Rac1 Leads to Phosphorylation-dependent Increase in Stability of the p66shc Adaptor Protein: Role in Rac1-induced Oxidative Stress

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The rac1 GTPase and the p66shc adaptor protein regulate intracellular levels of reactive oxygen species (ROS). We examined the relationship between rac1 and p66shc. Expression of constitutively active rac1 (rac1V12) increased phosphorylation, reduced ubiquitination, and increased stability of p66shc protein. Rac1V12-induced phosphorylation and up-regulation of p66shc was suppressed by inhibiting p38MAPK and was dependent on serine 54 and threonine 386 in p66shc. Phosphorylation of recombinant p66shc by p38MAPK in vitro was also partly dependent on serine 54 and threonine 386. Reconstitution of p66shc in p66shc-null fibroblasts increased intracellular ROS generated by rac1V12, which was significantly dependent on the integrity of residues 54 and 386. Overexpression of p66shc increased rac1V12-induced apoptosis, an effect that was also partly dependent on serine 54 and threonine 386. Finally, RNA interference-mediated down-regulation of endogenous p66shc suppressed rac1V12-induced cell death. These findings identify p66shc as a mediator of rac1-induced oxidative stress. In addition, they suggest that serine 54 and threonine 386 are novel phosphorylatable residues in p66shc that govern rac1-induced increase in its expression, through a decrease in its ubiquitination and degradation, and thereby mediate rac1-stimulated cellular oxidative stress and death.

INTRODUCTION

Oxidative stress is a phenomenon that represents the imbalance between the production and elimination of reactive oxygen species (ROS). The small GTPase rac1 is an important regulator of ROS generation in a broad range of cell types. Activation of rac1 triggers the production of ROS by a number of intracellular sources. In immune cells, the plasma membrane NADPH oxidase responsible for the microbicidal oxidative burst is a principal target of active rac1 (DeLeo *et al.*, 1999). Many nonimmune cells also express many of the subunits of this oxidase (Bokoch and Knaus, 2003). However, active rac1 can also lead to ROS generation through mechanisms that are independent of the NADPH oxidase. Such mechanisms, including ROS generation as by-products of mitochondrial electron transport, play a major role in leading to oxidative stress resulting from activation of rac1 (Deshpande *et al.*, 2003).

P66shc is an adaptor protein that is modified in response to oxidative stress and that seems to play a pivotal role in mediating cell death induced by triggers of oxidative stress (Migliaccio *et al.*, 1999). Furthermore, emerging evidence suggests that the expression of p66shc itself governs cellular levels of reactive oxygen species. P66shc expression increases ambient intracellular hydrogen peroxide levels by

inhibiting forkhead-mediated transcription of catalase, one of the enzymes responsible for the breakdown of peroxide (Nemoto and Finkel, 2002). In addition, p66shc mediates the production of oxidants in the mitochondria induced by the tumor suppressor p53 (Trinei *et al.*, 2002). Thus, p66shc may regulate both the production and elimination of intracellular ROS.

P66shc is widely, although not ubiquitously expressed. In some cell types, p66shc is not expressed under basal conditions, but when challenged with oxidative or apoptotic stimuli, its expression is dramatically up-regulated (Pacini *et al.*, 2004). The mechanisms that lead to such up-regulation are not completely understood. In particular, although reports have emerged about the transcriptional control of p66shc expression (Ventura *et al.*, 2002), the role of posttranslational modifications in governing p66shc expression have not been studied.

P66shc is a modular protein consisting of an N-terminal collagen homology domain (CH2), a phosphotyrosine-binding domain, a second collagen homology domain (CH1), and a C-terminal src homology-2 domain. Together with p52shc and p46shc, p66shc comprises the shcA family of adaptor proteins (Bonfini *et al.*, 1996). Structurally, p66shc differs from p52shc and p46shc, by virtue of its 110-amino acid CH2 domain. This unique domain also confers upon p66shc its distinct functional characteristics, namely, its role in ROS regulation. Serine 36 in the CH2 domain is phosphorylated in response to oxidative stress, and this post-translational modification impacts upon the unique redox-regulatory function of p66shc (Migliaccio *et al.*, 1999). However, the effect of S36 phosphorylation on the expres-

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sion of the protein is not known. Moreover, the effect of phosphorylation of residues other than S36, both in and outside of the CH2 domain, on p66shc expression has not been investigated.

The relationship between rac1-regulated oxidative stress and p66shc is not known. Intrigued by the possibility of cross-talk between the proteins in the execution of their functions, we investigated the role of rac1 in regulating p66shc expression, and the importance of p66shc in governing rac1-induced oxidative stress.

MATERIALS AND METHODS

Cell Lines and cDNAs

PC12 and COS-7 cells were obtained from American Type Culture Collection (Manassas, VA). P66shc^{-/-} mouse embryo fibroblasts (MEFs) and the XPress-tagged p66shcWT cDNA in pcDNA3.1/His A (Invitrogen, Carlsbad, CA) expression vector have been described previously (Nemoto and Finkel, 2002). The rac1V12 expression vector, a generous gift from A. Hall (University College, London, United Kingdom), has also been described previously (Irani *et al.*, 1997). Site-directed mutations were created in p66shcWT using QuikChange (Stratagene, La Jolla, CA), and all mutations were verified by sequencing. Cells were transfected using Lipofectamine 2000 (Invitrogen) or electroporated using Nucleofector (Amaxa, Gaithersburg, MD). After 24 h, the transfected cells were further treated or harvested for the specified assay.

Antibodies, Immunoprecipitations, and Western Blotting

Antibodies to XPress tag (Invitrogen), shcA (H-108; Santa Cruz Biotechnology, Santa Cruz, CA), rac1 (UBI), hemagglutinin (HA) (Roche Diagnostics, Indianapolis, IN), and c-myc (9E10; Santa Cruz Biotechnology) were purchased. Immunoprecipitations were typically carried out by incubating 2 μ g of antibody with 1 mg of cell lysate overnight, followed by 40 μ l of protein A-Sepharose slurry (GE Healthcare, Little Chalfont, Buckinghamshire, United Kingdom) for 2 h. After washing, immunoprecipitates were boiled in SDS-PAGE gel loading buffer, subjected to SDS-PAGE, transferred to nitrocellulose filter, and probed with the specified primary antibody and the appropriate peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology). Western blotting of 50 μ g of whole cell lysates was similarly performed. Chemiluminescent signal was developed using Super Signal West Femto substrate (Pierce Chemical, Rockford, IL), blots imaged with a Gel Doc 2000 ChemiDoc system (Bio-Rad, Hercules, CA), and bands were quantified using Quantity One software (Bio-Rad).

Bacterial Expression of Proteins and In Vitro Phosphorylation Assays

The p66shcWT and p66shcS54A/T386A cloned into pGEX4T-2 (GE Healthcare) were expressed and induced with isopropyl β -D-thiogalactoside (1 mM) in BL21-DE3 (Stratagene) bacterial host strain. Expressed proteins were purified over a glutathione S-transferase (GST)-agarose column (GE Healthcare). Ten micrograms of recombinant proteins was incubated in kinase assay buffer (25 mM Tris-HCl, pH 7.5, 1.2 mM EGTA, 15 mM MgCl₂, 4 mM 3-(N-morpholino)propanesulfonic acid [MOPS], 5 mM β -glycerol phosphate, 200 μ M sodium orthovanadate, 200 μ M dithiothreitol [DTT], and 100 μ M ATP), with 200 μ Ci/ml [γ -³²P]ATP, with and without 50 ng of active p38MAPK (p38 α /SAPK2a; Upstate Biotechnology, Lake Placid, NY), at room temperature for 10 min. For the c-Jun NH₂-terminal kinase (JNK) assay, 5 μ g of recombinant proteins was incubated in assay buffer (20 mM MOPS, pH 7.2, 25 mM β -glycerol phosphate, 5 mM EGTA, 15 mM MgCl₂, 1 mM sodium orthovanadate, 1 mM DTT, and 100 μ M ATP), with 200 μ Ci/ml [γ -³²P]ATP, with and without 20 ng of active JNK1 α 1/SAPK1c (Upstate Biotechnology), at room temperature for 10 min. The reaction mixes were subjected to SDS-PAGE and autoradiographed using a PhosphorImager (GE Healthcare).

Adenoviruses

Recombinant adenoviruses encoding myc-tagged rac1V12 (Adrac1V12) and the inert *Escherichia coli* LacZ gene (Ad β gal) have been described previously (Deshpande *et al.*, 2000). Adrac1V12 was used at 100 multiplicity of infection (moi) to express rac1V12 in PC12 cells. Ad β gal at the same moi was used in the controls. All adenoviruses were purified on double cesium gradients and titered before use. Cells were typically infected with viruses for 4–6 h, and assays performed after 24–36 h.

Apoptosis

Apoptosis was quantified with the Cell Death Detection ELISA kit (Roche Diagnostics), as per manufacturer's recommendations. Measured OD₄₀₅ was normalized for cell number (protein content).

ROS Measurements

Intracellular ROS levels were measured using the redox-sensitive fluorophore 2',7'-dichlorofluorescein diacetate DCF-DA (Invitrogen), as described previously (Angkeow *et al.*, 2002). Intracellular DCF-DA fluorescence was detected and quantified by fluorescence-activated cell sorter (BD Biosciences, San Jose, CA). Representative histograms of DCF-DA fluorescence or quantification of mean fluorescence are shown.

RNA Interference

A 19-mer sequence corresponding to bases 45–63 of the cDNA of p66shc was cloned as a hairpin loop into the pSilencer 2.1-U6 neo vector (Ambion, Austin, TX) as per manufacturer's recommendation. The vector encoding a scrambled sequence was used as a control. Nucleotides 45–63 are in the 330 base pair coding region of the N-terminal CH2 domain and are unique to p66shc.

Pulse-Chase Experiments

PC12 cells grown in methionine-free medium were pulse labeled with [³⁵S]methionine at 100 μ Ci/ml (GE Healthcare) for 30 min. After washing away labeling medium, the cells were incubated in unlabeled medium for the specified chase time. Cells were then harvested, and their lysates were immunoprecipitated with XPress antibody, subjected to SDS-PAGE, and autoradiographed. Densitometric quantification of bands was done using ImageQuant software (GE Healthcare).

In Vivo Phosphorylation Assays

PC12 cells grown in phosphate-free medium were labeled with ³²PO₄³⁻ at 1 mCi/ml (GE Healthcare) for 4 h. After removing labeling medium and washing, the cells were harvested and lysed. Lysates were immunoprecipitated with XPress antibody, subjected to SDS-PAGE, autoradiographed with a PhosphorImager, and immunoblotted with XPress antibody.

Ubiquitination

COS-7 cells were cotransfected with HA-ubiquitin and (His)₆-p66shcWT, with and without rac1V12. P66shcWT was purified from whole cell lysates with a Ni²⁺-NTA column. Eluted p66shcWT, after normalization for protein expression, was subjected to SDS-PAGE and immunoblotting with shcA and HA antibodies. Whole cell lysate was immunoblotted with myc antibody for detection of myc-rac1V12.

Reagents

All other reagents and chemicals were purchased from Sigma-Aldrich. The p38MAPK inhibitor 4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)1H-imidazole (SB203580) was used at 30 μ M for 4 h and the pan-JNK inhibitor SP600125 at 20 μ M for 4 h.

Statistical Analysis

Data are shown as mean $\pm$ SEM. Statistical analyses were performed by *t* test, and *p* < 0.05 was considered statistically significant. Representative experiments that were reproduced twice are shown.

RESULTS

Activated rac1 Increases Expression of p66shc

To understand the relationship between rac1 activity and p66shc expression, endogenous p66shc protein was examined in PC12 cells in which the constitutively activated mutant of rac1 (rac1V12) was expressed. In comparison with control cells, rac1V12 expression resulted in an increase in p66shc protein levels (Figure 1A). Similar to endogenous protein, ectopic p66shcWT expression was also up-regulated in response to rac1V12 (Figure 1A). In contrast, p46shc and p52shc levels were unchanged in rac1V12-expressing cells. Therefore, activation of rac1-regulated signaling leads to a selective increase in expression of p66shc protein.

Serine 36 in the CH2 domain of p66shc has been well characterized. Phosphorylation of this residue is critical for p66shc-mediated cell death (Migliaccio *et al.*, 1999) This prompted us to determine the importance of serine 36 in rac1-induced up-regulation of p66shc expression. P66shcWT, or p66shcS36A (S $\rightarrow$ A mutation of the phosphoacceptor S36 residue), with and without rac1V12, were expressed in PC12 cells. Rac1V12 resulted in an increase in p66shcS36A to a magnitude similar to that of p66shcWT (Figure 1B), suggest-

Figure 1. Activation of rac1 up-regulates p66shc protein in PC12 cells. (A) Immunoblot showing expression of ectopic p66shcWT in PC12 cells, in the presence and absence of rac1V12. Expression of endogenous shcA proteins is also shown. Expression of myc-tagged rac1V12 is shown at bottom. (B) Immunoblot showing expression of ectopic p66shcWT and indicated p66shc mutated constructs in PC12 cells, in the absence and presence of rac1V12. Expression of myc-tagged rac1V12 is shown at bottom.

ing that phosphorylation of serine 36 was not critical to rac1-induced up-regulation of p66shc expression.

We were intrigued by the possibility that residues other than serine 36 may be important to the regulation of p66shc expression. Many oxidative stimuli lead to activation of the proline-dependent, stress-activated protein kinases JNKs, and p38MAPK. Moreover, rac1 is believed to lie upstream of, and mediate the activation of JNKs and p38MAPK (Coso *et al.*, 1995; Bakin *et al.*, 2002), both of which have been implicated in phosphorylating p66shc on serine 36 (Le *et al.*, 2001). Having observed that this residue is not critical to rac1V12-stimulated increase in expression of p66shc, we then hypothesized that these stress-activated kinases, in addition to phosphorylating S36, also phosphorylate residues in p66shc other than serine 36. Furthermore, we speculated that phosphorylation of these alternative residues may mediate rac1-induced up-regulation of p66shc expression. A search of the amino acid sequence of p66shc revealed several putative phosphorylation sites for p38MAPK and JNKs. Among these, serine 36, serine 54, and threonine 384 lie within strong p38MAPK and JNK consensus phosphorylation motifs. Because expression of p66shcWT and p66shcS36A was up-regulated by rac1V12 to a similar degree, we narrowed our search to serine 54 and threonine 386. These putative phosphoacceptor residues were mutated to alanine individually, and the p66shcS54A and p66shcT386A were separately expressed in PC12 cells. Adenoviral coexpression of rac1V12 led to a significant up-regulation of both p66shcS54A and p66shcT386A (Figure 1B), suggesting that, similar to serine 36, phosphorylation of serine 54 or threonine 386, alone, could not be responsible for rac1-induced up-regulation of p66shc expression. In addition, the S54A and T386A mutations, in the context of the S36A mutation (p66shcS36A/S54A and p66shcS36A/T386A double mutants), also did not abrogate rac1-stimulated up-regulation of p66shc (our unpublished data). In contrast, rac1V12 in-

duced up-regulation of p66shc mutated at both serine 54 and threonine 386 (p66shcS54A/T386A) to a considerably less degree than it did p66shcWT (Figure 1B). This shows that rac1-stimulated up-regulation of p66shc protein expression requires both S54 and T386, suggesting that modifications of these residues on the same molecule may be necessary for the governance of p66shc expression by rac1.

P38MAPK Phosphorylates p66shc on S54 and T386 and Mediates rac1-stimulated Up-Regulation of p66shc Expression

We then turned our attention to demonstrating that these residues are targets of stress-activated, proline-dependent kinases. First, we examined the effect of inhibiting p38MAPK on rac1-induced up-regulation of p66shc in PC12 cells. Pretreatment of cells with SB203580, a specific pharmacologic inhibitor of p38MAPK, significantly reduced rac1V12-stimulated increase in p66shcWT protein expression (Figure 2A). Moreover, inhibition of p38MAPK also suppressed rac1V12-stimulated up-regulation of p66shcS36A (Figure 2A), suggesting that p38MAPK-mediated up-regulation of p66shc is independent of S36. We also determined the effect of SP600125, a pan-JNK inhibitor, on rac1V12-stimulated p66shc expression. Similar to SB203580, treatment with SP600125 also suppressed rac1V12-stimulated up-regulation of both p66shcWT and p66shcS36A (Figure 2B), implicating JNKs as well in mediating rac1V12-induced up-regulation of p66shc expression.

Next, we examined phosphorylation of p66shc *in vivo*. Radionuclide phospholabeling of PC12 cells showed that rac1V12 induced phosphorylation of p66shcWT (Figure 2C). Rac1V12-triggered phosphorylation of p66shcWT was significantly blunted in cells pretreated with SB203580. In addition, p66shcS54A phosphorylation *in vivo* was also triggered by rac1V12, although to a much lesser degree than p66shcWT. Finally, *in vivo* phosphorylation of p66shcS54A/T386A induced by rac1V12 was completely blunted compared with that of p66shcWT. These results indicate that rac1-stimulated phosphorylation of p66shc *in vivo* is mediated through p38MAPK and that this phosphorylation occurs largely on S54 but also on T386.

To demonstrate that S54 and T386 are direct phosphorylation targets of p38MAPK, we also performed *in vitro* phosphorylation assays using recombinant p66shc and active p38MAPK. p38MAPK efficiently phosphorylated p66shcWT (Figure 2D). However, when p66shcS54A/T386A was substituted for p66shcWT as a substrate for p38MAPK, phosphate incorporation was considerably lower. In contrast, *in vitro* phosphorylation induced by a member of the JNK family (JNK α 1) was not different between p66shcWT and p66shcS54A/T386A. This suggests that S54 and T386 are good targets for p38MAPK phosphorylation, but not for JNK α 1. Together, the *in vitro* and *in vivo* phosphorylation data demonstrate that phosphorylation of these residues by p38MAPK, and possibly members of the JNK family, mediates rac1-stimulated up-regulation of p66shc expression.

Rac1 Leads to a Decrease in Turnover of p66shc Protein

That mutations at S54 and T386 altered rac1-stimulated phosphorylation and expression of p66shc suggested to us that rac1 activation might lead to a change in the stability of p66shc protein. To investigate this possibility, we examined the stability of p66shcWT protein in the absence and presence of rac1V12. [35 S]Methionine pulse-chase experiments in PC12 cells revealed a significant increase in the stability of p66shc protein with the expression of rac1V12 (Figure 3A).

Figure 2. Activation of rac1 induces p38MAPK-dependent phosphorylation and up-regulation of p66shc in vitro and in vivo. Immunoblots showing expression of ectopic p66shcWT and p66shcS36A in PC12 cells, in the presence and absence of rac1V12, and with and without pretreatment with SB203580 (A) or SP600125 (B). Expression of endogenous and myc-tagged rac1V12 is shown at bottom. (C) Autoradiograph showing incorporation of radiolabeled phosphate into ectopic p66shcWT and indicated p66shc mutated constructs in PC12 cells, in the absence and presence of rac1V12, and with and without pretreatment with SB203580. Immunoblot of expression of ectopic proteins is shown at bottom. Values represent ratio of phosphorylated/total protein. (D) Autoradiograph showing incorporation of radiolabeled phosphate group into recombinant p66shcWT or p66shcS54A/T386A, in the presence or absence of active p38MAPK or active JNK α 1. Coomassie staining of recombinant proteins is shown at bottom.

The half-life of p66shcWT was $\sim$ 4.5 and 8 h in the absence and presence of rac1V12, respectively.

We then asked whether the S54 and T386 were important to rac1-stimulated increase in the half-life of p66shc. We compared the stabilities of p66shcWT and p66shcS54A/T386A in PC12 cells, with and without rac1V12. In the presence of rac1V12, the decline in presynthesized p66shcWT was significantly lower than that of p66shcS54A/T386A (Figure 3B), suggesting that S54 and T386 play an

Figure 3. Activation of rac1 decreases the turnover of p66shc protein. (A) [35 S]Methionine pulse-chase experiments showing stability of p66shcWT protein in PC12 cells, in the presence and absence of rac1V12 ($n = 3$). (B) Quantification of [35 S]methionine pulse-chase experiments showing decline in ectopically expressed p66shcWT and p66shcS54A/T386A proteins, in the absence and presence of rac1V12. * $p < 0.05$ versus p66shcWT + rac1V12 and # $p = NS$ versus p66shcWT + rac1V12 ($n = 3$). (C) Immunoblot showing incorporation of ubiquitin moieties into ectopically expressed p66shcWT, in the absence and presence of rac1V12. Immunoblots of total p66shcWT and myc-tagged rac1V12 expression are shown at bottom.

important part in the rac1V12-induced decrease in turnover of p66shc. Moreover, the decline in p66shcS54A/T386A protein in the presence of rac1V12 was similar to that of p66shcWT protein in the absence of rac1V12 (Figure 3B). These findings indicate that activation of rac1 leads to an increase in stability of p66shc protein and that residues 54 and 386 mediate this decrease in turnover.

Because many cellular proteins are targeted for degradation by polyubiquitination, we investigated whether p66shc is ubiquitinated and whether rac1V12 affects the ubiquitination of p66shc. P66shc was polyubiquitinated in vivo. Furthermore, expression of rac1V12 led to a decrease in p66shc

Figure 4. P66shc mediates active rac1-induced oxidative stress and cell death. (A) Histograms showing intracellular ROS levels in p66shc^{-/-} MEFs expressing ectopic p66shcWT, in the absence and presence of rac1V12. (B) Change in intracellular ROS levels with rac1V12 in p66shc^{-/-} MEFs expressing p66shcWT or p66shcS54A/T386A. Values represent percent increase compared with cells not expressing rac1V12. **p* < 0.05 versus p66shcWT (*n* = 3). (C) Programmed cell death induced by rac1V12 in PC12 cells, with and without RNAi-mediated down-regulation of p66shc. Values are expressed relative to apoptosis in cells not expressing rac1V12. **p* < 0.05 versus scrambled RNAi + rac1V12 (*n* = 3). Immunoblot of shcA proteins is shown at bottom. (D) Programmed cell death induced by rac1V12 in PC12 cells with and without expression of ectopic p66shcWT and p66shcS54A/T386A. Values are absolute optical densities (measure of apoptosis) normalized for protein content. **p* < 0.05 versus p66shcWT + rac1V12 (*n* = 3).

ubiquitination (Figure 3C). Together, these findings show that activation of rac1 leads to a decrease in the ubiquitination and degradation of p66shc and that residues 54 and 386 mediate this stabilization of the protein.

P66shc Mediates rac1-stimulated Oxidative Stress and Cell Death: The Roles of S54 and T386

In many cell types, constitutive activation of rac1 results in oxidative stress (Deshpande *et al.*, 2003). We wondered whether rac1-stimulated oxidative stress is mediated by p66shc. We first measured the rac1V12-stimulated increase in intracellular reactive oxygen species in MEF cells derived from p66shc^{-/-} mice. Expression of rac1V12 led to a modest increase in intracellular ROS (Figure 4A). We then measured intracellular ROS levels in p66shc^{-/-} MEF coexpressing rac1V12 and p66shcWT. The amplitude of ROS increase induced by rac1V12 was markedly higher with coexpression of p66shcWT (Figure 4A).

Next, we sought to determine whether the S54 and T386 are important to p66shc-mediated oxidative stress induced by active rac1. We compared the increase in intracellular ROS induced by rac1V12 expression in p66shc^{-/-} MEF coexpressing either p66shcWT or p66shcS54A/T386A. Rac1V12-induced increase in intracellular ROS was significantly decreased in cells coexpressing p66shcS54A/T386A, compared with cells coexpressing p66shcWT (Figure 4B). Therefore, the mediating role of p66shc in rac1-stimulated intracellular ROS production is dependent on S54 and T386.

Oxidative stress is associated with cell death. We therefore examined the role of endogenous p66shc in mediating rac1-induced cell death. Endogenous p66shc was down-regulated with an RNAi approach. Down-regulation of p66shc resulted in significant reduction in rac1V12-induced cell death (Figure 4C). Therefore, endogenous p66shc partly mediates rac1-induced cell death.

Figure 5. Hypothetical model showing the regulation of p66shc protein turnover by rac1, p38MAPK, and the proteasome.

We then examined the effect of p66shc overexpression on rac1V12-induced cell death. In PC12 cells, p66shcWT overexpression increased rac1V12-induced apoptosis, compared with cells not overexpressing p66shc (Figure 4D). In contrast, expression of p66shcS54A/T386A did not augment rac1V12-induced cell death. These findings support the contention that p66shc promotes rac-stimulated cell death and that S54 and T386 in p66shc mediate this effect.

DISCUSSION

The identification of S54 and T386 as residues in p66shc that are likely targets of phosphorylation raises the question as to how such phosphorylation affects the stability of the protein. Many cellular proteins that are regulated at the posttranslational level are ubiquitinated and degraded by the 26S proteasome. There are two known sequences that mark proteins for proteasomal degradation: 1) a PEST sequence (Rechsteiner and Rogers, 1996) and 2) a destruction box (Fenteany and Schreiber, 1998). We did not find a destruction box within the sequence of p66shc, but interestingly, p66shc does possess two strong PEST motifs encompassing amino acids 14 through 64 in the CH2 domain and amino acids 328 through 347 in the CH1 domain.

PEST sequences are motifs that are hydrophilic and are enriched in proline (P), glutamic acid (E), serine (S), and threonine (T). They are flanked by lysine (K), arginine (R), or histidine (H) residues, but they have no positively charged residues within them. PEST sequences can either be constitutive proteolytic signals, or conditional signals. With regard to the latter, there are several examples in which phosphorylation and dephosphorylation of serines and threonines controls the metabolic stability of PEST-containing proteins, such as G₁ cyclins (Yaglom *et al.*, 1995). In the case of p66shc, serine 54 lies within the 51-amino acid PEST motif in the CH2 domain of p66shc, whereas threonine 386 flanks the 20-amino acid PEST sequence in the CH1 domain. This raises the intriguing possibility that phosphorylation of these residues serves to mask their respective PEST sequences from recognition by the ubiquitin-26S proteasome degradation pathway (Figure 5).

P66shc, unlike very short-lived proteins such as HIF-1, is not unstable. This is evident from its half-life of ~4.5 h. At

face value, the 50% increase in this half-life (from 4.5 to 8 h) induced by rac1V12 may not seem dramatic. However, this modest decrease in protein turnover would be expected to lead to a severalfold increase in expression over a 30-h time frame, provided synthesis does not change. It is noteworthy that in our experiments (in which rac1V12 was expressed over 24–36 h), we did observe a mean increase of two- to threefold in protein levels (Figure 1, A and B), consistent with the magnitude of increase in half-life calculated from the pulse-chase experiments. It is important to note, however, that although turnover of p66shc is regulated by rac1, we did not evaluate the effect of rac1V12 on transcription of p66shc, and therefore we cannot rule out the possibility that rac1V12 also may affect synthesis of p66shc.

p38MAPK and JNKs belong to the family of proline-dependent serine/threonine kinases, and they share the same minimal S/TP target motif (Kyriakis and Avruch, 1996). Indeed, serine 36 on p66shc can be phosphorylated by JNKs as well as p38MAPK (Le *et al.*, 2001). Therefore, it is likely that S54 and T386, in addition to being targets of p38MAPK, are phosphorylated by JNKs. This prediction is supported by our data that a pan-JNK inhibitor was capable of partially inhibiting rac1V12-stimulated up-regulation of both p66shcWT and p66shcS36A. Our finding that in vitro phosphorylation of p66shcS54A/T386A by active JNKα1 was not significantly different from that of p66shcWT, speaks against a role for this specific JNK in phosphorylation of these residues, but it does not exclude the possibility that other members of the JNK family may target these residues. In addition to sharing the target motif at the phosphoacceptor site, many, but not all, known substrates of proline-dependent, stress-activated kinases (p38MAPK and JNKs but not extracellular signal-regulated kinases) also share other motifs that facilitate binding to the kinases. Principal among these is the docking domain, consisting of hydrophobic residues several amino acids downstream from a region containing several basic residues (Sharrocks *et al.*, 2000). However, there are significant differences in the composition and spacing for substrates of docking domains for different classes of proline-dependent kinases. Docking domains typically lie 50–100 amino acids N-terminal to the phosphoacceptor residue, although there is considerable variation in this distance as well. Variations notwithstanding, it is inter-

esting to note that a stretch of residues N-terminal of serines 36 and 54 on p66shc (7-KPKYNPLRNESLSSL-21; bold and italicized letters show basic and hydrophobic residues, respectively) fits the general requirements of a docking domain. Such a consensus domain is lacking upstream of the T386 phosphoacceptor residue. However, it is possible that effective docking at one site may suffice to phosphorylate an acceptor residue on the same molecule distant from the docking site. This model likely is responsible for the JNK-induced phosphorylation of JunD (which lacks an effective JNK docking domain) when present as a heterodimer with c-Jun or JunB (that do possess effective JNK docking sites) (Kallunki *et al.*, 1996). The presence of a putative mitogen-activated protein kinase (MAPK) docking domain on p66shc also raises the possibility that p66shc may function as a scaffolding protein and may serve to recruit these MAPKs into signaling complexes. One can certainly envision p66shc, with its multiple protein binding domains, acting in such a manner.

Definitive proof that S54 and T386 are phosphoacceptor residues for p38MAPK, and possibly for members of the JNK family, would require phosphopeptide mapping. Our findings that rac1V12- and p38MAPK-stimulated phosphate incorporation was significantly diminished in p66shcS54A/T386A, compared with p66shcWT, strongly suggests, but does not unequivocally prove, that these residues are phosphorylated by p38MAPK in response to rac1 activation. A global conformational change of the protein induced by these mutations could also indirectly lead to a decrease in phosphorylation. However, this seems unlikely, given that these mutations decreased phosphorylation by p38MAPK but not by JNK α 1 (Figure 2D).

Unlike the CH2 domain, the CH1 domain is common to all isoforms of the shcA family. Therefore, phosphorylation of T386 by p38MAPK or JNKs would also be expected to occur on p46shc and p52shc. The fact that expression of p52shc and p46shc was not increased by rac1V12, suggests that phosphorylation of T386 alone, although necessary, is not sufficient to alter the turnover of shcA proteins. An alternative explanation for the lack of up-regulation of p52shc and p46shc expression may be that T386, although phosphorylated by p38MAPK on p66shc, is not phosphorylated on the other two shcA isoforms. This explanation seems plausible considering the absence of a CH2 domain (with its putative MAPK docking domain) in these isoforms.

A careful examination of our findings also suggests that of the two residues that we targeted, serine 54 is likely to be, by far, the more important and physiologically relevant residue. Serine 54 is conserved among p66shc of different species, whereas threonine 386 is not. In addition, rac1V12-stimulated phosphorylation of p66shcS54A was markedly (but not entirely) blunted (Figure 2C). It is also tempting to hypothesize that, because of its location in the CH2 domain, and because it is evolutionarily conserved, the physiological relevance of serine 54 phosphorylation, may be manifested as changes in the unique and fundamental cellular functions of p66shc, independent of its effect on p66shc expression.

It is informative to consider where p66shc and intracellular ROS may lie with respect to rac1. Rac1-induced increase in p66shc expression is at least partly responsible for rac1-stimulated oxidative stress. However, although expression of p66shc expression correlates with the extent of oxidative stress induced by rac1, p66shc was not essential for rac1-stimulated increase in ROS levels (Figure 4A). Furthermore, rac1-stimulated increase in expression of p66shc is dependent on intracellular ROS (our unpublished data), suggesting that ROS generated by rac1 activation may also lie

upstream of p66shc. These observations together suggest that 1) activation of rac1 can lead to generation of reactive oxygen species independent of p66shc and 2) rac1-stimulated increase in p66shc expression, which depends on p38MAPK, but may also depend on rac1-induced, p66shc-independent ROS production, serves to further increase ROS levels. Thus, p66shc may amplify, rather than trigger, rac1-stimulated reactive oxygen species production. This positive feedback between p66shc and ROS would ultimately be expected to lead to intracellular oxidative stress (Figure 5).

In conclusion, our findings suggest that p66shc expression is governed by rac1, through posttranslational decrease in protein turnover. Moreover, our data also suggest that this up-regulation of p66shc expression by rac1 is an important factor that contributes to rac1-induced oxidative stress and consequent cell death. In addition to its importance in cell death, this relationship among rac1, ROS, and p66shc may also contribute to other p66shc-mediated phenotypes, such as cellular aging.

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