The tyrosine phosphatase CD148 interacts with the p85 regulatory subunit of phosphoinositide 3-kinase

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CD148 is a transmembrane tyrosine phosphatase that has been implicated in the regulation of cell growth and transformation. However, the signalling mechanisms of CD148 are incompletely understood. To identify the specific intracellular molecules involved in CD148 signalling, we carried out a modified yeast two-hybrid screening assay. Using the substrate-trapping mutant form of CD148 (CD148 D/A) as bait, we recovered the p85 regulatory subunit of PI3K (phosphoinositide 3-kinase). CD148 D/A, but not catalytically active CD148, interacted with p85 in a phosphorylation-dependent manner in vitro and in intact cells. Growth factor receptor and PI3K activity were also trapped by CD148 D/A via p85 from pervanadate-treated cell lysates. CD148 prominently and specifically dephosphorylated p85 in vitro. Co-expression of CD148 reduced p85 phosphorylation induced by active Src, and attenuated the increases in PI3K activity, yet CD148 did not alter the basal PI3K activity. Finally, CD148 knock-down by siRNA (short interfering RNA) increased PI3K activity on serum stimulation. Taken together, these results demonstrate that CD148 may interact with and dephosphorylate p85 when it is phosphorylated and modulate the magnitude of PI3K activity.

Key words: CD148, protein tyrosine phosphatase, p85, phosphoinositide 3-kinase (PI3K).

INTRODUCTION

CD148 (also named DEP-1/PTPθ) is a transmembrane PTP (protein tyrosine phosphatase) that is composed of an extracellular segment of eight fibronectin type-III repeats, a transmembrane domain and a single intracellular PTP domain [1]. CD148 is expressed in various cell types including vascular endothelial cells [2] and duct epithelia in thyroid, mammary and gastrointestinal tissues [3–5]. Growing evidence indicates a prominent role for CD148 in negative regulation of cell proliferation and transformation. The homoygous CD148 mutant mice die at mid-gestation due to vascularization failure accompanied by aberrant endothelial cell proliferation and vessel growth [6]. CD148 is down-regulated in cancer cell lines, correlated with their malignant phenotype [7–9]. and introduction of CD148 prominently suppresses tumour cell growth in vitro and in vivo [3,8,10]. Furthermore, ptrpj (CD148) has been implicated as a tumour suppressor in human cancers [11]. Loss of heterozygosity at the ptrpj locus is frequently observed in human cancers [11,12]. However, the intracellular signalling molecules or pathways that directly couple with CD148 are still incompletely understood.

The PI3K (phosphoinositide 3-kinase) cascade has been linked to many important cellular functions, including cell growth and survival [13]. In addition, abnormal activation of this pathway was shown to be involved in increased cell growth and malignant phenotype of tumours [13,14]. The well-characterized class IA PI3K is a heterodimeric protein composed of a 110 kDa catalytic subunit (p110) and a 85 kDa regulatory subunit (p85). Under resting conditions, p85 SH2 (Src homology 2) domains stabilize the p85–p110 complex and inhibit PI3K activation [15]. This inhibition is relieved by binding of the SH2 domains of p85 to the YYXM (where X is any amino acid) motif in tyrosine-phosphorylated receptors or adaptor proteins. Furthermore, recent studies have demonstrated that tyrosine phosphorylation of p85 may also alter PI3K activity [16,17].

To determine the specific intracellular proteins through which CD148 signals, we here conducted a modified yeast two-hybrid assay using a ‘substrate trapping’ mutant form of CD148 (CD148 D1205A; referred to throughout as CD148 D/A) as bait. The present study demonstrates the first evidence that CD148 may interact with the p85 regulatory subunit of PI3K and modulate the level of PI3K activity.

MATERIALS AND METHODS

Reagents

Antibodies: anti-HA (haemagglutinin) (12A5) was from Roche (Indianapolis, IN, U.S.A.); anti-HA (16B12) was from Babco (Berkeley, CA, U.S.A.); and anti-FLAG (M5) was from Sigma (Saint Louis, MO, U.S.A.). Polyclonal anti-Src and anti-PDGFRβ [PDGF (platelet-derived growth factor) receptor β] were from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). Polyclonal anti-p85, monoclonal anti-phosphotyrosine (4G10) and rabbit anti-p85, monoclonal anti-phosphotyrosine (4G10) and rabbit polyclonal anti-PTEN (phosphatase and tensin homologue deleted on chromosome ten) were from Upstate Biotechnology (Lake Placid, NY, U.S.A.). Plasmids: pcDNA3 p85αε was provided by Dr L. Cantley (Harvard University, Cambridge, MA, U.S.A.); pcDNA3 pcDNA3 p85ε was provided by Dr L. Cantley (Harvard University, Cambridge, MA, U.S.A.).

Abbreviations used: CD148 D/A, CD148 D1205A; CHO, Chinese hamster ovary; FBS, fetal bovine serum; GFP, green fluorescent protein; GLEPP1, glomerular epithelial protein 1; GST, glutathione transferase; HA, haemagglutinin; HEK-293 cells, human embryonic kidney cells; NP-40, Nonidet P40; PI3K, phosphoinositide 3-kinase; PIP, phosphatidylinositol phosphate; PTP, protein tyrosine phosphatase; PDGF, platelet-derived growth factor; PDGFR, PDGF receptor; PTEN, phosphatase and tensin homologue deleted on chromosome 10; siRNA, short interfering RNA; SAP-1, stomach cancer-associated PTP-1; SH2 domain, Src homology 2 domain; SHP, SH2 domain-containing PTP; VE-PTP, vascular endothelial PTP; WT, wild-type.

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Yeast two-hybrid interaction assay

Yeast two-hybrid screening was performed using the Saccharomyces cerevisiae strain L40 which harbours the reporter genes HIS3 and LacZ under the control of an upstream LexA-binding site, pBridgeLexA CD148(D/A)/v-Src, and the human placenta cDNA library was expressed as a fusion protein with GAL4 in the pACT2 vector (Clontech), as described previously [18]. Approz. 1.0 × 10^7 clones were screened and positive clones were selected on plates lacking leucine, tryptophan and methionine, and verified by a β-galactosidase filter-lift liquid assay. The clones that showed an increase in blue colour development by v-Src induction were selected as previously described [18]. The clones that showed an increase in blue colour development by v-Src induction were selected as previously described [18]. The clones that showed an increase in blue colour development by v-Src induction were selected as previously described [18]. The clones that showed an increase in blue colour development by v-Src induction were selected as previously described [18].

GST fusion proteins

GST fusion proteins were prepared as previously described [19]. The fusion proteins were prepared by subcloning the cDNA fragments encoding the entire intracellular region of CD148 (WT, C/S, D/A) into the pGEX-3X vector (Amersham Biosciences). Plasmids for GST–PTP [mouse VE-PTP (vascular endothelial PTP), human SAP-1 (stomach cancer-associated PTP-1) and human GLEPP1 (glomerular epithelial protein 1)] fusion proteins were prepared by subcloning PCR-amplified intracellular sequences of the PTP into pGEX-3X vector. All constructs were confirmed by DNA sequencing.

In vitro dephosphorylation assay

HEK-293 cells were transiently co-transfected with pCMV-FLAG-p85 and pSrc527F. Cells were treated with 0.1 mM pervanadate for 20 min, rinsed with PBS, and lysed in lysis buffer [20 mM Tris/HCl, pH 7.6, 150 mM NaCl, 1 mM EDTA and 1% (v/v) NP-40 (Nonidet P40)] containing 5 mM NaF, 1 mM PMSF and 10 μg/ml aprotinin. Iodoacetate acid (5 mM) was added to lysates to inhibit cellular PTPs irreversibly. After incubation on ice for 5 min, diithiothreitol (10 mM) was added to the lysates to inactivate unreacted iodoacetate acid. The cleared lysates and GST-fusion proteins were incubated overnight at 4 °C, and glutathione–agarose beads (Amersham Biosciences) were then added and incubated for 1 h. After washing with lysis buffer, bound proteins were separated by SDS/PAGE (reducing condition), and subjected to immunoblot analysis. For the vanadate competition assay, 2 mM Na2VO4 was added to cell lysates. GST fusion proteins were also pre-incubated in lysis buffer (without EDTA) with or without 2 mM Na2VO4. The GST-pull down study was also performed with the PDGF-stimulated cells. CHO (Chinese hamster ovary) cells were transfected with PDGFβ plasmid and, 24 h after transfection, the cells were starved in culture medium supplemented with 0.1% FBS for 24 h and treated with or without 20 ng/ml of human PDGF-BB (R & D Systems) for 10 min. The cells were lysed and subjected to GST-pull down assay as described above.

Cell Culture and Transfection

HEK-293 cells and CHO cells were cultured and transfected using Lipofectamine™ Plus (Invitrogen) or FuGENE™ HD (Roche) reagent according to the manufacturers’ protocols.

Immunoprecipitation and immunoblot analysis

Cells were lysed in lysis buffer [20 mM Tris/HCl, pH 7.6, 150 mM NaCl, 1 mM EDTA and 1% (v/v) NP-40] containing 5 mM NaF, 1 mM PMSF and aprotinin (10 μg/ml). The cleared cell lysates were incubated with antibodies overnight at 4 °C, and then Protein A or Protein G–Sepharose beads (Amersham Biosciences) were added and incubated for 1 h at 4 °C. The beads were washed with lysis buffer, and the immunoprecipitated proteins were separated by SDS/PAGE, transferred to PVDF membranes (Amersham Biosciences), and immunoblotted with antibodies. The immunoreactions were visualized using the ECL® (enhanced chemiluminescence) detection system (Amersham Biosciences).

Cell Stain

CHO cells were plated on glass coverslips and transiently co-transfected with pSRα CD148 (D/A)–HA and pGFP-p85. Cells were washed with PBS 24 h after transfection, fixed with 4% formaldehyde for 20 min and permeabilized with 0.02% (v/v) saponin (Sigma) in PBS containing 0.1% BSA for 30 min. The cells were immunolabelled with anti-HA antibodies (16B12; 2 mg/ml) for 1 h at room temperature (25°C), washed with PBS, and then incubated with rhodamine-conjugated secondary antibodies for 30 min. After washing with PBS, the cells were observed under a confocal microscope (LSM410; Carl Zeiss).

In vitro dephosphorylation assay

HEK-293 cells were transiently co-transfected with pCMV-FLAG-p85 and pSrc527F. Cells were treated with 0.1 mM pervanadate for 20 min, rinsed with PBS and lysed in lysis buffer [50 mM Tris, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1.0%...
(v/v) NP-40 and 0.5% sodium deoxycholate] containing 1 mM Na3VO4 and protease inhibitors. p85 was immunoprecipitated using anti-FLAG or anti-p85 antibodies and the beads were washed with lysis buffer and subsequently with reaction buffer (40 mM imidazole, 0.1 μg/ml BSA and 2 mM dithiothreitol) and incubated with GST proteins for 20 min at 37°C. The immunoprecipitates were separated by SDS/PAGE and immunoblotted with anti-phosphotyrosine (4G10) antibodies. For the vanadate competition assay, 1 mM Na3VO4 was added to the reaction buffer.

**PI3K assay**

Anti-FLAG or anti-phosphotyrosine (4G10) immunoprecipitates were washed with lysis buffer followed by PI3K reaction buffer (20 mM Tris/HCl, pH 7.4, 100 mM NaCl and 0.5 mM EGTA) and suspended in reaction buffer containing 0.1 mg/ml of phosphatidylinositol (Sigma). The reactions were initiated by adding a MgCl2/ATP mixture (20 mM MgCl2 and 20 μM ATP) containing 5 μCi of [γ-32P]ATP and performed for 20 min at 25°C. The reactions were terminated by adding chloroform/methanol/11.6 M HCl (100:200:2). After addition of chloroform, the organic phase was separated by centrifugation and washed with methanol/1 M HCl (1:1). After evaporation, the pellets were resuspended in chloroform, spotted onto a silica gel plate, and developed in chloroform/methanol/28% ammonium hydroxide/water (43:38:5:7). The PIP (phosphatidylinositol phosphate) was visualized by autoradiography and semi-quantified using image software (Adobe Photoshop 6.0). The intensity of PIP was measured by subtracting the background of the adjacent area.

**RNA interference**

siRNA (short interfering RNA) duplexes were synthesized by Dharmacon. The sequences of the CD148 oligonucleotides were 5'-GCAGUACAGCAGAAUCUUCGU-3' and 5'-UUGGACGUGUGCAUAGGAA-3'. Annealed siRNA duplexes (100 nM) were transfected to the cells using Gene Eraser (Stratagene) according to the manufacturer’s protocol. A scrambled siRNA duplex (Dharmacon) was used as a control.

**RESULTS**

**Yeast two-hybrid interaction between CD148 and p85**

To identify the relevant substrate for CD148, we conducted a substrate-trapping yeast two-hybrid assay [18]. This system is based on the yeast-two hybrid system, with two essential modifications: conditional expression of v-Src tyrosine kinase to tyrosine-phosphorylate prey proteins and use of the substrate-trapping D/A bait construct, suggesting that the interaction requires protein specificity of the interaction. Both N-terminal and C-terminal SH2 domains in p85 were mapped as binding sites of CD148 D/A (results not shown), indicating the specificity of the interaction. Both N-terminal and C-terminal SH2 domains in p85 were mapped as binding sites of CD148 D/A (Supplementary Figure S1, at http://www.BiochemJ.org/bj/413/ bj4130193add.htm).

**CD148 D/A traps p85 and PI3K**

We next performed a series of pull-down experiments using GST fusion proteins of the CD148 (WT, D/A) cytoplasmic domain. As shown in Figure 2(A), GST-CD148 D/A, but not GST-CD148 WT, pulled down p85 in a phosphorylation-dependent manner. Furthermore, incubation of GST–CD148 D/A with vanadate, a competitive inhibitor that binds to the catalytic site of PTP, abrogated the binding of p85 to GST–CD148 D/A (Figure 2B), suggesting interaction of p85 with the catalytic site of CD148. Since treatment of cells with pervanadate may result in phosphorylation of proteins on numerous sites that are not physiologically relevant, we investigated further the interaction of p85 with GST–CD148 D/A on PDGF stimulation. As shown in Figure 2(C), the binding of p85 to GST–CD148 D/A was promoted...
by PDGF as well as by pervanadate treatment, suggesting that the interaction between CD148 and phospho-p85 may occur in the context of cell signalling.

PI3K activity in the pulled-down protein complexes was also examined. As shown in Figure 2(D), PI3K as well as p85 was trapped by GST–CD148 D/A in a phosphorylation-dependent manner. To determine whether the trapping of PI3K by CD148 D/A is mediated by p85, we carried out a further series of experiments using a dominant-negative mutant form of p85, p85DN, which lacks the p110 binding site. As shown in Figure 2(E), overexpression of p85DN almost completely abrogated the binding of endogenous p85 to GST–CD148 D/A. Similarly, the interaction of PI3K with GST–CD148 D/A was remarkably suppressed by p85DN expression (Figure 2F).

**CD148 D/A interacts with p85 bound to PDGFR**

As PI3K activity was recovered with CD148 D/A, we also asked whether CD148 D/A interacts with p85 that is recruited to YXXM proteins. For this, we used PDGFRβ as a model, as CD148 has previously been shown to interact with PDGFRβ [20,21]. The interactions of CD148 D/A with WT PDGFR and the mutant PDGFR in which YXXM motifs are mutated to YQTI (PDGFR-YQTI) (Figure 3A) were investigated. This mutant PDGFR was shown to undergo PDGF-stimulated autophosphorylation but not bind to p85 [22]. As shown in Figure 3(B), CD148 D/A, but not WT CD148, trapped WT PDGFR in a phosphorylation-dependent manner, whereas the interaction with the mutant PDGFR (YQTI) was much weaker. The results suggest that CD148 may interact with p85, recruited to PDGFR through the phospho-YXXM motifs.

**Association of CD148 with p85 in mammalian cells**

To validate the interaction of CD148 with p85 in mammalian cells, HA-tagged CD148 WT or CD148 D/A were transiently transfected into HEK-293 cells. The cells were treated with pervanadate before harvest to enhance tyrosine phosphorylation of the cellular proteins. Figure 4(A) shows CD148 D/A, but not WT CD148, was co-immunoprecipitated with p85 from pervanadate-treated cell lysates, suggesting a tyrosine-phosphorylation-dependent interaction between p85 and CD148 D/A. Furthermore, we examined subcellular localization of CD148 D/A and p85 to verify overlapped distribution of these proteins in mammalian cells. CHO cells were co-transfected with HA-tagged CD148 (D/A or WT) and GFP–p85. CD148 D/A (Figure 4B) and CD148 WT (results not shown) distributed to plasma membranes and co-localized with p85, indicating a possible interaction of these proteins in intact cells.
Figure 3  CD148 D/A traps PDGFR, but is less able to interact with the mutant PDGFR that lacks p85 binding

(A) The altered YXXM sequences in the mutant PDGFR. (B) Immunoblot analysis of PDGFR trapped by GST–CD148 D/A (DA). HEK-293 cells were transfected with PDGFR-WT or mutant PDGFR (PDGFR-YQTI) plasmids. The cells were treated with (+) or without (−) pervanadate (PV), and GST–CD148 WT (WT) or GST–CD148 D/A proteins were incubated with the cell lysates. The trapped proteins were subjected to immunoblotting (IB) using anti-PDGFR antibodies (top). The same amount of total protein lysates from the same samples were subjected to immunoblotting using anti-PDGFR antibodies (bottom).

CD148 reduces tyrosine phosphorylation of p85 and attenuates PI3K activity

The ability of CD148 to dephosphorylate p85 was examined in vitro and in intact cells (Figure 5). As shown in Figure 5(A), p85 was prominently dephosphorylated by GST–CD148 WT but not by GST–CD148D/A in vitro and this p85 dephosphorylation was inhibited by vanadate. The specificity of CD148 dephosphorylation of p85 was examined using GST fusion proteins of type III receptor PTPs, including VE-PTP, SAP-1 and GLEPP1. Prominent dephosphorylation of p85 was observed with CD148 as compared with other type III receptor PTPs (Figure 5A, bottom panels), indicating the specificity of the CD148 activity. For an in vivo test, we used the constitutively active mutant of Src tyrosine kinase (Src 527F) to enhance p85 phosphorylation for the following reasons. First, CD148 was shown to increase Src tyrosine kinase activity by dephosphorylating the suppressive tyrosine in Src [20,23]. Secondly, CD148 is known to suppress activity of various growth factor receptors [20,24]. As shown in Figure 5(B), expression of Src 527F resulted in tyrosine phosphorylation of p85, and co-expression of WT CD148 remarkably reduced the phospho-tyrosine content of p85 without affecting phosphorylation of Src (Figure 5B, middle panels), whereas the CD148 D/A mutant showed no effects. Reduction of p85 phosphorylation was also observed in the CD148 transfected cells on growth factor stimulation (results not shown).

Figure 4  CD148 D/A interacts with p85 in intact mammalian cells

(A) Phosphorylation-dependent interaction of CD148 D/A (DA) and p85 in mammalian cells. Mock or HA-tagged CD148 WT (WT) or CD148 D/A and p85 were co-transfected into HEK-293 cells. The cells were treated with or without pervanadate (PV; 0.1 mM, 20 min) prior to lysis. Anti-p85 immunoprecipitates (upper panels) and total cell lysates (lower panels) were subjected to immunoblot (IB) analysis using anti-HA and anti-p85 antibodies. Normal mouse IgG was used as the control immunoprecipitation. (B) Subcellular co-localization of CD148 D/A and p85. CHO cells were co-transfected with HA-tagged CD148 D/A and GFP–p85 plasmids and stained with anti-HA antibodies as described in the Materials and methods section. Subcellular localization of CD148 D/A and GFP–p85 were analysed by confocal microscopy.

To elucidate the significance of CD148 and p85 interaction, we next examined the effect of CD148 expression on PI3K activity (Figure 6A). HEK-293 cells were transfected with Src 527F, FLAG-tagged p85, and CD148 WT or CD148 D/A. Anti-FLAG immunoprecipitates were subjected to the PI3K assay. Co-expression of Src 527F increased p85-associated PI3K activity by 25%, and the expression of CD148 WT completely blocked the increases in PI3K activity whereas CD148 D/A showed no effect (Figure 6A). Consistent with this finding, an increase in Akt phosphorylation by Src527F was inhibited by CD148 (results not shown). It is noteworthy that CD148 expression did not reduce basal PI3K activity (Figure 6A). As a final test, we examined the effect of CD148 knock-down on PI3K activity using a CHO cell line [25] stably transfected with HA-tagged CD148. Knockdown of CD148 expression in the siRNA-transfected cells was
assessed by immunoblot analysis. As shown in the upper panel of Figure 6(B), CD148 siRNA almost completely suppressed the CD148 expression, whereas reduction of CD148 was not observed in the cells transfected with control siRNA. We also confirmed the unaltered expression of PTEN, a well-known negative regulator of PI3K, in the siRNA-transfected cells. The lower panel of Figure 6(B) demonstrates the PI3K activity in the siRNA-transfected cells. CD148 gene knock-down increased PI3K activity by 30% on serum stimulation, whereas control siRNA showed no effects. In aggregates, these results demonstrate that CD148 may interact with p85 when it is phosphorylated and attenuates PI3K activity.

DISCUSSION

p85 interacted with the ‘substrate-trapping’ D/A mutant of CD148 in yeast and mammalian cells. The interaction was not observed in the absence of v-Src or without pervanadate or PDGF treatment, indicating that the protein interaction requires tyrosine phosphorylation of either p85, CD148 or both. As p85 interacts with tyrosine-phosphorylated proteins through the YXXM motif, it is possible that p85 binds to phosphorylated CD148 D/A, but not as a substrate. However, we think this is unlikely. First, p85 does not interact with the CD148 C/S mutant that exhibits a similar phosphorylation pattern to CD148 D/A. Second, the interaction between p85 and CD148 D/A is abrogated by vanadate. Lastly, the tyrosine motif (YXXM) that mediates p85 binding does not exist in the CD148 cytoplasmic domain. Taken together, these results suggest that CD148 D/A binds to p85 through interaction between the phosphorylated p85 and the catalytic pocket of the D/A mutant.

Because the direct association of p85 and c-Src or v-Src has been demonstrated [26,27], it is also possible that p85 binds to CD148 D/A through c-Src. However, we think this is even less likely. First, SH2 domains in p85 were sufficient to interact with CD148 D/A (Supplementary Figure S1), yet p85 was shown to bind to v-Src through a proline-rich region [27]. Secondly, c-Src was not co-immunoprecipitated with CD148 D/A from HEK-293 cell lysates under the same experimental conditions (results not shown). Together with the finding that p85 is prominently dephosphorylated by CD148, our results demonstrate that p85 interacts with CD148 as a substrate.

CD148 expression blocked an increase in PI3K activity induced by active Src. The finding suggests that CD148 inhibits excessive PI3K activation caused by p85 phosphorylation. The data agree with previous reports indicating that CD148 expression moderately reduces PI3K activity and Akt phosphorylation on PDGF stimulation [20,28]. It is known that the tyrosine phosphorylation level of p85 is quite low in intact cells. As was reported previously [29], we could detect p85 tyrosine phosphorylation in intact cells only upon co-expression with Src. On the other hand, relatively high levels of p85 tyrosine phosphorylation and PI3K activity have been observed in transformed cells [30,31]. This indicates that tyrosine phosphorylation of p85 is strictly controlled at a very low level by PTPs in physiological situations and that
The recent studies have shown p85 functions which are independent of the p110 catalytic subunit, including regulation of gene transcription [35,36] and cytokinesis [37]. Given the important roles of p85, our findings should facilitate exploration of the role of CD148.

We thank Dr Tom Daniel for discussion, Dr Jon Backer for the PDGFRβ-YQTI plasmid, Dr Dietmar Vestweber (Max-Planck-Institute of Molecular Biomedicine, Münster, Germany) for VE-PTP cDNA, Dr Takashi Matozaki (Laboratory of Biosignal Sciences, Gunma University, Japan) for SAP-1 cDNA and Dr Roger Wiggins (Nephrology Division, University of Michigan, U.S.A.) for GLEPP1 cDNA. This work was supported by grants from the National Institute of Health (DK38517, CA68485), Immunex and the Juvenile Diabetes Foundation (#2-2000-147).

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