Affinity between TBC1D4 (AS160) phosphotyrosine-binding domain and insulin-regulated aminopeptidase cytoplasmic domain measured by isothermal titration calorimetry

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INTRODUCTION

Uptake of circulating glucose into the cells happens via the insulin-mediated signalling pathway, which translocates the glucose transporter 4 (GLUT4) vesicles from the intracellular compartment to the plasma membrane. Rab·GTPases are involved in this vesicle trafficking, where Rab·GTPase-activating proteins (RabGAP) enhance the GTP to GDP hydrolysis. TBC1D4 (AS160) and TBC1D1 are functional RabGAPs in the adipocytes and the skeletal myocytes, respectively. These proteins contain two phosphotyrosine-binding domains (PTBs) at the amino-terminus of the catalytic RabGAP domain. The second PTB has been shown to interact with the cytoplasmic region of the insulin-regulated aminopeptidase (IRAP) of the GLUT4 vesicle. In this study, we quantitatively measured the $\sim$$\mu$M affinity ($K_d$) between TBC1D4 PTB and IRAP using isothermal titration calorimetry, and further showed that IRAP residues 1-49 are the major region mediating this interaction. We also demonstrated that the IRAP residues 1-15 are necessary but not sufficient for the PTB interaction. [BMB Reports 2012; 45(6): 360-364]

Other than the RabGAP domain, two amino-termini phosphotyrosine binding domains (PTBs) predicted from the amino acid sequence also exist in TBC1D4 and TBC1D1 (PTB1, amino-terminal PTB; PTB2, carboxyl-terminal PTB, Fig. 1). The boundaries of the domains were identified using the web-based program, Simple Modular Architecture Research Tool (SMART, (7)). Serine and threonine residues within PTB1 and also be- between PTB2 and the RabGAP domain have been shown to un- dergo Akt-mediated phosphorylation, which in turn decreases the RabGAP activity (5). PTBs in general function as adaptors or scaffolds near the biomembrane to recruit signalling com- plexes by binding to peptides and phosphatidylinositides. The -60 human proteins encoding the PTBs can be divided into three groups based on the structure and evolutionary analysis (phosphotyrosine-dependent Shc-like, phosphotyrosine-dependent IRS-like and phosphotyrosine-independent Dab-like), with Dab-like PTBs representing nearly 75% of the PTBs (8).

Peptide sequences favored by the PTBs in each group revealed that the “NPXpY (or Y)” (where X is any amino acid) sequence is the canonical PTB recognition motif. Structural analysis of the PTBs bound to the peptide ligand have indicated that the peptides form an anti-parallel pseudo $\beta$-sheet with type I $\beta$-turn formed by the NPXY motif at the distal end.

The two TBC1D4 PTBs (Fig. 1) were previously classified in-
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Fig. 1. Domains of TBC1D4 (AS160) and IRAP, and the protein constructs used for the ITC measurements. (A) TBC1D4 (also known as AS160) is a multi-domain protein with two amino-terminal PTBs and a catalytic RabGAP domain. The RabGAP domain enhances the hydrolysis rate of Rab-bound GTP to GDP. PTB proteins used in this study are described with residue boundaries. The boundaries of the domains were identified using the web-based program, Simple Modular Architecture Research Tool (SMART). (B) IRAP is a marker for GLUT4 vesicle, which consists of an amino-terminal 109-residue cytoplasmic region and a carboxyl-terminal extracellular/intravesicular catalytic domain connected by a single trans-membrane (TM) region. IRAP proteins used in this study are described with residue boundaries.

RESULTS AND DISCUSSION

TBC1D4 and IRAP interaction is mediated by TBC1D4 PTB2

Based on the previously reported study showing that TBC1D4 containing the second PTB (PTB2) immuno-precipitates with the cytoplasmic region of IRAP (1-109 residues) (9), we quantitatively measured the affinity between these two partners using isothermal titration calorimetry (ITC). First, IRAP1-109 was titrated into several PTB containing TBC1D4 proteins to measure the affinity between the proteins (Fig. 1 and Table 1). IRAP1-109 was shown to bind to two PTBs linked together (PTB1-PTB2) with KD = 8.5 (± 1.4) μM (Fig. 2A), and the affinity slightly decreased when the same IRAP1-109 was titrated into PTB2 (KD = 23.3 [± 3.6] mM, Fig. 2B). However, IRAP1-109 showed no measurable interaction to PTB1 by itself (KD > 1 mM, Supplementary figure), suggesting that PTB2 is mainly responsible for the IRAP interaction. It is possible that certain regions of PTB1 form a minor IRAP binding site, which may explain the enhanced IRAP interaction of PTB1-PTB2 compared to that of PTB2. Of note, mixtures of PTB1 and PTB2 showed the same IRAP binding as measured with only PTB2 (result not shown).

Interestingly, when the carboxyl-terminal residues of PTB1-PTB2 (residues 30-449) were extended to -536 or -609, the

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**Table 1. ITC measured interaction of various TBC1D4 (AS160) and IRAP constructs**

| Various TBC1D4 PTB constructs | Affinity with IRAP1-109 (Kd, μM) | Various IRAP constructs | Affinity with TBC1D4 PTB2 (Kd, μM) |
|--------------------------------|----------------------------------|-------------------------|----------------------------------|
| PTB1-PTB2 (30-449)            | 8.5 (± 1.4)                      | IRAP1-49                | 47.0 (± 15.0)                     |
| PTB1 (30-192)                 | N.D.                             | IRAP1-28-75             | N.D.                             |
| PTB2 (193-449)                | 23.3 (± 3.6)                     | IRAP1-73                | N.D.                             |
| PTB1-PTB2 (30-536)            | N.D.                             | IRAP1-109               | N.D.                             |
| PTB1-PTB2 (30-609)            | N.D.                             | IRAP1-15                | N.D.                             |

*N.D.: non-detectable (>1 mM) interaction.*

IRAP affinity decreased significantly (Table 1 & Supplementary figure). Since these proteins behaved normal during the purification steps, these results suggest that the lack of binding was not from improper folding of the protein, but rather from inhibition of the IRAP interaction by the carboxyl-terminal residues of PTB2. However, previously reported co-immunoprecipitation experiments with the full-length TBC1D4 showed IRAP binding (9), indicating that the inhibition observed in our case for the truncated protein may not be relevant in the context of the full-length protein inside the cell. Probing the IRAP interaction using a more extended construct of PTB1-PTB2 or the full-length TBC1D4 could not be performed due to low protein expression levels.

**IRAP of residue 1-49 is the major site of TBC1D4 PTB2 interaction**

Various PTBs mostly recognize ∼15 amino acid sequences on their counterpart proteins (8). Since our ITC measurement illustrated that the TBC1D4 PTB2 and IRAP1-109 interact with Kd = 23.3 (± 3.6) μM (Fig. 2B), we further attempted to define a shorter IRAP region involved in this interaction. Since the sequence of IRAP1-109 has no canonical NPXY motif, we subdivided and designed three IRAP1-109 constructs with intermittent sequence overlap (IRAP1-49, IRAP28-75, IRAP51-109). Among these IRAPs, which were tested for PTB2 binding, only IRAP1-49 showed Kd = 47.0 (± 15.0) μM (Fig. 2C) which was slightly lower binding compared to IRAP1-109. No binding was observed for IRAP28-75 (Supplementary figure), and IRAP51-109 could not be tested for PTB2 binding due to low protein expression. Although regions of IRAP resides 50-109 may partly play a role in the interaction or be required for proper folding of IRAP, we concluded that the IRAP residues of 1-49 was the major site of the PTB2 interaction.

To search for the shortest amino acid IRAP sequence (~15
residues) that could interact with PTB2, PTB2 binding using several more IRAP constructs was evaluated. Neither IRAP<sub>10-75</sub> nor IRAP<sub>20-75</sub> showed measurable PTB2 binding (Supplementary figure), suggesting that 1-15 residues of IRAP are necessary for the PTB2 interaction. However, when synthesized IRAP<sub>1-15</sub> was titrated into PTB2, no interaction was observed (Supplementary figure). Therefore, we concluded that the IRAP region within residues 1-15 was needed but not sufficient for the normal PTB2 interaction. It is possible that IRAP 1-15 may be required for the proper folding of IRAP; however, other regions of IRAP 1-15 maybe required for the proper folding of IRAP 1-15 in regards to PTB2 binding.

In conclusion, we determined that the cytoplasmic region of IRAP (IRAP<sub>1-109</sub>) has the strongest affinity to TBC1D4 PTB1-PTB2 with $K_D = 8.5 \pm 1.4 \mu M$. The IRAP<sub>1-109</sub> affinity to TBC1D4 PTB2 decreased slightly ($K_D = 23.3 \pm 3.6 \mu M$), but no measurable IRAP<sub>1-109</sub> interaction to TBC1D4 PTB1 was observed, which confirmed that IRAP and TBC1D4 binding is mainly mediated by PTB2. Furthermore, truncated IRAP<sub>1-49</sub> bound to TBC1D4 PTB2 ($K_D = 47.0 \pm 15.0 \mu M$), indicating that region within residues 1-49 contains the major site of interaction, and IRAP<sub>20-109</sub> maybe a minor interaction site.

Our results clearly suggest that IRAP is highly compartmentalized with the first half of the cytoplasmic region dedicated to the TBC1D4 interaction. Multiple physiological functions of the cytoplasmic IRAP have been suggested in various studies focusing on the latter half of the cytoplasmic region. IRAP being a membrane protein that contains two dileucine motifs with a preceding acidic cluster (40EPDEVEYEPRGSRLL<sup>64</sup>, 40DEEDEEDYESSAKLL<sup>77</sup>), injection of the IRAP<sub>55-82</sub> induced GLUT4 translocation in 3T3-L1 adipocytes, suggesting that IRAP interacts with a sorting and targeting protein regulating GLUT4 translocation (10-12). IRAP<sub>55-82</sub> has also been shown to associate with acyl-coenzyme A dehydrogenase (13) and formin homolog over-expressed in spleen (14). Furthermore, IRAP<sub>1-109</sub> has been shown to interact with p115 (15), and a defined sequence (89RQSPDG<sup>105</sup>) has been shown to be involved in binding to the ankyrin repeats of tankyrase (16). All of these results strongly indicate the complex involvement of the cytoplasmic IRAP with the machinery of GLUT4 vesicle trafficking, which is an important component under insulin regulation.

Although PTBs mostly recognize ~15 amino acid sequences on their counterpart proteins, we failed to identify a shorter sequence of IRAP<sub>1-49</sub> that binds to the TBC1D4 PTB2. Overall, our results suggest that either the TBC1D4 PTB2/IRAP interaction is mediated by multiple cytoplasmic IRAP regions within residues 1-49, or that various IRAP regions are required for the proper folding of the protein. Three dimensional structure determination of the TBC1D4 PTB2/IRAP<sub>1-109</sub> (or IRAP<sub>1-49</sub>) complex may allow us to better understand the molecular boundary of IRAP and PTB2 at the binding interface. Although we attempted to crystallize the complex, we have been unsuccessful thus far.

### MATERIALS AND METHODS

**Protein expression and purification**

Fragments of cDNA encoding human TBC1D4 PTB domains (PTB1: 30-192, PTB2: 193-449, PTB1-PTB2: 30-449, 30-536, 30-609; DNA from Takahiro Nagase, Kazusa DNA Research Institute) and various rat IRAP regions (1-109, 1-49, 51-109, 28-75, 10-75, 20-75) were PCR-cloned into the pET28a (Novagen) vector. The proteins were expressed with a His6-tag in the E. coli strain BL21 (DE3) (Stratagen) using kanamycin selection (25 μg/ml). The IRAP 51-109 was not expressed at levels high enough for the calorimetry experiment. The recombinant proteins were isolated on Nickel-NTA columns, and the His6-tag was removed with thrombin (Roche) cleavage as previously reported (6). The proteins were further purified using a Superdex 200 sizing column (GE Healthcare) in GF buffer (50 mM TRIS pH 7.5 and 150 mM NaCl), and concentrated by centrifugation (Amicon Centriprep). Protein concentrations were estimated by absorption at λ = 280 nm by estimating the molar extinction coefficient using the method described by Gill and von Hippel (17).

**Isothermal titration calorimetry**

Calorimetric measurements were carried out using either VP-ITC or ITC<sub>200</sub> calorimeters (MicroCal) at room temperature. Before titration, proteins were dialyzed against the same GF buffer supplemented with 5 mM DTT. Lyophilized IRAP peptide (IRAP<sub>1-15</sub>; MEPTNDRLQLPRNM<sup>15</sup>; Peptron, Inc.) was dissolved in the same buffer supplemented with 5 mM DTT. Lyophilized IRAP peptide (IRAP<sub>1-15</sub>; MEPTNDRLQLPRNM<sup>15</sup>; Peptron, Inc.) was dissolved in the same buffer. ITC measurements were carried out using TBC1D4 concentrations ranging from 90-120 μM and IRAP concentrations ranging from 0.8-2.0 mM. Binding constants were calculated by fitting the data using the ITC data analysis module of Origin 7.0 (OriginLab).

**Acknowledgements**

We thank Dr. Byung-Chul Oh and Ok-Hee Kim (Lee Gil Ya Cancer and Diabetes Institute, Gachon University of Medicine and Science) for kindly allowing us to use their calorimeter.

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