Association of v-ErbA with Smad4 Disrupts TGF-β Signaling

Richard A. Erickson and Xuedong Liu

Department of Chemistry and Biochemistry, University of Colorado-Boulder, Boulder, CO 80309

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Disruption of the transforming growth factor-β (TGF-β) pathway is observed in the majority of cancers. To further understand TGF-β pathway inactivation in cancer, we stably expressed the v-ErbA oncoprotein in TGF-β-responsive cells. v-ErbA participates in erythroleukemic transformation of cells induced by the avian erythroblastosis virus (AEV). Here we demonstrate that expression of v-ErbA was sufficient to antagonize TGF-β-induced cell growth inhibition and that dysregulation of TGF-β signaling required that v-ErbA associate with the Smad4 which sequesters Smad4 in the cytoplasm. We also show that AEV-transformed erythroleukemia cells were resistant to TGF-β–induced growth inhibition and that TGF-β sensitivity could be recovered by reducing v-ErbA expression. Our results reveal a novel mechanism for oncogenic disruption of TGF-β signaling and provide a mechanistic explanation of v-ErbA activity in AEV-induced erythroleukemia.

INTRODUCTION

The transforming growth factor-β (TGF-β) signaling pathway is a major signaling network that controls cell proliferation, differentiation, and tumor suppression (Massague et al., 2000; Attisano and Wrana, 2002; Wakefield and Roberts, 2002). The TGF-β cytokine signals through hetero-oligomeric complexes of type I and type II serine/threonine kinase receptors, TGF-β type I (TβRI) and type II (TβRII) receptors (Heldin et al., 1997; Massague et al., 2000). On ligand binding to the TβRII receptor, TβRII phosphorylates TβRI and activates the TβRI kinase (Wrana et al., 1992; Chen and Weinberg, 1995). The signal is further propagated by phosphorylation of the downstream signal transducers Smad2 and Smad3 by the type I receptor kinase (Heldin et al., 1997). Phosphorylated Smad2 and Smad3 form a complex with Smad4 and translocate into the nucleus (Massague et al., 2000). These Smad complexes then interact with other transcription factors and bind the promoter regions of TGF-β–responsive genes to regulate gene expression (Massague et al., 2000).

Insensitivity to antigrowth signals such as TGF-β is common in cancer cells (Hanahan and Weinberg, 2000; Massague et al., 2000). Multiple genetic mutations are observed in TGF-β–insensitive tumor cells, and mechanisms have been identified that explain the disruption of TGF-β’s cytotatic activity. These mechanisms include the down-regulation of TGF-β receptors, mutations in TGF-β signaling components such as Smad4, and mutations in other downstream targets (Hanahan and Weinberg, 2000; Massague et al., 2000). Overexpression of oncoproteins is also implicated in the disruption of TGF-β signaling. Tumor cells insensitive to TGF-β are known to overexpress diverse oncoproteins such as Cdk4, Ski, Evi-1, Ras, Mdm2, E6/E7, E1A, and PLZF-RARα (Pietenpol et al., 1990; Ewen et al., 1993; Datto et al., 1997; Kurokawa et al., 1998; Sun et al., 1998; Hanahan and Weinberg, 2000; Massague et al., 2000; La et al., 2003; Luo, 2004). Overexpression of HPV-16 E7 and E1A, for example, is shown to cause TGF-β resistance in skin keratinocytes by preventing TGF-β–induced down-regulation of c-myc transcription (Pietenpol et al., 1990). Expression of E1A is also known to overcome TGF-β–regulated growth inhibition by blocking induction of the cyclin-dependent kinase inhibitors, p15Ink4B and p21Cip1 (Datto et al., 1997). Although some of the mechanisms by which tumor cells are rendered insensitive to TGF-β are known, additional mechanisms likely remain unidentified.

Although the oncogenic mechanism of erythroleukemia induced by the avian erythroblastosis virus (AEV) involves the dysregulation of a yet unidentified signaling pathway, mechanisms involving cell-growth inhibition from TGF-β pathway activation have not been previously demonstrated (Damm and Evans, 1993). AEV effectively blocks erythroid differentiation and suppresses erythrocyte-specific gene transcription (Graf and Beug, 1983; Rietveld et al., 2002). AEV carries two oncogenes, v-erbA and v-erbB, that work cooperatively to induce erythroleukemia and sarcomas (Vennstrom et al., 1980; Graf and Beug, 1983). v-erbB, a mutated and truncated viral variant of the epidermal growth factor receptor, retains its transforming activity when expressed alone (Lin et al., 1984). v-ErbA, on the other hand, is derived from the fusion of a fragment of the retroviral group antigen (gag) region and a mutated variant of thyroid hormone receptor α (TRα/c-ErbA; NR-1 subfamily; Damm et al., 1989; Zenke et al., 1990). This gag sequence is required for its biological activity (Hayman et al., 1979; Zenke et al., 1990). Though v-ErbA lacks the ability to transform cells when expressed alone, it is often described as a potent oncogenic enhancer of cellular transformation because of its apparent ability to block cell differentiation and proliferation pathways (Graf and Beug, 1983; Sap et al., 1986; Weinberger et al., 1986). Though the molecular mechanism underlying the function of v-ErbA in cellular transformation remains elusive (Thormeyer and Banaihmard, 1999; Rietveld...
et al., 2001), investigators speculate that v-ErbA antagonizes a major tumor suppressor pathway to enhance cellular transformation (Hayman et al., 1979; Vennstrom and Bishop, 1982).

The purpose of this study was to determine whether v-ErbA, when stably overexpressed, dysregulates the TGF-β signaling pathway. We demonstrate that v-ErbA antagonizes TGF-β-induced cell-growth inhibition and that pathway dysregulation requires that v-ErbA complex with Smad4 and disrupt Smad4 nuclear localization. Expression of v-ErbA was found to confer TGF-β resistance in HD3 erythroleukemic cells and that increased TGF-β sensitivity was observed in these cells upon reduction of v-ErbA expression. Mutations in the zinc-finger region of v-ErbA were found to abolish v-ErbA/Smad4 association and were unable to dysregulate TGF-β signaling. We believe our work uncovers a mechanism of v-ErbA onco-negativity that underscores the importance of TGF-β signal dysregulation in oncogenic transformation.

MATERIALS AND METHODS

Tissue Culture and TGF-β-induced Growth Inhibition Assays

PE25, SW480, and 293T cell lines were incubated at 37°C at 5% CO₂ and cultured in DMEM medium supplemented with 10% fetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 mM l-glutamine. For growth inhibition assays, PE25 cells were plated at low density (approximately 15K cells/well) on six-well plates and after 8–18 h were subjected to 0, 1, 5, 10, or 50 pM concentrations of TGF-β. Medium and TGF-β were replaced every 3–4 d. After approximately 2 wk, the cells were washed with phosphate-buffered saline (PBS), fixed with 70% ethanol for 1 h, and stored in water with 10% glycerol. HD3 cells were incubated at 37°C at 5% CO₂ and cultured in DMEM medium supplemented with 8% fetal bovine serum, 2% heat-inactivated chicken serum (1:1 at 50°C), 10 mM HEPES (pH 7.4), 100 U/ml penicillin, and 100 μg/ml streptomycin.

Retroviral-mediated Gene Transfer and Cell Sorting

Retroviral supernatants were produced by cotransfecting the pREX-CD2-IRE5-GFP, pRex-v-ErbA-IRE5-GFP, pRex-delzF-IRE5-GFP, pRex-C308A-IRE5-GFP, pRex-GFP-Smad4, pRex-GFP-Smad4-euc, pRex-v-ErbA-mC-CD2, pRex-delzF-mC-CD2, or pRex-C308A-mC-CD2 retroviral packaging vectors with pCl-Amp (Naviaux et al., 1996) into 293T cells using FuGene 6 (Roche, Indianapolis, IN) and collecting medium 48, 72, and 96 h after transfection. PE25 or SW480 cells were plated at low density and incubated with retroviral supernatant and 10–20 μg/ml polybrene for 8–16 h. The supernatant was replaced with fresh medium. Infected cells were selected using a Dako Cytomation MoFl Cell sorter (Carpinteria, CA) using green fluorescent protein (GFP) or CD2 reporter expression to approximately two orders of magnitude above background fluorescence levels of uninfected cells.

Reporter Gene Construct and Luciferase Assays

The PE25 cell line used in this study was stably transfected with pGL3-PE2.1 (Hua et al., 1998). This construct contains two tandem copies of a 36-base pair fragment containing the Smad-binding elements (SBEs) of the human PAI-1 promoter as previously described (Hua et al., 1999). Infected and enriched PE25 cells were counted and plated at 4 × 10⁵ cells/well in triplicates on 12-well plates. After 8–18 h, medium was replaced with low-serum (0.5%) DMEM supplemented with 1% penicillin, 1% streptomycin, 10 mM HEPES, and 1 mM NaF and cultured for another 24 h. Cells were transfected with 100 pM of pGL3 construct using the Lipofectamine 2000 (Invitrogen, Carlsbad, CA) transfection reagent. Infected and enriched PE25 cells were plated at low density (approximately 15K cells/well) on six-well plates and after 8–18 h were subjected to 0, 1, 5, 10, or 50 pM concentrations of TGF-β. Medium and TGF-β were replaced every 3–4 d. After approximately 2 wk, the cells were washed with phosphate-buffered saline (PBS), fixed with 70% ethanol for 1 h, and stored in water with 10% glycerol. HD3 cells were incubated at 37°C at 5% CO₂ and cultured in DMEM medium supplemented with 8% fetal bovine serum, 2% heat-inactivated chicken serum (1:1 at 50°C), 10 mM HEPES (pH 7.4), 100 U/ml penicillin, and 100 μg/ml streptomycin.

In Vitro Binding Assay

The coding sequence of v-ErbA was cloned into the pRKS vector using standard cloning techniques to provide a DNA template for in vitro transla-

Fluorescence Microscopy and Nuclear-Cytoplasmic Analysis

Cells plated on glass coverslips were treated as described, fixed with 4% paraformaldehyde in PBS for 1 h, rinsed in water, and mounted using Vectashield mounting medium with DAPI (Vector Laboratories, Burlingame, CA). Images were acquired with a Nikon inverted microscope (Melville, NY) with a 60× 1.4 NA immersion objective, GFP/YPF/RFP dichroic mirror, and single-band excitation and emission filters (Chroma Technology, Brattle-

Statistical Analysis

One-way analysis of variance (ANOVA) was used to compare differences among groups or three or more followed by Tukey’s post hoc test to assess differences within groups. Significance between two groups was demon-

Statistical Analysis

One-way analysis of variance (ANOVA) was used to compare differences among groups or three or more followed by Tukey’s post hoc test to assess differences within groups. Significance between two groups was demonstrated by one-tailed t test.
v-ErbA Expression Dysregulates TGF-β Signaling

It is well documented that a majority of cancer cells have lost their sensitivity to TGF-β. To gain further insight into the mechanism by which tumor cells evade the action of TGF-β, we performed a genetic screen to isolate cDNAs, that when ectopically expressed, rendered cells insensitive to TGF-β. Unexpectedly, we isolated an ear2 sequence in a cDNA library screen that, when linked in frame to remnants of a gag sequence of the Moloney murine leukemia virus, exhibited resistance to TGF-β-induced cell-growth inhibition (data not shown). The nuclear orphan receptor, ear2 (erbA related gene 2), is a member of the COUP-TF (NR2) subfamily of nuclear hormone receptors and shares homology with TRα1 (Miyajima et al., 1988). Whereas gag-ear2 fusion is artificially created during the construction of cDNA library, fusion proteins between a viral gag sequence and nuclear hormone receptors have been identified in nature (Grignani et al., 1998; Lin and Evans, 2000; La et al., 2003). An example is the oncprotein v-ErbA, which is composed of an N-terminal gag sequence fused to a mutated form of thyroid hormone receptor α (TRα/c-ErbB; Vennstrom and Bishop, 1982). We were prompted to test whether v-ErbA might disrupt TGF-β signal transduction activity when we isolated an ear2 sequence in a cDNA library screen that, when linked in frame to remnants of a gag sequence of the Moloney murine leukemia virus, exhibited resistance to TGF-β-induced cell-growth inhibition. The nuclear orphan receptor, ear2 (erbA related gene 2), is a member of the COUP-TF (NR2) subfamily of nuclear hormone receptors and shares homology with TRα1 (Miyajima et al., 1988). Using retroviral gene-mediated transfer technique (Figure 1B; Liu et al., 1997; Hua et al., 1998). Immunoblot analysis confirmed expression of the fusion protein (Figure 1A). A control cell line was also created using PE25 cells by stably expressing a fragment of CD2 and was growth inhibited above 1 pM TGF-β demonstrating that TGF-β sensitivity was not disrupted by the retroviral gene-mediated transfer technique (Figure 1B; Liu et al., 1997). In contrast, PE25 cells expressing v-ErbA were resistant to TGF-β at concentrations of 10 pM (Figure 1B). Thus, stable expression of v-ErbA but not the CD2 control enables cells to escape TGF-β-induced growth arrest.

We hypothesized that resistance to TGF-β growth inhibition upon expression of v-ErbA may result from interference of TGF-β-mediated transcriptional regulation. To determine whether expression of v-ErbA affects TGF-β–regulated gene expression, we measured the luciferase activity in the PE25 cells stably expressing either CD2 (control) or v-ErbA in the absence or presence of TGF-β. The luciferase reporter gene stably transfected in the PE25 cell line is driven by a promoter containing two Smad-binding elements (SBES; Hua et al., 1998). We emphasize that this reporter is stably integrated into the genome, thus ensuring that the behavior of the reporter’s promoter is similar to that of an endogenous promoter within the native chromatin environment. As expected, we observed a large increase in luciferase activity in the control cells upon TGF-β treatment (Figure 1C, lane 1 vs. lane 2). In cell lines expressing v-ErbA, the luciferase activity was greatly diminished in both untreated and TGF-β–treated conditions (lanes 3 and 4). These data show that the activity of the TGF-β–responsive reporter correlates well with the escape of TGF-β–induced growth inhibition upon v-ErbA expression (Figure 1B). Specifically, the CD2 control cells were growth inhibited in the presence of TGF-β and had high levels of transcriptional activity. In contrast, cells expressing v-ErbA were both resistant to TGF-β treatment and exhibited a greatly reduced transcriptional response.

c-v-ErbA Expression Causes TGF-β Resistance in Erythroblasts

v-ErbA was originally isolated from chicken erythroblast cells unable to terminally differentiate in the presence of AEV (Frykberg et al., 1983). Because growth inhibition is an essential step in erythroblast differentiation that leads to erythrocyte formation, we hypothesized that erythroblasts expressing AEV would be insensitive to TGF-β. HD3 cells were considered ideal to investigate this hypothesis because they are chicken erythroblasts transformed by AEV and express both native v-ErbA and v-ErbB (Beug et al., 1982). To investigate the hypothesis that HD3 erythroblasts expressing AEV would be insensitive to TGF-β, we performed a thymidine incorporation assay in the presence or absence of TGF-β in HD3 cells. Cells were either left untreated or treated with 100 pM TGF-β for 24 h and then radiolabeled with [3H]thymidine before harvesting. HD3 cells treated with TGF-β showed levels of thymidine incorporation statistically similar to untreated cells (Figure 2A). Therefore, the unchanged levels of thymidine incorporation in the presence or absence of TGF-β indicated that HD3 cells were TGF-β resistant.

Figure 1. v-ErbA expression dysregulates TGF-β signaling. (A) Immunoblot demonstrating expression of v-ErbA-Flag-tagged protein expressed in PE25 mink lung cells. (B) PE25 mink lung cells stably expressing either CD2 (control) or v-ErbA plated at low density and grown in the presence of 0, 1, 5, 10, 25, or 50 pM TGF-β for approximately 10 d. Cells were fixed and stained with crystal violet. CD2 (control) cells were unable to proliferate in the presence of 5 pM or greater TGF-β. Cells expressing v-ErbA proliferated in the presence of higher TGF-β concentrations. (C) Luciferase assay using cells (PE25) with a stably transfected reporter responsive to TGF-β. Cells lines additionally expressed CD2 (control) or v-ErbA. Means (bars) and SDs (error bars) are shown. Increased reporter response observed in CD2 (control) cells (lane 1 vs. lane 2) in the presence of TGF-β (24 h at 100 pM). Cells expressing v-ErbA demonstrated basal activity near background levels (lane 3) and greatly reduced reporter response in the presence of TGF-β (lane 4).
We further hypothesized that if v-ErbA expression was reduced in the AEV transformed HD3 cells, the cells would both revert to a growth-inhibited state and become sensitive to TGF-β. We used siRNA-mediated gene silencing to down-regulate v-erbA mRNA expression. The siRNA was targeted to the gag sequence of v-erbA and produced using a published method (Myers et al., 2003). We chose to target only the gag region to avoid potential degradation of endogenous TRα or other nuclear receptors that likely share similar sequence homologies. To demonstrate v-erbA down-regulation, HD3 cells were transiently transfected with either a negative-control siRNA or the siRNA duplexes targeted to v-erbA. Expression levels of v-erbA mRNA were reduced by 31% in transiently transfected cells compared with HD3 cells transfected with an equivalent amount of control siRNA (Figure 2B). Thus, endogenous expression of v-erbA in an AEV-transformed cell line was down-regulated with the gag-targeted siRNA. We then used a thymidine incorporation assay to measure the effect of this partial depletion of v-erbA on the proliferation of HD3 cells in the presence or absence of TGF-β. Control siRNA or gag-targeted siRNA-transfected cells were either untreated or treated with 100 pM TGF-β for 24 h and then radiolabeled with [3H]thymidine before harvesting. As expected, the proliferation of HD3 cells transfected with control siRNA remained statistically unchanged upon TGF-β treatment (Figure 2C, lanes 1 and 2). In contrast, cells transfected with gag siRNA demonstrated a significant 41% reduction in thymidine incorporation under basal conditions (lane 1 vs. lane 3) and a significant 58% reduction in thymidine incorporation upon TGF-β treatment (lane 2 vs. lane 4). The reduction in thymidine incorporation upon gag siRNA transfection without TGF-β treatment may arise from a combination of v-ErbA’s overall oncogenic activities. One contributing effect may be that upon v-ErbA reduction, the TGF-β pathway becomes reactivated by basal or autocrine signaling. Additionally, the observed reduction in thymidine incorporation may reflect a reduction of oncogenicity from other known pathways affected by v-ErbA such as constitutive transcriprional repression of target gene expression upon v-ErbA binding to v-ErbA response elements (Ciana et al., 1998). More importantly, we observed a significant 28% decrease in thymidine incorporation upon TGF-β treatment in gag siRNA-transfected cells, suggesting that HD3 cells acquire TGF-β sensitivity when v-erbA levels are reduced (lane 3 vs. lane 4).

In summary, we show that HD3 cells are insensitive to TGF-β and that TGF-β sensitivity is recovered in these cells by down-regulating expression of v-erbA. These results support our hypothesis that v-ErbA expression dysregulates TGF-β signaling.

**v-ErbA Associates with Smad4 and Disrupts Smad4 Nuclear Localization**

We hypothesized that the mechanism of TGF-β pathway disruption observed in cells expressing v-ErbA depends on the ability of this fusion protein to associate with one or more of the Smad transcription factors. To address this possibility, we performed direct protein-binding assays in...
which GST-tagged, recombinant Smad2, Smad3, and Smad4 proteins were incubated with $^{35}$S-radiolabeled v-ErbA prepared by in vitro translation (IVT). Little or no binding was observed between a GST-only negative control and v-ErbA and thus eliminated the possibility that v-ErbA could interact with the GST-tag of the recombinant Smad proteins (Figure 3A, lane 2). We also observed that GST-Smad2 and GST-Smad3 bound minimally to v-ErbA (Figure 3A, lanes 3 and 4). In contrast, v-ErbA efficiently bound Smad4 at a level greater than the 10% control input (lane 5 vs. lane 1). To confirm that the gag region was essential for Smad binding to v-ErbA, an ErbA-only (gag deleted) construct was created and radiolabeled. As we observed with full-length v-ErbA protein, ErbA-only demonstrated minimal association with GST-only, Smad2, and Smad3 (Figure 3A, lanes 2-4). In contrast to v-ErbA, increased binding was not observed between ErbA-only and Smad4 and thus suggests that the gag region is necessary for v-ErbA/Smad4 association (lane 5). Taken together, these in vitro experiments indicate that v-ErbA binds Smad4 and that v-ErbA/Smad4 association requires the presence of the gag region of v-ErbA.

Although the in vitro binding experiments imply that v-ErbA/Smad4 association may be involved in dysregulation of TGF-β signaling, this result does not suggest an obvious mechanism by which this interaction may convey its influence. A recent study revealed that v-ErbA is predominantly localized in the cytosol because of the presence of a CRM1-dependent nuclear export sequence within the C-terminal portion of the p10 region of gag (DeLong et al., 2004). Because v-ErbA is predominately restricted to the cytoplasm, we wondered if v-ErbA/Smad4 association may disrupt Smad4 nuclear localization. Previous work demonstrates that v-ErbA, when fused to a fluorescent protein, provides an appropriate probe for studying v-ErbA localization (DeLong et al., 2004). For this study we C-terminally fused mCherry to v-ErbA (v-ErbA-mC) and used mCherry alone (mC-only) as a control. Moreover, we fused both Smad4 and a Smad4 variant that predominantly accumulates in nucleus as a result of mutations in the nuclear export signals (NES) to GFP and use these fusion proteins to characterize their subcellular localizations (Xiao et al., 2000, 2003). Combinations of Smad4 and v-ErbA expression vectors were then stably expressed in colon-cancer–derived SW480 cells. We used SW480 cells because they are a Smad4-deleted cell line that regains TGF-β pathway activity upon exogenous expression of Smad4 (Schwarte-Waldhoff et al., 1999). To confirm that GFP-Smad4 exhibited the subcellular localization characteristics of endogenously expressed Smad4, we first examined GFP-Smad4 in the presence of the stably expressed mC-only control with or without TGF-β treatment or with leptotynycin B (LMB), a known CRM1 inhibitor (Figure 3B, a-I). As expected, we observed GFP-Smad4 in both the nucleus and cytoplasm in the absence of TGF-β treatment (Figure 3B), and upon TGF-β or LMB treatment GFP-Smad4 accumulated in the nucleus (Figure 3B, f and j). Thus, GFP-Smad4 nucleo-cytoplasmic distribution is similar to that of the endogenous Smad4 observed in TGF-β–responsive cell lines (Pierreux et al., 2000; Xiao et al., 2003). To quantitatively characterize GFP-Smad4 localization, we created nuclear and cytoplasmic masks of cell images, measured fluorescence in each region, and calculated the nuclear:cytoplasmic ratio (see Materials and Methods for details).

As shown in Figure 3C (lane 1 vs. lanes 3 and 5), there are statistically significant 1.24- and 1.89-fold increases in the nuclear:cytoplasmic ratio of GFP-Smad4 upon TGF-β and LMB treatments, respectively. GFP-Smad4-nuc cells exhibited a stronger nuclear localization compared with TGF-β–treated GFP-Smad4 cells (Figure 3Bn), and we quantitatively confirmed this observation by measuring a statistically significant 2.07-fold increase in the nuclear:cytoplasmic ratio when compared with untreated cells (Figure 3C, lane 1 vs. lane 7). In agreement with the observations of others using YFP-only as a control (DeLong et al., 2004), we also saw slight nuclear localization of the mCherry control for all treatment conditions. However, neither TGF-β treatment nor the expression of GFP-Smad4-nuc affects the subcellular localization of mCherry as the nuclear:cytoplasmic ratios of mCherry control are essentially the same under either condition (Figure 3, B, c vs. g and o, and D, lane 1 vs. lanes 3 and 7). On LMB treatment there is small, but statistically significant, 1.34-fold increase the nuclear:cytoplasmic ratio for mCherry only cells (Figure 3, B, c vs. k, and D, lane 1 vs. lane 5), similar to what has been reported for YFP-only control (DeLong et al., 2004). Thus GFP-Smad4, just like the endogenous Smad4, translocates into the nucleus upon TGF-β or LMB treatments, and expression of either wild-type Smad4 or Smad4-nuc does not affect the nuclear:cytoplasmic ratio of mCherry fluorescent protein.

To investigate whether v-ErbA/Smad4 association may disrupt Smad4 nuclear localization, we analyzed Smad4 and v-ErbA subcellular localization in SW480 cells expressing both proteins under various conditions. Without TGF-β treatment, GFP-Smad4 was predominantly localized in the cytoplasm in both v-ErbA-mC and mCherry control cells (Figure 3B, b vs. r), although there was a slight decrease in the nucleo-cytoplasmic ratio of v-ErbA-mC compared with mCherry alone (Figure 3C, lane 1 vs. lane 2). v-ErbA suppressed the ability of GFP-Smad4 to translocate into the nucleus upon TGF-β treatment (a 37.1% decrease in the nucleo-cytoplasmic ratio compared with the mCherry control cells; Figure 3B, f vs. v, and C, lane 3 vs. lane 4). Unexpectedly, v-ErbA also blocks GFP-Smad4 translocation in response to LMB (a 54.5% decrease in the nucleo-cytoplasmic ratio; Figure 3, B, j vs. z, and C, lane 5 vs. lane 6). Furthermore, v-ErbA causes a significant increase in cytoplasmic accumulation of GFP-Smad4-nuc in cells expressing both v-ErbA-mC and GFP-Smad4-nuc (a 67.9% decrease in the nucleo-cytoplasmic ratio; Figure 3B, n vs. dd, and C, lane 7 vs. lane 8). We also examined the nucleo-cytoplasmic distribution of v-ErbA-mC and found no significant difference upon TGF-β or LMB treatment or when Smad4-nuc was expressed (Figure 3B, s vs. w, aa, and ee, and D, lane 2 vs. lanes 4, 6, and 8). We did observe that v-ErbA-mC is consistently more cytoplasmic than mCherry control, which shows a rather diffused localization pattern and that v-ErbA-mC subcellular localization is not affected by TGF-β or LMB treatments or the expression of Smad4-nuc (Figure 3, B and D).

Because nuclear export of both Smad4 and v-ErbA occurs through a CRM1-mediated pathway, the inability of LMB treatment to increase the nuclear localization of these proteins was unexpected (Pierreux et al., 2000; DeLong et al., 2004). To confirm that the v-ErbA-mCherry construct used in this investigation retained CRM1-dependent characteristics similar to wild-type v-ErbA, SW480 cells stably expressing v-ErbA-mCherry but not Smad4 were examined. As expected, v-ErbA-mCherry remains predominately cytoplasmic in the presence or absence of TGF-β (Figure 3E, B, e and f, and D, lanes 1 and 2). In contrast, LMB treatment induces nuclear accumulation of v-ErbA-mCherry (Figure 3, Eh and F, lane 3). Thus, in the absence of Smad4, inhibition of CRM1 by LMB treatment disrupts nuclear export of v-ErbA.

Taken together, the data support that upon expression of v-ErbA that 1) in the absence of TGF-β treatment, the nuclear-cytoplasmic distribution of Smad4 is similar to that of control cells, 2) when cells are treated with TGF-β or LMB,
Figure 3. v-ErbA/Smad4 association and nucleo-cytoplasmic distribution of Smad4 and v-ErbA. (A) Recombinant GST, GST-Smad2, GST-Smad3, or GST-Smad4 fusion proteins precipitated using GST agarose beads after incubation with in vitro–translated, 35S-labeled v-ErbA or ErbA-only proteins. v-ErbA associated with Smad4 (lane 5) with greater affinity than with Smad2 or Smad3 (lanes 3 and 4). Only background binding was observed with the ErbA-only fragment to Smad2/3/4 proteins (lanes 3–5). (B) SW480 cells stably expressed GFP-Smad4 or GFP-Smad4-nuc and mCherry-only or v-ErbA-mCherry as indicated. Cells were either untreated, treated with TGF-β (100 pM, 1 h), or treated with LMB (50 ng/ml, 1 h) as indicated. Cells were stained with DAPI to identify nuclei. Images are representative of nucleo-cytoplasmic distribution of GFP-Smad4, GFP-Smad4-nuc, mCherry-only, or v-ErbA-mCherry for each condition. (C) SW480 cells and treatments are as indicated and as described in B. Ratios of the average nuclear and cytoplasmic fluorescence for GFP-Smad4 and GFP-Smad4-nuc were calculated for n ≥ 20 cells for each condition to quantify changes in nucleo-cytoplasmic distribution. Means (bars) and SDs (error bars) are shown. One-way ANOVA indicated statistically significant differences (p < 0.05). Differences within the group assessed by Tukey’s post hoc test and denoted as follows: *p < 0.01; **p < 0.05. Smad4 nuclear localization increased in cells expressing mCherry control upon TGF-β or LMB treatment or when GFP-Smad4-nuc was expressed (lane 1 vs. lanes 3, 5, and 7). Smad4 nuclear localization was abolished upon TGF-β or LMB treatment and when GFP-Smad4-nuc was expressed in the presence of v-ErbA (lane 2 vs. lanes 4, 6, and 8). (D) Cells, treatment, and analysis methods are identical to C, but ratios of the average nuclear
Sma4 is less effectively translocated to the nucleus, 3) even when the strongly nuclear-localizing Sma4 variant is expressed, Sma4-nuc is transported into the cytoplasm, and 4) regardless of whether cells were treated with TGF-β or LMB or whether Sma4-nuc is expressed, the nuclear-cytoplasmic distribution of v-ErbA-mCherry remains unchanged but overall is more cytoplasmic than the mCherry control.

v-ErbA Mutants Failing to Associate with Sma4 Are Unable to Antagonize TGF-β-induced Cell-Growth and Sma4 Nuclear Localization

To confirm that the interaction between Sma4 and v-ErbA is critical for TGF-β signal disruption, we sought to identify a v-ErbA mutant unable to bind Sma4. As with other nuclear hormone receptors, v-ErbA contains a DNA-binding domain, a hinge region, and a ligand/transactivation domain (Figure 4A; Damm and Evans, 1993; Thornmeyer and Banaihamad, 1999). To identify the region of v-ErbA involved in Sma4 binding, we generated four v-ErbA C-terminal-deletion mutants and then tested their ability to bind Sma4 in vitro. We found binding to GST-Sma4 was retained upon deletion of residues 404–639, but observed minimal binding when residues 329–639 were deleted (Figure 4B, lane 7 vs. lane 6). These results suggest that a site located between residues 329–404 of v-ErbA, a region that includes a small fragment of the hinge region and the DNA-binding domain, is required for Sma4 association.

Having identified the general region of Sma4/v-ErbA interaction, we chose to further isolate this interaction by creating two constructs possessing mutations within the DNA-binding domain. As in TRα and other nuclear hormone receptors such as the glucocorticoid and retinoic acid receptors, v-ErbA has two tandemly repeated zinc finger motifs (Privalsky et al., 1988). Each of these zinc fingers contain four invariant cysteine residues that coordinate with a single zinc ion to form a structure that enables the DNA-recognition helix within the first zinc finger to complex with its cis-acting element (Bonde and Privalsky, 1990). The first v-ErbA mutant deleted both zinc fingers, delZF, by eliminating residues 291–348. The second construct contained a single-point mutation, C308A, which substituting an alanine at the third Zn²⁺-coordinating cysteine within the first zinc finger. These mutants were selected based on a study using site-directed mutagenesis within the DNA-binding domain that abolishes v-ErbA’s ability to induce erythroid colony formation (Bonde and Privalsky, 1990). Using these delZF and C308A v-ErbA mutants, we repeated in vitro binding assays to investigate altered Sma4 association. We found both the delZF and the C308A mutants to have reduced association with Sma4 compared with the intact v-ErbA control (Figure 5A, lane 3). The data therefore suggest that for the first zinc finger within DNA-binding domain of v-ErbA is required for Sma4 association.

If v-ErbA/Sma4 association is essential for TGF-β signal dysregulation, we would anticipate that both the delZF and C308A mutants, shown to have impaired binding to Sma4, would recover TGF-β sensitivity. On stable expression of these mutants in PE25 cells (Figure 5B), we found that though some cell growth was observed in the 5 pM-TGF-β wells, both cell line cells exhibited growth inhibition greater than cells expressing v-ErbA (Figure 5C). We also repeated the luciferase assay incorporating the PE21-TGF-β-responsive promoter using both the delZF and C308A mutants stably expressed in PE25 cells. The results demonstrate restored promoter activity in cells expressing these v-ErbA mutants (Figure 5D, lanes 5–8 vs. lanes 1–4). Thus, the data show that in contrast to cells expressing wild-type v-ErbA, cells expressing the delZF and C308A v-ErbA mutants are sensitive to TGF-β-induced growth inhibition and no longer repress the activity of a TGF-β-responsive promoter.

If, as proposed, TGF-β signaling is disrupted though the mechanism of v-ErbA/Sma4 interaction and perturbation of Sma4 nuclear accumulation, we would anticipate that v-ErbA mutants that are unable to associate with Sma4 would also be unable to alter Sma4 nuclear-cytoplasmic localization. To investigate this hypothesis a mCherry fusion construct was created for the C308A mutant, C308A-mCherry, and stably expressed in the SW480 cell lines also expressing either GFP-
v-ErbA mutants fail to associate with Smad4 and are unable to antagonize TGF-β-induced cell growth. (A) Recombinant GST and GST-Smad4 fusion proteins precipitated using GST agarose beads after incubation with in vitro–translated, 35S-labeled delZF and C308A v-ErbA mutants and v-ErbA (positive control) proteins. v-ErbA (positive control) demonstrated association with Smad4, but greatly reduced GST-Smad4 affinity observed with the delZF and C308A mutants (lane 3). (B) Immunoblot demonstrating stable expression of C-terminally Flag-tagged v-ErbA mutants in PE25 mink lung cells. (C) PE25 mink lung cells stably expressing the indicated v-ErbA mutants were plated and treated as described in Figure 1B. Cells expressing either the delZF or C308A mutants demonstrated increased sensitivity to TGF-β compared with cells expressing v-ErbA and sensitivity to TGF-β, similar to cells expressing CD2 (control). (D) PE25 cells with the luciferase reporter responsive to TGF-β treatment, or upon Smad4-nuc expression, localization control cells not expressing v-ErbA (Figure 6A, b, f, j, and n, vs. B, b, f, j, and n). In support of these qualitative observations, changes in the nuclear:cytoplasmic ratios of Smad4 for cells expressing C308A-mC in the untreated condition were compared with those with TGF-β and LMB treatments and with cells expressing Smad4-nuc. We observed statistically significant increases of the nuclear:cytoplasmic ratios of 1.62-, 2.02-, and 2.28-fold (Figure 6B, lane 1 vs. lanes 2-4, respectively). Therefore, upon expression of the C308A-mC mutant, the localization behavior of Smad4 both qualitatively and quantitatively reverted to that observed in control cell lines that did not express v-ErbA.

We postulated that the localization of the C308A mutant might be similar to that of v-ErbA because the NES of v-ErbA is located in the gag region and therefore should be unaffected by the single point mutation within the zinc finger. Because the C308A mutation was within the DNA recognition helix of the zinc finger, we also considered that the balance between nuclear retention (due to DNA binding) and the nuclear export could change. Cell lines expressing C308A-mC with GFP-Smad4 or with GFP-Smad4-nuc enabled us to observe the localization behavior of this mutant. We found that in cells without or with TGF-β and in cells expressing GFP-Smad4-nuc, the cytoplasmic localization of C308A-mC was similar to that of v-ErbA-mC (Figure 6A, c, g, i, and n, vs. B, s, w, and ee). Also subcellular localization of C308A-mC is indistinguishable from v-ErbA-mC (Figure 6C, lanes 1, 2, and 4). Thus, the localization behavior of the C308A mutant is similar to that of nonmutated v-ErbA for untreated, TGF-β-treated, and in Smad4-nuc–expressing cells.

For cells expressing C308-mC and treated with LMB, the nuclear-cytoplasmic ratio was similar to that of cells expressing only v-ErbA-mC (Figure 6A vs. 3Eh, and Figure 6C, lane 3, vs. 3F, lane 3). This result contrasts with cells expressing both GFP-Smad4 and v-ErbA-mC, where v-ErbA nuclear localization was not increased upon LMB treatment (Figure 3Baa). These results agree with our observation that Smad4-v-ErbA interaction also disrupts nuclear localization of v-ErbA upon CRM1 inhibition by LMB. In summary, cells expressing a v-ErbA mutant that is unable to interact with Smad4 demonstrate increased nuclear localization upon LMB treatment.

Together, the results of these mutation experiments support the hypothesis that the v-ErbA oncoprotein antagonizes TGF-β signaling through its interaction with Smad4 and that v-ErbA association with Smad4 is essential for the dysregulation of TGF-β signaling. These observations additionally support a model in which v-ErbA/Smad4 interaction in conjunction with v-ErbA’s predominately cytoplasmic localization disrupts Smad4 cyto-nuclear shuttling necessary for a functional TGF-β pathway.

**DISCUSSION**

More than two decades have passed since v-ErbA was identified as the independent oncogenic product of AEV responsible for potentiating erythroblast transformation (Hayman et al., 1979; Vennstrom and Bishop, 1982). The cellular homolog of v-ErbA, TRα (c-ErbA), positively regulates target gene expression when bound to its cognate ligand, T3, and negatively regulates TRα/T3 responsive promoters in the absence of ligand. In contrast, the v-ErbA oncoprotein acts as a constitutive transcriptional repressor and is known to interfere with the function of endogenous thyroid hormone receptor as a dominant negative form of TRα (Damm et al., 2012).
This dominant negative activity, though, does not fully explain v-ErbA oncogenicity and has lead researchers to propose that v-ErbA most likely disrupts an unidentified pathway involved in the repression of genes required to prevent cellular transformation (Damm and Evans, 1993). TGF-β-regulated Growth Inhibition Is Disrupted by v-ErbA

In this study we hypothesized that the unidentified pathway disrupted by v-ErbA was the TGF-β signal pathway. The TGF-β pathway is a logical candidate because it regulates cell proliferation and differentiation (Massague et al., 2000; Attisano 1989).
and Wrana, 2002; Wakefield and Roberts, 2002), and disruption of TGF-β signaling is observed in the majority of cancers (Hanahan and Weinberg, 2000; Massague et al., 2000). Additionally, growth hormones play a critical role in erythroid progenitors in maintaining a balance between self-renewal and terminal differentiation into erythrocytes. Expression of onco-
genesis is often linked to the disruption of this delicate balance and results in leukemic transformation with sustained self-
terminal differentiation into erythrocytes. Expression of onco-
tionally, growth hormones play a critical role in erythroid

Propagation of TGF-β signaling within the cell depends on a highly regulated combination of phosphorylation, complex formation, and nuclear translocation of the Smad proteins (Massague et al., 2000). Our data demonstrate v-ErbA/Smad4 association and suggest that this interaction is involved in the dysregulation of TGF-β signaling and escape of growth inhibition. v-ErbA also remains predominately cytoplasmic due to a nuclear export signal located in the gag region (DeLong et al., 2004). We therefore hypothesize that TGF-β signaling is dysregulated upon the disruption of normal cyto-nuclear shuttling of Smad4 by v-ErbA’s strong cytoplasmic localization and its association with Smad4 (Figure 6D). In support of this hypothesis we demonstrated that indeed, Smad4 nuclear translocation was disrupted upon v-ErbA expression. Although in vitro binding assays clearly show v-ErbA/Smad4 association and fluorescence microscopy images show that Smad4 remains predominately cytoplasmic in the presence of v-ErbA, demonstration of direct v-ErbA/Smad4 interaction using in vivo immunoprecipitation techniques were inconsistent (results not shown). The variable outcomes of these immunoprecipitation experiments suggest several possible scenarios: One, that unidentified conditions are required to consistently observe direct v-ErbA/Smad4 binding in vivo, or two, that the v-ErbA/Smad4 interaction may be transient and occur only in the nucleus or during translocation. Another possibility is that interaction of Smad4 and v-ErbA requires integrity of cytoskeleton, which is often disrupted upon cell lysis. These potential scenarios as well as other possibilities await further research efforts and may provide further understanding of v-ErbA/Smad4 interactions.

To further characterize v-ErbA/Smad4 association, we investigated two v-ErbA mutations: 1) delZF, a mutant where both zinc-fingers in the DNA-binding domain were deleted; 2) C308A, a previously discovered, single-point mutant that prevents cysteine coordination and hence structural formation of the first zinc finger in TR-α (Bonde and Privalsky, 1990). The zinc finger domain is known to mediate other protein–protein interactions. A recent publication confirms that mutations in the zinc finger domain of the MDM2 oncoprotein disrupts ribosomal protein interaction and attenuates MDM2-induced p53 degradation (Lindstrom et al., 2007). Both the delZF and C308A mutations were unable to associate with Smad4 and cells stably expressing either delZF or C308A regained TGF-β-induced cell growth inhibition and TGF-β-responsive promoter activity.

Additionally, though C308A remained predominantly cytoplas-


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