Degradation of Incretins and Modulation of Blood Glucose Levels by Periodontopathic Bacterial Dipeptidyl Peptidase 4

Yuko Ohara-Nemoto,[a #] Manami Nakasato,[b, c] Yu Shimoyama,[b] Tomomi T. Baba,[a] Takeshi Kobayakawa,[a] Toshio Ono,[a] Takashi Yaegashi,[c] Shigenobu Kimura,[d] and Takayuki K. Nemoto[a]

Department of Oral Molecular Biology, Course of Medical and Dental Sciences, Nagasaki University Graduate School of Biomedical Sciences, Nagasaki, Japan[a]; Division of Molecular Microbiology, Iwate Medical University, Yahaba, Iwate, Japan[b]; Division of Periodontology, Iwate Medical University School of Dentistry, Morioka, Iwate, Japan[c]; Department of Oral Hygiene, Kansai Women's College, Kashiwara, Osaka, Japan[d]

Running title: Incretin degradation by oral bacterial DPP4

Address correspondence to: Yuko Ohara-Nemoto, ynemoto@nagasaki-u.ac.jp

Keywords: blood glucose, diabetes mellitus, GIP, GLP-1, incretins, periodontitis, *Porphyromonas gingivalis*, *Prevotella intermedia*, *Tannerella forsythia*

ABSTRACT

Severe periodontitis is known to aggravate diabetes mellitus, though molecular events related to that link have not been fully elucidated. *Porphyromonas gingivalis*, a major pathogen of periodontitis, expresses dipeptidyl peptidase 4 (DPP4), which is involved in regulation of blood glucose levels by cleaving incretins in humans. We examined the enzymatic characteristics of DPP4 from *P. gingivalis* as well as two other periodontopathic bacteria, *Tannerella forsythia* and *Prevotella intermedia*, and determined whether it is capable of regulating blood glucose levels. Cell-associated DPP4 activity was found in those microorganisms, which was effectively suppressed by inhibitors of human DPP4, and molecules sized 73 kDa in *P. gingivalis*, and 71 kDa in *T. forsythia* and *P. intermedia* were immunologically detected. The k_{cat}/K_m values of recombinant DPP4s ranged from 721 ± 55 to 1283 ± 23 $\mu M^{-1}sec^{-1}$ toward Gly-Pro-4-methylcoumaryl-7-amide (MCA), while those were much lower for His-Ala-MCA. MALDI-TOF MS analysis showed the His/Tyr-Ala dipeptide release from the N-termini of incretins, glucagon-like peptide-1 (GLP-1) and glucose-dependent insulinotropic polypeptide, respectively, with the action of microbial DPP4. Moreover, intravenous injection of DPP4 into mice decreased plasma active GLP-1 and insulin levels, accompanied by a substantial elevation in blood glucose over the control after oral glucose administration. These results are the first to show that periodontopathic bacterial DPP4 is capable of modulating blood glucose levels the same as mammalian DPP4, thus the incidence of periodontopathic bacteremia may exacerbate diabetes mellitus via molecular events of bacterial DPP4 activities.

INTRODUCTION

Periodontitis is a highly prevalent type of chronic inflammation caused by a complex of oral bacteria, with greater than 47% of adults in the United States aged 30 years and older reported to suffer from this disease (1). Foremost among periodontopathic microbes are three Gram-negative anaerobic rods: *Porphyromonas gingivalis*, *Tannerella forsythia*, and *Treponema denticola*, as they are most frequently isolated in subgingival dental plaque samples obtained from diseased sites (2–4). This inflammatory disease is a major cause of permanent tooth loss in adults (5), resulting in decrement in overall quality-of-life, especially for elderly individuals. In addition, it is related to increased risk for systemic diseases. Particularly, multiple studies starting from the 1930s have shown a link between periodontitis and hyperglycemia and/or diabetes mellitus. It has been reported that severe periodontal disease often coexists with severe diabetes mellitus and that diabetes is a risk factor for severe periodontal disease, thus their 'two-way' relationship is recognized (for reviews see 6–9). Meta-analyses have found that periodontal disease adversely affects diabetes outcomes, while its successful treatment leads to improvement of glycemic control in type 2 diabetic patients (10, 11). To date, one model linking inflammation to diabetes and periodontal infections has been proposed, in which chronic inflammation ascribed to periodontitis elevates the blood concentrations of pro-inflammatory cytokines, such as TNF-α and IL-1β resulting in magnitude increases in advanced glycation end-product amplification and insulin resistance (10, 12, 13). However, despite a large number of epidemiological studies, few have focused on the more direct relationship between oral microbiota and diabetes.

DPPs are exopeptidases that liberate a dipeptide from the N-terminus of oligo- and

poly-peptides. We recently identified novel dipeptide-producing exopeptidases in *P. gingivalis*, including DPP11 that specifically releases Xaa-Asp and Xaa-Glu (14), DPP5 with preference for the penultimate Ala and hydrophobic residues from the N-terminus (15) and acylpeptidyl oligopeptidase (AOP) mainly for the P1-position hydrophobic residue (16). Besides, *P. gingivalis* express two additional DPPs and gingipains: DPP4 releases mainly Xaa-Pro but also Xaa-Ala and His-Ser (17, 18), DPP7 preferentially cleaves dipeptides with both P1 and P2 hydrophobic residues (19, 20), and Lys- and Arg-gingipains (Kgp and Rgp, respectively) exhibit Lys and Arg specific dipeptidyl peptidase activities besides endopeptidase activities (15). These peptidases are thought to cover most combinations of P2 and P1 amino acid residues, and therefore, efficiently produce N-terminal dipeptides from polypeptides (21). This dipeptide liberating potential is of crucial importance for asaccharolytic *P. gingivalis*, which incorporates amino acids mainly as dipeptides, not single amino acids (22, 23), and utilizes them exclusively as carbon and energy sources (24). In addition, from the viewpoint of niche differentiation, dipeptide-incorporating and amino acid-utilizing properties are likely to provide a benefit for *P. gingivalis* during symbiosis in the complex of subgingival microbiota, since, for instance, *Prev. intermedia* and *Fusobacterium nucleatum* incorporate single amino acids (25), and *Prev. intermedia*, *Trep. denticola*, and *Aggregatibacter actinomycetemcomitans* are saccharolytic.

Besides *P. gingivalis*, DPP4 (EC: 3.4.14.5) is also found in mice and humans. Human DPP4 (hDPP4) is considered to be one of the major factors necessary for postprandial glycemic control (26, 27), since it inactivates incretin peptides, such as glucagon-like peptide-1(7-37) [GLP-1(7-37)] and glucose-dependent insulinotropic polypeptide [GIP(52-93)] via cleavage at the second Ala-third Glu bond from the N-terminus (28). GLP-1(7-37) and GIP(52-93) are active forms that interact with their membrane receptors in the islet, resulting in insulin

91 secretion (29). In circulation, the half-life of active incretins is 1 to 1.5 min (30).

92 The amino acid sequence of *P. gingivalis* DPP4 (PgDPP4) is 31% identical to hDPP4.
93 PgDPP4 potently hydrolyzes Gly-Pro-4-methycoumaryl-7-amide (MCA) and Lys-Ala-MCA as
94 well at a much lower level of activity (31), the same manner as mammalian DPP4, and that
95 activity is efficiently suppressed by hDPP4 inhibitors (15). These similarities between PgDPP4
96 and hDPP4 led us to speculate that PgDPP4 mimics the role of hDPP4 in glycemic control
97 during recurrent bacteremia in periodontal patients, which may explain the link between severe
98 periodontitis and diabetes mellitus. Previous studies have reported that PgDPP4 hydrolyzes
99 substance P, IL-1β, IL-2 (18), RANTES, and MCP1 (32). However, whether PgDPP4 degrades
100 incretins and other bioactive peptides *in viv*o, thereafter causing a pathological response, has yet
101 to be elucidated. In addition, since periodontitis is an infectious disease caused by multiple
102 microorganisms, it is reasonably inferred that DPP4 from other periodontopathic bacteria also
103 contribute to degradation of incretins.

104 In the present study, we report that the major periodontopathic bacteria *P. gingivalis*, *T.*
105 *forsythia*, and *Prev. intermedia* express DPP4 that degrades GLP-1(7-37) and GIP(52-93), and
106 *i.v.* administration of bacterial DPP4 induced hyperglycemia *in vivo* in mice, accompanied by
107 decreases in plasma active GLP-1 and plasma insulin levels. This is the first report to directly
108 demonstrate the relationship between periodontopathic bacterial peptidases and host blood
109 glucose levels.

110

111

112 **MATERIALS AND METHODS**

113

Materials. pQE60 (Qiagen Inc., Chatsworth, CA) and pTrcHis2-TOPO (Invitrogen, Carlsbad, CA) were used as expression vectors. Restriction and DNA-modifying enzymes were purchased from New England Biolabs (Ipswich, MA), while TALON metal affinity resin was from Takara Bio (Kusatsu, Japan) and KOD-Plus-Neo DNA polymerase from Toyobo (Tokyo, Japan). Oligonucleotide primers were synthesized by FASMAC (Atsugi, Japan). Gly-Pro-MCA, His-Ala-MCA, and [(2*S*, 3*S*)-3-carboxyoxirane-2-carbonyl]-L-leucine (4-guanidinobutyl) amide hemihydrate (E-64) were from the Peptide Institute (Osaka, Japan). Human GLP-1 and GIP, *N*-acetylmuramic acid, Nα-tosyl-L-lysine chloromethyl ketone (TLCK), aprotinin, sitagliptin phosphate monohydrate, and α-cyano-4-hydroxycinnamic acid were obtained from Sigma-Aldrich (St. Louis, MS). Isoleucine thiazolidide hemi-fumarate (P32/98) was from FOCUS Biomolecules (Plymouth Meeting, PA) and vildagliptin from LKT Laboratories (St. Paul, MN). Low-molecular-weight and full-range rainbow molecular weight markers, and an ECL plus western blotting detection system were from GE Healthcare (Little Chalfont, UK).

Bacterial strains and culture. Periodontopathic bacteria were grown anaerobically at 37˚C (80% N_2, 10% CO_2, 10% H_2). *P. gingivalis* ATCC 33277, KDP136 (33), and NDP200 (15) were cultured in enriched brain-heart infusion broth (Becton Dickinson, Franklin Lakes, NJ) supplemented with 0.1% cysteine, 5 μg/ml of hemin, and 0.5 μg/ml of menadione in the absence and presence of appropriate antibiotics (ampicillin, erythromycin, tetracycline, chloramphenicol) for KDP136 and NDP200, as previously described (15). *Prev. intermedia* ATCC 25611 and *T. forsythia* ATCC 43037, provided by the RIKEN BRC through the National Bio-Resource Project of MEXT, Japan, were cultured in anaerobic bacteria culture media (Eiken Chemical, Tokyo, Japan) supplemented with 1% cysteine and 0.5 μg/ml of menadione.

137 *N*-Acetylmuramic acid (15 μg/ml) was further added to the medium for *T. forsythia*.
138 *Streptococcus mutans* ATCC 25175 was cultured in Todd Hewitt Broth (Becton Dickinson).
139 Bacterial cells in the early stationary phase were harvested by centrifugation at 6,000 x *g* for 10
140 min at 4˚C, washed once with ice-cold phosphate-buffered saline (PBS) at pH 7.4, then
141 re-suspended in PBS. Absorbance of each bacterial cell suspension at 600 nm was adjusted to
142 2.0 or 10.0.
143
144 **Expression and purification of recombinant DPP4**. Genomic DNA from *T. forsythia* and
145 *Prev. intermedia* was prepared as previously reported (34). A DNA fragment encoding *T.*
146 *forsythia* DPP4 (TfDPP4) Val^{18}–Leu^{722} was amplified by PCR using KOD-Plus-Neo DNA
147 polymerase, genomic DNA as a template, and a set of primers
148 (GTTGTCAGCGCTCAGCAGCGGGTGGA and GAGATTTTCCAGTACAAAATTCGTCA),
149 which were designed based on the gene (KEGG entry, BFO_1659) assigned as S9A/B/C family
150 peptidase in *T. forsythia* ATCC 43037. That for *Prev. intermedia* DPP4 (PiDPP4) Cys^{8}–Lys^{731}
151 was amplified with a set of primers
152 (CAAGCCGGATCCTGTGCAGCGTTATTAGCAACGTCTA and
153 ATCTCTGGATCCCTTCAAGTTCTGAACAAACCAATCG, *Bam*H1 sites are underlined)
154 designed according to the sequence (GenBank accession no. AB127116). The PCR fragment of
155 TfDPP4 was cloned into pTrcHis2 TOPO and that of PiDPP4 digested with *Bam*H1 was inserted
156 into the *Bam*H1 site of pQE60. The expression plasmid of PgDPP4 Asp^{23}–Leu^{723} was previously
157 reported (31). All constructs were confirmed by DNA sequencing. *Escherichia coli* XL-1 Blue
158 cells carrying an expression plasmid were cultured in Luria-Bertani broth supplemented with 75
159 μg/ml ampicillin at 37˚C. Recombinant proteins tagged with a histidine hexamer at the

160 C-terminus were induced with 0.2 mM isopropyl-thiogalactopyranoside at 30˚C for 4 h, then
161 purified from the bacterial cell lysate using TALON affinity chromatography, as previously
162 described (14).
163
164 **Peptidase activity**. Peptidase activity was determined as previously described (15).
165 Generally, the reaction was started by addition of a bacterial cell suspension (1 – 5 µl) or
166 recombinant DPP4 (0.5 – 100 ng) in a reaction mixture (200 µl) composed of 50 mM
167 Na-phosphate (pH 7.5), 5 mM EDTA, and 20 µM peptidyl MCA. After 30 min at 37˚C,
168 fluorescence intensity was measured with excitation at 380 nm and emission at 460 nm. An
169 enzyme unit was defined as enzyme activity that catalyzed the conversion of 1 µmol of substrate
170 into the product in 1 min. To determine enzymatic parameters, an aliquot of DPP4 (0.5 ng DPP4
171 for Gly-Pro-MCA; 10 ng PgDPP4 or 100 ng TfDPP4 and PiDPP4 for His-Ala-MCA) was
172 incubated with various concentrations of peptidyl MCA. Values determined from four
173 independent measurements are presented as the average ± SE. Data were analyzed using a
174 nonlinear regression curve fitted to the Michaelis-Menten equation with the GraphPad Prism
175 software program (San Diego, CA).
176
177 **MALDI-TOF MS**. *P. gingivalis* wild type and NDP200 (8 µl of cell suspension, A_{600} = 2.0)
178 organisms were pre-incubated in 80 µl of 50 mM Na-phosphate buffer, pH 7.5, containing 5 mM
179 EDTA, 0.5 mM TLCK, and 30 µM E-64 for 10 min at 0˚C. A reaction was started by addition of
180 20 µl of GLP-1(7-37) or GIP(52-93) (20 µM) at 37˚C, then MS analysis was performed as
181 previously reported (14). GLP-1(7-37) hydrolysis was further examined in the presence of 2%
182 heat-inactivated fetal calf serum (56˚C for 20 min) with *P. gingivalis* (8 µl of cell suspension,

A_{600} = 2.0), *T. forsythia*, *Prev. intermedia*, and *S. mutans* (8 µl of cell suspension, A_{600} = 10.0). The reaction was stopped at the appropriate time point by addition of trifluoroacetic acid (0.1%), then hydrolyzed products were adsorbed to a Millipore ZipTip-C18, washed with 0.1% trifluoroacetic acid, and eluted with 50% acetonitrile containing 5 mg/ml of α-cyano-4-hydroxycinnamic acid. Hydrolysis of 20 µM GLP-1(7-37) or GIP(52-93) was also carried out with aliquots of DPP4 (5 – 100 ng). The molecular masses of the products were determined by mass spectrometry using a Voyager DE-Pro (Applied Biosynstems, Foster City, CA) and a Bulker Ultraflex III (Billerica, MS).

Glucose tolerance test. All animal experiments were approved by the animal ethics committee of Nagasaki University (no. 0911170797). C57BL/6N female mice were purchased from Charles River Lab (Fukuoka, Japan) and maintained in a specific pathogen-free facility under a 12 h-light, 12 h-dark cycle, then subjected to experiments at the age of 10 to 13 weeks. Purified recombinant DPP4 was dialyzed against PBS at 4˚C, then sterilized with a membrane filter (pore size = 0.22 µm). DPP4 activity in each fraction was determined using Gly-Pro-MCA. DPP4 (0.2 –1 U) in a volume of 50 or 100 µl was injected via the tail vein into mice after 15-h fasting. An identical volume of sterilized PBS was injected as control. A glucose tolerance test was performed according to previous reports (35, 36). Briefly, 2 min after injection of DPP4, a glucose solution (3 mg/g body weight) was orally administrated and blood glucose levels were measured at 0, 10, 30, 60, and 120 min using an OneTouch Ultravue device (Jonson & Jonson, New Brunswick, MJ). For measurement of plasma GLP-1(7-37) (active form) and plasma insulin, blood specimens were obtained from the heart at 15 min after glucose administration, and 100 KIU of aprotinin, 2 µM P32/98, and 2 mM EDTA were immediately added to the

specimens. Plasma was collected by centrifugation at 1,200 × *g* at 4˚C and stored at -80˚C until use. Concentrations of plasma GLP-1(7-37) were measured using an ELISA kit from Shibayagi (Shibukawa, Japan), while those of insulin were measured with an Ultrasensitive Mouse/Rat Insulin ELISA kit (Morinaga Institute of Biological Science, Yokohama, Japan), according to the manufacturers' protocols. As these ELISA kits were differently affected by the extent of hemolysis, several plasma samples were excluded from the analyses. Data are presented as the mean ± SE and were analyzed using GraphPad Prism software. Statistical significance was determined for parametric data by an unpaired Student's *t*-test with Welch's correction. A *P* value of < 0.05 was considered to indicate statistical significance.

SDS-PAGE and immunoblotting analysis. Rabbit anti-PgDPP4 antiserum was prepared using purified PgDPP4 according to a previously reported method (14). For immunoblotting, proteins from bacterial whole cell lysates or recombinant DPP4 was separated by SDS-PAGE using 10% polyacrylamide gels, then transferred onto polyvinylidene difluoride membranes (Life Sciences, Billerica, MA) and incubated with anti-PgDPP4 antiserum (10^3-10^4-fold dilution). DPP4 was detected with alkaline phosphatase-conjugated anti-rabbit IgG, nitro blue tetrazolium, and 5-bromo-4-chloro-3-indolyl phosphate. An ECL plus western blotting detection system was also used for detection of bacterial endogenous DPP4. Low Molecular Weight Calibration and Full-Range Rainbow Molecular Weight Markers (GE Healthcare, Chicago, IL) were used as standards.

RESULTS

DPP4 activity in periodontopathic bacteria. Periodontitis is a chronic inflammation caused by a complex of subgingival microorganisms. Then, firstly, distribution of DPP4 in oral bacteria was studied. Orthologs of the *P. gingivalis dpp4* gene (PGN_1469) encoding 723 amino acids classified as peptidase S9 were searched in the KEGG Orthology database (37), and 546 bacterial genes with higher than 30% identity were found. Among them, 37 bacterial species were oral bacteria (Table 1), which are members out of 688 taxa nominated in the Human Oral Microbiome Database (38). Twenty-one bacteria are anaerobic, *i.e.*, the genera *Bacteroides*, *Porphyromonas*, *Prevotella*, and *Tannerella*, which are mainly present in subgingival plaque. The genes from two major periodontopathic bacteria, *T. forsythia* BFO_1659 and *Prev. intermedia* AB127116, demonstrated amino acid identities of 61.1% and 43.1%, respectively. In contrast, *dpp4* orthologs were absent in the other periodontopathic bacteria, *i.e.*, *Trep. denticola* and *F. nucleatum*, while *A. actinomycetemcomitans* had a truncated gene D11S_0680 encoding 320 amino acids that is annotated as peptidase S15. Thus, the present study focused on DPP4 of *P. gingivalis*, *T. forsythia*, and *Prev. intermedia*.

Alignment of their amino acid sequences shows the catalytic triad (Ser^{593}, Asp^{668}, His^{700} in PgDPP4 numbering) of the serine protease and an N-terminal conserved sequence motif with two adjacent Glu residues (Glu^{195}, Glu^{196}), which are essential for dipeptidyl peptidase activity in hDPP4 (39) (Fig. 1). The deduced M_r of the full-length forms were 81,939 for PgDPP4, 81,875 for *T. forsythia* DPP4 (TfDPP4), and 82,457 for *Prev. intermedia* DPP4 (PiDPP4).

Gly-Pro-MCA hydrolyzing activity was observed with the three periodontopathic bacterial cells, with the highest activity per cell seen for *P. gingivalis*, followed by *T. forsythia* and *Prev. intermedia* (Fig. 2). These activities were markedly abrogated in the presence of the hDPP4

252 inhibitor P32/98, suggesting that Gly-Pro-MCA-hydrolyzing activity is mediated solely by
253 DPP4. In fact, the *dpp4*-disrupted *P. gingivalis* strain NDP200 completely lost its activity, and *S.*
254 *mutans*, a pathogen of dental caries, which does not possess the *dpp4* gene showed no
255 hydrolyzing activity either. On the contrary, the activity was increased in strain KDP136
256 (Δ*kgp-rgpA-rgpB*) as reported previously (15).

257 Three recombinant DPP4 were expressed and purified to homogeneity (≥ 95%) judged by
258 SDS-PAGE (Fig. 2). Recombinant PgDPP4 migrated at 72 kDa, and TfDPP4 and PiDPP4 at 76
259 kDa. Immunoblotting using antiserum against recombinant PgDPP4 demonstrated a gradual
260 decrease in band intensities from PgDPP4, TfDPP4 to PiDPP reflecting their sequence identities.
261 In the bacterial cells, 73-kDa PgDPP4 was accompanied by a partially degraded 66-kDa species.
262 Endogenous TfDPP4 was convincingly detected at 71 kDa and PiDPP4 was detected at around
263 71 kDa by ECL detection. Increased expression of PgDPP4 in KDP136 was confirmed by
264 immunoblotting, and no band was observed in NDP200 and *S. mutans*. These results confirmed
265 the expression of DPP4 in the three microorganisms. Furthermore, similar masses between
266 recombinant and native DPP4 suggested that major post-translational modifications did not
267 occur in bacterial entities. Indistinguishable activities between native and recombinant PgDPP4s
268 were reported previously (18, 32). In addition, molecular masses of recombinant and native
269 forms of DPP4 were estimated approx. 9% smaller than those of the deduced sequences. This
270 difference was not fully explained by the deletion of their signal sequences, however, it seems
271 intrinsic properties of bacterial DPPs migrating on SDS-PAGE, since, the reduction of apparent
272 masses on SDS-PAGE have been commonly observed on *P. gingivalis* DPP5 (15%) (15), DPP7
273 (10%) (31), and DPP11 (8%) (14).
274

Peptidase inhibitor efficiency is summarized in Table 2. One mM of PMSF, a serine protease inhibitor, showed slight inhibition, whereas no inhibition toward bacterial DPP4 was observed with 1 mM of EDTA or 0.1 mM of leupeptin. On the other hand, the activities were completely inhibited by human DPP4 inhibitors including P32/98, sitagliptin, and vildagliptin, though the effect of the latter on PiDPP4 was lower. These results suggest configuration similarities for the active site of serine peptidase between bacterial and human DPP4s.

Enzymatic parameters are shown in Table 3. The K_m and k_{cat}/K_m values of TfDPP4 and PiDPP4 toward Gly-Pro-MCA were comparable to those of PgDPP4. Hydrolyzing activity was also measured with His-Ala-MCA, since the penultimate amino acid residue at the N-terminus is Ala in GLP-1(7-37) (His^7-Ala^8-Glu^9-…-Gly^{37}) and GIP(52-93) (Tyr^{52}-Ala^{53}-Glu^{54}-…-Gln^{93}). Although PgDPP4 cleaved His-Ala-MCA, the k_{cat}/K_m value was one-seventieth that for Gly-Pro-MCA. This activity was also detected in TfDPP4 and PiDPP4 at a substrate concentration of 20 μM, though the k_{cat}/K_m values were too low to be evaluated.

N-terminal truncation and inactivation of incretins by periodontopathic bacterial DPP4. The potential of bacterial DPP4 to cleave human incretins was examined by MALDI-TOF MS analysis. Following the incubation of GLP-1 (His^7-Gly^{37}, HAEGTFTSDVSSYLEGQAAKEFIAWLVKGRG, M_r 3355.7) with *P. gingivalis* wild-type, three major products with M_r of 2098.2 (His^7-Lys^{26}), 1890.0 (Glu^9-Lys^{26}), and 1005.2 (Glu^{27}-Lys^{34}), which were possibly produced by Kgp and DPP4, were demonstrated (Fig. 3). In addition, a peak with 1970.0 corresponding to His^7-Ala^{25} seemed to be produced by C-terminal one-amino acid truncation from His^7-Lys^{26} by an unidentified carboxypeptidase. However, in the presence of 2% heat-inactivated fetal calf serum (FCS), nearly all degradation products disappeared, but

298 instead, a peak of 3147.5 (Glu^{9}-Gly^{37}) was detected, indicating clipping a dipeptide His^{7}-Ala^{8}
299 from the N-terminus of GLP-1. These results suggested the potential of PgDPP4 for incretin
300 degradation in the blood, and also the presence of serum inhibitors for Kgp. In fact, previous
301 studies demonstrated that antithrombin III (40) and α2-microglobulin (41) inhibit gingipain
302 activities. Similarly, release of the N-terminal His^{7}-Ala^{8} was demonstrated with *T. forsythia* and
303 *Prev. intermedia* cells, whereas hydrolyzing of GLP-1 was not observed with *S. mutans*.
304 To study incretin degradation under the defined conditions, we searched gingipain
305 inhibitors and found that Rgp and Kgp activities toward
306 *t*-butyloxycarbonyl-L-Phe-Ser-Arg-MCA and benzyloxycarbonyl-L-His-Glu-Lys-MCA,
307 respectively, were abrogated in the presence of 0.5 mM TLCK and 30 μM E-64 (data not shown).
308 Then, MS analysis was performed in the presence of TLCK and E-64. Under these conditions, *P.*
309 *gingivalis* wild type cells evidently produced an N-terminal dipeptide-shortened peak,
310 Glu^{9}-Gly^{37} (Fig. 4). Its production was also observed with KDP136 cells without inhibitors in a
311 time-dependent manner, while the peak was scarcely detected with NDP200. Taken together,
312 these results clearly show that PgDPP4 degrades active GLP-1 to the inactive form. The rate of
313 limited hydrolysis of GLP-1(7-37) was approximately 1×10^{-12} U/cell, which was
314 semi-quantitatively calculated with KDP136.
315 Hydrolysis at the Ala^{8}-Glu^{9} peptide bond of GLP-1(7-37) as well as at the Ala^{53}-Glu^{54} bond
316 of GIP(52-93) (Y^{52}AEGTFISDYSIAMDKIHQQDFVNWLLAQKGKKNDWKHNITQ^{93}, M_r
317 4983.6) was observed with recombinant PgDPP4 (Fig. 5). The degradation rates of GLP-1 and
318 GIP by PgDPP4 were 2.0 and 2.4×10^{-3} U/μg protein, respectively. Similarly, N-terminal
319 dipeptide truncations of GLP-1(7-37) and GIP(52-93) were demonstrated with TfDPP4 and
320 PiDPP4.

Modulation of blood glucose, plasma active GLP-1, and insulin levels in mice. To evaluate the degradation of incretins by periodontopathic bacterial DPP4 *in vivo*, a glucose tolerance test was performed in mice. Sterilized PgDPP4 or PBS was injected via the tail vein in 10- to 13-week-old C57BL/6N mice after fasting. Blood glucose levels were monitored for 120 min following oral administration of glucose. As shown in Fig. 6, hyperglycemia was observed in the control mice from 10 to 60 min, after which the blood glucose concentration gradually returned to a normal level within 120 min. Under these conditions, PgDPP4 injection (0.3 U/mouse) prior to glucose administration markedly enhanced the level of hyperglycemia observed from 10 to 60 min. Similarly, enhancement of blood glucose level was also demonstrated with PiDPP4 and TfDPP4 in a concentration-dependent manner. These observations strongly suggest the possible involvement of periodontopathic bacterial DPP4 in modulation of postprandial hyperglycemia.

We found that recombinant TfDPP4 (pI = 6.1) was soluble and adequately recovered from dialysis against PBS at pH 7.4 at 4˚C, whereas large portions of PgDPP4 (pI = 7.4) and PiDPP4 (pI = 7.9) were precipitated during dialysis. Accordingly, subsequent animal experiments were performed with TfDPP4 due to its yield. The blood glucose concentration at 15 min after glucose administration was substantially higher in the group that received TfDPP4 (1 U/mouse) as compared to the control group given PBS ($P < 0.001$) (Fig. 6). Concomitantly, significant decreases in the plasma concentrations of both active GLP-1(7-37) and insulin were demonstrated ($P < 0.05$). These results clearly showed that periodontopathic bacterial DPP4 can function *in vivo* as a modulator of blood glucose level.

DISCUSSION

The present study is the first to demonstrate expression of DPP4 in *T. forsythia* and *Prev. intermedia*, which exhibited enzymatic properties similar to those of *P. gingivalis*. Taken together with the present and previous observations of PgDPP4, including the kinetic parameters of enzymatic reactions, optimal pH, inhibitor profiles, and substrate preference for Pro and less for Ala (15, 18, 31), we concluded that the enzymatic properties of bacterial DPP4 substantially resemble those of the human entity (28, 42). In fact, the present findings revealed release of the N-terminal dipeptide from the incretin peptides GLP-1 and GIP by bacterial DPP4. In addition, decreased concentrations of plasma active GLP-1 and plasma insulin were demonstrated following administration of bacterial DPP4, which concomitantly occurred with increased hyperglycemia in the mouse model. Therefore, when these microorganisms enter the bloodstream via daily activities (recurrent bacteremia), bacterial DPP4 may decrease the concentrations of incretins in the host.

Periodontitis is a polymicrobial inflammatory disease caused by subgingival complex microbiota, among which the three bacterial species *P. gingivalis*, *T. forsythia*, and *Trep. denticola* are thought to be primarily responsible (3). When we designed the present investigation, the *dpp4* orthologs were found in *T. forsythia* and *Prev. intermedia* in a search of the KEGG Orthology and HOMD databases. These three orthologs are classified as peptidase subfamily S9, the same as vertebrate DPP4. On the other hand, the truncated one in *A. actinomycetemcomitans* (D11S_0680) is listed as an S15 family member and termed Xaa-Pro DPP in the MEROPS peptidase database (43). Although we found scant Gly-Pro-MCA

hydrolyzing activity in *A. actinomycetemcomitans* ATCC 33384, as well as in *Trep. denticola* ATCC 33520 and *Fusobacterium nucleatum* subsp. nucleatum ATCC 25586 under our experimental conditions (Y. S., Y. O-N., T.K. N., unpublished observation), it is interesting to determine whether *A. actinomycetemcomitans* possesses incretin degradation activity.

Bacterial DPP4 activity was cell associated, while no activity was detected in the culture supernatants of the three microorganisms. Our previous studies of DPP5 (15), DPP11 (14), and AOP (16) indicated that these exopeptidases are localized as soluble forms in the periplasmic space. Hence, PgDPP4 seems to be also located in the periplasmic space. Periplasmic/cytosol localization of PgDPP4 was postulated in a proteome analysis (44). Furthermore, periplasmic localization of DPP4 is supported by lack of a trans-membrane region for the inner membrane and the conserved C-terminal domain, which is essential for outer membrane localization (45).

Accordingly, peptide substrates seem to pass through the outer membrane via either nonspecific porin or specific channels (46). Since GLP-1 (M_r 3355.7) and GIP (M_r 4983.6) are likely metabolized in the periplasmic space, molecules with a mass smaller than 5,000 permeate through the outer membrane. Similarly, this and previous examinations have demonstrated interactions among synthetic oligopeptidyl MCA substrates (M_r 320 ~ 764), including hydrophobic, anionic, and cationic amino acid residues at the P1 and P2 positions, and *P. gingivalis* periplasm-localized exopeptidases (14–16).

The present results showed the *in vivo* activity of bacterial DPP4. The capability of bacterial enzymes was also supported by their high turnover rate against the synthetic substrate Gly-Pro-MCA. We think that the k_{cat}/K_m value for Gly-Pro-MCA of PgDPP4 (1,300 sec^{-1} μM^{-1}) is adequate for efficient function *in vivo* in comparison with that of other enzymes (0.1 – 1000 sec^{-1} μM^{-1} at 25˚C) (47). Although the activity for His-Ala-MCA was quite low compared with

390 Gly-Pro-MCA, hydrolyses at the Ala^{8}-Glu^{9} bond of GLP-1(7-37) and Ala^{53}-Glu^{54} bond of
391 GIP(52-93) were clearly demonstrated with both *P. gingivalis* cells and recombinant bacterial
392 DPP4s in MS analysis. More effective hydrolysis of oligopeptides with Ala^{2} than dipeptide
393 *p*-nitroanilide substrates has been already reported in regard to hDPP4 by Bongers *et al.* (48),
394 who suggested that conformation and peptide length may greatly affect the cleavage efficiency
395 of DPP4.

396 Oral microorganisms' bacteremia is initiated by activities of daily living, such as chewing
397 and tooth brushing, as well as periodontal treatments. Notably, periodontitis lesions allow entry
398 into the bloodstream of oral bacteria that have colonized the gingival sulcus. When these
399 microorganisms enter the circulation and escape from innate immunity, they may be
400 prophlogistic for systemic diseases. In fact, we previously reported a case of infective
401 endocarditis caused by *Granulicatella elegans* derived from supragingival plaque in a patient
402 with poor oral hygiene who suffered from severe periodontitis (49). Furthermore, the incidence
403 of bacteremia in adults has been reported to increase up to 75% with elevated periodontitis
404 severity, while *P. gingivalis* and *Prev. intermedia* have been reported to be isolated from the
405 blood of more than one-third of periodontitis patients after toothbrushing (50, 51). Therefore, it
406 is likely that dietary bacteremia caused by periodontopathic bacteria with DPP4 increases the
407 long-term risk of glucose tolerance and aggravates diabetes. In accordance with this speculation,
408 a recently published 5-year cohort study conducted in Japan of 13,070 subjects reported that
409 dental plaque accumulation is an independent risk factor for developing diabetes mellitus in
410 males and dyslipidemia in females (52).

411 The present results is developing great interest for investigations to determine whether oral
412 bacteria directly modulate the regulation systems of human metabolism via their peptidases

targeting bioactive peptides, including chemokines, neuropeptides, and peptide hormones. Furthermore, since hDPP4 is known as a multifunctional peptidase that functions not only with hydrolase, but is also involved in T-cell activation, lymphocyte-epithelial cell adhesion, and the pericellular proteolysis of the extracellular matrix (53–56), bacterial DPP4 might have additional roles in periodontopathic patients. In conclusion, we propose a novel molecular mechanism of periodontitis-diabetes interaction, in which periodontopathic bacterial DPP4 adversely impacts glycemic control.

ACKNOWLEDGMENTS

This study was supported by KAKENHI grants from JSPS (16K11481 to Y.O.-N., 15K11047 to T.K.N., 15K11020 to S.K.). The authors declare no competing financial interests.

Author contributions for this study were as follows: conceptualization (Y.O-N., T.K.N.), methodology (Y.O-N., T.K.N., T.T.B., S.K., T.Y.), formal analysis (Y.O-N., T.K.N.), investigation (Y.O-N., T.K.N., M.N., Y.S., T.T.B., T.K., T.O.), writing of original draft (Y.O-N.), review and editing (Y.O-N., T.K.N.), and funding acquisition (Y.O-N., T.K.N., S.K.).

REFERENCES

1. **Eke PI, Dye BA, Wei L, Thornton-Evans GO, Genco RJ.** 2012. Prevalence of periodontitis in adults in the United States: 2009 and 2010. J Dent Res **91:**914–920.
2. **Seida K, Saito A, Yamada S, Ishihara K, Naito Y, Okuda K.** 1992. A sensitive enzymatic method (SK-013) for detection of *Treponema denticola*, *Porphyromonas gingivalis* and *Bacteroides forsythus* in subgingival plaque samples. J Periodontal Res **27:**86–91.
3. **Socransky SS, Haffajee AD, Cugini MA, Smith C, Kent RL Jr.** 1998. Microbial complexes in subgingival plaque. J Clin Periodontol **25:**134–144.
4. **Holt SC, Ebersole JL.** 2005. *Porphyromonas gingivalis*, *Treponema denticola*, and *Tannerella forsythia*: the "red complex", a prototype polybacterial pathogenic consortium in periodontitis. Periodontol 2000 **38:**72–122.
5. **Armitage GC.** 1999. Development of a classification system for periodontal diseases and conditions. Ann Periodontol **4:**1–6.
6. **Grossi SG, Genco RJ.** 1998. Periodontal disease and diabetes mellitus: a two-way relationship. Ann Periodontol **3:**51–61.
7. **Taylor GW.** 2001. Bidirectional interrelationships between diabetes and periodontal diseases: an epidemiologic perspective. Ann Periodontol **6:**99–112.
8. **Lalla E, Papapanou PN.** 2011. Diabetes mellitus and periodontitis: a tale of two common interrelated diseases. Nat Rev Endocrinol **7:**738–748.
9. **Preshaw PM, Alba AL, Herrera D, Jepsen S, Konstantinidis A, Makrilakis K, Taylor R.** 2012. Periodontitis and diabetes: a two-way relationship. Diabetologia **55:** 21–31.
10. **Teeuw WJ, Gerdes VE, Loos BG.** 2010. Effect of periodontal treatment on glycemic control of diabetic patients: a systematic review and meta-analysis. Diabetes Care **33:**421–427.
11. **Borgnakke WS, Ylöstalo PV, Taylor GW, Genco RJ.** 2013. Effect of periodontal disease on diabetes: systematic review of epidemiologic observational evidence. J Periodontol **84 (4 Suppl):**S135–152.
12. **Takano M, Nishihara R, Sugano N, Matsumoto K, Yamada Y, Takane M, Fujisaki Y, Ito K.** 2010. The effect of systemic anti-tumor necrosis factor-α treatment on

Porphyromonas gingivalis infection in type 2 diabetic mice. Arch Oral Biol **55:**379–384.

13. **Soorya KV, Suchetha A, Lakshmi P, Sapna N, Apoorva SM, Divya B, Darshan BM.** 2014. The effect of scaling and root planing on glycaemic control, periodontal status and gingival crevicular fluid TNF-α levels in an Indian population- To reveal the ambivalent link. J Clin Diagn Res **8:**ZC22–26.
14. **Ohara-Nemoto Y, Shimoyama Y, Kimura S, Kon A, Haraga H, Ono T, Nemoto TK.** 2011. Asp- and Glu-specific novel dipeptidyl peptidase 11 of *Porphyromonas gingivalis* ensures utilization of proteinaceous energy sources. J Biol Chem **286:**38115–38127.
15. **Ohara-Nemoto Y, Rouf SM, Naito M, Yanase A, Tetsuo F, Ono T, Kobayakawa T, Shimoyama Y, Kimura S, Nakayama K, Saiki K, Konishi K, Nemoto TK.** 2014. Identification and characterization of prokaryotic dipeptidyl-peptidase 5 from *Porphyromonas gingivalis*. J Biol Chem **289**:5436–5448.
16. **Nemoto TK, Ohara-Nemoto Y, Bezerra GA, Shimoyama Y, Kimura S.** 2016. A *Porphyromonas gingivalis* periplasmic novel exopeptidase, acylpeptidyl oligopeptidase, releases N-acylated di- and tripeptides from oligopeptides. J Biol Chem **291:**5913–5925.
17. **Kiyama M, Hayakawa M, Shiroza T, Nakamura S, Takeuchi A, Masamoto Y, Abiko Y.** 1998. Sequence analysis of the *Porphyromonas gingivalis* dipeptidyl peptidase IV gene. Biochim Biophys Acta **1396:**39–46.
18. **Banbula A, Bugno M, Goldstein J, Yen J, Nelson D, Travis J, Potempa J.** 2000. Emerging family of proline-specific peptidases of *Porphyromonas gingivalis*: purification and characterization of serine dipeptidyl peptidase, a structural and functional homologue of mammalian prolyl dipeptidyl peptidase IV. Infect Immun **68**:1176–1182.
19. **Banbula A, Yen J, Oleksy A, Mak P, Bugno M, Travis J, Potempa J.** 2001. *Porphyromonas gingivalis* DPP-7 represents a novel type of dipeptidylpeptidase. J Biol Chem **276:**6299–6305.
20. **Rouf SM, Ohara-Nemoto Y, Ono T, Shimoyama Y, Kimura S, Nemoto TK.** 2013. Phenylalanine 664 of dipeptidyl peptidase (DPP) 7 and Phenylalanine 671 of DPP11 mediate preference for P2-position hydrophobic residues of a substrate. FEBS Open Bio **3:**177-183.
21. **Nemoto TK, Ohara-Nemoto Y.** 2015. Exopeptidases and gingipains in *Porphyromonas*

gingivalis as prerequisites for its amino acid metabolism. Jpn Dent Sci Rev **52:**22–29.

22. **Tang-Larsen J, Claesson R, Edlund MB, Carlsson J.** 1995. Competition for peptides and amino acids among periodontal bacteria. J Periodontal Res **30:**390–395.

23. **Takahashi N, Sato T, Yamada T.** 2000. Metabolic pathways for cytotoxic end product formation from glutamate- and aspartate-containing peptides by *Porphyromonas gingivalis*. J Bacteriol **182:**4704–4710.

24. **Coykendall AL, Kaczmarek FS, Slots J.** 1980. Genetic heterogeneity in *Bacteroides asaccharolyticus* (Holdeman and Moore 1970) Finegold and Barnes 1977 (Approved Lists, 1980) and proposal of *Bacteroides gingivalis* sp. nov. and *Bacteroides macacae* (Slots and Genco) comb. nov. Int J Syst Bacteriol **30:**559–564.

25. **Takahashi N, Sato T.** 2002. Dipeptide utilization by the periodontal pathogens *Porphyromonas gingivalis*, *Prevotella intermedia*, *Prevotella nigrescens* and *Fusobacterium nucleatum*. Oral Microbiol Immunol **17:**50-54.

26. **Ahrén B, Holst JJ, Mårtensson H, Balkan B.** 2000. Improved glucose tolerance and insulin secretion by inhibition of dipeptidyl peptidase IV in mice. Eur J Pharmacol **404:**239-245.

27. **Weber AE.** 2004. Dipeptidyl peptidase IV inhibitors for the treatment of diabetes. J Med Chem **47:**4135–4141.

28. **Mentlein R, Gallwitz B, Schmidt WE.** 1993. Dipeptidyl-peptidase IV hydrolyses gastric inhibitory polypeptide, glucagon-like peptide-1(7-36)amide, peptide histidine methionine and is responsible for their degradation in human serum. Eur J Biochem **214:**829–835.

29. **Seino Y, Yabe D.** 2013. Glucose-dependent insulinotropic polypeptide and glucagon-like peptide-1: Incretin actions beyond the pancreas. J Diabetes Invest **4:**108–130.

30. **Deacon CF, Pridal L, Klarskov L, Olesen M, Holst JJ.** 1996. Glucagon-like peptide 1 undergoes differential tissue-specific metabolism in the anesthetized pig. Am J Physiol **271:**E458–464.

31. **Rouf SMA, Ohara-Nemoto Y, Hoshino T, Fujiwara T, Ono T, Nemoto TK.** 2013. Discrimination based on Gly and Arg/Ser at position 673 between dipeptidyl-peptidase (DPP) 7 and DPP11, widely distributed DPPs in pathogenic and environmental gram-negative bacteria. Biochimie **95:**824–832.

519 32. **Kumagai Y, Konishi K, Gomi T, Yagishita H, Yajima A, Yoshikawa M.** 2000. Enzymatic
520 properties of dipeptidyl aminopeptidase IV produced by the periodontal pathogen
521 *Porphyromonas gingivalis* and its participation in virulence. Infect Immun **68:**716–724.
522 33. **Shi Y, Ratnayake DB, Okamoto K, Abe N, Yamamoto K, Nakayama K.** 1999. Genetic
523 analyses of proteolysis, hemoglobin binding, and hemagglutination of *Porphyromonas*
524 *gingivalis*: construction of mutants with a combination of *rgpA*, *rgpB*, *kgp*, and *hagA*. J Biol
525 Chem **274**:17955–17960.
526 34. **Ikeda Y, Ohara-Nemoto Y, Kimura S, Ishibashi K, Kikuchi K.** 2004. PCR-based
527 identification of *Staphylococcus epidermidis* targeting *gseA* encoding the
528 glutamic-acid-specific protease. Can J Microbiol **50:**493–498.
529 35. **Uysal KT, Wiesbrock SM, Marino MW, Hotamisligil GS.** 1997. Protection from
530 obesity-induced insulin resistance in mice lacking TNF-α function. Nature **389:**610–614.
531 36. **Mizokami A, Yasutake Y, Gao J, Matsuda M, Takahashi I, Takeuchi H, Hirata M.** 2013.
532 Osteocalcin induces release of glucagon-like peptide-1 and thereby stimulates insulin
533 secretion in mice. PLoS One **8:**e57375. doi: 10.1371/journal.pone.0057375.
534 37. **Kanehisa M, Furumichi M, Tanabe M, Sato Y, Morishima K.** 2017. KEGG: new
535 perspectives on genomes, pathways, diseases and drugs. Nucleic Acids Res **45(D1):**D353–
536 D361. doi: 10.1093/nar/gkw1092. Epub 2016 Nov 28.
537 38. **Chen T, Yu WH, Izard J, Baranova OV, Lakshmanan A, Dewhirst FE.** 2010. The
538 human oral microbiome database: a web accessible resource for investigating oral microbe
539 taxonomic and genomic information. Database(Oxfird) doi: 10.1093/database/baq013.
540 39. **Abbott CA, McCaughan GW, Gorrell MD.** 1999. Two highly conserved glutamic acid
541 residues in the predicted β propeller domain of dipeptidyl peptidase IV are required for its
542 enzyme activity. FEBS Lett **458:**278–284.
543 40. **Curtis MA, Slaney JM, Carman RJ, Remberton PA.** 1993. Interaction of a trypsin-like
544 enzyme of *Porphyromonas gingivalis* W83 with antithrombin III. FEMS Microbiol Lett
545 **108:**169–174.
546 41. **Grøn H, Pike R, Potempa J, Travis J, Thogerson IB, Enghild JJ, Pizzo SV.** 1997. The
547 potential role of α2-macroglobulin in the control of cysteine proteinases (gingipains) from
548 *Porphyromonas gingivalis*. J Periodontal Res **32:**61–68.

42. **Leiting B, Pryor KD, Wu JK, Marsilio F, Patel RA, Craik CS, Ellman JA, Cummings RT, Thornberry NA.** 2003. Catalytic properties and inhibition of proline-specific dipeptidyl peptidases II, IV and VII. Biochem J **371(Pt 2):**525–532.
43. **Rawlings ND, Barrett AJ, Finn RD**. 2016. Twenty years of the MEROPS database of proteolytic enzymes, their substrates and inhibitors. Nucleic Acids Res **44**:D343–D350.
44. **Veith PD, Chen YY, Gorasia DG, Chen D, Glew MD, O'Brien-Simpson NM, Cecil JD, Holden JA, Reynolds EC.** 2014. *Porphyromonas gingivalis* outer membrane vesicles exclusively contain outer membrane and periplasmic proteins and carry a cargo enriched with virulence factors. J Proteome Res **13:**2420–2432.
45. **Slakeski N, Seers CA, Ng K, Moore C, Cleal SM, Veith PD, Lo AW, Reynolds EC.** 2011. C-terminal domain residues important for secretion and attachment of RgpB in *Porphyromonas gingivalis*. J Bacteriol **193:**132–142.
46. **Nikaido H.** 2003. Molecular basis of bacterial outer membrane permeability revisited. Microbiol Mol Biol Rev **67:**593–656.
47. **Wolfenden R, Snider MJ.** 2001. The depth of chemical time and the power of enzymes as catalysts. Acc Chem Res **34:**938–945.
48. **Bongers J, Lambros T, Ahmad M, Heimer EP.** 1992. Kinetics of dipeptidyl peptidase IV proteolysis of growth hormone-releasing factor and analogs. Biochim Biophys Acta **1122:**147–153.
49. **Ohara-Nemoto Y, Kishi K, Satho M, Tajika S, Sasaki M, Namioka A, Kimura S.** 2005. Infective endocarditis caused by *Granulicatella elegans* originating in the oral cavity. J Clin Microbiol **43:**1405–1407.
50. **Silver JG, Martin AW, McBride BC.** 1977. Experimental transient bacteraemias in human subjects with varying degrees of plaque accumulation and gingival inflammation. J Clin Periodontol **4:**92–99.
51. **Forner L, Larsen T, Kilian M, Holmstrup P.** 2006. Incidence of bacteremia after chewing, tooth brushing and scaling in individuals with periodontal inflammation. J Clin Periodontol **33:**401–407.
52. **Kuwabara M, Motoki Y, Sato H, Fujii M, Ichiura K, Kuwabara K, Nakamura Y.** 2016. Low frequency of toothbrushing practices is an independent risk factor for diabetes mellitus

in male and dyslipidemia in female: A large-scale, 5-year cohort study in Japan. J Cardiol S0914-5087(16)30264–30267. http://dx.doi.org/10.1016/j.jjcc.2016.10.008.

53. **Park JE, Lenter MC, Zimmermann RN, Garin-Chesa P, Old LJ, Rettig WJ.** 1999. Fibroblast activation protein, a dual specificity serine protease expressed in reactive human tumor stromal fibroblasts. J Biol Chem **274:**36505–36512.

54. **Durinx C, Lambeir AM, Bosmans E, Falmagne JB, Berghmans R, Haemers A, Scharpé S, De Meester I.** 2000. Molecular characterization of dipeptidyl peptidase activity in serum: soluble CD26/dipeptidyl peptidase IV is responsible for the release of X-Pro dipeptides. Eur J Biochem **267:**5608–5613.

55. **Ikushima H, Munakata Y, Ishii T, Iwata S, Terashima M, Tanaka H, Schlossman SF, Morimoto C.** 2000. Internalization of CD26 by mannose 6-phosphate/insulin-like growth factor II receptor contributes to T cell activation. Proc Natl Acad Sci U.S.A. **97:**8439–8444.

56. **Gines S, Marino M, Mallol J, Canela EI, Morimoto C, Callebaut C, Hovanessian A, Casado V, Lluis C, Franco R.** 2002. Regulation of epithelial and lymphocyte cell adhesion by adenosine deaminase-CD26 interaction. Biochem J **361:**203–209.

595 **TABLE 1** Distribution of *dpp4* orthologs in the Human Oral Microbiome Database (HOMD)
596

No.	Class	Genus	Species in HOMD	Gene	Definition	%
1	Alp	*Porphyrobacter*	*Porphyrobacter neustonensis*	A9D12_13280	peptidase S9	30.2
2	Alp	*Sphingomonas*	*Sphingomonas melonis*	BJP26_07260	peptidase S9	30.0
3	Alp	*Caulobacter*	*Caulobacter crescentus* CB15	CC_2154	peptidase S9	30.0
			Caulobacter crescentus NA1000	CCNA_02237	DPP4	32.8
4	Alp	*Caulobacter*	*Caulobacter henricii*	AQ619_12080	Prolyl tripeptidyl peptidase	32.6
5	Alp	*Caulobacter*	*Caulobacter segnis*	Cseg_1267	DPP4	32.2
6	Alp	*Caulobacter*	*Caulobacter* sp. K31	Caul_3418	peptidase S9	31.1
7	Alp	*Sphingomonas*	*Sphingomonas* sp. MM-1	G432_00900	peptidase S9	30.9
8	Alp	*Sphingomonas*	*Sphingomonas taxi*	MC45_04785	peptidase S9	32.7
9	Bct	*Bacteroides*	*Bacteroidales bacterium* CF	BRDCF_p911	peptidase S9	30.9
10	Bct	*Bacteroides*	*Bacteroides cellulosilyticus*	BcellWH2_02945	DPP4	50.1
11	Bct	*Bacteroides*	*Bacteroides dorei* HS1_L_1_B_010	EL88_17320	DPP4	47.7
			Bacteroides dorei HS1_L_3_B_079	GV66_06260	Prolyl tripeptidyl peptidase	47.7
12	Bct	*Bacteroides*	*Bacteroides fragilis* 638R	BF638R_0958	DPP4	50.7
			Bacteroides fragilis BOB25	VU15_00050	Prolyl tripeptidyl peptidase	50.9
			Bacteroides fragilis NCTC9343	BF9343_0861	DPP4	50.9
			Bacteroides fragilis YCH46	BF0977	DPP4	50.7
13	Bct	*Bacteroides*	*Bacteroides helcogenes*	Bache_0782	DPP4	49.4
14	Bct	*Bacteroides*	*Bacteroides ovatus*	Bovatus_04481	170 kDa melanoma membrane-bound gelatinase	49.5
15	Bct	*Bacteroides*	*Bacteroides salanitronis*	Bacsa_1666	peptidase S9	49.2
16	Bct	*Bacteroides*	*Bacteroides thetaiotaomicron* 7330	Btheta7330_0504	peptidase S9B DPP4 domain	49.7

			Bacteroides thetaiotaomicron VPI-5482	BT_4193	peptidase S9	50.1
17	Bct	*Bacteroides*	*Bacteroides vulgatus*	BVU_3876	DPP4	47.6
18	Bct	*Bacteroides*	*Bacteroides xylanisolvens*	BXY_33000	DPP4	49.7
19	Bct	*Capnocytophaga*	*Capnocytophaga canimorsus*	Ccan_17390	peptidase S9	42.3
20	Bct	*Capnocytophaga*	*Capnocytophaga haemolytica*	AXF12_00665	peptidase S9B DPP4 domain	42.5
21	Bct	*Capnocytophaga*	*Capnocytophaga ochracea*	Coch_1057	peptidase S9B DPP4 domain	43.6
22	Bct	*Capnocytophaga*	*Capnocytophaga* sp. oral taxon 323	AM608_08390	peptidase S9	43.6
23	Bct	*Pedobacter*	*Pedobacter cryoconitis*	AY601_0660	peptidase S9	30.5
24	Bct	*Pedobacter*	*Pedobacter* sp. PACM 27299	AQ505_24800	peptidase S9	30.5
25	Bct	*Pedobacter*	*Pedobacter steynii*	BFS30_11275	DPP4	31.2
26	Bct	*Porphyromonas*	*Porphyromonas asaccharolytica*	Poras_1656	DPP4	55.6
27	Bct	*Porphyromonas*	*Porphyromonas gingivalis* ATCC 33277	PGN_1469	DPP4	100.0
			Porphyromonas gingivalis TDC60	PGTDC60_1620	DPP4	99.0
			Porphyromonas gingivalis W83	PG_0503	dipeptidyl aminopeptidase	98.6
28	Bct	*Prevotella*	*Prevotella dentalis*	Prede_1703	peptidase S9	42.0
29	Bct	*Prevotella*	*Prevotella denticola*	HMPREF9137_1131	peptidase S9	43.4
30	Bct	*Prevotella*	*Prevotella enoeca*	AS203_03985	peptidase S9	42.3
31	Bct	*Prevotella*	*Prevotella fusca*	ADJ77_09950	peptidase S9	43.3
32	Bct	*Prevotella*	*Prevotella intermedia*	AB127116	peptidase S9	43.1
33	Bct	*Prevotella*	*Prevotella melaninogenica*	HMPREF0659_A557	DPP4	42.8
34	Bct	*Prevotella*	*Prevotella ruminicola*	PRU_0634	peptidase S9	40.7
35	Bct	*Tannerella*	*Tannerella forsythia*	BFO_1659	peptidase S9	61.1
36	Bct	*Tannerella*	*Tannerella* sp. oral taxon HOT-286	BCB71_04900	DPP4	57.1

37	Gam	*Stenotrophomonas*	*Stenotrophomonas maltophilia* D457	SMD_4050	putative exported DPP4	32.8
			Stenotrophomonas maltophilia JV3	BurJV3_3912	peptidase S9B DPP4 domain	33.0
			Stenotrophomonas maltophilia K279a	Smlt4503	peptidase S9	32.8
			Stenotrophomonas maltophilia R551-3	Smal_3865	peptidase S9	32.6

Ninety-four human oral bacterial species listed in the HOMD (38) possess orthologs of the *P. gingivalis dpp4* gene (PGM_1479). Among them, 48 orthologs in 37 species higher than 30% identity are presented. Identical species are shaded. Alp, Alphaproteobacteria; Bct, Bacteroidia; Gam, Gammaproteobacteria.

601

602 **TABLE 2** Inhibition profile of periodontopathic bacterial DPP4[a]

603

Inhibitor	Concn (mM)	Residual activity (%)		
		PgDPP4	TfDPP4	PiDPP4
P32/98	0.25	0.3	0.2	0.5
Sitagliptin	0.25	1.6	1.6	7.5
Vildagliptin	0.25	0	0.3	27.3
PMSF	1	52.3	71.2	40.8
EDTA	1	102.0	103.6	117.0
Leupeptin	0.1	104.3	102.4	94.4

604

605 [a]The specific activities of PgDPP4, TfDPP4, and PiDPP4 against Gly-Pro-MCA were 0.93 ± 0.02,

606 0.67 ± 0.01, and 0.44 ± 0.01 U/mg of protein, respectively.

607 **TABLE 3** Enzymatic parameters of bacterial DPP4

608

Substrate	DPP	k_{cat} (sec^{-1})	K_m (μM)	k_{cat}/K_m (sec^{-1} μM^{-1})
Gly-Pro-MCA	PgDPP4	121,752 ± 2,974	94.9 ± 2.1	1,283.3 ± 22.5
	TfDPP4	104,189 ± 30,036	142.9 ± 48.4	740.0 ± 42.5
	PiDPP4	950,097 ± 17,404	133.8 ± 33.4	720.7 ± 54.6
His-Ala-MCA	PgDPP4	14,322 ± 12,393	> 3.2	< 18.6

609

610 **Figure Legends**

611

612 **FIG 1** Alignment of deduced amino acid sequences of DPP4 from *P. gingivalis* (Pg, PGN_1469),
613 *T. forsythia* (Tf, BFO_1659), and *Prev. intermedia* (Pi, AB127116). Hyphens represent gaps
614 introduced for maximal matching. Common amino acid residues are marked with asterisks and
615 those matched between two DPPs are indicated by dots. Potential sets of three residues (Ser^{593},
616 Asp^{668}, His^{700} in PgDPP4), forming the essential triad of serine peptidases, are noted in red. The
617 conserved N-terminal motif with two adjacent essential Glu residues (Glu^{195}, Glu^{196} in blue) is
618 boxed.

619

620 **FIG 2** Expression of DPP4 in periodontopathic bacteria. (A) Aliquots (5 µl) of bacterial cell
621 suspensions (A_{600} = 2.0) of *P. gingivalis* wild type (Pg), *T. forsythia* (Tf), *Prev. intermedia* (Pi), and
622 *P. gingivalis* KDP136, NDP200, and *S. mutans* (Sm) were incubated with 20 µM Gly-Pro-MCA in
623 the absence (-) and presence (+) of 0.25 mM P32/98. Values are shown as the mean ± S.D. (n=3).
624 (B) Recombinant DPP4 [0.5 µg for CBB and 0.1 µg for immunoblotting (IB)] were separated by
625 SDS-PAGE and visualized. (C) Whole bacterial cell lysates were separated by SDS-PAGE and
626 detected by IB (30 µg of proteins). Lysates of *P. gingivalis* (10 µg), *T. forsythia* (15 µg), *P.*
627 *intermedia* (45 µg), and *S. mutans* (45 µg) were subjected to IB ECL plus detection.

628

629 **FIG 3** Degradation of GLP-1 by *P. gingivalis* cells. (A) MALDI-TOF MS analysis was
630 performed with 20 µM GLP-1(7-37) incubated with 2% heat-inactivated fetal calf serum (FCS) or
631 *P. gingivalis* wild type (Pg) for 30 min at 37˚C. Analysis was also performed with *P. gingivalis*, *T.*
632 *forsythia* (Tf), *Prev. intermedia* (Pi), and *S. mutans* in the presence of 2% FCS. Data are
633 representative of at least three independent experiments. (B) Amino acid sequence of active form
634 of GLP-1 (His^{7}-Gly^{37}) and average molecular masses of its degradation products appearing in

635 panel A and Fig. 4 are summarized. Blue arrowhead indicates a site of cleavage by DPP4, while red
636 and black arrowheads are sites for Kgp and an unidentified carboxypeptidase, respectively (see text
637 for details).
638
639 **FIG 4** MALDI-TOF MS analysis of GLP-1 hydrolysis by *P. gingivalis*. *P. gingivalis* wild type
640 (wt) and NDP200 were pre-incubated with 0.5 mM TLCK and 30 μM E64 at 0˚C for 10 min as
641 described in MATERIALS AND METHODS. (A) GLP-1(7-37) (20 μM). GLP-1(7-37) (20 μM)
642 was incubated with (B) *P. gingivalis* wild type, (C) NDP200, or (D) KDP136 cells. After 30 min at
643 37˚C, resultant products were analyzed using mass spectrometry. Figures (3355.7, 3147.5, 1275.5)
644 correspond to GLP-1(7-37), (9-37), and (27-37), respectively. (E) GLP-1(7-37) was incubated with
645 KDP136 and the reaction was stopped at indicated times. The percent intensity of GLP-1(9-37) was
646 plotted over time. MS data are representative of at least three independent experiments.
647
648 **FIG 5** MALDI-TOF MS analysis of truncation of GLP-1 and GIP by recombinant DPP4. (A)
649 Twenty μM GLP-1(7-37) was incubated with 5 ng PgDPP4 at 37˚C. After 30 min, resultant
650 products were analyzed using mass spectrometry. (B) The amounts of the truncated forms
651 GLP-1(9-37) are plotted over time. GLP-1 was incubated with 20 ng of (C) TfDPP4 and (D)
652 PiDPP4. (E) Twenty μM GIP(52-93) (M_r = 4983.6) was incubated with 5 ng PgDPP4 at 37˚C, and
653 (F) the amounts of GIP(54-93) (M_r = 4749.3) are plotted. GIP was incubated with 20 ng of (G)
654 TfDPP4 and (H) PiDPP4. Data are representative of at least three independent experiments.
655
656 **FIG 6** Modulation of glucose tolerance, and changes in concentrations of plasma insulin and
657 active GLP-1 by periodontopathic bacterial DPP4. PBS (square) or recombinant PgDPP4 (triangle,
658 0.3 U) (n = 4), (B) PiDPP4 (triangle, 0.3 U) (mean, n = 3), or (C) TfDPP4 (circle, 0.2 U; triangle, 1
659 U) (n = 8) was injected *i.v.* into 10- to 13-week-old mice, as described in MATERIALS AND

METHODS. Blood glucose concentrations were measured at various times following oral administration of glucose. Bars indicate the mean ± S.E. Concentrations of (C) blood glucose, (D) plasma active GLP-1(7-37), and (E) plasma insulin at 15 min were measured with injection of TfDPP4 (0.5 U). Bars indicate mean values. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ vs. control (Student's *t* test).

Figure 1

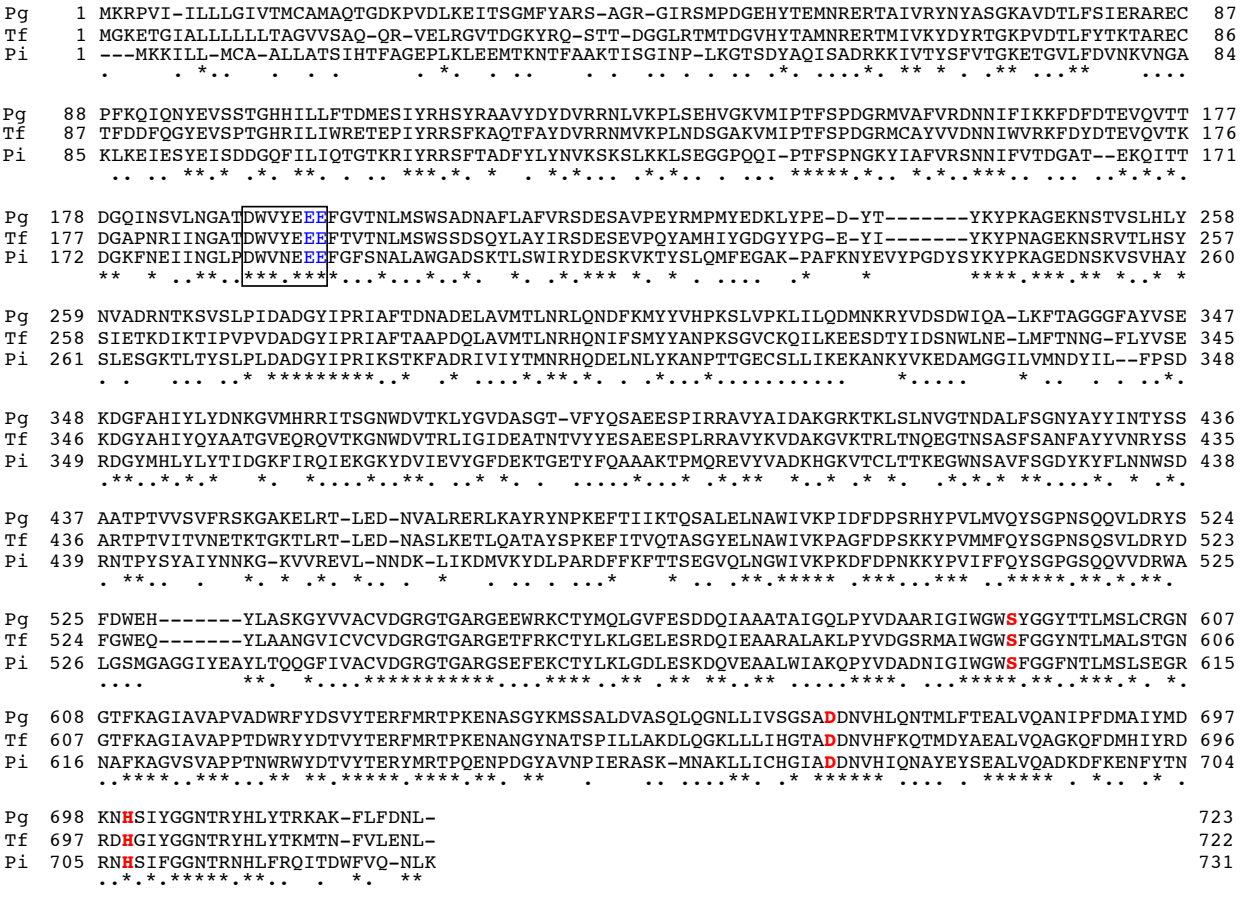

Pg 1 MKRPVI-ILLLGIVTMCAMAQTGDKPVDLKEITSGMFYARS-AGR-GIRSMPDGEHYTEMNRERTAIVRYNYASGKAVDTLFSIERAREC 87
Tf 1 MGKETGIALLLLLLTAGVVSAQ-QR-VELRGVTDGKYRQ-STT-DGGLRTMTDGVHYTAMNRERTMIVKYDYRTGKPVDTLFYTKTAREC 86
Pi 1 ---MKKILL-MCA-ALLATSIHTFAGEPLKLEEMTKNTFAAKTISGINP-LKGTSDYAQISADRKKIVTYSFVTGKETGVLFDVNKVNGA 84
Pg 88 PFKQIQNYEVSSTGHHILLFTDMESIYRHSYRAAVYDYDVRRNLVKPLSEHVGKVMIPTFSPDGRMVAFVRDNNIFIKKFDFDTEVQVTT 177
Tf 87 TFDDFQGYEVSPTGHRILIWRETEPIYRRSFKAQTFAYDVRRNMVKPLNDSGAKVMIPTFSPDGRMCAYVVDNNIWVRKFDYDTEVQVTK 176
Pi 85 KLKEIESYEISDDGQFILIQTGTKRIYRRSFTADFYLYNVKSKSLKKLSEGGPQQI-PTFSPNGKYIAFVRSNNIFVTDGAT--EKQITT 171
Pg 178 DGQINSVLNGATDWVYEEFGVTNLMSWSADNAFLAFVRSDESAVPEYRMPMYEDKLYPE-D-YT-------YKYPKAGEKNSTVSLHLY 258
Tf 177 DGAPNRIINGATDWVYEEFTVTNLMSWSSDSQYLAYIRSDESEVPQYAMHIYGDGYYPG-E-YI-------YKYPNAGEKNSRVTLHSY 257
Pi 172 DGKFNEIINGLPDWVNEEFGFSNALAWGADSKTLSWIRYDESKVKTYSLQMFEGAK-PAFKNYEVYPGDYSYKYPKAGEDNSKVSVHAY 260
Pg 259 NVADRNTKSVSLPIDADGYIPRIAFTDNADELAVMTLNRLQNDFKMYYVHPKSLVPKLILQDMNKRYVDSDWIQA-LKFTAGGGFAYVSE 347
Tf 258 SIETKDIKTIPVPVDADGYIPRIAFTAAPDQLAVMTLNRHQNIFSMYYANPKSGVCKQILKEESDTYIDSNWLNE-LMFTNNG-FLYVSE 345
Pi 261 SLESGKTLTYSLPLDADGYIPRIKSTKFADRIVIYTMNRHQDELNLYKANPTTGECSLLIKEKANKYVKEDAMGGILVMNDYIL--FPSD 348
Pg 348 KDGFAHIYLYDNKGVMHRRITSGNWDVTKLYGVDASGT-VFYQSAEESPIRRAVYAIDAKGRKTKLSLNVGTNDALFSGNYAYYINTYSS 436
Tf 346 KDGYAHIYQYAATGVEQRQVTKGNWDVTRLIGIDEATNTVYYESAEESPLRRAVYKVDAKGVKTRLTNQEGTNSASFSANFAYYVNRYSS 435
Pi 349 RDGYMHLYLYTIDGKFIRQIEKGKYDVIEVYGFDEKTGETYFQAAAKTPMQREVYVADKHGKVTCLTTKEGWNSAVFSGDYKYFLNNWSD 438
Pg 437 AATPTVVSVFRSKGAKELRT-LED-NVALRERLKAYRYNPKEFTIIKTQSALELNAWIVKPIDFDPSRHYPVLMVQYSGPNSQQVLDRYS 524
Tf 436 ARTPTVITVNETKTGKTLRT-LED-NASLKETLQATAYSPKEFITVQTASGYELNAWIVKPAGFDPSKKYPVMMFQYSGPNSQSVLDRYD 523
Pi 439 RNTPYSYAIYNNKG-KVVREVL-NNDK-LIKDMVKYDLPARDFFKFTTSEGVQLNGWIVKPKDFDPNKKYPVIFFQYSGPGSQQVVDRWA 525
Pg 525 FDWEH-------YLASKGYVVACVDGRGTGARGEEWRKCTYMQLGVFESDDQIAAATAIGQLPYVDAARIGIWGWSYGGYTTLMSLCRGN 607
Tf 524 FGWEQ-------YLAANGVICVCVDGRGTGARGETFRKCTYLKLGELESRDQIEAARALAKLPYVDGSRMAIWGWSFGGYNTLMALSTGN 606
Pi 526 LGSMGAGGIYEAYLTQQGFIVACVDGRGTGARGSEFEKCTYLKLGDLESKDQVEAALWIAKQPYVDADNIGIWGWSFGGFNTLMSLSEGR 615
Pg 608 GTFKAGIAVAPVADWRFYDSVYTERFMRTPKENASGYKMSSALDVASQLQGNLLIVSGSADDNVHLQNTMLFTEALVQANIPFDMAIYMD 697
Tf 607 GTFKAGIAVAPPTDWRYYDTVYTERFMRTPKENANGYNATSPILLAKDLQGKLLLIHGTADDNVHFKQTMDYAEALVQAGKQFDMHIYRD 696
Pi 616 NAFKAGVSVAPPTNWRWYDTVYTERYMRTPQENPDGYAVNPIERASK-MNAKLLICHGIADDNVHIQNAYEYSEALVQADKDFKENFYTN 704
Pg 698 KNHSIYGGNTRYHLYTRKAK-FLFDNL- 723
Tf 697 RDHGIYGGNTRYHLYTKMTN-FVLENL- 722
Pi 705 RNHSIFGGNTRNHLFRQITDWFVQ-NLK 731

Figure 2

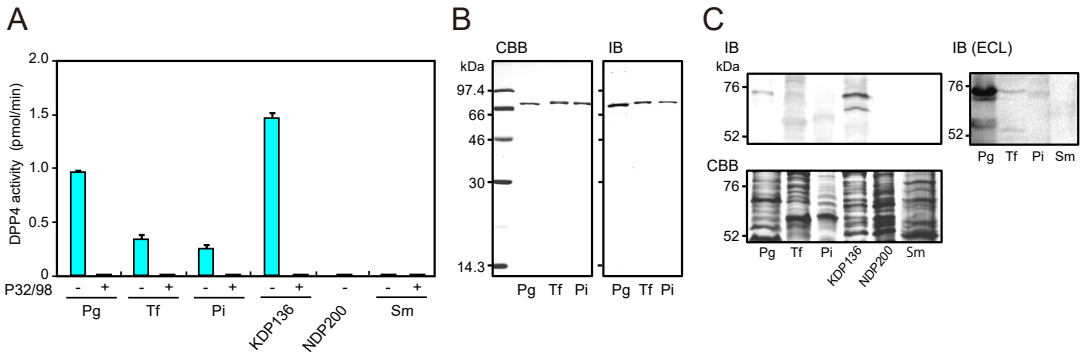
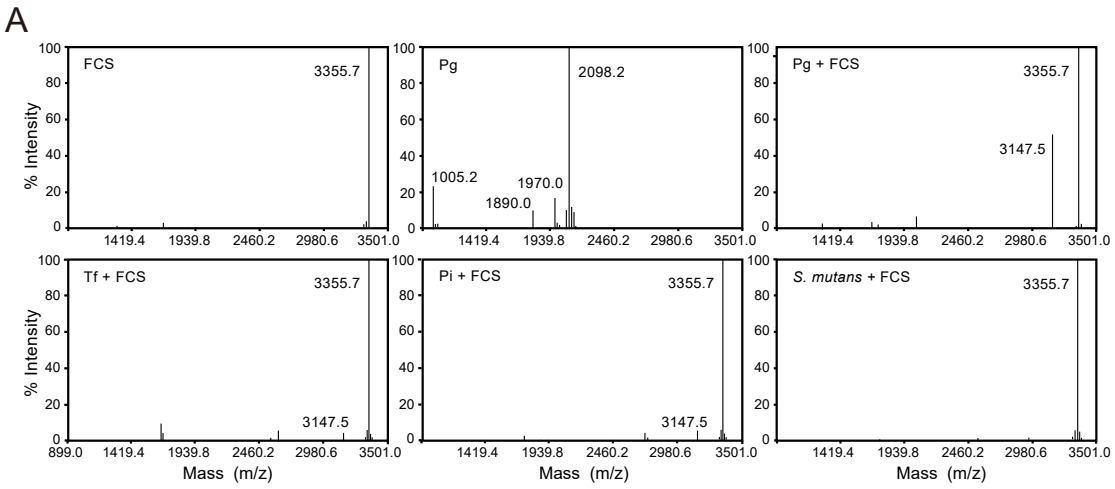

A
DPP4 activity (pmol/min)
2.0
1.5
1.0
0.5
0
P32/98
- + - + - + - + - - + -
Pg
Tf
Pi
KDP136
NDP200
Sm
B
CBB
IB
kDa
97.4
66
46
30
14.3
Pg Tf Pi
Pg Tf Pi
C
IB
IB (ECL)
kDa
76
52
Pg Tf Pi Sm
CBB
76
52
Pg Tf Pi KDP136 NDP200 Sm

Figure 3


A
FCS
3355.7
Pg
2098.2
1005.2
1970.0
1890.0
Pg + FCS
3355.7
3147.5
Tf + FCS
3355.7
3147.5
Pi + FCS
3355.7
3147.5
S. mutans + FCS
3355.7
% Intensity
Mass (m/z)
899.0
1419.4
1939.8
2460.2
2980.6
3501.0

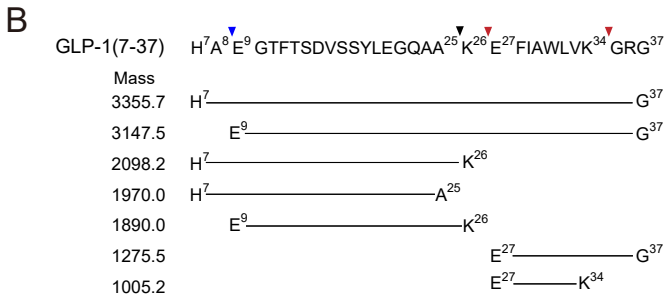

B
GLP-1(7-37) H7A8E9GTFTSDVSSYLEGQAA25K26E27FIAWLVK34GRG37
Mass
3355.7 H7—G37
3147.5 E9—G37
2098.2 H7—K26
1970.0 H7—A25
1890.0 E9—K26
1275.5 E27—G37
1005.2 E27—K34

Figure 4

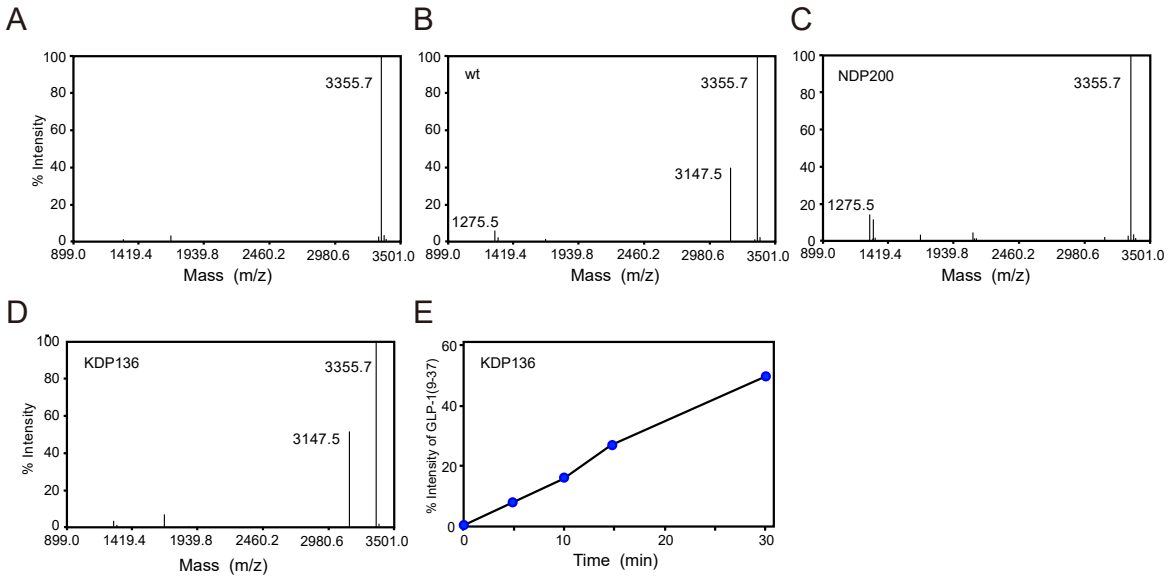

A
B
C
D
E
wt
NDP200
KDP136
KDP136
3355.7
3147.5
1275.5
% Intensity
Mass (m/z)
% Intensity of GLP-1(9-37)
Time (min)
899.0
1419.4
1939.8
2460.2
2980.6
3501.0

Figure 5

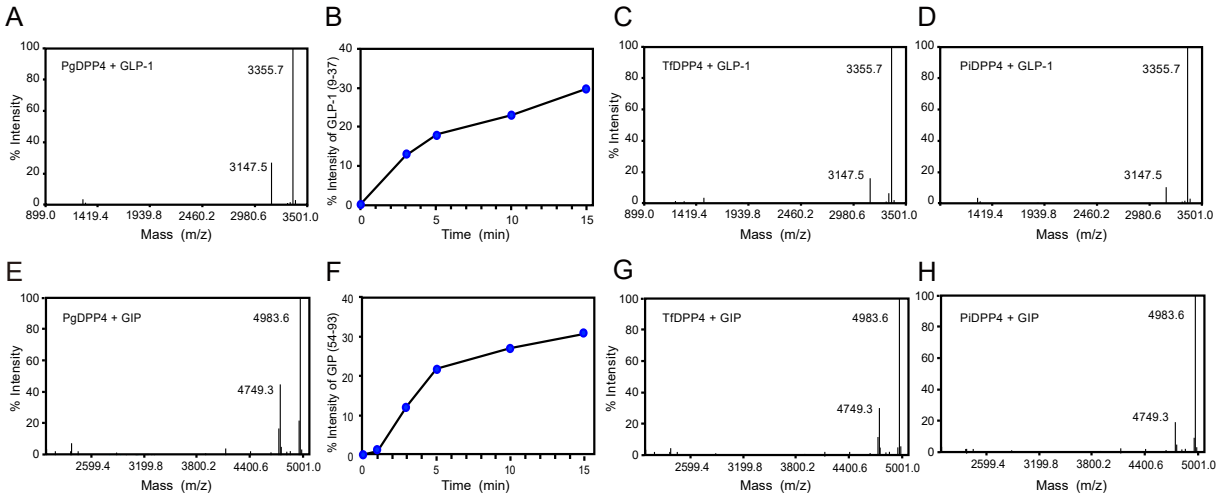
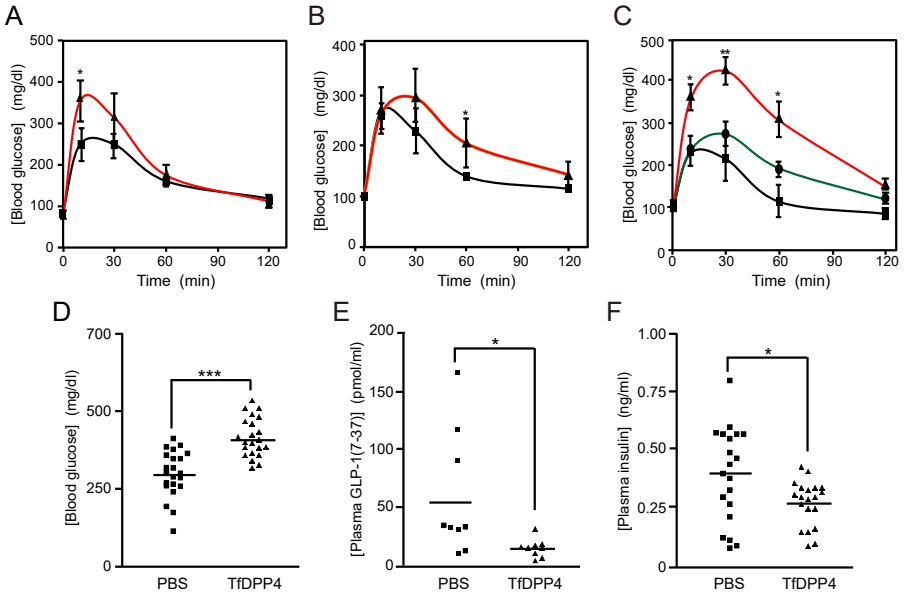

A
PgDPP4 + GLP-1
3355.7
3147.5
% Intensity
Mass (m/z)
B
% Intensity of GLP-1 (9-37)
Time (min)
C
TfDPP4 + GLP-1
3355.7
3147.5
% Intensity
Mass (m/z)
D
PiDPP4 + GLP-1
3355.7
3147.5
Mass (m/z)
E
PgDPP4 + GIP
4983.6
4749.3
% Intensity
Mass (m/z)
F
% Intensity of GIP (54-93)
Time (min)
G
TfDPP4 + GIP
4983.6
4749.3
% Intensity
Mass (m/z)
H
PiDPP4 + GIP
4983.6
4749.3
Mass (m/z)

Figure 6


A
B
C
[Blood glucose] (mg/dl)
Time (min)
D
E
F
[Plasma GLP-1(7-37)] (pmol/ml)
[Plasma insulin] (ng/ml)
PBS
TfDPP4