A New Ligand for Immunoglobulin G Subdomains by Screening of a Synthetic Peptide Library

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By screening a synthetic peptide library of general formula (NH_2-Cys1-X2-X3-X4)$_2$-Lys-Gly-OH, a disulfide-bridged cyclic peptide, where X2-X3-X4 is the tripeptide Phe-His-His, has been selected as a ligand for immunoglobulin G (IgG). The peptide, after a preliminary chromatographic characterization, has proved useful as a new affinity ligand for the purification of polyclonal as well as monoclonal antibodies from biological fluids, with recovery yields of up to 90% (90% purity). The ligand is able to bind antibody fragments containing both Fab and Fc from different antibody isotypes, a fact suggesting the presence of at least two different antibody-binding sites. While the recognition site on Fab is unknown, comparative binding studies with Fc, in association with the striking similarities of the peptide (named Fc-receptor mimetic, FcRM) with a region of the human FcγRIII receptor, strongly indicate that the peptide could recognize a short amino acid stretch of the lower hinge region, which has a key role in autoimmune disease triggering. The unique properties make the ligand attractive for both the purification of antibody fragments and as a lead for the generation of Fc-receptor antagonists.

Introduction

Antibodies are among the most used classes of proteins in research, diagnostic, and clinical applications. They are a group of bifunctional glycoproteins with unique structural features (for a comprehensive review on antibody structure, see ref. [1]), and they play a central role in the regulation and functioning of the immune system of all mammals. Due to their remarkable properties, immunoglobulins are routinely used in biochemical and biological research as analytical reagents for the qualitative and quantitative determination of molecules in a variety of assays and as biotherapeutic molecules. In fact, both polyclonal (intravenous immunoglobulin, hyperimmune immunoglobulin G (IgG)[2]) and monoclonal antibodies have become the basis for standard therapies in a number of malignancies.[3–5] The rising need for highly purified immunoglobulins, as well as for other biotherapeutic agents, as injectable drugs has been accompanied by the exponential growth of regulatory restrictions applied to their production and this has led to the pursuit of new solutions in order to reduce times and costs.

Although antibodies of the G class can be conveniently purified by affinity chromatography by using immobilized protein A or G, even on large scale,[6–10] the use of synthetic ligands would be worthwhile,[11] since, as well as being less expensive, they would pose fewer problems from both the stability and regulatory points of view and with regard to the possibility of their sanitation and regeneration under very stringent conditions. A number of synthetic derivatives, mainly for the G class, have been proposed for this purpose, including amino acids,[12] thiols,[13] dyes,[14] triazine-based ligands,[15] modified peptides,[16–20] and peptides.[21–22] Due to the multifunctional nature of the antibody molecule, ligands could also be effective as therapeutic agents, according to their recognition sites. In fact, antigen:antibody complexes can activate a wide range of biological responses that promote their elimination or destruction. The principal antibody ligands for the primary regulation (activation) of the immune response and clearance mechanisms (inflammatory reactions) are cell-surface receptors for the Fc region of antibodies.[23,24] Antibodies from all classes bind and activate a number of corresponding Fc receptors (FcRs).[25–27] They are glycoproteins expressed on hematopoietic cells, with activating (ITAM) or inhibiting (ITIM) intracytoplasmatic domains whose combinations can produce activation or inhibition of immune system regulatory pathways, with a strong influence on autoimmune reactions and cancer, a stronger influence than antibody-mediated enhancement of viral infections, allergic reactions, and asthma.[25–27] Thus, the position of many FcRs as a gateway to both cellular and humoral aspects of the immune cascade makes them attractive targets for therapies

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based on antibody/receptor antagonists.[28] For IgGs, at least four distinct class of receptors have been described, FcγRI, FcγRII, FcγRIII, and FcRn,[23–25,27,29] which have been largely functionally and structurally characterized.[30–36] While FcRn binds on the same site as that recognized by protein A/G,[32] the FcγRI–III receptors contain two or three extracellular Ig-like domains, which bind Fc with different affinities across the C_H2 domain and the highly flexible hinge region.[30,31,33–36] Binding determinants on both antibodies and receptors have been precisely mapped by crystallographic studies on Fc:FcR complexes, as well as on a number of isolated Fc units and receptors,[30–36] and some peptide-based antagonists have been investigated and proposed for both IgE and IgG.[28,37–39] Nevertheless, none of these has been used as an affinity ligand or has yet been proposed as a possible therapeutic agent.

To identify new conformationally restrained ligands for antibodies of the G class, we have synthesized, characterized, and screened a cyclic dimeric peptide library.[40–43] The dimeric structures, selected to increase the molecular surface and at the same time to simplify the synthesis work, have been produced by using a lysine residue as a branching unit and have been cyclized by formation of a disulfide bridge between two cysteines at the N-terminal ends of each monomer. Through the screening, we have identified a peptide that is able to bind a large variety of both monoclonal and polyclonal IgGs and fragments thereof; the peptide has been named Fc-receptor mimetic (FcRM). The new molecule has been fully investigated by ELISA, affinity chromatography, and NMR spectroscopy in order to characterize its recognition properties and to define its structure and conformation properties in solution.

Experimental Section

Materials: HPLC columns were from Phenomenex (Torrance, CA). The MALDI-TOF Voyager DE mass spectrometer was from Applied Biosystems (Monza, Italy). Polypropylene syringes (8 mL) endowed with filtration septa were from Alltech SpA (Sedriano, Italy). 9-Fluorenylmethoxycarbonyl-glycine-4-hydroxymethylphenoxyacetic (Fmoc-Gly-HMP) derivatized polystyrene resin (PS) for solid-phase peptide synthesis was purchased from Novabiochem (Laufelfingen, Switzerland), while all Fmoc-derivatized amino acids (purity >99%) were from Inbios (Pozzuoli, Italy) and Chem-Impex (Wood Dale, IL). HPLC-grade dichloromethane (DCM), *N*-methylpyrrolidone (NMP), methanol, trifluoroacetic acid (TFA), diethyl ether, water, and acetonitrile (ACN) were from LabScan (Dublin, Ireland). Reagents used as scavengers during cleavage of peptides from resin, such as phenol, thioanisol, and triisopropylsilane, the nitrocellulose membrane (usually 9×12 cm), all anti-IgGs, anti-Fc peroxidase, anti-Fab peroxidase, anti-Ig peroxidase, the recombinant soluble mouse tumor necrosis factor (TNF) receptor (TNFR), papain, pepsin, and all other chemicals for library screening and for other assays were purchased from Sigma–Aldrich (Milan, Italy), unless otherwise stated. 0.50 M stock solutions of all protected residues were prepared by dissolving 2.5 mmol of each amino acid in 5.0 mL of dry *N*,*N*-dimethylformamide (DMF). The solutions were then stored at −20 °C until used. The prepacked recombinant protein A/Sepharose Fast Flow (rPA/SFF) and HiTrap Desalting columns were from Amersham-Biosciences (Uppsala, Sweden). 3 M Emphaze Biosupport medium with azalactone groups was purchased from Pierce (Rockford, IL). The BIO-DOT apparatus was from Bio-Rad, (Milan, Italy). Deuterated solvents were from Isotec Inc. (Milwaukee, WI). Monoclonal antibodies 7H3, 4E10, 9B11, and ST2146 and Kaptiv-GY columns were from Tecnogen (Piana di MonteVerna, CE, Italy).

Synthesis of peptide libraries:
Synthesis of the dimeric tripeptide library (Cys1-X2-X3-X4)$_2$-Lys-Gly (mother library): Libraries were synthesized manually by applying the portioning–mixing method[44,45] and using 8 mL polypropylene reaction vessels endowed with filtration septa. The synthesis was performed from Fmoc-Lys(Fmoc)-Gly-HMP-PS resin (200 mg) previously prepared by coupling Fmoc-Lys(Fmoc)-OH to PS-HMP resin (120 mg; substitution = 0.99 mmol g^{-1}). The resin was dispensed into 18 tubes after suspension in a mixture of DMF/DCM (1:1). Resins (around 6 μmol each) were washed 3 times with of DMF/DCM (1:1; 1.0 mL) and 2 times with dry DMF (1.0 mL). After Fmoc deprotection (15 min, 30% piperidine in DMF (1.0 mL)) and DMF washes, a different amino acid was coupled to each resin (30 min, RT) by using a 5-fold excess. Amino acids (0.50 M in DMF stock solutions) were activated in situ with benzotriazol-1-yloxytripyrrolidinophosphonium hexafluorophosphate (PyBOP) in DCM (1 equiv) and diisopropylethylamine (DIEA; 2 equiv). 18 natural L-amino acids were used, excluding cysteine and tryptophan to avoid oxidation side reactions. All of the resins were recombined and, after Fmoc deprotection and washes, were again split into 18 equal samples. The coupling with the 18 amino acids and the mix–split procedure were repeated. The tubes were then labeled and the contents were separately coupled again with the 18 amino acids, deprotected with piperidine, and finally coupled with Fmoc-L-Cys-(Trt)-OH (Trt = trityl = triphenylmethyl; 5-fold excess, 30 min, RT). Once the the Fmoc groups were removed, all resins were washed with DMF (2.0 mL, 3×), DCM (2.0 mL, 3×), MeOH (2.0 mL, 3×), and Et_2O (2.0 mL, 3×), then dried under vacuum for 20 min.

Since three positions were randomized by using 18 building blocks, theoretically 5832 different dimeric peptides were produced (18^3), arranged in 18 separated sublibraries, each containing 324 different molecules. The dimeric peptides were cleaved from the solid support by treatment with TFA/H_2O/thioanisol/phenol/ethandithiol mixture (86:3:3:4.5:1.5, *v*/*v*; 900 μL per tube) for 5 h at RT in the same reaction vessels as were used for the solid-phase synthesis. The resins were filtered off and the peptides precipitated in cold Et_2O (5.0 mL). The white precipitates were washed once with diethyl ether, dissolved in 50% ACN and 0.1% TFA, and lyophilized. Products were repeatedly lyophilized until no thiol odor was detected. The dimeric peptide pools were subsequently cyclized by dissolving them at a concentration of 0.1 mg mL^{-1} in 50 mM NH_4HCO_3 (pH 8.5) and stirring for 72 h. All solutions were then acidified to pH 2 with concentrated HCl, frozen, and lyophilized.

Resynthesis of the sublibrary (Cys1-Phe2-X3-X4)$_2$-Lys-Gly: This sublibrary, selected in the first screening round, was resynthesized as described before for the mother library on a scale of approximately 100 μmol from Fmoc-Lys(Fmoc)-Gly-PS resin (200 mg). The procedure was repeated until the first mix–split step, then the tubes were labeled and the resins were coupled with the 18 different Fmoc-protected amino acids. After removal of the Fmoc groups, Fmoc-Phe-OH and Fmoc-Cys(Trt)-OH were subsequently coupled to all resins as described. The resins were dried and the libraries were cleaved and cyclized following the procedures described earlier.

Resynthesis of sublibrary (Cys1-Phe2-His3-X4)$_2$-Lys-Gly: This library, composed of 18 single peptides, was prepared by performing a

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parallel synthesis of the molecules on a 5 μmol per peptide scale. The peptides were cleaved from the resins, cyclized, and characterized in the open and cyclic forms by RP-HPLC and MALDI-TOF mass spectrometry. Single cyclic peptides were purified by semipreparative RP-HPLC (5 mg aliquots) on a 25×1.0 cm ID RP18 Jupiter column (Phenomenex) by applying a gradient of 5→60% of ACN with 0.1% TFA over 30 min. The fractions corresponding to the main peaks were collected and lyophilized. Characterization was achieved by RP-HPLC and MALDI-TOF mass spectrometry. By following similar procedures, monomeric Phe-His-His-Gly peptide (LIN) and dimeric noncyclic FcRM (NC-FcRM, $(Phe\text{-}His\text{-}His)_2$-Lys-Gly) were also prepared.

Characterization of peptide libraries: The mother library was characterized by pool amino acid analysis following hydrolysis of peptide mixtures as reported elsewhere.[48] Less complex peptide mixtures (18 components) were also analyzed by RP-HPLC (125× 4.6 mm C8 Zorbax column, linear gradients from 3→80% ACN with 0.1% TFA over 25 min, flow 1 $mL\,min^{-1}$), by also checking (in some cases) the shifts of retention times observed upon disulfide-bridge formation,[46–48] and by MALDI-TOF mass spectrometry. All single peptides were produced by using the same procedures as for the synthesis of the libraries; the products were fully characterized in terms of purity, molecular weight, and disulfide-bridge formation.

After cyclization, the solutions were acidified up to pH≈4 and repeatedly lyophilized. The resulting peptide mixtures were redissolved in dimethylsulfoxide (DMSO) to obtain stock solutions at a concentration of 5 $mg\,mL^{-1}$.

Screening of libraries by ELISA-like assay: Peptide libraries at a concentration of 5 $mg\,mL^{-1}$ in DMSO were diluted in 50 mM NH_4HCO_3 at pH 8.5 (coating buffer) to a final concentration of 50 $\mu g\,mL^{-1}$. These solutions (100 μL) were dispensed into the plate wells in duplicate, while some wells were filled with coating buffer only (100 μL, blanks). The plate was left overnight at 4 °C and then washed 3 times with phosphate-buffered saline (PBS) to remove the unbound material. Blocking solution (3% bovine serum albumin (BSA) in PBS; 200 μL per well) was added, then the plate was incubated for 2 h at 37 °C. The plate was washed 3 times with PBS and a 20 $\mu g\,mL^{-1}$ solution of the 7H3 monoclonal antibody, directed against TNF receptor I, was dispensed into separate wells. After incubation for 1 h at 37 °C, the plate was washed 6 times with PBS containing Tween 0.05% (PBS-T) with 0.5% BSA. A goat anti-mouse horseradish peroxidase (GAM-HRP) solution in PBS-T with 0.5%BSA at 1000-fold dilution was prepared and added to the wells, then the plate was incubated again for 1 h at 37 °C. After 6 washes with PBS-T containing 0.5% BSA, freshly prepared *o*-phenylendiammine (OPD) solution (100 μL per well, 4 mM in citrate buffer at pH 5.0) with catalytic amounts of H_2O_2 was added and the plate was left in the dark for 15 min to allow color development. After addition of 3.0 M H_2SO_4 (25 μL per well), the absorbance at 492 nm in all wells was determined by using a microplate reader. Data, obtained as optical density (OD) values in each well, were elaborated, with duplicates averaged and corresponding blank lines subtracted. Data were reported as bar plots. All assays were carried out at least twice and the results expressed as an average.

Screening of libraries by nitrocellulose adsorption: Pieces of nitrocellulose membrane of proper size were wetted with 100 mM tris(hydroxymethyl)aminomethane (Tris) containing 150 mM NaCl at pH 7 (binding buffer) and allowed to soak for 10 min. The Bio-Dot apparatus was assembled as described by manufacturer; then, after placing the membrane on it, 100 mM Tris containing 150 mM NaCl at pH 7.5 (100 μL) was applied to all 96 sample wells by using a multichannel pipette. The buffer was allowed to filter through the membrane by gravity flow (30–40 min), then a slight vacuum was applied to complete removal of the solution. In the same way, library aliquots (100 μL) were applied. After rapid washing with buffer, the membrane was removed from the apparatus and cut in order to obtain only the piece containing samples (usually 3× 8 cm). Sample positions were lightly marked with a pencil for labeling. The membrane was incubated for 1 h in binding buffer containing 3% BSA (blocking buffer), then rapidly (1 min) washed with binding buffer. The membrane was incubated with the antibody target diluted in binding/blocking buffer at a final concentration of 30 $\mu g\,mL^{-1}$ for 2 h. The membrane was washed 3 times for 5 min in binding buffer, then it was incubated for 2 h with the alkaline phosphatase (AP) labeled anti-mouse IgG (secondary antibody) solution diluted 1:5000 in binding/blocking buffer. After the membrane was washed 3 times in binding buffer containing 0.5% BSA (binding/blocking buffer), it was incubated with the color substrate solution (3-ethyl benzothiazoline-6-sulphonic acid (ABTS) solution (45 μL) and phosphate buffer (35 μL), thereby allowing color development to proceed in the dark. When the desired intensity spots had developed, the reaction was stopped by washing the membrane several times with redistilled water and then drying it on paper towels.

ELISA: Dose-dependent assays were carried out as described in the previous section, by using different peptide-coating (1, 10, and 50 $\mu g\,mL^{-1}$) and antibody (0–20 $\mu g\,mL^{-1}$) concentrations. In a further experiment, three different monoclonal antibodies, 7H3, 4E10, and 9B11, were tested, all against the TNF receptor. A competition experiment with increasing amounts of TNF receptor (5–250 $ng\,mL^{-1}$) was carried out by immobilizing FcRM at 5 $\mu g\,mL^{-1}$ with a constant concentration of 7H3 of 5 $\mu g\,mL^{-1}$. Incubation times, washings, secondary antibody concentrations, and detection conditions were the same as those reported for the binding experiments.

Binding experiments with Protein A purified polyclonal antibodies were performed as described for the screening assays by using the corresponding anti-antibody antibodies and with a peptide-coating concentration of 1 $\mu g\,mL^{-1}$. Sera from rabbit, rat, mouse, and goat were used in these experiments.

For the mouse- and human-IgG determination in the crude material and the bound and unbound fractions, polystyrene microtiter plates were incubated overnight at 4 °C or for 2 h at room temperature, in a humid covered box, with a solution of anti-Ig (100 μL per well) in PBS (5–10 $\mu g\,mL^{-1}$). After 5 washings with the PBS solution, the wells were saturated with PBS (200 μL) containing 3% (*w/v*) dried milk (PBS-M) for 1 h at room temperature, to block the uncoated plastic surface. Plates were washed again with PBS-T and filled with standard immunoglobulins to a concentration in the range of 5–0.01 $\mu g\,mL^{-1}$, and with crude, unbound, and bound materials at varying concentrations, previously diluted with PBS containing 0.5% (w/v) BSA (PBS-BSA). After 1 h incubation, the plates were washed 5 times with PBS-T. For antibody detection, wells were filled with a horseradish peroxidase labeled anti-Ig solution (100 μL) diluted 1:1000 with PBS-M. The plates were left for 1 h at room temperature, washed 5 times with PBS-T, and then filled with chromogenic substrate solution consisting of 1 $mg\,mL^{-1}$ 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (Sigma) in 0.10 M sodium citrate phosphate buffer (pH 5.0) containing 5 mM hydrogen peroxide. The absorbance at 405 nm was determined with a model 3550 EIA microplate reader (Bio-Rad).

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Similarly, with 7H3 only, comparative binding and inhibition assays were carried out by using FcRM, NC-FcRM, and the monomeric LIN. As we were unable to directly adsorb the monomeric peptide onto the ELISA wells, the binding experiment was performed by using coated keyhole limpet hemocyanin (KLH) conjugated peptides. KLH–FcRM and KLH–NC-FcRM were coated at a fixed concentration of 20 $\mu g\,mL^{-1}$, whereas KLH–LIN was coated at 10 $\mu g\,mL^{-1}$ (the molecular weight of monomeric LIN is about half that of the dimeric peptides). 7H3 was used at a range of concentrations of 1–50 $\mu g\,mL^{-1}$ total protein. The competition assay with free peptides (at a range of concentrations of 0.1–100 $\mu g\,mL^{-1}$ for competitors and a fixed concentration of 4 $\mu g\,mL^{-1}$ for 7H3) was carried out to obtain an estimation of analogues relative affinities toward 7H3. Conjugation of the peptides to the carrier was carried out by using the same molar ratio of peptide:protein under the same conditions. To assess binding specificity, we also set up competition assays by using 7H3-derived Fc and Fab fragments. Fragments were prepared as described below and, after extensive dialysis, used as competitors in a range of concentrations of 0–180 $\mu g\,mL^{-1}$. Detection was carried out by using anti-Fc and anti-Fab secondary antibodies in Fab and Fc competition, respectively. Inhibition of 7H3:FcRM interaction was carried out by using immobilized peptide (1 µg, about 0.8 nmol) and a constant concentration of 7H3 (approximately 2.7 pmol). Soluble mouse TNFR (0–250 ng, 0–7 pmol) was used as a competitor.

Affinity resin preparation: The FcRM peptide was coupled to an Emphaze matrix (polyacrylamide/azalactone-activated gel), as recommended by the manufacturers' protocols. Peptide (5.0 mg) was dissolved in 200 mM $NaHCO_3$ containing 600 mM sodium citrate (pH 8.0, 5.0 mL) and incubated with dry preactivated matrix (130 mg, corresponding to 1.0 mL). The suspension was incubated for several hours at room temperature under gentle agitation and the extent of peptide incorporation was monitored by RP-HPLC analysis at different times. The coupling yield was always $>90\%$. After washing with 100 mM Tris (pH 8.5) to deactivate residual active groups, the resins were finally packed into a 100×6.6 mm i.d. glass column.

Affinity purification: Samples were desalted on a G25 column, dialyzed or diluted 1:4 (*v/v*) with the starting buffer, and loaded onto the FcRM/Emphaze column (1.0 mL) equilibrated at a flow rate of 0.50 $mL\,min^{-1}$ with the selected buffer. After elution of unbound material, the eluent was changed to 100 mM acetic acid (pH 2.7) to elute the adsorbed material. Bound fractions were immediately neutralized with a few drops of 1.0 M Tris (pH 9.5) and characterized by ELISA, UV analysis, sodium dodecylsulfate (SDS) PAGE, Western blotting, and gel-permeation analysis in order to determine IgG recovery, the purity, and the binding properties of FcRM peptide for IgG and fragments. In a preliminary experiment, purified 7H3 monoclonal IgG (5.0 mg) at a concentration of 1.0 $mg\,mL^{-1}$ was loaded onto a FcRM/Emphaze column (1.0 mL) at a flow rate of 0.50 $mL\,min^{-1}$ in 25 mM sodium phosphate buffer (pH 7.0). Bound fractions were characterized in terms of IgG recovery by UV analysis by using $\varepsilon_{1\%}=13.4\,cm^{-1}$. The same experiment was performed with Tris (25 mM, pH 7.5) and bis(2-hydroxyethyl)-iminotris(hydroxymethyl)methane (Bis-Tris; 25 mM, pH 6.5) as binding buffers, to investigate the dependence of the loading capacities on the loading buffer and pH value. Further affinity experiments were carried out with other monoclonal and polyclonal immunoglobulins from biological fluids. To this aim, cellular supernatants of monoclonal 7H3 and ST2146 and samples of human serum were used. Monoclonal supernatants were first subjected to a desalting step on a G25 column, then samples (5–10 mL, corresponding to about 4–6 mg of IgG) were loaded at a flow rate of 0.50 $mL\,min^{-1}$ and eluted as described above. Serum (0.50 mL) was diluted 5-fold with buffer and applied to the column at the same flow rate. 25 mM Bis-Tris (pH 6.5) was used as the binding buffer throughout all the experiments with sera and supernatants. The purity and concentration of the recovered fractions were assessed by ELISA, SDS PAGE, Western blotting, and gel filtration analysis. Similar protocols were followed for the affinity experiments with antibody fragments, again using 25 mM Bis-Tris (pH 6.5) as the running buffer. Bound and unbound fractions were collected and analyzed by site-exclusion chromatography (SEC) HPLC, SDS PAGE, and Western blotting to identify retained and unretained IgG fragments. Similar procedures were used for affinity purification of human serum on a Kaptiv-GY column (1.0 mL), as recommended by the manufacturers' protocols.[17]

Protein A purification of antibodies and fragments thereof was carried out on a rPA/SFF column (1.0 mL) as recommended by the manufacturers' protocols. Retained antibodies were eluted by lowering the pH value with 0.10 M Gly to pH 2.7 and were neutralized with 1.0 M Tris (pH 9.5). All fractions derived from the Kaptiv and protein A purifications were again characterized by ELISA, SDS PAGE, Western blotting, and gel-permeation analysis.

SDS PAGE analysis: Characterization of the bound fractions from the affinity columns was performed by SDS PAGE analysis under nonreducing conditions, on a 12% and 4–20% gradient gel (Bio-Rad, Hercules, CA) of acrylamide/bisacrylamide solution. About 7 µg of total proteins were analyzed by performing the electrophoretic runs on the Mini-Protean II apparatus (Bio-Rad), following the manufacturers' instructions. Detection of the protein bands was performed with the Brilliant Blue Coomassie R-250 (Merck) staining method, and the degree of purity was determined by electronic scanning and densitometric analysis of the gel with the IMAGE PRO-PLUS software.

IgG fragmentation: The three mouse monoclonal antibodies 7H3 (IgG1), 9B11 (IgG2a), and ST2146 (IgG2b) were subjected to fragmentation with papain and pepsin according to their different susceptibilities toward the proteolytic enzymes.[46,47] 7H3 and 9B11 (4–6 $mg\,mL^{-1}$) were incubated with papain in presence of 5.0 mM cysteine (reducing conditions) and 2.0 mM ethylenediamine tetraacetate (EDTA) in 50 mM sodium phosphate (pH 7.0) at 37 °C by using an enzyme/IgG ratio of 1:100 (*w/w*) for IgG1 and 1:200 (*w/w*) for IgG2a. The reactions, monitored by SEC-HPLC on a Superdex HR200 10/30 column (Amersham), were complete in 2–4 h and the enzyme was then inactivated by addition of 10 mM iodoacetamide to avoid further degradation. ST2146 was digested in absence of reducing agent. In this case, papain was preactivated with 10 mM cysteine in 50 mM sodium phosphate containing 2.0 mM EDTA (pH 7.0) for 30 min at 37 °C, and the reducing agent was rapidly removed by a desalting step in the same buffer. The antibody (6 $mg\,mL^{-1}$) was then incubated in 50 mM sodium phosphate containing 2.0 mM EDTA (pH 7.0) at 37 °C with an enzyme/IgG ratio of 1:200, while the progression of the reaction was monitored by SEC HPLC. After 3 h, the reaction was stopped as previously described. In the same way, a second papain digestion was also carried out on 7H3, with the reaction time lengthened to 15 h. Pepsin digestion of 7H3 and 9B11 was carried out according to the protocol of Parham.[46] The two antibodies (4–6 $mg\,mL^{-1}$) were incubated with pepsin at 37 °C with an enzyme/IgG ratio of 1:50 (*w/w*) in 0.10 M sodium citrate at pH 3.5 and 4.2, respectively. The reactions were monitored for 8–10 h by SEC HPLC until the formation of the desired products (3 h for 9B11 and 10 h for 7H3) and they were then stopped by raising the pH value to 7.5–8.0 with 1.0 M Tris (pH 9.5).

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7H3 Fab and Fc were produced as described above by preactivated papain treatment and purified by a two-step procedure with protein A to remove Fc and size-exclusion chromatography to separate Fab and $F(ab)'_2$. Fragments were dialyzed and quantified by using a Bio-rad kit.

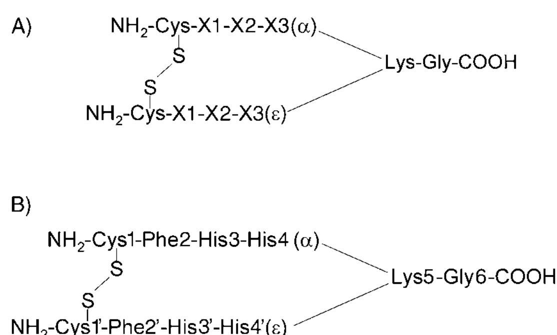
Western-blotting analysis: Standard human immunoglobulins (IgG, IgM, and IgA) and bound and unbound materials (2 μg of total proteins) were run on a SDS PAGE gel, as previously described, and transferred to a nitrocellulose filter by the electroblotting method. After protein transfer, the filter was incubated overnight at 4 °C in 100 mM Tris with 0.15 M NaCl and containing 5% dried milk (blocking buffer B1). After being washed 5 times with B1, the membrane was incubated for 1.5 h at RT with goat anti-human Ig-HRP diluted 1:1000 with B1. The membrane was then left for 1 h at room temperature, washed 3 times with water and then soaked with a chromogenic substrate solution consisting of 0.7 $mg\,mL^{-1}$ 3,3'-diaminobenzidine and 0.17 $mg\,mL^{-1}$ urea hydrogen peroxide in 60 mM Tris (Sigma-Aldrich, Milan, Italy). This substrate produces an intense brown-black precipitate at the site of enzyme binding.

Gel filtration analysis: Gel permeation analysis was performed by using a Superdex HR 10/30 GF column (300×10 mm, Amersham, Milan, Italy) equilibrated at a flow-rate of 0.75 $mL\,min^{-1}$ with PBS and 10 mM NaN_3 (pH 6.8) with monitoring of the effluent at 280 nm. About 400 μg of total protein deriving from crude and unbound material or 150 μg of standard or FcRM/Emphaze affinity column purified immunoglobulins were filtered (0.22 μm) and applied to the column.

NMR analysis: The peptide FcRM was dissolved in a H_2O/[D6]DMSO mixture (500 μL, 20:80 *v/v*) at a concentration of 2.0 mM. NMR experiments were acquired at 25 °C by using a 600 MHz Varian Inova spectrometer. All 2D spectra were recorded by the States–Haberkorn method and water suppression was obtained by the Watergate PFG technique. Spin-system identification and assignment of individual peptide resonances were carried out by using a combination of 2D-TOCSY[48] and DQF-COSY[49] spectra. Mixing times for 2D-NOESY experiments were set at 50, 100, 150, 200, 250, and 300 ms to determine NOE build-up rates, which were found to be linear up to 250 ms. 2D-TOCSY experiments were recorded with mixing times of 30 and 70 ms and 2D-ROESY[50] spectra were recorded with a mixing time of 150 ms. The data were apodized with a square sine window function and zero-filled to 1 K in f1 prior to Fourier transformation. Chemical shifts were referenced to residual DMSO ($\delta = 2.49$ ppm) and measurements of coupling constants were obtained from 1D and DQF-COSY spectra. Data were transformed with the standard Varian software and processed with the XEASY program.[51] Experimental distance restraints for structure calculations were derived from cross-peak intensities in NOESY spectra at 250 ms. NOESY cross-peaks were manually integrated by using the XEASY program and converted into upper distance constraints by using the CALIBA module of the DYANA program.[52] Distance constraints were then used by the GRIDSEARCH module to generate a set of allowable dihedral angles, and the structure calculation was carried out with the macro ANNEAL by using the torsion-angle dynamics. The 20 structures with the lowest target functions were selected. The analysis modules of the MOLMOL program were used for structure analysis.

Results

A cyclic dimeric tripeptide library (Scheme 1 A) composed of 18 natural amino acids has been prepared by solid-phase peptide



Scheme 1. Schematic representation of A) the cyclic tripeptide library and B) the selected peptide. The peptide labeling used in the NMR analysis is reported in (B).

synthesis,[40–43] by applying the mix-split method.[44,45] After on-resin assembly, cleavage, and lyophilization, an average of 5.5 mg of the sublibraries were obtained. Peptide pools were cyclized by spontaneous oxidation of the two N-terminal cysteine residues[40–43] in a slightly alkaline aqueous solution buffered with ammonium bicarbonate[41] to ensure partial salt removal over lyophilization. The library contained a theoretical total number of $18^3 = 5832$ different peptides, arranged in 18 different subpools, each theoretically containing 324 cyclic peptides. The pools, given the high complexity, were characterized only by amino acid analysis,[42,43] with comparison of the data obtained with those expected by an equimolar distribution of all components. Data deriving from this analysis were highly consistent with the expected values. Less complex peptide mixtures composed of 18 molecules, prepared throughout library deconvolution, were submitted to a more extensive characterization by reversed-phase (RP) HPLC analysis, MALDI-TOF mass spectrometry, and amino acid analysis.[42] The characterization, carried out comparatively on reduced and oxidized pools, evidenced that almost all molecules were cyclized and that only minor fractions of polymerized derivatives were present in the final mixtures[42] (data not shown). Single peptides produced for the last screening round were characterized by analytical RP HPLC, with the observation of an average purity level of around 85% after cyclization. The screening assays, carried out by both ELISA and nitrocellulose adsorption, evidenced that the sublibrary best able to bind the 7H3 antibody was that carrying phenylalanine residues on the positions next to the cysteines. The ELISA assay (Figure 1 A) showed that other sublibraries also efficiently bound the antibody, but these were not considered for resynthesis as they were only slightly active in the nitrocellulose assay (data not shown). After this first screening, the sublibraries (NH_2-Cys1-Phe2-X3-X4)$_2$-Lys-Gly were synthesized, cyclized, and screened as described for the mother library. The only sublibrary show-

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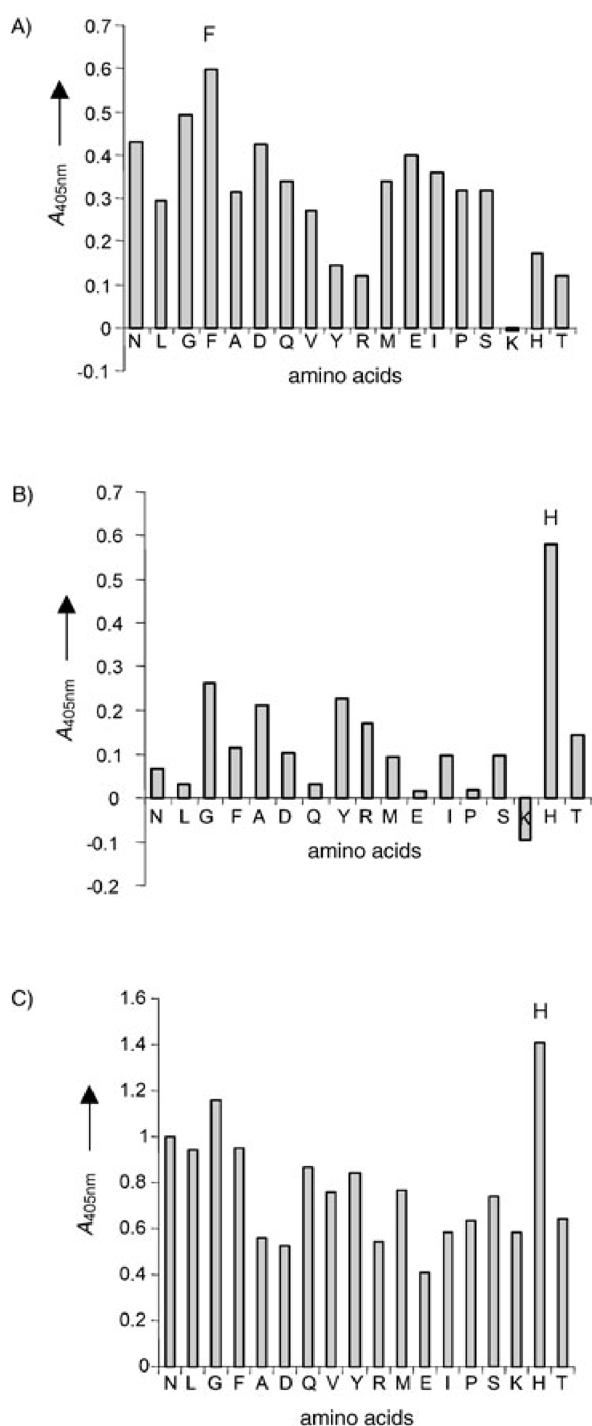

Figure 1. Screening assays carried out to deconvolute the tripeptide library (NH_2-Cys1-B2-X3-X4)$_2$-Lys-Gly. A) In the first screening, a theoretical number of $18^3=5832$ peptides, arranged in 18 pools of 324 peptides each, were screened and the pool of general formula (NH_2-Cys1-Phe2-X3-X4)$_2$-Lys-Gly was selected as that giving the best binding. B) In the second screening, 18 pools composed of 18 peptides of general formula (NH_2-Cys1-Phe2-X3-X4)$_2$-Lys-Gly were assayed and the pool with X3=His was selcted. C) In the third screening, 18 single molecules were assayed and the peptide (NH_2-Cys1-Phe2-His3-His4)$_2$-Lys-Gly was identified as that able to give best binding to 7H3.

ing affinity for the monoclonal antibody was that carrying histidine on the third position (Figure 1B) and the nitrocellulose assay confirmed this outcome (data not shown). After the 18 single peptides were produced and cyclized, they were submitted to screening. At least 5 different peptides, carrying His, Leu, Asn, Gly, or Phe in position 4, (Figure 1C), bound the 7H3 antibody, but the His peptide was definitely the most active. The nitrocellulose counterproof was not carried out in this last case. The cyclic peptide (NH_2-Cys1-Phe2-His3-His4)$_2$-Lys-Gly (Scheme 1B), named FcRM, was then selected as the best under these conditions and submitted to further characterization.

To this aim, dose-dependent assays were carried out in which different amounts of immobilized peptide, different concentrations of 7H3, and different monoclonal antibodies were tested. In a first experiment, the peptide was immobilized on microtiter wells at 1.0, 10, and 50 $\mu g mL^{-1}$ and different amounts of 7H3 monoclonal antibody (mAb) were added. As shown in Figure 2A, the peptide was capable of binding the antibody in a dose-dependent manner, with a high affinity even at a peptide coating of 1.0 $\mu g mL^{-1}$. In a second experiment, the peptide was coated at 1.0 $\mu g mL^{-1}$ and three different monoclonal antibodies, including 7H3, were tested at concentrations ranging of 0.1–200 $\mu g mL^{-1}$ (Figure 2B). The three selected antibodies recognized the immobilized peptide, although the 4E10 (IgG2a type) displayed appreciable binding only for concentrations above 10 $\mu g mL^{-1}$ (not shown). The 7H3 (IgG1 type) and 9B11 (IgG2a type) antibodies showed similar, stronger binding capacity. Although the 9B11 and 4E10 antibodies are also raised against a TNF receptor,[53] they most likely recognize different protein epitopes, a fact suggesting that the peptide was not an epitope mimic but that it binds to a different antibody site. This hypothesis was further investigated by carrying out binding assays with protein A purified polyclonal antibodies from different animal sources and by competition experiments with the soluble TNFR. As shown in Figure 2C, the polyclonal antibodies were all well recognized by the immobilized peptide in a dose-dependent manner, while TNFR, when used up to a concentration of 250 $ng mL^{-1}$, was unable to inhibit the binding of the immobilized peptide to the MAb (data not shown).

A comparative analysis between the FcRM peptide and the linear and noncyclic analogues (LIN and NC-FcRM) evidenced that, once linked to a carrier protein, the simplified variants maintain the capability to bind to 7H3 (see the Supporting Information, Figure S1), although the original cyclic molecule exhibited a stronger affinity. Competition experiments with the free (not bound to KLH) peptides were also carried to obtain an estimation of relative affinities. While the LIN peptide was unable to decrease binding when used up to a concentration of 200 $\mu g mL^{-1}$, the two dimeric variants (FcRM and NC-FcRM) showed a very similar behavior, with 50% competition at about 25 $\mu g mL^{-1}$ (≈ 20 μM for both peptides; see the Supporting Information, Figure S2). Similar competition experiments were also carried out with 7H3 fragments obtained by enzyme treatment; only Fab abolished recognition of the immobilized peptide to the intact molecule, while Fc did not when used up to a concentration of 180 $\mu g mL^{-1}$ (see the Supporting Information, Figure S3).

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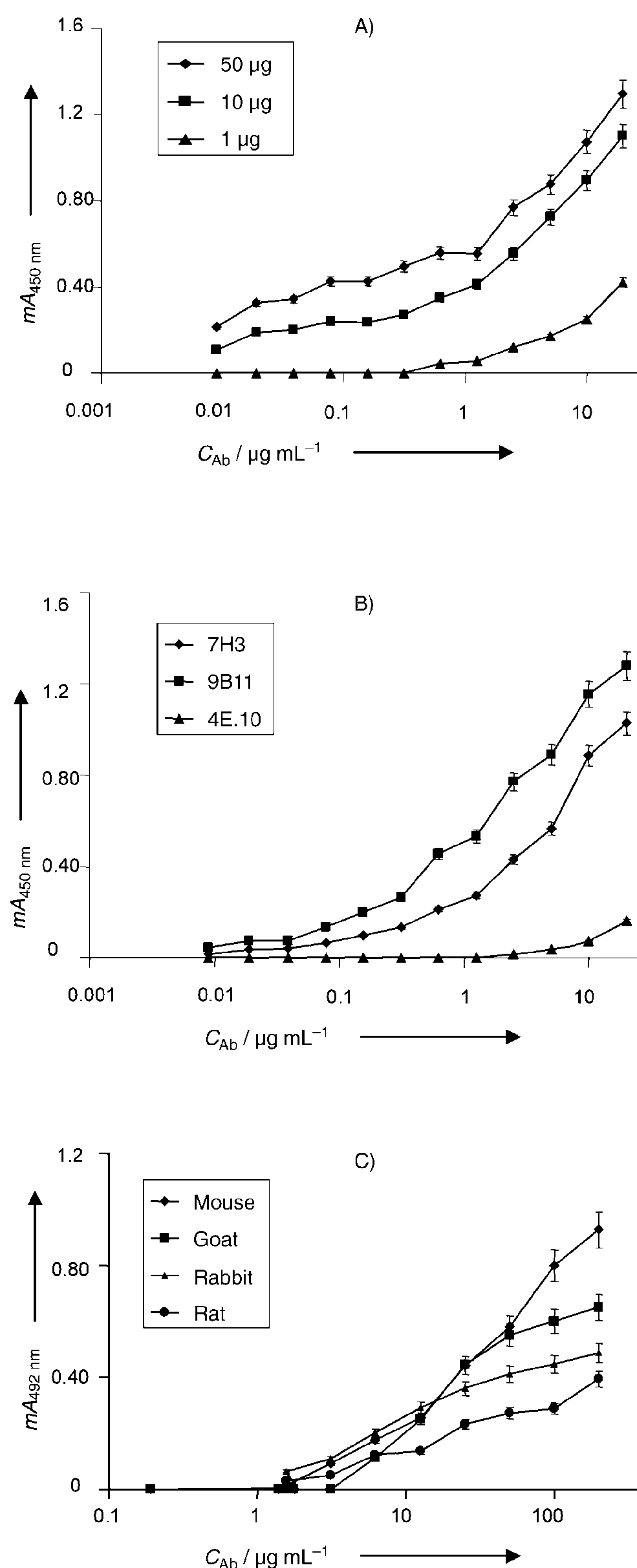

Figure 2. A) Dose-dependent assay with immobilized FcRM. The peptide coated at 1.0, 10, and 50 µg mL^{-1} was able to bind the mAb 7H3 in a dose-dependent fashion. B) Dose-dependent binding of immobilized FcRM (1.0 µg mL^{-1}) to different anti-TNFR mAbs. The peptide binds to all antibodies, with a stronger affinity showed by 7H3 and 9B11. C) Dose-dependent binding of immobilized FcRM (1.0 µg mL^{-1}) to polyclonal antibodies from different animal sources.

To evaluate the affinity properties of FcRM in chromatography applications, the peptide was immobilized on a solid support. Preliminary experiments were carried out by loading fractions of pure 7H3 monoclonal IgG (5.0 mg) in 25 mM sodium phosphate (pH 7.0) buffer onto a FcRM/Emphaze column (1.0 mL). After elution of the unretained material, bound IgG was recovered by changing the eluent to 100 mM acetic acid (pH 2.7). Analysis of the bound fractions showed that about 50% of the loaded IgG was retained, thereby demonstrating the effectiveness of FcRM to bind IgG even when covalently bound on a solid surface. The same experiment, when repeated with 25 mM Tris (pH 7.5) and 25 mM Bis-Tris (pH 6.5) as binding buffers, showed that the highest recovery was obtained with the Bis-Tris buffer at pH 6.5 (>70%), while only a minor fraction (30%) of 7H3 was retained with the pH 7.5 buffer. Subsequent experiments were then performed by using this buffer system.

The capacity and selectivity of the FcRM ligand to purify immunoglobulins were further assessed in affinity chromatography experiments on IgG-containing biological fluids. In a first purification, cellular supernatant of monoclonal 7H3 (5–10 mL, corresponding to about 6–7 mg of IgG; produced as previously described[54]) was loaded onto the FcRM/Emphaze column (1.0 mL) and processed as reported in the Experimental Section. Subsequently, human serum (0.50 mL) was diluted five times with the starting buffer (25 mM Bis-Tris, pH 6.5) and applied to the column. The results, expressed as IgG recovery and purity, are shown in Table 1 and compared with data obtained by performing human-serum purifications on protein A and Kaptiv-GY columns (1.0 mL).[17] The purity of the retained IgGs was very high (>90%), as determined by SEC HPLC and SDS PAGE (Figure 3 A–D), while other matrix components were revealed only in the flowthrough fraction, along with the remaining antibodies. The identification of IgA and IgM in the bound material of the human-serum purification was only possible by Western-blotting analysis (data not shown), a result indicating a much lower specificity of FcRM for these antibody classes. The amounts of recovered IgG, determined by ELISA assays, ranged between 4.5 (hIgG) and 5.6 mg (mIgG1; Table 1) and corresponded to 67–90% of the loaded immunoglobulins.

In order to identify IgG domain(s) recognized by FcRM, comparative affinity chromatography experiments with immobilized-FcRM and -protein A columns were carried out. Antibody fragments were produced by papain and pepsin digestion by following standard protocols. As reported,[46,47] pepsin degradation of the 7H3 antibody, belonging to the mouse IgG1 subclass, produced only F(ab')$_2$ and completely degraded Fc (Figure 4C). By contrast, the digestion with papain in both reducing and nonreducing conditions produced F(ab')$_2$ and Fc, but the preactivated enzyme (reducing conditions) also gave rise to some Fab[46,47] (Figure 4B and Table 2). The action of pepsin on 9B11 (IgG2a type) produced F(ab')$_2$, but with some Fab and degraded Fc as contaminants, while the treatment with papain gave Fab, some F(ab')$_2$ impurities, and Fc (Table 2). The fragmentation of ST2146 (IgG2b type) with papain produced, with a short incubation time, Fab, Fc, and a Fab/c fragment (Table 2), where Fab/c is a fragment formed by the Fc region

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Table 1. Purification of antibodies by Emphaze-immobilized FcRM and comparison with protein A and Kaptiv-GY columns for affinity purification of G-type immunoglobulins from human serum.

Sample	IgG content[a] [mg]	FcRM/Emphaze recovery[b] [mg]		FcRM/Emphaze purity[c] [%]	rpA/SFF recovery[b] [mg]		rpA/SFF purity[c] [%]	Kaptiv-GY recovery[b] [mg]		Kaptiv-GY purity[c] [%]
		B	UB		B	UB		B	UB	
7H3	6.2	5.6	0.5	>90	n.d.[d]	n.d.[d]	n.d.[d]	n.d.[d]	n.d.[d]	n.d.[d]
human	6.7	4.5	2.1	>90	5.5	1.2	>90	6.0	0.6	>90

[a] IgG content in loaded sample. [b] IgG determination carried out by ELISA. B = Bound material, UB = unbound material in the flowthrough fraction. [c] IgG purity of bound fraction as evaluated by SDS-PAGE. [d] n.d. = not determined.

plus one Fab arm.[46] After enzyme treatment, antibody fragments were not separated but were loaded as mixtures onto the columns. Figure 4 A–C show the SDS PAGE analysis of fractions recovered after affinity chromatography of fragmented 7H3 with Emphaze-immobilized FcRM peptide. As can be deduced, the resin retained the Fab and $F(ab')_2$ fragments derived from papain and pepsin cleavage with high affinity, while the Fc fragment, obtained by papain digestion in both reducing and nonreducing conditions, was not recognized and was recovered in the flowthrough fractions. Similar results, summarized and compared with each other in Table 2, were obtained with the 9B11 and ST2146 fragments, but in these cases the Fc fragments were also retained. As expected, protein A was only able to bind those structures containing the Fc region (Fc and Fab/c), while the peptide bound all fragments (Fab, $F(ab')_2$, Fab/c, and Fc) except the Fc derived from the IgG1 7H3 which, in the hinge region, shows striking sequence differences with the other Fc fragments (Figure 5). It is worth noting that the papain cleaves IgG1 at Thr232 (human numbering as given in Figure 5) under both reducing and nonreducing conditions,[47] thereby leaving all residues of the lower hinge on the Fc region (Figure 5). The 9B11 Fc (IgG2a type) is obtained by cleavage of papain at Lys230 (human numbering[46,47]) and thus, apart from the lower region, it also contains the core hinge residues. The Fc region produced from the IgG2b by papain starts from Thr216 or Ile217[47] and contains the entire hinge region (upper, core, and lower; Figure 5).

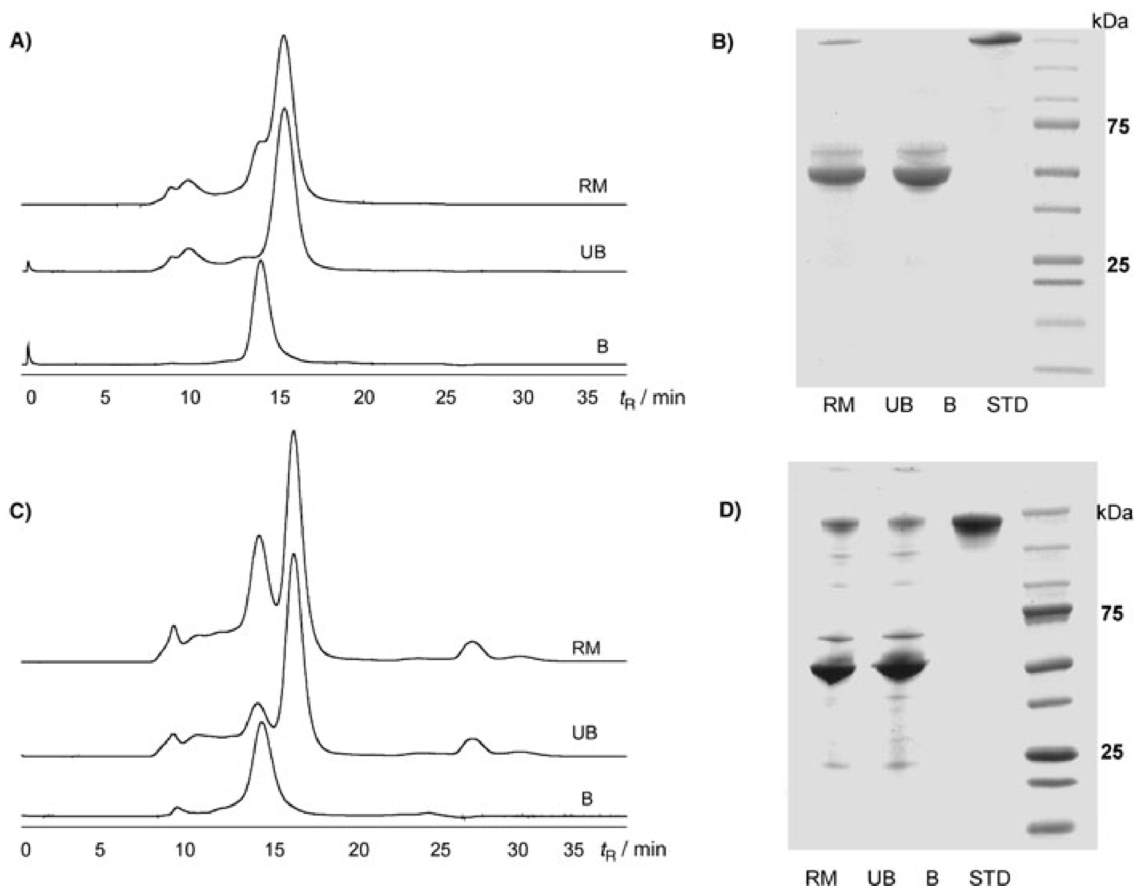

Figure 3. A) Affinity purification of 7H3 from crude hybridoma supernatants. B) The same experiment was also carried out with normal human serum. Both experiments were carried with a column (1 mL) with ligand (3 mg) immobilized on EMPHAZE. Fractions were characterized by gel filtration chromatography (A and C) and SDS PAGE (B and D) as described in the Experimental Section. STD = standard molecular weight, RM = raw material, B = bound material, UB = unbound material.

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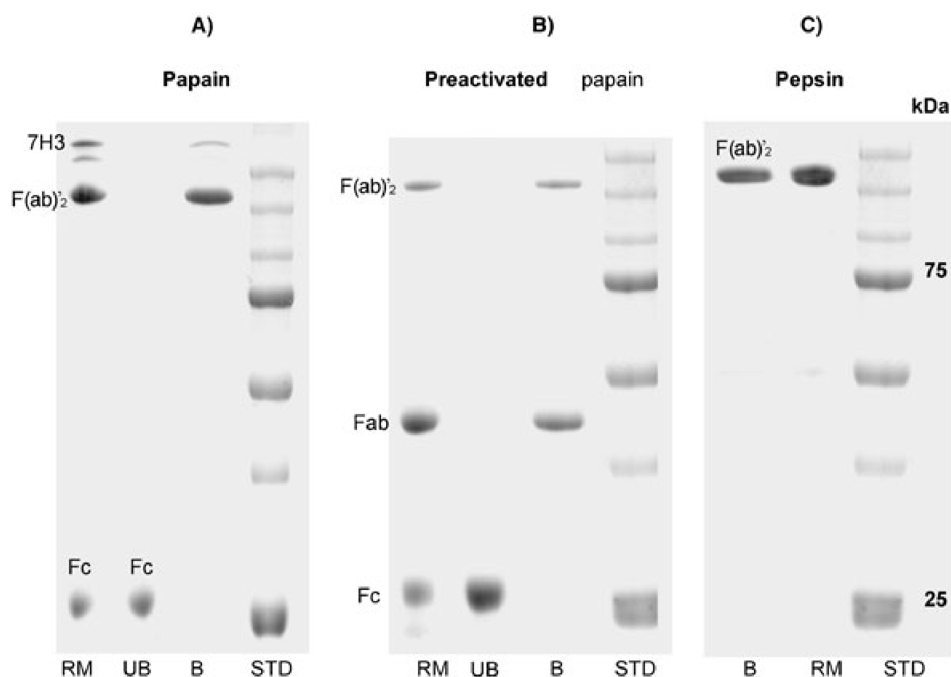

Figure 4. A) SDS PAGE analysis of affinity-purified 7H3 fragments derived upon papain (A and B) and pepsin (C) digestion. F(ab)′$_2$ and Fab fragments were fully retained by the columns, while Fc was not.

rent field of the adjacent aromatic amino acid residues (Phe2 and Phe2′). Measurements of NH–Hα and Hα–Hβ coupling constants allowed us to estimate the ranges of the φ and χ_1 torsion angles. $J_{NH-H\alpha}$ coupling constants (data not shown), extracted from the 1D and the DQF-COSY spectra, indicate an extended conformation for the peptide. The measurements of NH–Hα coupling constants for His3, His4, Lys5 (NHε), His3′, and His4′, as well as the diastereotopic assignments of the β protons of all residues (except for residues Phe2 and Phe2′), could not be achieved because the signals overlap in both the 1D and DQF-COSY spectra. Moreover, the spin systems of Cys1 and Cys1′, of His3 and His4, and of His3′and His4′ have the same values of chemical shifts and therefore the pseudosymmetry of the molecule also generated a certain ambiguity in the NOE assignments. We were unable to assign NOEs between 8.02–4.42 ppm because they could either belong to the couples His4′CαH–His4′NH, His3′CαH–His4′NH, His3′CαH–His3′NH, or His3′NH–Phe2′CαH, or they could be the resultants of different contributions of these. For simplicity, the molecule has been ideally dissected in three separate regions. A first region, comprising Cys1 and Cys1′, in which the spin systems are overlapping, a second region, comprising His4 and His4′, in which the spin systems are distinctly separated, and a third region comprising amino acids Phe2, His3, Phe2′, His3′, where NOEs were assigned only with a marked uncertainty. A total of 52 observed NOEs (Table 4), mostly sequential or intraresidue, were used in preliminary structure calculations. Distance restraints derived from these NOEs were introduced in simulated annealing (SA) torsion-space calculations performed by using the DYANA package and the best 20 structures in terms of target functions were selected from among 200 structures sampled in torsion-space simulated-annealing calculations. Figure 6 shows the superposition of backbones of amino acids 2, 3, and 4 (blue) and the corresponding amino acids 2′, 3′, and 4′ (white) obtained in these 20 structures. In this preliminary low-resolution model, we could observe two regions encompassing residues 2′–4′ and 2–3 with well-defined backbone conformations, while the region involving the lysine and cysteine residues was quite undetermined. The overall data suggest that the disulfide bridge and the lysine side chain (Figure 6) are characterized by a marked flexibility and work as a sort of hinge around which both copies of the rigid Phe-His-His tripeptide can freely fluctuate.

Table 2. Comparative binding of enzyme-fragmented monoclonal antibodies by immobilized protein A and FcRM.[a]

	Recognition by FcRM/pA				
	papain fragments			pepsin fragments	
mAb	F(ab′)$_2$	Fab/c	Fab	Fc	F(ab′)$_2$
7H3 (mouse IgG1)	+/−	n.o.	+/−	−/+	+/−
9B11 (mouse IgG2a)	+/−	n.o.	+/−	+/+	+/−
ST2146 (mouse IgG2b)	n.o.	+/+	+/−	+/+	

[a] The + symbol indicates binding by the corresponding ligand, whereas the − symbol indicates no binding. n.o. = not obtained. Experimental conditions are reported in the Experimental Section. Abbreviations for fragments are explained in the text.

NMR analysis

The preferred conformations of the FcRM peptide and the region of highest flexibility were analyzed by NMR techniques. In order to define the best experimental conditions, preliminary experiments were performed by using peptide solutions in different solvent systems and acquiring 1D spectra at different temperatures. The 1D spectrum acquired at 25 °C in a H_2O/[D$_6$]DMSO (20:80 *v*/*v*) mixture showed the sharpest and best-resolved resonances and consequently all spectra were acquired under these conditions. It is worth noting that the amine NH_2 protons of Cys1 and Cys1′ resonate at low field (see Scheme 1 for amino acid labeling and Table 3 for chemical-shift assignments); this effect is explained by the local ring-cur-

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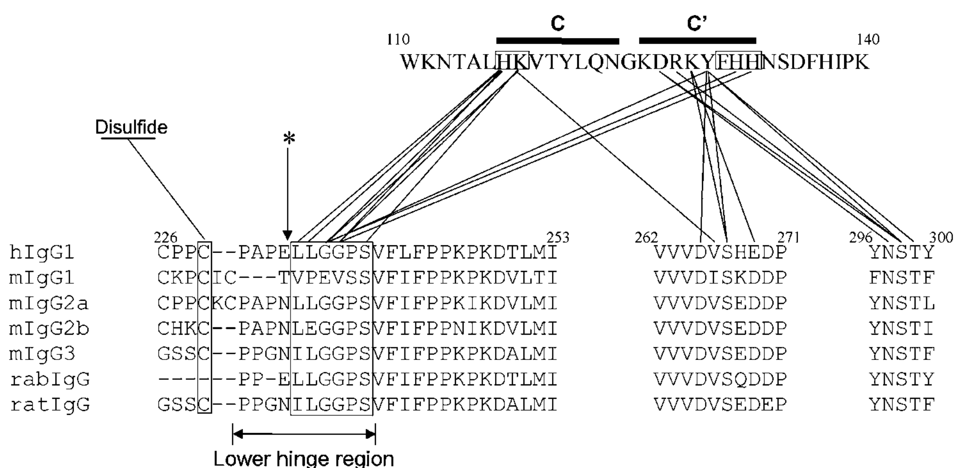

Figure 5. Multiple alignment of human IgG1, mouse IgG1, IgG2a, and IgG2b, and rabbit, sheep, and rat IgG. In the upper part of the figure, the sequence of human FcγRIIIA, along with binding contacts between the receptor and the human Fc are depicted.[36] The lower hinge region, the tripeptide Phe130-His131-His132 (FHH), and the dipeptide His116-Lys117 (HK) on the receptor sequence are boxed. The asterisk indicates the position of papain cleavage.

Table 3. The chemical shifts (δ) of peptide protons. Values are given in ppm relative to internal [D$_6$]DMSO (2.49 ppm).

Proton	Chemical shift	Proton	Chemical shift
Cys1 NH_2	8.86	Cys1′ NH_2	8.83
Cys1 Hα	4.54	Cys1′ Hα	4.52
Cys1 Hβ	3.04/2.72	Cys1′ Hβ	3.04/2.72
Phe2 NH	8.69	Phe2′ NH	8.73
Phe2 Hα	4.43	Phe2′ Hα	4.43
Phe2 Hβ	3.03/2.92	Phe2′ Hβ	3.03/2.92
Phe2 H2 + H6	7.17	Phe2′ H2-H6	7.17
Phe2 H3 + H5	7.24	Phe2′ H3-H5	7.24
Phe2 H4	7.20	Phe2′ H4	7.20
His3 NH	7.99	His3′ NH	8.02
His3 Hα	4.51	His3′ Hα	4.42
His3 Hβ	3.10/3.01	His3′ Hβ	3.02/2.90
His3 H2	–	His3′ H2	7.14
His3 H4	–	His3′ H4	8.57
His4 NH	7.99	His4′ NH	8.02
His4 Hα	4.51	His4′ Hα	4.42
His4 Hβ	3.10/3.01	His4′ Hβ	3.04/2.91
His4 H2	8.62	His4′ H2	8.57
His4 H4	7.18	His4′ H4	7.16
Lys5 NH	8.20		
Lys5 Hα	4.21		
Lys5 Hβ	1.67/1.47		
Lys5 Hγ	1.17		
Lys5 Hδ	1.30		
Lys5 Hε	3.06/2.92		
Lys5 NH	7.95		
Gly6 NH	8.35		
Gly6 Hα	3.81/3.72		

Table 4. The relevant unambiguous backbone NOEs[a] observed for the peptide.

NOE	Intensity	NOE	Intensity
Phe2 NαH–His3 NαH	m	His4 CαH–Lys5 NαH	s
His4 NαH–Lys5 NαH	m	Lys5 CαH–Lys5 NαH	w
Lys5 CαH–Gly6 NαH	m	Lys5 NαH–Gly6 NαH	m
Lys5 NαH–His4 Hβl	w	Lys5 NαH–His4 Hβh	m
Lys5 NαH–His4′ H4	m	Lys5 NαH–His4 H4	m
Gly6 NαH–Lys5 Hβl	w	Gly6 NαH–Lys5 Hβh	m
Gly6 NαH–Lys5 Hγ	w	His4′ CαH–Lys5 NεH	m
Phe2′ NαH–His3′ NαH	m		

[a] The NOEs corresponding to distances of 2.5 Å are classified as strong; those corresponding to distances of 2.5–3.5 Å are classified as medium; those corresponding to distances of 3.5–4.5 Å are classified as weak.

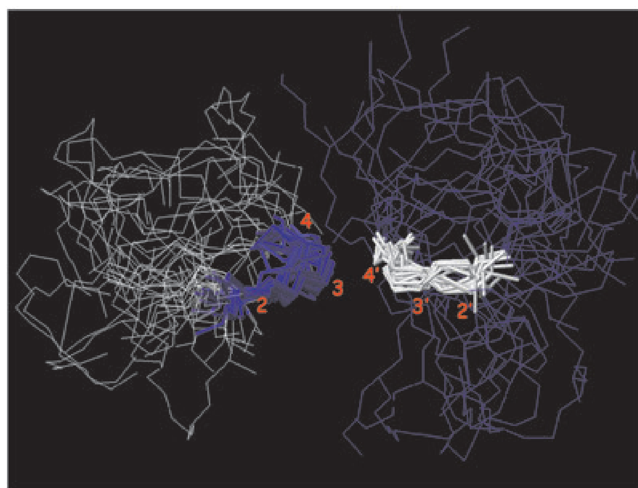

Figure 6. Structural models of the peptide as obtained by the NMR analysis. Superposition of amino acids 2, 3, and 4 (blue) and the corresponding residues 2′, 3′, and 4′ (white) from the best 20 structures. The numbering scheme is shown in Scheme 1 B.

Discussion

By screening a peptide library of general formula (NH_2-Cys1-X2-X3-X4)$_2$-Lys-Gly, where the molecules have been cyclized by bridging the thiol groups of the two cysteine residues, we have identified a peptide able to specifically bind immunoglobulins of the G class. The selected ligand is able to recognize a number of monoclonal antibodies of different isotypes, as well

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as polyclonal antibodies from different sources (human, mouse, rabbit, sheep, and rat) with good affinity but without an apparent specificity. These properties clearly suggest that the binding site on the antibodies must be localized outside the antigen-combining site and must include structural features common to all recognized IgGs. This deduction is strengthened by the observation that binding to a set of mAbs (7H3, 4E10, 9B11), all against the TNFR,[53] could not be displaced by soluble TNFR. Comparative studies by affinity chromatography carried out with fragmented antibodies and immobilized-FcRM columns (Table 2) have proven that the peptide recognizes both the Fab and Fc fragments from all immunoglobulins, except IgG1. For this subtype, only Fab was shown to interact with the immobilized ligand, while Fc was entirely recovered in the flowthrough fraction (Figure 4 A and B).

Fab binding has also been assessed by ELISA competition assays (see the Supporting Information, Figure S3), thereby confirming the specificity of this interaction.

The peptide contains two copies of the Cys-Phe-His-His tetrapeptide sequence, where the cysteine residues, introduced into the general library structures to achieve cyclization, do not contribute to binding since they present in all library components. Interestingly, the tripeptide sequence Phe-His-His is present on an exposed loop (the loop C′[30,31]) of the human FcγRIII peptide (Phe130–His132, Figure 5) that, as evidenced in the crystallographic structure of its complex with a human Fc fragment,[30,31] is heavily involved in Fc binding. From inspection of this structure, it emerges that the two histidine residues, together with the His116–Lys117 dipeptide on an adjacent loop (loop C[30,31]), form a patch of four amino acids, dispersed on the corner of an almost perfect square with an edge of around 5 Å, whose side chains perpendicularly protrude toward the Fc and make many contacts with amino acids from the lower hinge region, namely Lys234–Ser239[30] (Figure 5). These two adjacent loops closely resemble the ligand structure, which, in place of a lysine bears the similarly basic amino acid histidine. The structure analogy between the peptide ligand and the receptor site is further supported by the NMR analysis, which has evidenced a very high flexibility around the lysine and the disulfide bond but a noticeable rigidity in the two tripeptide backbones, a result could favor the mobility of the side chains. As can be seen in Figure 5, the lower hinge sequence, which is the N-terminal part of the Fc domain, is highly conserved in mouse IgG2a, IgG2b, and IgG3 and in rabbit, sheep, and rat IgG (all recognized by the peptide), while it is fully substituted by the sequence VPEVSS in mouse IgG1, from which is derived the only Fc fragment not interacting with immobilized FcRM (Figure 4 A and B; see also the Supporting Information, Figure S3). These observations strongly suggest that the lower hinge region is involved in peptide binding; the peptide, in turn, could work as a mimic of the receptor binding site. From our experiments, no hypothesis or predictions can be made about the second interaction site on the Fab region.

The apparent global dissociation constant relative to 7H3 and FcRM is about 20 μM (as measured in a competition ELISA assay), a value that is comparable to that similarly evaluated for the NC-FcRM. The LIN peptide, by contrast, is incapable of interfering with the FcRM-7H3 interaction, a fact suggesting that the minimal unit able to efficiently recognize the antibody is provided by the dimeric structure and that the N-terminal cysteines are actually not involved in recognition or give only a small contribution. Furthermore, cyclization is not a stringent requisite for binding, since the two variants exhibit only tiny differences, as evidenced mainly in binding experiments (see the Supporting Information, Figure S1).

The peptide has proved useful as an affinity ligand for the purification of IgG, since it is able to extract human polyclonal antibodies from serum and monoclonal antibodies from crude hybridoma supernatants (Figure 3 A–D). Although the chromatographic properties of the peptide have not yet been optimized (experiments are underway) and the column capacities are still not comparable to those attainable with protein A or other available synthetic ligands, the recovered antibodies are highly pure with only very small protein contaminants derived from the IgM or IgA fractions. As with the Kaptiv-GY system,[17] FcRM offers the advantage of being synthetic and therefore indifferent to denaturation or unfolding. It can be reused many times, even after treatment under the strong conditions required for cleaning and removal of pyrogens (sanitation), which could be an advantage over protein A. The data suggest that the peptide could bind Fc by mimicking the FcγRIII binding site,[30] but this hypothesis, which is appealing for the generation of Fc/FcR antagonists, needs further investigations.

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Keywords: affinity purification · antibodies · immunoglobulins · ligand identification · peptides

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