FEBS Letters 494 (2001) 48–53 FEBS 24743

Phosphorylation of neurofibromin by cAMP-dependent protein kinase is regulated via a cellular association of N^G,N^G-dimethylarginine dimethylaminohydrolase

Hiroshi Tokuo[a,b], Shunji Yunoue[a], Liping Feng[a], Masumi Kimoto[c], Hideaki Tsuji[c], Tomomichi Ono[b], Hideyuki Saya[a], Norie Araki[a,*]

[a] *Department of Tumor Genetics and Biology, Kumamoto University School of Medicine, 2-2-1 Honjo, Kumamoto 860-0811, Japan*
[b] *Department of Dermatology, Kumamoto University School of Medicine, 2-2-1 Honjo, Kumamoto 860-0811, Japan*
[c] *Department of Nutritional Science, Okayama Prefectural University, Kuboki, Soja, Okayama 719-1137, Japan*

Received 21 February 2001; accepted 28 February 2001

First published online 20 March 2001

Edited by Julio Celis

Abstract The neurofibromatosis type 1 (NF1) tumor suppressor (neurofibromin) is thought to play crucial roles in cellular Ras- and cAMP-dependent kinase (PKA)-associated signals. In this study, we identified a cellular neurofibromin-associating protein, N^G,N^G-dimethylarginine dimethylaminohydrolase (DDAH) that is known as a cellular NO/NOS regulator. The interaction of DDAH was mainly directed to the C-terminal domain (CTD) and to the cysteine/serine-rich domain (CSRD) of neurofibromin, coinciding with the regions containing specific PKA phosphorylation sites. DDAH increased PKA phosphorylation of native neurofibromin in a dose-dependent manner, especially affecting the phosphorylation of CSRD. These findings suggest that the PKA accessibility of neurofibromin was regulated via DDAH interaction, and this regulation may modulate the cellular function of neurofibromin that is implicated in NF1-related pathogenesis. © 2001 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

Key words: Neurofibromatosis type 1; Neurofibromatosis Type 1 (NF1); Neurofibromin; cAMP-dependent protein kinase (PKA); N^G,N^G-dimethylarginine dimethylaminohydrolase (DDAH)

1. Introduction

Neurofibromatosis type 1 (NF1) is a dominantly inherited human disease affecting one in 2500–3500 individuals [1,2]. The NF1 phenotype is highly variable with several organ systems being affected, including bones, skin, iris, and the central nervous system as manifested in learning disabilities and gliomas [2]. The hallmark of NF1 is the development of benign tumors of the peripheral nervous system [1,2], and the increased risk of developing malignancies [3,4]. The *NF1* gene lies on chromosome 17q11.2 [5,6], and the great majority of patient mutations prevent the expression of the intact NF1 product, neurofibromin [7]. Neurofibromin contains a central domain homologous to a family of proteins known as Ras-GTPase-activating proteins (Ras-GAPs), which function as negative regulators for Ras proteins [8]. Ras-GAPs attenuate signaling from Ras, thus blocking the transmission of signals leading to increased growth or differentiation. However, the precise function of cellular neurofibromin, on either Ras-dependent signals or on NF1-related pathogenesis, has not been clearly elucidated.

Recently some interesting studies have reported that *Drosophila* NF1 acts as a possible activator of the cAMP pathway that involves the *rutabaga* (*rut*)-encoded adenylyl cyclase [9,10]. The mechanism of the NF1-dependent activation of the cAMP pathway was suggested being essential for mediating *Drosophila* learning and memory. From this evidence, neurofibromin has been thought to play a crucial role in cellular functions linked to cAMP-dependent protein kinase (PKA)-associated signals as well as to Ras-GAP signals in major cellular pathways.

Izawa et al. reported that neurofibromin is constitutively phosphorylated, and that the specific phosphorylation lie on the cysteine/serine-rich domain (CSRD) and the C-terminal domain (CTD) of neurofibromin [11]. in vitro and in gel kinase assays suggest that PKA is a candidate for the neurofibromin kinase [11]. Although the biological significance of the PKA phosphorylation of neurofibromin has not been clear, these findings prompt us to speculate that cellular neurofibromin is regulated post-translationally by several cellular factors.

Most of the *NF1* mutations are nonsense, frame shift, or truncating mutations leading to premature termination codons [7,12], and a relative high frequency of the mutation has been reported down stream of the GAP-related domain (GRD) to the C-terminal region [12]. This suggests that the C-terminal region of neurofibromin is important in regulating the tumor suppressive function of neurofibromin. In this context, identification and analysis of cellular proteins that are bound to C-terminal neurofibromin is thought to be crucial to elucidate the biological significance of neurofibromin.

In this study, we attempted to purify a cellular neurofibromin-interacting protein from a bovine brain cytosolic fraction using affinity column immobilized CTD of neurofibromin, and identified it as N^G,N^G-dimethylarginine dimethylaminohydrolase (DDAH). The binding region of DDAH was found on the CSRD as well as CTD that was happened to coincide with the region containing the specific PKA phosphorylation sites of neurofibromin. Thus, we analyzed the effects of DDAH in PKA phosphorylation of neurofibromin, and showed that DDAH clearly increased the PKA phosphoryla-

*Corresponding author. Fax: (81)-96-373 5120.
E-mail: nori@gpo.kumamoto-u.ac.jp

0014-5793/01/$20.00
PII: S0014-5793(01)02309-2

tion accessibility of neurofibromin. Consequently, we postulated that the cellular binding proteins of neurofibromin, such as DDAH, might modulate both the PKA-related signals and the Ras-associated signals of neurofibromin.

2. Materials and methods

2.1. Preparation of GST fusion neurofibromin fragments

PGEX-2TH plasmids harboring NF1-domain cDNAs, which generate GST–GRD fusion protein (residues 1168–1545), CSRD: 543–909, leucine repeat domain (LRD: 1530–1950), and CTD: 2262–2818 respectively, were constructed as described previously [11]. For pGEX-CTD1000 (2262–2619), a fragment of the 3′-end region in the CTD insert was cut off from the pGEX-CTD with the double digestion of the *Eco*RI and *Hin*dIII sites, blunt-ended by T4 DNA polymerase, self-ligated and subcloned. For pGEX-CTD600 (2620–2818), 600 bases of fragment containing the 3′-end of the CTD was cut out by the double digestion of *Eco*RI and *Aat*II sites from pGEX-CTD, inserted into the *Eco*RI and *Aat*II sites of pGEX-2TH and subcloned. We confirmed the entire sequences of the insert of all newly constructed plasmids were identical to the reported sequences by DNA sequencing analysis. Expression and purification of each GST fusion protein were performed according to the original protocol as described previously [13,14].

2.2. Expression and purification of DDAH

A native DDAH was purified from rat kidney as described previously [15]. GST–DDAH was prepared from pGEX-DDAH according to the original protocol as previously described [13]. A mammal expression vector pbj-Myc-DDAH was constructed with an insertion of *Bam*H1 fragment from pGEX-DDAH to a pbj-Myc *Bam*H1 cloning site and cloned. To express myc-tagged DDAH, the pbj-Myc-DDAH was transfected into COS7 cells by the liposome-mediated gene transfection method.

2.3. Antibodies

Antibodies against GST–CSRD and GST–GRD type 1 fusion proteins were raised and purified from male Fisher 344 rats sera. Antibody against C-terminal (D) neurofibromin was purchased from Santa Cruz Biotechnology. Mouse monoclonal antibody against native rat DDAH was prepared as described previously [16]. The anti-Myc monoclonal antibody was prepared from 9E10 cells.

2.4. Preparation of cytosolic fraction from bovine and rat brain

Cytosolic fraction of bovine brain was prepared as previously described [17]. In brief, bovine brain gray matter was cut into small pieces and suspended in homogenizing buffer A (25 mM Tris–HCl, pH 7.5, 1 mM dithiothreitol, 5 mM EGTA, 10 mM $MgCl_2$, 10% sucrose), homogenized with a Potter–Elvehjem Teflon glass homogenizer, and centrifuged at 12 000 rpm for 30 min. Solid ammonium sulfate was added to the supernatant to 40% saturation, and the mixture was centrifuged. To the supernatant of the centrifugates, solid ammonium sulfate was subsequently added to 80% saturated, and then centrifuged. The precipitate was dialyzed against buffer A, and used as the cytosolic 40–80% fraction. For the rat brain cytosolic fraction, rat brain was homogenized with buffer B (20 mM Tris–HCl, pH 7.5, 1 mM EDTA, 5 mM $MgCl_2$, 150 mM NaCl, 0.1% NP-40, 1 mM 4-(2-aminoethyl)-benzenesulfonyl fluoride hydrochloride (p-ABSF), 20 μg/ml aprotinin, 1.5 μM pepstatin A, 10 μg/ml leupeptin, and 500 μM sodium orthovanadate) in a dance type homogenizer. The homogenized sample was centrifuged at 14 000 rpm for 20 min, and the supernatant was used for the experiments. All experiments described were performed at 4°C or on ice.

2.5. Detection of binding proteins from brain cytosolic fraction by GST–CTD affinity chromatography

The GST–CTD fusion protein immobilized on GSH-agarose was packed into a column, and equilibrated with buffer C (30 mM Tris–HCl, pH 7.5, 1 mM EDTA, 5 mM $MgCl_2$, 1 mM dithiothreitol). Bovine brain cytosolic fraction, pre-cleared by passing it through a GSH column, was then loaded onto the GST–CTD column. After washing the column with buffer C, the protein bound to the column was eluted by the addition of buffer D (buffer A containing 0.5 M NaCl), and subjected to SDS–PAGE, followed by silver staining.

2.6. Purification of p35 and determination of peptide

To purify a 35-kDa protein (p35), the binding proteins eluted from the column were dialyzed against distilled water. After concentration of the solution by evaporation, the sample was subjected to SDS–PAGE, transferred onto a PVDF membrane (ABI), and stained with Ponceau S. A stained band of p35 was cut out from membrane, reduced *S*-pyridylethylated, and digested by *Achromobacter* protease I (Wako) for 48 h. After sonication and centrifugation, the supernatant was loaded onto a reverse-phase HPLC (Hitachi L655-15) using a μ-Bondasphere C8 column (Waters). The HPLC fractions were subjected to amino acid sequencing using the 492 Procise protein sequencing system (PE Applied Biosystems). Amino acid sequences obtained were subjected to SwissProt, EST-DATA base and BLAST sequence homology search analyses.

2.7. Interaction of DDAH with neurofibromin in vitro

Various GST fusion fragments of neurofibromin or GST immobilized to GSH-agarose beads were incubated with DDAH purified from GST–DDAH after PreSission protease digestion or COS7 cell lysates overexpressing myc-DDAH, for 2 h at 4°C. After washing three times with buffer B, the protein bound to the beads were eluted, subjected to SDS–PAGE, electroblotted onto the membrane, and then probed with the anti-DDAH or anti-myc antibodies. After reaction with the HRP-conjugated secondary antibodies, the reacted protein band on the membrane was visualized by an ECL detection system (Amersham).

2.8. Interaction of DDAH with cellular endogenous neurofibromin

Subconfluently grown SH-SY5Y cells were lysed in buffer B, and centrifuged at 14 000 rpm for 30 min at 4°C. The supernatant was incubated with GST or GST–DDAH immobilized GSH beads. COS7 cells over expressing myc-DDAH were lysed in buffer B after 48 h of transfection. The lysates were centrifuged at 14 000 rpm for 30 min at 4°C, and the supernatant immunoprecipitated with an anti-myc monoclonal antibody, or mouse IgG as a negative control. The proteins bound on each beads were separated through SDS–PAGE, electroblotted onto the membrane and detected by the immunoblotting being probed with the anti-neurofibromin antibody (D). After reaction with HRP-conjugated secondary antibodies, the reacted protein band on the membrane was visualized by an ECL detection system.

2.9. Phosphorylation assay of neurofibromin by PKA

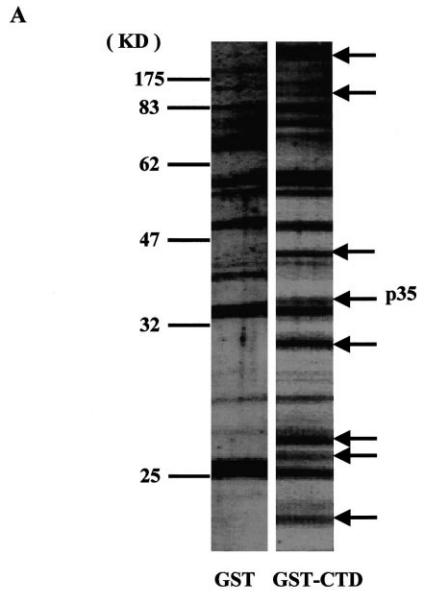
Confluently grown HeLa cells were lysed in buffer B containing 10 mM NaF. After centrifugation of the cell lysates, the supernatant was incubated with rat anti-GRD polyclonal antibody conjugated on CNBr-activated Sepharose 4B beads (Amersham Pharmacia) at 4°C for 2 h. After the incubation, the bound neurofibromin on the beads was washed three times with buffer B, once with the starting buffer (20 mM Na-phosphate buffer, pH 7.0), and eluted with the elution buffer (100 mM glycine HCl pH 2.7), followed by the neutralization with 1 M Tris–HCl. In vitro kinase reactions for the eluted endogenous neurofibromin were carried out in a reaction buffer of a final volume of 50 μl, containing 25 mM sodium acetate, 2 mM $MgCl_2$, 10 μCi of [γ-^{32}P]ATP (3000 Ci/mmol), and 5 μl neurofibromin (1 μg) with 2 units of PKA catalytic subunit at 37°C for 10 min without or with PKA inhibitor. The reaction mixtures were then subjected to SDS–PAGE (6%), and [^{32}P]phosphate-labeled neurofibromin on the gel was detected by autoradiography using a BAS 2000 (Fuji Photo Film) with Image Reader Ver. 1.3 and Mac Bas V2.5 software.

3. Results

3.1. Identification of neurofibromin-associating proteins

To purify neurofibromin-binding proteins, bovine brain cytosolic fraction was loaded on GSH columns to which GST–CTD or GST were immobilized, and the proteins bound to the columns were eluted with a high salt buffer. Eight proteins with molecular masses of 175, 97, 45, 35, 30, 27, 26 and 19 kDa were obtained from the GST–CTD column, but none of them were obtained from the GST column (Fig. 1A).

To identify the 35-kDa protein (p35), p35 was purified and digested by lysyl end-peptidase. After peptide separation of



B

```
1 MAGLSHPSVF GRATHAVVRA PPESLCRHAL RRSQGEEVDF ARAERQHQLY
  VGVLGSKLGL QVVQLPADES LPDCVFVEDV AVVCEETALI TRPGAPSRRK
  EVDMMKEALE KLQLNIVEMK DENATLDGGD VLFTGREFFV GLSKRTNQRG
  AEILADTFKD YAVSTVPVAD SLHLKSFCSM AGPNLIAIGS SESAQKALKI
  MQQMSDHRYD KLTVPDDMAA NCIYLNIPSK GHVLLHRTPE EYPESAKVYE
  KLKDHLLIPV SNSEMEKVDG LLTCCSVFIN KKTDS 285
```

Fig. 1. Detection of Neurofibromin-interacting proteins (A), and homology analysis of amino acid sequences of p35 vs. rat DDAH (B). A: Brain proteins bound to the affinity columns, either immobilized GST–CTD or GST, were subjected to SDS–PAGE followed by silver staining. The molecular mass (kDa) is indicated on the left. The arrows indicate the positions of the specific binding proteins. B: Internal amino acid sequences of p35 identified are underlined in the complete amino acid sequence of rat DDAH [18].

the digested p35 with reverse-phase HPLC (C_8), they were subjected to amino acid sequencing. Six peptide sequences derived from p35 were determined. These were EALEK, LQLNIVEMK, XTNQXGAEIL, DYAVSTVPV, SFCSMAGPN, IMQQMS, and GHVLLHRTPE (Fig. 1B). All these identified sequences were found in the sequences of rat DDAH [18] (Fig. 1B). The calculated molecular mass of rat DDAH was 31 121 Da, which was close to the apparent molecular mass of p35 estimated by SDS–PAGE. We further confirmed that p35 is DDAH by immunoblot analysis using an antibody raised against rat DDAH [16]. Purified rat DDAH was used for a positive authentic control. An immunoreactive band was detected at 35 kDa in the eluates from the GST–CTD column subjected to the bovine brain extracts but not in the eluates from the GST column (Fig. 2A). The human DDAH (human DDAH has 98% homology to rat DDAH) has been recently cloned by Kimoto et al. [19]. DDAH is known as a regulator for MMA (N^G-monomethyl-L-arginine) and DMA (N^G,N^G-dimethyl-L-arginine), substrate analogues of NO synthase (NOS), and is thought to be a NOS/NO endogenous regulator, controlling the metabolism of MMA and DMA in a variety of cells and tissues [19]. The other neurofibromin-interacting proteins identified in this experiment will be described elsewhere.

3.2. Interaction of DDAH and neurofibromin in vitro and in vivo

To evaluate interaction of between DDAH and neurofibromin, bacterially expressed and purified recombinant DDAH was incubated with GSH beads, which immobilized either GST–CTD or GST. Recombinant DDAH were retained only on the GST–CTD beads but not on the GST beads (Fig. 2B). These results suggest the direct interaction of DDAH and neurofibromin in vitro. To address whether DDAH could also interact with endogenous neurofibromin, human neuroblastoma SH-SY5Y cell lysates were analyzed by the pull-down assay using GSH beads, which immobilized either GST–DDAH or GST. The neurofibromin (240 kDa) was precipitated only with the GST–DDAH beads, but not with the GST beads (Fig. 2C), suggestive of the apparent interaction of endogenous neurofibromin and DDAH. To ensure an interaction of neurofibromin and DDAH in vivo, cell extracts prepared from COS7 cells that overexpressed myc epitope-tagged DDAH (myc-DDAH) were precipitated with the antibody against myc-epitope (9E10). Immunoblot analysis of the immunoprecipitates using the anti-neurofibromin antibody revealed that endogenous neurofibromin was co-precipitated with myc-DDAH (Fig. 2D, lane 3). These results indicate that neurofibromin and DDAH form a complex in vivo as well as in vitro.

3.3. Specific binding sites of DDAH on neurofibromin

To determine which region of neurofibromin interacts with DDAH, various fragments of neurofibromin fused to GST (Fig. 3A) immobilized on GSH-beads were prepared, and reacted with myc-DDAH overexpressed in COS7 cells. As shown in Fig. 3B and C, myc-DDAH strongly interacted with GST–CTD 600, slightly with GST–CSRD and GST–

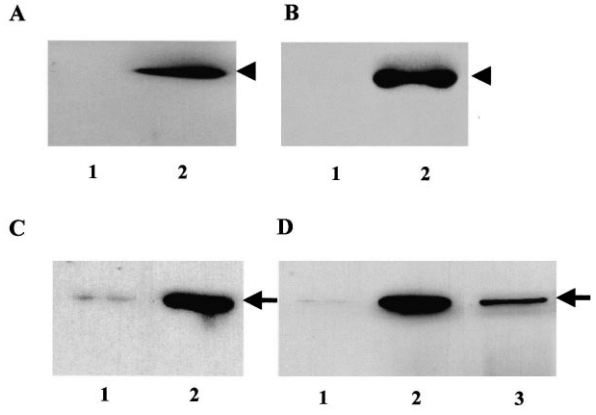


Fig. 2. Interaction of neurofibromin and DDAH. A: Immunoreactivity of anti-DDAH antibody against p35 in the brain proteins eluted from GST–CTD (lane 2) or GST column (lane 1). B: The recombinant DDAH purified from GST–DDAH after PreScission protease digestion was incubated with the GST–CTD (lane 2) or GST (lane 1) beads, and the interacted DDAH on each beads was detected with anti-DDAH antibody. The arrowheads indicate the position of DDAH. C: The lysates of SH-SY5Y cells were incubated with GST (lane 1) or GST–DDAH (lane 2) beads, and the interacted DDAH on each beads was detected with an anti-neurofibromin antibody. D: The cell lysates prepared from COS7 cells expressing myc-DDAH were immunoprecipitated with mouse IgG (lane 1), anti-NF1-GRD antibody (lane 2) or anti-myc antibody (lane 3). The immunoprecipitates were analyzed by immunoblotting probed with an anti-neurofibromin (D) antibody. The arrows indicate the position of endogenous neurofibromin.

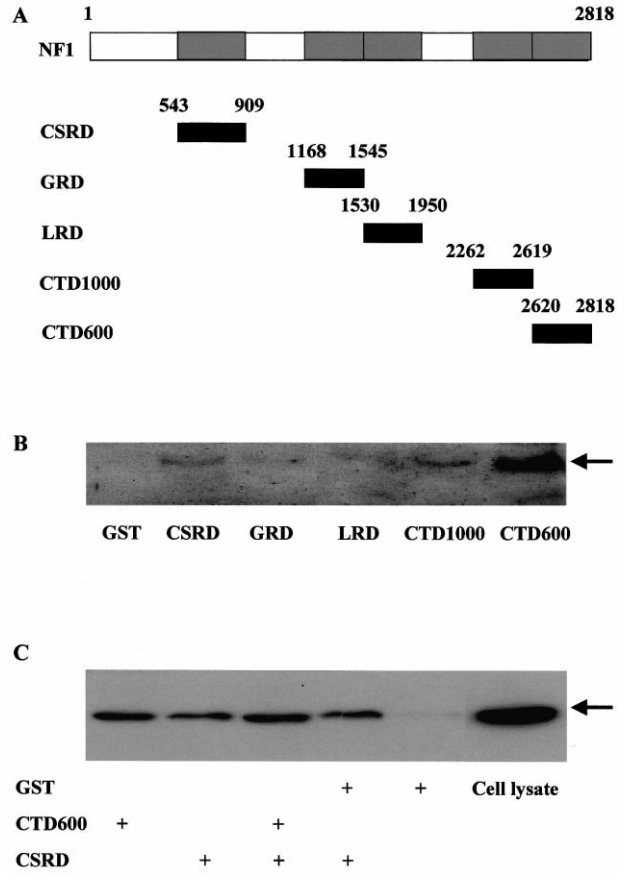


Fig. 3. Interaction of neurofibromin fragments with DDAH. A: The GST–neurofibromin fragments used are schematically represented. B, C: Myc-DDAH over expressed in COS7 cells were reacted with GST or GST fusion neurofibromin fragments (GST–CSRD, GRD, LRD, CTD1000, CTD600) immobilized onto GSH-agarose beads. Myc-DDAH bound on the beads was detected by immunoblotting probed with an anti-myc monoclonal antibody. The arrows indicate the position of myc-DDAH.

CTD 1000, but not with other domains or GST. These results suggest that DDAH could interact not only with CTD but also with CSRD. The DDAH-binding activity to both CSRD and CTD in their mixture was accountable without any additional increase or decrease (Fig. 3C), suggesting that CTD and CSRD interact independently with DDAH in this experimental condition.

3.4. *Effect of DDAH on PKA phosphorylation of neurofibromin*

Interestingly, the DDAH-binding regions of neurofibromin (CSRD, CTD) were found to coincidently contain the sites for PKA phosphorylation [11]. This prompted us to speculate that DDAH acts as a regulator for PKA phosphorylation of neurofibromin. To evaluate the significance of DDAH interaction with neurofibromin, we next analyzed the effect of DDAH on PKA phosphorylation of cellular neurofibromin purified from HeLa cells by an in vitro kinase reaction using PKA catalytic fragments. The results showed that neurofibromin was significantly phosphorylated by PKA, and the phosphorylation was increased in the presence of GST–DDAH in a dose-dependent manner (Fig. 4A). This PKA phosphorylation was completely inhibited by the presence of PKA inhibitor (data not shown). However, neither GST (Fig. 4A) nor BSA (data not shown) could increase neurofibromin phosphorylation. The maximum effect of GST–DDAH on neurofibromin phosphorylation was up to 12.5 times higher than that of GST or BSA (Fig. 4B). No effects of GST–DDAH on the increase of PKA phosphorylation on myelin basic protein (MBP), an authentic substrate used in typical PKA kinase assays, were observed (Fig. 5). These results strongly suggest that DDAH has no direct effects on PKA kinase activity, but up-regulates the PKA phosphorylation accessibility of neurofibromin.

Finally, to further evaluate the PKA phosphorylation of neurofibromin, we analyzed the phosphorylation region that would be affected by the DDAH interaction. The PKA phosphorylation was observed only on CSRD and CTD, and not on other domains such as GRD (Fig. 5A); consistent with the results reported by Izawa et al. [11]. However, the dose-dependent effect of DDAH on the increase of PKA phosphorylation was significantly observed on CSRD but little on CTD (Fig. 5). These results suggest that the interaction of DDAH with neurofibromin affects the accessibility of PKA phosphorylation, especially on CSRD.

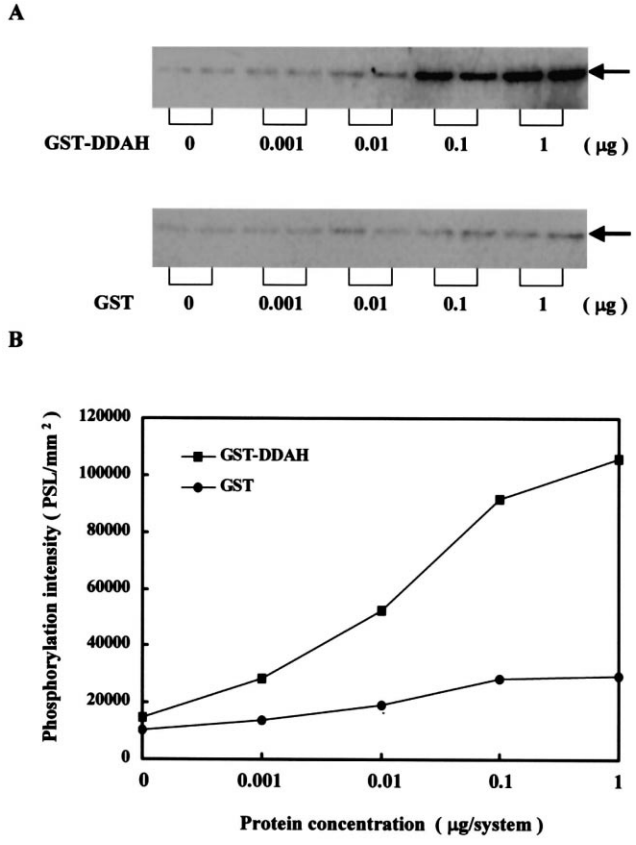


Fig. 4. Effects of DDAH on PKA phosphorylation of cellular neurofibromin. A: Cellular endogenous neurofibromin prepared from HeLa cell lysates was incubated with various concentrations of GST–DDAH or GST, and reacted with PKA catalytic subunits in the presence of [γ-^{32}P]ATP. The phosphorylated proteins were separated through 6% SDS–PAGE, and analyzed by autoradiography. The arrows indicate the position of neurofibromin. B: Relative intensities of phosphorylation rates of neurofibromin in each sample tested were calculated by Mac Bass, and plotted on the graph. Data represent mean of duplicate experiments, and the figure is a representative of three independent experiments.

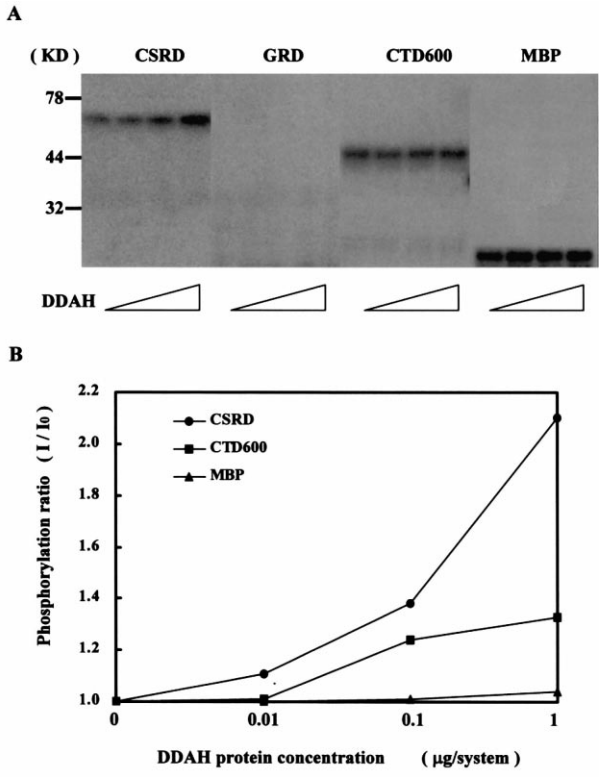


Fig. 5. Effects of DDAH on the PKA phosphorylation of CSRD, GRD, CTD600, and MBP. A: In vitro kinase reactions of PKA against GST fusion fragments CSRD, GRD, CTD600, and MBP were performed with various concentrations of GST–DDAH (lane 1, 0; lane 2, 10 ng; lane 3, 0.1 μg; lane 4, 1.0 μg/system). After the reaction was stopped, the phosphorylated proteins were analyzed by autoradiography. B: Relative intensity ratios (I/I_0) of PKA phosphorylation on GST–CSRD (●), CTD (■), and MBP (▲) (I/I_0; PKA phosphorylation intensity of sample reacted with DDAH (I)/PKA phosphorylation intensity of samples reacted without DDAH (I_0)). Data represent mean of duplicate experiments, and the figure is a representative of three independent experiments.

4. Discussion

In this study, we demonstrate a new neurofibromin-binding protein, DDAH, that regulates the specific PKA phosphorylation of neurofibromin. At the first step of this study, we identified DDAH as a cellular associating protein of the C-terminal region of neurofibromin, since we used the CTD affinity column to detect and purify it. Because most of the NF1 mutations cause the C-terminal truncated type of neurofibromin [7,12], it is suggestive that the C-terminal region of neurofibromin is crucial in the neurofibromin function, and the CTD-binding proteins such as DDAH may work as functional regulators of neurofibromin. Indeed, in this study, DDAH was demonstrated as a regulatory factor to increase the PKA accessibility of neurofibromin.

Surprisingly, several binding assays indicate that DDAH could associate not only with CTD but also with CSRD (Fig. 3). Fahsold et al. recently reported that unlike truncating mutations, missense or single amino acid deletion mutations were identified clustered in CSRD and GRD, and so, they assumed that CSRD is a second functional domain of the neurofibromin molecule [7]. The CSRD comprises amino acid residues 543–909, in which the ATP-binding domain and three potential PKA recognition sites (residues 586T, 818S, 876S) are contained [20]. The specific PKA phosphorylation of CSRD was confirmed in this study as well as in the previous report [11]. Under the presence of DDAH, CSRD was the most sensitive region for the PKA phosphorylation in the neurofibromin fragments (Fig. 5), although the binding activity of CSRD to DDAH was much weaker than that of CTD (Fig. 3). The increase of PKA phosphorylation on CSRD was independent of the PKA catalytic activation by DDAH, because DDAH could not increase PKA phosphorylation of other PKA substrates such as MBP (Fig. 5). These findings led us to conclude that neurofibromin-binding protein DDAH increases the PKA phosphorylation accessibility of the CSRD. Since a small region (residues 815–834) of the CSRD sequence similar to MAP-2 and tau were reported to associate with microtubules [21], it is speculated that the regulation of PKA phosphorylation by DDAH as well as DDAH interaction on this region might modulate the neurofibromin association to microtubules, or other cellular factors. The increase of the PKA phosphorylation of the purified cellular neurofibromin by DDAH was more significantly observed than that of GST–CSRD (Fig. 4). Thus, it is speculated that DDAH binding to CTD may cause alteration of the tertiary structure of the endogenous neurofibromin to increase the PKA accessibility on CSRD.

DDAH is an enzyme that is known to metabolize endogenous NOS inhibitors such as MMA and DMA to citrulline. The expression of DDAH protein was widely distributed in rat, particularly, in kidney, pancreas, liver, brain and aorta at high concentrations [16]. Methylarginines including MMA and DMA are now known to exist endogenously in immune cells and in the brain [16,22], and to inhibit NOS [23,24], suggesting that DDAH contributes to the control of NOS and NO in vivo. The subcellular localization of DDAH has not been revealed precisely, except that it is localized in the cytoplasm of kidney and brain cells that was analyzed immunocytochemically [25] and biochemically [26]. This was also evident in this study where DDAH was detected in the cytoplasmic fraction of rat and bovine brain (Figs. 1 and 2). On the other hand, evidence for the neurofibromin localization in cytoplasm has been reported [27–31] with some controversial pieces of results on its precise localization. We have detected the endogenous neurofibromin biochemically in the cytoplasmic fraction from rat and bovine brains, mouse fibroblasts, HeLa cells, and human neuroblastoma cells. These results suggest that neurofibromin and DDAH may co-localize at least in the cellular cytoplasmic region.

Drosophila NF1 acts as a possible activator of the *rut*-encoded adenylyl cyclase, and the mechanism of the NF1-dependent activation of cAMP pathway was suggested being essential for mediating *Drosophila* learning and memory [10]. Our results, however, suggest that activation of cAMP pathway regulates the neurofibromin function, since neurofibromin is specifically phosphorylated by PKA. Thus, it is speculated that neurofibromin function may involve at least two cAMP-related pathways, namely, the neurofibromin-dependent activation of cAMP pathway and the cAMP-dependent activation of the neurofibromin signal pathway. Further study would be able to make clear the precise mechanism of the PKA relation to the neurofibromin functions. Particularly, it would be important to study the effect of PKA phosphorylation and its regulation of neurofibromin on cellular Ras-MAPK signals.

Our preliminary study using embryonic fibroblast cells of *Nf1* gene knock-out mouse showed that DDAH catalytic activity to convert MMA to citrulline in those cells was significantly higher than that in *Nf1* gene wild type mouse cells (unpublished data). We speculate that the functional regulation of DDAH as a NO/NOS regulator may also have some links to neurofibromin function and neurofibromin-related signals, although those mechanisms have to be further analyzed more precisely.

While the functional significance of the PKA phosphorylation of neurofibromin is still unclear, the effect of the regulation of PKA phosphorylation on cellular neurofibromin by DDAH may be an important cellular event controlling physiological states. The results of this study provide new insight into the mechanisms of the functional regulation of cellular neurofibromin that is strongly linked to NF1-related tumor generation and various other pathogeneses.

Acknowledgements: We thank Dr. I. Izawa, Aichi Cancer Center Research Institute, and Dr. T. Nishi, Department of Neurosurgery, Kumamoto University School of Medicine for valuable discussions, and K. Vrinda, T. Arino and Y. Fukushima for secretarial assistance. This work was supported by grants for Brain Research, Cancer Research, and Kiban Research from the Ministry of Education, Science and Culture of Japan (N.A.), the Ministry of Health and Welfare of Japan (H.S.).

References

[1] Marchuk, D.A., Saulino, A.M., Tavakkol, R., Swaroop, M., Wallace, M.R., Andersen, L.B., Mitchell, A.L., Gutmann, D.H., Boguski, M. and Collins, F.S. (1991) Genomics 11, 931–940.
[2] Feldkamp, M.M., Gutmann, D.H. and Guha, A. (1998) Can. J. Neurol. Sci. 25, 181–191.
[3] Ducatman, B.S., Scheithauer, B.W., Piepgras, D.G. and Reiman, H.M. (1984) J. Neurooncol. 2, 241–248.
[4] Zoller, M.E., Rembeck, B., Oden, A., Samuelsson, M. and Angervall, L. (1997) Cancer 79, 2125–2131.
[5] Viskochil, D., Buchberg, A.M., Xu, G., Cawthon, R.M., Stevens, J., Wolff, R.K., Culver, M., Carey, J.C., Copeland, N.G. and Jenkins, N.A. et al. (1990) Cell 62, 187–192.
[6] Wallace, M.R., Marchuk, D.A., Andersen, L.B., Letcher, R., Odeh, H.M., Saulino, A.M., Fountain, J.W., Brereton, A., Nicholson, J. and Mitchell, A.L. et al. (1990) Science 249, 181–186.
[7] Fahsold, R., Hoffmeyer, S., Mischung, C., Gille, C., Ehlers, C., Kucukceylan, N., Abdel-Nour, M., Gewies, A., Peters, H., Kaufmann, D., Buske, A., Tinschert, S. and Nurnberg, P. (2000) Am. J. Hum. Genet. 66, 790–818.
[8] Boguski, M.S. and McCormick, F. (1993) Nature 366, 643–654.
[9] Guo, H.F., The, I., Hannan, F., Bernards, A. and Zhong, Y. (1997) Science 276, 795–798.
[10] Guo, H.F., Tong, J., Hannan, F., Luo, L. and Zhong, Y. (2000) Nature 403, 895–898.
[11] Izawa, I., Tamaki, N. and Saya, H. (1996) FEBS Lett. 382, 53–59.
[12] Korf, B.R. (1998) The NF1 genetic analysis consortium. In: Upadhyaya, M., Cooper, D.N. (Eds.) Neurofibromatosis type 1: from genotype to phenotype. BIOS Scientific Publishers, Oxford, pp. 57–63.
[13] Smith, D.B. and Johnson, K.S. (1988) Gene 67, 31–40.
[14] Frangioni, J.V. and Neel, B.G. (1993) Anal. Biochem. 210, 179–187.
[15] Ogawa, T., Kimoto, M. and Sasaoka, K. (1989) J. Biol. Chem. 264, 10205–10209.
[16] Kimoto, M., Tsuji, H., Ogawa, T. and Sasaoka, K. (1993) Arch. Biochem. Biophys. 300, 657–662.
[17] Yamamoto, T., Matsui, T., Nakafuku, M., Iwamatsu, A. and Kaibuchi, K. (1995) J. Biol. Chem. 270, 30557–30561.
[18] Kimoto, M., Sasakawa, T., Tsuji, H., Miyatake, S., Oka, T., Nio, N. and Ogawa, T. (1997) Biochim. Biophys. Acta 1337, 6–10.
[19] Kimoto, M., Miyatake, S., Sasagawa, T., Yamashita, H., Okita, M., Oka, T., Ogawa, T. and Tsuji, H. (1998) Eur. J. Biochem. 258, 863–868.
[20] Maru, Y. and Witte, O.N. (1991) Cell 67, 358.
[21] Gregory, P.E., Gutmann, D.H., Mitchell, A., Park, S., Boguski, M., Jacks, T., Wood, D.L., Jove, R. and Collins, F.S. (1993) Somat. Cell Mol. Genet. 3, 265–274.
[22] Ueno, S., Sano, A., Kotani, K., Kondoh, K. and Kakimoto, Y. (1992) J. Neurochem. 6, 2012–2016.
[23] Hibbs Jr., J.B., Vavrin, Z. and Taintor, R.R. (1987) J. Immunol. 138, 550–565.
[24] MacAllister, R.J., Parry, H., Kimoto, M., Ogawa, T., Russell, R.J., Hodson, H., Whitley, G.S. and Vallance, P. (1996) Br. J. Pharmacol. 119, 1533–1540.
[25] Tojo, A., Welch, W.J., Bremer, V., Kimoto, M., Kimura, K., Omata, M., Ogawa, T., Vallance, P. and Wilcox, C.S. (1997) Kidney Int. 52, 1593–1601.
[26] Nakagomi, S., Kiryu, S.S., Kimoto, M., Emson, P.C. and Kitama, H. (1999) Eur. J. Neurosci. 11, 2160–2166.
[27] Gutmann, C.H. and Collins, F.S. (1992) Ann. Neurol. 31, 555–561.
[28] Gregory, P.E., Gutmann, D.H., Mitchell, A., Park, S., Boguski, M., Jacks, T., Wood, D.L., Jove, R. and Collins, F.S. (1993) Somat. Cell Mol. Genet. 19, 265–274.
[29] Daston, M.M., Scrable, H., Nordlund, M., Sturbaum, A.K., Nissen, L.M. and Ratner, N. (1992) Neuron 8, 415–428.
[30] Nordlund, M., Gu, X., Shipley, M.T. and Ratner, N. (1993) J. Neurosci. 13, 1588–1600.
[31] Roudebush, M., Slabe, T., Sundaram, V., Hoppel, C.L., Golubic, M. and Stacey, D.W. (1997) Exp. Cell Res. 236, 161–172.