Development and application of cytotoxic T lymphocyte-associated antigen 4 as a protein scaffold for the generation of novel binding ligands

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Abstract We have explored the possibilities of using human cytotoxic T lymphocyte-associated antigen 4 (CTLA-4) as a single immunoglobulin fold-based scaffold for the generation of novel binding ligands. To obtain a suitable protein library selection system, the extracellular domain of CTLA-4 was first displayed on the surface of a filamentous phage as a fusion product of the phage coat protein p3. CTLA-4 was shown to be functionally intact by binding to its natural ligands B7-1 (CD80) and B7-2 (CD86) both in vitro and in situ. Secondly, the complementarity determining region 3 (CDR3) loop of the CTLA-4 extracellular domain was evaluated as a permissive site. We replaced the nine amino acid CDR3-like loop of CTLA-4 with the sequence XXX-RGD-XXX (where X represents any amino acid). Using phage display we selected several CTLA-4-based variants capable of binding to human αvβ3 integrin, one of which showed binding to integrins in situ. To explore the construction of bispecific molecules we also evaluated one other potential permissive site diametrically opposite the natural CDR-like loops, which was found to be tolerant of peptide insertion. Our data suggest that CTLA-4 is a suitable human scaffold for engineering single-domain molecules with one or possibly more binding specificities.

Key words: Cytotoxic T lymphocyte-associated antigen 4; Phage display; Scaffold; Integrin

1. Introduction

Antibodies are the evolutionary solution to nature's requirement for binding ligands to combat disease. The immune system is composed of a huge repertoire of antibodies with diverse molecular surfaces, which are capable of recognizing essentially any antigen. The generation of small recombinant versions of antibodies is of fundamental importance to the field of targeted therapy and imaging [1,2]. Significant advances in this field have been made using protein engineering and display technologies, which allow the rapid isolation of recombinant antigen-specific antibodies in vitro. Large 'single pot' repertoires of recombinant antibody fragments have been generated, pre-dominantly using filamentous phage as the display vehicle [3] which provide a source of recombinant single-chain Fv or Fab fragments to any chosen target [4–6].

Despite single chain Fv antibodies (scFv) and Fab antibodies being the most frequently used binding ligands, they can be seen to have certain disadvantages. Firstly, they are composed of two domains, which require complicated cloning schedules for the pairing of antibody variable genes and in some cases may lead to the unstable association of the two polypeptide chains. Secondly, the restraining of randomized peptide sequences is complicated by the fact that immunoglobulins (Igs) carry six variable loops brought into close proximity by the association of two separate domains. It is apparent that simpler molecular scaffolds, based on a single domain, may offer potential advantages for combinatorial library engineering.

Efforts to study single Ig-domain molecules as binding ligands initially focused on the production of single VH (variable region of an antibody heavy chain) domains [7]. However, these molecules generally have a tendency to aggregate due to the exposed hydrophobic surface normally buried at the VH/VL (variable region of an antibody light chain) interface [8,9]. There are, however naturally occurring single domain molecules based on the immunoglobulin V_HH domain from camelids, which are devoid of light chains [10]. Initial problems with the production of human single VH domains have been addressed by 'camelizing' which involves mimicking residues in the naturally occurring single domain camel V_HH antibodies on a human framework [11]. A number of novel single domain scaffolds have been described which have a defined structure in which permissive sites have been identified. These include protein Z, tendamistat (for a review see [12]), fibronectin [13] and lipocalin [14]. The properties of a scaffold which could be deemed important are that it should; (i) be human if therapeutic applications are envisaged; (ii) be a small single domain molecule and be amenable to engineering into multivalent or multispecific molecules; (iii) have a known three-dimensional structure to allow easy loop modeling; (iv) fold correctly and be stable both in vitro and in vivo and (v) have permissive site(s)/surfaces for introducing diversity which are sufficiently large for high affinity binding and which have the potential to be affinity/specificity matured.

The Ig protein fold has been used by nature to solve many

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Abbreviations: CTLA-4, cytotoxic T lymphocyte-associated antigen 4; CDR, complementarity determining region; Ig, immunoglobulin; VH, variable region of an antibody heavy chain; VL, variable region of an antibody light chain; ScFv, single chain Fv antibody

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of the requirements of biomolecular recognition. The Ig fold occurs in functionally diverse proteins which includes matrix proteins, receptors, chaperones, enzymes and of course antibodies (for a review see [15]). In searching for a small human molecule based on an Ig fold that will be amenable to engineering of its ligand binding site, we have evaluated cytotoxic T lymphocyte-associated antigen 4 (CTLA-4) [16,17]. CTLA-4 is normally present on the surface of T-cells and mediates their activity by interactions with its co-receptors B7-1 and B7-2 on antigen-presenting cells [18].

In contrast to Igs, the CTLA-4 extracellular domain has two intra-molecular disulfide bridges and can exist as a single domain. Albeit, on the surface of T-cells it exists pre-dominantly as a disulfide-bridged homodimer [19,20]. The production of monomeric CTLA-4 extracellular domain has been reported previously [20,21] and required the removal of the C-terminal cysteine residue employed in the dimerization on the surface of T-cells. The three dimensional structure of CTLA-4 has recently been elucidated [22] and has confirmed that despite dimerization on the surface of T-cells, CTLA-4 exists essentially as two single domain molecules which do not interact with each other via a hydrophobic face as is the case for Fv-type molecules. The existence of three naturally occurring loops analogous to complementarity determining region (CDR) loops present in Igs suggests that it would be structurally feasible to replace these loops with heterologous sequences.

In this study we have generated a phage displayed repertoire of CTLA-4 variants in which the CDR3-like loop has been replaced by an RGD motif which mediates binding to integrin, presented at the apex of a randomized sequence. From this repertoire CTLA-4 variants specific for αvβ3 integrin were selected. Furthermore, with the aim of building bispecific single Ig-domains, a loop opposite the natural binding surface of CTLA-4 was also investigated as a potential permissive site.

2. Materials and methods

2.1. Construction of a recombinant CTLA-4 scaffold molecule, mutant derivatives and repertoires for display on filamentous phage

The sequence corresponding to the extracellular part of the human CTLA-4 (residue A^{1}MHV–DPEP^{120}) minus the C-terminal cysteine residue (C^{121}) was cloned into the phage display vector pCES-1 [6]. Human CTLA-4 was amplified by polymerase chain reaction (PCR) from 0.1 ng of pBSK(+)hCTLA-4 (Innogenetics, Gent, Belgium) using primers CTLA-4 (reverse) (5′-GGCATGCAATGCAGTGCACAGGCAATGCACGTGGCCCAGCCTGCT-3′) and CTLA-4 (forward) (5′-GCCGCAGTTGCAAGCGGCCGCCGGTTCTGGATCAA TTACATAAAT-3′) to append *Apa*L1 and *Not*1 restriction sites. The PCR product was cloned into pCES-1 and transformed into the *Escherichia coli* strain TG1.

The SGGGS sequence was inserted into the loop A′B (β strands are designated according to [22]) between S^{14}R. The pCES-1 CTLA-4 template was amplified using primers #11209 (5′GATTACGCCAAGCTTTGGAGC-3′) and #11071 (5′ GTCACCACGAG AGCCACCGCCACCGCTGCTGGCCAGTACCACAG-3′) which amplifies the 5′ end of the CTLA-4 gene. A second PCR was performed using primers # 11072 (5′-CGTGGT GACGGTGGCGGTGGCTCTCG AGGCATCGCCAGCTTTG-3′) and #11210 (5′-ATGCGGCCCCATTCAGATC-3′) which amplifies the 3′end of the CTLA-4 gene. Both products were gel purified and an additional PCR with primers #11209 and #11210 was performed. The resulting PCR fragment was digested with *Apa*L1 and *Not*1 and cloned into pCES-1 CTLA-4 to give pCES-1 CTLA-4 (A′B).

For the construction of a CTLA-4 (X_3-RGD-X_3) repertoire, a fragment corresponding to CTLA-4 was recovered as an *Apa*L1/*Not*1 fragment and was subjected to PCR with oligonucleotides CTLA-4 (reverse) and CTLA-4 (RGD) (5′-AATCTGGGTT CCGTTGCCTATGCCMNNMNNMNNATCGCCACGMNNMNNMNNCTCCACCTTGCAGATGTAGAG-3′). The PCR product was then purified and reamplified with CTLA-4 (reverse) and CTLA-4 (AMP) (5′-GCGGCCGCCGGTTCTGGATCAATTAC ATAAATCTGGGTTCCGTTGCC-3′). This fragment DNA was purified, digested with *Apa*L1 and *Not*1, cloned into pCES-1 and transformed into *E. coli* TG1. All CTLA-4 scaffold derivatives were confirmed by DNA sequencing.

2.2. Selection of phage displayed CTLA-4 RGD repertoire

Immunotubes (Nunc) were coated overnight at 4°C with 10 μg/ml of αvβ3 integrin (Chemikon) in coating buffer (0.1 M carbonate buffer, pH 9.6). After three washes with phosphate-buffered saline (PBS; 0.15 M NaCl, 8 mM Na_2HPO_4 and 1.56 mM KH_2PO_4, pH 7.4) tubes were blocked with 2% dried skimmed milk in PBS (2% M-PBS) for 1–2 h at room temperature. Selection of the (X_3-RGD-X_3) repertoire was performed as in [6].

2.3. Screening of selected clones by bacteriophage and soluble enzyme-linked immunosorbent assay (ELISA)

Clones from the second and third round of selection were picked randomly and both soluble molecules and phage displayed molecules were produced as described previously [5].

For initial detection of positive binding phage clones, ELISA plates were coated with 1 μg/ml αvβ3 integrin (Chemikon), 1μg/ml B7.1 Ig (Innogenetics) and 1 μg/ml bovine serum albumin (BSA) and incubated overnight at 4°C in coating buffer. To test for binding to B7, ELISA was performed on B7-1 Ig, B7-2 Ig (B7 fused to Fc fragment) and B7-2 His (B7.2 fused to histidine tag), purified from baculovirus (Innogenetics) and coated onto ELISA plates at 5 μg/ml. Screening of soluble CTLA-4-derived molecules was performed in 96 well ELISA plates coated with antigens B7-1 Ig (1 μg/ml), αvβ3 integrin (1 μg/ml), B7-2 Ig (1μg/ml) and B7-2 His (10μg/ml) as in [5].

2.4. Protein purification sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and Western blot analysis

Protein samples were analyzed by SDS–PAGE through 12.5% Tris/glycine gels. Samples for Western blotting analysis were transferred to nitrocellulose membranes. Prior to immunostaining, blots were incubated with PBS plus 0.1% Tween and 2% non-fat milk powder (M-PBST). Antibodies for immunoblotting were diluted in M-PBST to 1 μg/ml for the anti-CTLA-4 antibody (Innogenetics) and 1:2000 for anti c-Myc. Membranes were washed with PBS and PBS plus 0.1% Tween and visualized using staining with diamino benzidine (0.5 mg/ml diamino benzidine in 0.05 M Tris pH 7.6 and 0.015% H_2O_2).

Purified proteins were prepared as in [5] and analyzed on a precalibrated Superdex 75 column (Pharmacia) on a Bio-Logic HPLC machine (BioRad). Fractions were collected and stored at −20°C for further analysis.

2.5. Binding of phage CTLA-4 derivatives to cells by flow cytometry

The binding of phage displaying CTLA-4 and CTLA-4 scaffold derivatives was measured by flow cytometry (Becton Dickinson, Oxnard, USA). The EBV-transformed cell line RPMI 8866 (Innogenetics) was used to test binding of wild-type CTLA-4 phage to cells expressing B7-1 and B7-2. About 500 000 cells were used per experiment. Cells were washed once in PBS and resuspended in 2% M-PBS plus 0.1% BSA and 0.01% azide. To test the binding of phage displaying CTLA-4 RGD scaffold derivatives to cell surface integrins we used flow cytometric analysis of human umbilical vein endothelial cells (HUVECS) [23].

3. Results

3.1. Display of the extracellular domain of human CTLA-4 on the surface of filamentous phage

We displayed the extracellular domain of CTLA-4 on the surface of filamentous phage. We used the variant from A^{1}MHV to DPEP^{120} (numbered according to [20]) minus C^{121}, which is required for dimer formation on the surface of T-cells, fused to the N-terminus of the phage coat protein

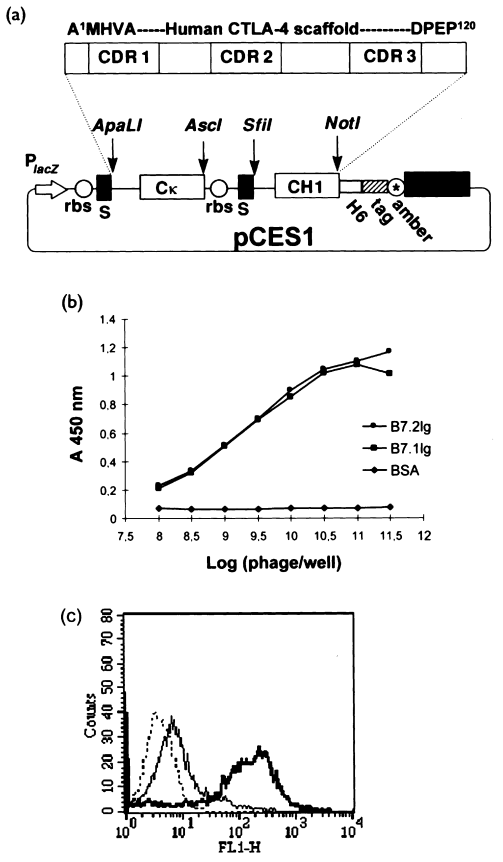

Fig. 1. Functional display of CTLA-4 extracellular domain. a: A figure of the phagemid vector construct displaying the extracellular domain of CTLA-4 from A^1MHV to $DPEP^{120}$, the backbone vector is pCES-1 [6] which contains the following features: antibody genes C_{H1} and C_κ of the constant heavy and light chains; PlacZ, promoter; rbs, ribosome binding site; S, signal sequence; H6, six histidines; tag, c-*myc*-derived tag; amber, amber codon that allows production of soluble Fab fragments in non-suppressor strains, gIII, gene encoding one of the minor coat proteins of filamentous phage. Restriction sites used for cloning are indicated. b: Binding of phage displaying the CTLA-4 scaffold is shown binding to its natural ligands B7-1 Ig (■) and B7-2 Ig (●) but not to the non-relevant antigen, BSA (♦). A graph of log (phage/well) versus absorbance at 450 nm is shown. Strong specific binding is seen decreasing proportionately with the amount of phage present (c). Binding of phage (5×10^{11} cfu/ml) displaying CTLA-4 to B7-1 and B7-2 present on a cell membrane. Flow cytometry on RPMI 8866, that strongly expresses both B7-1 and B7-2 is shown. The results show binding of wild-type CTLA-4 scaffold displaying phage (bold line) whereas phage derived from empty pCES-1 (thin line) showed non-specific binding and cells with only control showed autofluorescence (dotted line).

p3. Fig. 1a shows the phagemid CTLA-4 vector construct. The recent report by Nuttall et al. (1999) describing CTLA-4 as a scaffold molecule for the presentation of somatostatin describes a slightly shorter scaffold molecule from A^1MHV to $QIYV^{115}$ [24]. We have found that the inclusion of an extra five residues of the CTLA-4 extracellular domain has not affected the ability to display functional molecules on phage. ELISA with this CTLA-4 phage showed specific binding to B7-1 Ig and B7-2 Ig, furthermore, the signal decreased proportionally with the amount of phage present (Fig. 1b). Binding of soluble CTLA-4 product could also be seen in ELISA (data not shown). Further evidence of ligand-specific binding was shown by competition with soluble B7-1 Ig and B7-2 His (purified recombinant B7-2 having a histidine purification tag rather than an Fc) in ELISA (data not shown). This clearly demonstrated that CTLA-4 was displayed on phage and was correctly folded for recognition of its natural ligands B7-1 and B7-2. Immunoblotting showed a soluble product of the expected size of 16 kDa, detected using both antibodies to the c-*myc* epitope tag and a monoclonal antibody to CTLA-4 (data not shown, Fig. 5). A further confirmation of the high specificity of the CTLA-4 scaffold molecule was shown by binding to cells transfected with B7-1 and B7-2. Phage displaying the CTLA-4 extracellular domain showed strong binding whereas only background binding was seen with wild-type phage particles (Fig. 1c). Thus CTLA-4, despite being a glycoprotein in eukaryotic cells, can be expressed in a non-glycosylated form in *E. coli* and be functionally displayed by bacteriophage.

3.2. Design of a CTLA-4 scaffold library with a propensity to bind integrins

Both sequence alignment and structural analysis showed CTLA-4 to be most similar to Ig superfamily variable domains, with eight β strands providing the framework for three complementarity determining region-like loops, CDR1, CDR2 and CDR3 [22] (Fig. 2). We predicted that these loops would be ideal permissive sites for randomization and that the underlying protein framework containing two disulfide bridges would be sufficiently stable to accommodate amino acid changes. As a proof of concept we randomized the CDR3 loop of CTLA-4 ($L^{97}MYPPPYYL^{105}$) since mutational analysis had shown this loop as being almost exclusively responsible for binding to the natural ligands B7-1 and B7-2 [17]. We chose to replace the CDR3 of CTLA-4 with the RGD motif, which is known to be essential for binding to αvβ3 integrin [25], at the apex of a randomized loop. We replaced the nine amino acids of the CDR3-like loop of CTLA-4 ($L^{97}MYPPPYYL^{105}$) with XXX-RGD-XXX, where X represents any amino acid and X being encoded by (NNK) at the nucleotide level. Mutagenesis was restricted to nine amino acids to respect the sequence similarity to the natural

Table 1
Panning of the phage displayed CTLA-4-RGD scaffold library on αvβ3 integrin

Selection round	Input phage[a]	Output phage[a]	Input/output	Enrichment factor (fold)	Percentage binders (αvβ3)	Percentage binders (B7-1 Ig)
1	1e13	6.9e4	6.9a−9	–	nd	nd
2	1.9e13	3.1e5	1.6e−8	2.3	0	0
3	1.8e13	2.2e6	1.6e−7	10	65	0

nd: not determined.
[a]The input and output phage titrations are given as colony forming units (cfu).

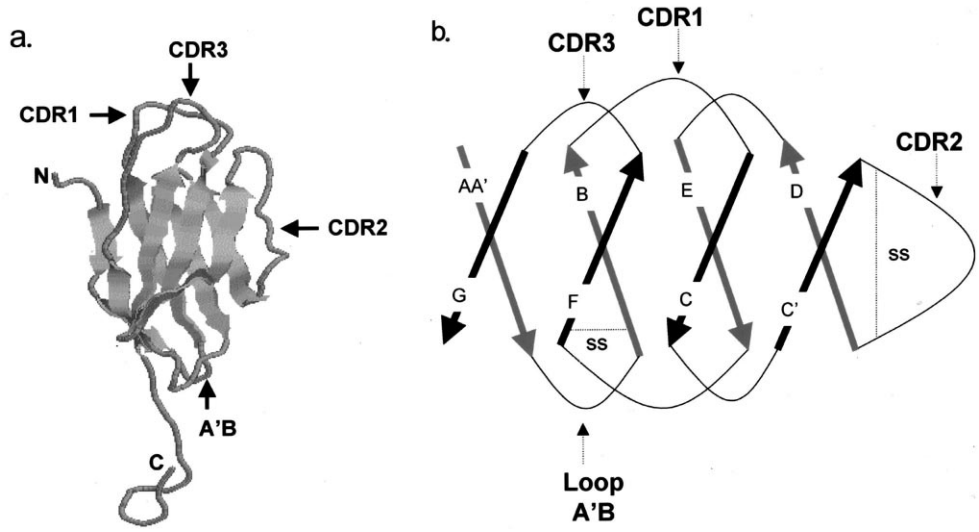

Fig. 2. Structure of CTLA-4 extracellular domain. a: Nuclear magnetic resonance structure of CTLA-4 [22] showing the position of the CDR-like loops, loop A′B and the N and C terminus of CTLA-4 (b). Schematic representation of the CTLA-4 scaffold molecule. The eight β strands are indicated as A, A′, B, C, C′, D, E, F and G. These anti-parallel β strands constitute two β sheets. The loops connecting the β strands are represented by a thin curved line. The natural CDR3-like loop of CTLA-4 is indicated and the loop A′B is indicated as a potential alternative permissive site on the opposite side of the CTLA-4 Ig fold to the natural binding surface. Both the Ig-type disulfide bridge and the extra disulfide bridge are also shown by the symbol SS. Adapted from [22].

CDR3-like loop. Based on this design, oligonucleotides were used to build a CTLA-4 (X_3-RGD-X_3) repertoire of 1.3×10^7 independent clones.

3.3. Selection of a phage displayed CTLA-4 (X_3-RGD-X_3) scaffold repertoire

The CTLA-4 scaffold repertoire was selected on purified human αvβ3 integrin. After three rounds of selection, 65% of clones showed binding to αvβ3 integrin with no detectable binding to B7-1 Ig or BSA (Table 1). Twelve binding clones were sequenced, revealing six different peptides in which the CDR3 (L^{97}MYPPPYYL^{105}) sequence was replaced by the RGD motif flanked by selected sequences (Table 2). Clone RGD-7B was the dominant clone being recovered five times, followed by clone RGD-8H which was recovered three times, all other clones were identified once in the group sequenced. Clone RGD-7B showed binding as a soluble product in our high throughput screening ELISA. We reasoned that this clone was our optimum binding clone for further analysis, as the other five clones did not give binding in this assay of low sensitivity (data not shown).

The clones RGD-8H, RGD-9B, RGD-8E and RGD-11B contained the RGD motif presented as expected, flanked by three different selected amino acids on either side. The clones, RGD-7B and RGD-11C, contained an RGD motif flanked by three selected amino acid residues on the 5′ side (of the RGD sequence) and five selected residues on the 3′ side (of the RGD motif). The is probably an artifact of the oligonucleotide synthesis but suggests that larger peptide sequences than the naturally occurring nine residue CDR3-like loop may be constrained within the CTLA-4 scaffold molecule.

Table 2
Selected heterologous RGD peptide sequences in the permissive CDR3-like loop of CTLA-4

Clone	Protein sequence		
Wild-type CTLA-4	L M Y	P P P	Y Y L
RGD-7B:	F L P	**R G D**	D Y P R R
RGD-8H:	I L D	**R G D**	S Y Y
RGD-11C:	V Q* G	**R G D**	S H P A I
RGD-9B:	H P C	**R G D**	C L A
RGD-8E:	K P D	**R G D**	Y A P
RGD-11B:	T T A	**R G D**	S D K
Fibronectin	V T G	**R G D**	S P A S S

The alignment of isolated clones which demonstrated binding to αvβ3 integrin. Wild-type CTLA-4 scaffold sequence corresponding to the CDR3 loop (L^{97}MYPPPYYL^{105}) is shown. The corresponding 9 and 11 amino acid RGD loop substitutions (shown in bold) for the six heterologous peptide sequences isolated, RGD-7B, 8C, 11C, 9B, 8E and 11B are shown. RGD-11C has an amber codon indicated by * which when suppressed becomes a glutamine residue. The relevant sequence of fibronectin responsible for binding to αvβ3 integrin is also indicated.

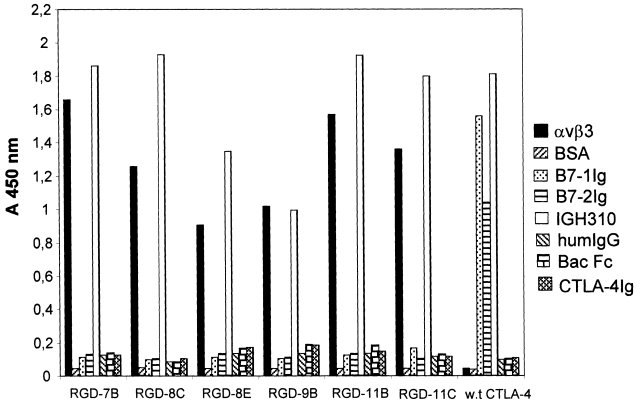

Fig. 3. Specificity ELISA of specific CTLA-4 scaffold derivatives. Specificity phage ELISA of CTLA-4 scaffold derivatives on a monoclonal antibody to CTLA-4 (IGH310), B7-1 Ig, B7-2 Ig, αvβ3 integrin, BSA, CTLA-4 Ig, human IgG and baculovirus produced Fc. Clones RGD-7B, RGD-8C, RGD-8E, RGD-9B, RGD-11B and RGD-11C are CTLA-4 scaffold variants presenting different RGD peptide loops in the CDR3-like loop of CTLA-4.

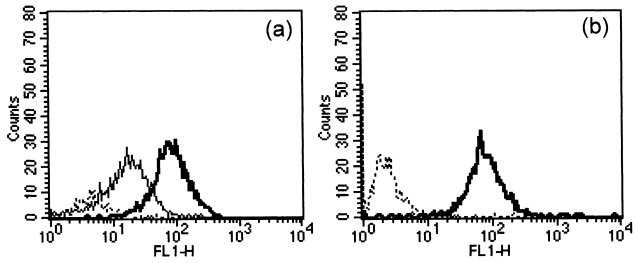

Fig. 4. Binding of CTLA-4 scaffold RGD containing clone to integrins in situ. Binding of phage (5×10^{11} cfu/ml) displaying RGD-7B molecule to (8.75×10^{4} cells) human umbilical vein endothelial cells using flow cytometry. a: Binding can be seen with phage prepared from RGD-7B (bold line). Phage displaying wild-type CTLA-4 showed some binding to a lower level than RGD-7B (normal line). Empty phage containing pCES-1 showed background binding (thin line). b: The positive control antibody LM609 also showed binding on HUVECS.

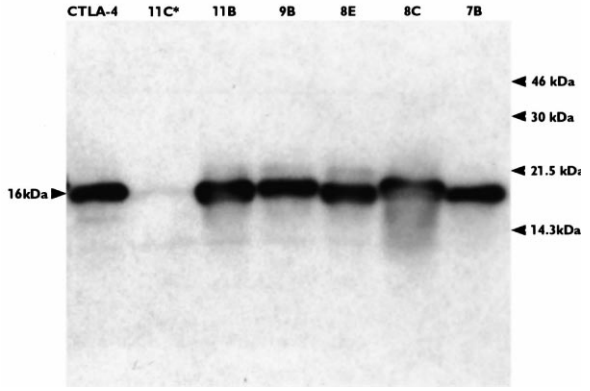

Fig. 5. Western blot analysis of CTLA-4 (RGD) scaffold variants. Periplasmic preparations of the CTLA-4 (RGD) scaffold variants in the non-suppressor strain HB2151. Detection with anti-c-Myc antibody of RGD-7B, RGD-8C, RGD-8E, RGD-9B, RGD-11B, RGD-11C* and wild-type CTLA-4 shows a product of the expected molecular weight of 16 kDa.

3.4. Analysis of the selected CTLA-4 RGD variants

To test the absolute specificity of the six different selected CTLA-4 (RGD) clones, ELISA was performed on αvβ3 integrin, BSA, B7-1 Ig, B7-2 Ig, CTLA-4 Ig, human IgG, baculovirus produced-Fc and a conformational-specific antibody to CTLA-4 (Fig. 3). The clones RGD-7B, RGD-8C, RGD-8E, RGD-9B, RGD-11B and RGD-11C were specific for αvβ3 integrin with no demonstrated residual binding to B7-1 Ig or B7-2 Ig. Furthermore, strong binding to a conformational-specific antibody indicated that the underlying protein framework and general structure of CTLA-4 appeared intact, despite the incorporation of a heterologous peptide sequence.

To test whether RGD-7B was able to recognize integrins in situ, we performed flow cytometric analysis with HUVECS. It can be seen that RGD-7B phage showed binding to HUVECS whereas wild-type phage showed only background binding (Fig. 4). Phage displaying wild-type CTLA-4 showed some binding which probably represents endogenous binding to B7 on HUVECS. This clearly demonstrates that CTLA-4 (RGD) variants can recognize cell surface integrins in their normal context.

The expression of soluble CTLA-4 (RGD) scaffold molecules was assessed by immunoblotting and it was seen that a homogeneous band of the expected size of 16 kDa was recovered for all clones except RGD-11C which gave no protein product (this was due to the stop codon present in this clone which was not suppressed in the bacterial strain HB2151 (Fig. 5). The wild-type CTLA-4 scaffold protein and the CTLA-4 variant, RGD-7B, were isolated on a larger scale from the bacterial periplasm and purified using immobilized metal affinity chromatography. The proteins were analyzed by gel filtration on a pre-calibrated Superdex 75 gel filtration column. Wild-type CTLA-4 recombinant protein gave predominantly two species corresponding to dimeric (approximately 30 kDa; 16.32 min) and aggregated material (11.24 min) (Fig. 6a). RGD-7B also gave dimeric (approximately 30 kDa; 16.16 min) and some aggregated material (11.31 min)(Fig. 6b), however, for RGD-7B there was an increase in the amount of dimeric material present relative to aggregated material. Only very low levels of monomeric protein could be detected. This is in agreement with the recent report of Nuttall et al. (1999) who noted that dimers were the dominant molecular weight species particularly when there was a delay before purification of periplasmic extracts [24]. The isolated peaks were analyzed by SDS–PAGE and it was shown that both dimers and aggregated material could be reduced to the monomeric form (data not shown).

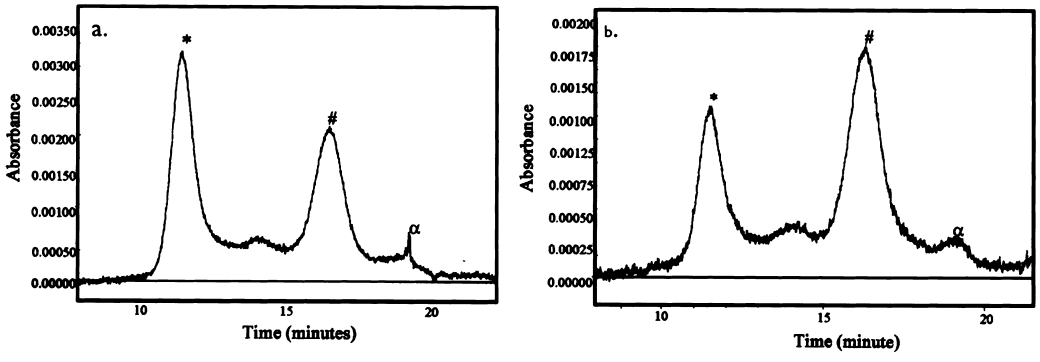

Fig. 6. Analysis of CTLA-4 and CTLA-4 variant RGD-7B by gel filtration. a: Gel filtration of metal chelate affinity-purified recombinant CTLA-4 variants on a pre-calibrated Superdex 75 column. For wild-type CTLA-4 scaffold molecule two dominant protein species are present corresponding to dimeric ('#') and aggregated material ('*'). b: RGD-7B also gave dimeric ('#') and aggregated material ('*'). Monomeric CTLA-4 protein production is indicated by ∝.

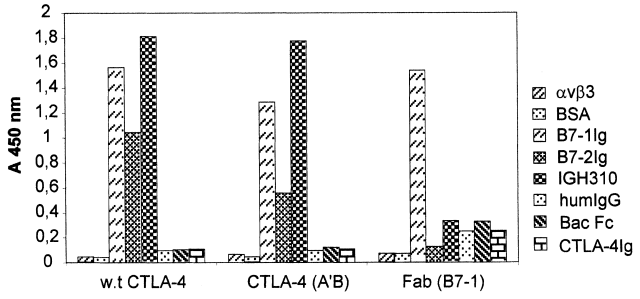

Fig. 7. Evaluation of loop A′B as a permissive site. Binding in phage ELISA of CTLA-4 (A′B) to anti-CTLA-4 (IGH310), B7-1 Ig, B7-2 Ig, αvβ3 integrin, BSA, CTLA-4 Ig, human IgG, baculovirus produced Fc. CTLA-4 (A′B) refers to the insertion of the sequence SGGGS into the loop A′B. Phage displaying unmodified CTLA-4 extracellular domain is shown by wild-type CTLA-4. Fab (B7-1) refers to control phage displaying a Fab antibody specific for B7-1.

3.5. Evaluation of a potential permissive site opposite the CDR-like loops

Graphical analysis of the CTLA-4 scaffold molecule suggested an alternative permissive site in CTLA-4 to the natural binding surface. The CTLA-4 molecule is stabilized by two disulfide bridges and, as such, may be tolerant of insertion or substitution at loops of the CTLA-4 framework opposite to the natural CDR-like loops. The loop formed by the β strands A′B (between $S^{14}R$) was chosen for investigation (Fig. 2). To validate this loop the relatively inert sequence SGGGS was inserted between residues $S^{14}R$ to give CTLA-4 (A′B).

To evaluate the structural and functional integrity of this molecule, we performed a specificity ELISA with phage displayed product on αvβ3 integrin, B7-1 Ig, B7-2 Ig, BSA, anti-CTLA-4, human IgG, baculovirus produced-Fc and CTLA-4 Ig. CTLA-4 (A′B) showed binding to the conformational-specific anti-CTLA-4 antibody and also showed binding to the natural ligands B7-1 Ig and B7-2 Ig (Fig. 7). This demonstrated that CTLA-4 (A′B) was most likely folded and displayed correctly, and despite peptide insertion within loop A′B, remained functionally intact.

4. Discussion

We report the successful phage display of the extracellular domain of CTLA-4. Furthermore, we describe the selection of CTLA-4 binding variants with integrin binding activity from a focussed repertoire and the definition of an alternative permissive site distinct from the natural binding surface of CTLA-4.

The expression of non-glycosylated CTLA-4 in *E. coli* has been shown previously to lead to aggregation [20]. CTLA-4 is normally a glycoprotein and mutation of the glycosylation site (N^{78}) leads to aggregation with an accompanying reduction in binding activity [22]. This suggested that glycosylation is essential for maintaining the monomeric status of CTLA-4. Both mammalian cells [20] and the yeast *Pichia pastoris* [21] have been used to produce glycosylated and functional monomers of CTLA-4 extracellular domain. We report here that the extracellular domain of CTLA-4 can be produced by secretion from *E. coli* and be displayed on the surface of filamentous phage. Furthermore it can be produced as a mixture of soluble monomers, dimers and aggregated material. The lack of a carbohydrate moiety on the hydrophobic BED face of CTLA-4 probably accounts for some of the aggregation. The substitution of the hydrophobic MYPPPY loop of CTLA-4 with a heterologous peptide, however, also shifts the equilibrium from aggregated material towards the dimeric form. Although both wild-type and CDR3-substituted CTLA-4 molecules showed clear binding in soluble ELISA, it is unclear if the active species is a dimer or the very low level of monomer that can be detected. This suggests that it is a combination of both the hydrophobic BED face of CTLA-4 exposed in the absence of glycosylation and the hydrophobic MYPPPY loop that lead to significant aggregation in prokaryotic expression systems which has also been reported by others. The successful display of CTLA-4 scaffold molecules on filamentous phage has presumably limited the aggregation and allowed the presentation of both structurally and functionally intact molecules fused to the phage p3 coat protein. The issue of whether CTLA-4 is a monomer or dimer displayed on phage is open to speculation and cannot easily be resolved. If dimers are displayed then their presence, if they are functional, could increase the selection efficiency as is often the case with dimerizing scFv antibodies. It should be emphasized that this issue has not prevented the use of CTLA-4 as a protein scaffold. Furthermore, we have clearly demonstrated that CTLA-4 repertoires can be generated from which lead binding molecules can be isolated for further downstream development.

We have semi-randomized the CDR3 loop of the CTLA-4 scaffold and showed that one can select novel molecules which bind to αvβ3 integrin, by means of constrained heterologous peptide sequences. We selected six different peptide sequences grafted into the CDR3-like loop of the CTLA-4 scaffold. The dominant clone was RGD-7B, which suggests that five residues on the 3′ side of the RGD motif may be preferential to three residues indicating that a larger loop can be constrained within the CTLA-4 than that of the natural CDR3 loop. This may be beneficial in that the generation of a large extended loop may have specific applications for binding into clefts present in enzyme active sites [25,26], and receptors, as is the case for camel V_HH antibodies. This is in contrast to Igs from other mammals, in which antibodies are selected for binding to larger flatter surfaces similar to those found on the surface of proteins [25]. Indeed, structure-based modeling of the extracellular domain of CTLA-4 has shown significant structural similarity with camel V_HH domain antibodies suggesting that loops from CTLA-4 could be grafted onto a V_HH framework and vice versa (data not shown).

Heterologous binding sequences within the CTLA-4 scaffold were recovered with identity to recognized αvβ3 integrin binding peptides and also to fibronectin, a natural ligand of αvβ3 integrin. The clone RGD-11C showed identical flanking residues (to the RGD motif) that exist in the natural target, fibronectin (Table 2), possibly indicating the relative independence of the peptide from the scaffold or structural conservation between fibronectin and CTLA-4 which are both members of the Ig superfamily. The residue C-terminal to the RGD motif in fibronectin is a serine residue and in three of the selected clones (RGD-8H, RGD-11C and RGD-11B) a serine residue is present in the equivalent position. This indicates that CTLA-4 is an excellent scaffold and has allowed the isolation of an RGD loop, which is homologous to the 'naturally' optimized loop within fibronectin. The CTLA-4 scaffold derivative, RGD-9B, is directly analogous to the RGD-

4C peptide isolated by the group of Ruoslahti [27], which specifies an RGD determinant constrained by two disulfide bridges. This peptide has been reported to be highly selective for αvβ3 integrin in vivo and it could be expected that the similarly constrained peptide within the context of the CTLA-4 scaffold (with the RGD motif constrained by one disulfide motif and also the CTLA-4 framework), will have similar specificity. The equivalence between selected peptide sequences, and peptide sequences selected within the context of CTLA-4 suggests that, in some cases, CTLA-4-derived peptide loops may bind as individual peptides.

We also describe an alternative permissive site which is separate from the natural binding surface of CTLA-4. Insertion of a five residue inert peptide sequence at the loop formed by β strands A and B′ did not affect binding to the natural ligand B7. The underlying Ig framework can be assumed to be intact as previous efforts to both isolate constrained peptide-based binding ligands based on the MYPPPY motif and to present within a permissive site of hirudisin, failed to generate B7 binding ligands [28]. Future experiments will involve the generation of repertoires with both an insertion and a replacement randomized loop so that ligands with the desired binding properties can be selected using display technology. The further development of this loop may allow the generation of a new type of bispecific molecule, which is analogous to the antibody-derived χ bodies described by [29].

The potential of CTLA-4 as a scaffold molecule for the generation of a new family of binding ligands is clear. Its human origin means that it is less likely to elicit an immune response than non-human binding ligands after therapeutic administration. The current development of CTLA-4-Ig for the treatment of host versus graft disease suggests easy engineering of CTLA-4 scaffold derivatives into immunoadhesins [30] and also demonstrates the potential to develop novel CTLA-4-based immunomodulatory reagents with modified binding activity and specificity. Furthermore, the engineering of multivalent, multispecific molecules based on a single Ig fold may overcome some of the difficulties associated with the production of higher-order derivatives of conventional antibodies such as diabodies and triabodies [8]. Future research will also determine whether binding molecules to diverse molecular targets can be isolated from a large 'single pot' non-biased repertoire. It will also be interesting to explore the possibility of certain target type complementarity. The structural similarity between CTLA-4 and camel V_HH domains suggests that engineered CTLA-4 derivatives may be suitable for binding to enzyme active sites or receptor binding pockets as is noted for camel V_HH single domain antibodies [25].

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