Biochemical and Biophysical Research Communications 438 (2013) 116–121

Contents lists available at SciVerse ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

p130Cas controls the susceptibility of cancer cells to TGF-β-induced growth inhibition

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ARTICLE INFO

Article history:
Received 8 July 2013
Available online 18 July 2013

Keywords:
p130Cas
TGF-β
Smad
Cancer
EMT

ABSTRACT

Transforming growth factor-beta (TGF-β) suppresses the initiation of tumorigenesis by causing arrest at the G1 phase of the cell cycle. The loss of the antiproliferative function of TGF-β is a hallmark of many cancers. Here we report that p130Cas plays a role in determining the cellular responsiveness to TGF-β-induced growth inhibition in some cancer cells. An analysis of the tyrosine phosphorylation levels of p130Cas revealed higher levels of phosphorylation in cancer cell lines (MCF7 and A375) than in corresponding normal cell lines (MCF10A and MEL-STV). In contrast to normal cells, the cancer cells showed resistance to not only TGF-β-induced Smad3 phosphorylation and p21 expression, but also growth inhibition. However, silencing p130Cas using siRNA was sufficient to restore Smad3 phosphorylation and p21 expression, as well as the susceptibility to TGF-β-induced growth inhibition. Interestingly, the stable overexpression of p130Cas accelerated TGF-β-induced epithelial–mesenchymal transition. Our results suggest that elevated expression and tyrosine phosphorylation of p130Cas contributes to the resistance to TGF-β-induced growth inhibition, and thus to the initiation and progression of human cancers that harbor an active integrin signal.

1. Introduction

p130Cas (Crk-associated substrate, 130 kDa), the product of the breast cancer anti-estrogen resistance 1 gene, is an adaptor protein that is a tyrosine-phosphorylated protein by *v*-Src-dependent cellular transformation and the *v*-Crk oncogene [1,2]. p130Cas has multiple protein–protein interaction domains, and has a great influence on actin cytoskeleton regulation, cell migration, cell survival, and apoptosis, and especially on the cooperation of integrin with receptor tyrosine kinases, including EGF, estrogen, and VEGF receptors [3–6]. Extensive work has shown that p130Cas is critical for the initiation of tumorigenesis and maintenance of the proliferative capacity of human cancers [7]. The overexpression of p130Cas promotes mammary tumorigenesis and metastasis [7–9], and is correlated with poor prognosis in breast cancer patients [10,11]. Recent studies have extended this notion to the transforming growth factor (TGF)-β signaling pathway [12,13].

TGF-β signals are transduced by receptor serine/threonine kinases (RS/TKs) and intracellular effectors called Smads [14], which are implicated in a variety of cellular functions, such as proliferation, apoptosis, and differentiation [15,16]. In response to TGF-β stimulation, Smad2 and Smad3 are phosphorylated at the C-terminal $S^{422}SXS^{425}$ motif by the TGF-β type I receptor (TβRI) [14]. Phosphorylated Smad2/3 forms a complex with Smad4, and then translocates into the nucleus, in which the complex leads to the transcription of target genes that include cell cycle inhibitor p21 [17,18]. As a consequence, TGF-β induces growth arrest at the G1 phase in most cell types,including normal epithelial, endothelial, and hematopoietic cells, as well as primary fibroblasts of embryonic origin [19,20]. Thus, the TGF-β/Smad cascade has been regarded as a tumor suppressor pathway, and reducing the responsiveness of cancer cells to its tumor suppressor activity is critical for tumorigenesis in epithelia [21].

The loss of the growth-inhibitory function of TGF-β is a hallmark of many cancers. It has been well known that TGF-β also has pro-oncogenic effects, including enhanced epithelial-mesenchymal transition (EMT) and increased invasiveness, coupled with a resistance of the cells to TGF-β-induced growth inhibition and an increased production of TGF-β [21]. Several studies suggest that this transition of the TGF-β signal toward oncogenesis may result from changing thebalance between canonical and noncanonical pathways [13,21]. However, the detailed molecular mechanism

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http://dx.doi.org/10.1016/j.bbrc.2013.07.037

has not been firmly investigated. Recently, we reported that integrin signaling counteracts the inhibitory effects of TGF-β on cell growth, and that p130cas acts as an important mediator for integrin-dependent TGF-β suppression through its direct interaction with Smad3 [12]. This inhibitory role of p130Cas in the TGF-β signaling pathway was further confirmed in another study, in which it was shown that p130Cas is required for mammary tumor growth and TGF-β-mediated metastasis through the regulation of Smad2/3 activity [13]. Here, we tried to determine whether the p130Cas-mediated control of TGF-β signaling contributes to changing the net cellular readout of the TGF-β signal from tumor suppression to tumor promotion,contributing to the initiation and progression of human cancers.

2. Materials and methods

2.1. Cell culture and transfections

Human keratinocyte (HaCaT), human breast cancer (MCF-7), and human melanoma cells (SK-MEL-2, SK-MEL-28 and A375) were cultured in Dulbecco's modified Eagle medium (DMEM) with 10% fetal bovine serum. MEL-STV immortalized melanocytes were cultured in DMEM with 5% fetal bovine serum [22]. MCF10A normal breast epithelial cells were cultured in DMEM/F12 supplemented with 5% horse serum, 10 μg/ml insulin, 20 ng/ml EGF, 0.5 μg/ml hydrocortisone, and 100 ng/ml cholera toxin (all from Sigma). All transfections were carried out using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) or GeneExpresso 8000 (InnoVita, Gaithersburg, MD, USA) according to the manufacturer's instructions. HaCaT cell clones stably expressing p130Cas were obtained by transfection with pcDNA3.0-p130Cas, followed by selection for 1–2 weeks in G418 (GIBCO BRL). TGF-β1 was purchased from R&D Systems (Minneapolis, MN).

2.2. Immunoprecipitation and western blot analysis

Cells incubated with or without TGF-β1 (2 ng/ml) were lysed in modified RIPA buffer (50 mM Tris–HCl at pH 7.4, 150 mM NaCl, 1% NP-40, 0.25% sodium deoxycholate, 10 mM NaF, 1 mM PMSF, 1 mM sodium orthovanadate, 10 μM leupeptin, 1.5 μM pepstatin and 10 μg/ml aprotinin). For the analysis of endogenous Smad3–Smad4 and Smad3–p130Cas interaction, the lysates were immunoprecipitated with anti-Smad3 N Ab [17], followed by western blotting with anti-Smad4 mAb (Santa Cruz Biotechnology) or anti-p130Cas mAb (BD Transduction Laboratories, San Jose, CA). The tyrosine phosphorylation of p130Cas was analyzed by immunoprecipitation with anti-Cas2 Ab, followed by immunoblotting with anti-phosphotyrosine mAb (4G10, Upstate Biotechnology, Lake Placid, NY). Anti-Cas2 was obtained from Dr. Hismaru Hirai. Western blot analysis was carried out using anti-Smad3 Ab (Zymed Laboratories, South San Francisco, CA), anti-phospho-Smad3 Ab (a kind gift of Edward B. Leof, Mayo Clinic Cancer Center, MN), anti-tubulin mAb (Sigma), and anti-p21 mAb (BD Transduction Laboratories).

2.3. Silencing of endogenous p130Cas with small interfering RNA (siRNA)

Cells were depleted of p130Cas using siRNA corresponding to nucleotides 2366–2384 (CCCACAAGCUGGUGUUACU dT dT) of human p130Cas (Bioneer, Seoul, Korea) [17]. Cells were transiently transfected with siRNA for p130Cas in Opti-MEM I medium (Invitrogen) using Lipofectamine 2000 reagent (Invitrogen) following the manufacturer's instructions. Scramble siRNA (Bioneer) was transfected as a negative control. Four hours later, the Opti-MEM I medium was replaced with complete culture medium. TGF-β1 was added to the cultures 24–36 h after the transfection, and the effects of the down-regulation of p130Cas were assessed at 48–60 h.

2.4. Immunofluorescence

Cells were treated withTGF-β, fixed, permeabilized, blocked, and incubated with anti-E-Cadherin (BD Bioscience Transduction Laboratories, Lexington, KY) and anti-β-Catenin (BD Bioscience Transduction Laboratories), followed by staining with Alexa Fluor-conjugated secondary Ab (Invitrogen). Actin was visualized by Alexa 488-conjugated phalloidin (Invitrogen). Images were obtained using an Olympus confocal microscope FV1000 (Olympus, Tokyo, Japan) with FV10-MSASW software.

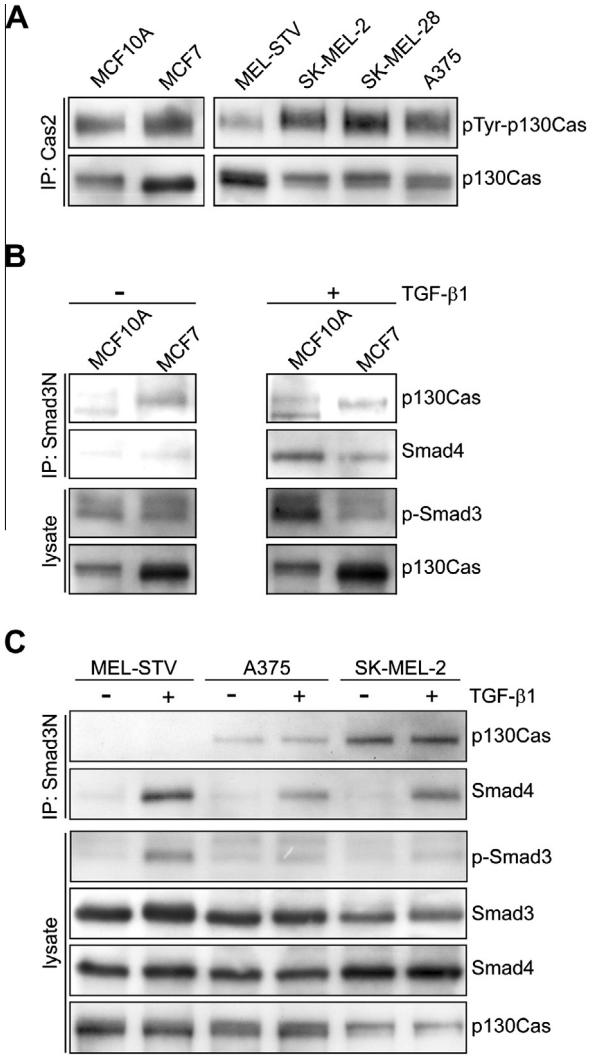

Fig. 1. Expression and phosphorylation levels of p130Cas and Smad3 in normal and cancer cell lines. (A) Levels of total (p130Cas) and tyrosine-phosphorylated p130Cas (pY-p130Cas) were determined by immunoprecipitation using anti-Cas2 Ab, followed by western blotting with anti-Cas mAb or anti-phosphotyrosine mAb (4G10) in normal cells (MCF10A and MEL-STV) and their corresponding cancer cells (MCF7, SK-MEL-2, SK-MEL-28 and A375). (B) MCF10A and MCF7 cells were treated with or without TGF-β (2 ng/ml) for 30 min, and the cell lysate was subjected to immunoprecipitation and western blotting for the indicated proteins. (C) MEL-STV, A375, and SK-MEL-2 cells were treated with or without TGF-β for 30 min, and the cell lysate was subjected to immunoprecipitation and western blotting for the indicated proteins.