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ABSTRACT

Melanopsin is the mammalian photopigment that primarily mediates non-visual photoregulated physiology. So far, this photopigment is poorly characterized with respect to structure and function. Here, we report large-scale production and purification of the intact long isoform of mouse melanopsin (melanopsin-L) using the baculovirus/insect cell expression system. Exploiting the baculoviral GP67 signal peptide, we obtained expression levels that varied between 10–30 pmol/10^6 cells, equivalent to 2–5 mg/L. This could be further enhanced using DMSO as a chemical chaperone. LC–MS analysis confirmed that full-length melanopsin-L was expressed and demonstrated that the majority of the expressed protein was N-glycosylated at Asn^{30} and Asn^{34}. Other posttranslational modifications were not yet detected. Purification was achieved exploiting a C-terminal deca-histag, realizing a purification factor of several hundred-fold. The final recovery of purified melanopsin-L averaged 2.5% of the starting material. This was mainly due to low extraction yields, probably since most of the protein was present as the apoprotein. The spectral data we obtained agree with an absorbance maximum in the 460–500 nm wavelength region and a significant red-shift upon illumination. This is the first report on expression and purification of full length melanopsin-L at a scale that can easily be further amplified.

Introduction

Melanopsin is an integral membrane protein that belongs to the family of the GPCRs. Melanopsin was first discovered in the dermal melanophores of *Xenopus leavis* [1], and subsequently the mammalian ortholog was found to be expressed in a small subset of retinal ganglion cells (RGCs)[1] that project to the central pacemaker, the suprachiasmatic nucleus in the anterior hypothalamus [2], and to deeper parts of the brain [3–5]. This photopigment is responsible for non-visual photic responses such as photo-entrainment of the circadian system, the sustained response of the pupillary light reflex [6–8], and sleep – awake processes [9]. Most studies report a peak sensitivity of its action spectrum around 480 nm [10]. The melanopsin-expressing RGCs were shown to be intrinsically photosensitive (ipRGCs) with a spectral dependence that corresponds well with that observed for non-visual responses in several mammalian species [11–13]. A series of elegant studies with transgenic mice led to the current view that all three photoreceptor systems (rod, cone and ipRGCs) contribute to non-visual photophysiology [6–8,14,15], and that melanopsin is in particular required for the response at higher light intensities and for sustained activity [11]. Interestingly, melanopsin has greater sequence similarity to invertebrate opsins [1,16] and, in contrast to the vertebrate visual opsins, shows large interspecies differences in size and sequence of the N- and C-terminal tails [16]. Recently, it has been shown that a long and a short isoform of melanopsin, mainly differing in the length of their C-terminals, are differentially expressed in the mammalian retina [17].

Our current understanding of the molecular properties of melanopsin (3D structure, photochemistry, signaling) is still very limited, mainly because of its low natural abundance. Due to the paucity of melanopsin-expressing cells in the mammalian retina purification from natural sources is very laborious [18–20] and does not provide sufficient material for molecular studies. The alternative, therefore, would be expression in a heterologous system.

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[1] *Abbreviations used:* ASB-C8φ, 4-n-octylbenzoylamido-propyl-dimethylammonio-sulfobetaine; ASB-14, 3-[N,N-dimethyl(3-myristoylaminopropyl)ammonio]propane-sulfonate; ASB-14-4, tetradecanoylamidopropyl-dimethylammonio-butanesulfonate; CHAPS, 3-[(3-cholamidopropyl) dimethylammonio]-1-propane sulfonate; CTAC, hexadecyltrimethylammonium chloride; Cymal7, n-cyclohexyl-heptyl-β-D-maltoside; C12E8, octaethylene glycol monododecyl ether; DDM, n-dodecyl-ß-D-maltoside; DHPC, 1,2-dihexanoyl-sn-glycero-3-phosphocholine; DM, n-decyl-ß-D-maltoside; DMSO, dimethylsulfoxide; DPC, n-dodecylphosphocholine; Dpi, days post-infection; ER, endoplasmic reticulum; GPCR, G protein-coupled receptor; Gtα, transducin α subunit; Gtßγ, transducin ß and γ subunit; ipRGC, intrinsically photosensitive retinal ganglion cells; MOI, multiplicity of infection; PNGase F,peptide-N-glycosidase F; RGC, retinal ganglion cells; TDM, n-tridecyl-ß-D-maltoside.

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Heterologous expression of membrane proteins has been accomplished in many systems such as *Escherichia coli* [21], yeast [22], insect cell lines [23], cell-free conditions [24] and mammalian cell lines as well as "*in vivo*" in *Caenorhabditis elegans* [20] and in mouse photoreceptor cells [25,26], but with varying degree of success because of the different conditions in the host cell environment. For a recent review see Midgett and Madden [27].

Successful heterologous expression of mammalian melanopsin was reported in several mammalian cell lines such as HEK293 [13], Neuro 2A [12] and D407 [28] and in *Xenopus* oocytes [29], but because of the very low expression levels only limited information on ligand type (retinal), action spectrum (around 480 nm) and signaling (Gq protein; Ca^{2+} transients) has become available. Terakita and coworkers [30] succeeded in expressing and purifying melanopsin of the cephalochordate Amphioxus *Branchiostoma belcheri* from HEK293 cells, but only after truncation of the C-terminal. They reported a λ_{max} of 485 nm and a, typical for invertebrate rhodopsins, stable photoproduct (metamelanopsin; λ_{max} about 520 nm) that could be photoregenerated into the melanopsin parent state. Recently, Matsuyama et al. [31] described expression of mouse melanopsin in HEK293 cells, but functional purification was again only achieved after nearly completely truncating the C-terminal tail. Remarkably, they report a peak sensitivity at 467 nm for this recombinant melanopsin and about 476 nm for the meta photoproduct. These data clearly deviate from earlier estimates based upon action spectra of about 480 and 587 nm, respectively [10,32].

Successful amplified production of vertebrate visual opsins has been achieved with the baculovirus/insect cell expression system yielding functional expression levels of up to 5 mg/L cell culture [33–37]. Therefore, we investigated the potential of this system for production and purification of the intact long isoform of mouse melanopsin (melanopsin-L). The long isoform was selected, since the long C-terminal probably is involved in signaling processes.

Here we report large-scale production and purification of mouse melanopsin-L using recombinant baculovirus-driven expression in the S*f*9 insect cell line. A major effort was invested in a search for conditions that would improve functional expression and stability during purification.

Materials and methods

Materials

Buffer A: 20 mM Bis/Tris propane, 1 mM dithioerythritol (DTE), 1 mM benzamidine, 5 mM $MgCl_2$, 5 μM leupeptin (pH 7.6); Buffer B: 20 mM Bis/Tris propane, 1 M NaCl, 1 mM histidine, 5 μM leupeptin, (pH 7.2); Buffer C: 20 mM Pipes [piperazine-N,N′-bis(2-ethanesulfonic acid)], 0.1 mM EDTA, 130 mM NaCl, 10 mM KCl, 3 mM $MgCl_2$, 2 mM $CaCl_2$ (pH 6.5); Buffer D: 20 mM Pipes, 30 mM EDTA (pH 7.0); Buffer E: buffer B, with DPC, DDM, DHPC or ASB-14-4 either single or in combination at 20 mM pro detergent, 20 mM imidazole (pH 7.2); Buffer F: same as buffer E, but with 50 mMimidazole (pH 7.2); Buffer G: same as buffer E, but with 200 mM imidazole (pH 7.2); Buffer H: same as buffer E, but with 400 mM imidazole (pH 6.5). Penicillin and streptomycin were from Gibco-BRL, Breda, The Netherlands. Leupeptin was obtained from MP Biomedicals, CA, USA. Grace's insect medium and Insect-Xpress medium were obtained from Lonza, USA. Mouse anti tetra-His tag monoclonal antibody was obtained from Qiagen, Hilden, Germany. The polyclonal antibodies CERN9412 and CERN911 were raised against Gtαβγ in albino New Zealand rabbits as described before [38,39] and were selective for the α and β subunit, respectively, of trimeric G-proteins (Bovee-Geurts and DeGrip, unpublished). The goat anti-rabbit (IR Dye®700 CW) and goat anti-mouse (IR Dye®800 CW) secondary antibodies were obtained from Li-Cor biosciences, U.S.A. Monoclonal anti-FLAG® M2 antibody was from Sigma–Aldrich Chemie B.V., The Netherlands and rabbit polyclonal anti-calnexine (C-20)-R sc-6465 was obtained from Santa Cruz Biotechnology, CA, USA. Rabbit polyclonal anti-ninaA was a kind gift from Dr. Jacques Janssen (Ophthalmology, Radboud University Nijmegen Medical Centre, The Netherlands). All-*trans* retinal was obtained from Sigma–Aldrich Chemie B.V., and 11-*cis* retinal was a generous gift from Dr. Rosalie Crouch (Medical University of South Carolina, Charleston, USA) through financial support from NEI. DDM and digitonin were obtained from Serva Electrophoresis GmbH, Heidelberg, Germany. ASB-14, ASB-14-4 and ASB-C8φ were obtained from Calbiochem, an affiliate of Merck KGaA, Darmstadt, Germany. DHPC was obtained from Avanti polar lipids Inc, Alabama, USA. DPC, CTAC, C12E8, Cholate, Cymal7, CHAPS and TDM were obtained from Anatrace Inc, Ohio, USA. DM was prepared and purified as described before [40]. The plasmids with FLAG-tagged β-arrestin-1 and β-arrestin-2 were kindly donated by Dr. Robert Lefkowitz (Duke University Medical Center, Durham, N.C., USA) [41]. The corresponding recombinant baculoviruses were generated as described below for the recombinant mel-baculovirus. The baculoviruses expressing ninaA and calnexin were a kind gift from Dr. Chris Tate (MRC Laboratory of Molecular Biology, Cambridge, UK) [42]. The baculoviruses expressing Gqα, Gβ1 and his-tagged Gγ2 were kindly provided by Dr. Ted McMurchie (CSIRO, Adelaide Laboratories, Australia) [43].

Methods

Construction of recombinant baculovirus for expression of melanopsin-L

The baculovirus transfer vector pAcGP67 (BD Biosciences Clontech, Palo Alto, CA, USA) was modified by introduction of a synthetic sequence encoding a 10x histidine tag preceded by a BamHI and SpeI site in frame and followed by a stop codon and an EcoR1 site. Mouse melanopsin-L cDNA (Genbank accession no.: AAF24979.1) was then excised from the pCDNA3 construct [28] and ligated into pAcGP67-10xhis using the BamHI and SpeI sites. The resulting pAcGP67-Mel-10x his vector was then used to generate recombinant baculovirus in the *Spodoptera frugiperda* derived Sf9 cell line (ATCC: CRL-1711) employing the Baculogold homologous recombination system (BD Biosciences Clontech, Palo Alto, CA, USA) according to the manufacturer's instructions. This results in recombinant mel-baculovirus with the melanopsin-L cDNA under control of the strong late-phase polyhedrin promoter. The correct insertion and
sequence of the His-tagged melanopsin-L in the mel-baculovirus genome was verified by cycle sequencing of baculovirus DNA isolated from Sf9 cell nuclei as described before [44].

Sf9 cell culture and melanopsin-L expression

The *S. frugiperda* (S*f*9) cell line was used for generation and amplification of the mel-baculovirus and for the amplified production of melanopsin-L protein. Although of the insect cell lines tested S*f*9, S*f*-21 and High-Five yield similar expression levels of recombinant protein in culture flasks (monolayers), this is volumetric quite restricted and does not produce sufficient recombinant protein for purification and characterization. In suspension culture (shaker flasks), S*f*-21 performed less well than S*f*9, and High-Five grew and produced poorly, hence S*f*9 was selected for all here described experiments. The cells were grown as monolayers at 27 °C in Grace's insect medium for generation and amplification of the baculovirus. High titer stocks (>10^7 pfu) were produced by three rounds of virus amplification, and the optimal multiplicity of infection (MOI) for recombinant protein expression was determined

empirically by infection of Sf9 cells with different MOIs of high-titer virus stock. The protease inhibitor leupeptin was routinely added to the cell culture (5 μM final concentration) to suppress proteolysis of melanopsin. For production of melanopsin-L, serum-free and protein-free Insect-Xpress medium was utilized. For large-scale production (400–1000 mL culture volume), S*f*9 cells were inoculated at 0.5×10^6 cells/mL and grown in suspension culture in Erlenmeyer flasks (*Corning, USA*) using Insect-Xpress medium, with addition of penicillin and streptomycin to 5 i.u./mL and 5 μg/mL respectively, under constant rotation of 100 rpm at 27 °C. Under these conditions, the cell doubling time was typically 20–24 h. Total cell counts were performed with a haemocytometer (VWR International Ltd.) hence with an accuracy of ±10%. When the cell density had reached approximately 2×10^6 S*f*9 cells/mL, the culture was infected with mel-baculovirus at a MOI = 0.1. The cells were routinely harvested at 4 dpi.

Cell culture with biological and chemical chaperones

Production tests of melanopsin-L with biological or chemical chaperones and combinations were performed in the same Insect-Xpress medium. DMSO and glycerol were added at 1–2% v/v directly to the culture medium through a 0.2 μm filter (Whatman GmbH, Dassel, Germany). 11-*cis* and all-*trans* retinal were added under dim room light as a solution in DMF through a 0.2 μm filter to a final concentration of 6 μM. The recombinant baculoviruses for FLAG-tagged ß-arrestin-1 or -2 were either added separately or in combination to the culture medium at various MOIs (0.01, 0.05, 0.1, 0.5) together with the mel-baculovirus (MOI = 0.1). Expression of ß-arrestins was confirmed in immunoblots using anti-FLAG primary antibody.

The same approach was taken for the Gqα, Gß1 and Gγ2 subunits, where the baculoviruses for the three subunits were added together at four different MOIs (0.1, 0.3, 0.5, 1). Only Gγ2 is his-tagged and the expression of this subunit was confirmed via immunoblot with the anti-his antibody. The expression of Gqα and Gß1 was confirmed via immunoblotting with polyclonal antibodies raised against Gtαßγ (CERN9412 and CERN911, respectively). The recombinant baculoviruses for ninaA and calnexin were added to the culture medium together with the mel-baculovirus, all at a MOI = 0.1. Calnexin expression was confirmed by immunoblot using the polyclonal rabbit anti-calnexin antibody (1:500) and ninaA expression was confirmed using the rabbit anti-ninaA antibody (1:100).

Incubation of melanopsin-L with ligand

At 4 dpi the infected S*f*9 cells were collected by centrifugation (10 min, 3000*g*, 4 °C). The cell pellet was re-suspended at a density of 50×10^6 cells/ml in buffer A. The cells were subsequently homogenized with 3 strokes (5 s per stroke) of a polytron homogenizer (IKA®T10 basic, Ultra-Turrax, Staufen, Germany) at 4 °C. The cell homogenate was centrifuged (20 min, 35,000*g*, 4 °C) and the resulting membrane pellet was re-suspended in buffer B at a density equivalent to 10^8 cells/ml. All subsequent manipulations were performed in the dark or under dim red light ($\lambda > 620$ nm, RG620 cut-off filter, Schott, Menden, Germany). The ligand 11-*cis* retinal was added as a freshly prepared concentrated solution in dimethylformamide to a final concentration of 30 μM and the suspension was incubated for 1–2 h under an argon atmosphere with constant rotation at ambient temperature. The membrane suspension was then directly used for solubilization tests or purification of melanopsin-L, as described below.

Screening of detergents for solubilization of recombinant melanopsin-L

Detergents were selected either from the Solution Master Detergent Kit (Anatrace) which were pre-dissolved at a concentration of 10%, or added as stock solution in buffer B to the membrane suspension in a final concentration of 20 mM per detergent. Detergents and, if required, buffer B were added to the membrane suspension containing the ligand-treated melanopsin-L such that the final membrane density was equivalent to $10–20 \times 10^6$ cells/mL. The samples were incubated for 1 h at 4 °C with constant rotation and supernatants were collected after centrifugation (20 min, 40,000*g*, 4 °C). For SDS–PAGE analysis, 2.5 μl of the supernatant, corresponding to approximately 20,000 cells originally, were mixed with 2.5 μl of SDS–PAGE solubilisation buffer (Laemmli sample buffer, Bio- Rad, CA, USA), incubated for 15 min at ambient temperature, and applied to the gel-slot. After electrophoresis, proteins were visualized by silver staining (see below), or transferred onto nitrocellulose membrane for immunoblot analysis (see below).

Solubilization and purification of recombinant melanopsin-L

After treatment with ligand, the melanopsin-L containing membrane suspension was diluted with buffer B to a density equivalent to $10–20 \times 10^6$ cells/mL. Stock solutions of detergents in buffer B and ß mercaptoethanol were added to a final concentration of 20 mM per detergent and 5 mM, respectively. After incubation for at least 1 h at 4 °C with constant rotation under an argon atmosphere, the unsolubilized material was removed by centrifugation (20 min, 80,000*g*, 4 °C). The supernatant was applied to superflow Ni^{2+}-nitrilotriacetic acid (NTA) beads (Ni^{2+}-NTA, Qiagen, Hilden, Germany) for batch-wise purification. The beads were pre-equilibrated with buffer B. The extract from originally 6×10^8 cells was applied to 0.5 mL of settled beads. After overnight incubation under rotation at 4 °C, the beads were allowed to settle and the supernatant was carefully removed. The loaded beads were then washed sequentially with 5 bead volumes (BV) of buffer B and 0.5 BV of buffer E. Elution of melanopsin was then initiated with twice 0.5 BV of buffer F, followed by twice 0.5 BV of buffer G, and completed with twice 0.5 BV of buffer H. The fractions containing melanopsin-L, as shown by immunoblot, were stored at −80 °C. They were assayed for purity by SDS–PAGE followed by silver staining and quantified by immunoblot.

UV/visible spectroscopy

Spectra were recorded at ambient temperature from 250 to 700 nm on a Lambda-15 spectrophotometer (Perkin-Elmer, Norwalk, CT, U.S.A.) equipped with an end-on photomultiplier tube. Photoproducts were generated by illumination for 15 min through a 430 nm cut-off filter (Schott). If "dark state" spectra without interference of photoproducts were required, hydroxylamine was added to a final concentration of 50 mM before illumination.

Immunoblot analysis

Proteins were separated on a 12.5% SDS-PAGE [45] and transferred to a nitrocellulose membrane (0.2 μm iBlot™ Dry Blotting System, Invitrogen). The nitrocellulose membranes were washed with 50 ml of buffer D and then blocked by incubation for 1 h at RT with gentle shaking in 10 ml of Odyssey blocking buffer (Li-Cor Biosciences, USA) diluted 1:1 with PBS. For the primary antibody step, the membranes were incubated for 1 h at RT under gentle shaking with 10 ml blocking buffer containing 0.05% tween-20 and mouse anti-his-tag monoclonal antibody diluted 1:10,000, followed by 3 washes for 10 min with 50 ml PBS containing 0.05% tween-20 (washing buffer). In the secondary antibody step, the primary-antibody loaded membranes were soaked for 1 h under gentle shaking in 10 ml blocking buffer containing 0.05% tween-20 and goat anti-mouse (IR Dye® 800 CW) antibody diluted 1:10,000, followed by 3 washes for 10 min with 50 ml washing buffer. After drying, the blots were scanned with the Odyssey imaging system (Infrared imaging system, application software

version 3.0, Li-Cor biosciences, USA). The signal intensities were quantified using the Odyssey imaging system software.

Silver staining

Proteins were separated on a 12.5% SDS-PAGE [45] and the gel was subsequently treated with fixative (40% ethanol, 10% acetic acid and 50% deionized water) under constant shaking for 1 h at RT or overnight at 4 °C. Thereupon the gel was incubated under shaking for 20 min at RT, first three times in 30% ethanol in deionized water and finally once in distilled water. The gel was then treated for 1 min at RT with sensitizing solution (0.02% $Na_2S_2O_3$), washed three times for 20 s at RT with deionized water, and finally stained for 20 min at RT in silver nitrate reagent solution (0.2% $AgNO_3$ + 0.02% formaldehyde 37% in deionized water) under protection from light. After staining, the gel was washed again withdeionized water (three times 30 s at RT) and subsequently incubated in developer solution (3% Na_2CO_3, 0.05% formaldehyde (37%), 0.0005% $Na_2S_2O_3$ in deionized water) until the desired intensity was reached. Stop solution (0.5% glycine) was then added to stabilize the image, and the image was digitized with an imaging densitometer (Bio-Rad, Molecular Analyst™ version 1.5, CA, USA).

Preparation for LC–MS

Purified melanopsin-L prepared as described above, was subjected to enzymatic N-linked deglycosylation with PNGase F (Roche diagnostics GmbH, Germany). In brief, 4.5 pmol of purified melanopsin-L was incubated for 1.5 h at 37 °C with 2 μl of PNGase F in 0.1 ml containing 0.5% (w/v) SDS, 2 μM leupeptin. A control sample of purified melanopsin-L was treated in the same way without addition of the enzyme. Samples were prepared for SDS–PAGE as described above and 0.43 pmol (±22 ng) of melanopsin was applied to a 12.5% SDS–polyacrylamide gel. Following electrophoresis the gel was washed with milliQ (three times, 2 min) and incubated with 50 ml of Coomassie blue stain (Bio-Safe Coomassie G250 Stain, Bio-Rad, USA) for 1 h at room temperature under constant shaking. The gel was washed subsequently until the optimal signal/background ratio was reached. Samples were prepared for LC–MS analysis by cutting bands out of the gel. From an untreated sample, the upper and the middle band were kept together and the lower band was cut out separately (see results). From the enzyme-treated sample the two bands were cut out separately. The samples were analyzed at the Proteomics Facility Nijmegen by LC–MS.

Results

Construct for expression of recombinant histidine-tagged melanopsin-L

Our aim to produce melanopsin in insect cells required the construction of a baculovirus vector containing the melanopsin-L cDNA under control of a strong baculoviral promoter [46]. For this purpose, we chose the baculovirus transfer vector pAcGP67, which leads to protein expression driven by the strong late-stage polyhedrin promoter, and results in fusion with the signal peptide of the baculoviral protein GP67. GP67 is a glycoprotein of the viral envelope that is required for the uptake of the virus by the host cell via adsorptive endocytosis. Once the cell is infected, a large amount of the GP67 protein is produced and anchored to the virus peplomers, which renders it the most effective baculovirus-encoded signal sequence. This signal peptide is cleaved off in the endoplasmic reticulum (ER) during or after protein translation. Placing the GP67 signal peptide in front of the rhodopsin c-DNA indeed leads to a significant increase in expression level, while the signal sequence was properly removed (Bovee-Geurts and DeGrip, unpublished data).

For easy purification, a tag consisting of ten histidine residues was introduced at the C-terminus of melanopsin-L. There is ample evidence that the extension of the C-terminus of GPCRs with a his-tag does not interfere with their functional properties [35,47,48], and expression of C-terminally his- or eYFP tagged melanopsin in mammalian cells indeed generated the typical downstream signaling effects of melanopsin upon illumination (transient increase in intracellular Ca^{2+} level) [28].

Expression and production of decahis-tagged Melanopsin-L in the Sf9 insect cell line

Recombinant baculovirus encoding mouse melanopsin-L was used to infect *Sf*9 cells as described under Methods. In order to monitor the expression of melanopsin, membranes isolated from *Sf*9 cells expressing the recombinant his-tagged melanopsin, were analyzed by SDS–PAGE and probed with anti-his antibody (Fig. 1). Calibrated amounts of purified recombinant bovine his-tagged rhodopsin [49] were applied as an internal standard. The immunostaining clearly demonstrates that the melanopsin protein is expressed and migrates with the expected molecular weight of approximately 57 kDa. The presence of the C-terminal his-tag is confirmed by the positive reaction with the antibody, which is a strong indication that full-length melanopsin is produced. Consistently, three separate bands running closely together are observed, most likely due to different (extents of) posttranslational modification (see below). Proteomic analysis unambiguously identified all three bands as representing the full-size melanopsin-L protein (see below).

Production of melanopsin was maximal at 3–4 days post infection and this level subsequently decreased slightly. This time course is typical for genes under control of the polyhedrin promoter. The expression level ranged between 10 and 30 pmol/10^6 -cells, equivalent to 2–5 mg/L, which is in the same range as the visual pigments produced in this system without the GP67 signal peptide [44,50].

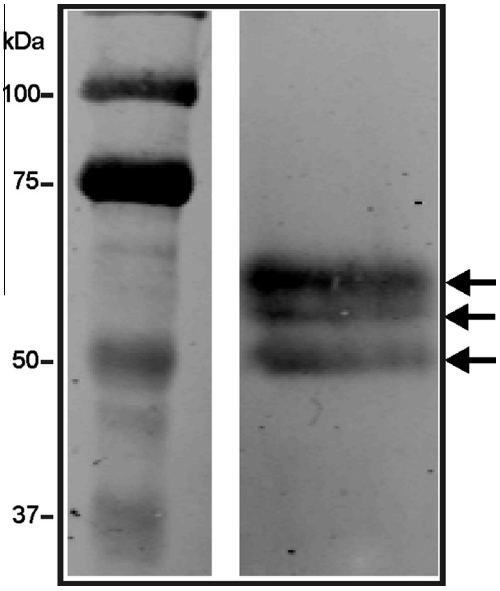

Fig. 1. Immunoblot detection of mouse melanopsin-L expression in *Sf*9 cells. Blot was screened with anti-histag antibody. Left lane presents calibration proteins (Mw in kD). Right lane represents the membrane fraction of *Sf*9 cells expressing melanopsin-L. Typically three bands are detected around 55 kD with the upper two often merging.

Effect of chaperones on melanopsin-L production

In case of vertebrate visual pigments, addition of the ligand 11-*cis* retinal to the S*f*9 membrane fraction, followed by solubilization in a mild detergent and spectral analysis, already results in a discernable absorbance peak that can be quantified by difference spectroscopy [46]. This was not the case for melanopsin; we found no obvious difference in spectral properties of membranes from melanopsin-producing S*f*9 cells or control cells upon solubilization in the mild detergents DDM or CHAPS. Also, we observed no effect of illumination (data not shown). This could be due to a low level of functionally folded protein or to a low stability of melanopsin in detergent solution, as suggested in earlier studies [31,51]. Unfortunately, we could not use the downstream assay, we applied in mammalian cells [28], since as reported before [52], this did not work properly in insect cells.

Therefore, our first objective was to investigate the effect of a variety of chemical and biological chaperones on the expression level of melanopsin-L. It has been demonstrated that the presence of such chaperones can enhance the total and functional expression level of GPCR's, although their activity is usually very selective [53,54]. Therefore, all subsequent expression trials were performed in the presence of one or more chaperones, either added to the culture upon infection of the insect cells or co-expressed from their individual baculovirus.

Fig. 2 presents an overview of the results of these studies. The expression levels in the presence of chaperones (pmole/10^6 cells) were normalized against those obtained under the standard condition included in each experiment (Sf9 cells infected with the melanopsin virus at a MOI = 0.1). The chemical chaperones used in this study were dimethylsulfoxide (DMSO) and glycerol, added at 1–2% v/v to the culture medium, and the ligands 11-*cis* and all-*trans* retinal, added to a concentration of 6 μM. Evidence has been presented that addition of DMSO to cell cultures can increase the yield of recombinant GPCRs [55], while glycerol is an established stabilizer of tertiary protein structures [56,57]. Ligands in general may promote correct protein folding [58] and in this specific case an increase in functional level was observed upon addition of 11-*cis* or all-*trans* retinal to mammalian cell lines or oocytes expressing melanopsin [12,13,28,29].

The only chemical chaperone that, as a single compound, significantly enhanced (up to 3-fold) the expression level of melanopsin-L in S*f*9 cells was DMSO (Fig. 2). In combinations this effect was always reduced. It is noteworthy that while glycerol and the retinals individually did not have a significant effect, combinations clearly promoted expression of melanopsin-L (Fig. 2).

In addition a number of proteins for which scattered evidence is available that they can support heterologous expression of GPCR's, were tested both individually and in combination with other chaperones. For instance, co-expression with the signaling partner β-arrestin-2 stabilized recombinant chicken melanopsin [59] and enhanced the light-evoked response of *Xenopus* oocytes expressing mouse melanopsin [29]. *Drosophila* nina A is a photoreceptor-specific cyclophilin required for the biogenesis of the visual pigment, rhodopsin. It is an integral membrane glycoprotein and forms a stable complex with *Drosophila* rhodopsin, acting as a chaperone-like molecule escorting rhodopsin through the secretory pathway [60,61]. In addition, it is reported to support functional expression of cone visual pigments in COS cells [62]. Finally, calnexin is an universal biological chaperone in the E.R. assisting protein folding and quality control, such that only properly folded and assembled proteins proceed further along the secretory pathway [63,64].

Co-expression with the β-arrestins had no stimulatory effect (Fig. 2). In fact, at a higher infection level (MOI = 0.5) it strongly suppressed melanopsin-L expression. Co-expression with Gq also had an inhibitory effect at a higher MOI, but a slightly stimulatory effect at the low MOI of 0.1. The combination of ninaA and 11-*cis* retinal enhanced melanopsin-L expression, as well. The largest effect in this category came from the combination of DMSO, 11-*cis* retinal and β-arrestin-2, but this combination still was not as effective as DMSO by itself.

Screening of detergents for solubilization of recombinant melanopsin-L

Solubilization from the host cell membrane using detergents is essential for purification and subsequent characterization of membrane proteins. In earlier studies, melanopsin was reported to only survive solubilization when using very mild detergents such as DDM or CHAPS [31,51]. However, these detergents performed very poorly in extracting melanopsin-L from insect cell membranes (8 ± 8% and 10 ± 10%, respectively) (Fig. 3). Therefore we investigated the efficacy of fourteen individual detergents and several combinations in the extraction of recombinant his-tagged melanopsin-L. The selection spanned the different classes (anionic, cationic, zwitter-ionic, non-ionic) and was based on their efficacy in extracting a variety of membrane proteins [35,47,65,66]. The detergent concentrations used for extraction were 20 mM for individual detergents, adding up to 40 mM for a combination of two detergents. Crude membrane fractions from melanopsin-L expressing S*f*9 cells were incubated for at least 1 h at 4 °C with the selected detergent (combination).

Unsolubilized material was precipitated by centrifugation and the amount of melanopsin in the supernatant was quantified from the intensity of the corresponding bands in Western blots of the extraction mixture before and after centrifugation. To ensure maximal possible extraction at the moderate detergent concentration of 20 mM, the density of the membrane suspension was kept relatively low (equivalent to 10–20 × 10^6 cells/ml). Of the individual detergents, only DPC displayed a good solubilization capacity (50 ± 20%, n = 12; Fig. 3). Although phosphocholines have been reported to be effective in solubilizing several types of GPCR's [65,67], including vertebrate visual pigments, solubilization and purification using DPC did not yield a light-sensitive absorbance band in the 450–550 nm region in the purified fraction. This need not be surprising, since invertebrate visual pigments are notoriously unstable in most detergent solutions, except very mild ones like digitonin and DDM [51,68]. Unfortunately, as mentioned before, mild detergents such as DDM and CHAPS were very ineffective in solubilizing melanopsin-L. Therefore, we tested several detergent combinations. The combination of DDM with the more aggressive detergents DPC or DHPC gave reasonable extraction yields in the range 20–30% (Fig. 3). A new mild detergent, ASB-14-4, shown to be efficient in solubilization of complex membrane proteins [66], performed second best as a single detergent with an extraction efficiency of more than 20%.

Hence, we selected DPC, ASB-14-4 and the combination of DDM with DPC or DHPC to test solubilization and purification of melanopsin-L from insect cell membranes.

Purification of recombinant melanopsin-L

Several conditions yielding a good production of melanopsin-L in small-scale culture were amplified to a larger scale, usually in units of 400–500 ml culture volume. In all cases crude membrane fractions isolated from S*f*9 cells, harvested 4 days post-infection, were incubated with 11-*cis* retinal at a final concentration of 30 μM. To gain on time-consuming centrifugation and column loading, we attempted to use a 5–10-fold more concentrated membrane suspension (equivalent to 10^8 cells/ml) for extraction of melanopsin. This resulted however in very low yields of solubilized protein, even at higher detergent concentrations, and we further routinely used less concentrated suspensions, similar to the detergent screens. For purification by immobilized metal-affinity chromatography (IMAC), melanopsin was extracted using the detergent selection described in the previous section, and loaded

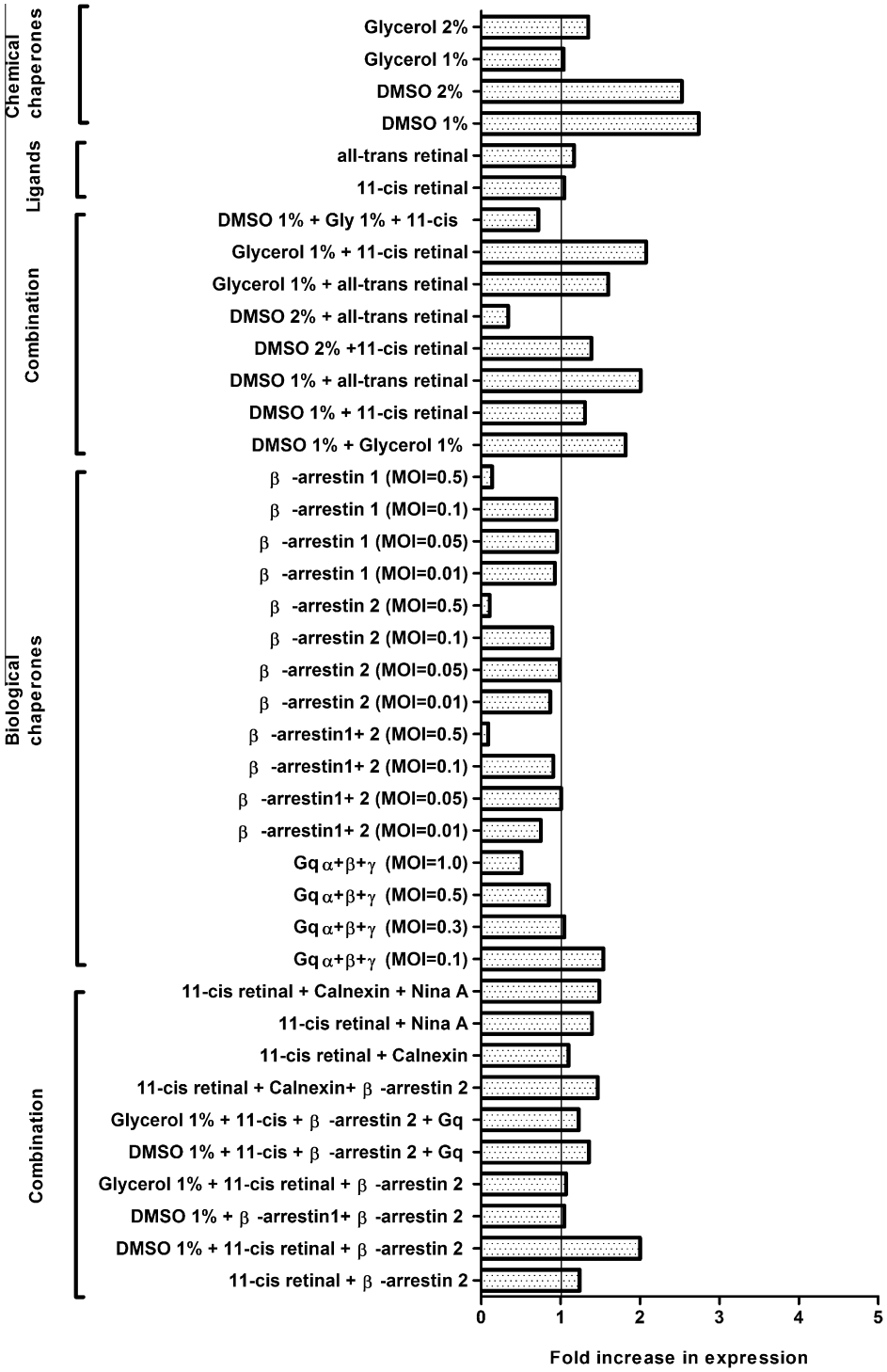

Fig. 2. Normalized expression level of mouse melanopsin-L in Sf9 cells in the presence of chemical or biological chaperones and various combinations. Cells were grown in Insect-Xpress medium. Chemical chaperones were added upon infection, biological chaperones were co-expressed with melanopsin. The expression level (pmole/10^6 cells) was quantified on immunoblots using his-tagged rhodopsin for calibration and normalized against standard conditions. Standard conditions contain melanopsin virus at a MOI = 0.1 without chaperones and are represented by the vertical line at $x = 1$. The number of experiments per condition varies between 1 and 4 with an average range in the levels ±30%.

onto superflow Ni^{2+}-NTA agarose columns. Imidazole was used to elute bound melanopsin, since this proved to be more effective in eluting deca-histagged membrane proteins [48]. Typical results are presented in Fig. 4 and quantitative data are summarized in Table 1. The melanopsin-L bands on the blots were quantified using a calibration curve of purified his-tagged rhodopsin, run on the same gel. As is evident from Table 1, on the average, approximately 50% of the extracted melanopsin becomes bound to the affinity column (Table 1 and Fig. 4). Additional washing steps with only detergent elute a complex protein population (Fig. 4B, lane 4), probably representing non-specifically or very weakly bound proteins. Most of the low affinity contamination could be removed by washing with 20 mM imidazole (Fig. 4B; lane 5), The major part of the bound melanopsin was eluted upon raising the imidazole concentration

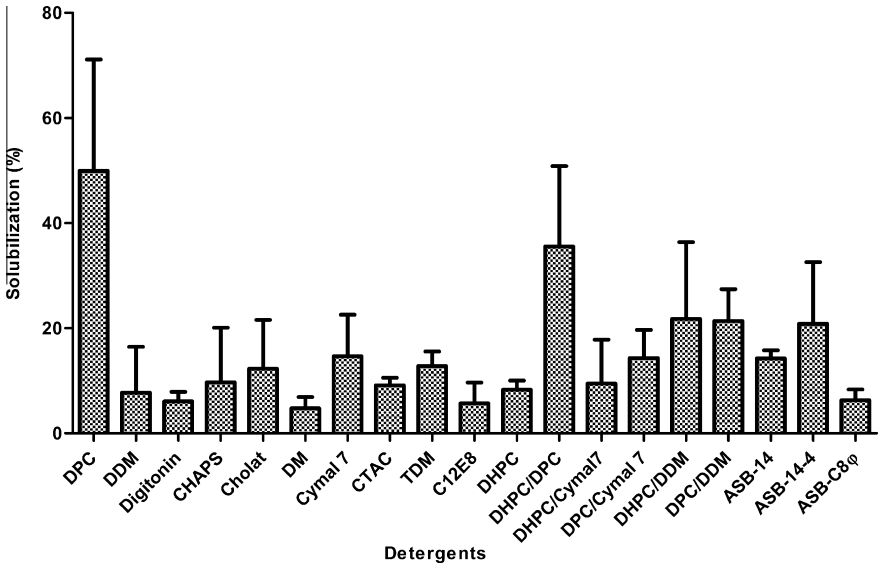

Fig. 3. Solubilization potential of detergents versus recombinant mouse melanopsin-L produced in insect cells. The extraction capacity is given as the percentage of melanopsin-L in the cellular membrane fraction that is detected in the supernatant after centrifugation of the extraction mixture. The amounts are quantified on Western blot as detailed in the Methods section. Bars represent the average with S.D. of 3–12 experiments.

from 20 to 200 mM (Fig. 4A, lanes 5 and 6). A small amount of melanopsin remained bound to the column and could be eluted by raising the imidazole concentration to 400 mM (Fig. 4A, lane 7). Based upon silver staining, melanopsin-L accounted for at least 70% of the protein in the purified fractions, indicating a purification factor of several hundred-fold. Upon purification the same triad band pattern was maintained that was observed in the membrane fraction (Fig. 1): a major band, positioned at 57 ± 5 kD, representing two closely migrating species, and a minor band at 50 ± 5 kD (Fig. 4B, lanes 6 and 7; Fig. 5, lane 3). Table 1 shows the destination of melanopsin-L through the purification steps. On average, the final recovery in the purified fractions accounted for 2.5% of the amount of melanopsin-L in the starting material. The yield of purified melanopsin-L protein averaged about 0.13 mg/L culture volume (Table 1).

When the membrane fraction was stored at −80 °C prior to purification, degradation of melanopsin-L was observed, resulting in a further loss of recovery. Therefore, purification was always performed with freshly prepared membranes from freshly harvested melanopsin-L producing insect cells.

Posttranslational modification of recombinant melanopsin-L

To verify that the three protein bands around 55 kD obtained after purification indeed represent full-length mouse melanopsin-L, an LC–MS analysis was performed on the combined upper bands that were difficult to isolate separately, and the lower band. Several dominantly present peptides were obtained in both samples that all derived from the melanopsin-L sequence (Fig. 6). Since this includes a near N-terminal peptide (Val^{9}-Arg^{38}), and since all three bands are detected by the anti-histag antibody, i.e. contain the C-terminal his-tag, we conclude that all three bands contain the full-length melanopsin-L protein. Therefore, the different migration of the three bands is probably due to secondary factors, such as posttranslational modification. We first investigated potential N-glycosylation, since mouse melanopsin-L contains two sequences (Asn^{30}-Gly-Thr and Asn^{34}-Ser-Val) within its extracellular amino terminal domain, and one (Asn^{87}-Leu-Thr) in its putative first membrane helix that obey the consensus sequence for *N*-linked glycosylation. It was demonstrated before that rat melanopsin could be glycosylated at the equivalent N-terminal positions Asn-31 and Asn-35 [69]. To determine whether mouse melanopsin-L is glycosylated, purified melanopsin was incubated with N-glycosidase F, which specifically cleaves N-glycan chains from glycoproteins. Upon incubation with N-glycosidase F, the upper band of the melanopsin triad disappears and the middle band increases in intensity. The lowest band was unaffected with respect to relative position and intensity (Fig. 5). We conclude that the upper band is N-glycosylated and upon deglycosylation comigrates with the middle band. The middle and lower band are not N-glycosylated.

The main band obtained after deglycosylation was also examined by LC–MS, since upon N-glycosidase F treatment the glycosylated Asn residue is deamidated, which can be detected by MS. This LC–MS analysis indicated that Asn30 and Asn34 in peptide Val^{9}–Arg^{38} both had become subject to deamidation. The very long amphipatic peptide containing Asn^{87} (Ala^{39}–Arg^{96}) was not detected, but Asn^{87} most likely resides in a transmembrane helix [1] and will not be glycosylated. At present, it is not clear why the middle and lower band migrate with a different apparent molecular weight. It has been reported that melanopsin can be phosphorylated at several sites [70,71], but so far we have found no evidence from our LC–MS analyses that this also occurs in the insect cell line. Also, these analyses did not find evidence for thiopalmitoylation (data not shown), which was confirmed by prolonged incubation with 1 M hydroxylamine, that would release palmitoyl chains [72], but did not change the triad pattern.

Spectral analysis of purified melanopsin-L

While following IMAC purification of vertebrate visual pigments, the obvious absorbance bands were reproducibly obtained [36,49], the purified melanopsin-L fractions did not present a consistent pattern. As expected from the SDS–PAGE analyses, a clear protein band at 280 nm was always observed, but often an absorbance band in the 450–550 nm region, was not discernable. Difference spectroscopy (subtracting the spectrum taken after illumination from the one before) was not of much help, since changes in scattering perturbed the baseline such that any other small differences were usually completely overruled. In case an absorbance band in the region mentioned was detected, it was always of low intensity, which rendered it difficult to estimate the absorbance maximum, but this was

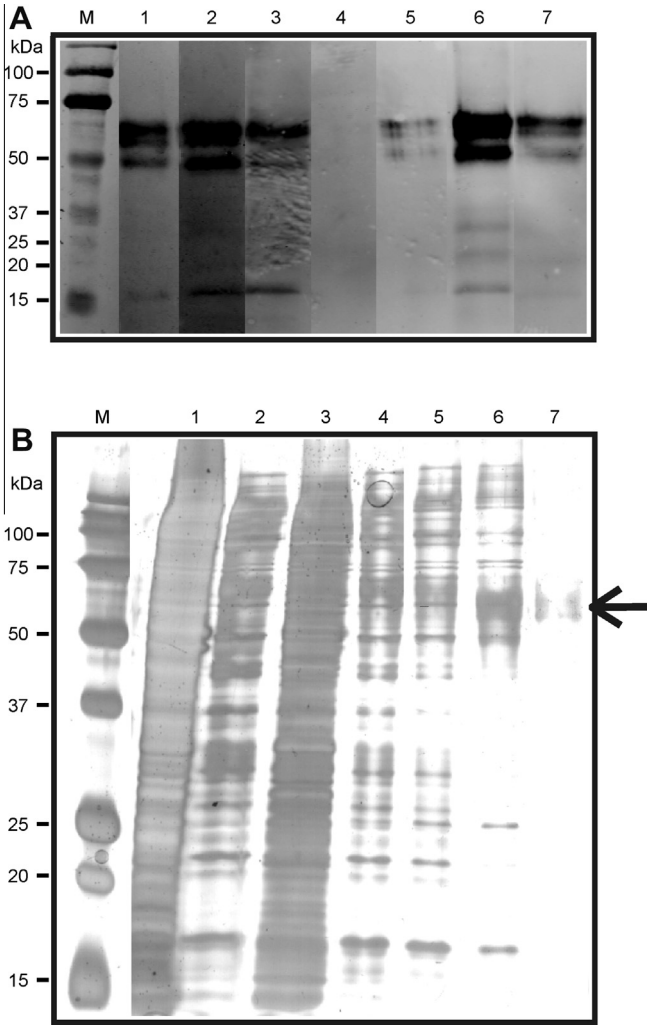

Fig. 4. Typical purification profile of mouse melanopsin-L using IMAC. Fractions were analyzed by SDS–PAGE, followed by immunoblotting (A) or silver-staining (B). The immunoblot was screened with anti-histag antibody. Lane 1 represents the Sf9-cell membrane fraction. Lane 2 represents the solubilized extract loaded onto the Ni^{2+}-NTA-agarose column. The nonbound fraction is shown in lane 3. Lane 4 and 5 represent the fractions obtained upon washing with only detergent and detergent with 20 mM imidazole, respectively. The strongly bound fractions, eluted from the column using 200 mM and 400 mM imidazole, respectively, are shown in lane 6 and 7. Arrow shows position of melanopsin-L in panel B.

Table 1
Yield of mouse melanopsin-L from each step upon IMAC purification as percentage of the starting amount of melanopsin-L. Quantification was done on immunoblots using the Odyssey fluorodetection and analysis software. Values given are mean ± S.D. of five experiments. The whole cell lysate is taken as 100%.

Step	Melanopsin-L %	% Of total membrane protein
Whole cell lysate	≡100	<0.5
Detergent extract	14.3 ± 6.7	<0.5
Non-bound fraction	7.3 ± 5.1	
Wash fraction	1.4 ± 2.6	
Purified fraction	2.5 ± 1.9	⩾70

confined to the 460–500 nm range. An example is presented in Fig. 7. Difference spectra were of very poor quality, as explained above. The best one is shown in the insert of Fig. 7 and suggests that upon illumination the absorbance band red-shifts, peaking between 540 and 570 nm. While a variety of culture conditions, including the use of promising chaperones, were used for production and various

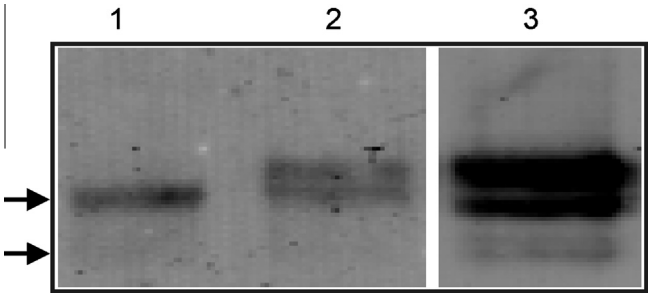

Fig. 5. Effect of N-glycosidase F treatment on the band pattern of purified melanopsin-L. Lane 1 shows the pattern after incubation with PNGase F and lane 2 is the control, incubated without the enzyme. Lane 3 represents the original triad pattern. Upon enzyme treatment the upper band disappears and comigrates with the middle band. Arrows point at the position of the middle and lower band in the melanopsin-L triad.

mdspsgpr**VL** **SSLTQDPSFT** **TSPALQGIWN** **GTQNVSVR**aq
llsvspttsa hqaaawvpfp tvdvpdhahy tlgtvillvg
ltgmlgnltv iytfcrnrgl r**TPANMFIIN** **LAVSDFLMSV**
TQAPVFFASS **LYK**kwlfget gcefyafcga vfgitsmitl
taiamdr**YLV** **ITRPLATIGR** gskrrtalvl lgvwlyalaw
slppffgwsa yvpeglltsc swdymtftpq vraytmllfc
fvfflpllii ifcyififra iretgr**ACEG** **CGESPLR**qr**R**
QWQRLQSEWK makvalivil lfvlswapys tvalvafagy
shiltpymss vpaviak**ASA** **IHNPIIYAIT** **HPK**yr**VAIAQ**
HLPCLGVLLG **VSGQRSHPSL** **SYR**sthr**STL** **SSQSSDLSWI**
SGRkrqeslg sesevgwtdt ettaawgaaq qasgqsfcsq
nledgelk**AS** **SSPQVQR**skt pk**VPGPSTCR** **PMK**gqgarps
slrgdqkgrl avctglsecp hphtsqfpla fleddvtlrh
lhhhhhhhhh h

Fig. 6. Amino acid sequence of deca-histagged mouse melanopsin-L (*Mus musculus*). The peptides identified in our LC–MS analyses are shown in bold and upper case. Potential N-glycosylation sites are shown in italics and are highlighted in gray.

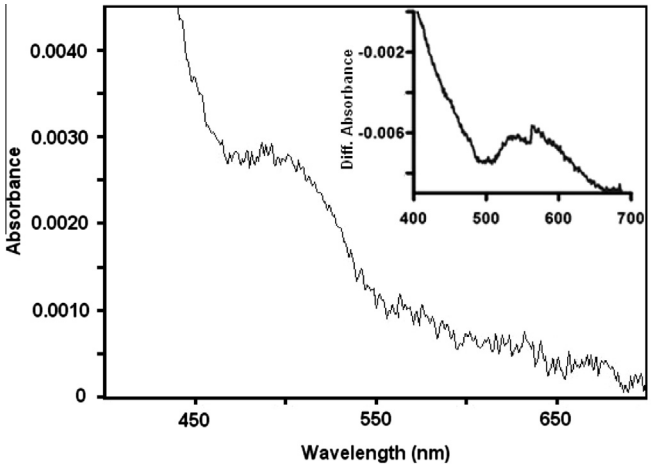

Fig. 7. UV–visible spectrum of a purified melanopsin-L fraction, showing an absorbance band with a maximum in the 480–500 nm range. The difference spectrum in the insert was obtained by subtracting the spectrum recorded after 10 min of illumination with blue light (432 nm) from the "dark" spectrum. Our interpretation is that upon illumination the absorbance band red-shifts to a photoproduct with a maximal absorbance between 540 and 570 nm.

detergents or combinations were used for purification of melanopsin-L, we did not find a correlation with observing a detectable absorbance band in the visible region.

Discussion

Melanopsin is a not until 1998 identified photoreceptor protein, belonging to the rhodopsin subfamily of the GPCRs [1]. Melanopsin provides photosensitivity to a minor subclass of ganglion cells in the mammalian retina that are committed to non-visual photo-regulated tasks [10]. This vertebrate "non-visual" pigment is already noteworthy in that it is most homologous to invertebrate visual pigments [1,16]. In spite of the broad interest in melanopsin, relatively little is known about its structure and its functional characteristics such as its spectral properties, photochemical trajectory, mechanism of photo-activation and signaling partners. Biophysical and structural studies of membrane proteins require milligram quantities of protein. To obtain such quantities of sufficiently pure and functional membrane protein still is a major bottleneck. Here we report a successful application of the baculovirus expression system to produce and purify up to mg quantities of melanopsin-L protein.

Expression of melanopsin-L in the baculovirus-insect cell system

Because of the low abundance of melanopsin in the mammalian retina, overexpression of recombinant melanopsin offers the only way to purify and investigate the protein in more detail. So far, functional expression and purification of melanopsin has been achieved only in a C-terminally truncated form at low yield in HEK293 cells [30,31]. This should provide at least some information on its spectral sensitivity, but unfortunately these studies targeted different species (Amphioxus and mouse, respectively) and report a different λ_{max} (485 and 467 nm, respectively).

The baculovirus-insect cell expression system has proven to be quite effective in the expression of relatively large amounts of a variety of GPCRs with properties conforming to those of the native proteins, including visual pigments (e.g. [73]). Hence, we selected this system and here we present the first baculovirus-mediated expression and purification of full-length mouse melanopsin-L. Using the GP67 signal peptide, which strongly enhanced the expression level of rhodopsin to about 30 mg/L, we could boost the production level of melanopsin-L to 2–5 mg/L. In HEK293 cells the expression level of melanopsin also was about ten-fold lower than that of rhodopsin [31]. In addition, we applied C-terminal histidine tagging, since this allows, in combination with immobilized metal affinity chromatography, rapid and relatively inexpensive large-scale purification of recombinant visual pigments without significant functional side-effects and allows simple identification with anti-histag antibodies [47]. A deca-histag was selected for mouse melanopsin-L, since this affords stronger binding to the affinity matrix and more easy disposal of contaminating proteins [48]. The expressed melanopsin-L was indeed detectable by immunoblot using a histag specific antibody (Fig. 1). This identification, together with the apparent Mw (ca 55 kD), is a strong indication that the full-length protein is expressed, for which a Mw of about 57 kD is expected. However, the immunoblot in fact reveals three anti-histag positive bands with two major ones at 57 ± 5 kD running closely together and a third minor one at 50 ± 5 kD (Fig. 5). Hence, we resorted to analyze these bands by limited proteolysis followed by LC–MS. In this way it became obvious that the major peptides identified in these bands indeed derive from melanopsin-L (Fig. 6) and include a near-N-terminal sequence (Val^{8}–Arg^{38}). This leads us to conclude that all three bands represent full-length melanopsin-L. Their different migration behavior can be partly explained by posttransitional modification (see below).

Evaluation of chaperones

Expression of melanopsin-L under standard conditions, followed by incubation with the ligand, 11-*cis* retinal, did not produce a detectable absorbance band in the 500 nm range. This might originate in destabilization upon solubilization with detergent or in a high extent of improper folding upon and following translation. As we used DDM for solubilization, a fairly stable condition for melanopsin according to earlier reports [30,31], we first investigated the effect of a variety of biological and chemical chaperones, that were reported to enhance functional and/or total expression of recombinant membrane proteins in several expression systems. Most chaperones, single or in combination, had little effect on the expression level of melanopsin-L, sometimes even reducing it (Fig. 2). The major exception was DMSO that by itself enhanced expression levels up to threefold. The impact of DMSO on cellular structures and metabolism is complex [74], and the mechanism involved in enhancing recombinant protein production is not very well understood [75,76]. A study in *S. cerevisiae* [77] showed that DMSO treatment leads to membrane proliferation and affects membrane integrity in yeast. Thus, DMSO may enhance the membrane pool for membrane proteins and/or increase membrane dynamics [78] and/or influence genetic factors that control the rate of membrane protein translation and folding. The second best condition in stimulating melanopsin-L expression was the combination of glycerol and 11-*cis* retinal (Fig. 2), two chemical chaperones that individually had no significant effect. Glycerol is a recognized stabilizer of protein tertiary structure and possibly folding intermediates [56,58], while the presence of ligand can increase the production of intact recombinant protein, as not only reported for melanopsin [12,13,28,29,31] but for GPCRs in general [75]. Why only the combination of these chaperones had a stimulatory effect is presently unexplained, in particular since it still did not result in a detectable absorbance band around 500 nm after solubilization of cellular membranes with DDM. The combination of ß-arrestin and 11-*cis* retinal with DMSO also showed a significant stimulatory effect, but this was presumably mainly the influence of DMSO.

The negative effect of the ß-arrestins at higher expression level, upon melanopsin-L expression (Fig. 2) is surprising, considering earlier reports that co-expression of mouse as well as chicken melanopsins with ß-arrestins in *Xenopus* oocytes leads to large and consistent photocurrents [29,59]. ß-arrestins can also target GPCRs for lysosomal or proteasomal degradation [78,79] and possibly this is the preferred pathway for recombinant mammalian melanopsin in insect cells, in particular when its folding is slow or incomplete.

Taken this all together, the presence of any chaperone did still not result in a detectable photosensitive absorbance band around 500 nm after solubilization in DDM. Hence, our next effort was to screen detergents for optimal solubilization and purification of recombinant melanopsin-L.

Screening of detergents for solubilization of recombinant melanopsin-L

We set out to test the efficacy of a number of detergents or detergent mixtures in solubilizing melanopsin-L. Detergents were selected on the basis of our experience with visual pigments (e.g. [80]) and literature data on GPCRs (e.g. [73,81]). DPC turned out to be most effective in solubilizing melanopsin-L. This detergent indeed is reported to efficiently solubilize hydrophobic and amphipathic α-helices [82,83] and has been used in quite a few GPCR studies [65,67,81]. However, following solubilization and

purification of melanopsin-L in DPC, we did not detect any light-sensitive absorbance band in the 450–550 nm region. This suggests that DPC destabilizes melanopsin-L upon solubilization, which would agree with the reported poor detergent stability of Amphioxus melanopsin [30] and invertebrate visual pigments in general [68]. Consequently, we resorted to the second best options, solubilizing 20–30% of the expressed melanopsin-L using mild detergents such as ASB-14–4 and DDM in combination with DPC and DHPC.

We also tested some reportedly stabilizing additives such as NaCl (0.5 M), arginine (50 mM), glutamine (50 mM) and cholesterol hemisuccinate (CHS) (0.2% w/v), but neither of these did specifically result in higher yields of solubilized melanopsin-L (data not shown).

A striking observation was the poor extraction of melanopsin-L with DDM and CHAPS, detergents that were successfully employed in solubilizing C-terminally truncated melanopsin [30,31]. One possible explanation could be that the large C-terminal tail of melanopsin-L strongly interacts with other components or perturbs the micellar structure. However, we consider it more likely that most of the melanopsin may be present as the apoprotein, due to intrinsic poor uptake of free ligand and/or to suboptimal folding. The retinal apoproteins (“opsins”) are notoriously less stable in detergent micelles than the holoproteins (e.g. [81]), and have a tendency to aggregate in mild detergents. For instance, upon solubilization of visual rhodopsin, produced with the baculovirus-insect cell system, by means of DDM or NG most of the apoprotein stays behind in the pellet (Bovee-Geurts and DeGrip, unpublished).

Purification of melanopsin-L

Being able to produce mg quantities of full-length melanopsin-L, the next step is to achieve sufficient purification. In view of melanopsin-L taking up <0.5% (w/w) of the membrane protein population in the insect cells and of the instability of melanopsin in a micellar environment, this requires a rapid high-affinity procedure that can easily be scaled up. IMAC in combination with a histag satisfies these conditions (e.g. [49]). This approach worked satisfactorily and yielded comparable results, irrespective of the culture conditions (with or without chaperones) and the detergent (mixture) used for solubilisation. During purification the same triad band pattern was conserved that was observed in the original membrane fraction, consisting of two closely migrating major bands at 57 ± 5 kD, and a minor band at 50 ± 5 kD (see above). Based upon protein staining, these three bands together account for $\geqslant$70% of the total protein in the purified fraction, which entails a satisfactory purification factor of several hundred-fold. The recovery is problematic, since the 20% of the extracted melanopsin-L that is recovered in the purified fraction, accounts for only 2.5% of the original content in the cellular membrane fraction (Table 1). Still, this opens the way to production in the baculovirus/insect cell system of adequate quantities of purified melanopsin for functional studies, provided the purified protein would be fully functional.

However, the second enigma we encountered was the low level of functional protein, as represented by an absorbance band around 500 nm in the purified fraction. Based on a molar absorbance similar to that of squid rhodopsin (35,000 M^{-1} cm^{-1} [84]), the percentage of spectrally intact melanopsin varied in the purified fraction from not detectable to approximately 10%.

Presumably, both symptoms, low recovery and low level of functional protein, originate from poor uptake of the ligand 11-cis retinal, resulting in a high level of apoprotein that is much less stable in detergent micelles. The poor uptake of free ligand may be due to incomplete folding and/or to an intrinsic property of an invertebrate-like opsin [68,85]. If incomplete folding would be the major factor, we would expect that this at least partly could be remedied by employing chaperones. For instance, the presence of ligand (9-*cis*, 11-*cis* or all-*trans* retinal) has been reported to enhance the photo-activity of melanopsin expressed in mammalian cell lines [12,13,28,29,31], as does co-expression with ß-arrestins in *Xenopus* oocytes [29,59]. Furthermore, folding and targeting of visual pigments can be enhanced upon co-expression with the chaperones ninaA or calnexin [60,62,86]. However, neither of these or any other of the chaperones we tested did significantly and consistently increase the level of holo-melanopsin-L. It might be an option to search for more specific chaperones, e.g. possibly operating in the ipRGCs. On the other hand, 50–70% of the expressed melanopsin-L is N-glycosylated, which in the baculovirus-insect cell system usually is equivalent to correct folding [87,88]. Hence, we postulate that the major problem lies in poor uptake of free ligand. In fact, in the cephalopod photoreceptor cell 11-*cis* retinal is provided to the visual opsin by a relay system consisting of retinochrome, a photo-isomerase producing 11-*cis* retinal from all-*trans* retinal, and a specialized retinal-binding protein (RALBP) that takes up 11-*cis* retinal from retinochrome and shuttles it to opsin [89,90]. Since multi-protein co-expression is easily established in the baculovirus expression system [91], this seems the best option in further trials to improve large-scale functional expression of melanopsin-L. The spectral properties of melanopsin still are a matter of debate [10]. Although our data are not very specific, they are consistent with a maximal absorbance around 480 nm and a significant red-shift upon photoconversion to metamelanopsin.

Posttranslational modification

Proteins can be subject to a variety of posttranslational modifications (disulfide bridge formation, methylation, acetylation, targeted proteolysis, N- and *O*-glycosylation, thiopalmitoylation, phosphorylation, ubiquitination, sumoylation a.o.), that are involved in a variety of processes like quality-control in the endoplasmic reticulum, targeting, complex formation, signal transduction and modulation, stabilization and degradation. Insect cell lines can also execute such modifications, except that N-glycosylation mostly involves simpler oligomannose chains [92–94]. Visual pigments are N-glycosylated in their extracellular N-terminus, which is not a functionally relevant modification, but probably a stabilizing factor [95–97]. A similar role was recently inferred for two glycosylation sites in rat melanopsin[69]. Mouse melanopsin-L contains two corresponding glycosylation consensus sequences within its extracellular N-terminal domain (Asn^{30}–Gly–Thr and Asn^{34}–Ser–Val). Via treatment of purified melanopsin-L with N-glycosidase F, in combination with LC–MS analysis, we could demonstrate that the upper band of the melanopsin triad is glycosylated at both sites and that the two lower bands are not glycosylated. This explains the difference in apparent Mw between the upper and the middle band.

Visual pigments are usually also thiopalmitoylated, which anchors a short C-terminal helical segment to the membrane [98,99]. This probably has a stabilizing function as well [100]. In fact, rhodopsin produced in the baculovirus system is thiopalmitoylated [87]. Melanopsin-L contains several cysteine residues in its large C-terminal domain, of which Cys-364 probably corresponds to the thiopalmitoylated residues in visual pigments. The peptide containing this residue (Val^{356}–Arg^{375}) was identified in our LC–MS analysis (Fig. 6), but no evidence was found for palmitoylation in any of the triad bands. Furthermore, mouse melanopsin was reported to be phosphorylated in its C-terminal by a GR-kinase and on Thr-186 and Ser-287 by protein kinase A [70,71]. Our LC–MS-identified peptides covered some serine and threonine residues in the C-terminal, as well as Ser-287 (Fig. 6), but also here we did not find evidence for modification. Since phosphorylation

probably is triggered upon photo-activation, it is possible that we would not detect this modification because of the low level of holo-melanopsin. On the other hand, Thr-186 and most of the C-terminally located serine and threonine residues were not covered by the peptides we identified. Finally, we did not yet search for *O*-glycosylation, but it was reported that octopus rhodopsin is *O*-glycosylated at two N-terminal sites [101]. Further analysis combined with a higher coverage of the melanopsin-L sequence with LC–MS introduced peptides, using the approach reported elsewhere [102], may detect further modifications explaining the different migration of the middle band of the triad and the lower band.

In conclusion, we successfully expressed his-tagged full length mouse melanopsin-L using the baculovirus expression system. Immunoblot analysis revealed a triad band pattern, consisting of two closely migrating major bands at 57 ± 5 kD, and a minor band at 50 ± 5 kD. All bands represent full-length melanopsin-L. The upper band accounts for 50–70% of the triad protein content and is *N*-glycosylated at Asn-30 and Asn-34. The expression level of melanopsin-L ranged between 2–5 mg/L culture volume. Out of a variety of chemical and biological chaperones tested, only DMSO significantly enhanced the total expression level, whereas β-arrestins had a surprisingly strong inhibitory effect. None of the tested conditions resulted in a consistently detectable photosensitive absorbance band around 500 nm after solubilization in the mild detergent DDM. Better solubilization was achieved with DPC, ASB-14-4 or combinations of DDM with DPC or DHPC. Purification of the recombinant melanopsin-L using IMAC yielded fractions containing at least 70% melanopsin-L on a protein basis. However, the recovery of melanopsin is quite low after purification (2.5 ± 1.9%) resulting in a yield of purified protein of about 0.13 mg/L. The yield of spectrally intact protein is even lower, maximally 10% of the purified protein, which we currently impute to a combination of incomplete folding and poor uptake of free ligand.

To our knowledge this is the first report on production and purification of full-length mouse melanopsin-L at a scale that can easily be further put up. The next step should investigate stabilization of the protein either by co-expression with a specialized chaperone and/or with ligand-bearing RALBP. Large-scale production of functional melanopsin-L then will trail-blaze the much-awaited unraveling of its structural and functional properties.

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