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Regulation of Serum Response Factor-Dependent Gene Expression by Proteasome Inhibitors

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ABSTRACT

Serum response factor (SRF) is activated by contractile and hypertrophic agonists, such as endothelin-1 (ET1) to stimulate expression of cytoskeletal proteins in vascular smooth muscle cells (VSMCs). While studying the regulation of smooth muscle α-actin (SMA) expression at the level of protein stability, we discovered that inhibition of proteasome-dependent protein degradation by *N*-benzoyloxycarbonyl (*Z*)-Leu-Leu-leucinal (MG132) or lactacystin (LC) did not enhance the levels of SMA, but, unexpectedly, attenuated SMA expression in response to ET1, without affecting the viability of VSMCs. Down-regulation of SMA protein by MG132 or LC occurred at the level of SMA transcription and via the inhibition of SRF activity. By contrast, MG132 and LC potentiated the activity of activator protein-1 transcription factor. Regulation of SRF by MG132 was not related to inhibition of nuclear factor-κB, an established target of proteasome inhibitors, and was not mediated by protein kinase A, a powerful regulator of SRF activity. Signaling studies indicate that inhibition of ET1-induced SRF activity by MG132 occurs at the level downstream of heterotrimeric G proteins Gq/11 and G13, of small GTPase RhoA, and of actin dynamics but at the level of SRF-DNA binding. MG132 treatment did not result in ubiquitination or accumulation of SRF. By contrast, the levels of c-Jun were rapidly increased upon incubation of cells with MG132, and ectopic overexpression of c-Jun mimicked the effect of MG132 on SRF activity. Together, these data suggest that inhibition of proteasome results in down-regulation of SMA expression via up-regulation of c-Jun and repression of SRF activity at the level of DNA binding.

Programmatic gene expression is an important phenomenon in developing and differentiating cells. In vivo, vascular smooth muscle cells (VSMCs) are thought to the have the ability to "toggle" programmatic gene expression to assume either a contractile (differentiated) or synthetic (proliferative) phenotype (Miano, 2003). The contractile phenotype is characterized by high levels of expression of smooth muscle contractile proteins, such as smooth muscle α-actin (SMA), SM22, smooth muscle (SM)-calponin, and others, whereas the synthetic phenotype is characterized by down-regulation of contractile protein expression and higher proliferative capacity, in response to various external stimuli and injury. This switch is partly controlled by the activity of the transcription factor serum response factor (SRF), which is required for the expression SM genes.

Endothelin-1 (ET1) is a contractile and growth-promoting vasoactive agonist that is released in response to endothelial injury and is thought to be important in the dysregulation of smooth muscle cell growth in disease states such as angioplasty-induced restenosis and pulmonary hypertension (Tahara et al., 1991; Giaid et al., 1993). ET1 stimulates SRF activity via the heterotrimeric G proteins $G_{q/11}$ and $G_{12/13}$, with subsequent downstream activation of the small GTPase RhoA, polymerization of monomeric G-actin to filamentous F actin, and subsequent transactivation of SRF (Gohla et al., 1999; Sotiropoulos et al., 1999).

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ABBREVIATIONS: VSMC, vascular smooth muscle cell; SMA, smooth muscle α-actin; SM, smooth muscle; SRF, serum response factor; ET1, endothelin-1; NF-κB, nuclear factor-κB; AP1, activator protein 1; Ub, ubiquitin; LC, lactacystin; VASP, vasodilator-stimulated phosphoprotein; ERK, extracellular signal-regulated protein kinase; TNF, tumor necrosis factor; SRE, serum response element; WKY, Wistar-Kyoto; HEK, human embryonic kidney; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; PMSF, phenylmethylsulfonyl fluoride; TUNEL, terminal deoxynucleotidyl transferase dUTP nick-end labeling; PBS, phosphate-buffered saline; FITC, fluorescein isothiocyanate; CMV, cytomegalovirus; PKA, protein kinase A; GST, glutathione *S*-transferase; RBD, Rho-binding domain; DTT, dithiothreitol; EMSA, electrophoretic mobility shift assay; IP, immunoprecipitation; IκBαm, IκBα phosphorylation-deficient mutant; dnPKA, dominant negative protein kinase A; Jasp, jasplakinolide; CChD, cytochalasin D; YY1, Yin Yang 1; HDAC, histone deacetylase; MG132, *N*-benzoyloxycarbonyl (*Z*)-Leu-Leu-leucinal.

We and others have shown that ET1 stimulates SMA expression in VSMCs (Andrawis et al., 1996), partially in an SRF-dependent manner (Davis et al., 2003a). In this study, while examining the contribution of protein stability for the mechanism of ET1-induced SMA expression, we unexpectedly found that two proteasome inhibitors, MG132 and lactacystin (LC), completely blocked SMA protein increase in response to ET1. The 26S proteasome is a primary site of the rapid intracellular protein degradation, and it controls the rate of cell proliferation, survival, and gene expression by regulating the levels of many signaling molecules, including NF-κB inhibitor IκB, cyclin inhibitor $p27^{kip1}$, $p21^{waf1}$, and p53, and others (Adams, 2004). These proteins are targeted for degradation by the addition of polyubiquitin tails via the ubiquitin (Ub) ligases E1, E2, and E3 (Goldberg, 2003).

Given the novelty of our finding that inhibition of the proteasome prevents SMA expression in response to ET1, we sought to examine the mechanism by which this inhibition occurs. In this study, we show for the first time 1) that proteasome inhibition results in inactivation of SRF, 2) that this effect of proteasome inhibitors occurs at the level downstream of actin dynamics and at-or upstream of SRF-DNA binding, and 3) that inhibition of proteasome results in upregulation of c-Jun, a negative regulator of SRF-dependent gene transcription.

Materials and Methods

DNA and Reagents. The luciferase reporter plasmids for smooth muscle α-actin promoter (−125 base pairs) and for SRF (SRE.L) were described previously (Davis et al., 2003a; Hogarth et al., 2004). The activator protein 1 (AP1) luciferase reporter plasmid was from Stratagene (La Jolla, CA). The expression plasmid for $G\alpha_{11}QL$ was provided by Dr. Hiroshi Itoh (Tokyo Institute of Technology, Yokohama, Japan) and was used previously (Dulin et al., 1999). The expression plasmids for $G\alpha_{13}QL$ and RhoA-V14 were gifts from Dr. Tatyana Voyno-Yasenetskaya (University of Illinois at Chicago, Chicago, IL). The expression plasmid for vasodilator-stimulated phosphoprotein (VASP) was provided by Dr. Darren Browning (Medical College of Georgia) and was used previously (Dulin et al., 2001; Davis et al., 2003a). The expression plasmid for c-Jun was provided by Dr. Anning Lin (The University of Chicago, Chicago, IL). The antibodies were from the following sources: anti-SM-α-actin and anti-β-actin were from Sigma-Aldrich (St. Louis, MO); anti-phospho-ERK1/2 and anti-β-catenin were from Cell Signaling Technology Inc. (Beverly, MA); anti-RhoA and anti-SRF were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA); and anti-ERK2 was a gift from Dr. Michael Dunn (Medical College of Wisconsin, Milwaukee, WI). Endothelin-1, MG-132, epoxomicin, lactacystin, calpeptin, PD150606, and TNF-α were from EMD Biosciences (San Diego, CA).

Cell Culture. The WKY7 vascular smooth muscle cell line maintaining endogenous receptors to various vasoactive ligands was described previously (Davis et al., 2003a). In brief, primary aortic SMC derived from Wistar-Kyoto (WKY) rats were subcloned by serial dilution. Clone-7 (WKY7) was selected for the responsiveness to various vasoactive ligands, subcloned again to ensure its purity, and characterized for the expression of SM-specific proteins. The HeLa cells and human embryonic kidney (HEK) 293 cells were from American Type Culture Collection (Manassas, VA). The cells were grown in DMEM supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 100 U/ml streptomycin, 250 ng/ml amphotericin B, and 100 U/ml penicillin. The cells were serum-deprived using DMEM containing 0.2% calf serum and 2 mM L-glutamine. All stimulations were performed in DMEM containing 0.1% bovine serum albumin, and 2 mM L-glutamine.

Transient Transfection of DNA. Transient transfections were performed using LipofectAMINE-PLUS reagent (Invitrogen, Carlsbad, CA) following the manufacturer's recommendations. Cells were incubated with DNA-liposome complexes in FBS- and antibiotic-free DMEM for 3 h, followed by FBS supplementation to a final concentration of 10% and incubation for additional 6 h. The cells were then starved in DMEM containing 0.2% calf serum overnight. Stimulations with desired agonists were carried out in DMEM containing 0.1% bovine serum albumin.

Western Blotting. After stimulation of quiescent cells with desired agonists, cells were lysed in radioimmunoprecipitation assay buffer containing 25 mM HEPES, pH 7.5, 150 mM NaCl, 1% Triton X-100, 0.1% SDS, 2 mM EDTA, 2 mM EGTA, 10% glycerol, 1 mM NaF, 200 μM sodium orthovanadate, and protease inhibitors (1 μg/ml leupeptin, 1 μg/ml aprotinin, and 1 mM PMSF). The lysates were cleared from insoluble material by centrifugation at 20,000*g* for 10 min, boiled in Laemmli buffer, subjected to polyacrylamide gel electrophoresis, analyzed by Western blotting with desired primary antibodies followed by horseradish peroxidase-conjugated secondary antibodies (Calbiochem, San Diego, CA), and developed by enhanced chemiluminescence reaction (Pierce Chemical, Rockford, IL).

Apoptosis Assay. DeadEnd TUNEL assay kit (Promega, Madison, WI) was used for the assessment of DNA fragmentation as described previously (Dulin et al., 2000). In brief, cells grown on four-chamber slides were washed with PBS, fixed in 4% paraformaldehyde, and permeabilized in 0.2% Triton X-100/PBS for 5 min. The 3′-OH DNA ends were labeled with fluorescein isothiocyanate (FITC)-labeled-12-dUTP using terminal deoxynucleotidyl transferase (TdT), as described in the manufacturer's standard protocol. The slides were then washed with PBS and mounted in 4,6-diamidino-2-phenylindole-containing mounting medium (Vector Laboratories, Burlingame, CA). The FITC-positive apoptotic cells were visualized under the fluorescent microscope. For statistical analysis, at least six fields (each containing approximately 20 cells) from each experiment were analyzed and expressed as percentage of apoptotic cells (FITC-positive) over the total population of cells determined by 4,6-diamidino-2-phenylindole fluorescence of nuclei.

Luciferase Assay. The cells grown in 24-well plates were transfected with 100 ng/well desired luciferase reporter plasmid, 50 ng/well CMV-driven LacZ plasmid and 100 ng/well empty vector or the plasmid of interest. After 5-h stimulation with desired agonists, cells were washed with PBS, lysed in protein extraction reagent, and the cleared lysates were assayed for luciferase and β-galactosidase activity using the corresponding assay kits (Promega). To account for differences in transfection efficiency, luciferase activity of each sample was normalized to β-galactosidase activity and expressed as -fold of control (unstimulated cells transfected with empty vector).

Nonradioactive in Vitro Assay for Protein Kinase A Activity. After stimulation with desired agonists, the cells (grown in 12-well plates) were lysed in 0.15 ml/well lysis buffer containing 25 mM HEPES, pH 7.5, 0.5% Nonidet P-40, protease inhibitors (1 μg/ml leupeptin, 1 μg/ml aprotinin, and 1 mM PMSF), and phosphatase inhibitors (1 mM NaF and 200 μM Na-orthovanadate). The lysates were cleared from insoluble material by centrifugation at 20,000*g* for 10 min, and 5 μl of cleared lysates was subjected to a kinase reaction with the fluorescence-labeled PKA substrate kemptide (Promega), following the manufacturer's protocol. The reaction was stopped by boiling the samples for 10 min. The phosphorylated kemptide was separated from nonphosphorylated kemptide by 0.8% agarose electrophoresis. The fluorescent images were taken by luminescent image analyzer LAS-3000 (Fujifilm, Tokyo, Japan).

RhoA Activity Assay. Rho activation was determined by a pull-down assay using Rho-binding domain of rhotekin (GST-RBD). Cells (one 10-cm dish/condition) were stimulated with ET1 for 2 min and lysed in 600 μl of buffer containing 50 mM Tris-HCl, pH 7.5, 500 mM NaCl, 10 mM $MgCl_2$, 1 mM EDTA, 1 mM DTT, 1% Nonidet P-40, and protease inhibitors as described above. Cell lysates were centrifuged at 15,000*g* for 5 min at 4°C. The supernatant was then incubated

with 20 μg of GST-RBD/glutathione-Sepharose beads for 1 h at 4°C. The beads were washed four times with 800 μl of washing buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 10 mM $MgCl_2$, 1 mM EDTA, 1 mM DTT, 0.5% Nonidet P-40, and protease inhibitors as described above). The proteins were eluted from the beads with SDS-sample buffer, boiled, and subjected to Western blot analysis with RhoA antibodies.

Electrophoretic Mobility Shift Assay (EMSA). The cell nuclear extracts were prepared according to a published method (Dignam et al., 1983) with some modifications. Unless indicated otherwise, all procedures were performed at 4°C. In brief, 10 million cells/condition were harvested by centrifugation and washed twice with ice-cold Dulbecco's PBS buffer. The pellet was resuspended in 4× packed cell volume of buffer A (10 mM HEPES, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM PMSF, and 0.5 mM DTT) and incubated on ice for 10 min. The supernatant was discarded after centrifugation at 1500 rpm for 7 min, and 1× original packed cell volume of buffer A was added. The cell suspension was transferred to a 50-ml tube and centrifuged at 11,000*g* for 20 min. The supernatant was removed and set aside as the cytoplasmic extract. The pellet was gently washed with buffer A an additional time and resuspended in 1× original packed cell volume of buffer C (20 mM HEPES, pH 7.9, 25% glycerol, 1.5 mM $MgCl_2$, 420 mM KCl, 0.2 mM EDTA, 0.5 mM PMSF, and 0.5 mM DTT). The suspension was stirred on a rocking platform for 30 min, homogenized using a Dounce pistol B, and then centrifuged at 20,000*g* for 30 min. The supernatant was collected without disturbing the pellet and placed in dialysis tubing (Invitrogen). Dialysis was performed for 1 h against three changes of 200 ml of buffer D (20 mM HEPES, pH 7.9, 20% glycerol, 100 mM KCl, 0.2 mM EDTA, 0.5 mM PMSF, and 0.5 mM DTT). After dialysis, the nuclear extract was clarified by centrifugation at 14,000*g* for 20 min in an Eppendorf microcentrifuge tube. Protease inhibitors including leupeptin, chymostatin, and pepstatin A (Sigma-Aldrich) were added immediately (5 μg/ml each) to extracts before saving them at −80°C.

EMSA was performed as described previously (Beg et al., 1993) with some modification. Double-stranded human SM-Actin CArG synthetic oligonucleotides 5′-AAGGGGACCAAATAAGGCAAGGTGG-3′ were purchased from Promega and end-labeled with [γ-^{32}P]ATP (Amersham Biosciences Inc., Piscataway, NJ) and T4 polynucleotide kinase (New England Biolabs, Beverly, MA). Binding reactions were performed on ice in a total volume of 15 μl. DNA probe (2000 cpm, 1–5 fmol) was preincubated for 15 min with 1.5 μl of binding buffer (50 mM HCl, pH 7.5, 20% Ficoll, 375 mM KCl, 5 mM EDTA, and 5 mM DTT) and 1 μg of poly(dI-dC) (Promega). DNA-protein binding was initiated by adding 4 μg of nuclear extract. A 200-fold excess of unlabeled CArG probe and unrelated oligonucleotide probes for CArG were used to assess the specificity of the DNA binding reaction. Electrophoresis was performed for 2 h at 100 V in 0.5× Tris-borate-EDTA running buffer in a 4°C cold room. The dried gel was visualized via exposure to high-performance autoradiography film. The supershift analyses were performed by incubating the DNA binding reactions with antibodies against SRF (1 μg/reaction) for an additional 20 min on ice before electrophoresis.

Immunoprecipitation. VSMCs (one 10-cm dish/condition) were lysed in 1 ml of nondenaturing immunoprecipitation (IP) buffer containing 25 mM HEPES, pH 7.5, 150 mM NaCl, 0.5% Triton X-100, 2 mM EDTA, 2 mM EGTA, and protease inhibitors (1 μg/ml leupeptin, 1 μg/ml aprotinin, and 1 mM PMSF). The insoluble material was precipitated by centrifugation at 20,000*g* for 5 min, and the supernatant was "precleaned" by incubation with 10 μl of protein A-conjugated agarose beads (20 mg/ml IgG binding capacity) for 1 h at 4°C. A 50-μl aliquot of precleaned cell lysates was set aside, and the rest of the lysates (950 μl) was incubated with desired primary antibodies (5 μg/ml) overnight, followed by incubation with 10 μl of protein A-conjugated agarose beads for 2 h. The beads were then washed three times with 1 ml of IP buffer, resuspended in Laemmli buffer, and boiled for 5 min.

Results

Regulation of α-SM-Actin Protein Expression by Proteasome Inhibitors. To elucidate the regulation of SMA expression at the level of stability in VSMCs, we examined the effect of two lysosome inhibitors, calpeptin and PD150606, and two proteasome inhibitors, MG132 and LC, on SMA protein levels. As shown in Fig. 1, a 24-h treatment of VSMCs with calpeptin and PD150606 had no effect on the levels of SMA protein. By contrast, MG132 and LC abolished the ET1-induced expression of SMA, without affecting the basal level of SMA or the amount of ubiquitous β-actin. Given that proteasome inhibitors can stimulate apoptosis of some cells, we examined whether the down-regulation of SMA protein levels by MG132 and LC was a result of VSMC apoptosis. Treatment of cells with MG132 or LC for 24 h had no significant effect on VSMC apoptosis, as assessed by TUNEL assay (Fig. 1C). This suggests that the effect of MG132 and LC on SMA expression is not caused by VSMC apoptosis. In addition, inhibition of caspases, which are commonly activated during apoptosis, did not prevent the regulatory effect of MG132 and LC on SMA expression (data not shown).

Down-Regulation of α-SM-Actin Protein Expression by Proteasome Inhibitors Occurs at the Level of SRF-Dependent Transcription. To examine whether the regulation of SMA protein expression by proteasome inhibitors occurs at the level of transcription, we assessed the effect of MG132 and LC on the activity of luciferase reporter driven by a proximal (−125 base pair) SMA promoter (−125SMA-Luc), which was previously shown by us and others to be sufficient for induction by angiotensin II (Hautmann et al., 1997) or by

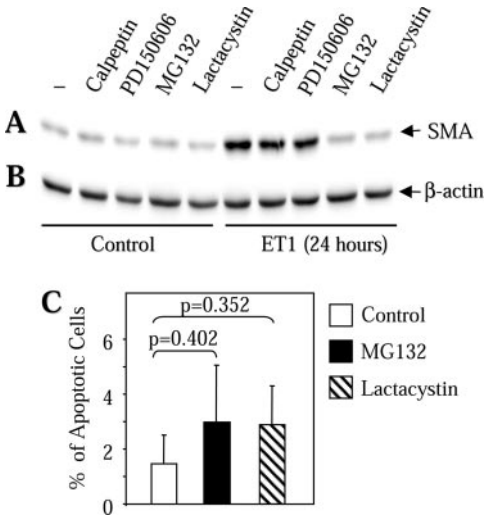

Fig. 1. Proteasome inhibitors attenuate ET1-induced expression of SM-α-actin without inducing apoptosis in quiescent VSMCs. Serum-starved VSMCs were preincubated with 10 μM calpeptin, 10 μM PD150606, 3 μM MG132, or 10 μM lactacystin for 1 h, followed by stimulation with 100 nM ET1 for 24 h (without washing out the inhibitors). A and B, cells were lysed, and the equal amounts (per protein) of cell lysates were analyzed by Western blotting with antibodies against SM-α-actin (SMA, A) or β-actin (B). Shown are the representative blots from three independent experiments. C, apoptosis of quiescent VSMCs after treatment with 3 μM MG132 or 10 μM lactacystin for 24 h, as measured by TUNEL assay. The percentage of apoptotic cells is shown with the standard error for pooled proportions. A Fisher's exact test was performed to determine the *p* values for the difference in apoptotic percentages between control and treatment conditions.

ET1 (Davis et al., 2003a). As shown in Fig. 2A, both MG132 and LC decreased the basal as well as ET1-induced activities of −125SMA-Luc reporter. In contrast, the activity of a constitutive CMV promoter was not affected by theses agents. Moreover, MG132 and LC slightly increased AP1-driven transcription (Fig. 2B), which is in accord with the previously published results (Nakayama et al., 2001). Together, these results demonstrate that proteasome inhibitors at the level of transcription regulate the expression of SMA protein.

The transcription of many SM-specific genes is induced by SRF, which binds the CArG box sequences of their promoters (Miano, 2003). We have previously shown that ET1-induced transcription of SMA is also mediated by SRF (Davis et al., 2003a). Therefore, we hypothesized that the regulation of SM gene expression by proteasome inhibitors is a result of attenuation of SRF activity. To test this hypothesis, we examined the effect of three proteasome inhibitors, MG132, epoxomicin, and LC, on the activity of SRF-luciferase reporter (SRE.L), as we did previously (Davis et al., 2003a). Figure 3A shows the dose-dependent inhibition of ET1-induced SRE.L activity by MG132, epoxomicin, and LC, with maximal inhibition occurring at 3 μM MG132, 3 μM epoxomicin, and 10 μM LC, respectively. The dose dependence of SRE.L inhibition by these compounds roughly corresponds to their reported effects on proteasome inhibition (Palombella et al., 1994; Fenteany et al., 1995; Meng et al., 1999). Together, these data suggest that the regulation of SMA protein expression by proteasome inhibitors occurs at the level of transcription, via inhibition of SRF activity. To elucidate whether the regulation of SRF activity by proteasome inhibitors is specific for VSMCs, we examined the effect of MG132 on SRE.L reporter activity in the cells of epithelial origin, namely, HeLa cells and HEK293 cells, cotransfected with ET1 receptor (type A) cDNA. As shown in Fig. 3, B and C, MG132 inhibited basal and ET1-induced SRF activity in both cell types, suggesting that this effect is not cell-specific.

Regulation of SRF Activity by MG132 Is Not Mediated by Inhibition of NF-κB-Dependent Gene Transcription. We next examined the mechanism by which proteasome inhibition results in the regulation of SRF activity, using SRE.L reporter, and MG132 as a representative proteasome inhibitor. It is established that the activity of NF-κB is induced in a proteasome-dependent manner by phosphorylation and degradation of NF-κB inhibitor IκB (Palombella et al., 1994). This may suggest that the regulation of SRF activity by MG132 could be a result of NF-κB inhibition. If so, then inhibition of NF-κB by other means should mimic the effect MG132 on SRF activity. To test this possibility, we used the IκBα phosphorylation-deficient mutant (IκBαm), which cannot be degraded and which behaves as superrepressor of NF-κB activity. As shown in Fig. 4A, transient overexpression of IκBαm blocked TNF-α-induced NF-κB activity, as was expected. However, IκBαm failed to attenuate, and, in fact, enhanced SRF activity, especially during ET1 stimulation (Fig. 4B). This indicates that regulation of SRF by MG132 is not mediated by stabilization of IκBα or by inhibition of NF-κB-driven gene expression. The potentiation of SRF activity by IκBαm is an interesting new observation, but it is not a focus of this study, because it does not explain the mechanism of MG132 action on SRF.

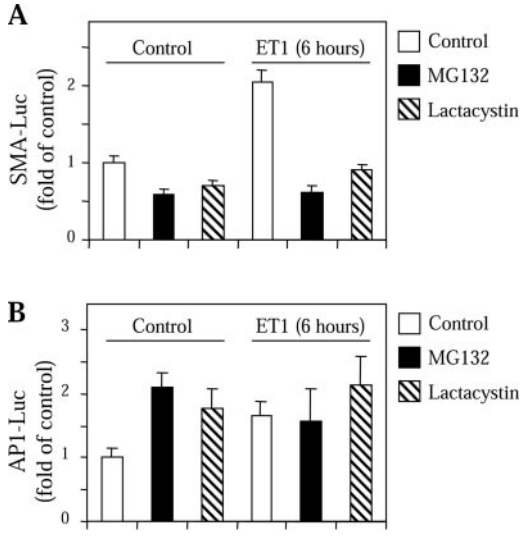

Fig. 2. Proteasome inhibitors attenuate ET1-induced activity of the proximal SM-α-actin promoter. VSMCs were transfected with luciferase reporter constructs for SM-α-actin promoter (SMA-Luc, A), or AP1 binding elements (AP1-Luc, B), together with CMV-driven LacZ plasmid, as described under *Materials and Methods*. Cells were serum-starved for 24 h, preincubated with 3 μM MG132, or 10 μM lactacystin for 1 h, followed by stimulation with 100 nM ET1 for 6 h (without washing out the inhibitors). Luciferase activity in cell lysates was then measured, normalized to β-galactosidase activity and expressed as -fold of control (mean ± S.D. from three independent experiments performed in quadruplicate).

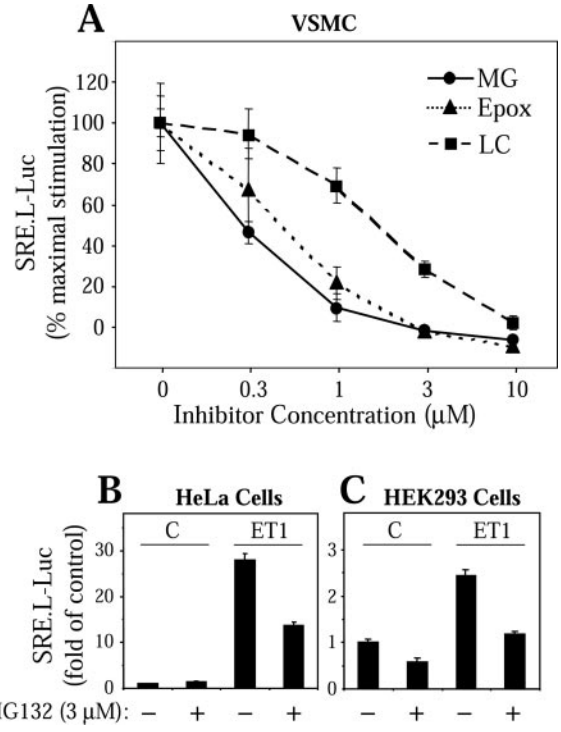

Fig. 3. Regulation of SRF-dependent gene transcription by proteasome inhibitors. VSMCs (A), HeLa cells (B), or HEK293 cells (C) were transfected with SRF-luciferase reporter construct (SRE.L-Luc) together with CMV-driven LacZ plasmid and ET1 receptor cDNA (B and C only), as described under *Materials and Methods*. Cells were serum-starved for 24 h and preincubated with indicated concentrations of MG132, epoxomicin, or lactacystin for 1 h, followed by stimulation with 100 nM ET1 for 6 h (without washing out the inhibitors). Luciferase activity in cell lysates was then measured, normalized to β-galactosidase activity, and expressed as percentage of maximal response (A) or as -fold of control (B and C) [mean ± S.D. from three (A) or two (B and C) independent experiments performed in quadruplicate].

Regulation of SRF Activity by MG132 Is Not Mediated by Activation of PKA. We have previously shown that SRF-dependent gene transcription is highly sensitive to regulation by PKA and that PKA activation by ET1 limits the extent of SRF stimulation by this agonist (Davis et al., 2003a), presumably caused by the phosphorylation and inhibition of small GTPase RhoA (Lang et al., 1996). Thus, it was possible that MG132 could also stimulate PKA activity resulting in SRF inhibition. Consistent with our previously published results (Davis et al., 2003a), ET1 stimulated PKA activation in VSMCs, as measured by in vitro kinase assay (Fig. 5A). However, preincubation with MG132 did not result in measurable PKA activation but rather slightly attenuated ET1-induced PKA activity. The latter effect is consistent with partial regulation of PKA by IκB, as we and others have reported previously (Zhong et al., 1997; Dulin et al., 2001). In contrast, MG132 alone slightly increased basal phosphorylation of ERK1/2 without modifying the effect of ET1, as assessed by Western blotting with phospho-ERK1/2 antibodies or by electrophoretic mobility shift assay for the phosphorylated ERK2 (Fig. 5, B and C). To further elucidate the potential role PKA in the effect of MG132, we used a dominant negative PKA mutant, whose efficiency and specificity in PKA inhibition we confirmed previously (Davis et al., 2003a). If PKA mediates the regulatory effect of MG132 on SRF activity, then this effect should be reversed by inhibition of PKA. As shown in Fig. 5D, overexpression of dnPKA increased the basal SRF activity and further potentiated the effect of ET1, which is consistent with our previous results (Davis et al., 2003a). However, dnPKA overexpression did not reverse the effect of MG132, suggesting that this agent does not involve PKA in the regulation of SRF activity.

MG132 Inhibits SRF Activity at the Level Downstream of Actin Dynamics. Having excluded the abovementioned two possibilities for the mechanism of MG132 action on SRF activity, we sought to systematically explore where in the signaling of ET1 to SRF activation MG132 acts. In fibroblasts, the SRF activation by ET1 is mediated by $G\alpha_{q/11}$ and $G\alpha_{12/13}$ heterotrimeric G proteins, both of which converge their signaling to a small GTPase RhoA (Mao et al., 1998). To investigate whether MG132 inhibits SRF activity at the level of these molecules, we used their corresponding constitutively active mutants, $G\alpha_{11}$QL, $G\alpha_{13}$QL and RhoA-V14. As shown in Fig. 6A, transient overexpression of $G\alpha_{11}$QL, $G\alpha_{13}$QL and RhoA-V14 all resulted in significant induction of SRF activity as expected. In the presence of MG132, each of their effects was completely blocked, suggesting that MG132 acts at the level or downstream of RhoA. We then examined whether MG132 inhibits RhoA activity. Figure 6B shows that ET1 stimulates a rapid activation of RhoA in VSMCs, as assessed by rhotekin RBD binding assay. Pre-

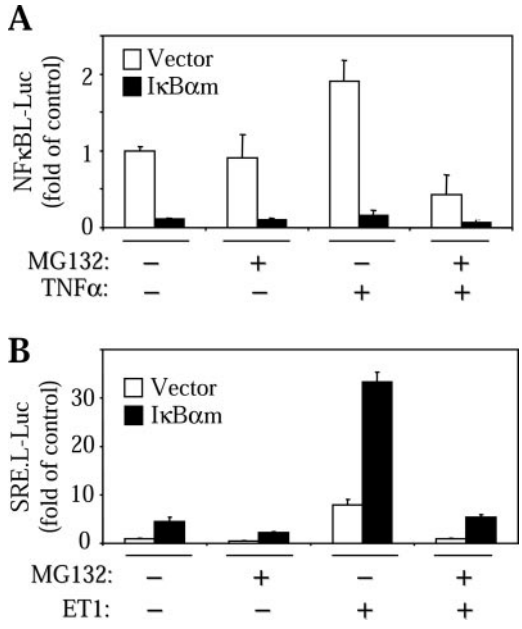

Fig. 4. Regulation of SRF activity by MG132 is not related to inhibition of NF-κB signaling. VSMCs were transfected with luciferase reporter constructs for NF-κB (NFκB-Luc, A) or for SRF (SRE.L-Luc, B), with CMV-driven LacZ plasmid, together with vector alone (open columns) or with the plasmid encoding IκBα-phosphorylation-deficient mutant IκBαm (closed columns). Cells were serum-starved for 24 h and preincubated with 3 μM MG132 for 1 h, followed by stimulation with 25 ng/ml TNF-α (A) or 100 nM ET1 (B) for 6 h (without washing out MG132). Luciferase activity in cell lysates was then measured, normalized to β-galactosidase activity, and expressed as -fold of control (mean ± S.D. from three independent experiments performed in triplicate).

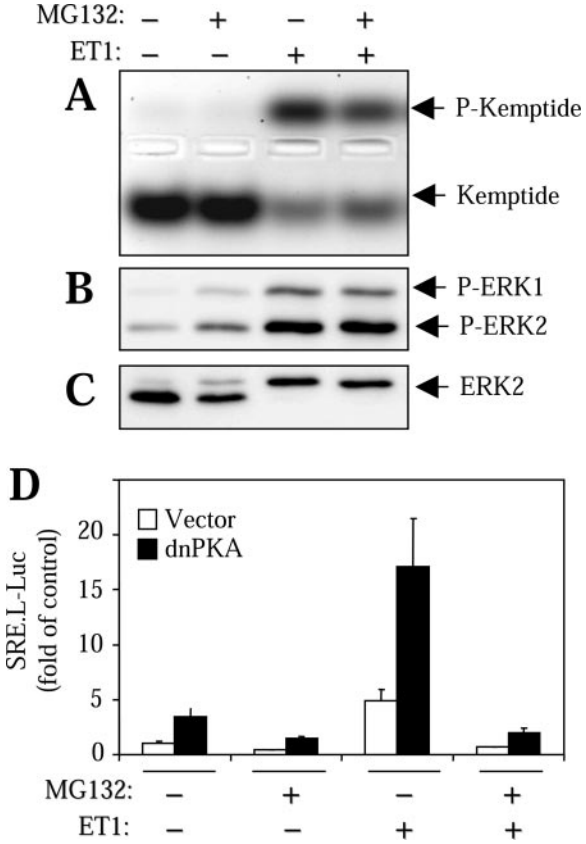

Fig. 5. Regulation of SRF activity by MG132 is not mediated by PKA activation. A to C, quiescent VSMCs were preincubated with 3 μM MG132 for 1 h, followed by stimulation with 100 nM ET1 for 5 min. Cells were lysed and subjected to the in vitro PKA assay (A) or to Western blotting with antibodies against phosphorylated ERK1/2 (B) or against total ERK2 (C). D, VSMCs were transfected with SRF-luciferase reporter construct (SRE.L-Luc), with CMV-driven LacZ plasmid, together with vector alone (open columns) or with the plasmid encoding dominant-negative PKA mutant dnPKA (closed columns). Cells were serum-starved for 24 h and preincubated with 3 μM MG132 for 1 h, followed by stimulation with 100 nM ET1 for 6 h (without washing out MG132). Luciferase activity in cell lysates was then measured, normalized to β-galactosidase activity and expressed as -fold of control (mean ± S.D. from three independent experiments performed in triplicate).

incubation of cells with MG132 did not affect the ET1-induced RhoA activation, suggesting that MG132 acts downstream of RhoA.

The mechanism of RhoA-dependent SRF activation involves actin polymerization (F-actin formation), and as a result, a depletion of monomeric G-actin, which sequesters the SRF-coactivators, such as MAL1 (Miralles et al., 2003) and maybe myocardin (Wang et al., 2001). In light of this, we investigated whether the effect of MG132 on SRF activity occurs at the level of actin dynamics. Jasplakinolide (Jasp) promotes actin polymerization by inhibiting its depolymerization (Bubb et al., 1994), whereas the VASP cooperates with RhoA signaling to promote the formation of F-actin (Walders-Harbeck et al., 2002). Application of Jasp or overexpression of VASP is sufficient for SRF induction in fibroblasts (Grosse et al., 2003). By contrast to jasplakinolide and VASP, cytochalasin D (CChD) promotes F-actin disassembly through the interaction with (and stabilization of) the monomeric G-actin. Nevertheless, CChD still induces SRF activity independent of actin dynamics, presumably by interfering with the ability of G-actin to sequester the SRF coactivators (Miralles et al., 2003). As shown in Fig. 6C, SRF activation induced by jasplakinolide, by VASP overexpression, or by CChD, was inhibited by MG132. This suggests that MG132 acts not at the level of actin dynamics, but downstream of it, to inhibit SRF activity. In accord with this suggestion, no significant decrease in F-actin, or in VSMC shape were observed during MG132 treatment, such as those induced by PKA activators or by the F-actin-depolymerizing agent latrunculin B, all of which inhibit SRF activity by promoting actin depolymerization (data not shown).

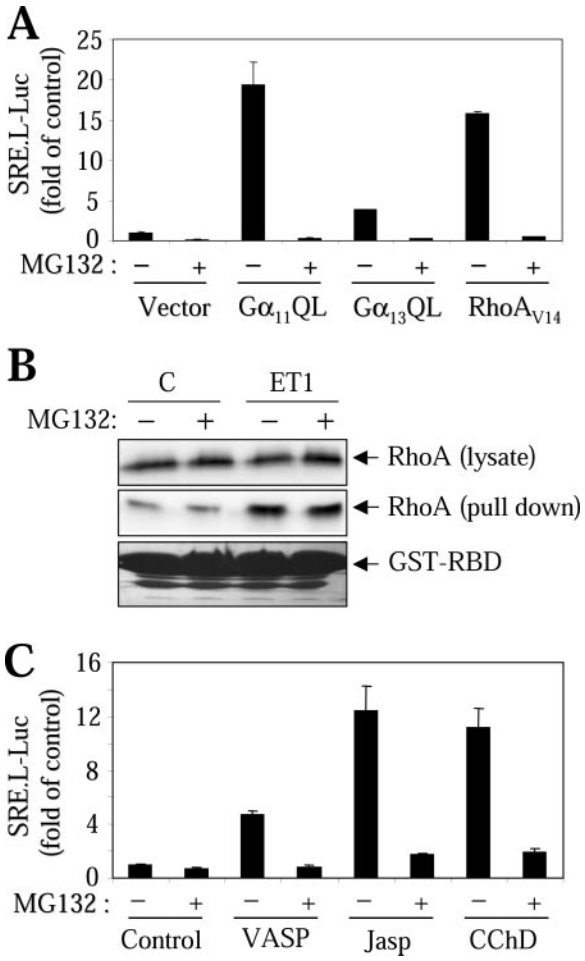

Fig. 6. MG132 inhibits SRF activity at the level downstream of actin dynamics. A and C, VSMCs were transfected with SRF-luciferase reporter plasmid (SRE.L-Luc), with CMV-driven LacZ plasmid, together with vector alone or with expression plasmids for constitutively active mutants of $G\alpha_{11}$ ($G\alpha_{11}$QL), $G\alpha_{13}$ ($G\alpha_{13}$QL), RhoA ($RhoA_{V14}$), or for VASP, as indicated. Cells were serum-starved for 24 h and preincubated with or without 3 μM MG132 for 1 h, followed by stimulation with or without 500 nM Jasp or 2 μM CChD for 6 h (without washing out MG132), as indicated. Luciferase activity in cell lysates was then measured, normalized to β-galactosidase activity, and expressed as -fold of control (mean ± S.D. from three independent experiments performed in triplicate). B, serum-starved VSMCs were preincubated with 3 μM MG132 for 1 h followed by stimulation with 100 nM ET1 for 2 min. Cells were then lysed, and the cell lysates were subjected to GST-RBD pull-down RhoA activity assay as described under *Materials and Methods*. The amounts of total (lysate) and RBD-bound (pull down) RhoA were assessed by Western blotting with RhoA antibodies. The equal amounts of GST-RBD were confirmed by Ponceau S staining.

MG132 Inhibits SRF Binding to CArG Box. We then examined whether treatment of VSMCs with MG132 affects SRF binding to its DNA target, CArG box, by EMSA using the radiolabeled CArG consensus sequence from smooth muscle α-actin. As shown in Fig. 7A, stimulation of WKY cells with ET1 for 1 h resulted in a significant increase in

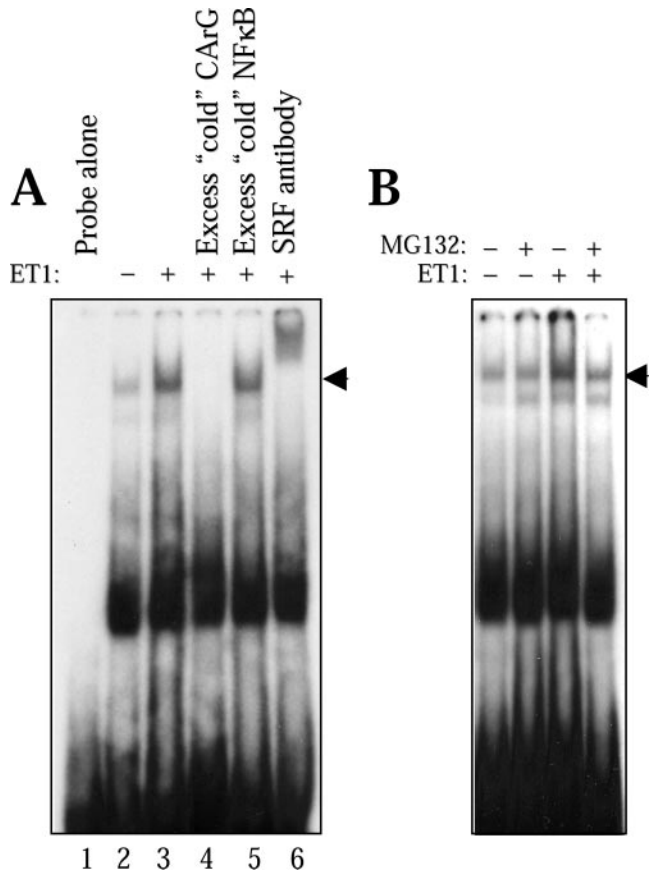

Fig. 7. MG132 inhibits SRF binding to CArG box. Serum-starved VSMCs were preincubated with or without 3 μM MG132 for 1 h, followed by stimulation with or without 100 nM ET1 for additional 1 h (without washing out MG132). The nuclear extracts were then prepared and the SRF binding to radiolabeled CArG probe was assessed by EMSA as described under *Materials and Methods*. A, specificity of EMSA for SRF-CArG binding was confirmed by competition with 50× excess of unlabeled CArG probe but not unlabeled NF-κB probe and by supershift observed in the presence SRF antibody. B, pretreatment of cells with MG132 inhibits ET1-induced binding of SRF to CArG probe.

SRF binding to radiolabeled CArG probe. The specificity of SRF binding was confirmed by 1) inhibition of binding (competition) in the presence of a 50-fold excess of unlabeled CArG probe (lane 4), 2) the lack of competition with a 50-fold excess of the irrelevant unlabeled NF-κB probe (lane 5), and 3) a supershift in the presence of SRF antibodies (lane 6). As shown in Fig. 7B, pretreatment of VSMCs with MG132 attenuated the ET1-induced SRF binding to CArG probe. It is noteworthy that addition of MG132 during the binding reaction had no effect on SRF binding to CArG probe (data not shown). Together, these results suggest that MG132 inhibits SRF-dependent gene transcription at the level or upstream of SRF binding to CArG box.

SRF Is Not Ubiquitinated in VSMCs. Given that proteasome inhibition results in accumulation of ubiquitinated proteins, we examined whether MG132 treatment of VSMCs leads to ubiquitination of SRF, which could affect its binding to DNA. As shown in Fig. 8A, a 2-h treatment of VSMCs with MG132 resulted in accumulation of ubiquitinated proteins, as assessed by Western blotting of cell lysates with antibodies against Ub. Stimulation of cells with ET1 for 1 h did not significantly affect the total ubiquitination profile, regardless of the presence of MG132 (data not shown). Immunoprecipitation of SRF from cell lysates followed by Western blotting with Ub antibodies revealed the presence of at least three ubiquitinated proteins in SRF-immune complex with approximate molecular masses of >250, 170, and 100 kDa. These ubiquitinated proteins were not detected when normal IgG was used for immunoprecipitation. Western blotting of immune complexes with SRF antibodies confirmed the successful and specific SRF immunoprecipitation and revealed that none of these ubiquitinated bands represent SRF. In contrast to SRF, β-catenin, which is known to be ubiquitinated and degraded by proteasome (Aberle et al., 1997), accumulated upon MG132 treatment in mono- and oligo-ubiquitinated forms, as viewed by a "ladder" following Western blotting of β-catenin immunoprecipitates with Ub antibodies and especially with β-catenin antibodies (Fig. 8B). Together, these data suggest that SRF is not ubiquitinated in VSMCs, but it may interact with ubiquitinated proteins of unknown nature. However, the amounts of these SRF-bound proteins were not altered by MG132, and therefore the interaction of SRF with these proteins cannot explain the inhibition of SRF activity by MG132.

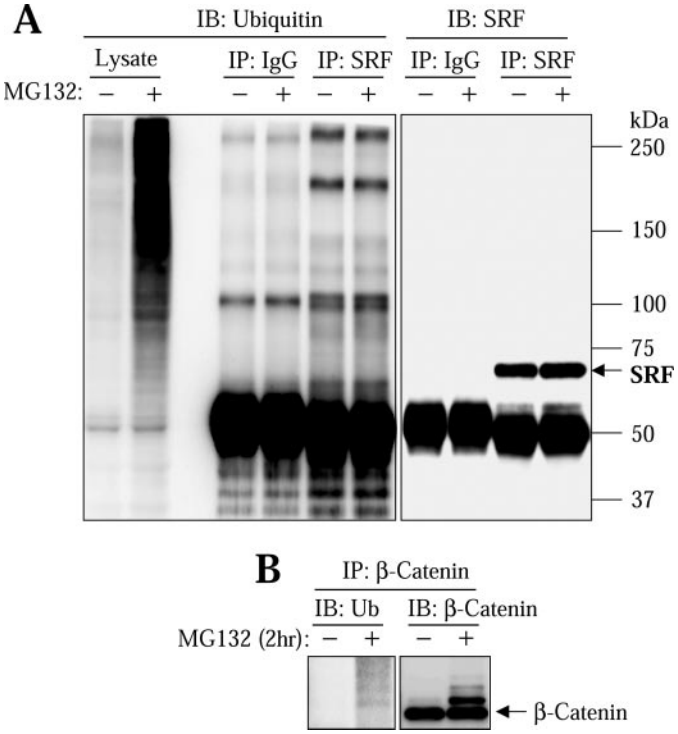

Fig. 8. SRF is not ubiquitinated in VSMCs. Serum-starved VSMCs were incubated with or without 3 μM MG132 for 2 h, lysed, and the cleared cell lysates were subjected to immunoprecipitation with normal rabbit IgG or with anti-SRF antibodies (A) or with anti-β-catenin antibodies (B) as described under *Materials and Methods*. The corresponding immune complexes (IP) or total cell lysates (1/20th of those used for IP) were immunoblotted (IB) with antibodies against ubiquitin, SRF, or β-catenin as indicated. Shown are the representative blots from three (A) and two (B) independent experiments with similar results.

Accumulation of c-Jun upon MG132 Treatment and Regulation of SRF Activity by c-Jun. We then examined whether treatment of cells with MG132 could lead to accumulation of other transcription factors that were previously reported to repress SRF-dependent transcription, especially that of smooth muscle genes. The member of ternary complex factor Elk1 competes with myocardin for SRF binding and inhibits RhoA-mediated, SRF-dependent transcription of smooth muscle genes (Murai and Treisman, 2002; Wang et al., 2004). Yin Yang 1 (YY1) competes with SRF for CArG binding (Gualberto et al., 1992). Finally the components of AP1 complex, c-Fos and c-Jun, attenuate SM-α-actin promoter activity, presumably at the level of CArG-mediated transcription (Bushel et al., 1995). As shown in Fig. 9A, the amounts of Elk1, YY1, and c-Fos proteins were not significantly affected by MG132 treatment. By contrast, the levels of c-Jun increased by approximately 3-fold after 2-h incubation with MG132 (Fig. 9A). This accumulation of c-Jun was not accompanied by an ubiquitinated "ladder" formation, as seen with β-catenin upon MG132 treatment (Fig. 8B), which is consistent with previous report showing that ubiquitination is not an absolute requirement for degradation of c-Jun protein by proteasome (Jariel-Encontre et al., 1995). If accumulation of c-Jun mediates SRF inhibition by MG132 treatment, then ectopic overexpression of c-Jun should mimic the effect of MG132. Figure 9B shows that transient overexpres-

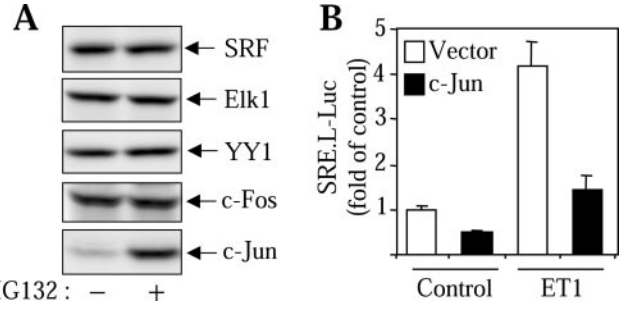

Fig. 9. Accumulation of c-Jun upon MG132 treatment and inhibition of SRF activity by c-Jun overexpression. A, serum-starved VSMCs were incubated with or without 3 μM MG132 for 2 h, lysed, and the cleared cell lysates were analyzed by Western blotting with antibodies against SRF, Elk1, YY1, c-Fos, and c-Jun as indicated. B, VSMCs were transfected with SRF-luciferase reporter construct (SRE.L-Luc), with CMV-driven LacZ plasmid, together with vector alone (open columns) or with c-Jun expression plasmid (closed columns). Cells were serum-starved for 24 h, followed by stimulation with 100 nM ET1 for 6 h. Luciferase activity in cell lysates was then measured, normalized to β-galactosidase activity, and expressed as -fold of control.

sion of c-Jun in VSMCs attenuates both basal and ET1-induced activity of SRF reporter. Thus, accumulation of c-Jun upon MG132 treatment may explain the regulation of SRF activity by proteasome inhibitors.

Discussion

Inhibition of the proteasome pathway commonly leads to cell death in transformed or malignant cell cultures. This is in contrast to normal cell phenotypes, which can be resistant to the proapoptotic effects of proteasome inhibitors, with certain cell types, such as neural tissue, responding with a differentiated phenotype. The potential therapeutic effect of proteasome inhibitors on malignancies has recently been demonstrated with the agent bortezomib, leading to the first approved use for treatment of multiple myeloma (Adams and Kauffman, 2004). Regarding the cardiovascular system, MG132 was recently shown to attenuate restenosis after balloon injury in vivo, presumably via inhibition of inflammatory response and possibly via apoptosis of proliferating VSMCs (Meiners et al., 2002). Our results indicate that, at least within 24 h in culture, MG132 does not stimulate apoptosis or reduce viability of quiescent VSMCs.

The mechanism by which proteasome inhibitors induce cell death of transformed cells may include 1) accumulation of misfolded proteins that are normally degraded by proteasome (Goldberg, 2003); 2) inhibition of survival mechanisms, such as NF-κB pathway (Adams, 2004); and 3) inhibition of cell cycle progression, for example, via stabilizing p27kip or other inhibitors of cyclins (Chen and Lin, 2004). This study demonstrates for the first time that proteasome inhibition results in attenuation of SRF activity in quiescent VSMCs, HeLa cells, and HEK293 cells. In VSMCs, SRF is critical for the expression of cytoskeletal proteins, such as SMA and others (Hautmann et al., 1997), which contribute to the VSMC hypertrophy in response to the vasoactive peptide angiotensin II (Turla et al., 1991). Thus, down-regulation of SRF activity by MG132 could represent additional mechanism by which proteasome inhibitors attenuate neointima formation in response to vascular injury (Meiners et al., 2002).

Our study indicates that MG132 inhibits SRF activity not via the down-regulation of NF-κB signaling, an established target of proteasome inhibitors. Thus, overexpression of IκB super-repressor (IκBαm) did not mimic the effect of MG132 on ET1-induced SRF activity, whereas it blocked TNF-α-induced NF-κB activation as was expected (Fig. 4). Moreover, overexpression of IκBαm increased both basal and ET1-induced SRF activity, an interesting observation that we cannot explain at this moment but which does not relate to the mechanism of SRF inhibition by MG132. Likewise, PKA, a powerful regulator of SRF signaling, as we showed recently (Davis et al., 2003a; Hogarth et al., 2004), is also not involved in the mechanism of SRF inhibition by MG132, because 1) MG132 did not stimulate PKA activity and 2) inhibition of PKA did not reverse the effect of MG132 (Fig. 5).

Our signaling studies suggest that MG132 inhibits ET1-induced SRF signaling at the level downstream of actin dynamics and at or upstream of SRF-DNA binding (Figs. 6 and 7). At this level, SRF activity could be regulated 1) by dynamic nuclear/cytoplasmic distribution or 2) by other proteins that may compete with SRF for CArG binding, or directly interact with SRF at the sites critical for SRF transcriptional activity. Our additional experiments suggest the following. First, regulation of SRF activity by MG132 cannot be explained by changes in SRF localization, because SRF localized predominantly to the nucleus, regardless of the presence or absence of MG132 for up to 6 h, as assessed by immunofluorescence (data not shown). Second, SRF-dependent gene transcription can be regulated via modulation of chromatin structure by histone deacetylases (HDACs) (Davis et al., 2003b). However, regulation of SRF by MG132 is unlikely to be mediated by recruitment (or stabilization) of HDAC, because inhibition of HDAC by trichostatin A did not reverse the effect of MG132, whereas it increased the basal SRF activity as expected (data not shown).

Having excluded these mechanisms for SRF regulation at the level of transcription, we hypothesized that inhibition of proteasome could lead to accumulation of ubiquitinated protein(s) that down-regulate SRF activity either by direct binding to SRF or by interfering with CArG-dependent transcription and that are normally degraded by proteasome. Our coimmunoprecipitation experiments show that although SRF itself is not ubiquitinated, it exists in a complex with at least three ubiquitinated proteins of unknown nature (Fig. 8A). This is an interesting new observation but does not explain the mechanism of regulation of SRF activity by proteasome inhibitors, because no significant changes in the amounts of those SRF-bound proteins were detected upon MG132 treatment. By contrast, a dramatic increase in the levels of c-Jun protein was observed after 2-h incubation of VSMCs with MG132 (Fig. 9A). This result is consistent with the established regulation of c-Jun levels by proteasome-mediated degradation (Acquaviva et al., 2002), and it may explain the potentiation of AP1 activity by proteasome inhibitors, observed by us (Fig. 2B) and others (Nakayama et al., 2001). Furthermore, we found that ectopic overexpression of c-Jun down-regulates SRF activity (Fig. 9B), which is consistent with the previous study that has reported the repression of α-SM-actin promoter activity by c-Jun overexpression (Bushel et al., 1995). Together, our data and the results of others suggest that the mechanism by which proteasome inhibitors attenuate α-SM-actin expression may involve accumulation of c-Jun, leading to a repression of SRF-dependent, CArG-mediated transcription of α-SM-actin. The mechanism by which c-Jun down-regulates the SRF-dependent transcription of SMA and other SM-specific genes is a focus of our future studies.

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