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Antiapoptotic Action of Focal Adhesion Kinase (FAK) Against Ionizing Radiation

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ABSTRACT

Focal adhesion kinase (FAK) has an antiapoptotic role in anchorage-dependent cells *via* an unknown mechanism. To elucidate the role of FAK in the antiapoptosis, we have demonstrated that FAK-overexpressed (HL-60/FAK) cells have marked resistance against various apoptotic stimuli. That is, HL-60/FAK cells were highly resistant to hydrogen peroxide or etoposide-induced apoptosis compared with the vector-transfected cells. In this study, we demonstrated that HL-60/FAK cells were highly resistant to ionizing radiation (IR)-induced apoptosis. IR at 10–40 Gy induced significant DNA fragmentation, activation of caspase-3 and -8, the processing of a proapoptotic BID, and mitochondrial release of cytochrome *c* in the parental or HL-60/Vect cells, whereas no significant DNA fragmentation or no other concurring events were observed in the HL-60/FAK cells. Of note is that, in the HL-60/FAK cells, phosphatidylinositol 3′-kinase–Akt survival pathway was activated, accompanied with significant induction of inhibitor-of-apoptosis proteins (cIAP-2, XIAP). Finally, constructs of FAK mutants revealed that the central kinase domain (K454), autophosphorylation site (Y397), as well as focal adhesion target regions (Y925), were prerequisite for the FAK function. These results indicated that mitochondria pathway is required for IR-induced apoptosis, and FAK overexpression prevents this pathway, thus rendering antiapoptotic states. *Antioxid. Redox Signal.* 4, 491–499.

INTRODUCTION

REACTIVE OXYGEN SPECIES (ROS) are presumed to be important regulators of apoptosis. Production of ROS is found to be stimulated by tumor necrosis factor-α (TNF-α) (1), lipopolysaccharide (42), ceramide (11), growth factor withdrawal (3), HIV infection (8), ionizing radiation (IR), or p53-induced apoptosis (17, 31). In contrast, overexpression of thioredoxin (16), manganese superoxide dismutase (43), or Bcl-2 (40, 41) can delay apoptosis. The mechanism of these antioxidant molecules to suppress apoptosis has been extensively elucidated so far.

Focal adhesion kinase (FAK) has been implicated in the integration of signals from integrins, oncogenes, and neuropeptides (5, 35). FAK has also been shown to play an important role in the cell survival of anchorage-dependent cells (10). Proteolytic cleavage of FAK by caspase-3 has been reported during growth factor deprivation-induced apoptosis in human umbilical vein endothelial cells (21), which implies an association between FAK and its antiapoptotic function.

We found that FAK was tyrosine-phosphorylated by oxidative stress before the onset of apoptosis (37). Further, PKB/Akt, which has been implicated in the pathway of survival signal, was serine-phosphorylated following tyrosine-phosphorylation of FAK. Thus, we assumed that FAK has some antiapoptotic capacity. Actually, we observed that whereas parental HL-60 cells are sensitive to various apoptosis-inducing reagents, FAK-overexpressed cells acquired resistance to oxidative-stress, etoposide-induced apoptosis, with concomitant inhibition of caspase-3 protease (38). In addition, we exhibited that the phosphatidylinositol 3′-kinase (PI3-kinase)–Akt survival pathway and nuclear factor-κB

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(NF-κB) activation with concomitant increase of inhibitory apoptosis proteins (IAPs) are involved in the FAK-induced resistance to apoptosis (38). Thus, we claimed that FAK activates a signal pathway linking NF-κB and IAPs.

In this study, we explored whether the FAK overexpression endows resistance to the IR-induced apoptosis as well, because IR induced apoptosis by severely damaging DNA, mitochondrial membranes, and its membrane potential (20, 24). We demonstrated here that the antiapoptotic action by FAK occurs in multiple steps, including the suppression of caspase-8 expression and activation, BID protein cleavage, and cytochrome *c* release. This is the first demonstration that FAK overexpression might interfere with an apoptotic signal by inhibiting the expression of caspase-8, a first molecule initiating apoptosis.

EXPERIMENTAL PROCEDURES

Cells and materials

HL-60 cells were suspended in RPMI 1640 medium containing 5% fetal bovine serum (Nippon Bio-Supply Center, Tokyo, Japan). For the radiation experiments, cells were subcultured at a density of 2×10^5 cells/ml in medium containing 1% fetal bovine serum. Anti-FAK monoclonal antibody (mAb), anti-caspase-3 mAb, anti-BID mAb, and anti-XIAP mAb were purchased from Transduction Laboratories (Lexington, KY, U.S.A.), and anti-phosphotyrosine (PY) mAb (4G10) and rabbit anti-PI3-kinase (p85) antibody (Ab) from Upstate Biotechnology Inc. (Lake Placid, NY, U.S.A.). Anti-human caspase-8 mAb, anti-human cytochrome *c* mAb, and anti-cIAP-1 mAb were obtained from Pharmingen (San Diego, CA, U.S.A.), anti-cleaved caspase-3 and -8 mAbs from Cell Signaling (Beverly, MA, U.S.A.), and the horseradish peroxidase-conjugated secondary Ab from DAKO (Denmark). Anti-β-tubulin mAb and enhanced chemiluminescence reagents were obtained from Amersham–Pharmacia–Biotech (Tokyo, Japan). Substrates for protease activity, Ac-YVAD-7-amino-4-methylcoumarin (AMC) (caspase-1), Ac-DEVD-AMC (caspase-3), Ac-VEID-MCA (caspase-6), Ac-IETD-MCA (caspase-8), Ac-LEHD-MCA (caspase-9), and anti-cIAP-2 Ab were obtained from R&S (Minneapolis, MN, U.S.A.).

Transfection and irradiation

Hemagglutinin-tagged FAKcDNA and mutated FAKcDNA subcloned into the plasmid pRcCMV were transfected into HL-60 cells using electroporation with a Gene Pulser (Bio-Rad) at 0.35 kV, and stable transformants were selected in the presence of 0.5 mg/ml geneticin, as described elsewhere (38). Representative clones of HL-60/Vect, HL-60/FAK, and HL-60/mutated FAK were used in this study. Gamma-irradiation was done using Gamma Cell 40 (1.3 Gy/min from a ^{137}Cs source; Atomic Energy of Canada Ltd.).

Electrophoresis and immunoblotting

For the preparation of cell lysate, 1×10^6 packed cells were lysed with lysis buffer as described elsewhere (38). Ab dilutions were used as follows: Anti-FAK mAb, anti-caspase-3 mAb, anti-XIAP mAb, anti-PY mAb, anti-caspase-8 mAb, anti-cleaved caspase-3 mAb, anti-cleaved caspase-8 mAb, rabbit PI3-kinase Ab, and horseradish peroxidase-conjugated secondary Ab were used at 1:1,000 dilution. Anti-BID mAb, anti-cytochrome *c* mAb, anti-IAP-1 mAb, and rabbit anti-IAP-2 Ab were used at 1:500 dilution.

DNA fragmentation and cell viability assay

DNA fragmentation assay was performed as described previously (37). In brief, cells were gently lysed for 30 min at 4°C in a buffer containing 5 m*M* Tris buffer (pH 7.4), 20 m*M* 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, Wako Pure Chemicals, Tokyo, Japan), followed by proteinase K (20 μg/ml) digestion. DNA fragments were precipitated in 99% ethanol. Samples were then electrophoresed on a 2% agarose gel and visualized with 0.1% ethidium bromide. Cell viability was determined by trypan blue dye exclusion, and cell recovery was assayed by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method (37).

Assay of caspase-1, -3, -6, -8, and -9 activity

Cells were washed with phosphate-buffered saline, and cell lysate was prepared as described by Nicholson *et al.* (28). Cell lysate (50 μg of protein) was incubated at 37°C with 50 μ*M* Ac-DEVD-AMC, Ac-VEID-AMC, Ac-IETD-AMC, and Ac-LEHD-AMC as caspase-3, -6, -8, and -9 substrates, respectively, for 30 min or Ac-YVAD-AMC as a caspase-1 substrate for 60 min. The amount of 7-amino-4-methylcoumarin (AMC) released was measured using a fluorescence spectrofluorometer (Hitachi F-4000, Tokyo, Japan), with excitation at 380 nm and emission at 460 nm. Caspase activities were expressed as picomoles per minute per milligram of protein.

Measurement of cytochrome c *release from mitochondria*

Cytosolic extracts from cells were prepared by the procedure described by Kulik *et al.* (19). Cells (1×10^7) were washed with phosphate-buffered saline and suspended in 300 μl of buffer [20 m*M* HEPES-KOH (pH 7.5), 10 m*M* KCl, 1 m*M* EDTA, 1 m*M* EGTA, 1.5 m*M* $MgCl_2$, 1 m*M* dithiothreitol, 0.1 m*M* phenylmethylsulfonyl fluoride] containing 250 m*M* sucrose. Cells were homogenized with a Teflon homogenizer, and the homogenates were centrifuged at 10,000 *g* for 15 min at 4°C. The supernatants were further centrifuged at 100,000 *g* for 1 h at 4°C, and the resulting supernatants were analyzed by blotting with the anti-cytochrome *c* mAb.

RESULTS

Inhibition of IR-induced apoptosis by the FAK overexpression

To induce apoptosis, cells were γ-irradiated with doses of 5–40 Gy, and cell viability was assessed by the MTT assay method. As shown in Fig. 1, parental HL-60 and HL-60/Vect

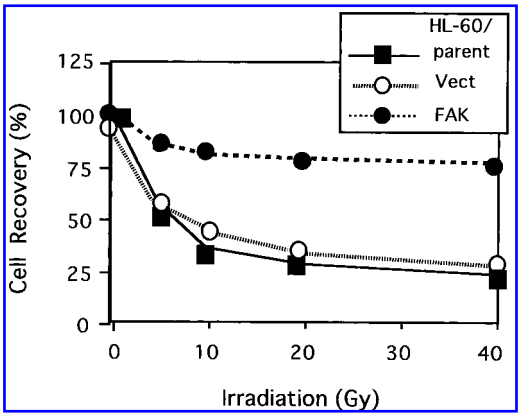

FIG. 1. Induction of cell death by the IR. Cells (2×10^5/ml) were treated with IR at 5–40 Gy and incubated for 24 h. Recovery of viable cells was assessed by the MTT method.

cells showed marked cell death by the IR over 10 Gy, whereas HL-60/FAK cells were highly resistant to the IR-induced cell death, as assessed at 24 h after IR. Parental HL-60 and HL-60/Vect cells showed >50% cell death 24 h or 48 h after the 20 Gy IR, whereas HL-60/FAK cells did not exhibit significant cell death upon 48-h incubation periods (Fig. 2A). IR-induced cell death was assumed to be apoptotic, because typical DNA fragmentation was induced by the IR treatment in parental and HL-60/Vect cells (Fig. 2B). In contrast, no significant DNA fragmentation was observed in HL-60/FAK cells under the same condition.

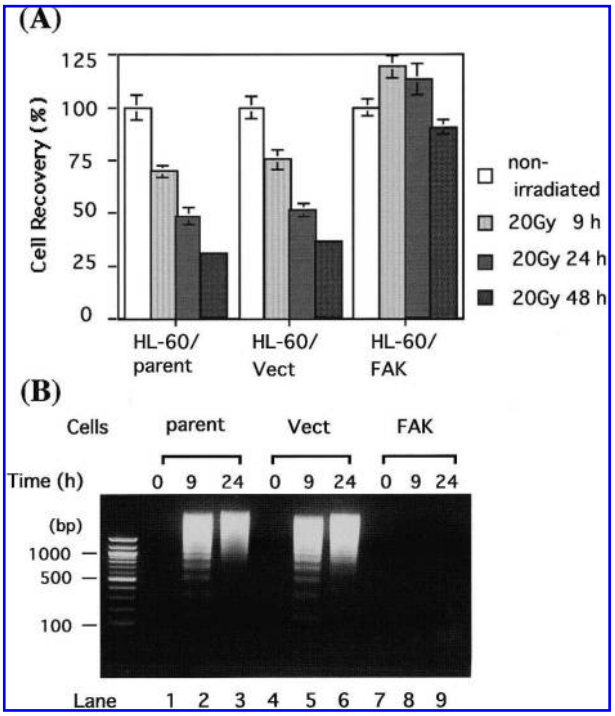

FIG. 2. Time course of viable cell recovery after IR treatment and detection of apoptotic DNA fragmentation. Cells (2×10^5/ml) were treated with IR at 20 Gy and incubated for 9, 24, and 48 h. (**A**) Recovery of viable cells was assessed by the MTT method. (**B**) Soluble fragmented DNA was extracted, electrophoresed, and visualized after staining with ethidium bromide.

Tyrosine phosphorylation of FAK and serine phosphorylation of Akt and the association with PI3-kinase in HL-60/FAK cells

We have previously described that the FAK–PI3-kinase–Akt survival pathway was activated before the initiation of apoptosis by hydrogen peroxide in a human glioblastoma cell line, T98G cells. To investigate whether these signal pathways are involved as well in the antiapoptotic action of FAK-overexpressed cells, we examined the tyrosine phosphorylation in these cells. HL-60/FAK and HL-60/Vect cells were treated with 20 Gy IR, incubated for 3 h, and lysed and immunoblotted with anti-PY mAb. As shown in Fig. 3A, tyrosine phosphorylation on several protein bands was detected in the IR-treated or nontreated HL-60/FAK cells, indicating that FAK induced some protein phosphorylation irrespective of IR treatment. We identified the 125-kDa band to be FAK by immunoprecipitation with anti-FAK mAb, followed by blotting with anti-PY mAb (Fig. 3B). In addition, to investigate the association of FAK with PI3-kinase, cell lysates of HL-60/Vect and HL-60/FAK cells were immunoprecipitated by anti-FAK mAb, followed by the detection of its associated PI3-kinase, a p85 subunit of PI3-kinase. As shown in Fig. 3C, significant PI3-kinase activity was found in the anti-FAK immunoprecipitates in the HL-60/FAK, but not in parental or HL-60/Vect cells. These results indicated that PI3-kinase associates directly with tyrosine-phosphorylated FAK and activates PI3-kinase activity. Thus, the survival pathway of FAK–PI3-kinase–Akt was assumed to be activated in HL-60/FAK cells constitutively.

Furthermore, we explored whether Akt was activated following PI3-kinase activation. To study Akt activation, serine phosphorylation of Akt was examined using anti-phospho-Akt Ab. As shown in Fig. 3B, serine phosphorylated-Akt was consistently detected in HL-60/FAK cells, whereas no phospho-Akt was detected in HL-60/Vect cells with or without IR treatment. The above results confirmed that the FAK–PI3-kinase–Akt survival pathway was constitutively activated in HL-60/FAK cells.

FAK inhibits the activation of caspase-3

Recent studies have shown that members of the protease caspase family play pivotal roles in apoptosis initiation and execution. To examine which caspase family members are involved in the IR-induced apoptosis, we measured caspase-1, -3, -6, -8, and -9 protease activities using specific peptide substrates in cells treated with IR. Although no caspase-1 and minimal caspase-9 activities were detected at 24 h after IR, caspase-3 activity was markedly elevated and caspase-8 was moderately activated with IR-treated parental HL-60 and HL-60/Vect cells (Fig. 4A). In contrast, no significant caspase-3 and -8 activities were induced in HL-60/FAK cells, suggesting that enhanced FAK expression suppresses caspase-3 and -8, resulting in the inhibition of IR-induced apoptosis.

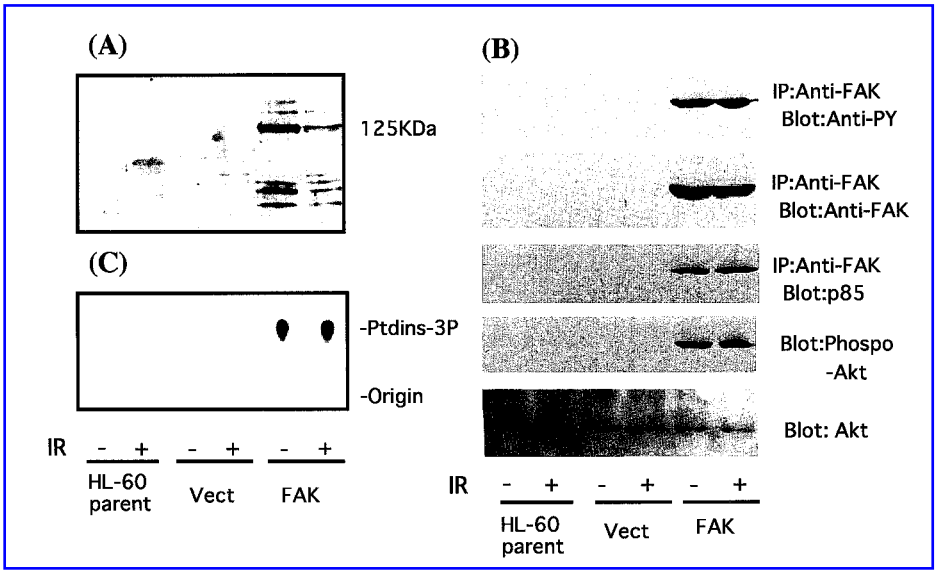

FIG. 3. Detection of activated FAK, Akt, and PI3-kinase in the HL-60 transfectants, and the effect of IR. (**A**) Detection of tyrosine-phosphorylated proteins in HL-60, HL-60/Vect, and HL-60/FAK cells. Cells were treated with IR at 20 Gy, lysed after 24 h, and analyzed by western blotting with anti-PY mAb. (**B**) HL-60/FAK cells induced tyrosine phosphorylation of p125FAK. Tyrosine phosphorylation was analyzed by immunoprecipitation using anti-FAK mAb followed by immunoblotting with anti-PY mAb. Association of PI3-kinase with FAK was analyzed by western blotting with anti-PI3-kinase (p85) Ab of anti-FAK immunoprecipitates. Activation of Akt was analyzed by western blotting with rabbit anti-phospho-Akt Ab or rabbit anti-Akt Ab. (**C**) To measure the PI3-kinase activity, cell lysates were prepared and immunoprecipitated by anti-FAK mAb, and the associated PI3-kinase activity was assayed using thin-layer chromatography.

To confirm further the activation of caspase-3 and -8, we detected procaspase-3 and -8 and their mature fragments, because caspase-3 and -8 are synthesized as a 32-kDa and 55-kDa inactive precursors, which are proteolytically cleaved to produce corresponding mature fragments, respectively. As shown in Fig. 4B, the 32- and 55-kDa procaspase-3 and -8 protein bands disappeared and the active fragments (17 kDa and 10 kDa) appeared upon IR-induced apoptosis in parental and HL-60/Vect cells. In contrast, procaspase-3 and -8 proteins remained uncleaved, and no mature fragments were observed in IR-treated HL-60/FAK cells. It should be noted that total caspase-8 expression decreased in HL-60/FAK cells compared with the parental or HL-60/Vect cells, but the reason for this remains undefined. These results indicated that IR induces activation of caspase-8 and, to a lesser extent, caspase-9, which leads to the activation of caspase-3 in parental and HL-60/Vect cells, whereas FAK suppresses the activation of both caspase-8 and caspase-3.

IR-mediated cytochrome c *release and cleavage of BID are blocked in HL-60/FAK cells*

The apoptotic pathway consists of two pathways: one is dependent on the proapoptotic factors, *i.e.,* cytochrome *c* and Apaf-1 from mitochondria, and the other is an independent pathway. To explore whether the IR-induced apoptosis in HL-60 cells is dependent on the proapoptotic factors, we examined mitochondrial cytochrome *c* release in the cytosolic extracts at 24 h after the cells were treated with IR. As shown in Fig. 5A, IR induced significant cytochrome *c* release in the parental and HL-60/Vect cells, but not in HL-60/FAK cells. Thus, IR induced cytochrome *c* release, which was totally inhibitable by the overexpression of FAK, suggesting that FAK acts upstream of the mitochondria pathway.

As it is recently known that caspase-8 cleaves BID, a proapoptotic Bcl-2 family member, and the cleaved fragment of BID induces mitochondorial cytochrome *c* release (19), we also tested cleavage of BID. As shown in Fig. 5A, a significant decrease of a proapoptotic BID (p22) was observed in parental and HL-60/Vect cells, presumably degraded into p15 and p13 fragments, but not in HL-60/FAK cells. Thus, IR-induced BID cleavage was also blocked in HL-60/FAK cells, suggesting that FAK acts upstream of the BID.

Induction of IAP-family protein expression in HL-60/FAK cells

We assumed that the inhibition of IR-induced apoptosis by FAK may be ascribed to the expression of the survival genes, such as inhibitory apoptosis protein (IAP), and/or the FLICE-like inhibitory protein (FLIP) (32). The IAP family proteins interfere in the caspase cascade, particularly the proteolytic activation of caspase-3, -7, and -9 (36). Therefore, we examined the expression of the IAP family proteins, cIAP-1,2 and XIAP by immunoblotting (Fig. 5B). Expression of cIAP-2 and XIAP in HL-60/FAK cells was markedly higher than in HL-60/Vect cells, and their levels were sustained by the IR treatment. In contrast, cIAP-2 and XIAP expression in HL-60 and HL-60/Vect cells was significantly decreased by the IR treatment. As FLIP is known as an inhibitor of caspase-8, we examined the expression of this protein. The level of FLIP protein was not changed in these cells with or without IR

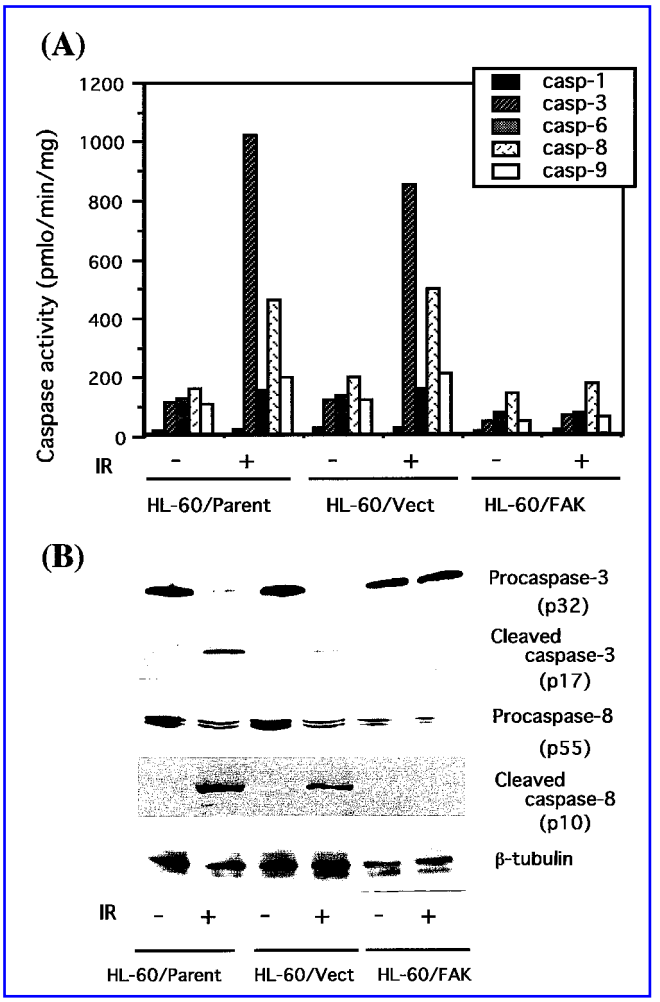

FIG. 4. Activation of caspase and detection of procaspase-3 and -8 and cleaved caspase-3 and -8 after treatment with IR at 20 Gy. (**A**) Cells were treated with IR at 20 Gy, and cell lysates after 24 h were used for the assay of caspase activity. (**B**) Detection of procaspase and mature form of caspase-3 and -8 in each cell lysate (100 μg/lane), which were analyzed by western blotting, using respective Abs, as described in Experimental Procedures. Each Ab dilution used was 1:500. Equal application of each cell lysate was confirmed by using anti-β-tubulin mAb.

treatment (data not shown). These results suggested that elevated levels of cIAP-2 and XIAP suppress caspase-3, -7, and -9 processing, thus inhibiting apoptosis in HL-60/FAK cells.

Antiapoptotic effect in mutated-FAK transfected cells

FAK consists of a central kinase domain, N-terminal autophosphorylation site, C-terminal proline-rich regions, and paxillin binding focal adhesion targeting region. We prepared several mutated FAKcDNA constructs and established stable transformants to determine the FAK regions responsible for the antiapoptotic function. One is a kinase-inactive FAK in which ATP binding site Lys^{454} is replaced with Arg (K454R). The second is a mutation form of FAK in which the autophosphorylation site Tyr^{397} was replaced with Phe (Y397F), and the third is a mutated FAK of the tyrosine-phosphorylation site Tyr^{925} replaced with Phe (Y925F). As shown in Fig. 6, these three mutated-FAK-transfected cells all failed to exhibit antiapoptotic activity, indicating that at least these three regions are prerequisite for the antiapoptotic function of FAK.

DISCUSSION

In this article, we demonstrated that overexpression of FAK endowed HL-60 cells to protect against apoptosis induced by IR. There have been several reports describing the antiapoptotic roles of FAK in various apoptosis-inducing systems. Hungerford *et al.* (13) reported that anchorage-dependent cells became apoptotic when cells were microinjected with anti-FAK Ab, or with a peptide corresponding to the portion of the β1-integrin cytoplasmic domain presumed to be required for β1 integrin–FAK interaction. In another study, Frisch *et al.* reported that constitutively activated FAK protected MDCK cells from apoptosis consequent upon the loss of matrix contact (10). Furthermore, Xu *et al.* (45) reported that attenuation of FAK expression leads to apoptosis in tumor cells. Interestingly, Ilić *et al.* (15) reported that the extracellular matrix survival signals via FAK suppressed a p53-regulated apoptosis by serum withdrawal in anchorage-dependent cells. Whether FAK has an antiapoptotic effect on other stresses or drug-induced apoptosis has not been explored, and how the FAK mediates antiapoptotic function remains to be determined. Recently, we presented evidence that FAK has an antiapoptotic role in the apoptosis induced by oxidative stress, as well as etoposide, in anchorage-independent cells, HL-60. Etoposide, as well as hydrogen peroxide, is known to produce ROS (2). In this study, we further demonstrated evidence that FAK has an important role in the antiapoptotic function in the IR-induced apoptosis, as well as in the oxidative stress-induced apoptosis.

Why we used human leukemic HL-60 cells for the transfection of FAK should be commented. First, HL-60 cells express virtually no FAK, and the antiapoptotic function of FAK can easily be evaluated. Second, HL-60 cells are relatively sensitive to IR-induced apoptosis, whereas they become resistant when differentiated into adherent macrophage-like cells with substantial expression of FAK (data not shown).

HL-60 lacks p53 protein, which is one of the major regulators of the apoptotic response to IR, suggesting that a p53-independent pathway is operating in HL-60 cells. The cellular response to IR includes activation of DNA repair, cell-cycle arrest, and lethality. The signaling pathway responsible for these events still remains unclear. Recent studies have shown that IR induces two different mitogen-activated protein (MAP) kinase cascades that converge on c-Jun N-terminal kinase (JNK) and p38 MAP kinase (p38) (44). Apoptosis signal-regulatory kinase 1 (ASK1) is known as a MAP kinase kinase kinase that activates the JNK and p38 (14). Overexpression of ASK1 induced apoptosis in epithelial cells

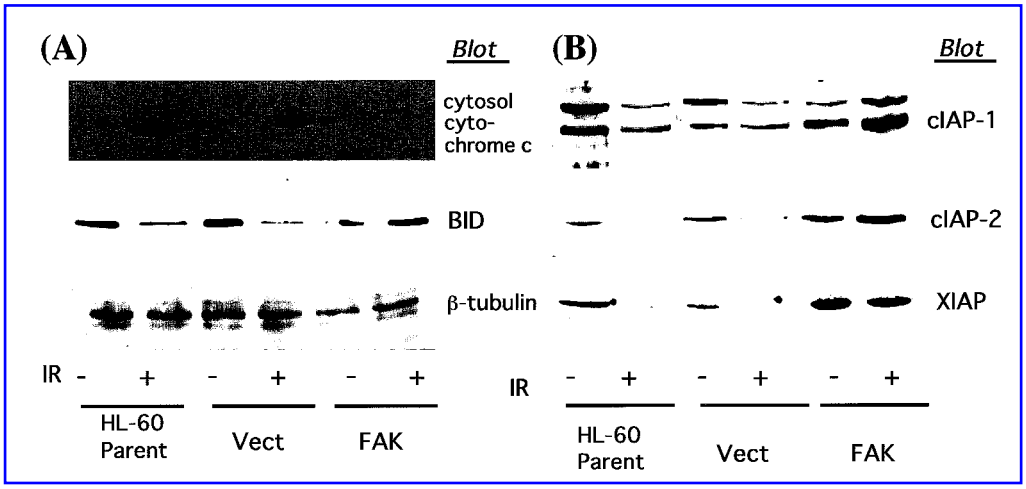

FIG. 5 IR-induced cytochrome *c* release into cytosol, BID cleavage, and IAP family proteins. (**A**) Cells were treated with IR at 20 Gy, and cytosolic fractions prepared after 24 h, as described in Experimental Procedures, were analyzed by western blotting, using anti-cytochrome *c* mAb. (**B**) Cellular levels of BID and IAP family proteins were also determined by western blotting, using anti-BID mAb and anti-IAP-1 and anti-IAP-2 Abs. Each Ab dilution used was 1:500. Equal application of each cell lysate was confirmed by using anti-β-tubulin mAb.

and ovarian cancer cells, and that was a mitochondria-dependent apoptosis pathway with caspase-9 and -3 activation (12). Taken collectively, these results and our results suggested that ASK1 might be potentially involved in signal transduction of IR-induced apoptosis. In resting cells, ASK1 forms an inactive complex with thioredoxin. Upon treatment of cells with TNF or ROS, thioredoxin is oxidized and then ASK1 is dissociated from thioredoxin and activated (22, 34). While Bcl-2 is phosphorylated and inactivated by the coexpression of ASK1 and JNK, the ASK1–JNK pathway promotes mitochondria-dependent apoptosis by Bcl-2 phosphorylation (47).

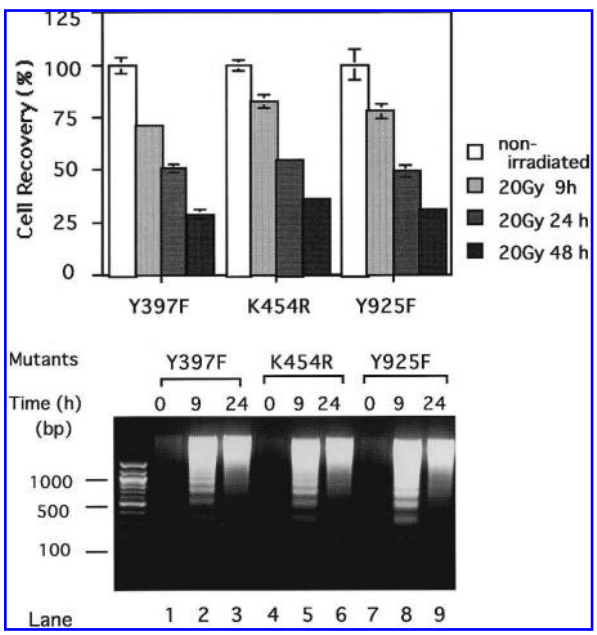

FIG. 6. Induction of cell death by the IR in mutated FAK-transfected cells. Cells (2×10^5/ml) were cultured and treated with IR at 20 Gy. (**A**) After 24 h, recovery of viable cells was assessed by the MTT method. (**B**) Soluble fragmented DNA was extracted, electrophoresed, and visualized after staining with ethidium bromide.

Caspase-3 is a critical downstream protease in the caspase cascade (46), responsible for the cleavage of important substrates such as poly(adenosine diphosphate ribose) polymerase (PARP) (27) and inhibitor of caspase-activated deoxyribonuclease (ICAD) (23). The caspase family controls apoptosis by multiple stimuli including Fas ligand and TNF-α(25, 26). We found that caspase-8 and -3 activation, BID cleavage, and cytochrome *c* release by IR were interrupted in the HL-60/FAK cells. Caspase-9 is also an important regulator for the mitochondria-dependent apoptotic signal in association with cytochrome *c* and Apaf-1. Actually, significant cytochrome *c* release was observed in our system (Fig. 5A), and caspase-9 activity was low compared with that of caspase-3 (Fig. 4A). We assume that the minimally activated caspase-9 may be critical to the downstream caspase-3 pathway. Alternatively, we determined caspase-9 activity at 24 h after IR; therefore, the optimal activation occurred earlier than that of other caspases. More detailed kinetic study needs to be performed. Thus, FAK interacts directly or indirectly with caspase-8 and caspase-9, upstream of caspase-3 activation. It should be noted that expression of caspase-8 protein was decreased in HL-60/FAK cells. This might also account for the antiapoptotic function of HL-60/FAK cells. Here, we depicted the outline of caspases and related molecules involved in the IR-induced apoptosis and antiapoptotic pathway concerned with FAK (Fig. 7).

Antiapoptotic proteins, CrmA and p35, have recently been shown to inhibit apoptosis by directly inhibiting ICE family proteases (39), most likely by functioning as substrates for, and as competitive inhibitors of ICE family proteases. In contrast, IAPs bind directly to caspases and inhibit its activity. Expression of the IAP proteins (c-IAP2, XIAP) were decreased in HL-60/Vect cells, but remained in HL-60/FAK

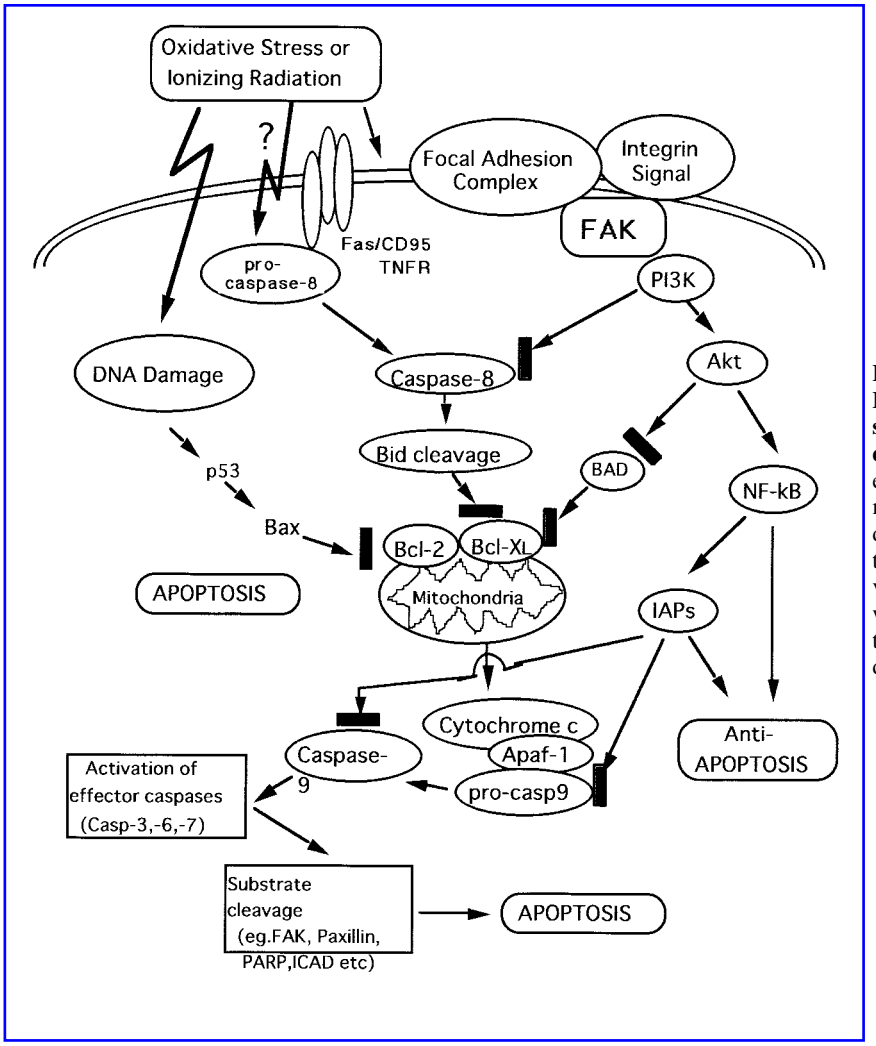

FIG. 7. Outlook of the cross-talk of FAK-induced PI3-kinase–Akt survival signals and oxidative stress or IR-induced apoptosis signals. Signaling molecules shown in this figure contain only molecules involved in the apoptosis inducers, executioners for the apoptosis by the oxidative stress or the IR, and the survival factors by the FAK-mediated pathways. The details are described in the text. → indicates activation and →▮ indicates inactivation or inhibition.

cells during IR treatment. Furthermore, basal expression of cIAP-2 and XIAP proteins increased in HL-60/FAK cells. Thus, our data indicated that FAK induces expression of IAP proteins and decreases expression of caspase-8. Further study on the regulation of caspase-8 expression may require the delineation of the exact role of the antiapoptotic function of FAK. We showed here that the FAK–PI3-kinase–Akt survival pathway is consistently activated in HL-60/FAK cells. Akt mediates phosphorylation of IKK, BAD, CREB, caspase-9, and Forkhead (4, 6, 9, 30, 33). A recent report indicated that Akt phosphorylates ASK1 and inactivates it (18), thus, we might depict that activated Akt via the FAK–PI3-kinase pathway inactivates ASK1 through its phosphorylation.

FAK Tyr^{397} is an autophosphorylation site and a high-affinity binding site for Src homology 2 (SH2) domains of Src family kinases (29). PI3-kinase and phospholipase Cγ also interact with this site. FAK Lys^{454} is essential for kinase activity, and FAK Tyr^{925} is a binding site for the Grb2 SH2 domain, and this interaction contributes to integrin-stimulated activation of Ras. However, transfection with FAK mutants (Y397F, K454R, Y925F) indicated that tyrosine residues at 397, 454, and 925 were all essential for the antiapoptotic effect. These results suggested that the signals through Src family kinase, PI3-kinase, Grb2, and kinase activity are essential for antiapoptotic function in HL-60/FAK cells. It should be mentioned that constitutive and elevated NF-κB activation was observed in the FAK-overexpressed HL-60 cells (38). NF-κB regulates many genes including IAPs. How FAK regulates downstream genes following NF-κB activation should be of great interest to delineate the antiapoptotic pathway. In conclusion, we indicated here that HL-60 and HL-60/Vect cells induced apoptosis by IR stimulation via a mitochondria pathway, caspase-8 activation, BID cleavage, and cytochrome *c* release, leading to the caspase-3 activation. In HL-60/FAK cells, in contrast, no such events have been observed, but decreased expression of caspase-8 and increase of IAP proteins were observed. Down-regulation of caspase-8 and resulting signals to caspase-3 activation was suppressed in the FAK-overexpressed cells, thus protecting IR-induced apoptosis. To our knowledge, the data presented here provide the first line of evidence for the regulation of caspase-8 and IAP proteins by FAK.

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ence of Japan. We express thanks to Dr. Steven Hanks, Vanderbilt University, for generously providing FAK and FAK mutant cDNA.

ABBREVIATIONS

Ab, antibody; AMC, 7-amino-4-methylcoumarin; ASK1, apoptosis signal-regulatory kinase-1; FAK, focal adhesion kinase; FLIP, FLICE-like inhibitory protein; IAP, inhibitor-of-apoptosis protein or inhibitory apoptosis protein; ICAD, inhibitor of caspase-activated deoxyribonuclease; IR, ionizing radiation; JNK, c-Jun N-terminal kinase; mAb, monoclonal antibody; MAP, mitogen-activated protein; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NF-κB, nuclear factor-κB; PARP, poly(adenosine diphosphate ribose) polymerase; PI3-kinase, phosphatidylinositol 3′-kinase; PY, phosphotyrosine; ROS, reactive oxygen species; SH2, Src homology 2; TNF-α, tumor necrosis factor-α.

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