Endothelin-1 Couples β Pix to p66Shc: Role of β Pix in Cell Proliferation through FOXO3a Phosphorylation and p27^{kip1} Down-Regulation Independently of Akt

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The phosphorylation of forkhead transcription factor FOXO3a by Akt is critical regulator of cell proliferation induced by serum. We show that endothelin-1 (ET-1) stimulation of primary human mesangial cells (HMCs) induces β Pix and p66Shc up-regulation, resulting in the formation of the β Pix/p66Shc complex. In transformed HMCs, ET-1 induces a biphasic phosphorylation of p66Shc and FOXO3a. The second phase leads to p27^{kip1} down-regulation independently of Akt. Depletion of β Pix blocks the second phase of p66Shc and FOXO3a phosphorylation and prevents p27^{kip1} down-regulation induced by ET-1. Depletion of either β Pix or p66Shc inhibits ET-1-induced cell proliferation. The expression of β_1 Pix induces FOXO3a phosphorylation through activation of Rac1, ERK1/2, and p66Shc. Using either p66Shc- or Akt-depleted cells; we show that β_1 Pix-induced FOXO3a phosphorylation requires p66Shc but not Akt. β_1 Pix-induced p27^{kip1} down-regulation was blocked by U0126 but not by wortmannin. Endogenous β Pix and FOXO3a are constitutively associated with endogenous p66Shc. FOXO3a and p66Shc binding requires β_1 Pix homodimerization. Expression of β_1 Pix homodimerization deficient mutant abrogates β_1 Pix-induced p27^{kip1} down-regulation and cell proliferation. Our results identify p66Shc and FOXO3a as novel partners of β_1 Pix and represent the first direct evidence of β_1 Pix in cell proliferation via Erk/p66Shc-dependent and Akt-independent mechanisms.

INTRODUCTION

β_1 Pix (Pak-interacting exchange factor) is a guanine nucleotide exchange factor (GEF) for Rac 1 and Cdc42 (Manser *et al.*, 1998; Daniels *et al.*, 1999). GEF proteins contain a pleckstrin homology (PH) domain and the catalytic Dbl homology (DH) domain. In addition to these domains, β_1 Pix contains a Src homology 3 (SH3) domain that binds with high affinity to a polyproline stretch in Pak1 (p21-activated kinase; Manser *et al.*, 1998). β_1 Pix also binds GIT1 through a GIT1-binding domain (Zhao *et al.*, 2000). The leucine zipper (LZ) domain at the C-terminus mediates β_1 Pix homodimerization (Kim *et al.*, 2001). There is mounting evidence showing that Pix functions as an integrator of signaling pathways controlling adhesion and cytoskeletal organization. Recently we have shown that endothelin-1 (ET-1) induces β_1 Pix translocation to focal adhesions through a PKA-dependent pathway (Chahdi *et al.*, 2005). Pix was found to form a complex with paxillin, Pak, and p95PKL, which regulates cytoskeletal remodeling (Turner *et al.*, 1999). Moreover, the Pak-Pix-GIT

complex has been shown to target and regulate focal adhesions (Zhao *et al.*, 2000). Recent study showed that the same complex plays a role in a different location where GIT1 functions as a scaffold protein to target Pix and Pak to the centrosome resulting in the activation of the centrosomal kinase Aurora-A (Zhao *et al.*, 2005).

The adaptor protein Shc exists in three isoforms with relative molecular masses (K_d) of 46, 52, and 66 (Pelicci *et al.*, 1992). They consist of a phosphotyrosine binding domain (PTB), a collagen homology domain (CH1) and a C-terminal Src homology 2 domain (SH2). In addition, p66Shc has a collagen homology domain 2 (CH2) at the N-terminus in which Ser36 is located. The serine 36 is phosphorylated in response to oxidative stress and appears to be critical for coupling Shc to stress response leading to apoptosis (Migliaccio *et al.*, 1997). p66Shc knockout mice had a 30% increase in average lifespan when compared with control animals (Migliaccio *et al.*, 1999). This increased longevity has been linked to enhanced resistance to oxidative stress. We have previously demonstrated the crucial role of p52Shc tyrosine phosphorylation in ET-1-mediated Ras activation in mesangial cells (Foschi *et al.*, 1997). This phosphorylation enables and stabilizes the formation of a Shc-Grb2 complex and its subsequent association with Ras exchange factor Sos (Son of sevenless) facilitates the biphasic activation of Ras (Foschi *et al.*, 1997). p46Shc has also been shown to bind Sos and Grb2 and mediate signals from receptor tyrosine kinase to the MAPK pathway (Pelicci *et al.*, 1992; Egan *et al.*, 1993). Although it can be tyrosine-phosphorylated (Bonfini *et al.*, 1992; Migliaccio *et al.*, 1997), serine 36 phosphorylation appears to be the main mechanism of p66Shc activation. Recent study established a link between p66Shc and FOXO3a where serine 36 phosphorylation has been shown to regulate

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Abbreviations used: AFX, All 1 Fused gene chromosome X; ET-1, endothelin-1; DH, Dbl homology; FOXO3a, forkhead box class O3a; GEF, guanine nucleotide exchange factor; GIT1, G protein-coupled receptor kinase (GRK)-interacting targets; LZ, leucine zipper; Pak, p21-activated kinase; β_1 Pix, Pak-interacting exchange factor; PH, pleckstrin homology; PKL, paxillin kinase linker; p27^{kip1}, cyclin-dependent kinase inhibitor; SH3, Src homology 3.

FOXO3a phosphorylation (Nemoto and Finkel, 2002). In addition, oxidative stress no longer stimulated phosphorylation of FOXO3a in p66Shc deficient cells.

FOXO3a is a member of a large family of forkhead transcription factors that also contains FOXO1 and FOXO4. In the nematode *Caenorhabditis elegans*, the gene encoding DAF-16 (FOXO3a in mammals) promotes cell survival in response to nutrient starvation (Lin *et al.*, 2001). Forkhead transcription factors are phosphorylated by Akt resulting in their export from the nucleus to the cytoplasm and, hence, a decrease in transcriptional activity of their target genes and enhanced cell survival and proliferation (Brunet *et al.*, 1999; Kops *et al.*, 1999). These forkhead factors inhibit cell cycle progression at the G1/S transition by controlling transcription of the cyclin-dependent kinase inhibitor p27^{kip1} (Brunet *et al.*, 2001; Nakamura *et al.*, 2000), although a p27^{kip1}-independent mechanism may exist (Medema *et al.*, 2000).

The elevated expression of both β_1 Pix and p66Shc in breast cancer cells (Jackson *et al.*, 2000; Bae *et al.*, 2005) suggests that both proteins may be involved in regulating cell proliferation, although p66Shc has been shown recently to inhibit T-cell proliferation (Pacini *et al.*, 2004). In addition to its vasoactive property, ET-1 also controls cell proliferation and genes expression (Sugimoto *et al.*, 2001). We found that ET-1 stimulation increases β Pix and p66Shc expression. The relationship between β Pix and p66Shc is not known. In this study we investigated the physical and functional coupling between β Pix and p66Shc and the role of β Pix in cell proliferation. Our data suggest the existence of novel signaling pathway initiated by ET-1-dependent formation of trimeric signaling complex β Pix/p66Shc/FOXO3a, resulting in previously unknown Akt-independent mechanism of FOXO3a inactivation which promotes cell proliferation.

MATERIALS AND METHODS

Cell Culture, Transfection, and Plasmids

Previously characterized SV40-transformed human mesangial cells (HMCs; Sraer *et al.*, 1996) and primary HMCs were cultured in RPMI 1640 supplemented with 10% fetal bovine serum, penicillin (100 U/ml), and streptomycin (100 μ g/ml) in a 37°C humidified incubator with 5% CO₂. Transient transfection of HMCs with mammalian expression vectors were performed using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions.

Rat β_1 Pix, β_1 PixDHm(L238R, L239S), β_1 PixSH3m(W43K), β_1 Pix Δ PH, β_1 Pix Δ ERD, β_1 Pix Δ (547-586), β_1 Pix Δ (587-626), β_1 Pix Δ (597-616), and β_1 Pix Δ (602-611) plasmids have been described (Chahdi *et al.*, 2005; Chahdi and Sorokin, 2008). β_1 Pix(SH3m, DHm), β_1 Pix Δ (605-608), and β_1 Pix Δ (603-608) were generated using QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA).

Mouse p66Shc cDNA cloned into pRK5-p66Shc (kindly provided by Dr. Ben Margolis, University of Michigan Medical School) was subcloned into FLAcpCMV vector (Stratagene). p66ShcS36A was generated using QuikChange site-directed mutagenesis kit. All the expression constructs were verified by DNA sequencing.

Small Interfering RNAs

pSUPER-p66Shc shRNA (5'-TGAGTCTCTGTCATCGCTG-3') was kindly provided by Dr. M. F. Lin (Department of Biochemistry and Molecular Biology, University of Nebraska Medical Center). p66Shc small interfering RNAs (siRNAs; 5'-CGATAGTCCCACTACCTG-3') was from Ambion (Austin, TX). Both target sequences of p66Shc siRNA were within the CH2 region.

Akt siRNA#1 (5'-TGCCCTTCTACAACCAGGA) was from Cell Signaling (Beverly, MA) and Akt siRNA#2 (5'-GGCTCCCTCAACAACCTC-3') was from Ambion. The human β Pix siRNA has been described previously (Park *et al.*, 2004).

Immunoprecipitation and Western Analysis

HMCs were transfected with the appropriate construct for 24 h. Cells were washed twice in phosphate-buffered saline (PBS) and lysed in lysis buffer containing 20 mM Tris, pH 7.5, 100 mM NaCl, 5 mM MgCl₂, 1 mM EDTA, 1% Triton X-100, 1 mM sodium fluoride, 1 mM sodium vanadate, 1 mM phenylmethylsulfonyl fluoride, 1 μ g/ml pepstatin, and 1 μ g/ml leupeptin. Equal

amount of proteins were separated by using 7.5% SDS-PAGE, electrophoretically transferred onto a PVDF membrane (Millipore, Bedford, MA), immunoblotted with the appropriate antibody, and visualized by enhanced chemiluminescence (ECL, Amersham Pharmacia Biosciences, Piscataway, NJ). For immunoprecipitation (IP), antibodies against p66Shc (Upstate Biotechnology, Lake Placid, NY), FLAG (M2, Sigma, St. Louis, MO), or c-Myc (9E10, Santa Cruz Biotechnology, Santa Cruz, CA) were added to the cell lysate (500 μ g) for a 2-h incubation, followed by addition of protein A or protein G agarose beads for an additional hour. The beads were washed three times in PBS. The immunoprecipitated proteins were released from the beads by boiling in 1× sample buffer for 5 min and subsequently were analyzed by Western blot. Total cell lysate (TCL) was run to assess the equal overexpression of the constructs. Expression of recombinant proteins was verified by immunoblotting with antibodies to the Myc or FLAG epitope tag.

Kinase inhibitors U0126 (10 μ M), a MAP/ERK kinase (MEK) inhibitor, or wortmannin (100 nM), a PI-3K inhibitor, were added 4 h after cell transfection. Equal amounts of cell lysate were subjected to Western blot analysis using antibodies against phospho-S36p66Shc (Calbiochem, La Jolla, CA); phospho-ERK1/2, ERK1/2, phospho-Akt(Ser473), Akt, phospho-FOXO3a(Thr32), and FOXO3a, (Cell Signaling); p27^{kip1} (Santa Cruz); β Pix (Chemicon, Temecula, CA); or actin (Sigma).

Cell Cycle Analysis

HMCs were seeded at a density of 12×10^4 /well in six-well plates in the regular medium for 2 d and then transfected with 2 μ g of empty vector, β_1 Pix, or β_1 Pix Δ (603–608) for 48 h. Cells were trypsinized, harvested, and washed twice in PBS containing EDTA (2 mM) and BSA (0.5%). Cells were treated with 70% ethanol at 4°C overnight and then washed with PBS. The DNA of ethanol-fixed cells was stained by propidium iodide (50 μ g/ml) containing DNase-free RNase A (100 U/ml) at 4°C for 30 min. The determination of cell cycle distribution for single cells was carried out using fluorescence-activated cell sorter (FACSCalibur, Becton-Dickinson) at the MCW Flow Cytometry Core Facility.

Cell Proliferation

HMCs were counted and seeded into 96-well culture plates (6×10^3 cells/well). After 24 h, the cells were transfected with 100 ng of empty vector, β_1 Pix, or β_1 Pix Δ (603–608) for 48 h. The cells were pulsed with 5 μ Ci/ml [³H]thymidine during the last 4 h of culture. Before harvesting, the cells were incubated (20 min at 37°C) with trypsin to induce detachment of the cells from the microtiter plates. The cells were washed and lysed with distilled water and collected on filters using an automatic cell harvester (Skatron, Sterling, VA). The filters were placed in ScintiSafe Econo 1 scintillation fluid (Fisher Scientific, Pittsburgh, PA) and counted with the use of a TRI-CARB 2100TR liquid scintillation counter (Packard, Meriden, CT).

Rac1 Activation Assay

Detection of GTP-Rac1 was performed by using a GST-Pak binding domain pulldown following the manufacturer's instructions (Upstate Biotechnology). Lysates from cells expressing Myc-tagged constructs β_1 Pix, β_1 PixSH3m(W43K), or β_1 PixDHm(L238R, L239S) were incubated with 10 μ g of the Pak-PBD agarose for 60 min at 4°C and washed three times with lysis buffer, and samples were separated by SDS-PAGE and immunoblotted with anti-Rac1 antibody (Upstate Biotechnology).

Statistical Analysis

All experiments were performed in triplicate and the p values were determined using the paired Student's t test.

RESULTS

Endothelin-1 Induces an Increase in β Pix and p66Shc Expression, Resulting in the Formation of β Pix/p66Shc Complex in Primary HMCs

We have previously shown that short exposure of mesangial cells to ET-1 stimulation induces p66Shc (Foschi *et al.*, 2001) and β_1 Pix activation (Chahdi *et al.*, 2005). In this study, we investigated the effect of ET-1 stimulation at different time points on p66Shc and β Pix expression in primary HMCs. As shown in Figure 1A, the level of β Pix and p66Shc increased 12 h after ET-1 stimulation and further increased at 24 h. The effect of ET-1 stimulation was specific to p66Shc because it did not affect the levels of p46Shc or p52Shc.

Shc interacts with a number of phosphorylated proteins and tyrosine kinase receptors by means of SH2 and phosphotyrosine-binding domains (Gu *et al.*, 2000). To examine if

Figure 1. Endothelin-1 induces β_1 Pix and p66Shc up-regulation in primary human mesangial cells. (A) Cells were serum-starved for 24 h before endothelin-1 (100 nM) was added for the indicated times. Equal amounts of proteins were probed by immunoblotting with anti- β_1 Pix, anti-SHC, or anti-actin antibodies. (B) Cells were serum-starved for 24 h before ET-1 (100 nM) was added for 24 h. Cell lysates were immunoprecipitated with anti-p66Shc antibody and the membranes were probed with the indicated antibodies.

p66Shc associates with β_1 Pix, p66Shc was first immunoprecipitated from quiescent and ET-1-treated primary HMCs using isoform-specific anti-p66Shc antibodies. Western blot analysis with anti-phospho-p66ShcSer36 showed that ET-1 induces not only an increase in p66Shc expression but also p66Shc activation after 24-h stimulation (Figure 1B). Importantly, β_1 Pix was present in this anti-p66Shc immune complex. These data taken together demonstrate that ET-1 stimulation induces the formation of β_1 Pix/p66Shc complex in vivo.

p66Shc Binding Requires β_1 Pix Homodimerization

Even though β_1 Pix and p66Shc are expressed in transformed HMCs, ET-1 was able to slightly increase their expression (unpublished data). The Rac1/Cdc42 guanine nucleotide exchange factor β_1 Pix contains central DH and PH domains and an SH3 domain located at the N-terminus. In addition, β_1 Pix contains a glutamic acid-rich domain (ERD, residues 511-634) within the carboxyl-terminal region. Using coimmunoprecipitation experiments in transformed HMCs, we found that endogenous β_1 Pix is associated with endogenous

p66Shc (Figure 2A). This result confirms the interaction found between these proteins in primary HMCs (Figure 1B). β_1 Pix was absent when a control IgG was used instead of anti-p66Shc antibody. To characterize the p66Shc binding site in β_1 Pix, a series of truncated β_1 Pix constructs were created. We first confirmed that Myc- β_1 Pix was readily detected in endogenous p66Shc immunoprecipitates, but not with IgG control (unpublished data). Coimmunoprecipitation studies showed that both β_1 Pix Δ DH and β_1 Pix Δ PH bound p66Shc, whereas β_1 Pix Δ ERD did not (Figure 2B), indicating that p66Shc binding site lies within the ERD domain of β_1 Pix. To further delineate the p66Shc binding site, we prepared different truncated β_1 Pix ERD domains, and found that deletion of amino acid 547-586 retained the ability to interact with p66Shc. No binding was observed between β_1 Pix Δ (587-626) or β_1 Pix Δ (597-616) and p66Shc (unpublished data). To identify the minimal p66Shc interaction sequence on β_1 Pix, we generated additional deletions within amino acids 597-616 of β_1 Pix. Our result showed that deletion of amino acids 605-608 significantly diminished the binding of p66Shc to β_1 Pix, whereas deletion of amino acids 603-608 or 602-611 completely abolished the ability of p66Shc to interact with β_1 Pix (Figure 2C). On the basis of the fact that the amino acid sequence 603-608 is located within the LZ domain, which mediates β_1 Pix homodimerization (Kim *et al.*, 2001, Chahdi and Sorokin, 2008), we tested the hypothesis that the deletion of this amino acid sequence would also prevent β_1 Pix homodimerization. To answer this question we transfected HMC cells with Flag- β_1 Pix, Myc- β_1 Pix, or both. We found that Myc- β_1 Pix coimmunoprecipitated with Flag- β_1 Pix only when both epitope-tagged forms of β_1 Pix were coexpressed (Figure 2D, lane 4), confirming that β_1 Pix forms dimers (Kim *et al.*, 2001, Chahdi and Sorokin, 2008). However, expression of Flag- β_1 Pix and Myc- β_1 Pix Δ (603-608) failed to form a dimer (Figure 2D, compare lanes 4 and 5). This result indicates that p66Shc binds only to the dimeric form of β_1 Pix and that deletion of these residues, 603-608, is sufficient to abolish β_1 Pix homodimerization and p66Shc binding.

Figure 2. p66Shc binding requires in β_1 Pix homodimerization. (A) Endogenous p66Shc was immunoprecipitated with anti-p66Shc antibody, and associated endogenous β_1 Pix was detected by anti- β_1 Pix antibody. (B) Myc-tagged β_1 Pix or its mutants β_1 Pix Δ DH, β_1 Pix Δ PH, or β_1 Pix Δ ERD were expressed in HMCs, and anti-p66Shc antibody was used for immunoprecipitation. (C) Cell lysates expressing Myc-tagged β_1 Pix, β_1 Pix Δ (605-608), β_1 Pix Δ (603-608), or β_1 Pix Δ (602-611) were immunoprecipitated with p66Shc antibody followed by immunoblotting as above. (D) Cells were transfected with Myc- β_1 Pix, Flag- β_1 Pix, combination of both, or Flag- β_1 Pix and Myc- β_1 Pix Δ (603-608), and then cell lysates were immunoprecipitated with anti-Flag antibody followed by immunoblotting as indicated. These results are representative of four independent experiments.

Figure 3. Kinetics of ET-1 stimulation on ERK1/2, p66Shc, FOXO3a phosphorylation, and p27^{kip1} down-regulation. HMCs were serum-starved for 48 h and then stimulated with ET-1 (100 nM) for 0, 15 min, 4 h, or 24 h. Cells lysates were probed by immunoblotting using specific antibodies as indicated. These results are representative of three independent experiments.

Kinetics of ERK1/2, p66Shc, Akt, and FOXO3a Phosphorylation, and p27^{kip1} Expression in ET-1-treated HMCs

We have previously shown that short exposure of rat mesangial cells to ET-1 stimulation induces ERK1/2 and p66Shc activation (Foschi *et al.*, 2001). In this study, we investigated the effect of ET-1 stimulation at different time points on HMCs. Our results showed that ET-1 stimulation induced ERK1/2 activation at 15 min and stayed active up to 24 h as indicated by using a phospho-ERK1/2 specific antibody (Figure 3, top). Interestingly, ET-1 stimulation resulted in biphasic activation of p66Shc and FOXO3a phosphorylation. The initial increase in phosphorylation at 15 min is followed by a decrease in baselines at 4 h and subsequently by a second increase in p66Shc and FOXO3a phosphorylation. This is in contrast with the time course of Akt activation. Indeed, ET-1 induced Akt activation at 15 min and 4 h, followed by a decrease in baselines at 24 h (Figure 3). FOXO3a transcription factor, which is one of Akt substrate, was phosphorylated at 24 h, whereas Akt was completely inactive, suggesting that the second phase of FOXO3a phosphorylation induced by ET-1 is independent of Akt.

We next examined the effect of ET-1 stimulation on p27^{kip1} protein expression. p27^{kip1} protein is a cyclin-dependent kinase inhibitor and a target gene for FOXO3a transcription factor (Dijkers *et al.*, 2000). Total cell lysates were prepared from cells stimulated with ET-1 (100 nM) for the times indicated. Immunoblot analysis showed that the expression level of p27^{kip1} was elevated in quiescent HMCs and in cells treated with ET-1 for 15 min and 4 h. However, the p27^{kip1} protein level significantly decreased in response to ET-1 at 24 h (Figure 3, bottom). Altogether our results suggest that the second phase of ET-1-induced p66Shc phosphorylation, FOXO3a inactivation, and p27^{kip1} down-regulation takes place independently of Akt.

β Pix Is Essential for the Second Phase of ET-1-induced p66Shc and FOXO3a Phosphorylation and p27^{kip1} Down-Regulation

We have shown that ET-1 increased p66Shc and β Pix expression, which resulted in the formation of β Pix/p66Shc complex. To examine the role of β Pix in ET-1 signaling as shown above, we used β Pix siRNA to knockdown endogenous β Pix expression. β Pix siRNA strongly suppressed β Pix expression in HMCs (Figure 4A, bottom). β Pix siRNA had no effect on ET-1-induced ERK1/2 activation, suggesting that ERK1/2 can be activated independently of β Pix. However, depletion of β Pix prevented the second phase of p66Shc and FOXO3a phosphorylation induced by ET-1 compared with cells treated with negative control siRNA (Figure 4A). β Pix depletion also strongly blocked ET-1-induced p27^{kip1} down-regulation at 24 h.

p27^{kip1} is a cyclin-dependent kinase inhibitor that control cell cycle progression at the G1/S transition preceding cell proliferation. The observation that β Pix depletion blocked p27^{kip1} down-regulation in response to ET-1, prompted us to examine the role of β Pix and p66Shc in cell proliferation. Stimulation of quiescent HMCs with ET-1 induced a strong increase in [³H]thymidine incorporation (Figure 4B). However, siRNA depletion of either β Pix or p66Shc completely inhibited ET-1-induced cell proliferation compared with cells treated with negative control siRNA, which had no effect. This result suggests that β Pix/p66Shc complex plays an essential role in ET-1-induced cell proliferation.

β_1 Pix-induced p66Shc Phosphorylation on Serine 36 Is ERK1/2-dependent, but Akt-independent

We have shown that β Pix is essential for the second phase of phosphorylation of p66Shc and FOXO3a and for p27^{kip1} down-regulation induced by ET-1 by using β Pix siRNA. We have also found that ET-1 induces an increase in β Pix expression. Taken together these results support our hypothesis that the increase in β Pix expression is responsible for the activation of the second phase in ET-1 signaling. To confirm this hypothesis the effect of β_1 Pix overexpression in HMCs was investigated. Using phospho-specific antibody against phosphorylated serine 36, we showed that β_1 Pix induced p66Shc phosphorylation, an effect that was inhibited by the MEK inhibitor U0126 but not by phosphoinositide 3-OH kinase (PI-3K) inhibitor wortmannin (Figure 5A, top). This result confirms that the second phase of p66Shc activation by ET-1 (Figure 3) or β_1 Pix-induced p66Shc activation does not involve Akt. We next assessed the effect of β_1 Pix on ERK1/2 activation using phospho-specific ERK1/2 antibody. In cells expressing β_1 Pix, ERK1/2 was strongly activated compared with cells transfected with empty vector (Figure 5A, bottom). Using U0126 to inhibit MEK, a kinase that directly phosphorylates and activates ERK1/2, we found that U0126 completely abolished β_1 Pix-mediated ERK1/2 activation, whereas PI-3K inhibitor wortmannin had no effect. Previous reports have demonstrated the role of Pak in Rac1-mediated activation of ERK1/2 (Renshaw *et al.*, 1997; Del Pozo *et al.*, 2000). Because it has been shown that activation of Rac1 can mediate ERK1/2 activation through Pak1-MEK1 (Frost *et al.*, 1997; Eblen *et al.*, 2002), we examined the ability of β_1 Pix to regulate Rac1 activity in HMCs. In agreement with previous reports (Park *et al.*, 2004), cells expressing β_1 Pix demonstrated a marked increase in GTP-Rac1 compared with cells transfected with empty vector (Figure 5B). However, the expression of β_1 PixSH3m(W43K) or β_1 PixDhM(L238R, L239S) failed to activate Rac1. Thus the GEF activity of β_1 Pix mediated by the DH domain and the ability of β_1 Pix to bind

Figure 4. Effect of β_1 Pix depletion on ET-1 signaling. (A) Cells were transfected with β_1 Pix siRNA (50 nM) for 24 h in complete RPMI and serum-starved for an additional 24 h. Cells were then stimulated with ET-1 (100 nM) for the indicated times, and cell lysates were immunoblotted with different antibodies as indicated. (B) Cells were transfected with 5 nM of β_1 Pix siRNA, p66Shc siRNA, or negative control siRNA for 24 h and serum-starved for an additional 24 h before addition of ET-1 (100 nM) for 24 h. [³H]thymidine incorporation was performed as described in *Materials and Methods*. Values represent the mean $\pm$ SEM from four independent experiments performed in triplicates. Symbols above a column indicate a statistical comparison between the indicated sample and nonstimulated cells (NS). * $p < 0.05$.

Pak1 through its SH3 domain are essential for β_1 Pix-induced Rac1 activation. Next, we want to show that β_1 Pix-mediated Rac1 activation is necessary for ERK1/2 activation. β_1 Pix

strongly increased ERK1/2 phosphorylation, whereas β_1 PixSH3m(W43K) and β_1 PixDHm(L238R, L239S) had only minor stimulatory effect on ERK1/2 phosphorylation (Fig-

Figure 5. β_1 Pix-induced p66Shc phosphorylation on serine 36 is mediated by Rac1 and ERK1/2 activation. (A) HMCs were transiently transfected with empty vector or Myc-tagged β_1 Pix. Four hours after transfection cells were treated with vehicle, U0126 (10 μ M), or wortmannin (100 nM) for 20 h. Endogenous p66Shc was immunoprecipitated from cell lysates followed by immunoblotting with anti-phospho-S36p66Shc or anti-p66Shc antibodies. Portions of the cell lysates were immunoblotted with anti-phospho-ERK1/2 or anti-ERK1/2 antibodies. (B) Cell lysates overexpressing β_1 Pix or β_1 Pix mutants were subjected to GST-Pak pulldown. Rac1-GTP bound to GST-Pak (top) and Rac1 in total lysates (middle) were detected by immunoblotting with anti-Rac1. The quantitative analysis of Rac1-GTP (active) was obtained by densitometry (bottom panel). (C) Lysates from cells expressing β_1 Pix, β_1 PixSH3m, β_1 PixDHm, or β_1 Pix(SH3m, DHm) were analyzed using anti-phospho-Erk1/2 or anti-Erk1/2 antibodies. (D) Myc-tagged β_1 Pix was expressed alone or with Flag-tagged p66Shc or Flag-tagged p66ShcS36A. Cell lysates were immunoprecipitated with Flag antibody followed by Western blotting analysis as indicated. Anti-Flag and anti-myc antibodies show the expression levels of Flag-tagged p66Shc and Myc-tagged β_1 Pix, respectively (bottom). Results are representative of at least three independent experiments.

ure 5C). In cells expressing β_1 Pix double mutant, β_1 Pix(SH3m, DHm), ERK1/2 phosphorylation was significantly decreased, indicating that both SH3 and DH domains are required for β_1 Pix-mediated ERK1/2 activation. Taken together, our results indicate that p66Shc phosphorylation on serine 36 is mediated by β_1 Pix-induced Rac1 and ERK1/2 activation. To confirm our finding, wild-type Flag-tagged p66Shc and p66ShcS36A (in which serine 36 has been mutated to alanine) were transiently cotransfected with Myc- β_1 Pix into HMCs. Anti-Flag immunoprecipitates demonstrate that expression of β_1 Pix results in p66Shc phosphorylation on serine 36. As expected, β_1 Pix was unable to induce phosphorylation of p66ShcS36A (Figure 5D). Cumulatively, these results demonstrate that β_1 Pix stimulates ERK1/2-dependent phosphorylation of p66Shc on serine 36. It must be pointed out, however, that we cannot completely exclude the presence of other phosphorylated residues on p66Shc.

β_1 Pix-induced FOXO3a Phosphorylation Requires p66Shc But Not Akt

FOXO3a protein, like other forkhead-related transcription factors, is phosphorylated (inhibited) by activated Akt and then exported from the nucleus to the cytoplasm, resulting in inhibition of transcription of its target genes (Brunet *et al.*, 1999; Dijkers *et al.*, 2000). A recent study provided compelling evidence that p66Shc serine 36 phosphorylation plays a major role in oxidative stress-induced FOXO3a inhibition (Nemoto and Finkel, 2002). Because we showed that β_1 Pix physically interacts with p66Shc and induces its phosphorylation on serine 36 through ERK1/2 activation, we investigated whether Akt and/or FOXO3a respond to β_1 Pix over-

Figure 6. β_1 Pix induces p27^{kip1} down-regulation through ERK/p66Shc/FOXO3a pathway. (A) Cells transfected with β_1 Pix were either left untreated or treated with U0126 (10 μ M) or wortmannin (100 nM) 4 h after transfection. Cell lysates were then analyzed by Western blotting as indicated using phospho-Akt, phospho-FOXO3a, or p27^{kip1} specific antibodies. FOXO3a phosphorylation was quantified by densitometry (bottom panel). (B) Nontransfected cells were serum-starved overnight before stimulation with 10% serum in the presence or absence of wortmannin (100 nM). Cell lysates were analyzed by Western blotting as indicated above. Similar results were obtained from three independent experiments.

Figure 7. Serum-induced FOXO3a phosphorylation is Akt-dependent and p66Shc-independent. Cells were transfected with 100 nM of each Akt siRNA (A) or 100 nM of p66Shc siRNA or pSUPERp66Shc shRNA (8 μ g; B) for 24 h. The cells were serum-starved for an additional 24 h before stimulation with 10% of serum for 20 min. The cell lysates were blotted as indicated. These results are representative of three independent experiments.

expression in HMCs. Akt phosphorylation status was not affected by β_1 Pix or by U0126 treatment. However, wortmannin treatment resulted in a complete inhibition of Akt phosphorylation induced by the serum present in the medium (Figure 6A, top). β_1 Pix induced strong FOXO3a phosphorylation compared with the cells transfected with empty vector (Figure 6A). This phosphorylation was significantly inhibited by U0126, whereas wortmannin had a less inhibitory effect. The sensitivity to wortmannin inhibition is due to the Akt-dependent FOXO3a phosphorylation induced by the presence of serum. This finding implies that activation of β_1 Pix signaling induces FOXO3a phosphorylation through ERK1/2-dependent but Akt-independent mechanism(s). Furthermore, β_1 Pix expressing cells showed a significant down-regulation of p27^{kip1} protein, a cyclin-dependent kinase inhibitor and a target gene for FOXO3a (Dijkers *et al.*, 2000; Stahl *et al.*, 2002). This down-regulation was blocked by U0126 but not by wortmannin (Figure 6A, bottom). In nontransfected cells, however, both Akt and FOXO3a phosphorylation induced by stimulation with serum were completely inhibited by wortmannin (Figure 6B, top). Moreover, wortmannin completely blocked serum-induced p27^{kip1}

Figure 8. β_1 Pix-induced FOXO3a phosphorylation is p66Shc-dependent and Akt-independent. Cells were transfected with 100 nM of each Akt siRNA (A) or 100 nM of p66Shc siRNA or pSUPERp66Shc shRNA (8 μ g) (B) for 24 h before transfection with empty vector or β_1 Pix for another 24 h. The cell lysates were blotted as indicated. These results are representative of three independent experiments.

Figure 9. FOXO3a binding, like p66Shc, requires β_1 Pix homodimerization. (A) Lysates from cells expressing β_1 Pix or β_1 Pix Δ (603-608) were immunoprecipitated with anti-myc antibody followed by Western blotting with anti-phospho-FOXO3a or anti-FOXO3a. (B) Lysates from nontransfected cells were immunoprecipitated with anti-p66Shc or IgG control followed by immunoblotting with anti-phospho-FOXO3a or anti-FOXO3a.

Figure 10. β_1 Pix/p66Shc/FOXO3a is required for p27^{kip1} down-regulation and cell proliferation. (A) Lysates from cells expressing β_1 Pix or β_1 Pix Δ (603-608) were analyzed by Western blot using anti-p27^{kip1}. (B) Cells were transfected with empty vector, β_1 Pix, or β_1 Pix Δ (603-608) for 48 h. [³H]thymidine incorporation was performed as described in *Materials and Methods*. Values represent the mean $\pm$ SEM from four independent experiments performed in triplicates. Symbols above a column indicate a statistical comparison between the indicated sample and cells transfected with β_1 Pix. **p* < 0.05. (C) Cells expressing β_1 Pix or β_1 Pix Δ (603-608) were trypsinized, fixed in 70% ethanol, and stained with propidium iodide. Cells were analyzed with a flow cytometer for the distribution of cell cycle. The experiment shown is representative of four independent experiments performed in triplicates.

down-regulation (Figure 6B, bottom). Altogether, our results show that in HMCs β_1 Pix, unlike serum, induced FOXO3a phosphorylation and p27^{kip1} down-regulation through ERK/p66Shc-dependent but Akt-independent mechanisms. To confirm this finding, we examined the effect of Akt knockdown by RNA interference on FOXO3a phosphorylation induced by serum or β_1 Pix. We first confirmed that both Akt siRNAs specifically knocked down the expression of Akt (Figures 7A and 8A). Our results showed that Akt depletion by two different siRNAs strongly inhibited FOXO3a phosphorylation induced by serum (Figure 7A) but not by β_1 Pix (Figure 8A).

We showed that p66Shc physically interacts with β_1 Pix and the expression of β_1 Pix resulted in p66Shc activation. We therefore sought to investigate the role of p66Shc in serum- and β_1 Pix-induced FOXO3a phosphorylation by using p66Shc siRNA. To knock down p66Shc, we used siRNAs against two different targets of p66Shc. HMCs were transiently transfected with pSUPER-p66Shc shRNA vector (Veeramani *et al.*, 2005) or with p66Shc siRNA. The cells transfected with each of p66Shc siRNAs exhibited a significant reduction in the abundance of endogenous p66Shc protein level but showed no change in the level of p46 or p52 isoforms of Shc (Figures 7B and 8B). Both p66Shc siRNAs strongly inhibited FOXO3a phosphorylation induced by β_1 Pix (Figure 8B) but had no effect on serum stimulation (Figure 7B), indicating that p66Shc is required for β_1 Pix-induced FOXO3a phosphorylation, but p66Shc is not involved in mediating serum signaling.

β_1 Pix/p66Shc/FOXO3a Complex Is Required for p27^{kip1} Down-Regulation and Cell Proliferation

The fact that endogenous β Pix interacts with endogenous p66Shc and β_1 Pix overexpression induces FOXO3a phosphorylation raised the question of whether FOXO3a interacts with β_1 Pix. To assess the interaction between β_1 Pix and endogenous FOXO3a, lysates were obtained from cells transfected with Myc-tagged β_1 Pix or β_1 Pix homodimerization deficient mutant that cannot interact with p66Shc, β_1 Pix Δ (603–608), and were immunoprecipitated with anti-myc antibody. Endogenous FOXO3a was readily detected in the immunoprecipitate, whereas deletion of amino acids 603–608 abolished the interaction between FOXO3a and β_1 Pix (Figure 9A). This result indicates that like p66Shc, FOXO3a binding requires β_1 Pix homodimerization. Anti-phospho-FOXO3a showed that FOXO3a associated with β_1 Pix is already phosphorylated, which may indicate a phosphorylation-dependent binding of endogenous FOXO3a to β_1 Pix. Furthermore, we found that endogenous FOXO3a coimmunoprecipitates with endogenous p66Shc (Figure 9B), indicating that endogenous p66Shc forms a complex with both endogenous β Pix (Figure 2A) and FOXO3a.

We next examined the functional relevance of the β_1 Pix/p66Shc/FOXO3a complex on downstream signaling. It is well established that FOXO3a controls p27^{kip1} protein level (Dijkers *et al.*, 2000). β_1 Pix overexpression induced p27^{kip1} down-regulation, whereas the expression of β_1 Pix Δ (603–608) unable to form β_1 Pix/p66Shc/FOXO3a complex blocked p27^{kip1} down-regulation (Figure 10A). p27^{kip1} is a well-known regulator of the G1/S transition through its cyclin-dependent kinase inhibitory activity, which blocks the cell in G1 phase by preventing cdk-dependent phosphorylation of pRb (Dijkers *et al.*, 2000). The expression of β_1 Pix induced a strong increase in cell proliferation compared with cells transfected with empty vector as evidenced by increase in the incorporation of [³H]thymidine (Figure 10B). This in-

crease in cell proliferation correlates with a significant increase of the percentage of cell population in the S phase of the cell cycle resulting from a decrease in the number of cells in G1 phase (Figure 10C). The expression of β_1 Pix routinely elicited a more than 60% increase in the cell number in S phase. The expression of β_1 Pix Δ (603–608) that blocks p27^{kip1} down-regulation also abolished the increase in cell proliferation (Figure 10B) and reduced cell percentage in S phase of the cell cycle induced by β_1 Pix (Figure 10C), confirming that β_1 Pix plays a critical role in regulating FOXO3a transcriptional activity and cell proliferation. These findings confirm the results obtained showing that β_1 Pix is essential in cell proliferation induced by ET-1. Because β_1 Pix interacts with and activates p66Shc, we sought to determine the role of p66Shc in β_1 Pix signaling by examining the effect of p66Shc depletion on cell proliferation induced by β_1 Pix. Using two different siRNAs to knockdown p66Shc, we found that the depletion of p66Shc completely abolished β_1 Pix-induced cell proliferation (Figure 11). This functional link between β_1 Pix and p66Shc also confirms the physical interaction between these two proteins and their role in cell proliferation.

DISCUSSION

Our data represent the first demonstration of ET-1-induced β Pix and p66Shc up-regulation leading to the formation of β Pix/p66Shc complex *in vivo*. Further, we provide evidence that ET-1 stimulation results in a biphasic phosphorylation of p66Shc and FOXO3a in which the secondary response induces cell proliferation through p27^{kip1} down-regulation. The binding of both p66Shc and FOXO3a to β_1 Pix requires

Figure 11. p66Shc is necessary for β_1 Pix-induced cell proliferation. Cells were transfected with p66Shc siRNA, pSUPERp66Shc shRNA, or control siRNA for 24 h before transfection with empty vector or β_1 Pix for another 24 h. [³H]thymidine incorporation was performed as described in *Materials and Methods*. Values represent the mean $\pm$ SEM from three independent experiments performed in triplicate. Symbols above a column indicate a statistical comparison between the indicated sample and cells transfected with β_1 Pix. *p < 0.05.

β_1 Pix homodimerization. Furthermore, depletion of either β Pix or p66Shc strongly inhibits cell proliferation induced by ET-1. The proposed model of β Pix-mediated control of cell proliferation in ET-1-dependent manner is depicted in Figure 12. In quiescent primary glomerular mesangial cells the endogenous level of β Pix protein is low. After 24 h of incubation with ET-1, β Pix and p66Shc expression is up-regulated resulting in the formation of β Pix/p66Shc complex (Figure 1). FOXO3a is recruited to this complex where it is phosphorylated and retained leading to p27^{kip1} down-regulation (Figures 9, 4A, and 6). Based on the findings of this study, a pool of FOXO3a is unphosphorylated and localizes to the cytoplasm where it can bind to β Pix/p66Shc complex resulting in FOXO3a inactivation via ERK and p66Shc-dependent, but Akt-independent phosphorylation.

A previous study implicated PI-3K activation in mediating growth factor-induced ROS production through activation of β Pix and Rac1 but independently of Pak (Park *et al.*, 2004). Here we show that β_1 Pix-induced Rac1 activation requires both β_1 PixGEF function and SH3 domain as a Pak binding site (Figure 5B). Activation of Rac1 leads to the activation of ERK1/2 (Figure 5C) through mechanism involving Pak1 phosphorylation of MEK1 (Frost *et al.*, 1997; Eblen *et al.*, 2002). Activated ERK1/2 phosphorylates p66Shc (Hu *et al.*, 2005) and mediates β_1 Pix-induced serine 36 phosphorylation of p66Shc (Figure 5A). β Pix has been shown to induce ROS production (Park *et al.*, 2004), resulting in p66Shc activation (Migliaccio *et al.*, 1999; Nemoto and Finkel, 2002). In our case we could not detect any change in intracellular level of ROS in cells expressing β_1 Pix, and

β_1 Pix-induced p66Shc phosphorylation was unaffected by treatment with the antioxidant ebselen (unpublished data).

The inability of β_1 Pix to activate Akt (Figure 6A) as well as the inability of Akt siRNAs to block β_1 Pix-induced FOXO3a phosphorylation (Figure 8A) indicates that Akt is not involved in β_1 Pix signaling. This is in agreement with our result showing that ET-1 stimulation (through β Pix up-regulation) results in FOXO3a phosphorylation independently of Akt (Figure 3). However, in line with previous reports (Hu *et al.*, 2005; Nemoto and Finkel, 2002) Akt is required for serum-stimulated FOXO3a phosphorylation and p27^{kip1} down-regulation (Figures 6B and 7A). In the light of this result, we explored whether ERK1/2 and p66Shc are important in mediating β_1 Pix effects. Blocking ERK1/2 activation (and p66Shc) inhibits both FOXO3a phosphorylation and p27^{kip1} down-regulation (Figure 6A). The inhibition of FOXO3a phosphorylation by p66Shc depletion (Figure 8B) confirms the importance of p66Shc in mediating FOXO3a phosphorylation, although the kinase responsible for this phosphorylation is presently unknown. Recent study showed that I κ B kinase directly phosphorylates FOXO3a and promote cell proliferation (Hu *et al.*, 2004). It is possible that FOXO3a is either directly phosphorylated by ERK1/2 or indirectly through another kinase. Interestingly, ERK1/2 has been found to form a complex with p66Shc (Hu *et al.*, 2005). Another possibility is that Pak1, a well-characterized β_1 Pix effector, may phosphorylate FOXO3a. This hypothesis is supported by a previous report showing that Pak1 binds to and phosphorylates FOXO1 (Mazumdar *et al.*, 2003). The third possibility may involve a yet unidentified kinase. Our

Figure 12. Schematic representation of proposed ET-1 signaling through β_1 Pix/p66Shc/FOXO3a complex in glomerular mesangial cells. Endothelin-1 (ET-1), acting via its G-protein-coupled receptor, induces expression of β Pix and p66Shc, leading to the formation of β Pix/p66Shc complex, which then recruit cytoplasmic FOXO3a to form a trimeric signaling complex. β Pix expression, through sequential activation of ERK and p66Shc results in FOXO3a phosphorylation via Akt-independent pathway. Akt-dependent FOXO3a phosphorylation in the nucleus is also shown.

results indicate clearly that unlike serum and ROS, β_1 Pix utilizes ERK/p66Shc/FOXO3a signaling pathway that does not require Akt.

Despite the lack of response to β_1 Pix, Akt is still active in our experimental setting and induces FOXO3a phosphorylation (Figure 6). This implies that even in β_1 Pix-expressing cells, FOXO3a is responsive to Akt signaling. Indeed, a pool of endogenous FOXO3a associated with β_1 Pix and p66Shc is already phosphorylated (Figure 9), suggesting that Akt signaling is responsible for the cytosolic localization of (phosphorylated) FOXO3a. This conclusion is further supported by the finding that Akt-independent phosphorylation mediates AFX-induced transcriptional activity and cell proliferation, whereas AFX subcellular localization is regulated by Akt (De Ruiter *et al.*, 2001). These results are consistent with our data that disruption of β_1 Pix/p66Shc/FOXO3a complex induced by β_1 Pix Δ (603–608) inhibits p27^{kip1} down-regulation, cell proliferation and cell cycle progression into S phase induced by β_1 Pix (Figure 10). It is interesting that both p66Shc and FOXO3a bind only to the dimeric form of β_1 Pix (Figure 2D), indicating that β_1 Pix brings p66Shc and FOXO3a together, thus providing a unique platform to regulate cell proliferation. We have recently shown that 14-3-3 β binding also requires β_1 Pix homodimerization resulting in the modulation of its GEF activity (Chahdi and Sorokin, 2008). Therefore, we can rightly wonder whether dimerization is a general mechanism by which β_1 Pix interacts with all of its known partners and whether its GEF activity is affected by these interactions.

What is the role of β_1 Pix-induced p27^{kip1} down-regulation? Down-regulation of p27^{kip1} results in cell cycle progression into S phase. Decrease in p27^{kip1} protein level is found in many human cancers characterized by uncontrolled cell proliferation (Alkarian and Slingerland, 2004). Rac3, which is a downstream effector of β_1 Pix, is hyperactive in breast cancer cells and requires Pak to induce cell proliferation (Mira *et al.*, 2000). Moreover, recent study showed Pak–Pix interaction to be essential for Pak activation in some breast cancer cell lines (Stofega *et al.*, 2004). Interestingly, β_1 Pix expression is significantly increased in human breast cancer tissues (Ahn *et al.*, 2003). Therefore, p27^{kip1} down-regulation induced by β_1 Pix may contribute to cell proliferation in some breast cancer cells.

It is tempting to suggest that scaffolding activity of β Pix facilitates serine 36 phosphorylation of bound p66Shc because the ability of β Pix and p66Shc to recruit ERK1/2 has been shown previously (Lim *et al.*, 2003; Hu *et al.*, 2005). Thus formation of signaling complex including β Pix, ERK1/2, p66Shc, and FOXO3a is a novel signaling module that provides additional mechanistic insight into the network of signaling pathways activated by ET-1 through β Pix, a molecule possessing both scaffolding and enzymatic properties. Indeed, siRNA depletion of either β Pix or p66Shc clearly blocked ET-1-induced cell proliferation, demonstrating that p66Shc/ β Pix interaction plays an essential role in cell proliferation. The ability of ET-1 to up-regulate β Pix and p66Shc expression combined with the ability of these proteins to form a complex with FOXO3a at the endogenous level further strengthens the physiological relevance of such multimolecular complex.

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