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Proinsulin C-peptide interaction with protein tyrosine phosphatase 1B demonstrated with a labeling reaction

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ABSTRACT

Based on nickel-catalyzed cross-labeling where binding partners become biotinylated, we have studied molecular interactions with an N-terminally fused *GGH*-tag proinsulin C-peptide. Since C-peptide has been reported to influence phosphatase activity in intact cells, we employed this method to study possible binding of the peptide to protein tyrosine phosphatase 1B (PTP-1B). C-peptide was found to interact with PTP-1B (and for control, also with antibodies to C-peptide), as did also the N- and C-terminal fragments of C-peptide which have sequence similarities with PTP-1B binding proteins. The labeling data combined with enzyme activity analysis indicate a functional interaction between acidic regions of C-peptide and specific sites of PTP-1B. Results highlight the importance of possible phosphatase/C-peptide roles in diabetes, and the usefulness of the cross-labeling reaction also for acidic peptides like C-peptide.

Introduction

Proinsulin C-peptide is secreted into the blood stream together with insulin [1]. The fact that it is secreted and circulates in concentrations higher than insulin made it important to study it for bioactivity. Accordingly, C-peptide has been reported to have observable effects in patients with type 1 diabetes (T1D) [2], to bind to cell membranes [3], and to influence cellular signaling pathways [4–6]. It is now considered as a bioactive peptide [7]. In addition to the receptor-mediated effects on signaling pathways, C-peptide has also been reported to interact with different molecules, including insulin [8,9], and also to become internalized into cells [10], like an intracrine factor with defined functions. One of the further effects reported for C-peptide has been inhibition of intracellular protein tyrosine phosphatase (PTP) activity, giving increased autophosphorylation of the insulin receptor (IR) [11]. We therefore now investigated if C-peptide could directly bind to a specific phosphatase and influence its activity. To do this, we studied a biotinylating cross-labeling reaction, described in the literature [12–16] but not used with acidic peptides like C-peptide. The reaction results in biotinylation of interacting partners via a chemical reaction mediated by activated Ni^{2+} bound to a Gly-Gly-His (*GGH*) N-terminal tag attached to C-peptide. The biotinylation allows for affinity purification and identification of protein C-peptide binding partners.

We found conditions for labeling of C-peptide interacting partners, and then studied its interaction with protein tyrosine phosphatase 1B (PTP-1B), a phosphatase implicated as a negative regulator of IR signaling [17]. This phosphatase also dephosphorylates the epidermal growth factor receptor (EGFR) [18,19]. We further found that the N- and C-terminal acidic fragments of C-peptide also interact with PTP-1B, as evaluated by their promotion of cross-labeling, like C-peptide itself.

The results are concluded to indicate specific C-peptide/PTP-1B charge-based interactions. Together with previous reports, they also show a general applicability of the cross-labeling reaction.

Materials and methods

Synthetic C-peptide and its N- and C-terminal pentafragments (EAEDL and EGSLQ, respectively) with the Gly-Gly-His tripeptide added N-terminally were purchased from CASLO Laboratory ApS (Lyngby, Denmark). The pGEX-PTP403 plasmid was a kind gift from J. Chernoff (Fox Chase Cancer Center, Philadelphia, USA). DADEpYL was purchased from Bachem AG (Bubendorf, Switzerland). Tyramine HCl, $NiAc_2$, biotinyl-6-aminohexanoic acid NHS ester (Sigma, St Louis, USA), magnesium monoperoxyphthalic acid (MMPP) (Fluka/Sigma) and 2-mercaptoethanol (VWR, West Chester, USA) were of analytical grade and solutions were prepared in ultrapure deionized water. $NiAc_2$ and MMPP solutions were prepared freshly, while peptides were diluted in water, aliqouted and stored at −20 °C after quantification by amino acid analysis.

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Expression and purification of GSH-tagged protein tyrosine phosphatase 1B (GST-PTP-1B). The plasmid pGEX-PTP403 was amplified in *Escherichia coli* XL1blue, expressed in *E. coli* BL21 (Stratagene, Agilent) and transformed into *E. coli* cells as described by the manufacturer. Plasmid DNA was purified using a Flexiprep kit (GE Healthcare, Uppsala, Sweden) according to the manufacturer instructions. Briefly, isopropyl β-D-1-thiogalactopyranoside (IPTG) stimulated *E. coli* BL21 was pelleted and dissolved in PBS containing EDTA-free complete protease inhibitor cocktail (Roche, Basel, Switzerland). The suspension was sonicated for a total of 5 min in pulses of 20 s. Cell debris was removed by centrifugation (1 h, 20,000 rpm, 4 °C) and Triton X-100 added to a final concentration of 1% (v/v). The supernatant containing recombinant GST-PTP-1B was incubated with a GST-Sepharose slurry (GE Healthcare) for 1.5 h, 4 °C. Protein was serially eluted with 20 mM glutathione in 20 mM Tris–HCl, pH 7.5, followed by centrifugation at 4000 rpm, and the protein content in each fraction was analyzed on an SDS–PAGE gel. Fractions containing GST-PTP-1B were pooled and further purified on a PD-10 column (GE Healthcare).

Biotinylation of tyramine. Biotinyl-6-aminohexanoic acid NHS ester was dissolved in 50 mM borate buffer (pH 8) and tyramine HCl added. The mixture was kept on agitation overnight and filtered through a 45 μM syringe filter. The biotinylation of tyramine was verified by MALDI-MS and blotting analysis. 10% (v/v) DMSO was added upon usage.

pH-dependence of GGH-C-peptide nickel complex formation. GGH-C-peptide was diluted in ammonium acetate buffer, pH 4–8, Ni^{2+} was added in three-fold molar excess, and samples were incubated for 30 min on ice. Samples were introduced at a flow rate of 5–10 μL/min into an LCQ ion trap mass spectrometer (ThermoFinnigan, San Jose, USA), operated in negative ion mode with data acquisition over a mass range of m/z 150–2000.

Oxidative cross-labeling. Cross-labeling reactions were performed in a total volume of 15 μL. Concentrations in brackets denote final concentrations. *GGH*-peptide (20 μM) diluted in ammonium acetate (50 mM, pH 8.5) was incubated with $NiAc_2$ (100 μM) on ice for a minimum of 1 h. The samples were diluted in cross-labeling buffer (50 mM NaCl, 150 mM Na phosphate, pH 7) and mixed with additives (antibodies and/or proteins, as indicated) and biotinylated tyramine. Cross-labeling was initiated by the addition of magnesium monoperoxyphthalic acid (MMPP) (100 μM) and quenched after 2 min with 4 × NuPage LDS sample buffer (Invitrogen, Carlsbad, USA). The samples were boiled, separated on SDS–PAGE gradient gels (4–12%, NuPage, Invitrogen) and transferred to PVDF membranes using the iBLOT™ system (Invitrogen). The bands were visualized with an anti-biotin avidin-horse radish peroxidase (HRP) antibody (Calbiochem/Merck, Darmstadt, Germany) and enhanced chemiluminescence (ECL; GE Healthcare, Uppsala, Sweden). A low molecular weight calibration kit for electrophoresis (GE Healthcare) containing phosphorylase b (94 kDA), bovine serum albumin (BSA; 67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), trypsin inhibitor (20 kDa) and α-lactalbumin (14 kDa) was added at different concentrations (5–20 ng/μL), as indicated.

Labeling of a C-peptide antibody. Preparations of *GGH*-fused C-peptide, or *GGH*-tag only, and antibody was activated and allowed to react as described above. A monoclonal anti-C-peptide antibody was used to establish the protocol (Medix Biochemika, Kaunianen, Finland) and a randomly chosen antibody (anti-LL37, Inovagen, Lund, Sweden) was added under the same conditions to verify specificity of the biotin-labeling.

Oxidative cross-labeling of PTP-1B. GST-PTP-1B (70–260 nM) was added to the cross-labeling reaction as described above. The interaction between *GGH*-tagged C-peptide and PTP-1B was challenged by addition of the 6-protein mixture described above. In the competition experiments, peptides were added at concentrations equimolar with those of *GGH*-peptides.

PTP-1B activity assay. C-peptide and C-peptide fragments (N-terminal EAEDL, and C-terminal EGSLQ) were mixed with enzyme in pre-heated (37 °C) buffer (50 mM NaCl, 0.1 mM EDTA, 1 mM DTT, 50 mM Hepes, pH 7.5). The generic phosphatase substrate, p-nitrophenol phosphate (pNPP), was added to a final concentration of 10 mM, and the reaction was allowed to proceed for 60 min at 37 °C. The reaction was quenched by the addition of NaOH, and the amount of product (*p*-nitrophenol) formed was calculated from the absorbance at 405 nm.

Affinity purification. The cross-labeling reaction of *GGH*-C-peptide (20 μM) and GST-PTP-1B (130 nM) was performed as described above for the cross-labeling in general with the exception that the reaction was quenched with 2-mercaptoethanol (0.25 M). The reaction mixture was incubated with magnetic streptavidin beads (Dynabeads M-280, Invitrogen, Carlsbad, USA) pre-equilibrated with cross-labeling buffer. The mixture was kept on tilted rotation for 30 min at RT, and the beads were then washed three times with cross-labeling buffer. Protein/protein complexes bound to the beads were eluted in 4 × NuPage LDS sample buffer and boiled (5 min). The protein/bead mixture was added onto SDS–PAGE gradient gels and after electrophoresis bands were visualized with SilverQuest (Invitrogen), an MS-compatible silver-staining kit. Protein bands were manually excised and destained (according to the manufacturer protocol) in a Massprep robotic system (Waters, Milford, USA), with addition of three steps: dehydration with 100% methanol for 5 min; rehydration in 30% methanol for 5 min; washing with ultrapure water for 2 × 10 min). Subsequently, proteins were digested with trypsin in the Massprep system [20]. Tryptic digests were concentrated by solvent evaporation under a stream of nitrogen and were then analyzed by liquid chromatography tandem MS (LC-MS/MS) using Waters CapLC and Q-Tof Ultima API instruments (Waters) [20]. Database matching used the Protein Lynx Global Server (PLGS) 2.2.5 (Waters) with a fragment tolerance of ±0.1 Da. Uniprot 1.0 was used as databank.

Results

Influence of pH on GGH-C-peptide nickel-binding

Since C-peptide is acidic, we investigated the ability of *GGH*-C-peptide to coordinate nickel ions at different pHs. Solutions of *GGH*-C-peptide and Ni^{2+} were incubated at pH 4–8, and analyzed by electrospray ionization (ESI) MS to assess the nickel-binding. The binding was found to be optimal at pH 8. Consequently, we used pH 8 in the experiments below.

Specificity in labeling a C-peptide interacting protein by a nickel-mediated reaction

To test whether this cross-labeling works for proteins interacting with the highly acidic C-peptide, the labeling of a monoclonal C-peptide antibody was first studied. Preparations of *GGH*-C-peptide and antibody were activated and allowed to react. The antibody and peptide were separated by SDS–PAGE and transferred to PVDF membranes probed with a streptavidin antibody. One band corresponding to the labeled antibody was seen (Fig. 1A, lane 1). The antibody was thus biotinylated by cross-labeling via its interaction with *GGH*-C-peptide. This labeling of the antibody was used to titrate the reagents necessary for adequate labeling and control the specificity of the reaction.

When either of the reaction partners, MMPP, nickel or biotinylated tyramine, were omitted in different experiments, cross-labeling did not occur, and exclusion of MMPP from the reaction was

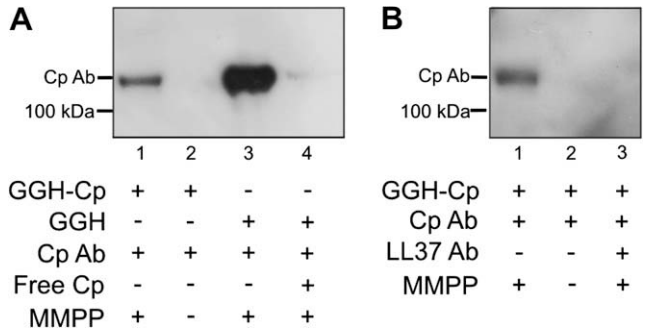

Fig. 1. Cross-labeling with *GGH*-C-peptide biotinylates a C-peptide antibody. (A) All three reagents; MMPP, Ni^{2+} and biotinylated tyramine (BT) are necessary to mediate cross-labeling of the C-peptide antibody (lanes 1 and 2). Control of interaction between C-peptide antibody and the *GGH*-tag shows a signal (lane 3) that is completely abolished upon addition of free C-peptide (lane 4). (B) Addition of a control antibody (anti-LL37) does not give a signal.

chosen as a control. The ability of the *GGH*-tag itself to interact with proteins was investigated with Ni-activated *GGH* and the C-peptide antibody. A signal corresponding to biotinylated antibody was detected (Fig. 1A, lane 3), but this was observed regardless of which protein or antibody was added to the reaction, indicating an unspecific binding to *GGH*. This is consistent with the observation that when free C-peptide is added to the cross-labeling of the C-peptide antibody, the signal is abolished as C-peptide binds to the antibody more efficiently than *GGH* (Fig. 1A, lane 4). To check that C-peptide could not induce cross-labeling of another antibody, anti-LL37, randomly chosen and with no known recognition motif to C-peptide, was added under the same conditions. No cross-labeled product could be detected (Fig. 1B, lane 3), demonstrating specificity of C-peptide induced cross-labeling.

C-peptide interactions with PTP-1B

Since C-peptide has been shown to increase autophosphorylation of the IR via inhibition of PTP activity in cells [11], we tested whether the *GGH*-tagged C-peptide could cross-label a specific PTP, PTP-1B, with biotin using the conditions found. PTP-1B was chosen as it is implicated in IR signaling [13]. Cross-labeling of PTP-1B was indeed found, both with the pure phosphatase and in a protein mixture (Fig. 2B, lane 1, 3–5). The cross-labeling was shown to be specific, and no labeling of PTP-1B occurred without tagged C-peptide (Fig. 2C, lane 1). Testing for enzymatic activity of PTP-1B in mixtures with C-peptide and its fragments showed that C-peptide had no effect on the PTP-1B activity (Fig. 2D). A scrambled C-peptide, with the same composition but different sequence, also had no effect (data not shown). However, a small decrease in the activity was seen with the C-terminal pentapeptide of C-peptide, and the activity of PTP-1B was reduced further with the N-terminal pentapeptide of C-peptide (Fig. 2D). The cross-labeling reaction was studied with these *GGH*-tagged peptides as well, and both were found to label PTP-1B (Fig. 3, lane 3 and 4). Interestingly, we also find sequence similarities between the acidic C-peptide fragments and known *in vivo* PTP-1B substrates and inhibitors (Fig. 2A). Addition of the *in vivo* PTP-1B substrate to the cross-labeling reaction affected the labeling of PTP-1B only to a minor extent (Fig. 3, lane 5 and 6). The cross-labeling of PTP-1B with C-peptide and the influence of the C-peptide acidic end fragments indicate that charge interactions are important for the C-peptide/PTP-1B interaction.

Mass spectrometry compatible workflow

Identification of interaction partners is of importance to gain insight into C-peptide biological functions. Since the results show

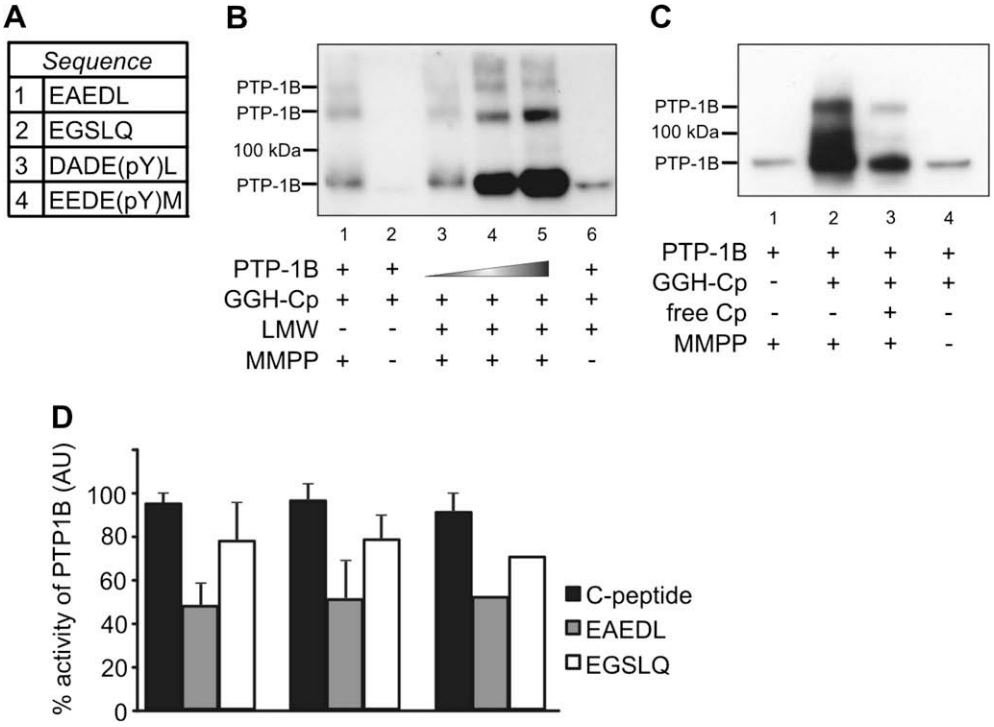

Fig. 2. C-peptide and its analogs interact with PTP-1B. (A) Sequence similarity of (1) C-peptide N-terminal pentapeptide, (2) C-peptide C-terminal pentapeptide, (3) *in vivo* PTP-1B substrate EGFR (residues 1012-17) and (4) a super-inhibitor of PTP-1B [9]. (B) Cross-labeling of *GGH*-C-peptide and PTP-1B. Lane 1 shows cross-labeling of PTP-1B, lane 2 as control, lane 3–5 cross-labeling of increasing amounts of PTP-1B in the presence of a low molecular weight (LMW) protein mixture, with lane 6 as control. (C) PTP-1B is not cross-labeled in the absence of *GGH*-C-peptide (lane 1) and the interaction with *GGH*-C-peptide is specific as excess free C-peptide reduces labeling (lane 2 and 3, with lane 4 as control). (D) Enzymatic activity of PTP-1B upon treatment with C-peptide and its N- and C-terminal analogs. Arbitrary units (*AU*) after normalization to PTP-1B activity.

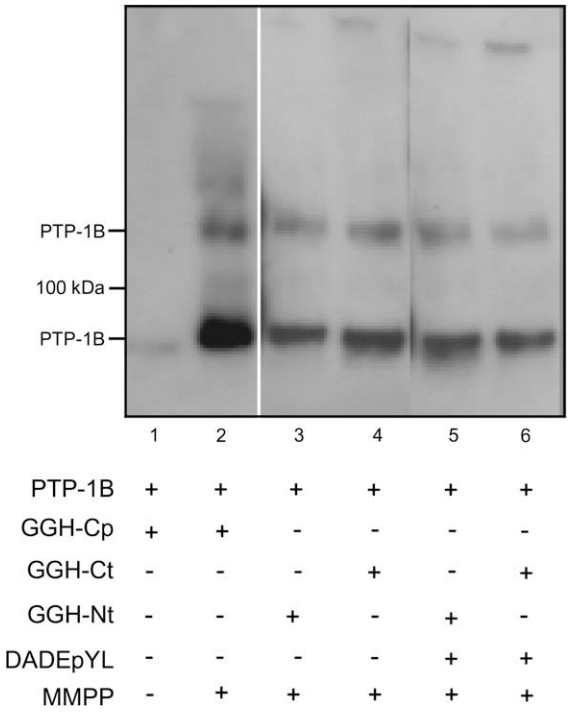

Fig. 3. Cross-labeling of the interaction between PTP-1B and *GGH*-C-peptide (panel 2), *GGH*-N-terminal C-peptide fragment (panel 3) and *GGH*-C-terminal C-peptide fragment (panel 4). The addition of a free EGFR binding motive, DADEpYL, did not affect the binding of the *GGH*-N-terminal (panel 5), but slightly reduced the interaction signal with the *GGH*-C-terminal (panel 6).

that cross-labeling is possible to achieve also with charged molecules like C-peptide, we introduced an affinity capture step, which, coupled with subsequent trypsin digestion and mass spectrometric identification, would allow protein partners in peptide interactions to be accessible to identification. To demonstrate this, cross-labeled PTP-1B was affinity purified on magnetic streptavidin-coated beads, separated by SDS–PAGE, in-gel digested with trypsin, and the fragments identified by tandem mass spectrometry (LC-MS/MS). This resulted in identification of 5 peptides with 9+ residues each. Non-cross-labeled PTP-1B, submitted to the same treatment by direct application to the gel was identified with only one more peptide. Thus, there is virtually no loss of fragments or sensitivity in this workflow of cross-labeling affinity purification compared with direct analysis.

Discussion

We demonstrate the applicability of the Ni-mediated cross-labeling reaction for an acidic peptide like C-peptide, and identify PTP-1B as an interaction partner of C-peptide. PTPs in general have been implied in C-peptide actions [11], but not a direct interaction with a specific phosphatase like PTP-1B, which is a negative regulator of IR [17] and EGFR [18] signaling. C-peptide is now found to induce specific labeling of PTP-1B (Fig. 2B), and two acidic end fragments of C-peptide are shown to inhibit PTP-1B activity in a phosphatase activity assay (Fig. 2A and D). These acidic end fragments are found to have sequence similarities with the site of EGFR that is dephosphorylated by PTP-1B, suggesting that C-peptide interactions with PTP-1B are relevant and compatible with other PTP-1B interactions [21,22]. We therefore now have a direct link between C-peptide and a regulatory protein, PTP-1B, for further studies in relation to diabetes of both types 1 and 2. This interaction is of additional interest, since C-peptide has recently been demonstrated to be an intracrine factor capable of entering several types of cell [10].

The second aspect of the results is the fact that interactions can be chemically studied with an acidic peptide like C-peptide. The use of this cross-labeling reaction in previous [12–16] studies and now with small and acidic peptides suggest that oxidative Ni-mediated introduction of biotin in interacting partners may be suitable to study many biological systems. The underlying mechanism of cross-labeling is suggested to proceed via a chain of electron transfers introducing the biotin moiety in proteins proximal to the bait [12–16,23,24]. The labeled partner protein could now also be identified in an MS-compatible workflow, with the cross-labeling and affinity purification on magnetic streptavidin-coated beads, followed by gel separation, in-gel tryptic digestion and mass spectrometric fragment identification. The binding partner was identified with nearly the same sequence coverage as for non-cross-labeled PTP-1B. This allows for analysis of protein/bead mixtures, gives minimal material losses and eliminates the need of immunoblotting-based identification of interacting partners. The separation on the gel gives size information that is useful for confident identification of interacting proteins. The beads remain in the well, while the detached proteins migrate and become available for identification by mass spectrometry. In conclusion, we show that C-peptide interacts with PTP-1B, and that an MS-compatible workflow allows for partner identification in cross-labeling.

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