Utilization of in situ ELISA method for examining Trk receptor phosphorylation in cultured cells

Hanna Antila, Henri Autio, Laura Turunen, Kirsi Harju, Päivi Tammela, Kristi Wenerberg, Jari Yli-Kauhaluoma, Henri J. Huttunen*, Eero Castrén and Tomi Rantamäki*

*Neuroscience Center, P.O. Box 56 (Viikinkaari 4), FI-00014 University of Helsinki, Finland.
*Division of Pharmaceutical Chemistry, Faculty of Pharmacy, P.O. Box 56 (Viikinkaari 4), FI-00014 University of Helsinki, Finland.
*Centre for Drug Research, Faculty of Pharmacy, P.O. Box 56 (Viikinkaari 4), FI-00014 University of Helsinki, Finland.

Abstract

Background: Trk receptor tyrosine kinases regulate multiple important neuronal processes during the development and in the adulthood. Tyrosine phosphorylation of Trk serves as the initial step in the Trk signaling pathway and indicates receptor’s autocatalytic activity. However, methods allowing simple and large-scale Trk phosphorylation analyses in cultured cells are lacking.

Methods: The addition of a novel agonistic anti-Trk ELISA (enzyme-linked immunosorbent assay) method where cell culture, receptor stimulation and Trk phosphorylation analysis are all performed on the same multwell plate.

Results: In situ phospho-Trk ELISA readily and specifically detects neurotrophin-induced Trk receptor phosphorylation in cultured cells. A proof-of-concept small molecule screening of a library composed of 2000 approved drugs and other bioactive compounds was carried out using this novel method.

Conclusions: We describe a novel method that can be efficiently used to examine Trk receptor phosphorylation in cultured cells. Principally similar methods can be developed to examine the levels and signaling of any intracellular protein.

Keywords: BDNF; TrkB phosphorylation; ELISA; in situ

1. Introduction

Tropomyosin-related kinases, Trks, serve as cognate receptors for neurotrophins: nerve growth factor (NGF) binds preferentially to TrkA, brain-derived neurotrophic factor (BDNF) and neurotrophin-4 (NT-4) to TrkB and neurotrophin-3 (NT-3) to TrkC (Huang and Reichardt, 2001). Trk receptor signaling regulates several important neuronal processes and abnormal Trk signaling is implicated in some cancers and in numerous nervous system disorders, including mood disorders, neurodegenerative disorders and pain (Rantamäki and Castrén, 2008; Thiele et al., 2009). Thus, pharmacological blockade of the receptor would hold significant therapeutic potential. Supporting this concept, the therapeutic actions of antidepressant drugs have been associated with their ability to activate Trk receptors in the brain (Saarelainen et al., 2003; Rantamäki et al., 2007; Rantamäki and Castrén, 2008). Furthermore, experimental Trk receptor agonists provide neuroprotective support and ameliorate the neuroanatomical and behavioral outcomes in animal models of neurodegenerative disorders (Longo and Massa, 2013).

Upon activation, TrkB receptor is phosphorylated at specific intracellular tyrosine residues that further initiate intracellular signaling (Huang and Reichardt, 2001). TrkB phosphorylation is tightly associated with the receptor’s catalytic activity and is thus widely used as an indicator of TrkB activation (Segal et al., 1996). The main purpose of this study was to set up and validate an in situ ELISA (enzyme-linked immunosorbent assay) method that can be efficiently used to examine Trk receptor phosphorylation in cultured cells. In this method the cells are directly incubated (treated) on multwell plates coated with the capturing antibody and, upon cell lysis, all subsequent assay steps are performed in situ. We describe several conditions that significantly increase the sensitivity of in situ ELISA to detect ligand induced Trk phosphorylation. In order to further validate the developed method and investigate its suitability for large-scale analyses, we performed a small molecule screening with the Spectrum Collection, a chemical library with compounds of wide range of biological activities and structural diversity suitable for testing de novo assays.

2. Materials and methods

2.1. Reagents and chemicals

The Spectrum Collection compounds were purchased from MicroSource Discovery Systems, Inc. (Gaylordsville, CT, USA) and stored at -80°C in DMSO stocks of 20 mM. The library consisted of 2000 compounds divided in three major groups: 50% drug or drug-like compounds, 30% natural products and 20% other bioactive compounds (for complete list visit: http://www.msdiscovery.com/spectrum.html). BDNF was purchased from Peprotech (Rocky Hill, NJ, USA), diluted in PBS (phosphate buffered saline; pH 7.4) containing 0.1% BSA (bovine serum albumin; Sigma Aldrich, Finland, Helsinki, Finland) and stored in aliquots at -80°C. NGF (Promega, Madison, WI, USA) was kindly provided by Dr. Urmans Arumade (University of Helsinki). The synthesis of INMPP1 (1-(1-dimethylhydraz-3)- [1-naphthalenylmethyl]-1H-pyrazolo[3,4-d]pyrimidin-4-amine) has been described previously (Sallent et al., 2009). Optocart® solution (catalog nro. C10202) was purchased from AlerChek, Inc. (Springvale, MA, USA). ProteoJET™ membrane protein extraction kit (catalog nro. K0321), chemiluminescence reagent (Pierce; catalog nro. 32106) and streptavidin-HRP (catalog nro. 21126) were purchased from Thermo Fisher Scientific Oy (Vantaa, Finland). Trk kinase inhibitor K252a (Sigma Aldrich) was kindly provided by Dr. Heikki Rauvala (University of Helsinki). Detergents were purchased from Sigma Aldrich and prepared as 1% (all, except Brj 35) or 0.1% solutions in buffer consisting of 137 mM NaCl, 20 mM Tris, 10% glycerol, 50 mM NaF, 2 mM NaVO₃ and protease inhibitors (Complete inhibitor tablets, F. Hoffmann-La Roche Ltd., Basel, Switzerland).

2.2. Cell culture

MGB8-trkB fibroblasts (Vesa et al., 2000) kindly provided by Dr. Eric Shooter, Stanford University) were cultured in Dulbecco’s Modified Eagle’s Medium (DMEM) (supplemented with 10% fetal bovine serum, 1% penicillin/streptomycin, 1% L-glutamine and 400 µg/ml G418). PC12 cells were cultured in DMEM/F12 media (1:1; Gibco 31300-038) supplemented with 10% horse serum, 5% fetal calf serum, 10 mM HEPEs (pH 7.2), 5 mM L-glutamine, 1% penicillin/streptomycin, 20 mM sodium bicarbonate). The cell lines were maintained in a cell incubator (5% CO₂, 37°C) until reaching confluence after which the cells were divided or used for experiments.

For the primary neuronal cultures, hippocampi or cortex was dissected from E18 rat embryos or E16 TrkB(E19) mouse embryos (kindly provided by Dr. David Ginty, Johns Hopkins University) and the tissue dissociated in papain solution (2 mg/ml in 10 DL-Cystein-HCl, pH 8, 20% BSA, 250 µg/ml papain, 1 mM EDTA, 50 µg/ml penicillin/streptomycin, 1% L-glutamine and 400 µg/ml G418). The cells were cultured in Neurobasal medium (supplemented with 2% B27, 1% penicillin/streptomycin and 1% L-glutamine) and supplemented with fresh medium every 3rd day. TrkB(E19) mice carry a mutation near the ATP binding of TrkB rendering the receptor susceptible for inhibition by the receptor-specific kinase inhibitor INMPP1 (Chen et al., 2005; Sallent et al., 2009).

2.3. ELISA

Conventional sandwich phospho-Trk ELISA was performed essentially as described earlier (Rantamäki et al., 2011). Specifically, cell homogenates (lysate volume 25-75µl) were transferred directly from culture plates into pre-coated (sc-11-R, Santa Cruz Biotechnology, Santa Cruz, CA, USA; 1:500-1000 in Optocart®; overnight at 4°C) and pre-blocked (2% BSA/PBS-T; 2 h at room temperature) white 96-well Optiplate® (PerkinElmer Oy, Espoo, Finland) plates and 3% BSA/PBS-T (∼2 µM Na₂VO₃ to inhibit tyrosine phosphatases) added ad 200 µl. After overnight incubation at 4°C the wells were washed with PBS-T (4×300 µl) and anti-phosphotyrosine antibody added to the wells (in house biotinylated PY20; AbD Serotec, Kidlington, UK; 1:1000 in 2% BSA/PBS-T, overnight at 4°C). Following sequential washes and HRP-coupled streptavidin antibody incubation (1:10000 in 2% BSA/PBS-T; O/N at 4°C) 100 µl of ECL substrate mix was added to the wells and luminescence measured with Variskan Flash plate reader (Thermo Fisher Scientific Oy).

For the in situ phospho-Trk ELISA, dissociated cells were directly plated onto UV-sterilized ELISA plates pre-coated and pre-blocked with sterile-filtered antibody and blocking solution, respectively. Following experimental treatment of cells, the plates were put immediately on ice, medium discarded and lysate buffer applied (10-25 µl). Next, the plates were rigorously shaken in a multi-rotor at 30-60 min (800 rpm, Labsystems Wellmix, Thermo Fisher Scientific Oy) after which 3% BSA/PBS-T (∼2 µM Na₂VO₃, to inhibit tyrosine phosphatases) solution was applied ad 200 µl (96-multwell) or ad 50 µl (384-multwell). After overnight incubation at 4°C, the wells were washed and ELISA assay continued as described above.
In situ phospho-Trk ELISA

Next we sought to test whether the conventional sandwich phospho-Trk ELISA could be transferred to a well-plate format that would allow cell culturing, stimulation, lysis and phosphorylation analysis in the same plate (Figure 2a). Such in situ ELISA set-up has been previously utilized to examine the levels of secreted factors, including TrkB ligand BDNF (Balkowiec and Katz, 2000), but to the best of our knowledge not to examine intracellularly resident proteins such as transmembrane receptors. In our method, cells were directly cultured on pre-coated and pre-blocked ELISA plates. Following cell stimulations, the plates were immediately put on ice, medium discarded and cells lysed in situ by rigorous shaking in a small volume of lysis buffer (ProteoJET™ membrane protein extraction kit). Then 2 % BSA/PBS-T (supplemented with tyrosine phosphatase inhibitor sodium vanadate) was added to the wells and immunocapturing of Trk receptors was achieved during overnight incubation; after this, the wells were washed and ELISA assay continued as described above.

In situ phospho-Trk ELISA readily detected BDNF-induced TrkB phosphorylation in MGG8-trkB fibroblasts in both 96- and 384-multiwell formats; however, S/B was significantly higher in the former format (Figure 2b). The method also allowed detecting small but significant BDNF-induced (5 ng/ml, 15 min) change in TrkB phosphorylation in cultured E18 rat primary cortical cultures (6 days in vitro) (Vehicle: 100.00±4.70% vs. BDNF: 127.79±2.48%; n=8-16; P<0.005, two-tailed Student t-test).

Next we performed side-by-side analyses with conventional and in situ phospho-Trk ELISAs to directly compare the performances of these methods. Briefly, the MGG8-trkB cells were grown and stimulated with BDNF in 96-well cell culture plates and transferred manually into 96-well ELISA plates or the cells were directly grown and stimulated in 96-well ELISA plates. Subsequent ELISA steps were performed identically thereafter. Although the in situ method is mechanistically more straightforward, the S/B was significantly better with the conventional ELISA (Figure 2c).

Results

3.1. Conventional sandwich phospho-Trk ELISA

We have previously developed a phospho-Trk sandwich ELISA based on commercial antibodies and reagents and used this assay to examine Trk phosphorylation in cell homogenates (Rantamäki et al., 2011). Briefly, lysed cell homogenates (reference lysis buffer: NP buffer) were transferred to plates previously coated with commercial antibodies and reagents (reference antibody: sc-11-R, rabbit polyclonal; reference coating solution: phosphate buffered saline). Next, the plates were sequentially washed and incubated with biotinylated phospho-tyrosine antibody, streptavidin-HRP and chemiluminescence reagent, after which emitted light is quantified. Under these reference conditions the assay readily detected BDNF-induced TrkB phosphorylation in MGG8-trkB fibroblasts (Figure 1a), NGF-induced TrkA phosphorylation in PC12 cells (Figure 1b) and BDNF-induced Trk phosphorylation in primary mouse and rat neuronal cultures (Figure 1c-d), whereas no such neurotrophin induction was seen in MG87 parental cells or in TrkB expressing cells pre-treated with Trk kinase inhibitors or if the capturing antibody was not present in the assay (Figure 1a, b-d). We further optimized the sampling and assay conditions by evaluating the influence of different lysis buffers, antibodies and coating buffers on signal-to-background ratio (S/B) achieved with BDNF stimulation. The highest S/Bs were observed when the ProteoJET™ membrane protein extraction kit was used for the cell lysis (Figure 1e) and when the polyclonal rabbit pan-Trk antibody in Optacoat™ solution was used for Trk immunocapturing (Figure 1f-g). However, small lysate levels produced significantly lower S/B than higher lysate levels (S/B: 50 µg < 100 µg = 200 µg) (Figure 1h). Under optimized assay conditions, the S/B remained unchanged for 7 minutes after application of chemiluminescence reagent (Figure 1i) and results obtained after 5 minutes were used for all described analyses.

3.2. In situ phospho-Trk ELISA

Next we sought to test whether the conventional sandwich phospho-Trk ELISA could be transferred to a well-plate format that would allow cell culturing, stimulation, lysis and phosphorylation analysis in the same plate (Figure 2a). Such in situ ELISA set-up has been previously utilized to examine the levels of secreted factors, including TrkB ligand BDNF (Balkowiec and Katz, 2000), but to the best of our knowledge not to examine intracellularly resident proteins such as transmembrane receptors. In our method, cells were directly cultured on pre-coated and pre-blocked ELISA plates. Following cell stimulations, the plates were immediately put on ice, medium discarded and cells lysed in situ by rigorous shaking in a small volume of lysis buffer (ProteoJET™ membrane protein extraction kit). Then 2 % BSA/PBS-T (supplemented with tyrosine phosphatase inhibitor sodium vanadate) was added to the wells and immunocapturing of Trk receptors was achieved during overnight incubation; after this, the wells were washed and ELISA assay continued as described above.

In situ phospho-Trk ELISA readily detected BDNF-induced TrkB phosphorylation in MGG8-trkB fibroblasts in both 96- and 384-multiwell formats; however, S/B was significantly higher in the former format (Figure 2b). The method also allowed detecting small but significant BDNF-induced (5 ng/ml, 15 min) change in TrkB phosphorylation in cultured E18 rat primary cortical cultures (6 days in vitro) (Vehicle: 100.00±4.70% vs. BDNF: 127.79±2.48%; n=8-16; P<0.005, two-tailed Student t-test).

Next we performed side-by-side analyses with conventional and in situ phospho-Trk ELISAs to directly compare the performances of these methods. Briefly, the MGG8-trkB cells were grown and stimulated with BDNF in 96-well cell culture plates and transferred manually into 96-well ELISA plates or the cells were directly grown and stimulated in 96-well ELISA plates. Subsequent ELISA steps were performed identically thereafter. Although the in situ method is mechanistically more straightforward, the S/B was significantly better with the conventional ELISA (Figure 2c).

Figure 1. Characterization and optimization of sandwich phospho-Trk ELISA (a) The assay readily detects BDNF-induced (5 ng/ml, 15 min) TrkB phosphorylation in fibroblasts expressing Trk (MGG8-trkB) but not in parental cells (MGG8) or if the capturing antibody is not present in the assay (n=3-4/group). (b) NGF-induced TrkA phosphorylation in PC12 cells (n=3-10/group) (c) The assay readily detects BDNF-induced (5 ng/ml, 2 min) TrkB phosphorylation in E18 rat primary neuronal cultures whereas no signal is detected when the cultures are pretreated with kinase inhibitor k252a (200 nM, 15 min) (n=6/group) (d) The assay readily detects BDNF-induced (5 ng/ml, 15 min) TrkB phosphorylation in E18 mouse primary neuronal cultures derived from Tkdβ−/− mutant mice whereas no signal is generated when the cultures are pretreated with mitogen-specific kinase inhibitor 1NPP1 (100 nM; 24 hours) (n=4-group) (e) The impact of tested cell lysis buffers on signal-to-background ratio compared to reference buffer (n=6-group) (f) The impact of tested capture antibodies on signal-to-background ratio compared to reference antibody (n=3-group) (g) The impact of tested coating solutions on signal-to-background ratio compared to reference coating (n=3-group) (h) Signal-to-background ratio (n=3-group) at 3, 5 and 7 minutes after addition of the chemiluminescence reagent. **P<0.01, ***P<0.001, ****P<0.0005 of vehicle or reference condition, one-way ANOVA followed by Newman-Keuls post hoc test (a, e, f, g, h, i). Abbreviations: Ab=antibody, PBS=phosphate buffered saline, CB=carbonate buffer.
4. Discussion

The main aim of this study was to test whether our previously developed (Rantamäki et al., 2011) and further optimized conventional sandwich phospho-TrkB ELISA (Figure 1) can be developed into an in situ format, i.e. a method that would allow the cells to be directly cultured, receptors stimulated and TrkB phosphorylation assayed on the same multiwell plate. As a single-plate assay this approach would encompass several advantages over conventional sandwich ELISA. Most importantly, it would make unnecessary to scrape and transfer cell homogenates from plate to plate and therefore would reduce the time, manpower and resources needed for performing the analysis. Moreover, Balkowiec and Katz (2000, 2002) have previously developed in situ ELISA that allowed detecting neurally released BDNF with higher sensitivity than the conventional method. The in situ phospho-TrkB ELISA readily detected BDNF-induced phosphorylation of TrkB receptors in fibroblasts expressing TrkB in 96- and 384-multiwell formats, albeit the S/B ratio was lower in latter format, a finding that may be related to lower lysate amount (see Figure 1h) and thus absolute levels of TrkB protein in the 384-well format. BDNF-induced TrkB phosphorylation was also detected in rat primary neuronal cultures, but the S/B ratio was less than expected based on data obtained with BDNF not treated E. 5 µg/ml, 15 min) and further optimization is needed for primary neuronal cultures employed for this approach. We also compared the S/B achieved by submaximal BDNF stimulation in conventional and in situ TrkB ELISA methods. Although the in situ method is mechanistically more straightforward and thus readily allows implementation to robotics, the S/B was significantly better with the conventional ELISA.

In order to further validate the developed in situ phospho-TrkB method and investigate its suitability for large-scale analyses, including drug screening, we carried out semi-automated small-scale compound screening in 96-multiwell format. The method produced highly reproducible data in the screening setup. Although we did not primarily aim at identifying novel compounds targeting TrkB signaling, we found several potential inhibitors and activators of TrkB from the Spectrum Collection compounds, a selected set of drug and drug-like chemicals, natural products and other bioactive compounds. Importantly, some of the compounds represent a chemical or pharmacological class that has been previously shown to increase TrkB signaling (e.g. synthetic glucocorticoid betamethasone valerate (Jeanneteau et al., 2008), gedunin derivative epoxygedunin (Jang et al., 2010)). Majority of potential TrkB inhibitors belong to anthelmintics (benzadine derivatives), antibiotics (e.g. gramicidin) or antiviral drugs (e.g. podophyllins). Interestingly, depending on dose, 5 compounds produced either inhibition or facilitation of BDNF-induced TrkB activation.

In conclusion, we describe a novel method that can be used to examine TrkB receptor phosphorylation in cultured cells directly plated on antibody-coated multiwell plates. Principally similar methods can be developed to examine the levels and signaling of any cellular protein. Indeed, our preliminary studies indicate that the in situ ELISA approach can be utilized for the detection of phosphorylated Tau (data not shown), a microtubule assembly protein whose hyperphosphorylation has been implicated in the pathophysiology of neurodegenerative disorders.

Competing interests

H.H. is a co-founder and Chief Scientific Officer at Hermo Pharma Ltd. E.C. is a co-founder and shareholder of Hermo Pharma Ltd.

Acknowledgments

We would like to thank Outi Ninkilä and Anna Lehto for their excellent technical assistance.

Financial disclosure

This study was financially supported by the Finnish Funding Agency for Technology and Innovation (TEKES) (E.C.), Drug Discovery and Chemical Biology Network (Biocentra Finland) (L.T., P.T., K.W.), Doctoral Program Brain & Mind (H.A.) and Hermo Pharma Ltd (E.C., T.R.).

References

Huang EJ, Reichardt LF. Neurotrophins: roles in neuronal development and function. Annu Rev Neurosci 2001;24:677-736
Jeanneteau F, Garabedian MJ, Chao MV. Activation of Trk neurotrophin receptors by glucocorticoids provides a neuroprotective effect. PNAS 2008;105:8462-8467