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Induction of CRMP-2 by GDNF and analysis of the CRMP-2 promoter region☆

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Abstract

Collapsin response mediator protein-2 (CRMP-2) is a mammalian homologue of UNC-33 of *Caenorhabditis elegans*. Mutations of CRMP-2 result in abnormal axon termination. Recently, it was demonstrated that CRMP-2 binds to tubulin heterodimers to promote microtubule assembly that is critical for axonal differentiation and growth during development. Here we show that glial cell line-derived neurotrophic factor (GDNF) enhances CRMP-2 expression in TGW human neuroblastoma cells via activation of RET receptor tyrosine kinase. GDNF-mediated CRMP-2 expression was regulated mainly by the extracellular regulated kinase (ERK) pathway, but was independent of activation of phosphatidylinositol 3-kinase and Src family kinases. Analysis of the promoter region of the CRMP-2 gene revealed that the region 214–48 bp upstream of the transcriptional start site is important for CRMP-2 expression. The SP1, E2F, and GATA1/2 binding sites appeared to play some roles in regulation of CRMP-2 expression. As expected, the CRMP-2 protein accumulated in extended neurites of TGW cells treated with GDNF. However, neuritogenesis of TGW cells was mostly dependent on Src family kinase activity and not ERK activity, indicating that the increased expression of CRMP-2 alone was not sufficient for neuritogenesis.

Keywords: CRMP-2; GDNF; RET; Neuritogenesis; Promoter; Transcription

CRMP-2 is one of the five CRMP gene family members (CRMP-1–5) and is highly expressed in the developing nervous system [1–3]. The mutation of *unc-33*, which is a CRMP-2 homologue of *Caenorhabditis elegans* (*C. elegans*), leads to abnormal axon termination of various neurons [4–6]. CRMP-2 is enriched in the distal part of the growing axon of cultured hippocampal neurons, and overexpression of CRMP-2 induces the formation of multiple axons. In contrast, dominant negative mutations of CRMP-2 inhibit the formation of the primary axon, indicating an important role for CRMP-2 in axonal growth [7]. Recently it was demonstrated that CRMP-2: (1) binds to tubulin heterodimers to promote microtubule assembly and (2) regulates polarized Numb-mediated endocytosis of L1 [8,9].

The *RET* gene was originally identified as an oncogene activated by DNA rearrangement [10]. *RET* encodes a receptor tyrosine kinase; mutations of the *RET* gene cause several human diseases such as papillary thyroid carcinoma, multiple endocrine neoplasia type 2A and 2B, and Hirschsprung's disease [11]. These mutations result in RET activation or inactivation by various mechanisms. Physiologically, RET is activated by ligands of the glial cell line-derived neurotrophic factor (GDNF) family, including GDNF, neurturin, artemin, and persephin [12]. However, unlike ligands for other receptor tyrosine kinases, the GDNF family ligands (GFLs) do not bind to RET directly, but require

☆ *Abbreviations:* CRMP-2, collapsing response mediator protein-2; GDNF, glial cell line-derived neurotrophic factor; ERK, extracellular regulated kinase; PI3K, phosphatidylinositol 3-kinase; GFLs, GDNF family ligands; GFRα, GDNF family receptor α; NCAM, neural cell adhesion molecule; NGF, nerve growth factor.

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glycosyl-phosphatidylinositol (GPI)-anchored coreceptors, called GDNF family receptor αs (GFRαs), as ligand binding components [13–15]. The formation of this multicomponent receptor complex is responsible for activation of a variety of intracellular signaling pathways such as RAS/ERK, PI3K/AKT, p38MAPK, and JNK pathways [11]. Through activation of RET tyrosine kinase, GFLs promote the survival and differentiation of various neurons such as peripheral autonomic and sensory neurons as well as central motor and dopaminergic neurons [12,16–18]. In addition, gene ablation studies revealed that the GDNF/RET signaling pathway plays a crucial role in development of the kidney and the enteric nervous system [19–23].

To date, only a few reports have shown a link between CRMP-2 and neurotrophic factors. It was reported that CRMP-2 expression increased in PC12 cells treated with nerve growth factor (NGF), although the mechanism of this action was not described in detail [24,25]. Another report showed that CRMP-2 expression increased after nerve injury and accelerated axon regeneration in rat motor neurons [26]. Injury to motor nerves also increases the expression of GDNF in the denervated muscle, of GDNF and GFRα1 in affected Schwann cells, and of RET and GFRα1 in the injured motor neurons [12]. On the basis of these findings, it is interesting to investigate whether GDNF can enhance CRMP-2 expression in neuronal cells. In addition, elucidating the mechanism of GDNF-mediated CRMP-2 induction may contribute to understanding of neuronal differentiation and regeneration. In this study, we found that CRMP-2 expression is induced through activation of the GDNF/RET signaling pathway and that ERK activation is important for enhancement of CRMP-2 expression. We also performed an analysis of the promoter region of the human CRMP-2 gene and found that several transcription factors such as Sp1, E2F, and GATA1/2 may play a role in GDNF-dependent CRMP-2 transcription.

Materials and methods

Cell culture. TGW human neuroblastoma cells were maintained in RPMI 1640 supplemented with 10% fetal bovine serum.

Western blotting. TGW cells were stimulated with GDNF (100 ng/ml) for 10 min, 2, 8 or 24 h after overnight-starvation and lysed in sodium dodecyl sulfate (SDS) sample buffer (20 mM Tris–HCl, pH 6.8, 2 mM EDTA, 2% SDS, 10% sucrose, 20 μg/ml bromophenol blue, and 80 mM dithiothreitol). The resulting lysates were ultrasonicated and boiled for 5 min. Their protein concentration was determined using a DC protein assay kit (BIO-RAD, Hercules, CA, USA) and equal amounts of total protein were applied to each lane on the gel. After they were subjected to SDS–polyacrylamide gel electrophoresis, proteins were transferred to polyvinylidene difluoride membranes (Immobilon PVDF; Nihon Millipore, Tokyo, Japan). Membranes were blocked in 3% albumin in phosphate-buffered saline (PBS) containing 0.05% Tween 20 and probed with the primary antibody. After washing, they were incubated with the secondary antibody for 1 h and specific binding was detected by the enhanced chemiluminescence system (ECL; Amersham Biosciences, Piscataway, NJ, USA).

Inhibitors and antibodies. A MEK inhibitor, PD98059, a PI3K inhibitor, LY294002, a Src kinase family inhibitor, PP2, and a negative control for PP2, PP3 [27] were purchased from Calbiochem (San Diego, CA, USA). A PI3K inhibitor, wortmannin, was purchased from SIGMA–ALDRICH (Tokyo, Japan). Anti-CRMP-2 antibody was previously described [28].

Construction of luciferase reporter plasmids. Approximately 1 kb of the CRMP-2 promoter region was isolated by PCR and inserted into the pGL3Basic luciferase reporter plasmid (Promega, Madison, WI, USA). Site-directed mutagenesis of the CRMP-2 promoter was carried out by the overlap elongation method [29]. Primers for introduction of mutations of the E2F, Sp1, Oct-1, GATA-1/2, and p300 binding sites were as follows: E2F, 5′-CACTGCCGCATTTAACGCTCTCGCGCCGC-3′; Sp1, 5′-GCTCTCGCGCCGCGCCCCTTCCCACCGGCC-3′; Oct-1, 5′-GCCTAAATTTGCCCATCCCAGGATCGCGGC-3′; GATA-1/2, 5′-GTCTCTCTCGAAGCGGCCGGCTTTTGCCTG-3′; and p300, 5′-CCTGAGAGGAAAGTTAGTGGCTGGCGGCGC-3′. Mutations introduced are underlined.

Transfection and luciferase assay. Cells were grown to 50% confluency and transfected with 1 μg of reporter plasmid DNA on 24-well plates with Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). pRL-TK plasmid (0.1 μg) (Promega) was cotransfected to normalize for transfection efficiency. After transfection, cells were treated with or without 100 ng/ml GDNF for 10 min. Luciferase gene expression was measured 24 h later with a luminometer and normalized by *Renilla* luciferase activity. The luciferase assays were performed in triplicate, and relative fold activation of luciferase activity was calculated.

Immunostaining. TGW cells were cultured on coverslips, fixed in PBS containing 4% paraformaldehyde for 10 min at room temperature, and washed twice in PBS. They were permeabilized for 10 min in −20 °C methanol, and blocked with 3% albumin in PBS. The cells were incubated with anti-CRMP-2 antibody or normal rabbit immunoglobulin (DAKO, Glostrup, Denmark), followed by incubation with the FITC-conjugated secondary antibody.

Results

Upregulation of CRMP-2 expression by GDNF

We investigated the effect of GDNF on CRMP-2 expression in TGW human neuroblastoma cells expressing RET and GFRα1. Western blot analysis revealed that CRMP-2 expression increased after GDNF stimulation in a time-dependent manner. Forty-eight hours after GDNF stimulation, levels of CRMP-2 expression had increased by approximately fivefold (Fig. 1A).

Regulation of CRMP-2 expression by the ERK pathway

GDNF can activate a variety of signaling pathways such as the RAS/ERK and PI3-K/AKT pathways [11]. In addition, GDNF treatment leads to activation of Src family kinases in neuronal cells [30,31]. To investigate which pathway is responsible for the increase of CRMP-2 expression, TGW cells were treated with 50 μM PD98059 (a MEK inhibitor), 100 nM wortmannin, 30 μM LY294002 (PI3K inhibitors) or 2 μM

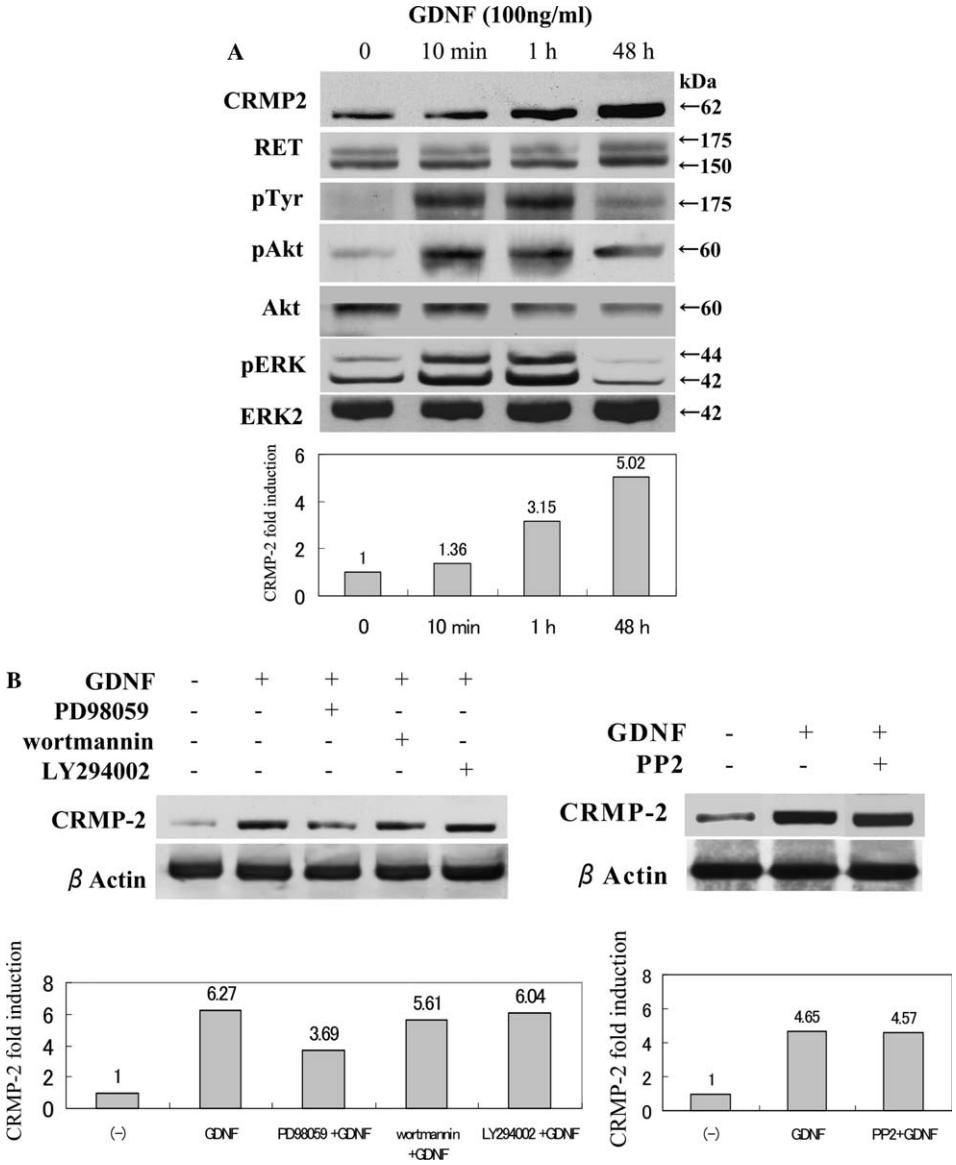


Fig. 1. GDNF-induced CRMP-2 expression via the ERK pathway. (A) GDNF-mediated CRMP-2 expression. Total cell lysates of TGW cells treated with GDNF (100 ng/ml) for 10 min, 1 or 48 h were analyzed by Western blotting with the indicated antibodies (upper panel). Analysis by densitometry showed that CRMP-2 expression increased approximately fivefold after 48 h of GDNF stimulation (lower panel). (B) Regulation of GDNF-mediated CRMP-2 expression by the ERK pathway. TGW cells were incubated in the presence or absence of PD98059 (50 μM), wortmannin (100 nM), LY294002 (30 μM) or PP2 (2 μM) for 30 min and stimulated with GDNF (100 ng/ml) for 24 h. Inhibitors other than wortmannin were added together with GDNF. Wortmannin was removed before addition of GDNF because its action as an inhibitor was irreversible. Each signaling pathway was specifically inhibited by these concentrations of inhibitors as described previously [32,41].

PP2 (a Src family kinase inhibitor). The concentrations of these inhibitors little affected the viability of TGW cells at least for 24 h. As shown in Fig. 1B, PD98059 treatment significantly suppressed GDNF-induced CRMP-2 expression. In contrast, treatment with wortmannin, LY294002 or PP2 had little effect.

To confirm the role of the ERK pathway in CRMP-2 expression, we next investigated whether CRMP-2 promoter activity was suppressed by PD98059. We isolated the CRMP-2 promoter region, which is an approximately 1-kb fragment upstream of the CRMP-2 transcriptional start point (Fig. 2A). The sequence information for this region was obtained from the NCBI site (Human Genome Resources; http://www.ncbi.nlm.nih.gov/genome/guide/human/). The CRMP-2 promoter region is highly GC-rich, and a variety of putative transcription factor binding sites were identified using TFSEARCH (http://molsun1.cbrc.aist.go.jp/research/db/TFSEARCH.html). Such putative sites included the binding sites for Sp1, E2F, Oct-1, GATA1/2, and p300 (Fig. 2A). When the transcriptional activity of this

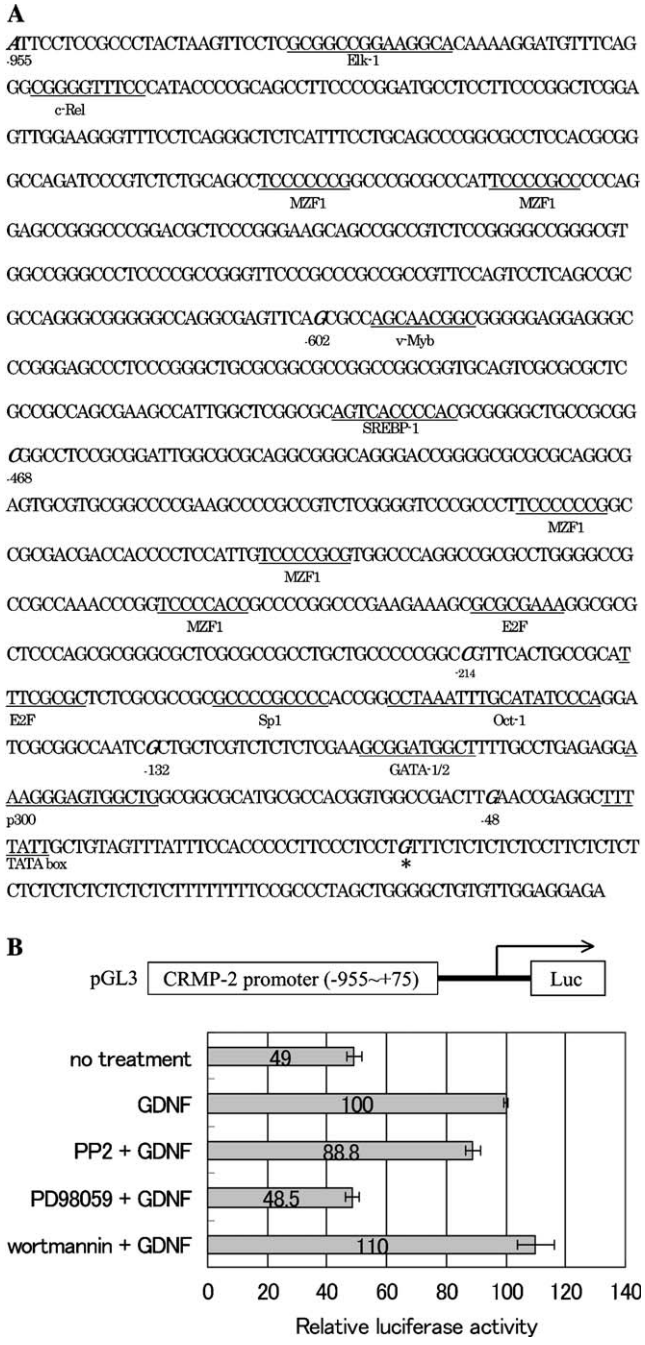


Fig. 2. Regulation of the transcriptional activity of the CRMP-2 promoter region by the ERK pathway. (A) Isolation and nucleotide sequence of the CRMP-2 promoter region. The putative transcription factor binding sites are underlined (from TFSEARCH, http://molsun1.cbrc.aist.go.jp/reseach/db/TFSEARCH.html). The region of −214 to −48 includes binding sites for several transcriptional factors such as E2F, Sp1, Oct-1, GATA-1/2, and p300. (B) Effects of kinase inhibitors on the transcriptional activity of the CRMP-2 promoter region. The CRMP-2 promoter region was inserted into the pGL3-basic luciferase vector. TGW cells were transfected with this luciferase vector plus pRL-TK plasmid. After transfection, the cells were cultured for 24 h, incubated in the presence of each kinase inhibitor for 30 min, and stimulated with GDNF for 24 h. All values were normalized by the activities of the *Renilla* luciferase gene. Results represent averages from at least three independent experiments and bars indicate the standard errors.

region was analyzed using a luciferase reporter-gene assay, the activity showed a twofold increase in GDNF-treated TGW cells as compared with GDNF-untreated cells (Fig. 2B). Addition of PD98059 reduced the activity in GDNF-treated cells to the level in GDNF-untreated cells, confirming the importance of the ERK pathway for GDNF-dependent CRMP-2 expression. In contrast, wortmannin and PP2 did not significantly affect the transcriptional activity of the CRMP-2 promoter region (Fig. 2B).

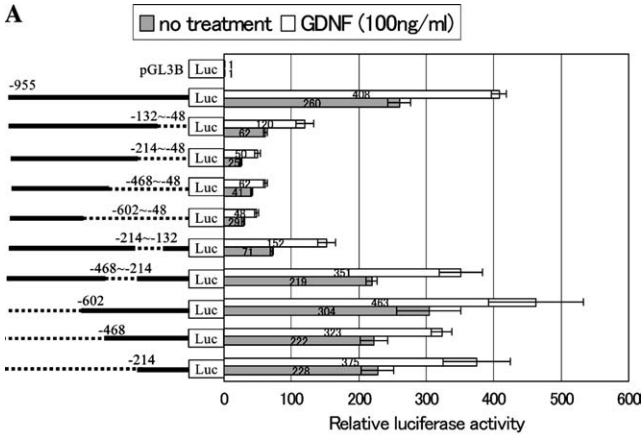
Analysis of the promoter region responsible for CRMP-2 transcription

We next tried to determine which part of the CRMP-2 promoter region was responsible for transcription. We constructed various deletion mutants and examined their promoter activities using a luciferase reporter-gene assay. This analysis showed that the region between 214 and 48 bp upstream of the transcription start point is important for CRMP-2 transcription (Fig. 3A). When this region was deleted, transcriptional activity was reduced by about 90% as compared with the unaltered sequence. The sequence upstream of −214 had only a small effect on transcriptional activity. In addition, the transcriptional activity of each construct in GDNF-treated TGW cells was approximately 1.5- to 2.1-fold higher than that in GDNF-untreated cells.

Because a variety of putative transcription factor binding sites were identified, including the binding sites for E2F, Sp1, Oct-1, GATA1/2, and p300 (Fig. 2A), we made constructs in which mutations were introduced in the binding site for each transcription factor. We then examined the effect of these constructs on transcriptional activity. In cells treated with GDNF, the constructs containing mutated E2F, Sp1 or GATA1/2 binding sites exhibited transcriptional activity that was reduced by about 35% in comparison to the original constructs. In cells untreated with GDNF, the mutant constructs exhibited transcriptional activity that was 22–30% reduced when compared to the original constructs. These results suggest that these transcription factors may be involved in regulation of CRMP-2 transcription. The activities of the constructs with mutated Oct-1 or p300 binding sites were slightly suppressed in GDNF-treated cells but not in GDNF-untreated cells (Fig. 3B).

Role of CRMP-2 expression in neuritogenesis of TGW cells

GDNF induces neurite extension in TGW cells. Thus, we compared the subcellular localization of CRMP-2 between GDNF-treated or untreated cells. Immunostaining with anti-CRMP-2 antibody revealed CRMP-2



B

	binding site	mutation (underlined)
E2F	5'-TTTCGCGC-3'	5'-TTTAACGC-3'
Sp1	5'-GCCCCGCCCC-3'	5'-GCCCCTTCCC-3'
Oct-1	5'-CCTAAATTTGCATATCCCA-3'	5'-CCTAAATTTGCCCATCCCA-3'
GATA-1/2	5'-GCGGATGGCT-3'	5'-GCGGCCGGCT-3'
p300	5'-AAAGGGAGTGGCTG-3'	5'-AAAGTTAGTGGCTG-3'

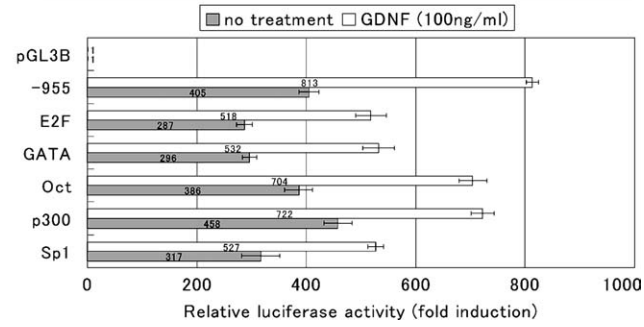


Fig. 3. Characterization of the CRMP-2 promoter region. (A) Detection of the promoter region responsible for CRMP-2 expression. A variety of deletion mutants of the CRMP-2 promoter region were generated and used for the luciferase reporter-gene assay. Deleted regions are shown by broken lines. The luciferase reporter-gene assay was performed as described in the legend of Fig. 2. Results represent averages from at least three independent experiments and bars indicate the standard errors. (B) Effects of mutations in transcription factor binding sites on the transcriptional activity of the CRMP-2 promoter region. Mutations introduced in each binding site and the luciferase activity of each mutant construct are shown in upper and lower parts, respectively.

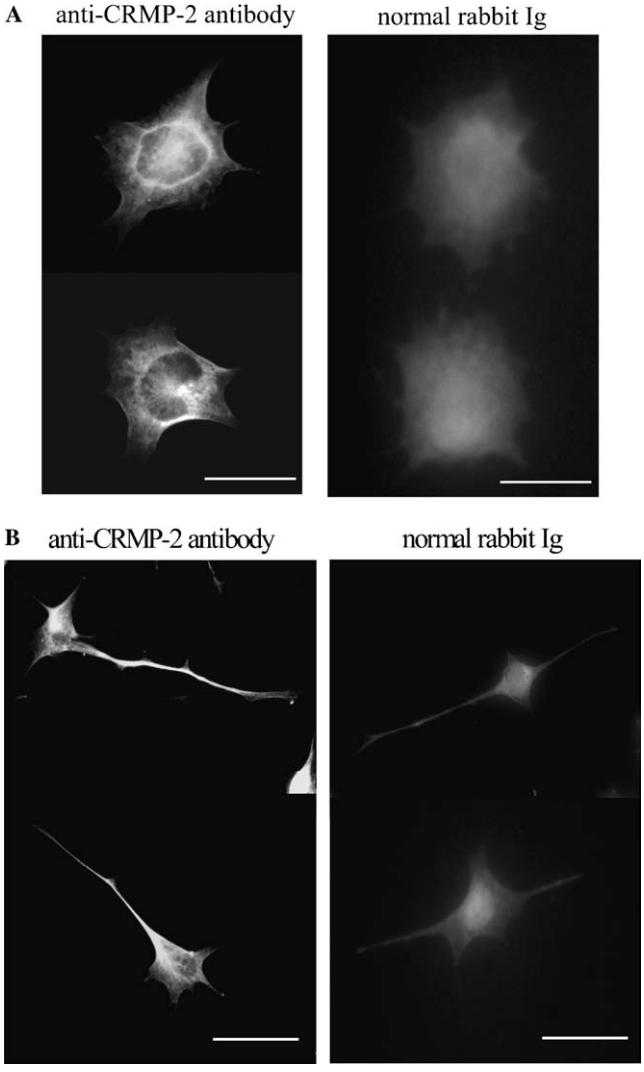


Fig. 4. Localization of CRMP-2 in TGW cells. TGW cells untreated or treated with GDNF were immunostained with an anti-CRMP-2 antibody (left panel) or with normal rabbit immunoglobulin (right panel). TGW cells were cultured in the absence of fetal bovine serum during GDNF stimulation. (A) CRMP-2 immunostaining occurred diffusely in cytoplasm without GDNF stimulation. Bar = 25 μm. (B) After 24 h GDNF stimulation, a high level of CRMP-2 immunostaining was observed along the extended neurites. Bar = 50 μm.

immunostaining diffusely in the cytoplasm before GDNF treatment (Fig. 4A). After 24 h GDNF stimulation, a high level of CRMP-2 immunostaining was observed along the extended neurites (Fig. 4B). This result is consistent with the finding that CRMP-2 was enriched in the growing axons of cultured hippocampal neurons [7].

We next investigated the effects of PD98059, wortmannin, and PP2 on GDNF-induced neurite extension. Interestingly, PP2 largely inhibited the neurite outgrowth induced by GDNF, whereas the effect by PD98059 and wortmannin was small (Figs. 5B–E and G). In addition, PP3, a negative control for PP2 [27], showed no effect on neurite extension (Figs. 5F and G). Because PP2 did not significantly affect the GDNF-dependent increase of CRMP-2 expression (Fig. 1B), these findings suggest that increased expression of CRMP-2 alone is not sufficient for neurite outgrowth in TGW cells.

Discussion

We have shown that GDNF enhances CRMP-2 expression in TGW cells. A MEK inhibitor, PD98059, significantly suppressed GDNF-mediated CRMP-2 expression whereas the PI3K inhibitors wortmannin and LY294002 did not. PD98059 also suppressed the transcriptional activity of the CRMP-2 promoter region, suggesting that GDNF-mediated CRMP-2 expression

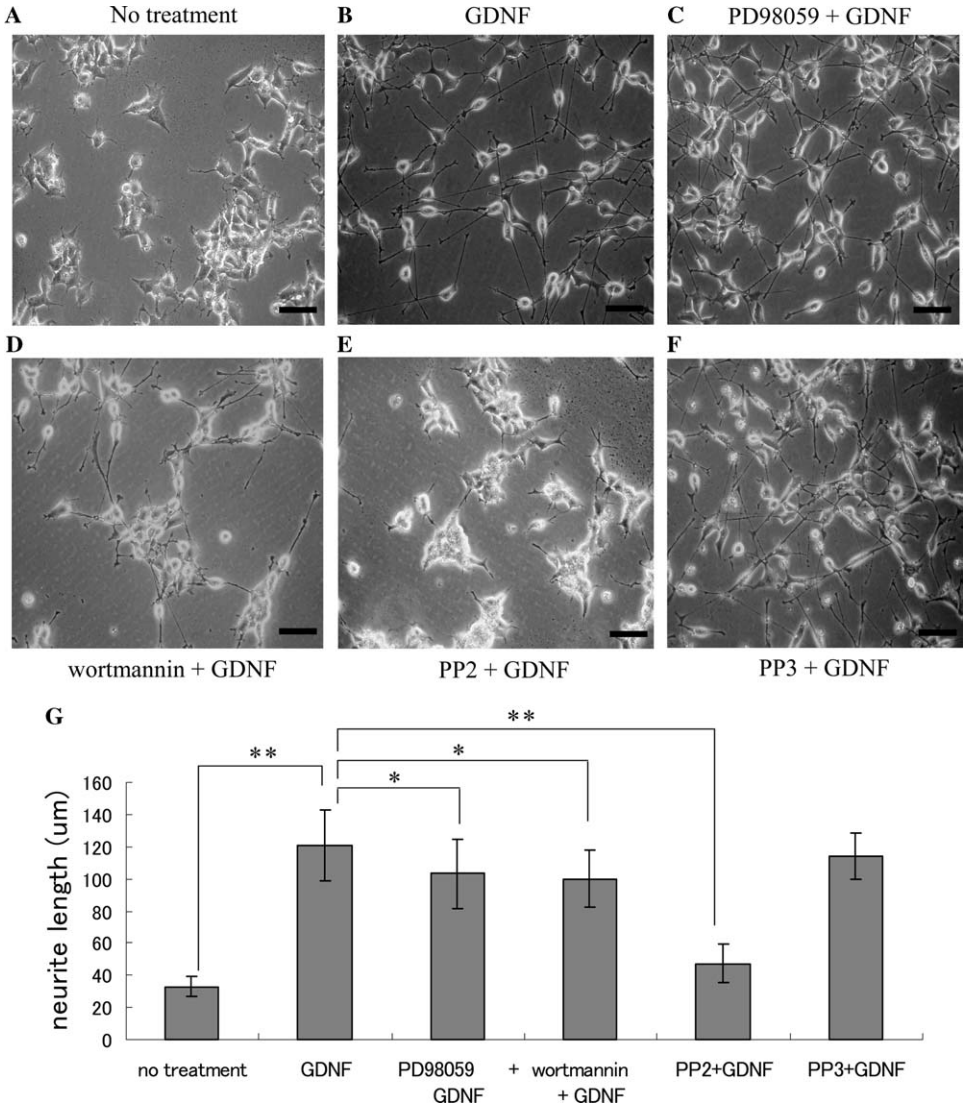


Fig. 5. Effects of kinase inhibitors on GDNF-induced neurite extension in TGW cells. TGW cells were incubated in the absence (A) or presence (B–F) of GDNF for 24 h. Before GDNF stimulation, the cells were treated with PD98059 (C), wortmannin (D), PP2 (E) or PP3 (F) for 30 min. Inhibitors other than wortmannin were added together with GDNF. Wortmannin was removed before addition of GDNF because its action as an inhibitor was irreversible. All experiments were performed in the absence of fetal bovine serum. Bar = 50 μm. (G) Quantitative analysis of neurite extension in TGW cells. The longest neurites of 100 cells were measured. PP2 treatment markedly reduced neurite extension of TGW cells whereas the effects of PD98059 and wortmannin were small. Differences were analyzed by using a Student's t test and significant difference was indicated with asterisks ($^{*}P < 0.05$ and $^{**}P < 0.001$).

depends mostly on ERK activity in the GDNF/RET signaling pathway.

It was recently reported that NCAM is an alternative signaling receptor for GDNF family ligands. GDNF binds to NCAM resulting in activation of Fyn (a member of the Src kinase family) and induction of axonal growth in hippocampal neurons. This activity was independent of the RET signaling pathway [31,32]. Because TGW cells express endogenous NCAM, we investigated whether PP2 (an inhibitor of the Src kinase family) treatment affected GDNF-mediated CRMP-2 expression. Although both RET and NCAM can activate Src family kinase, RET has other signaling pathways independent of Src [30,31,33]. PP2 had only minor effects on CRMP-2 expression and transcriptional activity of the CRMP-2 promoter region, suggesting that GDNF-induced CRMP-2 expression is mediated via RET rather than NCAM.

We analyzed the CRMP-2 promoter region to identify the transcription factors that mediate GDNF-induced CRMP-2 expression. We found several transcription factor binding sites that were necessary for CRMP-2 transcription. The 5′-flanking region consisting of base pairs −214 to −48 from the transcriptional start point of the CRMP-2 gene was important for its transcription. This region included several putative

transcription factor binding sites including Sp1, E2F, Oct-1, GATA-1/2, and p300. By introducing mutations into these binding sites, we demonstrated that Sp1, E2F, and GATA-1/2 may play roles as CRMP-2 transcriptional enhancers.

Sp1 is a ubiquitously expressed transcription factor that recognizes a GC-rich sequence present in the regulatory regions of numerous housekeeping genes. Sp1 is susceptible to modification such as phosphorylation and glycosylation [34]. For example, ERK directly phosphorylates threonines 453 and 739 of Sp1, and this phosphorylation increases the DNA binding activity of Sp1 [35–37]. Because ERK is responsible for GDNF-mediated CRMP-2 expression, it is reasonable to speculate that GDNF promotes the DNA binding activity of Sp1, thereby enhancing CRMP-2 promoter activity. When mutations were introduced into the Sp1 binding site, the transcriptional activity was suppressed in TGW cells with or without GDNF treatment. In addition, the degree of suppression was slightly higher in GDNF-treated TGW cells in comparison to GDNF-untreated cells (35 vs. 22% in Fig. 3B). Thus, Sp1 might be a GDNF-response element in the CRMP-2 promoter.

E2F is a critical factor in cell cycle progression, arrest, and in apoptosis. E2F4 was previously shown to be highly upregulated after neuronal differentiation of PC12 cells treated with NGF. Its expression was also found to increase in the developing rat brain, whereas E2F1, E2F3, and E2F5 were partially downregulated [38]. In addition to E2F4, E2F2 increased in PC12 cells in response to NGF and this increase was associated with the differentiated, postmitotic state and its maintenance [39]. Our finding that mutation of the E2F binding site reduced the transcriptional activity of the CRMP-2 promoter region suggests a possible involvement of E2F4 or E2F2 in the regulation of CRMP-2 transcription.

GATA-2 is known to play a crucial role during early stages of neuronal differentiation in the mouse [40]. It is interesting to note that GATA-2 and CRMP-2 have partially overlapping distributions, and CRMP-2 appears to be expressed slightly later than GATA-2 during mouse embryonic development [24,40]. These observations suggest that GATA-2 can function as an enhancer of CRMP-2 transcription in neuronal cells.

CRMP-2 binds to tubulin heterodimers to promote microtubule assembly that is responsible for axonal growth [8]. Thus, the increased CRMP-2 expression may also contribute to GDNF-induced neurite elongation of TGW cells. However, our analysis using kinase inhibitors revealed that the increased expression of CRMP-2 via the ERK pathway was not strongly correlated with neurite outgrowth in TGW cells. Rather, modification of CRMP-2 function may be important for neurite outgrowth. For example, Arinuma et al. [28] reported that Rho kinase phosphorylates CRMP-2 at threonine-555 and reduces the CRMP-2 activity, leading to growth cone collapse or retraction. Although we investigated phosphorylation of CRMP-2, a significant change in the phosphorylation level at threonine-555 was not observed when cells were treated with GDNF (data not shown). In addition, tyrosine phosphorylation of CRMP-2 was not induced by GDNF stimulation (data not shown).

Interestingly, PP2, an inhibitor of Src family kinases, largely inhibited GDNF-induced neurite elongation. It was previously shown that Src activity is required for GDNF-mediated neurite outgrowth [30], and that Fyn activity via the GDNF/NCAM signaling pathway promotes axonal growth of hippocampal neurons [32]. Thus, Src family kinases may modulate CRMP-2 function responsible for neuritogenesis without accompanying the increase of CRMP-2 expression. It is also possible that Src family kinases modulate the function of factors involved in neuritogenesis other than CRMP-2.

In summary, GDNF enhances CRMP-2 expression through the GDNF/RET signaling pathway, and this CRMP-2 induction depends mostly on the ERK pathway. CRMP-2 promoter analysis showed that transcription factors such as Sp1, E2F, and GATA-1/2 may play a role in regulation of CRMP-2 transcription.

Acknowledgments

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