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Biochemical characterization of riboflavin carrier protein (RCP) in prostate cancer

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1. ABSTRACT

Riboflavin carrier protein (RCP) is a growth- and development-specific protein. Here, we characterized the expression of this protein in prostate cancer by polyclonal and monoclonal antibodies against chicken RCP. RCP was localized to both androgen-dependent and independent prostate cancer cell lines. Compared to controls, RCP was over-expressed in all 45 prostate adenocarcinomas, irrespective of the Gleason's score or the stage of the disease. The identified RCP had a molecular weight of 38 kDa, similar to RCP purified from chicken. Presence of this protein was also confirmed by siRNA inhibition analysis. Antibodies to chicken RCP inhibited incorporation of tritiated thymidine into DNA and prevented riboflavin uptake in PC3 prostate cancer cells, suggesting a critical function of this protein in prostate cancer cell growth. These data suggest that RCP can be used as a tumor biomarker in prostate cancer.

2. INTRODUCTION

Vitamin carrier proteins are a class of proteins synthesized in the liver to perform the function of transporting the respective vitamin to distant organs. Specific carrier proteins have been demonstrated for Folic acid, Riboflavin, Thiamin, Retinol and Cyanocobalamin (vitamin B12). Folate Binding Protein has been shown to be over expressed in tumor tissue of several cancers.

Riboflavin carrier protein (RCP) is a growth- and development-specific protein proven to be highly conserved through amphibians, reptiles and birds (1-3). Chicken RCP (cRCP) is a 37 kDa phosphoglycoprotein found in chicken egg white, egg yolk, and the plasma of egg laying hens. The protein transports riboflavin (vitamin B2) from the maternal circulation to the yolk, a nutritional storehouse for the developing embryo.

The cRCP is composed of two domains, the larger riboflavin binding and the smaller oocyte receptor binding domain. Results from a recent study indicate that although the ligand binding domain assumes near-native conformation, it does not bind riboflavin, suggesting that an interdependence of the two domains is necessary for proper organization of the riboflavin binding pocket (4).

cRCP has approximately 30% homology with human folate binding protein (hFBP) (3). Studies of bonnet macaques have shown that unlike hFBP, which is constitutively expressed, RCP is inducible in estrogen-stimulated liver (5). Active immunization with cRCP and passive immunization with an antibody to cRCP can terminate pregnancy in rats and monkeys (6-8). This suggests that a homolog of cRCP exists in mammalian systems. However it has not been fully characterized in mammalian systems.

Using an antibody to cRCP we have previously reported an over expression of RCP protein in breast adenocarcinoma tumors. This was accompanied by high serum levels, suggesting its potential use as a marker for early detection of breast cancer (9, 10). Furthermore, we have localized an RCP-like protein in ovarian adenocarcinoma, endometrial adenocarcinoma (unpublished results) and hepatocellular carcinoma (11).

Here, we report, for the first time, the identification and biochemical characterization of RCP in human prostate cancer cells and tumors, and further determine whether it can be used as a tumor marker for prostate cancer.

3. MATERIALS AND METHODS

3.1. Antibody production and characterization

Polyclonal antibodies to cRCP were produced by immunizing New Zealand white rabbits with a highly purified chicken RCP preparation. The antibody was made monospecific by selective absorption with chicken serum. We have characterized this antiserum and shown it to be free of cross-reaction with milk hFBP or human retinol binding protein (RBP) using a sensitive radioimmunoassay (10). Monoclonal antibodies to highly purified cRCP were made in mice using standard techniques. Two of the monoclonal antibodies, 6B2C12 and 6A4D7, have been used in Western blot analysis. In addition, polyclonal antibodies were produced in rabbits against two synthetic peptides of cRCP. Of these, peptide HACQKKLLKFEALQQEEGEE represented the C-terminal sequence of cRCP and peptide GENHCKSKCYPYSEMYAN was originated from a region of cRCP previously shown to be involved in riboflavin binding. Gamma globulin fractions of each of these polyclonal anti-sera were produced by ammonium sulfate fractionation and used in the present investigation.

3.2. Immunohistochemistry

Human prostate cancer cell lines (LNCaP, PC3, and DU-145) were purchased from ATCC and grown in tissue culture on chamber slides. The cells were fixed in 5% buffered formalin, permeabilized and stained for RCP. To determine the presence of surface localization of RCP, cells were released by trypsinization, washed, and formalin fixed. They were smeared on poly L-lysine-coated slides and subjected to immunohistochemical staining without permeabilization. A commercially available histoarray of 45 human prostate cancer tissues (Imgenex Corp., San Diego, CA) was used for tissue localization studies. An avidin-biotin-peroxidase kit (VECTASTAIN®; Vector Laboratories, Burlingame, CA) was used for immunostaining of RCP; staining was performed according to the manufacturer's protocol using diaminobenzedine as a chromogen.

3.3. ^{3}H Thymidine uptake assay

Duplicates samples of cells were seeded, into 25 cm^2 flasks (10^6 cells/flask) and incubated for 24 hrs. Cells were then incubated overnight in serum-free DMEM medium. The medium (3 ml DMEM/flask) was replaced with 10% serum-containing medium; ^{3}H thymidine (1microCi/ml) was added in the presence of either non-immune rabbit gamma globulin (control samples) or polyclonal anti-cRCP rabbit globulin (600 micrograms/flask, treated samples), and cells were grown for 24 hrs. The cells were subsequently rinsed with PBS and collected using a cell scraper. ^{3}H-thymidine incorporation was measured in 10% TCA-precipitable fraction of cell homogenates, using a scintillation spectrometer.

3.4. ^{3}H-Riboflavin uptake assay

Duplicate samples of PC3 cells were seeded at 6 x 10^5 cells per flask; following attachment, cells were washed twice, with both serum-free medium and serum-free, riboflavin-free AIM-V medium (Invitrogen, Corp., Carlsbad, CA). ^{3}H-riboflavin (25 microCi/mM, specific activity) was added at 2 microCi/ml, followed by 400 micrograms of either non-immune globulin (control samples) or anti-cRCP immunoglobulin (treated samples), and cells were grown for an additional 24 hrs. Cells were then harvested, washed three times with medium to remove any free ^{3}H-riboflavin, and lysed in 0.25M NaOH. Incorporated radioactivity was measured using a scintillation counter.

3.5. Immunoprecipitation

Cells were rinsed twice with cold PBS, harvested and centrifuged at 6000 rpm at 4ºC. The pellet was lysed in 200 microliters of lysis buffer (50 mM HEPES, 10 mM NaCl, 1 mM EDTA, 0.5% NP-40, 1 microliter protease inhibitor cocktail, pH 7; Sigma-Aldrich Co., St. Louis, MO). The lysate (supernatant) was collected by centrifugation at 6000 rpm. Lysates were pre-cleared by the addition of protein A agarose beads (Protein A Agarose Fast Flow, Millipore Corp., Billerica, MA), which had been washed in lysis buffer and prepared according to manufacturer's protocol. Immunoprecipitation was initiated by treating the supernatant with anti-RCP globulin (15 micrograms) at 4ºC overnight. Washed and prepared protein A agarose beads were added and incubated for two hours on a rocking platform. The beads were collected by centrifugation at 10,000 xg for 30 seconds, washed twice with lysis buffer, and prepared for electrophoresis by

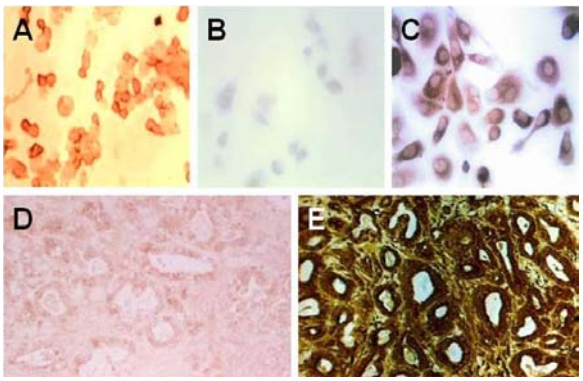

Figure 1. Immunohistochemical localization of RCP in PC3 prostate cancer cells and tissues - a) immunolocalization without permeabilization, showing surface localization; b) control treated with non-immune serum; and c) specific cytoplasmic localization with RCP antibody (light microscopy, magnification 100x). d) normal prostate, and e) adenocarcinoma (Gleason Score 4, with no metastasis).

heating for four minutes in a boiling water bath with 200 microliters of Laemmli sample buffer. We have also used a second method of immunoprecipitation where the anti-RCP globulin was covalently coupled to solid phase support through primary amino groups (Reacti-Gel™, Thermo Scientific, formerly Pierce Biotechnology, Rockford, IL), in order to ensure no free globulins contaminated the western blots. This coupling procedure was performed according to the manufacturer's instructions. After washing to remove any uncoupled globulin, the Reacti-gel beads were mixed and incubated with cell lysates overnight in order to effect attachment of any RCP like proteins to the solid phase support. This was followed by washing and treatment of beads with lysis buffer, and treatment with Laemmli buffer as described above.

3.6. Western Blot

The immunoprecipitated samples were separated by 12% SDS-PAGE and blotted on to nitrocellulose membranes. The membranes were blocked in 5% BSA in Tris-glycine buffer, incubated overnight with anti-RCP globulin, followed by horse radish peroxidase-conjugated goat anti-rabbit or anti-mouse IgG (Millipore Corp.) and developed using the SuperSignal West Pico chemiluminescent kit (Thermo Scientific) according to manufacturer's instructions.

3.7. siRNA-mediated depletion of RCP:

Three specific RCP siRNAs (predesigned oligonucleotides S1: 5'-CCCUCUUUGCUGUCAUCACtt-3'; S2: 5'-GCCAAACAUGCAUGAAUGCtt-3'; and S3: 5'-GGACAUGGUGGCAAUCAAGtt-3') and a *Silencer*® Negative Control #1 siRNA were synthesized commercially (Applied Biosystems/Ambion; Austin TX). Cells were seeded and grown to 70% confluency and washed twice in sterile PBS. The cells were incubated with a transfection cocktail comprising of Opti-MEM® 1 reduced serum medium (Invitrogen), Lipofectamin™ 2000 transfection reagent (Invitrogen), and the three siRNA together or scrambled oligonucleotides at a final concentration of 100 nM for 6 hrs at 37°C. Cells were replenished in 20% (v/v) FBS-enriched growth media and incubated at 37°C for 48 hrs before harvesting and experimentation. Depletion of RCP expression in cells was confirmed by immunoblotting.

4 RESULTS

4.1. Immunohistochemical localization of RCP

In order to RCP expression in prostate cancer cells and prostate adenocarcinomas, we performed immunohistochemical staining on established prostate adenocarcinoma cell lines as well as on a histoarray comprised of 45 prostate adenocarcinoma samples.

We observed specific RCP localization in the cytoplasm of androgen-insensitive PC3 cells grown in culture (Figure 1b, 1c). The pattern varied from uniform localization to heterogeneous localization, with no nuclear staining. The localization was observed in > 95% of the cells grown in culture, with some cells exhibiting more intense localization than others. A similar pattern of localization was also observed in androgen-insensitive DU-145 cells as well as in androgen-sensitive LNCaP cells (pictures not shown). This pattern was not observed when the primary antibody was omitted or absorbed with excess of RCP indicating its specificity (Figure 1b). Formalin fixed non-permeabilized cells showed surface localization of RCP (Figure 1a). This is in line with the known transport function of RCP as a carrier protein for its ligand, riboflavin.

Histoarray analysis showed that RCP localization was prominent in each of the 45 samples of prostate adenocarcinoma, regardless of the stage of cancer and Gleason score. In earlier stages (stages 1 to 3), RCP immunolocalization was predominantly seen in the tubular epithelium, whereas in later stages, a robust but more widely dispersed localization was evident despite the breakdown of tissue organization. Typical localization patterns in normal prostate and a cancer tissue sample are shown in Figures. 1d and 1e, respectively.

To determine whether the staining intensity correlated with adenocarcinoma stage, we developed an arbitrary RCP-staining scale to score the tissues. In this scale, we used a score of 1+ to denote the least intense staining and 4+ to indicate the most intense RCP staining among prostate cancer samples. Whereas normal prostate tissue scored at 1+, 100% of the cancer tissue scored from 3+ to 4+. These data are summarized in Table 1. It can be seen that out of a total of 33 tumor samples with Gleason scores of more than 7, 21 showed exhibited the highest RCP score of 4+ and 11 samples exhibited a 3+ score. While immunolocalization was observed primarily in the cytoplasm, a prominent nuclear localization was discovered in one sample (figure not shown). The significance of this nuclear localization is not clear.

4.2. ^{3}H-Thymidine uptake studies

We observed that ^{3}H-thymidine incorporation into cultured PC3 cells was markedly inhibited by addition

Table 1. Intensity of riboflavin carrier protein immunohistochemical-staining on prostate adenocarcinoma histoarray

Gleason score	Total #	Number of samples with 4+	% of samples with 4+	% of samples with 3+
3-6	6	5	83%	17%
7	9	8	89%	11%
8	6	3	50%	50%
9	23	15	65%	35%
10	4	3	75%	25%

The tissues were scored on an arbitrary scale of 1+ (least intense) to 4+ (most intense)

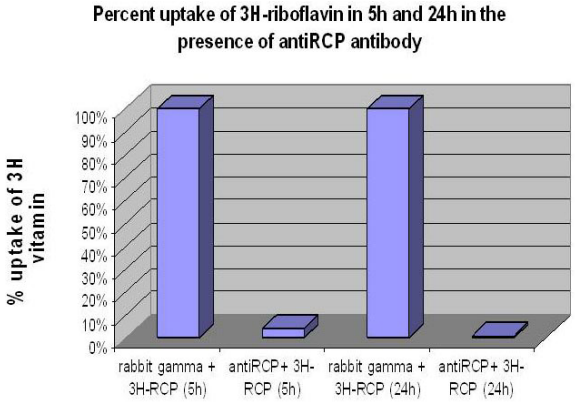

Figure 2. Inhibition of ^{3}H riboflavin uptake by anti-RCP globulin. Riboflavin uptake was inhibited by 95.6% as compared to controls (treated with non-immune globulin) by 5 hours and by 99.0% 24 hours following addition of polyclonal anti-RCP globulin.

of anti-RCP globulin to the culture medium as compared to controls (with no addition of globulin or addition of non-immune globulin). The uptake was reduced to 21.3% of the controls by addition of anti-RCP globulin, indicating inhibition of DNA synthesis. This inhibition was specific, since addition of non-immune globulin did not inhibit uptake of ^{3}H-thymidine.

4.3. ^{3}H Riboflavin uptake studies

In immunoneutralization studies performed to observe 3H-riboflavin uptake by cultured PC3 cells, we found that riboflavin uptake was inhibited by 95.6% as compared to controls (treated with non-immune globulin) by five hours. This inhibition was increased to 99.0% by 24 hours following addition of polyclonal anti-RCP globulin (Figure 2).

4.4. Western-blot studies

As there is very little information available about human RCP in prostate cancer, immunoprecipitation of RCP from different tissues (normal mouse liver, human liver adenocarcinoma) and prostate cell lines (LNCaP, PC3 and DU-145 cells) was carried out. Western blots of lysates from prostate cancer cell lines and various tissues were analyzed using a variety of antibody combinations for immunoprecipitation and probing (Figure 3). Equal protein loading was confirmed by re-probing the membrane for beta-actin expression and the signal was quantified by densitometry. Data values were expressed as percentage of expression when compared to control.

Using two different monoclonal antibodies to cRCP, PC3-prostate cancer cell lysates immunoprecipitated with protein A, showed a single band at approximately 38 kDa, similar to purified cRCP (Figure 3a). Immunoprecipitation using monoclonal and polyclonal antibody covalently cross-linked to Reacti-Gel CDI-activated, cross-linked beaded agarose (Thermo Scientific, formerly Pierce Protein Research Products, Rockford, IL) also yielded similar results (Figure 3a). However, immunoprecipitation of cultured NIH 3T3 cells and a human umbilical vein endothelial cell line used as controls (non-cancer cell lines) did not yield any bands (data not shown). Similarly, no bands were observed if non immune globulin was used during immunoprecipitation step, indicating the specificity of the reaction with anti-RCP globulin (Figure 3a).

Western blot analysis of immunoprecipitated lysates from mouse liver, human liver adenocarcinoma, and the three prostate cancer cell lines LNCaP, DU-145 and PC3 revealed a 38 kDa band similar to that observed for purified cRCP (Figure 3b).

4.5. siRNA studies

To confirm that the 38kDa signal observed in the western blots (Figure 3b) is indeed the RCP like protein, we utilized RNAi method. Combined treatment with three specific chicken siRNAs (predesigned oligonucleotides based on chicken RCP sequence) for 48 hrs resulted in a 70% reduction of RCP synthesis in both PC3 and Hep G2 cell line (Figure 3c). As the three siRNAs used were designed based on the sequence of chicken RCP, this indicates a significant structural homology of this human analog with its avian counterpart. This is in agreement with the electrophoretic mobility of the band at 38kDa seen in the western blot analysis (Figure 3a and 3b).

5. DISCUSSION

This investigation demonstrates for the first time the expression of an RCP-like protein in prostate cancer cells and tumor tissues. The protein localization in the cytoplasm of these cancer cells is similar to that we reported in breast cancer tissues (9). In other studies, we observed over-expression of RCP in reproductive cancers of the ovary and endometrium (unpublished data) as well as in hepatocellular carcinoma (11). In breast and hepatocellular carcinomas, this over-expression was accompanied by significant elevation of serum levels of RCP, suggesting that RCP may be useful for detection of early stage adenocarcinomas of these two organs. It is well known that the prostate gland is a target for estrogen, which is formed by aromatization of androgens. Even though RCP has been shown to be an estrogen-induced protein in

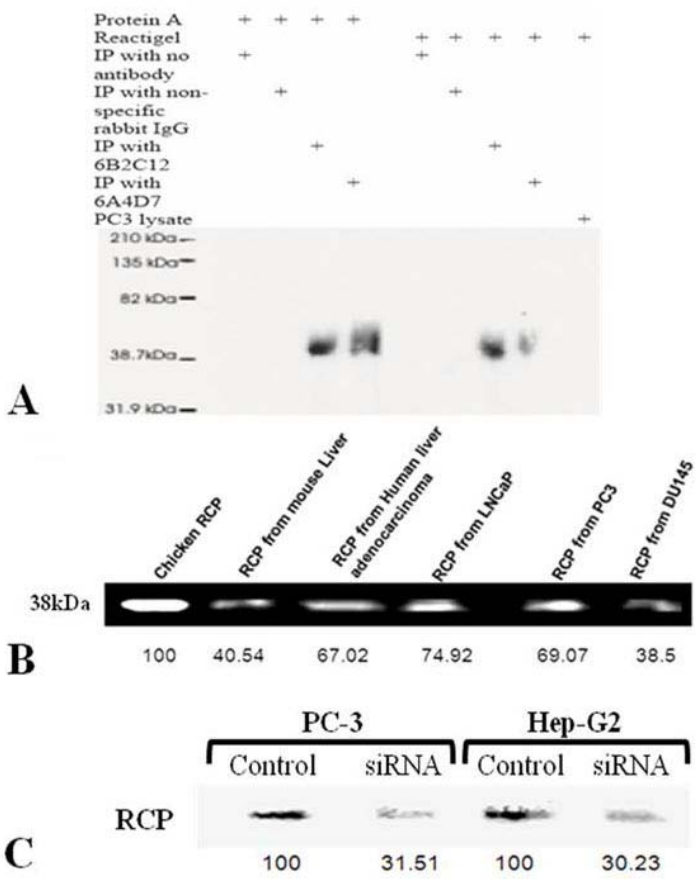

Figure 3. Western blot analysis of immunoprecipitates using antiRCP globulin. a) immunoprecipitation of RCP from PC3 cell lysates using monoclonal antibodies 6B2C12 and 6A4D7 coupled to protein A or to Reactigel. This was followed by western blot analysis using monoclonal antibodies; b) immunoprecipitation of RCP from different tissues (normal mouse liver, human liver adenocarcinoma) and prostate cell lines (LNCaP, PC3 and DU-145 cells) using polyclonal antibody followed by western blot analysis with a monoclonal antibody; and c) immunoblot illustrating specific RNAi-mediated suppression of RCP expression in PC-3 and Hep G2 (positive control of RCP) cancer cell lines transfected with specific RCP oligonucleotides. Equal protein loading was confirmed by re-probing the membrane for beta-actin expression and the signal was quantified by densitometry. Data values were expressed as the percentage of expression when compared to control.

the liver, its expression was seen in androgen sensitive LNCaP, as well as androgen insensitive PC3 and DU-145 prostate cancer cell lines. This finding indicates an uncoupling of RCP from the hormonal regulation in prostate cancers, similar to that we reported for hormone receptor-positive or -negative breast cancers (10).

While RCP has been well characterized in the reptilian and avian species, relatively little is known about the characteristics of mammalian RCP. Polyclonal antibodies produced against chicken RCP have been shown to terminate pregnancy in rats and bonnet monkeys (8, 12-15). Immunological cross-reactivity between chicken egg white RCP and an RCP-like molecule isolated from sera of pregnant monkeys has been demonstrated. The isolated primate RCP shared physico-chemical characteristics, such as isoelectric point, electrophoretic mobility, and molecular weight (~ 36 kDa) with its chicken analogue. In the present studies, using Western blotting, a similar band of about 38kDa was observed when human prostate cancer cell line extracts were immunoprecipitated and probed with monoclonal cRCP antibodies. In addition, the expression of this protein band could be significantly inhibited by siRNA treatment. These findings indicate the presence of a molecule with physico-chemical and immunological similarity to the avian RCP in several human cancers. Previous investigations have shown specific riboflavin-binding activity (determined by HPLC) to be associated with albumin and globulin fractions of serum from pregnant women and cows (16,17). Our observations that antibodies to chicken RCP almost completely inhibited ^{3}H-riboflavin uptake by PC3 cells indicate that a carrier protein may be obligatory for riboflavin transport into these cells. Recently, Mason *et al.* have shown that chicken RCP antibodies inhibited riboflavin uptake by BeWo cells (18). Taken together, these observations indicate an obligatory involvement of RCP in the transport of riboflavin and its internalization into the cells. Its internalization may involve a receptor mediated endocytic pathway in several adenocarcinomas, including that of prostate, similar to

placental trophoblast cells (18). However, extensive NCBI BLAST searches of the human and mammalian genome data bases did not reveal any sequences with significant homology to chicken RCP.

An RCP-like molecule has been isolated from human amniotic fluid and antibodies to this molecule recognized five epitopic sequences from the cRCP (residues 37-42, 73-76, 133-140, 174-176 and 200-207 of chicken RCP)(19). These findings suggest the presence of a similar protein in the primates. It is possible this region is being amplified in various cancers, leading to over-expression of this protein in adenocarcinomas of breast, prostate, and liver, as well as that of the ovary (unpublished data). Further biochemical and immunological characterization of the bands reacting with antibody to cRCP in the Western blot analysis is in progress and should yield definitive information on identification of this molecule in the human cancers.

Finally, overexpression of RCP in breast and hepatocellular carcinomas was accompanied by elevated serum levels that might be used as markers of early detection of these cancers (9-11). Studies using sera from prostate cancers of various stages will be necessary to determine the potential of this molecule to serve as a serum marker for early detection of this cancer also.

6. SUMMARY & PERSPECTIVE

Here, we have demonstrated for the first time, over expression of RCP in prostate cancer cell lines and tumor tissues. Further, we have shown its similarity to cRCP. Our observations on the effects of the antibody to cRCP on prostate cancer cells make this protein a worthy candidate for the development of a vaccine and possibly for immunotherapy of prostate cancer.

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Abbreviations: RCP: riboflavin carrier protein, cRCP: chicken riboflavin carrier protein, hFBP: human folate binding protein, RBP: retinol binding protein.

Key Words: Prostate Cancer, Riboflavin Carrier Protein, Tumor Marker, Antibody

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