

Signalling of GPI-anchored CD157 via focal adhesion kinase in MCA102 fibroblasts

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Abstract CD157, a glycosylphosphatidylinositol-anchored protein, has previously been shown to mediate tyrosine phosphorylation of a 130 kDa protein (p130) in several cell lines. In this study, we have identified the p130 protein to be focal adhesion kinase (FAK or pp125^{FAK}). FAK undergoes phosphorylation at Tyr-397 and Tyr-861 in intact MCA102 cells stably transfected with CD157 (MCA/CD157). MCA/CD157 cells, which displayed a rounded and compact cell morphology, exhibited a dispersed distribution, in contrast to a more closely associated and elongated spindle cell shape in the vector-transfected cells. MCA/CD157 cells proliferated at a rate 20–25% slower than the control cells. Our results demonstrate, for the first time, that FAK is a downstream signalling molecule of CD157. © 2001 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.

Key words: CD157; Focal adhesion kinase; Localization; Morphology; Proliferation; Tyrosine phosphorylation

1. Introduction

CD157/BST-1, a glycosylphosphatidylinositol (GPI)-anchored glycoprotein, was first identified as an antigen having increased expression on bone marrow stromal cell lines derived from rheumatoid arthritis patients [1,2]. The deduced amino acid sequence of CD157 shares about 30% identity with that of CD38, and both CD157 and CD38 possess ADP-ribosyl cyclase as well as cyclic ADP ribose (cADPR) hydrolase activity [3–5]. cADPR has been found to activate calcium release from intracellular stores via an inositol trisphosphate-independent pathway [6].

Increasing evidence suggests that CD157 has a receptor and/or ligand-like function. CD157, expressed on the bone marrow stromal cell lines, was found to be responsible for the augmented growth of a pre-B cell line, DW34 [7]. The growth support of nurse-like bone marrow cells to the peripheral B lymphocytes decreased in the presence of anti-CD157 antibody [8]. Okuyama et al. [9] demonstrated that when CD157 was cross-linked with an anti-CD157 antibody, a 130 kDa protein (p130) was tyrosine-phosphorylated in CHO/CD157, U937 and THP-1 cells; a 100 kDa protein

was tyrosine-dephosphorylated in CHO/CD157 cells. In our previous study, tyrosine phosphorylation of a p130 was also observed in CHO/CD157, MCA102/CD157 (MCA/CD157), COS-7/CD157 cells and monocytes (CD157-positive) differentiated from HL-60 cells [10]. The identification of the p130(s) seems important to elucidate and understand CD157 function. In this work, we purified and sequenced the tyrosine-phosphorylated p130 from MCA/CD157 cells. The protein was identified to be focal adhesion kinase (FAK or pp125^{FAK}), a cytoplasmic protein playing key roles in integrating signals regulating cell functions. We further demonstrated that two tyrosine residues of FAK, Tyr-397 and Tyr-861, were phosphorylated in intact cells. FAK was located at the focal contacts in MCA/vector cells, but was localized to perinuclear regions in MCA/CD157 cells. The site-specific tyrosine phosphorylation of FAK and its alteration in localization might contribute to the observed changes in cellular morphology and a reduced proliferation rate of MCA/CD157 cells.

2. Materials and methods

2.1. Materials

The CD157 cDNA was provided by Toshio Hirano, Osaka University Medical Institute, Japan. The MCA102 (mouse fibrosarcoma) cell line was obtained from Yunn Hwen Gan from our Department originally established by others [11]. Monoclonal anti-phosphotyrosine agarose was purchased from Sigma (St. Louis, MO, USA). Monoclonal anti-phosphotyrosine antibody (PY20) was from PharMingen International (San Diego, CA, USA). Polyclonal anti-FAK antibody (C-20) was obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Polyclonal anti-FAK [pY³⁹⁷], [pY⁴⁰⁷], [pY⁵⁷⁶], [pY⁵⁷⁷], [pY⁸⁶¹], [pY⁹²⁵] antibodies were obtained from Biosource International. Sources of other reagents have been specified appropriately.

2.2. Cell culture and stable transfection

MCA102 cells were grown in RPMI 1640 medium (Sigma) containing 10% fetal bovine serum and appropriate L-glutamine, penicillin and streptomycin supplements (Gibco BRL, Life Technologies). The full-length coding sequence of human CD157 was inserted into the multiple cloning sites of vector pXJ41 [12]. The recombinant pXJ41-CD157 construct was introduced into MCA102 cells using Lipofectamine reagent (Gibco BRL) following the protocol of the manufacturer. Stable transfectant clones were selected in the culture medium containing 200 µg/ml G418 (Sigma). The CD157 expression was analyzed by immunoblotting.

2.3. Tyrosine phosphorylation assay

MCA/vector and MCA/CD157 cells were grown to about 80% confluence. Then cells were incubated in serum-free RPMI 1640 medium for 45 min and harvested using phosphate-buffered saline (PBS) containing 1.0 mM ethylenediaminetetraacetic acid. After spinning at 200 × g for 5 min, the cellular pellets were resuspended in serum-free RPMI 1640 at 2 × 10⁶ cells/ml. An aliquot of 50 µl of the suspension was preincubated at 37°C for 2–3 min and then stimulated with or

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Abbreviations: GPI, glycosylphosphatidylinositol; FAK, focal adhesion kinase; cADPR, cyclic ADP ribose; uPAR, urokinase-type plasminogen activator receptor

without 10 $\mu\text{g}/\text{ml}$ affinity-purified polyclonal anti-CD157 antibodies for 10 min. 1.0 ml ice-cold PBS containing 1.0 mM sodium vanadate was then added to stop the reaction. The cell suspension was kept on ice for 5 min. Cells were then lysed in 40 μl Laemmli sample buffer and boiled for 5 min after being spun down at $1000\times g$ for 1.0 min. The tyrosine-phosphorylated proteins were detected by immunoblotting as described below.

2.4. Immunoblotting

Protein samples were subjected to SDS-PAGE and transferred to nitrocellulose membrane using a semi-dry transelektroblotting system (Bio-Rad). The membrane was blocked in TBS-T (20 mM Tris, 137 mM NaCl, pH 7.5, 0.1% Tween 20) containing 5% bovine serum albumin (BSA) for 1 h. After incubating in appropriate primary antibody for 1–2 h at room temperature, the membrane was incubated in horseradish peroxidase-conjugated secondary antibody for 45 min. The immunoreactive bands were detected by enhanced chemiluminescence (ECL) reagent.

2.5. Purification and sequencing of the tyrosine-phosphorylated p130

MCA/CD157 cells (50×10^6) were grown to 80% confluence and lysed in lysis buffer (PBS containing 1% Triton X-100, 0.1 mM phenylmethylsulfonyl fluoride, 10.0 $\mu\text{g}/\text{ml}$ of aprotinin and leupeptin, 50.0 $\mu\text{g}/\text{ml}$ of soybean trypsin inhibitor and 1 mM sodium vanadate) on ice for 1 h. The lysate was centrifuged at $10000\times g$ for 15 min at 4°C . Anti-phosphotyrosine agarose beads (30 μl) were added to the recovered supernatant and binding was allowed to proceed for 3 h at 4°C on a rotating platform. The beads were washed three times with the lysis buffer. Laemmli sample buffer was added to the beads and boiled for 5 min before being loaded to SDS-PAGE and stained with Coomassie blue or subjected to anti-phosphotyrosine immunoblotting. The 130 kDa protein band in Coomassie blue-stained gel was excised for in-gel tryptic digestion followed by peptide sequencing using MALDI-TOF mass spectrometry.

2.6. Immunoprecipitation

Anti-FAK antibody (10 $\mu\text{g}/\text{ml}$) was added to the clarified lysate of 10×10^6 MCA/CD157 and MCA/vector cells, and incubated for 3 h followed by the addition of 20 μl protein G agarose and binding was performed for another 3 h at 4°C on the rotating platform. After washing with lysis buffer, FAK bound to the beads was probed with either anti-FAK or anti-phosphotyrosine antibodies.

2.7. Immunofluorescence analysis

Cells grown on coverslips were fixed in 3.7% paraformaldehyde for 15 min at room temperature, and then permeabilized with 0.1% Triton X-100 in PBS containing 0.5% BSA for 15 min. Coverslips were incubated in blocking buffer (3% BSA, 0.01% Triton X-100 in PBS) for 1 h, followed by incubation with 10 $\mu\text{g}/\text{ml}$ anti-FAK antibody and then FITC-conjugated secondary antibody (1:100) for 1 h. Nuclei were counterstained with 4.0 $\mu\text{g}/\text{ml}$ propidium iodide for 5 min and rinsed with buffer. Cells were mounted with Vectashield mounting medium (Vector Laboratories, Burlingame, CA, USA) and sealed with nail polish. The fluorescence image was analyzed by confocal microscopy.

2.8. Analysis of cell proliferation

Cells (0.1×10^6) were seeded in a six well plate and grown in a 37°C humidified incubator with 5% CO_2 until reaching $\sim 75\%$ confluence. Cells were harvested and counted with a hemocytometer. The following formula was used to calculate the number of generations: $2^n = N_f / N_i$, where N_i and N_f were the initial and final cells counts, respectively. The doubling time was obtained by dividing the incubation time by the number of generations.

3. Results

The expression of CD157 in three transfectant clones was determined by immunoblotting with anti-CD157 antibody (data not shown). MCA/CD157 cells in all of the analyzed clones displayed a rounded and compact morphology and were easily detachable from the culturing flask, whereas MCA/vector cells retained a typical fibroblast spindle shape

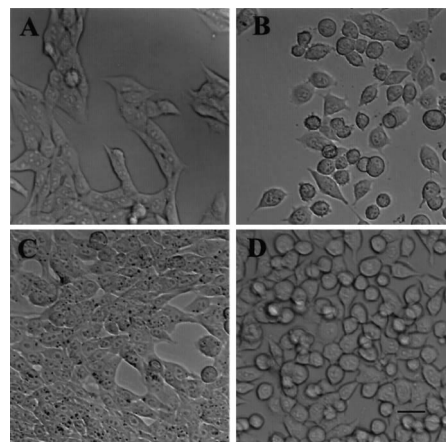


Fig. 1. Changes in the morphology of MCA102 cells stably transfected with vector pXJ41 (A and C) and pXJ41-CD157 (B and D). The morphology of cells was observed at subconfluent (A and B) and confluent (C and D) states. The MCA/vector cells exhibited a typical elongated spindle cell shape whereas the MCA/CD157 cells developed a rounded and compact morphology. Bar = 20 μm .

and exhibited a strong adherence to the flask wall (Fig. 1). Cell proliferation assays showed that the growth rate of MCA/CD157 clones (doubling time 21.8 h) was 21% slower than that of MCA/vector clones (doubling time 17.9 h). The results suggest that CD157 may participate in signalling pathways regulating cell morphology and proliferation.

Immunoblotting of cell proteins showed elevated tyrosine phosphorylation of a 130 kDa protein (p130) in MCA/CD157 cells, and cross-linking of CD157 with polyclonal anti-CD157 antibodies could not further increase the p130 tyrosine phosphorylation (Fig. 2). All three MCA/CD157 clones examined showed similar results (data not shown). The p130 was then affinity-purified using anti-phosphotyrosine beads and subjected to tryptic digestion and purification of peptides. Three of the recovered peptides were sequenced and the deduced amino acid sequences were NLLDVIDQAR, AQLSTILLEEK and FFEILSPVYR. A search of the SwissProt protein database identified all three sequences to be FAK of various species including human, mouse, rat, etc. The identity of p130 as FAK was further confirmed by immunoprecipitation with anti-FAK antibodies and immunoblotting with either anti-FAK or anti-phosphotyrosine antibodies. Similar levels of FAK protein were found in MCA/CD157 and MCA/vector cells, however, the level of tyrosine phosphorylation of FAK was much higher in MCA/CD157 cells (Fig. 3). Multiple tyrosine residues in FAK, including Tyr-397, 407, 576, 577, 861 and 925, have been identified to undergo autophosphorylation or phosphorylation by Src family kinases [13,14]. To address the CD157-mediated phosphorylation sites of FAK, we used phosphorylation state-specific antibodies. The results of immunoblotting with the different antibodies showed that Tyr-397 and Tyr-861 were significantly phosphorylated in MCA/CD157 cells (Fig. 4), while no detectable phosphorylation was observed for other tyrosine residues, Tyr-407, 576, 577, and 925 (data not shown). In addition, immunofluorescence revealed that FAK localized at the peripheral focal contacts of MCA/vector cells; by contrast, FAK molecules moved away from focal adhesion and located at the perinuclear region in MCA/CD157 cells (Fig. 5).

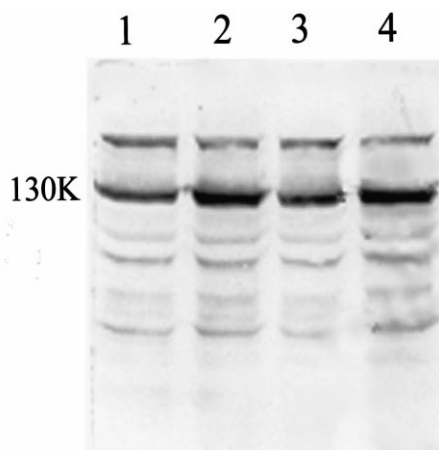


Fig. 2. CD157-mediated tyrosine phosphorylation of p130 in MCA102 cells. 0.1×10^6 MCA/vector (lanes 1, 3) and MCA/CD157 (lanes 2, 4) cells were stimulated with (lanes 3, 4) or without (lanes 1, 2) anti-CD157 antibodies for 10 min. Protein tyrosine phosphorylation was detected with a monoclonal anti-phosphotyrosine antibody. An increase in the tyrosine phosphorylation of p130 was observed in MCA/CD157 cells; cross-linking of CD157 could not obviously further increase the p130 phosphorylation in MCA/CD157 cells.

4. Discussion

A large number of GPI-anchored proteins have been reported to be able to transduce cellular activation signals, although they lack transmembrane or cytoplasmic sequences [15]. Antibody cross-linking of these membrane surface molecules leads to protein tyrosine phosphorylation, intracellular calcium mobilization and activation of transcription factors of cells. The physiological contexts of such activation, however, remain poorly understood. There are reports that some GPI-anchored proteins locate at microdomains (also known as rafts), which are enriched in sphingolipids and cholesterol, within the cell membrane [16]. It is thought that antibody cross-linking or overexpression of GPI-anchored proteins may lead to coalescence of the microdomains, thereby clustering and activating microdomain-associated Src family protein

tyrosine kinases to phosphorylate the downstream substrates [16,17]. Urokinase-type plasminogen activator receptor (uPAR), a GPI-anchored protein, has been shown to associate with members of the integrin family. FAK was shown to undergo tyrosine phosphorylation upon binding of uPAR with its ligand, uPA [18]. In MCA102/CD157 cells, CD157 was not uniformly distributed on the cell surface but was sporadically distributed in specific regions of the cell surface membrane, possibly the microdomains (data not shown). The CD157-mediated FAK phosphorylation was ligand-independent in MCA/CD157 cells since the cross-linking of CD157 with its antibody could not further increase the phosphorylation. Time course studies showed that there was not much change in the level of FAK phosphorylation after the antibody was added within a time range of 30 s to 30 min in the MCA/CD157 clones (data not shown). This might be due to an overexpression of CD157 molecules on the cell surface such that these molecules, due to their close proximity on the cell membrane, could mimic a clustered formation and were able to transmit signals to downstream molecules. However, the precise mechanism of how CD157 relays its signal to FAK remains to be investigated.

FAK is a cytoplasmic tyrosine kinase that can be phosphorylated and activated by many factors [19]. All available information suggests that FAK is at a crossroad of multiple signal pathways, and may play important roles in the assembly of signalling complexes that regulate diverse cellular functions including morphogenesis, anchorage, migration, proliferation, etc. [20,21]. Our observed phenotype alteration and a decrease in the rate of proliferation in MCA/CD157 cells might be due to the phosphorylation of FAK. Most evidence so far indicates that FAK phosphorylation is associated with its accumulation at the focal contacts and an increase in cell proliferation [22,23]. Interestingly, our findings showed that the CD157-mediated phosphorylation of FAK correlated with its dispersal from the focal contacts and a decrease in cell proliferation. The phenotypic differences may be attributed to a differential phosphorylation of FAK.

FAK has six tyrosine phosphorylation sites that have been identified so far: Tyr-397, 407, 576, 577, 861 and 925. Tyr-397 is recognized as the major autophosphorylation site and its phosphorylation generates a high-affinity site for the SH2 do-

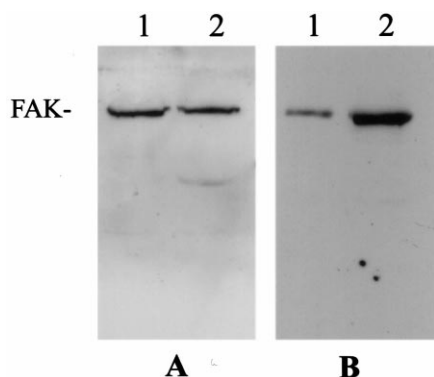


Fig. 3. CD157-dependent tyrosine phosphorylation of FAK in MCA102 cells. FAK was immunoprecipitated from the lysate of 10×10^6 MCA/vector (lane 1) and MCA/CD157 (lane 2) cells using a monoclonal anti-FAK antibody. The immunoprecipitates were immunoblotted with either a polyclonal anti-FAK (A) or a monoclonal anti-phosphotyrosine antibody (B). For a similar amount of protein, FAK was phosphorylated much more in MCA/CD157 cells.

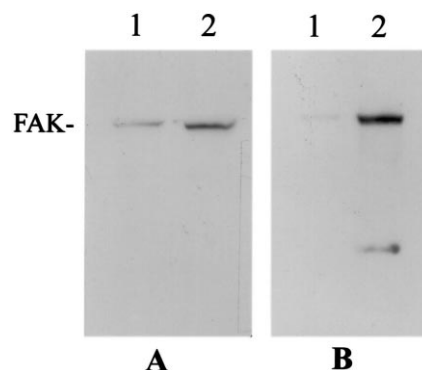


Fig. 4. CD157-mediated tyrosine phosphorylation of Tyr-397 and 861 in intact MCA102 cells. FAK was immunoprecipitated from the lysate of 10×10^6 MCA/vector (lane 1) and MCA/CD157 (lane 2) cells and the immunoprecipitates were immunoblotted with phosphorylation state-specific antibodies of FAK, anti-pY397 (A) and anti-pY861 (B). FAK exhibited an increase in phosphorylation at Tyr-397 and Tyr-861 in MCA/CD157 cells.

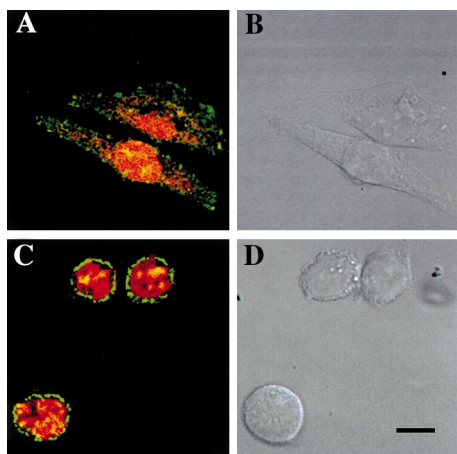


Fig. 5. Immunofluorescence of FAK (green) and staining of nuclei with propidium iodide (red) in MCA/vector (A) and MCA/CD157 (C) cells. B and D: Phase contrast photomicrographs of the cells in A and C, respectively. FAK was localized predominantly at the focal contacts in MCA/vector cells, but was localized at the perinuclear region in MCA/CD157 cells. Bar = 5 μ m.

main of Src family kinases [24]. The interaction of Src with FAK could lead to the phosphorylation of Tyr-407, 576, 577 and 861, which would further elevate the kinase activity of FAK *in vivo* [13,25]. The phosphorylation of Tyr-407, which is close to Tyr-397 and contains a proline at the +3 position, may help to recruit additional SH2 domain-containing proteins [26]. Tyr-576 and 577 lie in the catalytic domain and their phosphorylation is required for the maximum kinase activity of FAK [25]. The phosphorylation of Tyr-925 creates a Grb binding site and therefore links FAK to the Ras-mitogen-activated protein kinase pathway [27]. In MCA/CD157 cells, we found increased phosphorylation of Tyr-397 and 861 on FAK. The phosphorylated Tyr-861 and its flanking amino acids (Y⁸⁶¹-Q-P-V) may represent a binding site for SH2 domain-containing proteins and/or regulate the interaction of SH3 domain-containing proteins with the proline-rich motifs nearby (863–913), thereby regulating signalling pathways. The downstream signal transduction mechanism as a consequence of CD157-dependent FAK phosphorylation at Tyr-397 and 861 remains to be clarified.

In our previous work, we showed a CD157-dependent p130 tyrosine phosphorylation in several cell lines including CHO/CD157, COS-7/CD157, MCA/CD157 and monocytes (CD157-positive) differentiated from HL-60 cells [10]. While we have shown that the p130 in MCA/CD157 was indeed FAK, it was determined to be not FAK in other cell lines (data not shown). This indicates a cell type specificity of the CD157-mediated signalling mechanism. It is possible that different signalling molecules are recruited by CD157 depending on cell types. However, the questions remain: what are the identities of the CD157-dependent p130 in other cell lines? Do the distinct CD157-dependent p130s share a similar signal transduction mechanism in different cell lines? The answers to these questions are essential for further elucidation of CD157 function in the signalling process. Our results demon-

strate, for the first time, that FAK is a signal transducer of CD157 in one type of cell and this finding is likely to pave the way for further investigations into the function and regulation of CD157 in normal and diseased cells.

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