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Role of *N*-Cadherin- and Integrin-Based Costameres in the Development of Rat Cardiomyocytes

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Abstract Costameres, vinculin-containing structures found in skeletal and cardiac muscle, are thought to anchor the Z-discs of the peripheral myofibrils to the sarcolemma. Several lines of evidence indicate that two different sets of costameres, integrin- and *N*-cadherin-based, are present in cardiac muscles. In this study, immunoblot analysis was used to study the expression of *N*-cadherin, α-catenin, β-catenin, vinculin, talin, and laminin in rat cardiac muscles at embryonic days 15 and 19, the day of birth (postnatal day 0), postnatal weeks 1, 2, 3, and 4, and in the adult. Double immunofluorescence microscopy was performed to study the spatial and temporal distribution of these two sets of costameres in rat cardiomyocytes. Costameric staining for *N*-cadherin, codistributed with β-catenin, was strong from embryonic day 15 up to postnatal week 2, gradually decreased after postnatal week 3, and was undetectable at postnatal week 4 and in the adult. Confocal microscopy showed that *N*-cadherin colocalized with α-actinin at cortical myofibrils. Double-labeling of β-catenin and talin indicated the coexistence of *N*-cadherin/catenin- and integrin/talin-based costameres in rat cardiac muscle. Although β-catenin and vinculin were co-localized at the costamere of cardiomyocytes from embryonic day 15 to postnatal week 3, staining for β-catenin or talin was mutually exclusive at all stages examined. These results demonstrate the simultaneous, but mutually exclusive, existence of *N*-cadherin/catenin- and integrin/talin-based costameres in rat cardiomyocytes between late embryonic stages and postnatal week 3, while only integrin/talin-based costameres were found in adult rats. The *N*-cadherin/catenin-based costameres in rat cardiac muscles may play a role in myofibrillogenesis similar to that of their counterparts in cultured cardiomyocytes. J. Cell. Biochem. 84: 717–724, 2002.

Key words: talin; β-catenin; α-actinin; costamere; cardiomyocyte

In cardiac muscle, the Z-discs of cortical myofibrils make contacts with the sarcolemma to form costameres. Costameres are prominent features of the sarcolemma and were originally identified as vinculin-containing, rib-like structures distributed at the sarcolemma of skeletal and cardiac muscles [Koteliansky and Gneushev, 1983; Pardo et al., 1983a,b]. In addition to myofibril anchoring, vinculin has been suggested to be involved in myofibril alignment [Tokuyasu, 1989; Hilenski et al., 1991]. Treatment of cardiomyocytes with antisense oligonucleotides complementary to vinculin mRNA perturbs the alignment of myofibrils and results in incomplete assembly of the sarcomere [Shiraishi et al., 1997]. Spectrin [Craig and Pardo, 1983], talin [Belkin et al., 1986], α-actinin [Danowski et al., 1992], and integrins [Terracio et al., 1989; Carver et al., 1994; McDonald et al., 1995; Imanaka-Yoshida et al., 1999] are all components of the costamere complex.

Integrins are transmembrane proteins composed of heterodimeric α and β subunits that bind to the extracellular matrix (ECM) or to counter-receptors on the cell surface [for review, see Hynes, 1992]. Individual integrins bind via their extracellular domains to various ECM proteins, such as collagen, fibronectin, vitronectin, and laminin. The cytoplasmic domain of the

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integrin β chain is directly associated with cytoskeletal proteins, such as talin, α-actinin, filamin, and paxillin [for review, see Hemler, 1998]. The presence of integrin/talin complexes in costameres suggests that they play a role in linking cortical myofibrils to the extracellular milieu. In vitro studies of adult rat and chick embryonic cardiomyocytes cultured on flexible silicon rubber substrata have indicated that integrin-based costameres are myofibril attachment sites, which transmit forces from the contraction apparatus to the ECM [Danowski et al., 1992; Imanaka-Yoshida et al., 1999]. In vitro analyses have also suggested that integrin-based costameres are essential in myofibrillogenesis. Treatment of cardiomyocytes with either a Ca^{2+} channel blocker to inhibit contractile activity or with C3 exoenzyme to inactivate rho A protein results in the loss of integrin-based costameres and the disassembly of myofibrils [Sharp et al., 1997; Wang et al., 1997].

Cadherins, a superfamily of molecules that are responsible for specific cell–cell adhesion, exist as Ca^{2+}-dependent dimers which associate with cadherin dimers on other cells [for review, see Nollet et al., 2000]. The highly conserved cytoplasmic portion of cadherins interacts with catenins, which link the cadherin to the actin cytoskeleton [Ozawa et al., 1989]. In cardiac muscle, the major transmembrane component of the adherens junction in the intercalated discs is *N*-cadherin [Volk and Geiger, 1984, 1986; Geiger et al., 1990], which is associated with catenins [Wheelock and Knudsen, 1991]. In addition to the integrin-based costameres, there is evidence for the existence, on the dorsal cell surface of cultured cardiomyocytes, of *N*-cadherin-containing costameres that are also involved in myofibrillogenesis [Goncharova et al., 1992; Wu et al., 1999]. However, the free dorsal cell surface does not exist in the cardiac muscle in situ but is an artifact created by plating cardiomyocytes onto a plane surface and does not make contact with the plating matrix. Although *N*-cadherin-based costameres are found in cultured cardiomyocytes, the presence of this set of costameres in rat cardiac muscles has not been proven. In this study, the spatial and temporal distribution of proteins associated with *N*-cadherin- or integrin-based costameres in embryonic, neonatal, young, and adult rat cardiac muscle was determined. Relationships between these two sets of costameres and between the *N*-cadherin-based costamere and vinculin during cardiomyocyte development were explored.

MATERIALS AND METHODS

Animals and Tissue Preparation

Timed pregnant female Wistar rats were purchased from the Facility for Research Animals of the National Taiwan University. The maintenance and use of the animals were in accordance with the "Guide for the Care and Use of Laboratory Animals" published by the US National Institutes of Health (NIH publication No. 85-23, revised 1985). Rats were entrained to a 12 h light/12 h dark cycle with free access to food and water. For prenatal studies, pregnant rats were sacrificed at various time-points during pregnancy and the embryos collected for isolation of hearts. The day on which the vaginal plug was identified was designated as embryonic day 0 (E0) and the day of birth as postnatal day 0 (D0). For postnatal studies, rats were sacrificed under anesthesia (intraperitoneal injection of 70% chloral hydrate) on D0 or at 1, 2, 3, or 4 weeks of age or as adults, and the hearts collected for embedding and sectioning or homogenization. All the data presented in this study were obtained from at least three independent experiments.

Immunoblot Analysis

Homogenization of tissues, polyacrylamide gel electrophoresis (PAGE), and electrophoretic transfer of protein to nitrocellulose membranes were performed as previously described [Wu et al., 1993]. Nitrocellulose membranes were blocked for 1 h at room temperature using 5% skimmed milk and 0.1% Tween 20 in phosphate-buffered saline, pH 7.4 (PBS). The blots were then incubated overnight at 4°C with a 1:1,000 dilution of hybridoma supernatant containing mouse antibody against *N*-cadherin (13A9; a kind gift from Dr. Margaret J. Wheelock and Dr. Keith R. Johnson, University of Toledo, OH) [Knudsen et al., 1995] or of ascites fluid containing mouse antibody against vinculin or talin (V-9131 and T-3287, both from Sigma Chemical Co., St. Louis, MO), or a 1:3,000 dilution of rabbit antiserum against α-catenin, β-catenin, or laminin (C-2081, C-2206, or L-9393; all from Sigma Chemical Co.). They were then incubated for 2 h at room temperature with a 1:10,000 dilution of alkaline phosphatase-conjugated,

goat anti-rabbit IgG antiserum or goat anti-mouse IgG antiserum (Promega Corporation, Madison, WI), as appropriate. Detection of immunoreactive bands was performed by substrate development using nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate in 100 mM NaCl, 100 mM Tris-base, 5 mM $MgCl_2$, pH 9.5.

Immunofluorescence and Confocal Microscopy

Cardiac ventricles were collected, embedded in O.C.T. compound (Miles, Inc., Elkhart, IN), and frozen in liquid nitrogen. Cryostat sections (7 μm thick) of the frozen tissues were fixed for 5 min with cold acetone at −20°C. After rehydration with PBS, sections were blocked for 15 min at room temperature with 5% non-fat dry milk in PBS. The sections were incubated for 1 h at 37°C with a 1:100 dilution of mouse hybridoma supernatant (anti-*N*-cadherin, 13A9) or of ascites fluid (anti-vinculin [V-9131] or anti-talin [T-3287]) or a 1:200 dilution of rabbit antiserum against α-catenin (C-2081), β-catenin (C-2206), or laminin (L-9393). Bound primary antibodies were detected by incubation for 1 h at 37°C with a 1:100 dilution of Texas red-conjugated, goat anti-mouse IgG or FITC-conjugated, goat anti-rabbit IgG (Vector, Burlingame, CA), as appropriate. The sections were then washed with PBS and mounted in 2% n-propyl gallate and 60% glycerol in 0.1 M PBS, pH 8.0. Double labeling was performed using a mixture of mouse monoclonal antibody and rabbit polyclonal antibody, followed by a mixture of the secondary antibodies. The sections were examined under a Reichert Polyvar 2 microscope (Leica, Vienna, Austria), equipped with epifluorescence, and photographed using Kodak T-Max 400 films. For confocal microscopic analysis, the sections were double-labeled for *N*-cadherin and α-actinin and observed using a Leica TCS SP2 laser scanning microscope (Leica, Heidelberg, Germany), equipped with both agron ion and He–Ne lasers as previously described [Wu et al., 1999].

RESULTS

Immunoblot Analysis of Costamere-Related Proteins in Rat Cardiomuscles

Immunoblot analysis was performed to confirm the specificity of the antibodies and to determine the level of expression of the proteins at various stages during cardiomyocyte development. The specificity of the antibodies was confirmed by their binding only to the appropriate band on blots, although some bands of lower molecular weights, probably due to degradation, were also detected on the vinculin and talin blots. As shown in Figure 1, all the proteins tested were present at all stages, but, as the animals became mature, vinculin expression increased, whereas expression of *N*-cadherin, α-catenin, β-catenin, talin, and laminin did not change significantly.

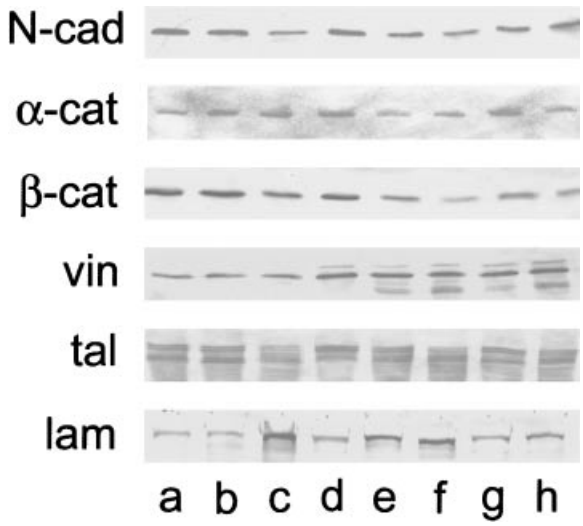

Fig. 1. Immunoblot analysis of costamere-related proteins in developing rat cardiac muscle. Cardiac muscle homogenates from rats at E15 (**lane a**), E19 (**lane b**), D0 (**lane c**), postnatal weeks 1 (**lane d**), 2 (**lane e**), 3 (**lane f**) and 4 (**lane g**), and adult (**lanes h**) were separated by electrophoresis in 10% polyacrylamide gels. The separated proteins were transferred onto nitrocellulose membranes and probed with antibodies specific for *N*-cadherin (N-cad), α-catenin (α-cat), β-catenin (β-cat), vinculin (vin), talin (tal), or laminin (lam). The same amount of protein (30 μg) was applied to each lane.

Distribution of *N*-cadherin and Catenins in Rat Cardiac Muscles

Indirect immunofluorescence microscopy was used to study the distribution of *N*-cadherin in rat cardiac muscle. Strong costameric staining of *N*-cadherin was detected at the cell membrane of cardiomyocytes at E15 (Fig. 2A), E19 (Fig. 2B), and D0 (Fig. 2C) and persisted at postnatal weeks 1 (Fig. 2D) and 2 (Fig. 2E), decreased at week 3 (Fig. 2F), and became undetectable at week 4 (Fig. 2G) and in the adult (Fig. 2H). Staining of *N*-cadherin on the intercalated disc was clearly detected in postnatal week 1 cardiomyocytes (Fig. 2D) and became stronger as the cardiomyocytes matured

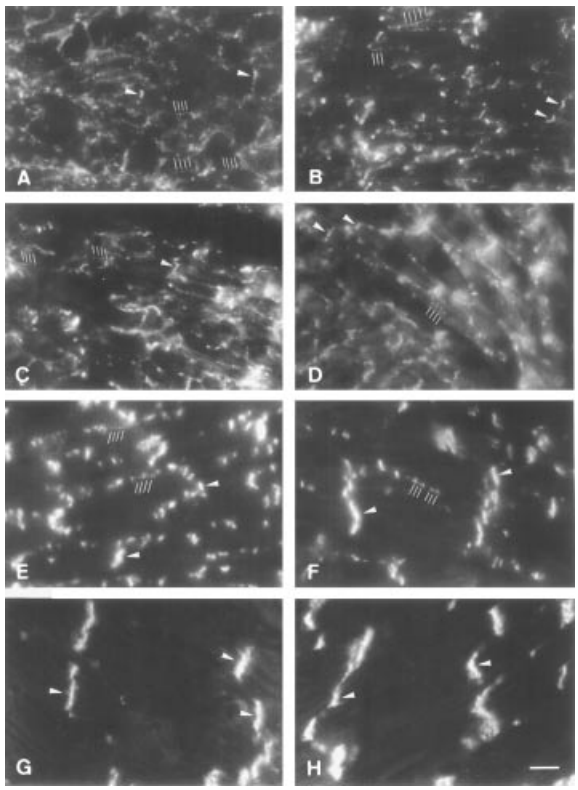

Fig. 2. Localization of *N*-cadherin in developing and mature rat cardiac muscles. Cryostat sections from E15 (**A**), E19 (**B**), D0 (**C**), postnatal weeks 1 (**D**), 2 (**E**), 3 (**F**), and 4 (**G**), and adult (**H**) rat cardiac muscles were immunolabeled for *N*-cadherin. The arrowheads indicate *N*-cadherin staining of the intercalated discs, while the short bars indicate *N*-cadherin staining of the costamere. Bar = 10 μm.

(Fig. 2E). Confocal microscopy was performed to determine the relationship of *N*-cadherin to costameric α-actinin. When the focal plane was scanned through the tangentially cut cardiac muscle cell surface, staining for *N*-cadherin and for α-actinin was superimposed in the cortical myofibrils (Fig. 3). Double immunofluorescence showed that both α-catenin (data not shown) and β-catenin were co-localized with *N*-cadherin in both the costamere and intercalated disc in E15 (Fig. 4A,B), postnatal week 2 (Fig. 4C,D), and adult (Fig. 4E,F) cardiomyocytes.

Distribution of Two Different Sets of Costameres in Rat Cardiac Muscles

Since the cytoplasmic portion of integrins is linked directly to talin [Hemler, 1998], in order to explore the relationship between integrin- and *N*-cadherin-based costameres, rat cardiac muscles were double-labeled for talin (Fig. 5A,C,E,G) and β-catenin (Fig. 5B,D,F,H). Talin was detected in the cell margins of

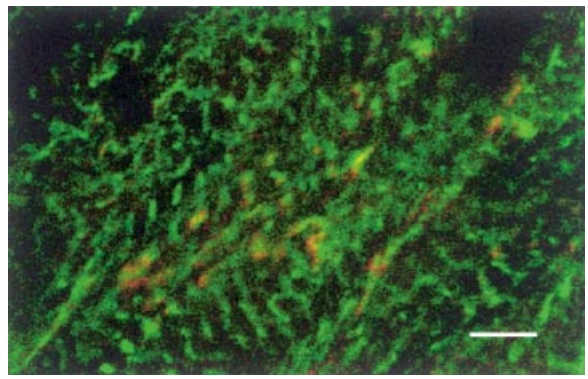

Fig. 3. Confocal micrograph of *N*-cadherin and α-actinin in developing rat cardiac muscles. Cryostat sections from postnatal day 3 rat cardiac muscles double-labeled for *N*-cadherin (red) and α-actinin (green) with the focal plane scanning through the tangentially-cut cardiomyocyte surface. *N*-cadherin staining overlies α-actinin staining (yellow spots), indicating colocalization of *N*-cadherin and α-actinin. Bar = 5 μm.

cardiomyocytes at E15 (Fig. 5A) and E19 (Fig. 5C) and distinct costameric staining for talin was seen in postnatal week 2 cardiomyocytes (Fig. 5E) and persisted in the adult (Fig. 5G). A mutually exclusive staining pattern for talin and β-catenin (compare Fig. 5A and B,

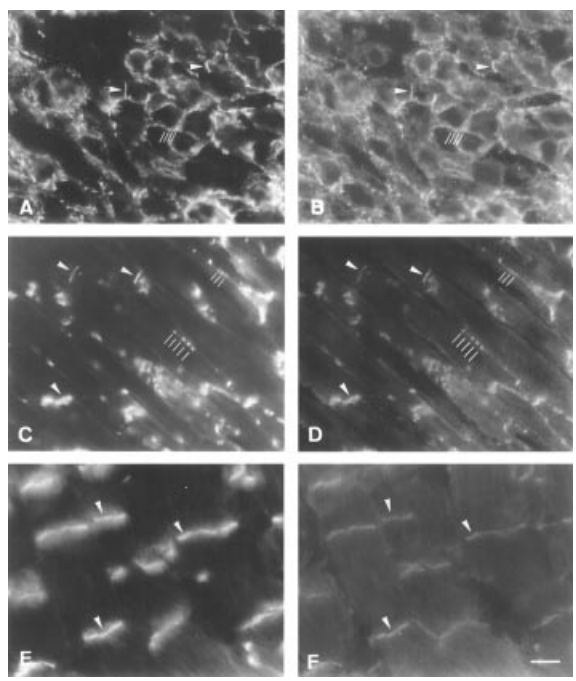

Fig. 4. Co-localization of *N*-cadherin and β-catenin in rat cardiac muscles. Cryostat sections from E15 (**A**, **B**), postnatal week 2 (**C**, **D**), and adult (**E**, **F**) rat cardiac muscles were double-labeled for *N*-cadherin (A, C, E) and β-catenin (B, D, F). *N*-cadherin and β-catenin are colocalized at the intercalated discs (arrowheads) and costameres (short bars). Bar = 10 μm.

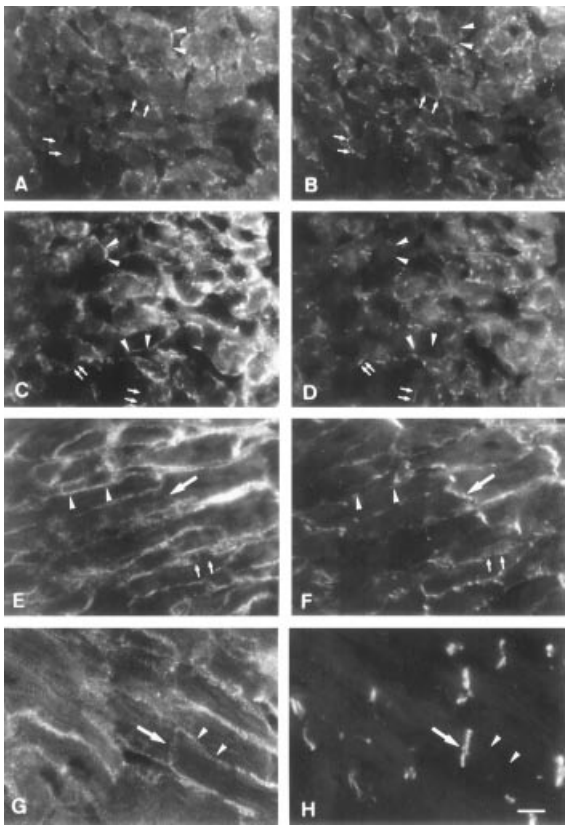

Fig. 5. Localization of talin and β-catenin in rat cardiac muscles. Cryostat sections from E15 (**A**, **B**), E19 (**C**, **D**), postnatal week 2 (**E**, **F**), and adult (**G**, **H**) rat cardiac muscles were double-labeled for talin (A, C, E, G) and β-catenin (B, D, F, H). The arrowheads indicate costameres stained for talin but not for β-catenin, while the small arrows indicate costameres stained for β-catenin but not for talin. The large arrows indicate staining of the intercalated discs for talin (E, G) and β-catenin (F, H). Bar = 10 μm.

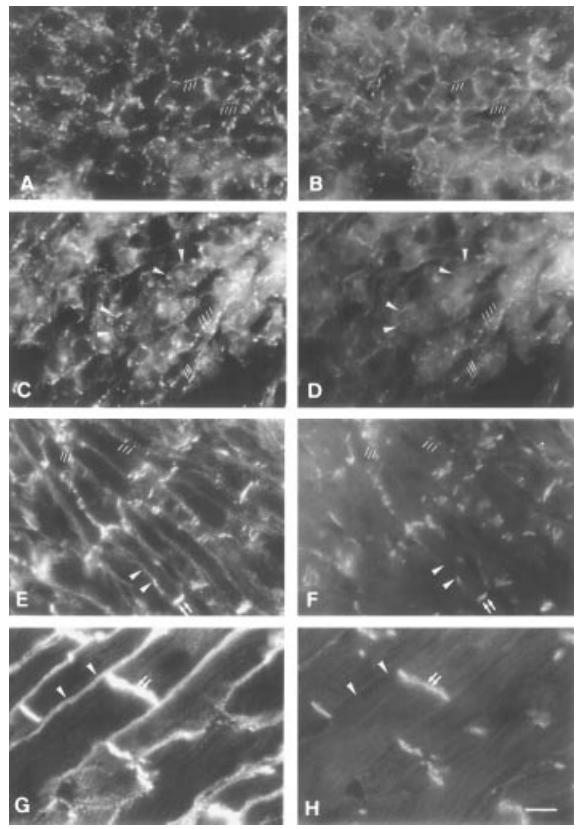

Fig. 6. Localization of vinculin and β-catenin in rat cardiac muscles. Cryostat sections from E15 (**A**, **B**), E19 (**C**, **D**), postnatal week 2 (**E**, **F**), and adult (**G**, **H**) rat cardiac muscles were double-labeled for vinculin (A, C, E, G) and β-catenin (B, D, F, H). Vinculin and β-catenin are co-localized at the costameres (short bars) and intercalated discs (double arrow). The arrowheads indicate costameres stained for vinculin (C, E, F), but not for β-catenin (D, B, H). Bar = 10 μm.

C and D, E and F, G and H) was detected in the lateral plasma membrane of cardiomyocytes at E19 (Fig. 5D) and at postnatal week 2 (Fig. 5E). Although the intercalated disc in adult cardiomyocytes was strongly stained for β-catenin, talin staining was barely detectable (large arrows in Fig. 5E,G).

Vinculin and β-Catenin in Rat Cardiac Muscles

Since vinculin is a major component of the costamere [Pardo et al., 1983a] and is suggested to be a component of the cadherin/catenin complex [Knudsen et al., 1998], the relationship between vinculin and β-catenin in the costamere was further investigated by double-labeling immunofluorescence microscopy of rat cardiomyocytes. Vinculin and β-catenin were co-localized at costameres at E15 (Fig. 6A,B), E19 (Fig. 6C,D), and at postnatal week 2 (Fig. 6E,F). However, costameric staining for vinculin without codistribution of β-catenin was also detected in E19 (Fig. 6C,D) and postnatal week 2 (Fig. 6E,F) cardiomyocytes. In adult cardiomyocytes, distinct staining of costameres for vinculin (Fig. 6G) but not β-catenin (Fig. 6H), was seen, and codistribution of vinculin and β-catenin was only detected in the intercalated discs (Fig. 6G,H).

Relationship Between Laminin and the Two Different Sets of Costamere

To study the relationship between ECM proteins and the integrin/talin- and *N*-cadherin/catenin-based costameres, double-labeling was performed for laminin and either talin (Fig. 7) or *N*-cadherin (Fig. 8). Although less extensive staining was detected in embryonic than in postnatal hearts, laminin was widely

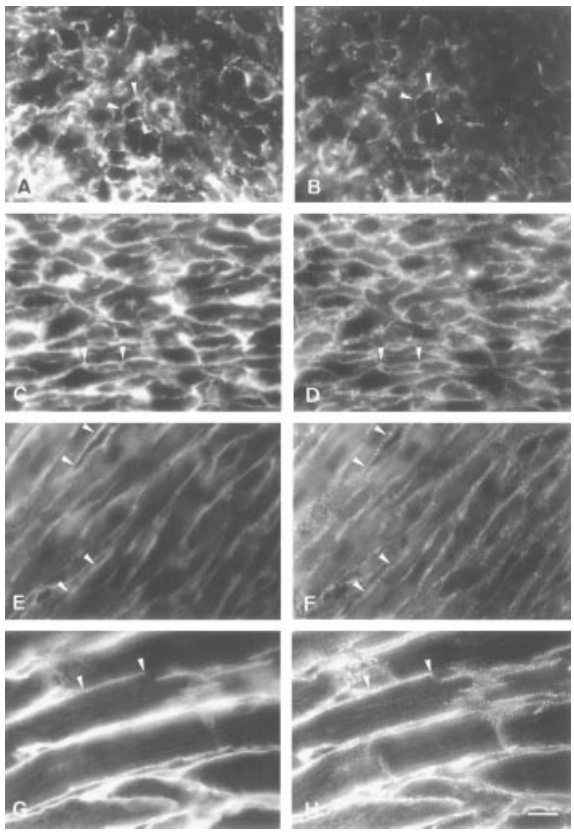

Fig. 7. Localization of laminin and talin in rat cardiac muscles. Cryostat sections from E15 (**A**, **B**), D0 (**C**, **D**), postnatal week 2 (**E**, **F**), and adult (**G**, **H**) rat cardiac muscles were double-labeled for laminin (A, C, E, G) and talin (B, D, F, H). The arrows show examples of the close association of talin and laminin staining in the costamere. Bar = 10 μm.

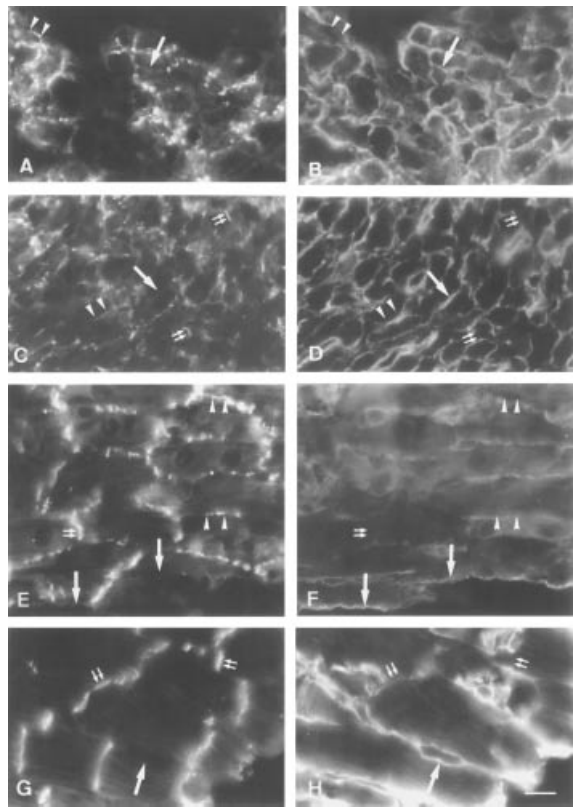

Fig. 8. Localization of *N*-cadherin and laminin in rat cardiac muscles. Cryostat sections from E15 (**A**, **B**), D0 (**C**, **D**), postnatal week 2 (**E**, **F**), and adult (**G**, **H**) rat cardiac muscles were double-labeled for *N*-cadherin (A, C, E, G) and laminin (B, D, F, H). At the boundaries of cardiomyocytes, costameric staining for *N*-cadherin (arrowheads) is not superimposed on that for laminin (large arrows). The small arrows indicate *N*-cadherin staining of the intercalated discs. Bar = 10 μm.

distributed in the extracellular areas of rat cardiac muscles (Figs. 7A,C,E,G and 8B,D,F,H). At all ages studied, the distribution of laminin was very similar to that of talin (Fig. 7), but not that of *N*-cadherin (Fig. 8). Areas in the lateral borders of cardiac muscles lacking apposed laminin were occasionally detected (Fig. 8D,F).

DISCUSSION

The non-intercalated disc distribution of *N*-cadherin in cardiac muscles was first demonstrated in the lateral sarcolemma of chicken and mammalian cardiomyocytes [Volk and Geiger, 1984, 1986; Geiger et al., 1990]. In cultured chicken cardiomyocytes, the *N*-cadherin/catenin-containing costamere is localized at the dorsal cell surface [Goncharova et al., 1992; Wu et al., 1999]. In the present study, *N*-cadherin was co-localized with costameric α-actinin at the lateral sarcolemma in rat cardiomyocytes and both α- and β-catenin co-localized with the *N*-cadherin-containing costameres. Taken together, these results suggest the existence of a set of *N*-cadherin/catenin-based costameres in rat cardiomyocytes and are consistent with the catenin/cadherin-based cell-adhesion costameres found in frog heart muscle [Kurth et al., 1996].

The expression of *N*-cadherin/catenin-based costameres in rat cardiomyocytes is developmentally regulated. A previous study of chicken hearts showed that *N*-cadherin is distributed throughout the myocyte cell membrane in embryos and is concentrated in the intercalated discs in adults [Ong et al., 1998]. In a developmental study, dispersed spots of *N*-cadherin were transiently expressed at the lateral plasma membrane of rat cardiomyocytes between postnatal days 1 and 5, and *N*-cadherin eventually concentrated in the intercalated discs in the

adult rat heart [Angst et al., 1997]. In the present study, staining for *N*-cadherin/catenin-based costameres was strong from the late embryonic stage up to postnatal week 2, decreased after postnatal week 3, and became undetectable after postnatal week 4. In adult rat cardiac muscle, the *N*-cadherin/catenin complex was only present in the intercalated discs. The absence of *N*-cadherin/catenin-based costameres in adult rat cardiomyocytes in this study is consistent with the results of a previous study showing that catenin-containing costameres are not present in adult guinea pig cardiac muscle [Kurth et al., 1996].

The presence of *N*-cadherin-containing costameres in rat cardiomyocytes is also consistent with their role in cell–cell adhesion during early stages of development [Kurth et al., 1996]. In vitro studies on cultured cardiomyocytes suggested a key role for the *N*-cadherin/catenin complex in cardiac myofibrillogenesis [Goncharova et al., 1992; Soler and Knudsen, 1994; Imanaka-Yoshida et al., 1998; Wu et al., 1999]. Perturbation of the myofibril-sarcolemma linkage by the introduction into the cardiomyocyte cytoplasm of antisera specific for *N*-cadherin, α-catenin, or β-catenin results in myofibril disassembly and fragmentation [Wu et al., 1999]. In addition to cell–cell adhesion, the *N*-cadherin-containing costameres in rat cardiac muscle cells may play a role in myofibrillogenesis similar to that of their counter-parts in cultured cardiomyocytes.

Double-labeling for β-catenin and talin indicated the coexistence of *N*-cadherin/catenin- and integrin/talin-based costameres in rat cardiomyocytes, although staining for β-catenin and talin was mutually exclusive. On going from the embryo stage to the adult, staining of the *N*-cadherin/catenin-based costamere in rat cardiac muscle decreased at postnatal week 3 and was no longer seen on the lateral plasma membrane at postnatal week 4. By contrast, staining of the integrin/talin complex gradually increased and distinct costamere staining for talin was detected at postnatal week 2. The distribution of laminin around the circumferential areas of cardiomyocytes was developmentally regulated and correlated well with the distribution of talin on the sarcolemma at all stages studied. However, areas in the lateral borders of cardiomyocytes lacking laminin staining were detected, and cardiomyocytes were not completely surrounded by laminin until after postnatal week 3, the time when costameric *N*-cadherin staining decreased. After postnatal week 3, the talin-containing costamere became the prevalent costamere in rat cardiomyocytes. As the animals grew older, ECM proteins accumulated around the cardiomyocytes and the *N*-cadherin/catenin-based costameres were gradually replaced by integrin/talin-based costameres for cell matrix adhesion.

The observed codistribution of β-catenin and vinculin also suggests that vinculin is a component of the *N*-cadherin/catenin-based costamere in cardiac muscles. However, the distribution of vinculin was more extensive than that of β-catenin, since costameres containing vinculin but not β-catenin were also detected in cardiac muscles in late embryonic (Fig. 6C,D) and early postnatal (Fig. 6E,F) stages. Since *N*-cadherin/catenin- and integrin/talin-based costameres coexist in rat cardiac muscles, it is tempting to speculate that the β-catenin-free vinculin is complexed with the talin-containing costamere. Although double-labeling for talin and vinculin was not possible in the present study because the antibodies against these two molecules were generated in the same species, talin forms a complex with vinculin in the plasma membrane [Burridge and Mangeat, 1984], and both talin and vinculin are localized in the costamere [Koteliansky and Gneushev, 1983; Pardo et al., 1983a,b; Belkin et al., 1986]. We conclude that vinculin is associated with both *N*-cadherin/catenin- and integrin/talin-based costameres in rat cardiomyocytes. This association would place vinculin in a pivotal position in anchoring myofibrils to the sarcolemma [Geiger et al., 1980; Koteliansky and Gneushev, 1983; Pardo et al., 1983a,b] via either set of costameres and is consistent with a role for vinculin in sarcomere assembly [Shiraishi et al., 1997]. In conclusion, we have demonstrated the coexistence of *N*-cadherin/catenin- and integrin/talin-based costameres in rat cardiomyocytes from late embryonic stages to postnatal week 3 and that only integrin/talin-based costameres persist in adult rat cardiac muscles.

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