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Isolation and identification of heterogeneous nuclear ribonucleoproteins (hnRNP) from purified plasma membranes of human tumour cell lines as albumin-binding proteins

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Abstract

Since albumin is being developed as a drug carrier to target tumours the search for albumin-binding proteins (ABPs), which play a role in cell surface binding and endocytosis of native and conjugated albumins becomes more and more interesting. We isolated five different proteins from purified plasma membranes from three different human tumour cell lines (CCRF-CEM, MV3 and MCF7) by albumin affinity chromatography and identified them as four members of the heterogeneous nuclear ribonucleoproteins (hnRNP) family and calreticulin by matrix-assisted laser desorption ionisation time-of-flight mass spectrometry. Contamination of the plasma membrane preparation by nuclear membranes was excluded with anti-nucleopore antibodies. Western blot analyses of plasma membranes showed ABPs with the same molecular weights as the albumin-affinity isolates. Tryptic digestion of intact cells was used to determine the sidedness of the albumin-binding property, which is oriented to the exterior of the cell. Localisation to the plasma membrane and albumin binding is a novel property of hnRNP.

Keywords: Albumin-binding proteins (ABPs); Heterogeneous nuclear ribonucleoproteins (hnRNP); Calreticulin; Crosslinking; Albumin affinity chromatography; MALDI-TOF-MS

1. Introduction

We are interested in albumin as a carrier for target-directed tumour therapy, since albumin accumulates in tumours [1]. This accumulation depends on the degree of modification of albumin. If more than three molecules of ligand per molecule albumin are bound, the drug–albumin conjugate is eliminated by the reticuloendothelial system of the liver [2]. We could show endocytosis of methotrexate-human serum albumin (MTX-HSA) into cells of the human leukaemia line CCRF-CEM and release of MTX by these cells [3]. Since we postulate endocytosis to be receptor-mediated, we are interested in finding ABPs on the plasma membranes of human tumour cells which would mediate this process.

ABPs for denatured or modified albumin have been reported in different tissues and cell lines. High affinity binding sites for formaldehyde-treated albumin have been detected in liver sinusoidal and renal plasma membranes [4,5]. Receptors for modified albumin molecules have been isolated from liver plasma membranes [6], and specific binding of these modified albumins but not of native albumin was shown. It was presumed that these ABPs belong to a family of scavenger receptors identified in macrophages or macrophage derived cells [7]. Ligand blotting experiments and crosslinking studies which proved the existence of 18 and 31 kDa scavenger receptors for modified albumin on vascular endothelial cell surfaces [8] and MDA-MB-453 tumour cell surfaces [9] confirmed

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Abbreviations: ABP, albumin-binding protein; DPBS, Dulbecco's phosphate-buffered saline; hnRNP, heterogeneous nuclear ribonucleoproteins; HSA, human serum albumin; MALDI-TOF-MS, matrix-assisted laser desorption ionisation time-of-flight mass spectrometry; PSD, post source decay; SASD, sulfosuccinimidyl-2-(*p*-azidosalicylamido)ethyl-1,3′-dithiopropionate.

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this assumption. These receptors are also expressed in fibroblasts and smooth muscle cells. Two proteins with the same masses were identified in membrane enriched fractions of Raji B lymphoma cells by their binding to nitrocellulose albumin [10]. It has been suggested that the binding of modified albumins, e.g. formaldehyde treated albumin and albumin–gold complexes to these receptors may initiate endocytosis, and the ligand may then be degraded by lysosomal proteases [11]. Native albumin can also couple to these ABPs in endothelial cells upon which transcytosis of HSA ensues [12,13]. Tiruppathi *et al.* prepared antibodies against gp60, a 60 kDa glycoprotein from bovine pulmonary microvascular endothelial cells. The antibody recognised three endothelial cell membrane proteins (57–60, 43 and 36 kDa) which are binding sites for native albumin [14]. Therefore ABPs play a role in endocytosis of albumin in various cell types. While ABPs seem to be present on the plasma membranes of several normal cells, no conclusive data are available on the occurrence of ABP for native albumin on the plasma membranes of human cancer cells and none of the ABPs have been characterised. We therefore set out to isolate and characterise ABP from highly purified plasma membranes from three human cell lines, CCRF-CEM (T-cell leukaemia), MV3 (melanoma) and MCF7 (breast carcinoma). ABPs were isolated by affinity chromatography on HSA columns and the isolated proteins characterised by MALDI-TOF-MS. Albumin was crosslinked to its putative binding proteins in intact cells and ABPs shown in Western blots of plasma membrane proteins exposed to albumin and anti-albumin antibodies.

2. Materials and methods

2.1. Chemicals and antibodies

Mouse anti-human nuclear pore monoclonal antibody (NA10L) was from Calbiochem, rabbit anti-HSA antibody (P0356) and alkaline phosphatase-conjugated rabbit anti-mouse immunoglobulin, horseradish peroxidase-conjugated pig anti-rabbit immunoglobulin and rabbit peroxidase–anti-peroxidase were obtained from DAKO. HSA essentially globulin free, soybean trypsin inhibitor type I-S, α-cyano-4-hydroxycinnamic acid, colloidal Coomassie blue, bis-benzimid Hoechst No. 33258, and *n*-octyl-β-D-glucopyranoside were from Sigma. CNBr-activated sepharose 4 fast flow, Na ^{125}I and PD-10 gel filtration columns were from Amersham Biosciences. Poly-Prep chromatography columns were from BioRad. Complete protease inhibitor cocktail tablets were from Roche. Accutase was from PAA. Curix RP 1.000 G X-ray films were from Agfa. SASD and IODO-GEN pre coated iodination tubes were obtained from Pierce Chemical Co. Centricon YM-3 centrifugal filter devices were from Milipore. Cell culture media and sera were from PAN, or Biochrom, NuPage 4–12% bis–tris gels were from Invitrogen. Sequencing grade modified trypsin was from Promega.

2.2. Cell culture

CCRF-CEM (human T-cell leukaemia) which were kindly provided by J.J. McGuire (Rosswell Park Cancer Institute Buffalo) and MV-3 (human melanoma) cells from D. Schadendorf (DKFZ) were adapted to RPMI 1640 medium with 50% of the usual amino acid content supplemented with 2.5% human serum and additional HSA to achieve an overall albumin content of ∼2 mg mL^{-1} [3]. MCF7 cells (human breast carcinoma) from our in house tumour bank were cultivated in DMEM medium supplemented with 10% fetal bovine serum. Both media were buffered with 25 mM HEPES. Cells were seeded at a density of 5×10^4 mL^{-1} and passaged every 3–4 days. Cells were regularly checked for the absence of mycoplasms with bis-benzimid.

2.3. Synthesis of ^{125}I-ASD-HSA

SASD is a crosslinker which contains an iodinatable benzene ring with a photoactivable azide and a disulfide bond which can be cleaved by SH-reagents to liberate the ligand, in our case albumin. All steps with SASD, ^{125}I-SASD and ^{125}I-ASD-HSA were performed in the dark under red light. Iodination of SASD with IODO-GEN and conjugation of ^{125}I-SASD with HSA were carried out with modifications according to the procedure supplied by Pierce Chemical Co. SASD (1 mg) was dissolved in 3690 μL DPBS. The ^{125}I-SASD was synthesised by incubating 1 mL of this SASD solution with 200 μCi Na ^{125}I dissolved in 50 μL DPBS in an IODO-GEN tube for 30 s. By dissolving 6.8 mg HSA in 2 mL DPBS a 50 μM solution was obtained. To produce ^{125}I-ASD-HSA 1450 μL of the HSA solution was transferred into a 15 mL Falcon tube and incubated with 1050 μL ^{125}I-SASD on a shaker for 30 min at 23°. Unreacted Na ^{125}I was separated from the labelled HSA using a PD-10 gel filtration column, which was previously equilibrated with DPBS. The eluted ^{125}I-ASD-HSA was diluted with DPBS to 10 mL.

2.4. ^{125}I-ASD-HSA crosslinking studies

Two 175 cm^2 tissue culture flasks with MCF7 and two with MV3 cells grown for 72 hr were pre-treated with 2% polyvinylpyrrolidone in DPBS for 1 hr at 37° and washed twice with DPBS. The cells from each flask were covered with 5 mL of the ^{125}I-ASD-HSA solution and incubated for 15 min at 37°. Photolysis was achieved by exposing the cell layers to UV light of 254 nm with a 10 W UV hand lamp held at a distance of 10 cm for 5 min after cutting off the top of the flask. After photolysis the supernatant was removed and the cell layers were scraped from the flasks using a teflon cell scraper and washed twice with DPBS

containing 1 tablet complete protease inhibitor per 30 mL. After centrifugation (350 *g*, 10 min, 4°) the cell pellet was dissolved in 150 μL sample buffer [15] containing 10% SDS and 5% 2-mercaptoethanol. Release of the HSA from the crosslinked complex was achieved by boiling at 100° for 5 min in a water bath. The labelled proteins were separated on a 4–12% gel by SDS–PAGE according to the method of Laemmli [15]. The gel was dried and autoradiography was performed for 96 hr at −80°.

2.5. Plasma membrane isolation

Isolation of plasma membranes from cultivated MCF7, MV3 and CCRF-CEM cells was performed by a combination of differential and sucrose density gradient centrifugation adapted from methods described for rabbit enterocytes [16,17] and for Caco-2 cells [18].

MCF7 or MV3 cells from twenty 175 cm^2 tissue culture flasks grown for 4 days (approximately 2×10^8 cells) were washed with DPBS containing 1% Na_2EDTA, and then detached with Accutase to prevent plasma membrane proteins from being damaged. Approximately 2×10^8 CCRF-CEM cells were washed twice with DPBS and pelleted. The cell pellet was immediately frozen in liquid nitrogen and stored at −80° or used directly for plasma membrane isolation. All further operations were carried out at 4° or less to keep proteolytic activity low. The cells were disintegrated in a micro-dismembrator (B. Braun Biotech) in liquid nitrogen for 1 min at 3000 rpm. The resulting cell powder was suspended in 10 mL of buffer A (250 mM sucrose, 12 mM Tris–HCl, pH 7.4 with 1 tablet complete protease inhibitor per 30 mL). The suspension was homogenised with 30 strokes in a Dounce homogeniser. Microscopic examination showed almost quantitative cell breakage. The procedure shown in Fig. 1 was followed. The homogenate was centrifuged for 10 min at 270 *g*. The resulting supernatant (SN1) was centrifuged for 10 min at 920 *g*. The supernatant (SN2) was collected, $MgCl_2$ was added to 10 mM, stirred on ice for 15 min and the endoplasmic reticulum pelleted for 15 min at 2300 *g*. The supernatant (SN3) was centrifuged for 45 min at 100,000 *g* in a 70.1 Ti rotor in a Beckman L5-50B ultracentrifuge. The crude membrane pellet (P4) was resuspended in 0.5 mL buffer B (250 mM sucrose, 5 mM Na_2EDTA, 12 mM Tris–HCl, pH 7.4) with 10 strokes in a Dounce homogeniser and layered on top of the following discontinuous sucrose density gradient (w/w): 1.5 mL 45%; 1.5 mL 35%; 1.5 mL 30% in a 13 mm × 51 mm Beckman polyallomer tube. The sucrose solutions were buffered with 10 mM Tris–HCl, pH 7.4 and their density

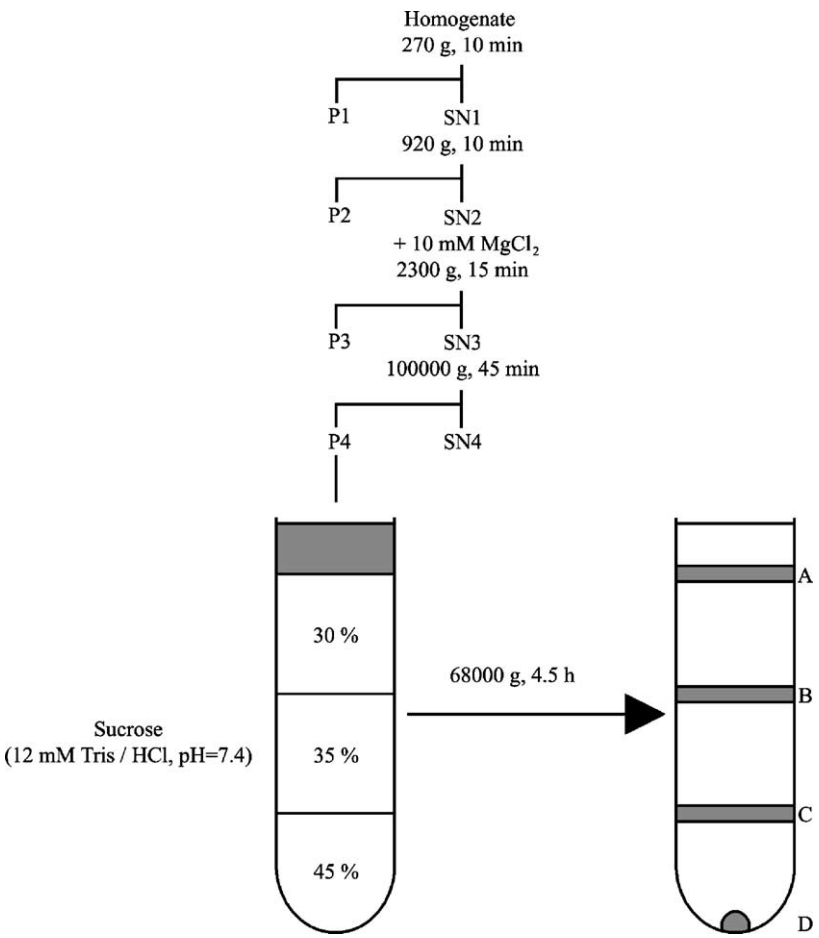

Fig. 1. Flow diagram for the isolation of plasma membrane fractions from CCRF-CEM, MV3 and MCF7 cells.

was checked with a refractometer (Carl Zeiss). Centrifugation was for 4.5 hr in a 50.1 swing out rotor in the ultracentrifuge. The bands at the interfaces of the gradient and the pellet (designated as: A, overlay/30%; B, 30%/35%; C, 35%/45%; D, pellet) were collected with a syringe and a needle and diluted to 8% sucrose with buffer A. The fractions were pelleted separately by centrifugation for 45 min at 100,000 *g* in a 70.1 Ti rotor. To prepare the samples for albumin affinity chromatography the pellets were solubilised with slight modifications of the method of Lisanti *et al.* [19]. A solubilisation buffer was obtained by adding 175 mg *n*-octyl-β-D-glucopyranoside, 100 μL Triton X-100 and 1/3 tablet complete protease inhibitor to 10 mL of buffer C (150 mM NaCl, 10 mM Tris–HCl, pH 7.5). Each pellet was homogenised with 2 mL solubilisation buffer with 30 strokes in a Dounce homogeniser and solubilised in an overhead shaker at 4° for 16 hr. Insoluble particles were removed by centrifugation for 45 min at 100,000 *g* in a 70.1 Ti rotor. The supernatants were used for affinity chromatography. If the pellets were used for Western blots they were taken up in 150 μL sample buffer [15]. Transmission electron microscopic examination and energy-filtering TEM for phosphorous showed no nucleic acids in fractions A–C and only vesicular structures.

2.6. Preparation of the HSA affinity columns and affinity chromatography

The coupling of HSA to the CNBr activated sepharose was carried out with modifications of the procedure supplied by Amersham Biosciences. All liquids were removed from the columns by means of a peristaltic pump (Pharmacia Biotech P-1). All incubations were performed in an overhead shaker at 23° if nothing else is mentioned. A Poly-Prep chromatography column was filled with 250 μg CNBr sepharose and soaked for 15 min with 1 mM HCl. After sucking off the HCl the bed was washed five times with 1 mM HCl. After removal of the HCl the sepharose was incubated for 2 hr with 5 mL coupling buffer (0.1 M $NaHCO_3$, 0.5 M NaCl, pH 8.3) which contained 2 μg HSA. Then unreacted binding sites of the sepharose were deactivated by incubating twice with deactivation buffer (0.5 M ethanolamine, 0.5 M NaCl, HCl, pH 8.3) for 1 hr. To remove non-covalently bound HSA the column was washed three times alternately with acetate buffer (0.1 M NaOAc, 0.5 M NaCl, AcOH, pH 4.0) and washing buffer (0.5 M NaCl, 0.1 M Tris–HCl, pH 8.0), then conditioned with buffer C. The HSA affinity columns were incubated with the solubilised plasma membrane fractions for 16 hr at 4°. After removal of the liquid phase the columns were washed three times with buffer C. ABPs were eluted from the columns by incubating these with 2 mL buffer C containing 8 M urea for 15 min.

To lower the urea concentration to less than 1 M and to reduce the buffer volume the eluted fractions from the affinity columns were transferred in Centricon YM-3 tubes with a 3000 Da cut-off. Filtration was performed at 4300 *g* in a Heraeus fixed angle rotor for 5 hr at 4°. The remaining volume above the filter (approximately 200 μL) was diluted to 2 mL with buffer C and centrifuged again. The remaining volume above the filter and the filter were transferred into an Eppendorf tube. One-fifth of the volume of five times sample buffer with 5% 2-mercaptoethanol was added and the tubes were boiled at 100° for 5 min in a water bath. Separation of the ABPs was done by SDS–PAGE on a 4–12% gel [15]. Gels were stained with colloidal Coomassie blue.

2.7. Western blot techniques

Demonstration of ABPs by Western blot was adapted from a method described by Tiruppathi *et al.* [14]. All operations were performed at 23°. After electrophoresis of the fractions from the density gradients the proteins were transferred onto a nitrocellulose membrane. Non-specific binding sites were blocked by incubating the membrane with 2.5% Tween-20 in DPBS for 16 hr. Then the membrane was washed and cut into two halves. One-half was incubated with 0.6 mg of HSA per mL DPBS for 2 hr, and the other half was incubated without HSA. After washing three times with DPBS the strips were incubated with rabbit anti-HSA antibodies for 2 hr. The membranes were washed and incubated with pig anti-rabbit IgG for 2 hr, then with a rabbit peroxidase–anti-peroxidase complex for 2 hr. To visualise the proteins the membranes were incubated with staining solution (33 mg 4-chloro-1-naphtol dissolved in 10 mL methanol, DPBS to 50 mL, 16.5 μL H_2O_2, 35%) until bands appeared.

To check for nuclear pore proteins as a marker for nuclear membrane the nitrocellulose membrane blots were washed three times with DPBS then incubated for 2 hr with mouse anti-nuclear pore antibody, followed by alkaline phosphatase-conjugated rabbit anti-mouse IgG for 2 hr. The proteins were visualised after staining with 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium salt.

2.8. Trypsinisation of intact cells

To analyse whether the albumin binding site of ABPs are exposed on the cell surface an experiment was performed with cells which after harvesting were treated with trypsin. The protocol was similar to a procedure published for HeLa cells [20]. Approximately 2×10^8 MCF7 cells were harvested as described above. After centrifugation the cells were resuspended in 5 mL DPBS containing 0.01% trypsin (w/v) and incubated in a water bath at 37° for 5 min. The digestion was terminated by addition of a 100-fold excess (50 mg) of soybean trypsin inhibitor and two washing steps with DPBS. Plasma membranes and nuclear fraction (P1, Fig. 1) were isolated and subjected to albumin affinity chromatography as described above.

2.9. Tryptic digestion of proteins in SDS gels

Protein bands were excised from the Coomassie blue stained gels and cut into 1 × 1 mm pieces. These were washed twice with deionised water, acetonitrile/water 1:1 and acetonitrile. Proteins were digested with sequencing grade modified trypsin in 40 mM NH_4HCO_3 at 37° overnight. The reaction was stopped by freezing.

2.10. MALDI-TOF mass spectrometry (MALDI-TOF-MS)

For the mass spectrometric analysis of tryptic digests samples were prepared on thin film spots [21]. Briefly, aliquots of 0.3 μL of a saturated solution of α-cyano-4-hydroxycinnamic acid in acetone containing nitrocellulose were deposited onto individual spots on the target. Subsequently, 0.8 μL 10% formic acid and 0.4 μL of the protein digests were loaded onto the thin film spots and allowed to dry slowly at ambient temperature. To remove salts from the digestion buffer the spots were washed with 10% formic acid and with water.

MALDI-TOF mass spectra were recorded in the positive ion reflector mode with delayed extraction on a Reflex II time-of-flight instrument (Bruker-Daltonik) equipped with a SCOUT multiprobe inlet and a 337 nm nitrogen laser. Ion acceleration was set to 26.5 kV, the reflector was set to 30.0 kV and the first extraction plate was set to 20.6 kV. Mass spectra were obtained by averaging 50–200 individual laser shots. Calibration of the spectra was performed internally by a two-point linear fit using the autolysis products of trypsin at *m/z* 842.50 and 2211.10.

2.11. Post source decay analysis

Sample preparation for peptide sequencing by PSD analysis was achieved by co-crystallisation of matrix with samples concentrated on ZipTips C18 (Millipore). Briefly, the peptides in the supernatant of the in-gel digestions were absorbed to a prewashed (50% acetonitrile/water) and equilibrated (0.1% trifluoroacetic acid/water) Zip Tip C18 by repetitive pipetting steps. Following washing of the Zip Tip C18 with equilibration buffer the peptides were eluted from the Zip Tip with 1 μL of matrix (α-cyano-4-hydroxycinnamic acid saturated in 50% acetonitrile/water).

PSD analysis was performed in the positive ion reflector mode with delayed extraction by setting an ion gate width of 40 Da around the ion of interest. Data were acquired in 14 segments by decreasing the reflector voltage in a stepwise fashion. For each segment 100–200 individual laser shots were accumulated. The fragment ion spectrum was obtained by pasting together all segments to a single spectrum using the FAST software provided by Bruker. Fragment ion calibration was performed externally with the fragment masses of the adrenocorticotropic hormone 18–39 clip.

2.12. Database search

Singly charged monoisotopic peptide masses were used as inputs for database searching. Searches were performed against the NCBInr database using the ProFound search algorithm (http://129.85.19.192/prowl-cgi/ProFound.exe) and the Protein Prospector software developed at the University of California, San Francisco (http://prospector.ucsf.edu). Isoelectric points were allowed to range from 0 to 14, and the oxidation of methionine was included as possible modification. Up to one missed tryptic cleavage was considered, and the mass tolerance for the monoisotopic peptide masses was set to ±100 ppm or ±0.1 Da.

Searches with fragment masses from PSD experiments were performed against the NCBInr database using the MS-Tag search algorithm provided by the Protein Prospector software package. Parent mass tolerance was set to ±0.1 Da and fragment ion tolerance was set to ±0.7 Da.

3. Results

3.1. ^{125}I-ASD-HSA crosslinking studies

With the crosslinking experiments we found ABP expressed on both human tumour cell lines, MCF7 and MV3, used for this study. Crosslinking of HSA to its putative receptor proteins was achieved with SASD, a heterobifunctional, iodinatable, photoactivable and reductively cleavable reagent. In the crosslinking experiments the MCF7 and MV3 cells were exposed to HSA conjugated with on average 6.9 mol ^{125}I-SASD per mol HSA. After SH-mediated cleavage of the crosslinker the proteins to which albumin had bound are iodine labelled and visible in the autoradiographs of the electrophoretically separated cell homogenates (Fig. 2). In both, MV3 and MCF7 preparations 18 and 40 kDa bands were observed. In MCF7 cell homogenates additional minor bands with 28, 31, 36, 44 and 50 kDa were detected. These findings indicate that at least two ABPs are expressed on both melanoma and breast cancer cells.

3.2. Isolation of ABPs by albumin affinity chromatography

In order to identify the proteins plasma membranes from MV3, MCF7 and CCRF-CEM cells were isolated by density gradient centrifugation and the fractions A–D (Fig. 1) were then subjected to albumin affinity chromatography. The eluates from the columns were separated on SDS–PAGE. Bands of ABP appeared mainly in the fractions A and B, whereas in fraction C the bands were weaker and fraction D did not show any ABPs. SDS–PAGE of the isolates obtained from fractions B of the plasma membranes of the three cell lines are shown in Fig. 3.

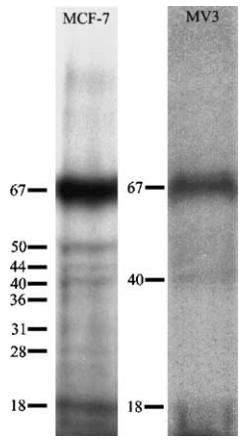

Fig. 2. Albumin-binding proteins visualised with ^{125}I-SASD. MCF7 and MV3 cells were incubated with ^{125}I-ASD-HSA for 15 min at 37°. After washing cells were solubilised, ^{125}I-ASD-HSA hydrolysed and the proteins separated by SDS–PAGE. The resulting gels were dried and autoradiography was performed for 96 hr. For MCF7 a major 18 kDa band and minor bands with 28, 31, 36, 40, 44 and 50 kDa were detected (left lane). For MV3 two faint bands at 18 and 40 kDa appeared on the autoradiogram (right lane). The band at 67 kDa is residual albumin. Molecular weights are indicated in kDa.

Artefactual binding of intracellular proteins from dead cells to the plasma membranes was excluded by exposing cells to a homogenate of the same number of dead cells. No further or more intense bands were detected in the plasma membranes isolated from the live cells after albumin affinity chromatography.

3.3. Identification of isolated proteins by MALDI-TOF-MS

MALDI-TOF-MS analyses were performed on bands isolated from plasma membrane fractions B, because the yields were highest in these fractions. The 36 kDa bands isolated from plasma membranes of MV3, MCF7 and CCRF-CEM cells were excised, digested and analysed by MALDI-TOF-MS. From CCRF-CEM isolates the 50, 40, 31, and 18 kDa bands were also cut out and the tryptic digests analysed by MALDI-TOF-MS and selected tryptic fragments sequenced by PSD. Figure 4 shows the sequences of the identified proteins with the regions of the tryptic fragments analysed in the three cell lines indicated. Of the five proteins identified, four were members of the hnRNP, of which at least two occur in 2 isoforms (hnRNP A2/B1 and hnRNP C1/C2). For two proteins the characterised tryptic fragments allowed the unequivocal identification of the isoforms, namely the hnRNP B1 isoform and the hnRNP C1 isoform (see figure legend for details). The fifth protein was identified as calreticulin. The identified proteins are listed in Table 1. The band at 67 kDa in Fig. 3 is HSA.

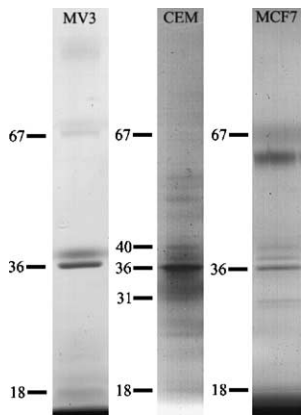

Fig. 3. SDS–PAGE of proteins isolated by albumin affinity chromatography from purified plasma membrane fractions (fraction B in Fig. 1) from MV3 (left lane), CCRF-CEM (centre lane) and MCF7 (right lane) cells. Gels are 4–12% gradient gels and were stained with colloidal Coomassie blue. Molecular weights are indicated in kDa.

Table 1
Albumin-binding proteins identified with MALDI-TOF-MS and PSD

Size on SDS-PAGE in kDa	Source	Identified protein	Swiss-Prot number
50	CCRF-CEM	Calreticulin	P27797
40	CCRF-CEM	hnRNP A3	P51991
36	CCRF-CEM, MV3, MCF7	hnRNP A2/B1	P22626
36	CCRF-CEM	hnRNP C1	P07910
36	MCF7	hnRNP A1	P09651
31	CCRF-CEM	hnRNP A2/B1	P22626
31	CCRF-CEM	hnRNP A1	P09651
18	CCRF-CEM	hnRNP B1	P22626

3.4. Western blot analyses

To analyse directly ABP in the plasma membrane with native albumin, fractions A–D of the density gradient centrifugation from MCF7 and CCRF-CEM cells were separated by SDS–PAGE and blotted onto nitrocellulose membranes (Fig. 5). The blots were cut in two, one-half of each blot was incubated with HSA and the other half without HSA. To make the bands visible the membranes were developed with anti-HSA antibodies as described in the methods. The MCF7 plasma membrane blot incubated with albumin showed bands at 36 and 50 kDa and several bands between 31 and 18 kDa mainly in fractions A and B (lanes 7 and 8). The CCRF-CEM plasma membrane blot showed several bands between 31 and 18 kDa in fractions B and C (lanes 2 and 3) and weaker bands in fraction A (lane 1). Even without prior incubation with HSA the same bands were faintly visible after reaction with the antibodies (lanes 1 and 2 for MCF7, lanes 7–9 for CCRF-CEM). It is not clear why this happened.

The hnRNP identified are considered nuclear proteins, therefore contamination of the plasma membranes by nuclear proteins had to be excluded. To this end the plasma membrane fractions A–D (Fig. 1) from CCRF-CEM and MCF7 cells were separated by SDS–PAGE. After blotting

Fig. 4. Amino acid sequences of the identified proteins. Underlined sequences were identified by MALDI-TOF-MS in proteins isolated from CCRF-CEM, dashed sequences from MCF7 and plus symbols sequences from MV3 cell membranes. Only the 18 kDa protein of CCRF-CEM isolates yielded a fragment with the hnRNP B1 isoform specific sequence shown in bold letters, in addition to the fragments shown for hnRNP A2/B1. The hnRNP C1 isoform was identified by a tryptic fragment which spanned amino acids 100–117. The C1 isoform lacks amino acids 108–120 present in isoform C2 (see Table 1 for a list of the identified proteins).

the gels onto nitrocellulose membranes the blots were incubated with anti-nuclear pore antibody and bands were made visible as described in Materials and Methods. The pellets P1, P2 and P3, which are the main fractions containing nuclei, served as positive controls. Nuclear pore proteins were only visible in fractions P1, P2 and P3 (Fig. 6). By further developing the same blots with albumin, anti-HSA and the corresponding antibody cascade described in Materials and Methods to make the HSA-binding proteins visible, the sensitivity for the nuclear pore proteins was increased, because the anti-rabbit IgG also detected rabbit anti-mouse IgG the antibody, which bound to the anti-nuclear pore antibody. Due to this enhancement it was possible to detect vestiges of nuclear pore protein in fraction C (for CCRF-CEM) and D (for both cell lines) only, but not in fractions A and B from which the ABP were

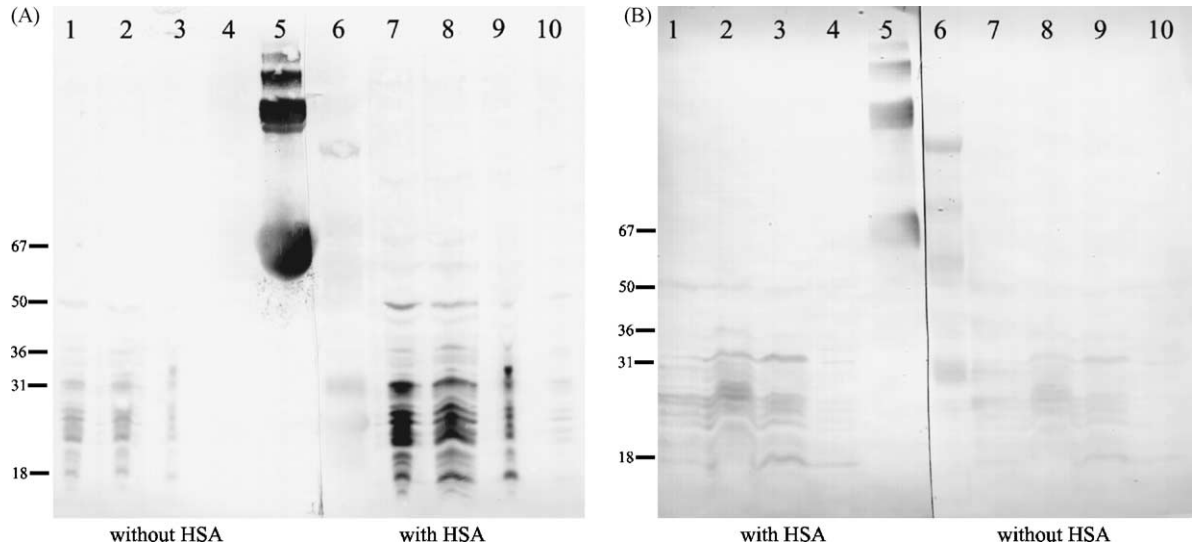

Fig. 5. Western blot analyses of albumin-binding proteins in plasma membranes. Plasma membrane fractions A–D from MCF7 cells (A) and CCRF-CEM (B) were separated by SDS–PAGE and the proteins blotted onto nitrocellulose. Each blot was cut in two, one-half was incubated with HSA the other without HSA. Then all blots were incubated with mouse anti-HSA antibody as described in Materials and Methods. In both blots lanes 1 and 7 are fraction A, lanes 2 and 8 are fraction B, lanes 3 and 9 are fraction C and lanes 4 and 10 are fraction D. Lanes 5 are HSA and lanes 6 are molecular weight markers. The inner halves were incubated with HSA, the outer halves without HSA. Molecular weights are indicated in kDa.

isolated and characterised. In fraction B from MCF7 cells the characterised hnRNP bands at 36 and 31 kDa are clearly visible in the blots reacted with HSA. ABP are also visible in the fractions containing nuclei, especially of CCRF-CEM cells.

3.5. Trypsinisation of intact cells

To analyse the sidedness of the albumin-binding property of the isolated proteins MCF7 cells were treated with trypsin to digest surface proteins. From these cells fraction P1, which contained the nuclei, and plasma membrane fractions A–D were isolated. The same fractions were isolated from untreated cells. All fractions were subjected to albumin affinity chromatography and separated by SDS–PAGE. The fractions A–D from the trypsin treated cells did not contain any ABPs whereas the fractions A–D from untreated cells had a normal ABP pattern. There was no significant difference in the band patterns of the P1 fractions from trypsin treated and untreated cells (Fig. 7). Trypsin did therefore not affect intracellular proteins, namely those on the nucleus. This experiment shows that the albumin binding site of the ABP is exposed to the extracellular side of the plasma membrane.

4. Discussion

Our investigations show that albumin binds to distinct proteins on the surface of different human cancer cell lines, namely CCRF-CEM T-cell leukaemia cells, MCF7 breast cancer cells and MV3 melanoma cells. With the cross-linking method using intact MCF7 and MV3 cells an 18 and a 40 kDa protein were labelled on cells of both lines. This experiment and the tryptic digestion of intact cells indicate that ABPs appear to be distinct membrane components exposed on the cell surface. By affinity chromatography of purified plasma membranes from CCRF-CEM, MCF7 and MV3 cells five proteins were isolated and identified as members of the hnRNP family and calreticulin by MALDI-TOF-MS analyses of tryptig digests. These experiments are the first to characterise and identify the ABPs on the cell surface and to show hnRNP to be associated with plasma membranes of tumour cells and to bind to albumin.

The protein with 50 kDa in the CCRF-CEM isolates was identified as calreticulin. This protein was initially described as an endoplasmic reticulum chaperone and Ca^{2+} signalling protein, but has since also been found on the cell surface and is involved in cell adhesion, but has also many other functions [22]. Recently, calreticulin was found on the surface of human platelets where it is described to be associated with collagen receptors on the cell membrane, and might play a role in the modulation of the platelet–collagen interaction [23].

The 36 kDa protein detected in all three cell lines was identified as hnRNP A2/B1. The 36 kDa band from CCCRF-CEM cells also contained hnRNP C1. The difference between hnRNP A2 and B1 and between C1 and C2 consists of short peptide inserts in an otherwise identical sequence [24]. The 18 kDa peptide isolated from CCRF-CEM membranes contained the B1 isoform specific sequence, which was not detected in any other of the hnRNP A2/B1 tryptic digests. The hnRNP C1 isoform was identified by the lack of the 13 amino acids in a tryptic fragment typical for isoform C2. The 31 kDa band

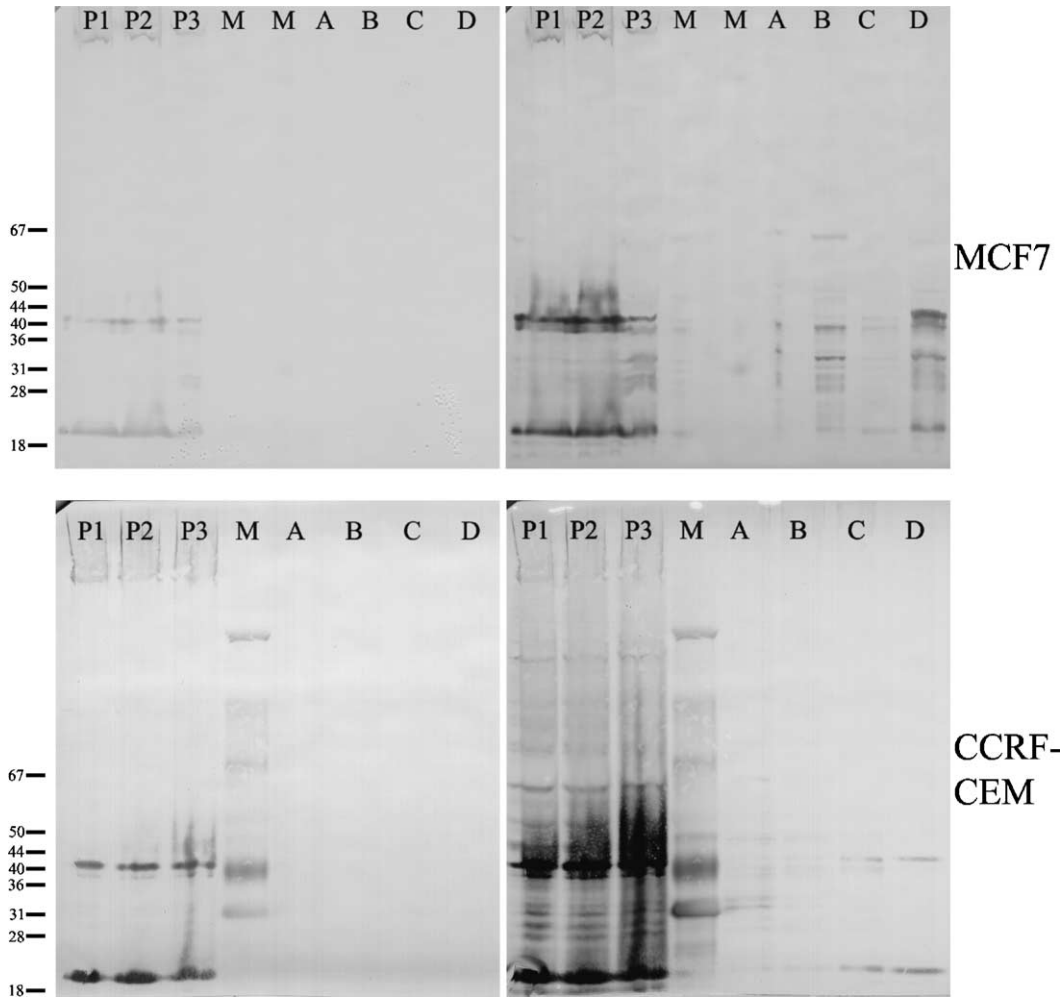

Fig. 6. Western blot with anti-nuclear pore antibodies. Solubilised pellets P1, P2 and P3 from the differential centrifugation procedure and fractions A–D from the sucrose density gradients from MCF7 cells (above) and CCRF-CEM cells (below) were run on SDS–PAGE and the proteins blotted onto nitrocellulose. In a first step the nuclear pore proteins were visualised with anti-nuclear pore antibody (left images). Afterwards the same blots were incubated with HSA and anti-HSA antibody. The antibody cascade used for visualisation of ABPs enhanced the sensitivity for nuclear pore proteins (right images) (see text for details). Molecular weights are indicated in kDa.

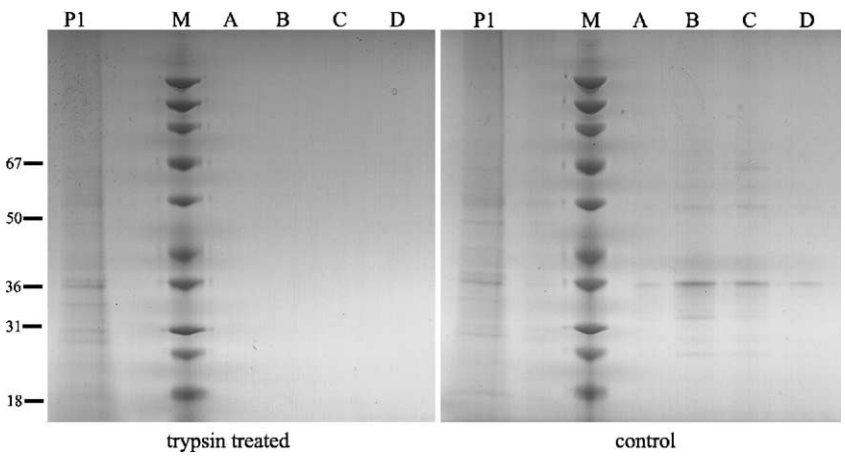

Fig. 7. Coomassie blue stained SDS–PAGE gels of fractions from trypsin treated cells. Fractions P1, A, B, C and D from MCF7 cells which were incubated with trypsin (left gel) and control cells without trypsin treatment (right gel) were subjected to albumin affinity chromatography and proteins were separated with SDS–PAGE. Molecular weights are indicated in kDa.

from CCRF-CEM cells contained hnRNP A2/B1 and hnRNP A1, this protein was also identified in MCF7 cells. HnRNP A3 was found to be the 40 kDa protein in CCRF-CEM cells. All hnRNP contain one or two sequence type RNA-binding domains and are either basic (A and B groups) or acidic (C group). Their function is often deduced from similarity in the amino acid sequence to that of hnRNP A1, rather than from functional studies (see Swiss-Prot entries to the proteins: http://us.expasy.org/sprot/).

We do not know in which form hnRNP are associated with the plasma membrane. We excluded an artefactual contamination with other cell organelles by the following observations: Albumin affinity chromatography of a crude plasma membrane preparation (100,000 *g* sediment) did not yield higher amounts of hnRNP. Further plasma membrane purification by the protocol described herein did not result in loss of hnRNP although other contaminants such as endoplasmic reticulum constituents disappeared. Electron microscopic analyses showed no nucleic acids and only vesicular structures in the density gradient fractions from which hnRNP were isolated. Contaminations with nuclear membranes were excluded in a Western blot with anti-nuclear pore antibodies.

Several authors have shown proteins to occur in several organelles of a cell. Members of different classes of proteins like cytoskeletal components, kinases, transmembrane proteins and cell surface proteins have been identified in other cellular compartments than those they were originally detected in [25]. In many cases cell surface receptors turned out to be identical to intracellular proteins. For example, a 67-kDa laminin-binding protein was identified in a ribosomal subunit [26,27].

The same is true for members of the hnRNP family, which we have here identified by their binding to albumin. Most members of the hnRNP family like hnRNP A2/B1 are nuclear RNA-binding proteins involved in pre-mRNA processing [28]. It was suggested that the up-regulation of hnRNP A2/B1 plays an important role in early stages of carcinogenesis [29]. The overexpression especially of hnRNP B1 has recently been considered to be a marker of cancer in an early stage [30–32]. The molecular functions of many hnRNP proteins have not yet been very extensively studied. Kamma *et al.* [33] showed hnRNP A2 to be distributed not only in the nucleus but also in the cytoplasm of post-mitotic cells. The arginine residues of hnRNP A1 can be methylated and this post-translational modification changes the properties of the proteins [34]. Binding affinity of the methylated form for single stranded DNA decreased. Arginine methylation also facilitates the shuttling of certain hnRNPs out of the nucleus but is not necessary for all hnRNPs [35]. Recently, the Ewing sarcoma (EWS) protein which is a member of the family of RNA-binding proteins that includes hnRNP was reported to be found on the cell surface and not only in the cytosol and the nucleus [36].

An 18 and 31 kDa protein were identified by others as HSA or α-fetoprotein receptor in plasma membrane enriched fractions of leukaemic cells [10]. Antibodies raised against an 18 kDa ABP isolated from endothelial cells cross reacted with a 31 kDa protein before the antibody was purified by affinity chromatography [37]. We found the 18 kDa ABP to be hnRNP B1. This observation could explain the cross reactivity seen by those authors. Studies with antibodies against hnRNP might show a reduced HSA uptake. HSA endocytosis is however a slow process *in vitro* [3,38] and antibodies themselves are often endocytosed, and the receptors recycled, therefore such experiments would be difficult to interpret. Also a further confirmation of the hnRNP on the cell surface with antibody staining is not possible due to the low levels of proteins detected (see Fig. 3 which shows the protein bands isolated from 2×10^8 cells).

We do not yet know what the biological significance of the albumin-binding property of hnRNP proteins is and whether they are involved in albumin endocytosis. The finding however, that hnRNP are localised on the surface of tumour cells is interesting, especially in view of the observation of others [30–32] that hnRNP A2/B1 are overexpressed in lung cancer. The results also underline the growing evidence that many proteins are not only active in a single organelle but appear also in unexpected locations [25].

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