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CELLULAR & MOLECULAR BIOLOGY LETTERS
http://www.cmbl.org.pl

Received: 07 August 2013
Final form accepted: 20 January 2014
Published online: January 2014

Volume 19 (2014) pp 77-97
DOI: 10.2478/s11658-014-0181-9

Research article

CALRETICULIN AFFECTS CELL ADHESIVENESS THROUGH DIFFERENTIAL PHOSPHORYLATION OF INSULIN RECEPTOR SUBSTRATE-1

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Abstract: Cellular adhesion to the underlying substratum is regulated through numerous signaling pathways. It has been suggested that insulin receptor substrate 1 (IRS-1) is involved in some of these pathways, via association with and activation of transmembrane integrins. Calreticulin, as an important endoplasmic reticulum-resident, calcium-binding protein with a chaperone function, plays an obvious role in proteomic expression. Our previous work showed that calreticulin mediates cell adhesion not only by affecting protein expression but also by affecting the state of regulatory protein phosphorylation, such as that of c-src. Here, we demonstrate that calreticulin affects the abundance of IRS-1 such that the absence of calreticulin is paralleled by a decrease in IRS-1 levels and the unregulated overexpression of calreticulin is accompanied by an increase in IRS-1 levels. These changes in the abundance of calreticulin and IRS-1 are accompanied by changes in cell-substratum adhesiveness and phosphorylation, such that increases in the expression of calreticulin and IRS-1 are paralleled by an increase in focal contact-based cell-substratum adhesiveness, and a decrease in the expression of these proteins brings about a decrease in cell-substratum adhesiveness. Wild type and calreticulin-null mouse embryonic fibroblasts (MEFs) were cultured and the

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Abbreviations used: CRT – calreticulin; ECM – extracellular matrix; ER – endoplasmic reticulum; FAK – focal adhesion kinase; IRS-1 – insulin receptor substrate 1; KO – knockout; MEF – mouse embryonic fibroblast, ROCK – Rho-associated kinase; WT – wild type

IRS-1 isoform profile was assessed. Differences in morphology and motility were also quantified. While no substantial differences in the speed of locomotion were found, the directionality of cell movement was greatly promoted by the presence of calreticulin. Calreticulin expression was also found to have a dramatic effect on the phosphorylation state of serine 636 of IRS-1, such that phosphorylation of IRS-1 on serine 636 increased radically in the absence of calreticulin. Most importantly, treatment of cells with the RhoA/ROCK inhibitor, Y-27632, which among its many effects also inhibited serine 636 phosphorylation of IRS-1, had profound effects on cell-substratum adhesion, in that it suppressed focal contacts, induced extensive close contacts, and increased the strength of adhesion. The latter effect, while counterintuitive, can be explained by the close contacts comprising labile bonds but in large numbers. In addition, the lability of bonds in close contacts would permit fast locomotion. An interesting and novel finding is that Y-27632 treatment of MEFs releases them from contact inhibition of locomotion, as evidenced by the invasion of a cell's underside by the thin lamellae and filopodia of a cell in close apposition.

Keywords: Calreticulin, Insulin receptor substrate-1, Adhesion, Focal contacts, Close contacts, Motility

INTRODUCTION

Cellular adhesion to its surrounding matrix has for some time been recognized as critical in defining cell motility and migration [1]. Cellular migration is essential for development, as embryonic cells must alter their adhesive properties in order to involute and form the germ layers, namely the ectoderm, mesoderm and endoderm [2]. These developmental events are calcium regulated, which involves a spectrum of calcium-binding proteins [3–6]. One such protein is the endoplasmic reticulum-resident, calcium-binding chaperone, calreticulin. This multifunctional protein participates in calcium homeostasis via its high calcium storage capacity, in protein "quality control" via its chaperoning activity, and in cell adhesion via pathways that are still not entirely clear [7, 8]. It is known that the level of calreticulin expression affects adherens-type adhesions i.e., focal contacts and zonula adherens [9], by regulating the expression of their structural proteins, namely the focal contact protein vinculin, the zonula adherens protein, N-cadherin and the ECM protein, fibronectin [10–15]. Clustering of the integrin $\alpha_5\beta_1$ (fibronectin receptor) is also affected by the level of calreticulin expression [14].

Many cytoplasmic proteins, such as focal adhesion kinase (FAK), paxillin, talin, α-actinin and vinculin, localize to focal contacts [16–18], where they have a role in stabilizing the focal contact and in signal transduction through the integrins clustered there. Signaling pathways are activated by cell binding to extracellular matrix-associated proteins, such as fibronectin, leading to increased cellular protrusions and adhesion, and thus to motility. Interestingly, integrin-based signaling itself may be modulated by cytosolic signaling pathways. Insulin

receptor substrate-1 (IRS-1) is involved in the insulin signaling pathway, but it has also been found to associate with $\alpha_V\beta_3$ integrins to promote signaling [19]. Furthermore, Goel et al. [20] showed that IRS-1/β1 integrin signaling affected cell-substratum adhesion via integrin activation. Conversely, it has been reported that adhesion to the substratum affects IRS-1 level and phosphorylation [21–24]. Since calreticulin is involved in integrin-dependent cell-substratum adhesion via focal contacts, in this study we set out to explore how this ER-resident protein may play a role in adhesion through the regulation of the insulin pathway-related molecule, IRS-1.

MATERIALS AND METHODS

Cell culture

Mouse embryonic fibroblasts (MEFs) were isolated and established from calreticulin-deficient (K42) and wild-type embryos (K41) by Nakamura et al. [25]. Calreticulin-null (K42) MEFs were transfected with full-length calreticulin to create an additional cell line, K42CRT. These MEF cell lines were previously described [25, 26]. For the sake of simplicity, here we refer to the calreticulin-containing wild-type MEFs as WT, the calreticulin-deficient MEFs as KO, and calreticulin-null MEFs transfected with full-length calreticulin as KO•CRT. MEFs were cultured in Dublecco's Modification Eagle's Medium (DMEM, Wisent), with 10% FBS (Wisent), 1% non-essential amino acids and 1% antibiotics (both from Gibco, Canadian Life Technologies) at 37ºC and 5% CO_2 for 48 h before treatments. For treatment with Y27632 ROCK-inhibitor (Sigma Aldrich), MEFs were plated in the medium described above with the addition of the inhibitor at a working concentration of 1 mM for a period of 4 h prior to either lysis or ultrasonication.

SDS-PAGE and western blot analysis

Cells in a semi-confluent state were collected for western blotting following overnight growth periods, harvested in a lysis buffer (Cell Signaling), and sonicated. Sample protein concentrations were quantified using Bio-Rad Protein Assay solutions and an absorbance plate reader at a 750 nm wavelength. Protein samples were loaded at 30 μg per lane, separated by SDS-PAGE, and transferred to nitrocellulose membrane. To determine the level of matrix fibronectin, the acellular ECM fraction was isolated by sequential washes with 1) PBS; 2) 3% Triton X-100 in PBS; 3) 50 mM Tris (pH 7.4), 10 mM $MnCl_2$, 1 M NaCl; 4) 2% deoxycholate in 50 mM Tris (pH 8.8), 10 mM EDTA. All washes were carried out at room temperature for 3 min in the presence of 1 mM PMSF. The material that remained after the washes was considered to be ECM and was scraped in 1% SDS and boiled for 5 min. Western blot analysis was carried out with the following antibodies in 1% milk PBS: Genetex anti-phosphoIRS-1 Y896 (1:500), Abcam anti-phosphoIRS-1 S636 (1:500), Abcam anti-IRS-1 (1:500), Abcam anti-calreticulin (1:600), Abcam anti-GAPDH (1:600), Sigma

anti-fibronectin (1:1000). Horseradish peroxidase-conjugated secondary antibodies used were Alexa Fluor 488 anti-mouse and Sigma Aldrich anti-rabbit (both 1:3000). Nitrocellulose membranes were blocked overnight in 5% milk PBS at 4ºC. Primary antibodies were applied at room temperature for 1 h, followed by washing in 1% milk PBS (3 times for 5 min). Secondary antibodies were applied for 1 h, followed by washing (3 times for 5 min). Immunoreactive bands were detected with a chemiluminescence ECL Western blotting system (GE Healthcare). Western blots were normalized using anti-GAPDH antibodies.

Immunolabeling and microscopy

Cells were grown overnight (16 h) before processing unless otherwise indicated. For immunolocalization, cells on coverslips were fixed in 3.7% formaldehyde in PBS for 10 min. After washing (3 times for 5 min) in PBS, the cells were permeabilized for 2 min with 0.1% Triton X-100 in buffer containing 100 mM PIPES, 1 mM EGTA and 4% (w/v) polyethylene glycol 8000, then washed (3 times for 5 min) and incubated with primary antibodies for 30 min at room temperature. Anti-vinculin (SIGMA cat. no. V4506), anti-paxillin (Millipore cat. no. 05-417), anti-talin (SIGMA cat. no. T3287) and anti-fibronectin (SIGMA cat. no. F7387) antibodies were all used at 1:50 dilution in PBS, and Texas Red Phalloidin (Invitrogen/Molecular Probes) was used at 1:10 dilution in PBS.

After being washed (3 times 5 min) in PBS, the cells were stained with appropriate fluorescent secondary antibodies for 30 min at room temperature. All of the secondary antibodies (Jackson Immunoresearch Labs Inc.) were used at 1:50 in PBS. For double labeling, incubations with appropriate antibodies were done sequentially.

After the final wash (3 times for 5 min), the slides were mounted in Fluorescent Mounting Medium (DAKO) to prevent photobleaching. A Bio-Rad MRC-600 laser scanning confocal fluorescence microscope equipped with a Nikon 60/1.40 Plan Apochromatic oil immersion objective and a krypton/argon laser was used for fluorescence imaging at room temperature. Bio-Rad COMOS software was used for image acquisition. For focal contact counting, cells were single-labeled with anti-vinculin, anti-paxillin or anti-talin, followed by secondary antibodies, and the brightly fluorescent patches were manually counted.

For Interference Reflection Microscopy (IRM), cells were fixed in 4% glutaraldehyde for 10 min, washed with PBS, and mounted in PBS. IRM was conducted using a Bio-Rad MRC-600 laser scanning confocal fluorescence microscope equipped with a reflection filter module. Spurious reflections were cleaned up in final images by subtracting the background images using Adobe Photoshop (Image Calculations) or by using the Pseudo Flat Field plugin of the freely available ImageJ software [http://rsbweb.nih.gov/ij/].

Motility assays

Phase contrast images were captured using a Nikon Coolpix 4500 digital camera along with a programmable Nikon MC-EU1 remote cord connected to a Nikon Diaphot-TMD inverted microscope using a 20x objective. An incubator was attached to the microscope hood and set to 37ºC. A low light with a green filter was used to illuminate the dish to minimize light-induced reactions. Buffered DMEM (15 mM HEPES, L-glutamine, 10% FBS, 1% NEAAs, 1% antibiotics, 2 g/l sodium bicarbonate) was used to culture cells during phase contrast microscope photography [27]. Sodium pyruvate was added to a final concentration of 2 mM to prevent hydrogen peroxide toxicity caused by light exposure, as recommended by the University of Alberta, Faculty of Medicine Imaging Centre [http://www.microscopy.med.ualberta.ca/techniques/2011/09/hepes-buffered-media-for-live-cell-imaging/]. Paraffin oil (NidaCon NidOil from Somagen Diagnostics Inc.) was applied to the medium surface to prevent evaporation during microscopy.

Images were captured at 2 min intervals for 6 or more h. To analyze motility differences between cell lines, ImageJ software was used to compile image sequences into films. The nuclei of at least 30 cells of each type in at least three different experiments were tracked using the MTrackJ plugin for ImageJ from Erik Meijering, Erasmus University Medical Center Rotterdam, The Netherlands [http://www.imagescience.org/meijering/software/mtrackj/] and translocation was quantified for displacement and directionality.

Ultrasonic adhesion strength assays

Our assay follows that of Menssen et al. [28] and while for convenience's sake, we refer to it as a measurement of the strength of adhesion, in reality it is a measure of the energy required to dislodge cells from the substratum. The cells were first plated on 0.1% gelatin-coated tissue culture dishes and grown to sub-confluence to avoid cell-cell interactions. For ROCK inhibition, the cells were incubated in Y27632-treated medium for 4 h. The cell culture medium was changed immediately prior to sonication to ensure that no detached cells would be already present in the medium prior to the adhesion assay. Plates were sealed with Parafilm, immobilized to the center of a ultrasonicator well, and sonicated for 4 min using a VWR Ultrasonicator 97043-960 at 35 kHz and 48 W. The medium containing the detached cells was removed from the plates and centrifuged. The supernatant medium was removed and the remaining detached cells were treated with 500 µl trypsin/EDTA for 4 min in order to disrupt any intercellular adhesions, after which the solution was mixed with trypan blue dye at a ratio of 30 µl cell isolate to 10 µl trypan blue. 40 µl of the cell isolate/dye mix was transferred to a counting slide and living cells (those not permeated with blue dye) were counted under an inverted phase contrast microscope.

RESULTS AND DISCUSSION

Calreticulin affects focal contact-based cell-substratum adhesiveness

We previously established that in a variety of cell types, changes in the level of calreticulin expression affect cell-substratum adhesiveness via the regulation of the number and prominence of focal contacts [29]. Indeed, double labeling of mouse embryonic fibroblasts (MEFs) for actin and paxillin, an important focal contact protein, clearly demonstrates the scarcity of focal contacts in calreticulin-deficient (KO) cells in comparison to calreticulin-containing (WT) cells (Fig. 1A, B). Transfection of calreticulin KO cells with the full-length calreticulin construct restores the number and prominence of focal contacts (Fig. 1C).

Fig. 2 shows counts of the focal contacts per cell after staining with vinculin, paxillin and talin. Their protein composition is far from uniform [30], but a few general rules may apply. Talin is a cell-substratum attachment protein that is most widespread amongst the different contact types. Soon after integrin clustering occurs, talin and paxillin are recruited followed by recruitment of vinculin [31, 32]. Still, as shown in Fig. 2, the presence or absence of calreticulin affects the number of focal contacts but not their composition. It is worth noting here that in cells that are re-transfected with calreticulin, the calreticulin vector is driven by a viral promoter and thus is not regulated [26]. This leads to exaggerated restoration of many (but not all) calreticulin-dependent features such as the number and prominence of focal contacts in KO•CRT cells as compared to WT cells.

IRS-1 and its phosphorylation

It was reported at the turn of the century that a reciprocal relationship may exist between cell adhesion and the abundance and phosphorylation of IRS-1 [20–22, 33]. We assessed if the presence or absence of calreticulin affect IRS-1 abundance and the phosphorylation of its two potentially adhesion-related residues, serine 636 and tyrosine 896 [34]. Western blotting shows that the presence or absence of calreticulin has no substantial effect on either the level of total IRS-1 or its phosphorylation at tyrosine 896. In stark contrast, the phosphorylation of IRS-1 at serine 636 increases dramatically in the absence of calreticulin (Fig. 3).

Serine 636 of IRS-1 is phosphorylated by ROCK (Rho-associated kinase, Rho-kinase, ROK) [24, 35]. Expression of activated ROCK induces IRS-1 serine phosphorylation [36] and ROCK physically associates with IRS-1 [37]. ROCK is a kinase involved in the regulation of cell contractility via the formation of stress fibers and the formation or maturation of focal contacts [38, reviewed in 17, 39]. ROCK is specifically inhibited by Y-27632 [40], a treatment that decreases the phosphorylation of serine 636 of IRS-1 [24, 35]. Fig. 4 shows by Western blotting that, predictably, treatment of the MEF lines with Y-27632 universally decreased IRS-1 serine 636 phosphorylation. Y-27632 exposure did not affect phosphorylation of IRS-1 tyrosine 896. Furthermore, Y-27632 exposure substantially decreased the level of total IRS-1 in all cell lines (Fig. 4). This was unexpected because to date there is no report of such an effect of Y-27632.

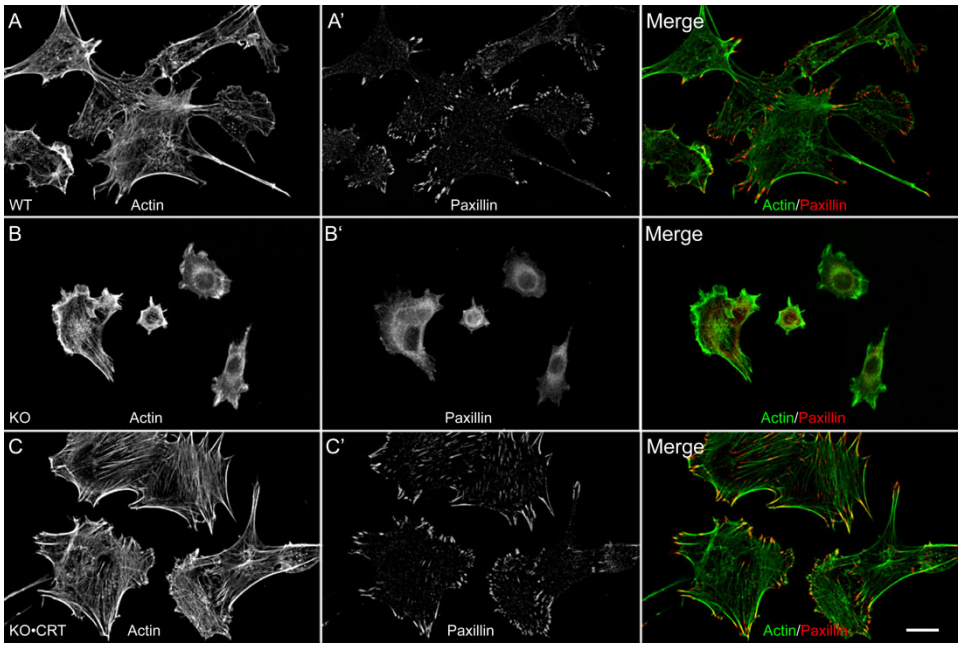

Fig. 1. Fluorescence localization of actin- and paxillin-containing structures in MEFs with or without calreticulin. Calreticulin wild-type (WT) and rescued cells (KO•CRT) have prominent stress fibers. Calreticulin-null cells (KO) are rounded and lack stress fibers (A, B, C). Paxillin-containing focal contacts in the KO cells (B') are scarce compared to the calreticulin-expressing (WT and KO•CRT) cells (A', C'). Scale bar – 20 μm.

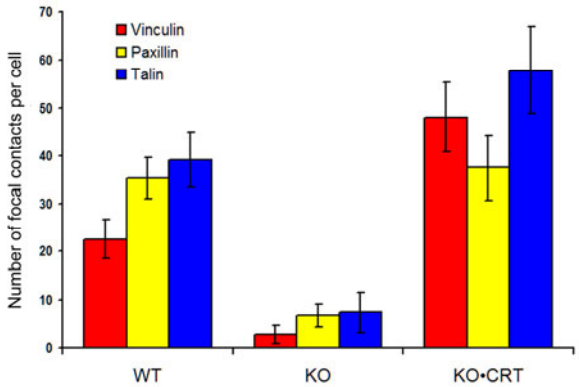

Fig. 2. The absence of calreticulin is paralleled by a reduction in the number of focal contacts. Reintroduction of the protein restores their number. This bar graph shows that in spite of the changes in the number of focal contacts between the MEF lines, their composition remains almost the same: the relative drop in paxillin level is barely significant. Error bars show SDs.

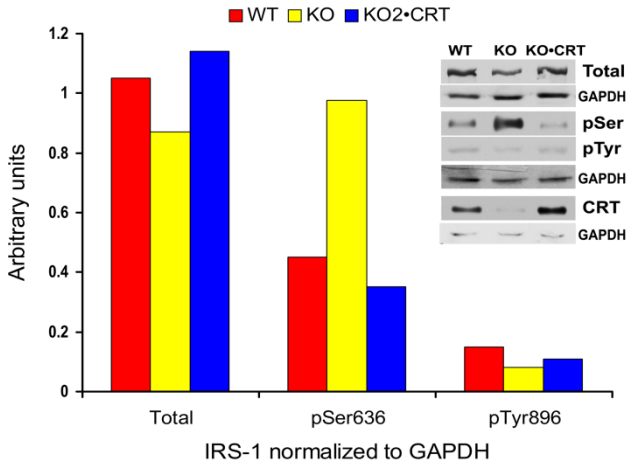

Fig. 3. Phosphorylation of IRS-1 on serine 636 increases in the absence of calreticulin. the graph shows the densitometry of the western blot lanes shown in the insert normalized to GAPDH (pSer and pTyr show the same membrane stripped and re-blotted). These are: total IRS-1 (Total), serine 636-phosphotylated IRS-1 (pSer636), tyrosine 896-phosphorylated |IRS-1 (pTyr896) and calreticulin (CRT). This pattern of IRS-1 content and phosphorylation was maintained in 5 separate experiments.

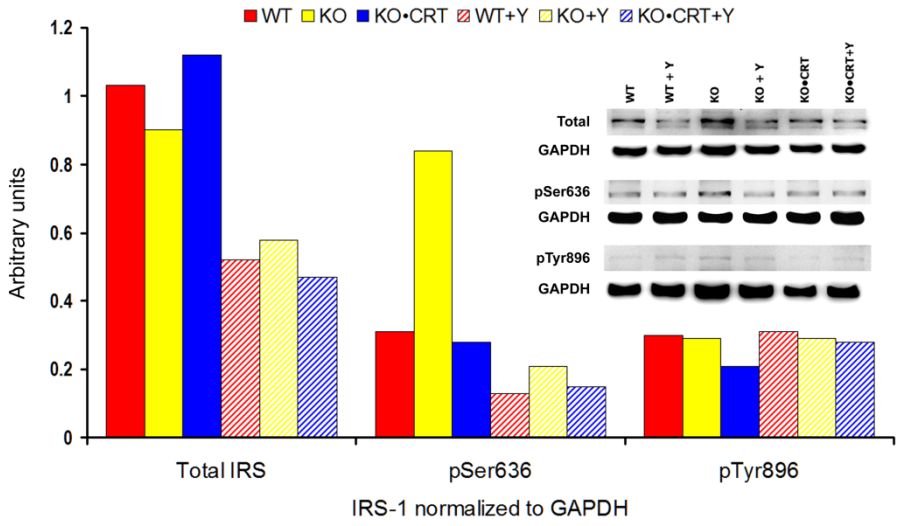

Fig. 4. Inhibition of phosphorylation of serine 636 of IRS-1 causes its downregulation. The graph shows the contents of total IRS-1 (Total IRS), serine 636-phosphotylated IRS-1 (pSer636) and tyrosine 896-phosphorylated IRS-1 (pTyr896) in the three MEF lines with (+ Y, striped bars) or without (solid bars) Y-27632 treatment. Each lane was normalized to GAPDH. The insert shows the corresponding western blot lanes. This pattern of IRS-1 content and phosphorylation was maintained in 5 separate experiments.

IRS-1 inhibition and types of cell-substratum adhesions

IRS-1 is affected by focal contact-dependent adhesion, which is mediated by β_1 integrins [20, 41]. Focal contact-dependent, β_1 integrin-mediated adhesion is modulated by calreticulin [10-14, 42]. Having noted differential phosphorylation of IRS-1 on serine 636 upon differential calreticulin expression and Y-27632 treatment, it was important to examine its morphological consequences. Immunofluorescence with specific antibodies was used to reveal the presence and arrangement of focal contact-related proteins, while interference reflection microscopy (IRM) was used to produce an overview of the topography of a cell underside adherent to the substratum. IRM takes advantage of the interference of wavefronts reflected at the phase boundaries created by the thin layer of culture medium that separates the cell and the glass substratum to which it adheres. In monochromatic light, grey levels in the resultant fringe image are indicative of the distance of separation between the cell and substratum [43-46]. In general, white indicates a separation distance over 100 nm, light to dark greys are generated by a separation of 70-20 nm and black is generated by a gap of less than 15 nm. In terms of adhesion, whites are considered non-adhesive areas of the cell underside, greys are referred to as close contacts and regarded as weakly adhesive, and blacks denote strongly adhesive focal contacts (focal adhesions). Using actin labeling with phalloidin and vinculin immunofluorescence localization, we found that ROCK inhibition with Y-27632 caused all MEFs to lose stress fibers and focal contacts (Fig. 5, actin and vinculin columns) and assume the bizarre shapes that have been noted previously [47–49]. This is particularly striking in WT and KO•CRT cells (Fig. 5). Cell-substratum adhesions were visualized in the same cells using IRM and it appears that the black images of focal contacts are missing from the photomicrographs of both untreated KO cells and all Y-27632-treated MEF cell lines (Fig. 5, IRM column).

An informative IRM image is formed by the interference of incident light wavefronts reflected from the ventral plasma membrane/medium and medium/glass interfaces. The IRM images shown in Fig. 5 are not of the best quality for two reasons. First, antibody labeling requires cell permeabilization with detergents, which destroy the reflectivity of the plasma membrane. Second, mounting media used to prevent fluorescence quenching tend to have an increased refractive index, which attenuates the reflectivity of the interfaces. Such an increase in the refractive index of a substance intervening between the surfaces of a cell and the glass may in fact reverse the contrast of IRM images of focal contacts [50], as in the case of fibrillar (ECM) contacts [13]. To avoid problems with IRM imaging of formaldehyde-fixed and permeabilized cells used for immunolocalization, glutaraldehyde-fixed, non-permeabilized, PBS-mounted cells can be used [51]. Fig. 6 shows the IRM images of such cells and it is immediately apparent that the untreated KO cells are nearly devoid of focal contacts and that Y-27632 exposure causes total elimination of the focal contacts from all of the MEF lines. It also becomes apparent that unlike the undulating,

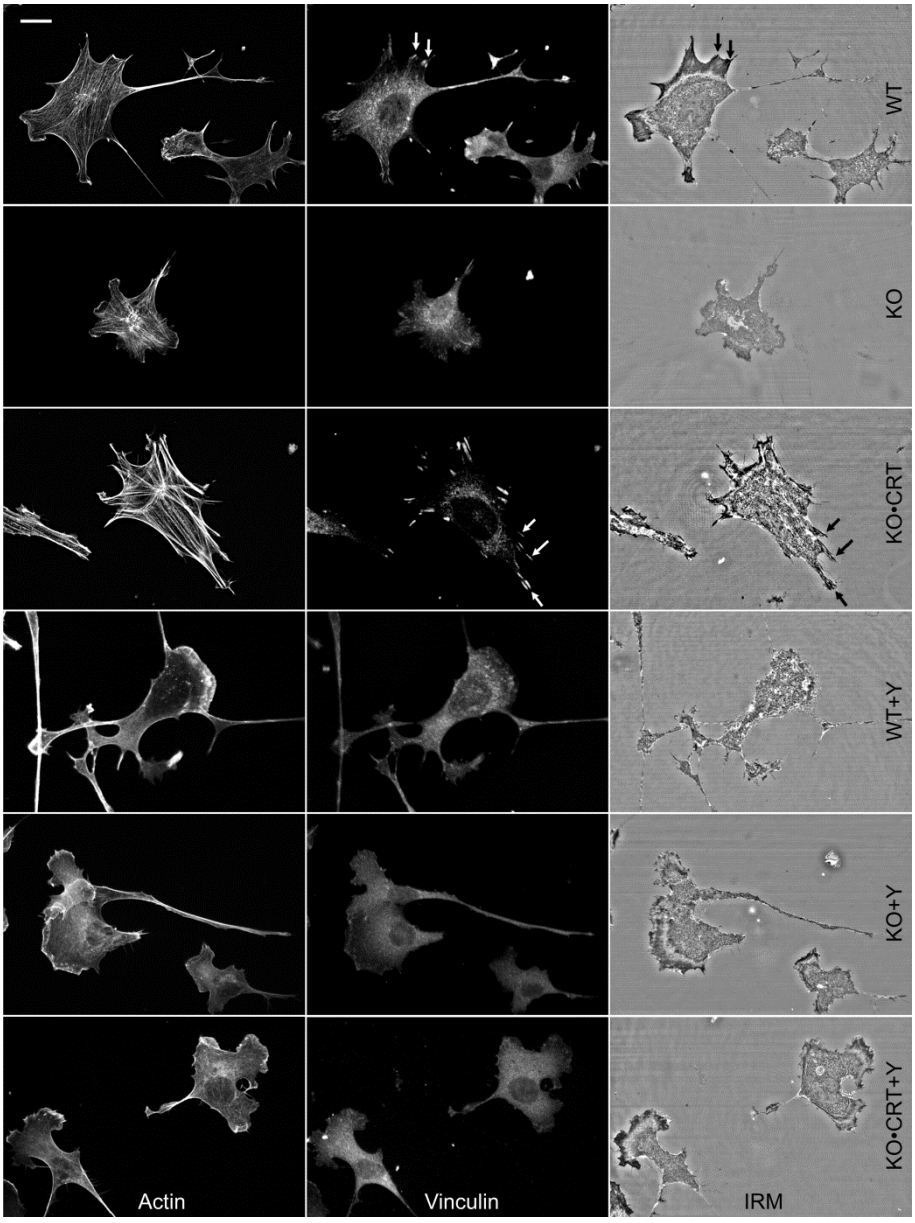

Fig. 5. Triple imaging for actin and vinculin fluorescence vis-à-vis IRM of MEFs following Y-27632 treatment. All MEF lines after Y-27632 exposure (+ Y rows) undergo profound shape changes concomitant with the loss of stress fibers and vinculin-containing focal contacts. Arrows point to examples of focal contacts in WT and KO•CRT cells. IRM column shows that while untreated cells and calreticulin-containing cells (WT and KO•CRT rows) have diversified underside, as shown by full gamut of greys in the IRM images, the cells treated with Y-27632 appear more uniformly grey. Scale bar – 20 μm.

diversified underside of the untreated cells (Fig. 6A-C), the underside of the Y-27632-treated cells is uniformly dark grey, with the exception of the white cell edges and spurious reflections from the nuclei (Fig. 6 A'-C'). This uniformly spread dark greyness of the undersides of all Y-27632-treated cells indicates that they all adhere to the substratum with fairly flat and extensive close contacts.

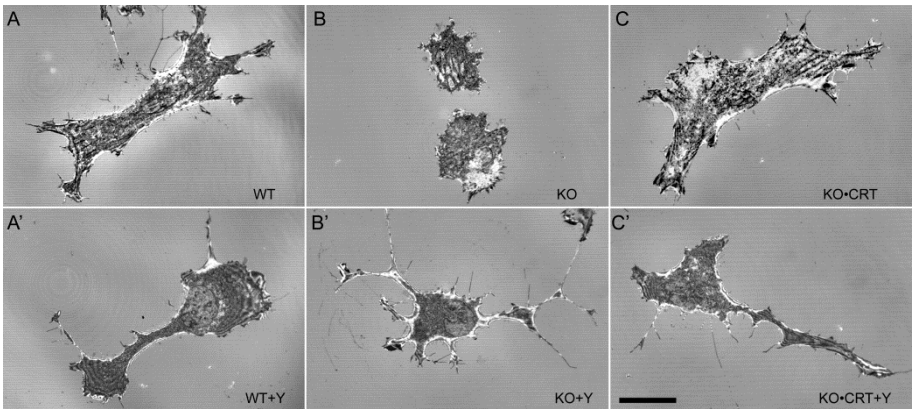

Fig. 6. Y-27632 treatment causes the disappearance of focal contacts and flattening of the underside of cells. IRM imaging of MEFs fixed with glutaraldehyde and mounted in PBS shows that their ventral surfaces are fairly undulating (A, B, C). By contrast, the undersides of Y-27632-treated cells (A', B', C') produce large and uniform grey reflections except for the cell edges that are whiter. This indicates fairly flat apposition between the cell plasma membrane and the substratum. Scale bar – 20 μm.

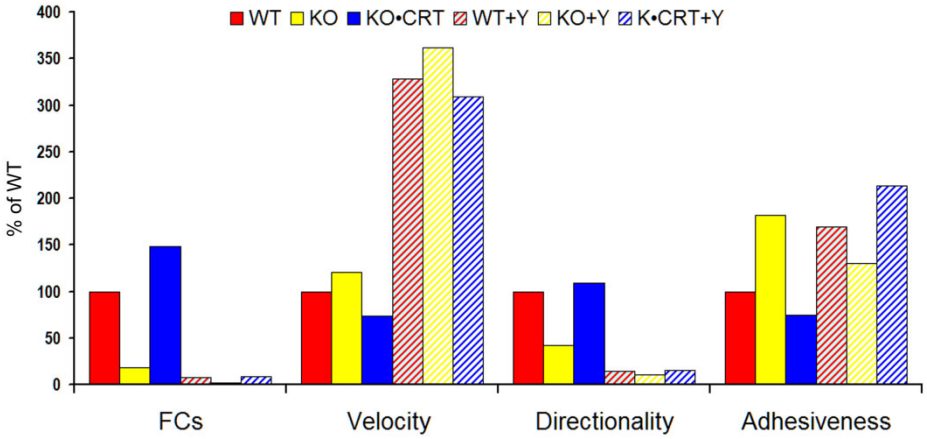

Fig. 7. Inhibition of phosphorylation of serine 636 of IRS-1 affects motile phenomena. In Y-27632-exposed cells (+ Y, striped bars) the number of focal contacts decreases and the speed of locomotion increases. The cells move faster but their migration lacks directionality. Unexpectedly, the adhesion strength, as measured by the energy required to dislodge the cell from the substratum, increases after Y-27632 treatment. The numerical value of each parameter for WT cells was taken as 100%.

We are the first research group to directly image the adhesive properties of Y-27632-treated cells with IRM. The images show all types of cell-substratum adhesions, not only the antibody-selected ones. Thus, unlike previous reports, these images provide a more global view of cell-substratum adhesions [52].

Functional consequences of IRS-1 inhibition

In line with the impression given by fluorescence imaging, focal contact counts show a massive reduction in their number in all MEF lines treated with Y-27632 (Fig. 7). Thus, the profoundly destructive effect of Y-27632 on focal contacts is paralleled by a decrease in the total IRS-1 level. Interestingly, KO cells that have a reduced number of focal contacts (Figs 2 and 7) also show a small but discernible decrease in the total IRS-1 level (Figs 3 and 4). Conversely, KO•CRT cells overexpressing calreticulin controlled by an unregulated CMV promoter [26] show an overabundance and prominence of focal contacts [42] and have a similar increase in the total IRS-1 level. Inhibition of FAK-mediated adhesion was shown to correlate with downregulation of total IRS-1 [22]. Assuming that FAK-mediated adhesion is largely realized via focal contacts, our data are concur with those of Lebrun et al. [22], who directly showed that a relationship exists between focal contact-mediated adhesion and the total IRS-1 level.

The reciprocal relationship of IRS-1 regulation and focal adhesion formation has been previously demonstrated [21, 22, 24]. IRS-1 can be phosphorylated on serine and tyrosine residues [34]. Serine phosphorylation of IRS-1 is calreticulin dependent, while that on tyrosine is not. It has been suggested that IRS-1 signaling is turned on in its tyrosine-phosphorylated state, and off in the serine state [53]. Furthermore, serine and tyrosine phosphorylation of IRS-1 appear to be mutually inhibitory [24, 53]. Literature data on cell adhesiveness in relation to the phosphorylation state of IRS-1 are scarce and contradictory [21, 23, 24, 54]. Unfortunately, our data do not allow the formulation of a clear-cut notion of that unless one assumes that there is an optimal level of phosphorylation of the serine residues of IRS-1 necessary to maintain functional focal contacts. A significant increase in IRS-1 phosphorylated at serine 636 might then functionally impair focal contact formation (as in untreated calreticulin KO cells), but a similar effect is seen when this isotype is present at low levels (as in Y-27632-treated cells). Previous studies showed that when tyrosine phosphorylation of IRS-1 was stimulated, focal contacts were decreased. This was suggested to be regulated via focal adhesion kinase [54]. These results are in concert with the notion that phosphorylation of the IRS-1 residues is mutually inhibitory.

In parallel to the loss of focal contacts, the speed of locomotion of all cells treated with Y-27632 increases dramatically, while the directionality of their movement is lost (Fig. 7). Fig. 8 illustrates the meaning of directionality of cell movement and how it is measured [55]. The relationship between the number of

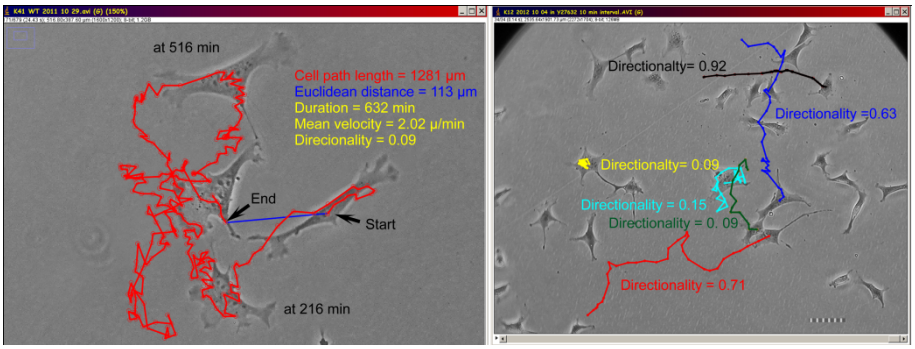

Fig. 8. Directionality (persistence) of cell locomotion can be defined as a quotient of cell displacement (the Euclidean distance) divided by the total length of the cell path. This is illustrated in panel A, which shows four screen grabs of the same cell at different time points during translocation. The Euclidean distance is shown as a straight blue line, while the cell path is shown in red. The directionality of movement of this cell is 0.09. Panel B shows a superimposed collage of cell paths and directionalities covering a range of 0.09 to 0.92. B also shows that the total length of a cell's path does not affect directionality. One of the cells in B has a directionality of 0.09, but has covered a much smaller distance than the cell shown in A.

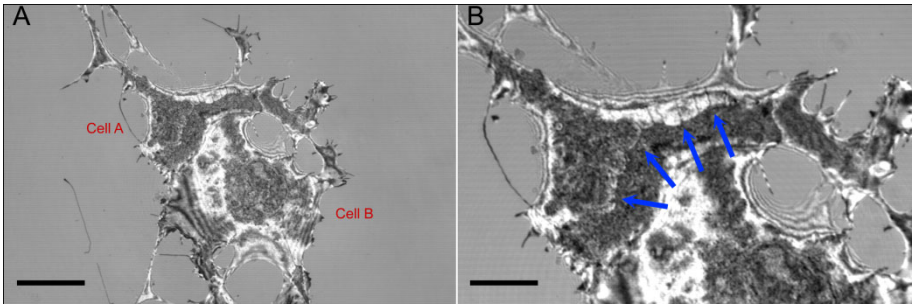

Fig. 9. Inhibition of ser636 phosphorylation of IRS-1 releases cells from contact inhibition of locomotion. IRM images of two Y-27632-treated KO•CRT cells apposing each other (A, Cell A and Cell B). Cell B invades the underside of Cell A with thin lamellae and filopodia (B, blue arrows). This is a clear indication of a lack of contact inhibition of locomotion. Scale bars: A – 20 µm, B – 10 µm.

focal contacts and the cell movement, speed and directionality shown in Fig. 7 concurs with much earlier data showing that cells slow down as the number of focal contacts [56–58] and directionality of their movement increases [59, 60]. Indeed, this is already evident in calreticulin KO cells, which still have some albeit much less numerous stress fibers and focal contacts. It becomes quite dramatic in Y-27632-treated cells that are devoid of stress fibers and focal contacts altogether. Furthermore, it was elegantly established over thirty years ago that both focal contacts and the stress fibers that associate with them [61–64] are instrumental in establishing the directionality of cell movement

while slowing cells down [56, 59, 65]. Our observation that Y-27632 treatment accelerates cell movement with an accompanying loss of directionality is also in line with reports showing that RhoA/ROCK inhibition affects both the speed and directionality of cell movement by causing degradation of focal contacts and stress fibers [66–68].

The number and prominence of focal contacts have all been associated with an increase in the strength of cell-substratum adhesion [56, 57, 65, 69–72]. It thus came as a surprise that our MEFs that are devoid of focal contacts and stress fibers adhere more strongly to the substratum (Fig. 7). Counterintuitive though it may seem, this observation is not, in fact, unique. The formation of focal contacts takes time and it has been observed that cells develop considerable adhesion strength and tractional force before any focal contacts are formed [56, 57, 73]. In fact several cell types never form focal contacts and still adhere and move effectively e.g., *Dictyostelium* amoebae [74, 75], keratinocytes [76, 77] and leukocytes [58, 78]. How can relatively strong adhesion be accomplished by such close contacts, which are nondescript structures without any specific ECM receptors attributable to them? It has been proposed that cell adhesion can be accomplished by non-specific interactions of the charged surface of a cell with that of a substratum, according to the DLVO theory and via long distance van der Waals attraction forces operating at a 10–30 nm distance of separation between the surfaces [79, 80]. While focal contact adhesion strength per unit area is greater than any other part of the cell undersurface [69, 70, 81, 82], it is apparent from imaging of the ventral surface of cells that, in normal fibroblasts, its topography is very diversified, with the area of focal contacts covering only a small portion of a cell's undersurface. This contrasts with the Y-27632-treated cells shown in Figs. 5 and 6, in which dark close contact areas cover nearly the entire undersurface. Close contacts associate with the cortical meshwork of actin microfilaments [57, 83, 83, 84], so it is plausible that locally controlled contractility modulates cell surface geometry on a nanoscale, thus further contributing to cell adhesiveness [69, 70, 72, 79, 85]. It has also been observed that coated pits filled with ruthenium red-positive material seem to concentrate in areas of close contacts [86, 87] possibly providing a wealth of charged molecules dangling from the cell surface. While putative adhesion bonds in close contacts are more labile and weaker than those in focal contacts [69, 70, 81, 88, 89], their large numbers in extensive close contacts would account for increased adhesion strength. On the other hand, the lability of bonds in close contacts would permit fast locomotion as shown in Fig. 7 and in an earlier study [80].

Finally, IRM images of Y-27632-treated MEFs clearly show cells in apposition invade each other's underside with thin lamellae and filopodia (Fig. 9). When fibroblasts with normal motility collide, they undergo temporary paralysis of movement and then divert their paths to avoid contact in a phenomenon known as contact inhibition of locomotion, which was first described by Michael Abercrombie and Joan Heaysman in the early 1950s [90, 91]. The cell overlap

shown by IRM here indicates that ROCK inhibition causes a release from contact inhibition of locomotion, as reported before [92–94], and reviewed by Mayor and Carmona-Fontaine [95]. A consequence of contact inhibition of locomotion is that sparse cells will translocate faster than more numerous cells due to a lesser number of collisions [90]. It should be noted that the loss of contact inhibition of locomotion is not merely due to a decrease in the number of focal contacts, as the KO cells, which lack prominent focal contacts, do show contact inhibition of locomotion, unlike the ROCK-inhibited MEFs.

Acknowledgements. Sylvia Papp received a Canada Graduate Scholarship from the Canadian Institutes of Health Research (CIHR). Michal Opas is a member of the Heart & Stroke/Richard Lewar Centre of Excellence and the Ontario Stem Cell Initiative. This study was supported by grants from CIHR (MOP-106461 and MOP-102549) awarded to Michal Opas.

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