Increased Resistance of Tumor Cells to Hyperthermia Mediated By Integrin-linked Kinase[1]

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ABSTRACT

Purpose: **Integrin-linked kinase (ILK) is a serine-threonine kinase associated with anchorage-independent growth and tumorigenic transformation. Previous studies indicate that overexpression of ILK is common among several types of tumors, and it is involved in the regulation of tumor cell survival under stress. In this study, we examined the effects of ILK expression on tumor cellular response to hyperthermia.**

Experimental Design: **We used an adenovirus-mediated approach to overexpress the *ILK* gene in a prostate cancer cell line and examine its effects on heat stress-induced cell death. Clonogenic survival, as well as apoptosis, was evaluated in cells that overexpress ILK. In addition, the ability to form tumors *in vivo* was examined in syngeneic hosts. Finally, potential molecular mechanisms of ILK-mediated resistance to heat were examined by determining the status of a variety of signal transduction pathways.**

Results: **ILK overexpression made tumor cells significantly more resistant to the cell-killing effects of hyperthermia. This was correlated at the molecular level with the down-regulation of hyperthermia-induced activation of stress-activated protein kinase/c-Jun-NH_2-terminal kinase, p38 mitogen-activated protein kinase activities, and caspase 9. The overexpression of ILK was also shown to induce a more rapid tumor growth in a murine prostate cancer cell line**

Conclusion: **ILK plays an important role in tumor growth and tumor response to hyperthermia treatment.**

INTRODUCTION

At the molecular genetic level, cancer cells are characterized by an extensive array of mutations in their genome. Among the mutations are ones that allow a normal cell to become sequentially hyperproliferative, tumorigenic, and eventually metastatic (1). A tumor cell must confront a myriad of adverse growth conditions, such as hypoxia, low pH, and a general lack of nutrients (2–5). Tumor cells grow despite these conditions. Many, especially those that are malignant or metastatic, thrive under these conditions. This is possible because they are often genetically unstable, which enables them to undergo a Darwinian selection process so that the "fittest" cells with the right set of genetic alterations can survive (6). Examples of these genetic alterations include mutations in the *p53* gene, which makes tumor cells more tolerant of genetic instability (7, 8) and less susceptible to various apoptotic signals (9); amplifications of the myc oncogene, which enable tumor cells to escape the G_0-G_1 control (10–13); amplification of the *bcl*-2 genes, which makes tumor cells more resistant to cell death signals (14); and constitutive expression of otherwise tightly controlled angiogenic genes, such as vascular endothelial cell growth factor, by virtue of mutations in the *VHL* gene, which enables the overexpression of the HIF1 transcription factor, which in turn up-regulates an array of angiogenic genes (15).

ILK[3] is an ankyrin repeat containing serine-threonine protein kinase that binds to the cytoplasmic domain of β(1) and β(3) integrin subunits and promotes anchorage-independent growth (16). ILK is overexpressed in a number of human malignancies. ILK overexpression can suppress apoptosis, promote anchorage-independent cell cycle progression, and induce tumorigenesis and invasion. These properties are shown to be associated with the ability of ILK to phosphorylate [at Ser (473)] and activate PKB/Akt kinase (17). Inhibition of ILK has been shown to promote stress-induced apoptosis (18, 19), and the overexpression of it can protect cells from stress-induced apoptosis (20, 21). ILK overexpression promotes anchorage-independent tumor growth (22, 23). ILK has been shown to be overexpressed in colon (24) and prostate tumors (25). Consistent with the role of ILK being a survival factor for cancer cells, ILK was found to be up-regulated in hypoxic hepatocarcinoma cells (26). In this study, we attempt to address the role of ILK expression on tumor cell response to hyperthermia, a potentially important cancer therapeutic modality that has shown positive results in Phase III clinical trials (27). Our results indicate that

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[3] The abbreviations used are: ILK, integrin-linked kinase; PKB, protein kinase B; SAPK, stress-activated protein kinase; JNK, c-Jun-NH_2-terminal kinase; GFP, green fluorescence protein; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5- diphenyltetrazolium bromide; ATF, activating transcription factor; MAPK, mitogen-activated protein kinase; MOI, multiplicity of infection; CMV, cytomegalovirus.

ILK overexpression can significantly increase tumor cellular resistance to hyperthermia treatment.

MATERIALS AND METHODS

Cell Culture. The 293 cells for adenovirus expansion were obtained from American Type Culture Collection (Manassas, VA). The Tramp-C mouse prostate cancer cell line was obtained from Dr. Norman Greenberg of Baylor College of Medicine. Both cell lines were cultured in DMEM (Life Technologies, Inc.) with 10% fetal bovine serum (Hyclone), 100 units/ml penicillin, and 100 μg/ml streptomycin at 37°C with 5% CO_2.

Plasmid Construction and Adenovirus Production. The AdEasy system of adenovirus packaging, including plasmid pAdtrack-CMV, pAdeasy-1, and the *Escherichia coli* BJ5183 cells, were kindly provided by Drs. T-C. He and B. Vogelstein (Ref. 28; The Johns Hopkins Medical School, Baltimore, MD). The human *ILK* gene was amplified by reverse transcription-PCR from total RNA extracted from FADU (a human squamous cell carcinoma cell line) cells. The forward primer sequence is 5′-G*CTCGAG*ACTATGGACGACATTTTCACTCAG-3′, and the reverse primer is 5′-G*GATATC*CTAAGCATAATCTG-GAACATCATATGGATACTTGTCCTGCATCTTCTC-3′. They were designed according to GenBank sequences (accession no. HSU40282). An *Xho*I and *Eco*RV site (italicized) were engineered into the forward and reverse primers, respectively. The human *ILK* gene was subsequently cloned into the *Xho*I/*Eco*RV sites of pAdtrack-CMV vector to produce the pAdtrack-CMV/ILK plasmid. Packaging and production of a recombinant adenovirus carrying the human *ILK* gene were achieved by use of the AdEasy system according to published protocols (28).

Western Blot Analysis. An anti-ILK antibody (Upstate Biotechnology, Lake Placid, NY) was used to detect ILK protein expression. An anti-β-actin antibody was used as a loading control (NeoMarkers Corp., Fremont, CA). Anti-phospho-Akt and anti-caspase 9 antibodies were from New England Biolabs (Beverly, MA). Anti-*bcl*-2 antibody was purchased from PharMingen (San Diego, CA). Densitometric analysis of the Western blot analysis was carried by use of a UMAX ASTRO4000 scanner and the Adobe PhotoShop image analysis/software. The total expression of a protein was quantified as the total number of the pixels in its band (on an autoradiograph) multiplied by the intensity of the bands. All of the expression was normalized by the expression of the β-actin, the highest expression level on each Western blot.

Clonogenic Assay. Tramp-C cells at 50–60% confluence were infected with AdILK or AdGFP viruses at a MOI of 5. Proliferation experiments showed that AdILK and AdGFP infection at this MOI caused no growth delay in Tramp-C cells. Fifteen hours later, the cells were heated at 44°C for 10–50 min. They were then plated immediately at various densities in 10-cm Petri dishes so that 50–200 colonies/dish would grow. Ten to 14 days later, cells on the dishes were fixed in methanol and stained with 2% Giemsa solution. Colonies with ≥50 cells were counted. Five replicate dishes were plated for each thermal dose. The surviving fraction (SF) was calculated for each thermal dose as follows:

SF = number of colonies/(total number of cells plated) × plating efficiency.

Plating efficiency of Tramp-C cells was determined by plating unirradiated cells at 100–200 cells/plate and counting emerging colonies 10–14 days later.

MTT Assay for Acute Cell Death. The MTT assay for acute cell death in hyperthermia-treated TRAMP-C cells was carried out by an established protocol (29). The MTT assay examines the activity of metabolic enzymes in the mitochondria of live cells. MTT was purchased from Sigma (St. Louis, MO). The catalogue number was M-5655. Tramp-C cells at 50–60% conferency were infected with AdILK or AdGFP (MOI of 5). Fifteen hours later, the cells were heated for 30 min at 44°C. Twenty-four hours later, live cells in the Petri dishes were analyzed by MTT assay. The values were normalized using nontreated cells.

Hoechst Staining for Apoptotic Cells. Cellular apoptosis induced by heat was observed by Hoechst 33342 (Calbiochem, San Diego, CA) staining. A published method for Hoechst staining was adopted (30). This method allows for the observation of nuclear condensation, which is a hallmark of cellular apoptosis. Tramp-C cells at 50–60% confluency were infected with AdILK or AdGFP (MOI of 5). Fifteen hours later, the cells were heated and fixed in 100% methanol another 4 h later, stained with Hoechst 33342, and evaluated for apoptosis. Those cells that showed clear condensation and bright Hoechst 33342 staining were counted as apoptotic. To get a quantitative value, four randomly chosen areas were counted and averaged for each sample.

Assay for the Activity of SAPK/JNK and P38 MAPK. SAPK/JNK and p38 MAPK kinase activities in heated and nonheated cells with or without adenovirus-mediated ILK expression were measured by use of commercially available assay kits (Cell Signaling Technology, Beverly, MA; Ref. 30). Cell lysates (200 μg) were incubated overnight with immobilized phospho-antibody to p38 MAPK. SAPK was pulled down by NH_2-terminal c-Jun fusion protein bound to glutathion-Sepharose beads. Samples were centrifuged for 30 s at 4°C, and the resultant pellets were washed twice with lysis buffer and kinase buffer, resuspended in kinase buffer, and incubated with appropriate substrates in the presence of ATP for 30 min. ATF-2 fusion protein was used for p38 MAPK, whereas c-Jun was used for SAPK. Reactions were terminated by adding SDS sample buffer and boiling. Samples extracts were loaded onto SDS-PAGE and blotted into nylon membranes after electrophoresis. The membranes were then probed with a polyclonal antibody to phospho-ATF-2 and c-Jun.

Tumor Growth Studies. Animal care and experimental procedures were carried out in accordance with institutional guidelines. About 3×10^6 Tramp-C cells in 50 μl of PBS were injected s.c. in the right flank of C57BL/6 mice by use of a 30-gauge needle. The mice were held in a restrainer to facilitate the injection. Before inoculation, the cells were infected with AdILK or AdGFP (an adenovirus encoding a constitutively expressed GFP) viruses at a MOI of 5 for 15 h. Each treatment group consisted of 6–10 animals. Growth curves are derived by plotting the mean treatment group tumor volume ±SE *versus* time. The following formula was used to calculate tumor volume (31):

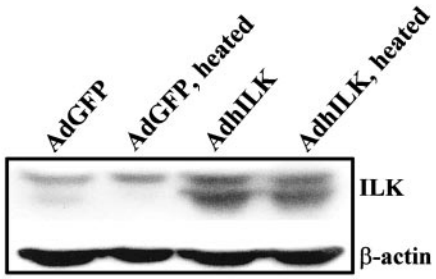


Fig. 1 Adenovirus-mediated expression of the ILK protein. Western blot analysis of the ILK protein levels in Tramp-C cells. Tramp-C cells at 50–60% confluence were infected with AdILK and AdGFP, respectively, at a MOI of 5 for 15 h. The cells were then heated for 30 min at 44°C. Four hours later, total cellular protein was extracted and analyzed by use of an anti-ILK antibody. A total of four ILK bands was detected. Shown are two major bands at M_r 50,000 and 59,000. β-actin was used as a loading control.

$V = (1/2) \times W^2 \times L$ (W, the shortest dimension; L, the longest dimension).

RESULTS

To evaluate the function of ILK, an adenovirus encoding the human *ILK* gene under the control of CMV promoter was engineered. When this virus was used to infect TRAMP-C prostate cancer cells, it mediated efficient expression of the *ILK* gene. In control AdGFP-infected cells, heat treatment (44°C, 30 min) resulted in a reduced intensity of one of the lower ILK bands. The exact implication of this reduction was unknown. In AdILK-infected cells, identical heat treatment did not affect the expression of ILK (Fig. 1).

To evaluate the effects of the *ILK* gene expression on cellular response to hyperthermia treatment, the cells were infected with AdGFP (control) and AdILK at a MOI of 5. Fifteen hours later, the cells were treated at 44°C for different lengths of time. Afterward, three assays were used to evaluate the effects of ILK expression on cellular response to hyperthermia in these cells.

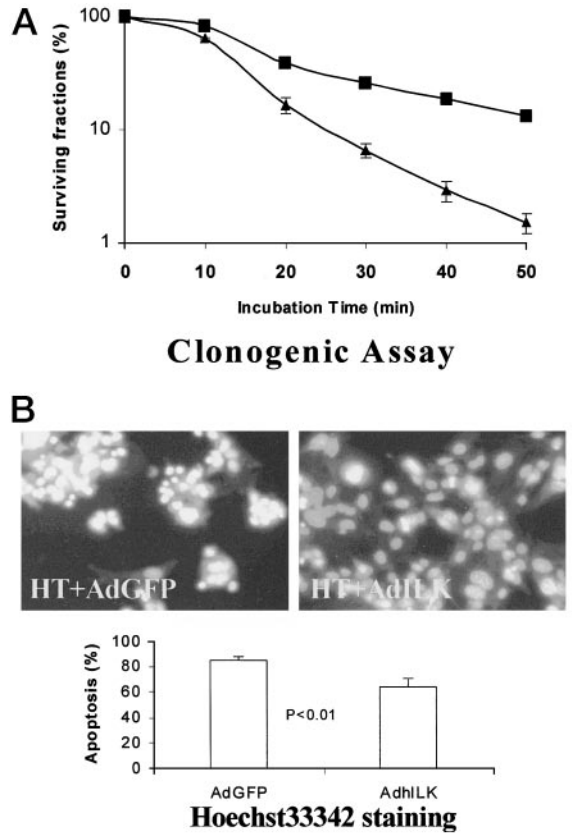
Fig. 2*A* shows the results of a clonogenic assay, which evaluates the long-term cellular survival after heat treatment. Compared with cells that have been infected with AdGFP (control), which had a similar survival rate as that of noninfected cells (data not shown), the AdILK-infected cells showed clear survival advantage, with significantly higher survival fractions when hyperthermia treatment was conducted for ≥20 min. When AdGFP-infected TRAMP-C cells were treated for 50 min, cellular survival was <2%, whereas those infected with the AdILK adenovirus had a survival rate of >10%.

Fig. 2*B* shows the results of Hoechst 33342 staining, which was used to quantify apoptosis in AdILK-infected and heat treated cells. The Hoechst 33342 dye stains the nucleus of mammalian cells. Apoptotic cells are typically identified as those cells that possess significantly smaller, condensed, and fragmented nuclei under a fluorescence microscope. Adenovirus-mediated expression of the reporter gene (*GFP*) had no effect on hyperthermia-mediated cell death (Fig. 2*C*). About 85.4 ± 2.3% of hyperthermia-treated (44°C for 30 min) cells appeared apoptotic (similar to uninfected cells). By comparison, TRAMP-C cells infected with AdILK showed a significant reduction in hyperthermia-mediated cell death with 64.3 ± 6.3% of the cells showing signs of apoptosis.

Fig. 2*C* shows the results of the MTT [3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide] assay. The MTT assay allows one to quantify the amount of live cells by detecting active enzymes in the mitochondria. It is based on the conversion of the yellow tetrazolium salt (MTT) to the blue formazan derivative by mitochondrial enzymes in viable cells. We used an improved version of the assay (29). AdILK infection protected the cells from hyperthermia-induced cytotoxicity significantly (Fig. 2*B*). When cells were treated at 44°C for 30 min, 16.4% of the AdGFP-infected TRAMP-C cells survived from the heat treatment, whereas 38.2% of the AdILK-infected cells survived from the same treatment.

The ability of certain genes to protect tumor cells under stress will often give them survival advantages *in vivo* as the microenvironment of tumors are often hypoxic, acidic, and nutrition deprived. Overexpression of ILK may enhance tumor growth *in vivo*. Indeed, when AdILK-infected TRAMP-C cells were used to inoculate s.c. into syngeneic C57/BL6 mice, tumors from the cells grew much faster than those from AdGFP-infected cells (Fig. 3). This was consistent with previous findings that ILK overexpression prevented cells from apoptosis (20). It is also consistent with the finding in our laboratory that ILK overexpression prevented cells from oxidized low-density lipoprotein (21), free radical (H_2O_2), or hypoxia-induced cell death (data not shown). Because AdGFP infection has no effect on tumor growth (data not shown), these results indicate that ILK overexpression can promote tumor growth as well as enhance its resistance to hyperthermia treatment.

What is the molecular basis for these observations? The PKB/Akt signal transduction pathway, along with other stress-activated signal transduction pathways, have been implicated downstream of the *ILK* gene. We carried out a series of experiments to determine whether ILK overexpression changed the status of PKB/Akt and a number of other related molecular factors. The results were shown in Fig. 4. The phosphorylation status of PKB/Akt was analyzed by use of Western blot and an antibody specific for phosphorylated PKB/Akt. Results indicate no obvious changes in PKB/Akt phosphorylation levels in control AdGFP, as well as in AdILK-infected TRAMP-C cells (Fig. 4, *A* and *F*). In addition, the kinase activities of SAPK/JNK (Fig. 4*B*), as well as p38 MAPK(Fig. 4, *B* and *F*), were examined. Both have been implicated in stress-induced apoptosis (32–35). SAPK/JNK has specifically been implicated in heat-induced apoptosis (36–38). As shown in Fig. 4, *B* and *F*, hyperthermia induced a significant increase in c-Jun kinase activity in control cells that have been infected with AdGFP by 30 min after heat treatment. However, in AdILK-infected cells, this increase was suppressed. Interestingly, the nonheated AdILK-infected cells had lower c-Jun kinase activities than nonheated AdGFP-infected cells, indicating the ability of ILK to suppress c-Jun kinase activities even under nonstressful conditions. Fig. 4, *C* and *F* shows the activities of p38 MAPK under different conditions. Hyperthermia treatment induced p38 MAPK and SAPK/JNK kinase activities in control AdGFP-infected cells, consistent with the reported involvement of it in heat-induced cell



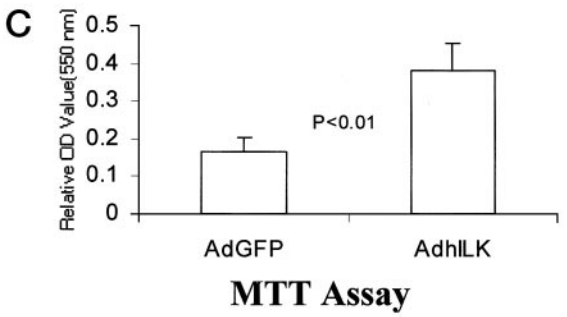


Fig. 2 Increased cellular resistance to heat stress mediated by ILK overexpression. *A,* clonogenic assay of cellular survival after heat treatment. *B,* apoptosis in Tramp-C cells as evaluated by Hoechst 33342 staining. *Top panels,* fluorescence photomicrographs of Hoechst 33342-stained AdGFP- and AdILK-infected cells that had been heated. The bars in each photograph represent 50 μm. *Bottom panel,* quantitative analysis of apoptosis. *C,* MTT assay for acute cell death in heat-treated and ILK-transduced cells.

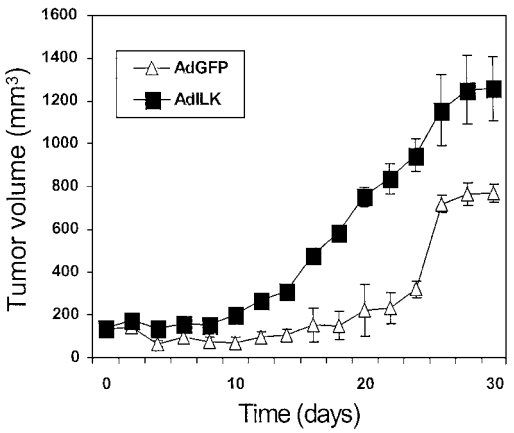


Fig. 3 Tumor growth from cells that have been transduced with the *ILK* gene. Tramp-C cells were first infected with either AdGFP or AdILK at a MOI of 5. Fifteen hours after infection, about 3×10^6 tumor cells were injected s.c. into the flanks of C57/BL6 mice. The measurement of tumor sizes was then conducted every other day.

death. When AdILK was overexpressed, the induction of p38 MAPK kinase activities was reduced, indicating that ILK functioned upstream from these two factors to block their activation. Again, the overall level of p38 MAPK kinase activities was lower in AdILK-infected cells, indicating general suppression by an ILK overexpression.

Subsequently, we examined the level of caspase 9, an effector of apoptosis. We used a caspase antibody that can detect both the inactive full-length and active cleaved forms. The results were shown in Fig. 4, *D* (top two bands) and *F*. In AdGFP-infected cells, hyperthermia treatment caused a significant increase in the cleavage of caspase 9 (bottom band), indicating its activation. This heat-induced cleavage was reduced significantly in AdILK-infected cells. Finally, we examined the level of *bcl*-2, a negative regulator of apoptosis (Fig. 4, *E* and *F*). *bcl*-2 expression showed no discernable changes in AdGFP-infected cells that have been heated for 30 min. Neither was its levels changed in AdILK-infected cells.

DISCUSSION

Many cancer cells are in a general state of genetic instability. This instability allows the emergence of various subclones that are resistant to internal and external stress exposures. Understanding the genetic factors that are involved in cancer cellular resistance to therapeutic treatment offers the potential to develop novel therapeutics that counter the treatment resistance in tumors. This study was conducted with the purpose of elucidating the potential role that ILK may play in cellular response to hyperthermia, an experimental cancer treatment. ILK was chosen because it was a known signal transduction molecule that has been involved in cellular stress response. In addition, it was known to be overexpressed in a number of tumor types.

Our studies indicate that the overexpression of the *ILK*

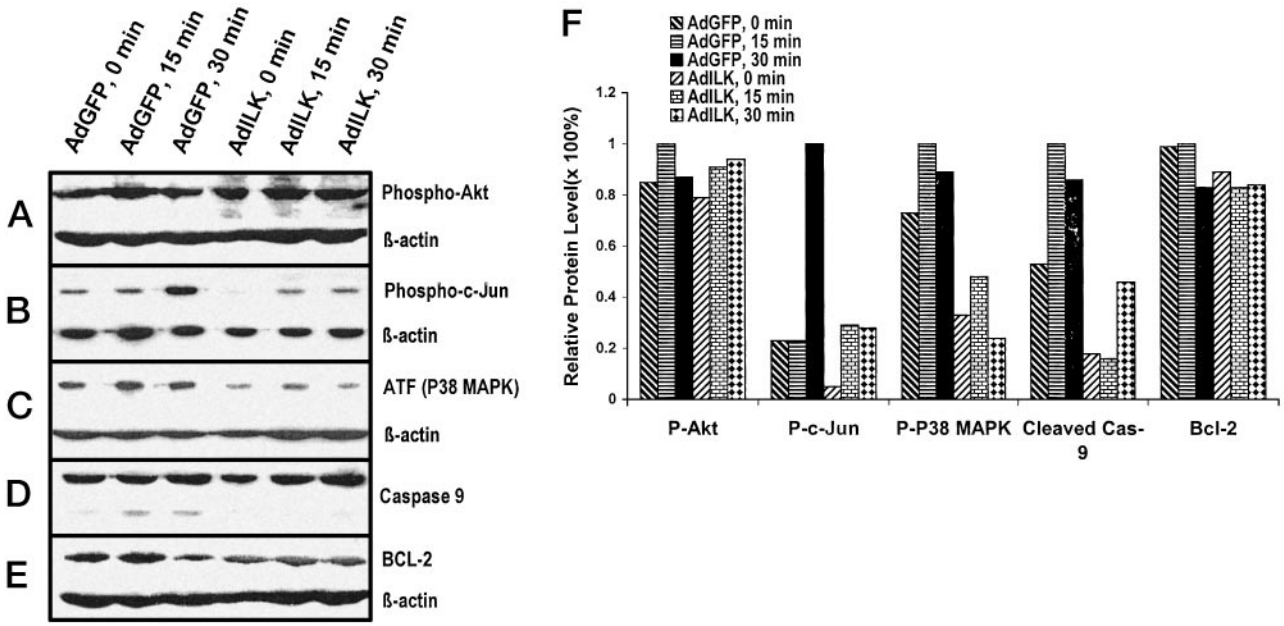


Fig. 4 Molecular analysis of proteins in the apoptosis signal transduction pathways. Tramp-C was infected with control AdGFP or AdILK for 15 h at a MOI of 10. The cells were then heated at 44°C for 15 or 30 min. Four hours later, cell lysate was prepared for analysis. *A,* Western blot analysis of the amount of phospho-Akt. It was detected by use of an antibody that specifically binds phosphylated Akt (at Ser473). *B,* Western blot analysis of phosphorylated c-Jun protein. It was detected by use of an antibody that specifically bound phosphorylated c-Jun (at Ser63, mediated by SAPK). The results were obtained after unphosphorylated c-Jun fusion protein beads were incubated with SAPK that were selectively "pulled down" from the lysates. *C,* Western blot analysis of the amount of phospho-ATF-2 (Thr71), which had been phosphorylated by immunoprecipitated p38 MAPK from the lysates. The results were obtained after unphosphorylated ATF-2 was incubated with immunoprecipiated p38 (derived by use of an immobilized monoclonal antibody to p38 MAPK). β-actin protein was used as protein loading control. *D,* Western blot analysis of caspase 9. For caspase 9, the top band represents the M_r 49,000 intact form, and the bottom band represents the M_r 37,000 cleaved form. Notice the difference (for the cleaved forms) between the AdGFP-infected cells *versus* the AdILK-infected cells. β-actin protein was used as protein loading control. *E,* Western blot analysis of *bcl*-2. *F,* densitometric analysis of the Western blot results in *A–E.*

gene can substantially prevent heat-induced cell death. The suppression of SAPK/JNK kinase and p38 MAPK kinase activity and other effectors by ILK in cellular apoptotic machinery is likely the main mechanism. Taken together with the fact that the *ILK* gene is abundantly expressed in some tumor tissues and an important regulator of cell-extracellular matrix interactions that promotes cell survival, it is likely that ILK plays crucial roles in both tumor development and response to hyperthermia treatment.

There are additional important issues that remain to be resolved, *e.g.*, what is the relationship between ILK activity and the more conventional stress response proteins, such as heat shock proteins? Preliminary data in our laboratory indicate that the overexpression of ILK can lead to an increased level of hsp70 in heat-treated cells (data not shown). More experiments need to be carried out to decipher the precise molecular links between ILK and heat shock proteins.

Our studies also indicate that ILK may be a valuable target for hyperthermia cancer treatment. Compounds that specifically inhibit the function of ILK may enhance the cytotoxic activities of hyperthermia on cancer treatment. We will test this idea initially by use of a dominant-negative version of the ILK protein to inhibit ILK activity in select cancer cells and evaluate the effects of ILK inhibition on heat-induced cell death. Positive results from these experiments will lead to the development of novel therapeutics that enhances hyperthermia cancer treatment.

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