Functional role of death associated protein 3 (DAP3) in anoikis

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ABSTRACT

Detachment of adherent epithelial cells from the extracellular matrix induces apoptosis, known as anoikis. Integrin stimulation protects cells from anoikis, but the responsible mechanisms are not well known. Here, we demonstrate that a pro-apoptotic GTP-binding protein, DAP3 (death associated protein 3), is critical for induction of anoikis. Downregulation of DAP3 expression by antisense oligonucleotides inhibited anoikis. Conversely, over-expression of DAP3 augmented cell death and caspase activation induced by cell detachment. Furthermore, association of DAP3 with FADD and activation of caspase-8 were induced by cell detachment. We also show that DAP3 is phosphorylated by kinase Akt (PKB), and active Akt can nullify apoptosis induction by DAP3. Mutation of a consensus Akt phosphorylation site in DAP3 renders it resistant to suppression by active Akt in cells. Integrin ligation stimulates Akt activation and phosphorylation of DAP3 in intact cells, as well as suppressing the ability of DAP3 over-expression to augment anoikis. Involvement of DAP3 in anoikis signaling demonstrates a novel mechanism role for this GTP-binding protein in apoptosis induction caused by cell detachment.
INTRODUCTION

The process of apoptosis is critical for the development and maintenance of all multicellular metazoans. Dysregulated apoptosis is germane to many human diseases, including cancer, immunodeficiency, and neurodegeneration. At least two major pathways for apoptosis induction have been identified, commonly known as the extrinsic and intrinsic pathways (reviewed in (1,2)). The extrinsic pathway is triggered by Tumor Necrosis Factor (TNF)-family cytokines, and involves the adapter protein FADD, which recruits pro-caspases-8 and –10 to death receptor complexes, resulting in activation of these cell death proteases and initiation of the apoptosis process. The intrinsic pathway, in contrast, is triggered by myriad stimuli that impinge on mitochondria, causing these organelles to release apoptogenic proteins into the cytosol, such as cytochrome c, which binds caspase-9-activator Apaf-1, initiating the apoptosis mechanism.

DAP3 (death associated protein 3) was identified as a pro-apoptotic protein during a functional screen based on tumor cell transfection with an antisense cDNA expression library and screening for rescue from cytokine-induced apoptosis (reviewed in (3,4)). It was subsequently shown that DAP3 antisense protects tumor cells from
death induced by certain cytokines, including TNF\(\alpha\), FasL, and TRAIL (5). DAP3 is a protein of 46 kDa that carries a "P-loop" motif capable of binding GTP (6,7). Mutagenesis studies suggest that GTP binding is critical for the pro-apoptotic function of DAP3 (7). Two intracellular pools of DAP3 have been identified, including a mitochondrial pool of DAP3 molecules which undergo proteolytic processing upon import into these organelles (8), and a cytosolic pool (9). Functional mapping of the apoptosis pathway pertinent to DAP3 by use of antisense and dominant-negative mutants indicates that DAP3 is involved in the so-called “extrinsic” cell death pathway activated typically by TNF\(\alpha\), Fas, TRAIL, and related TNF-family death ligands and receptors (5,7). However, it is also shown recently that DAP3 is involved in the process of mitochondrial fragmentation during cell death and mitochondrial maintenance (10).

Anoikis is apoptosis that results from cell detachment from extracellular matrix, and it has been linked to the extrinsic pathway, typically involving FADD and caspase-8 (reviewed in (11,12)). We provide evidence here that DAP3 is at least partially required for anoikis, and that cell detachment induces DAP3 interaction with FADD, correlating with caspase-8 activation. Furthermore, we present evidence that DAP3 is a substrate of the kinase, Akt (PKB), with Akt opposing the pro-apoptotic action of DAP3. The
findings provide novel insights into the mechanisms responsible for anoikis regulation, and thus may have relevance to pathophysiological situations where cell detachment from matrix is involved, such as tumor metastasis and wound healing.

Materials and Methods

Plasmids And Antisense Oligodeoxynucleotides

The DAP-3 mutants, DAP3(T237A) and DAP3(T237E), were generated by polymerase chain reaction (PCR) mutagenesis from a human DAP3 cDNA (gift from Kimchi A.) and subcloned into pcDNA3-FLAG plasmid. The sequence of 237 threonine (ACA) was mutated to the sequence of alanine (GCC) for DAP3(T237A) or glutamate (GAA) for DAP3(T237E). DAP3(T237A) cDNA was cloned into pET21d-N vector for bacterial expression. Antisense oligodeoxynucleotides for human DAP3 were generated with the sequences 5’-CATCATCCTTGCACGTGA-3’(AS1) and 5’-TTTCAGCATCATCCTTGCA-3’(AS2). Control sense oligodeoxynucleotides were synthesized with the sequence a 5’-ATGATGCTGAAAGGAATA-3’. These oligodeoxynucleotide was transfected into HEK293 cells as reported previously (7).
Anoikis Assays

Anoikis assays were performed using established procedures (13). For cell viability assays or caspase 8 assays, cells were detached and maintained in suspension as described above in HEMA-coated culture plates. After 48 hours, cells were harvested and the percentage of dead cells was assayed by trypan blue staining and caspase 8 activity of cell lysates was assayed by hydrolysis of fluorogenic substrate Ac-IETD-AFC (Calbiochem, Inc.), as described below.

Caspase 8 Activity Assays

Caspase 8 activity was measured in cell lysates as described (7) at 37°C using a fluorometric plate reader (Perkin-Elmer, LS50B) in the kinetic mode with excitation and emission wave lengths of 400 and 505 nm, respectively. Activity was measured by the release of 7-amino-4-trifluoromethyl-coumarin (AFC) (RFU) from the synthetic substrate Ac-IETD-AFC (PharMingen) after 30 min incubation.
Immunoprecipitation Assays

HEK293 cells (1X10^6) in 10 cm plates were transiently transfected with 10 µg (total) of plasmid DNA. Cells were cultured in attached or detached (in suspension culture) conditions as described above. After 24 or 48 hours, cells were suspended in lysis buffer containing 0.1% NP-40, 20 mM Tris-HCl pH 7.5, 2 mM MgCl₂, 1 mM EGTA, 130 mM NaCl and protease inhibitors (Boehringer Mannheim). After pre-clearing with 10 µl protein A or protein G-agarose, immunoprecipitations were performed using 10 µl of anti-FLAG antibody M2-conjugated agarose (Sigma) or each antibody at 4°C for 4 hours. After extensive washing by lysis buffer, immune complexes were analyzed by SDS-PAGE or immunoblotting using indicated antibodies, followed by HRPase-conjugated antibodies and detection using an enhanced chemiluminescence (ECL) system (Amersham, Inc.).

In Vitro Kinase Assays

Plasmid of pCMV6-myrAkt-HA, pCMV6-Akt(E40K) or pCMV6-dn-Akt was used for the expression of each Akt mutant, Akt-myr, Akt-E40K or Akt-DN (14). 293T cells (10^7) were transiently transfected with 25 µg of plasmid DNA, normalizing total DNA using pcDNA3
(control) plasmid. After 24 hours, cells were lysed in 1.5 ml of 20 mM Tris-HCl (pH 7.4), 140 mM NaCl, 1% NP-40, 10 mM NaF, 1 mM Na$_3$V$_2$O$_4$, 1 mM EDTA, and protease inhibitors. After normalizing for protein concentration, lysates were precleared with protein G-Sepharose and preimmune serum for 1 hour and then incubated at 4°C with 0.5 µg of rat high-affinity mAb to hemagglutinin (HA) (Boehringer-Mannheim), followed by addition of 10 µl of protein G-Sepharose (Pharmacia) for 1 hour. Immunoprecipitates were washed three times in lysis solution and two times in kinase solution [20 mM Hepes (pH 7.2), 10 mM MgCl$_2$, 10 mM MnCl$_2$, 1 mM DTT, and 3 µM ATP]. In vitro kinase reactions were performed using these immunoprecipitates with [$^{32}$P]ATP and purified His$_6$-tagged DAP3 (7) or His$_6$-tagged DAP3(T293A) substrate proteins. His$_6$-tagged proteins were expressed with NH$_2$-terminal His$_6$-tags from pET21d in BL21 cells and affinity-purified with Ni-NTA spin-columns (Qiagen).

**Antibody Production**

A rabbit antiserum to DAP3 phosphopeptides was raised against a purified peptide, CRVRNA(T)DAVGIV, in which threonine (T) was phosphorylated. The first cysteine(C) residue was bound to the maleimide-activated carrier proteins (Pierce, Inc.) through its sulfhydryl group. The antiserum was purified using a phosphopeptide-conjugated column.
RESULTS

**DAP3 is required for anoikis.** To explore the requirement of DAP3 for anoikis, we used antisense oligodeoxynucleotide-based gene silencing. Two different antisense (AS) oligodeoxynucleotides (ODN) targeting the human DAP3 mRNA, AS1 and AS2, were effective at reducing levels of endogenous DAP3 protein when transfected into HEK293 cells, whereas various control ODNs (including a sense sequence) did not (Figure 1A). We then tested the effects of transfected ODNs on cell death induced by cell detachment from matrix. AS-ODNs reduced cell death by approximately half, compared to control untreated cells or sense ODN-treated cells (Figure 1B). Thus, expression of endogenous DAP3 appears to be required, at least in part, for cell death induction by cell detachment.

**Cell detachment promotes DAP3 interaction with FADD.** Previously we showed that DAP3 associates with FADD (7). We therefore explored the effects of cell detachment on interaction of DAP3 with FADD, using co-immunoprecipitation assays. In addition, we compared control-transfected cells with cells transfected with plasmids encoding constitutively active mutants of the protein kinase, Akt, because of its
previously documented ability to suppress anoikis (15).

In attached cells, little endogenous DAP3 associated with endogenous FADD. In contrast, when cells were suspended, then association of DAP3 and FADD increased, as determined by co-immunoprecipitation assay (Figure 2A). In cells expressing the active forms of Akt, namely myristoylated Akt or Akt (E40K), less FADD was recovered with immunoprecipitated DAP3, suggesting that Akt suppresses the interaction of these proteins. Levels of FADD and DAP3 were not altered by either cell detachment or Akt activity, excluding differences in protein expression as a trivial explanation for these findings (Figure 2A).

As expected by a previous report (16), cell detachment correlated with elevations in caspase-8 protease activity, as measured in cell lysates by a fluorogenic substrate, Ac-IETD-AFC (Figure 2B). Akt suppressed caspase-8 activation induced by cell detachment, consistent with prior reports (16).

**Akt phosphorylates DAP3 and regulates the pro-apoptotic activity of DAP3.** The ability of Akt to suppress DAP3 association with FADD in suspended cells prompted us to explore the possibility that DAP3 might be a direct or indirect substrate of this protein.
kinase. Akt phosphorylates proteins on serine or threonine residues within the sequence motif RXRXX(S/T) (17). Interestingly, we found that DAP3 contains a site that conforms to the consensus Akt phosphorylation motif at Thr237 (RVRNAT).

To examine whether DAP3 is directly phosphorylated by Akt, active Akt was immunoprecipitated from HEK293T cells transfected with plasmids encoding myr-Akt or Akt (E40K), and in vitro kinase (IVK) assays were performed with [$^{32}$P]γATP and recombinant, purified His$_6$-DAP3 as a candidate substrate (Figure 3A). Incubation of immune-complexes containing active forms of the kinase, Akt-myr or AktE40K, phosphorylated DAP3 in vitro. In contrast, His$_6$-DAP3 was not phosphorylated in vitro when using immune-complexes containing a kinase-dead mutant of Akt, previously shown to have dominant-negative (DN) effects on endogenous Akt (Figure 3A). By comparison, a variety of control kinases did not cause in vitro phosphorylation of DAP3, including JNK and Ask1 (data not shown). Thus, Akt or an Akt-associated kinase can directly phosphorylate DAP3 in vitro.

Next, to determine whether the predicted phosphorylation site in DAP3 is required for phosphorylation by Akt, we compared His$_6$-tagged wild-type DAP3 with mutant DAP3, DAP3(T237A), in which threonine 237 was mutated to alanine. These
proteins were produced in bacteria and purified for use as in vitro substrates in kinase assays with active Akt. Wild-type DAP3 was phosphorylated in vitro by active immunoprecipitated Akt, whereas DAP3(T237A) was not (Figure 3B).

To explore the functional significance of Akt-mediated phosphorylation of DAP3 on anoikis, we performed transfection experiments where DAP3 was over-expressed in cells at levels insufficient to induce apoptosis of attached cells but adequate for enhancing apoptosis of detached cells. Using this approach, we compared the effects of active Akt on cell death when cells were transfected with either wild-type or T237A mutant DAP3 (Figure 3C). Whereas myr-Akt suppressed cell death associated with expression of wild-type DAP3 in detached cells, it did not reduce cell death in DAP3 (T237A)-expressing cells. These data thus suggest that Akt can inhibit the death-promoting effect of DAP3 in the detached cells, but not when the presumptive Akt phosphorylation site within DAP3 has been ablated. Consistent with this hypothesis, we observed that anoikis was not augmented when HEK293 cells were transfected with a plasmid encoding DAP3 in which a phospho-mimicking mutation was engineered (e.g., T237E) (Figure 3C).

Similar results were obtained when measuring caspase-8 activity in detached
cells transfected with wild-type versus mutant version of DAP3. Using amounts of
plasmid DNA insufficient to affect attached cells (not shown), over-expression of
wild-type DAP3 augmented the detachment-induced increase in caspase-8 protease
activity. Co-expression of active (e.g. Akt-myr or AktE40K) but not inactive (e.g.,
Akt-DN) Akt profoundly suppressed caspase-8 activity in detached DAP3-expressing
cells (Figure 3D). In contrast, active versions of Akt were less effective at suppressing
caspase-8 activity in detached cells expressing DAP3(T237A), compared to cells
expressing wild-type DAP3. The residual Akt-mediated suppression of caspase-8
activity in DAP3(T237A)-expressing cells may be due to the effects of Akt on
endogenous DAP3 within these cells. Regardless, comparisons of wild-type and
DAP3(T237A) reveal a quantitative difference in their Akt sensitivity. In contrast to
DAP3(T237A), the phospho-mimic mutant, DAP3(T237E) was far less potent than
wild-type DAP3 at promoting increases in caspase-8 activity in detached cells.
Immunoblot analysis showed that comparable levels of DAP3, DAP3(T237A), and
DAP3(T237E) were produced in transfected cells, excluding differences in protein
expression as an explanation for these results (not shown).
Integrin ligands induce phosphorylation of DAP3 and suppress DAP3-induced apoptosis. Integrin signaling inhibits anoikis induction (11,12,15,18). Thus, we examined whether integrin ligands could modulate DAP3-induced apoptosis. First, we documented the ability of integrin ligand vitronectin to rescue detached cells from anoikis and to control Akt activity. For these experiments, HEK293 cells were detached and then stimulated by vitronectin for 48 hours. Anoikis induction was strongly inhibited by vitronectin in a concentration-dependent manner (Figure 4A), as expected from prior reports (19). Stimulation by vitronectin also induced Akt phosphorylation even after cell detachment (Figure 4B). Similar results were obtained using osteopontin, as an alternative integrin ligand (not shown). Thus, integrin ligands induce Akt activity and rescue detached HEK293 cells from anoikis.

Next, we tested the effects of integrin ligands on apoptosis of cells transfected with DAP3-encoding plasmid. Apoptosis induced by over-expression of DAP3 in cultures of attached cells was inhibited by addition of vitronectin (Figure 4C). Also, DAP3-mediated enhancement of apoptosis induced by cell detachment was also markedly inhibited by addition of vitronectin to cultures (Figure 4C). Thus, integrin ligand vitronectin suppressed the pro-apoptotic effects of DAP3 over-expression.
Because Akt was demonstrated to phosphorylate DAP3 in vitro, we next explored whether the phosphorylation status of DAP3 changes in cells in accordance with cell attachment and detachment. To this end, we first produced a phospho-specific antibody using a synthetic phosphopeptide corresponding to amino-acids 231 to 243 of human DAP3. To confirm the ability of this antibody to recognize T237 phosphorylated DAP3, HEK293 cells were transfected with plasmids encoding active or inactive forms of Akt, then endogenous DAP3 was recovered by immunoprecipitation and analyzed by immunoblotting using the phospho-specific antibody. As shown in Figure 4D, antibody binding to DAP3 was markedly increased in cell expressing active versions of Akt. Conversely, antibody binding was reduced in cells expressing Akt-DN, compared to control-transfected cells (Figure 4D). Blotting of each sample with an antibody that recognizes DAP3 independently of its phosphorylation state confirmed loading of equivalent amounts of DAP3 protein for all samples.

Using this validated phosphospecific antibody, we then evaluated the in vivo phosphorylation status of DAP3 in cells before and after detachment. As shown in Figure 4E, cell detachment was associated with reduced phosphorylation of
endogenous DAP3. Addition of integrin ligand, vitronectin, to detached cells increased DAP3 phosphorylation. Blotting of each sample with an antibody that reacts with DAP3 irrespective of phosphorylation confirmed loading of equivalent amounts of DAP3 protein for all samples, and demonstrated that the total levels of DAP3 protein are not altered by cell detachment or integrin ligation.

**DISCUSSION**

By manipulating the levels of DAP3 expression using antisense and gene transfer methods, we uncovered a role for this pro-apoptotic protein in anoikis. Reducing endogenous DAP3 expression using antisense ODNs decreased apoptosis caused by cell detachment, while over-expressing DAP3 increased the percentage of cells undergoing apoptosis per time following detachment. Anoikis is thought to play a variety of important roles in normal development, ensuring that cells survive only when they reach their correct positions in the body, as well as in disease scenarios such as tumor metastasis and wound healing at epithelial surfaces (reviewed in (20-22)). Thus, a role for DAP3 in anoikis places this protein into a biological context of relevance to normal development and disease.
Previously, the DAP3 protein has been shown to have dual roles in normal mitochondrial physiology and cell death regulation, not unlike other apoptosis-relevant proteins such as cytochrome c, which also displays dual functions in normal cell physiology and cell death. An intra-mitochondrial pool of DAP3 apparently operates as a subunit of the mitochondrial ribosome, presumably involved in translation of mitochondria genome-derived transcripts (23-25), while an extra-mitochondrial pool of DAP3 participates in apoptosis signaling within the extrinsic pathway (5-7). Unlike cytochrome c, however, there is no evidence thus far that DAP3 must be released from mitochondria to perform its cell death function (10). Rather, incomplete import of DAP3 into mitochondria seems to generate an extra-mitochondrial pool of this pro-apoptotic protein, the amount of which varies among cell types and cell lines (9). Cytochrome c and DAP3 also differ in that the former resides in the space between the inner and outer mitochondrial membranes, while the latter resides in the matrix (23).

A role for DAP3 in extrinsic pathway signaling was first demonstrated by antisense, gene transfer, and dominant-negative experiments, where DAP3 was shown to modulate apoptosis induction by TNFα, TRAIL and Fas (5). Subsequent studies showed physical interaction of DAP3 with FADD and other TNF-family death receptor...
components, suggesting that this GTP-binding protein may participate in assembly of so-called “Death-inducing signaling complexes” (DISCs) that trigger caspase-8 activation (7). Anoikis has been shown to be dependent on extrinsic pathway participants FADD and caspase-8, but not on components of the intrinsic pathway (16,26). Thus, a role for DAP3 in anoikis is consistent with evidence that this pro-apoptotic protein participates in the extrinsic pathway. Interestingly, we observed that cell detachment resulted in an increase in the association of DAP3 with FADD, without altering the total levels of either of these proteins. This observation suggests an inducible protein interaction, which could be related to the recent observation that FADD is often sequestered in the nuclei of epithelial cells (27), undergoing translocation into the cytosol upon cell detachment.

Akt is recognized for its role in anoikis suppression and anchorage-independent survival of transformed cells (reviewed in (11,12,28-32)). The mechanism(s) by which Akt suppresses anoikis however has(have) been unclear, particularly given the dearth of targets of this kinase in the extrinsic pathway. We observed that activity of Akt and phosphorylation of endogenous DAP3 modulate in concert in response to changes in cell attachment/detachment and in response to integrin ligands. Furthermore, gene
transfer-mediated increases in Akt activity correlated with increased in vivo phosphorylation of DAP3, while Akt-DN reduced basal phosphorylation of DAP3. Finally, Akt-containing immune-complexes were capable of inducing phosphorylation of recombinant DAP3 in vitro, suggesting that Akt or a kinase tightly associated with Akt directly phosphorylates DAP3. In this regard, DAP3 contains a sequence motif typical of Akt substrates, and mutation of the candidate phospho-acceptor site within this motif abolished phosphorylation of DAP3 in vitro by Akt and correlated with reduced sensitivity of DAP3 to Akt-mediated apoptosis suppression. Conversely, a T237E phospho-mimic displayed reduced pro-apoptotic activity in transfection experiments, implying an important role for this motif in regulating DAP3 function. Taken together, these observations suggest that Akt-mediated phosphorylation of DAP3 on threonine 237 represents a potential mechanism by which Akt suppresses anoikis. Future studies will determine the range of cell-types and pathophysiological settings where this Akt-mediated mechanism for anoikis suppression prevail.

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Figure Legends

Figure 1. Antisense-mediated gene silencing demonstrates a role for DAP3 in anoikis. (A) HEK293 cells (1X10^6) were transfected with two different DAP3-targeting antisense (AS) or a control sense ODN. After 48 hours, transfected cells were lysed, and immunoprecipitations were performed using anti-DAP3 (top) or anti-Akt (bottom) antibodies, followed by SDS-PAGE/immunoblot analysis with anti-DAP3 or anti-Akt antibody, and ECL-based detection. (B) HEK293 cells (1X10^6) were transfected with AS or sense ODN. After 48 hours, transfected cells were cultured in HEMA-coated dishes for 48 hours. The percentage of dead cells was assayed by trypan blue staining (mean
Figure 2. Detachment induces DAP3 association with FADD, which is blocked by Akt. (A) Plasmids (10\(\mu\)g/each sample) encoding Akt mutants were transfected into HEK293 cells (1X10\(^6\)). After 24 hours, transfected cells were cultured in HEMA-coated dishes for 24 hours (“detached”). Cells were recovered and lysed for immunoprecipitation with anti-DAP3 or anti-FADD antibody, followed by SDS-PAGE/immunoblot analysis with anti-FADD or anti-DAP3 antibody. Alternatively, lysates were normalized for protein content and loaded directly in gels, for detection of HA-tagged Akt mutants by anti-HA antibody (bottom panel). (B) Control plasmid or plasmids (10\(\mu\)g/each sample) encoding for Akt mutants were transfected into 293 cells (1X10\(^6\) cells). After 24 hours, transfected cells were cultured in HEMA-coated dishes for 24 hours. Cells were recovered, lysed and caspase 8 activity was analyzed based on cleavage of Ac-IETD-AFC (100 \(\mu\)M/final concentration), after normalization for total protein content. Data are expressed as relative fluorescence units (RFU) (mean \(\pm\) SD; \(n = 3\)).
Figure 3. Akt phosphorylates DAP3 and suppresses pro-apoptotic effect of DAP3 on anoikis. (A) Purified His<sub>6</sub>-tagged DAP3 protein (200 ng/each sample) was used for in vitro kinase assays (IVK) employing P<sup>32γ</sup>-ATP as a phospho-donor and immune-complexes prepared with anti-HA antibody using lysates from HEK293T cells transfected with control plasmid or plasmids encoding various HA-tagged Akt mutants (top). An aliquot of the IVK mixtures was analyzed by SDS-PAGE/immunoblotting using anti-DAP3 (middle) or anti-HA (bottom) antibody. (B) Purified His<sub>6</sub>-tagged wild-type (WT) DAP3 or mutant DAP3(T237A) proteins (200 ng/each sample) were used for IVK assays (top panel) with P<sup>32γ</sup>-ATP and either control anti-HA immune-complex prepared from control transfected cells (-) or immunoprecipitated active Akt mutant, Akt-myr (+). An aliquot of the IVK mixtures was analyzed by SDS-PAGE/immunoblotting using anti-His (middle) or anti-HA (bottom) antibody. (C) Plasmids encoding DAP3 or various DAP3 mutants were co-transfected with control plasmid or plasmids encoding active Akt mutant HA-myr-Akt (10 µg/total DNA used for each sample) into HEK293 cells (1X10<sup>6</sup> cells), maintaining total DNA constant by addition of control pcDNA3 plasmid. After 24 hours, transfected cells were cultured in HEMA-coated dishes for 48 hours (detached). The percentage of dead cells was measured by trypan blue staining (mean ± SD; n = 3).
(D) Cells were transfected and cultured as above, then after 24 hours, lysates were prepared, normalized for total protein content, and caspase 8 activity was measured based on cleavage of Ac-IETD-AFC, expressing data as relative fluorescence units (RFU) (mean ± SD; n = 3).

**Figure 4. Integrin ligand vitronectin modulates Akt activity, DAP3 phosphorylation, and pro-apoptotic effect of DAP3 on anoikis.**  
(A) HEK293 cells (5X10^5 cells) were detached, cultured in HEMA-coated dish and treated with vitronectin at the indicated concentrations. After 48 hours, cell viability was determined by trypan blue cell staining (mean ± SD; n = 3).  
(B) 293 cells (1X10^6 cells) were detached, cultured in HEMA-coated dishes, and then treated with vitronectin (100 µg/mL). After 24 hours, cells were recovered, lysed, and immunoprecipitations were performed with anti-Akt antibody, followed by SDS-PAGE/immunoblot analysis using anti-phospho-Akt (top) or anti-Akt (bottom) antibody.  
(C) Control or DAP3-encoding plasmids (10 µg) were transfected into 293 cells (1X10^6 cells). After 24 hours, cells were left attached or cultured detached in HEMA-coated dishes, with (+) or without (-) vitronectin (100 µg/mL). After 48 hours, cell viability was determined by trypan blue cell staining (mean ±
SD; n = 3). (D) HEK293 cells were transfected with control plasmid or plasmids encoding various Akt mutants. After 24 hours, cell lysates were prepared and used for immunoprecipitation of DAP3, followed by SDS-PAGE/immunoblot analysis using anti-phosphopeptide antibody (top) or anti-DAP3 antibody (bottom). (E) 293 cells were cultured attached or detached, without (-) or with (+) vitronectin (100 µg/mL). Lysates were prepared 24 hours later, and used for immunoprecipitation of DAP3, followed by SDS-PAGE/immunoblot analysis using anti-phosphopeptide antibody (top) or anti-DAP3 antibody (bottom).
FIGURE 1

A

Control  AS1  AS2  Sense

DAP3

IP:anti-DAP3  Blot:anti-DAP3

Akt

IP:anti-Akt  Blot:anti-Akt

B

Cell Death (%)

Control  AS1  AS2  Sense

ATTACH

DETACH
**FIGURE 2**

**A**

- **FADD**
  - IP: anti-DAP3
  - Blot: anti-FADD

- **DAP3**
  - IP: anti-DAP3
  - Blot: anti-DAP3

- **Akt-myr**
  - IP: anti-DAP3
  - Blot: anti-Akt-myr

- **Akt-E40K**
  - Blot: anti-Akt-E40K

**B**

- **CASP8 ACTIVITY (RFU)**
  - Attach
  - Control
  - Akt-myr
  - Akt-E40K

**DETACH**
**FIGURE 4**

**A**

![Graph showing cell death (%) in response to different concentrations of vitronectin.](image)

**B**

![Western blot images showing phosphorylated Akt under various conditions.](image)

**C**

![Graph showing cell death (%) under different conditions involving DAP3 and vitronectin.](image)

**D**

![Western blot images showing phosphorylated DAP3 under various conditions.](image)

**E**

![Western blot images showing DAP3 under various conditions.](image)
