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Proinsulin C-peptide Activates α -enolase: Implications for C-peptide – Cell Membrane Interaction

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Running title: Proinsulin C-peptide Activates α -enolase

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Summary

Proinsulin C-peptide shows beneficial effects on microvascular complications of type 1 diabetes. However, the possible occurrence of membrane C-peptide receptor(s) has not been elucidated. The aim of this study was to identify and characterize membrane proteins to which C-peptide binds. The enzyme α -enolase was co-immunoprecipitated with C-peptide after chemical cross-linking to HL-60 cell surface proteins, and identified by mass spectrometry. Recombinant α -enolase activity was modulated by C-peptide, with a significant decrease in K_m for 2-phosphoglycerate without affecting V_{max} . The enzyme modulation by C-peptide was abolished when C-terminal basic lysine residue (K434) of the enzyme was replaced by neutral alanine or acidic glutamate, but not with basic arginine. The enzyme modulation by C-peptide was reproduced with the C-peptide fragments containing glutamate corresponding to position 27 (E27) of the full-length C-peptide. Addition of a lysine analogue to the assay and A31 cell culture abrogated the enzyme modulation and MAP kinase activation by C-peptide, respectively. The results indicate that C-peptide has the capacity to activate α -enolase via a specific interaction between E27 of the peptide and K434 of the enzyme. Since α -enolase plays a role as a cell surface receptor for plasminogen, it may conceivably also serve as a receptor for C-peptide *in vivo*.

Key words: C-peptide, α -enolase, ENO1, MAP kinase, plasminogen

C-peptide is a connecting segment of proinsulin and is secreted from pancreatic β -cells into the circulation along with insulin after the cleavage of proinsulin. Its blood levels are inversely related to the development of diabetic complications including microvascular disturbances and neuropathy, and recent studies have suggested that C-peptide possesses several beneficial effects on diabetic complications of patients with type 1 diabetes mellitus [1-5]. When given to patients or animals with type 1 diabetes mellitus, C-peptide decreases glomerular hyperfiltration [6-10], diminishes urinary excretion of albumin [7-10], reduces urinary sodium waste [11] and induces body weight gain regardless of hyperglycemia and glycosuria [11, 12]. Moreover, C-peptide lowers the leakage of albumin or fluorescein across the blood-retina barrier [13], increases glucose uptake in skeletal muscle [14, 15] and improves autonomic nerve and microvascular functions [6, 7, 13, 14, 16-18].

More recently, anti-inflammatory properties of C-peptide have been demonstrated. For example, C-peptide prevents insulin-induced neointima formation [19] and reduces hyperglucose-induced proliferation of vascular smooth muscle cells [3, 20]. Additionally, C-peptide replacement reduces diabetes-induced upregulation of RAGE expression, and activation of NF- κ B and of pro-inflammatory factors in hippocampi [21]. C-peptide treatment improves the survival rate after acute endotoxemia, with reduction of pro-inflammatory cytokines [22].

Beneficial functions of the C-peptide are supposed to be mediated by a specific receptor for C-peptide. Using the fluorescence correlation spectroscopy method, Rigler *et al.* have shown the specific binding of rhodamine-labeled C-peptide to cell membranes of renal tubular cells, fibroblasts and endothelial cells, with an equilibrium association constant, K_{ass} , of $2\sim 3.3 \times 10^9 \text{ M}^{-1}$ [23]. The binding is displaced by

unlabeled native C-peptide (L-form) and its C-terminal pentapeptide (EGSLQ), but not by an all D-amino acid C-peptide, scrambled C-peptide, insulin, proinsulin or insulin-like growth factor I or II [23]. In line with their results, we have found that native C-peptide at sub-nM concentration, but not D-form and retro-sequenced C-peptide, enhances phosphorylation of p44/p42 mitogen-activated protein kinase (MAPK, Erk1/2) in fibroblasts and endothelial cells in culture [24-26]. As pertussis toxin treatment abolishes the C-peptide binding to membrane and also Erk phosphorylation [23, 24], it is most probable that one of the putative receptors for C-peptide may be a G protein-coupled type of receptor. However, attempts to demonstrate the nature of the binding and the possible receptor structure have not been successful so far.

In the present study, we aimed to examine the molecular mechanisms of C-peptide's cell membrane interaction in an attempt to identify and characterize membrane proteins to which C-peptide binds. We chose a human promyelocytic leukemia cell line to identify the proteins of interest, on the basis of the findings that C-peptide induces chemotaxis for monocytes and neutrophils *in vitro* [27, 28], and found that the enzyme α -enolase selectively interacted with C-peptide.

Material and Methods

Chemotaxis assay with HL60 cells

HL60 cells, a human promyelocytic leukemia cell line, were maintained in RPMI1640 (Invitrogen Co., Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS, Trace Scientific Ltd., Melbourne, Australia). Chemotaxis assay was performed under serum-free conditions in a 96-well cell migration chamber (QCM™ 3 µm pore, Chemicon, Temecula, CA, USA). In brief, HL60 cells (1×10^6 cells/ml) were incubated in the serum-free medium for 16 hours and then aliquots (100 µl each) were further incubated for 1 h in upper chamber wells, which were set on lower chamber wells containing the serum-free medium with phosphate-buffered saline (PBS), 10% FBS, 10 nM human C-peptide (purity >95%, Eli Lilly Co., Indianapolis, IN, USA), 10 nM reverse sequence (retro)-human C-peptide (purity >95%, Bio-Synthesis, Inc., Lewisville, TX, USA) and 1 µg/ml interleukin-8 (Wako Pure Chemical Co., Osaka, Japan), respectively. The number of cells that migrated into the lower chamber wells was counted under a light microscope, according to the instructions provided.

Identification of the proteins specifically co-precipitated with C-peptide after chemical cross-linking

HL60 cells were extensively washed with the cold serum-free medium, and two sets of cells (10^6 cells each) were incubated with 10 nM human C-peptide at 4 °C for 1 h. Then, each set of cells was further incubated with either bis-[b-(4-azidosalicylamido)ethyl]disulfide (BASED, Pierce, Rockford, IL, USA) or dimethylformamide (DMSO) at 4 °C for 1 h. The cells were recovered by centrifugation at 4 °C and lysed in the CHAPS buffer consisting of 20 mM Hepes (pH 7.4), 115 mM

NaCl, 24 mM NaHCO₃, 4.7 mM KCl, 1.26 mM CaCl₂, 1.2 mM KH₂PO₄, 1.2 mM MgSO₄, 11.1 mM glucose, 0.5% bovine serum albumin (BSA), 2% CHAPS (Sigma-Aldrich, St. Louis, MO, USA) and protease inhibitor mixture (Complete™, Boehringer Mannheim, GmbH, Germany) for 30 min on ice. The supernatant was recovered by centrifugation at 15,000 x g for 15 min at 4 °C. Protein concentration was determined by the method of Lowry et al. using BSA as a standard [29].

The supernatants (700 µg of protein each) were first incubated with 20 µl of protein G-sepharose (1:1 slurry, GE Healthcare, Little Chalfont, Buckinghamshire, UK) for 2 h at 4 °C. After removal of the beads, the supernatants were incubated with anti-human C-peptide antibody (Biogenesis, Oxford, UK) for 16 h at 4 °C, followed by incubation with 20 µl of protein G-sepharose for 2 h at 4 °C. After washing the beads, they were incubated with 200 µl of the solution consisting of 8 M urea, 2% CHAPS, 0.5% ampholyte and 20 mM dithiothreitol for 30 min. The proteins were recovered by centrifugation at 5,000 x g for 10 min at 4 °C.

The proteins were first separated by isoelectric focusing using ZOOM IPG Strip (pH 3-10, non-linear, Invitrogen). The proteins were then separated by SDS-polyacrylamide gel electrophoresis and stained with silver staining kits (Wako for analysis and Pierce Silver SNAP for mass spectrometry) according to the instructions provided. The separated proteins in the two-dimensional gels were analyzed with PDQuest software (BioRad, Hercules, CA, USA). Mass spectrometric analysis of the protein of interest was outsourced to APRO Science (Tokushima, Japan).

RT-PCR

Total cellular RNA was isolated from the cultured cells and mouse tissues by the

guanidine-isothiocyanate method using RNAiso reagent (Takara Bio, Shiga, Japan). The RNA (2 µg) was treated at 76 °C for 10 min and reverse-transcribed using 100 units Moloney murine leukemia virus reverse transcriptase (Invitrogen), 50 pmol poly(dT) primer and 20 nmol dNTP in a total volume of 20 µl at 37 °C for 1 h. After heating at 95 °C for 5 min, PCR amplification was performed with 2.5 units Taq polymerase (Ampliqon, Herlev, Denmark), 3 mM MgCl₂ and 50 pmol forward and reverse primers specific to the respective genes in a total volume of 25 µl. Denaturation and annealing were performed at 94 °C and 62 °C for 30 sec, respectively, while extension was performed at 72 °C for 60 sec. The primers used are summarized in Supplementary Table 1. The PCR products were analyzed by electrophoresis in 2% agarose gel and stained with ethidium bromide.

Assessment of MAP kinase (Erk) activation

A31 cells, a mouse fibroblastic cell line, were maintained in Dulbecco's modified Eagle's medium (DMEM, Wako), supplemented with 10% FBS. The cells were treated with DMEM supplemented with 1% FBS for 48 h before stimulation with C-peptide or plasminogen (Enzyme Research, South Bend, IN, USA) for 5 min. When A31 cells were treated with either carboxypeptidase B (CpB, 20 mg/ml, Calbiochem, San Diego, CA, USA) or pertussis toxin (PTX, 100 ng/ml, List Biological Laboratories, Campbell, CA, USA), the cells were incubated with either CpB for 30 min or PTX for 2 h before the stimulation. When ε-aminocaproic acid was given (EACA, 10 mM, Sigma), it was added simultaneously with C-peptide or plasminogen.

After stimulation with C-peptide or plasminogen, A31 cells were lysed in ice-cold buffer consisting of 50 mM HEPES (pH 7.5), 150 mM NaCl, 5 mM EDTA, 10 mM Na

pyrophosphate, 2 mM NaVO₃, protease inhibitor mixture and 1% Nonidet P-40. The lysate was kept on ice for 30 min and centrifuged at 15,000 x g for 20 min at 4°C. Aliquots of the resulting cell lysate (15 µg protein) were resolved by SDS-PAGE (10% gel) under reducing conditions, and the proteins produced were electroblotted onto a PVDF membrane (Immobilon™, Millipore, Bedford, MA, USA). The membrane was first blocked for 1 h at room temperature in 5% (w/v) skimmed milk in 20 mM Tris/HCl (pH 7.5), 0.15 M NaCl and 0.01% Tween 20, before being incubated with antibodies against phospho-specific p44/42 MAPK (1:3000, Erk 1/2, Thr-202/Tyr-204) (Cell Signaling Technology: CST, Beverly, MA, USA) and total p44/42 MAPK (1:5000, CST) for 1 h at 4 °C. The membrane was then washed 5 times with 20 mM Tris/HCl (pH 7.5), 0.15 M NaCl and 0.01% Tween 20 and incubated with horseradish peroxidase-conjugated secondary goat anti-rabbit IgG antibody (1:10000, Zymed Laboratories, Inc., South San Francisco, CA, USA) for 1 h at room temperature. Visualization was performed using an enhanced chemiluminescence detection system (Millipore) according to the manufacturer's instructions. The intensity of chemiluminescence of the corresponding proteins was analyzed by NIH Image, a public-domain image processing and analysis program.

Detection of cell surface α -enolase after its biotinylation

A31 cells were grown in 10 cm dishes to confluence and the cells were washed three times with ice-cold phosphate-buffered saline (PBS). The medium was replaced with PBS and cells were treated with 0.5 mg/ml EZ Link Sulfo-NHS-SS-Biotin (a membrane-impermeable biotinylation reagent, Pierce) or dimethylformamide (DMF) as a negative control for 30 min at room temperature. The cells were washed three times

with 100 mM ice-cold glycine and scraped for lysis in CHAPS buffer for 30 min at 4°C. The lysate was recovered by centrifugation at 15,000 x g for 15 min at 4°C, and the biotinylated proteins were obtained as follows: The lysate (200 µg of protein) was incubated with 40 µl of NeutrAvidin Agarose Resin (1:1 slurry, Pierce) for 16 h at 4 °C. After washing the beads, they were heated in 30 µl of the SDS sample buffer (50 mM Tris/HCl pH 6.8, 2% SDS, 10% glycerol, 1% β-mercaptoethanol, 12.5 mM EDTA and 0.02 % bromophenol blue) for 3 min, and the supernatant was recovered by centrifugation at 5,000 x g for 10 min at 4 °C. The biotinylated proteins and the lysate (15 µg of protein) as an input were subjected to SDS-PAGE (10% gel) under reducing conditions and Western blot analysis to detect α-enolase protein using its specific antibody (1.25 µg/ml, Avita System Biology, San Diego, CA, USA).

Assay of α-enolase activity

A31 cells were scraped and homogenized in the buffer containing 250 mM sucrose, 20 mM HEPES (pH 7.5), 10 mM KCl, 1 mM MgCl₂, 1 mM EGTA, 1 mM EDTA, 1 mM dithiothreitol and 1 mM PMSF. The homogenate was then centrifuged at 15,000 x g for 15 min at 4 °C, and the resulting supernatant was recovered for the enzymatic assay of α-enolase. In brief, an aliquot (30 µl) of the supernatant was incubated with the reaction buffer (50 µl) containing 20 mM imidazole/HCl (pH 7.0), 400 mM KCl and 1 mM magnesium acetate, in either the presence or the absence of human C-peptide for 30 min, and the absorbance of the solution at 240 nm was recorded at room temperature. Subsequently, 2-phosphoglycerate (2-PG, 30 µl) was added to the solution to initiate the enzyme reaction and the absorbance at 240 nm was measured at 5 min after the addition of 2-PG. Changes in the absorbance reflect the conversion of

2-PG to phosphoenolpyruvate (PEP) by α -enolase, where the molecular extinction coefficient for PEP ($4.42 \times 10^5 \text{ M}^{-1}\text{cm}^{-1}$) was used for calculation. For recombinant α -enolase protein, the enzyme was first incubated with the reaction buffer containing various concentrations and types of C-peptide including scrambled C-peptide as a control and/or EACA for 30 min, and then the rate of conversion of 2-PG to PEP was measured at 5 min after addition of increasing concentrations of 2-PG.

Preparation of recombinant native and mutant α -enolase proteins

Full-length human α -enolase cDNA was cloned from HL60 cells and its sequence was found to be identical to the reported sequence (GenBank accession number NM_001428.2). The α -enolase cDNA was inserted into pDESTTM 17 vector (Invitrogen) and N-terminal His-tagged α -enolase protein was expressed in the competent cells (*Escherichia coli* strain KRX, Promega, Madison, WI, U.S.A.) after incubation with 0.1% L-rhamnose for 16 hours at 20 °C. The cells were recovered by centrifugation for 15 min and lysed using BugBuster solution (Merck KGaA, Darmstadt, Germany) containing 0.2 mg/ml lysozyme, 20 $\mu\text{g}/\text{ml}$ DNase, 1 mM MgCl_2 and 1 mM PMSF. The solubilized fraction was applied to HisTrap FF crude kit (GE) pre-equilibrated with 20 mM imidazole/HCl (pH 7.0). After extensive washing, purified His-tagged α -enolase was eluted from the column with 500 mM imidazole/HCl and dialyzed against 20 mM imidazole/HCl, 400 mM KCl and 1 mM magnesium acetate. Aliquots of the purified protein were stored at -80 °C.

The cDNA for mutated α -enolase proteins (K434A, K434E, K434R) was generated by PCR using specific reverse primers and native human α -enolase cDNA as a template. Respective His-tagged mutated α -enolase proteins were produced as

described above. The primers used are summarized in Supplementary Table 2.

Statistical analyses

The results are expressed as means \pm S.E.M. Statistical analyses were performed using ANOVA for experiments and Fischer's post hoc test and Student's *t* test, with $p < 0.05$ being considered statistically significant.

Results

To examine whether HL60 human promyelocytic leukemia cells respond to C-peptide, chemotaxis assay with the cells was performed. The cells migrated across the 3 μ m membrane to the lower chamber containing 10% FBS and IL-8 as a chemotactant, but not to the medium alone (control) (Fig. 1). The cells also migrated to the lower chamber containing human C-peptide, while they did not respond to retro-sequenced C-peptide, suggesting that HL-60 cells expressed a molecule that distinguished native and retro-sequenced C-peptides. To identify the molecule that selectively elicits C-peptide-mediated chemotaxis, we undertook immunoprecipitation with anti-C-peptide antibody after chemical cross-linking of C-peptide to HL60 cell surface proteins at 4 °C. Analyses by two-dimensional electrophoresis of the precipitates with or without chemical cross-linking revealed that there was a protein present only after chemical cross-linking, which had a molecular weight of around 45 kDa and a pI value of 7.0 (Supplementary Fig. 1). MALDI TOF-MS analysis suggested that the protein was human α -enolase (ENO1), as six peptides (9 to 21 amino acids in length) matched with internal amino acid sequence of human α -enolase and covered 21% (93/434 amino acids) of the protein. In addition, cell surface expression of α -enolase in HL60 cells has been reported [30] and its mRNA expression was confirmed by RT-PCR (Supplementary Fig. 2).

α -enolase is a rate-limiting enzyme in the glycolytic pathway, and therefore mainly located in the cytosol. However, recent studies have shown that α -enolase is also expressed on the cell surface of hematopoietic, epithelial and endothelial cells as a plasminogen receptor [31, 32]. In addition, it has been shown that the lysine residue present at the carboxy (C)-terminal end of α -enolase is important for

α -enolase-plasminogen interactions [31, 32]. Thus, we next examined site-specific ERK phosphorylation by C-peptide in A31 mouse fibroblastic cells, in which plasminogen activated the ERK pathway [33]. A31 cells expressed α -enolase mRNA, but not β - and γ -enolases (ENO3 and ENO2) (Supplementary Fig. 2), and α -enolase was labeled with membrane-impermeable biotinylation reagent (Supplementary Fig. 3). Treatment of the cells with either C-peptide or plasminogen activated ERK phosphorylation, which was abrogated by both prior treatment of carboxypeptidase B to remove C-terminal amino acid residues and inclusion of EACA as a competitor for lysine residue (Fig. 2). These results suggested that cell surface molecule(s) containing basic amino acid(s) at the C-terminal, such as α -enolase, mediated signal transduction of C-peptide. In addition, ERK phosphorylation induced by C-peptide or plasminogen was abolished by prior treatment with pertussis toxin (Supplementary Fig. 4).

Interestingly, C-peptide slightly enhanced α -enolase activity in A31 cell lysate (Supplementary Fig. 5). Therefore, we measured recombinant α -enolase activity stoichiometrically in either the presence or the absence of C-peptide to examine the interaction between C-peptide and α -enolase. Native sequenced α -enolase (15 nM) converted 2-phosphoglycerate (2-PG) to phosphoenolpyruvate with K_m of 1.14 ± 0.38 mM and maximal velocity (V_{max}) of 29.3 ± 8.4 pmol/min. Human C-peptide (15 nM), but not scrambled C-peptide, increased α -enolase activity at the 2-PG concentrations tested, with a significant decrease in K_m value to 0.54 ± 0.02 mM, without affecting V_{max} (Fig. 3A and Table 1). Dose-response experiments of C-peptide revealed that an equimolecular amount of C-peptide to α -enolase was sufficient to fully modify the enzyme activity, suggesting that one molecule of C-peptide interacts with one molecule of the enzyme (Figs. 3B and 3C, and Table 1). This assumption was confirmed by the

fact that the addition of half the amount of C-peptide to α -enolase reduced K_m value to 0.85 ± 0.12 mM as if only half the amount of the enzyme were modified by binding with C-peptide.

To determine whether the C-terminal lysine residue of α -enolase was involved in its interaction with C-peptide, the enzyme activity was measured in the presence or absence of C-peptide and EACA. Inclusion of EACA abolished C-peptide-induced enzyme modification, while it did not affect basal activity (Fig. 4A), supporting the hypothesis. Furthermore, the mutant enzymes in which C-terminal lysine (basic amino acid, K434) was replaced with alanine (neutral amino acid, K434A) or glutamate (acidic amino acid, K434E) exhibited similar catalytic activity to the wild-type enzyme, although C-peptide failed to modify these enzyme activities (Figs. 4B and 4C). However, the activity of the mutant enzyme with arginine (basic amino acid, K434R) replacing C-terminal lysine was enhanced by C-peptide, as observed in the wild-type enzyme (Fig. 4D). Thus, the results indicate that the C-terminal lysine residue of α -enolase is essential for the stimulatory effect of C-peptide.

The glutamate residue at position 27 (E27) of C-peptide is suggested to be important for C-peptide binding to cell membranes [34]. To determine whether E27 of C-peptide was involved in its interaction with α -enolase, the enzyme activity was measured in the presence of C-peptide fragments. Among the fragments, peptides containing E27 (F2, F4 and F7) were as effective as the full-length peptide, although peptides without E27 (F5: E27 deletion and F6: E27A) were not (Figs. 3 and 5, Tables 1 and 2). In addition, peptides replaced E27 with aspartate and lysine (E27D and E27K) were also failed to modulate the enzyme activity (data not shown). The fragments F1 and F3 containing a glutamate residue at positions of 1 and 3 tended to increase

α -enolase activity slightly, with a tendency for a decrease in both K_m and V_{max} values (Fig. 5 and Table 2). Actually, there were differences in V_{max} values between F1/F3 fragments and F2 pentapeptide (Table 2). Furthermore, the retro-sequenced C-peptide as well as the fragment F3 increased wild-type and mutant (K434R) α -enolase activity, to a much lesser extent than that of the native C-peptide (Supplementary Fig. 6). These results suggested that glutamate residues of C-peptide are involved in the modulation of the enzyme and that the glutamate residue at position 27 is essential for the stimulatory effect of C-peptide on α -enolase.

Discussion

In the present study, we have demonstrated for the first time that C-peptide enhances α -enolase activity through the specific interaction between E27 of the peptide and K434 of the enzyme. This is based on the following findings: 1) α -enolase activity was enhanced by full-length C-peptide, accompanied by a significant decrease in K_m for the substrate without affecting V_{max} , 2) excess amounts of C-peptide over the amounts of α -enolase (molar basis) were unproductive for the stimulatory effect of the peptide, 3) enhancement of α -enolase activity by C-peptide was abolished when the C-terminal basic lysine residue (K434) of the enzyme was replaced with neutral alanine or acidic glutamate residue, while enhancement by C-peptide was observed for an enzyme in which the lysine residue had been replaced by arginine, 4) the enhancement by C-peptide was also abrogated by the presence of EACA (a lysine analogue), and 5) the peptides containing glutamate at position 27 (E27) of full-length C-peptide, but not the other peptides tested, enhanced α -enolase activity in the same manner as full-length peptide.

Other than C-peptide, the peptide having the sequence of GMQGPAGSGWEEGSGSPPGVTP LFSP from acetylcholine esterase (AChE) variant R has been shown to increase α -enolase activity, but its level of activation is only 12% at 16-fold excess amounts of the peptide over the enzyme [35]. The study did not provide any detailed information on the AChE peptide- α -enolase interactions, thus the mechanism of the activation remains unknown. In contrast, two peptides (VGGSEI and LPQLTP) derived from C-reactive protein inhibit α -enolase activity by an unknown mechanism [36]. C-peptide at equi-molar ratio to α -enolase apparently enhanced the enzyme activity by almost 50% at the maximal substrate concentration tested, but

actually decreased K_m for the substrate of α -enolase without affecting the maximal velocity. It is reported that active site of α -enolase are composed of S39, D244, E292, and D317 in human α -enolase [37]. In addition, the mutant enzymes in which C-terminal lysine residue (K434) was replaced with alanine or glutamate exhibited similar catalytic activity to the wild-type enzyme either in the absence or presence C-peptide. Since the assay system of the enzyme contains only α -enolase, 2-phosphoglycerate (2-PG), and C-peptide, it is unlikely that C-peptide directly affect the active site of α -enolase and the substrate itself. Thus, it is the most probably that C-peptide-induced decrease in K_m is due to a conformational change of α -enolase by electrostatic specific interaction between E27 and K434, resulting in increase of apparent affinity to 2-PG. To clarify conformational changes in the enzyme structure by C-peptide like an allosteric activator, future studies involving the structural analysis of α -enolase in the presence of C-peptide will be necessary.

Human C-peptide consists of 31 amino acid residues. The N-terminal region of C-peptide is shown to be essential for disaggregation of insulin oligomers and intramolecular chaperone-like activity for proinsulin folding [38-40]. The central region of C-peptide (i.e. residues 4-24 of human C-peptide) is suggested to be important for non-chiral membrane interaction [13]. This region consists largely of nonpolar amino acids flanking proline at position 16 and residues 9-12, 15-18 and 22-25 show tendencies to form β -bends, while the N-terminal region forms a type I β -turn [40]. C-terminal pentapeptide of human C-peptide (residues 27-31) presents the most well-defined structure of the whole molecule, including a type III' β -turn [41], and is highly conserved in mammalian species, although the amino acid sequences and number of residues of the C-peptides from different species are quite variable [42]. This

C-terminal region is critical for the C-peptide binding to cell membrane [23] and subsequent activation of intracellular effectors, such as MAPK (Erk) as well as Na⁺, K⁺-ATPase [42, 43], although the other regions also contribute to activation of these effectors to some extent [42, 43]. In the present study, the peptides containing E27 including full-length C-peptide enhanced α -enolase activity by altering the apparent affinity for substrate without affecting maximal velocity, whereas the peptides containing N-terminal region of C-peptide having 2-3 glutamate residues (E1, E3, E11) and retro-sequenced C-peptide (E5, E21, E29, E31) modulated α -enolase activity in a different manner from that of the peptides containing E27. Thus, these results, taken together with the structural features of C-peptide, suggest that the structure of C-terminal pentapeptide is important for interaction between C-peptide and α -enolase, in addition to the electrostatic interaction between E27 and K434.

Other than α -enolase, C-peptide has been reported to interact with following proteins: insulin [38-40], α -chain of spectrin, myosin light chain kinase, cytoskeletal keratin type II, calcium/calmodulin-dependent serine protein kinase [44], protein tyrosine phosphatase 1B [45] and histone H4 [46]. However, the molecular bases of these interactions remain obscure, with the exception of C-peptide-insulin interaction as described above [38-40], and none of these is likely to engage with C-peptide-membrane interactions, although these proteins might be involved in the C-peptide signals and functions such as cell proliferation [46]. In contrast, it was previously shown that α -enolase is expressed on the cell surface of hematopoietic, epithelial and endothelial cells and serves as a plasminogen receptor [30-33], as well as on A31 cells in this study. Moreover, lysine residue present at the C-terminal of α -enolase including K434 is suggested to be important for plasminogen interaction with

α -enolase and its cellular signaling. The present study showed that α -enolase was co-immunoprecipitated by C-peptide antibody, and that both C-peptide and plasminogen activated ERK phosphorylation in A31 cells, which was abrogated by prior treatments of carboxypeptidase B and pertussis toxin and by inclusion of EACA. These results, taken together with the localization of α -enolase and its specific interaction with C-peptide, suggest that cell surface α -enolase is critical for pertussis toxin-sensitive signal transduction of C-peptide.

It is unlikely that the cell surface α -enolase plays role in extracellular glycolytic activity, because 2-PG, the substrate of the enzyme, might be deficient in extracellular space. As suggested above, C-peptide interaction with α -enolase the most probably alters the enzyme conformation. Therefore, we assume that similar conformational changes of the enzyme in the absence of the substrate might be induced by C-peptide at the cell surface, and that such a conformational change in α -enolase triggers signaling events for C-peptide. Unfortunately, our trials such as knockdown the enzyme expression in A31 and HL60 cells failed (data not shown), because α -enolase is the rate-limiting enzyme in the glycolytic pathway and, consequently, these cells did not grow. Thus, it is not possible to claim that α -enolase is the C-peptide receptor on the basis of the present evidence. Moreover, as several proteins such as p11 and TIP49a are also known as plasminogen receptors [47, 48], some proteins other than α -enolase might also be involved in C-peptide-membrane interaction. Of course, there is a possibility that an unidentified pertussis toxin-sensitive G-protein-coupled receptor serves as a C-peptide receptor. However, the present results strongly suggest that α -enolase is a novel candidate for C-peptide receptor.

In summary, we have demonstrated that C-peptide modulates α -enolase activity

through a specific interaction between E27 of the peptide and K434 of the enzyme. This C-peptide- α -enolase interaction is likely to occur on the cell surface, and might be of importance for signal transduction and the effects of C-peptide.

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Table 1. C-peptide lowers Km value for the substrate of recombinant α -enolase

	Km (mM)	Vmax (pmol/min)
Control	1.14 \pm 0.38	26.7 \pm 7.6
C-peptide 3 nM	1.00 \pm 0.12	27.2 \pm 4.5
C-peptide 7.5 nM	0.85 \pm 0.12	28.5 \pm 3.0
C-peptide 15 nM	0.54 \pm 0.02 *	26.9 \pm 0.6
C-peptide 30 nM	0.60 \pm 0.04 *	29.4 \pm 1.2

The α -enolase activity of the recombinant native enzyme protein (15 nM) was assayed in a total volume of 110 μ l with increasing concentrations of 2-phosphoglycerate (2-PG, 0-1 mM) as a substrate in the presence or absence (control) of increasing concentrations of C-peptide as shown in Fig. 3B. The K_m and V_{max} values were calculated under the double reciprocal plots. Results are mean \pm S.E.M. for three independent experiments.

* $p < 0.05$ compared with control.

Table 2. C-peptide fragments containing Glu at amino acid position 27 lower K_m value for the substrate of recombinant α -enolase, without affecting V_{max} value

	K_m (mM)	V_{max} (pmol/min)
Control	1.11 ± 0.37	28.5 ± 8.1
F1: human C-peptide (1-26)	0.62 ± 0.05	$23.6 \pm 1.2^\dagger$
F2: human C-peptide (27-31)	$0.54 \pm 0.02^*$	30.0 ± 0.6
F3: human C-peptide (1-5)	0.58 ± 0.13	$21.9 \pm 3.2^\dagger$
F4: human C-peptide (6-31)	$0.49 \pm 0.08^*$	27.5 ± 3.1
F5: human C-peptide (28-31)	1.40 ± 0.59	33.8 ± 10.4
F6: human C-peptide (27-31) E27A	1.41 ± 0.39	25.5 ± 3.4
F7: rat C-peptide (27-31)	$0.43 \pm 0.08^*$	28.5 ± 8.1

The α -enolase activity of the recombinant native enzyme protein (15 nM) was assayed in a total volume of 110 μ l with increasing concentrations of 2-phosphoglycerate (2-PG, 0-1 mM) as a substrate in the presence or absence (control) of various C-peptide fragments (15 nM each) as shown in Fig. 5. The K_m and V_{max} values were calculated under the double reciprocal plots. Results are mean \pm S.E.M. for three independent experiments. *, $p < 0.05$ compared with control. \dagger , $p < 0.05$ compared with F2.

Figure legends

Fig. 1 C-peptide, but not retro-sequenced C-peptide, is a chemotactic factor for HL60 cells

HL60 cells (10^5 cells) in RPMI1640 medium were put in the upper chamber wells, while the medium alone (control), the medium containing 10% FBS, human C-peptide (10 nM), retro-sequenced C-peptide (10 nM) and IL-8 (10 μ g/ml), respectively, were put in the lower chamber wells. Cell migration into the lower chamber was evaluated after 1 h incubation. The number of cells migrated are shown as means \pm S.E.M. of three independent experiments. * $p < 0.05$ vs. control.

Fig. 2 C-peptide and plasminogen cause Erk1/2 phosphorylation in A31 cells, and both are abolished by prior treatment with carboxypeptidase B or by the presence of lysine mimetic

A31 cells were stimulated with either C-peptide (Cp, 10 nM) or plasminogen (Pla, 2 μ g/ml) for 5 min. In A, the cells were treated with or without carboxypeptidase B (CpB, 20 mg/ml) for 30 min before the stimulation. In B, ϵ -aminocaproic acid (EACA, 10 mM) was added simultaneously with the stimulation. The cell lysates were analyzed for site-specific phosphorylation and total amounts of ERK1/2. Representative blots and densitometric analyses from three independent experiments are shown. * $p < 0.05$ vs. control (no stimulation), ** $p < 0.05$ vs. without CpB or EACA treatment.

Fig. 3 C-peptide enhances α -enolase activity

The α -enolase activity of the recombinant native enzyme protein (15 nM) was assayed in a total volume of 110 μ l with increasing concentrations of 2-phosphoglycerate (2-PG,

0-1 mM) as a substrate. When included, human C-peptide (3-30 nM) and scrambled C-peptide (15 nM) were incubated with the enzyme solution for 30 min and then the rate of conversion of 2-PG to phosphoenolpyruvate was measured at 5 min after addition of 2-PG. Results are means of three independent experiments. Error bars in each substrate concentration are hidden by respective symbols. In C, the dose response effect of C-peptide on α -enolase activity was evaluated at 1 mM 2-PG. Results are means \pm S.E.M. of three independent experiments.

Fig. 4 C-terminal lysine residue of α -enolase is essential for the stimulatory effect of C-peptide

The α -enolase activity of the recombinant wild-type and mutated enzyme proteins (15 nM) was assayed in a total volume of 110 μ l with increasing concentrations of 2-phosphoglycerate (2-PG, 0-1 mM) as a substrate. (A) Assay was performed with native enzyme in either the presence (+) or the absence (-) of ϵ -aminocaproic acid (EACA, 10 mM) and C-peptide (15 nM). (B, C, D) Assay was performed with mutated enzyme with alanine (B, K434A), glutamate (C, K434E) or arginine (D, K434R) replacing the C-terminal lysine in either the presence or the absence of C-peptide (15 nM). Results are mean \pm S.E.M. for three independent experiments.

Fig. 5 Glutamate at amino acid position 27 of C-peptide is essential for the enhancement of the α -enolase activity

(A) Primary sequences of the fragments used are shown: F1, human C-peptide (1-26); F2, human C-peptide (27-31); F3, human C-peptide (1-5); F4, human C-peptide (6-31); F5, human C-peptide (28-31); F6, human C-peptide (27-31) with Ala substituting Glu;

F7, rat C-peptide (27-31). (B) The α -enolase activity of the recombinant native enzyme protein (15 nM) was assayed in a total volume of 110 μ l with increasing concentrations of 2-phosphoglycerate (2-PG, 0-1 mM) in either the presence or the absence (cont) of various C-peptide fragments (15 nM each). Results are mean \pm S.E.M. for three independent experiments.

Supplements

Supplementary Table 1. Primer list

Gene (Accession No.)	Forward primer sequence Reverse primer sequence (PCR product size)
ENO1 (NM_001428.2)	5'-TGCCCTGGTTAGCAAGAAAC-3` 5'-GGTTGTGGTAAACCTCTGCT-3` (+366 to + 725, 360 bp)
ENO2 (NM_001975.2)	5'-ATCCCAGCCATCATGTCCAT-3` 5'-CGATAAAACTCTGAGGCAGC-3` (+211 to +980, 770 bp)
ENO3 (NM_001976.4)	5'-GCCTTCAATGTGATCAACGG-3` 5'-AATCCTCTTGGGGTTGGTGA-3` (+583 to +1122, 540 bp)

Supplementary Table 2. Primer list

Common forward primer:

GGGGACAAGTTTGTACAAAAAAGCAGGCTTCCATGTCTATTCTCAAGATCCA
TGCCA

Reverse primer for native human α -enolase (K434):

GGGGACCACTTTGTACAAGAAAGCTGGGTTTTACTTGGCCAAGGGGTTTCTG
AAG

Reverse primer for K434R:

GGGGACCACTTTGTACAAGAAAGCTGGGTTTTACCTGGCCAAGGGGTTTCT
GAAGTTCCTGCCGGC

Reverse primer for K434E:

GGGGACCACTTTGTACAAGAAAGCTGGGTTTTACTCGGCCAAGGGGTTTCT
GAAGTTCCTGCCGGC

Reverse primer for K434A:

GGGGACCACTTTGTACAAGAAAGCTGGGTTTTACGCGGCCAAGGGGTTTCT
GAAGTTCCTGCCGGC

Supplementary figure legends

Supplementary Fig. 1 Representative results of two-dimensional gel electrophoresis of the proteins co-immunoprecipitated by anti-human C-peptide antibody after chemical cross-linking C-peptide to cell surface proteins

HL60 cells were incubated with C-peptide with or without chemical cross-linker (BASED) at 4 °C. Subsequently, the proteins immunoprecipitated by anti-human C-peptide antibody were subjected to two-dimensional gel electrophoresis. Representative results of silver-stained gels from three independent experiments are shown. Circle indicates the protein of interest. Detailed data of MALDI TOF-MS analysis suggesting that the protein was human α -enolase (ENO1) are shown.

Supplementary Fig. 2 Expression of α -enolase (ENO1) mRNA in HL60 and A31 cells

The mRNA expressions of α -enolase (ENO1), γ -enolase (ENO2) and β -enolase (ENO3) in HL60 and A31 cells were determined by RT-PCR. The mRNAs from HL60 cells in A and from A31 cells in B were used in lanes 1, 3 and 5, while mRNAs from mouse brain and skeletal muscle were used in lanes 2 and 4, and lane 6, respectively, as positive controls for the expression of enolase isoforms. α -enolase, γ -enolase and β -enolase cDNAs were amplified in lanes 1 and 2, lanes 3 and 4, and lanes 5 and 6, respectively. Representative results are shown.

Supplementary Fig. 3 α -enolase was labeled with membrane-impermeable biotinylation reagent in A31 cells

A31 cells were incubated with or without membrane-impermeable biotinylation reagent

(bio or cont, respectively). Cell surface biotinylated proteins were isolated with NeutrAvidin Agarose Resin (IP). Western blot analysis was performed using anti- α -enolase antibody.

Supplementary Fig. 4 Prior treatment of pertussis toxin inhibited C-peptide- and plasminogen-induced Erk1/2 phosphorylation in A31 cells

A31 cells were stimulated with either C-peptide (Cp, 10 nM) or plasminogen (Pla, 2 μ g/ml) for 5 min, with or without prior treatment of pertussis toxin (PTX, 100 ng/ml) for 2 h. The cell lysates were analyzed for site-specific phosphorylation and total amounts of ERK1/2. Representative blots and densitometric analysis from three independent experiments are shown. * $p < 0.05$ vs. PBS (no stimulation), † $p < 0.05$ vs. corresponding stimulation.

Supplementary Fig. 5 C-peptide enhances enolase activity in A31 cell lysate

The enolase activity in the A31 cell lysate was assayed in a total volume of 110 μ l with 2-phosphoglycerate as a substrate. When included, human C-peptide (Cp, 1 nM, 10 nM) and plasminogen (Pla, 2 μ g/ml) were added to the lysate 30 min before addition of 2-phosphoglycerate. * $p < 0.05$ vs. control.

Supplementary Fig. 6 Glutamate at amino acid position 27 of C-peptide is essential for the full enhancement of the α -enolase activity

The α -enolase activity of the recombinant wild-type (K434) or mutant (K434R) enzyme protein (15 nM) was assayed in a total volume of 110 μ l with increasing concentrations of 2-phosphoglycerate (2-PG, 0-1 mM) as a substrate. Assay was performed in either the

presence or the absence of native human C-peptide (1-31), F3 fragment of human C-peptide (1-5) and retro-sequenced human C-peptide (31-1). Results are mean \pm S.E.M. for three independent experiments.

Figure 1

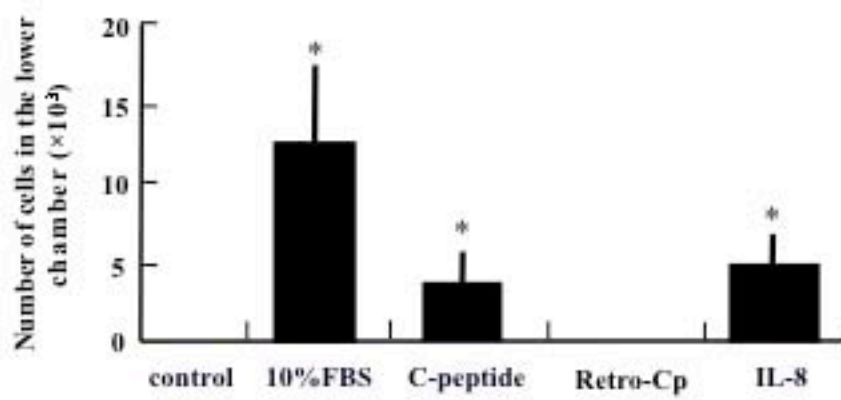


Figure 2

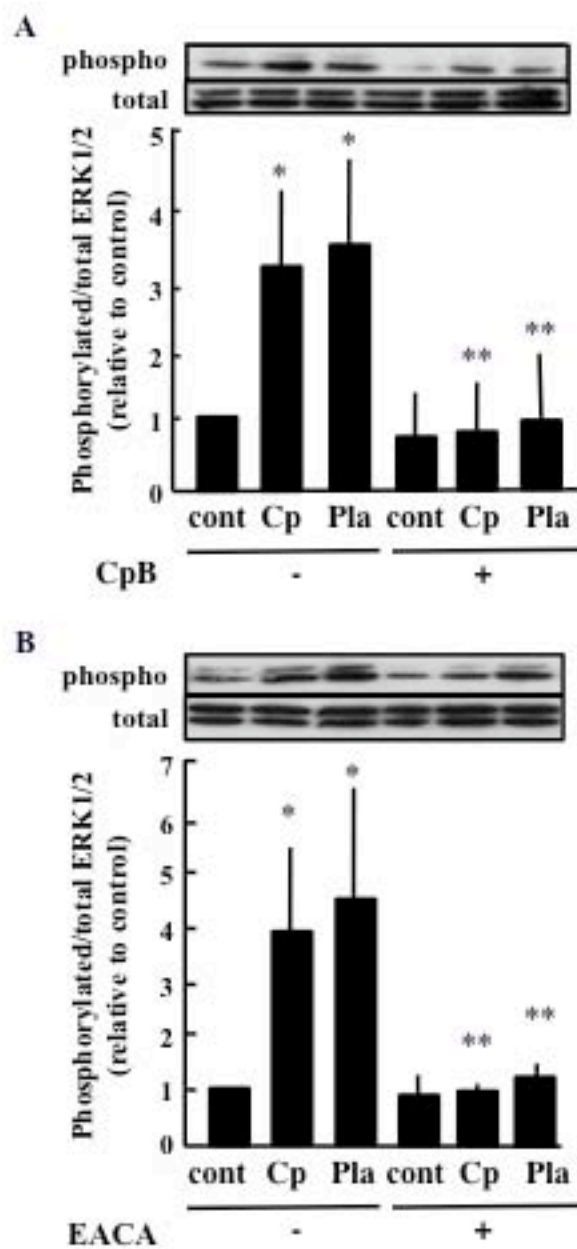


Figure 3

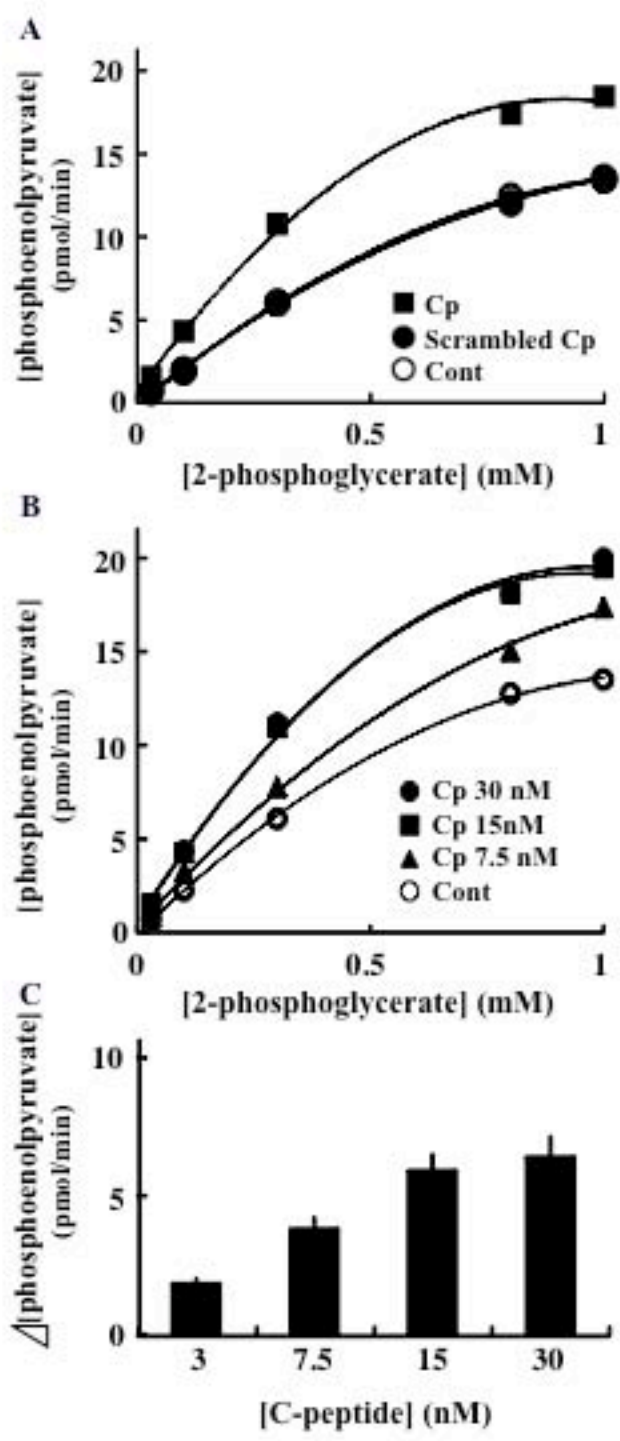


Figure 4

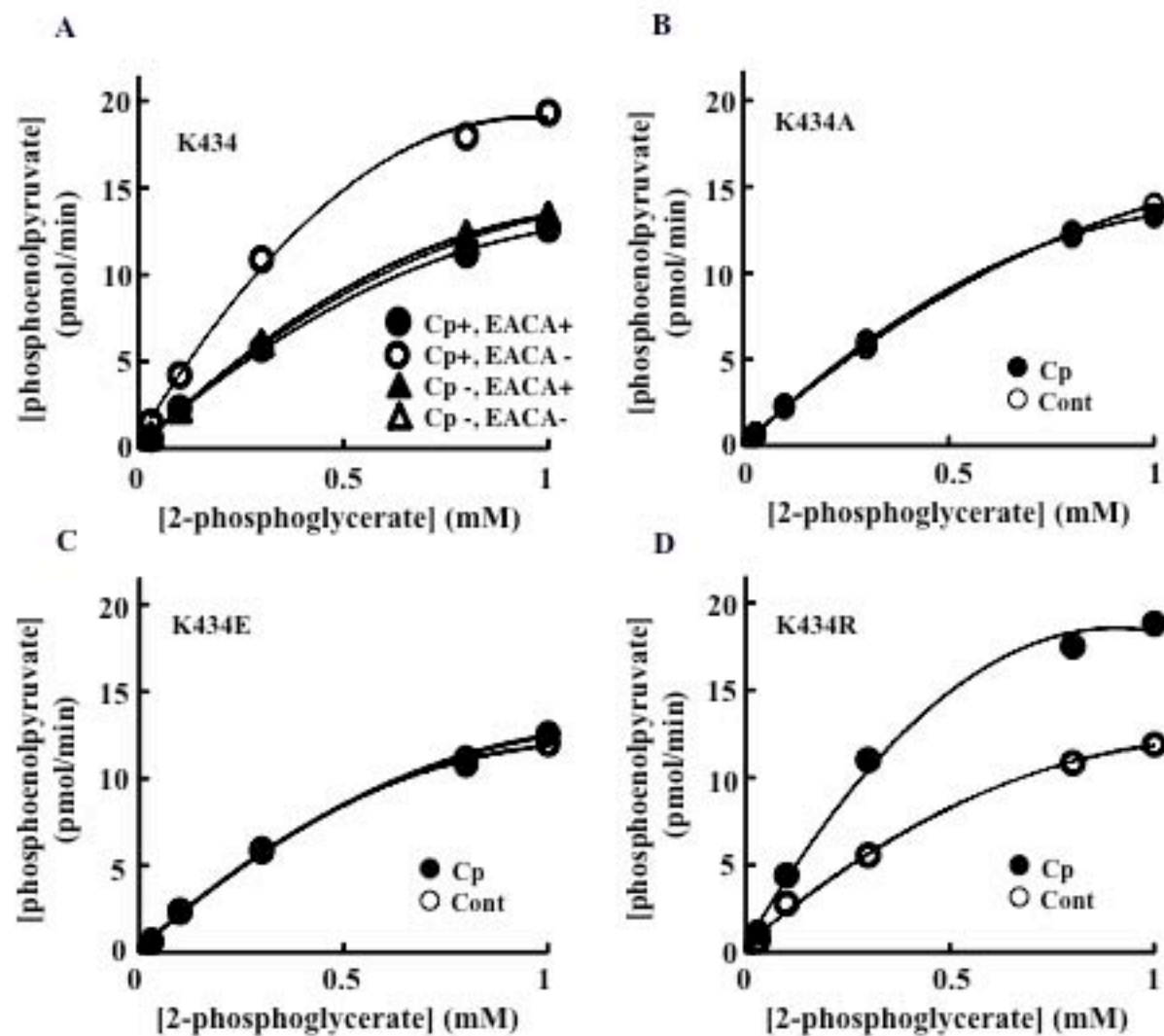
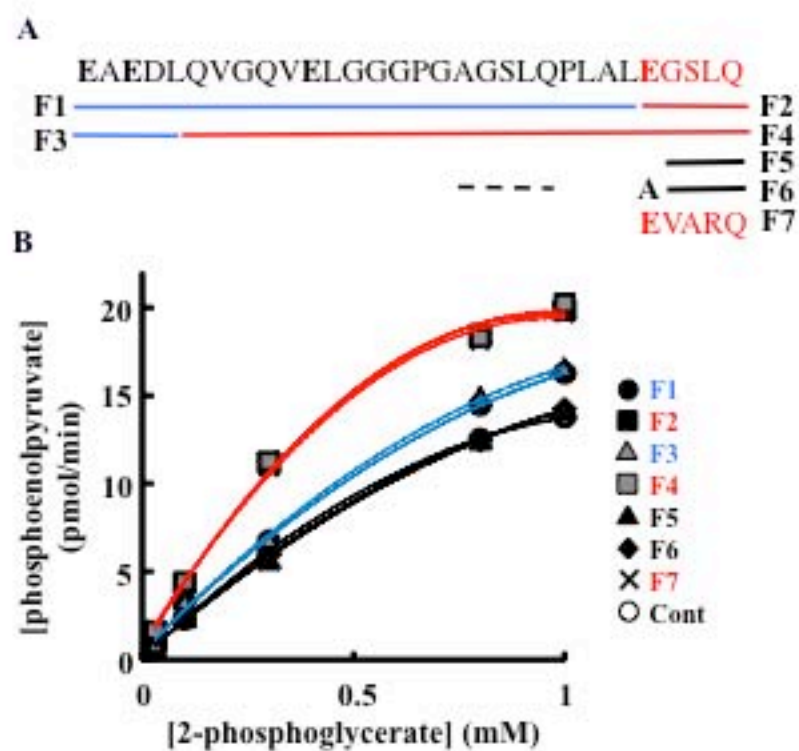
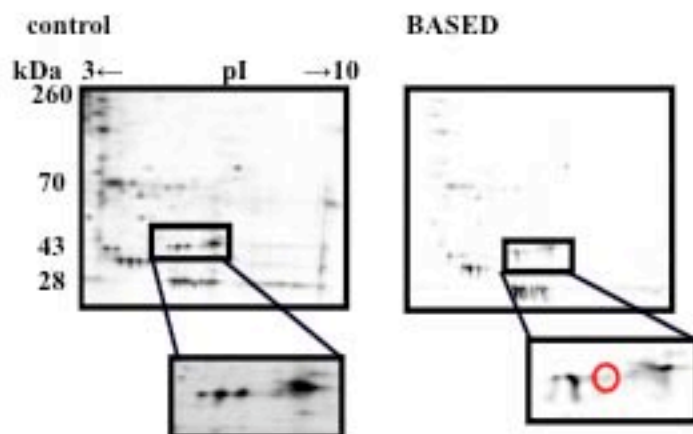


Figure 5



Supplementary Figure 1



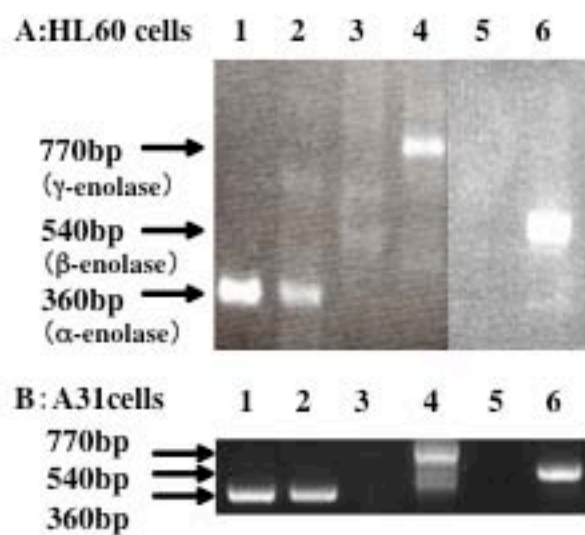
The suggested protein of interest:

Homo Sapiens enolase 1 varriant (Accession # 62897945, MW 47141.2Da, pI 7.01)

The matched peptides shown below cover 21% (93/434 amino acids) of the protein.

m/z Submitted	MH+ matched	start/end	Peptide sequence
871.5124	871.5253	72/80	(K)TIAPALASK(K)
1525.7808	1525.7698	359/372	(K)LAQANGWGVMVSHR(S)
1540.7836	1540.7834	240/253	(K)VVIGMDVAASEFFR(S)
1804.9560	1804.9445	33/50	(R)AAVPSGASTGIYEALR(D)
1907.9869	1907.9876	163/179	(K)LAMQEFMILPVGAANFR(E)
2510.1200	2510.1152	286/306	(K)DYPVVSIEDPFDQDDWGAQK(F)

Supplementary Figure 2



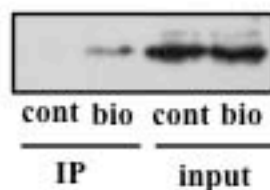
Source of cDNA

Lane 1, 3, 5 : mRNA from the HL60 cells in A and A31 cells in B

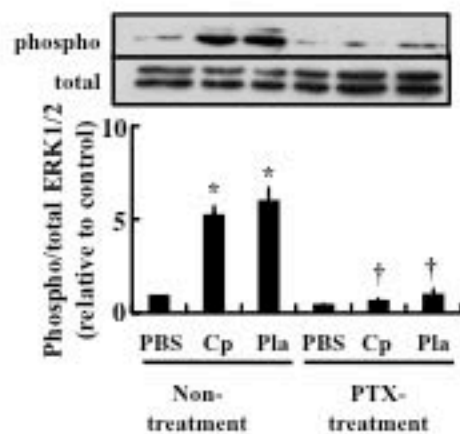
Lane 2, 4 : mRNA from mouse brain

Lane 6 : mRNA from mouse skeletal muscle

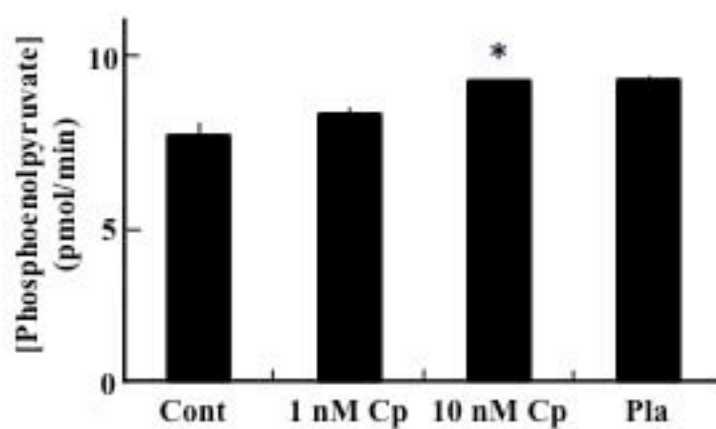
Supplementary Figure 3



Supplementary Figure 4



Supplementary Figure 5



Supplementary Figure 6

