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Cloning and expression of human apoptotic Daxx gene in human kidney cells

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ABSTRACT

The Fas/Fas ligand system is a key signal pathway involved in the regulation of apoptosis, which is a genetically controlled process for the elimination of the cells. Human Daxx was first identified as a Fas-binding protein and acts as a pro-apoptotic protein that enhances Fas-mediated apoptosis. However, the anti-apoptotic function of Daxx is suggested. Therefore, the precise role of Daxx still needs further investigation. The purpose of this study was to clone, and verify the expression of human Daxx in human kidney HEK293T cells. Total RNA was extracted from human lymphocytes and converted to cDNA by RT-PCR. Daxx was then amplified with primers designed according to the published sequence of Daxx in Genbank. The PCR product was inserted into pCDNA3.1 Hygro expression vector and the recombinant plasmid, namely pCDNA3.1 Hygro-Daxx, was further verified by restriction endonuclease analysis and DNA sequencing. Furthermore, plasmid pCDNA3.1 Hygro-Daxx was transfected into HEK293T cells and the protein expression was verified by flow cytometry analysis. The results showed that the entire coding region of human Daxx gene was cloned, and expressed in HEK293T cells. The clone will be used to investigate the detailed molecular mechanisms of human Daxx in apoptotic process of the cells.

Keywords: cloning, protein expression, human Daxx, human kidney HEK293T cells

INTRODUCTION

Apoptosis is characterized by typical morphological and biochemical changes including cell shrinkage, nuclear DNA fragmentation and membrane blebbing (Hengartner, 2000). Initiation of the apoptosis response leading to caspase activation follows activation of either the death receptor pathway or the mitochondrial pathway. Death receptors are members of the TNF receptor gene superfamily, which consists of more than 20 proteins with a broad range of biological functions. The best-characterized death receptors comprise Fas receptor, TNF receptor 1 (TNFRI), TRAIL-receptor 1 and TRAIL-receptor 2. The corresponding ligands of the TNF superfamily comprise death receptor ligands such as Fas ligand, TNFa, and TRAIL, respectively (Debatin and Krammer, 2004).

Daxx was originally identified as a protein that specifically binds to Fas receptor and potentiates Fas-induced apoptosis (Yang *et al.*, 1997). In the cytoplasm, Daxx interacts with Fas receptor at the plasma membrane (Yang *et al.*, 1997). Stimulation of Fas signaling causes translocation of Daxx from the nucleus to the

cytoplasm, where it interacts with apoptosis signal-regulating kinase 1 (ASK1) and promotes Jun N-terminal kinase (JNK) activation (Chang *et al*., 1998). A recent study employed RNA interference to down-regulate Daxx in primary fibroblasts. Remarkably, Daxx-depleted cells are resistant to cell death induced by both UV irradiation and oxidative stress. Furthermore, the down-regulation of Daxx results in impaired JNK activation. This is the first evidence that Daxx promotes cell death and JNK activation in physiologic conditions (Khelifi *et al*., 2005).

Daxx is present predominantly in the nucleus and upon induction of apoptosis by Fas stimulation, Daxx sometimes does not translocate out from the nucleus to the cytoplasm (Ishov *et al*., 1999; Torii *et al*., 1999). The induction of apoptosis by Daxx in the cytoplasm may not be entirely correct. Daxx-mediated potentiation of Fas-mediated apoptosis was therefore suggested to occur from its nuclear location as well as from the cytoplasm (Torii *et al*., 1999). In the nucleus, Daxx shuttles between two different sub-nuclear structures including nucleoplasm and the promyelocytic leukemia nuclear bodies (Pml) (Ishov *et al*., 1999; Zhong *et al*., 2000). In the nucleoplasm, Daxx acts as a transcriptional repressor (Li *et al*., 2000a; Li *et al*., 2000b). It associates with proteins that are critical for transcriptional repression, such as histone deacetylase II, constituents of chromatin such as core histones H2A, H2B, H3 and H4, and Dek, a chromatin-associated protein reported to change the topology of DNA in chromatin (Hollenbach *et al*., 2002). In addition, Daxx interacts with p53 in the nucleus and enhances p53-mediated apoptosis (Kim *et al*., 2003; Gostissa *et al*., 2004).

The anti-apoptotic function of Daxx has been demonstrated as well as the pro-apoptotic function of Daxx. In the embryo, Daxx appears to be anti-apoptotic because disruption of the Daxx gene in the mouse results in early embryonic lethality with extensive apoptosis (Michaelson *et al*., 1999). RNAi for Daxx in cell lines was reported to increase apoptosis and to sensitize cells to the apoptosis induced by Fas ligand, UV and TNF-α (Chen and Chen, 2003; Michaelson and Leder, 2003). Furthermore, Daxx inhibits CD43-induced cell death in myeloid progenitor (Cermak *et al*., 2002).

The aim of this study was to clone and express human apoptotic Daxx gene in human kidney HEK293T cells. The expressed clone will be further used to clarify the precise role of human apoptotic Daxx protein in the apoptotic process of the cells.

MATERIALS AND METHODS

PCR amplification

Human cDNA was synthesized by SuperScript III First-Strand Synthesis system for RT-PCR (Invitrogen) from Thai human lymphocytes. The full-length Daxx was amplified by nested-PCR using Platinum Pfx DNA polymerase enzyme (Invitrogen), which has 3'-5' proofreading activity. The PCR reaction was carried out in a GeneAmp PCR System 9700 (Applied Biosystems) by using nested-PCR with outer primers including NTDaxF (5'CCA TGC GAG GTT CTG AG 3') and NTDaxR (5'CAC CAA AAG GGG ATT AG 3'), which started with denaturation at 94°C for 2 minutes, followed by 30 cycles of denaturation (94°C for 30 seconds), annealing (50°C for 1 minute) and extension (72°C for 4 minutes), and final extension at 72°C for 7 minutes. For inner primers including DaxBamHA (5'GTA GGA TCC GAC ATG TAC CCA TAC GAC GTC CCA GAC TAC GCT GCC ACC GCT AAC AGC 3') and DaxXbaR (5'CTC TAG ACT AAT CAG AGT CTG AG 3'), which started with denaturation at 94°C for 2 minutes, followed by 30 cycles of denaturation (94 °C for 30 seconds), annealing (59 °C for 1 minute) and extension (72 °C for 4 minutes), and final extension at 72 °C for 7 minutes.

***In vitro* DNA cloning and DNA sequencing**

The 2.3 kb PCR product containing human Daxx was purified and digested with restriction endonuclease enzymes; *BamH*I and *Xba*I. The mammalian expression vector, pCDNA 3.1/Hygro (+) (Invitrogen) was sequentially digested with the same restriction enzymes. The vector and insert were then ligated and transformed into a competent *E. coli* strain DH5α. The clones, which grew on LB plate containing 100 μg/ml ampicilin, were screened by colony PCR and restriction enzyme digestion. A positive clone, containing the full-length Daxx, namely pCDNA 3.1Hygro-Daxx, was obtained, verified by DNA sequencing and aligned with database from GenBank.

Verification of protein expression by flow cytometry analysis

Plasmid pCDNA 3.1Hygro-Daxx was transfected into human kidney HEK293T cells using lipofectamine (Invitrogen). Forty-eight hours post transfection, the transfected cells were collected and washed with PBS. The transfected cells were then fixed with 2% formaldehyde in PBS for an hour and permeabilized with 0.2 Triton X-100 in PBS for 20 minutes at room temperature. After washing three times with 0.1% Triton X-100 in PBS, the transfected cells were incubated with rabbit polyclonal antibody against Daxx protein (Santacruze Biotechnology) as a primary antibody for an hour. After washing, the transfected cells were further incubated with Cy3-conjugated donkey anti-rabbit Ig antibody (Jackson Immunoresearch Laboratories) as a secondary antibody at room temperature for 30 minutes. Finally, the samples were analyzed by flow cytometry (Becton–Dickinson).

RESULTS

We successfully constructed the entire coding region of human Daxx gene in an expression vector, pCDNA 3.1 Hygro and the protein expression of a clone expressing human Daxx was demonstrated. First, human Daxx was amplified by PCR. Second, the 2.3 kb PCR product was sub-cloned into pCDNA 3.1 Hygro. The plasmid isolated from a positive clone in *E. coli*, was designated pCDNA 3.1Hygro-Daxx. Third, the restriction enzymes digestions of pCDNA 3.1Hygro-Daxx by *BamH*I and *Xba*I resulted in the expected size of a 2.3 kb insert and 5.6 kb vector (Fig. 1, lane 4). In Figure 1 lane 3, pCDNA 3.1Hygro was digested with the same enzymes and gave the expected size of vector only. Fourth, the pCDNA 3.1Hygro-Daxx contained a human Daxx gene, which has the exact amino acid sequences to the published sequence of human Daxx in GenBank with protein ID AAC39853.1. Finally, pCDNA 3.1 Hygro-Daxx was transfected into HEK293T cells. Flow cytometry was subsequently performed to detect the amount of Daxx protein. We found the increased amount of fluorescence intensity in pCDNA 3.1Hygro-Daxx transfected HEK293T cells (gray line) compared to that of pCDNA 3.1Hygro HEK293T cells (black line) (Fig. 2). This suggests that the increased expression of Daxx is from pCDNA 3.1Hygro-Daxx.

DISCUSSION

Traditionally, *in vitro* DNA cloning consists of several steps. The first step is to cleave the total cellular DNA with specific restriction enzymes. The resulting DNA fragments are joined to DNA vector molecules to form hybrid molecules by ligase. Each hybrid recombinant DNA molecules then conveys its insert DNA into a single host cell, where it is replicated. As the host cell multiplies, copies of the same insert DNA fragment multiplies as well. The cloned DNA is eventually released from its vector by cleavage using appropriate restriction endonuclease and is isolated.

We successfully *in vitro* cloned the entire coding region of human Daxx gene and expressed

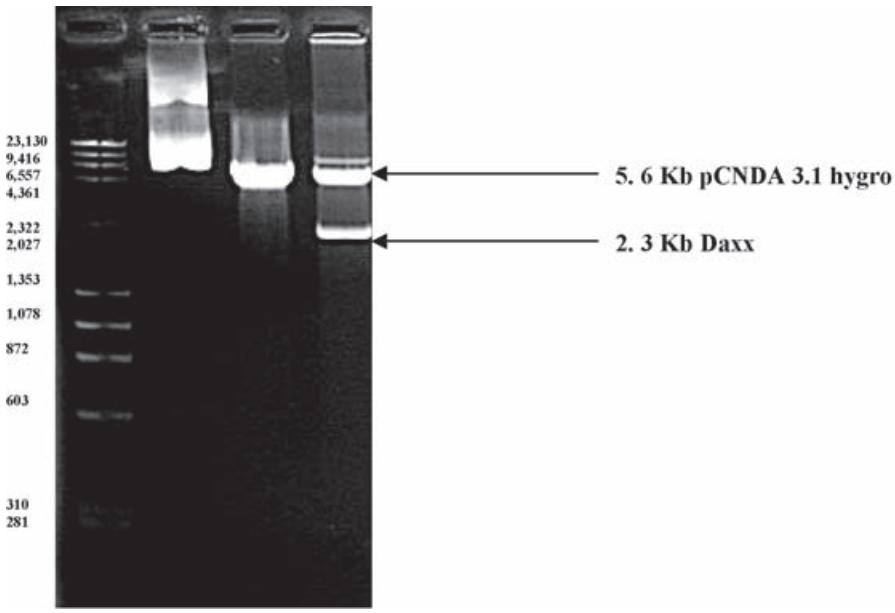

Figure 1 Verification of a positive clone by restriction enzyme digestion. The pCDNA 3.1 Hygro before digestion was showed in lane 2. Lane 3 was the pCDNA 3.1 Hygro, which was double-digested with *BamH*I and *Xba*I and gave the expected size of a vector only. The restriction enzyme digestion of pCDNA 3.1Hygro-Daxx by the same enzymes resulted in expected size of a 2.3 kb insert and 5.6 kb vector (lane 4).

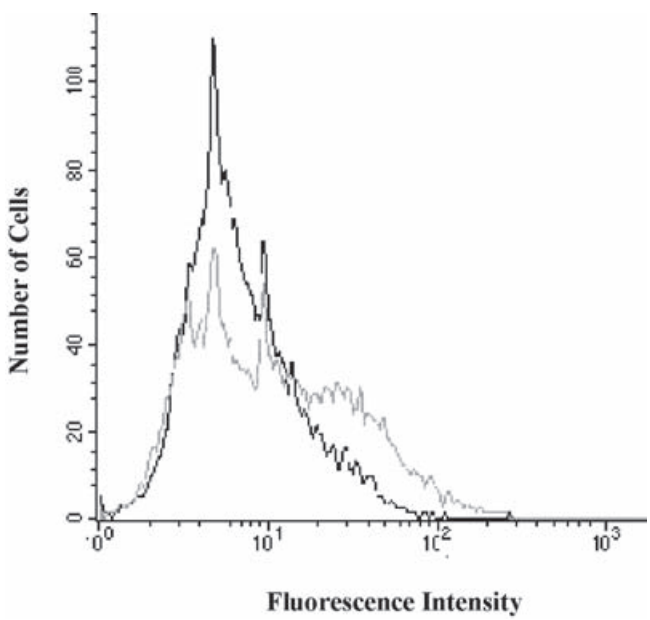

Figure 2 Increased fluorescence intensity of pCDNA 3.1Hygro Daxx-HA transfected HEK293T cells (gray line) compared with that of pCDNA 3.1Hygro transfected HEK293T cells (black line).

in human kidney cells. The clone expressing human Daxx will be used to identify the precise mechanism by which Daxx exerts its function both in physiologic and pathological condition. In the pathological condition, it is currently unclear whether Daxx is altered in human pathological conditions and what impact of Daxx functional inactivation has on the pathogenesis of neurodegenerative diseases although two recent studies showed that Daxx is strongly up-regulated in the hippocampus of Alzheimer's patients (Colangelo *et al*., 2002; Lukiw, 2002).

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