Published in final edited form as:

Mol Cancer Res. 2011 January ; 9(1): 78–89. doi:10.1158/1541-7786.MCR-10-0216.

Distinctive mechanism for sustained TGF- β signaling and growth inhibition: MEK1 activation-dependent stabilization of type II TGF- β receptors

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Abstract

There are multiple mechanisms by which cells evade TGF- β -mediated growth inhibitory effects. In this report, we describe a novel mechanism by which cells become resistant to TGF- β -mediated growth suppression. While having all the components of the TGF- β signaling pathway, different cell lines, RL, HaCaT, and BJAB, have different sensitivities towards TGF- β -induced growth suppression. The TGF- β resistance of RL, a B cell lymphoma cell line, was due to ligand-induced down-regulation of TGF- β receptor II (T β RII) and only transient TGF- β -induced nuclear translocation of Smad2 and Smad3. With low dose PMA or anti-IgM treatment, TGF- β sensitivity was restored by stabilizing T β RII expression and sustaining TGF- β signaling. The MEK inhibitor U0126 blocked both PMA- and anti-IgM-induced up-regulation of T β RII. In HaCaT and BJAB, two TGF- β -sensitive cell lines, which had higher basal levels of phospho-MEK and T β RII compared to RL, U0126 induced down-regulation of T β RII and blocked subsequent TGF- β signaling. Similar results were also obtained with normal B cells, where MEK1 inhibitor down regulated T β RII and subsequent TGF- β signaling. Constitutively active MEK1, but not constitutively active ERK2, induced up-regulation of T β RII. Furthermore, T β RII physically interacted with the constitutively active MEK1, but not with wild type MEK1, indicating involvement of active MEK1 in stabilizing T β RII. Collectively, our data suggest a novel mechanism for MEK1 in regulating the sensitivity to TGF- β signaling by stabilizing T β RII.

Keywords

Signaling; ERK; Lymphoma; Smad; MEK

INTRODUCTION

TGF- β signaling affects numerous cellular processes, including proliferation, differentiation, migration and apoptosis, depending on the cell type and its stage in malignant progression (1,2). TGF- β initiates signaling by binding to and bringing together type I (T β RI) and type II (T β RII) receptor serine/threonine kinases on the cell surface (3). This allows receptor II to phosphorylate the receptor I kinase domain, which then propagates the signal through phosphorylation of the receptor-regulated Smad protein (Smad2/3), which is directly phosphorylated and activated by the type I receptor kinase. It then undergoes

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homotrimerization and formation of heteromeric complexes with a common partner, Smad4. The activated Smad complexes translocate into the nucleus and, in conjunction with other nuclear cofactors, regulate the transcription of target genes. The Smad pathway is negatively regulated by Inhibitory Smad (Smad6/7) (4,5) and PPM1A phosphatase (6).

Low expression levels of T β RII were found in human B cell lymphoma cell lines (7) and breast cancers (8), and were thought to be responsible for the development of resistance to TGF- β -induced growth arrest. Mutations in the T β RII were also found in colon and gastric cancer with microsatellite instability (MSI) (9,10). T β RII frameshift mutations have been found in human gliomas (11). T β RII missense mutations have been observed in two head and neck carcinoma cell lines (12). TGF- β receptor expression may also be reduced in tumor cells through hypermethylation of CpG islands in the T β RII gene promoters (13), or from mutations in the T β RII promoter that interfere with transcription factor binding (14). The oncogene EWSR1 can repress T β RII expression by blocking T β RII promoter activity and may account for decreased responsiveness to TGF- β in some cancer cells (15).

T β Rs can interact with the pro-apoptotic adaptor protein Daxx (16), TGF- β -activated kinase 1 (TAK1) (17) and Rho GTPase (18), which lead to the induction of apoptosis or Epithelial/endothelial-Mesenchymal Transitions (EMT). In addition, several intracellular proteins have been shown to interact with the TGF- β receptor complex, including SARA (19), FKBP12 (20), STRAP (21), TRIP-1 (22) and chimeric tyrosine kinase ETV6-NTRK3 (23), to facilitate or suppress TGF- β signaling.

Although Smads are the most well-characterized target proteins, activated T β Rs can also lead to the activation of MAPKs, such as the ERK (24), c-Jun N-terminal kinase (25) and p38 MAP kinase (26), the extent and kinetics of which differ among different cell lines and types. The canonical mitogen activated protein kinase (MAPK) cascade, including the Ras-Raf-MEK-ERK module, is critically involved in the regulation of normal cell proliferation, survival and differentiation. Aberrant regulation of MAPK cascades contribute to cancer and other human diseases (27). MAPKs may also modulate TGF- β signaling. ERK has been shown to phosphorylate the linker region of Smad2/3, which results in blocking the nuclear translocation of activated Smad2/3 (28). ERK MAP kinase pathway has also been shown to downregulate TGF- β signaling by activating TACE-mediated ectodomain shedding of T β RI (29). Moreover, ERK, JNK and TAK1/p38 pathways were shown to regulate TGF- β signaling via Smad7 expression, depending on the cell type (30,31).

While having all the components of the TGF- β signaling pathway, RL, a germinal center-derived lymphoma cell line was resistant to TGF- β -induced growth arrest. We wanted to determine the mechanism of TGF- β resistance in RL cells, and examined the involvement of that underlying mechanism in TGF- β sensitive cell lines. In the present study we have demonstrated that the TGF- β resistance of RL was due to ligand-induced down-regulation of TGF- β receptor II (T β RII). Activated MEK1 stabilized T β RII, and a MEK1 inhibitor, U0126, induced down-regulation of T β RII in two TGF- β responsive cells lines and in normal human peripheral blood B cells with TGF- β resistance developing as a consequence. Moreover, we have shown that constitutively active, but not wild type MEK1 physically interacted with T β RII, indicating a role for active MEK1 in stabilizing T β RII. Our findings identify a novel mechanism by which tumor cells may evade TGF- β -induced growth suppression by controlling the stability of the T β RII.

MATERIALS AND METHODS

Reagents

For Western blot analysis and immunoprecipitation, rabbit polyclonal anti-T β RI antibody (sc-398), anti-T β RII (sc-220 and sc-400), phospho-MEK1/2 (sc-7995), MEK (sc-6250), phospho-ERK1/2 (sc-16982R), ERK1 (sc-93) and ERK2 (sc-154) were obtained from Santa Cruz Biotechnology Inc (Santa Cruz, CA, USA); rabbit polyclonal phospho-Smad2 antibody, phospho-Smad3 were purchased from Cell Signaling Technology Inc. (Beverly, MA, USA); Rabbit polyclonal anti-Smad2 antibody (51–1300) and anti-Smad3 (51–1500) were obtained from Zymed Laboratories Inc. (South San Francisco, CA, USA); anti-Nucleoporin p62 and anti-BiP/GRP78 were from BD Transduction Laboratories (San Jose, CA, USA); mouse monoclonal anti-FLAG (F1804) was purchased from Sigma (St. Louis, MO, USA); mouse monoclonal anti-HA (clone 12CA5) was purchased from Roche Applied Science (Indianapolis, IN, USA); β -Actin was purchased from Abcam (Cambridge, United Kingdom). All HRP conjugated secondary antibodies were purchased from GE Healthcare. Recombinant TGF- β 1 (240-B) and anti-human TGF- β RII-Phycoerythrin were purchased from R&D Systems (Minneapolis, MN, USA); phorbol 12-myristate 13-acetate (PMA), and Phorbol 12,13-dibutyrate (PDBu) were from Sigma-Aldrich (St. Louis, MO, USA); U0126 was from EMD Biosciences. DSP (dithiobis[succinimidylpropionate]), Sulfo-NHS-LC-Biotin and NeutrAvidin™ Agarose Resins were from Pierce (Rockford, IL, USA).

Cell culture

BJAB is an EBV-negative Burkitt's lymphoma cell line (Kindly provided by Dr. Jeffery Sample, St. Jude Children's Research Hospital). RL and BJAB cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), 2mM L-glutamine, 1,000 U of penicillin/mL, 100 μ g of streptomycin/mL. HEK293A and HaCaT cells were cultured in DMEM medium supplemented with 10% FBS, 2mM L-glutamine, 1,000U of penicillin/mL, 100 μ g of streptomycin/mL. No exogenous growth factors were added. Cells were grown at 37°C in 5% CO₂. Fresh growth medium was added to cells every 3 to 4 days. For growth observation, RL or RL/pWPI/HA-T β RII cells were stimulated with either 0.15ng/mL PMA or 2ng/mL recombinant TGF- β 1 and/or PMA in RPMI 1640 with 5% FBS. Human B cells were purified from peripheral blood mononuclear cells (PBMCs) isolated from normal donors' blood by negative selection using the B Cell Isolation Kit II (Miltenyi Biotec, Germany). Donors provided written informed consent. Freshly isolated B cells were cultured in RPMI 1640 medium supplemented with 1% fetal bovine serum (FBS), 2mM L-glutamine, 1,000 U of penicillin/ml, 100 μ g of streptomycin/ml. The routine purity of the isolated B cells was over 95%.

Cell surface Biotinylation

Biotinylation and Immunoprecipitation of cell surface TGF- β 1 receptors were performed according to the procedure reported by Liu et al.(29). Briefly, cells were washed twice with ice-cold PBS, and incubated with EZ-link Sulfo-NHS-LC-Biotin (0.5mg/ml) at room temperature for 30 minutes. Biotinylation was quenched with 0.1 M glycine and remove excess biotin reagent. Cells were lysed in lysis buffer (10mM Tris-Cl (pH 8.0), 150mM NaCl, 1mM MgCl₂, 5 μ g/mL E-64, 1mM PMSF, 2.5 mM Sodium pyrophosphate, 1 mM beta-glycerophosphate, 1 mM NaOV4, 1mM NaF and 1% NP-40). Biotinylated proteins were precipitated with NeutrAvidin™ Agarose Resins and analyzed by immunoblotting using anti-T β RI (V-22) antibody and anti-T β RII (L-21) antibody.

Flow cytometry

Cells were washed with cold PBS and blocked with 0.2% Human IgG for 30 minutes at 4°C. Cells were then incubated with polyclonal anti-human TBR1-Phycoerythrin for 30 minutes at 4°C, washed with PBS, and fixed in 2% paraformaldehyde before analyzed in FACSscan.

Cell lysis and Western Blot Analysis

Cytoplasmic and nuclear extracts were prepared according to the procedure reported earlier (32). Membrane preparation was carried out according to the previous procedure (13). Whole cell lysates were prepared according to the following procedure: harvested cells were resuspended in lysis buffer [10mM Tris-Cl (pH 8.0), 150mM NaCl, 1mM MgCl₂, 5μg/mL E-64, 1mM PMSF, 2.5 mM Sodium pyrophosphate, 1 mM beta-glycerophosphate, 1 mM NaO₄V, 1mM NaF and 1% Triton X-100] and were incubated on ice for 30 min. Samples were then homogenized, centrifuged, and the supernatants were collected. Protein concentrations were measured with Bio-Rad Protein Assay Dye reagent (Bio-Rad Laboratories, Hercules, California). Gel-electrophoresis was carried out using 4~12% SDS-PAGE under reducing conditions. After membrane transfer, bound antibodies were detected using chemiluminescent detection system (GE Healthcare).

Immunoprecipitation

Lysates were precleared with protein A/G plus-agarose (sc-2003, Santa Cruz Biotechnology Inc., Santa Cruz, California) for 30 min at 4 °C, and the pre-cleared lysates were incubated overnight with either anti-MEK1 or anti-Flag antibody and A/G plus-agarose beads. The agarose beads were washed three times with extraction buffer containing 25 mM MOPS (pH 7.2), 15mM MgCl₂, 137mM NaCl, 1mM PMSF, 15mM EGTA, 5μg/mL E-64, 1 mM sodium vanadate and 1mM NaF and 0.1% Triton X-100. The immune complexes were dissociated with loading buffer containing LDS and boiled for 5 min. Electrophoresis was carried out by 4~12% SDS-PAGE under reducing conditions.

Protein Cross-linking

Cross-linking using DSP was performed according to the manufacturer's protocol. Briefly, DSP was dissolved in dimethyl sulfoxide as a stock solution of 250 mg/ml. Samples were rinsed twice with cold PBS and incubated with PBS containing 1mg/ml DSP for 6 minutes at room temperature. The cross-linking reaction was quenched by adding 1M Tris-Cl pH8.0 to a final concentration of 100mM followed by 1 minute incubation in room temperature. Cells were rinsed once with cold PBS before lysates were prepared as described above.

Quantitative Real-time PCR

Total RNA was isolated with Rneasy Mini Kit (Qiagen, Valencia, CA). The complementary DNA was generated from 2μg of RNA by using the SuperScript VILO cDNA synthesis kit and random hexamers (Invitrogen). One hundredth of the samples were used in a real-time PCR reaction containing a 0.2μM concentration of both forward (tcactgacaacaacggtgc) and reverse (tgactttggagaagcgc) primers of TBR1 and SYBR green PCR master mix (Applied Biosystems, Foster City, CA). Quantitations of fold induction were normalized by β-actin and analyzed by the method described by Schmittgen et al (33).

Statistical analysis

Values were obtained from three independent experiments and were expressed as means ± SD. Statistical analysis was performed using Student's t-test and analyzed by two tailed test of paired samples. Values were considered significant (*) if 'p' values were <0.05.

DNA constructs and transfection

To generate Flag-tagged constructs, sequences corresponding to the wild type T β RI and T β RII were amplified from DNA constructs expressing HA-tagged T β RI or T β RII (generous gifts from Drs. Carl-Henrik Heldin and Joan Massague, respectively), and were subcloned into pENTRY/SD/D-TOPO vector (Invitrogen). The pENTRY/SD/D/Flag-T β RII was then used in recombination with pcDNA-DEST40 according to the manufacturer's protocol. Wild type and constitutively active form of MEK1 constructs were obtained from Dr. Natalie Ahn (University of Colorado at Boulder). Wild type and constitutively active form of ERK2 constructs were obtained from Dr. Melanie Cobb (University of Texas Southwestern Medical Center). Wild type and constitutively active form of ERK1 constructs were obtained from Dr. David Engelberg (Hebrew University of Jerusalem, Israel). In HEK293A cells, transient transfections were performed with Lipofectamine 2000 reagent according to the protocol provided by the manufacturer (Invitrogen). RL cells were transfected by electroporation as follows: Exponentially growing RL cells were resuspended in 100 μ l Kit V solution (Lonza, Gaithersburg, Maryland) containing 3 μ g plasmid. Cells were then exposed to electroporation (program S-18) using Nucleofector Device (Lonza, Gaithersburg, Maryland). After a 12-hour incubation, cells were transferred to fresh medium, and were further cultured for 24 hours.

Virus production and transduction

A DNA fragment corresponding to the wild type T β RII containing HA sequence was subcloned into lentiviral vector pWPI (kindly provided by Dr. Didier Trono, Global Health Institute, Lausanne, Switzerland). To introduce the human Cocksackie Adenovirus Receptor (hCAR) to the B cell lymphoma cell line, pHRCMV/hCAR-EGFP (generously provide by Dr. Mikko Mättö, University of Kuopio, Finland) was used. Lentiviral production with pWPI/HA-T β RII and pHRCMV/hCAR-EGFP, using the envelope vector pMD.G and the packaging vector pCMVR8.91 (kindly provided by Dr. Didier Trono) were carried out as described before (34). RL cells were seeded in a 12 well plate at 1×10^6 cells per well and virus was added with a multiplicity of infection (MOI) equal to 10. EGFP positive cells were isolated by limited dilution and checked by FACScan (BD Biosciences). Recombination between Flag-T β RII from pENTRY/SD/D and pAD/CMV/V5-DEST vector was done by LR Clonase II Enzyme Mix (Invitrogen) and preparation of adenoviruses bearing Flag-T β RII were done according to the manufacturer's instructions. RL/hCAR-EGFP cells were infected with adenovirus harboring Flag-T β RII with an MOI equal to 200. The culture medium was replaced with fresh medium the next day, and incubated for another 24h. Cells were then treated with PMA (10ng/ml) and U0126 (20 μ M), with or without TGF- β 1 (2ng/ml).

RESULTS

Rapid loss of TGF- β signaling accounts for the resistance of RL cells to TGF- β -mediated growth suppression

We have shown previously that a B cell lymphoma cell line RL is unresponsive to TGF- β -mediated growth suppression, whereas in the presence of a low dose of PMA, RL cells can be rendered responsive to TGF- β by up-regulating p21^{CIP/WAF1} and down-regulating c-Myc (Fig. 1A & B) (13, 35). RL cells can also be rendered responsive to TGF- β with regards to the growth suppression and up-regulation of p21^{CIP/WAF1} by biologically relevant stimulation delivered by anti-IgM treatment (Fig. 1C & D). To understand the mechanism behind the lack of TGF- β responsiveness, RL cells were treated with TGF- β in the presence or absence of PMA for various time periods, and the TGF- β signaling was monitored by the nuclear translocation of phospho-Smad2. As shown in Fig. 1E, although RL cells had all the components of the TGF- β pathway, the TGF- β signaling was short lived. The levels of

nuclear phospho-Smad2 were decreased by 12 hours and were further down-regulated by 24 hours after TGF- β treatment. By contrast, in the presence of low dose PMA, TGF- β signaling was sustained at 24 hours and growth inhibition was observed. These data suggested that the transient TGF- β signaling might be responsible for the unresponsiveness of RL cells to TGF- β .

TGF- β 1-induced down-regulation of T β RII was responsible for transient TGF- β 1 signaling in RL cells

To explore whether ligand-induced down-regulation of TGF- β receptors (T β Rs) accounts for the transient signaling, we examined the status of T β RI and T β RII upon treatment with TGF- β 1 in the presence or absence of PMA for various time periods. As shown in Fig. 2A (Upper panel), TGF- β 1 alone induced down-regulation of T β RII at 24 hours, whereas in the presence of PMA, T β RII levels were sustained for at least 48 hours. In contrast, the levels of T β RI were unchanged throughout the time points tested. To determine the status of the cell surface T β RI and T β RII, protein biotinylation was used according to the procedure described by Liu et al. (29). As shown in Fig. 2A (lower panel), cell surface expression of T β RII was also down-regulated upon TGF- β 1 stimulation and the down-regulation was prevented by the PMA treatment. Like the total T β RI level, cell surface T β RI was unaffected by the TGF- β treatment in the presence or absence of PMA. Cell surface status of T β RII was also examined by fluorochrome-conjugated anti-T β RII antibody and analyzed by fluorescence activated cell sorter (FACS) (Supplemental Fig. 1A). TGF- β 1-induced down-regulation of T β RII, both total and cell surface, was also blocked by the proteasome inhibitor MG132 (Fig. 2B, and Supplemental Fig. 1B). This stability of T β RII along with the stability of Smad2 by MG132 (data not shown; (36)) resulted in the sustained phosphorylation of Smad2 upon TGF- β 1 treatment (Fig. 2C). To verify further whether ligand-induced down-regulation of T β RII was responsible for transient TGF- β 1 signaling, we generated RL cells stably over-expressing HA-tagged T β RII (Supplemental Fig. 2). As shown in Fig. 2D, RL cells over-expressing T β RII were rendered responsive to TGF- β 1-mediated growth suppression, and the growth suppression was correlated with higher surface expression of T β RII, sustained activation of Smad2, and expression of p21^{CIP/WAF1} (Fig. 2E and Supplemental Fig. 3). These data suggested that ligand-induced down-regulation of T β RII was responsible for transient TGF- β 1 signaling, and this down regulation was prevented by a proteasome inhibitor and PMA treatment.

MEK activation is involved in the stabilization of T β RII

To determine the kinase responsible for PMA-induced T β RII stabilization, we investigated the phosphorylation status of MEK1/2 and ERK1/2. As shown in Fig. 3A, dose-dependent increases in phosphorylation of both MEK1/2 and ERK1/2 were observed upon PMA treatment of RL cells. We did not observe any phosphorylation of PKC, as determined by the phospho-specific (pan) PKC antibody, at the low PMA concentration used here (data not shown). Low dose PMA-induced phosphorylation of MEK1/2 and ERK1/2 correlated with increased total and cell surface T β RII levels in RL cells (Fig. 3B and Supplemental Fig. 4A). To evaluate whether MEK activation was the cause of the T β RII stabilization induced by PMA, the MEK inhibitor, U0126, was used. As shown in Fig. 3B, although U0126 had no effect on the phosphorylation status of MEK1/2 (since U0126 only affects MEK1/2 kinase activity), it blocked the phosphorylation of its downstream target ERK1/2. U0126 treatment resulted in the blockage of PMA-induced increase in T β RII level, indicating the possible involvement of MEK1/2 kinase activity in PMA-induced T β RII stabilization in RL cells (Fig. 3B). U0126 treatment also inhibited PMA-induced increase in cell surface T β RII (Supplemental Fig. 4A). The activated MEK-induced up-regulation of cell surface T β RII level was also observed in case of anti-IgM treated RL cells, and the up-regulation was sensitive to U0126 (Supplemental Fig. 4B). Down-regulation of T β RII by U0126 resulted in

a decrease in PMA/TGF- β 1-induced phosphorylation of Smad2 (Fig. 3C). Interestingly, treatment with U0126 also caused down-regulation of the T β RII levels and subsequent TGF- β 1 signaling in two TGF- β 1-responsive cell lines, HaCaT and BJAB (Fig. 3B, D, Supplemental Fig. 4C, & data not shown). The basal level of phospho-MEK1/2 was significantly higher in HaCaT and BJAB in comparison to RL cells, which correlated with higher basal levels of T β RII in these cell lines (Fig. 3B, Supplemental Fig. 4, & data not shown).

To determine whether the relation between the level of active MEK and T β RII status was unique to cell lines, freshly isolated normal human peripheral blood B cells were pre-treated with U0126 followed by the treatment with TGF- β , and the total and cell surface T β RII levels were analyzed (Fig. 3E and Supplemental Fig. 4D). The pre-treatment with U0126 caused down regulation of total T β RII levels and subsequent blockage in the phosphorylation of Smad2. Surprisingly, the levels of cell surface T β RII was increased upon U0126 treatment in the presence or absence of TGF- β 1, indicating the involvement of MEK1 in T β RII levels in normal B cells is different than the immortalized cell lines (Supplemental Fig. 4D).

To investigate the effect of TGF- β treatment on the levels of T β RII and subsequent TGF- β signaling in TGF- β sensitive and insensitive cell lines, RL, HaCaT and BJAB cells were treated with TGF- β for various time periods, and the levels of T β RII and phospho-Smad2 were determined by western blot analysis, and the cell surface T β RII was determined by FACS. Although TGF- β treatment caused down regulation of T β RII in RL cells in a time-dependent manner, the levels of T β RII were unaffected in TGF- β sensitive cell lines HaCaT and BJAB (Supplemental Fig. 5A, B, C, & data not shown). The T β RII status was correlated with the TGF- β signaling in these cell lines (Supplemental Fig. 5A). These data suggested that active MEK1/2 plays an important role in T β RII stabilization.

Constitutively active MEK1 up-regulates T β RII via protein stabilization

If the activation of MEK1/2 was responsible for the up-regulation of T β RII, ectopically expressed active MEK1 should up-regulate T β RII and convey TGF- β 1-responsiveness to the RL cells. We co-transfected RL cells with T β RII and either a wild type (MEK1-WT/ERK-WT) or a constitutively active form of MEK1 or ERK2 (MEK1-CA/ERK2-CA), and then checked the expression of T β RII by western blot analysis and cell surface staining. As shown in Figure 4A, the constitutively active, but not the wild type MEK1, up-regulated both total as well as cell surface T β RII level (lane 3 vs. 1, and lane 2 vs. 1, and Supplemental Fig. 6A). Interestingly, neither wild type nor constitutively active ERK2 had any effect on T β RII levels (lanes 4 & 5 vs. 1). Similar results were also obtained with wild type and constitutively active ERK1 (Supplemental Fig. 7), suggesting that the up-regulation of T β RII was independent of ERK activation. To determine whether this phenomenon was unique to RL cells, the same experiment was performed in HEK293A cells. As shown in Figure 4B, only constitutively active, but not wild type MEK1, up-regulated both total as well as cell surface T β RII in HEK293A cells (lane 3 vs. 1, and lane 2 vs. 1, and Supplemental Fig. 6B). These data suggested that the activation of MEK1, but not ERK1/2, was mainly responsible for the up-regulation of T β RII.

To determine whether the up-regulation of T β RII was due to a transcriptional or a post-transcriptional mechanism, we first tested the effect of PMA in the presence or absence of U0126 on the mRNA levels of T β RII by real-time PCR. Treatment with PMA in the absence or presence of U0126 did not have any effect on the T β RII mRNA levels throughout the time points tested, whereas these treatments affected the T β RII protein levels as observed in Fig. 3B (Supplemental Fig. 8). To investigate whether MEK1 activation was involved in the stabilization of T β RII protein, HEK293A cells were co-transfected with T β RII and either

wild type or constitutively active MEK1. After 24 hours, cells were exposed to cycloheximide (CHX) for different periods of time as indicated. At each time point, cells were collected and the levels of T β RII were determined by western blot analysis. As shown in Figure 4C (upper panel), CHX treatment resulted in the rapid down-regulation of the basal T β RII levels (lanes 1–5), whereas constitutively active MEK1-induced T β RII levels were sustained for longer periods of time (lanes 6–10). To calculate the half lives ($t_{1/2}$) of T β RII in the presence of either wild type or constitutively active MEK1, densitometric analysis of the autoradiographs within the linear range of the films were performed. As shown in Fig. 4D (lower panel), $t_{1/2}$ for wild type MEK1 was 64 ± 6.9 minutes, whereas $t_{1/2}$ for constitutively active MEK1 was 136 ± 27.7 minutes ($p < 0.01$, $n = 3$). These data indicate that the up-regulation of T β RII by active MEK1 was via stabilization of T β RII protein.

Constitutively active MEK1 physically interacts with T β RII

In order to determine whether MEK1 stabilizes T β RII via physical interaction, co-immunoprecipitation was performed using lysates prepared from HEK293A cells co-transfected with FLAG-tagged T β RII and either wild type or constitutively active form of MEK1. As shown in Fig. 5A, anti-MEK1 antibody was able to pull down T β RII only from the cells expressing the constitutively active form of MEK1, but not the wild type MEK1 (left panel). We were also able to demonstrate the interaction in reverse immunoprecipitation, where anti-FLAG antibody co-immunoprecipitated the constitutively active form of MEK1 (right panel). Although, ERK was found to be associated with active MEK1 during immunoprecipitation with anti-MEK1 antibody, immunoprecipitation with anti-FLAG antibody failed to pull down ERK, indicating ERK was not the effectors for MEK1-mediated T β RII stabilization. Also, we did not observe any interaction between T β RI and active MEK (data not shown). Next, we wanted to know whether the MEK inhibitor, U0126, had any effect on the interaction between T β RII and active MEK1. To evaluate that question, HEK293A cells were co-transfected with T β RII and the constitutively active form of MEK1, and after 24 hours, cells were treated with U0126 for different periods of time. As shown in Fig. 5B, U0126 blocked the interaction between T β RII and the constitutively active form of MEK1 in a time-dependent manner without affecting the T β RII levels (Fig. 5B). As a consequence of blocking the interaction of MEK1 and T β RII, U0126 prevented the up-regulation of T β RII induced by both exogenous and endogenous MEK1 in HEK293A and RL cells, respectively (Fig. 5C and Supplemental Fig. 6A & B).

To determine whether interaction between T β RII and MEK occurs endogenously, RL cells were stimulated with either medium alone or PMA for 24 hours. Cells were then treated with U0126 for different time periods, after which cross-linker was used to monitor the interaction. As shown in Fig. 5D (left panel), anti-T β RII antibody was able to pull down endogenous phospho-MEK1 in absence of U0126. Treatment with U0126 affected the interaction in a time-dependent manner. Similarly in reverse immunoprecipitation analysis, anti-MEK1 antibody was also able to pull down endogenous T β RII in the absence of U0126 (right panel). We were unable to demonstrate the interaction between T β RII and MEK1 in the absence of any cross-linker indicating the fact that either the interaction is transient, or the lysis conditions are not optimal to preserve the interaction. Our data indicate that the endogenous T β RII comes in contact with phospho-MEK1 upon PMA treatment, and the MEK1 inhibitor U0126 prevents the interaction between T β RII and phospho-MEK1. Finally, to determine whether biologically relevant stimulation can induce interaction between T β RII and active MEK, T β RII over-expressing stable RL cells were stimulated with either medium alone or anti-IgM for 1 hour. Cells were then treated with U0126 for indicated time periods. Following cross-linking by DSP, whole cell lysates were used for immunoprecipitation. As shown in Fig. 5E, anti-T β RII antibody was able to pull down

endogenous phospho-MEK1 in absence of U0126. Treatment with U0126 affected the interaction in a time-dependent manner. Collectively, these data strongly suggested that the interaction between T β RII and active MEK1 was essential for the up-regulation of T β RII.

DISCUSSION

Although the mechanism of activation of the TGF- β signaling pathway is well established, additional non-canonical components greatly influence the activity of this pathway. T β RII is responsible for the initiation of multiple TGF- β signaling pathways, and loss of its function is associated with many types of human cancers (9,10). In this report, we describe a novel mechanism by which cells become resistant to TGF- β -mediated growth suppression. A B-cell lymphoma cell line, RL, which had intact components of the TGF- β signaling pathway, was resistant to TGF- β -induced growth arrest due to ligand-induced down-regulation of TGF- β receptor II (T β RII) and only transient TGF- β -induced nuclear translocation of Smad2, without affecting T β RI. The down-regulation of T β RII was blocked by the proteasome inhibitor MG132, and mitogenic stimulation like low dose PMA or α IgM treatment. We have found that active MEK1 was involved in the stabilization of T β RII based on the following observations: (1) the MEK1/2 inhibitor U0126 blocked PMA-induced up-regulation of T β RII and subsequent TGF- β signaling in RL cells; (2) two TGF- β -responsive cell lines, HaCaT and BJAB, which had higher basal levels of phospho-MEK and T β RII (Fig. 3B), had attenuated TGF- β signaling upon U0126 treatment, which was associated with T β RII down-regulation; (3) in normal B cells, the MEK1/2 inhibitor U0126 down regulated T β RII and subsequent TGF- β signaling; (4) ectopically expressed active MEK1, but not active ERK1 or ERK2, was able to up-regulate T β RII; (5) both exogenous and endogenous active MEK1 physically interacted with T β RII; and (6) interference in the interaction between T β RII and active MEK1 by U0126 resulted in the inhibition of T β RII up-regulation. These data suggest that the stabilization of T β RII by active MEK is a general phenomenon.

The number of cell surface TGF- β receptors is tightly controlled by internalization, recycling and ubiquitin-dependent down-regulation, which contribute to the regulation of cell surface expression of TGF- β receptors. In mink lung epithelium, the half-life of T β RII has been shown to be 60 minutes, which was further reduced to 45 minutes in the presence of exogenous ligand (37). TGF- β receptors can be internalized through both caveolin and EEA1-positive vesicles, and reside in both lipid raft and non-raft membrane domains (38). Clathrin-dependent internalization in the non-raft membrane domain, EE1-positive endosomes, which are enriched in Smad2 and SARA, activates TGF- β signaling, whereas internalization in caveolin-containing raft domains, which are enriched in Smad7 and Smurf2, stimulates receptor turn over through the ubiquitylation pathway (38–40). Recycling occurs as receptors are internalized through clathrin-coated pits, and then returned to the plasma membrane via a rab11-dependent mechanism (41). Interestingly, internalization of T β RII is unaffected by ligand-receptor interaction (38,41). Our findings indicate an important role that MEK1 plays in determining the fate of T β RII possibly by redirecting T β RII to recycling as opposed to the degradation pathway. Whether MEK1 does this either by directly associating with T β RII or by modifying T β RII through phosphorylation is currently under investigation. The role of MEK1 in T β RII stabilization is not unique in B cell lymphoma cell lines as we have shown this effect in multiple cell types: BJAB (B cell lymphoma); HaCaT (keratinocyte), HEK293 (epithelial), and normal human peripheral blood B cells.

Active MEK-mediated T β RII stabilization may have significant physiological relevance to the conversion from a tumor suppressor to a tumor promoter role for TGF- β . As a case in point, epidermal growth factor receptor (EGFR) is expressed in a wide variety of human

tumors (42), and EGFR engagement triggers a number of downstream signaling pathways including Ras/Raf/MEK (43). In work that has similarity to ours, it has been shown that MEK1/2 was involved in the permissive role of epidermal growth factor in TGF- β signaling by stabilizing T β RII mRNA in prostate adenocarcinoma cells (44). Our data suggest that MEK-mediated modification may play an important role in maintaining T β RII level on the cell surface and prevent ligand-induced down-regulation of T β RII. These findings also suggest a mechanism by which tumor cells evade TGF- β -induced growth suppression by increasing the ligand-induced turnover of the T β RII. Furthermore, it is possible that an agent capable of activating MEK phosphorylation could restore TGF- β responsiveness to some lymphomas in vivo. Experiments to test this notion are underway.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This research was supported (in part) by the Intramural Research Program of the NIH, National Institute on Aging.

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Figure 1.

Effect of TGF-β1 on RL cells in the presence or absence of low dose PMA. (A) RL cells were plated at 0.125×10^6 cells/mL and treated with either medium alone, TGF-β1 (2ng/mL), PMA (0.15 ng/mL), or PMA plus TGF-β1 for various time periods. At the end of each time point, cell counts were performed. Results are representative of triplicate experiments. *

indicates the inhibition is statistically significant ($p < 0.01$). (B) RL cells were treated with either medium alone, TGF- β 1, PMA, or PMA plus TGF- β 1 for various time periods. Equal amounts of whole cell lysates were analyzed by western blot analysis. (C) Effect of TGF- β 1 on RL cells in the presence or absence of α IgM. RL cells were treated as in (A) except α IgM (1 μ g/ml) was used instead of PMA, and cell counts were performed at the end of each time point. * indicates the inhibition is statistically significant ($p < 0.01$). (D) RL cells were treated with either medium alone, TGF- β 1, α IgM, or α IgM plus TGF- β 1 for 24 hours. Equal amounts of whole cell lysates were analyzed by western blot analysis. (E) Time course of nuclear translocation of phospho-Smad2 induced by TGF- β 1 (2ng/mL) with or without PMA (0.15ng/mL) treatment. Equal amounts of nuclear lysates for the time points indicated were analyzed by western blot analysis. The membranes were probed and reprobed with different antisera after stripping, as indicated above. Bar graphs represent the quantification of the phospho-Smad2 and total Smad2 blots normalized with the nucleoporin bands.

Figure 2.

Restoration of T β R II renders RL cells responsive to TGF- β 1 mediated growth arrest. (A) Upper panel: RL cells were treated with either medium alone or with TGF- β 1 (2ng/mL) in the presence or absence of PMA (0.15ng/mL) for various time periods. Equal amounts of membrane proteins were analyzed by western blot analysis. Lower panel: RL cells were treated with either medium alone, TGF- β 1, PMA, or PMA plus TGF- β 1 for 24 hours. Cell surface TGF- β receptors were detected by biotinylation, neutravidin bead adsorption and western blot analysis according to the procedure described in Methods and Materials. (B) RL cells were pretreated with PDBU (Phorbol 12,13-dibutyrate, a washable phorbol ester, 1.2ng/mL) for 24hrs, washed and resuspended in fresh medium before treatment with either medium alone or TGF- β 1 in the presence or absence of either MG132 (20 μ M) or PMA (0.15ng/mL) for 8 hrs. Equal amounts of membrane proteins were analyzed by western blot analysis. (C) RL cells were treated with either medium alone or TGF- β 1 in the presence or absence of MG132 for the indicated time points. Equal amounts of whole cell lysates were analyzed by western blot analysis. (D) RL/pWPI/HA-T β R II cells were plated at 0.125×10^6 cells/mL and treated with either medium alone or TGF- β 1 for various time periods. At the end of each time point, cell counts were performed. Results are representative of triplicate experiments. * indicates the inhibition is statistically significant ($p < 0.01$). (E) RL or T β R II over-expressing stable RL cell line (RL/pWPI/HA-T β R II) was treated with TGF- β 1 for different time periods. Equal amounts of whole cell lysates were analyzed for phospho- and total Smad2, p21^{Cip1/WAF1}, β -actin by western blot analysis.

Figure 3.

TβRII expression was correlated with MEK activation. (A) RL cells were treated with either medium alone or PMA (0.15ng/mL and 10ng/mL) for 6 hrs. Equal amounts of whole cell lysates were analyzed by western blot analysis. (B) RL, BJAB, and HaCaT cells were treated with either medium alone or PMA in the presence or absence of U0126 (20μM) for indicated time periods, and equal amounts of whole cell lysates were analyzed by western blot analysis. (C) RL cells were treated with medium alone or PMA plus TGF-β1 in the presence or absence of U0126 for indicated time periods, and equal amounts of whole cell lysates were analyzed by western blot analysis. (D) HaCaT and BJAB cells were treated with TGF-β1 in the presence or absence of U0126 for 6 hours, and equal amounts of whole cell lysates were analyzed for phospho- and total Smad3 by western blot analysis. (E) Human peripheral blood B cells were pretreated with U0126 for 6 hours before treatment with TGF-β1 for 1 hour. An equal amount of whole cell lysates were analyzed by western blot analysis.

Figure 4. Constitutively active MEK1 up-regulates TβRII expression through protein stabilization. (A) RL and (B) HEK293A cells were co-transfected with TβRII and either wild type MEK1 (MEK1-WT), constitutively active MEK1 (MEK1-CA), wild type ERK2 (ERK2-WT) or constitutively active ERK2 (ERK2-CA) constructs. After 24 hours, whole cell lysates were prepared and analyzed by western blot analysis. Constitutively active form of ERK2 (ERK2-CA) is a chimeric construct in which ERK2 was coupled to its upstream activator MEK1 resulting in a larger protein (45). (C) Upper panel: HEK293A cells were transfected with either wild type MEK1 (MEK1-WT, lanes 1–5) or constitutively active MEK1 (MEK1-CA, lanes 6–10) constructs. After 24 hours, cycloheximide (CHX, 20μg/ml) was added to the culture, and cells were collected at different time points indicated post CHX treatment. Equal amounts of whole cell lysates were analyzed by western blot analysis. Lower panel: Graphical representation of the densitometric analysis of the western blot from three independent CHX experiments. * indicates the statistical significance (p< 0.01).

A

B

Figure 5.

TβRII physically interacts with constitutively active, but not wild type MEK1. (A) HEK293A cells were co-transfected with TβRII and either wild type (MEK1-WT) or constitutively active MEK1 (MEK1-CA) constructs. After 24 hours, whole cell lysates were prepared, and equal amounts of whole cell lysates were used for immunoprecipitation with anti-TβRII or anti-MEK1 antibody, and the immunocomplexes were analyzed by western blot analysis. (B) HEK293A cells were co-transfected with TβRII and constitutively active MEK1 (MEK1-CA) constructs. After 24 hours, transfected cells were treated with either medium alone or U0126 for different times. Equal amounts of whole cell lysates were used for immunoprecipitation with anti-MEK1 antibody, and the immunocomplexes were analyzed by western blot analysis. (C) Left panel: HEK293A cells were co-transfected with TβRII and either wild type (MEK1-WT) or a constitutively active MEK1 (MEK1-CA) constructs. After 6 hours, transfected cells were treated with either medium alone or U0126 overnight. Equal amounts of whole cell lysates were analyzed by western blot analysis; Right panel: RL/hCAR-EGFP cells were infected with pAD/CMV/Flag-TβRII (MOI: 200). 48 hours later, cells were treated with either medium alone or PMA in the presence or absence of U0126 for 6 hours. Equal amounts of whole cell lysates were analyzed by western blot analysis. (D) RL cells were stimulated with either medium alone or PMA for 24 hours. Cells were then treated with U0126 for indicated time periods. Following cross-linking by DSP, whole cell lysates were prepared, and equal amounts of whole cell lysates were used for immunoprecipitation either with anti-TβRII or anti-MEK1 antibody, and immunocomplexes were analyzed by western blot analysis. (E) TβRII over-expressing stable RL cells were stimulated with either medium alone or IgM for 1 hour. Cells were then

treated with U0126 for indicated time periods. Following cross-linking by DSP, whole cell lysates were prepared, and equal amounts of whole cell lysates were used for immunoprecipitation with anti-T β RII antibody, and immunocomplexes were analyzed by western blot analysis.