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Identification of Receptor Protein Tyrosine Phosphatases (RPTPs) as Regulators of Receptor Tyrosine Kinases (RTKs) Using an RPTP siRNA-RTK Substrate Screen

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Abstract

Receptor tyrosine kinase (RTK) signaling exists in equilibrium between RTK tyrosyl phosphorylation and RTK tyrosyl dephosphorylation. Despite a detailed understanding of RTK tyrosyl phosphorylation, much less is known about RTK tyrosyl dephosphorylation. The receptor PTPs (RPTPs) are outstanding targets for the dephosphorylation of RTKs because of their mutual membrane proximity. In this chapter, we describe how to identify RPTPs that modulate the activity of RTKs using a siRNA screen and commercially available proteomic applications. The validation of putative RTKs as RPTP substrates using substrate-trapping approaches is detailed.

Keywords

Receptor protein tyrosine phosphatases; Receptor protein tyrosine kinases; siRNA screen; Tyrosine phosphatase substrate; Phosphatase substrate-trapping; Tyrosine dephosphorylation

1 Introduction

In the human genome, the protein tyrosine phosphatase superfamily comprises approximately 110 protein tyrosine phosphatases (PTPs) [1–3]. One group is the classical PTPs that specifically dephosphorylate phospho-tyrosine substrates. The classical PTPs are divided into 21 receptor-like PTPs (RPTPs) and 16 non-receptor PTPs [3, 4]. Although substantial insight towards understanding the function of many of the non-receptor PTPs has been made, in general much less is known about RPTPs and their substrates. RPTPs comprise variable extracellular domains, a single transmembrane domain, and a cytoplasmic domain containing two, and in some cases, a single, PTP domain [3, 5]. Typically, ligand binding to the extracellular domain of an RPTP induces dimerization that leads to the inhibition of RPTP catalytic activity [6–9]. In the dimeric state, reciprocal inhibition of the catalytically competent proximal D1 PTP domain occurs whereby the “wedge motif” of one D1 domain occludes the active site of the opposing D1 PTP domain in the dimer [3, 9]. The distal, D2 PTP domain is catalytically inactive although it provides important regulatory functions of RPTPs such as stabilizing substrate interactions, mediating protein-protein interactions and facilitating RPTP dimerization [7, 10–13].

There are 58 receptor tyrosine kinases (RTKs) in the human genome. Similar to that of the RPTPs, these RTKs also contain an extracellular domain, a single transmembrane domain, a juxtamembrane regulatory region, and a cytoplasmic region containing the protein tyrosine kinase domain [14]. In contrast to RPTPs, ligand binding to the extracellular domain of an

RTK, in some but not all cases, induces dimerization that results in the transphosphorylation of tyrosine residues that leads to the activation of downstream signaling [14].

RTKs are coordinately inactivated, through several mechanisms and these can include receptor downregulation and internalization as well as direct dephosphorylation by PTPs [14]. RTKs have been shown to be dephosphorylated by both non-receptor PTPs and RPTPs [15–18]. However, a complete understanding of how the RTKs are dephosphorylated has yet to be fully established. The most notable example of a non-receptor PTP dephosphorylating an RTK is that of PTP-1B which directly dephosphorylates the insulin receptor [15]. Several studies have shown that RPTPs also dephosphorylate RTKs. For example PTPRF (leukocyte common antigen related; LAR) dephosphorylates the insulin receptor [16, 17] and PTPRJ (density-expressed phosphatase; DEP-1) dephosphorylates the vascular endothelial growth factor receptor 2 (VEGFR2) [18]. Using approaches that we will describe here, this laboratory showed that LAR dephosphorylates EphA2 [19]. These observations indicate that RTKs can serve as direct substrates for both non-transmembrane PTPs and RPTPs. However, of the 58 RTKs in the human genome, the majority have no known counteracting PTP in which to account for their dephosphorylation.

Although both non-transmembrane PTPs and RPTPs are capable of dephosphorylating RTKs, we speculated that because of the relative location in the plasma membrane, RPTPs might play a major role in RTK dephosphorylation. Moreover, the identity of RPTP substrates remains poorly defined. Therefore, to gain insight into the actions of RPTPs, and to acquire a broader understanding of how RTKs are dephosphorylated, we developed a siRNA screen to identify RPTPs that function as RTK phosphatases. In this chapter, we detail how siRNA-screening approaches can be used to identify RPTPs that are capable of targeting RTKs. The initial value of the screen can be used to guide more detailed studies on the relationship between RPTPs and RTKs and as such one of these approaches involves validating whether the identified RPTP-RTK pair represents a bona fide enzyme-substrate relationship. To achieve this, we describe the approach of substrate-trapping to validate the RPTP-RTK enzyme-substrate relationship.

2 Materials

2.1 Cell Culture

1. Cell line for siRNA screen (*see Note 1*).
2. Cell line for substrate trapping experiment (*see Note 2*).
3. Growth medium.
4. Transfection medium: growth medium without antibiotics.
5. OPTI-MEM medium (Life Technologies, Carlsbad, CA, USA).

2.2 siRNA Transfection

1. Silencer[®] Select siRNAs (Life Technologies) were used for suppressing expression of human RPTPs. Other species and siRNA sources can be used.

2. DharmaFECT 3 (Thermo Fisher Scientific, Rockford, IL, USA) transfection reagent for siRNA transfection.
3. Appropriate siRNA transfection reagents can be used depending on cell type and siRNA.

2.3 Human Phospho-RTK Array

1. Proteome Profile™ antibody array for human phospho-RTKs (R&D Systems, Minneapolis, MN, USA) (*see Note 3*). This particular array kit contains the nitrocellulose membrane array which is spotted with duplicates of antibodies to the following human RTKs: Axl, Dtk, EGFR, EphA1, EphA2, EphA3, EphA4, EphA6, EphA7, EphB1, EphB2, EphB4, EphB6, ErbB2, ErbB3, ErbB4, FGFR1, FGFR2 α , FGFR3, FGFR4, Flt-3/Flk-2, HGF R/c-MET, IGF-IR, InsulinR/CD220, M-CSFR, Mer, MSPR/Ron, MuSK, PDGFR α , PDGFR β , c-Ret, ROR1, ROR2, SCFR/c-kit, Tie-1, Tie-2, TrkA, TrkB, TrkC, VEGFR1/Flt-1, VEGFR2/KDR, VEGFR3/Flt-4. Eight positive controls on the array are proprietary to the manufacture whilst the ten negative controls consisted of various non-specific IgGs. Both positive and negative controls are used for purposes of normalization. The assay kit provides the following proprietary buffers; array buffer 1, array buffer 2 and wash buffer and anti-phosphotyrosine-HRP antibody.
2. Enhanced chemiluminescence.

2.4 Plasmids

1. Mammalian expression vector encoding of full-length wild-type RPTP.
2. Mammalian expression vector encoding full-length substrate-trapping mutant.
3. Mammalian expression vector encoding full-length RTK.

2.5 Buffers and Solutions

1. Cell lysis buffer 1: 150 mM NaCl, 50 mM Tris-HCl (pH 7.8), 1 mM EDTA, 1 % Nonidet P-40, 2 mM sodium orthovanadate (Na_3VO_4), 10 mM sodium fluoride (NaF), 1 mM dithiothreitol (DTT), 1 mM benzamidine, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 $\mu\text{g}/\text{mL}$ pepstatin A, 5 $\mu\text{g}/\text{mL}$ aprotinin, and 5 $\mu\text{g}/\text{mL}$ leupeptin.
2. Cell lysis buffer 2: 150 mM NaCl, 50 mM Tris-HCl, pH 7.8, 1 mM EDTA, 1 % Nonidet P-40, 10 mM iodoacetic acid, 10 mM NaF, 1 mM DTT, 1 mM benzamidine, 1 mM PMSF, 1 $\mu\text{g}/\text{mL}$ pepstatin A, 5 $\mu\text{g}/\text{mL}$ aprotinin, and 5 $\mu\text{g}/\text{mL}$ leupeptin.
3. STE buffer: 100 mM NaCl, 10 mM Tris-HCl (pH 8.0), 1 mM EDTA.
4. ST buffer: 150 mM NaCl and 50 mM Tris-HCl (pH 8.0).
5. 10 mM Na_3VO_4 solution.

2.6 GST-Fusion Proteins

1. Glutathione S-transferase-fusion PTP wild type.
2. Glutathione S-transferase-fusion PTP substrate-trapping mutants.
3. Glutathione S-transferase (GST).

3 Methods

3.1 Receptor Protein Tyrosine Phosphatase-Receptor Tyrosine Kinase Screening

Figure 1 shows the schematic strategy of the siRNA RPTP-phospho-RTK screen. Each RPTP is knocked down in cells by siRNA, lysates are prepared and incubated with the phospho-RTK array. As a control, cells are transfected with non-targeting (NT) siRNA and these lysates are incubated with the array. Following incubation with lysates prepared from the RPTP siRNA-treated and NT-treated cells the arrays are incubated with anti-phosphotyrosine antibodies. The relative value of phosphotyrosine intensity for each RTK is quantitated as described in Subheading 3.1.5.

3.1.1 Characterization of human RPTP expression (See Note 4)—As a first step, the identity of the complement of RPTPs that are expressed in the target cell line should be determined using standard RT-PCR approaches. Because each human RPTP has different mRNA isoforms, primers against each RPTP should be designed within the common region of all isoforms. However, to amplify specific RPTPs, the primers should be targeted to the non-conserved RPTP region. The sequence of the primers should be then interrogated against the NCBI database to ensure that the sequences do not match to those of other genes. We identified 16 of 21 RPTPs that are expressed in MCF10A breast epithelial cells [19].

3.1.2 Optimization of siRNA to Knockdown Human RPTPs (See Note 5)—Various concentrations of siRNA from 5 to 100 nM and various times from 24 to 96 h post-transfection can be used to determine the optimal condition in which to perform siRNA RPTP knockdown. The efficiency of the knockdown can be initially examined by standard RT-PCR or quantitative PCR. Ideally, one should confirm the efficiency of the siRNA knockdown by immunoblotting for expression of each of the RPTPs. However, this is extremely costly and the efficiency of many of the antibodies to the RPTPs are marginal making this exercise somewhat inefficient at this early stage in the procedure.

3.1.3 Cell Preparation for Phospho-RTK Array

1. Prepare cells at approximately 70 % confluence in 150 mm culture dishes (approximately 1×10^7 cells per dish).
2. Mix optimized concentration of siRNA in 1 mL OPTI-MEM and optimized volume of transfection reagent in 1 mL of OPTI-MEM and incubate at RT for 25 min.
3. While incubating siRNA-transfection reagent complex, replace growth medium with 15 mL of transfection medium.
4. Drop the siRNA-transfection reagent complex into the 150 mm culture dishes.
5. After 16-h incubation replace transfection medium with 25 mL of growth medium.

6. Lyse cells with 1 mL of cell lysis buffer 1 at the time previously determined to confer the optimized knockdown efficiency.

3.1.4 Phospho-RTK Array—All steps are performed at room temperature.

1. Block the array with 2 mL of array buffer 1 on a rocking shaker for 1 h.
2. Incubate the array with diluted lysates (500 µg) approximate volume of 1.5 mL with array buffer 1 on a rocking shaker for 2 h.
3. Wash the array with 2 mL of wash buffer three times on a rocking shaker for 10 min.
4. Incubate the array with 1.5 mL of diluted HRP-conjugated phosphotyrosine antibodies (1: 5,000 dilution) with array buffer 2 on a rocking shaker for 2 h.
5. Wash the array in 2 mL of wash buffer three times on a rocking shaker for 10 min.
6. Visualize using enhanced chemiluminescence.

3.1.5 Quantitative Analysis of RPTP-RTK Screen—For quantitative analysis of the RPTP-RTK screen, the arrays are scanned and intensities of immunoblotted “spots” in the array are measured using image J or other comparable densitometric software programs. The relative values of phosphotyrosyl RTK intensities derived from duplicate phospho-RTK “spots” following knockdown by an individual RPTP is calculated as follows: average intensity of duplicated RTK spots is measured and subtracted by average intensity of ten negative control spots (subtraction of background); this value is normalized to the average intensity of eight positive control spots which is subtracted by the average of ten negative control spots as described in the following equation:

$$\text{The relative values of phosphotyrosyl RTK} = \frac{(\text{average intensity of duplicated RTKs spots}) - (\text{average intensity of 10 negative control spots})}{(\text{average intensity of 8 positive control spots}) - (\text{average intensity of 10 negative control spots})}$$

The relative change in each phosphotyrosyl RTK intensity for an individual RPTP targeting siRNA is calculated by subtraction from the corresponding RTK intensity for non-targeting siRNA. Positive values represent hypertyrosyl phosphorylated RTKs and negative values hypotyrosyl phosphorylated RTKs. The phosphorylation levels of the RTKs are normalized across the 12 RPTPs to yield Z scores that are plotted on a heat map using the g-plot package in R (Version 2.13) (Fig. 1).

3.2 Validation of RTK as a Direct Substrate of RPTP

The quantitative analysis of the siRNA screen will show both hypo- and hypertyrosyl phosphorylated RTKs upon RPTP knockdown. By definition, putative RTKs that serve as RPTP substrates will be hyper tyrosyl phosphorylated. However, those that are indirectly regulated by RPTPs will be hypo-tyrosyl phosphorylated. Several studies have shown that RPTPs can activate protein tyrosine kinases. For example PTP α (PTPRA) activates Src and Fyn [20, 21] and PTP ϵ (PTPRE) knockout mice exhibit decreased Syk kinase activity [22].

All hyper-tyrosyl phosphorylated RTKs should be validated using the substrate-trapping approach discussed below.

RPTP substrate-trapping mutants can be generated in order to determine whether the putative hyper-tyrosyl phosphorylated RTK is a direct RPTP substrate. PTP substrate-trapping mutants stably bind their cognate substrate but are unable to catalyze their dephosphorylation [23, 24]. Epitope-tagged substrate-trapping RPTP mutants for immunoprecipitation representing substitutions at the conserved aspartic acid (D) residue in the active site of the PTP-D1 domain to alanine (A) (D/A) and a cysteine (C) to serine (S) mutation (C/S) can be generated. Cells are transfected with these epitope-tagged substrate-trapping mutants and cell lysates are immunoprecipitated with the antibodies against the epitope tag of the trapping mutant. The substrate-trapping mutants form stable interactions with the endogenous putative RTK, whereas wild type RPTP interacts to a much lesser extent. If the RPTP mutants are not able to form a stable complex with the putative RTK, this is interpreted to indicate that the RTK is an indirect substrate of the RPTP.

3.2.1 Substrate-Trapping in Cells (See Note 6)

1. Prepare cells at approximately 70 % confluence in 100 mm culture dishes.
2. Transfect cells with 10 µg of epitope-tagged wild type RPTP, substrate trapping mutants (C/S or D/A) and control vector.
3. Lyse cells with 1 mL of cell lysis buffer 2 for 30 min on ice.
4. Immunoprecipitate 1 mg of cell lysates using antibodies against its epitope tag.
5. After washing immunoprecipitates with 1 mL of cell lysis buffer 2 three times then wash with 1 mL of STE and resolve protein complex on SDS-PAGE and detect putative substrate using RTK and phosphotyrosine antibodies.

3.2.2 Vanadate Competition Assay—If the complex between wild-type RTK and the RPTP substrate-trapping mutant is direct, this interaction should be disrupted by the PTP catalytic site inhibitor vanadate. This experiment can be performed by affinity precipitation assays using a purified RPTP-PTP domain substrate-trapping mutant that is incubated either in the absence or presence of vanadate, along with lysates prepared from RTK expressing cells. The substrate-trapping PTP domain mutant of the RPTP should form an enzyme-substrate complex with the RTK in the absence of vanadate; in contrast this complex should be disrupted in the presence of vanadate.

1. Incubate 10 µg of GST-RPTP-CS fusion proteins with 10 mM of Na₃VO₄ for 10 min at 4 °C and wash GST-fusion proteins with 1 mL of PBS one time.
2. Resuspend GST-fusion proteins with 1 mg of lysates which is lysed with cell lysis buffer 2 and incubate on a rocking shaker for 3 h at 4 °C.
3. After washing the protein complex with 1 mL of cell lysis buffer 2 without iodoacetic acid three times, wash with 1 mL of ST. Finally, resolve the protein complex on SDS-PAGE and detect putative substrate using RTK antibodies.

4 Notes

1. The selection of the cell line to use is an important component of the siRNA RPTP-RTK screen. Cell lines should satisfy the users' specific purpose of investigation. The choice of cell lines is also dictated by the ease in which siRNA transfection and knockdown efficiency can be achieved. Although the cell type of choice should be driven by the questions of the investigator there are other important aspects of cell line choice to consider. The first is the state of RTK tyrosyl phosphorylation in the particular cell line. If a cell line has a high level of tyrosyl-phosphorylated RTKs, RPTP knockdown is unlikely to generate levels of RTK hyper tyrosyl phosphorylation that are readily detected in the screen. Therefore, it is preferable to identify cell lines that exhibit generally low levels of basal RTK tyrosyl phosphorylation. If this is not possible alternative avenues such as manipulating culture conditions in order to achieve a low basal RTK activity can be attempted.
2. In general, the cell lines used for the screen if possible should also be used to perform the substrate-trapping experiments. Preferably, cells should express the endogenous RTK that is to be validated for substrate-trapping. In cases where the RTK is not detected cells can be co-transfected with wild-type RTK along with the RPTP substrate-trapping mutants. In some cases, the basal level of tyrosyl phosphorylation of RTK is not easily detected under cell-proliferating conditions in this situation; cells can be stimulated with the appropriate RTK ligand or in the absence of an available ligand, pervanadate.
3. A mouse phospho-RTK array is also available at R&D Systems.
4. RPTP PTP-OST is a pseudogene in humans and is not expressed.
5. Before optimizing for the knockdown condition a fluorescence-labeled siRNA can be used to determine the optimal condition for transfection efficiency in cells.
6. Users should establish the level of tyrosyl phosphorylated RTK under study. If it is not detectable, cells should be stimulated with the cognate ligand for the RTK or treat cells with pervanadate in order to induce RTK tyrosyl phosphorylation.

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Fig. 1. Schematic strategy of the siRNA RPTP-phospho-RTK screen