Glucose-6-phosphate dehydrogenase (G6PD), the rate-limiting enzyme of the oxidative pentose phosphate cycle, regulates the NADPH/NADP+ ratio in eukaryotic cells. G6PD deficiency is one of the most common mutations in humans and is known to cause health problems for hundreds of millions worldwide. Although it is known that decreased G6PD functionality can result in increased susceptibility to oxidative stress, the molecular targets of this stress are not known. Using a Chinese hamster ovary G6PD-null mutant, we previously demonstrated that exposure to a thiol-specific oxidant, hydroxyethylisulfide, caused enhanced radiation sensitivity and an inability to repair DNA double strand breaks. We now demonstrate a molecular mechanism for these observations: the direct inhibition of DNA end binding activity of the Ku heterodimer, a DNA repair protein, by oxidation of its cysteine residues. Inhibition of Ku DNA end binding was found to be reversible by treatment of the nuclear extract with dithiothreitol, suggesting that the homeostatic regulation of reduced cysteine residues in Ku is a critical function of G6PD and the oxidative pentose cycle. In summary, we have discovered a new layer of DNA damage repair, that of the functional maintenance of repair proteins themselves. In view of the rapidly escalating number of roles ascribed to Ku, these results may have widespread ramifications.

Reactive oxygen species (ROS)1 produced during oxidative stress are widely believed to cause cell death or late effects such as cancer, aging related diseases, etc. (1). Several studies have indicated that ROS would be even more damaging if it were not for the protective role of intracellular superoxide dismutase, catalase, and glutathione (2, 3). Superoxide dismutase, catalase, and GSH are the most widely investigated modifiers of oxidative stress are widely believed to cause cell death or late effects such as cancer, aging related diseases, etc. (1). Several studies have indicated that ROS would be even more damaging if it were not for the protective role of intracellular superoxide dismutase, catalase, and glutathione (2, 3). Superoxide dismutase, catalase, and GSH are the most widely investigated modifiers of oxidative stress. The effects of these agents are presumed to be due to the interaction of ROS with macromolecular targets such as DNA, lipid, and protein, but the precise molecular targets are not well defined (17–19).

Glucose-6-phosphate dehydrogenase (G6PD) activity can increase several hundredfold, producing ROS directly and/or reducing oxidatively modified macromolecules. NADPH, a major cofactor required for the maintenance of GSH during oxidative stress, is produced by the oxidative limb of the pentose phosphate cycle, i.e. OPPC (4–7). Glucose-6-phosphate dehydrogenase (G6PD) is the rate-limiting enzyme for OPPC (4). At low levels of oxidative stress, G6PD activity can increase several hundredfold, producing NADPH necessary to reduce/repair the oxidized molecules (4, 7). Severe oxidative stress has been shown to cause the inactivation of G6PD that correlated with oxidative damage (8–10). Therefore, NADPH, the major cofactor produced by G6PD/OPPC, is vital to cellular defense against oxidative stress.

Genetic defects in G6PD, an X-linked gene, are extremely common, occurring in more than 400 million people throughout the world (11–13). These G6PD genetic deficiencies result in an impaired ability to deal with oxidative stress and can cause various health problems such as cancer, aging related diseases, etc. (14–16). The best known example for the immediate effect is hemolytic anemia caused by treatment of G6PD-deficient patients with antimarial drugs (17). In addition, several studies using cells from G6PD-deficient patients have demonstrated an increased sensitivity of these cells to oxidants, toxins, and oxidative stress (17). The effects of these agents are presumed to be due to the interaction of ROS with macromolecular targets such as DNA, lipid, and protein, but the precise molecular targets are not well defined (17–19).

A recent study has demonstrated that the majority of patients with acute non-lymphocytic leukemia have decreased G6PD levels (16). This study also reported the association of a high percentage of chromosomal abnormalities, lower survival, and higher remission rates in patients having decreased G6PD activity versus normal, respectively. Although some studies have indicated that there was no difference in cancer risk among G6PD-deficient versus G6PD-normal subjects, this study showed a significant increase in mortality from non-Hodgkin's lymphoma in G6PD-deficient subjects (14). In a related animal study, Wells et al. (15) demonstrated that G6PD-deficient mice had enhanced embryopathies, indicating a teratological role for endogenous oxidative stress caused by the absence of G6PD. In their review, Martini and Ursini (20) have suggested that the high prevalence of G6PD deficiency in various ethnic populations may allow the putative role of G6PD deficiency in carcinogenesis and aging to be ascertained.

We have recently demonstrated that the Chinese hamster ovary (CHO) G6PD-null mutant has enhanced radiation sensitivity and an inability to repair DNA double strand breaks (DSBs) after oxidative stress induced by hydroxyethylisulfide (HEDS), a thiol-specific oxidant (21). Here we demonstrate that a reasonable molecular mechanism for its defective DNA DSB repair is the inhibition of the DNA end binding activity of the
Ku heterodimer, a DNA repair protein, due to the oxidation of its cysteine residues. Our reason for investigating the Ku protein is that it plays a central role in DNA damage recognition and non-homologous DNA end joining by binding to DNA ends in mammalian cells (22–24). Ku is also believed to be involved in the regulation of apoptosis and telomere fusion (25, 26). Our present results demonstrate that G6PD deficiencies cause not only susceptibility to oxidative damage in general but also the specific oxidation of proteins critical for maintaining proper cellular functions, such as DNA repair.

**EXPERIMENTAL PROCEDURES**

**G6PD-null Mutants and Cell Transfections**—The cell lines were derived from the K1 clone of CHO cells as described previously (27). Briefly, G6PD-null mutants of CHO cells were isolated after mutagenesis with a minimally toxic concentration of ethylmethanesulfonate by means of the selection technique coupled with histochemical staining for enzyme activity. Cells transfected to reintroduce the G6PD gene were carried out by the electroporation of G6PD-null mutant cells with hamster G6PD-cDNA (GenBank™ accession number AF044676) inserted into pcDNA3.1 constructs and selected in G418 medium. Target clones were identified by histochemical staining for G6PD activity and Northern analysis using G6PD-cDNA (25). The A1A cells are stably transfecined with a vector resistant to G418. We have also selected several clones with differing and similar G6PD activity to normal cells (27).

**HEDS Treatment and Preparation of Nuclear Extract—**Cells (0.8 × 10⁶) in 4 ml of medium were plated in 60-mm dishes on the day before the cells with 1 ml of fresh medium plus 50 μM PMSF, and pepstatin (1 μg/ml) were added. Cells were incubated with HEDS for the desired time. These cells were incubated with HEDS for the desired time. These cells were then washed three times with ice-cold PBS and then treated with SSA as above. This treatment precipitates cellular macromolecules in place without loss of protein. The acid was removed, and dishes were washed two more times with 5 ml of SSA, which was removed by aspiration. Under these experimental conditions, only non-protein sulphydryl groups were washed off the cells. Washed cells were then incubated with 1 ml of 100 mM phosphate buffer (pH 7.4) containing 1.5 mM 5,5'-dithiobis(2-nitroanionic acid) for 15 min at 37 °C, and the absorbance was read at 412 nm. Protein thiols were then estimated using an absorption coefficient for reduced 5,5'-dithiobis(2-nitroanionic acid) of 13,600 at 412 nm. The protein thiol level in untreated cells was 0.40 ± 0.05 μmol/cell.

**DNA Probe Preparation—**A 144-base pair probe was prepared from a pUC18 plasmid using PvuII and EcoRI digestion and purified by 10% preparative gel electrophoresis (29). The appropriate 144-base pair fragment was excised and eluted from the gel into 100 mM NaCl in TE buffer (10 mM Tris (pH 8.0), 0.1 mM EDTA). The eluted DNA was extracted with phenol/chloroform/isoamyl alcohol (25:24:1), ethanol-precipitated, and resuspended in TE buffer. The 144-bp fragment was labeled with [α-32P]dATP (PerkinElmer Life Sciences) using the Klenow fragment of DNA polymerase I. Unincorporated nucleotide was removed by chromatography on Sephadex G-50 spin columns.

**DNA Mobility Shift and Antibody Supershift Assays—**Mobility shift mixtures containing 2 μg of nuclear protein, 1 ng of [α-32P]-labeled 144-bp probe, and 1 μg of pUC18 (specific DNA competitor). The final volume of 20 μl of binding buffer (10 mM Tris-HCl (pH 8.0), 0.1 mM EDTA, 150 mM NaCl, 1 mM PMSE, leupeptin (2.5 μg/ml) and pepstatin (1 μg/ml), and 10% v/v glycerol) were incubated for 15 min at room temperature (29). For mobility supershift assays, a Ku70 antibody (29) was incubated with the nuclear extract for 30 min at the desired concentration after the 15-min incubation with the radioactive probe and pUC18. The mixtures were electrophoresed on a 6% polyacrylamide gel at 20–25 mA in TBE buffer (45 mM Tris-HCl (pH 8.0), 45 mM boric acid, 1 mM EDTA), and the gel was dried and subjected to autoradiography or phosphorimaging and NIH image analysis for quantitation (Bio-Rad).

**Western Blot Analysis—**A total cellular Ku70 protein was quantified using an immunoblot and NIH image analysis. Cells were washed with PBS three times and mixed with 200 μl of room temperature-cooled, preboiled (2 min) Laemmli buffer containing 10 mM dithiothreitol (DTT). Cells in Laemmli buffer were removed using a Teflon scraper and transferred to an Eppendorf tube using a micropetitte. The cells were homogenized using a 1-ml syringe (18-gauge needle), and samples were stored at 80 °C. Protein extracts (15 μg/ml) in sample buffer were electrophoresed on a 7.5% precast gel from Bio-Rad at 150 V for 75 min in TBE buffer (45 mM Tris-HCl (pH 7.8), 45 mM boric acid, 1 mM EDTA). The proteins were transferred to nitrocellulose by electrophoresis at 100 V for 60 min. The nitrocellulose paper was incubated in 10 ml of blocking buffer for 1.5 h at room temperature on a rocker and stored in the cold room overnight. The nitrocellulose paper was washed five times with TBST (20 mM Tris base (pH 7.6), 137 mM NaCl, 0.05% Tween 20) and incubated with 30 μl of primary Ku70 antibody (Ab-4, clone NSH10, Neomarkers) in 15 ml of blocking buffer for 2 h at room temperature. The nitrocellulose paper was washed 4 times with TBST before incubation with the secondary antibody for 1 h per the manufacturer’s instruction (Amersham Biosciences, Inc.). The bands were detected and quantified using a standard ECL kit and NIH image analysis, respectively.

**RESULTS**

The NADPH value for untreated G6PD-null mutant E89 cells (~60 pmol/10⁶ cells) was about 40% lower than that of wild-type K1 (~90 pmol/10⁶ cells) and transfectant A1A (~100 pmol/10⁶ cells) cells (Fig. 1). NADH values (~800 pmol/10⁶ cells) were the same in all three cell lines (Fig. 2). Incubation of these cells with 5 mM HEDS for 1 h caused a 5-fold decrease in NADH in E89 cells with little change for K1 and A1A cells (Fig. 2). This treatment almost completely eliminated the NADPH level in E89 cells (Fig. 1) and decreased that of K1 and A1A cells by 90% (Fig. 1).

To measure bioreductive capacity, we quantified the extracellular mercaptoethanol produced by cells treated with 5 mM HEDS immediately after a 1-h incubation (Fig. 3). The wild-type K1 cells released 522 nmol of ME into the extracellular medium. G6PD-deficient E89 cells released 53 nmol of ME, which is a 10-fold decrease in bioreductive capacity compared...
with K1 cells. The G6PD-transfected A1A cells released 815 nmol of ME, indicating the recovery of bioreductive capacity due to the transfection of the wild-type G6PD gene into the null mutant cells. The intracellular GSH level was almost the same in untreated wild-type (13 nmol), null mutant (18 nmol), and transfectant (16 nmol) cells (Fig. 4). The incubation of HEDS for 1 h did not significantly affect the GSH levels in wild-type (12 nmol) or transfectant (17 nmol) cells. However, the GSH levels in G6PD-null mutant cells decreased from 18 to 2.0 nmol (9-fold decrease) after a 1-h incubation with 5 mM HEDS. The effect of HEDS on protein thiols was measured by using Ellman’s reagent (Fig. 5). All three untreated cell lines had similar amounts of intracellular PSH (42 nmol/10^6 cells). G6PD-null mutants treated with 5 mM HEDS showed a significant decrease (30%) in PSH compared with untreated E89 cells. However, wild-type K1 and A1A transfectants showed an insignificant decrease (7%) in protein thiols after HEDS treatment (Fig. 5).

To test whether cellular redox changes caused by HEDS treatment (Figs. 1–5) affect Ku binding to DNA ends, we used a modified method of the standard mobility gel shift procedure (29). This assay contains a 32P-labeled 144-bp DNA probe, a nuclear extract, and an excess of circular plasmid DNA. Under these assay conditions, proteins that bind to internal DNA sequences will be bound by the excess unlabeled plasmid DNA that does not contain ends. Thus, only proteins that specifically bind to DNA ends will bind to the labeled probe. DTT, a strong reducing agent, is normally added to protein extracts to protect against spontaneous oxidation. To measure the effect of oxidation on end binding activity in vivo, it was necessary to determine whether DTT could be eliminated from the assay because its presence would be likely to reverse such oxidation. Therefore, we compared DNA end binding activity with and without DTT for nuclear proteins extracted from wild-type (K1) cells, G6PD-null mutant (E89) cells, and G6PD-null mutant cells stably transfected with a cDNA encoding the wild-type hamster G6PD (A1A). The NIH image analysis of labeled bands (Fig. 6, plots 2–7) shows that similar amounts of DNA end binding
activity (Ku-DNA complex) were observed in nuclear extracts isolated from cells in the presence or absence of DTT. Authentication of Ku as the DNA end binding species was made by demonstrating a supershift of the bound probe upon addition of an anti-Ku70 antibody to the reaction mixture (Fig. 6, lanes 8–10).

Having demonstrated that Ku binding was not affected by the absence of DTT in this assay, we examined the effect of HEDS treatment on Ku binding in the same three cell lines. HEDS treatment inhibited Ku DNA end binding activity by 70% in the G6PD-null mutant E89 cells (Fig. 7, lane 5). In contrast, HEDS had no effect on Ku DNA end binding activity in the wild-type cell line K1 or the G6PD+ transfectant A1A (Fig. 7, lanes 3 and 7).

To ensure that the inhibition of Ku DNA end binding observed in HEDS-treated cells was not due to a decrease in the Ku protein, we measured the amount of Ku70 by Western blot analysis using a monoclonal anti-Ku70 antibody. A single ~70-kDa immunoreactive band of equal intensity was observed in total cell lysates extracted from all three cell lines treated with and without HEDS (Fig. 8). This indicates that treatment with 5 mM HEDS had no measurable effect on the amount of Ku70 in any of the CHO cell lines. These results support the hypothesis that HEDS-induced oxidation of Ku leading to reduced end binding activity was responsible for the previously observed increase in radiation sensitivity and reduction in DSB repair in G6PD-null mutant cells (21).

To determine whether the effect of HEDS on Ku extracted from G6PD-null mutant cells was a direct consequence of thiol oxidation, we treated the nuclear extract with the thiol-specific reductant, DTT. Ku DNA end binding activity was fully restored by treating the lysates from HEDS-treated G6PD-null mutant cells with 5 mM DTT for 10 min (Fig. 9, lane 5). Ku DNA end binding activity in lysates from G6PD-null mutant cells that had not been exposed to HEDS was not affected by DTT. Taken together, the results indicate that HEDS blocks Ku DNA binding activity in vivo through the oxidation of cysteine residues in the Ku heterodimer without affecting the protein level because this oxidation can be reversed by reduction of cysteine residues by DTT. The fact that Ku was not affected by HEDS treatment in G6PD-proficient cells suggests that homeostatic regulation of cysteine residues in Ku is a critical function of G6PD and the oxidative pentose cycle.

**DISCUSSION**

A major biochemical function of G6PD is that it acts as the rate-limiting enzyme of the oxidative pentose phosphate cycle. The enzymes of the OPPC are highly conserved and ubiquitously expressed in prokaryotic and eukaryotic cells, indicating the importance of this pathway for cell growth and function (5, 8–10). The oxidative limb of the pentose cycle catalyzes the oxidation of G6P to NADPH, ribulose-5-phosphate, and CO2 (7). NADPH is utilized in the reductive biosynthesis of fatty acids, cholesterol, steroids, and the prenyl groups of mem-
brane-bound proteins (30). NADPH is also utilized in the reduction of nucleotide diphosphates to deoxyribonucleic acids and is therefore necessary for de novo synthesis of DNA (30).

G6PD-deficient and null cells remain viable because of the availability of NADPH produced by pathways such as malic enzyme, isocitrate dehydrogenase, and NADH/NADPH transhydrogenase (30). In addition, the ribose necessary for DNA and RNA synthesis is available from the transketolase and transaldolase enzymes of the nonoxidative limb of the pentose cycle (31, 32). Furthermore, many of the nucleotides can also be scavenged from the matrix (33). Although cells can survive in the absence of G6PD by using reducing equivalents produced by G6PD-independent pathways, G6PD becomes more important for cell survival during oxidative stress. In wild-type cells, the oxidative limb of the pentose cycle is activated by oxidative stress, producing the reducing equivalents to maintain the redox status of the cells (7). As the capability of the protective function declines, cells become progressively susceptible to oxidative stress (10, 34, 35). We showed that cells deficient in G6PD were sensitive to a variety of toxins thought to act by induction of oxidative damage (27, 34, 36). Cells deficient in G6PD become progressively susceptible to radiation-induced apoptosis (27). In addition, a slightly slower DNA repair kinetics was observed for G6PD-deficient cells compared with wild-type cells after radiation damage (21). Our unpublished results indicated that malic enzyme and isocitrate dehydrogenase were not modified in these cell lines. In view of the sensitivity of G6PD-null cells to oxidative stress, this suggests that G6PD plays the major role in the cellular response to oxidative damage.

One type of molecular damage that can be expected from G6PD deficiency during oxidative stress is the oxidation of protein thiols because G6PD-deficient cells lack production of reducing equivalents necessary to maintain protein cysteine residues in their reduced form. Cysteine residues in proteins are important for the structure and functions of proteins (37, 38). Although it is clear that a change in the normal redox status of protein cysteine thiols can alter function, protein redox is well regulated except when cells are subjected to oxidative stress or potentially toxic oxidative agents such as hydrogen peroxide, menadione, etc. (39-43). However, these agents are 80% nonspecific that it is impossible to determine whether protein thiol oxidation plays a major role in the cytotoxic response. Indeed, it has not been possible to identify the critical targets of damage (e.g., membrane, mitochondria, DNA, protein) using these agents (44). Part of the reason for such nonspecificity is that cells massively resist oxidative attack, utilizing the reducing capacity provided by the oxidative limb of the pentose cycle (NADPH) or respiration (NADH) (34, 45, 46). Therefore, the amount of oxidant required must be strong enough to overwhelm the protective defenses of the cell, thereby causing massive damage to multiple cellular targets (41, 46).

To better define the molecular nature of thiol-specific oxidative damage in G6PD-null mutant cells, we used the disulfide HEDS, which is relatively non-reactive with any molecule other than thiols. The fast bioreduction of HEDS (−500 nmol of ME per million cells in 1 h) by wild-type cells suggests that HEDS may be reduced by intracellular reductants to produce ME as shown in Reaction 1.

\[
2 \text{HX} + \text{RSSR} (\text{HEDS}) \leftrightarrow 2 \text{HSH} + \text{RSSXR} \quad \text{REACTION 1}
\]

Although the G6PD-null mutant cells have almost the same initial amounts of GSH, PSH, NADPH, and NADH, the conversion of HEDS into ME is much lower than that of G6PD normal cells. HEDS may be reduced by any one or all of the intracellular reductants such as GSH, cysteine, and even protein thiols either directly or through enzymatic reduction using NADPH and NADH as cofactors. The loss of NADPH in both G6PD-null mutant and control cells indicates that HEDS consumes NADPH (Fig. 1). This suggests that cells require OPPC to produce enough reducing equivalents (NADPH) to reduce 5 mM HEDS. A significant amount of NADPH remaining in wild-type cells during HEDS treatment suggests that G6PD maintains NADPH through recycling of NADPH by the oxidative pentose phosphate cycle, which is shown in Reaction 2.

\[
\text{OPPC} + 12 \text{NADPH} \rightarrow 12 \text{NADPH} + 6 \text{CO}_2 + 12 \text{H}^+ \quad \text{REACTION 2}
\]

\(^2\) J. E. Biaglow, unpublished data.
This is consistent with the bioreductive capacity of E89 cells, which is 6- and 12-fold lower than that of the K1 and A1A cells, respectively.

The ability of wild-type and transfectant cells to maintain normal levels of GSH, PSH, and NADH despite a 10-fold reduction in steady state NADPH attests to the adaptability of this pathway and cellular bioreductive capacity. However, for the G6PD-null mutant cells, the near total loss of NADPH results in a cascade of other oxidations, including the loss of NADH, GSH, and PSH (Figs. 1–5).

Our results showed that of 54 nmol of ME produced by E89 cells, only 28 nmol is accounted for by GSH (16 nmol), PSH (12 nmol), G6PD-independent NADPH (0.06 nmol), and NADH (0.6 nmol). The remaining 26 nmol of reductants involved in the reduction of HEDS may have come from NADH or G6PD-independent NADPH (0.06 nmol), G6PD-independent NADPH (0.06 nmol), and G6PD-independent NADPH (0.06 nmol), and NADH (0.6 nmol). The remaining 26 nmol of reductants involved in the reduction of HEDS in E89 cells may have come from NADH or G6PD-independent NADPH cycling. It has been shown that NADH produced by respiration can reduce thiols, albeit at a much slower rate compared with NADPH (47).

The complexity of these reactions warrants considerable additional study to identify the enzymes that use NADPH as cofactor to reduce oxidized PSH.

We have previously reported that DNA DSB rejoining is inhibited by HEDS in G6PD-null mutant cells (21). Because G6PD-null mutant cells have a higher level of oxidized protein thiols after HEDS treatment, it is possible that the inhibition of DNA DSB rejoining may have resulted from the oxidation of cysteine residues in one or more of the proteins involved in DSB repair. Biochemical and genetic studies using null mutant rodent cell lines sensitive to ionizing radiation have identified at least four genes, XRCC4, XRCC5, XRCC6, and XRCC7, that are required for non-homologous end joining (NHEJ) (22–24).

Our results showed that of 54 nmol of ME produced by E89 cells, only 28 nmol is accounted for by GSH (16 nmol), PSH (12 nmol), G6PD-independent NADPH (0.06 nmol), and NADH (0.6 nmol). The remaining 26 nmol of reductants involved in the reduction of HEDS in E89 cells may have come from NADH or G6PD-independent NADPH cycling. It has been shown that NADH produced by respiration can reduce thiols, albeit at a much slower rate compared with NADPH (47).

The simplest explanation for the loss of GSH, NADPH, and NADH in G6PD-null mutant cells would be that HEDS may oxidize GSH and NADPH independently, either by chemical or enzymatic reduction. However, it is more likely that all of these oxidations are highly inter-dependent. It has been demonstrated that the reduction of HEDS is facilitated by thiol transferase-linked reactions (48–50). The thioltransferase enzyme utilizes GSH in the reduction of disulfides to stimulate the second step of Reaction 3 (51).

2 GSH + RSSR (HEDS) ⇔ GSH + RSH + RSSG

REACTION 3

We know from our studies and those of others that NADPH is required to recycle GSSG back to GSH, as shown in Reaction 4.

GSSG + NADPH + H⁺ ⇔ 2 GSH + NADPH

REACTION 4

GSSG produced as shown in Reaction 3 may in turn oxidize protein thiol, as shown in Reaction 5.

2 PSH + GSSG ⇔ PSH + GSH + GSSP ⇔ 2 GSH + PSSP

REACTION 5

However, PSH may also be oxidized through direct oxidation by HEDS, as depicted in Reaction 6.

2 PSH + RSSR (HEDS) ⇔ PSH + RSH + RSSP ⇔ 2 RSH (ME) + PSSP

REACTION 6

In G6PD-containing cells, oxidized protein thiol is reduced back to PSH presumably by enzyme-linked and/or direct NADPH reduction of PSSR to PSH as shown below in Reaction 7.

Glutaredoxin/thioredoxin reductase

PSSR + GSH → PSH + RSSR

Thioredoxin reductase

PSSR + NADPH + H⁺ → PSH + RSH + NADPH

REACTION 7

The complexity of these reactions warrants considerable additional study to identify the enzymes that use NADPH as cofactor to reduce oxidized PSH.
radiation; no further sensitization occurred in Ku-deficient cells by HEDS even though 30% of the total protein thiols were oxidized.

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