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ORIGINAL ARTICLE

Minetake Kitagawa · Daisuke Goto · Mizuko Mamura
Isao Matsumoto · Satoshi Ito · Akito Tsutsumi
Takayuki Sumida

Identification of three novel peptides that inhibit CD40–CD154 interaction

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Abstract The CD40–CD154 interaction is an attractive target for therapeutic intervention in various autoimmune disorders, including rheumatoid arthritis, systemic lupus erythematosus (SLE), multiple sclerosis, and myasthenia gravis. In this study, to develop a new disruption strategy of the CD40–CD154 interaction, we screened for peptides with inhibitory effects on such ligation. 2×10^{11} phage display libraries displaying liner peptides of 12-mer amino acids were screened by CD40-Ig binding assay and eight phages which expressed a different respective peptide (40BP-1 to -8) were able to specifically bind to CD40. Competitive inhibition analyses showed that 3 of the 8 peptides (40BP-N1-1 – APELPNMTPSWT; 40BP-N1-2 – APRPHTSYSPLP; and 40BP-N1-3 – GMTAPPPPRLTQ) blocked CD40–CD154 interaction when used at high concentrations. A consensus sequence (APxPPxxT) was conserved in these three peptides. These peptides may constitute a useful and novel strategy for the inhibition of the interaction between CD40 and CD154 molecules.

Key words Autoimmune disease · CD40 · CD154 · Peptide · Phage display

Introduction

CD40 is a 48 kDa membrane glycoprotein expressed on B cells, monocytes, dendritic cells, and thymic epithelium, as well as on certain carcinomas. It belongs to the tumor necrosis factor receptor superfamily, a group of type I transmembrane molecules. The ligand for CD40 (CD154) belongs to the tumor necrosis factor family and is predominantly expressed on activated T cells, although variable expression has been reported on mast cells, B cells, monocytes, and basophils. The CD40–CD154 interaction plays a central role in the control of both humoral and cellular immunity.[1,2]

Blockade of the CD40–CD154 interaction with a monoclonal antibody directed against CD154 has been shown to inhibit autoimmune responses in vivo in various murine models of autoimmune disease, including rheumatoid arthritis,[3] systemic lupus erythematosus (SLE),[4] and multiple sclerosis.[5,6] It has been proposed that disruption of CD40–CD154 could be a useful strategy in the treatment of autoimmune diseases.

In the present study, we screened the phage libraries to find peptides that could inhibit CD154–CD40 interaction. We found eight phage peptides that could specifically bind to CD40, and three of them inhibited the binding of CD40 and CD154 molecules. A consensus sequence (APxPPxxT) was identified in the three phages that showed inhibitory activity. Based on the inhibitory action of these regulatory peptides on CD40–CD154 interaction, we discuss the possible use of such strategy for the treatment of autoimmune diseases.

Materials and methods

Fusion proteins

CD40-Ig, which is fused to the extracellular domain of human CD40 and the human IgG1 Fc region, were prepared by constitutive transfection of HEK293 cells and purified with Hitrap-rProtien A affinity chromatography (Pharmacia Biotech, Uppsala, Sweden).[7,8] Soluble CD154 (sCD154), consisting of the extracellular domain of murine CD8α tagged with FLAG and the extracellular domain of human CD154, were also obtained using HEK293 cells and were purified with anti-FLAG M2-agarose affinity resin (Sigma, St. Louis, MO, USA) as described previously.[9] Bioactivity of sCD154 was checked by the induction of CD95

M. Kitagawa · D. Goto · M. Mamura · I. Matsumoto · S. Ito · A. Tsutsumi · T. Sumida (✉)
Department of Internal Medicine, Institute of Clinical Medicine, University of Tsukuba, 1-1-1 Tennodai, Tsukuba 305-8575, Japan
Tel. +81-298-53-3221; Fax +81-298-53-3222
e-mail: tsumida@md.tsukuba.ac.jp

on Daudi lymphoma cells, which constitutively express functional CD40.

Selection of CD40-binding phages

M13 phage display libraries displaying linear peptides of 12-mer amino acids (Ph.D. System, New England Biolabs, Beverly, MA, USA) were used to select CD40-Ig binding phages (40BP). Panning was performed as described in the product manual with the following modifications. CD40-Ig was diluted to 50μg/ml in 0.1M $NaHCO_3$ (pH 8.6) and adsorbed onto 96-well microtiter plates (Falcon 1172, non-tissue culture treated) for 12h at 4°C. After blocking for 3h with 0.5% bovine serum albumin in 0.1M $NaHCO_3$ (pH 8.6), aliquots of the phage display libraries (2×10^{11} plaque-forming units) were diluted in Tris-buffered saline (TBS) (150mM NaCl, 50mM Tris–HCl, pH 7.5) containing 0.1% Tween-20, and phages were allowed to bind for 1h at room temperature. Unbound phages were removed by washing with TBS containing 0.5% Tween-20, and bound phages were eluted with 250μg/ml sCD154 in TBS containing 0.5% Tween-20, and amplified in *Escherichia coli* ER2537. Phages were prepared from the culture supernatant by standard polyethylene glycol/NaCl precipitation and used for the next round of panning. After the third round of panning randomly selected phage plaques were amplified, and single-strand DNA was sequenced using the Amplitaq FS sequencing kit (Applied Biosystems, Foster City, CA, USA) with a primer (5′-CCCTCATAGTT AGCGTAACG-3′).

Phage-binding assay

CD40-Ig or human IgG_1 (Sigma, I-8640) was coated on 96-well plates as described above. Phages amplified from single plaques and purified by polyethylene glycol/NaCl precipitation were serially diluted in TBS containing 0.5% Tween-20 and allowed to bind to CD40-Ig for 2h at room temperature. After washing, bound phages were detected with a horseradish peroxidase-conjugated anti-M13 monoclonal antibody (Amersham Pharmacia Biotech, Piscataway, NJ, USA). Tetramethylbenzidine peroxidase substrate mixture (Sumitomo Bakelite, Akita, Japan) was added and the absorbance was measured at 450nm by a microplate reader (SPECTRAmax190; Molecular Devices, Sunnyvale, CA, USA).

Recombinant phage protein

Recombinant g3p N1 fragments displaying distinct 12-mer peptides of 40BP (40BP-N1) were constructed using pET-20b(+) His-tagging expression vector. The polymerase chain reaction (PCR) 5′-primer was GATCCCATGGGTA TGAAAAAATTATTATTCGCAATTCC and the 3′-primer was GAATCTCGAGTTCAGGGATAGCAAGC CCA. The PCR products were cleaved with *Nco*I and *Xho*I and inserted into pET-20b(+). 40BP-N1 were obtained using *Escherichia coli*. BL21(DE3) transformed with the constructed vector and 40BP-N1 production was induced by 0.5mM isopropyl-β-D-thiogalactopyranoside. The produced 40BP-N1 in periplasmic and cytoplasmic fraction was purified with Ni affinity chromatography. The amino acid sequences of each 40BP-N1 were confirmed by a peptide sequencer. 12-mer peptide was identified on the N-end of each 40BP-N1.

CD40–CD154 binding assay

sCD154 was iodinated with ^{125}I-labeled Bolton-Hunter reagent (NEN Life Science Products, Boston, MA, USA) as described in the product manual. The binding of [^{125}I]sCD154 to CD40-Ig was performed using protein-A scintillation proximity assay (SPA) regent, type I (Amersham Life Science, Buckinghamshire, UK) in 96-well Opti-plate. CD40-Ig (0.2μg/ml) diluted in TBS containing 10% fetal bovine serum was incubated with 10nM [^{125}I]sCD154 for 30min. Then, SPA reagent (4mg/ml) was added and the plate was incubated for 1h with agitation. The binding of [^{125}I]sCD154 was counted with scintillation counter (Packard, Meriden, CT, USA). All procedures were carried out at room temperature. The competition assay was performed using the purified 40BP-N1. The serially diluted 40BP-N1 was incubated with CD40-Ig for 10min before the addition of [^{125}I]sCD154. The subsequent procedures were the same as described above.

Results

CD40 binding phage peptides

By CD40 binding activity using CD40-Ig-coated plates, 2×10^{11} phage display libraries displaying linear peptides of 12-mer amino acids were screened. After the third round of panning, eight phage plaques were randomly selected. These CD40 binding phages (40BP-1, -2, -3, -4, -5, -6, -7, and -8) had distinct 12-mer amino acid on their N-terminal of g3p (Fig. 1). The sequences of all 40BPs did not show homology to CD40 and CD154 at the amino acid level. To confirm the specificity of their CD40 binding, these phages were applied to CD40-Ig-coated plates or control human IgG-coated plates. As control, wild-type phages (WT) and the phages randomly selected from the plaques before the first panning (NP-1, -2, and -3) were also examined. As shown in Fig. 1, 40BPs bound to CD40-Ig but not to human control IgG. On the other hand, WT, NP-1, -2, and -3 did not bind to both CD40-Ig and human IgG. 40BP-1, -2, -3, and -6 bound to CD40-Ig more preferably than the other 40BPs.

Inhibitory effects of 40BP peptides for CD40–CD154 interaction

To examine whether the peptides expressed on 40BPs could inhibit the binding between CD40 and CD154 molecules,

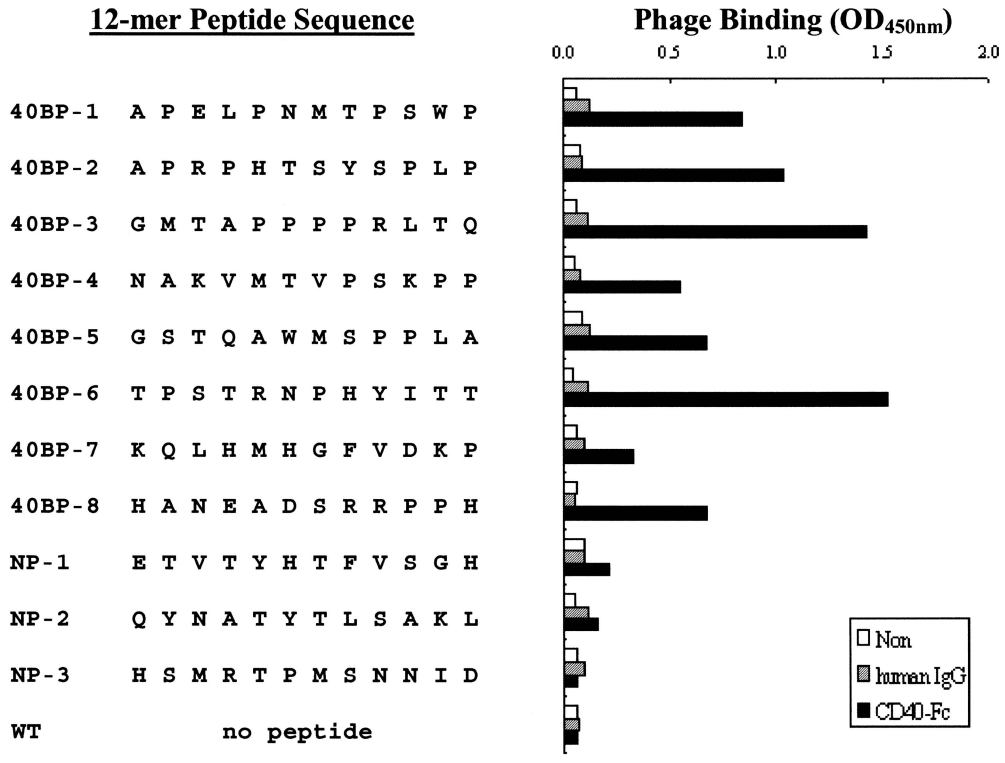

Fig. 1. Specificity of phage peptides. Eight CD40 binding phages (40BP-1 to -8) selected from the plaques after the third panning were able to bind to CD40-Ig fusion protein but not to human IgG. The three other phages randomly selected from the plaques before the first panning (NP-1 to -3) are control peptides that do not have binding activity to CD40-Ig. *Wt*, wild-type phages with no peptides

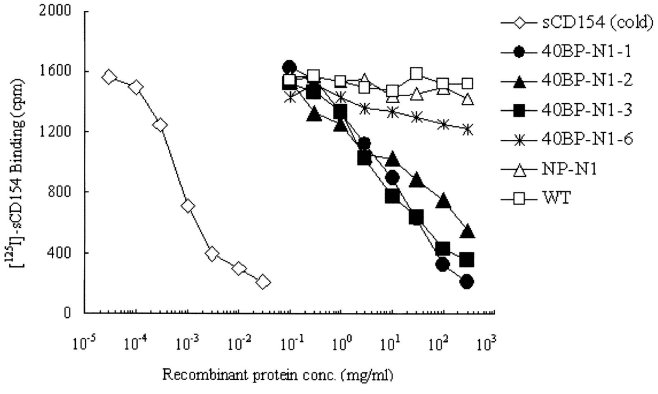

Fig. 2. Competitive inhibition of CD40–sCD154 binding. CD40-Ig and [^{125}I]sCD154 binding was competitively inhibited by cold sCD154 (positive control), recombinant phage proteins (40BP-N1-1, -2, -3, -6, NP-N1), and WT phage g3p N1 fragment (WT, negative control). Three peptides (40BP-N1-1, -2, and -3) with a consensus sequence interfered with CD40–sCD154 interaction, whereas 40BP-N1-6 with no consensus sequence, NP-N1, and WT with no binding activity to CD40-Ig did not inhibit CD40–CD154 binding

the recombinant fusion proteins consisting of 12-mer peptide and N1 fragment of phage g3p from 40BP-1, -2, -3, and -6 were prepared (40BP-N1-1, -2, -3, and -6, respectively), and the soluble CD154 (sCD154) and CD40-Ig binding inhibition assay was carried out. The purity of recombinant proteins was analyzed by the sodium dodecyl sulfate–polyacrylamide gel electrophoresis method (data not shown). As shown in Fig. 2, the binding of CD40-lg and [^{125}I]sCD154 was competitively inhibited by using cold sCD154 at concentrations of 0.001–0.01 mg/ml. The binding of [^{125}I]sCD154 to CD40-Ig was inhibited by 40BP-N1–1, -2, and -3, when they were used at high concentrations of 1–300 mg/ml. In contrast, 40BP-N1-6, NP-N1 (the recombinant fusion proteins consisted of 12-mer peptide and N1 fragment of phage g3p from NP-1), and WT (wild-type phage g3p N1 fragment) did not show such an inhibitory effect even at a higher concentration (up to 300 mg/ml). A consensus sequence (APxPPxxT) was identified among the three inhibitory peptides, whereas no motif was identified in the other peptides that had no inhibitory effect.

Discussion

We have screened eight phages that could specifically bind to CD40. These phages have expressed a different 12-mer peptide on their N-terminal of g3p fragment protein, respectively. Four phages (40BP-1, -2, -3, and -6) bound to CD40-Ig more preferably than the other 40BPs. In the present study we provided evidence for the inhibitory effects of three novel peptides (40BP-N1-1 – APELPNMTPSWT; 40BP-N1-2 – APRPHTSYSPLP; and 40BP-N1-3 – GMTAPPPPRLTQ) on CD40–CD154 interaction when used at high concentrations, and that a consensus sequence (APxPPxxT) was conserved in these three peptides. These peptides did not show any homology with CD40 and its ligands CD154 by computer analyses at the amino acid level. Thus, we conclude that these peptides with the APxPPxxT motif could be considered as novel inhibitory molecules for the CD40–CD154 interaction.

Interestingly, 40BP-N1-6 constructed from 40BP-6 phage, which preferably bound to CD40, did not inhibit CD40–CD154 interaction. A multidimensional protein structure of 12-mer peptide and full-length g3p protein (phage minor capsid protein) may contribute to the binding of the 40BP-6 phage to CD40. The structure could not retain in 40BP-N1-6 recombinant protein, which is composed of only 12-mer peptide and g3p N1 fragment, a part of g3p protein.

The efficacy of blocking the CD40–CD154 interaction therapeutically with an anti-CD154 monoclonal antibody has been shown in animal models for many autoimmune diseases, including rheumatoid arthritis, SLE, multiple sclerosis, and myasthenia gravis.[3–6,10] Therefore, disruption of the CD40–CD154 pathway has been proposed as a strategy for treating various human autoimmune diseases mediated by T cells and autoantibodies. At present, antibody against CD154 or CD40 is the only way that has been shown to block the CD40–CD154 interaction. In this study, we have devised a new strategy to inhibit CD40–CD154 interaction by peptides. What is the difference between anti-CD154 antibodies and inhibitory peptides identified in the present study? First, there are major differences in the molecular weight. IgG antibody is a molecule of about 150 kDa, while the inhibitory peptides are only 12-mer amino acids and are estimated to be 1–2 kDa molecules. Thus, it is very easy to produce these peptides and it is difficult to generate antibodies against these molecules in vivo. Secondly, the affinity of the antibody to CD154 may be higher than that of the peptides to CD40 molecule. The K_d is 10^{-3} M in the case of antigen–antibody interaction, whereas the peptide–CD40 interaction might be less than the K_d of T-cell receptors and peptides in the context of the major histocompatibility complex (10^{-4}–10^{-5} M).[11] Thus, several peptides are required to regulate the CD40–CD154 interaction. Further studies are also necessary to clarify the inhibitory activity of these peptides on the CD40–CD154 interaction in vitro and in vivo.

In conclusion, we have described three novel peptides that could specifically inhibit the CD40–CD154 interaction, suggesting that these might constitute a novel therapeutic method in autoimmune disease mediated by CD40–CD154.

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