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Focal Adhesion Kinase and p130Cas Mediate Both Sarcomeric Organization and Activation of Genes Associated with Cardiac Myocyte Hypertrophy

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Hypertrophic terminally differentiated cardiac myocytes show increased sarcomeric organization and altered gene expression. Previously, we established a role for the nonreceptor tyrosine kinase Src in signaling cardiac myocyte hypertrophy. Here we report evidence that p130Cas (Cas) and focal adhesion kinase (FAK) regulate this process. In neonatal cardiac myocytes, tyrosine phosphorylation of Cas and FAK increased upon endothelin (ET) stimulation. FAK, Cas, and paxillin were localized in sarcomeric Z-lines, suggesting that the Z-line is an important signaling locus in these cells. Cas, alone or in cooperation with Src, modulated basal and ET-stimulated atrial natriuretic peptide (ANP) gene promoter activity, a marker of cardiac hypertrophy. Expression of the C-terminal focal adhesion-targeting domain of FAK interfered with localization of endogenous FAK to Z-lines. Expression of the Cas-binding proline-rich region 1 of FAK hindered association of Cas with FAK and impaired the structural stability of sarcomeres. Collectively, these results suggest that interaction of Cas with FAK, together with their localization to Z-lines, is critical to assembly of sarcomeric units in cardiac myocytes in culture. Moreover, expression of the focal adhesion-targeting and/or the Cas-binding proline-rich regions of FAK inhibited ANP promoter activity and suppressed ET-induced ANP and brain natriuretic peptide gene expression. In summary, assembly of signaling complexes that include the focal adhesion proteins Cas, FAK, and paxillin at Z-lines in the cardiac myocyte may regulate, either directly or indirectly, both cytoskeletal organization and gene expression associated with cardiac myocyte hypertrophy.

INTRODUCTION

Cardiac hypertrophy is an adaptive response of the heart to a variety of intrinsic and extrinsic stimuli, including hypertension, myocardial infarction, and contractile abnormalities resulting from mutant sarcomeric proteins (Hunter and Chien, 1999). Because cardiac myocytes lose the ability to divide soon after birth, the increase in cardiac mass results from an increase in size of individual myocytes without an increase in cell number (i.e., hypertrophy rather than hyperplasia). Although initially beneficial, prolonged hypertrophy can become deleterious, resulting in cardiomyopathy, heart failure, and sudden death (Hunter and Chien, 1999).

At the cellular level, hypertrophy is thought to develop in response to a combination of mechanical (e.g., strain) and neurohormonal (e.g., endothelin [ET], angiotensin II [Ang II], or α-adrenergic agonist phenylephrine) stimuli. In cultured neonatal cardiac myocytes these stimuli effect a series of changes that closely resemble those seen with hypertrophy in vivo (Schaub *et al.*, 1997). These include activation of the immediate early gene family (c-*jun*, c-*fos* and *egr*), followed by up-regulation of the so-called fetal gene atrial natriuretic peptide (ANP), β-myosin heavy chain (β-MHC), and α-skeletal actin. These changes in turn are followed by enhanced expression of the sarcomeric contractile proteins (e.g., myosin light chain and cardiac actin) that leads to the assembly of highly organized myofibrils (Chien *et al.*, 1993). The development of hypertrophy in cultured myocytes is usually monitored experimentally by following reactivation of fetal gene expression (e.g., ANP) and by analyzing cell

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Abbreviations used: Ang II, angiotensin II; ANP, atrial natriuretic peptide; BNP, brain natriuretic peptide; β-MHC, β-myosin heavy chain; CAT, chloramphenicol acetyltransferase; Cas, p130Cas; ET, endothelin; FAK, focal adhesion kinase; FAT, focal adhesion targeting domain; FCS, fetal calf serum; GAPDH, glyceraldehyde phosphate dehydrogenase; GFP, green fluorescent protein; HA, hemagglutinin; MAP, mitogen-activated protein; PBS, phosphate-buffered saline; PR-1, proline rich region-1; Pfu, plaque-forming units;.

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morphology for increased cell size and increased sarcomeric density and organization. From a biological standpoint, the accumulation of sarcomeres is perhaps the most important feature of hypertrophy because it addresses the myocyte's need to increase contractile force. More than 50 different proteins, ordered in near crystalline array, are found in the sarcomere (Vigoreaux, 1994). Z-line–associated structures are responsible for alignment of myofibrils and lateral anchorage at *N*-cadherin- and vinculin-containing costamers (Simpson *et al.*, 1993). The formation of perfectly aligned myofibrils, a prerequisite for productive contraction in cardiac muscle, represents an extreme example of supramolecular assembly in eukaryotic cells. Although the molecular mechanisms underlying immediate early gene induction and reactivation of fetal genes have been studied extensively (Kovačič-Milivojević and Gardner, 1992; Chien *et al.*, 1993; Sadoshima and Izumo, 1997), the mechanisms underlying increased myofibrillogenesis are understood only to a limited degree. Furthermore, little is known about how these two processes (gene transcription and sarcomeric assembly) are coordinated in the cardiac myocyte. For example, are there common receptor-proximal control points that synchronize the changes in gene expression with the changes in organization of sarcomeric structure that characterize hypertrophy?

ET is one of the most potent hypertrophic stimuli in the neonatal myocyte system (Shubeita *et al.*, 1990; Chien *et al.*, 1991). ET-1 binds to a specific heterotrimeric G protein-coupled receptor that is linked to a number of well-defined intracellular signaling pathways (Chien *et al.*, 1993; Bogoyevitch *et al.*, 1994). ET has been shown to stimulate tyrosine kinase activity in several systems (Zachary *et al.*, 1993; Cazaubon *et al.*, 1997) and, more recently, has been linked directly to activation of c-Src in neonatal ventricular myocytes (Fuller *et al.*, 1998; Kovačič *et al.*, 1998). ET is closely tied to activation of ANP gene expression (Kovačič *et al.*, 1998) and, by inference, to the development of myocyte hypertrophy in this system. These effects appear to be Src dependent. ET also promotes phosphorylation of Src substrates, including the adaptor protein p130Cas (Cas), in Swiss 3T3 cells (Casamassima and Rozengurt, 1997), and focal adhesion kinase (FAK) in cardiac myocytes (Eble *et al.*, 2000), mesangial cells (Haneda *et al.*, 1995), and primary astrocytes (Cazaubon *et al.*, 1997).

Both Cas and FAK are localized at sites of integrin-receptor clustering. The localization of FAK is dependent on its C-terminal focal adhesion-targeting domain (FAT; Hildebrand *et al.*, 1993). The FAT region of FAK interacts with integrin-associated proteins such as paxillin (Bellis *et al.*, 1995; Schaller and Parsons, 1995) and talin (Chen *et al.*, 1995). After integrin receptor ligation, or stimulation by growth factors or G protein-linked receptors, FAK undergoes (auto)phosphorylation at Tyr397, creating a binding site for Src-family kinases and other proteins, as described in the review by Schlaepfer *et al.* (1999). The proline-rich region 1 (PR-1) of FAK (residues 712–718) provides a binding site for the SH3 domain of Cas (Harte *et al.*, 1996; Polte and Hanks, 1997). This interaction leads to localization of Cas in focal contacts and phosphorylation by either FAK or Src (Nakamoto *et al.*, 1997; Polte and Hanks, 1997; Nakamura *et al.*, 1998). Whereas the phosphorylated protein is found tightly associated with the cytoskeleton (Polte and Hanks, 1997), nonphosphorylated Cas localizes mainly to the cytoplasm (Sakai *et al.*, 1994). The FAK-Cas complex provides the backbone for many of the multimolecular complexes that form around integrin cytoplasmic tails at focal adhesion sites. These complexes may include a number of proteins that, in combination, transduce signals leading to the mitogen-activated protein (MAP) kinase effector cascades (Schlaepfer *et al.*, 1999). These effects, in turn, regulate basic cellular processes such as growth, differentiation, migration, and survival. Although tyrosine phosphorylation of FAK and Cas appears to be linked to G protein-coupled receptor stimulation in cardiac myocytes, the biological role(s) for these phosphorylation events remains unclear (Sadoshima *et al.*, 1996; Taylor *et al.*, 2000). The current study focuses on the potential link between ET-dependent phosphorylation of the FAK-Cas complex in cardiac myocytes and the relationship of this signaling pathway to the cytoskeletal changes and alterations in gene expression that accompany myocyte hypertrophy.

MATERIALS AND METHODS

Plasmids and Adenovirus Production

The −1150hANP chloramphenicol acetyltransferase (CAT) reporter (LaPointe *et al.*, 1988) and expression vector for c-Src (Kovačič *et al.*, 1998) have been described previously. Construction of green fluorescent protein (GFP)-FAT has been described (Ilić *et al.*, 1998), as has construction of GFP-paxillin (Turner *et al.*, 1999). To construct a fragment containing only the major Cas-binding region of FAK, bound to GFP (GFP-hunter), the *Sna*BI-*Xho*I fragment from a GFP-FRNK expression vector (Ilić *et al.*, 1998) was isolated and subcloned into the new pEGFP C1 plasmid (Clontech, Palo Alto, CA) opened with *Sna*BI-*Xho*I. Hemagglutinin (HA)-tagged wild-type and mutant Cas, cloned in the mammalian expression vector pSSRα, were provided by T. Nakamoto and H. Hirai, Tokyo University, Tokyo, Japan (Nakamoto *et al.*, 1997). For adenovirus production, an *Nde*I-*Bam*HI fragment from pEGFP C1 or pEGFP-FAT (Ilić *et al.*, 1998) containing GFP- or GFP-FAT–coding sequences, respectively, were inserted into the pAdlox adenoviral shuttle vector (Hardy *et al.*, 1997). The viruses were established by transfecting the ligated adenoviral genome constructs with replication-defective ψ5 virus into CRE 8 cells that stably express Cre-recombinase. Positive clones were expanded in HEK293 cells. After recombination, plaque-purified virus (10^{10} plaque-forming units [pfu]/ml) was isolated and purified as described elsewhere (Hardy *et al.*, 1997).

Cell Culture, Transfection, Viral Infection, and CAT Assay

Primary cultures of rat ventricular cardiac myocytes from 1- to 2-d–old rats were prepared as described before (Kovačič-Milivojević *et al.*, 1997) and plated on fibronectin-coated plates (10 μg/ml) in DME-H21/10% fetal calf serum (FCS). Cells were cultured under conditions that promote a high level of sarcomeric organization. After cells attached (~18 h), they were rinsed twice in serum-free DME-H21 and placed in fresh DME-H21/10% FCS. All experiments were performed 48 h postplating unless otherwise stated. For transient transfections, 10^7 cells/group were resuspended in 400 μl of phosphate-buffered saline (PBS)/0.1% glucose containing 25 μg of reporter plasmid together with varying concentrations of individual expression vectors, as described in the figure legends. The DNA concentration for all samples was adjusted to 40 μg with pUC18. Cells were electroporated with the use of the Gene Pulsar (Bio-Rad, Hercules, CA) at 280 V/250 μF. Independent measurements of transfection efficiency, with the use of pRSV β-galactosidase, typically showed <15% variation within a given experi-

ment. After transfection each group was plated onto 6-cm dishes (15×10^4 cells/cm^2) in DME-H21/10% FCS. The cells were harvested at 48 h posttransfection and the cell lysates were assayed for CAT activity as described previously (Kovačič-Milivojević *et al.*, 1996). For adenoviral infection, cells were infected with wild-type replication-defective ψ5 adenovirus or with adenovirus encoding either GFP or GFP-FAT. Infection was carried out with ~10–20 pfu/cell, either at the time of plating or after 72 h of culture with DME-H21 containing 10% FCS. Three hours later cells were washed with PBS and cultured in DME-H21 supplemented with 10% FCS, glutamine, sodium pyruvate, nonessential amino acids, 10^{-4} M β–mercaptoethanol, and penicillin/streptomycin for the times indicated.

Isolation of RNA and Northern Blot Analysis

Total RNA was isolated from ventricular cardiocytes with the use of RNAzol B (Tel-Test, Friendswood, TX). Eight micrograms of ventricular RNA was size fractionated on 1% agarose containing 2.2 M formaldehyde, transferred by capillary action to GeneScreen Plus Hybridization Transfer Membrane (NEN Research Products, Boston, MA) in 10× standard saline citrate (1.5 M sodium chloride and 0.15 M sodium citrate) for 8–16 h, and fixed to the membrane by UV irradiation (DNA transfer lamp; Fotodyne, New Berlin, WI). An 840-bp *Eco*RI/*Hin*dIII fragment of rat ANP cDNA or a 640-bp *Eco*RI fragment of rat brain natriuretic peptide (BNP) cDNA was labeled with [γ-^{32}P]dCTP with the use of the random primer technique. Hybridizations were performed in Rapid-Hyb buffer (Amersham, Arlington Heights, IL) according to instructions provided by the manufacturer. Blots were later stripped and reprobed with a radiolabeled glyceraldehyde phosphate dehydrogenase (GAPDH) cDNA to control for differences in RNA loading and transfer among samples.

Antibodies

To detect full-length FAK, but not FAT, we used mouse monoclonal anti-FAK antibodies (Transduction Laboratories, Lexington, KY) and rabbit or goat polyclonal A-17 antibodies (Santa Cruz Biotechnology, Santa Cruz, CA). Full-length FAK and FAT were detected at the same time by rabbit polyclonal JF1 antibody increased against the C-terminal part of FAK (Ilić *et al.*, 1995). FAK phosphorylation on Tyr397 was assessed with rabbit anti-phosphoY397FAK antibody (BioSource International, Camarillo, CA). Where indicated, polyclonal anti-FAK antibodies against C-terminal FAK (C-20) were also used (Santa Cruz Biotechnology). GFP and GFP fusion proteins were detected with monoclonal C163 anti-GFP antibody (Zymed, San Francisco, CA). Monoclonal anti-HA (HA.11) antibody was purchased from BAbCo (Richmond, CA), anti-paxillin was purchased from Zymed, anti-tensin was purchased from Transduction Laboratories, anti-sarcomeric α-actinin was purchased from Sigma (St. Louis, MO), anti-phosphotyrosine (4G10) was purchased from UBI (Lake Placid, NY), and anti–β-MHC was purchased from Chemicon International (Temecula, CA). Polyclonal anti-Src was from Santa Cruz Biotechnology. Polyclonal anti-Cas antibodies N-17 and C-20 (Santa Cruz Biotechnology) were used for immunoprecipitations and monoclonal anti-Cas (Transduction Laboratories) was used for Western blots except where indicated. All secondary antibodies were purchased from Jackson ImmunoResearch (West Grove, PA).

Immunoprecipitation and Western Blot Analysis

Cardiac myocytes were lysed in modified radioimmunoprecipitation assay lysis buffer containing freshly added protease and phosphatase inhibitors (50 mM HEPES, pH 7.4, 150 mM NaCl, 10% glycerol, 1.5 mM $MgCl_2$, 1 mM EGTA, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 0.25 mM Na_3VO_4, 10 mM sodium pyrophosphate, 100 mM NaF, 10 μg/ml each of leupeptin and aprotinin, and 1 mM phenylmethylsulfonyl fluoride). After insoluble material was removed by centrifugation for 15 min at 15,000 × *g*, lysates were precleared with 20 μl of protein G-Sepharose (Amersham Pharmacia Biotech, Piscataway, NJ) for 30 min. For immunoprecipitation, normalized samples (1 mg protein/0.5 ml) were incubated on a rotating platform for 2 h at 4°C with 2–4 μg of appropriate antibodies per sample. Immune complexes were collected after addition of 25 μl of protein G-Sepharose beads and incubation for 45–60 min at 4°C. The pellets were washed once with lysis buffer and three times with buffer A (20 mM Tris, pH 7.4, 1 mM EDTA, 0.1% Nonidet P-40, 10% glycerol, and 5 mM β-mercaptoethanol) before separation on 8% SDS-PAGE gels. Precleared whole lysates were separated on 4–20% SDS-PAGE gradient gels. Proteins were transferred from the gels to a nitrocellulose membrane (Schleicher & Schuell, Keene, NH). Membranes were blocked for 2 h at room temperature with either 5% skim milk/TBST buffer (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.05% Tween 20) or, for anti-phosphotyrosine blots, with 5% bovine serum albumin/TBST. Membranes were exposed to primary antibodies overnight at 4°C, washed in TBST, and exposed to horseradish peroxidase-conjugated secondary antibodies in 0.5% skim milk/TBST for 1 h at room temperature. Immunoreactive bands were visualized by enhanced chemiluminescence (Amersham Pharmacia Biotech). When necessary, membranes were stripped with 2% SDS in 62.5 mM Tris-HCl, pH 6.7, in the presence of 200 mM β-mercaptoethanol (freshly added) for 60 min at 50–60°C. After overnight blockade in 1% skim milk/TBST, membranes were reblotted with appropriate antibodies. Signals were identified and quantified with the use of NIH Image (National Institutes of Health, Bethesda, MD).

Immunocytochemistry

Cells were plated in DME-H21 supplemented with 10% FCS on fibronectin-coated coverslips and, at the indicated time, fixed as described in the figure legends. Immunocytochemistry was carried out essentially as described by Kovačič *et al.* (1998). Streptavidin conjugates with fluorescein isothiocyanate and rhodamine were purchased from Vector Laboratories (Burlingame, CA) and Hoechst 33342 was purchased from Molecular Probes (Eugene, OR).

RESULTS

Cas Is Colocalized with FAK and Paxillin at Z-Lines in Cardiac Myocytes

Cas is part of the supramolecular complex that links extracellular matrix, through integrins, to the cytoskeleton at focal adhesion sites (Polte and Hanks, 1997; Almeida *et al.*, 2000; O'Neill *et al.*, 2000). It has been shown that Cas plays an important role in cardiac development and growth during embryogenesis. Honda *et al.* (1998) showed that deletion of Cas by homologous recombination resulted in abnormal heart development and embryonic lethality. Cas colocalizes with Z-lines in wild-type cardiac muscle, whereas Cas-deficient cardiac muscle displays a perturbed sarcomeric organization with severely disrupted Z-lines (Honda *et al.*, 1998). In several cell types Cas is found diffusely spread throughout the cytoplasm with only a fraction concentrated in focal contacts (Sakai *et al.*, 1994; Nakamoto *et al.*, 1997; Polte and Hanks, 1997). The intracellular distribution of Cas in primary cardiac myocytes is not known. We therefore examined the subcellular localization of endogenous Cas in cardiac myocytes grown on fibronectin-coated coverslips (48 h in DME-H21/10% FCS) by immunostaining with several commercially available anti-Cas antibodies. All showed similar highly repetitive striations resembling well-defined structural components of the sarcomeres. Accumulations of

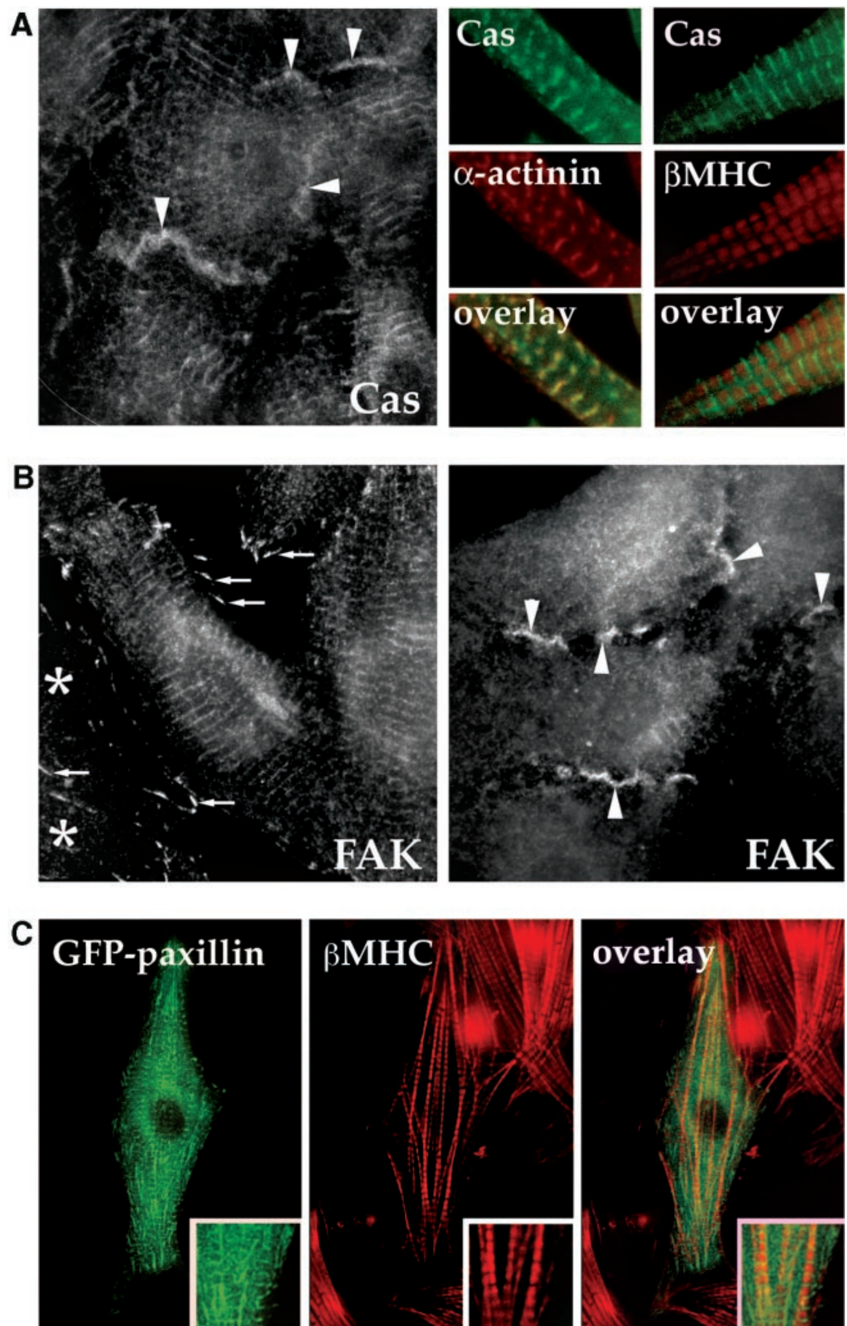

Figure 1. Cas is colocalized with FAK and paxillin at Z-lines in cardiac myocytes. (A) Cas is localized at Z-lines in cardiac myocytes. Cells were grown for 24 or 48 h on fibronectin-coated glass coverslips, fixed with ethanol at −20°C for 30 min, and exposed to acetone at room temperature for 1 min. Cells were double stained with monoclonal antibodies against Cas (detected by fluorescein isothiocyanate) and α-actinin or β-MHC (detected by rhodamine). The merged image shows that Cas staining localizes between sarcomeric A-bands in a pattern that overlies the Z-lines of the sarcomere. Arrowheads identify Cas at intercalated disks. (B) Subcellular localization of FAK in cardiac myocytes. Cells were grown on fibronectin-coated glass coverslips and then fixed for 10 min in cold acetone. Left, FAK was stained with rabbit polyclonal JF1 antibody. Asterisks (*) indicate neighboring nonmyocyte cell. Arrows indicate focal adhesions on both fibroblasts and cardiomyocytes. Right, FAK in intercalated disks (arrowheads) was demonstrated with a combination of rabbit and goat polyclonal A-17 and C-20 antibodies (Santa Cruz Biotechnology). (C) Expression of GFP-paxillin in cardiac myocytes. Freshly isolated cells transiently transfected with a GFP-paxillin expression vector were plated on fibronectin-coated glass coverslips for immunofluorescence. At 48 h posttransfection cells were fixed with paraformaldehyde, permeabilized with acetone, and stained with anti–β-MHC antibodies (detected with rhodamine, middle). GFP-paxillin–expressing cells were visualized by fluorescence microscopy (left). The merged image (right) shows that paxillin localizes between sarcomeric A-bands in a pattern corresponding to the Z-lines of the sarcomere.

Cas were also detected at intercalated disks, structures involved in cell-cell contact and organization of actin filaments in myofibrils of adjacent cells (Figure 1A, left). We used an antibody against sarcomeric α-actinin, a protein found in the Z-lines of the sarcomere, as well as an antibody against β-MHC, a component of the sarcomeric A-band, to define the precise localization of Cas. Immunostaining with these antibodies revealed a well-organized pattern of repetitive striations for both β-MHC and α-actinin (Figure 1A, right). Overlaying images of cells stained for Cas with those for β-MHC or α-actinin suggested that Cas is located at or very near to the Z-line.

Because the SH3 domain of Cas forms a high-affinity interaction with the PR-1 domain of FAK and colocalizes with FAK at focal adhesions (Nakamoto *et al.*, 1997), we asked whether Cas and FAK are colocalized in cardiac myocytes. As expected, FAK was found in classic focal adhesion sites at the edges of myocytes and nonmyocytes present in the cultures (Figure 1B). FAK, like Cas, was also detected in Z-lines and intercalated discs. Paxillin, a third component of

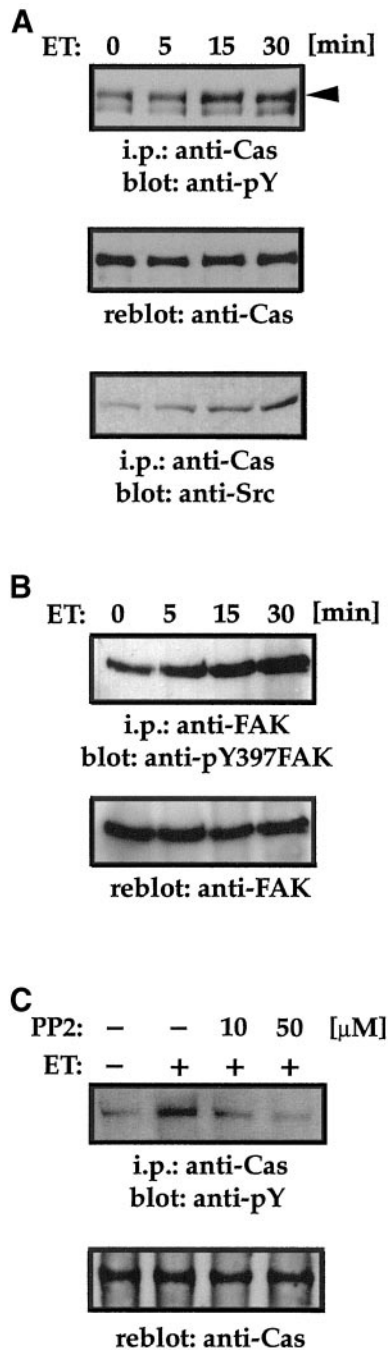

Figure 2. Association of Src with Cas is required for maximal Src-dependent activation of the ANP promoter. (A) Time course for ET-induced tyrosine phosphorylation of Cas and association with Src. Freshly prepared cardiac myocytes were plated as described in MATERIALS AND METHODS. At 48 h postplating, cells were stimulated with 100 nM ET-1 for the indicated times. Cell lysates were immunoprecipitated (i.p.) with monoclonal anti-Cas antibodies. Immunoprecipitates were subjected to SDS-PAGE and Western blot analysis for phosphotyrosine residues with 4G10 antibody (top) or c-Src with rabbit anti-Src antibody (bottom). Arrowhead indicates position of p130Cas protein. Membranes were then stripped and reblotted with N-17 goat anti-Cas antibodies to confirm the identity of the 130-kDa phosphotyrosine band and to control for protein loading (middle). (B) Time course for ET-induced tyrosine phosphorylation of FAK. Myocytes were prepared as in A above. At 48 h postplating, cells were stimulated with 100 nM ET-1 for the indicated times and lysates were immunoprecipitated with a combination of A-17 and C-20 goat anti-FAK. Immunoprecipitates were subjected to SDS-PAGE and Western blot analysis. FAK (auto)phosphorylation levels were detected with phosphospecific anti-pY397 FAK antibodies (top). The membrane was reprobed with monoclonal anti-FAK antibodies (bottom). (C) Src activity is required for ET-induced Cas phosphorylation. At 48 h postplating myocytes were pretreated with 10 or 50 μM PP2. At 30 min after addition of PP2, cells were treated with 100 nM ET-1 for 15 min. Cell lysates were immunoprecipitated with monoclonal anti-Cas antibodies. Immunoprecipitates were subjected to SDS-PAGE and Western blot analysis. Phosphotyrosine (anti-pY) was detected by antibody 4G10. The membrane was stripped and reblotted with N-17 goat anti-Cas antibodies to confirm the identity of the 130-kDa phosphotyrosine band and to control for protein loading (bottom).

focal adhesion sites, was also detected in Z-lines of the myocytes (Figure 1C). Collectively, these data suggest that Z-lines may be important signaling centers for regulating and/or coordinating the changes in sarcomeric organization in these cells that occur during myocyte hypertrophy.

Src Substrates, Cas and FAK, Are Part of the ET- and Src-dependent Signaling Pathway That Promotes Cardiac Myocyte Hypertrophy

Tyrosine phosphorylation is a critical covalent modification driving protein-protein interactions required for cytoskeletal reorganization and focal-adhesion assembly (Clark and Brugge, 1995; Giancotti and Ruoslahti, 1999). We reported previously that ET stimulation of neonatal rat ventricular myocytes results in increased Src activity (Kovačič *et al.*, 1998). To determine whether the Src substrates, Cas and FAK, are part of the ET-dependent signaling pathway, we examined tyrosine phosphorylation levels of Cas and FAK immunoprecipitated from cardiac myocytes after stimulation with 100 nM ET. Enhanced tyrosine phosphorylation of Cas was observed within 5 min, with increased phosphorylation (3.15-fold ± 0.95 [mean ± SD], n = 3) detected 15–30 min after addition of ET (Figure 2A). The time course of ET-induced FAK phosphorylation (2.9-fold ± 0.87, n = 3) was similar to that of Cas phosphorylation (Figure 2B). The amount of Src present in Cas immunoprecipitates increased after ET treatment (Figure 2A, bottom), paralleling the increase in Cas phosphorylation levels. This observation suggests that Src might be responsible for the increase in Cas phosphorylation. We tested this possibility by exposing cells for 30 min to increasing concentrations of the Src-tyrosine kinase family inhibitor PP2 (Hanke *et al.*, 1996). Cells were then stimulated with ET for 15 min. Tyrosine phosphorylation of Cas decreased in a dose-dependent manner, with substantial inhibition at 10 μM and nearly complete inhibition at 50 μM PP2. Subsequent blotting with anti-Cas antibody demonstrated that Cas protein precipitated equally across all lanes (Figure 2C).

Given the evidence linking ET to activation of Src and phosphorylation of Cas and FAK (Figure 2), we sought to determine whether Cas is linked mechanistically to downstream markers of hypertrophy in cardiac myocytes. To address this issue, we

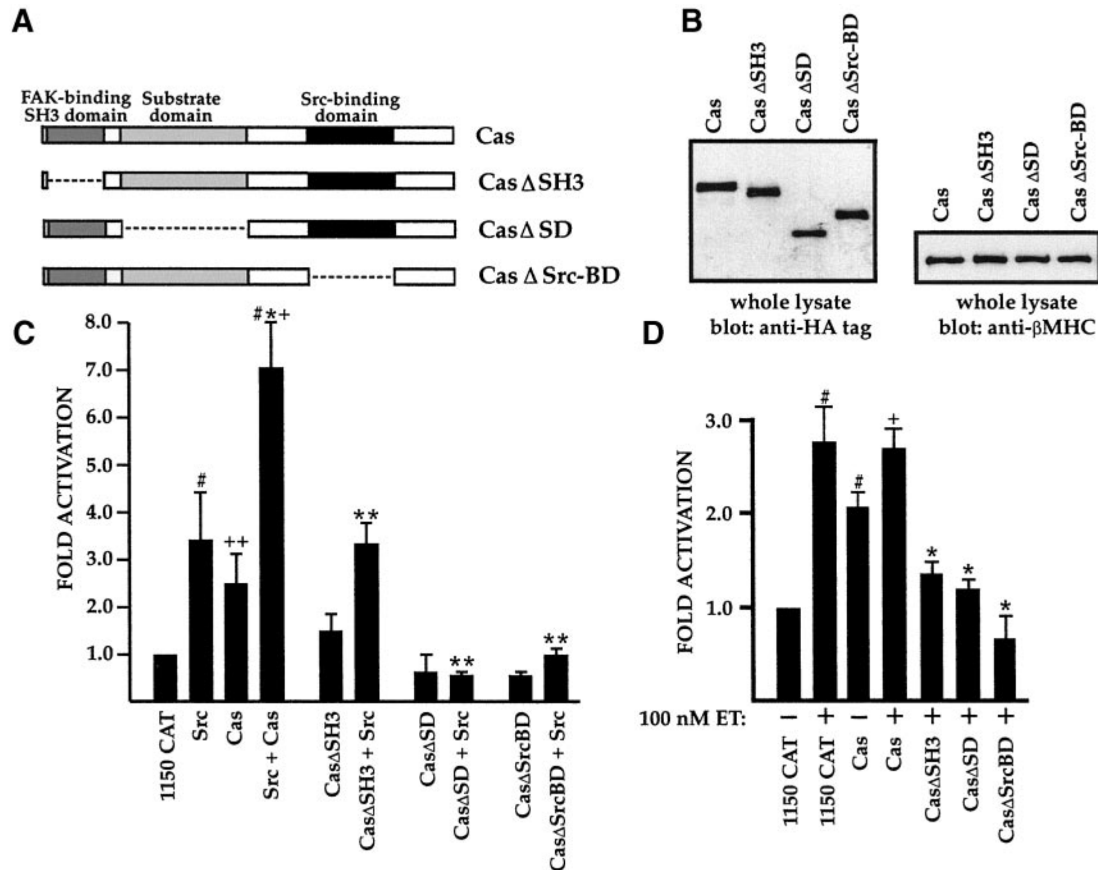

Figure 3. Cas is required for maximal Src-dependent activation of the ANP promoter. (A) Schematic diagram representing the various Cas constructs used. HA-tagged Cas constructs were introduced into pSSRα vector. Details of construction of each mutant form are given in MATERIALS AND METHODS. (B) Cardiac myocytes were transfected with 5 μg of expression vector encoding HA-tagged wild-type Cas or the indicated mutant Cas forms, except for CasΔSH3, for which 15 μg was used throughout all experiments to achieve a protein level similar to Cas wild-type and other mutants. After 48 h immunoblotting was performed on lysates (20 μg) with anti-HA antibody to show that all transfected vectors were expressed at same level (left), and with anti–β-myosin antibody to show equal loading in each lane (right). (C) Cas-dependent activation of ANP gene. Neonatal cardiac myocytes were transfected with −1150hANP CAT (20 μg) alone or together with 1 μg of c-Src, 5 μg of Cas, or 1 μg of the mutant Cas constructs or Src and Cas constructs in combination. Cells were plated on fibronectin and incubated for 48 h. Cell lysates were processed for Western blot analysis and for CAT assay as described in MATERIALS AND METHODS. CAT activity was measured in equivalent amounts of soluble proteins (100 μg) for each reaction. Results are expressed as fold activation over basal −1150hANP CAT activity and represent the means ± SD of three to nine separate experiments done in triplicate. #, $p < 0.01$ compared with basal −1150CAT activity; ++, $p < 0.05$ compared with basal −1150CAT activity; **, $p < 0.01$ compared with Src-stimulated −1150CAT activity; *, $p < 0.05$ compared with Src-stimulated −1150CAT; +, $p < 0.01$ compared with Cas-stimulated −1150CAT as determined by Student's *t* test. (D) Inhibition of ET-dependent activation of −1150hANP CAT with different mutant Cas constructs. Neonatal cardiac myocytes were transfected with −1150hANP CAT (20 μg) alone or together with 5 μg of Cas or 1 μg of the mutant Cas constructs. Cells were plated on fibronectin in 10% serum for 24 h. Plates were then washed in PBS and cells were grown for an additional 24 h in serum-free medium in the absence or presence of 100 nM ET. CAT activity was measured in 150 μg of soluble protein for each reaction. Results are expressed as fold activation over basal −1150hANP CAT activity and represent the means ± SD of four separate experiments done in triplicate. #, $p < 0.01$ compared with basal −1150CAT activity; *, $p < 0.01$ compared with ET-stimulated −1150CAT activity; +, $p < 0.05$ compared with Cas-stimulated −1150CAT activity in the presence of ET.

transiently transfected cardiac myocytes with ANP promoter-driven CAT reporter (−1150hANP CAT), a marker that has been shown to respond to a number of different hypertrophic stimuli, including ET, in this in vitro model (Shubeita *et al.*, 1990; Knowlton *et al.*, 1991). Overexpression of Cas led to a twofold increase in −1150hANP CAT activity, whereas overexpression of Src increased promoter activity 3.4-fold. Coexpression of Src and Cas together resulted in a sevenfold increase in reporter activity, implying a cooperative interaction of these two proteins in regulation of the ANP promoter (Figure 3C). To identify Cas domain(s) required for induction of ANP promoter activity, we carried out studies with several Cas deletion constructs. These included Cas lacking the SH3 domain (CasΔSH3), substrate domain (CasΔSD), or Src-binding domain (CasΔSrc-BD) (Figure 3, A and B). Cotransfection with CasΔSH3 resulted in a modest increase in basal −1150hANP

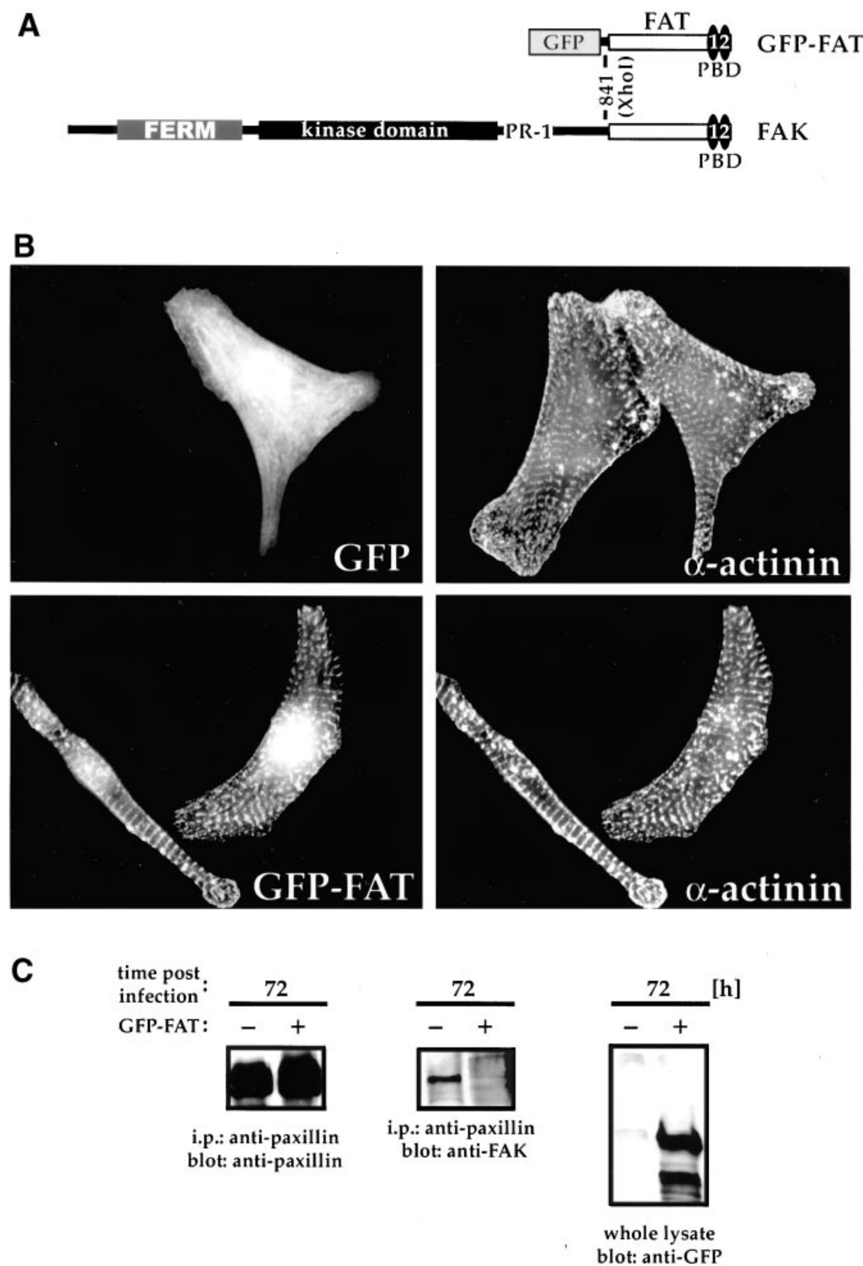

Figure 4. Changes in cellular morphology during cardiac myocyte development in uninfected and GFP-FAT–infected cells. (A) Diagram of wild-type FAK and dominant-negative FAK mutant, GFP-FAT. PBD, paxillin-binding domains 1 and 2. (B) Subcellular localization of dominant-negative FAK mutant, GFP-FAT, in cardiac myocytes. Freshly isolated cells infected with adenovirus expressing GFP or GFP-FAT were plated on fibronectin-coated glass coverslips for immunofluorescence. At 48 h postinfection cells were fixed in 3.8% paraformaldehyde/PBS for 20 min, permeabilized in cold acetone for 10 min, and stained with anti–α-actinin antibodies (right). GFP- and GFP-FAT–expressing cells were visualized by fluorescence microscopy (left). (C) Expression of GFP-FAT disrupts endogenous FAK-paxillin binding. Cell extracts were prepared from control (−) or GFP-FAT–infected (+) cardiac myocytes plated on fibronectin and cultured for 48 h in the presence of serum. Paxillin was immunoprecipitated (i.p.) with monoclonal anti-paxillin antibodies (Zymed). Immunoprecipitates were separated by 8% SDS-PAGE and transferred to a nitrocellulose membrane. The membrane was first blotted with monoclonal anti-paxillin antibodies (top), stripped, and reprobed with monoclonal anti-FAK (Transduction Laboratories) antibodies (bottom). Western blot analysis of whole cell lysate (20 μg) with anti-GFP antibodies shows expression level of GFP-FAT. (D) Freshly isolated cardiac myocytes, control or infected with adenovirus expressing GFP or GFP-FAT, were plated on fibronectin-coated glass coverslips. Cells were cultured for 24, 48, or 72 h, fixed in 3.8% paraformaldehyde/PBS for 20 min, permeabilized in cold acetone for 10 min, and stained with anti–α-actinin antibodies. (E) Controls showing levels of GFP, GFP-FAT, and endogenous FAK. Immunoblotting was performed on lysates (20 μg) from control, GFP-, and GFP-FAT–infected cells with the use of antibodies specific for GFP (top) or for the C terminus of FAK (bottom). (F) Quantification of the apoptotic index at indicated time points (percentage of cells containing condensed, fragmented nuclei, as detected by Hoechst 33342) for control and GFP- or GFP-FAT–infected cells. Results are means ± SD of three independent experiments.

CAT activity but had no significant effect on Src-activated ANP promoter activity. This suggests that endogenous Cas interacts with transfected Src in the presence or absence of CasΔSH3. Inability of CasΔSH3 to compete with endogenous Cas for binding to the PR-1 region of FAK suggests that CasΔSH3 is functionally neutral in this context, neither amplifying nor inhibiting Src-dependent activity. Expression of either CasΔSD or CasΔSrc-BD resulted in minor repression of basal ANP CAT activity and complete inhibition of the Src-dependent induction. These results imply that both these mutants interfere with endogenous Cas, perhaps by functioning as dominant negative mutants (e.g., by competing with endogenous Cas for binding to the PR-1 region of FAK; Figure 3C). All constructs were expressed at similar levels in transfected myocytes (Figure 3B). These results support the conclusion that full Cas function in promoting Src-stimulated activity of the ANP promoter involves a combination of distinct interactions mediated by all three of its major domains: the SH3-, substrate-, and Src-binding domains. Cas deletion constructs also exhibited the same effect on ET-stimulated reporter activity. ET treatment resulted in an approximately threefold increase in reporter activity that was significantly reduced (~40–50%) in the presence of CasΔSH3 or CasΔSD and completely inhibited by cotransfection with CasΔSrc-BD (Figure 3D). Furthermore, ET treatment promoted a modest (~30%), but statistically significant, increase in Cas-dependent activation of ANP gene promoter

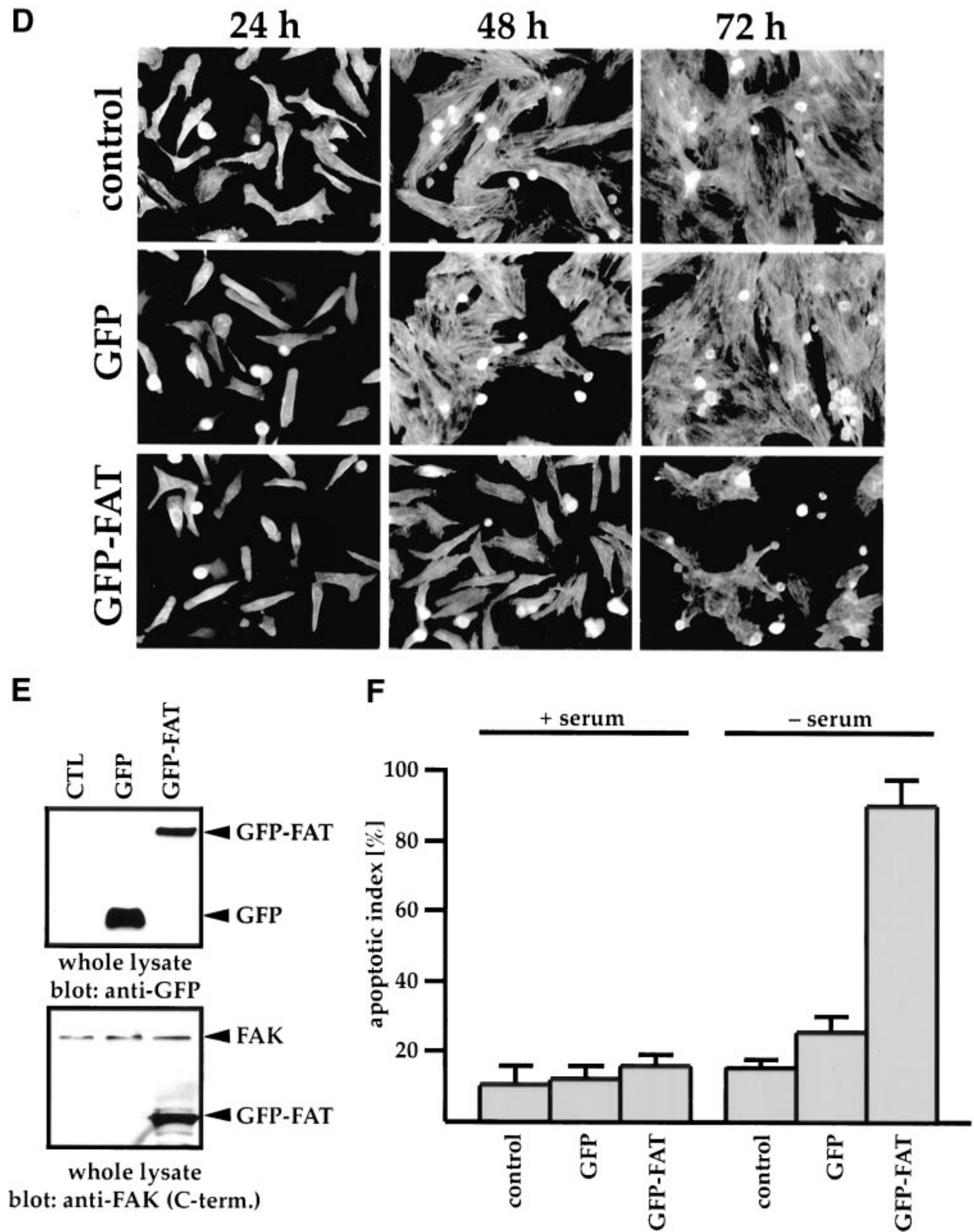

Figure 4 (cont).

activity. Taken together, these findings suggest that Cas plays an active role in Src- and ET-dependent activation of the ANP gene promoter. Preservation of the multiple protein interaction motifs of Cas is required for maximal Src-dependent activity.

Undermining FAK Function by Expressing GFP-FAT Disrupts the Changes in Cytoskeletal Architecture Associated with Myocyte Hypertrophy in Culture without Affecting Myocyte Survival

Next, we investigated whether disruption of FAK localization and function impairs the cytoarchitecture of cardiac myocytes undergoing hypertrophy in culture. We expressed a GFP-linked FAT (GFP-FAT; Figure 4A) that has been shown in several systems to displace endogenous FAK from focal adhesion sites and to act as a dominant-negative inhibitor of FAK function by impairing assembly of FAK-containing molecular complexes at focal adhesion sites (Gervais *et al.*, 1998; Ilić *et al.*, 1998; Xu *et al.*, 1998; Shen and Schaller, 1999; Almeida *et al.*, 2000). Because this dominant-negative strategy requires a high percentage of FAT-expressing cells to observe changes in FAK signaling at a biochemical level, we generated replication-defective adenovirus encoding GFP-FAT or GFP alone. GFP and GFP-FAT were efficiently expressed in ~90% of cardiac myocytes <12 h after infection. At that time, GFP was uniformly distributed

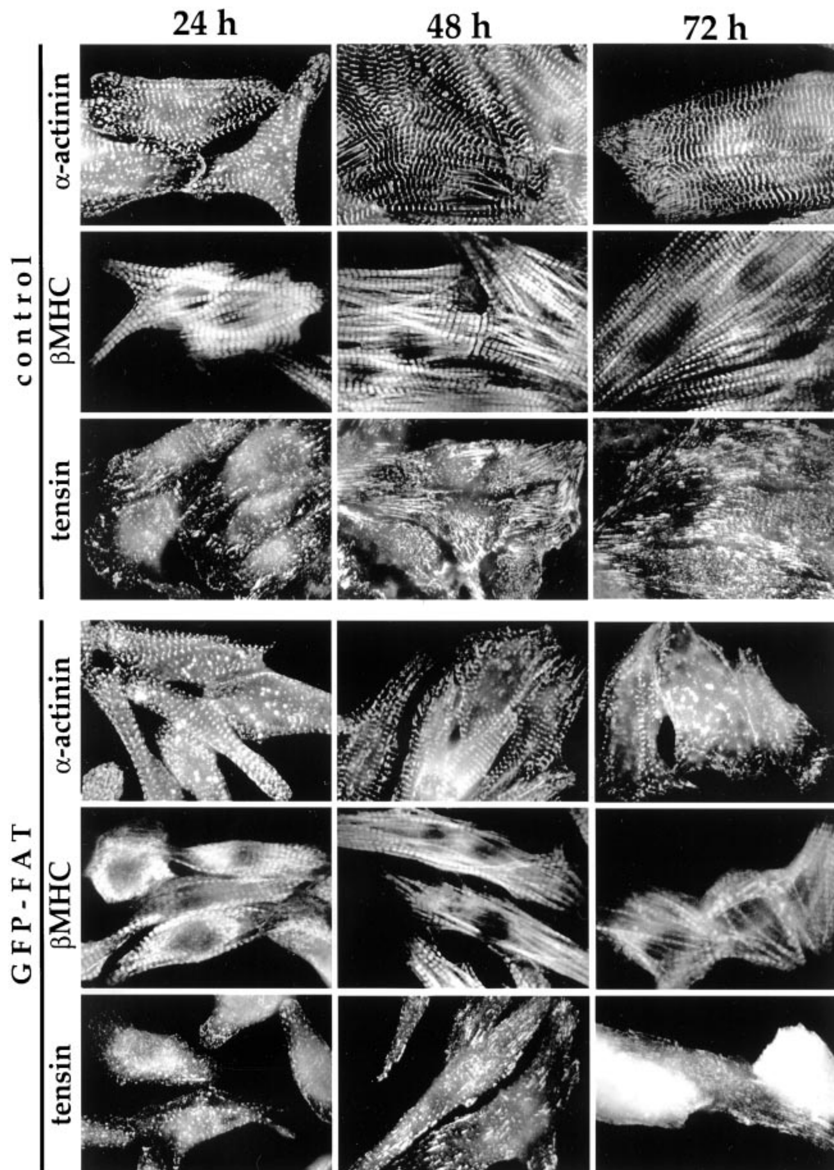

Figure 5. Analysis of sarcomeric organization in untreated (control) and GFP-FAT–infected cardiac myocytes at 24, 48, and 72 h postinfection. Paraformaldehyde-fixed, acetone-permeabilized cells were stained with antibodies against α-actinin, β-MHC or tensin.

throughout the cytoplasm and nucleus, whereas GFP-FAT distribution, like that of endogenous FAK, was detected primarily at Z-lines, colocalizing with α-actinin immunoreactivity (Figure 4B). GFP-FAT was also found in granular material that was rich in α-actinin. Because physical interactions between the C-terminal domain of FAK and proteins such as paxillin promote the targeting of FAK to focal contact sites (Tachibana *et al.*, 1995; Brown *et al.*, 1996; Turner, 1998), we assessed the association of endogenous FAK with paxillin to determine whether expression of GFP-FAT had disrupted endogenous FAK function. Extracts from uninfected cells or GFP-FAT–infected cells were immunoprecipitated with antibody against paxillin and examined for the presence of FAK. Paxillin-FAK complex formation was completely inhibited in GFP-FAT–expressing cells (Figure 4C).

We then determined how expression of GFP-FAT affected the development of the hypertrophic phenotype in cultured cardiac myocytes. Uninfected (control), GFP-, and GFP-FAT–infected cardiac myocytes were examined over a period spanning 24–72 h after plating (Figure 4D). At 24 h, cells in all three cultures displayed a similar spindle or triangular shape, without obvious differences in size (Figure 4D, left). After 48 h (Figure 4D, middle), uninfected and GFP-infected cells were larger and fully spread on the culture surface and had started grouping together. At the same time, GFP-FAT–infected cardiac myocytes were much smaller than the uninfected cells. Cell aggregates were also smaller and sparser, consisting of only two to four cells/group. This difference was particularly striking 72 h after infection (Figure 4D, right). Uninfected and GFP-infected cardiac myocytes appeared similar and formed an almost continuous layer of myocytes with myofibrils of adjacent spindle-shaped cells aligned across cell boundaries. In contrast, almost all GFP-FAT–infected cells remained triangular in shape and small, and they formed only poorly organized

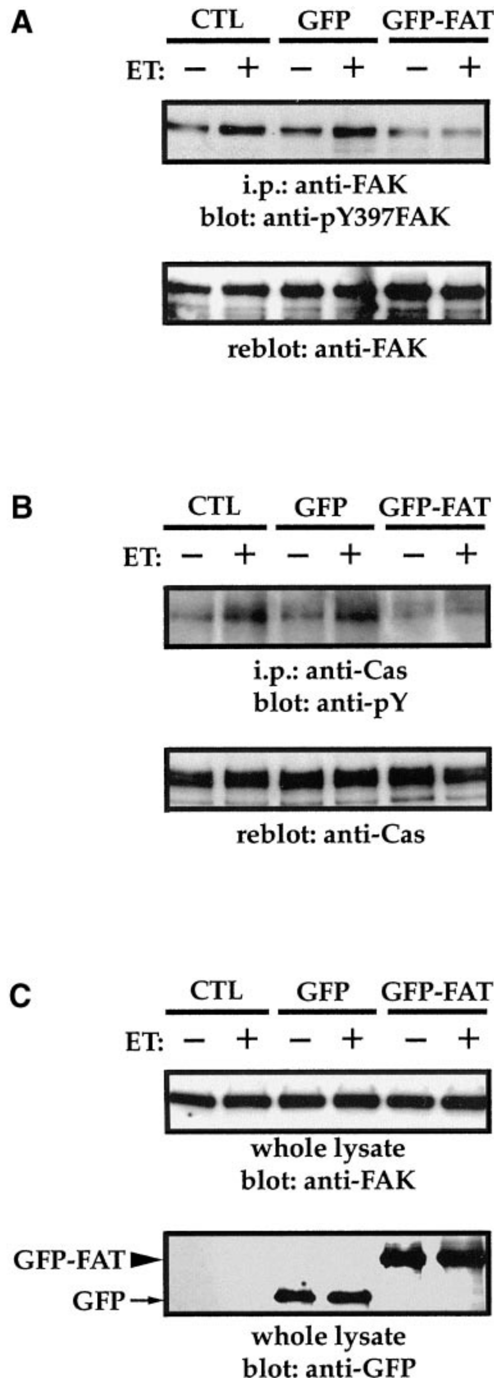

Figure 6. Expression of GFP-FAT interferes with phosphorylation of Cas. Uninfected (CTL) and GFP- or GFP-FAT–infected cardiac myocytes plated on fibronectin were cultured for 48 h in the presence of serum. Subsequently cells were transferred to serum-free medium for 4 h. After this period cells were either left untreated (−) or they were treated for 15 min with 100 nM ET (+). (A) Endogenous FAK was immunoprecipitated (i.p.) with a combination of goat polyclonal N-17 (Santa Cruz Biotechnology) and monoclonal (Chemicon) anti-FAK antibodies. Immunoprecipitates were subjected to SDS-PAGE and analyzed by Western blotting. The membrane was first immunoblotted with rabbit antibody to active pY397 FAK, stripped, and reblotted with monoclonal anti-FAK (Transduction Laboratories). (B) Cas was immunoprecipitated with a combination of N-17 and C-20 rabbit polyclonal anti-Cas antibodies (Santa Cruz Biotechnology). Immunoprecipitates were subjected to SDS-PAGE and analyzed by Western blotting. The membrane was first immunoblotted with 4G10 anti-phosphotyrosine antibody, stripped, and reprobed with monoclonal anti-Cas (Transduction Laboratories). (C) Western blot analysis of whole lysates (20 μg) with anti-FAK antibodies (top) shows equal expression of endogenous FAK in all three cell populations. Reblot of same membrane shows expression levels of GFP and GFP-FAT ~48 h postinfection.

cell aggregates. The GFP-FAT construct was expressed at similar or lower levels than GFP in infected myocyte cultures, indicating that the process of infection or production of protein per se did not trigger the suppression of the hypertrophic response observed in the GFP-FAT–infected cells (Figure 4E). Furthermore, expression of GFP or GFP-FAT appeared to have no significant effect on endogenous FAK levels (Figure 4E).

Disruption of cytoskeletal structure may promote cell death in anchorage-dependent cells. Moreover, GFP-FAT expression reportedly leads to a high level of apoptosis in cultures of adhesion-dependent cells when serum is withdrawn (Ilić *et al.*, 1998; Almeida *et al.*, 2000). Therefore, it was important to assess whether expression of GFP-FAT in cardiac myocytes affected their survival, even though serum as well as fibronectin was always present. First, we assessed the integrity of nuclear structure after staining with Hoechst 33342. The percentage of cells that contained condensed/fragmented nuclei was determined as the apoptotic index for each culture. By 48 h after plating on fibronectin in the presence of serum, uninfected, GFP- and GFP-FAT–infected cultures of cardiac myocytes all displayed a similar 10–20% level of apoptotic cells (Figure 4F). Next, we assessed the ability of unfixed GFP and GFP-FAT–infected cardiac myocytes to bind biotinylated annexin (annexin v-biotin) (Martin *et al.*, 1995). The level of apoptosis was 10–20% in this assay as well, thereby confirming by a different method that expression of GFP and displacement of FAK by GFP-FAT do not influence survival of cardiac myocytes in these serum-containing cultures. However, when cells were plated on fibronectin in the absence of serum, ~85% of the cardiac myocytes expressing GFP-FAT displayed signs of apoptosis only 16 h after plating. At later times GFP-FAT expression led to cell rounding and detachment. Under the same serum-free conditions, uninfected and GFP-infected cells showed a low level of apoptosis (~15–25%) similar to the levels found for cultures grown on fibronectin in the presence of serum (Figure 4F).

FAK Plays a Key Role in the Assembly of Sarcomeric Structures in Cardiac Myocytes

To understand the effects of GFP-FAT on cytoskeletal and sarcomeric architecture at the subcellular level, we examined Z-lines, sarcomeres, and focal adhesions of GFP-FAT–infected cells at higher magnification over the same 24- to 72-h time period (Figure 5). Because there was no obvious difference between uninfected and GFP-infected cardiac myocytes (Figure 4C), we present comparisons only between uninfected and GFP-FAT–infected cells (Figure 5). By 24 h the Z-line marker α-actinin was found

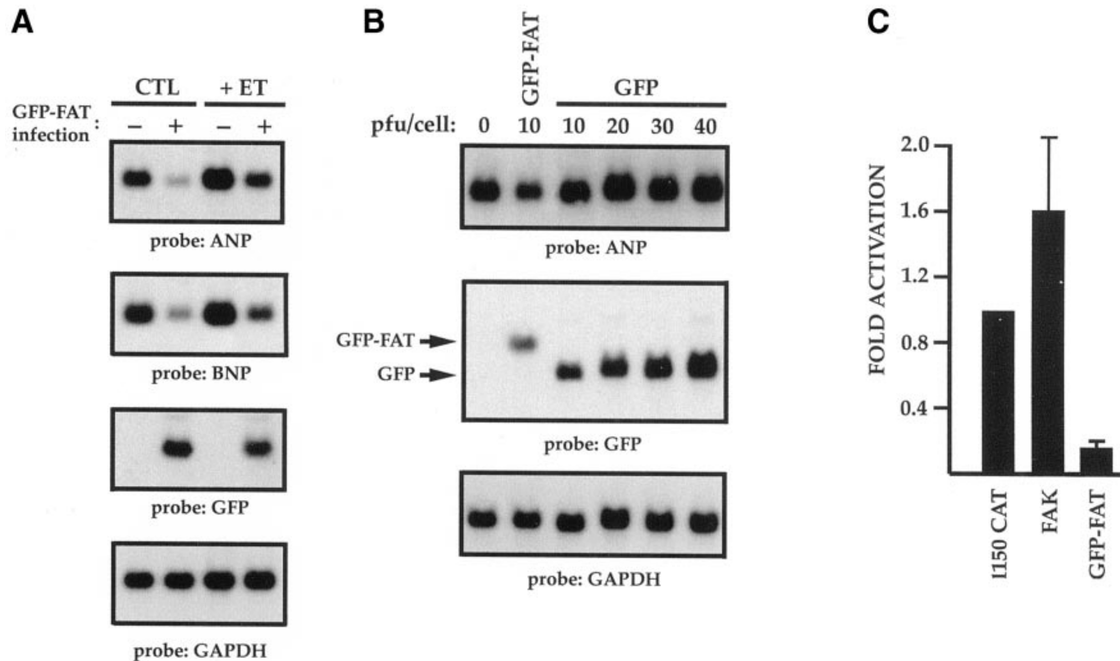

Figure 7. Role of FAK in regulation of ANP gene expression. (A) Dominant-negative mutant FAK (GFP-FAT) attenuates ET-induced ANP and BNP gene expression. Freshly isolated uninfected (−) or GFP-FAT–infected (+) cardiac myocytes (20 pfu/cell) were plated on fibronectin. At 24 h postinfection, cells were treated for 24 h with either vehicle (CTL) or 100 nM ET-1 (+ET). Eight micrograms of total RNA were analyzed by Northern blot analysis. Membranes were hybridized with radiolabeled rat ANP or BNP cDNA. After autoradiography, membranes were stripped and reprobed with radiolabeled GAPDH cDNA. (B) Inhibition of ANP and BNP gene expression is not a consequence of adenoviral infection or GFP expression. Isolated cardiac myocytes were infected with adenovirus expressing GFP-FAT (10 pfu/cell) or with increasing concentrations of adenovirus expressing GFP alone (10–40 pfu/cell). Total RNA was analyzed 48 h postinfection. Membrane was hybridized with radiolabeled ANP cDNA, stripped, and reprobed with radiolabeled GFP cDNA. Finally, the same membrane was probed with GAPDH cDNA to control for equal RNA loading. (C) Expression of dominant-negative FAK inhibits basal activity of ANP gene promoter. Neonatal cardiac myocytes were transfected with −1150hANP CAT (20 μg) alone or together with 5 μg of FAK (wild-type) or GFP-FAT expression vector. Cells were cultured and processed for CAT activity. Data were averaged from six separate experiments: values shown are means ± SD.

in Z-lines, as well as in numerous granular structures, in both uninfected and GFP-FAT–infected cells. At 48 h Z-lines were still detectable by anti–α-actinin staining in GFP-FAT–infected cells, but by 72 h most α-actinin was diffusely distributed in the cytoplasm or concentrated in dense granules. Immunostaining for cardiac-specific β-MHC revealed well-organized repetitive A-bands in uninfected cells. In GFP-FAT–infected cells, myofibrils and A-bands became disordered with kinetics similar to those observed for the disappearance of Z-lines. We also used tensin as a marker for focal adhesions (Lo *et al.*, 1994). In uninfected cardiac myocytes, tensin staining was detected not only in "classic" focal adhesions and fibrillar adhesions at the periphery of myocytes but also at Z-lines in a pattern that resembled the distribution of FAK and Cas. Between 24 and 48 h, GFP-FAT–infected cells began to show less pronounced tensin staining in focal adhesions, and myocytes lost the Z-line–like tensin staining pattern. Collectively, these observations strongly support the idea that correct localization of FAK in Z-lines and in focal adhesions is required for proper sarcomeric organization in myocytes undergoing hypertrophy.

FAK-mediated Signaling Is Involved in ET-dependent Tyrosine Phosphorylation of Cas

Having shown that GFP-FAT both undermined endogenous FAK function, as assessed by FAK's ability to bind paxillin (Figure 4C), and interfered with sarcomeric assembly associated with hypertrophy (Figure 5), we investigated the effect of disrupting FAK function on ET-dependent phosphorylation of FAK and Cas. Cardiac myocytes were infected with GFP or GFP-FAT and treated with 100 nM ET (15 min) 48 h postinfection. GFP-FAT inhibited both basal and ET-stimulated phosphorylation of endogenous FAK at Tyr397, as compared with uninfected and GFP-infected cells (Figure 6A). Subsequent blotting with anti-FAK antibody demonstrated that FAK was present at similar levels in all samples. Similar immunoprecipitation experiments were then performed to examine the effect of FAT overexpression on ET-dependent Cas phosphorylation. GFP-FAT almost completely inhibited basal and ET-dependent phosphorylation of Cas compared with uninfected or GFP-infected myocytes. Again, Cas levels were similar in all immunoprecipitates (Figure 6B). Both GFP and GFP-FAT were expressed at

similar levels and did not alter the expression of endogenous FAK (Figure 6C). Taken together, these results indicate that disruption of FAK function in cardiac myocytes interferes effectively with phosphorylation of Cas.

FAK Plays a Key Role in Regulation of ANP Gene Expression

To further define the role that FAK plays in the hypertrophic response, we examined the effect of overexpressing either full-length FAK or the dominant-negative FAK construct GFP-FAT on expression of genes associated with hypertrophy. Myocytes were infected with GFP- or GFP-FAT–expressing adenovirus for 24 h and then treated with 100 nM ET for an additional 24 h. RNA was isolated and analyzed by Northern blot hybridization for expression of the hypertrophic marker genes ANP and BNP. As expected, ET promoted a strong increase in ANP and BNP mRNA levels in control cells. Infection of myocytes with GFP-FAT resulted in significant inhibition of both basal and ET-stimulated expression of ANP and BNP mRNA (Figure 7A). To confirm that the inhibition of ANP and BNP gene expression was not a consequence of adenoviral infection or expression of GFP, we examined expression of the ANP transcript in the presence of an increasing number of GFP-expressing adenovirus pfu per cell. Results presented in Figure 7B indicate that infection with adenovirus encoding GFP did not interfere with the expression of the ANP gene or that of a housekeeping gene encoding GAPDH. Taken together, these data implicate FAK in the regulation of these hypertrophic marker genes. To extrapolate this finding to the level of transcription, we examined the effect of GFP-FAT overexpression on hANP gene promoter activity in a transient transfection assay. Cotransfection of −1150hANP CAT with increasing concentrations (0.1–20 μg) of expression vector encoding wild-type FAK did not significantly alter basal activity of the reporter (1.6 ± 0.38; Figure 7C). However, expression of FAT resulted in almost complete inhibition of promoter activity, implying that the effects on steady state transcript levels noted above are derived, at least in part, from reduced transcriptional activity.

Interaction Between Cas and FAK Plays a Critical Role in Sarcomeric Assembly in Cardiac Myocytes

To perturb the function of FAK in a different way and to determine whether association of Cas with FAK is critical for proper Z-line formation and sarcomeric organization in cardiac myocytes, we expressed the small fragment of FAK (residues 638–841), C-terminal to the kinase domain and N-terminal to the FAT domain, that contains PR-1. This PR-1–containing FAK fragment, which we named hunter, for hunting Cas protein, was expressed as a GFP-tagged protein (Figure 8A). It has been clearly shown that the SH3 domain of Cas binds preferentially and with high affinity to the PR-1 region of FAK (Polte and Hanks, 1995). Therefore, the hunter fragment of FAK should act as a sink for Cas and other proteins that would ordinarily bind to the PR-1 region of endogenous FAK.

GFP-hunter was introduced into cardiac myocytes either by adenovirus infection or by transient transfection. First, we examined the effect of GFP-hunter overexpression on cardiac myocyte cytoarchitecture. Cardiac myocytes were plated on fibronectin in the presence of serum and at high density, conditions that promote rapid myofibrillar organization. GFP-hunter expression was easily detected as early as 12 h postplating. At 24 h the distribution of the GFP-hunter fusion protein resembled that of endogenous Cas and FAK (Figure 1, A and B). Thus, it was detected primarily at Z-lines, colocalizing with α-actinin immunoreactivity (Figure 8B)

To investigate the effects of GFP-hunter expression on sarcomeric cytoarchitecture at the subcellular level, we examined Z-lines in GFP-hunter–infected cells over a 24- to 72-h period. By 24 h, the Z-line marker α-actinin was found in organized repetitive Z-lines in both uninfected and GFP-hunter–infected cells (Figure 8C, left). At 48 h, Z-lines were still detectable by α-actinin staining in the GFP-hunter–infected cells (arrowheads in Figure 8C, middle), but by 72 h most of the α-actinin staining was diffusely distributed in the cytoplasm or in numerous cable-like structures (arrowheads in Figure 8C, right). At the same time, staining of uninfected cells in the same culture revealed a high content of well-organized sarcomeric units. In a series of three experiments >1000 cells were examined over the period spanning 24–72 h to quantify the effects of GFP-hunter expression. Infected cells displayed three distinct phenotypic patterns with regard to the organization of their sarcomeric units (Figure 8D). At 24 h postplating, ∼ 45% of cells displayed typical sarcomeric organization throughout the entire cellular compartment; ∼25% of hunter-positive cells showed partial and discontinuous staining pattern of α-actinin; the remaining GFP-hunter–infected cells showed very limited staining for α-actinin. By 48 h, the population of cells that displayed highly organized myofibrils with well-organized sarcomeres dropped to 10%, whereas the percentage of cells containing low-level sarcomeric organization increased to 60%. The remainder (∼30%) displayed a nearly complete loss of sarcomeric organization. By 72 h, ∼90% of the cells infected with GFP-hunter displayed severely defective sarcomeric organization. About half of these cells showed complete loss of sarcomeres in the cytosolic compartment, whereas the other half displayed only a very low number of sarcomeric units still present in the cell. The remaining 10% of the GFP-hunter–infected cells displayed well-organized myofibrils. These observations strongly support the idea that the interaction of the SH3 domain of Cas with PR-1 of FAK plays a critical role in the formation of Z-lines and assembly of sarcomeric units in the cardiac myocytes.

Expression of GFP-Hunter Inhibits ET- and Cas-stimulated ANP Gene Expression

Next, we examined the effect of GFP-hunter on regulation of ANP gene expression in cardiac myocytes undergoing hypertrophy. Uninfected and GFP-hunter–infected cardiac myocytes were plated for 24 h and then treated with ET for an additional 24 h. RNA was isolated and analyzed by Northern blot hybridization. As expected, ET stimulation resulted in a strong increase in ANP mRNA levels in uninfected cells. Expression of GFP-hunter resulted in almost complete inhibition of basal as well as ET-stimulated expression of ANP mRNA (Figure 8E). To extrapolate this finding to the level of transcription, we examined the effect of GFP-hunter expression on hANP gene promoter activity. Cotransfection of −1150hANP CAT with 1 or 5 μg of a full-length Cas-containing expression vector led to an approximately twofold increase

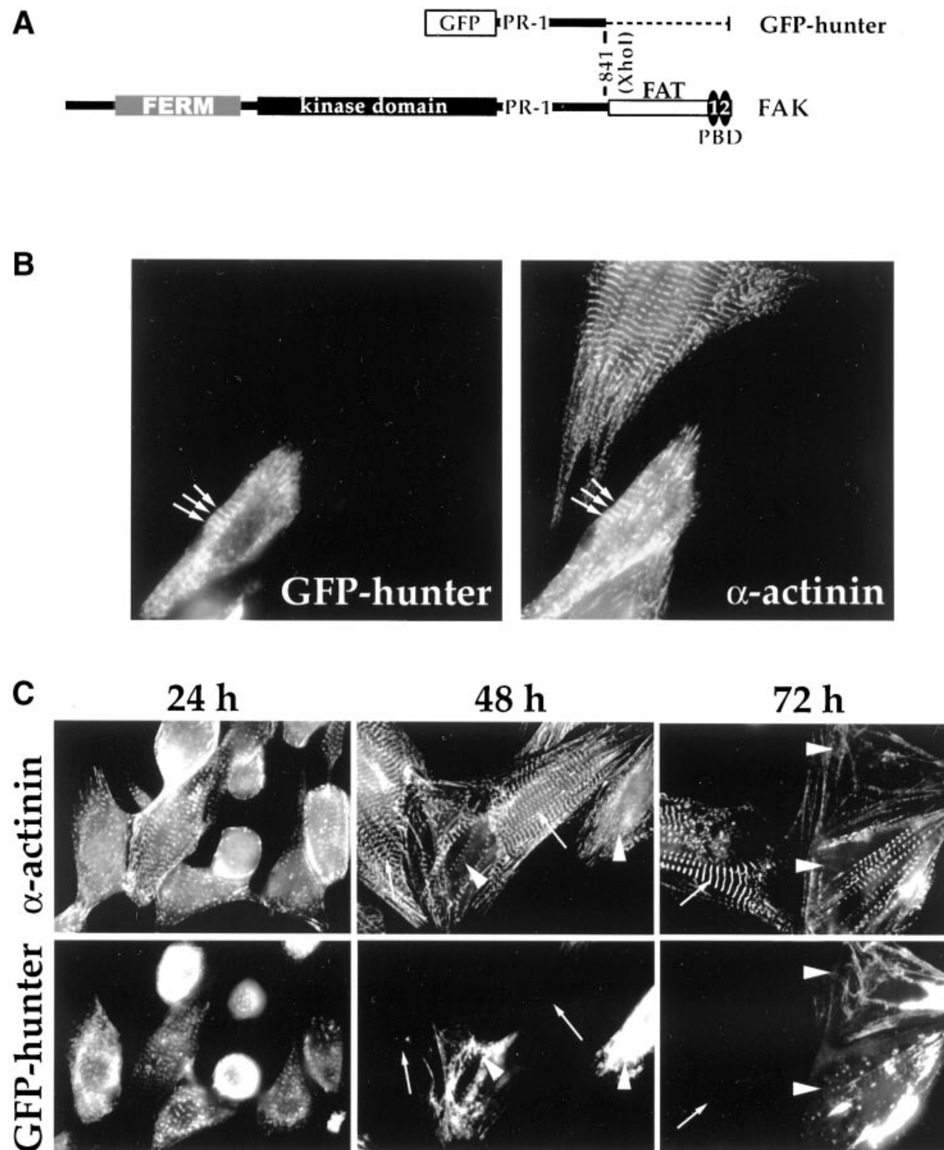

Figure 8. Effects of GFP-hunter expression in cardiac myocytes. (A) Diagram of GFP-hunter construct. PBD, paxillin-binding domains 1 and 2. (B) Localization of GFP-hunter in cardiac myocytes. Freshly isolated cardiac myocytes infected with adenovirus expressing GFP-hunter were plated on fibronectin-coated glass coverslips for immunofluorescence. At 48 h postinfection cells were fixed in 3.8% paraformaldehyde/PBS for 20 min, permeabilized in cold acetone for 10 min, and stained with anti–α-actinin antibodies (right). GFP-hunter–expressing cells were visualized by fluorescence microscopy (left). (C) Expression of GFP-hunter disrupts the sarcomeric structure in infected cardiac myocytes. Paraformaldehyde-fixed, acetone-permeabilized cells were stained with antibodies against α-actinin (top). GFP-hunter–expressing cells were visualized by fluorescence microscopy (bottom). (D) Analysis of sarcomeric organization in GFP-hunter–infected cardiac myocytes at 24, 48, and 72 h postinfection. In a series of three experiments, >1000 individual GFP-hunter–infected cells were examined for the presence of sarcomeric structures. (E) GFP-hunter attenuates ET-induced ANP gene expression. Freshly isolated uninfected (−) or GFP-hunter–infected (+) cardiac myocytes were plated on fibronectin. Twenty-four hours postinfection cells were treated for 24 h with either vehicle (CTL) or 100 nM ET-1 (+ET). Eight micrograms of total RNA were analyzed by Northern blot analysis. Membrane was hybridized with radiolabeled rat ANP cDNA. After autoradiography, membranes were stripped and reprobed with radiolabeled GAPDH cDNA. (F) Expression of dominant-negative FAK inhibits basal activity of ANP gene promoter. Neonatal cardiac myocytes were transfected with −1150hANP CAT (20 μg) alone or together with 1 or 5 μg of Cas, in the presence or absence of the indicated concentration of GFP-hunter expression vector. Cells were cultured and processed for CAT activity. Data were averaged from three separate experiments done in triplicate; values shown are means ± SD.

when compared with the basal activity of the −1150hANP CAT promoter (Figure 8F). Addition of increasing concentrations (0.5–10 μg) of the GFP-hunter expression vector resulted in a dose-dependent inhibition of both basal and Cas-stimulated CAT activity, implying that the observed reduction in ANP transcript levels is derived, at least in part, from suppressed transcriptional activity.

DISCUSSION

Protein phosphorylation plays a central role in the regulation of many cellular events, including growth and cell division, and it is widely believed that these processes participate in myocyte hypertrophy (Sugden and Clerk, 1998; Clerk and Sugden, 1999). Previously we demonstrated that ET-dependent activation of Src kinase leads to initiation of the hypertrophic program in cardiac myocytes (Kovačič *et al.*, 1998). The present study shows that FAK and Cas play an important role in integrating the cellular signaling machinery that controls the enhanced sarcomeric organization and gene expression program associated with hypertrophy. Our data suggest several conclusions: 1) stimulation of cardiac myocytes with the hypertrophic agonist ET results in increased tyrosine phosphorylation of Cas and FAK; 2) Cas,

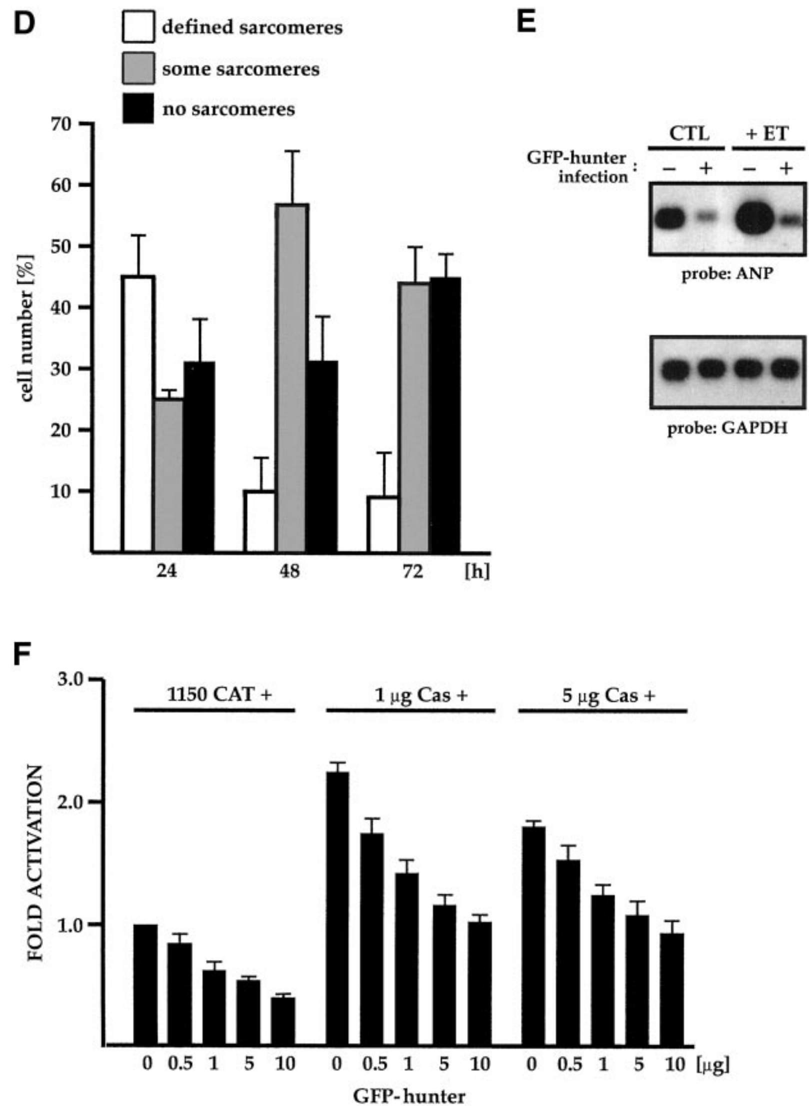

Figure 8 (cont).

FAK, paxillin, and tensin all localize to sarcomeric Z-lines, implying that these structures represent important sites of biochemical signal transduction in the cardiac myocyte; 3) introduction into cultured myocytes of GFP-FAT, a dominant-negative C-terminal fragment of FAK required for the proper targeting of FAK, inhibits ET-dependent phosphorylation of Cas and FAK. Furthermore, expression of either GFP-FAT or GFP-hunter fragment, which encodes the isolated Cas-binding region of FAK, promotes disorganization of sarcomeres and disruption of sarcomeric Z-lines; 4) both Cas and FAK are also involved, directly or indirectly, in promoting expression of the hypertrophic response genes, ANP and BNP. Thus, ectopic expression of wild-type but not mutant Cas amplified the ET- and Src-dependent stimulation of ANP promoter activity, whereas expression of dominant-negative FAT or the GFP-hunter construct almost completely abrogated ANP gene promoter activity and expression of the endogenous ANP gene. Collectively, these findings implicate Cas and FAK as key players in regulating both the increased sarcomeric organization and the gene expression program associated with cardiac myocyte hypertrophy.

Functional and Structural Similarities of Focal Adhesions and Cardiac Myocyte Z-Lines

One of the hallmarks of myocyte hypertrophy is an increase in sarcomeric organization (Hunter and Chien, 1999). Previous studies have shown that phenylephrine, ET, and AngII each induce sarcomeric assembly in cultured cardiac myocytes within 0.5–4 h after agonist stimulation (Aoki *et al.*, 1998, 2000; Clerk *et al.*, 1998). Thus, the rapid (2–5 min) ET-induced phosphorylation of Cas, FAK, and Src (Kovačič *et al.*, 1998) must precede sarcomeric assembly and may represent the primary stimulus for Z-line organization. Recruitment of three important components of the focal adhesion assembly (i.e., Cas, FAK, and paxillin), and possibly Src as well (Kovačič *et al.*, 1998), to the Z-line complex in cardiac

myocytes highlights an unexpected functional similarity between these two structures, as proposed first by Brugge (1998). This conclusion has intriguing implications. It implies that the Z-line represents a site of signal transduction involving Cas-FAK-paxillin–containing complexes that may participate in the control of contractile and/or hypertrophic responses of the myocyte. It is important to recall that tubular invaginations of sarcolemmal membranes (i.e., costameres) are located at the level of the Z-lines (Terracio *et al.*, 1989). These inward extensions of extracellular space couple cellular excitation to contraction and may provide a venue for FAK-Cas-paxillin activation by signaling molecules present in the extracellular space. The Z-line–associated complexes are also ideally positioned for regulation of sarcomeric contractile force, cytoskeletal organization, and transduction of signals from extracellular matrix to the cytoplasmic and nuclear compartments, functions that are analogous to those largely subserved by focal adhesion sites in other cell types. This raises the intriguing possibility that these complexes may serve as regulatory control centers in the myocytes, sensing and integrating biochemical and mechanical signals that ultimately lead to downstream changes in gene expression and sarcomeric organization.

What Role Does the FAK-Cas-Src Complex Play in Sarcomeric Organization?

Our studies of myocytes infected with the dominant-negative mutant GFP-FAT indicate that FAK plays a critical role in the regulation of myocyte growth and sarcomeric assembly. Sarcomeric disorder in FAT-expressing myocytes may result from blockade of nascent myofilament assembly. Alternatively, it could arise from disruption of preexisting sarcomeric units. Neonatal cardiac myocytes adhere within 3 h to the surface of a fibronectin matrix. In the presence of 10% FCS, cells maintain morphological features characteristic of intact myocytes. They resume spontaneous rhythmic beating within 12–18 h and enlarge to two to three times the original cell volume within 72 h of culture. The enlarging cells seek contact with one another and eventually form a confluent two-dimensional layer that beats spontaneously and in a synchronous manner. By comparison, GFP-FAT–infected cells, although able to attach to the matrix, preserve none of the properties mentioned above. Immunocytochemical analysis demonstrated that the earliest detectable structural response to GFP-FAT expression was seen within the α-actinin component of the Z-lines. Loss of α-actinin from Z-lines was observed as early as 24 h after infection. At that point sarcomeric proteins such as MHC were still retained in an organized array. After longer periods of GFP-FAT expression, we observed the dissociation of β-MHC from sarcomeres together with a general deterioration of Z-lines and sarcomeric structure. We propose that targeting FAT to the site of the FAK/Cas-containing Z-line–signaling complex triggers conformational changes, which, in turn, perturbs sarcomeric assembly in the myocyte. Because focal adhesion sites also appear to be altered in cells expressing GFP-FAT (see especially the loss of tensin in Figure 5), it is possible that disruption of signals from focal adhesions also contributes to the disassembly of cytoarchitecture and the gene expression program. Further studies will be needed to clarify their particular contribution.

We have shown here that FAK plays an important role in linking hypertrophic stimuli to the cellular machinery that promotes sarcomeric assembly in cardiac myocytes undergoing hypertrophy. Codistribution of FAK with several important signaling molecules (e.g., Cas, paxillin, and tensin) at sarcomeric Z-lines suggests that these proteins form physical and/or functional associations during Z-line assembly. This is further supported by the observed disruption of the interaction of FAK with paxillin by GFP-FAT. In addition, changes in phosphorylation status of Cas and FAK that are consistently seen after ET stimulation were abolished after infection with GFP-FAT (Figure 6). Finally, titration of Cas with GFP-hunter resulted in significant disruption of sarcomeric organization, implying that Cas itself plays a key role in supporting this process. Progressive assembly of paxillin, Cas, FAK, and other proteins, possibly including a newly described focal contact and Z-line–associated protein, paladin (Parasat and Otey, 2000), in the Z-line complex appears to be required to establish both functional and structural competence in this subcellular structure.

Several intracellular signaling molecules have been proposed to mediate sarcomeric organization. These include the small G-proteins Ras (Thorburn *et al.*, 1993), Rac (Pracyk *et al.*, 1998; Sussman *et al.*, 2000), and Rho (Hoshijima *et al.*, 1998), members of the extended MAP kinase family (Clerk *et al.*, 1998; Clerk and Sugden, 1999), as well as myosin light chain kinase (Aoki *et al.*, 2000). Overexpression of constitutively active forms of Ras, Rho, and Rac has been shown to mediate both increased sarcomeric organization and increased gene expression after exposure to hypertrophic stimuli (Pracyk *et al.*, 1998). Studies in other systems have documented functional interactions of a number of these signaling molecules with FAK and/or Cas (Schlaepfer *et al.*, 1999, and references therein; O'Neill *et al.*, 2000). Dissection of these functional interactions should now be feasible with the use of Cas or FAK mutant constructs that are selectively impaired in each of these signal transduction pathways

Does FAK or Cas Play a Critical Role in Sarcomeric Assembly and Activation of Hypertrophy-dependent Gene Expression In Vivo?

A conditional knock-out of cardiac FAK or Cas in adult heart would be required to address the in vivo relevance of our findings. However, several pieces of data are consistent with our in vitro conclusions. Deletion of Cas by homologous recombination results in embryonic lethality, with Cas-deficient embryos showing marked systemic congestion and growth retardation (Honda *et al.*, 1998). Histological examination of hearts from these embryos revealed poorly developed myocardia accompanied by disorganized myofibrils and disrupted Z-lines, a phenotype similar to that seen after FAT or hunter expression in our in vitro system.

Activation of FAK and its association with Cas and Src appears to be necessary for the generation of ET-dependent hypertrophy in vitro. ET increases phosphorylation of FAK and Cas in cultured myocytes (see Figures 1 and 3). AngII (Eble *et al.*, 1999), phenylephrine (Taylor *et al.*, 2000), hypoxia (Seko *et al.*, 1999a), and mechanical stretch (Seko *et al.*, 1999b) also enhance tyrosine phosphorylation of FAK in cardiac

myocytes. Collectively, these data suggest that activation of FAK and its assembly into a complex with Cas may be a shared requirement for activation of the hypertrophy program. In vivo support for this model comes from the studies of Kuppuswamy *et al.* (1997) who demonstrated association of tyrosine-phosphorylated Src, FAK, and β_3-integrin with cytoskeletal structures in a pressure overload model of right ventricular hypertrophy. More recently they extended these studies by demonstrating association of the adapter proteins Cas, Shc, and Nck with cytoskeletal structures in the same animal model of hypertrophy (Laser *et al.*, 2000). Additional studies are needed to characterize the intermediate signaling pathways positioned downstream of the FAK-Cas-paxillin complex. Their identification should be of assistance in establishing the molecular mechanisms that promote individual components of the hypertrophic phenotype.

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REFERENCES

Almeida, E.A., Ilić, D., Han, Q., Hauck, C.R., Jin, F., Kawakatsu, H., Schlaepfer, D.D., and Damsky, C.H. (2000). Matrix survival signaling: from fibronectin via focal adhesion kinase to c-jun NH(2)-terminal kinase. J. Cell Biol. *149*, 741–754.

Aoki, H., Izumo, S., and Sadoshima, J. (1998). Angiotensin II activates RhoA in cardiac myocytes: a critical role of RhoA in angiotensin II-induced premyofibril formation. Circ. Res. *82*, 666–676.

Aoki, H., Sadoshima, J., and Izumo, S. (2000). Myosin light chain kinase mediates sarcomere organization during cardiac hypertrophy in vitro. Nat. Med. *6*, 183–188.

Bellis, S.L., Miller, J.T., and Turner, C.E. (1995). Characterization of tyrosine phosphorylation of paxillin *in vitro* by focal adhesion kinase. J. Biol. Chem. *270,* 17437–17441.

Bogoyevitch, M.A., Glennon, P.E., Andersson, M.B., Clerk, A., Lazou, A., Marshall, C.J., Parker, P.J., and Sugden, P.H. (1994). Endothelin-1 and fibroblast growth factors stimulate the mitogen-activated protein kinase signaling cascade in cardiac myocytes: the potential role of the cascade in the integration of two signaling pathways leading to myocyte hypertrophy. J. Biol. Chem. *269*, 1110–1119.

Brown, M.C., Perrotta, J.A., and Turner, C.E. (1996). Identification of LIM3 as the principal determinant of paxillin focal adhesion localization and characterization of a novel motif on paxillin directing vinculin and focal adhesion kinase binding. J. Cell Biol. *135*, 1109–1123.

Brugge, J.S. (1998). Casting light on focal adhesions. Nat. Genet. *19*, 309–311.

Casamassima, A., and Rozengurt, E. (1997). Tyrosine phosphorylation of p130(cas) by bombesin, lysophosphatidic acid, phorbol esters, and platelet-derived growth factor: signaling pathways and formation of a p130(cas)-Crk complex. J. Biol. Chem. *272*, 9363–9370.

Cazaubon, S., Chaverot, N., Romero, I.A., Girault, J.A., Adamson, P., Strosberg, A.D., and Couraud, P.O. (1997). Growth factor activity of endothelin-1 in primary astrocytes mediated by adhesion-dependent and -independent pathways. J. Neurosci. *17*, 6203–6212.

Chen, H.C., Appeddu, P.A., Parsons, J.T., Hildebrand, J.D., Schaller, M.D., and Guan, J.L. (1995). Interaction of focal adhesion kinase with cytoskeletal protein talin. J. Biol. Chem. *270*, 16995–16999.

Chien, K.R., Knowlton, K.U., Zhu, H., and Chien, S. (1991). Regulation of cardiac gene expression during myocardial growth and hypertrophy: molecular studies of an adaptive physiologic response. FASEB J. *5*, 3037–3046.

Chien, K.R., Zhu, H., Knowlton, K.U., Miller-Hance, W., van-Bilsen, M., O'Brien, T.X., and Evans, S.M. (1993). Transcriptional regulation during cardiac growth and development. Annu. Rev. Physiol. *55*, 77–95.

Clark, E.A., and Brugge, J.S. (1995). Integrins and signal transduction pathways: the road taken. Science *268*, 233–239.

Clerk, A., Michael, A., and Sugden, P.H. (1998). Stimulation of the p38 mitogen-activated protein kinase pathway in neonatal rat ventricular myocytes by the G protein-coupled receptor agonists, endothelin-1 and phenylephrine: a role in cardiac myocyte hypertrophy? J. Cell Biol. *142*, 523–535.

Clerk, A., and Sugden, P.H. (1999). Activation of protein kinase cascades in the heart by hypertrophic G protein-coupled receptor agonists. Am. J. Cardiol. *83*, 64H–69H.

Eble, D.M., Qi, M., Strait, J.B., and Samarel, A.M. (1999). Contraction Dependent Hypertrophy of Neonatal Rat Ventricular Myocytes: Potential Role for Focal Adhesion Kinase, Boston, MA: Kluwer Academic.

Eble, D.M., Strait, J.B., Govindarajan, G., Lou, J., Byron, K.L., and Samarel, A.M. (2000). Endothelin-induced cardiac myocyte hypertrophy: role for focal adhesion kinase. Am. J. Physiol. Heart Circ. Physiol. *278*, H1695–1707.

Fuller, S.J., Gillespie-Brown, J., and Sugden, P.H. (1998). Oncogenic src, raf, and ras stimulate a hypertrophic pattern of gene expression and increase cell size in neonatal rat ventricular myocytes. J. Biol. Chem. *273*, 18146–18152.

Gervais, F.G., Thornberry, N.A., Ruffolo, S.C., Nicholson, D.W., and Roy, S. (1998). Caspases cleave focal adhesion kinase during apoptosis to generate a FRNK-like polypeptide. J. Biol. Chem. *273*, 17102–17108.

Giancotti, F.G., and Ruoslahti, E. (1999). Integrin signaling. Science *285*, 1028–1032.

Haneda, M., Kikkawa, R., Koya, D., Shikano, T., Sugimoto, T., Togawa, M., and Shigeta, Y. (1995). Endothelin-1 stimulates tyrosine phosphorylation of p125 focal adhesion kinase in mesangial cells. J. Am. Soc. Nephrol. *6*, 1504–1510.

Hanke, J.H., Gardner, J.P., Dow, R.L., Changelian, P.S., Brissette, W.H., Weringer, E.J., Pollok, B.A., and Connelly, P.A. (1996). Discovery of a novel, potent, and Src family-selective tyrosine kinase inhibitor: study of Lck- and FynT-dependent T cell activation. J. Biol. Chem. *271*, 695–701.

Hardy, S., Kitamura, M., Harris-Stansil, T., Dai, Y., and Phipps, M.L. (1997). Construction of adenovirus vectors through Cre-lox recombination. J. Virol. *71*, 1842–1849.

Harte, M.T., Hildebrand, J.D., Burnham, M.R., Bouton, A.H., and Parsons, J.T. (1996). p130Cas, a substrate associated with v-Src and v-Crk, localizes to focal adhesions and binds to focal adhesion kinase. J. Biol. Chem. *271*, 13649–13655.

Hildebrand, J.D., Schaller, M.D., and Parsons, J.T. (1993). Identification of sequences required for the efficient localization of the focal adhesion kinase, pp125FAK, to cellular focal adhesions. J. Cell Biol. *123*, 993–1005.

Honda, H., Oda, H., Nakamoto, T., Honda, Z., Sakai, R., Suzuki, T., Saito, T., Nakamura, K., Nakao, K., Ishikawa, T., Katsuki, M., Yazaki, Y., and Hirai, H. (1998). Cardiovascular anomaly, impaired actin bundling and resistance to Src-induced transformation in mice lacking p130Cas. Nat. Genet. *19*, 361–365.

Hoshijima, M., Sah, V.P., Wang, Y., Chien, K.R., and Brown, J.H. (1998). The low molecular weight GTPase Rho regulates myofibril formation and organization in neonatal rat ventricular myocytes: involvement of Rho kinase. J. Biol. Chem. *273*, 7725–7730.

Hunter, J.J., and Chien, K.R. (1999). Signaling pathways for cardiac hypertrophy and failure. N. Engl. J. Med. *341*, 1276–1283.

Ilić, D., Almeida, E.A., Schlaepfer, D.D., Dazin, P., Aizawa, S., and Damsky, C.H. (1998). Extracellular matrix survival signals transduced by focal adhesion kinase suppress p53-mediated apoptosis. J. Cell Biol. *143*, 547–560.

Ilić, D., Furuta, Y., Kanazawa, S., Takeda, N., Sobue, K., Nakatsuji, N., Nomura, S., Fujimoto, J., Okada, M., Yamamoto, T., and Aizawa, S. (1995). Reduced cell motility and enhanced focal adhesion contact formation in cells from FAK-deficient mice. Nature *377*, 539–544.

Knowlton, K.U., Baracchini, E., Ross, R.S., Harris, A.N., Henderson, S.A., Evans, S.M., Glembotski, C.C., and Chien, K.R. (1991). Co-regulation of the atrial natriuretic factor and cardiac myosin light chain-2 genes during alpha-adrenergic stimulation of neonatal rat ventricular cells: identification of *cis* sequences within an embryonic and a constitutive contractile protein gene which mediate inducible expression. J. Biol. Chem. *266*, 7759–7768.

Kovačič, B., Ilić, D., Damsky, C.H., and Gardner, D.G. (1998). c-Src activation plays a role in endothelin-dependent hypertrophy of the cardiac myocyte. J. Biol. Chem. *273*, 35185–35193.

Kovačič-Milivojević, B., and Gardner, D.G. (1992). Divergent regulation of the human atrial natriuretic peptide gene by c-jun and c-fos. Mol. Cell. Biol. *12*, 292–301.

Kovačič-Milivojević, B., Wong, V.S., and Gardner, D.G. (1996). Selective regulation of the atrial natriuretic peptide gene by individual components of the activator protein-1 complex. Endocrinology *137*, 1108–1117.

Kovačič-Milivojević, B., Zlock, D.W., and Gardner, D.G. (1997). Ras inhibits Jun-activated human atrial natriuretic peptide gene transcription in cultured ventricular myocytes. Circ. Res. *80*, 580–588.

Kuppuswamy, D., Kerr, C., Narishige, T., Kasi, V.S., Menick, D.R., and Cooper, G.T. (1997). Association of tyrosine-phosphorylated c-Src with the cytoskeleton of hypertrophying myocardium. J. Biol. Chem. *272*, 4500–4508.

LaPointe, M.C., Wu, J.P., Greenberg, B., and Gardner, D.G. (1988). Upstream sequences confer atrial-specific expression on the human atrial natriuretic factor gene. J. Biol. Chem. *263*, 9075–9078.

Laser, M., Willey, C.D., Jiang, W., Cooper, G., Menick, D.R., Zile, M.R., and Kuppuswamy, D. (2000). Integrin activation and focal complex formation in cardiac hypertrophy. J. Biol. Chem. *275*, 35624–35630.

Lo, S.H., Weisberg, E., and Chen, L.B. (1994). Tensin: a potential link between the cytoskeleton and signal transduction. Bioessays *16*, 817–823.

Martin, S.J., Reutelingsperger, C.P., McGahon, A.J., Rader, J.A., van Schie, R.C., LaFace, D.M., and Green, D.R. (1995). Early redistribution of plasma membrane phosphatidylserine is a general feature of apoptosis regardless of the initiating stimulus: inhibition by overexpression of Bcl-2 and Abl. J. Exp. Med. *182*, 1545–1556.

Nakamoto, T., Sakai, R., Honda, H., Ogawa, S., Ueno, H., Suzuki, T., Aizawa, S., Yazaki, Y., and Hirai, H. (1997). Requirements for localization of p130cas to focal adhesions. Mol. Cell. Biol. *17*, 3884–3897.

Nakamura, I., Jimi, E., Duong, L.T., Sasaki, T., Takahashi, N., Rodan, G.A., and Suda, T. (1998). Tyrosine phosphorylation of p130Cas is involved in actin organization in osteoclasts. J. Biol. Chem. *273*, 11144–11149.

O'Neill, G.M., Fashena, S.J., and Golemis, E.A. (2000). Integrin signaling: a new Cas(t) of characters enters the stage. Trends Cell Biol. *10*, 111–119.

Parasat, M., and Otey, C. (2000). Characterization of palladin, a novel protein localized to stress fibers and cell adhesion. J. Cell Biol. *150*, 643–655.

Polte, T.R., and Hanks, S.K. (1995). Interaction between focal adhesion kinase and Crk-associated tyrosine kinase substrate p130Cas. Proc. Natl. Acad. Sci. USA *92*, 10678–10682.

Polte, T.R., and Hanks, S.K. (1997). Complexes of focal adhesion kinase (FAK) and Crk-associated substrate (p130(Cas)) are elevated in cytoskeleton-associated fractions following adhesion and Src transformation: requirements for Src kinase activity and FAK proline-rich motifs. J. Biol. Chem. *272*, 5501–5509.

Pracyk, J.B., Tanaka, K., Hegland, D.D., Kim, K.S., Sethi, R., Rovira, II, Blazina, D.R., Lee, L., Bruder, J.T., Kovesdi, I., Goldshmidt-Clermont, P.J., Irani, K., and Finkel, T. (1998). A requirement for the rac1 GTPase in the signal transduction pathway leading to cardiac myocyte hypertrophy. J. Clin. Invest. *102*, 929–937.

Sadoshima, J., and Izumo, S. (1997). The cellular and molecular response of cardiac myocytes to mechanical stress. Annu. Rev. Physiol. *59*, 551–571.

Sadoshima, J., Qiu, Z., Morgan, J.P., and Izumo, S. (1996). Tyrosine kinase activation is an immediate and essential step in hypotonic cell swelling-induced ERK activation and c-fos gene expression in cardiac myocytes. EMBO J. *15*, 5535–5546.

Sakai, R., Iwamatsu, A., Hirano, N., Ogawa, S., Tanaka, T., Mano, H., Yazaki, Y., and Hirai, H. (1994). A novel signaling molecule, p130, forms stable complexes in vivo with v-Crk and v-Src in a tyrosine phosphorylation-dependent manner. EMBO J. *13*, 3748–3756.

Schaller, M.D., and Parsons, J.T. (1995). pp125FAK-dependent tyrosine phosphorylation of paxillin creates a high-affinity binding site for Crk. Mol. Cell. Biol. *15*, 2635–2645.

Schaub, M.C., Hefti, M.A., Harder, B.A., and Eppenberger, H.M. (1997). Various hypertrophic stimuli induce distinct phenotypes in cardiomyocytes. J. Mol. Med. *75*, 901–920.

Schlaepfer, D.D., Hauck, C.R., and Sieg, D.J. (1999). Signaling through focal adhesion kinase. Prog. Biophys. Mol. Biol. *71*, 435–478.

Seko, Y., Takahashi, N., Sabe, H., Tobe, K., Kadowaki, T., and Nagai, R. (1999a). Hypoxia induces activation and subcellular translocation of focal adhesion kinase (p125(FAK)) in cultured rat cardiac myocytes. Biochem. Biophy. Res. Commun. *262*, 290–296.

Seko, Y., Takahashi, N., Tobe, K., Kadowaki, T., and Yazaki, Y. (1999b). Pulsatile stretch activates mitogen-activated protein kinase (MAPK) family members and focal adhesion kinase (p125(FAK)) in cultured rat cardiac myocytes. Biochem. Biophys. Res. Commun. *259*, 8–14.

Shen, Y., and Schaller, M.D. (1999). Focal adhesion targeting: the critical determinant of FAK regulation and substrate phosphorylation. Mol. Biol. Cell *10*, 2507–2518.

Shubeita, H.E., McDonough, P.M., Harris, A.N., Knowlton, K.U., Glembotski, C.C., Brown, J.H., and Chien, K.R. (1990). Endothelin induction of inositol phospholipid hydrolysis, sarcomere assembly, and cardiac gene expression in ventricular myocytes: a paracrine mechanism for myocardial cell hypertrophy. J. Biol. Chem. *265*, 20555–20562.

Simpson, D.G., Decker, M.L., Clark, W.A., and Decker, R.S. (1993). Contractile activity and cell-cell contact regulate myofibrillar organization in cultured cardiac myocytes. J. Cell Biol. *123*, 323–336.

Sugden, P.H., and Clerk, A. (1998). Cellular mechanisms of cardiac hypertrophy. J. Mol. Med. *76*, 725–746.

Sussman, M.A., Welch, S., Walker, A., Klevitsky, R., Hewett, T.E., Price, R.L., Schaefer, E., and Yager, K. (2000). Altered focal adhesion regulation correlates with cardiomyopathy in mice expressing constitutively active rac1. J. Clin. Invest. *105*, 875–886.

Tachibana, K., Sato, T., D'Avirro, N., and Morimoto, C. (1995). Direct association of pp125FAK with paxillin, the focal adhesion-targeting mechanism of pp125FAK. J. Exp. Med. *182*, 1089–1099.

Taylor, J.M., Rovin, J.D., and Parsons, J.T. (2000). A role for focal adhesion kinase in phenylephrine-induced hypertrophy of rat ventricular cardiomyocytes. J. Biol. Chem. *275*, 19250–19257.

Terracio, L., Gullberg, D., Rubin, K., Craig, S., and Borg, T.K. (1989). Expression of collagen adhesion proteins and their association with the cytoskeleton in cardiac myocytes. Anat. Rec. *223*, 62–71.

Thorburn, A., Thorburn, J., Chen, S.Y., Powers, S., Shubeita, H.E., Feramisco, J.R., and Chien, K.R. (1993). HRas-dependent pathways can activate morphological and genetic markers of cardiac muscle cell hypertrophy. J. Biol. Chem. *268*, 2244–2249.

Turner, C.E. (1998). Paxillin. Int. J. Biochem. Cell Biol. *30*, 955–959.

Turner, C.E., Brown, M.C., Perrotta, J.A., Riedy, M.C., Nikolopoulos, S.N., McDonald, A.R., Bagrodia, S., Thomas, S., and Leventhal, P.S. (1999). Paxillin LD4 motif binds PAK and PIX through a novel 95-kD ankyrin repeat, ARF-GAP protein: a role in cytoskeletal remodeling. J. Cell Biol. *145*, 851–863.

Vigoreaux, J.O. (1994). The muscle Z band: lessons in stress management. J. Muscle Res. Cell Motil. *15*, 237–255.

Xu, L.H., Yang, X., Craven, R.J., and Cance, W.G. (1998). The COOH-terminal domain of the focal adhesion kinase induces loss of adhesion and cell death in human tumor cells. Cell Growth Differ. *9*, 999–1005.

Zachary, I., Sinnett-Smith, J., Turner, C.E., and Rozengurt, E. (1993). Bombesin, vasopressin, and endothelin rapidly stimulate tyrosine phosphorylation of the focal adhesion-associated protein paxillin in Swiss 3T3 cells. J. Biol. Chem. *268*, 22060–22065.