# Calreticulin Modulates Cell Adhesiveness via Regulation of Vinculin Expression

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***Abstract.*** Calreticulin is an ubiquitous and highly conserved high capacity $Ca^{2+}$-binding protein that plays a major role in $Ca^{2+}$ storage within the lumen of the ER. Here, using L fibroblast cell lines expressing different levels of calreticulin, we show that calreticulin plays a role in the control of cell adhesiveness via regulation of expression of vinculin, a cytoskeletal protein essential for cell–substratum and cell–cell attachments. Both vinculin protein and mRNA levels are increased in cells overexpressing calreticulin and are downregulated in cells expressing reduced level of calreticulin. Abundance of actin, talin, $\alpha_5$ and $\beta_1$ integrins, pp125 focal adhesion kinase, and α-catenin is not affected by the differential calreticulin expression. Overexpression of calreticulin increases both cell–substratum and cell–cell adhesiveness of L fibroblasts that, most surprisingly, establish vinculin-rich cell–cell junctions. Upregulation of calreticulin also affects adhesion-dependent phenomena such as cell motility (which decreases) and cell spreading (which increases). Downregulation of calreticulin brings about inverse effects.

Cell adhesiveness is $Ca^{2+}$ regulated. The level of calreticulin expression, however, has no effect on either the resting cytoplasmic $Ca^{2+}$ concentration or the magnitude of FGF-induced $Ca^{2+}$ transients. Calreticulin, however, participates in $Ca^{2+}$ homeostasis as its level of expression affects cell viability at low concentrations of extracellular $Ca^{2+}$. Consequently, we infer that it is not the $Ca^{2+}$ storage function of calreticulin that affects cell adhesiveness. Neither endogenous calreticulin nor overexpressed green fluorescent protein–calreticulin construct can be detected outside of the ER. Since all of the adhesion-related effects of differential calreticulin expression can be explained by its regulation of vinculin expression, we conclude that it is the ER-resident calreticulin that affects cellular adhesiveness.

Cells adhere to substrata and to each other with two subclasses of adherens-type adhesions: focal contacts (cell–substratum adhesions) (Burridge et al., 1988) and zonulae adherens (cell–cell adhesions) (Franke et al., 1988). Both types of adhesions universally share an adhesion-specific cytoskeletal protein, vinculin (Otto, 1990). Although vinculin is essential for formation and function of these adhesions, very little is known about its regulation. Focal contacts link the cytoskeleton to proteins of the extracellular matrix (Singer, 1979; Hynes et al., 1982; Singer et al., 1984; Burridge and Fath, 1989; Hitt and Luna, 1994). This transmembrane linkage can be realized by several proteins (Luna and Hitt, 1992), of which the integrin superfamily of heterodimeric cell surface receptors has been the most studied (Ruoslahti, 1991; Hynes, 1992). To function as adhesion receptors, integrins must be clustered and immobilized into a focal contact (Schwartz et al., 1991; McNamee et al., 1993; Yamada and Miyamoto, 1995). Experiments with truncated integrins (Ylänne et al., 1993; O'Toole et al., 1994; Williams et al., 1994) show that a conserved amino acid sequence, KxGFFKR, found in the cytoplasmic region of the α integrins plays an important role in regulation of cell adhesiveness. The GFFKR motif also appears in the DNA-binding domain of the steroid receptors (Laudet et al., 1992). In vitro experiments showed that the GFFKR motif, present in the two different cytoplasmic loci, is bound by an ER-resident protein, calreticulin (Rojiani et al., 1991).

Calreticulin, a 60-kD $Ca^{2+}$-binding protein, is a major ER component of nonmuscle cells (Ostwald and MacLennan, 1974; Fliegel et al., 1989*a*,*b*; Treves et al., 1990; Baksh and Michalak, 1991; Opas et al., 1991; Michalak et al., 1992). The protein is synthesized with an $NH_2$-terminal signal sequence, and it terminates with the KDEL sequence (Fliegel et al., 1989*a*,*b*) that is responsible for localization of proteins within the lumen of the ER (Pelham, 1989). Calreticulin plays a central role in intracellular $Ca^{2+}$

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homeostasis: it regulates agonist-sensitive, rapidly exchangeable $Ca^{2+}$ storage, and controls $Ca^{2+}$ influx via the plasma membrane and $Ca^{2+}$ release via the inositol-trisphosphate receptor/$Ca^{2+}$ channel of the ER membrane (Pozzan et al., 1994; Bastianutto et al., 1995; Camacho and Lechleiter, 1995; Mery et al., 1996). Furthermore, calreticulin modulates gene expression (Burns et al., 1994; Dedhar et al., 1994; St-Arnaud et al., 1995; Wheeler et al., 1995) and has a chaperone activity (Nigam et al., 1994; Nauseef et al., 1995; Peterson et al., 1995; Wada et al., 1995; Otteken and Moss, 1996). Most importantly, calreticulin affects cell adhesion (Leung-Hagesteijn et al., 1994; Coppolino et al., 1995) as transient downregulation of calreticulin reduces attachment of cells to extracellular matrix substrata. It has been postulated that this may be mediated by direct interaction, which was shown to occur in vitro, between calreticulin and the KxGFFKR sequence of α integrins (Rojiani et al., 1991; Dedhar, 1994; Leung-Hagesteijn et al., 1994; Coppolino et al., 1995). Consequently, to functionally affect integrins clustered in focal contacts in vivo, calreticulin should be present in the cytoplasm, but, as of yet, there is no direct evidence of this finding.

To investigate mechanisms of calreticulin-dependent modulation of cell adhesiveness, we used mouse L fibroblasts differentially expressing calreticulin generated and extensively characterized previously (Burns et al., 1994; Mery et al., 1996). Here we show that changes in the level of calreticulin expression evoke corresponding changes in the expression of vinculin. Expression of no other adhesion-related proteins examined so far is affected. Consequently, we postulate that the adhesion-related effects of differential expression of calreticulin are vinculin mediated. As we find no evidence for the presence of calreticulin in either cytoplasm or the focal contacts, we hypothesize that calreticulin in vivo performs its adhesion-related functions from within the ER lumen.

## Materials and Methods

### Materials

Human recombinant basic fibroblast growth factor (bFGF)[1], geneticin (G-418 sulfate), tissue-culture media, trypsin, trypsin/EDTA, and restriction endonuclease were from Gibco (Canadian Life Technologies, Burlington, Ontario, Canada). FBS was from ICN Biomedicals (Costa Mesa, CA). pSVL was from Pharmacia (Baie D'Urfe, PQ, Canada). pRc/CMV plasmid was from Invitrogen (San Diego, CA). Plasmid purification kit was purchased from QIAGEN Inc. (Chatsworth, CA). A human glyceraldehyde 3-phosphate dehydrogenase cDNA probe was from Clontech Laboratories (Palo Alto, CA). Human fibronectin was from Collaborative Research, Inc. (Bedford, MA). [$^{32}$P]CTP and Hybond N nylon membranes were from New England Nuclear (Mandel Inc., Mississauga, Ontario, Canada). pGFP10.1 was a gift from Dr. M. Chalfie (Columbia University, New York). All of the electrophoresis reagents were purchased from BioRad Laboratories (Richmond, CA). Chemiluminescence ECL Western blotting system was from Amersham (Oakville, Ontario, Canada). A mouse mAb against vinculin was from ICN ImmunoBiologicals (Montreal, PQ, Canada); a mouse mAb against α-catenin was from Transduction Laboratories (Lexington, KY); a rabbit polyclonal antibody against pp125 focal adhesion kinase was from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA); mouse mAbs against actin and cadherin were from Sigma Chemical Co. (St. Louis, MO). Anti-talin was from Dr. K. Burridge (University of North Carolina); anti-$\beta_1$ integrin was from Dr. M. Ginsberg (Scripps Research Institute); and anti-$\alpha_5$ and anti-$\beta_1$ integrins were from Dr. B. Chan (University of Western Ontario, Canada). All secondary antibodies were from Jackson Immunoresearch Laboratories, Inc. (West Grove, PA). Chelex 100 resin and Coomassie brilliant blue were from Bio Rad Laboratories (Mississauga, Ontario, Canada). Indo-1/AM, Indo-1 $Na^+$, Calcium Calibration Kit 1, and rhodamine phalloidin were from Molecular Probes (Eugene, OR). Cycloheximide and trypan blue were from Sigma Chemical Co. Sudan black B and liquid paraffin were from BDH Chemicals Ltd. (Poole, UK). Naphthol blue black was from Industrial Chemicals Division. Sodium-pantothenate was from Aldrich Chemical Co. (Milwaukee, WI). Poly Mount was from Polysciences Inc., (Warrington, PA). Vinol 205S was from St. Lawrence Chemical (Toronto, Ontario, Canada). All chemicals were of the highest grade commercially available.

### Plasmid Construction

To construct the calreticulin expression vector, the DraI/SmaI restriction DNA fragment (nucleotides 20–1653) of pcDx-CRT (GenBank accession number J05138) was first inserted into the SmaI-digested pSVL vector to generate pSCR-1. Next, the full-length calreticulin cDNA was excised from the pSCR-1 and cloned into the XbaI site of pRc/CMV vector. Two constructs were generated and designated pRCR-DT-1 and pRCR-DT-2, which contain calreticulin cDNA in the sense and antisense orientations, respectively (Burns et al., 1994). These vectors were used to stably transfect L fibroblasts. Another calreticulin expression vector, pSCRGF, containing the green fluorescent protein (GFP) tag was also constructed to study the localization of calreticulin in the cells. The GFP DNA was prepared by PCR amplification using pGFP10.1 (which contains the EcoRI fragment encoding the GFP cDNA in pBluscript II KS [Chalfie et al., 1994]) as the template. In the PCR reaction the 5′ primer (5′-TATAGCGGCCGCGATGAGTAAAGGAGAAG-3′) was designed to incorporate the NotI site at the 5′ end of the GFP. The 3′ primer (5′-GCATGGATGAACTATACAAAGACGAGCTGTAGAGCTCTATA-3′) was designed to include the KDEL sequence and incorporate a SacI site at the 3′ end of GFP. The PCR product was then cut with the two enzymes NotI and SacI and gel purified. To facilitate the insertion of GFP tag, the NotI/SacI DNA fragment of pSCR-1 was removed (removing the KDEL ER retention sequence), and the PCR fragment was then cloned into the NotI/SacI site of pSCR-1, generating the pSCRGF vector. To generate the control GFP vector (pSGF), the EcoRI fragment of pGFP10.1 was first filled at the ends, and then cloned into the SmaI site of the pSVL plasmid. These vectors were used to stably transfect A10 smooth muscle cells.

### Stable Transfection

For transfection experiments, all plasmids were purified using Mega-plasmid preparation and columns (QIAGEN Inc.) as recommended by the manufacturer. Mouse L fibroblasts were transfected with 20 μg of pRCR-DT-1, pRCR-DT-2, or pRc/CMV plasmid by electroporation (1500 V/cm, 25 μF). Cells were then selected for resistance to geneticin (200 μg/ml) for 14 d. The clones obtained were then screened for expression of calreticulin. Two cell lines designated KAB-8 and BAK-4 expressed elevated (~2.0-fold KAB) and reduced (0.5-fold BAK) levels of calreticulin as determined by Western blot analyses (Burns et al., 1994; Mery et al., 1996). KAB cells (referred to as calreticulin overexpressers) and BAK cells (referred to as calreticulin underexpressers), together with a mock-transfected L fibroblast cell line (transfected with pRc/CMV vector, designated PGK, and referred to as control), were selected for use in the present report. To create cell lines expressing GFP-calreticulin, A10 smooth muscle cells were cotransfected with 3 μg of pSCRGF or pSGF and 6 μg of pSVRNeo using the calcium phosphate method and a BES buffer (Ausubel et al., 1989). After selection of the cells for resistance to geneticin (150 μg for 14 d), two stable cell lines were generated and designated ACGF and AGF. ACGF cells expressing GFP-calreticulin and AGF control A10 cells expressing GFP alone were used in the present work.

### Cell Culture

Cells were grown in high glucose (HG) DME supplemented with 10% FBS and with geneticin at a concentration of 100 μg/ml (50 μg/ml for PGK cells).

$Ca^{2+}$-free medium was prepared in the same manner as HG DME except for the omission of $CaCl_2$, replacement of vitamin D–calcium pan-

1. *Abbreviations used in this paper*: bFGF, basic FGF; $[Ca^{+2}]_c$, cytosolic $Ca^{+2}$ concentration; GFP, green fluorescent protein; HG, high glucose.

tothenate by sodium-pantothenate, and the use of chelated distilled water. Chelation of water was carried out with Chelex 100 resin at 5 g/10 liter for 1 h with stirring. FBS was chelated with 5 g of Chelex per 100 ml for 1 h with stirring, after which Chelex was filtrated and the pH was adjusted to 7.2. $Ca^{2+}$ concentration was measured with a fluorescence spectrophotometer (F-2000; Hitachi Ltd., Tokyo, Japan) using 1.2 μM Indo-1 $Na^+$ dissolved in DMSO. Calibration curve of $Ca^{2+}$ concentrations (0.0–39.8 μM) was made using Calcium Calibration Kit 1. The final $Ca^{2+}$ concentration of total medium (HG DME plus serum) was calculated to be ~20 nM.

## Cell Proliferation and Viability

The L cell lines were plated in tissue-culture dishes (10,000 cells per 50-mm Φ dish) in normal (i.e., containing 2 mM $Ca^{2+}$) medium. After 48 h, the medium for each cell type in one half of the dishes was replaced with $Ca^{2+}$-free medium. At selected time intervals, nonadherent cells were washed off the dishes with a brief rinse, and the remaining adherent cells were trypsinized and counted (in triplicate) on a Coulter counter (Hialeah, FL). Viability was measured for each point of the growth curve (data not shown except for final point) by adding trypan blue solution to medium at a final concentration of 0.04%. Dead (i.e., stained) cells were counted in 20 areas, each of 0.635 $mm^2$, under a light microscope.

## Cell Attachment

Substrata of fibronectin and glass were used to measure cell attachment. Fibronectin substratum was made by coating 30-mm Φ dishes with 100 μg/ml human fibronectin diluted in water. Cells used for attachment were trypsinized with EDTA-free trypsin and plated onto fibronectin and glass substrata at 400,000 cells per 30-mm Φ dish. Cells were allowed to attach for various times (data shown are for 2.5 h) before washing, trypsinization, and counting on a Coulter counter. In some experiments, cycloheximide (0.5 μg/ml) was added to the cultures 1 h before the experiment and present throughout.

## Cell Motility

Cells were plated in HG DME and grown at a density of ~250,000 cells per 50-mm Φ cell culture dish. Cells were prepared for filming by changing medium for HG DME plus 10% Hepes buffer, and by covering medium surface with liquid paraffin to prevent evaporation. Time-lapse recordings were done in a chamber kept at 37°C with a Hitachi CCD camera connected to an inverted phase-contrast microscope (CK; Olympus Corp. of America, New Hyde Park, NJ). Images were collected at 2-min intervals over a 16 h period using a frame grabber and digital image processor (Image-1; Universal Imaging Corp., West Chester, PA) on a Compaq 386/25 computer. Average velocity of cells was calculated by tagging and tracking cells (i.e., nuclear positions) for measurements of distances traveled over unit time. For each cell line, 60 cells were followed over three 2-h intervals taken from three different filming sessions, after which ANOVA statistical analysis of the data was performed.

## Cell Morphometry

Cells growing on coverslips were fixed with 3.8% formaldehyde in PBS, washed in PBS, and then stained with 1% Coomassie brilliant blue R-250 and 1% Naphthol blue black in PBS for 60 min. After washing for 15 min in PBS, cells were stained with 0.5% Sudan black B in 70% ethanol. Coverslips were washed with ethanol and mounted in Poly Mount. Phase-contrast images were fed into the Image-1 digital processor. Measurements of cell area and shape factor were computed for 300 cells of each of the L cell lines, after which ANOVA statistical analysis of the data was performed. The cell shape factor is a measure of divergence of the cell shape from a circle (i.e., the shape factor of a circle = 1; the shape factor of a line = 0). The shape factor of a given object is calculated as:

$$4\pi \times A/P^2, \quad (1)$$

where $A$ = object area and $P$ = object perimeter.

## Immunostaining and Fluorescence Microscopy

Cells on coverslips were fixed in 3.8% formaldehyde in PBS for 10 min. After washing (three times for 5 min) in PBS, the cells were permeabilized with 0.1% Triton X-100 in buffer containing 100 mM PIPES, 1 mM EGTA, and 4% (wt/vol) polyethylene glycol 8000 (pH 6.9) for 2 min, washed three times for 5 min in PBS, and then incubated either with goat polyclonal anti–calreticulin antibody (diluted 1:50 in PBS) (Michalak and MacLennan, 1980; Fliegel et al., 1989*a*,*b*; Opas et al., 1991) or with a mouse monoclonal anti–vinculin antibody (diluted 1:50 in PBS) for 30 min at room temperature. After washing (three times, 5 min) in PBS, the cells were stained with appropriate secondary antibodies for 30 min at room temperature. The secondary antibodies were as follows: FITC-conjugated donkey anti–mouse IgG(H+L) (diluted 1:30 in PBS), Texas red–conjugated donkey anti–mouse ($F(ab')_2$ used at 1:30 dilution), and dichloro-triazinylamino-fluorescein–conjugated donkey anti–goat IgG(H+L) (diluted 1:30 in PBS). For double labeling, incubations with appropriate antibodies were done sequentially. After the final wash (three times, 5 min), the slides were mounted in Vinol 205S that 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 μM rhodamine phalloidin in methanol was diluted 1:10 in PBS and incubated with fixed and permeabilized cells for 20 min at room temperature. GFP-calreticulin fluorescence was recorded from either living A10 cells or cells fixed with 2.5% glutaraldehyde in PBS. A Bio Rad MRC-600 confocal fluorescence microscope equipped with a krypton/argon laser was used for fluorescence, phase contrast, and interference reflection microscopy.

## Measurements of Cytosolic $Ca^{2+}$ Concentration

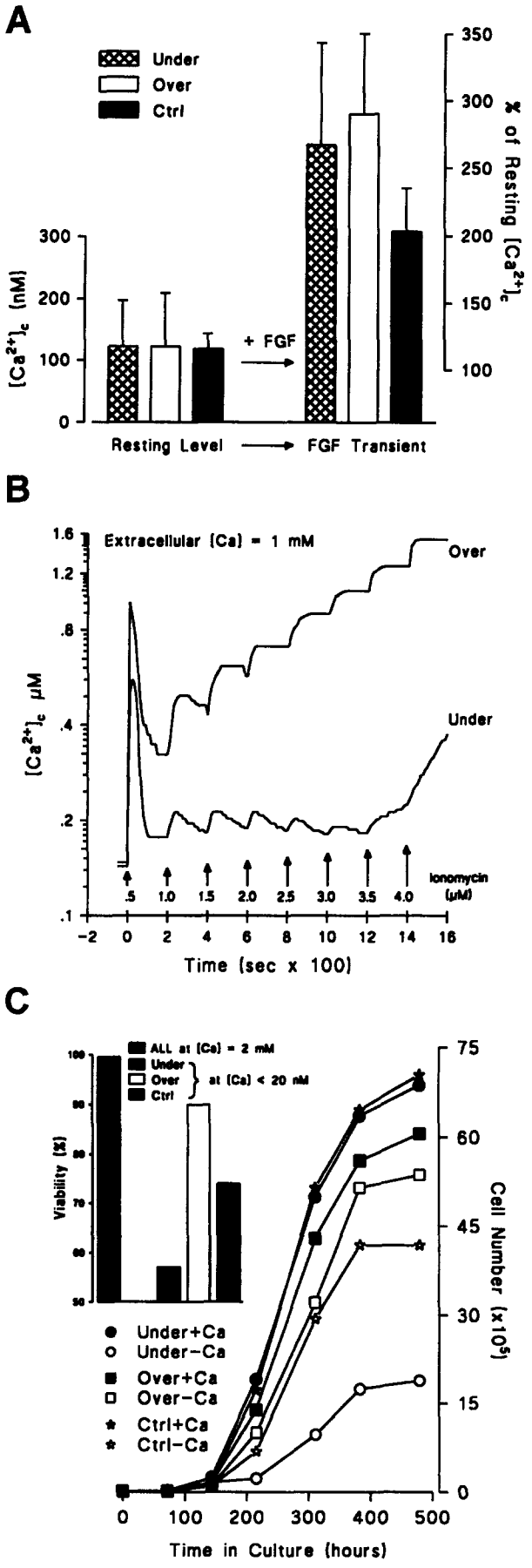
The cells were trypsinized with EDTA-free trypsin and counted, and 5–10 × $10^6$ cells were loaded with 1.5 μM Indo-1/AM in $Ca^{2+}$-containing medium (140 mM NaCl, 4 mM KCl, 10 mM glucose, 10 mM Hepes, 1 mM $MgCl_2$, 1 mM $CaCl_2$, pH 7.4) for 40 min at room temperature. To prevent precipitation of solid Indo-1/AM, 0.02% pluronic acid was included in the incubation medium. Next, cells were pelleted by centrifugation, rinsed with the same medium, and left for 40 min at room temperature for Indo-1/AM deestrification to occur. For cytoplasmic $Ca^{2+}$ concentration measurements, $10^6$ cells were suspended in 1.5 ml of $Ca^{2+}$-containing medium in a 1-cm cuvette at 37°C and allowed to equilibrate for 5 min with continuous stirring. To measure bFGF-induced $Ca^{2+}$ transients, recordings were performed 10 s after addition of 5 ng/ml of bFGF to the cuvette. The Indo-1 fluorescence was monitored in a Hitachi F-2000 fluorescence spectrophotometer with excitation and emission wavelengths of 355 and 410 nm, respectively. Calibration was performed on the same cells by adding 10 μM ionomycin and 4 mM $CaCl_2$ to the cuvette to obtain the maximal fluorescence value ($F_{max}$), followed by 2 mM $MnCl_2$ to obtain the manganese-quenched fluorescence value ($F_{Mn}$). The cytosolic $Ca^{2+}$ concentration ($[Ca^+]_c$) was determined using the equation $[Ca^{2+}]_c = K_d[(F\text{-}F_{min})/(F_{max}\text{-}F)]$ where $F$ was the observed fluorescence, and the minimal fluorescence, $F_{min}$, was calculated from the equation $F_{min} = [(F_{max}\text{-}F_{Mn})/12]+F_{Mn}$ (Grynkiewicz et al., 1985).

## SDS-PAGE and Western Blotting

Cells were homogenized in lysis buffer (50 mM Tris-HCl, 120 mM NaCl, 0.5% NP-40, pH 8.0) and frozen at −70°C. The amount of proteins in these extracts was determined by the method of Bradford (1976). Protein samples (10 μg per lane for extracts and 2 μg per lane for molecular weight markers) were subjected to SDS-PAGE as described by Laemmli (1970). Subsequent transfer of proteins to nitrocellulose sheets and blocking of nonspecific sites with skim milk powder were carried out as described by Towbin et al. (1979). Nitrocellulose sheets with bound proteins were incubated with primary antibodies for 1 h at room temperature followed by incubation with HRP-conjugated donkey anti–mouse IgG(H+L) diluted 1:10,000 for 1 h at room temperature. The primary antibodies were used at the following dilutions in PBS: actin, 1:100; pp125 focal adhesion kinase, 1:200; cadherin, catenin, integrins, talin, 1:1,000; vinculin, 1:1,500. Immunoreactive bands were detected with a chemiluminescence ECL Western blotting system. The protein bands in each blot were scanned two dimensionally using a densitometer (UltroScanXL; Pharmacia), and areas under the curves were calculated using Gel Scan XL software.

## RNA Isolation and Northern Blotting

Total RNA was extracted from calreticulin overexpressers, calreticulin underexpressers, and control cells as described previously (Burns et al., 1994). 30 μg of RNA was separated on an agarose gel, transferred to a Hybond N nylon membrane, and hybridized as described (Burns et al., 1994). For vinculin mRNA, 572-bp EcorRI fragment of Vinc 1020 cDNA (generously donated by Dr. Sue Craig, Johns Hopkins University, Baltimore,




MD) was used as a probe (Coutu and Craig, 1988). The blots were normalized with a human glyceraldehyde 3-phosphate dehydrogenase cDNA probe. The relative abundance of mRNA was determined using a Fujiex BAS1000 Phosphorimager (Fuji Photo Film Co., Ltd., Tokyo, Japan).

## Results

### Changes in Levels of Calreticulin Expression Affect Cellular $Ca^{2+}$ Only in Adverse $Ca^{2+}$

*Conditions.* Cytosolic $Ca^{2+}$ concentration stayed the same irrespective of the level of calreticulin expression and was 122 nM (± 74) for calreticulin underexpressers, 121 nM (± 88) for calreticulin overexpressers, and 117 nM (± 25) for control cells. Furthermore, stimulation of cells with bFGF (5 ng/ml) induced a $Ca^{2+}$ transient that was similar in all cell lines (Fig. 1 *A*). This is in agreement with previous reports (Bastianutto et al., 1995; Mery et al., 1996) and indicates that the level of calreticulin expression does not manifest itself as an altered $[Ca^{2+}]_c$ under normal culture conditions. Effects of the differential calreticulin expression were immediately evident, however, when $[Ca^{2+}]$-buffering capability of cells was challenged by the ionomycin-induced $Ca^{2+}$ influx. Stepwise addition of the $Ca^{2+}$ ionophore, ionomycin, caused stepwise influx of $Ca^{2+}$ that was efficiently clamped down by calreticulin overexpressers but not by the underexpressers (Fig. 1 *B*).

Sustained cell growth requires full intracellular $Ca^{2+}$ stores (Ghosh et al., 1991; Short et al., 1993); hence, we next examined if stable differential expression of calreticulin affects cell growth and viability. All the L cell lines grown in normal media containing 2 mM $Ca^{2+}$ showed similar proliferation kinetics (Fig. 1 *C*). When growth of the cell lines was examined under low ($[Ca^{2+}]$ <20 nM) extracellular $Ca^{2+}$ concentrations, the differences in proliferative capacities between calreticulin overexpressers and calreticulin underexpressers became prominent. At low $Ca^{2+}$ concentration, calreticulin underexpressers barely grew,

*Figure 1.* Overexpression of calreticulin does not affect normal cellular $Ca^{2+}$ homeostasis but enhances its maintenance in adverse conditions. (*A*) Neither resting $[Ca^{2+}]_c$ nor bFGF-induced $Ca^{2+}$ transients are affected by the differential calreticulin expression. The transient was measured 10 s after stimulation of cells with 5 ng/ml of bFGF. (*B*) Overexpression of calreticulin enhances $[Ca^{2+}]$-buffering capability of cells. Calreticulin overexpressers are much more efficient in maintaining $[Ca^{2+}]_c$ homeostasis in the face of ionomycin-induced $Ca^{2+}$ influx than the underexpressers. $Ca^{2+}$ influx was induced by stepwise addition of ionomycin in 0.5 μM increments. The graph shows two typical traces. (*C*) Calreticulin overexpressers can grow at low $[Ca^{2+}]$ in the medium. In contrast to the underexpressers, calreticulin overexpressers show the same growth rate in medium with either high or low $[Ca^{2+}]$. (*Inset*) Viability of the cells at day 20 of culture in either high (2 mM) or low (<20 nM) extracellular $[Ca^{2+}]$. There are no viability differences between the cell lines at high $[Ca^{2+}]$; they are shown as a bar marked *ALL*. Each data point is the mean of nine measurements. Error bars are hidden for the sake of clarity, as most of them are smaller than the symbols. *Under*, calreticulin underexpressers; *Over*, calreticulin overexpressers; *Ctrl*, control mock transfectants; *+Ca*, cells grown in standard medium containing 2 mM $Ca^{2+}$, *−Ca*, cells grown in $Ca^{2+}$-depleted medium containing no more than 20 nM $Ca^{2+}$.

while growth of calreticulin overexpressers was almost unimpaired. When the viability of the cell lines was examined, no differences were detected at normal extracellular $Ca^{2+}$ concentration. At low extracellular $Ca^{2+}$ concentration, however, ~90% of calreticulin overexpressers were viable in contrast with only 57% of calreticulin underexpressers. Thus, the differential level of calreticulin expression in our L cell lines manifests itself functionally as varying cell ability to withstand adverse $Ca^{2+}$ conditions.

### *Calreticulin Overexpression Enhances Cell Attachment Efficacy*

Transient downregulation of calreticulin reduces attachment of cells to extracellular matrix substrata (Leung-Hagesteijn et al., 1994). Hence, we examined attachment efficacy in the stable L cell lines differentially expressing calreticulin. Calreticulin overexpressers attached much more avidly to fibronectin-coated substrata than either calreticulin underexpressers or the control, mock-transfected cells (Fig. 2). We observed no significant differences between the calreticulin underexpressers and the control cells throughout all of the attachment assays. The presence or absence of serum, as well as the presence or absence of cycloheximide (used to inhibit synthesis of endogenous extracellular matrix proteins that might have been deposited onto the substratum), was without effect on the distribution of attachment efficacy between the cell lines.

### *Changes in Levels of Calreticulin Expression Affect Cell Shape, Motility, and the Cytoskeleton*

Morphometry showed that calreticulin overexpression caused L cells to be more spread and to have more cell projections (e.g., filopodia) than the control cells (Fig. 3). Conversely, calreticulin underexpression caused cells to be rounder and smoother than the control cells. This was measured as a cell shape factor. The cell shape factor is a measure of divergence of the cell shape from a circle (i.e., the shape factor of a circle = 1; the shape factor of a line = 0). It is quite clear that there is a straight relationship between calreticulin expression and the cell area, and an inverse relationship between calreticulin expression and the shape factor in the transfectants. Functionally, a time-lapse analysis of moving cells showed that calreticulin underexpressers move with an average speed of 0.974 ± 0.116 μM per min, which is almost twice as fast as calreticulin overexpressers (0.498 ± 0.117 μM per min). Control cells moved with an average speed of 0.751 ± 0.129 μM per min.

Staining with an F-actin–specific probe, phalloidin revealed that the abundance of stress fibers and the number of filopodia increased in calreticulin overexpressers in comparison with either calreticulin underexpressers or control cells (Fig. 4). Collectively, differential level of expression of calreticulin in our L cell lines is reflected in alterations in cell adhesiveness and adhesion-mediated cell functions.

[2]Optical sectioning along the optical axis of the microscope uses a stack of optical sections collected in a plane parallel to the substratum (called XY sections) to electronically reconstruct optical cross-sections (called XZ sections) of the same cells as if taken perpendicularly to the substratum.

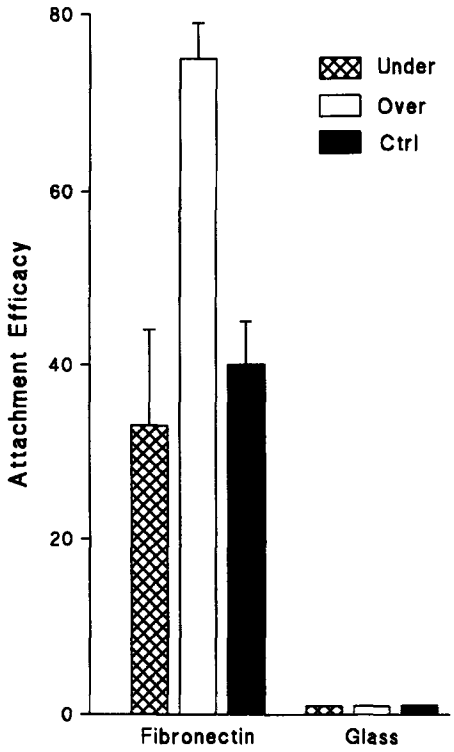



*Figure 2.* Overexpression of calreticulin increases efficacy of cell attachment on fibronectin. *Attachment Efficacy*, number of cells attached to dishes 2.5 h after plating, expressed as percentage of the number of plated cells. *Under*, calreticulin underexpressers; *Over*, calreticulin overexpressers; *Ctrl*, control mock transfectants.

### *Calreticulin Is Not Present in Focal Contacts or Integrin Clusters*

It has been postulated (Dedhar, 1994; Coppolino et al., 1995) that adhesion-related effects of differential calreticulin expression may be mediated by direct interaction, which was shown to occur in vitro between calreticulin and the GFFKR sequence (Rojiani et al., 1991). Hence, to functionally affect integrins by direct protein–protein interaction, calreticulin must be present in the focal contacts.

Double immunolabeling of calreticulin and vinculin failed to demonstrate any calreticulin in association with focal contacts (Fig. 5). Next, we investigated if calreticulin colocalized with integrins after they became clustered by incubation of living cells with specific anti-integrin antibodies. Using antibodies against either subunit of the major extracellular matrix receptor in L fibroblasts, the $\alpha_5$ and $\beta_1$ integrins, we detected no colocalization of calreticulin with integrin clusters. In an alternative approach, we used interference reflection microscopy to identify focal contacts in cells that overexpressed GFP-calreticulin. Interference reflection microscopy is a form of incident light interferometry in which radiance of an image of an adherent cell is a derivative of the width of the gap separating the cell's undersurface from the substratum (Curtis, 1964; Gingell and Todd, 1979). Focal contacts appear as black streaks in interference reflection images (Izzard and Lochner, 1976). Another common form of cell attachment is a

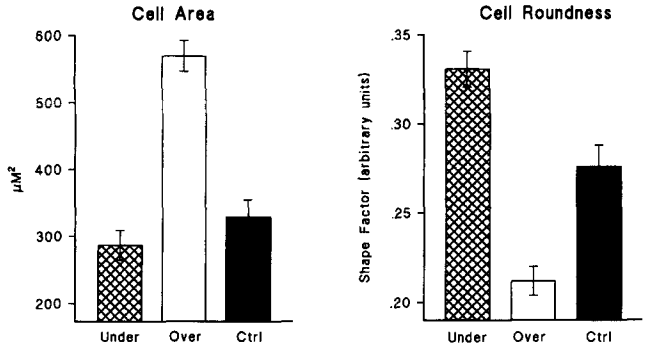



*Figure 3.* Overexpression of calreticulin enhances cell spreading and formation of cell protrusions. Morphometric measurements were taken 48 h after plating. The shape factor is a measure of the perimeter regularity (i.e., roundness) and, for a circle, shape factor = 1. *Under*, calreticulin underexpressers; *Over*, calreticulin overexpressers; *Ctrl*, control mock transfectants.

close contact appearing gray by interference reflection. By using vital technologies to both identify focal contacts and to detect endogenous fluorescent signal of GFP-calreticulin, we avoided problems commonly associated with double immunolabeling. Fig. 6 shows that GFP-calreticulin, while abundant in the ER, could not be detected in either the cytoplasm or focal contacts.

### *Calreticulin Overexpression Causes Formation of Vinculin-rich Contacts by L Fibroblasts*

Stable overexpression of calreticulin induced a dramatic increase in the number and prominence of vinculin-containing adhesions (Fig. 7). Fig. 7 shows a comparison of vinculin distribution in calreticulin overexpressers with that in calreticulin underexpressers. The most obvious differences were observed between densely populated long-term cultures. Calreticulin underexpressers did not attach well to each other and had only a few vinculin-positive focal contacts and even fewer cell junctions (Fig. 7 *A*). Optical sectioning along the optical axis of the microscope (XZ sectioning)[2] showed that calreticulin underexpressers had a tendency to multilayer. In contrast, calreticulin overexpressers flattened out and, by developing abundant vinculin-rich zonulae adherens, formed a monolayer that closely resembled an epithelial cell sheet. In sparse cultures (Fig. 7, *C* and *D*), calreticulin underexpressers had very few vinculin-positive focal contacts and remained round (Fig. 7 *C*). Calreticulin overexpressers spread extensively, had abundant focal contacts, and developed many vinculin-rich cell–cell junctions (Fig. 7 *D*). Differences in vinculin distribution between calreticulin underexpressers and the control cells were not as striking.

### *Calreticulin Induces Changes in Vinculin Expression*

Next, using Western and Northern blotting, we tested the L cell lines differentially expressing calreticulin for the expression of vinculin protein and mRNA, respectively. Furthermore, we examined expression levels of several adhesion-related proteins in these cell lines. Results of densitometric analysis are shown in Fig. 8. No change in abundance of either actin, talin, $\alpha_5$ and $\beta_1$ integrins, pp125 focal adhesion kinase, or α-catenin was detected by Western blotting. To detect α-catenin bands in the blots, however, the exposure time had to be increased 120 times. Amount of vinculin is dramatically elevated in calreticulin overexpressers and somewhat reduced in calreticulin underexpressers in comparison with control cells. These changes in vinculin protein level were further supported by Northern blotting analysis. Total RNA was isolated from the L cell lines and assayed for the vinculin mRNA abundance. Fig. 8 shows that, comparatively, vinculin mRNA abundance in calreticulin overexpressers, underexpressers, and control cells follows a similar pattern to that of the protein (Fig. 8).

## *Discussion*

In the present paper we show that the level of calreticulin expression affects cell adhesiveness via coordinate regulation of vinculin expression. Stable calreticulin overexpression increases cell–substratum attachment of L fibroblasts, while stable calreticulin underexpression suppresses it. L cells overexpressing calreticulin flatten out, develop strong cell–substratum adhesions, reorganize their actin into stress fibers and, most surprisingly, establish striking epithelial-looking, vinculin-rich, cell–cell junctions. We suspect that the lack of effect of calreticulin underexpression on the efficacy of cell attachment is due to a rather small suppres-

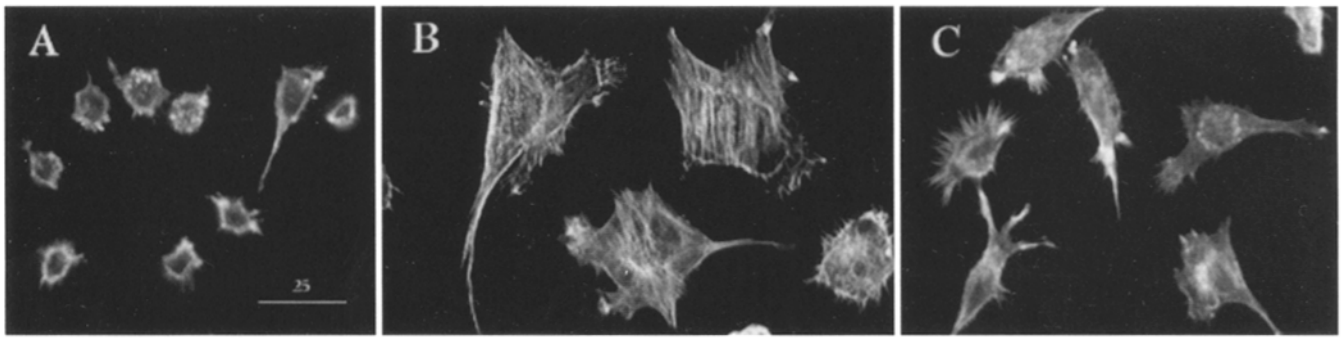



*Figure 4.* Overexpression of calreticulin is associated with the appearance of prominent stress fibers and numerous filopodia as revealed by staining with a fluorescent F-actin probe, phalloidin. (*A*) Calreticulin underexpressers. (*B*) Calreticulin overexpressers. (*C*) Control mock transfectants. Bar, 25 μM.

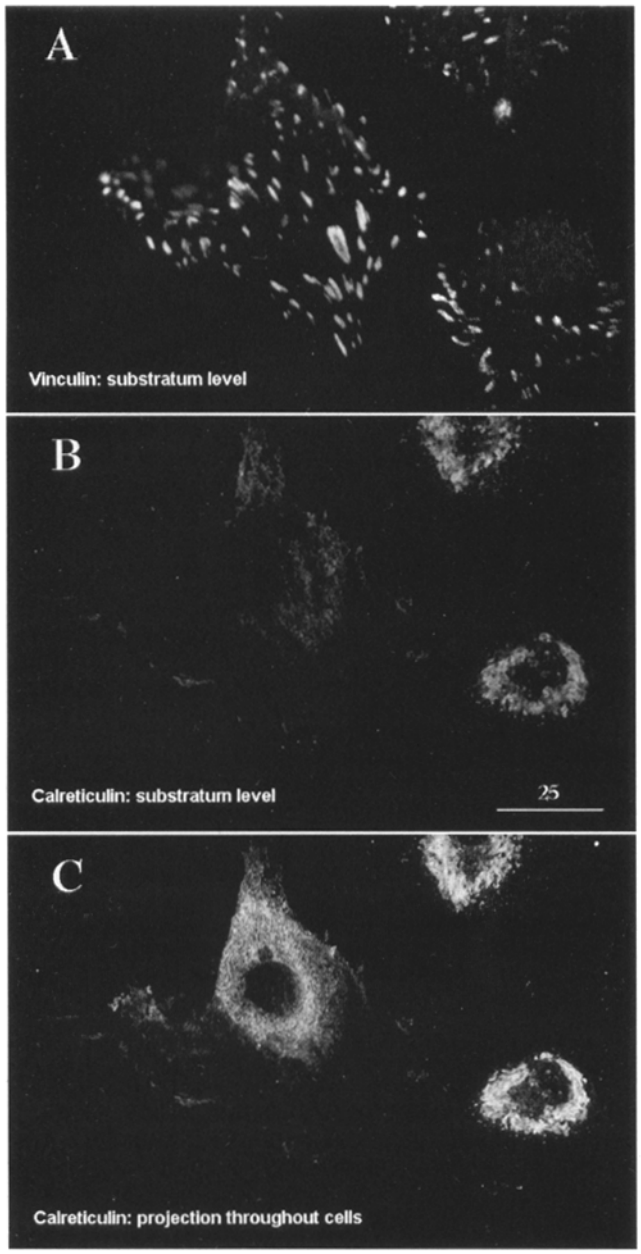



*Figure 5.* Calreticulin is not detectable in calreticulin-overexpressing cells in focal contacts by immunofluorescence after double labeling with antibodies against vinculin (*A*) and calreticulin (*B* and *C*). Confocal optical sectioning detects vinculin label in focal contacts at the substratum level (*A*). At the same optical plane, calreticulin label, although detectable, follows an entirely different pattern of distribution (*B*). *C* is a projection of six confocal sections to show cellular calreticulin distribution in its entirety. Bar, 25 μM.

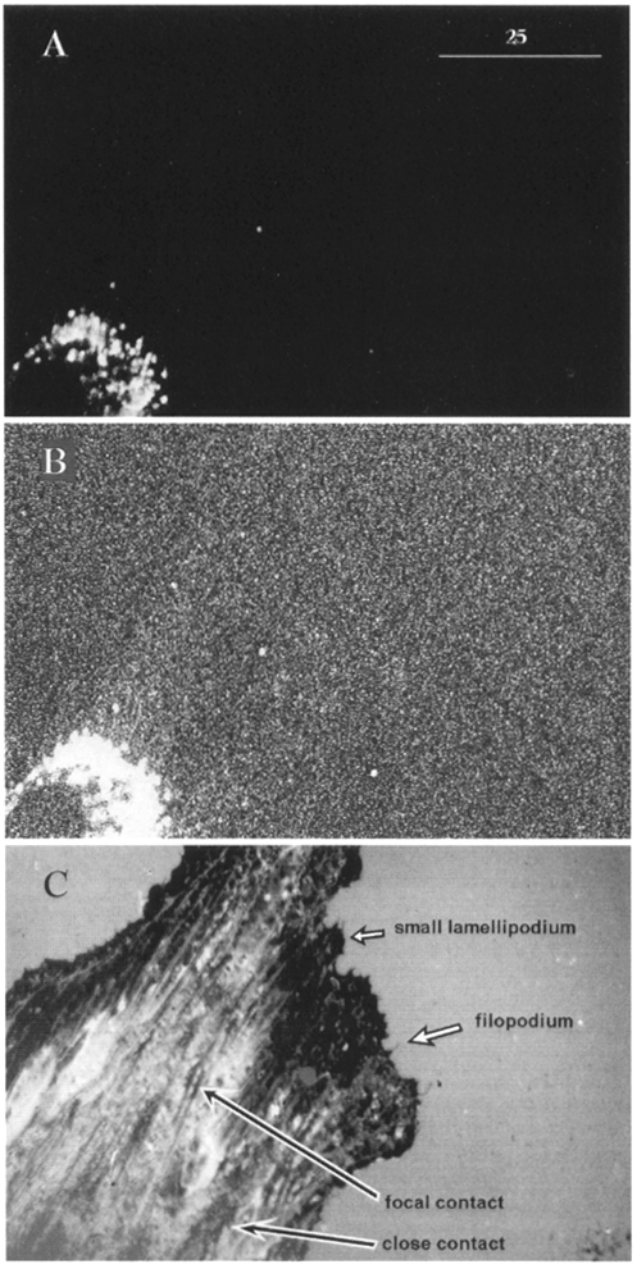



*Figure 6.* GFP-calreticulin is not detectable in focal contacts. Confocal microscopy of GFP-calreticulin fluorescence (*A* and *B*) shows that the label is confined to the ER irrespective of whether normal (*A*) or maximal gain (*B*) detection mode of photomultiplier is used. *C* shows interference reflection image of the same cell with a variety of adhesive/motile organelles indicated by arrows. Bar, 25 μM.

sion effect (~0.5-fold) that may be insufficient to attenuate a complex process such as adhesion. Nevertheless, effects of calreticulin underexpression are detected in adhesion-dependent phenomena such as cell motility (which increases) and cell spreading (which decreases). Relatively modest extents of enhancement and suppression of calreticulin expression have in fact been found advantageous (Mery et al., 1996) as (*a*) problems associated with severe over/under production of protein are avoided, and (*b*) levels of calreticulin expression in our L cell lines are still within the range reported for a variety of tissues (Khanna and Waisman, 1986).

### *How Can Calreticulin Affect Cell Adhesiveness?*

For the apparent multifunctional nature of calreticulin (Nash et al., 1994; Michalak, 1996) and complexity of cell adhesion, several scenarios have to be considered.

***Calreticulin in its Function of $Ca^{2+}$ Store Controls $[Ca^{2+}]_c$ and, Indirectly, Cell Adhesiveness.*** This is unlikely because

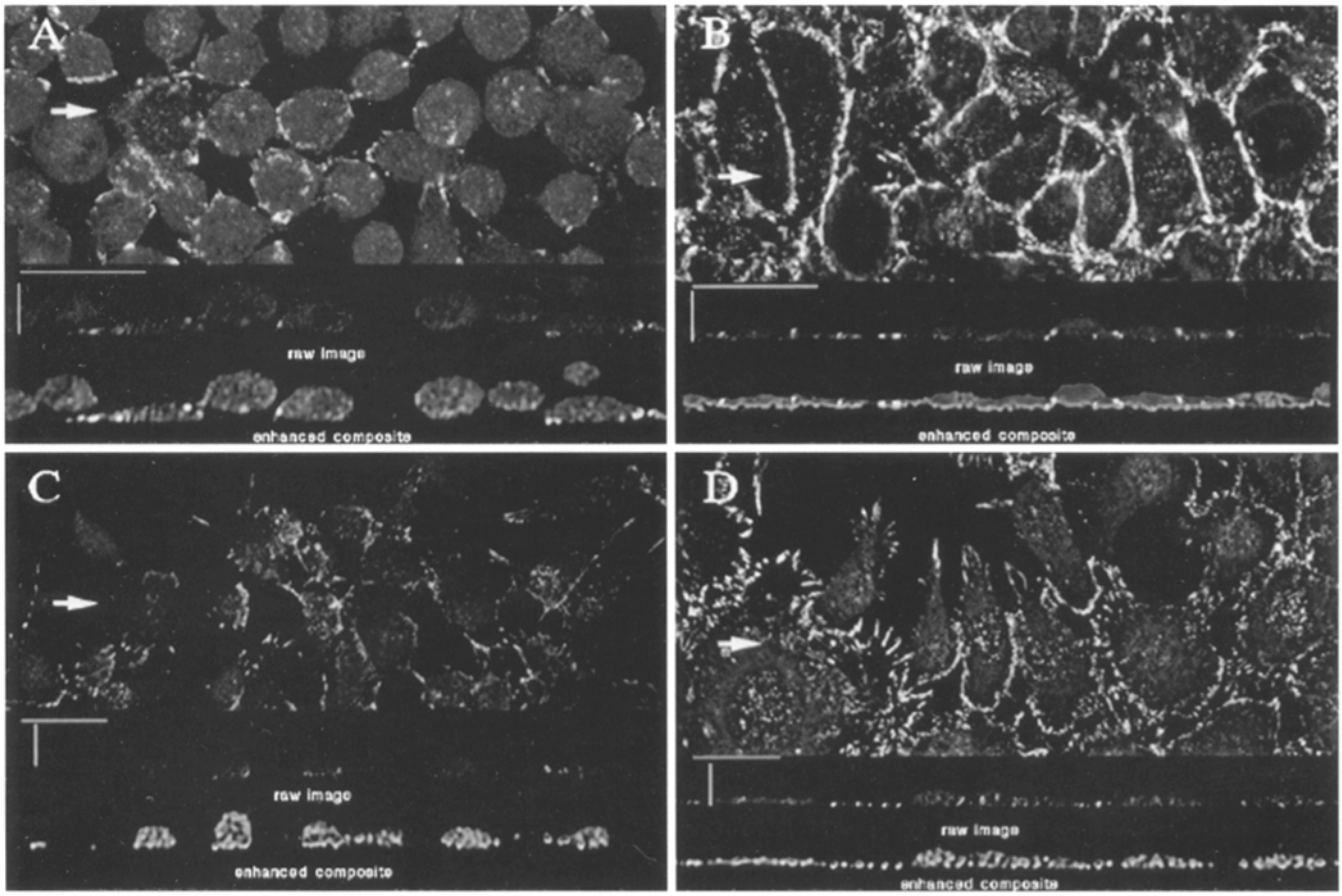



*Figure 7.* Calreticulin overexpression induces epithelial-like vinculin-rich cell–cell junctions in transformed fibroblasts. This figure shows immunolocalization of vinculin in crowded (*A* and *B*) and sparse (*C* and *D*) cultures of calreticulin underexpressers (*A* and *C*) and overexpressers (*B* and *D*). The bottom part of each panel shows two XZ sections taken along the line indicated by the arrows. The top XZ section (*raw image*) shows an unenhanced image, while the bottom XZ section (*enhanced composite*) is an enhancement of nonspecific cytoplasmic background to show the cell height plus the exaggerated vinculin image. In crowded cultures, calreticulin underexpressers (*A*) are round and can pile up. This is especially apparent in XZ sections taken perpendicularly to the substratum. Crowded calreticulin overexpressers (*B*) are much flatter and develop abundant vinculin-rich cell–cell junctions, thus assuming an epithelial cell sheet morphology. In sparse cultures, calreticulin underexpressers form vinculin-positive focal contacts but remain round. Sparse calreticulin overexpressers are well spread and have abundant vinculin-rich cell–cell junctions (*D*). Focal contacts of calreticulin overexpressers are especially evident in a large cell shown at the left side of the photograph, and their cross-sections are discernible in the corresponding part of the XZ section (a string of dots in the left part of the raw image). Bars: (*horizontal*) 25 μM; (*vertical*) 10 μM.

neither we nor others detect any correlation between resting $[Ca^{2+}]_c$ and calreticulin abundance (Bastianutto et al., 1995; Mery et al., 1996). Furthermore, a single application of stimulant (bFGF) to the cell lines differentially expressing calreticulin does not reveal differences in $[Ca^{2+}]_c$ between them. We also show that the level of calreticulin expression, while not affecting resting $[Ca^{2+}]_c$ in normal conditions, will nevertheless have a profound effect on $[Ca^{2+}]_c$ homeostasis in the face of high $[Ca^{2+}]$ insult. A transient downregulation of calreticulin decreases viability of cells exposed to ionomycin (Liu et al., 1994). Here we show that effects of differential calreticulin expression manifest themselves as growth and viability enhancement only at adversely low extracellular $Ca^{2+}$ concentration. Collectively, these data imply that calreticulin acts as an emergency $Ca^{2+}$ store but it does not affect the resting $[Ca^{2+}]_c$ under normal circumstances.

***Overexpression of Calreticulin Compromises its ER Retention Mechanisms and the Protein Finds Its Way to the Outer Aspect of the Cell Surface Where It Plays an Adhesive Role.*** The presence of cell surface–associated calreticulin has been reported (Gray et al., 1995; White et al., 1995). We, however, do not see calreticulin at the surface of L cells, and the presence of antibodies against calreticulin in the medium does not affect these cells in any measurable manner (Jass, G.K., and M. Opas, unpublished data).

***The Increase in Cell Adhesiveness Concomitant with Calreticulin Overexpression Is Related to a Chaperone Function of Calreticulin.*** This is unlikely as— of the adhesion-related proteins examined so far— not the cell surface integrins, but the cytoskeletal protein vinculin and its RNA, are regulated coordinately with calreticulin.

***Calreticulin Regulates Adhesion by Direct Binding to α Integrins.*** For this to occur, there should be focal contact–associated (or at least cytoplasmic) calreticulin. However, to date, neither we nor others (Bastianutto et al., 1995) could detect any cytoplasmic or focal contact–associated calreticulin using immunolocalization with different anti-

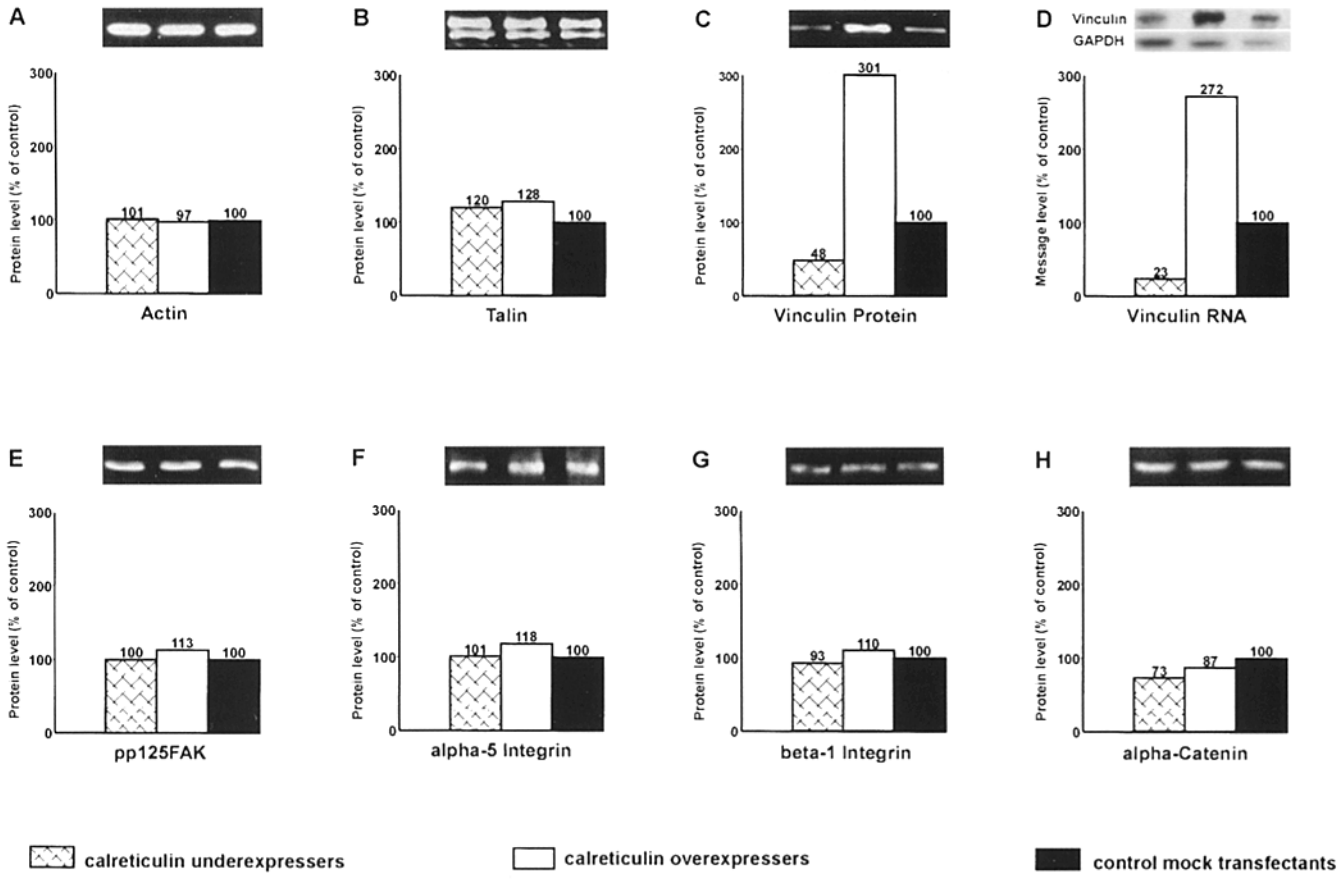



*Figure 8.* Effect of the differential expression of calreticulin on expression of adhesion-related proteins. The densitometric data are shown as the percentage of values obtained for the control cells. The insets show blots in which positions of lanes correspond to the graph bars. Only abundance of vinculin and its messenger RNA change coordinately with the level of calreticulin expression. As a result of extreme susceptibility of talin to proteolysis (Beckerle et al., 1986, 1987) its band appears as a doublet.

bodies and in numerous cell types. We are unable to detect any colocalization of calreticulin and integrins when the latter are clustered by incubation of L cells with specific antibodies. Targeting of calreticulin to the cytoplasm either by microinjection (unpublished data) or by expression of a leaderless calreticulin (Michalak et al., 1996) has no effect on cell morphology or cell adhesion. Finally, using a combination of interference reflection microscopy to locate focal contacts and the detection of endogenous GFP-calreticulin fluorescence, we are still not able to detect calreticulin in any cell compartment other than the ER. Hence, we conclude that cytoplasmic calreticulin, if it exists, is both not detectable and nonfunctional in terms of regulating cell adhesion.

***Calreticulin Exerts Its Adhesion-related Effects via Regulation of Vinculin from the ER Lumen.*** This scenario derives indirect support from the fact that, in vivo, only the ER form of calreticulin inhibits steroid-mediated gene expression, while its cytosolic variant is ineffective (Michalak et al., 1996). In the L cells differentially overexpressing the full-length ER form of calreticulin, changed levels of vinculin expression are sufficient to account for all adhesion-related effects observed so far (Rodríguez Fernández et al., 1992, 1993; Coll et al., 1995; Goldmann et al., 1995; Volberg et al., 1995). This does not exclude involvement of other proteins, although a few obvious candidates (actin, talin, $\alpha_5$ and $\beta_1$ integrins, and pp125 focal adhesion kinase) have been eliminated by us. What remains unclear is how calreticulin overexpression translates into the increased intercellular adhesiveness. Nevertheless, in light of our data, none of the adhesion-related effects of differential calreticulin expression requires the protein to be present at the cytoplasmic face of the plasma membrane. Therefore, we conclude that calreticulin in vivo performs its adhesion-related functions from within the ER lumen.

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