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A neutralizable epitope is induced on HGF upon its interaction with its receptor cMet

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Abstract

A new conformational neutralizable epitope is created on heptocyte growth factor (HGF), when it interacts with its receptor, cMet. By immunizing rabbits with HGF–cMet complex, we successfully generated a monoclonal antibody (SFN68) that inhibits HGF–cMet interaction, and blocks the biological function mediated by HGF. To define the epitope, we screened out an epitope-mimicking peptide, KSLSRHDHIHHH, from a phage display of combinatorial peptide library. In molecular mimicry this peptide bound to cMet and inhibited HGF–cMet interaction. No humoral response was induced to this epitope-mimicking peptide when immunization was done with HGF alone.

Keywords: HGF; cMet; Antibody; Phage display

Hepatocyte growth factor (HGF) has been considered as a motogen, mitogen, and morphogen [1]. HGF has been shown to induce the development and progression of cancer and angiogenesis via cMet, its only known functional receptor [1]. Implication of HGF and cMet in cancer progression has generated great interest in the development of agents capable of neutralizing HGF and cMet. Several HGF-neutralizing monoclonal antibodies significantly suppress tumor growth in the xenograft model but their epitopes were not defined [2,3]. Since antibodies to different epitopes show distinct clinical activities [4], it is necessary, for development of an optimal immunotherapeutic reagent, to generate and test the clinical efficacy of antibodies to diverse panel of epitopes. As a few monoclonal antibodies were generated serendipitously to a receptor induced binding site (RIBS) on ligand [5–7], we hypothesized that a new epitope might be induced on HGF during its complex formation with cMet. We generated a recombinant monoclonal antibody, SFN68, to this epitope by immunizing HGF–cMet complex. We showed that SFN68 is a neutralizing antibody that inhibited HGF–cMet interaction, cMet activation, and, cell scattering and proliferation mediated by HGF. As the epitope of SFN68 is characterized to be non-linear in Western blot analysis, we screened a phage display of combinatorial library, for a peptide specifically reactive to SFN68 and confirmed that this peptide is epitope-mimicking by showing that it bound to cMet and inhibited HGF–cMet interaction. We further proved that this epitope is formulated by its interaction with cMet by showing that the humoral response to this epitope-mimick-

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ing peptide is elicited only by immunizing HGF in combination with cMet, and not by immunizing HGF alone.

Materials and methods

Materials. Recombinant human HGF and cMet/Fc chimera were purchased from R&D Systems (Minneapolis, MN). HRP (horseradish peroxidase)-conjugated anti-human Fab antibody and HRP-conjugated rabbit anti-human Fc antibody were purchased from Pierce (Rockford, IL). Microtiter plate was purchased from Costar (Cambridge, MA).

Construction of antibody library, selection of binders, and characterization of the epitope. A mixture of 3.2 μg recombinant human HGF and 5 μg cMet/Fc chimera was dissolved in 2 ml phosphate-buffered saline (PBS), incubated at 37 °C for 30 min, emulsified with MPL + TDM + CWS adjuvant (Sigma, St. Louis, MO) and used for immunization of New Zealand white rabbits. After four booster injections on a 3-week interval schedule, mRNA was prepared from the spleen and the bone marrow and cDNA was synthesized using this mRNA. From this cDNA, a rabbit/human chimeric Fab library was constructed using phage display as described previously [8]. Fab clones were selected from the library, through biopanning on HGF coated-microtiter plates. For further analysis, selected Fabs were over-expressed and purified as described previously [9]. For characterization of the epitope, HGF was dissolved in reducing and non-reducing sample buffers (Invitrogen, Carlsbad, CA) and loaded into 4–12% bis–tris sodium dodecyl sulfate–polyacrylamide gel (Invitrogen). After gel electrophoresis, the proteins were transferred onto a nitrocellulose membrane. After blocking with Tris-buffered saline (TBS) with 3% bovine serum albumin (Sigma, blocking buffer), the membrane was incubated with 1.5 ng/ml SFN68 Fab in blocking buffer at room temperature for 12 h and HRP-conjugated anti-human Fab antibody diluted in blocking solution (1:5000) at room temperature for 1 h, with intermittent washing with 0.05% Tween in TBS (TTBS). An electrochemiluminescent kit (Pierce) was used for visualization of the band.

Real-time interaction analysis. The kinetic parameters of the interaction between the Fab and HGF were determined using the BIAcore system (Biacore AB, Uppsala, Sweden) [10]. HGF was immobilized on a CM5 dextran sensor chip (Biacore AB) in 10 mM sodium acetate buffer (pH 4.0) at a flow rate of 5 μl/min using the amine coupling kit. SFN68 Fab in PBS containing 0.005% surfactant P20 was injected over 2 min at a flow rate of 30 μl/min at 25 °C and the surface was regenerated with 1 M NaCl/50 mM NaOH. To monitor the neutralizing effect of SFN68 Fab, cMet/Fc chimera was immobilized on a CM5 sensor chip as described above HGF was applied to the chip at a flow rate of 30 μl/min for 2 min at 25 °C in the presence of either Fab (0–1.5 μM) or soluble cMet/Fc chimera (0–600 nM).

Competition enzyme immunoassay. To monitor the neutralizing effect of SFN68 Fab, a microtiter plate was coated with HGF and blocked with blocking buffer. Twenty-five microliters of 2 nM cMet/Fc chimera in TBS-B and 25 μl of 0–200 nM SFN68 Fab in blocking buffer were applied to the wells. The amount of bound cMet/Fc chimera was determined using HRP-conjugated rabbit anti-human Fc antibody and ultra-TMB substrate solution (Pierce).

Expression of SFN68 IgG_1. The variable heavy chain gene with signal sequence was amplified by PCR with two sense primers (5′-NNNAAGCTTATGGAATGGAGTTGGATATTTCTCTTTCTCCTGTCAGGAACTGCAG-3′, 5′-CTCTTTCTCCTGTCAGGAACTGCAGGTGTCCACTCTCAGCAGCAGCTGGTGGAGTCC-3′) and an antisense primer (5′-TGAGGAGACGGTGACCAGGGT-3′) using SFN68 Fab DNA as a template. The IgG_1 constant region gene was amplified by PCR with a sense primer (5′-ACCCTGGTCACCGTCTCCTCAGCCAGCACGAAGGGCCCAT-3′) and an antisense primer (5′-NNGGATCCTCATTTACCCGGGGACAGGGAG-3′) using human bone marrow cDNA as a template The variable kappa chain with signal sequence was amplified by PCR with two sense primers (5′-NNNNNNNNGCGGCCGCATGGAGACAGACACACTCCTGCTATGGGTACTGCTGCTCTGGGTTCCA-3′, 5′-GGTACTGCTGCTCTGGGTTCCAGGTTCCACTGGTGACGAGCTCGATCTGACCCAGACTCC-3′) and an antisense primer (5′-GAAGACAGATGGTGCTGCAGCCACAGT-3′) using SFN68 Fab DNA as a template. The kappa chain constant region gene was amplified by PCR with a sense primer (5′-CGAACTGTGGCTGCACCATCTGTC-3′) and an antisense primer (5′-NNNNNTTCGAATTAACACTCTCCCCTGTTGAAGCTCTT-3′) using pComb3XTT DNA [8] as a template. All the above PCR was cycled 30 times at 94 °C for 15 s, 56 °C for 30 s, and 72 °C for 90 s. The heavy chain gene was generated by a linker PCR using a variable region gene and constant region gene as templates using a sense primer (5′-NNNAAGCTTATGGAATGGAGTTGGATATTTCTCTTTCTCCTGTCAGGAACTGCAG-3′) and an antisense primer (5′-TGAGGAGACGGTGACCAGGGT-3′). The kappa chain genes was generated by a linker PCR using a kappa variable and constant region genes using a sense primer (5′-NNNNNNNNGCGGCCGCATGGAGACAGACACACTCCTGCTATGGGTACTGCTGCTCTGGGTTCCA-3′) and an antisense primer (5′-NNNNNTTCGAATTAACACTCTCCCCTGTTGAAGCTCTT-3′). The linker PCR was carried for 30 cycles at 94 °C for 15 s, 56 °C for 30 s, and 72 °C for 2 min. *Hin*dIII (New English Biolabs, Beverly, MA) and *Bam*HI (NEB) restriction enzymes were used for cloning of heavy chain and *Not*I (NEB) and *Bst*BI (NEB) restriction enzymes were used for cloning of kappa chain gene, respectively, into pBudCE4.1 expression vector (Invitrogen). The expression vector was transfected into Human embryonic kidney (HEK) 293F cells (Invitrogen) using 293fectin (Invitrogen). After culture of the cells in Freestyle™ 293 expression medium (Invitrogen) at a 37 °C with 8% CO_2 for 48 h, SFN68 IgG_1 in the media was purified by protein A column chromatography.

MDCK-2 scattering and cell proliferation assays. DMEM containing a mixture of 25 pM HGF and 1.25 nM SFN68 IgG_1 was prepared, pre-incubated for 1 h at 37 °C and applied to MDCK-2 cells. After overnight incubation, the cells were fixed as described previously [11] and monitored under an inverted light microscope. For cell proliferation assay, DMEM with 10% FBS containing 625 pM HGF with or without 62.5 nM SFN68 IgG_1 was applied to HepG2 cells. Periodically, the number of viable cells was determined using the CellTiter 96® AQueous One Solution Cell Proliferation Assay (Promega, Madison, WI).

ERK1/2 phosphorylation assay. HepG2 cells were treated with mixtures of 250 pM HGF with either 25 nM SFN68 IgG_1 or irrelevant human IgG for 5 min after incubation for 24 h in serum-free medium, lysed in a lysis buffer (1% Triton X-100, 0.5% Nonidet P-40, pH 7.5, 50 mM Tris, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM $NaVO_4$, 1 mM NaF, 25 mM β-glycerophosphate, 1 mM phenylmethylsulfonyl fluoride, 5 mg/L leupeptin inhibitor, and 5 mg/L pepstatin A inhibitor) and clarified by centrifugation at 13,000 rpm for 5 min. Cell lysate was subjected to 4–12% bis–tris sodium dodecyl sulfate–polyacrylamide gel (Invitrogen) under reducing conditions and transferred onto a nitrocellulose membrane. After blocking with blocking buffer for 30 min, blots were incubated with either anti-ERK1/2 rabbit antibody (Santa Cruze Biotechnology Inc., Santa Cruze, CA) or anti-phospho-ERK1/2 rabbit antibody (Cell Signaling Technology Inc., Beverly, MA) diluted in blocking buffer (1:1000) overnight at room temperature and HRP-conjugated mouse anti-rabbit secondary antibody (Pierce) diluted in blocking buffer (1:5000) for 1 h at room temperature. The immunoreactive proteins were detected with the Supersignal West Pico Substrate (Pierce).

Identifying the epitope-mimicking peptide of SFN68. Binders were selected from Ph.D.-7, C7C and 12 phage display peptide libraries (NEB) through four rounds of biopanning on SFN68 Fab immobilized to the microtiter plates via mouse anti-HA antibody as described previously [12]. Chemical synthesis of the peptide was performed by Peptron (Daejeon, South Korea). Specific reactivity of phages displaying the KSLSRHDHIHHH peptide to SFN68 Fab and cMet/Fc chimera was tested using the microtiter plates coated with each protein and anti-M13 phage antibody (Amersham Biosciences, Uppsala, Sweden) as described previously [12]. Binding of SFN68 Fab and cMet/Fc chimera to the synthetic KSLSRHDHIHHH peptide was tested in an enzyme immunoassay using a microtiter plate coated with the peptide. The amounts of SFN68 Fab and cMet/Fc chimera bound to the microtiter plate were determined using HRP-conjugated goat anti-human Fab antibody and HRP-conjugated rabbit anti-human Fc antibody, respectively. Inhibition of HGF–cMet interaction by the peptide was tested

using a microtiter plate coated with HGF and HRP-conjugated rabbit anti-human Fc antibody.

Immunization and analysis of humoral response. New Zealand white rabbits ($n = 3$ per group) were immunized with 3.2 μg HGF, 5 μg cMet/Fc chimera or their mixture in parallel as described above. Sera were collected before immunization and after four booster injections given on a 3 week interval schedule. The reactivity of sera with KSLSRHDHIHHH, HGF and cMet/Fc chimera was tested by an enzyme immunoassay using a microtiter plate coated with HGF, cMet/Fc chimera or the mimetic peptide and HRP-conjugated mouse anti-rabbit IgG antibody (Pierce).

Results

Generation and characterization of recombinant antibodies to HGF

After immunization with HGF–cMet complex, a rabbit/human chimeric Fab library was constructed using phage display with a complexity of 3.3×10^9. Twenty-nine positive clones were selected via biopanning on HGF coated on a microtiter plate. Eight clones encoded SFN68 Fab gene, and 21 clones encoded SFN61 Fab gene (Fig. 1A). These two Fabs were highly homologous with each other and the amino acid residues of the variable region of the heavy (V_H) and light (V_L) chains differed by only 12 amino acid residues.

The kinetics of interaction of SFN68 Fab with HGF was examined by real-time interaction analysis. The affinity of SFN68 Fab for immobilized HGF on a sensor chip was calculated from the serial Fab dilutions. The kinetic parameters of the interaction were as follows: $k_a = 7.6 \times 10^3\ M^{-1}s^{-1}$; $k_d = 9.46 \times 10^{-5}\ s^{-1}$; $K_D = 1.24 \times 10^{-8}$ M (Fig. 1B). In enzyme immunoassay, both SFN61 Fab and SFN68 Fab reacted with the native form of HGF coated on a microtiter plate (data not shown). In Western blot analysis, both Fabs failed to recognize the reduced form of HGF, but did react with the non-reduced form (Fig. 1C). This selectivity indicated that the epitope of these two Fabs is conformation-dependent and non-linear.

SFN68 neutralizes HGF

In real-time interaction analysis, SFN68 Fab inhibited binding of HGF to immobilized cMet/Fc chimera in a dose-dependent manner with an IC_{50} of 533 nM (Fig. 2A). Soluble cMet/Fc chimera, used as a positive control, blocked binding of HGF to immobilized cMet/Fc chimera with an IC_{50} of 192 nM (Fig. 2B). In a competition enzyme immunoassay SFN68 Fab specifically blocked binding of cMet/Fc chimera to immobilized HGF in a dose-dependent manner (Fig. 2C). An unrelated chimeric Fab did not show any inhibition in both studies. In MDCK-2 cell scattering assay complete scattering of cells were observed when cells were incubated with 25 pM HGF overnight (Fig. 2D). When SFN68 IgG_1 was added

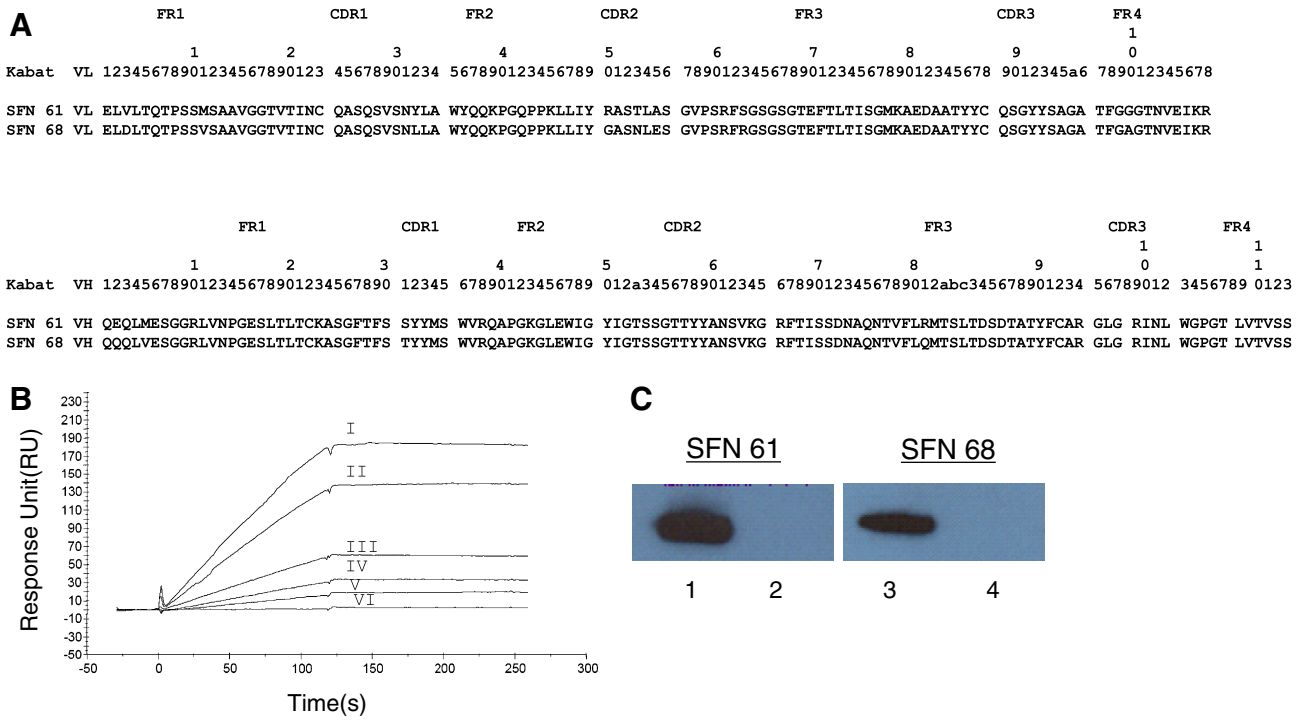

Fig. 1. Generation and characterization of SFN 68 and SFN61. (A) Amino acid sequences of SFN68 and SFN61. (B) SFN68 (I: 600 nM, II: 400 nM, III: 200 nM, IV: 100 nM, and V: 50 nM) bound to HGF immobilized on a CM5 sensor chip in a dose-dependent manner while an unrelated Fab (VI: 600 nM) did not. (C) In Western blot analysis both SFN61 and SFN68 reacted with unreduced form of recombinant HGF (lanes 1 and 3) but did not recognize the reduced form (lanes 2 and 4).

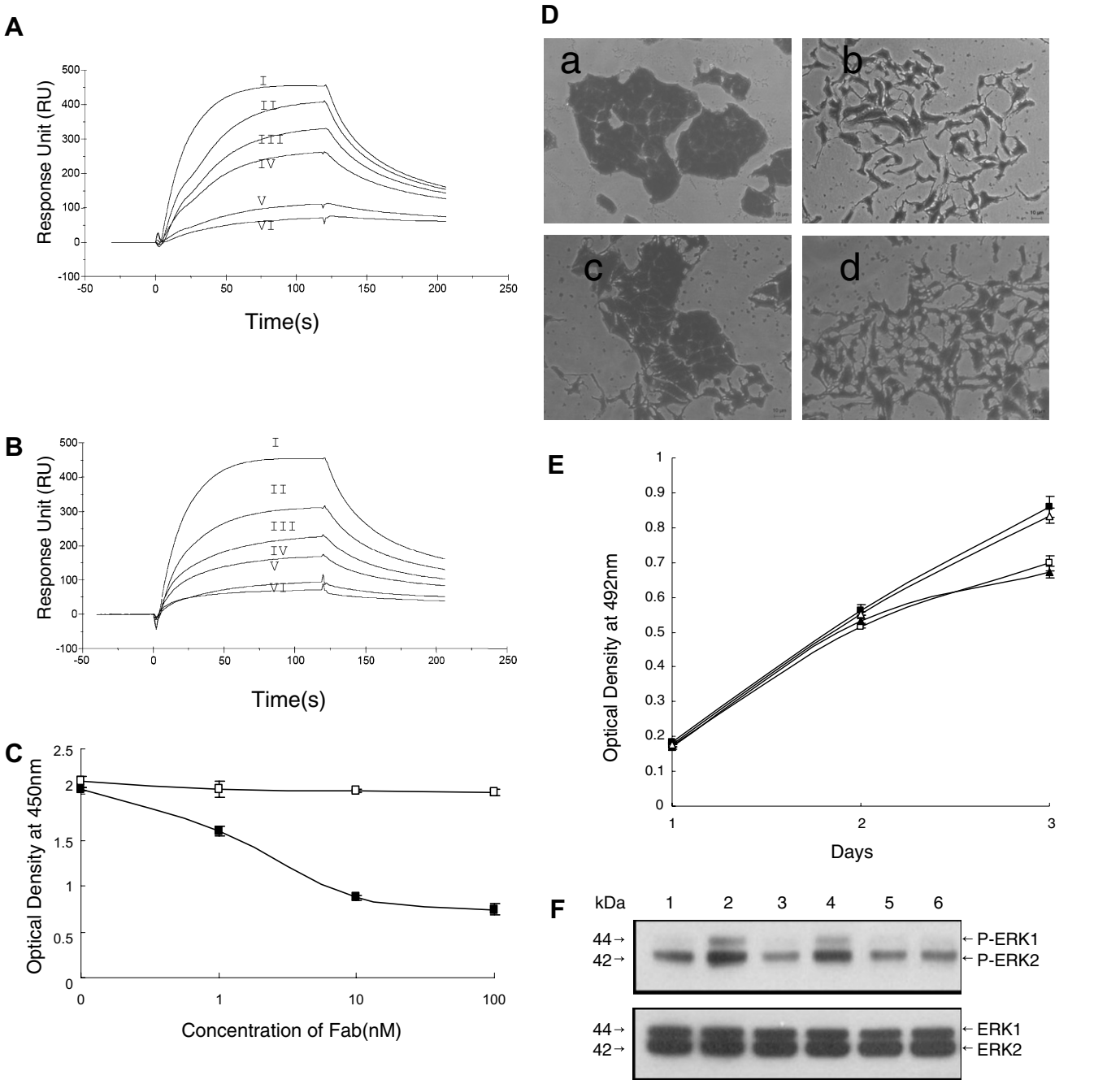

Fig. 2. Neutralization of HGF by SFN68. (A and B) In real-time interaction analysis, SFN68 Fab (A, I: 0 nM, II: 50 nM, III: 250 nM, IV: 500 nM, V: 1 μM, and VI: 1.5 μM) and soluble cMet/Fc chimera (B, I: 0 nM, II: 50 nM, III: 100 nM, IV: 200 nM, V: 400 nM, and VI: 600 nM) inhibited binding of HGF to the cMet/Fc chimera conjugated on CM5 chip in a dose-dependent manner. The sensograms are presented after subtracting the background signal obtained by injecting the sample over unconjugated CM5 sensor chip. (C) In a competition enzyme immunoassay, the cMet/Fc chimera was mixed with either SFN68 Fab (■) or an unrelated chimeric Fab (□) (0–100 nM) and applied to the HGF coated on plate. All data points represent means ± SD of triplicate determinations. (D) MDCK-2 cell cells were incubated overnight with media (a), the media with 25 pM HGF (b), the media with 25 pM HGF and 1.25 nM SFN68 IgG_1 (c) or the media with 25 pM HGF and 1.25 nM unrelated human IgG (d). (E) HepG2 cells were treated with control media (□), 625 pM HGF (■), 625 pM HGF and 62.5 nM SFN68 IgG_1 (▲), or 625 pM of HGF and 62.5 nM unrelated human IgG (Δ). All assays were performed in triplicate and reported as means ± SD. (F) HepG2 cells were treated with the following components and analyzed with a Western blot analysis using anti-phosphoERK1 and anti-ERK1 antibodies: lane 1, medium only; lane 2, medium with 250 pM HGF; lane 3, medium with 250 pM HGF and 25 nM SFN68 IgG_1; lane 4, medium with 250 pM HGF and 25 nM unrelated human IgG; lane 5, medium with 25 nM SFN68 IgG_1; lane 6, medium with 25 nM unrelated human IgG.

to the media at the final concentration of 1.25 nM, cell scattering was inhibited (Fig. 2D), which was dose-dependent (data not shown). Irrelevant human IgG did not show any inhibitory effect at the same concentration (Fig. 2D). In cell proliferation assay (Fig. 2E), the growth of HepG2 cells was significantly stimulated by HGF at 625 pM

($p < 0.05$). This HGF-enhanced proliferation was inhibited by SFN68 IgG_1 at 62.5 nM ($p < 0.05$) while irrelevant human IgG showed no inhibition. ERK1/2 phosphorylation in HepG2 cell induced by adding 250 pM HGF in medium was inhibited by adding 25 nM SFN68 IgG_1 while irrelevant human IgG showed no inhibition (Fig. 2F).

The epitope of SFN68 Fab is mimicked by the synthetic peptide, KSLSRHDHIHHH

To define the epitope of SFN68 Fab, we enriched peptide encoding phages from the phage display of a combinatorial peptide library via biopanning on SFN68 Fab-coated

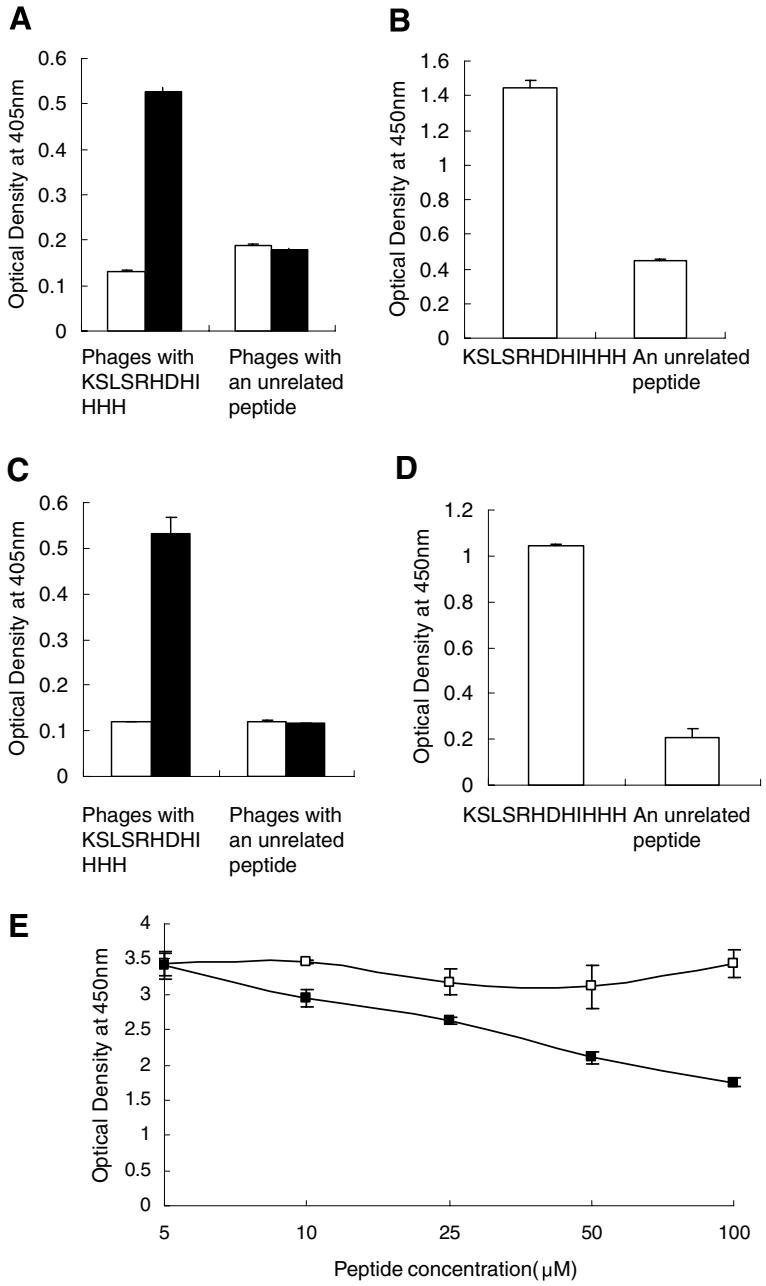

Fig. 3. Confirmation that the synthetic peptide, KSLSRHDHIHHH mimicks the epitope. (A) Microtiter plate wells coated with either with SFN68 (■) or with an unrelated Fab (□) were allowed to react to phages displaying KSLSRHDHIHHH or unrelated phages. The amount of bound phages was determined using HRP-conjugated anti-M13 antibody. (B) SFN68 was added to microtiter plate wells coated with KSLSRHDHIHHH or an unrelated peptide and detected by HRP-conjugated anti-human Fab antibody. (C) Microtiter plate wells were coated with cMet/Fc chimera and blocked (■) or blocked without coating (□). Wells were incubated either with phages displaying KSLSRHDHIHHH or with phages encoding an unrelated peptide. The amount of bound phages was determined using HRP-conjugated anti-M13 antibody. (D) The cMet/Fc chimera bound to microtiter plate wells coated with KSLSRHDHIHHH or an unrelated peptide was detected by HRP-conjugated anti-human Fc antibody. (E) The cMet/Fc chimera was mixed either with KSLSRHDHIHHH (■) or with an unrelated peptide (□) (0–100 μM) and added to microtiter plate wells coated with HGF. The amount of bound cMet/Fc chimera was determined using HRP-conjugated anti-human Fc antibody. Data are presented as means ± SD of triplicate determination (A–E).

Table 1
Specificity of rabbit sera to immobilized HGF, cMet/Fc chimera, and KSLSRHDHIHHH

Antigen used for immunization	Antigen coated on a microtiter plate		
	HGF	cMet/Fc chimera	KSLSRHDHIHHH
HGF–cMet/Fc chimera complex			
Pre-immune	0.167 ± 0.008	1.272 ± 0.019	0.220 ± 0.015
Immune	4.075 ± 0.152	4.220 ± 0.318	3.070 ± 0.168
HGF			
Pre-immune	0.499 ± 0.042	1.661 ± 0.149	0.240 ± 0.090
Immune	5.066 ± 0.833	1.177 ± 0.069	0.158 ± 0.005
cMet/Fc chimera			
Pre-immune	0.294 ± 0.042	1.344 ± 0.018	0.150 ± 0.013
Immune	0.221 ± 0.036	4.568 ± 1.240	0.148 ± 0.002

The optical density of wells is measured and reported as means ± SD. A typical data representing each group ($n = 3$ per group) is presented.

microtiter plate wells. Of 14 clones with specific reactivity, four shared the amino acid sequence KSLSRHDHIHHH. The phages displaying KSLSRDHIHHHH and the chemically synthesized peptide bound specifically to SFN68 Fab (Fig. 3A and B). The cMet/Fc chimera coated on a microtiter plate specifically reacted with phages displaying KSLSRHDHIHHH, but not with phages encoding an unrelated peptide (Fig. 3C). The synthetic KSLSRHDHI HHH peptide coated on a microtiter plate also specifically interacted with cMet/Fc chimera (Fig. 3D). When the mixture of cMet/Fc chimera and KSLSRHDHIHHH was added to HGF-coated microtiter plate coated with HGF, KSLSRHDHIHHH but not an unrelated peptide inhibited cMet/Fc chimera binding to HGF in a dose-dependent manner (Fig. 3E).

Antibody response to KSLSRHDHIHHH is elicited only by immunization of HGF–cMet complex

In enzyme immunoassay (Table 1), rabbits immunized with HGF alone showed increased antibody titers only to HGF, but not to KSLSRHDHIHHH. Rabbits immunized with the HGF–cMet/Fc chimera complex showed elevated antibody titers to both HGF and KSLSRHDHIHHH, while immunization with the cMet/Fc chimera alone did not induce the humoral response to HGF and KSLSRHDHIHHH.

Discussion

We found that antibody response to the KSLSRHD HIHHH peptide mimicking the epitope of a HGF-neutralizing antibody SFN68 was elicited only when HGF–cMet complex was the immunizing agent, but not when HGF or cMet alone was used as the immunogen (Table 1), indicating that the SFN68 epitope emerges on HGF when it forms a complex with cMet. HGF was previously reported to have at least two cMet binding sites, namely a high affinity binding site in the α-chain (k_d: 50–70 pM) and a low affinity binding site in the β-chain (k_d: 90 nM) [13,14]. Due to this extremely high affinity it might be possible that the complex persists *in vivo* long enough to induce an immune response specific to the newly formed conformational structures on HGF.

Since SFN68 bound to HGF that was not in complex with cMet (Fig. 1B and C), it is clear that the SFN68 epitope is induced by molecular interaction between HGF and SFN68. But how SFN68 could block the formation of the complex is not clear. A similar phenomenon, neutralization of a receptor by an antibody to a ligand induced binding site (LIBS) on integrin $\alpha_{IIb}\beta_3$, was previously reported but the underlying mechanism was not studied [15]. Two possible mechanisms might be invoked to explain this phenomenon. First, molecular interaction between HGF and the SFN68 may induce conformational changes which not only create the epitope but also neutralize both binding sites. Second, there is a chance that the epitope of SFN68 is located on one of the binding sites and directly inhibits HGF interaction with cMet by allosteric interference at one binding site and indirectly inhibit cMet interaction at the other binding site by inducing conformational changes. This possibility is strongly supported by our observation that the epitope-mimicking peptide KSLSRHDHIHHH binds to cMet (Fig. 3D) and inhibited the latter's interaction with HGF (Fig. 3E).

In summary, we show here that immunization with HGF–cMet complex induces antibody response to a new epitope specific to the complex. Currently, we do not know whether this immunization approach can be applied to other protein complexes. However, it may be tried when antibodies specific to new epitopes not present in the interacting components are required, if there is evidence that structural changes take place during the complex formation and the reacting components have high affinity for each other.

Acknowledgments

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