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Non-Natural CBP2 Binding Peptides and Peptomers Modulate Carcinoma Cell Adhesion and Invasion

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Abstract A combinatorial approach that utilized a repertoire of bacteriophage-peptides has identified a number of non-natural CBP2 binding peptides. Moreover, co-localization of some of these peptides with CBP2 in a number of tumor cell lines demonstrated that the peptides were directed to an intracellular location spatially coincident with the normal distribution of CBP2 [Sauk et al., 2000]. From among these sequences WHYPWFQNWAMA and LDSRYSLQAAMY were the most effective CBP2 binding peptides and best fulfilled the combinatorial motif containing deep hydrophobic pockets. When the hydropathic profiles of collagen α1(IV) and α2 (IV) were compared with these dodecapeptides, the hydropathic profiles of WHYPWFQNWAMA and LDSRYSLQAAMY closely matched those of α1(IV) 414–452 and α1(IV)531–543. These peptides were shown to be functional peptidomimics and possessed the ability to alter cell adhesion and invasion of human squamous cell carcinoma cell lines. Peptomers were formed of these non-natural peptides to explore the role that a repetitive peptide may have on cell adhesion. The enhanced cell adhesion observed with the peptomers required both CBP2 antibodies and integrin antibodies for inhibition. The enhanced adhesion observed even in the face of combined antibody inhibition was consistent with such complexes possessing correspondingly slower dissociation rates. Thus, suggesting that peptomers may function in a like manner to multimeric peptide MHC complexes (tetramers) binding more than one cell receptor on a specific cell. These findings evoke both peptidomimics of native ligands and their peptomers as potential reagents by which to target tumor cells for chemotherapy, imaging, or retargeting viral vectors for gene therapy. J. Cell. Biochem. 82: 145–154, 2001.
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Key words: CBP2; colligin2; Hsp47; integrin; carcinoma; bacteriophage; targeting

The development of regulatory peptides and peptidomimics as tools to visualize and treat malignant tumors is an important focus of interest [Behr et al., 1999]. For example, the development of stable somatostatin analogs and conjugates for diagnostic and therapeutic applications has opened new horizons in oncology [Behr et al., 1999; Otte et al., 1999]. Other regulatory peptides, such as gastrin-releasing peptide/bombesin derivative [Safavy et al.,

Abbreviations used: CBP2, collagen binding protein 2; Hsp, heat shock protein; Hsp47, heat shock protein 47; SCC, squamous cell carcinoma; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; EDTA, ethylenediaminetetraacetic acid; ER, endoplasmic reticulum; HEPES, N-2-hydroxyethpiperazine-N'-2-ethanesulfonic acid; BCECF-AM, 2′, 7′-bis-[2-carboxyethyl-]-5-[6]-carboxyfluorescein-acetoxymethylester; NEM, N-ethylmaleimide; TBS, Tris-buffered saline; PBS, phosphate buffered saline; BSA, bovine serum albumin; NFDM, non-fat dry milk; NGS, normal goat serum; DMEM, Dulbecco's Modified Eagle's Medium; PEG, polyethylene glycol; mAbs, monoclonal antibodies; rCBP2, recombinant CBP2; aa, amino acid; ATCC, american type culture collection; I_g, immunoglobulin; DTSSP, 3′,3′-dithiobis(sulfo-succinimidyl propionate); DSP, dithiobis(sulfo-succinimidyl propionate); SA, streptavidin.

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1999], vasoactive intestinal polypeptide [Raderer et al., 1998, 2000], epidermal growth factor receptor [Blackwell et al., 1999], and substances P [Reubi, 1997; Reubi and Horisberger, 1998], have also become evident as potentially useful candidates for in vivo scintigraphy, radiopeptide therapy, immunotherapy, and retargeted gene therapy.

The pursuit to discover a favorable target for head and neck cancers and squamous cell carcinoma in particular has identified CBP2 as a potential candidate [Sauk et al., 2000]. CBP2 derived from various sources is a heat inducible glycoprotein [Nagata et al., 1988; Nandan et al., 1990]. Normally, the CBP2 gene product is limited to the ER–Golgi where it is first associated with procollagen chains at a very early point during translation of nascent chains [Sauk et al., 1994] and then later with properly folded procollagen [Koide et al., 2000; Tasab et al., 2000]. CBP2 is retained within these cellular compartments by recycling of the erd2 gene product, KDEL receptor, which associates with the COOH-terminus sequence RDEL of CBP2 [Sauk et al., 1998]. However, in some tumor cell lines CBP2 is expressed independent of its chaperone properties and eludes or leaks from this surveillance mechanism and manifests on the cell surface. The precise mechanism of anchorage of this protein in the plasma membrane, in that ionic and covalent interactions with other proteins can be excluded, appears to favor a hydrophobic association with other cell surface membrane proteins [Hebert et al., 1999].

CBP2 has been shown to possess a limited number of natural intracellular ligands [Nakai et al., 1989; Nandan et al., 1990]. Previous studies have defined the Hsp47 binding to a region defined by the anti-propeptide antibody SP1.D8 [Hu et al., 1995], to a region of procollagen to *N*-propeptides of the $\alpha1$(I)-chains between residues 23–151, gelatin [Nagata et al., 1988], fetulin [Nakai et al., 1989], (Pro, Pro, Gly)n peptides [Koide et al., 2000], and triple helical procollagen[Tasab et al., 2000].

In an effort to characterize the binding characteristics of this protein, we recently reported on a combinatorial approach that utilized a repertoire of bacteriophage-peptides obtained from panning experiments with CBP2. Co-localization of some of these peptides with CBP2 in a number of tumor cell lines demonstrated that the peptides could be directed to an intracellular location spatially coincident with the normal distribution of CBP2 [Sauk et al., 2000].

Here, we explore the properties of two of these peptides on modulating carcinoma cell adhesion and invasion. In particular, we show that peptomers containing repetitive binding motifs enhance cell adhesion and as such appear to function in a like manner to tetrameric peptide MHC complexes [Altman et al., 1996]. These findings evoke both peptidomimics of native ligands and their peptomers as potential reagents by which to target tumor cells for chemotherapy, imaging, or retargeting viral vectors for gene therapy.

MATERIALS AND METHODS

Cell Lines and Cell Culture

Studies were performed using established cell lines of human oral squamous cell carcinomas [SCC-4, -9, -15, and -25] obtained from ATCC. In all of the studies presented here, cells were cultured in a 1:1 mixture of Ham's F12 and Dulbecco's modified Eagle's medium containing 10% fetal calf serum, hydrocortisone (0.4 μg/ml, Sigma) at 37°C in a 5% CO_2 air atmosphere. Cells were subcultured by disaggregation with trypsin (0.1%)-EDTA (0.01%) in phosphate buffered saline [PBS] at pH 7.5.

Design, Synthesis, and Transfection of Antisense/Scrambled Oligonucleotides

Antisense and sense phosphorothioate oligonucleotides were designed using the *CBP2* cDNA nucleotide sequence and a scrambled oligonucleotide was designed to act as an additional control. The oligonucleotides were synthesized by Oligos etc. (Wilsonville, OR), as follows: *CBP2* antisense (5′-CAGGAGGGAG-CGCAT-3′), *CBP2* sense (5′-ATGCGCTCCCT-CCTG-3′), scramble (5′-CTGGACGCAGGG-CTT-3′). The transfection reagent, lipofectin (Life Technologies, Paisley, Scotland), was used to introduce oligonucleotides into the carcinoma cell lines. For these studies 2×10^5 cells were seeded in 2 cm^3 tissue culture dishes and the cells were incubated under normal conditions for 24 h. Cells were transfected using 0.2 μM oligonucleotides and 5 μg of lipofectin according to the manufacturer's instructions. Twenty-four hours later, quantitative Real-Time RT-PCR, using CBP2- specific primers, assessed the cells for CBP2 mRNA expression. The CBP2

oligonucleotide primers, (5′-TGCTGAGCCCGGAAACTC-3′ and 5′-TTTCAGGGCAGGCAGAATG-3′), were selected using Primer Express software (PE Biosystems, Foster City, California). All reactions within each experiment were prepared using the same master PCR mix. The PCR reaction was carried out using a SYBR® Green PCR kit (PE Applied Biosystems, Foster City, California). The amplification was carried out as follows: 30 min at 48°C, 10 min at 95°C, followed by 40 cycles of 15 sec at 95°C and 1 min at 60°C. Thermal cycle and fluorescence detection were performed using an ABI 5700 Prism (PE Applied Biosystems, Foster City, California). Amplified CBP2 PCR products were quantified by comparison with a sample of the same cDNA amplified using gene-specific primers for the housekeeping gene, glyceraldehydes-3-phosphate dehydrogenase. The normalized amount of each of the genes present in tumor tissue was determined by designating the normal tissue as a calibrator using a comparative Ct method using PE Biosystems Protocols. All analyses were performed in triplicate. In addition, CBP2 production was determined by Western blot as previously described [Sauk et al., 2000].

Antibodies

For these studies, the monoclonal antibody SPA-470 to CBP2 (StressGen, Victoria, BC) and a CBP2 rabbit polyclonal antibody, prepared against a 22-mer peptide corresponding to the *N*-terminal sequence of mouse CBP2, were used [Sauk et al., 1994]. Also, purified mouse monoclonal antibodies prepared against integrin subunits α_1, α_2, α_V, and β1 were obtained from Chemicon International, Inc. (Temecula, CA).

Affinity Panning of a Library of Peptides

To study the binding specificity of CBP2, we utilized a bacteriophage library with dodecapeptide (Ph.D.-12, New England Biolabs; Beverly, MA) inserts at the *N*-terminus of pIII protein. The Ph.D.-12 library consisted of $\sim 2.7 \times 10^9$ electroporated sequences amplified once to yield ~55 copies/10 μl of supplied phage [Sauk et al., 2000].

Selection of CBP2-Binding Bacteriophages by Affinity Panning and Screening for CBP2-Binding Bacteriophages

Bacteriophages displaying peptides recognized by CBP2 were identified using the Ph.D.-12 kit (New England Biolabs; Beverly, MA) and the protocols modified for a biotinylated target [Sauk et al., 2000]. Screening for CBP2 binding bacteriophages was likewise performed as previously described [Sauk et al., 2000].

Determination of the Sequence of Bacteriophage-Displayed Peptides

Single-stranded bacteriophage DNAs were purified and sequenced as -96 primer an oligonucleotide (5′-CCCTCATAGTTAGCGTAACG-3′). Sequencing reactions were carried out using an ABI Prism Model 373 Version 3.0 [Sauk et al., 2000].

Peptide Synthesis

Selected dodecapeptides and LHepIII peptides were prepared by continuous flow solid-phase synthesis and analyzed by high-pressure liquid chromatography and mass spectrophotometry as described in previously [Cwirla et al., 1990; Li and Srivastava, 1993].

Peptomers were synthesized after the methods of Robey et al. [1995] and Frey et al. [1997]. Basically, a chloroacetyl moiety was added to the *N*-terminus using the same reaction conditions that were preprogrammed into the instrument for coupling glycine. The peptides were synthesized as the *C*-terminal amides using *p*-methyl-PAM resin purchased from Applied Biosystems, Inc. Following purification of the *N*-chloroacetylated, cystein-containing peptide using preparative reverse phase HPLC, the peptomer was formed by dissolving the peptide in 0_2-free H_2O and then adding a sufficient volume of 0.1 M Tris and 0.01 M EDTA, pH 8.0. The reaction was stirred at 25°C for 3 h, at which time it appeared that most of the detectable free sulfhydryl groups had been consumed. The reaction was continued for an additional 18 h and terminated by dialysis against H_2O at 4°C and then dialysis against 0.1 M $NaHCO_3$. The peptomer was end-capped by reacting it with 0.1 ml of β-mercaptoethanol in 10 ml followed by excess of idoacetamide. The peptomer was finally dialyzed against 0.1 M sodium acetate followed by deionized H_2O. Following lyophilization, the peptomer in the sodium acetate form was obtained as a dry white powder and stored desiccated at 4°C. Formation of peptomers with a range of Mrs = ~15–17k was verified by SDS-PAGE of the Coomassie Blue-stained gels. Helicity of the peptides was confirmed from

circular dichroism spectra obtained from a Jasco CD spectropolarimeter (model J-500A/DP-501N) and predicted using Provencher's spectral deconvolution program [Schiffer and Edmundson, 1967a, 1967b].

Cell Adhesion Assays

Squamous cell carcinoma adhesion assays were performed as described previously [Li et al., 1997]. In essence, cells were radiolabeled with [^{35}S] methionine, and microtitre plates were coated and incubated at 37°C overnight with the proteins or peptides of desired concentrations. Nonspecific binding sites were blocked with NFDM reconstituted in PBS for 2 h at 37°C. Cells were released from tissue culture flasks with trypsin-EDTA and washed with adhesion medium [DMEM], 2mg/ml bovine serum albumin (BSA), and 20 mM HEPES (pH 7.4)]. Cells (100 µl) were added into each well of a 96 well plate and incubated for 30 min at 37°C. Nonadherent cells were removed by washing with PBS containing Ca^{2+} and Mg^{2+}, and then the plates were counted and percent of cells attached determined. All assays were run in triplicate. Inhibition of cell adhesion was determined in like manner, except that the cells were preincubated with various concentrations of mAbs or peptides for 30 min at 37°C after they had been harvested. The cells were added to the wells to evaluate the adhesion to coated peptides or proteins in the continued presence of mAbs or peptides [Li et al., 1997]. Cells were allowed to adhere for 30 min at 37°C, and cell adhesion was quantified as described above.

Tumor Cell Invasion Assays

To assess tumor cell invasion an in vitro assay was modified after that described previously by Chu et al. [1993], utilizing Matrigel®, a reconstituted basement membrane. In essence, a modified Boyden chamber containing an 8 µm-porosity polyvinylpyrolidone-free polycarbonate filter was precoated with Matrigel® (Collaborative Biomedical Products, Becton Dickinson, Bedford, MA). The lower well of the chamber was then filled with serum free medium containing 500 µl of 3T6 cell-conditioned medium as a chemoattractant. The upper well was then seeded with 200 µl of cell suspension at 1.0×10^4 cells/chamber plus additives as indicated. The chambers were then incubated at 37°C for 24 h. Noninvasive cells are removed from the upper surface of the membrane with a cotton swab and the chamber incubated in 3 ml of Dispase (Collaborative Biomedical Products, Becton Dickinson, Bedford, MA) for 2 h and the reaction stopped with 10 mM EDTA. The resulting cells contained in Matrigel®, as well as the cells in the lower chamber, were counted in a Coulter counter. Data were expressed as the percent invasion through the matrix and membrane relative to the migration through the control membrane. The "Invasion Index" was expressed as the ratio of the percent invasion of a test cell over the percent invasion of a control cell.

Statistical Analysis

Overall treatment and group effects were assessed using an analysis of variance (ANOVA), with post-hoc comparisons based on the Newman-Keuls test ($P \leq 0.05$).

Subcellular Fractionation of Plasma Membranes

The method for fractionating plasma membranes was modified after the methods described by Weber et al. [1988] and Hebert et al. [1999]. In essence, 5 ml of cells [$(2-5) \times 10^6$ cells/ml] suspension was centrifuged at 100g for 60 sec at room temperature. The cell pellets were suspended in 10 ml of Tris/EDTA/sucrose buffer (20 mM Tris/HCl, 1mM EDTA and 255 mM sucrose, pH 7.4) at 18–20°C. The pellet was resuspended in 500 µl of Tris/EDTA/sucrose buffer by using a glass-Teflon homogenizer, layered on a 600 µl cushion of 1.2 M sucrose in 20 mM Tris/1mM EDTA buffer (pH 7.4), and centrifuged in a Beckman TLS55 rotor at 8150g at 4°C for 30 min. Plasma membranes collected at the cushion interface were suspended in 2.5 ml of Tris/EDTA/sucrose buffer and centrifuged in a Beckman TLA100.3 rotor at 41,0000g at 4°C for 20 min. The final plasma membrane pellet was resuspended in 60 µl of buffer. The samples were then treated with bacterial collagenase to eliminate the possibility of cytoplasmic derived procollagen-colligin/Hsp47 binding to the cell surface integrin receptors as a result of cell fractionation.

The membrane fractions were characterized by the distribution of 5′-nucleosidase activity [Avruch and Wallach, 1971]. Protein was measured with the BCA protein assay kit (Pierce, Rockford, IL). Triton solubilized plasma membranes were incubated with non-natural peptides, which were immobilized on nitrocellulose, at 4°C for 24 h. Subsequently, the membranes

were then washed extensively with 10 mM Tris-HCl pH 7.4, 0.9 mM NaCl and subjected to Western analysis. For Western blots, proteins immobilized on nitrocellulose paper were blocked with 10% NFDM in 10 mM Tris-HCl pH 7.4, 0.9 mM NaCl (TBS) for 2 h and then in TBS/NFDM with 2% NGS (GIBCO, Grand Island, NY). Antiserum or preimmune serum was diluted 1:2000 in the same buffer and incubated with gentle shaking overnight. The nitrocellulose was then rinsed three times for 5 min in TBS/Tween.

Homology With Type IV Collagen

The homology of peptides derived from bacteriophage panning experiments with CBP2 to that of Collagen IV was assessed using National Center for Biotechnology Information Blast 2 sequences database [Tatusova and Madden, 1999]. The hydropathic profiles of two proteins were assessed using the Weizmann Institute of Science Genome and Bioinformatics database for hydrophilicity/hydrophobicity searches and comparisons.

RESULTS

To elucidate the binding motifs of Hsp47, we utilized a bacteriophage library with random dodecapeptide inserts at the *N*-terminus of pIII protein. The sequences of dodecapeptides displayed by CBP2-binding bacteriophages obtained by panning were determined by DNA sequence analyses of the corresponding region of the bacteriophage genome. These sequences constituted part of the database used previously in determining the general binding motifs for CBP2 [Sauk et al., 2000]. From among these sequences WHYPWFQNWAMA and LDSRYSLQAAMY were the most effective CBP2 binding peptides and best fulfilled the combinatorial motif containing deep hydrophobic pockets [Sauk et al., 2000].

Recognizing that collagen IV is a natural ligand for CBP2, BLAST program analyses were performed to assess the sequence homology between collagen α1(IV) and α2(IV) chains and the selected dodecapeptides. Little specific homology was observed between the collagen IV and the peptides obtained from panning. However, when the hydropathic profile of collagen α1(IV) and α2 (IV) were compared with the dodecapeptides, the hydropathic profiles of WHYPWFQNWAMA and LDSRYSLQAAMY possessed a profile 70 and 80% identical to those of α1(IV) 414–452 and α1(IV)531–543 respectively (Fig. 1).

In that these two regions of collagen IV have been associated with cell attachment and migration, we determine if these peptides might have a modulating effect on tumor cell adhesion and invasion. Figure 2 depicts the effects of WHYPWFQNWAMA and LDSRYSLQAAMY as well as L-Hep III, the collagen α1(IV) 531–543 sequence known to mediate cell adhesion and migration in a variety of tumor cell lines [Li et al., 1997]. These data revealed that plates coated with peptides WHYPWFQNWAMA and LDSRYSLQAAMY enhanced binding of the SCC4 cell line better than L-HepIII or variant peptide sequences. These data were consistent for all of the cell lines tested, which have been shown to manifest cell surface CBP2 [Sauk et al., 2000] with some minor variance between WHYPWFQNWAMA and LDSRYSLQAAMY,

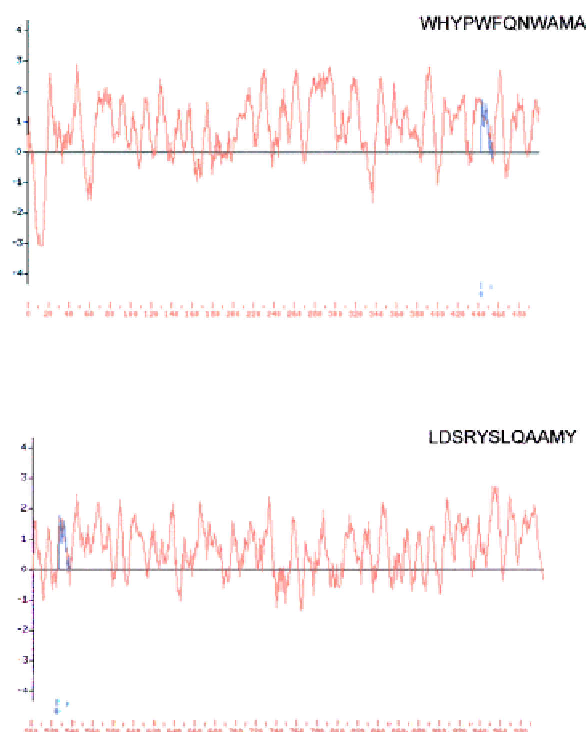

Fig. 1. Comparison of the hydropathic profile of CBP2 binding peptides and α1(IV) collagen. The hydropathic profiles were calculated and compared using the Kyte-Doolitle methods with a window of seven and favored matches over short fragments. Peptides depicted in each panel are indicated in the upper right. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

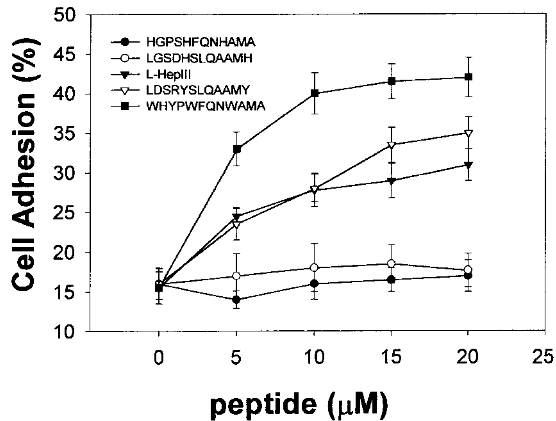

Fig. 2. Adhesion of SCC4 cells to non-natural CBP2 binding peptides. LDSRYSLQAAMY and WHYPWFQNWAMA, variant peptide controls HGPSHFQNHAMA and LGSDHSLQAAMH, and L-HepIII. Cells were allowed to adhere to peptide-coated plates for 30 min at 37°C. Data represent the means of triplicate points±SEM.

and between LDSRYSLQAAMY and L-HepIII. When similar experiments were performed on collagen IV coated plates and peptides were added to the cells prior to plating and included in the medium, the results were the converse of what was observed in adhesion experiments. That is with increasing peptide concentrations peptides WHYPWFQNWAMA, LDSRYSLQAAMY, and L-HepIII inhibited cell adhesion to collagen IV (Fig. 3).

To assess the role of cell surface CBP2 in mediating cell adhesion competitive inhibition experiments were also performed using antibodies directed against CBP2, α_1, α_2, α_V, and β_1 integrin subunits. These studies revealed that antibodies against both CBP2 and the α_1 integrin subunit and to a lesser degree the α_2 integrin subunit inhibited tumor cell adhesion, while the β_1 and α_V antibodies were slightly less effective (Fig. 4). To further evaluate these observations carcinoma cells were as before treated with CBP2 antisense oligonucleotides to significantly reduce the expression and production of CBP2 (Fig. 5). Cells treated in this manner were then tested in cell adhesion experiments outlined above. These studies showed that reduction of CBP2 dramatically reduced tumor cell adhesion to WHYPWFQNWAMA and LDSRYSLQAAMY. Interestingly, a significant number of CBP2 antisense treated cells still adhered to WHYPWFQNWAMA and LDSRYSLQAAMY peptides, however, the adhesion of these cells could be inhibited by α integrin antibodies (Fig. 6). To verify the binding of CBP2 and integrins to peptides, purified proteins were shown by Western blot analysis to bind to immobilized WHYPWFQNWAMA (Fig. 7) and LDSRYSLQAAMY (not shown)

In that WHYPWFQNWAMA and LDSRYSLQAAMY peptides were most effective in mediating cell adhesion and apparently could bind

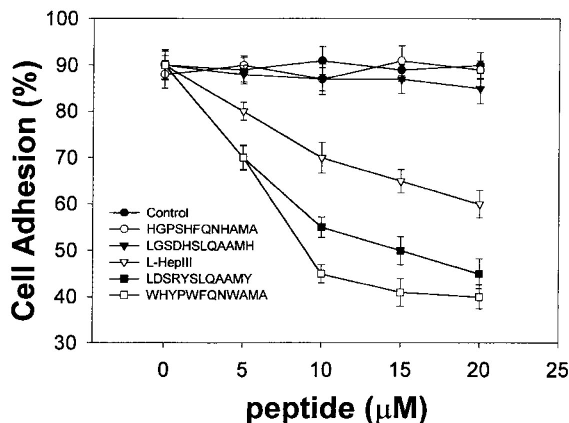

Fig. 3. Inhibition of SCC4 cell adhesion to 5 μg/ml collagen IV by non-natural CBP2 binding peptides LDSRYSLQAAMY and WHYPWFQNWAMA, variant peptide controls HGPSHFQNHAMA and LGSDHSLQAAMH, and L-HepIII. Cells were preincubated with the peptides for 30 min and then added to the wells in the presence of the peptides for 30 min incubation at 37°C. The data represent the means of triplicate points±SEM.

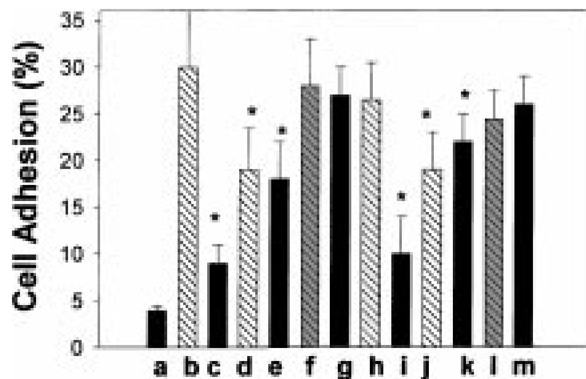

Fig. 4. Adhesion of SCC4 cells to **a:** plastic, **b:** 10 μm of WHYPWFQNWAMA, and **c:** 10 μm of LDSRYSLQAAMY. Effect of **c, i:** CBP2 antibodies, **d, j:** α1 integrin antibodies. **e, k:** α2 integrin antibodies, **f, l:** αV integrin antibodies, and **g, m:** β1 integrin antibodies on the adhesion of SCC4 cells to 10 μm of WHYPWFQNWAMA and LDSRYSLQAAMY peptides. Cells were preincubated with antibodies (3 μg/ml) for 30 min and then added to the wells in the presence of the antibodies for 30 min incubation at 37°C. The data represent the means of triplicate points±SEM. *designates significant difference from peptide controls, $P=.001$

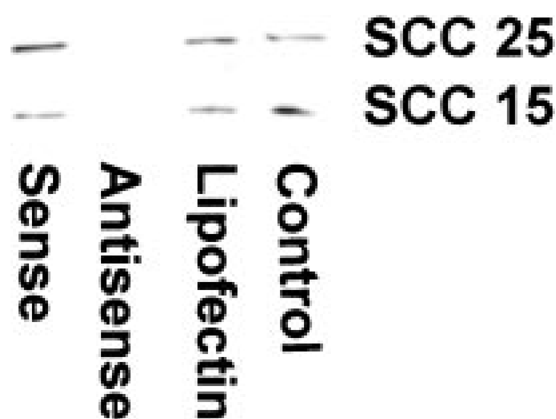

Fig. 5. Effect of antisense on the production of CBP2 demonstrated by Western blot analysis in SCC25 and SCC15 cell lines.

Fig. 7. The binding of purified integrins and CBP2 to WHYPWFQNWAMA immobilized on nitrocellulose following Western blot analysis with integrin and CBP2 antibodies.

both α integrin subunits as well as CBP2, we increased the chain length of the peptides through formation of peptomers to ascertain whether making available repetitive substrate sequences and imparting helicity might promote adhesion by CBP2 and integrin subunits. Figure 8 depicts this experiment, which showed that peptomers formed with WHYPWFQN-WAMA peptide dramatically increased cell adhesion. The adherence to these peptomers was not inhibited to a great extent by antibodies against CBP2, or by individual α integrin subunit antibodies alone. However, CBP2 antibodies and anti- α integrin subunit antibodies added together did reduce cell adhesion. Moreover, carcinoma cells that were treated with CBP2 antisense oligonucleotides, showed a significant decrease in adhesion to peptomers. However, the remaining adhering cells were in good measure displaced by α integrin antibodies (Fig. 8). Similar results were also obtained with LDSRYSLQAAMY (not shown).

In that L-Hep III, WHYPWFQNWAMA, and LDSRYSLQAAMY peptides could modulate cell adhesion the effects of these peptides on cell invasion into a reconstituted basement membrane matrix were assessed. These studies are

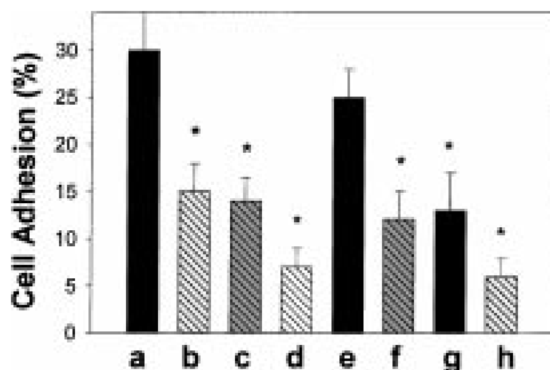

Fig. 6. **a:** Adhesion of SCC4 cells to 10 μm of WHYPWFQN-WAMA. **b:** Effect of CBP2 antibodies on adhesion to peptide. **c:** CBP2 antisense treated cells on WHYPWFQNWAMA. **d:** Effect of α1 integrin antibodies on antisense treated cell adhesion to peptide. **e:** Adhesion of SCC4 cells to 10 μm of LDSRYSL-QAAMY. **f:** Effect of CBP2 antibodies on adhesion to peptide binding depicted in e. **g:** CBP2 antisense treated cells on LDSRYSLQAAMY. **h:** Effect of α1 integrin antibodies on antisense treated cell adhesion to adhesion to peptide depicted in e. Cells were preincubated with the antibodies for 30 min and then added to the wells in the presence of the antibodies for 30 min incubation at 37°C. The data represent the means of triplicate points±SEM. *designates significant difference from peptide controls, $P = .001$.

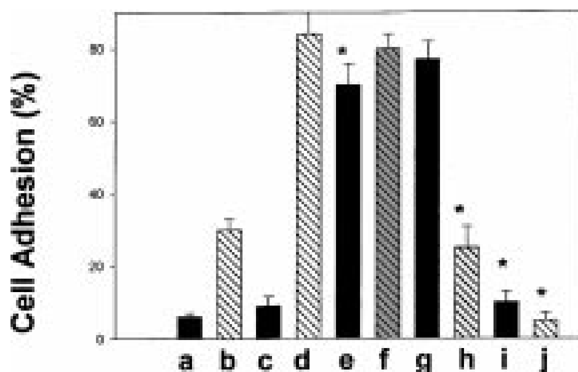

Fig. 8. Adhesion of SCC4 cells to **a:** plastic, **b:** 10 μm of WHYPWFQNWAMA peptide, and **c:** 10 μm of WHYPWFQN-WAMA + CBP2 antibodies and **(d)** WHYPWFQNWAMA peptomer. **e:** Effect of CBP2 antibodies on peptomer adhesion. **f:** Effect of α1 integrin antibodies on peptomer adhesion. **g:** Effect of α2 integrin antibodies on peptomer adhesion. **h:** CBP2 + α1 integrin antibodies on the adhesion of SCC4 cells to 10 μm of WHYPWFQNWAMA peptomer. **i:** Effect of CBP2 antisense on peptomer adhesion. **j:** The effect of α1 integrin antibodies on CBP2 antisense treated cell adhesion to peptomer. Cells were preincubated with the antibodies for 30 min and then added to the wells in the presence of the antibodies for 30 min incubation at 37°C. The data represent the means of triplicate points±SEM. *designates significant difference from peptomer controls, $P = .001$.

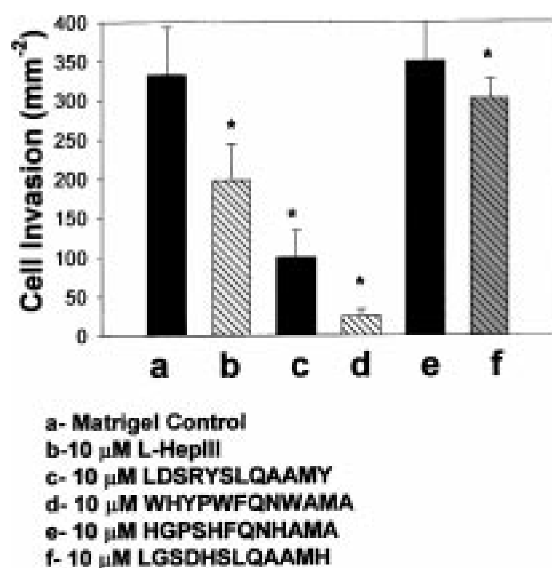

Fig. 9. Inhibition of SCC4 cell invasion through Matrigel. **a:** Matrigel control. **b:** 10 μm L-HepIII. **c:** 10 μm LDSRYSLQAAMY. **d:** 10 μm WHYPWFQNWAMA. **e:** 10 μm HGPSHFQNHAMA. **f:** 10 μm LGSDHSLQAAMH. Cells were mixed with various peptides and tested for their ability to invade through a reconstitute basement membrane matrix. The data represent the means of triplicate points±SEM. *designates significant difference from Matrigel controls, $P = .001$.

depicted in Figure 9, which revealed that 10 μm concentrations of WHYPWFQNWAMA, LDSRYSLQAAMY, and L-HepIII all inhibited tumor cell invasion into a basement membrane matrix while variant peptide sequences had no effect. These results with minor variations in degree were consistent for all four of the SCC cell lines.

DISCUSSION

Previously we showed that affinity panning of a library of peptides displayed on bacteriophages revealed a number of peptides that bind CBP2. In the present investigation we recognized that two of these peptides WHYPWFQNWAMA and LDSRYSLQAAMY, the strongest CBP2 binding peptides, best resembled the binding motif determined by combinatorial chemistry. This motif is hallmarked by hydrophobic pockets that are flanked by regions that contain charged residues at position 1,4, 5, and 7–10 in dodecapeptides [Sauk et al., 2000]. Both of these peptides were shown to modulate cell adhesion and invasion in squamous cell carcinoma cell lines expressing CBP2. Further support for these findings was that pretreatment of cells with CBP2 antisense phosphorothioate oligonucleotides diminished the effects on adhesion and invasion. Interestingly, the cells remaining adherent to these peptides, following antisense treatment, were displaced in great part by α integrin antibodies. The binding of both CBP2 and α integrins to these peptides was verified by affinity binding to immobilized non-natural peptides.

Although, no specific sequence homology was recognized between these CBP2 binding peptides and type IV collagen, these peptides possessed hydropathic profiles with close similarity to α1(IV) 531–543 and α1(IV) 414–452 peptides. The sequence $[\alpha1(IV)\ 414–452]_2\alpha2(IV)$ 432–469 is acknowledged as a $\alpha_1\beta_1$ related cell adhesion site [Li et al., 1997]. Moreover, the peptide fragment α1(IV) 531–543 has been shown to promote $\alpha_2\beta_1$ and $\alpha_3\beta_1$ integrin-mediated cell adhesion of corneal epithelial cells [Maldonado and Furcht, 1995] and $\alpha_2\beta_1$ integrin-mediated keratinocyte motility [Kim et al., 1994]. Additionally, α1(IV) 531–543 fragments have been shown to bind the $\alpha_3\beta_1$ and promote melanoma and ovarian cell adhesion [Miles et al., 1995; Li et al., 1997]. Even though the complete substitution of hydrophobically equivalent amino acids in peptides maintains and sometimes increments their peptide-receptor mediated physiological potency [Kaiser and Kezdy, 1983, 1984]. The mechanism for binding of these peptides to integrin subunits is problematic, since none of these non-natural peptides possess putative RGD binding sequences. However, examination of both of these peptide motifs reveals deep hydrophobic domains that are occupied by phenylalaine (F) and tryptophan (W). Both of these amino acids have been established as key elements in cation-π interactions, which are an important general force for molecular recognition in biological receptors. As a general rule proteins can use aromatic side-chains to stabilize bound cationic ligands. However, recently the reciprocal process in which a cationic protein side-chain stabilizes a neutral aromatic ligand has been demonstrated [Pellequer et al., 2000]. Moreover, now there is strong evidence that in the platelet fibrinogen receptor, gpIIb-gIIIa, π-electrons may substitute for the carboxyl group present in other RGD-types of ligands [Thorpe et al., 1999]. Although the weight of evidence indicates that cation-π interactions are among the many noncovalent forces that contribute to biological structures

[Dougherty, 1996; Mecozzi et al., 1996; Gallivan and Dougherty, 1999]. The implication of such interactions here will require both structural and energy based analyses for these peptides and their purported receptors.

The utilization of WHYPWFQNWAMA and LDSRYSLQAAMY peptomers was designed to explore the role that a repetitive peptide binding motif may have on carcinoma cells. The formation of peptomers usually produces secondary structure in the form of a α-helices in proportion to chain length [Robey et al., 1995]. The enhanced cell adhesion observed with the WHYPWFQNWAMA peptomer is intriguing in that previous studies have indicated that triplical helical conformations of the [α1(IV) 414–452]$_2$ α2(IV)432–469 collagen fragment is required for optimal activity [Li et al., 1997]. However, in that a combination of CBP2 and α integrin subunit antibodies were both required to inhibited adhesion of the cells plated on WHYPWFQNWAMA peptomes suggests that more than one receptor on a cell binds to the peptomer. In contrast to [α1(IV) 414–452]$_2$, α2(IV)432–469 collagen fragments, helical conformation does not significantly increase the adhesion-promoting activity of α1(IV) 531–543 peptides [Li et al., 1997]. Thus, it was not expected that LDSRYSLQAAMY peptomers, which share hydropathic profiles with this region of α1 (IV) collagen fragments, would promote additional cell adhesion to equivalent concentrations of monomers. However, in that this binding was likewise inhibited by both CBP2 antibodies and integrin antibodies suggests that peptomers constructed from these non-natural peptides may in a like manner to multimeric peptide MHC complexes bind more than one cell receptor on a specific cell [Altman et al., 1996]. Furthermore, the enhanced adhesion observed even in the face of combined antibody inhibition would be consistent with such complexes possessing correspondingly slower dissociation rates [Altman et al., 1996]. These studies thus, evoke such non-natural ligands, and their peptomers as prospective reagents to target tumor cells for chemotherapy, imaging, and/or retargeting viral vectors for gene therapy.

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