Anti-calreticulin Antibody Binds to a Membrane Protein in Caveolae

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Caveolae have been reported to contain a number of proteins related to Ca²⁺-mobilization, including an inositol 1,4,5-trisphosphate receptor-like protein, and a plasmalemmal calcium pump. In search of other Ca²⁺-related proteins, we found that a polyclonal antibody to calreticulin (CRT), an endoplasmic reticulum (ER) Ca²⁺-binding protein, binds to the cytoplasmic surface of caveolae. By immunofluorescence microscopy of cultured fibroblasts, labeling by the anti-CRT antibody was found colocalized with caveolin-1. Immunogold electron microscopy of ultrathin frozen sections showed that the antibody decorates the cytoplasmic surface of caveolae. The caveolar labeling could be due to CRT that leaked out of the ER, but the labeling persisted even when isolated plasmalemmal preparations were treated with 10 mM EDTA, 1 M NaCl, or 0.1 M Na₂CO₃ (pH 11). Furthermore, in SDS-digested freeze-fracture replicas, labeling was associated with caveolae in the P face of the plasma membrane. However, in cells transfected with hamagglutinin (HA)-tagged cDNA, the labeling by anti-HA was observed only in the ER and was not found in caveolae. Taken together, the results suggest that a protein structurally similar to CRT is localized in caveolae as an integral membrane protein, and that the protein could be functionally related to the intracellular Ca²⁺ homeostasis.

Key words: caveolae, calreticulin, calcium, fibroblast, immunocytochemistry

I. Introduction

Caveolae were first defined morphologically as invaginations of the plasma membrane [54]. Caveolin-1, 2, and 3 were identified as major components of caveolae [42, 43, 47, 52]. Many kinds of receptors and signaling molecules have been found to be associated with caveolae, and therefore caveolae have been hypothesized as a signal transduction domain at the cell surface [1, 31, 41]. On the other hand, caveolae have been shown to contain a number of proteins involved in Ca²⁺ translocation: an inositol 1,4,5-trisphosphate receptor (IP₃)-like protein [16, 18], a plasmalemmal Ca²⁺-pump ATPase (PMCA) [17], and transient receptor potential channels, Trp1 [33, 51], and Trp4 [50]. The IP₃-like protein and Trps are assumed to mediate the Ca²⁺ influx, while PMCA is to extrude Ca²⁺ from the cytoplasm. In addition, caveolae in the endothelium have been shown as the site of Ca²⁺ wave initiation and Ca²⁺ entry [26–28]. These results indicate that caveolae are related to Ca²⁺ transport through the plasma membrane and important for intracellular Ca²⁺ homeostasis.

The endoplasmic reticulum (ER) is considered to be the major intracellular Ca²⁺ pool [44] and harbors membrane proteins for discharge and retrieval of Ca²⁺, including IP₃Rs and sarcoplasmic/endoplasmic Ca²⁺-pump ATPases (SERCAs) [25]. The topology and function of IP₃Rs and SERCAs are analogous to the caveolar IP₃-like protein and PMCA. In addition, the ER contains Ca²⁺-binding proteins in its lumen for Ca²⁺ storage [35, 39]. Interestingly, the caveolar lumen was reported to contain concentrated Ca²⁺ [46]. We presumed that a protein(s) for Ca²⁺ storage could also exist in caveolae, if caveolae are engaged in the Ca²⁺ homeostasis. In searching for Ca²⁺-related proteins in caveolae, we found that a polyclonal antibody raised to recombinant human calreticulin (CRT) binds to caveolae in human fibroblasts. CRT is a Ca²⁺-binding chaperone in the ER lumen, and has many low affinity Ca²⁺-binding sites and one high affinity Ca²⁺-binding site [21, 36, 38]. CRT has been

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reported to exist in diverse locations, including the nuclear matrix [7, 10], focal contact [30], the cell surface [2, 22, 40], and the extracellular milieu [53]. Thus it is not surprising if CRT exists in caveolae. But contrary to our expectation, the labeling by the CRT antibody was found on the cytoplasmic surface of caveolae. Furthermore, by analysis of tagged CRT, we found that the caveolar molecule is not the same as CRT in the ER lumen. The results indicated that a protein similar to CRT exists in caveolae, but that it is not CRT itself. The implications of these results were discussed in relation to the caveolar functions in Ca^{2+} translocation.

II. Materials and Methods

Cells and tissues

Human fibroblasts explanted from biopsied normal human skin were grown in Dulbecco’s modified Eagle’s medium (Nihonseiyaku Co., Tokyo) supplemented with 10% fetal bovine serum, 50 U/ml penicillin and 0.05 mg/ml streptomycin at 37°C in 5% CO_2. Human oviducts were obtained from women undergoing total hysterectomy due to squamous cell carcinoma of the uterine cervix with informed consent.

Antibodies and probes

Rabbit anti-CRT antibody (PA3-900, Affinity Bio-reagents, Inc., Golden, CO), mouse anti-caveolin-1 antibody (clone 2234, BD Transduction Laboratories, Lexington, KY), rabbit anti-caveolin-1 antibody (sc-894, Santa Cruz Biotechnology, Inc., Santa Cruz, CA), mouse anti-HA antibody (clone 12CA5, Roche Diagnostics, Indianapolis, IN), Alexa 488- and Alexa 568-conjugated anti-rabbit IgG and anti-mouse IgG antibodies (Molecular Probes, Inc., Eugene, OR), HRP-conjugated anti-rabbit IgG antibody (Jackson ImmunoResearch Lab., West Grove, PA), and Alexa 546-phalloidin (Molecular Probes, Inc.) were purchased. The anti-CRT antibody was raised against recombinant human CRT produced in the Baculovirus insect cell system.

The specificity of the anti-CRT antibody was tested by Western blotting using total lysates of cultured human fibroblasts and rat liver homogenate as antigens. The reaction was visualized by the ECL detection system (Amersham, Buckinghamshire, UK) according to the manufacturer’s instruction.

Production and transfection of hemagglutinin (HA)-tagged CRT

cDNA encoding HA-tagged CRT was cloned by PCR and inserted into pcDNA3.1 vector (Invitrogen, Carlsbad, CA). Because CRT has a signal sequence at the N-terminus and an ER retention signal at the C-terminus, the HA tag was inserted into the middle portion of CRT molecule as described [4]. The construct was transfected to cells by FuGENE 6 (Roche Diagnostics) according to the manufacturer’s instruction.

Immunofluorescence microscopy

Cells cultured on glass coverslips were fixed with 3% formaldehyde in 0.1 M sodium phosphate buffer, pH 7.4, for 10 min and permeabilized with 0.1% Triton X-100 in phosphate-buffered saline (PBS) for 5 min and treated with 3% bovine serum albumin (BSA) in PBS for 10 min before immunolabeling. Procedures after fixation were done at room temperature. The specimens were observed by a Zeiss LSM510 laser confocal scanning microscope or by a Zeiss Axiosvert microscope equipped with Apotome.

In some experiments, the basal plasma membrane was isolated by the following protocol done on ice. Cells cultured on coverslips were washed extensively by PBS, then by a cytoplasmic buffer (70 mM KCl, 5 mM MgCl_2, 3 mM EGTA, 10 mM PIPES), and finally by the cytoplasmic buffer diluted to one third by distilled water. Prewet nitrocellulose paper was placed on the cells, and gently pressed by a finger. The upper plasma membrane and most cytoplasm were removed by lifting the paper; the remaining basal plasma membrane specimens were fixed immediately. In one experiment, the plasma membrane preparation was treated with either 10 mM EDTA, 1 M NaCl, or 0.1 M Na_2CO_3 (pH 11) in the cytoplasmic buffer for 15 min before fixation. Fixation was done in 3% formaldehyde in 0.1 M sodium phosphate buffer, pH 7.4, for 10 min. The samples were treated with 3% BSA in PBS for 10 min, and subjected to immunofluorescence labeling.

Immunoelectron microscopy

For ultrathin frozen sections, cells were fixed with 3% formaldehyde and 0.1% glutaraldehyde for 30 min, infiltrated with 2.3 M sucrose in 0.1 M phosphate buffer, pH 7.4 [49], and frozen in liquid nitrogen. Frozen sections were prepared in a Reichert Ultracut S ultramicrotome attached with a cryochamber FC-4 and processed for immunogold electron microscopy as described before [16, 23]. The specimens were observed by JEOL 100CX or 200CX electron micro-

Fig. 1. Immunoblotting. Lane A, human fibroblast; lane B, rat liver homogenate. Cell and tissue lysates were subjected to SDS-PAGE (10% acrylamide gel), transferred to nitrocellulose, and incubated with the anti-CRT antibody. In both samples, a single band at Mr of 56–58 kDa was observed.
scopes operated at 80 kV.

For immunolabeling of freeze-fracture replicas, cells were processed as described previously [20]. Briefly, cells grown on gold foil were frozen rapidly and freeze-fracture replicas were prepared. The replicas were treated with 2.5% SDS in PBS overnight, rinsed with PBS, pretreated with 3% BSA in PBS, and incubated sequentially with the rabbit anti-CRT antibody and 10 nm colloidal gold-conjugated goat anti-rabbit antibody (Amersham) as described [14]. The labeling density was compared between caveolae and non-caveolar membrane areas. In printed electron micrographs, a circle of 100 nm in diameter was drawn for each caveola and gold particles within the perimeter were counted as labeling in caveolae.

Fig. 2. Double immunofluorescence microscopy by anti-CRT (A, D, G) and anti-caveolin-1 (B, E, H) antibodies. (A–C) Small human fibroblast with a narrow cytoplasm; labeling by the anti-CRT antibody was observed in a dense reticular pattern in the cytoplasm, whereas labeling for caveolin-1 was seen as patches and along the cell edge. Whether the labeling by the anti-CRT antibody colocalized with caveolin-1 could not be discerned. (D–I) Large human fibroblasts with a wide cytoplasm; labeling by the anti-CRT antibody was seen in discrete patches as well as in a reticular pattern. The patches clearly colocalized with caveolin-1. Bar=20 μm.
III. Results

Specificity of the anti-CRT antibody

Total lysates of human fibroblasts and rat liver homogenate were electrophoresed in a 10% polyacrylamide gel, transferred to nitrocellulose filter, and probed with the rabbit anti-CRT antibody. Both samples showed a single band at Mr of 56–58 kDa (Fig. 1), which matched the mobility of CRT reported previously [36]. The result indicates the mono-specificity of the antibody in human fibroblasts used in the present experiment.

Immunofluorescence microscopy

Human fibroblasts were doubly labeled by the anti-CRT and the anti-caveolin-1 antibodies. In relatively small cells with a narrow cytoplasm, labeling by the anti-CRT antibody was seen as a dense network in the cytoplasm (Fig. 2A, C); the distribution was similar to that of ER observed by other probes [34, 48]. Labeling for caveolin-1 was seen as longitudinal patches and along cell edges (Fig. 2B, C). Since the labeling by the anti-CRT antibody was seen intensely in the cytoplasm, its correlation with the caveolin-1 labeling could not be observed clearly in small cells.

In relatively large cells, labeling by the anti-CRT antibody was seen not only in a reticular pattern in the perinuclear region, but also as discrete patches in the peripheral cytoplasm (Fig. 2D, F, G, I). The latter labeling by the anti-CRT antibody colocalized with that of the anti-caveolin-1 antibody (Fig. 2D–I). The result showed that the anti-CRT antibody labels caveolae.

Treatment on isolated plasma membrane preparation

CRT is known to exist in the lumen of ER as a soluble protein [21, 36, 38]. Even though CRT was reported to exist in the cell surface in some cell types [2, 22, 40], the anti-CRT antibody did not label human fibroblasts when it was applied without membrane permeabilization (data not shown). Thus we speculated that the anti-CRT antibody binds to the cytoplasmic surface of caveolae. This result might be caused by CRT molecules that leaked from the ER lumen and adhered to caveolae during the labeling procedure. To examine this possibility we prepared a basal plasma membrane preparation and treated it with various solutions that should remove peripheral membrane proteins. If the caveolar labeling by the anti-CRT antibody was caused by leaked CRT molecules, it should be extinguished by the procedure. The plasma membrane preparation was treated with either 10 mM EDTA, 1 M NaCl, or 0.1 M Na₂CO₃ (pH 11), fixed, and then doubly labeled by the anti-CRT antibody and Alexa 546-phalloidin. Alexa 546-phalloidin was used to label F-actin, which is attached to the membrane noncovalently and should be removed by the above treatments. Labeling for F-actin remained after the EDTA treatments, while it was extinguished completely by NaCl or Na₂CO₃ (Fig. 3E–H). In contrast, labeling by the anti-CRT antibody remained as discrete spots and was not abolished by any of the treatments (Fig. 3A–D). The results suggest that the labeling by the anti-CRT antibody is not caused by leaked CRT molecules, but is caused by some integral membrane component.

![Fig. 3](image-url) Immunofluorescence microscopy of the basal plasma membrane preparation of human fibroblasts. The membrane specimens on coverslips were treated for 15 min on ice with the cytoplasmic buffer alone (A, E), or the same buffer containing 10 mM EDTA (B, F), 1 M NaCl (C, G), or 0.1 M Na₂CO₃ (pH 11) (D, H). After fixation the specimens were doubly labeled by the anti-CRT antibody (A–D) and Alexa 546-phalloidin (E–H). The F-actin labeling was extinguished by NaCl (G) and 0.1 M Na₂CO₃ (H), but the labeling by the anti-CRT antibody persisted after any treatment (A–D). Bar=20 μm.
**Immunoelectron microscopy of ultrathin frozen sections**

Localization of the anti-CRT antibody labeling was examined by immunogold electron microscopy of ultrathin cryosections. In cultured human fibroblasts, immunogold particles were observed densely in the lumen of ER and the nuclear envelope, but they were also seen consistently in the cytoplasmic surface of caveolae (Fig. 4A). The labeling was not seen in the non-caveolar portion of the plasma membrane. To determine if the same labeling occurs in cells in vivo, the human oviduct was examined; immunolabeling by the anti-CRT antibody was observed in fibroblasts in vivo in exactly the same manner as their in vitro counterpart: immunogold particles were localized both in the ER lumen and along the caveolar membrane (Fig. 4B). The caveolar labeling was also seen in smooth muscle cells (Fig. 4C). In contrast, caveolae in the capillary endothelium were hardly labeled by the anti-CRT antibody (Fig. 4D). The results of immunoelectron microscopy confirmed that the anti-CRT antibody binds to the cytoplasmic surface of caveolae, and that the labeling occurs preferentially in fibroblasts and smooth muscle cells, but not in capillary endothelial cells.

**Immunoelectron microscopy of freeze-fracture replicas**

Freeze-fracture replicas of rapidly-frozen cultured human fibroblasts were immunolabeled by the anti-CRT antibody. The experiment had two purposes: one was to examine the ultrastructural distribution of the labeling by a method of different principle; the other was to confirm that the labeling was not caused by peripheral membrane proteins. Even when treated with SDS, integral membrane proteins and membrane lipids remain in the platinum/carbon replicas and can be labeled by antibodies, whereas peripheral membrane proteins are solubilized and cannot be detected [14, 15].

We previously showed that caveolae are observed as small round indentations in the P face of the plasma membrane heavily decorated by anti-caveolin-1 antibody [19, 20]. Labeling by the anti-CRT antibody was found on or very close to the indentations, although its labeling density was lower than that of caveolin-1 (Fig. 5). Only a few gold particles were observed in the flat non-caveolar membrane region. Distribution density of gold particles in caveolae (43.6±27.0/μm²) was significantly higher than that of the non-caveolar membrane (1.8±0.82/μm²; p<0.0001). The result was another evidence that the anti-CRT antibody binds to an integral membrane protein(s) localized to caveolae.

**Immunofluorescence microscopy of human fibroblasts expressing HA-tagged CRT**

To examine whether the caveolar protein labeled by the anti-CRT antibody and CRT in the ER are the same molecule, we transfected HA-tagged CRT cDNA to human fibroblasts and observed whether the tagged molecules is targeted to caveolae. The tagged molecule was designed to preserve the ER retention signal, KDEL, at the carboxyl terminus, and shown to localize in the ER [4]. By immunofluorescence labeling by anti-HA antibody, the tagged CRT was observed in a reticular pattern similar to the endogenous CRT, but it was never seen to colocalize with caveolin-1 (Fig. 6). The result definitely showed that the caveolar protein recognized by the anti-CRT antibody and CRT in the ER are not the same molecule.

**IV. Discussion**

In the present study, we found that a rabbit anti-CRT antibody specifically binds to the cytoplasmic surface of caveolae. Because the labeling was not abolished by various procedures to remove peripheral proteins, and could be observed in SDS-treated freeze-fracture replicas, it was most likely caused by an integral membrane protein(s) in caveolae. On the other hand, when HA-tagged CRT was expressed in the human fibroblast, the tagged molecule was only observed in the ER, and not targeted to caveolae. This last observation could indicate that the caveolar labeling by the anti-CRT antibody is due to nonspecific reaction. But we think it unlikely that the anti-CRT antibody used in the present experiment reacted with totally unrelated proteins, because it was raised to recombinant human CRT obtained in the Baculovirus insect cell system, reacted with a single band at the expected molecular mass in Western blotting (Fig. 1), and has been applied successfully to different kinds of cells [8, 11, 24, 45, 53]. Based on these findings, we surmised that an integral membrane protein(s) homologous to CRT is localized in caveolae.

CRT has been characterized extensively as a Ca²⁺-binding chaperone in the ER lumen, and is thought to be engaged in the regulation of intracellular Ca²⁺ homeostasis and the quality control of secretory proteins (for a review, see [21]). CRT has also been reported to exist in other cellular locations and assumed to have diverse functions. For example, CRT in the nuclear matrix was shown to regulate the glucocorticoid-sensitive gene transcription [6, 10, 38]; at the focal contact, CRT was speculated to bind the cytoplasmic domain of integrins and modulate the cell-matrix adhesion [29, 30]. CRT was also reported to be enriched in the caveolin-1-positive detergent-resistant membrane fraction isolated from smooth muscle cells [9]. These reports suggest that the same CRT molecule could exist not only in the ER lumen, but also in other locations.

On the other hand, when a large amount of tagged CRT was expressed by cDNA transfection, the protein was found only in the ER and not in other locations [4, 12, 13]. A similar result was also reported after transfection of native CRT cDNA [37]. Our present observation on the HA-tagged CRT is in accord with these reports. These results raise questions whether the same CRT molecule could really exist in locations other than the ER lumen. But in view of the cell-type specificity of the caveolar labeling by the anti-CRT antibody, the discrepant results may be reconciled by considering differences of the cells and tissues used.

What kind of functions could the CRT-like protein
Fig. 4. Immunogold electron microscopy of ultrathin cryosections. Human fibroblast in culture (A); fibroblast (B), smooth muscle cell (C), and fibroblast and capillary endothelium (D) in the human oviduct in vivo. (A–C) Immunogold particles were found in the ER lumen (large arrows) and along the cytoplasmic surface of caveolae (arrowheads). (D) The labeling in caveolae (arrowheads) was seen in the fibroblast (fb), but was scarce in the capillary endothelium (en) (caveolae are shown by small arrows); the ER and nuclear envelope were labeled in both cells. Bars=200 nm.

Fig. 5. Immunogold electron microscopy of SDS-digested freeze-fracture replica of human fibroblasts in culture. Caveolae, observed as round indentations in the P face of the replica, were labeled positively by the anti-CRT antibody (arrowheads). Bar=200 nm.
have in caveolae? Previously, we reported that an IP₃R-like protein, a PMCA, and Trp4 are localized in caveolae, and proposed that caveolae may be involved in the influx and extrusion of Ca²⁺ through the plasma membrane [16, 17, 50]. Trp1 was also found in caveolae [33, 51], and the components for store-operated Ca²⁺ entry are thought to be organized in caveolae [28]. If the caveolar molecule recognized by the anti-CRT shares the Ca²⁺ storage domain of CRT and has the affinity for Ca²⁺, it may modulate Ca²⁺ concentration of the cytoplasm just beneath the caveolar membrane.

CRT in the ER has two kinds of Ca²⁺-binding sites: one of them binds one mole of Ca²⁺/mole of protein with high affinity (Kd=10⁻⁶ M), while the other binds 25 moles of Ca²⁺/mole of protein with low affinity (Kd=2.5×10⁻⁴ M) [36]. Because the concentration of Ca²⁺ in the general cytoplasm is far below that of the ER lumen, even if the caveolar CRT-like molecule has the Ca²⁺-binding sites with a similar affinity as CRT, it may seem unlikely that it could be functional in the cytoplasmic surface of caveolae. But a Ca²⁺-sensor protein, yellow cameleon, fused to caveolin-1 revealed that caveolae are preferred sites of the store-operated Ca²⁺ entry, and that the subplasmalemmal Ca²⁺ concentration exhibits a change quite different from that in the bulk cytoplasm [28]. Other studies showed that the Ca²⁺ concentration right beneath the plasma membrane could reach the order of 10⁻⁴ M [3, 32]. These results suggest that the Ca²⁺ concentration in the vicinity of caveolae may temporarily reach the level at which the low affinity sites of CRT could bind Ca²⁺. Under these circumstances, the caveolar CRT-like protein may function as a buffer to prevent an excessive increase and decrease of Ca²⁺ concentration near caveolae.

The mechanism that regulates Ca²⁺ translocation through the plasma membrane is important for various cellular functions. The presence of a CRT-like protein in caveolae strengthens the hypothesis that caveolae are involved in the Ca²⁺ translocation. Molecular identification of the caveolar CRT-like protein and its functional analysis should be important in understanding the caveolar function in this respect.

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Fig. 6. Immunofluorescence microscopy of human fibroblasts transfected with cDNA of HA-tagged CRT. Labeling by anti-HA antibody was seen as a dense reticular pattern in the cytoplasm (A, D), and did not show any colocalization with caveolin-1 (B, C, E, F). Bars=20 μm.
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