

Ankyrin-based clusters of Na channels persist in Trembler mutant mice with defective myelin (228). These clusters could have partial function in saltatory conduction of the action potential and could explain the relatively mild phenotype of trembler and the related disease of Marie-Charcot-Tooth type 1A in humans.

The molecular events leading to assembly of ankyrin-G and other components at nodes of Ranvier and axon initial segments remain to be deciphered. Neurofascin and NrCAM have been proposed to direct assembly, first, of ankyrin at nodes of Ranvier and other sites, followed by the localization of Na channels (228). However, the finding that neurofascin is not distributed normally in the absence of ankyrin-G (453) suggests that ankyrin-G is required for the concentration of neurofascin as well as the Na channel, at least at axon initial segments. The domains required for restriction of GFP-tagged 270-kDa ankyrin-G to axon initial segments of cultured dorsal root ganglion neurons have been examined with the conclusion that the membrane-binding domain is not sufficient (449). Moreover, both the spectrin-binding and tail domains of ankyrin-G exhibit activity in targeting GFP constructs to the plasma membrane of neurons. These findings suggest that ankyrin-G functions as an integrated unit requiring all of its domains and imply unidentified ankyrin-binding protein(s) upstream in the pathway to formation of axon initial segments.

E. Targeting of Ca²⁺-Release Channels to the Ca²⁺ Compartment of the ER

The ER of metazoan cells is a continuous system of intracellular membranes segregated into subdomains with specialized functions (407). One ER compartment with particular relevance for cell signaling is devoted to Ca²⁺ homeostasis and is a major determinant of intracellular Ca²⁺ levels (278). An ER Ca²⁺ homeostasis compartment is present in many cell types but is morphologically best defined in striated muscle and the nervous system (26, 129, 171). The ER Ca²⁺ compartment of striated muscle, termed the sarcoplasmic reticulum (SR), is intimately integrated with sarcomeres. In heart muscle, this configuration allows generation of Ca²⁺ waves that generate periodic cycles of contraction and relaxation by the contractile apparatus. Ryanodine receptors mediate Ca²⁺...
release from SR stores and are localized adjacent to voltage-sensitive Ca\(^{2+}\) channels in t tubules, which are extensions of the plasma membrane (129). The SR/ER Ca\(^{2+}\)-ATPase (SERCA) is responsible for subsequent Ca\(^{2+}\) uptake into the SR lumen and is localized over actomyosin in the A-band at sites distinct from ryanodine receptors (198). The specialized neuronal ER involved in Ca\(^{2+}\) homeostasis is located in dendritic spines and growth cones of axons and resembles the SR found in muscle as visualized by electron microscopy (171). The Ca\(^{2+}\) compartment of dendritic spines may have a role in cellular memory (26), while the same compartment in axon growth cones modulates axon pathfinding (378).

Evidence for an essential role of ankyrin-B in targeting Ca\(^{2+}\)-release channels to the SR of striated muscle has come from disruption of the ankyrin-B gene in mice (397). Knock-out of the ankyrin-B gene was initially characterized in the nervous system, where the 440-kDa ankyrin-B isoform is required for postnatal survival of premyelinated axons (258) (see below). Ankyrin-B-deficient mice subsequently were discovered to have musculoskeletal defects, elevated serum creatine kinase levels, as well as localized sites of disorganized sarcomeres in skeletal muscle (397). Ankyrin-B (−/−) cardiomyocytes have highly irregular cytosolic Ca\(^{2+}\) waves, with reduced frequency and rate of uptake of Ca\(^{2+}\). Moreover, ryanodine receptors were no longer distributed in a striated pattern associated with sarcomeres in ankyrin-B (−/−) cardiomyocytes and skeletal muscle. IP\(_3\) receptors also exhibited altered localization in cardiomyocytes. The SR and SR/t-tubule junctions are apparently preserved in a normal distribution in ankyrin-B (−/−) skeletal muscle based on electron microscopy and the presence of a normal pattern of triadin and the voltage-gated Ca\(^{2+}\) channel (dihydropyridine receptor). The abnormal localization of IP\(_3\) and ryanodine receptors therefore represents a defect in intracellular sorting of these proteins in striated muscle. Ankyrin-B (−/−) mice also exhibit thymic atrophy as well as reduced expression of IP\(_3\) receptors in thymic lymphocytes (397). The requirement for ankyrin-B for proper targeting of Ca\(^{2+}\)-release channels therefore is likely to be a feature of other cell types with a specialized Ca\(^{2+}\) compartment.

Several considerations support the hypothesis that ankyrin-B operates at the level of transport and/or segregation of Ca\(^{2+}\)-release channels into vesicles. Ankyrin-B is not localized with SERCA or ryanodine receptors in striated muscle (397) but is associated with a novel class of vesicles in striated muscle as well as brain (J. Q. Davis and V. Bennett, unpublished data). Exchange of proteins between ER and Golgi is well known to involve a cellular machinery for recruitment of specific cargo proteins into vesicles and transfer of these vesicles between organelle compartments. A comparable system may also mediate transfer of functionally defined proteins from the ER to specialized sites within the SR. In this case, the basic defect in ankyrin-B (−/−) cells underlying missorting of multiple Ca\(^{2+}\)-release channels could be a failure in some step required for segregation of these proteins and/or their transport between the ER and SR.

Ankyrin-B is a multifunctional protein that could participate in ER protein sorting at several levels. The multivalent ankyrin membrane-binding domain could bind to and laterally segregate selected proteins within the plane of the ER membrane (281, 282). Ankyrin-B also could participate in coupling vesicles to transport systems through interactions of the spectrin-binding domain or the microtubule-association site (75, 88, 173). Finally, ankyrin has recently been reported to associate with the terminal domain of clathrin (283) and could therefore interact with the coat proteins of certain vesicles.

F. Orientation of Mitotic Spindles in Asymmetric Germ Cell Division in Drosophila

Germ line cell division in Drosophila as well as most insects results in a stem cell, which remains undifferentiated, and a daughter cystoblast, which will form the oocyte as well as nurse cells. The cystoblast subsequently experiences four synchronized rounds of mitosis, with the unusual feature of incomplete cytokinesis, resulting in a 16-cell syncytium connected through incompletely closed cleavage furrows. These cells, termed cystocytes, are interconnected by a cytoplasmic structure called the fusome, which was first visualized by light microscopy (85, 275).

Fusomes are comprised of two components: membrane skeletal proteins and membrane vesicles and cisternae proposed to represent a specialized form of the ER (233). Membrane skeletal proteins identified so far in fusomes include adducin (encoded by the huli-tao shao gene), α-spectrin, and β-spectrin (85, 243). β-H spectrin as well as protein 4.1 and other members of the ERM family are not present in fusomes (85, 272). Fusomes originate from a structure in the stem cell termed the spectrosome, which is enriched in ankyrin as well as α/β-spectrin and adducin (243). The membrane compartment of fusomes includes a fly homolog of the transition ER ATPase (TER94) (233). TER94 is believed to have a role in vesicle fusion in assembly of ER cisternae, and the presence of this protein in the fusome implies that fusome membranes are derived from the ER and undergo fusion events.

Mutations in the huli-tao shao (hts) gene, named from the Chinese for too-little nursing (444), and in α-spectrin (85) establish the essential role of these proteins in assembly of fusomes and the spectrosome. Moreover, the phenotypes of these mutants establish that the fusome/spectrosome is required for several steps in successful maturation of oocytes. Hts mutants lack both the
membrane and protein components of fusomes (243, 444). Adducin (or more accurately, the alternatively spliced adducin isoform expressed in germ cells; see below) therefore is required for assembly of both membrane skeletal proteins as well as membrane components of fusomes. α-spectrin mutations targeted to germ cells result in loss of fusome-associated β-spectrin and adducin, but to a lesser extent than the hts mutants (85). α-Spectrin mutants were not examined by electron microscopy, and the state of membrane vesicles has not been determined.

Detailed analysis of hts mutants has demonstrated several roles of the fusome in oogenesis (90, 243). A striking observation is that the spectrosome is required for orientation of the mitotic spindle in the first asymmetric cell division that produces the cystoblast and a renewing stem cell. Spindle orientation is a key event in asymmetric cell division events in generation of neuroblasts as well as other examples (reviewed in Ref. 242). It will be of interest to determine if adducin and other fusome components have a broader role in asymmetric cell division events in addition to insect germ cells. Other consequences of the hts mutation that are likely to be specific for germ cells included loss of synchronized cell division in cyctocytes and loss of polarized microtubule networks (90).

Repasky and colleagues (28, 344) have discovered an intracellular spectrin-rich structure in lymphocytes that has interesting parallels with the fusome/spectrosome of D. melanogaster germ cells. The lymphocyte “spectrosome” also contains membrane vesicles (28), as well as ankyrin (153). Other features of the lymphocyte spectrosome include association with a protein kinase C isoform and relocation in response to extracellular signals. Ankyrin-B is expressed in thymocytes, and ankyrin-B (−/−) mice exhibit thymic atrophy (397), although the spectrosome of ankyrin-B-deficient lymphocytes has not been examined. The role of the spectrosome in lymphocyte physiology thus remains to be evaluated.

The relationship between the isoform(s) of adducin expressed in germ cells and spectrin is not clear. Four adducin transcripts are expressed in D. melanogaster ovaries, and only the isoform found in somatic follicular cells contains the COOH-terminal MARCKS-related domain that associates with spectrin and actin, while the isoforms present in spectrosomes lack this domain and presumably cannot interact directly with spectrin (423). All adducin spliced variants contain a conserved globular head domain, which at this point has an unknown function. The observation that vesicle components are missing in hts mutants suggests the possibility that the head domain of adducin participates in organization of intracellular membranes and that spectrin and ankyrin are secondarily recruited via interactions with adducin-associated membranes.

G. A Role for Protein 4.1 in Selective and Directed Membrane Protein Accumulation

1. The 4.1 proteins and the neurexin superfamily

A striking common feature of D. melanogaster Coracle and mammalian 4.1 proteins is their requirement for the stable accumulation of proteins related to glycoporphin D/C and neurexins in plasma membranes. Both human glycoporphin C and D. melanogaster neurexin IV are trapped and essentially immobile in complexes with 4.1 proteins. The binding site for 4.1 on glycoporphin C has been mapped (266) and shows strong sequence similarity to a juxtamembrane sequence in the cytoplasmic domain of neurexin IV (15).

The neurexins are a family of three classes of CAMs (15, 288, 335, 443). One class is the “classical” neurexins I-III, which have roles in neuron-neuron adhesion. The second class is the “NCP” group, which have roles in mammalian brain in axon-glia contact, and in fly epithelia in septate junction formation. The third class is represented so far only by the fly protein axotactin, a secreted form of neurexin with a glia-neuronal signaling function. The 4.1-binding sequence identified in glycoporphin C and D. melanogaster neurexin IV is also represented in all the transmembrane members of the neurexin superfamily (335).

In mammals, the junctions of axons and paranodal loops from Schwann cells at the paranodal regions of myelinated axons have been likened to septate junctions (15, 113, 406). During development of myelinated nerves, electron-dense junctions progressively form between the Schwann cell loops and axons; these are incomplete at day 10 of the postnatal life of a rat and may require up to day 31 to assume their final form, substantially after the paranodal complexes have been formed (433). The NCP-group protein CASPR/paranodin is found in these junctions (113, 279) and is localized to the paranodes coincident with their formation (339). CASPR/paranodin associates with protein 4.1 both from red blood cells and brain extracts (279). Given that the neurexin IV/coracle complex seems to be required for the formation of a transepithelial barrier in fly epithelium (417, 418), it seems probable that an analogous function is performed by a paranodin/4.1 complex at the junction of axons with myelinating cells. The paranodal junction makes a partial barrier to the diffusion of small ions and a complete barrier to large molecules, and it will be important to test in the 4.1R null mice whether there is evidence of breakdown of this barrier that might underlie their observed neurological defects. CASPR was identified as a ligand in cis on the neuronal cell surface for the GPI-linked molecule contactin (324); contactin is required for cell surface expression of CASPR (115). Contactin-null mice have a
severe ataxic phenotype (25), reminiscent of (but more extreme than) part of the 4.1R-null phenotype (411).

Neighboring to the paranodal regions are the juxtaparanodes, in which another NCP protein CASPR2 (335) is found. Here the Shaker-like K⁺ channels Kv1.1, Kv1.2, and their β-subunit Kvβ2 are clustered and precisely colocalized with CASPR2. The COOH termini of CASPR2 and these K⁺ channels contain potential binding sites for the PDZ domains of MAGUK proteins; CASPR2 contains a putative 4.1 binding sequence. It seems likely that these channels and CASPR2 are cross-linked in a complex with MAGUKs and 4.1. These K⁺ channels are thought to modulate conduction failures at branch points of complex axonal trees and to stabilize transition zones in myelinated axons (58), so it will be important to establish whether these functions are compromised in 4.1R null mice.

The classical neurexins are presynaptic adhesion proteins (38, 288) that retain the conserved 4.1 binding sequence (15). A cytoplasmic interaction of neurexin I with a MAGUK protein, CASK, has been identified in yeast two-hybrid assays (167). CASK is the human homolog of a C. elegans MAGUK, LIN-2, a protein required for vulval differentiation (183). CASK interacts with 4.1R (61), raising the possibility of a three-way neurexin/MAGUK/4.1 complex in presynaptic regions. Although such an idea is not supported by fly genetics (since Coracle has not been reported in presynaptic regions), rat 4.1N has been reported to be abundant in presynaptic terminals (311).

2. The 4.1 proteins and syndecans

Syndecans are a major class of cell surface heparan sulfate proteoglycan (HSPG), of which syndecan-2 is one of the major neuronal representatives in the forebrain. CASK and syndecan-2 interact in two-hybrid assays, are coexpressed and colocalize in brain development, and can be communoprecipitated from brain extracts (61, 185). 4.1R interacts with CASK in vitro, and so is suggested to generate a tripartite complex (61); however, simultaneous binding of the three proteins to each other has not yet been demonstrated, even though syndecan-2 contains a putative 4.1-binding motif.

Bearing in mind that in flies Coracle is essential for full expression of EGF receptor activity, the interaction with syndecans has added interest in terms of signaling. HSPGs are often considered to be coreceptors for heparin-binding factors: by binding these molecules, with lower affinity and specificity than their cognate high-affinity tyrosine kinase receptors, they are regarded as increasing their effective concentration in the vicinity of the plasma membrane (45). In vertebrates, neuregulin/ARIA is a heparin-binding secreted factor that acts on EGF receptor-like tyrosine kinases ErbB3 and ErbB4 (455). Neuregulin/ARIA is concentrated in synaptic clefts of neuronal-neuronal synapses and in the basal lamina of neuromuscular junctions (150, 355) and has a role in inducing transcription of receptors for acetylcholine, N-methyl-d-aspartate, and GABA (117, 314, 347). Syndecan-2 is concentrated in synapses and has been suggested to be the coreceptor for ARIA/neuregulin (185). Both syndecans and ErbB3/4 bind MAGUK proteins (61, 133, 185) and seem likely to be coclustered in postsynaptic regions by MAGUK interactions. Whether 4.1 has a role in linking this receptor/HSPG complex to the spectrin cytoskeleton and thereby stabilizing the mechanics of synaptic junctions, or whether 4.1 is required for the stable accumulation of either receptor or HSPG at the synapse remains to be seen. CASK has a specific signaling role in brain development (184), thus adding an extra dimension to the potential significance of the 4.1-interactive proteins in brain.

IV. CLINICAL IMPLICATIONS

A general lesson from results of gene knock-outs in model organisms of spectrin and ankyrin-related proteins is that these genes are not essential for fundamental cellular function, but act at the level of integration of cells into tissues. A corollary is that mutations in these proteins may be compatible with survival but with impaired physiological function, and therefore are candidates to cause disease in humans. Gene knock-outs of ankyrins, protein 4.1 family members, and adducin in mice are now providing clues regarding general classes of phenotypes to be expected as well as specific lesions. Moreover, these studies suggest the presence of pathways that involve additional proteins and potential disease candidates. A summary of currently known disorders due to abnormalities of spectrin, ankyrin, and associated proteins are discussed below (see Table 2).

A. Functional Channelopathies Due to Defects in Ankyrin-Dependent Targeting

Ankyrin-G and ankyrin-B-deficient mice described above both suffer from missorting of ion channels that are the equivalent in phenotype to mutations in the channels themselves. For example, mice missing cerebellar ankyrin-G exhibited severe ataxia associated with loss of ability of Purkinje neurons to fire action potentials (453). Moreover, ankyrin-G (−/−) mice and mice with a mutation in the SNC6 Na⁺ channel subunit both exhibited progressive degeneration of Purkinje neurons (32, 215). Ankyrin-G is encoded by a complex set of tissue and developmentally specific transcripts (93, 178, 219, 327). Mutations could occur that would target specific tissues as in the case of the cerebellar-restricted gene knock-out. The potential channels targeted by ankyrin-G may include the Na⁺-/K⁺-ATPase (297, 304, 385), the Na⁺/Ca²⁺ ex-
TABLE 2. *Metazoan disorders due to abnormalities of spectrin, ankyrin, and associated proteins*

<table>
<thead>
<tr>
<th>Disorder</th>
<th>Organism</th>
<th>Protein(s)</th>
<th>Reference Nos.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hereditary hemolytic anemias</td>
<td>Human, mouse</td>
<td>Spectrin: α, β; AE1; ankyrin-R; protein 4.1R; glycoprotein C</td>
<td>393</td>
</tr>
<tr>
<td>Ataxia</td>
<td>Mouse</td>
<td>β-adducin</td>
<td>143</td>
</tr>
<tr>
<td>Muscular dystrophy/myopathy</td>
<td>Mouse</td>
<td>Ankyrin-R; ankyrin-G</td>
<td>326, 453</td>
</tr>
<tr>
<td>Morphogenetic defects</td>
<td><em>C. elegans</em></td>
<td>β-G spectrin</td>
<td>157, 292</td>
</tr>
<tr>
<td>Nervous system developmental defects</td>
<td><em>Drosophila</em></td>
<td>β-H spectrin</td>
<td>276</td>
</tr>
<tr>
<td>Abnormal cognitive function</td>
<td>Mouse</td>
<td>Ankyrin-B</td>
<td>358</td>
</tr>
<tr>
<td>Female infertility</td>
<td><em>Drosophila</em></td>
<td>Adducin</td>
<td>444</td>
</tr>
<tr>
<td></td>
<td><em>Drosophila</em></td>
<td>β-H spectrin</td>
<td>388</td>
</tr>
<tr>
<td></td>
<td><em>Drosophila</em></td>
<td>α-Spectrin</td>
<td>231</td>
</tr>
</tbody>
</table>

The specialized neuronal ER involved in Ca\(^{2+}\) homeostasis is located in dendritic spines and growth cones of axons and resembles the SR found in muscle as visualized by electron microscopy (171). The 220-kDa ankyrin-B is expressed in neuron cell bodies and dendrites in the postnatal brain in a time frame that approximates development of the dendritic spine apparatus of hippocampal and Purkinje neurons (225), whereas 440-kDa ankyrin-B is targeted to axons early in development (49, 224). These results suggest that 220-kDa ankyrin-B is a good candidate to participate in targeting Ca\(^{2+}\)-release channels to their physiological sites in dendrites of neurons, whereas 440-kDa ankyrin-B may have a similar function in axons.

B. Use-Dependent Dystrophies of Muscle and Nerves Due to Defects in the Spectrin-Based Transcellular Mechanical Coupling Pathway

Consequences of spectrin deficiency in *C. elegans* (159, 292) include degeneration of muscles and abnormalities in the nervous system that could be attributed to mechanical stresses. Prediction of the phenotype in vertebrates resulting from defects in spectrin are complicated by the multiple closely related genes that may overlap in function. However, truncations of β-spectrin can act in a dominant-negative fashion by poisoning α/β-spectrin dimers and preventing formation of tetramers (186). Such mutations conceivably could exhibit penetrance even in the presence of other normal β-spectrin genes.

Examples of acquired disorders due to loss of mechanical support include unexplained myopathies and pe-
 peripheral neuropathies. Demyelinated peripheral axons would be particularly vulnerable to trauma if axo-glia contacts were weakened. It is of interest in this regard that a major defect in multiple sclerosis is severing of axons at sites where myelin is interrupted (391). A potential therapeutic strategy in this case would be to restore expression of 440-kDa ankyrin-B and of L1, which are normally lost with myelination, and may play a role in stabilizing premyelinated axons.

C. Abnormal Nervous System Development Due to L1 CAM Defects

The L1 CAM family comprises a major class of cell adhesion molecules in the adult mammalian nervous system and has been implicated in diverse functions ranging from axon fasciculation to synaptogenesis (39). Disorders related to defects in L1 have provided initial insight into the clinical implications of these functions. Mutations in the human L1 gene, located on the X chromosome, are responsible for a syndrome termed CRASH for corpus callosum agenesis, mental retardation, adducted thumbs, spastic paraplegia, and hydrocephalus (128, 212). Over 35 mutations in L1 have currently been characterized, and these are distributed in all domains including several in the cytoplasmic domain. Disruption of the L1 gene in mice results in abnormal axon guidance in the corticospinal tract, brain malformations including dilated ventricles, and behavioral deficits (61, 73).

Several considerations implicate ankyrin and/or phospho-FIGQY signaling in the pathophysiology of L1 defects. L1 and 440-kDa ankyrin-B are colocalized in premyelinated axon tracts in the developing nervous system and are both downregulated after myelination (358). Ankyrin-B (−/−) mice exhibit a phenotype similar to, but more severe than, L1 (−/−) mice and share features of human patients with L1 mutations (358). For example, ankyrin-B (−/−) mice exhibit hypoplasia of the corpus callosum and pyramidal tracts, dilated ventricles, all features of the CRASH syndrome associated with L1 mutations. One missense mutation in the L1 cytoplasmic domain results in conversion of FIGQY 1229 to H and clinical disease (405). The Y1229H mutation of the FIGQY tyrosine would abolish both ankyrin-binding activity (450) as well as phospho-FIGQY tyrosine signaling. One or both of these activities thus is essential for L1 function in nervous system development.

Diseases have not yet been attributed to other L1 CAM family members. These genes are autosomal, in contrast to L1, and presumably would exhibit a recessive pattern of inheritance. It also is possible that mutations could result in expression of dominant/negative forms of polypeptides, as has been observed with LAD-1 in C. elegans (Chen and Bennett, unpublished data). It is conceivable that mutations in L1 CAM family members may have subtle consequences at the level of complex neural function resulting in problems such as learning disabilities, thinking disorders, and overall mental retardation. These activities are difficult to assess in mice, but could be significant in a human context.

V. SUMMARY AND PERSPECTIVES

Molecular dissection of the spectrin-based membrane skeleton of the humble mammalian erythrocyte has provided biologists with a set of proteins with diverse roles in organization and survival of cells in metazoan organisms. The lack of an essential function for membrane skeletal proteins in generic cells grown in culture and the absence of their genes in the yeast genome have, until recently, limited advances in understanding roles of these proteins outside of erythrocytes. However, characterization of genes and alternatively spliced variants combined with completion of the genomes of simple metazoans and application of homologous recombination in mice now are providing the first glimpses of the full scope of physiological roles for spectrin, ankyrin, and their associated proteins. These functions now promise to include organization of specialized compartments within the plasma membrane and endoplasmic reticulum, mechanical stabilization at the tissue level based on transcellular protein assemblies, participation in epithelial morphogenesis, and orientation of mitotic spindles in asymmetric cell divisions.

These studies, in addition to stretching the erythrocyte paradigm beyond recognition, also are revealing new pathways essential for metazoan life that may not be present in fungi or plants. Examples include ankyrin-dependent targeting of proteins to the specialized compartments within the plasma membrane and ER, and components and function of the phospho-FIGQY tyrosine pathway of L1 CAMs. A broader issue is the evolution of these pathways, beginning with spectrin and ankyrin, and their associated proteins. These functions now promise to include organization of specialized compartments within the plasma membrane and endoplasmic reticulum, mechanical stabilization at the tissue level based on transcellular protein assemblies, participation in epithelial morphogenesis, and orientation of mitotic spindles in asymmetric cell divisions.

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