FEBS Letters 582 (2008) 2887–2892

Double-membrane gap junction internalization requires the clathrin-mediated endocytic machinery

Anna M. Gumpert, Joseph S. Varco, Susan M. Baker, Michelle Piehl[1], Matthias M. Falk*

Department of Biological Sciences, Lehigh University, 111 Research Drive, Iacocca Hall, Bethlehem, PA 18015, USA

Received 17 June 2008; revised 26 June 2008; accepted 14 July 2008

Available online 24 July 2008

Edited by Lukas Huber

Abstract Direct cell–cell communication mediated by plasma membrane-spanning gap junction (GJ) channels is vital to all aspects of cellular life. Obviously, GJ intercellular communication (GJIC) requires precise regulation, and it is known that controlled biosynthesis and degradation, and channel opening and closing (gating) are exploited. We discovered that cells internalize GJs in response to various stimuli. Here, we report that GJ internalization is a clathrin-mediated endocytic process that utilizes the vesicle-coat protein clathrin, the adaptor proteins adaptor protein complex 2 and disabled 2, and the GTPase dynamin. To our knowledge, we are first to report that the endocytic clathrin machinery can internalize double-membrane vesicles into cells.

Keywords: Annular gap junction; Clathrin; Connexin; Endocytosis; Gap junction; RNAi

1. Introduction

The role of clathin in vesicle endocytosis is well documented. Clathrin forms a typical curved lattice around endocytic vesicles that are internalized at the plasma membrane (PM) [1]. However, clathrin has also been implicated in the internalization of viruses, pathogenic bacteria, and even latex beads [2–4]. We have discovered an additional clathrin-mediated endocytosis (CME) process that results in the internalization of double-membrane vesicles from the PMs of cells that are coupled by gap junctions (GJs) [5].

GJs consist of transmembrane channels that bridge the PMs to provide direct intercellular communication. GJ channels cluster into so-called ''plaques'' containing a few to tens of thousands of channels. The channels facilitate diffusion of ions and small molecules, thus coupling the connected cells electrically and metabolically. GJ function has been determined vital for embryonic development, regulating differentiation and growth, and in maintaining tissue homeostasis. The essential role of GJ intercellular communication (GJIC) is manifested by numerous known mutations in GJ proteins, which have been linked to deafness, neuropathies, cataracts, skin disorders, and defects in cranio-facial development.

Two half-channels, or connexons, each synthesized by one of the coupled cells, dock head-to-head in the extracellular space to form the complete GJ channel. Six transmembrane proteins, termed connexins (Cxs), oligomerize around a central hydrophilic pore to shape the connexon. Connexin proteins represent a large gene-family with connexin 43 (Cx43) being most ubiquitously expressed. It is evident, that GJIC needs to be regulated precisely for proper cellular function. GJ channel activity is regulated by both, channel opening and closing (gating) in response to physiological parameters, such as intracellular pH, Ca^{2+} concentration, and Cx phosphorylation [6–8], and by controlled channel biosynthesis and internalization [9,10]. Regulated GJ channel internalization, for example, is vital to cell migration and wound healing, as well as for cell division, when cells uncouple at beginning of mitosis, and recouple at cytokinesis [11]; and misregulation of GJ channel internalization potentially leads to severe pathological conditions, such as cancer metastasis, pulmonary edema, ischemia, and hemorrhagic fevers.

We discovered that cells internalize their GJs in response to various endogenous and exogenous stimuli, including exposure to inflammatory mediators and non-genomic carcinogens [5] (Gilleron et al., manuscript submitted; Baker et al., manuscript in preparation). GJ internalization results in the formation of cytoplasmic double-membrane GJ vesicles [earlier termed annular gap junctions (AGJs)] that are degraded by lysosomal pathways. Interestingly, the GJ vesicle lumen and the inner vesicle membrane are derived from cytoplasm and PM of the neighboring cell, while the outer vesicle membrane is derived from the PM of the internalizing cell [5,12]. We further found that clathrin, and clathrin-associated proteins colocalize with internalizing GJs and GJ vesicles, suggesting a role for these proteins in GJ internalization [5]. Knocking down protein expression levels using RNA interference (RNAi), we now show that cellular depletion or functional inhibition of clathrin, the clathrin-adaptor protein complex 2 (AP-2) and disabled 2 (Dab2), and of the GTPase dynamin significantly inhibits GJ internalization.

2. Materials and methods

2.1. Cell culture and connexin constructs

HeLa cells (CCL 2, American Type Culture Collection, Manassas, VA) were cultured under standard conditions as described [13]. For all experiments cells were grown on round 22 mm diameter glass cover slips coated with poly-L-lysine (Sigma–Aldrich, St. Louis, MO).

*Corresponding author. Fax: +1 610 758 4004.
E-mail address: MFalk@lehigh.edu (M.M. Falk).

[1]Current address: MB Research Labs, 1765 Wentz Road, Spinnerstown, PA 18968, USA.

Abbreviations: AGJ, annular gap junction; AP-2, adaptor protein complex 2; CHC, clathrin heavy chain; CME, clathrin-mediated endocytosis; Cx, connexin; Dab2, disabled 2; GJ, gap junction; KD, knockdown; PM, plasma membrane; RNAi, RNA interference; Trfn, transferrin

0014-5793/$34.00
doi:10.1016/j.febslet.2008.07.024

Cx43-GFP is described [13]. Cx43-mApple was constructed by replacing GFP by mApple cDNA [14].

2.2. RNAi knockdown (KD) procedures

All RNAi oligonucleotides (oligos) were purchased from Dharmacon RNA Technologies (Lafayette, CO). Clathrin heavy chain (CHC) oligos were SMARTpool option CLTC (M-004001-00). AP-2 α-adaptin subunit RNAi oligos were SMARTpool option AP2 A1 (M-012492-00). Dab2 RNAi oligos were sequence 5′–UUCUU-UAAGAGAAAAUCCAUU–3′ published in [15]. Dynamin2 RNAi oligos were SMART pool option DNM2 (L-004007-00).

Previously described KD procedures were followed [5]. Briefly, oligonucleotides were transfected into HeLa cells using Oligofectamine (Invitrogen, Carlsbad, CA), followed 48 h later by Cx43-GFP cDNA [13] using Superfect (Qiagen, Valencia, CA) as recommended by the manufacturers. Oligo transfection efficiency was tested using a fluorescent-labeled control (siGLO RISC-Free fluorescently-labeled, non-targeting oligonucleotide, Dharmacon) and was more than 90% efficient. Cells were assayed 72 h after oligo transfection. Endocytosis inhibition was monitored by incubation in medium containing 10 μg/ml Alexa-Fluor 488-labeled transferrin (Trfn) (5 mg stock solution in 1× PBS; Molecular Probes) for 3 min, fixation, and microscopic examination.

2.3. Immunoblot analyses

Verification of protein KD was assessed by Western blotting 72 h post-RNAi transfection. Cells were scraped into PBS/protease inhibitor cocktail (Sigma–Aldrich). Cells were lysed in standard 5× sample buffer and boiled for 5 min. Samples were resolved on 10% Bis/Acrylamide (1:29) gels and transferred onto nitrocellulose membrane (pore size 0.2 μm, Millipore Corporation, Bedford, MA). Following blocking, membranes were incubated in primary antibodies (1:1000 in 4% BSA in PBST) for 1 h at RT or o/n at 4 °C. Membranes were washed in PBST for 15 min and incubated with horseradish peroxidase (HRP) – conjugated anti-mouse and anti-rabbit secondary antibody (1:5000 in 4% BSA in PBST, Zymed Laboratories) for 1 h at RT or o/n at 4 °C. Immunoreactive bands were detected using an Immun-star HRP chemiluminescent kit (BioRad Laboratories, Hercules, CA). The signal was quantified by scanning developed films using SCION software (NIH).

2.4. Antibodies and immunofluorescence analyses

Mouse mAB anti-clathrin heavy chain (HCH) X22, a rabbit polyclonal antiserum directed against the β-subunit of AP-2 (GD/1, anti β1/β2), mouse mAB anti-α-Adaptin (AP-2 α-subunit) (MA1-64; Affinity BioReagents, Golden, CO), mouse mAB anti-α-Adaptin (610501; BD Biosciences, San Jose, CA), mouse mAB anti-Dab2 (p96, BD Biosciences), and mouse mAB anti-Dynamin2 (clone 41; BD Biosciences) were used at dilutions of 1:50–1:250 in 10% FBS/PBS. Secondary antibodies and immunofluorescence analyses have been described previously [5]. KD of proteins was evaluated by comparing the fluorescence intensity signals measured along lines on images taken under identical exposure conditions from treated and untreated cells.

2.5. Dynamin assays

Cells were transfected with Cx43-GFP, and 14 h later dynamin function was inhibited by treating cells with 10 μM GTPγS (Sigma, 10 mM stock in water, for 5 h), or with 40 μM dynasore 14 h post-transfection and again with 20 μM dynasore 17 h post-transfection (Tocris Bioscience, Ellisville, MO, 20 mM stock in DMSO, for a total of 5 h). Additionally, cells were cotransfected with Cx43-mApple, and GFP-tagged dominant negative Dynamin2-K44A mutant. Number and size of GJs and GJ vesicles (AGJs) was evaluated 19 h after transfections as described for the KD assays.

2.6. Statistical analyses

AGJs and GJs were counted on images taken of KD, drug treated and control cells in at least three independent experiments each. Only clearly defined GJs (a line of fluorescent puncta, or elongated plaques located between cell pairs), and AGJs (bright fluorescent spherical structures located in the cytoplasm $\geqslant 0.5$ μm in diameter, see Ref. [5]) were considered. Statistical analyses were performed using Microsoft Excel's analysis of variance (ANOVA) "Single Factor and Descriptive Statistics" functions of the data analysis package. For statistical analyses, the total number of AGJ vesicles was divided by the number of cell pairs that were positive for Cx43-GFP/-mApple expression and clearly coupled by GJs. Similarly, the total number and length of GJ plaques was determined and divided by the number of cell pairs. In all analyses, a P-value $\leqslant 0.05$ was considered statistically significant.

3. Results

3.1. Depletion of clathrin significantly reduces GJ internalization

Using live-cell imaging, our lab and others have shown previously that cells internalize entire GJ plaques and plaque portions in a highly regulated process, with one cell of a pair preferentially budding GJs outward, and the other cell preferentially internalizing the double-membrane spanning GJ plaques [5,12]. We further found that internalizing GJs specifically colocalized with clathrin, the alternative clathrin adaptor Dab2, dynamin2, myosin-VI, and actin filaments, using both, monoclonal and polyclonal antibodies. Additionally, we found that cultivating Cx43-GFP transfected HeLa cells in hypertonic medium, a treatment described to prevent clathrin and adapter proteins from interacting [16], or depleting cells of clathrin using CHC RNAi significantly reduced GJ internalization by 56% and 55%, respectively [5]. Together, these investigations suggested a critical role for clathrin in the internalization of Cx43-based GJs. However, since clathrin is a major component of cellular metabolism, depletion of this protein could have reduced unspecifically Cx biosynthesis and trafficking, and thus GJ formation. If clathrin mediates the internalization of GJs, then clathrin depletion should correlate with a reduction of AGJ vesicles and a concomitant increase in GJ plaque size and/or number. To test this, we repeated RNAi-mediated CHC KD experiments in HeLa cells. Significant CHC depletion (>90%) at 72 h post-RNA-oligo transfection was confirmed by Western blot analyses (Fig. 1A). In addition, immunofluorescence and fluorescence intensity analyses revealed significantly reduced CHC staining (Fig. 1B and C), and functional studies showed an almost complete inhibition of Trfn uptake (Fig. 1D and E), indicating efficient CHC depletion and CME inhibition.

CHC-depleted or control treated HeLa cells were transiently transfected with Cx43-GFP cDNA 48 h post-RNAi transfection. In HeLa cells (that do not express endogenous Cxs), GJ plaques form only between transfected groups of cells (Fig. 2A, arrows). Cells were allowed to make and internalize GJ plaques for an additional 24 h before fixation. Fields with clearly defined GJ plaques between cells were imaged (Fig. 2A, arrows), and AGJ and GJ plaque number and size were quantified (see Section 2, Fig. 2B and C). As in our previous study [5], we observed a significant reduction in the number of GJ vesicles that were internalized under CHC KD conditions (50% compared to control cells; Fig. 2B and C). In addition, we found a significant increase in the size and number of PM GJs (97% and 30% increase compared to control cells, Fig. 2B and C). Together, a decrease in internalized GJ vesicles, and a concomitant increase in GJ plaque size and number strongly suggests that GJ internalization is a clathrin-mediated process.

3.2. Depletion of clathrin adaptors significantly reduces GJ internalization

CME requires adaptor proteins that bind to both cargo and cargo receptors, and to clathrin to form endocytic vesicles. The