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Solid-phase Synthesis and Cyclization of a Large Branched Peptide from IgG Fc with Affinity for FcγRI

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Abstract: A solid phase approach has been used to synthesize a large branched disulphide peptide from IgG Fc, Ac-F-C*-A-K-V-N-N-K-D-L-P-A-P-I-E-K(Ac-E-L-L-G-G-P-S-V-F)-C*-I-NH_2. This peptide combines the lower hinge region of IgG and a proximal β-hairpin loop, both implicated in binding to FcγRI. Solid phase $Tl(tfa)_3$ cyclization of the linear branched peptide resulted in a poor yield of cyclic hinge–loop peptide (11%) most likely due to steric hindrance caused by the branch. However, if addition of the branch was preceded by solid phase $Tl(tfa)_3$ cyclization of the loop, the yield was excellent at 75%. Cyclic hinge–loop peptide was active in displacing IgG2a from FcγRI expressed on monocyte cell lines with an IC_{50} of 40 μM, whereas the linear form of this peptide was inactive. The Fc hinge–loop peptide demonstrates the potential for a non-mAb high affinity, immunomodulatory ligand for FcγRI. Copyright © 1999 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: FcγRI; Fc peptides; thallium trifluoroacetate; disulphide; branched cyclic peptide

INTRODUCTION

A high affinity ligand for the type I Fc receptor for IgG (FcγRI, CD64), which is expressed in high levels on monocytes and macrophages, would have a number of immunomodulatory applications, as has been demonstrated using mAb against FcγRI. Conjugation of an FcγRI ligand to vaccines or antagonistic T cell epitopes would allow targeting to APC cells and concomitant T cell activation or anergy respectively (reviewed in [1]). In addition, an FcγRI ligand could therapeutically block normal Fc binding to FcγRI in inflammatory disorders, such as the autoimmune disorder, immune thrombocytopenic purpura (ITP) [2].

Studies with mutagenized or chimeric antibodies have isolated two sites of the Fc portion of IgG which are involved in binding to FcγRI. These are a lower hinge region motif, and an adjacent β hairpin loop in the C_H2 domain of Fc, proximal to the hinge [3–10, and reviewed in 11]. Critical residues in the lower hinge are a Leu-Leu-Gly-Gly sequence (residues 234–237 in IgG1, 247–250 in IgG2a), and in the loop, a proline (residue 331 in IgG1, 350 in IgG2a). The highest affinity IgG isotype is murine IgG2a (Figure 1), a crystal structure of which has recently been published [12]. Using this structure as a template, we rationally designed a branched-disulphide peptide that combines the known critical residues of both regions (Figure 1a, b). In this hinge–loop peptide, lower regions of the β strands

Abbreviations: Acm, acetamidomethyl; APC, antigen presenting cell; ^{t}Bu, *tert*-butyl; DCM, dichloromethane; Dde, 1-(4,4-dimethyl-2,6-dioxocyclohex-1-ylidene)ethyl; DIEA, *N*,*N*-diisopropylethylamine; DMF, *N*,*N*-dimethylformamide; FACS, fluorescence activated cell sorting; FCS, foetal calf serum; Fmoc, 9-fluorenylmethoxycarbonyl; HPLC, high-performance liquid chromatography; MALDITOF-MS, matrix assisted laser desorption time of flight mass spectrometry; mAb, monoclonal antibody; PAL, tris(alkoxy) benzylamide linker; PyBOP, benzotriazole-1-yl-oxy-tris-pyrrolidino-phosphonium hexafluorophosphate; TFA, trifluoroacetic acid; $Tl(tfa)_3$, thallium trifluoroactetate; TNBS, 2,4,6-trinitrobenzene sulfonic acid; amino acid symbols denote the L-configuration; U937 and THP-1 are monocytic cell lines expressing FcγRI; *, denotes disulphide bonded cysteines.

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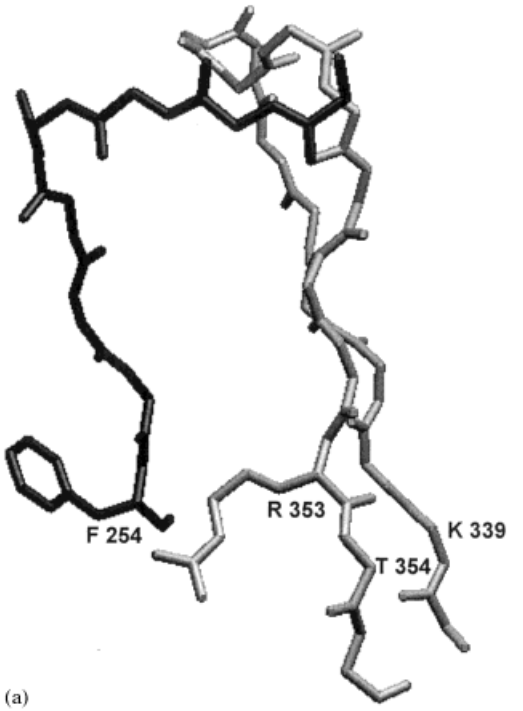
of the loop were included to allow a branched amide bond between the ε-amino of a lysine in the loop and the hinge peptide carboxy-terminus. The disulphide bond was placed at a non-hydrogen bonding position of the loop to improve the chance of β hairpin mimicry [13]. To promote solubility, the hinge peptide was from the human IgG sequence which has a Glu residue where muIgG2a has Asn.

To synthesize the hinge–loop peptide a solid-phase strategy was chosen. Cyclic disulphide peptides may be obtained in good yields and purities when oxidations are carried out on the solid-phase. This takes advantage of the pseudo-dilution phenomenon which favours intramolecular cyclization [14,15]. However, this strategy has mostly been applied to cyclic disulphide peptides of small to medium size. Our target peptide has an intramolecular disulphide involving 16 residues (50-membered ring), thus testing the applicability of the strategy to larger ring sizes [see also 16]. To form the disulphide bond we chose $Tl(tfa)_3$ as a mild oxidant, which despite its toxicity can give higher yields than alternatives such as iodine [17,18]. The absence of methionine residues made the peptide suitable for $Tl(tfa)_3$ oxidation [17,19–21]. The peptide was tested for binding to FcγRI expressed on monocyte cell lines.

EXPERIMENTAL PROCEDURES

General

Reagents: $Tl(tfa)_3$ (Sigma/Aldrich, UK), Fmoc-amino acids (PE Applied Biosystems, UK and Alexis Corporation, UK), PyBOP (Calbiochem-Novabiochem (UK) Ltd, UK), hydrazine monohydrate (Sigma/Aldrich), 1% TNBS/DMF and acetic anhydride (Fluka Chemicals, UK). All solvents (and DIEA) were purchased from Rathburn Chemicals, UK. Solid phase Fmoc/^{t}Bu methodology was used on pre-swelled Fmoc-PAL-PEG-PS resin (0.19 mmol/g) (PE Applied Biosystems) in DMF under continuous flow, on a Milligen 9050 machine programmed to perform Fmoc-amino acid/PyBOP/DIEA couplings for 45 min, and Fmoc deprotection reactions (in 20% piperidine in DMF, v/v) for 9 mins. Fmoc deprotection was monitored at 365 nm. Where required, couplings or acetylations were judged complete with



Lower Hinge

246 254

N-L-L-G-G-P-S-V-F (IgG2a murine)

E-L-L-G-G-P-S-V-F (IgG1 human)

Proximal Loop

338 350 355

F-K-C-K-V-N-N-K-D-L-P-A-P-I-E-R-T-I (IgG2a murine)

F-**C**-**A**-K-V-N-N-K-D-L-P-A-P-I-E-**K**-**C**-**I** (altered proximal loop)

Hinge-Loop Peptide

Ac-E-L-L-G-G-P-S-V-F

(b) Ac-F-C-A-K-V-N-N-K-D-L-P-A-P-I-E-K-C-I-NH_2

Figure 1 (a) The hinge–loop excised from a murine IgG2a crystal structure (1IGT.PDB [12]). The loop is shown in grey and the hinge in black. Residue positions involved in cyclization and branching are labelled. (b) The hinge–loop peptide and sequences used in its construction. Residues substituted in the IgG2a hinge–proximal loop for cyclization and branching are shown in bold, as are those in the hinge sequence to promote solubility. Residue numbering is with respect to IgG2a.

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TNBS [22]. Selective deprotection of Lys(Dde) was done under continuous flow, using 2% hydrazine/DMF (v/v) at 3 ml/min through a 1 cm diameter reaction column. Dde removal was monitored at 365 nm, confirmed with TNBS, and the peptide-resin then washed with DMF. Final cleavage from the resin and removal of TFA-labile protecting groups used a cocktail of 95% TFA/2.5% TIS/2.5% H_2O (v/v) for 2 h at r.t. Cleaved resin was removed by filtration, washed twice with 4 ml of neat TFA and the combined filtrates evaporated to 2 ml. Ice-cold diethyl ether (40 ml) was added to cause peptide precipitation and the mixture centrifuged at 1000 rpm for 5 min. The ethereal solution was decanted and four further diethyl ether extractions were performed. Analogously, small scale cleavages used 20 mg of dry peptide-resin and 2 ml of TFA cleavage cocktail for 2 h at r.t. The TFA–peptide filtrate and a further 2×1 ml TFA resin washes were reduced to ~ 0.25 ml with N_2 sparging. Peptide precipitation and four further ether extractions used 8-ml volumes of ice-cold ether. Analytical HPLC was performed on a Phenomenex Nucleosil 5 C8 300 (150×4.6 mm) column with gradients of solvents, A = 0.1% aq. TFA and B = 0.1% TFA/acetonitrile (v/v), run at 1 ml/min with 215 nm UV detection. Preparative HPLC used the same solvents on a Vydac C4 214TP1022 column (Hichrom Ltd, UK), run at 10 ml/min with 225 nm UV detection. The extents of reaction completions were estimated from the HPLC area. The accurate final yields of the hinge–loop peptide were determined by comparing the HPLC peak area of crude peptide to that of a standard of pure peptide of known concentration (determined by amino acid analysis of the pure peptide). Co-injection of pure peptide with crude material was used to assure the identity and integrity of the major product.

Preparation of Linear Loop Peptide

Ac-F-C(Acm)-A-K(Boc)-V-N(Trt)-N(Trt)-K(Boc)-D(O^{t}Bu)-L-P-A-P-I-E(O^{t}Bu)-K(Dde)-C(Acm)-I-PAL-PEG-PS. To 0.2 mmol of resin, Fmoc-amino acids/PyBOP/DIEA were sequentially coupled in the proportions 4/4/8 w.r.t. mmol peptide-resin. The first Ile was double-coupled to the resin, as were the Cys(Acm) residues which had low solubility in DMF. After removal of the final Fmoc group, the resin was transferred to a sintered funnel and manually acetylated with 20 ml 20% acetic anhydride/DMF (v/v), for 2 h with N_2 bubbling. Acetylation was judged complete with TNBS. The resin was washed $\times 3$ with DMF, DCM, and MeOH and dried under high vacuum for 5 h. This protected peptide-resin was used as starting material for the two synthetic routes to the hinge–loop peptide. The product of a small scale TFA cocktail cleavage of 20 mg of peptide-resin gave a major peak by HPLC (84% by HPLC area, gradient = 5–70% B in A over 25 min, $R_t = 17.85$ min). This peak was confirmed by MALDITOF-MS as the required material Ac-F-C(Acm)-A-K-V-N-N-K-D-L-P-A-P-I-E-K(Dde)-C(Acm)-I-NH_2 ($[M + Na]^+ = 2372$).

Synthesis Route 1 to Cyclic Hinge–Loop Peptide

Part 1. Oxidation with Tl(tfa)$_3$. Ac-F-C(Acm)-A-K(Boc)-V-N(Trt)-N(Trt)-K(Boc)-D(O^{t}Bu)-L-P-A-P-I-E(O^{t}Bu)-K(Dde)-C(Acm)-I-PAL-PEG-PS (0.065 mmol, 0.6 g) was suspended in DMF (10 ml) and treated with $Tl(tfa)_3$ (42 mg, 0.078 mmol, 1.2 equivalents) for 80 min at 0°C. The oxidized peptide-resin was washed with 3×20 ml DMF, and a sample of the oxidized peptide-resin was removed, washed, dried and TFA-cocktail cleaved (see small scale cleavage above). The resultant crude peptide gave two peaks by HPLC (10–60% B in A over 25 min). The first peak ($R_t =$ 18.8 min, MALDITOF MS $[M + H]^+ = 2353$) was the linear precursor and the second ($R_t = 20.7$ min) was the oxidized product (MALDITOF MS $[M + H]^+ =$ 2208). HPLC area indicated the oxidation was 70% complete. Therefore the reaction was completed with a further addition of $Tl(tfa)_3$ (42 mg, 0.3×0.078 mmol) for 80 min at 0°C. The oxidized peptide-resin was washed with 3×20 ml DMF, 3×20 ml DCM, 3×20 ml MeOH, and dried under high vacuum for 5 h. The final yield by HPLC area was 86%.

Part 2. Addition of hinge peptide to cyclic loop peptide. Ac-F-C*-A-K(Boc)-V-N(Trt)-N(Trt)-K(Boc)-D(O^{t}Bu)-L-P-A-P-I-E(O^{t}Bu)-K(Dde)-C*-I-PAL-PEG-PS (0.05 mmol, 0.457 g, * denotes disulphide) was swollen in DMF and selective deprotection of Lys(Dde) performed under continuous flow as described above. Amino acids were then sequentially coupled (Fmoc-amino acids/PyBOP/DIEA, 8/8/16 w.r.t. mmol peptide-resin) under continuous flow. The first hinge amino acid (Fmoc-Phe) was double coupled and a few beads of resin were TNBS tested to confirm coupling (colourless beads). After removal of the final Fmoc group, the resin was transferred to a sintered funnel and manually acetylated for 2×2 h with 10 ml 20% acetic anhydride/DMF (v/v) and N_2 bubbling. Acetylation was judged complete with TNBS. The resin was washed $\times 3$ with DMF, DCM, and MeOH and dried under high vacuum for 5 h. Final cleavage and isolation of the crude peptide was achieved using the

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TFA cocktail and diethyl ether trituration as above. Crude peptide mass yield (excluding resin sampling) was 101 mg, 72%. Analytical HPLC (20–50% B in A over 30 min) gave a very predominant peak ($R_t = 22.8$ min) of the expected MW (MALDITOF MS $[M + H]^+ =$ 2986). Accurate final yield of cyclic hinge loop was 75%. Preparative HPLC was performed using a gradient of 25–45% B in A over 30 min.

Synthesis Route 2 to Cyclic Hinge–Loop Peptide

Part 1. Addition of hinge peptide to linear form of loop peptide. Ac-F-C(Acm)-A-K(Boc)-V-N(Trt)-N(Trt)-K(Boc)-D(O^{t}Bu)-L-P-A-P-I-E(O^{t}Bu)-K(Dde)-C(Acm)-I-PAL-PEG-PS (0.068 mmol, 0.635 g), was swollen in DMF and selective deprotection of Lys(Dde) performed under continuous flow as described above. Amino acids were then sequentially coupled (Fmoc-amino acids/PyBOP/DIEA, 6/6/12 w.r.t. mmol peptide-resin) under continuous flow. The first hinge amino acid (Fmoc-Phe) was double coupled and a few beads of resin were TNBS tested to confirm coupling (colourless beads). After removal of the final Fmoc group, the resin was transferred to a sintered funnel and manually acetylated for 2 h with 20 ml 20% acetic anhydride/DMF (v/v) and N_2 bubbling. Acetylation was judged complete with TNBS. The resin was washed $\times 3$ with with DMF, DCM, and MeOH and dried under high vacuum for 5 h. The product of a small scale TFA cleavage of 20 mg of peptide-resin gave a major peak by HPLC (67% by HPLC area, gradient = 20–50% B in A over 30 min, $R_t = 20.25$ min). This peak was confirmed by MALDITOF-MS as the required material Ac-F-C(Acm)-A-K-V-N-N-K-D-L-P-A-P-I-E-K(Ac-E-L-L-G-G-P-S-V-F)-C(Acm)-I-NH_2, $[M + H]^+ = 3130$. Preparative HPLC was performed using a gradient of 25–40% B in A over 30 min.

Part 2. Oxidation with Tl(tfa)$_3$. Ac-F-C(Acm)-A-K(Boc)-V-N(Trt)-N(Trt)-K(Boc)-D(O^{t}Bu)-L-P-A-P-I-E(O^{t}Bu)-K(Ac-E(O^{t}Bu)-L-L-G-G-P-S(^{t}Bu)-V-F)-C(Acm)-I-PAL-PEG-PS (0.0295 mmol, 0.3 g) was suspended in DMF (5 ml) and treated with Tl(tfa)$_3$ (20 mg, 0.036 mmol, 1.2 equivalents) for 80 min at 0°C. The oxidized peptide-resin was washed with 3×20 ml DMF, and a sample of the oxidized peptide-resin was removed, washed, dried and TFA-cocktail cleaved (see small scale cleavage above). The resultant crude peptide gave three major peaks by HPLC (20–50% B in A over 30 min) indicating incomplete oxidation. MALDITOF MS of the lyophilized peaks showed that the peaks were: $R_t = 20.4$ min (39% by HPLC area) linear precursor, $R_t = 22.8$ min (10%) required cyclic peptide, $R_t = 25.24$ mins (16.5%) dimer. Repeated treatment of the peptide-resin with Tl(tfa)$_3$ (20 mg) for 80 min at 0°C, gave the same peaks with areas: $R_t = 20.4$ min (30%) linear precursor, $R_t = 22.8$ min (15%) required cyclic peptide, $R_t = 25.24$ mins (21%) dimer. Crude peptide mass yield (excluding resin sampling) was 75%. Accurate final yield of cyclic hinge loop was 11%.

Molecular Modelling

The structure of the IgG2a intact monoclonal antibody Mab231 was obtained from the Brookhaven Protein Data Bank, access code 1IGT [12]. Rasmol version 2.6-beta-2 (©Roger Sayle) and Sybyl version 6.1 (Tripos Inc., USA) packages were used for visualization and modelling of molecules.

Cellular Assays

U937 cells and THP-1 cells were cultured in RPMI with glutamax (Life Technologies, UK), 50 μg/ml gentamicin and 5% FCS at 37°C, 5% CO_2. Cells were supplemented with 100 U/ml or 150 U/ml recombinant human γ-interferon (R&D Systems) for 2 days before use for binding assays. Purified mouse IgG2aλ(HOПX-1) or huIgG4 (30 μg) (Sigma-Aldrich) were radioiodinated with ^{125}I (0.5 mCi; Amersham International) with chloramine T (50 μg) for 45 s, quenched with tyrosine (0.1 mg), and desalted on Sephadex G25 suspended in PBS/1% bovine serum albumin. Peptides (or huIgG1) in PBS/1% bovine serum albumin (0.3 ml) were incubated with 5×10^6 cells and ^{125}I-antibodies (2.5 μg/ml; 1.1×10^6/ml) at 0°C for 40 min. Cells were washed in PBS/1% bovine serum albumin (2×0.8 ml) by centrifugation at 1000 rpm for 10 min in a Sorvall RT6000D at 4°C, and counted in an LKB 1282 Compugamma. Means and standard deviations of triplicate assays were calculated.

5(6)-Carboxy-fluoresceine *N*-hydroxysuccinimide (0.015 ml of 10 mg/ml in DMF) was reacted with 0.3 mg IgG2a in 0.3 ml 0.2 M sodium phosphate (pH 8.6) for 2 h, quenched with glycine (1 mg) and desalted on Sephadex G25 in PBS/1% bovine serum albumin. THP-1 cells (0.5×10^6) were incubated with fluoresceine-IgG2a (0.67 μg/ml) and peptides on ice for 1 h, washed in PBS/1% bovine serum albumin, and incubated in 2% paraformaldehyde before analysis. FACS analyses were performed on FACScan flow cytofluorimeter (Becton Dickinson) using a 488 laser, and monitoring at 530 nm.

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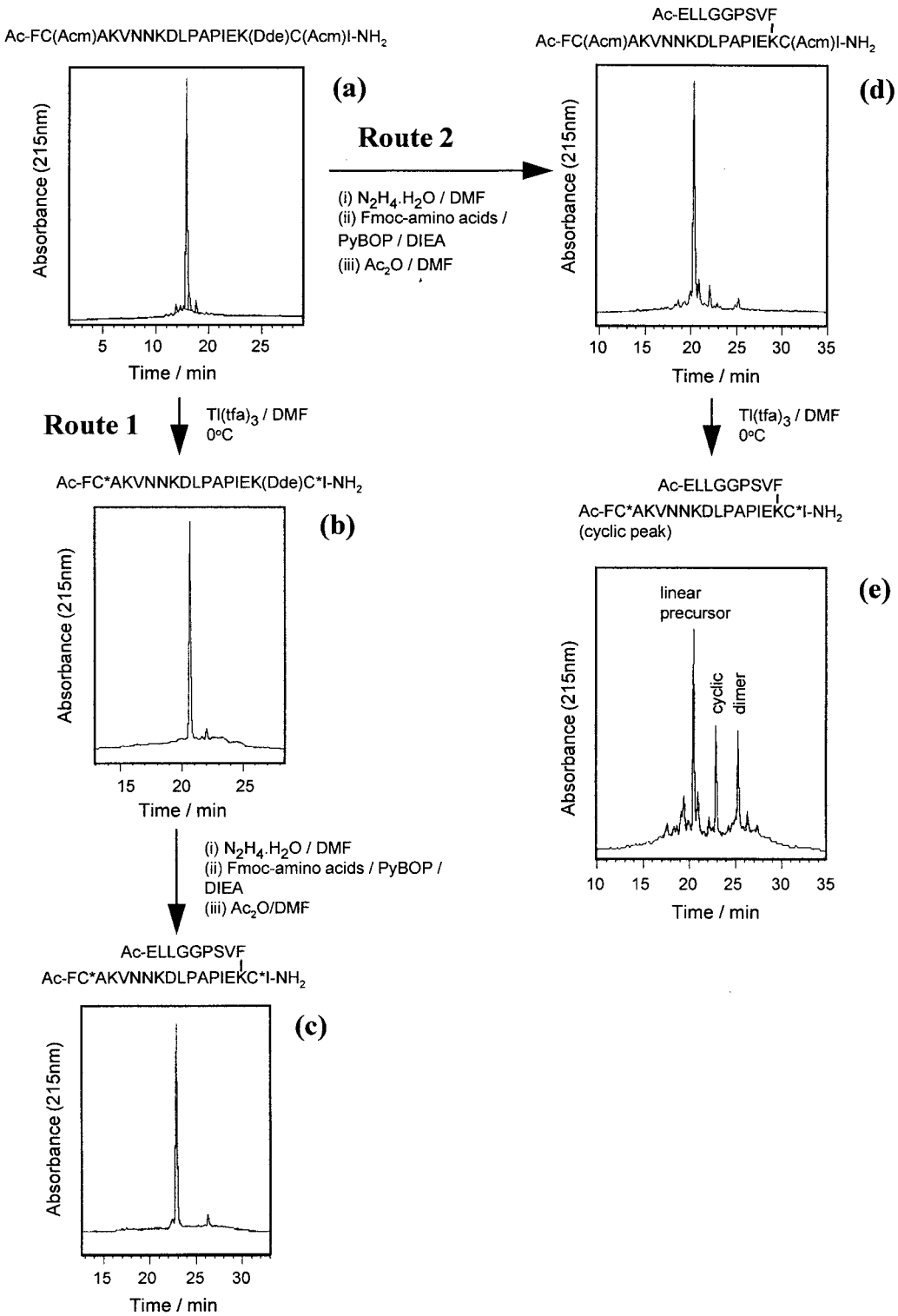

Figure 2 Schemes 1 and 2 for the synthesis of the cyclic hinge–loop peptide of Fc. Disulphide bonding cysteines are starred (*). The analytical HPLC of intermediates and final product is shown. Conditions: Phenomenex C8 (150 × 4.6 mm) column and HPLC buffers, A = 0.1% aq. TFA and B = 0.1% TFA/acetonitrile (v/v) run at 1 ml/min. Gradients: (a) 5–70% B in A over 25 min, (b) 10–60% B in A over 25 min, (c), (d) and (e) 20–50% B in A over 30 min. Intermediates and final products were confirmed by MALDITOF-MS.

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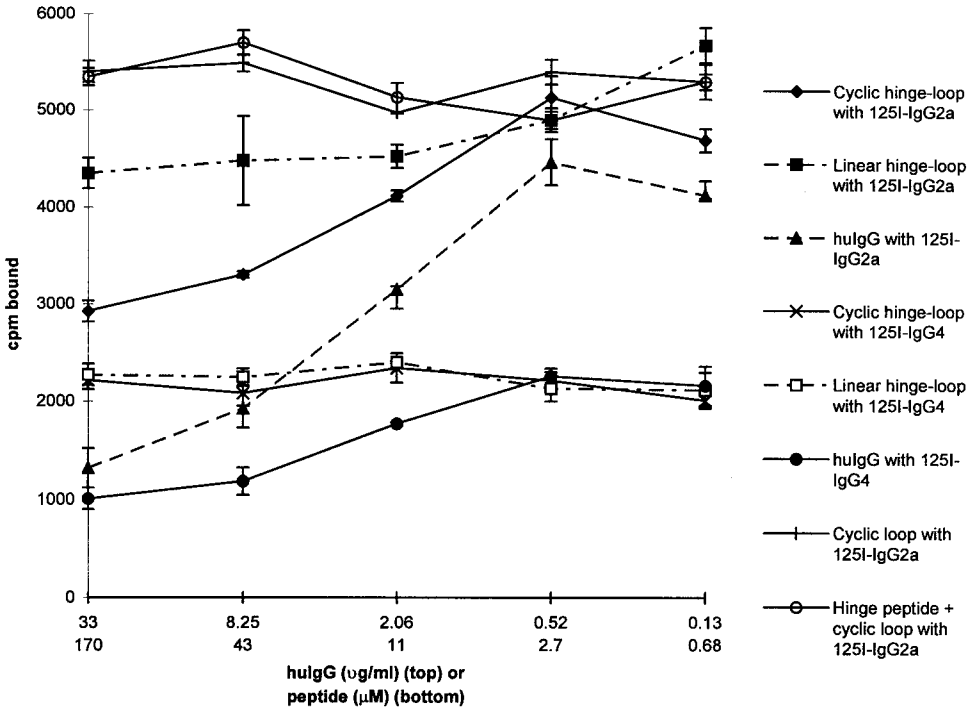

Figure 3 Binding of peptides to FcγRI on U937 cells previously incubated with γ-interferon was measured by competitive displacement of ^{125}I-mIgG2a or ^{125}I-huIgG4.

RESULTS AND DISCUSSION

The two synthetic routes to the hinge–loop peptide are outlined in Figure 2 with analytical HPLC traces of the final product and intermediates. Both strategies used Fmoc/^{t}Bu methodology on the TFA-labile PAL linker for peptide assembly and solid-phase Tl(tfa)$_3$ oxidations in DMF at 0°C in accordance with Reference [18]. Residues of the hinge peptide were sequentially coupled after selective deprotection of Lys(Dde) in the loop peptide. Intermediates and final products were confirmed by MALDITOF-MS (see 'Experimental Procedures').

The starting point for both synthetic routes was fully-protected linear loop peptide-resin Ac-F-C(Acm)-A-K(Boc)-V-N(Trt)-N(Trt)-K(Boc)-D(O^{t}Bu)-L-P-A-P-I-E(O^{t}Bu)-K(Dde)-C(Acm)-I-PAL-PEG-PS. This intermediate was obtained in high yield and purity (84% by HPLC area, Figure 2(a)). In route 1, the next step is the solid-phase Tl(tfa)$_3$ oxidation of fully-protected linear loop peptide-resin. The resultant cyclic loop was again obtained in high yield and purity (86% by HPLC area), with only minimal dimerization (at $R_t = 21.9$) (Figure 2(b)). After Lys(Dde) deprotection, addition of the hinge peptide proceeded smoothly giving the branched-disulphide peptide in excellent purity (accurate yield 75%, Figure 2(c)).

In route 2, selective Lys(Dde) deprotection on the linear loop peptide-resin and addition of the hinge peptide gave the linear version of the hinge–loop peptide with good purity (67% by HPLC area, Figure 2(d)). However, attempts at Tl(tfa)$_3$ oxidation of this intermediate to the cyclic hinge–loop peptide met with only limited success (accurate yield 11%, Figure 2(e)). By-products were the unreacted linear hinge–loop peptide and a dimeric peptide. As the hinge peptide is attached to the side chain of a Lys which is adjacent to the *C*-terminal Cys(Acm) residue, steric hindrance is likely during disulphide bond formation, resulting in the low yield of the monomeric cyclic peptide by this route.

The cyclic hinge–loop peptide was shown to possess affinity for FcγRI, both by its ability to displace radiolabelled IgG2a from U937 cells (Figure 3), and by its ability to displace fluoresceine-labelled IgG2a from THP-1 cells (Figure 4). The IC$_{50}$ value of the peptide was 40 μM, whereas the IC$_{50}$ value of huIgG was 27 nM. No displacement of ^{125}I-huIgG4 by the peptides was seen. HuIgG4 contains a phenylalanine in place of the first hinge leucine in the highest affinity IgG isotypes such as muIgG2a and huIgG1.

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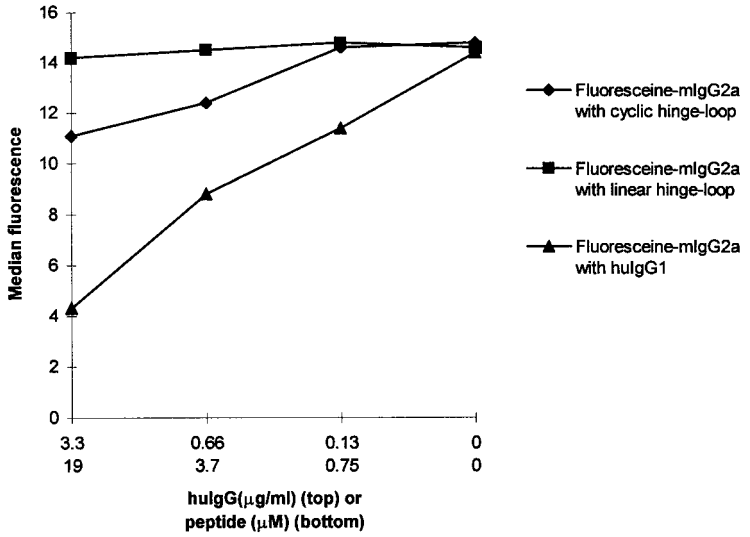

Figure 4 Median fluorescence from FACS analysis of THP-1 cells incubated with fluoresceine-mIgG2a and cyclic or linear forms of the hinge–loop peptide, or huIgG1, are shown.

In addition, the crucial proline of muIgG2a (Pro350) and huIgG1 (Pro331) is replaced by serine in IgG4. However, simultaneous mutation of both of these residues in IgG4 to those of high affinity IgG isotypes causes a decrease in affinity [6]. The possibility remains that IgG4 binds in a different way to muIgG2a and huIgG1. The use of higher concentrations of peptide was prevented by the limited solubility in aqueous conditions. Neither the cyclic nor the linear forms of the loop peptide minus the hinge displaced IgG2a binding from the cells (data not shown). The lower hinge–loop peptide, Ac-ELLGGPS-NH_2, synthesized and purified by the general methods described above, did not displace IgG2a either by itself, or together in equimolar amounts with the cyclic loop peptide (Figure 3). The linear form of the hinge–loop was inactive at the same concentrations, indicating that a degree of structural annealing together with the hinge sequence is required for binding to FcγRI.

The Fc hinge–loop peptide represents a lead for the design of a high affinity ligand for FcγRI, and represents an improvement from the inactive peptide derived solely from the hinge region [8]. Molecular dynamics and ^{1}H-NMR are being used to investigate if the peptide has structure in solution, and whether the structure resembles that of the analogous regions in the crystal structure of muIgG2a. We aim to use selective residue substitution of this larger peptide to further isolate the important FcγRI-binding epitopes. The chirality at the disulphide bond is a first candidate for alteration, as are alternative conformational constraints in this region of the peptide. Ultimately, we aim to anneal the peptide into smaller peptides and non-peptides encompassing the pharmacophore represented by the hinge and loop regions. In addition, recent support for a 1:2 binding ratio of Fc to FcγRI receptor suggests potential for a dimeric FcγRI ligand [23].

CONCLUSION

Solid-phase $Tl(tfa)_3$ oxidation has provided a facile route to the synthesis of a rationally designed, large-branched disulphide peptide from the Fc region of IgG. The high yield and purity of the peptide adds further support to solid-phase intramolecular disulphide formation and its application to peptide disulphides of large ring size.

Previous approaches for targeting or blocking FcγRI have utilized mAb or mAb fusion proteins against FcγRI. The hinge–loop peptide we have synthesized represents a lead for the design of high affinity peptide and non-peptide immunomodulatory ligands against FcγRI.

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