Vinculin modulation of paxillin–FAK interactions regulates ERK to control survival and motility

M. Cecilia Subauste, Olivier Pertz, Eileen D. Adamson, Christopher E. Turner, Sachiko Junger, and Klaus M. Hahn

1Department of Cell Biology, The Scripps Research Institute, La Jolla, CA 92037
2The Burnham Institute, La Jolla Cancer Research Center, La Jolla, CA 92037
3Department of Cell and Developmental Biology, State University of New York, Upstate Medical University, Syracuse, NY 13210

Cells lacking vinculin are highly metastatic and motile. The reasons for this finding have remained unclear. Both enhanced survival and motility are critical to metastasis. Here, we show that vinculin null (vin−/−) cells and cells expressing a vinculin Y822F mutant have increased survival due to up-regulated activity of extracellular signal-regulated kinase (ERK). This increase is shown to result from vinculin’s modulation of paxillin–FAK interactions. A vinculin fragment (amino acids 811–1066) containing the paxillin binding site restored apoptosis and suppressed ERK activity in vin−/− cells. Both vinY822F and vin−/− cells exhibit increased interaction between paxillin and focal adhesion kinase (FAK) and increased paxillin and FAK phosphorylation. Transfection with paxillin Y31FY118F dominant-negative mutant in these cells inhibits ERK activation and restores apoptosis. The enhanced motility of vin−/− and vinY822F cells is also shown to be due to a similar mechanism. Thus, vinculin regulates survival and motility via ERK by controlling the accessibility of paxillin for FAK interaction.

Introduction

Adhesion complexes (i.e., focal adhesions and focal complexes) and cell–cell contacts are specialized structures that harbor a large number of cytoskeletal proteins and one of the highest concentrations of signaling molecules in cells (Ruoslahti and Obrink, 1996). These multiprotein complexes are capable of capturing and integrating many signals from the extracellular as well as intracellular environments (Rosales and Juliano, 1995). Such signals are indispensable for the coordinated control of fundamental cellular processes including differentiation, cell cycle control, apoptosis, and motility (Ruoslahti and Obrink, 1996; Huang and Ingber, 2000). Coordinating these processes is critical for the maintenance of tissue homeostasis (Huang and Ingber, 2000). In fact, disruption in the regulation of motility and survival is a major step in the pathogenesis of cancer (Parise et al., 2000).

Disregulation in the control of multiple cellular processes is seen in cells with altered expression and/or activation of molecules in focal adhesions and cell–cell contacts. Such molecules include paxillin, FAK (Rodina et al., 1999), and vinculin (Raz and Geiger, 1982; Lifschitz-Mercer et al., 1997). Cancer cells devoid of vinculin (Rudiger, 1998) are highly metastatic (Raz and Geiger, 1982; Lifschitz-Mercer et al., 1997) and motile (Coll et al., 1995; Xu et al., 1998a,b). Transfecting vinculin back into vinculin null cells remarkably represses their metastatic capacity (Rodriguez-Fernandez et al., 1992) as well as their enhanced motility (Coll et al., 1995; Xu et al., 1998b). The mechanisms through which vinculin controls motility and represses metastatic capacity remain unclear.

Highly metastatic cells, such as those lacking vinculin (Rodriguez-Fernandez et al., 1992), are usually very motile (Parise et al., 2000). Enhanced cell motility has been associated with increased survival (Frisch and Francis, 1994). Recent evidence has shown the existence of signaling cascades that coordinate these two processes (Cho and Klemke, 2000). Thus, vinculin could affect signaling through multiple proteins that mediate both survival and motility and are found in focal adhesions and/or cell–cell contacts. These proteins include paxillin, FAK (Rodina et al., 1999), and vinculin (Raz and Geiger, 1982; Lifschitz-Mercer et al., 1997). Cancer cells devoid of vinculin (Rudiger, 1998) are highly metastatic (Raz and Geiger, 1982; Lifschitz-Mercer et al., 1997) and motile (Coll et al., 1995; Xu et al., 1998a,b). Transfecting vinculin back into vinculin null cells remarkably represses their metastatic capacity (Rodriguez-Fernandez et al., 1992) as well as their enhanced motility (Coll et al., 1995; Xu et al., 1998b). The mechanisms through which vinculin controls motility and represses metastatic capacity remain unclear.

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proteins include p130 Crk-associated substrate (Cas) and Crk-II adaptor proteins (Nakamoto et al., 1997; Cho and Klemke, 2000), extracellular signal–regulated kinase (ERK)1/2 (Klemke et al., 1997; Fincham et al., 2000), phosphatidylinositol-3 kinase (PI-3K; Watton and Downward, 1999; Sasaki et al., 2000), and FAK (Hauck et al., 2002). FAK is a particularly important signaling molecule, as it controls multiple fundamental cellular processes by regulating the assembly of different multiprotein complexes (Tachibana et al., 1995; Schwartz, 2001). Interaction between FAK and the adaptor protein paxillin is critical for the activation of signaling cascades involved in the control of cell survival and motility (Turner, 2000). FAK binds to paxillin through its COOH-terminal focal adhesion targeting (FAT) region (Hayashi et al., 2002). This FAT region is also contained within FAK-related nonkinase (FRNK), a naturally occurring fragment of FAK (Richardson and Parsons, 1996). Transfection with FRNK blocks the interaction of endogenous FAK with paxillin at focal adhesions, decreasing survival and motility (Richardson and Parsons, 1996; Hauck et al., 2000, 2002). Mutagenesis studies have shown that this interaction requires the sequence of the FAT region in FRNK (Hauck et al., 2002). Interestingly, the structure of the FAT region resembles that of vinculin’s COOH-terminal “tail domain” (Bakolitsa et al., 1999; Hayashi et al., 2002). Furthermore, the FAT region and vinculin tail bind to overlapping repeats of leucine-rich sequences in paxillin named LD motifs (Turner et al., 1999; Turner, 2000; Tumbarello et al., 2002), suggesting that vinculin too may inhibit important FAK interactions. These observations led us to examine whether or not the highly metastatic and motile vinculin null cells have enhanced survival, and whether or not enhanced survival and the enhanced motility previously reported were due to disregulation of paxillin–FAK interactions. In this paper, we describe a novel pathway whereby vinculin controls cell survival and motility through regulation of paxillin–FAK interactions to alter ERK1/2 activation.

**Results**

**Vinculin null cells are resistant to apoptosis**

To test whether or not vinculin is important in apoptosis, we first examined apoptosis induced by serum withdrawal. Apoptosis was compared in wild-type mouse F9 embryonal
Vinculin regulation of apoptosis requires its hinge-tail fragment

Vinculin has multiple binding sites for interaction with components of cell contact sites (Rudiger, 1998). It consists of a large amino-terminal head and a rodlike tail connected by a flexible hinge region (840–857 aa; Rudiger, 1998). To address which regions of vinculin are important for control of apoptosis, cell death was examined in F9 cells (Fig. 1, E and F). The effect of camptothecin on caspase-3 activity was similar in wild-type and F9 vin⁻⁻ cells. The rate of camptothecin-induced apoptosis was also determined using morphological criteria (blebbing and cell fragmentation; Subauste et al., 2000). As shown in Fig. 1 G, this rate was considerably reduced in F9 vin⁻⁻ cells compared with WT F9 or F9 vin⁻⁻ rescue cells. We also found that vinculin null embryonal fibroblasts are resistant to various apoptotic stimuli (unpublished data). Together, these findings show that vinculin regulates apoptosis induced by multiple different stimuli.

carcinoma cells (WT F9), their vinculin null counterparts (F9 vin⁻⁻), and F9 vin⁻⁻ rescue cells (stable cell lines in which vinculin had been reintroduced into the F9 vin⁻⁻ cells). Caspase-3 activation was assessed as an indicator of irreversible commitment to apoptosis (Nicholson et al., 1995). WT F9 cells exhibited caspase-3 activation reflected by the presence of cleaved caspase-3 after 10 h of serum withdrawal (Fig. 1 A). In contrast, F9 vin⁻⁻ cells showed a remarkable inhibition of caspase-3 activation, and caspase-3 activity was restored in the F9 vin⁻⁻ rescue cells (Fig. 1 A). N-acetyl-Asp-Glu-Val-Asp-7-amino-4-methylcoumarin (DEVD-AMC) cleavage quantifies the activity of caspase-3 as well as caspase-7 (Nicholson et al., 1995; He et al., 1998). Activation of caspase-7 is also an indicator of irreversible commitment to apoptosis (He et al., 1998; Riedl et al., 2001). DEVD-AMC cleavage was inhibited by 70% in F9 vin⁻⁻ cells compared with WT F9 cells after 10 h of serum withdrawal and was fully restored to wild-type levels in the F9 vin⁻⁻ rescue cells (Fig. 1 B).

The role of vinculin in anoikis, apoptosis induced by detachment from the cellular substrate (Frisch and Francis, 1994), was also examined. Caspase-3 activation and DEVD-AMC cleavage were remarkably inhibited in F9 vin⁻⁻ cells compared with WT F9 cells (Fig. 1, C and D). Here too, both caspase-3 activation and DEVD-AMC cleavage were restored to wild-type levels in F9 vin⁻⁻ rescue cells.

Finally, caspase-3 activity and DEVD-AMC cleavage induced by 2-h incubation with camptothecin (Hertzberg et al., 1989), a cytotoxic drug widely used in cancer therapy, was also inhibited in F9 vin⁻⁻ cells compared with WT F9 cells (Fig. 1, E and F). The effect of camptothecin on caspase-3 activity was similar in wild-type and F9 vin⁻⁻ rescue cells. The rate of camptothecin-induced apoptosis was also determined using morphological criteria (blebbing and cell fragmentation; Subauste et al., 2000). As shown in Fig. 1 G, this rate was considerably reduced in F9 vin⁻⁻ cells compared with WT F9 or F9 vin⁻⁻ rescue cells. We also found that vinculin null embryonal fibroblasts are resistant to various apoptotic stimuli (unpublished data). Together, these findings show that vinculin regulates apoptosis induced by multiple different stimuli.
Vinculin affects apoptosis by modulating ERK1/2 activity

Our results showed that vinculin controls apoptosis. Because vinculin also affects cellular motility (Coll et al., 1995), we searched for a signaling pathway through which vinculin might regulate both processes. ERK1/2 regulates motility and apoptosis and localizes in focal adhesions (Klemke et al., 1997; Fincham et al., 2000; Howe et al., 2002). Furthermore, ERK1/2 activity is elevated in cells that are highly metastatic and resist anoikis (Howe et al., 2002). Therefore, we assessed whether or not vinculin affected apoptosis by regulating ERK1/2 activity, concentrating on anoikis because of its significance in cancer biology (Frisch and Francis 1994; Howe et al., 2002).

As shown in Fig. 5 A, treatment of F9 vin−/− cells with ERK antisense oligonucleotides restored anoikis-induced caspase-3 activation. Antisense treatment was shown to reduce ERK protein levels, scrambled antisense oligonucleotide controls had no effect, and blotting with anti-Erk Abs demonstrated that each cell line had similar Erk concentrations before antisense treatment (Fig. 5, A and B).

ERK1/2 is activated upon phosphorylation of its regulatory threonine and tyrosine residues by MAPK kinase (MEK; Ahn et al., 1991). The MEK inhibitor PD98059 restored caspase-3 activation to wild-type levels in F9 vin−/− and F9 vin−/− Y822F cells (Fig. 5 C), and this increase was largely eliminated by MEK inhibition (Fig. 5 D). This finding indicated that anoikis resistance was mediated by activation of the MEK–ERK pathway.

We also examined ERK1/2 activity in the apoptosis-resistant F9 vin−/− and F9 vin−/−–Y822F cells, and the apoptosis-sensitive F9 vin−/−–hinge-tail and WT F9 cells. Lysates from these cells were probed with Abs specific for the active, phosphorylated forms of ERK1/2 at different times after cellular detachment (Fig. 5 E). In both F9 vin−/− and F9 vin−/−–Y822F cells, detachment led to ERK1/2 activation substantially above that in WT F9 cells. In contrast, F9 vin−/−–hinge-tail cells showed ERK1/2 activation inhibited to levels seen in the wild-type cells. In F9 vin−/− cells stably transfected with vinculin, ERK1/2 activity after detachment was the same as in WT F9 cells (Fig. 5 F).

ERK1/2 was recently shown to down-regulate apoptosis by inhibiting activation of caspase-9 (Allan et al., 2003). Caspase-9 is an initiator protease that activates caspase-3 and other downstream caspases to trigger apoptosis, and it has been found to be particularly important in anoikis (Gross-
munn et al., 2001). Therefore, we examined caspase-9 activation after cellular detachment. WT F9, rescue, and F9 vin⁻/⁻ hinge-tail cells showed cleaved caspase-9 30 min after cellular detachment (Fig. 5 G). In contrast, no cleaved caspase-9 was observed in F9 vin⁻/⁻ and F9 vin⁻/⁻/Y822F cells (Fig. 5 G). Together, these data indicate that vinculin affects apoptosis by regulating ERK1/2 activity through a mechanism requiring the hinge-tail region.

PI-3K is another signaling molecule that localizes into focal adhesions and affects both motility and apoptosis (Wat-}

Vinculin regulates ERK1/2 activity through modulation of paxillin–FAK interaction

We found that vinculin regulates apoptosis via the ERK1/2 pathway, and that this regulation requires the vinculin hinge-tail region. The hinge-tail fragment is targeted to focal adhesions, where it colocalizes with the adapter protein paxillin (Xu et al., 1998b). Furthermore, the vinculin tail domain and the FAT region of FAK bind to overlapping paxillin LD motifs (Turner et al., 1999; Turner, 2000; Tumbarello et al., 2002). FAK is an adapter protein whose kinase activity is involved in the activation of ERK1/2, cell survival, and motility (Schwartz, 2001; Hauck et al., 2002).

Therefore, we tested if the paxillin–FAK interaction is affected by vinculin.

To gauge the level of paxillin–FAK binding in different cell lysates, either paxillin or FAK were immunoprecipitated, and Western blotting was used to probe for the presence of the binding partner (Fig. 6, A and B). Paxillin–FAK interaction was enhanced relative to WT F9 cells in vin⁻/⁻ and vin⁻/⁻/Y822F cells. In contrast, paxillin–FAK binding is at wild-type levels in F9 vin⁻/⁻/hinge-tail and rescue cells (Fig. 6, A and B).

FAK interaction with paxillin, coupled with phosphorylation of both proteins, creates docking sites for signaling molecules involved in cell motility and survival (Turner, 2000; Schwartz, 2001; Hauck et al., 2002). Phosphorylation of FAK at tyrosine 397 (Y397) is critical for the induction of downstream effects (Hauck et al., 2000, 2002; Schwartz, 2001), including phosphorylation of paxillin at Tyrosine 118 (Y118; Turner, 2000). We assessed whether or not the enhanced paxillin–FAK interaction in F9 vin⁻/⁻ and F9 vin⁻/⁻/Y822F cells correlated with increased phosphorylation of FAK-Y 397 and paxillin-Y118. The data in Fig. 7 (A and B) show that these cells do in fact have constitutively increased FAK-Y397 and paxillin-Y118 phosphorylation. After cellular detachment, phosphorylation at these sites rapidly decreased (Fig. 7, A and B). In contrast, phosphorylation at both sites is at wild-type levels in F9 vin⁻/⁻/hinge-tail and rescue cells (Fig. 7, A and B), both constitutively and during anoikis.

Finally, we examined cells overexpressing paxillin mutated at the residues phosphorylated upon FAK interaction (paxil-lin Y31FY118F; Turner, 2000). This mutant disrupts important signaling events activated by the paxillin–FAK interaction (Petit et al., 2000). Overexpression of paxillin Y31FY118F restored anoikis in F9 vin⁻/⁻ and F9 vin⁻/⁻/Y822F cells to wild-type levels (Fig. 8 A). Importantly, this mutant also inhibited the enhanced ERK1/2 activity during cellular detachment (Fig. 8 B). Together, these data indicate
that vinculin controls cell survival via ERK by regulating paxillin–FAK interaction.

**Vinculin regulates motility via paxillin and ERK1/2 activation**

A previously published paper showed that the enhanced motility of F9 vin<sup>−/−</sup> cells was inhibited by expression of the vinculin hinge-tail fragment (Xu et al., 1998b). Because cellular motility and survival can be regulated by the same signaling pathways (Cho and Klemke, 2000), we assessed whether or not vinculin controls motility as well as apoptosis via ERK1/2 activation, and whether or not this is also dependent on paxillin–FAK interaction. In a Boyden chamber assay, WT F9 cells migrated significantly less than either F9 vin<sup>−/−</sup> cells or F9 vin<sup>−/−</sup>/Y822F cells. Incubation with the MEK inhibitor PD 98059 led to a 50–55% inhibition of F9 vin<sup>−/−</sup> migration rates and decreased the enhanced migration of F9 vin<sup>−/−</sup>/Y822F cells to wild-type levels (Fig. 9 A). The drug had little effect on the migration of WT F9 cells.

Vinculin has a well characterized function as a cytoskeletal protein within adhesion complexes (Rudiger, 1998), but much of its role in signaling remains obscure. Tissue homeo-
Vinculin, paxillin, and FAK regulate apoptosis and motility.

Apoptosis resistance in vin−/− and vin−/−Y822F cells was ERK dependent. This conclusion is based on the fact that apoptosis of both F9 vin−/− and F9 vin−/−Y822F cells could be restored to wild-type levels using ERK antisense oligonucleotides or the MEK inhibitor PD98059. Moreover, recovery of apoptosis in F9 vin−/− hinge-tail cells was associated with a reduction of ERK1/2 activity to wild-type levels, and apoptosis-resistant F9 vin−/− and F9 vin−/−Y822F cells showed increased ERK1/2 activation after cellular detachment. Increased ERK1/2 phosphorylation after cellular de-

Figure 6. F9 vin−/− and F9 vin−/−Y822F cells have an increased paxillin–FAK interaction. (A) Cell extracts from WT F9, F9 vin−/−, rescue, F9 vin−/−Y822F, and F9 vin−/− hinge-tail cells were immunoprecipitated with a mouse anti-paxillin Ab, followed by immunoblotting with Ab against FAK. (B) Cell extracts from WT F9, F9 vin−/−, rescue, F9 vin−/−Y822F, and F9 vin−/− hinge-tail cells were immunoprecipitated with a mouse anti-FAK Ab, followed by immunoblotting with Ab against paxillin. The results shown are representative examples from three independent experiments.

Figure 7. F9 vin−/− and F9 vin−/−Y822F cells have increased FAK-Y397 and paxillin-Y118 phosphorylation. (A) A representative immunoblot showing FAK phosphorylated at Y397 in WT F9, F9 vin−/−, rescue, F9 vin−/−Y822F, and F9 vin−/− hinge-tail cells after disruption of cell–matrix interactions. (B) An immunoblot showing paxillin phosphorylated at Y118 in WT F9, F9 vin−/−, rescue, F9 vin−/−Y822F, and F9 vin−/− hinge-tail cells after disruption of cell–matrix interactions. The results shown are representative of three independent experiments.
Attachment has been shown before in apoptosis-resistant lung adenocarcinoma cells (Wei et al., 2001). However, in contrast to our work, apoptosis resistance in these tumoral cells was ERK1/2-independent (Wei et al., 2001). Differences in the experimental design may account for this discrepancy. Specifically, in the present work, we inhibited ERK activity with PD98059 and ERK antisense oligonucleotides before cellular detachment, whereas in the previous work (Wei et al., 2001), ERK1/2 activity was inhibited with PD98059 after cellular detachment. This might be an important issue because basal activity levels might determine if a cell survives or dies upon apoptosis induction (Howe et al., 2002). However, consistent with our work, there was no difference in the constitutive ERK1/2 phosphorylation levels among apoptosis-resistant and apoptosis-sensitive cells (Wei et al., 2001). It is possible that only a small fraction of the total cellular ERK is involved in this regulation. ERK1/2 is known to be compartmentalized in various subcellular locations (Reffas and Schlegel, 2000; Pouyssegur et al., 2002; Smith et al., 2004).

Combining our own data with previous studies suggested that vinculin regulates motility and survival by affecting paxillin–FAK interaction. Expression of a vinculin hinge-tail fragment in F9 vin\(^{-/-}\)/H11002 cells reduced FAK-Y397 phosphorylation, cell motility (Xu et al., 1998b), and ERK1/2-dependent cell survival. These effects are very similar to those produced by transfection with FAK fragments containing the sequence of the FAT region, which also inhibits FAK-Y397 phosphorylation and downstream effects, including cell motility and survival (Richardson and Parsons, 1996; Hauck et al., 2000). Interestingly, the vinculin tail domain and the FAT region bind to overlapping paxillin LD motifs (Turner et al., 1999; Turner, 2000; Tumbarello et al., 2002). The FAT region contains two distinct paxillin binding subdo-
main (Turner et al., 1999; Tumbarello et al., 2002), which bind to paxillin LD2 and LD4 motifs (Hayashi et al., 2002). The tail domain of vinculin is capable of binding paxillin LD1, LD2, or LD4 motifs (Turner et al., 1999; Tumbarello et al., 2002). Vinculin oligomers (Rudiger, 1998) could bind simultaneously to paxillin LD2 and LD4 motifs from the same paxillin molecules, thus interfering with FAK binding. We showed that the highly motile and apoptosis-resistant F9 vin\(^{+/−}\) cells did in fact exhibit enhanced paxillin–FAK interaction, and this was inhibited to wild-type levels upon expression of the vinculin hinge-tail region.

Paxillin–FAK interaction leads to paxillin phosphorylation at Y31 and Y118 (Turner, 2000). We addressed whether or not paxillin phosphorylation at these sites is mediating the increased motility and survival in F9 vin\(^{+/−}\) and F9 vin\(^{−/−}\)/Y822F cells. Expression of a paxillin Y31FY118F mutant in these cells led to a substantial reduction in motility and survival mediated by ERK1/2. The link between paxillin–FAK binding and ERK activation may be through Crk, an important adaptor molecule involved in ERK1/2 activation (York et al., 1998). A previous paper demonstrated that paxillin Y31 and Y118 phosphorylation creates docking sites for Crk (Petit et al., 2000).

We also found that vinculin with a Y822F point mutation was unable to restore normal apoptosis induction. These profound effects from a point mutation strongly suggest that enhanced survival is not due to nonspecific cytoskeletal alterations, but rather from specific effects in survival signaling pathways (Judson et al., 1999; Rodina et al., 1999; Sattler et al., 2000; Turner, 2000; Hauck et al., 2002). Previous works indicate that the observed effects were not due simply to an inability of the mutant to localize in focal adhesions (Goldmann et al., 1998). Y822 is very close to the flexible hinge region of vinculin, so the mutation could affect the equilibrium between the open and closed form of the protein. When vinculin is in its open, active conformation it localizes into focal adhesions (Rudiger, 1998). Upon binding to phosphatidylinositol(4,5)-biphosphate (Steimle et al., 1999) at the cell membrane, vinculin unfolds, exposing its talin binding sites (Johnson and Craig, 1994). The talin binding sites are also critical for vinculin’s focal adhesion localization in cells (Goldmann et al., 1998). In contrast, paxillin interactions are not sufficient to cause vinculin localization to focal adhesions in vivo (Goldmann et al., 1998), despite the fact that paxillin–vinculin interaction in vitro occurs for both open and closed conformations (Gilmore and Burridge, 1996). The Y822F mutation could decrease the stability of vinculin’s open conformation, affecting an early stage of this complex process, before paxillin interaction.

Vinculin can be tyrosine phosphorylated (Sefton et al., 1981), and Y822 is a putative site for phosphorylation (Jockusch and Rudiger, 1996), but a physiological role for Y822 phosphorylation has not been demonstrated (Jockusch and Rudiger, 1996). Phosphorylation may be important for maintaining the open conformation, as for the cytoskeletal protein ezrin (Berryman et al., 1995). Thus, vinculin Y822F could be more prone than wild-type vinculin to adopt a closed conformation after its incorporation into focal adhesions. Elucidating the precise mechanism through which vinculin Y822F affects interactions with paxillin may be a valuable route to further understanding of cell motility and survival.

Understanding how cell motility and survival are regulated is an important fundamental goal in cell biology. Motility and survival are controlled by multimolecular signaling complexes created upon dynamic interaction between paxillin and FAK. Our results identify vinculin as a key molecule regulating these cellular processes and provides evidence of an important signaling mechanism tying vinculin to carcinogenesis.

### Materials and methods

#### Cell culture

The following cell lines were used: WT F9 mouse embryonal carcinoma cells and vinculin-null F9 cells (F9 vin\(^{+/−}\) clones y229 and y227; Coll et al., 1995); F9 vin\(^{−/−}\) cells stably transfected with plasmid vectors expressing full-length vinculin (F9 vin\(^{−/−}\) rescue); a fragment containing the sequences of the head portion of vinculin (F9 vin\(^{−/−}\) head, 1 to 821 aa); a vinculin fragment 243–1066 in which the talin binding sites were deleted (F9 vin\(^{−/−}\)Δ talin bd); a full-length vinculin containing a Y822F point mutation (F9 vin\(^{−/−}\)Y822F); and expressing a vinculin hinge-tail fragment (F9 vin\(^{−/−}\) hinge-tail, amino acid residues 811 to 1066), which was Flag M2-tagged (Xu et al., 1998b). These cells were cultured as described previously (Coll et al., 1995; Xu et al., 1998b).

#### Anoikis assay

Anoikis assays were performed according to a published protocol (Folkman and Moscona, 1978). In brief, 6-well plates (Costar) were coated with polyHEMA to create a nonadhesive surface. The wells were allowed to dry for 48 h and washed with PBS (GIBCO BRL) before experiments. Cells were added in each well at a concentration of 5 × 10^4 cells ml\(^{−1}\), and the extent of apoptotic cell death was determined 3 h later.

#### Live cell microscopy for apoptosis

Immediately before the experiment, the medium was changed to PBS supplemented with 10% FBS. Gelatin-coated coverslips with adherent F9, F9 vin\(^{+/−}\), and F9 vin\(^{−/−}\) rescue cells were mounted in a sealed Dvorak chamber (Nicholson Instruments) held in a temperature-controlled stage (20/20 Technologies). Microscopy was performed using a microscope (model Axiovert 100 TV; Carl Zeiss Microimaging, Inc.) modified with automated stage and filter wheels (LEP Ltd.) and a 40x NA 1.3 objective with differential interference contrast optics (Carl Zeiss Microimaging, Inc.). Control of microscope automation and image analysis were performed using Inovision ISSE software. The extent of apoptotic cell death was assessed during the first 6 h after addition of 2 μM camptothecin (Sigma-Aldrich). As described previously (Subauste et al., 2000), individual cells were logged in using an automated microscope and scored for changes in morphology over time. Previously reported studies confirmed that the morphological features assayed (contraction and blebbing leading to cell fragmentation) were indicative of apoptosis (Subauste et al., 2000).

#### Caspase activity assay

The synthetic tetrapeptide fluorogenic substrate DEVD-AMC from Enzyme Systems Products was used as described previously (Subauste et al., 2000).

#### Preparation of protein extracts, immunoblotting, and immunoprecipitation

For immunoblotting analyses, cells were lysed in a buffer containing 20 mM Tris-HCl, pH 8, 100 mM NaCl, 10% glycerol, 1% Triton X-100, 50 mM NaF, 1 mM sodium vanadate, 1 mM benzamidine, 5 μg ml\(^{−1}\) leupeptin, and 1 mM PMSF. The cell lysates were clarified at 10,000 g for 10 min at 4°C. Samples were separated on SDS–polyacrylamide gels (Bio-Rad Laboratories). Proteins were transferred to Immobilon-P transfer membranes (Millipore) and blocked and probed with the following Abs: rabbit anti-ERK1/2 Ab, rabbit anti-phospho ERK1/2 (Thr202/Tyr204) Ab, rabbit anti-MEK Ab, rabbit antiphospho paxillin (Yyr118) Ab, and rabbit anti–Caspase-3 Ab from Cell Signaling Technology; rabbit anti–FAK Ab from Santa Cruz Biotechnology, Inc.; mouse monoclonal antipaxillin Ab; mouse monoclonal antiphospho FAK (Yyr397) Ab from Becton Dickinson; rabbit anti–Caspase-9 Ab from BD Biosciences; and mouse monoclonal antivinculin Ab, mouse monoclonal anti-myc Ab, and mouse monoclonal anti-FLAG M2
Ab from Sigma-Aldrich. Round Ab was detected by incubation with secondary Abs conjugated with HRP (rabbit and mouse HRP–derived Ab were obtained from Cell Signaling) and visualized by ECL (Pierce Chemical Co.). Equal loading in each lane was verified by assessing their ERK1/2 concentration.

Immunoprecipitation studies that assessed the interaction of FAK with paxillin were performed according to procedure described previously (Tachibana et al., 1995) with some minor modifications using the mouse monoclonal antipaxillin Ab as well as the rabbit anti-FAK Ab (the concentration used for both Abs was of 1/500).

Cell migration assays
Modified Boyden chambers (8 μm pore size; Transwell Costar Corporation) were used in cell migration assays. The filter inserts were coated with 10 μg ml⁻¹ collagen type I (Upstate Biotechnology) for 2 h at 37°C, rinsed with PBS, and the inserts were placed into the lower chamber containing 0.5 ml of α-MEM media containing 10% FBS (GIBCO BRL). 2 × 10⁵ cells that were previously serum starved (1% FBS) for 10 h were added to the top of each migration chamber and allowed to migrate to the underside of the top chamber for 8 h. Nonmigratory cells on the upper membrane surface were removed with a cotton swab, and the migratory cells attached to the bottom surface of the membrane were fixed with methanol and stained with 0.1% crystal violet in 0.1 M borate (Sigma-Aldrich), pH 9, and 2% ethanol for 20 min at RT. The number of migratory cells per membrane was counted with an inverted microscope (Nikon) using a 10× objective. Each determination represents the average of two individual wells. Background migration was assessed on BSA (1%)-coated membranes and subtracted from all data.

Inhibition of p42 and p44 MAPK (ERK1 and ERK2) with Erk antisense oligonucleotides
Depletion of MAPK (ERK1 and 2) from F9 embryonal carcinoma cells was performed using phosphorothioate-protected oligonucleotides according to the procedure of Sale et al. (1995) with some modifications. Erk antisense (5'-GCC GCC GCC GCC GCC AT-3') and control oligonucleotides (5'-GCC GCC GCC GCC GCC CC-3') were HPLC purified (Operon Biotechnology). Cells were grown to 30% confluency in 6-well tissue culture plates. Before transfection, lipofectin (GIBCO BRL) was diluted into 400 μl of α-MEM at a concentration of 30 μg/ml for 40 min at RT. The diluted lipofectin was mixed with 400 μl α-MEM containing 6.0 μg ERK antisense or control phosphorothioate oligonucleotides for 15 min at RT. This mixture was added to the cells. The final concentrations of lipofectin and oligonucleotides were 15 μg/ml and 3 μM, respectively. Cells were incubated for 8 h at 37°C in the presence of 5% CO₂. They were rinsed, incubated for another 40 h in fresh culture medium containing oligonucleotides, and tested as described in Anokias assays. An aliquot of the aforementioned cells was analyzed in parallel for changes in ERK protein levels using ERK-specific Abs and immunoblotting as described in Preparation of protein extracts, immunoblotting, and immunoprecipitation. As a control for nonspecific depletion of proteins, MEK concentration of each sample was assessed by immunoblotting.

Plasmids and transfection
pCdNA3 wild-type and Y31FY118F paxillin constructs were previously described (Petit et al., 2000). To mcg tag these constructs at their NH₂ terminus, they were excised with BamHI and EcoRI enzymes and subcloned in a pTriExx4 His-myc vector. A monomeric red fluorescent protein (mRFP) subcloned in the same vector was used as a control. Transient transfections were done using lipofectamine and Plus Reagent (GIBCO BRL) following the manufacturer’s instructions. In brief, F9 cell lines were transfected with 8 l lipofectamine, 16 l of Plus Reagent, and 7 μg of the plasmids. Cells were used 48 h after transfection. The plasmids expressions were confirmed with a mouse anti-myc Ab (Sigma-Aldrich).

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