FAK Is the Upstream Signal Protein of the Phosphatidylinositol 3-Kinase-Akt Survival Pathway in Hydrogen Peroxide-induced Apoptosis of a Human Glioblastoma Cell Line*

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Protein phosphorylation in a human glioblastoma cell line, T98G, was examined after exposure to oxidative stress in vitro. Hydrogen peroxide (1 mM) markedly induced tyrosine phosphorylation of focal adhesion kinase (FAK) and serine phosphorylation of Akt at 1 h after stimulation. Concomitantly, the association of FAK with phosphatidylinositol 3'-OH-kinase (PI 3-kinase) was also observed by the hydrogen peroxide stimulation. When T98G cells were incubated with wortmannin, a PI 3-kinase inhibitor, both PI 3-kinase activity and phosphorylation of Akt were inhibited, whereas apoptosis by oxidative stress was accelerated. Concomitant with apoptosis, elevated level of CPP32 protease activity (caspase-3) was observed, with decreases in Bcl-2 protein and increases in Bax protein. These results suggested that in the signal transduction pathway from FAK to PI 3-kinase, Akt promotes survival. Thus, it became apparent that FAK is the upstream signal protein of the PI 3-kinase-Akt survival pathway in hydrogen peroxide-induced apoptosis in T98G cells.

Phosphorylation and dephosphorylation on tyrosine residues play critical roles in the signal transduction pathways that regulate cell activation, proliferation, and differentiation. Reactive oxygen species (ROS)1 have been reported to induce increased tyrosine phosphorylation of several proteins, such as p77

Many growth factors and cytokines promote cell survival, including insulin-like growth factor 1 (5) and platelet-derived growth factor (6). Phosphatidylinositol 3'-OH-kinase (PI 3-kinase) has recently been shown to be involved in cell survival. Growth factors activate PI 3-kinase, and p85 subunit of PI 3-kinase associates with specific phosphotyrosine either on the cytoplasmic domain of growth factor receptors or on receptor-associated adapter proteins. One target of PI 3-kinase is the serine-threonine kinase Akt, also named PKBα (5). Akt is a general mediator of growth factor-induced survival and has been shown to suppress apoptotic death by a variety of stimuli (5). Signaling via growth factor receptor activation leads to the sequential activation of PI 3-kinase and Akt. Recently, Datta et al. (7) reported that Akt phosphorylates BAD in vitro and in vivo and blocks BAD-induced death of primary neurons. In eukaryotes, Bcl-2 family are central to the regulation of cell death. Several members of the Bcl-2 family (Bcl-2, Bcl-XL, MCl-1, and A1) promote survival, whereas other members (Bcl-Xs, BAD, Bax, Bak) promote cell death (8–13). Bcl-2 family proteins homo- and heterodimerize, and the balance between homo- and heterodimers appears to be critical to the maintenance of cell survival and cell death. The mechanisms of Bcl-2 family members function yet remains to be determined.

In a previous study (14), we reported that hydrogen peroxide markedly induced rapid tyrosine phosphorylation of focal adhesion kinase (FAK) followed by the decrease of phosphorylation concomitant with apoptosis. Further, the inhibition of tyrosine phosphorylation of FAK by herbimycin A, a tyrosine kinase inhibitor, accelerated apoptosis, and antisense oligonucleotides of FAK decreased cell viability. From these studies, we proposed an anti-apoptotic role of FAK in hydrogen peroxide-induced apoptosis. Based on these findings, we hypothesized FAK may be an upstream signal protein in the PI 3-kinase and Akt pathway and promotes cell survival against stresses in some cell types. Therefore, we examined the relationships between FAK, PI 3-kinase, Akt, Bcl-2 family proteins, and CPP32 protease (caspase-3) using a human glioblastoma cell line, T98G.

To investigate the role of PI 3-kinase and Akt in hydrogen peroxide-induced apoptosis, T98G cells were treated with hydrogen peroxide (1 mM) which caused tyrosine phosphorylation of FAK and serine phosphorylation of Akt. We also found that the association of FAK with PI 3-kinase was stimulated by hydrogen peroxide. Interestingly, the PI 3-kinase inhibitor wortmannin accelerated apoptosis and inhibited serine phosphorylation of Akt. Decreases in Bcl-2 protein and increases in Bax protein and CPP32 protease activity were observed concomitantly with apoptosis. These data suggested that the signal transduction from FAK to PI 3-kinase and Akt exerts an anti-apoptotic effect on apoptosis induced by oxidative stress and FAK locates in the upstream signal of the PI 3-kinase-Akt survival pathway in hydrogen peroxide-induced apoptosis of T98G cells.

EXPERIMENTAL PROCEDURES

Cells and Materials—T98G cells were suspended in RPMI 1640 medium containing 5% fetal bovine serum (Nippon Bio-Supply Center, Tokyo, Japan). For oxidative stress experiments, growing cells were subcultured at a density of 2 × 10^5/ml cell in medium containing 1% fetal bovine serum. Monoclonal anti-phosphotyrosine antibody (mAb: 4G10) and rabbit anti-PI 3-kinase (p85) Ab were purchased from Upstate Biotechnology Inc. (NY), anti-FAK mAb from Transduction Laboratories (KY), goat anti-Bcl-2 Ab, rabbit anti-Bax Ab, and goat anti-CP32 Ab from Santa Cruz Biotechnology, rabbit anti-Akt and phospho-Akt Abs from New England Biolabs. Inc. (MA), and the horse-radish peroxidase-conjugated secondary Ab from DAKO (Denmark).
**RESULTS**

**Tyrosine Phosphorylation of FAK and Serine Phosphorylation of Akt and the Association with PI 3-Kinase in Hydrogen Peroxide-treated T98G Cells**—We have previously described that hydrogen peroxide induced significant tyrosine phosphorylation of p125FAK in a human glioblastoma cell line, T98G (14). To investigate the signal transduction from FAK, we examined whether FAK associates with PI 3-kinase, which has been reported to associate with FAK by stimulation of platelet-derived growth factor in NIH3T3 mouse fibroblast (18). T98G cells were treated with or without 1 mM hydrogen peroxide for various times. Lysates were prepared from these cells and immunoprecipitated by anti-FAK mAb, followed by the detection of its associated PI 3-kinase activities, as well as the p85 subunit of PI 3-kinase, and the FAK tyrosine phosphorylation.

In response to hydrogen peroxide stimulation, a significant increase of PI 3-kinase activity (i.e. increases of 3-fold at 1 h and 10-fold at 2–4 h) was found in the anti-FAK immunoprecipitates (Fig. 1A). The product was confirmed as PtdIns 3-P by PtdIns 3-phosphate (PtdIns 3-P) by a comparison with the product of kinase assay using the immunoprecipitates with anti-PI 3-kinase Ab. The formation of PtdIns 3-P was completely inhibited (> 95% inhibition) by the 0.5 mM wortmannin (data not shown).

The PI 3-kinase activity gradually increased and reached maximal at 2 h. Western blotting of anti-FAK immunoprecipitates with anti-p85 Ab paralleled with this observation (Fig. 1B). Simultaneously, tyrosine phosphorylation of the anti-FAK immunoprecipitates were determined by using anti-phosphotyrosine mAb, indicating that FAK phosphorylation increased significantly at 1–2 h and maintained constant till 4 h after incubation. Thus, the tyrosine phosphorylation of FAK preceded the PI 3-kinase association with FAK to response to hydrogen peroxide stimulation. Of note is that blotting with anti-FAK mAb revealed the same amounts of FAK precipitated from all five samples (Fig. 1D). These results suggested that PI 3-kinase associates clearly with tyrosine-phosphorylated FAK. It was recently reported that insulin-like growth factor 1 promotes cell survival by activating PI 3-kinase and its downstream target, the serine-threonine kinase Akt (5). We therefore examined the effect of hydrogen peroxide on serine phosphorylation of Akt using anti-phospho-Akt Ab. The cell lysates were subjected to immunoblotting with anti-phospho-Akt or anti-Akt Abs. When lysates of hydrogen peroxide-treated cells were electrophoresed and immunoblotted with anti-Akt Ab, the corresponding band of Akt was consistently detected up to 4 h after stimulation with hydrogen peroxide (Fig. 2). Immunoblotting using anti-phospho-Akt Ab, which recognizes phosphorylated Ser-473 of Akt, revealed phosphorylation of Akt at 1 h after

**PI 3-Kinase-Akt Survival Pathway in H2O2-induced Apoptosis**

**FIG. 1. Induction of FAK-PI 3-kinase association by hydrogen peroxide in T98G cells**. T98G cells were treated with hydrogen peroxide (1 mM) with or without wortmannin (Wow) (0.5 mM) for various periods. Lysates were prepared and analyzed by Western blotting using anti-phospho-Akt Ab or anti-Akt Ab.

**FIG. 2. Time course of Akt phosphorylation.** T98G cells were treated with hydrogen peroxide (1 mM) with or without wortmannin (Wow) (0.5 mM) for various periods. Lysates were prepared and analyzed by Western blotting using anti-phospho-Akt Ab or anti-Akt Ab.

**Nicholson et al. (17).** Cell lysate (50 μg of protein) was incubated at 37 °C with 50 mM DEVD-MCA as a substrate for apopain/CPP32, for 30 min, or TVAD-MCA as a substrate for ICE, for 60 min. The amounts of released 7-amino-4-methylcoumarin were measured with fluorescence spectrophotometer (Hitachi F-4000, Tokyo, Japan), with excitation at 380 nm and emission at 460 nm.

**Opti-MEM, Lipofectin reagent, and prestained molecular marker were obtained from Life Technologies, Inc. (Gibco). Enhanced chemiluminescence reagent were obtained from Amersham Pharmacia Biotech (Tokyo, Japan). Substrates for protease activity, YVAD-MCA and DEVD-MCA, were obtained from Peptide Institute, Inc. (Osaka, Japan).

**PI 3-Kinase Activity in Immunoprecipitates—**T98G cells (5 × 10⁶ cells) were incubated with hydrogen peroxide at various times, washed once with ice-cold phosphate-buffered saline, and lysed with lysis buffer as described previously (15). Insoluble material was removed by centrifugation at 4 °C for 20 min at 10,000 × g. The supernatants were incubated with anti-FAK mAb at 4 °C overnight. Immunocomplex was precipitated with protein G-Sepharose (Amersham Pharmacia Biotech) and washed as described previously (15). The immunoprecipitates were incubated with phosphatidylinositol (PtdIns) and 1 αC (γ-32P)PATP in the reaction buffer (20 mM Tris-HCl, pH 7.5, 10 mM MgCl₂, 10 mM ATP, 200 μM phosphatidylserine) at 25 °C for 10 min and terminated by addition of 100 μl of 1 M HCl. Phospholipids were then extracted with 200 μl of CHCl₃/MeOH (1:1). The organic layer was spotted onto a silica gel 60 plate (Merck, Darmstadt, Germany) pretreated with 1% potassium oxalate. Thin-layer chromatography plates were developed with CHCl₃/MeOH/acetic acid/CH₃COOH/H₂O (7:5:2:2:2), dried, visualized, and analyzed by Fuji image analyzer (Tokyo, Japan).

**Electrophoresis and Immunoblotting—**For the preparation of cell lysate, 1 × 10⁶ packed cells were lysed with lysis buffer as described previously (16). After centrifugation, Laemmli sample buffer was added to the cell lysate. Samples were boiled for 5 min, and equal amounts of protein were separated by SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. The membranes were blocked in 3% bovine serum albumin in phosphate-buffered saline for 1 h and then incubated with primary Ab for 1 h at room temperature. After incubation with secondary Ab coupled to horseradish peroxidase, detection was made using the enhanced chemiluminescence system (Amersham Pharmacia Biotech). Molecular sizes were determined by the relative mobilities of prestained molecular weight markers. Densitometric analysis was performed on a Macintosh computer using the public domain NIH Image program.

**Analysis of DNA Fragmentation—**DNA fragmentation study was performed as described elsewhere (14). In brief, cells were gently lysed for 30 min at 4 °C in a buffer containing 5 mM Tris buffer (pH 7.4), 20 mM EDTA, and 0.5% Triton X-100. After centrifugation at 15,000 rpm for 15 min, supernatants containing soluble fragmented DNA were collected and treated with RNase (20 μg/ml), followed by proteinase K (20 μg/ml). DNA fragments were precipitated in ethanol. Each sample was then electrophoresed on a 2% agarose gel and visualized by staining with 0.1% ethidium bromide.

**Quantitation of Apoptosis—**Cell viability was determined by Trypan blue dye exclusion, and the existence of apoptotic cells was confirmed by the appearance of sub-G₁/G₀ peak fractions in cell cycle analysis. For the cell cycle analysis, ethanol-fixed cells were stained with propidium iodide (50 μg/ml) in the presence of RNase A (100 μg/ml, Wako Pure Chemical, Osaka, Japan) and then analyzed by fluorescence-activated cell sorter Calibur using a CELLQuest program (Becton Dickinson, CA).

**ICE (-like) and CPP32 (-like) Protease Activity—**After washing with phosphate-buffered saline, cell lysate was prepared as described by...
stimulation with hydrogen peroxide, which increased markedly till 4 h. Pretreatment with wortmannin (0.5 μM), a specific PI 3-kinase inhibitor for 1 h, completely inhibited Akt phosphorylation. These findings suggested that FAK-associated PI 3-kinase activity is prerequisite for Akt activation.

**Wortmannin Accelerates Hydrogen Peroxide-induced Apoptosis in T98G Cells**—We demonstrated previously that 1 mM hydrogen peroxide treatment for 15 h induced apoptosis in T98G cells (14). When T98G cells were treated with 1 mM hydrogen peroxide for various periods, less than 12% of the cells died within 4 h by the estimation with trypan blue dye exclusion (Fig. 3). To investigate the role of PI 3-kinase in apoptosis, we examined the effects of wortmannin. When T98G cells were pretreated with 0.5 μM wortmannin for 1 h, followed by treatment with 1 mM hydrogen peroxide, more than 34% of the cells died within 4 h. Simultaneously, we estimated the DNA content of the cells using flow cytometry after staining with PI. DNA histogram of the PI-stained cells in Fig. 4 indicated that 10% of hydrogen peroxide-treated cells (4 h) had hypodiploid DNA (Fig. 4A), indicative of apoptosis, whereas untreated control cells contained less than 4% in this area. In the presence of wortmannin, hydrogen peroxide treatment for 4 h induced 40% of the cells with a hypodiploid DNA pattern, indicating that wortmannin enhanced hydrogen peroxide-induced apoptosis significantly (Fig. 4B). Wortmannin alone did not induce apoptosis during these incubation periods (data not shown). In addition, when DNA fragmentation was analyzed, marked DNA ladder was observed after pretreatment with wortmannin for 1 h followed by hydrogen peroxide for 4 h (Fig. 5B), suggesting that these cells were apoptotic. No significant DNA fragmentation was induced by treatment with wortmannin alone (data not shown).

**Hydrogen Peroxide in the Presence of Wortmannin Down-regulates the Amount of Bcl-2 Protein and Up-regulates the Amount of Bax Protein**—We next examined whether hydrogen peroxide-induced apoptosis is modulated by Bcl-2 family proteins. Western blotting indicated the amounts of Bcl-2 and Bax in hydrogen peroxide-treated T98G cells were constant until 4 h after stimulation with hydrogen peroxide (Fig. 6). In the presence of wortmannin, however, Bcl-2 gradually decreased to one-tenth at 4 h, whereas Bax showed a 15-fold increase at 4 h. Thus, there appears to be an inverse correlation between expression of Bcl-2 and Bax in hydrogen peroxide- and wortmannin-induced apoptosis of T98G cells. The above data confirmed that Bcl-2 is down-regulated, whereas apoptosis-inducing Bax protein is up-regulated during apoptosis in some cell types (19, 20).

**Hydrogen Peroxide in the Presence of Wortmannin Activates CPP32 Protease**—ICE family proteases have been reported to be activated in apoptosis (21). To examine the possible involvement of ICE family proteases in hydrogen peroxide-induced apoptosis, we measured the activity of ICE protease and CPP32 protease using peptide substrates in cells treated with hydrogen peroxide, hydrogen peroxide and wortmannin, or wortman-
Expression of Bcl-2 family (Bcl-2 and Bax) proteins in the hydrogen peroxide-treated cells with or without wortmannin. Cells were treated with wortmannin for 1 h and then followed by hydrogen peroxide treatment for indicated periods, lysed, and analyzed by Western blotting using anti-Bcl-2 Ab or anti-Bax Ab. Proteins (40 μg) were separated on 12% SDSPolyacrylamide gel electrophoresis and analyzed as described under “Experimental Procedures.” Densitometric analysis revealed that the Bcl-2 band reduced to one-fifth (3 h) and to one-tenth (4 h), whereas Bax increased up to 15-fold at 4 h, compared with time 0.

Discussion

We have reached the following conclusions in this paper. 1) Hydrogen peroxide stimulated the association of FAK with PI 3-kinase. 2) Wortmannin accelerated hydrogen peroxide-induced apoptosis in T98G cells. 3) Hydrogen peroxide stimulated the phosphorylation of Akt. 4) When apoptosis occurred, CPP32 protease was activated, concomitant with the decrease of Bcl-2 protein and increase of Bax protein. 5) Phosphorylation of Akt is inhibited by wortmannin. Recently, we reported the anti-apoptotic role of FAK in hydrogen peroxide-induced apoptosis (14). In this study, we demonstrated that tyrosine phosphorylation of FAK, the association of FAK with PI 3-kinase, as well as serine phosphorylation of Akt occur in T98G cells after exposure to hydrogen peroxide. It should be mentioned that in the presence of wortmannin, PI 3-kinase activity and serine phosphorylation of FAK were inhibited with increasing apoptosis rate. Putative downstream effectors of PI 3-kinase are the PKA and PKC families of protein kinases. Akt is also homologous to the PKA and PKC families of protein kinases. Akt is involved in the promotion of cell survival through inhibition of apoptosis, possibly playing a role in PI 3-kinase-mediated neuronal cell survival (5). As a mode of survival signaling from Akt to BAD, Datta et al. (7) proposed that in the case of insulin-like growth factor 1 stimulation, Akt is activated via PI 3-kinase and activated Akt phosphorylated BAD, which dissociates from Bcl-XL or Bcl-2. Then, phosphorylated BAD is sequestered in the cytosol bound to 14–3–3 (22). As a result, Bcl-2 homodimer or Bcl-2-Bcl-XL heterodimer was formed, thereby leading to cell survival.

The above idea appears to be consistent with our observation on the oxidative stress-induced apoptosis as shown in this study. In T98G cells, after stimulation with hydrogen peroxide, FAK was tyrosine-phosphorylated followed by the association and activation of PI 3-kinase. Activation of PI 3-kinase leads to the activation of Akt. Although we could not determine whether the target of Akt in the oxidative stress is BAD or not, Akt might regulate the balance of Bcl-2 family by phosphorylation of apoptosis-related proteins. In this study, the presence of Bcl-2 led to survival, whereas the increase of Bax, a BAD homolog, led to apoptosis. Although the mechanism of the function of Bcl-2 and Bax in apoptosis remains to be determined, Aritomi et al. (23) performed crystallographic studies indicating that Bax possesses a greater potential for membrane insertion than either Bcl-2 or Bcl-XL, and thus Bax is likely to form membrane pores. They proposed that the roles of Bcl-XL and Bcl-2 are to inhibit pore formation of Bax or other pore-forming proteins through heterodimerization. Taken together, we speculated that in signal transduction from FAK to PI 3-kinase, Akt plays a survival role by regulating the balance of apoptosis-blocking protein (Bcl-2) and apoptosis-inducing factor (Bax).

In this study, Akt was translocated to the plasma membrane after stimulation with hydrogen peroxide, and this translocation was sensitive to wortmannin (data not shown). We propose the following model for FAK, PI 3-kinase, Akt, in the apoptosis pathway. After exposure to hydrogen peroxide, FAK is activated by tyrosine phosphorylation, followed by PI 3-kinase activation and translocation of Akt to membrane. Akt is activated by serine phosphorylation and phosphorylates its target proteins in the cytosol, leading to the regulation of the balance of Bcl-2 family. Akt phosphorylation almost disappeared by the transfection of FAK antisense phosphorothioate oligonucleotides as previously employed (14) (data not shown). Taken collectively, FAK is an upstream signal protein of the PI 3-kinase-Akt survival pathway in hydrogen peroxide-induced apoptosis. Further analysis of T98G transfectants expressing active FAK or depletion of the
FAK gene should provide more information on the role of FAK in apoptosis.

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