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Phosphorylation of neurofibromatosis type 1 gene product (neurofibromin) by cAMP-dependent protein kinase

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Abstract The critical function of the neurofibromatosis type 1 (*NF1*) gene product (neurofibromin) is not well defined except that neurofibromin has homology with a family of the GTPase-activating proteins (GAPs). In this study, we confirmed that neurofibromin is constitutively phosphorylated and detected kinase activities which specifically phosphorylate the cystein/serine-rich domain and the C-terminal domain of the neurofibromin in cell lysate. In vitro and in-gel kinase assays strongly indicated that cAMP-dependent protein kinase (PKA) is a candidate for the neurofibromin kinase. The biological significance of the phosphorylation of neurofibromin is unclear at present, but we speculate that neurofibromin plays a crucial role in cellular function since it links the two major cellular pathways which are the GAP-ras and PKA-associated signals.

Key words: Neurofibromatosis type 1 (NF1); Neurofibromin; Phosphorylation; cAMP-dependent protein kinase

1. Introduction

Neurofibromatosis type 1 (*NF1*) gene was identified by positional cloning [1,2], and regarded as one of the tumor suppressor genes [3,4]. Analysis of the predicted protein sequence of the *NF1* gene product (neurofibromin) revealed that a 360-amino acid region in the center of the protein has significant homology with a family of the GTPase-activating proteins (GAPs) including mammalian ras-GAP, budding yeast IRA1 and IRA2, fission yeast sar1, and *Drosophila* GAP1 [5–8]. Neurofibromin has more extensive sequence homology with *Saccharomyces cerevisiae* IRA proteins [9–11] than with other GAPs. This GAP-related domain of neurofibromin (NF1-GRD) has been found to possess GTPase-stimulating activity and complement the loss of IRA function in *S. cerevisiae*, indicating that neurofibromin is the mammalian homologue of IRA1 and IRA2 [5,12,13]. In addition, it has been reported that there are two types of NF1-GRD transcripts, type I and type II, generated by the alternative splicing mechanism [14,15]. The NF1-GRD type II transcript contains an insertion of 63 nucleotides (21 amino acids) in the center of the NF1-GRD region, and the relative abundance of the type I versus the type II form is modulated differentially during brain development and can be affected by stimulation with retinoic acid [14].

To date, the function of NF1-GRD is fairly well characterized [12,14–17], but that of other domains remains to be analyzed. As a clue to elucidate further the function of neurofibromin, we directed our attention to reports suggesting that neurofibromin is heavily phosphorylated on serine and threonine residues in response to growth factors [3,18]. In this study, we detected kinase activities which specifically phosphorylate neurofibromin in cell extracts and defined that cAMP-dependent protein kinase (PKA) is a candidate for the neurofibromin kinase.

2. Materials and methods

2.1. Cell culture and preparation of cell extracts

SH-SY5Y human neuroblastoma cells and SV40-transformed human fibroblast (VA13) cells were cultured in a 1:1 mixture of Eagle's minimum essential medium (MEM) and Ham's F12 nutrient medium supplemented with 5% (v/v) heat-inactivated fetal calf serum in a humidified CO_2 incubator. Confluently grown SH-SY5Y cells and VA13 cells were incubated in serum-free medium for 12 and 48 h, respectively, and then treated with 100 nM phorbol 12-myristate 13-acetate (PMA) for 15 min, and 50 μM forskolin for 20 min. VA13 cells were also stimulated with 40 nM epidermal growth factor (EGF) for 15 min. After the treatments, the cells were scraped into ice-cold extraction buffer consisting of 20 mM Tris (pH 7.4), 10 mM $MgCl_2$, 5 mM EGTA, 2 mM dithiothreitol, 50 mM β-glycerophosphate, 0.1 mM NaF, 1 mM Na_3VO_4, 1 mM phenylmethylsulfonyl fluoride (PMSF), 10 μg/ml aprotinin, and 10 μg/ml leupeptin. The collected cells were lysed by sonication for 10 s, and then centrifuged at 100 000×*g* for 30 min at 4°C. The supernatant was used immediately as cell extracts.

2.2. Plasmid construction

A pGEX-2TH bacterial expression vector and a pGEX-2TH plasmid harboring NF1-GRD type I cDNA which generates GST-GRD type I fusion protein (residues 1168–1545) were generously provided by Dr. H. Maruta. A GST-GRD type II expression plasmid was constructed by ligation of the NF1-GRD type II cDNA [14] into the multi-cloning site of the pGEX-2TH vector. Furthermore, we generated three other GST-neurofibromin domain fusion proteins corresponding to sequences of residues 543–909, 1530–1950, and 2262–2818 of neurofibromin, which were tentatively designated cysteine/serine-rich domain (CSRD), leucine repeat domain (LRD), and COOH-terminal domain (CTD), respectively. The cDNA fragments corresponding to the various domains were amplified by RT-PCR using three sets of oligonucleotide primers that contained appropriate restriction sites flanking the domains of interest (CSRD sense primer 5′-CAGAGAATTCCAGGAAGCAATGGAGGCTCTGC-3′, antisense primer 5′-TTTGAAGCTTCACTTTCTCATGGTTACACAC-3′; LRD sense primer 5′-TCCTGGATCCCACAAACCTGTGGCA-GATACACA-3′, anti-sense primer 5′-GAATAAGCTTAACTCT-TTGTCGTTTGGCATCAT-3′; CTD sense primer 5′-AAAAA-GATCTGACACTTACAACAGTCAAGTT-3′, anti-sense primer 5′-AAGCAAGCTTCACACGATCTTCTTAATGCTA-3′) and single strand cDNA prepared from poly(A)$^+$ RNA of SH-SY5Y cells [14]. The amplified DNA fragment was digested by restriction enzymes and cloned into the pGEX-2TH plasmid. The entire sequences of the inserts were confirmed to be identical to the previously reported sequences by DNA sequencing analysis.

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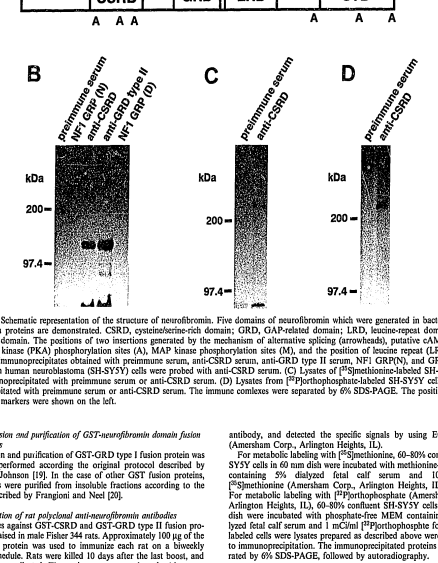

Fig. 1. (A) Schematic representation of the structure of neurofibromin. Five domains of neurofibromin which were generated in bacterial cell as GST fusion proteins are demonstrated. CSRD, cysteine/serine-rich domain; GRD, GAP-related domain; LRD, leucine-repeat domain; CTD, C-terminal domain. The positions of two insertions generated by the mechanism of alternative splicing (arrowheads), putative cAMP dependent-protein kinase (PKA) phosphorylation sites (A), MAP kinase phosphorylation sites (M), and the position of leucine repeat (LR) are indicated. (B) Immunoprecipitates obtained with preimmune serum, anti-CSRD serum, anti-GRD type II serum, NF1 GRP(N), and GRP(D) antibodies from human neuroblastoma (SH-SY5Y) cells were probed with anti-CSRD serum. (C) Lysates of [^{35}S]methionine-labeled SH-SY5Y cells were immunoprecipitated with preimmune serum or anti-CSRD serum. (D) Lysates from [^{32}P]orthophosphate-labeled SH-SY5Y cells were immunoprecipitated with preimmune serum or anti-CSRD serum. The immune complexes were separated by 6% SDS-PAGE. The positions of molecular size markers were shown on the left.

2.3. Expression and purification of GST-neurofibromin domain fusion proteins

Expression and purification of GST-GRD type I fusion protein was essentially performed according the original protocol described by Smith and Johnson [19]. In the case of other GST fusion proteins, the proteins were purified from insoluble fractions according to the method described by Frangioni and Neel [20].

2.4. Generation of rat polyclonal anti-neurofibromin antibodies

Antibodies against GST-CSRD and GST-GRD type II fusion proteins were raised in male Fisher 344 rats. Approximately 100 μg of the GST-fusion protein was used to immunize each rat on a biweekly injection schedule. Rats were killed 10 days after the last boost, and the sera were collected. The antiserum was precleared with excess GST conjugated glutathione (GSH)-agarose beads (Sigma) before use.

2.5. Immunoprecipitation, immunoblotting and metabolic labeling

Confluently grown SH-SY5Y cells were lysed in RIPA buffer consisting of PBS (pH 7.4) with 1% NP-40, 0.1% SDS, 0.5% sodium deoxycholate, 1 mM PMSF, 20 μg/ml aprotinin, and 10 μg/ml leupeptin. The supernatant of the lysate was used for immunoprecipitation with rat preimmune serum, rat anti-CSRD and anti-GRD type II polyclonal antibodies, and commercially available rabbit anti-NF1 GRP(N) and NF1 GRP(D) antibodies (Santa Cruz Biotechnology, Inc., CA). The immunoprecipitated proteins were separated by 6% SDS-PAGE (Schleicher & Schuell), and transferred onto a nitrocellulose membrane. The membrane was immunoblotted with anti-CSRD antibody, and detected the specific signals by using ECL system (Amersham Corp., Arlington Heights, IL).

For metabolic labeling with [^{35}S]methionine, 60–80% confluent SH-SY5Y cells in 60 mm dish were incubated with methionine-free MEM containing 5% dialyzed fetal calf serum and 100 μCi/ml [^{35}S]methionine (Amersham Corp., Arlington Heights, IL) for 6 h. For metabolic labeling with [^{32}P]orthophosphate (Amersham Corp., Arlington Heights, IL), 60–80% confluent SH-SY5Y cells in 60 mm dish were incubated with phosphate-free MEM containing 5% dialyzed fetal calf serum and 1 mCi/ml [^{32}P]orthophosphate for 4 h. The labeled cells were lysates prepared as described above were subjected to immunoprecipitation. The immunoprecipitated proteins were separated by 6% SDS-PAGE, followed by autoradiography.

2.6. In vitro kinase assay

Assays were conducted at 25°C for 20 min in a final volume of 50 μl containing 20 mM Tris (pH 7.4), 10 mM $MgCl_2$, 10 μCi of [γ-^{32}P]ATP (3000 Ci/mmol) (New England Nuclear), 15 μl of cell extracts, and 5 μg of GST-neurofibromin domain fusion protein with or without 2 μM cAMP-dependent protein kinase (PKA)-inhibitor peptide (PKI[6–22]amide, GIBCO-BRL). For the termination of reaction and separation of phosphorylated GST fusion proteins, each reaction mixture was immediately chilled and mixed with 60 μl of GSH-agarose beads (50% slurry) and 1 ml of ice-cold TNE buffer containing 20 mM Tris (pH 7.4), 1% NP-40, 10 mM EDTA, 100 mM NaCl, 10 mM NaF, 10 mM β-glycerophosphate, 1 mM Na_3VO_4, 1 mM PMSF, 10 μg/ml aprotinin, and 10 μg/ml leupeptin. followed by rock-

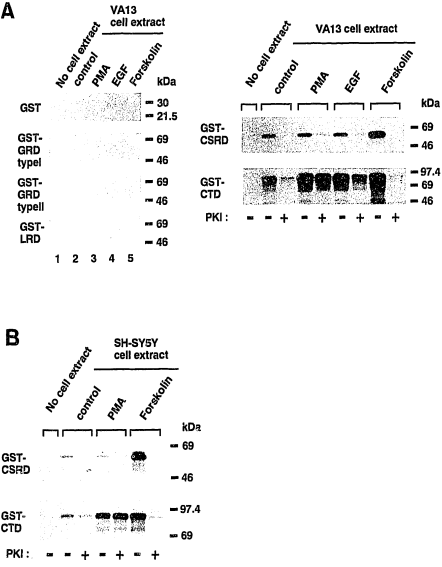

Fig. 2. Detection of neurofibromin kinase activities in cell extracts. Control (serum-starved), PMA-, and forskolin-treated VA13 and SH-SY5Y cell extracts and EGF-treated VA13 cell extracts were incubated for 20 min at 25°C with each GST-neurofibromin domain fusion protein and [γ-^{32}P]ATP in the absence (−) and presence (+) of PKA inhibitor (PKI). (A) There was no prominent kinase activity detected in the VA13 cell extracts against GST, GST-GRD type I, GST-GRD type II, and GST-LRD fusion proteins. PKI-sensitive kinase activities stimulated by forskolin in VA13 cell extracts against GST-CSRD and GST-CTD fusion proteins were detected. There were PKI-insensitive kinase activities toward GST-CTD fusion protein in PMA-, EGF-treated VA13 cell extracts. (B) SH-SY5Y neuroblastoma cell extracts also had both PKI-sensitive and-insensitive kinase activities toward GST-CSRD and GST-CTD.

ing for 20 min at 4°C. The GSH-agarose beads were then washed three times with TNE buffer, and heated in 30 μl of Laemmli sample buffer to elute the GST fusion proteins. The sample was resolved by 8–16% SDS-PAGE. The gel was stained with Coomassie brilliant blue, dried, and radioactive bands were detected by autoradiography.

2.7. In-gel kinase assay

Detection of protein kinase activity on polyacrylamide gels containing substrate proteins after SDS-PAGE [21] was performed according to the method of Kameshita and Fujisawa [22]. The substrate for phosphorylation by protein kinases in cell extracts was included in 10% SDS-polyacrylamide gel prior to polymerization. The gel contained GST (0.5 mg/ml), GST-CSRD (0.1 mg/ml), GST-CTD (0.1 mg/ml), or no substrate for each detection. 20 μl of cell extracts obtained after several treatments, or 2 μl (10 units) of cAMP-dependent protein kinase (PKA) catalytic subunit from bovine heart (Sigma) was loaded on each lane. After electrophoresis, denaturation with 6 M guanidine HCl, and renaturation, protein kinase activity was detected by incubating the gel at 25°C for 60 min with 40 mM HEPES (pH 7.5), 2 mM dithiothreitol, 0.1 mM EGTA, 5 mM $MgCl_2$, 2.5 μCi/ml [γ-^{32}P]ATP (3000 Ci/mmol). The incorporation of phosphate was then analyzed by autoradiography of the dried gel.

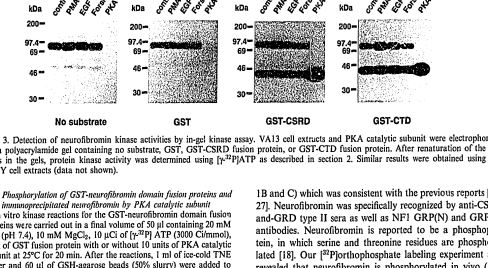

Fig. 3. Detection of neurofibromin kinase activities by in-gel kinase assay. VA13 cell extracts and PKA catalytic subunit were electrophoresed on a polyacrylamide gel containing no substrate, GST, GST-CSRD fusion protein, or GST-CTD fusion protein. After renaturation of the proteins in the gels, protein kinase activity was determined using [γ-^{32}P]ATP as described in section 2. Similar results were obtained using SH-SY5Y cell extracts (data not shown).

2.8. Phosphorylation of GST-neurofibromin domain fusion proteins and immunoprecipitated neurofibromin by PKA catalytic subunit

In vitro kinase reactions for the GST-neurofibromin domain fusion proteins were carried out in a final volume of 50 μl containing 20 mM Tris (pH 7.4), 10 mM $MgCl_2$, 10 μCi of [γ-^{32}P] ATP (3000 Ci/mmol), 5 μg of GST fusion protein with or without 10 units of PKA catalytic subunit at 25°C for 20 min. After the reactions, 1 ml of ice-cold TNE buffer and 60 μl of GSH-agarose beads (50% slurry) were added to each reaction mixture, followed by rocking for 15 min at 4°C. The GST-fusion protein was eluted by boiling for 5 min in 30 μl of Laemmli sample buffer. The samples were separated by 8–16% SDS-PAGE, and autoradiography was performed.

For examining the phosphorylation of native neurofibromin by PKA catalytic subunit, confluently grown SH-SY5Y cells (1×10^8) were lysed with 1 ml of RIPA buffer. The lysates were immediately incubated with anti-CSRD or rat preimmune serum for 4 h, and then with Protein G-agarose beads (Oncogene Science Inc., Manhasset, NY) for 1 h at 4°C. The beads were washed and resuspended in a 36 μl of solution containing 25 mM sodium acetate, 2 mM $MgCl_2$, and 20 μCi of [γ-^{32}P]ATP (3000 Ci/mmol). Kinase reaction was started by adding 20 units of PKA catalytic subunit. After the incubation at 37°C for 20 min, the beads were washed and boiled in Laemmli sample buffer to elute the GST fusion proteins. The sample was separated on 4–12% gel, and autoradiography was performed.

3. Results

3.1. Identification of neurofibromin as a phosphoprotein

To elucidate the function of neurofibromin, we produced five GST-neurofibromin domain fusion proteins. Fig. 1A illustrates the domains of neurofibromin. GRD type II has 21 amino acid inserts in GRD type I, which are generated by the mechanism of RNA alternative splicing [14]. CSRD is a cysteine-and serine-residue-rich domain, in which three cysteine pairs (residues 622/632, 673/680, and 714/721) may be comparable to one that Maru et al. [23] suggested as the ATP-binding domain of BCR protein. LRD has a leucine repeat which is present beginning at residue 1834, and not predicted to be in an α-helical conformation due to the presence of proline in the middle of the repeat [24]. CTD has two putative MAP kinase phosphorylation sites, and another alternative splicing was reported in this region [24]. Marchuk et al. [24] suggested six potential cAMP-dependent protein kinase (PKA) phosphorylation sites of neurofibromin (Fig. 1A).

Neurofibromin was detected by both immunoblot and immunoprecipitation assays as an approx. 250 kDa protein (Fig. 1B and C) which was consistent with the previous reports [25–27]. Neurofibromin was specifically recognized by anti-CSRD and-GRD type II sera as well as NF1 GRP(N) and GRP(D) antibodies. Neurofibromin is reported to be a phosphoprotein, in which serine and threonine residues are phosphorylated [18]. Our [^{32}P]orthophosphate labeling experiment also revealed that neurofibromin is phosphorylated in vivo (Fig. 1D). Phosphoamino acid analysis of the phosphorylated neurofibromin revealed that serine and threonine residues were phosphorylated (data not shown).

3.2. Detection of neurofibromin kinase activity

To identify the cellular kinases which specifically phosphorylate neurofibromin, we performed in vitro kinase reaction using cell extracts as sources of the enzyme and various GST-neurofibromin domain fusion proteins as the substrates (Fig. 2). Among these domains, only CSRD and CTD were significantly phosphorylated by VA13 cell extract (Fig. 2A). SH-SY5Y neuroblastoma cell extract also phosphorylated CSRD and CTD (Fig. 2B). The phosphorylation of CSRD

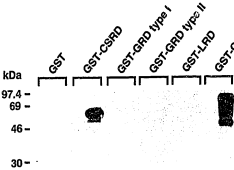

Fig. 4. In vitro phosphorylation of GST-neurofibromin domain fusion proteins by PKA catalytic subunit. GST and GST-neurofibromin domain fusion proteins were incubated for 20 min at 25°C with [γ-^{32}P]ATP in the absence (−) and presence (+) of PKA catalytic subunit as described in the text.

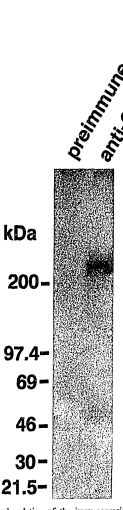

Fig. 5. Phosphorylation of the immunoprecipitated native neurofibromin by PKA catalytic subunit. Immunoprecipitates obtained with preimmune serum and anti-CSRD serum from SH-SY5Y human neuroblastoma cells were incubated for 20 min at 30°C with [γ-^{32}P]ATP and PKA catalytic subunit as described in section 2.

was enhanced by the extract of the cells treated with forskolin which is an activator of adenylate cyclase, and this reaction was inhibited by PKI. This result indicated that PKA is involved in the phosphorylation of CSRD. The phosphorylation of CTD was enhanced not only by the forskolin-treated cell extract but also by PMA-or EGF-treated cell extract. However, the phosphorylation of CTD by PMA-or EGF-treated cell extract was not effectively inhibited by PKI. This finding suggested that PMA and EGF treatments induce the other kinase activities which phosphorylate CTD protein.

For further characterization of these neurofibromin kinases, we employed in-gel kinase assay (Fig. 3). A protein kinase with relative molecular mass of 41 kDa was specifically detected on both GST-CSRD and GST-CTD substrate gels, while it was not found in the absence of substrate or when GST was used as substrate. The size of the neurofibromin kinase was comparable to that of PKA catalytic subunit, and the purified PKA catalytic subunit was actually detected to phosphorylate both CSRD and CTD substrate gels at the same size as the neurofibromin kinase. In addition, the activity of the kinase was not changed by various treatments including PMA, EGF, and forskolin. Since regulatory and catalytic subunits of PKA were separated on SDS-polyacrylamide gels, the in-gel activity of PKA catalytic subunit should not change by various treatments without the regulatory subunit. These lines of evidence strongly suggested that the 41 kDa neurofibromin kinase detected on the substrate gels is the catalytic subunit of PKA.

Furthermore, we examined whether PKA catalytic subunit can phosphorylate immunoprecipitated native neurofibromin as well as the GST fusion proteins. As shown in Fig. 4, GST-CSRD and GST-CTD fusion proteins were well phosphorylated by PKA catalytic subunit in vitro whereas other domains were not. In addition, endogenous neurofibromin that was immunoprecipitated by using anti-CSRD antiserum was also phophorylated by PKA catalytic subunit in vitro (Fig. 5). Autophosphorylation of GST-neurofibromin domain fusion proteins and immunoprecipitated endogenous neurofibromin was not detected under the conditions tested.

4. Discussion

In this study, we identified neurofibromin as an approx. 250 kDa protein on SDS-polyacrylamide gel using rat polyclonal antibodies raised against GST-neurofibromin domain fusion proteins, and also confirmed that the endogeneous neurofibromin is constitutively phosphorylated on its serine/threonine residues. Among the five domains of the neurofibromin which we generated in bacterial cells as fusion proteins with GST, CSRD and CTD were significantly phosphorylated by PKI-sensitive kinase that exists in cell extracts. Moreover, the kinase activity in the cell extract was enhanced by forskolin treatment of the cells. Any other remarkable kinase activities against CSRD and CTD were not detected in serum-starved cell extracts. To identify the apparent mass of the neurofibromin kinases, we employed the in-gel kinase assay. A prominent kinase activity migrating with the electrophoretic mobility identical to PKA catalytic subunit was detected on the gels containing CSRD and CTD as substrates. Moreover, PKA catalytic subunit could indeed phosphorylate CSRD, CTD and immunoprecipitated native neurofibromin. Direct evidence that PKA phosphorylates neurofibromin in vivo has not been obtained thus far [28]. Since the basal phosphorylation level of neurofibromin is high in the cultured cells, it may be difficult to observe the effect of forskolin or dibutyryl cAMP on the phosphorylation of neurofibromin in vivo. However, our results strongly indicate that PKA is a most likely neurofibromin kinase. Additionally, we demonstrated the existence of PKI-insensitive kinase activity against CTD in PMA-and EGF-stimulated cell extracts, which should be further elucidated.

The functional significance of phosphorylation of neurofibromin by PKA is still unclear, but we presume that neurofibromin has a fundamental function in cells since it is involved in both the GAP-ras signaling pathway and the physiological response to intracellular second messenger cAMP. Recently, the biochemical link between the second messenger cAMP and the ras signaling pathway has been reported [29–33]. Wu et al.

[33] have suggested that in certain cell types, cAMP prevents transmission of signals from ras to Raf-1, possibly due to phosphorylation of Raf-1 by PKA, and thus inhibits the activation of the MAP kinase cascade. Although a role of neurofibromin in ras-related signaling pathway has not been clearly understood, the biochemical consequence of neurofibromin phosphorylation by PKA should be taken into account in the ras-cAMP connection.

Phosphorylation of ras-related proteins and their relatives as a mechanism of regulation has been tested in a number of cases [34], in which rap1/Krev-1/smgp21 has been implicated in the cAMP-mediated inhibition of platelet metabolism, and appeared to be identical to thrombolamban, a major substrate for PKA in the cells [35–38]. Phosphorylation of rap1 in response to hormones that elevate intracellular cAMP correlates with translocation of rap1 from a membrane to a cytosolic fraction [37]. In addition, it has been shown that rap1-GAP is also phosphorylated by PKA [39]. The rap1/Krev-1/smgp21 has been known to bind with high affinity to ras-GAP, and it has been suggested that this competition may account for its ability to revert the ras-transformed phenotype in 3T3 cells [39]. The interaction between rap1/Krev-1/smgp21 and GRD of neurofibromin has not been fully examined.

As for the subcellular localization of neurofibromin, there have been some controversial pieces of evidence reported. Neurofibromin is demonstrated to reside in both the cytosolic and particulate fractions [26,40], in which it may associate with tubulin [41,42], smooth endoplasmic reticulum [43], and cytoplasmic structures that are distinct from actin or tubulin filaments [26]. Gregory et al. [42] suggested sequence similarity between a small region of neurofibromin (residues 815–834) and two other proteins that associate with microtubules, MAP-2 and tau, and hypothesized that phosphorylation of this region might regulate the association of neurofibromin and microtubules. This putative microtubule-associated region (20 amino acids) resides in CSRD, and contains a consensus sequence which can be phosphorylated by PKA (on Ser-818). Nordlund et al. [43] demonstrated that neurofibromin was associated with smooth vesiculotubular elements and cisternal stacks, and with multivesicular bodies in the cell bodies and dendrites of neurons of the central nervous system (CNS), and hypothesized that some, if not all, of the CNS manifestations of NF1 might result from the altered expression of neurofibromin in neurons, perhaps through disruption of Ca^{2+} signaling, translocation of organelles, or endocytic pathways.

Since the cloning of the gene defective in NF1, there has been remarkable progress in dissecting the mechanism of the disease, although the pivotal role of neurofibromin in cells is still unclear as described above. The cumulative evidence including the presence of GAP activity, the localization of neurofibromin to the nervous system, and the potential phosphorylation by PKA and other kinases enables us to infer that neurofibromin plays a key role in cell growth and differentiation, and especially in the cellular process of neurons. Therefore, better understanding of the function of neurofibromin will provide the fascinating insights into the intracellular biological network as well as the therapy for NF1 and malignant tumors.

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