Targeting the *cis*-dimerization of LINGO-1 with low MW compounds affects its downstream signalling

L Cobret¹, M L De Tauzia¹, J Ferent², E Traiffort¹, I Hénaoui¹,4, F Godin¹, E Kellenberger⁵,6, D Rognan⁵,6, J Pantel⁷, H Bénédetti¹ and S Morisset-Lopez¹

¹Centre de Biophysique Moléculaire, Département biologie cellulaire et cibles thérapeutiques, CNRS, UPR 4301, University of Orléans and INSERM, Orléans, France, ²Institut de Recherches Cliniques de Montréal, Montréal, Quebec, Canada, ³INSERM-Université Paris Sud U788, Le Kremlin-Bicêtre, France, ⁴Institut de Pharmacologie Moléculaire et Cellulaire, Valbonne, France, ⁵Laboratoire d’Innovation Thérapeutique, UMR 7200 CNRS, Université de Strasbourg, Illkirch, France, ⁶MEDALIS Drug Discovery Center, Illkirch, France, and ⁷Centre de Psychiatrie et Neurosciences, INSERM U894, Paris, France

BACKGROUND AND PURPOSE

The transmembrane protein LINGO-1 is a negative regulator in the nervous system mainly affecting axonal regeneration, neuronal survival, oligodendrocyte differentiation and myelination. However, the molecular mechanisms regulating its functions are poorly understood. In the present study, we investigated the formation and the role of LINGO-1 *cis*-dimers in the regulation of its biological activity.

EXPERIMENTAL APPROACH

LINGO-1 homodimers were identified in both HEK293 and SH-SY5Y cells using co-immunoprecipitation experiments and BRET saturation analysis. We performed a hypothesis-driven screen for identification of small-molecule protein–protein interaction modulators of LINGO-1 using a BRET-based assay, adapted for screening. The compound identified was further assessed for effects on LINGO-1 downstream signalling pathways using Western blotting analysis and AlphaScreen technology.

KEY RESULTS

LINGO-1 was present as homodimers in primary neuronal cultures. LINGO-1 interacted homotypically in *cis*-orientation and LINGO-1 *cis*-dimers were formed early during LINGO-1 biosynthesis. A BRET-based assay allowed us to identify phenoxybenzamine as the first conformational modulator of LINGO-1 dimers. In HEK-293 cells, phenoxybenzamine was a positive modulator of LINGO-1 function, increasing the LINGO-1-mediated inhibition of EGF receptor signalling and Erk phosphorylation.

CONCLUSIONS AND IMPLICATIONS

Our data suggest that LINGO-1 forms constitutive *cis*-dimers at the plasma membrane and that low MW compounds affecting the conformational state of these dimers can regulate LINGO-1 downstream signalling pathways. We propose that targeting the LINGO-1 dimerization interface opens a new pharmacological approach to the modulation of its function and provides a new strategy for drug discovery.

Abbreviations

DSP, Dithiobis[succinimidyl] propionate; EGFR, EGF receptor; ER, endoplasmic reticulum; HA, haemagglutinin-tag; IP, immunoprecipitation; OPCs, oligodendrocyte precursor cells; RLuc, Renilla luciferase; SMPPIM, small-molecule protein–protein interaction modulator; trkB, tropomyosin-related kinase B; YFP, yellow fluorescent protein
Tables of Links

<table>
<thead>
<tr>
<th>TARGETS</th>
<th>LIGANDS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Catalytic receptors</td>
<td>Phenoxycobenzamine</td>
</tr>
<tr>
<td>EGFR, EGF receptor</td>
<td></td>
</tr>
<tr>
<td>p75NTR, nerve growth factor receptor (TNFRSF16)</td>
<td></td>
</tr>
<tr>
<td>TrkB, neurotrophic receptor tyrosine kinase</td>
<td></td>
</tr>
<tr>
<td>TROY, TNF receptor (TNFRSF19)</td>
<td></td>
</tr>
<tr>
<td>Enzymes</td>
<td>EGF</td>
</tr>
<tr>
<td>Akt</td>
<td></td>
</tr>
<tr>
<td>ERK1/2</td>
<td></td>
</tr>
<tr>
<td>GPCR</td>
<td></td>
</tr>
<tr>
<td>5-HT4 receptor</td>
<td></td>
</tr>
</tbody>
</table>

These Tables list key protein targets and ligands in this article which are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Pawson et al., 2014) and are permanently archived in the Concise Guide to PHARMACOLOGY 2013/14 (Alexander et al., 2013a,b,c).

Introduction

The transmembrane protein LINGO-1 is a new therapeutic target in several neurodegenerative diseases including Parkinson’s disease, chronic glaucoma, amyotrophic lateral sclerosis and multiple sclerosis (Mi, 2008; Rudick et al., 2008; Bessero and Clarke, 2010; McDonald et al., 2011). This single transmembrane protein composed of 620 amino acids displays a large extracellular region consisting of 12 leucine-rich repeat motifs followed by an immunoglobulin domain, and a short cytoplasmic tail. LINGO-1 is selectively expressed in brain and spinal cord on both oligodendrocytes and neurons, and it is not detectable in non-neural tissues (Carim-Todd et al., 2003; Mi et al., 2004; Okafuji and Tanaka, 2005; Barrette et al., 2007; Llorens et al., 2008).

LINGO-1 is a potent negative regulator of neuritic growth and neuronal survival. The combination of four different approaches to reducing endogenous LINGO-1 function in neurons (LINGO-1 RNAi, dominant negative LINGO-1, LINGO-1-Fc and LINGO-1 knockout) demonstrated that the inhibition of LINGO-1 improves survival of dopaminergic neurons (Inoue et al., 2007) and increases neuronal differentiation (Loov et al., 2012), neurite outgrowth (Mi et al., 2004) and axonal sprouting (Ji et al., 2006a).

LINGO-1 also plays a key role in the negative regulation of oligodendrocyte differentiation as well as myelination and remyelination. LINGO-1 functions as a constitutive inhibitor of the differentiation of oligodendrocyte precursor cells (OPCs) into mature oligodendrocytes. All of the strategies for reducing endogenous LINGO-1 function in OPCs invariably result in the observation of morphological changes that are characteristic of highly differentiated and mature cells of the oligodendroglial lineage (Mi et al., 2005; 2009). By contrast, the over-expression of full-length LINGO-1 had the opposite effects (Mi et al., 2005; Lee et al., 2007; Zhao et al., 2007).

Although LINGO-1 was discovered over 10 years ago (Carim-Todd et al., 2003), the molecular mechanisms involved in the regulation of its functions have remained elusive. Nevertheless, LINGO-1 has been shown to interact homotypically and heterotypically with other membrane proteins. LINGO-1 physically interacts likely in cis (i.e. on the same plasma membrane) with the Nogo-A receptor (NgR1) and the p75 neurotrophin receptor (p75NTR; Mi et al., 2004). The formation of the ternary complex LINGO-1/NgR1/p75NTR on neuronal growth cones is essential for the inhibition of neurite regeneration and axonal outgrowth (Mi et al., 2004). The ligands of this complex, Nogo-66, myelin-associated glycoprotein and oligodendrocyte-myelin glycoprotein, are myelin components that each bind to NgR1, using its coreceptors to transduce the activation of the RhoA pathway thereby leading to actin depolymerization and then the collapse or retraction of neurites (Yamashita et al., 2005). LINGO-1 inhibitors (LINGO-1-Fc, LINGO-1-ΔCter) block RhoA activation and reverse the growth inhibitory effects of myelin components (Mi et al., 2004). LINGO-1 also interacts with and mediates the action of TNF receptor orphan Y (TROY). The higher expression of TROY compared with p75NTR in the brain, particularly after birth suggests that TROY can substitute for p75 NTR in the LINGO-1/NgR1 complex to activate the RhoA pathway upon interaction with NgR1 ligands (Park et al., 2005; Shao et al., 2005). However, the existence of additional signalling co-factors has been suggested based on the absence of p75 and TROY on LINGO-1 expressing neurons projecting to the spinal cord (Barrette et al., 2007). Zhang and collaborators have identified several candidates co-factors that directly interact with LINGO-1 using the intracellular domain of Lingo-1 as bait, including the serine threonine kinase With No Lysine K (WNK1; Zhang et al., 2009). In addition, LINGO-1 is also able to directly interact with the EGF receptor (EGFR) thereby inhibiting its downstream signalling and leading to the decrease of neuronal survival (Inoue et al., 2007). Until recently, it was considered unlikely that LINGO-1 functioned as a stand-alone receptor because its cytoplasmic domain did not appear to contain any signalling motifs, and no known soluble ligand has yet been identified. However, a recent study demonstrated that LINGO-1 functions both as a ligand and a receptor through trans self-interactions (i.e. between two cells) to inhibit myelination (Jepson et al., 2012). Interestingly, it has been proposed that the secreted glycoprotein myocilin interacts with LINGO-1 and may be considered as a LINGO-1 ligand (Kwon et al., 2014).

The role of dimerization as a step for receptor activation is generally accepted for several classes of receptors. There is some evidence that some RTKs are activated by ligand-induced dimerization (Schlessinger, 2002; Simi and Ibanez, 2010). An increasing number of studies also indicate that some RTKs and GPCRs are present as pre-formed dimers and
are activated by a conformational change within the dimers upon agonist addition (Ferre et al., 2014; Maruyama, 2014). Receptor dimerization is recognized as a crucial step in the activation of many plasma membrane receptors (Simi and Ibanez, 2010). Therefore, the aim of the present study was first to explore whether LINGO-1 could interact homotypically in cis-oriented orientation and then, to identify low MW compounds able to bind to these oligomeric complexes, to modulate their conformation and subsequently LINGO-1-mediated signalling. Using the BRET method, we demonstrate that LINGO-1 forms homodimers through cis-orientation in living cells. We report that this constitutive dimerization occurs both in endoplasmic reticulum (ER) and on the plasma membrane. We also demonstrate that a LINGO-1 BRET-based assay may be used as a robust and a mainstreamed platform for screening of LINGO-1 ligands and/or small-molecule protein–protein interaction modulators (SMPPIMs). Finally, the screening of approximately 1000 chemical compounds allowed us to identify phenoxymethylsulfonylmethylsulfonyl fluoride as a low MW compound able to enhance LINGO-1-mediated intracellular signalling in HEK-293 cells.

Methods

Antibodies

For Western blot analysis, polyclonal anti-LINGO-1 was purchased from R & D Systems (Abingdon, UK); anti-haemagglutinin-tag (HA) from Roche Diagnostics (Meylan, France), anti-Flag from Sigma Aldrich (St Louis, MO, USA), a living colour anti-YFP antibody from BD Bioscience Clontech; anti Na/K ATPase from Tebu-bio (Le Perray-en-Yvelines, France) and anti-calnexin from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Polyclonal anti-phospho-EGFR (Tyr1173), phospho-Akt (Ser 473) and phosphorylated ERK1/2 (ppERK1/2) were obtained from Cell Signaling Technology (Danvers, MA, USA). Goat anti-mouse and goat anti-rat-IgG HRP-linked whole antibodies were obtained from Invitrogen Life Technologies.

Plasmid constructs

The human HA-tagged LINGO-1 M1-I620 (LINGO-1-HA) was amplified by PCR from the plasmid corresponding to the clone IMAGE ID (GenBank accession number NM_032808) with specific primers. Primers were designed to introduce the sequence of the HA tag in the C-terminal part of LINGO-1. Then, the PCR product was subcloned into pcDNA3 (Invitrogen) using HindIII and EcoRI restriction sites to generate 5-HT6 receptors with C-terminal fusions of YFP (5-HT6-YFP) and RLuc (5HT6-RLuc). A truncated LINGO-1, M1-W582 (LINGO-1-ACter), containing the extracellular and the transmembrane domain of LINGO-1, was cloned into the HindIII and Nhe1 sites of the pcDNA3. All constructs were checked by direct DNA sequencing. Plasmid containing the trkB sequence was obtained from Yves-Alan Borde (Bibel et al., 1999; Addgene plasmid 39978).

Animals

All animal care was in accordance with the principles of laboratory animal care of guidelines published by the French Ethical Committee (laboratory agreement n° C 45-234-9) and under the supervision of the authorized investigator (n°75-1331). All experimental procedures in this study complied with the animal welfare guidelines of the European Community and were approved by the local ethical committee CECCO 03. Studies involving animals are reported in accordance with the ARRIVE guidelines for reporting experiments involving animals (Kilkenny et al., 2010; McGrath et al., 2010). A total of 6 animals were used in the experiments described here.

Cell cultures and transfections

HEK-293 cells were grown in DMEM supplemented with 10% (vol/vol) FBS, 1 g L−1 glucose, 100 U mL−1 penicillin, 0.1 mg mL−1 streptomycin and 1 mM glutamine. For BRET experiments and for the generation of stable cell lines, cells were transfected with the calcium phosphate precipitation method. Stably transfected HEK-293 cell lines co-expressing LINGO-1-RLuc and LINGO-1-YFP were maintained in the medium described above with the addition of 2 mg mL−1 of G418.

Cortical neurons were prepared from rat embryos at embryonic day 18. Neurons were plated on polyornithine-coated plastic culture dishes (50 000 cells per cm2). Cultures were grown in neurobasal medium (Invitrogen) supplemented with B27 (Invitrogen) and 2 mM L-glutamine.

Cell fractionation studies

Stably transfected cells co-expressing LINGO-1-RLuc and LINGO-1-YFP were washed with PBS, scraped off and lysed with cold lysis buffer (20 mM HEPES, pH 7.4, 2 mM EDTA, 2 mM EGTA, 6 mM MgCl2, 1 mM PMSF and protease cocktail inhibitor). Cell suspensions were sonicated (on ice, three times for 30 s), and lysates were centrifuged at 10 000×g for 5 min. The supernatant was collected and 2 M sucrose was added to achieve a final concentration of 0.2 M. Cell lysates were applied to the top of a discontinuous sucrose step gradient (5 mL per step), made at 0.5, 0.9, 1.2, 1.35, 1.5 and 2.0 M sucrose in lysis buffer. The samples were centrifuged in a Beckman SW28 rotor (27 000×g for 16 h). Fractions were then submitted to fluorescence/luminescence and BRET analysis. The identification of plasma membrane and...
endoplasmic reticulum (ER)-enriched fractions was achieved by Western blot analysis.

**BRET measurements**

Forty-eight hours after transfection, HEK-293 cells or cultured cortical neurons were detached with versene (Invitrogen) and resuspended in HBSS saline buffer (Invitrogen). Intact cells or membranes were distributed in 96-well microplates (Optiplate, Perkin Elmer) and incubated for 15 min at 25°C in the absence or presence of the indicated ligands. Coelenterazine H substrate (Molecular Probes) was added at a final concentration of 5 μM, and reading was performed with a Mithras LB 940 Multitreader (Berthold, Bad Widbad, Germany), which allows the sequential integration of luminescence signals detected with two filter settings (RLuc filter, 485 ± 10 nm; YFP filter, 530 ± 12 nm). Emission signals at 530 nm were divided by emission signals at 485 nm. The BRET ratio was defined as the difference between the emission ratio obtained with co-transfected RLuc and YFP fusion proteins and that obtained with the RLuc fusion protein alone. The results were expressed in milliBRET units (mBU, with 1 mBU corresponding to the BRET ratio values multiplied by 1000). BRET\textsubscript{max} is the maximal BRET signal obtained in milliBRET units and BRET\textsubscript{50} represents the ratio of acceptor and donor receptors (acceptor/donor) yielding 50% of the maximum BRET signal. All BRET, luminescence, fluorescence measurements were performed at 21°C using a Mithras LB 940 microplate analyser (Berthold, Bad Widbad, Germany).

**Western blotting**

The cell lysates, immunoprecipitates or membranes from HEK-293 transfected cells or cortical neurons were separated by electrophoresis on SDS/PAGE (8% or 10% gels) and transferred to PVDF membranes (GE Healthcare Life Sciences). Blots containing HA or YFP-tagged proteins were probed with a rat anti-HA antibody (1:5000), or a rabbit anti-BD living colours full-length polyclonal antibody (Clontech). Immunoblots were also probed with a goat anti-LINGO-1 polyclonal antibody (LifeSpan, Biosciences, Seattle, WA, USA). For the analysis of EGF signalling pathways, membranes were incubated with antibodies against phospho-EGF\textsubscript{R} (Tyro3, Bki, Sprouty), phospho-Akt (Ser473) or pERK1/2. HRP-conjugated rabbit anti-goat, anti-mouse or anti-rat antibodies (1:33 000) were used as secondary antibodies. Immunoreactive bands were detected using the Pico or Dura detection kit. For quantification, blots were analysed using Genetools software (Syngene, Frederick, MD, USA).

**AlphaScreen assays**

The AlphaScreen SureFire phospho-ERK assay (Perkin Elmer) was also used to quantify pERK1/2. HEK-293 cells were transfected using Lipofectamine 2000 with LINGO-1-YFP (HEK-LINGO-1) or with peYFP-N1 plasmids (control cells). At 24 h after transfection, cells were detached and plated in 24-well plates (2 × 10\textsuperscript{4} cells per well). Then, the medium was replaced with medium without serum 16 h before assays. At 48 h after transfection, cells were washed with HBSS buffer and then incubated with vehicle or EGF (5 ng·mL\textsuperscript{-1}) in the absence or presence of phenoxybenzamine (10 μM or specified concentrations) diluted in HBSS. Cells were lysed 7 min after EGF stimulation in lysis buffer (from Perkin Elmer kit) and pERK1/2 was quantified according to the manufacturer’s instructions.

**Data analysis**

Results are expressed as means ± SEM. All data analyses were carried out with the GraphPad Prism 4 software for Windows (GraphPadSoftware Inc, San Diego, CA, USA). Concentration-response curves were fitted by nonlinear regression and saturation curves by a hyperbolic one-binding site equation. The method provided estimates for EC\textsubscript{50} values, BRET\textsubscript{max} and BRET\textsubscript{50} values and their SEM. Statistical analysis of the difference of the BRET\textsubscript{max} values or BRET\textsubscript{50} Values was determined using Student’s t test. Statistical significance of the difference in the detected BRET signals between assays with or without protein competitors was determined using one-way ANOVA using GraphPad Prism software. Statistical significance of the effect of phenoxybenzamine observed in HEK control cells compared to those obtained in HEK-LINGO-1 cells was analysed by two-way ANOVA followed by Newman-Keuls multiple comparisons tests, using GraphPad Prism software.

**Materials**

Tris-HCl, Tween-20, Triton X100 were obtained from Sigma Aldrich (St Louis, MO, USA). PBS was purchased from Euromedex (Souffelweyersheim, France). Coelenterazine substrate was obtained from Interchim (Montluçon, France). A protease inhibitor cocktail was obtained from Roche (Mannheim, Germany). PVDF membranes, CL-X film were purchased from GE Healthcare (Chalfont St. Giles, UK). Dithiobis-succinimidyl propionate (DSP), SuperSignal West Pico and SuperSignal extended Dura chemiluminescent substrates were purchased from Thermo Fisher Scientific Inc. (Rockford, IL, USA); the plasmids peYFP-N1 was purchased from BD Bioscience Clontech (San Jose, CA, USA). Platinum Taq high fidelity DNA polymerase was obtained from Invitrogen™, Life Technologies (Carlsbad, CA, USA). AgeI, HindIII, KpnI, BsrGI and EcoR1 restriction enzymes were obtained from New England Biolabs France (Evry, France). Recombinant human EGF was obtained from Peprotech (Rocky Hill, CT, USA). Clones IMAGE ID coding for LINGO-1 was from BioValley (Conches, France). We thank M Cazorla for kindly providing the tropomyosin-related kinase B (trkB)-yellow fluorescent protein (YFP) plasmid. All media for cell cultures were obtained from PAA Laboratories (Les Mureaux, France). The Prestwick Chemical Library was purchased from Prestwick Chemical Inc (Illkirch, France).
Results

Self-interaction of LINGO-1 in HEK-293 cells and cortical neurons

To determine whether LINGO-1 can interact with itself in a cellular system, we performed co-IP analyses of HA- and YFP-tagged LINGO-1 transiently expressed in HEK-293 cells. We tested whether LINGO-1-YFP could be co-immunoprecipitated with the HA-tagged construct by using specific HA antibodies. As a control, the analysis was also carried out with cells expressing LINGO-1-YFP and empty pcDNA3-HA vector only. Under these conditions, YFP-tagged LINGO-1 was specifically co-immunoprecipitated with HA-tagged LINGO-1, indicating an interaction between the two proteins (Figure 1A). To further prove the specificity of our approach, we performed the same experiment in reverse by immunoprecipitating the LINGO-1-YFP with anti-YFP antibodies. As shown in Figure 1B, LINGO-1-HA was specifically co-immunoprecipitated. These results show that LINGO-1 is able to form homotypic interactions in cell lysates.

We next examined this self-interaction by testing the formation of complexes using in situ cross-linking in living HEK-293 cells and cortical neurons. For this purpose, we used an anti-LINGO-1 antibody, and its specificity was first evaluated by Western blotting on HEK-293 cell lysates overexpressing LINGO-1-HA and compared with the results obtained with the anti-HA antibody. Both antibodies labelled two prominent immunoreactive species at approximately 80 and 100 kDa (Figure 2A), corresponding to different states of LINGO-1 glycosylation (Zhong et al., 2009; Supporting Information Fig. S1) and thereby demonstrating the specificity of the anti-LINGO-1 antibody used here. Following chemical cross-linking, cell lysates were subjected to SDS/PAGE under reducing conditions. Western blotting was performed with an anti-LINGO-1 antibody so that oligomeric complexes stabilized by chemical cross-linking could be visualized. This allowed us to detect additional immunoreactive bands at approximately 200 and 250 kDa, corresponding to homotypic interactions of LINGO-1 in living HEK-293 cells (Figure 2B). After the cross-linking of cortical neurons, the specific LINGO-1 antibody labelled two prominent immunoreactive species of 90 and 180 kDa, consistent with monomeric and dimeric forms of the native LINGO-1 protein respectively (Figure 2C). Because chemical cross-linkers have only a limited efficiency, the actual proportions of LINGO-1 monomers, dimers or higher order oligomeric species cannot be determined with this method. Together, these data indicate that LINGO-1 can self-interact in both HEK-293 cells and cortical neurons.

Constitutive cis-dimerization/ oligomerization of LINGO-1 using BRET analysis

To exclude artefacts generated by the solubilization of membrane-bound receptors, we also studied LINGO-1 self-interaction in intact living cells using BRET analysis. The BRET approach involves the transfer of energy resulting from the degradation of coelenterazine by RLuc, to a YFP, which in turn emits fluorescent light. BRET is strictly dependent on the molecular proximity (10–100 Å) between the energy donor (RLuc) and acceptor (YFP), making it ideal for studying protein–protein interactions in living cells at ‘physiological’ expression levels. The major advantage of this technique over biochemical methods is that protein–protein interactions can be monitored without disrupting the natural environment, which is frequently altered by detergents and membrane preparations. In addition, this method allows for the examination of whether the self-interaction of LINGO-1 that we observed by biochemical approaches occurs in cis- or trans-orientation because a BRET signal is measured only if a cis-interaction takes place (Figure 3A). For this purpose, the homodimerization of LINGO-1 was examined in living HEK-293 and SH-SY5Y cells in suspension using quantitative BRET analysis, with the proper controls required for this approach (James et al., 2006; Bouvier et al., 2007; Bacart et al., 2008; Figure 3B). BRET signals were spontaneously generated by

![Figure 1](https://example.com/figure1.png)

**Figure 1**
Homotypic interactions of LINGO-1 in HEK-293 cells. (A and B) The HA-tagged LINGO-1 was expressed transiently in HEK-293 cells in the presence or absence of YFP-tagged-LINGO-1. In all cases, 1/25 of each lysate was used as an input control (lysates). Cell lysates were immunoprecipitated with the indicated antibodies (IP) and then analysed by 10 or 8% SDS/PAGE and immunoblotted for YFP and HA-tagged constructs. For each experiment, control cells were transfected with empty plasmid controls, pcDNA3.
constitutive LINGO-1 dimers in which the C-terminus of one subunit contains YFP (LINGO-1-YFP) and the other contains RLuc (LINGO-1-RLuc). The specificity of this LINGO-1 oligomerization was shown in both cell lines by the very low energy transfer measured in cells co-expressing LINGO-1-RLuc with the trkB receptor, a neurotrophin receptor that it also expressed at the plasma membrane (Figure 3B inset). Data analysis revealed that the BRETmax value obtained in the SH-SYSY preparation (1288 ± 51 mBU) was lower than that obtained in HEK-293 cells (1725 ± 89 mBU; \( P < 0.01; n = 4 \)).

We further analysed the distribution of LINGO-1 dimers by cell fractionation studies. The distributions of the fluorescent and luminescent LINGO-1 both indicated that the vast majority of total LINGO-1 was exported from the ER to the plasma membrane (Figure 4). The BRETmax values were reached not only at the plasma membrane, but also in the ER (Figure 4), indicating that the homo-dimerization of LINGO-1 occurs early during its biosynthesis.

**Validation of BRET-based assays as a screening platform**

Because the present study demonstrated that LINGO-1 formed cis-dimers at the plasma membrane, we speculated that the manipulation of this dimerization process may represent an innovative strategy to modulate LINGO-1 function. The identification of proteins and/or molecules interfering with this process may help in further understanding the physiological role of LINGO-1 cis-dimerization. To identify such molecules, we speculated that they may induce conformational changes within LINGO-1 dimers leading to detectable modifications in the measured BRET signals. These modifications may represent changes in the quaternary structure of LINGO-1 (dimerization state of the protein), or its tertiary structure (conformational changes within protomers). To validate the proof of this hypothesis, we tested whether the BRET signal of LINGO-1 dimers could be modulated by low MW compounds as well as by protein partners.

First, competitive BRET assays were performed using various HA-tagged proteins. Interestingly, we demonstrated that the truncated construct lacking the intracellular terminal loop of LINGO-1 (LINGO-1-ΔCter) was able to significantly and specifically reduce the basal BRET signal (Figure 5B) in a manner similar to that observed with the full-length LINGO-1 (Figure 5A). Furthermore, the BRET signal was not modified by increasing concentrations of trkB, a neurotrophin receptor used as a negative control (Figure 5C). We then confirmed that LINGO-1 BRET-based assays could be used for screening aimed at identifying LINGO-1 ligands and SMPPIMs by monitoring conformational changes within dimers and/or dimer disruption. To determine the performance (\( Z' \) score) of the assay, we measured the BRET signal in 384-well microplates from cells transiently expressing LINGO-1-RLuc alone (monomer) or co-expressing LINGO-1-RLuc (constant amount) and increasing concentrations of LINGO-1-YFP yielding various BRET levels (from 15 to 60% of BRETmax). Consistent with excellent assay performance, the test runs established a \( Z' \) score within the 0.8-0.9 range, underlying the high quality and suitability of the assay for screening (Figure 6A).

Before starting to screen a large collection of compounds, we tested the signal reproducibility of the assay using 160 compounds belonging to the Prestwick Chemical Library. The screening was performed on a single day with two different transiently transfected cell lines expressing LINGO-1-RLuc and LINGO-1-YFP to reach 50% of BRETmax. As shown in Figure 6B, the LINGO-1 BRET signals obtained by the screening of two different transiently transfected cell lines overlapped well, indicating that the assay is fully reproducible.
We applied the conditions described above to screen a total of 1263 compounds from the Prestwick chemical library to identify LINGO-1 ligands and/or SMPPIMs of LINGO-1. The primary hit rate of compounds that induced an increase or a decrease of more than three standard deviations of the basal BRET signal was \( \sim 2\% \) allowing the selection of 23 compounds (Table 1). However, some molecules interfering with the absorption properties of the BRET-based assay (fluorescent or coloured compounds, molecules with toxic effects or having quenching effects) may give false positives. To confirm the specificity of these potential LINGO-1 ligands/inhibitors, we tested the effect of these selected compounds on the basal BRET signal produced by the dimerization of an unrelated protein, the 5-HT\(_6\) receptor (Table 1). Among the 23 compounds selected in the primary screen, only six were found to be specific for LINGO-1 because they did not modify the BRET signal from 5-HT\(_6\) receptor dimers (Table 1). We decided to follow up one compound, phenoxybenzamine (Figure 7A), which produced the strongest decrease (around \(-40\%\) ) of the basal LINGO-1 BRET signal, to test the biological relevance of this hit. Using BRET saturation analysis (Figure 7B), we demonstrated that phenoxybenzamine (10 \( \mu \)M) produced a rightward shift of the BRET saturation curves in cells expressing LINGO-1, whereas it did not affect the BRET signal in cells expressing 5-HT\(_6\) receptors (Figure 7C). Because the energy transfer depends on both the relative orientation and the distance between the BRET tags, two parameters (BRET\(_{50}\) and BRET\(_{\text{max}}\)) can be modulated upon addition of ligands. The
BRETmax level was significantly modified in the presence of phenoxybenzamine (BRETmax = 1629 ± 54 mBU) compared with conditions without phenoxybenzamine (BRETmax = 1805 ± 67 mBU; Figure 7B; P < 0.05). The BRET50 increased to 7.5 ± 1 in the presence of phenoxybenzamine compared with that obtained in its absence (BRET50 = 2.5 ± 0.6; P < 0.01, n = 4; Figure 7B and D). This indicates that phenoxybenzamine leads to a change in the relative affinity between LINGO-1 monomers promoting a dissociation of LINGO-1 performed dimers, mostly upon conformational changes as indicated by the decrease of the BRETmax value. In addition, the phenoxybenzamine-induced decrease of the basal BRET of LINGO-1 dimers was dose dependent with an EC50 = 4 μM, whereas it did not modify the basal BRET of the 5-HT6 receptor dimers (Figure 7E). Finally, although the LINGO-1 BRET signal stayed stable in the presence of buffer, the addition of phenoxybenzamine (20 μM) markedly decreased the BRET signal in a time-dependent manner (Figure 7F). Together, these results indicate that phenoxybenzamine may be considered the first specific SMPPIM of LINGO-1 cis-dimers.

Phenoxybenzamine behaves as a positive modulator of LINGO-1 downstream signalling

In Cos-7 cells, LINGO-1 activities may be mediated through the inhibition of EGFR downstream signalling (Inoue et al., 2007), a signalling pathway previously documented to be involved in neuronal survival and axonal regeneration as well

Figure 4

Constitutive BRET signals between LINGO-1-RLuc and LINGO-1-YFP in plasma membrane and ER. HEK-293 cells stably co-expressing LINGO-1-RLuc and LINGO-1-YFP (basal BRET signal = 175 ± 10 mBu) were lysed and subcellular fractions were resolved on a sucrose gradient. Individual fraction were subjected to fluorescence/luminescence and BRET analysis and resolved by SDS-PAGE and Western blot analysis using anti-NaK-ATPase or anti-calnexin antibodies used as markers of the plasma membrane (fractions 1–15) or the RE (fractions 16–30) respectively.

Figure 5

BRET competition analysis. The basal BRET signal obtained after the transient co-expression of LINGO-1-RLuc and LINGO-1-YFP in HEK-293 cells (yielding to around 50% of the BRETmax) is significantly reduced by the transfection of increasing concentrations (0 to 2600 ng) of both the full length of LINGO-1 (A) and its truncated form (LINGO-1-ΔCter) (B). The specificity of these inhibitions has been demonstrated by the lack of effect of trkB on the basal BRET signal (C). Values were expressed as the percentage of BRET signal measured in the absence of protein competitors (control) from three independent experiments read in triplicate. The expressions of the transfected HA-tagged competitors were confirmed by Western blot analysis (upper panels). The average of basal BRET signal value from three experiments was 915 ± 31 mBU. *P < 0.05; **P < 0.01, ***P < 0.001, compared with no competitor.
as oligodendrocyte differentiation (Grimm et al., 2009). Therefore, we decided to explore the effect of phenoxybenzamine on several signalling pathways downstream of EGFR, such as the phosphorylation of EGFR (measured as phosphorylation of Tyr1173), the activation of the MAPK pathway (measured as phosphorylation of ERK 1/2) and the activation of the PI3K pathway (measured as phosphorylation of Akt) in HEK-LINGO-1 cells compared with control cells (Figure 8). To avoid the constitutive activation of these pathways by growth factors contained in the medium, the cells were grown in the absence of serum for 16 h prior to experiments. EGF treatment (5 ng·mL\(^{-1}\)) for 7 min induced a strong increase in the phosphorylation of EGFR (+366%; \(P < 0.0001\)) and ERK 1/2 (+430%; \(P < 0.0001\)), but a lower and non-significant increase in Akt phosphorylation (+15%). After EGF treatment, we observed a significant reduction in EGFR phosphorylation on Tyr1173 of approximately 45% in LINGO-1-transfected cells compared with control cells. Similarly, the levels of Akt and ERK 1/2 phosphorylation were reduced by 35% (\(P < 0.05\)) and by 38% (\(P < 0.01\)), respectively, in LINGO-1-transfected cells compared with control cells. To elucidate the role of phenoxybenzamine in LINGO-1 function, we first examined its effect in control cells treated with or without EGF treatment. Phenoxybenzamine did not significantly modify either the phosphorylation of EGFR, or the phosphorylation of studied kinases in mock-transfected cells. In contrast, in LINGO-1-transfected cells after EGF treatment, phenoxybenzamine induced a strong and significant decrease of EGFR phosphorylation (73%, \(P < 0.01\)) and ERK 1/2 phosphorylation (54%, \(P < 0.01\)) but no significant modification of Akt phosphorylation of Tyr1173, the activation of the MAPK pathway (measured as phosphorylation of ERK 1/2) and the activation of the PI3K pathway (measured as phosphorylation of Akt). Therefore, we decided to explore the effect of phenoxybenzamine on several signalling pathways downstream of EGFR, such as the phosphorylation of EGFR (measured as phosphorylation of Tyr1173), the activation of the MAPK pathway (measured as phosphorylation of ERK 1/2) and the activation of the PI3K pathway (measured as phosphorylation of Akt) in HEK-LINGO-1 cells compared with control cells (Figure 8). To avoid the constitutive activation of these pathways by growth factors contained in the medium, the cells were grown in the absence of serum for 16 h prior to experiments. EGF treatment (5 ng·mL\(^{-1}\)) for 7 min induced a strong increase in the phosphorylation of EGFR (+366%; \(P < 0.0001\)) and ERK 1/2 (+430%; \(P < 0.0001\)), but a lower and non-significant increase in Akt phosphorylation (+15%). After EGF treatment, we observed a significant reduction in EGFR phosphorylation on Tyr1173 of approximately 45% in LINGO-1-transfected cells compared with control cells. Similarly, the levels of Akt and ERK 1/2 phosphorylation were reduced by 35% (\(P < 0.05\)) and by 38% (\(P < 0.01\)), respectively, in LINGO-1-transfected cells compared with control cells. To elucidate the role of phenoxybenzamine in LINGO-1 function, we first examined its effect in control cells treated with or without EGF treatment. Phenoxybenzamine did not significantly modify either the phosphorylation of EGFR, or the phosphorylation of studied kinases in mock-transfected cells. In contrast, in LINGO-1-transfected cells after EGF treatment, phenoxybenzamine induced a strong and significant decrease of EGFR phosphorylation (73%, \(P < 0.01\)) and ERK 1/2 phosphorylation (54%, \(P < 0.01\)) but no significant modification of Akt

**Table 1**

Compounds inducing BRET changes in cells expressing LINGO-1 (primary screen) or 5-HT\(_6\) receptors (counterscreen)

<table>
<thead>
<tr>
<th>Hits</th>
<th>Screen variation of LINGO-1 BRET signal (% of control)</th>
<th>Counterscreen variation of 5-HT(_6)R BRET signal (% of control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BRET signal inhibitors</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phenoxybenzamine</td>
<td>57 ± 6</td>
<td>100 ± 2</td>
</tr>
<tr>
<td>Ethacrynic acid</td>
<td>87 ± 1</td>
<td>100 ± 3</td>
</tr>
<tr>
<td>Cyanocobalamin</td>
<td>84 ± 3</td>
<td>100 ± 2</td>
</tr>
<tr>
<td>Chicago sky blue</td>
<td>25 ± 3</td>
<td>56 ± 6</td>
</tr>
<tr>
<td>Myricetin</td>
<td>80 ± 4</td>
<td>85 ± 4</td>
</tr>
<tr>
<td>Amphotericin B</td>
<td>79 ± 5</td>
<td>107 ± 4</td>
</tr>
<tr>
<td>BRET signal enhancers</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bambuterol</td>
<td>127 ± 9</td>
<td>100 ± 2</td>
</tr>
<tr>
<td>Clofazimine</td>
<td>122 ± 4</td>
<td>100 ± 1</td>
</tr>
<tr>
<td>Pinacidil</td>
<td>119 ± 3</td>
<td>100 ± 2</td>
</tr>
<tr>
<td>Nitrofurantoin</td>
<td>110 ± 2</td>
<td>113 ± 2</td>
</tr>
<tr>
<td>Balsalazide</td>
<td>116 ± 2</td>
<td>118 ± 2</td>
</tr>
<tr>
<td>Perhexiline</td>
<td>117 ± 5</td>
<td>108 ± 3</td>
</tr>
<tr>
<td>Nimesulide</td>
<td>125 ± 4</td>
<td>119 ± 2</td>
</tr>
<tr>
<td>Fendiline</td>
<td>125 ± 7</td>
<td>118 ± 4</td>
</tr>
<tr>
<td>Quinacrine</td>
<td>127 ± 2</td>
<td>140 ± 8</td>
</tr>
<tr>
<td>Azapropazone</td>
<td>134 ± 7</td>
<td>129 ± 7</td>
</tr>
<tr>
<td>Phenaoppyridine</td>
<td>137 ± 3</td>
<td>141 ± 6</td>
</tr>
<tr>
<td>Dantrolene</td>
<td>140 ± 5</td>
<td>147 ± 5</td>
</tr>
<tr>
<td>Harmolol</td>
<td>148 ± 10</td>
<td>117 ± 2</td>
</tr>
<tr>
<td>Riboflavine</td>
<td>153 ± 10</td>
<td>140 ± 5</td>
</tr>
<tr>
<td>Nifurtlimox</td>
<td>154 ± 8</td>
<td>148 ± 2</td>
</tr>
<tr>
<td>Rifampicin</td>
<td>159 ± 9</td>
<td>143 ± 2</td>
</tr>
<tr>
<td>Hycanthone</td>
<td>185 ± 15</td>
<td>156 ± 8</td>
</tr>
</tbody>
</table>

Cells transiently co-expressing LINGO-1-RLuc and LINGO-1-YFP (screen) or 5-HT\(_6\)R-RLuc and 5-HT\(_6\)R-YFP (counterscreen) were resuspended in physiological buffer and distributed in 96-well white plates. Compounds (20 \(\mu\)M in 0.5% of DMSO) from the Prestwick Chemical Library were incubated for 15 min at room temperature. BRET measurement was performed on a Mithras LB940. Results are expressed in percent change of the respective BRET control signal and are the mean ± SEM of two experiments.
phosphorylation level (Figure 8B–D). To further characterize the effect of phenoxybenzamine on LINGO-1 function, we measured ERK 1/2 activation in HEK-293 cells using the highly sensitive AlphaScreen Phospho-ERK assay. We first verified that this assay gave results similar to those from the Western blot analysis for ERK 1/2 activation (Figure 9A). As expected, EGF-stimulated ERK 1/2 activation was inhibited by 29% after LINGO-1 overexpression compared with control cells. We also confirmed that phenoxybenzamine did not significantly modify the basal ERK 1/2 phosphorylation in HEK-293 cells compared with control cells. We also confirmed that phenoxybenzamine did not significantly modify the basal ERK 1/2 phosphorylation in HEK-293 cells using the highly sensitive AlphaScreen Phospho-ERK assay. We first verified that this assay gave results similar to those from the Western blot analysis for ERK 1/2 activation (Figure 9A). As expected, EGF-stimulated ERK 1/2 activation was inhibited by 29% after LINGO-1 overexpression compared with control cells. We also confirmed that phenoxybenzamine did not significantly modify the basal ERK 1/2 phosphorylation either in control or in LINGO-1-transfected cells. In contrast, whereas phenoxybenzamine did not significantly inhibit EGF-stimulated ERK activation in control cells, it induced a strong and significant decrease of ERK 1/2 phosphorylation in LINGO-1-transfected cells (−46%, P < 0.001) compared with control cells (−16%; non-significant). A dose-response curve for the effect of phenoxybenzamine was also examined on both basal and EGF-induced ERK1/2 activation. Again, phenoxybenzamine induced a dose-dependent decrease of EGF-induced ERK phosphorylation levels, only in cells expressing LINGO-1, with an EC50 of 4 μM and does not modify the BRET signal resulting from 5-HT6 receptor dimerization. (F) Whereas the LINGO-1 dimers BRET signal stays stable in the presence of buffer, the addition of phenoxybenzamine (10 μM) markedly decreases the basal BRET signal in a time-dependent manner.

Discussion

Numerous studies have reported negative regulatory functions of LINGO-1 in axonal regeneration, neuronal survival, oligodendrocyte differentiation and myelination (Mi et al., 2008). However, the molecular mechanisms that control these functions are poorly understood.

Within neurons, LINGO-1 has been found to be associated with other membrane proteins typically in a co-receptor function (Mi et al., 2004). Studies based on IP assays after cell
Figure 8
Phenoxybenzamine (PHEN) enhances the LINGO-1 induced inhibition of EGF-stimulated downstream pathways in HEK-293 cells. HEK-293 cells were transiently transfected with peYFP-N1 control plasmid (control cells) or with YFP-tagged LINGO-1 plasmid (LINGO-1 cells). Forty-eight hours after transfection, cells were stimulated for 7 min with EGF (5 ng·mL⁻¹) in presence of phenoxybenzamine (10 μM) or the vehicle (0.5% DMSO) in HBSS. After cell lysis, EGFR downstream signalling (pEGFR, pERK, pAkt) was analysed by Western blot analysis (A) and were quantified by densitometry (B–D). Equal protein load was verified by analysing the samples with a tubuline antibody. The data shown are the means ± SEM of values from at least three independent experiments. Two-way ANOVA statistical analysis of pEGFR (B) assays was performed as follows: factor cells transfection $F(1, 8) = 57.18, P = 0.0001$; factor treatment $F(3, 8) = 84.5, P < 0.0001$; and interaction $F(3, 8) = 42.5, P < 0.0001$. Then Newman–Keuls multiple comparisons were performed. Two-way ANOVA statistical analysis of pAkt (C) assays was performed as follows: factor cells transfection $F(1, 8) = 27.07, P = 0.0008$; factor treatment $F(3, 8) = 0.9154$; and interaction $F(3, 8) = 0.2396, P = 0.0415$. Then Newman–Keuls multiple comparisons were performed. Two-way ANOVA statistical analysis of pErk (D) assays was performed as followed: factor cells transfection $F(1, 8) = 44.35, P < 0.0001$; factor treatment $F(3, 8) = 52.05, P < 0.0001$; and interaction $F(3, 8) = 5.114, P = 0.0289$. Then, Newman–Keuls multiple comparisons were performed. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$ versus respective control.

Figure 9
Characterization of the effect of phenoxybenzamine (PHEN) on pERK1/2 signalling. HEK-293 cells were transiently transfected with peYFP-N1 control plasmid (control cells) or with YFP-tagged LINGO-1 plasmid (LINGO-1 cells). Forty-eight hours after transfection, cells were stimulated for 7 min with EGF (5 ng·mL⁻¹) in presence of phenoxybenzamine at 10 μM (A) or at the indicated concentration (B) or the vehicle (0.5% DMSO) in HBSS. After cell lysis, pERK1/2 was quantified using the AlphaScreen assays. Means ± SEM of values from four separate experiments. (A) Two-way ANOVA statistical analysis was performed as follows: factor cells transfection $F(1, 25) = 44.17, P < 0.0001$; factor treatment $F(3, 25) = 148.7, P < 0.0001$; and interaction $F(3, 25) = 11.94, P = 0.0415$. Then, Tukey’s multiple comparisons were performed, ***$P < 0.001$ versus respective control. (B) Tukey’s multiple comparisons were performed, ***$P < 0.001$ versus EGF effect in control cells.
Moreover, because the known LINGO-1 partners p75 NTR, new strategy to modulate its functions, particularly because of LINGO-1 targeting to the cell surface may represent a consistent with the model based on the crystal structure of LINGO-1 ectodomain, showing that it forms a tetrameric structure (Mosyak et al., 2006). By ensuring that the cells were in suspension and present as single cells when the measurements were performed, we minimized the possibility that the observed cis-dimers were secondary to trans-interactions. Moreover, because the known LINGO-1 partners p75 and NgR1, TROY (Mi et al., 2004), trkA and trkC (Mandai et al., 2009) are not expressed in HEK-293 cells, our results provide evidence that interactions with these proteins are not necessary for the formation of LINGO-1 dimers. Furthermore, their expression in both cortical neurons and SH-SY5Y cells does not prevent LINGO-1 oligomer formation. Indeed, although the maximal BRET value obtained in SH-SY5Y cells was lower than that observed in HEK-293 cells, this difference does not reflect a lower number of dimers formed in these cells but is more likely to be due to differences in the relative orientation of the dimers. Moreover, protein binding partners of LINGO-1, as well as the lipid composition of membranes, may influence the BRET parameters (Couturier and Jockers, 2003). The detection of BRET signals both at the cell surface and in the ER after cell fractionation on a sucrose gradient indicates that receptors exist as dimers/oligomers in both compartments. Oligomeric assembly within the ER is a general feature of the export system that controls the quality of several plasma membrane proteins such as ion channels (Ma et al., 2001) and GPCRs (White et al., 1998; Bulenger et al., 2005). The decrease in the LINGO-1 BRET signal induced by LINGO-1-ΔCter is likely to reflect a reduction in the formation of wild-type LINGO-1 dimers as a consequence of the formation of heterodimers (LINGO-1/LINGO-1-ΔCter). More importantly, by forming heterodimers with full-length LINGO-1 in the ER, this truncated form prevents LINGO-1 from reaching the plasma membrane (Supporting Information Fig. S2). Thus, we propose that the increased neuronal survival and/or the higher myelination competence previously reported to be induced by LINGO-1-ΔCter overexpression (Mi et al., 2005; Inoue et al., 2007) may reflect an alteration of LINGO-1 maturation and/or the targeting of functional dimers to the cell surface. Therefore, the inhibition of LINGO-1 targeting to the cell surface may represent a new strategy to modulate its functions, particularly because an up-regulation of LINGO-1 expression has been observed in pathological conditions such as in animal models of Parkinson disease (Inoue et al., 2007) or spinal cord injury (Gerin et al., 2011).

A recent study demonstrated that LINGO-1 was capable of self-association in trans interactions and that such intercellular interactions are involved in the inhibition of OPC differentiation and myelination. It has been proposed that the disruption of such protein–protein interactions may be useful to reduce LINGO-1 function and improve myelination (Jepson et al., 2012). Due to the relatively large surfaces that are typically involved in protein–protein pairing, attempts to modulate such interactions with drug-like, low MW compounds have historically been considered challenging (Arkin and Wells, 2004; Thompson et al., 2012). However, a number of recent successful examples have generated increased interest in this approach (Fry, 2008), and protein–protein interaction disruptors in signalling networks have been recently identified, and their therapeutic properties have been demonstrated in addiction, pain (Ji et al., 2006b; Liu et al., 2008; Pichon et al., 2010) and cancer (Qi et al., 2013). Together, these data suggest that the manipulation of protein–protein interactions represents an innovative strategy to modulate protein functions and therefore that SMPPIMs may be considered as compounds with promising therapeutic applications.

Our observation that LINGO-1 forms cis-homodimers in living cells using a BRET-based homogenous assay allowed us to suggest that this simple and rapid method could be applied to the screening of LINGO-1 ligands and/or SMPPIMs (Couturier and Deprez, 2012). Because BRET depends strictly on the molecular proximity and the orientation between donor and acceptor, it is theoretically applicable to study conformational changes as a consequence of ligand binding (De Vries et al., 2010; Couturier and Deprez, 2012). Our present data demonstrate that our LINGO-1 BRET-based assay is of high quality and suitable for use in screening (Z′ score > 0.85) and the screening of around 1000 chemical compounds led us to identify one molecule, phenoxybenzamine, that specifically and dose-dependently decreased the LINGO-1 BRET signal. Phenoxybenzamine is an irreversible subtype-nonselective α-adrenoceptor antagonist and has been used to treat hypertension and as a peripheral vasodilator with a long duration of action. Site-directed mutation studies on the α2-adrenoceptor revealed that the covalent binding of phenoxybenzamine to the receptor involves the solvent-exposed cysteine residue in the third transmembrane domain (Frang et al., 2001). Based on the crystal structure of LINGO-1 (Mosyak et al., 2006), all of the cysteine residues in the extracellular domain of the protein are engaged either in disulfide bridges or buried in the protein hydrophobic core, and they are thus unlikely to react with phenoxybenzamine (Supporting Information Fig. S3). We also exclude the role of the transmembrane cysteine CS75 in the action of phenoxybenzamine as shown by BRET saturation curves experiments (Supporting Information Fig. S4). Moreover, the reactivity of phenoxybenzamine depends on the formation of a reactive aziridinium intermediate, which in turn depends on steric features of the N-benzyl groups that are released when phenoxybenzamine is bound to adrenoceptors. Consequently, structural and mechanistic considerations eliminate the pos-
sibility of covalent binding of phenoxybenzamine to LINGO-1, consistent with our experimental findings (Supporting Information Fig. S5). We therefore propose that phenoxybenzamine induces conformational changes within the LINGO-1 dimer, promoting a dissociation of preformed LINGO-1 dimers.

To evaluate whether phenoxybenzamine modulated biological processes downstream of LINGO-1, we tested its effect on EGFR signalling in HEK-293 cells overexpressing LINGO-1 compared with control cells. The EGFR-induced activation of EGFR occurs through receptor dimerization, which induces the activation of several transduction pathways, including MAPK (Ras, Raf, MEK, Erk) and the PI3K pathways (Ras, PI3K, Akt; Jorissen et al., 2003). A previous study demonstrated that the overexpression of LINGO-1 inhibits signalling downstream of EGFR and that some LINGO-1 inhibitors (LINGO-1-Fc, LINGO-1-ΔCter or anti-LINGO-1 antibody) can suppress the inhibitory effects of LINGO-1 on EGFR activity and PI3K/Akt signalling (Inoue et al., 2007). Our data demonstrated that the overexpression of LINGO-1 not only significantly inhibited the EGFR-induced MAPK pathway (Akt phosphorylation), as previously reported (Inoue et al., 2007), but also the EGFR-induced activation of ERK. Although the mechanisms by which LINGO-1 mediates inhibition of the ERK pathway should be further characterized, it is possible that WNK1 is involved, as this kinase is known to physically interact with LINGO-1 (Zhang et al., 2009) and to activate downstream cascades in response to EGF (Xu et al., 2004; Huang et al., 2008).

We also demonstrated that phenoxybenzamine acts as a positive modulator of LINGO-1 by increasing the inhibitory action of LINGO-1 on EGFR downstream signalling. Phenoxybenzamine is a known blocker of α-adrenoceptors (Frang et al., 2001). However, several arguments lead us to conclude that its binding to α-adrenoceptors is not involved in its inhibitory effect on EGFR downstream signalling. First, α1-adrenoceptors are not endogenously expressed in HEK-293 cells (Theroux et al., 1996). Second, even if α1 adrenoceptors were endogenously expressed in HEK-293 cells (Cooper et al., 1997), phenoxybenzamine does not modify the EGFI-induced MAPK/ERK activation when tested in control cells (transfected with empty vector, pEYFP-N1). Because EGFR itself plays a crucial role in the regulation of cell proliferation and is involved in many aggressive human cancers, novel strategies to inhibit EGFR activation could represent novel potential therapeutic approaches. Interestingly, phenoxybenzamine has recently been identified as a low MW inhibitor of glioblastoma cell growth (Wang et al., 2012).

Given the emerging role of LINGO-1 as a negative regulator of several degenerative diseases, targeting the functions of LINGO-1 is intensively investigated for the treatment of neurodegenerative and demyelinating diseases. Here, we have demonstrated that LINGO-1 can form dimers or higher order oligomers in living cells under physiological conditions. We took advantage of this property to use a BRET-based assay for the screening of ligands and/or SMPPIMs of LINGO-1. The identification of the first chemical compound able to promote conformational changes within the dimers of LINGO-1 allowed us to propose that this method will be particularly useful to identify innovative pharmacological inhibitors or activators of LINGO-1. Such compounds will serve as tools to decipher the role of LINGO-1 dimerization in its downstream signalling and its relevance to physiological processes.

Acknowledgements

We thank Professor Tobias Hevor and Philippe Moreau (Neurobiology Laboratory, Orleans) for their contribution to neuronal cultures. We thank David Gosset (CBM, Orleans) for his technical help to obtain clonal cell lines. We also thank Béatrice Vallee and Michel Doudeau (CBM, Orléans) for all discussions of the results and for their technical advices.

This work was supported by a Trampoline grant (15058) from the AFM (Association Française contre les Myopathies), by the CNRS structure of valorization and transfer (03247-02), the Ligue contre le Cancer (Loiret and Cher comities) and a FEDER grant (N° 5789/39115) from Region Centre. This work has been published within the LABEX ANR-10-LABX-0034_Medalis and received a financial support from French government managed by ‘Agence National de la Recherche’ under ‘Programme d’investissement d’avenir’.

Author contributions

L.C. performed the BRET experiments, drug screening, Western blot analysis and AlphaScreen experiments. M.-L.de T., I.H. and E.G. generated the plasmid constructs. J.F performed the functional assays on primary culture. E.K. and D. R. performed the 3D modelling of LINGO-1 and contributed to writing the manuscript. E.T., J.P. and H.B. contributed to scientific discussion and to writing the manuscript. S.M.-L. designed experiments and wrote the manuscript.

Conflict of interest

All authors declare no competing financial interests.

References


Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher’s web-site:

http://dx.doi.org/10.1111/bph.12945

Figure S1 Deglycosylation of LINGO-1-HA with peptide-N-glycosidase (PNGase F). Lysates from HEK-293 cells transiently transfected with the LINGO-HA were incubated in buffer B (50 mM sodium citrate, pH 7.5, 1% Triton, cocktail of protease inhibitor) with or without PNGase F for 2 h at 37°C. The reaction was stopped by adding loading sample buffer. Samples were separated using 8% SDS-PAGE and analysed by immunoblotting with the anti-BD living colours antibody (BD Biosciences). Treatment of membranes with PNGase F reduced the 80 kDa and 100 kDa mature forms to about 60 kDa, which represented the deglycosylated forms of Functional relevance of LINGO-1 cis-dimers

BJP

LINGO-HA. These data demonstrate that recombinant LINGO-1 expressed in HEK-293 cells is N-glycosylated.

**Figure S2** Effect of LINGO-1ΔCter on the distribution and dimerization of wild-type LINGO-1. HEK-293 cells were transiently transfected with LINGO-1-RLuc and LINGO-1-YFP with or without LINGO-1ΔCter. Forty-eight hours after transfection, cells were lysed and subcellular fractions were resolved on a sucrose gradient. Individual fractions were subjected to luminescence and BRET analysis.

**Figure S3** Solvent accessibility of cysteine residues in human LINGO-1 and human α2-adrenoceptor. The protein backbone is represented with grey ribbon. Only cysteine residues are shown, their side chain being depicted using CPK-coloured ball and sticks. A. The three-dimensional structure of LINGO1 ecto-domain (residues 1–476) was solved by X-ray crystallography (PDB: 2IDS) and revealed a tetrameric organization (doi: 10.1074/jbc.M607314200). Each protomer contains 13 cysteine residues, 10 engaged in disulfide bridges and the remaining three burying their side chain into the core structure of the leucine-rich repeats. B. The three-dimensional structure of α2-adrenoceptors was modelled by homology with human α2-adrenoceptors (PDB: 3POG). Details of modelling: The template file was chosen because of sequence similarity between the protein (25.8% identity) as well as shape similarity between phenoxybenzamine and the ligand bound to transmembrane cavity of the receptor. The model was built using MOE v2011.10 (Chemical Computing Group Inc) based on sequence alignment guided by GPRC constraints. The 3D structure of phenoxybenzamine was generated with Corina v3.1 (Molecular Network GmbH). Model of complex between phenoxybenzamine and the α2-adrenoceptor was obtained from covalent docking of phenoxybenzamine into the transmembrane cavity (defined in a radius of 10 Å centred on OE1 atom of ASP3.32) using GOLD v5.2 (The Cambridge Crystallographic Data Centre). The best scored pose selected with ASP scoring function closely reproduces the binding mode of ligand bound to the template receptor.

**Figure S4** BRET analysis on LINGO1C575A. The Cys575 residue is located is the transmembrane domain of LINGO-1. In order to investigate its potential role in the action of phenoxybenzamine, we performed two constructions containing the mutated LINGO fused to Renilla luciferase (LINGO-1-C575A-RLuc) or with yellow fluorescent protein (LINGO-1-C575AYFP). BRET saturation curves analysis demonstrates that the mutation does modify neither the ability of LINGO-1 to form dimers (A) nor the ability of phenoxybenzamine to decrease BRET signal (B).

**Figure S5** Phenoxybenzamine binds LINGO-1 in a reversible manner. BRET experiments were performed in adherent HEK-293 cells transiently transfected with LINGO-1-RLuc alone or with LINGO-1-RLuc and LINGO-1-YFP. BRET signal was determined in the presence of phenoxybenzamine immediately before and immediately after extensive washing of the cell culture. After the washing, the BRET signal reached a value not significantly different from the control value. Data are the mean ± SEM of two independent experiments. ***P < 0.001.