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Surface Expression and CEA Binding of hnRNP M4 Protein in HT29 Colon Cancer Cells

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Abstract. *Carcinoembryonic antigen (CEA) has been shown to participate in the progression and metastatic growth of colorectal cancer. However, its biological function remains elusive. Recently, we found that CEA protects colon cancer cells from undergoing apoptosis, suggesting a complex role that includes signal transduction activity. Additionally, it was reported that CEA binds to Kupffer cells and macrophages to a membrane-anchored homolog of heterogeneous nuclear protein M4 (hnRNP M4), which subsequently was named CEA-receptor (CEAR). Cytoplasmatic and membranous expression of CEAR in CEA-positive colon cancer tissues prompted us to analyze the CEA-CEAR interaction in HT29 colon cancer cells. Both, CEA and CEAR were found on the cell surface of HT29 cells, as demonstrated by confocal microscopy. Imaging analysis suggested co-localization and, thus, interaction of both molecules. To confirm this observation, immunoprecipitation experiments and Western blot analysis were performed and indicated binding of CEA and CEAR. Immunoprecipitation of CEA resulted in a pull down of CEAR. The pull down of CEAR correlated with the amount of CEA as demonstrated by ribozyme targeting of CEA. Finally, external treatment of HT29 cells with soluble CEA induced tyrosine phosphorylation of CEAR, suggesting a CEA-dependent role of CEAR in signal transduction. Future experiments will elucidate whether the CEA-CEAR interaction is involved in CEA's antiapoptotic role and mediates the prometastatic properties of CEA in colon cancer cells.*

Abbreviations: CEA, carcinoembryonic antigen; Rz, ribozyme; hnRNP, heterogeneous nuclear ribonucleoprotein; CEAR, CEA-receptor; RT, room temperature.

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Colorectal cancer is the third most common malignancy and the second leading cause of cancer death in the United States, with an incidence of 160,000 new patients per year. It has a high 5-year mortality rate due to metastatic disease (1). Surgery remains the most effective cure. The first line drug for chemotherapy, 5-Fluoruracil (5-FU), shows 20-30% response rates in metastatic patients (2) and eliminates tumor cells *via* induction of apoptosis (3).

Carcinoembryonic antigen (CEA) is overexpressed in a variety of carcinomas including cancer of the colon, breast and lung. CEA is the most useful marker for monitoring the therapeutic effectiveness of surgery in colorectal cancer. Elevated levels of CEA in the blood are associated with the metastatic progression of colon cancer after an apparently curative surgical intervention (4).

Experimental and clinical studies suggest that CEA participates in the development of liver metastasis from colorectal cancer. High preoperative CEA serum levels correlate with a poor clinical outcome in colorectal (5), gastric (6), lung (7) and breast cancer (8). Loss of apical CEA expression and diffuse cytoplasmic staining of CEA in colon cancer is also associated with metastatic disease (9), as is CEA expression by circulating colon cancer cells (10).

CEA is a glycoprotein of approximately 180 kDa, belonging to the immunoglobulin supergene family, which is anchored in the cell membrane *via* a glycosyl phosphatyl inositol moiety (11). Despite of its wide clinical use, CEA's biological function in colon cancer remains elusive.

Ordonez *et al.* have shown that CEA overexpression can protect tumor cells from anoikis, *i.e.* apoptosis induced by loss of cell contact with the extracellular matrix (12). We demonstrated that CEA protects colon cancer cells from apoptosis induced by different agents like UV-light, γ-Interferon and 5-FU (13). We assume that CEA might protect colon cancer cells from the hostile conditions they are exposed to during progression. Thus, CEA-expressing colon cancer cells may have a growth advantage because of this CEA antiapoptotic function (14).

Recently, it has been shown that CEA binds to a receptor at the membrane of Kupffer cells and rodent macrophages. CEA binding caused endocytosis of CEA, initiating a series of signaling events in macrophages and the release of various cytokines (15).

This CEA-receptor (CEAR) has been identified as a homolog of the heterogeneous nuclear ribonucleoprotein M4 (hnRNP M4), a protein so far known to function as a pre-mRNA binding protein (16). hnRNP M4 binds avidly to poly(G) and poly(U) RNA homopolymers *in vitro*, serves as a nuclear mRNA-transporter and is involved in mRNA splicing (17, 18).

Based on these data, we herein use the term CEAR for the hnRNP M4 homolog and hypothesized that CEAR mediates CEA signaling, not only in macrophages but also in cancer cells. We have investigated CEAR expression in human colon cancer tissue cells and analyzed its interaction with CEA in human HT29 colon cancer cells.

Materials and Methods

Immunohistochemistry of CEAR. For immunohistochemistry, we investigated formalin-fixed, paraffin-embedded sections derived from 5 colon cancer patients . Tissues were obtained according to standardized tissue collection procedures of the Cancer Research Institute Indivumed, Germany. Tissues were collected from the central area of the tumor, formalin-fixed within 15 min following tumor resection and paraffin embedded. Tissue section (5 μm) were deparaffinized and heated in a microwave. After washing with DPBS, nonspecific binding was blocked with 10% goat serum. After three washes, the slides were incubated with 1:125 diluted anti-CEAR antibody (Upstate Biotechnology, Lake Placid, NY, USA) for 30 min at RT. After washing, incubation of the secondary antibody for 30 min at RT followed, using biotinylated secondary rabbit anti-goat antibodies (Vector Laboratories, Burlingame, USA). The immunocomplexes were highlighted using the ABC phosphatase system (Vectastain Elite ABC kit, Vector Laboratories). Counterstaining was performed by hematoxylin solution (Sigma, Deisenhofen, Germany).

Cell cultures. HT29 human colon cancer cells and the mouse alveolar macrophage P388D1 cell line were obtained from the American Type Culture Collection, (Rockville, MD, USA).

The cells were cultivated in IMEM culture medium (Life Technologies, Inc., Gaithersburg, MD, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS, Mediatech Cellgro, VA, USA) and 1% L-glutamine at 37˚C in a 5% CO_2 humidified incubator.

The HT29 Rz4 cell line has have been extensively described previously (13). These cells are derived from HT29 colon cancer cells and stably express a CEA-targeted ribozyme under control of the tet-off promoter system. In summary, plasmids expressing the tetracycline transactivating (tTA)/VP16 fusion protein (pUHG15-1) and the tTA/heptameric operator binding site (tet-O; pUHC13-3) (19) were obtained from Dr. Bujard (Heidelberg, Germany). The ribozyme expression plasmid (pTET) was derived from pUHC13-3 and modified as described (20). The following ribozyme coding sense and antisense oligonucleotides were annealed and ligated into the HindIII- and NotI-restriction site of pTET: [5'-agctt**TGCTCTT***CTGATGAGTCCGTTAGGACGAA***ACTATGGA**ggg cc-3'] (sense) and [5'-c**TCCATAGT***TTCGTCCTAACGGACTCA TCAG***AAGAGCA**a-3'] (anti-sense) with lower case letters indicating HindIII-/NotI restriction site overhangs, bold capital letters showing CEA specific antisense regions and italic capital letters indicating the hammerhead ribozyme core sequence. The resulting ribozyme expression plasmid pTET/Rz2113 contains CEA-specific antisense flanking regions of 7 nucleotides (nt) on 5' and 8 nt on 3' ends of the 22 nt catalytic hammerhead ribozyme core sequence, that target it to the B3 domain of CEA and is specific for CEA according to blast search. An *in vitro* cleavage assay (Figure 1a) demonstrated specific cleavage of CEA mRNA, as shown previously (13). Western blot analysis (Figure 1b) showed, in HT29 Rz4 cells, a tetracycline-dependent reduction of CEA levels by approximately 50% and recovery of CEA levels by tetracycline within 12 h.

Western blot analysis. Fifty % confluent HT29 colon cancer cells were washed with cold phosphate-buffered saline, pH 7.4 and then lysed at 4˚C in a buffer containing 50 mM Tris-HCl, pH 8, 150 mM NaCl, 40 mM β-glycerophosphate, 1 mM EGTA, 0.25% sodium deoxycholate, 1% Nonident P-40, 50 mM sodium fluoride, 20 mM sodium pyrophosphate, 1 mM sodium orthovanadate and protease inhibitors (2 μg/ml leupeptin, 2 μg/ml aprotinin, 1 μg/ml pepstatin, and 100 μg/ml pefabloc). Cellular debris was removed by centrifugation at 14,000 rpm for 15 min. Cell lysates assayed for total protein content were fractionated on 8% gradient polyacrylamide SDS–PAGE gels (ISC BioExpress, UT, USA) and electrophoretically transferred to PVDF membranes (Millipore, Bedford, MA, USA). After blocking the membranes with 5% (w/v) non-fat dry milk or 2% Bovine Serum Albumin in Tris-buffered saline, pH 7.4, containing 0.5% Tween-20 (TBS-T) for 3 h with gentle rocking at room temperature, the membranes were incubated with antibodies conjugated to horseradish peroxidase (Amersham Pharmacia Biotech, Piscataway, NJ, USA) at 1:10,000–1:20,000 dilution in TBS-T for 1 h and the protein bands were visualized using ECL (Supersignal WestPico, Pierce, Rockford, IL, USA) according to the manufacturer's directions. For staining we used either a rabbit polyclonal C-terminal anti-CEA receptor antibody (1:1000, Upstate Biotechnology, Lake Placid, NY, USA), a rabbit polyclonal anti-actin antibody (1:3,000, Sigma-Aldrich, MO, USA) or a mouse monoclonal anti-CEA antibody (1:500, Cymbus Biotechnologies Ltd., Chandlers Ford, Hants, UK). Equivalent protein loading was achieved by adjusting the amount of protein according to photometric quantification and was controlled by total protein staining of the membrane using Ponceau S (Fisher Biotech, Fair Lawn, NJ, USA) or additional staining for control proteins such as actin.

Fluorescence-activated cell sorting (FACS). Cells were detached using 0.05% Trypsin- EDTA in PBS, washed with ice-cold PBS and diluted to a density of $2x10^6$ cells/ml using cold PBS. Aliquots of 100 μl ($2x10^5$ cells) were spun at 14,000 rpm for 5 min at 4˚C, supernatants were discarded and the cells were suspended in 100 μl of anti-CEA Receptor antibody (10 μg/ml Upstate Biotechnology) and incubated for 45 min at 4 ˚C. The cells were then washed twice with PBS and incubated for an additional 45 min with 1:100 diluted anti-rabbit-fluorescein isothiocynate (FITC) conjugated secondary antibody (Jackson Immunoresearch Laboratories, PA, USA) at 4˚C in the dark.

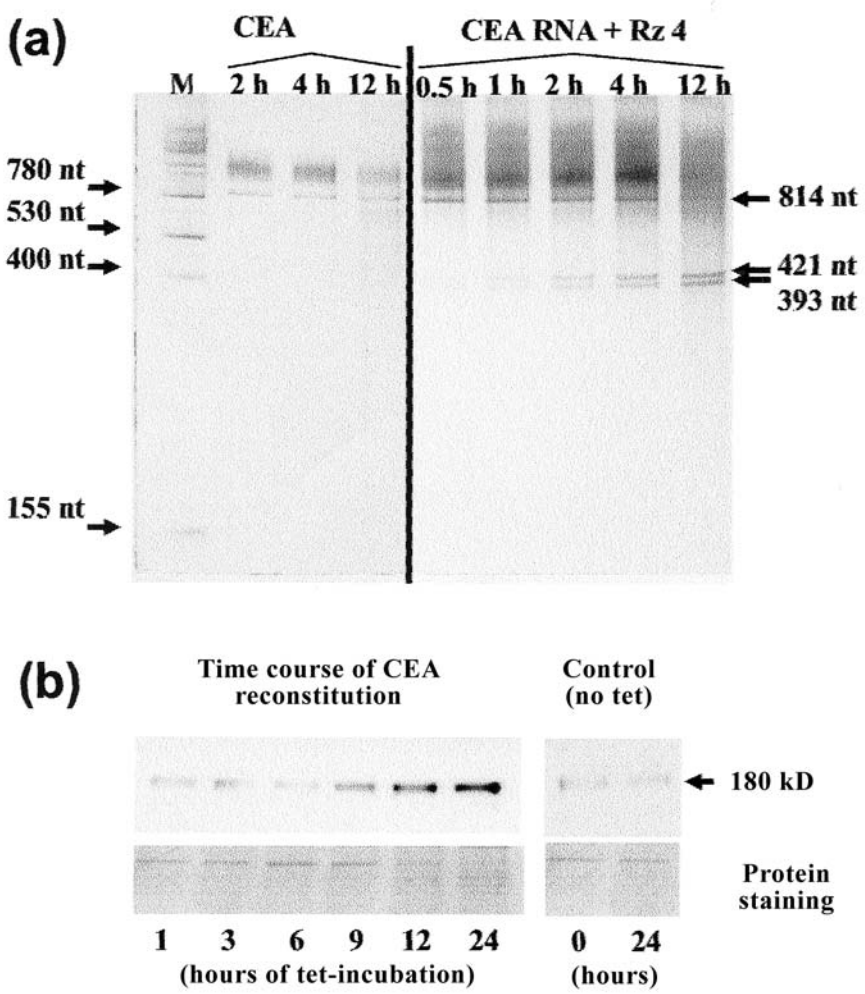

Figure 1. *(a) In vitro cleavage assay to determine the activity of CEA-targeted ribozyme Rz2113. A CEA transcript of 814 nt was co-incubated with 100-fold excess of Rz2113 for various time intervals (right 5 lanes). The expected cleavage products of 421 nt and 393 nt length became visible after 1 hour. As a control (left) served CEA RNA without additon of Rz2113 (M=molecular weight marker). (b) Western blot analysis to determine the time kinetic of CEA reconstitution after inhibition of ribozyme expression by 1 μg/ml tetracycline. The lower panel shows as a control HT29/Rz4 cells (stable expressing Rz2113 ribozyme), which were kept in culture in the absence of tetracycline (- tet).*
For details see (13).

After two final washings, the cells were suspended in 500 μl 4% paraformaldehyde. Unlabelled cells and cells labelled with secondary antibody alone served as negative controls. The mean values of fluorescence intensity of 10,000 cells were determined by FACS analysis (FACStar plus, Becton Dickinson).

Confocal microscopy. HT29 cells were cultured on glass coverslips ($7x10^4$ per 18 mm coverslip). The cells were incubated overnight at 37˚C before treatment with 1 mg/ml tetracycline, for 24 hours. The cells were fixed and labelled by indirect immunofluorescence.

Colocalization was examined by immunostaining with a rabbit C-terminal anti-CEAR antibody (1:400, Upstate Biotechnology) and a mouse anti-CEA antibody (1:25, Cymbus Biotechnologies Ltd.).

As secondary antibody we used a FITC-conjugated anti-rabbit and a Texas Red-conjugated anti-mouse antibody (Jackson Immunoresearch Laboratories), respectively. All secondary antibodies were applied in dilutions of 1:200. Coverslips were mounted, using the ProLong antifade kit (Molecular Probes, OR, USA). Confocal microscopy was carried out using an Olympus Fluoview confocal microscope with a 60X/1.4 N.A. objective lens. Imaging was performed at the Lombardi Cancer Center Microscopy and Imaging Shared Resources facility, U.S.A.

Immunoprecipitations. Immunoprecipitation was performed as described previously (21). Proteins were immunoprecipitated from the cleared lysate by incubation with 20 μl of rabbit polyclonal anti-CEA antibody (Cymbus Biotechnologies Ltd.), overnight at 4˚C with Sepharose-bound Protein G beads (Gammabind Plus Sepharose, Amersham/Pharmacia Biotech) for 2 h at 4˚C.

Immunocomplexes were recovered by centrifugation and washed four times with cold lysis buffer and twice with TN buffer (50 mM Tris, pH 7.5 and 150 mM NaCl). Samples were suspended with

25 μl of 1x Laemmli's buffer and heated for 5 min at 95˚C. Immunoprecipitates were separated by SDS-PAGE 8% polyacrylamide gel, transferred onto BioMax MultiBlot kit for proteins (Kodak, IKA Works, New Haven, CT, USA) and analyzed by immunoblot analysis.

Results

CEAR immunohistochemistry of colon cancer tissue. Tissue sections of CEA-positive colon cancer tissue from five different patients were immunostained for CEAR. All five sections showed comparable staining patterns. As shown in Figure 2, CEAR receptor was found in the nuclear region of tumor cells and stroma cells such as lymphocytes and fibroblasts. In addition, CEAR was found in the cytoplasm and membrane of cancer cells.

Expression of CEAR in colon cancer cells. To study CEAR and CEA interaction in detail, HT29 colon cancer cells were used. The expression of CEAR was determined by Western blot and FACS analysis. Mouse P388D1 macrophages served as a control cell line and confirmed specific staining of the expected 78 and 80 kDa proteins (data not shown). For staining, the C-terminal anti-CEAR antibody was used, which can detect at least two CEAR splice variants (78-kDa and 80-kDa) (22).

As seen in the inset of Figure 3, HT29 colon cancer cell lines expressed high levels of both CEAR splice variants. FACS analysis of viable cells indicated cell surface expression of CEAR (Figure 3). This finding supports the assumption that CEAR not only functions as a nuclear protein, but might also play a role as a surface receptor in colon cancer cells.

CEA and CEA-receptor co-localize in the membrane. To localize CEAR and CEA, confocal microscopy was performed using CEA and CEAR antibodies linked to two different fluorophores (Figure 4). As expected, the majority of CEAR was found in the nuclear region of HT29 colon cancer cells (strong green color). However, a distinct expression of CEAR was also visible on the cell surface, demonstrating the membrane-anchorage of hnRNP M4.

CEA was found almost exclusively in the membrane (red color). Most interestingly, membranous CEAR co-localized with CEA (yellow color). The merging of red and green, *i.e.* the yellow color, indicates an overlapping location and suggests interaction of CEA and CEAR.

CEA immunoprecipitation pulls down CEA receptor. Immunoprecipitation of CEA was used, to further demonstrate binding and interaction of CEA and CEAR (Figure 5). If endogenous CEA, which is exclusively found on the cell surface, binds to CEAR, immunoprecipitation of CEA should pull down CEAR. We were fortunate to have a cell model available which allowed reduction of endogenous CEA levels by tetracycline-regulated CEA-targeted ribozymes. In this model, the addition of tetracycline inhibited ribozyme expression and, subsequently, restored CEA levels within 12-24 h (13).

Immunoprecipitation of CEA followed by staining for CEAR demonstrated a pull down of CEAR. Furthermore, CEAR binding to CEA depends on the amount of endogenous CEA. HT29Rz4 cells with reduced CEA levels (CEA ribozyme expression) showed a significantly lower amount of CEAR in the Western blot analysis, while cells with normal CEA levels (ribozyme expression inhibited by tetracycline) presented high CEAR levels. The amount of co-immunoprecipitated CEAR correlated with the amount of endogenous CEA.

External treatment with CEA induced phosphorylation of CEAR. Protein phosphorylation is a common feature of molecules which are involved in signal transduction. Therefore, we analyzed if CEAR can be phosphorylated by CEA treatment.

HT29 cells were treated with 20 ng/ml soluble CEA. Immunoprecipitation with CEAR antibody was performed after various time points (Figure 6) and blotted with anti-phosphotyrosine antibody. After 3 h of CEA treatment, an increase in the phosphorylation of CEAR was observed. Maximal phosphorylation levels were achieved after 6 h before they returned to starting levels.

Discussion

Numerous clinical and experimental studies indicate that carcinoembryonic antigen (CEA), the oldest and most widely used clinical tumor marker in colorectal cancer, actively supports metastatic cancer progression. However, the biological function of CEA remains elusive. We had experimental evidence that CEA can protect colorectal cancer cells from undergoing apoptosis (13) and promotes the release of various cytokines from rat Kupfer cells (15). CEA, a member of the immunoglobulin superfamily, is a GPI-anchored glycoprotein located on the cell surface but also released into the extracellular space from cancer cells (11). Because of its biological effects and biochemical properties, we hypothesized that CEA interacts with a receptor-like molecule to be able to transmit signal transduction activity. Because CEA overexpression occurs in more than 90% of colorectal cancer (4) and in 60% of breast, lung, gastric and pancreatic cancer (5-8), identification of the receptor for CEA which mediates its prometastatic activities would have great impact on cancer therapy.

In this study, we analyzed hnRNP M4 regarding its potential function as a surface receptor for CEA. Originally, hnRNP family members were described as nuclear proteins

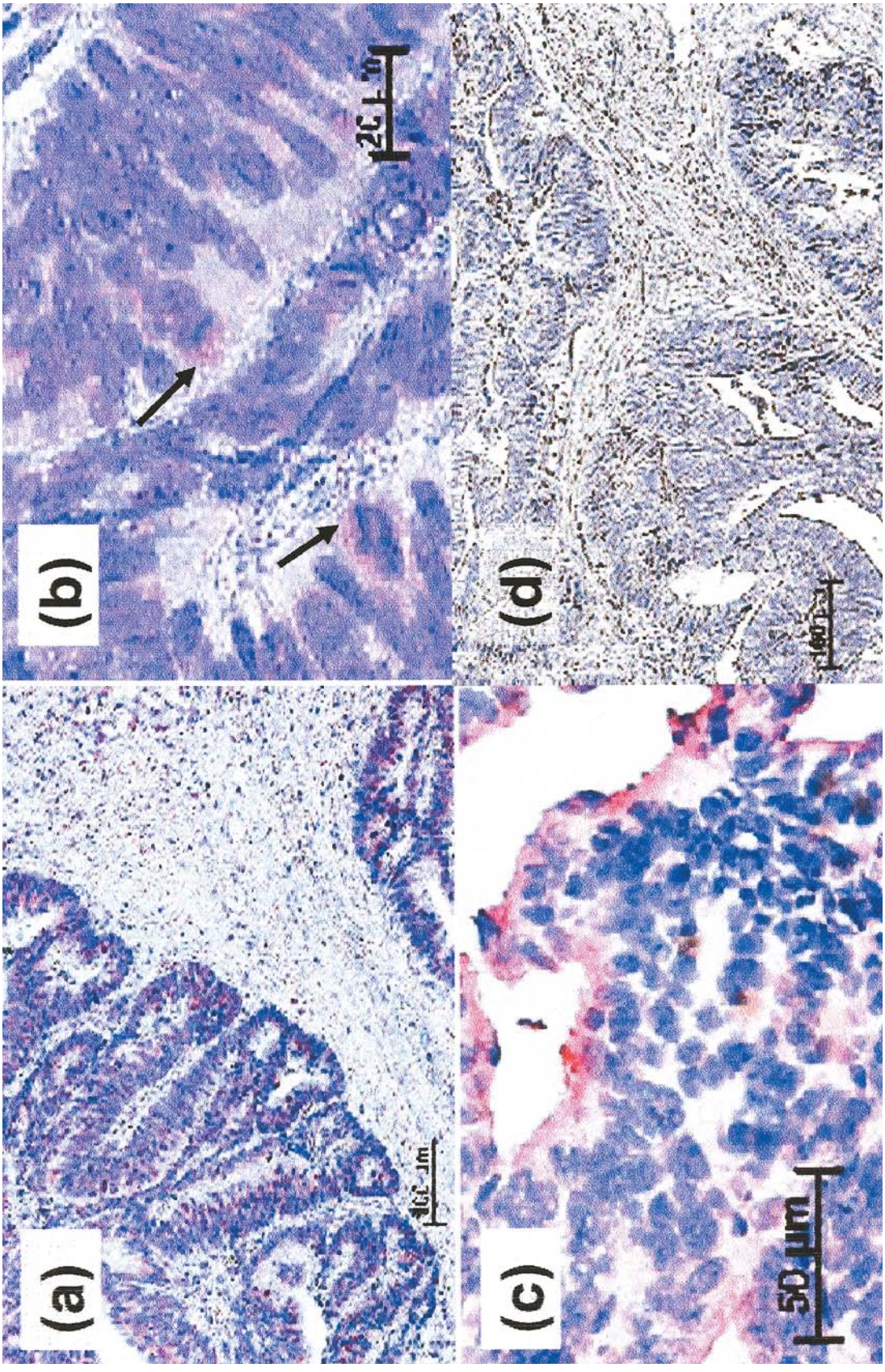

Figure 2. *Immunostaining of CEAR and CEA in colon cancer tissue. Shown are a microscopic overview (a) and an enlarged area (b). Arrows mark regions of strong CEAR expression (arrows). (c) Shows corresponding CEA staining. As negative control, tissue section were incubated with secondary antibodies only (d).*

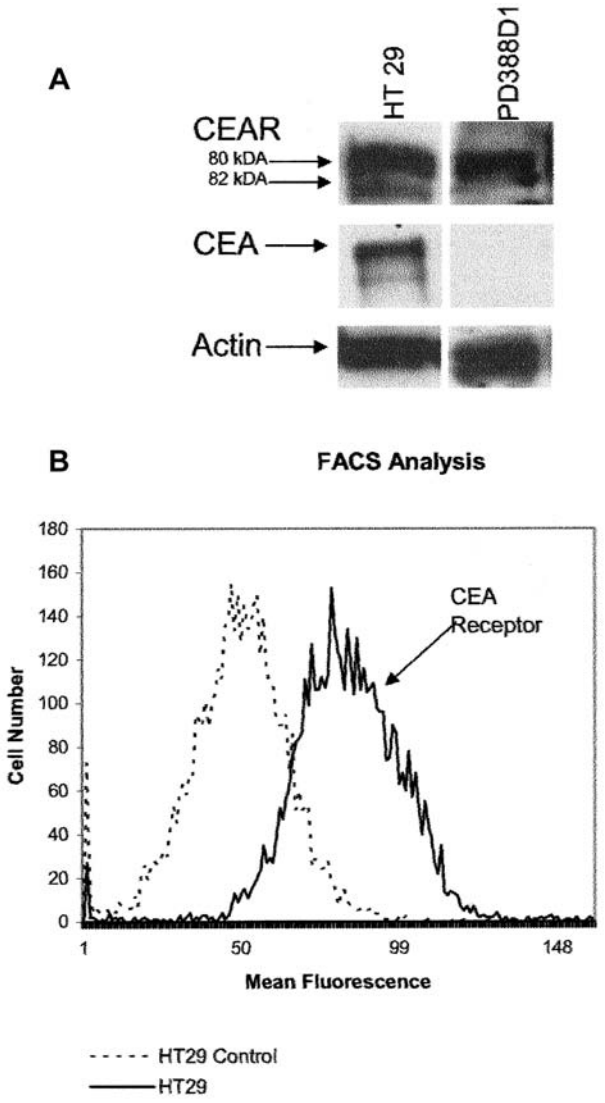

Figure 3. *FACS analysis of CEAR expression in HT29 cells (solid line). To control for background staining, cells were exclusively incubated with fluorescence-labelled secondary antibody (dotted lines).*
Inset: Western blot analysis to determine expression of 80 and 78 kDa CEAR-splice variants (upper panel) and CEA (middle panel) in HT29 colon cancer cells. Actin staining was used as loading control (lower panel).

functioning in RNA biogenesis, but emerging evidence indicates additional functions of hnRNP proteins. Several studies strongly suggest that these proteins are multifunctional and can also be involved in cell signaling (23-28). Furthermore, hnRNP family members have been found to act as surface receptors such as hnRNP U, which shuttles between the cytoplasm and nucleus, providing a mechanism for extracellular regulation of cell growth and virus infection (29, 30). Human full length hnRNP M4 was described as a receptor for thyroglobulin (30). Kupffer cells express a hnRNP M4 homolog on the surface (16). This hnRNP M4 protein has two splice variants, the full-length protein of 80 kDa and the 78 kDa, and binds human CEA (22). This protein was named the CEA-receptor (CEAR) because binding of CEA to this receptor caused release of various cytokines (31). Furthermore, CEAR contains a tyrosine kinase phosphorylation site in between aa 100-106 of its intracellular domain (16), which is a typical characteristic of cell signaling surface receptors.

Initially, we studied CEAR expression in CEA-positive colon cancer tissue obtained from 5 different patients. All samples showed a similar pattern of CEAR expression. CEAR was not only detected in the nuclear region but also in the cytoplasm and membrane of tumor cells. These data supported reports, as cited above, which suggested that hnRNP molecules are multifunctional (23-28). It prompted us to study the CEA-CEAR interaction in CEA-expressing HT29 colon cancer cells.

Both splice variants, the long 80 kDa and the short 78 kDa, were detected in HT29 cells using Western blot analysis and FACS analysis of viable cells, suggesting CEAR expression on the cell surface. To confirm this finding and to determine the localization of CEAR and CEA in colon cancer cells, confocal microscopy was performed using different fluorescence colors for both proteins. As expected, the highest amount of the heteronuclear protein CEAR was detected in the nucleus while CEA was exclusively found on the cell surface. However, distinct CEAR staining was also observed on the cell membrane. In several areas of tumor cells, CEA and CEAR colors merged suggesting overlapping localization and interaction of both proteins. This finding supports our hypothesis that CEAR serves as a receptor for CEA in human colon cancer cells. To confirm CEA binding to CEAR, we used a previously established HT29 colon cancer cell model which allowed us to modify endogenous CEA levels. These HT29 Rz4 cells represent a CEA-expressing subclone which contained a CEA-targeted ribozyme under control of the tet-off system (13). Previous studies have demonstrated the specificity of this approach to regulate CEA protein expression (13). CEA expression is modified by treatment with low levels of tetracycline, which switched off the tet-promoter, blocked expression of CEA-targeted ribozymes and, subsequently, restored the expression of CEA within 12 h (see Figure 1b).

Using this model, we demonstrated that immunoprecipitation of CEA at normal CEA levels pulls down CEAR. In addition, we showed that reduction of endogenous CEA levels also resulted in a decrease of CEAR binding. In cells with normal CEA levels (ribozyme expression switched off), more CEAR was co-immunoprecipitated compared to cells with reduced CEA levels (ribozyme switched on). The correlation of CEA binding with endogenous CEA levels was an important observation. First, it

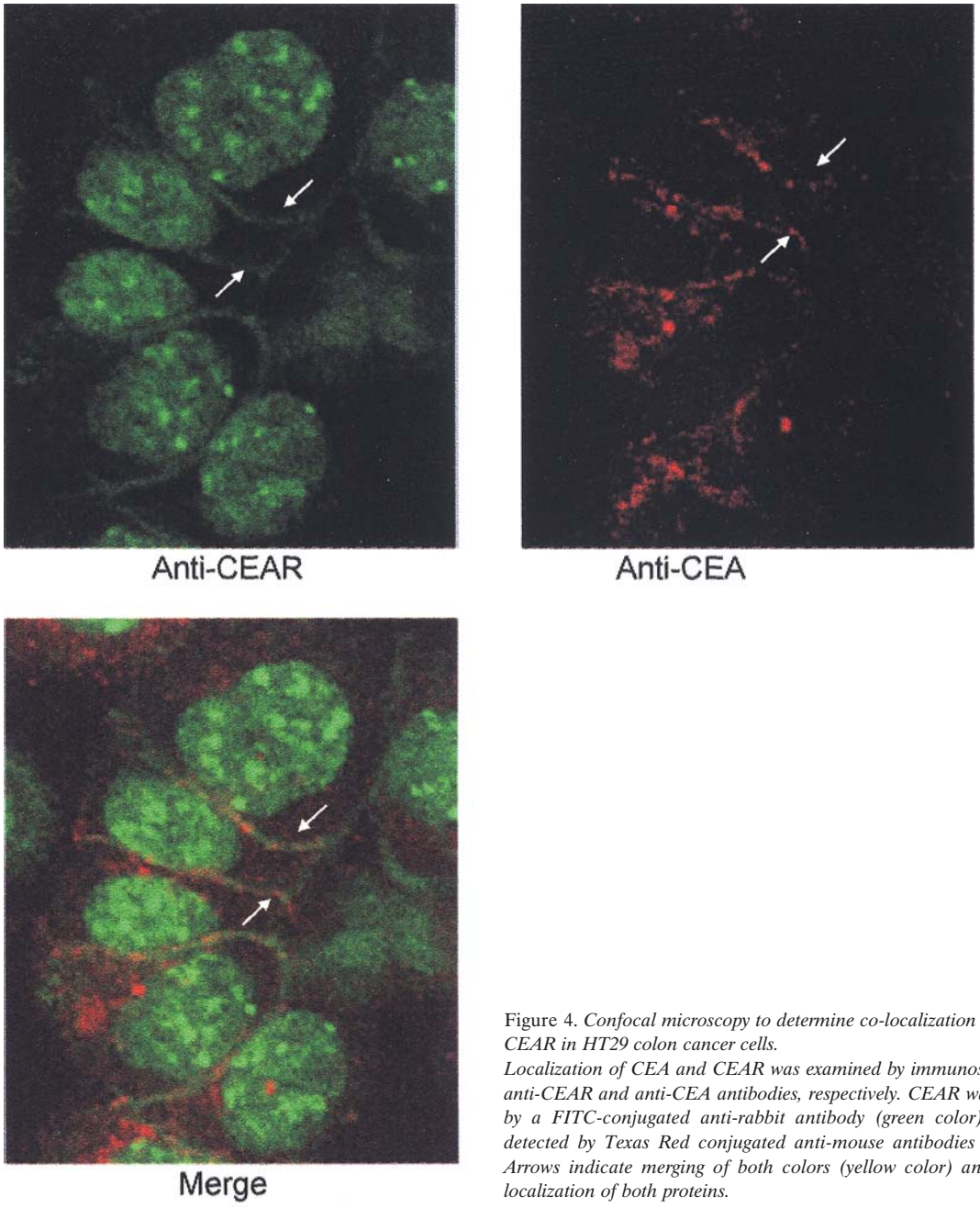

Figure 4. *Confocal microscopy to determine co-localization of CEA and CEAR in HT29 colon cancer cells.*
Localization of CEA and CEAR was examined by immunostaining with anti-CEAR and anti-CEA antibodies, respectively. CEAR was visualized by a FITC-conjugated anti-rabbit antibody (green color), CEA was detected by Texas Red conjugated anti-mouse antibodies (red color). Arrows indicate merging of both colors (yellow color) and, thus, co-localization of both proteins.

underlined that co-immunoprecipitation was a specific event. This was helpful because the immunoprecipitation experiment could not be performed with anti-CEAR due to the high amount of nuclear hnRNPM4/CEAR. The amount of co-immunoprecipitated, membranous CEA was too small to allow its detection.

Second, the dose-dependent co-immunoprecipitation of CEAR and the exclusive finding of CEA on the cell surface (co-localized with CEAR as demonstrated by confocal microscopy) strongly suggest direct binding of CEA and CEAR on the cell surface. It could be explained by heterodimerization of both membrane-anchored proteins. However, binding of released CEA to CEAR still remains an alternative mechanism.

As an initial experiment to study the CEA-related biological activity of CEAR, we analyzed and found phosphorylation of CEAR following external CEA treatment of HT29 cells. Because receptor-mediated

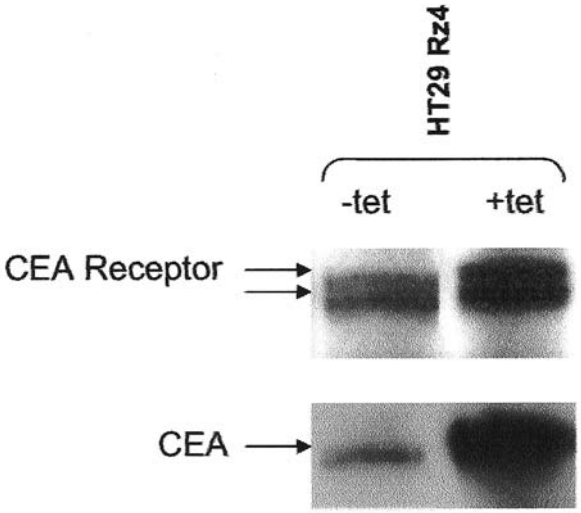

Figure 5. *Immunoprecipitation of CEA using HT29/Rz4 cells (which express tetracycline-controlled anti-CEA ribozymes) followed by Western blot analysis detecting CEAR and CEA. To modify CEA levels, HT29/Rz4 cells were cultured for 24 hours in the presence (+tet=normal CEA level) and absence (-tet=diminished CEA level) of tetracycline. Shown is a Western blot analysis and staining for CEAR (80 and 78 kDA band) and CEA.*

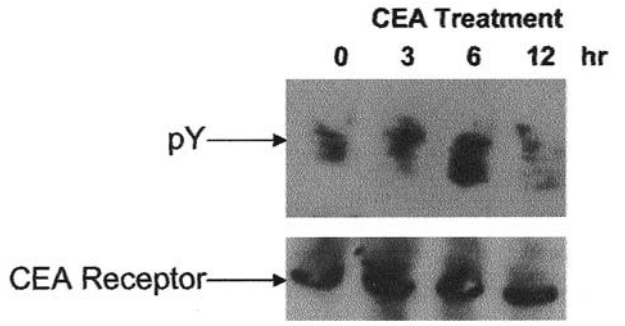

Figure 6. *CEA treatment of HT29 colon cancer cells to determine CEA-related phosphorylation of CEAR. After exposure to 20 ng/ml of CEA for various time periods, lysates of HT29 cells were subjected to immunoprecipitation for CEAR and analyzed by Western blot analysis and stained for tyrosine-phophorylation (upper panel). CEAR staining served as loading control (lower panel).*

signaling occurs most frequently *via* tyrosine-phosphorylation, these data suggest that CEAR serves as a functional signaling receptor in cancer cells. Significant phosphorylation of CEAR was observed within 3 to 6 h following external CEA treatment. Additional studies will elucidate this interaction and the biological role of CEAR in activating signaling cascades.

From a clinical point of view, targeting of the CEA pathway is extremely attractive because of the wide distribution of this tumor-associated antigen in various malignancies and only remote expression in normal tissues. Several studies have shown that antibody targeting of CEA and inhibition of endogenous CEA expression using ribozymes inhibit metastatic growth in mice (14). Assuming that CEA induces tumor progression by activation of an unknown signaling cascade, identification of this signaling event and subsequent inhibition seems to be an extremely attractive approach.

Our study strongly suggests that CEAR (hnRNP M4) could serve as a receptor in colon cancer cells mediating the prometastatic effects of CEA. Subsequent studies are under way to identify and elucidate the CEA-mediated signaling events and, finally, to develop a suitable therapeutic agent.

Acknowledgements

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