TGFβ and IGF1R signaling activate protein kinase A through differential regulation of ezrin phosphorylation in colon cancer cells

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ABSTRACT

Aberrant cell survival plays a critical role in cancer progression and metastasis. We have previously shown that ezrin, a cAMP-dependent A-kinase anchoring protein (AKAP), is upregulated in colorectal cancer (CRC) liver metastasis. Phosphorylation of ezrin at threonine 567(T567) activates ezrin and plays an important role in CRC cell survival associated with XIAP and survivin upregulation. In this study, we demonstrate that, in FET and GEO colon cancer cells, knockdown of ezrin expression or inhibition of ezrin phosphorylation at T567 increases apoptosis through PKA activation in a cAMP-independent manner. TGFβ signaling inhibits ezrin phosphorylation in a Smad3-dependent and Smad2-independent manner and regulates pro-apoptotic function through ezrin-mediated PKA activation. On the other hand, ezrin phosphorylation at T567 by IGF1R signaling leads to cAMP-dependent PKA activation and enhances cell survival. Further studies indicate that phosphorylated ezrin forms a complex with PKA RII and dephosphorylated ezrin dissociates from the complex and facilitates the association of PKA RII with AKAP149, both of which activate PKA yet lead to either cell survival or apoptosis. Thus, our studies reveal a novel mechanism of differential PKA activation mediated by TGFβ and IGF1R signaling through regulation of ezrin phosphorylation in CRC resulting in different cell fate. This is of significance because TGFβ and IGF1R signaling pathways are well characterized tumor suppressor and oncogenic pathways respectively with important roles in CRC tumorigenesis and metastasis. Our studies indicate that they crosstalk and antagonize each other’s function through regulation of ezrin activation. Therefore, ezrin may be a potential therapeutic target in CRC.

INTRODUCTION

Colorectal cancer (CRC) is the third leading cause of cancer related death in the United States. Stage I and II cancers that are confined within the wall of the colon are curable by surgical resection; however, the survival rate of cancer patients is drastically reduced when cancer metastasizes to distant organ sites such as liver and/or lungs (1,2). Despite significant improvements in early diagnosis and treatment of CRC, metastasis and recurrence of the disease remain the main cause of cancer death (1,3,4). Genetic and epigenetic changes pivotal for metastasis are acquired at late stages of CRC during progression to advanced disease (1). Therefore, identification of these changes and understanding the underlying mechanisms are critical for the development of novel anti-metastatic therapies.

Our recent studies have demonstrated that expression of ezrin is upregulated in liver metastases when compared to primary tumors in an orthotopic model of colon cancer; and that ezrin expression is increased in primary tumors from
colon cancer patients (5). Ezrin, a member of the ezrin radixin moesin (ERM) family (6-9), exists in two conformations, an active open form mainly localized at the plasma membrane and a dormant closed form largely resides in the cytoplasm. Inactive ezrin forms oligomers where the C-terminal domain folds back and binds tightly to the FERM domain, masking several of its active sites (8,10,11). Phosphorylation at threonine 567 (T567) transitions ezrin from inactive oligomers into active monomers by unmasking the active sites through dissociation of FERM and C-terminal domains (11-15). Ezrin plays an important role in cell motility and invasion and has been implicated in metastasis of several types of cancer including CRC (16-21). In addition, ezrin plays a key role in cell survival of colon cancer cells and regulates expression of Inhibitor of Apoptosis Proteins (IAP), XIAP and survivin, which have been shown to be involved in cell survival and metastasis (5,22). We have previously shown that IGF1R signaling regulates ezrin phosphorylation at T567 (5). Other studies have shown that ezrin is a downstream effector of the PI3K/AKT pathway (23,24). Nevertheless, little is known of the mechanism of ezrin-mediated cell survival.

Dransfield et al. characterized ezrin as a cyclic AMP (cAMP)-dependent A-kinase anchoring protein (AKAP) (25). There are more than 50 AKAPs identified. Protein kinase A (PKA) consists of catalytic subunits and inhibitory regulatory subunits and plays a dominant role in the integration of multiple signal transduction networks (26). AKAPs interact with the regulatory subunits of PKA and target these supramolecular complexes to specific subcellular localizations, where they regulate phosphorylation of specific substrates and execute different functions (27,28). For example, AKAP149/PKA contributes to the disruption of the XIAP/survivin complex through phosphorylation of survivin at serine 20, leading to proteasome-mediated degradation of XIAP (29,30).

In this study, we demonstrate that knockdown of ezrin expression or inhibition of ezrin phosphorylation in GEO and FET cells increases apoptosis through activation of PKA in a cAMP-independent manner. AKAP149 plays an important role in this process. In addition, we show that TGFβ inhibits ezrin phosphorylation at T567 in a Smad2-independent and Smad3-dependent manner, resulting in PKA activation and induction of apoptosis. On the other hand, phosphorylation of ezrin at T567 by IGF1R signaling leads to cAMP-dependent PKA activation and increased cell survival. Further studies indicate that phosphorylated ezrin displays more association with PKA RII than dephosphorylated ezrin; hypophosphorylation of ezrin facilitates complex formation of PKA RII and AKAP149 whereas hyperphosphorylation of ezrin reduces their association. Therefore, our studies uncover a novel mechanism of differential activation of PKA mediated by TGFβ and IGF1R signaling through regulation of the phosphorylation status of ezrin, which leads to different cell fates. Given the importance of TGFβ and IGF1R signaling in CRC, it implies that ezrin may be a potential therapeutic target in CRC.

RESULTS

Knockdown of ezrin expression activates PKA and induces apoptosis in colon cancer cells

Recently, we demonstrated that transient knockdown of ezrin using siRNA leads to downregulation of XIAP and survivin expression (5). To understand the underlying molecular mechanism, stable knockdown (KD) of ezrin expression was performed using GIZF lentiviral shRNA#1 and #3 in GEO and FET colon cancer cells. A non-targeting shRNA (NT sh) was used as a control. Ezrin expression was significantly reduced by ezrin shRNAs in both cell lines (Figure 1A). As a result, DNA fragmentation assays showed an approximately 2-fold increase in apoptosis in ezrin KD cells when compared to the NT sh control cells (Figure 1B). In addition, cleaved caspase 7 was higher in ezrin KD cells than in NT sh cells (Figure 1C), confirming the results of DNA fragmentation assays. Expression of XIAP and survivin was downregulated in both GEO and FET cells (Figure 1C). These results indicated that inhibition of ezrin expression leads to increased apoptosis associated with reduced XIAP and survivin expression in colon cancer cells. PKA activation has been shown to disrupt XIAP/survivin complex formation, leading to degradation of XIAP and survivin and induction of apoptosis (30). We next determined whether ezrin KD had any effect on PKA activity. PKA activity
assays showed that ezrin KD increased PKA activation by 2-3 fold relative to NT sh control (Figure 1D). Phosphorylation of CREB, a direct target of PKA (31), was increased in ezrin KD cells, and pretreatment with H89, a specific PKA inhibitor which binds to the PKA catalytic α subunit to inhibit its activation, completely abrogated ezrin KD-mediated increase in CREB phosphorylation (Figure 1E), supporting the ability of ezrin KD to activate PKA. Further studies showed that ezrin KD dissociated PKA catalytic α subunit from its inhibitory regulatory subunit RII (Fig. 1F). Since cAMP binds to the PKA regulatory subunits and dissociates them from the catalytic subunits to activate PKA (29,30), we next determined whether ezrin KD increased cAMP production. cAMP levels were measured using a non-radioactive cAMP enzyme immunoassay. Forskolin, a PKA activator, was used as a positive control. Treatment of forskolin led to a significant increase in cAMP levels (Figure 1G). In contrast, ezrin KD was unable to increase cAMP production in both GEO and FET cells (Figure 1G). These results indicate that ezrin KD activates PKA in a cAMP-independent manner.

To determine whether ezrin KD induces apoptosis through PKA activation, expression of the PKA catalytic α subunit was knocked down by a shRNA in FET cells (designated PKACatα KD) (Figure 2A). As expected, PKACatα KD markedly reduced endogenous PKA activity and blocked ezrin KD-induced PKA activation (Figure 2B). There was no difference in ezrin phosphorylation at T567 and expression of total ezrin between PKACatα KD and scrambled shRNA control cells (Figure 2A). Although knockdown of PKACatα did not affect XIAP and survivin expression (Figure 2A), it blocked ezrin KD-mediated downregulation of XIAP and survivin expression (Figure 2C). Consequently, PKACatα KD did not induce apoptosis, but blocked apoptosis induced by ezrin KD (Figure 2D). These results demonstrated that ezrin KD mediates apoptosis through PKA activation.

### Inhibition of ezrin phosphorylation at T567 leads to PKA activation and induction of apoptosis.

Ezrin is present in an inactive and closed conformation in the cytoplasm and phosphorylation at T567 activates ezrin (11). Previously, we have shown that ezrin is hyperphosphorylated at T567 in CRC liver metastasis when compared to primary tumors (5). We, therefore, hypothesized that inhibition of ezrin phosphorylation at T567 would inactivate ezrin, leading to PKA activation and induction of apoptosis. To test this hypothesis, site directed mutagenesis was performed. An ezrin phospho-deficient mutant (designated as T567A), in which threonine 567 was replaced by alanine, was generated. GFP-tagged ezrin T567A was introduced into ezrin KD cells and GFP-tagged wild type ezrin (designated as WT) was used as a control (Figure 3A). Expression of ezrin T567A led to downregulation of XIAP and survivin expression (Figure 3A), increased apoptosis (Figure 3A & 3B) and enhanced PKA activation (Figure 3C), whereas restoration of WT ezrin increased XIAP and survivin expression (Figure 3A), decreased apoptosis (Figure 3A & 3B) and reduced PKA activation (Figure 3C) to levels observed in control cells. Ezrin T567A-mediated PKA activation was independent of cAMP since cAMP levels remained unchanged in ezrin T567A- or WT ezrin-expressing cells (Figure 3D). These results indicate that inhibition of ezrin phosphorylation at T567 activates PKA and induces apoptosis in colon cancer cells.

We next determined the effects of NSC668394 (designated as NSC), a small molecule inhibitor which inhibits ezrin phosphorylation at T567 (32), on PKA activation and apoptosis of colon cancer cells. Treatment of GEO and FET cells with increasing concentrations of NSC showed a dose-dependent inhibition of ezrin phosphorylation at T567, with no change in the levels of total ezrin (Figures 4A). Expression of XIAP and survivin were downregulated by NSC in a dose-dependent manner (Figure 4A). In addition, a 2 to 4- and 3 to 5- fold increase in apoptosis was observed after NSC treatment for 2 and 6 hours respectively (Figure 4B). Moreover, PKA activity assays showed a significant time-dependent activation of PKA by NSC treatment in GEO and FET cells, which was abrogated by pretreatment with H89 (Figure 4C). Forskolin was included as a positive control (Figure 4C). As expected, NSC-mediated PKA activation was independent of cAMP (Figure 4D). These results indicate that inhibition of phosphorylation of ezrin at T567 by NSC activates PKA and induces
apoptosis in colon cancer cells. Importantly, knockdown of the PKA catalytic α subunit in FET cells blocked NSC-mediated downregulation of XIAP and survivin expression (Figure 4E), prevented NSC-induced apoptosis (Figure 4F) and abrogated NSC-mediated PKA activation (Figure 4G). These results demonstrate that inhibition of phosphorylation of ezrin at T567 by NSC leads to apoptosis through PKA activation.

**TGFβ inhibits ezrin phosphorylation at T567 resulting in PKA activation and apoptosis**

TGFβ signaling plays an important role in tumorigenesis and metastasis in many cancers, including CRC (33). It has been shown that abrogation of TGFβ signaling promotes cell survival under stress and increases metastatic potential of colon cancer cells (34). We have previously shown that TGFβ activates PKA in a cAMP-dependent manner leading to apoptosis, and that this activation is dependent on Smad3 (30,35). TGFβ signaling also downregulates XIAP and survivin expression (30). These results suggest that TGFβ may function through ezrin regulation. We therefore determined whether TGFβ signaling regulates ezrin phosphorylation and activation. Treatment of FET cells with TGFβ1 resulted in a time-dependent decrease in ezrin phosphorylation at T567 (Figure 5A). To further determine whether TGFβ-mediated inhibition of ezrin phosphorylation is Smad-dependent, expression of Smad2 and Smad3 was knocked down individually in FET cells by shRNAs specific for Smad2 or Smad3 (Figure 5B). Knockdown of Smad2 or Smad3 had little effect on the basal levels of ezrin phosphorylation at T567 (Figure 5C). However, TGFβ-mediated inhibition of ezrin phosphorylation was prevented in Smad3 knockdown cells but not in Smad2 knockdown cells (Figure 5C). These results indicate that TGFβ inhibits ezrin phosphorylation in a Smad3-dependent and Smad2-independent manner.

Previous studies have shown that TGFβ signaling induces apoptosis in colon cancer cells in a Smad3-dependent manner (30,35). To demonstrate that TGFβ activates PKA and induces apoptosis through inhibition of ezrin activation, ezrin expression was knocked down in FET cells (Figure 1A). While TGFβ markedly increased PKA activity in NT sh control cells, it activated PKA to a much lesser degree in ezrin KD cells (Figure 5D). Consistently, inhibition of ezrin phosphorylation by NSC treatment attenuated TGFβ-induced PKA activation (Figure 5E). Furthermore, knockdown of ezrin expression reduced TGFβ–induced apoptosis (Figure 5F). These results indicate that TGFβ activates PKA and increases apoptosis at least partially through ezrin inhibition.

Of note, knockdown of ezrin had no effect on expression of TGFβRI and TGFβRII (Figure 5G), expression of Smad3 or TGFβ-mediated Smad3 phosphorylation (Figure 5H), indicating that ezrin does not affect canonical TGFβ signaling.

**Phosphorylation of ezrin at T567 leads to PKA activation and cell survival**

IGF1R signaling plays an important role in colon cancer cell survival (36,37). Whereas its inhibition leads to downregulation of XIAP and survivin and induction of apoptosis (36), its activation by transferrin and insulin (TI) increases cell survival (37). We have previously shown that inhibition of IGF1R decreases ezrin phosphorylation at T567 (5), indicating that IGF1R-mediated signaling activates ezrin by increasing T567 phosphorylation. Consistently, treatment of GEO cells with TI, which activates IGF1R signaling (37), increased ezrin phosphorylation at T567 and concurrently enhanced expression of XIAP and survivin and reduced apoptosis (Figure 6A, B). Knockdown of ezrin expression abrogated TI-mediated pro-survival effects (Figure 6A, B). Importantly, TI treatment activated PKA and increased cAMP production (Figure 6C & 6D). These results suggest that phosphorylation of ezrin at T567 activates PKA in a cAMP-dependent manner. To determine whether this is the case, an ezrin phosho-mimetic mutant (designated as T567D), in which threonine 567 was replaced by aspartic acid, was generated. GFP-tagged ezrin T567D was introduced into ezrin KD cells and GFP-tagged WT ezrin was used as a control (Figure 6E). Analysis of PKA activity showed that expression of WT ezrin reversed the stimulative effect of ezrin KD on PKA activation whereas expression of ezrin T567D further increased PKA activation (Figure 6F) and enhanced production of cAMP (Figure 6G). It indicates that, in contrast to ezrin T567A-mediated PKA activation which is cAMP-
independent (Figure 3D), ezrin T567D activates PKA in a cAMP-dependent manner. Moreover, overexpression of WT ezrin increased expression of XIAP and survivin (Figure 6E) and prevented apoptosis induced by ezrin KD (Figure 6H) whereas overexpression of ezrin T567D not only blocked ezrin KD-induced apoptosis but further increased cell survival as compared NT sh control (Figure 6H). Taken together, these studies indicate that phosphorylation of ezrin at T567 activates PKA and enhances cell survival.

AKAP149 contributes to ezrin inhibition-mediated PKA activation

It has been shown that AKAP/PKA interaction is a prerequisite for targeting PKA and its substrate complexes to specific subcellular locations (27). To determine whether AKAP is involved in ezrin inhibition-mediated PKA activation, a pan-AKAP inhibitor Ht31 was utilized. Pretreatment with Ht31 abrogated NSC-mediated PKA activation in FET and GEO colon cancer cells (Figure 7A). These results indicate that one or more AKAPs contribute to ezrin inhibition-mediated PKA activation. It has been previously shown that TGFβ/Smad3-mediated PKA activation is dependent upon AKAP149 (30,35). We therefore used siRNA knockdown strategies to determine whether AKAP149 is required for PKA activation by ezrin inhibition. Knockdown of AKAP149 had little effect on ezrin expression and phosphorylation, and ezrin KD did not affect AKAP149 expression (Figure 7B). While AKAP149 KD had no effect on XIAP and survivin expression in the control cells, it slightly increased their expression in ezrin KD cells (Figure 7B). In addition, AKAP149 KD reduced apoptosis in ezrin KD cells but not in the control cells (Figure 7C). Interestingly, knockdown of AKAP149 did not affect PKA activation in the control cells; however, it completely abrogated ezrin inhibition-mediated PKA activation by NSC (Figure 7D). These results indicate that AKAP149 is essential for PKA activation when ezrin activation is inhibited. However, it has little effect on PKA activation when ezrin is activated. Given that AKAPs bind the regulatory subunits of PKA (PKA RI or PKA RII), leading to the dissociation of regulatory subunits from the catalytic α subunit and activation of PKA, and that AKAP149 binds PKA RII (30), we next determined the complex formation of PKA RII and AKAP149 by immunoprecipitation (IP) analysis. As shown in Figure 7E, ezrin KD increased PKA RII/AKAP149 complex formation. While expression of ezrin T567A further increased the association of PKA RII and AKAP149, expression of ezrin T567D markedly decreased their association (Figure 7E). These results indicate that hypophosphorylation of ezrin facilitates complex formation of PKA RII and AKAP149 whereas hyperphosphorylation of ezrin prevents their association. It suggests that phosphorylation of ezrin enhances the complex formation of ezrin and PKA RII. To investigate whether this is the case, PKA RII/ezrin complex formation was determined by IP analysis. The results showed that ezrin T567D displays more association with PKA RII than ezrin T567A (Figure 7F). These studies indicate that phosphorylated ezrin forms a complex with PKA RII and dephosphorylated ezrin dissociates from the complex and facilitates the association of PKA RII with AKAP149.

Taken together, our studies suggest a novel model of differential activation of PKA mediated by the TGFβ/Smad3/AKAP149 or IGF1R/ezrin signaling axis (Figure 8). In the presence of TGFβ, TGFβ/Smad3 inhibits ezrin phosphorylation at T567, leading to the association of AKAP149 with PKA RII to activate PKA in a cAMP-independent manner, suppress XIAP and survivin expression and induce apoptosis. In the context of IGF1R activation, ezrin is hyperphosphorylated at T567, which results in the association of ezrin with PKA RII and PKA activation. This activation of PKA is cAMP-dependent, increases expression of XIAP and survivin and enhances cell survival.

DISCUSSION

We have shown in this study that ezrin mediates cell survival through PKA activation. Ezrin knockdown or inhibition leads to PKA activation in a cAMP-independent manner and induces apoptosis associated with downregulation of XIAP and survivin expression. Further studies indicate that PKA activation by ezrin inhibition is dependent upon AKAP149, knockdown of which abrogated ezrin inhibition-mediated PKA activation and induction of apoptosis. In addition, activation of TGFβ signaling dephosphorylates ezrin at T567 in a Smad3-dependent and Smad2-
Independent manner and TGFβ/Smad3 induces its pro-apoptotic effects through inhibition of ezrin phosphorylation. On the other hand, ezrin hyperphosphorylation at T567 mediated by IGF1R signaling leads to PKA activation in a cAMP-dependent manner, promoting cell survival associated with XIAP and survivin upregulation. These findings suggest that TGFβ and IGF1R signaling antagonize each other by differentially regulating ezrin phosphorylation, which activates PKA through different mechanisms and mediates the equilibrium between cell survival and apoptosis. Therefore, PKA signaling plays an important role in the integration of multiple signal transduction pathways in colon cancer cells. Of note, serine/threonine kinases such as protein kinase B/Akt2, Rho-kinase, protein kinase Cα (PKCα), PKC0, G protein-coupled receptor kinase 2 (GRK2) and Cdc42 have been implicated as mediators of ezrin phosphorylation at T567 (38-43). In addition, myosin phosphatase and type 2C protein phosphatase (PP2C) have been suggested to dephosphorylate T567 in ERM proteins (12,40). Further studies are needed to determine whether TGFβ and IGF1R signaling regulate these kinases and/or phosphatases to modulate ezrin phosphorylation and activation.

Previous studies have shown that deregulation of AKAPs contributes to cancer (44,45). We have shown that ezrin is hyperphosphorylated at T567 in CRC liver metastasis when compared to primary tumors (5). Our present studies indicate that phosphorylation of ezrin at T567 enhances the complex formation of ezrin and PKA RII to activate PKA in favor of cell survival whereas dephosphorylation of ezrin at T567 facilitates the association of AKAP149 and PKA RII, which activates PKA and leads to induction of apoptosis. These results suggest that phosphorylation of ezrin at T567 acts as a signaling node, determining differential PKA activation accomplished by different AKAP association with PKA RII, leading to different cell fate. This adds to the complexity of the context-dependent AKAP-PKA interaction, which mediates different PKA function by directing PKA supramolecular complexes to various substrates, activating different downstream signaling. While ezrin-PKA association may enable downstream signaling pathways that promote survival, AKAP149-PKA association likely activates the apoptotic pathways to induce apoptosis. Determining the dynamics of the context-dependent interaction between PKA and different AKAPs, and the mechanisms underlying the activation of different downstream signaling pathways will be critical for understanding the function of AKAPs in regulating cell survival and metastasis of colon cancer cells.

XIAP and survivin are anti-apoptotic proteins important for cell survival. Independent of their cytoprotective function, XIAP and survivin also play important roles in tumor cell invasion and metastasis (22). It has been shown that PKA/AKAP149 signaling contributes to the disruption of the association of XIAP/survivin through phosphorylation of survivin at serine 20, leading to proteasome-mediated degradation of XIAP and induction of apoptosis (29,30). This regulation involves protein phosphatase 2A (PP2A)-mediated dephosphorylation of Akt (30). However, it is not clear how ezrin/PKA enhances expression of XIAP/survivin. In addition to regulation of its expression, it has been shown that, under stress, pro-apoptotic proteins, XAF1, Smac and Omi, interact with XIAP to inhibit its anti-apoptotic function (46). Given the importance of XIAP and survivin in cancer metastasis and relative lack of knowledge of their regulation, more studies are needed to determine the mechanisms of regulation of their expression and function.

In summary, we have made novel observations that TGFβ/Smad3 and IGF1R signaling activate PKA through differential regulation of ezrin phosphorylation and activation, leading to induction of apoptosis or promotion of cell survival (Figure 8). Our studies uncover a novel mechanism by which TGFβ and IGF1R signaling crosstalk to mediate apoptosis and cell survival. This is of significance because TGFβ and IGF1R signaling pathways are well characterized tumor suppressor and oncogenic pathways respectively with important roles in CRC tumorigenesis and metastasis. Since they both function through regulation of ezrin activation, ezrin could be used as a potential therapeutic target in CRC treatment.

**EXPERIMENTAL PROCEDURES**

**Cell lines**
FET and GEO cell lines were originally derived from primary tumors obtained from two different colon cancer patients (47). Both cell lines harbor mutations in APC/β-catenin and K-Ras (48) and have been used by various groups to investigate colon cancer pathways (49-56). Cells were maintained at 37°C in a humidified atmosphere, using chemically defined serum-free medium consisting of McCoy’s 5A medium (Sigma-Aldrich) supplemented with amino acids, pyruvate, vitamins, antibiotics, and growth factors transferrin (4μg/ml; Sigma-Aldrich), insulin (20μg/ml; Sigma-Aldrich), and EGF (10ng/ml; R&D Systems). Supplemented McCoy’s medium (“SM”) is McCoy’s 5A medium supplemented with antibiotics and nutrients but lacking growth factors. Cells were routinely sub-cultured with 0.25% trypsin (Invitrogen) and 0.1% EDTA.

**Antibodies**

The following primary antibodies were obtained from Cell Signaling Technology (Danvers, MA): Survivin rabbit polyclonal antibody (catalog#2803), XIAP rabbit monoclonal antibody (catalog#2045), AKAP149 rabbit monoclonal antibody (catalog#5203), PKACα rabbit monoclonal antibody (catalog#5842), TGFβRII rabbit polyclonal antibody (catalog#3712), Smad2 rabbit monoclonal antibody (catalog#3122), Smad3 rabbit monoclonal antibody (catalog#9523), p-Smad3 rabbit monoclonal antibody (catalog#9520), CREB rabbit polyclonal antibody (catalog#9197), p-CREB rabbit monoclonal antibody (catalog#9198) and Caspase 7 rabbit polyclonal antibody (catalog#9492). Ezrin mouse monoclonal antibody (catalog#sc-71082) and TGFβRII mouse monoclonal antibody (catalog#sc-17791) were obtained from Santa Cruz Biotechnology (Dallas, TX). P-ezrinT567 rabbit polyclonal antibody (catalog#ab47293) and PKARIII mouse monoclonal antibody (catalog#ab124400) were obtained from Abcam (Cambridge, MA). Actin rabbit antibody (catalog#A2066) was obtained from Sigma-Aldrich (St. Louis, MO).

**Pharmacological antagonists**

Ezrin small molecule inhibitor NSC668394 (NSC) was purchased from Millipore.

TGFβ1 was purchased from R&D Systems. PKA inhibitor H89 (catalog#9844) and forskolin (catalog#3828) were obtained from Cell Signaling Technology. Pan-AKAP inhibitor Ht31 (catalog#V8211) was obtained from Promega.

**Transfection studies**

Ezrin GIPZ lentiviral shRNAs and on-TARGETplus SMARTpool AKAP149 siRNA were purchased from Dharmacon (Thermo Fisher Scientific Inc., USA) and knockdowns were performed according to the manufacturer protocols. Smad2, Smad3 and PKACα shRNAs were obtained from Santa Cruz Biotechnology and knockdowns were performed as described previously (30,33). Ezrin wild type (WT), T567A (phospho-deficient) and T567D (phospho-mimetic) mutants were kindly provided by the Khanna Laboratory, NIH (16). Stable transfections were performed in GEO cells using lipofectamine 2000 as described previously (16).

**Cell lysate preparation**

GEO and FET cells were harvested at 70-80% confluency. Cells were washed in cold 5% PBS and collected in lysis buffer [50mmol/L Tris (pH 7.4), 100 mmol/L NaCl, 1% NP40, 2 mmol/L EDTA, 0.1% SDS, 50 mmol/L NaF, 10 mmol/L Na3VO4, 1 mmol/L phenylmethylsulfonyl fluoride, 25 μg/ml β-glycerophosphate, and one protease inhibitor cocktail tablet from Roche]. Crude cell lysates were homogenized using a 21 gauge needle to shear DNA and lysed for 30 minutes on ice at 4°C. Cell lysates were then cleared by centrifugation at 13000 rpm for 20 minutes at 4°C. Protein concentrations were determined by the Pierce bicinchoninic acid protein assay (Pierce Biotechnology).

**Western Blotting and Immunoprecipitation**

Protein (30-100μg) was fractionated by SDS-PAGE and transferred onto a nitrocellulose membrane (Amersham Biosciences) by electroblotting. The membrane was blocked with 5% nonfat dry milk in 1X TBST (50 mM Tris, pH 7.5, 150 mM NaCl, 0.05% Tween20) for 1 hour at room temperature or overnight at 4°C. The membrane was then incubated in primary antibodies for 1½ hour at room temperature or...
overnight at 4°C with 5% nonfat dry milk in 1X TBST or 5% bovine serum albumin (BSA) in 1X TBST according to the antibody manufacturer’s directions. The membrane was washed three times with 1X TBST for 10 minutes each and incubated with horseradish peroxidase-conjugated secondary antibody (Amersham Biosciences) for 1 hour at room temperature. This was followed by washing in 1X TBST and proteins were detected by enhanced chemiluminescence system (Amersham Biosciences). Immunoprecipitation was performed with 500 µg protein aliquots using magnetic beads (Millipore) according to the manufacturer’s instructions. Quantification of Western blots was performed using image J.

Apoptosis assay

Apoptosis was measured by the Cell Death Detection ELISA Plus kit (Roche) as described previously (57). Inhibition of cell proliferation was assessed by the MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay as described previously (57,58).

PKA activity assay

PKA activity was measured using the PepTag nonradioactive protein kinase assay kit from Promega (catalogue#V5340) using kemptide (LRRASLG) in the absence of cAMP according to the manufacturer’s protocol as described previously (30).

Cyclic AMP activity assay

For quantitative determination of cAMP, a non-radioactive Direct Cyclic AMP Enzyme Immunoassay kit from Enzo Life Sciences (catalogue#ADI-901-066) was utilized and the assay was performed according to the manufacturer’s protocol as described previously (30).

Statistical analysis

Statistical significance was determined using one-way ANOVA analysis and student t-test with a p value less than 0.05 using the Graphpad Prism 7 software. All the experiments were repeated three times independently, for the ANOVA analysis. The results were expressed as mean ± SE for each treatment.

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Conflict of Interest

The authors declare that they have no conflicts of interest with the contents of this article.

Author contributions

PDL, MGB, JDB and JW conceived and coordinated the study. PDL and JW wrote the manuscript. PLD performed the experiments. PDL and JW designed and analyzed the experiments. JDB provided critical review of the manuscript. All authors except Dr. Brattain reviewed the results and approved the final version of the manuscript.
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FOOTNOTES

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*Deceased

The abbreviations used are: AKAP, A-kinase anchoring protein; CRC, colorectal cancer; CREB, cAMP response element binding protein; ERM, ezrin radixin moesin; FERM, four-point-one, ezrin, radixin, moesin; IAP, inhibitor of apoptosis; IGF1R, insulin-like growth factor 1 receptor; PP2A, protein phosphatase 2A; XAF1, XIAP-associated factor 1; XIAP, X-linked inhibitor of apoptosis.
FIGURE LEGENDS

Figure 1. Knockdown of ezrin expression activates PKA and induces apoptosis in colon cancer cells. A, GEO and FET colon cancer cells with stable expression of ezrin shRNA (sh#1 and sh#3) showed a significant reduction in ezrin protein expression. Non-targeting sh RNA (NT sh) was used as a control (left). Quantification of Western blots was performed as described in Experimental Procedures (right). **p<0.01, n=3. B, Ezrin KD cells showed an approximately 2-fold increase in apoptosis, as determined by DNA fragmentation assays. **p<0.01, n=3. C, Ezrin KD led to downregulation of XIAP and survivin and an increase in cleaved caspase 7 (left). Quantification of Western blots is shown on the right. **p<0.01, ***p<0.001, n=4. D, Ezrin KD GEO and FET cells exhibited increased PKA activation compared to NT sh cells. Forskolin (10µM) was used as a positive control. **p<0.01, n=3. E, Ezrin KD-mediated increase of p-CREB is abrogated by PKA inhibitor H89 (15µM) (left). Quantification of Western blots is shown on right. **p<0.01, ***p<0.001, NS, not statistically significant, n=2. F, Immunoprecipitation (IP) assays showed the decreased association of PKARI with PKACata in ezrin KD cells compared with NT sh control cells (left). Relative levels of PKACata to PKARI were analyzed and results are shown on right. ***p<0.001, n=2. G, Production of cAMP was not increased in ezrin KD cells as determined by cAMP assay. Forskolin was used as a positive control. NS, not statistically significant, n=3.

Figure 2. Knockdown of ezrin expression induces apoptosis through PKA activation. A, Expression of PKA catalytic α subunit (PKACata) was knocked down in FET cells, with no change in ezrin phosphorylation at T567 and XIAP and survivin expression (left). Quantification of Western blots is shown on right. **p<0.01, NS, not statistically significant, n=3. B, PKACata KD blocked endogenous as well as ezrin KD-mediated PKA activation. **p<0.001, NS, not statistically significant, n=3. C, PKACata KD abrogates ezrin KD-mediated XIAP and survivin downregulation (left). Quantification of Western blots is shown on right. **p<0.01, NS, not statistically significant, n=3. D, PKACata KD abrogates ezrin KD-induced apoptosis. ***p<0.001, NS, not statistically significant, n=3.

Figure 3. Dephosphorylation of ezrin at T567 regulates PKA activation and apoptosis in GEO cells. A, Ezrin T567A downregulated XIAP and survivin expression and increased caspase 7 cleavage (left). Quantification of Western blots is shown on right. **p<0.01, n=3. B, Ezrin T567A increased apoptosis. **p<0.01, n=3. C&D, Ezrin T567A activated PKA with no increase in cAMP production. Forskolin was used as a positive control. **p<0.01, NS, not statistically significant, n=3.

Figure 4. Inhibition of ezrin phosphorylation at T567 by NSC668394 induces apoptosis through PKA activation. A&B, GEO and FET colon cancer cells treated with the ezrin inhibitor NSC668394 (NSC) showed a dose-dependent decrease in phosphorylation of ezrin at T567, downregulation of XIAP and survivin expression (A, upper) and induction of apoptosis (B). Lower panels show quantification of Western blots. **p<0.01, ***p<0.001, n=3. C, PKA activity assays showed PKA activation by NSC (20µM) treatment. PKA inhibitor H89 (15µM) pretreatment abrogated endogenous and NSC-induced PKA activation. Forskolin was used as a positive control. **p<0.001, NS, not statistically significant, n=3. D, NSC-induced PKA activation is independent of cAMP production. NS, not statistically significant, n=3. E, PKACata KD blocked NSC-mediated XIAP and survivin downregulation (upper). The lower panel shows quantification of Western blots. **p<0.01, NS, not statistically significant, n=3. F&G, PKACata KD abrogated NSC-mediated apoptosis (F) and PKA activation (G). **p<0.01, NS, not statistically significant, n=3.

Figure 5. TGFβ inhibits ezrin phosphorylation at T567, resulting in PKA activation and inhibition of XIAP and survivin expression. A, TGFβ1 treatment of FET cells decreased ezrin phosphorylation at T567 in a time-dependent manner (left). Relative levels of p-ezrin T567 to total ezrin were analyzed on and results are shown on right.
TGFβ and IGF1R activate PKA differently

Quantification of Western blots is shown on right. ***p<0.001, n=3. B, Expression of Smad2 and Smad3 was knocked down in FET cells (left). Quantification of Western blots is shown on right. **p<0.01, n=2. C, Smad3KD, but not Smad2KD, prevented TGFβ1-mediated inhibition of ezrin phosphorylation (left). Relative levels of p-ezrin T567 to total ezrin were determined and are shown on right. **p<0.01, NS, not statistically significant, n=3. D, TGFβ1 treatment increased PKA activation to a much lesser degree in ezrin KD cells than in NT sh control cells. **p<0.01, ***p<0.001, n=3. E, NSC attenuated TGFβ-induced PKA activation. ***p<0.001, n=3. F, Ezrin KD reduces TGFβ1-induced apoptosis. **p<0.01, n=2. G&H, Ezrin KD has no effect on TGFβRI or TGFβRII expression (G), or on Smad3 expression or TGFβ-mediated Smad3 phosphorylation (H, left). Quantification of Western blots is shown on right. **p<0.01, ***p<0.001, NS, not statistically significant, n=3.

Figure 6. Hyperphosphorylation of ezrin at T567 activates PKA and promotes survival.
A, Transferrin and insulin (TI) treatment increased phosphorylation of ezrin at T567 and expression of XIAP and survivin together with an increased level of apoptosis in NT sh control cells. However, the increase was abrogated in ezrin KD cells (left). Quantification of Western blots is shown on right. **p<0.01, NS, not statistically significant, n=3. B, TI treatment reduced apoptosis in NT sh control cells, and this inhibition was abrogated in ezrin KD cells. **p<0.01, NS, not statistically significant, n=2. C & D, TI activated PKA (C) and increased cAMP production (D) ***p<0.001, ****p<0.0001, n=3. E, Ectopic expression of ezrin T567 phosho-mimetic mutant (T567D) or wild type ezrin (WT) increased XIAP and survivin expression in GEO cells (left). Quantification of Western blots is shown on right. **p<0.01, n=3. F&G, Ezrin T567D mutant enhanced PKA activation (F) concomitant with increased cAMP production (G). Forskolin is used as a positive control. **p<0.01, n=3. H, Ectopic expression of ezrin T567D and ezrin WT reduced apoptosis. *p<0.05 and **p<0.01, ***p<0.001, n=3.

Figure 7. PKA activation induced by ezrin inhibition is dependent on AKAP149 expression.
A, Pre-treatment with a pan AKAP inhibitor Ht31 (25 μM) abrogated NSC-mediated PKA activation in FET and GEO cells. **p<0.01, ***p<0.001, NS, not statistically significant, n=3. B, AKAP149 KD had no effect on expression of ezrin, p-ezrinT567, XIAP or survivin but restored XIAP and survivin expression by ezrin KD (upper left panels). Ezrin KD had no effect on AKAP149 expression (upper right panel). Quantification of Western blots is shown in the lower panel. **p<0.01, NS, not statistically significant, n=3. C, AKAP149 KD abrogated ezrin KD-mediated apoptosis. **p<0.01, NS, not statistically significant, n=3. D, AKAP149 KD blocked NSC-mediated PKA activation. **p<0.01, NS, not statistically significant, n=3. E, Reciprocal IP analysis with AKAP149 or PKARII antibodies showed increased association of AKAP149 with PKARII in ezrin T567A cells and decreased association in ezrin T567D cells (upper panel). Relative levels of PKARI and PKARII were analyzed and results are shown in the lower panel. *p<0.05, **p<0.01, n=2. F, IP analysis with ezrin or PKARII antibodies indicated that there was more PKARII associated with ezrin in T567D cells than in T567A cells (upper panel). The relative levels of PKARI and ezrin are shown in the lower panel. *p<0.05, **, p<0.01, n=2.

Figure 8. Proposed model of crosstalk between TGFβ/Smad3 and IGF1R signaling pathways.
TGFβ/Smad3 inhibits ezrin phosphorylation at T567, leading to the association of PKA RII with AKAP149 to activate PKA in a cAMP-independent manner, which suppresses XIAP and survivin expression and induces apoptosis. With IGF1R activation, ezrin is hyperphosphorylated at T567, resulting in association of ezrin with PKA RII and PKA activation in cAMP-dependent manner leading to XIAP and survivin upregulation and cell survival.
TGFβ and IGF1R activate PKA differently

Figure 1

A. GEO and FET with Ezrin shRNA.

B. Fold change in DNA frag.

C. Indicated proteins/Actin for Ezrin, XIAP, Survivin, Cleaved Cas 7.

D. Fold change in PKA activation.

E. Indicated proteins/Actin for Ezrin, p-CREB, CREB.

F. IP PKARII and IB PKACα/β and PKARII.

G. cAMP (pmol/ml) for GEO and FET.
TGFβ and IGF1R activate PKA differently

Figure 2

A

B

C

D

Fold change in PKA activation

Fold change in DNA frag
Figure 3

A

B

C

D

TGFβ and IGF1R activate PKA differently

GFP-Ezrin
Ezrin
XIAP
Survivin
Cas 7
Cleaved Cas 7
Actin

Fold change in DNA frag

Fold change in PKA activation

cAMP(pmol/ml)

NT sh
Ezrin sh#3+EV
Ezrin sh#3+T567A
Ezrin sh#3+WT

NT sh
Ezrin sh#3+EV
Ezrin sh#3+T567A

NT sh
Ezrin sh#3+WT
Ezrin sh#3+T567A
Forstokin

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NS

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Figure 4

Figure 4
Figure 5

TGFβ and IGF1R activate PKA differently
Figure 6

TGFβ and IGF1R activate PKA differently
Figure 7

TGFβ and IGF1R activate PKA differently
Figure 8
TGFβ and IGF1R signaling activate protein kinase A through differential regulation of ezrin phosphorylation in colon cancer cells
Premila D. Leiphrakpam, Michael G. Brattain, Jennifer D. Black and Jenny Jing Wang

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