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Quantitative analysis by surface plasmon resonance of CD28 interaction with cytoplasmic adaptor molecules Grb2, Gads and p85 PI3K

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CD28 surface receptors provide co-stimulatory signals that are required for full T cell activation. The CD28 cytoplasmic region has one YMNM and two PXXP motifs as a functional motif. Upon CD28 ligation, Grb2, Gads, and the p85 subunit of PI3 kinase are recruited to the CD28 cytoplasmic region. Here, the interactions between these adaptor proteins and CD28 cytoplasmic domains were analyzed using a Biacore surface plasmon resonance biosensor. For all three adaptor proteins, entire molecules bound more tightly to CD28 than did their isolated SH2 domains. For each adaptor, different outcomes of mutation of CD28's PXXP motifs on binding affinity indicated that only the SH3 domain of Grb2 bound directly. Regarding binding of SH2s to CD28, the SH2 domains of p85 bound more strongly than those of both Grb2 and Gads. Since intact p85 had a 50-fold higher binding affinity than its fragments, and yet the p85-CD28 interaction does not involve SH3-PXXP binding, binding of both N-terminal and C-terminal SH2s to YMNM may create an "avidity" effect. In contrast, when Grb2 and Gads interact with CD28, binding of their SH3 domains may be important. These results suggest that all these interactions are multivalent, through both SH2 and SH3 domains.

Keywords Adaptor molecule, affinity, CD28, co-stimulation, interaction

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INTRODUCTION

In addition to the signaling provided by recognition of antigen-major histocompatibility complex by the T cell receptor, other receptor-ligand interactions play critical roles for full activation of T cells by providing co-stimulatory signals (Mueller et al., 1989). The best-characterized pairs are the CD28 receptor and its ligands, CD80 (B7-1) and CD86 (B7-2) (Harding et al., 1992; Sharpe & Freeman, 2002). It has been postulated that upon CD28 engagement, protein tyrosine kinases (PTKs) are recruited to the CD28 cytoplasmic tail, which consists of 41 amino-acid residues and contains four tyrosine residues, and phosphorylate the tail. Using transgenic mice expressing mutant CD28, we recently demonstrated that among 4 tyrosine residues, Tyr^{189} within a YMNM motif is critical for phosphorylation of CD28. We proposed that the YMNM motif plays an important role in the co-stimulatory signaling pathway (Ogawa et al., 2013).

The cytoplasmic region of CD28 also contains two proline-rich regions that are required for association with tyrosine kinases and co-stimulation. Mutagenesis documented the importance of the C-terminal PxxP motif in regulating a variety of CD28-dependent T-cell responses, which include cytokine production, proliferation, regulatory T-cell development, help for antibody production, and autoimmune disease (Andres et al., 2004; Burr et al., 2001; Friend et al., 2006; Tai et al., 2005; Tai et al., 2007).

When CD28 is ligated, it recruits the Grb2 family proteins Grb2 and Gads and p85 to its YMNM motif, depending on phosphorylation. CD28 also activates MAPK, Akt, NFAT, and NF-κB, which play critical roles in CD28-mediated co-stimulation such as T cell proliferation. They also induce functional differentiation, cytokine production and anti-apoptosis (Rudd & Schneider, 2003).

For binding of adaptor molecules to CD28, the key regions in Grb2, Gads and p85 may be their SH2 domains and SH3 domains. Grb2 contains a central SH2 domain flanked by two SH3 domains (Lowenstein et al., 1992), while Gads contains two SH3 domains with an intervening SH2 domain and a unique insert region containing a Pro/Glu-rich sequence. Grb2 is expressed ubiquitously in mammalian cells, whereas Gads is expressed predominantly in lymphoid tissue and hematopoietic cells, particularly T cells (Asada et al., 1999; Law et al., 1999; Liu & McGlade, 1998).

The associations between these molecules have been investigated mainly in antigen-specific signaling pathways in lymphocytes. The SH2 domains of Grb2 and Gads showed similar binding specificities for several target molecules (such as Shc, M-CSF receptor, and LAT), but the SH3 domains bind much more specifically. For example, Grb2 binds to Sos and Cbl3, but Gads does not bind to these molecules. The Gads SH3 domain has higher affinity for SLP-76 than does the Grb2 SH3 domain (Asada et al., 1999; Liu et al., 2001; Liu & McGlade, 1998). The association of these Grb2 family proteins with the CD28 cytoplasmic tail upon CD28 ligation, and their important role in CD28-mediated co-stimulation, are well known (Schneider et al., 1995a; Schneider & Rudd, 2008), but little is known about the molecular mechanisms.

The p85 subunit of PI3K has two SH2 domains, N-terminal (N-SH2) and C-terminal (C-SH2), and an SH3 domain at the N-terminus. The binding

of SH2 to YMNM motifs has been well documented (Pages et al., 1996; Prasad et al., 1994; Raab et al., 1995), but the SH3 binding site is not yet identified.

Grb2, Gads and p85-PI3K play distinct roles downstream of CD28-mediated co-stimulatory signaling. Grb2 stimulates CD28-mediated IL-2 promoter activation through the Vav1-NFAT/AP-1 signaling pathway (Schneider & Rudd, 2008). Previously, we demonstrated that the binding of Gads to CD28 is essential for CD28-mediated NF-κB activation (Harada et al., 2003; Takeda et al., 2008; Watanabe et al., 2006). p85-PI3K has both positive and negative effects on CD28-mediated co-stimulation (Harada et al., 2001). It has been established that after CD28 ligation, all three signaling molecules somehow interact with the CD28 cytoplasmic tail, but the details of these interactions are not yet known.

In this study, our aim was to define the molecular details of interactions between CD28's cytoplasmic domain, and the three adaptor proteins. The interactions were analyzed using a Biacore surface plasmon resonance (SPR) biosensor. Binding affinities were measured for intact adaptor proteins and for isolated SH2 domains. We compared adaptor molecules' affinities for wild-type CD28, and for CD28 with mutated PXXP motifs. Finally, we discuss possible mechanisms of binding to the CD28 cytoplasmic region.

MATERIALS AND METHODS

Preparation of GST-Grb2, GST-Grb2-SH2, GST-Gads-SH2, and GST-p85

Plasmids containing the human Grb2 and Gads genes were kindly provided by K. Sugamura (Tohoku University) and human p85 was a kind gift from Y. Fukui (The University of Tokyo). These genes were sub-cloned into expression vector pGEX-4T-1, resulting in fusion of *Grb2, Grb2-SH2, Gads-SH2,* and *p85* to Glutathione S-transferase (GST). These constructs were expressed in *E. coli* BL21 (DE3) strain and fusion protein was purified by affinity chromatography on glutathione-agarose beads (GE Healthcare) and anion-exchange chromatography (except for GST-p85). Each elute of SH2 was dialyzed overnight at 4 °C. GST-Grb2 and GST-p85 were finally purified by gel chromatography in buffer A [PBS, 0.005 % Tween-20].

Preparation of His-tagged-Gads

PCR was performed using cDNA forward primer TTACACCAGGCAGAG AACTTACTC, and reverse primer, TTTGAATTCCTGGAAGTTCTGTTCCA GGGGCCCATGGAAGCTGTTGCCAAGTTTG. The amplified products were sub-cloned into expression vector pET32a, resulting in fusion of the N-terminus of Gads to thioredoxin (Trx), six-histidines (6 × His) and a PreScission protease recognition sequence. Gads was expressed in *E. coli* Origami (DE3) strain. The recombinant protein was isolated from supernatant using High Performance Ni-Sepharose (GE Healthcare) according to the manufacturer's recommendation with buffer B [20 mM Tris, 200 mM NaCl, 3 mM DTT]. Trx was cut off using a PreScission Protease kit (GE Healthcare), in buffer C [20 mM Tris, 200 mM NaCl, 1 mM DTT]. Gads was separated by gel chromatography in buffer D [PBS, 0.005 % Tween-20, 100 mM Arginine].

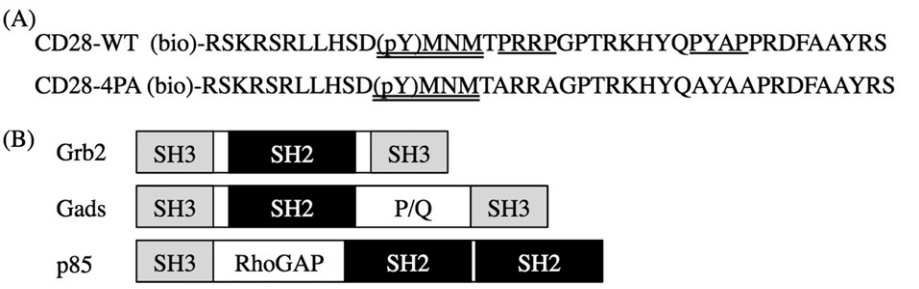

Figure 1. The synthetic peptides, CD28-WT and CD28-4PA: sequence and domain organization. (A) Amino acid sequence of human CD28 cytoplasmic region. The phosphorylated tyrosine was incorporated at a specific position within the YMNM motif. SH2 and SH3 binding motifs are indicated by double and single underline, respectively. (B) Domain organization of Grb2, Gads and p85. P/Q, proline/glutamine-rich motif; RhoGAP, RhoGAP domain.

Synthesis of fragment peptides for CD28

Peptides listed in Figure 1(A) were prepared by the Fmoc solid-phase method with a PSSM8 peptide synthesizer (Shimadzu). The C-termini are carboxyamide groups prepared with Fmoc-NH-SAL-PEG resin (Watanabe Chemicals). Chain elongation was performed with Fmoc amino acids and activation reagents 2-(1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium-hexafluorophosphate, 1-hydroxybenzotriazole, and *N*-methylmorpholine (1: 1: 2 equivalent for Fmoc amino acid). To remove the Fmoc group without causing side reactions with aspartic acid residues for example (Watanabe et al., 2006), we used *N,N*-dimethylformamide (DMF) solution containing 1,8-diazabicyclo[5.4.0]-7-undecene, piperidine, and 1-hydroxybenzotriazole (1.2%: 8%: 2.5%).

Phosphorylated tyrosine was incorporated at a specific position by using *O*-monobenzyl-protected Fmoc-phosphotyrosine (Fmoc-Tyr(PO(OBzl)OH)-OH) as for general amino acids (Harada et al., 2001). The N-terminal amino acids were biotinylated by standard coupling procedures. After completion of chain-elongation, products were cleaved using a mixture of trifluoroacetic acid, 1,2-ethanedithiol, tri-isopropyl silane, and water (86%: 6%: 6%: 2%). Peptides were precipitated with diethyl ether, purified by reverse-phase HPLC using YMC-Pack-Pro-C18 column (YMC), and identified by ESI-MS (Shimadzu QP-8000α). Phosphorylation was confirmed by an 8 nm blue-shift of the absorption band for tyrosine.

Surface plasmon resonance

A Biacore 2000 (GE Healthcare) was used to measure interaction between signalling proteins and CD28. The detection system uses SPR, a quantum mechanical phenomenon that detects changes in the refractive index at the surface of a sensor chip. To immobilize CD28, Biacore sensor chip SA surfaces with streptavidin pre-immobilized to dextran were used. Biotinylated CD28 peptides diluted in buffer A were applied to the chip at 5 μl/min, which resulted in the capture of 250–300 response units. To measure association, signal proteins diluted in buffer A were injected over the immobilized CD28 at 20 μl/min for 180 s at 25 °C. Dissociation was measured by injecting buffer

A alone. The surface of the chip was regenerated by 10 mM glycine, pH 2.3 for 45 s. Analysis was performed using BIAevaluation version 3.1. The equilibrium dissociation constant, K_d, was calculated from the association and dissociation rate constants, k_a and k_d, as $K_d = k_d/k_a$.

Isothermal titration calorimetry

Experiments were carried out on a MicroCal MCS-ITC calorimeter interfaced with a microcomputer. The CD28 peptide, SD(pY)MNMTPRRPG, was titrated into the SH2 domain. Each titration consisted of a preliminary 2-μl injection followed by 19 subsequent 5-μl additions.

RESULTS

Preparation of whole proteins, SH2 domains of Grb2 and Gads, the p85 subunit of PI3K, and biotinylated CD28

Intact proteins Grb2, Gads, p85 and their SH2 domains (Grb2-SH2, Gads-SH2, P85-N-SH2 and P85-C-SH2) were expressed in *E. coli* and purified. Gads deteriorated rapidly, probably due to aggregation and precipitation (Figure 2A left). We examined whether L-arginine (Arg) could inhibit this, because a previous report demonstrated that Arg influenced the folding and stability of recombinant soluble proteins (Rudolph & Lilie, 1996). We measured the soluble form of Gads in buffer A containing 100–500 mM Arg for 0–72 h following purification, with 100 mM Arg found to inhibit aggregation (Figure 2A). Figure 2(B) shows the purified proteins analyzed on SDS-PAGE.

The cytoplasmic domain of CD28 with N-terminal biotin was synthesized chemically to generate the site-specific phosphorylated Tyr. N-terminal biotin immobilizes CD28 on the sensor-chip so that all amino acid sites are available for binding to adaptor proteins. Considering the density on the chip surface of immobilized CD28, which is linked to the chip by long flexible dextran molecules, multiple CD28 molecules can form complexes with adaptor proteins if needed.

SPR analysis of the binding of CD28 to Grb2 and Gads

Previous reports demonstrated that the CD28 cytoplasmic domain interacts directly with several cytoplasmic proteins, some of which bind specifically to the tyrosine residue. To understand how the signaling molecules interact with CD28, we investigated interactions through the pYMNM motif and PXXP motifs. To verify a relationship between these interactions and biological function, we first investigated whether CD28 associations with isolated SH2 domains, and CD28 associations with intact proteins, are of similar strength. This was done by monitoring the profiles of their binding affinities in real time. Grb2 (0.1–6.4 μM), Grb2-SH2 (0.2–6.4 μM), Gads (0.8–3.2 μM), Gads-SH2 (0.2–6.4 μM), p85 (0.025–1.6 μM), p85-N- SH2 (0.2–3.2 μM), p85-C-SH2 (0.2–3.2 μM) were injected across sensor surfaces, with typical sensorgrams shown in Figure 3. K_d values were calculated from the two rate constants, k_a and k_d, determined by the global fitting, and are summarized in Table 1. The $K_{d,\ ITC}$ values for SH2 domains were similar to those obtained using isothermal titration calorimetry (Table 1).

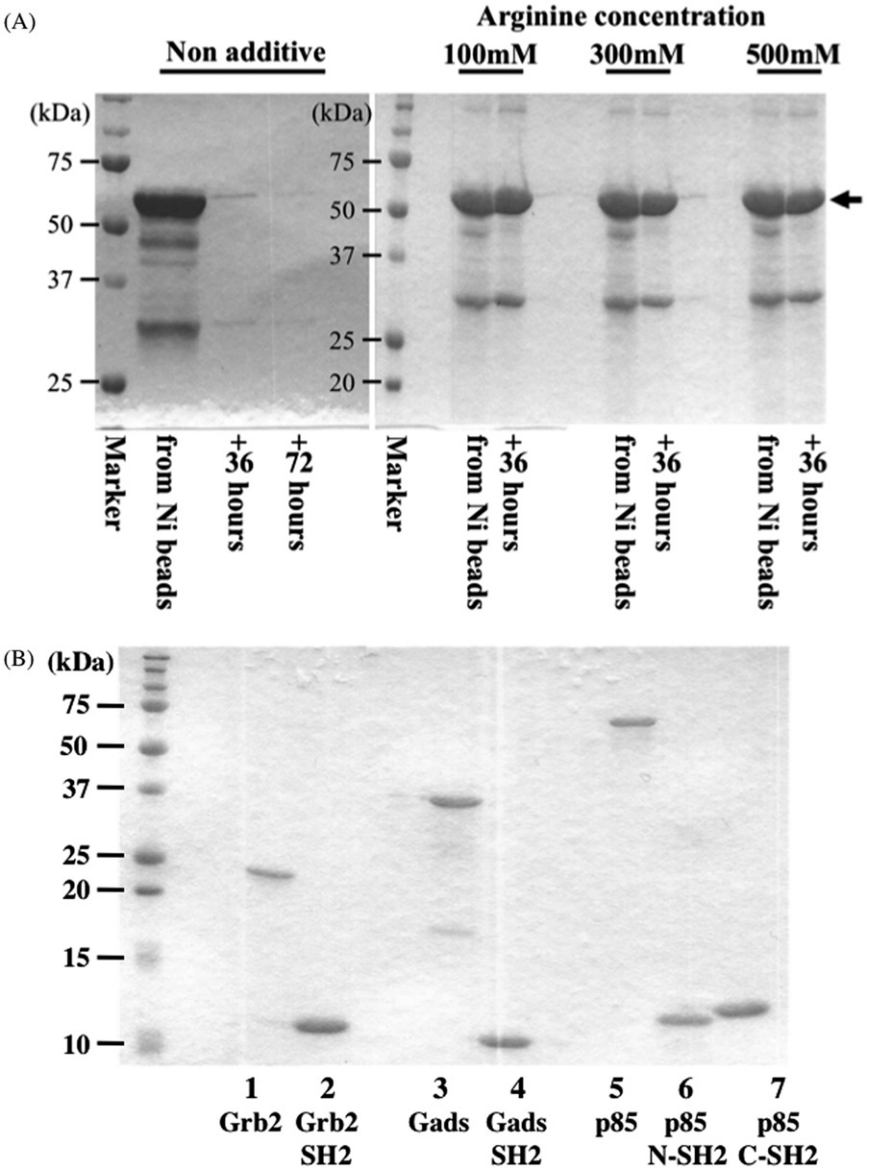

Figure 2. Expression and purification of whole proteins or fragments containing the SH2 domains: Grb2, Gads and the p85 subunit of PI3K. The expressed proteins were purified and subjected to SDS-PAGE with Coomassie Brilliant Blue staining. (A) GST-Gads protein aggregated during purification (Showing eluted GST-Gads analyzed immediately or after 36 hours; black arrow, GST-Gads). To prevent aggregation, we used 100 mM arginine as an aggregation inhibitor. (B) Lanes 1, Grb2; 2, Grb2-SH2; 3, Gads; 4, Gads-SH2; 5, p85; 6, p85-N-SH2; 7, p85-C-SH2.

For Grb2, the affinity between the Grb2 whole molecule and CD28 is 10–20 times higher than that between Grb2-SH2 and CD28. The affinity of the Gads whole molecule is also >20 times higher than that of Gads-SH2. The lower K_d values for isolated SH2 domains are due to higher dissociation rates, which are compensated only partly by higher association rates. These results suggest that regions of Grb2 and Gads outside the SH2 domain also interact with CD28.

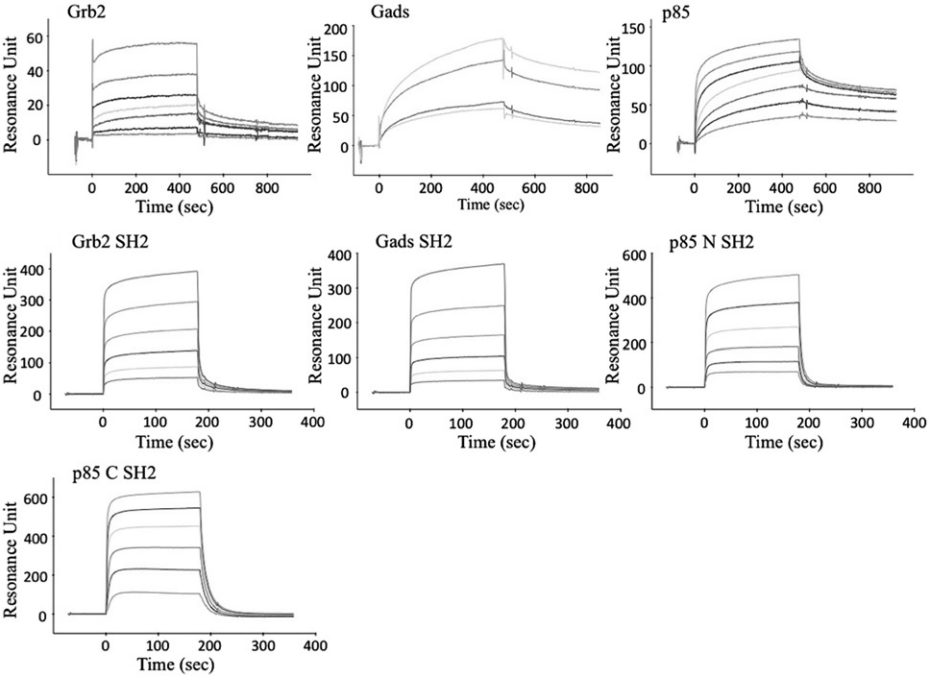

Figure 3. Representative Biacore sensorgrams of interaction between CD28 and signal proteins. Various concentrations of signal proteins were injected across the chip surface, and binding was monitored by SPR. Concentrations of signal proteins were 0.1–6.4 μM (Grb2); 0.1, 0.2, 0.8, 1.6 μM (Gads); 0.025–1.6 μM (p85); 0.2–6.4 μM (Grb2-SH2); 0.2–6.4 μM (Gads-SH2); 0.1–3.2 μM (p85-N-SH2); and 0.1–3.2 μM (p85-C-SH2).

Table 1. Binding kinetics and dissociation constants for the interactions of Grb2, Gads, p85 and their SH2 domains with CD28 and its mutant at 25 °C.

Analyte	Ligand	k_a (1/Ms)	k_d (1/s)	k_d (M)	k_d, ITC (M)
Grb2-SH2	CD28-wt	3.44×10^4	1.38×10^{-1}	4.01×10^{-6}	2.03×10^{-6}
Grb2	CD28-wt	9.52×10^3	2.03×10^{-3}	2.13×10^{-7}	
Grb2	CD28-4PA	1.30×10^3	2.01×10^{-3}	1.55×10^{-6}	
Gads-SH2	CD28-wt	5.41×10^4	2.59×10^{-1}	4.79×10^{-6}	2.48×10^{-6}
Gads	CD28-wt	4.93×10^3	1.02×10^{-3}	2.07×10^{-7}	
Gads	CD28-4PA	4.80×10^3	1.07×10^{-3}	2.23×10^{-7}	
p85-N-SH2	CD28-wt	6.94×10^4	1.13×10^{-1}	1.63×10^{-6}	
p85-C-SH2	CD28-wt	6.25×10^4	5.20×10^{-2}	8.32×10^{-7}	
p85	CD28-wt	1.12×10^5	1.61×10^{-3}	1.44×10^{-8}	
p85	CD28-4PA	8.54×10^4	7.64×10^{-4}	0.89×10^{-8}	

It has been reported that SH3 domains of Grb2 also bind to C-terminal proline-rich motifs of CD28 (Kim et al., 1998). Consistent with this, mutation of C-terminal proline-rich PXXP motifs significantly reduced CD28-mediated co-stimulation of proliferation and IL-2 production, as well as the *in vivo* immune response (Friend et al., 2006; Tai et al., 2005; Tai et al., 2007).

Thus we next examined the role of CD28's PXXP motifs in the interaction with Grb2 or Gads. We generated a mutant, CD28-4PA, in which all Pro residues in the putative SH3 binding sites were mutated to Ala, and then

analyzed its binding to Grb2 and Gads. As shown in Figure 3 and Table 1, the affinity of Grb2 for CD28-4PA is approximately 7-fold weaker than that for CD28-WT. This indicates that Grb2's association with CD28 involves Grb2's SH3 domains and CD28's PXXP motifs.

In contrast, the affinity of Gads for CD28-4PA was similar to that for CD28-WT, indicating that Gads' SH3 domain does not bind to CD28's PXXP motifs one-to-one. Since intact Gads has much higher affinity to CD28 than the Gads-SH2 domain alone, we assume that Gads' SH3 interacts with CD28's cytoplasmic region, but at locations other than PXXP motifs.

SPR analysis of the binding of the p85 subunit of PI3K to CD28

Grb2, Gads and the p85 subunit of PI3K share a YMNM binding site motif at their SH2 domain: pYXXM for p85 and pYXNX for Grb2 or Gads. p85 has two SH2 domains (Figure 1), and the C-terminal SH2 has twice the affinity to CD28 of the N-terminal SH2 (Figure 3 and Table 1). SPR analysis revealed that the interaction between CD28 and p85 whole molecule was 50–100 times stronger than the interaction between CD28 and p85's SH2 domain alone.

Next, the role of the p85 SH3 domain was investigated by measuring the affinity between p85 and CD28-4PA. As shown in Figure 3 and Table 1, the K_d value for CD28-4PA was slightly different from that for CD28-WT, indicating that association of the SH3 domain to PXXP motifs has a limited role in p85 binding to CD28.

Finally, we compared affinities of CD28 to whole molecules of p85, Grb2 or Gads. p85 has a 14 times higher affinity than Grb2 or Gads. The decreased K_d is mainly due to an increased k_a. p85's two SH2 domains each have a higher affinity to pYMNM, than Grb2's SH2 domain (p85-N-SH2/Grb2-SH2, 2.5 times; p85-C-SH2/Grb2-SH2, 4.5 times) or Gads' SH2 domain (p85-N-SH2/Gads-SH2, 3 times; p85-C-SH2/Gads-SH2, 5.8 times). These results indicate that the high affinity binding of p85 to CD28 is the result of avidity through both N- and C-terminal SH2 domains.

DISCUSSION

After ligation of CD28 externally, the CD28 cytoplasmic tail recruits p85, Grb2 and Gads, and initiates a signaling cascade, resulting in CD28-mediatated co-stimulation. We recently reported, on the basis of results from primary $CD4^+$ T cells from mutant CD28 transgenic mice (Ogawa et al., 2013), that the YMNM motif of CD28, which is a binding motif for these adaptor molecules, is critical for CD28-mediated co-stimulatory functions, such as increasing T cell proliferation and IL-2 production.

The mechanisms by which these adaptor proteins associate with CD28 have been extensively studied by many groups including ourselves (Rudd & Schneider, 2003). The studies were carried out by precipitation experiments, such as immune-precipitation and pull-down assays. Since the experiments used diverse materials, cell populations, conditions and methods, the findings have been somewhat inconsistent. In this report, we studied molecule-to-molecule interaction by SPR analysis.

Consistent with previous immune-precipitation studies of whole molecule interaction, the affinity of p85 to CD28 was much higher (14 times) than

affinities of Grb2 or Gads, which bind CD28 at a similar affinity. For all three adaptors, intact molecules showed significantly higher binding affinities than their isolated SH2 domains. They presumably increase their affinity by interaction outside the YMNM motif which is SH2's binding site. Furthermore, our study revealed that these three molecules interact with CD28 in strikingly different ways. For Grb2, alteration of CD28's two canonical SH3 binding motifs dramatically decreased affinity, indicating that interaction between SH3 domains of Grb2 and PXXP motifs of CD28 is involved in the association of Grb2 with CD28. This confirms the previous report by Okkenhaug & Rottapel (1998) who used precipitation experiments to show that Grb2's SH3 domains bind to CD28's cytoplasmic C-terminal proline-rich motif, in addition to binding of Grb2's SH2 domain to CD28's phosphorylated tyrosine. McDonald et al. (2008) demonstrated that Grb2 exists as a monomer-dimer in solution. Grb2 dimerization may lead to rapid signal amplification upon receptor engagement. In our study, dimerization perhaps elevated the affinity of intact Grb2 compared to the Grb2-SH2 domain.

In contrast, the affinity of Gads for CD28-4PA was similar to that for CD28-WT, indicating that Gads-SH3 domain does not bind to CD28's PXXP motifs one-to-one. Since intact Gads has much higher affinity to CD28 than the Gads-SH2 domain alone, we assume that Gads' SH3 interacts with CD28's cytoplasmic region, but at locations other than PXXP motifs. R/KXXR/K motifs are also known as non-canonical motifs for SH3. Berry et al. (2002) demonstrated that SH3 domains of Grb2 and Gads bind to non-canonical RXXK motifs of SLP-76. Lewitzky et al. (2004) and Harkiolaki et al. (2009) showed that the C-terminal SH3 domain of Gads binds to SLP-76, HPK1 and Gab1, through a non-canonical PX(V/I)(D/N)RXXKP motif. CD28's cytoplasmic tail has an RXXR motif just beneath the plasma membrane (Figure 1), and Gads-SH3 could bind to it. Otherwise, given that Gads possesses an SH3 - SH2 - SH3 domain structure, and shares homology with Grb2, we speculate that Gads could form homodimers which may increase affinity with CD28; however, the dimerization of Gads has not yet been reported. Finally, Gads could have an unknown binding domain other than SH2 and SH3. The proline- and glutamine- (P/Q) rich region flanked by SH2 and the C-terminal SH3 domain is a candidate, but seems unlikely as Ellis et al. (2000) previously showed that it did not interact with CD28. For a better understanding of these interactions, we are currently examining the structure of Grb2-CD28 and Gads-CD28 complexes.

Using precipitation experiments, we have previously shown that the N-terminal PXXP motif of CD28 interacts with Gads, but not with Grb2 (Watanabe et al., 2006). The cell lysate used in immuno-precipitation or pull-down assays contains various cytoplasmic proteins. Thus multi-protein signaling complexes may form and influence the interaction of adaptors with CD28's cytoplasmic region. In contrast, SPR measures the affinity of one purified molecule for another (here, purified whole Gads, or its SH2 domain, to purified phosphorylated CD28 cytoplasmic region). Thus Gads' SH3 might need other molecule(s) to interact with the PXXP motif. On the other hand, for Grb2, other molecules are known, which inhibit the association of Grb2's SH3 domain to PXXP motifs in precipitation experiments. Sos, Vav, Gab2, and Themis all block the interaction in pull-down assays (Harkiolaki et al., 2009;

Jones & Kazlauskas, 2001; Nishida et al., 2001; Patrick et al., 2009). We are currently trying to identify these molecules by phage display methods.

Although the importance of PI3 kinase in CD28-mediated co-stimulatory signaling and the binding of its p85 subunits to CD28 have been well-documented, the binding mechanism remained unknown. p85 has two SH2 domains, and in one-to-one binding conditions either SH2 bound to CD28 at higher affinity than Grb2 or Gads SH2 (Figure 3, Table 1). Since the SH2 domains share less than 20% homology, the affinities of p85's SH2 domains for CD28 may be quite different from the affinities of Grb2's domain, or of Gads' domain for CD28. It has been previously shown in precipitation experiments that both N- and C-terminal SH2 domains of p85 are able to bind the target molecule (Reedijk et al., 1992; Schneider et al., 1995b). The N-terminal SH2 domain of p85 is critical for interaction with several molecules, such as SLP-76 (Shim et al., 2004), whereas C-terminal SH2 is used for binding with Vav1, CTLA-4, PDGFR, and CSF-1 R (Ramalingam et al., 1995; Reedijk et al., 1992; Schneider et al., 1995b; Songyang et al., 1994). As CD28 forms homo-dimers (Abe et al., 1995), tandem SH2 domains of p85 might bind to each pYMNM to create an avidity effect, which results in high affinity binding.

Based on the results from SPR analysis, we propose that CD28 interacts with the three adaptor molecules as follows (Figure 4):

(1) In the resting state, Grb2 and Gads SH3 domains bind with CD28, at CD28's PXXP and non-PXXP motifs. Dimerization of Grb2 (and possibly also Gads) may increase binding. Alternatively, in the ligand-free condition, the CD28 cytoplasmic domain may be located in close proximity to the membrane, making it difficult for adapter molecules to access the CD28.
(2) Upon CD28 ligation, CD28 is phosphorylated and Grb2 and Gads bind through their SH2 domains to CD28's Y(p)MNM motif. Conformational changes to the CD28 cytoplasmic tail induced by ligand interaction may be important for tyrosine phosphorylation of CD28 through association with a protein tyrosine kinase such as Lck.
(3) Next, p85 uses its two SH2 domains to bind strongly to CD28's tyrosine-phosphorylated YMNM motif.

This multi-step model is consistent with a previous report, which demonstrated that Gads - CD28 association peaked 1 min after activation, whereas the p85 - CD28 association increased gradually over 30 min (Ellis et al., 2000). Furthermore, p85's slower association with CD28 may regulate CD28-mediated co-stimulatory signaling through PIP_3 production, and through inhibition downstream of CD28-mediated Grb2 and Gads signaling pathways. Otherwise, when Grb2's and Gads' SH3 domains bind to CD28, they may cause a conformational change of CD28's cytoplasmic region rendering CD28's phosphorylated YMNM motif accessible to Grb2's and Gads' SH2 domains. This conformational change may make CD28 slightly inaccessible to p85.

Furthermore, the interaction of other molecules to CD28 is important for CD28-mediated co-stimulation. For example, PKCθ co-localized with CD28 (Yokosuka et al., 2008) and Lck and/or Rltpr is involved in the recruitment of PKCθ to CD28 (Kong et al., 2011; Liang et al., 2013). Further studies will be

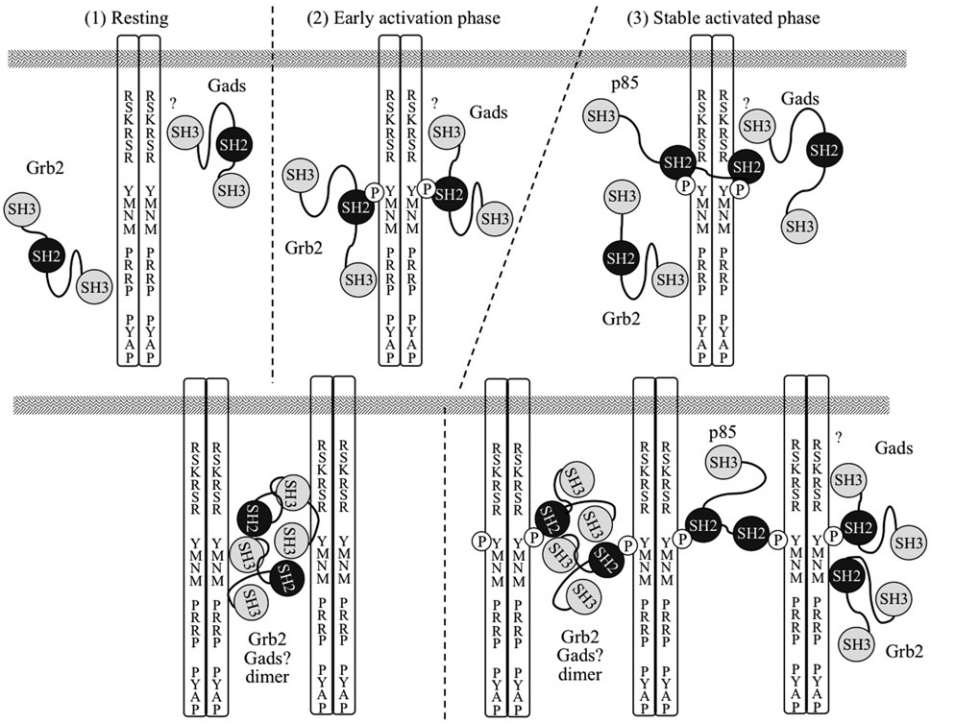

Figure 4. Multi-step and multi-protein model for interaction between adaptor molecules and CD28. The top panel shows the monomer interaction model, and the bottom panel shows the Grb2 (Gads) dimerization model and p85 bridge model between two CD28 receptors. In the resting state, Grb2 and Gads SH3 domains can interact with the PXXP motif, and outside the PXXP region respectively, within the cytoplasmic tail of CD28. Dimerization of Grb2 (and perhaps Gads) may induce conformational change and increase affinity. They could bind to CD28 even in the resting state or soon after engagement. These interactions are quickly enhanced by their SH2 domains binding, following tyrosine phosphorylation of CD28's YMNM motif. Then, the two SH2 domains of p85 interact with the phosphorylated YMNM motif. This p85 interaction with CD28 is strong compared to Grb2 and Gads, mainly due to the bivalent effect, in addition to the high affinity SH2 – CD28 interaction.

needed to elucidate the precise interactions and stoichiometric and allosteric changes within the multi-molecular complex that allow for CD28-mediated signal transduction.

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DECLARATION OF INTEREST

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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