

**REQUIREMENTS FOR THE LOCALIZATION OF p130 Cas TO Z-LINES
IN CARDIAC MYOCYTES**

BRANKA KOVAČIČ-MILIVOJEVIĆ¹, CAROLINE C. DAMSKY²,
DAVID G. GARDNER^{1,3} and DUŠKO ILIĆ²

¹Metabolic Research Unit and Department of ²Stomatology and ³Medicine,
University of California, San Francisco, California 94143, USA

Abstract: The vertebrate heart responds to hemodynamic load with the enlargement of postmitotic, terminally differentiated cardiac myocytes. Such hypertrophic changes are characterized by alterations in sarcomeric organization and gene expression. Previously, we established a role for a nonreceptor tyrosine kinase, focal adhesion kinase, in signaling the changes in cytoskeletal organization associated with hypertrophy [1]. Here, we report on data supporting a key role for p130Cas in this process. In neonatal cardiac myocytes FAK, Cas and paxillin are located in sarcomeric Z-lines, suggesting that the Z-line is an important signaling locus in these cells. The expression of different Cas mutants results in a nearly complete loss of sarcomeric organization in these myocytes. Moreover, expression of the C-terminal focal adhesion-targeting domain of FAK both disrupted sarcomeric organization and interfered with the localization of endogenous Cas to Z-lines. These findings suggest that the association of FAK and Cas and the preservation of multiple protein-interaction motifs of Cas are required for the correct assembly of sarcomeres in cardiac myocytes.

Key Words: Cardiac Myocyte Hypertrophy, Z-Lines, Cas, FAK

INTRODUCTION

The heart adapts to increased demand for cardiac work by increasing cardiac muscle mass through the activation of a hypertrophy. This is a consequence of alterations in specific signaling molecules and downstream pathways in individual cardiac myocytes. At the cellular level, hypertrophy is thought to develop in response to a combination of biomechanical and neurohumoral stimuli. Cultured neonatal cardiocytes respond to these stimuli with phenotypic changes that parallel those seen with hypertrophy *in vivo*. At the level of gene expression, this includes the activation of immediate early genes (*c-jun*, *c-fos*) followed by the reactivation of a fetal gene program (ANP, β -MHC and α -

Abbreviations used: ANP, atrial natriuretic factor; β -MHC, β -myosin heavy chain; Cas, p130Cas; ECM, extracellular matrix; FAK, focal adhesion kinase; FAT, focal adhesion targeting domain; GFP, green fluorescent protein; MAPK, mitogen-activated protein kinases.

skeletal actin) and subsequent upregulation of sarcomeric contractile proteins (myosin light chain-2 and cardiac actin). These changes are in turn followed by an increase in cell size sarcomeric organization and density. The assembly of contractile proteins into organized sarcomeric units is the most distinctive feature of cardiac hypertrophy. In culture, the development of cardiac myocytes is characterized by myofibrillogenesis, a process that organizes myofibrils into sarcomeric structures containing thick and thin filaments with appropriately spaced Z-lines. Z-line associated structures are responsible for the lateral alignment of myofibrils and the production of active contraction in cardiac muscle. The signaling mechanisms underlying this process are understood only to a limited degree.

Cas is part of the supramolecular complex that links ECM through integrins to cytoskeletal structures at focal adhesion sites. Cas is organized into a number of structural domains associated with unique functional activities.

Proceeding from the amino to the carboxy terminus of the molecule, one can identify (1) the SH3 domain, (2) the substrate domain (SD) with multiple SH2 binding sites, and (3) the so-called Src-binding domain (Src-BD) with SH2 and SH3 domain-binding motifs for Src family kinases [2]. FAK is a well-documented binding partner for Cas. The proline-rich (PR-1) region of FAK provides a binding site for the SH3 domain of Cas. When cells attach to ECM, FAK participates in Src-mediated tyrosine-phosphorylation of Cas, through its physical interaction with Src. Integrin-mediated stimulation of the FAK-Cas pathway elicits the activation of MAP kinases that regulate basic cellular processes such as growth, differentiation, migration and cell survival [3].

We established previously that FAK, Cas and paxillin are localized in the sarcomeric Z-line [1]. We also showed that the expression of the FAT domain of FAK interfered with the localization of endogenous FAK to the Z-line. Moreover, expression of the Cas-binding PR-1 region of FAK hindered the association of Cas with endogenous FAK and impaired the structural stability of sarcomeres [1]. In light of the important role that Cas appears to play in the structural stability of sarcomeric Z-lines, this study was undertaken to identify those domains that are required for the proper subcellular distribution of Cas in cardiac myocytes. We demonstrate that the SH3 and substrate binding domains, as well as Src-BD, are important for the localization of Cas to the Z-line, and each plays a pivotal role in cytoskeletal organization in cardiac myocytes.

MATERIAL AND METHODS

Plasmids

The construction of GFP-FAT has been described previously [1]. Hemagglutinin (HA)-tagged wild-type and mutant Cas, cloned in the mammalian expression vector pSSr α , were provided by T. Nakamoto and H. Hirai of Tokyo University (Tokyo, Japan) [2].

Antibodies

Monoclonal anti-HA (HA.11) antibody was purchased from BabCo (Richmond, CA) and monoclonal anti-Cas was from Transduction Laboratories (San Diego, CA).

Cell culture, transfection and viral infection

Primary cultures of neonatal rat ventricular cardiac myocytes were prepared from 1-2 day old rats as described previously [1]. Freshly isolated cells were transfected with eukaryotic expression plasmids as described in the individual figure legends and plated on fibronectin-coated coverslips. All the experiments were performed 48h post-plating. Adenoviral infection of cardiac myocytes was carried out essentially as described [1].

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 as described [1].

RESULTS AND DISCUSSION

To determine the requirements for targeting Cas to Z-lines, we carried out studies with several HA-tagged deletion mutants of Cas. These included Cas lacking the SH3 domain, (Cas Δ SH3), substrate domain (Cas Δ SD), or Src-binding domain (Cas Δ Src-BD) (Fig. 1A). These variants were transiently transfected into cardiac myocytes, and their association with sarcomeric Z-lines was evaluated by indirect immunofluorescence. First, we examined the subcellular localization of full-length Cas by immunostaining with anti HA-tag antibodies. As shown in Fig. 1B (upper left panel) the majority of wild-type Cas was organized in a highly repetitive striated pattern suggesting that ectopically expressed Cas associates with one or more of the well-defined structural components of the sarcomere. Moreover, this kind of highly repetitive banding pattern was identical to that determined for endogenous Cas in our earlier study [1]. We conclude that the majority of transiently expressed wild-type Cas is appropriately targeted to Z-lines. We next searched for the domain or domains that are necessary for proper localization of Cas to the Z-lines. 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 [4], we asked whether a functional SH3 domain was necessary for targeting Cas to Z-lines. HA-tagged Cas Δ SH3 was expressed in cardiac myocytes. Unlike in the case of wild-type Cas, Cas Δ SH3 transfected myocytes lost their Z-line staining pattern. HA-Cas Δ SH3 was found diffusely distributed throughout the cytoplasm and showed no association with any type of cytoskeletal structure in the myocytes (Fig. 1 B, upper right panel). This data indicates that deletion of the SH3 domain, which promotes the interaction of Cas with FAK and other SH3 binding proteins, and abrogates its localization to Z-lines in cardiac myocytes. We also examined the

distribution of the Cas Δ SD mutant in cardiac myocytes. As shown in Fig. 1B (lower right panel), deletion of the SD domain resulted in a similar phenotype and severely attenuated the ability of Cas to localize to Z-lines. The substrate domain of Cas, with its numerous tyrosine phosphorylation sites, provides a docking station for assembly of additional SH2-domain containing proteins creating a network of signaling molecules capable of processing information generated at the cell surface [6]. Expression of the Cas Δ Src-BD mutant, which (such as SH2 domain kinases, adaptor proteins or phosphatases), thereby lacks a

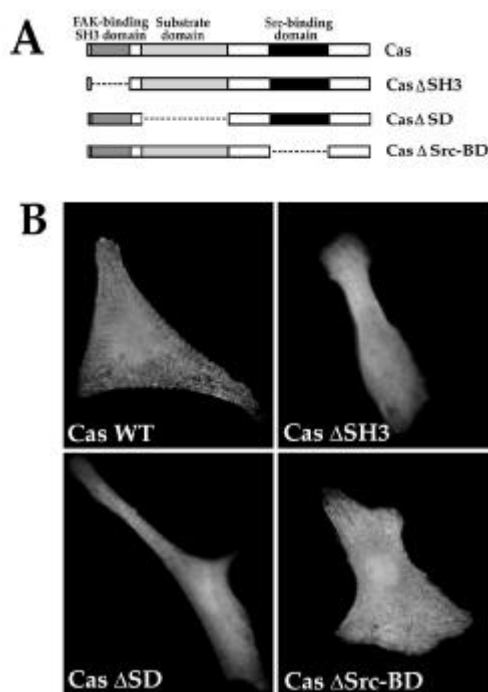


Fig. 1. Localization of various ectopically expressed Cas mutants. (A) Schematic diagram representing various constructs used. Details for each mutant are given described in [2]. (B) Cardiac myocytes were transfected with 5 μ g of expression vector encoding HA-tagged wild-type Cas or the indicated mutant Cas forms, except for Cas Δ SH3 where 15 μ g was used throughout all the experiments to achieve a protein level similar to Cas wild-type and other mutants. Cells were grown for 48h on fibronectin-coated coverslips, fixed with ethanol at -20°C for 30 min and exposed to acetone at room temperature for 1 min. Fixed cells were stained with monoclonal anti-HA-tagged antibodies (detected with FITC).

region containing the Src SH2 and SH3 binding sites, resulted in an almost complete loss of sarcomeric organization (Fig. 1B, lower right panel). This observation suggests that the association of Cas with Src, or other SH domain-containing signaling molecules, is required for proper targeting of Cas to Z-lines. The effectiveness of all three mutants in disrupting myocyte cytoskeletal organization suggests that Cas participates in sarcomeric organization through multiple independent protein-protein interactions.

To confirm that the association of Cas with FAK is critical for proper Z-line formation and sarcomeric organization, we interfered with the normal localization of the FAK-Cas complex by expressing a replication-defective adenovirus encoding GFP-FAT (Fig. 2 A). GFP-FAT acts as a dominant-negative inhibitor of FAK activity through the displacement of endogenous FAK from focal adhesion contacts [1]. Eighteen hours after infection GFP-FAT was effectively expressed in ~90% of myocytes in culture (data not shown) and demonstrated a specific subcellular localization. Examination of GFP-FAT-transduced cells revealed that a portion of GFP-FAT was localized to Z-lines (similar to the localization of endogenous FAK), as well as in areas with decreased myofibrillar density which were filled with a granular material rich in α -actinin staining (Fig. 2 B).

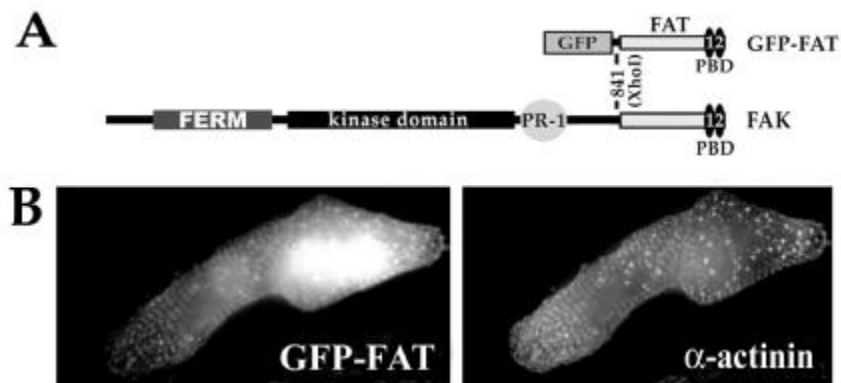


Fig. 2. Localization of GFP-FAT in cardiac myocytes. (A) Schematic diagram of wild-type FAK and GFP-FAT. PR-1, proline rich region; PBD, paxillin-binding domain 1 and 2. (B) Freshly isolated cardiac myocytes infected with adenovirus expressing GFP-FAT were plated on fibronectin-covered glass coverslips. 18h post-infection, sarcomeric Z-lines were stained with anti α -actinin (left panel). GFP-FAT expressing cells were visualized by fluorescence microscopy (right panel).

To obtain further insight into the Cas-FAK association, endogenous Cas was immunolocalized in cardiac myocytes not exposed to the virus (Fig. 3, left panel), as well as in GFP-FAT transduced cells (Fig. 3, right panel). Endogenous Cas was detected in the well-organized and repetitive pattern of the myofibrillar Z-lines. In GFP-FAT-transduced cultures, most Cas was diffusely distributed in the cytoplasm or organized in small dense granular aggregates. We propose that targeting of GFP-FAT to the Z-lines interferes with the signals required for the FAK-Cas interaction. Such GFP-FAT-mediated displacement of the FAK-Cas complex is, in turn, associated with the disruption of the sarcomeric units.

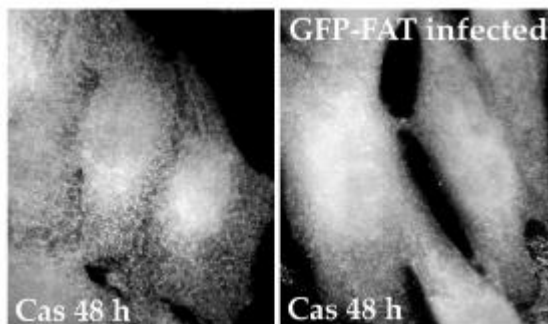


Fig. 3. Effects of GFP-FAT expression on the subcellular localization of endogenous Cas. Freshly isolated cardiac myocytes infected with adenovirus expressing GFP-FAT were plated on fibronectin-covered glass coverslips. 48h post-infection, the cells were fixed as described in the legend of Fig. 1 and stained with monoclonal antibodies against Cas (detected with FITC) as described in Material and Methods.

An initial clue as to the importance of Cas for Z-line formation in cardiac myocytes comes from mice with a homozygous null mutation of the *cas* gene [4]. Deletion of *cas* by homologous recombination resulted in embryonic lethality. Histological examination of the hearts revealed a poorly developed myocardium accompanied by disorganized myofibrils and disrupted Z-lines, a phenotype similar to those seen with our studies of disrupted Cas function in cultured cardiac myocytes. Our results support the hypothesis that conserved regions of Cas provide a docking station for the assembly of signaling and structural proteins that coordinate the development of the signal transduction machinery with the physical process of sarcomerogenesis in the cardiac myocyte. Further studies should identify these proteins and illuminate their role in Cas signaling.

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