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DAN GENE PRODUCT HAS AN AFFINITY FOR Ni^{2+}

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Summary: Differential screening-selected gene aberrative in neuroblastoma (*DAN*) encodes a protein which possesses metal binding motifs. Glutathione S-transferase DAN fusion protein had an ability to bind to Ni^{2+}-immobilized affinity resin. Truncation of the C-terminal region including a $(HX)_n$ repeat of DAN caused a loss of binding ability to the affinity resin, suggesting that this region is essential for Ni^{2+}-binding. DAN produced in cultured rat cells also had an affinity for Ni^{2+}. Cross-linking experiments demonstrated that the C-terminal region might function as a protein-protein interacting domain.

Metal ions can be bound by a large number of proteins. Such protein-metal interactions occur within specific amino acid residues called metal binding motifs. The motifs that putatively bind metal ions preferentially contain clusters of Cys and/or His (1). Among these, $(HX)_n$ repeat has been reported to confer a strong affinity for Ni^{2+} (2-4). In eukaryotes, several transcription factors (5), an inhibitor of protein kinase C (6), and a chymotrypsin inhibitor (4) contain $(HX)_n$ repeat. This motif may function as a metal-dependent protein-protein interaction domain (3, 5).

DAN was originally cloned from a rat fibroblast 3Y1 library by a differential screening method (7). The expression level of *DAN* is significantly reduced in 3Y1 cells transformed with v-*src*, v-*mos* and SV40, respectively (7). The level of *DAN* mRNA is also decreased in several human neuroblastoma cell lines (8).

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Abbreviations:
GST, glutathione S-transferase; LB medium, Luria-Bertani medium; PBS, phosphate-buffered saline; DMEM, Dulbecco's modified Eagle's medium; SDS-PAGE, SDS-polyacrylamide gel electrophoresis; TBS, Tris-buffered saline.

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Biological activity of DAN has been investigated in transformed and non-transformed cell lines. Constitutive expression of DAN in v-*src* transformed 3Y1 cells suppressed tumorigenicity (9). Overexpression of DAN in non-transformed 3Y1 cells caused a retardation of the entry into the S phase, suggesting a cell cycle-regulatory role of DAN (10).

Protein encoded by *DAN* has two possible metal-interacting domains. One locates in the central region and has a zinc finger motif (7). The other is in the C-terminal region and has a $(HX)_n$ repeat motif. We found that GST DAN fusion protein had an affinity for Ni^{2+}-immobilized resin. Truncation of the C-terminal region including $(HX)_n$ repeat sequence caused a complete loss of binding ability to the affinity resin. DAN produced in mammalian cell lines also showed the ability to bind to Ni^{2+} affinity resin. In addition, function of $(HX)_n$ repeat was investigated by cross-linking of GST fusion proteins.

Materials and Methods

Production and purification of fusion protein. A full-length rat *DAN* cDNA was deleted from its 5' terminus with exonuclease III and filled in using Klenow enzyme. The cDNA fragment was subcloned into pGEX2T(Pharmacia). One of the clones that lost 41 base pairs from start codon (pGDΔ41) was used to obtain GST fusion protein. Construction of C-terminal truncated fusion protein was carried out by the following procedure. pGDΔ41 was digested with BamHI and PstI. The resultant cDNA fragment was subcloned into the BamHI/PstI site of pBluescript KS-(Stratagene), redigested with BamHI and EcoRI and recloned into the BamHI/EcoRI site of pGEX2T to obtain pGDΔ41ΔHX. Protein encoded by pGDΔ41 (GST DAN) contains residues 14 through C-terminus of DAN and protein encoded by pGDΔ41ΔHX (GST DANΔ) contains residues 14 to 117 of DAN. Over-night culture of *Escherichia coli* JM109 harboring either pGEX2T, pGDΔ41, or pGDΔ41ΔHX was diluted 1:10 in LB medium and grown for additional 2 hours with shaking at 37°C. The synthesis of fusion protein was induced with 0.3 mM isopropyl-1-thio-β-D-galactopyranoside, and the cells were grown for additional 4 hour at 37°C. The cells were harvested and 1/100 cultured volume of PBS containing 1% Triton X-100, 1 mg/ml lysozyme was added to the pellet. The suspension of the cells was sonicated and centrifuged at 20,000 × g for 20 min. The supernatant was loaded on glutathione Sepharose 4B (Pharmacia) column. The column was first washed with PBS, then the fusion protein was eluted with PBS containing 10 mM glutathione.

Ni^{2+} affinity chromatography. Ni^{2+}-immobilized resin (Probond Resin; Invitrogen) was washed with basal buffer(20 mM phosphate buffer, pH 5.0, 0.5 M NaCl, 0.05% Tween 20) containing 10 mM imidazole and used for affinity chromatography. Partially purified GST fusion proteins were mixed with the resin and proteins were eluted in batch wise using basal buffer containing 20, 30, 40, 100 mM imidazole, respectively. The use of pH non-adjusted imidazole resulted in a better separation of the samples. Fractions were subjected to SDS-PAGE analysis (11) and stained with Coomasie Blue.

Western blot analysis. Rat fibroblast 3Y1, v-*src* transformed 3Y1 (SR 3Y1) and DAN overexpressing 3Y1 clone, S12 (10), were grown in DMEM (Nissui) supplemented with 10% heat-inactivated fetal bovine serum (ICN) and antibiotics. Exponentially growing cells were washed with ice cold PBS and lysed in an SDS sample buffer followed by sonication. Protein concentrations were measured with protein assay kit (Bio-Rad). Cell lysate protein (20 mg) was incubated with Ni^{2+}-immobilized resin. Thereafter, imidazole-eluted fractions were subjected to analysis in 12% SDS-PAGE and transferred to nitrocellulose membrane (12). After blocking with 5% nonfat dry milk in TBS, the membrane was probed with affinity-purified anti-DAN rabbit polyclonal antibody (10). After washing with TBS with 0.05% Tween 20, the membrane was incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG (GIBCO-BRL). Signals were detected with ECL kit (Amersham).

Cross-link of fusion proteins. 1 mg of each fusion protein was cross-linked with 0.01% glutaraldehyde for 1 hr in PBS containing 1 mM EDTA and 2 mM $NiCl_2$. Samples were subjected to SDS-PAGE analysis and stained with Coomasie Blue.

Results

Affinity of GST DAN fusion protein for Ni^{2+}-immobilized resin. Deduced amino acid sequence of *DAN* gene contains two putative metal-interacting domains (Fig. 1A). One is in the central region and has a putative zinc finger motif which is shown to be homologous to that of MEL-18 gene product (7). The other is in the C-terminal region and has a $(HX)_n$ repeat motif. The presence of these metal binding motifs suggests that DAN could have an affinity for metal-immobilized resin. To examine the metal-binding ability of DAN, we have constructed GST DAN and C-terminal region truncated GST DANΔ (Fig. 1B). Partially purified GST fusion proteins were mixed with Ni^{2+}-immobilized resin and successively eluted by increasing concentrations of imidazole. GST alone was eluted with 20 mM imidazole. However, GST DAN was eluted with 100 mM imidazole (Fig. 2A). Under the optimized condition, GST DANΔ,

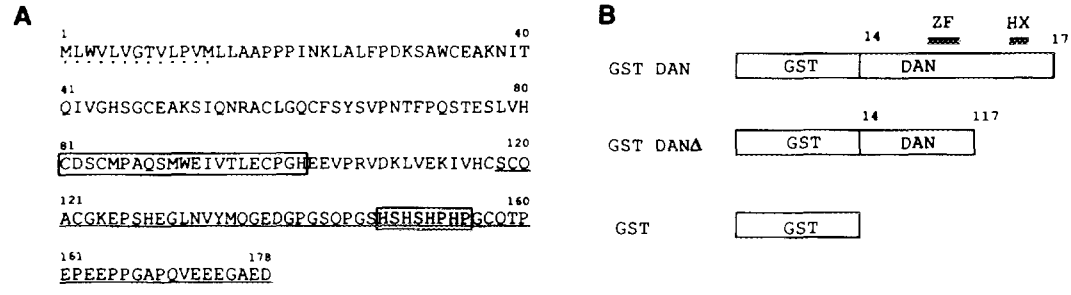

Figure 1. (A) Deduced amino acid sequence of rat DAN. First 13 residues with dots are deleted in GST fusion proteins. Underlined residues are deleted in GST DANΔ. Open and shaded boxes indicate putative zinc finger motif and $(HX)_n$ repeat motif, respectively. (B) Schematic representation of GST DAN fusion proteins. Black bars indicate zinc finger motif (ZF) and $(HX)_n$ repeat motif (HX).

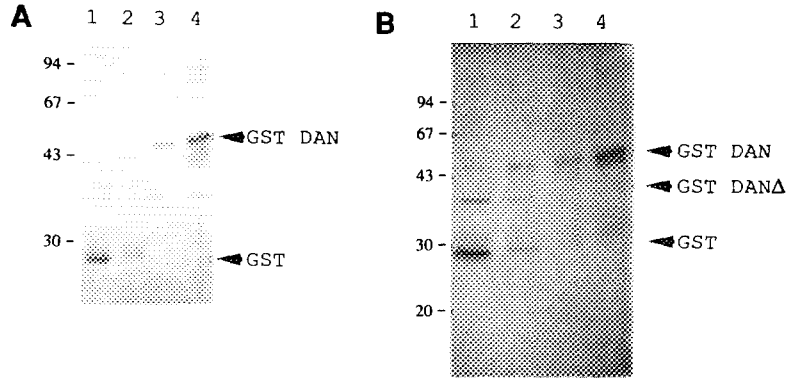

Figure 2. Binding of GST fusion proteins with Ni^{2+}-immobilized resin. (A) Difference in affinity between GST and GST DAN. A mixture of GST and GST DAN was subjected to affinity chromatography. Fractions eluted with imidazole (Lanes: 1, 20 mM; 2, 30 mM; 3, 40 mM; 4, 100 mM) were analyzed by SDS-PAGE. Left, molecular weight markers in kDa. (B) Difference of affinity between GST DAN fusion proteins. A mixture of GST, GST DAN and GST DANΔ was subjected to affinity chromatography. Analysis was performed as in (A).

which lacks $(HX)_n$ repeat motif, was eluted in 20 mM imdazole fraction (Fig. 2B). Therefore, the elution behavior of GST DANΔ on the affinity resin was quite similar to that of GST.

Affinity of DAN produced in mammalian cell for Ni^{2+}-immobilized resin. To investigate whether DAN expressed in mammalian cells also has an affinity for Ni^{2+}-immobilized resin, cell extract from DAN-expressing cells was subjected to affinity chromatography. Fractions after separation on the affinity resin were subjected to SDS-PAGE. Western blot analysis was performed using an anti-DAN polyclonal antibody as a probe. DAN with a molecular mass of 27 kDa was detected in 3Y1 cell lysate (Fig. 3, lane 2 and 3) and a higher amount of DAN can be seen in DAN-overexpressing clone, S12 (Fig. 3, lane 8 and 9). As expected, in the case of SR 3Y1 cells that do not express *DAN* mRNA (7), no signal was detected. These results indicate that DAN derived from mammalian cells has a Ni^{2+}-binding activity.

Cross-linking of fusion proteins with glutaraldehyde. $(HX)_n$ repeat motif was suggested to function as a metal-dependent protein-protein interaction domain (3, 5). To investigate whether $(HX)_n$ repeat motif in DAN could also function as a metal-dependent protein-protein interaction domain or not, the fusion proteins were cross-linked with glutaraldehyde in the presence or absence of EDTA and $NiCl_2$. As shown in Fig. 4, slowly migrating smears were

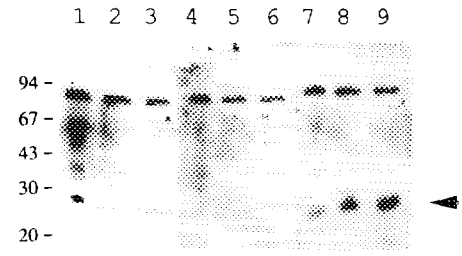

Figure 3. Ni^{2+} affinity of mammalian-produced DAN. Cell lysate from 3Y1 (Lanes 1, 2, 3), SR 3Y1 (Lanes 4, 5, 6), S12 (Lanes 7, 8, 9) was subjected to affinity chromatography. Total cell lysate (Lanes: 1, 4, 7) and fractions eluted with imidazole (Lanes: 2, 5, 8; 40 mM. 3, 6, 9; 100 mM) were analyzed by SDS-PAGE and probed with anti-DAN antibody. Arrowhead indicates the positon of DAN.

detected in GST DAN cross-linked with glutaraldehyde, suggesting that GST DAN exists as dimer/oligomered forms. However, in the case of GST DANΔ, predominantly a monomer form was detectable in all the lanes as in GST. In all the cases, dependence of metal ion was not observed.

Discussion

In the present study we demonstrated that recombinant as well as natural DAN has an affinity for Ni^{2+}. Although DAN has a consensus sequence for N-glycosylation and casein kinase II phosphorylation (7), such mammalian-specific modifications seems to have no effects on the ability of DAN to bind to the affinity resin. Reduced affinity for Ni^{2+} in the case of C-terminal region

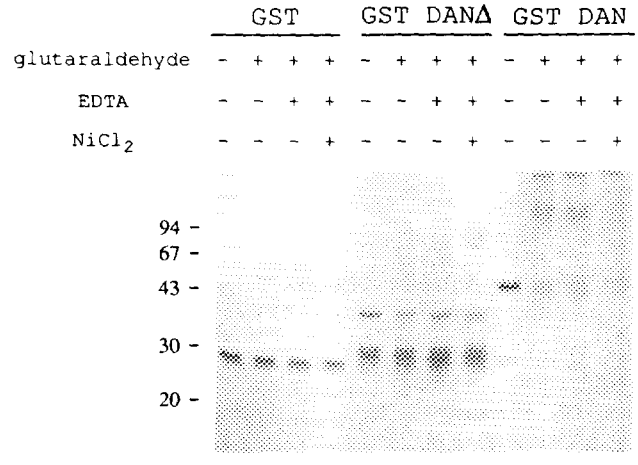

Figure 4. Cross-linking of GST fusion proteins. Fusion proteins were incubated with 0.01% glutaraldehyde, 1 mM EDTA and 2 mM $NiCl_2$ as indicated. Samples were analyzed by SDS-PAGE. Left, molecular weight markers in kDa.

deleted GST DANΔ suggests that (i) $(HX)_n$ repeat motif has an essential role in binding Ni^{2+}, (ii) zinc finger motif alone does not contribute to Ni^{2+} binding ability. As suggested by Janknecht *et al.* (5), cross-linking experiment demonstrated that the C-terminal region of DAN containing $(HX)_n$ repeat could function as a protein-protein interaction domain. However, dimer/oligomerization of GST DAN was not inhibited by the presence of 1 mM EDTA (Fig. 4). One possibility is that dimer/oligomerization of DAN is C-terminal region-dependent but metal-independent (*i.e.* dimer/oligomerization via Cys residues). The other is that depletion of metal ion from protein with 1 mM EDTA was ineffectual. Against the latter possibility, Wülfing *et al.* reported that Ni^{2+} or Zn^{2+} bound to *E. coli* protein with $(HX)_n$ repeat can successfully depleted with 1 mM EDTA (13). Nonetheless, DAN obviously exists as dimer/oligomerized forms in the cell via C-terminal region.

At present, how the Ni^{2+}-binding nature of DAN is related to its tumor-suppresive function is unknown. Also, we do not know whether DAN exists as a Ni^{2+}-bound form *in vivo* or not. Concerning the ability of metal ions to be bound to $(HX)_n$ repeat, it has been reported that Ni^{2+}, Zn^{2+}, Co^{2+} and Cu^{2+}, but no Mg^{2+}, Fe^{2+} or Ca^{2+} can be bound to the above mentioned *E. coli* protein. However, only Ni^{2+} and Zn^{2+} were found to be bound to the purified protein (13). Therefore, it is probable that Ni^{2+} and Zn^{2+} are ions bound to DAN *in vivo*. We are now trying to purify DAN from rat cells to answer these questions.

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