Focal adhesion kinase (FAK) has been implicated to play a role in suppression of apoptosis. In this study, we have demonstrated that UV irradiation induced cleavage of FAK and two of its interacting proteins Src and p130Cas in Madin-Darby canine kidney cells, concomitant with an increase in cell death. The cleavage of these proteins upon UV irradiation was completely inhibited by ZVAD-FMK, a broad range inhibitor of caspases, and apparently delayed by Bcl2 overexpression. To examine if FAK plays a role in suppressing UV-induced apoptosis, stable Madin-Darby canine kidney cell lines overexpressing FAK were established. Our results showed that a marked (30–40%) increase in cell survival upon UV irradiation was achieved by this strategy. In our efforts to determine the mechanism by which FAK transduces survival signals to the downstream, we found that a FAK mutant deficient in binding to phosphatidylinositol 3-kinase failed to promote cell survival. Moreover, the expression of the Src homology 3 domain of p130Cas, which competed with endogenous p130Cas for FAK binding, abrogated the FAK-promoted cell survival. Together, these results suggest that the integrity of FAK and its binding to phosphatidylinositol 3-kinase and p130Cas are required for FAK to exert its antiapoptotic function.

Focal adhesion kinase (FAK), a 125-kDa cytoplasmic tyrosine kinase localized in focal adhesions, is a key component in integrin-mediated signal transduction pathways (1–3). So far, FAK has been implicated to play an important role in regulating at least three aspects of cellular functions, including cell migration (4, 5), cell cycle progression (6), and cell survival (7–10). The ability of FAK to transduce signals to the downstream is believed to be dependent on its ability to interact with several intracellular signaling molecules including Src family kinases (11, 12), phosphatidylinositol 3-kinase (PI3K; Ref. 13), Grb2 (14), and p130Cas (15). It has been shown recently that two simultaneous bindings of PI3K and p130Cas are required for FAK to promote cell migration on extracellular matrix proteins (16, 17). However, it is not clear if any of these molecules are responsible for the function of FAK in cell cycle or cell survival.

Tyr-397 has been identified as the major site of FAK autophosphorylation (18) and the binding site for the Src homology 2 (SH2) domains of Src (11, 12) and PI3K (19). The binding site for the SH2 domain of Grb2 has been mapped to Tyr-925 (20). The proline-rich sequence region of FAK (residues 712–718) has been identified as the major binding site for the SH3 domain of HSP27 (20). To directly analyze the effect of the loss of PI3K binding on FAK’s functions, a FAK mutant deficient only in PI3K binding has recently been introduced by a substitution of Asp-395 with Ala (17).

Several lines of evidence have suggested that FAK may have a function in promoting cell survival. Inhibition of FAK in fibroblasts by microinjection with anti-FAK antibodies or with a peptide corresponding to a region of β1 integrin cytoplasmic domain thought to be required for FAK binding results in apoptosis (7). Similarly, attenuation of FAK expression by antisense oligonucleotides induces apoptosis in tumor cells (8).

In this report, we have demonstrated that FAK is a target of caspases during UV-induced apoptosis and that overexpression of the wild-type (WT) FAK is capable of suppressing cell death upon UV irradiation. Moreover, we have found that inhibition of the association of FAK with PI3K or p130Cas abrogates the ability of FAK to promote cell survival. Taken together, these results strongly suggest that signals transmitted through the associations of FAK with PI3K and p130Cas are important for cell survival.

**EXPERIMENTAL PROCEDURES**

**Expression Plasmids and Antibodies**—The mammalian expression plasmid pKH3 encoding hemagglutinin (HA) epitope-tagged WT FAK or its mutants (D395A, Y397F, P712A/P715A, and Y925F) have been described previously (17). The plasmid pKH3-CasSH3 encoding the HA epitope-tagged SH3 domain of p130Cas was kindly provided by Dr. J.-L.
Role of FAK in UV-induced Apoptosis

Guarn (Cornell University, Ithaca, NY) and described previously (16).

The anti-FAK mAb (clone 77) was purchased form Transduction Laboratories (Lexington, KY). The rabbit polyclonal anti-p130Cas (C-20) was purchased from Santa Cruz Biotec, Inc. (Santa Cruz, CA). The anti-Src monoclonal antibody (95–184) was purchased from Upstate Biotechnology, Inc. (Lake Placid, NY). The rabbit polyclonal anti-p85 and the monoclonal antibody 12CA5 (anti-HA) have been described previously (19).

Cells and Transfections—MDCK cells, clone 3B5, were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (Life Technologies, Inc.). To generate cells stably expressing HA epitope-tagged FAK, subconfluent MDCK cells grown on 60-mm dishes were co-transfected with 2 μg of pKH3 plasmid encoding WT FAK or its mutant and 0.2 μg of pSV2neo using 10 μl of LipofectAMINE (Life Technologies, Inc.). Two days after transfection, cells were detached and replated on 100-mm dishes at an appropriate density in the medium containing 0.5 mg/ml of G418 (Calbiochem). After approximately 14 days, neomycin-resistant cell clones were picked using cloning cylinders and screened for exogenous FAK expression by immunoblotting using monoclonal antibody anti-HA. Multiple positive clones were obtained for further analysis for each FAK construct. Stable MDCK cells overexpressing Bcl2 have been described previously (22) and were maintained in G418-containing medium.

To generate MDCK cells stably expressing both FAK and CasSH3, WT cells were transfected as described above using LipofectAMINE, 2 μg of pKH3CasSH3, and 0.2 μg of pREP3. Clones were selected in growth medium containing 0.5 mg/ml G418 and 125 units/ml Hygromycin B (Calbiochem) and screened for FAK and CasSH3 expression by immunoblotting with anti-HA.

UV Irradiation, Treatment of Caspase Inhibitors, and Determinations of Cell Survival and Apoptotic Index—For UV irradiation, MDCK cells were plated at 10⁵ per 60-mm culture dish in growth medium. After 12 h, the medium was reduced to 1 ml/dish, and culture dishes were uncovered in a UV cross-linker (model UVC-508, ULTRA-LUM Inc., Carson, CA). UV irradiation was carried out with 10 mJ/cm² for 1 min. Following irradiation, 2 ml of growth medium was added, and the cells were incubated at 37 °C for the indicated times in a CO₂ incubator. For some experiments, cells were pretreated with 40 μM caspase inhibitor ZVAD-FMK or DEVD-CHO (Takara Shuzo Co., Shiga, Japan) for 1 h. Following UV irradiation, cells were incubated in growth medium with freshly added caspase inhibitor for 12 h.

Cell viability was determined by trypan blue exclusion. Briefly, UV-irradiated cells were collected and washed once with phosphate-buffered saline. Cell suspensions in phosphate-buffered saline were mixed with an equal volume of 0.4% trypan blue (Sigma), and cells excluding dye were counted with a hemocytometer. Cell survival was expressed as the percentage of live cells remaining after UV irradiation compared with the cell number at the time right before UV irradiation.

For nuclear staining, cells (10⁶) were plated on glass coverslips for 16 h and then exposed to UV radiation. After 4 h, coverslips were washed with phosphate-buffered saline, fixed in 4% paraformaldehyde, and stained with 0.5 μg/ml Hoechst 33258 (Sigma). Normal nuclei and apoptotic nuclei comprising those with fragmented nuclei and condensed chromatin were counted in 10 randomly chosen fields counted under a fluorescence microscope at ×10 magnification. Apoptotic index was expressed as the percentage of apoptotic nuclei in total counted (200–300) nuclei.

Immunoprecipitations, Immunoblotting, and in Vitro Kinase Assays—To analyze protein cleavage, UV-irradiated cells were collected and lysed in 1% Nonidet P-40 lysis buffer containing protease inhibitors as described previously (23). An equal amount (50 μg) of lysates was resolved by SDS-polyacrylamide gel electrophoresis and analyzed by immunoblotting with anti-FAK, anti-p130Cas, anti-Src, anti-p85, or anti-HA using the Amersham Pharmacia Biotech chemiluminescence system for detection.

To determine the PI3K activity associated with ectopically expressed FAK proteins in MDCK cells, epo-tope-tagged FAK proteins were immunoprecipitated with anti-HA from MDCK cell lysates (700 μg) and subjected to an in vitro PI3K assay as described previously (13).

To detect the association of epo-tope-tagged FAK with endogenous p130Cas in MDCK cells, lysates (500 μg) were incubated with polyclonal anti-p130Cas. The immunocomplexes were washed with 1% Nonidet P-40 lysis buffer and analyzed by immunoblotting with anti-HA.

RESULTS

A proteolytic cleavage of FAK has been observed during apoptosis induced by serum deprivation (24), the addition of Fas ligand/Apo-2L (25), overexpression of c-Myc (26), and treatment of certain chemicals such as staurosporine (27). In this report, we have examined if FAK cleavage also occurs during UV-induced apoptosis. Similar to other apoptotic stimuli as described above, UV irradiation induced a sequential cleavage of FAK into two fragments in MDCK cells, which was concomitant with a decrease in cell survival (Fig. 1). The first cleavage product of the 85-kDa fragment was detected as early as 6 h after UV irradiation, and the second cleavage product of 77-kDa fragment was detected 3 h later. Twenty-four hours after UV irradiation, dead cells accounted for more than 95% of the total cell population in which the intact FAK was not detected, and only the 77-kDa fragment remained. In addition to FAK, we also examined if other cellular proteins that are known to interact with FAK undergo cleavage during UV-induced apoptosis (Fig. 1). Among these proteins, we found that the amount of p130Cas was gradually decreased 6 h after UV irradiation, presumably due to a proteolytic cleavage, and finally disappeared at 24 h. In addition, the cleavage of Src upon UV irradiation was found to generate an approximately 50-kDa fragment. Interestingly, a significant amount of Src remained intact 24 h after UV irradiation, indicating that Src was only partially cleaved during the apoptotic response. In contrast, the level of four other FAK-interacting proteins including the p85 subunit of PI3K (Fig. 1), Grb2, paxillin, and talin had no detectable change during UV-induced apoptosis (data not shown).

To examine if caspases are involved in the cleavage of FAK, p130Cas, and Src during UV-induced apoptosis, UV-exposed MDCK cells were incubated with tetrapeptide caspase inhibitors, ZVAD-FMK or DEVD-CHO (Fig. 2A). ZVAD-FMK, a general inhibitor of cysteinyl proteases, completely blocked cleavage of FAK, p130Cas, and Src, and significantly (~30%) increased cell survival upon UV irradiation. DEVD-CHO, a specific inhibitor of caspase-3, at a concentration of 40 μM was able to completely block Src cleavage but only partially inhibit the cleavage of FAK and p130Cas. These results suggest that the cleavage of FAK and p130Cas is likely to be mediated by...
more than one member of caspase family during UV irradiation-induced apoptosis.

The expression of Bcl2 has been shown to suppress apoptosis by inhibiting the activation of caspases (28, 29). To determine whether Bcl2 blocks FAK cleavage in response to UV irradiation, stable MDCK cell lines overexpressing Bcl2 were established (22). The expression level of ectopic Bcl2 in MDCK cells was at least 10-fold higher than endogenous Bcl2 (data not shown). The Bcl2-overexpressed (Bcl2) cells and control (Neo) cells were exposed to UV radiation and then analyzed for FAK cleavage after various time intervals. The results showed that overexpression of Bcl2 in MDCK cells apparently delayed FAK cleavage upon UV irradiation (Fig. 2B). The first FAK cleavage product of the 85-kDa fragment appeared at 12 h after UV irradiation in Bcl2-overexpressed cells, which was an approximately 6-h delay compared with control cells. Consistent with the results from parental MDCK cells (Fig. 1), no intact FAK could be detected in Neo control cells 24 h after UV irradiation. Notably, at this time point, a significant amount of intact FAK still remained in Bcl2-overexpressed cells.

To examine if FAK plays a role in preventing UV-induced cell death, stable MDCK cell lines overexpressing HA-tagged FAK (WT) were established. The expression level of ectopic FAK in MDCK cells was approximately 2.5-fold of endogenous FAK (Fig. 3A). Cells from three WT clones and a control (Neo) clone were exposed to UV radiation, and their survival rates were determined after various time intervals. The results showed that, 9, 12, or 24 h after UV irradiation, the survival rate of WT cells was significantly (30–40%) higher than that of control cells (Fig. 3B). To further confirm the antiapoptotic effect of FAK in this system, other apoptotic characteristics were analyzed. The Hoechst staining showed that, 4 h after UV irradiation, control cells contained a higher number of apoptotic nuclei, manifested by shrunken and fragmented nuclear morphology and condensed chromatin, than WT cells (Fig. 3C). The DNA laddering assays indicated that control cells also exhibited a more severe DNA fragmentation than WT cells upon UV irradiation (data not shown). Together, these data indicate that overexpression of FAK is able to suppress UV-induced apoptosis. A simple explanation for WT cells being more resistant to UV irradiation is that more intact FAK proteins retained in WT

**Fig. 2.** Effect of caspase inhibitors or Bcl2 expression on cleavage of FAK, p130Cas, and Src during UV-induced apoptosis. A, MDCK cells were pretreated with (+) or without (−) ZVAD-FMK (40 μM) or DEVD-CHO (40 μM) for 1 h prior to UV irradiation. The cells were further incubated with or without ZVAD-FMK or DEVD-CHO for 12 h. Cell survival and the cleavage of FAK, p130Cas, Src, and p85 were analyzed as described in the legend to Fig. 1. Survival of cells without UV irradiation and inhibitor treatment is defined as 100%. Values are the average of two independent experiments. B, an equal amount (50 μg) of lysates prepared from a control (Neo) clone or a MDCK cell clone overexpressing Bcl2 were exposed to UV radiation. After indicated times, the cleavage of FAK was analyzed as described in the legend to Fig. 1.

**Fig. 3.** Suppression of UV irradiation-induced apoptosis by FAK overexpression. A, an equal amount (50 μg) of lysates prepared from a control (Neo) clone or three independent MDCK cell clones overexpressing HA-tagged WT FAK (designated WT-1, WT-2, and WT-3) was analyzed by immunoblotting with anti-HA or anti-FAK. B, control and WT cells were exposed to UV radiation, and cell survivals were measured as described in the legend to Fig. 1. Data (means ± S.E.) are from five independent experiments. C, control and WT cells were exposed to UV and then visualized with Hoechst 33258 dye for nucleus 4 h after UV irradiation, as described under “Experimental Procedures.” Apoptotic nuclei comprising those with fragmented nuclei (arrows) and condensed chromatin (arrowheads) were counted. The apoptotic index is expressed as the percentage of apoptotic nuclei in total counted nuclei. Values are the average of two independent experiments. D, control and WT cells were exposed to UV radiation and then analyzed for FAK cleavage 9 h afterwards, as described in the legend to Fig. 1.
Role of FAK in UV-induced Apoptosis

In this report, first we showed a sequential cleavage of FAK during UV-induced apoptosis. Because FAK cleavage is also triggered by other apoptotic stimuli in various cell types (24–27), it is possible that this proteolytic event is a general phenomenon in apoptosis and plays an important role in the execution of the suicide pathway. The cleavage of FAK during UV-induced apoptosis was completely inhibited by a general caspase inhibitor ZVAD-FMK and apparently delayed by Bcl2 expression, suggesting an involvement of caspases in this event. In fact, several members of the caspase family have been shown to directly cleave FAK in vitro (25, 27). Notably, the patterns of FAK cleavage in various cell types undergoing apoptosis induced by UV irradiation (Fig. 1) or other stimuli (24–27) were very similar, indicating that a similar, if not the same, set of caspases may be responsible for FAK cleavage in response to various apoptotic stimuli.

Among known FAK-interacting proteins, we found that Src and p130Cas were cleaved during UV-induced apoptosis. The cleavage of Src could be completely blocked by a caspase-3-specific inhibitor and delayed by Bcl2 expression, suggesting a role of caspase-3 in the cleavage of Src in this system. The cleavage of Src was also inhibited by a general caspase inhibitor ZVAD-FMK, suggesting that caspase-3 is involved in the cleavage of Src in this context. However, the involvement of caspases in the cleavage of FAK is still unresolved and requires further investigation.

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PI3K, Src, p130Cas, or Grb2. Surprisingly, these ectopically overexpressing FAK or its mutants deficient in binding to p130Cas in FAK-promoted cell survival. We showed that expression of the SH3 domain of p130Cas was able to interfere with FAK-p130Cas association and to suppress cell survival because of the fast decay rate of this mutant during UV-induced apoptosis (Fig. 4). Thus, we employed another strategy to examine the potential role of p130Cas binding as described before, which only partially inhibit cleavage of FAK and p130Cas (Fig. 2). These results suggest that only caspase-3-like caspase(s) may be responsible for Src cleavage during UV-induced apoptosis. Moreover, our results showed that a substantial amount of Src remained intact 24 h (Fig. 1) or even 36 h (data not shown) after UV irradiation, indicating that Src cleavage occurred only in part of its fractions. Src is mainly a membrane protein, anchored via an N-terminal myristic acid and neighboring positively charged amino acids (30). To examine if membrane association protects Src from caspase-mediated cleavage, we carried out subcellular fractionation for UV-irradiated MDCK cells. Our preliminary results showed that Src cleavage was observed in cytosol fractions, but not in membrane fractions (data not shown). These results raise an intriguing possibility that, in addition to the presence of specific motifs in substrates for caspase recognition, subcellular localization and/or membrane anchorage of caspase substrates may have some impact on the execution of the proteolysis.

In this study, we have established stable MDCK cell lines overexpressing FAK or its mutants deficient in binding to PI3K, Src, p130Cas or Grb2. Surprisingly, these ectopically expressed FAK proteins exhibited different rates in their decay during UV-induced apoptosis (Fig. 4), presumably due to a caspase-mediated cleavage. It appeared that Y397F, P712A/P715A, and Y925F mutants had a faster decay rate than both WT FAK and D395A mutant, which were similar in their rates of decay. Although the reason for this is unclear at present, it is possible that protein-protein interactions may lead to a mask of caspase recognition motifs on FAK or block the access of caspase to FAK, thereby temporarily protecting FAK from cleavage. Alternatively, the amino acid substitutions in FAK mutants Y397F, P712A/P715A, or Y925F may cause a change in protein conformation, leading to protein less stable regardless of UV irradiation. The latter possibility was excluded by our observation that all FAK mutants used in this study had a turnover rate similar to WT without UV irradiation (data not shown). It is noteworthy that, like WT, all FAK mutants described in this report targeted to focal adhesions (data not shown), rendering it unlikely that a faster decay rate for certain FAK mutants is due to an incorrect subcellular localization.

Despite the fact that expression of the constitutively activated form of FAK rescues MDCK cells from apoptosis induced by the disruption of cell-matrix interactions (10), here we show the first time that expression of WT FAK can significantly (30–40%) promote cell survival upon UV irradiation (Fig. 3). In addition to UV irradiation, these FAK-overexpressed cells were also found to be more resistant to some tested apoptotic stimuli, such as treatment of cyclohexamide and loss of cell-matrix adhesion (data not shown). Using this system, we further showed that a PI3K binding-deficient mutant (D395A) that had a decay rate similar to WT FAK upon UV irradiation failed to promote cell survival (Fig. 4). Because, except for deficiency in PI3K binding, no other changes could be detected for the D395A mutant, including the ability to bind other FAK-interacting proteins and the level of tyrosine phosphorylation and in vitro kinase activity, our results strongly suggest that PI3K binding is required for FAK to promote cell survival. In fact, an increased association of FAK with PI3K and a subsequent activation of Akt have been observed in hydrogen peroxide-induced apoptosis in human glioblastoma cells (31). This and our results are consistent with other work showing the importance of PI3K in promoting cell survival (32–34).

Although a FAK mutant (P712A/P715A) deficient in p130Cas binding failed to promote cell survival upon UV irradiation, we could not conclude that p130Cas binding is required for FAK to promote cell survival because of the fast decay rate of this mutant during UV-induced apoptosis (Fig. 4). Thus, we employed another strategy to examine the potential role of p130Cas in FAK-promoted cell survival. We showed that expression of the SH3 domain of p130Cas was able to interfere with FAK-p130Cas association and to suppress cell survival promoted by FAK overexpression (Fig. 5). These results suggest that, in addition to PI3K, p130Cas binding is also required for FAK to promote cell survival. Ilic et al. (9) showed that two FAK C-terminal constructs, FAK-related nonkinase and the FAT domain, were localized in focal adhesions and displaced endogenous FAK from these sites, but only the FAT domain was able to abrogate the function of FAK to transduce survival signals from fibronectin. These results suggest that FAK-related nonkinase, but not the FAT domain, may contain sequences required for FAK to promote cell survival. A comparison between these two constructs revealed that an N-terminal region of FAK-related nonkinase comprising a proline-rich sequence for p130Cas binding was not present in the FAT domain, supporting the notion that p130Cas binding is required for FAK to promote cell survival. In fact, it has been proposed that the initial cleavage of FAK at Asp-772 by caspase-3-like proteases may generate a FAK C-terminal fragment corresponding to the
FAT domain, which may act as a competitive inhibitor for the remaining intact FAT during apoptosis (27).

The adaptor protein p130Cas was originally identified as a major tyrosine-phosphorylated protein in cells transformed by either v-src (35–37) or v-erb-B (38–40). Its association with another adaptor protein Crk has been shown to play an important role in promoting cell death (41). Our results in this report imply that p130Cas may also have a function in regulating cell survival. It has recently been shown that p130Cas-Crk association further leads to activation of c-Jun N-terminal kinase via small GTP-binding protein Rac (42, 43). It will be of interest to determine the potential connection between the c-Jun N-terminal kinase activation and p130Cas-mediated cell migration and/or cell survival.

Because the effect of FAK on the suppression of UV-induced apoptosis is limited (∼30%), apparently other survival factors against UV-induced apoptosis are present. In fact, the first cellular response detectable in UV-irradiated cells is the tyrosine phosphorylation of different cell membrane growth factor receptors (44). It has been shown that UV rapidly induces activation of Ras, Src, and other molecules located at or near the plasma membrane (45, 46) and inhibition of tyrosine phosphatases (47), leading to signaling and to transcription of UV-responsive genes. Recently, the atypical protein kinase C (PKC) isoforms, PKCζ and PKCα, have been suggested to play a protective role in UV-induced apoptosis (48, 49). It was found that overexpression of PKCζ and PKCα inhibited UV-induced cell death, whereas exposure of cells to UV radiation leads to a dramatic reduction of PKCζ activity (48). Furthermore, it is known that UV irradiation potently activates c-Jun N-terminal kinase (50). The UV-induced c-Jun N-terminal kinase activation has also been suggested to trigger a protective response through the activation of genes coding for protective proteins (45, 50, 51). Therefore, the signaling cascades induced by UV radiation appear to be complex and involve many different molecules. Some of them promote survival, whereas others promote cell death. The relationship between FAK and other survival factors in UV-induced apoptosis remains to be investigated.

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