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Cellular Localization of $\alpha 3\beta 1$ Integrin Isoforms in Association with Myofibrillogenesis during Cardiac Myocyte Development in Culture

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The cellular localization of $\alpha 3\beta 1$ integrin isoforms was examined in cultured neonatal myocytes at selected times during development using double immunofluorescence assays. The distribution of $\alpha 3$A subunits began as diffuse and patternless, but as the cells matured, the distribution assumed a sarcomeric banding pattern, and $\alpha 3$A appeared to be localized in costameres – sarcolemmal regions adjacent to the Z-disks. α-actinin, a component of the Z-disk, was localized in the same intracellular regions. Temporal analysis of the incorporation of the $\alpha 3$A subunit and other myofibrillar proteins into sarcomeres revealed that $\alpha 3$A was integrated into sarcomeres following incorporation of α-actinin and myosin heavy chain (MHC) but prior to that of desmin. This suggests that $\alpha 3$A integrins are incorporated into a pre-existing myofibrillar structure, and it is unlikely that $\alpha 3$A integrins participate in the initial assembly of myofibrillar proteins. The $\alpha 3$B, $\beta 1$A and $\beta 1$D subunits were also localized in costameres, where they formed $\alpha 3$A$\beta 1$A, $\alpha 3$A$\beta 1$D and $\alpha 3$B$\beta 1$A heterodimers. The $\alpha 3$B$\beta 1$D heterodimer, however, was not found in cardiac myocytes. The antisera raised against the cytoplasmic domains of $\alpha 3$A, $\alpha 3$B, $\beta 1$A and $\beta 1$D caused disruption of sarcomere structure. Thus, the myofibril–extracellular matrix linkages mediated by isoforms of $\alpha 3\beta 1$ integrin may play a crucial role in the stabilization of myofibril assembly and in the maintenance of sarcomere structure. Co-immunoprecipitation experiments revealed that $\beta 1$A, but not $\beta 1$D, interacts with the Nck signaling protein, suggesting that Nck participates in downstream signaling triggered by $\beta 1$A and that the $\beta 1$A-mediated signaling pathway is distinct from that of $\beta 1$D.

Keywords: Integrin, myocyte, myofibrillogenesis

INTRODUCTION

The interaction of cells with extracellular matrix (ECM) proteins plays a prominent role in the normal development of cardiac muscle. During development of the heart, ECM components in the basal lamina exhibit specific spatial distributions and patterns of expression, and they form the cardiac

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jelly that surrounds the embryonic endocardium. Among the many ECM components, interstitial collagen, in particular, is organized into three dimensional networks that interconnect myocytes and attach to costameres, where myofibrils are anchored to sarcolemma (Borg *et al.*, 1983; Terracio, 1989); disruption of this collagen networks causes abnormal heart function (Dawson *et al.*, 1982).

ECM proteins play important roles in the processes of cell morphogenesis, adhesion, differentiation and tissue development. These cellular responses are initiated by the binding of ECM proteins to their respective cell surface receptors, called integrins. Integrins are heterodimeric proteins, each composed of an alpha (α) and a beta (β) glycoprotein subunit. The 10 α and 16 β subunit isoforms combine to form at least 25 integrins having varying degrees of specificity for different ECM proteins.

Vertebrate skeletal and cardiac muscles express a variety of integrins during development. In skeletal muscle, α7 integrin is found at myotendinous junctions and appears to participate in the distal and lateral cohesion of muscle fibers needed for the generation of force and movement (Bao *et al.*, 1993; Song *et al.*, 1993). αV integrin is found at specific sarcolemmal sites associated with Z-lines, which represent a site to transmit the mechanical force of contraction across the sarcolemma (McDonald *et al.*, 1995; Terracio *et al.*, 1991). Because the intercellular transmission of force requires tight cell adhesion, integrin-mediated cytoskeleton–ECM linkage likely contributes to the maintenance of muscle structure integrity. Indeed, *Drosophila* embryos that do not express βPS integrin form defective Z-bands (Volk *et al.*, 1990). During development of the heart, expression of α6 integrin is developmentally regulated, and its graded expression across myocardium may contribute to the formation of heart tube (Collo *et al.*, 1995; Hierck *et al.*, 1996b). The importance of integrin expression in association with heart development is even more evident in the α4 integrin null mouse, in which epicardium is absent resulting in cardiac hemorrhage (Yang *et al.*, 1995).

Interactions between integrins and ECM take place continuously throughout sarcomere assembly. In culture, the development of cardiac myocytes is characterized by myofibrillogenesis in which myofibrils are organized into sarcomere structures; this process underlies the progressive assembly of thick and thin filaments into sarcomeres with appropriately spaced Z-disks (Lin *et al.*, 1989). In addition, collagen is laterally attached to specific sites at the sarcolemma near Z-disks where myofibrils also come into contact with the sarcolemma (Borg *et al.*, 1983; Terracio *et al.*, 1991). Collagen cell adhesion molecule is found at this site in the cultured rat cardiac myocytes (Terracio, 1989).

β1 integrin is predominantly expressed in costameres of cardiac myocytes, where the cells attach to the collagen network (Belkin *et al.*, 1996), and forms strong attachment sites required by highly contractile cells. Moreover, Fassler *et al.* (1996) provided a compelling evidence that organization of sarcomere structure in cardiac myocytes is crucially dependent on the presence of β1 integrin. Despite these studies, the α subunits associated with β1 integrin in cardiac myocytes have not yet been identified, and their functional properties remain unclear. Expression of α1 and α3 subunits has been shown in myocytes isolated from neonatal rat heart (Terracio *et al.*, 1991), and they have been identified as collagen receptors; nevertheless, little is known about their specific cellular localization or their molecular interactions with myofibrillar proteins. For this reason, we have endeavored to determine which integrin α subunits are associated with β1 subunits at the costameres of neonatal cardiac myocytes in culture. The present study demonstrated that α3 integrin is localized at costameres and is associated with the β1 subunit, suggesting that the α3 subunit functions in the organization and stabilization of myofibrils. Alternate isoforms in the cytoplasmic domains of α3 and β1 subunits were also identified, and their cellular distribution and signal properties were examined.

MATERIALS AND METHODS

Materials

Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), antibiotics (penicillin/

streptomycin), complete Freund's adjuvant, incomplete Freund's adjuvant, and trypsin were obtained from GIBCO BRL (Grand Island, NY). Monoclonal anti-α-actinin antibody (Clone EA-53) was purchased from Sigma Chemical Co (St. Louis, MO). Monoclonal anti-human desmin antibody (Clone D33) was purchased from DAKO (Glostrup, Denmark). Rabbit polyclonal anti-α3A integrin antibody raised against the cytoplasmic domain of α3A integrin was purchased from Chemicon International Inc. (Temecula, CA) and all secondary antibodies were from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA). Protein A-sepharose was purchased from Pharmacia Biotech (Uppsala, Sweden).

Cell Cultures

Myocytes were isolated according to the methods of Borg *et al.* (1984) and Simpson *et al.* (1994). Briefly, hearts from 12–25 neonatal rats (2–3 day-old) were excised and placed into ice-cold Moscona's saline (136.8 mM NaCl, 28.6 mM KCl, 11.9 mM $NaHCO_3$, 9.4 mM glucose, 0.008 mM NaH_2PO_4, pH 7.4). The tissues were then transferred into ice-cold Krebs Ringer Buffer-I solution (KRB-I: 118.4 mM NaCl, 2.4 mM $MgSO_4$, 4.7 mM KCl, 23.8 mM $NaHCO_3$, 1.5 mM KH_2PO_4 plus 1 mg/ml fraction V bovine serum albumin (BSA), 2 mg/ml glucose, 100 units/ml penicillin and 100 units/ml streptomycin, pH 7.4), cut into small pieces, and then rinsed with fresh KRB-I. The minced tissues were placed in 50 ml flasks and incubated in KRB-II (KRB-I plus 20 mg/ml BSA, 2 mg/ml glucose, 100 units/ml penicillin, 100 units/ml streptomycin, and 100 units/ml collagenase Type II) at 37°C for a series of 9 min intervals. After each interval, the supernatant was taken and pipetted several times to mechanically release partially dissociated cells. The supernatant was then withdrawn and diluted 1 : 2 with ice-cold KRB-III (KRB-I plus 20 mg/ml BSA, 2 mg/ml glucose, pH 7.4); the remaining tissues were allowed to settle. To enrich the myocytes, the supernatant from the first dissociation was discarded. Myocytes isolated from several successive dissociation cycles were pooled in ice-cold KRB-III and filtered through Nitex membrane (250 μm). The cells were then resuspended in DMEM supplemented with 10% FBS, seeded onto collagen-coated dishes (50 μg/ml) at a concentration of $1-2 \times 10^6$ cells/ml, and grown in a humidified atmosphere containing 5% CO_2 at 37°C. C2C12 mouse skeletal myoblasts were cultured in DMEM with 10% FBS. To switch cells to myodifferentiation, 10% FBS was replaced with 3% horse serum (HS) in DMEM.

Antibodies

Integrin cytoplasmic domain peptides were synthesized at Research Genetics, Inc. (Huntsville, AL); a 13 amino acid peptide (AVTTVVNPKYEGK) corresponding to the β1A cytoplasmic domain; a 16 amino acid (PINNFKNPNYGRKAGL) peptide corresponding to the β1D cytoplasmic domain; and a 15 amino acid peptide (RIREEERYPPPGSTL) corresponding to the α3B cytoplasmic domain were obtained. An NH_2-terminal cystein was added to all peptides and coupled to carrier protein, keyhole limpet hemocyanin (KLH). Rabbits were subcutaneously immunized with 1 mg of the conjugate in complete Freund's adjuvant and boosted with 0.5 mg of the conjugate in incomplete Freund's adjuvant after 4 weeks. Additional boosts were then administrated every 4 weeks. Anti-Nck antiserum was prepared from rabbit injected with purified Nck (Park, 1997). Antibody production was tested by enzyme-linked immunosorbent assay (ELISA). The ELISA plates (96 well) were coated with 5 μg/ml of antigen peptides and incubated with test serum and horseradish peroxidase (HRP)-conjugated goat anti-rabbit immunoglobulin. O-phenylenediamine (OPD), which is a substrate of horseradish peroxidase, was added to the plate, and the color change was observed using ELISA reader (Bio-Rad, Hercules, CA).

Immunoglobulin was isolated by applying immunserum to a sepharose-column which is immobilized with the synthetic antigen peptides. The column was then extensively washed with 50 mM Tris-HCl (pH 7.0) and eluted with 100 mM glycine (pH 3.0). The pH of elutes was immediately readjusted to pH 8.0 with 1 M Tris (pH 9.0). The affinity-purified

immunoglobulin fraction was extensively dialyzed against phosphate buffered saline (PBS, pH 7.4) and stored at −70°C.

Immunofluorescence

Cardiac myocytes grown on collagen (50 μg/ml)-coated coverslips were rinsed twice with PBS and fixed with 3.7% (w/v) paraformaldehyde for 10 min. They were permeabilized with 0.5% Triton X-100 in PBS for 5 min, and then incubated for 45 min at room temperature with the primary antibody that had been diluted in PBS. The cells were washed several times with PBS and then stained with fluorescein isothiocyanate (FITC) and/or tetramethyl rhodamine isothiocyanate (TRITC)-conjugated goat anti-immunoglobulin for 45 min at room temperature. The coverslips were mounted in glycerol/PBS (9/1: v/v; pH 8.5) containing 10 mM p-phenylenediamine and examined under a Leica DMRBE microscope equipped with a 63X PL APO objective and a HBO 100 W mercury lamp. Images were photographed with T-max 3200 ASA film (Kodak, Rochest, NY).

Immunoprecipitation, Electrophoresis and Immunoblot Analysis

Cardiac myocytes grown on collagen (50 μg/ml)-coated culture dishes were washed three times with cold PBS and extracted for 1 h at 4°C in 1 ml of extraction buffer containing 200 mM N-octyl β-D glucopyranoside, 50 mM Tris (pH 7.5), 150 mM NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 2 mM PMSF, 0.02 mg/ml aprotinin and 0.0125 mg/ml leupeptin. The lysates were then centrifuged for 10 min at 10,000*g* at 4°C. The protein concentration of the supernatants was determined and 1 mg of total protein was subsequently immunoprecipitated with anti-α3A, anti-α3B, anti-β1A or anti-β1D antiserum (1 : 100 dilution), followed by protein A-sepharose beads. The beads were extensively washed with the same extraction buffer, and the immunoprecipitates were boiled and then electrophoresed on 8% SDS-polyacrylamide gels (PAGE). Proteins were transferred onto PVDF membrane, and the membranes were blocked with 5% nonfat milk, 0.1% Tween 20 in TBS (50 mM Tris-HCl, 150 mM NaCl, pH 7.5) for 1 h. The membranes were incubated with anti-β1A, anti-β1D antiserum (1 : 500 dilution) or anti-Nck antiserum (1 : 100 dilution) followed by peroxidase-conjugated goat anti-rabbit immunoglobulin. The membranes were extensively washed with TBS plus 0.1% Tween 20 and then finally developed using ECL+ reagents (Amersham, Buckinghamshire, England).

Cell Permeablization and Antibody Internalization

Cardiac myocytes grown on collagen-coated dishes for 7 days were washed twice with DMEM and exposed to streptolysin O (80 μg/ml) at 37°C for 30 min (Pacifici *et al.*, 1994). The cells were washed three times with DMEM and incubated with growth medium containing normal rabbit immunoglobulin, affinity-purified anti-α3A, anti-α3B, anti-β1A or anti-β1D antibody (200 μg/ml). After incubation for 1, 24 or 48 h at 37°C, the cells were washed extensively with DMEM and subjected to immunofluorescence assay.

RESULTS

Cellular Localization of α3A Integrin in Cultured Cardiac Myocytes

To determine the cellular localization of α3A integrin during neonatal myocyte development, cardiac myocytes isolated from neonatal rat hearts were cultured and subjected to indirect immunofluorescence assays. Freshly isolated neonatal myocytes displayed a round morphology. Once cultured, these cells attached, spread, and underwent dramatically morphological changes during the first week. By the time attachment was completed, the cells were flattened and spread into polygonal forms of various sizes and shapes. Individual myocytes retained contractile activity, and cell populations within cultures beat in an organized fashion. During these processes, the distribution of α3A integrin,

in particular, dramatically changed from a diffuse pattern to a definitively striated sarcomeric banding pattern (Fig. 1).

To temporally and spatially resolve incorporation of α3A integrin into sarcomeres relative to incorporation of α-actinin, one of the earliest proteins to be incorporated into sarcomere structures, double immunofluorescence analysis was performed. In 2–3 day cultures, α3A integrin was diffusely distributed on the surface of myocytes and did not display any particular pattern of distribution, while α-actinin was diffused throughout the cytoplasm (Fig. 1A and B). As myocytes became more flattened after 4–5 days in culture, the assembly of myofibrillar proteins into sarcomere structures was initiated: α-actinin assembled into longitudinal arrays of stress fiber-like structures (SFLSs), and α3A integrin appeared to localize along the SFLSs (Fig. 1C and D). In this way, the SFLSs might provide nucleation sites for the myofibrillar proteins. As the cells matured further (6–8 days in culture), the sarcomeric banding pattern was revealed by α-actinin antibodies (Fig. 1E), and shortly thereafter, the A bands stained by anti-myosin heavy chain (MHC) antibodies were clearly seen within sarcomeres (Fig. 1H). After MHC appeared, anti-α3A integrin antibodies exhibited a distinct pattern of binding in sarcolemmal regions adjacent to the Z-disks (Fig. 1G). Thus, the spatial distribution of α3A integrin was similar to that of α-actinin which is a component of the Z-disk (Fig. 1E and F). Moreover, the intermediate filament, desmin, appeared to be incorporated into the Z-disk after α3A integrin (Fig. 1I and J).

Cellular Localization of $\alpha 3\beta 1$ Isoforms

Vertebrate muscles express a variety of integrins, and expression of the various isoforms of the cytoplasmic domain is developmentally regulated (Hynes, 1992). In particular, alternative splicing of integrin results in isoform switching during skeletal muscle differentiation: α7B and β1A integrin subunits are progressively replaced by α7A and β1D (Belkin *et al.*, 1996; Song *et al.*, 1993). These observations led us to examine cellular localization of several isoforms of the $\alpha 3\beta 1$ cytoplasmic domain during development of neonatal cardiac myocytes. Immunofluorescence analysis showed that the β1A isoform was predominantly found in sarcolemmal regions and formed sarcomeric banding patterns (Fig. 2A(c)). In addition, β1A appeared to colocalize with α-actinin (data not shown). This means that the α3A subunit is likely to be associated with the β1A subunit – a conclusion that was further confirmed in immunoprecipitation experiments (Fig. 2B).

Immunofluorescent staining using the antibodies raised against the cytoplasmic domains of the α3B and β1D subunits revealed that they were distributed in a striated pattern in sarcolemmal regions likely associated with Z-disks in mature cardiac myocytes (Fig. 2A(b) and (d)); a similar pattern of distribution was also observed for α-actinin. These staining patterns were completely eliminated by preincubation of the primary antibodies with antigenic peptides (data not shown). These data suggest that all α3 and β1 integrin isoforms are present at sarcolemmal regions and also form heterodimers at those locations. In addition, the α3A subunit was found in the M-line of the sarcomere in fully mature myocytes, but other isoforms were not (Fig. 2A(a)). Thus, the α3A subunit may be associated with other β subunits in the M-line of the sarcomere. To assess which α3 subunits (α3A and α3B) were associated with which β1 subunits (β1A and β1D), lysates obtained from 8 days cultures of myocytes were immunoprecipitated with antibodies raised against α3A or α3B cytoplasmic domains and immunoblotted with anti-β1A or anti-β1D antibodies. As shown in Fig. 2B, the α3A subunit immunoprecipitated with both β1A and β1D subunits, suggesting that both $\alpha 3A\beta 1A$ and $\alpha 3A\beta 1D$ heterodimers are formed in cardiac myocytes. On the other hand, the α3B integrin was immunoprecipitated with β1A, but not with β1D. Thus, the $\alpha 3B\beta 1D$ heterodimer does not appear to be present in cardiac myocytes.

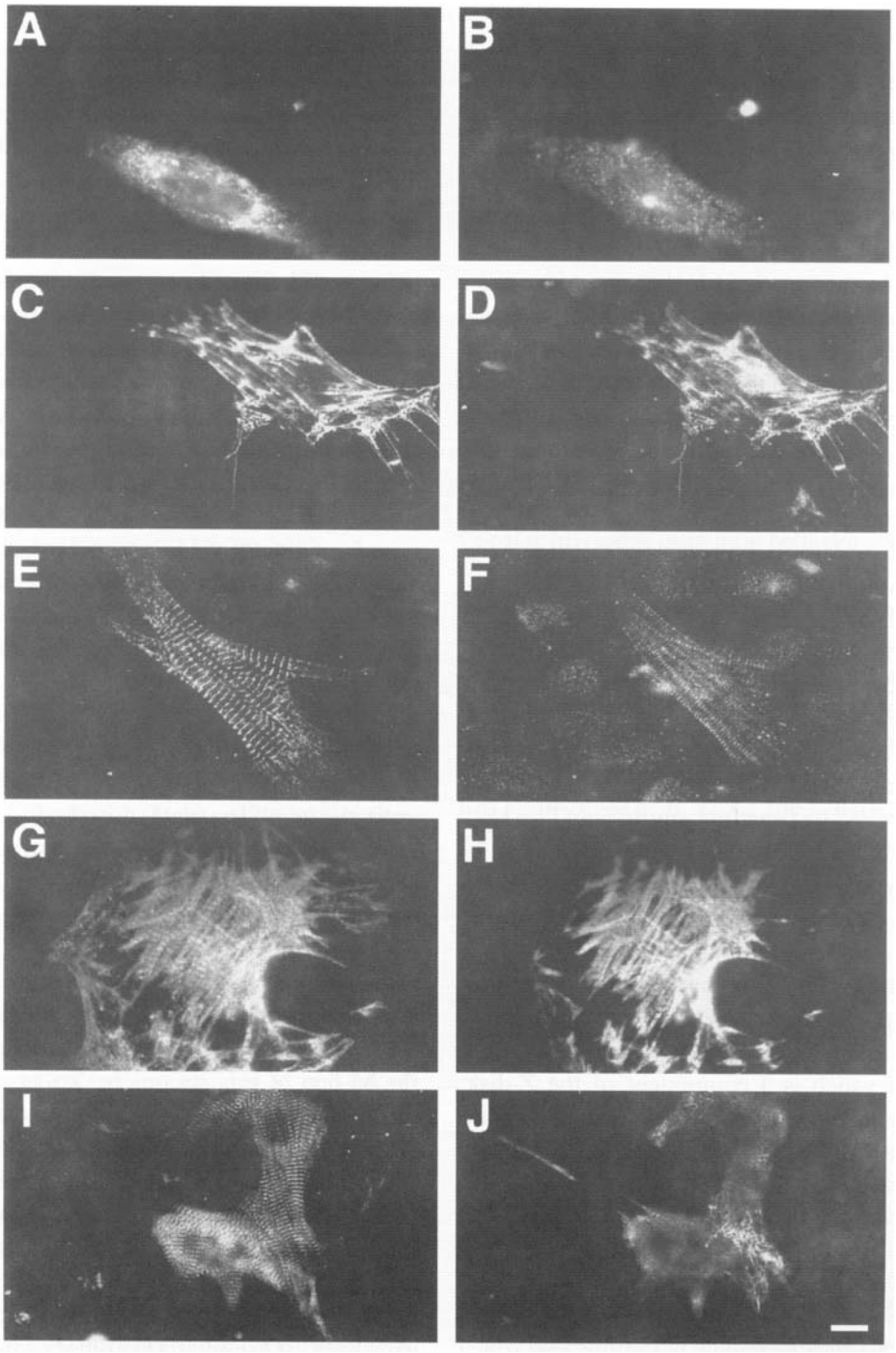

FIGURE 1 Changes in the cellular distribution of the α3A integrin during cardiac myocyte development. Neonatal rat myocytes were cultured for 2 days (A and B), 4 days (C and D), 8 days (E, F, G and H) or 10 days (I and J) and then immunostained with monoclonal anti-α-actinin (A, C and E), polyclonal anti-α3A (B, D, F, G and I), monoclonal anti-MHC (MF-20, H) or monoclonal anti-desmin (D33, J) antibodies, and then followed by FITC-conjugated goat anti-rabbit immunoglobulin (B, D, F, G and I) and TRITC-conjugated goat anti-mouse immunoglobulin (A, C, E, H and J). The images in panels A, C, E, G and I depict alternate staining of the same cells shown in panels B, D, F, H and J, respectively. During development, the pattern of α3A integrin staining changed dramatically from a diffuse distribution to a sarcomeric banding pattern. The sarcomeric banding pattern of α3A staining was visible only after the appearance of α-actinin and MHC (E, F, G and H), but it preceded the appearance of desmin (I and J). Bar, 10 μm.

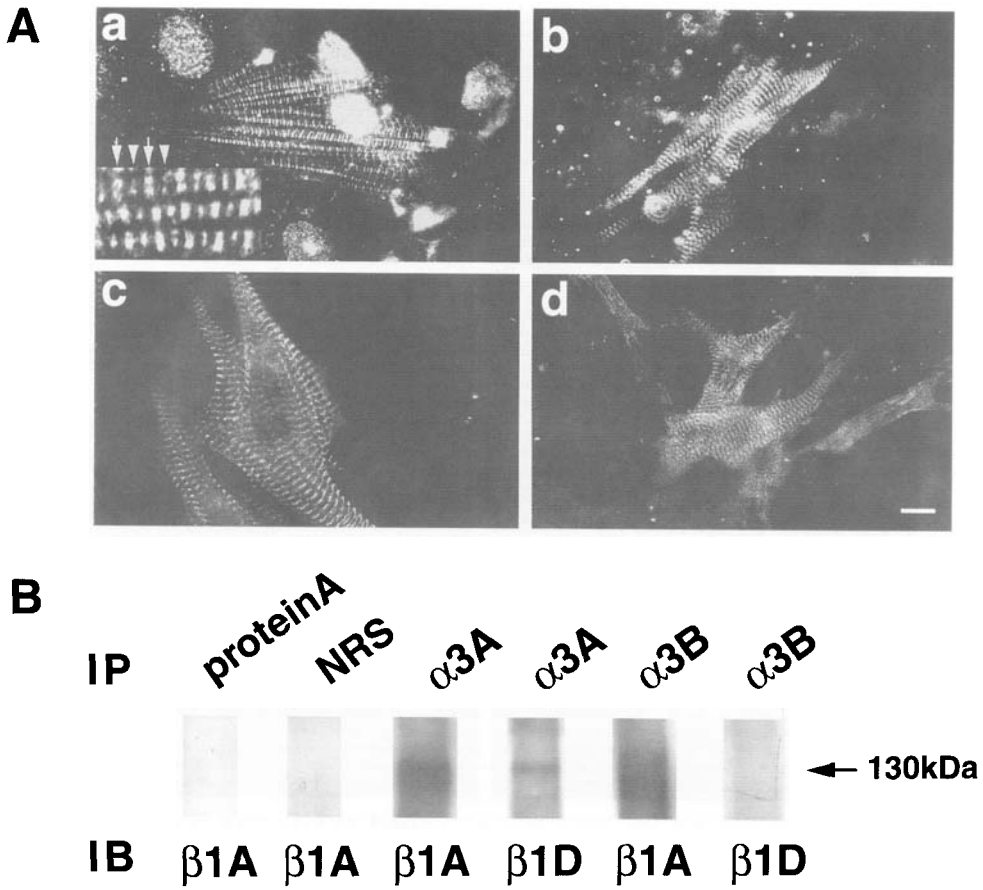

FIGURE 2 Cellular distribution of α3 and β1 subunit isoforms in cardiac myocytes. (A) Cardiac myocytes were cultured for 8 days, immunostained with antibodies raised against the cytoplasmic domain of α3A (a), α3B (b), β1A (c) or β1D (d), and then visualized with FITC-conjugated goat anti-rabbit immunoglobulin. All of these isoforms were found at the costameres and were distributed in sarcomeric banding patterns; the α3A isoform was found in the M-line as well. The arrows indicate the positions of Z-disks, and the arrowheads point to the M-lines. (B) To assess which α3 subunits were associated with β1 subunits, myocyte lysates were immunoprecipitated (IP) with either anti-α3A or α3B antibodies and then immunoblotted (IB) with anti-β1A or β1D antibodies. Note that α3Aβ1A, α3Bβ1A and α3Bβ1A heterodimers were found in the myocyte lysates, but α3Bβ1D was not. Protein A and normal rabbit serum (NRS) were used as controls. Bar, 10 μm.

Disruption of Sarcomere Structure by Introducing Integrin Antibodies into Cells

The localization of α3β1 integrin isoforms in sarcolemmal regions associated with Z-disks suggested a potential role for α3β1 integrin isoforms in the organization of sarcomere structure. To further clarify their roles in the organization of sarcomere structure, antibodies against α3β1 integrin cytoplasmic domains were introduced into cardiac myocytes (7 days cultures) that had been permeabilized with streptolysin O, and their effect on sarcomere structure was examined by immunofluorescence assay using anti-α-actinin antibody.

Cells cultured for 7 days exhibited typical sarcomere structure (Fig. 3A). Streptolysin O, by itself, did not affect cell viability, which was consistent with previous reports (Coppolino *et al.*, 1995; Pacifici *et al.*, 1994). In 1 day cultures that had been exposed to normal rabbit immunoglobulin (NRIgG), typical sarcomeric patterns were observed when cells were stained with anti-α-actinin antibodies (Fig. 3B). In contrast, introduction of anti-α3A, α3B, β1A, or β1D antibodies elicited

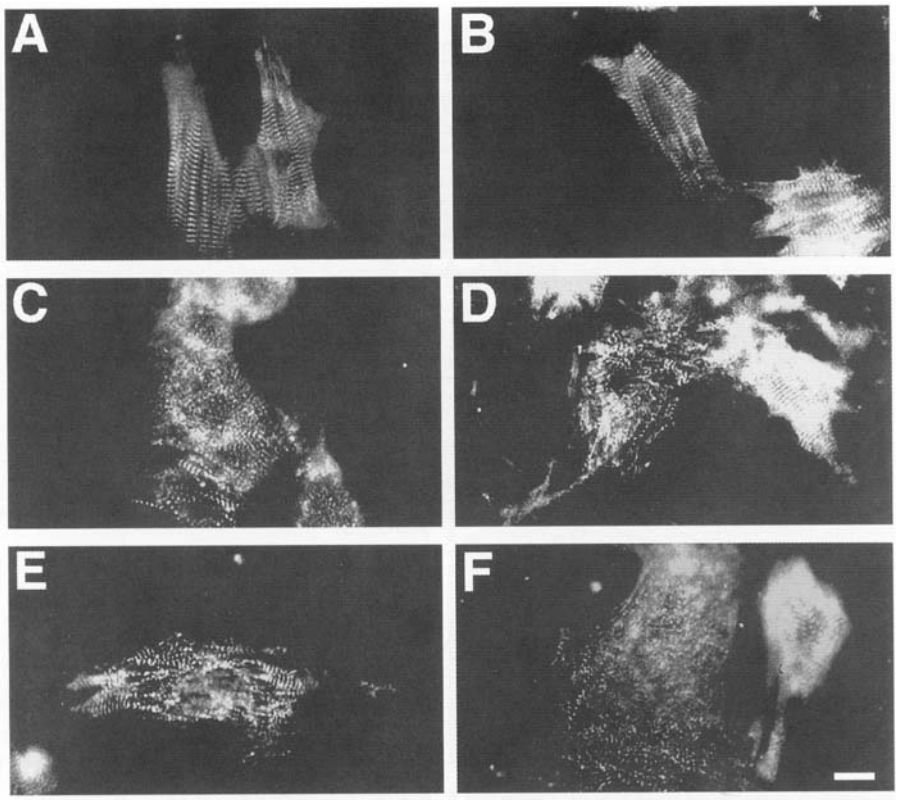

FIGURE 3 Antibodies against the cytoplasmic domains of the $\alpha 3$ and $\beta 1$ subunit isoforms caused disruption of sarcomere structure. Myocytes were cultured for 7 days and then treated with streptolysin O for 30 min to permeabilize the cell membrane. To visualize changes in sarcomere structure, after antibody treatment for 24 h, the cells were stained with anti-α-actinin antibodies and visualized by FITC-conjugated goat anti-mouse immunoglobulin. Cells cultured for 7 days exhibited typical sarcomere structure (A). Normal rabbit immunoglobulin (NRIgG), which did not affect the disruption of sarcomere structure, was used as a control (B). Antibodies against $\alpha 3$A (C), $\alpha 3$B (D), $\beta 1$A (E) and $\beta 1$D (F) all caused clear alternations in sarcomere structure. Bar, 10 μm.

obvious alterations in sarcomere structure (Fig. 3C–F). After 1 h, myofibrillar organization appeared to be compromised and α-actinin began to form cortical-actinin containing bodies (CABs) which were comprised of α-actin and sarcomeric α-actinin. This indicates that as with the earlier findings by Lin *et al.* (1989) the I–Z–I complexes had been disrupted. After 1 day, most cells contained CABs, and the cells detached from the substratum within 2 days of antibody treatment.

The percentage of sarcomeres disrupted by antibody treatment was calculated from the mean number of cardiac myocytes containing disrupted sarcomeres (Fig. 4). Streptolysin O and normal rabbit immunoglobulin had little effect on sarcomere structure; in the absence of antibodies, ~8% of cells exhibited disrupted sarcomeres. In contrast, treatment with integrin antibodies had a dramatic effect; substantial increases in the numbers of cells exhibiting disrupted sarcomeres were detected within 1 h (Fig. 4, hatched bar), and within 24 h, ~50% of cells showed disrupted sarcomeres (Fig. 4, solid bar). These data suggest that the interaction of integrin cytoplasmic domain with other proteins plays an important role in the maintenance of sarcomere structure and/or the assembly of myofibrils into sarcomeres.

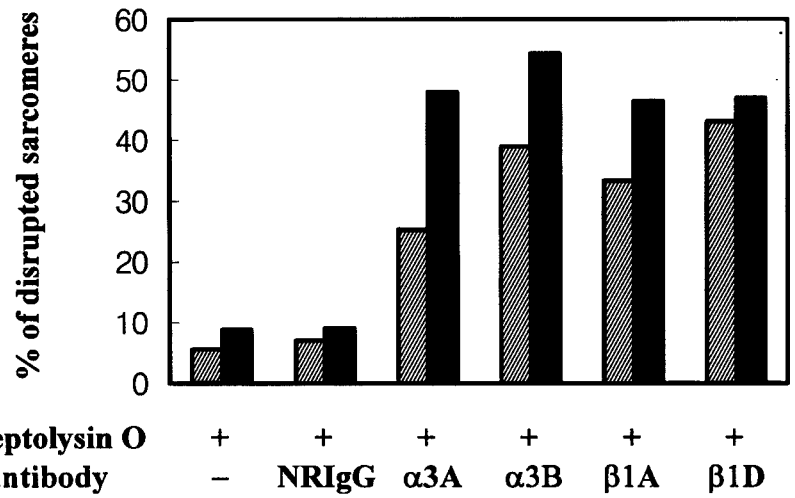

FIGURE 4 The percentage of disrupted sarcomeres was calculated from the mean number of myocytes in which disrupted sarcomeres were observed. Note that the antibodies elicited a strong effect, disrupting ~50% of sarcomeres, whereas in the presence of streptolysin O and NRIgG, the degree of sarcomere disruption remained at basal levels (~8%). Hatched and solid bars depict the percentage of disrupted sarcomeres observed after 1 and 24 h of antibody treatment, respectively.

Association of Nck with β1A Integrin Cytoplasmic Domain but not with β1D *In Vitro*

The cytoplasmic domains of integrins interact with many other proteins, and this interaction is thought to be important for intracellular signal transduction. To investigate proteins associated with the cytoplasmic domains of α3β1 integrin isoforms, immunofluorescence analysis was performed using antibodies against signal molecules. Interestingly, Nck, an adaptor molecule containing 1 SH2 and 3 SH3 domains (Park and Rhee, 1992), exhibited a staining pattern characterized by sarcomeric banding and colocalized with α-actinin at the Z-disks in cardiac myocytes (Fig. 5A(a) and (b)). Immunoprecipitation and immunoblot analysis demonstrated that β1A integrin co-immunoprecipitated with Nck, suggesting that Nck may participate in the downstream of signaling pathway via α3β1A integrin in the sarcomeres of cardiac myocytes (Fig. 5B). Nck, however, was not associated with the β1D isoform, and the signal pathway of β1A seemed to be distinct from that of β1D.

DISCUSSION

Vertebrate muscles express a variety of integrins depending upon developmental stages (Hynes, 1992). Previous studies have demonstrated the expression of α1 and α3 integrins as collagen receptors and α7 as a laminin receptor in cultured cardiac myocytes (Kaufman *et al.*, 1985; Simpson *et al.*, 1994; Terracio *et al.*, 1991). Other integrins including α4, α5, α6, β1 and β4 have been reported to be expressed during heart development (Collo *et al.*, 1995; Yang *et al.*, 1995; Fassler *et al.*, 1996; Hierck *et al.*, 1996a,b; Terracio *et al.*, 1991). Thus, it is likely that many integrin α and β subunits are expressed during heart development and are critically involved in the developmental process. Nevertheless, with the exception of β1 integrin found in costameres, the cellular localization of integrins, which should provide important clues about their function, has not yet been reported. In the present study, we examined the cellular localization of several α3β1 integrin isoforms and their roles in sarcomere assembly during neonatal rat cardiac

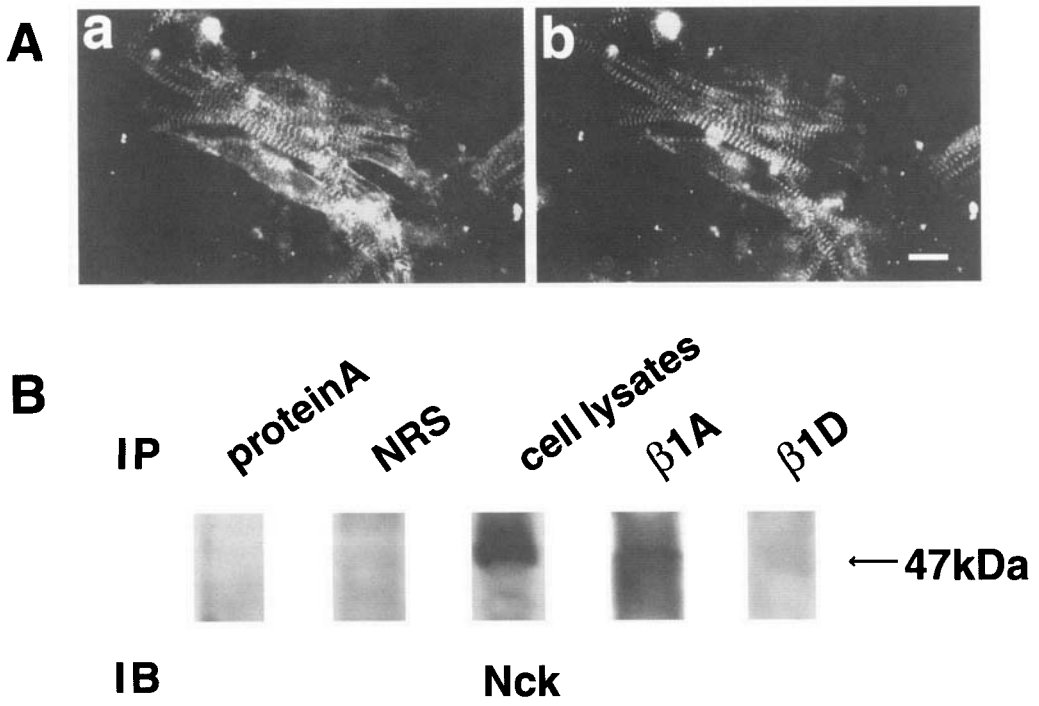

FIGURE 5 (A) Myocytes grown for 8 days were stained with anti-Nck (a), or anti-α-actinin (b) antibodies and visualized by FITC-conjugated goat anti-rabbit (a) or TRITC-conjugated goat anti-mouse immunoglobulin (b). The image in panel (a) depicts alternate staining of the same cell shown in panel (b), respectively. (B) The cell lysates obtained from 8 days cultures of cardiac myocytes were immunoprecipitated (IP) with either anti-β1A or β1D antibodies and immunoblotted (IB) with anti-Nck antibody. Nck (47 kDa) co-immunoprecipitated with β1A, but not with β1D integrin. Bar, 10 μm.

myocyte development in culture. The observations described demonstrate that the cellular localization of α3β1 integrin, in particular, dramatically changed from a diffuse distribution to a sarcomeric banding pattern during the development of cardiac myocytes, and their localization in sarcolemmal regions associated with Z-disks closely correlated with myofibril assembly and organization of sarcomere structure.

The localization of α3β1 integrin in costameres, where the myofibrils are laterally linked to the sarcolemma, reflects the specialized function of α3β1 integrin in myofibril organization. α3 integrin and α-actinin colocalize mainly along the Z-disks of peripheral myofibrils that are closely associated with the cell membrane, where most collagen fibrils appear to be anchored (Borg *et al.*, 1983; Terracio *et al.*, 1991). In the context of the role of integrins as transmembrane receptors mediating linkage between the cytoskeleton, cell membrane, and the ECM (Hynes, 1992), α3 integrin may mediate myofibril–collagen fibril linkages, and such linkages are likely to maintain overall sarcomere structure. Earlier findings lend further support to the notion that integrins help to coordinate myofibrillar assembly: *Drosophila* embryos carrying the lethal myospheroid mutant gene do not express βPS integrin and undergo defective differentiation leading to defective Z-bands in muscle (Volk *et al.*, 1990). Moreover, adhesion-perturbing antibodies against β1 integrin caused a significant regression in myofibrillar organization in skeletal muscle cells (McDonald *et al.*, 1995). The data presented in this report document the disruption of sarcomere structure caused by the introduction into cardiac myocytes of antibodies raised against integrins.

With respect to the sequence of myofibrillar assembly, our double immunofluorescence experiments showed that the sarcomeric banding patterns of $\alpha 3$ integrins were visible after the appearance of α-actinin and MHC but prior to the appearance of desmin. This sequence of events is consistent with that seen with incorporation of αV integrin into sarcolemmal regions associated with Z-lines in skeletal muscle (McDonald *et al.*, 1995), and it implies that incorporation of integrins into costameres is dependent upon the presence of myofibrillar proteins such as α-actinin and MHC. It also indicates that integrins probably do not participate in the initiation of myofibrillar assembly. Because nascent myofibrils seem to be primarily assembled in close proximity to the sarcolemma and then are extended into the cell interior, it is conceivable that $\alpha 3$ integrin takes part in the stabilization of developing myofibrils.

Many intracellular proteins interact with integrin cytoplasmic domains either directly or indirectly. For example, cytoskeletal proteins such as the actin-binding proteins (α-actinin, talin, vinculin, and tensin) have long been known to bind the $\beta 1$ integrin cytoplasmic domain (Dedhar and Hannigan, 1996; Lewis and Schwartz, 1995; Otey *et al.*, 1990). The highly conserved KXGFFKR sequence of the integrin α subunit interacts with an intracellular calcium-binding protein, calreticulin (Dedhar 1994; Rojiani *et al.*, 1991). Signaling molecules such as FAK, PI3K, Rho, Ras, Shc, Grb2, SOS, Src, CAS are targeted to adhesion sites where they come into contact with the integrin cytoplasmic domain (Machesky and Hall, 1996; Nojima *et al.*, 1995; Schaller *et al.*, 1995; Schlaepfer *et al.*, 1997; Wary *et al.*, 1996; Yamada and Geiger, 1997; Zhu and Assoian, 1995).

The spatial relationships and interactions between signaling molecules and the α and β subunit cytoplasmic domains can alter the binding affinity of integrins for their respective ligands in the ECM. For instance, mutation of an NPXY motif of the $\beta 1$ cytoplasmic domain appears to completely inhibit binding of PAC1, which recognizes integrins in the activated state (O'Toole *et al.*, 1994; 1995). In the present study, the antibodies introduced into cardiac myocytes presumably prevented the integrin cytoplasmic domain from interacting with signaling molecules. This likely altered the binding affinity of the integrin extracellular domain for ECM proteins, thereby weakening myofibril–ECM linkage and, consequently, disrupting sarcomere structure. In addition, anti-$\beta 1$A and $\beta 1$D antibodies raised against the C-terminal amino acid sequence containing the conserved NPXY motif may contribute to maintaining integrins in an inactivated state.

We report the presence of three $\alpha 3\beta 1$ integrin isoforms, $\alpha 3A\beta 1A$, $\alpha 3B\beta 1A$, and $\alpha 3A\beta 1D$, at the costameres of cardiac myocytes. Structural differences in these isoforms are restricted to alternate forms of the cytoplasmic domain arising from alternative splicing. The reasons why three integrins capable of binding the same ligand are present at the same site in cardiac myocytes are not clear. Perhaps the diversity in the cytoplasmic domains contributes to the varied capacities of integrin isoforms to transduce inwardly directed signals initiated by extracellular interactions; signals arising within the cell and directed outward via integrin activation may be similarly affected. The present data also suggest that alternate forms of the cytoplasmic domain selectively transduce distinct signals. Co-immunoprecipitation and immunofluorescence experiments using anti-Nck and anti-$\beta 1$ isoform antibodies revealed that Nck was localized at Z-disks and interacted with the $\beta 1$A isoform. It is reasonable to hypothesize, therefore, that $\beta 1$A interacts directly with Nck, because the $\beta 1$A cytoplasmic domain contains two tyrosines (Y783 and Y795) and a conserved NPXY motif that upon phosphorylation may provide a binding site for the SH2 domain of Nck. On the other hand, the possibility that Nck interacts with the $\beta 1$A integrin cytoplasmic domain in cooperation with other effector molecules is also supported by earlier findings. Several effector molecules such as SOS, Cbl, WASP, PAK (serine/threonine kinase) family, FAK and CAS which interact with the SH3 domains of Nck, have been identified (Bagrodia *et al.*, 1995; Galisteo *et al.*, 1996; Hu *et al.*, 1995;

Lu *et al.*, 1997; Rivero-Lezcano *et al.*, 1994; Schlaepfer *et al.*, 1997; Su *et al.*, 1997). Among these molecules, CAS is likely a candidate for linking β1A and Nck, because CAS is spatially associated with the β1 integrin cytoplasmic domain in focal adhesion sites (Schaller *et al.*, 1995; Schlaepfer *et al.*, 1997; Turner and Miller, 1994). Recent report that CAS is localized at Z- disks in adult cardiac muscle further supports this (Honda *et al.*, 1998). However, the signal pathway in Z-disks via β1 integrin has not been elucidated yet. It was recently reported that Arg-BP2 is localized in Z-disks of cardiac muscle (Wang *et al.*, 1997); Erk-1 is predominantly expressed in Z-disks as well (unpublished data). To address the question whether these proteins are involved in signal transduction initiated by the interaction of $\alpha 3\beta 1$ integrin with ECM proteins, detailed studies of the cellular localization of these proteins as well as biochemical analysis will be required.

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