Study of the interaction of the medium chain μ 2 subunit of the clathrin-associated adapter protein complex 2 with cytotoxic T-lymphocyte antigen 4 and CD28

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The medium chain μ 2 subunit (AP50) of the clathrin-associated adapter protein complex 2 (AP-2) interacts specifically with the tyrosine-based signals of several integral membrane proteins through the consensus sequence YXX Φ , where X can be any residue and Φ is a large hydrophobic residue. Using surface plasmon resonance combined with structural information, we have analysed the interaction of AP50 with peptides derived from the cytoplasmic tail of cytotoxic T-lymphocyte antigen 4 (CTLA-4). The crystal structure of AP50 in complex with a CTLA-4-derived peptide was determined to 3.6 Å (1 Å = 0.1 nm) resolution. The binding domain of AP50 (residues 164–435) was expressed in *Escherichia coli* and purified. In agreement with previous reports, the AP50 domain bound to residues 152–174 of CTLA-4, but not to the same peptide that was phosphorylated at the single tyrosine residue (position 165). The interaction

exhibited fast kinetics with rapid on and off rates and a K_d of 0.7 μ M. In order to further understand why AP50 binds to CTLA-4, but not to the homologous receptor CD28, a comparison of binding of AP50 with five peptides with single changes in and around the YXX Φ motif to the equivalent residues of CD28 was made. T162H greatly reduced binding, whereas T161L had little effect. Mutations G163S, V164D and K167N all exhibited reduced binding. Modelling of the single amino acid changes using structural information, was in broad agreement with the binding data, demonstrating that residues outside of the YXX Φ motif are also important in the interaction of membrane proteins with AP50.

Key words: co-stimulation, endocytosis, tyrosine motif.

INTRODUCTION

CD28 and cytotoxic T-lymphocyte antigen 4 (CTLA-4) are structurally related cell surface receptors found primarily on T-cells [1–3]. They both bind to the structurally related ligands CD80 and CD86 on antigen-presenting cells to affect T-cell activation. CD28 provides a major co-stimulatory signal for T-cell activation [4–6]. CTLA-4, in contrast, is believed to have a critical inhibitory function [7–9]. CD28 is constitutively expressed at the cell surface. In contrast, CTLA-4 is expressed only transiently at the cell surface, peaking at 48–72 h following T-cell activation. The vast majority of CTLA-4 in all conditions is found in intracellular endosomal compartments [10,11]. Several studies have shown that this intracellular location of CTLA-4 is the result of an interaction between the cytoplasmic tail of CTLA-4 and the medium chain μ 2 subunit (AP50) of the clathrin-associated adapter protein complex 2 (AP-2) [12–15]. This interaction is negatively regulated by phosphorylation of Tyr¹⁶⁵ in the cytoplasmic tail of CTLA-4 [12,15]. AP50 has been shown to recognize a tyrosine-based sorting signal with the consensus sequence YXX Φ , where X can be any residue and Φ is a bulky hydrophobic amino acid [16]. X-ray crystallographic data have revealed some of the details of the interactions between AP50 and YXX Φ in the cytoplasmic tail of both the epidermal growth factor (EGF) receptor and the *trans*-Golgi network (TGN)

receptor [17]. Specificity is conferred by two hydrophobic pockets that bind the tyrosine and Φ residues. The structures illustrated that a phosphorylated tyrosine would be incapable of binding to AP50; the pocket is too small to accommodate a phosphate without causing steric clash with Asp¹⁷⁶ in AP50.

CTLA-4 and CD28 both contain a tyrosine-based motif (YVKM in CTLA-4 and YNM in CD28). However, a number of studies have shown that, despite the sequence similarities within the cytoplasmic tail, CTLA-4 binds AP50, whereas CD28 does not, thus accounting for their different cellular locations. This suggests that residues outside of the consensus sequence can influence the interaction with AP50. Bradshaw et al. [12] made several chimaeric molecules between the cytoplasmic tails of CD28 and CTLA-4 which suggested that residues N-terminal to the key tyrosine residue influenced the interaction with AP50.

In order to further understand the requirements for the interaction of CTLA-4 with AP50 and the lack of binding of CD28, we have changed five residues within the CTLA-4 cytoplasmic domain to the corresponding residues in CD28. Using surface plasmon resonance (SPR), we have then looked at the binding of the mutated peptides to AP50. We show that most of the changes have a deleterious effect on binding, but particularly pronounced was the effect of changing a threonine residue at position 162 to a histidine residue; this almost completely abolished binding. In addition, we have determined

Abbreviations used: AP-2, adapter protein complex 2; AP50, medium chain μ 2 subunit of the AP-2 complex; CTLA-4, cytotoxic T-lymphocyte antigen 4; DTT, dithiothreitol; EGF, epidermal growth factor; pY, phospho-tyrosine; RU, resonance units; SPR, surface plasmon resonance; tAP50, tagged AP50; TGN, *trans*-Golgi network.

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the crystal structure of AP50 complexed with a peptide derived from the cytoplasmic tail of CTLA-4 that further clarifies structural relationships in the interaction. The single amino acid changes to the equivalent CD28 residues were modelled to provide further information on interactions outside of the tyrosine motif. These data all indicate that CD28 residues outside the YXXΦ motif are likely to exert a negative influence on binding to AP50, thereby preventing internalization and enabling CD28 to be localized at the cell surface rather than in intracellular vesicles.

EXPERIMENTAL

Expression and purification

Human AP50 encoding amino acids 164–435 was generated by PCR using a whole brain cDNA clone (Image clone 44395; Image Consortium, <http://image.llnl.gov/>) as the template and the primers 5'-GAATAATATTGAGGGTATCAAGTATCGT-CGGAAT-3' (forward) and 5'-GAATGCGGCCGCTAGCAG-CGAGTTTCATAAATGCC-3' (reverse). The PCR product was digested with *Ssp*I and *Not*I and cloned into an inducible T7 expression vector, based on that described by Studier et al. [18], in frame with a His₆-c-Myc fusion tag. The recombinant plasmid was verified by DNA sequencing and transformed into *Escherichia coli* BL21(DE3). His₆-c-Myc-AP50(164–435), i.e. tagged AP50 (tAP50), was produced by growing *E. coli* in a 20 litre fermentor at 25 °C. Expression was induced with 0.5 mM isopropyl β-D-thiogalactoside at an absorbance at 550 nm of 18, and after 5 h the cells were harvested by centrifugation and immediately frozen at –80 °C.

Centrifuged *E. coli* cells (250 g) were thawed and suspended in 2.5 litres of 50 mM Tris/HCl (pH 7.9), 0.5 M NaCl, 5 mM imidazole, 5 mM 2-mercaptoethanol and protease inhibitors (1 µg/ml leupeptin, 1 µg/ml pepstatin and 1 mM PMSF). Lysis of the cells was achieved using a Manton–Gaulin homogenizer. The lysate was centrifuged at 20000 *g* for 45 min and the supernatant was frozen at –80 °C. The lysate supernatant (500 ml) was thawed slowly at 4 °C and purified on a Ni²⁺-nitrilotriacetate-agarose column (Qiagen), equilibrated with 50 mM Tris/HCl (pH 7.9), 0.5 M NaCl and 5 mM imidazole (running buffer). After washing with ten column volumes of running buffer the column was eluted with running buffer containing 0.2 M imidazole. Fractions containing tAP50 were pooled and dialysed into 50 mM Tris/HCl (pH 7.9), 0.5 M NaCl and 5 mM dithiothreitol (DTT) to remove the imidazole. Dialysed material was loaded on to a Superdex™ 75 column (1.6 cm × 55 cm; Amersham Pharmacia Biotech) and eluted with 50 mM Tris/HCl (pH 7.9), 0.15 M NaCl and 5 mM DTT. Fractions were collected and analysed using SDS/PAGE, under reducing conditions, and Coomassie Brilliant Blue staining. Fractions containing tAP50 were pooled, sterile-filtered and stored at 4 °C.

The concentration of tAP50 was determined using amino acid analysis and, using this data, the molar absorption coefficient was calculated for the fusion protein. This permitted the determination of the concentration of further samples of tAP50 using the absorbance at 280 nm.

tAP50 was analysed by gel-filtration chromatography on an Amersham Pharmacia Biotech SMART system. A Superdex 75 PC 3.2/30 column was equilibrated in running buffer [50 mM Tris/HCl (pH 7.9), 0.5 M NaCl and 1 mM DTT]. The system was calibrated with BSA, chymotrypsinogen, RNase A and ovalbumin made up to 1 mg/ml in running buffer.

Table 1 Cytoplasmic tail sequences of CTLA-4 and CD28 containing the tyrosine motif

Receptor	C-terminal sequence containing the YXXΦ motif
CTLA-4(152–174)	¹⁵² KMLKKRSLTTGVYVKMPPTPEE
CD28(152–174)	¹⁵² WVRSKRSRLHSDYMNMTPRRPG

Table 2 Peptide amides used for SPR binding studies

All peptides were produced by Peninsula and were biotinylated at the N-terminal end. The **bold** residue is the single amino acid change from the CTLA-4 sequence to the corresponding residue of CD28.

Peptide code	Peptide sequence
CTLA-4(152–174)	KMLKKRSLTTGVYVKMPPTPEE-NH ₂
[Y165pY]CTLA-4(152–174)	KMLKKRSLTTGVpYVKMPPTPEE-NH ₂
T161L	KMLKKRSL L TGVYVKMPPTPEE-NH ₂
T162H	KMLKKRSL H GVYVKMPPTPEE-NH ₂
G163S	KMLKKRSL S TVYVKMPPTPEE-NH ₂
V164D	KMLKKRSLTT D YVKMPPTPEE-NH ₂
K167N	KMLKKRSLTTGVY N MPPTPEE-NH ₂

BIAcore analysis

SPR experiments (time course of the SPR signal) were conducted at 25 °C with a BIAcore™ biosensor to analyse molecular interactions. The running buffer used for all of the experiments was 50 mM Tris/HCl (pH 7.9), 0.5 M NaCl, 1 mM DTT and 0.005 % polysorbate 20, and binding responses were measured in resonance units (RU). On the completion of each cycle, the surface was regenerated with a pulse of 100 mM HCl. Streptavidin was covalently coupled to a research grade CM5 sensor chip through its primary amine groups, using the Amino Coupling Kit (BIAcore) according to the supplied instructions. BIAcore streptavidin-coated chips were used where indicated in the Results section.

Binding analysis

Peptide C-terminal amides biotinylated at the N-terminus were purchased from Peninsula (San Carlos, CA, U.S.A.). Each peptide consisted of 23 residues and was based on the CTLA-4 C-terminal tail sequence CTLA-4(152–174), with single amino acid changes to that of CD28(152–174) (Tables 1 and 2). The biotinylated peptides were immobilized by injection of the peptide at 10 µg/ml over the streptavidin surface for 1 min at a flow rate of 30 µl/min. Prior to each cycle, a fresh injection of biotinylated peptide at 10 µg/ml was made to ensure a consistent and saturated amount of the biotinylated peptide. When conducting experiments with multiple cycles over the same surface an identical sample was injected at the start and end of the experiment to demonstrate a consistent degree of binding throughout.

Crystallization and data collection

Complex crystals were grown at 15 °C by using vapour diffusion techniques under conditions similar to those described in Owen and Evans [17]: the drops contained a 1:1 (v/v) mixture of complex solution [6 mg/ml protein and 1 mM 11mer peptide

Table 3 Summary of crystallographic analysis

RMSD, root-mean-square deviation.

	Overall	Highest shell
Data collection statistics		
Space group	P6 ₄	
Cell dimensions (Å)	a = b = 126.8, c = 74.2	
Resolution (Å)	48.2–3.6	3.8–3.6
R _{merge} (%)	13.3	35.4
Number of measurements	14633	2081
Unique reflections	6681	992
Completeness (%)	83.2	85.9
Multiplicity	2.2	2.1
I/sigI	4.0	1.7
Refinement statistics		
Resolution (Å)	8.0–3.6	3.8–3.6
R-factor (%)	27.8	62.6
R _{free} (%)	31.7 (603 reflections)	56.6
Model parameters		
Average B for protein atoms (Å ²)	39.1	
Average B for peptide atoms (Å ²)	50.8	
Residues in disallowed regions of the Ramachandran plot	none	
Deviations from ideality		
RMSD bond lengths (Å)	0.008	
RMSD bond angles (°)	1.4	

(TTGVYVKMPPT) solution in 20 mM Hepes (pH 7.5) and 1 mM DTT and reservoir buffer [0.1 M Mes (pH 6.2–7.0), 1.4–2.4 M NaCl, 0.4 M sodium/potassium phosphate and 15% (v/v) glycerol]. The crystals are isomorphous with those described in Owen and Evans [17]. X-ray diffraction data to 3.6 Å (1 Å = 0.1 nm) resolution (Table 3) were collected at the Synchrotron Radiation Source, Daresbury, U.K. (station 9.6; wavelength 0.87 Å). Data were collected with a CCD (charged couple device) detector (Area Detector Systems Corporation, Poway, CA, U.S.A.) at 100 K from a single crystal using the crystallization mixture as cryoprotectant. These images were evaluated using MOSFLM [18a]. The CCP4 suite of programs [19] was used for the data reduction and subsequent data handling.

Structure determination, model building and crystallographic refinement

The structure was determined using difference Fourier techniques using the co-ordinates of the published AP50 structure (PDB code 1BXX [17]), omitting the TGN38-derived peptide. The resulting difference electron density maps showed the location of the CTLA-4-derived peptide. The new peptide was modelled into density using the program Quanta98 (Molecular Simulations, San Diego, CA, U.S.A.). Out of eleven peptide residues ten were ordered and clearly visible in the electron density maps. Only weak electron density was visible for the side chain of Lys¹⁶⁷, and the side chains of Thr¹⁶¹ and Thr¹⁶² were also less well ordered and were difficult to model accurately. The structure was subjected to torsion angle refinement and a few cycles of positional and grouped B-factor refinement, with a bulk-solvent correction applied using the programs X-PLOR and CNX [20,21]. R_{free} was carefully monitored to avoid over fitting. The final model comprises protein residues 166–218, 262–375 and 384–435, and the 10 peptide residues TTGVYVKMPPT, missing

the final C-terminal threonine residue. Peptide mutations were modelled using the program Quanta 98 (Molecular Simulations, Inc.) using the most common rotamers for the side chains.

RESULTS AND DISCUSSION

Expression and purification

At harvest, tAP50 accounted for 24% of the total microbial protein of which approx. 35% was in the soluble fraction (Figure 1, lane A). The soluble tAP50 was purified using Ni²⁺-nitrilotriacetate (Figure 1, lane B) and gel-filtration chromatography (Figure 1, lane C). SDS/PAGE under reducing conditions indicated that the final product was approx. 90% pure (Figure 1, lane C). There is a minor species at 70–80 kDa. However, this represented less than 10% of the total and there was no evidence for such a species under native conditions on an analytical gel-filtration column (Figure 2). It may be that the 70–80 kDa species seen on SDS/PAGE was an artefact of the conditions used, but in any case it only represented a minor species. The gel-filtration column showed a major peak with a molecular mass of

Figure 1 Expression and purification of tAP50

SDS/PAGE analysis with Coomassie Brilliant Blue staining for visualization. Tris/glycine Novex pre-packed cassette gel (4–20%) showing: lane A, *E. coli* lysate supernatant; lane B, nickel chelate purification (step 1), elution pool in 0.2 M imidazole; and lane C, tAP50 after gel-filtration chromatography (step 2). Molecular-mass markers are indicated on the left-hand side (in kDa).

Figure 2 Analytical gel-filtration chromatography of purified tAP50

The sample was analysed on a Pharmacia Biotech SMART system and a Superdex 75 PC 3.2/30 column using 50 mM Tris/HCl (pH 7.9), 0.5 M NaCl and 1 mM DTT as the buffer. tAP50 is retained as a single peak at 30 min with an estimated molecular mass of 34 kDa compared with standard proteins (results not shown).

Figure 3 SPR of binding of tAP50 to phosphorylated and non-phosphorylated peptides

Streptavidin (1650 RU) was immobilized on flow cell 1 and tAP50 (4 μ M) was injected at a flow rate of 30 μ l/min over this surface to give a control sample. Biotinylated non-phosphorylated peptide (25 μ g/ml) (1A) was bound to flow cell 1 and the tAP50 injection was repeated. Streptavidin was then immobilized (1480 RU) to flow cell 2 and used to bind phosphorylated peptide (1B). A further identical tAP50 sample was injected over this surface.

34 kDa along with a shallow peak eluting later on, consistent with a very low molecular mass material. The purity was therefore considered to be adequate for BIAcore studies.

On analysis of purified tAP50, using analytical gel-filtration chromatography, a single peak was observed with a molecular

mass equivalent to approx. 34 kDa (Figure 2). This indicates that tAP50 is a monovalent protein, essential for valid affinity and kinetic measurements [22]. Identity was confirmed by MS and N-terminal sequencing.

BIAcore analysis

A binding comparison of tAP50 with biotinylated peptides CTLA-4(152–174) and [Y165pY]CTLA-4(152–174) (where pY stands for phospho-tyrosine) (Table 2) using SPR is shown in Figure 3. tAP50 was initially injected over a control surface with only streptavidin immobilized. A small increase in response (approx. 25 RU) was observed during the injection phase of tAP50, due to the change in refractive index of the injected sample compared with the running buffer. tAP50 was then injected over the streptavidin surface to which a biotinylated peptide had been bound. Clear binding of tAP50 was observed with non-phosphorylated peptide CTLA-4(152–174) (Figure 3). A rapid increase of tAP50 to a binding maximum of 540 RU was reached within 30 s, and during the dissociation phase half of the tAP50 was released after just 12 s. Both characteristics indicate fast-binding kinetics with rapid association and dissociation rates. SPR had previously been used to examine the interaction between AP50 and peptides based on the cytoplasmic tail of TGN38 [23]. These authors [23] examined the binding of native AP-2 complex to a fusion protein of glutathione S-transferase fused to a triple repeat of the sequence SDYQRL which contains

Figure 4 Measuring the affinity of tAP50 binding to CTLA-4 non-phosphorylated peptide by equilibrium binding

(A) tAP50 at 6, 3, 1.5, 0.75 and 0.375 μ M was injected sequentially for 1 min at 30 μ l/min over a control streptavidin-immobilized surface (1300 RU) (no peptide bound). (B) This was repeated over the same flow cell with a fresh injection of CTLA-4 peptide at 10 μ g/ml for 30 s between each tAP50 injection. (C) The equilibrium response at each concentration for the control was measured ($\blacktriangle$) and subtracted from that of the peptide binding ($\blacksquare$) to give corrected actual binding ($\square$). The actual binding yields a K_d of 0.5 μ M and a maximum binding of 940 RU. (D) A Scatchard plot of the same data with a linear regression fit yields a K_d of 0.4 μ M and shows a maximum binding of 960 RU.

Figure 5 Ribbon diagram of the crystallographic dimer of AP50 with bound CTLA-4 peptide

The peptides form additional β -strands, forming one continuous β -sheet across the dimer. The peptides are shown in all-atom representation. The N- and C-termini of the protein and peptide chains are indicated. The Figure was generated using BOBSCRIPT [28].

the YXX Φ motif from TGN38. In their case the dissociation rate was too low to be determined. This very low observed dissociation rate may have been due, in part, to multivalent interactions reflecting avidity rather than affinity. This seems a possibility as not only was a triple repeat of the sequence SDYQRL used but it was attached to a protein that is normally a dimer. It is also possible that the difference may be due to using full-length AP50 in a complete AP2 complex rather than truncated AP50 in isolation, as used in the present study.

In contrast with the clear binding we observed to the unphosphorylated peptide, tAP50 had little or no affinity for the same peptide phosphorylated on the tyrosine residue (Figure 3). This confirms previous studies in which little, or no, binding to CTLA-4 was observed when Tyr¹⁶⁵ in the cytoplasmic tail was phosphorylated [12,14,15]. As discussed in previous studies [14], this phosphorylation is likely to determine whether CTLA-4 is either present on the membrane to deliver its negative signal or internalized.

Affinity measurements

The affinity of tAP50 for CTLA-4(152–174) was determined using equilibrium binding analysis. This avoids the many problems associated with determining kinetic measurements

using SPR [24–26]. To determine a background response, increasing concentrations of tAP50 were injected over sensor surfaces to which streptavidin had been immobilized (Figure 4A). The binding response was determined by injections of tAP50 over the same surface to which the biotinylated peptide CTLA-4(152–174) had been bound (Figure 4B). For each tAP50 concentration, the corrected response was calculated by subtracting the background response from the binding response. A plot of these indicates saturable binding (Figure 4C). Fitting a standard Langmuir binding isotherm to the data gave a K_d of 0.5 μ M and a maximum binding of 940 RU (Figure 4C). A Scatchard plot of the data (Figure 4D) gave a K_d of 0.4 μ M and a maximum binding of 960 RU. The mean K_d ($\pm$ S.D.) value from six independent determinations was 0.7 ± 0.3 μ M.

Crystal structure of AP50 in complex with a CTLA-4-derived peptide

We have determined the crystal structure of AP50 in complex with a CTLA-4-derived peptide to 3.6 Å resolution (Figure 5). This represents the first structural observation of the interaction between AP50 and CTLA-4. Despite the limited resolution, the electron density maps clearly reveal the bound peptide, which can be readily modelled. To date, there have been no AP50 crystals that have afforded high quality, high resolution

Figure 6 Stereo view of the binding site for the CTLA-4 peptide using ball-and-stick representation

Stereo view of the binding site for the CTLA-4 peptide using ball-and-stick representation. Top panel: A $(2F_{\text{obs}} - F_{\text{calc}})$ electron density for the CTLA-4 peptide bound to AP50. Only selected residues from one monomer and residues Thr¹⁶¹ to Tyr¹⁶⁵ of the peptide are shown. The binding pocket for Tyr¹⁶⁵ is too small to accommodate a phosphorylated tyrosine residue. Bottom panel: Details of the interactions made by the two CTLA-4 peptides with the crystallographic dimer. Residues from the symmetry-related molecules are denoted by #. Modelling the T162H, G163S and V164D mutations indicates potential steric clashes. The Figure was generated using BOBSCRIPT [28].

diffraction. The overall crystal structure of the protein–peptide complex (Figure 5) is similar to those previously described for AP50–EGF receptor and AP50–TGN receptor complexes [17]. Details of the interaction are shown in Figure 6. Tyr¹⁶³ of the peptide interacts with the protein as described for the EGF and TGN receptor complexes and, as predicted, the hydrophobic pocket for the Y + 3 position easily accommodates Met¹⁶⁸ of the CTLA-4 (or CD28) receptor YXXΦ motif [17]. Notably, from peptide library screening studies, there is a strong preference for arginine at peptide position 167 [27]. In the published AP50 complex with the TGN38 receptor-derived peptide [17], Arg¹⁶⁷ forms hydrophobic interactions mainly with protein residue Trp⁴²¹, with its guanidinium group exposed to solvent and making a hydrogen bond between Ne and the carbonyl of Lys⁴²⁰. In the CTLA-4 peptide complex, there is only weak electron density for the lysine at position 167. It is likely that the hydrophobic interactions with Trp⁴²¹ are maintained, but no hydrogen bond can be formed with the carbonyl of Lys⁴²⁰, as is the case for the equivalent alanine of the peptide in the EGF

receptor peptide–AP50 complex [17]. Peptide residue Val¹⁶⁴ is in a hydrophobic pocket formed by Trp⁴²¹, Val¹⁶⁴ (from the symmetry-related peptide) and Leu¹⁶⁰ (this peptide residue is visible in a 3.7 Å structure of an AP50–20mer CTLA-4 peptide complex, results not shown). Gly¹⁶³ (of CTLA-4) packs quite closely against Arg⁴²³ (of AP50), indicating a preference for a small side chain at position 163. The orientations of side chains 161 and 162 of the peptide are difficult to model accurately, but Thr¹⁶² is within salt-bridging distance of Gln³¹⁸ (Figure 6).

Binding comparison using SPR and modelling data of tAP50 with CTLA-4 peptides with single amino acid changes to resemble CD28

AP50 binds CTLA-4 leading to a predominantly intracellular location, however, it does not bind to CD28, which is consequently localized exclusively at the cell surface. This behaviour exists despite the fact that both receptors contain the YXXΦ motif in their cytoplasmic tail. We used SPR measurements combined with modelling data to investigate this distinction.

Five peptides were made with single amino acid changes from the CTLA-4 residue to the equivalent residue in CD28 (Table 2). The rationale for choosing these five residues is as follows. Two previous studies had indicated that residues N-terminal to the YXXΦ might have an effect on binding to AP50 [12,29]. Thus four of the changes were made to analyse the effect on binding to AP50 of residues N-terminal to the YXXΦ motif in CD28. The fifth change, K167N, was made because of previous studies that suggested the Y + 2 residue can play a role in recognition [27,30]. In addition, the crystal structure of AP50 with the peptide DYQRLN showed significant interactions between the arginine at Y + 2 and AP50 [17]. No changes were made at position 166 because of previous work that indicated that AP50 had little preference at this position [30], and in the published structure there was no significant interaction between Y + 1 and AP50 [17].

tAP50 was then injected at three different concentrations over the five biotinylated peptides immobilized on streptavidin (Figure 7). In the same experiment, tAP50 was also injected over a control surface with no peptide and over peptides CTLA-4(152–174) and [Y165pY]CTLA-4(152–174). The results were compared with modelling the mutations into the AP50–CTLA-4, protein–peptide complex X-ray structure (Figure 6). As described in the SPR experiments above, phosphorylation of Tyr¹⁶⁵ reduced the binding to levels only slightly above background (Figure 7). On modelling, it is clear that, when phosphorylated, the tyrosine residue would be unable to fit into the tyrosine pocket, as reported for the AP50–EGF receptor and AP50–TGN38 protein–peptide interactions [17]. The T161L mutant had almost no effect on SPR binding (Figure 7), in good agreement with our modelling results. In contrast, modelling the T162H change indicates a steric clash with the side chain of Gln³¹⁸. This has been confirmed by SPR in which the T162H change almost completely abolished binding to AP50 to levels similar to the phosphorylated peptide. The three changes G163S, V164D and K167N all reduced binding to a similar degree, between 30–50% of that of the non-mutated CTLA-4 sequence (Figure 7). Of these, the larger effect was observed with V164D, followed by K167N and then G163S. Modelling indicates potential steric clashes of G163S and V164D. Our data suggest that these steric clashes at positions 163 and 164 would both give a modest reduction in affinity, rather than abolishing binding altogether, in agreement with SPR data. The K167N SPR result gave a response that was less than 50% of that with CTLA-4. Although this is a consistent observation, it is difficult to explain

Figure 7 Binding of tAP50 to different peptides, demonstrating the effect of changing a single CTLA-4 residue to a corresponding CD28 residue

All experiments were performed at 25 °C and at a flow rate of 30 μ l/min. To ensure equivalent levels of streptavidin on the chip surface, SA chips (BIAcore®) (preimmobilized with streptavidin) were used. Background effects with each concentration of tAP50 were determined for each new chip prior to injection of biotinylated peptide. Corrected maximum binding response was calculated by subtracting the background response from the maximum binding measured after injection for 1 min.

why this should be by modelling, due to the low density for Lys¹⁶⁷.

A similar type of study has previously been performed by Bradshaw et al. [12], in which several chimaeric molecules were made between CTLA-4 and CD28, in addition to some point mutations, but generally not to the equivalent residues in CD28. The interaction with AP50 was then analysed via a yeast two-hybrid assay linked to β -galactosidase activity.

Bradshaw et al. [12] changed Thr¹⁶¹ to alanine in the YXX Φ motif and like our data for this residue observed no corresponding effect. Residue Thr¹⁶² was changed to alanine and led to a 90% reduction in binding, which agrees well with our T162H mutant SPR and modelling data. Modelling indicated that Thr¹⁶² may form a salt bridge; mutation to alanine would result in loss of this salt bridge. In contrast, Bradshaw et al. [12], determined that changing Gly¹⁶³ and Val¹⁶⁴ residues abolished binding. Our SPR and modelling data show that these mutations lead to a significant steric effect; however, binding was not completely abolished. Changing the Lys¹⁶⁷ residue was noted to have a negative effect on binding by both Bradshaw et al. [12], and our SPR data. Although this is a consistent observation, as previously mentioned it is difficult to explain this by modelling, due to the poor electron density for Lys¹⁶⁷.

In summary, our data show that while several residues both within and outside the YXX Φ motif of CTLA-4 and CD28 can influence binding to AP50, the change of Thr¹⁶² in CTLA-4 to a histidine in CD28 is likely to be one of the most important reasons why CD28 does not bind to AP50 and consequently localizes to the cell surface.

We thank Dr Roger Camble for assistance. We also thank Mrs Rachael Rowlinson and Ms Janice Young for N-terminal sequencing, and Mr Derek Barratt for MS.

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Received 5 February 2001/11 June 2001; accepted 1 August 2001