Ecto-F_0/F_1 ATPase as a novel candidate of prothymosin α receptor in $G\alpha_i$-rich lipid rafts of retinal cells

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ABSTRACT

Objectives: Prothymosin α (ProTα) was reported to inhibit the neuronal necrosis by facilitating the plasma membrane localization of endocytosed glucose transporter 1/4 through an activation of putative G_i-coupled receptor. The present study aims to identify a novel ProTα target, which may lead to an activation of G_i-coupled receptor.

Methods: We used G_i-rich lipid rafts fraction of retinal cell line N18-RE-105 cells for affinity cross-linking. The biological confirmation that F_0/F_1 ATPase is a target protein complex was performed by cell-free experiments using ELISA-based binding assay, surface plasmon resonance assay and quartz crystal microbalance assay, and cell-based experiments to measure extracellular ATP level in the HUVECs culture.

Results: From the cross-linking study and above-mentioned protein-protein interaction assays, ATP5A1 and ATP5B, F_1 ATPase subunits were found to ProTα binding target proteins. In the culture of HUVEC cells, furthermore, ProTα increased the extracellular ATP levels in a reversible manner by anti-ATP5A1- and ATP5B-antibodies.

Conclusion: The present study suggests that ProTα may activate ecto-F_0/F_1 ATPase and produced ATP. This study leads to next subjects whether produced ATP and its metabolites, ADP or adenosine may activate corresponding G_i-coupled receptors.

Introduction

Prothymosin α (ProTα) is a highly acidic nuclear protein, which was discovered from thymosin fraction V of calf thymus tissue[1]. ProTα is found in the nuclei of virtually all mammalian cells. ProTα is generally thought to be an oncoprotein that is correlated with cell proliferation by sequestering anti-coactivator factor, a repressor of estrogen receptor activity, in various cells. In view of the history of discovery, a lot of immune-related studies on ProTα have been reported[2, 3, 4]. Independently, we have first identified ProTα from the conditioned medium of primary culture of cortical neurons as an anti-necrosis factor[5]. In this study, we demonstrated the evidence for mechanisms underlying the unique necrosis-inhibition by ProTα. Under the serum-free starving condition, there was a rapid decrease in cellular ATP levels and underlying glucose influx due to transporter 1/4 endocytosis. ProTα reverses these mechanisms through an activation of putative G_i-coupled receptor, phospholipase C and protein kinase C β2. Based on these findings, we have attempted to search for the G_i-coupled ProTα receptor by use of affinity cross-linkage strategy, but it was not successful. Instead, it was found and we confirmed that toll-like receptor 4 (TLR4) is a functional and binding target for ProTα[6, 7]. Significant, but not complete protective actions of ProTα against the retinal ischemia-induced damage were found to be TLR4 and TRIF (downstream Toll, interleukin-1 receptor and resistance protein domain-containing adaptor inducing interferon-β)-mediated[8], being consistent to with the report using macrophage[6]. As the post-ischemic treatment of ProTα shows complete protective action through a necrosis inhibition, as seen in the case with primary culture of cortical neurons[5] or in vivo retinal model[9], however, we speculated that ProTα may cause G_i-coupled receptor functions through indirect mechanisms. This speculation has been accelerated by the preceding report that ecto-F_1 ATPase could be a receptor candidate of thymosin β4 (Thβ4), which is also a member of thymosin 5 peptides as well as ProTα. Freeman et al. (2011) reported that Thβ4 binds to and increases extracellular ATP levels through

ecto-F_1 ATPase in HUVEC cells, and they proposed that Thβ4 migrates HUVEC cells through an action of ATP on P2X4 receptor[10]. In the present study, we attempted to see whether ProTα may also use this target, since ProTα also have beneficial action against vascular damage[11], like in the case with vascular actions of Thβ4.

Keywords: prothymosin α, ecto-F_0/F_1 ATPase, N18-RE-105 cells, HUVEC, protein-protein interaction

Materials and methods

Cell lines

We used N18-RE105 cell line (passage 9), which had been purchased from JCRB Cell Bank of National Institute of Biomedical Innovation, Health and Nutrition (Osaka, Japan) to identify the binding target for ProTα, since we have observed that ProTα shows the translocation of glucose transporter 4 to plasma membranes of the same cell line under the ischemic condition[12], being consistent to the original finding using cortical neurons from the embryonic rat brain. The culture of mouse neuroblastoma clone N18TG-2 x Fisher rat 18-day embryonic neural retina hybrid N18-RE105 cells were carried out in DMEM with 5% fetal bovine serum (FBS) and 100 mM hypoxantine, 1 mM aminopterine, 16 mM thymidine at 37ºC in 5% CO_2 atmosphere. We also used human umbilical vein endothelial cells (HUVECs) on the analogy of vascular actions of Thβ4. HUVEC cells (passage 8), which had been purchased from Kohjin Bio (Saitama, Japan) were cultured in an RPMI-1640 medium with 10% FBS, 10 ng/mL of EGF and 70 ng/mL of ECGF at 37°C were plated in a number of 1 x 10^4 cells/well of a 96 well plate one day before the ATP measurement assay.

Preparation of membrane proteins from $G\alpha_i$-containing lipid rafts

Purification of lipid rafts from N18-RE-105 cells in the absence of detergent was carried out using OptiPrep™ (60% iodixanol solution; AXIS-SHIELD, Oslo, Norway), and method was in accordance with its Application Sheet S34, 4th edition. Briefly, the cells were washed once in PBS and twice isolation medium (0.25 M sucrose, 1 mM $CaCl_2$, 1mM $MgCl_2$, 20 mM Tris-HCl, pH7.8), and then scraped into isolation medium. Cells were pelleted by centrifugation for 2 min at 250 g and resuspended in 1 mL isolation medium containing protease inhibitor cocktail (Nacalai Tesque, Kyoto, JAPAN). The cells were then lysed by passage through at 22G syringe needle 20 times.

Lysates were centrifuged at 1,000 *g* for 10 min. The postnuclear supernatant was collected and transfer to a new tube. The pellet was again lysed by the addition of 1 ml isolation medium containing protease inhibitors. Second postnuclear supernatant was combined with the first. OptiPrep™ density gradient centrifugation was performed, according to the report demonstrating the preparation of detergent-free lipid rafts[13]. Gradients (0% – 20% iodixanol) were centrifuged for 90 min at 52,000 g using an SW-41 rotor in a Beckman ultracentrifuge. After centrifugation, gradients were fractionated and the distribution of $G\alpha_i$ in fractions was assessed by Western blotting. The $G\alpha_i$-positive fractions were numbered from 1 to 16.

Pull-down assay

Membrane proteins in these fractions were solubilized in 1% CHAPS and used for the binding experiment of rat ProTα by use of sulfosuccinimidyl-2-[6-(biotin-amido)-2-(p-azidobenzamido) hexanoamido]ethyl-1,3′-dithiopropionate (Sulfo-SBED, Pierce™, IL), which is a a biotin-encapsulated reagent capable of cross-linking with the target proteins. SBED-ProTα was incubated with the $G\alpha_i$-positive fractions for 4 h at 4ºC. SBED-ProTα-target proteins complex was cross-linked by UV irradiation for 10 min. This cross-linking is cleaved in the reduction process and biotin is transferred to the target protein. Target protein was purified and isolated with streptavidin sepharose (GE Healthcare Bio-Science Corp.). For ProTα-binding protein solution in each fraction, fractions 1-5, 6-11, and 12-16 were combined and concentrated by ultrafiltration. ATP5B, but not ATP5A1 was identified by using one-dimensional electrophoresis with in-gel digestion of bands followed by MALDI-TOF using an Ultraflex™ TOF/TOF system (Bluker Daltonics Inc, MA).

ELISA-based binding assay

Anti-Flag (MBL Co., Ltd. Nagoya, JAPAN) was immobilized on a 96 well ELISA plate to 100 µL/well with 2 µg/ml (in 0.05 M Sodium carbonate buffer, pH 9.6) at 4ºC for O/N. The plate was washed 3 times with interaction buffer (50 mM Tris-HCl pH 7.6, 15 mM NaCl, 140 mM KCl, 0.5 mM $MgCl_2$). Host proteins: Flag-tagged human ATP5A1 (Flag-ATP5A1), and Flag-tagged human ATP5B (Flag-ATP5B) and their complex proteins were kindly provided from Dr. S. Yokoyama (RIKEN Structural Biology Laboratory, Kanagawa, JAPAN). Flag-ATP5A1 (5 µg/mL, 100µL) or Flag-ATP5B (5 µg/mL, 100 µL) or their complex (10µg /mL, 100µL) was immobilized on the plate for 2 h at RT and unbound host proteins were washed 3 times with interaction buffer. Various concentration of SBED-human ProTα, a guest protein was treated into the plate, incubated for 2 h at RT and washed 3 times with interaction buffer. Host-guest proteins complex was cross-linked by UV irradiation for 10 min. After 1 h blocking with 1% BSA/interaction buffer, Streptavidin-HRP (1/2000 dilution in interaction buffer) was incubated for 2 h at RT and washed 3 times with interaction buffer. The amount of protein binding to ProTα was measured using o-phenylenediamine as a substrate, and the absorbance at 490 nm was read with an ARVO^{TM}MX 1420 Multilabel Counter (PerkinElmer JAPAN Co., Ltd, Kanagawa, JAPAN). Binding unit (BU) represents the value calculated from the observed absorbance at 490 nm (Abs_{490}) in the presence of guest protein – Abs_{490} in the absence of guest protein. For kinetic analysis, K_D was calculated form the Double-Reciprocal Plot: $1/BU = K_D/BU_{max} \times 1/[\text{Conc. of hProT}\alpha] \times BU_{max}$.

Surface Plasmon Resonance (SPR) assay

Experiments to measure surface plasmon resonance (SPR) were performed using a BIACORE® X (GE Healthcare Bio-Science Corp. Piscataway, NJ) with a running buffer of HBS-EP (10 mM HEPES, 150 mM NaCl, 3 mM EDTA, and 0.005% Tween-20). GST-human ATP5A1 (GST-ATP5A1, Novus Biologicals, CO, USA) immobilized onto sensor chip was used as an

analyte and run at 25 ± 1ºC. All sensorgram data of interaction between human ProTα and GST-ATP5A1 show the resonance unit (RU) value. For the kinetic analysis, the changes in RU value induced by applied different concentrations of ProTα were fitted to the formula. K_D (dissociation constant) was calculated form the Double-Reciprocal Plot: $1/RU = K_D/RU_{max} \times 1/[\text{Conc. of ProT}\alpha] + 1/RU_{max}$.

Quartz crystal microbalance (QCM) assay

Real-timely protein-protein interactions were detected using AffinixQ system (Initium Inc., Tokyo, JAPAN), a quartz crystal microbalance (QCM) sensor device. Briefly, AT-cut quartz crystals coated with a thin gold surface layer with a fundamental frequency of 27 MHz were used. Immediately before use, the gold surface of the quartz resonator was cleaned with piranha solution (H_2SO_4: 30% H_2O_2=3:1) for 5 min, and thoroughly washed with double-distilled water. Anti-Flag was applied to the resonator for 30 min to obtain a layer for direct immobilization. Next, the resonator was rinsed with interaction buffer, immersed in interaction buffer (8 ml). The protein-protein interactions were determined from the frequency changes (ΔF in Hz) owing to changes in the mass on the electrode at the sub-nanogram level, upon application of a small volume of protein solution. Based on the Sauerbrey formula, a decrease of 1 Hz is calculated as an interaction of 30.38 pg of a molecule with the biosensor. In all immobilizations of Flag-human ATP5A1/5B on the resonator, it was confirmed that ~83 femto moles of ATP5A1/5B was immobilized as an absolute amount (a decrease of Hz: 300 Hz). All experiments were carried out at 25 ± 1°C. All sensorgram data show the ΔF value following the association phase. For kinetic analysis, frequency changes induced by each concentration of human ProTα were curve-fitted to the formula $\Delta F/X = -K_D \times \Delta F + \Delta F_{Max}/K_D$ (Scatchard plot). K_D and X denote dissociation constant and concentration of the added protein, respectively.

Measurement of extracellular ATP level in the HUVECs culture

For the ATP measurement assay, the cells were washed 3 times with serum-free RPMI and then incubated in a volume of 50 μL for 4 h at 37°C. After the total supernatant of culture was replaced with fresh serum-free RPMI with or without mouse ProTα[11], the cells were incubated for 10 min. The supernatant (20 μL) from each well was used for the measurement of ATP level by bioluminescence using ATP lite 1 step (PerkinElmer JAPAN Co., Ltd., Kanagawa, Japan). *D*-luciferin-luciferase signals were detected in ARVO^{TM}MX 1420 Multilabel Counter (PerkinElmer JAPAN Co., Ltd, Kanagawa, JAPAN). In some experiments 1 μg of anti-ATP synthase alpha/ATP5A1 or anti-ATP synthase beta/ATP5B antibody (Thermo Fisher Scientific, Inc., MA) was added to the incubation medium 4 h before the addition of ProTα.

Statistical analysis

Results are shown as means ± standard error of the mean (S.E.M.). The differences in multiple groups were compared using Dunnett multiple comparison post hoc test after a one-way ANOVA. $p<0.05$ was considered statistically significant.

Results

F_1 ATPase as a ProTα binding protein

In our previous attempts to identify GPCR-type ProTα binding proteins by use of affinity-cross-linkage to brain membranes, we have failed to detect any GPCR-like protein bands from brain membranes. In the present study, we chose the $G\alpha_i$-rich lipid rafts preparation as the source of ProTα-target proteins of N18-RE105 cells. Fig. 1A showed that $G\alpha_i$-like immunoreactivities were abundantly found in lanes 6-11. On the analogy of previous report on Thβ4 protein, we performed the western blotting experiments of streptavidin sepharose-purified biotinylated ProTα-binding proteins by use of anti-ATP5A1 or anti-ATP5B antibody. For this purpose we used Sulfo-SBED, a trifunctional cross-linking reagent having biotin covalently attached to a heterobifunctional reagent. In this reaction, the first step was the replacement of sulfonated N-hydroxysuccinimide moiety with ProTα followed by the photo-affinity crosslink with target binding proteins for ProTα. After the reduction of reacted products, biotinylated target binding proteins were separated by SDS-PAGE. As seen in Fig. 1B, significant immunoreactive signals by anti-ATP5B antibody were observed at 58 kDa in lanes 13-16, while any significant signals by anti-ATP5A1 antibody were not observed at ~55 kDa in these lanes. However, the possibility that ProTα may bind to other target proteins cannot be excluded, since no attempt to detect immunoreactive biotinylated proteins has been done. Pooled samples (fractions 1-5, 6-11 and 12-16), which have been pulled-down by use of streptavidin sepharose were separated on SDS-PAGE and silver-stained. A major band at ~55 kDa was digested by trypsin and analyzed by MALDI-TOF-MS/MS. From the data of 14 digested peptides (Fig. 1D), it was identified to be F_1-ATPase β-subunit ATP5B.

ELISA-based interaction between ProTα and F_1 ATPase subunits

As F_1-ATPase contains three copies each of the alpha and beta subunits that form the catalytic core [14], we tested whether ProTα has an affinity to β-subunit as well as α-subunit by use of ELISA-based assay as described under Materials and methods, the kinetic analysis between SBED-ProTα and Flag-hATP5A1, Flag-hATP5B or their complex was performed (Fig. 2A). The K_D value for the interaction was 67.98 ± 9.89, 241.33 ± 15.93 or 55.78 ± 11.87 nM (n=4, each), respectively (Fig. 2B).

SPR method-based interaction between ProTα and ATP5A1

In the SPR method, the addition of various concentrations of hProTα at 0.1 – 3 μM to GST-hATP5A1 immobilized to the biosensor, the resonance signal immediately increased in a concentration manner, and it was rapidly declined after the replacement with vehicle-containing solution (washout process), suggested the fast dissociation kinetics between proteins (Fig. 3). The K_D was calculated as 0.16 ± 0.02 μM (n=6). However, the binding of ProTα to ATP5A1 was retained in a concentration-dependent manner even in the washout process. The residual binding levels may be related to the time consuming for enzyme activity control, though further studies to evidence this speculation will be the next subject.

QCM-based interaction between ProTα and F_1 ATPase α/β-subunit complex

As shown in Fig. 4A, the reduction of fundamental frequency derived from quartz crystal is dependent on the mass of protein or protein complex loaded on the electrode at the sub-nanogram level, upon application of a small volume of protein solution. The addition of aliquots (8 μL) of ProTα solution to Flag-ATP5A1/5B immobilized on the electrode showed a concentration-dependent decrease in the frequency (Fig. 4B). Scatchard plot analysis revealed the K_D was 28.08 ± 0.40 nM (n=4).

ProTα-induced ATP production in HUVEC cells

On the analogy of the previous report that Thβ4 induces ATP production, which was reversed by the addition of neutralizing antibody against F_1 ATPase α or β-subunit[10], we attempted to see effects of ProTα on ATP production in the HUVEC cell culture. From the time course study of ProTα increased the extracellular levels of ATP in the conditioned medium, we adopted the time for incubation at 10 min. As shown in Fig. 5A, ProTα at 0.01 – 1 μM concentration-dependently increased the ATP levels. The ProTα (1 μM)-induced increase of ATP levels were significantly inhibited by further addition with 1 μg of antibody against anti-ATP5A1- or ATP5B-antibody (Fig. 5B).

Discussion

We have reported that TLR4-TRIF system is involved in the mechanisms underlying the preconditioned ProTα-induced protection from retinal ischemia-reperfusion-induced damage[8], but the mechanisms underlying TLR4-independent and more potent protective action by ProTα given after the retinal ischemia have remained elusive. Furthermore, we have demonstrated that ProTα-induced inhibition of neuronal necrosis is explained by the plasma membrane transport of glucose transporter1/4 through an activation of putative G_i-coupled receptor, phospholipase C and protein kinase C β2[5], though underlying receptor mechanisms have remained to be determined.

In the present study, we observed the findings that ProTα binds to F_1-ATPase on the analogy of the case with the Thβ4-binding protein[10]. The successful study was enabled by use of G_i-rich fractions derived from lipid rafts of N18-RE-105 cells, where ProTα stimulation transfers the GLUT4 internalized by the ischemic stress into plasma membranes[12], as described above in the case of primary culture of neurons[5]. The pull-down and ELISA studies using sulfonyl-SBED-modified ProTα reagent indicated that modified ProTα first binds to ATP5A1, an α subunit of F_1 ATPase, followed by the second photo-affinity cross-linking to ATP5B, its β subunit through an azide reaction. The lower K_D value (28.08 nM) for the interaction between ProTα and ATP5A1/ATP5B complex may support that ProTα-binding pocket is located in between ATP5A1 and ATP5B. The rapid action of ProTα to increase the extracellular levels of ATP in HUVEC cells was blocked by the neutralizing anti-ATP5A1 or ATP5B antibody, suggesting that ProTα may cause an ATP synthesis, as proposed in the case with Thβ4[10]. In the case with Thβ4, it was suggested that produced ATP causes a HUVEC cell migration through P2X4 activation. On the other hand, we are interested in the G_i-coupled receptor functions, as above-mentioned. Therefore it is very interesting to speculate that unknown ecto-ATPases may convert the produced ATP to ADP or adenosine, which in turn stimulates G_i-coupled $P2Y_{12/13}$ or adenosine A_1-receptors. Alternatively,

we should also consider the possibility of TLR4-involvement in the G_i-mediated neuronal necrosis inhibition, since there is a report that constitutively active TLR4-induced activation of ERK1/2-phosphorylation was abolished by pertussis toxin-treatment or dominant negative G_i-expression in HEK 293 cells or RAW 264.7 cells[5]. To investigate this possibility, we need to perform in further studies, whether ProTα also induces ATP production in N18-RE105 cells, TLR4- or G_i-inhibition affects the ProTα-induced ATP production in N18-RE105 cells or HUVECs, and ATP5A1- or ATP5B inhibition affects the ProTα-actions on ischemia-induced damage of N18-RE105 cells and HUVECs. In our previous study, we observed that ProTα showed TLR4-mediated beneficial actions against the retinal ischemia-induced damage when it was given 2 days before the ischemia[8]. However, we have preliminary finding that TLR4 is not involved in the survival activity by post-ischemic treatment with ProTα (in preparation). Accordingly, further studies to see involvements of F_1-ATPase, TLR4 and G_i in ProTα-induced biological activities should wait for the next in vivo and in vitro studies.

In conclusion, the present study demonstrates the successful identification of ProTα-binding protein by use of G_i-rich lipid rafts of N18-RE-105 cells. In addition, it was revealed that ProTα increased extracellular ATP levels in HUVEC cells through an activation of F_0/F_1-ATPase, which was reversed by anti-ATP5A1- or ATP5B-antibody.

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Author contribution

HU and HM designed the whole study and wrote the draft manuscript. HU, HM, YM, SM, RI were involved in the main study and gave substantial inputs to the plan for analysis. SY and MS prepared recombinant proteins. All authors read and approved the final manuscript.

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Legends

Fig. 1 Identification of F_1 ATPase β-subunit as a ProTα binding protein candidate in $G\alpha_i$-rich retinal lipid raft membrane preparations

A: $G\alpha_i$ expression in lipid rafts fractions of N18-RE-105 cell membranes. **B**: ATP5B expression in the samples after pull-down treatment. Proteins in each fraction were cross-linked with SBED-rat ProTα by UV irradiation, followed by removal of ProTα by reduction. Biotinylated ProTα-target proteins were separated by use of streptavidin sepharose, applied to SDS-PAGE and detected by western blot using anti-ATP5B antibody. **C**: Pooled samples were applied to SDS-PAGE and detected by silver stain. **D**: MALDI-TOF-MS/MS analysis of the protein at 58 kDa. Details are described in the text.

Fig. 2 Evidence for direct binding between ProTα and F_1 ATPase subunits in ELISA-based binding assay

A: Schematic model for ELISA-based binding assay between ProTα and F_1 ATPase subunits. **B**: Double reciprocal plots for the interaction between SBED-human ProTα and Flag-tagged human ATP5A1, Flag-tagged human ATP5B or Flag-tagged human ATP5A1/ATP5B. Details are described in the text.

Fig. 3 Evidence for direct binding between ProTα and ATP5A1 in the SPR assay

Results show representative traces of SPR assay-based interaction between immobilized GST-human ATP5A1 and various concentrations of human ProTα.

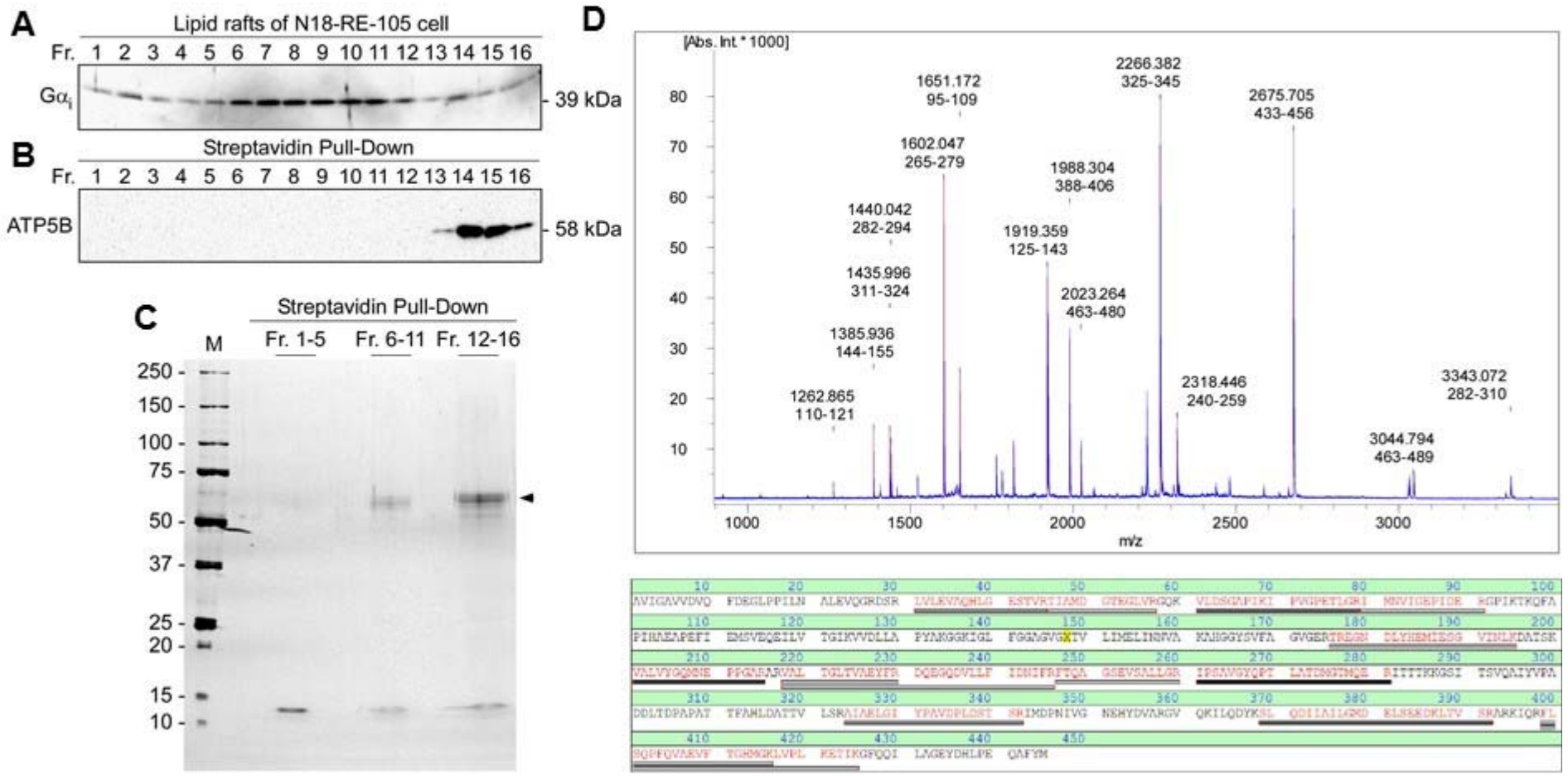
Fig. 4 High-affinity interaction between human ProTα and Flag-human ATP5A1/5B by QCM assay

A: Representative traces of ProTα concentration-dependent decrease in quartz frequency. **B**: Scatchard plot analysis. Results show charts of representative data. More details are described in the text.

Fig. 5 ATP5A1 or ATP5B-mediated ATP production by ProTα in the culture of HUVEC cells

A: Concentration-dependent increase in extracellular ATP levels by ProTα. Results represent the % increase of vehicle control levels in each experiment, as mean ± S.E.M. The number of experiments is indicated in the parenthesis. **B**: Blockade of ProTα (1μM)-induced increase in ATP levels by further addition of 1 μg of anti-ATP5A1 (anti-α) or anti-ATP5B (anti-β) antibody to the culture of HUVEC cells. Data are means ± S.E.M., *P<0.05.

Fig. 1


A
Lipid rafts of N18-RE-105 cell
Fr. 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
Gαi
39 kDa
B
Streptavidin Pull-Down
Fr. 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
ATP5B
58 kDa
C
Streptavidin Pull-Down
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250
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325-345
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463-489
m/z

Fig. 2

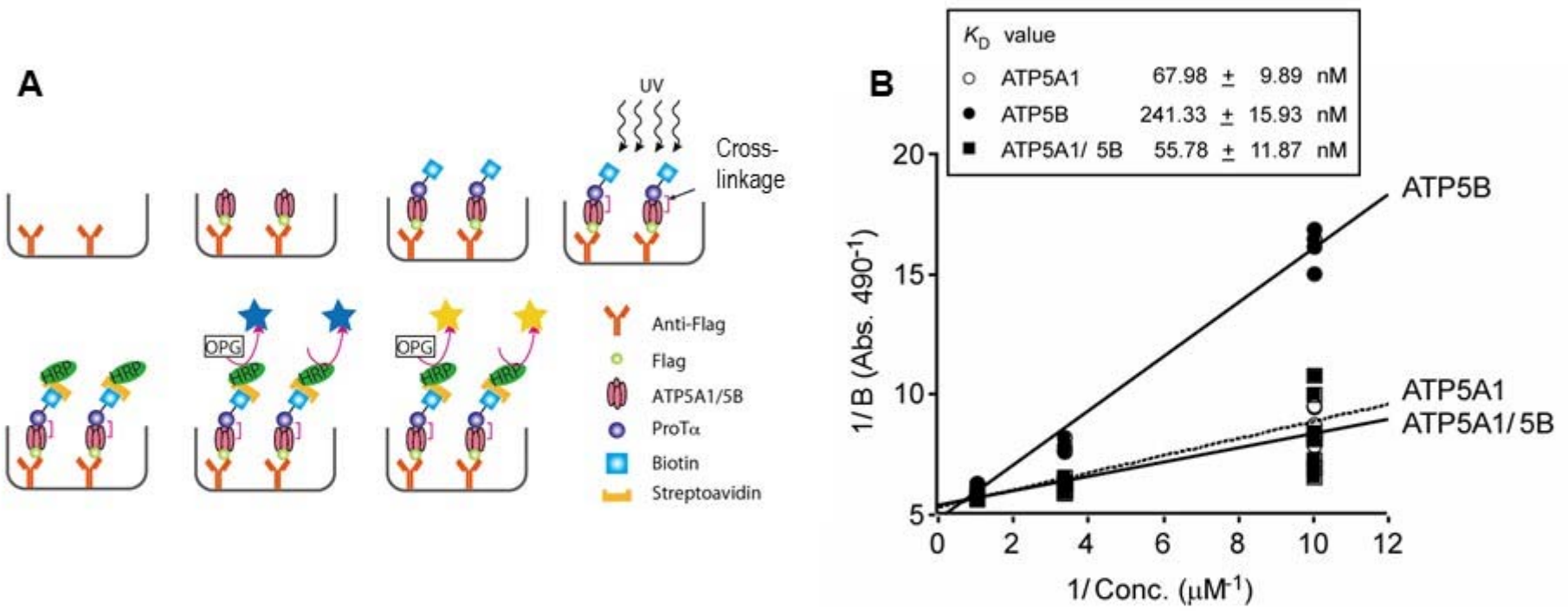

A
UV
Cross-linkage
OPG
HRP
Anti-Flag
Flag
ATP5A1/5B
ProTα
Biotin
Streptoavidin
B
K_D value
ATP5A1 67.98 ± 9.89 nM
ATP5B 241.33 ± 15.93 nM
ATP5A1/ 5B 55.78 ± 11.87 nM
ATP5B
ATP5A1
ATP5A1/5B
1/B (Abs. 490^{-1})
1/Conc. (μM^{-1})
20
15
10
5
0
2
4
6
8
10
12

Fig. 3

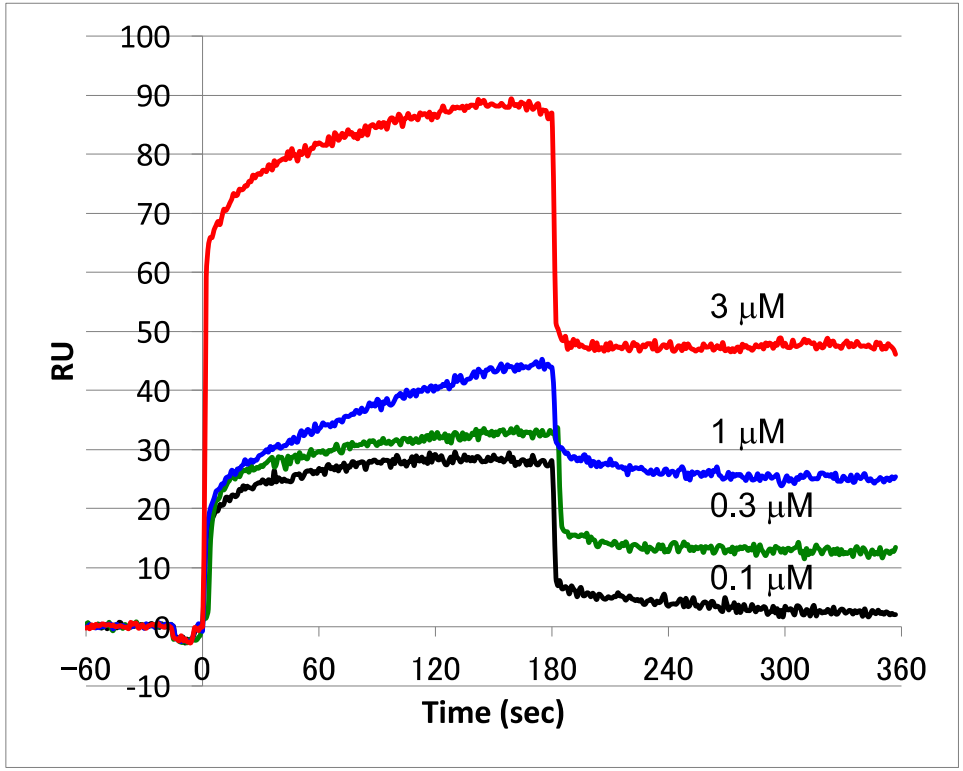

100
90
80
70
60
50
40
30
20
10
0
-10
RU
−60
0
60
120
180
240
300
360
Time (sec)
3 μM
1 μM
0.3 μM
0.1 μM

Fig. 4

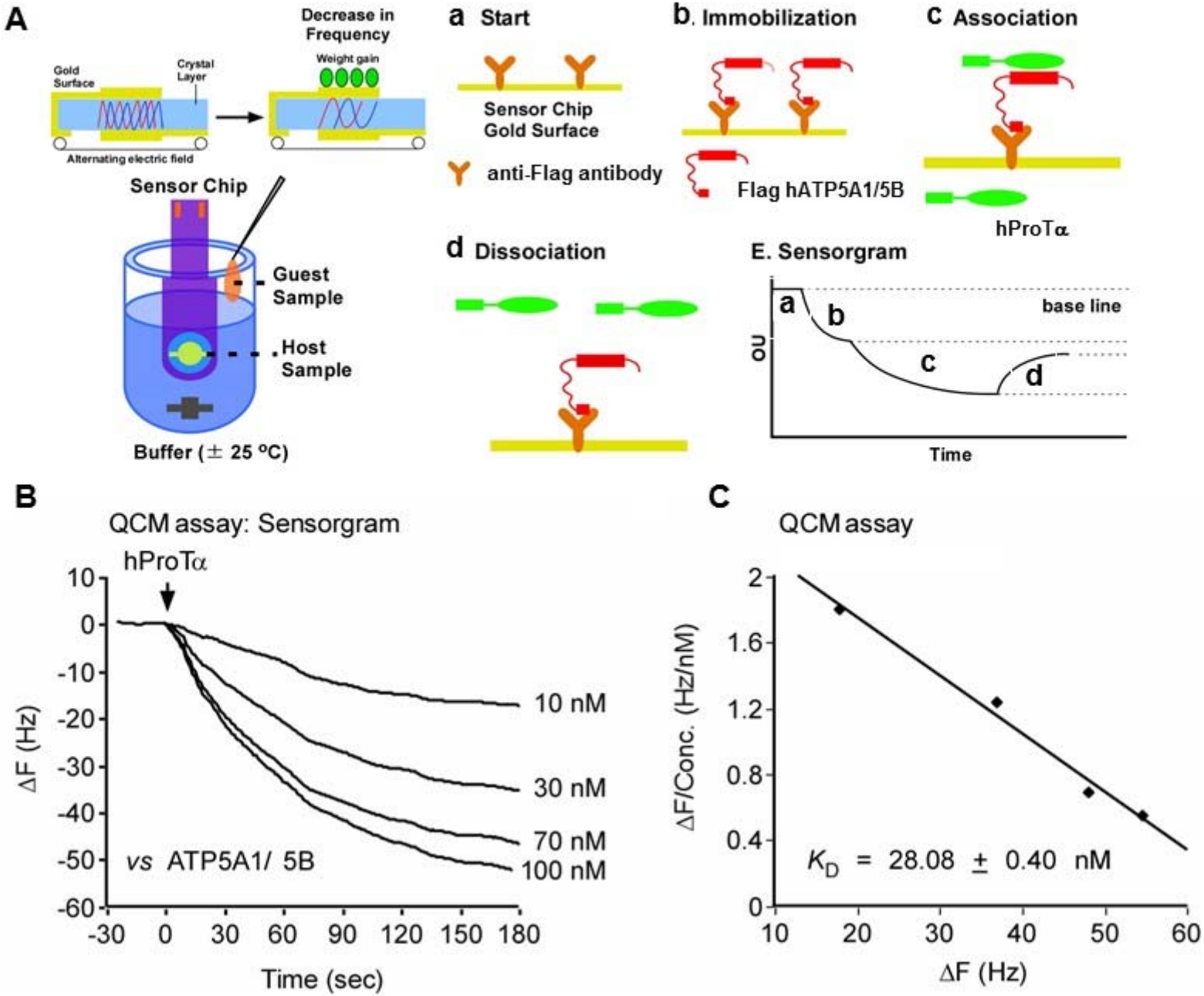
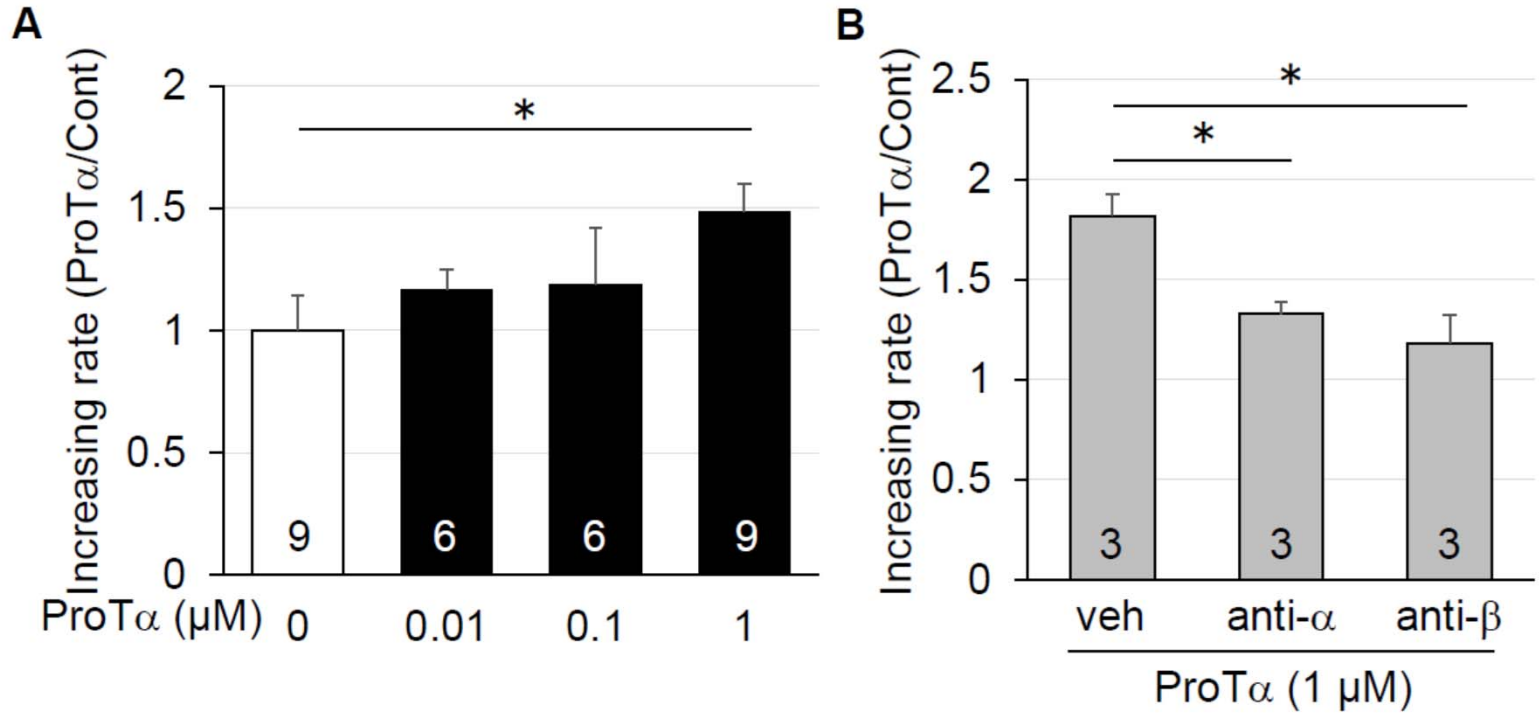

A
Decrease in Frequency
Gold Surface
Crystal Layer
Weight gain
Alternating electric field
Sensor Chip
Guest Sample
Host Sample
Buffer (± 25 ºC)
a Start
Sensor Chip Gold Surface
anti-Flag antibody
b. Immobilization
Flag hATP5A1/5B
c Association
hProTα
d Dissociation
E. Sensorgram
a
b
c
d
base line
ou
Time
B
QCM assay: Sensorgram
hProTα
10 nM
30 nM
70 nM
100 nM
vs ATP5A1/ 5B
ΔF (Hz)
Time (sec)
C
QCM assay
ΔF/Conc. (Hz/nM)
K_D = 28.08 ± 0.40 nM
ΔF (Hz)

Fig. 5


A
Increasing rate (ProTα/Cont)
2
1.5
1
0.5
0
*
9
6
6
9
ProTα (μM)
0
0.01
0.1
1
B
Increasing rate (ProTα/Cont)
2.5
2
1.5
1
0.5
0
*
*
3
3
3
veh
anti-α
anti-β
ProTα (1 μM)

Supplementary Figure 1

Structure and strategy of affinity cross-link of target binding proteins for ProTα using sulfo-SBED (sulfosuccinimidyl-2-[6-(biotinamido)-2-(p-azidobenzamido) hexanoamido]ethyl-1,3´-dithiopropionate)