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PKA phosphorylation and 14-3-3 interaction regulate the function of neurofibromatosis type I tumor suppressor, neurofibromin

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Abstract **Neurofibromin, a neurofibromatosis type I (NF1) tumor suppressor gene product, has a domain acting as a GTPase activating protein and functions in part as a negative regulator of Ras. Loss of neurofibromin expression in NF1 patients is associated with elevated Ras activity and increased cell proliferation. Therefore, regulation of the function of neurofibromin is heavily involved in cell growth and differentiation. In the present study, we identified a novel cellular neurofibromin-associating protein, 14-3-3, which belongs to a highly conserved family of proteins that regulate intracellular signal transduction events in all eukaryotic cells. The interaction of 14-3-3 is mainly directed to the C-terminal domain (CTD) of neurofibromin, and the cAMP-dependent protein kinase (PKA)-dependent phosphorylation clustered on CTD-Ser (2576, 2578, 2580, 2813) and Thr (2556) is required for the interaction. Interestingly, the increased phosphorylation and association of 14-3-3 negatively regulate the function of neurofibromin. These findings indicate that PKA phosphorylation followed by 14-3-3 protein interaction may modulate the biochemical and biological functions of neurofibromin.**

Key words: Neurofibromatosis type I; Neurofibromin; cAMP-dependent protein kinase; Tumor suppressor; 14-3-3 protein; GTPase activating protein

1. Introduction

Neurofibromatosis type I (NF1), or von Recklinghausen neurofibromatosis, is one of the most common autosomal dominantly inherited disorders, with an incidence of approximately 1 in 3500 individuals [1], and is characterized by the development of benign tumors of the peripheral nervous system and increased risk of developing malignancies [2–5]. The phenotype of NF1 is highly variable with several organ systems being affected, including bones, skin, irises and central nervous system, and manifests as neurofibroma, gliomas and learning disabilities [3]. The *NF1* gene was identified by positional cloning and found to encode a large 250-kDa cytoplasmic protein product, neurofibromin. Sequence analysis of neurofibromin revealed a region of homology with the Ras GTPase activating protein (GAP) family, including vertebrate p120-GAP, yeast *ira1* and *ira2*, and *Drosophila* Gap1 [2]. Ras-GAPs are considered to attenuate signaling from Ras, thus blocking the transmission of signals leading to increased growth or differentiation [6].

Since the Ras-GAP domain of neurofibromin comprises only 10% of the whole polypeptide, we speculated that other regions of this protein are crucial for modulating cell growth or other processes related to NF1-related disease. In *Drosophila*, homozygous null mutation of the *NF1* gene causes a reduction in body size, which is an obvious sign of perturbed Ras-mediated signaling, and this phenotype can be rescued by expression of activated cAMP-dependent protein kinase (PKA) [7]. Neurofibromin and PKA thus appear to interact in a pathway that controls the growth of *Drosophila*. Additionally, we previously reported that neurofibromin is constitutively phosphorylated and that the specific phosphorylation lies on the cysteine/serine-rich domain (CSRD) and the C-terminal domain (CTD) of neurofibromin [8]. In vitro and in gel kinase assays suggest that PKA is a candidate for the neurofibromin kinase. All these observations led to the hypothesis that some NF1-associated proteins, which are phosphorylation-dependent, may have regulatory effects on the function of neurofibromin. Searching for NF1-associated proteins is therefore of particular interest as it may lead to the identification of novel cellular components that play important roles in Ras regulation, and should also further our understanding of the mechanism of NF1-related pathogenesis.

In a recent report, we sought to identify a cellular neurofibromin-interacting protein from a bovine brain cytosolic fraction using affinity column-immobilized CTD of neurofibromin, and characterized it as N^G,N^G-dimethylarginine dimethylaminohydrolase (DDAH), which is known as a cellular NO/NO synthase regulator [8]. DDAH increased PKA phosphorylation accessibility of neurofibromin via the CSRD and CTD [8], suggesting that phosphorylation of neurofibromin is regulated by its associating proteins and controls the cellular function of neurofibromin.

In this study, we use the same approach and identify 14-3-3 as another CTD binding protein. PKA-mediated phosphorylation of neurofibromin promotes association with 14-3-3.

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Abbreviations: NF1, neurofibromatosis type I; GAP, GTPase-activity protein; PKA, cAMP-dependent protein kinase; CSRD, cysteine/serine-rich domain; CTD, C-terminal domain; GRD, GAP-related domain

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Furthermore, we demonstrate that 14-3-3 association negatively regulates NF1-GAP activity by using a newly developed NF1-GAP assay method. These findings suggest that 14-3-3 modulates the biochemical and biological functions of neurofibromin through PKA-mediated phosphorylation.

2. Materials and methods

2.1. Preparation of glutathione S-transferase (GST) fusion proteins

We generated four GST-neurofibromin domain fusion proteins corresponding to sequences of residues 543–909, 1168–1530, 1545–1950, and 2260–2818 of neurofibromin, which were designated CSRD, GAP-related domain (GRD), leucine repeat domain (LRD), and CTD, respectively [9]. GST-14-3-3η was produced by polymerase chain reaction (PCR) amplification and cDNA cloning into the pGEX-3X vector as described [10]. Expression and purification of each GST fusion protein were performed according to our previously described protocol [8].

2.2. Plasmid construction

The cDNA for myc-tagged 14-3-3η was generated by PCR amplification as described [10]. The PCR fragment was digested with *Mlu*I and *Bam*HI, and then inserted into the cloning site of myc tag vector pMUM1. The myc-tagged 14-3-3η cDNA was then digested with *Hin*dIII and *Xba*I, and inserted into the mammalian expression vector pcDNA3 (Invitrogen) [10].

2.3. Antibodies

Antibodies against the C-terminus of neurofibromin [anti-GRP (D)] and 14-3-3 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The anti-myc-tag monoclonal antibody was purchased from MBL (Nagoya, Japan). The anti-GST goat antibody was purchased from Amersham. An anti-NF1-GAP antibody was generated as previously described [11].

2.4. Preparation of cytosolic fraction from rat brain

Cytosolic fraction of rat brain was prepared as previously described [8]. In brief, rat brain was homogenized with lysis buffer A (20 mM Tris–HCl, pH 7.5, 1 mM EDTA, 5 mM $MgCl_2$, 150 mM NaCl, and 0.1% NP-40), containing 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 Na_3VO_4 in a Dounce 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 and identification of binding proteins from rat brain cytosolic fraction by GST-CTD affinity chromatography

GST-CTD fusion protein immobilized on GSH-agarose was packed into a column and equilibrated with buffer B (30 mM Tris–HCl, pH 7.5, 1 mM EDTA, 5 mM $MgCl_2$, and 1 mM dithiothreitol (DTT)). Rat brain cytosolic fraction, pre-cleared by passing through a GSH column, was then loaded onto the GST-CTD column. After washing the column with buffer B, the proteins bound to the column were eluted by the addition of buffer C (buffer A containing 0.5 M NaCl), and subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), followed by silver staining. For identification of the bound protein, the protein band detected in SDS–PAGE was cut and sliced into small pieces. The gels were then desalted in 50% methanol and 100% acetonitrile, dried in a Speed-Vac, and soaked again with digestion buffer containing trypsin. After overnight protein digestion, the supernatant was again desalted by Zip tips (Millipore Corporation, Bedford, MA, USA) and used for sequence analysis by nano infusion ESI tandem mass spectrometry (Qstar pulsar i, Applied Biosystems Japan). The amino acid sequence obtained from mass-fit and product ion analysis was used to search for sequence similarity against the PIR International database and/or SwissProt using MASCOT search engine. Alternatively, the proteins separated with the SDS–PAGE were electroblotted onto a nitrocellulose membrane, and then probed with the anti-14-3-3 antibodies. After reaction with the horseradish peroxidase (HRP)-conjugated secondary antibodies, the reacted protein bands on the membrane were visualized by an ECL detection system (Amersham).

2.6. Cell culture and transfection

COS-7 cells were cultured in Dulbecco's modified Eagle's medium nutrient mixture F-12 Ham (Sigma, St. Louis, MO, USA) with 10% fetal bovine serum. To transiently express myc-14-3-3η, pcDNA3-myc-14-3-3η was transfected into COS-7 cells with Fugene 6 (Roche) according to the manufacturer's protocol. PC12 cells were grown on collagen-coated tissue culture plates in RPMI 1640 medium supplemented with 10% heat-inactivated horse serum and 5% fetal bovine serum, as described previously [11]. PC12 cells permanently expressing myc-14-3-3η (termed PC12 14-3-3η cells) were prepared as described [10].

2.7. Immunoprecipitation assays

Immunoprecipitation was conducted on lysates of COS-7 or PC12 cells transfected with or without pcDNA3 myc-14-3-3η. Cells were lysed with buffer A containing 1 mM p-ABSF, 5 μg/ml aprotinin, 1 μg/ml pepstatin A, 1 μg/ml leupeptin, 10 mM NaF, 2 mM Na_3VO_4, and 1 μM okadaic acid, and passed through a 25-G syringe 15 times. The lysates were centrifuged at 14 000 rpm for 20 min at 4°C, the protein concentrations of the supernatant were determined using the BCA protein assay reagent (Pierce). The lysates were normalized in the same protein concentration, mixed with 4 μg of anti-myc antibody (MBL) or anti-neurofibromin [GRP (D)] (Santa Cruz) and protein G plus/protein A agarose (Oncogene Science), and then rocked for 2 h at 4°C. Immunoprecipitates were washed with buffer A and boiled in 30 μl of 2×SDS loading buffer. Samples were subjected to SDS–PAGE and electro-transferred onto membranes, and then probed with anti-myc or anti-neurofibromin [GRP (D)]. After reaction with the HRP-conjugated secondary antibodies, the immunoreactive proteins on the membrane were visualized by an ECL detection system (Amersham).

2.8. GST pull-down assay for the interaction of various GST fusion fragments of neurofibromin

COS-7 cells were transfected with or without pcDNA3-myc-14-3-3η, and lysed with cell lysis buffer as described above. The cell lysates were incubated with GST alone or various GST-neurofibromin domain fusion proteins immobilized to GSH-agarose for 2 h at 4°C. The protein complexes with agarose beads were recovered by centrifugation, washed three times with cell lysis buffer, and subjected to SDS–PAGE followed by immunoblotting analysis as described above.

2.9. Phosphorylation assay of GST-CTD by PKA

GST-CTD fusion protein (100 μg) was incubated with 50 μl of 50% GSH-agarose in phosphate-buffered saline (PBS) for 2 h at 4°C. The GST-CTD fusion protein immobilized on GSH-agarose was incubated with or without 30 U PKA (Sigma) in a total volume of 100 μl kinase assay buffer (20 mM Tris–HCl (pH 7.4), 10 mM $MgCl_2$, 50 mM KCl, and 0.1 mM ATP) at 37°C for 30 min. The reaction was terminated by washing with cold PBS containing 0.1 mM DTT. Then the immobilized protein was incubated with cell lysates of PC12 14-3-3η in the presence or absence of 2 μM PKA-inhibitor peptide (PKI-[6–22] amide, Sigma).

2.10. Assay for NF1-GAP activity

The GAP activity assay was performed as previously described [11]. In brief, cell lysates in the presence or absence of anti-NF1-GAP antibody were incubated with GST-H-Ras preincubated with [γ-^{32}P]-GTP, and the sample was passed through a nitrocellulose filter (0.45 μm; Schleicher and Schuell). After washing, the radioactivity of the filter was counted. Specific NF1-GAP activity was obtained from the difference between the sample radioactivities after the reaction with and without anti-NF1-GAP IgG. We also analyzed the binding efficiency of NF1 protein to Ras-GTP for evaluating the GAP activity of endogenous NF1 protein. To prepare the $GTP\gamma S$- (or $GDP\beta S$-) bound form of Ras, 200 μg of GST-H-Ras was preincubated with 80 μl of 50% GSH-agarose in PBS for 2 h at 4°C in 200 μl of GAP assay buffer (0.25 M Tris–HCl, pH 8.0, 25 mM EDTA, 0.25 M $MgCl_2$ and 0.25 M DTT), and washed twice with the same buffer, and then once with GST loading buffer (20 mM Tris–HCl, pH 7.5, 5 mM $MgCl_2$, 20 mM EDTA, 100 mM NaCl, 10% glycerol, and 2.5 mg/ml bovine serum albumin). The GST-H-Ras beads were then incubated with 10 μl of 10 mM $GTP\gamma S$ or $GDP\beta S$ for 30 min at 37°C, and washed three times with buffer A. Cell lysates of PC12 or PC12 14-3-3η cells were incubated with the GST-Ras-$GTP\gamma S$ or GST-Ras-$GDP\beta S$ for 2 h at 37°C with rocking, and washed three times with buffer A. The

bound form of cellular neurofibromin (active form) on the beads was separated with the SDS–PAGE, electroblotted onto the membrane, and detected with the anti-neurofibromin [GRP (D)] antibody, followed by reaction with the HRP-conjugated secondary antibodies and visualization by an ECL detection system (Amersham). To study the effect of PKA phosphorylation on GAP activity of cellular neurofibromin, 50 μM forskolin was used to treat PC12 14-3-3η cells for 20 min at 37°C, and then the binding efficiency of neurofibromin with 14-3-3η or with GST-H-Ras $GTP_{\gamma}S$ was compared with that of cells not treated with forskolin, as described above.

2.11. Site-directed mutagenesis of GST-CTD

Mutants of GST-CTD were prepared as described [11]. Briefly, GST-CTD was used as a template for PCR amplification with Pfu DNA polymerase (Stratagene).

For mutations of the 14-3-3 binding motifs, we used the following primers: S2576A-sense, 5′-CGTAAAGTTGCAGTGTCTGAATCAAA-3′, S2576A-antisense, 5′-TGATTCAGACACTGCAACATTTTTACGTAA-3′; S2578A-sense, 5′-CGTAAAGTTGCAGTGGCCGAATCA-3′, S2578A-antisense 5′-TGATTCGGCCACTGCAACTTTACG-3′, S2580A-sense, 5′-CGTAAAGTTGCAGTGGCCGAAGCAAA-3′, S2580A-antisense, 5′-GAGAACATTTGCTTCGGCCACTGCAA-3′. For mutations of the PKA potential phosphorylation sites we used the following primers: T2556A-sense, 5′-GTTTCAGTGGCCGAATCAAATGTTCTC-3′, T2556A-antisense, 5′-GAGAACATTTGATTCGGACACTGAAAC-3′; T2813A-sense, GTTTCAGTGTCTGAAGCAAATGTTCTC-3′, T2813A-antisense, 5′-GAGAACATTTGCTTCAGACACTGAAAC-3′. The sequences of the mutant plasmids were confirmed using a BigDye Terminator sequence kit (Applied Biosystems) and an ABI Prism 310 Genetic Analyzer (Applied Biosystems).

3. Results

3.1. Identification of 14-3-3 as a neurofibromin-associating protein

To isolate proteins that physically associate with neurofibromin, rat brain cytosolic fraction was loaded on a GSH column to which GST-CTD or GST was immobilized, and the proteins bound to the columns were dissociated with high salt elution buffer. As shown in Fig. 1A, ten proteins with molecular masses between 175 and 10 kDa were reproducibly obtained from the GST-CTD column, but none of them was detected from the GST column. Analysis of the protein band with a molecular mass of 31 kDa (p31) by mass spectrometry revealed that six specific $[M]^{+}$ signals selected in the tryptic peptide masses obtained from p31 (1189.6, 907.5, 669.3, 615.3, 1205.6, 1047.3) were well matched with the calculated masses of tryptic fragments of rat 14-3-3 protein β/α/γ/η/ζ/δ (protein kinase C inhibitor protein-1). Two tryptic fragment masses with 1205.6 and 1315.6 were sequenced and identified as the 14-3-3 γ peptides of 217-226 (DSTLIMQLLR including an oxidation form of ^{219}Met) and 132-143 (YLAEVATGEK), respectively. Identification of p31 as 14-3-3 protein was further confirmed with Western blot analysis using anti-14-3-3 antibodies (Fig. 1B).

Biochemical interactions between 14-3-3 and endogenous neurofibromin were tested by GST pull-down assay. Rat brain lysates were incubated with GST or GST-14-3-3η and precipitated with GSH beads. Endogenous neurofibromin significantly coprecipitated with GST-14-3-3η but not with GST protein (Fig. 2). These results indicate that GST-14-3-3η interacts with endogenous neurofibromin.

3.2. CTD of neurofibromin interacts with 14-3-3

To determine the 14-3-3 binding site of neurofibromin, various fragments of neurofibromin fused to GST were immobilized on GSH beads, and then incubated with myc-tagged 14-3-3η (myc-14-3-3η) overexpressed in COS-7 cells. This pull-down assay (Fig. 3) showed that myc-14-3-3η strongly interacts with GST-CTD, but not with other domains or GST alone. We therefore concluded that 14-3-3η interacts only with the CTD of neurofibromin.

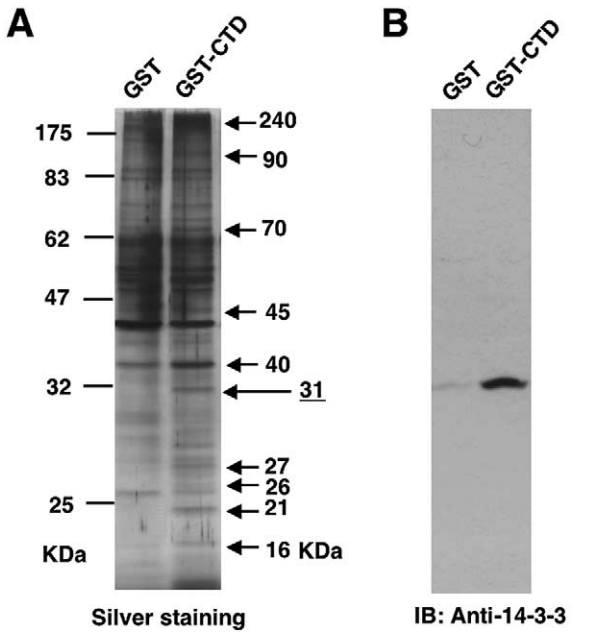

Fig. 1. Identification of neurofibromin-associating proteins. A: Rat brain proteins bound to the immobilized GST or GST-CTD are analyzed by SDS–PAGE (12% PAGE gel, silver staining). The molecular weight markers are shown in kDa on the left and arrows indicate the positions of the specific binding proteins. The 31-kDa protein indicated corresponds to 14-3-3. B: Rat brain proteins bound to the immobilized GST or GST-CTD are immunoblotted with anti-14-3-3 antibodies.

3.3. Phosphorylation of neurofibromin enhances association with 14-3-3

The 14-3-3 family proteins bind to various phosphorylated proteins, such as Raf, Bcr, BAD, etc., via phosphorylated serine residues [12]. Given that neurofibromin has been reported to be constitutively phosphorylated on its serine/threonine residues [9], we speculated that phosphorylation of neurofibromin may affect the interaction with 14-3-3. To examine this possibility, we analyzed the interaction of myc-14-3-3η with cellular endogenous neurofibromin. Myc-14-3-3η overexpressed in COS-7 cells was solubilized in the presence or absence of phosphatase inhibitors (10 mM NaF, 1 μM okadaic acid). Lysates were immunoprecipitated with anti-myc or non-immune mouse IgG, subjected to SDS–PAGE and immunoblotted with anti-GRP (D). Neurofibromin precipitation with myc-14-3-3η in the presence of the phosphatase inhibitors increased by up to four times compared with that in their absence (Fig. 4). Reciprocally, immunoprecipitates with anti-NF1-GRP (D) contained five times higher amounts of myc-14-3-3η in the presence of the phosphatase inhibitors compared with that in their absence. These results suggest that phosphorylation of neurofibromin enhances interaction with 14-3-3.

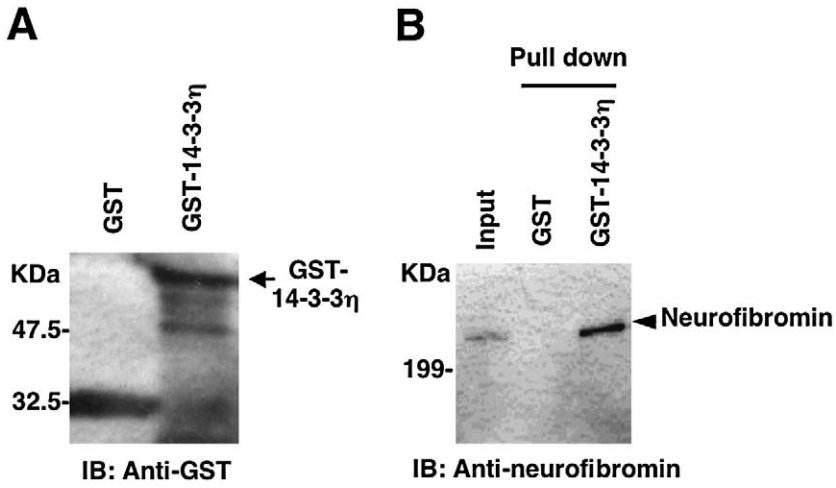

Fig. 2. GST pull-down assay showing that 14-3-3η interacts with endogenous neurofibromin in rat brain. Cell lysates of rat brain were incubated with GST or GST-14-3-3η immobilized onto GSH-agarose for 2 h at 4°C with rocking. A: GST or GST-14-3-3η immobilized onto the agarose beads is analyzed by immunoblotting with anti-GST. B: Neurofibromin bound on the beads is detected by immunoblotting with anti-neurofibromin antibody [GRP (D)]. The arrowhead indicates the endogenous neurofibromin. The molecular weight markers are shown in kDa on the left.

3.4. *Phosphorylation of neurofibromin by PKA activates its binding to 14-3-3*

It was previously reported that PKA is responsible for the constitutive phosphorylation of neurofibromin [9]. Since the experiment shown in Fig. 4 suggested that 14-3-3η binding to neurofibromin potentially increases in a neurofibromin phosphorylation-dependent manner, we tested the possibility that the phosphorylation of neurofibromin by PKA may involve the enhancement of interaction between 14-3-3η and neurofibromin. We previously reported that PKA catalytic subunit phosphorylates the immunoprecipitated native neurofibromin as well as the GST-CTD fusion proteins. To assess whether the PKA phosphorylation of GST-CTD fusion protein activates its binding to 14-3-3, we performed binding assay between 14-3-3η and GST-CTD in the presence or absence of PKA catalytic fragments. As shown in Fig. 5, 14-3-3η bound to GST-CTD fusion protein phosphorylated by PKA catalytic fragments much more efficiently than that unphosphorylated. Furthermore, association of the endogenous 14-3-3η with GST-CTD was inhibited by the addition of the inhibitor protein of cAMP-dependent protein kinase (PKI) (Fig. 5). These results suggest that PKA phosphorylation of CTD is important for regulation of the interaction between neurofibromin and 14-3-3.

3.5. *Binding of 14-3-3η negatively regulates the neurofibromin GAP activity*

Next, we examined whether the phosphorylation-mediated binding of 14-3-3η regulates the GAP activity of neurofibromin. To measure the GAP activity of endogenous neurofibromin, we first established a pull-down assay system as described in Section 2. Cell lysates were mixed with GST-H-Ras-GTP$_\gamma$S or GST-H-Ras-GDP$_\beta$S that was immobilized on GSH beads, and the bound fractions on beads were identified by immunoblotting using antibody against neurofibromin. As shown in Fig. 6A, neurofibromin could bind to GST-H-Ras-

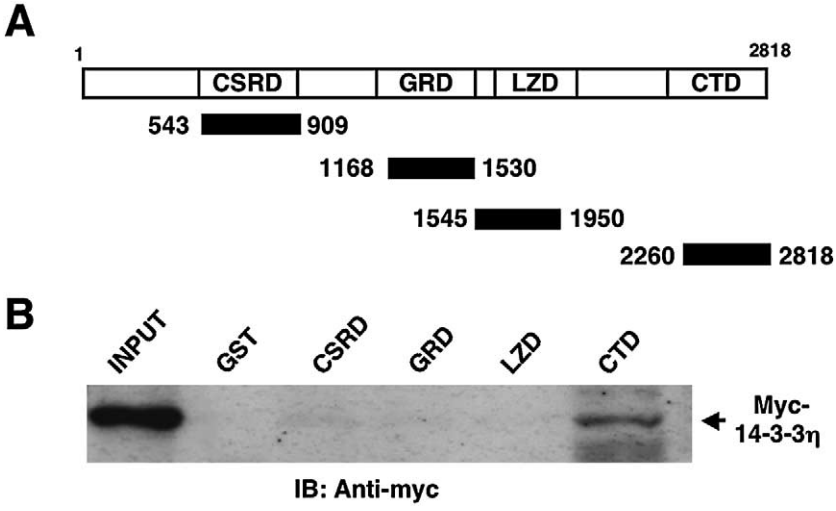

Fig. 3. Determination of 14-3-3η binding domain in neurofibromin. A: Schematic diagram of four neurofibromin domains. B: Myc-14-3-3η was transfected into COS-7 cells, and the cell lysates were incubated with GST or GST-neurofibromin domain fusion proteins (GST-CSRD, GRD, LRD, and CTD) immobilized onto GSH-agarose. Myc-14-3-3η bound on the beads was immunoblotted with anti-myc antibody. The arrow indicates the position of myc-14-3-3η.

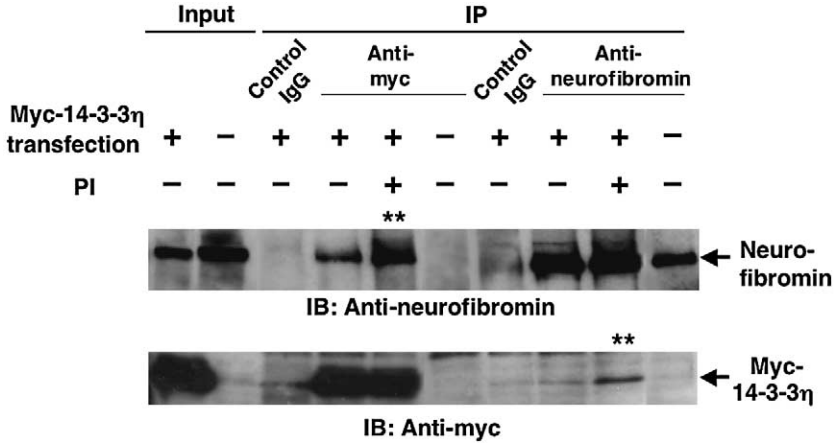

Fig. 4. Effects of phosphorylation of neurofibromin on the interaction with 14-3-3η. COS-7 cells transfected with or without myc-14-3-3η were lysed with lysis buffer A in the presence or absence of phosphatase inhibitors (phosphatase inhibitor (PI): 10 mM NaF, 1 μM okadaic acid). Cell extracts are either immunoprecipitated with anti-myc antibody or anti-neurofibromin [GRP (D)], resolved by SDS–PAGE, and immunoblotted with anti-neurofibromin [GRP (D)] or anti-myc antibody, respectively. The arrows indicate the positions of endogenous neurofibromin (upper panel) and transfected myc-14-3-3η (lower panel). Note that interaction between myc-14-3-3η and neurofibromin was enhanced by the presence of phosphatase inhibitors (**). Similar findings were observed in five independent experiments.

$GTP_\gamma S$ in a dose-dependent manner, but not to GST-H-Ras-$GDP_\beta S$. To confirm that this binding activity corresponds to that obtained from the original GAP assay, the same cell lysates were subjected to the GAP filtration assay using specific NF1 antibody, as described [11]. As shown in Fig. 6B, the NF1-GAP activity significantly increased in a cell extract dose-dependent manner, and this was correlated to the results obtained from the pull-down assay using GST-H-Ras-$GTP_\gamma S$ (Fig. 6A,B). These results indicate that the amount of neurofibromin bound to GST-H-Ras-$GTP_\gamma S$ could reflect the pool of neurofibromin that is available to act on Ras, and is therefore an indirect measurement of cellular NF1-GAP activity, as suggested by several GAP studies reported elsewhere [13–15]. By using this system, we found that neurofibromin GAP is higher in PC12 cells than that in PC12 14-3-3η cells, although the total amount of neurofibromin in the lysates is equal in both cells (Fig. 6C). This was also confirmed by the filtration GAP activity assay (Fig. 6D). These results suggest that 14-3-3 could negatively regulate neurofibromin function, perhaps by affecting its ability to interact with Ras-GTP.

To further verify whether the PKA phosphorylation of neurofibromin enhances 14-3-3η binding to neurofibromin and decreases the GAP activity of neurofibromin in living cells, PC12 14-3-3η cells were treated with forskolin, an activator of PKA, and its effects on the 14-3-3 interaction and GAP activity in vivo analyzed. Forskolin treatment enhanced the interaction of the endogenous neurofibromin with 14-3-3η by $320 \pm 5.1\%$, and suppressed the ability of neurofibromin to bind Ras-GTP by $63.7 \pm 5.4\%$ compared to the control treatment (Fig. 6E,F). These results strongly suggest that neurofibromin GAP activity is regulated via PKA phosphorylation and 14-3-3 binding on CTD of neurofibromin.

3.6. *Multiple substitutions of the potential phosphorylation sites for 14-3-3 binding on CTD significantly affects the interaction of 14-3-3 with neurofibromin*

To find out the phosphorylated sites of CTD affecting the binding between 14-3-3 and neurofibromin, several CTD mutant proteins were prepared whose putative phosphorylation sites of PKA or 14-3-3 binding motif (2576 Ser, 2578 Ser, 2580 Ser, 2556 Thr, and 2813 Ser) were mutated to Ala (point, double, triple, and quintuple mutations) (Fig. 7), and tested for their binding abilities. As shown in Fig. 7, none of the point/double/triple mutations (lanes 1–8) affected the 14-3-3η binding with GST-CTD. However, only the quintuple mutations on CTD significantly affected the binding activity with 14-3-3 protein (Fig. 7, lanes 9 and 10). These results indicate that a multiple cluster of phosphorylation on CTD (2576 Ser, 2578 Ser, 2580 Ser, 2554 Thr, and 2813 Ser) is necessary for the binding of 14-3-3 and neurofibromin.

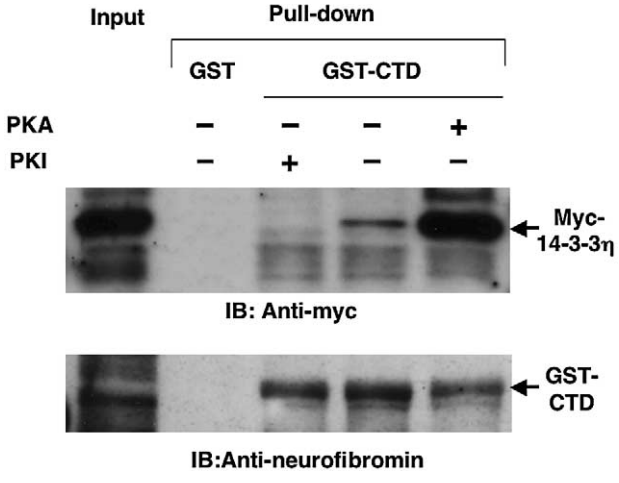

Fig. 5. In vitro phosphorylation of GST-CTD fusion protein by PKA catalytic subunit activates its binding to 14-3-3η. GST-CTD fusion protein (100 μg) was incubated with GSH-agarose for 2 h at 4°C. The immobilized GST-CTD was then incubated with or without 30 U PKA (Sigma) in a total volume of 100 μl kinase assay buffer at 37°C for 30 min. The treated GST-CTD protein was mixed with cell lysates of PC12 14-3-3η in the presence or absence of 2 μM PKI[6–22] amide (Sigma). The amount of myc-14-3-3η binding to GST-CTD fusion protein was detected by immunoblotting using anti-myc antibody. Similar findings were observed in five independent experiments.

4. Discussion

Neurofibromin, with a predicted size of 2818 amino acids, holds a central GRD of 360 amino acids. Besides GRD, the *NF1* gene product has no clearly defined functional domains [16]. Given that most C-terminal mutations result in production of the C-terminally truncated form of neurofibromin, it can be speculated that the C-terminus of NF1 is crucial for modulating functions of neurofibromin. Here, we identified that 14-3-3 interacts with the CTD of neurofibromin in vitro and in vivo, and negatively regulates the ability of neurofibromin to interact with Ras-GTP, consequently affecting its GAP activity.

We previously found that PKA is responsible for the constitutive phosphorylation of neurofibromin [8]. Three lines of evidence obtained in this study suggest that the PKA-dependent phosphorylation of neurofibromin allows binding of 14-3-3, which consequently inhibits the GAP activity of neurofibromin. First, the phosphorylation of GST-CTD by PKA activated its binding to 14-3-3η and this interaction is abolished by PKI treatment. Second, the GAP activity of neurofibromin was lower in 14-3-3η-overexpressed PC12 cells than in parental PC12 cells. Third, treatment with forskolin, an activator of adenylate cyclase, enhanced the interaction between endogenous neurofibromin and 14-3-3η, and suppressed the binding activity of neurofibromin to Ras-GTP. We believe that this is the first evidence that GAP activity is regulated by PKA phosphorylation and followed by 14-3-3 binding.

The 14-3-3 proteins, in many but not all cases, bind to phosphorylated form proteins. Phosphoserine binding motifs for 14-3-3 proteins like RSXpSXP and RXXpSXP have been identified by Rittinger et al. using oriented phosphopeptide libraries [14]. Additionally, a novel 14-3-3 binding motif displaying $RX_{1-2}SX_{2-3}S$ has also been identified in Cbl protein

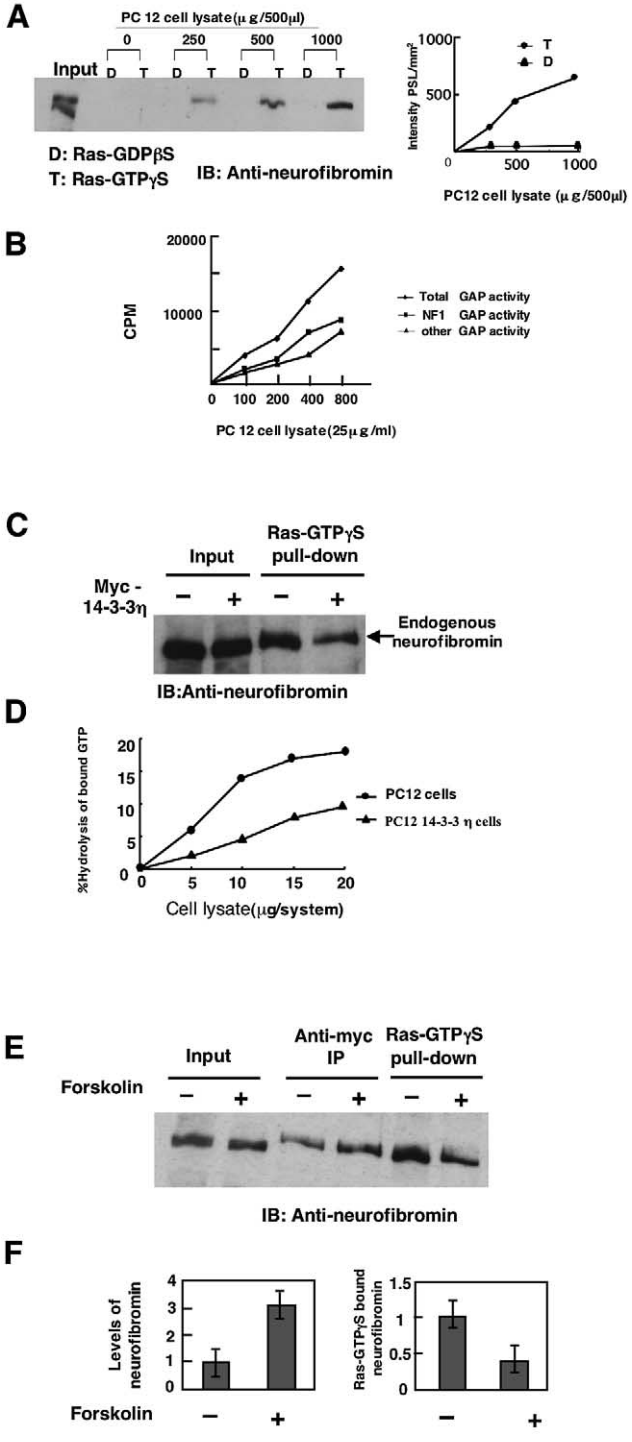

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Fig. 6. 14-3-3η and forskolin negatively regulate NF1-GAP activity. A: GAP activity assay of neurofibromin in PC12 cells by the GST-Ras pull-down method. PC12 cells were lysed with buffer A, and indicated amounts of the cell lysates were applied to the beads immobilized with GST-Ras-GTP$_\gamma$S or GST-Ras-GDP$_\beta$S and the bound neurofibromin on the beads was separated by the SDS–PAGE and detected by immunoblotting using anti-GRP (D) as described in Section 2. A representative result is shown in the left panel, and the averaged intensities of each band in five independent experiments are plotted on the graph (right panel). B: GAP activity assay of neurofibromin in PC12 cells by the filtration assay method [11]. The indicated amounts of cell lysates were incubated with GST-Ras-[γ-^{32}P]GTP in the presence of NF1-GAP antibody or preimmune IgG for 2 h on ice, and passed through a filter membrane. After washing, the radioactivity of the filter was counted. The total GAP activities were obtained from the radioactivity differences between the total Ras-[γ-^{32}P]GTP radioactivity and the sample radioactivity that hydrolyzed after the GAP reaction. The specific NF1-GAP activity was obtained from the radioactivity difference between the sample radioactivity after the reaction with and without anti NF1-GAP IgG. The activity of the other GAP activity was obtained from the subtraction of the NF1-GAP activity from the total GAP activity. C: Comparison of the GAP activities of neurofibromin between PC12 cells and PC12 14-3-3η cells by the GAP pull-down assay method using GST-Ras-GTP$_\gamma$S and GST-Ras-GDP$_\beta$S. D: Comparison of the GAP activities of neurofibromin between PC12 cells and PC12 14-3-3η cells by the GAP filtration assay method using GST-Ras-[γ-^{32}P]GTP. E: Forskolin enhances the neurofibromin association with 14-3-3η but decreases the neurofibromin binding activity to GST-Ras-GTP$_\gamma$S. PC12-14-3-3η cells were treated with or without 50 μM forskolin for 20 min at 37°C. Cell extracts were immunoprecipitated with anti-myc antibody, and the precipitates were immunoblotted with anti-neurofibromin antibody (two lanes in the middle). For the GST-Ras-GTP$_\gamma$S pull-down assay, the same extracts were incubated with GST-Ras-GTP$_\gamma$S and immunoblotted with anti-neurofibromin antibody (two lanes on the right). The total cellular lysates were applied to the immunoblotting using anti-neurofibromin and also used for the loading controls (two lanes on the left). The figure shows representative data from five separate identical experiments. F: Relative intensities of the results shown in E were quantitated and are demonstrated as bar graphs. Data represent means ± S.D. values from five experiments performed in duplicate.

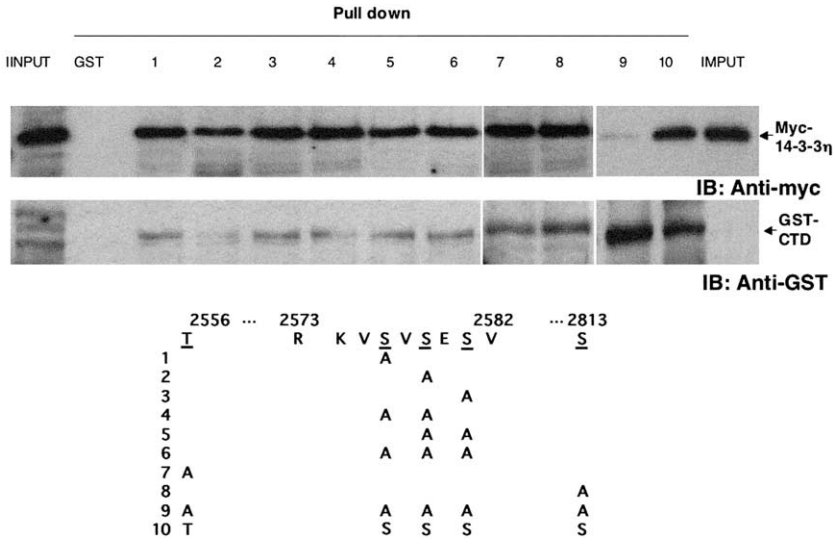

Fig. 7. Multiple substitutions of serine/threonine with alanine on the 14-3-3 binding motif and putative PKA phosphorylation sites on GST-CTD significantly affected the interaction of 14-3-3 with neurofibromin. Nine GST-CTD mutant proteins harboring point/double/triple/quintuple mutation sites of Ser2576Ala, Ser2578Ala, Ser2580Ala, Thr2554Ala, and Ser2813Ala were prepared and tested for their binding activity to myc-14-3-3 protein using PC12 14-3-3 cell lysates by the pull-down assay as described in Section 2. The mutation sites in each mutant CTD are presented in the lower panel.

[17]. Neurofibromin is constitutively phosphorylated and the specific phosphorylation sites were determined in its CSRD and CTD. Since one of the 14-3-3 binding motifs ($RX_{1-2}SX_{2-3}S$) is present on the C-terminus of neurofibromin (2573/2580 RKVSVSES), we tested the binding of 14-3-3η to GST-CTD having substitution of three serine residues (2576 Ser, 2578 Ser, 2580 Ser) to alanine. However, those mutant forms GST-CTD interacted with 14-3-3η in a similar manner as the wild-type GST-CTD. The GST-CTD also contains two potential PKA recognition sites (2556 Thr, 2813 Ser), but neither the threonine nor the serine mutation form could affect the 14-3-3 association with it. However, surprisingly, when all of the mutations (Ser2578Ala, Ser2579Ala, Ser2581-Ala, Thr2554Ala, and Ser2813Ala) were assembled on the CTD, the interaction with 14-3-3 was almost abolished. These results indicate that phosphorylation on all of the putative PKA phosphorylation sites in addition to the 14-3-3 binding motif on CTD is necessary for the association between 14-3-3 and neurofibromin, and this phosphorylation may regulate the neurofibromin function via the 14-3-3 binding. More precise study will be needed to clarify the mechanism of this interaction between 14-3-3 and neurofibromin in detail.

The interaction of 14-3-3 protein is known to modulate target proteins. These functions can be assembled into five categories: (1) alteration of the ability of the target protein to interact with other partners; (2) relocalization of the cytoplasmic/nuclear partition of the protein partner; (3) protection of the target protein from proteolysis and/or dephosphorylation; (4) serving as a phosphorylation-dependent adapter/scaffold to bridge two targets; and (5) inhibition or enhancement of the intrinsic catalytic activity of the target protein [12]. Various enzymes such as serotonin *N*-acetyltransferase, and the ASK-1 kinase activity, have been found to have their activities modified by interaction with 14-3-3 protein [12]. However, no reports have described the modification of NF1 function by 14-3-3. Our present data propose a novel function wherein 14-3-3η interacts with neurofibromin and negatively regulates its function.

In *Drosophila*, the mechanism of *NF1*-dependent activation of the Rut-adenylyl cyclase pathway is essential for mediating learning and memory [18,19]. In a mouse model of NF1, the learning deficits may be caused by excessive Ras activity, which leads to impairments in long-term potentiation caused by increased GABA-mediated inhibition [16]. Manifestation in NF1 patients is in learning and memory disorders; and it seems likely that 14-3-3 might be related to the NF1-related learning and memory disorders because it decreases the function of neurofibromin. However, further studies will be needed to prove this hypothesis.

We recently reported that NF1-GAP activity is increased in PC12 cells when neuron-like differentiation is induced by nerve growth factor (NGF) treatment [11]. This NF1-GAP increase, which is required for neurite extension, was caused by the alternative splicing changes in the NF1-GAP domain; and we suggested that NGF stimulation induces the splicing of NF1-GRD type I isoform which possesses higher GAP activity than the type II isoform, and regulates the cellular GAP activity via a Ras phosphoinositide 3′-kinase signal induction [11]. In the present study, we have shown that NF1 function is regulated not only by the splicing alteration in the GAP domain but also by phosphorylation of the C-terminal region followed by interaction with 14-3-3. Given that we previously demonstrated that neurofibromin is constitutively phosphorylated by PKA [9], GAP activity might be lower in normal growing cells. It can be speculated that external or internal signals which inhibit PKA activity may prevent 14-3-3 from interacting with neurofibromin, resulting in activation of NF1 function, which leads to suppression of the Ras-related signal pathways inducing cell cycle arrest. We believe this is the first report to support that PKA phosphorylation regulates neurofibromin function via 14-3-3 binding. Further information based on this study should give new insights into NF1-related pathogenesis.

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