Antibody-induced dimerization of HARPTPα–EGFR chimera suggests a ligand dependent mechanism of regulation for RPTPα

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Received 3 October 2000; revised 12 October 2000; accepted 13 October 2000

First published online 26 October 2000

Edited by Gianni Cesareni

Abstract We developed a system to study the function of the ectodomain of RPTPα, a transmembrane protein-tyrosine phosphatase, by fusing the HA-epitope tagged ectodomain of RPTPα to the transmembrane and intracellular domain of the epidermal growth factor receptor, EGFR, a receptor protein-tyrosine kinase that is activated by dimerization. Although the use of chemical crosslinkers shows that preformed HARPTPα–EGFR dimers exist, bivalent anti-HA-tag antibody activated HARPTPα–EGFR chimeras, suggesting this system may be used to study regulation of dimerization. We used this system to show that newborn calf serum may contain (a) potential ligand(s) for RPTPα. Our results suggest that RPTPα dimerization and thus activity may be affected by ligand binding. © 2000 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.

Key words: Protein tyrosine phosphatase α; Dimerization; Regulation; Ligand; Epidermal growth factor receptor; Crosslinking

1. Introduction

Communication between cells in multicellular organisms is an absolute requirement for the organism to develop normally. Propagation of many extracellular signals is mediated by tyrosine phosphorylation through the receptor protein tyrosine kinases (RPTKs). The accepted model is that ligand-induced dimerization brings the two kinase domains to close proximity, allowing transphosphorylation of regulatory tyrosine residues and activation [1]. RPTK activation results in phosphorylation of target proteins and initiation of signaling cascades. One of the most studied and best characterized RPTKs is the epidermal growth factor receptor (EGFR) whose activation leads to multiple downstream events, including MAPK activation.

The antagonists of the PTKs are the protein tyrosine phosphatases (PTPs) [1–3]. Intuitively, PTPs are as important for the regulation of phosphotyrosine levels in proteins as the PTKs. Receptor PTPs (RPTPs) are transmembrane proteins forming a large subgroup of the PTP family. RPTPs are distinguished by their ectodomains but all have a single transmembrane domain and most RPTPs have two tandem PTP domains. Interestingly, the majority of the catalytic activity is retained in the membrane proximal PTP domain (RPTP-D1) while the membrane distal domain (RPTP-D2) contains no or very little activity [4,5]. RPTP-D2s are thought to regulate RPTP-D1 either directly [6] or by modulating the dimerization state of RPTP-D1s [7,8].

In contrast to the RPTKs, little is known about the mechanism of regulation of the RPTPs. To date, ligands have been found for only a few RPTPs. RPTPμ, RPTPκ and RPTPδ form homophilic interactions, in that they serve as their own ligand [9–13]. Contactin is a ligand for RPTPβ/ζ and surprisingly was also shown to interact in *cis* with RPTPα [14,15]. The laminin–nidogen complex bound a specific splice variant of LAR [16]. Finally, pleiotrophin is a ligand of RPTPβ [17]. Interestingly, pleiotrophin is the first and, to date, the only ligand shown to influence the catalytic activity of a RPTP [17].

Functional [18–20] and structural [21] evidence suggests that RPTPs may be negatively regulated by dimerization. Furthermore, RPTPα dimers were detected in vivo using different techniques, including chemical cross-linking [22] and fluorescence resonance energy transfer between RPTPα fusion proteins fused to GFP mutants (L. Tertoolen, C. Blanchetot, G. Jiang, J. Overvoorde, T. Hunter and J. den Hertog, submitted). However, whether dimers are regulated (and how) remains unknown.

Here, by fusing the ectodomain of RPTPα to the EGFR transmembrane and kinase domain, we developed a system to test the involvement of the ectodomain of RPTPα, as well as potential ligands, in the regulation of dimerization. We show, using an antibody recognizing the HA-tag in the ectodomain of RPTPα, that the system is dependent on dimerization. The presence of preformed dimers, detected using chemical crosslinkers, suggests that the antibody-induced activation may be mediated by changes in the topology of the ectodomain of RPTPα that are conferred to the intracellular tyrosine kinase. Furthermore, in a first attempt to find potential ligands of RPTPα, we show that component(s) of newborn calf serum (NCS), but not fetal calf serum (FCS) affected the dimerization state of the chimeric construct through the ectodomain of RPTPα, suggesting that RPTPα dimerization may be modulated by ligands that bind to the ectodomain.

2. Materials and methods

2.1. Constructs and reagents

PSG5-13-HARPTPα–EGFR was made by PCR with the ectodomain of HARPTPα (*Xho*I/*Sph*I, aa 1–142) [23] and the transmembrane and intracellular domain of human EGFR (*Sph*I/*Sma*I, aa 646–1212). YFP fusion proteins were made by introducing yellow fluorescent protein (YFP) by PCR at position 702 of HARPTPα–

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PII: S0014-5793(00)02165-7

EGFR or at position 200 of HARPTPα. Anti-phosphotyrosine antibodies (PY20), goat anti-mouse (GAM), GAM-HRP, and goat anti-rabbit-HRP were from Transduction Laboratories, anti-Mapk antibody were from SantaCruz. Rabbit anti-EGFR antibodies (281-7) were a gift from B. Defize. 12CA5 was purified and concentrated to an estimated final concentration of 1 mg/ml. A final concentration of approximately 1 μg/ml was used for 12CA5 stimulation. 12CA5-Fab fragments were made from 12CA5 antibody using the Immunopure Fab kit (Pierce). Concanavalin A was from Sigma.

2.2. Cell cultures and transfections

293 cells were routinely grown in DF medium supplemented with 7.5% FCS. Cells were transfected using the standard calcium-phosphate method [23]. Briefly, 10-cm dishes were transfected with a total of 20 μg of DNA. The next day, the medium was refreshed, and left another 16 h before harvesting. In the case of 12CA5 stimulation, the cells were serum-starved overnight. For serum stimulation, the cells were left overnight in medium containing 7.5% FCS to maintain relatively high basal tyrosine phosphorylation, allowing detection of stimulus-induced changes. Similar results were found after serum starvation, although low basal phosphorylation of RPTPα–EGFR made it difficult to see the effect of NCS (data not shown).

2.3. Immunoprecipitation and Western blotting

Sub-confluent transfected cells were stimulated as indicated, washed twice with ice cold PBS, and lysed with cell lysis buffer CLB (50 mM HEPES 7.4, 150 mM NaCl, 1 mM $MgCl_2$, 10% glycerol, 1% Triton X-100, 1 mM Vanadate and protease inhibitors [23]) for 20 min on ice, harvested and centrifuged at $14\,000\times g$ for 15 min to remove the insoluble fraction. 12CA5 or anti-EGFR antibodies were added to the supernatant for 2 h. The beads were carefully washed 4× with HNTG buffer (20 mM HEPES pH 7.4, 150 mM NaCl, 0.1% Triton X-100, 10% glycerol), mixed with Laemmli sample buffer and loaded on a 7.5% SDS–PAGE gel. The proteins were transferred to PVDF membrane using a semi-dry transfer system. After Coomassie staining, the membrane was blocked for 1 h with 5% milk in TBST (50 mM Tris pH 8.0, 150 mM NaCl, 0.05% Tween-20), incubated with the first antibody for 1.5 h, washed 4× with TBS–Tween, incubated with secondary antibody for 1 h, washed 4× with TBST, and developed using enhanced chemiluminescence (ECL).

2.4. Crosslinking

Transfected cells were washed twice with ice cold PBS and left 30 min on ice with PBS containing 1 mg/ml of BS^3 (Sigma). After incubation, cells were carefully washed 3× with PBS and lysed in a Tris-based CLB for 20 min on ice. After harvesting, insoluble particles were spun down, protein concentration was measured before loading equal amounts on gel.

3. Results and discussion

We wanted to develop a system to study the role of the ectodomain of RPTPα and the effect of potential ligands. To this end, we made fusion proteins with the HA-tagged ectodomain of RPTPα fused to the transmembrane and intracellular domain of the human EGF receptor (EGFR), HARPTPα–EGFR (Fig. 1A). EGFR is well known to be activated by dimerization (Fig. 1A). Binding of EGF to the ectodomain of EGFR, leads to the stabilization of dimers. After ligand binding, EGFR is activated by transphosphorylation (Fig. 1A). Since RPTPα may be regulated by dimerization, EGFR seemed to be a good choice to fuse to RPTPα.

3.1. Constitutive dimerization of HARPTPα–EGFR chimeras mediated by the transmembrane domain

To test the dimeric state of the fusion protein, we used the chemical crosslinker BS^3. Since BS^3 does not cross the plasma membrane, only complexes that interact extracellularly are detected. A band at the expected size for a dimer was detected in cells transfected with HARPTPα–EGFR after crosslinking

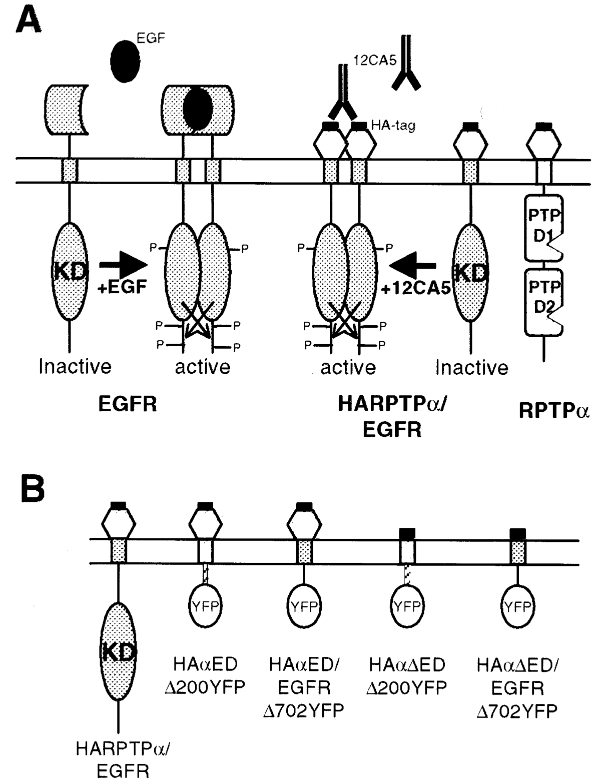

Fig. 1. Model of dimerization-induced transphosphorylation of the EGFR and chimeric constructs. A: Model for dimerization of the EGFR by EGF (left) and model of 12CA5-induced dimerization of the HARPTPα–EGFR chimera (right). KD: kinase domain. B: Deletion constructs used for the crosslinking experiments (see Section 2).

(Fig. 2A), suggesting the presence of in vivo dimers. To test which part of the protein was involved in dimerization, we made a panel of deletion mutants. The high molecular weight of HARPTPα–EGFR made the detection of dimer difficult. Therefore, we replaced the large intracellular domain of HARPTPα–EGFR by YFP in order to detect the protein and dimers more easily (Fig. 1B). Dimers were efficiently detected when a large part of the intracellular domain of the EGFR was replaced by YFP (HAαED–EGFRΔ702YFP, Fig. 2B). Dimers were also detected when a large part of the ectodomain of RPTPα was further deleted (HAαΔED–EGFR-Δ702YFP), suggesting that the transmembrane domain of the EGFR mediated dimerization in vivo (Fig. 2B). The equivalent constructs of RPTPα (HAαEDΔ200YFP and HAα ΔEDΔ200YFP) also formed dimers (Fig. 2B) [24]. It is difficult to compare the differences in band intensity for the dimers since the crosslinking efficiency will depend on the orientation and accessibility of tertiary amine-containing amino acids. Furthermore, technical reasons may be responsible for the difference, since in our hand higher complexes were always weakly detected, presumably due to poor blotting (data not shown). A similar approach has been used successfully to study the role of the different domains in RPTPα dimerization [22] and our results are consistent with reports showing that the transmembrane domain of RPTPα and of EGFR form stable dimers in vivo [22,24]. In conclusion, our results indicate that the HARPTPα–EGFR chimera formed dimers to some extent.

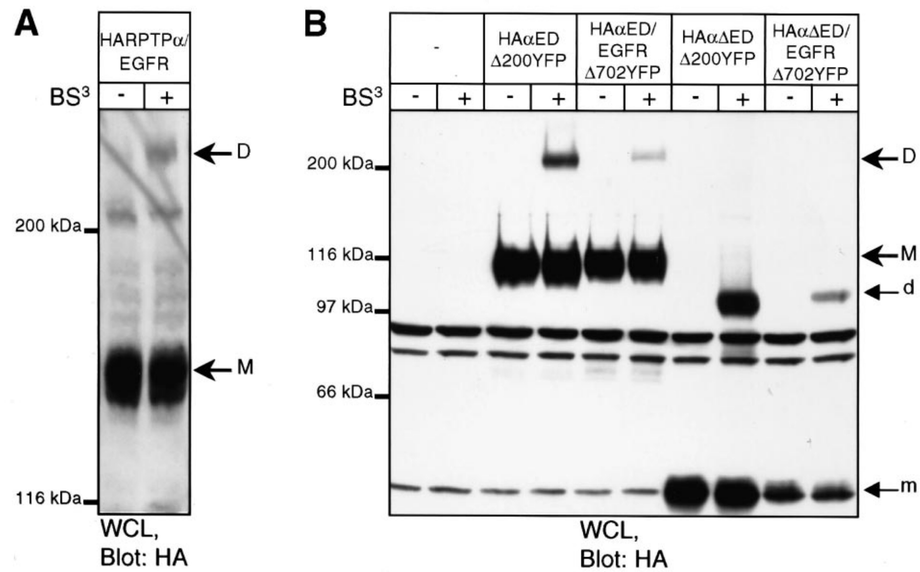

Fig. 2. Constitutive HARPTPα–EGFR dimers. A: 293 cells were transiently transfected with HARPTPα–EGFR, and crosslinked with BS^3 (+) (see Section 2) or left in PBS (−). Equal amounts of proteins were resolved on SDS–PAGE and probed with 12CA5 antibody. B: 293 cells transiently transfected with the indicated constructs with the ectodomain of RPTPα (αED) or without (αΔED) were crosslinked using BS^3 (+) or not (−). Equal amounts of proteins were resolved on SDS–PAGE and probed with 12CA5 antibody. The monomers are indicated on the right as 'm' and 'M' with the corresponding dimers indicated as 'd' and 'D' respectively. Molecular weight markers are indicated on the left.

3.2. Activation of HARPTPα–EGFR chimeras by 12CA5 is dependent on the bivalence of the antibody

We investigated if HARPTPα–EGFR was activated by ligands. We postulated that the HA-tag in the ectodomain of RPTPα could be used to induce dimers by addition of bivalent 12CA5 anti-HA-tag antibody, thus simulating a potential ligand by forming a bridge between two chimeras (Fig. 1A). Indeed, 12CA5 antibody induced activation of the HARPTPα–EGFR chimeras as detected by tyrosine-phosphorylation of the receptor (Fig. 3A). The same effect was found in transiently transfected 293 cells as in stable NIH-3T3 cell lines, expressing different levels of fusion proteins, although basal levels of tyrosine phosphorylation varied from experiment to experiment (data not shown). The effect was specific since the same 12CA5 antibody had no effect on EGFR (Fig. 3B) and untagged chimera (RPTPα/EGFR, Fig. 5C and data not shown) and was sustained for at least 1 h (Fig. 3C). Furthermore, addition of the 12CA5 antibody led to MAPK activation (Fig. 3C), indicating functional activation of HARPTPα–EGFR. In conclusion, we show that HARPTPα–EGFR can be activated by an antibody recognizing the HA-tag in the ectodomain of the fusion protein.

We further investigated if the activation was due to dimerization or to aggregation. We made use of Fab fragments from the 12CA5 antibody. Fab fragments are monovalent, but are still able to bind to the HA epitope, although may be less efficiently. 12CA5-Fab fragments by themselves did not induce HARPTPα–EGFR tyrosine phosphorylation (Fig. 4A). However, preincubation of stably expressing HARPTPα–EGFR cells with 12CA5-Fab fragments reduced the 12CA5-induced activation in a concentration dependent manner (Fig. 4A). This indicates that the 12CA5-Fab fragments are able to compete with the antibody by binding to the HA-tag of the HARPTPα–EGFR chimeras. Furthermore, although 12CA5-Fab fragments were unable to induce activation, preincubation of the Fab fragments with GAM antibody (restoring bivalence) induced activation of HARPTPα–EGFR (Fig. 4B). Other agents inducing aggregation of glycoproteins like concanavalin A or wheat germ agglutinin had no effect on the tyrosine phosphorylation of HARPTPα–EGFR (Fig. 4C and data not shown). Nevertheless, concanavalin A still induced MAPK activation (Fig. 4C), presumably by activating receptors other than HARPTPα–EGFR, by aggregation. Our results clearly indicate that activation of HARPTPα–EGFR by 12CA5 is dependent on the bivalence of the antibody, suggesting that HARPTPα–EGFR is regulated by dimerization. The bivalent antibody may not induce dimerization de novo, but instead may induce a change in the relative orientation of the two monomers in preformed dimers. In general, the orientation of two receptors relative to each other within the dimer may affect receptor activity [25]. The best-characterized example is the erythropoietin receptor (EpoR) for which a specific orientation between the two monomers, driven by ligand binding, is required for full activation of downstream events [26]. The same may be applicable to the EGFR and may explain the existence of low and high affinity receptors [27,28]. Furthermore, in the context of RPTPα, conformational changes in preformed dimers may be favored in response to ligands because of the intrinsic potential of RPTPα to dimerize in vivo (Fig. 2B) [22].

The use of antibody to induce dimerization and activation is not new for RPTKs. Many examples exist that used specific antibodies for the ectodomain of the RPTK in question, for instance the EGFR and the EpoR [26,27]. We developed a system that applies the widely used HA-epitope-tag and 12CA5, the specific monoclonal antibody that recognizes the HA-tag. Such a system has a few advantages. First, it does not require the production of receptor specific antibodies [26,27]. Second, it allows specific dimerization of the HA-tagged receptor in the presence or absence of endogenous receptor. Finally, it is presumably completely cell type independent. Taken together, the system should allow the study of any receptor in any cell type. Limitations are that the epitope tag may by itself induce conformational changes in the ectodomain, and may not be optimal for antibody induced dimerization. However, we did not encounter problems with RPTPα in these respects, but in general, this approach may require receptor specific optimization.

Although 12CA5 binding to HARPTPα–EGFR brought the two kinase domains to close proximity allowing activation, we do not know what the intracellular effect on HARPTPα would be. Addition of the antibody may bring close or pull apart the intracellular PTP domain. Furthermore, it is not known if antibody binding would be sufficient to disrupt or change the configuration of the transmembrane dimer and/or the D1–D1 dimer [20,22]. However, the fact that dimerization of RPTPα ectodomain can be modulated by ligand binding provides the first evidence that ligand(s) may affect RPTPα.

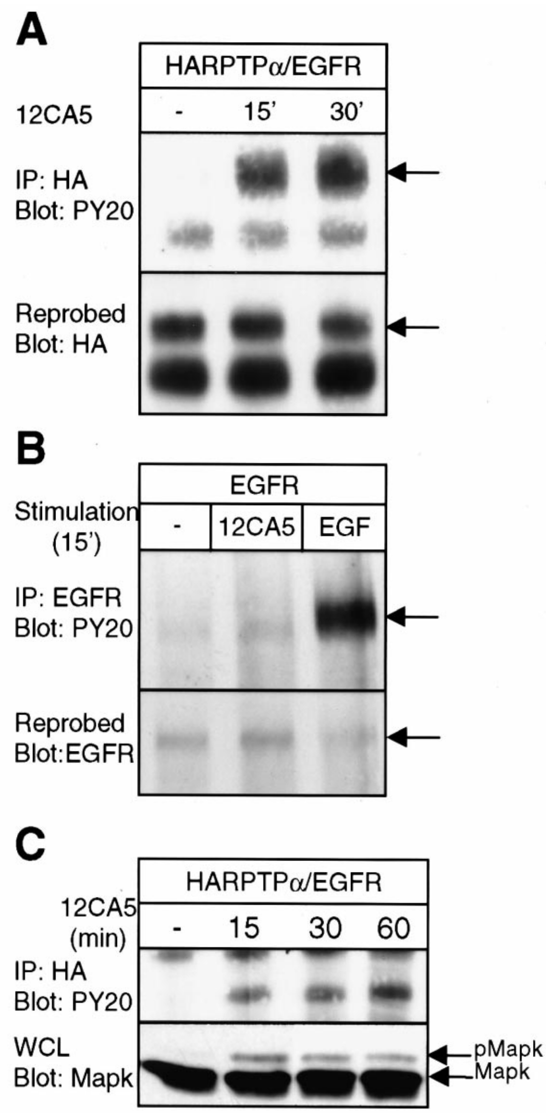

Fig. 3. Activation of the HARPTPα–EGFR chimeras by addition of 12CA5 antibody. A: NIH3T3 cells stably expressing HARPTPα–EGFR were serum-starved and stimulated with 12CA5 antibodies (∼1 μg/ml) for 15 or 30 min as indicated. HARPTPα–EGFR was immunoprecipitated using 12CA5 antibody, and probed with PY20 antibody (top panel). After stripping, the same immunoblot was reprobed with 12CA5 antibody (bottom panel). B: NIH3T3 cells stably overexpressing EGFR were stimulated with nothing (−), 12CA5 or EGF (50 ng/ml) for 15 min. EGFR was immunoprecipitated with anti-EGFR antibody and analyzed for their phosphotyrosine content with PY20 (top panel). After stripping the same immunoblot was reprobed with anti-EGFR antibody (bottom panel). C: NIH3T3 cells stably expressing HARPTPα–EGFR were stimulated with 12CA5 for the indicated time. HARPTPα–EGFR was immunoprecipitated with 12CA5 antibody, and probed with PY20 antibody (top panel). Aliquots of the whole cell lysate was probed with anti-Mapk (bottom). Proteins of interest are indicated with an arrow. pMapk is phosphorylated, activated Mapk.

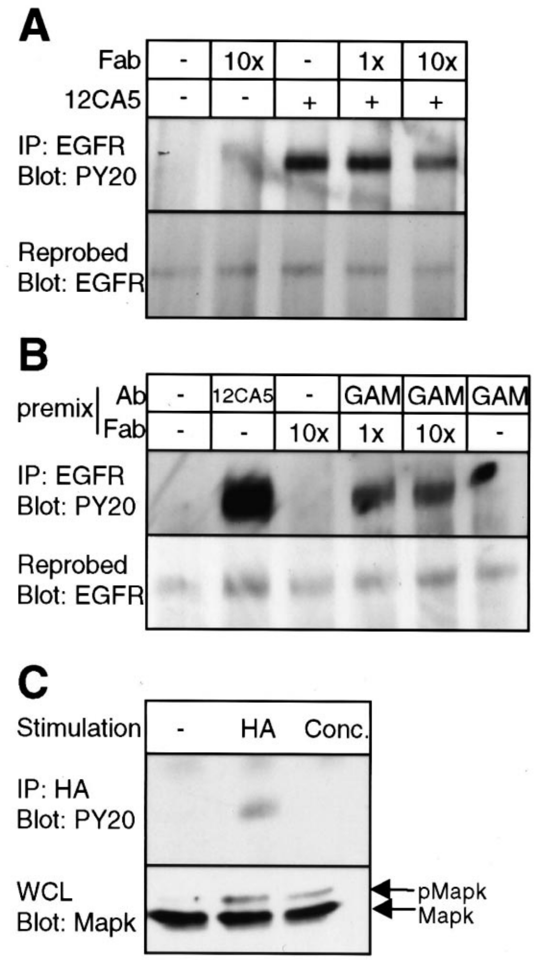

Fig. 4. HARPTPα–EGFR activation is dependent on the bivalence of the antibody. A: NIH3T3 cells stably expressing HARPTPα–EGFR were serum-starved and preincubated as indicated with nothing (−) or with 12CA5-Fab fragments for 15 min (1×, ∼0.5 μg/ml and 10×, ∼5 μg/ml) before addition of 12CA5 antibody (∼1 μg/ml) for another 30 min (HA) or not (−). After anti EGFR immunoprecipitation, the phosphotyrosine content of HARPTPα–EGFR was tested with PY20 (top panel). The same blot was reprobed with anti-EGFR antibody (bottom panel). B: NIH3T3 cells stably expressing HARPTPα–EGFR were serum-starved and incubated with 12CA5 antibody (12CA5), 12CA5-Fab fragments (Fab), GAM antibody or a mixture of pre-coupled 12CA5-Fab fragments and GAM antibody for 30 min. After anti-EGFR immunoprecipitation, the phosphotyrosine content of HARPTPα–EGFR was tested with PY20 (top panel). The same blot was reprobed with anti EGFR antibody (bottom panel). C: NIH3T3 cells stably expressing HARPTPα–EGFR were serum-starved and stimulated with 12CA5 antibodies (HA) or with Concanavalin A (50 μg/ml) for 15 min. After 12CA5 immunoprecipitation, the phosphotyrosine content was tested with PY20 (top panel), and whole cell lysate was probed with anti-Mapk (bottom panel).

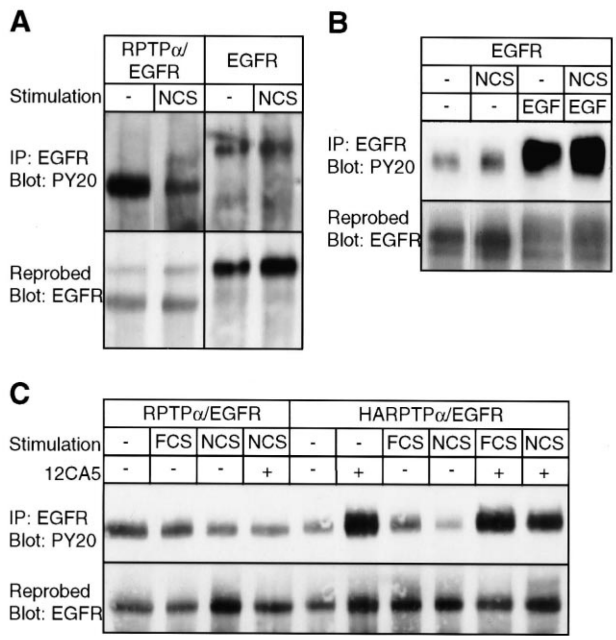

Fig. 5. NCS may contain ligand for RPTPα. A: NIH3T3 cells stably expressing RPTPα–EGFR (without HA-tag) or EGFR, were stimulated with 10% NCS (NCS) or not (−) for 30 min. After EGFR immunoprecipitation, the blot was probed with PY20 (top). The same blot was reprobed after stripping with anti-EGFR antibody (bottom). B: 293 cell transiently expressing RPTPα–EGFR or HARPTPα–EGFR were stimulated with 10% FCS or 10% NCS, and 12CA5 antibody (+) or not (−) for 30 min. The anti-EGFR immunoprecipitates were blotted and probed with PY20 (top panel) and reprobed with anti-EGFR antibody (bottom panel). C: 293 cell transiently expressing EGFR were stimulated with 10% NCS, and 50 ng/ml EGF (+) or not (−) for 30 min. The anti-EGFR immunoprecipitates were blotted and probed with PY20 (top panel) and reprobed with anti-EGFR antibody (bottom panel).

3.3. NCS may contain a ligand for RPTPα

The identification of ligand is crucial to understand the function of a receptor. However, ligand identification can be time consuming and fastidious. Having shown that the dimerization state of the HARPTPα–EGFR could be altered and detected by tyrosine phosphorylation, we used the chimeric construct to test potential ligand for the ectodomain of RPTPα. In a broad attempt to test for ligand(s) of RPTPα, we tested sera, NCS and FCS. Interestingly, effects were detected when NCS was added to the medium. Stimulation of growing cells with NCS led to a small but reproducible decrease in basal level tyrosine phosphorylation of RPTPα–EGFR, but not EGFR (Fig. 5A). Although the NCS-induced decrease in RPTPα–EGFR tyrosine phosphorylation is reproducible, the extent of the decrease is variable (cf. Fig. 5A,B), which is presumably due to the differences in experimental conditions. The same effects were detected in serum-starved cells, although the lower basal tyrosine phosphorylation of the receptor made the effect of NCS difficult to observe (data not shown). In addition, NCS had a negative effect on the 12CA5-induced activation of HARPTPα–EGFR (Fig. 5B), suggesting possible competition between the ligand in NCS and the antibody. The effect of NCS was specific for the ectodomain of RPTPα since NCS did not affect tyrosine phosphorylation of the EGFR in the absence (Fig. 5A) or in the presence of EGF (Fig. 5B) excluding an indirect effect of NCS on the receptor (activation of phosphatases, for example). Interestingly, stimulation with FCS had no effect on tyrosine phosphorylation of HARPTPα–EGFR or RPTPα–EGFR (Fig. 5C, data not shown). These results suggest that (a) component(s) in NCS (but not in FCS) are able to affect the dimerization state of RPTPα–EGFR through binding to the ectodomain of RPTPα, further suggesting that potential ligands for RPTPα are present in NCS. We do not know if ligand binding induced either monomerization of HARPTPα–EGFR or stabilization of an inactive dimeric state. Since RPTPα's activity may be regulated by dimerization [20], it is tempting to speculate that RPTPα activity may be regulated by external stimuli that affect its dimerization state and thus activity. Analysis of the effect of NCS on RPTPα activity is hampered by the fact that NCS contains too many factors that have too many direct and indirect effects on the cell (for example, Src, a known substrate of RPTPα may be regulated independently of RPTPα by specific NCS components [29]). Further purification and identification of the ligand will be required to test the direct effect of this ligand on RPTPα activity.

Little is known about the regulation of RPTP activity by ligand-induced dimerization. Here, we describe a system that may be used to characterize ligand(s) and/or to study RPTP signaling by 12CA5-induced dimerization. Moreover, it will be interesting to use similar systems to test the effect of potential ligands on dimerization and activity of other RPTPs, e.g. the effect of pleiotrophin on RPTPβ dimerization and activation. Recently, Contactin, a GPI-anchored protein was suggested to act as a ligand for RPTPα [15]. Contactin was shown to bind to RPTPα in *cis*, excluding Contactin as being the ligand in the NCS. It will be interesting to see the effect of Contactin on HARPTPα–EGFR dimerization.

In conclusion, using an antibody dependent dimerization system, we show that the ectodomain of RPTPα can mediate ligand binding, leading to intracellular signaling. Furthermore, we show that NCS contains endogenous ligand(s) for RPTPα, suggesting that RPTPα dimerization and thus activity may be regulated by extracellular signals.

Acknowledgements: We thank Bas Defize for anti-EGFR antibody (281-7), John Overvoorde for his technical assistance, Dr. Arjan Buist and Jaap van Hellemond for helpful discussions.

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