Identification of a new surfactant protein A binding protein at the cell membrane of rat type II pneumocytes

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Antibodies against a type II pneumocyte component were developed by an auto-anti-idiotypic approach using surfactant-associated protein A (SP-A) as the immunogen. The antibodies recognize an SP-A-binding protein (~ 170–200 kDa under non-reducing conditions, 55 kDa under reducing conditions) on the cell membrane of rat type II pneumocytes. One of the antibodies competes with SP-A for binding to type II cells. In immunization

assays *in vitro*, with this antibody as the antigen, anti-SP-A antibodies have been generated. The SP-A-binding cell membrane protein recognized by this auto-anti-idiotypic antibody may be involved in the interaction of extracellular SP-A with the type II pneumocyte and may play a role in the regulation of alveolar surfactant metabolism.

INTRODUCTION

The surfactant-specific glycoprotein, surfactant-associated protein A (SP-A), ~ 650 kDa in its native condition, 28–36 kDa as a monomer, enhances surfactant lipid uptake and inhibits lipid secretion by the type II pneumocyte [1–3]. It seems likely that these processes play a role in the processes regulating surfactant metabolism.

It has been suggested that the interaction of SP-A with the type II pneumocyte occurs through a specific high-affinity receptor on the type II cell membrane [3,4]. The nature of this putative receptor, however, remains controversial.

In this paper, we present the results of experiments using auto-anti-idiotypic antibodies generated by immunizing mice with SP-A. With this technique we identified an SP-A-binding protein, which we call bp55 (which stands for SP-A-binding protein, ~ 55 kDa) on the cell membrane of type II pneumocytes, which is likely to be involved in the interaction of extracellular SP-A with type II pneumocytes.

MATERIALS AND METHODS

Chemicals

Biotin compounds and Protein A- and Protein G-Agarose were obtained from Pierce, Rockford, IL, U.S.A.; streptavidin, ³[H]leucine and supplies for autoradiography were from Amersham, Braunschweig, Germany; elastase was from Boehringer Mannheim, Germany. Fluorescein isothiocyanate (FITC)-labelled Extravidin was from Sigma, St. Louis, MO, U.S.A. All other chemicals were of analytical grade and obtained from major suppliers.

Cell lines

COS-7 cells and HFL (human fetal lung) fibroblasts were obtained from American Type Culture Collection, Rockville,

MD, U.S.A. Both cell lines have been growing in our laboratory for several years. NS-1 myeloma cells and HepG2 cells were a gift from R. Tauber, Berlin. Alveolar macrophages were collected after centrifugation of rat lung lavage and differential adherence (adhering cell fraction) on IgG-coated plates. They were subsequently cultured for 3 days in RPMI + 10 % fetal calf serum.

Pulmonary SP-A

In most experiments purified human alveolar proteinosis SP-A, a gift from Dr. J. R. Wright (University of California at San Francisco) was used. Where indicated, sheep SP-A, purified as described by Hawgood et al. [5] from lavages of sheep lungs obtained from the slaughterhouse, was used.

The purity of each batch was analysed by SDS/PAGE and Western blot. As tested by ELISA and Western blotting with anti-human or anti-sheep IgG no contaminating IgG were detected.

SP-A was biotinylated using sulphosuccinimidobiotin (Sulfo-NHS-Biotin) at a molar ratio biotin:SP-A of 5:1 and a volume ratio of 1:50 at pH 6.3. Thereafter it was extensively dialysed against the appropriate buffers.

The biological activity of all SP-A and biotinylated SP-A (B-SP-A) preparations was tested by surfactant secretion assay as described by Dobbs et al. [1]. Biotinylation did not affect the activity of SP-A in this assay.

Type II cell preparations

Type II pneumocytes from Wistar rats were prepared by elastase digestion and differential adherence on IgG-coated plates according to Dobbs et al. [6]. The purity was 93 ± 3 % and the viability was 94 ± 2 %.

Auto-anti-idiotypic monoclonal antibody production

Three Balb/c mice were immunized with 10 µg of human SP-A in complete Freund's adjuvant each. After 6 weeks of alternate week immunizations with 10 µg of SP-A in incomplete Freund's

Abbreviations used: (B-) SP-A, (biotinylated) surfactant-associated protein A; FITC, fluorescein isothiocyanate; TBS, Tris-buffered saline (20 mM Tris, pH 7.5/150 mM NaCl); DMEM, Dulbecco's modified essential medium.

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adjuvant, the mice were boosted. Five days later their spleen cells were fused with NS-1 myeloma cells by standard techniques [7]. Screening for binding to type II cells was done by cell ELISA on fixed adherent 1 day-old type II cells, according to Harlow and Lane [7]. For screening, the plates were washed twice with PBS + $\text{Ca}^{2+}/\text{Mg}^{2+}$ and then incubated for 60 min in PBS + 1% BSA + 0.05% Tween-20. Thereafter they were washed once with PBS. Supernatant (50 μl) was applied to each well and incubated for 2 h. After washing, 50 μl of peroxidase-labelled anti-(mouse IgG) (Sigma), diluted 1:3000 in PBS + 2% BSA was applied and the plates incubated for 1 h. Staining was developed by applying diaminobenzidine/ H_2O_2 [7].

Positive clones that cross-reacted with SP-A in either ELISA, dot blot or Western blot, were rejected.

After four rounds of limiting dilutions, two clones were finally obtained. In this report results on one, 2H5, producing an IgG_{2a} monoclonal antibody, are presented. These hybridoma cells have been growing in our laboratory for 2 years.

Generation of anti-anti-idiotypic antibodies

2H5 was used as the antigen in immunization assays *in vitro*, as described by Vaux [8]. Briefly, 10 μg of 2H5 was added to a culture of freshly isolated spleen cells of a 3-week old Balb/c mouse and the culture left untouched for 5 days. On day 5, the supernatant was harvested, centrifuged free of cells and used for screening for anti-SP-A activity by both ELISA and dot blot. As controls, immunizations *in vitro* against no antigen or an irrelevant antibody of the same subclass as 2H5, were performed. All immunizations *in vitro* were done at least three times with different mice. In two assays, the loosely adhering cells of the immunization were fused to NS-1 myeloma cells to generate hybridomas as described above.

To screen the supernatants for binding to SP-A, ELISA plates were coated for 16 h at 4 °C with 10 $\mu\text{g}/\text{ml}$ SP-A in 0.1 M NaHCO_3 , pH 10. After blocking with 2% BSA in PBS-0.5% Tween, the wells were incubated with various dilutions of supernatants of immunizations *in vitro* overnight at 4 °C. In some experiments the supernatants were co-incubated with various concentrations of either SP-A or 2H5. After washing, 100 μl of peroxidase-labelled antibody was added. The assays were then processed as described above. The absorbance at 490 nm was measured. Values were corrected for a PBS-Tween blank. For specific details see the Results section and Figure legends.

Biotinylation of the cell membrane and cell solubilization

Cells were cooled to 4 °C on ice. They were then incubated for 20 min with sulphosuccinimidyl-6(biotinamido)-hexanoate (NHS-LC-biotin) (5 mg of biotin per 3×10^6 cells) in PBS at 4 °C to achieve selective labelling of the plasma membrane [9]. Thereafter, excess biotin was removed by washing in PBS + 1% BSA. The cells were lysed in solubilization buffer (20 mM Tris, pH 7.5/150 mM NaCl/1% Nonidet P-40/0.02% NaN_3) and subsequently centrifuged at 40000 g for 30 min.

Immunoprecipitation

A 200–400 μg amount of cell lysate protein and 50–100 μg of 2H5 were used per immunoprecipitation (and per lane) on an 8×11 cm gel (1.5 mm thick).

Preclearing of the cell lysates was done by incubating the supernatants with agarose-bound protein A for 1 h. After removal of the agarose by a short centrifugation, the supernatants were then incubated with 2H5 for at least 1 h at 4 °C. After

centrifugation for 15 min at 14000 g, the mixtures were incubated with agarose-bound protein A for 1 h at 4 °C. Afterwards, the beads were spun down and washed extensively, three times with solubilization buffer, then twice with 20 mM Tris, pH 7.5/150 mM NaCl [Tris-buffered saline (TBS)]. For electrophoresis they were then incubated with electrophoresis sample buffer, vortexed and boiled for 3 min, and then spun sharply at 13800 g in a microfuge. The resulting supernatant was then applied to the SDS/PAGE system [10], transferred onto nitrocellulose [11,12], incubated with alkaline phosphatase-labelled streptavidin (1:3000 in TBS) and visualized with Nitroblue Tetrazolium/bromochloroindolyl phosphate in the presence of 2 mM levamisole.

Ligand blot

For ligand blotting, type II cells were solubilized, immunoprecipitated with 2H5, and the immunoprecipitate applied to gel electrophoresis and blotting as described above. Then the paper was incubated for 30 min in 3% Nonidet P-40 in PBS, subsequently for 2 h in 3% albumin and then overnight with 5 μg of biotinylated SP-A in TBS containing 2 mM Ca^{2+} (pH 7.4). After careful washing of the membrane three times with TBS containing 2 mM Ca^{2+} , the membrane was incubated with streptavidin and bound SP-A was visualized as described above.

Immunoprecipitation of biotin-SP-A-binding proteins/cross-linking

To investigate whether SP-A would bind to other proteins on the cell membrane, 25×10^6 type II cells were incubated with 2 $\mu\text{g}/\text{ml}$ B-SP-A in Dulbecco's modified essential medium (DMEM) containing 2 mg/ml albumin for 60 min at 4 °C. Cells were then washed twice with ice-cold DMEM, centrifuged and incubated for 30 min with 1 mM bis(sulphosuccinimidyl)suberate (BS_3 ; Sigma). After two washes with DMEM, a membrane fraction was prepared as described above. A 300 μg portion of the protein of this cell preparation was used in an immunoprecipitation with 2H5 as described above. Another 300 μg was immunoprecipitated directly with agarose-bound streptavidin. Proteins in the samples were then separated by SDS/PAGE, transferred onto nitrocellulose, incubated with alkaline phosphatase-labelled streptavidin and visualized as described above.

Flow cytometry

For each measurement, 2 $\mu\text{g}/\text{ml}$ biotinylated SP-A, either alone or together with excess unlabelled SP-A (50 $\mu\text{g}/\text{ml}$), 2H5 (25 $\mu\text{g}/\text{ml}$) or an irrelevant antibody of the same subclass as 2H5 (25 $\mu\text{g}/\text{ml}$), were added in ice-cold DMEM + 0.1% BSA + 0.01% NaN_3 to 3×10^6 freshly isolated type II cells kept on ice. After allowing 45 min for binding, the cells were washed twice with medium and then incubated with FITC-labelled Extravidin added at a ratio 1:50 (avidin:medium). For the FITC-labelled antibody, 30 min later, a commercially available, formaldehyde diethylene glycol-based fixing and lysing solution (FACS Lysing Solution, Beckton-Dickinson, Heidelberg, Germany) was added for 10 min. Samples were processed according to the manufacturer's instructions and subjected to a final fixation with 1% paraformaldehyde. Cells (10000 per sample) were analysed on a FACScan flow cytometer (Becton-Dickinson) and net mean fluorescence intensities were calculated.

Protein assay

Protein content was measured by the Bradford assay (BioRad, Richmond, CA, U.S.A.).

Figure 1 (a) Binding of anti-2H5 supernatant to SP-A and (b) inhibition of binding of anti-2H5 supernatant to SP-A by 2H5

(a) SP-A-coated ELISA plates were incubated with the supernatants of immunizations *in vitro* with 2H5, diluted as indicated in PBS-Tween. As second antibody peroxidase-labelled goat anti-(IgM) (1:2000 in PBS-Tween) was added. Colour was developed with α -phenylenediamine and read at 490 nm. Values are absorbance values, means $\pm$ S.E.M. of triplicates, corrected for blanks. (b) SP-A-coated ELISA plates were coincubated with the supernatant of an immunization *in vitro* with 2H5, diluted 1:5 in PBS-Tween and increasing concentrations of 2H5. As second antibody, peroxidase-labelled goat anti-(IgM) (1:2000 in PBS-Tween) was added. Colour was developed with α -phenylenediamine and read at 490 nm. Values are absorbance values, means $\pm$ S.E.M. of triplicates, corrected for blanks.

RESULTS

A monoclonal antibody (2H5) recognizing a type II cell membrane component was generated after immunization with SP-A.

To demonstrate that 2H5 is an antibody generated by the immunized mouse against the idiotype of an anti-SP-A antibody, 2H5 was used as the antigen in three immunizations *in vitro*. The supernatants of these experiments recognized SP-A in ELISA and dot blots. Figure 1(a) shows titrations by ELISA of a supernatant from one representative experiment. The supernatant produced was able to bind SP-A as well as 2H5. Figure 1(b) shows the results of a representative experiment in which binding of the anti-2H5 supernatant to SP-A was inhibited by 2H5. At the highest concentration used, 58% inhibition was observed.

The supernatant of a control immunization *in vitro* with an irrelevant antibody of the same subclass as 2H5 did not show binding to SP-A (absorbance at all dilutions ≤ 0 , after subtraction of blank). Also, addition of another antibody of the same subclass as 2H5 did not mimic its inhibitory effect on binding of the supernatant to SP-A.

From two of these immunizations *in vitro*, after fusion, 13/120 and 14/120 clones respectively reacted with SP-A as well as with 2H5. From these, after three limiting dilutions three hybridomas could be retained which produced antibodies that strongly react with SP-A in ELISA and dot blot. While further characterization of these clones is still ongoing, this strongly suggests that the anti-type II cell membrane antibodies are directed against the

Figure 2 Identification of the protein bound by 2H5 as an SP-A-binding protein

(a) Western blot of biotinylated type II cell membrane proteins (arrows) after immunoprecipitation with monoclonal antibody 2H5. Lane 1, non-reducing conditions; lane 2, reducing conditions. (b) Ligand blot of the protein immunoprecipitated by 2H5 with biotinylated SP-A. Lane 3, non-reducing conditions; lane 4, reducing conditions. Molecular mass standards (kDa) are indicated.

idiotype of anti-SP-A antibodies made by the same animal that produced 2H5.

Identification of the antigen recognized by the antibodies

Figure 2 shows the results of the immunoprecipitation of biotinylated type II cells with 2H5. 2H5 recognizes a cell surface antigen with a molecular mass of 170–200 kDa under non-reducing conditions. Reduction of disulphide bonds shows that this protein complex is composed of subunits of approx. 55 kDa.

The protein recognized is not an immunoglobulin. All immunoprecipitations were done after careful preclearing of the sample with protein A and in some cases protein G. Also, immunoprecipitation without added antibody did not produce any bands.

To check whether the biotinylation procedure labelled all components of the protein identified, type II pneumocytes were cultured for 18 h in the presence of [^3H]leucine (3.2 μCi per 10^6 cells; 141 Ci/mmol). Preparation of the membrane fraction and immunoprecipitation and gel electrophoresis on SDS/PAGE were done as described above. Subsequent autoradiography showed no difference in labelling pattern between the experiments using biotinylated membrane fraction and those using radiolabel (results not shown).

Immunoprecipitation of unbiotinylated type II pneumocyte cell proteins with 2H5 produced no bands that could be visualized by alkaline phosphatase-labelled streptavidin after Western blot, demonstrating that the antibody does not recognize biotin (results not shown).

Control immunoprecipitations were also performed with (1) no antibody, (2) mouse serum, (3) an irrelevant antibody of the same subclass as 2H5 and (4) no avidin.

Cell specificity

Alveolar macrophages, HFL lung fibroblasts, NS-1 myeloma cells, kidney-derived COS-7 cells as well as liver-derived HepG2 cells were biotinylated, lysed in solubilization buffer and the

Figure 3 bp55 is the major SP-A-binding protein on type II cell membranes

Western blot of immunoprecipitated type II cell membrane proteins cross-linked to biotinylated SP-A. After incubation with type II cells, biotinylated SP-A was cross-linked to the bound membrane proteins. The cells were then solubilized and immunoprecipitated with either agarose-bound streptavidin (a) or 2H5 and agarose-bound protein G (b), as described in the Materials and methods section. After SDS/PAGE (15% polyacrylamide) under reducing conditions and Western blot, biotinylated SP-A linked to cell membrane components was visualized by streptavidin alkaline phosphatase. In both lanes the same band at 83–85 kDa is seen. In this particular blot, faint bands of higher molecular mass in both gels are the result of incomplete reduction of disulphide bonds in this sample. Molecular mass standards (kDa) are indicated.

lysates subjected to immunoprecipitation with 2H5 as described above. All experiments were repeated at least twice. No immunoprecipitating bands from these cells could be detected, suggesting type II cell specificity (results not shown).

SP-A binding

As shown in Figure 2, biotinylated SP-A bound to bp55 after blotting of the immunoprecipitated protein onto nitrocellulose.

In an alternative approach, type II cells were incubated at 4 °C with biotinylated SP-A. Bound SP-A was then cross-linked to the binding proteins on the cell membrane by addition of the cross-linker BS₃. Thereafter the cells were solubilized. Solubilized protein (300 µg) was immunoprecipitated with 2H5 and analysed by SDS/PAGE and Western blot. One single band with an apparent molecular mass of approx. 83–85 kDa, corresponding to bp55 linked to monomeric SP-A, was found. Since the cross-linking approach used was unspecific, to investigate whether SP-A would have been linked to other proteins another 300 µg of cell lysate protein was immunoprecipitated with streptavidin coupled to agarose. This approach selects for any biotinylated proteins. In the subsequent Western-blot assay, a band at 83–85 kDa was found, corresponding exactly to the band seen with 2H5. In some blots, a faint 28–36 kDa triplet, corresponding to monomeric SP-A, could be seen. In our assay, we could not detect other SP-A-linked proteins (Figure 3). Since the 85 kDa band was seen both by immunoprecipitation with 2H5 and streptavidin, it cannot be SP-A cross-linked to itself. The higher molecular mass bands seen at a similar position in both panels of Figure 3 were not seen on repeat blots with either the same starting material or from experiments with different preparations and are therefore most likely due to incomplete reduction of disulphide bonds in these particular samples.

Binding of labelled SP-A to the type II cell can be inhibited by 2H5. 2H5 (10 µg/ml) inhibited binding of labelled SP-A by 71 %, as demonstrated by the difference in fluorescence versus control (biotinylated SP-A bound to type II cells as detected by FITC-labelled avidin). Increasing the amount of 2H5 to 100 µg

Figure 4 2H5 competes with SP-A for binding to type II cells

Cells were incubated with biotinylated SP-A and different concentrations of 2H5 antibody. Subsequently, they were incubated with Extravidin and the fluorescence intensity was analysed by flow cytometry. Results are given as fluorescence intensity as a percentage of the value obtained with biotinylated SP-A alone after subtraction of the fluorescence intensity of avidin alone (means $\pm$ S.E.M., $n = 3$).

decreased the fluorescence further to 23 % of control (77 % inhibition) (Figure 4).

Simultaneously, controls were run for autofluorescence (no Extravidin added) and for unspecific binding of avidin (Extravidin added to cells without biotinylated SP-A). An irrelevant IgG decreased fluorescence by a maximum of 8.4 % at the highest concentration used (100 µg/ml).

DISCUSSION

To study ligand–receptor interactions in which little information on the receptor is available, such as the interaction of SP-A with the type II cell membrane, the anti-idiotypic antibody approach can be a powerful tool. Most often anti-idiotypic antibodies are raised by a sequential approach, first raising antibodies against the ligand, then immunizing with these antibodies. This way it can be checked that the second-generation antibody reacts both with the first-generation antibody and the ligand. The auto-anti-idiotypic antibody approach we chose in order to obtain type II cell-binding antibodies was pioneered by Cleveland and co-workers [13,14]. The theory behind this approach, first proposed by Jerne [15], states that upon exposure to a foreign antigen the immune system will generate, as part of a whole network of antibodies, not only antibodies against the antigen but also antibodies against the idio type of these first antibodies. Whereas under normal circumstances this anti-idiotypic response will be suppressed relatively early, Cleveland and co-workers demonstrated that by choosing the time of fusion carefully, this property can nevertheless be employed to generate autoanti-idiotypic antibodies.

The disadvantage of our approach was that the first-generation antibody against SP-A with the relevant idio type was not available. Therefore, to prove that the antibodies we obtained were indeed anti-idiotypic, in a second immunization round antibodies against 2H5 were raised in immunization assays *in vitro*. The fact that anti-SP-A activity could be demonstrated in the supernatants of the immunizations *in vitro* and monoclonal antibodies directed against SP-A could be generated from these immunizations, argues strongly that 2H5 is indeed directed against the idio type of an anti-SP-A antibody generated by the

same animal that made 2H5 and 5F2. It also suggests that the idiotype of 2H5 might be spatially similar to a binding site on SP-A for a type II cell membrane site.

The results of the ligand blotting as well as the binding experiments which found bp55 to be the only protein cross-linked to biotinylated SP-A, suggest that bp55 does bind to SP-A. This, together with the results from the flow cytometry experiments showing that the binding of SP-A to the type II cell can be competitively inhibited by 2H5, leads us to suggest that 2H5 does in fact bind to the same site on type II cells as SP-A. The anti-SP-A antibodies generated from the immunizations *in vitro* should help to localize the exact binding site on SP-A for bp55.

The antibody 2H5, which is directed against the SP-A-binding site on bp55, will immunoprecipitate this protein even if the latter has already bound SP-A. bp55 in its native state consists of several 55 kDa subunits. It is possible that more than one of these can bind to SP-A or 2H5, thus allowing simultaneous binding of 2H5 and SP-A to the same complex. The disulphide bonds, which link these subunits, in our assay were cleaved only after immunoprecipitation, which explains why labelled SP-A appears in our immunoprecipitation blots. The competition of 2H5 and B-SP-A for binding to type II cells is compatible with this model.

Recently, using a sequential approach, Strayer et al. identified another SP-A-binding protein [16]. The second major difference between their approach and ours was the choice of immunogen. While we used purified SP-A to generate our antibodies, they used a complex mixture of surfactant components. While for one of the antibodies they used SP-A as the original antigen, to develop the second antibody they used a porcine surfactant preparation (Curosurf) which lacks SP-A but contains the smaller hydrophobic proteins SP-B and SP-C. Nevertheless, after generating antibodies against these primary antibodies, they identified an SP-A-binding protein [17]. It is possible, but not yet shown, that the natural ligand of this binding protein is a complex of SP-A with one or both of the smaller proteins. The reported molecular mass (30 kDa) of this binding protein is different from that of bp55 (50–55 kDa).

In summary, we have identified an SP-A-binding protein on the cell membrane of type II pneumocytes which might be involved in surfactant metabolism. Investigations are under way

to elucidate the possible role of bp55 in surfactant metabolism. Preliminary evidence suggests that this SP-A-binding protein might be involved in surfactant endocytosis. The antibodies described should be valuable tools in helping us understand this fascinating system.

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