

PARTIAL REVERSAL OF TRANSFORMED FUSIFORM PHENOTYPE BY OVEREXPRESSION OF CALRETICULIN

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Abstract: Calreticulin, a Ca^{2+} -storage and chaperone protein of the ER, has also been shown to affect cell adhesiveness. To examine the effects of differential expression of calreticulin on cellular adhesiveness, we used L fibroblast cell lines stably expressing either elevated or reduced amounts of full length, ER-targeted calreticulin. Overexpression of calreticulin correlates with an increase in adhesiveness of L fibroblasts such that these transformed cells acquire epithelioid morphology and form an epithelial-cell sheet when crowded. Functionally, the “reversal” of transformed phenotype in L fibroblasts differentially overexpressing calreticulin can be accounted for by changes in levels of expression of N-cadherin and vinculin. Structurally, however, although the form and extent of cell-cell contacts in L fibroblasts overexpressing calreticulin mimicked those in normal epithelia, electron microscopical examination revealed that cell-cell junctions formed by these transformed cells bore only superficial resemblance to those of normal epithelia in culture. Our data imply that overexpression of calreticulin, while partially reverses fusiform transformed phenotype is in itself insufficient to re-establish *bona fide* zonulae adherens in transformed fibroblasts.

Key words: Calreticulin, Adhesion, Vinculin, N-Cadherin

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Abbreviations used: BSA – bovine serum albumin; DMEM – Dulbecco's modified Eagle's medium; EDTA – ethylenediaminetetraacetic acid; EGTA – glycol-bis(2-aminoethylether)-*N,N,N',N'*-tetraacetic acid; ER – endoplasmic reticulum; FITC – fluorescein isothiocyanate; PBS – phosphate buffered saline; SDS-PAGE – sodium dodecyl-polyacrylamide gel electrophoresis

INTRODUCTION

Calreticulin, a Ca^{2+} -binding protein of the ER of non-muscle cells, an important role in Ca^{2+} homeostasis *in vivo* and is a lectin-like chaperone [1, 2]. In addition to its Ca^{2+} storage and chaperone functions, calreticulin modulates expression of several genes, most notably those whose products are involved in cell adhesion [3]. Cell adhesion is, to a large extent, mediated by two subclasses of adherens-type adhesions: focal contacts (cell-substratum adhesions) and zonulae adherens (cell-cell adhesions) [4]. Both types of adhesions universally share the cytoskeletal protein, vinculin [5], expression of which is affected by the level of calreticulin expression [6, 7]. Changes in the level of calreticulin expression also affect expression of the intercellular junction protein, N-cadherin [8]. It is unclear whether the effects of calreticulin on cellular adhesiveness are mediated by its role in Ca^{2+} -homeostasis or by its chaperone function. It has been suggested that calreticulin may affect cellular adhesiveness by a direct binding to the KxGFFKR sequence conserved in the cytoplasmic tail of α -integrins [9, 10]. However, but the evidence for cytosolic calreticulin has been scarce so far [11, 12]. To affect integrins in focal contacts by direct binding calreticulin should be present there, but calreticulin has not yet been localized to focal contacts [6]. Bulk of calreticulin has been shown to reside in the ER [13-15]. Hence, an alternative has been proposed, that calreticulin may participate in signalling pathway(s) from the lumen of the ER to the cell surface that modulates cell adhesiveness [16].

In the present report, we used transformed L fibroblast cell lines stably and differentially expressing calreticulin [17]. Functionally, L fibroblasts over-expressing calreticulin efficiently aggregate in suspension and migrate as a sheet on solid substrata [8]. Here we show that stable overexpression of full length, ER-targeted calreticulin correlates with an induction of adhesive structures in these transformed L fibroblasts. The observed changes in cell-substratum adhesiveness are likely to be mediated by the induction of vinculin, while the changes in cell-cell adhesiveness are likely to be mediated by the induction of N-cadherin [18, 19].

MATERIALS AND METHODS

Materials

Tissue culture media, BSA, fetal bovine serum, trypsin, and trypsin/EDTA were from Gibco. All the electrophoresis reagents were purchased from Bio-Rad. The nitrocellulose membranes (0.22 μm pore size) were from Micron Separations Inc. Chemiluminescence ECL Plus Western Blotting system was from Amersham Biosciences. Vinol 205S was from St. Lawrence Chemical. All chemicals were of the highest grade commercially available.

A well-characterized goat anti-calreticulin antibody [14, 15, 20] was used to detect calreticulin. A rabbit polyclonal antibody against C-terminal amino acids of chick N-cadherin ("pan-cadherin" from Sigma) was used to detect N-cadherin

[21]. Mouse monoclonal antibodies against actin and β -catenin were from Sigma, while a mouse monoclonal antibody against α -catenin was from Transduction Laboratories. A mouse monoclonal antibody against vinculin was from ICN ImmunoBiologicals. All secondary antibodies were from Jackson ImmunoResearch Laboratories except a goat anti-rabbit IgG antibody conjugated to horseradish peroxidase for immunodetection of N-cadherins that was from Kirkegaard and Perry Laboratories.

Cell culture

Stably transfected cell lines of transformed mouse L fibroblasts generated and characterized before [17] were used. These cells express about 2.0 fold elevated calreticulin level (overexpressing cells) or 0.5 fold reduced calreticulin level (underexpressing cells) as determined by Western blotting. The cells were grown in high glucose DMEM supplemented with 10% fetal bovine serum and with geneticin at concentration of 100 μ g/ml.

Immunostaining and fluorescence microscopy

Cells on coverslips were fixed either in methanol for 10 min at -20°C or in 3.8% formaldehyde in PBS for 10 min at room temperature. After methanol fixation the cells were washed with PBS and blocked with 10% goat serum in 0.1% glycine for 20 min. After formaldehyde fixation the cells were washed in PBS and permeabilized with 0.1% Triton X-100 in buffer containing 100 mM PIPES, 1 mM EGTA and 4% (w/v), polyethylene glycol 8000 (pH 6.9) for 2 min, washed 3 times for 5 min in PBS and then incubated with primary antibodies for 30 min at room temperature. Methanol-fixed cells were used for labelling with a rabbit polyclonal anti-pan cadherin antibody (diluted 1:200 in 1% BSA and 2% goat serum, 1h of incubation at room temperature) as formaldehyde fixation abolished the specific staining. For labelling with remaining antibodies formaldehyde-fixed cells were used. Mouse monoclonal antibodies against vinculin (diluted 1:20 in PBS) were used in 30 min incubations at room temperature. After washing (3 times 5 min) in PBS the cells were stained with appropriate secondary antibodies for 30 min at room temperature. The ER was visualized with FITC-conjugated concanavalin A (Sigma) used at 20 μ g/ml in PBS [15]. After the final wash (3 times 5 min) the slides were mounted in Vinol 205S which contained 0.25% 1,4-diazabicyclo-(2,2,2)-octane and 0.002% p-phenylenediamine to prevent photobleaching. For actin staining, stock solution of 3.3 μ M rhodamine phalloidin (Invitrogen) in methanol was diluted 1:10 in PBS and incubated with fixed and permeabilized cells for 20 min at room temperature. A Bio-Rad MRC-600 confocal fluorescence microscope equipped with a krypton/argon laser was used for fluorescence microscopy.

Electron microscopy

L fibroblasts were plated at a density of 100,000 cells per 50 mm ϕ cell culture dish and allowed to grow for over a week until the cells were slightly over packed. The culture dishes were washed with PBS (3 x 5 min) before fixing with

2% glutaraldehyde and 2% paraformaldehyde for 30 minutes. Cells were treated with 0.5% osmium tetroxide for 30 minutes after washing. Cells were rinsed with distilled water after the next set of PBS washes to prepare them for 30 minute *en bloc* secondary fixation with 1% uranyl acetate. Cells were then rinsed and dehydrated with 5 min single washes with 10, 25, 50% alcohol, and 2 times 5 min washes with 70, 80, 90, 100% alcohol and then embedded in Epon. The coverslips were snapped off in liquid nitrogen. 80-90 nm sections were cut with diamond knife on microtome and placed on nickel grids. Structural staining with saturated uranyl acetate, washing, and lead citrate staining were 15 min each. The sections were then analyzed on a Hitachi-7000 electron microscope.

SDS-PAGE and Western blotting

Cells were homogenized in ice-cold lysis buffer (50 mM Tris-HCl, 120 mM NaCl, 0.5% NP-40, pH 8.0) and immediately boiled for 5 min. Protein samples normalized for DNA content (200 ng of DNA per lane) and molecular weight markers (2 µg/lane) were subjected to SDS-PAGE as described by Laemmli [22]. DNA levels were determined by measuring DNA fluorescence in the presence of Hoechst 33258 dye as described by Teixeira et al. [23]. The standard curve for DNA measurement was obtained using DNA Molecular Weight Markers III in concentrations 100-5000 ng of DNA. Hoechst fluorescence was monitored in Hitachi F-2000 fluorescence spectrophotometre. Nitrocellulose sheets with bound proteins were incubated with primary antibodies for 1 hr at room temperature followed by incubation with horseradish peroxidase-conjugated donkey anti-mouse IgG(H+L) diluted 1:10000 for 1 hr at room temperature. The primary antibodies were used at following dilutions in PBS: actin - 1:100, calreticulin - 1:500, N-cadherin and catenins - 1:1000 and vinculin - 1:1500. Immunoreactive bands were detected with a chemiluminescence ECL Western Blotting system.

RESULTS AND DISCUSSION

Transformed L fibroblasts stably overexpressing calreticulin flatten out and acquire focal contacts

Stable overexpression of calreticulin induced a substantial increase in cell spreading. Fig. 1 shows general features of L fibroblasts after double labelling with fluorescently tagged concanavalin A, to show the ER and an antibody to calreticulin. It is also evident that, at this level of level of detectability, calreticulin localizes to the ER in both calreticulin overexpressing and underexpressing cells. Double labelling of the cells with fluorescently tagged phalloidin to show F-actin cytoskeleton and an antibody to vinculin, to show focal adhesions (Fig. 2) revealed that actin cytoskeleton is underdeveloped in calreticulin underexpressers and their focal contacts are small and sparse. Most of calreticulin underexpressing L fibroblasts have fusiform morphology, characteristic of transformed cells [24]. This is in contrast to calreticulin overexpressing cells which are well spread and display actin stress fibres and prominent focal contacts.

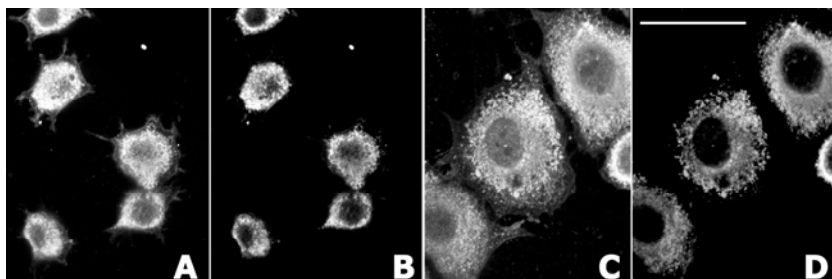


Fig. 1. Sparse L fibroblasts either underexpressing (A, B) or overexpressing (C, D) calreticulin after double labelling with fluorescently tagged concanavalin A (A, C) to show the ER and an antibody to calreticulin (B, D). Scale bar = 25 μ M.

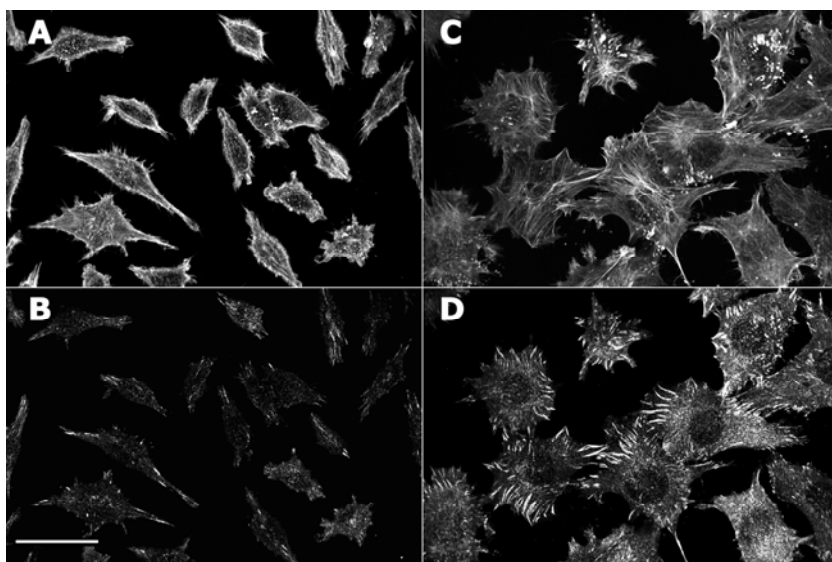


Fig. 2. Sparse L fibroblasts either underexpressing (A, B) or overexpressing (C, D) calreticulin after double labelling with fluorescently tagged phalloidin (A, C) to show the F-actin cytoskeleton and an antibody to vinculin (B, D) to show focal contacts. Scale bar = 25 μ M.

Induction of vinculin and N-cadherin accompanies overexpression of calreticulin

Western blotting (Fig. 3) confirmed that calreticulin overexpressing L fibroblasts indeed display elevated levels of calreticulin, compared to calreticulin underexpressers. Immunodetection of vinculin showed substantial (~3-fold) increase of its abundance in calreticulin overexpressers. In parallel, the abundance of N-cadherin increased in calreticulin overexpressers from a barely detectable level in calreticulin underexpressing L fibroblasts. In contrast, we

detected no substantial differences in the abundance of α -catenin, β -catenin and actin between calreticulin over- and under expressers. Lack of changes in actin abundance indicates that the dramatic difference between calreticulin over- and under expressers in the organization of actin cytoskeleton depends on swaying G/F-actin balance rather than on induction of actin synthesis. The lack of induction of β -catenin in parallel to N-cadherin in calreticulin overexpressers is somewhat surprising considering reports that overexpression of E-cadherin in L fibroblasts increases either the stability [25] or abundance [26] of β -catenin.

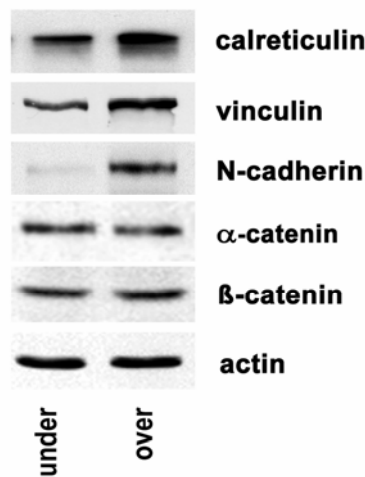


Fig. 3. Western blotting of total cell extracts from L fibroblasts either underexpressing (under) or overexpressing (over) calreticulin.

Nevertheless, this may be due to a rather weak induction of N-cadherin by calreticulin overexpression or it may simply reflect the biochemical difference between E- and N-cadherin. While we did not detect changes in expression of several other adhesion-related proteins (actin, talin, α -catenin, α_5 and β_1 integrins, and pp125 focal adhesion kinase) in calreticulin-associated changes of cell adhesiveness (this report and [6]), however, the involvement of other, yet unidentified proteins, cannot be excluded. Nevertheless, the observed changes in levels of expression of N-cadherin and vinculin in the L fibroblasts differentially overexpressing the full length, ER-resident form of calreticulin are sufficient to account for all the adhesion-related effects observed [27-30].

Transformed L fibroblasts stably overexpressing calreticulin acquire epithelial-like traits

Morphologically, L fibroblasts underexpressing calreticulin are fusiform when sparse or round, piling up when crowded. Reversal of such transformed morphology is one of most striking effect of calreticulin overexpression. Fig. 4 depicts the adhesion-related morphological consequences of differential

expression of calreticulin revealed by immunodetection of vinculin (A, B) and N-cadherin (C, D). This figure shows crowded cultures of L fibroblasts underexpressing (A, C) or overexpressing (B, D) calreticulin. The XZ (“profile” view) sections demonstrate that the calreticulin underexpressers do not spread well and have only scarce vinculin-rich focal contacts with the substratum (Fig. 4A).

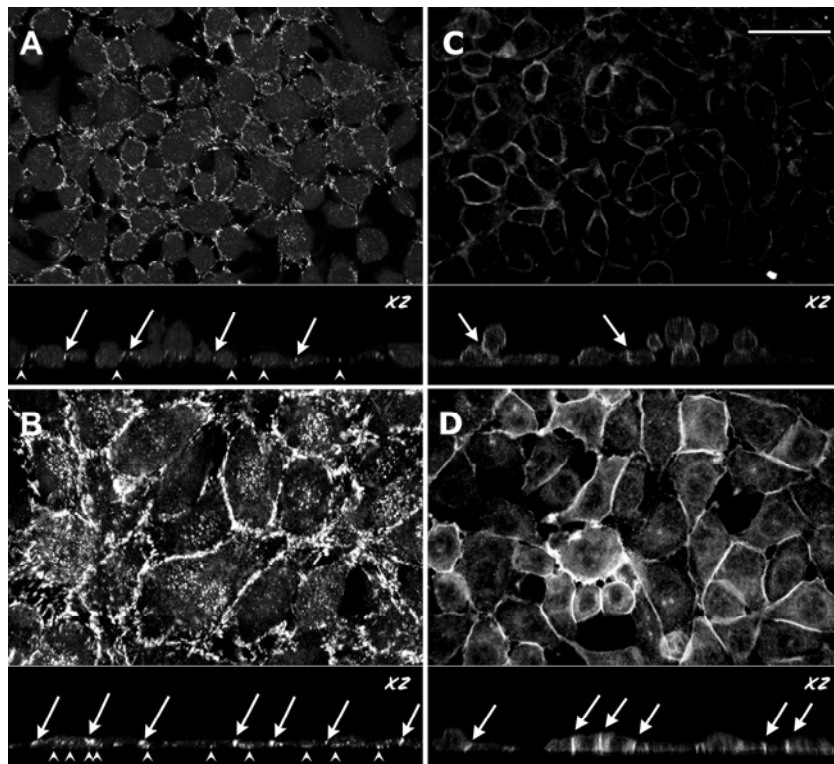


Fig. 4. Low magnification confocal images of crowded L fibroblasts either underexpressing (A, C) or overexpressing (B, D) calreticulin after labelling with antibodies to vinculin (A, B) or N-cadherin (C, D). Bottom of each panel shows an XZ optical section, i.e., an optical section reconstructed perpendicularly to the substratum. The arrowheads indicate the position of the most prominent focal contacts in the XZ sections, while arrows point to the most prominent cell-cell junctions. Scale bar = 25 μ M.

N-cadherin-rich cell-cell junctions are also sparse in these cells (Fig. 4C), which is especially clear in the XZ section. The ring-like appearance of N-cadherin signal is due to round shape of calreticulin underexpressing cells rather than any particular accumulation of the protein at the cell surface. From profile views it is also evident that L fibroblasts underexpressing calreticulin have tendency to form a multilayer. In stark contrast, calreticulin overexpressers have flattened out morphology and develop numerous vinculin-rich cell-cell junctions as well as numerous focal contacts (Fig 4B). These cells are

circumscribed by N-cadherin rich belts (Fig 4D), assuming thus epithelial cell sheet morphology. The presence of continuous belts of zonulae adherens circumscribing each cell is a morphological hallmark of cohesion of an epithelial cell sheets. Indeed, high magnification confocal microscopy of L fibroblasts (Fig. 5) shows that, in contrast to L fibroblasts underexpressing calreticulin (Fig. 5A, C), cells overexpressing calreticulin shows substantial accumulation of vinculin (Fig. 5A) and N-cadherin (Fig. 5B) at cell-cell borders that are highly reminiscent of zonulae adherens.

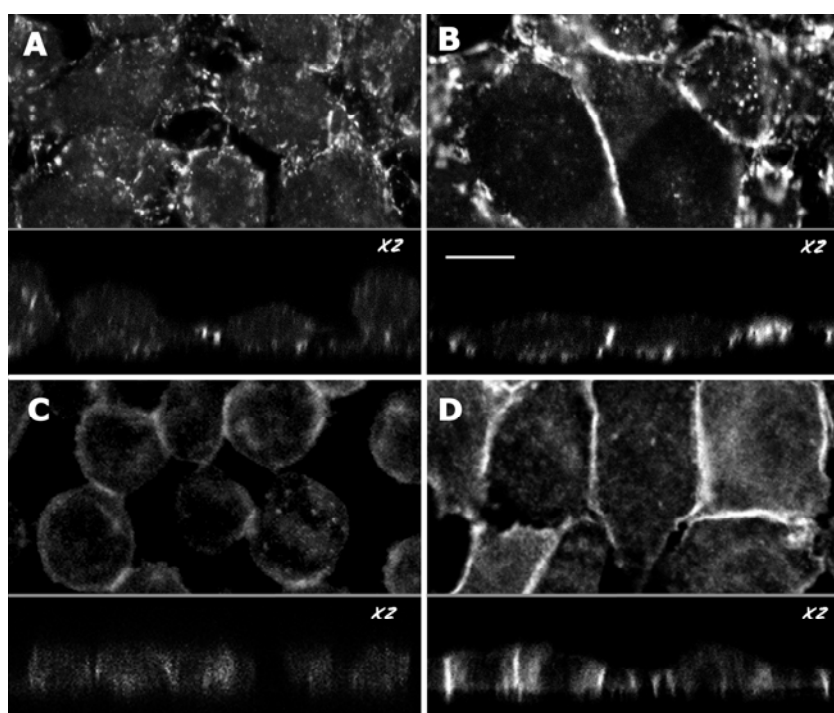


Fig. 5. High magnification confocal images of crowded L fibroblasts either underexpressing (A, C) or overexpressing (B, D) calreticulin after labelling with antibodies to vinculin (A, B) or N-cadherin (C, D). Bottom of each panel shows an XZ optical section, i.e., an optical section reconstructed perpendicularly to the substratum. Vinculin- and N-cadherin-positive intercellular contacts are displayed prominently in both XY (en face) and XZ (profile) optical cross-sections of monolayers of calreticulin overexpressers (B and D). Scale bar = 10 μ M.

How would changes in abundance of N-cadherin and vinculin modulate cellular adhesiveness of L fibroblasts? Cadherin-based cell-cell adhesion has been described in fibroblasts [31-36]. Linkage of the cadherin/catenin complex to the actin cytoskeleton is accomplished *via* α -actinin in WI-38 fibroblasts [34] and *via* vinculin in NRK fibroblasts [35]. With the exception of plakoglobin, L cells express most of the major proteins linking transmembrane adhesion receptors of

either the cadherin or integrin variety to the cytoskeleton [6, 34, 35, 37]. In the present case of L fibroblasts stably overexpressing calreticulin, the increased abundance of N-cadherin and vinculin is likely to account for the formation of more stable intercellular junctions. Hence, a β -catenin/vinculin complex may account for effective linkage of N-cadherin to the cytoskeleton [38, 39]; reviewed in [40, 41]. A decrease in phosphotyrosine level coincidental with an increase in calreticulin expression has been reported [7, 8]. The decreased tyrosine phosphorylation in L fibroblasts overexpressing calreticulin may decrease the rate of proteolytic cleavage of N-cadherin to NCAD90 [42], hence increasing the abundance molecules of the full-length, transmembrane form of N-cadherin [43, 44]. Consequently, an increase in the abundance of N-cadherin itself at the cell surface is sufficient to account for the increased intercellular adhesiveness of calreticulin overexpressers because cadherins that are either truncated [42, 45] or glycosyl phosphatidylinositol-anchored [46] are still effective in mediating intercellular adhesion. In terms of cell-substratum adhesion, the increase in vinculin abundance is sufficient to account for the observed effects [47-51].

Cell-cell contacts of transformed L fibroblasts stably overexpressing calreticulin are not *bona fide* zonulae adherens

High cohesion of epithelial cell sheets is achieved because each and every cell in a sheet is attached to all of its neighbours by a continuous belt of zonulae adherens. Do L fibroblasts overexpressing calreticulin make true zonulae adherens? The aforementioned light microscopical appearance of the cells after labelling with antibodies against either N-cadherin or vinculin suggested that, indeed, that might be the case. To determine if the combined increases in abundance of vinculin and N-cadherin caused the formation of zonulae adherens in fibroblasts overexpressing calreticulin, electron microscopy was utilized. Each cell in a monolayer made contacts with neighbouring cells and the cells were closely apposed. However, the hallmarks of typical adherens junctions were not detectable in fifteen different areas from five separate cultures. No substantial accumulation of electron-dense extracellular material could be detected in either L fibroblast cell line (Fig. 6). Although submembranous microfilament bundles are clearly present in a contact area in calreticulin overexpressers (Fig. 6D), they run in parallel to the membrane without any membrane-associated junctional dense material that serves as anchorage for microfilaments in typical zonulae adherens [52]. Collectively, neither traits of the typical zonulae adherens were detectable in either cell line nor did the cell-cell contact areas resemble cadherin-mediated contacts of NRK fibroblasts [35]. Hence, cohesion in monolayers of L fibroblasts overexpressing calreticulin has been achieved without the presence of a continuous lattice of zonulae adherens circumscribing each and every cell in the monolayer. A likely explanation for the failure of calreticulin overexpressers to form *bona fide* adherens junctions is that the modest increase in N-cadherin abundance (accompanied by low

abundance of the catenins) may be insufficient for the formation of zonulae adherens as the initial level of the zonula adherens proteins expressed in the L fibroblast is minimal [25, 26, 37, 53, 54].

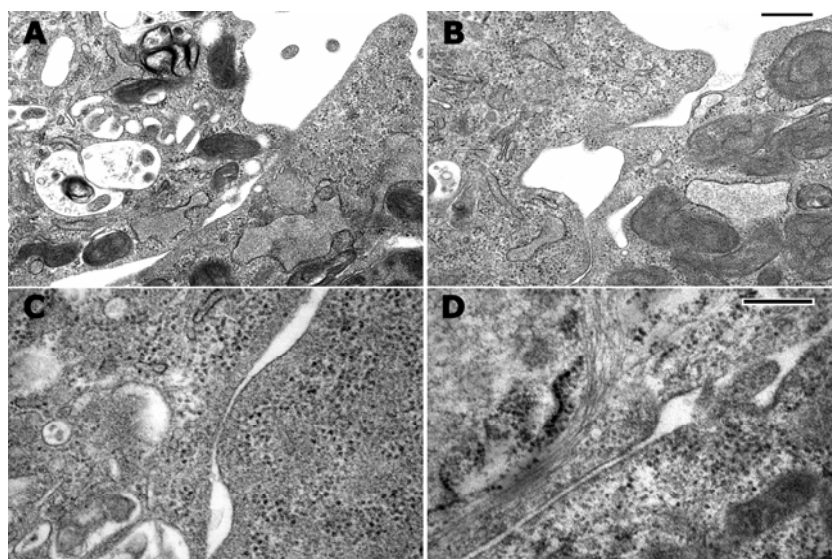


Fig. 6. Transmission electron micrographs of crowded L fibroblasts either underexpressing (A, C) or overexpressing (B, D) calreticulin. Low power images (A, B) show that the general arrangement of cell surfaces between apposed cells appears very similar in both calreticulin under- (A) and overexpressing (C) cell lines. C and D show higher magnification electron micrographs from which it is evident that the typical accumulations of intracellular membrane-associated dense material are not present in either cell line. Scale bars: A, B = 1 μ M, C, D = 0.2 μ M.

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