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Vinculin activators target integrins from within the cell to increase melanoma sensitivity to chemotherapy

Elke S. Nelson¹, Andrew W. Folkmann¹, Michael D. Henry², and Kris A. DeMali^{1,3}

¹Department of Biochemistry, Roy J. and Lucille A. Carver College of Medicine; 51 Newton Rd. Iowa City, IA 52242

²Department of Molecular Physiology and Biophysics, Roy J. and Lucille A. Carver College of Medicine; 51 Newton Rd. Iowa City, IA 52242

Abstract

Metastatic melanoma is an aggressive skin disease for which there are no effective therapies. Emerging evidence indicates that melanomas can be *sensitized* to chemotherapy by increasing integrin function. Current integrin therapies work by targeting the extracellular domain, resulting in complete gains or losses of integrin function that lead to mechanism-based toxicities. An attractive alternative approach is to target proteins, such as vinculin, that associate with the integrin cytoplasmic domains and regulate its ligand binding properties. Here we report that a novel reagent, denoted vinculin activating peptide or VAP, increases integrin activity from within the cell, as measured by elevated: (1) numbers of active integrins, (2) adhesion of cells to extracellular matrix ligands, (3) numbers of cell-matrix adhesions, and (4) downstream signaling. These effects are dependent on both integrins and a key regulatory residue A50 in the vinculin head domain. We further show that VAP dramatically increases the sensitivity of melanomas to chemotherapy in clonal growth assays and *in vivo* mouse models of melanoma. Finally, we demonstrate that the increase in chemosensitivity results from increases in DNA damage-induced apoptosis in a p53-dependent manner. Collectively these findings demonstrate for the first time that integrin function can be manipulated from within the cell and validate integrins as a new therapeutic target for the treatment of chemoresistant melanomas.

INTRODUCTION

Melanoma is the deadliest and most aggressive form of skin cancer. While early melanoma can often be cured by surgical intervention, advanced melanoma is notoriously resistant to chemotherapy. Genetic, functional and biochemical studies suggest that melanoma cells become resistant to chemotherapeutic drugs by reprogramming their proliferation and survival pathways. In recent years, proteins involved in the regulation and execution of melanoma apoptosis have been identified and provided insights into the molecular basis for melanoma resistance. The current challenge is now to identify targets that bypass these cell death defects and to devise strategies for manipulating their function.

One group of proteins that are dysfunctional in resistant melanomas is the integrins, cell surface receptors that mediate attachment of cells to the extracellular matrix on the outside

³To whom correspondence should be addressed: Department of Biochemistry, Roy J. and Lucille A. Carver College of Medicine, Iowa City, IA 52242 .

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of the cell and the actin cytoskeleton on the inside of the cell. In addition to physically tethering cells to the matrix, integrins send and receive molecular signals that regulate cell adhesion (reviewed in (1, 2) cell survival, proliferation, and migration (3-4). In some tumors, ligation of integrins to the extracellular matrix appears to regulate cell survival, as detachment rapidly induces apoptosis (5). In support of this notion, increases in the expression of $\beta 3$ integrins in melanomas fuel resistance to chemotherapy (6-7). Efforts based on this line of thinking have led to the development of a number of $\beta 3$ integrin antagonists (8-10). However, some of these reagents prevent binding of integrins to extracellular matrix ligands with an RGD motif; thus they are not necessarily specific for $\beta 3$ integrins. Moreover, although these reagents are thought to have antagonist properties, depending on the concentration employed they may instead agonize integrin function (11-13). While there has been some success in preventing tumor angiogenesis by employing such targets, these promising anti-cancer drugs have failed in the clinic. Thus, alternative mechanisms for targeting integrin function are needed.

Several studies have unexpectedly demonstrated that adherent cells are more sensitive than suspended cells to DNA-damaging agents (14-16). These findings suggest that, in addition to expressing higher levels of $\beta 3$, resistant melanomas have a defective integrin pathway. In support of this notion, melanomas treated with chemotherapeutics often persist when they have little contact to the extracellular matrix but rapidly succumb to chemotherapy when extracellular agonists are applied to them (16). Hence reagents that rescue defective integrin function are expected to improve treatment for melanoma resistance.

Some integrin-associated proteins (Rap1a, talin and paxillin) have perceived therapeutic potential (reviewed in (17)). However, mechanisms whereby they can be targeted to tumors have yet to be established, and the potential efficacy of targeting has thus not been explored. Vinculin is an actin-binding protein that is recruited to the integrin β cytoplasmic tail via its interaction with talin (18-19), and numerous pieces of evidence suggest that vinculin is a critical regulator of integrin-mediated adhesion and migration. For example, cells devoid of vinculin have fewer and smaller adhesions and close a wound more rapidly than vinculin-positive cells (20-23). Furthermore, recent work has shown that vinculin regulates the clustering, turnover and ligand binding properties of integrin-containing complexes (23-24).

Vinculin's regulation of integrin-mediated events depends on relief of an intramolecular interaction between the vinculin head and tail domains (24-26), and several proteins known to disrupt this interaction. Upon *Shigella* entry into the intestinal epithelium, the invasin protein IpaA binds and unfurls vinculin (27). We and others previously showed that IpaA has two functional domains: an N-terminal domain that induces the cytoskeletal rearrangements required for bacterial entry, and a non-invasive C-terminal motif that binds vinculin and induces conformational changes that lead to its activation (28-30). As a consequence of its role in unfurling vinculin's conformation, the C-terminal motif has been named a vinculin activating peptide or VAP. The unique properties of VAP suggest that it has potential as a tool to activate vinculin and enhance integrin function. In this study, we have tested the efficacy of VAP in increasing integrin function from within the cell and sensitizing melanomas to chemotherapy and have further investigated the mechanism for its effects.

RESULTS

The impetus of this work was to identify mechanisms for increasing integrin ligation to the extracellular matrix from within the cell and determine if they are effective in sensitizing melanomas to chemotherapy. Vinculin is recruited to the integrin cytoplasmic tail where it regulates integrin clustering, turnover and ligand binding properties (23-24). These

characteristics led us to investigate if integrin function can be modulated by promoting activating conformational changes in vinculin. To address this possibility, we employed VAP, a short peptide derived from the C-terminus of the *Shigella* invasion protein, IpaA, that activates vinculin by promoting conformational changes (28-30).

VAP increases integrin-mediated cell adhesion and signaling

We first examined the effects of VAP on integrin function. For this initial study, we used HeLa cells which have well characterized integrin profiles and responses. We allowed cells expressing GFP or a fusion of GFP and VAP to adhere to dishes coated with a wide range of fibronectin concentrations. More VAP expressing cells than control cells adhered; quantification revealed that the increase was maximal at 10 μ g/ml fibronectin, with $97 \pm 7\%$ of the VAP-expressing cells adhering compared to only $46 \pm 2\%$ of control cells (Fig. 1A). To determine if the effect of VAP on cell adhesion required integrins, we pre-incubated the cells with 6S6, a function-blocking antibody against $\beta 1$ integrins. We found that adhesion of both the control and VAP-treated cells was blocked to a similar extent (Fig. 1A), suggesting that integrin function is required for VAP to achieve its adhesive effect. We next examined if the effect of VAP on cell adhesion is limited to fibronectin by plating cells on different extracellular matrices. In the case of collagen, we found that $70 \pm 5\%$ of VAP-expressing cells, versus only $29 \pm 3\%$ of the GFP-expressing cells, adhered to coated surfaces (Fig. 1B). We obtained similar, though somewhat less dramatic, results for vitronectin (Fig. 1B).

To determine if the increase in adhesion affected cell spreading and/or the number of focal adhesions, we examined the cells by immunofluorescence microscopy. As anticipated, VAP largely colocalized with vinculin at focal adhesion sites (Fig 1C). Although the VAP-expressing cells consistently appeared to be more spread, an analysis of the average cell area revealed that these differences were statistically insignificant (Supplemental data Fig 1A). It also seemed that there were more focal adhesions in VAP-expressing cells than in the control cells, and in many instances the change appeared to be the consequence of an increase in the number of adhesion complexes on the cell interior (Fig 1C). To confirm these observations, we carefully counted the number of cell-matrix adhesions. Focal adhesions that were located on, or contacted, the cell border were scored as peripheral focal adhesions, whereas focal adhesions that did not contact the cell periphery were recorded as interior focal adhesions. Fig. 1D reveals that there was a significant increase in the number of focal adhesions in VAP-expressing cells that had been plated on fibronectin for 2 hours. This effect was attributed to an increase in the number of more centrally located focal adhesions following exposure to VAP, as the number of peripheral adhesions remained unchanged in this context (Fig. 1D). Similar results for focal adhesion numbers were obtained at six hours after plating on fibronectin (Supplemental data Fig. 1B).

As an independent measure of integrin function we assessed if VAP could increase the tyrosine phosphorylation of focal adhesion kinase (FAK), a potent downstream mediator of integrin signaling. In good agreement with findings from previous studies (31), there was an initial burst of FAK tyrosine phosphorylation followed by a more modest increase that was prolonged for at least the first four hours of plating after plating cells on fibronectin (Fig. 1E). In cells expressing GFP-VAP, FAK phosphorylation was increased to a similar extent initially but remained elevated to a greater extent and for a longer period of time than in cells expressing GFP only. Collectively, these results suggest that the VAP-mediated increases in integrin activation manifest itself in the form of more adhesions and elevated intracellular signaling.

VAP enhances the sensitivity of melanoma cells to chemotherapy

Adherent melanoma cells are more sensitive than suspended cells to chemotherapy. This observation, together with our finding that VAP enhances integrin function, led us to explore if VAP can modulate the sensitivity of M21 melanomas to chemotherapy. To this end, we generated M21 human melanoma cell lines stably expressing GFP, GFP-VAP, or a GFP-fusion with a non-binding version of VAP that lacks the vinculin binding sites (Δ VBS). In this setting, GFP-VAP but not the control proteins bound to (Supplemental Fig. 2A) and co-localized with vinculin at focal adhesions (Fig 2A). Furthermore, GFP-VAP increased adhesion of these cells to both fibronectin (Fig 2B) in a dose-dependent manner and also to collagen and vitronectin (Fig 2C). Cells expressing the control Δ VBS protein produced no significant increases in adhesion over the GFP-expressing cells (Supplemental Fig. 2B).

To examine if vinculin activation by VAP affects integrin ligation to the extracellular matrix, we measured the amount of active integrin on melanoma cells expressing VAP. To this end, we assessed the percentage of M21 cells stably expressing GFP or a fusion of GFP and VAP that stained positive for active β 1 integrins on the cell surface using antibodies that recognize the active conformation. In the presence of VAP there was a $15 \pm 4\%$ increase in cells expressing active integrin over control GFP-expressing cells (Fig. 2D) despite having similar levels of total β 1 integrin on the cell surface (Fig. 2E). Given that GFP-VAP was expressed at very low levels relative to GFP alone (data not shown), it is probable that the magnitude of the effect is even greater than evident in Fig 2D.

We wanted to ensure that the effects of GFP-VAP on the M21 melanomas were not limited to this cell type or an artifact of expression. To address these issues, we delivered VAP to a distinct melanoma cell line by fusing it to the basic domain (residues 47-57) of the HIV transcription factor TAT, as this peptide confers the ability to traverse cell membranes. Administration of TAT-VAP to M21 or A375 melanomas led to 2.0- and 2.3-fold increases in adhesion, respectively, over that observed in cells treated with TAT alone (Fig. 2F). These findings suggest that VAP increases integrin function independent of the melanoma cell line employed, and independent of its mode of delivery.

To assess whether VAP-mediated elevations in integrin function increase the sensitivity of M21 melanomas to chemotherapy, we performed colony-forming assays to examine sensitivity of the M21 cells to the chemotherapeutic drug 1- β -D-arabinofuranosylcytosine (araC). For these experiments, M21 cells were exposed to varying doses of araC for 24 hours, replated at low density in growth medium, and scored for subsequent clonogenic growth. Cells expressing either GFP or GFP- Δ VBS were largely resistant, exhibiting only partial decreases in clonogenicity even at high doses (Fig. 3A). By contrast, GFP-VAP-expressing melanoma cells formed very few colonies at drug concentrations near the IC_{50} for araC (Fig. 3A, (32)). This effect was not limited to cells treated with araC as similar effects were observed when the M21 cells were treated with dacarbazine, a FDA approved chemotherapeutic for the treatment of metastatic melanoma (supplementary Figure 3).

To test whether stimulating integrin function can enhance the response to chemotherapy *in vivo*, we examined the effects of GFP-VAP on melanoma responses to chemotherapy in a M21 mouse model. Mice bearing subcutaneous tumors expressing GFP or GFP-VAP were left untreated or received two treatments (low-dose) of araC approximately one week apart, and were monitored for tumor size. We found that control mice injected with GFP-expressing melanoma cells developed tumors with a speed consistent with that reported for the melanoma cells previously (16) and that administration of araC alone had no significant effect on tumor growth (Fig. 3B). In the mice injected with VAP-expressing melanoma cells, the onset of tumor formation and tumor size were greatly reduced relative to the same

parameters in the control mice. Moreover, treatment of these mice with araC resulted in a significant decrease in tumor size ($p < 0.05$) once tumors began to develop (Fig 3B).

VAP sensitizes melanomas to chemotherapy by stimulating apoptosis

VAP's effects on tumor growth *in vitro* and *in vivo* motivated us investigate the mechanism by which enhanced integrin function increases melanoma chemosensitivity. We first examined if VAP-expressing cells are more susceptible to chemotherapy-induced apoptosis than their control counterparts. Using flow cytometry, we measured the number of cells that stained positive for the apoptosis marker APC-Annexin V. The percentage of apoptotic cells among araC-treated, VAP-expressing melanoma cells was over 2-fold higher than that among araC-treated control melanoma cells (expressing either GFP or the non-binding Δ VBS mutant (Fig. 4A). As an independent measure of apoptosis, we examined the extent of caspase-9 cleavage. We consistently observed more cleaved caspase-9 in the VAP-expressing melanoma cells than in their GFP counterparts (Fig 4B). Moreover the stimulation of caspase-9 cleavage was triggered not only by araC; clinically relevant DNA damaging agents, including cisplatin, also induced caspase-9 cleavage, and to a much greater extent than araC (Fig 4C). Since VAP seemed to be having an anti-proliferative effect *in vivo*, we also examined if cell doubling was altered by VAP. However, we consistently found no differences in cell doubling (Fig 4D).

Previous studies suggest that the melanoma cells most susceptible to apoptosis may have elevated levels of p53 (15). We thus tested whether p53 levels were elevated in M21 cells expressing GFP or VAP. In the control cells, p53 levels were barely detectable, and the addition of a DNA damaging agent increased the levels as expected (Fig. 5A). In contrast, the GFP-VAP-expressing cells had a high basal level of p53. As in the control cells, DNA damage led to an increase in p53 levels (Fig. 5A). We determined if elevations in p53 are required for VAP's effects. For these studies, we inhibited p53 using RNA interference. When p53 levels were diminished using this approach (Fig. 5B), the control cells were still able to induce apoptosis to the same extent as the cells expressing wild-type levels of p53 (Fig. 5B). In contrast, apoptosis was reduced to basal levels in the VAP-treated cells. Hence elevations in p53 expression are required for VAP to induce apoptosis. Collectively these findings demonstrate that VAP enhances integrin function in M21 melanomas and this effect sensitizes them to DNA-damage induced apoptosis in a p53-dependent manner.

VAP enhances adhesion by binding to an amphipathic α -helix in the vinculin head

To ensure that VAP acts by targeting vinculin, we explored its effects in cells lacking vinculin. We wished to perform these experiments in melanoma cells lacking vinculin. However, only a small fraction of vinculin is required to maintain cell-matrix adhesion, and studies demonstrate that it cannot be depleted (using RNA interference) to an extent that affects cell-matrix adhesion (33-34), and S. Craig, personal communication). As an alternative approach, we examined VAP's effects in mouse embryo fibroblasts isolated from the vinculin null mouse. As anticipated, VAP did not co-precipitate (Fig. 6A) and exhibited diffuse cytoplasmic staining (Fig. 6B) in the absence of vinculin. When vinculin was re-introduced into these cells, it bound VAP (Fig. 6A) and largely limited VAP's localization to focal adhesions (Fig. 6B). The failure of VAP to localize to focal adhesions in the absence of vinculin supports the idea that vinculin is required for VAP's effects on cell adhesion. In agreement with this conclusion, the expression of GFP or VAP resulted in a similar level of cell adhesion to fibronectin-coated surfaces (Fig. 6C), and re-expression of vinculin increased adhesion (Fig. 6C), as has been previously reported (22, 35). Importantly, expression of GFP-VAP in the vinculin-rescued cells resulted in a further increase in the number of adherent cells, with the magnitude of the increase slightly lower than that observed in the HeLa cells (Fig. 6C and Fig. 1A).

We next investigated which region of vinculin is required for the effects of VAP on cell adhesion. Vinculin ligands, like VAP, that interact with the head domain have the potential to bind to two sites within it: a hydrophobic site in the N-terminal α -helical bundle and a more C-terminal site (36). To investigate if the N-terminal site is critical for VAP binding and enhanced adhesion, we used site-directed mutagenesis to generate a full-length vinculin harboring an A50I substitution. This mutant version of vinculin is well-characterized with respect to its ability to localize to focal adhesions (24, 26, 37). To determine if this substitution blocks VAP deposition at focal adhesions, and also binding to vinculin, we examined co-precipitation and co-localization of A50I vinculin and GFP-VAP in the vinculin-null MEFs. VAP did not readily bind to or co-localize with A50I vinculin (Figs 6A and 6B). This effect was not due to differences in the amounts of the two vinculins (Supplemental Fig. 4). Furthermore, in the presence of A50I vinculin, VAP was not able to elicit an increase in cell adhesion (Fig. 6C). Taken together, these results indicate that VAP enhances integrin-mediated events by engaging vinculin at amino acid A50.

DISCUSSION

The goal of this study was to discover novel mechanisms that might enable effective targeting of integrin function as a therapeutic strategy for sensitizing melanomas to chemotherapy. Given that cytoplasmic integrin-associated proteins regulate the affinity of integrins for the extracellular matrix (reviewed in (38-40)), we explored whether an activator of vinculin can be employed for such manipulation. Our discovery that VAP increases both integrin-mediated adhesion and signal transduction together with the observation that this effect is dependent on the presence of both integrins and vinculin suggests that such an approach is feasible. Furthermore, we have tested whether molecules such as VAP might be useful for enhancing integrin function in melanoma. We found that VAP sensitizes melanoma cells to cancer chemotherapy both *in vitro* and *in vivo*, and that it does so by increasing their susceptibility to DNA damage-induced apoptosis in a p53-dependent manner. Collectively, these findings suggest that molecules like VAP may have utility in sensitizing resistant melanomas to chemotherapy.

Notably, the level of VAP expressed in a particular cell line does not correlate strictly with the adhesion response it elicits. Despite the fact that all the cells examined express comparable levels of VAP, HeLa cells exhibit a 2-fold increase in adhesion to fibronectin whereas M21 cells show only a 1.5-fold increase. This difference may be due, in part, to the fact that the melanoma cells have adhesion defects (41). Hence it is likely that the effects of VAP on HeLa cell adhesion are greater because in that context VAP provides a boost to an adhesion mechanism that is already functioning. In contrast, when VAP is expressed in cells with adhesion defects, it can only partially enhance the abilities of a defective or dysregulated integrin. Nevertheless, the increase in integrin function in the M21 cells is sufficient to produce a dramatic increase in the sensitivity of these melanoma cells to the cancer chemotherapeutic araC.

Also notable is our observation that although VAP alone inhibits melanoma growth *in vivo*, it does not do so in the *in vitro* clonogenic growth assays. We consistently observed this effect, and believe that it may be a consequence of the fact that the clonogenic growth assays measure growth after 8-10 days, whereas growth *in vivo* is measured over a much longer time frame, i.e. 5-12 times longer. An additional possibility is that VAP has additional growth inhibitory effects *in vivo*. For example, VAP may inhibit interaction between the tumors and constituents of the stroma. Future work will focus on dissecting the underlying cause of the additional potency of VAP *in vivo*.

How might VAP elicit its effects on cell adhesion? Within the cell, vinculin exists in at least three pools: an inactive fraction in the cytoplasm, an active focal adhesion-bound fraction at the cell periphery, and an inactive focal adhesion-bound fraction in a gradient with the concentration increasing from the cell periphery to the cell interior (25). Whether VAP targets vinculin that is already associated with an adhesion complex or that is part of the non-associated and inactive cytoplasmic pool is not yet known. At least two pieces of evidence argue against the latter possibility. First, all available evidence suggests that vinculin is recruited to a focal adhesion complex prior to its activation (25). Thus, our observation that VAP does not localize to adhesion sites in the absence of vinculin (Figure 6) would suggest that VAP, by itself, cannot both recruit and activate vinculin. Secondly, if VAP were to target the inactive cytoplasmic vinculin fraction, we would expect to see intense cytoplasmic staining of GFP-VAP or vinculin. We have carefully examined our cells and find no evidence for such an increase. Hence, it seems more likely that this protein targets the pool of vinculin at a focal adhesion. Our observation that VAP induces an increase in the number of more centrally located focal adhesions supports the notion that VAP targets the inactive, focal adhesion-bound pool of vinculin (Figure 1). In this scenario, VAP could increase either the level or duration of vinculin activity. Our observation that VAP increases the duration of FAK tyrosine phosphorylation lends support to the notion that VAP increases the length of time for which vinculin is active. Depending on the conditions, such prolonged signaling could prevent the termination of adhesion signaling by inhibiting disassembly of the multiprotein complex, remodeling of the multiprotein complex, or sliding of the multiprotein complex.

While it remains to be established which pool of vinculin is targeted by VAP, our work provides information about the structural requirements for its effects. VAP binds vinculin, localizes to focal adhesions, and increases adhesion of cells that express the wild-type vinculin but not the A50I vinculin mutant (implying that VAP's occupation of the A50 site on vinculin is critical for increased adhesion). If VAP were to target vinculin at focal adhesions, then one might expect that the vinculin's A50 site is occupied by talin (the major head ligand in cell-matrix adhesions), and/or that talin competes for binding to this site. However, previous work demonstrated that VAP cannot displace talin from vinculin (30). Indeed, talin has recently been reported to occupy another more C-terminally located site in the vinculin head, and binding to this region is thought to lead to the recruitment of vinculin to focal adhesions (36). Thus, our data are indicative of a scenario in which talin recruits vinculin to adhesion sites via the more C-terminal site, and VAP activates vinculin by binding to the A50 site.

The observation that VAP increases integrin function suggests that VAP, or molecules like it, have potential as therapeutics for diseases caused by defects in integrin function. Melanomas are notoriously resistant to chemotherapy owing to their ability to survive detachment from the extracellular matrix (14, 16). Integrins are critical to this process, as M21 melanomas undergo apoptosis in response to chemotherapy when the cells are attached to an extracellular matrix but not when they are held in suspension. Further support for the notion that integrin-mediated substrate anchoring affects susceptibility to chemotherapeutics comes from the observation that agents mimicking interactions between integrins and the extracellular matrix increase the sensitivity of melanomas to such agents (16). Here we show that VAP enhances integrin-mediated adhesion in M21 melanomas. Thus targeting integrins from inside the cell has an effect that is similar to that of targeting them from the outside, and that may represent a novel means of avoiding mechanism-based toxicities that develop from global and chronic interference with all integrins in the body.

Our findings raise the question of whether VAP itself is suitable for use in patients. Certainly there is precedent for naturally occurring biological agents serving as potent

pharmaceutical agents. For example, several snake-venom toxins that inhibit integrin function by targeting the extracellular domain of the molecule are currently in use in the clinic to treat other diseases (reviewed in (42-43)). One potential concern with using VAP itself is that it may promote *Shigella* infection in treated individuals. However, this seems extremely unlikely given that VAP would be targeted to a melanoma rather than to the gut, and also because VAP lacks the IpaA N-terminus, which is required for bacterial entry into the host cell. Although immunogenicity could potentially limit the use of VAP in standard chemotherapy, the observation that VAP-when fused to the membrane-permeable peptide sequences from TAT-dramatically sensitizes melanomas to chemotherapy, suggests that this peptide may have therapeutic potential. In support of using a peptide in a therapeutic regimen, a broad spectrum of peptide drugs (either alone or as vectors for the delivery of ligands), has achieved clinical success. Alternatively, small molecule mimetics of VAP can be developed. More work is needed to establish whether VAP or small molecule mimetics of this peptide can be employed as adjuvants for melanoma chemotherapy. However, the work described here lays the foundation for the development of a new class of therapeutics that target integrin-associated proteins.

MATERIALS & METHODS

Cell lines

HeLa and Vin^{-/-} MEFs were obtained and maintained as previously described (35). M21 human melanoma cells were the generous gift of Paul Sondel, and were maintained in RPMI + 10% FBS + 1% penicillin/streptomycin. They are a subclone that was derived in the lab of Dr. Ralph Reisfeld (Scripps, La Jolla, CA) from the human melanoma line UCLA-SO-M21, which was originally provided by Dr. D. L. Morton (UCLA, Los Angeles, CA).

Constructs

GFP fusion proteins containing Ipa fragments (VAP and ΔVBS) were constructed by PCR amplification of the cDNA of interest from pEC15 (44), followed by subcloning into pLEGFP-C1 (Clontech laboratories) or pEGFP-C1 (Clontech Laboratories). Briefly, VAP (IpaA residues 500-633) was PCR amplified using the primers GGATCCGGAACACAAGAACGAGAGTTACAGG and GAATTCCTTAATCCTTATTGATATTCTTTAATACTTTTGATAGGG; the resulting PCR products were cloned into the BglII- EcoRI sites of pEGFP-C1. For cloning into pLEGFP-C1, VAP was PCR amplified using the primers AAACCTCGAGAAACAACTCCCTAAGTAATCTTATATC and TTTGGATCCTTAATCCTTATTGATATTCTTTAATAC, digested with XhoI and BamHI, and subsequently ligated into XhoI-BamHI digested vector. Two different mutant forms of VAP lacking the vinculin binding sites were also generated (ΔVBS1 and ΔVBS2). ΔVBS1 consists of IpaA residues 510-556, and was created using the following primers: AAACCTCGAGAAGGAACATAATCAGGAACACAAGAACG and GGGGGATCCGTTGGTTTCTGTTACGTAGTTTCTTATATTTGC. ΔVBS2 contains residues 590-611 of IpaA, and was generated using the primers TCGAGAAGAATTACTTAC AGATGATATATCTGATTTAAAAATAACAATGATATTACAGCTGAAAACAATAA TG and GATCCATTATTGTTTTTCAGCTGTAATATCATTGTTATTTTTTAAATCAGATATA TCATCTGTAAGTAATTCC. ΔVBS constructs were cloned into the XhoI and BamHI sites of pLEGFP-C1 as described for VAP. ΔVBS1 was employed in melanoma studies examining localization, adhesion, and clonal growth. ΔVBS2 was utilized in melanoma apoptosis experiments. Full-length vinculin in pCMV-myc has been previously described (35). GFP-vinculin was the generous gift of Ken Yamada (National Institutes of Health) and

has previously been described (45). The A50I substitutions were generated by site-specific mutagenesis according to the Quickchange Manual (Stratagene), using GFP-vinculin as a template and the following primers: CCCGTGTCGGCCGTGCAG GCCATTGTCAGCAACCTGGTGCGG and CCGCACCAGGTTGCTGACAATGGCCTGCA CGGCCGACACGGG.

Infections and transfections

Stable cell lines were generated as previously described (34), and selected in 0.5 mg/mL (HeLa) or 1.0 mg/ml (M21) G418. Transient transfections were performed as previously described (28, 35). M21 cells stably expressing GFP-VAP were sorted to identify a cell line expressing higher expression levels of GFP-VAP. For these studies, high expressing cells were isolated using fluorescence activated cell sorting on the Beckton Dickinson FACS DiVa flow cytometer housed in the University of Iowa Flow Cytometry Facility.

FAK phosphorylation assays

For analysis of FAK phosphorylation, HeLa cells expressing GFP or VAP were suspended in serum-free medium for 1 hour and then plated on fibronectin-coated dishes (10µg/ml) for 0-4 hours. Cells were washed and lysed as previously described (34). To immunoprecipitate FAK, cell lysates were incubated with a polyclonal antibody against FAK (the generous gift of Keith Burrige, University of North Carolina). The immune complexes were recovered using Protein A Sepharose (Sigma), washed 4x with lysis buffer (34) and subjected to SDS-PAGE and western blot analysis. The immune complexes were blotted with an anti-phosphotyrosine antibody (BD Biosciences), after which the blot was stripped and then reprobed with the polyclonal FAK antibody. The level of FAK phosphorylation for three independent experiments was determined using densitometry and ImageJ software.

Immunoprecipitation of GFP fusion proteins

These experiments were performed in essentially the manner as the FAK immunoprecipitation experiments. A monoclonal anti-GFP antibody (Roche) was used for the immunoprecipitation, and the bound proteins were recovered using Protein G Agarose (Sigma). The resulting immunoprecipitates were blotted with antibodies against vinculin (hvin1) or GFP (Roche).

Immunofluorescence

Cells were fixed, permeabilized and washed as previously described (35). To visualize vinculin, cells were blocked in 10% BSA in universal buffer (150mM NaCl, 50mM Tris-HCL pH 7.6, 0.01% NaN₃), incubated with a mixture of hVIN-1 (Sigma) and 7F9 (Millipore) at a 1:200 dilution, washed, and incubated with an anti-mouse Texas Red-conjugated donkey anti-mouse IgG (H+L) at a 1:750 dilution (Jackson ImmunoResearch Laboratories, Inc). Fluorescence images of mouse embryo fibroblasts were captured at room temperature with a confocal microscope (model LSM 510; Carl Zeiss MicroImaging, Inc.). We used a 63X oil objective (Carl Zeiss MicroImaging, Inc.) with a NA of 1.4. Images were obtained using the LSM Image Browser (Carl Zeiss MicroImaging, Inc.). All other cells were visualized at room temperature using an Axiovert 200M inverted microscope (Carl Zeiss MicroImaging, Inc.) and 63X oil objective (Carl Zeiss MicroImaging, Inc.) with a NA of 0.55. Cells were photographed using an ORCA-ER-1394 cooled charge-coupled device camera (Hamamatsu Corporation), and images were acquired using Axiovision 4.7 software (Carl Zeiss MicroImaging, Inc.). Magnified images were obtained using the crop function in Adobe Photoshop CS3. Minor adjustments of brightness and contrast were applied to entire images using Adobe Photoshop CS3 or Microsoft PowerPoint.

Adhesion assays

Human fibronectin was purified from outdated human platelets as previously described (46), rat tail collagen I was purchased from BD Biosciences, and vitronectin was obtained from Invitrogen. For adhesion assays, cells were lifted, washed and plated as previously described (DeMali et al., 2002), with minor modification to the number of cells plated. For experiments using HeLa cells or M21 melanomas, cells were plated at a concentration of 50,000 cells per well. For MEF experiments, either 25,000 or 50,000 cells per well were plated, depending on the numbers of transfected cells available. HeLa cells were allowed to adhere for 15 min, MEFs for 30 min, and M21 cells for 2h, after which the wells were washed 2 times to remove nonadherent cells. The average number of adherent cells in 10 fields of view from at least three independent experiments was plotted as a function of the % of total cells. p-values were determined using Graph Pad Instat Analysis software. For analysis of focal adhesion numbers and cell area, HeLa cells were plated onto fibronectin-coated dishes for 2 or 6h, and the cell area and number of focal adhesions in 20 cells was quantified. Cell area was measured using ImageJ software, and focal adhesions were counted using AxioVision 4.7 software. The results were averaged over 3 independent experiments.

Apoptosis assays

M21 cell lines that stably express GFP, GFP-VAP, or GFP- Δ VBS were left untreated or were treated with araC for 48 hours in growth medium. To quantify apoptosis using AnnexinV staining, cells were lifted, washed with PBS, and stained with APC-AnnexinV according to the manufacturer's protocol (BD Pharmingen). The number of apoptotic cells was scored as described above. The percentage of apoptotic, AnnexinV-positive cells before and after treatment is shown. Results are representative of at least three individual experiments, and error bars indicate the standard deviation. To examine apoptosis by assessing caspase 9 cleavage, M21 cells expressing the indicated GFP fusion proteins were treated with 20 μ M 1- β -D-arabinofuranosylcytosine or 5 or 20 μ g/mL cisplatin (SIGMA) for 48 h. The cells were lysed in 2x sample buffer, the samples were separated by SDS-PAGE, and proteins were immunoblotted with antibodies against caspase-9 (Cell Signaling). This antibody is directed against human caspase-9 and recognizes both full-length and cleaved fragments of caspase-9.

Analysis of p53 levels

M21 cells expressing GFP or GFP-VAP were treated for 18h with 25 μ M araC. Lysates were harvested and separated as described for the apoptosis assays. p53 levels were examined using a mouse monoclonal antibody against human p53 (Sigma clone BP53-12). Actin levels were examined using a pan-actin mouse monoclonal antibody (clone c4, Millipore). The levels of p53 and actin were quantified as described above for the analysis of FAK levels. Expression of p53 was accomplished using lentiviruses encoding shRNAs against human p53 (Santa Cruz Biotechnology).

Colony forming assays

Melanoma cells were treated with various concentrations of the chemotherapeutic drug 1- β -D-arabinofuranosylcytosine (araC) or dacarbazine (DTIC) in RPMI + 0.5% FBS at 37°C and 10% CO₂. Cells were treated with araC for 24 hours, after which 1×10^5 cells were plated in growth medium, maintained for 10 days, and then fixed and stained with Crystal Violet. Images of each plate were obtained using a Kodak EDAS 290 camera. Each condition was tested in triplicate, and clonal growth was quantified by densitometry using ImageJ software. For DTIC experiments, 5×10^5 cells were treated for 48 hours, with fresh drug added every 24 hours. Following treatment, cells were maintained for 5 days in fresh

growth medium, fixed, and stained with Crystal Violet. Each plate was scanned using a Canon CanoScan 5600F.

Mouse tumor growth

All procedures involving animals were performed in accordance to The University of Iowa Animal Care and Use Committee policies. Severe combined immunodeficient/ beige mice (Charles River Laboratories) were injected s.c. with 100,000 M21 cells expressing either GFP or GFP-VAP suspended in cold PBS. Seven to eight mice were used for each condition. Mice were anesthetized with isoflurane, and injection sites were prepped with alcohol pads before injection. Starting at week four, when tumors were becoming visible, mice were injected i.p. with araC at 100mg/kg. A second araC injection was performed at week five. Tumors were measured weekly and tumor volume was calculated using the formula $(\text{length} \times \text{width}^2)/2$ where length was the longest dimension and width was the dimension perpendicular to length. When the tumor length exceeded 1.5cm in any dimension, a tumor volume of 1721 cm² was recorded, and mice were euthanized via CO₂ gas asphyxiation and cervical dislocation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. VAP increases integrin-mediated cell adhesion and cell signaling

(A and B) VAP increases adhesion to various extracellular matrix ligands. HeLa cells expressing GFP or GFP-VAP (VAP) were plated for 15 minutes on surfaces coated with the indicated concentrations of fibronectin (A), 1 µg/ml rat tail collagen I (B) or 2.5 µg/ml vitronectin (B). The surfaces were washed gently, and the numbers of adherent cells from ten representative fields of view from three independent experiments were counted and expressed as a percentage of total cells ± the standard error of the mean. Where indicated, cells were pre-incubated with a function-blocking antibody against β1-integrins (anti-β1) prior to plating on 10 µg/mL fibronectin. * indicates p value < 0.001 and # demarks a p value of < 0.05. (C and D) VAP increases focal adhesion numbers. (C) Representative images of cells incubated on fibronectin for 2h, fixed, and stained with antibodies against vinculin (Texas red). Bottom images show a 7x magnification of the boxed region depicted in the corresponding image above and incorporates both a peripheral and interior region. White arrows indicate peripheral focal adhesions and arrow heads indicate interior focal adhesions. Top panel bar = 20 µm; bottom panel bar = 3 µm (D) The number of vinculin-containing focal adhesions (FAs) at the periphery (in contact with the cell border) or interior (not in contact with the cell border) in 20 cells were counted. The average number of focal adhesions ± standard error of the mean from three independent experiments. (E). VAP increases integrin-mediated signaling. The indicated cells were suspended in serum-free medium and plated on fibronectin-coated dishes for 0-4 h, after which FAK was immunoprecipitated. The resulting immunoprecipitates were subjected to Western blot analyses with an anti-phosphotyrosine antibody (pY-FAK). The blot was stripped and reprobed with an antibody against FAK to confirm that equal amounts of protein were recovered (upper panel). The amount of phosphorylated and total FAK was measured using densitometry, and the level of phosphorylated FAK from three independent experiments was expressed as a function of the FAK protein present ± standard error of the mean (lower panel).

Figure 2. VAP increases integrin function in melanoma cell lines independent of its mode of delivery

(A) VAP localizes to focal adhesions in melanoma cells. M21 cells expressing the indicated fusion protein were fixed, permeabilized, stained with antibodies against vinculin (Texas red), and analyzed by immunofluorescence microscopy. Bottom images show a 7x magnification of the boxed region depicted in the corresponding image above. White arrows indicate interior focal adhesions. Top panel bar = 20 μm; bottom panel bar = 3 μm. (B and C) VAP increases melanoma adhesion. M21 cells expressing the indicated proteins were incubated in wells coated with fibronectin (B), 1 μg/ml rat tail collagen (C) or 2.5 μg/ml vitronectin (C). The wells were washed gently, and the percentage of cells that adhered was quantified and analyzed as described in the legend of Figure 1. * indicates p value < 0.001 and # demarks a p value of < 0.05. (D and E) VAP increases the number of active integrins but not total integrin levels. Sub-confluent cultures of cells were lifted from tissue culture surfaces. The cells were stained with 12G10 (D, Active Integrin) or TS216 (E, Total Integrin) followed by a Texas Red-conjugated secondary antibody. Positive cells were scored using flow cytometry, and the average number of positive cells was expressed ± standard deviation. The means from three independent experiments were analyzed using a student's t-test. (F) VAP increases melanoma-cell adhesion independent of cell line employed or mode of delivery. M21 or A375 melanomas were treated with TAT or a fusion of TAT and VAP (TAT-VAP), lifted and examined for their ability to adhere to dishes coated with fibronectin as described in the legend of Figure 1. The numbers of adherent cells from ten representative fields of view from three independent experiments were counted and expressed as a percentage of total cells ± the standard error of the mean.

Figure 3. VAP sensitizes melanoma cells to chemotherapy

M21 cell lines stably expressing GFP, GFP-VAP, or GFP- Δ VBS were examined for sensitivity to the chemotherapeutic drug 1- β -D-arabinofuranosylcytosine (araC). Melanoma cells were treated with the indicated concentrations of araC for 24 hrs and were counted. (A) Clonal growth was assessed by culturing equal numbers of cells for ten additional days before staining with crystal violet. Images show colony growth at increasing araC concentration, and values below images represent density as a percent of the untreated cells. (B) SCID beige mice with subcutaneous M21 tumors expressing GFP or VAP were left untreated or were treated twice beginning at week 4 with araC (black arrows). Tumor volume was then followed. The individual points are the mean from 7-8 animals, and the error bars represent the standard error.

Figure 4. VAP potentiates apoptosis in response to DNA damage

(A) VAP increases the number of Annexin V-positive apoptotic cells. M21 cell lines stably expressing GFP, GFP-VAP (VAP), or a fusion of GFP and the non-binding version of VAP (Δ VBS) were left untreated (control) or treated with 20 μ M araC. Cells were lifted off dishes, washed with PBS, and stained with APC-AnnexinV, after which the number of positive cells was scored using flow cytometry. The percentage of AnnexinV-positive cells before and after treatment is shown. Results are representative of at least three individual experiments, and error bars indicate the standard deviation. (B and C) VAP causes an increase in caspase 9 cleavage. The same cell lines were treated with araC (B) as described above or with 0, 5, or 20 μ g/mL cisplatin (C), lysed and immunoblotted with antibodies against human caspase-9 or GFP. FL indicates full-length caspase 9. The white lines indicate regions where irrelevant lanes were removed from the blot. (D) VAP has no effect on cell proliferation. Defined numbers of cells were plated at low density and harvested and counted daily for 5 days.

Figure 5. VAP-mediated increases in apoptosis are p53-dependent

(A) VAP increases p53 levels. GFP or GFP-VAP expressing cells were lysed and p53 levels were examined by western blot analysis, with antibodies against p53 or actin (loading control). The amount of p53 and actin was quantified using densitometry. The graph below the blots depicts the levels of p53 normalized for the total protein levels $\pm$ standard error of the mean for at least three independent experiments. (B) p53 is required for VAP-induced apoptosis. p53 was inhibited by treating cells with viruses encoding shRNAs against the human p53. Cells were then left resting or treated with araC, lysed and probed for caspase 9 cleavage or p53 levels as described in the legend of Figure 4. All of the samples were prepared and analyzed in the same experiment; the white line indicates where the western blot was cut to remove some irrelevant lanes. The amount of cleaved caspase 9 and actin (loading control) from five independent experiments were measured using densitometry, and the level of cleaved caspase 9 was expressed as a function of the total protein present $\pm$ standard error of the mean (lower panel). * indicates p value < 0.01 and # indicates a p value of < 0.05.

Figure 6. VAP requires vinculin amino acid A50 for its effects on integrin-mediated events (A) Co-immunoprecipitation of VAP with wild-type (WT) and mutant (A50I) vinculin. Mouse embryonic fibroblasts isolated from the vinculin-null mouse were co-transfected with GFP-VAP as well as Myc, a fusion of either Myc and wild-type vinculin (Myc-Vin), or Myc and vinculin containing an alanine 50 to isoleucine substitution (Myc-A50IVin). The cells were lysed, and the GFP proteins were immunoprecipitated and analyzed by blotting using antibodies against vinculin or GFP. (B) Representative images of cells that were stained with antibodies against vinculin and examined by immunofluorescence microscopy. Bar = 20 μ m. (C) Adhesion assays of Myc or Myc-Vin expressing cells were incubated on fibronectin (10 μ g/ml) for 30 min and washed. The number of adherent cells in three independent experiments was quantified as described in the legend of Figure 1. * indicates p value < 0.001 and # indicates a p value of < 0.05.