GDNF triggers a novel Ret-independent Src kinase family-coupled signaling via a GPI-linked GDNF receptor α1

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Abstract Glial cell line-derived neurotrophic factor (GDNF) has potentially great clinical importance in the treatment of Parkinson's disease and several other neurodegenerative diseases, however its intracellular signaling mechanisms are poorly understood. Here we show that upon GDNF binding glycosyl-phosphatidylinositol (GPI)-linked GDNF receptor α1 (GFRα1) activates cytoplasmic Src family tyrosine kinase(s) in Ret tyrosine kinase-deficient cultured mouse dorsal root ganglion neurons and in two Ret-negative cell lines. GFRα1-mediated Src-type kinase activation subsequently triggers phosphorylation of mitogen-activated protein kinase, cAMP response element binding protein and phospholipase Cγ. We therefore conclude that GDNF can activate intracellular signaling pathways Ret-independently via GPI-linked GFRα1.

Key words: Glial cell line-derived neurotrophic factor; Glycosyl-phosphatidylinositol-linked receptor; Ret tyrosine kinase; Src family kinase; Mitogen-activated protein kinase; cAMP response element binding protein

1. Introduction

Glial cell line-derived neurotrophic factor (GDNF) [1] is a survival factor for embryonic midbrain dopaminergic neurons, spinal motor neurons, locus coeruleus noradrenergic neurons, and subpopulations of peripheral sensory, sympathetic, and parasympathetic neurons. The pattern of neurotrophic activity of GDNF is therefore promising for its potential use in the treatment of Parkinson's disease, Alzheimer's disease, motoneuron diseases and several other neurodegenerative diseases [2,3]. The biological importance of GDNF is illustrated by the phenotype of GDNF null mice which display deficits in primary sensory, sympathetic and motor neurons, fail to develop kidneys and most of the enteric nervous system, and die at birth [4–6]. Despite the potential clinical importance of GDNF, the intracellular mechanism of GDNF's action is far from understood. GDNF has been thought to act through a multicomponent receptor system including a glycosyl-phosphatidylinositol (GPI)-linked GDNF family receptor α1 (GFRα1) [7,8] and a transmembrane receptor tyrosine kinase, Ret [9,10]. GFRα1, lacking an intracellular domain, was originally assessed as a binding site for GDNF, serving only in the presentation of the GFRα1/GDNF complex to Ret [7,8,11]. Recent data, however, suggest that GFRα1 can evoke GDNF-induced intracellular signaling in the absence of Ret [12]. Ret and GFRα1 expression patterns, although similar, exhibit differences in many tissues [11,13,14], which may be a sign of the distinct signaling from GRFα receptors alone or in conjunction with Ret tyrosine kinase *in trans* [15]. We showed recently both in vitro and in vivo [16] that GDNF promotes survival of postnatal cochlear sensory neurons expressing GFRα1 mRNA but lacking Ret mRNA.

Here, we show that GDNF evokes Ret-independent signaling via GFRα1. We found that GDNF-stimulated GFRα1 activates Src family tyrosine kinases. In the absence of Ret GDNF-activated GFRα1/Src complex triggers phosphorylation of phospholipase Cγ (PLCγ), ERK1/ERK2 kinases (mitogen-activated protein kinase, MAPK) and cAMP response element binding protein (CREB) in Ret-deficient ($Ret^{-/-}$) dorsal root ganglion (DRG) neurons and in two Ret-negative cell lines.

2. Materials and methods

2.1. Neuronal cultures

DRG from embryonic day 18 (E18) mice were treated with trypsin (Worthington), non-neuronal cells removed by preplating, and about 95% pure neurons cultured on poly-ornithine-laminin-coated glass coverslips in Ham's F14 medium (Imperial Laboratories) with serum substitute containing nerve frowth factor (NGF), brain-derived neurotrophic factor (BDNF) and NT-3 (all from PeproTech, 2 ng/ml each) for at least 1 day before measurement. Neurotrophins were extensively washed out before GDNF application. GDNF was from PeproTech or donated by Cephalon, Inc. $Ret^{-/-}$ mice [17] were identified from heterozygote matings by the absence of kidneys and PCR-based genotyping.

2.2. RT-PCR analysis of GDNF receptors in the SHEP human neuroblastoma cell line

In the RT-PCR analysis of SHEP cells we used the primers for human GFRα1, GFRα2 and Ret. RT-PCR reaction was performed using a Titan One Tube RT-PCR kit (Boehringer Mannheim) according to the manufacturer's instructions. The primer sequences and PCR conditions were reported by Hishiki et al. [18].

2.3. Immunocomplex kinase assays and immunoblotting

DRG neurons (5×10^5), SHEP neuroblastoma cells (10^7) or

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Abbreviations: GDNF, glial cell line-derived neurotrophic factor; GFRα1, GDNF receptor α1; DRG, dorsal root ganglion; MAPK, mitogen-activated protein kinase; CREB, cAMP response element binding protein

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NIH3T3 cells (NIH3T3/pBpGFRα1) stably transfected with GFRα1 (10^7) were incubated in serum-free culture medium without (control) or with GDNF (GDNF-treated) for 0.5–15 min at 37°C. The cells were washed twice with cold phosphate buffered saline (PBS) containing 1 mM vanadate and lysed in TX-100 lysis buffer (50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 5 mM EDTA, 1% Triton X-100, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride and protease inhibitors (Boehringer)) on ice for 1 h. The postnuclear lysates (supernatants obtained after centrifugation of the whole lysate at 2000 rpm) were precleared with 50% protein G-Sepharose (1 h at +4°C). The lysates were incubated with GFRα1 antibodies (Santa Cruz Biotechnology) and immunocomplexes were collected with protein G-Sepharose. The immunocomplexes were washed twice with TX-100 lysis buffer without EDTA and twice with kinase buffer (25 mM HEPES, pH 7.4, 5 mM $MgCl_2$, 5 mM $MnCl_2$, 1 mM sodium orthovanadate). The immunocomplexes were incubated in a kinase buffer supplemented with 5 μCi of [γ-^{32}P]ATP for 20 min at 37°C. The samples were subjected to 10% SDS-PAGE and Western blotting. The radioactive signals were visualized with a Fuji Bioimage analyzer BAS 2000. For immunoblotting the membranes were probed with Src-2 antibodies (Santa Cruz Biotechnology) recognizing c-Src, Fyn and Yes.

2.4. Immunoprecipitation and Western blotting of PLCγ

PLCγ was immunoprecipitated as described in [19] from the cell lysate using anti-PLCγ antibodies (Upstate Biotechnology) and immunoblotted. The filter was probed with anti-phosphotyrosine 4G-10 antibodies (Upstate Biotechnology). The membranes were stripped, re-probed with anti-PLCγ antibodies and developed with ECL reagents.

2.5. MAPK and CREB phosphorylation assays

To assess GDNF-dependent MAPK and CREB phosphorylation in SHEP cells and NIH3T3/pBpGFRα1 fibroblasts, the cells were starved overnight in medium containing 0.5% serum and for 3 h in serum-free medium, and then GDNF was applied for the indicated time. For the analysis of MAPK and CREB activation in Ret$^{-/-}$ animals the DRG neurons were isolated from E18 mice and maintained in NGF-containing medium for 1 day. Then the neurons were deprived of NGF by placing them in NGF-free medium in the presence of blocking anti-NGF antibodies (Boehringer). After 2 h without NGF, the neurons were stimulated with GDNF. In the experiments involving Src kinase inhibition, a Src family kinase inhibitor, PP2, (Calbiochem) was added at the indicated concentrations to the cell monolayers 10 min before GDNF application. The proteins from whole cell lysates were resolved on SDS-PAGE and blotted. Western blots were probed with polyclonal antibodies (pAbs) to the activated forms of either MAPK (ERK1/2), JNK (Promega) or with an antibody that specifically recognizes the Ser-133-phosphorylated form of CREB (New England Biolabs). The blots were then reprobed with GFRα1 monoclonal antibodies (mAbs) (Transduction Laboratories) or CREB pAbs.

3. Results

3.1. GDNF stimulates GFRα1-coupled Src family kinases in SHEP neuroblastoma cells

Human neuroblastoma SHEP cells lack Ret and GDNF receptor α2 (GFRα2) mRNA but express GFRα1 mRNA (Fig. 1A). The presence of GFRα1 protein and the absence of Ret in these cells was further confirmed by Western blotting (data not shown). GPI-linked proteins have been shown to be localized to lipid rafts and directly associated with Src family kinases [20]. In SHEP cells, GDNF (100 ng/ml) increased the activity and protein amount of Src family kinases (Fig. 1B) detected in co-immunoprecipitates with anti-GFRα1 antibodies from Triton X-100 postnuclear lysates ($n = 5$ independent experiments). PLCγ has been shown to be a substrate for Src family kinases [19]. In the whole cell lysates GDNF rapidly activated PLCγ phosphorylation (Fig. 1C; $n = 3$ experiments). It has been shown that activation of endogenous

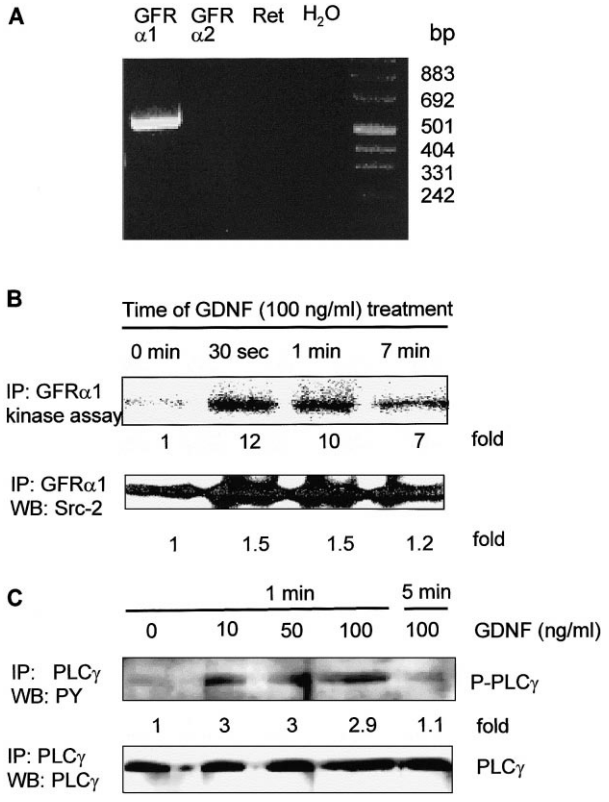

Fig. 1. GDNF activates Src family kinases associated with GFRα1 and induces PLCγ tyrosine phosphorylation in Ret-negative SHEP cells. A: RT-PCR analysis shows that SHEP cells express only GFRα1 (538 bp fragment), but not GFRα2 or Ret mRNA (expected fragments of 280 and 281 bp, respectively). H_2O lane represent a negative control without added mRNA. B: Upper panel: Triton X-100 postnuclear lysates of SHEP cells were immunoprecipitated (IP) with anti-GFRα1 antibodies and assayed for in vitro kinase activity ($n = 5$ independent experiments). The cells were non-treated (0 min) or treated with 100 ng/ml GDNF for the indicated time. The optical density of the bands was determined using a phosphoimager and a TINA program and is presented as fold increase relative to control (GDNF non-treated cells). Lower panel: After the kinase assay the samples were analyzed by Western blotting (WB) with Src-2 antibodies. C: Upper panel: SHEP cells were incubated with indicated concentrations of GDNF for 1 or 5 min. PLCγ proteins were immunoprecipitated from the lysates with anti-PLCγ antibodies. The immunocomplexes were analyzed for tyrosine-phosphorylated proteins by WB using 4G-10 antibodies (α-PY). Lower panel: The blot was re-probed with anti-PLCγ antibodies. In all panels the numbers below lanes indicate fold induction of PLCγ phosphorylation relative to control.

Ret in PC12 cells as well as activation of transiently or stably expressed Ret in COS-1 cells or NIH3T3 fibroblasts leads to the phosphorylation of c-Jun NH_2-terminal protein kinase (JNK) [20]. In our experiments GDNF (100 ng/ml) did not evoke JNK activation in SHEP cells lacking Ret ($n = 2$ experiments; data not shown).

3.2. GDNF activates ERK1/ERK2 and CREB

We further checked whether the activation of a GFRα1-coupled kinase would lead to phosphorylation of the MAPKs ERK1 and ERK2 in the absence of Ret, as Src kinases can activate MAPK [21]. In SHEP cells application of GDNF (100 ng/ml) evoked a rapid MAPK activation that lasted less than 1 h (Fig. 2A, $n = 4$ experiments). This pattern of MAPK activation was different from the elevation of

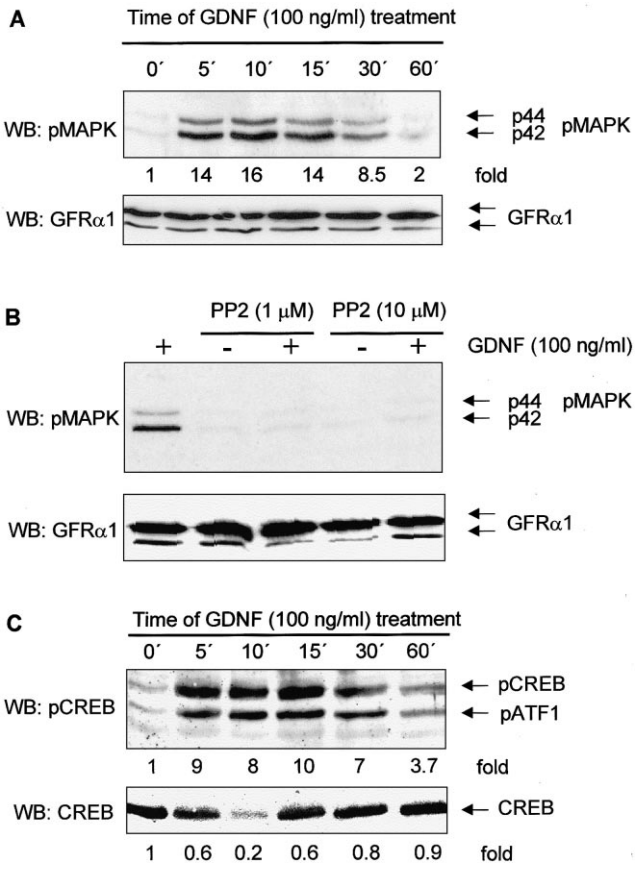

Fig. 2. GDNF evokes phosphorylation of MAPK, CREB and ATF-1 in the Ret-negative SHEP neuroblastoma cell line. A: Upper panel: Addition of GDNF evoked a transient and profound increase of p42/p44 MAPK phosphorylation. The numbers below the lanes indicate the fold induction of p42 phosphorylation relative to control. Lower panel: Re-probing of the same filter with anti-GFRα1 antibodies. B: Upper panel: GDNF-induced phosphorylation of p42/p44 MAPK was completely abolished by a 10-min pretreatment with PP2. Lower panel: The blot was re-probed with the anti-GFRα1 antibodies. C: Upper panel: GDNF treatment of SHEP cells resulted in potent induction of CREB Ser-133 phosphorylation as well as induced phosphorylation of the CREB-related protein ATF-1. The numbers below the lanes indicate the fold induction of CREB phosphorylation relative to control. Lower panel: Re-probing of the same filter with anti-CREB antibodies.

MAPK phosphorylation observed in Ret/GFRα1-expressing cells that lasted many hours [16] (Poteryaev, unpublished). The GDNF-dependent phosphorylation of MAPK was blocked by the Src-type kinase inhibitor PP2 already at a low concentration (1 μM) (Fig. 2B, $n=2$ separate experiments). In another Ret-negative but GFRα1-expressing cell line, 3T3NIH/pBpGFRα1 fibroblasts, GDNF (100 ng/ml) again increased phosphorylation of MAPK ($n=3$ experiments, data not shown), completely inhibitable by PP2. PP2 (0.1–10 μM) did not affect NGF-dependent TrkA-mediated activation of MAPK in PC12 (data not shown).

We further investigated whether GDNF affects phosphorylation of CREB and we found that GDNF potently induced phosphorylation of CREB at Ser-133 (Fig. 2C, $n=2$ experiments). Interestingly, GDNF also induced phosphorylation of the CREB-related protein ATF-1 (Fig. 2C). The dynamics of GDNF-induced Ret-independent CREB and ATF-1 phosphorylation was similar to that of GDNF-induced MAPK phosphorylation.

3.3. GDNF activates a GFRα1-coupled kinase, MAPKs and CREB in $Ret^{-/-}$ DRG neurons

To study Ret-independent signaling via GFRα1 in primary neurons, we used DRG neurons isolated from E18 $Ret^{-/-}$ mice. In vitro kinase assay of immunoprecipitates with GFRα1 antibodies from Triton X-100 postnuclear lysates of GDNF-stimulated $Ret^{-/-}$ DRG neurons revealed a major phosphoprotein of approximately 60 kDa, which corresponds to the M_r of several Src family tyrosine kinases (p59 Fyn, pp60 Src and p62 Yes) (Fig. 3A).

In these neurons application of GDNF (100 ng/ml) evokes a several-fold rapid activation of MAPK ($n=3$ experiments, Fig. 3B). BDNF has been shown to activate the Ras/ERK/pp90 ribosomal S6 kinase pathway that culminates in CREB phosphorylation [22]. We found that CREB phosphorylation

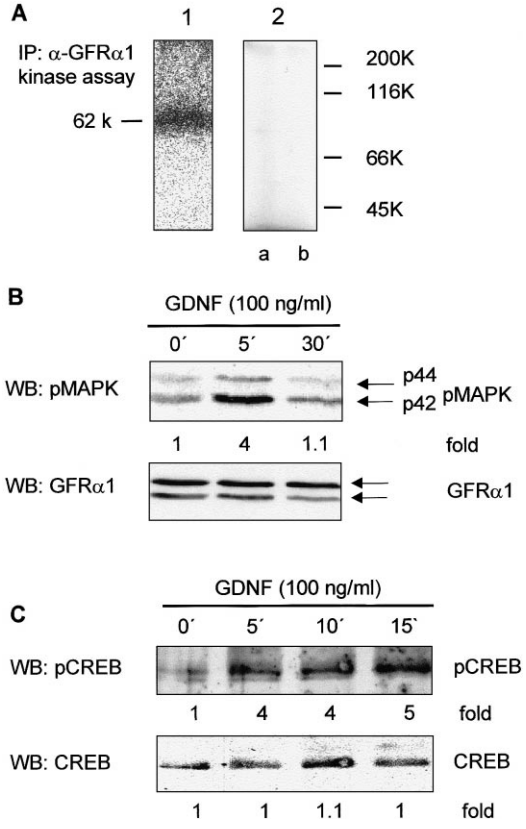

Fig. 3. GDNF induces phosphorylation of MAPKs and CREB in $Ret^{-/-}$ DRG neurons. A: Panel 1: Triton X-100 postnuclear lysates of GDNF (100 ng/ml, 5 min)-stimulated $Ret^{-/-}$ mouse DRG neurons were immunoprecipitated with GFRα1 antibodies, subjected to an in vitro kinase assay and revealed a major phosphorylated ~60 kDa band. Panel 2: This band was not seen in the control kinase assay performed in $Ret^{-/-}$ DRG neurons either with protein A-Sepharose alone (a) or with bFGF antibodies instead of GFRα1 antibodies (b). B: Upper panel: GDNF induces phosphorylation of p42 MAP kinases in $Ret^{-/-}$ DRG neurons. The numbers below the lanes indicate the fold induction of p42 band phosphorylation relative to control. Lower panel: Re-probing of the same filter with anti-GFRα1 mAb. C: Upper panel: GDNF induces an increase in CREB Ser-133 phosphorylation. The numbers below the lanes indicate the fold induction of CREB phosphorylation relative to control. Lower panel: Re-probing of the same filter with anti-CREB antibodies.

at Ser-133 increased substantially already at 5 min after GDNF treatment in $Ret^{-/-}$ DRG neurons (Fig. 3C). We did not observe GDNF-dependent phosphorylation of ATF-1 found in SHEP cells (Fig. 2C).

4. Discussion

In this study we have shown using $Ret^{-/-}$ DRG neurons and two different Ret-negative cell lines that GDNF activates Ret-independently Src family kinases and subsequently induces phosphorylation of ERK1/ERK2, CREB and ATF-1. GDNF also evokes PLCγ phosphorylation in SHEP cells. We therefore have demonstrated that GDNF can evoke potent intracellular signaling not only via established the GFRα1-Ret multicomponent receptor route [2,3,8–10], but also through a Ret-independent GFRα1-mediated pathway.

According to current understanding [23] GPI-anchored proteins, transmembrane tyrosine kinase proteins, G proteins and acylated tyrosine kinases of the Src family can all associate with so-called lipid rafts, a structure of sphingolipids and cholesterol packed into moving platforms within the liquid bilayer [23,24]. After Triton X-100 extraction, insoluble lipids and proteins remain in the form of detergent-insoluble glycolipid-enriched complexes or DIGs [23]. We found that Src family kinases can be co-immunoprecipitated with GFRα1 antibodies from DIGs of $Ret^{-/-}$ DRG neurons as well as from two different Ret-negative but GFRα1-expressing cell lines. In DIGs from SHEP neuroblastoma cells, GDNF evoked a potent transient activation of Src family kinases. These findings suggest that activation of Src family kinases by GDNF in Ret-negative DRG neurons, neuroblastoma cells and fibroblasts might occur within the lipid rafts.

As in the wild type DRG neurons, GDNF evoked potent activation of p42/p44 MAPK and CREB in the different Ret-deficient cell lines. GDNF-evoked phosphorylation of MAPK in SHEP cells was abolished with the selective Src kinase inhibitor PP2. Thus GDNF-evoked Ret-independent phosphorylation of MAPK is completely dependent on the GDNF-evoked activation of Src family kinases. Since the studies on neuronal survival showed that MAPK effectively phosphorylates CREB, it was of great interest to reveal the downstream targets of activated MAPK in Ret-negative neurons and cell lines. Indeed, we detected significant GDNF-dependent activation of CREB both in the $Ret^{-/-}$ DRG neurons and in the SHEP cells. Our results are therefore to some extent contradictory to those of Trupp et al. [12] who found that GDNF Ret-independently activates CREB phosphorylation but not the Ras/ERK pathway in the raphe nucleus-derived RN33B cell line. Interestingly, we also observed a robust GDNF-dependent activation of the CREB-related protein ATF-1 in Ret-negative SHEP cells but not in the $Ret^{-/-}$ DRG neurons. It is therefore possible that the discrepancies between our results and those reported by Trupp et al. [12] reflect the differences between the cells used. Ret-independent GDNF-evoked activation of the transcription factors of the CREB family can regulate gene expression. This can result in profound changes in neuronal plasticity [22]. Unfortunately, the study of the possible changes in neuronal plasticity invoked by GDNF in $Ret^{-/-}$- or GFRα1-deficient neurons is precluded by the early postnatal death of the knock-out animals. The morphological and biological consequences which might be triggered by GDNF-evoked Ret-independent signaling remain to be elucidated.

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