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Selection of a Peptide with Affinity for the Tumor-Associated TAG72 Antigen from a Phage-Displayed Library

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A hexapeptide phage library was used to select peptides with affinity for the tumor-associated TAG72 antigen. Twenty-one phage clones were selected after the third round of biopanning. Three phage clones with the same DNA insert of ARTLRF were found to bind more strongly to the TAG72 antigen than other phage clones and the wild-type phage. A synthetic decapeptide GAARTLRFGA with two conjunctive amino acid residues of the phage coat protein III on each side of the selected peptide was found to bind more strongly to the TAG72 antigen than to other antigens such as the mouse metallothionein. Furthermore, immunohistochemical studies revealed that this peptide displayed preferential binding to colonic adenocarcinomatous cells expressing the TAG72 antigen. Therefore, this anti-TAG72 peptide may be useful in serving as the starting point with regard to further designing peptidomimetics as potential pharmaceuticals.

The concept of phage displays was first documented by Smith (1) with the demonstration of the expression of an antigenic peptide on the surface of the filamentous bacteriophage fd. This concept subsequently led to experiments which set the stage for construction of libraries of random peptides (2,3) and protein domains (4,5) displayed on the phage surface. More and more evidence has shown that peptide libraries of phage display appear to be powerful tools for isolating peptide sequences that bind to target molecules. They can be used to identify epitopes and mimotopes recognized by antibodies (6,7). Furthermore, phage display technology has been used for the isolation of protein antagonists and inhibitors (8–10). In this study, the human tumor-associated TAG72 antigen was used as a target for screening a hexapeptide library of phage display. We demonstrated that it is possible to select small hexameric peptides with affinity for the TAG72 antigen.

MATERIALS AND METHODS

Materials. The hexapeptide library (11) and Escherichia coli K91kan were obtained from Dr. George Smith (University of Missouri, Columbia, MO). The human tumor-associated TAG72 antigen recognized by the B72.3 antibody was purified from LS174T xenografts in nude mice by B72.3 antibody-affinity chromatography (12). The mouse metallothionein and bovine serum albumin were purchased from SIGMA (St. Louis, MO). The B72.3 antibody was biotinylated according to the method as previously described (13). The biotinylated peptide GAARTLRFGA as well as the irrelevant biotinylated peptide WTWDQY were synthesized by the Research Genetics, Inc. (Huntsville, AL).

Biopanning. A 35mm petri dish was coated with 1ml of purified TAG72 antigen (1:300) by shaking in a cold room overnight; it was then blocked with bovine serum albumin (BSA, 5mg/ml). One milliliter of the hexapeptide library containing 10^8 phage particles was added to the petri dish. The dish was incubated by shaking in the cold room overnight and then washed 10 times with TBS (50mM Tris-HCl, pH 7.5, 150mMM NaCl), 0.5% Tween 20. The bound phages were eluted from the dish with 400μl of elution buffer (0.1N glycine-HCl, pH 2.2, 1mg/ml BSA). The eluates were neutralized and adjusted to pH 8.0 by adding 40ul of 1N Tris-HCl (pH 9.1), and then concentrated to 100μl with an Amicon Centricon-10 concentrator (Amicon Inc., Beverly, MA). Eluted phages were amplified as described (2). Briefly, the eluates were mixed with an equal volume of starved K91kan cells. The cells were added to 20ml Luria-Bertani (LB) medium containing 40 μg/ml tetracycline and grown by shaking at 300rpm at 37°C overnight. Phages from liquid cultures were

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obtained by clearing the supernatant twice by centrifugation at 8000rpm for 10 min at 4°C, and precipitated with 3% polyethylene glycol (PEG) 8000 (SIGMA, St. Louis, MO), 0.5N NaCl. Phage pellets were resuspended in TBS and used for second biopanning. This panning protocol was repeated twice. After three rounds of biopanning, the final eluates were titered and used for transfection of starved K91kan cells and plated onto LB plates containing 100μg/ml kanamycin and 40μg/ml tetracycline. The plates were incubated at 37°C overnight. The individual clones were then subjected to amplification and purification.

Amplification and purification of eluates. The selected clones were used for amplification. Briefly, each of the selected clones was inoculated into 5ml of LB medium containing 20μg/ml tetracycline and grown by shaking at 300rpm at 37°C overnight. Phages in supernatant were obtained by centrifugation and precipitation with PEG/NaCl solution as described above. These purified phages were then subjected to DNA sequencing and enzyme-linked immunosorbent assay (ELISA).

DNA sequencing of inserts. Phage pellets were dissolved in 50μl of TBS, extracted with phenol/chloroform and precipitated with ethanol. Pellets of phage DNA were then dissolved in 10μl of TE (10mM Tris-HCl, 1mM EDTA) for sequencing. The DNA sequence of the insert in each phage clone was verified using a Pharmacia T7 sequencing™ kit (Pharmacia Biotech., Milwaukee, Wisconsin) and a sequencing primer (5′ ccctc atagt tagcg taacg 3′) complementary to positions 1717–1736 of the wild-type gene III sequence by the dideoxy nucleotide sequencing method (14).

Enzyme-linked immunosorbent assay. The TAG72-binding ELISA (12) was performed in order to characterize the binding reactivity of phage clones and peptides. Briefly, 50μl of purified TAG72 antigen and mouse metallothionein (1μg/ml) were coated to each well of microtiter plates respectively. The plates were blocked with 5% BSA. In the TAG72-binding ELISA for characterization of phage clones, fifty microliters of phage clones containing 10^8 plaque forming units (PFUs) and their two-fold dilutions were added to each well and incubated at 37°C for 1 hour. After 3 washes, plates were incubated with biotinylated rabbit anti-phage antibody at 10μg/ml for another 1 hour. In the TAG72-binding ELISA for examination of peptides, fifty microliters of the biotinylated decapeptide GAARTLRFGA at 16.7μM and their two-fold dilutions were added to each well, and incubated at 37°C for 1 hour. An irrelevant biotinylated hexapeptide WTWDQY at 16.70μM and the biotinylated B72.3 antibody at 0.013μM, and their 2-fold dilutions were used as negative and positive controls, respectively. After 3 washes, plates were incubated with horseradish peroxidase conjugated avidin (1:2000) (Pierce, Rockford, IL) for 1 hour at 37°C. After another 3 washes, substrates (peroxidase substrate kit, BioRad Lab Ltd, Mississauga, Ontario, Canada) were added for the generation of color reaction. The optical density of each well was determined at 405nm in a Bio-Rad Model 3550 microplate reader.

Immunohistochemical study. The reactivity of peptide GAARTLRFGA on formalin-fixed tumor tissue sections was determined using a modification of the avidin-biotin-peroxidase complex (ABC) method as described previously (12). Briefly, human colon adenocarcinoma tissue sections were deparaffinized in xylene and rehydrated in graded alcohols. Endogenous peroxidase activity was quenched by incubating the slides with 0.3% hydrogen peroxide in methanol for 15 min followed by three PBS rinses. The slides were incubated for 20 min in 10% normal rabbit serum to reduce any nonspecific staining and then incubated with the biotinylated decapeptide GAARTLRFGA (167μM) or the biotinylated WTWDQY (167μM) as a control overnight at 4°C. All slides were washed 3 times in PBS and then incubated with the ABC reagent for 30 min. The peroxidase activity was developed with freshly prepared 0.06% 3′, 3′-diaminobenzidine containing 0.1% hydrogen peroxide. Hematoxylin was used as the counterstain.

RESULTS

Screening of a hexapeptide library for peptides with affinity for the TAG72 antigen

In order to identify peptides with affinity for the tumor-associated TAG72 antigen, a hexapeptide library of phage display was screened using the biopanning technique with the TAG72 antigen immobilized on a petri dish. The DNA samples from 21 individual phage clones recovered from the third panning were sequenced, and their deduced amino-acid sequences are shown in Table 1. Among them, there were 11 types of different hexapeptides. There was 3 phage clones (clone #1, Table 1) with the DNA insert of ARTLRF at the N-terminus of phage coat protein III showing stronger binding for the TAG72 antigen than the other phage clones and the wild-type phage (Table 1, Figure 1A). Therefore, ARTLRF may confer the TAG72-binding properties to this phage clone. In order to confirm this, we purchased a biotinylated decapeptide GAARTLRFGA. The latter includes two conjunctive amino acid residues of the phage coat protein III on each side of the selected peptide ARTLRF such that the conformation of ARTLRF may be maintained.

Characterization of the GAARTLRFGA peptide

The binding reactivity of peptide GAARTLRFGA for the TAG72 antigen was evaluated in the

TABLE 1
Deduced Amino-Acid Sequences of the Insert in Phages Isolated after Biopanning

Clone number	Sequence[a]	Frequency[b]	ELISA (OD405)[c]
1	ARTLRF	3/21	1.34
2	ERDLLR	3/21	0.77
3	WSWLSA	3/21	0.41
4	QERFPA	3/21	0.59
5	AQWENE	2/21	0.53
6	QAILTV	2/21	0.64
7	VTLVID	1/21	0.46
8	RIGTLD	1/21	0.43
9	YFVVNA	1/21	0.32
10	RRRFRA	1/21	0.38
11	SDGLSR	1/21	0.41

[a] Deduced amino-acid sequences of the insert at the N-terminus of phage coat protein III in different phage clones. The single letter codes for amino-acids are: A, Ala; W, Trp; Y, Tyr; F, Phe; V, Val; G, Gly; R, Arg; S, Ser; L, Leu; I, Ile; E, Glu; T, Thr; D, Asp; N, Asp; P, Pro; Q, Gln.

[b] Number of times each sequence was independently isolated.

[c] The average reading at OD405 represents the binding reactivity of each phage clone at 10^8 PFU to the TAG72 antigen in the TAG72-binding ELISA assay. The average readings at OD405 for the wild-type phage and for the PBS control are 0.25 and 0.19, respectively.

TAG72-binding ELISA. As depicted in Figure 1B, the GAARTLRFGA at 16.7μM showed the binding reactivity for the TAG72 antigen as did the biotinylated B72.3 antibody at 0.013μM, but not for the mouse metallothionein (Figure 1C). This indicates that the GAARTLRFGA peptide may have binding specificity for the TAG72 antigen. In order to confirm the TAG72 binding specificity,

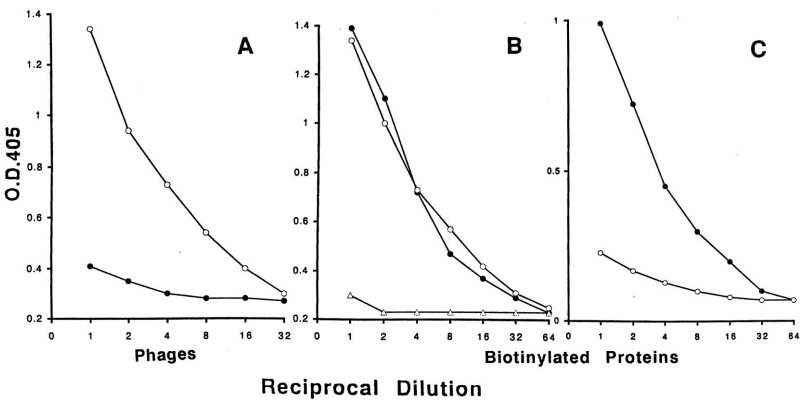

FIG. 1. TAG72-binding ELISA. (A) Titration curves of phage clones in the TAG72-binding ELISA. The phage clone #1 with the DNA insert of ARTLRF (○) and the phage clone #3 with the DNA insert of WSWLSA (●) (10^8 PFUs) and their twofold dilutions were added to the TAG72-coated plates. (B) Titration curves of peptides in the TAG72-binding ELISA. Fifty microliters of the biotinylated peptides GAARTLRFGA (○, 16.7μM), WTWDQY (Δ, 16.7 μM) and the biotinylated B72.3 (●, 0.013μM), and their twofold dilutions were added to the TAG72-coated microtiter plates. (C) The biotinylated peptide GAARTLRFGA (16.7μM) and its twofold dilutions were added to the microtiter plates coated with the TAG72 antigen (●) and the mouse metallothionein (○). The SD of each determination is less than 5%.

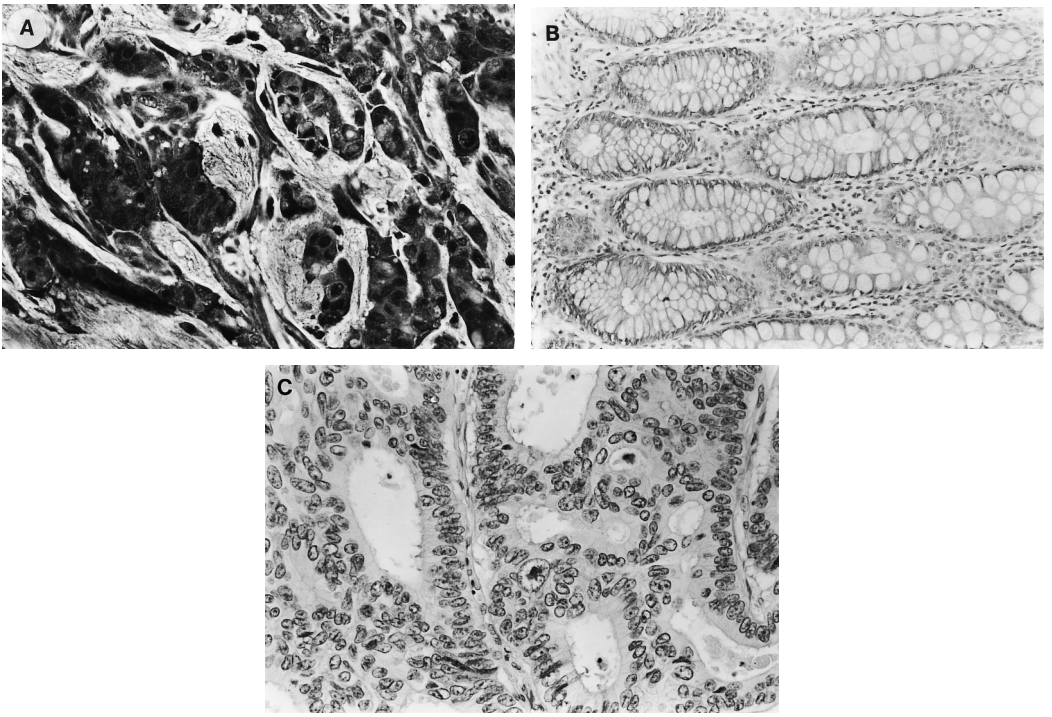

FIG. 2. Immunohistochemical studies of formalin-fixed, paraffin-embedded human colon adenocarcinoma by using the biotinylated peptide GAARTLRFGA. The adenocarcinomatous cells were positive for the biotinylated peptide GAARTRFGA (A), whereas normal colonic mucosa was negative (B); the positive control, biotinylated B72.3 reacted similarly. The irrelevant peptide WTWDQY was uniformly negative in both normal and carcinomatous cells (C). Magnification is X280 for A–C.

the biotinylated peptide GAARTLRFGA was further studied using immunohistochemistry on colonic adenocarcinoma tissue sections. As shown in Figure 2, the peptide GAARTLRFGA showed preferential binding for human colonic cancers which express the TAG72 antigen (Figure 2A) compared to the negative staining in normal colon mucosa (Figure 2B). The positive staining was seen on both surface as well as in the cytoplasm of the colon adenocarcinomatous cells. On contrary, the irrelevant peptide WTWDQY did not show any binding for the colonic adenocarcinomatous cells (Figure 2C).

DISCUSSION

The human tumor-associated TAG72 antigen is a polymorphic epithelial mucin originally defined by the B72.3 antibody (15). The TAG72 antigen has been found in more than 85% colorectal, gastric, breast and ovarian adenocarcinomas especially in their differentiated forms and only trace amounts of TAG72 were detected in normal tissues (16,17). The B72.3 antibody has thus been extensively used for immunodetection of malignancy in more than 1,500 patients, showing an 80% positive rate including 20% occult lesions (18,19). In general, however, one of the major limitations in the clinical application of antibodies as tumor imaging reagents is the inadequate uptake and the poor distribution of labelled antibodies in tumors (20,21). This is primarily due to physiological factors such as the heterogenous blood supply and interstitial hypertension, and also partially due to the large size (150KD) of antibody molecules (22). One approach to overcome this problem is the use of engineered antibody fragments of small size, since the small molecules often have higher

values of permeability and interstitial diffusion coefficients, and are able to penetrate deeper into tumors. Comparative pharmacokinetic studies have demonstrated that engineered FV antibody molecules (25KD) showed increased uptake in tumors, and higher ratios of tumor/normal tissues than the intact antibody molecule (23). In order to further reduce the size of binding molecules, the concept of small binding peptides, so-called the molecular recognition unit was proposed by Rodwell (24). Recently, it has been demonstrated that small synthetic linear peptides (<1KD) derived from the sequence of the antibody complementarity-determining region were able to bind to the antigen in a manner similar to that of the parental antibody (25,26).

In this study, we used the biopanning technique to screen a hexapeptide library of phage display in order to identify novel peptides with affinity for the human tumor-associated TAG72 antigen. We demonstrated that a phage clone with the peptide ARTLRF in the N-terminus of phage coat protein III was able to bind to the TAG72 antigen. In order to confirm that this peptide conferred the TAG72-binding property onto the selected phage clone, we synthesized the decapeptide GAARTLRFGA; the latter has two conjunctive amino acid residues of the phage coat protein III on each side of the selected peptide. Our data showed that this decapeptide at 16.7μg/ml was able to significantly bind to the TAG72 antigen as did the B72.3 antibody at 0.013μg/ml. This peptide showed a significant binding for the TAG72 antigen, but not for the mouse metallothionein. Furthermore, GAARTLRFGA showed staining positivity for human colonic cancer which express the TAG72 antigen and negativity for normal colonic mucosa. Although the binding reactivity of decapeptide GAARTLRFGA for the TAG72 antigen is relatively low, it should still be useful in serving as the starting point in further designing peptidomimetics as potential anti-TAG72 pharmaceuticals.

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REFERENCES

1. Smith, G. (1985) *Science* **228,** 1315–1317.
2. Cwirla, S., Peters, E., Barrett, R., and Dower, W. (1990) *Proc. Natl. Acad. Sci. USA* **87,** 6378–6382.
3. Devlin, J., Panganiban, L., and Devlin, P. (1990) *Science* **249,** 404–406.
4. Bass, S., Greene, R., and Wells, J. (1990) *Proteins* **8,** 309–314.
5. Markland, W., Robert, B., Saxena, M., Guterman, S., and Ladner, R. (1991) *Gene* **109,** 13–19.
6. Felici, F., Luzzaga, A., Folgori, A., and Cortese, R. (1993) *Gene* **128,** 21–27.
7. Folgori, A., Tafi, R., Meola, A., Felici, F., Galfre, G., Cortese, R., Monaci, P., and Nicosia, A. (1994) *EMBO J* **13,** 2236–2243.
8. Smith, G., Schultz, D., and Ladburg, J. (1993) *Gene* **128,** 37–42.
9. Yayon, A., Aviezer, D., Safran, M., Gross, J., Heldman, Y., Cabilly, S., Givol, D., and Katchalski-Katzir, E. (1993) *Proc. Natl. Acad. Sci. USA* **90,** 10643–10647.
10. Balass, M., Heldman, Y., Cabilly, S., Givol, D., Katchalski-Katzir, E., and Fuchs, S. (1993) *Biochemistry* **90,** 10638–10642.
11. Scott, J., and Smith, G. (1990) *Science* **249,** 386–390.
12. Xiang, J., Moyana, T., Kalra, J., Hamilton, T., and Qi, Y. (1992) *Mol. Biother.* **4,** 174–183.
13. Harlow, E., and Lane, D. (1988) Antibodies. A Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
14. Sanger, F., Nicklen, S., and Coulson, A. (1977) *Proc. Natl. Acad. Sci.* **74,** 5463–5467.
15. Thor, A., Ohuchi, N., Szpak, C., Johnston, W., and Schlom, J. (1986) *Cancer Res.* **46,** 3118–3124.
16. Nuti, M., Teramoto, Y., Mariani-Costantini, R., Horan, H., and Schlom, J. (1982) *Int. J. Cancer* **29,** 539–545.
17. Stramingononi, D., Bowen, R., Atkinson, B., and Schlom, J. (1983) *Int. J. Cancer* **31,** 543–552.
18. Renda, A., Salvatore, M., Sava, M., Landi, R., Lastoria, S., Coppola, L., Schlom, J., Colcher, D., and Zannini, G. (1987) *Dis. Colon Rectum.* **30,** 683–686.
19. Carrasquillo, J., Sugarbaker, O., Colcher, D., Reynolds, J., Esteban, J., and Bryant, G. (1988) *J. Nucl. Med.* **29,** 1022–1030.

20. Miraldi, F., Nelson, A., Kraly, C., and Ellery, S. (1986) *Radiology* **161,** 413–417.
21. Cohn, K., Welt, S., Banner, W., Harrinton, M., Yeh, S., Sakamoto, J., Cardon-Cardo, C., Daly, J., Kemeny, N., Cohn, A., Lloyd, K., DeCosse, J., Ottengen, H., and Old, L. (1987) *Arch. Surg.* **122,** 1425–1429.
22. Jain, R. (1990) *Cancer Res.* **50,** 814–819.
23. Yokata, T. (1992) *Cancer Res.* **52,** 3402–3408.
24. Rodwell, J. (1989) *Nature* **48,** 7022–7024.
25. Williams, W., Moss, D., Kieber-Emmons, T., Cohen, J., Myers, J., Weiner, D., and Greene, M. (1989) *Proc. Natl. Acad. Sci. USA* **86,** 5537–5541.
26. Berry, M., and Davies, J. (1992) *J. Chromatography* **597,** 239–245.