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Direct interaction of nerve growth factor receptor, TrkA, with non-receptor tyrosine kinase, c-Abl, through the activation loop

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Abstract The nerve growth factor receptor, TrkA, is essential for the survival and differentiation of neurons in the central and peripheral nervous systems. To understand the molecular principles underlying this differentiation step, we employed a yeast two-hybrid screening protocol using human TrkA as bait. We isolated c-Abl as a TrkA-interacting protein, in addition to known proteins such as phospholipase Cγ and SH2-B. This interaction was confirmed by an in vitro binding assay using glutathione *S*-tranferase–Abl fusion protein. Furthermore, we show here that c-Abl binds to phosphotyrosine residue(s) in the kinase activation loop of TrkA.

Key words: TrkA-interacting protein; c-Abl tyrosine kinase; SH2-B; Kinase activation loop

1. Introduction

Nerve growth factor (NGF) is a member of the family of neurotrophins and essential for the survival and differentiation of neurons in the central and peripheral nervous systems [1]. The binding of NGF to its receptor TrkA causes dimerization and activation of the receptor-associated tyrosine kinase leading to autophosphorylation of the cytoplasmic domain at multiple tyrosine residues. The newly formed phosphotyrosines constitute binding sites for Src homology 2 (SH2) domain- or phosphotyrosine binding (PTB) domain-containing cytoplasmic proteins which are thought to participate in the control of mitogenic or differentiation pathways, cell metabolism and/or cell morphology [2].

Autophosphorylation sites of human TrkA include the tyrosine residues Y670, Y674 and Y675, which are located within the activation loop of the tyrosine kinase domain and two tyrosine residues, Y490 and Y785, that lie outside of the kinase domain. The latter two tyrosine-residues provide binding sites for Shc and PLCγ, respectively [3,4]. In addition to these two proteins, the TrkA tyrosine kinase was reported to interact with several SH2 or PTB domain-containing proteins, such as Crk [5], SH2-B [6] and SNT/FRS-2 [7,8]. Activation of these signaling molecules culminates in downstream events involved in the promotion and maintenance of neuronal survival and differentiation. Shc and PLCγ are essential TrkA effector molecules. The integrity of both binding sites is required for the NGF induction of Ras-MAP kinase (MAPK) signaling and morphological differentiation of PC12 cells [3,9]. The other effector molecule, SH2-B, binds to Grb2 and may also mediate the Ras/MAPK pathway [6]. The association of TrkA with SH2-B plays a role for TrkA-mediated sympathetic neuron development [6] and PC12 cell differentiation [10].

To understand the molecular mechanisms of the TrkA-mediated differentiation pathways, we employed a yeast two-hybrid screening protocol based on the tyrosine-phosphorylated cytoplasmic domain of human TrkA as bait. We isolated c-Abl as a TrkA-interacting protein in addition to known proteins such as PLCγ and SH2-B. Furthermore, we show here that c-Abl binds to phosphotyrosine residue(s) in the kinase activation loop.

2. Materials and methods

2.1. Plasmid constructions and yeast two-hybrid screening

For expression of the cytoplasmic domains of wild-type (wt) and mutant TrkA and c-Fms as LexA-fusions, corresponding fusion genes were generated in pBTM116 and transformed into yeast strain L40 or YRN974 [11,12]. A single colony, selected for expression of the LexA–TrkA fusion-protein, was tested for TrkA autophosphorylation and used for transformation with a VP16 cDNA library derived from a 10–11 day mouse embryo [12–14]. GST–Abl, GST–SH2-B, GST–PLCγ and GST–TrkA fusion proteins were generated in the pGex system (Pharmacia, Freiburg, Germany).

2.2. Binding assay using the two-hybrid system

The qualitative and quantitative evaluations of various two-hybrid protein/protein interactions were described previously [11].

2.3. Cells and antibodies

NIH3T3 cells expressing the human *TrkA* gene (NIH3T3(TrkA)) were grown in Dulbecco's modified Eagle's medium supplemented with 10% FCS. Monoclonal antibody against phosphotyrosine (4G10) and polyclonal antibodies against PLCγ and TrkA were from Upstate Biotechnology Incorporated (Lake Placid, NY, USA) or from Santa Cruz Biotechnology (Santa Cruz, CA, USA), respectively.

2.4. Tyrosine kinase assays, immunoblotting

For tyrosine kinase assays, TrkA-specific immune complexes were incubated for 30 min at room temperature with 10 μCi of [γ-^{32}P]ATP (Amersham Buchler, Braunschweig, Germany) in the presence of 10 mM $MnCl_2$ and analyzed by SDS–PAGE. For the identification of proteins by immunoblotting, proteins were transferred onto PROTRAN nitrocellulose sheets (Schleicher and Schuell, Dassel, Germany) by a semi-dry blotting technique. Bound antibody was visualized by incubation of blots in 3 ml of 20 mM Tris–HCl, pH 7.6, containing 137 mM NaCl and 2 μCi of ^{125}I-labeled anti-species-specific immunoglobulin G (ICN, Eschwege, Germany). Bound radioactivity was quantified with a model BAS1500 bio-imaging analyzer (Fuji Photo Film Co., Kanagawa, Japan).

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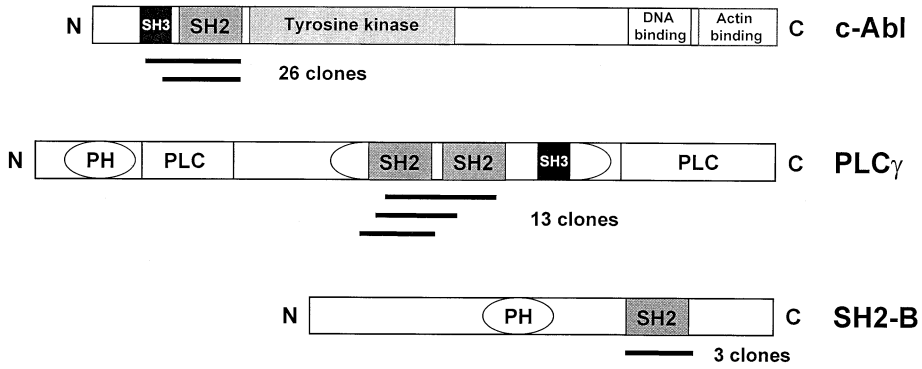

Fig. 1. Isolation of the SH2 domains of c-Abl, PLCγ and SH2-B as binding partners of TrkA tyrosine kinase. Schematic drawing of c-Abl, PLCγ and SH2-B. The various sizes of cDNAs encoding c-Abl, PLCγ and SH2-B obtained by the two-hybrid screening are presented as bars. PH: Pleckstrin homology domain; SH2: Src homology 2 domain; SH3: Src homology 3 domain.

2.5. In vitro transcription/translation

In vitro transcription/translation was performed using TNT coupled reticulocyte lysate systems (Promega, Madison, WI, USA).

2.6. GST-binding assay

Strain TKX-1 (TKX, Stratagene, La Jolla, CA, USA) was used for the isolation of phosphorylated GST–TrkA fusion proteins. Phosphorylated GST-fusion proteins were produced as recommended by the manufacturer [15]. The corresponding unphosphorylated molecular species were isolated from *Escherichia coli* strains DH5α or BL21(DE3)-pT-Trx (Trx) [16]. Purified GST-fusion proteins were bound for 1 h at 4°C to glutathione (GT) agarose beads (40 μl slurry; Pharmacia) suspended in binding buffer (50 mM HEPES, pH 7.5, 150 mM NaCl, 0.01% Triton X-100, 1% Trasylol and 400 μM sodium orthovanadate). Precharged beads were incubated overnight at 4°C with ^{32}P-labeled TrkA, as generated by in vitro autophosphorylation, or with ^{35}S-labeled c-Abl(SH2) as generated by in vitro transcription/translation in a total volume of 2 ml of binding buffer. Beads were washed five times with binding buffer and pellets were analyzed by SDS–PAGE.

3. Results and discussion

3.1. Isolation of c-Abl, SH2-B and PLCγ by yeast two-hybrid screening

To identify proteins which specifically interact with the cytoplasmic domain of the TrkA, we screened a mouse embryo cDNA library using a yeast two-hybrid system [13,17]. The bait consisted of the cytoplasmic domain of human TrkA fused to the C terminus of the DNA-binding and dimerization domain of LexA. Upon expression in yeast cells, dimerization leads to interchain tyrosine phosphorylation of the two TrkA receptor segments [14]. Screening of a cDNA library yielded altogether 52 cDNA clones of various length falling into four classes each of which encoded a different protein. Three of these classes encoded the previously identified SH2 domain containing proteins such as PLCγ, c-Abl and SH2-B (Fig. 1). In addition, we have isolated a novel binding partner, TR17, however, the biological significance of this protein remains to be studied. To our surprize, about 50% of cDNA encoded various sizes of c-Abl, all of which contain the c-Abl–SH2 domain (Fig. 1).

3.2. Autophosphorylated TrkA binds to GST–Abl fusion protein

We next wanted to confirm the interaction of these two proteins using GST–Abl (SH2 domain)-fusion protein. The ligand, ^{32}P-labeled TrkA polypeptide, was generated by in vitro autophosphorylation and was isolated from immunocomplexes by boiling in the presence of 1% SDS. Aliquots of the TrkA preparation were diluted with binding buffer and incubated with agarose beads that carried the GST fusion proteins. Bound material was analyzed by SDS–PAGE and autoradiography. As shown in Fig. 2, GST–Abl bound the TrkA rather stronger than GST–PLCγ, but no binding was observed with GST alone. However, we could not detect any significant association of TrkA and endogenous c-Abl by co-immunoprecipitation from PC12 cells as well as NIH3T3-(TrkA) in the absence or the presence of NGF, suggesting this interaction in vivo is rather weak or very transient.

3.3. TrkA binds directly to PLCγ, Abl, and Shc but not to Crk

To further analyze the TrkA/substrates interaction with other binding candidates such as the p85 subunit of PI-3′ kinase or Crk, we employed the yeast two-hybrid protocol

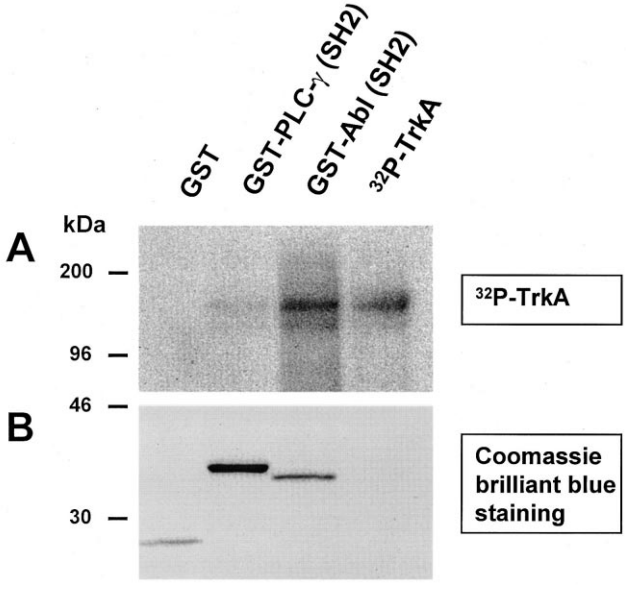

Fig. 2. GST–Abl (SH2) fusion protein binds to the tyrosine phosphorylated TrkA. Precharged beads with purified GST-fusion proteins were incubated overnight at 4°C with ^{32}P-labeled TrkA (^{32}P-TrkA), as generated by in vitro autophosphorylation. Following washing, bound protein was analyzed by SDS–PAGE (A). As a control, aliquots were analyzed by SDS–PAGE and stained with Coomassie brilliant blue (B).

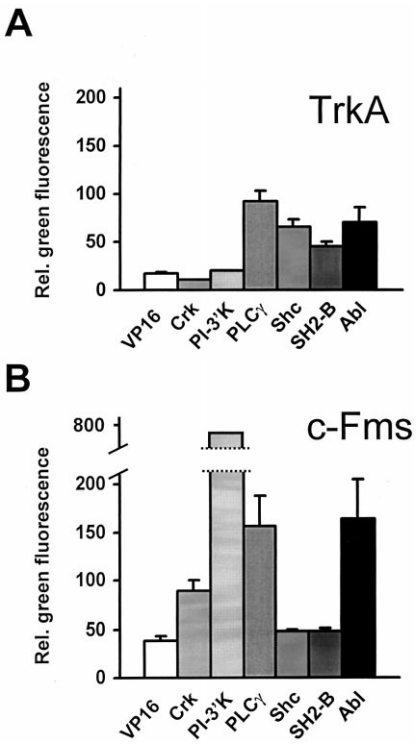

Fig. 3. TrkA binds directly to PLCγ, Shc, SH2-B and c-Abl, but not to Crk and p85 of PI-3 kinase. pBTM116 carrying cloned cDNAs encoding the cytoplasmic domains of either TrkA (A) or c-Fms (B) were co-transformed with pVP16 carrying the cDNA of the SH2 domains of Crk, p85 subunit of PI-3′ kinase, PLCγ, SH2-B or c-Abl, or the PTB domain of Shc in YRN974 [11]. 10 000 cells of four independent transformants were analyzed for fluorescence intensity using a Becton Dickinson FACScalibur flow cytometer. Values represent the mean values of four independent experiments. Rel. green fluorescence: relative green fluorescence.

using strain YRN974 containing a chromosomally integrated LexA-Operator-GFP cassette. As a control, we included c-Fms as bait [11,12]. Western blotting with anti-phosphotyrosine antibody clearly demonstrated that c-Fms as well as TrkA moieties were phosphorylated on tyrosine in the yeast two-hybrid assay (data not shown). As expected, Shc, PLCγ, SH2-B and c-Abl bind to TrkA, however, no association could be demonstrated between TrkA and the SH2 domain of p85 subunit of PI-3′ kinase and also Crk which associated with c-Fms in this system (Fig. 3), suggesting these proteins may form a complex with TrkA but do not associate directly (Fig. 3). It has been reported that NGF induces the complex formation of TrkA with Crk [5]. Hence, Crk may interact with TrkA via other binding proteins such as FRS-2 [8] or c-Abl [18].

3.4. Abl binds to the phosphorylated activation loop of TrkA

To study binding properties of the SH2 domain of Abl to TrkA, similar binding analyses were performed again using the yeast two-hybrid protocol with a series of TrkA mutants. We determined whether binding required the presence of activated TrkA molecules. As a control, we therefore included a kinase-inactive TrkA-molecule in which the ATP binding site K538 was replaced by a methionine residue (K538M). Association of TrkA with Abl was observed in kinase active TrkA exclusively (Fig. 4A). In addition, we generated several TrkA mutants including D668V-, Y490F-, Y785F- and its double mutant TrkAYY490/785FF. In agreement with previous data [3,4], replacement of Y490 or Y785 abolished the binding of Shc or PLCγ, respectively (Fig. 4A). All tyrosine-mutants, however, bind to c-Abl and SH2-B equally well (Fig. 4A), indicating that one of the Abl or SH2-B binding site(s) is different from these residues. SH2-B was found to associate with a TrkA mutant lacking all tyrosines except the three phosphotyrosine residues within the activation loop of the kinase domain, suggesting these core tyrosines in the activation loop may provide the binding site for SH2-B [6]. Since TrkA mutants lacking tyrosine residues within the activation loop are deficient in kinase activity, we generated tyrosine phosphorylated GST–TrkA (amino acid number: 667–686) fusion protein which contains three tyrosines, Y670, Y674 and Y675, within the activation loop. As a control, we generated the GST–TrkA (749–790) which contains the PLCγ binding site, Y785. Both fusion proteins were phosphorylated on tyrosine in the bacteria strain TKX (Fig. 4C). In agreement with previous data (Fig. 4A), only tyrosine phosphorylated GST–TrkA (749–790) associated with PLCγ (Fig. 4C). Interestingly, the SH2 domain of c-Abl binds to only tyrosine phosphorylated GST–TrkA (667–686) (Fig. 4C), indicating that at least one of the core tyrosine residues in the activation loop provides the binding site for c-Abl. Can a core phosphotyrosine be the binding site for its substrate in vivo? The members of the Trk-family and insulin receptor (IR)-family contain three tyrosine residues in the activation loop, all of which are phosphorylated [3,19–21]. Among these tyrosine residues, only the second tyrosine residue is the positional equivalent of core tyrosine residue in other receptor tyrosine kinases, such as the platelet-derived growth factor receptor, c-Fms, c-Kit, or the epidermal growth factor receptor. It has been shown that phosphotyrosine residues of IR in the activation loop bind to the SH2 domain of tyrosine phosphatase, SHP-2 [22] and the PTB domain of insulin receptor substrate-2 [23], suggesting the activation loop of this type of receptor provides the binding site for its substrate. These core tyrosine residues in the activation loop are phosphorylated prior to Y490 and Y785 upon stimulation with NGF [24]. Therefore, we asked next whether the binding of c-Abl modulates the tyrosine kinase activity of TrkA. In vitro autokinase reaction of TrkA in the presence or absence of the SH2 domain of c-Abl as well as SH2-B did not influence the degree of autophosphorylation of TrkA (data not shown). However, we cannot rule out that the binding of substrates at the activation loop of tyrosine kinase has an influence on phosphorylation of other sites in vivo.

Furthermore, we found that the replacement of conserved D668 into the V in the kinase activation loop strongly reduced the association of Abl, Shc, SH2-B and PLCγ (Fig. 4A), suggesting that tyrosine kinase activity was impaired by this replacement. This finding is different from other receptors such as c-Met, c-Fms or c-Kit. The mutation at the corresponding D was originally found in the c-Kit mutant that is expressed in mastocytoma [25]. This mutation causes the permanent activation of c-Kit tyrosine kinase, and also in the yeast two-hybrid system c-Kit D816V mutant was highly activated [12,26]. Except for TrkA tyrosine kinase, other types of receptor tyrosine kinases, such as c-Fms [27], c-Met [28] and c-Ron [29], were also activated by the corresponding point mutation. Is the structure of TrkA activation loop different from

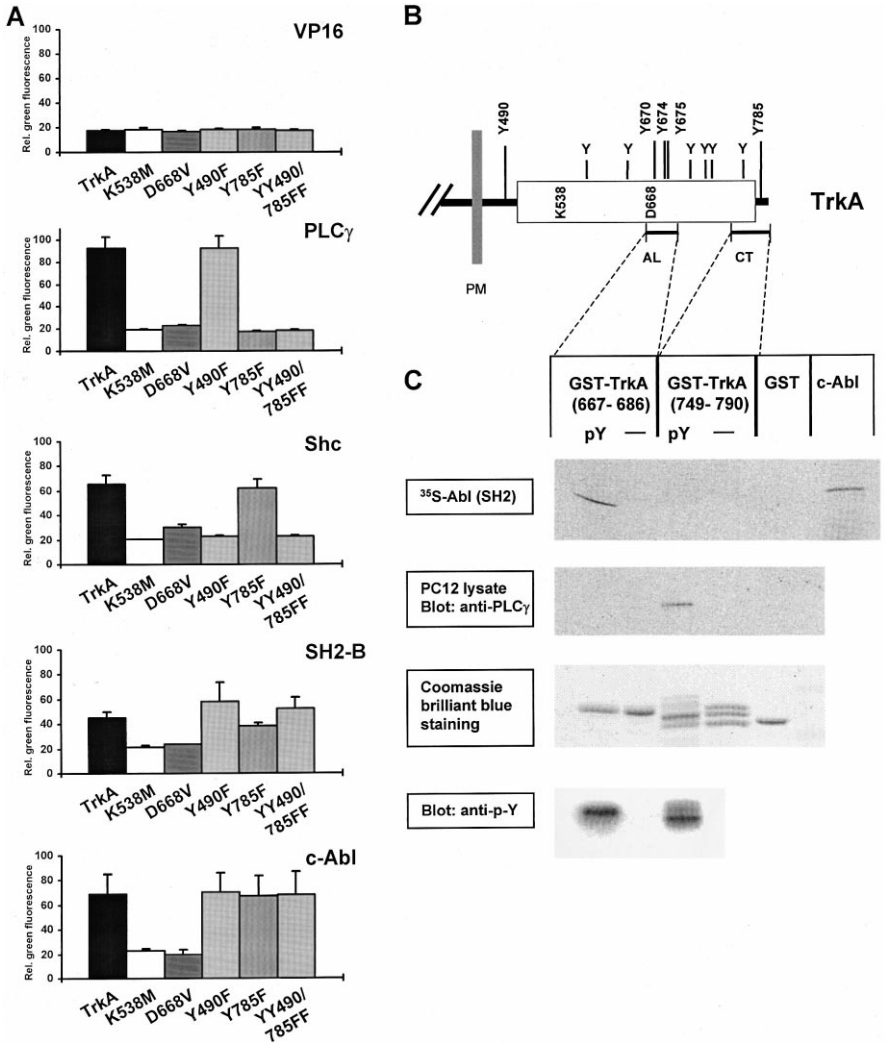

Fig. 4. The SH2 domain of c-Abl binds to the phosphorylated activation loop of the TrkA. A: Interaction of PLCγ, Shc, SH2-B and c-Abl with wild-type (wt) and mutant TrkAs. The SH2 domain of PLCγ, SH2-B and c-Abl and the PTB domain of Shc were co-expressed as VP16 fusion proteins together with the wild-type and mutant LexA-TrkA fusion proteins in YRN974. Mutants carry tyrosine/phenylalanine replacements as indicated. K538M: a kinase-negative TrkA mutant lacking the ATP-binding site. D668V: the conserved D668 was replaced by V. B: Schematic drawing of the cytoplasmic domain of TrkA. PM: plasma membrane; AL: activation loop; CT: C-terminal domain. Tyrosine residues, ATP-binding site K538 and conserved D668 in the activation loop are indicated. The open box represents kinase domain. C: GST–TrkA (667–686) containing three tyrosines, Y670, Y674 and Y675, in the activation loop binds to c-Abl. GST–TrkA (667–686), GST–TrkA (749–790), or GST alone, were produced in bacterial strains DH5(a), BL21(DE3)-pT-Trx (Trx) or TKX to generate either non-phosphorylated or phosphorylated TrkA-species, respectively. GST-fusion protein or GST were bound to GT-agarose beads and incubated with ^{35}S-labeled SH2 domain of c-Abl. Bound protein was analyzed by SDS-PAGE. As a control, cell lysate obtained from PC12 was incubated with GST-fusion proteins and bound proteins were analyzed by immunobloting using polyclonal antibody against PLCγ. To demonstrate tyrosine phosphorylation of TrkA (667–686) and TrkA (749–790) from strain TKX, aliquots of the same samples were analyzed by immunoblotting using an anti-phosphotyrosine monoclonal antibody. As a control, aliquots were analyzed by SDS–PAGE and stained with Coomassie brilliant blue.

those of these receptors? Recently, Cunningham and Greene [30] proposed a 'switch' model for Trk activation. These authors identified residues that form specific charged pairs with each of the three TrkA activation loop phosphotyrosines. These residues include H639 (for pY670), R676 and R680 (for pY674) and R643 and R667 (for pY675). Taken together, the replacement of D668 may cause conformational changes of the activation loop, modulating the multiple step-activation of Trk tyrosine kinase.

There are several potential roles of c-Abl which may involve the signal transduction pathways mediated by TrkA signaling. Firstly, an anti-proliferative effect of NGF was shown to be accompanied by the accumulation of cells in the G1 phase [31]. Here, events affecting the action of G1 regulatory proteins, such as c-Abl [32] may be involved. Secondly, since the interaction of Crk and c-Abl is well documented [18], Crk may interact with TrkA via c-Abl and be phosphorylated on tyrosine. Thirdly, both TrkA and c-Abl play roles in the integrin mediated signal pathways. NGF stimulates the accumulation of integrin at the tips of filopodia in the growth cones of sympathetic neurons [33] and it has been reported that integrin regulates c-Abl activity and cytoplasmic-nuclear transport [34]. Finally, recent evidence has accumulated indicating that c-Abl and its related kinase, Arg, play fundamental roles in the development and function of the mammalian central nervous system [32,35]. Furthermore, it has been well demonstrated in the *Drosophila* system that the Abl tyrosine kinase controls motor axon outgrowth [36,37]. Clearly, addi-

tional experiments are required to support the potential role(s) of c-Abl in TrkA mediated signal pathways.

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