Journal of Cellular Biochemistry 78:251–263 (2000)

Binding Motifs of *CBP2* a Potential Cell Surface Target for Carcinoma Cells

John J. Sauk,[1,2]* Ricardo D. Coletta,[3] Kathleen Norris,[1] and Carla Hebert[1]

[1]Department of Pathology, University of Maryland, Baltimore, Maryland 21201
[2]Dental School and UMB Greenebaum Cancer Center, University of Maryland, Baltimore, Baltimore, Maryland 21201
[3]Department of Oral Pathology, Campinas University Piracicaba, Šao Paulo, Brazil

Abstract Previously we have shown (Hebert et al. [1999] J. Cell Biochem. 73:248–258) that among many cell lines the *CBP2* gene product, Hsp47, eludes its retention receptor, *erd2P*, resulting in the appearance of Hsp47 on the cell surface associated with the tetraspanin protein CD9. Since Hsp47 possesses a highly restricted binding cleft, random peptide display libraries were used to characterize peptides binding to Hsp47 and then to target this protein on carcinoma cell lines in vitro. Comparison of the clones obtained from panning revealed little specific homology based on sequence alone. To determine whether carcinoma cells expressing Hsp47 could selectively take up the selected bacteriophages, traditional immunofluorescence and confocal microscopy were employed. These studies revealed that phage-displaying Hsp47 binding peptides bound to cell lines expressing Hsp47 and that the peptides were rapidly taken up to a location coincident with Hsp47 staining. These observations were confirmed by cytometric analyses. These data indicate that *CBP2* product may provide a molecular target for chemotherapy and/or imaging of malignancies. J. Cell. Biochem. 78:251–263, 2000.

Key words: CBP2; Hsp47; carcinoma; bacteriophage; targeting

There is an ongoing quest to identify specific cell surface targets by which to direct treatment or to image neoplastic cells. For example, one group of proteins that has been used as targets for drug delivery is chimeric molecules created by cancer-associated chromosomal abnormalities. However, such molecules are usually unique to a particular tumor [Baron et al., 1997; Diez de Medina et al., 1997]. In addition to the aforementioned proteins, potential sources of such targets are the products of genes that are amplified at a particular chromosome locus in malignancy. For example the chromosome locus 11q13→14 is commonly amplified in human cancers that include cancers of the head and neck, lung, esophagus, bladder, and breast [Schuuring et al., 1992]. This amplicon is large, spanning 2.5–5 Mb and harbors several genes with known oncogenic potential [Bekri, 1997]. In breast cancer, this locus is amplified in ~13% of primary tumors while in head and neck cancers amplification may represent 40–76% of tumors. A more detailed map of this region has evolved indicating that as many as five distinct amplification units exist on 11q13. Among these units is a genomic area encompassing the GARP gene at 11q13.5→q14.1. Assessment of this region, located telomeric to *CCDN1* and *EMS1*, has revealed a number of genes in the regional map, namely *CBP2* (*Hsp47*) and "spot 14" at chromosome locus 11q13.5 with, *CLNS1A*, *UVRAG*, and *PAK1* located telomeric to this later region, thus, narrowing the core of the 11q13→q14 amplicon to a 350-kb area encompassing D11S533 on its telomeric side [Bekri, 1997; Moncur, 1998].

In assessing this amplicon, it became evident that the gene product of *CBP2* was associated with, and may distinguish a group of malignant tumors [Morino et al., 1994, 1995, 1997;

Abbreviations used: CBP2 , collagen binding protein 2; ER, endoplasmic reticulum; ATCC, American Type Culture Collection; TBS, Tris buffered saline; PBS, phosphate buffered saline; PEG, polyethylene glycol, FITC, fluoresceinisothiocyanate; BSA, bovine serum albumin.

Grant sponsor: U.S.P.H.S.; Grant number: R01-DE12606.

*Correspondence to: John J. Sauk, Dental School and UMB Greenebaum Cancer Center, University of Maryland, 666 W. Baltimore Street, Baltimore, MD 21201. E-mail: jjs001@dental.umaryland.edu

Received 15 January 2000; Accepted 21 January 2000

Print compilation © 2000 Wiley-Liss, Inc.
This article published online in Wiley InterScience, May 2000.

Shirakami et al., 1995a–c], as well as being localized to extravillous cytotrophoblasts and decidual cells at the fetal maternal interface [Pak et al., 1997; Shirakami et al., 1995b,c; Morino et al., 1994, 1995, 1997a,b]. Interestingly, *CBP2*, mapped to chromosome 11q13.5, shares a locus with *Spot 14* a key gene expressed in lipogenic neoplasms [Moncur, 1998].

Normally, the *CBP2* gene product, Hsp47, 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]. Hsp47 is retained within these cellular compartments by recycling of the erd2 gene product, KDEL receptor, that associates with the COOH-terminus sequence RDEL of Hsp47 [Sauk et al., 1998]. However, in some tumors Hsp47 is expressed independent of its chaperone properties and eludes or leaks from this surveillance mechanism and manifests on the cell surface, but is not secreted into the medium. Although previous studies have demonstrated that Hsps such as gp96 are tightly surface-bound peripheral membrane proteins, the precise mechanism of anchorage was unclear, albeit ionic interactions with other proteins could be excluded [Tamura et al., 1997]. Hsp47, like gp96, lacks sequence characteristics for farnesylation, palmitation, isoprenyllation, or myristylation; consequently, covalent bonding also appears to be an unlikely anchoring mechanism [Tamura et al., 1997; Altmeyer et al., 1996]. Interestingly, Hsp47 has been shown to associate with the tetraspanin protein CD9 in some epidermoid carcinoma cell lines [Hebert et al., 1999]. Moreover, in these instances Hsp47 has been shown to be easily recovered, even without the use of cross-linking, from membrane immunoprecipitates utilizing anti-CD9 antibodies, suggesting the presence of hydrophobic interactions between these proteins [Hebert et al., 1999].

Hsp47 unlike many other chaperones has been shown to possess a limited number of intracellular ligands [Nakai et al., 1989]. 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 α1(I)-chains between residues 23-151 [Hu et al., 1995], and to gelatin [Nagata et al., 1988]. Most recently, it has been shown that a typical collagen model sequence, $(Pro\text{-}Pro\text{-}Gly)_n$, possesses sufficient structural information required for recognition by Hsp47 [Koide et al., 1999].

Here we report on a repertoire of bacteriophage-peptides obtained from panning experiments with Hsp47. Co-localization of some of these peptides with Hsp47 in a number of tumor cell lines demonstrates that the peptides can be directed to an intracellular location spatially coincident with Hsp47. These results indicate that cell surface Hsp47 is not permanently anchored to the cell surface and that this protein undergoes recycling with an intracellular pool. In addition, these studies suggest that expression of the *CBP2* in tumors may provide a target for phage-directed gene therapy, a mechanism by which to deliver drugs, or a means of imaging occult disease and metastases.

MATERIALS AND METHODS

Cell Lines

Studies were performed using a number of established cell lines of human oral squamous cell carcinomas (SCC4, SCC9, SCC15, and SCC25) obtained from ATCC, and a transformed normal oral keratinocyte cell line GMSM-K was courtesy of Dr. V. Murrah (UNC, Chapel Hill, NC). Breast carcinomas (HTB126, HTB127), and HTB125 normal breast cells were likewise obtained from ATCC. In addition, prostate cell lines PC-3, LNCaP, and PZ-HPV were courtesy of Dr. R. Franklin (UM, Baltimore, MD). In 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, St. Louis, MO) at 37°C in a 5% CO_2 air atmosphere. For breast carcinoma cell lines, 10 μg/ml of insulin was added to the medium as prescribed by ATCC and PZ-HPV cells were grown in KSF medium. Cells were subcultured by dissaggregation with trypsin (0.1%)-EDTA (0.01%) in phosphate buffered saline (PBS) at pH 7.5.

Antibodies

The monoclonal antibody SPA-470 to Hsp47 (StressGen, Victoria, BC) was used, as well as, a Hsp47 rabbit polyclonal antibody [Sauk et al., 1994]. The anti-M13 monoclonal antibody was procured from Amersham Pharmacia Biotech (Piscataway, NJ). Monoclonal antibodies

for cytometric analyses were directly conjugated with fluorescein using 5(6) carboxyfluorescein-N-hydroxy succinimide ester kit (Boehringer Mannheim, Indianapolis, IN) or labeled with SA-Red670™ following biotinylation of the antibody using EZ-Link™ Sulfo-NHS-LC-Biotinylation kit (Pierce, Rockville, IL).

Affinity Panning of a Library of Peptides

To study the binding specificity of Hsp47, we utilized two bacteriophage libraries with random septapeptide (Ph.D.-7, New England Biolabs, Beverly, MA) or dodecapeptide (Ph.D.-12, New England Biolabs) inserts at the N-terminus of pIII protein. The Ph.D.-7 library consists of $\sim 2.8 \times 10^9$ electroporated sequences amplified once to yield ~70 copies of each sequence in 10 μl of supplied phage. 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.

Selection of Hsp47-Binding Bacteriophages by Affinity Panning

Bacteriophages displaying peptides recognized by Hsp47 were identified using the Ph.D.-7 or Ph.D.-12 kits (New England Biolabs) and the protocols modified for a biotinylated target. In essence, 96 wells of a microtitration plate were coated with 15 μl of streptavidin (1 mg/ml) in 135 μl of 0.1M $NaHC0_3$ pH 8.6, with gentle agitation in a humidified chamber overnight at 4°C . After removal of the streptavidin solution the wells were washed three times with TBS containing 0.05% Tween 20 (TBS-Tween) and then blocked by incubation for 1 h at 37°C with 1% BSA in PBS containing 0.1 μg/ml streptavidin.

Next, phage was precomplexed with biotinylated Hsp47 by adding 0.1 μg biotinylated Hsp47 and 2×10^{11} pfu of the input phage in 400 μl TBS-Tween and incubated for 60 min. The phage-target complex solution was then added to the washed blocked plates and incubated at room temperature for 10 min. Biotin was added to a final concentration of 0.1 mM and incubated for 5 min to displace streptavidin-binding phage. Non-binding phage were discarded and the plates washed 10 × with TBS-Tween (0.1%). The wells were then treated with 15 μl of 1 M Tris-HCl; pH 2.2, containing 1 mg/ml BSA for 5 min to elute Hsp47-phage. The samples were neutralized with 1 M Tris-HCl; pH 9.1, and amplified by adding to 20 ml of ER 2537 culture incubated at 37°C with vigorous shaking for 4.5 h. The cultures were then transferred to fresh tubes centrifuged at 10,000 rpm, 4°C, for 10 min. The supernatants were transferred to fresh tubes and phage precipitated with 1/6 volume of PEG/NaCl at 4°C, overnight. To 4 ml of LB media a single colony of ER2537 was inoculated and then incubated at 37°C with vigorous shaking until the culture reached mid-log phase ($O.D._{600}$ ~0.5). A pellet was obtained by centrifugation for 15 min at 10,000 rpm, 4°C. The pellet was suspended in 1 ml of TBS and centrifuged for 5 min, 10,000 rpm, 4°C. The supernatant was transferred to a fresh tube and precipitated with 1/6 volume of PEG/NaCl on ice for 1 h. Following centrifugation the pellet was resuspended in 200 μl TBS. The eluate was titered and plated onto LB/IPTG/XGal plates and incubated overnight. Since the library phage are derived from the common cloning vector M13mp19, which carries the *lacZ*α gene, phage plaques appear blue when plated on media containing Xgal and IPTG. Blue colonies were selected for sequencing or used for a second round of panning. As a control streptavidin was used as a target and after three round of panning a to verify a consensus sequence for streptavidin-binding peptides.

Screening for Hsp47-Binding Bacteriophages

Clones of blue colonies from plates containing 50–200 colonies were transferred to nitrocellulose filters. The bacteria were washed from the filters with PBS containing 0.05% Tween 20 and 1% bovine serum albumin, and the filters were then incubated for 30 min in the same buffer before washing three times with PBS-Tween. Following incubation for 1 h in 5 ml of biotinylated Hsp47 (0.1–2 μg/ml in PBS-Tween), the filters were again washed three times in PBS-Tween. The positions of the clones that had secreted Hsp47-binding bacteriophages were then located by one of two methods: the filters were incubated with 5 ml of alkaline phosphatase-conjugated streptavidin (1/10,000 dilution in PBS-Tween; Pierce, Rockford, IL) for 1 h at room temperature before extensive washing with PBS-Tween, or the filters were incubated for 1 h in 5 ml of an anti-biotin antibody (1/50,000 dilution in PBS-Tween; Pierce, Rockford, IL), washed, and incubated with a rabbit anti-goat immunoglobulin antibody conjugated to alkaline phosphatase (1/5,000 dilution in PBS-Tween; Pierce,

Rockford, IL). In each case, alkaline phosphatase activity was revealed using a mixture of nitroblue tetrazolium/and 5 bromo-4-chloro-3-indolylphosphate tolidium (Bethesda Research Labs, Rockville, MD) as substrate.

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.

Program to Score Hsp47-Binding Peptides

To characterize peptides that bind to Hsp47, we compared the starting and selected libraries in a position-dependent manner. A statistical analysis was performed by maximum likelihood and bootstrap resampling. This revealed the distribution of residues by position in Hsp47-binding peptides compared to peptides in the original library. To codify the preferences obtained above, we used a scoring system previously described for BiP [Cwirla et al., 1990] that would be used to predict Hsp47-binding sites in synthetic and naturally occurring polypeptides. In so doing a score is given to each of the possible 20 amino acids at each position of the seven-residue core sequence. These scores were derived from the fold difference in the overall abundance of each residue in the peptides displayed by the selected and nonselected bacteriophage populations. The scoring for each of the seven residue sequences present in each septapeptide was determined as previously described [Cwirla et al., 1990].

To validate our scoring set of Hsp47-binding sequences, a second set of Hsp47-binding sequences not part of the database used to generate the scoring matrix is necessary. To accomplish this a second library consisting of independent recombinant bacteriophages displaying random dodecapeptides was used (Ph.D-12, New England Biolabs). Sequences were then compared by position specific iterated BLAST, pattern Hit Initiated BLAST, and BLAST 2 sequences against each other (NCBI). In addition, the hydropathic profiles of two peptides were compared using the Weizmann Institute of Sciences Genome and Bioinformatics database.

Peptide Synthesis

Septapeptides and dodecapeptides were prepared by continuos flow solid-phase synthesis and analyzed by high-pressure liquid chromatography and mass spectrophotometry as described in previously [Cwirla et al., 1990].

Cytometric Analyses

Cytometric analyses were performed after the methods of Hebert et al. [1999]. In essence, cells grown in vitro, as described above, washed and blocked with Polyglobin N [Takeshita et al., 1995]. Next, 50 ml of the cell suspension (1×10^6 cell/ml) was incubated with 2.5 ml (1 mg) of antibodies, conjugated with fluorescein, or SA-Red670™ (GibcoBRL, Gaithersburg, MD). To assess intracellular Hsp47 and M13 bacteriophage, cells were first permeabilized with 0.1% Saponin as previously described [Tang et al., 1993, 1994]. Samples were then analyzed on a FACScan flow cytometer (Becton Dickinson, San Jośe, CA) as described previously [Hebert et al., 1999].

Immunofluorescence and Confocal Microscopy

Immunofluorescence microscopy was carried out after the method of Tang et al. [1993, 1994]. To visualize cell surface Hsp47 or M13 bacteriophage, the cells were not permeabilized but treated and fixed with 1% paraformaldehyde as described for cytometric analyses. However, to prevent non-specific binding, the cells were blocked with 10% pig serum in PBS for 1 h. The cells were then incubated with anti-Hsp47 or anti-M13 bacteriophage antibodies (Amersham Pharmacia-Biotech, Piscataway, NJ), washed with PBS, and incubated for 1 h with goat anti-rabbit IgG conjugated with either fluorescein or Texas red. Coverslips were mounted in mounting media containing an antibleaching agent (Kirkegaard & Perry Laboratories, Inc.; Gaithersburg, MD). Cells were examined under a Zeiss II photomicroscope equipped with epifluorescence. Cells untreated with primary antibodies were used as negative controls.

Confocal images were collected at the same focal plane from ~1 μM thick samples using a Zeiss LSM410 confocal microscope. A FT of 488/568 with a barrier filter of 590 was used to detect Texas red staining and a FT of 560 with a barrier at 515–540 were used to generate fluorescein labeled images. Digital images were collected on a ZIP drive and figures gen-

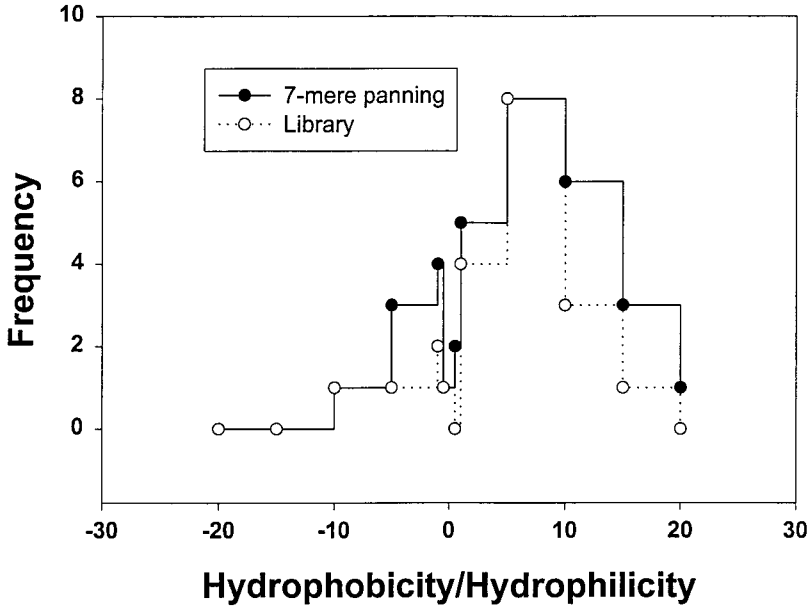

Fig. 1. Frequency distribution of hydophobicity scores computed for peptides displayed by bacteriophages selected from the phd-7 library following panning against hsp47 compared with frequency distribution from a random library. The frequency distribution of overall hydrophobicity scores calculated for 54 peptides using the hydropathy scale of Kyte and Doolittle [1982] are shown for displayed by bacteriophages obtained from Hsp47 binding following three rounds of panning. These scores are compared with the frequency of hydrophobicity/hydrophilicity of 70 peptides from a random library.

erated using Adobe Photoshop 3.0 software (Adobe Systems Inc. Mountain View, CA). No fluorescence was associated with cells after incubation with secondary antibodies alone.

To label the lysosomal compartment, cells were incubated with 1 mg/ml lysine-fixable FITC-dextran (Molecular Probes; Eugene, OR) in growth medium for 4 h at 37°C in 5% $C0_2$. After washing, cells were incubated an additional 30 min to chase the dextran from the early endosomal to the lysosomal compartments. For identification of the early and recycling endosomal compartments, cells were incubated in serum-free medium containing 50 μg/ml FITC-transferrin (Molecular Probes; Eugene, OR) for 30 min at 37°C in 5% $C0_2$. After treatments to identify the specific subcellular compartments, cells were fixed and processed for immunofluorescence and/or confocal microscopy.

Subcellular Fractionation of Plasma Membranes

The method for fractionating plasma membranes was modified after the methods described by Weber et al. [1988]. The membrane fractions were characterized by the distribution of 5'-nucleosidase activity, a marker of plasma membrane [Avruch and Wallach, 1971]. For immunoprecipitation, cells were first labeled with ^{35}S-methionine as previously described and the immunoprecipitates were directly subjected to PAGE and autoradiography [Hebert et al. 1999; Sauk et al., 1998].

RESULTS

To elucidate the binding motifs of Hsp47, we utilized two bacteriophage libraries with random septapeptide or dodecapeptide inserts at the N-terminus of pIII protein. Because peptides containing at least seven or eight residues are generally required for efficient binding, we first chose a library of bacteriophages that displayed septameric peptides (Ph.D.-7, New England Biolabs). To begin to characterize these peptides, 70 individual bacteriophage clones were picked at random from the unselected library, and the amino acid sequences of the variable septapeptide inserts were deduced from the nucleotide sequences of the corresponding coding region. All amino acids are represented, although their frequencies do not always correspond to those expected from the relative numbers of codons encoding each residue.

The sequences of septapeptides displayed by 54 Hsp47-binding bacteriophages obtained by panning were determined by DNA sequence analysis of the corresponding region of the bacteriophage genome. The peptide sequences from the 54 clones that bound Hsp47 were first considered as a single population and compared with those from the 70 clones picked randomly from the starting library (Fig. 1).

Comparison of the overall amino acid composition of the two populations, selected and unselected, of septapeptides revealed that aspar-

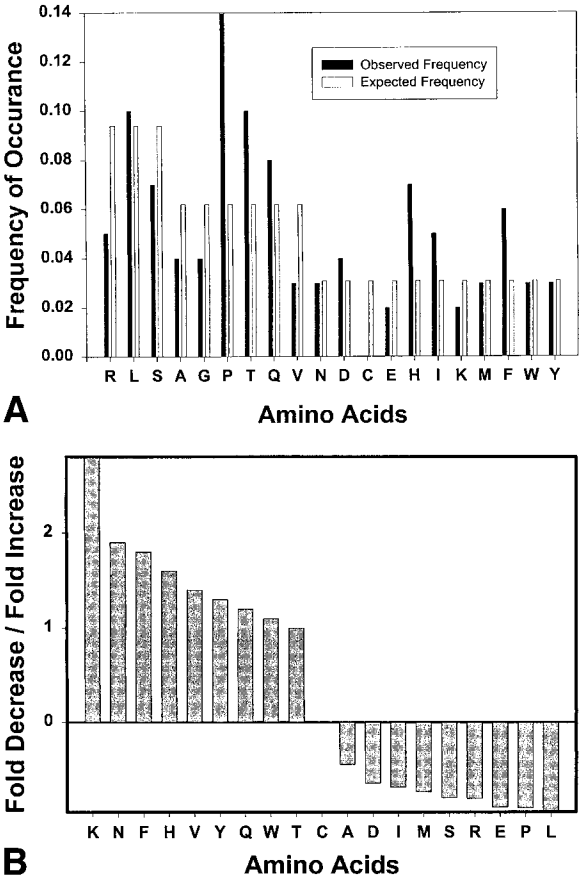

Fig. 2. Relative abundance of the 20 amino acids in septapeptides displayed by the library of Phd-7 bacteriophages. **A:** Single-stranded DNA was purified from 70 bacteriophage clones from the PhD library, and the sequences of the septapeptides displayed by these bacteriophages were deduced from the DNA sequence of the corresponding region of the bacteriophage genome. The figure shows the frequency of occurrence of each amino acid calculated as the number observed divided by the total number of residues in the 70 septapeptides. The amino acids are grouped according to the number of codons that specify them, and the frequency expected for each group if all codons were utilized with equal efficiency is shown by open bars [Cwirla et al., 1990] . **B:** The overall amino acid composition of 54 septapeptides from the Hsp47-binding PhD-7 bacteriophages was compared with that of 70 septapeptides from clones picked randomly from the PhD-7 starting library. The fold increases or fold decrease in the abundance is shown.

agine (N), threonine (T), tyrosine (Y), and proline (P) were particularly enriched, while phenylalanine (F), aspartic acid (D), and arginine (R) were significantly depleted (Fig. 2a,b). However, when considered individually, the hydrophobic group of peptides was noticeably enriched in tryptophan and leucine as well as valine and alanine.

The five-residue spacer linking the variable septapeptides to the mature pIII protein con-

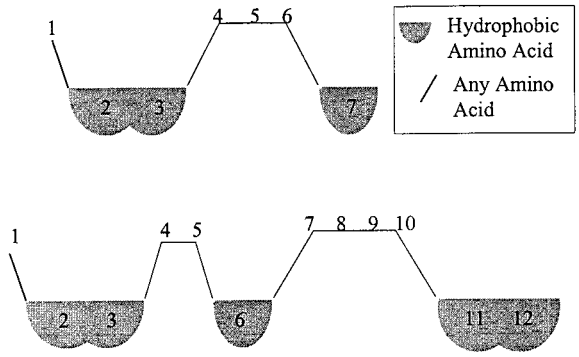

Fig. 3. Schematic model of the peptide-binding site of Hsp47. The peptide backbone is shown as an extended chain. The side chains of two adjacent residues extend into deep pockets in the peptide-binding site that have overall preferences for large hydrophobic or aromatic side chains. The data indicate that amino acids at position 2 and 3 in septa peptides and 2 and 3 and/or 11 and 12 make favorable contacts with Hsp47 side chains. The hydrophobic pockets are flanked by regions that containing charged residues at position 4,5,6 in septapeptides and positions 4 and 5 and 7,8,9, and 10 in dodecapeptides.

tained no enriched residues, thus, indicating that the spacer residues are unlikely to contribute to the binding activity of selected bacteriophages, allowing us to look only within the variable septapeptide sequences for the presence of a binding motif. Inspection of the selected hydrophobic peptides revealed that a residue motif is best described as XHyHyXXX-HyHy, where Hy is a large hydrophobic amino acid (usually W, L, or F) and X is any amino acid. This core motif is also identified in a selected residue from a Ph.D-12 library represented by a motif XHyHyXXHyXXXXHyHy. Taken together these data suggest that the peptide-binding site contains a deep hydrophobic pocket separated by charged hydrophilic residues (Fig. 3). The peptide motif described as XXHyHyXXX best described the selected hydrophilic peptides selected from the library. Interestingly, a similar core motif (HyXXXHy-HyXXHyXXX) could also be identified in selected resides from the dodecapeptide library.

Recognizing that procollagen I was a natural ligand for Hsp47, BLAST program analyses were performed to assess the sequence homology between bacteriophage-displayed peptides and procollagen I and the selected dodecapeptides. Interestingly, little specific homology was observed based on sequence alone. However, when the hydropathic profile of procollagen I (1) was compared with the dodecapeptides obtained from three rounds of panning,

using Kyte-Doolittle [1982] method of calculating hydrophilicity over a window length of 7, all of the phage displayed peptides were represented by specific regions within the procollagen molecule. Interestingly, most of the hydrophobic peptides were localized to regions within the *N*-propeptide region (residues 59–71) or the *C*-propeptide region (residues 1344–1445). Conversely, the most of the hydrophilic peptides were localized to the regions with the helical region of procollagen (residues: 283–295, 470–482, 666–678, 727–739, 1040–1052, and 1087–1099) with only one peptide localized to a sequence within the *N*-propeptide region (residues 100–112) (Fig. 4).

To gain insight into the subcellular localization of Hsp47-binding phage (HBP) on tumor cells, we incubated 25 μl of HBP and a similar sample of unselected phage from the library with 10^6 cells (SCC4, SCC9,SCC15, SCC25, HTB126, HTB127, PC-3, and LNCaP) in media for 1 h at 37°C. The cells were then washed 3 times with TBS-Tween and the bacteriophage stained with FITC-anti-M13 antibodies and analyzed by flow cytometry. These studies revealed that non-permeabilized tumor cell lines (SCC4, SCC9, SCC15, SCC25, HTB126, and PC-3) possessed varying levels of M13 phage staining on their cell surfaces. Furthermore, if the cells were permeabilzed prior to the addition of antibody, then cell surface staining coupled with internalized phage revealed enhanced staining. However, GMSM-K, an established epithelial cell line, treated in a like manner, revealed little or no staining. Figure 5 depicts a representative tumor cell line, SCC4 cells, compared to GMSM-K cells. Results similar to those obtained for GMSM-K cells were also obtained for HTB125 and PZ-HPV normal beast and prostate cell lines, respectively. Furthermore, when samples of unselected bacteriophage from the random library were used in place of HBP, these samples showed little (3.0–5.0%) or no staining in any of the cell lines examined. The presence of Hsp47 cell surface staining was likewise absent from GMSM-K, HTB, and PZ-HPV cells (not shown).

To verify the association of M13-binding phage with specific cell lines, ^{35}S-methionine labeled SCC4 and GMSM-K cells were treated with an HBP for 1 h, then washed 3 times with TBS-Tween and the plasma membranes were isolated and immunoprecipitated with anti-M13 antibodies. Following, autoradiography two protein bands were identified, one band with a Mr = 47k and another band with a Mr = 27k (Fig. 6).

Confocal microscopy was also employed to determine the fate and co-localization of HBP. These studies revealed that when tumor cell lines were treated with HBP, there was a distribution of staining at the cell surface, in microvesicles and an intense staining in a perinuclear region that was coincident with the ER [Hebert et al., 1999]. Cells were then treated with M13 bacteriophage followed by double staining with Texas red-anti-Hsp47 antibodies and FITC-M13 antibodies. The localization of Hsp47 was similar to that of M13 phage staining within the same focal plane, and co-localization of antibodies revealed superimposition, yellow hues, of both M13 staining and Hsp47 staining patterns (Fig. 7). Cells treated with bacteriophage from an unselected random library showed no staining or uptake characteristic of the HBP (not shown).

Next, we determined whether HBP localized to the late endosomal/lysosomal compartment. To label the lysosomal compartment, cells were cultured in the presence of FITC-conjugated dextran, followed by a chase period of 30 min to remove the dextran from early endosomal compartments, before fixation and immunostaining with the Texas red conjugated anti-M13 antibodies. SSC4 cells, which were representative of the other cell lines, demonstrated a clear identification of FITC-dextran to vesicular structures. However, the HBP staining was primarily limited to punctate vesicles in the cytoplasm and a perinuclear zone (Fig. 8). To verify that HBP was not significantly targeted to lysosomes, SCC4 cells were fed latex beads and HBP and then fixed and processed by for immunofluorescence using anti-M13 antibodies. In SCC4 cells, M13 signal could not be located at the periphery of the bead, suggesting that there was minimal association with the phagosomes.

Since we had previously shown that Hsp47 was expressed on the cell surface we considered whether HBP bound to cell surface Hsp47 might be present in recycling endosomes. To label these compartments, SCC4 and GMSM-K cells were cultured in the presence of FITC-conjugated or Texas red-conjugated transferrin before fixation and staining with anti-M13 antibodies. Analysis by confocal microscopy indicated that transferrin stained both the plasma membrane (ring staining at the edge of cells) and recycling endosomes (subcellular punctate

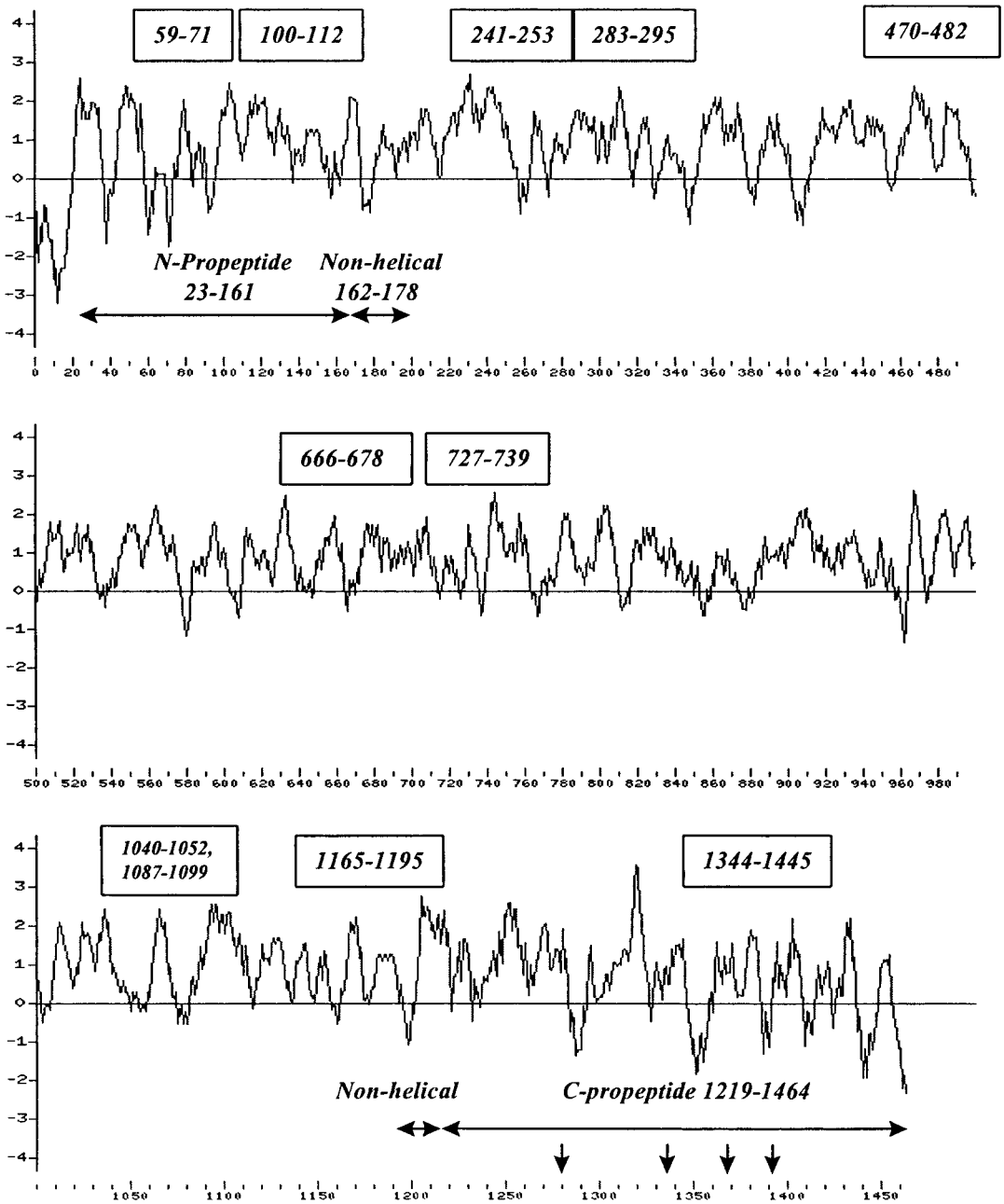

Fig. 4. The hydropathic profile of procollagen I compared with selected peptides from the PhD-12 library. Using the Kyte Doolittle [1982] method of calculating hydrophilicity over a window length of 7, selected phage from the PhD-12 library were compared with procollagen I alpha chains. The resides indicated in the boxes above the procollagen I profile indicate the residues similar to Hsp47 binding peptides selected from the PhD-12 library after three rounds of panning. Arrows at the C-propeptide regions indicate amino acids critical for the association and assembly of procollagen [Chessler, 1993a,b; Lees, 1994, 1997; Oliver, 1996]

staining). A very similar and overlapping pattern was observed for M13 antibody staining, consequently superimposition of the two images indicated colocalization (yellow hues) of the two signals at the punctate subcellular region and plasma membrane (Fig. 9). Noteworthy was that GMSM-K cells provided like patterns of staining with conjugated dextran and transferrin but were not stained by anti-M13 antibodies (not shown).

Based on the sequence of peptides isolated by panning, septapeptides and dodecapeptides

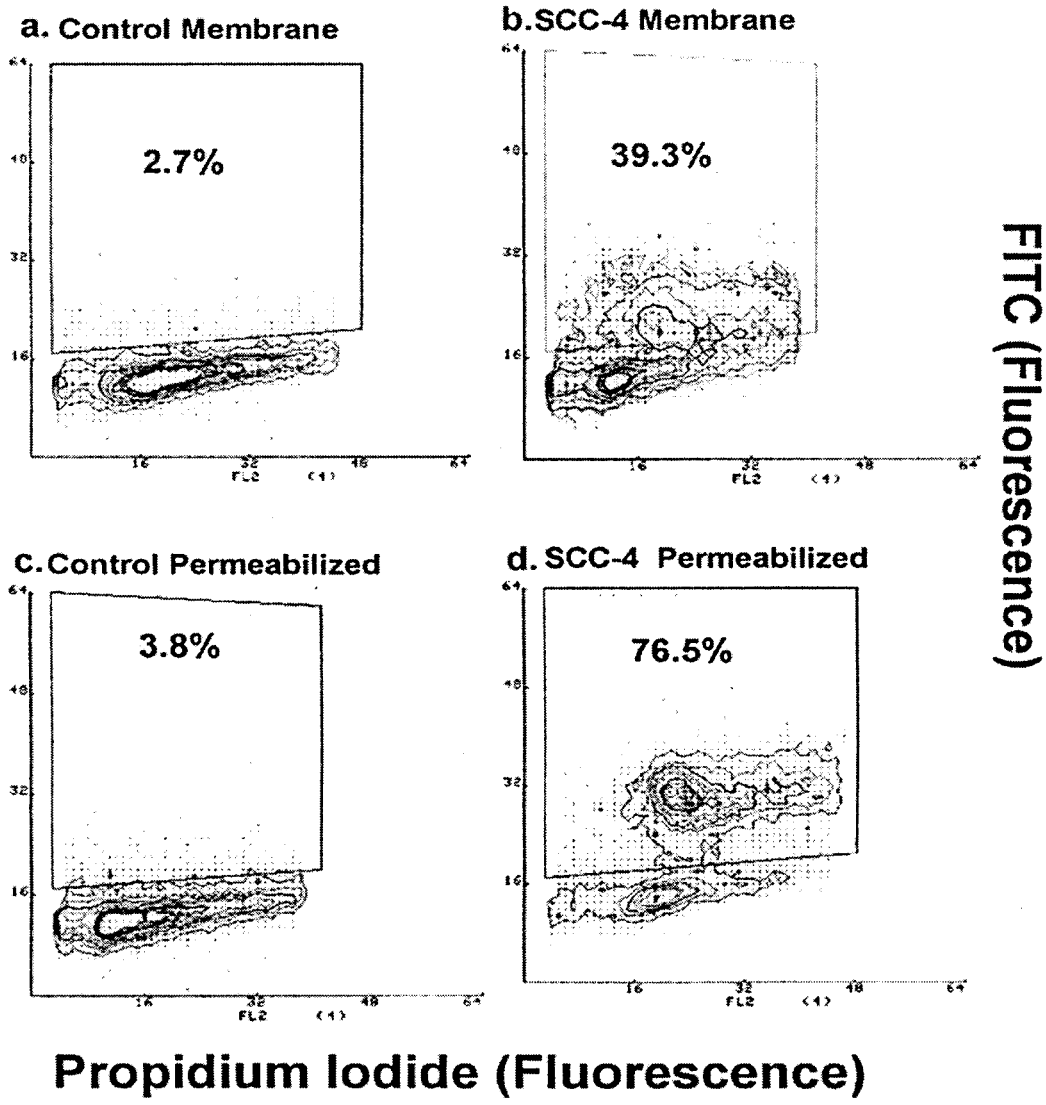

Fig. 5. FITC-antiphage M13 staining of GMSM-K (Control) and SCC-4 Cells. **a** and **b** represent control GMSM-K cells and SCC-4 cells respectively depicting membrane staining. **c** and **d** represent GMSM-K cells and SCC-4 cells, respectively, in which the cells have been permeabilized to demonstrate intracellular staining. The areas within the boxes represent FITC staining for phage-peptide.

consistent with the predicted models obtained from scoring were synthesized, labeled with FITC, and incubated with SCC cells that were shown to bind and take-up HBP. Although none of the cell lines took up the septapeptides, all of the synthesized dodecapeptides could be noted in the cytoplasm of tumor cells after 15 min of incubation (Fig. 10).

DISCUSSION

The use of random peptide libraries has been shown to be a valuable tool for identifying novel therapeutic molecules. The libraries represent an enormous number of peptide sequences displayed either on the virion surface of filamentous phage clones or on a solid phase synthetic support. Central to this strategy is the observation that peptides isolated by affinity selection from such libraries typically interact with biologically relevant domains of the target proteins [Smith and Scott, 1993; Kitamura et al., 1992; Burkhardt et al., 1991; Smith et al., 1995; Pasqualini et al., 1996; Pasqualini and Ruoslahti, 1996; Ruoslahti, 1996, 1997; Hutchcroft et al., 1992; Borst et al., 1993]. This strategy often results in a number

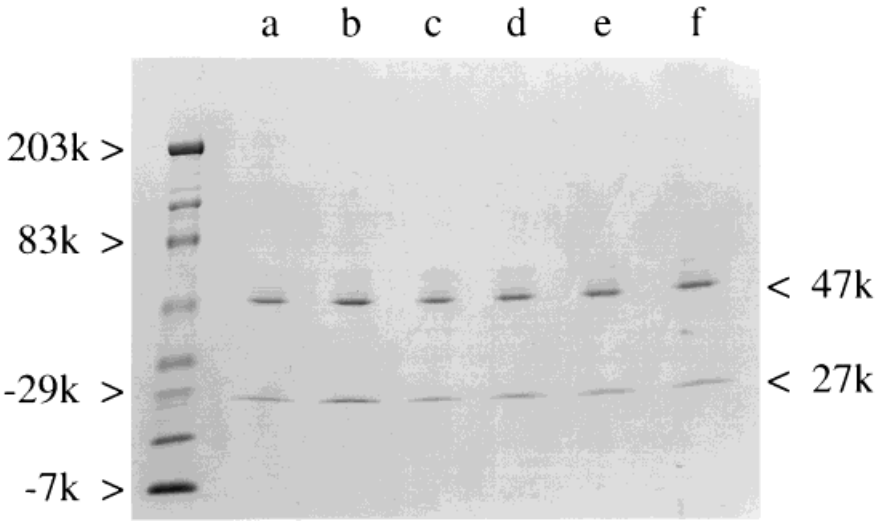

Fig. 6. Immunoprecipitation of membrane proteins by anti-phage M13 antibodies. Bands represent anti-M13 phage binding proteins immunoprecipitated as described in Materials and Methods. **Lanes a–f:** M13 clones selected from PhD-7 and Ph-D12 libraries. **Lanes a–c:** PhD-7 clones 3,5, and 7, respectively. **Lanes d–f:** PhD-12 clones 2, 5, and 9, respectively.

of peptides that apparently bind to a single domain of a protein or receptor. Curiously, the peptides often lack sequence similarity and are thus reminiscent of the discovery of mimotopes [Bowditch et al., 1996; Partidos et al., 1997], where short peptides bind to the antigen-binding site of antibodies even though they differ in sequence from the antigen. Clearly, this appears to be the case here, where myriads of peptides were localized from both libraries. The enrichment of valine and alanine as well as tryptophan and leucine in peptides displayed by this selected group of bactiophages is consistent with the affinity of Hsp47 to a region that is determined by its hydrophobic character. In addition, the demonstration of HBP binding by dodecapeptides with a motif of XXXXXHyXXHyXHyHy indicates that the hydrophobic domains of the ligand need not be located at the pIII terminus. Thus, our data suggest that these selected peptides describe a peptide binding site for Hsp47 that contains a long pocket that can accommodate the side chains of large hydrophobic and aromatic amino acids and that contain adjacent regions that can interact with charged residues. These conclusions are supported by three-dimensional structural modeling studies that reveal that mature Hsp47 possesses a binding region as a long, deep cleft, at physiologic pH, where the base is formed by a β-sheet with sides formed by helices. The helices project hydrophobic amino acid residue side chains in toward the cleft while β-sheet project hydrophobic amino acid residue side chains up from the bottom [Davids et al., 1995]. Moreover, this model explains the ability of Hsp47 to bind to hydrophobic regions within the *N*-propeptide as described previously [Hu et al., 1995] and likewise to the backbone of denatured collagen (gelatin) [Nagata et al., 1988] and $(Pro\text{-}Pro\text{-}Gly)_n$ collagen peptide analogs [Koide et al., 1999]. An interesting discovery was that regions within the C-propeptide region of procollagen might also provide a binding site for Hsp47, since carboxyterminal association of procollagen chains are implied in chain assembly [Chessler, 1993a,b; Lees, 1994, 1997; Oliver, 1996].

To gain further understanding into the nature and fate of Hsp47 displayed on the cell surface of cancer cells we localized M13-phage selected from the bacteriophage libraries to the cell surface of a number of oral cancer, breast, and prostate cell lines utilizing flow cytometric analyses. In so doing, we demonstrated that permeabilization of the cells prior to antibody staining enhanced the FACS signaling, suggesting that M13 was internalized into the cells. Immunoprecipitation of the plasma membranes of the tumor cells exposed to selected bacteriophages displaying Hsp47 binding peptides revealed that Hsp47 was immunoprecipitated with the M13 bacteriophage antibodies as well as another 27 kD protein, which may represent CD9 [Hebert et al., 1999], thus, substantiating that the selected HBP were associated with cell surface Hsp47 complexes. Confocal imaging was then employed to begin to localize the fate of the internalized selected

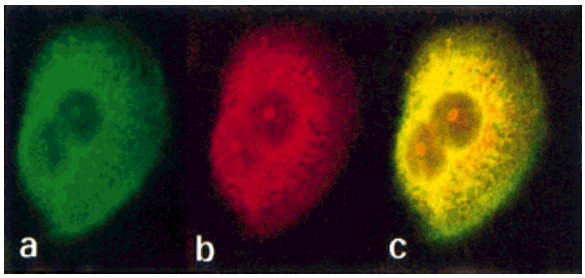

Fig. 7. Confocal microscopic images of SCC-4 cells stained with anti-Hsp47 and anti-M13 antibodies. **a:** Tumor cell stained with FITC conjugated anti-M13 antibodies. **b:** Texas red conjugated anti-Hsp47 antibodies. **c:** Spatial co-localization, yellow hues, of FITC and Texas red staining.

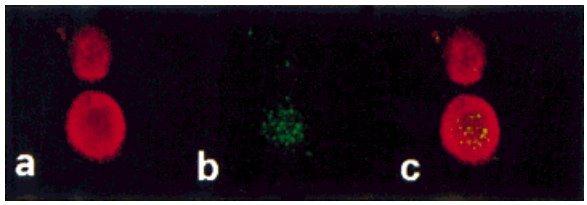

Fig. 8. Confocal microscopic images of SCC-4 cells stained with FITC-Dextran and Texas red-Anti-M13 antibodies. **a:** Tumor cell stained with FITC conjugated Dextran. **b:** Texas red conjugated anti-M13 antibodies. **c:** Spatial co-localization of FITC and Texas red staining. Only a minor portion of anti-M13 antibodies co-localizes with FITC-Dextran, yellow hues.

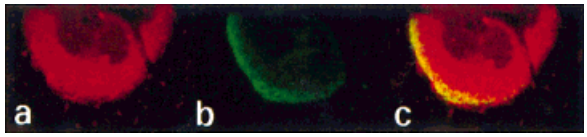

Fig. 9. Confocal microscopic images of SCC-4 cells stained with FITC-transferrin and Texas red-Anti-M13 antibodies. **a:** Tumor cells stained with FITC conjugated Transferrin. **b:** Texas red conjugated anti-M13 antibodies. **c:** Spatial co-localization, yellow hues, of FITC and Texas red staining.

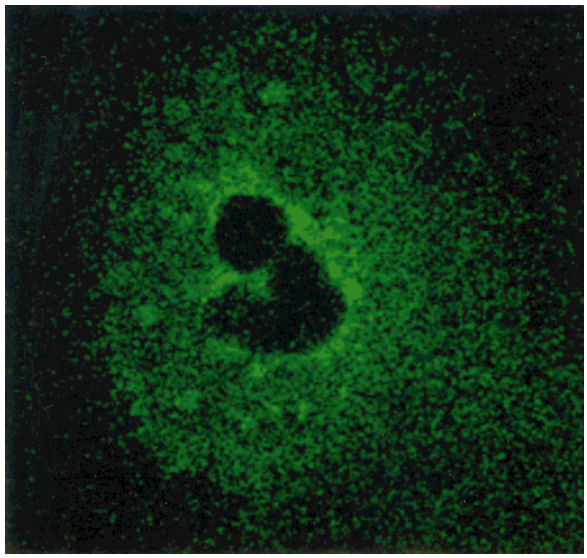

Fig. 10. Fluorescein isothiocyanate conjugated dodecapeptide uptake in SCC cells. Dodecapeptide corresponding to a cloned peptide was synthesized and conjugated to fluorescein isothiocyanate as described in Materials and Methods. The peptide was then incubated with SCC-4 cells for 30 min, washed, and processed for fluorescence microscopy. The green fluorescence depicted about the nuclear area and in punctate staining represents cellular distribution of the peptide uptake.

peptide displaying bacteriophage. These studies revealed that short-term incubation resulted in selected peptides being localized to the cell surface and recycling endosomes. This was not unexpected since membrane turnover of cell surface proteins and receptors normally follow such a course. However, surprisingly only a small amount of the internalized peptides could be identified in lysosomes distinguished by FITC-dextran staining. Antithetically, when the cells were stained for both M13 displayed HBP and Hsp47 there was a concurrent localization, yellow hues, of both antibodies to coincident intracellular locations represented by recirculating endosomes and the ER.

Collectively, these data indicate that unlike cell surface peptide hormone receptors that undergo internalization by endocytosis upon binding to ligands, which are then sorted in endosomes to lysosomes where they are presumably degraded [Gruenheid, 1999], cell surface ER proteins are routed with their ligands back to the ER with only a small amount of the ligand destined for lysosomal processing. Conceivably, the use of an ER protein with a binding capacity to a restricted number of ligands will permit targeting of drug conjugates or imaging compounds directly to tumor cells expressing these proteins. Such targeting offers the possibility of minimizing nonselective toxic effects. Moreover, one would expect that multidrug resistance would be less important in the case of cell surface ER-mediated transport of the drug across the cell membrane [Czerwinski, 1998]. Although these in vitro data indicate that a cell surface expressed Hsp47 contain elements that might be able to deliver a very selective tumor agent, elements that may have importance in determining this activity have yet to be tested in this model. These elements include the type of toxic moiety that

could deliver its effect in the ER and the necessary level of Hsp47 expressed on the cell surface to deliver drug and the ability to recycle for additional ligand binding and transport [Czerwinski, 1998]. Septapeptides displayed at the pIII terminus of bacteriophage were taken up by tumor cells while their synthetic homologues were not internalized, and this indicates that the use of such peptides would necessitate peptide linkers that are stable in the circulation, capable of displaying the peptides for binding and are yet able to be processed following internalization. Ongoing research in our laboratory is directed to resolving these issues and to the application of our findings to clinically relevant tumors.

ACKNOWLEDGMENTS

This work was supported by in part by a grant to J. Sauk from U.S.P.H.S., R01-DE12606.

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