Muscle ring finger protein-1 inhibits PKCε activation and prevents cardiomyocyte hypertrophy

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Much effort has focused on characterizing the signal transduction cascades that are associated with cardiac hypertrophy. In spite of this, we still know little about the mechanisms that inhibit hypertrophic growth. We define a novel anti-hypertrophic signaling pathway regulated by muscle ring finger protein-1 (MURF1) that inhibits the agonist-stimulated PKC-mediated signaling response in neonatal rat ventricular myocytes. MURF1 interacts with receptor for activated protein kinase C (RACK1) and colocalizes with RACK1 after activation with phenylephrine or PMA. Coincident with this agonist-stimulated interaction, MURF1 blocks PKCε translocation to focal adhesions, which is a critical event in the hypertrophic signaling cascade. MURF1 inhibits focal adhesion formation, and the activity of downstream effector ERK1/2 is also inhibited in the presence of MURF1. MURF1 inhibits phenylephrine-induced (but not IGF-1–induced) increases in cell size. These findings establish that MURF1 is a key regulator of the PKC-dependent hypertrophic response and can blunt cardiomyocyte hypertrophy, which may have important implications in the pathophysiology of clinical cardiac hypertrophy.

Introduction

Myocardial hypertrophy is an early milestone during the clinical course of pressure overload–induced heart failure and by itself is an important risk factor for subsequent cardiac morbidity and mortality. The heart adapts in response to mechanical and hemodynamic stimuli by initiating a hypertrophic response and increasing muscle mass (Hunter and Chien, 1999). At the cellular level, cardiac myocytes respond to diverse types of biomechanical stress by initiating several different cytoplasmic signal transduction cascades that lead to hypertrophic gene expression and growth of individual myocytes (Molkentin and Dorn, 2001). Blockade of specific intracellular signaling pathways in the heart can dramatically affect the orchestration of the entire hypertrophic response and effectively diminish cardiac enlargement.

Ventricular myocytes represent an excellent model system for the study of the myocyte hypertrophic response (Chien et al., 1991). Agonists that induce a hypertrophic response phenotype in cultured cells include phorbol esters (Allo et al., 1992), endothelin-1 (Shubeita et al., 1990), and α-adrenergic

receptor (α-AR) agonists such as phenylephrine (PE) and norepinephrine (Lee et al., 1988; Iwaki et al., 1990). One mechanism underlying α-AR–induced cardiac hypertrophy is the activation of PKC isoforms that can be regulated by selective translocation from the cytosolic to the membrane compartment (Mochly-Rosen, 1995). Translocation and activation of the Ca^{2+}-dependent PKCα and Ca^{2+}-independent PKCε occur in response to α-AR stimulation in rat hearts (Clerk et al., 1994; Puceat et al., 1994; Rybin and Steinberg, 1994; Korzick et al., 2001) and are, in turn, mediated by PKC isoform-specific associations with membrane anchoring proteins termed receptors for activated protein kinase C (RACKs) (Mochly-Rosen et al., 1991; Disatnik et al., 1994a; Mochly-Rosen and Gordon, 1998). RACK1, a 36-kD WD40 repeat protein, was originally identified in a screen for proteins that bind activated PKCβII (Mochly-Rosen et al., 1991). RACK1 is not a substrate for PKCβII; however, in its presence substrate phosphorylation by PKCβII is increased (Ron et al., 1994), suggesting that the PKCβII–RACK1 complex may be the active form of the enzyme. In addition to PKCβII, RACK1 has also been found in association with PKCε in hypertrophied heart lysates, demonstrating a potential functional role for PKCε–RACK1 interactions in the myocardium (Mochly-Rosen et al., 2000; Pass et al., 2001a,b).

RACK1 is also an adaptor for other signaling enzymes including phospholipase Cγ (Disatnik et al., 1994b), Ras-GAP

Figure 1. **MURF1 interacts with RACK1 in vivo and in vitro.** (A) In a yeast two-hybrid screen the NH$_2$ terminus of MURF1 was used as bait to screen an adult heart library. (B) To confirm the yeast two-hybrid interaction, COS 7 cells were transfected with RACK1 and/or Myc-MURF1 as indicated. The cell lysates were immunoprecipitated with anti-Myc antibody followed by Western blotting with RACK1 antibody. (C) To test the interaction between MURF1 and RACK1 in vitro, GST fusion proteins (GST alone, GST-RACK1, or GST-DB1 as a negative control) were incubated with COS 7 cell lysates that express Myc-MURF1. The protein complexes were immunoblotted with anti-Myc or anti-RACK1 antibody. (D) GST fusion proteins (GST alone or GST-MURF1) were incubated with COS 7 cell lysates expressing the indicated HA-tagged RACK1 deletions (The rapidly migrating HA-immunoreactive bands in cell lysates probably represent RACK1 degradation products). The protein complexes were immunoblotted with anti-HA, -PKCε, or -GST antibody. (E) NRVM were infected with Ad.GFP or Ad.MURF1 at the indicated multiplicities of infection (MOI), and lysates were immunoprecipitated with an antibody against PKCε. Immunoprecipitates were then blotted for the presence of RACK1 and MURF1 (using an anti-Myc antibody).
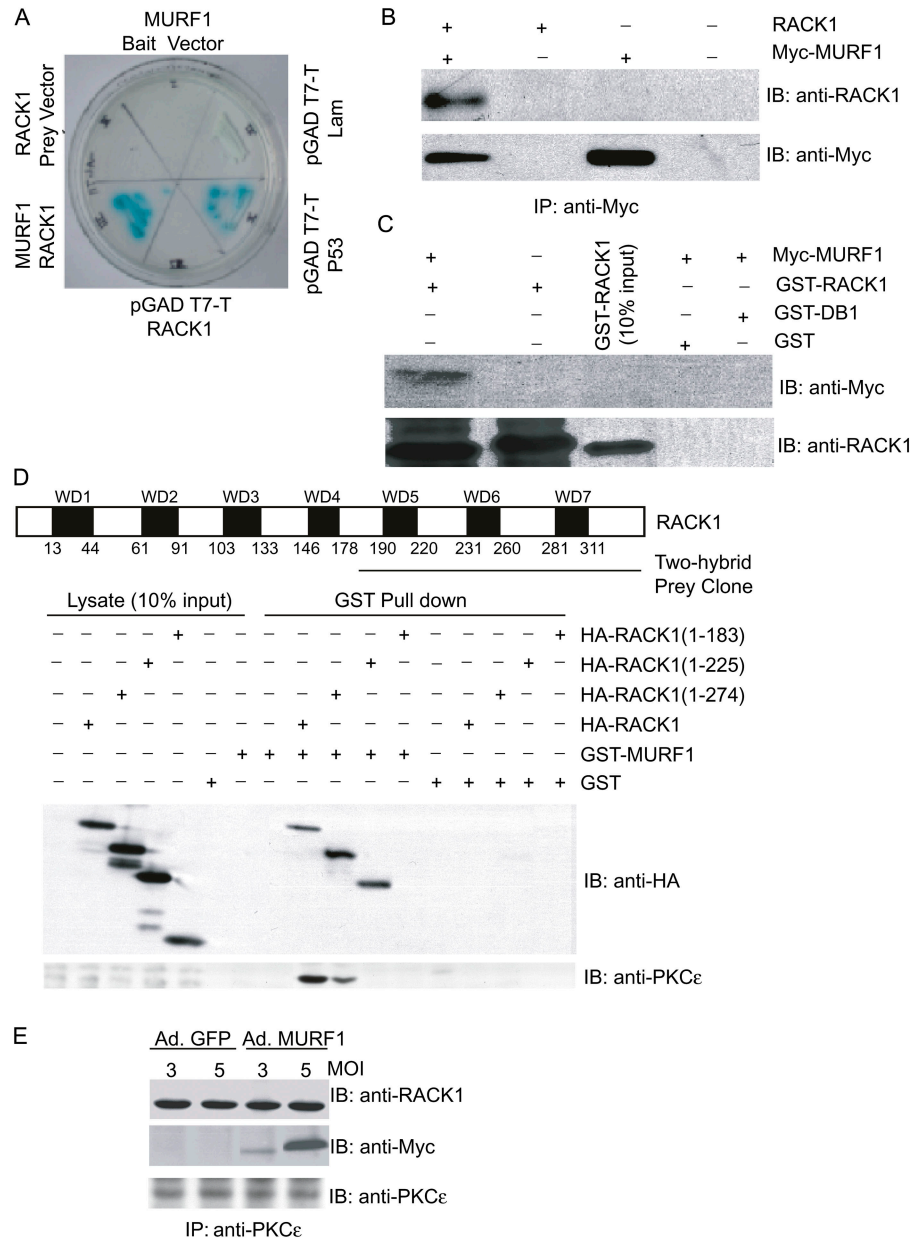
(Chang et al., 1998), dynamin-1 (Lin and Gilman, 1996; Rodriguez et al., 1999), Src (Luttrell et al., 1996, 1997; Chang et al., 1998, 2001), and the β subunit of integrins (Liliental and Chang, 1998; Buensuceso et al., 2001). Recently, Besson et al. (2002) showed that phorbol ester stimulation of human glioma cells increases focal adhesion formation and coimmunoprecipitation of PKCε, RACK1, and β$_1$ and β$_5$ integrins. Interestingly, FAK and PKCε colocalize to focal adhesions in neonatal rat ventricular myocytes (NRVM), suggesting a functional link between these two kinases (Heidkamp et al., 2003). Like PKCε, FAK has a requisite role in α-AR agonist–induced cardiac hypertrophy (Fluck et al., 1999; Laser et al., 2000; Taylor et al., 2000). In spite of all this, the factors that could potentially inhibit these pathways and thus α-AR signaling in cardiac myocytes are not well known. In addition, it is undetermined what effector molecules regulate the complex interactions between PKC isoforms and RACK1.

Recently, muscle ring finger protein-1 (MURF1) was identified as a protein up-regulated in skeletal muscle atrophy (Bodine et al., 2001b). MURF1 expression is exquisitely restricted to cardiac and skeletal muscle. MURF1 binds the sarcomeric protein titin (Centner et al., 2001) and overexpression of MURF1 disrupts the portion of titin that binds MURF1, suggesting that MURF1 regulates the stability of this large structural protein (McElhinny et al., 2002). Structurally, MURF family members contain a Zn^{2+}-binding RING finger domain at their extreme NH$_2$-terminal ends, a MURF family-specific conserved region, a B-box domain, coiled-coil motifs, and an acidic tail (Spencer et al., 2000; Centner et al., 2001; Dai and Liew, 2001). MURF2 and MURF3 are two other proteins that share a high degree of homology to MURF1 (Spencer et al., 2000; Centner et al., 2001). The RING finger B-box coiled-coil (RBCC) family of proteins to which the MURFs belong have critical roles in cellular processes including signal transduction,

A   Ad. GFP − phenylephrine

B   Ad. GFP + phenylephrine

C   Ad. MURF1 − phenylephrine

D   Ad. MURF1 + phenylephrine

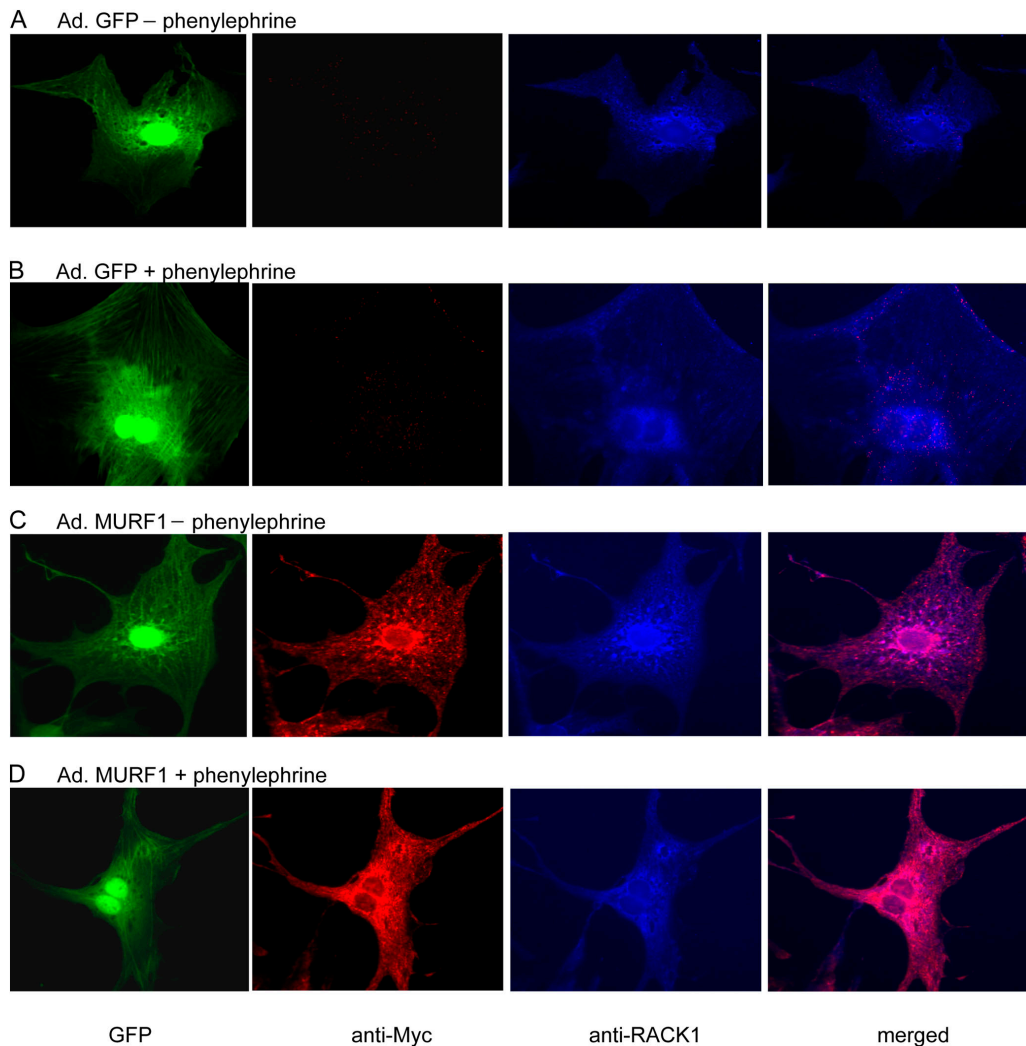GFP   anti-Myc   anti-RACK1   merged

Figure 2. **MURF1 colocalizes with RACK1 in cultured cardiac myocytes.** Rat cardiac myocytes were infected with recombinant adenovirus Ad.GFP (A and B) or Ad.MURF1 (C and D) for 24 h in serum free medium followed by induction with PE (B and D) for 48 h. Immunostaining was done with anti-Myc and anti-RACK1 antibodies. This was followed by secondary antibody incubation with anti–rabbit Alexa 568 (red) and anti–mouse AMCA (blue). The green color represents GFP expression.

gene transcription, ubiquitination, and differentiation (Borden, 2000; Freemont, 2000).

To date, very little is known about the cellular roles of MURF1. In vitro binding studies indicate that MURF family members homo- and hetero-oligomerize (Centner et al., 2001). MURF3 associates with microtubules and may participate in myogenic differentiation and microtubule stabilization through undetermined mechanisms (Spencer et al., 2000). MURF1 interacts with small ubiquitin-related modifier3 (Dai and Liew, 2001), a member of a ubiquitin-related class of proteins implicated in subcellular targeting and nuclear import (Melchior, 2000), and glucocorticoid element binding protein-1 (McElhinny et al., 2002), a nuclear protein implicated in transcriptional regulation (Zeng et al., 2000). Consistent with this finding, MURF1 is detected in nuclei in addition to its sarcomeric and cytoplasmic localization (Dai and Liew, 2001). Despite these recent studies, the exact physiological role(s) of the MURF family members have remained elusive.

Results

MURF1 interacts with the signaling scaffold protein RACK1

To identify proteins that bind to MURF1, we screened a human heart cDNA library with a GAL4 fusion construct containing aa 1–143 of MURF1 (which contains ring finger and MURF-specific domains, but not the B-box domain known to interact with titin) using a yeast two-hybrid approach. After selection in high stringency conditions, three interacting cDNAs were isolated and sequenced. One of the cDNAs contained the COOH terminus of RACK1 (475–1144 nucleotides) in frame with GAL4. The selectivity of the MURF1–RACK1 interaction in the yeast two-hybrid reaction was confirmed by retesting in a lacZ reporter assay (Fig. 1 A).

To confirm the binding between MURF1 and RACK1 in vivo, COS 7 cells were transfected with vector alone, RACK1, or RACK1 with full-length Myc-MURF1. Proteins immunoprecipitated with anti-Myc antibody were immuno-
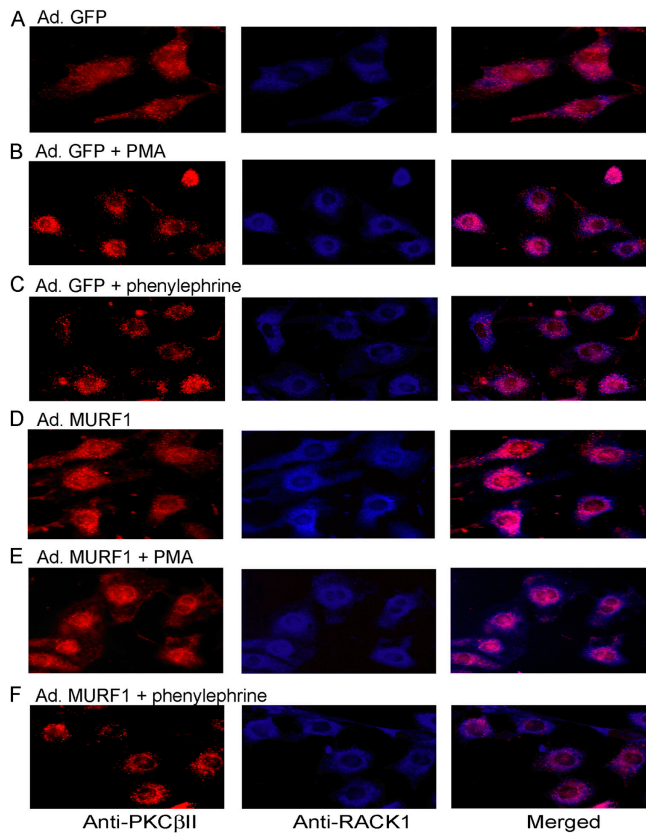
A Ad. GFP

B Ad. GFP + PMA

C Ad. GFP + phenylephrine

D Ad. MURF1

E Ad. MURF1 + PMA

F Ad. MURF1 + phenylephrine

Anti-PKCβII   Anti-RACK1   Merged

G

	Ad. GFP	+	+	+	−	−	−
	Ad. MURF1	−	−	−	+	+	+
	PMA	−	+	−	−	+	−
	phenylephrine	−	−	+	−	−	+

Figure 3. **MURF1 does not alter PE- and PMA-induced translocation of PKCβII from cytosol to the perinuclear region in NRVM.** NRVM were infected with Ad.GFP (A–C) or Ad.MURF1 (D–F) for 24 h in serum-free medium followed by induction with PE or PMA for 15 min. The cells were fixed and incubated with anti-RACK1 (blue) and anti-PKCβII (red) for 2 h. (G) Quantitative determination of perinuclear PKCβII staining under the indicated conditions.

blotted with an antibody specific for RACK1 (Fig. 1 B). RACK1 was detected only in cell lysates expressing both Myc-MURF1 and RACK1, indicating efficient association between these two proteins in vivo. To further confirm the interaction of MURF1 with RACK1, we incubated a GST-RACK1 fusion protein together with COS 7 cell lysates expressing Myc-MURF1. Protein complexes were collected on glutathione-agarose beads and assayed for MURF1 binding by immunoblot analysis with anti-Myc antibody (Fig. 1 C). MURF1 was pulled down by GST-RACK1 only, and not by GST or GST-DB1 (a nonspecific control).
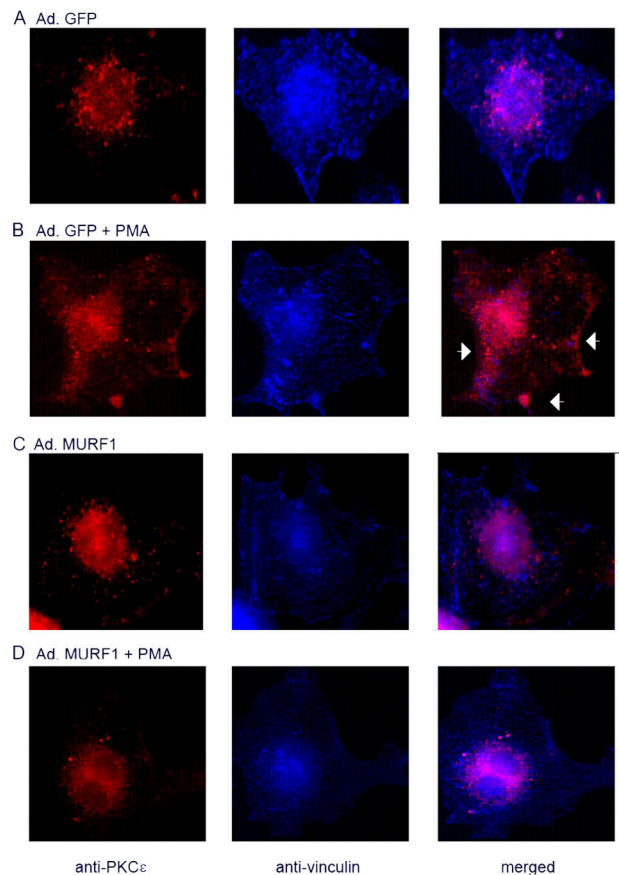
A Ad. GFP

B Ad. GFP + PMA

C Ad. MURF1

D Ad. MURF1 + PMA

anti-PKCε   anti-vinculin   merged

Figure 4. **MURF1 inhibits PMA-induced translocation of PKCε from the perinuclear region to focal adhesion complexes in NRVM.** NRVM were infected with Ad.GFP (A and B) or Ad.MURF1 (C and D) for 24 h in serum-free medium followed by induction with PMA (B and D) for 15 min. The cells were fixed and incubated with anti-vinculin (blue) and anti-PKCε (red) for 2 h. Arrows indicate focal adhesions.

RACK1 contains seven repeats of the WD40 motif (Ron et al., 1994; Schechtman and Mochly-Rosen, 2001), a domain involved in protein–protein interactions that regulates multiple cellular functions (Neer et al., 1994). Because our two-hybrid screen indicated that MURF1 binds to the COOH-terminal 174–317 amino acids of RACK1, we generated serial COOH-terminal RACK1 deletions of WD40 repeats to delineate the binding site of MURF1 on RACK1. GST or GST-MURF1 fusion proteins were incubated with HA-tagged RACK1 deletions expressed in COS 7 cells. Using this approach, we found that MURF1 binding requires residues in the WD5 repeat region of RACK1 (183–225 amino acids; Fig. 1 D). This stands in contrast to previous reports that implicate the adjacent domain WD6 in PKC binding to RACK1 (Mochly-Rosen et al., 1991; Ron et al., 1994). Indeed, we also found that the presence of RACK1 markedly increases the amount of PKCε in GST-MURF1 precipitates, but only in constructs containing the WD6 domain. We cannot exclude the possibility that MURF1 binds RACK1 through additional points of contact in addition to WD5, but its binding mechanism appears to be distinct from that of PKCε.
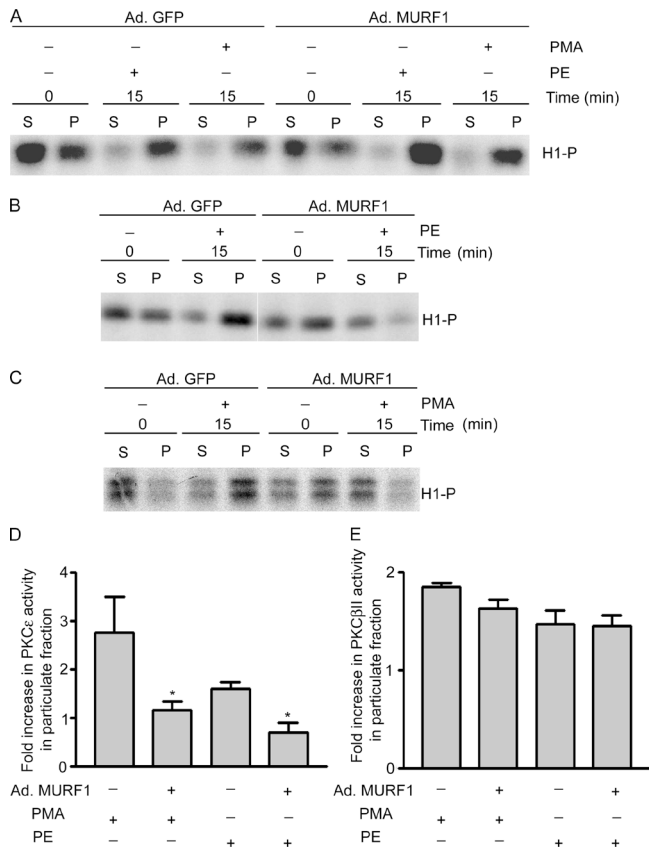
Figure 5. **MURF1 inhibits adrenergic agonist-induced PKCε activity (but not PKCβII activity) in the particulate fraction of NRVM.** (A–C) After infection with Ad.GFP or Ad.MURF1 for 24 h in serum-free medium, NRVM were induced with PE or PMA for 15 min and subjected to subcellular fractionation. The detergent-soluble (S) and particulate (P) fractions were immunoprecipitated with anti-PKCβII (A) or anti-PKCε antibody (B and C) and were subjected to in vitro kinase assays using histone H1 and γ[^{32}P]ATP. (D and E) The results from densitometric scanning of kinase assays from three independent experiments are presented as means ± SEM of PKCε (D) or PKCβII (E) activity in the particulate fraction compared with soluble fraction. *, P < 0.05 compared with control.

To determine whether MURF1 forms a ternary complex with RACK1 and PKC isoforms, we infected cardiomyocytes with Ad.MURF1 and immunoprecipitated PKCε. We then probed these immunoprecipitates for RACK1 and MURF1 (using a Myc antibody; Fig. 1 E). MURF1 is present in PKCε immunoprecipitates (indicating that a ternary complex is formed), and the amount of RACK1 in these immunoprecipitates is unaffected by the amount of MURF1 that is present (which suggests that there is neither cooperation nor competition among these proteins).

MURF1 colocalizes with RACK1 in cardiac myocytes

Previous papers have shown that MURF1 localizes in the cytosol, sarcomeres, and nuclei of myocytes (McElhinny et al., 2002), whereas RACK1 is present in the cytosol (Ron et al., 1995, 1999). However, after activation with PMA, RACK1 is localized in perinuclear structures (Ron et al., 1995, 1999). We also observed similar staining of MURF1 and RACK1 in
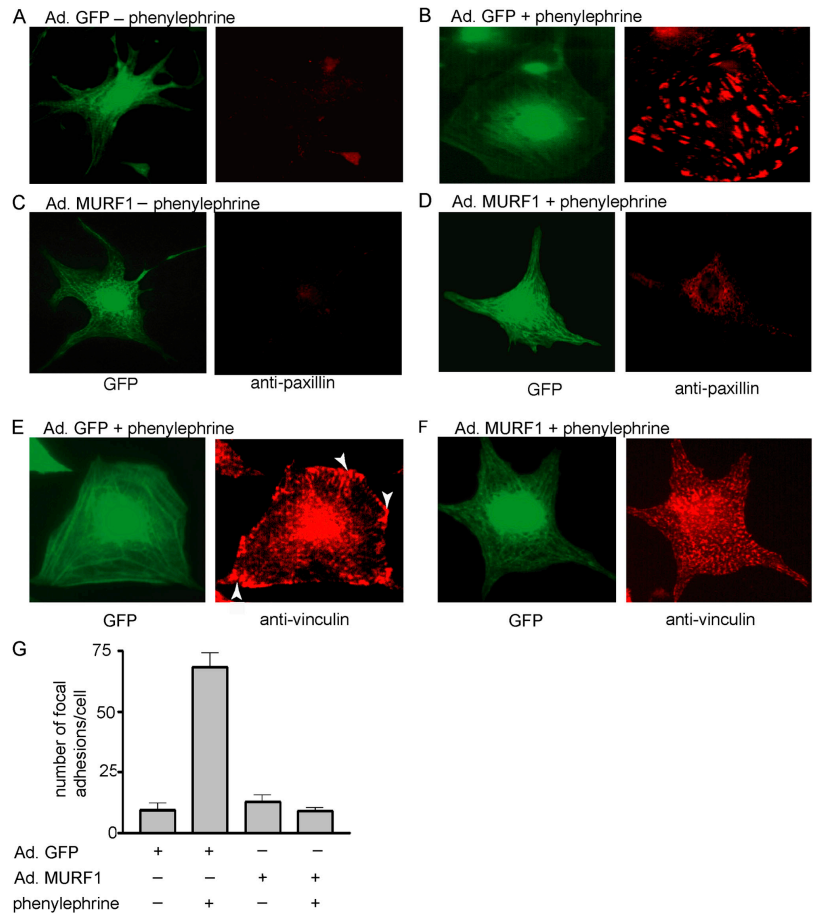
Ad.MURF1-infected NRVM (Fig. 2, A–C). In addition, we observed enhanced colocalization of MURF1 with RACK1 in the perinuclear region after activation with PE (Fig. 2 D). Stimulation with PMA resulted in similar colocalization of MURF1 with RACK1 from the cytosol and sarcomere to the perinuclear region and nucleus (unpublished data). These data indicate that MURF1 colocalizes with RACK1 after activation in NRVM, placing MURF1 in the right place and time to modulate RACK1-dependent signaling.

MURF1 specifically inhibits the translocation of PKCε, but not PKCβII

Because RACK1 is known to regulate targeting of PKC isoforms, we examined the translocation of PKC isoforms in NRVM in the presence of MURF1. In nonstimulated myocytes, PKCβII is associated with fibrillar structures and after activation it translocates to the nuclear and cell periphery, whereas before stimulation, PKCε is observed in the nucleus and perinucleus but translocates to cross-striated structures and focal adhesions in stimulated cells (Disatnik et al., 1994a; Ron et al., 1995; Johnson et al., 1996; Heidkamp et al., 2003). PKC activation induces the association and colocalization of RACK1 with PKCβII (Ron et al., 1999); MuRF1 modestly increased perinuclear localization of PKCβII under unstimulated conditions and did not inhibit PE- or PMA-dependent PKCβII translocation (Fig. 3). PKCβII moved from cytosol to perinuclear structures after activation with PE and PMA, and colocalized with RACK1 in control as well as Ad.MURF1-infected NRVM. Several recent reports have also shown colocalization of PKCε and RACK1 in response to stimulation with PE and PMA (Pass et al., 2001a,b; Besson et al., 2002). Using an anti-PKCε antibody, we observed the translocation of PKCε from perinuclear structures to focal adhesions upon activation with PE or PMA in NRVM infected with Ad.GFP, as indicated by costaining with the focal adhesion marker vinculin. Surprisingly, this translocation was inhibited after infection with Ad.MURF1 (Fig. 4). PKCε translocation occurred in 22 ± 6% of Ad.MURF1-infected cells treated with PMA compared with 82 ± 8% of control cells. After stimulation with PMA, PKCε was still observed in the perinuclear structures and nuclei in these cells. Similar patterns were observed after stimulation with PE (unpublished data). These data indicate that MURF1 specifically inhibits the movement of the PKCε isoform after agonist-induced stimulation.

To further define the function of MURF1 in NRVM, PKC activity was measured with an in vitro kinase assay. NRVM were treated with PE or PMA in the presence or absence of ectopic MURF1 expression. The lysates were subsequently fractionated into soluble and particulate components. After immunoprecipitation with either anti-PKCε or anti-PKCβII antibody, the immunocomplexes were mixed with histone H1 as an exogenous substrate. Consistent with our immunofluorescence data, MURF1 did not inhibit PKCβII activity in response to PE or PMA treatment. The majority of PKCβII phosphotransferase activity (72.5% ± 5) was observed in the particulate fraction of Ad.GFP- and Ad.MURF1-infected cells after PE or PMA treatment (Fig. 5 A), and there was no signifi-

Figure 6. **MURF1 inhibits focal adhesion formation in NRVM.** NRVM were infected with Ad.GFP (A, B, and E) or Ad.MURF1 (C, D, and F) for 24 h in serum-free medium followed by induction with PE for 48 h (B and D). The cells were fixed in 3.7% formaldehyde followed by incubation with anti-paxillin (A–D) or anti-vinculin (E and F) antibodies. Arrows indicate focal adhesions. (G) Quantitative determination of paxillin-positive focal adhesions per cell under the indicated conditions.

A Ad. GFP – phenylephrine
B Ad. GFP + phenylephrine
C Ad. MURF1 – phenylephrine   GFP   anti-paxillin
D Ad. MURF1 + phenylephrine   GFP   anti-paxillin
E Ad. GFP + phenylephrine   GFP   anti-vinculin
F Ad. MURF1 + phenylephrine   GFP   anti-vinculin

G number of focal adhesions/cell

Ad. GFP	+	+	−	−
Ad. MURF1	−	−	+	+
phenylephrine	−	+	−	+

cant effect of MURF1 on PKCβII activity (Fig. 5 E). However, PKCε activity was observed in the soluble fraction even after PE (62.5% ± 5) or PMA (68.5% ± 2) treatment in Ad.MURF1-infected cells, and translocation to the particulate fraction was inhibited (Fig. 5, B–D). We cannot determine whether these effects are totally dependent on the ability of MURF1 to interact with RACK1; nevertheless, these data indicate that MURF1 specifically inhibits the translocation and activity of PKCε in the particulate fractions of NRVM.

MURF1 inhibits focal adhesion formation in NRVM

Previous reports indicate that PE-induced hypertrophy in isolated neonatal cardiomyocytes requires signaling through FAK (Taylor et al., 2000), and activation of FAK, in turn, depends on PKCε (Eble et al., 2000; Heidkamp et al., 2003). To determine whether the effects of MURF1 on PKCε translocation and activation influence focal adhesion assembly, we first examined the localization of paxillin, a resident focal adhesion protein, by immunostaining with anti-paxillin antibody after PE treatment in NRVM. Paxillin staining was dispersed in untreated cells (Fig. 6 A), but after PE treatment staining appeared in distinct focal complexes in the Ad.GFP-infected cells (Fig. 6 B). In contrast, the subcellular redistribution of paxillin in response to PE was markedly inhibited in the presence of Ad.MURF1 (Fig. 6, C and D). Similarly, vinculin staining revealed characteristic staining

of Z-disc structures and peripheral focal adhesions after PE treatment, whereas cells infected with Ad.MURF1 lost the focal adhesion pattern but retained Z-disc–tethered vinculin expression. A quantitative analysis of paxillin-positive focal adhesions per cell is shown in Fig. 6 G. These data suggest that MURF1 inhibits PE-induced focal adhesion formation in NRVM.

Tyrosine phosphorylation is a major covalent modification driving protein–protein interactions required for focal adhesion assembly. To determine the kinetics and extent of tyrosine phosphorylation of focal adhesion–associated proteins, FAK was immunoblotted with a phospho-FAK antibody that specifically recognizes the phosphorylation of tyrosine 397 (Schaller et al., 1994), which is crucial for FAK activation. An increase in tyrosine phosphorylation of FAK was observed after 15–20 min of PE treatment in control cells (Fig. 7 A). This phosphorylation of tyrosine 397 was blocked by increasing levels of MURF1. In addition, we immunoprecipitated paxillin from PE-treated cells and tested its phosphorylation status by Western blotting with anti-phosphotyrosine antibody. As expected, paxillin phosphorylation occurred rapidly after PE or PMA treatment, and MuRF1 inhibited the phosphorylation of paxillin by PE or PMA (Fig. 7 B). Previous papers have shown that tyrosine phosphorylation and activation of FAK can activate ERK1/2 signaling cascades (Govindarajan et al., 2000), and that ERK2 is the downstream target of FAK that is activated during assembly of focal adhesion proteins in cardiac
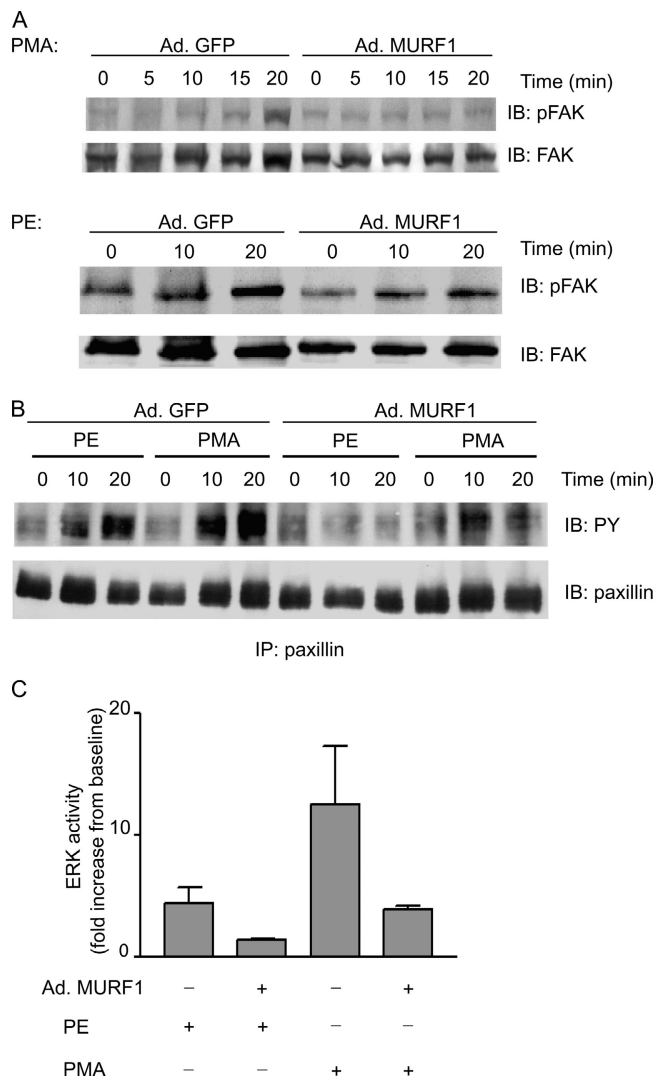
A
PMA:

IB: pFAK
IB: FAK

PE:

IB: pFAK
IB: FAK

B

IB: PY
IB: paxillin

IP: paxillin

C

Figure 7. **Effects of MURF1 on components of the FAK signaling pathway.** (A) NRVM were infected with Ad.GFP or Ad.MURF1 for 24 h followed by induction with PE or PMA. NRVM lysates were separated by SDS-PAGE. Blots were incubated with anti-pFAK (397Y) antibody and anti-FAK antibody (loading control). (B) NRVM cell lysates treated as in A were immunoprecipitated with anti-paxillin antibody followed by immunoblotting with anti-phosphotyrosine (PY) antibody. (C) To test for activation of MAP kinase signaling, NRVM cell lysates were analyzed by Western blotting with anti-pERK antibody and anti-ERK antibody (loading control). The cumulative results are presented as fold increase in ERK activity from basal level ± SEM.

myocytes (Taylor et al., 2000). Similar to the inhibition of FAK phosphorylation, we observed inhibition of ERK2 phosphorylation in Ad.MURF1-infected cardiomyocytes after 15–20 min of PE or PMA treatment (Fig. 7 C). Together, these observations indicate that the ability of MURF1 to inhibit activation of PKCε leads to impaired focal adhesion assembly and arrests signaling downstream of FAK in PE- or PMA-stimulated NRVM.

MURF1 antagonizes PE- and PMA-dependent cardiomyocyte hypertrophy in vitro

Activation of both PKC and FAK leads to cellular changes indicative of hypertrophy, including increased cell size, sarco-

meric organization, and induction of hypertrophic markers such as atrial natriuretic factor (ANF). To test the hypothesis that MURF1 antagonizes initiation of the hypertrophic response, we infected NRVM with Ad.GFP or Ad.MURF1, followed by stimulation with PE for 48 h. Immunohistochemical analysis indicated that ANF was induced after PE treatment in the perinuclear region in the Ad.GFP-infected NRVM as expected, but this effect was inhibited in Ad.MURF1-infected cells (Fig. 8 A). Similar results were observed after treatment with PMA (unpublished data). Next, we examined the expression of ANF, α-actin, and β-myosin heavy chain mRNA in cardiomyocytes treated with PE, angiotensin-II, endothelin-1, insulin-like growth factor 1 (IGF-1), or serum, in the presence and absence of Ad.MURF1 by RT-PCR. The basal level of these mRNAs was detected in myocytes as reported previously (Sekiguchi et al., 1999). Exposure to each of these agonists resulted in up-regulation of these hypertrophic markers, and in each case (except for IGF-1) up-regulation was blocked by increased expression of MURF1 (Fig. 8 B). These data indicate that MURF1 inhibits G protein–coupled receptor-dependent expression of hypertrophic markers in cardiomyocytes.

To further characterize the effects of MURF1 on cardiomyocyte hypertrophy, we examined how the activity of MURF1 modulated cell size and sarcomeric organization in NRVM. As expected, PE treatment of GFP-infected cardiomyocytes led to increased cell size compared with untreated cells (Fig. 9 A). In the presence of Ad.MURF1, the response to PE was dramatically reduced. To quantitate the extent of hypertrophy, a total of 150–200 cells in each treatment were scored for their cell surface areas. Treatment of cardiomyocytes with PE for 48 h resulted in a threefold increase in cell size. MURF1 completely abolished the PE- and PMA-induced increase in myocyte size, but not the changes induced by IGF-I (Fig. 9 B). The specificity of MURF1 effects on hypertrophy was further substantiated by studying the effect of a similar ring finger ubiquitin ligase protein, CHIP. CHIP had no effect on agonist-induced increases in cell size (Fig. 9 B).

NRVM cultured in serum-free conditions displayed thin and rudimentary sarcomeric structure (Fig. 9 C), and treatment of myocytes with PE or PMA caused a reorganization of sarcomeres typical of the hypertrophic response as shown by immunostaining with α-actinin antibody (Fig. 9 D). However, Ad.MURF1 expression completely abolished PE- or PMA-induced sarcomere reorganization (Fig. 9, C and D). Together, these morphologic, immunohistochemical, and molecular data demonstrate that MURF1 blocks PE- and PMA-induced hypertrophic signaling pathways and indicate a functional role of MURF1 in inhibiting initiation of cardiomyocyte hypertrophy.

To demonstrate that endogenous expression of MURF1 has a role in the regulation of hypertrophy, we knocked down MURF1 expression in NRVM by generating small interfering RNA (siRNA) specific to MURF1. MURF1 siRNA inhibited expression of MURF1 in COS 7 cells by >70% (Fig. 10 A). MURF1 siRNA was cotransfected in NRVM along with EGFP to mark transfected cells. A total of 150–200 cells per condition were scored for their cell surface areas. When MURF1 expression was knocked down, cells were 2.5-fold larger than the
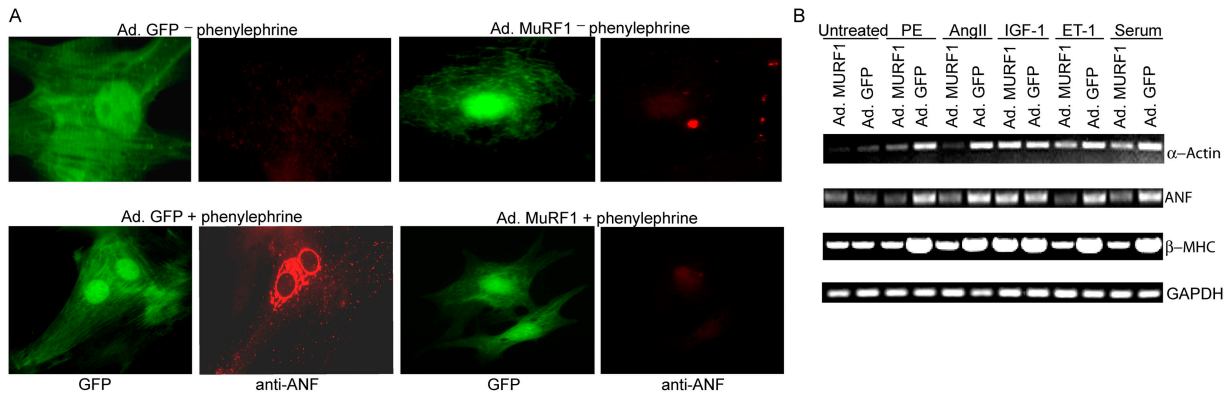
Figure 8. **MURF1 inhibits adrenergic agonist-induced cardiac gene expression.** (A) NRVM were infected with Ad.GFP or Ad.MURF1 for 24 h followed by induction with PE for 48 h. The cells were followed by immunostaining with rabbit anti-ANF antibody (red). (B) After adenoviral infection for 24 h in serum-free medium, cells were induced with PE, Ang II, IGF-1, endothelin-1 (ET-1), and serum for 24 h. mRNA was isolated and subjected to RT-PCR using primers specific to skeletal α-actin, ANF, β-MHC, and GAPDH.

control cells under quiescent conditions and 1.5-fold higher after treatment with PE or PMA (Fig. 10 B). To further confirm the affect of endogenous MURF1 on hypertrophy, we examined the expression of ANF in cells transfected with MURF1 siRNA and counted the number of cells expressing ANF. We observed a 20-fold increase in cells expressing ANF under basal conditions and enhancement of PE- and PMA-induced ANF expression (Fig. 10 C). These data indicate that endogenous MURF1 regulates NRVM cell size tonically and also after stimulation with hypertrophic agonists.

Discussion

Cardiac hypertrophy is viewed as a compensatory response to increased load common to diverse clinical settings such as hypertension, valvular disease, and inherited cardiomyopathies (Sadoshima and Izumo, 1997; MacLellan and Schneider, 2000). Myocytes respond to pressure overload with the addition of sarcomeres, resulting in an increase in ventricular wall thickness, which itself is an independent risk factor for cardiovascular mortality (Mathew et al., 2001; Verdecchia et al., 2001). Hence, blunting hypertrophic growth might be beneficial to cardiac function, and previous studies of genetically engineered mice disrupting certain hypertrophic pathways confirm this prediction (Esposito et al., 2002; Sano and Schneider, 2002). However, we still do not know which pathways hold greatest potential for therapeutic benefit. Although the signaling pathways that activate hypertrophy have been described in detail (McKinsey and Olson, 1999; Molkentin and Dorn, 2001), still very little is known of mechanisms that attenuate hypertrophic growth. In spite of well-defined genetic models and essential mediators of hypertrophy (Adams et al., 1998; Molkentin et al., 1998; Bueno et al., 2000; Antos et al., 2002; Minamino et al., 2002), the distal effectors that execute myocyte and heart enlargement remain uncertain. To this end, we have characterized a novel anti-hypertrophic signaling pathway that operates in part through inhibition of PKC-mediated signaling in myocytes, indicating that MURF1 plays a critical role in tuning the balance of hypertrophic and anti-hypertrophic signaling within myocytes.

Evidence supports the notion that activation of PKC may be a critical trigger of cardiac hypertrophy and failure (Wakasaki et al., 1997; Pass et al., 2001b). Transgenic mice expressing cardiac-specific PKCβII exhibit enhanced troponin I phosphorylation and develop hypertrophy (Takeishi et al., 2000). Activation of PKCε also contributes to $G_{\alpha q}$ overexpression-induced cardiac hypertrophy (D'Angelo et al., 1997). In addition, PKCε association with RACK1 is linked to the genesis of cardiac hypertrophy and failure (Pass et al., 2001b). Thus, our observations that MURF1 interacts with RACK1 and inhibits PE- and PMA-induced PKCε translocation demonstrate the existence of a previously unrecognized anti-hypertrophic mechanism in cardiac myocytes. It is possible that the ability of MURF1 to antagonize hypertrophy may be due to regulation of a class switch between PKCβII–RACK1 and PKCε–RACK1 interactions. Previous reports have shown that RACK1 interacts with the $G_{\beta\gamma}$ dimer and $G_{\alpha\beta\gamma}$ trimer of G protein and both $G_{\beta\gamma}$ and $G_{\alpha\beta\gamma}$ compete with the binding of activated PKC to RACK1 (Dell et al., 2002). Thus, MURF1 may also play a role in coordinating the interactions between PKC isoforms and $G_{\alpha q}$ with RACK1.

FAK, a primary mediator of integrin signaling, plays a role in PE- and endothelin-1–induced hypertrophy and adhesive response of NRVMs (Eble et al., 2000; Taylor et al., 2000), and FAK is activated by PKCε (Heidkamp et al., 2003). Moreover, increased focal adhesion association and coimmunoprecipitation of PKCε, RACK1, and β_1 and β_5 integrins in human glioma cells suggest a functional and structural link between these two pathways (DePasquale and Izzard, 1991; Guan and Shalloway, 1992; Romer et al., 1994; Defilippi et al., 1997; Heidkamp et al., 2003). We demonstrate that in addition to inhibition of PKCε translocation to focal adhesions, MURF1 inhibits the tyrosine phosphorylation of FAK and paxillin leading to inhibition of focal adhesion formation. Furthermore, MURF1 significantly inhibits the adrenergic activation of ERK1/2. This observation is consistent with reports from other groups showing that a dominant inhibitor of FAK blunts ERK1/2 activation and ERK-dependent hypertrophy (Ross et al., 1998; Taylor et al., 2000). Together, our data suggest that
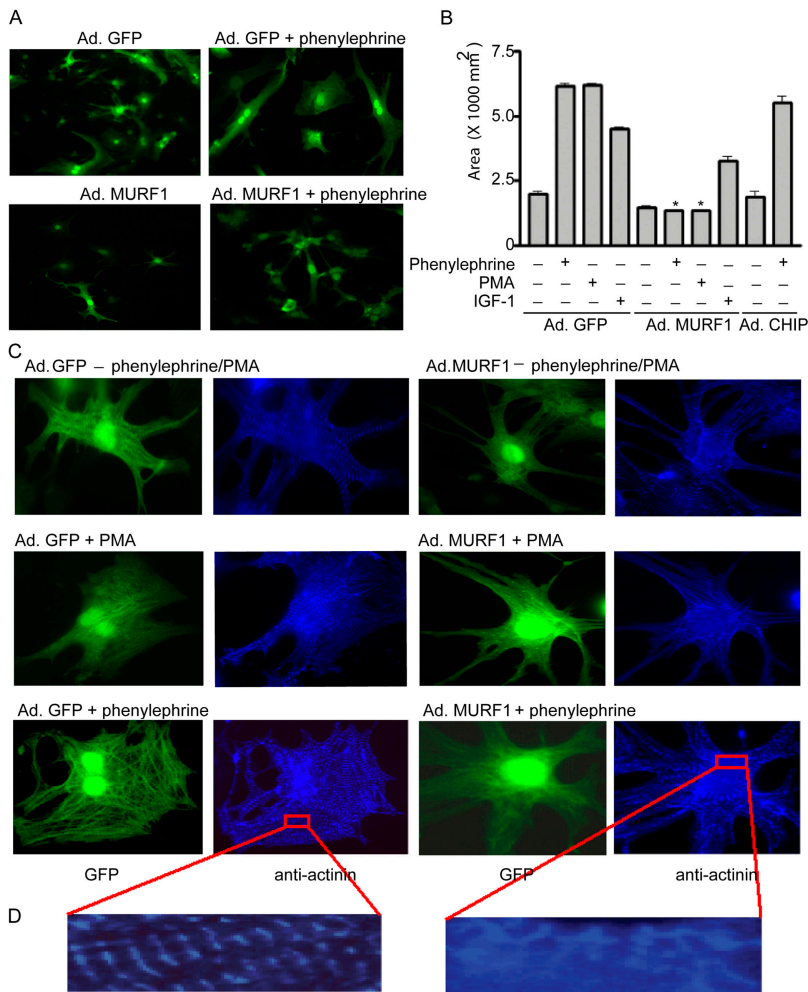
Figure 9. **Effects of MURF1 on cardiac cell size and sarcomere organization.** (A) NRVM were infected with Ad.GFP or Ad.MURF1 for 24 h followed by induction with PE or PMA for 48 h. The cells were observed under a fluorescent microscope with a 20× objective lens. (B) Approximately 150–200 cells per condition were measured for cell surface area using the ImageJ program. The data are presented as means ± SEM. *, $P < 0.05$ compared with control. (C) After induction with PE or PMA, cells were fixed and immunostained with mouse anti-α-actinin antibody (blue). (D) Insets reveal sarcomeric organization in myocytes.

MURF1 plays a central role in regulating PKC-dependent focal adhesion assembly and activation of downstream signaling pathways that lead to hypertrophy.

We found that MURF1 antagonizes the hypertrophic effects of PMA and PE, which induces cardiomyocyte hypertrophy in a PKCε-dependent fashion (Pass et al., 2001a,b; Heidkamp et al., 2003). Remarkably, MURF1 overexpression has no effect on IGF-1–dependent hypertrophic marker gene expression (Fig. 8) or cardiomyocyte hypertrophy (Fig. 9). Because IGF-1–dependent hypertrophy requires signaling via the phosphoinositide 3-kinase/Akt axis rather than through PKC-dependent mechanisms (Kozma and Thomas, 2002), these observations indicate that, at least at the level of intracellular signaling, there is a degree of specificity to the actions of MURF1. In light of recent observations indicating that signaling pathways mediating pathological versus physiological hypertrophy may be distinct (Wilkins et al., 2004), it is tempting to speculate that MURF1 is better positioned to inhibit the former, but not the latter. Further studies will be needed to address this interesting possibility.

Our data and that of other groups indicate that MURF1 is localized in multiple sites within the cell. In the cytosol MURF1 associates with microtubules, and in the sarcomere MURF1 is the only MURF family member that binds Titin

(Centner et al., 2001). We have also observed increased immunostaining of MURF1 in the perinuclear region after agonist stimulation in NRVM (Fig. 2). These data indicate that MURF1 may inhibit hypertrophy at multiple steps and may participate in discrete multiprotein complexes that reside in different places within the cell. In fact, MURF1 has been shown to interact with ubiquitin-conjugating enzyme 9, isopeptidase T-3, and glucocorticoid modulatory element binding protein-1 (McElhinny et al., 2002). In the nucleus, MURF1 may regulate processes such as the signal-dependent transcriptional activation of cardiac genes involved in hypertrophy or the import and export of proteins. Further studies are needed to determine whether MuRF1 in the nuclear compartment directly regulates the transcription of cardiac-specific genes, which might also contribute to its anti-hypertrophic activity.

The presence of a ring finger raises the possibility that MURF1 has E3 ubiquitin ligase activity. In fact, MURF1 catalyzes the assembly of ubiquitin chains in vitro (Bodine et al., 2001a). Our studies indicate that neither RACK1 nor PKCε are likely to be substrates for MURF1's ubiquitin ligase activity, as we did not observe changes in steady-state levels or accumulation of multiubiquitinated forms of RACK1 by MURF1. These data indicate that the anti-hypertrophic effects of MURF1 may be mediated, at least in part, independently of ubiquitin ligase
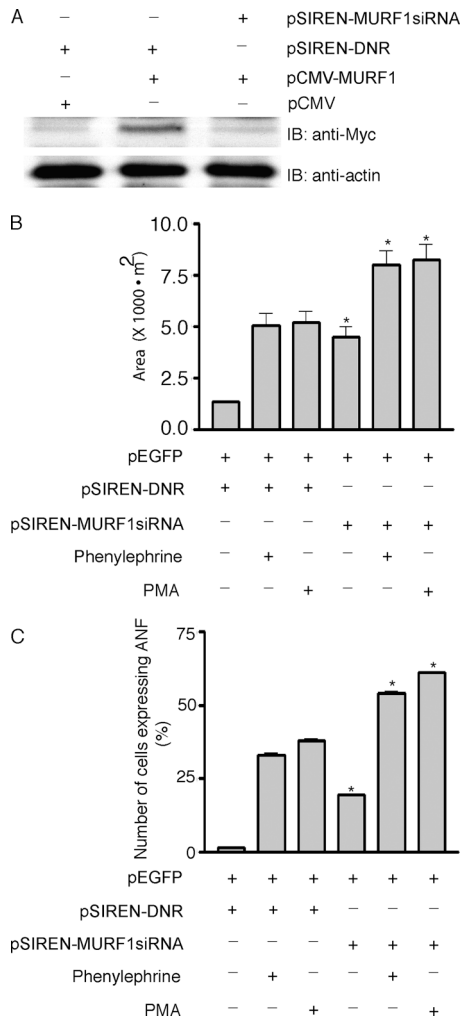
Figure 10. **Effect of endogenous MURF1 on cardiac cell size and ANF expression.** (A) COS 7 cells were transiently transfected with pCMV-Myc-MURF1 and pSIREN-MURF1 siRNA or with control vectors as indicated. The cell lysates were subjected to SDS-PAGE analysis followed by Western blotting with anti-Myc antibody. (B) NRVM cells were transfected with GFP and MURF1 siRNA for 36 h followed by induction with PE or PMA for 48 h. The cells were observed with a fluorescent microscope with a 60× objective lens. 150–200 cells per condition were measured for cell surface area using the ImageJ program. The data are presented as means ± SEM. *, P < 0.05 compared with control. (C) After induction with PE or PMA, cells were fixed and immunostained with rabbit anti-ANF antibody. 200 cells per condition were counted for ANF expression. The data are presented as means ± SEM. *, P < 0.05 compared with control.

activity. However, other studies from our laboratory indicate that MURF1 interacts with troponin I and elicits its degradation via ubiquitin ligase activity (unpublished data), indicating that MURF1 does have ubiquitin ligase activity in vivo. The association with troponin I may account for the fraction of MURF1 that is localized to the sarcomere, and suggests that the anti-hypertrophic activity of MURF1 may result from coordinated events in several cellular compartments.

Previous studies have identified genetic pathways that are activated during hypertrophy and induce cardiac growth. Our studies highlight the importance of molecular pathways that repress hypertrophic responses. Recently, homeodomain protein (Hop; Kook et al., 2003) and myocyte-enriched calcineurin-

interacting protein (MCIP; Rothermel et al., 2001) have also been demonstrated to regulate anti-hypertrophic gene responses. Hop inhibits the expression of anti-hypertrophic genes by associating with histone deacetylases and MCIP inhibits the calcineurin signaling pathway. Moreover, both of these proteins inhibit cardiac hypertrophy in genetic models of cardiomyopathy. Our studies indicate that MURF1 may represent another mechanism to regulate cardiac hypertrophy induced via G protein–coupled receptor signaling pathways. The extent to which these three proteins mediate their effects through common mechanisms is a promising topic for future research.

In the present paper we have demonstrated a new mechanism to regulate hypertrophy. Our present observations are limited to cultured cardiomyocytes, which limits their generalizability; nevertheless, cultured cardiomyocytes have proven extremely effective in revealing mechanisms of cardiac hypertrophy (Hunter and Chien, 1999). We speculate that blunting the hypertrophic response by MURF1-dependent inhibition of PKCε signaling may serve as a major adaptive strategy in the face of ongoing hypertrophic stimulation. MURF1 is a potential target for pharmacological intervention because it may regulate parallel and interconnected signaling pathways in cardiac muscle cells. Still, several key issues remain to be understood. How are the different signaling systems that evoke a hypertrophic response interconnected? When does MURF1 regulate the cascade of events leading to hypertrophy? The challenge now lies in elucidating the targets of MURF1 that are upstream of RACK1 and the downstream effector molecules inside the nucleus that regulate cardiac gene expression, and how MURF1 regulates hypertrophic responses in vivo.

Materials and methods

Cell culture
COS 7 cells were cultured in DME and transiently transfected using Fu-GENE (Roche) as described previously (Jiang et al., 2001). NRVM were isolated using the neonatal cardiomyocyte isolation kit (Worthington) and were plated on laminin. NRVM cell size was measured using ImageJ. The calculated cell areas from 150–200 cells were used to determine the average cell size of NRVM. Adenoviruses expressing MURF1 or control viruses were constructed with the Ad-Easy system, which express GFP and cargo proteins from a bicistronic message. Cultures were routinely infected at a multiplicity of infection of 3 with an infection efficiency of >98%.

Yeast two-hybrid screen
The NH_2 terminus of MURF1 (aa 1–143) was cloned into pGBKT7 in frame with the GAL4 DNA binding domain and screened against human heart library cloned into prey-vector pACT2. The candidate clones were rescued on medium-stringency medium (SD-His, Leu, Trip/X-B-gal) and later screened on high-stringency medium (SD-Leu, Trip, His, Ade/X-a-gal).

Immunoprecipitation
COS 7 cells were harvested 36–48 h after transfection and lysed with modified RIPA buffer (Garcia-Cardena et al., 1996) supplemented with protease inhibitors. Cell lysates were clarified by centrifugation at 16,000 g for 10 min and protein concentration was determined. Immunoprecipitated proteins or cell lysates were mixed with SDS sample buffer and were separated by SDS-PAGE.

GST pull-down assays
GST fusion protein beads were incubated with cell lysates of myc-MURF1 or HA-tagged deletions of RACK1 for 3 h. Protein complexes were washed four times in buffer containing 0.5% Nonidet P-40, 20 mM Tris, pH 8.0, 100 mM NaCl, and 1 mM EDTA, and were boiled in SDS sample buffer. Proteins were resolved by SDS-PAGE and subjected to immunoblot analysis.

Ventricular myocyte subcellular fractionation and immune complex kinase assays

Myocytes were infected with GFP or MURF1 adenovirus for 24 h in serum-free medium before induction with PE or PMA for 15 min. Cells were fractionated as described before (Clerk et al., 1994). In brief, cells were washed in ice-cold PBS and lysed by extraction in 500 μl buffer A (12.5 mM Tris-HCl, 2.5 mM EGTA, 1 mM EDTA, 100 mM sodium fluoride, 5 mM DTT, and 300 μM PMSF) containing 0.05% digitonin for 5 min. The soluble fraction was retained. The particulate fraction was washed in 500 μl buffer A, centrifuged, and resuspended in the same volume of buffer A containing 1% Triton X-100. The PKC isozyme-specific kinase assay was performed as described previously (Chen and Faller, 1999). The subcellular fractions (250 μg) were immunoprecipitated with the corresponding anti-PKC antibody and collected by absorption to protein A/G–Sepharose. The immunocomplexes bound to protein A/G–Sepharose were washed with buffer A twice and kinase buffer twice (50 mM Tris-HCl, pH 7.4, 10 mM sodium fluoride, 1 mM Na_3VO_4, 0.5 mM EDTA, 0.5 mM EGTA, 2 mM $MgCl_2$, 10 μg/ml leupeptin, and 1 mM PMSF). Subsequently, the immunocomplexes bound to the beads were resuspended in reaction buffer (20 mM Tris-HCl, pH 7.4, 10 mM $MgCl_2$, 10 μM cold ATP, 0.4 mg/ml histone H1, and 2.5 μCi γ[^{32}P]ATP [6,000 Ci/mmol]) and incubated at 30°C for 15 min.

Generation of MURF1 siRNA

MURF1 siRNA was generated using BD Knockout RNAi systems (BD Biosciences). MURF1 oligonucleotide (nt 616–638) was cloned in pSIREN vector and was sequence confirmed. pSIREN-DNR containing a scrambled oligo insert served as a control. COS7 cells and NRVM were transfected using FuGENE 6 (Invitrogen).

Image analysis

Cells were mounted in Fluoromount G and were viewed at RT with the 60× objective of an inverted microscope (Eclipse E800; Nikon) using NA 1.40. Pictures were taken with the QImaging RETIGA camera and digitized with Qcapture software (W. Nunsbaum, Inc.) The images were then converted to grayscale and were pseudocolored using IP Lab software (VayTek). Goat anti–mouse aminomethylcoumarin-labeled and goat anti–rabbit Alexa 568–labeled antibodies were used as fluorochromes.

Statistical methods

Results are presented as means ± SEM. Statistical significance was tested by two-tailed t test and was taken as being established at $P < 0.05$.

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