THE SPECTRIN-ASSOCIATED CYTOSKELETON IN MAMMALIAN HEART

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TABLE OF CONTENTS

1. ABSTRACT

The membrane-associated cytoskeleton of the cardiac muscle cell is emerging as an important element in the maintenance of normal cell functioning. Recently it was shown that when proteins (betaII-spectrin, muscle Lim-only protein, ankyrin-B, ankyrin-G) of this system are defective or deficient, cardiac malfunction ensues. It is well-established that the spectrin cytoskeleton is associated with the plasma membrane, but it was only lately demonstrated that its components also lie on internal cell membranes. This is particularly apparent in muscle cells of the heart which contain specialised intracellular membrane compartments particular to this cell type such as the sarcoplasmic reticulum and T-tubules. Cardiomyocytes are subjected to constant mechanical stress. Since their mechanics are controlled through coordination of calcium fluxes mediated *via* cell membrane-based assemblies, it is imperative that these essential elements withstand the displacement forces of contraction. Cardiomyocyte spectrin locates the multifunctional spectrin/actin-binding and membrane-binding component, protein 4.1, and they act together on the plasma membrane as well as on internal membranes. We have found that cardiac protein 4.1 links to the calcium handling apparatus whilst spectrins connect with the sarcomeric contractile elements of the cell. Overall this assembly fulfils roles in stabilising cardiomyocyte cell membranes and in coordinating the macromolecular protein accumulations which regulate and accomplish cardiac molecular crosstalk, whilst at the same time enabling the muscle cells to resist extreme forces of contraction.

2. INTRODUCTION

Unlike skeletal muscle, the cardiac cell never takes a rest and equally never tires. Cardiomyocytes have incorporated special adaptations over and beyond that of the skeletal muscle cell in order to coordinate, over extended periods, the external/internal signalling with contraction elements. We now know that these needs are enabled by components of the spectrin-based cytoskeleton. We review the current state of knowledge of this emerging subject and consider the relationship of the cardiac muscle cell cytoskeleton with that of cardiac dysfunction.

3. THE SPECTRIN-ASSOCIATED CYTOSKELETON IN CARDIAC AND SKELETAL MUSCLE CELLS – A HISTORIC PERSPECTIVE

In the early 1980s it became clear that spectrin, far from being restricted to red cells as earlier supposed (1), is widely expressed in mammalian tissues. Polypeptides that cross-react immunologically with spectrin were identified in many tissues (2-5), including cardiac myocytes (4). The proteins identified in all these analyses appeared to be spectrin on the basis of their apparent size (240 kDa and 235 kDa), comparative peptide fingerprints and appearance in the electron microscope. In neurons, axonally transported polypeptides of similar size to spectrin were named fodrin (6). Subsequent cDNA analyses, gene sequencing, and finally the sequencing of animal genomes have revealed that the 'non-erythroid' spectrins are indeed all related to erythrocyte spectrin, and that all derive from prototypical spectrins that arose with early metazoans (7). Erythrocyte spectrin in mammals derives from the alphaI and betaI genes. The most abundant of the non-erythroid spectrins are alphaII and betaII gene products (these are still sometimes referred to as fodrin).

The first high resolution analyses of spectrin in intact muscle revealed spectrin to be concentrated in an array on the cytoplasmic face of skeletal myocytes (8). This array was named ëcostameresí (Latin costa, rib; Greek meros, part) because of its appearance that was reminiscent of ribs. Costameres contain many other structural proteins, among them vinculin and gamma-actin. Vinculin is a component of the apparatus that links receptors for the extracellular matrix (integrins) to the cytoskeleton; beta1-integrin is found in costameres, as is the adaptor protein talin. gamma-actin is a non-sarcomeric actin, indicating that costameres represent a cytoskeletal compartment distinct from the sarcomere. Costameres align over the Z-disk, and are now thought to represent sites of force transmission across the plasma membrane. Intermediate filaments are thought to provide the link between the Z-disk and costameres and it is through them that contractile force is passed.

Ankyrin colocalises with spectrin at skeletal muscle costameres (9). A major transmembrane binding partner for ankyrin at the plasma membrane is Na/K-ATPase (10). Ankyrin and spectrin in epithelial cells are required for correct organisation of this pump (11; 12), and this is also the case in skeletal muscle (13). Mohler and Bennett describe in detail elsewhere in this volume the significance of ankyrin for sodium pump and Na/Ca-exchanger function in cardiomyocytes. In brief, ankyrinB (ankB) is required for the normal operation of the fast Ca^{2+}-clearance system that resets the cytoplasmic Ca^{2+} concentration between beats. This system comprises the Na/Ca-exchanger, NCX1, and the Na/K-ATPase: In the absence of ankB these do not correctly accumulate at the plasma membrane. The Ca^{2+} therefore takes longer to clear after each beat, resulting in a long QT cardiac arrhythmia (14).

Spectrin in cardiomyocytes is also localised at costameres (15), and additionally at intercalated discs (16) – structures not present in skeletal muscle. Isayama et al. (16) noted a distinct segregation of spectrin isoforms in heart. Using polyclonal antibodies to erythrocyte spectrin they found reactivity in all regions of the plasma membrane. Antibodies to brain spectrin (presumably mainly to epitopes in alphaIIbetaII spectrin) reacted most strongly with intercalated discs. The presence of betaI at lateral membranes was confirmed by Flick and Konieczny (17). These authors showed that in heart betaI mRNA and protein was expressed, and that it was in the costameres. They did not note any accumulation at intercalated discs. Similarly, Kostin et al. (18) noted costameric spectrin using an antibody cross-reactive between erythrocyte alphaI- and betaI-spectrin, and did not note any intercalated disc spectrin. Thus, it seems that betaI is restricted to lateral membranes, and is absent from costameres. Kostin et al. also noted immunoreactivity in T-tubules.
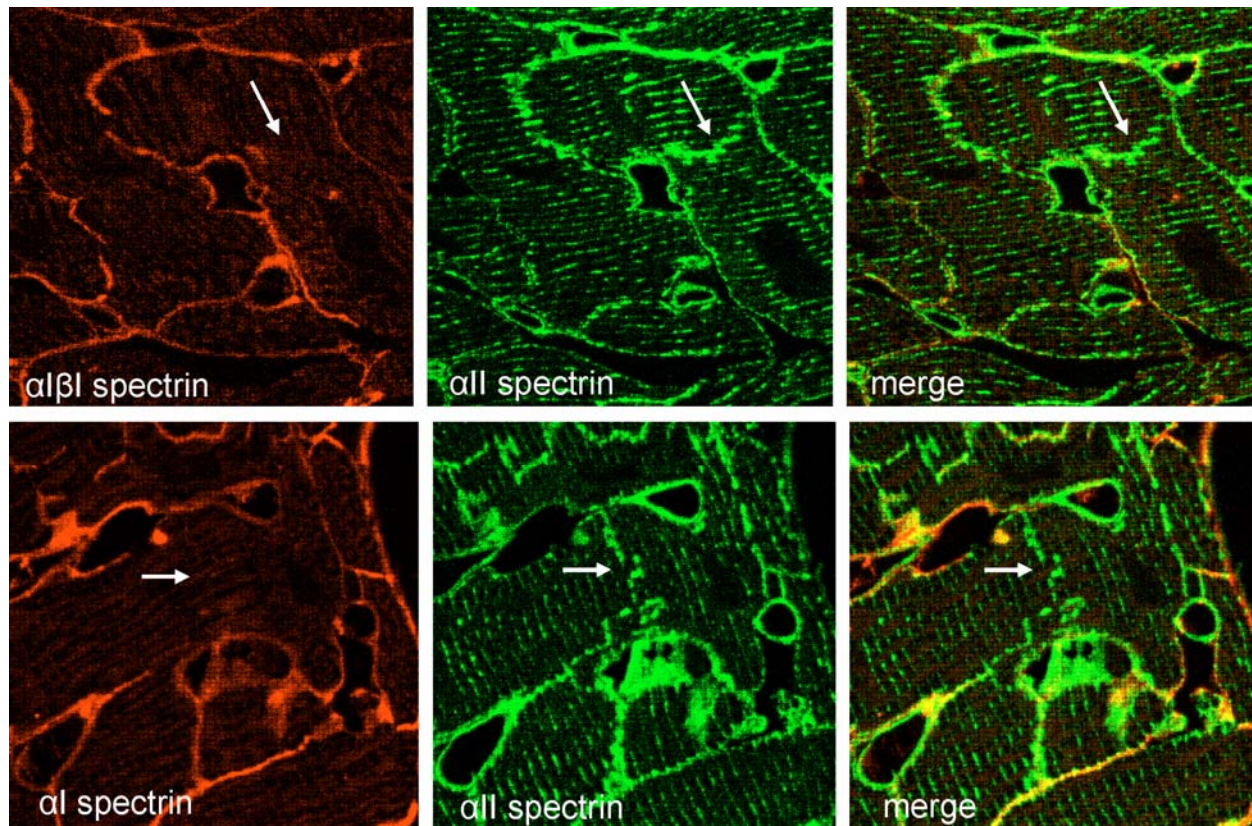
Protein 4.1 has also been described in skeletal muscle. Analysis of 4.1R gene expression revealed a splice variant containing an exon (17A) that was essentially muscle-specific (19). Kontrogianni-Konstantopoulos et al. (20) described 4.1R immunoreactive proteins in skeletal muscle: principally, these were in register with the A-band. Significantly, this is different to the costameric location noted for spectrin and ankyrin. In the A band it is possible that 4.1R binds directly to myosin since the spectrin/actin-binding domain (SAB; present in skeletal muscle isoforms) binds myosin (21). The mRNAs for 4.1R, 4.1G and 4.1N are expressed in rat heart (22), but no previous descriptions of their localisation exist.

4. CARDIAC SPECTRIN-ASSOCIATED CYTOSKELETON GENES AND PROTEINS

4.1. Spectrins in heart: isoforms, locations and tetramers
4.1.1. alphaI-spectrin

Isotype-specific monoclonal antibodies are available to alphaI-spectrin (23). We have used them to reveal the specific location of alphaI-spectrin in cardiac muscle (Figure 1). Note that label is at the lateral plasma membrane, and that it also gives a faint internal stain. Strikingly, the intercalated discs are not labelled. Thus, both the 'erythroid' spectrin subunits are selectively associated with the lateral membrane.

Figure 1. Comparative localisation of alpha-spectrins in cardiac muscle. Immunofluorescence images showing the comparative locations of spectrin polypeptides in mouse cardiac muscle (0.2 μm sections). Antibodies that react with alphaIbetaI-spectrin, alphaI-spectrin and alphaII spectrin all recognise lateral plasma membranes. alphaII-spectrin is in intercalated discs (arrowed), which are devoid of reactivity with the other antibodies. Cross-striations recognised by alphaII antibodies are Z-discs (24). Antibodies used are monoclonal SB-SP1 (Sigma, anti-alphaIbetaI); monoclonal 17C7 ((23), anti-alphaI); polyclonal anti-alphaII (24). Image kindly provided by Pauline Bennett and Alison Maggs (King's College London). All appropriate consents and approval for the conduct of these experiments have been obtained.

4.1.2. alphaII-spectrin

We have used an isotype-specific polyclonal antibody in both immunofluorescence and immunogold microscopy to evaluate the location of alphaII-spectrin in mouse heart (24). We found that alphaII is abundant at the lateral plasma membrane (Figure 1). Since we also found alphaI here, isoforms of spectrin in costameres might be described as ({alphaI,alphaII}betaISigma2)$_2$. The existence of a mixed alphaI,alphaII-containing heterotetramer has yet to be proven by immunoprecipitation (IP) from cardiac membranes, but IP from skeletal muscle supports the existence of such tetramers (25).
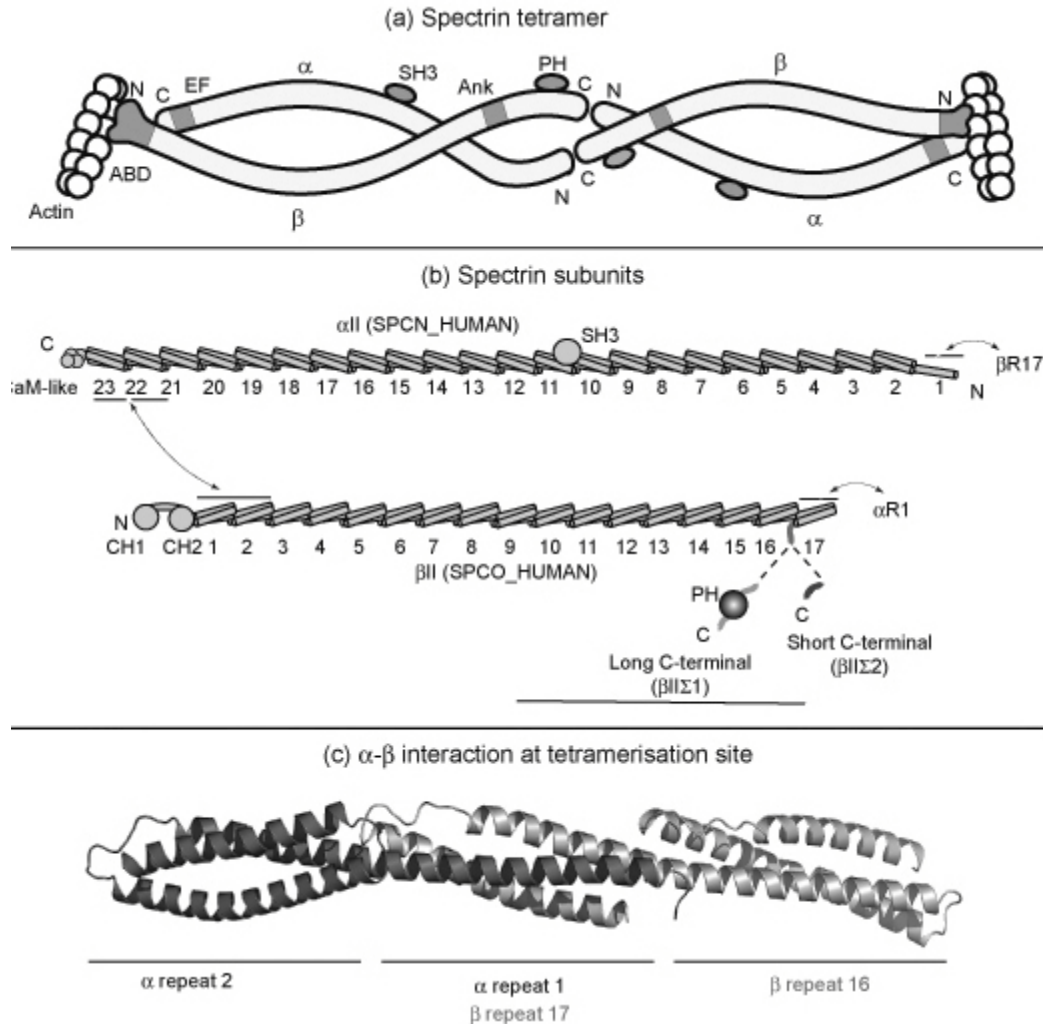
One of our most striking findings is the enrichment of alphaII at intercalated discs (24). This clarifies and extends the earlier data of Isayama *et al*. (16) who showed that antibody to brain spectrin (*i.e.* polyclonal antibodies enriched in anti-alphaII/betaII antibodies) reacted strongly with these structures.

4.1.3. betaI-spectrin

Analysis of cDNAs for skeletal muscle betaI-spectrin revealed a major splice variation near the C-terminus. The prototypical betaI-spectrin of erythrocytes (subtype 1, or betaISigma1) has a short stretch of sequence on the C-terminal side of the last spectrin repeat. This is a sequence rich in phosphorylation sites. In muscle the phosphorylation sites are lost, and replaced by a longer region which links the last repeat to a pleckstrin homology (PH) domain (subtype 2, or betaISigma2). This domain is a membrane binding site since it can interact with phosphorylated inositol lipids. Flick and Konieczny (17) demonstrated that this isoform is also expressed in cardiac muscle, so it is most likely that the major spectrin isoform of cardiac costameres contains betaISigma2-spectrin. Strikingly, this isoform is not present at intercalated discs, giving a strong indication of functional compartmentalisation of membrane skeleton proteins on the plasma membrane.

Flick and Konieczny (op.cit.) showed that the muscle Lim protein (MLP) is a binding partner for betaI-spectrin at plasma membrane: it interacts with repeat 7 of betaI. Repeat 7 is not well conserved in evolution (26), and the suggestion is that this is a comparatively recent evolutionary adaptation of beta-spectrin. Since MLP binds

Figure 2. Spectrin tetramers and their mechanism of formation. (a) Spectrin tetramers arise by head-to-head association of alpha-beta dimers. Each dimer contains anti-parallel alpha and beta subunits. The majority of each polypeptide is made up from repeating ~106 amino acid units: exceptions (indicated) are an SH3 domain in alpha; two EF-hands (EF) near the C-terminus of alpha; an actin- and protein 4.1-binding domain (ABD) near the N-terminus of beta; and a pleckstrin homology domain near the C-terminus of beta. Ankyrin binds repeats 14/15 of beta. (b) The domain structure of alphaII- spectrin and betaII-spectrin. ~106 amino acid repeats are numbered. Near the C-terminus of alpha is a calmodulin (CaM)-like domain which contains two functional Ca^{2+}-binding EF hands. The actin/4.1-binding domains comprise a pair of calponin homology domains (CH1 and CH2). The PH domain can be variably spliced to give long (betaIISigma1) or short C-terminal (BIISigma2) variants. The short C-terminal variant lacks the PH domain. (c) Tetramerisation site. Repeat 1 of alpha represents just one helix of the three in a typical spectrin repeat. It interacts with repeat 17 of beta which contains two helices. The single helix of alpha and two of beta recapitulate an entire repeat. Variations in sequence between the different alpha and beta polypeptides give rise to interactions of varying affinity, as described in the text.

alpha-actinin through its LIM1 domain (27) and spectrin through its LIM2 domain, Flick and Konieczny (op cit.) suggested that MLP bridges betaI-spectrin to alpha-actinin. This would provide a direct bridge between costameric spectrin and the sarcomeric Z-disc.

4.1.4. betaII-spectrin

We have described a novel splice variation at the C-terminus of betaII-spectrin (28). As with betaI-spectrin, it turns out that there are long and short C-terminal variants

(Figure 2). The long C-terminal (*i.e.* with the PH domain) was the first to be discovered (29; 30), accordingly it is subtype 1 (betaIISigma1). The short C-terminal variant (betaIISigma2) lacks the PH domain, but has instead a short segment rich in potential phosphorylation sites. Although there is no substantial sequence identity between the short C-terminal regions of betaI and betaII spectrins, it is noticeable that they are both potential regulatory domains in that they contain multiple phosphorylation sites.

The cardiac cytoskeleton

Table 1. Location of spectrin and related proteins in Mouse cardiac muscle cells

	Z/I-band	M-line	Lateral Plasma membrane	ID	Nucleus	Reference
4.1R	✓	✓	✓	✓	✓	42
4.1G	✓	✗	✗	✗	✓	42
4.1N	✓	✗	✓	✓	✗	42
4.1B	✗	✗	✗	✗	✗	42
alphaI-spectrin	✗	✗	✓	✗	✗	Our unpublished data
alphaII-spectrin	✓	✗	✓	✓	✓	24
alphaIbetaI-spectrin (using alphaI/betaI monoclonal antibody)	✗	✗	✓	✗	✗	Our unpublished data
betaII-spectrin	✓	✓ (Sigma2 only)	✗	✓		28
AnkB	✗	✓	✗		✗	14
AnkR	✓	✓		✓		
AnkG	✓		✓	✓	✗	76

✓ denotes present at this subcellular location; ✗ denotes absent at this subcellular location

Antibodies to both betaIISigma1 and betaIISigma2 react with intercalated discs (28), so we may assume that intercalated discs contain (alphaIIbetaIISigma1)$_2$ and (alphaIIbetaIISigma2)$_2$ tetramers. Neither of the betaII splice variants is detected to any extent in costameres.

4.1.5. Intracellular spectrins

In addition to the expected plasma membrane locations, alphaII and both betaII variants are found intracellularly, close to sarcomeres. We showed that betaIISigma1 is primarily intracellular, and enriched in Z-disks, so that at the level of resolution of the confocal microscope it colocalises with alpha-actinin (28). The same is true of betaIISigma2, but additionally there is an M-line component for this variant. AnkyrinB is also present at M-lines of neonatal cardiomyocytes, and it is required for recruitment of betaII-spectrin to this point (31). betaII immunoreactivity was also found to run longitudinally, *i.e.* parallel to the sarcomeres in a manner reminiscent of sarcoplasmic reticulum.

alphaII-spectrin also has a sarcomere-associated component. We used quantitative immunogold electron microscopy to define the location of alphaII relative to the sarcomere (24). We found that it is closely associated with Z-discs, and is most abundant towards the perimeter of the Z-discs where they appose the SR. Additionally some gold labelling was found to run along the edge of the myofibril in the I-band again at a position where it would appose the SR. However, there is no labelling at M-lines. We can conclude therefore that spectrin is a component of sarcomeric Z-discs, and that along the I-band spectrin exists between sarcomeres and the SR. The isoforms here are (alphaIIbetaII)$_2$ tetramers, with both long and short C-terminal variants of betaII in this tetramer. At the M-line, there appears to be betaIISigma2 without any alpha-spectrin. Interestingly, in skeletal muscle, betaI has been reported to exist at the plasma membrane overlaying the M-line, again in the absence of alpha-chains (32).

In summary, there is evidence for multiple, spatially segregated populations of spectrin in cardiomyocytes: their compositions, locations and properties are summarised in Table 1.

4.2. The biochemical nature of differentially located spectrin tetramers

Based on the differential locations of spectrin polypeptides, there must be at least four types of spectrin dimer: namely, alphaIbetaISigma2, alphaIIbetaISigma2, alphaIIbetaIISigma1 and alphaIIbetaIISigma2. What does this say about the nature of tetramers formed by interaction of these dimers?

We know that the erythrocyte tetramer (alphaIbetaISigma1)$_2$ undergoes dynamic rearrangements in blood circulation. The relatively low affinity of the interaction of alphaI with betaI (K_D 800-900 nM) allows dissociation of tetramers to dimers under the stresses of circulation: this seems to be essential for physiological deformation of red cell membranes (33). But in heart, the requirements of spectrin are different. We have noted that spectrin is present at sites of force transduction in cardiomyocytes. In this case, perhaps dissociation of tetramers might not be so desirable. Kennedy *et al* (34) showed that betaISigma1 and betaISigma2 interacted with alphaI identically at the tetramerisation site, so there is no reason to suppose that splice variation of betaI affects tetramer formation.

Figure 2c indicates the mechanism of interaction of alpha and beta at the tetramerisation site. We investigated the interaction of alphaI, alphaII, betaISigma1, betaIISigma1 and betaIISigma2 at the tetramerisation site (35) and found that the betaII isoforms interact with both alpha chains much more strongly than betaI. The characteristics of the interaction are given in detail in Bignone and Baines (op. cit.), but relevant K_D values for interaction of the monomeric fragments are: alphaII-betaISigma1, 280 nM; alphaII-betaIISigma1, 5 nM;

Table 2. Nature of cardiac spectrin tetramers and their locations

Intracellular location	Spectrin isoforms	Nature of alpha-beta interaction (35)
Plasma membrane	alpha1-beta1 (red cell spectrin)	weak
	alphaII-betaI	intermediate
Z-disc and SR	alphaII-betaII (fodrin)	strong
Intercalated disc	alphaII-betaII	strong
M-line	betaIIsigma2	No interaction with alpha

alphaII-betaIISigma2, 9 nM. Tetramers comprised of alphaIIbetaII are therefore hugely more stable than alphaIIbetaI, especially bearing in mind that in a tetramer, each constituent dimer is bivalent for another dimer. So, we can suggest that where longitudinal force is exerted (intercalated discs, Z-discs) spectrin tetramers are adapted to resist dissociation. Costameric alphaIIbetaI represents an intermediate form, neither as readily dissociable as alphaIbetaI, nor as resistant as alphaIIbetaII. Perhaps at costameres the great membrane distortions that occur on contraction require some limited dynamic rearrangement of protein organisation, but not to the extent dictated in erythrocytes (c.f. Table 2).

4.3. Protein 4.1 in heart: isoforms and locations

Multiple tissue Northern blots reveal expression of several 4.1 genes in heart. Yamakawa *et al*. (22) could detect 4.1R, 4.1G and 4.1N (KIAA0338) mRNA in rat heart, but their antibodies scarcely detected any protein. 4.1B (KIAA0987) was absent by both Northern and Western blotting.

More recently, we have investigated 4.1 isoforms in both human and mouse heart by cDNA cloning and antibody reactivity (36). In human, mRNAs for 4.1R, 4.1G and 4.1N are expressed, but not 4.1B, in agreement with the rat data of Yamakawa *et al*. (22). On the other hand in mouse, 4.1B cDNAs are also detectable. In each of these cases, the major protein 4.1 functional domains: that is, the **f**our-point-one/**e**zrin/**r**adixin/**m**oesin (FERM), SAB and the C-terminal domain (CTD) are all expressed.

The presence of the SAB is highly unusual in the context of the broad tissue expression of 4.1R and 4.1G. The SAB of 4.1R is characteristic of the erythroid lineage, and is expressed as a gain of function during the final stages of red cell development (37). The fact that the 4.1R SAB is expressed in heart indicates a requirement for strong interaction of spectrin with the actin cytoskeleton. Likewise, with cardiac 4.1G which also retains the SAB (as in one of the three dominant 4.1G forms in brain, isoform A – NCBI: CAD62689). By contrast, in brain two further dominant isoforms C (NCBI:CAD62252) and D (NCBI:CAD62253) lack the SAB. Therefore, in cardiac tissues both 4.1R and 4.1G are able to cross-link transmembrane proteins via their FERM and CTD, and link them with high affinity to the spectrin-actin cytoskeleton. It seems probable that this requirement reflects the continuous stress of beating, something not felt in, for example, brain. 4.1N also retains the SAB, but in this case the SAB cannot function in promoting spectrin-actin interaction (38).

By immunofluorescence using an anti-SAB antibody, 4.1R is abundant at the plasma membrane both at costameres and at intercalated discs (potential complexes at these locations are summarised in Figs 3a and 3b). It is also present internally at Z-line and longitudinal locations. The location of 4.1R is almost exactly analogous to alphaII-spectrin. Since alphaII forms tetramers with high affinity at the tetramerisation site (see next section below), it seems probable that at all the major points to which force is transduced from the actomyosin apparatus (*i.e.* the Z-disc, costameres and intercalated disc) strong spectrin tetramers form. At these points, high affinity interactions occur with actin in a reaction promoted by 4.1R and 4.1G.

Interestingly, there is also M-line reactivity, *i.e.* there is a membrane compartment coincident with the M-line that contains 4.1R, betaIISigma2 and ankyrinB (summarised in Figure 3c). This seems to be a most unusual alpha-free complex. AnkB is required at this location to localise SR IP$_3$ receptors and betaII spectrin (39-41). Mechanisms underlying the mutual recruitment of this complex are far from clear. Why betaII and no alpha-spectrin is assembled here is not at all clear: presumably betaII can bind a protein other than alpha at M-lines, so blocking spectrin heterodimer formation. Equally, the nature and functional significance of 4.1R-membrane protein interactions here are not known.

Like 4.1R, 4.1G has an intracellular location, close to Z-discs and also in a lateral array paralleling the edge of myofibrils (42). However, in mouse heart, 4.1G is absent from the plasma membrane as judged both by immunofluorescence and subcellular fractionation.
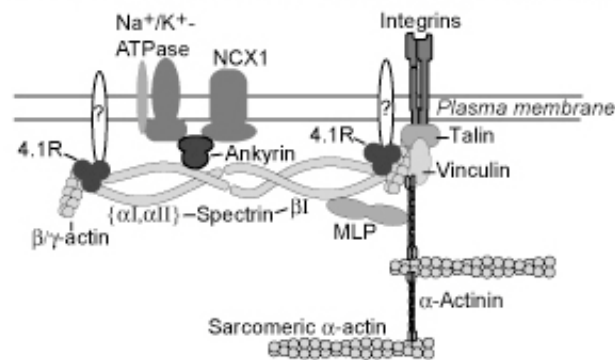
4.1N is most abundant at plasma membrane, being particularly concentrated at IDs. There is also an intracellular component, which appears to be in T-tubules (42). 4.1N has recently been shown to be required for membrane targeting and retention of IP$_3$-receptors in both neurons and epithelial cells (43-45). In both atrial and ventricular cardiomyocytes, a major fraction of IP$_3$-receptor is located at intercalated disc (46). We speculate that a function for cardiac 4.1N is in retaining IP$_3$-receptor at intercalated discs.

Heart muscle cells therefore compartmentalise 4.1 proteins, just as they compartmentalise spectrin subunits and ankyrins (Table 1).

4.4. Protein 4.1 and calcium pumps

In investigating cardiac 4.1R and 4.1G, it became clear that there is overlap with the location of the

(a) Protein interactions at the plasma membrane in costameres

(b) Protein interactions in the intercalated disc

(c) Protein interactions near the M-line

(d) Protein interactions near the Z-disc close to SR

Figure 3. Speculative spectrin-containing complexes in heart. The figure shows different protein complexes of the spectrin-associated cytoskeleton in cardiomyocytes. (a) Costameric complexes. (b) Intercalated disc complexes (c) M-Line complexes (d) Z-disc complexes.

sarcoplasmic reticulum Ca^{2+}-ATPase, SERCA2 (42). Double immunofluorescence revealed that at the Z-disc location these proteins were coincident, but that the 4.1G-containing lateral compartments, lying parallel to the myofibrils, were devoid of SERCA2. A complex of 4.1G and SERCA2 was revealed in immunoprecipitation. But this complex does not contain 4.1R. The compartmentalisation of 4.1 proteins evident at the light microscope level (see previous section) must extend to the sub-micron scale; functionally separate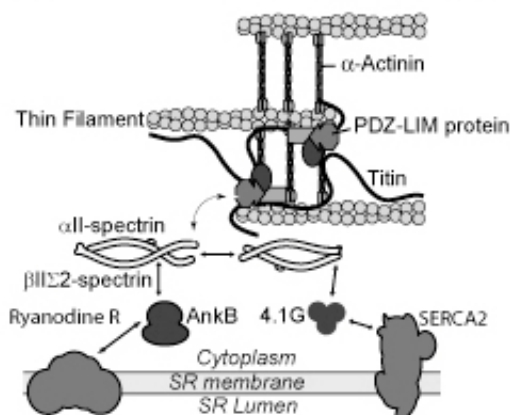 complexes characterised by differing 4.1 isoforms exist extremely close to each other. The SERCA2-4.1G complex also contains alphaII-betaII spectrin (Figure 3d). We predict that spectrin and 4.1G are required for recruitment and retention of SERCA2 at points close to the Z-disc; however, testing this hypothesis will probably have to wait until 4.1G knockout mice become available.

We will describe below (section 4.5) evidence linking 4.1R to plasma membrane Ca^{2+}-ATPase. Since 4.1G interacts with SERCA, and 4.1R interacts with the plasma membrane calcium ATPase (PMCA), we suggest that there is a general role for 4.1 proteins in Ca^{2+} homeostasis through the selective recruitment to – and retention of calcium pumps at – requisite points within the cardiomyocyte.

4.5. Function revealed through natural mutations and gene knockouts

What do we know of the function of the cardiac spectrin-cytoskeleton from genetic approaches? Approaches to this in mammals must necessarily be complicated by potential redundancy of the genes concerned (*e.g.* among the four conventional beta-spectrins).

4.1.5. alphaI-spectrin mutation: dilated cardiomyopathy?

The earliest descriptions of spectrin mutations that might affect heart were the naturally occurring mouse mutations *ja* (jaundiced) and *sph* (spherocytic). These are mutations in betaI-spectrin and alphaI-spectrin, respectively. Homozygous mice with either of these mutations develop to birth but tend to die in the early post-natal period. Clearly, alphaI- and betaI-spectrin are dispensable for heart development. To prolong their life sufficiently to allow detailed laboratory investigations, *ja/ja* mice need to be sustained with transfusions. Hearts of sustained animals are greatly enlarged compared with controls and have dilated heart chambers (47), suggesting that alphaI deficiency gives rise to dilated cardiomyopathy. However, a confounding factor in this analysis is the severe anaemia in these animals: enlargement of the hearts could be aggravated by anaemia.

The possibility that betaI-spectrin deficiency gives a cardiomyopathy has yet to be explored in more detail. The heart phenotype of *sph/sph* mice has yet to be reported in substantial detail.

No alphaII-spectrin knockout has yet been reported.

4.5.2. betaII-spectrin is required for normal heart development

The gene encoding betaII-spectrin has been knocked out in mice (48). As might have been predicted from its very widespread tissue expression, betaII -/- mice die in mid-gestation. They have multiple defects, including failure to fully develop the heart. This was attributed to failure of TGF-beta signalling via the SMAD signalling pathway. betaII-spectrin interacts with SMAD3/4 and is required for correct localisation of these proteins.

4.5.3. Protein 4.1R and organisation at intercalated discs

Protein 4.1R was knocked out by Shi *et al*. (49). Their knock-out strategy involved inserting beta-galactosidase into the 4.1R gene, giving them a read-out of promotor activity. 4.1R -/- individuals are viable, but severely anaemic. Like the erythrocyte spectrins, 4.1R is dispensable for heart formation. However Shi *et al*. (49) noted strong lacZ activity in heart sections indicating strong 4.1R promotor activity.

We have recently analysed 4.1R -/- mice. Morphologically their hearts appear generally normal, but at the electron microscope level there are alterations to the general appearance of the intercalated discs (P.M. Bennett, A. Maggs, AJB and JCP, in preparation). Immunofluorescence reveals that the plasma membrane Ca^{2+} pump PMCA2 is lost from the discs (42). 4.1R and PMCA2 also co-immunoprecipitate.
These data indicate a complex exists between 4.1R and PMCA2 which is required to recruit and retain this pump at the ID. 4.1R is likely to recruit and retain other PMCA isoforms, since PMCA4 and 4.1R also co-IP.

As yet detailed physiological analyses of the Ca^{2+} metabolism in 4.1R -/- animals have not been reported, nor is there an analysis of potential downstream effects of the 4.1R deficiency such as arrhythmia or effects on cell size.

4.5.4. Ankyrins G and B: roles in arrhythmia

The roles of ankB and ankG, and what has been learned from genetic approaches is reported elsewhere in this volume (Mohler and Bennett). The proteins are required for selective targeting and retention of Na/K-ATPase and NCX1 (ankB) and the intercalated disc/T-tubule Na channel, $Na_{V1.5}$ (ankG). Defects in either the ankyrin or the channels lead to arrhythmia.

4.6. The spectrin-associated cytoskeleton and Ca^{2+} metabolism

We suggest that an emerging role for the spectrin cytoskeleton in heart is the control of the major elements of the Ca^{2+}-handling apparatus. It is clear from the work of Mohler *et al*. (14; 39-41) that ankB directs the stable positioning – and therefore activity – of the fast Ca^{2+}-clearance apparatus, *i.e.* Na/K-ATPase and NCX1. AnkB is also required for positioning of a ligand-gated Ca^{2+} channel, the IP_3 receptor that modulates the contraction frequency of cardiomyocytes (31).

The 4.1 proteins have an analogous role in the positioning of the mechanism that maintains basal Ca^{2+}_i: 4.1G interacts with SERCA2 and 4.1R with PMCA (42).

Spectrin forms complexes with each of these, and presumably acts as an 'accumulation machine' to stabilise ankyrin/4.1/channel/pump complexes (see Figure 3). Spectrin knockout mice have not yet been analysed for the disposition and function of channels and pumps in heart. However, an accumulation machine role is supported by the observation that in skeletal muscle, Na/K-ATPase is mislocalised in *ja/ja* mice (13).

In assembling these complexes, as far as we can tell, ankyrin functions in the initial steps. Ankyrin is required for spectrin assembly at M-lines at least (31), and spectrin recruitment requires ankB. In this, the system parallels the erythrocyte, where ankR is required for recruitment of spectrin (50). Of the spectrins, erythrocyte beta binds ankyrin; alpha assembly is then limited by beta availability (51). Protein 4.1 is essential for the stability of the assembly (52). We view the synthesis and assembly of the cardiac spectrin cytoskeleton as analogous to the erythrocyte in this respect.

4.7. Is there a role for the spectrin-associated cytoskeleton in mechanical properties of cardiomyocyte membranes?

The erythrocyte paradigm suggests that the major role for spectrin is in mechanical integrity of the membrane. Spherocytic red cells that are deficient in spectrin spontaneously lose membrane by blebbing (for an example, see 53). The deformability of red cells requires a fully functioning cytoskeleton too (54). Furthermore, in the worm *C. elegans*, spectrin is required for stabilising the junctions of muscles with the body wall against the forces of contraction.

So is there evidence for a mechanical role in heart? At first glance, the answer so far is not much. None of the knockouts reported so far show gross evidence of mechanical failure in terms of either blebbing or loss of cell adhesion. While there are developmental defects in betaII -/- mice these seem to result not from mechanical failure but from biochemical signalling defects. There is a suspicion of dilated cardiomyopathy in the alphaI deficient mouse, but more detailed analysis will be needed.

In cases of spectrin/4.1/ankyrin knockout or deficiency, where the mice survive beyond birth, the intercalated discs, which transmit the forces of contraction and bear the brunt of mechanical stress, do not seem to fail. Where there are ID defects these relate to targeting and retention of channels or pumps.

The conclusion so far must be that the prime function of the spectrin-associated cytoskeleton relates to controlling the systems that regulate Ca^{2+} metabolism; evidence for further mechanical roles may well come from analysis of knockout mice, but this is not yet in hand.

5. CARDIAC BIOLOGY

5.1. Defects of the mammalian heart

12% of reported longstanding illnesses in the UK population occur with the heart and circulatory system. Of these, coronary heart disease and heart failure account for around 75% of all human heart conditions. After this, arrhythmias – disorders of the regular rhythmic beating of the heart - cause 10% and cardiomyopathies 2%. For a historical perspective on cardiac biology over the last ten years, see Olson (55).

Cardiomyopathy is the term which describes a heterogeneous group of diseases that affect the heart muscle. It is caused by many factors that may be induced by viruses, diet or alcohol, as well as those that are genetic in origin. Hereditary cardiomyopathies and arrhythmias are frequently fatal and for the former frequently go undiagnosed. Cardiomyopathies fall into two general groups: 'familial dilated cardiomyopathy' (FDC) and 'familial hypertrophic cardiomyopathy' (FHC). The prevalence of FDC is 1:2500. Recent studies using non-invasive scanning suggest that the incidence of FHC is very much greater than previously thought and may affect as many as 1 in 500 (56). It is the chief cause of sudden cardiac death in young people.

In FHC the heart muscle becomes considerably thickened reducing the capacity of the ventricles and, in some cases obstructing the flow of blood from the heart. Conversely, in FDC the heart muscle thins, becomes weak and loses its elasticity. This causes the heart and the left ventricle, especially, to become dilated and ineffectual.

In only 40% of cases is the genetic lesion known. As a general rule, FHCs are attributed to defects in proteins of the sarcomeric contractile system *e.g.* beta-myosin heavy chain, cardiac troponin T, myosin-binding protein C and others (reviewed in (57)), whereas FDCs arise from defects or deficiencies in non-sarcomeric proteins *e.g.* desmin, lamin A/C, muscle LIM protein, phospholamban, the PDZ protein *Cypher/ZASP* and several others (reviewed by Shaw (58)). However the mechanism by which the mutant proteins generate disease is uncertain. Over one hundred mutations are known in beta-cardiac myosin and they are the root cause of almost half of the cases of human FHC. As far as human beta-cardiac myosin functioning is concerned, there is little clue as to how and why mutations at so many widely different topological sites disturb the protein function. A defect in myocyte contractility had been proposed, but *in vitro* studies of force generation have subsequently demonstrated opposing results. An alternative hypothesis of "energy compromise" resulting from inefficient utilization of adenosine triphosphate (ATP) has been suggested and a recent study supports a proposed link between altered cardiac energetics and development of the disease phenotype (59). So whilst mutations in the sarcomeric proteins are being documented, explanations of more than half of all cases of cardiomyopathy remain elusive. Suggestions are now being made by the clinicians that elements of the calcium-handling apparatus may play key roles in maintaining normal cardiac function (60).

5.2. A role for the spectrin-based cytoskeleton in cardiac dysfunction

Lately proteins of the spectrin-based cytoskeleton have been identified as causative factors in heart dysfunction. The following currently known examples

Table 3. Human spectrin and 4.1 gene locus and cardiac defects

Gene	Protein	Gene locus	Other genes at this locus linked with cardiac disease	Reference
EPB41	4.1R	1p33-p32	laminin-5,beta-3	64
			plasma membrane calcium ATPase 4; myogenin; fibromodulin; renin	77
EPB41L1	4.1N	20q11.2-q12	None found	
EPB41L2	4.1G	6q23	laminin alpha-2; laminin alpha-4; triadin; phospholamban	64
			epicardin implied, but no mutations found	67
			Unknown gene causing congenital heart defects	65
SPTA	alphaI-spectrin	1q21	Multi-gene deletions in: acid phosphatase 6, lysophosphatidic;	78
			gap junction proteins; connexin 40 and connexin 50 lamin A/C	79
SPTAN1	alphaII-spectrin	9q33-q34	Osler-Weber-Rendu Syndrome; multiorgan arteriovenous malformations; Echocardiogram may show "high-output" heart failure	80
SPTB	betaI-spectrin	14q23-q24.2	Unidentified gene for arrhythmogenic right ventricular cardiomyopathy/dysplasia (ARVD)	81
				82
				83
SPTBN1	betaII-spectrin	2p21	None found	
SPTBN2	betaIII-spectrin	11q13	Unidentified gene	84
			Unknown gene causing presbycusis	85
SPTBN4	betaIV-spectrin	19q13.13	Isolated conduction blocks	80
			Krüpple-like zinc-finger gene *ZNF382*	86
SPTBN5	betaV-spectrin	15q21	Alpha-tropomyosin	80
			fibrillin-1	87

alphaIbetaI is the red cell form of spectrin; alphaIIbetaII is also known as fodrin or 'brain spectrin'

illustrate this: Mouse betaII-spectrin knockout homozygous mice die in mid-gestation due to impaired signalling during development. Along with defects in other tissues they display an abnormal heart phenotype (61). A mutation in an isoform of the spectrin-binding protein, ankyrin (ankB) has been shown to cause type 4 long-QT cardiac arrhythmia and sudden cardiac death in humans (14). Additionally, a transgenic null for MLP, the **m**uscle-specific **L**IM-only **p**rotein which binds to betaI-spectrin shows dilated cardiomyopathy (62) and morphological alterations at the intercalated disc (ID) (Ehler *et al.* (63)). Ehler *et al.* (63) have now suggested that dilated cardiomyopathy is a disease of the ID.

An examination of the genetic loci of alpha- and beta-spectrins and the three paralogous 4.1s showing activity in mammalian cardiac tissue is presented in Table 3. This reveals coincidence of two of the 4.1s (4.1R and 4.1G) and several of the spectrin subunits (alphaII, betaI and others) with loci linked with cardiac defects. However some of the genetic loci identified coincide with regions of known cardiomyopathy-linked genes as also identified in Table 3. We surmise that the contribution of 4.1s and spectrins to cardiomyopathy may have been overlooked for this reason. For example, Messina *et al.* (64) and Erdel *et al.* (65) noted the existence of an additional locus (a suspected laminin) on 6p, a region significantly associated with myopathy. Thierfelder (66) also identified 6q23 as a dominant disease locus. Later, Schonberger *et al.* (67) described sensorineural hearing loss together with FDC at 6q23 and suspected the involvement of a transcription factor, epicardin. Their implications proved to be unfounded as no mutations were found in the gene. We note, however, that 6q23 is the genetic locus of EPB41L2 (4.1G).

As yet, no specific investigations of 4.1s as causes of cardiac diseases have yet been reported, but these are no doubt to come.

6. WHAT IMPAIRED INTERACTIONS ARE IMPLICATED IN CYTOSKELETAL PROTEIN-BASED HEART DEFECTS?

Simplistically, impaired protein interactions may be brought about in many ways such as: a) a protein mutation which results in altered affinity (raised or lowered) of interacting proteins for each other; b) a mutation which leads to missense translation and/or premature stop resulting in reduced protein translation and/or retention; c) altered post-transcription modifications (*e.g.* phosphorylation) due to *in vivo* effectors such as hormones acting on signalling pathways; d) and others (see (68)). Here we summarise what is currently known as well as what might be expected of an impaired cardiac cytoskeleton protein/partner interaction. The intracellular locations of cardiomyocyte cytoskeletal proteins are identified in Table 1 and potential protein complexes are indicated in Figure 3. In Table 3 we compile a list of the genetic loci for spectrin and protein 4.1, and correlate these with known loci for cardiac defects.

6.1. Spectrin

Spectrin activities are dominated by the interactive properties of the beta-chains as well as through self-associations between the alpha- and beta-spectrin chains forming dimers and that of dimers forming tetramers (see Figure 2). The effects of spectrin defects and

deficiencies have been studied in great detail in the red cell (69; 70) and much of what has been learned from this 'model' system may be interpreted and applied for spectrins in nonerythroid cells (71). The larger part of each spectrin polypeptide chain is made up of triple-helical repeats of ~106 amino acids (72). These show low amino acid identity between repeats (26).

By analogy with the red cell system, we might expect that mutations such as those that cause elliptocytosis by disrupting formation of alphaI-betaI dimers or 4.1R function might affect the functionality of the spectrin network. Similarly spherocytosis mutations that reduce the targeting of alphaI-betaI spectrin or ankR at the plasma membrane might be manifest phenotypically in heart function. However, the paucity of reports of heart defects in patients with severe spherocytosis or elliptocytosis is surprising. Two issues confound such analysis: first, the evident redundancy of spectrin and 4.1 proteins in heart, and second the fact that conditions such as congestive heart failure are consequent upon the anaemia (e.g. 73). Even where reports of spherocytosis/elliptocytosis are associated with cardiomyopathy, in general they are not yet associated with specific genes (e.g. reference 74).

6.2. Protein 4.1

Protein 4.1 products occur with spectrin at many subcellular locations (Table 1). Molecular cloning revealed that virtually all cardiac transcripts encoding 4.1s constitutively encode the spectrin/actin-binding domain (36). Gimm et al. (38) have shown that the strength of the spectrin/actin/4.1 ternary interaction which cross-links the spectrin network is strongest for 4.1R; that for 4.1G and 4.1B is found to be relatively weaker though still physiologically relevant. However the equivalent molecular region of 4.1N is non-functional in spectrin/actin-binding and probably fulfils an, as yet, undefined alternative role. From this we can say that cardiac forms of 4.1R and 4.1G probably interact with the spectrin cytoskeleton. Table 1 shows that this is entirely possible at the locations identified and this in turn implies that targeting and retention of these cardiac 4.1s results from the 'accumulation attributes' of the spectrin cytoskeleton (75). Interestingly only 4.1R is targeted to the M-line and we surmise that at this intracellular position it is targeted by the truncated form of betaII-spectrin. The work of Bennett's group has shown that ankB is also targeted to the M-line through betaII-spectrin (14; 41).

7. SUMMARY AND PERSPECTIVE

Cytoskeletal proteins form a network on all membranes of cardiomyocytes. It affords strength and resilience to the membrane systems whilst also massing the functional protein complexes associated with calcium-handling, organisation and coordination of activity through the cell's signalling cascades of the contractile apparatus. It is also involved with transferring the forces of contraction and preventing the cells from tearing apart during contraction. It locates transmembrane connections to the extracellular matrix.

At the plasma membrane it locates the costameres and thence the Z-discs, contributing to retaining internal contractile protein order. In regions close to the Z-disc it organises the membranes of the SR and is involved with interactions with SERCA. At the IDs it functions in assembling and retaining PMCA. At this location it probably also coordinates cross-talk between adjacent cells potentially facilitating the passage of small molecule messages. In this location it contributes to the structural organisation of the desmosomes, gap junctions and adherens junctions.

It is not surprising that such ubiquitous presence impinges on wide varieties of the cardiac muscle cell's activities and it is also not surprising that this complex network of cytoskeletal proteins is involved in heart defects. That it should have been overlooked for so long is probably due to the invisible nature of the structures – they are small and only relatively recently have the appropriate tools, such as antibodies, for its dissection become available.

We can anticipate expansion of research into this field which has the capacity to define, in molecular terms, aspects relating to disposition and retention of calcium signalling proteins in normal cells as well as defects in cell functioning. At the same time we may look forward to better diagnosis of heart malfunctions, understanding the progression of disease and new methods for treatment.

8. ACKNOWLEDGEMENTS

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Abbreviations: FDC: familial dilated cardiomyopathy; FHC: familial hypertrophic cardiomyopathy; SERCA: sarcoplasmic reticulum calcium ATPase; PMCA; plasma membrane calcium ATPase; SR: sarcoplasmic reticulum; ID: intercalated disc

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