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Contortrostatin Activates ERK2 and Tyrosine Phosphorylation Events via Distinct Pathways

Matthew R. Ritter and Francis S. Markland, Jr.[1]
Department of Biochemistry and Molecular Biology and Norris Comprehensive Cancer Center, University of Southern California, Keck School of Medicine, Los Angeles, California 90033

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We report that cells adhering to contortrostatin show transient increases in activation of Extracellular signal Regulated Kinase 2 (ERK2). The kinetics and degree of activation are similar to cells adhering to fibronectin or vitronectin. We have recently shown that contortrostatin induces tyrosine phosphorylation in tumor cells. Contortrostatin is shown here to stimulate activation of ERK2 in suspended cells, but this activation follows a different dose-response pattern than contortrostatin-induced tyrosine phosphorylation. Since contortrostatin induces tyrosine phosphorylation via $\alpha v\beta 3$, we explored the effects of an $\alpha v\beta 3$-blocking antibody, 7E3, on contortrostatin-stimulated ERK2 activation. While 7E3 completely blocks the effect of contortrostatin on tyrosine phosphorylation, this antibody had no effect on activation of ERK2. In cells lacking expression of $\alpha v\beta 3$, tyrosine phosphorylation was unaffected by contortrostatin treatment, but ERK2 was activated. This is strong evidence that contortrostatin is regulating tyrosine phosphorylation events and ERK2 activation via separate pathways and through different integrin receptors.

Key Words: **integrin; disintegrin; MAP kinase; vitronectin receptor; tyrosine phosphorylation.**

Integrins not only provide cells with anchorage to the extracellular matrix, but also transduce signals that influence cell survival, division, differentiation and motility (1). The MAP kinase family of serine/threonine kinases is an important group of molecules regulated by integrins (2). The MAP kinases lie on the well-characterized Ras pathway and have been shown to be involved in regulating a multitude of cellular events. Growth factors initiate signaling to MAP kinase by binding to receptor tyrosine kinases at the cell surface. The receptors undergo dimerization and tyrosine phosphorylation at specific residues which create binding sites for SH2 domain-containing adapter protein Grb2, which can then recruit SOS, a guanine nucleotide exchange factor that causes exchange of GDP for GTP on Ras (3). GTP-bound Ras is the active form and binds directly to Raf, a serine/threonine kinase. Raf phosphorylates MEK, a dual specificity kinase which binds and phosphorylates ERK, a member of the MAP kinase family, on threonine 183 and tyrosine 185, resulting in its full activation (4). ERK is found in two isoforms, ERK1 (44 kDa) and ERK2 (42 kDa) both of which affect gene expression by directly phosphorylating transcription factors (5). ERK2 can also directly regulate the function of myosin light chain kinase (MLCK), an enzyme tightly associated with the control of cellular motility (6). Myosins are ATPases that are activated by actin and are capable of translational movement along actin filaments, which is important in generating force needed for cell motility. Myosin II is largely responsible for performing this role in non-muscle cells and is composed of two heavy chains (200 kDa) and two sets of light chains (16–20 kDa) (7). The function of myosin II is regulated by phosphorylation of the light chains by MLCK. Thus, ERK2 regulates cell motility indirectly through its ability to influence the activity of MLCK. Integrins are able to regulate the activity of the MAP kinases by sharing components of the Ras pathway (8). Integrin dimerization causes *trans*-autophosphorylation of FAK on a tyrosine residue that is recognized by the SH2 domain of Src. Src phosphorylates another tyrosine residue on FAK which becomes a binding site for Grb2, which propagates downstream signals via the Ras cascade (9). Grb2 can also bind Shc, a protein tyrosine phos-

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[1] To whom correspondence should be addressed at University of Southern California Keck School of Medicine, Cancer Research Laboratories #106, 1303 N. Mission Rd., Los Angeles, CA 90033. Fax: 323-224-7679. E-mail: markland@hsc.usc.edu.

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phorylated by FAK (10). Thus, the MAP kinases are situated at a point of convergence of multiple signaling pathways where they participate in the regulation of important cellular processes.

Disintegrins are potent integrin-binding proteins isolated from the venom of various species of snakes. This growing family was originally identified as inhibitors of platelet aggregation through blockage of the αIIbβ3 integrin, and later found to interact with β1 and β3 integrins on several cell types (11, 12). Disintegrins classically contain an RGD (Arg-Gly-Asp) sequence which allows them to act as competitive inhibitors for cell binding to RGD-containing extracellular matrix proteins such as fibronectin and vitronectin. Most disintegrins are monomeric with a single integrin binding motif. However, a subclass is emerging which is made up of dimeric disintegrins (13–15). The first dimeric disintegrin to be described was contortrostatin, isolated from the venom of the southern copperhead snake (*Agkistrodon contortrix contortrix*) (16). This protein is a 13.5 kDa disulfide-linked homodimer with two integrin-binding RGD motifs and has been shown to be an effective inhibitor of angiogenesis and cancer progression *in vivo* (17–19).

Investigations into the effects of contortrostatin on integrin signaling revealed that this disintegrin activates pathways leading to the tyrosine phosphorylation of FAK and CAS (20, 21). Although contortrostatin binds other integrins (16, 17, 19, 22), the αvβ3 integrin is the exclusive mediator of contortrostatin-induced tyrosine phosphorylation events and requires the activity of the Src family tyrosine kinases. Since monomeric disintegrins are incapable of stimulating tyrosine phosphorylation, it is concluded that the homodimeric structure of contortrostatin confers this activity. The functional consequence of contortrostatin signaling has recently been correlated with a massive disruption of cellular structure with a collapse of the actin cytoskeleton and disassembly of focal adhesion complexes (23). Contortrostatin is a potent inhibitor of tumor and endothelial cell motility (18, 19, 23), an activity which is likely related to the cytoskeletal disruptions. These findings at the molecular and cellular levels provide a mechanistic basis for the observed inhibition of angiogenesis and cancer progression *in vivo*.

This report describes the influence of contortrostatin on the activation of the MAP kinase, ERK2, and provides evidence for a pathway leading to activation of this molecule that is distinct from the contortrostatin-mediated, αvβ3-dependent pathway involving tyrosine phosphorylation of FAK and CAS. Contortrostatin-induced activation of ERK2 may be a separate but cooperative event negatively affecting cell motility and/or cell growth, and sheds new light on the activities of the disintegrin family.

METHODS

Materials. T24 human bladder carcinoma cells were purchased from ATCC (Rockville, MD). OVCAR-5 human ovarian carcinoma cells were a gift from Dr. Thomas Hamilton (Fox Chase Cancer Center, Philadelphia, PA). Contortrostatin was purified from the venom of the southern copperhead (*Agkistrodon contortrix contortrix*) as described previously (16, 17). The general protease inhibitor cocktail used in lysis buffers were obtained from Sigma (St. Louis, MO). The Src family kinase inhibitor, PP1, was from Calbiochem (La Jolla, CA). Anti-phosphotyrosine monoclonal antibody (mAb) PY99 was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). The activated form of ERK2 was detected using the Anti-ACTIVE MAPK polyclonal antibody from Promega (Madison, WI). 7E3 mAb was provided by Dr. Marian Nakada (Centocor, Malvern, PA).

Cell culture, preparation, and stimulation. Cells were maintained in RPMI 1640 medium containing 5% fetal bovine serum at 37°C and 5% CO_2. Cells were washed with phosphate-buffered saline (PBS) and starved in serum-free medium for 6 h at 37°C. Cells were detached by brief treatment with 0.05% trypsin/0.02% EDTA in PBS and collected by centrifugation, resuspended in soybean trypsin inhibitor (1 mg/ml in serum-free medium), and washed in 2% bovine serum albumin/serum-free medium. Cells were maintained in suspension for 1 h in 2% bovine serum albumin/serum-free medium at 37°C with end-over-end agitation. Quiescent cells (3×10^6/ml) were allowed to adhere to the indicated substrates after overnight immobilization of proteins and blocking with 2% BSA/PBS, or were treated with disintegrins or other reagents while in suspension.

Lysate preparation and immunoprecipitation. Suspended or adherent cells were washed twice with cold PBS and lysed in cold lysis buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 1% Nonidet P-40, 0.5% deoxycholate, 0.1% SDS, protease inhibitor cocktail, 1 mM sodium pyrophosphate, 1 mM sodium orthovanadate, 50 mM sodium fluoride). After 10–15 min incubation on ice, insoluble material was removed by centrifugation at 14,000 rpm in a microcentrifuge for 15 min. Supernatants were collected and total protein concentrations standardized by the BCA protein assay (Pierce, Rockford, IL). Whole cell lysates (30 μg total protein) were resolved by SDS–polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes.

Immunoblotting. Membranes were blocked with 5% nonfat milk/Tris-buffered saline/0.1% Tween 20 (blocking buffer) 1 h at room temperature or overnight at 4°C. Primary antibody incubations were performed in blocking buffer for 1 h at room temperature. After washing in Tris-buffered saline/0.1% Tween 20, membranes were incubated with horseradish peroxidase-conjugated secondary antibody in blocking buffer 1 h at room temperature. Membranes were washed extensively. Immunoblots were developed using Super Signal West Pico Chemiluminescent Substrate from Pierce.

RESULTS

Tumor cell adhesion to immobilized contortrostatin results in enhanced activation of ERK2. To test how T24 cells respond when adhering to immobilized contortrostatin we compared ERK activation in these cells to cells adhering to fibronectin or vitronectin. It was observed that T24 cells express much higher levels of ERK2 compared to ERK1. Lengthy exposure of blots probed for activated ERK1/2 revealed a faint band corresponding to ERK1 (44 kDa) that was slightly larger than the major band (ERK2, 42 kDa). It was found that T24 cells responded with ERK2 activation in a similar manner irrespective of the substrate to

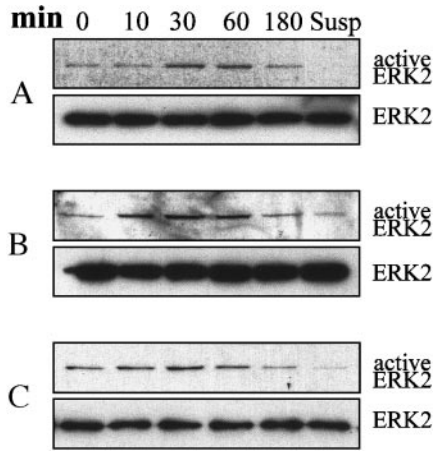

FIG. 1. ERK2 is transiently activated in T24 cells adhering to immobilized contortrostatin (5 μg/ml) (A) as well as in cells adhering to fibronectin (20 μg/ml) (B) or vitronectin (7 μg/ml) (C). Whole cell lysates were probed with an antibody that specifically recognizes the activated form of ERK1/2. ERK2 is the isoform predominantly expressed in T24 cells. Time 0 represents cell lysates prior to adhesion to the various substrates. Lysates from cells held in suspension for 180 min (Susp) are shown. Corresponding loading control blots are shown in the lower halves of each panel. Experiments were repeated to confirm results.

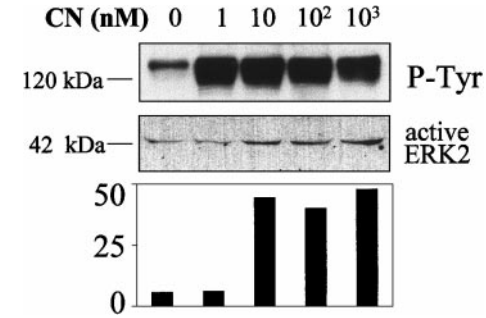

FIG. 2. Dose response of contortrostatin-induced signaling events in suspended T24 cells. Whole cell lysates were immunoblotted for phosphotyrosine (P-Tyr) or activated ERK2 after treatment with the indicated concentrations of contortrostatin (CN) for 10 min at 37°C. Since protein levels are not expected to change during the short 10 min incubation, ERK loading control blots are excluded. A densitometry plot with arbitrary units is shown for ERK2. Results shown are representative of three similar experiments.

which they adhered (Fig. 1). Cells bound to contortrostatin, as well as fibronectin and vitronectin, showed a peak in ERK2 activation at 30 min and declining activation at 180 min. These findings are consistent with other reports indicating that ERK2 activation following adhesion to extracellular matrix proteins is transient (8, 24) and suggests that cells are unable to distinguish contortrostatin from other RGD-containing ligands under these conditions.

Contortrostatin treatment of suspended T24 cells activates tyrosine phosphorylation and ERK2 with distinct dose-response patterns. Quiescent cells were treated for 10 min while in suspension with various concentrations of contortrostatin and protein tyrosine phosphorylation content was measured by anti-phosphotyrosine immunoblot. As previously reported (20) it was observed that contortrostatin-treated cells displayed dramatic activation of tyrosine phosphorylation of proteins in the size range of 120–140 kDa (Fig. 2). With higher concentrations of contortrostatin, a reduction in tyrosine phosphorylation levels was observed. ERK2 was also found to be modestly activated in response to contortrostatin treatment, but the dose response was distinct from that of the tyrosine phosphorylation events, suggesting that these two phenomena are regulated by different pathways.

Contortrostatin-induced alterations in tyrosine phosphorylation, but not ERK2 activation, are mediated by $\alpha v\beta 3$. Our previous work had shown that the $\alpha v\beta 3$ integrin is the exclusive mediator of tyrosine phosphorylation events stimulated by contortrostatin (20). In order to determine if $\alpha v\beta 3$ is also mediating contortrostatin-induced ERK2 activation, T24 cells were pretreated with various concentrations of 7E3, a monoclonal antibody generated against αIIb$\beta 3$ that has equal reactivity to $\alpha v\beta 3$ (25). T24 cells do not express αIIb$\beta 3$ as shown by a lack of staining with a 10E5, a specific antibody for αIIb$\beta 3$ (data not shown). We found that 7E3 at 1000 nM was able to completely block the tyrosine phosphorylation effects of 10 nM contortrostatin treatment (Fig. 3). The existence of multiple pathways regulated by contortrostatin was again evidenced by the observation that 7E3 had no effect on ERK2 activation in the same lysates. The involvement of the Src family of tyrosine kinases was explored in these experiments through use of a specific Src family inhibitor, PP1 (26). Inhibition of Src family kinase activity

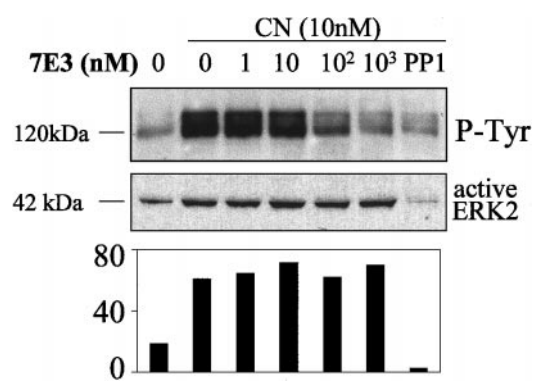

FIG. 3. Contortrostatin-induced tyrosine phosphorylation is mediated by the $\alpha v\beta 3$ integrin, but ERK2 activation is not. Whole cell lysates were probed for phosphotyrosine (P-Tyr) content or activated ERK2. Cells were pretreated with anti-$\alpha v\beta 3$ mAb (7E3) or the Src family kinase inhibitor (PP1) for 10 min prior to additional 10 min incubation at 37°C with 10 nM contortrostatin (CN). A densitometry plot with arbitrary units is shown for ERK2 activation. Results are representative of two similar experiments.

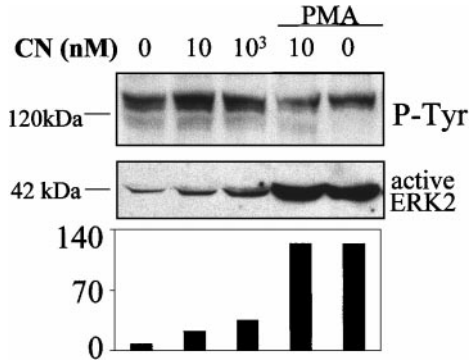

FIG. 4. Contortrostatin activates ERK2 in $\alpha v\beta 3$-negative cells but fails to stimulate tyrosine phosphorylation (P-Tyr). OVCAR-5 cells, which lack expression of $\alpha v\beta 3$, were treated with the indicated concentrations of contortrostatin (CN) for 10 min at 37°C, or were pretreated with the PKC activator, PMA at 10 nM for 30 min prior to treatment with contortrostatin. A densitometry plot with arbitrary units is shown for ERK2 activation. Results are representative of two similar experiments.

with 10 μM PP1 blocked the effects of 10 nM contortrostatin on tyrosine phosphorylation in these cells. From this result it can be concluded that contortrostatin-induced changes in tyrosine phosphorylation levels depend on the activity of Src family kinases. Interestingly, contortrostatin activation of ERK2 was also completely abrogated in the presence of PP1. Thus, these results support earlier findings that the $\alpha v\beta 3$ integrin mediates contortrostatin-induced tyrosine phosphorylation (20) and indicate that contortrostatin activation of ERK2 is independent of this receptor. Both pathways appear to require activation of the Src family kinases since inhibition of these enzymes completely eliminates contortrostatin-induced effects on both tyrosine phosphorylation and ERK2 activation.

Contortrostatin activates ERK2 in $\alpha v\beta 3$-negative cells, but tyrosine phosphorylation is unaffected. Further investigations into the different pathways regulated by contortrostatin were performed in OVCAR-5 human ovarian cancer cells. These cells were found to lack expression of the $\alpha v\beta 3$ integrin, but do express $\alpha v\beta 5$, an integrin to which contortrostatin has been shown to bind (22). This provided another opportunity to distinguish the role of $\alpha v\beta 3$ from other integrins in mediating contortrostatin signals. When contortrostatin-treated OVCAR-5 cell lysates were immunoblotted for phosphotyrosine, little change was observed (Fig. 4), clearly contrasting with the effects of contortrostatin on tyrosine phosphorylation in $\alpha v\beta 3$-expressing cells (Fig. 2). However, when ERK2 activation was measured, contortrostatin was found to cause a modest, dose-dependent activation of ERK2 in these cells.

It has been reported that signaling mediated by the $\alpha v\beta 5$ integrin is dependent on the activity of protein kinase C (PKC) (27). With the knowledge that contortrostatin binds $\alpha v\beta 5$, and since PKC is likely inactive under the serum-free conditions used here, we pretreated cells for 30 min with phorbol ester (PMA) to determine if PKC activation would effect contortrostatin-induced tyrosine phosphorylation or ERK2 activation. PMA pretreatment (10 nM) had no enhancing effect on tyrosine phosphorylation levels in the presence of contortrostatin (Fig. 4). When the same lysates are examined for ERK2 activation, PMA treatment alone caused dramatically increased activation, but had no effect on ERK2 activation in contortrostatin-treated cells. Thus the ability of contortrostatin to affect tyrosine phosphorylation events and ERK2 activation appears to be independent of the activation status of PKC.

DISCUSSION

The study of integrins as signaling molecules has gained the interest of researches in the fields of tumor biology and angiogenesis. Work in our laboratory on the antitumor and antiangiogenic properties of contortrostatin suggested the possibility that this disintegrin would have effects on integrin signaling, contributing to its inhibition of these processes. Indeed, contortrostatin does effect the levels of tyrosine phosphorylated proteins within tumor cells, and it has been shown that the $\alpha v\beta 3$ integrin is solely responsible for mediating these changes (20). In an earlier study conducted in platelets, contortrostatin treatment was shown to have $\alpha IIb\beta 3$-mediated effects on tyrosine phosphorylation distinct from that of a monomeric disintegrin (28). The most notable difference was that contortrostatin treatment caused an increase in tyrosine phosphorylation of several proteins, while the monomeric disintegrin did not, suggesting that the unique homodimeric structure of contortrostatin imbues it with added function.

More recent studies have shown that contortrostatin activates tyrosine phosphorylation of FAK and CAS in tumor cells (20). Other investigators have demonstrated that the activity of ERK2 is regulated by integrin signaling (2, 8). Our study of this important signaling molecule revealed that contortrostatin can positively affect ERK2 activation, but appears to do so through a pathway different than the pathway regulating tyrosine phosphorylation. These findings distinguish contortrostatin as an active regulator of integrin function and show that it acts as more than a passive integrin-blocking agent. The downstream events that may be effected by contortrostatin are numerous and include disruptions in motility and cell cycle progression. Other work in our laboratory has shown that contortrostatin inhibits the migration of tumor cells (18, 20, 22, 23) and is an inhibitor of angiogenesis (19). Activation of ERK2 is often associated with enhanced MLCK activity and increased migration (6). However,

our findings suggest that activation of ERK2 in this context may have a negative effect on migration. A possible reconciliation for this may be found when considering that precise temporal and spatial regulation of ERK2 activity might be necessary for its ability to function as a positive regulator of motility. Contortrostatin might thus be causing a dysregulation of ERK2 leading to decreased migratory capacity. Many other cellular functions are controlled by the MAP kinases, including proliferation. The fact that contortrostatin acts as an inhibitor of angiogenesis and cancer progression raises the likelihood that contortrostatin may also have negative effects on the ability of cells to divide, a hypothesis that remains to be investigated.

Although we have shown that the $\alpha v\beta 3$ integrin is responsible for mediating contortrostatin-induced tyrosine phosphorylation, the receptor(s) involved in transmitting the signal leading to activation of ERK2 remains unidentified. This diverging pathway may be controlled by another vitronectin receptor, $\alpha v\beta 5$. Previous work has identified four integrins to which contortrostatin binds: $\alpha IIb\beta 3$, $\alpha 5\beta 1$, $\alpha v\beta 3$ and $\alpha v\beta 5$ (16–18, 22). Since the monomeric disintegrin echistatin inhibited contortrostatin-induced tyrosine phosphorylation effects by blocking $\alpha v\beta 3$ (20, 21) but was unable to block ERK2 activation (data not shown), and since there are no reports of echistatin binding to $\alpha v\beta 5$, it is possible that this integrin mediates contortrostatin-induced activation of ERK2. This idea is supported by our findings with ovarian cancer cells (OVCAR-5) which lack expression of $\alpha v\beta 3$ but do express $\alpha v\beta 5$. Contortrostatin influenced ERK2 activation but not tyrosine phosphorylation in these cells. The role of $\alpha v\beta 5$ can be tested by using an antibody that blocks contortrostatin binding to this integrin, however we have not found an antibody that is capable of this. Epitope incompatibilities are the likely cause of the inability of these antibodies to block contortrostatin binding to $\alpha v\beta 5$. A similar situation exists with respect to the $\alpha 5\beta 1$ integrin. We have previously reported that contortrostatin interacts with $\alpha 5\beta 1$, enabling it to block adhesion to fibronectin (17). However, reports of the existence of different $\alpha 5\beta 1$ conformations (29–31) lead us to believe that contortrostatin may bind to only one specific conformation. In previous solid phase cell adhesion studies, we have observed contortrostatin binding to $\alpha 5\beta 1$ in K562 leukemia cells, but we observed no binding of contortrostatin to this integrin in KSY-1 Kaposi's sarcoma cells. Identifying the receptor(s) responsible for transmitting contortrostatin-initiated signals to ERK2 remains an area of interest in our laboratory. The most likely resolution to this problem lies in the engineering of cells with the desired integrin expression profile that are not only capable of binding contortrostatin but also have intact (nonmutated) signaling pathways leading to ERK2 activation.

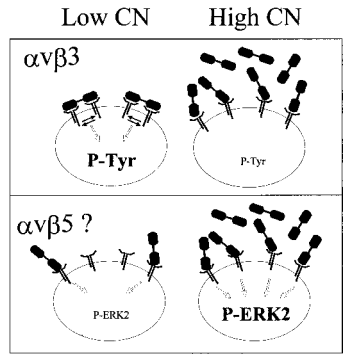

FIG. 5. A hypothetical model depicting two mechanisms leading to induction of tyrosine phosphorylation (P-Tyr) and activation of ERK2 (P-ERK2). (Upper panel) At low concentrations, contortrostatin (CN) is able to crosslink $\alpha v\beta 3$ integrins and initiate a pathway leading to tyrosine phosphorylation events. At high concentrations, each subunit of the dimer competes for binding sites, leading to a distinct binding orientation that fails to initiate tyrosine phosphorylation. (Lower panel) It is proposed that the $\alpha v\beta 5$ (or possibly $\alpha 5\beta 1$) integrin mediates contortrostatin-induced activation of ERK2. This model depicts ERK2 activation as being independent of integrin crosslinking, where increased activation coincides with increased dose.

It is well established that simple dimerization of integrins is sufficient to initiate tyrosine phosphorylation events (32). This has been accomplished with crosslinked anti-integrin antibodies (33) and multimeric integrin ligands (34). When taking into account that contortrostatin is composed of two identical subunits, both containing the integrin-binding RGD motif (17), it is probable that the ability of contortrostatin to effect tyrosine phosphorylation in tumor cells is directly related to its ability to crosslink individual $\alpha v\beta 3$ integrins. The reduction in tyrosine phosphorylation seen at higher contortrostatin concentrations is a consistent observation (20) and might be explained by imagining two binding orientations of the contortrostatin dimer, one in which each subunit is bound by an integrin, and the other where only one subunit is bound (Fig. 5). At low concentrations, each subunit would be given an opportunity to bind to an integrin, bringing two integrins into close proximity and allowing for the initiation of a signaling cascade. At high concentrations, all contortrostatin subunits will, in effect, compete with each other for binding sites. These conditions will force many of the contortrostatin dimers into a binding orientation with only one subunit bound, and it would be expected that a reduction in the effects on tyrosine phosphorylation would be observed. Although it is likely that integrin dimerization is essential for contortrostatin-induced tyrosine phosphorylation, the role of dimerization in influencing ERK2 activation is not clear. It has been suggested that integrin ligation, in the absence of dimerization, can initiate signals as well (35), and it is possible that this

mechanism is operating during contortrostatin activation of ERK2 (Fig. 5).

This work extends earlier findings that contortrostatin functions as more than a simple integrin antagonist, activating integrin signals leading to tyrosine phosphorylation of specific proteins. ERK2 is identified as an important signaling molecule activated by contortrostatin and provides a basis for further work into the downstream signaling effects of this unique disintegrin. Evidence is provided that, due to its ability to bind multiple integrins, contortrostatin influences more than one signaling pathway. The significance of the $\alpha v\beta 3$ and $\alpha v\beta 5$ integrins in angiogenesis and cancer progression suggest that contortrostatin may be an valuable tool for the further investigation of the roles of these receptors in these important biological processes.

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