Serine 36 in Response to Ultraviolet Irradiation*

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kinase; CH1/2, collagen-homologous region 1/2; CHO, Chinese ham-

Mice lacking expression of the p66 isoform of the ShcA
adaptor protein (p66ShcA) are less susceptible to oxidative
stress and have an extended life span. Specifically,
phosphorylation of p66ShcA at serine 36 is critical for the
cell death response elicited by oxidative damage. We
sought to identify the kinase(s) responsible for this
phosphorylation. Utilizing the SH-SY5Y human neuro-
blastoma cell model, it is demonstrated that p66ShcA
is phosphorylated on serine/threonine residues in re-
ponse to UV irradiation. Both c-Jun N-terminal kinases
(JNKs) and p38 mitogen-activated protein kinases are
activated by UV irradiation, and we show that both are
capable of phosphorylating serine 36 of p66ShcA in vitro.
However, treatment of cells with a multiple lineage ki-

nase inhibitor, CEP-1347, that blocks UV-induced JNK
activation, but not p38, phosphorylidinositol 3-kinase, or
MEK1 inhibitors, prevented p66ShcA phosphorylation in
SH-SY5Y cells. Consistent with this finding, transfected
activated JNK1, but not the kinase-dead JNK1, leads to
phosphorylation of serine 36 of p66ShcA in Chinese ham-
ster ovary cells. In conclusion, JNKs are the kinases that
phosphorylate serine 36 of p66ShcA in response to UV
irradiation in SH-SY5Y cells, and blocking p66ShcA phos-
phorylation by intervening in the JNK pathway may
prevent cellular damage due to light-induced oxidative
stress.

The accumulation of oxidative cellular damage has been
noted in a number of aging and neurodegenerative diseases,
such as Alzheimer’s, Parkinson’s, and amyotrophic lateral scele-
rosis (1–4). The molecular mechanisms leading to oxidative cell
death have not been not fully elucidated; however, recent studies
suggest that phosphorylation cascades may be involved in
events triggered by free radical overproduction. For example,
members of the mitogen-activated protein kinase (MAPK)1
family, including p38 and JNKs, are activated upon exposure of
cells to DNA-damaging agents, hydrogen peroxide, UV radi-

ation, and hypoxia (5–9). However, it is not clear what events
downstream of kinase activation contribute to the ultimate
damage induced by oxidative stress. In this regard, it is of

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1 The abbreviations used are: MAPK, mitogen-activated protein ki-

nase; JNK, c-Jun N-terminal kinase; ERK, extracellular signal-regu-
lated kinase; MEKK, mitogen activated protein kinase kinase kinase;
MKK, mitogen activated protein kinase kinase; MLK, mixed lineage
kinase; CH1/2, collagen-homologous region 1/2; CHO, Chinese hamster ovary;
CBP, calmodulin-binding protein; MBP, myelin basic protein; wt,
wild type; IGF-1, insulin-like growth factor-1.

interest that phosphorylation of the p66ShcA adaptor protein
has been shown to play an important role in signaling events
leading to cell death in response to oxidative damage (10).

The mammalian ShcA adaptor protein has three isoforms:
p46, p52, and p66 (11). All Shc isoforms contain a common
structure with a C-terminal SH2 (Src homology 2) domain, a
proline- and glycine-rich region, collagen-homologous region 1
(CH1), and an N-terminal PTB (phosphotyrosine binding) do-
main (12). In addition, there is a unique collagen homologous
region 2 (CH2) domain at the N terminus of the p66ShcA iso-
form. It is well established that the Shc proteins are phos-
phorylated at tyrosine residues in response to stimulation by a
variety of growth factors and cytokines (12–15, 16). The p46
and p52 isoforms transmit signals from receptor tyrosine ki-
nases to the Ras/MAPK pathway by forming a stable complex
involving Grb2 and a Ras exchange factor, SOS (Son of Seven-
less) (12, 17–21). However, p66ShcA appears functionally differ-
tent from the p46 and p52 isoforms. Unlike the other isoforms,
p66 does not transform mouse fibroblasts, nor does it induce
MAPK activation, although p66ShcA is transiently phosphoryl-
ated at tyrosine residues in response to growth factor stimulation
(22, 23).

The p66 protein is phosphorylated at serine/threonine resi-
dues in response to insulin, Taxol, UV, or H2O2 treatment (10,
24, 25). It has recently been demonstrated that, in response to
UV and H2O2 treatment, p66ShcA is phosphorylated mainly at
serine 36 in the N-terminal CI2 domain (10). p66ShcA-null
mouse fibroblast cells have enhanced cellular resistance to
oxidative damage (10). Wild type p66ShcA, but not a phos-
phorylation-defective mutant, can restore the normal stress re-
response in the p66ShcA-null mouse fibroblast cells. Moreover,
mice lacking expression of p66ShcA are less susceptible to chemi-
ical induced oxidative damage by paraquat and have an ex-
tended life span (10). Therefore, p66ShcA, particularly its phos-
phorylation at serine 36, appears to be important for the cell
death response upon oxidative stress, and the prevention of
this phosphorylation may have therapeutic impact on diseases
that are associated with oxidative damage.

We sought to identify the kinase(s) responsible for phospho-
ylating serine 36 of p66ShcA. We report here that JNK1, JNK2,
and JNK3 phosphorylate p66ShcA in vitro and further demon-
strate that blocking JNK activation prevents p66ShcA phos-
phorylation in UV-irradiated SH-SY5Y human neuroblastoma
cells.

EXPERIMENTAL PROCEDURES

Reagents—The kinase inhibitors SB203580, LY294002, and
PD98059 were purchased from BIOMOL Research Laboratories (Plym-
outh Meeting, PA). CEP-1347, also known as KT7515, is a semi-syn-
thetic derivative of K-252a and was provided by Kyowa-Hakko (Tokyo)
(26). All stocks of inhibitors were dissolved in dimethyl sulfoxide
(Me2SO) and stored at −20 °C in amber glass vials. Further dilutions
were made in appropriate solutions containing 0.05% bovine serum
albumin (Fraction V, Protease-free). Pefabloc and bovine serum albu-

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min were purchased from Roche Molecular Biochemicals. Dulbecco’s modified Eagle’s medium, Neural Basal media, and B-27 serum-free supplement were purchased from Life Technologies, Inc. Aprotinin and protease inhibitor mixture were purchased from Sigma. Recombinant mixed lineage kinases (MLKs) and JNK2 were made as described elsewhere (29). Recombinant MEKK1, MKK4, MKK7, JNK1x1, JNK2x2, p38, ERK1, and immunoprecipitating p66Shcα/A antibody were purchased from Upstate Biotechnology, Inc. (Lake Placid, NY). Western blotting p66Shcα/A antibody was purchased from Transduction Laboratories (Lexington, KY). The Grb2 antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Phospho-JNK and JNK antibodies were purchased from Cell Signaling Technology (Beverly, MA). Myc antibody was purchased from Babco (Richmond, CA). IGF-1 was purchased from Calbiochem (San Diego, CA).

Cell Culture—The human neuroblastoma cell line SH-SY5Y, kindly provided by Dr. June Biedler (Memorial Sloan-Kettering Cancer Center, Rye, NY) and the Chinese hamster ovary cells (CHO; ATCC CCL-61) from the American Type Culture Collection (Manassas, VA) were maintained in Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum at 37°C in 10% CO2. Cells were detached for passage by adding 0.25% trypsin. For all experiments in the presence of compounds the cells were maintained in Neural Basal media supplemented with B27 serum-free nutrient.

UV Treatment of SH-SY5Y Cells—In experiments utilizing MAPK inhibitor treatments, cells were pretreated with vehicle (0.2% dimethyl sulfoxide), 15 μM SB203580, 0.5 μM CEP-1347, 10 μM LY294002, or 50 μM PD98059 for 1 h at 37°C. Cells in a minimal amount of medium were irradiated in a UV Stratalinker (Stratagene, La Jolla, CA) for varying times as indicated. The medium was then replaced, and the cells were returned to the incubator for varying amounts of time before further analysis.

Immunoblots and Immunoprecipitations—Cell extracts were prepared by suspending cells in lysis buffer containing 10 mM Tris, 50 mM NaCl, 1% Triton X-100, 2 mM sodium vanadate, 0.2 mM 4-(2-aminoethyl)benzenesulfonyl fluoride (AEBSF, Roche Molecular Biochemicals), and proteinase inhibitor mixture (Sigma). Immunoblots and immunoprecipitations were performed according to the procedure of Harlow and Lane (29). Cell lysates or immunoprecipitates were separated on precast 10% Tris/glycine SDS-PAGE gels (Invitrogen, San Diego, CA), transferred to polyvinylidene difluoride membranes (Millipore, Chicago, IL), and probed with antibodies as indicated in buffer containing 10 mM Tris-HCl (pH 7.2), 150 mM NaCl, 0.1% Triton X-100, and 3% bovine serum albumin. After incubating with alkaline phosphatase-conjugated secondary antibody, blots were developed by the ECF reagent (Amersham Biosciences, Inc.) and analyzed on the STORM PhosphorImager System (Molecular Dynamics). The degree of p66 ShcA mobility shift as indicated by the separate lanes was determined for each sample on the STORM PhosphorImager system (Molecular Dynamics).

RESULTS

UV Irradiation Results in Serine/Threonine Phosphorylation of the 66-kDa Shc Isoform in SH-SY5Y Cells—p66Shcα/A is serine/threonine-phosphorylated in response to growth factors, such as epidermal growth factor and insulin, and to cellular stress induced by UV irradiation, hydrogen peroxide, or Taxol treatment (30, 24, 10, 25). To determine whether p66ShcA was also phosphorylated on serine/threonine residues in response to cellular stress in a neuronal environment, the human neuroblastoma SH-SY5Y cell line was UV-irradiated, and electrophoretic mobility was assessed. A retarded electrophoretic mobility shift of p66ShcA was observed in response to UV irradiation after 3 h of post-treatment incubation (Fig. 1A and B, lanes 1 and 2). In contrast, the mobilities of the p52 and p46 isoforms were not affected. The decreased mobility of p66ShcA is characteristic of post-translational modifications, such as protein phosphorylation. Indeed, this decreased mobility was abolished by serine/threonine protein phosphatase 2A1 treatment (Fig. 1A, lanes 4 and 5). These data support the observation that p66ShcA is phosphorylated at serine/threonine residue(s) after UV irradiation in SH-SY5Y cells, similar to previous results obtained in other cell systems (10, 24, 25, 30).

Inhibition of JNK Activation Prevents Phosphorylation of p66ShcA in SH-SY5Y Cells upon UV Irradiation—MAPKs, most notably JNKs and p38, have been shown to play an important role in response to oxidative damage in neuronal environments (31–34). To investigate whether the MAPKs were responsible for p66ShcA phosphorylation, several known kinase inhibitors were used to determine whether p66ShcA phosphorylation in response to UV irradiation. SH-SY5Y cells were treated with CEP-1347, an MLK inhibitor, SB203580, a p38 inhibitor, LY294002, a phosphatidylinositol 3-kinase inhibitor, or PD98059, a MEK1 inhibitor, followed by UV irradiation. The SB203580, LY294002, and PD98059 compounds were effective in blocking UV-induced phosphorylation of their respective downstream targets, ATF-2, AKT, and ERK1/2 (data not shown); however, they had no effect on the UV-induced p66ShcA phosphorylation.
mobility shift (Fig. 1B). In contrast, at a concentration at which CEP-1347 blocked JNK phosphorylation (data not shown), p66ShcA mobility shift was also inhibited to the same degree as protein phosphatase 2A1 treatment (Fig. 1, A and B). As previously reported, both MLK3 and JNK activation are blocked in the presence of CEP-1347 in SH-SYSY cells after UV irradiation (27). These results suggested that MLKs or kinase(s) regulated by MLKs might be responsible for p66ShcA phosphorylation upon UV irradiation.

Phosphorylation of p66ShcA at Serine 36 Is Regulated by the JNK Pathway—The major site of serine phosphorylation of p66ShcA after hydrogen peroxide treatment of mouse embryonic fibroblasts is serine 36, and overexpression of a phospho-deficient serine 36 mutant of p66ShcA in p66ShcA-null fibroblasts attenuates hydrogen peroxide induced cell death (10). To determine whether the MLK/JNK pathway affected p66ShcA phosphorylation as suggested by use of the kinase inhibitors in the whole cell studies described above (Fig. 1B) and in an effort to identify the kinase(s) responsible for its phosphorylation, kinases in the MLK/JNK pathway were co-expressed with either wild type (wt p66ShcA) or a serine 36 to alanine (S36A) mutant of p66ShcA and electrophoretic mobility was assessed. The wild type and mutant S36A cDNAs were tagged with a myc epitope and expressed in CHO cells along with MLK3 and JNK1. As described previously (27), overexpression of MLK3 led to phosphorylation/activation of JNK (Fig. 2A, lower panel). As shown in Fig. 2A, in the absence of an upstream activator of JNK1, co-expression of JNK1 with either wt p66ShcA or S36A p66ShcA did not alter the mobility of the Shc proteins. In contrast, co-expression of MLK3, JNK1 and wt p66ShcA, but not S36A p66ShcA protein, induced a decreased electrophoretic mobility of p66ShcA (Fig. 2A). Interestingly, the decreased mobility of wt p66ShcA was not observed when MLK3 and kinase-dead JNK1 (JNK1KD) were co-expressed (Fig. 2B). It was concluded from these results that the Shc kinase was not MLK3 or kinase(s) downstream of MLK3 and upstream of JNK. These data suggested that JNK, when activated either by UV treatment or MLK3 expression, was able to phosphorylate p66ShcA in whole cells.

Both JNKs and p38 Can Phosphorylate Serine 36 of p66ShcA in Vitro—Whole cell analysis indicated that the JNKs might be responsible for p66ShcA phosphorylation. An in vitro kinase assay was used to test candidate kinases directly. To generate recombinant wt p66ShcA and S36A p66ShcA proteins, wild type or mutant p66ShcA cDNAs were fused to the CBP sequence. Shc proteins were expressed as CBP fusion proteins and purified on a calmodulin affinity column. The recombinant Shc proteins were then used as substrates in a kinase assay to determine whether proteins in the MAPK pathway could phosphorylate Shc specifically at serine 36 in vitro.

As shown in the Fig. 3, A and B, all kinases tested were capable of phosphorylating both themselves and MBP, indicating that all of the kinases tested were active in the kinase reactions. ERK1 (Fig. 3B) and MEKK1 (data not shown) were not able to phosphorylate Shc proteins, whereas MLK1, MLK2, MLK3, MLK4, and MKK7 appeared to phosphorylate wt p66ShcA with no selectivity at serine 36 (Fig. 3C). In contrast, only ~10% of radioactivity was incorporated into S36A p66ShcA relative to wt p66ShcA when JNK1 and JNK2 were incubated with Shc proteins (Fig. 3D, B and C). Similarly, JNK3 and p38 also demonstrated some selectivity to the serine 36 site but to a lesser extent than JNK1 and JNK2. Furthermore, there was a decreased electrophoretic mobility of p66ShcA protein detected
JNK Phosphorylates Shc upon UV Irradiation

**Discussion**

The p66ShcA protein appears to be a key protein in mediating cellular responses upon oxidative stress. P66ShcA knock-out mice are less susceptible to the pesticide paraquat and also live 30% longer than their normal counterparts (10). Of particular interest is the demonstration that phosphorylation of p66ShcA at serine 36 is important for the normal cell death response due to oxidative damage. Blocking phosphorylation of serine 36 prevents hydrogen peroxide-induced cell death in fibroblasts (10). In consideration of these observations, it was of interest to determine the kinase(s) that mediated serine 36 phosphorylation of p66ShcA in response to UV irradiation.

In a UV-irradiated SH-SY5Y model, it is first shown that an inhibitor of the MLK pathway, CEP-1347, prevents p66ShcA phosphorylation (Fig. 1). This inhibition appears to be specific because p38, phosphatidylinositol 3-kinase, and MEK1 inhibitors have no effect. CEP-1347 has been previously demonstrated to inhibit members of the MLK family, which are upstream activators of MKK4/7 leading to JNK activation. Thus, our initial results implicated members of the MLK family or kinases downstream of these proteins as potential Shc kinases. Phosphorylation of p66ShcA at serine 36 is blocked in cells overexpressing MLK3 and a kinase-dead JNK1 (Fig. 2) suggesting that JNK1 is an Shc kinase. In support of these findings, three JNK isoforms can phosphorylate p66ShcA predominantly at serine 36, as determined in an isolated kinase assay (Fig. 3). It is concluded from both the whole cell and in vitro analyses that we have identified JNKs as candidate Shc kinases mediating phosphorylation at serine 36 of p66ShcA.

The JNKs (also called stress-activated protein kinases (SAPKs)) are an integral part of the signal transduction pathways that transmit extracellular signals of stress and injury to a variety of cellular responses including cell death (5–7). JNK is activated upon DNA and oxidative damage due to UV irradiation, hypoxia, or apoptotic agents such as Taxol (35–37). By both molecular and pharmacological approaches, it has been demonstrated that blocking JNK activation can prevent apoptosis after UV irradiation and oxidative stress (9, 38–40). One substrate of JNKs is the transcription factor c-Jun. In primary embryonic superior cervical ganglia and hippocampal neurons, activation of c-Jun after trophic withdrawal or potassium depolarization can lead to neuronal death, whereas blocking c-Jun either by neutralizing antibody, antisense oligonucleotides, or expression of dominant-negative proteins prevents cell death (41–44). In support of the cell culture results, the hippocampal neurons from either JNK3 knock-out or phospho-null c-Jun mice are protected from kainate-induced cell death (45, 46). Although it is clear that blocking c-Jun is sufficient to prevent certain types of cell death, the discovery that p66ShcA is also a substrate of the JNKs suggests that perhaps more than one pathway is triggered by activation of JNKs leading to cell death. Whether p66ShcA phosphorylation and c-Jun activation represent two unique or redundant events leading to cell death remains to be elucidated.

It has been reported that half of p66ShcA protein is phosphorylated at serine/threonine residues and associates with Grb2 protein upon epidermal growth factor stimulation (30). The serine/threonine-phosphorylated form of p66 competes with the p52 and p46 Shc protein for the limited pool of Grb2 and thus dominantly inhibits epidermal growth factor receptor downstream signaling events. We tested whether phosphorylated p66ShcA protein acts as a sink for Grb2 binding and thus may dominantly interfere or attenuate the survival pathway. Rather than an increase in p66ShcA associated Grb2 binding, UV irradiation resulted in a decreased amount of p66ShcA protein associated with Grb2. These results are consistent with the lack of Grb2 binding to Taxol-induced serine-phosphorylated p66ShcA (25). Although these studies do not distinguish the specific serine phosphorylated sites induced by the various stimuli, overall the results suggest that serine phosphorylation...
JNK Phosphorylates Shc upon UV Irradiation

may lead to distinct downstream events dependent perhaps upon the site of phosphorylation and the upstream kinases involved.

Both JNKs and p38 are activated by UV irradiation and are capable of phosphorylating p66Shc in vitro (Fig. 3). However, only JNKs appear to phosphorylate p66Shc in SH-SY5Y cells, because inhibition of JNKs by CEP-1347 prevented p66Shc phosphorylation, whereas inhibition of p38 kinase by SB25038 had no effect after UV irradiation (Fig. 1). Nevertheless, it is possible that the choice of kinase employed to phosphorylate p66Shc may depend upon the cellular context.

The genetic data from the p66Shc knockout mice suggest that serine phosphorylation of this protein may have profound effects on the extent of oxidative damage either directly incited or incurred with aging (10). Our data link the phosphorylation of p66Shc to the JNKs, a family well established as involved in oxidative stress responses. It will be of further interest to determine the downstream effects of p66Shc phosphorylation and the extent to which this protein contributes to JNK-mediated cell death.

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