

行政院國家科學委員會專題研究計畫 期中進度報告

TGF- β 對於肝癌細胞死亡的調控與機制之研究(4/5)

計畫類別：個別型計畫

計畫編號：NSC91-2321-B-002-001-

執行期間：91年08月01日至92年07月31日

執行單位：國立臺灣大學醫學院分子醫學研究所

計畫主持人：陳瑞華

計畫參與人員：蔡育庭、陳俊豪、王琬菁、郭津岑、陳忻怡、林峰麒、何宣宗、
林裕敏

報告類型：精簡報告

報告附件：出席國際會議研究心得報告及發表論文

處理方式：本計畫可公開查詢

中 華 民 國 92 年 5 月 26 日

DAP-kinase induces apoptosis by suppressing integrin activity and disrupting matrix survival signals

Won-Jing Wang,* Jean-Cheng Kuo,* Chung-Chen Yao,[‡] and Ruey-Hwa Chen*

*Institute of Molecular Medicine, [‡]Department of Orthodontics, College of Medicine, and [‡]Department of Dentistry, NTU Hospital, National Taiwan University, Taipei, Taiwan

Address correspondence to Ruey-Hwa Chen, Institute of Molecular Medicine, College of Medicine, National Taiwan University, Taipei, Taiwan. Tel: 886-2-23123456 Ext. 5700. Fax: 886-2-23957801. E-mail: rhchen@ha.mc.ntu.edu.tw

JCB 200204050R

Number of characters: 49998

Running Title: DAPK induces apoptosis by inhibiting adhesion

Key Words: DAP-kinase, integrin, apoptosis, adhesion, anoikis

Abstract

Death associated protein kinase (DAP-kinase) is a calcium/calmodulin-dependent serine/threonine kinase, and participates in various apoptosis systems. However, its apoptosis-promoting mechanism is poorly understood. Here, we demonstrate that DAP-kinase suppresses integrin-mediated cell adhesion and signal transduction, whereas dominant-negative interference of this kinase promotes adhesion. This effect of DAP-kinase is neither a consequence of apoptosis, nor a result of decreased expression of integrins. Rather, DAP-kinase downregulates integrin activity through an inside-out mechanism. We present evidence indicating that this adhesion-inhibitory effect accounts for a major mechanism of the apoptosis induced by DAP-kinase. First, in growth-arrested fibroblasts, DAP-kinase triggers apoptosis in cells plated on fibronectin, but does not affect the death of cells on poly-L-lysine. Second, in epithelial cells, DAP-kinase induces apoptosis in the anoikis-sensitive MCF10A cells, but not in the anoikis-resistant BT474 cells. Most importantly, the apoptosis-promoting effect of DAP-kinase is completely abolished by enforced activation of integrin-mediated signaling pathways from either integrin itself or its downstream effector FAK. Finally, we show integrin or FAK activation blocks the ability of DAP-kinase to upregulate p53. Our results indicate that DAP-kinase exerts apoptotic effect by suppressing integrin functions and integrin-mediated survival signals, thereby activating a p53-dependent apoptotic pathway.

Introduction

Interactions of cells with the extracellular matrix (ECM) regulate a variety of cellular functions, such as growth, differentiation, migration and survival. Most normal cells require adhesion to correct ECM for their survival, and inadequate or inappropriate cell-matrix interaction often causes apoptosis, a phenomenon known as anoikis (Frisch and Ruoslahti, 1997; Frisch and Screaton, 2001). Such apoptotic mechanism is crucial for maintaining homeostasis, organization, and architecture of epithelia of different organs, and resistance to anoikis results in anchorage-independence, a hallmark of malignant tumor cells. This anchorage-independence permits tumor cells that display altered adhesion and migratory properties to survive and grow in inappropriate environments, thus promoting metastasis (Frisch and Ruoslahti, 1997; Frisch and Screaton, 2001).

Cell adhesion to ECM is primarily mediated by integrins. In resting cells, integrins are present on the cell surface in an inactive state. Activation of integrins can be mediated by intracellular signals, termed inside-out signals, which induce a high affinity and/or avidity state of integrin (Ginsberg et al., 1992). The avidity state is regulated by integrin clustering, which can be triggered by remodeling of the cytoskeletal linkages or alterations of the receptor diffusion rate within cell membrane (Schoenwaelder and Burridge, 1999; van Kooyk and Figdor, 2000). The affinity state of integrin, however, is regulated by its conformational changes induced by a number of signaling pathways or proteins that interact with the cytoplasmic domains of integrins (Hughes and Pfaff, 1998). Structural studies suggest that such inside-out modulation of integrin affinity involves changes in the spatial relationship of the cytoplasmic and/or transmembrane domains of the α and β subunits, thereby inducing

a long-range conformational changes to affect the affinity of the ligand binding domains (Vinogradova et al., 2000; Takagi et al., 2001).

Besides connecting ECM with cytoskeleton, integrins are also capable of inducing various signaling pathways upon ligand binding. Engagement of several types of integrins has been shown to play an important role in cell survival (Giancotti and Ruoslahti, 1999). One initial step of integrin-mediated survival pathway involves the phosphorylation and activation of intracellular tyrosine kinases, such as FAK (Frisch and Ruoslahti, 1997; Frisch and Sreaton, 2001). Constitutive activation of FAK protects epithelial cells from anoikis (Frisch et al., 1996), whereas interference of FAK function in primary fibroblasts and endothelial cells triggers apoptotic death via a p53-dependent mechanism (Hungerford et al., 1996; Ilic et al., 1998). Besides FAK, ligation of a subset of integrins also leads to the recruitment of ILK and Shc. These three integrin-interacting molecules mediate ECM-dependent survival by activating a number of signaling pathways, including the PI 3-kinase/Akt, Raf/ERK and JNK pathways (for review see Frisch and Sreaton, 2001). Detachment of cells from ECM not only blocks these matrix-transduced survival pathways, but also results in a significant alteration of cytoskeletal structures. Evidence has emerged that such cytoskeletal changes can regulate signaling molecules that affect anoikis and/or induce the release of pro-apoptotic factors (Marti et al., 1997; Puthalakath et al., 1999; Otto et al., 2000; Puthalakath et al., 2001).

Death associated protein kinase (DAP-kinase) is an actin filament-associated pro-apoptotic protein (Cohen et al., 1997). This calcium/calmodulin-dependent serine/threonine kinase was identified by a functional cloning system based on its involvement in the interferon- γ -induced apoptosis (Deiss et al., 1995). Subsequent

studies revealed that DAP-kinase functions as a positive mediator of apoptosis induced by a variety of stimuli, such as Fas, TNF- α , TGF- β , ceramide and oncogenes c-myc and E2F (Deiss et al., 1995; Cohen et al., 1997; Inbal et al., 1997; Cohen et al., 1999; Pelled et al., 2001; Raveh et al., 2001; Jang et al., 2002; Yamamoto et al., 2002). Furthermore, overexpression of DAP-kinase induces apoptotic death and apoptotic morphological changes, such as cell rounding and shrinkage in a number of cell systems (Cohen et al., 1997; Pelled et al., 2001). In addition to its apoptosis-promoting activity, several lines of evidence indicate that DAP-kinase plays an important role in tumor suppression. First, the expression of DAP-kinase is frequently lost in various tumor cell lines and tissues (Raveh and Kimchi, 2001). Second, DAP-kinase is capable of suppressing c-myc- and E2F-induced oncogenic transformation (Raveh et al., 2001). Third, in an experimental metastasis model, the expression level of DAP-kinase is inversely correlated with the metastatic activities of the tumors, and reintroducing DAP-kinase into the highly metastatic tumors suppresses their ability to form metastases in mice (Inbal et al., 1997), implying a role of DAP-kinase in anoikis. However, despite such broad involvement in various apoptotic systems, very little is known about the mechanism by which DAP-kinase executes the pro-apoptotic function. A recent study demonstrated that DAP-kinase activates a p53-mediated apoptotic pathway through a mechanism that requires p19^{ARF} (Raveh et al., 2001). How cytoskeleton-localized DAP-kinase transduces a pro-apoptotic signal to activate p19^{ARF}, however, is currently unknown.

In this study, we investigated the pro-apoptotic mechanism of DAP-kinase. We found DAP-kinase functions as a negative regulator of integrin activity and cell adhesion. This effect of DAP-kinase diminishes integrin-mediated survival signals and plays a causative role in DAP-kinase-induced apoptosis. Furthermore, we

showed that the apoptotic inducibility of DAP-kinase is completely lost in the anoikis-resistant cells. Finally, integrin or FAK activation blocks DAP-kinase-induced upregulation of p53. We conclude that DAP-kinase exerts pro-apoptotic activity by suppressing integrin activity and integrin-mediated survival signals, thereby activating a p53-dependent apoptotic pathway.

Results

DAP-kinase induces apoptosis-independent morphological changes in 293T cells.

To unravel the pro-apoptotic mechanism of DAP-kinase, in particular, the mechanism by which DAP-kinase activates p53, we thought to determine the biochemical pathways and/or biological effects induced by DAP-kinase in cells lacking functional p53. We reasoned that, in such cells, the apoptotic effect of DAP-kinase would be greatly attenuated, thus allowing a longer period for the observation of DAP-kinase-induced events that act upstream of p53. 293T cells, carrying inactive p53, were used for transfection with various DAP-kinase expression vectors and the morphologies of transfected cells were determined 48 h post-transfection. As shown in Fig. 1B, overexpression of a constitutively active DAP-kinase mutant (Δ CaM), in which the catalytic activity is no longer regulated by calcium/calmodulin (Cohen et al., 1997), led to a dramatic morphological change, as evident by ~30% of the transfected cells showing a round cell shape. Such phenotype was also observed in cells overexpressing the wild type DAP-kinase, albeit with a slightly lower frequency. In cells transfected with a kinase-defective mutant of DAP-kinase (K42A) or the vector backbone, however, no significant cell rounding could be detected. Noted that all

three DAP-kinase proteins were expressed at comparable levels in 293T cells (Fig. 1A). Despite the cell rounding phenotype seemingly resembles apoptosis-like morphological changes, no other apoptotic morphological characteristics were detected, including membrane blebbing, apoptotic body formation (Fig. 1B), and nuclear condensation/fragmentation (data not shown). Furthermore, cells overexpressing DAP-kinase or Δ CaM did not display DNA fragmentation (Fig. 1C, D), were stained negatively by Annexin V (Fig. 1E), and showed normal mitochondrial localization of cytochrome c at 48 h post-transfection (data not shown). This insensitivity of 293T cells to DAP-kinase-induced apoptosis is most likely due to the inactivation of p53 by SV40 T-Ag. Altogether, these results indicate that DAP-kinase triggers morphological changes in 293T cells and that this effect is not a consequence of apoptosis.

DAP-kinase suppresses integrin-mediated cell adhesion and signal transduction.

Next, we investigated the molecular basis of DAP-kinase-induced cell rounding. We first tested whether interference of cell-matrix interaction could account for this phenotype. Indeed, 293T cells overexpressing DAP-kinase displayed a decreased adherence to fibronectin, as comparing to cells transfected with the vector backbone (Fig. 2A). Furthermore, expression of the active Δ CaM mutant caused a greater reduction in the adhesive capability. This impaired adhesion was not reversed by pretreatment of cells with a broad-spectrum caspase inhibitor, zVAD-FMK, further demonstrating that this effect is not due to DAP-kinase-induced apoptosis. Since 293T cells expressed a detectable amount of endogenous DAP-kinase (Fig. 1A), a dominant negative mutant, K42A, was introduced to block the function of the endogenous protein. This dominant-negative interference of DAP-kinase led to a significant acceleration of cell adhesion to fibronectin, as ~80 % of the transfectants

were already attached at 25 min after plating. To determine whether the anti-adhesive function of DAP-kinase was restricted to fibronectin, 293T transfectants were tested for their attachment to laminin. The results obtained from cell adhesion to laminin remarkably resembled those from cell attachment to fibronectin (Fig. 2B), suggesting DAP-kinase as a general inhibitor of cell-ECM interactions. In contrast, adhesion to poly-L-lysine, which does not engage integrins, was not affected by DAP-kinase (Fig. 1C). Thus, DAP-kinase specifically inhibits integrin-mediated cell adhesion.

To investigate whether the anti-adhesive effect of DAP-kinase is accompanied by a blockage of signals elicited by ECM, we evaluated tyrosine phosphorylation status of FAK, a key mediator of ECM growth and survival signals (Frisch et al., 1996; Hungerford et al., 1996; Ilic et al., 1997). When 293T cells carrying only the control vector or the K42A mutant were allowed to adhere to fibronectin for 1 h, a significant amount of tyrosine phosphorylated FAK was detected (Fig. 2D). However, the level of phosphorylated FAK was markedly reduced in cells expressing either DAP-kinase or the Δ CaM mutant. Furthermore, tyrosine phosphorylation of paxillin, a downstream target of FAK (Turner, 2000), was similarly downregulated by expression of DAP-kinase or Δ CaM (Fig. 2D). Together, our results demonstrate that DAP-kinase inhibits cell adhesion to ECM, thereby disrupting integrin-mediated signaling transduction.

DAP-kinase suppresses integrin function through an inside-out mechanism. To gain insight into the possible mechanism of the anti-adhesive role of DAP-kinase, we investigated whether this effect of DAP-kinase is due to a downregulation of integrin expression or activity. 293T cells require α 5 β 1 and α 6 β 1 for their attachment to

fibronectin and laminin, respectively (Bodary and McLean, 1990; and Fig. S1). However, flow cytometry analysis revealed that overall cell surface expressions of the $\alpha 5$, $\alpha 6$, and $\beta 1$ integrins were not significantly altered by the expression of DAP-kinase, Δ CaM or K42A (Fig. S1). Thus, DAP-kinase is likely to affect cell adhesion by modulating integrin activities. Conformational changes in integrins are suggested to underlie the modulation of their affinity for ECM components (O'Toole et al., 1994). If DAP-kinase did inhibit cell adhesion by converting integrins into an inactive conformation, treatment of cells with the integrin-activating antibody, which locks integrin in an active conformation, should be able to reverse this inhibitory effect. Indeed, when 293T cells transfected with DAP-kinase or Δ CaM were plated on fibronectin in the presence of TS2/16 (van de Wiel-van Kemenade et al., 1992), an activating antibody to $\beta 1$ integrin, the anti-adhesion effect of DAP-kinase or Δ CaM was totally eliminated (Fig. 3A). Similarly, treatment of these 293T transfectants with Mn^{2+} , which induces a conformational change of $\beta 1$ integrin in favor of the high affinity state (Gailit and Ruoslahti, 1988), abrogated the ability of DAP-kinase to suppress cell attachment (Fig. 3B). These results suggest that the adhesion inhibition by DAP-kinase involves a mechanism that converts integrin into an inactive conformation.

To further ascertain that DAP-kinase indeed affects the activity of integrin, we used the monoclonal antibody B44 recognizing a specific epitope in $\beta 1$ integrin, which is of very limited accessibility under normal conditions and is expressed upon ligand occupancy. Therefore, expression of the B44 epitope would be an indication of the presence of an active state of $\beta 1$ integrin on cell surface (Ni et al., 1998). Notably, ectopic expression of DAP-kinase or Δ CaM significantly reduced the level of B44 binding, which was completely reversed by pretreatment with Mn^{2+} (Fig. 3C).

Similar results were observed with another monoclonal antibody to activated $\beta 1$ integrin, that is, the HUTS-21 antibody (Table S1). Altogether, we conclude that DAP-kinase suppresses cell adhesion by inside-out modulation of integrin activity.

The anti-adhesion function of DAP-kinase is not dependent on cell types. Next, we investigated whether regulation of cell adhesion and integrin activity by DAP-kinase is restricted to 293T cells, which display a relatively weak adhesive capability and a highly transformed morphology. NIH3T3 mouse fibroblast was chosen for our study because of its low transforming activity and well spread morphology. Furthermore, NIH3T3 cells contain wild type p53 and are able to undergo apoptotic death by ectopic expression of DAP-kinase (Raveh et al., 2001), thus allowing the extension of our analysis to cells sensitive to the apoptotic effect of DAP-kinase. We generated NIH3T3 derivatives that express DAP-kinase, Δ CaM, or K42A by retrovirus-mediated gene transfer. Under normal culture conditions, the Δ CaM-expressing cells started to die at 6 to 7 days after infection, and were virtually eliminated by 9 days after infection (data not shown). To rule out the possibility that any adhesive change detected was a consequence of apoptosis, we only used cells at 4 days post-infection for our analysis. By this time, the expression of wild type and mutant DAP-kinase proteins was readily detected (Fig. 4A), yet apoptosis was not occurred in these cell populations (Fig. 4B). Notably, unlike what was observed in 293T cells, expression of DAP-kinase or Δ CaM did not significantly change the morphology of NIH3T3 cells (data not shown), presumably due to a higher affinity of NIH3T3 cells than 293T cells to the substratum. However, when these NIH3T3 derivatives were replated onto fibronectin, we found that expression of various forms of DAP-kinase affected the adhesion of NIH3T3 cells in the same fashion as they did in 293T cells (Fig. 4C). Furthermore, the adhesion inhibitory effect of DAP-kinase

or ΔCaM was reversed by preincubation of cells with Mn^{2+} (Fig. 4D) or a mouse $\beta 1$ activating antibody, 9EG7 (data not shown), indicating that DAP-kinase utilizes a similar inside-out mechanism to downregulate integrin activity in NIH3T3 cells. Altogether, our data support that the effect of DAP-kinase on anti-adhesion and integrin inactivation is a global mechanism and is not restricted to a small subset of cell types.

DAP-kinase promotes apoptosis by blocking ECM survival signals. Many types of cells undergo apoptotic death when they are deprived of integrin-mediated ECM survival signals (Frisch and Ruoslahti, 1997; Frisch and Screaton, 2001). The finding that DAP-kinase downregulates cell adhesion and integrin activity prompted us to investigate whether these effects of DAP-kinase could account for its apoptosis inducibility. We first utilized the NIH3T3 stable cell system described above. Again, cells at 4 days post-infection were plated onto fibronectin or poly-L-lysine. To study the effect of ECM signals independently of signals from other extracellular sources, such as growth factors, these cells were incubated in culture medium without serum. Twelve hours after plating, the apoptotic index of each cell population was determined by ELISA assays measuring the events of DNA fragmentation. As shown in Fig. 5A, when the four populations of cells were plated on poly-L-lysine, they displayed similarly high levels of apoptosis. However, in NIH3T3 cells carrying the control vector, plating onto fibronectin-coated dishes significantly reduced apoptosis. This survival-promoting activity of fibronectin was virtually eliminated in cells expressing DAP-kinase or the ΔCaM mutant. Conversely, the protection effect of fibronectin was slightly increased in the K42A-expressing cells comparing to the control cells, most likely due to a dominant-negative inhibition of endogenous DAP-kinase by this mutant. When these four populations of cells plated

on fibronectin were incubated in serum-containing medium, each of them showed a background level of apoptosis, with an apoptotic index below 0.1 (data not shown). This latter result is consistent with the observations from a number of cell types (Ilic et al., 1998; Valentinis et al., 1998), and highlights an overlapping role of ECM and growth factors in promoting the survival of fibroblasts. In addition to DNA fragmentation assays, we also performed caspase 3 activity assays and obtained a similar result (Fig. 5B). In conclusion, our data strongly suggest that interference of ECM survival signals is a major mechanism of DAP-kinase-induced apoptosis.

To further confirm the suppression of ECM survival signals as a mechanism of DAP-kinase-induced apoptosis, we thought to test whether this death-promoting activity could be blocked by enforced activation of ECM survival signals. FAK is a key mediator of these signals and its activation rescues apoptosis caused by loss of ECM attachment (Frisch et al., 1996). We thus investigated the effect of FAK activation on DAP-kinase-induced apoptosis. NIH3T3 cells were transfected with expression vectors for various DAP-kinase proteins and/or CD2-FAK (Chan et al., 1994), a constitutively active form of FAK. These transfected cells expressed comparable levels of the corresponding DAP-kinase proteins and the CD2-FAK (Fig. 5C). As observed previously (Chan et al., 1994), the CD2-FAK transfectants contained a slightly higher level of FAK (Fig. 5C), presumably due to degradation of the fusion protein. Two days after transfection, cells were tested for their survival on fibronectin under serum-starved conditions. In the absence of CD2-FAK, we found that the apoptotic response of each of these DAP-kinase transfectants was reminiscent of that seen in virally infected cells (Fig. 5D). Remarkably, when CD2-FAK was introduced into these cells, it was capable of decreasing the apoptotic index of each of these transfectants to a level that is similar to what was observed with the

K42A-expressing cells. Furthermore, CD2-FAK could no longer reduce the apoptotic level of K42A-expressing cells, indicating that the observed anti-apoptotic effect of CD2-FAK is specific to DAP-kinase. These data thus reinforce our conclusion that the apoptotic effect of DAP-kinase is caused by its interference of FAK-mediated ECM survival signals.

Next, we tested whether DAP-kinase-induced apoptosis could be rescued by enforced activation of integrin-mediated signaling pathway from further upstream, that is, the integrin itself. The activating antibody to mouse β 1-integrin, 9EG7, not only was capable of blocking the anti-adhesion effect of DAP-kinase (data not shown), but also restored FAK phosphorylation at tyrosine 397 in NIH3T3 cells expressing DAP-kinase or Δ CaM and plated on fibronectin (Fig. 6A). Most importantly, this activating antibody (Fig. 6B), but not a non-activating antibody to mouse β 1 integrin (Fig. 6C), significantly reduced the apoptotic inducibility of DAP-kinase or Δ CaM in NIH3T3 cells. Altogether, our studies clearly demonstrate the blockage of integrin-mediated survival signal as a major mechanism of DAP-kinase-induced apoptosis.

DAP-kinase induces an anoikis-like apoptosis in epithelial cells, which is reversed by integrin activation. Having demonstrated the apoptotic mechanism of DAP-kinase in fibroblasts, we next decided to broaden our analysis using epithelial cells, which are well known to undergo detachment-induced apoptosis, namely, anoikis. MCF10A human mammary epithelial cells were chosen for our study because of their high sensitivity to anoikis (Li et al., 1999). We introduced various forms of DAP-kinase into MCF10A cells by retrovirus-mediated gene transfer. Since prolonged culture of the DAP-kinase or the Δ CaM stable populations caused

apoptosis and eventually a complete loss of cells carrying the retroviral vectors, freshly infected cells were used for analyzing their apoptosis levels under attached or suspension condition. By the time they were assayed for apoptosis, each stable population still expressed the corresponding DAP-kinase protein at a much higher level than the endogenous level seen in cells carrying the control vector (Fig. 7A). The ability of these DAP-kinase- or Δ CaM-expressing cells to survive few days in culture is presumably due to the presence of a high concentration of epidermal growth factor (EGF) in the culture medium (see Materials and Methods), as EGF was demonstrated to rescue anoikis in epithelial cells (Jost et al., 2001). To assess the apoptotic effect of DAP-kinase, the four populations of cells were plated on dishes coated with either fibronectin or the non-adhesive polyhydroxyethylmethacrylate (poly-HEMA) and incubated in culture medium without addition of EGF. As shown in Fig. 7B, the various retrovirus-infected cells exhibited similar sensitivities to anoikis, when they were plated on poly-HEMA. However, when they were seeded on fibronectin, cells expressing DAP-kinase or Δ CaM displayed not only a reduced adhesive capacity (data not shown), as what was observed from 293T and NIH3T3 cell systems, but also an increased apoptotic level, comparing to cells carrying the control vector or the K42A mutant (Fig. 7B). Most importantly, this apoptosis-promoting effect of DAP-kinase or Δ CaM was completely abrogated by incubating cells with the TS2/16 human β 1 activating antibody, again demonstrating that the DAP-kinase-induced apoptosis can be prevented by an outside-in activation of integrin function. Noted that in cells carrying the control vector or even the K42A mutant, TS2/16 was still able to decrease the apoptotic index, presumably due to its ability to further promote the adhesion of these cells (data not shown). Nevertheless, this antibody did not protect suspension-induced apoptosis in cells plated on poly-HEMA, demonstrating that this antibody did not nonspecifically inhibit

apoptosis. Taken together, these data indicate that DAP-kinase is able to trigger an anoikis-like process in epithelial cells.

If DAP-kinase indeed induces anoikis in epithelial cells, it should not evoke apoptosis in cells that are resistant to anoikis. To test this hypothesis, we utilized the BT474 breast carcinoma cell line, which was shown to be able to grow in an anchorage-independent fashion (Xu et al., 2000). Similarly, cells transfected with various DAP-kinase expression constructs were tested for apoptosis by plating onto fibronectin or poly-HEMA. In contrast to the findings with MCF10A cells, DAP-kinase or Δ CaM did not induce apoptosis in either attached or detached BT474 cells (Fig. 8B), even though the various forms of DAP-kinase were expressed at high levels in these transfectants (Fig. 8A). Altogether, our data support DAP-kinase induces an anoikis-like apoptosis in epithelial cells.

Activation of integrin or FAK blocks DAP-kinase-induced upregulation of p53.

DAP-kinase was recently reported to link oncogenes myc and E2F to a p53-mediated apoptotic checkpoint (Raveh et al., 2001); thus activation of p53 represents one mechanism by which DAP-kinase exerts its pro-apoptotic effect. To address how DAP-kinase leads to p53 activation, we examined whether this activation is a consequence of DAP-kinase-induced anti-adhesion and whether enforced activation of ECM survival signals could abolish this activation. We first measured p53-dependent transcriptional activation in a reporter assay. Consistent with previous findings in mouse embryonic fibroblasts (Raveh et al., 2001), transfection of NIH3T3 cells with DAP-kinase or Δ CaM caused a significant induction of the transcription from a p53-responsible reporter, p53-TA-luc (Fig. 9A, B). Importantly, this DAP-kinase-induced reporter activity, but not the basal reporter activity, was

almost completely abrogated by either co-transfection of CD2-FAK (Fig. 9A) or treatment with the 9EG7 β 1 activating antibody (Fig. 9B), indicating that suppression of cell adhesion accounts for a mechanism by which DAP-kinase activates p53. Next, we determined whether these alterations in p53 activity are correlated with changes in the endogenous p53 protein level. Western blot analysis revealed that DAP-kinase or Δ CaM caused an increase in the p53 protein level, and, again, this elevation of p53 was abolished by CD2-FAK (Fig. 9C). Thus, the blockage of ECM survival signals by DAP-kinase is responsible for its induction of p53. In summary, we conclude that DAP-kinase triggers apoptosis by suppression of integrin-mediated cell adhesion, thereby activating a p53-dependent apoptotic pathway.

Discussion

In this study, we demonstrate an inhibitory role of DAP-kinase in integrin-mediated cell adhesion and ECM signal transduction. This effect of DAP-kinase is unlikely to be restricted to a small set of cell types or specific ECM components, as it was observed in several cell systems and on both fibronectin and laminin matrixes. Furthermore, this adhesion suppressive effect is not a consequence of DAP-kinase-induced apoptosis and can be readily detected in 293T cells, which are relatively insensitive to the pro-apoptotic activity of DAP-kinase due to the inactivation of p53. Even in NIH3T3 cells, which undergo apoptotic death by ectopic expression of DAP-kinase, this effect of DAP-kinase occurs prior to the onset of apoptosis. In accordance with this notion, the anti-adhesive function of DAP-kinase is not affected by pretreatment of cells with the broad-spectrum caspase

inhibitor. These findings lead to a hypothesis that DAP-kinase-induced downregulation of cell adhesion attenuates the survival signals transduced from ECM and plays a causative role in the pro-apoptotic function of DAP-kinase. Indeed, several lines of evidence are presented to support this notion. First, in serum-deprived NIH3T3 fibroblasts, which rely on ECM as the source of survival signals, DAP-kinase exerts its apoptosis-promoting effect on cells plated on fibronectin but not those plated on poly-L-lysine. Second, in epithelial cells, DAP-kinase efficiently induces apoptosis when expressed in the anoikis-sensitive MCF10A cells, but not in the anchorage-independent BT474 cells. Finally, and most importantly, the apoptotic inducibility of DAP-kinase is abolished by enforced activation of integrin-mediated ECM survival signals from either integrin itself or its downstream effector FAK. Thus, downregulation of cell adhesion, which leads to a suppression of ECM survival signals, accounts for at least one mechanism of DAP-kinase-induced apoptosis. In this regard, DAP-kinase may be considered as an anoikis inducer in anchorage-dependent cell types.

Disruption of cell-ECM interactions in serum-deprived endothelial cells and fibroblasts causes the activation of a p53-mediated apoptotic pathway (Ilic et al., 1998). Cell attachment to fibronectin suppresses this apoptosis through the activation of FAK (Almeida et al., 2000). Interestingly, DAP-kinase was reported to induce apoptosis via a p53-dependent mechanism (Raveh et al., 2001). In this study, we demonstrate that enforced activation of either integrin or FAK blocks the ability of DAP-kinase to upregulate p53. This finding indicates that induction of p53 by DAP-kinase is primarily mediated by its adhesion inhibitory effect; thus places p53 downstream of integrin inactivation along the apoptotic pathway of DAP-kinase. Previous study reveals the involvement of cPLA₂ and PKC λ/ι in the p53 activation in

response to the deprivation of both growth factors and matrix attachment (Ilic et al., 1998). Additional studies are required to determine the involvement of these signaling molecules in DAP-kinase-induced p53 activation.

We have examined the mechanism underlying the anti-adhesive effect of DAP-kinase. We found that DAP-kinase does not affect cell surface expression of the integrins that are responsible for ECM attachment, but, rather, inhibits the activities of these integrins by converting them into an inactive conformation. How DAP-kinase transduces an inside-out signal to affect the conformation of integrins is currently unclear. Nevertheless, the requirement of DAP-kinase catalytic activity for the anti-adhesion effect implies an involvement of phosphorylating specific substrates for DAP-kinase. In a separate study, we have identified an *in vivo* substrate for DAP-kinase, i.e., the regulatory light chain of myosin II (MLC). However, unlike other kinases that lead to MLC phosphorylation, such as myosin light chain kinase and Rho-kinase, DAP-kinase triggers the disruption rather than assembly of focal adhesions in fibroblasts and this effect does not lead to a concomitant loss of stress fibers (Kuo, J.-C., J.-R. Lin, J. M. Staddon and R.-H. Chen, submitted). We predict such uncoupling of the stress fiber and focal adhesion formation would lead to a perturbation of the balance between contractile and adhesion forces, which in turn triggers an anoikis-like event. Indeed, in this study, we clearly demonstrate that DAP-kinase exerts its pro-apoptotic activity by suppressing cell adhesion and disrupting adhesion-dependent survival signals. Taken these findings together, we propose a model that depicts the pathway by which DAP-kinase induces apoptosis (Fig. 9D).

It is noteworthy that the conclusion from this study does not rule out the possible

existence of other mechanisms by which DAP-kinase promotes apoptosis. Indeed, prolonged expression of DAP-kinase in certain cells induces membrane blebbing (Inbal et al., 2002), a characteristic of apoptosis. Accordingly, we found that when a human hepatoma cell line Hep3B was transfected with a GFP-fused DAP-kinase and maintained in the presence of drug for selection of the transfectants, the GFP expressing cells formed small colonies and eventually died with extensive membrane blebbing (Jang, C.-W. and R.-H. Chen, unpublished data). Noted that the Hep3B cells are p53-null (Jang et al., 2002) and should be insensitive to the p53-mediated, matrix-detachment-induced apoptosis. In addition to membrane blebbing, DAP-kinase also triggers autophagic (type II) cell death in cells lacking functional p53 (Inbal et al., 2002)

We show that DAP-kinase specifically blocks matrix survival pathways and does not significantly affect apoptosis in cells without receiving ECM signals. In contrast, it was previously reported that loss of DAP-kinase expression protects cells from matrix-detachment-induced apoptosis (Inbal et al., 1997). These seemingly contradictory results may be attributed, at least in part, to the different assay systems used. In the present study, the death-promoting effect of DAP-kinase was assayed in cells deprived with other signals that play a redundant role with ECM signals for cell survival. Under such conditions, DAP-kinase rapidly triggers cell death. However, in the previous study, the role of DAP-kinase in detachment-induced apoptosis was determined by soft agar colony forming assays (Inbal et al., 1997), which evaluate the long-term growth and survival of cells in semi-solid, growth factor-containing culture conditions. Thus, the finding that loss of DAP-kinase promotes colony formation in soft agar suggests that DAP-kinase expression is detrimental to the long-term growth and survival of cells.

DAP-kinase is the prototype of a family of several related kinases, i.e., the DAP-kinase family (Cohen and Kimchi, 2001). Although the homology of their sequences is restricted only to the kinase domains, each of these family members was reported to exert an apoptosis-promoting activity (Kawai et al., 1998; Sanjo et al., 1998; Inbal et al., 2000). The present unraveling of the pro-apoptotic mechanism of DAP-kinase would have an impact on our understanding of the functional basis of other family members. Interestingly, most of these proteins are capable of inducing cell rounding and detachment, when overexpressed in certain cell systems (Kawai et al., 1998; Sanjo et al., 1998; Inbal et al., 2000). We thus propose that suppression of cell adhesion and blockage of ECM survival signals might be a common mechanism of apoptosis induced by the DAP-kinase family proteins. Additional studies are required for testing this hypothesis.

Materials and Methods

Plasmids. The expression vectors pRK5-DAPK and pRK5-DAPK42A were described previously (Jang et al., 2002). The cDNA for DAPK Δ CaM (Cohen et al., 1997) was constructed by the Quick-Change site-directed mutagenesis kit (Stratagene) and then cloned to pRK5. The cDNAs for DAPK, DAPK42A and DAPK Δ CaM were cloned to pBabepuro3 to generate retroviral vectors. The expression vector for CD2-FAK (Chan et al., 1994) was a gift from Gena Whitney.

Cell culture, transformation and retroviral infection. 293T and NIH3T3 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum (FCS). BT474 cells were cultured in DMEM/F12 medium containing 5%

FCS. MCF10A cells were grown in DMEM/F12 medium supplemented with 5% horse serum, 5 $\mu\text{g/ml}$ of insulin, 0.01 $\mu\text{g/ml}$ of EGF, 0.1 $\mu\text{g/ml}$ of cholera toxin, and 0.5 $\mu\text{g/ml}$ of hydrocortisone. Transfection was performed by the calcium phosphate method. Generation of recombinant retrovirus and infection of NIH3T3 cells was carried out following procedures described previously (Tsai et al., 2000). For infection of MCF10A cells, the viral stock was supplemented with 2 $\mu\text{g/ml}$ of polybrene and incubated with cells for 18 h. Two days after the beginning of the infection, cells were selected in culture medium containing 1 $\mu\text{g/ml}$ of puromycin. Two days after selection, the uninfected cells were virtually eliminated and the surviving cells were used for various analyses.

Antibodies and reagents. The polyclonal antibody to DAP-kinase was described previously (Jang et al., 2002). The hybridoma for TS2/16 was from ATCC and the antibody was purified by affinity chromatography using HiTrap protein G column (Amersham Pharmacia Biotech). The mouse $\beta 1$ integrin activating antibody 9EG7, the antibody HUTS-21 for activated human $\beta 1$, antibodies to human $\alpha 5$ -integrin (Vc5), $\alpha 6$ -integrin (GoH3), and p53 were from Pharmigen. The human $\beta 1$ -integrin antibody AIIB2 was a gift from Caroline Damsky. The antibody B44 for the activated human $\beta 1$ integrin and the antibody MB1.2 for mouse $\beta 1$ integrin were from Chemicon. Antibodies to FAK, paxillin and phosphotyrosine were from Transduction Laboratories, whereas antibody to FAK phosphorylated at tyrosine 397 was from Biosource.

Adhesion assays. Assays of cell adhesion on plates coated with 10 $\text{ng}/\mu\text{l}$ of fibronectin, 30 $\text{ng}/\mu\text{l}$ of laminin, 2 mg/ml of poly-L-lysine or 1% BSA in phosphate-buffered saline (PBS) were performed essentially as described (Mould et

al., 1995). For experiments with blocking or activating antibodies, cells were pre-incubated with 5 $\mu\text{g}/\text{ml}$ of purified antibodies on ice for 30 min, and adhesion assays were performed in the presence of antibodies. For experiments with Mn^{2+} , 2 mM MnCl_2 was added into cell suspension before the adhesion assays. To estimate the reference value for 100 % attachment, cells were seeded on plates pre-coated with 20 $\text{ng}/\mu\text{l}$ of fibronectin or 50 $\text{ng}/\mu\text{l}$ of laminin and incubated for 3 to 4 h at 37°C. After incubation, cells were fixed immediately and approximately 90 to 100 % of input cells were recovered.

Flow cytometry analysis. To assess cell surface expression of specific integrins, cells were washed with PBS, and resuspended in blocking solution containing 5 % dissociation buffer (Invitrogen) and 2% goat serum in PBS. Cells were then incubated with anti-integrin antibody for 1 h at 4°C, washed with blocking solution and labeled with FITC-conjugated secondary antibody for 30 min at 4°C. Cells were then washed and analyzed on a Becton Dickison FACScan device. For detecting the activated $\beta 1$ integrin, cells transfected with various constructs were incubated in blocking solution with or without 2 mM MnCl_2 at 37°C for 30 min., followed by incubating with B44 or HUTS-21 antibody for 45 min at 4°C, and then labeled with secondary antibody as described above.

Immunoprecipitations. Cells were lysed in buffer containing 50 mM Tris (pH 7.5), 150 mM NaCl, 1% NP40, 1% sodium deoxycholate, 0.1% SDS, supplemented with 1 mM PMSF, 10 $\mu\text{g}/\text{ml}$ aprotinin, 10 $\mu\text{g}/\text{ml}$ leupeptin, 1 mM sodium vanadate, 4 mM sodium pyrophosphate, and 20 mM NaF. Lysates containing equal amount of proteins were subjected to immunoprecipitations as described previously (Tsai et al., 2000).

Apoptosis and caspase activity assays. Cells transfected with various DAP-kinase expression constructs or infected with recombinant retrovirus expressing DAP-kinase or its mutants were seeded on plates pre-coated with 25 ng/ μ l of fibronectin, 0.1% poly-L-lysine or 0.5 ml of 12 mg/ml poly-HEMA and cultured for various time periods in the presence or absence of integrin activating antibody. For all apoptosis-related assays, both detached and adherent cells were harvested and combined. DNA fragmentation and Caspase 3 activities were measured as described previously (Jang et al., 2002). For determining population of cells with sub-G1 DNA content, cells were stained with propidium iodide and then analyzed by flow cytometry. Annexin V staining was performed using the Annexin V-FITC Apoptosis Detection Kit (Oncogene).

Reporter assays. To analyze transcriptional activity of p53, the p53-TA-luc reporter (Clontech) and the pRK5- β gal plasmid were transfected into NIH3T3 cells in the presence or absence of various DAP-kinase expression constructs. Cells were detached at 36 h after transfection, replated onto dishes coated with fibronectin and cultured under serum-starved conditions for 12 h. For experiments using the 9EG7 antibody, cells were fed with serum-free medium at 36 h after transfection. Eight hours later, cells were detached and pre-incubated with 5 μ g/ml of 9EG7 in DMEM containing 1% BSA at 37°C for 30 min. Cells were then plated on fibronectin and cultured for another 4 h. In all cases, both attached and detached cells were harvested and combined. Luciferase and β -galactosidase activities were quantitated as described (Jang et al., 2002).

Supplemental materials available

Table S1 shows the levels of HUTS-21 epitope on cells transfected with various forms of DAP-kinase. Fig. S1 shows that DAP-kinase does not affect cell surface expression of integrins.

Acknowledgements

We thank Gena Whitney for CD2-FAK, Caroline Damsky for AIIB2 antibody, Lillian Shum for p53 reporter, Hong-Chen Chen for discussion and Randall Kramer for critical reading the manuscript. This work was supported by NSC Frontier Grant 90-2321-B-002-004 to R.-H. C, and Grants NSC 89-2134-B-002-349 and NTUH 91-N020 to C.-C. Yao.

Abbreviations

DAP-kinase, death associated protein kinase

DMEM, Dulbecco's modified Eagle's medium

ECM, extracellular matrix

EGF, epidermal growth factor

FCS, fetal calf serum

MLC, regulatory light chain of myosin II

PBS, phosphate buffered saline

Poly-HEMA, polyhydroxyethylmethacrylate

References

- Almeida, E.A., D. Ilic, Q. Han, C.R. Hauck, F. Jin, H. Kawakatsu, D.D. Schlaepfer and C.H. Damsky. 2000. Matrix survival signaling: from fibronectin via focal adhesion kinase to c-Jun NH(2)-terminal kinase. *J Cell Biol* 149: 741-54.
- Bodary, S.C. and J.W. McLean. 1990. The integrin beta 1 subunit associates with the vitronectin receptor alpha v subunit to form a novel vitronectin receptor in a human embryonic kidney cell line. *J Biol Chem* 265: 5938-41.
- Chan, P.Y., S.B. Kanner, G. Whitney and A. Aruffo. 1994. A transmembrane-anchored chimeric focal adhesion kinase is constitutively activated and phosphorylated at tyrosine residues identical to pp125FAK. *J Biol Chem* 269: 20567-74.
- Cohen, O., E. Feinstein and A. Kimchi. 1997. DAP-kinase is a Ca²⁺/calmodulin-dependent, cytoskeletal-associated protein kinase, with cell death-inducing functions that depend on its catalytic activity. *Embo J* 16: 998-1008.
- Cohen, O., B. Inbal, J.L. Kissil, T. Raveh, H. Berissi, T. Spivak-Kroizaman, E. Feinstein and A. Kimchi. 1999. DAP-kinase participates in TNF-alpha- and Fas-induced apoptosis and its function requires the death domain. *J Cell Biol* 146: 141-8.
- Cohen, O. and A. Kimchi. 2001. DAP-kinase: from functional gene cloning to establishment of its role in apoptosis and cancer. *Cell Death Differ* 8: 6-15.
- Deiss, L.P., E. Feinstein, H. Berissi, O. Cohen and A. Kimchi. 1995. Identification of a novel serine/threonine kinase and a novel 15-kD protein as potential mediators of the gamma interferon-induced cell death. *Genes Dev* 9: 15-30.
- Frisch, S.M. and E. Ruoslahti. 1997. Integrins and anoikis. *Curr Opin Cell Biol* 9: 701-6.

- Frisch, S.M. and R.A. Screaton. 2001. Anoikis mechanisms. *Curr Opin Cell Biol* 13: 555-62.
- Frisch, S.M., K. Vuori, E. Ruoslahti and P.Y. Chan-Hui. 1996. Control of adhesion-dependent cell survival by focal adhesion kinase. *J Cell Biol* 134: 793-9.
- Gailit, J. and E. Ruoslahti. 1988. Regulation of the fibronectin receptor affinity by divalent cations. *J Biol Chem* 263: 12927-32.
- Giancotti, F.G. and E. Ruoslahti. 1999. Integrin signaling. *Science* 285: 1028-32.
- Ginsberg, M.H., X. Du and E.F. Plow. 1992. Inside-out integrin signalling. *Curr Opin Cell Biol* 4: 766-71.
- Hughes, P.E. and M. Pfaff. 1998. Integrin affinity modulation. *Trends Cell Biol* 8: 359-64.
- Hungerford, J.E., M.T. Compton, M.L. Matter, B.G. Hoffstrom and C.A. Otey. 1996. Inhibition of pp125FAK in cultured fibroblasts results in apoptosis. *J Cell Biol* 135: 1383-90.
- Ilic, D., E.A. Almeida, D.D. Schlaepfer, P. Dazin, S. Aizawa and C.H. Damsky. 1998. Extracellular matrix survival signals transduced by focal adhesion kinase suppress p53-mediated apoptosis. *J Cell Biol* 143: 547-60.
- Ilic, D., C.H. Damsky and T. Yamamoto. 1997. Focal adhesion kinase: at the crossroads of signal transduction. *J Cell Sci* 110: 401-7.
- Inbal, B., O. Cohen, S. Polak-Charcon, J. Kopolovic, E. Vadai, L. Eisenbach and A. Kimchi. 1997. DAP kinase links the control of apoptosis to metastasis. *Nature* 390: 180-4.
- Inbal, B., G. Shani, O. Cohen, J.L. Kissil and A. Kimchi. 2000. Death-associated protein kinase-related protein 1, a novel serine/threonine kinase involved in apoptosis. *Mol Cell Biol* 20: 1044-54.
- Inbal, B., S. Bialik, I. Sabanay, G. Shani and A. Kimchi. 2002. DAP kinase and

DRP-1 mediated membrane blebbing and the formation of autophagic vesicles during programmed cell death. *J Cell Biol* 157: 455-68.

Jang, C.W., C.H. Chen, C.C. Chen, J.Y. Chen, Y.H. Su and R.H. Chen. 2002. TGF-beta induces apoptosis through Smad-mediated expression of DAP- kinase. *Nat Cell Biol* 4: 51-8.

Jost, M., T.M. Huggett, C. Kari and U. Rodeck. 2001. Matrix-independent survival of human keratinocytes through an EGF receptor/MAPK-kinase-dependent pathway. *Mol Biol Cell* 12: 1519-27.

Kawai, T., M. Matsumoto, K. Takeda, H. Sanjo and S. Akira. 1998. ZIP kinase, a novel serine/threonine kinase which mediates apoptosis. *Mol Cell Biol* 18: 1642-51.

Li, G., R. Fridman and H.R. Kim. 1999. Tissue inhibitor of metalloproteinase-1 inhibits apoptosis of human breast epithelial cells. *Cancer Res* 59: 6267-75.

Marti, A., Z. Luo, C. Cunningham, Y. Ohta, J. Hartwig, T.P. Stossel, J.M. Kyriakis and J. Avruch. 1997. Actin-binding protein-280 binds the stress-activated protein kinase (SAPK) activator SEK-1 and is required for tumor necrosis factor-alpha activation of SAPK in melanoma cells. *J Biol Chem* 272: 2620-8.

Mould, A.P., S.K. Akiyama and M.J. Humphries. 1995. Regulation of integrin alpha 5 beta 1-fibronectin interactions by divalent cations. Evidence for distinct classes of binding sites for Mn²⁺, Mg²⁺, and Ca²⁺. *J Biol Chem* 270: 26270-7.

Ni, H., A. Li, N. Simonsen and J.A. Wilkins. 1998. Integrin activation by dithiothreitol or Mn²⁺ induces a ligand-occupied conformation and exposure of a novel NH₂-terminal regulatory site on the beta1 integrin chain. *J Biol Chem* 273: 7981-7.

O'Toole, T.E., Y. Katagiri, R.J. Faull, K. Peter, R. Tamura, V. Quaranta, J.C. Loftus, S.J. Shattil and M.H. Ginsberg. 1994. Integrin cytoplasmic domains mediate inside-out signal transduction. *J Cell Biol* 124: 1047-59.

Otto, I.M., T. Raabe, U.E. Rennefahrt, P. Bork, U.R. Rapp and E. Kerkhoff. 2000. The p150-Spir protein provides a link between c-Jun N-terminal kinase function and actin reorganization. *Curr Biol* 10: 345-8.

Pelled, D., T. Raveh, C. Riebeling, M. Fridkin, H. Berissi, A.H. Futerman and A. Kimchi. 2001. Death-associated protein- (DAP) kinase plays a central role in ceramide- induced apoptosis in cultured hippocampal neurons. *J Biol Chem* 277: 1957-61.

Puthalakath, H., D.C. Huang, L.A. O'Reilly, S.M. King and A. Strasser. 1999. The proapoptotic activity of the Bcl-2 family member Bim is regulated by interaction with the dynein motor complex. *Mol Cell* 3: 287-96.

Puthalakath, H., A. Villunger, L.A. O'Reilly, J.G. Beaumont, L. Coultas, R.E. Cheney, D.C. Huang and A. Strasser. 2001. Bmf: a proapoptotic BH3-only protein regulated by interaction with the myosin V actin motor complex, activated by anoikis. *Science* 293: 1829-32.

Raveh, T., G. Droguett, M.S. Horwitz, R.A. DePinho and A. Kimchi. 2001. DAP kinase activates a p19ARF/p53-mediated apoptotic checkpoint to suppress oncogenic transformation. *Nat Cell Biol* 3: 1-7.

Raveh, T. and A. Kimchi. 2001. DAP kinase-a proapoptotic gene that functions as a tumor suppressor. *Exp Cell Res* 264: 185-92.

Sanjo, H., T. Kawai and S. Akira. 1998. DRAKs, novel serine/threonine kinases related to death-associated protein kinase that trigger apoptosis. *J Biol Chem* 273: 29066-71.

Schoenwaelder, S.M. and K. Burridge. 1999. Bidirectional signaling between the cytoskeleton and integrins. *Curr Opin Cell Biol* 11: 274-86.

Takagi, J., H.P. Erickson and T.A. Springer. 2001. C-terminal opening mimics 'inside-out' activation of integrin alpha5beta1. *Nat Struct Biol* 8: 412-6.

Tsai, Y.T., Y.H. Su, S.S. Fang, T.N. Huang, Y. Qiu, Y.S. Jou, H.M. Shih, H.J. Kung and R.H. Chen. 2000. Etk, a Btk family tyrosine kinase, mediates cellular transformation by linking Src to STAT3 activation. *Mol Cell Biol* 20: 2043-54.

Turner, C.E. 2000. Paxillin interactions. *J Cell Sci* 113 Pt 23: 4139-40.

Valentinis, B., K. Reiss and R. Baserga. 1998. Insulin-like growth factor-I-mediated survival from anoikis: role of cell aggregation and focal adhesion kinase. *J Cell Physiol* 176: 648-57.

van de Wiel-van Kemenade, E., Y. van Kooyk, A.J. de Boer, R.J. Huijbens, P. Weder, W. van de Kastele, C.J. Melief and C.G. Figdor. 1992. Adhesion of T and B lymphocytes to extracellular matrix and endothelial cells can be regulated through the beta subunit of VLA. *J Cell Biol* 117: 461-70.

van Kooyk, Y. and C.G. Figdor. 2000. Avidity regulation of integrins: the driving force in leukocyte adhesion. *Curr Opin Cell Biol* 12: 542-7.

Vinogradova, O., T. Haas, E.F. Plow and J. Qin. 2000. A structural basis for integrin activation by the cytoplasmic tail of the alpha IIb-subunit. *Proc Natl Acad Sci U S A* 97: 1450-5.

Xu, L.H., X. Yang, C.A. Bradham, D.A. Brenner, A.S. Baldwin, Jr., R.J. Craven and W.G. Cance. 2000. The focal adhesion kinase suppresses transformation-associated, anchorage-independent apoptosis in human breast cancer cells. Involvement of death receptor-related signaling pathways. *J Biol Chem* 275: 30597-604.

Yamamoto, M., T. Hioki, T. Ishii, S. Nakajima-Iijima and S. Uchino. 2002. DAP kinase activity is critical for C2-ceramide-induced apoptosis in PC12 cells. *Eur J Biochem* 269: 139-147.

Figure Legends

Fig. 1. DAP-kinase induces apoptosis-independent morphological changes in 293T cells. (A) 293T cells were transiently transfected with expression vectors for various DAP-kinase proteins or the vector backbone. The transfection efficiency was 90 %. Expression of the corresponding proteins was determined by Western blot analysis. (B) Morphologies of the 293T transfectants. Two days after transfection, cell morphology was examined by phase-contrast microscopy and the percentage of cells with round morphology is indicated as mean \pm S.D. Bar, 10 μ M. (C, D, and E) Apoptotic analyses of the 293T transfectants. Two days after transfection, cells as described in (A) were harvested and analyzed for their sub-G1 DNA content by flow cytometry analysis (C), DNA fragmentation by Cell-Death Detection ELISA (D), and binding to Annexin V (E). As a control, 293T cells were irradiated with UV at 0.04 J/cm² followed by 24 h of incubation, and then subjected to the same analyses.

Fig. 2. DAP-kinase inhibits integrin-mediated cell adhesion and ECM signal transduction. (A and B) 293T cells were transfected with various expression vectors as in Fig. 1. Immediately after transfection, cells were incubated with or without 50 μ M of zVAD-FMK throughout the culture and assay periods. Two days post-transfection, cells were replated on fibronectin (A), or laminin (B) and allowed to adhere for 40 or 25 min as indicated. The percentage of adhesion was calculated as described in Materials and Methods. Data from three separate experiments are presented as mean \pm S.D. (C) Cells as in (A) were assayed for their attachment to poly-L-lysine. Adhesion assays were performed as in (A). (D) Effect of DAP-kinase on tyrosine phosphorylation of FAK and paxillin. 293T transfectants as described in (A) were plated on fibronectin for 1 h. Cells in suspension and attached

were combined and then lysed. FAK or paxillin tyrosine phosphorylation was detected by immunoprecipitations with anti-FAK or anti-paxillin antibody as indicated, followed by Western blotting with antibody to phosphotyrosine.

Fig. 3. DAP-kinase suppresses integrins activity. (A and B) Activation of β 1-integrin abrogates the anti-adhesion effect of DAP-kinase. 293T cells transfected with DAP-kinase, Δ CaM or vector control were detached at 2 days after transfection. Cells were incubated with the TS2/16 anti- β 1 activating antibody (A), or $MnCl_2$ (B) and then assayed for their attachment to fibronectin. (C) Effect of DAP-kinase on the expression of the active conformation of β 1 integrin. 293T transfectants were incubated with antibody B44 specific to the activated β 1 integrin or buffer alone (control), followed by labeling with the FITC-conjugated secondary antibody. Alternatively, cells were pre-incubated with 2 mM $MnCl_2$ for 20 min at 37°C (Mn), and then subjected to the same immunostaining. Fluorescence intensity was determined by FACS analysis, and results of a representative experiment are shown. Mean fluorescence intensity (\pm S.D.) calculated from three independent experiments is listed on the right (*, $P < 0.05$; **, $P < 0.005$ compared with the vector control without receiving Mn).

Fig. 4. DAP-kinase inhibits adhesion of NIH3T3 cells by inside-out modulation of integrin activity. (A) NIH3T3 cells were infected with retroviruses carrying various DAP-kinase or the control virus and expression of the corresponding DAP-kinase proteins was determined at 4 days post-infection by Western blot analysis. (B) Cells as in (A) were assayed for their sub-G1 DNA content. As a control, NIH3T3 cells were irradiated with UV at 0.02 J/cm² followed by 24 h incubation. (C) Cells as described in (A) were assayed for their attachment to fibronectin. Cells were

allowed to adhere for 40 or 25 min and adhesion assays were performed as described in Fig. 2. (D) Mn^{2+} treatment abrogates the inhibitory role of DAP-kinase in cell adhesion. Cells as in (A) were pre-incubated with or without $MnCl_2$ before assaying for their attachment to fibronectin.

Fig. 5. DAP-kinase promotes apoptosis by blocking ECM survival signals. (A) DAP-kinase induces apoptosis in cells plated on matrix. NIH3T3 cells infected with various retroviruses at 4 days post-infection were plated on fibronectin (FN) or poly-L-lysine (PLL) and cultured in the absence of serum for 12 h. Cells were harvested and apoptotic cells were determined by Cell-Death Detection ELISA. Data from triplicate experiments are presented as mean \pm S.D. (B) NIH3T3 cells infected, plated and cultured as in (A) were subjected to caspase 3 activity assays. (C) NIH3T3 cells were transiently transfected with the combinations of expression vectors as indicated. The transfection efficiency was around 50%. Expression of various proteins was assayed by Western blot analyses with antibodies to DAP-kinase (upper panel), FAK (middle panel) and tubulin (bottom panel). (D) CD2-FAK prevents DAP-kinase-induced apoptosis. NIH3T3 transfectants as in (C) were plated on fibronectin and cultured for 12 h. under serum-starved conditions. Apoptotic cells were assayed as described in (A).

Fig. 6. Activation of integrin restores FAK tyrosine phosphorylation and protects cells from DAP-kinase-induced apoptosis. (A). NIH3T3 cells were transfected with various DAP-kinase expression constructs as indicated. Thirty-six hours after transfection, cells were cultured in serum-free medium for 8 h. The cells were then detached, incubated with or without 5 μ g/ml of 9EG7 in DMEM containing 1% BSA for 30 min at 37°C, and plated onto fibronectin for 1 h. Cells were then harvested,

lysed and subjected to Western blot analysis with antibodies to FAK (lower panel) and FAK phosphorylated at tyrosine 397 (upper panel). (B and C) NIH3T3 cells transfected, cultured as in (A) were incubated with the activating antibody 9EG7 (B) or the non-activating antibody MB1.2 (C). Cells were then plated on fibronectin and cultured in serum-starved conditions in the presence of the corresponding antibody for 7 h. Apoptotic cells were quantitated as described in Fig. 5.

Fig. 7. DAP-kinase induces an anoikis-like apoptosis in epithelial cells. (A) MCF10A cells were infected with various DAP-kinase expressing retroviruses or the control virus. Infected cells were selected by puromycin and then harvested at 4 days post-infection. Expression of various forms of DAP-kinase was detected by Western blot analysis. (B) Activation of $\beta 1$ integrin rescues the survival of DAP-kinase- or Δ CaM-expressing cells. MCF10A derivatives as in (A) were plated on fibronectin or poly-HEMA and cultured in EGF-deprived medium with or without TS2/16 for 24 h. Apoptotic cells were quantitated as described in Fig. 5.

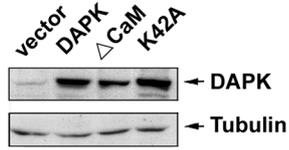
Fig. 8. DAP-kinase can no longer promote apoptosis in cells resistant to anoikis. (A) Expression of the various forms of DAP-kinase in BT474 breast carcinoma cells. BT474 cells were transiently transfected with expression vectors for various DAP-kinase proteins or a control vector. The transfection efficiency was around 60%. Two days after transfection, cells were lysed and subjected to Western blot analyses with antibodies as indicated. (B) BT474 transfectants as in (A) were plated on poly-HEMA or fibronectin and cultured for 24 h under serum-free conditions. Apoptotic cells were assayed as described in Fig. 5.

Fig. 9. Integrin or FAK activation blocks the induction of p53 by DAP-kinase. (A)

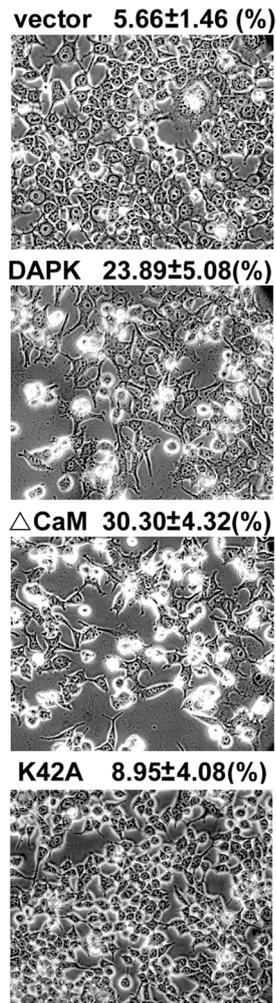
NIH3T3 cells were co-transfected with p53-TA-luc and pRK5- β gal, in the presence or absence of various DAP-kinase expression constructs and CD2-FAK. At 36 h post-transfection, cells were plated on fibronectin and cultured in serum-free medium for 12 h. and then lysed for measuring reporter activities. Values are mean \pm S.D. of triplicate assays. (B) NIH3T3 cells were co-transfected with p53-TA-luc and pRK5- β gal, in the presence or absence of various DAP-kinase expression constructs. Cells were then serum-starved, incubated with or without 9EG7, and plated on fibronectin as described in Materials and Methods. Reporter activities were determined as in (A). (C) NIH3T3 cells transfected as indicated were cultured for 36 h in growth medium and then plated on fibronectin-coated dishes. Cells were cultured under serum-free conditions for 6 h and subjected to Western blot analysis with antibodies as indicated. (D) A model illustrating the mechanism by which DAP-kinase induces apoptosis (see text).

Fig. 1

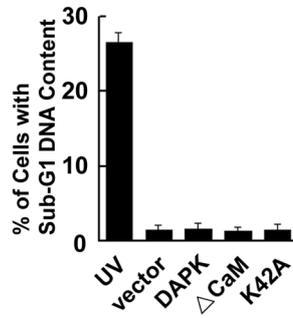
A.



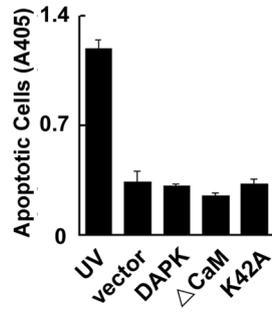
B.



C.



D.



E.

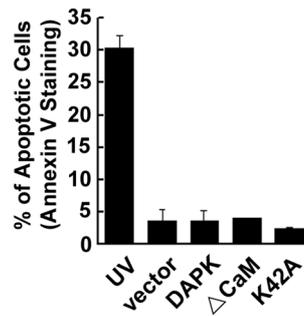


Fig. 2

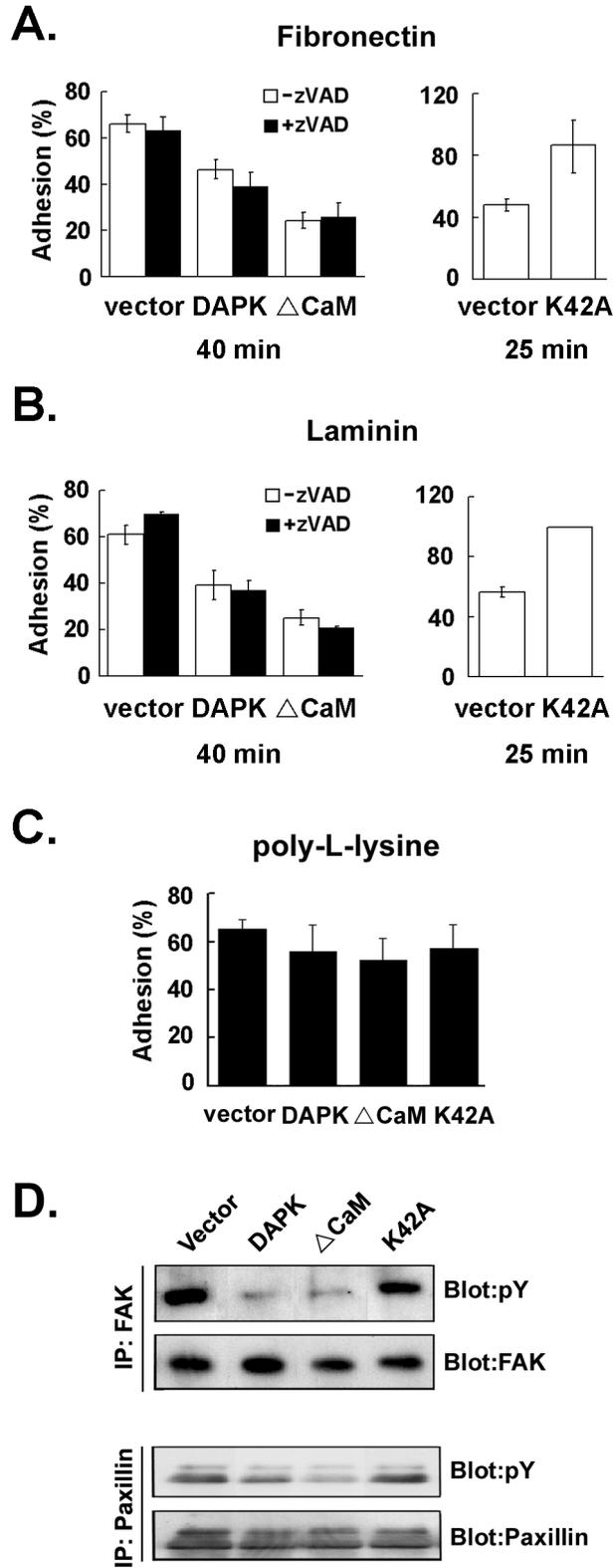


Fig. 3

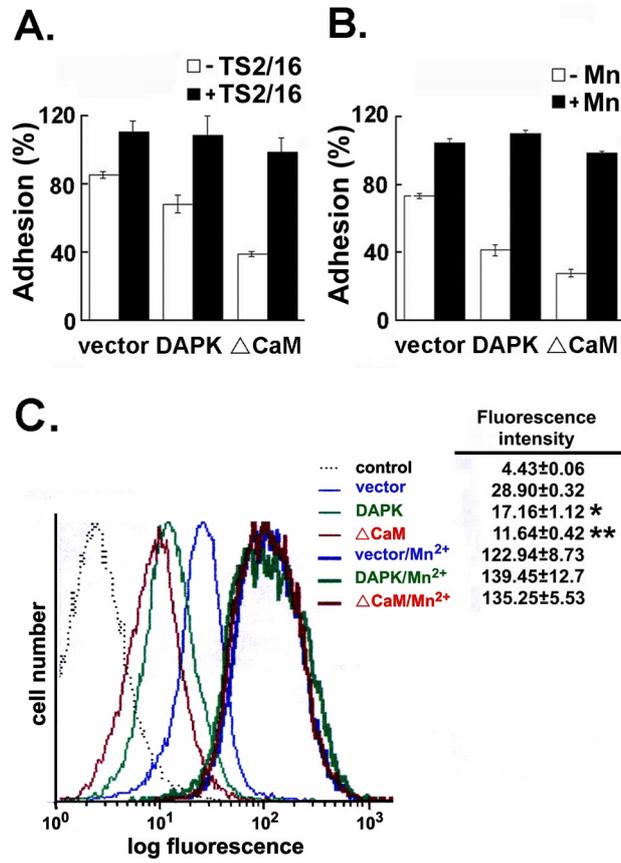
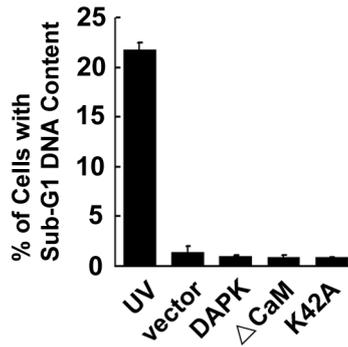


Fig. 4

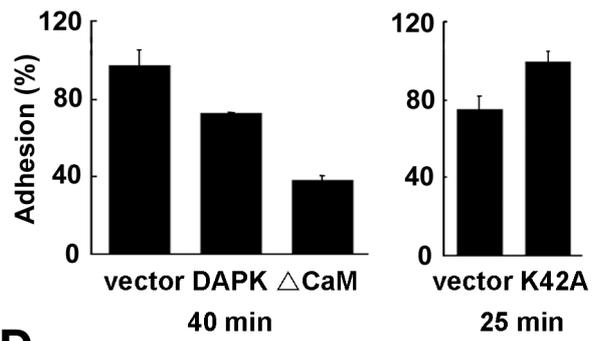
A.



B.



C.



D.

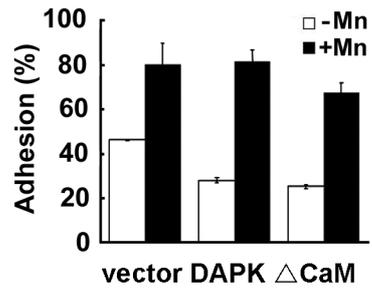
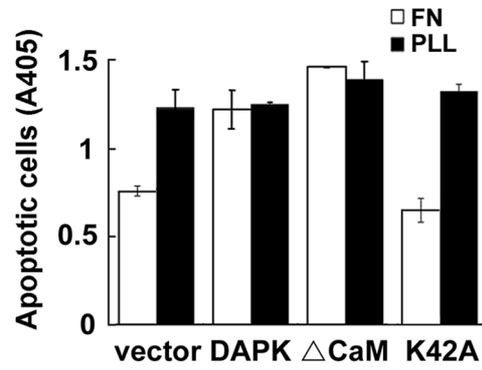
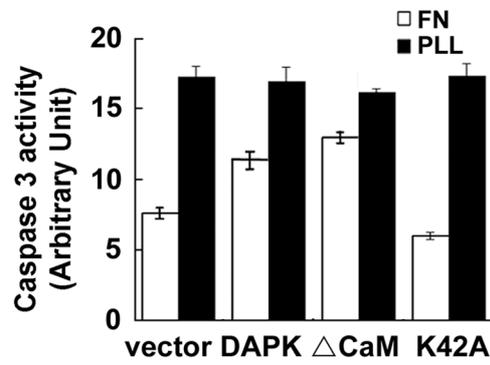


Fig. 5

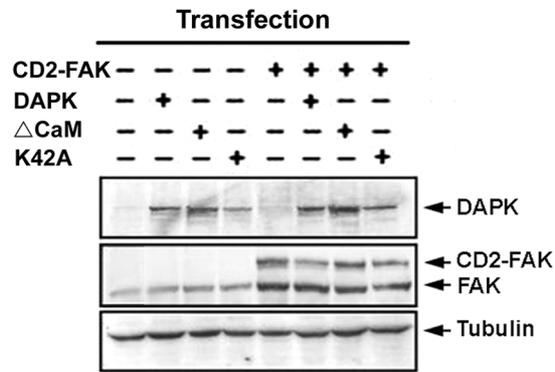
A.



B.



C.



D.

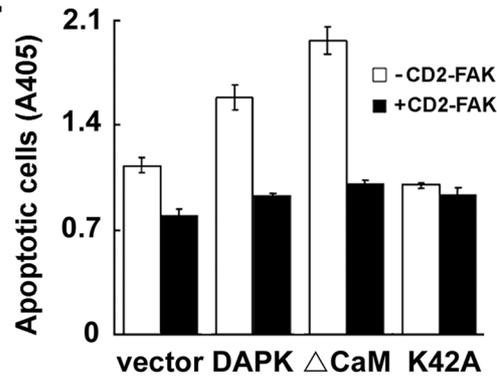
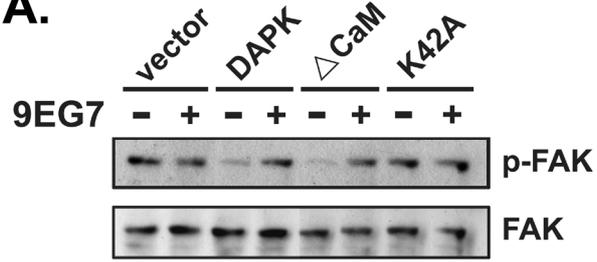
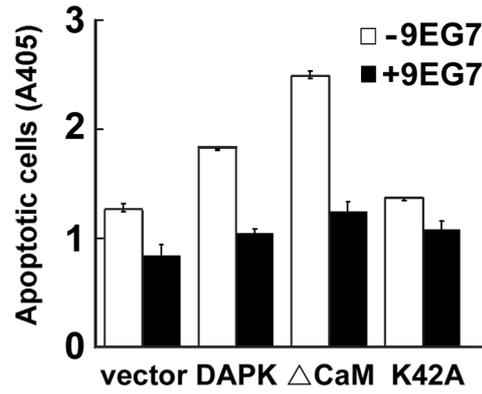


Fig.6

A.



B.



C.

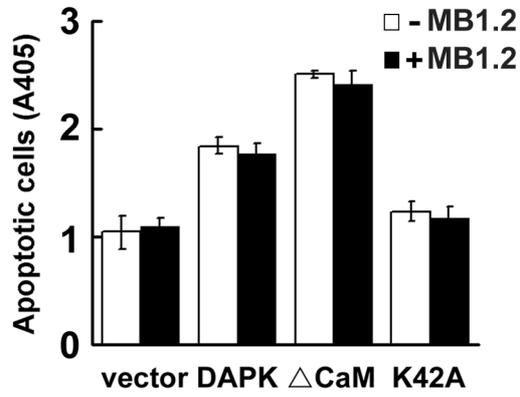


Fig. 7

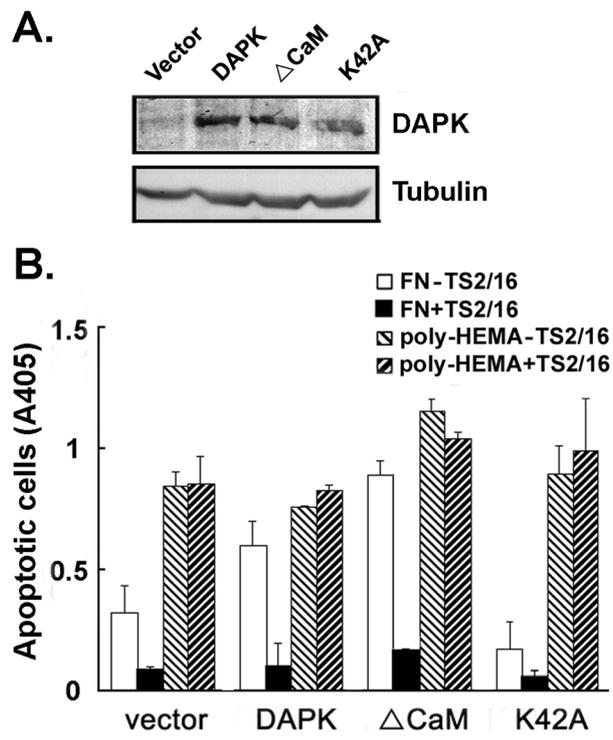


Fig. 8

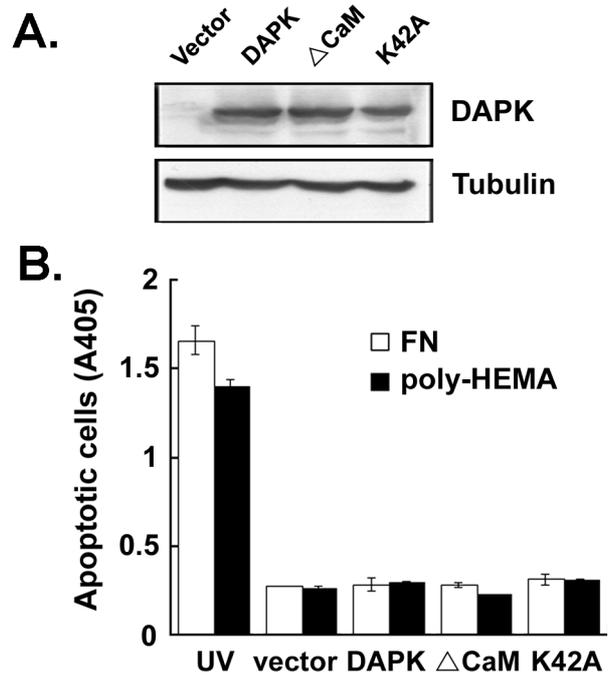
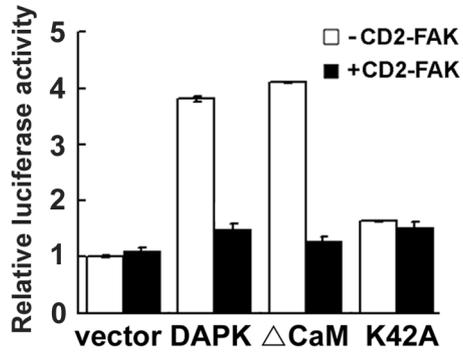
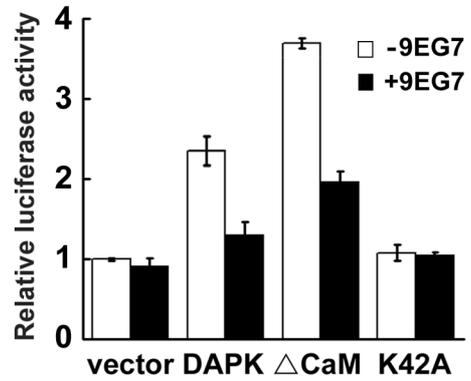


Fig. 9

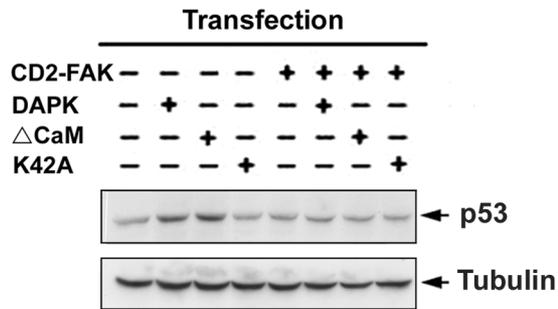
A.



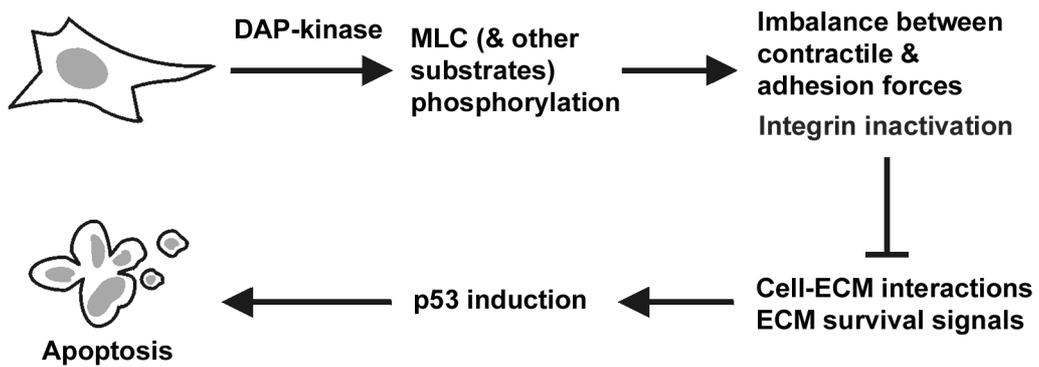
B.



C.



D.



Supplementary Table

Table S1: Expression of HUTS-21 epitope on 293T cells transfected with various constructs and incubated with or without Mn²⁺

Antibody	Transfection	Mn²⁺	Fluorescence intensity
control	vector	-	1.74±0.20
		-	10.63±0.87
		+	25.98±2.19
HUTS-21	DAPK	-	5.00±0.18*
		+	27.13±3.64
	△CaM	-	4.33±0.08**
		+	31.76±0.46

*, P<0.05; **, P<0.005 compared with vector control without Mn²⁺

Supplementary Figure

Fig. S1

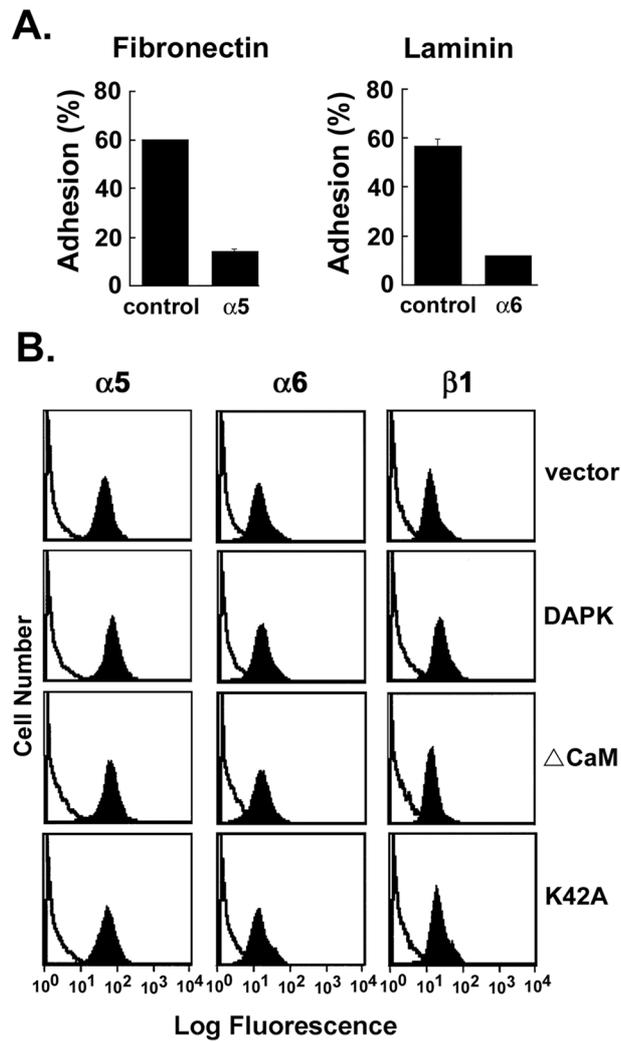


Fig. S1. DAP-kinase does not affect cell surface expression of the fibronectin and laminin receptors. (A) Effect of the anti- $\alpha 5$ and anti- $\alpha 6$ integrin blocking antibodies on cell adhesion to fibronectin and laminin, respectively. 293T cells were detached and incubated with the blocking antibody or a control antibody for 30 min and then subjected to adhesion assays. (B) Effect of DAP-kinase on the surface expression of $\alpha 5$, $\alpha 6$ and $\beta 1$ integrins. 293T cells transiently transfected with various forms of DAP-kinase were harvested at 2 days post-transfection. Cells were immunostained with antibody to the corresponding integrin and subjected to FACS analysis. White areas indicate the background fluorescence, whereas black areas represent cell surface expression of the corresponding integrin.