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Helix-Loop-Helix Protein p8, a Transcriptional Regulator Required for Cardiomyocyte Hypertrophy and Cardiac Fibroblast Matrix Metalloprotease Induction▽†

Sandro Goruppi,[1]* Richard D. Patten,[1] Thomas Force,[2] and John M. Kyriakis[1]*

Molecular Cardiology Research Institute, Tufts-New England Medical Center, Department of Medicine, Tufts University School of Medicine, 750 Washington Street, Boston, Massachusetts 02111,[1] and Center for Translational Medicine, Thomas Jefferson University, College Building Suite 316, 1025 Walnut Street, Philadelphia, Pennsylvania 19107[2]

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Cardiomyocyte hypertrophy and extracellular matrix remodeling, primarily mediated by inflammatory cytokine-stimulated cardiac fibroblasts, are critical cellular events in cardiac pathology. The molecular components governing these processes remain nebulous, and few genes have been linked to both hypertrophy and matrix remodeling. Here we show that p8, a small stress-inducible basic helix-loop-helix protein, is required for endothelin- and α-adrenergic agonist-induced cardiomyocyte hypertrophy and for tumor necrosis factor-stimulated induction, in cardiac fibroblasts, of matrix metalloproteases (MMPs) 9 and 13—MMPs linked to general inflammation and to adverse ventricular remodeling in heart failure. In a stimulus-dependent manner, p8 associates with chromatin containing c-Jun and with the cardiomyocyte atrial natriuretic factor (*anf*) promoter and the cardiac fibroblast *mmp9* and *mmp13* promoters, established activator protein 1 effectors. p8 is also induced strongly in the failing human heart by a process reversed upon therapeutic intervention. Our results identify an unexpectedly broad involvement for p8 in key cellular events linked to cardiomyocyte hypertrophy and cardiac fibroblast MMP production, both of which occur in heart failure.

The progression to heart failure involves an initial phase of pathological cardiomyocyte hypertrophy, which develops as a consequence of excess hemodynamic work load and may be triggered by α-adrenergic agents, angiotensin II, and/or endothelin. Pathological cardiomyocyte hypertrophy is followed by left ventricular decompensation, characterized by cardiomyocyte loss, and interstitial fibrosis—direct contributors to adverse ventricular remodeling. Ultimately, the contractile properties of the heart are compromised, resulting in heart failure (17, 18, 27). The molecular components and cellular events required for heart failure remain incompletely understood, and few genes have been linked to both pathological hypertrophy of cardiomyocytes and matrix remodeling (9, 17, 18, 27).

Post-myocardial infarction, in addition to hypertrophy of surviving cardiomyocytes, remodeling of the extracellular matrix occurs, particularly within the territory of the infarct, as lost myocytes are replaced by fibrous tissue (22, 23). Key to this remodeling process is the production and release of matrix metalloproteases (MMPs) from both resident cells, especially cardiac fibroblasts, and infiltrating leukocytes. Inflammatory cytokine production (especially tumor necrosis factor [TNF], interleukin-1 [IL-1], and IL-6 family members) by these cells is believed to be the major trigger for induction of MMP expression.

* Corresponding author. Mailing address: Molecular Cardiology Research Institute, Tufts-New England Medical Center, 750 Washington Street, Box 8486, Boston, MA 02111. Phone: (617) 636-5190. Fax: (617) 636-5204. E-mail for Sandro Goruppi: sgoruppi@tufts-nemc.org. E-mail for John M. Kyriakis: jkyriakis@tufts-nemc.org.

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Studies employing broad-spectrum inhibitors of MMPs have shown that cytokine (TNF)-stimulated upregulation of the expression of MMPs is a central factor leading to left ventricular dilation post-myocardial infarction, a harbinger of heart failure (28, 32, 48). Studies of mice with a targeted deletion of *mmp9* clearly implicate this factor in not only left ventricular dilation but also in inhibition of neo-angiogenesis postinfarct (1, 10, 15, 22, 23, 38). Other studies suggest MMP13 may also be important in late progression of remodeling (46).

In spite of the clear importance to the progression of cardiac pathology of cardiomyocyte hypertrophy and MMP production, both of these processes are still incompletely understood at the cellular and molecular level. The signal transduction and gene regulatory mechanisms that underlie the development of cardiomyocyte hypertrophy and the upregulation of MMPs are of considerable interest as potential avenues for therapy. Maladaptive cardiomyocyte hypertrophy is thought to be initiated upon recruitment of Gαq and Gα11, calcium mobilization, protein kinases C, the phosphatidylinositol 3′-OH kinase (PI-3-kinase), and mitogen-activated protein kinase (MAPK) signaling pathways (9), as well as transcription factors of the nuclear factor of activated T cells (NFAT) family (45). The molecular mechanisms governing MMP induction in cardiac fibroblasts during remodeling are less well understood (1, 15, 38). A critical unanswered question is whether or not such a diverse set of stress inputs, acting on cardiomyocytes and cardiac fibroblasts, recruits a common set of genes necessary at the cellular level to coordinate and integrate pathological cellular responses.

p8 (also called *candidate of metastasis 1* [*com1*] and *nuclear protein 1* [*nupr1*]) is an attractive candidate for a gene with multiple functions in cardiac and inflammatory pathology. *p8*

encodes an 8-kDa nuclear basic helix-loop-helix (bHLH) protein strongly induced in a mouse model of acute pancreatitis and implicated in several diverse functions, including transcriptional regulation, cell cycle control, stress responses, and diabetic renal hypertrophy (2, 16, 26, 30, 39, 41), as well as apoptotic regulation (2, 25).

Here we show that p8 is a transcriptional regulator critical to two key cellular events in heart failure: cardiomyocyte hypertrophy and cardiac fibroblast MMP expression.

MATERIALS AND METHODS

Human tissue. Biopsies of nonfailing left ventricular (LV) hearts were obtained at autopsy from individuals with no evidence of cardiac disease. Failing human myocardial samples were obtained consecutively from heart patients who had undergone heart transplantation because of severe heart failure consequent to LV systolic dysfunction. Myocardial samples were obtained first during placement of a ventricular assist device (VAD) and, a for a subset of the patients, a second sample was obtained subsequently at the time of heart transplant, after VAD support (29). This protocol was approved by the Institutional Review Board for Human Studies at Tufts-New England Medical Center.

Cells and treatments, immunofluorescence and hypertrophy assays, and small interfering RNA (siRNA)-dependent RNA inhibition (RNAi). Primary neonatal rat LV cardiomyocytes and fibroblasts were isolated, cultured, and assayed for hypertrophy as described elsewhere (4). 293, HeLa, and U2OS cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS). TNF, phenylephrine (PE), endothelin 1 (ET-1), MG132, lactacystin, LY294002, U0126, SB203580, and JNK inhibitor were from Calbiochem. Transfection methods, immunofluorescence staining, pCMV Myc-tagged Ub, and pcDNA His5- (human) p8 have been previously described (13). pCMV-c-Jun was from Origene; pCMV-FLAG-c-Fos was from Addgene.

The siRNAs for rat p8 were from Dharmacon and were derived from the rat p8 cDNA sequences: sense, 5′-GGACGTACCAAGAGAGAAGCT; antisense, 5′-CTTCTCTCTTGGTACGTCCTT. The cognate human control sequences were sense, 5′-GGTCGCACCAAGAGAGAAGCT, and antisense, 5′-AGCTT CTCTCTTGGTGCGACC (12, 13). Deprotection and annealing were performed immediately before use, according to the manufacturer's instructions. RNAi for rat *p8* was performed as described previously (12, 13) with some modifications. Thus, cardiomyocytes were allowed to attach for 16 to 24 h prior to oligofection. A 100 nM concentration of the appropriate double-stranded siRNA diluted in F10 medium with no serum or antibiotics added was mixed with 10 μl Oligofectamine (Invitrogen), also diluted in F10 medium in a total of 2.5 ml. The mixture was then added to the cardiomyocytes for 4 to 6 h. After this time, 2.5 ml of F10 medium, containing serum but no antibiotics, was added to the cells. The next day the cardiomyocytes were washed in F10 serum-free medium and then serum starved for an additional 24 h before stimulation. Figure S7 in the supplemental material is a diagram of this procedure.

Gene silencing in rat cardiac fibroblasts was performed as follows: the day before oligofection, an asynchronous population of fibroblasts was trypsinized, counted, and seeded at 4×10^4 cells/cm^2 in DMEM–10% FBS without antibiotics. siRNA/Oligofectamine complexes were prepared as for cardiomyocytes, except that DMEM was used as the medium. After 6 h of exposure to siRNA—carried out in DMEM under serum-free conditions—the cells were incubated overnight in DMEM containing 10% FBS. The next day, the cells were serum starved (0.1% FBS; 24 h) before stimulation with the appropriate agonists.

For immunocytochemical imaging, images were obtained using a Nikon E800 microscope coupled with a Hamamatsu digital camera. The software used was Openlab 3.0.7 on a Mac OS 9 platform. Mounting medium used for the images was Advantage permanent mounting medium (Axell, Westbury, NY). Objectives used for image capture were 40× and 60× (Nikon); imaging was performed at room temperature. Images were transferred to Adobe Photoshop 7.0 for preparation of the final figures.

RT-PCR and Northern blot analysis. Total RNA was isolated from cardiomyocytes and cardiac fibroblasts as described elsewhere (12). Reverse transcription-PCR (RT-PCR) was performed using the Titan one-tube RT-PCR kit (Roche). The β-actin gene and *gapdh* served as internal controls. Primer sequences were as follows (5′ to 3′): *p8* forward, ATGGCCACCTTCCCACCAGC; *p8* reverse, TCAGCGCCGTGCCCCTCGCT; *mmp9* forward, AAGGATGGTCTACTG GCA; *mmp9* reverse, AGAGATTCTCACTGGGGC; *mmp13* forward, CCTGG GATTTCCAAAAGAGGT; *mmp13* reverse, TAACACCACAATAAGGAA TTT; *anf* forward, CCATATTGGAGCAAATCCTG; *anf* reverse, CGGCATC TTCTCCTCCAGG; β-*actin* forward, CCAAGGCCAACCGCGAGAAGAT GAC; β-*actin* reverse, AGGGTACATGGTGGTGCCGCCAGAC. Northern analysis of *p8* expression was performed as described previously (12).

Transient-transfection and luciferase assays. A luciferase reporter plasmid containing the −638 to +62 sequence of the rat *anf* promoter, pANF, was a gift of Gordon Huggins, Tufts-New England Medical Center, while a luciferase reporter plasmid containing the −714 to +16 sequence of the human *mmp9* promoter, pMMP9, was kindly provided by Takahashi Kobayashi, Chiba University. HeLa cells were seeded in 12-well dishes and grown to 50% confluence. Assays were carried out in triplicate. Cells were transfected using Lipofectamine 2000 reagent (Invitrogen) with the reporter plasmids (pANF or pMMP9 luciferase reporter plasmids) alone (200 ng/well each) or together with pcDNA p8 (0.3 or 1 μg/well) or pCMV Jun encoding (1 μg/well). Empty pcDNA was used to balance plasmid levels. A plasmid encoding β-galactosidase, pCMV-β-gal (2 ng/well), was added to enable normalization of transfection efficiency. The cells were subsequently serum starved, and the luciferase assays were performed the following day using the luciferase assay system (Promega), as indicated by the manufacturer. Briefly, the cells were washed in phosphate-buffered saline and lysed in 160 μl lysis buffer (Promega) at room temperature. After centrifugation, 20 μl of total cell lysate were assayed for firefly luciferase activity, while a separate 20 μl of cell lysate was assayed for β-galactosidase expression. The changes in firefly luciferase activity were calculated and plotted after normalization to the β-galactosidase activity in the cognate sample. At least three independent transfection assays were performed for each reporter construct.

For assays of the TPA response element (TRE), a reporter containing six copies of the TRE, pAP1-Luc, from the PathDetect collection, was purchased from Stratagene. Assays were performed as described above.

ChIP. Cardiomyocytes were serum starved and treated with PE as indicated. Cardiac fibroblasts were serum starved and treated with vehicle or TNF for 15 h. 293 cells were kept in 10% FBS until harvest. Chromatin immunoprecipitation (ChIP) was performed as described elsewhere (3) using the EZ ChIP kit (Upstate). Endogenous p8 or c-Jun was immunoprecipitated from soluble chromatin as described elsewhere (13). For c-Jun ChIP, the antibody used was from Santa Cruz Biotechnology (H-79). For ChIP of transfected p8 pcDNA, His-tagged p8 was expressed in 293 cells. Recombinant p8 was isolated on Ni-nitrilotriacetic acid resin (QIAGEN). Copurified DNA was amplified using the following primers (two primer sets, for the distal and proximal activator protein 1 [AP-1] sites implicated in regulation of *mmp9* expression [1, 15, 24, 34, 38], dAP-1 and pAP-1, respectively, were used to amplify segments of the cardiac fibroblast *mmp9* promoter): dAP-1 *mmp9* forward, TGTCCCCTTTACTGCCCTGA; dAP-1 *mmp9* reverse, ACTCCAGGCTCTGTCCTCTT; pAP-1 *mmp9* forward, TGAC CCCTGAGTCAGCACTT; pAP-1 *mmp9* reverse, CTGCCAGAGGCTCATGG TGA; *mmp13* forward, CTCAAATTCTACCACAAACC; *mmp13* reverse, GA AGGCAGCCAGGACCCCTG. For the *anf* promoter, the following primers were used: *anf* forward, GGCCAGAGGTCCACCCACGA; *anf* reverse, CCAG ACCCTCAGCTGCAAGA. In parallel experiments, the chromatin containing recombinant p8 isolated from untransfected and transfected 293 cells was analyzed by immunoblotting with anti-Jun and -p8 antibodies after chemical reversal of the cross-linker.

In-gel zymography. MMP activity was assayed as described elsewhere (37).

Immunoblotting, protein immunoprecipitations, and pull-down assays. p8 antibodies and Western blotting have been described (12, 13). Antibodies for P-ERK, P-JNK, P-S6, P-Akt, and total Jun were from Cell Signaling Technologies; antibodies for total Akt, extracellular signal-regulated kinase (ERK), serum/glucocorticoid-inducible kinase, and green fluorescent protein (GFP) were from Santa Cruz; anti-FLAG and antiactin antibodies were from Sigma. For kinase inhibitor studies, cells were pretreated either with vehicle, SB203580 (20 μM), JNK inhibitor peptide (10 μM), LY294002 (10 μM), or U0126 (10 μM) for 30 min. Cells were then stimulated with 30 ng/ml TNF, 100 nM ET-1, or 100 μM PE for the times indicated in the figures. Cell lysates were then analyzed by immunoblotting.

For ubiquitination and sumoylation studies, 293 cells were transfected with the indicated plasmids (pcDNA-p8, His-tagged; pCMVMyc-Ub; pGFP-SUMO1) and treated for 3 h with 30 ng/ml of TNF before addition of MG132 (10 μM) or lactacystin (10 μM) for an additional 16 h. Cells were then lysed in 8 M urea, 100 mM Tris, pH 8, and p8 protein was isolated (13). Quantification of immunoblots was performed using ImageJ software (http://rsb.info.nih.gov/ij) and Microsoft Excel on an Apple OSX platform. Sumoylated p8 was detected with anti-GFP antibodies.

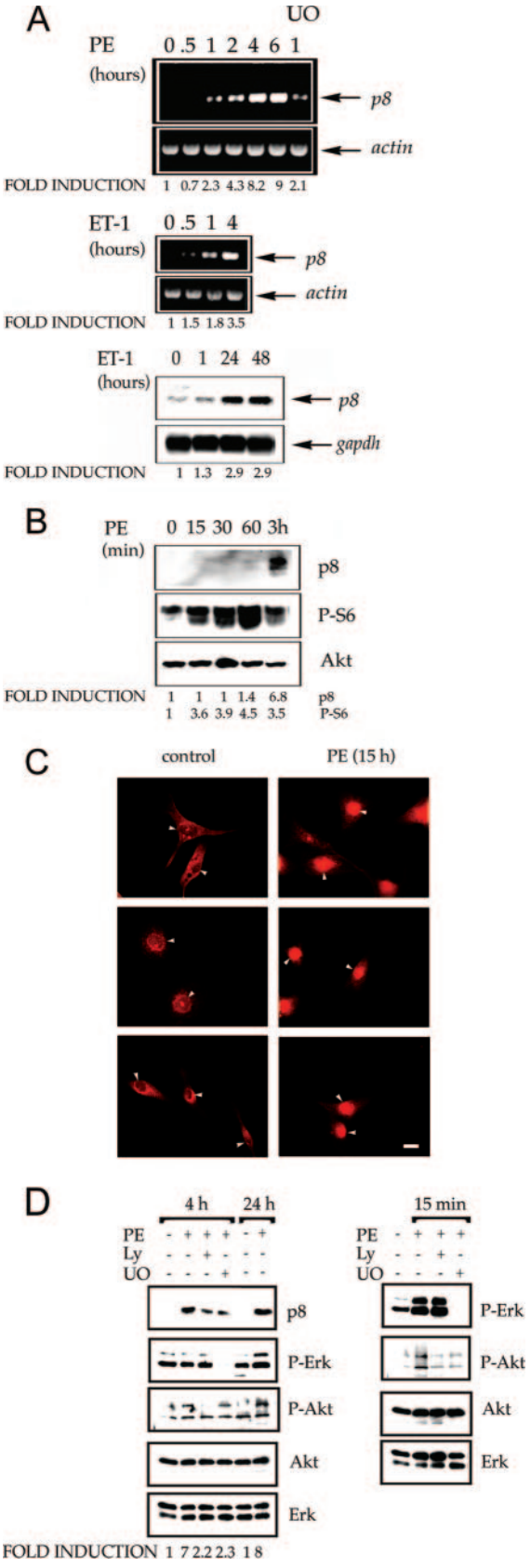


FIG. 1. Induction of primary rat cardiomyocyte *p8* mRNA and protein by PE and ET-1. Primary cultures of neonatal rat cardiomyocytes were treated with PE or ET-1 (A) or with PE (B to D) for the indicated times. (A) *p8* mRNA was detected by RT-PCR (top two panels) or Northern blotting (bottom panel). RNA levels were quantitated by densitometry (*p8* induction is indicated below the figures). (B and C) Endogenous p8 protein levels were detected by immunoblotting (and quantitated by densitometry, with p8 induction indicated below the figures) (B) or by immunocytochemistry (C). In panel C, the white arrowheads indicate cardiomyocyte nuclei. Bar, 5 μm. Note that p8 protein accumulates in the nucleus. For panel A, the level of *actin* (top two panels) or *gapdh* (bottom panel) mRNA served as a loading control; for panel B, levels of Akt served as a loading control, and an immunoblot of phosphorylated ribosomal S6 protein (P-S6) served as an indicator of the efficacy of PE treatment. (D) PE provokes expression of the cardiomyocyte p8 protein (quantitated by densitometry, with p8 induction indicated below the figures) in an Akt- and ERK-dependent manner. Neonatal rat cardiomyocytes were treated with PE or vehicle as indicated, for the times shown, either with or without prior treatment with U0126 or LY294002, as indicated. Cell extracts were subjected to immunoblotting with the indicated antibodies.

RESULTS

PE and ET-1 induce cardiomyocyte *p8* mRNA and p8 protein. p8 is required for PE- and ET-1-stimulated cardiomyocyte hypertrophy. ET-1 and PE couple to G-protein-coupled receptors (GPCRs) and can trigger cardiomyocyte hypertrophy in vivo and in tissue culture (4, 5, 9). ET-1 and PE stimulate a strong induction of cardiomyocyte endogenous *p8* mRNA first detected at 1 h and reaching a maximum by 4 to 6 h (maximal 9-fold induction for PE and 3.5-fold induction for ET-1). Induction of the mRNA appears not to be significantly affected by the ERK inhibitor U0126 (Fig. 1A). By contrast, the PE-stimulated appearance of p8 protein is first seen by 3 h of stimulation (Fig. 1B), with protein levels remaining elevated at six- to eightfold above basal for at least 24 h (Fig. 1D). Immunocytochemical analysis reveals that PE stimulates the accumulation of endogenous p8 protein, which is localized in the nucleus (Fig. 1C). p8 protein levels are modestly reduced upon inhibition of either ERK with U0126, a specific inhibitor of MAPK/ERK kinase 1, a direct upstream activator of ERK (7, 19), or inhibition of PI-3-kinase with the specific pharmacologic agent LY294002 (7) (Fig. 1D). Thus, in cardiomyocytes, the accumulation of p8 protein in response to GPCR agonists involves initially an enhancement of *p8* transcription followed by the appearance of the protein. The level of the cardiomyocyte p8 protein is modestly ERK and PI-3-kinase dependent.

We found that PE- and ET-1-induced p8 is required for both PE- and ET-1-stimulated cardiomyocyte hypertrophy. We used RNAi mediated by siRNA to assess the consequences of depletion of endogenous p8 on PE- or ET-1-stimulated cardiomyocyte hypertrophy. Species-specific (rat), but not human, p8 siRNA substantially ablated detectable neonatal rat cardiomyocyte *p8* mRNA induced by ET-1 (Fig. 2A; ET-1 induction was reduced from 8.3- to 2.5-fold upon silencing *p8*) and p8 protein induced by PE (Fig. 2B; induction was reduced from 12.1- to 1.3-fold upon silencing *p8*).

Increased protein synthesis (detected as an increase in the incorporation of [^{3}H]leucine into acid-insoluble material) and the induction of the embryonic gene *atrial natriuretic factor* (*anf*) are two characteristic features of cardiomyocyte hyper-

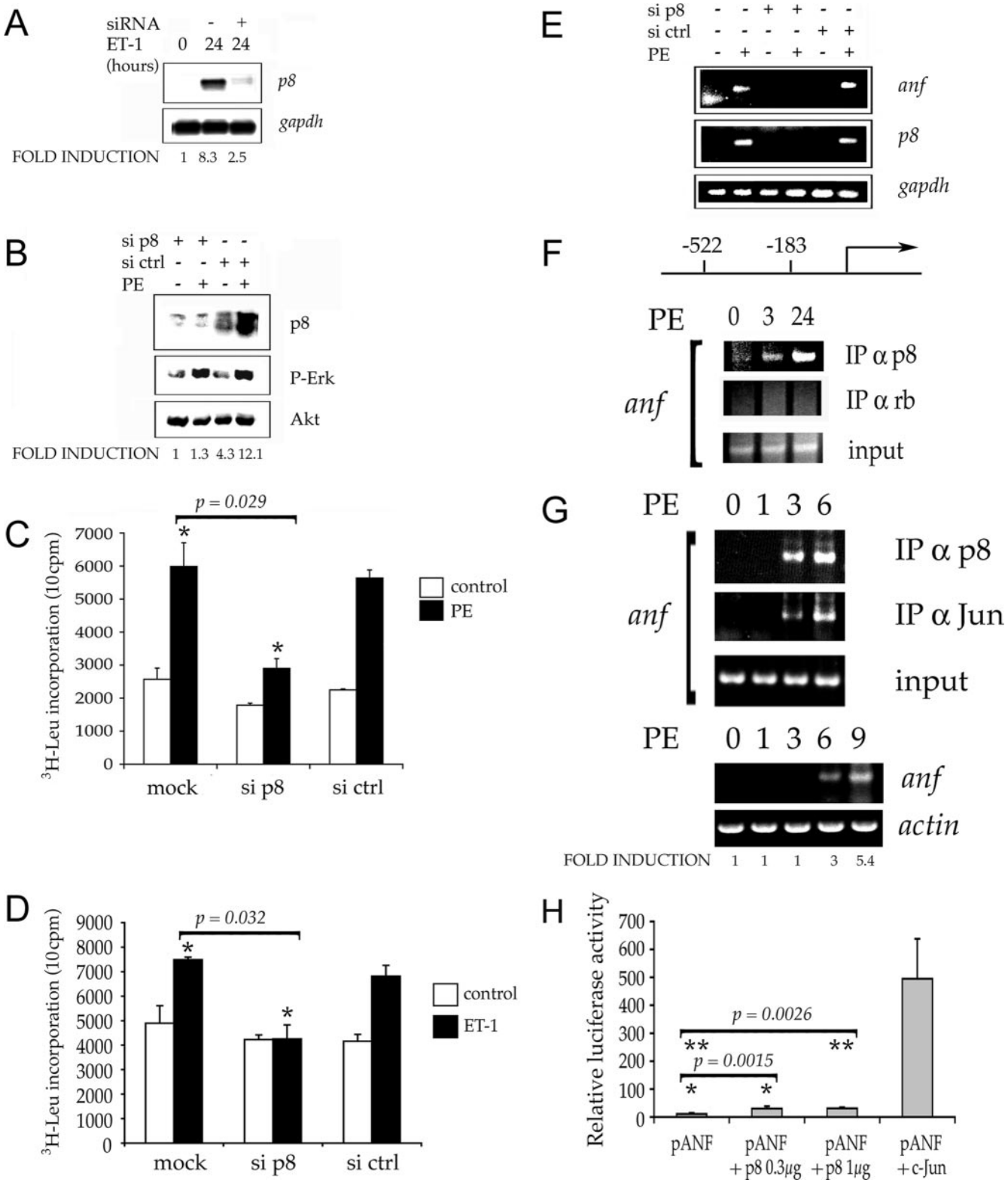


FIG. 2. p8 is required for ET-1- and PE-stimulated cardiomyocyte hypertrophy. p8 associates with chromatin that contains the *anf* promoter. (A and B) Efficacy of RNAi silencing of neonatal rat cardiomyocyte basal and agonist-induced *p8* mRNA (A) and protein (B), both of which were quantitated by densitometry (p8 induction is indicated below the panels). Primary cultures of neonatal rat cardiomyocytes were treated with specific rat, control nonspecific (human) p8 (ctrl) siRNA. Cells were then stimulated with ET-1 or PE as indicated. Samples were subjected to Northern blotting (A) or anti-p8 immunoblotting (B) to assess *p8* mRNA and protein levels, respectively. (C and D) Requirement for p8 for, respectively, PE- and ET-1-induced neonatal rat cardiomyocyte hypertrophy, as measured by stimulus-induced incorporation of [^{3}H]Leu into protein. Primary cultures of neonatal rat cardiomyocytes were treated with specific rat, control nonspecific (human) p8 (ctrl) siRNA, or oligofection agent (mock).

trophy (4, 5, 17, 18). We found that both processes require p8. Thus, either PE-stimulated (Fig. 2C) or ET-1-stimulated (Fig. 2D) neonatal rat cardiomyocyte protein synthesis requires p8 and can be blunted significantly ($P = 0.029$ and 0.032, respectively) with rat-specific, but not nonspecific control (human *p8* sequence), p8 siRNAs. Likewise, PE-stimulated induction of neonatal rat cardiomyocyte *anf* requires p8, and induction of *anf* can be reduced to undetectable levels by specific p8 siRNA (Fig. 2E). Consistent with previous observations (43) indicating that PE-stimulated cardiomyocyte protein synthesis is, at least in part, ERK dependent and with our finding that PE-stimulated induction of p8 protein requires, at least in part, ERK (Fig. 1D), we observed that inhibition of MEK/ERK signaling also suppresses PE-stimulated cardiomyocyte hypertrophy (see Fig. S1 in the supplemental material).

p8 associates with chromatin that contains the *anf* promoter. p8 localizes to the nucleus (Fig. 1C; see also Fig. 3B, below, and reference 13), where it may modulate transcription (16). We performed ChIP experiments to begin to identify the chromatin domains with which p8 associates. Endogenous p8 was immunoprecipitated from cardiomyocytes before and after treatment with PE. The immunoprecipitates were subjected to PCR with primers derived from an enhancer element in the *anf* promoter which contains a canonical AP-1 site (Fig. 2F, top illustration). Coincident with PE induction of p8 protein expression (first detectable after 3 h of stimulation) (Fig. 1B), we found that, in contrast to samples from untreated controls, starting at 3 h of PE treatment and continuing for 24 h of PE treatment, immunoprecipitates of endogenous p8 from cardiomyocyte chromatin contained substantial chromatin from the *anf* promoter region (Fig. 2F). Induction of *anf* is mediated by AP-1 (33, 42). We used ChIP to assess the kinetics of PE-stimulated p8 and c-Jun (an AP-1 component) binding to the *anf* promoter and compared this to PE induction of *anf* as measured by RT-PCR. PE-stimulated induction of p8 protein is first seen at 3 h (Fig. 1B). From Fig. 2G (and as quantitated in Fig. S6A of the supplemental material), it is evident that starting at 3 h of stimulation and reaching a maximum at 6 h, both p8 and c-Jun bind to chromatin that contains the same canonical AP-1 element used in Fig. 2F (Fig. 2G; see also the quantitation in Fig. S6 of the supplemental material). At 6 h, when p8 and c-Jun binding are maximal, PE-stimulated *anf* expression is first seen. p8 binding to the *anf* promoter, as well as induction of *anf*, continues for up to 24 h (Fig. 2E and F).

Inasmuch as the *anf* promoter contains an AP-1 site, we used a reporter construct wherein the *anf* promoter was fused to luciferase (p-*anf*-Luc), to determine if p8 or the AP-1 component c-Jun could drive transcription from the *anf* promoter. When *anf*-Luc and p8 are coexpressed in HeLa cells, we observe a small (twofold), but significant ($P = 0.0015$), induction of *anf*-Luc expression mediated by coexpressed p8 (Fig. 2H). c-Jun produces a more robust induction of *anf*-Luc (Fig. 2H). We did not observe a further, additive induction of *anf*-Luc upon coexpression with both p8 and c-Jun (data not shown). Thus, although p8 is required for *anf* expression, it is insufficient per se to drive maximal *anf* expression.

TNF induces cardiac fibroblast p8. p8 is required for TNF induction of cardiac fibroblast MMP9 and MMP13. Cardiac fibroblasts play an important role in heart failure, where they participate in pathological tissue remodeling. MMPs 9 and 13 have been linked to the proteolytic activation of latent transforming growth factor β, a strong inducer of interstitial fibrosis, and to postinfarct myocardial rupture. These MMPs are strongly induced in the heart by TNF and other proinflammatory stimuli present at elevated levels in the failing heart (1, 10, 15, 22, 23, 28, 32, 38, 48–50).

Our results indicate that, in primary cultures of cardiac fibroblasts, p8 is strongly and rapidly induced by TNF—with the accumulation of p8 protein preceding the appearance of *p8* mRNA. Thus, we stimulated primary cardiac fibroblasts with TNF and subjected the extracts to immunoblotting with anti-p8, anti-phospho-JNK, and anti-Akt (gel loading control). TNF induces a rapid increase in JNK activation (phospho-JNK immunoreactivity) first detectable at 5 min of stimulation and reaching a maximum at 15 min (Fig. 3A). TNF stimulates a rise in p8 protein levels, which follows closely the increase in JNK activation. Thus, increased p8 protein is first seen at 15 min of TNF stimulation (4.5-fold above basal) and remains at or just above this level for 6 hours (Fig. 3A, top panels). By contrast, *p8* mRNA is not detectable until 1 h of TNF stimulation and does not reach a maximum (~6-fold above basal) until 4 hours of TNF stimulation (Fig. 3A, bottom panel). Thus, it is likely that the rapid increase in p8 protein incurred by TNF is due to stabilization of the p8 polypeptide. Consistent with this, TNF stabilizes recombinant p8 protein expressed in transfected cells. Similarly, the proteasome inhibitor MG132 also stabilizes transfected p8 (Fig. 4; see also Fig. S2 in the supplemental material). These results fit with our previous findings (13) that p8 protein levels can be regulated by the ubiquitin proteasome. As with PE-treated cardiomyocytes, TNF stimulates the accu-

Cells were then treated with PE or ET-1 as indicated, and protein synthesis was measured as the uptake of [^{3}H]Leu into acid-insoluble material. Data shown are means ± standard deviations for triplicate samples. Where indicated, data were subjected to an unpaired Student's *t* test, as indicated. (E) PE induction of cardiomyocyte *anf*, a second indication of hypertrophy, also requires p8. Thus, *p8* was silenced as in panels A to D, and cells were treated with PE as indicated. Total RNA was prepared and subjected to RT-PCR with either *anf*-specific, *p8*-specific, or *gapdh*-specific (loading control) primers. (F) Endogenous cardiomyocyte p8 associates with chromatin domains that include the endogenous *anf* promoter. Neonatal rat cardiomyocytes were treated with PE as indicated. Specific anti-p8 antibodies (13) were used to immunoprecipitate endogenous p8 from chromatin. Immunoprecipitation with a nonspecific rabbit antibody (rb) served as a negative control. The chromatin immunoprecipitates or total chromatin (input) was subjected to PCR using specific primers that flank the AP-1 sites in the *anf* promoter. The top scheme indicates the positions of the oligonucleotides within the *anf* promoter. (G) Kinetics of endogenous cardiomyocyte p8 and endogenous c-Jun binding to chromatin containing the *anf* promoter. Cardiomyocytes were treated with PE for the indicated times. ChIP with anti-p8 or anti-c-Jun was performed as for panel F. In parallel, *anf* mRNA was detected by RT-PCR. (H) Induction of an *anf*-luciferase reporter construct by coexpressed c-Jun or p8. HeLa cells were transfected with the pANF reporter construct and either p8 or c-Jun as indicated. Luciferase activity was measured in triplicate and normalized to β-galactosidase activity. Means ± standard deviations are shown. Where indicated, data were subjected to an unpaired Student's *t* test to assess the statistical significance of the observed changes in luciferase activity.

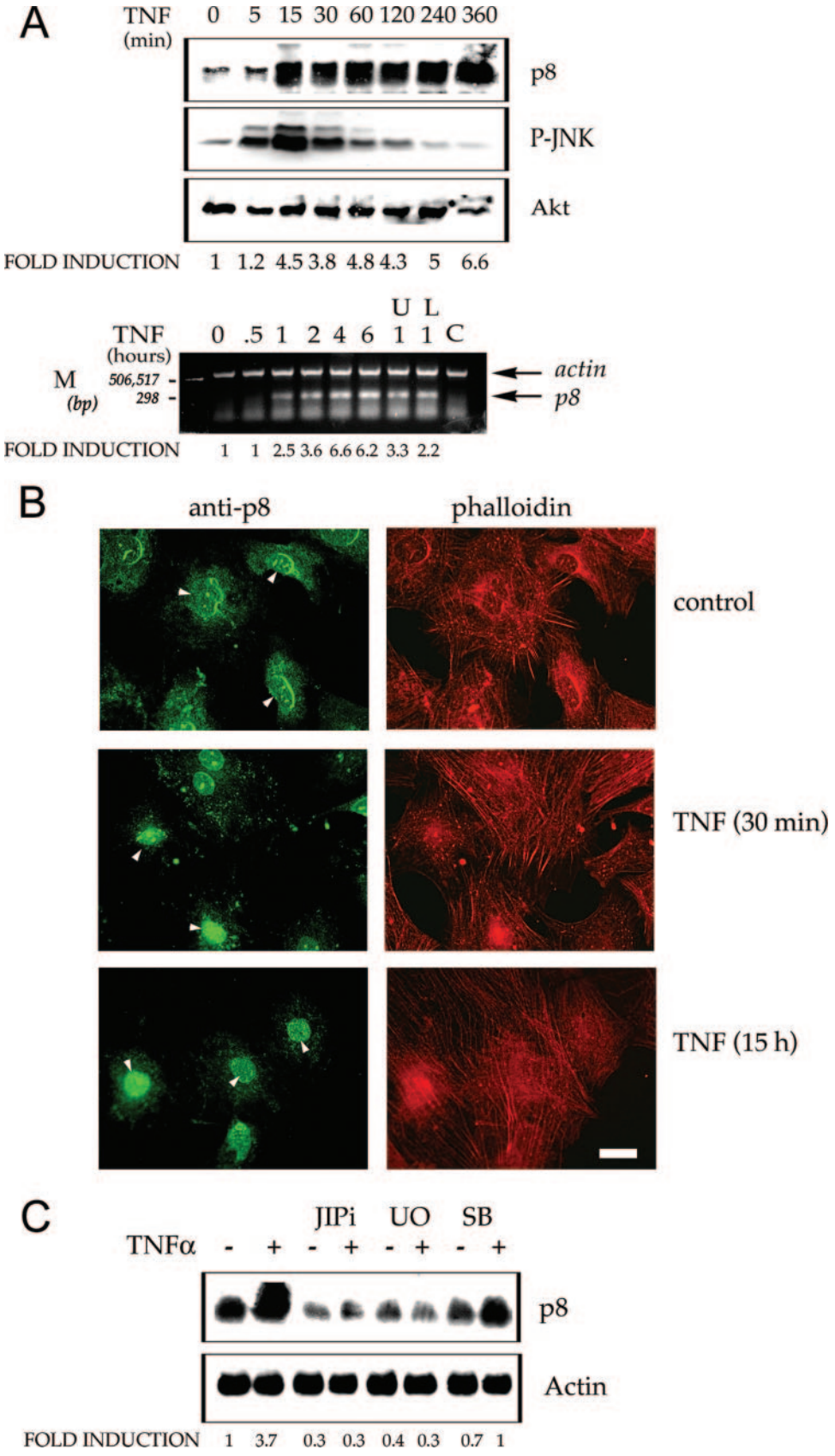


FIG. 3. TNF induction of primary rat cardiac fibroblast p8 protein precedes induction of the mRNA and is JNK and ERK dependent. (A) Primary cultures of neonatal rat cardiac fibroblasts were treated with TNF for the indicated times. In the top panel, as indicated, protein

mulation of endogenous p8 polypeptide, which is localized in the nuclei of primary cardiac fibroblasts (Fig. 3B).

The TNF-stimulated accumulation of p8 polypeptide requires the JNK and ERK pathways. Thus, JNK inhibitor 1, a highly specific cell-permeable peptide inhibitor of JNK based on the JNK binding domain of JNK interacting protein 1 (8), reduced both basal and TNF-stimulated endogenous cardiac fibroblast p8 protein levels, as did U0126. SB203580, a specific inhibitor of p38 (7), had a much more modest effect on p8 protein accumulation (Fig. 3C). We did not observe TNF activation of cardiac fibroblast Akt, making it unlikely that Akt contributes significantly to TNF induction of *p8* mRNA or p8 protein. The inhibitors exerted identical effects on p8 expressed from transfected plasmids (see Fig. S2 in the supplemental material), and the inhibitors produced the expected effects on MAPK signaling in situ (see Fig. S2), again implicating the JNK and ERK1/2 pathways in p8 protein stabilization. Finally, consistent with a role for JNK and ERK in p8 stabilization, transfected p8 was stabilized upon coexpression with upstream signaling kinases that recruit ERK1/2 (B-Raf), JNK (MAPK kinase 7, MAPK/ERK kinase 1 [MEKK1], or apoptosis signal-regulating kinase 1) or both (mixed-lineage kinase 3) (19, 20). An exception was MEKK3, which, upon overexpression, activates all known MAPKs (20). However, in the heart, MEKK3 likely functions more prominently in the regulation of p38 (47). Thus, under the conditions employed, MEKK3 may not have generated a sufficient ERK1/2 or JNK activation signal to stabilize p8. Serum/glucocorticoid-inducible kinase 1, which plays no known role in ERK or JNK signaling (21), failed to stabilize p8 (see Fig. S3 in the supplemental material). Thus, whereas PE and ET-1 induction of cardiomyocyte p8 likely proceeds largely through increased transcription, TNF induction of p8 involves an initial JNK/ERK-dependent stabilization of the protein followed by an increase in mRNA.

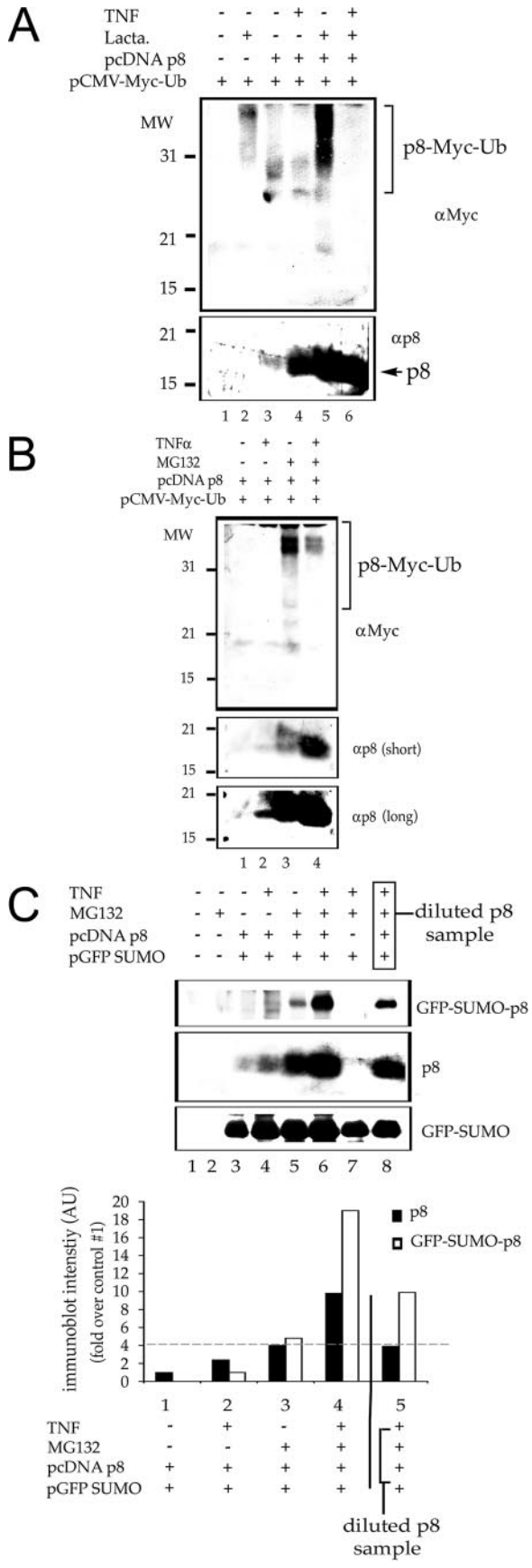
The rapid TNF-stimulated stabilization of p8 protein, coupled with the stabilization of p8 by inhibitors of the proteasome (see Fig. S2 in the supplemental material) (13) prompted us to ask if TNF could affect the level of p8 ubiquitination and degradation. To test this, we transfected 293 cells with Myc-tagged ubiquitin (Ub) plus either vector or His-tagged p8. Cells were then treated with either vehicle, TNF, lactacystin, or both. Recombinant p8 was isolated and immunoblotted with anti-p8 or, to detect Ub-p8, anti-Myc. From Fig. 4A, it is evident that TNF and lactacystin alone lead to comparably striking increases in p8 protein levels (lanes 3 versus 4 or lanes 3 versus 5). Although the amount of p8 isolated from cells increased with TNF treatment, the level of Myc-Ub immunoreactivity in these same samples decreased in a TNF-dependent manner, even if the cells were treated with lactacystin (Fig. 4A, compare lanes 3 and 4 or lanes 5 and 6). Thus, the stoichiometry of p8 ubiquitination decreases coincident with TNF stimulation. Similar results were obtained when another proteasome inhibitor, MG132, was used (Fig. 4B, lanes 3 versus 4).

Small ubiquitin-like modifier 1 (SUMO1) is one of three SUMO group proteins that share with Ub a similar secondary structure motif, the ubiquitin superfold (44). Covalent modification with SUMO can have wide-ranging effects on target proteins, including regulation of protein-protein interactions, subcellular localization, and antagonism of ubiquitination (35, 40, 44). Coincident with the TNF-dependent increase in p8 polypeptide levels and the decrease in p8 ubiquitination, we observed a TNF-stimulated increase in p8 sumoylation (with SUMO1). Thus, cotransfection of cells with p8 and SUMO1 (GFP tagged) results in a small degree of p8 sumoylation which, along with total p8 polypeptide, is increased with TNF (Fig. 4C, lanes 3 versus 4, or in the bar graph, bars 1 versus 2). Treatment with MG132 stimulates a large increase in p8 protein levels (Fig. 4C, lanes 3 versus 5, or in the bar graph, bars 1 versus 3). In the presence of both TNF and MG132 there is a further, more modest increase in total p8 protein accompanied by a striking increase in p8 sumoylation (Fig. 4C, lanes 5 versus 6, or in the bar graph, bars 3 versus 4). This increase in sumoylation is still observed if the levels of total p8 protein in samples from TNF plus MG132-treated cells are diluted such that the level of p8 present is equivalent to that in samples from cells treated with MG132 alone (Fig. 4C, lanes 5 versus 8 or, in the bar graph, bars 3 versus 5). Thus, TNF treatment coincides with increased p8 sumoylation and a reduction in the stoichiometry of p8 ubiquitination, which accompanies increased p8 protein levels. p8 is apparently neither a direct Akt nor a MAPK substrate (S. Goruppi, unpublished observations); accordingly, p8 stabilization arising via direct phosphorylation by these kinases in vivo is unlikely. Instead, these pathways may act indirectly either to promote deubiquitination or inhibit ubiquitination (perhaps via sumoylation), with reduced p8 ubiquitination enhancing p8 levels by preventing p8 degradation by the proteasome.

We used siRNA-mediated RNAi to evaluate the role of p8 in TNF stimulation of cardiac fibroblast MMP expression. TNF induction of rat cardiac fibroblast *p8* mRNA was unaffected by a human-specific *p8* siRNA. However, a rat-specific siRNA completely abolished detectable TNF induction of *p8* mRNA (Fig. 5A) and p8 protein (Fig. 5B).

Our RNAi findings indicate that p8 is required for TNF-stimulated expression of *mmp9* and *mmp13*. Thus, TNF stimulated a 4.6-fold induction of *mmp9* mRNA and a 3.2-fold

extracts were subjected to immunoblotting with anti-p8, anti-phospho-JNK (P-JNK), or anti-Akt (loading control) antibodies. The numbers at the bottom of the upper panel indicate the induction of p8 protein, as quantitated by densitometry. In the bottom panel, RNA from the treated cells was subjected to RT-PCR using p8 or actin (loading control) primers as indicated. M, molecular size. (B) TNF-stimulated rapid accumulation of endogenous p8 protein in the nuclei of primary rat cardiac fibroblasts. Cells were treated with vehicle (phosphate-buffered saline [control]) or TNF as indicated. Cells were stained with anti-p8 and phalloidin as indicated. White arrows denote cell nuclei. Bar, 5 μm. (C) TNF induction of p8 protein is JNK and ERK dependent, but not p38 MAPK dependent. Rat cardiac fibroblasts were pretreated with vehicle, a specific JNK inhibitor, the ERK pathway inhibitor U0126, or the p38 inhibitor SB203580, as indicated. Cells were then stimulated with TNF for 1 h, as indicated. Total protein extracts were subjected to immunoblotting with either anti-p8 (quantitated by densitometry; p8 induction is indicated below the figures) or anti-actin (loading control) as indicated.



induction of *mmp13* mRNA. Silencing of primary rat cardiac fibroblast p8 with a specific siRNA completely inhibited TNF-stimulated induction of *mmp9* and *mmp13* (Fig. 5C). Zymogram analysis indicated that silencing of primary rat cardiac fibroblast p8 siRNA also completely inhibited TNF-stimulated elaboration of functional MMP9 protein (Fig. 5D). Likewise, transfection of recombinant p8 into HeLa or U2OS cells resulted in an elevation in basal and TNF-stimulated MMP9 activity coincident with TNF stabilization of the recombinant p8 protein (see Fig. S4 in the supplemental material).

p8 associates with chromatin that contains the *mmp9* and *mmp13* promoters as well as with chromatin that contains the AP-1 component c-Jun. The ability of p8 to associate with the *anf* promoter and to affect PE-stimulated *anf* transcription, combined with the requirement for p8 in induction of *mmp9* and *mmp13*, prompted us to investigate if p8 associated with chromatin that contained the *mmp9* or *mmp13* promoter. We thus immunoprecipitated endogenous p8 from cardiac fibroblast chromatin and performed PCR on the copurified DNA. Use of either of two primers designed to flank consensus AP-1 sites in the *mmp9* promoter (34) identified both AP-1-containing regions of the endogenous *mmp9* promoter in the anti-p8 ChIP (Fig. 6A). Of note, within 24 h of treatment, TNF stimulated a dramatic increase in the amount of endogenous p8 associated with the *mmp9* promoter (Fig. 6A).

Moreover, ChIP isolates of recombinant p8 (expressed in 293 cells) contain the *mmp9* and *mmp13* promoters, as detected by PCR (Fig. 6B), indicating that p8 is capable of associating with the *mmp9* and *mmp13* promoters in different cell types. The induction by TNF of *mmp9* and *mmp13* is mediated in part by the AP-1 transcription factor (1, 24, 34, 36, 38). Consistent with this, we also observed that recombinant p8 associates with chromatin that contains the AP-1 component c-Jun (Fig. 6C). Taken together, these results indicate that endogenous and recombinant p8 associate with chromatin. In this capacity, p8 serves as a required component in PE induction of cardiomyocyte *anf* and TNF induction of cardiac fibroblast *mmp9* and *mmp13*, the expression of which is important to heart disease (1, 9, 10, 15, 28, 32, 38, 48).

Our results indicate that p8 is required for *mmp9* induction

FIG. 4. TNF-stimulated accumulation of p8 protein is accompanied by a suppression of p8 ubiquitination and concomitant p8 sumoylation. (A) TNF-stimulated p8 accumulation coincides with reduced ubiquitination. 293 cells were transfected with His5-p8 and Myc-Ub as indicated. Cells were then treated with TNF and/or lactacystin as indicated. p8 was isolated by nitrilotriacetic acid-agarose affinity chromatography, and the immunoblots were probed with anti-Myc or anti-p8 as indicated. (B) Same experiment as in panel A, except that MG132 was used. Two exposures (long and short durations) are provided for the anti-p8 immunoblots. (C) TNF-stimulated sumoylation of p8. 293 cells were transfected with His5-p8 and GFP-SUMO1 as indicated. Cells were then treated with TNF and/or MG132 as indicated. p8 was isolated as for panel A, and the immunoblots were probed with anti-GFP (to detect GFP-SUMO1-p8) and subsequently with anti-p8 antibodies. In the bottom panel, levels of p8 and of sumoylated p8 were quantified as a function of the control sample (lane and bar 1). For the diluted p8 sample indicated, p8 isolated from TNF/MG132-treated cells was diluted until the amount of p8 was identical to the level detected in cells treated with MG132 alone. The extract was then subjected to immunoblotting with the indicated antibodies.

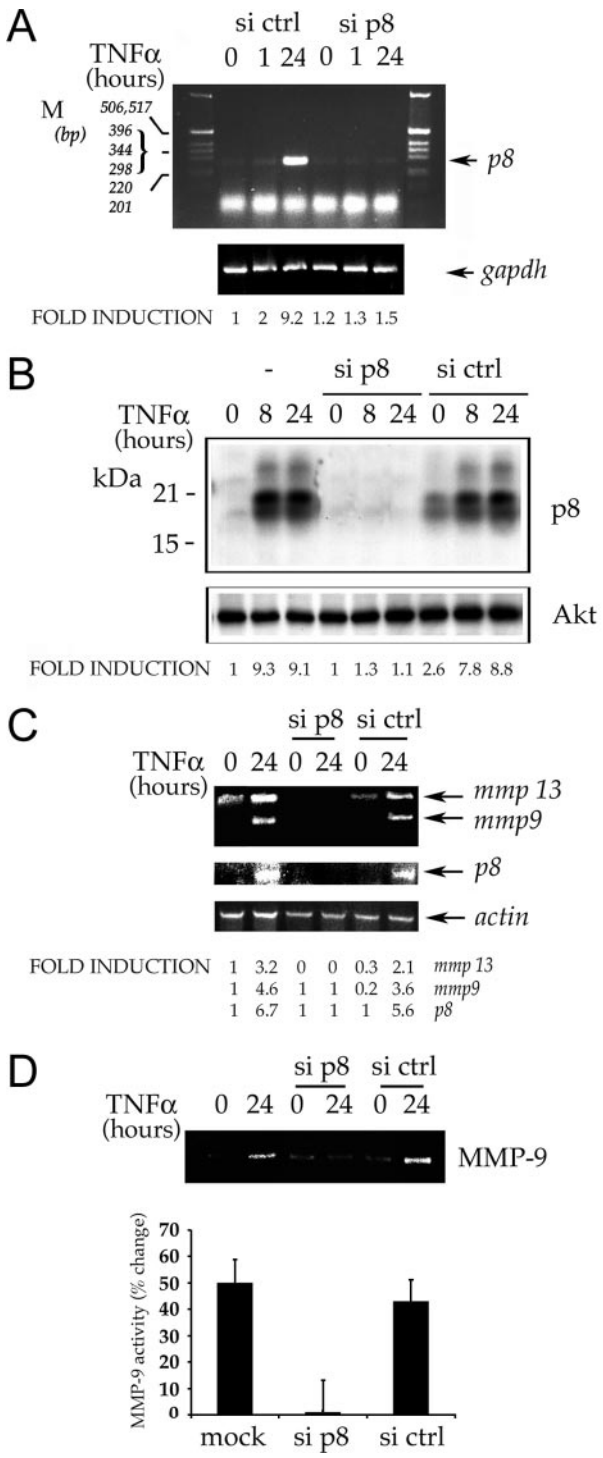


FIG. 5. p8 is required for TNF induction of cardiac fibroblast *mmp9* and *mmp13*. (A) Efficacy of siRNA-mediated RNAi of rat cardiac fibroblast p8. Cells were treated with rat p8 or human p8 (ctrl) siRNA. Cells were then stimulated with TNF for the indicated times. Total RNA from the cells was subjected to RT-PCR with *p8*-specific and *gapdh*-specific (loading control) primers. Induction of *p8* was quantitated by densitometry and is indicated below the figure. (B) TNF-induced p8 protein is stable for up to 24 h and can be silenced by RNAi. Cells were treated with siRNAs as for panel A and then with TNF as indicated. Protein extracts were probed with anti-p8 and anti-Akt (loading control). Induction of p8 was quantitated by densitometry and is indicated below the figure. (C) Silencing of *p8* suppresses TNF induction of cardiac fibroblast *mmp9* and *mmp13* mRNA. Cells were treated with rat- or human-specific p8 siRNA as in Fig. 2A and B. Cells were then stimulated with TNF for 24 h. RNA from the cells was subjected to RT-PCR as in Fig. 2G, using *mmp9*-, *mmp13*-, *p8*-, and *actin*-specific (loading control) primers. Induction of *p8*, *mmp9*, and *mmp13* was quantitated by densitometry and is indicated below the figure. Zero-fold induction indicates that no signal was detected. (D) Zymogram analysis of MMP9 production. TNF-stimulated production of rat cardiac fibroblast MMP9 requires p8. p8 RNAi and TNF treatment were as for panel C. Cell culture supernatants were subjected to zymogram analysis of MMP9 activity. The activity for the samples from TNF-treated cells is quantified in the bar graph (means ± standard deviations of triplicate samples).

by TNF; however, these assays were performed after 24 h of TNF stimulation. By contrast, TNF stimulates maximal p8 protein levels by 15 min, with significant nuclear accumulation of p8 by 30 min (Fig. 3A and B). We used ChIP to explore the kinetics of endogenous p8 and c-Jun binding to cardiac fibroblast chromatin containing the dAP-1 consensus AP-1 binding site of the *mmp9* promoter. Consistent with the rapid TNF-stimulated accumulation of p8 protein in the nucleus (Fig. 3A and B), at 30 min of TNF stimulation, binding of p8 to chromatin containing the *mmp9* promoter is first detectable. Binding reaches a maximum at 3 h and is sustained for 24 h (Fig. 6D; see quantitation of data in Fig. S6B in the supplemental material). TNF-stimulated c-Jun binding to chromatin containing the *mmp9* promoter was slower, as it was first detected at 1 h and reached a maximum at 3 h. As with p8 binding, c-Jun binding is sustained for 24 h (Fig. 6D; quantitated in Fig. S6B of the supplemental material). TNF-stimulated induction of *mmp9* mRNA, detected by RT-PCR, was first observed at 3 h, a time when both p8 and c-Jun are associated with chromatin containing the *mmp9* promoter. *mmp9* expression is sustained for 24 h (Fig. 6D). These results fit with the idea that p8 is a regulator of TNF-stimulated endogenous *mmp9* transcription.

We next used a reporter construct wherein the *mmp9* promoter (34) was fused to a luciferase cDNA (*mmp9*-Luc). In contrast to *anf*-Luc, which is, significantly, not strongly affected by coexpressed p8, expression of *mmp9*-Luc in HeLa and U2OS cells is enhanced strikingly ($P = 0.0022$) by coexpressed p8 (Fig. 6E). Coexpression in HeLa cells of c-Jun also induces *mmp9*-Luc, and expression of *mmp9*-Luc is further enhanced ($P = 0.03$) upon coexpression with both c-Jun and p8 (Fig. 6E). Thus, whereas p8 is necessary but not sufficient to stimulate transcription of an *anf* reporter and p8 does not significantly enhance c-Jun induction of an *anf* reporter (Fig. 2H), p8 alone can stimulate *mmp9*-Luc reporter expression and can enhance c-Jun-driven induction of *mmp9*-Luc.

Finally, consistent with the additive effects of p8 and c-Jun on *mmp9* reporter induction, high-efficiency transfection of p8 into U2OS cells enhances coexpressed c-Jun- or c-Fos-stimulated expression of endogenous MMP9 protein production (Fig. 6F).

Although our data show that p8 can modulate the expression of genes that are regulated in part by AP-1, we doubt that p8 directly influences AP-1 itself. Thus, we tested whether or not p8 alone, or together with c-Jun, could drive expression of a reporter construct consisting solely of six copies of a consensus

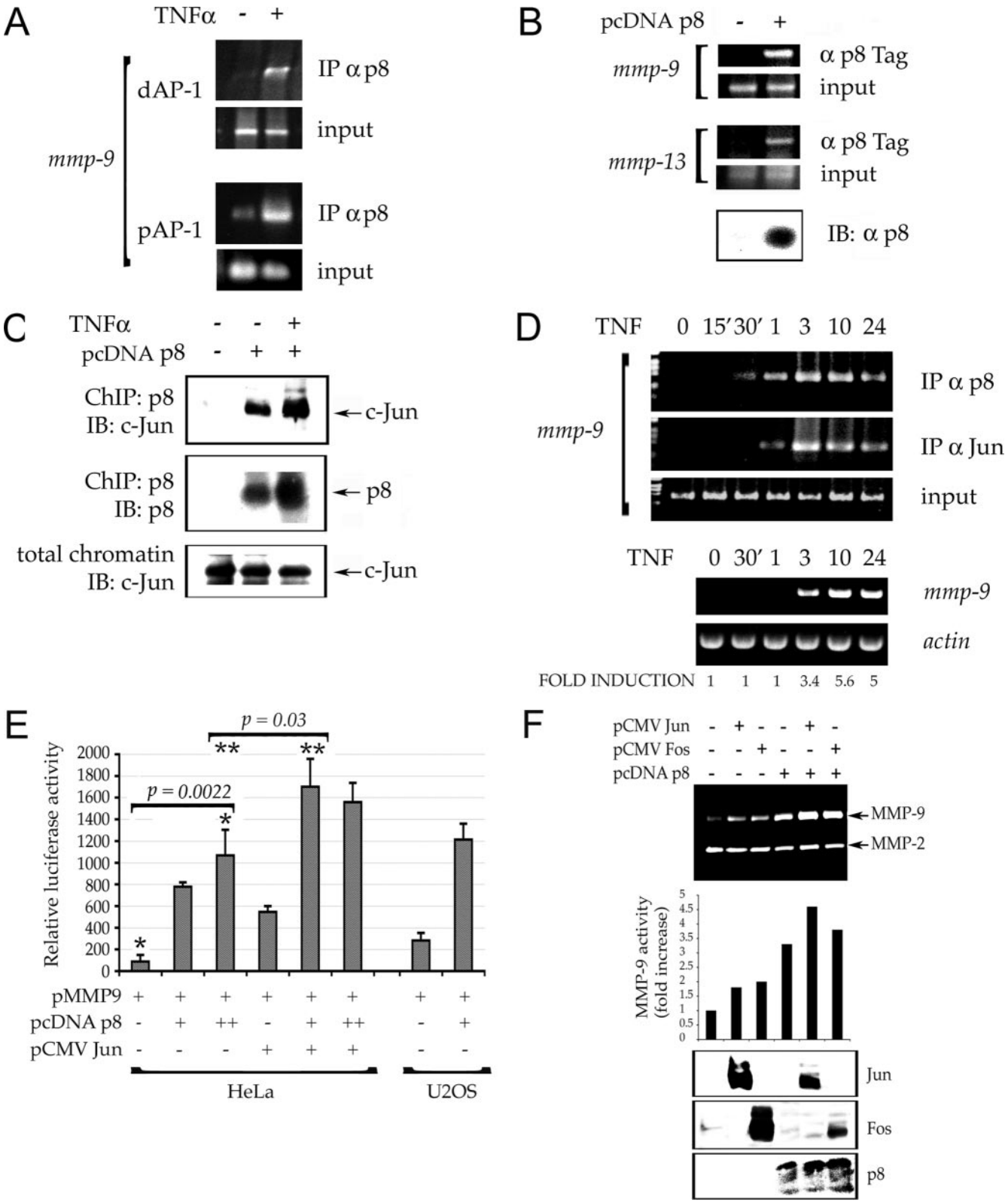


FIG. 6. In a stimulus-dependent manner, endogenous and recombinant p8 associate with chromatin containing regions of the *mmp9* and *mmp13* promoters that include canonical AP-1 binding sites. p8 also associates with chromatin that contains components of the AP-1 transcription factor. (A) Endogenous p8 was immunoprecipitated from chromatin preparations from cardiac fibroblasts before and after TNF treatment. Coprecipitated DNA (IP αp8), as well as total cellular chromatin (input) was subjected to PCR using either of two specific *mmp9* primers (dAP-1 and pAP-1). (B) Recombinant His-tagged p8 was isolated, along with associated material, from the chromatin of transfected 293 cells (transfected with vector or pcDNA p8 as indicated). Associated DNA was subjected to PCR with primers specific for the human *mmp9* or *mmp13* promoters

TRE, the canonical AP-1 *cis* element, fused to luciferase (pAP-1). From Fig. S5 in the supplemental material, it is evident that while c-Jun triggers robust induction of TRE-inducible luciferase, p8 coexpression is without effect and has no effect when administered together with c-Jun.

p8 protein levels increase strongly in human heart failure by a process reversed with therapeutic intervention. p8 protein is dramatically induced in the failing human heart (Fig. 7). We obtained myocardial samples from two sets of patients, the first (Fig. 7A) consisting of three individuals (at autopsy) without heart failure (nonfailing controls) and three individuals with severe heart failure secondary to left ventricular systolic dysfunction. The second sampling (Fig. 7B) included three patients with heart failure at autopsy and one without. Two myocardial samples were taken from the first set of heart failure patients (Fig. 7A): the first obtained at the time of VAD implantation and the second at the time of heart transplant, after mechanical unloading with VAD therapy. The samples were subjected to immunoblotting with an anti-p8 antibody. p8 protein levels were low to undetectable in the nonfailing heart controls. By contrast, myocardial samples taken from the heart failure patients showed a striking ($P = 0.003$) (Fig. 7C) increase in p8 protein levels (Fig. 7A and B, with the combined data quantitated in panel C). Interestingly, VAD therapy significantly reduced p8 protein levels (Fig. 7A), suggesting that mechanical unloading of the failing heart coincides with a reduction in p8 levels.

DISCUSSION

Our results suggest a model in which prohypertrophic agents acting on cardiomyocytes, and proinflammatory cytokines acting on cardiac fibroblasts, stimulate the accumulation of p8 protein—the former primarily via a relatively conventional process involving induction of the mRNA followed by translation of the protein, and the latter through both rapid stabilization of the p8 protein followed by a more modest induction of *p8* mRNA. The p8 protein then collaborates with transcription factors, notably AP-1, to reprogram gene expression, contributing to cardiomyocyte hypertrophy and cardiac fibroblast expression of MMPs, two key steps in the progression to heart failure (1, 9, 10, 15, 17, 18, 22, 23, 27, 28, 32, 38, 48–50). These findings provide insight into two important cellular events in cardiac pathology.

The appearance of p8 protein in response to GPCRs is likely cell specific. In renal mesangial cells, GPCR-mediated induction of *p8* mRNA is not accompanied by an increase in p8 protein. Instead, the mesangial cell p8 polypeptide is subject to degradation by the ubiquitin proteasome system, a process enhanced by the recruitment of PI-3-kinase-mediated inhibition of glycogen synthase kinase 3 (6, 13). Any ET-1- or PE-induced increases in mesangial cell *p8* transcription/translation are balanced by p8 protein degradation (13). By contrast, prohypertrophic stimuli in cardiomyocytes trigger increases in p8 protein, likely due in large part to increased de novo *p8* transcription. In mesangial cells, GPCR activation of ERK is comparably weak (12, 13), while in cardiomyocytes this activation is strong and, coupled with PI-3-kinase-stimulated *p8* transcription, may overcome PI-3-kinase-mediated destabilization of p8 in the long term. Accordingly, in cardiomyocytes, any p8 destabilization evoked by PE (as occurs in renal mesangial cells [13]) is likely superceded by mechanisms that act to increase p8 protein levels.

TNF induction of cardiac fibroblast p8 protein is quite rapid and precedes accumulation of *p8* mRNA. TNF-stimulated stabilization of p8 correlates with a stimulus-dependent decrease in the stoichiometry of p8 protein ubiquitination and an increase in sumoylation (Fig. 1, 3, and 4; see also Fig. S2 and S3 in the supplemental material). The molecular basis of TNF-stimulated p8 stabilization may coincide with a reduction in p8 ubiquitination (Fig. 3 and 4; see also Fig. S2), although we cannot infer a causal relationship between these processes. The relationship between p8 sumoylation (SUMO1 in this instance) and ubiquitination may be antagonistic, or p8 sumoylation may function to regulate p8 localization or the interactions between p8 and other proteins with which it functions to regulate transcription—functions already proposed for SUMO targets (35, 40, 44). Although TNF-stimulated p8 stabilization is JNK and ERK dependent, p8 is neither a direct MAPK substrate nor Akt substrate in situ (data not shown). Thus, it is reasonable to propose that other polypeptides that regulate directly p8 stability (either TNF-dependent inhibitors of p8 ubiquitination, promoters of p8 deubiquitination or, perhaps, regulators of p8 sumoylation) are targets for protein kinase signaling pathways.

p8 is a nuclear protein (11, 13, 26), and our ChIP and RNAi studies clearly implicate p8 in the regulation of transcription, especially the transcription of genes such as *anf* and *mmp9*, which are induced by AP-1-dependent mechanisms (24, 33, 36, 42). We have shown here that p8 and c-Jun bind to chromatin that contains consensus AP-1 sites in the *anf* and *mmp9* promoters and that this binding precedes the expression of both genes, consistent with the requirement for p8 for expression of *anf* and *mmp9*. Although p8 contains a bHLH domain and is

(top gels for each PCR). The bottom PCRs shown were run using total chromatin (input for the ChIP) as a template. (C) Recombinant p8 associates with chromatin containing c-Jun. Recombinant His-tagged p8 was isolated as for panel B (transfected with vector or pcDNA p8 as indicated and treated with vehicle or TNF as indicated). The immunoprecipitates were subjected to immunoblotting with anti-c-Jun or anti-p8. The bottom immunoblot represents total c-Jun present in the total chromatin input from the transfected cells. (D) Kinetics of endogenous cardiac fibroblast p8 and endogenous c-Jun binding to chromatin containing the *mmp9* promoter. Cardiac fibroblasts were treated with TNF for the indicated times. ChIP of p8 and c-Jun was performed as for panel A, using the dAP-1 primers. In parallel, *mmp9* mRNA was detected by RT-PCR. (E) Induction of an *mmp9*-luciferase reporter (pMMP9) by coexpressed p8 and c-Jun. HeLa or U2OS cells, as indicated, were transfected with pMMP9 and either p8, c-Jun, or both, as indicated. Luciferase activity was measured in triplicate samples and normalized to β-galactosidase activity. Means ± standard deviations are shown. As indicated, samples were subjected to an unpaired Student's *t* test to assess the significance of changes. (F) Ectopic expression of p8 induces endogenous MMP9 protein production. U2OS cells were transfected with p8, c-Jun, and/or c-Fos as indicated. MMP9 activity was measured by gelatin zymography of secreted proteins.

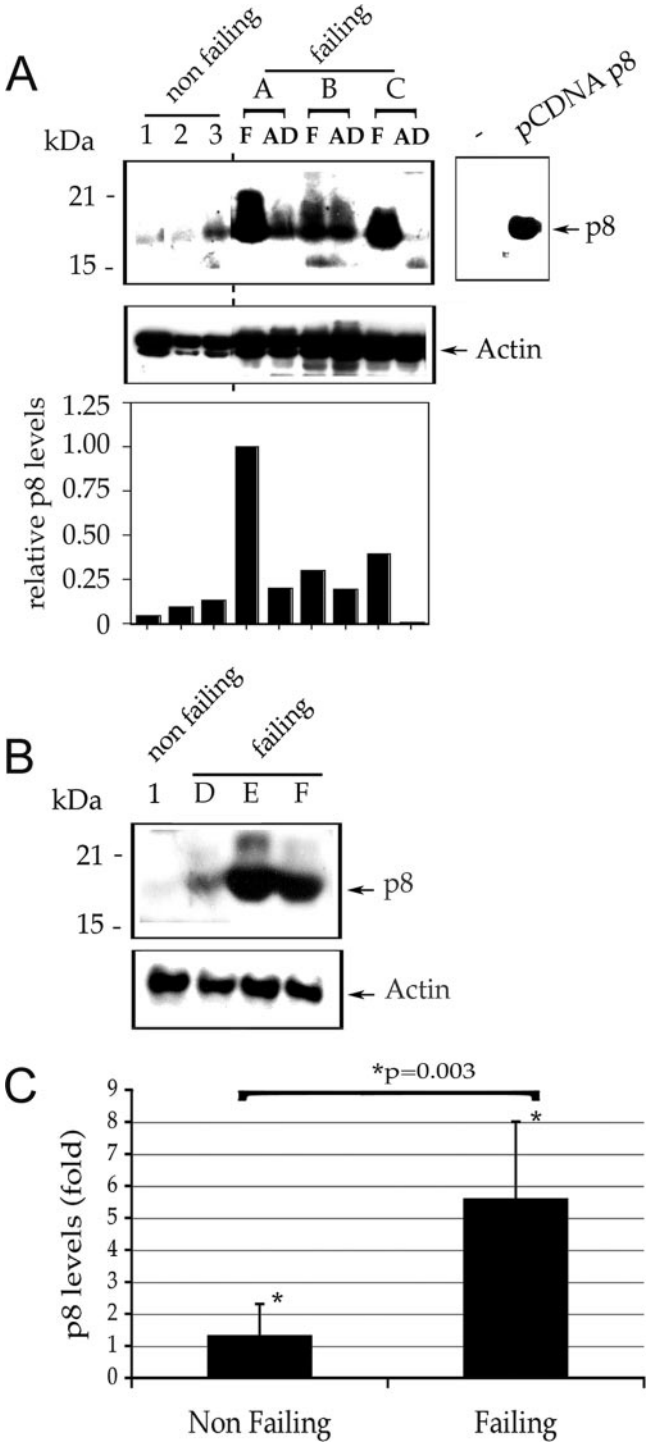


FIG. 7. Induction of p8 in human heart failure. (A) Protein extracts of myocardial samples from three control hearts (nonfailing; lanes 1, 2, and 3) and hearts from three patients with severe heart failure (failing; lanes A, B, and C) were normalized to equal protein levels (demonstrated in the actin immunoblot shown and parallel Coomassie staining of myocardial extract samples) and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis on the same gel and immunoblotting with anti-p8 antibody. To improve the presentation of the figure, after image capture the control samples were digitally moved to the position shown and small lines were placed between the control and heart failure samples. The specificity of the antibody is indicated in the right panel, in which either empty pcDNA vector or pcDNA-p8 was expressed in 293 cells and the extract was immunoblotted with anti-p8. For the samples from failing hearts, "F" indicates a sample taken at the time of VAD implantation. "AD" indicates a sample taken at the time of heart transplant, after a period of mechanical unloading with VAD therapy. The bar graph is a densitometric quantitation of the p8 levels in the heart samples. To obtain relative p8 levels in each sample, the quantitated p8 level was normalized to the quantitated level of actin in the cognate sample. (B) Same experiment as in panel A, except that the heart failure patient samples did not include material taken after VAD. (C) Quantitation of p8 protein induction for the control (nonfailing) and heart failure samples (without VAD unloading) shown in panels A and B. Mean (± standard deviation) p8 levels, as quantitated by densitometry, are shown (nonfailing, $n = 4$; failing, $n = 6$). Data were subjected to an unpaired Student's t test to assess the significance of changes in p8 protein.

modestly similar structurally to transcriptional regulators of the high-mobility group (HMG)-A/HMG-I/Y family (11), it lacks the conserved, so-called "AT hook" DNA binding motif (11, 26, 31). Accordingly, DNA binding by p8 is likely indirect.

In this regard, it is noteworthy that expression of p8 alone can trigger induction of reporter genes for *anf* and *mmp9*. In addition, p8 can augment induction of *mmp9*, but not *anf*, incurred by c-Jun. The reason for this discrepancy is unclear. We also do not yet understand how p8, a transcriptional regulator, can influence hypertrophy, which is associated with increased protein synthesis. It is possible that p8 can modulate the expression of genes associated with enhanced protein biosynthesis. Of note, both PE and ET-1 can trigger the expression of endogenous and reporter rRNA genes, and this induction contributes to cardiomyocyte hypertrophy (14).

We do not yet fully understand how p8 regulates transcription. Perhaps p8 functions in conjunction with other AT hook-containing HMG proteins to modulate gene transcription. HMG proteins do not bind DNA in a sequence-specific manner. It is thought that HMG-A proteins trigger allosteric changes in DNA or chromatin structure, which in turn affect locally the recruitment of transcription factors on the promoter regions of genes whose expression they modulate (31). Our finding that p8 binding to chromatin containing the *mmp9* or *anf* promoters precedes c-Jun binding is consistent with this idea. However, we did not observe coimmunoprecipitation of recombinant p8 and c-Jun or c-Fos, in spite of the fact that p8 ChIPs contain c-Jun. It is also unlikely that p8 acts directly at *cis* elements for sequence-specific DNA binding proteins, inasmuch as a reporter construct consisting solely of AP-1 sites is not *trans*-activated by p8, nor does p8 enhance c-Jun *trans*-activation of such a reporter (see Fig. S5 in the supplemental material). By contrast, induction of *mmp9*, which does require AP-1 (24), is augmented by p8. Taken together, these findings suggest that p8 is not a sequence-specific DNA binding protein per se, nor does it likely act at *cis* elements of sequence-specific transcription factors. Instead, p8 probably functions as an enabler or potentiator of transcription mediated by factors such as AP-1.

The requirement for p8 for cardiomyocyte hypertrophy and cardiac fibroblast MMP expression, coupled with the strong upregulation of p8 protein in heart failure (as well as the decrease in p8 levels coincident with mechanical unloading [Fig. 6]) is noteworthy. VAD therapy has been linked to the

regression of cardiomyocyte hypertrophy, and it is possible that the loss of p8 with mechanical unloading enables the regression of cardiomyocyte hypertrophy. Accordingly, it will be important to investigate the possibility that p8 might be a physiologically relevant biomarker for heart failure.

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