

# Aminoacyl-tRNA Synthetase-Interacting Multi-Functional Protein, p43, Is Imported to Endothelial Cells via Lipid Rafts

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**Abstract** An aminoacyl-tRNA synthetase subunit, p43, was previously demonstrated to be released from mammalian cells, and to function as an extracellular regulator of both angiogenesis and inflammatory responses (Ko et al., [2001] *J Biol Chem*, 276; 23028; Park et al. [2002], *J Biol Chem* 277; 45243). Here, we report that p43 is internalized to the endothelial cells via lipid rafts. Exogenous p43 was co-localized on bovine aorta endothelial cells with cholera toxin B (CTB), which binds to cholesterol-enriched lipid rafts. The p43 was rapidly internalized to the cells, as early as 5 min after binding to the surfaces of the cells. p43 bound to the isolated lipid rafts, and its interaction with the lipid rafts, was prevented by high salt content, but not by detergent. This suggests that ionic bonds are involved in the molecular association of p43 with the lipid rafts. Taken together, we conclude that p43 binds to the endothelial cell surface via lipid rafts. *J. Cell. Biochem.* 96: 1286–1295, 2005. © 2005 Wiley-Liss, Inc.

**Key words:** Lipid rafts; p43; cholera toxin B (CTB)

p43 is a non-enzymatic subunit of the mammalian aminoacyl-tRNA synthetase (ARS) complex. It consists of eight different ARS enzymes and three auxiliary subunits [Quevillon and Mirande, 1996; Quevillon et al., 1997, 1999]. p43 exhibits a tRNA binding capacity, which facilitates the aminoacylation of its bound enzyme, arginyl-tRNA synthetase [Park et al., 1999; Kim et al., 2000; Shalak et al., 2001]. In addition to its cytoplasmic function as a coactivator of arginyl-tRNA synthetase, p43 has been shown to be secreted in methylcholanthrene fibrosarcoma cells, 32D myeloid precursor cells, human prostatic adenocarcinoma cells, and

HEK 293 cells [Kao et al., 1992; Knies et al., 1998; Barnett et al., 2000; Ko et al., 2001]. Extracellular p43 induces the generation of chemokines and cytokines, including tumor necrosis factor (TNF), monocyte chemotactic protein 1 (MCP-1), macrophage inflammatory protein 1 (MIP-1), interleukin-1 $\beta$  (IL-1 $\beta$ ), and interleukin-8 (IL-8) in THP-1 cells [Ko et al., 2001]. It also regulates angiogenesis differentially, according to its concentration [Park et al., 2002b].

Lipid rafts, which exhibit two distinctive biochemical properties such as detergent insolubility and low density, due to cholesterol and glycosphingolipid in their structure, are plasma membrane platforms, which organize various receptors and their downstream molecules. Lipid rafts can also activate a variety of signal transduction pathways [Anderson, 1998; Galbiati et al., 2001; Munro, 2003]. Many receptors for growth factors, cytokines, and chemokines are principally localized in these lipid rafts. Therefore, the disruption of lipid rafts as the result of cholesterol deprivation leads to the disorganization of receptors and their downstream signaling molecules, and

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prevents a variety of signal transduction pathways. As lipid rafts concentrate many receptors, we attempted to identify receptors for orphan ligands from lipid rafts.

We have previously implicated ATP synthase subunit  $\alpha$  as a potential p43 receptor [Chang et al., 2002]. As p43 is a multi-functional cytokine, which exerts its effects on various cells, including macrophages, endothelial cells, and fibroblasts [Ko et al., 2001; Park et al., 2002a,b, 2005], there is a high probability that it may possess more than one functional receptor. In order to gain insight into the p43 receptor(s), we initially determined the localization patterns of p43 on the surfaces of endothelial cells. Interestingly, the exogenously administered p43 was found to have co-localized with cholera toxin B (CTB) in the cell surface, and was predominantly detected in the lipid raft fractions. Thus, it is quite likely that p43 receptors are localized in the lipid rafts.

## MATERIALS AND METHODS

### Cell Culture

Bovine aorta endothelial cells (BAECs) were isolated from descending thoracic aortas, and grown in Dulbecco's Modified Eagle Medium (DMEM) containing 20% fetal bovine serum. The cells used primarily in this study were between passages 5 and 10. HeLa and Raw264.7 cells were purchased from ATCC, and grown in DMEM containing 10% fetal bovine serum.

### Purification of p43, p43-N, and p43-C

p43 was expressed as a His tag fusion protein in *Escherichia coli* BL21 (DE3), and was purified by nickel affinity and Mono S ion-exchange chromatography. In order to remove lipopolysaccharide (LPS), the protein solution was dialyzed in pyrogen-free buffer (10 mM potassium phosphate buffer, pH 6.0, 100 mM NaCl). After dialysis, the p43 solution was loaded into polymyxin resin (Bio-Rad, Hercules, CA) pre-equilibrated with the same buffer, incubated for 20 min, and eluted. In order to further remove residual LPS, the protein solution was dialyzed against PBS containing 20% glycerol, and filtered through a Posidyne membrane (Pall-Gelman laboratory). The concentration of LPS in the p43 was below 20 pg/ml, as determined with the Limulus Amebocyte lysate QCL-1000 kit (BioWhittaker).

### Preparation of Polyclonal Rabbit and Monoclonal Mouse Antibodies Specific to p43

Purified recombinant human p43 (500  $\mu$ g) was then used to prepare polyclonal rabbit antibody, as previously described [Park et al., 1999]. The p43 was also used in the preparation of monoclonal mouse anti-p43 antibody, which was made by Komed (Seoul, Korea). It should be noted that polyclonal antibodies exhibit cross-reactivity to human, mouse, rat, and bovine p43, whereas monoclonal antibodies recognize only the human variant of p43. According to the findings of immunogold labeling electron microscopy, only the polyclonal antibody recognizes the recombinant p43 antigen.

### Immunofluorescence

BAECs on a 12 mm cover slip were washed briefly with cold phosphate buffer saline (PBS). Biotinylated p43 (at indicated concentration) was loaded onto the cover slip and incubated for 20 min at 4°C. After three washings with cold PBS, the BAECs were incubated with fluorescein isothiocyanate (FITC)-conjugated streptavidin (PIERCE) for 20 min at 4°C, then washed an additional three times with cold PBS. Cells were fixed with formaldehyde (3.7% w/v in PBS) for 10 min, washed thoroughly in PBS, and then mounted with anti-fading agent containing mounting solution (DAKO, Carpinteria, CA) and coverslipped. Cells were observed with a laser-scanning confocal microscope (Bio-Rad MRC-1024; Bio-Rad, Hercules, CA).

### Lipid Raft Isolation

Lipid rafts were isolated, as described previously [Kim et al., 2004]. In brief,  $5 \times 10^7$  cells were washed twice with cold PBS, then lysed with 1 ml of lysis buffer (25 mM HEPES, pH 6.5, 150 mM NaCl, 2% TX-100 or Brij 35, 1 mM EDTA, and 1 mM PMSF) containing protease inhibitor cocktail (Roche Molecular Biochemicals, Indianapolis, IN) for 20 min at 4°C. The lysates were then homogenized 20 times with a tight Dounce homogenizer (Kontes, Vineland, NJ). The extract was mixed with 1 ml of 80% sucrose, transferred to an SW 41 T: centrifuge tube, and overlaid with 7 ml of 30% sucrose solution, and 3 ml of 5% sucrose solution containing 25 mM HEPES, and 150 mM NaCl, at a pH of 6.5. The discontinuous sucrose gradients were centrifuged for 21 h at 4°C with an SW 41 T: rotor (Beckman Instruments, Palo

Alto, CA) at 39,000 rpm. The gradient was fractionated into 12 fractions, from bottom to top.

### In Vitro Binding of p43 to Lipid Rafts

Lipid rafts equivalent to 10  $\mu$ g of proteins were incubated with indicated concentrations of p43 for 20 min at 4°C in 1.5 ml tubes. The lipid rafts and p43 were mixed with equal volumes of 80% sucrose solution in phosphate buffer saline (PBS), and overlaid with 1 ml of 30% sucrose and 400  $\mu$ l of 5% sucrose solution in PBS. The discontinuous sucrose gradients were centrifuged at 14,000 rpm for 20 min at 4°C. The fraction between 5% and 30% sucrose gradients (200  $\mu$ l) were then collected and 1 ml of PBS was added, and then centrifuged at 14,000 rpm for 20 min at 4°C. The pellet was resuspended in 2X SDS-sample buffer, and the proteins were separated by SDS-PAGE.

### Electrophoresis, Silver Staining, and Immunoblots

Proteins were separated on 10% polyacrylamide gel, and visualized with a silver staining kit (No. 17-1150-01; Amersham Biosciences). For immunoblotting, the proteins were transferred onto a polyvinylidene difluoride membrane (Millipore), using SEMI-DRY transfer cells (Bio-Rad) with transfer buffers (25 mM Tris, 192 mM glycine, 20% methanol, 0.5% SDS, pH 8.3). The membrane was blocked in 1 X TBS, 5% nonfat dry milk and 0.5% Tween 20 for 1 h at room temperature, then allowed to react with a sequence of primary and secondary antibodies. The antigen signal was visualized with ECL reagents (Santa Cruz Biotech).

### Electron Microscopy

The BAECs were washed with washing solution (0.1% BSA in PBS) briefly, incubated with p43 (1  $\mu$ M) for 20 min at 4°C, and finally washed twice with washing solution. 0.1% BSA was used as a negative control. Polyclonal anti-p43 antibody (1:100) was incubated for 30 min at 4°C and washed twice with washing solution. Protein A-gold was incubated for 30 min at 4°C, then internalized at 30°C for 20 min. For ultrastructural examinations, pretreated BAECs were fixed for 1 h at 4°C in 2% paraformaldehyde, and 2.5% glutaraldehyde in 0.1 M sodium phosphate buffer (pH 7.4), then harvested with a cell lifter and washed with the same buffer, using a centrifuge at 3,000 rpm for 5 min. Cells

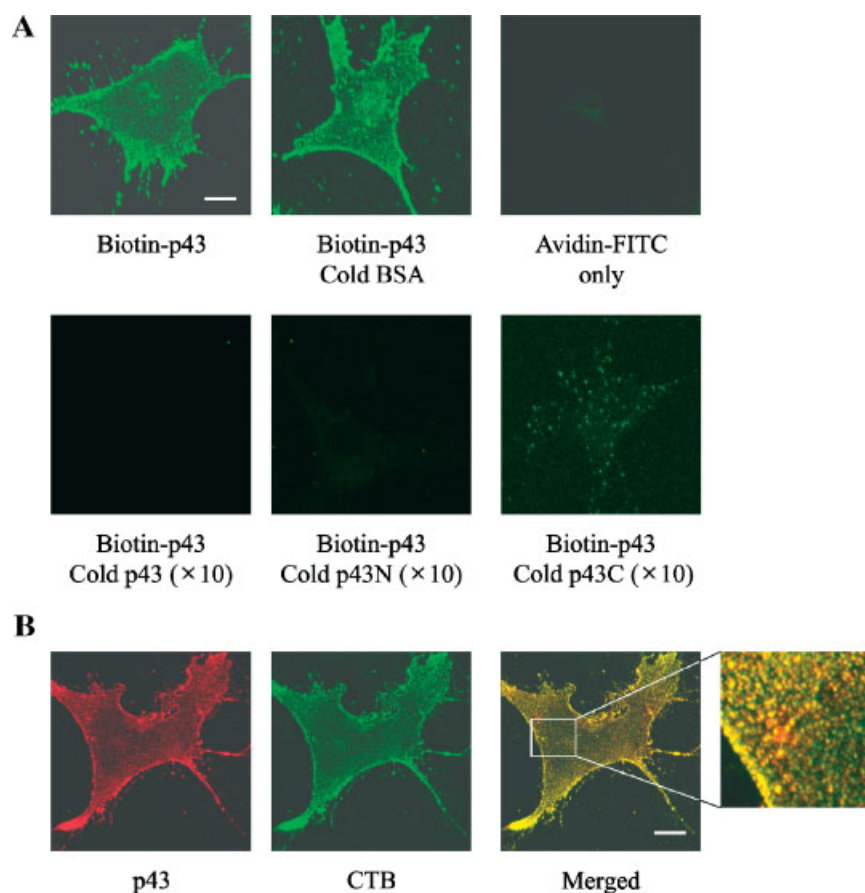
were then postfixed for 1 h in 1% OsO<sub>4</sub> in the same buffer, and subsequently embedded in Epon-Araldite. Thick and thin sections were prepared on an RMC MT-X ultramicrotome. The sectioned thick cells were then stained with 1% toluidin blue-borax solution. The thin sections were mounted on copper grids, and double-stained with uranyl acetate and lead citrate. The grids were visually examined with a JEM 100 CX-II electron microscope.

## RESULTS

### Punctated Cell Binding of p43

As full-length p43 activates various signal transduction pathways in endothelial cells, immune cells, and fibroblasts [Ko et al., 2001; Park et al., 2002b, 2005], we initially attempted to determine whether p43 binds to the surface of the bovine aorta endothelial cells (BAECs). A distinct punctate staining pattern was observed after the incubation of BAECs with biotin-labeled p43 and fluorescein isothiocyanate (FITC)-conjugated streptavidin at 4°C (Fig. 1A). As little as 50 nM of p43 is sufficient for observable positive staining, but the best resolution for immunofluorescence was achieved with 300 nM of p43. The punctate staining pattern disappeared after the addition of excess amounts (3,000 nM) of unlabeled p43, indicating that the p43 was binding specifically to the cells. On the contrary, the control cells treated with FITC-conjugated streptavidin exhibited only weak and nonspecific binding (Fig. 1A).

We have previously demonstrated that the various truncated p43 fragments retained the activities inducing inflammation, cellular migration, and wound healing [Ko et al., 2001; Park et al., 2002a,b, 2005]. To determine which domain of p43 is involved in cell surface binding activity, we prepared the 146-aa NH<sub>3</sub>-terminal (p43-N) and 166-aa COOH-terminal domains (p43-C) of p43, and BAECs were incubated with biotin-p43 (300 nM) in the presence of excess p43-N or p43-C (1500 nM, respectively). As shown in Figure 1A, the signal for biotin-p43 completely disappeared in the presence of unlabeled p43-N but weak signal was remained in the presence of unlabeled p43-C, suggesting that p43-N is more competitive to p43 in binding to cell surface than p43-C. The higher binding activity of p43-N to cell surface explain why p43-N has higher biological activities in inflammation, cellular migration, and wound healing



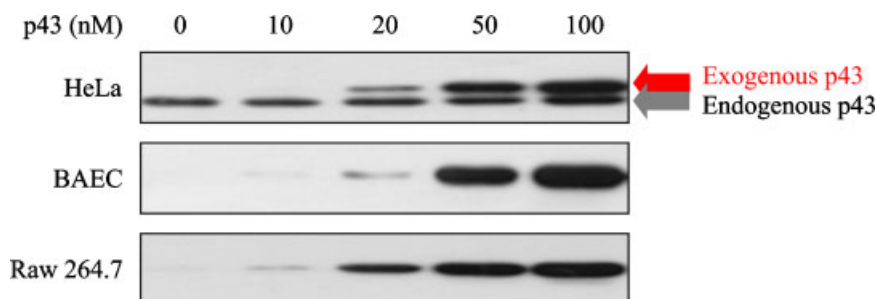
**Fig. 1.** p43 and CTB are co-localized in the endothelial cell surface. **A:** BAECs were incubated with biotin-p43 (300 nM) at 4°C. The biotin-p43 on the cell surface was identified with FITC-conjugated streptavidin. For determining binding domain of p43 to cell surface, the biotin-p43 was co-incubated with unlabeled p43, p43-N, p43-C, and bovine serum albumin (3 μM, respectively). **B:** The BAECs were incubated with biotin-p43 (300 nM) and rhodamine-conjugated cholera toxin B (CTB) (500 nM). (Bar size is 10 μm).

in comparison to p43-C [Ko et al., 2001; Park et al., 2002a,b, 2005].

Many glycosylphosphatidylinositol-anchored proteins and lipid raft marker proteins exhibit a typical punctate staining pattern with regard to their immunofluorescence [Harder et al., 1999]. As exogenous p43 also exhibited a typical punctate staining pattern, it is tempting to speculate that exogenous p43 binds to the lipid rafts of endothelial cells. In order to address this issue, we incubated the BAECs with rhodamine-conjugated cholera toxin B and biotin-p43 at 4°C for 20 min. As shown in Figure 1B, p43 and cholera toxin B were detected in the same cell surface location with a distinct punctate staining pattern, indicating that p43 was able to bind to the lipid rafts.

We re-confirmed that exogenous human p43 binds to cell surfaces via immunoblotting. After treating mammalian cells, including HeLa,

BAEC, and Raw264.7, with indicated concentrations of p43 at 4°C for 20 min, cellular proteins were analyzed by immunoblotting with anti-p43 monoclonal mouse antibodies, which only recognize human p43. Human cells, most notably HeLa cells, displayed two bands, representing endogenous and exogenous p43, whereas nonhuman cells, such as Raw264.7 and BAEC, showed only a single band, corresponding to exogenous p43 (Fig. 2). It should be noted that the monoclonal anti-p43 antibody recognizes only the human form of p43. Therefore, only exogenous p43 appears to exist in Raw 264.7 and BAEC, which are rat and bovine cells, respectively. As shown in Figure 2, as little as 20 nM of exogenous p43 was identified to bind to the surfaces of the cells. Importantly, the exogenous p43 was saturated at 100 nM, in order to bind to the cell surface. These immunofluorescent and immunoblotting data clearly



**Fig. 2.** The surface binding of p43 in BAEC, HeLa, and Raw264.7 cells. BAEC, HeLa, and Raw264.7 cells were incubated with various concentrations of p43 (0, 10, 20, 50, and 100 nM) for 20 min at 4°C, and then washed three times with cold-PBS. The cells were lysed with lysis buffer containing 1% TX-100, 1% deoxycholate, and 0.1% SDS. The whole cell lysates were analyzed with monoclonal anti-p43 antibody, which recognizes only the human form of p43. [Color figure can be viewed in the online issue, which is available at [www.interscience.wiley.com](http://www.interscience.wiley.com).]

indicated that p43 binds to mammalian cells, forming punctate structures.

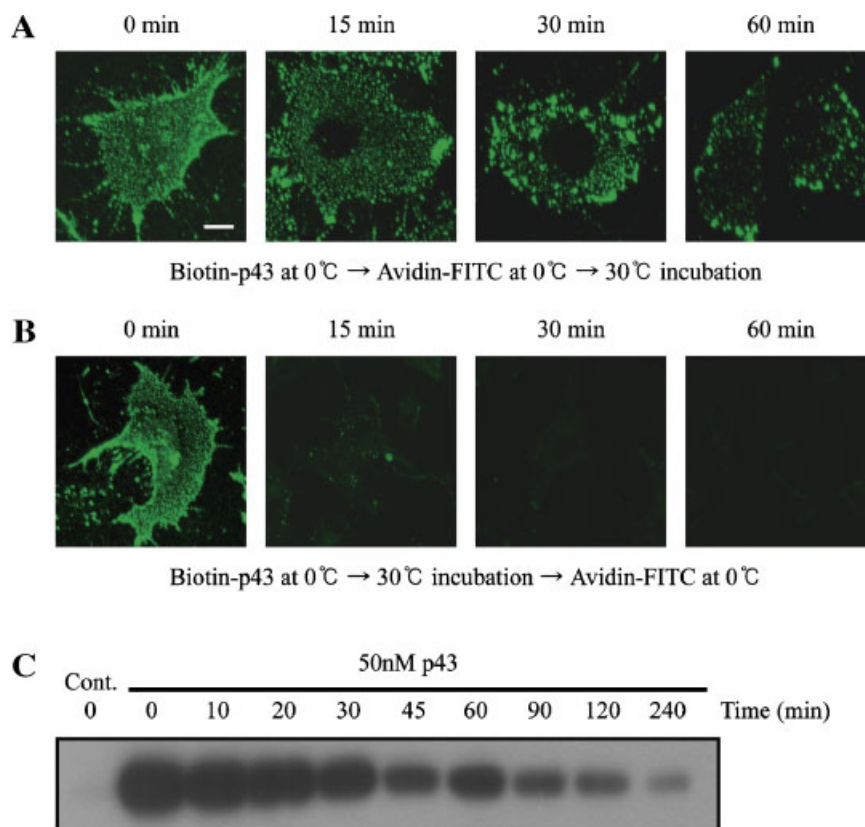
#### Internalization of p43

Next, we attempted to determine whether exogenous p43 is internalized to the cells after binding to cell surfaces. We incubated the BAECs with biotin-p43 and FITC-conjugated streptavidin at 4°C in sequence, followed by 30°C incubation to trigger p43 internalization. Figure 3A indicates that, under cellular incubation at 30°C for 30 and 60 min, the staining pattern appeared more aggregated or patchy than that which was observed with samples exposed to p43 at 4°C. Moreover, the p43 signal was significantly attenuated, as compared with that seen in cells which were not exposed to 30°C incubation, thereby suggesting that p43 might be degraded after aggregation.

In order to confirm whether aggregated or patchy staining represents the p43 internalized within the cells after 30°C incubation, we induced the cellular binding of biotin-p43 at 4°C, then incubated the sample at 30°C, and finally treated it with FITC-conjugated streptavidin at 4°C. If the p43 bound to the cell surface was internalized, the p43 signal would be unobservable, as the FITC-conjugated streptavidin cannot permeate the plasma membrane. Figure 3B shows that the p43 signal disappeared completely after 15 min of cellular incubation at 30°C, suggesting that the p43 signal observed in Figure 3A was an internalized signal. As the p43 signal dramatically diminished as early as 5 min after cellular incubation at 30°C (data not shown), it would appear that p43 is internalized almost immediately upon binding to the cellular surface.

As shown in Figure 3A, p43 signal was greatly reduced 60 min after internalization, suggesting that the exogenous p43 could be degraded. In order to address the issue, we incubated BAECs with exogenous p43 (50 nM) at 4°C for 20 min, and then at 30°C for various times. After cellular lysis, the exogenous p43 was monitored by immunoblotting with anti-p43 monoclonal mouse antibody that only recognizes exogenous human form of p43. Figure 3C shows that exogenous p43 was rapidly degraded after cellular internalization.

Immunogold electron microscopy was also used in the determination of the distribution of exogenous p43. Since anti-p43 mouse monoclonal antibody was not worked for immunogold EM, we used anti-p43 rabbit polyclonal antibody that recognizes both endogenous and exogenous p43. In order to chase the destination of exogenous p43 after its binding to the cell surface, we incubated BAECs with exogenous p43, and then treated anti-p43 rabbit antibody at 4°C for 20 min, respectively. Before or after cellular incubation at 30°C for 20 min, p43 was monitored by protein A-gold under electron microscopy. The gold labeling was principally associated with the cell surface, when the cells were treated with p43 at 4°C, whereas the gold labeling was found in the intracellular vesicles upon subsequent incubation at 30°C (Fig. 4A,B). It should be noted that there was no gold particle in cells without p43 incubation (Fig. 4C), suggesting that gold particles in Figure 4A,B indicate exogenous p43. These data compelled us to conclude that p43 is internalized after binding to the surfaces of the cells.



**Fig. 3.** p43 internalization within cells. **A:** BAECs were incubated with biotin-p43 (300 nM) for 20 min at 4°C, washed twice with PBS, and incubated with FITC-conjugated streptavidin for 30 min at 4°C. The cells were again washed with PBS, and incubated at 30°C for various durations (0, 15, 30, and 60 min). **B:** BAECs were incubated with biotin-p43 (300 nM) for 20 min at 4°C, washed twice with PBS, and incubated at 30°C for various durations (0, 15, 30, and 60 min). The cells were incubated with FITC-conjugated streptavidin. When biotin-p43 is internalized

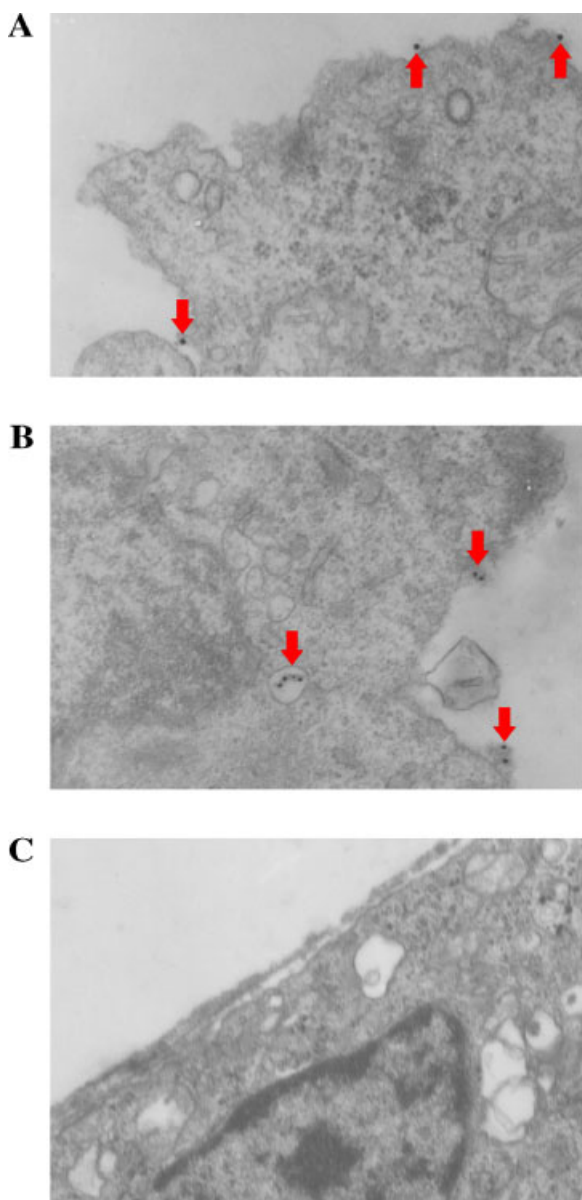
within the cells, there was no p43 signal, because FITC-conjugated streptavidin cannot penetrate the plasma membrane. (Bar size is 10  $\mu$ m). **C:** BAECs were incubated with exogenous p43 (50 nM) at 4°C for 20 min, washed with cold PBS, and incubated at 30°C for indicated times for inducing p43 internalization. After cell lysis, the p43 was monitored by anti-p43 mouse monoclonal antibody. [Color figure can be viewed in the online issue, which is available at [www.interscience.wiley.com](http://www.interscience.wiley.com).]

### Interaction of p43 With Lipid Rafts

As p43 binds to the cell surface with a distinct punctate staining pattern, and is found to be colocalized with cholera toxin B, it is tempting to speculate that exogenous p43 might recognize a receptor within the lipid rafts. To test this possibility, we attempted to determine whether the exogenous p43 was able to associate with the lipid rafts. We incubated the BAECs with exogenous p43, and isolated the lipid rafts via sucrose gradient ultracentrifugation, upon the basis of detergent insolubility and low density, as described in the Materials and Methods section. The sucrose gradient was fractionated into 12 fractions for further analysis. As expected, caveolin-1 was detected almost exclusively in the lipid raft fractions (Fig. 5A). Similar results were obtained by immunoblotting with

anti-p43 antibody. As shown in Figure 5A, almost all of the endogenous p43 was found in the bottom fractions, whereas a large amount of exogenous p43 was found in the lipid raft fractions.

In a set of parallel experiments, we initially isolated the lipid rafts from the BAECs, incubated the lipid rafts with p43, and ultracentrifuged the samples to pellet down the lipid raft membranes. The lipid raft proteins from the pellet were then visualized via Ponceau S staining, and analyzed by immunoblotting with anti-p43 antibody. Ponceau S staining, as shown in Figure 5B, revealed that the lipid rafts contained exogenous p43, suggesting that p43 has high affinity for the lipid rafts. Immunoblotting in Figure 5C also clearly indicated that large quantities of p43 (more than 2  $\mu$ g) could bind to lipid rafts



**Fig. 4.** Immunogold labeling revealed p43 internalization within cells. BAECs were incubated with p43 (300 nM) for 20 min at 4°C, washed with PBS, and incubated with anti-p43 polyclonal rabbit antibody for 20 min at 4°C (A). The cells were further incubated for 20 min at 30°C (B). Anti-p43 antibody was visualized by gold-labeled protein A under electron microscopy. For negative control (C), BAECs were incubated with anti-p43 polyclonal rabbit antibody for 20 min at 4°C in the absence of p43. After further incubation for 20 min at 30°C, the antibody was visualized by gold-labeled protein A. [Color figure can be viewed in the online issue, which is available at [www.interscience.wiley.com](http://www.interscience.wiley.com).]

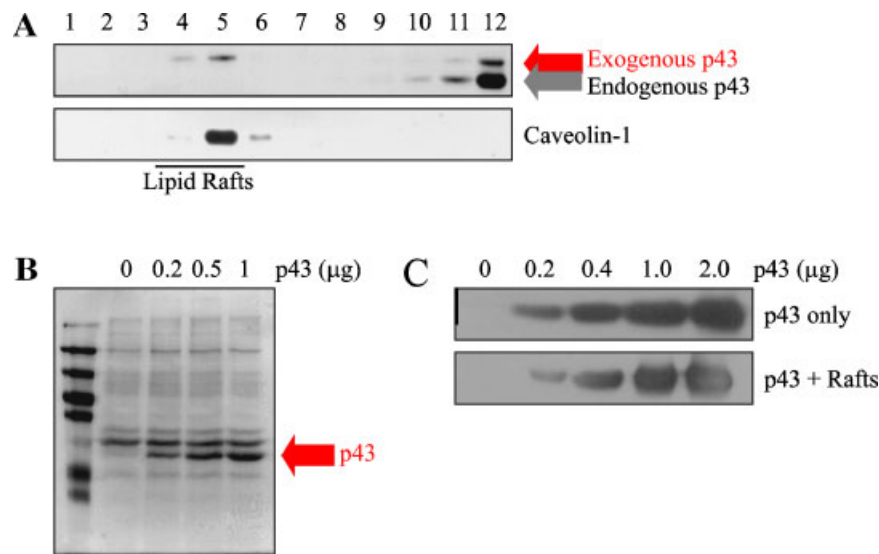
containing 40  $\mu$ g of proteins. Thus, we concluded that the lipid rafts possess a profound capacity for the accommodation of large quantities of p43.

To glean insight into the chemical identity of the potential p43 receptor in the lipid rafts, proteins present in the lipid rafts were inactivated by 20 min of heat treatment at 100°C, or by digestion with proteinase K at 37°C for 30 min, and then the resulting raft preparation was incubated with p43, in order to determine whether p43 was still able to bind to the lipid rafts. As shown in Figure 6A, p43 retained its ability to bind to the lipid rafts, even though lipid raft proteins had been denatured or degraded (as demonstrated by silver staining). This indicates that p43 may bind to non-protein components in the rafts.

In order to determine the physical properties of the molecular interaction occurring between p43 and the lipid rafts, we evaluated the effects of salt and detergent on this interaction. To our surprise, the interaction between p43 and lipid rafts was disrupted by high salts (more than 500 mM NaCl), but not by the non-ionic detergent, Brij 35 (Fig. 6B and C). This suggests that either ionic interaction, or a hydrogen bond between p43 and the lipid rafts, is important force for the binding of p43 to the lipid rafts.

## DISCUSSION

A COOH-terminal fragment of p43 (p43-C) called endothelial and monocyte activating polypeptide II (EMAP II) has been thought to be a cytokine to recruit monocytes for scavenging apoptotic cells because it is secreted from apoptotic cells and has an ability to induce monocyte migration and activation. [Kao et al., 1992, 1994; Knies et al., 1998; Shalak et al., 2001] However, EMAP II is non-selectively or passively secreted with other cellular proteins such as p18 and tubulin from cells that are already destroyed by apoptosis, suggesting that EMAP II could not be an active cytokine to recruit monocyte in the early stage of apoptosis [Ko et al., 2001]. EMAP II is rarely secreted whereas its precursor called p43 is highly secreted from Raw264.7 cells stimulated by tumor necrosis factor [Park et al., 2005], suggesting that p43 is a real cytokine for activating immune cells and endothelial cells. Previously, p43 has higher cytokine activity in immune and endothelial cells than EMAP II does [Park et al., 2002a,b]. Indeed, Figure 1A shows that EMAP II has weaker binding activity to cell surface than p43 and its NH<sub>3</sub>-terminal fragment, explaining why EMAP II



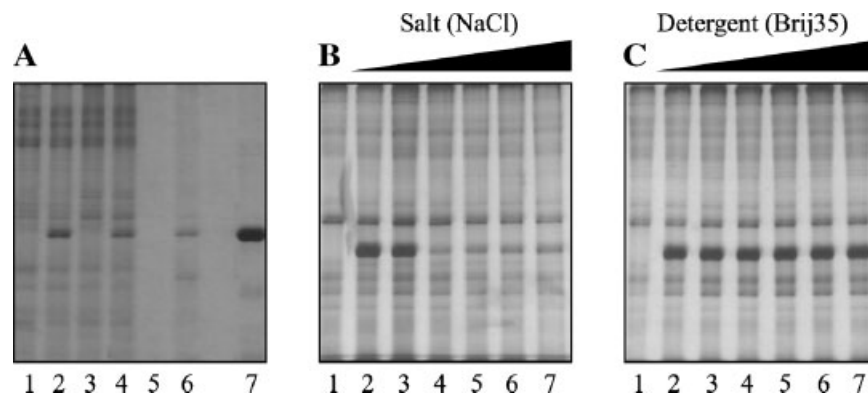
**Fig. 5.** p43 is associated with lipid rafts. **A:** BAECs were incubated with p43 (300 nM) for 20 min at 4°C, washed with cold PBS, and then extracted with 1% TX-100-containing lysis buffer. Lipid rafts were isolated via sucrose gradient ultracentrifugation. Each fraction was analyzed with anti-p43 and anti-caveolin-1 antibodies. **B:** Lipid raft fractions were isolated from the BAECs, and washed with washing buffer via ultracentrifugation. The lipid rafts (40 μg of proteins) were incubated with the indicated con-

centration of p43, and ultracentrifuged in order to pellet down lipid raft proteins. The lipid raft proteins were then analyzed by Ponceau S staining after SDS-PAGE. **C: Upper panel,** immunoblotting with anti-p43 antibody after loading different amounts of p43. **Bottom panel,** lipid raft-bound p43 in B was immunoblotted with anti-p43. It should be noted that almost all of p43 bound to the lipid rafts. [Color figure can be viewed in the online issue, which is available at [www.interscience.wiley.com](http://www.interscience.wiley.com).]

has lower cytokine activity than full length of p43. Since the p43 is highly expressed in skin wound area as well as in atherosclerotic region [Ko et al., 2001; Park et al., 2005], it could be an important cytokine regulating inflammation. Indeed, depletion of endogenous p43 in mice by gene disruption retarded wound repair, whereas exogenous supplementation of recombinant human p43 to the wound area stimulated

dermal fibroblast proliferation, collagen production, and wound closure [Park et al., 2005].

Here, we monitored the binding patterns and internalization of p43 on the surface of BAEC, HeLa, and Raw264.7 cells, using immunofluorescence, immunogold labeling, and immunoblotting. We determined that this may be internalized via lipid rafts. Our supporting evidence was acquired from different experimental



**Fig. 6.** p43 binds to the isolated lipid rafts in a hydrophilic manner. **A:** Lipid rafts (10 μg of raft proteins) from BAECs were boiled for 20 min (lanes 3 and 4) or treated with proteinase K for 24 h (lanes 5 and 6), and then the rafts were incubated either with or without 10 μM of p43. The rafts were re-isolated by discontinuous sucrose gradient centrifugation, as described in Materials and Methods. The mixture of p43 and rafts were visualized by silver staining. Lanes 1, 3, and 5 represent only rafts, and lanes 2,

4, and 6 represent mixtures of rafts and p43. Lane 7 shows the p43. Lipid rafts (10 μg of raft proteins) were incubated with p43 (10 μM) at a variety of salt concentrations (**B; lanes 2–7;** 0.1, 0.2, 0.5, 1.0, 2.0, and 5.0 M, respectively) and detergent concentrations (**C; lane 2 ~ lane 7;** 0, 0.1, 0.2, 0.5, 1.0, and 2.0%, respectively). Lane 1 in B and C shows rafts only. The mixture of p43 and rafts was detected by silver staining.



data. Firstly, biotin-p43 bound to the cell surface with a characteristic punctate staining pattern, which corresponds to the distribution of lipid rafts. Secondly, exogenous p43 was found in the lipid raft fractions when endothelial cells were incubated with exogenous p43 at 4°C. Thirdly, p43 evidenced profound binding activity for lipid rafts isolated from endothelial cells. As the lipid rafts have been assessed with regard to their functions as plasma membrane platforms, which accommodate different receptor types, it is not unreasonable to expect a potential p43 receptor to be present in the lipid rafts.

There are a couple of points, which suggest that non-protein components can function as a potential p43 receptor in the rafts. First, an extremely large quantity of p43 (more than 2 µg) is able to bind to lipid rafts, as shown in Figure 5C. Second, p43 was observed to bind to the lipid rafts after the component proteins had been inactivated by heat treatment or degraded by proteinase K (Fig. 6). As many lipids function as surface receptors for different ligands, and lipid rafts contain a large quantity of glycosphingolipids and cholesterol [Anderson, 1998; Galbiati et al., 2001; Munro, 2003], these molecules may constitute candidates for p43 receptors. For example, CTB, fibroblast growth factor 2 (FGF2), and myelin-associated glycoprotein (MAG) [Pang et al., 2004; Rusnati et al., 2002; Vinson et al., 2001] bind to the cell surface via gangliosides. This made it tempting to speculate that p43 can bind to gangliosides, which are enriched in the lipid rafts. In order to characterize the molecular association of p43 with the gangliosides, we prepared liposomes containing different ganglioside variants (GM<sub>1</sub>, GM<sub>3</sub>, GD<sub>1a</sub>, GD<sub>1b</sub>, GT<sub>1b</sub>, or sphingomyelin), along with cholesterol and dipalmitoyl phosphatidyl choline, and then incubated the liposomes with p43. However, p43 was not observed to bind to any liposomes, thereby suggesting that p43 does not bind to gangliosides (data not shown). In addition, ganglioside-free cells [Rusnati et al., 2002] retained a profound ability to bind to p43 (data not shown). p43 was also observed to bind to cell surfaces in which cholesterol had been removed with methyl-β-cyclodextrin, a cholesterol-removing drug (data not shown). Nonetheless, the strong preference of p43 to the lipid rafts still warrants additional systematic search for the potential receptor.

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## REFERENCES

- Anderson RG. 1998. The caveolae membrane system. *Annu Rev Biochem* 67:199–225.
- Barnett G, Jakobsen AM, Tas M, Rice K, Carmichael J, Murray JC. 2000. Prostate adenocarcinoma cells release the novel proinflammatory polypeptide EMAP-II in response to stress. *Cancer Res* 60:2850–2857.
- Chang SY, Park SG, Kim S, Kang C-Y. 2002. Interaction of the C-terminal domain of p43 and the alpha subunit of ATP synthase. Its functional implication in endothelial cell proliferation. *J Biol Chem* 277:8388–8394.
- Galbiati F, Razani B, Lisanti MP. 2001. Emerging themes in lipid rafts and caveolae. *Cell* 106:403–411.
- Harder T, Scheiffele P, Verkade P, Simons K. 1999. Lipid domain structure of the plasma membrane revealed by patching of membrane components. *J Cell Biol* 141:929–942.
- Kao J, Ryan J, Brett G, Chen J, Shen H, Fan YG, Godman G, Familletti PC, Wang F, Pan YC, Stern D, Clauss M. 1992. Endothelial monocyte-activating polypeptide II. A novel tumor-derived polypeptide that activates host-response mechanisms. *J Biol Chem* 267:20239–20247.
- Kao J, Houck K, Fan Y, Haehnel I, Libutti SK, Kayton ML, Grikscheit T, Chabot J, Nowygrod R, Greenberg S, Kuang W-J, Leung DW, Hayward JR, Kisiel W, Heath M, Brett J, Stern DM. 1994. Characterization of a novel tumor-derived cytokine. Endothelial-monocyte activating polypeptide II. *J Biol Chem* 269:25106–12519.
- Kim Y, Shin J, Li R, Cheong C, Kim K, Kim S. 2000. A novel anti-tumor cytokine contains an RNA binding motif present in aminoacyl-tRNA synthetases. *J Biol Chem* 275:27062–27068.
- Kim K-B, Kim S-I, Choo H-J, Kim J-H, Ko Y-G. 2004. Two-dimensional electrophoretic analysis reveals that lipid rafts are intact at physiological temperature. *Proteomics* 4:3527–3535.
- Knies UE, Behrendorf HA, Mitchell CA, Deutsch U, Risau W, Drexler HC, Clauss M. 1998. Regulation of endothelial monocyte-activating polypeptide II release by apoptosis. *Proc Natl Acad Sci USA* 95:12322–12377.
- Ko Y-G, Park H, Kim T, Lee J-W, Park SG, Seol W, Kim J-E, Lee W-H, Kim S-H, Park J-E, Kim S. 2001. A cofactor of tRNA synthetase, p43, is secreted to up-regulate proinflammatory genes. *J Biol Chem* 276:23028–23033.
- Munro S. 2003. Lipid rafts: Elusive or illusive? *Cell* 115:377–388.
- Pang H, Le P-U, Nabi IR. 2004. Ganglioside GM1 levels are a determinant of the extent of caveolae/raft-dependent

- endocytosis of cholera toxin to the Golgi apparatus. *J Cell Sci* 117:1421–1430.
- Park SG, Jung K-H, Lee J-S, Jo Y-J, Motegi H, Kim S, Shiba K. 1999. Precursor of pro-apoptotic cytokine modulates aminoacylation activity of tRNA synthetase. *J Biol Chem* 274:16673–16676.
- Park H, Park SG, Lee J-W, Kim T, Kim G, Ko Y-G, Kim S. 2002a. Monocyte cell adhesion induced by a human aminoacyl-tRNA synthetase-associated factor, p43: Identification of the related adhesion molecules and signal pathways. *J Leukoc Biol* 71:223–230.
- Park SG, Kang Y-S, Ahn Y-H, Lee S-H, Kim K-R, Kim K-W, Koh G-Y, Ko Y-G, Kim S. 2002b. Dose-dependent biphasic activity of tRNA synthetase-associating factor, p43, in angiogenesis. *J Biol Chem* 277:45243–45248.
- Park SG, Shin HS, Shin YK, Lee Y, Choi E-C, Park B-J, Kim S. 2005. The novel cytokine p43 stimulates dermal fibroblast proliferation and wound repair. *Am J Pathol* 166:387–398.
- Quevillon S, Mirande M. 1996. The p18 component of the multisynthetase complex shares a protein motif with the beta and gamma subunits of eukaryotic elongation factor 1. *FEBS Lett* 395:63–67.
- Quevillon S, Agou F, Robinson JC, Mirande M. 1997. The p43 component of the mammalian multi-synthetase complex is likely to be the precursor of the endothelial monocyte-activating polypeptide II cytokine. *J Biol Chem* 272:32573–32579.
- Quevillon S, Robinson JC, Berthonneau E, Siatecka M, Mirande M. 1999. Macromolecular assemblage of aminoacyl-tRNA synthetases: Identification of protein-protein interactions and characterization of a core protein. *J Mol Biol* 285:183–195.
- Rusnati M, Urbinati C, Tanghetti E, Dell'Era P, Lortat-Jacob H, Presta M. 2002. Cell membrane GM1 ganglioside is a functional coreceptor for fibroblast growth factor 2. *Proc Natl Acad Sci USA* 99:4367–4372.
- Shalak V, Kaminska M, Mitnacht-Kraus R, Vandenabeele P, Clauss M, Mirande M. 2001. The EMAPII cytokine is released from the mammalian multisynthetase complex after cleavage of its p43/proEMAPII component. *J Biol Chem* 276:23769–23776.
- Vinson M, Strijbos PJ, Rowles A, Facci L, Moore SE, Simmons DL, Walsh FS. 2001. Myelin-associated glycoprotein interacts with ganglioside GT1b. A mechanism for neurite outgrowth inhibition. *J Biol Chem* 276:20280–20285.