Available online at www.sciencedirect.com
ScienceDirect
Biochemical and Biophysical Research Communications 354 (2007) 783–788
BBRC
www.elsevier.com/locate/ybbrc

Identification of Dok-4b, a Dok-4 splice variant with enhanced inhibitory properties

Cindy Baldwin [a,b], Arda Bedirian [a], Hongping Li [a], Tomoko Takano [a], Serge Lemay [a,b,*]

[a] Department of Medicine, Division of Nephrology, McGill University Health Centre, Montreal, Que., Canada H3A 2B4
[b] Hormones and Cancer Research Unit, McGill University, Canada

Received 11 January 2007
Available online 19 January 2007

Abstract

Dok adapter proteins have been primarily implicated in negative regulation of tyrosine kinase signaling, but Dok-4 has been reported to exert both inhibitory and stimulatory effects. We have identified a splice variant of Dok-4, Dok-4b, which contains a 39 aa insert within the its C-terminal region. The ~45 kDa Dok-4b protein was detected in several human epithelial cell lines. Based on genomic sequences, Dok-4b was also predicted to exist in primates and possibly bovines, but not in rodents or other species. Compared to Dok-4, Dok-4b inhibited the tyrosine kinase-induced activation of both Erk and Elk-1 more strongly. Truncation of the C-terminal region of Dok-4 (Dok-4 ΔCT) also enhanced the inhibitory activity of Dok-4, whereas expression of the isolated C-terminal domain enhanced Elk-1 activation, suggesting that the N-terminus and C-terminus of Dok-4 possess opposing inhibitory and stimulatory properties, respectively, the balance of which is altered by alternative splicing of Dok-4 to Dok-b.
© 2007 Elsevier Inc. All rights reserved.

Keywords: Dok-4; Fyn; Ret; Epithelial growth factor receptor; Tyrosine kinases; Elk-1; Erk; Adapter molecules; Splice variant; Epithelial cells

The Dok family of adapter molecules comprises seven members (Dok-1 to 7) with a related amino-terminal tandem of PH and PTB domains and divergent C-terminal sequences. Dok-1, -2, and -3 are primarily expressed in hematopoietic cells [1–3], Dok-5 and -6 are mostly confined to the brain [4–6], Dok-7 is restricted to the heart and skeletal muscle [7], whereas Dok-4 is widely expressed in non-hematopoietic tissues [4,5]. In contrast to the structurally related IRS family and the PTB domain containing adapter proteins FRS and Shc, which are involved in signal amplification, Dok family molecules have been shown to function primarily as inhibitors of tyrosine kinase signaling [1,2,8–11]. It is believed that Dok proteins work as docking platforms for the formation of signaling complexes at the cell membrane with the PH and PTB domains acting cooperatively as membrane- and/or receptor-targeting modules and the C-terminal region acting as the principal site for recruitment of downstream molecules, through a series of SH2 and SH3 binding motifs [5,12–15]. The precise function of Dok protein C-terminal regions remains incompletely defined, especially in the case of Dok-4, for which no C-terminal domain partners have been identified to date.

While Dok-1 and Dok-2 have been consistently shown to inhibit Erk activation induced by a variety of tyrosine kinases [9,15–19], it remains controversial whether Dok-4 is a positive or a negative regulator of tyrosine kinase signaling. Grimm et al. and Cai et al. have shown that Dok-4 enhances the activation of Erk induced by GDNF [4] or insulin [20]. More recently, it was shown that Dok-4 enhanced GDNF-induced activation of Erk through the Rap1 GTPase and that this effect was dependent on tyrosine residues within the C-terminus of Dok-4 [21]. Similarly, we found that Dok-4 enhanced TNF-α-induced activation of NF-κB in endothelial cells [22]. In contrast, we also found that, in Caco-2 epithelial cells, Dok-4 inhibited activation of the transcription factor Elk-1 in response

* Corresponding author. Fax: +1 514 843 2815.
E-mail address: serge.lemay@mcgill.ca (S. Lemay).

0006-291X/$ - see front matter © 2007 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2007.01.054

to activated Fyn, or ligand-stimulated Ret or PDGFR-β [5]. Moreover, this inhibitory effect appeared to be independent of Dok-4 tyrosine phosphorylation.

In the current study, we identify a splice variant of Dok-4 characterized by the insertion of a 39 amino acid sequence within the C-terminal region and present evidence that this modification, as well as deletion of the entire Dok-4 C-terminal region enhances the inhibitory properties of Dok-4 in epithelial cells.

Methods

Cells and transfections. Caco-2 human colon cancer cells were cultured in α-MEM containing 10% FBS. Caco-2 cells stably overexpressing Dok-4bMyc were generated by selection in G-418 after transfection with pCND3.1-Dok-4bMyc/His. COS-1, T47D, HeLa, and 293 cells were cultured in DMEM/high glucose containing 10% FBS. Transfections were performed with the Lipofectamine 2000 reagent (Invitrogen).

cDNA's. The following expression constructs were described previously [5]: pCDNA3.1-Ret9 (human c-Ret), pME18S-FynY528F (activated mouse Fyn), pCDNA3.1-Dok-4Myc/His WT (Myc-tagged wild type Dok-4, amino acids 1–325), pCDNA3.1-Dok-4Myc/His ΔCT (lacking amino acids 234–325), pEGFP-Dok-4 WT and ΔPTB. Dok-4Myc CT, comprising only the C-terminal region of Dok-4 (amino acids 234–325) was generated by PCR. The sense oligonucleotide for this reaction (5′-ATAT AGAATTCAACC*ATG*CACAAGCGGGTCCTGCTG-3′) contained an *Eco*RI site (underlined) and an ATG (bold) preceded by the same five nucleotides (putative Kozak sequence) as the wild-type sequence. Human Dok-4-EGFP was obtained from Dr. J. Nunès. The human p52 Shc cDNA (in pXM139) was obtained from Dr. M. Park.

Antibodies and immunodetection. Generation of Dok-4 antiserum was described previously [5]. The following antibodies were purchased: anti-GFP from Santa Cruz, anti-α-tubulin from Sigma, anti-phospho-Erk-1/2, and anti-Erk-1/2 from Cell Signaling. Immunoprecipitations and immunoblotting were performed according to standard protocol, as previously described [5].

Cloning of Dok-4b. The sequence of the human splice variant of dok-4, dok-4b, was first inferred from assembling a consensus of the following EST's: BI838338, BU553542, BG683308, AL517428, BQ068620, BG752029, and BQ070913. The following oligonucleotides were then used to clone dok-4 from the human colon cancer cell line Caco-2: 5′-C CCAGCAGTCATCGGTG-3′ (hD4bRT) for reverse transcription; 5′-A ACCATGGCGACCAATTTCA-3′ (4–3) and 5′-AGCACTGTGGTC ACTGG-3′ (hD4b 3′) for amplification. Since this reaction amplified both forms of human dok-4, the larger of the two resulting fragments was gel purified and cloned with the PCR-Script kit (Stratagene). A Myc-tagged Dok-4b construct was produced by replacing the stop codon of Dok-4b with an in-frame *Hin*dIII site and subcloning the resulting product in the pCDNA3.1Myc/His vector using PCR with the following antisense oligonucleotide: 5′-ATATAAGCTTCTGGGATGGGGTCTTGGC-3′. The current accession number for full-length DOK-4b is CR610258.

Dok-4b-specific RT-PCR. Detection of Dok-4b mRNA by RT-PCR was performed with the human and mouse-compatible oligo 4–3 (see above) as sense primer and a Dok-4-specific oligo CTCAGAACTCAG AGCTAGGTC as antisense primer. To reduce the chance of missing non-human Dok-4 mRNA due to sequence mismatches, the Dok-4R oligo was 21 bp long and low-stringency conditions were used, i.e. 35 cycles with 50 °C as the annealing temperature.

RNA interference. Two Dok-4-specific siRNA oligonucleotides were used. The sequences targeted nucleotides 518–536 of human Dok-4 (hDok-4 siRNA #3) and nucleotides 4–22 (hDok-4 siRNA #4) and were both obtained from Dharmacon. As negative control, a non-targeting siRNA was used (siCONTROL #1, from Dharmacon). All siRNA were delivered into Caco-2 cells using an Amaxa Nucleofector device (200 pmol of siRNA for 2×10^6 cells). For experiments in transiently transfected 293 cells, Lipofectamine 2000 reagent was used to deliver Dok-4 plasmids and siRNA simultaneously. The final siRNA concentration for these was 4 nM.

Reporter gene assays. Caco-2 cells were transiently transfected in triplicates on 24-well plates. In addition to Dok-4 and/or kinase expression constructs, the pFR-Luc vector (Gal-4/luciferase) was transfected in combination with pFA2-Elk-1 (Elk-1/Gal-4 DNA-binding domain) (both from Stratagene). Transfection with pFR-Luc and pFC-dbd (Gal4 dbd) was used to determine background transactivation. Either the pRL-TK or phRL-null *Renilla* luciferase vector (Promega) was included for normalization. Empty vector was added where necessary to equalize the amounts of transfected DNA. Stimulation with ligands was performed overnight, starting 24 h after transfection. For Ret, the ligand was a combination of GDNF (100 ng/ml, from Biosource) and GFRα-1-Fc (800 ng/ml, from R&D Systems). EGF (Biosource) was used at a concentration of 100 ng/ml. Cells were lysed 48 h after transfection and luciferase activity was determined using the Dual-Luciferase Reporter system (Promega) and expressed as the ratio of firefly to *Renilla* luciferase activities (measured in relative light units or RLU) ± standard deviation.

Results

Evidence of a human splice variant of Dok-4

We had previously generated an antiserum directed specifically at the C-terminal region of Dok-4 and noted that, in addition to the expected 37 kDa band, it also detected an approximately 45 kDa band in Caco-2 intestinal epithelial cells [5]. Using this same antiserum, we performed anti-Dok-4 immunoprecipitation and immunoblotting in T47D and HeLa human epithelial cells. As shown in Fig. 1A, the same 45 kDa band was observed in these cells. High levels of this protein were also detected in TT human thyroid carcinoma cells (Fig. 1B). An analysis of available human EST's suggested the existence of a *dok-4* splice variant containing a 39 amino acid insertion in the C-terminal region (Fig. 2A). The predicted insert contained no homology to known proteins and no recognizable protein–protein interaction motifs. To confirm the existence of this

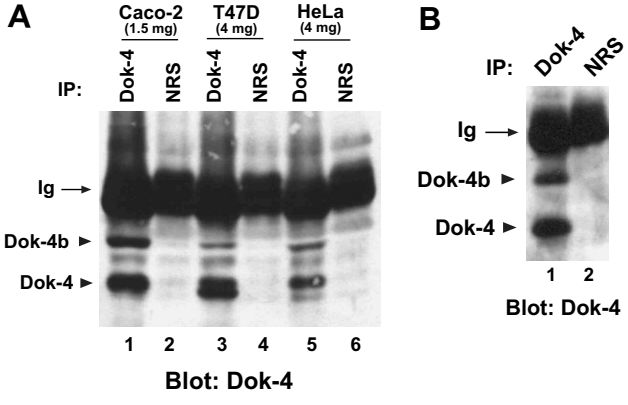

Fig. 1. Detection of Dok-4 and a putative splice variant in human epithelial cells. (A) Lysates of human Caco-2 (intestinal), T47D (mammary) and HeLa (cervical) epithelial cells were subjected to immunoprecipitation with Dok-4-specific antiserum or with normal rabbit serum (NRS), followed by anti-Dok-4 immunoblotting. The amount of lysate used for IP is indicated and was greater in T47D and HeLa cells because of lower expression levels in these cells. (B) Anti-Dok-4 immunoprecipitation in human TT thyroid carcinoma.

Download English Version:

https://daneshyari.com/en/article/1939304

Download Persian Version:

https://daneshyari.com/article/1939304

Daneshyari.com