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ARTICLES

Hpr6.6 Protein Mediates Cell Death From Oxidative Damage in MCF-7 Human Breast Cancer Cells

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Abstract Reactive oxygen species (ROS) cause cell death and are associated with a variety of maladies, from trauma and infection to organ degeneration and cancer. Cells mount a complex response to oxidative damage that includes signaling from transmembrane receptors and intracellular kinases. We have analyzed the response to oxidative damage in human breast cancer cells expressing the Hpr6.6 (*h*uman membrane *p*rogesterone *r*eceptor) protein. Although Hpr6.6 is related to a putative progesterone-binding protein, Hpr6.6 is widely expressed in epithelial tissues and shares close homology with a budding yeast damage response protein called Dap1p (*da*mage response protein related to membrane *p*rogesterone receptor). We report here that the Hpr6.6 protein regulates the response to oxidative damage in breast cancer cells. Expression of Hpr6.6 in MCF-7 cells sensitized the cells to death following long-term/low dose or short-term/high dose treatment with hydrogen peroxide. Cell death did not occur through a typical apoptotic mechanism and corresponded with hyperphosphorylation of the Akt and IκB proteins. However, inhibition of Akt activation and IκB degradation had no effect on Hpr6.6-mediated cell death, suggesting that Hpr6.6 regulates cell death through a novel oxidative damage response pathway. Our work indicates a key regulatory function for Hpr6.6 in epithelial tissues exposed to oxidative damage. J. Cell. Biochem. 90: 534–547, 2003.

Key words: breast cancer; oxidative damage; proliferation; cell death; signaling

Reactive oxygen species (ROS) are generated as a result of aerobic metabolism, with approximately 2% of all oxygen consumed by mitochondria being converted into ROSs [Cadenas and Davies, 2000]. These species attack membranes, proteins, and DNA, resulting in cell death from prolonged exposure. In addition to ROS production from normal metabolism, ROSs are generated by a variety of biological insults and responses to damage, including irradiation, hyperoxia, and exposure to chemical agents [reviewed by Mates and Sanchez-Jimenez, 2000]. ROSs are capable of generating genomic damage that can result in mutations that lead to cancer [Jackson and Loeb, 2001], and ROSs are a major source of cell death following ischemia from repurfusion of transplanted organs [reviewed by Serracino-Inglott et al., 2001], infection, and trauma.

Cells have developed a variety of pathways for responding to oxidative damage and removing cells with excessive damage from the population. These responses are specific for the type of tissue, the type of damage, and the amount of damage. As a result, oxidative damage can lead to proliferation, growth arrest, senescence, necrosis, or apoptosis in different cell types under different conditions. Because of the diversity of oxidative damage phenotypes, the proteins that regulate responses to oxidative

Abbreviations used: ERK1/2, extracellular-signal related kinases 1 and 2; Hpr6.6, human progesterone receptor; MAPR, membrane-associated progesterone receptor; MTT, 3-[4,5 dimethylthiazol-2-y]-2,5-diphenyltetrazolium bromide; PI3K, phosphoinositol-3 kinase; ROS, reactive oxygen species.

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damage in different cell types are of great interest.

Oxidative damage activates transmembrane receptor tyrosine kinases, which in turn activate cascades of intracellular tyrosine kinases [Martindale and Holbrook, 2002]. The epidermal growth factor receptor (EGFR) is activated by oxidative damage [Rossette and Karin, 1996], resulting in the stimulation of the phosphoinositol-3 kinase (PI3K) and the subsequent activation of Akt/protein kinase B [Wang et al., 2000]. Oxidative damage also leads to activation of the NF-κB transcription factor. NF-κB regulates the transcription of a broad variety of genes that regulate pro- and anti-apoptotic pathways [Richmond, 2002]. NF-κB is regulated by direct binding of the inhibitor protein IκB. In the IκB-bound form, NF-κB is sequestered in the cytoplasm [Richmond, 2002]. Phosphorylation of IκB targets the protein for degradation, releasing NF-κB to enter the nucleus. The role of NF-κB in the oxidative damage response is highly cell type-specific, suggesting pro-apoptotic [Luo et al., 1999; Vollgraf et al., 1999; Shou et al., 2000; Aoki et al., 2001] or anti-apoptotic [Li et al., 1997; Mattson et al., 2000; Yu et al., 2000; Kim et al., 2001] roles for NF-κB following oxidative damage [Clemens et al., 1997].

In this study, we have examined the effect of the Hpr6.6 protein on oxidative damage-induced cell death. The cDNA encoding Hpr6.6 was cloned [Gerdes et al., 1998] based on its homology to a porcine progesterone-binding protein that was purified from liver membrane fractions [Meyer et al., 1996]. Hpr6.6 shares the greatest identity with the rodent 25Dx proteins (Fig. 1A), although related proteins are found in evolutionarily distant organisms that include flowering plants, insects, worms, and both fission and budding yeast (Fig. 1A). Rat 25Dx was identified based on its induction in dioxin-treated liver tumors [Selmin et al., 1996], suggesting a role in cancer progression. 25Dx expression is repressed by progesterone and estrogen in the rat hippocampus during the female mating behavior lordosis and in murine fibroblasts [Krebs et al., 2000].

In spite of their apparent role in progesterone signaling, Hpr6.6 and 25Dx are most highly expressed in liver and kidney, with lesser expression in brain and pancreas [Selmin et al., 1996; Gerdes et al., 1998]. The liver and kidney are not well-characterized sites of progesterone action, although non-genomic effects of progesterone have been reported in liver and pancreatic cells [reviewed by Sutter-Dub, 2002]. In addition, the expression of Hpr6.6/25Dx homologues in organisms that do not signal through progesterone suggests other functions for this family of proteins. The sub-cellular locales of MAPR proteins also indicate complex roles in cellular function, as various MAPR proteins localize to the cell membrane or endoplasmic reticulum.

We identified the budding yeast homologue of Hpr6.6, called Dap1p for *da*mage-response protein related to *p*rogesterone-binding protein [Hand et al., 2003]. Loss of Dap1p function leads to extreme sensitivity to the DNA damaging agent MMS due to an inability to enter the cell cycle following damage [Hand et al., 2003]. Cells lacking Dap1p also have a partial arrest in sterol synthesis, elongated telomeres, and diminished mitochondrial function [Hand et al., 2003]. MMS-induced damage has many of the characteristics of oxidative stress [Gasch et al., 2001], suggesting that disruption of Dap1p function may contribute to sensitivity to oxidative damage.

In the present study, we have cloned the human MAPR homologue Hpr6.6 and expressed it in the human breast cancer cell line MCF-7. Hpr6.6 sensitized cells to death following oxidative damage, and death occurred through a non-apoptotic mechanism. The IκB and Akt proteins were both hyperphosphorylated in Hpr6.6-expressing cells, but do not contribute to cell death mediated by Hpr6.6. We conclude that Hpr6.6 directs a novel cell death pathway in response to oxidative damage. Hpr6.6 is expressed most highly in epithelial tissues that include liver, kidney, pancreas, and breast, and our work supports a role for Hpr6.6 in regulating cell death from oxidative damage in those organs.

MATERIALS AND METHODS

Cell Culture

MCF-7 cells were maintained in Modified Eagle Media containing 10% fetal bovine serum (FBS), 2 mM L-glutamine, and penicillin and streptomycin and NIH 3T3 cells were grown in Dulbecco's Modified Eagle Media containing 10% FBS with penicillin and streptomycin. Control or Hpr6.6 plasmids were transfected using Lipofectamine (Life Sciences, Rockville,

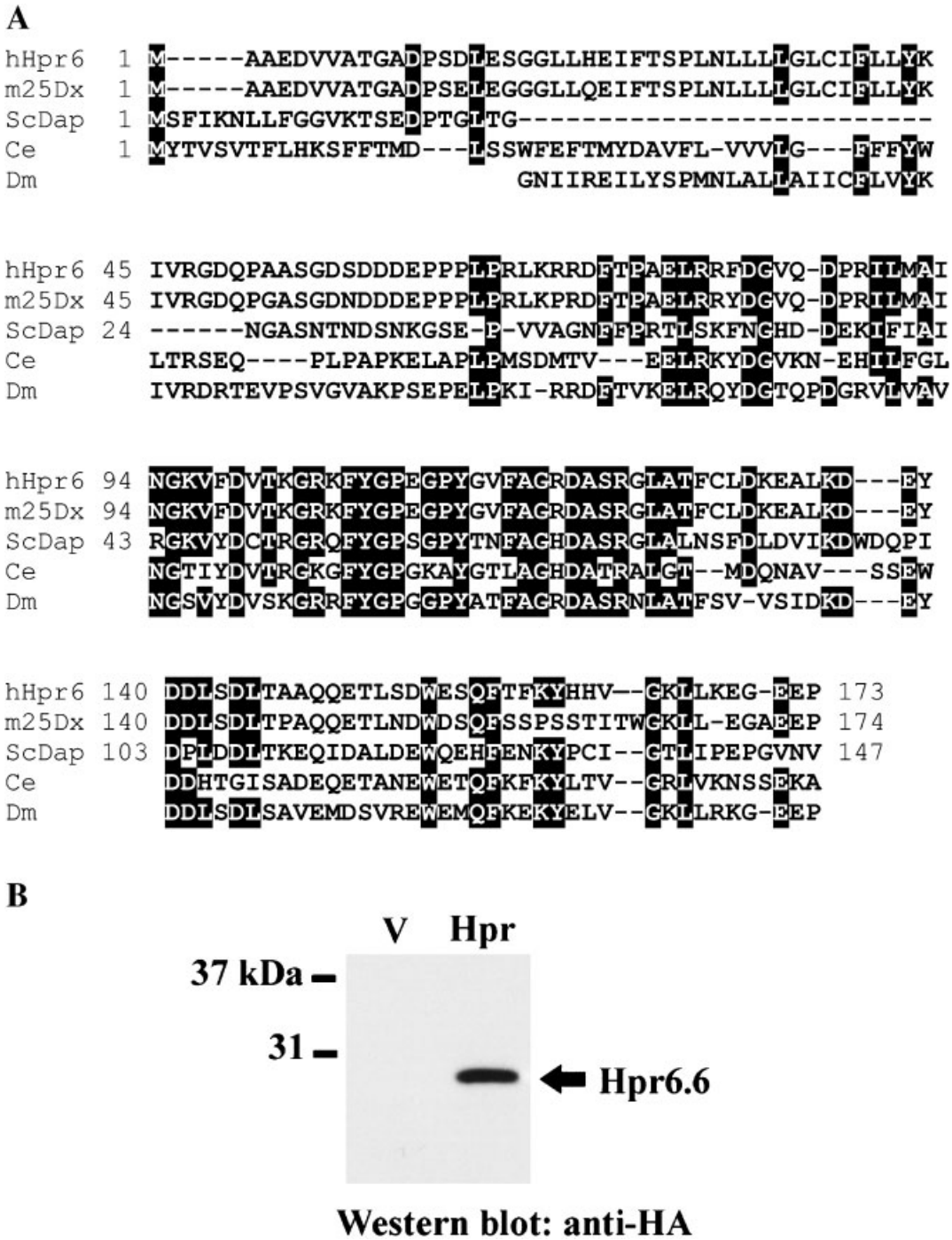

Fig. 1. Homology of Hpr6.6 to MAPR family proteins and expression in MCF-7 cells. **A**: Hpr6.6 shares extensive homology (black boxes) to proteins shared throughout eukaryotic species, with the highest degree of homology between residues 94–127 of Hpr6.6. The murine 25Dx (m25Dx), budding yeast Dap1p (ScDap), *Caenorhabditis elegans* K07E3.6 (CeK07), and *Drosophila melanogaster* LD12946p (DmLD) proteins are shown. **B**: Hpr6.6 was cloned in frame with an epitope tag sequence and expressed in MCF-7 human breast cancer cells. A western blot probed with an antibody to the HA epitope tag demonstrates expression and migration at 27 kDa.

MD) essentially as described [Xu et al., 1998], selected in Geneticin, and individual sub-clones were isolated and tested for Hpr6.6 expression by Western blot. Due to the inherent genetic instability of breast cancer cell lines, all experiments were performed within the first five passages after Hpr6.6-expressing isolates were identified.

Hydrogen peroxide (Sigma; St. Louis, MO) was added to cells in two regimens. First, cells were treated with 50–200 μM H_2O_2 in media for 20 h continuously, and metabolic activity, viability, and morphology were determined. Second, cells were treated with an "oxidative shock" of 1 mM H_2O_2 in media for 30–120 min, whereupon the H_2O_2-containing media was removed and replaced with normal growth media. Metabolic activity was determined after 20 h, while viability was measured after 1 h.

The PI3K inhibitor LY294002 (Sigma) was added to cells for 2 h at a concentration of 25 μM, then H_2O_2 incubation and viability assays were performed as described below. Cells were similarly pre-treated with cyclodextrin (Sigma) at a concentration of 7.5 mM. An adenovirus directing expression of a non-degradable form of the IκB protein [Wang et al., 1996; a gift from Matt Ewend, UNC Department of Surgery] or a matched control virus expressing *LacZ* were added to a final concentration of 5×10^7/ml. Cells were incubated with virus for 24 h prior to H_2O_2 exposure.

Plasmids

Hpr6.6 was cloned in two stages. First, the amino-terminal 109 amino acids were amplified from purified MCF-7 cell genomic DNA using the primers HPR1FHA and HPR330R. The former primer added a single HA epitope tag sequence (Asp-Tyr-Pro-Tyr-Asp-Val-Pro-Asp-Tyr-Ala-Glu-Ser) to the amino terminus of the Hpr6.6 open reading frame. The amplified product was sub-cloned into the TA cloning vector pCR2.1 (InVitrogen; Carlsbad, CA), then digested with *Bam*HI and *Kpn*I and ligated into the plasmid pBK-CMV (Stratagene; LaJolla, CA), forming the plasmid pRC38. A fragment containing the 3′ end of Hpr6.6 was amplified from the plasmid IMAGE 3254089 (Research Genetics; Huntsville, AL) using the primers HPR126F and HPR690RAPA, digested with *Apa*I, and inserted into the *Apa*I site of the plasmid pRC38, forming the plasmid pRC40. The integrity of all plasmid inserts was verified by automated sequencing.

Viability Assays

Viability following treatment with hydrogen peroxide was measured by two criteria. For MTT (3-[4,5 dimethylthiazol-2-y]-2,5-diphenyltetrazolium bromide, Sigma) assays, 5,000 cells were plated in appropriate wells of a 96-well culture dish. Cells were allowed to adhere to the dish overnight, and were then treated with various agents. Following treatment, media were removed and replaced with growth media containing 0.5 mg/ml MTT and incubated for 2 h. Following incubation, 80 μl of the MTT-containing media were removed and 100 μl of DMSO was added to each well. The cells were then incubated for 20 min on a rotating platform and the A_{595}/A_{650} was determined using a Molecular Devices Kinetic Microplate Reader microtiter dish reader. All readings were performed in triplicate and %viability was calculated as absorbance of treated cells divided by the average absorbance of three untreated samples. The results shown indicate representative results of at least three independent experiments.

For trypan blue assays, 100,000 cells/well were plated in 6-well tissue culture dishes, allowed to adhere overnight, and treated. At various time points, supernatants were collected, and the cells were washed with PBS, trypsinized, and pooled with the appropriate supernatants. Thus, we measured the number of viable cells in the adherent and non-adherent populations following treatment. Cells were centrifuged briefly and resuspended in 0.5 ml of PBS. A 0.1 ml of 0.4% trypan blue (Sigma) was added to each sample, and the cells were incubated for 5 min at room temperature. The number of blue and clear cells were then counted. All measurements were performed in triplicate, and the reported results are representative of at least three independent experiments.

Immunological Detection

Cells were lysed in NP-40 buffer (1% NP-40, 20 mM Tris, pH 7.4, 150 mM NaCl, 5 mM EDTA, 1 mM Na_3VO_4, and 10 μg/ml of the protease inhibitors aprotinin and leupeptin) essentially as described [Cance et al., 1994]. HA-tagged proteins were detected using the HA11 monoclonal antibody (BabCo; Berkeley, CA). Other antibodies used were anti-phosphoSer32/Ser36-IκB, anti-IκB, anti-Akt, and anti-phospho-Ser473-Akt from Cell Signaling, Inc. (Beverly, MA). Antibodies to tubulin were from Sigma; antibodies to phospho-Tyr204 of ERK1/2 were from Santa Cruz Biotechnology (Santa Cruz, CA); and anti-ERK1/2 was from Upstate (Waltham, MA). The monoclonal antibody to FAK was purchased from Upstate, Inc.,

and the anti-caspase-8 antibody was from Santa Cruz.

RESULTS

Hpr6.6 Sensitizes MCF-7 Cells to Cell Death Following Oxidative Damage

Hpr6.6 is a 27-kDa protein with homology to a family of putative membrane-associated progesterone-binding proteins. We expressed the Hpr6.6 protein fused to an HA epitope tag in MCF-7 breast cancer cells. The Hpr6.6 protein was readily detectable by western blot using an anti-HA monoclonal antibody (Fig. 1B) and migrated as a 27 kDa protein. We named the cell lines MCF-7/vector for cells transfected with the pBK-CMV control plasmid or MCF-7/Hpr6.6 for cells transfected with a plasmid encoding Hpr6.6.

Hpr6.6 caused cells to die when exposed to oxidative damage. MCF-7/Hpr6.6 cells were approximately 8-fold more sensitive to 100 μM H_2O_2 than control cells (Fig. 2A, right bars), while the difference was less extreme at a dose of 50 μM H_2O_2 (Fig. 2A, center bars). For these experiments, viability was analyzed by MTT assay, which measures mitochondrial activity. All measurements were performed in triplicate, and this result has been repeated more than ten times during the progress of this project.

While inability to metabolize MTT indicates a loss of mitochondrial activity, it is not a strict measurement of viability. In contrast, ability to exclude the compound trypan blue distinguishes cells with intact membranes from cells which have ruptured their membranes and are no longer viable. The trypan blue assay is also instant and does not rely on an extended incubation time, so we were able to use the trypan blue assay to determine the time at which cells died after oxidative damage. We compared MCF-7/vector and MCF-7/Hpr6.6 cells 7 and 24 h following treatment with 100 μM H_2O_2, and found that MCF-7/Hpr6.6 cells suffered a 2.3-fold decrease in viability within 7 h of treatment and a 13.5-fold loss at 24 h of treatment (Fig. 2B). We conclude that Hpr6.6-mediated cell death is rapid, occurring within 7 h of oxidative damage. As for the MTT assay, all measurements were performed in triplicate and have been repeated a number of times to date.

We then measured the effect of Hpr6.6 on oxidative damage in a different cell line system. Hpr6.6 was stably transfected into NIH 3T3 cells and Hpr6.6-expressing clones were tested for sensitivity to peroxide. As for MCF-7 cells, Hpr6.6 sensitized cells to death following either 100 or 50 μM H_2O_2 over a 20 h incubation (Fig. 2C). NIH 3T3 were more sensitive to lower doses of H_2O_2 than MCF-7 cells, so that there was a 10-fold loss of viability in Hpr6.6-expressing cells even at 50 μM H_2O_2 (Fig. 2C). We conclude that in two different cell types, Hpr6.6 causes increased cell death following oxidative damage.

Hpr6.6-Expressing Cells Do Not Die From Classic Apoptosis

Because reactive oxygen species trigger apoptotic cell death in response to a variety of conditions, we considered the possibility that Hpr6.6 was stimulating apoptosis following oxidative damage. However, Hpr6.6 expression caused cells to remain attached to the dish with disrupted membranes (Fig. 3A), appearing to disintegrate without losing adherence. This wasted cell morphology was also detected in Hpr6.6-expressing NIH 3T3 cells treated with hydrogen peroxide. This differs from the morphology of apoptotic cells, which detach from the dish, round and have condensed nuclei.

To confirm the lack of apoptosis in Hpr6.6-expressing MCF-7 cells, we examined a number of molecular endpoints associated with apoptosis. We did not observe cleavage of the apoptotic protein caspase 8 (Fig. 3B, upper panel) or poly (ADP ribose) polymerase following oxidative damage. We did not detect degradation of the focal adhesion kinase (Fig. 3B, middle panel), which is degraded to an 85-kDa amino-terminal fragment during apoptosis [Wen et al., 1997; Xu et al., 2000]. We did detect a decrease in FAK expression in Hpr6.6-expressing cells after oxidative damage (Fig. 3B, middle panel, lanes 6 and 8), suggesting that loss of FAK expression may be a feature of non-apoptotic cell death. For western blots, tubulin was probed as a control for loading (Fig. 3B, lower panel). In addition to expression of apoptotic regulatory proteins, we did not observe nuclear condensation with the DNA dye DAPI (4′,6′-diamidino-2-phenylindole), and we did not detect internucleosomal degradation of genomic DNA, or "laddering" in Hpr6.6-expressing cells after peroxide treatment (data not shown). We conclude that Hpr6.6 directs cell death through a non-apoptotic mechanism following oxidative damage.

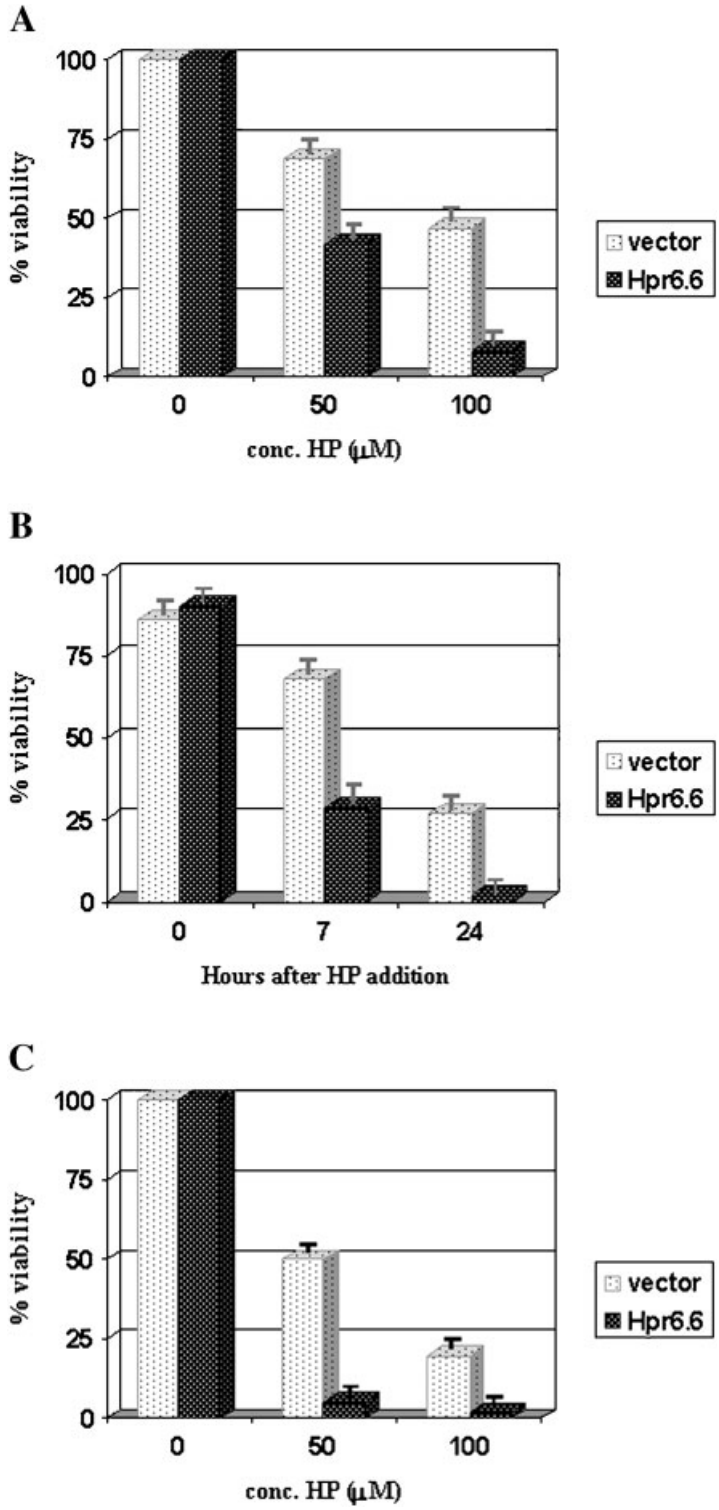

Fig. 2. Hpr6.6 sensitizes MCF-7 cells to cell death following oxidative damage. **A**: MCF-7 cells were treated for 20 h with 0–100 μM H_2O_2 and viability was measured by MTT assay. Cells expressing Hpr6.6 (black bars) were approximately 8-fold more sensitive to 100 μM H_2O_2 that control cells (gray bars). Standard deviations between triplicate measurements are indicated by error bars. **B**: MCF-7 cells were treated with 100 μM H_2O_2 for 7 and 24 h and viability was measured in triplicate by exclusion of the compound trypan blue. MCF-7/Hpr6.6 cells (black bars) underwent a marked loss in viability following peroxide treatment compared to MCF-7/vector cells (gray bars). The results shown are representative of three independent experiments. **C**: NIH 3T3/vector (gray bars) and NIH 3T3/Hpr6.6 cells (black bars) were treated with 50 and 100 μM H_2O_2 for 20 h., and viability was measured by MTT assay. Hpr6.6-expressing cells were significantly more sensitive to damage compared with control cells.

Hpr6.6 Sensitizes Cells to a "Peroxide Shock"

We analyzed the extent to which Hpr6.6 sensitized cells to a burst of oxidative damage. MCF-7/vector or MCF-7/Hpr6.6 cells were treated with 1 mM H_2O_2 for periods of 30–120 min before the media were removed and replaced with normal growth media. The cells were then incubated for 20 h and metabolic activity and viability were assayed by MTT and trypan blue assays, respectively. MCF-7/Hp6.6 suffered a 3.1-fold loss in metabolic activity (Fig. 4A) and a 2.6-fold loss in viability after a 90-min peroxide shock (Fig. 4B). We detected similar loss of viability in NIH 3T3 cells expressing Hpr6.6 after 30–60 min of H_2O_2 treatment (data not shown). Thus, Hpr6.6-expressing cells are sensitive to both prolonged and brief oxidative damage.

Oxidative damage initiates activation of the receptor for the epidermal growth factor and downstream activation of the phosphatidyl inositol-3 kinase (PI3K) and the protein kinases Akt, JNK, and ERK1/2 [Wang et al., 2000]. We considered the possibility that Hpr6.6 interfered with the activation of this cascade following oxidative damage, and that this disruption caused cell death. MCF-7 cells were treated with 0.4–1 mM H_2O_2 for 30 min, then lysed and analyzed by Western blot. Following treatment, MCF-7/Hpr6.6 cells underwent a fourfold higher induction of Akt phosphorylation than MCF-7/vector cells (Fig. 5, upper panel "p-Akt"), while the stability of the protein was unchanged (Fig. 5, second panel, "Akt"). The extent of phosphorylation in control cells was similar to levels previously reported in MCF-7 cells [Wang et al., 2000]. The hyperphosphorylation of Akt in MCF-7/Hpr6.6 was not due to a general increase in receptor-mediated phosphorylation, because ERK1/2 phosphorylation was not significantly altered in MCF-7/vector compared to MCF-7/Hpr6.6 cells (Fig. 5, third and fourth panels). In addition, JNK phosphorylation was induced following H_2O_2 treatment (data not shown), but was not elevated in Hpr6.6-expressing cells.

Wang et al. [2000] previously demonstrated that oxidative damage leads to elevated tyrosine phosphorylation of EGFR and that EGFR inhibitors block activation of Akt. We tested whether EGFR was activated by oxidative damage in MCF-7 cells, but we found that EGFR was undetectable in this cell line, as reported previously [Zajchowski et al., 1988]. This suggests that PI3K-Akt activation following oxidative damage occurs through a distinct pathway from that previously characterized in murine fibroblasts and HeLa cells.

Akt is activated indirectly by the phosphatidylinositol-3 kinase [PI3K, Cantley, 2002]. We inhibited PI3K with the compound LY294002 to block the activation of Akt. As previously reported for other cell types [Wang et al., 2000], we found that treatment of MCF-7 cells with LY294002 and subsequent oxidative damage inhibited Akt phosphorylation (Fig. 6A). Akt activation is characteristic of pro-survival, anti-apoptotic responses. We tested the formal possibility that Akt might have the opposite function when hyperactivated by Hpr6.6—that activated Akt might somehow stimulate cell death following oxidative damage. Pre-treatment of MCF-7/Hpr6.6 cells with LY294002 did not improve survival following a high dose H_2O_2 treatment (Fig. 6B). LY294002 treatment also failed to restore survival to MCF-7 cells treated with 100 μM H_2O_2 for 20 h (data not shown). We conclude that Akt hyperphosphorylation in MCF-7/Hpr6.6 cells does not cause cell death but is likely a means through which the cells attempt to suppress an apoptotic response.

NF-κB Inhibitor IκB Is Hyperphosphorylated in Hpr6.6-Expressing Cells

The transcription factor NF-κB regulates the transcription of a variety of genes that control survival and apoptosis [Wang et al., 2001]. NF-κB is regulated by an inhibitor protein called inhibitor of NF-κB (IκB), and IκB is regulated by phosphorylation on amino acids Ser32 and Ser36 [Brown et al., 1995]. We examined the phosphorylation of IκB using a phospho-specific antibody that recognizes only the Ser32–Ser36-phosphorylated form of IκB. The Ser32/Ser36 phosphorylated form of the protein was elevated in MCF-7/Hpr6.6 cells compared to MCF-7/vector cells (Fig. 7, upper panel, even vs. odd lanes). Phosphorylation of IκB targets the protein for degradation, but there was no difference in the expression levels of IκB in MCF-7/Hpr6.6 cells (Fig. 7, second panel, compare even and odd lanes), suggesting that the protein may be cycled more rapidly in Hpr6.6-expressing cells. The phosphorylation levels of IκB decreased following 30 min of H_2O_2 treatment (Fig. 7,

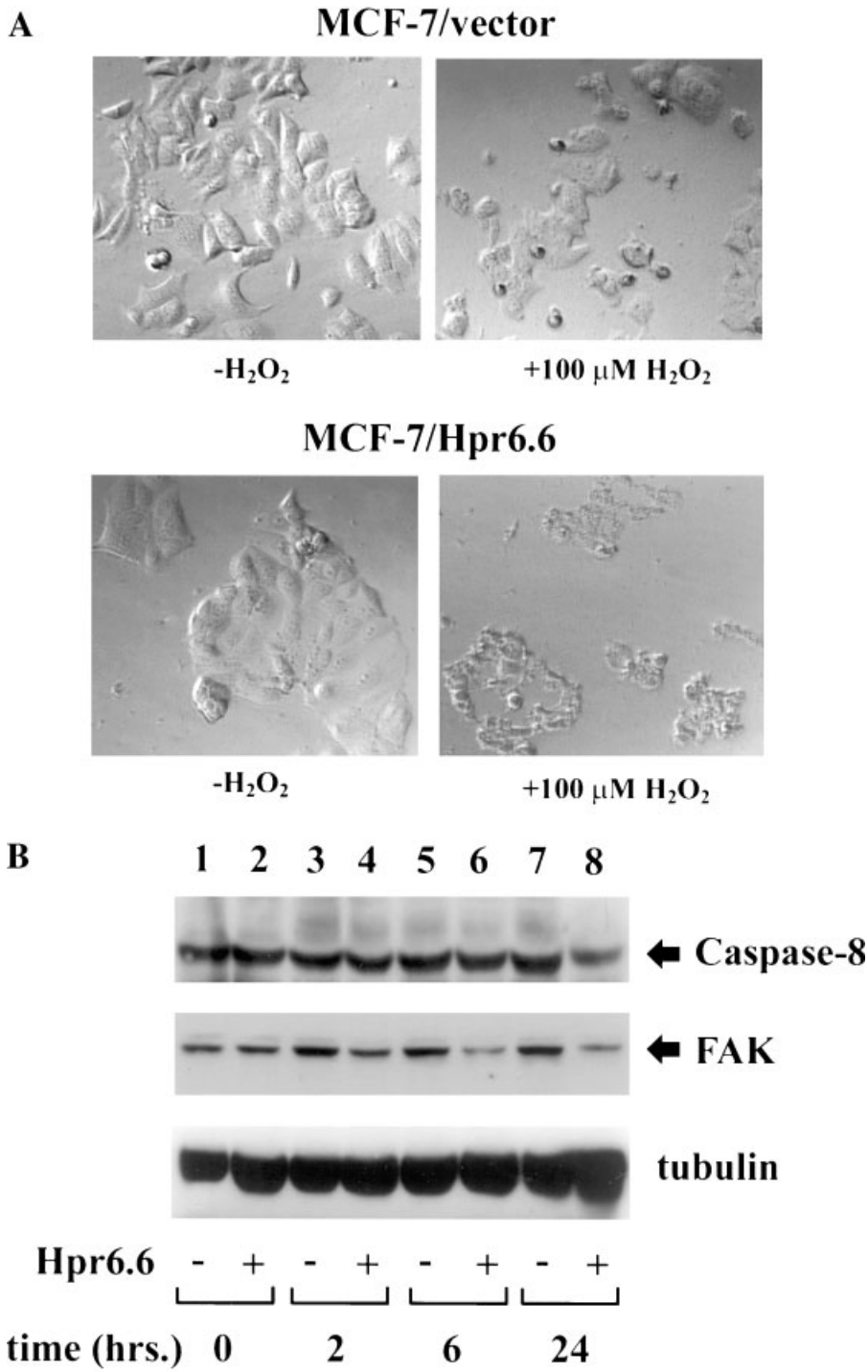

Fig. 3. Hpr6.6 sensitizes MCF-7 cells to non-apoptotic cell death. **A**: MCF-7/vector cells were photographed without (**upper left panel**) or with (**upper right panel**) a 20 h incubation with 100 μM H_2O_2. Peroxide damage caused little change in morphology, with a modest increase in the numbers of rounded cells (**upper right panel**). MCF-7/Hpr6.6 cells were photographed similarly. Peroxide-induced damage caused widespread degradation of the cells (**lower right panel**) but little cell rounding or loss of adhesion. **B**: Western blot analysis demonstrating lack of degradation of the apoptotic marker proteins Caspase-8 (**upper panel**) and focal adhesion kinase (**middle panel**), with tubulin as a control (**lower panel**). MCF-7/vector (odd lanes) and MCF-7/Hpr6.6 (even lanes) cells were treated with 100 μM H_2O_2 for 0–24 h, as indicated, then lysed and analyzed by western blot. Caspase-8 was not processed during the course of the experiments, and FAK decreased in expression but was not cleaved.

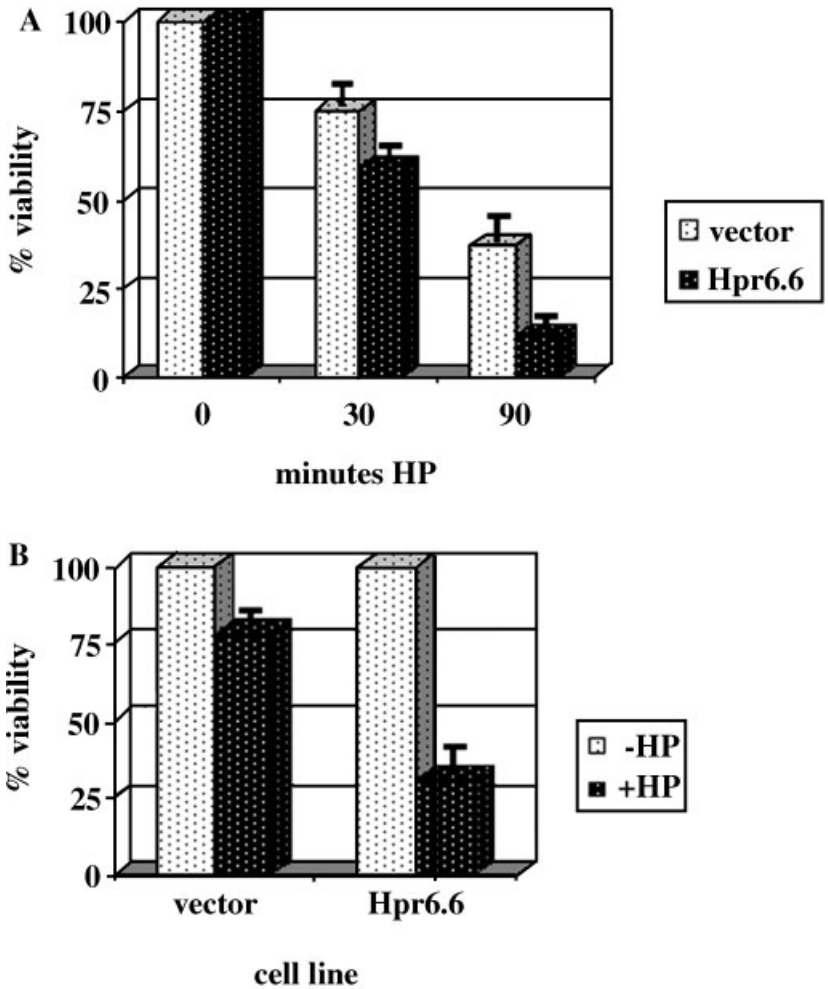

Fig. 4. Hpr6.6-mediated cell death following a peroxide shock. MCF-7/vector (gray bars) or MCF-7/Hpr6.6 (black bars) were treated with 1 mM H_2O_2 for 30 or 90 min then fed complete media without H_2O_2. Viability was measured after a 20 h incubation by MTT assay (**A**). For measurements of viability with trypan blue (**B**), cells were treated with 1 mM H_2O_2 for 90 min, the media were replaced with peroxide-free media, and trypan blue exclusion was measured 1 h later. Similar to a constant exposure to H_2O_2, we detected a 3.1-fold (A) or 2.6-fold (B) decrease in viability in Hpr6.6-expressing cells relative to control cells after H_2O_2 treatment using the MTT assay and trypan blue exclusion assay, respectively.

upper panel, lanes 2 and 6), but the relative phosphorylation of IκB in MCF-7/Hpr6.6 and MCF-7/vector cells remained constant following H_2O_2 treatment.

One potential mechanism for Hpr6.6-mediated cell death is through the constitutive activation of NF-κB, priming MCF-7 cells for death following H_2O_2 exposure. We tested the hypothesis that Hpr6.6 mediates cell death through IκB phosphorylation by overexpressing an IκB mutant that lacks the Ser32 and Ser36 phosphorylation sites. This IκB mutant is not phosphorylated on Ser 32 and Ser36 and is not degraded by the proteosome. As a result, it is called the IκB-SR because it acts as a "super repressor" that blocks NF-κB activation. We introduced the IκB-SR using an adenovirus, with a virus directing the expression of *LacZ* as a control. The expression of the IκB-SR protein in MCF-7/vector (odd lanes) and MCF-7/Hpr6.6 (even lanes) cells was confirmed by western blot with an antibody recognizing the phosphorylated and unphosphorylated forms of IκB (Fig. 8A).

The IκB-SR virus had no effect on viability in MCF-7/vector cells, but IκB-SR overexpression caused a significant decrease in viability after a 1 mM peroxide shock. We detected $33\% \pm 3$ viability for Ad-*LacZ* infected MCF-7/vector cells compared to $19\% \pm 7$ for IκB-SR-infected

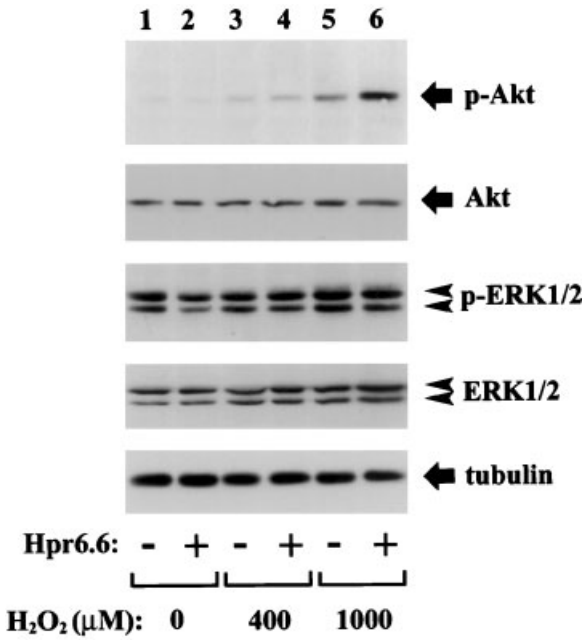

Fig. 5. Akt hyperactivation following oxidative damage in Hpr6.6-expressing cells. MCF-7/vector (odd lanes) and MCF-7/Hpr6.6 (even lanes) cells were either left untreated (**lanes 1** and **2**) or were treated with 400 μM (**lanes 3** and **4**) or 1,000 μM (**lanes 5** and **6**) H_2O_2 for 30 min. Cells were then lysed and analyzed by Western blot. Blots were probed with antibodies to phospho-Akt (**upper panel**), total Akt (**second panel**), phospho-ERK1/2 (**third panel**), total ERK1/2 (**fourth panel**), or tubulin (**bottom panel**). Hpr6.6 caused a hyperphosphorylation of Akt after H_2O_2 treatment, but had no effect on ERK1/2 phosphorylation.

cells (Fig. 8B). As expected, MCF-7/Hpr6.6 were more sensitive to a 1 mM H_2O_2 shock than MCF-7/vector cells ($6\% \pm 5$ for MCF-7/Hpr6.6 cells infected with control virus compared to $33\% \pm 3$ for similarly infected MCF-7/vector cells, $P = 0.005$). IκB-SR infection did not suppress loss of viability in H_2O_2-treated MCF-7/Hpr6.6 cells ($6\% \pm 5$ for cells infected with control virus compared to $1\% \pm 1$ for cells infected with the IκB-expressing virus, Fig. 8B, not statistically significant). Because a non-phosphorylated form of IκB can not suppress Hpr6.6-mediated cell death, we conclude that Hpr6.6 does not mediate cell death through phosphorylation of IκB.

Hpr6.6-Mediated Cell Death Is Not Reversed by Inhibition of Lipid Rafts

We examined the possibility that Hpr6.6 might sensitize cells to oxidative damage through the increased accumulation of lipid rafts (clustered sterols at the plasma membrane) for two reasons. First, the Hpr6.6 budding yeast homologue contributes to sterol synthesis [Hand et al., 2003]. Second, staining of lipid rafts in MCF-7 vector and MCF-7/Hpr6.6 cells indicated an increased intracellular accumulation of particles containing lipids. Lipid rafts are important in oxidative damage signaling because they are sites of receptor clustering, and disrupted lipid raft formation causes increased resistance to some types of cellular damage [Zhuang et al., 2002].

MCF-7/vector and MCF-7/Hpr6.6 cells were treated with the lipid raft inhibitor cyclodextrin for 2 h, then treated with a high dose of peroxide. Cells were then re-fed media without peroxide and incubated overnight. Cyclodextrin caused a slight decrease in metabolic activity in MCF-7/vector cells treated with H_2O_2 ($26\% \pm 3$ without cyclodextrin compared to $21\% \pm 2$ with cyclodextrin). In MCF-7/Hpr6.6 cells, cyclodextrin had no effect on metabolic activity after cyclodextrin treatment ($11\% \pm 3$ without cyclodextrin compared to $12\% \pm 2$ with cyclodextrin). Similar results were found with the lipid raft inhibitor filipin (data not shown). Thus, our results do not support a role for lipid raft formation in Hpr6.6-mediated cell death.

DISCUSSION

The pathways that regulate cellular responses to oxidative damage are important for a number of clinical conditions, and we have defined a novel pathway regulating these responses. The major results of this study are as follows: (1) Hpr6.6 sensitizes human breast cancer cells to cell death following oxidative damage; (2) Hpr6.6 hyperactivates the Akt protein kinase and IκB inhibitor protein; and (3) Hpr6.6-mediated cell death is independent of Akt and IκB. The ability to regulate cell death following oxidative damage is a novel function for the Hpr6.6 protein family and suggests a potential function for Hpr6.6 in its native sites of action in epithelial tissues.

Our data suggest that Hpr6.6 regulates the oxidative damage response through a novel pathway. The Akt protein kinase is phosphorylated in response to oxidative damage and acts acts as an anti-apoptotic signal [Wang et al., 2000]. Previous work demonstrated that Akt is hyper-phosphorylated in MCF-7 cells following a 30 min treatment with 1 mM H_2O_2 [Wang et al., 2000], and Akt is degraded following a 12 h treatment with 1–4 mM H_2O_2 [Martin

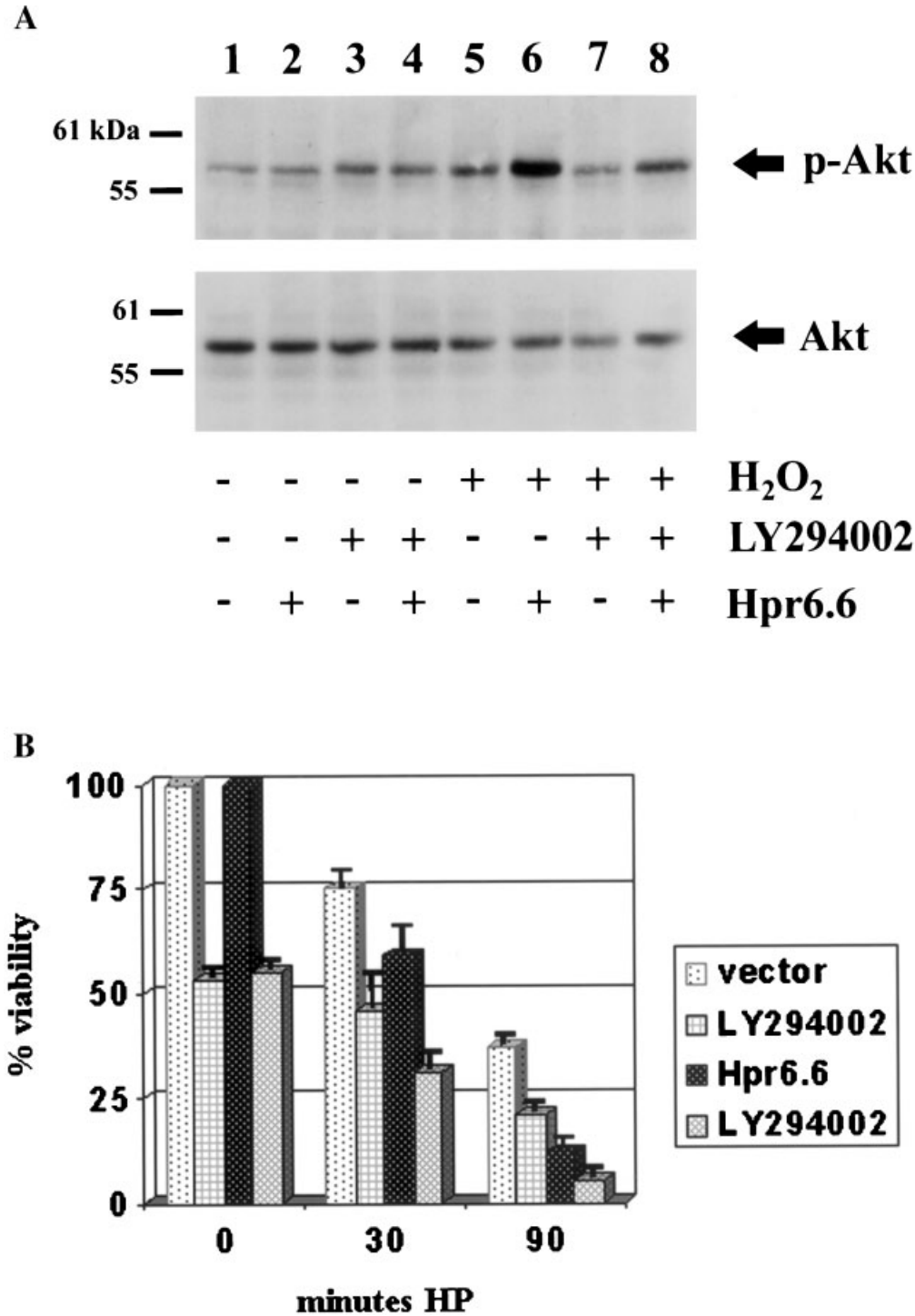

Fig. 6. Akt hyperactivation does not lead to cell death. **A**: Western blot demonstrating inhibition of Akt phosphorylation by the PI3K inhibitor LY294002. MCF-7/vector (odd lanes) and MCF-7/Hpr6.6 (even lanes) cells were treated with 1 mM H_2O_2 (**lanes 5–8**) or left untreated (**lanes 1–4**) in the absence (lanes 1, 2, 5, and 6) or presence (lanes 3, 4, 7, and 8) of LY294002. In lane 6, Hpr6.6 causes an elevation in Akt phosphorylation. In lane 8, this elevation is completely reversed by LY294002. **B**: Inhibition of Akt phosphorylation does not restore viability in Hpr6.6 expressing cells. MCF-7/vector (**two bars on left**) or MCF-7/Hpr6.6 (**two bars on right**) were treated with 1 mM H_2O_2 for 30 or 90 min. MCF-7/vector (**second bars from left**) and MCF-7/Hpr6.6 (**extreme right bars**) were also treated with LY294002. Viability was measured by MTT assay, and the values shown represent the average of triplicate measurements. The results shown are representative of three separate experiments.

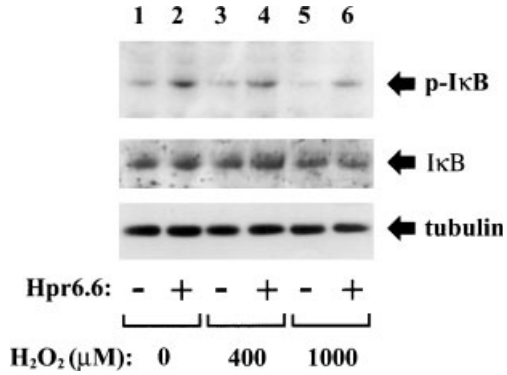

Fig. 7. IκB hyperphosphorylation in Hpr6.6-expressing cells. Western blots of MCF-7/vector (odd lanes) or MCF-7/Hpr6.6 (even lanes) cells treated with 0 (**lanes 1** and **2**), 400 μM (**lanes 3** and **4**), or 1,000 μM (**lanes 5** and **6**) H_2O_2 for 30 min. Cells were then lysed and proteins were analyzed by Western blot with antibodies to phospho-Ser32/Ser36-IκB (**top panel**), total IκB (**middle panel**), and tubulin (**lower panel**) as a loading control. IκB was phosphorylated at approximately fourfold higher levels in MCF-7/Hpr6.6 compared to MCF-7/vector cells.

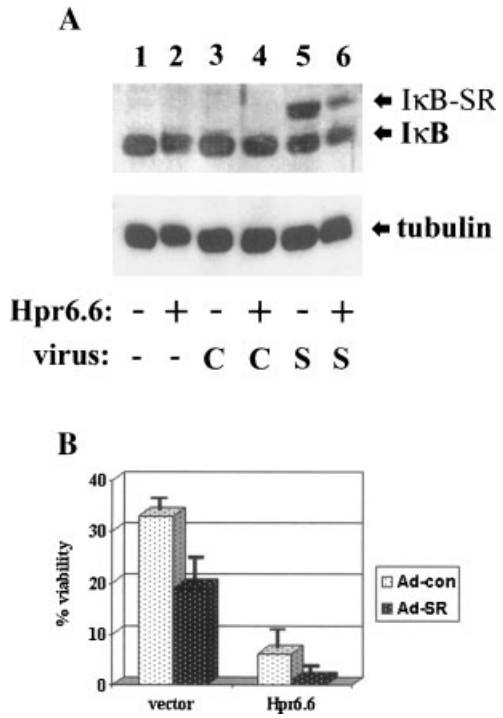

Fig. 8. A dominant-negative IκB mutant does not restore viability to Hpr6.6-expressing cells. **A**: Expression analysis of IκB-SR by Western blot. MCF-7/vector (odd lanes) or MCF-7/Hpr6.6 (even lanes) were uninfected (**lanes 1** and **2**), infected with a control adenovirus (''C'', **lanes 3** and **4**), or infected with an adenovirus directing the expression of IκB-SR (''S'', **lanes 5** and **6**). The blot was probed with an antibody to IκBα. Equal loading was assured by probing a Western blot of the same samples for tubulin. **B**: Effect of control adenovirus (gray bars) or IκB-SR (dark bars) on viability after a 1 mM peroxide shock. Viability was determined by MTT assay. Measurements of viability were determined in triplicate and error bars represent standard deviation. The results shown represent the results of three independent experiments.

et al., 2002]. Hpr6.6 leads to Akt hyperphosphorylation, but Hpr6.6-mediated cell death is Akt-independent. The ability of MCF-7 cells to undergo apoptosis is deficient due to the absence of Caspase-3, a feature that regulates their apoptotic response [Kim et al., 2000], and the activation of Akt appears to have little effect on the cell death response in this genetic background. Although Akt does not suppress Hpr6.6-mediated cell death following oxidative damage, it is likely that Akt modulates other functions of Hpr6.6 in other cell types.

While Hpr6.6 causes Akt hyperphosphorylation after oxidative damage, IκB is constitutively hyper-phosphorylated in Hpr6.6-expressing cells. NF-κB is implicated in death from oxidative damage, but IκB phosphorylation was not essential for Hpr6.6-mediated cell death. It is likely that hyperphosphorylation of Akt and IκB in Hpr6.6-expressing cells reflects other uncharacterized functions of Hpr6.6. NF-κB is generally associated with anti-apoptotic survival signaling, and our results suggest that under some circumstances Hpr6.6 might initiate pro-survival signaling. One caveat of this conclusion is that overexpression of some proteins mimics the phenotypes of cells lacking the protein. This is generally due to misregulation of the protein when overexpressed. Such a result is possible for our study and would suggest that Hpr6.6 does not normally promote cell death from oxidative damage, but inhibits it. Further work is required to understand Hpr6.6-mediated phenotypes completely.

Our results indicate that Hpr6.6 mediates cell death after oxidative damage through a novel pathway. The Hpr6.6 homologue in budding yeast functions by regulating sterol synthesis [Hand et al., 2003], and sterol-rich lipid rafts have been implicated in the damage response [Gniedecki et al., 2002]. However, our results suggest that lipid raft formation is not a major cause of cell death in Hpr6.6-expressing cells. Instead, we propose that Hpr6.6 alters an intracellular sterol pool that regulates the oxidative damage response.

The molecular mechanism of Hpr6.6 function is unknown, but Hpr6.6 and its homologues contain motifs common to heme-binding

proteins, suggesting a role in oxidative metabolism [Mifsud and Bateman, 2002]. Heme binding proteins play key roles in oxygen transport, and heme binding proteins are required for maintaining cellular stasis and detoxifying the cell. The homology between Hpr6.6 and heme-binding proteins suggests that Hpr6.6 might have a similar function, and that this function could contribute to the response to oxidative damage. However, heme binding has not been reported among Hpr6.6 or its homologues.

Hpr6.6 is most highly expressed in epithelial tissues, including cancer cell lines and tissues. The ability to respond to oxidative stress is a key feature of carcinogenesis. In addition, breast cancer cells are damaged by ROSs following chemotherapeutic and radiation treatments, and Hpr6.6 could contribute to cell death decisions in response to such treatments. Our results are consistent with a model in which Hpr6.6 regulates cell death in epithelial tissues, and suggest that Hpr6.6 may be an attractive target for therapeutic intervention in a variety of diseases.

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