p66\textsuperscript{shc} Inhibits Pro-survival Epidermal Growth Factor Receptor/ERK Signaling during Severe Oxidative Stress in Mouse Renal Proximal Tubule Cells*\textsuperscript{5}

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The fully executed epidermal growth factor receptor (EGFR)/Ras/MEK/ERK pathway serves a pro-survival role in renal epithelia under moderate oxidative stress. We and others have demonstrated that during severe oxidative stress, however, the activated EGFR is disconnected from ERK activation in cultured renal proximal tubule cells and also in renal proximal tubules after ischemia/reperfusion injury, resulting in necrotic death. Studies have shown that the tyrosine-phosphorylated p46/52 isoforms of the ShcA family of adaptor proteins connect the activated EGFR to activation of Ras and ERK, whereas the p66\textsuperscript{shc} isoform can inhibit this p46/52\textsuperscript{shc} function. Here, we determined that severe oxidative stress (after a brief period of activation) terminates activation of the Ras/MEK/ERK pathway, which coincides with ERK/JNK-dependent Ser\textsuperscript{36} phosphorylation of p66\textsuperscript{shc}. Isoform-specific knockdown of p66\textsuperscript{shc} or mutation of Ser\textsuperscript{36} to Ala, but not to Asp, attenuated severe oxidative stress-mediated ERK inhibition and cell death in vitro. Also, severe oxidative stress (unlike ligand stimulation and moderate oxidative stress, both of which support survival) increased binding of p66\textsuperscript{shc} to the activated EGFR and Grb2. This binding dissociated the SOS1 adaptor protein from the EGFR-recruited signaling complex, leading to termination of Ras/MEK/ERK activation. Notably, Ser\textsuperscript{36} phosphorylation of p66\textsuperscript{shc} and its increased binding to the EGFR also occurred in the kidney after ischemia/reperfusion injury in vivo. At the same time, SOS1 binding to the EGFR declined, similar to the in vitro findings. Thus, the mechanism we propose in vitro offers a means to ameliorate oxidative stress-induced cell injury by either inhibiting Ser\textsuperscript{36} phosphorylation of p66\textsuperscript{shc} or knocking down p66\textsuperscript{shc} expression in vivo.

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\textsuperscript{2}The on-line version of this article (available at http://www.jbc.org) contains a supplemental figure.

\textsuperscript{3}The abbreviations used are: MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase; ERK, extracellular signal-regulated kinase; EGFR, epidermal growth factor receptor; I/R, ischemia/reperfusion; EGF, epidermal growth factor; siRNA, small interfering RNA; JNK, c-Jun N-terminal kinase.
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reperfusion (I/R) injury), excess amounts of reactive oxygen species and free radicals (18, 19), including H₂O₂, are formed and have been postulated to play a crucial role in the pathogenesis of renal injury (19). During I/R injury, the EGFR is activated in the proximal tubules of the kidney, but ERK activation is absent, and the proximal tubules undergo necrotic death (20, 21). We made a similar observation in vitro: a high dose of H₂O₂ causes necrotic (oncotic) death of renal proximal tubule cells with concomitant activation of the EGFR but not ERK, and ectopic activation of endogenous ERK rescues the cells from injury (14, 15). On the other hand, during moderate stress (moderate dose of H₂O₂) in vitro, renal proximal tubule cells survive through activation of the EGFR and ERK (14, 15). These observations suggest that the activated EGFR could serve a prodeath function (22) in addition to its more widely accepted role of enhancing regeneration of the injured segments of the kidney (20, 23, 43, 44). These dual roles of the EGFR have been described previously, as reactive oxygen species-dependent activation of the EGFR leads to cell death in renal proximal tubule cells exposed to cisplatin (24), and functional inactivation of the EGFR in renal proximal tubular cells reduces tubular-interstitial lesions after renal injury (25). Because the expression of p66shc in the kidney has been demonstrated (26), we postulate here that activated p66shc inhibits the survival signaling pathway by disconnecting the activated EGFR from Ras/ERK activation depending on the extent of oxidative stress.

Accordingly, the aim of this study was to test the hypothesis that serine phosphorylation of p66shc during severe oxidative stress in renal proximal tubule cells inactivates ERK and leads to cell death. We sought means to manipulate either expression or Ser³⁶ phosphorylation of p66shc to restore ERK activation and survival.

MATERIALS AND METHODS

Antibodies—Anti-phospho-ERK1/2 (Thr²⁰²/Tyr²⁰⁴) and anti-ERK1/2 antibodies (Cell Signaling Technology, Beverly, MA), anti-total Shc and anti-SOS1 antibodies (BD Transduction Laboratories), anti-Grb2 antibody (Santa Cruz Biotechnology, La Jolla, CA), anti-phospho-p66shc (Ser³⁶) and anti-phospho-Shc (Tyr²³⁹/Tyr²⁴⁰) antibodies (Alexis Biochemicals/AXXORA, San Diego, CA), and anti-EGFR antibody and the ras activation assay kit (Millipore) were obtained from the indicated sources. Control IgG antibodies (sheep and rabbit) were obtained from Santa Cruz Biotechnology. Control IgG antibodies did not cause any cross-reaction with the various primary antibodies in the intraperitoneal studies (data not shown).

Cell Lines and Animals—The immortalized mouse proximal tubule line TKPTS was used as described (14, 15). Oxidative stress was induced by treatment of semiconfluent cells with 0.5 or 1 mM H₂O₂ for various time points. For in vivo experiments, 129Sv mice were used, and I/R injury was induced as described (14, 15).

Protein Isolation, Western Blotting, Immunoprecipitation, and Ras Activation—Kidneys were removed and homogenized in radioimmune precipitation assay buffer as described (14, 15). Similarly, monolayers of TKPTS cells were lysed in radioimmune precipitation assay buffer. Protein content was determined using a Bio-Rad protein determination assay. 100 µg of proteins from cell or tissue lysates were separated by SDS-PAGE and transferred to a polyvinylidene difluoride membrane (Bio-Rad). The filters were hybridized with the appropriate primary antibodies followed by an horseradish peroxidase-conjugated secondary antibody. The bands were visualized using the ECL method (Amersham Biosciences) and quantified by densitometry (UN-SCAN-IT, Silk Scientific, Inc., Orem, UT). For immunoprecipitation, 400 µg of total cell lysates were incubated with the appropriate primary antibody overnight at 4 °C using the Catch and Release Version 2.0 reversible immunoprecipitation system (Millipore). Immunoprecipitated proteins were resolved by SDS-PAGE as described above. In immunoprecipitation studies, negative control IgGs (rat or sheep) were also used to determine cross-reactivity. We found that these control antibodies did not cross-react upon Western blotting (data not shown). Activated ras was determined using an activation kit (Millipore) following the instructions of the manufacturer.

Elk1-Luciferase Reporter Transactivation Assay—The pFR-Luc (reporter) and pAF2-Elk1 (fusion transactivator) plasmids were purchased from Stratagene (La Jolla, CA). Plasmids were transiently transfected into TKPTS cells using GenePORTER 2 reagent (Promega, Madison, WI) together with a β-galactosidase plasmid (Invitrogen) in 6-well plates as described (27). Luciferase activity was determined using a luciferase assay kit (Promega, Madison, WI) as suggested by the manufacturer under control conditions and 6 h after treatment with either epidermal growth factor (EGF) or H₂O₂ (0.5 or 1.0 mM). This time point was chosen because cells show no obvious damage at this time. The relative luciferase activity was measured and normalized to the amount of activity detected for a cotransfected β-galactosidase plasmid.

Transient Transfection of shc Plasmids—The following plasmids were used: wild-type p66shc plasmid, p66shc small interfering RNA (siRNA)-expressing (pTERsisi66shc) plasmid, the p66shc(S36A) mutant plasmid (in which Ser³⁶ was mutated to Ala), and the p66shc(S36D) phosphomimetic mutant plasmid (in which Ser³⁶ was mutated to Asp). These plasmids were transiently transfected into TKPTS cells using GenePORTER 2 reagent in 6-well plates. Treatment protocols are described in the legends of the appropriate figures.

Establishment of a p66shc Knockdown Cell Line—TKPTS cells were stably transfected in a T-25 culture flask with either 6 µg of pTERsisi66shc plasmid or the appropriate vector using GenePORTER 2 reagent. After 48 h, the cells were split into 100-mm Petri dishes in the presence of 200 µg/ml Zeocin. 7–10 days later, the surviving colonies were picked up by Scienceware sterile cloning disks and serially propagated in 24-, 12-, and 6-well plates. The extent of p66shc knockdown was determined by Western blotting. For additional experiments, we used the control T18C and p66shc knockdown Tsi66-21 clones.

Statistical Analysis—Statistical differences between the treated and control groups were determined by Student’s paired t test. Differences between means were considered significant if p < 0.05. All analyses were performed using the Sigmaplot 3.5 software package.
RESULTS

Activation of the EGFR-mediated Ras/MEK/ERK Pathway Is Attenuated during Severe Oxidative Stress—TKPTS cells survive 0.5 mM H₂O₂ treatment via growth arrest, whereas they undergo necrotic (oncotic) death after treatment with 1 mM H₂O₂ (14, 15). Previously, we determined that both 0.5 and 1 mM H₂O₂ tyrosine-phosphorylate the EGFR, similar to EGF treatment (15), but that ERK activation is absent after treatment of the cells with 1 mM H₂O₂, whereas 0.5 mM H₂O₂-induced ERK phosphorylation is persistent for a longer time (14), similar to treatment with EGF (data not shown). Here, we wanted to determine whether the activated EGFR is connected to downstream elements of signaling such as Ras, MEK, and ERK. Accordingly, TKPTS cells were treated with EGF or H₂O₂ (0.5 mM or 1 mM) for various time points, and cell lysates were collected. Ras activation was determined by a kinase assay together with phosphorylation of MEK1 and ERK1/2 (Fig. 1). Surprisingly, we found that 30 min after treatment, the Ras/MEK/ERK pathway was activated regardless of the concentration of H₂O₂, similar to treatment with EGF. We also determined that the observed ERK activation at this time point required both the EGFR and ras, as pretreatment of the cells with AG1478 (an EGFR inhibitor) or infection with a dominant-negative ras adenovirus (28) inhibited ERK1/2 phosphorylation (data not shown). By contrast, activation of ras, as well as phosphorylation of MEK1 and ERK1/2, was greatly attenuated after 60 min of treatment with 1 mM H₂O₂, whereas EGF or 0.5 mM H₂O₂ treatment still sustained ras/MEK1/ERK activation.

Status of Shc Phosphorylation during Oxidative Stress in Vitro—The Shc adaptor proteins can be tyrosine-phosphorylated by various agents (11, 29, 45), whereas stress conditions such as H₂O₂ and UV irradiation can phosphorylate p66<sup>shc</sup> at Ser<sup>36</sup> (11). The tyrosine- or serine-phosphorylated Shc isoforms play opposite roles in EGFR-mediated ERK activation (8, 30). TKPTS cells were either pretreated with 50 μM U0126 or infected with dominant-negative JNK (dnJNK) adenovirus (multiplicity of infection = 25) prior to treatment with 1 mM H₂O₂. Tyrosine and serine phosphorylation (p) of ShcA isoforms were determined as described under “Materials and Methods.”

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FIGURE 1. Severe oxidative stress inhibits the Ras/MEK/ERK pathway time-dependently in renal proximal tubule cells. TKPTS cells were treated with 10 ng/ml EGF or 0.5 or 1 mM H₂O₂ for the time periods indicated. Activated ras, phosphorylated (p) ERK1/2 and MEK1, and total ERK1/2 and MEK1 were determined as described under “Materials and Methods.” Data shown are typical examples of three independent experiments.

FIGURE 2. Status of Shc phosphorylation during oxidative stress in renal proximal tubule cells. A, TKPTS cells were treated with 0.5 or 1 mM H₂O₂ for 30 min. Cell lysates were immunoprecipitated (i.p.) with an anti-Shc antibody and then immunoblotted (WB) with the indicated antibodies. pTyr<sub>shc</sub>, anti-phospho-Shc (Tyr<sup>239</sup>/Tyr<sup>240</sup>) antibody; pS<sup>36</sup><sub>shc</sub>, anti-phospho-p66<sup>shc</sup> (Ser<sup>36</sup>) antibody. Data shown are typical examples of three independent experiments. B, TKPTS cells were either pretreated with 50 μM U0126 or infected with dominant-negative JNK (dnJNK) adenovirus (multiplicity of infection = 25) prior to treatment with 1 mM H₂O₂. Tyrosine and serine phosphorylation (p) of ShcA isoforms were determined as described under “Materials and Methods.”
but not tyrosine phosphorylation of p66
ERK and JNK also attenuated Ser36 phosphorylation of p66
negative JNK, respectively (14). Surprisingly, inhibition of both
(phosphorylated) and can be inhibited by U0126 or dominant-
(Fig. 2A). Under this condition, both ERK and JNK are activated
phosphorylation of p66shc is determined by Western blotting in the Tsi66-21 cell line (derived
from TKPTS cells by stably transfecting a p66shc siRNA-expressing plasmid). C. shown is the status of ERK phosphorylation (p) in the control
(T18C) and p66shc knockdown (Tsi66-21) cell lines after treatment with a high
dose of H2O2 for 1 h. Data shown are typical examples of three inde-
pendent experiments.

**Figure 3.** p66shc attenuates ERK activation during severe oxidative stress in renal proximal tubule cells. A. TKPTS cells were transiently cotransfected with the Elk1-luciferase (Luc) reporter transactivation system, a β-galactosidase plasmid, and the various plasmids described under “Materials and Methods.” Luciferase activity was determined after treatment with EGF or 0.5 or 1 mM H2O2. cMEK, constitutively active MEK; si66shc, p66shc siRNA-expressing plasmid; S36Ap66shc, p66shc mutant, in which Ser36 is mutated to Ala; S36Dp66shc, p66shc mutant in which Ser36 is mutated to Asp. Data are expressed as the percent of the untreated control. Values are given as means ± S.D. (n = 3), *p < 0.001 compared with the untreated control. B. shown is the isoform-specific knockdown of p66shc as determined by trypan blue staining (Fig. 4A). In addition, survival of Tsi66-21 cells was significantly higher 6 or 24 h after treatment with 1 mM H2O2, as determined by trypan blue staining (Fig. 4A). In addition, transient transfection of HKPTS cells by a p66shc siRNA-expressing vector (34) significantly increased (by 2.5-fold) the number of surviving cells 24 h after treatment with 1 mM H2O2, as determined by trypan blue staining (Fig. 4A). In addition, transient transfection of the S36A mutant, but not the phosphomimetic mutant (S36D), also increased survival (by 2-fold). Similarly, survival of Tsi66-21 cells was significantly higher 6 or 24 h after treatment with a high dose of H2O2 (Fig. 4B) compared with that of its control counterpart. These results prove that through Ser36 phosphorylation, p66shc is necessary for ERK inactivation (Fig. 3) and the consequent cell death during severe oxidative stress.

**Involvement of p66shc and Ser36 Phosphorylation in Cell Death during Severe Oxidative Stress—**Previously, we showed that in the absence of ERK activation, TKPTS cells undergo necrotic death during severe oxidative stress but that ectopic activation of endogenous ERK rescues cells from that death (14). Thus, isoform-specific knockdown of endogenous p66shc or expression of its mutant (S36A) that restores ERK activation (Fig. 3) should ameliorate cell death under severe oxidative stress. Transient transfection of HKPTS cells by a p66shc siRNA-expressing vector (34) significantly increased (by 2.5-fold) the number of surviving cells 24 h after treatment with 1 mM H2O2, as determined by trypan blue staining (Fig. 4A). In addition, transient transfection of the S36A mutant, but not the phosphomimetic mutant (S36D), also increased survival (by 2-fold). Similarly, survival of Tsi66-21 cells was significantly higher 6 or 24 h after treatment with a high dose of H2O2 (Fig. 4B) compared with that of its control counterpart. These results prove that through Ser36 phosphorylation, p66shc is necessary for ERK inactivation (Fig. 3) and the consequent cell death during severe oxidative stress.

**p66shc Disrupts the EGFR-p52shc-Grb2-SOS1 Complex during Severe Oxidative Stress—**In the next step, we wanted to determine how p66shc uncouples the activated EGFR from ERK activation during severe oxidative stress. Accordingly, TKPTS cells were treated with EGF or H2O2 (0.5 or 1 mM) for different time points. Cell lysates were obtained, and protein complexes were immunoprecipitated with an anti-EGFR antibody, followed by immunoblotting with an anti-Shc, anti-Grb2, anti-SOS1, or anti-EGFR antibody. As shown in Fig. 5A, 30 min after treatment, the activated EGFR bound p46/52shc, Grb2, and SOS1 adaptor proteins regardless of the type of treatment. 60 min after treatment with 1 mM H2O2, however, binding of the p66shc isoform to the EGFR was also increased, with a concomitant decrease in SOS1 binding (Fig. 5B). By contrast, both EGF and 0.5 mM H2O2 retained the EGFR-p46/52shc, Grb2-SOS1

**Involvement of p66shc and Ser36 Phosphorylation in Inhibition of ERK Function during Severe Oxidative Stress—**We determined ERK activation/function using the Elk1-luciferase reporter transactivation system. Elk1 is a downstream target of ERK, and as such, its activation status reflects the activation status of ERK (33). Accordingly, TKPTS cells were transiently transfected with the Elk1-luciferase system together with a β-galactosidase plasmid and the plasmids indicated in Fig. 3A. As shown, a constitutively active MEK plasmid dramatically increased luciferase activity (8-fold) compared with the untreated control. EGF or 0.5 mM H2O2 treatment also increased luciferase activity, although to a lesser extent. By contrast, 1 mM H2O2 significantly suppressed Elk1-mediated luciferase activity. Notably, cotransfection of a p66shc siRNA-expressing plasmid or the p66shc(S36A) mutant (34) restored Elk1-luciferase activity after treatment with 1 mM H2O2. In contrast, the phosphomimetic S36D mutant did not rescue 1 mM H2O2-mediated inhibition of Elk1-luciferase activity.

We also used the Tsi66-21 cell line, which was derived from TKPTS cells by stably transfecting the p66shc siRNA plasmid, or its control vector-expressing counterpart (T18C). Fig. 3B shows significant knockdown of p66shc expression in Tsi66-21 cells. Treatment of this p66shc knockdown cell line with a high dose of H2O2 significantly increased phosphorylation of ERK, whereas the same treatment of the control cell line failed to demonstrate increased ERK phosphorylation (Fig. 3C). In addition, tyrosine phosphorylation of the p52shc isoform was longer lasting in the p66shc knockdown cell line than in the control cell line after treatment with H2O2 (data not shown). These results support the notion that Ser36-phosphorylated p66shc indeed attenuates ERK activation during severe oxidative stress.

**Involvement of p66shc and Ser36 Phosphorylation in Cell Death during Severe Oxidative Stress—**Previously, we showed that in the absence of ERK activation, TKPTS cells undergo necrotic death during severe oxidative stress but that ectopic activation of endogenous ERK rescues cells from that death (14). Thus, isoform-specific knockdown of endogenous p66shc or expression of its mutant (S36A) that restores ERK activation (Fig. 3) should ameliorate cell death under severe oxidative stress. Transient transfection of TKPTS cells by a p66shc siRNA-expressing vector (34) significantly increased (by 2.5-fold) the number of surviving cells 24 h after treatment with 1 mM H2O2, as determined by trypan blue staining (Fig. 4A). In addition, transient transfection of the S36A mutant, but not the phosphomimetic mutant (S36D), also increased survival (by 2-fold). Similarly, survival of Tsi66-21 cells was significantly higher 6 or 24 h after treatment with a high dose of H2O2 (Fig. 4B) compared with that of its control counterpart. These results prove that through Ser36 phosphorylation, p66shc is necessary for ERK inactivation (Fig. 3) and the consequent cell death during severe oxidative stress.

**p66shc Disrupts the EGFR-p52shc-Grb2-SOS1 Complex during Severe Oxidative Stress—**In the next step, we wanted to determine how p66shc uncouples the activated EGFR from ERK activation during severe oxidative stress. Accordingly, TKPTS cells were treated with EGF or H2O2 (0.5 or 1 mM) for different time points. Cell lysates were obtained, and protein complexes were immunoprecipitated with an anti-EGFR antibody, followed by immunoblotting with an anti-Shc, anti-Grb2, anti-SOS1, or anti-EGFR antibody. As shown in Fig. 5A, 30 min after treatment, the activated EGFR bound p46/52shc, Grb2, and SOS1 adaptor proteins regardless of the type of treatment. 60 min after treatment with 1 mM H2O2, however, binding of the p66shc isoform to the EGFR was also increased, with a concomitant decrease in SOS1 binding (Fig. 5B). By contrast, both EGF and 0.5 mM H2O2 retained the EGFR-p46/52shc, Grb2-SOS1
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**FIGURE 4.** p66$^{shc}$ mediates the death of renal proximal tubule cells during severe oxidative stress. A, TKPTS cells were transiently transfected with the plasmids indicated and treated with 1 mM H$_2$O$_2$. Cell survival was determined by trypan blue exclusion 24 h after treatment. Data are expressed as the percent of surviving untransfected or control plasmid-transfected cells after treatment with 1 mM H$_2$O$_2$. Values are given as means ± S.D. (n = 3), *, p < 0.001 compared with the 1 mM H$_2$O$_2$-treated cells. si66shc, p66$^{shc}$ siRNA-expressing plasmid. B, shown is the survival of the stably transfected control (T18C) and p66$^{shc}$ knockdown (Tsi66-21) cell lines after treatment with a high dose of H$_2$O$_2$. Cell survival was determined by trypan blue exclusion. Data are expressed as the percent of untreated cells. Values are given as means ± S.D. (n = 3), *, p < 0.001 compared with the untreated control; +, all T18C cells were dead.

These results suggest that p66$^{shc}$-bound Grb2 has decreased affinity for SOS1 during severe oxidative stress. Knockdown of p66$^{shc}$ expression decreased p66$^{shc}$ binding to the EGFR and restored SOS1 binding to the EGFR-p52$^{shc}$/p52$^{shc}$ complex (Fig. 6C). The results are summarized in Fig. 7. Accordingly, EGF, 0.5 mM H$_2$O$_2$, or even early 1 mM H$_2$O$_2$ treatment facilitates formation of the EGFR-p52$^{shc}$-Grb2-SOS1 complex, which leads to ras/ERK activation (Fig. 7A). In contrast, at a later time point (Fig. 7B), 1 mM H$_2$O$_2$ treatment induces binding of p66$^{shc}$ to the EGFR, resulting in Grb2 binding through p66$^{shc}$ and the consequent dissociation of the p52$^{shc}$-Grb2-SOS1 complex. Under this condition, ras and consequently ERK are not activated.

**I/R Injury in the Kidney Increases Phosphorylation of Shc Proteins and Their Binding to the EGFR—**First, protein lysates from kidneys of 129Sv mice that underwent I/R injury were immunoprecipitated with an anti-Shc antibody, followed by immunoblotting with an anti-phospho-p66$^{shc}$ (Ser$^{36}$), anti-phospho-Shc (Tyr$^{339}$/Tyr$^{340}$), or anti-Shc antibody. As shown in Fig. 8A, Ser$^{36}$ phosphorylation of p66$^{shc}$ was significantly increased 30 min and 24 h after reperfusion. Tyrosine phosphorylation of the p52$^{shc}$ isomorph that preceded p66$^{shc}$ Ser$^{36}$ phosphorylation also occurred in the kidney. Probing with an anti-EGF antibody showed that Shc proteins increasingly bound to the EGFR in the reperfusion phase. Immunoprecipitation of proteins with an anti-EGF antibody, followed by immunoblotting with an anti-Shc or anti-phospho-p66$^{shc}$ (Ser$^{36}$) antibody, revealed that the EGFR increasingly bound Ser$^{36}$-phosphorylated p66$^{shc}$ during reperfusion (Fig. 8A and B). In addition, there was a decline in the initial SOS1 binding to the EGFR complex 30 min after reperfusion that coincided with the increased binding of Ser$^{36}$-phosphorylated p66$^{shc}$ to the EGFR (Fig. 8A and B). Whether EGFR-bound and Ser$^{36}$-phosphorylated p66$^{shc}$ is localized to the proximal tubules that undergo necrotic death during I/R injury needs further studies. It is important to note that the EGFR is expressed mostly in renal proximal tubules (35). These observations are very similar to those shown in vitro after severe oxidative stress (Fig. 5B), suggesting a similar mechanism that inhibits the pro-survival ERK activation in vivo.
tyrosine-phosphorylated p46/52shc, i.e. inhibits coupling of the activated EGFR to downstream effectors such as Ras/MEK/ERK (8, 30). Lack of ERK activation could facilitate cell death during oxidative stress in renal tubular cells (14, 15, 37). This may be especially relevant under conditions in which phosphorylation of p66shc at Ser36 (11) prevents activation of ERK and increases cell death (8, 30).

Here, we have demonstrated that phosphorylation of ShcA proteins by H2O2 depends on the dose of the oxidant in renal proximal tubule cells in vitro: a moderate dose of H2O2 tyrosine-phosphorylates ShcA proteins (Fig. 2), similar to EGF treatment (data not shown). On the other hand, a high dose of H2O2 phosphorylates p66shc at Ser36, in addition to tyrosine phosphorylation of p52shc (Fig. 2A). This Ser36 phosphorylation of p66shc is MEK/ERK- and JNK-dependent (Fig. 2B), as shown previously (31, 32). Whether Ser36 phosphorylation of p66shc is a dose-dependent or all-or-none event will require further studies at the single-cell level rather than in a mixture of cells.

These results also suggest that the observed inhibition of the Ras/MEK/ERK pathway under severe oxidative stress (Fig. 1), the consequent death of these cells (14, 15), and the Ser36 phosphorylation of p66shc (Fig. 2A) are probably related and that the observed early ERK activation (Fig. 1), together with activation of JNK (14), serves as a negative feedback mechanism to shut down the ERK-dependent survival pathway during severe oxidative stress.

Indeed, siRNA-mediated knockdown of the p66shc isoform restored ERK function both in a transient reporter transactivation assay (Fig. 3A) and in a p66shc knockdown TKPTS cell line after treatment with a high dose of H2O2 (Fig. 3C). Similarly, knockdown of p66shc increased resistance to high dose H2O2-induced cell death (Fig. 4). Our results also show that substitution of Ser36 with Ala blunted high dose H2O2-induced inhibition of ERK function (Fig. 3A) and cell death (Fig. 4), whereas the phosphomimetic S36D mutant did not.

Furthermore, we sought to determine the mechanism by which Ser36-phosphorylated p66shc uncouples the activated EGFR from ras/MEK/ERK activation (Fig. 1) during severe oxidative stress. The activated EGFR recruits adaptor proteins such as Shc, Grb2, and SOS1 to activate ras (5). In this process, the tyrosine-phosphorylated p46/52shc isoforms play an important role, whereas p66shc could antagonize this process (30). It has been reported that p66shc and p52shc compete for the available Grb2 (8). Accordingly, we determined the assembly of the EGFR-Shc-Grb2-SOS1 complex by immunoprecipitation after
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Grb2 was restored during severe oxidative stress (Fig. 6B); this observation further supports the role of the Ser\textsuperscript{36} phosphorylation of \textit{p66\textsuperscript{shc}} in inhibition of ERK activation (Fig. 3) and cell death (Fig. 4). Khanday et al. (38) demonstrated that the N-terminal (CH2) domain of \textit{p66\textsuperscript{shc}} competes with the C-terminal region of SOS1 for the SH3 (Src homology 3) domain of Grb2, resulting in dissociation of the Grb2-SOS1 complex upon activation of \textit{p66\textsuperscript{shc}} during oxidative stress. This mechanism also implies reduced ras activation, as the Grb2-SOS1 complex formation is impaired. Okada et al. (8) showed that serine/threonine-phosphorylated \textit{p66\textsuperscript{shc}} is associated with Grb2 and competes for Grb2 binding with p52\textsuperscript{shc}, resulting in inhibition of EGFR function. Migliaccio et al. (30) demonstrated that the p66\textsuperscript{shc}-Grb2 complex does not activate ERK. Furthermore, the CH2 domain of \textit{p66\textsuperscript{shc}} contains Ser\textsuperscript{36}, the phosphorylation of which might affect p66\textsuperscript{shc}-Grb2 binding and the consequent dissociation of the Grb2-SOS1 complex, as our results suggest (Fig. 6B).

We hypothesize that during severe oxidative stress, EGFR-bound \textit{p66\textsuperscript{shc}} binds at least part of the available Grb2 and decreases the available Grb2-SOS1 complex, which would connect the activated EGFR to Ras/ERK activation.

We have also shown that in the \textit{p66\textsuperscript{shc}} knockdown cell line, the EGFR bound significantly less \textit{p66\textsuperscript{shc}} but more SOS1 compared with the control cell line during severe oxidative stress (Fig. 6C). Under these circumstances, the knockdown line also showed activation of ERK, whereas the control cell line did not (Fig. 3C).

Taken together, our data are consistent with the notion that severe oxidative stress (1 mM H\textsubscript{2}O\textsubscript{2}) disrupts the EGFR-p46/52\textsuperscript{shc}-Grb2-SOS1 complex through binding the available Grb2 to activated \textit{p66\textsuperscript{shc}}, as observed by others (8), resulting in the subsequent disruption of EGFR signaling to ERK. This process requires Ser\textsuperscript{36} phosphorylation of \textit{p66\textsuperscript{shc}}, as inhibition of Ser\textsuperscript{36} phosphorylation by the S36A mutant, but not the phosphomimetic S36D mutant, restored ERK function (Fig. 3) and increased survival (Fig. 4).

Notably, we found that the EGFR increasingly bound Ser\textsuperscript{36}-phosphorylated \textit{p66\textsuperscript{shc}} in addition to tyrosine-phosphorylated p46/52\textsuperscript{shc}. Grb2-SOS1 complex through binding the available Grb2 to activated \textit{p66\textsuperscript{shc}}, as observed by others (8), resulting in the subsequent disruption of EGFR signaling to ERK. This process requires Ser\textsuperscript{36} phosphorylation of \textit{p66\textsuperscript{shc}}, as inhibition of Ser\textsuperscript{36} phosphorylation by the S36A mutant, but not the phosphomimetic S36D mutant, restored ERK function (Fig. 3) and increased survival (Fig. 4).

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