A Novel Peptide Isolated from a Phage Display Peptide Library with Trastuzumab Can Mimic Antigen Epitope of HER-2

Received for publication, September 27, 2004, and in revised form, November 4, 2004  
Published, JBC Papers in Press, November 9, 2004, DOI 10.1074/jbc.M411047200

Beihai Jiang‡, Wenbin Liu‡, Hong Qu§, Lin Meng‡, Shumei Song‡, Tao Ouyang¶, and Chengchao Shou‡‡

From the ‡Department of Biochemistry and Molecular Biology, Beijing Institute for Cancer Research and Peking University School of Oncology, Beijing 100034, China, the §College of Life Sciences, Peking University, Beijing 100871, China, and the ¶Breast Cancer Center, Beijing Cancer Hospital, Beijing 100036, China

Trastuzumab, a humanized antibody to HER-2, has been shown to be effective in the treatment of breast cancer in which HER-2 overexpression and metastasis occurs. In our search for an effective mimic epitope of HER-2 binding with trastuzumab and to develop HER-2 peptide vaccine, we screened a phage display 12-mer peptide library with trastuzumab as the target. A mimetic peptide (mimotope) H98 (LLGPYELWELSH) that could specifically recognize trastuzumab was isolated. The DNA encoding peptide H98 was cloned and expressed as the fusion protein GST-H98 in Escherichia coli BL21. The purified GST-H98 could specifically bind to trastuzumab and block the binding of trastuzumab to HER-2 protein. Moreover, H98 could significantly block the function of trastuzumab inhibiting the growth of cancer cells. Mice that were immunized with GST-H98 made specific antibody to H98 as well as to HER-2. In addition, T-cell proliferation occurred in mice immunized with GST-H98. Although no sequence homology was found between H98 and HER-2, through the use of structure analysis we were able to determine that peptide H98 contributed to a conformational epitope of HER-2. Furthermore, we determined that the last two amino acids at the C terminus, and the third together with the fourth amino acid at the N terminus of peptide H98 are critical to the binding of H98 to trastuzumab. As a result, we conclude that peptide H98 has potential for being developed as a HER-2 vaccine for biotherapy of cancer with HER-2 overexpression.

HER-2 (also known as Neu or ErbB-2) is a member of the epidermal growth factor receptor (also known as HER) family of receptor tyrosine kinase (1). HER receptors are essential mediators of cell proliferation and differentiation in the developing embryo and in adult tissues (2). Overexpression of the HER-2 oncogene is a frequent molecular event in multiple human cancers, including breast, ovarian, gastric, and colorectal carcinomas (3). In patients with breast cancer, HER-2 overexpression is an independent predictor of survival. It is associated with poor prognosis, aggressive disease, resistance to chemotherapy and hormone therapy (4–6). The critical role of HER-2 in epithelial oncogenesis as well as its selective overexpression on malignant tissues makes it an ideal target for immunotherapy (7). Monoclonal antibodies directed to HER-2 induce phenotypic changes in tumor cells including down-modulation of the HER2 receptor, inhibition of tumor cells growth, reversion of cytokine resistance, restoration of E-cadherin expression levels, and reduction of vascular endothelial growth factor production (8). The humanized anti-HER-2/neu antibody trastuzumab (Herceptin; Genentech, Inc., South San Francisco, CA) has been proven to be effective in clinical trials in patients with HER-2-associated metastatic breast cancer (9), and has antitumor activities as both a single agent and in combination with chemotherapy (10). The molecular mechanisms underlying these growth inhibitory effects are not entirely clear. There is some evidence that antibodies to HER-2 may antagonize the constitutive growth signaling properties of the HER-2 system, enlist immune cells to attack the tumor target, and augment chemotherapy-induced cytotoxicity (8).

The clinical application of trastuzumab has shown efficacy. However, several important drawbacks (generation of antidiotypic antibodies, inadequate tissue distribution, levels necessitating multiple infusions and hence the associated cost) limit the usefulness of passive immunotherapy protocols (11). On the other hand, vaccination strategy is an alternative option that can elicit endogenous tumor inhibitory antibodies, stimulate immunologic memory, and accordingly provide long-term benefits to patients at lower costs. The use of a peptide or part of a protein rather than a whole protein as vaccines may circumvent tolerance against self-protein HER-2 and induce cross-reactive immunity.

Although many anti-HER-2 antibodies inhibit the proliferation of cancer cells, some actively stimulate cancer growth. It is well known that different biological effects are associated with the epitope specificity of the antibodies. Isolation of epitopes recognized by trastuzumab, an anti-tumor therapeutic monoclonal antibody directed against HER-2, can be useful in the development of peptide-based vaccines that are capable of stimulating an immune response directed to tumors. The screening of phage display libraries is a powerful technique that has been used in epitope or ligand mapping studies to define peptides that bind to a given antibody or receptor molecule (12–15). In this study, we used a phage display 12-mer peptide library to identify peptides that bind to trastuzumab, and tested their immune and biological activity. The screening of a 12-mer peptide library displayed on M13 filamentous phages led to the...
isolation of peptide H98 (LLGPYELWELSH). Further investigation showed that peptide H98, mimicking the binding epitope on HER-2 for trastuzumab, specifically blocked the binding of trastuzumab to HER-2 and elicited an anti-HER-2 antibody response as well as a cellular immune response in mice. Although no sequence homology was found between the mimotope and HER-2, given the known crystal structure of the binding domain of HER-2 and trastuzumab, we found that peptide H98 contributed to a conformational epitope of HER-2. Moreover, the last two amino acids at the C terminus, and the third together with the fourth amino acid at the N terminus of H98, were critical for the binding of H98 to trastuzumab. In addition, MutN1, a mutant of H98, enhanced the binding of trastuzumab to pro-peptide. The approach of using phage display peptide libraries to reveal conformational epitopes may play an important role in research of the immune activity of the HER-2 mimotopes. And the isolated peptide could become a candidate as a vaccine for the treatment of HER-2-associated cancers.

**EXPERIMENTAL PROCEDURES**

**Phage Library, Bacteria, Antibodies, Expression Vector, and Cells—** The Ph.D.-12 Phage Display Peptide Library Kit E8110S was purchased from New England Biolabs (Beverly, MA). The library contained 1.5 x 10^11 pfu/ml with a complexity of 2.7 x 10^10 transformants. The displayed peptides were expressed at the N terminus of the pIII coat protein of the filamentous coliphage M13. The library was stored in Tris-buffered saline, pH 7.5, with 50% glycerol. Phage was propagated in Escherichia coli strain ER2738, which was provided with the kit.

Antibody trastuzumab was provided by Breast Cancer Center at Beijing Cancer Hospital. Horseradish peroxidase (HRP)-conjugated anti-M13 antibody and the glutathione S-transferase (GST) gene fusion system were purchased from Amersham Biosciences. The dihydrofolate reductase (DHFR) gene fusion system was purchased from Qiagen (Valencia, CA).

**E. coli SKBR3 (American Type Culture Collection, Manassas, VA) was cultured in Dulbecco’s modified Eagle’s medium (Hyclone), supplemented with 10% fetal calf serum. NIH3T3-ErbB2 cell line (provided by Dr. S. L. Sun, Peking University School of Oncology) was cultured in RPMI 1640 medium (Hyclone) supplemented with 10% fetal calf serum.**

**Bio-panning and Selection of Phage—** Human normal IgG and trastuzumab were separately immobilized on protein A-agarose beads (Amersham Biosciences). Phages (1.5 x 10^11 pfu) were preabsorbed on beads containing immobilized human normal IgG to remove any phages that were not specifically reactive with trastuzumab. The phages, diluted in Tris-buffered saline containing 0.1% Tween 20, were incubated at 4 °C for 1 h with immobilized trastuzumab. The beads were washed 5 times with 0.1% Tween 20/Tris-buffered saline. Then the phages that bound with trastuzumab and beads complex were amplified by direct infection with E. coli ER2738. The amplified phages were purified by precipitation with 20% polyethylene glycol 8000 (PEG8000), 2.5 mM NaCl and used in the next cycle. Three rounds of selection were performed. After that, individual phages were picked up randomly and subjected to analysis by phage enzyme-linked immunosorbent assay (ELISA) and DNA sequencing, following amplification in E. coli ER2738.

**PHAGE ELISA—**In total, 300 phage clones were tested for reactivity with trastuzumab by ELISA. A single clone was grown for 4.5 h in 1 ml of Luria broth (LB) and shaken at 37 °C. Bacterial cells were spun with trastuzumab by ELISA. A single clone was grown for 4.5 h in 1 ml DNA sequencing, following amplification in and used in the next cycle. Three rounds of selection were performed.

**DNA Sequencing and Peptide Synthesis—** Single-stranded phage DNA was prepared from 26 immunopositive clones by standard techniques as described in the phage display peptide library kit and sequenced by Shenyou Biotech Inc. (Shanghai, China). Peptides representing the sequence of the positive clone H98 (LLGPYELWELISH) and an irrelevant control random peptide F56 (WHDTPPWWSWET) were synthesized by Bio-Scientific Inc. (Xian, China).

**Expression of Recombinant GST Peptide and DHFR Peptide Fusion Proteins in E. coli—** To detect the activity of peptide H98 simply and conveniently, GST-H98 and DHFR-H98 fusion proteins were prepared, respectively. To make the GST-H98 fusion protein, the sense (S’-GGAT- CTTTGTGGTGCTGTATGTTGAGCCCTTCTAGTGAACGCTTTG) and the antisense (5’-TGGCAAGCTTTTGGTCAAGAGAGACCC- CACAATCACTAGGACCAACAG) oligonucleotide fragments encoding the positive clone H98, with stop codon, HindIII site, and sticky ends of BamHI/Sall were shown by the underlined nucleotides, were synthesized together with pGEX-4T-1. Cells suspensions were snap-frozen at −80 °C for 1 min at a 20% pulse. After the lysate was cleared by centrifugation at 12,000 rpm for 10 min at 4 °C, the GST-H98 fusion protein was purified with glutathione beads according to the manufacturer's instructions (Amersham Biosciences).

To obtain DHFR-H98 fusion protein, the sense and antisense oligonucleotide fragments as described above were inserted into the BglII (the same as BamHI) and Sall sites of the pGEX-4T-1 vector (Amersham Biosciences). The ligated DNAs were used to transform E. coli BL21 cells. DNA of recombinants was identified by digestion with HindIII and EcoR V and then confirmed by DNA sequence analysis. All of these procedures were carried out as described by Sambrook et al. (16). Purification of DNA from agarose gels was performed with a QIAquick DNA purification kit (Qiagen, Valencia, CA).

For the preparation of GST-H98 fusion proteins, transformed bacteria were cultured in LB medium with ampicillin selection to an optical density of 0.8 at 600 nm. Next, 0.5 mM isopropyl-1-thio-β-galactopyranoside was added and the cultures were further incubated for 5 h at 30 °C. Cells were collected by centrifugation and resuspended in 20 ml of phosphate-buffered saline (PBS) with 1 mg/ml lysozyme and 0.1 mg/ml EDTA. Cell suspensions were sonicated for 1 min at a 20% pulse. After the lysate was cleared by centrifugation at 12,000 rpm for 10 min at 4 °C, the GST-H98 fusion protein was purified with glutathione beads according to the manufacturer's instructions (Amersham Biosciences).

**ELISA—** The reactivity of trastuzumab with GST and GST-H98 fusion protein was tested by ELISA, 96-Well microtiter plates were coated with 5 μg/ml GST or GST-H98 fusion protein in 0.1 M NaHCO3, pH 9.5, then blocked with 5% skim milk in PBS. The plates were added to dilutions of trastuzumab and held for 2 h at room temperature. Bound trastuzumab was detected using HRP-conjugated anti-human IgG antibody (Zhong Shan Co., Beijing, China) with 0.4 mg/ml o-phenylenediamine as a peroxidase substrate, as described under "Phage ELISA."

**Western Blot—** The reactivity of trastuzumab with GST fusion protein was tested by Western blot analysis. Two control GST, GST-H98, GST-H98, and DNA-Sequencing and Peptide Synthesis—Single-stranded phage was performed by lysing the cells in lysis buffer containing 1% Triton X-100, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, and 1 μg/ml aprotinin or GST-H98 fusion protein in 0.1 M NaHCO3, pH 9.5. Next, 0.5 μg/ml trastuzumab was mixed with dilutions of H98, F56, GST, GST-H98, and NIH3T3-ErbB2 cell lysate, and preincubated for 1 h at room temperature, and then transferred to the plates. Bound trastuzumab was detected by standard ELISA as described above. The peptides or proteins were tested at 0, 0.5, 5, 10, and 50 μg/ml. Each dilution was tested in duplicate. The inhibition was calculated by using the following formula: A \_test - \_control \times 100%. The experiment was repeated three times.

**Cell Proliferation Assay with MTT—** The human breast cancer cell line SKBR3 overexpressing HER-2 were seeded in 96-well microtiter plates at a density of 1 x 10^4 cells/well. After incubation overnight at 37 °C, Dulbecco’s modified Eagle’s medium containing either 1 μg/ml trastuzumab or human normal IgG with or without dilutions of peptides was added to the wells in a final volume of 150 μl. All treatments were...
were performed in triplicate. The plates were incubated at 37 °C for 3 days. Numbers of live cells were measured using a thiazolyl blue (MTT, Sigma) cell proliferation assay by reading A\text{492}. The proliferation inhibition rate was calculated by the formula as follow: (A\text{H92}−A\text{H98})/A\text{H92} × 100%

**Immunization**—Six- to 8-week-old BALB/c mice (purchased from the Animal Center of the Chinese Medical Academy) were immunized subcutaneously with 40 μg of GST-H98 or GST alone that was emulsified in complete Freund’s adjuvant the first time. Mice were boosted four times with the antigen emulsified in incomplete Freund’s adjuvant every 3 weeks. Sera were taken on day 0 (preimmune) and 7 days after the third and fifth immunization.

Antibody level in the sera of immunized mice was determined by ELISA. To test the antipeptide response, 96-well microtiter plates were coated with 5 μg/ml DHFR-H98 fusion protein in 0.1 M NaHCO₃, pH 9.5, and then blocked with 5% skim milk in PBS. A 1:1000 dilution of mice sera was added to the plates, which were then held for 2 h at room temperature. Bound antibody was detected by using HRP-conjugated anti-mouse IgG antibody with 0.4 mg/ml o-phenylenediamine as the peroxidase substrate. To test the anti-HER-2 response, 96-well microtiter plates were coated with 1 × 10⁴ cells/well of NIH3T3-ErbB2 and then blocked with 5% skim milk in PBS. A 1:100 dilution of mice sera was added to the plates and anti-HER-2 response was assessed by standard techniques as described above.

**T Cell Proliferation Assay**—Spleen cells from 2 randomly selected BALB/c mice, which were immunized 4 times with GST-H98 or GST other peptide, were isolated according to the standard techniques on day 7 post-immunization (17). 96-Well microtiter plates were coated with peptide H98 at different concentrations (0, 1, 10, and 100 ng/ml DHFR-H98) or GST-H98 fusion protein at 0.1 M NaHCO₃, pH 9.5, and then blocked with 5% skim milk in PBS. The splenocytes (2 × 10⁵/well) were cultured in 96-well plate and incubated at 37 °C for 3 days. The number of live cells were measured by using the thiazolyl blue (MTT, Sigma) cell proliferation assay by reading A\text{492}. The proliferation percentage was calculated by using the following formula: (A\text{H98}−A\text{mutated})/A\text{H98} × 100%. Each assay was performed at least in triplicate. The experiment was repeated twice.

**Structural Determinants of the Binding Sequence and Optimization of the Amino Acid Sequence**—To analyze the minimal requisites for binding activity, a variety of truncation and mutation peptides were made fused with GST (Table I). The corresponding GST-truncate, GST-mutant, and GST-cyclic H98 peptide fusion proteins were expressed and purified as described above. The reactivity of trastuzumab with these GST fusion peptides was tested by ELISA and Western blot.

**RESULTS**

**Phage Selection**—To select the positive clones that bind to trastuzumab, a random 12-mer phage display peptide library composed of 1.5 × 10¹⁵ independent phage clones was preabsorbed by human serum IgG and then specifically absorbed with trastuzumab. For each biopanning, phages were titrated for pfu in the inputs and outputs to determine the degree of selection. The total number of phages bound to trastuzumab was increased from 8.2 × 10⁴ pfu in the first round to 3.7 × 10⁶ pfu in the third round.

**Specificity of Peptides Binding to Trastuzumab and Their Sequences**—After 3 rounds of selection, roughly 13.7% (41/300) of the phage clones analyzed exhibited trastuzumab binding activity (data not shown). We sequenced 26 positive phage clones and 25 clones encoded the identical amino acid sequence. These two sequences are considerably different and have no obvious similarities in motif.

The binding of trastuzumab to the fusion protein GST-H98 was also demonstrated by ELISA and Western blotting. For this experiment, oligonucleotide encoding the peptide H98 was expressed as a C-terminal extension of the GST protein. The results of the ELISA showed that trastuzumab could specifically bind to GST-H98 but not to GST (Fig. 1). As shown in Fig. 1, trastuzumab reacted with the GST fusion protein containing the H98, whereas no reaction was obtained with GST. The results of Western blotting also suggested that trastuzumab could specifically bind to H98 (Fig. 2).

![Binding of trastuzumab to GST-H98](image1)

**Fig. 1.** Binding of trastuzumab to GST-H98. Microtiter wells were coated with 5 μg/ml GST-H98 or GST and then blocked with 5% skim milk. Trastuzumab was added to the wells. Bound trastuzumab was detected with HRP-conjugated anti-human IgG antibody and then with the substrate. The values shown are A\text{492} and the mean of triplicate samples. S.D. are indicated by error bars.

**Table I**

| Peptide | Amino acid sequence |
|---------|---------------------|
| H98     | LLGYELWELSH         |
| N10     | LLGYELWEL           |
| N8      | LLGYELW             |
| N6      | LLGYPE               |
| C10     | GPYELWELSH          |
| C8      | YELWELSH            |
| C6      | LWELSH               |
| M8      | GPYELWEL            |
| M6      | PYELWE               |
| MutN3−4 | LLVAELWELSH         |
| MutC1−2 | LLGPYELWELGA        |
| MutN1−2 | GAGPYELWELSH        |
| MutN1   | QLLGPYELWHEL         |
| Cyclic H98 | CLLGPYELWELSHC |

**Fig. 2.** Western blot analysis of binding of trastuzumab to peptide. Different GST fusion proteins were prepared as described under "Experimental Procedures." Equal amounts (2 μg) of proteins were subjected to SDS-PAGE and transferred to nitrocellulose membranes. The proteins were probed with anti-GST antibody (right panel) to detect the expression of GST fusion peptides and then probed with either trastuzumab (left panel) or human serum IgG (middle panel). Lane 1, GST; lane 2, GST-H98; lane 3, GST-HER-2; lane 4, DHFR-H98.

**GST-H98 and Peptide H98 Inhibition of Trastuzumab Binding to HER-2**—The results from the ELISA and Western blot above showed that peptide H98 bound to the variable regions of trastuzumab. However, to demonstrate that H98 resembled the epitope of HER-2, we had to show that H98 blocked binding between trastuzumab and HER-2. To perform these experiments, we used an ELISA in which antibody and inhibitors
The inhibition rate was calculated according to the following formula:

\[
\text{Inhibition rate} = \frac{A_{\text{Human normal IgG}} - A_{\text{Trastuzumab}}}{A_{\text{Human normal IgG}}} \times 100\%
\]

where \( A_{\text{Human normal IgG}} \) is the absorbance of human normal IgG, \( A_{\text{Trastuzumab}} \) is the absorbance of trastuzumab, and \( A_{\text{Peptide H98}} \) is the absorbance of peptide H98.

FIG. 3. Competitive inhibition activity of peptide H98 or GST-H98.

A, peptide H98 inhibited trastuzumab from binding to HER-2. Previously, 0.5 μg/ml trastuzumab was mixed with H98, F56, GST, GST-H98, and NIH3T3-ErbB2 cell lysate in the indicated concentrations, preincubated for 1 h, and then transferred to HER-2-coated plates. Bound trastuzumab was detected with HRP-conjugated anti-human IgG antibody and then to the substrate. B, HER-2 inhibited trastuzumab from binding to peptide H98. First, 0.5 μg/ml trastuzumab was mixed with H98, F56, GST, GST-H98, and NIH3T3-ErbB2 cell lysate in different concentrations. One hour later, the mixture was added to GST-H98-coated wells. Bound trastuzumab was detected with HRP-conjugated anti-human IgG antibody and then to the substrate. The inhibition rate was calculated according to the following formula:

\[
\text{Inhibition rate} = \frac{A_{\text{Trastuzumab}} - A_{\text{Trastuzumab with Protein}}}{A_{\text{Trastuzumab}}} \times 100\%
\]

No significant effect on cell growth inhibition. (GST-H98 or peptide H98) were preincubated and plated onto the microtiter plates coated with HER-2. This analysis revealed that GST-H98 and peptide H98 inhibited the binding of trastuzumab to HER-2 in a dose-dependent manner, but GST protein and irrelevant peptide F56 did not produce noteworthy inhibition on their interaction (Fig. 3A).

HER-2 Inhibiting Trastuzumab Binding to GST-H98—To demonstrate that the trastuzumab that bound to GST-H98 or peptide H98 was specific for HER-2, we used HER-2 to inhibit trastuzumab binding to GST-H98. Trastuzumab was preincubed with different dilutions of HER-2. The results show that HER-2 significantly inhibited trastuzumab binding to GST-H98 (Fig. 3B). In Fig. 3B, GST alone also demonstrated a concentration-dependent inhibition on trastuzumab binding to GST-H98, but the GST-H98 fusion protein doubled or tripled the inhibition effect, as compared with GST. The results demonstrated that peptide H98 played a critical role in inhibiting trastuzumab binding to GST-H98.

Peptide H98 Blocked the Function of Trastuzumab on Inhibiting Cell Growth—Trastuzumab is well known to inhibit the growth of breast cancer cells through interaction with HER-2. Because peptide H98 has the ability to block interaction between trastuzumab and HER-2, we therefore decided to test whether peptide H98 affects the ability of trastuzumab to inhibit cell growth. As shown in Fig. 4, the response of breast cancer cell SKBR3 to trastuzumab was significantly blocked by peptide H98 in a dose-dependent manner and the 50% block rate was reached when peptide H98 was present at a concentration of 60 μg/ml, whereas an equivalent concentration of the control peptide had no significant effect on cell growth inhibition.

Mice Immunized with GST-H98 Made Anti-HER-2 Antibodies—Peptide H98 was expressed as a GST fusion protein (GST-H98), and the immunogenicities were evaluated in mice. Four of 5 mice immunized with GST-H98 generated peptide-specific antibody (Fig. 5A) as well as antibody to HER-2 (Fig. 5B) after the fifth immunization. The anti-HER-2 response was lower than the anti-peptide response, and the anti-peptide H98 response could be detected earlier than the anti-HER-2 response. No antipeptide or anti-HER-2 response was observed in mice immunized with GST. One of 5 mice generated antibody to HER-2 that could be detected in an immunoprecipitation Western blot assay (data not shown).

Development of Cellular Immune Response—Cellular immunity induced in mice that were immunized with GST-H98 or GST-other peptide was evaluated with T-cell proliferation assay. The spleens of two mice were isolated for use in this assay. T-cell proliferation was assayed as described under “Experimental Procedures” using H98 as a stimulant. The results shown in Fig. 6 indicate that T-cell proliferation took place in mice immunized with GST-H98 but not in mice immunized with GST-other peptide.

Structural Determinants of H98 Binding to Trastuzumab—Decrease in peptide length may simplify the modifications of the peptide sequence, as well as provide an indication of the characteristics of the binding cleft. The ELISA and Western blot results showed that among 15 kinds of GST-H98 including GST-truncate and GST-mutant peptide fusion protein, only GST-H98, GST-C10, GST-MutN1–2, GST-MutN1, and GST-cyclic H98 were able to specifically bind to trastuzumab (Figs. 7 and 8).

As shown in Figs. 7 and 8, GST-MutN3–4 (mutant of N-terminal GP to VA) and GST-CS could not bind to trastuzumab, but GST-C10 could bind to trastuzumab. These differences in binding abilities imply that the third and fourth amino acids,
glycine (Gly) and Proline (Pro), at the N terminus were essential for the activity of peptide H98. Moreover, neither GST-MutC1–2 (mutant of C-terminal SH to GA) nor GST-N10 could bind to trastuzumab, which suggested that serine (Ser) and histidine (His) at the C terminus were also essential to retain complete peptide binding activity. Alternatively, the ability of GST-MutN1–2 to bind to trastuzumab was significantly impaired but not completely abolished, which implied that the first two leucines (LL) at the N terminus were not very important for the binding activity of H98 but were likely to have an effect on the marginal structure formation of the peptide. Further deletion of 4 or 6 residues at the N or C terminus completely abolished the ability of peptide H98 to bind to trastuzumab. Thus it appears that the peptide must be more than 8 amino acids in length to fulfill the requirements of binding.

According to the structure analysis of trastuzumab binding to HER-2 peptide H98 with the Docking program in the Insight II (2000) software package, the first amino acid of H98 at the N terminus was mutated from leucine (Leu) to glutamine (Gln), which is similar to HER-2<sup>2602Gln</sup> and more likely to form hydrogen bonds with trastuzumab (Fig. 9). The binding analysis verified that the interaction between this variant of H98 and trastuzumab was increased (Figs. 7 and 8, lane 14). Finally, to test the effect of cyclization on peptide recognition and binding, we introduced two cysteines (Cys) flanking the peptide at the N and C terminus, and found that the affinity of cyclic peptide to trastuzumab is significantly impaired rather than improved (Figs. 7 and 8, lane 15).

**DISCUSSION**

Her-2/neu (c-erbB2), as a 185-kDa glycoprotein (p185<sup>erbB2</sup>), is a member of the epidermal growth factor receptor family. The protein p185<sup>erbB2</sup> consists of three domains: a glycosylated extracellular domain with two cysteine-rich regions, a hydrophobic transmembrane domain, and an intracellular tyrosine kinase domain (18). HER-2 overexpression is directly associated with the malignant transformation of epithelial cells. The critical role of HER-2 in epithelial oncogenesis as well as its selective overexpression on malignant tissues makes it an ideal target for cancer biotherapy.
Humoral and cellular immunoreaction to HER-2 are known to occur naturally in patients with HER-2 positive tumors (7, 20, 21). In clinic trials, HER-2 vaccines were restricted to T-cell epitope peptides, which limited the use on some patients because of their major histocompatibility complex haplotype. So the use of B-cell epitope peptide vaccines may complement immunotherapy methods, such as using whole cells, glycosylated extracellular domain, DNA, or T-cell peptide epitopes. However, few studies have described the induction of epitope-specific B-cell immune responses to HER-2. Dakappagari et al. (11, 22) identified the B-cell epitopes from HER-2 by computer-aided analysis, and used this epitope to synthesize chimeras with a T-cell epitope from measles virus fusion protein. The chimeric peptide vaccines induced high levels of antibodies, which inhibited tumor cell growth, in outbred rabbits (11, 22). Although computer-aided analysis is one method available for finding epitopes, it does not always work well because the immunogenicity of tumor-associated antigen could be affected by glycosylation, which is difficult for a computer to predict. Knowing the crystal structure of trastuzumab binding with human HER-2, we synthesized a peptide epitope (PQCADPPFGDQ) containing vital amino acids for HER-2 binding with trastuzumab, but this peptide did not show any binding activity with trastuzumab in our experiments (data not shown).

Yip et al. (23) used the HER-2 antibody N21 as a target for screening a phage display library of HER-2 gene fragments, and isolated a peptide containing 55 amino acids, which elicited the active immune response to HER-2 in mice. Generally, the epitope can be a linear fragment or conformational region. Linear epitopes correspond to the ordered sequence of amino acids in the protein, whereas in conformational epitopes (mimotope), sequence from non-sequential protein regions in the primary structure or its associated carbohydrates contributes to a single tridimensional site. In view of the fact that phage-displayed HER-2 gene fragment libraries only select linear epitopes, screening phage display peptide libraries may be a simple and convenient way to find out not only linear epitopes but also conformational epitopes. To search for the peptides that could mimic the epitope of HER-2 and would have potential as a vaccine in immunotherapy, Riemer et al. (24) used trastuzumab as the target to screen the 10-mer phage display peptide library, and they obtained five candidate mimotopes that could induce an antibody response to HER-2 (24). Alternatively, we screened a 12-mer phage display peptide library by bio-panning also with the antibody trastuzumab as the target protein, and obtained a completely different peptide (LLGPYELWELSH) from Riemer’s (C-QMWAPQWGPD-C, C-KLYWADGEFT-C, etc.). In our procedure of screening the library, some improvements were introduced. First, to increase specificity with trastuzumab in our experiments (data not shown).
the contact between the phage-displayed peptides and the antibodies, we used solution bio-panning instead of the solid-phase bio-panning, which is more commonly used. Second, to remove the nonspecific clones, the original phage display library was first pre-absorbed with human serum IgG. Finally, instead of eluting the phages from the target proteins, the elution procedure was omitted in our work by infecting E. coli ER2738 directly with phages binding with trastuzumab and protein A complex. Twenty-six positive phage clones were selected from 300 clones and 25 clones were completely identical by DNA sequencing. To rule out the effects of phage protein in the binding, the GST peptide fusion protein was made. The clone GST-H98 showed binding activity to trastuzumab, but GST-H23 did not (data not shown). Further analysis demonstrated that the isolated mimotope H98 not only specifically inhibited the binding of trastuzumab to HER-2, but also blocked the function of trastuzumab in inhibiting the proliferation of cancer cells. Moreover, immunization analysis demonstrated that H98 could induce an active immune response to HER-2 in vivo. These results strongly suggested that H98 is similar to the antigen epitope of HER-2 that is recognized by trastuzumab.

Although no sequence homology was found between the mimotope (H98) and HER-2 by sequence alignment, our results appear that H98 mimics a conformational structure of the HER-2. To analyze the binding structure of peptide H98 with trastuzumab, we used the Insight II (2000) software package and a crystal structure of human HER-2 binding with trastuzumab at 2.5 Å (19). Docking program in Insight II (2000) was carried out to model the complex of H98 and trastuzumab. The interaction between HER-2 and trastuzumab buries 1,350 Å² of surface area over a long groove and possesses an unusually high shape complementarity for antigen-antibody interactions. The interaction was mediated by three regions of HER-2: loops 3, 19, and 25. The first and third loop were primarily electrostatic, whereas the second loop made mostly hydrophobic contacts in a pocket formed by the CDR3 loops of heavy and κ light chains of the antibody. The crystal structure of HER-2 with trastuzumab showed that HER-2560Asp is surface exposed and in juxtaposition to HER-2573Phe and H98-6Glu-HER-2572Pro, which are also surface exposed. These three amino acids, HER-2560Asp–HER-2573Phe–HER-2572Pro, form an epitope of HER-2 in structure and were well represented by the sequence “H98-6Glu-H98-5Tyr-H98-4Pro” in peptide H98. Amino acids HER-2560Asp and H98-6Glu are similar in structure, as are HER-2573Phe and H98-5Tyr. Moreover, HER-2560Asp could be replaced by H98-6Glu to form hydrogen bonds with heavy and κ light chains of trastuzumab (Fig. 9). This epitope structure analysis demonstrated that H98 could mimic the epitope of HER-2 binding to trastuzumab in structure, which is consistent with our experimental results. Moreover, knowing that HER-2560Gln could form hydrogen bonds with trastuzumab, we made a mutant (MutN1) in which the first amino acid of H98 was changed from leucine (Leu) to glutamine (Gln) (Fig. 9B). The mutant H98 achieved a higher binding activity to trastuzumab. To identify the critical amino acids in H98 for its binding to trastuzumab, we made truncation and mutation variants of the peptide H98 (see Table I). The binding assays showed that the last two amino acids at the C terminus and the third together with the fourth amino acid at the N terminus play an important role in the binding of H98 to trastuzumab.

In summary, peptide H98, which was isolated from the phage display peptide library with trastuzumab, could mimic the antigen epitope of HER-2 on a conformation structure and successfully elicit the humoral and cellular immune responses to HER-2. This peptide shows promise for development as a HER-2 vaccine for biotherapy of cancer that overexpresses HER-2.

Acknowledgment—We thank Dr. David Stollar for valuable advice on the discussion.

REFERENCES

1. Yardim, Y., and Slawkowski, M. X. (2001) Nat. Rev. Mol. Cell. Biol. 2, 127–137
2. Glatiyi, M. A., Neve, R. M., Lane, H. A., and Hynes, N. E. (2000) EMBO J. 19, 3159–3167
3. Wang, S. C., Zhang, L., Hortobagyi, G. N., and Hung, M. C. (2001) Semin. Oncol. 28, 21–29
4. Slamon, D. J., Godolphin, W., Jones, L. H., Holt, J. A., Wong, S. G., Keith, D. E., Levin, W. J., Stuart, S. G., Udove, J., Ullrich, A., and Press, M. F. (1989) Science 244, 707–712
5. Slamon, D. J., Clark, G. M., Wong, S. G., Levin, W. J., Ullrich, A., and McGuire, W. L. (1987) Science 235, 177–182
6. Nicholson, R. I., McClelland, R. A., Lee, J. M., Manning, P., Robertson, J. F., Ellis, I. O., and Blaimie, R. W. (1994) Breast Cancer Res. Treat. 29, 117–125
7. Dias, M. L., and Cheever, M. A. (1997) Adv. Cancer Res. 71, 343–371
8. Slawkowski, M. X., Lofgren, J. A., Lewis, G. D., Hotaling, T. E., Fendly, B. M., and Fox, J. A. (1999) Semin. Oncol. 26, Suppl. 12, 60–70
9. Nahta, R., Hortobagyi, G. N., and Esteva, F. J. (2003) Oncologist 8, 5–17
10. Nahta, R., Hortobagyi, G. N., and Esteva, F. J. (2003) Oncologist 8, 5–17
11. Coussens, L., Yang-Feng, T. L., Liao, Y. C., Chen, E., Gray, A., McGrath, J., Seeburg, P. H., Libermann, T. A., Schlessinger, J., Francke, U., Levinson, A., and Ullrich, A. (1985) Science 228, 1132–1139
12. Che, H. S., Mason, K., Ramyar, K. X., Stanley, A. M., Gabelli, S. B., Denney, D. W., Jr., and Leabhi, D. J. (2003) Nat. Biotechnol. 21, 29–43
13. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, 2nd Ed., pp. 5.3–6.59, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
A Novel Peptide Isolated from a Phage Display Peptide Library with Trastuzumab Can Mimic Antigen Epitope of HER-2
Beihai Jiang, Wenbin Liu, Hong Qu, Lin Meng, Shumei Song, Tao Ouyang and Chengchao Shou

J. Biol. Chem. 2005, 280:4656-4662.
doi: 10.1074/jbc.M411047200 originally published online November 9, 2004

Access the most updated version of this article at doi: 10.1074/jbc.M411047200

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 23 references, 11 of which can be accessed free at
http://www.jbc.org/content/280/6/4656.full.html#ref-list-1