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Cell Surface Colligin/Hsp47 Associates With Tetraspanin Protein CD9 in Epidermoid Carcinoma Cell Lines

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Abstract Hsps expressed on the cell surface have been associated with tumor invasiveness and used as targets for molecular surveillance. The present study utilized four human oral squamous cell carcinoma cells lines, SCC-4, SCC-9, SCC-15, SCC-25, the murine epidermoid carcinoma cell line LL/2, and primary cultures of human gingival fibroblasts to assess the cell surface expression of colligin/Hsp47, a proposed marker for malignancy. Immunoprecipitation studies following protein crosslinking revealed that Hsp47 was associated with a number of membrane proteins including the tetraspanin CD9. Cytometric analyses were performed to determine the distribution of cell surface colligin/Hsp47 during the phases of the cell cycle. These studies showed that colligin/Hsp47 was not limited to any phase of the cell cycle in epidermoid carcinoma cells. Boyden chamber tumor invasion assays and colloidal gold migration assays utilizing a reconstituted basement membrane (Matrigel®), collagen type I, and laminin-5 substrates revealed that cell lines expressing constitutive high levels of colligin/Hsp47 manifested the lowest invasion and migration indices. The incorporation of antibodies against Hsps into the migration and invasion assays, likewise, increased the invasion indices and the phagokinetic migration indices. These data indicate that colligin/Hsp47 is anchored to the cell membrane in a complex with CD9 where it moderates tumor cell invasion and motility possibly by acting as a serpin protein inhibitor or as a receptor for collagen. J. Cell. Biochem. 73:248–258, 1999.

Key words: Hsp47; CD9; tetraspanin; epidermoid carcinoma cells

Proteins that are residents of the endoplasmic reticulum contain a COOH-terminal sequence, which denotes that they intend to be retained in the ER [Lewis, 1992]. This management mechanism is not absolute and is acknowledged to be leaky [Takemoto et al., 1992]. Consequently, manifestations of ER resident proteins on cell surfaces, particularly of Hsps, have been suggested to represent the fortuitous adsorption of cellular components released from ruptured or damaged cells. However, the demonstration of the inability of living cells to absorb Hsps released by dead cells has dispelled this notion [Altmeyer et al., 1996]. Furthermore, this uncommon location of Hsps has been verified by flow cytometry, electron microscopy,

Abbreviations used: aa, amino acid; ATCC, American Type Culture Collection; BCECF-AM, 2′,7′-bis-[2-carboxyethyl-]-5-[6]-carboxyfluorescein-acetoxymethylester; BP, band pass; BSA, bovine serum albumin; DMEM, Dulbecco's modified Eagles medium; DSP, dithiobis(sulfo-succinimidyl propionate); DTSSP, 3′,3′-dithiobis(sulfo-succinimidyl propionate); EDTA, ethylenediaminetetraacetic acid; ER, endoplasmic reticulum; FACS, fluorescence activated cell sorter; FITC, fluorescein-isothiocyanate; FITC, fluorescein isothiocyanate; HEPES, N-2-hydroxyethpiperazine-N′-2-ethanesulfonic acid; Hsp, heat shock protein; Hsp47, heat shock protein 47; Hsp60, heat shock protein 60; Hsp70, heat shock protein 70; Hsp94, heat shock protein 94; I_g, immuno globulin; LL/2, Lewis Lung Carcinoma; LP, long pass; mAbs, monoclonal antibodies; NEM, N-ethylmaleimide; FDM, non-fat dry milk; NGS, normal goat serum; PAGE, polyacrylamide gel electrophoresis; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate buffered saline; pH_i, intracellular pH; PI, propidium iodide; SA, streptavidin; SDS, sodium dodecyl sulfate; TBS, tris-buffered saline; TM4SF, transmembrane 4 super family; TPCK, N-tosyl-L-leucine chromethyl ketone; TPCK, N-tosyl-L-phenylalanine chromethyl ketone.

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immunofluorescence, and subcellular membrane fractionation [Takemoto et al., 1992; Altmeyer et al., 1996; Ferrarini et al., 1992; Ullrich et al., 1986; Vanbuskirk et al., 1989; Freedman et al., 1992; Sauk et al., 1997]. However, the cell surface expression of Hsps is nevertheless regarded as anomalous, in that neither the mechanism for conveyance, nor docking of these proteins to the cell surface, is understood [Altmeyer et al., 1996]. In spite of the later, the surface expression of members of the Hsp70, Hsp90, and Hsp60 families has been associated with malignancy [Feige and van, 1996; Karopoulos et al., 1995; Kaufmann, 1994] or various autoimmune conditions [Albers et al., 1996]. As such, these proteins serve as potential targets of molecular surveillance and immunological recognition [Srivastava, 1993, 1997; Udono and Srivastava, 1993a, 1994b; Li and Srivastava, 1993].

Colligin/Hsp47, a heat inducible protein, is associated with procollagen chains at a very early point during translation of nascent chains [Sauk et al., 1994] and tenaciously binds to the pro α(l) amino-propeptide region as well as Gly-Xaa-Yaa sequences [Hu et al., 1995]. Analysis of the amino acid sequence deduced from cDNA reveals that colligin/Hsp47 also belongs to the serpin superfamily [Hirayoshi et al., 1991; Takechi et al., 1992; Jain et al., 1994]. Moreover, colligin/Hsp47 has been shown to be more effective than TPCK, TLCK, or leupeptin in inhibiting the proteolysis of procollagen chains [Jain et al., 1994]. As such, colligin/Hsp47 has been ascribed as a chaperone that is associated with procollagen from the ER to the *cis*-Golgi where it dissociates from its substrate and is recycled back to the ER [Takechi et al., 1992; Sauk et al., 1997]. To accomplish the later, colligin/Hsp47 binds to its receptor, erd2P, by its COOH-terminus RDEL peptide sequence. However, 3T6 mouse cells subjected to conditions of stress or lowering of pH_I will effect colligin/Hsp47 to elude its retention receptor to be expressed on the cell surface [Sauk et al., 1997].

The studies reported here reveal that colligin/Hsp47 is variably distributed on the cell surface of both human and murine epidermoid carcinoma cell lines. As such, colligin/Hsp47 is closely associated with the tetraspanin protein CD9. The relationship with CD9 provides a basis, distinct from integrins, for tumor cells to interact with extracellular matrices. In so doing, colligin/Hsp47 modulates tumor cell invasion, motility, and offers a possible target to which drugs or contrast agents may be directed for chemotherapy or imaging.

MATERIALS AND METHODS

Cell Lines and Cell Culture

Studies were performed using established cell lines of human oral squamous cell carcinomas [SCC-4, SCC-9, SCC-15, and SCC-25] and a murine epidermoid cell line, Lewis Lung Carcinoma (LL/2), obtained from ATCC. In addition, a primary gingival fibroblast cell line was used as a control for some studies [Sauk et al., 1997]. In all of the studies presented here, cells were cultured in a 1:1 mixture of Ham's F12 and Dulbecco's modified Eagles medium (DMEM) containing 10% fetal calf serum, hydrocortisone (0.4 μg/ml, Sigma Chemical Co., St. Louis, MO) at 37°C in a 5% CO_2 air atmosphere. Cells were subcultured by dissaggregation with trypsin (0.1%)-EDTA (0.01%) in phosphate buffered saline [PBS] at pH 7.5.

Antibodies

For these studies, the monoclonal antibody SPA-470 to colligin/Hsp47, (StressGen, Victoria, BC, Canada) and a colligin/Hsp47 rabbit polyclonal antibody, prepared against a 22-mer peptide corresponding to the N-terminal sequence of mouse colligin/Hsp47, were used [Sauk et al., 1994]. Also, antibodies to CD9 (KMC8) and antibodies to CD81 (2F7; PharMingen, San Diego, CA) were utilized. 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).

Cytometric Analyses

Cells grown in vitro, as described above, were washed and incubated in a 0.5% solution of Polyglobin N to block unsaturated Fc receptors and reduce nonspecific binding of monoclonal antibodies [Takeshita et al., 1995]. Next, 50 ml of the cell suspension (1×106 cell/ml) was incubated with 2.5 ml (1 mg) of antibodies, conjugated with fluorescein, or SA-Red670™ (GibcoBRL, Gaithersburg, MD). After washing, the cell pellet was resuspended in PBS contain-

ing BSA for flow cytometric assay. To assess intracellular colligin/Hsp47, 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 José, CA). The 488 nm Argon laser was run at 15 nW of power. The data from fluorescein conjugates were collected after a 530/30 BP filter. For two-color flow cytometric analysis either fluorescein or Red670™ were employed with propidium iodide. The filters used were 600 nm dichroic SP; 525 ± 15 nm BP (fluorescein) and 645 LP (Red670™).

Propidium iodide was used to assess cell cycle and stain for dead cells. For these studies a hypotonic citrate solution containing PI was added to $\sim 1 \times 10^6$ washed cells to a concentration of 1 mM. Cells were labeled for 20 min, then analyzed on the FACScan in their staining solution. Orange PI fluorescence was collected after a 585/42 nm BP filter.

Electronic compensation was used among fluorescence channels collecting emissions to remove residual spectral overlap. Fluorescence data were displayed on a 4-decade long scale. A minimum of 10,000 events was collected on each sample. Analysis of the data was performed with LYSYS II software (Becton Dickinson, Mansfield, MA). Fluorescence dual parameter contour plots were used for exclusion of debris and clumps. This method of gating allowed ready discrimination of debris from dead cells (low forward light scatter and high PI fluorescence). The percentage of cells with a $G_{1/0}$, S, of G_2/M DNA complement was determined from a DNA histogram by region integration using onboard Multicycle® data analysis routines (Phoenix Flow Systems, San Diego, CA).

Immunofluorescence Studies

Immunofluorescence microscopy was carried out after the method of Tang et al. [1993, 1994]. To visualize cell surface colligin/Hsp47, the cells were not permeabilized but treated and fixed with 1% paraformaldehyde as described for cytometric analyses. The cells were then stained with anti-colligin/Hsp47 antibodies as a primary antibody followed by FITC goat-anti-rabbit or anti-mouse IgG.

Subcellular Fractionation of Plasma Membranes

The method for fractionating plasma membranes was modified after the methods described by Weber et al. [1988]. In essence, after incubating 5 ml of cells ($2–5 \times 10^6$ cells per ml) with or without 1 mM amiloride, the cell suspension was centrifuged at 100*g* for 60 sec at room temperature. The cell pellets were suspended in 10 ml of tris/EDTA/sucrose buffer (20 mM Tris/HCl, 1 mM 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/1 mM EDTA buffer (pH 7.4), and centrifuged in a Beckman TLS55 rotor at 8,150*g* 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 410,000*g* 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 initial supernatant was centrifuged in a Sorvall SS34 rotor at 48,000*g* at 4°C for 15 min and the high-density microsome pellet was resuspended in 40 µl of buffer. The supernatant was further centrifuged in a Beckman 70.1 rotor at 300,000*g* at 4°C for 75 min and the low-density microsome pellet was resuspended in 60 µl of buffer.

The membrane fractions were characterized by the distribution of 5′-nucleosidase activity, a marker of plasma membrane [Avruch and Wallach, 1971]. Protein was measured with the BCA protein assay kit (Pierce). Plasma membranes were directly subjected to PAGE and Western analysis. For Western blots, proteins run on SDS-PAGE were immediately electrotransferred to nitrocellulose paper and blocked with 10% NFDM in 10 mM Tris-HCl pH 7.4, 0.9 mMNaCl (TBS) for 2 h and then in TBS/NFDM with 2% NGS (GIBCO, Grand Island, NY). Antiserum or perimmune serum was diluted 1:2,000 in the same buffer and incubated with gentle shaking overnight. The nitrocellulose was then rinsed three times for 5 min in TBS/Tween. Hsp47 was detected with [^{125}I]-labeled protein A (New England Nuclear, Boston, MA).

Cell surface labeling and immunoprecipitation. To demonstrate complexes between colligin/Hsp47 and TM4SF we used methods previously described for the characterization of TM4SF complexes with integrins [Berditchevski et al., 1996]. In essence, cells were labeled

with NHS-LC-biotin (Pierce) according to kit protocol, and lysed in immunoprecipitation buffer (1% Brij 96, 25 mM Hepes, pH 7.4, 150 mM NaCl, 5 mM $MgCl_2$, 2 mM PMSF, 20 µg/ml apotinin, and 10 µg/ml leupeptin). Immune complexes were collected on protein A beads prebound with antibodies, followed by four washes with immunoprecipitation buffer. For more "stringent" conditions, the immunoprecipitation buffer was supplemented with 0.2% SDS. Immune complexes were eluted from protein A beads with Laemmli elution buffer and proteins were separated by SDS-PAGE. Proteins were transferred to nitrocellulose membranes and visualized with HRPO-conjugated ExtrAvidin (Sigma Chemical Co.) using Renaissance Chemiluminescent Reagents (NEN, Cambridge, MA). Reprecipitations were preformed from Brij 96 lysates prepared from surface-biotinylated SCC cells. After five washes with the immunoprecipitation buffer, the protein complexes were dissociated with 0.5% SDS added to the immunoprecipitation buffer. The eluates were subsequently diluted 1:1 with immunoprecipitation buffer and re-precipitated with the appropriate antibodies directly coupled to Sepharose beads. The samples were then processed as described above.

For crosslinking cells were treated with DTSSP, a membrane impermeable crosslinker or DSP. After solubilization in immunoprecipitation buffer supplemented with 0.2% SDS protein complexes were immunoprecipitated as above and analyzed under reducing conditions.

Binding of cell surface Hsp47 to $_{\alpha}$1 (I)-N-propeptide. The cDNA sequences encoding the pro α1(I) N-propeptide globular domain (NP1; residues 23–108), and the globular domain + propeptide GlyXaaYaa domain (NP2; residues 23–151) were prepared as GST-fusion proteins as previously described by us [Hu et al., 1995]. Fusion protein expression was induced by 0.1 mM IPTG after bacteria reached midlog phase. The fusion proteins were purified by glutathionine-Sepharose 4B beads (Pharmacia, Piscataway, NJ) according the manufacturer's instructions. The proteins were characterized by SDS-PAGE and Western blot analysis as previously described [Hu et al., 1995].

NP1 and NP2 affinity beads were prepared after the method of Hu et al. [1995]. In essence, GST-fusion proteins were treated with thrombin, dialyzed, and the GST protein removed from each reaction mixture by passing it over a column of glutathione-Sepharose. The eluates were collected and lyophilized and coupled to CNBr activated Sepharose. The final beads contained 1–2 mg peptide per 250 µl. Hsp47 surface binding experiments were carried out by mixing 250 µl of a 50% (v/v) suspension of peptide-Sepharose (Pharmacia), with a suspension of plasma membranes from surface-biotinylated SCC cells. After incubation at 4°C for 1 h the beads were collected by centrifugation and washed three times with an equal volume of Laemmli Buffer. The beads were extracted with 250 µl Laemmli electrophoresis sample buffer by boiling the sample for 5 min. Following separation by SDS-PAGE, the proteins were transferred to nitrocellulose membranes and visualized with HRPO-conjugated ExtrAvidin (Sigma Chemical Co.) using Renaissance Chemiluminescent Reagents (NEN).

To demonstrate the availability of cell surface colligin/Hsp47 to bind procollagen propeptides in living cells, GST-fusion proteins (1 µg/ml) were added to the culture medium of $\sim 1 \times 10^6$ SCC cells, plated in chambered slides (Nalgene NUNC, Milwaukee, WI), for 10 minutes at 37°C. The cells were then washed in PBS fixed in paraformaldehyde as before, and cell associated fusion proteins (NP1, NP2) were identified with HRPO-conjugated anti-GST antibodies (Sauk et al., 1994).

Colloidal Gold Migration Assay

Tumor cells were plated on chamber slides precoated with a mixture of 80 µg/ml type I collagen, 100 µg/ml Matrigel®, or 100 µg of laminin-5, and colloidal gold particles and incubated in medium with or without various antibodies. Colloidal gold-coated chamber slides were prepared as described by Albrecht-Buehler [1977] with modification for keratinocytes and the inclusion of matrix proteins [Woodley et al., 1988; Kim et al., 1994a, b]. SCC or LL/2 cells were added to each chamber, and 20 min later nonadherent cells were removed and the medium replaced. Cultures were maintained for 24 h and then fixed in 1× Histochoice (Amresco, Solon, OH) for 1 min, washed in PBS, and dehydrated through graded ethanols. Areas devoid of gold particles identified the phagokinetic tracks. A migration index was determined using image analysis software by measuring the area of phagokinetic tracks associated with cells in random fields under dark field illumination at 100× [Pilcher et al., 1997]. All cells in a

field were counted and 25 cells were counted for each experiment. For each experiment, all conditions were done in triplicate.

Tumor Cell Invasion Assays

To assess tumor cell invasion an in vitro assay and 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) 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 ($P \leq 0.05$). The association of mean HSP47 fluorescence labeling with the invasion index was evaluated using the nonparametric Spearman's rank-order correlation coefficient (rho).

RESULTS

Expression of Colligin/Hsp47 at Surface of Oral Epidermoid Carcinoma Cells

Flow-cytometric analysis of SCC cell lines, the LL/2 murine cell line, and human gingival fibroblasts revealed that all of the cell lines possessed intracellular colligin/Hsp47 (Fig. 1, Table I). Cell cycle analysis further revealed that colligin/Hsp47 expression was not limited to any phase of the cell cycle in either gingival fibroblasts or epidermoid carcinoma cells (Table I). However, when cell cycle analysis was performed only for cell surface expression, colligin/Hsp47 was only limited to epidermoid carcinoma cell lines. Furthermore, there was variance in both the percentage and fluorescence intensity of cells expressing colligin/Hsp47 on cell surfaces in all of the carcinoma cell lines (Fig. 2, Table I).

Previously, we had shown that treatment with amiloride forced the surface expression of colligin/Hsp47 on mouse 3T6 cells [Sauk et al., 1997]. Consequently, tumor cells were treated with 1 mM amiloride and analyzed by flow cytometry. This resulted in amiloride dramatically increasing the anti-colligin/Hsp47 staining of the cell surface. Immunofluorescence microscopy of nonpermeabilized cells, prepared for flow-cytometric analysis, confirmed anti-colligin/Hsp47 staining and the enhancement following treatment with 1 mM amiloride (Fig. 3A–D). Likewise, subcellular membrane fractionation of amiloride and control cells to obtain a plasma membrane enriched fraction, followed by Western blot analysis confirmed the later by colligin/Hsp47 staining in the plasma membrane 5′-nucleosidase fraction (Fig. 3E).

Cell Surface Labeling and Immunoprecipitation

In studying the possible mechanism for anchoring colligin/Hsp47 to the cell membrane we found that other membrane surface proteins were consistently co-precipitated with anti-colligin/Hsp47 antibodies. Recognizing that tetraspanins were associated with a number of integrin receptors [Berditchevski et al., 1996], we sought to determine whether Hsp47 was localized to similar membrane complexes. To explore this possibility, we performed cross-linking experiments. For these studies, SCC and LL/2 cells were first pretreated with a cleavable cross-linker, DSP, then surface labeled with biotin or [^{125}]-I, and subsequent immunoprecipitation was carried out under stringent conditions to disrupt the noncovalent association between Hsp and the tetraspanin protein. A colligin/Hsp47-CD9 complex was immunoprecipitated using either anti-CD9 mAbs or anti-colligin/Hsp47 antibodies. A characteristic 47K band was readily detected in all anti-CD9 immunoprecipitates and protein band of

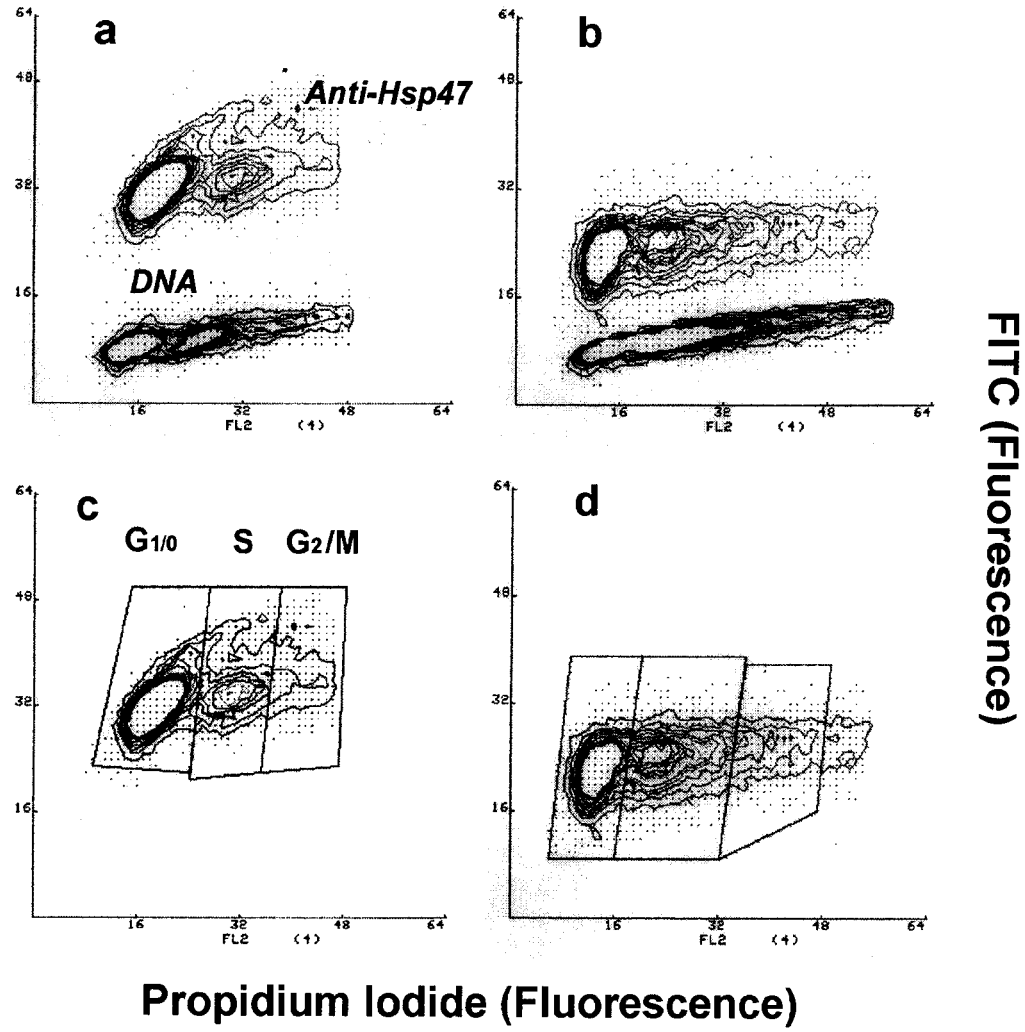

Fig. 1. General method of cell-cycle fractionation using DNA and Hsp47 analysis with Propidium Iodide and FITC-anti-Hsp47. Dual-parameter dot plot showing analysis of human gingival fibroblasts **(a)** and SCC cells **(b)** stained with Propidium Iodide (lower array) and FITC-anti-Hsp47 (upper array). Cell cycle analysis of Hsp47 staining of human gingival fibroblasts **(c)** and SCC cells **(d)** at various phases based on DNA distribution (see Materials and Methods).

TABLE I. Percent Distribution of Cells in Cell Cycle Phases (*cc*), Percent Distribution Total Cellular Staining (*tc*), and Cell Surface Staining (*cs*) of Colligin/Hsp47 Among Epidermoid Carcinoma Cell Lines[a]

	$G_{1/0}$			S			G_2/M		
Cell line	*cc*	*tc*	*cs*	*cc*	*tc*	*cs*	*cc*	*tc*	*cs*
SCC4	49 ± 9	51 ± 12	13 ± 8	23 ± 10	22 ± 11	15 ± 5	28 ± 8	26 ± 9	7 ± 2
SCC9	39 ± 5	40 ± 9	35 ± 5	47 ± 8	47 ± 9	30 ± 8	13 ± 4	13 ± 5	12 ± 2
SCC15	54 ± 9	55 ± 11	14 ± 2	40 ± 9	40 ± 8	13 ± 1	6 ± 1	6 ± 2	3 ± 1
SCC25	47 ± 11	49 ± 13	40 ± 7	48 ± 8	45 ± 6	33 ± 7	5 ± 2	5 ± 2	6 ± 6
LL/2	54 ± 10	56 ± 12	11 ± 9	33 ± 8	32 ± 11	15 ± 11	13 ± 7	12 ± 6	9 ± 3
GF	74 ± 2	73 ± 3	0 ± 0	20 ± 2	21 ± 2	0 ± 0	6 ± 2	5 ± 4	0 ± 0

[a]Data are the mean percent for three determinations ± SEM.

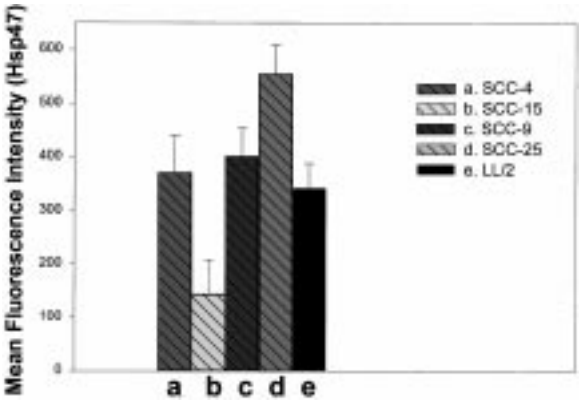

Fig. 2. Mean fluorescence intensity of cell surface anti-Hsp47 obtained by FACS analyses of unpermeabilized SCC and LL/2 cells stained with FITC-anti-Hsp47. Data represent the mean ± SEM of three determinations. Significant differences in mean intensity (Ps ≤ 0.05) were found between cell lines SSC-15 versus SCC-9 and SCC-25), although there was a trend towards a lower mean fluorescence for SCC-15 compared to SCC-4 and LL/2 these differences were not significant.

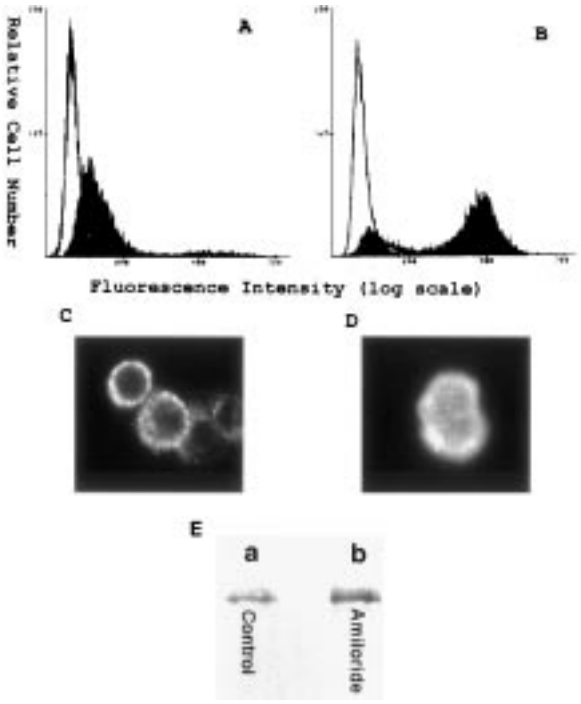

Fig. 3. **A,B:** FACS analyses of Hsp47 expression in SCC cells. The histogram containing the uncolored zone represents unstained cells, the histograms designated by the darkened zones are cells stained with FITC-anti-Hsp47. A represents control cells, B represents cells treated with 1 mM amiloride. **C,D:** Unpermeabilized cells from FACS analysis stained with anti-Hsp47. C represents control cells; D depicts amiloride treated cells. **E:** A Western blot of plasma membrane fraction revealing anti-Hsp47 staining in untreated control **(a)** and amiloride **(b)** treated cells.

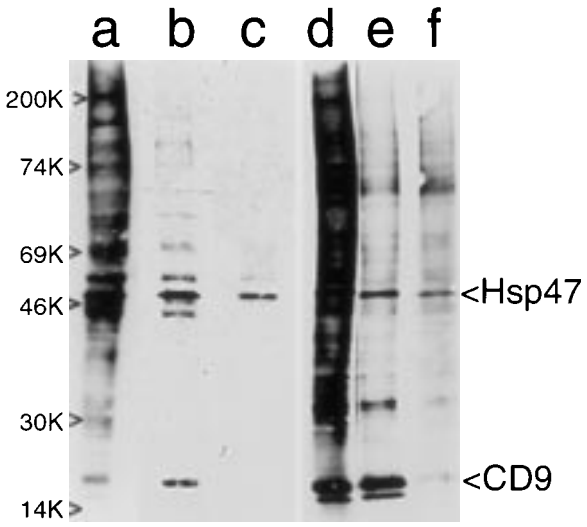

Fig. 4. Coimmunoprecipitation of surface proteins with CD9 and Hsp47. SCC-9 cells were labeled with NHS-LC-biotin (lanes a–c), and lysed in immunoprecipitation buffer. Immune complexes were collected on protein A beads prebound with anti-CD9 antibodies, followed washes with immunoprecipitation buffer supplemented with 0.2% SDS. Immune complexes were eluted from protein A beads and proteins were separated by SDS-PAGE. Proteins were transferred to nitrocellulose membranes and visualized with HRPO-conjugated ExtrAvidin (Sigma Chemical Co., St. Louis, MO) using Renaissance Chemiluminescent Reagents. Reprecipitations with anti-Hsp47 were performed from Brij 96 lysates prepared from surface-biotinylated SCC or LL/2 cells. For cross-linking (lanes d–f), cells were treated with DTSSP. After solubilization in immunoprecipitation buffer supplemented with 0.2% SDS, protein complexes were immunoprecipitated as above and analyzed under reducing conditions. **a:** Cell surface homogenate; **b:** CD9 immunoprecipitate; **c:** Hsp47 reimmunoprecipitate of b. Following crosslinking the lanes were **(d)** cell surface homogenate; **e:** CD9 immunoprecipitate; **f:** anti-Hsp47 reimmunoprecipitate of e.

~22K, similar in size to CD9/or CD81, were coprecipitated with anti-colligin/Hsp47 (Fig. 4). However, when similar experiments were performed with anti-CD81 mAbs, colligin/Hsp47 was not identified in the immunoprecipitates. However, reimmunoprecipitation of the CD9 immunoprecipitates with anti-colligin/Hsp47 resulted in a 47 K band in all SCC cells and LL/2 cell lines. To verify whether extracellular regions of Hsp47 and CD9 interact directly, we preformed similar crosslinking experiments using DTSSP, a membrane impermeable crosslinker. Treatment of intact SCC and LL/2 cells with either DSP or DTSSP resulted in coimmunoprecipitation of CD9 and colligin/Hsp47. Although these results were obtained in untreated cells, treatment with 1 mM amiloride greatly enhanced the amount of colligin/Hsp47 recovered following immunoprecipitation with-

out altering the profile of proteins precipitated (not shown).

Binding of Cell Surface Colligin/Hsp47 to $_{\alpha}$1 (I)-N-Propeptide

Binding experiments performed with biotin labeled plasma membranes and Sepharose bound pro α1(1) globular domain (residues 23–108) or pro α1(1) globular domain + propeptide GlyXaaYaa domain (residues 23–151) gave similar results. In both instances bands migrating at 46K and 47K were identified. Western blot analysis confirmed that both the 46K, unglycosylated colligin/Hsp47 [Hirayoshi et al., 1991], and 47 K bands reacted with anti-Hsp47 antibodies (Fig. 5). These findings were consistent for all of the cell lines.

Immunoperoxide cytochemical staining of GST-fusion proteins bound to cells in culture revealed that cells lines with a higher percentage of cells expressing surface colligin/Hsp47 displayed a higher percentage of bound GST-NP1 (Fig. 6) and GST-NP2 (not shown) fusion proteins.

Tumor Cell Invasion and Phagokinesis

Using modified Boyden chambers to assess tumor cell invasion revealed that SCC cells and LL/2 cells showed a significant variance in the invasion indices among the cell lines (Fig. 7). This variance was associated with the level of colligin/Hsp47 expressed on the cell surface

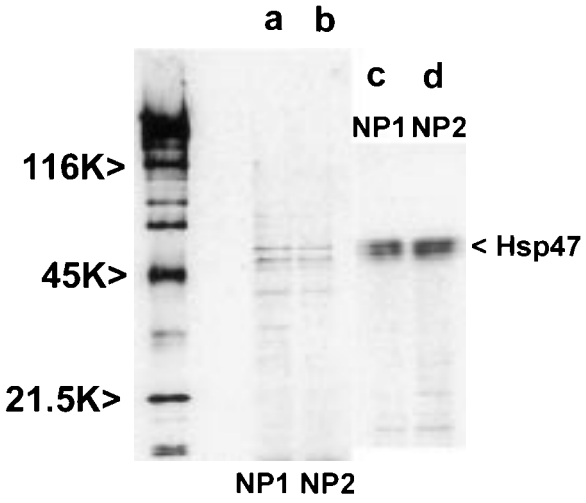

Fig. 5. Binding of GST-fusion proteins NP1 and NP2 to the cell surface of SCC-25 cells. **a,b:** Immunoprecipitated GST-fusion peptides bound to biotinylated cell membranes stained with Avidin-HRP, **c,d:** Western blots of lanes a and b using anti-Hsp47 antibodies. Similar results were obtained with SCC-4, SCC-9, and SCC-15 cells.

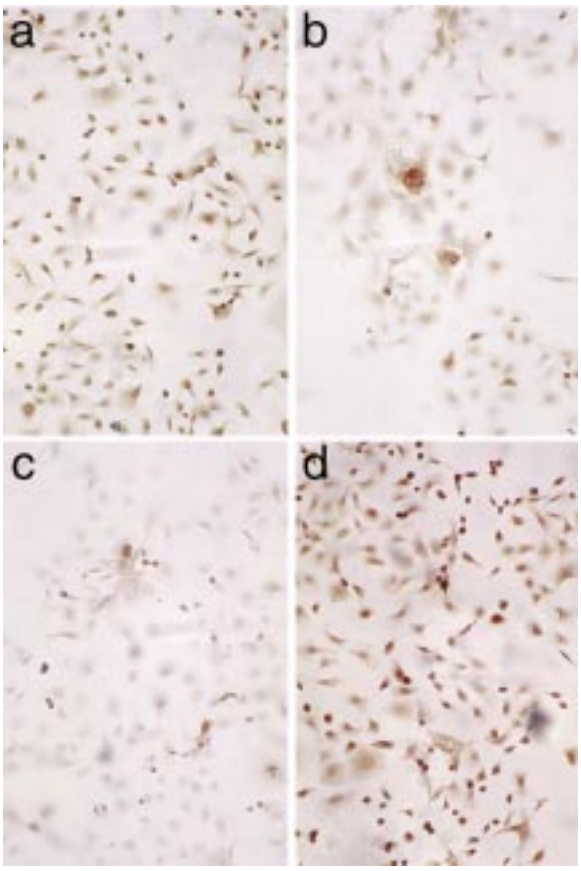

Fig. 6. Comparison of GST-NP1 fusion peptide binding to SCC cell lines. **a:** SCC-4; **b:** SCC-9; **c:** SCC-15; **d:** SCC-25 cells stained with immunoperoxidase utilizing HRP-conjugated anti-GST antibodies (see Materials and Methods).

(Figs. 2, 7). Chamber assays were then performed where the cells were incubated in the presence of antibodies directed to CD9, CD81, and colligin/Hsp47. Cells incubated in Matrigel® chambers with anti-colligin/Hsp47 resulted in an increase in the invasion index, while incubation with anti-CD9 or anti-CD81 antibodies were without affect and similar to controls (Fig. 8). Treatment with amiloride in each instant dramatically decreased the invasion index in all tumor cell lines. However, amiloride treated cells were unaffected by treatment with anti-colligin/Hsp47 antibodies.

The results of SCC cell migration on colloidal gold assays paralleled the results obtained from Boyden chamber assays but was dependent upon whether the colloidal gold contained Matrigel®, collagen, or laminin-5. The phagokinetic migration index of SCC cells was greatest on laminin-5 followed by collagen and Matrigel® (Fig. 9). In particular, the migration tracks were broader and longer on laminin-5 coated

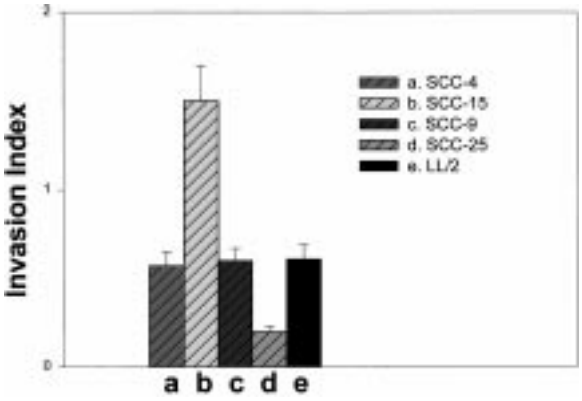

Fig. 7. Comparative Invasion Indices for SCC and LL/2 cell lines utilizing modified Boyden chamber assay. Data represent the mean ± SEM of three determinations. The mean Invasion Index for SCC-15 was significantly higher than for all other cell lines ($P \leq 0.05$). For SCC-25, the mean Invasion Index was notably lower than for SCC-4, SCC-9, and LL/2, although the difference was not statistically significant. Mean Hsp47 fluorescence was negatively correlated with the Invasion Index across cell lines (Spearman's $P = -0.90$, $P \leq 0.05$).

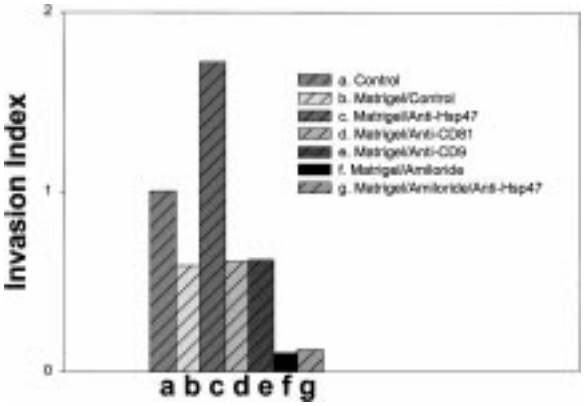

Fig. 8. Comparative effect of anti-CD9, anti-CD81, and anti-Hsp47 antibodies on the Invasion Index utilizing the modified Boyden chamber assay. Representative data are reported for SCC-9 cells. Cells grown on collagen in the presence of anti-Hsp47 demonstrated significantly greater invasion indices than cells under other culture conditions ($P \leq 0.05$). In contrast, cells grown on Matrigel in the presence of amiloride, with or without anti-Hsp47, exhibited similar but significantly lower mean Invasion Indices than cells under other culture conditions ($P \leq 0.05$).

colloidal gold than those observed on collagen or Matrigel®. The phagokinetic migration indices on both collagen and Matrigel® matrices were noted to increase following treatment with anti-colligin/Hsp47 antibodies, but was unaffected following treatment with anti-CD9 antibodies (not shown). The phagokinetic indices were unaffected following treatment with anti-colligin/Hsp47 on laminin-5 coated surfaces (Fig. 9).

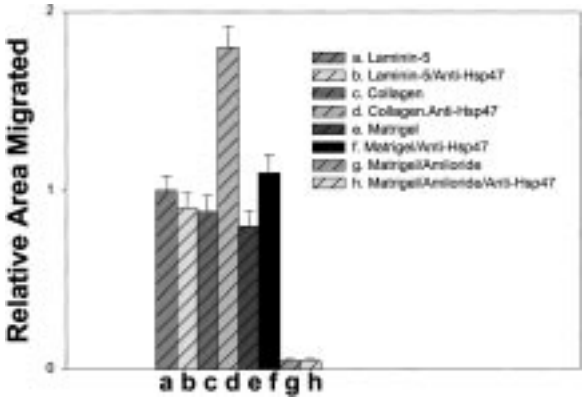

Fig. 9. Comparative effects of anti-Hsp47 and amiloride on the phagokinetic index for tumor cells plated on Laminin-5, Collagen, and Matrigel™. Representative data are reported for SCC-9 cells. Cells grown on collagen in the presence of anti-Hsp47 demonstrated significantly greater migration than cells under other culture conditions ($P \leq 0.05$). In contrast, cells grown on Matrigel in the presence of amiloride, with or without anti-Hsp47, exhibited similar but significantly lower mean areas of migration than cells under other culture conditions ($P \leq 0.05$).

DISCUSSION

The data reported here provide additional support for true expression, as opposed to fortuitous binding, of colligin/Hsp47 to the cell surface of epidermoid carcinoma cells. Although previous studies have demonstrated that Hsps such as gp96 are tightly surface-bound peripheral membrane proteins. The precise mechanism of anchorage was unclear; nevertheless, ionic interactions with other proteins could be excluded [Altmeyer et al., 1996]. Since colligin/Hsp47, like gp96, lacks sequence characteristics for farnesylation, palmitation, isoprenyllation, or myristylation, covalent bonding also appears to be an unlikely anchoring mechanism [Altmeyer et al., 1996; Clarke and Sanwal, 1992]. The association of colligin/Hsp47 shown here, with at least one member of the tetraspanin family, CD9, provides a membrane anchor for this Hsp. However, the mechanism for anchoring remains an uncertainty. The basic structure of tetraspanins include the presence of four hydrophobic, putative transmembrane domains, forming a small and a large extracellular loop which have been shown by epitope mapping and glycosylation to be hydrophilic [Hemler et al., 1996; Berditchevski et al., 1996]. In spite of the latter, colligin/Hsp47 was easily recovered, even without the use of crosslinking, from anti-CD9 membrane immunoprecipitates, suggesting the presence of hydrophobic interactions, albeit extracellular, be-

tween these proteins. This relationship in some ways parallels that between CD9 and some β_1 integrins in that deletion or exchange of integrin α chain cytoplasmic and transmembrane domains has been shown to not cause a loss of interaction with TM4SF proteins, suggesting that specificity was determined extracellularly [Hemler et al., 1996].

The ability of plasma membrane associated colligin/Hsp47 to bind NP1 and NP2 procollagen peptides indicates that this procollagen binding domain of Hsp47 is available to interact with procollagen peptides when colligin/Hsp47 is expressed on the cell membrane. Nonetheless, the recognized negative effects of CD9 on tumor cell motility and metastasis in a number of subsets of cancers raises the possibility that CD9 rather than colligin/Hsp47 might be responsible for modulating SCC and LL/2 cell behavior [Hemler et al., 1996]. This modulation of tumor cell behavior has been attributed to the relationship between CD9 and integrins. Accordingly, transfection of CD9 into poorly motile cells has been shown to enhance migration, while transfection into motile cells downregulated their motility [Maecker et al., 1997]. However, both in the Boyden chamber invasion assay and the colloidal gold migration assays antibodies directed to the tetraspanins CD9 and CD81 were without significant effect in either assay. Conversely, inhibitory antibodies to colligin/Hsp47 were shown to enhance tumor cell invasion and phagokinetic cell motility on Matrigel® and collagen. In that anti-colligin/Hsp47 antibodies were only effective on collagen containing matrices and were ineffective on laminin-5, indicates that the effects of colligin/Hsp47 were likely associated with collagen interactions.

The effect of limiting tumor invasion and motility, when expressed on the cell surface may stem from colligin/Hsp47 acting as a classical serpin. The basis for considering colligin/Hsp47 such resides in the homology colligin/Hsp47 shares with other serpins within the P_1-P_1' positions in the reactive site loop (RSL) region, and the downstream region from the RSL [Clarke and Sanwal, 1992]. Noteworthy, however, is that the "hinge region" upstream (P_1-P_{15}) varies from most inhibitory serpins and may preclude colligin/Hsp47 from acting as a pseudo-substrate for the target protease. If such is the case, then alternatively we anticipate that colligin/Hsp47 may function in a manner similar to maspin, a serpin family tumor suppressor, and bind elements, e.g., collagen, and act as a cellular anchor in regulating cell motility [Sager et al., 1996].

The cell surface display of colligin/Hsp47 among cell lines of SCC and LL/2 parallels the cell surface expression of Hsp70 and gp96 observed in other cancer cell lines and malignancies [Takemoto et al., 1992; Altmeyer et al., 1996; Ferrarini et al., 1992; Ullrich et al., 1986; Vanbuskirk et al., 1989; Freedman et al., 1992]. The high level of expression in cells with low invasion and motility indices may account for colligin/Hsp47 being regarded as a marker for extended survival for a number of malignancies [Morino et al., 1994, 1995, 1997a, b; Shirakami et al., 1995]. The unique cell surface expression of colligin/Hsp47 among epidermoid carcinoma cell lines and the specific peptide binding property on living cells, demonstrated here with GST-fusion proteins, also raises the possibility that colligin/Hsp47 may have utility as a therapeutic or imaging target in patients with minimal residual disease.

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