Galectin-3 secretion and tyrosine phosphorylation is dependent on the calpain small subunit, Calpain 4

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ABSTRACT

Cell adhesion and migration are important events that occur during embryonic development, immune surveillance, wound healing and in tumor metastasis. It is a multi-step process that involves both mechanical and biochemical signaling that results in cell protrusion, adhesion, contraction and retraction. Each of these events generates mechanical forces into the environment measured as traction forces. We have previously found that the calpain small subunit, Calpain 4, is required for normal traction forces, and that this mechanism is independent of the catalytic activities of the holoenzymes that are formed between Calpain 4 and each of the proteolytic heavy chains of Calpain 1 and 2. To define a potential mechanism for the Calpain 4 regulation of traction force, we have evaluated the levels of tyrosine phosphorylation, a hallmark of force dependent signaling within focal adhesions. Using 2D gel electrophoresis we compared tyrosine phosphorylation profiles of Calpain 4 deficient mouse embryonic fibroblasts (MEFs) to the levels in wildtype MEFs and MEF's deficient in the large catalytic subunits, Capn1 and Capn2. Of particular interest, was the identification of Galectin-3, a galactose binding protein known to interact with integrins. Galectin-3 has previously been shown to regulate cell adhesion and migration in both normal and tumor cells; however its full mechanism remains elusive. We have found that Calpain 4 is essential for the tyrosine phosphorylation of galectin-3, and its ultimate secretion from the cell, and speculate that its secretion interferes with the production of traction forces.

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1. Introduction

Galectins are a family of lectin proteins that bind to β-galactoside carbohydrate structures through their carbohydrate recognition domains (CRDs). Galectin-3 is a unique member of this family because it is chimeric, containing one CRD and a long N-terminal sequence rich in serine phosphorylation sites and glycine repeats, in addition to a tyrosine and proline rich collagen-like sequence (reviewed in [1]). Galectin-3 is ubiquitously expressed in most normal adult tissues and also found in a variety of tumor cell types. In tumor cells, galectin-3 expression varies with cell type and stage of cancer progression (reviewed in [2–4]). Within the cell, galectin-3 is localized to the nucleus, cytoplasm and cell surface and is also known to be secreted (reviewed in [5]). Cytoplasmic and nuclear localized galectin-3 is involved in apoptosis, cell proliferation, splicing, and Wnt/β-catenin signaling pathway, while cell surface and extracellular galectin-3 modulate cell adhesion (reviewed in [1,3,5]). Extracellular galectin-3 binds to extracellular matrix (ECM) proteins such as fibronectin, laminin, collagen IV, elastin, hensin, tenascin-C and -R (reviewed in [4]). Interestingly, galectin-3 also binds to a variety of integrin receptors located on the cell surface (reviewed in [4]).

Despite knowing that galectin-3 has multiple interactions in the extracellular environment, its function in this niche is not clear, and the exact mechanism by which galectin-3 is secreted is not understood. It is known that galectin-3 is secreted by a non-classical secretion pathway which bypasses the ER/Golgi, it also lacks a conventional secretion signal sequence ([6,7]). However, there are indications that the N-terminal 11-amino acids introduce structural changes in the protein that may mediate secretion [8–10]. There is some evidence of vesicle mediated secretion of galectin-3 and it has been found associated with exosomes [10,11]. Nonetheless, how extracellular galectin-3 effects cell adhesion is ambiguous. Previous studies have described either an enhancement or abrogation of cellular adhesion and spreading, dependent on cell type and galectin-3 concentration (reviewed in [4]). For example, extracellular galectin-3 may form a lattice along with Mgat5 to induce clustering and activation of β1 integrin. On the other hand, it has also been shown that extracellular galectin-3 is needed for internalization of the β1 integrin receptor [12–14]. A better understanding of how galectin-3 is secreted and what purpose it serves in the extracellular environment is needed. In this paper we have found a previously unknown link between secretion of galectin-3 and the calpain small subunit, calpain 4.

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Calpains are a family of intracellular calcium-dependent proteases involved in a plethora of physiological processes including cell migration, apoptosis, and cell proliferation to name a few (reviewed in [15]). The calpain system includes the Calpain 1 and 2 holoenzymes, and their endogenous inhibitor calpastatin. Calpain 1 and 2 holoenzymes are comprised of the large catalytic subunits calpain 1 and 2 and a common small regulatory subunit, calpain 4, often referred to as Css-1 (reviewed in [16]). This specific system impacts cell adhesion and migration, likely by controlling the turnover of focal adhesions (reviewed in [16–19]). We have previously found that the small subunit not only acts as a regulatory protein, but also functions independent of the catalytic activity of the large subunits to produce mechanical forces on the ECM, known as traction forces [20]. Calpain 4 deficient mouse embryonic fibroblasts (MEF) produce less traction force in comparison to wildtype MEF, MEF deficient in the large catalytic subunits or MEF overexpressing the endogenous inhibitor calpastatin.

Exactly how traction forces are produced is currently an area of intense research in which mechanisms involving tyrosine phosphorylation have become a focus. Early studies established that enhanced tyrosine phosphorylation occurs at focal adhesions upon the application of mechanical stress [21,22]. Subsequent studies have substantiated these original observations (as reviewed in [23]). To further elucidate the force generation pathway, we have compared the tyrosine phosphorylation patterns of intracellular proteins from calpain 4 deficient MEFs to wildtype MEFs. We have identified galectin-3 as differentially tyrosine phosphorylated in these cells. Most significantly, we have made the unique observation that calpain 4 alone, and not the catalytic subunits of the Calpain 1 and 2 holoenzymes, as essential for galectin-3 secretion.

2. Materials and methods

2.1. Cell culture and plasmids

Mouse embryonic fibroblasts (MEF) cells, $Capn4^{-/-}$ MEFs, $Capn1$ knockdown MEFs, $Capn2$ knockdown MEFs were used in this study [24–26]. All cell lines were cultured and maintained in Dulbecco's Modified Eagle's Medium – high glucose (Sigma), supplemented with 10% fetal bovine serum (Hyclone) and 1% penicillin/streptomycin/glutamine (Gibco) and incubated at 37 °C under 5% humidified CO_2. 0.1% Trypsin–EDTA was used for cell passages, never exceeding eight passages for a given cell line. Calpain 4 deletion was rescued by nucleofection of $Capn4^{-/-}$ MEFs with the plasmid pSBC-r28 kDa encoding the full-length rat calpain small subunit as described by Dourdin et al. [25]. Calpastatin was over-expression in wildtype MEF cells from the plasmid hrEGFP-calpastatin [27].

2.2. Protein extraction and collection of conditioned media

Proteins were extracted from each cell line with triple detergent lysis buffer (100 mM Tris–Cl, 300 mM NaCl, 0.5% sodium deoxycholate, 0.2% SDS, 2% NP 40) containing Protease Inhibitor Cocktail (Sigma) and also Halt[TM] Phosphatase Inhibitor Cocktail (Thermo Scientific). Protein from cells grown to 80% confluency on three 100 mm cell culture dishes were extracted, concentrated and further prepared for two-dimensional polyacrylamide gel electrophoresis as described below.

To test for the secretion of galectin-3, conditioned media was collected from two 80% confluent 60 mm culture dishes containing 2.5 ml of culture media. Equal volume of conditioned media from each cell type was loaded onto standard 4–20% Tris–HEPES–SDS polyacrylamide gels and used for western blot or for Coomassie Brilliant Blue staining to ensure equal loading.

2.3. Two-dimensional polyacrylamide gel electrophoresis (2-D PAGE)

The protocol used for 2-D PAGE has been described in detail with minor modification to the sample preparation [28]. Briefly, the protein extracts from each cell line was concentrated using Amicon Ultra-4 5 K filter units of 5000 Da Nominal Molecular Weight Limit (NMWL). The concentrated proteins were then solubilized in sample buffer (8 M Urea, 50 mM DTT, 4% CHAPS, 0.2% Carrier ampholytes, 0.0002% Bromophenol Blue). 200 micrograms of protein for each sample was rehydrated into isoelectric focusing strips with a pH range of 3–10 (Bio-Rad). Isoelectric focusing was then performed at 35,000 V h in a PROTEAN IEF Cell (Bio-Rad). Following this, second-dimension SDS PAGE was performed using 4–12% Bis–Tris precast polyacrylamide gels (Bio-Rad).

2.4. Western blot

Protein samples resolved either by the 2-D PAGE method or by standard SDS–PAGE (4–20% gradient Tris–HEPES–SDS precast polyacrylamide gel system, Pierce) were transferred (semi-dry) onto PVDF membranes (Bio-Rad). Buffers used for the transfer have been previously described [29]. The blots were probed with one of the following antibodies; anti-phosphotyrosine antibody clone PY20 (Millipore), monoclonal rat anti-Galectin-3 antibody and polyclonal rabbit anti-Galectin-3 antibody (gifts from Dr. A. Raz, Karmanos Cancer Institute, MI). Commercially available HRP conjugated secondary antibodies were detected with ECL Plus Western Blotting Detection Reagents (Amersham).

2.5. Identification of differentially tyrosine phosphorylated proteins

Protein samples from MEF cells and $Capn4^{-/-}$ MEF cells were resolved by 2-D PAGE. The proteins were partially electro transferred onto PVDF membranes. As mentioned above, the blots were probed using anti-phosphotyrosine antibody clone PY20 (Millipore). The gel containing residual proteins were stained using SYPRO-Ruby Protein Gel Stain (Bio-Rad). Images from the immunoblot and stained gels were superimposed to select protein spots that were differentially phosphorylated in the two cell types (described previously by Kang et al. [28]). These spots were then excised and identified by mass spectrometric analysis by the Protein Core Facility, Columbia University.

2.6. Immunofluorescence and microscopy

Cultured cells were fixed for immunofluorescence using 4% paraformaldehyde and 0.1% Triton X-100 for 10 min, blocked for one hour with 5% BSA in PBS, followed by anti-Galectin-3 antibodies and the species appropriate secondary Alexa Fluor 546 antibody. All images were acquired using an Olympus IX81 ZDC inverted microscope. Images were captured using a Diagnostic Instruments Boost EM-CCD-BT2000 back-thinned camera driven by IPLab software.

3. Results

3.1. Identification of Galectin-3 as a non-tyrosine phosphorylated protein in Calpain 4 deficient cells

Protein lysates were prepared from four different cell lines; wildtype MEF cells, MEF cells deficient in either the large Calpain 1 or 2 subunits, and MEF's deficient in the small subunit Calpain 4. These lysates were resolved by two-dimensional gel electrophoresis as described in the materials and methods section. Western blotting with an anti-phosphotyrosine antibody revealed numer-
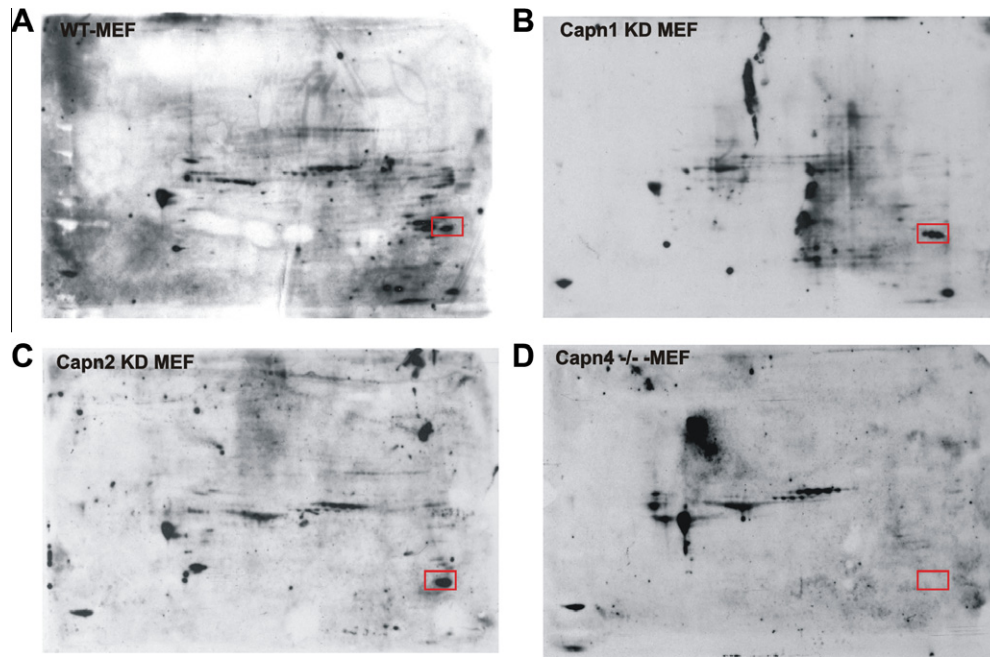
Fig. 1. Galectin-3 is not tyrosine phosphorylated in the absence of the calpain small subunit, calpain 4. Total cellular protein from wildtype MEF cells (A), *Capn1* silenced MEF cells (B), *Capn2* silenced MEF cells (C) and *Capn4*$^{-/-}$ MEF cells (D) were resolved by two-dimensional gel electrophoresis, transferred onto a PVDF membrane and probed for tyrosine phosphorylated proteins. Numerous protein spots are differentially phosphorylated in the four different cellular backgrounds. However, one prominent protein spot (red rectangular box) was phosphorylated in all cell types except in *Capn4* silenced MEF cells. The protein spot was identified as the 30 kDa protein galectin-3 by mass spectrometric analysis. (For interpretation of the references in color in this figure legend, the reader is referred to the web version of this article.)

ous protein spots that differed in their levels of tyrosine phosphorylation between the four cell lines (Fig. 1). A total of six protein spots were selected based on their differential presence on the four gels and analyzed by mass spectrometry. An isolated protein spot within the 30 kDa range which was present in wildtype cells and Capn1 and 2 deficient cells, but absent in *Capn4*$^{-/-}$ lysates was identified as galectin-3. Using an anti-Galectin-3 antibody, we confirmed that the protein was expressed in *Capn4*$^{-/-}$ cells and at levels equivalent to its expression in wildtype MEF cells (Fig. 2). Expression levels of galectin-3 were also unchanged when the two large catalytic subunits, Capn1 and Capn2, were silenced (data not shown). These results strongly indicate that calpain 4 is essential for the tyrosine phosphorylation of galectin-3.

3.2. The non-tyrosine phosphorylated form of Galectin-3 fails to localize to the cell periphery

Post-translational modifications of proteins are known to affect numerous facets of a protein including its function, interacting partners, localization and stability. We tested if the intracellular localization of the non-tryosine phosphorylated form of galectin-3 differed in the Calpain 4 deficient cells. In our immunofluorescence studies we used four different antibodies each recognizing a distinct region of the protein. We observed that the non-tyrosine phosphorylated form of Galectin-3 does not localize to the periphery of the *Capn4*$^{-/-}$ cell (Fig. 3). However, when tyrosine phosphorylated, as is the case in wildtype MEF cells and cells deficient in the catalytic heavy chains, galectin-3 is ubiquitously expressed in the cell and is not excluded from the cell periphery. This prompted us to ask if secretion of galectin-3 was disrupted in the Calpain 4 deficient cells.

3.3. Calpain 4 is essential for secretion of Galectin-3

Galectin-3 can be found on the surface of the cell and is also known to be secreted, although the mechanism is currently unknown. Galectin-3's extracellular activities are known to impact cell adhesion and migration in both normal and tumor cells. Since
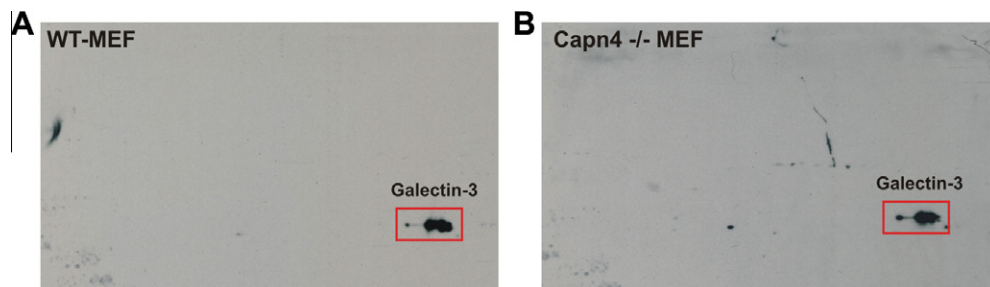

Fig. 2. Galectin-3 expression is normal in the absence of calpain 4. Total cellular protein from wildtype MEF cells (A) and *Capn4*$^{-/-}$ MEF cells (B) were resolved by two-dimensional gel electrophoresis, transferred onto a PVDF membrane and probed using an anti-Galectin-3 antibody. A distinct Galectin-3 spot (red rectangular box) was obtained in both cell types, indicating that Galectin-3 expression is normal in the absence of Capn4. (For interpretation of the references in color in this figure legend, the reader is referred to the web version of this article.)
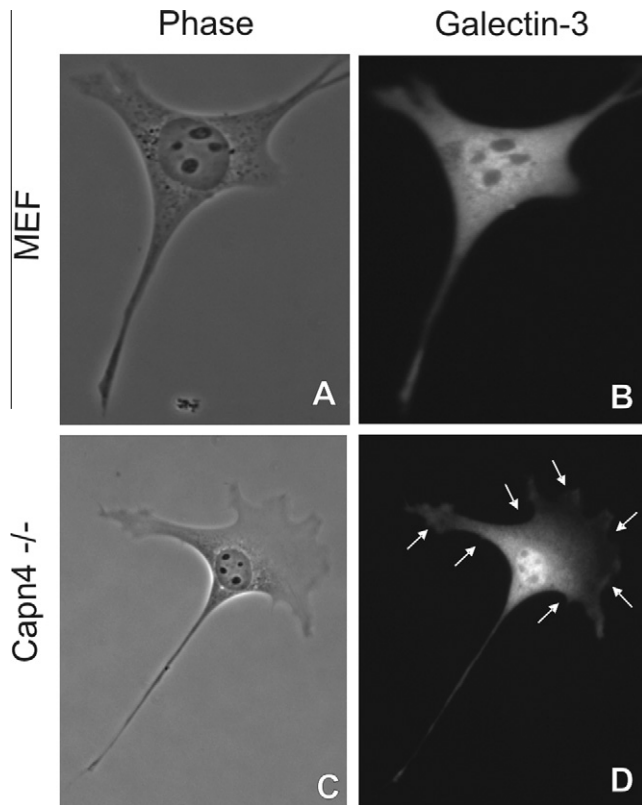
Fig. 3. Non-tyrosine phosphorylation Galectin-3 affects its localization in $Capn4^{-/-}$ cells. Wildtype MEF cells and $Capn4^{-/-}$ MEF cells were fixed and immunofluorescence was performed using a monoclonal anti-Galectin-3. Galectin-3 is present uniformly throughout the wildtype cell. However, in a $Capn4^{-/-}$ MEF cell, Galectin-3 was localized within the nucleus and in the peri-nuclear region, but absent from the cell periphery as indicated by white arrows around the cell periphery.

we were seeing an absence in the localization of galectin-3 to the cell periphery in Calpain 4 deficient cells, we tested for its secretion into the media of these cells. Conditioned media was collected on day 2 from cultures of all four cell lines; wildtype MEF, Capn1 and 2 deficient cultures and Calpain 4 deficient cell cultures. Consistent with our immunofluorescence results, we found that galectin-3 was not being secreted from the $Capn4^{-/-}$ cells, cells in which galectin-3 was not tyrosine phosphorylated (Fig. 4A). In addition to the four cell lines, we also tested the conditioned media of cultured cells in which an endogenous inhibitor (calpastatin) of the calpain holoenzymes was overexpressed, thereby ensuring that the catalytic activity of both the large subunits would be abrogated. As expected, conditioned media from these cell cultures also showed an abundance of secreted Galectin-3 (Fig. 4B). To further support our observation, we rescued $Capn4^{-/-}$ cells using a recombinant rat $Capn4$ sequence. Conditioned media from these cells showed that galectin-3 was now being secreted by these cells indicating that the calpain small subunit (calpain 4), but not the catalytic activities of calpain 1 and 2, is essential for galectin-3 secretion (Fig. 4B).

4. Discussion

Cell migration is a complex process and both biochemical and mechanical components of the environment impact how a cell migrates. Environmental information is transmitted into the cell through transmembrane receptors, such as GPCRs, hormone receptors, and integrins to name a few. Activation of a complex system of overlapping signaling cascades ultimately lead to altered cytoskeletal and focal adhesion dynamics necessary for cell spreading or migration.

As a cell migrates it generates its strongest traction forces at the leading edge of a migrating cell resulting in the maturation of focal complexes into focal adhesions [30]. Much of how traction forces are generated in a migrating cell remains to be elucidated though previous studies have identified proteolytic and phosphorylation activities as significant events. As part of identifying the role of calcium-dependent proteases in mechanical signaling, our group has previously established that the calpain small subunit (calpain 4) regulates traction forces and strengthening of adhesions, independent of the catalytic activity of the large subunits [20]. Furthermore, various studies have identified Src family kinases, focal adhesion kinase, the SH2 domain-containing phosphates and receptor-like protein tyrosine phophatases as important components of the force-dependent signal transduction pathways [22,23]. Based on this evidence we compared the tyrosine phosphorylation levels of proteins extracted from wildtype MEF cells and MEF cells in which the expression of each of the three subunits of the Calpain 1 and 2 holoenzymes were silenced independently. From this screen galectin-3 was found to be differentially phosphorylated.

Until recently, phosphorylation of galectin-3 was believed to occur only on Serine residues 6 and 12 at the amino terminus of galectin-3. A single study suggested that galectin-3 was phosphorylated on tyrosine residues at the N-terminal PGAY or PXXY motifs [10]. It was recently confirmed that galectin-3 is also phosphorylated on tyrosine residues 79, 107 and 118 and suggested that c-Abl kinase is the responsible kinase [31,32]. However, the functional significance of tyrosine phosphorylation of galectin-3 residues has not yet been established until now.

Many studies have confirmed the importance of this protein in the extracellular environment of cells and its impact on cell adhesion and migration under both normal and disease conditions. It has been shown in vivo that circulating galectin-3 promotes tumor progression [33]. However the literature is conflicting on how extracellular galectin-3 is influencing migration. For example, one study finds that in vitro galectin-3, along with caveolin-1, bind to N-glycans that have been modified by Mgat5 and recruit conformationally active $\alpha5\beta1$ integrin to adhesions, resulting in the activation of FAK and PI3K, hence enhancing the formation of adhesions [13,14]. Conversely, a second study found that galectin-3 is involved in internalization of $\beta1$ integrin, thereby working against the formation of adhesions [12]. Despite these different roles attributed to extracellular galectin-3 in cell adhesion and migration, a potential mechanism for the secretion of galectin-3 has not been identified.

Galectin-3 is not secreted by the classical secretion pathway and adopts a non-classical mechanism [6,7]. It has been reported, that the first 11 amino acids of galectin-3 act to regulate of the localization of galectin-3, as truncation of this region eliminates secretion and nuclear localization [8]. However little else is known about how this protein is secreted. In this study we have found a previously unknown link between calpain-4 and the secretion of galectin-3. More specifically, we have made the novel observation that the tyrosine phosphorylation status of galectin-3, indirectly modulated by calpain-4, influences its secretion. We speculate that phosphorylation of tyrosine residues 79, 107 and 118 [32] alters the quaternary structure of galectin-3, making the N-terminal 11 amino acids unavailable for mediating galectin-3 secretion. Nonetheless, the lack of galectin-3 secretion serves to explain the defects observed in traction forces and migration found in calpain 4 deficient cells and further experimentation is currently underway to establish this fact.
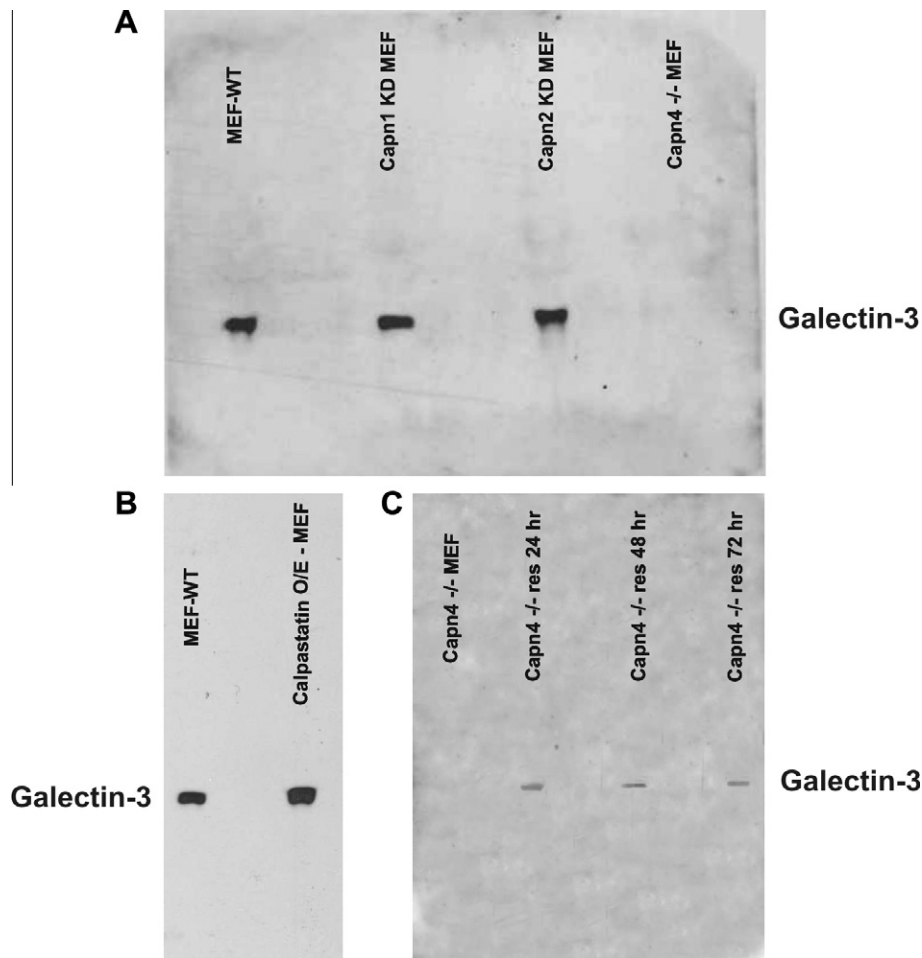
Fig. 4. Calpain 4 is essential for Galectin-3 secretion. (A) Western blot of Galectin-3 from media of wildtype MEF (MEF-WT), *Capn1* silenced MEF (*Capn1* KD MEF), *Capn2* silenced MEF (*Capn2* KD MEF) and *Capn4$^{-/-}$* MEF cell (*Capn4$^{-/-}$* MEF). (B) Calpastatin overexpressing MEF cells secrete Galectin-3 at levels similar to mock-nucleofected MEF cells (lanes labeled as MEF-WT and Calpastatin O/E–MEF). (C) *Capn4$^{-/-}$* MEF cells rescued by exogenous expression of Calpain 4 secrete Galectin-3 as compared to *Capn4$^{-/-}$* cells indicated as lane *Capn4$^{-/-}$* MEF. Lanes indicated as *Capn4$^{-/-}$* res 24 h, *Capn4$^{-/-}$* res 48 h, *Capn4$^{-/-}$* res 72 h shows results from conditioned media collected from Calpain 4 rescued cells 24, 48 and 72 h post nucleofection. The amount of secreted Galectin-3 decreases as the proportion of Calpain 4 rescued cells is overgrown by *Capn4$^{-/-}$* cells.

Acknowledgments

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