Endothelin-1 (ET-1) is a vasoconstrictor peptide known to be a potent mitogen for glomerular mesangial cells (GMC). In the current study, it is demonstrated that ET-1 treatment of GMC results in serine phosphorylation of the 66-kDa isoform of the adapter protein Shc (p66Shc). ET-1-induced serine phosphorylation of p66Shc requires activation of the mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK) signaling module and is efficiently inhibited by both a MAPK/ERK kinase (MEK)-selective inhibitor and adenovirus-mediated transfer of a dominant interfering MEK1 mutant. Furthermore, adenovirus-mediated transfer of a constitutively active MEK1 mutant was found to markedly increase p66Shc serine phosphorylation. Adenoviruses encoding constitutively active mutants of MAPK kinases 3 and 6 (upstream kinases of p38MAPK) and 7 (upstream kinase of c-Jun NH2-terminal kinase) failed to inhibit ET-1-induced phosphorylation of p66Shc resulting in association with the serine binding motif-containing protein SH3 domain of p110α. ET-1-induced phosphorylation of a serine encompassed in the 14-3-3 binding motif of p66Shc was confirmed in experiments employing anti-phospho-14-3-3 binding motif antibodies. These studies are the first to demonstrate that G protein-coupled receptors stimulate serine phosphorylation of p66Shc and the first to report the formation of a signaling complex between p66Shc and 14-3-3.

Endothelin-1 (ET-1) is the best characterized member of the endothelin family of vasoactive peptides. ET-1 binds to specific G protein-coupled receptors and evokes a wide variety of cellular responses via activation of tyrosine and serine-threonine protein kinases. ET-1-derived intracellular signals are also transduced by channel and transport proteins, as well as by various phospholipases, all of which cooperate in amplifying the biological properties of this peptide (1–3).

Phosphorylation of tyrosine, serine, and threonine residues in target molecules generates recognition motifs for a number of protein-protein interaction domains, thereby promoting the formation of multisubunit signaling complexes and constituting the basis of a complicated network of intracellular signaling pathways (4). Phosphorylation of tyrosine residues generates recognition motifs for structurally related Src homology 2 (SH2) and phosphotyrosine binding domains, whereas phosphorylation of serine residues produces recognition motifs for phosphoserine binding modules (5). Stable and finely regulated protein-protein interactions have been extensively investigated, and the fact that adaptor proteins and protein signaling modules play a central role in signal transduction has been widely established (5, 6).

The ubiquitously expressed adaptor protein Shc (7) exists in three isoforms with relative molecular masses of 46, 52, and 66 kDa (p46Shc, p52Shc, and p66Shc). p46Shc and p52Shc arise from the use of alternative translation initiation sites within the same transcript, whereas p66Shc contains an unique N-terminal region and is believed to be generated as a result of alternative splicing. Although all three Shc proteins are tyrosine-phosphorylated in response to many growth factors and all contain a recognition site for the SH2 domain of the adaptor protein Grb2, a number of studies have suggested distinct physiological roles for the three isoforms (8, 9). Different intracellular localization patterns have been reported for each (10, 11); moreover, it has been shown that p66Shc and p46Shc/p52Shc exert counter-regulatory effects on the c-fos promoter (8). In addition, p66Shc alone has been reported to act as a negative regulator of the epidermal growth factor (EGF)-stimulated ERK pathway (9). Remarkably, p66Shc has been shown to regulate both the oxidative stress response and life span in mammals. Migliaccio et al. (10) have demonstrated that p66Shc–/– mice exhibit enhanced cellular resistance to oxidative stress and a 30% increase in life span. Retrovirally mediated p46Shc/p52Shc overexpression failed to compensate for p66Shc deficiency, whereas, as expected, overexpression of p66Shc restored normal cellular responses to oxidative stress (10). Interestingly, the p66Shc-mediated response to oxidative stress was highly dependent on serine phosphorylation, as demonstrated by the observation that a non-serine-phosphorylated mutant form of p66Shc was unable to restore the normal stress response in p66Shc–/– cells (10). Recently, p66Shc has been hypothesized to be involved in phenoptosis (programmed death of the orga-
nism), which results from the reactive oxygen species-dependent apoptosis of a vital organ (12).

We have previously demonstrated the crucial role of p50Shc tyrosine phosphorylation in ET-1-mediated Ras activation in GMC (13). The persistent tyrosine phosphorylation of p50Shc enables and stabilizes the formation of a Shc-Grb2 complex, the subsequent association of which with the nonphosphorylated form of son-of-sevenless facilitates the biphasic GTP-loading of Ras (13). Significantly, p66Shc does not undergo tyrosine phosphorylation in response to ET-1 in GMC (13), providing additional evidence that p66Shc plays an isoform-specific role in this signaling network.

In the present study, evidence is presented that ET-1 induces MEK/ERK-dependent p66Shc serine phosphorylation and in this manner promotes the interaction of p66Shc with 14-3-3. The fundamental and unique role of the ERK pathway in affecting p66Shc serine phosphorylation was confirmed in studies employing MAPK-specific adenosival MEK/MKK constructs. Evidence is also provided that ET-1-induced serine phosphorylation of p66Shc generates a serine recognition motif that may be responsible for the direct association of p66Shc with 14-3-3.

MATERIALS AND METHODS

Reagents—purified human ET-1 was from Calbiochem-Novabiochem Corp. (La Jolla, CA). RPMI 1640 medium and routine tissue culture reagents were from Invitrogen (Carlsbad, CA). Fetal bovine serum was from Hyclone (Logan, UT). ECL reagent was from Amersham Pharmacia Biotech (Uppsala, Sweden). PD 98059 was kindly provided by Dr. S. Saltiel (Parke-Davis, Ann Arbor, MI). All other reagents were from Sigma.

Cell Culture and DNA Transfection—Primary GMC from Harlan Sprague-Dawley rats were isolated, characterized, and cultured as previously reported (13, 14). SV-40-transformed human mesangial cells (HMC) were kindly provided by J. D. Sraer (Hopital Tenon, Paris, France) and cultured as previously reported (15, 16). All experiments were performed with cells cultured in 100-mm dishes and made quiescent in serum-free medium for an additional 90 min incubation with 1.5 mCi/ml [32P]Pi (Amersham Pharmacia Biotech) at 37°C. Cells were stimulated with ET-1 (100 nM) for 15 min and then washed five times with ice-cold phosphate-buffered saline prior to lysis in 400 μl of lysis buffer containing 20 mM NaF and 1 μM mycophenolic acid. Cell lysates were immunoprecipitated with anti-Shc antibodies as described above, separated by means of SDS-PAGE and transferred to PVDF membrane. The PVDF membranes were exposed to an Rx film (Fuji Medical System, Tokyo, Japan), and the radioactive bands corresponding to p66Shc were excised and hydrolyzed in 6 N HCl at 110°C for 1 h. The hydrolysates were dried and resuspended in 6 μl of water and spotted onto TLC plates. The samples were then electrophoresed, together with phosphoamino acid standards, in pH 3.5 buffer at 1000 V for 90 min, and the plates were exposed to Rx film.

Methionine Labeling—Quiescent GMC were washed twice with methionine-free culture medium and incubated for an additional 6 h in serum-free medium containing 100 μCi/ml [35S]methionine. Cells were stimulated with ET-1 (100 nM) for 15 min, washed five times with ice-cold phosphate-buffered saline prior to lysis in 400 μl of lysis buffer containing 20 mM NaF. Cell lysates were immunoprecipitated with anti-p66Shc antibody, separated by means of SDS-PAGE, and transferred to PVDF membrane. The PVDF membrane was exposed to Rx film.

RESULTS

**ET-1 Induces Serine Phosphorylation of the Adaptor Protein p66Shc in GMC—p66Shc, in common with the other Shc isoforms, possesses three tyrosine residues that undergo phosphorylation in response to extracellular stimuli. However, whether phosphorylation of p46Shc, p52Shc, or p66Shc occurs depends upon the cell type and the applied stimuli. For example, EGF induces tyrosine phosphorylation of all three Shc isoforms in many cell types. By contrast, angiotensin II and platelet-derived growth factor have been shown to stimulate tyrosine phosphorylation of p66Shc in vascular smooth muscle cells, with minimal phosphorylation of p52Shc and p46Shc (21). Conversely, insulin predominantly promotes p52Shc, tyrosine phosphorylation in 3T3-L1 adipocytes and in Chinese hamster ovary (CHO) cells expressing the insulin receptor (22). As we have shown previously, ET-1 induces tyrosine phosphorylation of both p52Shc, and p46Shc in GMC (13). Mobility shifts detected by SDS-PAGE generally indicate modification(s) in the shifted molecule, and in many cases reflect a change in phosphorylation state. In the present study, ET-1 stimulation of quiescent GMC was found to result in the delayed mobility of p66Shc on SDS-PAGE, most evident between 2 and 15 min (Fig. 1). This was not due to tyrosine phosphorylation, because phosphotyrosine-specific antibodies failed to cross-react with p66Shc immunoprecipitated from ET-1-stimulated GMC lysates (not shown). It must be pointed out, however, that a negative result was immunoprecipitated with 2 μl of p42ERK antibody for 1 h at 4°C prior to the addition of protein A-Sepharose and incubation for an additional 1 h. Immunoprecipitates were washed twice in lysis buffer and twice in 20 mM Hepes, pH 7.4, 10 mM MgCl2, and 0.2 mM sodium orthovanadate and then incubated for 15 min at 30°C in 20 mM Hepes, pH 7.4, 10 mM MgCl2, and 0.2 mM sodium orthovanadate, containing 50 μM ATP, 5 μCi of [γ-32P]ATP and 0.25 mg/ml myelin basic protein. The reaction was terminated by the addition of Laemmli buffer, and the proteins were subjected to electrophoresis on a 12.5% SDS-polyacrylamide gel and visualized by autoradiography.

**Transduction with Recombinant Adenoviral Vectors—**Quiescent GMC were infected for 24 h at a multiplicity of infection (MOI) of 60, with the recombinant adenovaliral vectors expressing the constitutively active (Ad-MEK6α) and the catalytically inactive (Ad-MEK6) forms of MEK1, respectively (13). In experiments employing Ad-MK7 and Ad-MKK7E/Ad-MKK6E (adenoviruses expressing the constitutively active forms of MKK7, MKK3, and MKK6, respectively) quiescent GMC were infected for 36 h at a MOI of 100 and 50/50, respectively (18–20). Control cells were infected with an adenovirus encoding β-galactosidase (Ad-LaZ) at a MOI of 100, as described previously. Following infection, cell lysates were collected, and the resultant cell lysates were analyzed by Western analysis.

**Phosphoamino Acid Analysis—**Quiescent GMC were washed twice in phosphate-free medium and incubated for 90 min in serum-free, phosphate-free medium prior to an additional 90-min incubation with 1.5 mCi/ml [32P]Pi (Amersham Pharmacia Biotech) at 37°C. Cells were stimulated with ET-1 (100 nM) for 15 min and then washed five times with 14C-ice-cold phosphate-buffered saline prior to lysis in 400 μl of lysis buffer containing 20 mM NaF and 1 μM mycophenolic acid. Cell lysates were immunoprecipitated with anti-Shc antibodies as described above, separated by means of SDS-PAGE, and transferred to PVDF membrane. The PVDF membranes were blotted and exposed to Rx film.
following immunoblotting with phosphotyrosine-specific antibodies cannot completely exclude the presence of phosphorylated tyrosine residues on target proteins, because recognition of phosphorylated tyrosines by specific antibodies may be dependent on the amino acid sequence in which these residues are encompassed.

Previous papers have reported serine phosphorylation of p66Shc in response to hydrogen peroxide in mouse embryo fibroblasts (10), in response to EGF in CHO cells expressing the human EGF receptor (9), in response to phorbol ester and fibroblast growth factor 2 in NIH 3T3 cells (23), and in response to insulin stimulation in 3T3L1 adipocytes and CHO cells expressing the insulin receptor (22). It has been hypothesized that serine and tyrosine phosphorylation of p66Shc might result in opposing cell fates, because cytoprotective growth factor stimulation is associated with tyrosine phosphorylation, whereas environmental stresses elicit serine phosphorylation of this Shc isoform (10).

In the present study, orthophosphate labeling clearly demonstrated that ET-1-induced phosphorylation of p66Shc, p54Shc and p46Shc (Fig. 2A). A MEK-dependent activation of Shc may be crucial for GMC proliferation in renal diseases (24). ET-1 has been reported to activate the Raf-MEK-ERK cascade by two principal mechanisms, via protein kinases C-dependent activation of Raf (25) and in a p21ras-dependent manner (13). The time course of ET-1-induced p66Shc mobility shift in GMC was found to correlate with the kinetics of ERK1/2 activation (Fig. 1). ERK1/2 activation was assessed using three different methodologies, Western blotting of GMC lysates with anti-phospho-ERK-specific antibodies, assessing the delayed mobility shift of phosphorylated ERK on SDS-PAGE, and measuring the ability of immunoprecipitated ERK to phosphorylate myelin basic protein (see under “Materials and Methods”) (Fig. 1). The results obtained with all three methods were fully concordant, showing an increase in ERK activity slightly preceding or simultaneous with the observed gel mobility shift of p66Shc.

ERK1 and ERK2 are activated by dual specificity kinases MEK1 and MEK2. MEK1 and MEK2 are themselves activated by phosphorylation on Ser218 and Ser222, with further activation sensitive to the α-helix encompassing residues 32–51 (26, 27). Substitution of residues 218 and 222 with glutamic acid and aspartate, respectively, combined with the deletion of residues 32–51, generates a constitutively activated MEK1 mutant (26, 27). Conversely, a catalytically inactive form of this kinase is achieved by substitution of the ATP binding site Lys97 with Met (26). Catalytically inactive MEK1, by competing with endogenous MEK for Raf, suppresses MEK-dependent ERK

**Fig. 1.** Kinetics of ET-1-induced p66Shc phosphorylation correlate with kinetics of ERK activation. Quiescent GMC were stimulated with ET-1 (100 nM) for the times indicated. The delayed mobility of the band identified by anti-p66Shc antibodies following SDS-PAGE is indicative of p66Shc phosphorylation (Anti-Shc). ERK activity was assessed using anti-phospho-specific ERK antibodies (Anti-phospho-ERK), ERK mobility shift indicative of phosphorylation (Anti-ERK), and ERK immunocomplex assay (bottom panel). C, control. Shown is a representative result; the experiments were repeated at least three times.

**Fig. 2.** ET-1 induces the serine phosphorylation of p66Shc. Following incubation with [32P]inorganic phosphate as described under “Materials and Methods,” GMC were left untreated or incubated with ET-1 (100 nM) for 15 min at 37 °C. Cell lysates were immunoprecipitated with anti-Shc polyclonal antibodies. Immunoprecipitates were separated by SDS-PAGE and transferred to PVDF membrane, and Shc isoforms were visualized by autoradiography (A, top panel) and with anti-Shc antibody (A, bottom panel). The radiolabeled band corresponding to p66Shc was then excised, hydrolyzed, resolved by TLC, and subjected to autoradiography (B). Ori, origin; pY, phosphotyrosine; pT, phosphothreonine; pS, phosphoserine. Phosphorimager quantification is recorded ± S.D (C). C, control; IP, immunoprecipitation. Shown is a representative result; the experiment was repeated three times.
ET-1 recruits 14-3-3 to serine-phosphorylated p66<sup>Shc</sup>

Activation. Similarly, the MEK-selective inhibitor PD 98059 facilitates the pharmacological blockade of the ERK cascade both in vitro and in vivo (27).

In the current study, the complete ablation of both the p66<sup>Shc</sup> gel shift and ERK activation was observed when GMC were pretreated with PD 98059 prior to addition of ET-1 (Fig. 3A). Because PD 98059 has been reported to have other effects in addition to MEK inhibition, significantly the direct inhibition of prostaglandin endoperoxide synthases 1 and 2 activity (28), adenovirus-mediated transfer of constitutively active and dominant interfering MEK1 mutants was also employed to address the putative involvement of MEK in p66<sup>Shc</sup> phosphorylation (Fig. 3A). In both cases, infection of GMC was carried out at MOI of 60, which resulted in 100% of transduced cells (as assessed by parallel infection of cells with Ad-LacZ, not shown) (13). The effectiveness of the transduced MEK1 mutants to modulate ET-1-mediated ERK1/2 activation was confirmed both by Western blotting with phospho-specific anti-ERK antibodies and by detecting the delayed mobility shift of phosphorylated ERK on SDS-PAGE (Fig. 3A). Infection of GMC with Ad-MEK<sub>C1</sub> inhibited both ET-1-induced ERK activation and the p66<sup>Shc</sup> mobility shift. By contrast, Ad-MEK<sub>CA</sub> infection resulted in significant ERK activation and p66<sup>Shc</sup> mobility shift in quiescent GMC (Fig. 3A). Taken together these data strongly support the involvement of MEK1, and by inference ERK, in ET-1-induced p66<sup>Shc</sup> serine phosphorylation.

In addition to ERK, the two other principal mammalian MAPK pathways are the JNK and p38<sup>MAPK</sup> cascades (29). In the current study, we show that ET-1 activates ERK and p38<sup>MAPK</sup> pathways in mesangial cells. It has also been shown that ET-1 activates JNK in mesangial cell with peak at 15 min (30), although we could not detect this activation by means of anti-phospho-specific JNK antibody. In analogy to the activation of ERK1/2 by MEK1/2, MKK3 and MKK6 are upstream activators of p38<sup>MAPK</sup>, whereas MKK7 is a positive regulator of JNK (31). MKK3, MKK6, and MKK7, like MEK1/2, are dual specificity kinases, capable of phosphorylating both serine/threonine and tyrosines on p38<sup>MAPK</sup> and JNK, respectively.

Having established the relevance of the MEK-ERK module in ET-1-induced p66<sup>Shc</sup> serine phosphorylation, we next endeavored to determine whether activation of the JNK and/or p38<sup>MAPK</sup> pathways contributed to p66<sup>Shc</sup> serine phosphorylation. To answer this question, a number of approaches were employed; stimulation of cells with anisomycin, a potent activator of both the p38<sup>MAPK</sup> and JNK signaling cascades; preincubation of cells with the selective p38<sup>MAPK</sup> inhibitor SB 203580 (32); and adenovirus-mediated transfer of constitutively active mutants of MKK3 (Ad-MKK3<sub>BE</sub>), MKK6 (Ad-MKK6<sub>BE</sub>), and MKK7 (Ad-MKK7<sub>BE</sub>). As expected, stimulation of quiescent GMC with anisomycin resulted in JNK and p38<sup>MAPK</sup> activation, as determined by Western blotting with anti-phospho-specific antibodies (Fig. 3B). Anisomycin was also effective in inducing the delayed mobility shift of p66<sup>Shc</sup> (Fig. 3B). Interestingly, pretreatment of GMC with SB 203580 completely prevented both ET-1- and anisomycin-induced activation of p38<sup>MAPK</sup> without affecting the p66<sup>Shc</sup> mobility shift (Fig. 3B), suggesting that p38<sup>MAPK</sup> is not involved in serine phosphorylation of this adaptor protein.

To further define the role of JNK and p38<sup>MAPK</sup> in p66<sup>Shc</sup> phosphorylation and to rule out possible nonspecific actions of SB 203559, HMC were infected with adenoviral vectors encoding constitutively active mutants of MKK3, MKK6, and MKK7. Transduction of HMC was carried out at total MOI of 100, which resulted in 100% of cells being positively transduced (confirmed by parallel infection with Ad-LacZ, data not shown). As determined by Western blotting with anti-phospho-JNK or anti-phospho-specific antibodies (Fig. 3B). In analogy to the activation of p38<sup>MAPK</sup> without affecting the p66<sup>Shc</sup> mobility shift (Fig. 3B). By contrast, infection of GMC with Ad-MEK<sub>C1</sub> inhibited both ET-1-induced ERK activation and the p66<sup>Shc</sup> mobility shift. By contrast, Ad-MEK<sub>CA</sub> infection resulted in significant ERK activation and p66<sup>Shc</sup> mobility shift in quiescent GMC (Fig. 3A). Taken together these data strongly support the involvement of MEK1, and by inference ERK, in ET-1-induced p66<sup>Shc</sup> serine phosphorylation.

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ET-1 Recruits 14-3-3 to Serine-phosphorylated p66Shc

In order to identify candidate proteins that might associate with serine-phosphorylated p66Shc, we first immunoprecipitated from methionine-labeled quiescent and ET-1-treated GMC using isoform-specific anti-p66Shc antibodies, and the resultant immunoprecipitates were analyzed by SDS-PAGE (Fig. 5A). Western blot analysis with anti-p66Shc antibodies confirmed that the immunoprecipitates contained equivalent amounts of p66Shc (Fig. 5B). Three major [35S]methionine-labeled bands were co-immunoprecipitated with p66Shc from ET-1-stimulated cells; these were not present in the immunoprecipitates from quiescent cell lysates. These bands had apparent molecular masses of 21, 45, and >203 kDa (Fig. 5A). ET-1 has been described as causing intracellular translocation of caveolin (34), a 22-kDa transmembrane adaptor protein that forms a hairpin structure in the plasma membrane (35). A subset of β1 and αv integrins that are recruited by caveolin is linked to the ERK pathway through Shc (35). All three Shc isoforms have been reported to be co-precipitated with caveolin following antibody-dependent clustering of cell β1 integrins (35). Although the Shc/caveolin association has been reported to be independent of Shc tyrosine phosphorylation, it has been shown to require an interaction between the SH3 domain of the cytoplasmic tyrosine kinase Fyn and the Shc proline-rich sequence (35). In view of these studies, and because one of the three major proteins detected in methionine-labeled GMC was approximately the same size as caveolin, experiments were undertaken to determine whether ET-1 promoted an association between this protein and p66Shc. As shown in Fig. 6A, p66Shc was not found to be associated with caveolin in lysates of either control, ET-1-treated, or anisomycin-treated GMC. Co-precipitation of caveolin with p52Shc however, was detected. Interestingly, the strongest association was observed with an unidentified protein, recognized by anti-Shc antibodies, with an apparent molecular mass of between 52 and 66 kDa. Whether this band represents a novel Shc isoform expressed in GMC or an unrelated protein recognized by Shc antibodies requires further investigation. To rule out the possibility that co-precipitation of p66Shc with caveolin was not

**Fig. 4.** p38MAPK and JNK activation are not responsible for p66Shc phosphorylation. HMC were stimulated with anisomycin (100 nM) or infected with recombinant adenoviral constructs expressing the constitutively active forms of MKK3, MKK6, and MKK7 for 36 h at a MOI of 100 (see under “Materials and Methods”). Whole cell lysates were subjected to immunoprecipitated using anti-Shc antibodies (asterisk (top panel) indicates the phosphorylated protein form), anti-phospho-JNK, anti-phospho-ERK, or anti-phospho-p38MAPK antibodies as indicated. In MKK3/MKK6-infected HMC, anti-phospho-JNK antibody recognizes an unspecific band that was not detected blotting lysates of other infected cell types (18). Shown is a representative result; the experiment was repeated three times.

**Fig. 5.** ET-1 induces association of p66Shc with three proteins of molecular masses of 21, 45, and >203 kDa. GMC were incubated for 6 h with serum-free medium in the presence of 100 μCi/ml [35S]methionine and were then either left untreated or stimulated with ET-1 (100 nM) for 15 min at 37°C as described under “Materials and Methods.” Cell lysates were immunoprecipitated with anti-p66Shc polyclonal antibody. Immunoprecipitates were analyzed by SDS-PAGE, transferred to PVDF membrane, and visualized by autoradiography. Arrows on the left indicate the relative positions of the molecular weight markers (Bio-Rad). Arrows on the right indicate the three major [35S]methionine-labeled proteins that were co-immunoprecipitated with p66Shc (A). Equal amounts of p66Shc were immunoprecipitated from control and ET-1 treated GMC as detected by blotting with anti-p66Shc polyclonal antibodies (B). In ET-1 treated cells, p66Shc shift was not detected due to the higher acrylamide concentration of SDS-PAGE prepared for the detection of low molecular weight proteins. C, control; IP, immunoprecipitation. Shown is a representative result; the experiment was repeated three times.

and anti-phospho-p38MAPK antibodies, p38MAPK and JNK were strongly and selectively activated in HMC infected with Ad-MKK3bE/MKK6bE and Ad-MKK7D, respectively (Fig. 4). ERK activation was not observed in response to adenosinergic transfer of either Ad-MKK3bE/MKK6bE or Ad-MKK7D (Fig. 4). Significantly, neither JNK nor p38MAPK activation resulted in delayed mobility shift of p66Shc (Fig. 4). Analysis of HMC lysates treated with anisomycin revealed a significant activation of ERK1/2 (Fig. 4), suggesting that the observed effect of anisomycin treatment on p66Shc was mediated by the MEK-ERK pathway. Anisomycin-induced activation of ERK1/2 was also reproduced in GMC, and the complete ablation of both the p66Shc gel shift and ERK activation was observed when GMC were pretreated with PD 98059 prior to addition of anisomycin (Fig. 3C). Interestingly, although it is not a feature of all cell types, anisomycin-induced ERK activation was observed in both primary GMC and HMC (Figs. 3C and 4). Taken together, these data provide further evidence that the MEK-ERK signaling cascade specifically regulates p66Shc serine phosphorylation in mesangial cells.

p66Shc Serine Phosphorylation Controls Its Association with 14-3-3 Family Proteins—Protein phosphorylation is a universal regulatory mechanism engaged by cells to achieve a large variety of biological goals (6). Protein phosphorylation may effect protein conformational change and create binding modules integrally involved in protein-protein interactions (6).

Shc interacts with a number of phosphorylated proteins and tyrosine kinase receptors by means of SH2 and phosphotyrosine binding domains present in all three isoforms and three tyrosine residues within recognition sequences for SH2/phosphotyrosine binding-containing proteins (33). In attempting to determine the cellular significance of p66Shc serine phosphorylation, we were particularly intrigued by proteins that have been described previously as interacting with Shc in a phosphotyrosine-independent manner.

In order to identify candidate proteins that might associate with serine-phosphorylated p66Shc, p66Shc was first immunoprecipitated from methionine-labeled quiescent and ET-1-treated GMC using isoform-specific anti-p66Shc antibodies, and it was reproducibly precipitated with caveolin in lysates of either control, ET-1-treated, or anisomycin-treated GMC. Co-precipitation of caveolin with p52Shc however, was detected. Interestingly, the strongest association was observed with an unidentified protein, recognized by anti-Shc antibodies, with an apparent molecular mass of between 52 and 66 kDa. Whether this band represents a novel Shc isoform expressed in GMC or an unrelated protein recognized by Shc antibodies requires further investigation. To rule out the possibility that co-precipitation of p66Shc with caveolin was not...
of caveolin from either ET-1 or anisomycin-treated cells.

The members of the highly conserved phosphoserine binding 14-3-3 family of proteins share the ability to bind a multitude of functionally diverse signaling intermediates including kinases, phosphatases, and receptors. Thus, 14-3-3 family proteins have been implicated in key regulatory processes such as apoptotic cell death and cell cycle control (38). These proteins recognize phosphoserine residues encompassed in the recognition motif Arg-Ser-X-Ser(P)-X-Pro, as exemplified by the 14-3-3 binding motif of Raf-1 (39). Variation in the above motif can reduce binding affinity or render it impossible (40). In view of these data, a putative ET-1-induced association between p66Shc and 14-3-3 was next investigated.

In order to examine the possible association of serine-phosphorylated p66Shc with 14-3-3 family proteins, p66Shc immunoprecipitates were immunoblotted with anti-14-3-3 antibodies. As can be clearly seen (Fig. 8A), 14-3-3 associates with p66Shc in quiescent cells, and moreover, this association is strongly increased following ET-1 and anisomycin stimulation. Maximum association was observed 15 min following the addition of either ET-1 or anisomycin. These data were further confirmed by immunoblotting 14-3-3 immunoprecipitates with anti-Shc antibodies; from these studies, it is also apparent that both ET-1 and anisomycin promote the association of 14-3-3 with p52Shc (Fig. 8B).

To further confirm the association of p66Shc with 14-3-3, p66Shc immunoprecipitates were subjected to immunoanalysis using anti-phospho-14-3-3 binding motif antibodies. These antibodies recognize the phosphorylated serine recognition motif to which 14-3-3 family proteins bind. As can be seen (Fig. 8C), both ET-1 and anisomycin increase the presence of 14-3-3 binding sites in p66Shc, strongly suggesting that the association between the two proteins is a direct one. It must be pointed out, however, that the level of 14-3-3 binding motifs generated in p66Shc by ET-1 and anisomycin does not strictly correlate with the extent of association between 14-3-3 and p66Shc. Although anisomycin apparently induces a stronger association between 14-3-3 and p66Shc than ET-1 (Fig. 8, A and B), it appears to elicit a weaker induction of serine-phosphorylated 14-3-3 binding motif (Fig. 8C). One possible reason for this apparent discrepancy might be the fact that the amount of phosphorylated 14-3-3 binding motif in p66Shc is not the major determinant for protein-protein association. Speculation aside, the above experiments provide novel evidence that ET-1 induces the serine phosphorylation of p66Shc and thus promotes the association of this adapter molecule with 14-3-3 family proteins.
ET-1 Recruits 14-3-3 to Serine-phosphorylated p66Shc

DISCUSSION

The activation of signal transduction pathways is highly cell type- and ligand-specific (41). The existence of a large variety of protein-protein interaction modules results in an all-embracing repertoire of adaptor proteins responsible for specificity and diversity in signal transduction (42).

The adaptor protein Shc, one of the major tyrosine-phosphorylated proteins in growth factor-stimulated cells, has been demonstrated to link tyrosine kinase receptors to the activation of MAPK cascades (33). We and others have produced evidence that the recruitment of Shc is required for G protein-coupled receptor-dependent Ras-Raf-MEK-ERK pathway activation (13, 43). In the present paper, we demonstrate that p66Shc undergoes serine phosphorylation in response to ET-1 in GMC and subsequent association with the 14-3-3 scaffold protein. Serine phosphorylation of p66Shc in response to EGF in CHO cells expressing the human EGF receptor has been described to occur subsequent to p66Shc tyrosine phosphorylation and was prevented by pretreatment of the cells with the pharmacological MEK inhibitor PD 98059 (9). Our data clearly show that tyrosine phosphorylation of p66Shc is not an essential precondition for serine phosphorylation of this protein in mesangial cells. Indeed, it may be speculated that the isoform-specific serine phosphorylation of p66Shc could account for the specificity in signaling processes elicited by ET-1 (and possibly other G protein-coupled receptor agonists) in these cells.

Which serine-threonine kinase is responsible for p66Shc phosphorylation remains to be determined. It has been reported that overexpression of the dual specificity phosphatase MKP-1 in CHO cells overexpressing the insulin receptor was unable to prevent insulin-induced p66Shc serine phosphorylation (22). Dual specificity phosphatases are established negative regulators of MAPK pathways (44), and we have reported previously that ERK inactivation is associated with ET-1-dependent MKP-1 induction in GMC (13). If it is assumed that forced expression of MKP-1 prevents ERK1/2 activation, to date the sole substrates for MEK1/2 (45), these data would seem to suggest that, in contrast to the findings of the current study, the MEK-ERK cascade is not responsible for p66Shc phosphorylation. This apparent contradiction can to some degree be explained by the observation that both MKP-1 and ERK exhibit differing intracellular localization patterns. Dual specificity phosphatases are largely located in the nucleus (46, 47), the principal site of target protein dephosphorylation. Although activated ERK1/2 translocates to the nucleus (31), in agonist-stimulated cells, ERK phosphorylation takes place in the cytoplasm. Therefore, both nuclear and cytoplasmic proteins are undoubtedly phosphorylated by ERK following its ligand-dependent activation (13, 24, 29). Considering that p66Shc is predominantly localized to a cytosolic fraction associated with endosomes and endoplasmic reticulum, in all likelihood its phosphorylation takes place in the cytoplasm, implying that the localization of MKP-1 in the nucleus may prevent it from inhibiting ERK-mediated p66Shc phosphorylation. Analysis of ET-1-driven p66Shc phosphorylation in cells overexpressing MKP-3, a cytoplasmically localized dual specificity phosphatase known to be specific for ERK (44, 48) would almost certainly shed some light on these apparent contradictions. Moreover, the existence of alternative targets of MEK phosphorylation, although not as yet identified, cannot be completely ruled out.

Our data represent the first evidence of the recruitment of 14-3-3 in the context of Shc signaling. Among the most noteworthy signaling proteins that act as 14-3-3 ligands are Raf, BAD, p53, and p130Cas (38). Because 14-3-3 acts in a dimeric manner, it has the capability to simultaneously bind two dif-

FIG. 8. ET-1-induced serine phosphorylation of p66Shc is responsible for its association with 14-3-3 protein. A, lysates from quiescent HMC stimulated with anisomycin (100 nM) or ET-1 (100 nM) for 15 min were immunoprecipitated with anti-p66Shc-specific antibodies. The resultant immunoprecipitates were resolved by SDS-PAGE and then immunoblotted with anti-Shc (upper panel) or anti-14-3-3 antibodies (lower panel). B, lysates from quiescent HMC stimulated with anisomycin (100 nM) or ET-1 (100 nM) for 15 min were immunoprecipitated with anti-p66Shc-specific antibodies, separated by SDS-PAGE, and immunoblotted with anti-phospho-14-3-3 binding motif antibodies. C, control; A, anisomycin; control; A, anti-Shc-specific antibodies, separated by SDS-PAGE, and immunoblotted with anti-phospho-14-3-3 binding motif antibodies. Shown is a representative result; the experiment was repeated three times.
ferent protein ligands. The binding of 14-3-3 to target proteins has been reported to result in two possible outcomes: a conformational change (as for Raf), or, alternatively, the reciprocal modulation of protein activities (38, 49).

BAD proteins promote apoptosis by binding to, and inhibiting, the anti-apoptotic effects of Bcl-XL and Bcl-2. When BAD is in a cytosolic complex with 14-3-3, it is effectively sequestered from mitochondrion-localized Bcl-XL/Bcl-2 and therefore unable to induce apoptosis (50). ET-1 inhibits apoptosis of vascular smooth muscle cells induced by nitric oxide and serum deprivation via activation of the ERK pathway (51). In conclusion, the findings of the present study provide evidence that in mesangial cells, ET-1 promotes MEK/ERK-dependent p66Shc serine phosphorylation and the subsequent formation of a p66Shc/14-3-3 complex.

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REFERENCES


