Pergamon

European Journal of Cancer, Vol. 33, No. 12, pp. 1986–1990, 1997

Printed in Great Britain
0959-8049/97 $17.00+0.00

PII: S0959-8049(97)00333-X

Original Paper

A Product of *DAN*, a Novel Candidate Tumour Suppressor Gene, is Secreted into Culture Medium and Suppresses DNA Synthesis

Y. Nakamura, T. Ozaki, A. Nakagawara and S. Sakiyama

Division of Biochemistry, Chiba Cancer Centre Research Institute, 666-2, Nitona, Chuoh-ku, Chiba 260, Japan

Our previous studies have shown that the *DAN* gene product possesses an ability to revert phenotypes of transformed rat fibroblasts and represents a candidate tumour suppressor gene for neuroblastoma. In the present study, characterisation of DAN was carried out using rat fibroblast 3Y1 cells and their DAN-overexpressor counterparts (S-9). The N-terminal region of DAN (amino acids 1–24) was highly hydrophobic and DAN protein was found to be secreted into the culture medium. When DAN was treated with PNGase F, a enzyme that cleaves most N-linked carbohydrate residues, the mobility of both cytoplasmic and secreted DAN was increased in SDS–polyacrylamide gel electrophoresis, suggesting DAN is N-glycosylated, irrespective of its localisation. When partially purified, DAN was able, when added to the culture, to suppress DNA synthesis of Rous sarcoma virus-transformed 3Y1 cells, which lack the expression of DAN. © **1997 Elsevier Science Ltd.**

Key words: secretion, N-glycosylation, DNA inhibition, v-*src*
Eur J Cancer, Vol. 33, No. 12, pp. 1986–1990, 1997

INTRODUCTION

THE *DAN* gene (previously named *N03*) has been isolated by differential hybridisation screening of cDNAs between rat fibroblasts (3Y1) and those transformed with a Schmidt–Ruppin strain of Rous sarcoma virus (SR-3Y1) [1]. The *DAN* gene represents one of the genes downregulated in SR-3Y1 compared with parental 3Y1 cells [2–6]. The reduction of *DAN* expression is not specific to *src*-transformants but also takes place in 3Y1 and NIH3T3 cells which were transformed by other oncogenes [1, 7]. An extensive homology search of the *DAN* sequence in available databases shows an absence of any homology. When *DAN* cDNA was overexpressed in SR-3Y1 or 3Y1 cells, several important findings were obtained: flat reversion of morphology; suppression of cell growth as measured by doubling time; suppression of colony formation efficiency in soft agar medium; loss of tumorigenicity in nude mice [8]; and delay of entry into the S phase [9]. These data collectively suggest that DAN protein, when overexpressed, possesses an ability to suppress transformed phenotypes.

Correspondence to S. Sakiyama.

Subsequently, human *DAN* cDNA was obtained from a normal lung cDNA library and the *DAN* gene was mapped to human chromosome 1 (1p36.11–p36.13) [10]. It has been well documented that a high incidence of chromosome 1p deletion is frequently associated with the genesis of human neuroblastoma [11, 12]. Genomic Southern blot analysis revealed that altered sizes of the band were indeed detected in some neuroblastoma cases using the full-length human cDNA as a probe, raising the possibility that the *DAN* gene could be one of the tumour suppressor genes for neuroblastoma [10].

In the present study, we examined some of the structural features and biological roles of DAN.

MATERIALS AND METHODS

Cell culture

The rat fibroblast 3Y1 cells, those transformed with a Schmidt–Ruppin strain of Rous sarcoma virus (SR-3Y1) and 3Y1 cells stably transfected with *DAN* cDNA (S-9) [9] were maintained at 37°C in Dulbecco's modified Eagle's minimal essential medium (DMEM) supplemented with 10% heat-inactivated fetal calf serum and kanamycin.

Western blot analysis

SDS–polyacrylamide gel electrophoresis (SDS–PAGE) was carried out under reducing condition as described by Laemmli [13]. An electrophoretically blotted nitrocellulose filter was incubated with 5% dry milk in TBS-T buffer (150 mM NaCl, 50 mM Tris-HCl, pH 7.4, 0.05% Tween 20) to block non-specific binding sites [14]. The filter was then incubated with rabbit anti-rat DAN antibody [9] in TBS-T buffer followed by incubation with goat anti-rabbit I_gG conjugated with horseradish peroxidase (Gibco-BRL, Grand Island, New York, U.S.A.). DAN was finally detected by the use of an enhanced chemiluminescence Western blotting kit (Amersham).

Localisation of DAN

3Y1 or S-9 cells were treated with a lysis buffer containing 100 mM Pipes (pH 6.9), 5 mM $MgCl_2$, 0.2 mM EGTA, 4 M glycerol, 1 mM PMSF and 0.1% Triton X-100 for 10 min at room temperature [15]. The lysates were centrifuged at 8000 *g* for 10 min and the supernatant and the precipitate served as cytoplasmic and nuclear fractions, respectively. The conditioned medium of 3Y1 or S-9 cells were collected and centrifuged at 3000 g for 15 min.

Deglycosylation of DAN

The cell lysates and the conditioned medium of S-9 cells were treated with PNGase F(0.5 U/85 µl, Boehringer, Mannheim, Germany) at 30°C overnight as described previously [16]. Thereafter, a portion of each reaction was subjected to SDS–PAGE and Western blot analysis.

V8-peptide mapping of DAN

S-9 cells were metabolically labelled with [^{35}S]methionine overnight and the cell lysates and the conditioned medium were immunoprecipitated with anti-DAN antibody. After localisation of the position of DAN on SDS–PAGE by autoradiography, the bands corresponding to DAN were cut out from the gel and subjected to digestion with V8 protease [17] at different concentrations. The sizes of the digested products were analysed by SDS–PAGE followed by autoradiography.

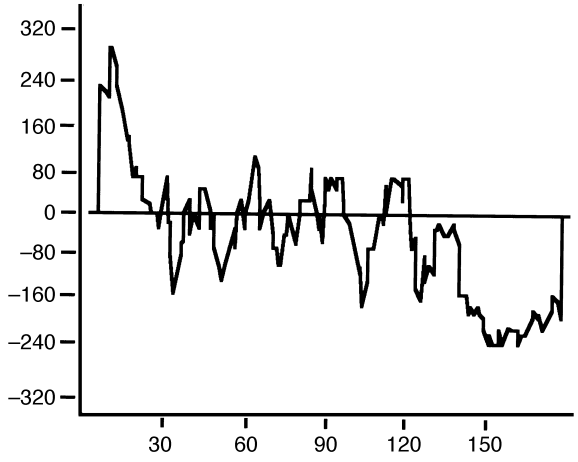

Figure 1. Hydropathicity of DAN protein. Hydropathicity of the deduced amino acid sequence of rat DAN protein was calculated using Kite and Doolittle algorithm. Calculated regions of hydrophobicity are positive in the Kite and Doolittle representation. The abscissa shows the numbers of amino acids residues of rat DAN.

Effect of DAN addition on DNA synthesis

DAN was partially purified by ammonium sulphate fractionation (30–50%). The fraction which was enriched with DAN was extensively dialysed against PBS and concentrated by ultrafiltration using Centricon-3 (Amicon Beverly, Massachusetts, U.S.A.). The DAN fraction thus obtained was added to the culture of SR-3Y1 cells at an approximate concentration of 100 ng/ml. Two days after the addition, cells were labelled with [^{3}H]thymidine (3.3 µCi/ml) for 2 h and the radioactivity incorporated into the DNA fraction was measured as previously described [18].

RESULTS

As previously reported, DAN possesses the ability to suppress cell growth and retard entry into the S phase of the cell cycle [8, 9]. Therefore, it is essential to examine how DAN protein can achieve these crucial events, which are related to tumour suppression. Although extensive database searches of DAN still show no significant overall homology to any known proteins or genes, the N-terminal region (amino acids 1–24) of DAN has been found to be highly hydrophobic and possibly to comprise the leader peptide [19] (Figure 1). Thus, we investigated whether DAN can be secreted or is present on a membrane fraction.

Initially, we investigated the intracellular localisation of DAN in 3Y1 and S-9 cells, the latter of which expresses a large amount of DAN due to transfection with *DAN* cDNA [9]. As shown in Figure 2, DAN was present as a molecular mass of 27 kDa in the cytoplasm, but was not detected in the nuclear fraction. We have previously shown by competition experiments that M_r 27 000 protein corresponds to DAN [9]. It has been predicted that DAN can be secreted outside the cell because of its hydrophobic region present at the N-terminal region. As shown in Figure 3, the presence of DAN was indeed clearly observed in the culture medium. The amount

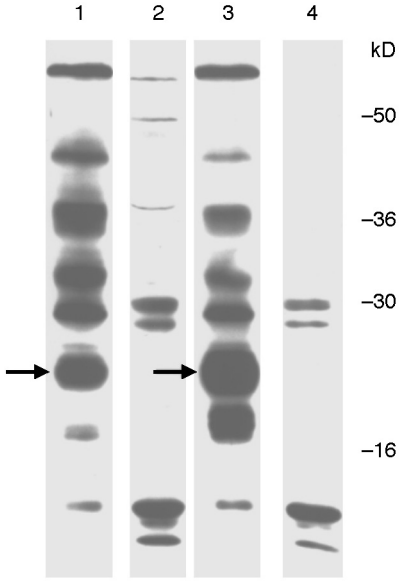

Figure 2. Subcellular distribution of DAN. The cell lysates of 3Y1 and SR-3Y1 cells were subfractionated into nuclear and cytoplasmic fractions as described in Materials and Methods. Each fraction (lanes 1 and 2, 3Y1 cells: lanes 3 and 4, S-9 cells: lanes 1 and 3, cytoplasmic fraction: lanes 2 and 4, nuclear fraction) was analysed for the presence of DAN by Western blotting. Arrows show the position of DAN.

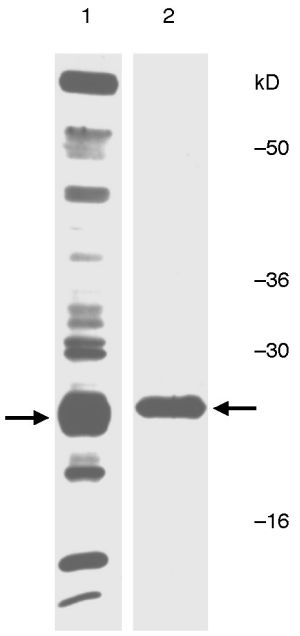

Figure 3. Secretion of DAN into the culture medium. Cell lysates and the conditioned medium derived from DAN-over-expressing 3Y1 fibroblast (S-9) were electrophresed on 10% SDS–PAGE. For cell lysates (1:4 dilution) and the conditioned medium (1:40 dilution) of the material derived from the culture dish (3.5 cm ϕ) were used for analysis. DAN was detected by the use of rabbit anti-DAN antibody. Lane 1, cell lysates: lane 2, conditioned medium. Arrows show the position of DAN.

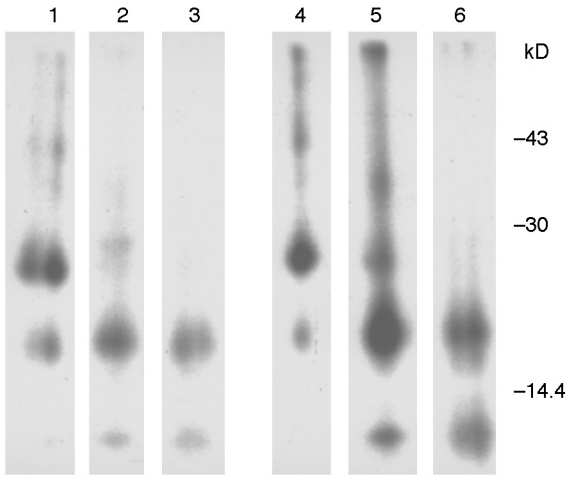

Figure 4. V8-peptide mapping of DAN. Cell lysates and the conditioned medium prepared from [^{35}S]methionine-labelled S-9 cells were immunoprecipitated with anti-DAN antibody. Each band corresponding to DAN (lanes 1–3, cell lysates: lanes 4–6, conditioned medium) was cut out and subjected to V8 protease digestion at different concentrations (lanes 1 and 4, 0: lanes 2 and 5, 1 μg/ml: lanes 3 and 6, 10 μg/ml). The products were analysed by SDS–PAGE followed by autoradiography.

of DAN secreted from the cells was calculated to be 80% of the total DAN protein, based on the sample volume applied for SDS–PAGE. The secreted form of DAN was also detected in 3Y1 cells, but the amount was less than that of S-9 (data not shown).

The mobility of extracellular DAN was found to be always slower than that of cytoplasmic DAN. Therefore, the identity of these two forms of DAN was examined by comparison of V8-protease maps of DAN prepared from cell lysates and culture medium. As shown in Figure 4, the peptide patterns generated by V8 digestion are basically the same for these two proteins. This observation indicates that the secreted form of DAN retains the principal peptide skeleton of DAN, but the molecular basis of the migration difference between cytoplasmic and extracellular DAN is still unknown.

As a putative N-glycosylation site of DAN is well conserved between human and rodents, this modification possibly has some biological meaning. One of the approaches to determine the presence of glycosyl residues is the susceptibility of protein toward glycosidase treatment. For this purpose, we examined the effect of PNGase F (an enzyme which hydrolyses most types of N-linked carbohydrate residues from glycoproteins at a point between the di-GlcNAc core and the Asn to which the carbohydrate is linked [16]) on the mobility of DAN in SDS–PAGE. Both intracellular and secreted DAN showed a significant reduction of the apparent mass of DAN (approximately 3000 Da) after treatment with PNGase F (Figure 5). These results imply that DAN can be glycosylated irrespective of its location.

In order to investigate the effect, if any, of DAN on DNA synthesis, DAN was partially purified from serum-free culture medium of S-9 by ammonium sulphate fractionation (30–50%). The material dialysed against PBS was regarded as crude DAN. We added the crude DAN to the SR-3Y1 cells which lack expression of DAN [1] at a concentration of 100 ng/ml. Two days after the addition of DAN, [^{3}H]thymidine incorporation into DNA fraction was measured. As shown in Table 1, the addition of DAN resulted in up to 40% inhibition of DNA synthesis. In this experiment, either PBS alone or the ammonium sulphate fraction prepared from DAN-negative cells (SR-3Y1) was used as a negative control. This inhibitory activity of DAN was abolished by heat treatment (100°C, 10 min).

DISCUSSION

In previous reports, we have shown that DAN has a variety of activities which include suppression of both anchorage-independent cell growth and tumorigenicity and a delay of

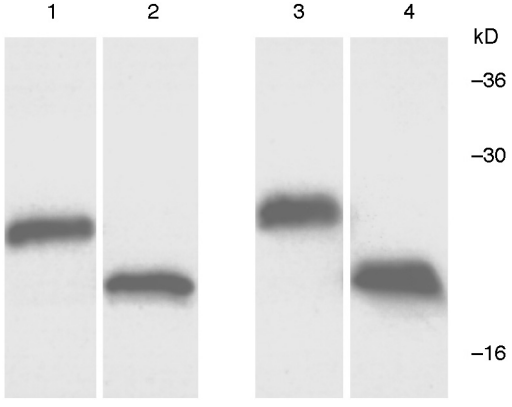

Figure 5. Deglycosylation of DAN. Cell lysates and the conditioned medium of S-9 cells were treated with PNGase F (0.5 U/85 μl) at 30°C overnight. Samples before (lanes 1 and 3) and after (lanes 2 and 4) digestion were analysed by Western blotting (lanes 1 and 2, lysates: lanes 3 and 4, conditioned medium).

Table 1. *Inhibition of DNA synthesis by secreted DAN*

Treatment	cpm (mean ± S.D.)	% Change
Experiment 1		
Control (10% FCS)	71 564 ± 391	—
Control (PBS)	65 476 ± 1908	−9
CM (from SR-3Y1)	60 826 ± 3603	−15
CM (from S-9)	41 488 ± 1265*	−42
Experiment 2		
Control (PBS)	40 830 ± 1639	—
CM (from S-9, heat treated)	42 623 ± 1061	+4
CM (from S-9)	31 880 ± 2867†	−22

*$P < 0.01$ CM (S-9) versus PBS and CM (SR-3Y1); cm, culture medium. †$P < 0.01$ versus PBS and CM (heat treated). DAN prepared from the culture medium of S-9 cells by ammonium sulphate fractionation (30–50%) was added to the culture of SR-3Y1 cells. The concentration of DAN was calculated to be approximately 100 ng/ml. In Exp. 1, 10% FCS, PBS or the equivalent fraction prepared from SR-3Y1 cells were used as a control. In Exp. 2, the crude DAN fraction was treated at 100°C for 5 min. Two days after the treatment, cells were labelled with [^{3}H]thymidine (3.3 μCi/ml) for 2 h and the radioactivity incorporated into DNA was measured. The numbers indicate the average of three dishes.

entry into the S phase [8, 9]. Therefore, DAN represents one of the proteins which suppress cell growth through negative regulation of cell cycle progression. The sequences of DAN protein, which consists of 178 and 180 amino acids for rat and human, respectively, have no significant homology with those of any known proteins, and it possesses several unique sequences and motifs. Those include a zinc finger-like domain, $(HX)_n$ repeat, N-linked glycosylation site, casein kinase II target sequences [1, 10, 20]. Another interesting feature is the presence of a highly hydrophobic stretch at the N-terminus of DAN. In the present report, we have shown that DAN is localised predominantly in the cytoplasm and can be secreted into culture medium. Indirect immunofluorescence microscopic examination, using affinity-purified antibody, failed to detect any significant distribution patterns of DAN due to a relatively high background staining.

Although precise molecular mechanisms underlying this phenomenon are yet to be studied, secretion of DAN is unique with respect to the mode of function of reported tumour suppressor gene products. Recently, Jensen and associates reported that BRCA1, a susceptible gene product for familial breast and ovarian cancer, is secreted into the medium [21]. Based on the subcellular localisation of DAN, the possibility that DAN functions as a DNA binding protein through its zinc finger-like domain can be considered to be negligible. DAN secreted can suppress DNA synthesis from outside the cells. It is also conceivable that a zinc finger-like domain and/or a $(HX)_n$ repeat can be utilised for protein–protein interaction(s).

It is likely that the N-terminal portion is retained in the secreted form of DAN. This is based on the observation of the mobility and V8 protease mapping of these two forms. It has been generally argued that the signal peptide of the secreted protein is cleaved when it is secreted. Determination and comparison of N-terminal sequences of both intracellular and secreted forms of DAN is necessary to prove this point.

The extent of the suppression of DNA synthesis never exceeded 40% in the present experiment. We presume that this is probably due to the impurity of the DAN preparation which might contain molecule(s) responsible for antagonistic action. Therefore, purification of DAN is the next crucial step in determining the significance of this important observation, and this is in progress in our laboratory.

It is possible that DAN, once secreted into the medium, can act as an inhibitor of DNA synthesis in an autocrine or paracrine manner. In this circumstance, the presence of an intermediate molecule, such as a receptor, which might be present on the membrane and transmit signal(s) from the outside to inside of cells can be speculated. As the search for target molecules of DAN is urgent and important, we are currently utilising a yeast two-hybrid system to search for candidate molecule(s).

1. Ozaki T, Sakiyama S. Molecular cloning and characterization of a cDNA showing negative regulation in v-*src*-transformed 3Y1 rat fibroblasts. *Proc Natl Acad Sci USA* 1993, **90**, 2593–2597.
2. Ozaki T, Sakiyama S. Lamin A gene expression is specifically suppressed in v-*src*-transformed cells. *FEBS Lett* 1992, **312**, 165–168.
3. Fagan JB, Sobel ME, Yamada KM, DE Crowbrugghe B, Pastan I. Effects of transformation on fibronectin gene expression using cloned fibronectin cDNA. *J Biol Chem* 1981, **256**, 520–525.
4. Joseph CK, Quereshi SA, Wallace DJ, Foster DA. MARCKS protein is transcriptionally down-regulated in v-*src*-transformed BALB/c 3T3 cells. *J Biol Chem* 1992, **267**, 1327–1330.
5. Pan J, Nakanishi K, Yutsudo M. *et al.* Isolation of a novel gene down-regulated by v-*src*. *FEBS Lett* 1996, **383**, 21–25.
6. Scholz G, Martinerie C, Perbal B, Hanafusa H. Transcriptional down regulation of the *nov* proto-oncogene in fibroblasts transformed by p60$^{v\text{-}src}$. *Mol Cell Biol* 1996, **16**, 481–486.
7. Ozaki T, Ma J, Takenaga K, Sakiyama S. Cloning of mouse *DAN* cDNA and its down-regulation in transformed cells. *Jpn J Cancer Res* 1996, **87**, 58–61.
8. Ozaki T, Sakiyama S. Tumor-suppressive activity of *N03* gene product in v-*src*-transformed rat 3Y1 fibroblasts. *Cancer Res* 1994, **54**, 646–648.
9. Ozaki T, Nakamura Y, Enomoto H, Hirose M, Sakiyama S. Overexpression of *DAN* gene product in normal rat fibroblasts causes a retardation of the entry into the S phase. *Cancer Res* 1995, **55**, 895–900.
10. Enomoto H, Ozaki T, Takahashi E, *et al.* Identification of human *DAN* gene, mapping to the putative neuroblastoma tumor suppressor locus. *Oncogene* 1994, **9**, 2785–2791.
11. White PS, Maris JM, Beltinger C, *et al.* A region of consistent deletion in neuroblastoma maps within 1p36.2-.3. *Proc Natl Acad Sci USA* 1995, **92**, 5520–5524.
12. Cheng NC, van Roy N, Chan A, *et al.* Deletion mapping in neuroblastoma cell lines suggests two distinct tumor suppressor genes in the 1p35–36 region, only one of which is associated with N-*myc* amplification. *Oncogene* 1995, **10**, 291–297.
13. Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (London)* 1970, **227**, 680–685.
14. Towbin H, Staehelin T, Gordon J. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some application. *Proc Natl Acad Sci USA* 1979, **76**, 4350–4354.
15. Lee SA, Karaszkiewicz W, Anderson WB. Elevated level of nuclear protein kinase C in multidrug-resistant MCF-7 human breast carcinoma cells. *Cancer Res* 1992, **52**, 3750–3759.
16. Nunez E, Aragon C. Structural analysis and functional role of the carbohydrate component of glycine transporter. *J Biol Chem* 1994, **269**, 16920–16924.
17. Cleveland DW, Fisher SG, Kirschner MW, Laemmli UK. Peptide mapping by limited proteolysis in sodium dodecyl sulfate and analysis by gel electrophoresis. *J Biol Chem* 1977, **252**, 1102–1106.
18. Mans RJ, Novelli GD. Measurement of the incorporation of radioactive amino acids into protein by a filter-paper disk method. *Arch Biochem Biophys* 1961, **94**, 48–53.

19. von Heijne G. Signal sequences. The limits of variation. *J Mol Biol* 1985, **184**, 99–105.
20. Kondo K, Ozaki T, Nakamura Y, Sakiyama S. *DAN* gene product has an affinity with Ni^{2+}. *Biochem Biophys Res Commun* 1995, **216**, 209–215.
21. Jensen RA, Thompson ME, Jetton TL. BRCA1 is secreted and exhibits properties of a granin. *Nature Genet* 1996, **12**, 303–308.

Acknowledgements—We thank Dr Imajoh-Ohmi, S, Institute of Medical Science, University of Tokyo, Japan, for helpful discussions. This work was supported in part by a Grant-in-Aid from the Ministry of Health and Welfare for a new 10-year strategy for Cancer Control, Tokyo, Japan and by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science and Culture, Japan.