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Functional expression of Ca^{2+} dependent mammalian transmembrane gap junction protein Cx43 in slime mold *Dictyostelium discoideum*

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ABSTRACT

In this paper, we expressed murine gap junction protein Cx43 in *Dictyostelium discoideum* by introducing the specific vector pDXA. In the first step, the successful expression of Cx43 and Cx43-eGFP was verified by (a) Western blot (anti-Cx43, anti-GFP), (b) fluorescence microscopy (eGFP-Cx43 co-expression, Cx43 immunostaining), and (c) flow cytometry analysis (eGFP-Cx43 co-expression). Although the fluorescence signals from cells expressing Cx43-eGFP detected by fluorescence microscopy seem relatively low, analysis by flow cytometry demonstrated that more than 60% of cells expressed Cx43-eGFP. In order to evaluate the function of expressed Cx43 in *D. discoideum*, we examined the hemi-channel function of Cx43. In this series of experiments, the passive uptake of carboxyfluorescein was monitored using flow cytometric analysis. A significant number of the transfected cells showed a prominent dye uptake in the absence of Ca^{2+}. The dye uptake by transfected cells in the presence of Ca^{2+} was even lower than the non-specific dye uptake by non-transformed Ax3 orf+ cells, confirming that Cx43 expressed in *D. discoideum* retains its Ca^{2+}-dependent, specific gating function. The expression of gap junction proteins expressed in slime molds opens a possibility to the biological significance of intercellular communications in development and maintenance of multicellular organisms.

1. Introduction

In the history of earth, multicellularity has evolved approximately 1 billion years ago. Multicellular organisms require intercellular communications for the structure formation as well as for the maintenance of their whole function. Except for the signal transduction pathways based on G-protein coupled receptors (GPCR) or receptor tyrosine kinase (RTK), gap junction is the machinery used throughout the development [1,2]. Gap junctions exist in nearly all metazoan cells, from simple cnidarians to complex mammals [3,4]. The purpose of gap junctions is to enable intercellular communication by regulating the diffusion of small signaling molecules including ions, metabolites, nucleotides such as cyclic AMP, cyclic GMP or small peptides up to ~1000 Da [5].

Gap junction proteins were firstly reported by Revel and Karnovsky [6] in intercellular junctions of mouse heart and liver cells. Hundreds of intercellular gap junction proteins are found in the membranous junctions, called as "gap junction plaques" [5]. Similar to innexins in invertebrates and pannexins in vertebrates, connexins are important gap junction proteins in mammalian cells. Connexins comprise a family of more than 20 members which confer different properties in terms of regulation [7]. One connexin gap junction is based on the end-to-end docking of two transmembrane connexons (called hemi-channels), and creates a pore spanning across the plasma membranes of two neighboring cells. One connexon hemi-channel is composed of six connexin subunits [8,9]. Among various connexin, homologues of Cx43 are ubiquitously expressed in a variety of cell types and organs [10]. To date, many studies have demonstrated that the dysfunction of Cx43 results in serious diseases such as deafness, skin disease, cardiac malformation, neurodegeneration and oculodentodigital syndrome [11–13].

One connexin polypeptide chain consists of four transmembrane helices, intracellular N- and C-termini, one intracellular loop, and two extracellular loops. The extracellular domains are thought to form a β-sheet secondary structure [14], which is essential for the tight docking of two adjacent cells and thus the formation of a pore channel. Furthermore, connexons interact with intracellular components to form a multi-protein signaling complex termed the "nexus" [15]. Potential interaction partners include cytoskeletal proteins, scaffolding proteins, protein kinases and phosphatases.

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This would explain channel-independent functions of connexins in cell migration, cell polarization and growth control [16]. An unpaired, single connexon can also function as a "hemi-channel" which is regulated via Ca^{2+} level and phosphorylation in mammalian cells as reviewed by [17,18]. Such hemi-channel functions have been demonstrated by the single channel recording [19] as well as by the uptake of carboxyfluorescein [20] or luciferin [21]. Albeit a number of studies have demonstrated the structures and molecular level functions of gap junction proteins, the significance of gap junctions and thus intercellular communications in the maintenance and development of multicellular organisms is not definitely understood.

In order to understand the significance of gap junctions in multicellularity, a commonly used strategy is to disturb or block the function of gap junction proteins in multicellular organisms. The second and more direct strategy is to express gap junction proteins in single cellular organisms and investigate if the expression causes any major change. In this study, we introduced the specific vector pDXA and expressed Cx43 in slime mold *Dictyostelium discoideum*, which has been used for the expression of myosins [22–24] and several transmembrane proteins [25–27]. Wild type *D. discoideum* does not express any gap junction protein, as demonstrated by numerous previous attempts to prove the existence [28] as well as by the recent genome analysis [29]. Therefore, the key questions that we would like to address in this paper are: (a) if the expression is possible, (b) if the expressed Cx43 retains its natural function, and (c) if the expression of Cx43 would have any impact on the fate of single *D. discoideum* cells.

2. Materials and methods

2.1. Plasmid construction, cloning, cell culture and sporulation

All procedures were performed according to established protocols of molecular cloning [30] and the recommendations of the manufacturers. Polymerase chain reaction (PCR) products were obtained using Phusion polymerase (New England Biolabs GmbH, Frankfurt, Germany), PCR fragments were phosphorylated by T4 PNK, and with T4 ligase inserted into the SmaI/CIP treated Stratagene pBluescript II SK−vector (Agilent Technologies, Waldbronn, Germany) prior to sequencing (MWG, Ebersberg, Germany). DNA purification kits were obtained from Qiagen (Hilden, Germany) and Macherey & Nagel (Düren, Germany). Synthetic oligonucleotides were obtained from MWG. The following computer resources were used: BCM [31]; BioEdit (Tom Hall, NCSU Raleigh, NC, USA); CDD [32] ; ExPASy [33]; NCBI-BLAST [34]; pDraw (AcaClone Software). Cx43 (NCBI Accession No. X62836) and Cx43-eGFP gene products from pMJ expression vectors (pMJ-mCx43-eGFP) were generous gifts from K. Willecke (Univ. Bonn), and pDXA-3H vector was from D. Manstein (Med. Univ. Hannover) [22]. Details of the vector construction, the overviews and nucleotide sequences of expression vectors pDXA_Cx43_His and pDXA_Cx43-eGFP_His are described in Supporting information (S1) available online. Transformations of *D. discoideum* Ax3 orf+ cells were performed according to established protocols (www.dictybase.org) with minor modifications. The detailed protocols used in this study are described in Supporting information (S2) available online. All experiments were carried out under sterile conditions with *D. discoideum* cultures which were started from freshly thawed spores.

2.2. Fluorescence microscopy, confocal laser scanning microscopy

Fluorescence images and differential interference contrast images were obtained using a Zeiss Axiovert 200 (Zeiss, Jena, Germany) equipped with a LD 63 Achroplan objective (N.A.: 0.75) and a 16 bit digital CCD camera Orca C4742-95-12ER (Hamamatsu, Herrsching, Germany). Confocal laser scanning microscopy images are collected using a LSM-510meta confocal laser scanning microscope (Carl Zeiss, Oberkochen, Germany) equipped with Plan-Neofluar 40×/1.3 Oil Ph3 objective and a PMT detector. The reference images were collected in a differential interference contrast (DIC) mode.

2.3. Whole mount immunostaining

Axenically grown *D. discoideum* cells (10^6) carrying the Cx43 expression vectors were resuspended in 100 µl PBS buffer. After adding 100 µl of 4% PFA solution (paraformaldehyde in PBS) cells were fixed for 10 min at room temperature and washed twice in PBS. Cells were perforated for 10 min in 0.2% Triton X100 and subsequently incubated in 10% BSA solution for 1 h to avoid unspecific antibody binding. Immunostaining was performed with a first rabbit anti-Cx43 polyclonal antibody (C-6219, Sigma, Deisenhofen, Germany) for 1 h, followed by a second FITC (fluorescein isothiocyanate) conjugated goat anti rabbit IgG antibody (F-1262, Sigma, Deisenhofen, Germany). Immunostained cells were subjected into glass cover slips for DIC and fluorescence imaging.

2.4. Flow cytometry

In order to quantitatively determine fluorescence signals from the Cx43-eGFP expression as well as the uptake of 5(6)-carboxyfluorescein (CF), were carried out with an analytical flow cytometer (FACScan), and a corresponding analysis software (CellQuest) both from Becton Dickinson, San Jose, USA [35]. The fraction of *D. disccoideum* cells was determined by the mean of flow cytometry. Staining with propidium iodide was performed to allow exclusion of nonviable cells. For the uptake of CF by connexon hemi-channels, the stationary grown cells were harvested and washed twice in HL5 medium with 10 mM EDTA. Each dye uptake assay was performed by incubating $(0.5–1) \times 10^6$ cells in HL5 medium with 10 mM EDTA and 0.5 mg/ml CF for 60 min at room temperature. As the control, cells were incubated in HL5 medium containing 2 mM $CaCl_2$ and 0.5 mg/ml CF. Cells were sedimented at 500*g* for 10 min and washed three times in CF-free HL5 medium containing 2 mM $CaCl_2$.

3. Results

3.1. Cloning strategy and generation of Cx43 expressing D. discoideum cell lines

As the starting point, we used the mammalian expression system, which is HeLa cell transformed with Cx43 and Cx43-eGFP (K. Willecke, Univ. Bonn). The insert of vectors pMJ-mCx43 and pMJ-mCx43-eGFP [36,37] was amplified by PCR. The insert was further equipped with restriction sites for cloning into pDXA-3H to attach six histidine residues for the purification with affinity chromatography (D. Manstein, Med. Univ. Hannover). Two different kinds of Cx43, with and without eGFP, were used for cloning and expression in *D. discoideum*. The mammalian signal peptide contained in the original mouse Cx43 was used without further modification for targeting of the heterologous fusion proteins into the cell membrane fraction. The ORF sequence matched exactly NCBI database records (NM_010288.3, GI:166091435 [38]), including the adjustment of the codon usage (30 base pairs) for the expression in *D. discoideum* (N-terminal) as well as for eGFP and histidine tags (C terminal). The respective FASTA sequences of the two plasmids are documented in Supporting information

(S1). Selected transformants of *D. discoideum* cells expressing either Cx43-His or Cx43-eGFP-His were subjected to sporulation. As we found that spores of the transformed cell lines were viable, spores from each cell line stored in glycerol at −80 °C were used throughout this study. The development from freshly thawed and seeded spores in HL5 medium to viable cells takes approximately 10–14 days.

3.2. Expression of Cx43 fusion proteins in D. discoideum membrane

The expression of transmembrane protein Cx43 and fusion protein Cx43-eGFP in *D. discoideum* was routinely identified by Western blot. Proteins of either membrane fractions or entire cells were first separated according to the procedure of Laemmli [39] on 10 wt.% acrylamide/bisacrylamide gels. Details of the membrane preparation and the protein analysis are described in Supporting information available online (S3). The heterologous expression of Cx43 was analyzed in cell lines after growing cells in HL5 medium for 3–4 days. The protein analysis was performed only for cells that firmly adhered onto the polystyrene culture flask. First, the presence of Cx43-His in (a) whole cell extracts, (b) cytosol extracts, and (c) membrane preparations normalized to the same volumes was checked by Western blot. Fig. 1 represents the Western blot of the membrane preparation labeled by polyclonal mouse anti Cx43 antibody (lane 1) and monoclonal mouse anti His-tag antibody (lane 2), clearly showing positive signals near the theoretically calculated molecular weight (MW ∼ 43 kDa). The fact that whole cell extracts showed almost identical patterns but no signal could be found for cytosol extracts implies that almost all Cx43-His were expressed in plasma membranes. The same experiments were performed for Cx43-eGFP-His using anti Cx43 antibody and rabbit polyclonal antibody against eGFP (Supporting information S2). Cx43-eGFP-His can be identified with both anti Cx43 and anti eGFP antibodies near the theoretically predicted position (MW ∼ 70 kDa), while Cx43-His was not recognized by anti eGFP. The obtained results confirmed the correct translation of mammalian Cx43-His and Cx43-eGFP-His in *D. discoideum* in terms of length and reading frame.

As the next step, the presence of Cx43 was analyzed by immunofluorescence imaging of the whole *D. discoideum* cells expressing Cx43-His. The adherent cells were transferred to a glass slide for fixation, and subjected to the antibody staining. Fig. 2 shows DIC and fluorescence micrographs of cells treated with polyclonal rabbit anti Cx43 antibody and secondary donkey anti rabbit IgG antibody conjugated with FITC. As presented in the figure, all the transfected cells exhibited fluorescent signals above the threshold, which was defined from non-transformed Ax3 orf+ (in the following also referred to as "wild type") control cells. Therefore, it has been concluded that the vast majority of transfected *D. discoideum* cells expresses the mammalian Cx43-His proteins.

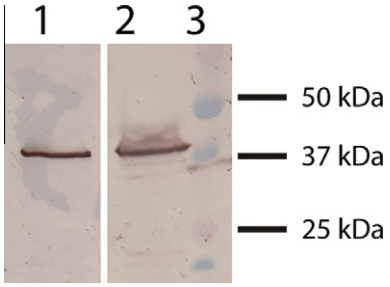

Fig. 1. Western blot analysis of membrane preparations from *D. discoideum* cells expressing Cx43-His (lane 1: polyclonal mouse anti Cx43 antibody; lane 2: monoclonal mouse anti histidine antibody; lane 3: molecular weight marker).

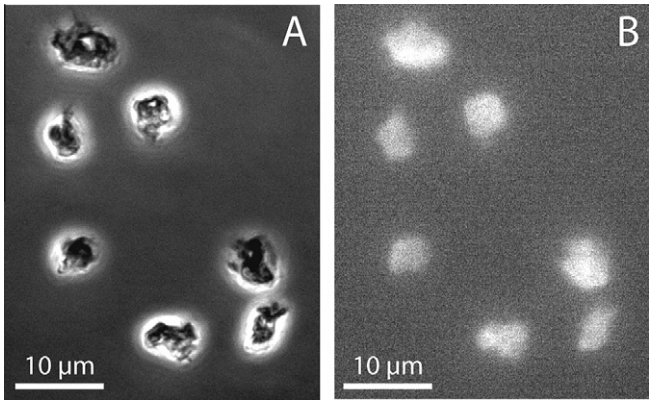

Fig. 2. (A) Differential interference contrast (DIC) image and (B) fluorescence image of *D. discoideum* cells. After the transfer and fixation of cells on glass slides, the cells were stained with polyclonal rabbit anti Cx43 antibody and secondary donkey anti rabbit IgG antibody conjugated with FITC. All the cells visualized by DIC exhibited fluorescence signals beyond the threshold level. Note that non-transformed Ax3 orf+ (wild type) cells after the same treatment did not show any fluorescence.

To verify the expression of Cx43-eGFP-His in *D. discoideum* cells we used the combination of using confocal laser scanning microscopy and FACS for live cells. Fig. 3A shows the overlay of DIC and fluorescence images of transfected cell line 7 days after the spore germination. Fluorescence signals from eGFP could be detected from more than one half of cells in the view area. In contrast to the immunofluorescence image presented in Fig. 2B, the zoom up of the confocal image (Fig. 3B) implies that Cx43-eGFP-His is localized in/near plasma membranes, forming clusters. To gain more

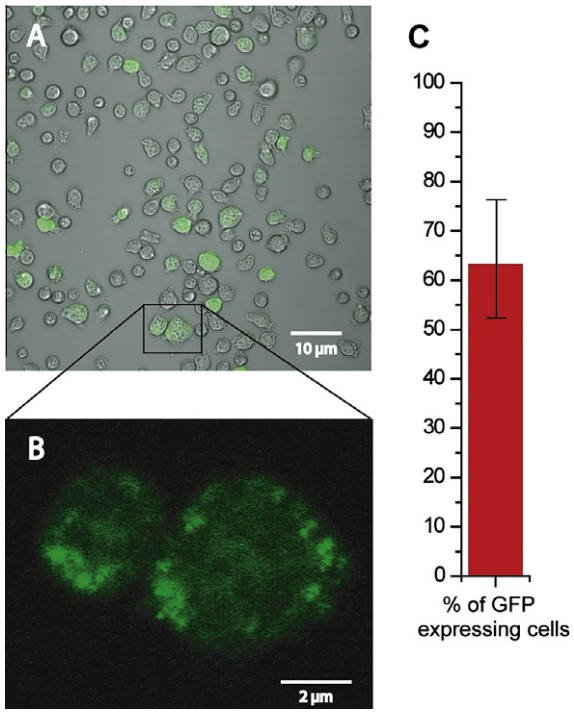

Fig. 3. Expression of Cx43-eGFP-His in *D. discoideum* cells confirmed by confocal laser scanning microscopy. (A) An overlay of DIC and fluorescence images suggests that more than one half of the cells in the view area expressed Cx43-eGFP-His fusion protein. (B) The zoom up image of the indicated region implies that the proteins are expressed in/near the plasma membranes and form clusters. (C) Statistical evaluation of protein expression using flow cytometric analysis, demonstrating that 64 ± 11% of *D. discoideum* cells adhered on the culture flask surface express Cx43-eGFP-His.

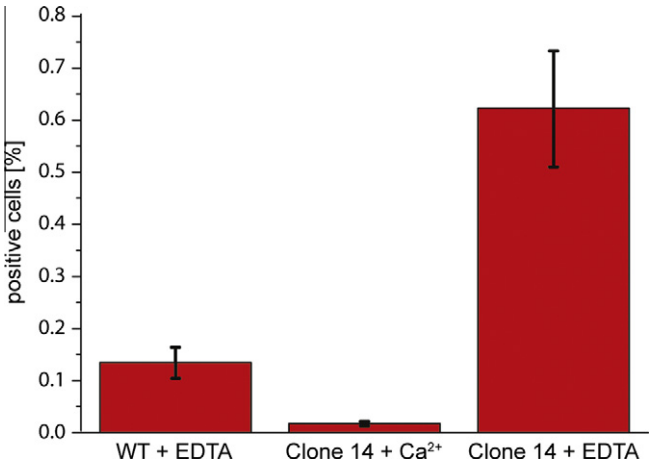

Fig. 4. Uptake of carboxyfluorescein (CF) by transfected *D. discoideum* cells expressing Cx43-His monitored by flow cytometry. After the incubation of 0.5 ml/mg CF solution at room temperature for 60 min, the cells were washed and analyzed on a FACScan. The uptake level of transfected cells in the absence of Ca^{2+} (0.60 ± 0.10%) was 50 times larger than that in the presence of 2 mM Ca^{2+} and five times larger than the control (non-transformed Ax3 orf+(wild type) *D. discoideum*), demonstrating that Cx43-His expressed in transfected *D. discoideum* maintain the Ca^{2+}-dependent hemi-channel function.

reliable statistics for the fraction of *D. discoideum* cells expressing Cx43-eGFP-His, we performed Flow cytometric analysis for the cells 7 days after spore germination and found that 64 ± 11% of adherent cells expressed Cx43-eGFP-His fusion protein (Fig. 3C).

3.3. Hemi-channel function of Cx43 expressed in D. discoideum cells

At the last step, we checked if Cx43 expressed in *D. discoideum* cells retains its native functions by monitoring the uptake of carboxyfluorescein (MW ~ 333). Fig. 4 represents the flow cytometric analysis of transfected *D. discoideum* cells without eGFP in the presence and absence of Ca^{2+}, and the control result from non-transformed Ax3 orf+ (wild type) *D. discoideum* cells. The incubation of transfected *D. discoideum* cells with 0.5 mg/ml of CF at room temperature for 60 min allows the cells for the passive uptake of CF molecules through Cx43 hemi-channels. First, in the presence of 2 mM Ca^{2+} ions, the fraction of transfected cells that have uptaken CF into cytoplasm was close to the detection limit (0.01 ± 0.004%). In fact, this level is about one order of magnitude lower than non-specific uptake of CF by non-transformed Ax3 orf+ (wild type) *D. discoideum* via passive diffusion across the plasma membranes (0.13 ± 0.03%). In contrast, once Ca^{2+} ions are depleted by EDTA, we found that the fraction of transfected *D. discoideum* cells that uptake CF is 60 times more (0.6 ± 0.1%) than the corresponding results in the presence of Ca^{2+}. In fact, the uptake level was distinctly higher (five times) than the reference level of non-transformed Ax3 orf+ (wild type) cells in the absence of Ca^{2+}. Thus, we have concluded that the Cx43-His expressed in *D. discoideum* cells retain their native hemi-channel function

4. Discussion

D. discoideum has a remarkable position in the evolutionary tree of life since it switches from a single cellular stage to multicellular aggregates in certain stages of its life cycle, e.g. prior to spore formation [40]. Its life cycle is divided into two mutually cycles, a first phase where single cells of the myxamoeba multiply by binary fission (feeding state), and a second phase, in which the cells associate and form multicellular aggregates (slug). Aggregating cells subsequently differentiate into either stalk cells or spore cells (starvation state). Taking a closer look at the multicellular stages of *D. discoideum*, one would expect that intercellular communications might be facilitated or enhanced by taking advantage of pore forming structures such as gap junctions which are common to every other multicellular organism. Previously, Johnson et al. [28] used a variety of fixation procedures at different stages of *D. discoideum* development and could not identify any gap junction forming structures. Later, Barbe et al. [41] described that gap junctions are built up by different proteins like innexins or pannexins in all multicellular organisms, except for the slime mold *D. discoideum*.

In this study, we successfully expressed Cx43-His and Cx43-eGFP-His in *D. discoideum* by using the high copy plasmid pDXA. We demonstrated that Cx43 can be expressed in single cells and facultative multicellular stages of the slime mold *D. discoideum* without disturbing its natural life cycle. For the storage, we enforced the transfected *D. discoideum* to stay at a multicellular, spore forming state by starvation, and started all the following cell cultures from freshly thawed spores. We conclude that the single connexin proteins are sufficient for the self-assembly of functional hemi-channels. The quantitative FACS test confirmed that the expression of only Cx43 is sufficient to retain Ca^{2+}-dependent, specific gating functions of hemi-channels. It should be noted that the cell progeny for transfected cells was reduced by a factor of 5–10 compared with the non-transformed Ax3 orf+ wild type cells, although we kept the cells under optimal conditions for vegetative growth to avoid the formation of multicellular slug aggregates. We also found that the expression level significantly decreased after 5–10 cell cycles, which approximately corresponds to the culture period of 3 weeks. After this time period, the expression level of Cx43 was reduced to the one below the detection limit. This low and non-stable expression of Cx43 seems to be in contrast to the previous accounts that reported the successful expression of plant aqua porin [26] human muscarin receptor [27], and mollusk myosin chitin synthase [25]. This finding suggests that the expression of Cx43 would cause stresses to cells due to the lack of other Cx43 regulatory proteins. Phenomenologically, this may be attributed to the fact that slime molds are commonly classified among the fungi, and gap junctions have not been observed in the plant kingdom.

It should be noted that we found no sign of permanent/stable cell–cell contacts on the single cellular level. This seems plausible, since connexon gap junctions alone are not capable of supporting stable cell–cell contacts in multicellular organisms. On the other hand, we also confirmed that the expression of Cx43 proteins do not cause any significant difference in both macroscopic morphology (stalks and slugs) and functions (production of viable spores) in the facultative multicellular stage. To understand the impact of Cx43 expression in *D. discoideum* on the multicellular level, further studies on (a) the time evolution of facultative multicellular structures (stalks and slugs) by wild type and transfected cells and (b) the precise localization of Cx43-eGFP during the formation of multicellular structures would be necessary. For this purpose, the use of expression promoters, such as discoidin [42], would be helpful to establish stable transfected cell lines.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2012.01.126.

References

[1] B. Alberts, A. Johnson, J. Lewis, et al., Molecular Biology of the Cell: Reference Edition, Garland Science, New York, USA, 2007.
[2] S.F. Gilbert, Developmental Biology, Sinauer Associates, Inc., Sunderland, MA, USA, 2010.
[3] J.P. Revel, The oldest multicellular animal and its junctions, in: E.L. Hertzberg, R.G. Johnson (Eds.), Modern Cell Biology, vol. 7: Gap Junctions, Alan R. Liss, New York, 1988, pp. 135–149.
[4] V.I. Shestopalov, Y. Panchin, Pannexins and gap junction protein diversity, Cell. Mol. Life Sci. 65 (2008) 376–394.
[5] N.M. Kumar, N.B. Gilula, The gap junction communication channel, Cell 84 (1996) 381–388.
[6] J.P. Revel, M.J. Karnovsky, Hexagonal array of subunits in intercellular junctions of the mouse heart and liver, J. Cell Biol. 33 (1967) C7–C12.
[7] G. Söhl, K. Willecke, Gap junctions and the connexin protein family, Cardiovasc. Res. 62 (2004) 228–232.
[8] D.L.D. Caspar, D.A. Goodenough, L. Makowski, et al., Gap junction structures. I. Correlated electron microscopy and X-ray diffraction, J. Cell Biol. 74 (1977) 605–628.
[9] L. Makowski, D.L.D. Caspar, W.C. Phillips, et al., Gap junction structures. II. Analysis of the X-ray diffraction data, J. Cell Biol. 74 (1977) 629–645.
[10] E.C. Beyer, T.H. Steinberg, Evidence that the gap junction protein connexin-43 is the ATP-induced pore of mouse macrophages, J. Biol. Chem. 266 (1991) 7971–7974.
[11] A.G. Reaume, P.A. de Sousa, S. Kulkarni, et al., Cardiac malformation in neonatal mice lacking connexin43, Science 267 (1995) 1831–1834.
[12] W.A. Paznekas, S.A. Boyadjiev, R.E. Shapiro, et al., Connexin 43 (GJA1) mutations cause the pleiotropic phenotype of oculodentodigital dysplasia, Am. J. Hum. Genet. 72 (2003) 408–418.
[13] W. Roscoe, G.I.L. Veitch, X.Q. Gong, et al., Oculodentodigital dysplasia-causing connexin43 mutants are non-functional and exhibit dominant effects on wild-type connexin43, J. Biol. Chem. 280 (2005) 11458–11466.
[14] C.I. Foote, L. Zhou, X. Zhu, et al., The pattern of disulfide linkages in the extracellular loop regions of connexin 32 suggests a model for the docking interface of gap junctions, J. Cell Biol. 140 (1998) 1187–1197.
[15] H.S. Duffy, M. Delmar, D.C. Spray, Formation of the gap junction nexus: binding partners for connexins, J. Physiol. Paris 96 (2002) 243–249.
[16] S. Olk, G. Zoidl, R. Dermietzel, Connexins, cell motility, and the cytoskeleton, Cell Motil. Cytoskel. 66 (2009) 1000–1016.
[17] D.A. Goodenough, D.L. Paul, Beyond the gap: functions of unpaired connexon channels, Nat. Rev. Mol. Cell Biol. 4 (2003) 285–294.
[18] J.L. Solan, P.D. Lampe, Key connexin 43 phosphorylation events regulate the gap junction life cycle, J. Membr. Biol. 217 (2007) 35–41.
[19] L.K. Buehler, K.A. Stauffer, N.B. Gilula, et al., Single channel behavior of recombinant beta gap junction connexons reconstituted into planar lipid bilayers, Biophys. J. 68 (1995) 1767–1775.
[20] H. Li, T.F. Liu, A. Lazrak, et al., Properties and regulation of gap junctional hemichannels in the plasma membranes of cultured cells, J. Cell Biol. 134 (1996) 1019–1030.
[21] A. Hofer, R. Dermietzel, Visualization and functional blocking of gap junction hemichannels (connexons) with antibodies against external loop domains in astrocytes, Glia 24 (1998) 141–154.
[22] D.J. Manstein, H.-P. Schuster, P. Morandini, et al., Cloning vectors for the production of proteins in *Dictyostelium discoideum*, Gene 162 (1995) 129–134.
[23] G. Tsiavaliaris, S. Fujita-Becker, D.J. Manstein, Molecular engineering of a backwards-moving myosin motor, Nature 427 (2004) 558–561.
[24] D.J. Manstein, K.M. Ruppel, J.A. Spudich, Expression and characterization of a functional myosin head fragment in *Dictyostelium discoideum*, Science 246 (1989) 656–658.
[25] V. Schönitzer, N. Eichner, H. Clausen-Schaumann, et al., Transmembrane myosin chitin synthase involved in mollusc shell formation produced in *Dictyostelium* is active, Biochem. Biophys. Res. Commun. 415 (2011) 586–590.
[26] F. Chaumont, W.F. Loomis, M.J. Chrispeels, Expression of an Arabidopsis plasma membrane aquaporin in *Dictyostelium* results in hypoosmotic sensitivity and developmental abnormalities, Proc. Natl. Acad. Sci. 94 (1997) 6202–6209.
[27] G. Voith, T. Dingermann, Expression of the human muscarinic receptor gene m2 in *Dictyostelium discoideum*, Nat. Biotechnol. 13 (1995) 1225–1229.
[28] G. Johnson, R. Johnson, M. Miller, et al., Do cellular slime molds form intercellular junctions?, Science 197 (1977) 1300.
[29] L. Eichinger, J.A. Pachebat, G. Glockner, et al., The genome of the social amoeba *Dictyostelium discoideum*, Nature 435 (2005) 43–57.
[30] J. Sambrook, E.F. Fritsch, T. Maniatis, Molecular Cloning, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, USA, 2001.
[31] R.F. Smith, B.A. Wiese, M.K. Wojzynski, D.B. Davison, K.C. Worley, BCM Search Launcher – an integrated interface to molecular biology data base search and analysis services available on the World Wide Web, Genome Res. 6 (1996) 454–462.
[32] A. Marchler-Bauer, J.B. Anderson, P.F. Cherukuri, et al., CDD: a Conserved Domain Database for protein classification, Nucleic Acids Res. 33 (2005) D192–196.
[33] E. Gasteiger, A. Gattiker, C. Hoogland, et al., ExPASy: the proteomics server for in-depth protein knowledge and analysis, Nucleic Acids Res. 31 (2003) 3784–3788.
[34] S.F. Altschul, T.L. Madden, A.A. Schäffer, et al., Gapped BLAST and PSI-BLAST: a new generation of protein database search programs, Nucleic Acids Res. 25 (1997) 3389–3402.
[35] J.B.R. DeGeorge, M. Rosenberg, V. Eckstein, et al., BMP-2 and FGF-2 synergistically facilitate adoption of a cardiac phenotype in somatic bone marrow c-kit+/Sca-1+ stem cells, Clin. Translational Sci. 1 (2008) 116–125.
[36] S. Maxeiner, O. Krüger, K. Schilling, et al., Spatiotemporal transcription of connexin45 during brain development results in neuronal expression in adult mice, Neuroscience 119 (2003) 689–700.
[37] S. Sonntag, G. Söhl, R. Dobrowolski, et al., Mouse lens connexin23 (Gje1) does not form functional gap junction channels but causes enhanced ATP release from HeLa cells, Eur. J. Cell Biol. 88 (2009) 65–77.
[38] H.J. Schwarz, Y.S. Chang, H. Hennemann, E. Dahl, P.A. Lalley, K. Willecke, Chromosomal assignments of mouse connexin genes, coding for gap junctional proteins, by somatic cell hybridization, Somat Cell Mol. Gen 18 (1992) 351–359.
[39] U.K. Laemmli, Cleavage of structural proteins during the assembly of the head of bacteriophage T4, Nature 227 (1970) 680–685.
[40] S. Annesley, P. Fisher, *Dictyostelium discoideum* – a model for many reasons, Mol. Cell. Biochem. 329 (2009) 73–91.
[41] M.T. Barbe, H. Monyer, R. Bruzzone, Cell–cell communication beyond connexins: the pannexin channels, Physiology 21 (2006) 103–114.
[42] M.L.W. Knetsch, G. Tsiavaliaris, S. Zimmermann, et al., Expression vectors for studying cytoskeletal proteins in *Dictyostelium discoideum*, J. Muscle Res. Cell M. 23 (2002) 605–611.