

Identification of cyclic peptides able to mimic the functional epitope of IgG1-Fc for human FcγRI

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ABSTRACT Identification of short, structured peptides able to mimic potentially protein-protein interfaces remains a challenge in drug discovery. We report here the use of a naive cyclic peptide phage display library to identify peptide ligands able to recognize and mimic IgG1-Fc functions with FcγRI. Selection by competing off binders to FcγRI with IgG1 allowed the isolation of a family of peptides sharing the common consensus sequence TX₂CXXΘPXLLGCFXE (Θ represents a hydrophobic residue, Φ is usually an acidic residue, and X is any residue) and able to inhibit IgG1 binding to FcγRI. In soluble form, these peptides antagonize superoxide generation mediated by IgG1. In complexed form, they trigger phagocytosis and a superoxide burst. Unlike IgG, these peptides are strictly FcγRI-specific among the FcγRs. Molecular modeling studies suggest that these peptides can adopt 2 distinct and complementary conformers, each able to mimic the discontinuous interface contacts constituted by the Cγ2-A and -B chains of Fc for FcγRI. In addition, by covalent homodimerization, we engineered a synthetic bivalent 37-mer peptide that retains the ability to trigger effector functions. We demonstrate here that it is feasible to maintain IgG-Fc function within a small structured peptide. These peptides represent a new format for modulation of effector functions.—Bonetto, S., Spadola, L., Buchanan, A. G., Jermutus, L., Lund, J. Identification of cyclic peptides able to mimic the functional epitope of IgG1-Fc for human FcγRI. *FASEB J.* 23, 000–000 (2009)

Key Words: effector functions • phage display • single-chain homodimer • molecular modeling • discontinuous interface • consensus sequence

THE GENERATION OF SMALL PROTEIN domains or peptides that are able to mimic protein-protein contact sites and show potent agonism or antagonism both *in vitro* and *in vivo* is a challenge in biological discovery. Although binding sites on enzyme surfaces and small ligand binding sites on receptor surfaces typically consist of a concave cleft shape, extracellular protein-protein interactions often involve large and relatively flat contact surfaces, lacking deep cavities and pockets that might provide compact binding sites for small molecules (1, 2). However, it may not be necessary for a small ligand to cover the entire protein-binding

interface. Of these many intermolecular contacts, a very limited number of residues, clustered in a centralized region, may account for up to 85% of the free energy of binding, hence contributing predominantly to the generation of high-affinity interactions (3). Many proteins are recognized by multiple partners. An important point is that these proteins tend to use the same binding “hot spots,” which are recognized in specific spatial orientations. Although in theory, potential ligands could bind to a protein anywhere on its solvent-exposed surface, most peptides recognize localized sites that appear to coincide with natural ligand binding sites, and consequently can function as agonists or antagonists (4). So, if proteins generally interact through compact functional epitopes, the task of identifying and designing small ligands may be achievable.

Since construction of a peptide phage library was described initially (5), over 1000 articles related to this strategy have been reported. Phage display libraries are commonly used to identify small ligands able to mimic natural binding partners with desired functional properties (6).

We used this technology to isolate cyclic peptides able to target the IgG-Fc (immunoglobulin G-fragment crystallizable) binding site on human FcγRI and mimic human IgG1 triggering of function. IgG antibodies are the predominant isotype in serum and interstitial fluids and are the format used almost exclusively for therapeutic antibodies. Antibody-dependent cell-mediated cytotoxicity (ADCC), phagocytosis, and superoxide generation are mediated through interaction of ICs (immune complexes) with FcγRs expressed on the surface of leukocytes, whereas CDC (complement-dependent cytotoxicity) occurs by interaction of ICs with the soluble complement system. Among the three classes of Fcγ receptor, FcγRI is a “high-affinity” receptor that interacts with monomeric, as well as complexed, human IgG1 or IgG3 (7). The physiological role of FcγRI is unclear, not least because FcγRI is always present in combination with other receptor classes. In order to signal, Fc receptors need to be collocated at the cell surface (8). Understanding the

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molecular basis of the Fc γ RI-Fc interaction has been the subject of detailed investigations largely based on comparative binding (9), activity studies of chimeric IgG (10), Ig-Fc modified by site-directed mutagenesis (11), and Fc peptide fragments (12). More recently, a peptide display library selection has successfully identified a specific ligand C6-2 to Fc γ RI able to promote receptor-mediated internalization (13). Unlike phage-derived peptides identified on the other classes of receptors (14–17), peptide C6-2 recognizes Fc γ RI at a site unrelated to IgG binding. Following crystallization of the human Fc γ RIII-Fc complex (18–20) and NMR spectroscopy studies of a mouse Fc γ RII-IgG2b complex (21), predominant interaction sites were identified within the C_H2 domains and shared by all Fc γ Rs, including the lower hinge sequence L₂₃₄LGGPS₂₃₉ from C γ 2-A and C γ 2-B chains and residues Leu328 to Pro329 from the C γ 2-A chain.

In the present study, we report the identification of an Fc-like peptide family that behaves as IgG, except it displays a strict specificity for Fc γ RI. To our knowledge, these are the first peptides able to antagonize effector functions mediated by Fc γ RI. Furthermore, we engineered a 37-residue single-chain homodimer peptide able to trigger a superoxide burst in a monocyte-like cell line. Therefore, we show that dimerization of monomeric Fc-mimetic peptides is sufficient to colocalize Fc γ RI on the cell surface. The results bring new molecular and structural insights to bear on the mechanism of IgG-receptor interaction and Fc γ R colocalization, and above all show that it is feasible to engineer a miniature Fc-like format able to mimic IgG-Fc mediation of effector function. These peptides may constitute the basis for the design of small ligands for the therapeutic treatment of cancer and inflammatory disorders, and suggest new opportunities for engineered antibodies.

MATERIALS AND METHODS

Cloning procedures, construction libraries, and phage library synthesis

Three cyclic phage-peptide libraries, X₂CX₁₀CX₂, X₃CX₈CX₃, and X₅CX₄X₅, were constructed by site-directed mutagenesis (22) using 3 oligonucleotides, 5'-GCTAAACA-CTTTCAACAGTTTCTGCGGCCGC(SNN)₂ACA(SNN)₁₀ACA(SNN)₂CTGTGCACTGTGAGAATAGAAGG-3'; 5'-GCTAAACA-CTTTCAACAGTTTCTGCGGCCGC(SNN)₃ACA(SNN)₈ACA(SNN)₃CTGTGCACTGTGAGAATAGAAGG-3'; and 5'-GCTAAACA-CTTTCAACAGTTTCTGCGGCCGC(SNN)₅ACA(SNN)₄ACA(SNN)₅CTGTGCACTGTGAGAATAGAAGG-3', where *n* = T, C, G, or A, and S = G or C, as described previously (23). Briefly, Fd-tet-Dog1 phage vector was used to transform *Escherichia coli* strain CJ236 cells (New England BioLabs, Beverly, MA, USA), and phage supernatant from a single colony was precipitated with 0.15 vol of solution of 20% (w/v) polyethylene glycol (PEG-8000) and 2.5 M NaCl. dUssDNA template was extracted with Qiaprep Spin M13 kit (Qiagen, Hilden, Germany), and 20 μ g was annealed with 0.7 μ g of each phosphorylated oligonucleotide (90°C for 2 min, 50°C for 3 min, 20°C for 5 min). Following elongation with T4 DNA ligase (30 U) and T7 DNA polymerase (30 U) (20°C,

3 h), cccDNA was purified with a Roche DNA purification kit (Roche Diagnostics GmbH, Mannheim, Germany) and transfected into *E. coli* strain TG1 by electroporation (2.5 kV, 200 Ω , 25 μ F in Bio-Rad micropulser; Bio-Rad, Hercules, CA, USA). Cells were plated on a large 2 $\times$ TY plate (12.5 μ g tetracycline/ml) and incubated for 16 h at 31°C. Phage libraries were prepared by PEG-8000, NaCl precipitation, and purified by CsCl gradient ultracentrifugation. The diversity of each library was estimated to be between 1 and 2.0×10^9 different clones, and their functionality was confirmed by biopanning on human serum albumin (HSA).

Phage-peptide panning selection

Each phage library was subject to 3 rounds of panning selection according to 3 different conditions (cond. 1, cond. 2, and cond. 3). Maxisorp microplates (Nunc A/S, Roskilde, Denmark) were coated with human Fc γ RIa (0.3 μ M in 0.1 M NaHCO₃, pH 8.6) (R&D Systems Inc., Minneapolis, MN, USA) for the first round, and for subsequent rounds at 0.3 μ M for cond. 1, 30 nM for cond. 2, and 0.1 μ M for cond. 3. The plates were blocked at each selection round with 0.3 nM Fc γ RI, supplemented alternatively with either 4% (w/v) powdered milk/0.1 M NaHCO₃ or 0.5% BSA (w/v)/0.1 M NaHCO₃ for 2 h at 4°C. Phage-peptide libraries [3.0×10^{11} plaque-forming units (PFU)/well] were equilibrated in PBS/4% powdered milk or 0.5% BSA for 1 h at 20°C and incubated on plates for 3 h at 24°C. After washing 10 times with PBS/0.1% Tween-20 and 10 times with PBS in cond. 1, 20 times with PBS/0.1% Tween-20 and 20 times with PBS in cond. 2, and 5 times with PBS/0.1% Tween-20 and 5 times with PBS in cond. 3, retained phage-peptides were eluted by competition with human IgG1 (2 μ M) for 1 h. The second round of selection was performed as previously, but using 5.0×10^{10} PFU in cond. 1, and 2.5×10^{10} in cond. 2 and 3. The third round was performed from 2.0×10^{10} PFU in cond. 1, 5.0×10^9 in cond. 2, and 1.0×10^{10} in cond. 3. After each selection round, the pools of eluted phage were amplified overnight at 37°C and then purified by precipitation for the next round of affinity selection.

Biotinylation of IgG1 antibody

Biotinylation was conducted in 200 μ l of 100 mM NaHCO₃ (pH 8.2) with 60 μ M of IgG1 and EZ-link NHS-LC-Biotin (Perbio/Pierce, Rockford, IL, USA) at a protein:biotin molar ratio of 1:4 for 15 min at 24°C. Biotinylation efficiency was ascertained by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF/MS) analysis (Ciphergen Biosystems Ltd, Guilford, UK). The reaction was stopped by gel filtration over a PD-10 column (Amersham Biosciences, Uppsala, Sweden) and the biotinylated protein concentration was determined by spectrophotometry at 280 nm ($\epsilon_{280} = 1.4 \text{ mg}^{-1} \text{ ml}^{-1}$).

Peptide synthesis and dimerization

Synthetic 18-mer peptides were produced in 1–3 mg amounts and characterized by analytical HPLC and LC/MS (Pepsan Systems, Lelystad, The Netherlands). Peptides were synthesized with a free N terminus and a biotinylated C terminus in the general form NH₂-AQX₃CX₈CX₃-K-biot in an oxidized state. Cyclic hinge-C_H2 peptide (sequence AQTAPCAPA PELLGCPVS) corresponding to the cyclic hinge sequence of native IgG1 constructed according to the format for cyclic octa-peptides, and peptide 22 (peptide 22 S₄S₁₃) with Ser substitutions replacing both Cys residues were also synthesized as described previously. The single-chain dimeric pep-

tides were synthesized and produced in 10-mg amounts (AMS Biotechnology, Oxon, England) using a standard solid-phase peptide synthesis on Wang resin with fluorenylmethoxycarbonyl (Fmoc) -protected amino acids. Briefly, homodimer peptides were synthesized as linear dimers, trityl (Trt) was used as a side-chain protecting group for the first pair of Cys residues on the first monomer, and S-acetamidomethyl aminoacetal (Acm) as a side-chain protecting group for the second pair of Cys residues on the second monomer. The resin and the protecting Trt groups were then cleaved by trifluoroacetic acid (TFA), and electrospray ionization-mass spectrometry (ESI-MS) analysis was used to confirm the correct structure of the peptide with Cys (Acm) intact. The unprotected Cys residues were air oxidized to form the first intramolecular disulfide bridge, and then purified by HPLC to achieve separation of high purity peptide containing the disulfide bridge. The correct mass was verified by ESI-MS analysis and lyophilized. The second intramolecular disulfide bridge was formed using iodine, which also directly removes the Acm protection group from the Cys side chain. After ESI-MS analysis to check for successful oxidation, the peptide was column purified and lyophilized. Finally, the peptide was analyzed for purity and the mass value of the correct bicyclic dimer was verified. For the assays, peptides were solubilized in either deionized water or in 2.5–50% CH₃CN or dimethylformamide (DMF) to a final concentration of 5 mM. Molecular masses of single-chain dimeric peptides were assessed by surface-enhanced laser desorption/ionization time-of-flight mass spectrometry (SELDI-TOF/MS) analysis according to the manufacturer's instructions (Ciphergen Biosystems Ltd). Briefly, peptides were diluted to 1 pmol/μl in ProteinChip EAM-I matrix (Bio-Rad) solubilized in 0.5% (v/v) TFA and 50% (v/v) acetonitrile. One picomole of this solution was spotted onto a ProteinChip NP20 array (Bio-Rad) and left to air-dry. The samples were analyzed in the positive ionization mode on a SELDI-TOF mass spectrometer (Ciphergen Biosystems). The m/z spectrum was calibrated using bovine ubiquitin standard (8564.8 Da) (Bio-Rad).

Production of soluble extracellular domains of human FcγRs

Human FcγRI extracellular domain was obtained from a commercial source (R&D Systems Inc.), which was expressed in the mouse NS0 cell line fused to the signal peptide of human CD33 and to a polyhistidine tag. To express the other human FcγR in soluble form, DNA was purchased that encoded human FcγRIIa, FcγRIIb, and FcγRIIIa extracellular domains (Geneservice, Cambridge, UK), cloned in fusion with a Flag-His-tag DNA sequence in pDEST12.2 (Invitrogen, Paisley, UK) and transfected into HEK-293 EBNA cells (6×10⁶ cells). Cells were grown on serum-free CD-CHO medium. The supernatant was harvested from 2–8 days and secreted protein was purified on an ÄKTA Explorer system (Amersham Pharmacia Biotech, Uppsala, Sweden) using a 5 ml HisTrap HP column (Amersham Biosciences). Protein was eluted with a linear gradient of imidazole (40–400 mM), and fractions were analyzed by SDS-PAGE on NuPAGE Novex 4–12% Bis-Tris gels (Invitrogen). Pooled samples were concentrated using an Amicon Ultra-15 30-kDa filter (Amicon Millipore, Watford, UK), loaded onto a Superdex S75 HR 10/30 gel Filtration Column (Amersham Pharmacia Biotech) and purified using an ÄKTA Explorer system. Fractions were analyzed by SDS-PAGE, pooled, and concentrated, and the concentration estimated spectrophotometrically (280 nm). The molecular mass was confirmed by MALDI-TOF/MS (Ciphergen).

Competitive ELISA to FcγRI

Microtiter plates (Maxisorp; Nunc) were coated with FcγRI (30 nM) in 0.1 M NaHCO₃ (pH 8.6) at 4°C for 16 h. Subsequently, plates were blocked for 2 h at 4°C using 0.7 nM FcγRI, 4% (w/v) powdered milk, 0.1 M NaHCO₃. Following washing with PBS, IgG1 (1.3 μM) was incubated with peptide at 50, 17, 6, or 2 μM for 2 h at 24°C. After washing, IgG1 binding was determined with a horseradish peroxidase-conjugated F(ab')₂ fragment goat α-IgG antibody (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) (1:5000) for 1 h and revealed with 3,3',5,5'-tetramethylbenzidine as substrate (Sigma-Aldrich Corp., St Louis, MO, USA). After quenching by addition of 50 μl of 0.5 M sulfuric acid, the absorbance was measured at 450 nm. The absence of competitor was equivalent to 100% binding, such that the absorbance measured in the presence of peptide gave the percentage of inhibition.

Peptide binding to FcγRs by AlphaScreen assay

The AlphaScreen technology (Perkin-Elmer BioSignal, Montreal, QC, Canada) was used to measure interaction between biotinylated peptides 22, 29, 30, and 33 (0.4 nM to 100 μM) bound to streptavidin donor beads and Fcγ receptors (20 nM) (FcγRI and FcγRIIb (R&D Systems Inc.), as well as FcγRIIa, FcγRIIb, and FcγRIIIa bound to nickel chelate acceptor beads. A control measurement with biotinylated IgG1 (0.19 nM to 137 nM) was performed in parallel. The reaction mixtures were incubated for 2 h in the dark and subsequently the luminescence was monitored on a Fusion-α microplate analyzer with excitation at 680 nm and emission at 600 nm (Perkin-Elmer BioSignal). The 3 greatest signals due to peptide binding were normalized relative to the 3 greatest signals due to IgG1 binding for each FcγR.

Superoxide burst activation and inhibition assays

To generate multimeric complexes, an excess of 5 nmol of peptide was incubated with 1 mg of streptavidin-coated paramagnetic beads (Dynabeads M-280 Streptavidin; Invitrogen) in PBS for 30 min at 24°C. Beads complexed with 0.5 nmol of biotinylated-IgG1 were also prepared. Superoxide bursts were measured as lucigenin-enhanced chemiluminescence, as described previously (24). U937 cells (100 μl) preincubated with γ-IFN, 10 μl of lucigenin (2.5 mM), and 10 μl of bead-peptide complexes (130 pM to 1.0 μM) or IgG1-bead complexes (10 pM to 11 nM), or single-chain dimeric peptides (70 nM to 170 μM) were incubated for 5 min at 37°C to initiate superoxide measurements. Chemiluminescence was monitored in 96-well plate format over a 60-min period at 37°C using a Berthold LB940 Luminometer (Berthold Technologies, Bad Wildbad, Germany). For the inhibition assay, either 10 μl of SRBCs (10⁹ cells/ml) derivatized using NIP-caproate-o-succinimide (0.23 mM) (Pierce) in borate buffered saline (100 mM boric acid, 25 mM sodium tetraborate, 75 mM NaCl, pH 8.1) for 1 h at 24°C and sensitized (1×10⁸ NIP-RBCs) with 450 nM α-NIP IgG1 for 1 h at 37°C or 10 μl of a suboptimal concentration of peptide 33 complexed on beads or homodimeric peptide 33 were incubated with 100 μl of cells and 10 μl of serially diluted soluble peptides (10 nM to 300 μM) or IgG1 (3 pM to 100 nM). Chemiluminescence was measured as described previously.

Rosetting assay

Binding of bead-peptide complexes to FcγRs expressed on U937 (previously stimulated with γ-IFN at 1000 U/ml), K562,

Daudi, and primary NK cells was determined by rosette formation. All human leukocyte cell lines were obtained from American Type Culture Collection (ATCC; Rockville, MD, USA). The cell lines were incubated in RPMI 1640 medium supplemented with 10% FBS, 100 U/ml penicillin and 100 µg/ml streptomycin at 37°C. NK cells were purified from blood packs by centrifugation on Ficoll gradients (Histopaque-1077; Sigma-Aldrich Company Ltd, Dorset, UK) and by negative selection using a magnetic bead isolation kit (human NK isolation kit II; Miltenyi Biotech Ltd., Surrey, UK). Bead complexes (6×10⁸ beads/ml) described in the previous section were incubated with each cell type (2×10⁶ cells/ml) at a ratio of 100:1 of beads to cells in a final volume of 100 µl of PBS. After 20 min at 24°C, 10 µl of acridine orange (66 µM) was added, and rosette formation was assessed under UV/visible illumination (Olympus BX61; Olympus, Tokyo, Japan). The ability of the cells to bind complexes was expressed as the percentage of cells that each bound 5 or more beads. For each condition 200 cells were counted in 3 replicates.

Phagocytosis and flow cytometry

U937 cells were preincubated with γ-IFN (1000 U/ml) for 48 h in RPMI 1640 medium supplemented with 10% FBS depleted of IgG, 100 U/ml penicillin, and 100 µg/ml streptomycin, and resuspended at 2 × 10⁶ cells/ml in HBSS/BSA. 5 nmoles of peptides or 500 nmole of biotinylated-IgG1 were incubated in PBS for 30 min at 24°C with 1 mg of streptavidin-coated paramagnetic beads and 0.5 or 50 nmoles of fluorescein-biotin respectively (Invitrogen). 100 µl of U937 cells were then incubated with 10 µl of fluorescent bead complexes for 2 h at 37°C. Parallel incubations were also performed in the presence of 10 µl of IgG1 (67 µM). Trypan blue (0.4%) was added to each sample for 1 h at 4°C to quench the extracellular fluorescence. The proportion of cells loaded with fluorescent bead complexes was determined by using a FACSCalibur flow cytometer (BD Biosciences, Oxford, UK), and the data were processed by CellQuest Pro Software. The relative fluorescence intensity of 10,000 cells was measured for each sample. In addition, internalization of bead-peptide complexes was visualized by Olympus BX61 epifluorescence microscopy.

Molecular modeling of the Fc mimetic peptide

A sequence similarity search was carried out for peptide 33 in the PDB database using the algorithm BLAST2. The most similar cyclic peptide (code 1s6w) corresponded to the synthetic peptide in hybrid white striped bass (25). The NMR structure of this peptide was used for building a homology model using the program PRIME (Schrödinger, LLC, Portland, OR, USA). After the initial model was built the program suite AMBER8 was used to perform a short molecular dynamics simulation in explicit water. The system was solvated by adding a cubic box of pre-equilibrated TIP3P water, and then minimized (conjugate gradient) and equilibrated using the Langevin temperature equilibration scheme and SHAKE constraints on hydrogen atoms. During the simulation temperature and pressure were maintained constant at 300 K and 1 atm. The simulation was run for a total of 2 ns, and the structure reached stability after 1.3 ns. The average structure over the final 700 ps was calculated and minimized. In parallel, a homology model of FcγRI was built and refined using the program PRIME and based on the sequence and structure of the receptor within the hFcγRIII-Fc complex (18). As a starting point to modeling FcγRI-peptide complexes, residues Leu10 to Gly12 of the

peptide were brought into proximity with FcγRI as per the motif Leu234-Gly237 of the B-chain of the Fc fragment in complex with FcγRIII. Similarly, residues Leu11 and Pro8 of the peptide were introduced to FcγRI as per the motif residue Leu235 and Pro239 of the A-chain of the Fc fragment in complex with FcγRIII. Subsequently, FcγRI-peptide complexes were optimized using standard molecular mechanics techniques (as implemented in MacroModel) to ensure consistent placement of side chain and to eliminate physically unreasonable steric interactions between them.

RESULTS

Selection of FcγRI binding phage-peptides

Unlike linear peptides, constrained peptides have restricted conformational freedom that often generates ligands with increased affinity and specificity (26). Phage display technology can introduce structural constraints by formation of covalent disulfide bonds between cysteine residues (27). Three naive 16-mer monocyclic libraries (CPEP-4, CPEP-8, and CPEP-10) were fused at the NH₂-terminal end of the pIII mature protein and displayed in polyvalent format on the M13 bacteriophage surface. To modify phage-peptide selection stringency, the receptor concentration, the phage input, and the number of washing cycles were varied according to three sets of conditions (conditions 1, 2, and 3). In addition, to select phage-peptides specific for the IgG1-Fc binding site, elution with IgG1 was performed. Following biopanning a significant increase in the number of eluted phage-peptides was observed only with the CPEP-8 library (~2000-fold after round 2 and under the first set of conditions). The phage-peptide sequences revealed seventeen unique sequences that shared strong homology and fitted the general consensus sequence TX₂CXXΘPXLLGCΦXE (Θ represents a hydrophobic residue which is often L or W, and Φ is usually an acidic amino acid residue) (Table 1). The peptide sequences varied according to selection conditions, which revealed that each biopanning condition was unique. Close to 70% of amino acid residues enclosed between the cystine residues display hydrophobic side chains. The C-terminal tripeptide sequence flanking the cyclic ring comprises one or more acidic residues. The linear mapping of the consensus onto the IgG1 primary sequence showed a strong similarity with the fragment 232–236 (EU numbering) overlapping the lower hinge at the C_H2 domain N terminus (Table 1).

Binding to FcγRI and activation of receptor-mediated functions

Initially we examined whether selected peptides could antagonize IgG1 binding for FcγRI. Thirteen of 17 peptides were able to compete significantly with IgG1 for binding to FcγRI in a dose-dependent way (Fig. 1). Inactive peptides were either uncyclized (peptides 35 and 37) or did not exhibit the dipeptide motif Leu10-Leu11 in the hydrophobic core (peptide 34). It is likely that the absence of both proline residues in the core and/or charged C-terminal end could contribute to

TABLE 1. Amino acid sequence and frequency of phage-peptides obtained from biopanning and amplification with library CPEP-8

Clone number	Peptide sequence ^a	Frequency (%) ^b	
		Round 2	Round 3
Cond.1			
cp 22	TDT C LMLP <u>LL</u> LG C DE <u>E</u>	0.00	31.16
cp 21	DPI C WYF <u>P</u> RL <u>L</u> LG C TTL	0.00	11.48
cp 23	WYP C YIY <u>P</u> RL <u>L</u> LG C DGD	0.00	18.01
cp 24	GNI C M <u>L</u> I <u>P</u> G <u>L</u> LG C S <u>Y</u> <u>E</u>	0.00	6.56
cp 33	VNS C LLL <u>P</u> N <u>L</u> LG C GDD	0.00	4.92
cp 25	<u>T</u> PV C ILL <u>P</u> S <u>L</u> LG C DTQ	0.00	13.12
cp 26	TVL C SLW <u>P</u> EL <u>L</u> LG C P <u>P</u> <u>E</u>	0.00	1.64
cp 27	TFS C LMW <u>P</u> W <u>L</u> LG C ES <u>L</u>	1.33	8.20
cp 32	FGT C YTW <u>P</u> W <u>L</u> LG C EG <u>F</u>	0.00	1.64
cp 34	SLF C RLL <u>L</u> TPV <u>G</u> C VSQ	2.66	0.00
p 35	HLL V <u>L</u> PRG <u>L</u> LG C TT <u>L</u> A	0.00	1.64
Total		3.99	98.37
Cond.2			
cp 28	TSL C SMF <u>P</u> D <u>L</u> LG C FNL	5.00	83.72
cp 29	SHP C GR <u>L</u> <u>P</u> M <u>L</u> LG C A <u>E</u> S	5.00	9.66
p 37	<u>T</u> ST C SMV <u>P</u> G <u>P</u> L <u>G</u> AV STW	0.00	3.22
Total		10.00	96.60
Cond.3			
cp 30	KDP C TRW <u>A</u> M <u>L</u> LG C D <u>G</u> <u>E</u>	0.00	32.34
cp 31	IMT C SVY <u>P</u> F <u>L</u> LG C VDK	0.00	38.22
cp 36	IHS C AHV <u>M</u> R <u>L</u> LG C WSR	3.13	2.94
Total		3.13	73.50
Consensus	TXX C XX <u>Θ</u> PXLLG C <u>Φ</u> XE		
hIgG1 ₂₂₅₋₂₄₀	TCP P CPAPELLG G PSV		

Peptides are depicted using the single-letter amino acid code. Θ , hydrophobic residue; Φ , acidic residue; X, any residue; cp, cyclic peptide; p, linear peptide. ^aHomologous amino acids in each position and those contributing to the consensus sequence are underscored. Consensus was aligned with the sequence 225–236 of human IgG1 (EU residue numbers). ^bFrequency (%) of each phage-peptide sequence is shown following sequencing of 100 clones from selection rounds 2 and 3. Total frequency of peptides identified for each condition is indicated.

their inactivity as well. The control peptide corresponding to the linear hinge-C_H2 sequence 227–244 from IgG1 (p hinge-C_H2) did not inhibit the binding of IgG1

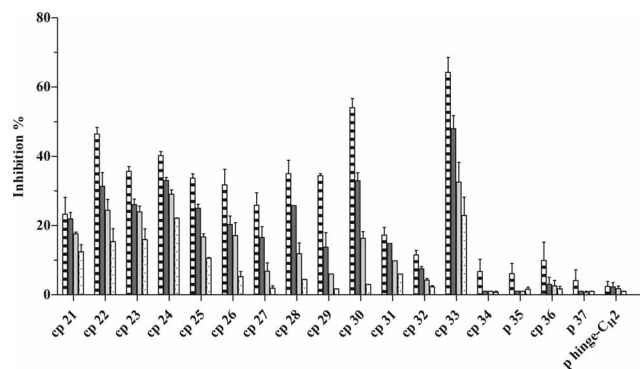


Figure 1. Ability of peptides to compete with IgG1 for binding to FcγRI. FcγRI coated onto microwells was incubated with 1.3 μM human IgG1 and 3-fold serial dilutions of soluble peptide from 50 μM (hatched bars), 16.6 μM (filled bars), 5.53 μM (gray bars), and 1.84 μM (stippled bars). Without peptide as inhibitor, the absorbance due to binding of IgG1 was normalized to 100%. IgG1 binding was detected by competition ELISA. The corresponding sequence of each peptide is given in Table 1. p hinge-C_H2, linear hinge-C_H2 peptide (sequence PPCAPELLGGPSVFLFP); cp, cyclic peptide; p 37, linear peptide 37. Results shown are the means of 4 separate experiments.

to the receptor over the concentration range tested. Similar observations had already been reported from studies carried out on human FcγRI with peptides G₂₃₃LLGGPYG₂₄₀ (28), E₂₃₃LLGGPSVF₂₄₁ (12), and C₂₃₃LLGGLGC₂₄₀ (13).

Receptor clustering induced by binding of IgG-antigen complexes triggers is known to trigger effector functions on leukocytes. Thus, we investigated the ability of peptide complexed on beads to induce both the release of reactive oxygen intermediates, and phagocytosis from IFN-γ stimulated U937 cells (29). Bead-IgG1 complexes were effective at inducing superoxide production (**Fig. 2**). Among the 17 bead-peptide complexes, 1 group (peptides 22, 30, and 33) showed a strong agonist activity and 2 groups exhibited reduced agonist activity (peptides 24, 29, and 31 and peptides 23, 25, and 32). All the other peptides tested, as exemplified by peptides 21, 27, and the linear hinge peptide, failed to trigger a superoxide burst. Interestingly, the cyclic hinge-C_H2 peptide (cp hinge-C_H2) lacked any functional properties. To examine the structural contribution of the disulfide bond, the covalent bridge in peptide 22 was omitted (peptide 22 S₄S₁₃). This double substitution caused complete loss of activity for peptide 22 (data not shown).

Phagocytic activity of complexes was examined by flow cytometry after quenching of external fluorescence (**Fig. 3A, B**), although internalized fluorescein

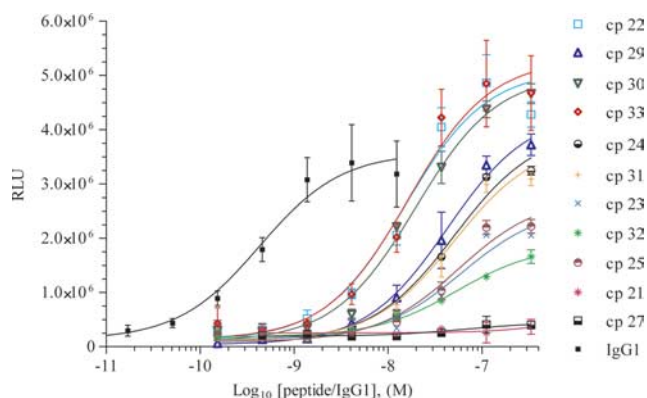


Figure 2. Ability of peptides to activate superoxide production from γ -IFN stimulated U937 cells. Cells (2×10^5) were activated with serial dilutions of either bead-peptide or bead-IgG complexes. After incubation with 2.5 mM lucigenin, luminescence was measured over a period of 60 min. RLU, relative luminescence units. Results shown are the means of 5 separate experiments.

may be underestimated at acidic pH. The internalized fluorescence of bead-peptide 22 complexes approached within 2-fold of that seen for bead-IgG1 complexes and was 10-fold greater than that detected with uncomplexed beads or beads complexed with an irrelevant peptide (Fig. 3C). In addition, in the presence of IgG1, the bead-peptide 22 signal intensity could be reduced to background (Fig. 3D). The results obtained with beads coupled with peptides 29, 30, or 33 were similar to those described for peptide 22 (data not shown).

Inhibition of superoxide burst triggered by IgG1 or bead-peptide 33 complexes

Fc γ RI recognition by the peptides was assessed by their ability to inhibit superoxide generation triggered by using IgG1-sensitized RBCs for the 4 strong agonist peptides (Fig. 4A) and alternatively by using peptide 33-sensitized beads for the nonagonist peptides (Fig. 4B) as a simpler and more controllable assay system where the response to peptide 33 as an inhibitor was equivalent in both assays (Fig. 4A, B). The 4 strong peptide agonists could inhibit superoxide generation in a dose-dependent manner, but were 3 orders of magnitude less effective as monomers relative to monomeric IgG1 (Fig. 4A). Thus, the apparent IC_{50} values were between 1 to 2 μ M for peptides 22, 30, and 33, and 20 μ M for peptide 29. Interestingly, in addition to competing IgG1 for binding to Fc γ RI (Fig. 1), some nonagonist peptides, especially peptides 21 and 27, could inhibit the activity of multimeric peptide 33 (Fig. 4B), with IC_{50} values of 15 and 7 μ M, respectively. In addition, cyclic and linear hinge- C_{H2} peptides and peptide 22 S_4S_{13} were only weakly able or unable to prevent receptor-mediated functions triggered by multimeric peptide 33. Taken together, these data indicate that unlike the hinge- C_{H2} control peptides, some cyclic peptides can mimic the interaction of IgG1 with Fc γ RI by triggering or inhibiting Fc γ RI-mediated effector

functions in complexed or soluble form, respectively. However, there was no strict correlation between the ability to trigger or inhibit effector functions for a given peptide.

Binding of peptides to Fc γ Rs

The affinity of monomeric IgG1 for Fc γ RI is high ($K_D = 10^{-8}$ – 10^{-9} M) relative to Fc γ RII and Fc γ RIII ($K_D = 10^{-5}$ – 10^{-7} M) (30). Under physiological conditions, complexed IgG1 binds effectively to all Fc γ Rs. The binding of peptides 22, 29, 30, and 33 to Fc γ RIIa, Fc γ RIIb, and Fc γ RIIIa was investigated using Alpha-Screen technology. Peptide binding to each receptor was compared with IgG1 binding (equivalent to 100%) (Fig. 5A). The binding assays showed clearly that the 4 peptides interacted efficiently with Fc γ RI but did not cross-react with the other receptor classes (while only the binding to Fc γ RIIIa is shown, the results were similar for the other low affinity receptors). Additionally, no significant binding to any Fc γ Rs was detected for the linear or cyclic hinge- C_{H2} peptides (data not shown). To corroborate these findings, binding specificity was also assessed by rosette-formation using different human cell lines (28) (Fig. 5B, C). Bead-IgG1 complexes set the standard (100% rosettes) for each

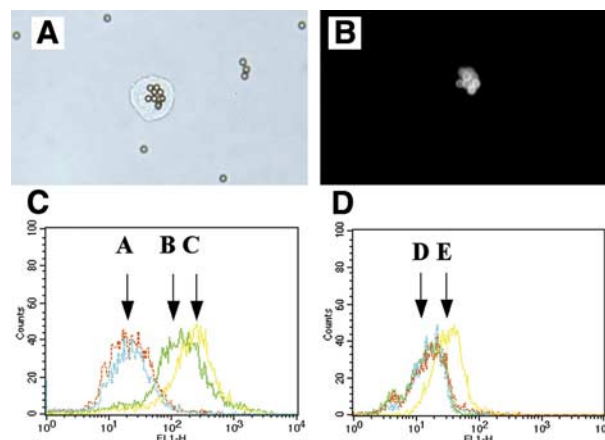


Figure 3. Phagocytosis of fluorescent bead-peptide complexes by γ -IFN stimulated U937 cells. Cells (2×10^5) were incubated with FITC-labeled beads saturated with peptide or IgG. Cells were then treated with trypan blue to quench the extracellular fluorescence. The ingested fluorescence was monitored by epifluorescence microscopy and using a FACScalibur flow cytometer. A, B) Same cell under nonfluorescent (A) and fluorescent (B) conditions. Internalization of fluorescent beads complexed with peptide 22 can be seen clearly; view $\times 400$. C, D) Flow cytometric profiles showing the fluorescence intensity of bead complexes without or with IgG1 (67 μ M) respectively. C) Profile A, beads not complexed (blue trace) and beads complexed with irrelevant peptide (sequence AQTHFDTC SWMYCWDGWW) (red trace). Profile B, beads complexed with peptide 22 (green trace). Profile C, beads complexed with IgG1 (yellow trace). D) Profile D, beads not complexed (blue trace), beads complexed with irrelevant peptide (red trace), and beads complexed with peptide 22 (green trace). Profile E, beads complexed with IgG1 (yellow trace). Results shown are representative of 3 separate experiments.

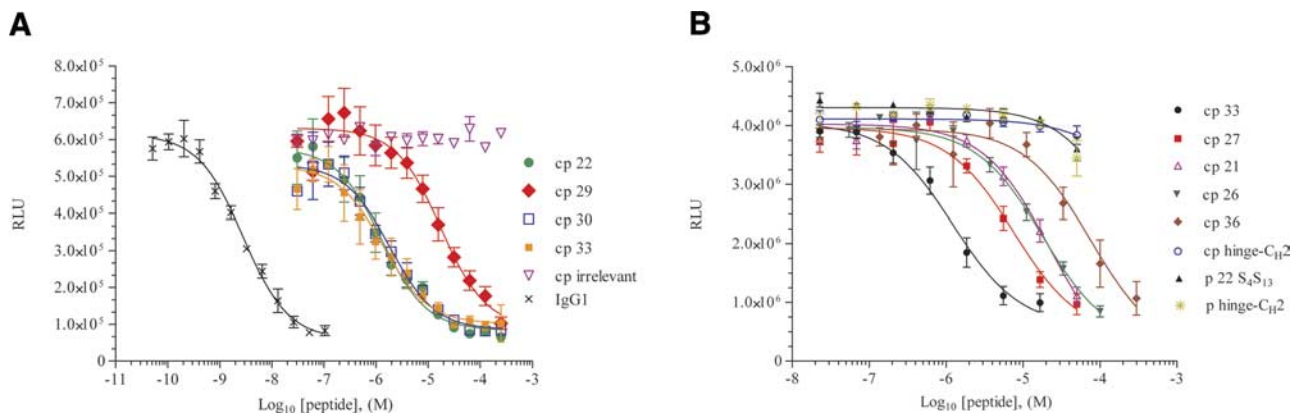


Figure 4. Peptide inhibition of immune or multimeric complex-mediated superoxide bursts by peptides from γ -IFN stimulated U937 cells. **A)** Cells (2×10^5) were activated by NIP-derivatized RBCs sensitized with α -NIP IgG1 and incubated with serial dilutions of IgG1 or soluble peptides. **B)** Cells were activated using a submaximal concentration of multimeric peptide 33 complexes and incubated with serial dilution of soluble peptides. Apparent peptide IC₅₀ values are defined as the peptide concentration required to inhibit 50% of the signal seen with the IgG immune complexes (**A**) or the multimeric peptide 33 complexes (**B**). cp hinge-C_H2, cyclic hinge-C_H2 peptide (sequence AQTAPCAPPELLGCPVS); p hinge-C_H2, linear hinge-C_H2 peptide (sequence PPCAPPELLGGPSVFLFP); p 22 S₄S₁₃, linear S₄S₁₃ peptide 22 (sequence AQTDTSLMLPLLGSDEE). Results shown are the means of 4 separate experiments.

cell type. The 4 peptide complexes were potent at rosette-formation mediated specifically *via* Fc γ RI. For the other cell lines the percentage rosetting was minimal, and did not exceed background levels.

Modeling of the Fc γ RI-peptide complex

The Fc γ R family shares a high degree of homology (50–96%) in the sequence and the structure of its ectodomains (8). Based on the crystal structure of human Fc γ 1 complexed to Fc γ RIII (19), a docked complex of peptide with Fc γ RI was generated. Peptide 33 was modeled on the structure of a referenced cyclic peptide and subsequently superimposed onto IgG-Fc residues predominant for recognition. As a result of alignment, energy minimization, and molecular dynamic simulation, 2 slightly different models for A-chain and B-chain peptide conformers were obtained (Fig. 6A, B, and Supplemental Fig. 6E). Pro8 residue of the A-chain conformer makes multiple hydrophobic contacts with residues Trp87 and Trp110 from Fc γ RI, resembling the interaction between Pro329 of C γ 2-A with these tryptophan residues in both the Fc γ RIII/Fc complex and the Fc γ RI/Fc model of Sonderman (19) (Fig. 6C). In addition, the hydrophobic interactions of Leu11 with Leu114 and Tyr116 resemble the interaction reported between Leu235 of the C γ 2-A lower hinge with Leu114 in the Fc γ RI/Fc model (19). The lack of favorable interactions that Leu11 makes with Fc γ RIII would contribute to the lack of binding of peptide 33 with Fc γ RIII (data not shown). In the B-chain conformer, residue Leu11 makes hydrophobic interactions with Tyr116, His131, and Trp132 of Fc γ RI (Fig. 6D). The hydrophobic region of Fc γ RIII defined by a cluster of histidine residues 116, 131, and 132 has been reported to interact closely with Leu235 of C γ 2-B (19). Additional potential hydrogen bonds are found between the backbone of Pro8 and Leu10 of the B-chain conformer and Arg159 and Asn117 of Fc γ RI, respectively.

Biological activity of single-chain homodimer peptide

Clustering of cell surface receptors is a powerful and flexible regulatory device in many signal transduction mechanisms, and particularly in the immune responses. Probably the most widely studied example is the cross-linking of Fc ϵ RI on mast cells (31). We investigated whether a covalent peptide homodimer could serve as the minimal unit able to induce release of reactive oxygen intermediates. Peptides 22 and 33 were selected for synthesis as antiparallel homodimer (carboxy-amino linked) and parallel (carboxy-carboxyl linked) homodimer peptides. Among the 4 dimeric peptides, only the dimeric parallel peptide 33 (Fig. 7A) was able to efficiently induce a superoxide burst with an apparent EC₅₀ value around 2 μ M (Fig. 7B). Mass spectrometric analysis confirmed the presence of dimeric peptide 33 at the anticipated molecular mass (4494 Da) (data not shown). As expected, IgG1 and soluble peptide 33 were both able to inhibit effector functions mediated by dimeric peptide 33 (Fig. 7C).

DISCUSSION

Many proteins function by binding to multiple partners. These interactions tend to share common hot-spot contacts, which are adaptive and energetically preferred sites (4). Structural analysis of IgG complexed with different binding partners has delineated several of these epitopes. Protein A, Protein G, rheumatoid factor, and neonatal receptor each have radically different scaffolds, yet bind an overlapping common site at the C γ 2/C γ 3 interface (32–36). Fc γ Rs and C1q recognize overlapping regions in the C γ 2 domain, within the f γ 2 β -strand, the b6 bend, and the lower hinge region, which are adjacent to each other in three-dimensional space (28, 37).

Characterization of molecular mechanisms by which

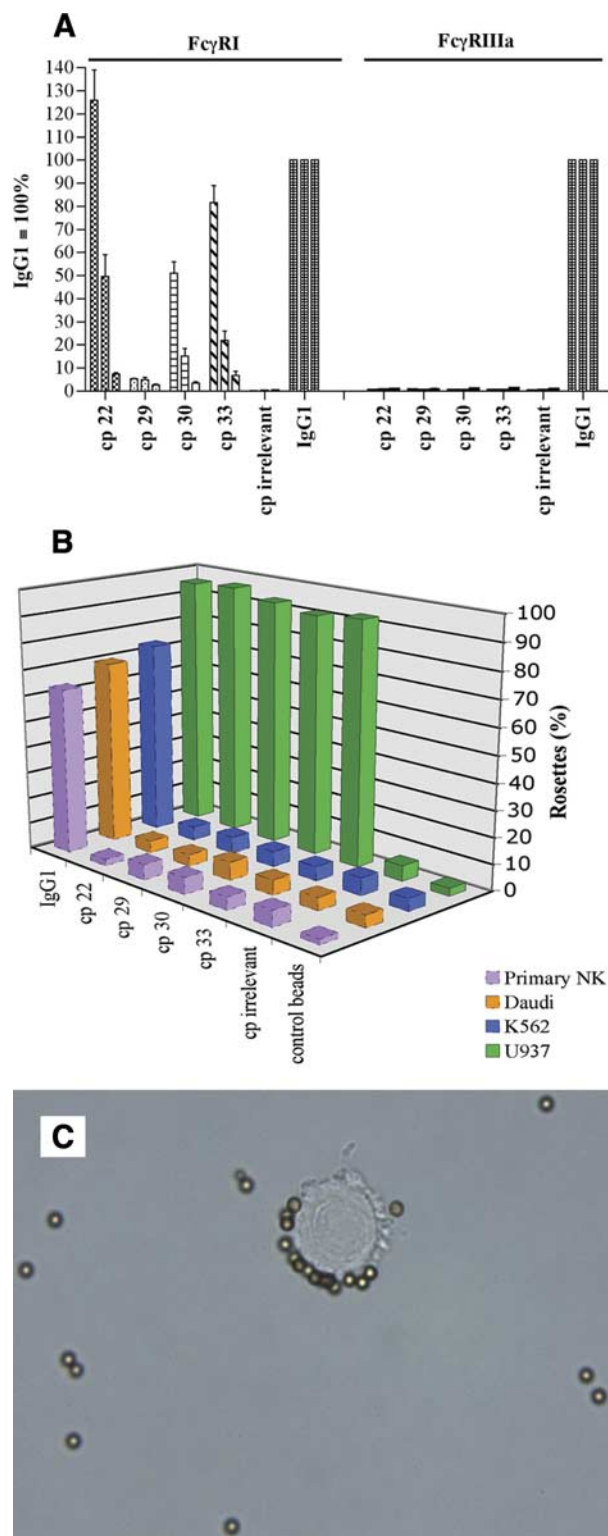


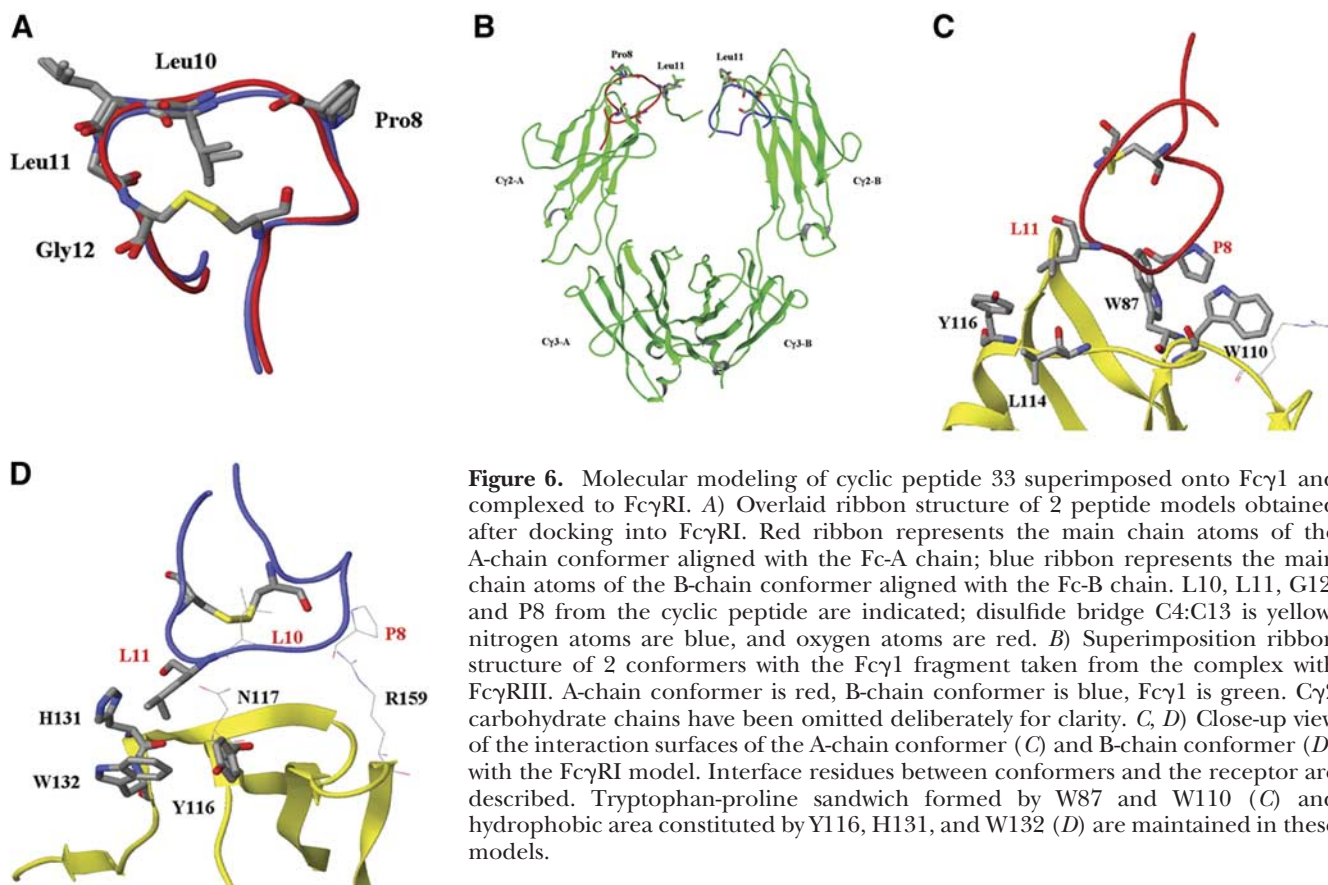
Figure 5. Peptide binding specificity for FcγRs assessed by AlphaScreen and rosette formation on FcγR bearing cells. **A)** Each biotinylated peptide was incubated with streptavidin donor beads, while the FcγRs were preincubated with nickel chelate acceptor beads. Signal due to peptide complexes added over a range of concentrations of 37, 111, and 333 nM was normalized relative to the signal due to IgG1 binding (equivalent to 100%) at 5, 15, and 46 nM for FcγRI, FcγRIIa, and FcγRIIb; and 15, 46, and 137 nM for FcγRIIIa and FcγRIIIb. Graph shows binding results for FcγRI, with results for FcγRIIIa shown as an example. Results shown were means

FcγRs recognize IgG is critical to understand better the antibody-dependent mechanism of immunoregulation (38) and may facilitate design of IgG and small molecule drugs (39). Within the last years, new and promising strategies have been undertaken to identify small “scaffolds” able to mimic FcR-Ig interactions. Using advanced techniques, including phage-peptide libraries (13–15, 17), combinatorial chemistry (40) or by compiling experimental data from the literature (12, 41), encouraging results have stimulated the development of small peptide ligands.

Our study identified a family of cyclic peptide able to mimic the functional epitope of Fcγ1 for FcγRI (Table 1). The 17 phage-octaopeptides share a strong sequence homology, such that approaching 90% of clones contain the tripeptide LLG within the hydrophobic core and the majority exhibit the LPXLLG motif. Most of them can inhibit IgG binding to FcγRI (Fig. 1). Some can trigger FcγRI-mediated effector functions efficiently (Figs. 2 and 3) and also inhibit IgG-mediated FcγRI activation within a micromolar concentration range (Fig. 4A). Conversely, some nonagonist peptides can also antagonize FcγRI activation (Fig. 4B). These latter peptides represent a class of “Fc nonmimetic peptides” that may lack sufficient avidity to trigger receptor-mediated effector function when in multimeric form, but can inhibit effector function at sufficiently high concentrations when in soluble form. Another explanation is that the Fc nonmimetic peptides may have a set of incomplete or unfavorable structural determinants, required to collocate FcγRI molecules appropriately *via* the Fc-binding site.

Many studies based on single amino acid mutations or on chimeric antibodies have reported the involvement of the sequence E₂₃₃LLGGP₂₃₈ of IgG1 in the recognition of all FcγR (42), and especially residues Leu234 and Leu235 for FcγRI (37). The solved crystal structure of FcγRIII-Fcγ1 shows that 60% of the interface area involves the 4 lower hinge residues L₂₃₄LGG₂₃₇ (18–20). This model also underlined the significant role of the loop at residues 327–333 and, more particularly, that of the Pro329 residue. In our study, the linear hinge-C_{H2} peptide corresponding to the hinge fragment sequence of IgG1, the cyclic hinge-C_{H2} peptide enclosing the hinge sequence according to the CPEP-8 format, and linear peptide 22 were unable to trigger a superoxide burst and showed only a weak ability to antagonize IgG-mediated effector functions. These observations suggest that the LLG motif is necessary but not sufficient to confer function. Consequently, we

of 4 independent experiments. **B)** Microbeads were sensitized with 10 μM peptide (22, 29, 30 or 33) or 400 nM IgG1 and incubated with 2×10^5 primary NK, Daudi, K562, or U937 cells at a ratio of 100:1 (molecule-bead complexes:leukocyte cells). Peptide or IgG-bead complex binding to cells was measured as rosette formation using a fluorescence microscope. Rosettes were defined as at least 5 beads clustered around 1 cell. Percentage rosetting indicates the ratio of rosetted cells to total cells counted. Control beads were not conjugated with any peptide. Results shown are 1 of 5 comparable experiments. **C)** Rosette formation with peptide 22 and a U937 cell visualized by light microscopy ($\times 400$).



conclude that the peptide core sequence does not correspond simply to the lower hinge sequence P₂₃₂ELLG₂₃₆ of IgG1, but in contrast is comprised of several noncontiguous determinants folded closely into a scaffold of 8 amino acid residues that are required to make the peptide functional.

Modeling studies of the FcγRI-peptide 33 complex reveal 2 complementary conformers able to mimic the functional interface of both Fc chains (Fig. 6A, B). The character of this interaction is principally hydrophobic and includes contributions by Pro8 and Leu11 of the A-chain conformer (Fig. 6C) and Leu11 of the B-chain conformer (Fig. 6D). This model suggests that Pro8 in the PXXLLG core motif does not match Pro232 from the lower hinge but rather Pro329 in the F/G loop of the C_H2 domain. This model is consistent with the view that the cyclic peptide comprises at least 2 separate discrete elements that actively take part in multiple interactions with FcγRI. However, to date we are not able to tell the relative contribution of each conformer to binding.

Furthermore, in contrast to IgG1, the cyclic peptides bind FcγRI specifically among the FcγR classes (Fig. 5), a result consistent with the modeling data where at least one of two conformers is disadvantaged for FcγRIII recognition. Even though all the receptors recognize a conserved theme in the IgGs, the reactivity profiles revealed the receptor bound overlapping but nonidentical sites (11, 18, 28, 37). Preliminary experiments have identified some specifically binding peptides for the low affinity receptors (17). Thus, a peptide consensus

with the motif PPXXLLG has been isolated by phage display on FcγRIII (unpublished results).

Screening of random peptide libraries has already reported the isolation of potent peptides able to spontaneously form covalent or noncovalent homodimers in solution (43, 44). By bivalent interaction with two receptors, the peptides inhibited or promoted receptor dimerization, a prerequisite to initiate cell signal transduction. FcγRs clustering at the leukocyte cell surface trigger signal activation in the cell. This mechanism is driven by IgG-antigen complexes under physiological conditions. We report here the synthesis of a single-chain dimeric peptide constituted by 2 covalently linked identical monomers able to elicit a biological response from the U937 cell surface membrane (Fig. 7). While the concept of the “antigen-driven receptor cluster” is recognized, its mechanistic aspects are less well defined, although colocalization of 2 receptors has been suggested (45). Unlike IgG1 where the stoichiometry with FcγRI is 1:1, it is plausible that the dimeric peptide binds FcγRI with a ratio 1:2 and so can elicit functional cross-linking of 2 FcγRI monomers.

Identification of such peptides could contribute to the characterization of the structural basis of the FcγRI-IgG interaction and help to better define FcγRI clustering mechanisms in their quantitative, structural, and mechanistic aspects. Possession of peptides specific for each FcγR may help resolve the contribution of individual receptors to immune functions. Conjugated to monovalent or multivalent IgG fragments, these

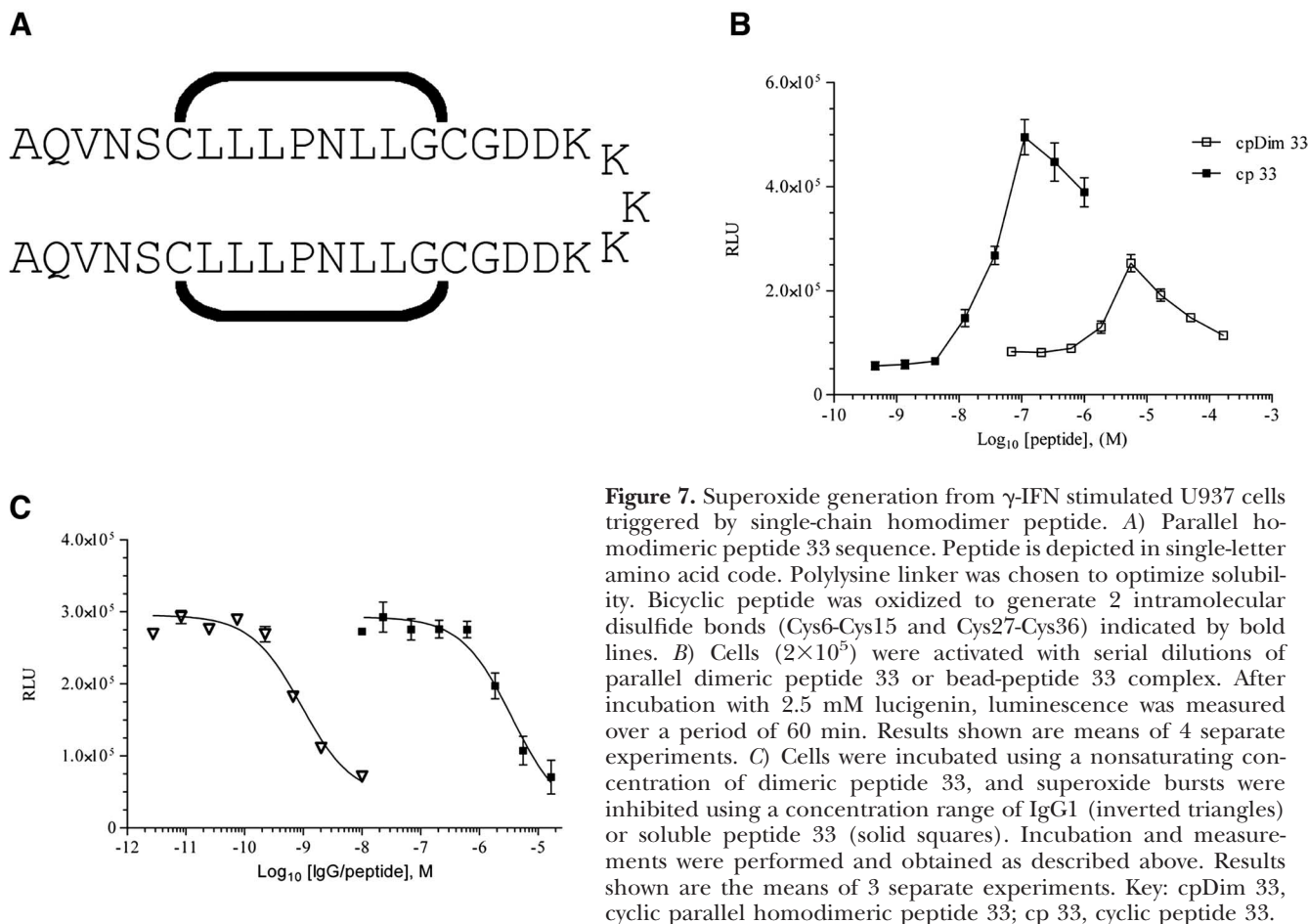


Figure 7. Superoxide generation from γ -IFN stimulated U937 cells triggered by single-chain homodimer peptide. **A)** Parallel homodimeric peptide 33 sequence. Peptide is depicted in single-letter amino acid code. Polylysine linker was chosen to optimize solubility. Bicyclic peptide was oxidized to generate 2 intramolecular disulfide bonds (Cys6-Cys15 and Cys27-Cys36) indicated by bold lines. **B)** Cells (2×10^5) were activated with serial dilutions of parallel dimeric peptide 33 or bead-peptide 33 complex. After incubation with 2.5 mM lucigenin, luminescence was measured over a period of 60 min. Results shown are means of 4 separate experiments. **C)** Cells were incubated using a nonsaturating concentration of dimeric peptide 33, and superoxide bursts were inhibited using a concentration range of IgG1 (inverted triangles) or soluble peptide 33 (solid squares). Incubation and measurements were performed and obtained as described above. Results shown are the means of 3 separate experiments. Key: cpDim 33, cyclic parallel homodimeric peptide 33; cp 33, cyclic peptide 33.

peptides could generate new antibody formats for the therapeutic treatment of tumors or infection, in addition to modulating specifically the activation-inhibition balance mediated by Fc γ R (46, 47). In soluble form, they may be used as candidates to block Fc γ R-mediated effector functions linked with inflammatory and autoimmune disorders. Conjugated to relevant IgG variable domains, the single-chain homodimer agonist peptide seems attractive when the clearance of univalent antigen is ineffective (48). However, it is clear that optimization strategies including rational design and molecular modeling will be required to improve the pharmaceutical properties of these peptides, both for potency and for stability to proteases. After a recent publication detailing the minimization of the antigen binding site (49) we here show additionally that specific functionalities of the antibody Fc moiety can be engineered into a substantially smaller peptide. Together, these data demonstrate that Fc-like peptide mimetics of antibody function can be generated opening new options for production, mode of administration, and tissue penetration. [F]

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Figure 6E. Molecular modelling of cyclic peptide 33 and FcγRI-peptide complex. Overview of peptide conformers complexed with FcγRI. The FcγRI model was built and refined based on the structure of FcγRIII in complex with the Fcγ1 fragment using the program PRIME. The A-chain conformer is shown in red, the B-chain conformer in blue, and FcγRI is in yellow.