Identification and Characterization of P63 (CKAP4/ERGIC-63/CLIMP-63), a Surfactant Protein-A Binding Protein on Type II Pneumocytes


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Running title: P63 is a functional binding protein for SP-A

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Abstract

Surfactant protein (SP)-A binds to alveolar type II cells through a specific high affinity cell membrane receptor, although the molecular nature of this receptor is unclear. In the present study we have identified and characterized an SP-A cell surface binding protein by utilizing two chemical cross-linkers: Profound Sulfo-SBED protein-protein interaction reagent and Dithiobis succinimidylpropionate (DSP). Sulfo-SBED-biotinylated SP-A was cross-linked to the plasma membranes isolated from rat type II cells and the biotin label was transferred from SP-A to its receptor by reduction. The biotinylated SP-A-binding protein was identified on blots by using streptavidin-labeled horseradish peroxidase. By using DSP, we cross-linked SP-A to intact mouse type II cells and immunoprecipitated the SP-A-receptor complex using anti-SP-A antibody. Both of the cross-linking approaches showed a major band of 63 kDa under reduced conditions that was identified as the rat homologue of the human type II transmembrane protein P63 (CKAP4/ERGIC-63/CLIMP-63) by matrix-assisted laser desorption ionization and nano-electrospray tandem mass spectrometry of tryptic fragments. Thereafter, we confirmed the presence of P63 protein in the cross-linked SP-A-receptor complex by immuno-probing with P63 antibody. Co-immunoprecipitation experiments and functional assays confirmed specific interaction between SP-A and P63. Antibody to P63 could block SP-A-mediated inhibition of ATP-stimulated phospholipid secretion. Both intracellular and membrane localized pools of P63 were detected on type II cells by immunofluorescence and immunoblotting. P63 co-localized with SP-A in early endosomes. Thus, P63 closely interacts with SP-A and may play a role in the trafficking or the biological function of the surfactant protein.

Key words: Lung, surfactant secretion, cross-linking, immunolocalization
Introduction

Pulmonary surfactant is a complex mixture of phospholipids and proteins. The major protein component, surfactant protein-A (SP-A), is a 34–36 kDa member of the calcium-dependent lectin family of proteins that facilitates the surface tension-lowering properties of surfactant phospholipids in the alveolus, regulates surfactant phospholipid synthesis, secretion, and recycling, and also plays an important role in pulmonary host defense (18). The mechanism of SP-A binding to lung type II cells is not completely understood, yet it appears to be critical for the cell-associated functions of SP-A. Binding of SP-A to type II cells was found both saturable and specific, suggesting that this interaction is mediated through a receptor on the cell surface (24, 55).

Thus far investigators have identified SP-A receptor(s) by utilizing either anti-idiotypic antibodies (43, 46) or SP-A affinity column chromatography (9, 21). Using an idiotypic approach, one group has identified three proteins with apparent $M_r$ of 30, 52, and 60 kDa (44) and the 30-kDa protein was shown to be associated with the regulation of secretagogue-stimulated surfactant secretion (45, 46)). On the other hand, another group using similar strategy identified a 55-kDa protein (43) shown to be involved in surfactant endocytosis by type II cells (53, 55). Using ligand affinity chromatography, three proteins with apparent $M_r$ of 65, 55, and 50 kD were identified by a third group on type II cell surface and the 50 kDa protein was shown to be involved in phospholipid uptake (21). A fourth group described a SP-A receptor from U937 macrophages with a molecular mass of 210 kDa using SP-A affinity column chromatography (9). This receptor was detected in both alveolar macrophages and type II epithelial cells and was found to block the SP-A-mediated inhibition of phospholipid secretion by type II cells. From the these studies, it seems that the SP-A receptor(s) on type II cell surface
consists of 30, 50-55, or 60-65 kDa polypeptides. The cDNA and the deduced amino acid sequence of the 30 kDa protein are described elsewhere (44, 46) however the molecular nature of the other components of the receptor(s) is still not clear. Thus far, it is also not completely understood how these proteins are related, if they are separate entities or/and components of a receptor complex.

The present investigation identifies a 63 kDa type II transmembrane protein as a functional SP-A receptor on type II cells. Unlike using an indirect idiotypic approach or chromatography we directly cross-linked SP-A to isolated plasma membranes as well as intact type II cells. We were able to successfully reproduce our cross–linking results by using two different cross linkers and identified the SP-A binding protein as P63/ERGIC 63 by LC-MS/MS analysis and NCBI database search. Co-immunoprecipitation experiments and functional assays provided firm evidences that reflected specific interaction between SP-A and P63. This is the first study that has utilized direct cross-linking of SP-A to isolated plasma membranes and intact type II cells and demonstrates direct involvement of P63 in SP-A mediated phospholipid homeostasis in type II pneumocytes.
Material and Methods

Isolation of pneumocytes

Type II cells were isolated from adult male Sprague-Dawley rat lungs according to the procedure of Dobbs et al. (11) as previously described (7, 8). Briefly, after perfusion via the pulmonary artery and lavage through a tracheal cannula, the lungs were digested with elastase and minced in the presence of DNase (Sigma-Aldrich) and fetal bovine serum (ICN Biochemicals). The cells were separated by filtration and enriched for type II cells by plating on rat IgG (Sigma-Aldrich) coated petri dishes that served to remove most contaminating macrophages. After overnight culture and removal of nonadhered cells, the purity of the type II cell preparation was 90-98%.

Type II cells were isolated from C57Bl6 mouse lungs using dispase as described previously (6, 50, 52). Briefly, perfused lungs were digested with dispase, treated with DNAase I and filtered through nylon mesh (100 µm, 40 µm, and 25 µm size). Macrophages were removed from the cell preparation by plating on mouse IgG-coated Petri dishes. The non-adherent cells were seeded on 100 mm cell culture dishes in 10% FBS at 37°C for 1 h twice to remove fibroblasts. The final cell isolates were seeded on Type I collagen-coated 35 mm dishes in Ham’s F12 culture medium supplemented with 15 mM HEPES, 0.8 mM CaCl2, 0.25% BSA, 5 µg/ml insulin, 5 µg/ml transferrin, 5 ng/ml sodium selenite, and 2% mouse serum. After 24 h, more than 95% of the isolated cells were type II cells.

Alveolar macrophages were isolated by bronchoalveolar lavage and centrifugation. The resultant preparation was > 98% macrophages (3).

Preparation of cell lysates
Type II cells or macrophages were lysed on ice in phosphate buffered saline, pH 7.4, containing 0.1% triton X-100 and protease inhibitor cocktail (Roche Diagnostics) for 10 min., and sonicated on ice for two 15-s cycles. Thereafter, cell lysates were stored at –20°C until use. Cell protein content was measured by the method of Lowry et al. (26) using BSA as a standard.

Isolation of plasma membrane proteins

Plasma membranes were isolated as described before (7, 15). Briefly, rat Type II cells were suspended in 0.32 M sucrose in cold HEPES-Tris buffer, pH 7.4 followed by three 15 sec. sonication cycles on ice at an interval of 5 min. Thereafter, cell lysates were layered over a discontinuous sucrose gradient containing 0.5, 0.7, 0.9, and 1.2 M sucrose, and centrifuged at 40,000 X g for 1 h at 4°C. Plasma membrane fraction was collected from the 0.9-1.2 M sucrose interface, diluted to 0.32 M sucrose with HEPES-Tris buffer, and centrifuged at 95,000 X g for 30 min (7, 15). The plasma membrane pellet was resuspended in PBS, pH 7.4 containing all above protease/phosphatse inhibitors and 0.1% Triton X-100 followed by vortexing. Aliquots were stored at -80°C until use.

Chemical cross-linking and immunoprecipitation

Chemical cross-linking using Profound Label Transfer Sulfo-SBED Protein-Protein Interaction reagent: All steps were performed under dark condition. 0.6 mM SP-A and 6mM Sulfo-SBED (Pierce Biotechnology) pre-dissolved in dimethyl sulfoxide were incubated in dark for 30 minutes under constant stirring. Bovine serum albumin (BSA) was used as a control protein for labeling. The reaction mixture was loaded on a desalting column (Bio-Spin P-6 from Bio-Rad) and quick spun for 5 sec. Collected filtrate was mixed with 100 µg of plasma
membrane proteins followed by illumination at 365 nm in the presence of 1.75mM CaCl₂ for 20 minutes. Cross-linked samples were analyzed thereafter by SDS-PAGE on 3-8% Tris-Acetate or 4-12% Bis-Tris gradient gels (Invitrogen) under reduced (100 mM DTT) and non-reduced conditions for both test and control (BSA) reactions followed by detection of biotinylated samples on Western blots using HRP-labeled streptavidin.

Chemical cross-linking using dithiobis succinimidylpropionate (DSP): Mouse type II cells cultured for 36 hrs in 35mm dishes (1.5 X 10⁶ cells~120µg protein/ dish) were washed three times with PBS, pH 7.4. SP-A (1 or 5µg) and CaCl₂ (2mM) were added to the washed cells and incubated at 37°C for 1 hr. After another round of washing with PBS, pH 7.4, cross-linking was performed by incubating with DSP (Pierce Biotechnology; 1 or 2 mM) pre-dissolved in dimethyl sulfoxide at room temp for 30 min. Preliminary experiments showed that both concentrations of SP-A and/or DSP were equally as effective. Thus 1µg SP-A and 1mM DSP were used in subsequent studies. After washing with PBS, cells were scraped in situ with 500 µl of radioimmune precipitation buffer (RIPA) containing 20 mM Tris HCl, pH 7.5, 150 mM NaCl, 2mM EDTA, 1% triton X-100 and protease inhibitor cocktail (Roche). The cell lysate was incubated with rabbit anti human SP-A antibody overnight at 4°C under gentle rotation. Thereafter, incubation mixture was centrifuged at 13, 000 rpm for 5 minutes at 4°C and Protein A immobilized agarose beads (Sigma) were added to the supernatant and incubated for 6 hrs in cold. After washing the beads three times with RIPA buffer, the bound proteins were eluted from the agarose beads by incubation and boiling with Laemmli (25) sample buffer in the presence 2-mercaptoethanol. The eluted proteins were loaded onto 4-12% Bis-Tris gradient SDS-PAGE gels (Invitrogen) for electrophoresis followed by Western blotting.
To investigate the interaction of P63 and SP-A in solution, co-immunoprecipitation experiments were performed as described above using protein A-agarose beads, rabbit anti-human SP-A (Chemicon), rabbit anti-human P63 or mouse anti-human P63 (40) antibodies.

SDS-PAGE and Western blotting

Proteins from the cell lysates or cross-linked samples were resolved on 3-8% Tris-Acetate or 4-12% Bis-Tris gradient gels (Invitrogen) by SDS-PAGE under reducing or non-reducing conditions (25). Proteins were electrophoretically transferred to nitrocellulose membrane at room temperature overnight (47, 48). The membrane was transiently stained with Ponceau S to monitor the transfer efficiency of the proteins and was blocked with 4% Carnation nonfat dry milk in Tris-buffered saline (TBS) at room temperature for 1 hr on a shaking platform. The nitrocellulose membrane was then incubated with primary antibodies. After washing with TBST (TBS with 0.1% Tween 20), blots were incubated with appropriate horseradish peroxidase labeled secondary antibodies (Amersham Biosciences). After washing three times with TBST, two times with TBS and a final rinse with de-ionized water, blots were incubated with ECL Chemiluminescence reagent (Amersham Biosciences) for 1 minute and developed on Hyperfilm ECL X-ray films (Amersham Biosciences). In order to reprobe the same membrane, blots were stripped by incubation at 55°C for 30 min in a buffer that contained 62.5mM Tris, 2% SDS and 100 mM 2-mercaptoethanol. After washing 4 times with TBST, blots were blocked with 4% milk and were re-probed with another antibody.

Western blot analysis by Odyssey was performed according to the manufacturers instruction (LI-COR Bioscience, Lincoln, Nebraska). Briefly, nitrocellulose membranes were blocked in 1:1 diluted Odyssey blocking buffer at room temperature for 1 hr. The blots were
incubated at 4°C overnight with P63 polyclonal antibody at 1:1000 dilution in blocking buffer containing 0.1% Tween-20. The membranes were washed four times in TBST and were incubated with IRDye 800-conjugated goat anti-rabbit antibody (Rockland Immunochemicals Inc, PA) for 1 hr at room temperature at a dilution of 1:5000. Membranes were scanned on the Odyssey infrared scanner after 5 washes in TBST buffer.

**Mass spectrometry and Database search**

SDS-PAGE gels were stained with mass spectrometry compatible silver stain (Silver Quest from Invitrogen) and the bands were excised. The gel pieces were washed, reduced, alkylated and digested in situ with trypsin. Peptides thus generated were injected into a nano capillary reverse phase HPLC coupled to a nano-electrospray ionization source of ThermoFinnigan LCQ quadrupole ion trap mass spectrometer. This mass spectrometer measured peptide masses and then fragmented individual peptides to produce MS/MS spectra of fragments that reflected the peptide sequence. The MS/MS spectra were run against NCBI non-redundant database. The resulting MS/MS matches and sequences were than reviewed for the fidelity of the matched spectra. This analysis was performed at the Wistar Institute Proteomics Facility of the University of Pennsylvania.

**Purification of SP-A**

Bronchoalveolar lavage fluid was obtained from lungs of alveolar proteinosis patients following therapeutic lavage at the Hospital of the University of Pennsylvania. Cellular material was removed by centrifugation, and surfactant was purified by density gradient centrifugation, followed by dialysis and lyophilization as described previously (2). We obtained SP-A
according to the method of Hawgood et al. (16), using 1-butanol and \(\text{-D-glucopyranoside}\) extraction, dialysis, and microconcentration, as described (2). The purity of the SP-A preparation was monitored by SDS-PAGE (25).

**Preparation of Liposomes**

Lipids were obtained from Avanti Polar Lipids (Birmingham, AL). Unilamellar liposomes were prepared from DPPC, egg PC, egg phosphatidylglycerol, and cholesterol (molar ratio, 10:5:2:3) using “The Extruder” (Lipex Biomembranes) according to the directions of the manufacturer.

**Phosphatidylcholine (PC) secretion**

After isolation, type II cells were incubated overnight with 0.5 \(\mu\text{Ci/dish}\) of \([\text{methyl-}^3\text{H}]\)-choline (Amersham, Arlington Heights, IL) in MEM containing 10% FCS to label cellular phospholipids. Cells were washed extensively and incubated for 30 min in MEM. One set of cells was harvested served as control for phospholipid secretion associated with the medium change, as described previously (4, 44). The remaining cells were pre-incubated with or without non-immune IgG or mAb P63 (100 \(\mu\text{g/ml}\), 15 min), followed by incubation without or with SP-A (0.1 \(\mu\text{g/ml}\), 15 min), followed by addition of adenosine triphosphate (ATP, 1mM, Sigma, St. Louis) for two hours. The media was removed and centrifuged to remove detached cells. Methanol was added to the cell monolayer, and the cells were scraped from the dish. The cells and the media were extracted using the Bligh and Dyer method (5, 9). The amount of phospholipid secretion was calculated as the percentage of lipid counts per minute in the
medium, relative to the total counts per minute lipid present in the cells plus the medium. All experiments were performed in duplicate or triplicate, and the values averaged.

**Immunofluorescence Confocal Microscopy**

The distribution of P63 was investigated in intact and permeabilized type II cells cultured for 24 hrs on glass cover slips as well as in mouse lung cryo-sections. Intact rat type II cells were rinsed with PBS and stained with wheat germ agglutinin Alexa 594 at 1 ug/ml for 15 min at room temperature and washed 3 times with PBS. After this, cells were fixed with 2% paraformaldehyde for 20 min washed and incubated with P63 antibody overnight at 4°C. The next day, the cells were washed and incubated for 1 hour with the secondary antibody labeled with Alexa 488 after which the cells were washed, mounted, and viewed by confocal microscopy.

Other preparations of type II cells were permeabilized by fixation with either cold methanol-acetone (1:1 in volume) for 5 min or 4% paraformaldehyde for 30 min at 4°C. After washing and treatment with sodium borohydride, the samples were blocked with a mixture of BSA and normal goat serum for 1 hr at room temp followed by incubation with non-immune mouse or rabbit IgG (control) or primary antibody at room temp for 2 h. The primary antibodies used were rabbit anti human SP-A(14); rabbit polyclonal antibody to P63 (kindly supplied by Dr. Jack Rohrer, Univ. of Zurich, Zurich, Switzerland); mouse monoclonal antibodies to P63 (Alexis Biochemicals, San Diego, CA (40); mouse anti-rat ABCA3 (monoclonal antibody 3C9, labels lamellar body membranes in type II cells, (ref. (29)) and anti-rat EEA-1 (early endosome marker, (34). The samples were washed three times with PBS and then incubated with Alexa 488 (green)
and/or Alexa 594 (red) labeled goat anti-mouse or -rabbit IgG (Molecular Probes) at room temp for 1 h. Thereafter, samples were observed under a confocal microscope (Bio-Rad).

**Statistics.**

Data is reported as mean + S.E. Statistical comparisons were performed with SigmaStat (Jandel Scientific) using a standard t-test. Results were reported as statistically significant differences at $P$ values < 0.05.
Results

Identification of SP-A receptor by chemical cross-linking

Chemical cross-linking followed by mass spectrometric analysis of the cross-linked species has been utilized successfully to elucidate ligand-receptor interactions (17, 50, 51). We cross-linked SP-A to type II cell plasma membranes in order to identify putative SP-A receptor proteins. Sulfo-SBED is a commercially available trifunctional crosslinking reagent that allows affinity-based enrichment of cross-linked species. Sulfonated NHS ester on this reagent reacted with primary amines on SP-A and, thereby, biotinylated SP-A in the first step. During the second step, photosensitive aryl azide moiety linked by a disulfide bond on this cross-linker captured the putative receptor from the plasma membrane lysate because of the receptor’s affinity and the closeness (within a distance of 10Å) to SP-A. After reduction of the disulfide bond with 100 mM DTT, biotin label was transferred to the captured protein(s) and revealed the presence of a protein with a molecular mass of 63 kDa and minor amounts of two proteins of 90 and 35 kDa (Figure 1, left). By re-probing the same blots with anti-rat SP-A antibody we confirmed that the 63 and 35 kDa bands did not represent the monomer or dimer of SP-A (Figure 1, right).

Another cross-linking agent, dithiobis succinimidylpropionate (DSP), was used to investigate an SP-A binding protein in intact mouse type II cells. This agent can be added directly to the tissue culture dishes and does not need UV illumination for cross-linking (50, 51). DSP, or Lomant’s reagent, is a homobifunctional cross-linker that has two NHS esters and a disulfide bond. Mouse cells were used since the mouse genome is characterized, facilitating the identity of proteins that might cross-link to SP-A. SP-A was cross-linked with intact mouse type II cells cultured on plastic dishes in the presence of calcium using DSP. The complex of SP-A
and bound protein(s) was immunoprecipitated with rabbit anti-human SP-A, and separated using gel electrophoretic procedures. Subsequent analysis of silver stained gels resulted in the identification of three proteins of 30, 50 and 63 kDa (Figure 2A, Lane 2). Western blots of similar gels probed with anti-SP-A antibody confirmed the 50 kDa band as the rabbit anti-SP-A IgG antibody as the band reacted with the goat anti-rabbit IgG secondary antibody (Figure 2B, Lanes 1 and 2, black arrow) whereas the 30 and 63 kDa silver stained bands (Fig 2A, Lane 2) were not labeled by the anti-SP-A antibody (Fig 2B, Lane 2). This identification of the 50 kDa band as rabbit IgG was further confirmed by separate immunoprecipitation experiments (Figure 3A, bottom, center lane, black arrow). The presence of a 63 kDa protein in the results of both cross-linking experiments indicated the specific interaction of this protein with SP-A. Mass spectrometry of the tryptic digest of the 63 kDa band and database search confirmed the presence of the transmembrane protein, P63 which is the mouse homologue of ERGIC 63 (endoplasmic reticulum and golgi intermediate compartment, Table 1). The six peptide sequences from a single database entry showed 100% match to the MS/MS spectra and confirmed the protein identity at a high confidence level. The 63 kDa protein band in the cross-linked complex was labeled with the polyclonal anti-P63 antibody (Fig. 2C, Lane 2), thus confirming the identity of the protein as P63/ERGIC 63.

**Co-immunoprecipitation of P63 and SP-A**

Protein-protein interaction between P63 and SP-A in vitro were studied by co-immunoprecipitation using rabbit polyclonal antibody to SP-A or mouse monoclonal antibody to P63. Anti-SP-A antibody immunoprecipitated P63 from rat type II cell lysates (Figure 3A, Top). Re-probing of the same blot with SP-A antibody showed a very strong 50-60 kDa band in the
type II cell lysate, likely a mixture of IgG and SP-A dimer (Figure 3A, Bottom, lane 2). Bands of 90, 120 and 150 may be higher molecular weight oligomers of SP-A, as shown in lanes containing isolated human SP-A (Fig 3A, Bottom, lane 3 and especially in Fig 3B, Top, lane 3). P63 monoclonal antibody immunoprecipitated SP-A from both isolated plasma membranes and whole type II cells (Figure 3B, Top) and re-probing of the same blot confirmed the presence of P63 in the immunoprecipitate (Figure 3B, Bottom).

Identification and localization of P63 in type II cells

Immunoblots of lysates from lung cells probed with polyclonal antibody to P63 revealed that P63 is expressed in type II cells but absent from alveolar macrophages (Figure 4). Reducing agents did not alter the mobility of the protein ruling out the presence of lower molecular weight disulfide-linked protein components.

In order for P63 to interact with SP-A in the alveolar space of the intact lung, P63 should be on the cell surface. As shown in Figure 4, right, and Fig 5A, plasma membranes isolated from type II cells demonstrated the presence of P63. Further experiments demonstrated that P63 also was present in the plasma membranes of A549 cell, a human lung adenocarcinoma with some type II cell characteristics (Fig 5B). Caveolin 1 is a protein enriched in the plasma membrane and is commonly used to validate the identity of this fraction. Although type II cells do not have caveolin, the plasma membrane fraction from the A549 cells contained caveolin (Fig 5B). Finally, P63 was demonstrated in the plasma membrane of pneumocytes by immunocytochemical techniques. Type II cells grown on glass slides incubated were incubated with wheat germ agglutinin to label the plasma membrane, then fixed and incubated with anti-
P63 antibody. Fig. 5 (C-F) demonstrates the co-localization of P63 and wheat germ agglutinin on the outer surface of type II cells.

Permeabilized rat type II cells showed an ER-like distribution of P63 which occasionally co-localized with ABCA3, a protein marker for lamellar body membranes (Figure 6, A-C). Since P63 is a putative receptor for SP-A, there should be intracellular compartments that are shared by both SP-A and P63. Double labeling of type II cells using antibodies against SP-A and P63 showed co-localization of the two on the outside of some vesicular structures (Figure 6, D-F). After uptake by type II cells, SP-A has been shown to transport towards early endosomal compartments (52). Using an antibody to EEA-1, a protein marker for early endosomal vesicles, we confirmed that SP-A colocalized with EEA-1 (Figure 7, D-F). In addition, P63 also was partially co-localized in the same early endosome intracellular compartment (Figure 7, A-C).

Expression and distribution of P63 in the lung was investigated by immunofluorescence and confocal microscopy in mouse whole lung cryosections. Monoclonal antibody 3C9 (mAb 3C9), which labels the lamellar body membrane ATP-binding cassette transporter A3 (ABCA3), was used as a marker of type II cells (29, 37). Type II cells, identified as ABCA3 positive, and other cells that did not stain for ABCA3, possibly type I cells, showed high intracellular expression of P63 (Figure 8, A-F).

*Antibody to P63 inhibited SP-A function*

SP-A has been shown to inhibit PC secretion through interaction with a specific cell surface receptor (22, 28). To determine whether a monoclonal antibody to P63 (mAb P63) would block the interaction of SP-A with its receptor, we examined the ability of the anti-P63 monoclonal antibody to interfere with one of the biologic activities of SP-A, the inhibition of
ATP-stimulated PC secretion from type II cells. Neither mAb P63 (Fig 9A and B) nor non-immune IgG (Fig 9B) affected basal or ATP-stimulated PC secretion when incubated with type II cells in the absence of added SP-A. P63 mAb inhibited the ability of SP-A to block secretagogue stimulated PC secretion in a dose-dependent manner (Figure 9A). SP-A (0.1 ug/ml) reduced ATP-stimulated PC secretion by 67%. Addition of mAb P63 resulted in a concentration dependent reversal of the effect of SP-A on stimulated secretion (Fig 9A). At 100 ug mAb/ml, the reversal of the SP-A affect was 50% while IgG had no effect (Fig. 9B).

Discussion

Earlier investigators have identified receptor(s) for SP-A using various approaches. The molecular nature and the functional roles played by this receptor, however, remain only partially understood. Unavailability of a highly specific antibody to the receptor and, size and stickiness of SP-A itself are some practical hindrances in designing an efficient experimental approach to reveal the molecular nature of the receptor. Among the different methods used to unravel ligand-receptor interactions, chemical cross-linking followed by identification of the cross-linked peptides by mass spectrometry has proven especially useful in dynamic and complex systems (10, 48, 51, 52, 55). Using this approach we identified P63 as an SP-A binding protein on type II cells. Several lines of evidence support this conclusion: 1) The cross-linking reagent Sulfo-SBED linked SP-A to a 63 kDa protein in a preparation of isolated rat type II cell plasma membranes. 2) Immunoprecipitation after cross linking of SP-A to intact mouse type II cells in culture identified a 63kDa protein in a Silver-stained gel. 3) Mass spectrometry of tryptic digests of the 63 kDa protein eluted from the gel followed by a data base search positively identified the peptides as segment components of P63/ERGIC 63 protein. 4) The
immunoprecipitate of the SP-A-mouse type II cell cross-linked preparation reacted with anti-P63 antibody confirming the identity of the cross-linked protein as P63/ERGIC63. 5) Treatment of type II cell lysates with anti-P63 antibody co-precipitated SP-A. 6) By immunohistchemical analysis, SP-A and P63 were co-localized and both proteins are found in the early endosome compartment of type II cells. 7) antibodies to P63 interfered with one of the biological activities of SP-A, the ability of SP-A to block secretagogue-stimulated DPPC secretion from type II cells. Since human SP-A was used with rodent type II cells in this study, possible affects due to species variation may influence the results. However, to date, human and rat SP-A interactions with rat type II cells felt to involve an SP-A receptor have shown no differences between the two species of SP-A. Thus, neither the binding characteristics of SP-A to type II cells (55) nor to the ability of SP-A to stimulate the uptake of phospholipid liposomes into type II cells (23) differed between SP-A isolated from humans or rats.

The P63/ERGIC63 protein is a non-glycosylated, reversibly palmitoylated type II transmembrane protein. Transmembrane proteins of type II have the N-terminus exposed to the cytoplasm while the C-terminus is in the lumen of the endomembrane compartment. P63 is also known as CKAP4 (cytoskeleton-associated protein 4, the Human Genome Organization gene name) and CLIMP-63 [cytoskeleton-linking membrane protein (20)]. At its amino-terminus, P63 has a 106 amino acid long cytosolic tail, a single transmembrane domain, and a large extracytoplasmic domain of 474 amino acids (38, 41). It was originally suggested that P63 is a resident protein of a membrane network interposed between the rough ER and Golgi apparatus or the ERGIC compartments (38, 39). Antibodies against ERGIC 63/P63 were first generated to elucidate the structural organization of the ER-to-Golgi pathway and to study the dynamics of its membrane elements (38, 40). The distribution of P63 was shown to overlap with the ER-Golgi
intermediate compartment as confirmed by another marker of ERGIC (ERGIC 53). Although original studies of P63 described it as an ER resident protein (40) and subsequently as an ER-microtubule linking protein (20), it is now known also to be expressed on the surface of vascular smooth muscle cells (35). The presence of normally ER resident proteins at the plasma membrane is not unusual, as illustrated by the integral membrane proteins kinectin (48, 49) and calnexin (32). Our results from immunocytochemical studies indicate that P63 is localized both intracellularly and on the surface of type II cells. Plasma membrane localization for P63 is supported by anti-P63 antibody labeling (by Western blots) of P63 in the plasma membrane fraction isolated from type II cell lysates; immunofluorescent staining of non-permeabilized type II cells with anti-P63 antibody and colocalization with wheat germ agglutinin, a marker of plasma membranes; cross-linking of SP-A and P63 in plasma membrane preparations as well as to P63 on the surface of live cells, and immunoprecipitation of P63 with anti-P63 from plasma membrane preparations. The inhibitory effect of an anti-P63 monoclonal antibody on SP-A-mediated inhibition of PC secretion, provides further evidence towards establishing that there are pools of P63 expressed on plasma membrane that can serve as specific binding sites for SP-A.

The cross-linking procedures utilized in the present report also identified a 30-32 kDa protein that complexed with SP-A. Previously, using anti-idiotypic antibody A2R, Strayer et al. identified SP-A binding proteins of 30 kDa as well as 56 and 60 kDa (44, 46, 47). The cDNA for the 30 kDa protein was identified and sequenced (44, 46). Using ligand blots of type II cell plasma membranes we showed that SP-A and A2R bound to proteins of approximately 30 and 60 kDa. Our studies demonstrated that the bulk of the proteins were intracellular and were transported to the cell surface with secretagogue treatment (7). What remains to be established is whether or not the 30 and 60 kDa protein in those studies are the same as the 30 kDa unknown
protein and P63 described in the current work. One contradictory point is that P63 does not seem to be a subunit of a larger protein complex as it remains at 63 kDa in both reduced and non-reduced gels. In our previous work using ligand blots with labeled A2R and SP-A, the bands of 30 and 60 kDa were labeled in reduced gels while a 210 kDa protein was labeled in non-reduced gels (7) indicating that the smaller proteins were components of a larger disulfide-bonded complex.

It has been demonstrated that although all three domains of the P63 protein are required to achieve complete intracellular retention, the cytoplasmic domain plays a dominant role. A truncation mutant of P63 which lacks this domain, Δ2-101AA has been shown to traffic exclusively to plasma membrane (40). COS-1 cell transfected with this mutant led to an increase in tPA (tissue plasminogen activator)-catalyzed plasminogen activation and thus served as the functional binding site for tPA on the surface of vascular smooth muscle cells (35).

The pathway whereby P63 reaches the plasma membrane is unclear; previous studies on trafficking and localization have shown subdomain-specific localization of P63 in the endoplasmic reticulum mediated by its luminal alpha-helical segment (20) and its possible role in the positioning of the rough ER along microtubules (13, 19). As discussed elsewhere, it is indeed possible that small pool of P63, when overexpressed, loses its association with microtubules and escapes its sub-domain-specific localization, freeing it to reach the plasma membrane (35). Another possibility is that P63 reaches the plasma membrane due to interactions with adaptor proteins such as 14-3-3. The 14-3-3 family of proteins consists of highly conserved acidic proteins of 30-33 kDa that are known to bind to nearly 200 target proteins in a phosphoserine/phosphothreonine depended manner and are involved in an impressively diverse range of functions, including receptor function and targeting of proteins to the plasma
membrane(27, 30). Most of the 14-3-3 proteins bind to target proteins via specific motifs and motif II [RXRXX(pS/pT)] is found in P63. Since P63 is located in the ER, of interest is the ability of 14-3-3 to override ER retention. In the case of many potassium channel proteins such as KCNK3 and KCNK9 (31), the di-arginine motif/s present on the N-terminus end binds to β-COP, a component of the coat protein complex I (COPI) (36). The forward transport of proteins retained in the ER by β-COP can be initiated by Ser/Thr phosphorylation of the protein’s 14-3-3 binding motif resulting in the subsequent binding of 14-3-3 to the protein and causing the release of β-COP by an unknown mechanism. This process has been demonstrated for channel proteins like KCNK3 and may be the case with P63, in view of the fact that several di-arginine (RR/RXR) motifs are present in P63, including one in the cytoplasmic amino terminus. Whether or not 14-3-3 accompanies P63 to the cell surface and/or has additional functions at that site is under investigation (12). However, it is intriguing that, in our P63-SP-A cross-linking studies, we identified an additional unknown protein of m.w. 30 kDa that cross-linked to SP-A at the type II cell surface. By Western blotting techniques, 14-3-3 was found to be present in type II cells (data not shown). The importance of the cytoplasmic tail of P63 for retention in the ER was demonstrated by Schweizer et al. (40). Deletion of the first 100 amino acid residues of P63 resulted in constitutive migration of P63 to the PM indicating that the di-arginine motif may be functionally important in retention of P63 in the ER (40). Thus, we hypothesize that 14-3-3 may play a role in the binding, transport or stability of P63 in the plasma membrane. Since P63 is found in cells that are not exposed to SP-A in vivo, P63 may have multiple, cell-specific functions. 14-3-3 and P63 may be part of a receptor complex specific for SP-A and functioning only in type II cells.
It is attractive to speculate that, although P63 is normally an ER resident protein, under specialized conditions of type II cells expressing SP-A or vascular smooth muscle cells expressing tPA, P63 would act not as an SP-A binding protein but as a chaperone that binds to a receptor complex and helps in its transport to or from the cell surface. P63 becomes reversibly palmitoylated in the presence of brefeldin A (protein transport blocker) (42) making palmitoylation a possible mechanism for protein transport by P63 as reported for transferrin receptors (1). It has been shown before that endocytosed SP-A is taken up in early endosomes (52, 54) that are located in the periplasm of the cells. Whatever the role of P63, our results clearly demonstrate the presence of both SP-A and P63 in early endosomes. Therefore, it is quite possible that P63 compartments are involved both in the internalization of secreted SP-A and in the exocytosis of newly synthesized SP-A that initially bypasses the lamellar body compartments but is eventually taken up from the extracellular space and assembled into the secretory organelle (6, 33).

To conclude, we have shown that cross-linking of SP-A to intact type II cells or isolated plasma membranes results in binding of the surfactant protein to a trans-membrane protein called P63. SP-A and P63 interact in vitro and in situ as revealed by co-immunoprecipitation and effects on PC secretion. Type II cells express P63 on the cell surface as well as in intracellular compartments where it co-localizes with SP-A. Thus P63 may play a role in SP-A recycling through transport of SP-A from the ER to the plasma membrane and/or in SP-A binding at the plasma membrane and subsequent internalization.
Acknowledgement

We sincerely thank Dr. Michael Koval for supplying EEA-1 antibodies and Drs. Jack Rohrer and Anja Schweizer for the gift of the polyclonal anti-P63 antibody. Drs. Sheldon Feinstein and David Spiecher were very helpful in suggesting experimental protocols for this project. This work was supported by NIH HL 19737.
References

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Table 1. Identification of P63.

<table>
<thead>
<tr>
<th>Peptides</th>
<th>Position in P63</th>
</tr>
</thead>
<tbody>
<tr>
<td>DELGQGLQGVEQK</td>
<td>128-140</td>
</tr>
<tr>
<td>VQSLQAAVGFESILR</td>
<td>141-156</td>
</tr>
<tr>
<td>DFTSLENTVEER</td>
<td>208-219</td>
</tr>
<tr>
<td>LALQALTEK</td>
<td>327-335</td>
</tr>
<tr>
<td>LLQSEESSSR</td>
<td>336-345</td>
</tr>
<tr>
<td>RLEEELQQLK</td>
<td>352-361</td>
</tr>
</tbody>
</table>

Tryptic peptides derived from the 63 kDa band from type II cell lysates were analyzed using microcapillary reverse phase HPLC nano-spray tandem mass spectrometry and searched against the non-redundant NCBI database. The six peptides were a 100% identical match with a transmembrane protein called either P63 or ERGIC 63, CKAP4 or CLIMP-63.
**Figure Legends.**

**Figure 1.** Cross-linking of SP-A with plasma membrane proteins isolated from rat type II cells using sulfo-SBED reagent. 4-12% Bis-Tris polyacrilamide gels, reducing conditions. (Left) Biotinylated samples were detected using HRP-labeled streptavidin. (Right) The same blot stripped and re-probed using anti-SP-A antibody.

**Figure 2.** Cross-linking of SP-A with intact mouse type II cells using DSP reagent and immunoprecipitation of the cross-linked proteins with anti-SP-A antibody. 4-12% Bis-Tris polyacrilamide gels, reducing conditions. Lane 1: Protein A beads + SP-A + anti-SP-A antibody; Lane 2: Protein A beads + cross-linked SP-A with type II cell membrane proteins + anti-SP-A antibody; Lane 3: Protein A beads alone. A. Silver stained gel. B. Immunoblot of gels in A probed with anti SP-A antibody. C. Immunoblot in B stripped and re-probed with polyclonal anti P63 antibody. White arrowhead, 63 kDa; grey arrowhead, SP-A (~34, 60, and 90 kDa); black arrowhead, unknown (~30 kDa); black arrows, IgG (~50kDa);

**Figure 3.** Interaction of P63 and SP-A by co-immunoprecipitation. A. Immunoprecipitation using rabbit anti-human SP-A antibody. Lane 1: Protein A beads alone. Lane 2: Protein A beads + rabbit anti-human SP-A antibody+ rat type II cell lysate. Lane 3: Human SP-A. (Top) Blot probed with mouse anti-human P63 showing presence of P63 (white arrow) in rat type II cells. (Bottom) Same blot stripped and reprobed with anti-human SP-A showing IgG (black arrow) and human SP-A (grey arrows). B. Immunoprecipitation using mouse monoclonal anti-human P63 antibody. Lane 1. Protein A beads + mouse anti-human P63 antibody + rat type II
cell plasma membrane lysates. Lane 2: Protein A beads + mouse anti-human P63 + rat type II whole cell lysates. Lane 3: Human SP-A. (Top) Blot probed with anti-human SP-A showing SP-A (grey arrows). (Bottom) Blot stripped and re-probed with anti-human P63 antibody showing IgG (black arrows) and P63 (white arrow). Protein A beads alone showed no bands.

**Figure 4.** P63 is found in type II cells and absent from macrophages. Western blot of type II (TII) and alveolar macrophage (MØ) cell lysates run under non-reduced (Left) and reduced (Center) conditions on 4-12 % Bis-Tris gels. (Right) The plasma membrane fraction from type II cell lysates run under reduced conditions. The blots are probed with mouse anti-human P63 polyclonal antibody.

**Figure 5.** P63 in the plasma membrane of type II cells. **Right.** Western blots (A) type II cell subcellular fractions probed with antibodies to P63. 18 ug protein/lane. (B) A549 cell subcellular fractions probed with antibodies to P63 or caveolin 1. 50 ug protein/lane. Two bands are alpha and beta subunits of caveolin 1. PM, plasma membrane. **Left.** Immunocytochemistry of type II cells grown on glass cover slips and fixed but not permeabilized. The cells were stained with anti-P63 antibody (C, green) and wheat germ agglutinin (D, red) as a plasma membrane marker. E. Co-localization of P63 and wheat germ agglutin (orange-yellow). F. Phase of C-E.

**Figure 6.** Immunolocalization of P63 in rat type II cells. Isolated type II cells after 24 hours of culture were permeabilized with triton X-100 and double labeled with anti-P63 antibody (A, D) and either anti-ABCA3 antibody (B) or anti SP-A antibody (E). C, merged view of A and B. F,
merged view of D and E. (G,H) Control cells treated similarly to cells in A-F but incubated with only secondary antibodies. (G, H) Fluorescence image. (I) Phase of G. Images show intracellular expression of P63, minimal co-localization (yellow) with ABCA3 within the cell (C), and significant co-localization with SP-A (F).

**Figure 7.** Partial co-localization of P63 and SP-A with the early endosome compartment of type II cells. Rat type II cells cultured for 24h and immunolabeled with EEA-1, a marker for the early endosomes and with either antibodies against P63 or SP-A. (A) anti-P63 antibody; (B, E) anti-EEA-1 antibody; (C) Merged view of A and B and phase; (D) anti-SP-A antibody; (F) Merged view of D and E and phase. Yellow indicates co-localization. A, B, and C are 40X. D, E, and F are 60X.

**Figure 8.** Immunolocalization of P63 in mouse lung. Lung cryosections double labeled with anti-P63 antibody and anti-ABCA3 antibody. ABCA3 is a protein specific for type II cell lamellar body membranes and serves as a marker for type II cells. (A,D) Anti-P63 antibody. (B,E) Anti-ABCA3 antibody. (C, F)Merged. (A-C) and (D-F) represent two different fields of the same lung.

**Figure 9.** Anti-P63 monoclonal antibody inhibits the biological activity of SP-A. Type II cell phospholipids were labeled by overnight incubation with [3H]-choline. A. The labeled cells were incubated without additions (no ATP, open triangles) or with ATP (1mM) alone (filled triangles), or together with SP-A (0.1 ug/ml, filled circles) or SP-A (0.25 ug/ml, open circles). Samples were incubated without or with increasing concentrations of monoclonal antibody to
P63. Antibodies were added for 15 minutes followed by SP-A for 15 min. Finally, ATP was added and the experiment continued for 2 h (see Methods). Data are means + range or + SE of duplicate or triplicate samples from 1-3 experiment as % phospholipid (phosphatidylcholine, PC) secretion. B. [3H]-phospholipid labeled type II cells were incubated with ATP(1mM), monoclonal antibody to P63 (100 ug/ml) or non-immune IgG (100 ug/ml) without or with SP-A (0.1 ug/ml) and PC secretion measured over a 2 h period. Data are means + SE of duplicate or triplicate samples from 3 experiments. a, significantly different from no SP-A; b, significantly different from no antibodies or IgG, p < 0.05.
Figure 1

HRP-Streptavidin

Anti-SP-A

kDa
210 —
111 —
71 —
55 —
41 —
26 —
front

kDa
210 —
111 —
71 —
55 —
41 —
26 —
front

SP-A trimer
SP-A dimer
SP-A monomer
Figure 2

A. IP: Anti-SP-A Silver-stain

B. IP: Anti-SP-A WB: Anti-SP-A

C. IP: Anti-SP-A WB: Anti-p63

- Human SP-A
- P63
- IgG
- Unknown
Figure 3

**A.**

**IP: Anti-SP-A**

- Anti-SP-A: - + -
- T II lysate: - + -
- SP-A: - - +

**WB: Anti-p63**

- SP-A trimer
- SP-A dimer
- SP-A monomer

**B.**

**IP: Anti-p63**

- Anti-p63: + + -
- T II PM: + - -
- T II lysate: - + -
- SP-A: - - +

**WB: Anti-SP-A**

- SP-A trimer
- SP-A dimer
- SP-A monomer
Figure 4
Figure 5

A. Type II cells
MW  PM  P63
64  50

B. A549 cells
MW  PM  P63  Caveolin 1
64  50  25  16
Figure 6

A. P63
B. ABCA3
C. Merge

D. P63
E. SP-A
F. Merge

G. mouse IgG
H. rabbit IgG
I. DIC
Figure 7
Figure 8

P63  
A  B  C

ABCA3  
D  E  F

Merge
Figure 9

A.

- ATP (1mM)
- ATP + SP-A (0.1 µg/ml)
- ATP + SP-A (0.25 µg/ml)
- No ATP

% PC Secretion vs. µg MAb P63

B.

% PC Secretion

ATP Antibodies - P63 IgG - P63 IgG + P63 + IgG +
SP- A - - - + + +

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