Identification of a Dominant Negative Functional Domain on DAPK-1 That Degrades DAPK-1 Protein and Stimulates TNFR-1-mediated Apoptosis*

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DAPK-1 is a stress-activated tumor suppressor protein that plays a role in both proapoptotic or antiapoptotic signal transduction pathways. To define mechanisms of DAPK-1 protein regulation, we have determined that DAPK-1 protein has a long half-life, and therefore its activity is primarily regulated at the protein level. Changes in DAPK-1 protein levels occur by a cathepsin B-dependent pathway, prompting us to evaluate whether cathepsin B plays positive or negative role in DAPK-1 function. The transfection of p55-TNF-1 induced complex formation between DAPK-1 and cathepsin B. Depletion of cathepsin B protein using small interfering RNA stimulated TNFR-1 dependent apoptosis. The minimal binding region on DAPK-1 for cathepsin B was mapped to amino acids 836–947. The transfection of the DAPK-1-(836–947) miniprotein acted in a dominant negative manner inducing endogenous DAPK-1 protein degradation in a TNFR-1-dependent manner. These data suggest that DAPK-1 forms a multiprotein survival complex with cathepsin B countering the rate of TNFR-1-dependent apoptosis and highlights the importance of developing DAPK-1 inhibitors as agents to sensitize cells to stress-induced apoptosis.

The tumor suppressor activity of p53 is linked to its function as a stress-responsive transcription factor (1). Mutations in the p53 gene occur commonly in approximately half of human cancers, resulting in attenuation in p53-mediated transactivation (2). Negative control of p53 is perhaps most well defined by a set of E3 ligases, including MDM2, PirH2, and COP-1 that accelerates ubiquitination and protein degradation (3). Depletion of the E3 ubiquitin ligases does not spontaneously activate p53, since genetic studies have shown that positive regulation of p53 occurs through a set of protein kinases, including ATM, CHK2, DAPK-1, LKB, AMPK, and PKR. The mechanisms whereby these kinases mediate p53 activation are only partly defined for the ATM-dependent DNA damage-activated checkpoint pathway. Current models highlight the ability of the ATM target, CHK2, to phosphorylate both p53 and E2F1, which in turn leads to an induction of gene expression by both of these transcription factors (1).

Although there are over 12 phosphorylation sites on p53 that alter transactivation, nuclear shuttling, and degradation, only two known biochemical activities are directly affected: sequence-specific DNA binding and p300 co-activator stability. Damage-stimulated phosphorylation of p53 at the C-terminal CK2 site (Ser395) forms a paradigm whereby sequence-specific DNA binding is stimulated by allosteric mechanisms (6, 7). Transgenic animals with an alanine substituted CK2 phosphoacceptor site have attenuated levels of UV-induced p53-dependent transcription in mouse skin and enhanced UV-induced skin cancer development (8). Phosphorylation of p53 not only modulates sequence-specific DNA-binding but also protein-protein interactions with the co-activator and acetyltransferase p300. B-cell lymphoma incidence increases in transgenic mice models with an alanine-substitution (S23A) in the equivalent murine phosphoacceptor site (9). The only known direct biochemical effect of Ser20 phosphorylation is to stabilize p300 binding (10–12). p300 anchoring to the phospho-LXXLL-activation domain in p53 is critical for p300-mediated acetylation of p53, explaining why the S23A transgenic mice have elevated cancer incidence; reduced levels of Ser20 phosphorylation could reduce p300 binding and attenuate p53 activity as a transcription factor.

In addition to the well characterized CHK2:p53 activation axis, other tumor-suppressing kinases of the calcium calmodulin kinase superfamily can activate p53-dependent processes including AMPK and DAPK-1. AMPK is a component of a metabolic checkpoint that activates p53-dependent gene expression in glucose-starved cells, although the mechanism whereby AMPK recruits p53 is undefined (13, 14). DAPK-1 is a component of an oncogene-activated checkpoint that activates p53 activity in an ADP-ribosylation factor-dependent manner (15). DAPK-1 also functions as a mediator of interferon-γ-induced apoptosis (16), TNFR-mediated cell death (17), stress-induced autophagy (18), integrin-mediated degradation during cell detachment (19), and myosin light chain phosphorylation and related cytoplasmic changes inducing membrane blebbing associated with cytoplasmic changes during apoptosis (20). Signaling pathways that modify DAPK-1 are beginning to be described and include an ERK2-dependent pathway that is required for DAPK-1-dependent apoptosis (21), a ribosomal

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2 The abbreviations used are: ERK, extracellular signal-regulated kinase; siRNA, small interfering RNA; PBS, phosphate-buffered saline; pIpC, polynosinic-polycytidylic acid; HA, hemagglutinin; GFP, green fluorescent protein; IP, immunoprecipitation; IRF, interferon-responsive factor; E3, ubiquitin-protein isopeptide ligase; TNF, tumor necrosis factor; GST, glutathione S-transferase; PARP, poly(ADP-ribose) polymerase; TUNEL, deoxynucleotidyldtransferase-mediated dUTP nick end labeling.
S6 kinase-mediated pathway that attenuates DAPK-1 function (22), and an interaction with the netrin receptor that controls dependence receptor apoptotic responses (23).

Given the overlapping role of both p53 and DAPK-1 in pro-apoptotic pathways, identifying fundamental mechanisms underlying DAPK-1 regulation will expand our understanding of the mechanisms of DAPK-1-mediated tumor suppression and cross-talk with the p53 pathway. In this report, we focus on developing an understanding of how DAPK-1 protein is regulated in cancer cell lines. Although previous results have shown that DAPK-1 protein can be regulated at the gene expression level, we have determined that DAPK-1 protein has a relatively long half-life, and its activity can be regulated post-translationally. In particular, transfection of TNFR-1-induced complex formation between DAPK-1 and cathepsin B that coincided with relocalization of both cathepsin B and DAPK-1 in the cytosol. The depletion of cathepsin B protein using siRNA-stimulated TNFR-1-dependent apoptosis suggesting that this DAPK-1-containing multiprotein complex can regulate negatively the rate of TNFR-1-dependent cell death pathways. This survival function of DAPK-1 under these conditions highlights the importance of developing DAPK-1 inhibitors that can sensitize cells to selected stress-activated apoptotic signals. Accordingly, we have mapped the cathepsin B binding domain on DAPK-1 and HA-DAPK-1 as a template. Transient transfections were performed using Lipofectamine 2000 (Invitrogen). Prior to transfection, Lipofectamine (~2 μl/μg of DNA or siRNA) was added to Optimum medium without fetal bovine serum. After a 30-min incubation at room temperature, the cells were washed six times by rotating them in 1 ml of IP dilution buffer with dry skimmed milk (5%, w/v) for 1 h at room temperature. Then after a quick wash with PBS-Tween 20, the membrane was incubated with the primary antibodies diluted in the milk for 1 h at room temperature or 4 °C overnight. Before incubation with the secondary antibodies for 1 h at room temperature, the membrane was washed three times for 5 min with PBS-Tween 20. After the incubation of secondary antibodies, the membrane was washed three times for 15 min. Finally, the membrane was incubated with ECL solution for 1 min, and the target proteins were detected by exposing the membrane to ECL hyperfilm.

**Immunoprecipitation (IP)**—Protein G beads from GE Healthcare were used in the immunoprecipitation reaction. The beads were kept in 70% ethanol in a 1:1 ratio. Each reaction requires 25 μl of beads, and before the immunoprecipitation, the beads were washed five times with 1 ml of IP dilution buffer (0.1% (w/v) Nonidet P-40, 25 mM Tris/HCl, 0.15 M KCl, 1 mM dithiothreitol, and 1× protease inhibitor). Proteins for IP were extracted by lysing the cells with IP lysis buffer (1% (w/v) Nonidet P-40, 25 mM Tris/HCl, 0.4 M KCl, 1 mM dithiothreitol, and 1× protease inhibitor). The protein concentrations of each sample were tested by Bradford assay. Equal amounts of proteins were added to 1 ml of IP of dilution buffer. After that, the antibodies for IP and 50 μl of protein G beads in suspension were added into the tubes, and the mixture was rotated overnight at 4 °C. After rotation, the beads were sedimented at 6000 rpm, and the supernatants were discarded. The beads were then washed six times by rotating them in 1 ml of IP dilution buffer for 5 min at room temperature.

**In Vitro Cleavage Assay**—35S-labeled HA-DAPK-1 was synthesized using the TNT® translation kit (Promega) according to the manufacturer’s protocols. 2 μl of the 35S-labeled HA-DAPK-1 was added to 3× cathepsin B reaction buffer (150 mM sodium acetate, pH 6.0, 12 mM EDTA, and 24 mM dithiothreitol) with 1 μl of purified cathepsin B (U.S. Biological) (0.02 units/μl), which was diluted in 50 mM sodium acetate, pH 5.0, with 1 mM EDTA prior to use. The final volume was brought to 15 μl with distilled water. The cleavage reaction was performed by incubation at 37 °C for times indicated. After incubation, 15 ml of 4× sample buffer was added to the samples, and then they...
were boiled and subjected to SDS-PAGE. The gels were then dried, and the signals were detected by autoradiography.

**TUNEL Assay**—HCT116 wild type cells were seeded directly onto coverslips in 6-well plates. 18 h later cells were transfected with the indicated amounts of expression vectors for a further 24 h. Following transfection cells were fixed in 1% paraformaldehyde for 10 min at room temperature. Apoptotic cells were labeled using Apoptag Plus fluorescein in situ apoptosis detection kit S7111 (Chemicon International) according to the manufacturer’s instructions and viewed by fluorescence microscopy.

**Annexin V Staining**—HCT116 wild type cells were seeded into 6-well plates. 18 h later, cells were transfected with the indicated amounts of expression vectors for a further 24 h. In order to include any floating cells, medium was collected into fluorescence-activated cell sorting tubes and centrifuged at 1700 rpm for 4 min to leave a cell pellet. Medium was then discarded from tubes. Petri dishes were washed with 2 ml of PBS, PBS was discarded, and then 1.5 ml of trypsin was added to each plate and incubated until cells became detached. Following cell detachment, 1.5 ml of McCoy’s 5A medium supplemented with 10% fetal calf serum was added to each plate to stop the trypsin. The cell suspensions were then added to each of the relevant cell pellets from the floating cells. Samples were centrifuged at 1700 rpm for 4 min, and pellets were resuspended in 1 ml of McCoy’s 5A medium supplemented with 10% fetal calf serum and incubated for 5 min. Samples were centrifuged for a further 4 min at 1700 rpm prior to resuspension in 1 ml of ice-cold PBS. Samples were centrifuged for a further 4 min at 1700 rpm. Apoptotic cells were detected using a TACS Annexin V-fluorescein isothiocyanate apoptosis detection kit (R&D systems) according to the manufacturer’s instruction and analyzed by flow cytometry.

**Immunofluorescence**—HCT116 wild type cells were seeded on the 4-well chamber slides. 18 h later, cells were transfected with the indicated expression vectors for a further 24 h. Then the slides were washed in 1X PBS and fixed in 4% paraformaldehyde for 15 min at room temperature and washed three times in 1X PBS afterward. The cells were then fixed in ice-cold methanol for 5 min and air-dried. After that, cells were permeabilized in freshly prepared 0.5% (w/v) Triton X-100 in 1X PBS, and slides were washed three with 1X PBS for 10 min. Following washes with 1X PBS, nonspecific sites were blocked for 1 h with 3% (w/v) bovine serum albumin/PBS in room temperature. After three washes in 1X PBS, the chambers from each slide were removed, and cells were incubated overnight with the primary antibody (1:30,000) diluted in 1% (v/v) bovine serum albumin/PBS at 4°C. Twenty-four hours postincubation, the slides were washed in 0.1% (v/v) PBS-Tween and incubated with the secondary antibody conjugated with Alexa-488 (Invitrogen) for 30 min at room temperature. After incubation, cells were washed three times in 1X PBS containing 0.1% (v/v) Tween. Nuclei were counterstained using 4',6-diamidino-2-phenylindole. Coverslips were mounted with Fluorescent mounting medium (DakoCytomation). The control for nonspecific binding of the secondary antibody was the 1% (w/v) bovine serum albumin/PBS, where no primary antibody was added. Microscopy analysis was performed on Zeiss microscope (Zeiss Axionplan Imaging Systems; kindly provided by the Medical Research Council, Human Genetics Unit).

**RNA Extraction and Real Time PCR**—mRNA was extracted from cells using the Qiagen RNeasy Mini kit following the manufacturer’s suggested procedures. The optional step of DNase treatment using the Qiagen RNase-free DNase set was also included. 1 μl of the sample RNA was diluted in 100 μl of water and loaded into a 96-well UV plate. The absorbance was detected by the Bio-Tek plate reader at 260 nm. After the extraction, the real time PCRs were performed in the Opticon 4 machine using the Qiagen QuantiTect SYBR Green one-step PCR kit. Equal amounts of mRNA were loaded in each reaction. The actin primers were used as follows: L, 5'-CTACGTCCCGCCCTGGACTTTGAGC-3'; R, 5'-GATGGAAGCCGCCGATCCACACGG-3’. The DAPK1 primers were as follows: L, 5'-CGAGGTGTAGGTGTTATGGTG-3'; R, 5'-CTGTGCTTTTGCTGGTGGA-3’.

**RESULTS**

In tumor cell lines with a defined p53 status, we set up experiments to determine whether DAPK-1 protein expression was controlled post-translationally. The mRNA and protein expressions of DAPK-1 in six different tumor cell lines were examined (Fig. 1A). In HCT116 cells, DAPK-1 protein is expressed in both 50% wild type (+/+ ) and p53 null (−/− ) cells (Fig. 1A), whereas in HeLa cells, which have an HPV-E6 attenuated wild type p53 pathway, DAPK-1 levels are relatively high (Fig. 1A). These data suggest that the basal expression of DAPK-1 protein is not p53-dependent, which is consistent with previous work (25). More interestingly, although in general the protein levels of DAPK-1 are consistent with the mRNA levels, HeLa cells actually possess more DAPK-1 protein compared with A549 cells, whereas A549 cells have the highest mRNA level (Fig. 1A). This indicates that DAPK-1 can be regulated post-translationally, and this regulation may be important for determining the basal protein levels of DAPK-1.

DAPK-1 was first discovered in interferon-γ-treated HeLa cells (16). Therefore, we treated cells with the virus mimetic reagent pIpC to determine whether it can trigger transcriptional or post-translational changes in DAPK-1 levels. The poststress expression of a series of endogenous proteins, including interferon-responsive factor-1 (IRF-1) and DAPK-1, were detected (Fig. 1B). The IRF-1 level increased as expected, but there were no significant changes in the steady-state expression of any DAPK family members or p53. We treated cells with combined serum starvation and pIpC treatment, because the cells respond better to such stresses as reported previously (26). Upon serum starvation, DAPK-1 mRNA levels decreased by ~50% in the HCT116 p53+/− cells, whereas no changes were observed in the HCT116 p53−/− cells (Fig. 1C). This suggests that factors in the serum may induce DAPK-1 mRNA expression through p53, supporting the idea that p53 can induce DAPK-1 expression during some stress conditions (25). Surprisingly, instead of increasing DAPK-1 level, pIpC treatment has an inhibitory effect on the DAPK-1 mRNA expression in both HCT116 cell lines (Fig. 1C). This might be because pIpC works through a specific receptor pathway not involving DAPK-1 (27). However, despite the changes in...
latent multiprotein complex in cells, since it is not rapidly
protein might be stored or assembled into a stable and possibly
ate, the treatment with cycloheximide further revealed a rela-
mRNA quantitation in cycling cells. Six different tumor cells, as indicated, were harvested, and each cell sample
was split into two halves. One was used for (i) mRNA level test using one-step SYBR green real time PCR (top); (ii) protein level quantitation using immunoblotting (middle); and (iii) loading control for immunoblotting (bottom). The relative mRNA level is depicted as a ratio of DAPK-1/actin as a function of cell line. WT, wild type. B, the pIpC response of the p53 pathway. HCT116 p53<sup>+/−</sup> cells were treated with pIpC for 6 h (lanes 1 versus lanes 2), and the endogenous protein expressions (as indicated; p53, DAPK-1, DAPK-2, DAPK-3, IRF-1, and loading control) were detected using the respective antibodies. C, DAPK-1 protein and mRNA quantitation in stress responses. HCT116 p53<sup>+/−</sup> and p53<sup>−/−</sup> were treated with pIpC for 6 h under normal culture or serum-starved conditions. Each cell sample was split into two halves. One was used for (i) mRNA level quantitation using real time PCR (top) and (ii) DAPK-1 protein quantitation using immunoblotting (bottom). The relative change in mRNA level is depicted as a ratio of DAPK-1/actin as a function of treatment.

mRNA levels, the DAPK-1 protein levels remain the same with or without treatment (Fig. 1C), suggesting again that gene transcription is not the key step for regulating DAPK-1 protein levels in cancer cell lines.

The half-life of DAPK-1 protein was subsequently examined in HCT116 p53<sup>+/−</sup> cells by performing a time course of cycloheximide, which inhibits the general translation machinery (Fig. 2, A and B). This was performed to determine whether DAPK-1 protein has a short half-life, like some signaling proteins, including p53 and IRF-1. In comparison with some short half-life proteins, such as p53 (Fig. 2A) and IRF-1 (Fig. 2A), DAPK-1 indeed has a much longer half-life (Fig. 2A). Further, in HeLa cells where DAPK-1-dependent signaling pathways operate, the treatment with cycloheximide further revealed a relatively long half-life (Fig. 2B). These data suggest that DAPK-1 protein might be stored or assembled into a stable and possibly latent multiprotein complex in cells, since it is not rapidly removed and resynthesized as are regulatory proteins like p53 and IRF-1.

These data are somewhat in contrast to a previous report that demonstrated that DAPK-1 can be controlled post-translationally through a proteasome-dependent pathway through the E3 ubiquitin ligase DIP-1 (28). We next tested the proteasome inhibitor MG132 on steady-state levels of endogenous and ectopically expressed DAPK-1 to determine whether DAPK-1 protein can be turned over by a proteasome-dependent pathway, despite the fact that it has a relatively long half-life. MG132 treatment increased slightly the DAPK-1 protein levels and rendered higher molecular mass adducts, which might be ubiquitinated DAPK-1 (Fig. 2C, lane 2 versus lane 1). However, as highlighted by the arrows, MG132 also unexpectedly depleted some lower mass fragments of DAPK-1. Ectopically expressed and tagged DAPK-1 protein also exhibited similar changes in higher and lower mass bands in the presence of MG132 (Fig. 2D). This suggests that DAPK-1 protein and higher molecular mass covalent adducts are clipped by a protease(s) that can be inhibited by MG132.

Several previous reports have described the appearance of DAPK-1 protein cleavage products (29, 30). These latter data are consistent with our data showing that DAPK-1 is cleaved by some proteases in vivo. Further, the accumulation of the 60-kDa cleavage band of DAPK-1 protein is specifically inhibited by a cathepsin B inhibitor (30). Moreover, since MG132 can also function as a general inhibitor of cathepsins (31), we compared the effect of cathepsin B inhibitor with MG132 on inhibition of the cleavage of DAPK-1 (Fig. 2E). The cathepsin B inhibitor depleted similar cleavage products as that observed using MG132. Further, the in vitro treatment of DAPK-1 with purified cathepsin B also induced a similar cleavage profile (Fig. 3A), suggesting that cathepsin B and not the proteasome is responsible for the accumulation of lower mass adducts seen in cells. Further, these data suggest that an interaction of cathepsin B with DAPK-1 in cells is inducing the proteolytic clipping of a small pool of the DAPK-1 protein.

Since the cathepsin B inhibitor can attenuate the proteolytic clipping of endogenous DAPK-1, we next investigated whether overexpression of cathepsin B can decrease the levels of
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DAPK-1. The transfection of a cathepsin B-GFP construct into HeLa cells did not have any effect on the expression of endogenous DAPK-1 (data not shown). Considering that DAPK-1 is a cytoskeleton-associated protein (16), there may be different pools of DAPK-1 localizing differently inside the cells, and therefore this pool of DAPK-1 might be refractory to interaction with transfected cathepsin B. Subsequently, we co-transfected HA-DAPK-1 and cathepsin B-GFP to determine whether cathepsin B can decrease ectopically expressed DAPK-1 protein levels (Fig. 3B). Distinct from endogenous DAPK-1, the HA-DAPK-1 protein levels were depleted with increasing amounts of transfected cathepsin B vector. This indicates that at least some pools of DAPK-1 protein can be depleted by cathepsin B.

Having shown that cathepsin B proteolytically clips and/or decreases DAPK-1 protein levels, suggesting an in vivo interaction between the two proteins, we set up siRNA to cathepsin B to determine whether the protein plays a role in regulating the rate of TNF-dependent apoptosis. The treatment of cells with TNF-\(\alpha\) resulted in apoptosis induction as defined by elevation in PARP cleavage (Fig. 3C, lane 2 versus lane 1). Further, siRNA to cathepsin B, which results in depletion of cathepsin B protein (Fig. 3C, lanes 5 and 6 versus lanes 2 and lanes 1) results in a stimulation of PARP cleavage (Fig. 3C, lane 6 versus lane 2). These data indicate that cathepsin B is a negative regulator of TNF-\(\alpha\)-dependent apoptosis. Further, DAPK-1 protein is depleted after combined treatment with cycloheximide and TNF-\(\alpha\) (Fig. 3C, lane 4 versus lanes 3 and 2) resulted in elevated PARP cleavage (Fig. 3C, lane 4 versus lane 2). These data suggest that both cathepsin B and DAPK-1 might be co-negative regulators of TNF-\(\alpha\)-dependent apoptosis. DAPK-1 protein depletion using TNF-\(\alpha\)-treated cells can also stimulate PARP cleavage (Fig. 3D, lane 6 versus lane 2). Further, DAPK-1 protein depletion does not block the potent PARP cleavage observed using TNF and cycloheximide combined treatment (Fig. 3D, lane 8 versus lane 2). This indicates that DAPK-1 is not required necessarily for TNF-mediated cell death and is consistent with a previous report highlighting that although DAPK-1 mediates interferon-\(\gamma\)-dependent apoptosis, DAPK-1 protein depletion using morpholino technologies sensitizes cells to TNF-dependent apoptosis (51).

Since cathepsin B and DAPK-1 appear to form an interaction in vivo, as defined by cathepsin B-sensitive proteolytic fragments (Fig. 2) and the two proteins together function as negative regulators of TNF-\(\alpha\)-dependent apoptosis (Fig. 3) (51), we set out to determine whether they directly bind to each other using co-immunoprecipitation assays. As determined using a cathepsin B antibody precipitation followed by a DAPK-1 protein probe (Fig. 3C), the binding of the endogenous cathepsin B and DAPK-1 is detectable but relatively weak (Fig. 4A, lane 3). Since both cathepsin B and DAPK-1 are reportedly to be involved in TNF apoptotic pathways (17, 32–34), we next determined whether TNFR-1 transfection altered the stability of the DAPK-
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1-cathepsin B immune complex. As shown in Fig. 4A, TNFR-1 transfection relative to vector control increased the binding of endogenous DAPK-1 and cathepsin B in lysates from three cell lines with a wild-type p53 status (Fig. 4, A–C, lane 4 versus lane 3). However, there was no detectable decrease in the total levels of DAPK-1 protein in direct lysates (Fig. 4, A–C, lane 5 versus lane 1). This suggests that the increase in immune complex formation might be due to relocalization of the two proteins, and this was examined (see below). In fact, the transfection of tagged DAPK-1 resulted in a striking TNFR-1-dependent relocalization of DAPK-1 from the plasma membrane to punctate cytosolic structures (Fig. 4D). We could not detect changes in localization of endogenous staining of DAPK-1, since the antibodies available can only detect by immunostaining transfected DAPK-1 protein (18, 20).

The fact that TNF can induce the permeabilization of lysosomes and thus release cathepsin B into cytosol (35) led us to consider that TNFR-1 triggered the binding between DAPK-1 and cathepsin B by relocalizing both of them to a different compartment. Therefore, we investigated the localization of DAPK-1 and cathepsin B with or without TNFR-1 transfection by subcellular fractionation. As expected, after TNFR-1 signaling, cathepsin B protein levels were elevated in the cytosolic fraction (Fig. 4E, lane 5 versus lane 1) and depleted in the “nuclear” fraction (Fig. 4E, lane 3 versus lane 7). Moreover, the levels of cytosolic DAPK-1 protein increased after TNFR-1 transfection (Fig. 4E, lanes 5 versus lane 1) and the cytoskeletal pool of DAPK-1 was depleted after TNFR-1 transfection (Fig. 4E, lane 4 versus lane 8). We further performed an immunoprecipitation from subcellular fractions 1–3 (Fig. 4F). Although basal DAPK-1 and cathepsin B complex can be detected in Fraction 3 (Fig. 4E, lane 4), TNFR-1-mediated signaling elevated the DAPK-1–cathepsin B complex in the cytosolic fraction 1 (Fig. 4E, lane 5 versus lane 2). TNFR-1-mediated depletion of the DAPK-1–cathepsin B immune complex occurred in fraction 3 (Fig. 4E, lane 7 versus lane 4). Subcellular fraction 2, having the highest levels of cathepsin B (Fig. 4D, lanes 2 and 6) did not yield formation of a detectable immune complex with DAPK-1 protein (Fig. 4E, lane 6 versus lane 3). These data suggest that the two proteins are assembled into a dynamic and specific multiprotein complex in subcellular fractions 1 and 3.

To define which region on DAPK-1 binds to cathepsin B, we created DAPK-1 mutants with deletion of key functional domains (Fig. 5B). First we examined whether TNFR-1 could also induce the binding of transfected full-length DAPK-1 (1432 amino acids) with endogenous cathepsin B, and a similar TNFR-1 dependence in complex formation was observed (Fig. 5A, lanes 3 versus lane 2). When co-transfecting the DAPK-1 deletion mutants (Fig. 5C, lanes 1–5) with TNFR-1 followed by immunoprecipitation with the cathepsin B antibody, only the longest DAPK-1 mutant (residues 1–1313) bound to endogenous cathepsin B (Fig. 5C, lane 10 versus lanes 6–9). These data suggest that the region between 836 and 1313 is important for the binding of cathepsin B to DAPK-1. A further set of deletion constructs were developed in order to map further the cathepsin B binding site. When co-transfecting the DAPK-1 deletion mutant (residues 1–835; Fig. 5D, lane 5) and (residues 1–947; Fig. 5D, lane 6) with TNFR-1 followed by immunoprecipitation with the cathepsin B antibody, only the DAPK-1 mutant (residues 1–947) bound to endogenous cathepsin B (Fig. 5D, lane 3 versus lane 2). These data suggest that the region
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FIGURE 4. TNFR-1 induces the relocalization of the endogenous cathepsin B and DAPK-1 multiprotein complex. A–C, TNFR-1 induces a complex between DAPK-1 and cathepsin B. A, HeLa; B, HCT116 p53<sup>−/−</sup>; C, AS49 cells were transfected with vectors encoding TNFR-1 for 18 h before harvesting. The lysates were subjected to immunoprecipitation with a cathepsin B antibody and then probed with anti-DAPK-1 antibody. Mouse secondary antibody was used in the control immunoprecipitation. A, no lysate control (lane NL); immunoprecipitation was also performed to check the background of the antibody. Lane 1, direct lysate with vector only transfection; lane 2, control immunoprecipitation; lane 3 and 4, immunoprecipitation in vector only or TNFR-1-transfected cells; lane 5, direct lysate with TNFR-1 transfection). D, TNFR-1 induces relocalization of DAPK-1. HCT116 p53<sup>−/−</sup> cells were transfected with vector encoding HA-DAPK-1 with and without TNFR-1 co-transfection for 24 h before immunostaining. The localization of HA-DAPK-1 was detected with HA antibody and the fluorescein isothiocyanate (FITC) mouse secondary antibody. 4',6-diamidino-2-phenylindole (DAPI) staining was used to identify the nucleus. E, TNFR-1 induces a DAPK-1 and cathepsin B mobilization to the cytosolic fraction. 1, HeLa cells were transfected with vectors encoding TNFR-1 for 18 h before harvesting. The subcellular fractionation was performed using a subcellular proteome extraction kit (Calbiochem; 1, cytosol; 2, membrane; 3, nuclear; 4, cytoskeletal). DAPK-1 was detected using DAPK-1 antibody and cathepsin B detected using a cathepsin B antibody. Lanes 1–4, DAPK-1 levels and cathepsin B levels in fractions 1–4 in vector-only transfectants; lanes 5–8, DAPK-1 levels and cathepsin B levels in fractions 1–4 in TNFR-1 transfectants. F, TNFR-1 induces a DAPK-1 and cathepsin B multiprotein complex to the cytosolic fraction 1. The lysates of each fraction were immunoprecipitated with cathepsin B antibody and then probed with DAPK-1 antibody. Mouse secondary antibody was used in the control IP. Lanes 2–4, DAPK-1 levels in fractions 1–3 in vector-only transfectants; lanes 5–7, DAPK-1 levels in fractions 1–3 in TNFR-1 transfectants.

between residues 836 and 947 is rate-limiting for the binding of cathepsin B to DAPK-1.

The use of small peptide domains derived from full-length polypeptides to manipulate cell signaling is an emerging field called chemical genetics or peptide therapeutics (50). Such minimodules often reveal novel insights into signaling as a result of disrupting a specific protein binding domain, and they can be expected to provide leads for drug design and development. To determine whether the minimal cathepsin B binding region of DAPK-1 acts in a gain-of-function or in a dominant negative fashion on TNFR-1-dependent apoptosis, the DAPK-1-(836–947) miniprotein was transfected into cells to evaluate effects on cell signaling. A dose-dependent production of DAPK-(836–947) miniprotein can be observed after transfection (Fig. 6A, lanes 1–3). Further, transfection of the cathepsin B expression vector results in reduction in levels of the DAPK-(836–947) miniprotein (Fig. 6B, lanes 4 versus lanes 3 and 2), indicating an interaction between the two proteins in cells. The transfection of the vector encoding the DAPK-(836–947) miniprotein resulted in a stimulation of TNFR-1-dependent PARP cleavage (Fig. 6C, lane 4 versus lanes 2 and 3). Further, similar to TNF-α signaling in the presence of cycloheximide (Fig. 3C, lane 4 versus lanes 1–3), endogenous DAPK-1 protein was depleted by the combined transfection of TNFR-1 and DAPK-(836–947) miniprotein (Fig. 6C, lane 4 versus lanes 1–3).

In order to determine whether changes in PARP cleavage correlated with changes in apoptosis, Annexin V staining and a TUNEL assay were used to quantify DAPK-(836–947) miniprotein changes in the apoptotic index. The transfection of TNFR-1 resulted in increased apoptosis as defined by Annexin V staining (Fig. 6D, TNFR panel) that was stimulated by the co-transfection of the DAPK-(836–947) miniprotein (Fig. 6D, TNFR + DAPK<sub>836–947</sub> panel). The transfection of TNFR-1 also resulted in apoptosis, as defined by TUNEL staining (Fig. 6E, TNFR panel), that was similarly stimulated by the co-transfection of DAPK-(836–947) miniprotein (Fig. 6E, TNFR + DAPK<sub>836–947</sub> panel; quantitated in Fig. 6F). This maximal level of apoptosis therefore correlated with PARP cleavage and reduction in endogenous DAPK-1 protein levels (Fig. 6C).

DISCUSSION

DAPK-1 is a member of the calcium calmodulin kinase superfamily whose many members play a genetic role in activating the p53 tumor suppressor pathway as well as other p53-independent proapoptotic and repair-triggered signaling pathways. Growing evidence points to distinct types of regulation of DAPK-1 protein function. The original data demonstrated that down-regulation of DAPK-1 by CpG methylation has been shown in a variety of tumors, providing tumors with growth advantages (36). However, more recent evidence suggests that there are some post-translational mechanisms controlling the activity of DAPK-1 in cells (28, 36, 37). These include ribosomal...
S6 kinase-dependent inactivation of DAPK-1 and ERK-dependent activation of the proapoptotic function of DAPK-1. Our observations show that cathepsin B can directly interact with DAPK-1 protein \textit{in vivo} and TNFR-1 transfection promotes relocalization into the cytosol fraction. Interestingly, similar to MG132, the cathepsin B inhibitor can also increase both the steady-state levels of DAPK-1 protein and the higher molecular mass adducts. Considering that the expression of the ubiquitin E3 ligase DIP-1 for DAPK-1 promotes TNF-α-induced apoptosis (28), there may be a link between the ubiquitination of DAPK-1, its interaction with cathepsin B, and TNF apoptotic responses.

DAPK-1 is known to bind to early responsive components in the TNF-α pathway, including TNFR-1 and FADD after brain seizures, although the function of these bindings is still unclear (32, 33). Whether these binding interactions are linked to the proapoptotic ERK pathway remains to be determined. Morpholino depletion of DAPK-1 protein sensitizes cells to TNF-induced apoptosis, whereas a depletion of DAPK-1 protein attenuates interferon-γ-induced apoptosis (Fig. 6G) (51). These data indicate that DAPK-1 can exhibit prosurvival as well as proapoptotic signaling properties. The discovery here of a TNFR-1-induced interaction between cathepsin B and DAPK-1 raises the question of whether this complex would be prosurvival or...
FIGURE 6. The cathepsin B binding region on DAPK-1-(836–947) stimulates TNF-1-induced apoptosis and the degradation of endogenous DAPK-1 protein. A, expression of DAPK-1-(836–947) in cells. HCT116 p53−/− cells were transfected with vectors encoding GST-DAPK-1-(836–947) for 36 h before harvest. The expression of GST-DAPK-1-(836–947) was detected by GST-antibody (Sigma). B, cathepsin B transfection depletes the DAPK-1-(836–947) miniprotein. HCT116 p53−/− cells were transfected with vectors encoding GST-DAPK-1-(836–947) and cathepsin B-GFP for 36 h before harvest. The expression of GST-DAPK-1-(836–947) was detected by GST-antibody, and cathepsin B was detected by GFP antibody. C, DAPK-1-(836–947) miniprotein induces TNFR-1-dependent DAPK-1 degradation in cells. HeLa cells were transfected with vectors encoding GST-DAPK-1-(836–947) and TNFR-1 for 24 h before harvest. The expression of endogenous DAPK-1 and PARP were detected by respective antibodies. D, annexin V quantitation of apoptosis stimulated by DAPK-1-(836–947). HCT116 p53−/− cells were transfected with the indicated expression vectors for 24 h before harvest. The apoptotic cells were then detected using the TACS Annexin V-fluorescein isothiocyanate apoptosis detection kit (R&D Systems) according to the manufacturer’s instruction and analyzed by flow cytometry. The percentages of the apoptotic cells are presented in bar graphs. E, TUNEL assay for quantifying apoptosis stimulated by DAPK-1-(836–947). HCT116 p53−/− cells were transfected with the indicated expression vectors for 24 h before staining. Apoptotic cells were labeled using an Apoptag Plus fluorescein in situ apoptosis detection kit S7111 (Chemicon International) according to the manufacturer’s instructions and viewed by fluorescence microscopy. F, the result of the TUNEL assay was analyzed by Image J, and the percentages of apoptotic versus total cells are presented in bar graphs. G, a model highlighting the two distinct pathways involving DAPK-1: a DAPK-1-dependent survival function antagonizing TNF-dependent apoptosis and the interferon-dependent proapoptotic function. H, a model highlighting the mechanism of DAPK-1-dependent attenuation of TNF-dependent apoptosis. Although cathepsin B has been reported to stimulate TNF-mediated apoptosis (38, 39), the binding of DAPK-1 to cathepsin B attenuates TNF-mediated apoptosis. From this current study, the depletion of either cathepsin B or DAPK-1 using siRNA can stimulate TNF-mediated PARP cleavage. The induction of a cathepsin B-DAPK-1 complex in the cytosol by TNF signaling suggests that the complex itself has such prosurvival activity. Further, the ability of the DAPK-1 binding region for cathepsin B (miniprotein amino acids 836–947) to degrade endogenous DAPK-1, thus stimulating apoptosis, suggests that this dimeric complex can counter rates of TNF-mediated apoptosis. We propose that the DAPK-1 domain from amino acids 836–947 can interact with an “anchoring” factor (like cathepsin B) that normally keeps DAPK-1 in a stable state with a very slow turnover (Fig. 2A). This region is required to maintain the DAPK-1–cathepsin B complex, and the DAPK-1 miniprotein (amino acids 836–947) can bind to this endogenous anchor (possibly cathepsin B), releasing cathepsin B from DAPK-1 and resulting in TNF-dependent DAPK-1 protein degradation and enhanced apoptosis. DAPI, 4′,6-diamidino-2-phenylindole.
proapoptotic. The stimulation of TNFR-1-induced apoptosis by either DAPK-1 or cathepsin B depletion using siRNA (Fig. 3) suggests that this complex could be regulating the rate of TNF-dependent apoptosis by virtue of attenuating the apoptotic program. Using the cathepsin B binding domain of DAPK-1 as a tool to further test the mechanism of TNF-α apoptotic signaling, we show that the DAPK-1 (836–947) miniprotein can function in a dominant negative manner, resulting in a stimulation of DAPK-1 protein degradation and an increase in TNF-dependent apoptosis (Fig. 6F). These data provide a genetic tool with which to dissect the mechanism of DAPK-1 links to TNF signaling in the future.

Our fractionation studies have demonstrated that there are distinct pools of DAPK-1 that may have distinct functions. It appears that TNFR-1 can induce a change in the localization of DAPK-1, and this correlates with the induction of a stable immune complex with cathepsin B. Since the majority of DAPK-1 in these fractionation experiments is nonresponsive to TNFR-1 signaling, these pools of DAPK-1 presumably will have distinct functions, possibly responding to ERK or other cytoskeleton-responsive signaling pathways.

Different from DAPK-1, although cathepsin B is known to be involved in TNF-α-induced apoptotic pathways, not much work has been done in investigating its binding partners in the classical TNF-α apoptotic pathways. Nevertheless, the role of cathepsin B in the TNF-α-induced apoptosis pathways in tumor cells is still controversial (38, 39), and the mechanism through which it kills the cells is still unclear. Our study may provide a new angle to investigate the function of cathepsin B in the TNFR-1–induced apoptotic pathways, since our data suggest that the DAPK-1 status can modify the cathepsin B apoptotic effect. More interestingly, cathepsin B has long been known to be involved in cell malignancy. Tumor cells tend to have higher expression of cathepsin B, and the secretion of high levels of cathepsin B results in the degradation of extracellular matrices (40). Recently, it was reported that DAPK-1 can inhibit cell motility by blocking the integrin-mediated polarity pathway (41). Moreover, DAPK-1 is also reported to be highly expressed in invading tumor-associated macrophages in colorectal cancer but not able to kill the cells (42). The interaction between DAPK-1 and cathepsin B may shed a light on explaining the dual function of both DAPK-1 and cathepsin B on the malignancy in tumors.

In addition to cathepsin B, several other proteases, such as cathepsin D and cathepsin L, are reported to be involved in TNF-α-induced apoptotic pathways (39, 43, 44). Further, cathepsin D also mediates the interferon-γ-activated programmed cell deathlike DAPK-1 (16, 44). Moreover, cathepsin L is a highly redundant functional homologue of cathepsin B, and only the mouse with double knock-out of both cathepsin B and L leads to severe brain atrophy associated with selective neuronal loss in the cerebral cortex and in the cerebellar Purkinje and granule cell layers (43, 45). A cell-specific function for the DAPK-1–cathepsin B complex needs to be considered. Considering the role of DAPK-1 in neuronal death (36) and our data indicating that cathepsin B can regulate basal DAPK-1 protein levels, it raises the possibility that the neuronal loss in the double knock-out mouse may be due to the overexpression of DAPK-1 and cathepsin B. It will be interesting to further investigate the group of proteases that can cleave DAPK-1 in addition to cathepsin B and the functions of these cleavage events.

DAPK-1 has been reported to bind to distinct proteins, such as ERK (21), ribosomal S6 kinase (RSK) (22), and myosin light chain (46), whereas the data from our studies indicate that cathepsin B is another new member of this large DAPK-1 multi-protein complex with a novel binding domain localized in the C-terminal domain of DAPK-1 between the ankyrin repeats and the death domain. Given that ability of TNF-dependent pathways to induce apoptosis (47, 48), studying how this cathepsin B–DAPK-1 complex integrates with MEK-ERK-DAPK-1 (52), RSK-DAPK-1, and DAPK-p53 signaling axes will shed light on signal transduction mechanisms that modify the apoptotic response (49).

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