Association of Tyrosine-phosphorylated c-Src with the Cytoskeleton of Hypertrophying Myocardium

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Given the central position of the focal adhesion complex, both physically in coupling integrins to the extracellular matrix and biochemically in providing an upstream site for anabolic signal generation, we asked whether the recruitment of non-receptor tyrosine kinases to the cytoskeleton might be a mechanism whereby cellular load could generate growth regulatory signals responsible for cardiac hypertrophy. Analysis revealed cytoskeletal association of c-Src, FAK, and β3-integrin, but no Fyn, in the pressure-overloaded right ventricle. This association was seen as early as 4 h after right ventricular pressure overloading, increased through 48 h, and reverted to normal in 1 week. Cytoskeletal binding of non-receptor tyrosine kinases was synchronous with tyrosine phosphorylation of several cytoskeletal proteins, including c-Src. Examination of cytoskeleton-bound c-Src revealed that a significant portion of the tyrosine phosphorylation was not at the Tyr-527 site and therefore presumably was at the Tyr-416 site. Thus, these studies strongly suggest that non-receptor tyrosine kinases, in particular c-Src, may play a critical role in hypertrophic growth regulation by their association with cytoskeletal structures, possibly via load activation of integrin-mediated signaling.

Hypertrophy is one of the major compensatory mechanisms available to the heart for sustaining chronic hemodynamic overloads. There are two types of hemodynamic overloads: (i) pressure overload (systolic stress), in which a normal blood volume is pumped during each cardiac cycle against an increased impedance; and (ii) volume overload (diastolic stress and strain), in which an increased blood volume is pumped during each cardiac cycle against a normal impedance (for review, see Ref. 1). The hypertrophic response to these hemodynamic overloads consists of both qualitative changes in the expression of specific proteins and a quantitative increase in total cardiocyte protein. It is well documented that compensatory hypertrophy of cardiac muscle in specific hemodynamic settings eventually results in a decrease in contractile performance, even prior to the onset of heart failure (2). Indeed, recent studies from this laboratory have shown that there is a cellular basis for contractile dysfunction of pressure-hypertrophied car-

diac muscle involving changes in the cardiocyte cytoskeleton (2, 3). However, a central question has not yet been answered: what are the mechanisms by which changes in cardiac load are coupled to the modulation of intracellular signals that are responsible for changes in cardiocyte mass and phenotype?

Since we know that the terminally differentiated adult cardiocyte is directly responsive to load input in terms of growth regulation (1), we have chosen to focus this study on potential mechanisms whereby cellular load input could activate growth regulatory signaling pathways. In this context, it is known that receptor-mediated protein tyrosine phosphorylation of various intracellular substrates plays a major role during both growth induction and the maintenance of differentiation. In addition to these established receptor-mediated regulatory pathways, there is recent evidence for synergistic growth regulation by integrin-mediated non-receptor tyrosine kinase pathways. Integrins are transmembrane proteins that provide tight adhesion of cells to extracellular matrix proteins at sites referred to as focal adhesions and that connect the extracellular matrix proteins to intracellular cytoskeletal proteins (4–10). Although integrins are transmembrane proteins bearing short cytoplasmic domains, they do not exhibit intrinsic tyrosine kinase activity. Therefore, a recently identified non-receptor tyrosine kinase known as FAK (focal adhesion kinase) has been proposed as a prime candidate for transmitting integrin-mediated signaling, and ligation of integrins with their extracellular ligands increases tyrosine phosphorylation of several proteins, including FAK (4–12). FAK has been shown to physically associate with at least two non-receptor tyrosine kinases, c-Src and Fyn, after autophosphorylation and activation (13). Interestingly, it has been shown in platelets that thrombin-induced platelet aggregation mediated by fibrinogen binding to integrins (glycoprotein IIb-IIIa) results in a redistribution of c-Src and Yes to cytoskeletal actin filaments, and this redistribution is associated with tyrosine phosphorylation of several cytoskeleton-associated proteins (14–16). Such phosphorylation has been suggested as a mechanism for the cytoskeletal rearrangement seen during platelet activation. In addition, cytoskeleton-membrane junctions are believed to contain binding domains for Src family kinases, and phosphorylation mediated by these kinases could be important for signal transmission between the extracellular matrix and the intracellular cytoskeleton (16). Thus, at least in terminally differentiated cells such as platelets, integrin-ligand interactions could cause cytoskeletal reorganization of several proteins, and Src family kinases could be a critical factor in such events.

We know that a specific cytoskeletal change involving tubulin up-regulation is one phenotypic consequence of cardiac hypertrophy (2, 3). In view of this fact and the information given above, we are now asking whether load-induced integrin-cy-
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Isolation of Cardiac Cytoskeleton—Cytoskeletal preparations were made as described for platelets by Fox et al. (16), with minor modifications. Briefly, 100 mg of LV or cytoskeletal pellet obtained from each control or pressure-overloaded hearts was minced and transferred into a tube of 2 ml of ice-cold Trips/Tris extraction buffer containing 100 mM Tris-HCl, pH 7.4, 2% Triton X-100, 0.5 mM phenylmethylsulfonyl fluoride, 10 μM leupeptin, 10 μM aprotinin, 2 μM pepstatin, 2 μM E-64, 1 mM dithiothreitol, and 10 mM EGTA. The tissue was immediately homogenized using a Tekmar Tissuemizer at a 50% power setting for 40 s; the Triton X-100-insoluble pellet (cytoskeletal fraction rich in actin filaments) was obtained by centrifugation at 15,000 × g for 5 min. The insoluble cytoskeletal pellet was re-extracted once with 1 ml of the same buffer and centrifuged as described above. To the pellet was then added 500 μl of Laemmli SDS sample buffer, after which it was vortexed and boiled for 5 min. After centrifugation, the supernatant, referred to as the Triton X-100-insoluble high-spin fraction, was saved for further analysis. The Triton X-100 supernatant from the original low-speed centrifugation was put in 10 ml Beckman tube and centrifuged at 100,000 × g for 2.5 h in a Ti-50 rotor. The pellet obtained after centrifugation was extracted in 1 ml of SDS sample buffer and processed as described above. This sample contains largely membrane skeleton (16). The supernatant, referred to as Triton X-100-soluble material, was mixed with equal volumes of SDS sample buffer and boiled for 5 min. The volumes of material obtained from 100 mg of tissue were as follows: 4 ml of Triton X-100-soluble material, 500 μl of Triton X-100-insoluble low-spin fraction (cytoskeleton), and 1 ml of Triton X-100-insoluble high-spin fraction (membrane skeleton).

Western Blot Analysis—For immunoblot analysis of Triton X-100-lysed fractions, 30 μl of samples boiled in SDS buffer was resolved by SDS-PAGE and transferred to Immobilon-P membranes. The blots were blocked for 2 h with 10% milk protein in TBST buffer (10 mM Tris, pH 7.4, 150 mM NaCl, and 0.1% Tween) and incubated with primary antibodies. The following primary antibodies were commercially obtained. Monoclonal antibodies were from Upstate Biotechnology, Inc for c-Src (GD11) and β3-integrin (CD29); Transduction Laboratory for Lyn (C42), FAK (C77), β3-integrin (C26), and annexin II (C5); and Chemicon International, Inc. for actin (1501). Polyclonal antibodies were from Santa Cruz for Fyn (FYN3) and Lyn and from Transduction Laboratory for YES. The monoclonal antibody against c-Src that specifically recognizes Tyr-527 unphosphorylated kinase was obtained as a generous gift from Dr. Koji Owada. Incubations with primary antibodies were done for either 2 h at room temperature or overnight at 4°C. Blots were washed five times with TBST buffer for 5 min and incubated for 1 h with horseradish peroxidase-conjugated secondary antibodies (Amer sham Corp.). After incubation, the blots were washed with TBST buffer once, and the tyrosine-phosphorylated proteins were detected by enhanced chemiluminescence (ECL, Amersham Corp.).

Cytoskeletal association of c-Src, FAK, and 3-integrin is seen as early as 4 h after pressure overloading and is maximal at 48 h. (ii) There is tyrosine phosphorylation of several cytoskeletal-associated proteins including c-Src. (iii) At least part of the cytoskeletal-associated c-Src exists in the active form. (iv) There is reversion of these early cytoskeletal changes to their base-line state after long-term pressure overloading when the hypertrophic growth response is complete. (v) The movement of c-Src to the cytoskeleton that was observed in cardiac tissue was also found in cardiocytes isolated from that tissue. (vi) Several other Src family members do not participate in this cytoskeletal association. Interestingly, c-Src, as a tyrosine-phosphorylated kinase, was exclusively present in the cytoskeletal fraction of pressure-overloaded myocardium and was completely absent in the normal heart.

Materials and Methods

Experimental Animal Models—Cats with two types of right ventricular pressure overload (RVPO), long-term and acute, were prepared as we have described before (17, 18). Long-term pressure overload of the right ventricle (RV) was induced by partially occluding the pulmonary artery with a 3.2-mm internal diameter band (17). Short-term RVPO (4 h) was induced by partial occlusion of the pulmonary artery by transvenous insertion of a balloon-tipped catheter (18). In both cases, RV pressure was more than doubled, while systemic arterial pressure remained unaltered. Controls, as appropriately specified, consisted of normal cats, sham-operated cats submitted to thoracotomy and pericardiotomy without hemodynamic intervention, or the normally loaded left ventricle (LV) from each animal having either short-term or long-term RVPO. All operative procedures were carried out in cats weighing 2.6–3.9 kg under full surgical anesthesia with meperidine (2.2 mg/kg) and atropine (0.2 mg/kg) and placed on oxygen. A left thoracotomy was performed, and the aorta was then cannulated; the coronary arteries were perfused with phosphate-buffered saline; and the free walls from the LV and RV were removed for immediate processing. Cardiocytes from normal and term RVPO. Alloperative procedures were carried out in cats weighing 2.6–3.9 kg under full surgical anesthesia with meperidine (2.2 mg/kg) and atropine (0.2 mg/kg). The samples were then mixed with or without anti-c-Src monoclonal antibody GD11 (1 μg) and incubated overnight at 4°C. Prewashed protein G beads (10 μl; Pierce) were then added, and incubation was continued for an additional 1 h. The immune complexes were washed three times with radioimmunoprecipitation assay buffer and boiled with 15 μl of SDS sample buffer. The proteins were separated by SDS-PAGE and transferred to Immobilon-P membrane for Western blot analysis with anti-tyrosine-phosphorylase c-Src antibody as described above. Immunoprecipitation and detection of tyrosine-phosphorylated c-Src were carried out as follows. 50 mg of ventricular tissue or cytoskeletal pellet was extracted with 0.5 ml of Laemmli SDS sample buffer containing 1 mM vanadate and boiled immediately for 5 min. After centrifugation, 100 μl of the supernatant was diluted in radioimmunoprecipitation assay buffer (20 mM Tris, pH 7.0, 150 mM NaCl, 1% Nonidet P-40, 1% sodium deoxycholate, 100 μg/ml aprotinin, 100 μg/ml phenylmethylsulfonyl fluoride, 1 mM EDTA, and 1 mM sodium orthovanadate, with the final SDS concentration being adjusted to 0.2%). The samples were then mixed with or without anti-c-Src monoclonal antibody GD11 (1 μg) and incubated overnight at 4°C. Prewashed protein G beads (10 μl; Pierce) were then added, and incubation was continued for an additional 1 h. The immune complexes were washed three times with radioimmuno precipitation assay buffer and boiled with 15 μl of SDS sample buffer. The proteins were separated by SDS-PAGE before Western blot analysis with anti-tyrosine-phosphorylated c-Src antibody as described above. For immunoprecipitating tyrosine-phosphorylated proteins, a similar protocol was followed to extract the proteins with SDS sample buffer, followed by dilution in radioimmunoprecipitation assay buffer. 100 μl of total lysate or Triton X-100-soluble fractions was diluted as described above, and biotin-conjugated anti-phosphotyrosine antibody (10 μl; Transduction Laboratory) was added and incubated for 1 h at 4°C. The immune complex then was precipitated with streptavidin-conjugated agarose beads (Upstate Biotechnology, Inc.) for 30 min and washed with radioimmunoprecipitation assay buffer. After boiling the immune complex with SDS sample buffer, the proteins were separated by SDS-PAGE and analyzed for c-Src as described above.

Measurement of Cytoskeleton-associated Kinase Activity—Cytoskeletal-associated kinase activity was assayed after suspending the Triton X-100-insoluble low-spin pellet (cytoskeleton) in kinase assay buffer (50 mM β-glycerophosphate, 1 mM dithiothreitol, and 1 mM EGTA); phosphorylation was performed as described by Kuppuswamy et al. (20) in the presence or absence of 2 mM synthetic peptide corresponding to amino acids 6–20 of p34 Arrestin (21).

Results

Cytoskeletal Redistribution of Non-receptor Tyrosine Kinases and Integrins in Pressure-overloaded Myocardium—Ventricular tissue samples obtained from either control cats or RVPO cats were homogenized in Triton X-100 buffer and centrifuged to obtain low-spin (15,000 × g) and high-spin (100,000 × g) pellets (actin-rich cytoskeleton and membrane skeleton) as well as high-spin supernatant (soluble fraction) as described under

1 The abbreviations used are: RVPO, right ventricular pressure overload; RV, right ventricle; LV, left ventricle; PAGE, polyacrylamide gel electrophoresis.
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**FIG. 1.** Western blot analysis for the detection of actin, c-Src, FAK, and β3-integrin in Triton X-100-lysed ventricular subfractions. Ventricular tissue samples were processed to obtain Triton X-100-lysed subfractions, namely soluble, low-spin (cytoskeleton), and high-spin (membrane skeleton) fractions as described under "Materials and Methods." The proteins were resolved by SDS-PAGE and Western-blotted with specific antibodies. In the case of c-Src, monoclonal antibody GD11, which recognizes all forms of c-Src, was employed. For RVPO, the pulmonary artery was partially occluded for various time periods either by inflating a balloon catheter within it for 4 h or by pulmonary artery-bandng for 48 h or longer. A represents the sham-operated control, and B–D represent 4 h, 48 h, and 1 week of RVPO, respectively. Lanes 1, 3, and 5 represent normally loaded same-animal control LV, and lanes 2, 4, and 6 represent either normally loaded RV or pressure-overloaded RV, as indicated.

"Materials and Methods." Fox et al. (16) have shown that both the Triton X-100-insoluble low-spin fraction (cytoskeleton) and the high-spin fraction (membrane skeleton) are rich in actin, released from long and short actin filaments, respectively. Western blots displaying the distribution of actin, c-Src, FAK, and β3-integrin for the sham-operated control (A), 4-h RVPO (B), 48-h RVPO (C), and 1-week RVPO (D) are shown in Fig. 1. The levels of these proteins in each fraction were compared between the normally loaded same-animal control LV and pressure-overloaded RV samples as well as fractions from sham-operated controls (Fig. 1A).

The low-spin pellets (cytoskeleton) contained higher amounts of actin relative to the high-spin pellets (membrane skeleton) or soluble fractions. This was true even after taking into account the volume of samples loaded for each Triton X-100-lysed subfraction. More important, in all of the experimental cats, the actin present in each set of LV and RV samples showed no observable differences, suggesting that the net amount of actin per unit mass of ventricular tissue is not changed by pressure overloading. Furthermore, it suggests that the extraction procedure was performed uniformly for both normal and pressure-overloaded ventricles. The presence of very low amounts of actin in the Triton X-100-soluble fractions indicates that the cytoskeletal and membrane skeletal proteins were not significantly disrupted during the isolation procedures.

The majority of c-Src, FAK, and β3-integrin was present in the Triton X-100-soluble fractions, just as has been reported by Fox et al. (16) for platelet preparations. They were either absent or present at very low levels in the Triton X-100-insoluble low- and high-spin fractions of all normally loaded ventricles. It should be noted that in sham-operated control cats, where both ventricles were normally loaded, we found no major difference between these two ventricles when the levels of any of these three proteins were compared for each set of Triton X-100-lysed subfractions.

When a similar analysis was done for a 4-h RVPO cat (Fig. 1B), the Triton X-100-soluble fraction showed no significant difference in the levels of c-Src, FAK, and β3-integrin when compared with the normally loaded same-animal control LV. However, the Triton X-100-insoluble low-spin fraction (cytoskeleton) prepared from the pressure-overloaded RV (lane 4) showed a significant amount of cytoskeleton-associated c-Src, which is completely absent in the similarly prepared and normally loaded control LV (lane 3). In addition, FAK and β3-integrin also showed increased association with the Triton X-100-insoluble fraction (cytoskeleton) in the pressure-overloaded ventricle.

Changes associated with 4-h RVPO were found to be more pronounced when RV pressure overloading was extended for 48 h (Fig. 1C). c-Src was significantly increased in the cytoskeletal fraction and was present as a double band (lane 4), probably representing variable sizes due to phosphorylation or partial proteolysis (22). Based on semiquantitation of the blots and the sample volume used for Western analysis, the cytoskeleton-associated c-Src in RVPO represents at least 30–40% of the total c-Src present in ventricular tissue. FAK association with the cytoskeleton also was found to be significantly increased upon pressure overloading for 48 h (Fig. 1C, compare lanes 4 and 3 for FAK), which represents 25% of the total ventricular FAK. However, in the case of β3-integrin, only a moderate increase in its association with the cytoskeletal fraction of the pressure-overloaded RV was noticed. On the other hand, a significant increase in the association of β3-integrin with the membrane skeleton due to pressure overloading was observed. All of these changes in the cytoskeletal redistribution of c-Src, FAK, and β3-integrin returned to their normal levels when RVPO was prolonged for 1 week (Fig. 1D), and the same finding was observed for RVPO durations as long as 5 weeks (data not shown).

The above experiments with RVPO for various time periods indicate that c-Src and FAK (and to a lesser degree, β3-integrins) redistribute to the Triton X-100-insoluble cytoskeletal pellet and that these changes are seen as early as 4 h, are maximal by 48 h, and return to their normal levels by 1 week of RVPO. These results were confirmed in at least three cats for each time point.

**Tyrosine Phosphorylation of Cytoskeleton-associated Proteins during Ventricular Pressure Overloading—**Both c-Src and FAK are members of the non-receptor tyrosine kinase group, and their cytoskeletal redistribution would be expected to cause phosphorylation of several proteins. Therefore, we examined the levels of tyrosine-phosphorylated proteins in the Triton X-100-lysed subfractions of a 48-h RVPO cat. The detergent-soluble fractions obtained from the normally loaded LV and the pressure-overloaded RV (Fig. 2, lanes 1 and 2, respectively) contained several tyrosine-phosphorylated proteins, although no significant changes between these two lanes were noticed. However, when such comparisons were made in the Triton X-100-insoluble low-spin fractions (cytoskeleton), the pressure-overloaded RV fraction showed several tyrosine-phosphorylated proteins of varying molecular sizes that were either completely absent or present at low levels in the control LV fraction (compare lanes 4 and 3). Two of the protein bands, indicated by arrows at 60 and 36 kDa, which were not present in detectable amounts as tyrosine-phosphorylated proteins in the Triton X-100-soluble fraction, were present in the Triton X-100-insoluble low-spin fractions. This indicated the presence of a high-molecular-weight tyrosine-phosphorylated protein that is distributed between the Triton X-100-soluble and -insoluble fractions of the cytoskeleton.

The above results indicate that tyrosine phosphorylation of various cytoskeletal proteins occurs upon pressure overloading. The presence of a high-molecular-weight tyrosine-phosphorylated protein, tyrosine phosphorylation of which is increased in the pressure-overloaded RV, suggests that tyrosine phosphorylation of cytoskeletal proteins may play a role in the functional and structural changes that occur during ventricular pressure overloading.
The major ~60-kDa tyrosine-phosphorylated protein band (Fig. 2, arrow) in the cytoskeletal fraction of the pressure-overloaded RV matches the size of c-Src shown in the previous experiment (Fig. 1). This would be consistent with the increase in cytoskeletal association of c-Src in pressure-overloaded myocardium. It is interesting to note that this band is present only in the cytoskeletal fraction, while it is completely absent both in the soluble and membrane skeleton fractions. Furthermore, in none of the subfractions of the normally loaded control LV was a ~60-kDa tyrosine-phosphorylated protein band present in detectable amounts. Such an observation raises two possibilities. First, phosphorylation of the protein(s) in the ~60-kDa band might be occurring only after its association with the cytoskeleton; and second, the phosphorylation might occur in other compartments prior to cytoskeletal binding in pressure-overloaded myocardium and then completely translocate to the cytoskeleton.

Although the results shown in Fig. 1C indicate significant amounts of FAK being translocated to the cytoskeleton of the 48-h pressure-overloaded RV, there was only a moderate increase in the 125-kDa tyrosine-phosphorylated protein band (Fig. 2, arrow). Finally, we wished to identify the 36-kDa tyrosine-phosphorylated band, also indicated by an arrow in Fig. 2. Annexin II is 36-kDa Ca\(^{2+}\)-dependent phospholipid-binding protein that recent studies (23, 24) have identified as a cellular substrate for c-Src. Western blot analysis with anti-annexin II antibody indicated that a small but significant amount (~5% of the total) of this protein was associated with the cytoskeletal fraction of the pressure-overloaded RV, but was almost absent in the normally loaded LV (data not shown).

c-Src Association with the Cytoskeleton Occurs Concurrently with Phosphorylation of a ~60-kDa Protein Band—Since cytoskeletal association of c-Src was seen maximally after 48 h of RVPO (Fig. 1), we asked whether the ~60-kDa tyrosine-phosphorylated protein band was also maximal during this time period. For this purpose, the cytoskeletal fractions obtained from control and pressure-overloaded ventricles for various time periods were examined for the presence of tyrosine-phosphorylated proteins. The ~60-kDa tyrosine-phosphorylated protein could be detected as early as 4 h after RVPO and was maximal 48 h after RVPO (Fig. 3). This change in the level of the ~60-kDa tyrosine-phosphorylated protein band reverted to a normal low level following a 1 week or longer (5 weeks) period of RVPO, when the hypertrophic response to a step increase in load is complete. During the entire time course, the level of the 42-kDa tyrosine-phosphorylated protein band that corresponds to actin did not vary appreciably between the two ventricular samples. More important, the time course for tyrosine phosphorylation of the ~60-kDa protein band parallels that of the cytoskeletal association of c-Src shown in Fig. 1. All of these experiments suggest that the cytoskeletal association of c-Src and the phosphorylation of the ~60-kDa protein band occur concurrently, with both being maximal after 48 h of pressure overloading.

Identification of c-Src in the Major ~60-kDa Tyrosine-phosphorylated Protein Band— Immunoprecipitation combined with Western blot analysis was done in total tissue extracts to confirm whether c-Src is present in the ~60-kDa tyrosine-phosphorylated protein band. Total extracts were prepared from 48-h pressure-overloaded RV and same-animal normal LV as well as control RV and LV. As can be seen in Fig. 4A, c-Src was present in substantial amounts in each extract. These total lysates were then immunoprecipitated with anti-c-Src antibody (clone GD11) and examined for tyrosine-phosphorylated c-Src by Western blotting with anti-phosphotyrosine antibody. A significant amount of tyrosine-phosphorylated c-
Src was present only in the pressure-overloaded RV (Fig. 4B, lane 4), while phosphorylated c-Src was undetectable in normally loaded ventricles (lanes 1–3). This observation was further confirmed by performing a reciprocal immunoprecipitation in which the total lysate was used to first immunoprecipitate all of the tyrosine-phosphorylated proteins and then examined for the presence of c-Src in the immune complex by Western blotting with anti-c-Src antibody. As anticipated, tyrosine-phosphorylated c-Src was present only in the pressure-overloaded ventricles (Fig. 4C, lane 4), while it was absent in all control ventricles (lanes 1–3). These results clearly show that c-Src is tyrosine-phosphorylated during pressure overloading. Furthermore, since in our previous experiment, a ~60-kDa tyrosine-phosphorylated protein band was present exclusively in the cytoskeletal fraction of pressure-overloaded myocardium (Fig. 2, lane 4), the results shown in Fig. 4 (B and C, lane 4) should represent cytoskeleton-bound tyrosine-phosphorylated c-Src. To confirm this, we isolated cytoskeletal fractions from normally loaded and pressure-overloaded ventricles and then examined the associated proteins with SDS sample buffer. From these samples, c-Src was immunoprecipitated and then probed for tyrosine-phosphorylated c-Src. As shown in Fig. 4D, tyrosine-phosphorylated c-Src was present in the cytoskeletal fraction of pressure-overloaded myocardium. All of these experiments clearly indicate that in pressure-overloaded myocardium, cytoskeleton-bound c-Src (Fig. 1C, lane 4) exists as a tyrosine-phosphorylated protein and that a significant amount of the ~60-kDa tyrosine-phosphorylated protein band shown in the previous experiment (Fig. 2, lane 4) represents tyrosine-phosphorylated c-Src. Since this antibody has been suggested to have weak affinity for SDS-denatured c-Src, immunodepletion experiments were not performed to find out whether the entirety of the ~60-kDa band is c-Src. Nevertheless, these experiments clearly suggest that c-Src is at least one of the major components present in the ~60-kDa tyrosine-phosphorylated protein band.

Association of tyrosine-phosphorylated c-Src with the cytoskeleton has been reported in activated platelets (14–16) and in growth factor-stimulated glioblastoma cells (25). Phosphorylation under these conditions has been shown to occur primarily at Tyr-416 by autophosphorylation, which in turn amplifies the kinase activity (26). However, in several tissues and cells under normal conditions, the majority of c-Src has been shown to be phosphorylated at Tyr-527 by a recently identified C-terminal Src kinase (Csk), and such phosphorylation suppresses c-Src kinase activity (27, 28). The absence of any such phosphorylation in normally loaded ventricular tissue further suggests that c-Src kinase might be differently regulated in the heart. These findings are not likely due to any artifact created by changes in tyrosine phosphatase activity since for the combined immunoprecipitation and Western blotting experiments described above (Fig. 4, B and C), tissue samples were prepared by direct extraction in Laemmli SDS sample buffer containing vanadate, and the homogenate was boiled immediately prior to centrifugation. It is unlikely that phosphatases remained active under these conditions.

**Demonstration of the Active Form of c-Src in the Cytoskeletal Fraction of Pressure-overloaded RV**—The finding that cytoskeleton-associated c-Src exhibits a high level of tyrosine phosphorylation could imply either activation or inactivation, depending upon whether the phosphorylation is at Tyr-416 or Tyr-527 (for review, Ref. 28). It was therefore important to determine whether cytoskeleton-bound tyrosine-phosphorylated c-Src is functionally active and thus potentially able to play a role in mediating hypertrophic growth. Unfortunately, we could only immunoprecipitate c-Src from the cytoskeletal fraction under denaturing conditions (see Fig. 4, B and D) and thus could not directly demonstrate c-Src kinase activity. Studies shown in Fig. 4 clearly demonstrate that a significant portion of the cytoskeleton-associated c-Src is tyrosine-phosphorylated. To determine whether cytoskeleton-associated c-Src is active in pressure-overloaded myocardium, we examined the tyrosine phosphorylation status of c-Src in normal and hypertrophied hearts. For this purpose, we employed a monoclonal antibody (clone 28) developed by Kawakatsu et al. (29) that reacts with c-Src only when the kinase is not phosphorylated at Tyr-527. Western blot analysis utilizing the clone 28 monoclonal antibody revealed that pressure overloading causes an association of a significant amount of the Tyr-527 unphosphorylated kinase relative to its low level in the normally loaded control LV (Fig. 5A, compare lanes 4 and 3). This population represents the unphosphorylated form of c-Src and/or c-Src phosphorylated at another tyrosine residue, which includes the other major Tyr-416 phosphorylation site. Therefore, we next attempted to demonstrate directly that at least a significant portion of the phosphorylated c-Src is due to phosphorylation of residues (e.g., Tyr-416) other than Tyr-527. The cytoskeletal fractions were first immunoprecipitated with anti-phosphotyrosine antibody, and then tyrosine-phosphorylated c-Src was detected by probing with the clone 28 anti-c-Src antibody. Fig. 5B clearly shows the presence of tyrosine-phosphorylated c-Src in the cytoskeletal fraction of pressure-overloaded myocardium that is not due to Tyr-527 phosphorylation and therefore most likely represents the active Tyr-416 phosphorylated species.
and the detergent-insoluble low-spin fraction (cytoskeleton). The cardiocytes were extracted with 2% Triton X-100 buffer, occurring at the level of individual cardiocytes, we isolated c-Src in the pressure-overloaded ventricles represent changes that the cytoskeletal association and phosphorylation of eton-associated c-Src is active.

Isolated from Pressure-overloaded Cat Ventricles—Due to pressure overloading. membraneskeletalfraction( (membrane skeleton) fractions as described under "Materials and Methods." For the experiment shown in A, the proteins were resolved by SDS-PAGE and Western-blotted with the clone 28 anti-c-Src monoclonal antibody. In B, aliquots of Triton X-100-lysed subfractions were used for immunoprecipitation with anti-phosphotyrosine antibody. The phosphorylated proteins present in the immune complex were resolved by SDS-PAGE and Western-blotted with the clone 28 anti-c-Src antibody. WB, Western blot; IP, immunoprecipitation; α-c-Src, anti-c-Src antibody (clone 28); α-PY, anti-phosphotyrosine antibody.

(see "Discussion"). In addition, we performed in vitro kinase reactions directly in the cytoskeletal pellets of control and pressure-overloaded myocardia. Such studies showed enhanced phosphorylation of several cytoskeletal proteins, including a major 60-kDa protein band in pressure-overloaded myocardium (data not shown). Furthermore, such kinase assays with the p34cdc2 kinase derived-substrate peptide (Lys-Val-Glu-Lys-Ile-Gly-Glu-Gly-Thr-Tyr-Gly-Val-Val-Tyr-Lys), a specific and efficient substrate of Src family tyrosine kinases (21), showed a 3-fold increase in phosphorylation by the cytoskeletal fraction prepared from pressure-overloaded ventricles (data not shown). In all these experiments, the cytoskeletal fractions from pressure-overloaded RVs and same-animal control LVs exhibited very similar levels of actin. Taken together, these data suggest that a significant proportion of the cytoskeleton-associated c-Src is active.

Since our earlier experiments revealed that tyrosine-phosphorylated c-Src was present only in the cytoskeletal fraction of pressure-overloaded myocardium and was completely absent in the normal heart, we anticipated that the clone 28 antibody would react with the c-Src present in the Triton X-100-soluble fractions. As shown in Fig. 5A, the detergent-soluble fractions of both normal and pressure-overloaded myocardia contain substantial amounts of c-Src recognized by the clone 28 anti-c-Src monoclonal antibody (lanes 1 and 2), and pressure overloading does not appear to change the level. In addition, this form of c-Src is also present in the Triton X-100-insoluble membrane skeletal fraction (lanes 5 and 6), showing no change due to pressure overloading.

Detection of the Myocardial ~60-kDa Tyrosine-phosphorylated Protein Band in the Cytoskeletal Fraction of Cardiocytes Isolated from Pressure-overloaded Cat Ventricles—To demonstrate that the cytoskeletal association and phosphorylation of c-Src in the pressure-overloaded ventricles represent changes occurring at the level of individual cardiocytes, we isolated these cells from both the LV and RV of a 48-h RVPO cat (19). The cardiocytes were extracted with 2% Triton X-100 buffer, and the detergent-insoluble low-spin fraction (cytoskeleton) was obtained for Western blot analysis with anti-phosphotyrosine antibody. The ~60-kDa tyrosine-phosphorylated protein band, which we have shown to contain c-Src, was detected only in the cardiocyte samples obtained from the pressure-overloaded RV (Fig. 6). Furthermore, Western blot analysis for the detection of c-Src (Upstate Biotechnology, Inc.) showed coplication of c-Src with this ~60-kDa protein band (data not shown). These data demonstrate that the changes seen with c-Src in pressure-overloaded myocardium occur at the level of individual cardiocytes.

Other Src Family Members Fail to Associate with the Cytoskeleton—the Src kinase family is composed of nine related non-receptor tyrosine kinases. Thus, we asked whether other Src family members were also translocated to the cardiac cytoskeleton during pressure overloading, as has been reported in platelets (16). The experiments in Fig. 7 show the levels of actin, c-Src, and Fyn in Triton X-100-soluble and -insoluble cytoskeletal and membrane skeletal fractions from another 48-h RVPO cat. As shown above, there was no significant difference in actin levels when comparing sets of Triton X-100-extracted LV and RV fractions, while there were substantial amounts of c-Src being recruited to the cytoskeleton during 48 h of pressure overloading. Western blot analysis of Fyn showed that both the detergent-soluble fractions (lanes 1 and 2) and the insoluble membrane skeletal fractions (lanes 5 and 6) contained substantial amount of Fyn. However, in contrast to c-Src, we did not see Fyn association with the cytoskeleton after 48 h of pressure overloading (Fig. 7, compare lanes 4 and 3 for Fyn and c-Src). Furthermore, we observed no tyrosine-phosphorylated protein band matching the size of Fyn in the cytoskeletal fraction of pressure-overloaded myocardium (Fig. 2 and data not shown). When such studies were performed for Lyn using a monoclonal antibody (Transduction Laboratory), we detected no protein band in any the Triton X-100-lysed samples. While a polyclonal antibody (Santa Cruz) detected low levels of this kinase in the Triton X-100-soluble fractions, we saw no association with the cytoskeleton of pressure-overloaded myocardium (data not shown). Similarly, we were unable to detect Yes in any of the Triton X-100-lysed subfractions when Western-blotted with a specific monoclonal antibody, although it is possible that antibody specificity prevented recognition of feline samples. Nonetheless, of the Src kinase family members that we examined, c-Src appears to be the only kinase
that demonstrated a shift in cytoskeletal association in pressure-overloaded myocardium.

**DISCUSSION**

Both skeletal and cardiac striated muscle cells undergo developmentally programmed terminal differentiation, with subsequent adaptation to increased demands for mechanical output being met by hypertrophy. Therefore, hypertrophic growth is considered to be a fundamental adaptive response to hemodynamic overloads. A series of studies from these laboratories (1, 2) has established that the cardiocyte is directly responsive to load input in terms of initiating and regulating this hypertrophic response. However, a critical unresolved question is that of how changes in mechanical loading of the myocardium are transformed into the intracellular chemical signals that eventually result in hypertrophic growth and any accompanying phenotypic changes. In this work, we asked whether the focal adhesion complex could function as a potential mediator through which changes in mechanical loading might be received from the interstitium and transmitted into chemical signaling via cardiocyte integrin-mediated pathways.

There are two broad views proposed for the mechanism of integrin-mediated signaling (for review, see Ref. 30). Since cytoplasmic domains of the integrin receptor family interact with cytoskeletal components (31, 32), one view suggests that integrins transmit signals by organizing the cytoskeleton and regulating cell shape. It is believed that such a change in cell shape and cytoskeletal organization might regulate the biosynthetic capabilities of the cell, thereby controlling cell growth and differentiation (33, 34). In the second view, integrins are considered to exert biochemical signals similar to growth factor receptor-mediated signaling pathways (35). Studies performed in platelets show that thrombin treatment coupled with mechanical stirring results in the cytoskeletal association of non-receptor tyrosine kinases, in particular c-Src (14–16, 36, 37). Under such conditions, Fox et al. (16) have further demonstrated that in addition to c-Src, glycoprotein Ib-IIIa (a major form of platelet integrin), FAK, and other Src family members are recruited to the cytoskeleton. Furthermore, cytoskeletal association of these signaling proteins has been shown to result in the phosphorylation of several cytoskeletal proteins and is suggested to be responsible for changes in cytoskeletal structure and thus for overall platelet shape changes. Interestingly, to show such observations in nucleated cells, Weernink and Rijksen (25) have recently shown translocation of c-Src to the cytoskeleton in A172 glioblastoma cells following treatment with platelet-derived growth factor and epidermal growth factor. Furthermore, in many of the above reports, ligation of integrins with extracellular matrix proteins has been shown to be necessary for the translocation of c-Src. Although Src family kinases are present at significant levels in cardiocytes, present knowledge of their role in proliferation, differentiation, and signaling processes as well as of their target proteins is limited. Our studies show for the first time mechanical load-induced movement of non-receptor tyrosine kinases (c-Src and FAK) and integrins to the cytoskeleton of the myocardium. Association of these signaling molecules with the cytoskeleton could be important for both the qualitative phenotypic changes and the quantitative increase in mass that constitute cardiac hypertrophy. While it is difficult to determine using in vivo models whether integrin-mediated signaling pathways are necessary for translocation of c-Src in pressure-overloaded myocardium, comigration of β3-integrin and FAK with c-Src suggests that integrin-mediated signaling events might be necessary for the cytoskeletal association of c-Src. Although β3-integrin was detected, β1-integrin appeared to be absent in all of the Triton X-100-lysed subfractions based on our studies using a specific monoclonal antibody against β1-integrin. Recent reports (38, 39) show a skeletal and cardiac muscle-specific isoform of the β-integrins, β1-D. It will be interesting to determine whether the cardiac muscle-specific β1-D integrin isoform is a major factor in coupling external mechanical signals to hypertrophic growth signals by translocating one or more potential non-receptor tyrosine kinases such as c-Src. In the second view of integrin-mediated signaling (35), FAK plays a major role in mediating mitogenic signaling pathways. In normal and pressure-overloaded adult feline myocardia, we did not observe a substantial level of a tyrosine-phosphorylated protein band corresponding to the size of FAK, although a significant amount of this kinase was found to be associated with the cytoskeleton between 4 and 48 h of RVPO. It is possible that an initial wave of FAK tyrosine phosphorylation might have occurred during the early time period of pressure overloading prior to FAK's cytoskeletal association. However, experiments with shorter time periods of RVPO are difficult to perform with in vivo animal models.

Because phosphorylation of c-Src is relatively high when compared with that of FAK, we focused our further studies on the cytoskeletal association and activation of c-Src. Cytoskeletal association of c-Src in platelets is not due to nonspecific trapping during platelet aggregation (14). Our study of the cytoskeletal association of c-Src further supports the idea that this translocation occurs as a specific event in response to cardiac pressure overloading for the following reasons. First, a closely related Src family member, Fyn, does not appear to migrate to the cytoskeleton in pressure-overloaded myocardium, although substantial amounts of this kinase are present both in Triton X-100-soluble as well as membrane skeleton fractions. Second, pressure overload-induced cytoskeletal association of c-Src was observed to be a slow and prolonged process, seen maximally after 48 h of RVPO and then declining to a normal level after 1 week of RVPO. This is quite different from the rapid recruitment of c-Src to the cytoskeleton seen within minutes after agonist-induced activation of either platelets (16) or glioblastoma cells (25), suggesting that the association of c-Src with the cardiocyte cytoskeleton might be a specific process occurring subsequent to changes in cytoskeletal structure and/or in c-Src kinase itself. Interestingly, this slow time course of c-Src recruitment to the cytoskeleton of the
pressure-overloaded ventricle precisely matches the time frame of the increase in ventricular mass in response to pressure overloading (3). Third, tyrosine-phosphorylated c-Src is not present in fractions other than the cytoskeletal fractions of pressure-overloaded cardiac tissue or isolated cardiocytes. If this association is due to non-specific trapping, at least some residual amount of tyrosine-phosphorylated c-Src would be expected to be present in the Triton X-100-soluble fraction, where this kinase is mostly present as a non-tyrosine-phosphorylated protein. Previous studies of the expression of the oncogenic form of Src (v-Src) in chicken embryonic fibroblasts showed a spontaneous association with the cytoskeleton, and such an association of the constitutively active form of this kinase has been linked to cellular transformation (40, 41). These studies support the idea that in pressure-overloaded myocardium, c-Src might play a key role in mediating changes in cardiac structure, composition, and function through its movement to the cytoskeleton and phosphorylation of associated proteins.

Interestingly, the complete absence of tyrosine-phosphorylated c-Src in the normal heart suggests tight cardiac regulation of this kinase. The C-terminal Src kinase (Csk), which phosphorylates c-Src at Tyr-527 and negatively regulates its activity, has been shown to be present in almost all tissues and cells. In addition, its level has been reported to be sufficient to phosphorylate even high amounts of overexpressed c-Src (28). Although we found substantial amounts of Csk in ventricular total lysates, we observed no tyrosine phosphorylation of Csk in the normal heart. This is further supported by the fact that the clone 28 monoclonal antibody specific for the Tyr-527 unphosphorylated form of the kinase detects substantial amounts of c-Src in the Triton X-100-soluble fraction. Furthermore, the complete absence of tyrosine phosphorylation of c-Src indicates that in addition to Tyr-527, another positive regulatory site, Tyr-416, is also not phosphorylated in the normal heart. In the context of this observation, a recent study from this laboratory shows the presence of a novel suppressor activity for Src family kinases in the adult cardiocyte. The presence of tyrosine-phosphorylated c-Src exclusively in the cytoskeletal fraction of pressure-overloaded myocardium further suggests that by moving to the cytoskeletal compartment, c-Src could be liberated from inhibitory constraints that might prevent its phosphorylation.

Our studies on cytoskeleton-bound c-Src in pressure-overloaded myocardium strongly suggest that the kinase is present in its active form. Earlier studies in platelets show that cytoskeleton-bound c-Src is phosphorylated at Tyr-416 upon stimulation with thrombin (26). In addition, expressed v-Src, which lacks the Tyr-527 negative regulatory site of c-Src, binds spontaneously to the cytoskeleton and becomes phosphorylated (40, 41). Similarly, binding of c-Src with polyoma middle antigen also results in the activation of c-Src (28). Our studies with the clone 28 antibody show the association of significant amounts of Tyr-527 unphosphorylated kinase in the cytoskeletal fraction of pressure-overloaded myocardium. In addition, at least a part of this form of kinase shows tyrosine phosphorylation distinct from Tyr-527, indicating that the kinase undergoes active phosphorylation in vivo, possibly at Tyr-416. Although we have not eliminated the possibility that some of the tyrosine phosphorylation of c-Src in the pressure-overloaded myocardium is at Tyr-527, it is important to note that negative regulation of c-Src by the Tyr-527 phosphorylated C-terminal residue requires intact SH2 and SH3 domains of c-Src (42). Furthermore, any alterations in these domains have been shown to result in the failure of negative regulation, and the kinase thus becomes activated even while Tyr-527 is phosphorylated by Csk (28, 42). Interestingly, the SH2 domain of c-Src has also been shown to be responsible for the cytoskeletal association of c-Src (43). Taken together, these studies indicate that interaction of the SH2 and/or SH3 domain of c-Src with the cytoskeletal protein could result in the loss of negative regulation mediated by the Tyr-527 phosphorylated residue. More important, our studies show that a significant proportion of the cytoskeleton-associated c-Src is phosphorylated at a residue other than Tyr-527. Thus, it is likely that this in vivo phosphorylation corresponds to Tyr-416 autophosphorylation. In this context, it is important to note that phosphorylation of any tyrosine residue other than Tyr-527 has not been implicated in negative regulation of c-Src (for review, see Ref. 28). Unfortunately, isolation of cytoskeleton-bound c-Src in pressure-overloaded myocardium required harsh denaturing conditions, such that we were unable to immunoprecipitate and show the kinase activity. Nevertheless, our studies on the in vitro kinase reaction using the cytoskeletal fraction of pressure-overloaded myocardium show strong phosphorylation of a 60-kDa protein band corresponding to the size of c-Src. In addition, studies using a synthetic peptide, employed as a specific substrate for Src family kinases (21), also suggest enhanced tyrosine kinase activity in the cytoskeletal fraction of pressure-overloaded myocardium. Finally, our in vivo studies reveal several newly tyrosine-phosphorylated proteins in the cytoskeletal fraction of pressure-overloaded myocardium, where this kinase is associated in substantial amounts. Taken together, these data further support our contention that the c-Src that is present in the cytoskeletal fraction of pressure-overloaded myocardium is functioning as an activated kinase.

The time course of the cytoskeletal association of c-Src and the appearance of a 60-kDa tyrosine-phosphorylated protein band containing c-Src suggests that both events occur concurrently. Two conclusions can thus be drawn from these observations. (i) In the context of hypertrophy, our earlier studies (3) have shown a similar time course for the pressure overload-induced increase in total RNA content. There is substantial evidence that mRNA and polysomes are associated with the cytoskeleton (44). As increased protein synthesis is the essential feature of hypertrophic cardiac growth (1), it is possible that cytoskeletal association of c-Src and phosphorylation of associated proteins might result in changes in the internal cellular architecture that could increase the size of the cytoskeleton-associated polysome pool. (ii) From a mechanistic point of view, we suggest that tyrosine phosphorylation of c-Src might occur subsequent to its cytoskeletal association. This is different from the view of Fox et al. (16), who, based on their platelet studies, suggest that tyrosine phosphorylation and activation of c-Src are necessary for its cytoskeletal association. Our interpretation is based on our observations that both c-Src phosphorylation and its cytoskeletal association occur concurrently in pressure-overloaded myocardium and that under these conditions, tyrosine-phosphorylated c-Src is exclusively present in the cytoskeletal fraction. This view is further supported by our unpublished observation that a non-ionic detergent extract obtained from adult cardiocytes exhibits strong c-Src suppressor activity, suggesting that kinase phosphorylation and activation may be prevented in the Triton X-100-soluble compartment.

We have shown here for the first time cytoskeletal association and activation of c-Src in response to cardiac pressure overloading. The role that c-Src plays during this very important physiological response is far from understood. But since many of the cytoskeletal changes in pressure-overloaded myocardium occur at the level of individual cardiocytes, one possi-
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