Physiological and Functional Interactions between Tcf4 and Daxx in Colon Cancer Cells*

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Daxx, a human cell death–associated protein, was isolated as a Tcf4-interacting protein, using a yeast two-hybrid screen. Co-immunoprecipitation in HEK-293T cells and yeast two-hybrid screen in Y190 cells were performed to identify the interaction between Tcf4 with Daxx and to map the binding regions of Tcf4. In the nucleus, Daxx reduced DNA binding activity of Tcf4 and repressed Tcf4 transcriptional activity. Overexpression of Daxx altered the expression of genes downstream of Tcf4, including cyclin D1 and Hath-1, and induced G1 phase arrest in colon cancer cells. A reduction in Daxx protein expression was also observed in colon adenocarcinoma tissue when compared with normal colon tissue. This evidence suggests a possible physiological function of Daxx, via interaction with Tcf4, to regulate proliferation and differentiation of colon cells.

Colorectal cancer is the most common cancer to afflict men and women in the United States (1, 2). Constitutive activation of the canonical Wnt signaling pathway is observed in a majority of colon cancers (3–5). The Wnt signaling pathway, also known as the APC/β-catenin/TCFs pathway, is one of the pivotal developmental and growth regulatory mechanisms of the cell. Wnt signaling appears to stabilize β-catenin by altering phosphorylation, initiating nuclear translocation, and association of β-catenin with TCFs transcriptional factor. The constitutive transcriptional activation of the β-catenin-TCFs complex is thought to underlie the biochemical mechanisms of colon tumorigenesis and other carcinogenesis like melanoma, hepatocellular carcinoma, and gastric carcinoma (3–8). These data strongly suggest that the dysregulation of the β-catenin/TCF signaling may stimulate the development of a broad range of human malignancies.

Human Tcf4 (also known as Tcf7L2) belongs to the TCFs family, which shares sequence homology with the N-terminal β-catenin binding domain and the C-terminal DNA binding domain (HMG box). Tcf4 is the predominant member of the TCFs family expressed in the colon epithelium (3). Previous studies have shown that cyclin D1 and Hath-1 are target genes of Tcf4 (9, 10). The consensus motif for the Tcf4–β-catenin binding site ((A/T)(A/T)CTCAAG) is present in the promoter region of cyclin D1. Cyclin D1 plays an important role in regulating progression of cells through the G1 phase of the cell cycle, and contributes to colonic abnormal growth and tumorigenicity (11, 12). Hath-1, a basic helix-loop-helix transcription factor is a critical positive regulator of terminal cell differentiation (13–16). Therefore, Wnt signaling, which deregulates expression of genes involved in cell cycle regulation, has been implicated in colon cancer and other types of cancer.

Daxx was first identified as a Fas-binding protein. It enhances Fas-mediated apoptosis via JNK activation (17, 18). The interaction of Daxx and Fas suggests that Daxx may function in the cytoplasm. However, Daxx is predominantly localized in the nucleus (19, 20) suggesting that Daxx has alternative roles in the nucleus and cytoplasm. Daxx has been detected in various human cell lines, is highly conserved, and is ubiquitously expressed (19, 20). In the nuclear compartment, the C-terminal of Daxx has been found to interact with several proteins including CENP-C, PML, ETS1, Pax3, Pax5, histone deacetylases, glucocorticoid receptor, and p53 family proteins (20–30). The N-terminal region has been shown to associate with DMAP1 or ATRX (31–33). Previous reports describe Daxx as a transcriptional repressor working through protein-protein interaction, distribution changes, protein modification or chromatin-remodeling (34–36).

In this present study, Daxx was isolated as a Tcf4-interacting protein through yeast two-hybrid screening. We sought to investigate the physiological significance of Daxx in regulating the transcriptional activity of Tcf4. Here we show that Daxx suppresses Tcf4 transcriptional activity and induces G1 arrest of colon cancer cells.

EXPERIMENTAL PROCEDURES

Plasmid Construction—For the yeast two-hybrid screening, Tcf4 and its derivatives were cloned by inserting the gene between the EcoRI and BamHI sites of pAS2-1 (Clontech). The Daxx gene was inserted between the BamHI and XhoI sites of pACT2 (Clontech) for cloning. For mammalian expression, the Tcf4 gene was inserted between the BamHI and XhoI sites of pCDNA3-HA2 (Invitrogen) for cloning. Daxx was inserted between the BamHI and EcoRI sites of pRK5-FLAG (BD Biosciences) for cloning. pGEX-1-Daxx was cloned by inserting the Daxx gene between the BamHI and EcoRI sites of pGEX-1 (Amersham Biosciences). His-Tcf4 was constructed by inserting the Tcf4 DNA fragment between the BamHI and XhoI sites of pCDNA3-His (Invitrogen).

Yeast Two-hybrid Screen—pAS2-1–Tcf4 with Gal4DB was used to screen a human HeLa cDNA library (Clontech). Approximately 5.5 × 106 transformants of the Y190 strain were screened according to the manufacturer’s protocol. Several cDNA clones from the activation domain library-encoded proteins that interacted with Tcf4 were isolated and sequenced. One of them contained a partial sequence of the Daxx gene. The full-length of Daxx was then cloned from a placental cDNA library (Clontech).

Cell Culture and Transfection—HEK-293T cells and human colon cancer cell lines including Hct116 with mutated APC, DLD1, or SW480 with mutated β-catenin were maintained in Dulbecco’s modified
Eagle’s medium with 10% fetal bovine serum. For the HEK-293T cells, 10^6 were seeded in a 6-well plate 12 h before transfection. Calcium phosphate-mediated DNA transfection was performed as described previously (37). Cell medium was refreshed after 12 h of transfection. For the human colon cancer cell lines, 2 x 10^5 Hct116, DLD1, or SW480 cells were seeded in 12-well plates 24 h before transfection. Lipofectamine 2000 (Invitrogen) was used according to the manufacturer’s protocol. Cells were transfected with DNA in incomplete medium and received fresh complete medium after 6 h of transfection.

Immunoprecipitation and Western Blot—After transfection, cells were lysed in lysis buffer (50 mM Tris-HCl, pH 7.8, 150 mM NaCl, 15 mM KCl, 0.5 mM EDTA, 0.5% Triton X-100, 0.1% sodium deoxycholate, and 0.5% Nonidet P-40). Co-immunoprecipitation and Western blotting were performed as described previously (38). Monoclonal antibodies anti-HA Ab (BAbCO), anti-FLAG Ab (Upstate, Sigma), anti-β-catenin Ab (Upstate), anti-cyclin D1 Ab, anti-P16 Ab, or polyclonal anti-Daxx Ab (Santa Cruz) were used. Polyclonal anti-GST Ab and anti-Tcf4 Ab were prepared from rabbits according to standard protocols (39).

Immunofluorescence—DLD1 and Hct116 cells were seeded on cover glass at ~30% confluence 24 h before transfection. After treatment, cells were fixed and stained as described previously (40). Confocal fluorescent microscopy with excitation at 488 and 543 nm, respectively, was used to record images.

Transactivation Assay—TOPFLASH with three repeat TCFs-binding sites, FOPFLASH with mutated TCFs-binding sites and a GSTK reporter with five repeat Gal4-binding sites were used to detect the transcriptional activity of Tcf4. The β-galactosidase expression vector pRK5-lacZ was included as an internal control. Luciferase activity was measured and quantified 24 h after transfection, following the manufacturer’s suggestions (Promega).

Protein Purification and In Vitro Translation—His-Tcf4 was expressed in HEK-293T cells and purified by nickel-chelating resins following the manufacturer’s protocol (Qiagen). The GST-Daxx fusion protein PGEX-1-Daxx was expressed in DE3pLys strain and purified according to standard protocols (41). In vitro translation was performed with the TnT system (Promega) according to the manufacturer’s instructions.

Electrophoretic Mobility Shift Assay—After transfection, nuclear fraction was prepared by NE-PER® Nuclear Extraction Reagents (Pierce) according to the manufacturer’s suggestions. For the electrophoretic mobility shift assay, 20 ng of the nuclear fraction and 0.01 pmol of 32P-labeled probe containing TCFs-binding sites (TBS) were incubated in 10 μl of reaction buffer containing 12.5 mM Hepes, pH 8.0, 12.5% glycerol, 60 mM KCl, 5 mM MgCl2, 0.1 mM EDTA, and 250 ng of poly(dI-dC)2 for 30 min. Electrophoresis was then performed at 4 °C in a 4% non-denaturing polyacrylamide gel containing 0.5× TBE buffer (45 mM Tris base, 45 mM boric acid, 1 mM EDTA, pH 8.0).

Quantitative Real Time PCR—Following the manufacturer’s protocol, RNA was extracted from transfected cells using TRIzol (Invitrogen). RNA was then reverse transcribed to cDNA using an oligo(dT)12–18 primer with Superscript II reverse transcriptase (Invitrogen). Appropriate dilutions of each cDNA for subsequent PCR amplification were determined with SYBR green labeled (SYBR® Green PCR Master Mix-and). Primer sequences were designed to comply with the Primer Express Operation Guide (Applied Biosystems). For cyclin D1 we used 5'-CCGAGAAGCCTGTGCTACAC-3' and 5'-GGTCCACCACTGACAC-3'. We used 5'-CCGAGAAGCCTGTGCTACAC-3' and 5'-GGTCCACCACTGACAC-3' for glyceraldehyde-3-phosphate dehydrogenase. For actin, 5'-TGTAGAAGGT-3' were used. All of the reactions were initially denatured at 94 °C for 10 min, followed by 40 cycles at 94 °C for 15 s, 60 °C for 1 s, and 72 °C for 1 s on an HT7900 sequence detection system (Applied Biosystems).
Cell Cycle Analysis—After starvation for 12 h, transfected cells were refreshed with complete medium for another 36 h. Cells were collected and stained with propidium iodide using a standard protocol (40). Stained cells were analyzed by flow cytometry using FACScalibur (BD Biosciences).

Colon Tissue Preparation—Colon tissue from each specimen was mechanically homogenized for 30 s at 2,000 rpm. Samples were then suspended in 0.5 ml of radioimmune precipitation assay buffer and centrifuged. Protein concentration were then measured and stored at −20 °C.

RESULTS

Identification and Characterization of Daxx as a Tcf4-interacting Protein—Yeast two-hybrid screening was performed to identify novel cellular partners of the Tcf4 protein. Fourteen positive colonies were harvested from the yeast and bacterial screens. After sequencing and blasting the human gene bank, 1 of the 14 positive colonies was identified as the human cell death associated protein, Daxx. Yeast two-hybrid was used to examine the specificity of the interaction between Daxx and Tcf4; only TCFs produced blue colonies when Tcf4 or Lef1ΔN105 was co-transformed with the full-length Daxx. Other transcription factors Nrf1, FoxP2, or the vector-only control did not produce any colonies.

To further characterize the physical association between Tcf4 and Daxx, transient transfection studies were performed in HEK-293T cells. HA-tagged Tcf4 (pCDNA3-HA-Tcf4) and FLAG-tagged Daxx (pRK5-Daxx-FLAG) were co-transfected into HEK-293T cells. Co-immunoprecipitation experiments revealed an interaction between Tcf4 and Daxx in HEK-293T cells (Fig. 1A). Confocal microscopy confirmed the co-distribution of Tcf4 and Daxx in the nucleus in vivo. In DLD1 and Hct116 cells, HA-Tcf4 and Daxx-FLAG were overexpressed as measured by immunofluorescence (Fig. 1B).

Because of the relatively strong affinity of Daxx for Tcf4, we sought to identify the region of Tcf4, which mediates the protein-protein interaction. First we used a yeast two-hybrid screen in Y190 cells. We trans...
formed the cells by pACT2-Daxx 1–740 mixed with pAS2-1-Tcf4 1–597, 1–200, 201–395, 396–597, or empty vector to identify the binding region of Tcf4. Only pAS2-1-Tcf4 1–597 and 201–395 could interact with Daxx and developed positive colonies (Fig. 1C). Next HEK-293T cells were transiently transfected with expression vectors encoding Daxx-FLAG and a series of HA-Tcf4 derivatives, Daxx could be immunoprecipitated with Tcf4 1–597 and 201–395 but not Tcf4 1–200, 396–597, or empty vector (Fig. 1C). These observations support the hypothesis that Daxx binds to the Tcf4 201–395 region in mammalian species.

**Daxx Represses Transcriptional Activity of Tcf4**—We used TOPFLASH to determine whether Daxx also modulated the transcriptional activity of Tcf4. When Tcf4 and β-catenin were overexpressed in HEK-293T cells, Daxx repressed the enhanced transcription activity of Tcf4 in a dose-dependent manner (Fig. 2A). An unrelated control protein, Mad2L1, did not have the same effect. Although TOPFLASH indicated that Daxx significantly down-regulated Tcf4 activity, it did not affect FOPFLASH or pGL3-LexA (Fig. 2B). The same pattern was found in Hct116 and SW480 cells, which had high endogenous levels of TCFs transcriptional activity. Daxx expression reduced the luciferase activity of TOPFLASH driven by endogenous Tcf4 in vitro. His-Tcf4 was purified from HEK-293T cells and β-catenin was in vitro translated by a TNT system at 30 °C for 90 min. His-Tcf4 and TNT-β-catenin were co-incubated with various concentration of GST-Daxx or GST purified from bacteria for 1 h in a 4 °C cold room. The β-catenin-Tcf4 protein complex was immunoprecipitated with anti-Tcf4 Ab and immunoblotted with anti-β-catenin Ab. GST fusion proteins were immunoblotted with anti-GST Ab.

**Daxx Does Not Influence the Formation of β-Catenin-Tcf4 Complexes**—To determine whether the mechanism by which Daxx represses the transcriptional activity of Tcf4 is inhibiting the formation of β-catenin-Tcf4 complexes, necessary to activate the transcription enhancement activity of Tcf4, we examined the efficacy of the complex formation using co-immunoprecipitation. In HEK-293T cells, HA-Tcf4 and β-catenin were overexpressed with or without Daxx-FLAG and precipitation of β-catenin/Tcf4 complexes was examined. We found no difference in the complex formation when Daxx was expressed dose-dependently (Fig. 3A). The same result was obtained in vitro (Fig. 3B). These results suggest that Daxx repressing transcriptional activity of Tcf4 is not through the disruption of β-catenin-Tcf4 complex formation.

**Daxx Reduces DNA Binding Activity of Tcf4**—We examined the effect of Daxx on the DNA binding activity of Tcf4 using a luciferase assay. We found that Daxx dose-dependently repressed β-catenin/Tcf4 binding of the DNA sequence for the TOPFLASH reporter (Fig. 4A, upper panel), but the control did not show any sensitivity. TOPFLASH was also activated by the Tcf4-VP16 fusion protein through Tcf4 binding, and expression of Daxx reduced this activation dose-dependently (Fig. 4A, bottom panel). These results support the prediction that Daxx reduces the DNA binding activity of Tcf4, resulting in repressed activity. An
Daxx resulted in an increase in the percentage of cells in G0/G1 phase compared to the control. Immunoblotted. Tubulin was used as an internal control.

Daxx-FLAG for 36 h. Endogenous cyclin D1 was detected by EEl staining after running on a 2% agarose gel. Cells were stained with propidium iodide and analyzed using flow cytometry. The values are averages of three independent experiments.

A 100-fold excess of 32P-unlabeled TBS cold probe could compete with this signal but not any other unrelated CC10 cold probe. This experiment confirmed the specificity of the probe to bind Tcf4 (Fig. 4B, lane E). A 100-fold excess of 32P-unlabeled TBS cold probe could compete with this signal but not any other unrelated CC10 cold probe. This experiment confirmed the specificity of the probe to bind Tcf4 (Fig. 4B, lane E).

DISCUSSION

Here we report evidence from both yeast and HEK-293T cells that Daxx interacts with Tcf4, leading to alteration of the DNA binding affinity of Tcf4 and, hence, reduced Tcf4 transcriptional activity. Daxx also induced G1 arrest in colon cancer cells and the expression levels of Daxx were reduced in colon cancer as compared with non-tumorous colon mucosa.

The increased expression of cyclin D1 has been detected in approximately one-third of colon tumors as an initial event during the multistage process of colon carcinogenesis (1, 2). Cyclin D1 may perturb cell cycle regulation in benign adenomas and thereby enhance tumor progression (9–12). Expression of antisense cyclin D1 abrogates the growth of SW480 colon cancer cells in nude mice (12). Hath-1, a critical positive regulator of cell differentiation is down-regulated in colon cancer. In colon cancer cells, Hath-1 inhibits cell proliferation, induces cell differentiation, and suppresses growth of colon cancer cells in animal models (13–16). In these experiments, Daxx represses the transcriptional activity of Tcf4, suppresses cyclin D1 expression, and arrests the cell cycle in the G1 phase. It also rescues G1 arrest in colon cancer cells induced by Daxx because of decreased cyclin D1 expression in colon cancer cells. However, Daxx had no effect on apoptosis enhancement (data not shown).

Daxx Protein Expression Is Reduced in Human Colon Adenocarcinoma—Tumor and adjacent tissue samples were collected from eight patients with colon cancer who underwent curative surgical resection at National Taiwan University Hospital and were pathologically diagnosed as having colon adenocarcinoma. Using Daxx antibody we observed a reduction of Daxx expression in colon adenocarcinoma as compared with normal colon tissue (Fig. 6).

FIGURE 5. Daxx alters the expression of genes downstream from Tcf4 and induces G1 arrest in colon cancer cells. A, Daxx alters the expression of downstream target genes of Tcf4. Hct116, DLD-1, and SW480 cells were transfected with Daxx-FLAG by Lipofectamine 2000. After 36 h of incubation, total RNA was reverse-transcribed to cDNA and amplified by real time PCR. Expression of these genes was normalized to an actin control and referenced to a control that was transfected with an empty vector (left). PCR products were detected by EtBr staining after running on a 2% agarose gel (right). B, Daxx reduces cyclin D1 protein expression in colon cancer cells. Western blot was used to detect endogenous cyclin D1 expression in HEK-293T, SW480, and Hct116 cells (top). Hct116 cells were transfected with or without Daxx-FLAG for 36 h. Endogenous cyclin D1 was immunoblotted. Tubulin was used as an internal control. C, Daxx induced G1 arrest in colon cancer cells. Hct116 cells were transfected with plasmid vectors (left). PCR products were detected by EtBr staining after running on a 2% agarose gel (right).
Interplay between Tcf4 and Daxx

**D1 expression (42, 43).** Mice deficient for the Tcf4 transcription factor completely lack proliferative cells in the fetal small intestinal epithelium (44). It is believed that proliferation and differentiation are intimately coupled in intestinal cells, and β-catenin and TCFs regulate these processes (42, 45). Indeed, most cells withdraw from the cell cycle to differentiate during the G1 phase (46). Hence, molecules that inhibit G1 phase progression have been considered to be excellent candidates for controlling the cell cycle and differentiation in developing tissues. Thus, the β-catenin-Tcf4 complex and Daxx may constitute the master switch that controls cell fate in healthy and malignant intestinal epithelial cells (42–48).

Daxx achieves its repressive effects through the reduction of Tcf4-DNA binding activity. These results are in line with evidence that the binding region of Tcf4 that interacts with Daxx, identified as AA201-395, contains the HMG box. However, the repressive effect of Daxx was only apparent in 30% of colon cancer cells, indicating that there could be a compensatory mechanism that uses other TCFs family members. As observed in recent reports, two isoforms of Tcf4 proteins (80 and 65 kDa) are detected in prostate cancer cells and CRC cells. These isoforms have been suggested to differ because of post-translational modifications. It has been indicated that the 80-kDa isoform of Tcf4 contributes the majority of activity (49, 50). In our investigation, there was a dose- and time-dependent increase in the ratio of 60- to 80-kDa Tcf4 when Daxx was overexpressed. The post-translational modification of Tcf4 may play a role in regulating its activity, and may be influenced by Daxx. Further work will be required to assess this possibility.

Daxx is a highly conserved mammalian gene with widespread expression in human tissue. Targeted deletion in the mouse reveals that Daxx is essential for embryonic development (51). Recently, Daxx has been reported to inhibit B lymphopoiesis induced by IFN-α. Antisense of Daxx rescues growth arrest and apoptosis induced by IFN-α in pro-B cells (52, 53). In our observation above, expression of Daxx protein was decreased in adenocarcinoma. Further studies of the role of Daxx in human physiological development will be important. This is the first report to demonstrate that Daxx interacts with Tcf4. The repressive effect of Daxx appears to affect the transcription of genes downstream of Tcf4, such as those for cyclin D1 and Hath-1. These results provide a physiological function of Daxx to alter Tcf4 activity and regulate cell proliferation and differentiation in colon cells.

**REFERENCES**

1. Jemal, A., Tiwari, R. C., Murray, T., Ghafoor, A., Samuels, A., Ward, E., Feuer, E. J., and Thun, M. J. (2004) CA-Cancer J. Clin. 54, 8–29
2. Jemal, A., Murray, T., Ward, E., Samuels, A., Tiwari, R. C., Ghafoor, A., Feuer, E. J., and Thun, M. J. (2005) CA-Cancer J. Clin. 55, 10–30
3. S.-L. Tzeng and J.-J. Kang, unpublished data.

**FIGURE 6. Reduction of Daxx protein in human colon adenocarcinoma.** Human colon tissue total lysate (30 μg) was separated in 10% SDS-PAGE. Expression of endogenous Daxx was recognized by anti-Daxx Ab and normalized by internal control. Daxx protein in human colon adenocarcinoma is decreased. The ratio indicates the Daxx expressing in colon adenocarcinoma tissue compared with normal colon tissue. Mean values of independent experiments (n = 3) are shown. pt, patient; N, normal tissue; T, tumor tissue.

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45. Batlle, E., Henderson, J. T., Beghtel, Harry, van den Born, M. M. W., Sancho, E., Huls, G., Meeldijk, J., Robertson, J., van de Wetering, M., Pawson, T., and Clevers, H. (2002) Cell 111, 251–263

46. Pardee, A. B. (1989) Science 246, 603–608

47. Bartkova, J., Thullberg, M., Slezak, P., Jaramillo, E., Rubio, C., Thomasen, L. H., and Bartek, J. (2001) Gastroenterology 120, 423–438

48. Cuff, M., Dyer, J., Jones, M., and Shirazi-Beechey, S. (2005) Gastroenterology 128, 423–438

49. Chesire, D. R., Ewing, C. M., Gage, W. R., and Isaacs, W. B. (2002) Oncogene 21, 2679–2694

50. Jung, C., Kim, R. S., Zhang, H., Lee, S. J., Sheng, H., Loehrer, P. J., Gardner, T. A., Jeng, M. H., and Kao, C. (2005) Br. J. Cancer 92, 2233–2239

51. Michaelson, J. S., Bader, D., Kuo, F., Kozak, C., and Leder, P. (1999) Genes Dev. 13, 1918–1923

52. Gongora, R., Stephan, R. P., Zhang, Z., and Cooper, M. D. (2001) Immunity 14, 727–737

53. Shimoda, K., Kamesaki, K., Numata, A., Aoki, K., Matsuda, T., Oritani, K., Tamiya, S., Kato, K., Takase, K., Imamura, R., Yamamoto, T., Miyamoto, T., Nagafuji, K., Gondo, H., Nagafuchi, S., Nakayama, K. I., and Harada, M. (2002) J. Immunol. 169, 4707–4711