Regulation of receptor protein-tyrosine phosphatase dimerization

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Abstract

Receptor protein-tyrosine phosphatases (RPTPs) are single membrane spanning proteins belonging to the family of PTPs that, together with the antagonistically acting protein-tyrosine kinases (PTKs), regulate the protein phosphotyrosine levels in cells. Protein-tyrosine phosphorylation is an important post-translational modification that has a major role in cell signaling by affecting protein–protein interactions and enzymatic activities. Increasing evidence indicates that RPTPs, like RPTKs, are regulated by dimerization. For RPTP\textsubscript{a}, we have shown that rotational coupling of the constitutive dimers in the cell membrane determines enzyme activity. Furthermore, oxidative stress, identified as an important second messenger during the past decade, is a regulator of rotational coupling of RPTP\textsubscript{a} dimers. In this review, we discuss the biochemical and cell biological techniques that we use to study the regulation of RPTPs by dimerization. These techniques include (co-) immunoprecipitation, RPTP activity assays, chemical and genetic cross-linking, detection of cell surface proteins by biotinylation, and analysis of RPTP\textsubscript{a} dimers, using conformation-sensitive antibody binding.

Keywords: (Receptor) protein-tyrosine phosphatases; Dimerization; Immunoprecipitation; Rotational coupling; Cross-linking

1. Introduction

Phosphorylation of proteins on tyrosine residues affects the function of many signal transduction proteins and is therefore an important determinant in cellular signaling. Phosphorylated levels are specifically and coordinately regulated by two antagonistically acting families of enzymes, the protein-tyrosine kinases (PTKs) and the protein-tyrosine phosphatases (PTPs). The classical PTP family can be subdivided into cytoplasmic and transmembrane PTPs [1], the latter being interesting because of their ability to signal across the membrane. The members of the transmembrane PTP family (reviewed in [2]), tentatively called receptor PTPs (RPTPs), differ mostly in their extracellular domain, varying from very small (e.g., RPTP\textsubscript{a}, 23 amino acids) to very large with multiple fibronectin type III-like and immunoglobulin domains (e.g., LAR). RPTPs have a single membrane-spanning domain and most RPTPs contain two cytoplasmic catalytic domains. The N-terminal membrane-proximal domain, RPTP-D1, contains most of the catalytic activity, while the C-terminal membrane-distal PTP domain, RPTP-D2, appears to play a regulatory role.

RPTP activity may be regulated by dimerization, analogous to their enzymatic counterparts, the receptor PTKs (RPTKs). The first evidence for dimerization as a regulatory mechanism of RPTP activity came from studies with a chimeric protein consisting of the extracellular domain of the epidermal growth factor receptor (EGFR, a proto-typical RPTK) and the intracellular domain of the RPTP CD45. Ligand-induced dimerization of EGFR/CD45 leads to functional inactivation of CD45 [3], suggesting that dimerization inhibits CD45 activity.
The crystal structure of the N-terminal membrane-proximal PTP domain of RPTP \(\alpha\) (RPTP-\(\alpha\)-D1) provided structural support for dimerization-induced regulation of RPTP activity [4]. A helix-turn-helix wedge-like segment to the N-terminal side of RPTP-\(\alpha\)-D1 that is conserved in RPTPs, but not in cytoplasmic PTPs, interacts with the dyad-related monomer, thus forming a dimer in which both catalytic sites are occluded [4]. For CD45, it was shown that mutation of a single residue in the corresponding wedge region abolishes dimerization-induced functional inactivation, strongly suggesting that the wedge plays a role in dimerization-induced inactivation of CD45 [5].

Forced dimerization of RPTP\(\alpha\) by insertion of a cysteine at position 137 in the extracellular domain, resulting in the formation of a disulfide bridge between two monomers, leads to inactivation of RPTP catalytic activity, which is dependent on an intact wedge [6]. However, introduction of cysteines at other positions in the extracellular domain (135, 139, and 141) leads to constitutive dimerization but not to inactivation of RPTP\(\alpha\), demonstrating that dimerization per se does not lead to inactivation. The positions of the disulfide bonds suggest that rotational coupling between the two monomers in a dimer is an important determinant for dimer activity.

Evidence is accumulating that RPTPs can be regulated by dimerization, as discussed above. However, do RPTPs indeed dimerize? It has been found that RPTPs dimerize constitutively in living cells [7–9]. Different domains in RPTP\(\alpha\) contribute to dimerization [7]. In addition, we have used fluorescence resonance energy transfer (FRET) to demonstrate dimerization of RPTP\(\alpha\) fused to cyan fluorescent protein and yellow fluorescent protein in living cells [8].

Since RPTP\(\alpha\) dimerizes constitutively on the cell surface and since the position of the two monomers in the dimer relative to each other determines catalytic activity [6], it is tempting to speculate that RPTP activity is regulated by small changes in rotational coupling as a result of ligand binding or post-translational modification. Although several ligand–RPTP pairs have been identified, including pleiotrophin binding to RPTP\(\beta\)/[10] and heparan sulfate proteoglycan binding to RPTP\(\sigma\) [11], a role for ligand binding in RPTP activity has not been established definitively. Oxidative stress, known to inhibit PTPs by oxidation of the catalytic cysteine, induces a conformational change in the C-terminal PTP domain (RPTP-\(\alpha\)-D2) [12] and leads to stabilization of the dimer, resulting in prolonged inactivation of RPTP\(\alpha\) after removing oxidative stress. Furthermore, the oxidative stress-induced conformational change in RPTP-\(\alpha\)-D2 leads to a change in the position of the extracellular domains relative to each other in the dimer [13]. These results suggest that the oxidative-stress sensitive C-terminal PTP domain serves as a regulator of rotational coupling of RPTP\(\alpha\) dimers. Furthermore, Xu and Weiss [9] demonstrated that different splice isoforms of CD45 dimerize differentially, indicating that there are multiple levels of regulation for RPTP dimerization.

Here, we review the methods used in our laboratory to study the regulation of RPTPs by dimerization. We have recently described the FRET-techniques to analyse intra- and inter molecular interactions in detail [14]. Therefore, we will focus on biochemical and cell biological techniques that we use routinely, including (co-) immunoprecipitation, RPTP activity assays, chemical and genetic cross-linking, detection of cell surface proteins by biotinylation, and analysis of RPTP\(\alpha\) dimers, using conformation-sensitive antibody binding.

2. Immunoprecipitation

At the basis of all the assays described here is immunoprecipitation under relatively mild conditions. In our laboratory, we use transiently or stably transfected 293 HEK, COS-1, SK-N-MC neuroepithelioma or mouse embryo fibroblast cells. To facilitate immunoprecipitation, constructs were generated with different epitope tags fused to the N-terminus, the Haemagglutinin (HA)-tag or Myc-tag. In stably transfected cells, we obtain two- to fivefold overexpression of RPTP\(\alpha\) and in transiently transfected cells 5–10-fold. We routinely use the following protocol: cells are washed twice in ice-cold phosphate-buffered saline (PBS) and lysed in cell lysis buffer (CLB: 50 mM Heps, pH 7.4, 150 mM NaCl, 1 mM MgCl\(_2\), 1 mM EGTA, 1% Triton X-100, 10% glycerol, and protease inhibitors: 10 U/ml aprotinin, 1 mM PMSF) 20 min on ice. The cell lysates are collected by scraping and cell debris is removed by centrifugation (10 min, 14,000 rpm in a microfuge at 4°C). The cleared lysates are incubated with antibody and protein A or protein G–Sepharose beads at 4°C for 2 h with gentle agitation (end-over-end rotation). The beads are washed 4× with ice-cold HNTG (50 mM Heps, pH 7.4, 150 mM NaCl, 0.1% Triton X-100, and 10% glycerol). Following final aspiration of the wash buffer, the beads are ready for use in PTP assays (see Section 4) or for sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). For SDS–PAGE, samples are resuspended in Laemmli sample buffer (2× Laemmli sample buffer: 125 mM Tris, pH 6.8, 20% glycerol, 4% SDS, bromophenol blue and 2% β-mercaptoethanol for reducing conditions), heated at 95°C for 5 min, and run on SDS–PAGE gels. Routinely, whole cell lysates are run in parallel to monitor expression of the proteins. The material on the gel is transferred to polyvinylidenefluoride (PVDF, Immobilon-P, Millipore) membrane by semi-dry blotting. The blots are stained with Coomassie blue, blocked for 1 h at room temperature in TBST (50 mM Tris, pH 8.0, 150 mM NaCl, and 0.05% Tween 20) containing 5% milk, and incubated for 1 h with anti-HA tag MAAb
Intramolecular FRET to show that RPTP domains may be regulated by external stimuli. We used Co-immunoprecipitation of proteins from cell lysates is generally seen as a strong evidence for specific in vivo protein–protein interactions. We have used co-immunoprecipitation experiments to investigate dimerization of full-length RPTPs [12,15] and, moreover, to map the regions in RPTPs that are involved in dimerization [12,16]. It has been found that RPTP-D2s bind to RPTP-D1s in an intra- and intermolecular fashion. Furthermore, intermolecular interactions between different RPTP types have been found [16,17], suggesting that crosstalk between RPTPs may be a shared mechanism of regulation. Interestingly, interactions between RPTP domains may be regulated by external stimuli. We used intramolecular FRET to show that RPTPα-D2 changes from a ‘closed’ to an ‘open’ conformation upon oxidative stress [12]. Co-immunoprecipitation experiments have shown that this change in conformation leads to a profound increase in intermolecular binding of RPTPα-D2 to other RPTP domains. Similar experiments with LAR-D2 show that oxidative stress induces a conformational change in LAR-D2 as well [15]. The function of RPTP-D2 binding to RPTP-D1 remains to be determined definitively. Whereas RPTPα-D2 binding to RPTPα-D1 leads to inactivation of RPTPα-D1 catalytic activity [17], CD45-D2 binding to CD45-D1 increases CD45-D1 activity [18]. The effect of PTP domain binding on RPTPα-D1 catalytic activity remains to be determined [15]. However, increased stability of RPTPα dimers upon oxidative stress leads to a reduction in RPTPα activity [12].

To investigate binding between a large panel of RPTP-D1s and RPTP-D2s, constructs were generated with different epitope tags, the HA-tag or the Myc-tag, fused to the N-terminus of the RPTP-D1s and RPTP-D2s. Cells are co-transfected with an HA-tagged and an Myc-tagged construct and 48 h after transfection, the cells are lysed in CLB. The HA-tagged proteins are immunoprecipitated from the cell lysates as described above and finally, the beads are resuspended in Laemmli sample buffer, heated at 95°C for 5 min, and run on SDS-PAGE gels. Material on gel is transferred to PVDF membrane and the blots are probed with anti-Myc tag MAb 9E10 and developed by ECL. Subsequently, the strips are stripped by rinsing 2× in H2O, incubation in 12CA5 (purified from hybridoma supernatant) or anti-Myc tag MAb (9E10, Santa Cruz) in TBST with 5% milk. The blots are washed three times with TBST and incubated with horseradish peroxidase-conjugated secondary antibody for 1 h. After washing with TBST, the immunoreactivity on the membranes is visualized using enhanced chemiluminescence (ECL) according to standard protocols.

3. Co-immunoprecipitation

Co-immunoprecipitation of proteins from cell lysates is generally seen as a strong evidence for specific in vivo protein–protein interactions. We have used co-immunoprecipitation experiments to investigate dimerization of full-length RPTPs [12,15] and, moreover, to map the regions in RPTPs that are involved in dimerization [12,16]. It has been found that RPTP-D2s bind to RPTP-D1s in an intra- and intermolecular fashion. Furthermore, intermolecular interactions between different RPTP types have been found [16,17], suggesting that cross-talk between RPTPs may be a shared mechanism of regulation. Interestingly, interactions between RPTP domains may be regulated by external stimuli. We used intramolecular FRET to show that RPTPα-D2 changes from a ‘closed’ to an ‘open’ conformation upon oxidative stress [12]. Co-immunoprecipitation experiments have shown that this change in conformation leads to a profound increase in intermolecular binding of RPTPα-D2 to other RPTP domains. Similar experiments with LAR-D2 show that oxidative stress induces a conformational change in LAR-D2 as well [15]. The function of RPTP-D2 binding to RPTP-D1 remains to be determined definitively. Whereas RPTPα-D2 binding to RPTPα-D1 leads to inactivation of RPTPα-D1 catalytic activity [17], CD45-D2 binding to CD45-D1 increases CD45-D1 activity [18]. The effect of PTP domain binding on RPTPα-D1 catalytic activity remains to be determined [15]. However, increased stability of RPTPα dimers upon oxidative stress leads to a reduction in RPTPα activity [12].

4. PTP activity assay

The PTP dependent formation of p-nitrophenol from p-nitrophenylphosphate is broadly used to measure PTP activity. This assay was used to investigate the differences in catalytic activity between purified GST-fusion proteins, encoding RPTPα-D1 and -D2 and, using D2 mutants, the amino acid residues were mapped that are responsible for the reduced activity of D2 [19]. Furthermore, the effect of oxidative stress on RPTPα activity was investigated by assessing PTP activity of immunoprecipitated RPTPα from cells treated with H2O2 [12].

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Fig. 1. Co-immunoprecipitation of Myc-tagged full-length RPTPα with HA-tagged full-length RPTPα following stimulation of cells with 1 mM H2O2 for 5 min. The top panel shows co-immunoprecipitating Myc-tagged RPTPα, the middle panel the total amount of immunoprecipitated HA-tagged RPTPα, and equal expression of Myc-tagged RPTPα in the lysates is monitored in the bottom panel.

The example in Fig. 1 shows co-immunoprecipitation of Myc-tagged RPTPα with HA-tagged RPTPα upon treatment of the cells with H2O2 for 5 min, which leads to stabilization of RPTPα dimers [12].

strip buffer (62.5 mM Tris, pH 6.8, 2% SDS, and 100 mM β-mercaptoethanol) for 45 min at 70°C, and washing 4× in H2O and 1× in TBST. The blots are reprobed with anti-HA tag MAb 12CA5 to monitor equal expression of the HA-tagged proteins.

The example in Fig. 1 shows co-immunoprecipitation of Myc-tagged RPTPα with HA-tagged RPTPα upon treatment of the cells with H2O2 for 5 min, which leads to stabilization of RPTPα dimers [12].
reaction without addition of GST-fusion protein or immunoprecipitated RPTPs.

5. Chemical cross-linking

Chemical cross-linkers have been widely used to investigate protein–protein interactions, including RPTP dimerization. For instance, Takeda et al. [20] used the thiol-cleavable and homobifunctional chemical cross-linker, dithiobis succinimidyl propionate (DSP, Pierce) to show dimerization of CD45. Non-cell permeable cross-linkers may be used to cross-link surface molecules on living cells. We have used the homobifunctional non-cell permeable cross-linker, bis[sulfosuccinimidyl]suberate (BS3, Pierce), on living cells to demonstrate cross-linking of RPTPζ [7,8]. A panel of deletion mutants and BS3-mediated cross-linking was used to map the site(s) of interaction in RPTPζ. Finally, we used BS3-mediated cross-linking with different experimental conditions to assess the effect of oxidative stress on RPTPζ dimerization [12]. Xu and Weiss [9] used a similar membrane-insoluble cross-linker, ethylene glycol bis[sulfosuccinimidyl]suceinate] (sulfo-EGS, Pierce), to show cross-linking of CD45. Taken together, chemical cross-linking using membrane-insoluble cross-linkers is a versatile method to show dimerization of RPTPs.

The cells are washed (2×) in ice-cold PBS and incubated with freshly prepared BS3 (1 mg/ml) in PBS for 1 h on ice. Subsequently, the cells are washed (2×) with PBS and lysed in Tris-buffered CLB (T-CLB; 50 mM Tris, pH 7.5, 150 mM NaCl, 1 mM MgCl2, 1 mM EGTA, 1% Triton X-100, 10% glycerol, and protease inhibitors: 10 U/ml aprotinin, 1 µM PMSF), quenching the BS3 in excess for 20 min on ice. Cells are scraped, lysates collected, and cell debris removed by centrifugation at 4°C. Aliquots of the cell lysates are loaded onto SDS–PAGE gels directly or RPTPζ is immunoprecipitated using anti-HA tag MAb 12CA5, directed against the HA-tag in the extracellular domain of RPTPζ, immediately C-terminal to the signal sequence of RPTPζ. After washing 4× with HNTG (50 mM Hepes, pH 7.4, 150 mM NaCl, 0.1% Triton X-100, and 10% glycerol), reducing Laemml sample buffer is added to the immunoprecipitates, and the samples are heated at 95°C for 5 min and loaded onto a 5% SDS–PAGE gel. After electrophoresis, the material on the gel is transferred to PVDF membranes by semi-dry blotting and probed with polyclonal anti-RPTPζ antibodies or MAb 12CA5. The blots are developed by ECL according to standard protocols.

6. Genetic cross-linking

Introduction of a cysteine residue in the extracellular juxtamembrane region of a transmembrane protein may lead to the formation of a disulfide bond if two molecules are close enough together. In some respects, this is similar to the action of chemical cross-linkers and therefore introduction of a disulfide bond may be viewed as genetic cross-linking. Sorokin et al. [21] found that introduction of a cysteine residue in the extracellular domain of the EGFR by itself is not sufficient to induce dimerization. However, EGF-treatment leads to disulfide bond formation of mutant EGFR, which persists after removal of EGF. We demonstrated that introduction of cysteine residues in the extracellular domain of RPTPζ induces constitutive dimerization, as assessed by SDS–PAGE on non-reducing gels [6]. We engineered four mutants with cysteine residues in the juxtamembrane domain of RPTPζ, just outside the plasma membrane at positions 135, 137, 139, and 141 (the first residue of the transmembrane domain being Ile143), ensuring that the four mutants cover at least one complete α-helical turn. All four mutants dimerize constitutively to a similar extent. Similarly, introduction of cysteine residues in the extracellular domain of three distinct splice isoforms of CD45 leads to constitutive dimerization as well [9].

Whether there is an optimal position for engineered disulfide bonds in transmembrane proteins remains to be determined. In the EGFR, RPTPζ and CD45 disulfide bonds were successfully introduced in the extracellular juxtamembrane domain—within the first 10 residues from the transmembrane domain—either by point mutation or by insertion of a short linker encoding a cysteine.

Detection of dimers after disulfide bond formation is done by non-reducing SDS–PAGE. The cells are transfected with mutants, containing cysteine residues in the extracellular domain, and the cells are harvested by lysis in CLB containing 20 mM iodoacetic acid (IAA) to protect the disulfide bond from reduction (20 min on ice). Monomers and dimers are detected by immunoblotting of the whole cell lysate, or the protein of interest is first immunoprecipitated. To avoid interfering bands of the intact antibody in the molecular weight range of monomeric or dimeric protein of interest, the antibodies are cross-linked to protein A–Sepharose beads. We routinely cross-link antibodies to protein A–Sepharose beads with dimethylpimelimidate (DMP), using an improved version of a protocol for cross-linking of glutathione-S-transferase fusion proteins to glutathione beads [22]: the antibody is allowed to bind to protein A–Sepharose beads for 1 h at room temperature; washed 3× with 0.2 M Na2B4O7 buffer (pH 9.0); cross-linked with 20 mM DMP (fresh) in Na2B4O7 buffer (30 min, room temperature); then washed 3× with 0.2 M ethanolamine (pH 8.0); incubated for 1 h at room temperature in 0.2 M ethanolamine (to quench excess DMP); and then washed 3× with Tris-buffered saline (TBS; 50 mM Tris, pH 7.5, 150 mM NaCl). Cross-linked antibodies may be stored for later use at 4°C in TBS containing 0.02% NaN3. The immunoprecipitates are washed 4× in HNTG and the beads are resuspended.
in an equal volume of 2× Laemmli sample buffer without β-mercaptoethanol (non-reducing conditions), or with β-mercaptoethanol (2% v/v; reducing conditions). The samples are heated at 95°C for 5 min and loaded onto separate 5% SDS–PAGE gels to avoid reduction of the disulfide bond in the non-reduced samples. Similarly, whole cell lysates are mixed with non-reducing or reducing sample buffer and loaded on to separate SDS–PAGE gels. After electrophoresis, the gels are blotted by semidry blotting and the blots are probed with antibodies and developed with ECL according to standard procedures.

7. Biotinylation of cell surface proteins

Biotinylation of cell surface proteins with non-cell permeable biotin is being done to test whether constructs are differentially expressed on the cell surface. We use it to test whether stimulation of cells, for instance with H2O2, leads to relocalization of RPTPα. For this, we wash subconfluent cells transfected with HA-tagged mutants of RPTPα 2× with ice-cold PBS and label them with 0.5 mg/ml non-cell permeable biotin (EZ-linked sulfo-NHS-LC-biotin; Pierce) in a non-amine containing borate buffer (10 mM boric acid, pH 8.0, 150 mM NaCl) ice-cold for 45 min. Thereafter, cells are washed 4× with ice-cold 15 mM glycine in PBS to quench the biotin and lysed in CLB. Total RPTPα is immunoprecipitated with anti-HA MAb 12CA5 and following washing the protein A–Sepharose beads are boiled in Laemmli sample buffer. The immunoprecipitates are separated on SDS–PAGE gels and transferred to PVDF membrane. Blots are washed three times with TBST and incubated with horseradish peroxidase-conjugated avidin–biotin complexes (ABCComplex, DAKO). After washing with TBST, the immunoreactivity of the membranes is visualized using ECL according to standard protocols. The amount of transfected RPTPα is visualized by stripping the blot afterwards and incubating it with anti-HA antibody and a horseradish peroxidase-conjugated secondary antibody. A typical example of a biotinylation experiment is depicted in Fig. 2, demonstrating that only the transmembrane full-length RPTPα, but not the cytoplasmically localized RPTPα-D1, is biotinylated.

8. Dimer conformation

Membrane localization of proteins with an epitope-tag in their ectodomain can be detected by incubating living cells expressing these constructs ice-cold with an antibody against the epitope tag. This method was used for studying membrane localization of RPTPα with an HA-tag in the N-terminal part of the ectodomain. We found that anti-HA antibody binding to transfected constitutive dimer mutants of HA-tagged RPTPα depends on rotational coupling within the RPTPα dimer, in that RPTPα–P137C, but not RPTPα–P135C, bound the anti-HA antibody [13]. Furthermore, we found that antibody binding to wild-type RPTPα is prohibited by dimer formation and can be increased by shortening the extracellular domain. These results prove that the HA-tag in the N-terminal part of one monomer in a dimer can be masked by the ectodomain of the dyad-related monomer and therefore that HA-tag accessibility is a read-out for the conformation of the dimer.

For these experiments, living cells transfected with RPTPα constructs with an HA-tag in the N-terminal part of the ectodomain are incubated for 1 h ice-cold with anti-HA MAb 12CA5 in DMEM/F12 medium. After incubation, cells are washed extensively (at least 3×) with ice-cold PBS and lysed in CLB. Lysates are pre-cleared by centrifugation at 14,000 g for 15 min and rotated for 2 h at 4°C with protein A–Sepharose beads to precipitate the antibody-bound fraction. The non-antibody-bound fraction can be precipitated by an additional incubation of the lysate with anti-HA antibody coupled to protein A-Sepharose. Both fractions are spun down and washed 4× with HNTG. Samples are boiled in Laemmli sample buffer and separated on 7.5% SDS–PAGE gels. The material on the gel is transferred to PVDF membrane and the blots are probed with anti-RPTPα antibody and developed by ECL.
Fig. 3 shows that the HA-tag in full-length RPTPα (FL) is accessible to the antibody only upon stimulation of cells with H2O2 (1 mM, 5 min). As a control, we used HA-tagged RPTPα-D1 which is cytoplasmically localized and is only detected in the non-accessible fraction, because the HA-tag is not exposed on the cell membrane.

### 9. Concluding remarks

Here, we describe the methods used in our laboratory to study the regulation of RPTPs by dimerization, including two methods to detect membrane localization of proteins and three methods to analyze dimerization. Although the methods are developed to detect similar phenomena, the results may differ between the different approaches. For example, the constitutive dimer mutants RPTPα-P135C and RPTPα-F137C seem to differ in subcellular localization upon ice-cold HA-antibody labelling of living cells, while biotinylation studies showed no difference. Similar experiments with a panel of deletion mutants demonstrate that the HA-tag antibody binding assay in living cells is actually a read-out for HA-tag accessibility and can be used to assess rotational coupling in RPTPα dimers. Furthermore, the methods described to study the dimerization vary in sensitivity and therefore may give different results. For instance, co-immunoprecipitation is a widely accepted evidence that proteins interact with each other. However, the affinity of the interaction has to be relatively high to allow detection of co-immunoprecipitating proteins. While cross-linking readily allows detection of full-length RPTPα dimers, co-immunoprecipitation of full-length RPTPα was rarely observed under control conditions [12]. Finally, although genetic cross-linking indicates that proteins have some affinity for each other, in that they have been in close proximity of each other to allow formation of the disulfide bond, it is not formal proof that the wild type proteins dimerize in living cells. Perhaps more importantly, these enforced dimers may be used as positive controls for chemical cross-linking and/or functional assays. Taken together, the combination of methods described here allows assessment of transmembrane protein dimerization and provides insight into protein conformation, localization, and interactions.

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### References