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Striated muscle-specific β_{1D}-integrin and FAK are involved in cardiac myocyte hypertrophic response pathway

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Pham, Can G., Alice E. Harpf, Rebecca S. Keller, Hoa T. Vu, Shaw-Yung Shai, Joseph C. Loftus, and Robert S. Ross. Striated muscle-specific β_{1D}-integrin and FAK are involved in cardiac myocyte hypertrophic response pathway. *Am J Physiol Heart Circ Physiol* 279: H2916–H2926, 2000.—Alterations in the extracellular matrix occur during the cardiac hypertrophic process. Because integrins mediate cell-matrix adhesion and β_{1D}-integrin (β1D) is expressed exclusively in cardiac and skeletal muscle, we hypothesized that β1D and focal adhesion kinase (FAK), a proximal integrin-signaling molecule, are involved in cardiac growth. With the use of cultured ventricular myocytes and myocardial tissue, we found the following: *1*) β1D protein expression was upregulated perinatally; *2*) α_1-adrenergic stimulation of cardiac myocytes increased β1D protein levels 350% and altered its cellular distribution; *3*) adenovirally mediated overexpression of β1D stimulated cellular reorganization, increased cell size by 250%, and induced molecular markers of the hypertrophic response; and *4*) overexpression of free β1D cytoplasmic domains inhibited α_1-adrenergic cellular organization and atrial natriuretic factor (ANF) expression. Additionally, FAK was linked to the hypertrophic response as follows: *1*) coimmunoprecipitation of β1D and FAK was detected; *2*) FAK overexpression induced ANF-luciferase; *3*) rapid and sustained phosphorylation of FAK was induced by α_1-adrenergic stimulation; and *4*) blunting of the α_1-adrenergically modulated hypertrophic response was caused by FAK mutants, which alter Grb2 or Src binding, as well as by FAK-related nonkinase, a dominant interfering FAK mutant. We conclude that β1D and FAK are both components of the hypertrophic response pathway of cardiac myocytes.

neonatal rat ventricular myocytes; heart; cell signaling; extracellular matrix; focal adhesion kinase

MECHANICAL LOADING OF THE POSTNATAL HEART leads to changes in cardiac gene expression as well as hypertrophic growth of the terminally differentiated cardiac myocyte (22, 52, 67). Whereas this process is initially compensatory, its progression will eventually lead to cardiac pump failure (43). The molecular pathways that orchestrate both the compensatory growth response as well as the transition to heart failure are not fully understood. As cardiac hypertrophy develops, changes in the cardiac extracellular matrix occur and have been suggested to play an important role in this process (15, 63).

The integrins compose a large family of heterodimeric cell surface receptors that are composed of α- and β-subunits and link the extracellular matrix to the cellular cytoskeleton. Intracellular signals modify integrin affinity for ligand through a process termed "inside-out signaling." After interaction with the extracellular matrix, signals are transmitted by integrins to the cell cytoplasm through "outside-in signaling." As such, the integrins function as bidirectional cell signaling molecules. Transmission of intracellular signals after integrin ligation is dependent upon integrin cytoplasmic domains, although the molecular basis of this mechanism as well as the full complement of signaling cascades that are activated by integrins remain poorly defined. Focal adhesion kinase (FAK) has been identified as the key cytoplasmic tyrosine kinase that transmits integrin-mediated signals in several cell types (37, 56). Several signaling events have been linked to the integrins, including modulation of cell growth and cytosolic Ca^{2+}, activation of p21 Ras, mitogen-activated protein kinases (MAPKs), and induction of immediate-early genes (10). In noncardiac cells, the integrins have also been found to act as mechanotransduction molecules, converting mechanical signals to biochemical ones (11, 31).

Alternative splicing of various integrin subunits has been identified, including α-subunits 3, 6, and 7 as well as β-subunits 3 and 4. Similarly, the β-integrin subunit that is dominantly expressed in cardiac tissue, β_1, has been identified to have at least four splice variants, with the variations found in the cytoplasmic/signaling domain of the molecule (17). The most highly expressed isoform that is detected in most tissues is termed β_{1A}. β_{1B} is expressed in high amounts only in the skin and liver, whereas the β_{1C}-isoform appears to be expressed ubiquitously but at low levels. The most recently identified integrin splice variant, termed β_{1D} (β1D), is ex-

pressed exclusively in the skeletal muscle and heart (66, 70).

Little is known about the function of integrins in the heart. We and others (48, 51, 63) have recently begun to characterize the role of integrins in cardiac hypertrophy. Our previous work linked β_1-integrins to the adrenergically induced hypertrophic response of cultured neonatal ventricular myocytes. We showed that overexpression of the ubiquitously expressed β_{1A}-integrin markedly augmented the phenylephrine (PE)-induced hypertrophic response and that disruption of normal integrin signaling caused downregulation of the stimulated atrial natriuretic factor (ANF) response before alteration of cellular morphology. These findings indicated that integrin adhesion and signaling play a role in the cardiac hypertrophic response pathway.

Because $\beta1D$ has restricted expression to striated muscle and little is known about its role in the cardiomyocyte, we studied its expression, function, and signaling in the cardiac cell. For these experiments, we utilized a well-characterized cell culture model of neonatal rat ventricular myocytes (NRVM) as well as cultured embryonic cardiac cells. $\beta1D$ became upregulated in the late fetal period and was highly expressed postnatally. Forced overexpression of $\beta1D$ in the fetal cardiomyocyte, which normally expressed little $\beta1D$, did not alter DNA synthesis. Adrenergically mediated hypertrophy of neonatal cardiac cells caused induction and cellular redistribution of $\beta1D$. Overexpression of $\beta1D$ stimulated hypertrophic marker gene expression and cellular reorganization and increased cell size. Expression of free cytoplasmic domains of $\beta1D$, which is known to inhibit integrin signaling, prevented adrenergically mediated cell organization and ANF expression. FAK was also examined and found to be a component of the hypertrophic signaling pathway. This was evidenced by coimmunoprecipitation of $\beta1D$ and FAK, hypertrophic marker gene induction by FAK overexpression, rapid and sustained phosphorylation of FAK by PE, and blunting of the adrenergically modulated hypertrophic response by FAK mutants. These results suggest that $\beta1D$ and FAK play roles in the hypertrophic growth of the cardiac muscle cell.

EXPERIMENTAL PROCEDURES

Cell cultures. NRVM from ventricles of 1- to 2-day-old Sprague-Dawley rats were cultured as previously described (51). Cell cultures with >95% myocytes, as assessed by immunofluorescence with myosin light chain-2 ventricular antisera, were obtained by discontinuous Percoll gradient purification. Myocytes were plated on various substrates as indicated at a density of 300 cells/mm^2. Plates were coated at least overnight with substrates at 4°C before plating. After isolation of the NRVM, we plated the cells and either maintained cells in the native state, transfected with plasmids (using previously described techniques; see Ref. 51), or infected cells with various recombinant adenoviral constructs as noted. Subsequent to the various procedures, cells were cultured in serum-free medium containing antibiotics (34 μg/ml ampicillin and 3 μg/ml gentamicin) and L-glutamine (2 mM) or antibiotics and glutamine plus 100 μM PE. Fetal

myocytes were isolated via similar procedures from embryos obtained from timed-pregnant female animals.

Transformed 293 human embryonic kidney cells CRL-1573 [American Type Culture Collection (ATCC), Manassas, VA] were cultured as advised by the supplier.

cDNAs and antibodies. Full-length wild-type and mutant FAK cDNAs were obtained from D. Schlaepfer (Scripps Research Institute, La Jolla, CA) (57). A 3,003-bp rat ANF promoter fused to a firefly luciferase cDNA reporter gene construct has been previously described (32). A −394 to +24 bp skeletal α-actin luciferase transgene, as previously described, was kindly supplied by Dr. R. MacLellan (46). The anti-human β_1-integrin monoclonal antibodies P5D2 and 102DF5 were obtained from the Developmental Studies Hybridoma Bank (University of Iowa, Iowa City, IA) and I. Virtanen (University of Helsinki, Helsinki, Finland), respectively. The anti-myosin monoclonal antibody MF-20 was also from the Developmental Studies Bank. Rabbit polyclonal rat antiatrial natriuretic peptide and sheep anti-5-bromo-2′-deoxyuridine (BrdU) were obtained from Research and Diagnostic Antibodies (Berkeley, CA). Monoclonal antibody 7G7/B6, used to detect the interleukin-2 receptor extracellular domain (TAC), was from the American Type Culture Collection. Rhodamine-conjugated phalloidin was obtained from Molecular Probes (Eugene, OR). FITC and rhodamine-labeled secondary antibodies were from Jackson Immuno-Research Labs (West Grove, PA). Anti-FAK rabbit polyclonal antibody and anti-phosphotyrosine mouse monoclonal (clone 4G10) were obtained from Upstate Biotechnology (Lake Placid, NY).

Preparation of anti-$\beta1D$ antibody. A 17-mer peptide sequence (CPINNFKNPNYGRKAGL), corresponding to the terminal 16 amino acids of β_{1D}-integrin and an NH$_2$-terminal cysteine to facilitate coupling to keyhole limpet hemocyanin, was synthesized. The 16 amino acids of $\beta1D$ represent a segment that is highly dissimilar to the COOH-terminus of β_{1A}-integrin. New Zealand White rabbits were immunized via subcutaneous injection with the carrier-hapten conjugate in Freund's complete adjuvant. This was followed with additional carrier-hapten conjugate injections in Freund's incomplete adjuvant at the recommended intervals. Test bleeds were utilized for analysis compared with serum obtained before the initial immunization.

Recombinant adenoviral expression constructs. Production of the full-length β_{1A}-integrin and β-galactosidase (lacZ) adenoviruses were as previously published (51). For production of the $\beta1D$ recombinant virus, the full-length $\beta1D$ cDNA fragment was cloned into the *Bam*HI site of the E1-deficient shuttle vector pacCMVpLpA (23). The TAC-$\beta1D$ adenovirus was produced in a similar manner. On the basis of known sequences (Genbank Accession U28252), the cytoplasmic domain of $\beta1D$ was amplified utilizing PCR techniques. This fragment was cloned in place of the β_{1A}-integrin cytoplasmic domain in the TAC-β_{1A}-integrin expression vector described previously (51). The TAC-$\beta1D$ chimeric construct was then excised with *Sna*B I and *Xba* I and ligated into pacCMV-pLpA. FAK-related nonkinase (FRNK) was PCR amplified from cDNA prepared from WI-38 human lung fibroblasts (ATCC CCL-75), cloned into pcDNA3 as an *Eco*R I/*Xba* I fragment, and then subcloned into the adenoviral shuttle vector pShuttle-CMV to prepare recombinant adenovirus utilizing the Ad-Easy system (25). In all cases, construct integrity was confirmed by restriction enzyme and sequencing analyses. Constructs in pacCMVpLpA vectors were cotransfected using the standard calcium-phosphate technique with the adenoviral plasmid JM17 into the E1-transformed cell line 293 (24). All viruses were clonally isolated. Recombina-

tion was verified by PCR analysis utilizing oligonucleotide primer sets present in the adenoviral sequences, the foreign gene of interest, or both. Viral production of recombinant protein was assayed by infection of Chinese hamster ovary (CHO) or NRVM cells for 48 h followed by immunostaining or flow cytometry. All viral stocks were titered using plaque assays. Cells were infected at matched multiplicities of infection.

Immunofluorescent studies. Cellular immunostaining was performed as described previously (51). Microscopic analysis was performed using a Nikon Diaphot microscope equipped with epifluorescent optics.

Measurement of protein content, luciferase activity, cell size, and ANF production. Protein content was determined using a modified Lowry assay (44) (Bio-Rad, Hercules, CA). Luciferase activity was determined from cell lysates via previously published techniques (51). Cell size was determined by microscopic digital acquisition of random fields of fixed cells followed by planimetry using SigmaScan software. ANF reactivity in culture medium was assayed using a competitive enzyme immunoassay kit as directed by the supplier (Peninsula Labs, San Carlos, CA).

Western blot and immunoprecipitation assays. Myocytes were washed twice with ice-cold PBS and lysed with modified radioimmunoprecipitation assay (RIPA) buffer [10 mM Tris (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 1 mM sodium meta-vanadate, 10 mM pyrophosphate, 1% sodium deoxycholate with 10 µg/ml aprotonin, 10 µg/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride]. Rat cardiac tissue was also homogenized with modified RIPA buffer. Both myocyte lysate and tissue homogenate were centrifuged (at 100,000 *g*) to remove insoluble debris. Protein concentration was determined, and equal amounts of total protein for immunoprecipitation was precleared with protein A-agarose (Roche Molecular Biochemicals, Indianapolis, IN) for 3 h at 4°C. The protein A-agarose was removed by centrifugation. Supernatant was transferred and then allowed to incubate with antibody overnight. The antigen-antibody immunocomplex was precipitated with protein A-agarose for at least 3 h at 4°C, collected by centrifugation, and then washed three times with RIPA buffer. The final immunoprecipitate was resuspended with Laemmli sample buffer.

Protein was resolved by SDS-PAGE. Semidry immunoblotting transfer was performed onto polyvinylidene fluoride Immobilon-P membranes (Millipore, Bedford, MA). Blots were subjected to a 1-h blocking step with blocking buffer (3% nonfat milk in 0.1% Tween 20-PBS). Primary antibody incubation was performed overnight. Blots were washed with 0.1% Tween 20-PBS for 30 min, and four additional washes of 5 min each were then perfomed. Blots were blocked with blocking buffer for 30 min before a 1.5-h incubation with horseradish peroxidase-conjugated secondary antibodies (Jackson ImmunoResearch Labs). Washes of blots were performed as above. Enhanced chemiluminescence (ECL), by Amersham Pharmacia Biotech (Arlington Heights, IL), was employed to detect bound secondary antibodies. When required, blots were stripped of primary and secondary antibodies and reprobed to detect a second protein species. Densitometric quantitation of protein bands was performed digitally with Alphaease software (Alpha Innotech, San Leandro, CA).

Monitoring of DNA synthesis. Assessment of cellular DNA synthesis was performed as described previously with minor modification (41). BrdU at a final concentration of 10 µmol/l was added to the control or infected cultures for the final 16 h of the culture period. Immunofluorescent staining was performed as above, using 4,6′-diamidino-2-phenylindole (DAPI) to locate all cell nuclei, anti-myosin antibody MF20 to localize myocytes, and anti-BrdU to evaluate for BrdU incorporation into the cells. BrdU-positive myocytes were scored by visually determining the number of BrdU-positive cells that also stained positively with the anti-myosin antibody. Scoring of control, control-infected, and integrin-infected groups was then compared.

RESULTS

β1D is expressed minimally during early fetal development and becomes dominantly expressed postnatally in cardiac cells. As a component of our study, we developed an anti-β1D isoform-specific polyclonal antibody. A synthetic peptide from the β1D cytoplasmic domain that distinguishes it from the other known β₁-integrin isoforms was used for preparation of polyclonal antisera in two rabbits. ELISA analysis showed high-titer reactions of both antisera against the β1D 17-mer peptide compared with preimmune sera (data not shown). Replication-defective recombinant adenoviruses were constructed that expressed the full-length β₁A-integrin or β1D isoforms. CHO cells were maintained in their native state or infected with the β₁A-integrin or β1D adenoviruses. These cells do not usually produce any β1D. Cell lysates from these specimens as well as NRVM and mouse tissue samples (from the lung and heart) were evaluated by Western blot analyses. Specificity of the antisera for β1D was confirmed as shown in Fig. 1*A*. The antibody detected the precursor and mature forms of β1D, as has been noted previously for other β₁-isoforms (26). Specificity of the antibody was confirmed because signals were only detected in CHO cells that had been infected with a β1D adenovirus as well as cardiac muscle cells or tissue but not β₁A-infected CHO cells or nonmuscle tissue. No signal was detected by preimmune control sera (data not shown).

With the use of this antibody, we evaluated the developmental expression pattern of β1D protein. Little β1D protein was expressed in the heart during fetal growth, but protein levels were significantly increased postnatally (Fig. 1*B*) Prenatal expression of β1D was generally <20% of the protein expression level in the adult ventricle. These results suggested that β1D could play a role in cell cycle arrest of the terminally differentiated myocyte. We cultured fetal myocytes at E 15.0 and infected them with recombinant adenoviruses expressing matched titers of either β1D or control (lacZ) transgenes. Despite increased β1D protein levels, no difference in the rate of DNA synthesis was found between β1D and control-infected cells (data not shown).

PE stimulation of NRVM causes induction and subcellular redistribution of striated muscle-specific β1D. Many proteins, including extracellular matrix components and integrins, are upregulated during the hypertrophic growth process. The β1D isoform is exclusively expressed in skeletal and cardiac muscle (66). Therefore, we next sought to evaluate the function of this integrin isoform in hypertrophic growth of the cardiac cell. We used the β1D-specific antibody to evaluate the
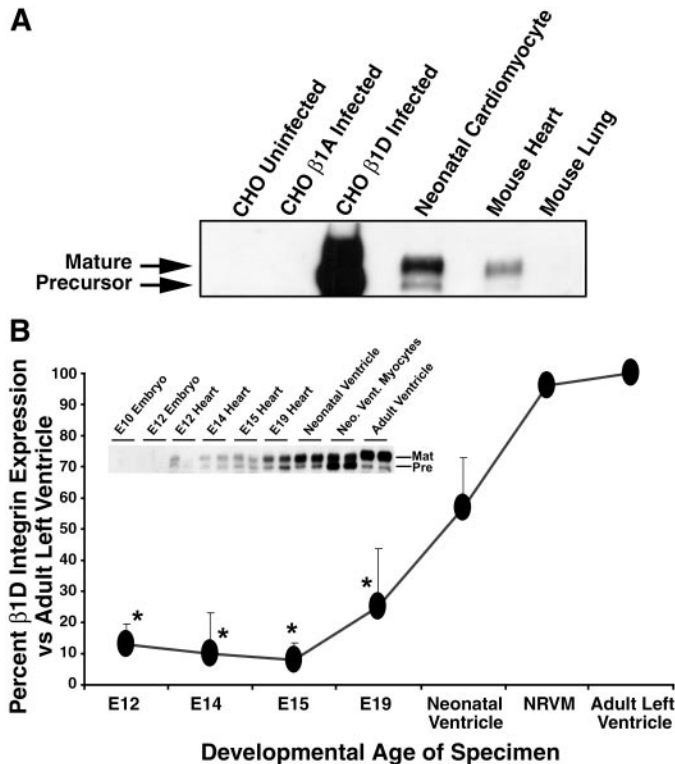
Fig. 1. β_{1D}-Integrin (β1D) protein is dominantly expressed in the late fetal and postnatal myocyte. *A*: polyclonal antibody specifically detects the β1D isoform. Chinese hamster ovary (CHO) cells are maintained uninfected or infected with recombinant adenoviruses that express either the β_{1A}-integrin or β1D isoform. Forty-eight hours after infection, we harvested the cells, and protein lysates were prepared. Lysates were also prepared from neonatal rat ventricular myocytes (NRVM), mouse heart, or mouse lung. Cellular or tissue lysates were utilized for SDS-PAGE and Western blotting procedures. Mature and precursor forms of the β1D protein were only detected in β1D-infected cells, cultured cardiac cells, or heart tissue. *B*: developmental expression of β1D protein. Whole rat embryos [embryonic (E) *days 10* and *12*], heart tissue, ventricular cells, and ventricular tissue samples were obtained and used for Western blot analysis with the polyclonal β1D antibody described above. Expression levels of total [both mature (Mat) and precursor (Pre) forms] of β1D protein of each specimen were determined and normalized to the total amount present in the adult ventricular sample, which was set to 100%. Because of the small standard error, error bars are not visible in the last two data points of the graph. *Inset*: representative Western blot. *$P < 0.05$ vs. protein levels in adult left ventricle.

lar apparatus. With the use of the β1D antibody, we assessed the localization of β1D in cells maintained in serum-free medium compared with cells stimulated with PE (Fig. 3). In the PE-treated cells, β1D was seen to shift its location over time, from punctate cytoplasmic staining to one colocalized with actin in the organizing myofibrils, most intensely at the Z line. No similar colocalization was seen in the cells cultured in serum-free medium.

Overexpression of β1D causes cellular organization, increases in endogenous ANF, induction of hypertrophic reporter genes, and increases in cell size. Because adrenergic stimulation alters β1D protein levels and subcellular localization, we examined whether overexpression of β1D would independently alter myocyte organization or expression of hypertrophic marker gene expression. NRVM were infected with matched titers of recombinant adenoviruses that express either human β1D or control (lacZ) transgenes. Dual immunostaining was used to evaluate F-actin and β1D localization. As shown in Fig. 4*A*, forced expression of β1D in myocytes cultured in the absence of serum caused increased cellular organization similar to that caused by adrenergic stimulation. These findings were distinct from cells infected with control virus in matched titer as well as uninfected cells.

To establish if β1D could augment hypertrophic marker gene expression, ventricular myocytes were transfected with either α-skeletal actin-luciferase or ANF-luciferase and, 16 h after transfection, infected with control or β1D recombinant adenoviruses (Fig. 4*B*). Increased β1D expression caused statistically significant induction of both reporter genes. Infection of myocytes with the control virus did not induce reporter
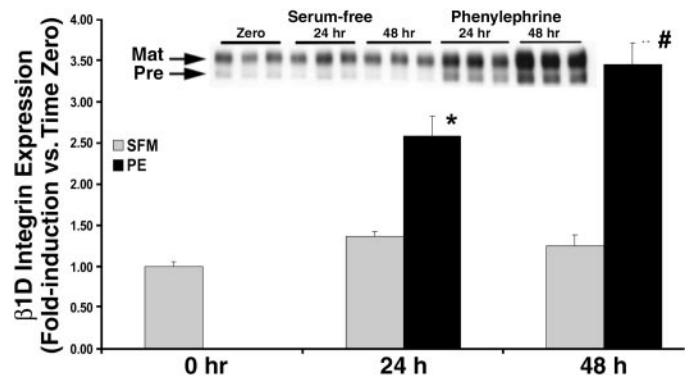
expression level of β1D in NRVM plated on collagen I and stimulated with the hypertrophic agonist PE. As shown in Fig. 2, β1D protein levels were increased by 100 μM PE treatment compared with cells maintained in serum-free medium. Whereas modest increases of the mature form of β1D were noted as soon as 15 min after agonist exposure (data not shown), substantial increases of both the precursor and mature forms were found when PE stimulation continued for 24–48 h, in accord with the hypertrophic response. Upregulation of the β1D protein was confirmed by Western blot analyses with loading of varied protein amounts from multiple independent experiments (data not shown).

PE stimulation of cultured NRVM orchestrates cell spreading and increases organization of the myofibril-

Fig. 2. Phenylephrine (PE) stimulation causes an increase in β1D protein. NRVM were cultured onto collagen I-coated plates and maintained in serum-free medium (SFM) for 24 h. After this, we harvested and lysed the cells (*time zero*) or stimulated cells with 100 μM PE for the defined time periods or maintained in cells serum-free conditions and then harvested. SDS-PAGE and Western blot analysis was performed with each of the samples using the β1D-specific polyclonal antibody described above. Densitometric analyses of protein blots from several independent experiments with replicate samples determined β1D expression level. Data are displayed as fold increase of β_1-integrin expression compared with the *time zero* samples. *Inset*: representative Western blot analysis. *$P < 0.001$ vs. 0 h SFM and #$P < 0.001$ vs. 24 h PE.

Phenylephrine

Fig. 3. PE stimulation causes redistribution of β1D in the cardiac myocyte. Neonatal rat ventricular myocytes were cultured onto collagen I-coated plates and maintained in SFM for 24 h. Cells were then fixed immediately (*time zero*; *A* and *B*) or stimulated with 100 μM PE for the defined time periods (2 h, *C* and *D*; 6 h, *E* and *F*; and 24 h, *G* and *H*) or maintained in serum-free conditions (2 h, *I* and *J*; 4 h, *K* and *L*; and 24 h, *M* and *N*) and then harvested. Dual immunostaining was performed with a rabbit polyclonal β1D-specific antibody and phalloidin to detect F-actin (*A*, *C*, *E*, *G*, *J*, *L*, and *N*) and β1D (*B*, *D*, *F*, *H*, *I*, *K*, and *M*), respectively.
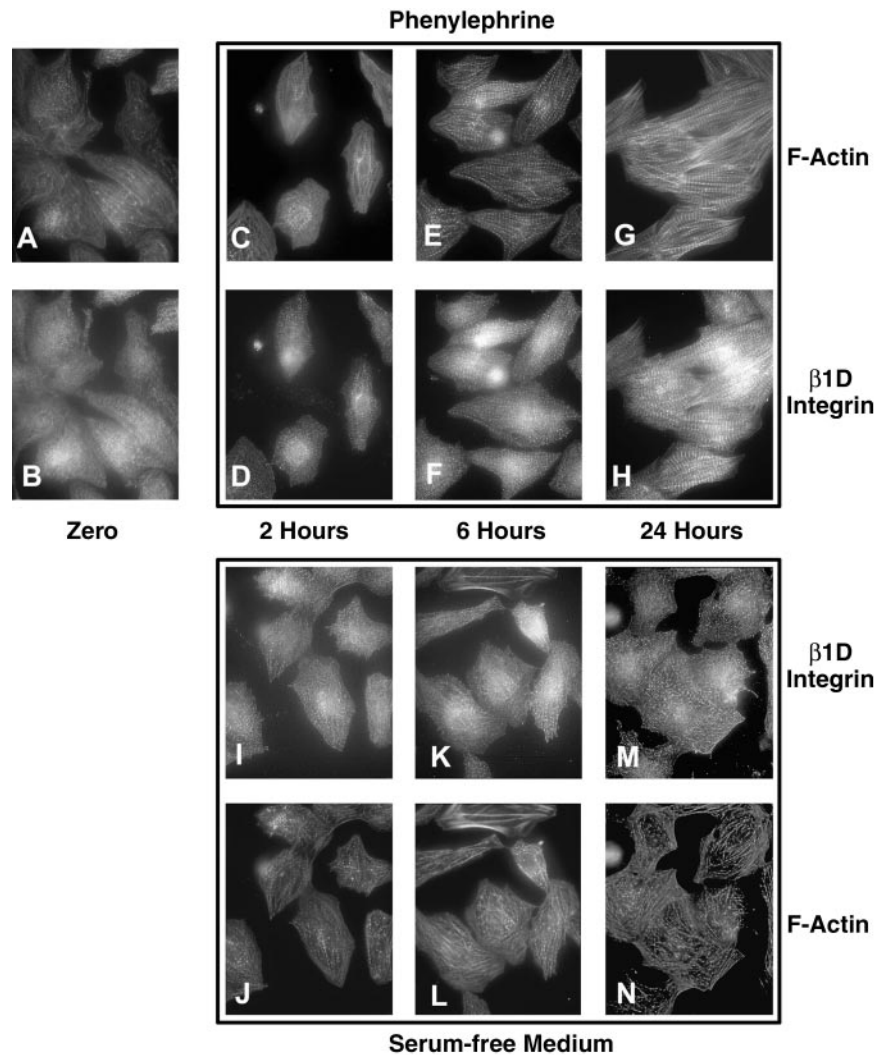
gene activity. Similarly, we evaluated the effect of β1D on endogenous ANF expression and cell size of NRVM infected with control or β1D recombinant adenoviruses. As shown in Fig. 4*C*, infection with the integrin virus resulted in induction of endogenous ANF, as detected by the perinuclear-staining pattern. Quantitative analysis of ANF-positive cells in each random high-power microscopic field detected an average of 8 ± 0.03-fold higher ANF-expressing cells in the β1D-infected groups compared with the control lacZ-infected cells infected at matched titers ($P < 0.01$). Similarly, we found that β1D overexpression increased cell size. β1D-infected cells were 1,573 ± 116 versus 620 ± 54 μm^2 ($P < 0.0001$) in the cells infected with matched titers of the control lacZ virus.

Expression of free cytoplasmic domains of β1D prevents adrenergically mediated NRVM organization or expression of ANF. We and others (2, 9, 45, 51) have utilized chimeric constructs, which express free β₁-integrin cytoplasmic domains to disrupt integrin signaling in myocytes as well as other cell types. To disrupt integrin signaling in NRVM, we constructed a recombinant adenovirus encoding a chimeric pro-

tein, which consisted of the extracellular and transmembrane domain of the TAC subunit of the interleukin-2 receptor fused to the β1D cytoplasmic domain (TAC-β1D). As shown in Fig. 5, this mutant reduced both PE-mediated NRVM cellular organization and ANF production, confirming the role of β1D in these events.

FAK is involved in hypertrophic response of neonatal ventricular myocytes. Integrins do not possess intrinsic tyrosine kinase activity. In noncardiac cells, integrin ligation has been demonstrated to activate the cytoplasmic tyrosine kinase FAK as well as the MAPK pathway (40). Previous studies (14, 62) have demonstrated that MAPK pathway components independent of FAK are involved in hypertrophic gene responses in cardiac cells, but little data is available about the function of FAK itself. We tested the hypothesis that FAK is also a component of the adrenergically mediated hypertrophic response pathway in neonatal ventricular myocytes.

Overexpression of FAK in myocytes cultured in serum-free medium caused upregulation of ANF luciferase activity (Fig. 6*A*), suggesting that this tyrosine
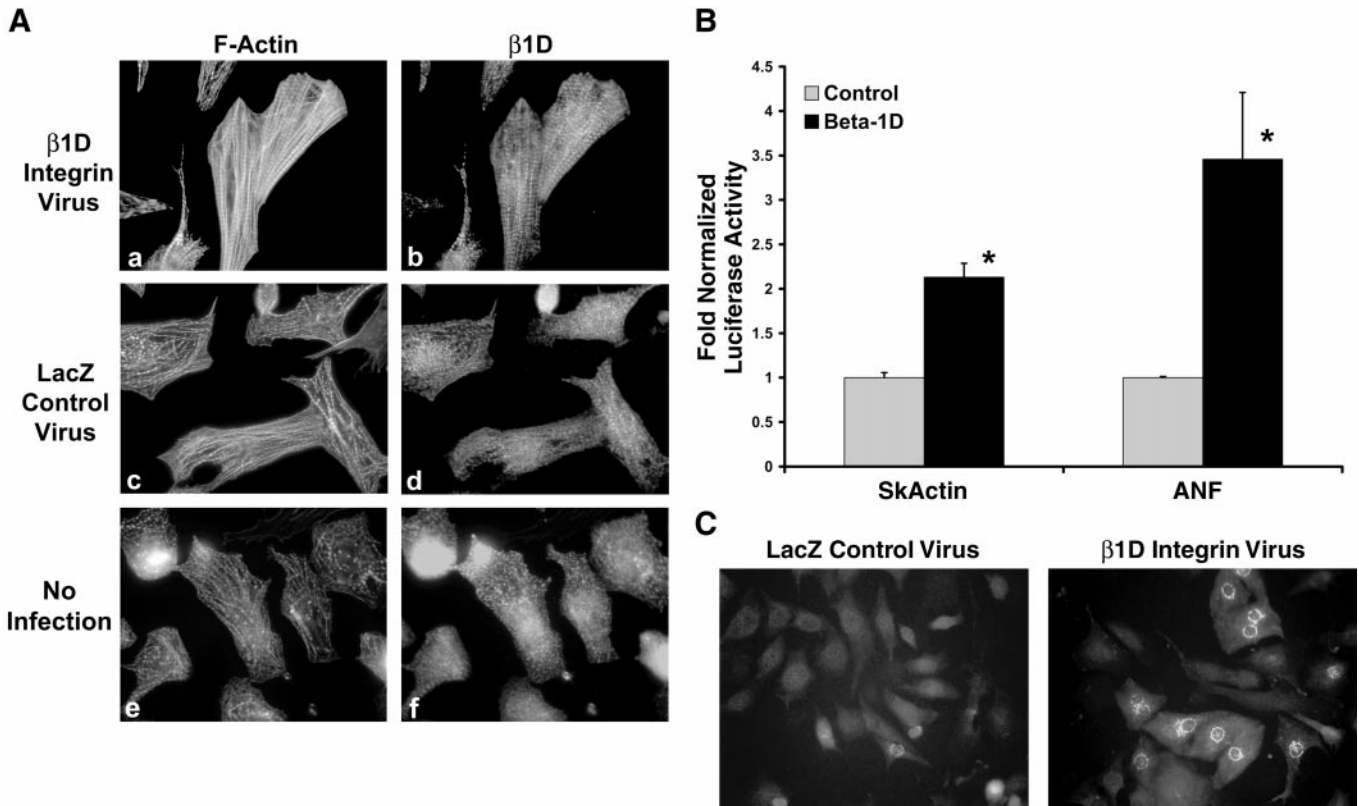
Fig. 4. β1D overexpression causes increased cellular organization, increased hypertrophic marker gene expression, and upregulation of endogenous atrial natriuretic factor (ANF) in cardiac myocytes. Cardiac myocytes were cultured onto collagen I-coated coverslips or plates. *A*: cells cultured in SFM were either infected with recombinant adenoviruses expressing β1D or β-galactosidase (lacZ) control protein or maintained in the uninfected state. After 36 h of infection, we fixed and stained the cells to localize F-actin (*a*, *c*, and *e*) and β1D (*b*, *d*, and *f*). *B*: cardiac myocytes were transfected with either ANF- (3,003 bp) or α-skeletal actin (420 bp; SkActin)-luciferase. Sixteen hours after transfection, we washed and maintained the cells in SFM or infected cells with matched amounts of either control or β1D-expressing adenoviruses. Cells were lysed and used for luciferase and protein assays. Results are displayed as fold induction compared with control infections of matched titers. *$P \leq 0.005$ vs. control. *C*: cardiac myocytes were infected with matched titers of β1D or control lacZ adenoviruses for 48 h. They were then fixed, and endogenous ANF was localized utilizing immunohistochemical techniques with a rabbit anti-rat polyclonal ANF antibody.

kinase was a component of the hypertrophic response pathway. We next evaluated the role of FAK in adrenergically mediated events in the cardiac cell. PE stimulation resulted in a rapid and sustained increase in FAK phosphorylation beginning by 15 min after PE induction and continuing for the 48-h duration of the stimulation (Fig. 6*B*). Direct interaction of β1D and FAK was detected through their coimmunoprecipitation, but viral-mediated β1D overexpression caused no significant increase in FAK phosphorylation when assessed from 24 to 48 h after viral infection (data not shown).

We next used two types of mutant FAK molecules to further elucidate its role in the adrenergically mediated hypertrophic response. Tyr-397 is the major site of FAK autophosphorylation and generates a binding site for Src family kinases and the formation of Src/FAK signaling complexes. Mutation of Tyr-925 (FAK-F925) disrupts Grb2 binding to FAK, which can effect FAK-induced activation of MAPK. Cells were transfected with ANF-luciferase as well as wild-type FAK or FAK mutants and then either maintained in serum-free medium or stimu-

lated with PE. Induction of ANF-luciferase in PE versus the serum-free culture was determined for each transfection condition. The ratio of PE to serum-free activity of ANF-luciferase when cotransfection was performed with a control (empty) backbone vector was set equal to 100%. As shown in Fig. 6*C*, overexpression of wild-type FAK did not augment the PE-stimulated induction of ANF-luciferase activity, suggesting that FAK was not a limiting factor in the context of this inductive pathway. In contrast, both mutant FAK molecules caused downregulation of the PE induction of ANF, suggesting that normal FAK signaling is necessary for the adrenergically mediated hypertrophic response. Furthermore, we utilized a well-characterized FAK inhibitor, FRNK, to disrupt FAK signaling (55, 59). As shown in Fig. 6*D*, expression of FRNK via a recombinant adenovirus caused a dose-responsive decrease in PE-mediated ANF expression by NRVM, in agreement with the inhibition orchestrated by the plasmid mutants discussed above. No similar decrease was detected in cells infected with equal titers of control viruses.
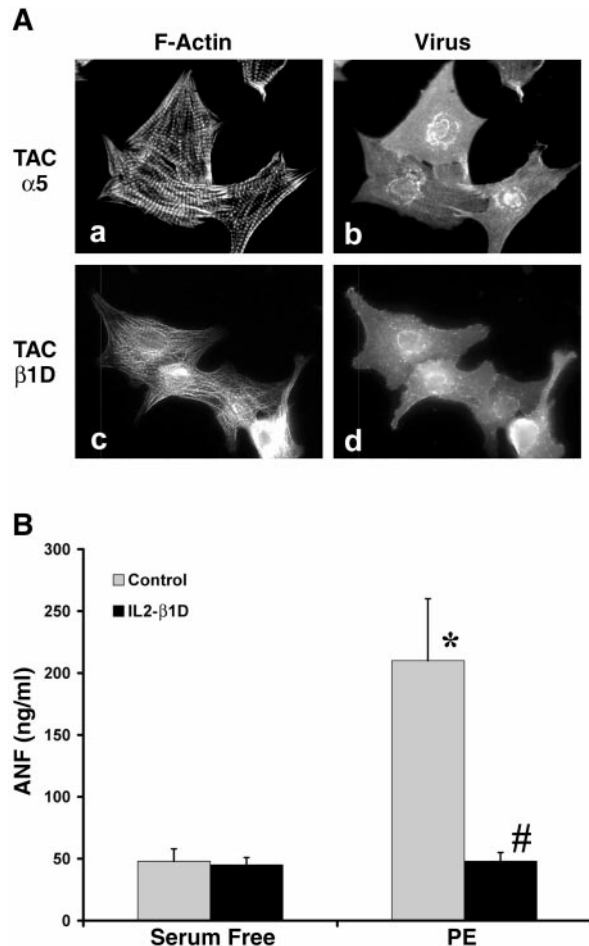
Fig. 5. Disruption of integrin signaling prevents adrenergically mediated NRVM organization and ANF production. *A*: NRVM were cultured and stimulated with 10^{-4} M PE. At the time of addition of PE, they were infected with matched titers of either the TAC-α_5 (control) virus (*a* and *b*) or the TAC-β1D virus (*c* and *d*). Cells were maintained for an additional 36 h, fixed, and stained for F-actin (*a* and *c*) or for expression of the virus (*b* and *d*) with an interleukin-2 specific antibody, which shows no signal on uninfected cells. *B*: cells were cultured and infected as described above, and ANF production was assessed via competitive ELISA. *$P \leq 0.001$ vs. SFM and #$P \leq 0.001$ vs. control PE.

DISCUSSION

In this study, we demonstrated that β1D, a splice variant that is specifically expressed in cardiac and skeletal muscle, participates in the hypertrophic response of NRVM. With the use of a β1D isoform-specific antibody, we found little β1D protein expression during early embryonic development, with significant upregulation near birth. Ectopic expression of β1D in embryonic rat ventricular myocytes did not alter the rate of fetal DNA synthesis compared with control viral infection. Adrenergic stimulation of neonatal myocytes caused increased levels of β1D protein and redirected its subcellular distribution. Overexpression of β1D via recombinant adenovirus *1*) increased ANF- and α-skeletal actin-luciferase activity, markers of the cardiac hypertrophic response; and *2*) promoted increased myocyte organization. Overexpression of free

β1D cytoplasmic domains altered PE-stimulated NRVM organization and ANF production. Results showed that FAK, which is an important mediator of integrin signaling in numerous cell types, was also involved in the hypertrophic response of NRVM because *1*) overexpression of wild-type FAK increased ANF-luciferase transgene activity, *2*) α-adrenergic stimulation induced rapid and sustained phosphorylation of FAK, *3*) FAK coimmunoprecipitated with β1D, and *4*) FAK mutants disrupted normal α-adrenergic induction of ANF.

Integrin-mediated cell adhesion to the extracellular matrix and integrin signaling are critical for cell survival, proliferation, migration, and differentiation (1, 47). As shown most dramatically by gene deletion experiments, normal integrin function has been found to be essential for these processes in cardiac cells and the intact heart (16, 20). Previous studies by our group and others (27, 48, 51) have shown that cardiac myocyte morphology and hypertrophic induction is influenced by attachment to extracellular matrices such as collagen, fibronectin, and laminin (27, 48, 51). Because cell matrix adhesion occurs via integrins, the integrin receptors expressed on cardiac myocytes are likely to be an important component of this response.

Our previous work (51) showed that the ubiquitously expressed β₁-integrin isoform, β₁A, was involved in the hypertrophic response of ventricular myocytes (51). The present study extends this work to specifically evaluate the role of the β₁D-isoform (66, 70). With the use of isoform-specific antibodies, we determined that little β1D expression was found in the prenatal cardiac muscle cells of the rat, in agreement with other reports in the mouse (6, 65). β1D has been found to inhibit cell cycle progression in cultured skeletal muscle cells (4). Fetal cardiac myocytes expressed little β1D, but forced expression of β1D did not alter BrdU incorporation compared with control-infected cells. In support of these results, Baudoin et al. (3) found no histologic abnormalities in "knockout" mice that did not express β1D. If β1D played a significant role in cardiac myocyte terminal differentiation, alteration in cardiac muscle mass would be anticipated in the mouse heart deficient in β1D. Because this was not found, the function of β1D in cardiac cells may be distinct from its role in skeletal muscle.

Our findings that PE stimulation increased β1D protein expression and altered its cellular distribution are consistent with the concept that this striated muscle-specific integrin may function to strengthen cytoskeletal-matrix interaction in the beating muscle cell. This would be of particular importance when the cardiac cell is pharmacologically stimulated or mechanically stressed during the process of hypertrophic induction in vitro or in vivo (61). β1D has been shown to bind more tightly to talin than the ubiquitously expressed β₁A-integrin (5, 50). Therefore, as the cardiac cell responds to stimuli that evoke hypertrophic responses in vitro or hemodynamic loading in vivo, β1D might provide for a more stable cytoskeletal structure through which contractile forces are transmitted. This concept
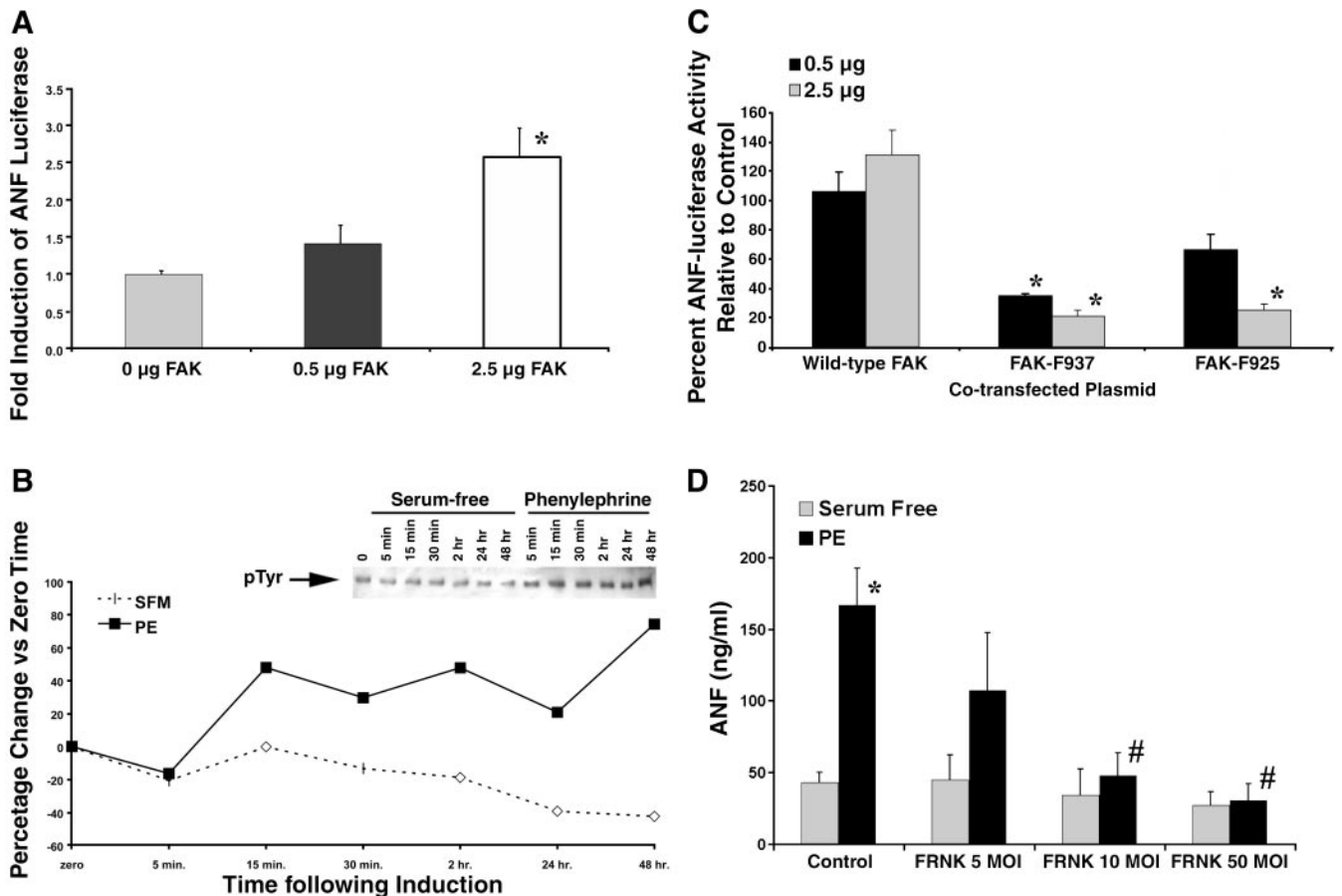
Fig. 6. Focal adhesion kinase (FAK) is involved in the neonatal ventricular cell hypertrophic response. *A*: wild-type FAK can independently increase ANF-luciferase expression in neonatal ventricular cells maintained in serum-free conditions. Cytomegalovirus-FAK and ANF (3,003 bp)-luciferase plasmids were cotransfected into neonatal ventricular myocytes, and luciferase assays were performed at 48 h after transfection. *P < 0.01 vs. 0 µg FAK. *B*: PE causes rapid and sustained increases in FAK phosphorylation. Myocytes were cultured in serum-free conditions on collagen I-coated plates and either maintained in serum-free conditions or stimulated with 100 µM PE for the defined periods. Cell lysates were then prepared, and equal amounts of protein were subjected to immunoprecipitation with an anti-FAK antibody. Western blotting was then performed to detect total and phosphorylated FAK. Data are displayed as phosphorylated FAK normalized to total FAK protein for each time point compared with zero time. *Inset*: representative Western blot of phosphorylated FAK. *C*: mutant FAK molecules can blunt PE induction of ANF-luciferase. ANF (3,003 bp)-luciferase plasmid was cotransfected with empty vector (control), wild-type FAK, or FAK molecules having mutations in both c-Src and Grb2 binding (FAK-F397) or Grb2 binding alone (FAK-F925). Cells were maintained in SFM or stimulated with 100 µM PE. Forty-eight hours after transfection, we lysed the cells for luciferase activity and protein measurements. Results are displayed as a ratio of luciferase activity of PE-stimulated cells to SFM cells, under the various transfected conditions, compared with the activity ratio in cells transfected with ANF (3,003 bp)-luciferase and control (empty) backbone plasmid. *P < 0.01 vs. control. *D*: FAK-nonrelated kinase (FRNK) can inhibit ANF production by PE-stimulated NRVM. Cells were cultured as described previously and stimulated with 10^{-4} M PE. At the time of stimulation, cells were infected with control virus at titers as indicated. Thirty-six hours after infection, we collected and utilized the medium in a competitive ELISA assay to determine ANF production by the myocytes. MOI, multiplicity of infection. *P ≤ 0.0005 vs. SFM control and #P ≤ 0.01 vs. PE control.

is supported by studies that show that talin accumulates at sites of mechanical loading in skeletal and cardiac muscle (18, 29). In preliminary studies, we have found that β1D protein levels are also upregulated in the hemodynamically loaded mouse heart (Ross et al., unpublished observations).

The integrins have been identified as mechanotransduction molecules in noncardiac cells converting mechanical signals to biochemical ones (30, 42). The integrins could thus transmit mechanical- or ligand-initiated signals from the extracellular matrix and

cause hypertrophic signaling events, as we detected in our study. Despite much investigation, no specific "stretch receptor" has been identified in the cardiac cell (33, 53, 62, 68). Integrins, and particularly β1D as the principal integrin in the postnatal cardiac myocyte, could be a component of the myocyte mechanotransduction pathway. Our results that show increased amounts of β1D protein after PE stimulation are in agreement with this concept.

We found that forced expression of β1D in the neonatal ventricular myocyte caused upregulation of ANF-

and α-skeletal actin-luciferase transgenes, endogenous ANF, increased cellular organization, and increased cell size. These results were of a less intense nature than those seen for agents such as serum, PE, or endothelin. Whereas this more modest result may be due in part to the culture conditions we utilized in our study, the role of β_1-integrins in myocyte organization and/or hypertrophy may be a cooperative one. We have previously shown (51) that β_1-integrin overexpression augments the hypertrophic induction effected by PE in NRVM, suggesting that there may be cross-talk between these pathways.

Studies (19, 21, 28, 34, 35, 54, 60, 64, 68, 69) have previously identified many molecules that are essential components of hypertrophic signaling in cardiac cells in vitro and in vivo, including extracellular signal-regulated kinase, p38, Ras, Rho, and Src. Thus alterations in the extracellular matrix could transmit both mechanical forces and through the integrin; these mechanical events could be converted to biochemical changes. Alternatively, stimuli (such as the adrenergic agents utilized in our study) could orchestrate intracellular signaling cascades that simultaneously impact upon hypertrophic signaling as well as integrin activation state and/or ligand-binding affinity, termed inside-out signaling (13). Whereas affinity state-specific antibodies that can recognize integrin heterodimers only in their activated state (9) are not available for use with rat β1D, we have consistently observed that PE-stimulated neonatal ventricular cells adhere more rapidly and spread to a greater extent on collagen-, laminin-, and fibronectin-coated plates compared with unstimulated control cells (Ross, unpublished results). This suggests that PE may well influence inside-out signaling through β1D. These results are in agreement with previous data, which found enhanced ligand binding and assembly of fibronectin in β1D-transfected nonmuscle cells (5) as well as recent data obtained with cardiac cells (48).

Whereas we noted increased expression of the ANF-luciferase transgene after overexpression of β1D, Baudoin et al. (3) found increased ANF and β-myosin heavy chain (βMHC) expression in male (though not female) mice deficient in β1D. Our results most likely relate directly to increased signaling through β1D-mediated pathways, whereas the upregulation of ANF and βMHC in the β1D knockout mice could be due to a secondary hypertrophic response in cells with "weakened" integrin-cytoskeletal interactions.

Adrenergic stimulation caused increased FAK activation, whereas transient overexpression of wild-type FAK increased hypertrophic marker gene response. FAK and β1D were coimmunoprecipitated from cardiac myocyte protein extract (data not shown), suggesting a direct association of signaling from β1D through FAK in the cardiac cells. FAK phosphorylation was modestly changed with PE stimulation, but mutant FAK expression blunted the PE-mediated hypertrophic response. Thus PE appears to at least partly signal through a FAK-mediated pathway. These results are in agreement with recent data, which showed that both

pulsatile stretch and vascular endothelial growth factor could alter FAK activation in the cardiac cell (58, 62). FAK has also been implicated in endothelin-mediated hypertrophic signaling in the cardiac cell (14). When we assayed at 48 h after β1D overexpression, we did not detect increased FAK phosphorylation. The cardiac myocytes require many hours to adhere to substrate, unlike many cell types, which do so in minutes. It is also known that changes in integrin activation state or clustering are necessary for modulation of FAK phosphorylation. It is likely that β1D causes transient activation of FAK at earlier time points, but, given our model system combined with the time necessary to express protein from recombinant adenoviral constructs, we were unable to assess these early events to directly link β1D expression levels to FAK activation. It is known that integrins can also signal through non-FAK pathways, which for the most part remain unknown (39). Several molecules that directly bind integrins, including ones specifically expressed in striated muscle, have been identified. These include melusin and muscle-specific β_1-integrin binding protein as well as integrin-linked kinase, integrin cytoplasmic domain activated protein 1, and receptor for activated protein kinase C (Rack-1) (7, 8, 12, 36, 38). It is possible that β1D could also signal through one of these newly identified integrin-binding molecules (49). Finally, it is also known that integrin signaling pathways may function uniquely in distinct cell types. Thus whereas our data fully support a role for FAK in cardiac myocyte hypertrophic signaling, additional studies are necessary and are in progress in our lab to more specifically define the pathways through which β1D signaling occurs in cardiac cells.

In summary, we have determined that the striated muscle-specific β1D and the cytoplasmic tyrosine kinase FAK are involved in postnatal hypertrophic growth responses of the cardiac myocyte. Further in vitro and in vivo experiments are necessary to determine the full biological function of these molecules in the myocardium.

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