Specialization at the Z line of cardiac myocytes

Thomas K. Borga,*, Edie C. Goldsmitha, Robert Pricea, Wayne Carvera, Louis Terracioa, Allen M. Samarelb

aDepartment of Developmental Biology and Anatomy, University of South Carolina School of Medicine, Columbia, SC 29208, USA
bCardiovascular Institute, Department of Medicine and Physiology, Loyola Medical School, Maywood, IL, USA

Received 14 October 1999; accepted 13 December 1999

Keywords: Cell culture/isolation; Developmental biology; Extracellular matrix; Myocytes

1. Introduction

The organization of any differentiated cell is not random but is the result of a dynamic integration of extracellular and intracellular signals. During the development of the heart, cardiac myocytes are round shaped cells that differentiate into a rod-shaped phenotype. During the different stages of commitment and morphogenesis, the myocyte organizes its internal structure by undergoing myofibrillogenesis. This process results in the precise arrangement of contractile elements, supporting cytoskeleton, and endoplasmic reticulum. Within the sarcolemma, specialized regions are defined for attachment to components of the extracellular matrix (ECM). This specialized site of the sarcolemma consists of the ECM–Receptor–Cytoskeleton complex (Fig. 1). These sites will integrate attachment to the ECM and the series of proteins necessary for the chemical and mechanical transmission of information. In this review, we will describe the evidence that indicates there is a specialization of the sarcolemma at the Z line for the clustering of various receptors for the ECM as well as the cytoskeleton.

2. Development of specialized regions of the sarcolemma

During development of the embryonic heart, two specialized regions of the sarcolemma develop: (1) intercalated disks for cell–cell interaction and (2) ECM–integrin–cytoskeletal connections for cell–ECM contacts. The formation of these regions appears to be a coordinated expression of ECM components, cell surface receptors, and signaling proteins [1–5]. The localization of fibronectin, integrins, cadherins, and kinase/phosphotase complexes, which are all important to myofibrillogenesis, appear as focal patches on the myocyte surface (Fig. 2). Interaction with the ECM occurs throughout development; however, the specialization to define the Z bands appears to be coordinated with myofibrillogenesis and the deposition of collagen. Fibroblasts appear to secrete collagen that is then attached to regions on the myocyte where the Z bands are forming internally [6]. Myofibrillogenesis appears to start in vivo near the internal margin of the sarcolemma [7,8]. This observation would be consistent with the presence of specific receptors in the sarcolemma prior to myofibrillogenesis necessary for the attachment of the myofibril to the Z band at the same site where collagen is attaching on the outside. These morphological data are consistent with the presence of the integrin receptors within the membrane at this time [9]. The formation of the collagenous ‘struts’ begins with attachment of a few collagen fibers which appear to increase in number (Fig. 2A). Associated with these attached fibers on the extracellular side are proteoglycans and glycoproteins [10]. What is not known is how the fibroblast ‘finds’ the myocyte to secrete the collagen at such a precise location. The presence of collagen receptors (integrins) on the myocyte at these sites indicates that these receptors may play a role in directing collagen strut formation [11] (Fig. 2B). Collagen struts do not appear to attach at precise sites on capillaries, but form a tight weave associated with the basement membrane of the capillary.

*Corresponding author. Tel.: +1-803-733-3115; fax: +1-803-733-1533. E-mail address: borg@med.sc.edu (T.K. Borg)

Time for primary review 28 days.
Fig. 1. A diagrammatic representation of the specialized regions of the sarcolemma. For cell–cell attachment, the extracellular regions of cadherin molecules exhibit homotypic binding while the cytoplasmic domains connect to catenins and actin. A variety of receptors including integrins and growth factors are found in a specialized region on the lateral margin of the myocyte at or near the Z band. Both complexes have direct associations with various signal transduction and cytoskeletal components. It appears that the lateral complexes are not associated with actin like the cadherin–catenin complex.

The temporal and spatial regulation involved in the formation of the connective tissue network is not clear. One explanation is that the connective tissue network, especially the endomysial weave network and perimysial cables, are laid down and then undergo remodeling as the myocytes and vasculature grow.

Remodeling of the ECM and its connection to the myocyte probably occurs whenever there is growth of the myocyte. During fetal and neonatal development, the need for forming and remodeling these connections is critical as growth is rapid. Modification of these ECM attachment sites in the adult is also important and related to normal growth and disease. During the physiological adaptation that causes the hypertrophic growth of the myocyte, these sites must change with the size of the myocyte. For example, as a myocyte grows in size or branches as seen in hypertrophy, the existing sites must change. Breakage of these sites of attachment could result in slippage or altered mechanics. In addition, release from the ECM can be a signal for apoptosis [12]. Theoretically, if too many of the sites are broken or released, the apoptic cascade could be initiated.

How does this remodeling take place? Integrins are also associated with proteases such as the serine proteases (urokinase) [13], metalloproteases [14] and A Disintegrin and Metallopeptase (ADAM) [15]. The MMPs are associated with integrins but their substrates are usually ECM components. However, the ADAMs have a potential role in the regulation of ECM–cell interaction through the metallopeptase domain and with integrins via a disintegrin domain [15,16]. For example, in sperm–egg interactions the disintegrin region of the ADAM protein has been shown to be critical to blocking the function of the integrin [17]. Peptides against the disintegrin on ADAM 2 have been shown to block αβ, a laminin receptor. In other studies, the metallopeptase region of ADAM 10 has been shown to proteolytically cleave collagen type IV [18]. This type of regulation of cell–ECM release and attachment would allow for changes in the attachment site on the Z band which would be necessary for growth.
Fig. 2. The localization of various proteins at the Z band of the sarcolemma of neonatal rat cardiac myocytes. Various proteins such as ECM, integrin receptors, signaling proteins and growth factor receptors have a similar localization. Panel A shows the formation and attachment of collagen to the region of the forming Z band (arrow). Panel B is the localization of β₁-integrin, a transmembrane receptor for collagen (arrow). Panel C shows the localization of angiotensin-II receptors in the sarcolemma at the Z line. Panel D shows the localization of PKC-ɛ on the cytoplasmic side of the sarcolemma. Together, these data indicate that the ECM, transmembrane receptors and signaling proteins form a complex that is located at the same region of the sarcolemma.

3. The ECM–integrin–cytoskeletal complex participates in the hypertrophic response of cultured cardiomyocytes

Isolated myocytes have been used to study cardiac hypertrophy in vitro but only recently have investigations incorporated the signaling pathways from the ECM via integrins. Recent studies provide strong evidence for an important modulatory role for the ECM–integrin–cytoskeletal complex in mediating growth and differentiation of neonatal cardiomyocytes in culture [19] (Fig. 2A–D).

Attachment to an antibody specific for β₁-integrin has been shown to augment cellular growth in response to the α₁-adrenergic agonists. Furthermore, over-expression of β₁-integrin using a replication defective adenovirus markedly increased phenylephrine-stimulated protein synthesis and ANF secretion as compared to either control cells, or cells infected with adenovirus encoding β-galactosidase. Conversely, over-expression of free β₁ integrin cytoplasmic domains markedly inhibited adrenergic mediated cell growth, indicating an essential role for integrin-dependent cell signaling pathways in mediating gene expression changes associated with cardiac hypertrophy (Fig. 3). Similarly, it has been shown that activation of focal adhesion kinase (FAK) is essential in mediating cell growth and hypertrophy in response to the hypertrophic peptide growth factor endothelin-1 [20]. Endothelin-induced FAK phosphorylation was markedly suppressed in cultured neonatal cardiomyocytes infected with Adv-FRNK, the C-terminal portion of FAK which contains the putative focal adhesion targeting sequence. Thus, FRNK functioned as a ‘dominant-negative’ inhibitor of FAK-dependent signaling. With FAK activation inhibited, endothelin stimulation failed to increase total protein/DNA or myosin heavy chain/DNA ratios, or to stimulate sarcomeric assembly. In contrast, endothelin-induced hypertrophy was unabated in uninfected cardiomyocytes or cardiomyocytes infected with adenovirus encoding β-galactosidase [20]. Thus both studies indicate that signals arising from the ECM–integrin–cytoskeletal complex are critical to cardiomyocyte growth induced by hypertrophic agonists.

The aforementioned studies highlight the close interrelationship between G-protein coupled receptor signaling and integrin-dependent signaling in modulating cardiomyocyte structure and function. An additional site of potential cross-talk between these pathways may occur via activation of the small GTPase rho during heterotrimeric G-protein coupled receptor stimulation (Fig. 3). For instance, phenylephrine-induced gene expression and sarcomeric assembly are both inhibited in cultured cardiomyocytes by blockade of rho function, perhaps mediated by the effects of rho kinase on cytoskeletal assembly [21]. Hypertrophic agonists that transduce signals via
Gq coupled receptors also cause rho translocation and GTP loading [22,23] and appear necessary for Gqα-induced ANF expression [22,24]. It is not clear, however, whether these signals are transmitted directly to downstream signaling cascades, or act indirectly via rho’s effects on stimulating focal adhesion formation, actin polymerization, and sarcomeric assembly [25]. One possibility is that rho activation by G-protein coupled receptors is required for integrin clustering to generate a three-dimensional ‘scaffold’ for the appropriate localization of signaling kinases and their substrates. Thus the specialized structures at the Z-line may function as more than just a structural site for sarcomerogenesis, but rather play an intimate role in transmitting signals from cell surface receptors to the nucleus and other organelles.

Although the ECM–integrin–cytoskeletal complex is a likely target for cell signaling involving extracellular growth factors, does mechanically ‘stressing’ cardiomyocyte integrin receptors directly activate cell signaling cascades? In other words, does the ECM–integrin–cytoskeletal complex actually perceive physical forces and trigger downstream signaling events? Schmidt et al. [26] recently showed that ‘dragging’ paramagnetic microbeads coated with anti-β1 and anti-α1 integrin antibodies across a monolayer of osteosarcoma cells directly activated MAPKS and increased the abundance of tyrosine-phosphorylated proteins physically anchored to the cytoskeleton. Similar studies have not been performed with cardiomyocytes; however it would be anticipated that an externally applied mechanical load to cultured neonatal cardiomyocytes would induce rapid FAK activation. However, cellular deformation may cause the release of autocrine or paracrine growth factors that are then capable of activating cell surface receptors leading to integrin clustering and FAK phosphorylation. Autocrine/paracrine release of angiotensin II, endothelin-1 and other growth factors in response to cell stretching has been described by several laboratories [27–29]. Whether integrins are involved in the stretch-induced growth factor release remains unknown.

4. Costameres and their role in mechanochemical signal transduction

The cardiomyocyte costamere is the site of co-localization of β1-integrins, α-actinin, vinculin and other cytoskeletal proteins. Their close association suggests that these membrane-associated proteins provide a structural link connecting components of the ECM to the cytoskeletal network of the cardiomyocyte [30]. Disruption of the
costameres with antisense oligonucleotides resulted in abnormal myofibrillar alignment [31]. In addition to their important structural role, there is growing evidence to suggest that this site also serves an important functional role in translating mechanical signals into biochemical signals that affect cellular function [32]. Insight into the role of specific membrane and cytoplasmic proteins involved in mechanochemical signal transduction has considerably increased over the past several years, due in large part to studies performed in cultured, adherent non-muscle cells. Of particular interest has been the role of the focal adhesion in mechanochemical signal transduction. Focal adhesions contain many of the same structural proteins that are found within the costamere of cardiomyocytes, but unlike their in vivo counterparts, focal adhesions are sites of close apposition of the cell membrane to the two-dimensional, rigid substratum provided by the artificial cell culture environment. Like costameres, focal adhesions are attachment sites where integrin receptors cluster and attach via their extracellular domains to specific peptide ligands contained within various ECM proteins.

Terracio et al. [33] demonstrated the presence of integrins at the costamere surface of freshly isolated adult cardiac muscle cells. Costamere-like structures are also found in cultured neonatal and adult cardiac myocytes [34]. In addition, cultured cardiomyocytes form focal adhesions similar to focal adhesions assembled by adherent non-muscle cells in culture during adhesion and cell spreading. Focal adhesions appear to be critical structures involved in assembly and maintenance of sarcomeres [35]. Both neonatal and adult cardiac myocytes in culture develop focal adhesions containing β1-integrins and vinculin, and their organization appears to be highly regulated by externally applied or intrinsically generated mechanical load [36,37].

In addition to their structural role, several groups have hypothesized that cardiac myocyte focal adhesions and costameres are major sites of mechanochemical signal transduction during cardiomyocyte growth and differentiation. Early studies showed that attachment of neonatal or adult cardiac myocytes to a collagen coated, rigid substrate stimulated cellular growth in culture even in the absence of additional mechanical load [38,39]. In retrospect, these findings were consistent with growth-promoting signals arising from focal adhesions as the isolated, quiescent cells attached and spread in culture, but the activation of cell signaling was not explored. However, the hypothesis that integrin receptors provide cytoplasmic growth signals for cardiac myocytes during mechanical loading is soundly based upon observations derived from non-muscle cells. When fibroblast integrin receptors bind to specific ECM components, they aggregate to form clusters. These integrin clusters also provide binding sites for several protein kinases which rapidly localize to focal adhesions in response to integrin engagement [12]. These cytoplasmic signaling kinases include the protein tyrosine kinases (PTKs) pp125FAK (FAK), pp60src (Src), and Csk, and one or more isoenzymes of protein kinase C (PKC) [40,41]. FAK is one member of a family of non-receptor PTKs [42], which also includes proline-rich tyrosine kinase2 (PYK2) [43]. FAK is expressed in a variety of cell types, including neonatal and adult cardiomyocytes [44,45]. In fibroblasts and other non-muscle cells, FAK binds to the cytoplasmic tail of β1-integrin through specific sequences located in its N-terminus. The C-terminal region of FAK (the so called focal adhesion targeting sequence) also binds directly to paxillin, a cytoskeletal protein that localizes to sites of integrin clustering. Once localized, FAK phosphorylates itself at a single tyrosine residue (Y-977) during integrin engagement [46]. This autophosphorylation site functions as a high affinity binding site (pYAEI motif) for the SH2 domain of Src-family PTKs [47]. Thus FAK autophosphorylation at Y-977 creates a binding site for other non-receptor PTKs to associate with FAK and other focal adhesion proteins via their SH2 domains. Once bound to FAK Src can then phosphorylate FAK at residues Y576 and Y577 within the catalytic domain, which augments FAK kinase activity toward exogenous substrates [48], and at Y-861 and Y-925 near its C-terminus [49]. The Y-925 phosphorylation site was recently found to promote the binding of Grb2, and perhaps other adapter proteins containing SH2 domains (e.g. Nck, Crk, and P13Kinase) to FAK [50,51]. Thus, FAK (or other PTKs bound to FAK during integrin clustering) can activate downstream signaling pathways in a manner similar to tyrosine autophosphorylation of a peptide growth factor receptor upon binding to its ligand. Recent data demonstrates that FAK phosphorylation in cardiac myocytes may also be regulated by [Ca2+]i, and PKC. For instance, the Ca2+ entry blockers nifedipine and verapamil reduce the amount of FAK and the number of focal adhesions in cultured neonatal rat ventricular myocytes [37,44], whereas endothelin-1 and PMA, potent activators of PKC-δ and PKC-ε, increase focal adhesion formation, along with FAK and paxillin phosphorylation in these cultured cells [20].

5. The ECM–integrin–cytoskeletal complex participates in cell signaling in vivo

Remarkably little is known about the role of costameres in regulating growth and function of cardiomyocytes in vivo. Numerous immunofluorescence studies have documented that a wide variety of components are localized to the Z band (Fig. 2A–D). On the outside of the sarcolemma, collagen attaches at specific locations [6,11] and laminin occurs in higher concentrations at these sites than elsewhere in the basement membrane [52]. At the level of the sarcolemma, integrin receptors are concentrated at these sites [9] as are cadherins [53], annexin [54], growth factor receptors [55], and proteases [56]. These molecules have both an extracellular and intracellular domain indicat-
ing their potential for both chemical and mechanical communication. On the cytoplasmic side of the sarcolemma at the Z band, several signaling molecules including FAK [44], PKC [57], paxillin [58], and vinculin [30] have been detected. These molecules represent a potentially important intracellular signaling pathway.

Recently, Kuppuswamy et al. [44] demonstrated that the association of FAK and Src with β₃-integrin is increased in tissue extracts of feline right ventricular myocardium subjected to 4 h of acute pressure overload in vivo. This study provides the first evidence that FAK and Src phosphorylation are increased in response to hemodynamic overload. What remains unclear, however, is whether the observed tyrosine kinase activation occurred within the cardiomyocyte population or within other cell types of the ventricular muscle. Furthermore, it is unclear whether the load activation of integrin-mediated signaling resulted from the load-dependent release of locally derived or circulating growth factors [59]. Additional studies which rely on targeted over-expression or down-regulation of specific components of the ECM–integrin–cytoskeletal complex in the intact animal will be required to precisely define the role of these components in cardiomyocyte mechanochanical signaling.

Inhibition of specific integrins, ECM and proteases have an affect on phenotype as well as signaling. Protein synthesis and other functions are altered when integrins are perturbed by a variety of methods, including mechanical tension, indicating that their function is essential in cell–ECM communication. The effects of modifying either the composition of the ECM, i.e. different components, or changing the glycosylation on ECM components can alter the cellular phenotype [60]. The addition of antisense oligodeoxynucleotides for ADAM-12 protease to cardiac myocyte cultures caused a radical alteration of the cellular phenotype (Carver, unpublished results). Together, these studies show that modification of any one aspect of the ECM–Receptor–Cytoskeleton complex at the Z band can change the cardiac phenotype.

Critical to mechanical signaling is the direct connection of transmembrane components, like integrins and cadherins, from the ECM to cytoskeletal components which then connect to the nuclear envelope. While the morphological evidence supports this concept, mechanistic evidence is not as easy to document. The continuity of the mechanical system may be important in a variety of functions such as the maintenance of cell shape, distribution of force from contraction as well as playing a role in the induction of genes. Perturbation studies of the cytoskeleton would likely affect all these functions and not be specific.

6. In vitro artifacts and relationship to in vivo

A significant question is do any of these alterations seen in vitro have any relation to physiological events in vivo? Major changes in the ECM occur when the physiology of the heart changes as in fetal development at the time of cardiac looping and valve formation, neonatal development and cardiac hypertrophy. In fetal development, these major changes in the composition of the ECM [61] are important to cellular migration, trabeculation, and valve formation. Changes in collagen and other ECM proteins are most evident during the rapid growth of neonatal development [62]. At these developmental times, numerous reports have documented the changes in ECM with cardiac hypertrophy and failure. In addition, developmental changes in integrins have been documented [34] as the myocytes undergo growth and branching in hypertrophy. Cardiac myocytes undergo dramatic phenotypic modifications during failure which may even result in apoptosis [63].

The progression of myofibrillogenesis in vivo is similar to that described in vitro [7,8,64–66]. Myofibrillogenesis is first associated with regions on the internal margin of the sarcolemma and is consistent with the presence of specific receptors in the sarcolemma prior to myofibrillogenesis for the attachment of the myofibril to the Z band at the same site where collagen is attaching on the outside. This observation is consistent with in vitro data on myofibrillogenesis and attachment of cells to various ECM substrates [64]. Yet it is not clear whether the focal adhesion sites seen in vitro are equivalent to the Z band attachment sites or to cell–cell attachment at the intercalated disc. Integrins and their associated signaling proteins are found at both locations in vivo, but in vitro they are located primarily at the site of actin filament insertion which is the focal adhesion. In most in vitro studies the myofibrils are located only on the bottom of the myocyte whereas in vivo the myofibril are associated with the sarcolemma in a three-dimensional manner. The use of aligned collagen thin gels as a culture substrate shows a more in vivo-like phenotype where the myocytes are rod-shaped and the myofibrils distributed around the cells rather than just on the basal surface [67].

Focal adhesions are complexes that occur in most cells when plated in vitro on ECM substrates. In myocytes, the forming myofibrils are inserted into these defined sites. In addition, numerous proteins including integrins, cadherins, and cytoplasmic signaling proteins have been localized to these sites in both myocyte and non-myocyte cultures [68]. These data have led to the speculation that the focal adhesion observed in vitro in cardiac myocytes is analogous to the cell ECM sites in vivo. However it is possible that the focal adhesion really represents a combination of the cell–ECM site and the intercalated disk. Since cadherins are primarily present in focal adhesions and actin fibers insert into these structures, they are more like the intercalated disk. The principal difference between a focal adhesion in vitro and the cell–ECM site in vivo, is that actin does not appear in the latter. In all in vitro focal adhesions, actin is part of the complex; however, it has not been detected as part of the ECM–Integrin–Cytoskeletal complex in vivo. The potential association of different
signaling proteins in the presence or absence of actin may explain the mechanical stimulation data from aligned myocytes [69]. Because of the difference in components located at complexes at cell–cell and cell–ECM connections, in vitro studies must be carefully interpreted.

The specialization of the Z band region of the sarcolemma to contain transmembrane receptors as opposed to the intercalated disc, or ends of the myocyte, might be related to ability of these regions to transmit different types of mechanical/chemical information. When isolated aligned myocytes were subjected to stretch (mechanical stimulation) in the long axis no significant protein synthesis occurred [69]. However, when the stimulation was applied perpendicular to the myocyte a significant increase in protein synthesis did occur. These data indicate that the site of stimulation is significant in hypertrophy and may also be related to the assembly of different signaling complexes at each site. As mentioned, these sites apparently lack actin and may have different signaling components associated with these sites. The ends of the myocytes have cadherin–catenin complexes compared to the integrin–FAK–paxillin–vinculin sites on the lateral margins of the myocyte.

Several mechanisms may potentially play a role in the establishment of these specialized regions of the sarcolemma. Insertion of specialized transmembrane proteins may be related to specialized assembly of particular lipids at these sites. Specialized membrane domains or lipid rafts consisting of complexes of sphingolipids and cholesterol have been documented with growth factor receptors in other systems, but not in cardiac myocytes [70]. Regulation of the insertion of specific proteins, such as integrins, cadherins and their associated signal molecules, might be directed by the transport of specific mRNAs to these defined sites near the sarcolemma. Experiments showing that the 3′ UTR region of the mRNA has specific information or ‘zipcode’ to direct it to the site of translation have been documented for proteins such as actin in myocytes [71], but not for signaling molecules or ECM receptors. In addition, specific docking proteins may play a significant role in the assembly of signaling molecules at specialized regions of the Z band [72].

7. Summary

Cardiac myocytes exist in a dynamic equilibrium between signals from the extracellular and the intracellular environment. Stimulation from the extracellular compartment by mechanical force, neurohormonal stimulation or both results in a response by the intrinsic genetic program that results in the up or down regulation of contractile protein synthesis and myofibrillar assembly or disassembly. Immunofluorescent localization of ECM receptors (integrins), ECM molecules and signaling proteins at precise regions of the sarcolemma indicates that there are specialized regions of the sarcolemma for the assembly of signaling molecule complexes for inside-out and outside-in communication. The integration of these signals plays a critical role in the adaptation of the cardiac myocyte to physiological and pathophysiological signals during development and disease.

Acknowledgements

The authors wish to express thanks to Dr Sergio Lavander, Dr Richard Hunt, Lisa Buchanan and Jeff Davis for their time and effort on this manuscript. This work was supported in part from grant BL-37669 and HL-58893.

References

[17] Yuan R, Primakoff P, Myles DG. A role for the disintegrin domain


[60] Reaves TA, Simpson DG, Terracio L, Borg TK. The role of N-linked oligosaccharides on cell adhesion and phenotype in cardiac myocytes and fibroblasts. J Mol Cell Cardiol 2000 (submitted).


