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New insights into the mechanism of light modulated signaling by heterotrimeric G-proteins: ENVOY acts on *gna1* and *gna3* and adjusts cAMP levels in *Trichoderma reesei* (*Hypocrea jecorina*)

Doris Tisch, Christian P. Kubicek, Monika Schmoll *

Research Area of Gene Technology and Applied Biochemistry, Institute for Chemical Engineering, Vienna University of Technology, Gumpendorfer Strasse 1a/1665, A-1060 Wien, Austria

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ABSTRACT

Sensing of environmental signals is often mediated by G-protein coupled receptors and their cognate heterotrimeric G-proteins. In *Trichoderma reesei* (*Hypocrea jecorina*) the signals transmitted via the G-protein alpha subunits GNA1 and GNA3 cause considerable modulation of cellulase transcript levels and the extent of this adjustment is dependent on the light status. We therefore intended to elucidate the underlying mechanism connecting light response and heterotrimeric G-protein signaling.

Analysis of double mutant strains showed that constitutive activation of GNA1 or GNA3 in the absence of the PAS/LOV domain protein ENVOY (ENV1) leads to the phenotype of constitutive G-alpha activation in darkness. In light, however the deletion-phenotype of Δ*env1* was observed with respect to growth, conidiation and cellulase gene transcription. Additionally deletion of *env1* causes decreased intracellular cAMP accumulation, even upon constitutive activation of GNA1 or GNA3. While supplementation of cAMP caused an even more severe growth phenotype of all strains lacking *env1* in light, addition of the phosphodiesterase inhibitor caffeine rescued the growth phenotype of these strains.

ENV1 is consequently suggested to connect the light response pathway with nutrient signaling by the heterotrimeric G-protein cascade by adjusting transcript levels of *gna1* and *gna3* and action on cAMP levels – presumably through inhibition of a phosphodiesterase.

1. Introduction

The adaptation of fungi to their environment is crucial for successful competition in their natural habitat. In this process, they respond to the availability of nutrients, light or the presence of a potential mating partner or competitor. Such key signals are received and transmitted via a complex machinery of signal transduction pathways, one of the most important among them being heterotrimeric G-protein signaling (Lengeler et al., 2000). G-protein signaling is widespread in mammalians as well as in fungi (Lafon et al., 2006; Li et al., 2007; Offermanns, 2003). In fungi, this signaling cascade is involved in many important processes, such as reproduction, virulence, stress responses and development (Hsueh et al., 2007; Ivey et al., 2002; Kays and Borkovich, 2004; Segers and Nuss, 2003; Yang et al., 2002). The main route of G-protein signaling is well studied (for review see Hamm, 1998; Li et al., 2007). G-proteins are composed of three subunits: alpha, beta and gamma. When the G-proteins are activated, a conformational change leads to the exchange of the G-alpha bound GDP to GTP. The associated beta and gamma subunits dissociate as a dimer. The free alpha subunit, as well as the betagamma-dimer are now able to bind and influence downstream factors. The genome of *Trichoderma reesei* comprises three G-alpha subunits (GNA1, GNA2 and GNA3), one beta and one gamma subunit (Schmoll, 2008). The number of different G-alpha subunits allows for many possibilities of combinations and hence response to various signals (Dohlman and Thorner, 1997). Interestingly, the transmitted signals do not necessarily lead to isolated or independent outputs: for example in *Cryptococcus neoformans* two G-alpha subunits were shown to act in opposite directions with respect to initiation of mating (Hsueh et al., 2007). The most fascinating element in this pathway is the fine-tuning mechanism, which allows the fungi to react immediately and appropriately to changes in the environment. This effect can be established by different proteins, which interact with G-proteins, such as the RGS domain proteins (Regulators of G-protein Signaling; Dohlman and Thorner, 1997) or other factors influencing abundance and/or activity of G-proteins (Li et al., 2007). Many of the factors which are involved in the regulation of G-protein signaling remain unknown.

* Corresponding author. Fax: +43 1 58801 17299.
E-mail addresses: doris.tisch@tuwien.ac.at (D. Tisch), ckubicek@mail.tuwien.ac.at (C.P. Kubicek), monika.schmoll@tuwien.ac.at (M. Schmoll).

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Recently it was shown that in the biotechnological workhorse *T. reesei* (*Hypocrea jecorina*), the heterotrimeric G-protein pathway impacts light dependent transmission of the cellulose signal and hence regulation of cellulase gene expression (Schmoll et al., 2009, 2010; Seibel et al., 2009). Since the rise in energy costs and the increased awareness of the imminent climate change at least in part caused by utilization of fossil fuels, development of more sustainable fuels, i.e. the so-called biofuels has become a focus of research. With respect to second generation biofuels derived from agricultural waste products (Rubin, 2008; Sticklen, 2008), *T. reesei* plays an important role as one of the most prolific producers of a highly efficient cellulase mixture (Schuster and Schmoll, 2010). In spite of its great potential to degrade plant material (cellulose and hemicellulose), the genome only comprises seven genes for cellulases (Martinez et al., 2008). Nevertheless, this fungus is known as a successful colonizer in its natural habitat, which may also be due to optimized use of resources by fine-tuning of the physiological response to the presence of nutrients in the environment. In this respect, the finding that ENVOY impacts the production of cellulases in a light dependent way (Schmoll et al., 2005) warrants further investigation in order to elucidate the underlying mechanism.

The modulation of cellulase gene transcription in *T. reesei* by light was found after screening for novel signaling factors involved in regulation of this process, which surprisingly identified a signaling factor (later named ENVOY for messenger) related to the light signaling machinery (Schmoll et al., 2004, 2005). ENVOY is a PAS/LOV domain protein, related to the *Neurospora crassa* photoreceptor VIVID (Brunner and Kaldi, 2008; Heintzen et al., 2001), but not conserved in all ascomycetes and thus far are absent outside of the ascomycete lineage (Schmoll et al., 2005, 2010; Idnurm et al., 2010; Rodriguez-Romero et al., 2010). PAS (PER, ARNT, SIM) domains are sensory domains and able to convey a protein/protein interaction (Taylor and Zhulin, 1999). "LOV" stands for sensing of light, oxygen and voltage (Cheng et al., 2003; Crosson et al., 2002) and LOV domains are characteristic for a subfamily of the PAS superfamily. Previously, it was shown that ENVOY is subject to an autoregulatory feedback loop (Schmoll et al., 2005). Moreover, constitutive activation of the Gα subunit GNA1 enhances *gna1* transcription levels upon growth on glycerol, hence suggesting a feedback loop (Seibel et al., 2009) and the constitutive activation of GNA3 leads to an increase in transcript levels of *gna3* on cellulose as carbon source in light. Interestingly, the light regulatory protein ENVOY turned out to influence the transcript levels of *gna3* (Schmoll et al., 2009).

Therefore the aim of this study was to investigate the underlying interplay of signal transduction pathways in order to understand the regulatory network constituting this fine-tuning mechanism between nutrient signaling and light response. The following study identified ENVOY as a central regulatory element in the fine-tuning mechanism of G-protein signaling.

2. Materials and methods

2.1. Strains, plasmids and culture conditions

T. reesei wild-type strains QM9414 (ATCC 26921) and TU-6 (ATCC MYA-256; uridine auxotroph; (Gruber et al., 1990)), Δ*env1* (Castellanos et al., 2010), GNA1QL (Seibel et al., 2009) and GNA3QL (Schmoll et al., 2009) strains were used in the present study. In the latter strains the Q-L mutation causes constitutive activation of the respective G-alpha subunit. Unless otherwise noted, all strains including those constructed for this study (see below) were grown on malt extract agar. For cultivations of the uridine auxotrophic strain TU-6, the medium was supplemented with 10 mM uridine (Merck, Darmstadt, Germany).

For Northern Blot analysis and qRT-PCR experiments, strains were grown in 1 L Erlenmayer shake flasks in constant darkness (DD), constant light (LL, 25 μmol photons m^{-2} s^{-1}; 1800 lux) or pregrown for 24 h in darkness and thereafter exposed to light (DL) at 28 °C on a rotary shaker (200 rpm). Harvesting of dark grown cultures was done under safe-red-light (darkroom lamp, Philips PF712E, red, E27, 15 W). For liquid culture Mandels–Andreotti minimal medium (Mandels and Andreotti, 1978) was used, supplemented with 0.1% (w/v) peptone to induce germination and with 1% (w/v) carbon source, i.e. glycerol (Merck, Darmstadt, Germany) or microcrystalline cellulose (# 1402; SERVA, Heidelberg, Germany), as indicated at the respective experiments. For inoculation 10^9 conidia per liter were used. For cAMP measurements strains were grown on Mandels–Andreotti medium, supplemented with 0.1% (w/v) peptone, 2% (w/v) agar–agar and 1% (w/v) of carboxymethylcellulose sodium salt (Roth, Karlsruhe, Germany) in constant light. *Escherichia coli* JM109 was used for the propagation of vector molecules and DNA manipulations (Yanisch-Perron et al., 1985).

2.2. Construction of T. reesei GNA1QL and GNA3QL strains lacking env1

For deletion of *env1* in GNA1QL and GNA3QL strains, plasmid pDELENV2 (Castellanos et al., 2010) conferring hygromycin B resistance was used. The deletion cassette was released from pDELENV2 by restriction enzymes *Acc65*I and BamHI. This linear fragment was used for the transformation of protoplasts of strains GNA1QL and GNA3QL (Gruber et al., 1990). Transformants were selected on plates containing 50 μg/mL hygromycin B (Roth, Karlsruhe, Germany). Stable transformants were obtained after two rounds of single spore isolation. Fungal DNA was isolated from transformants by standard protocols. To check for successful deletion, we performed two different control PCRs, using primers csfDEL5F and csfDEL3R as well as ENVSP1F and ENVNEU2R (for primer sequences see Table 1). These primers bind outside the deleted region, which enables verification of replacement of the *env1* gene with the hygromycin resistance cassette. Successful deletion, as expected, resulted in a significantly longer fragment than amplification from wild-type DNA as well as disappearance of the shorter wild-type fragment. These results were confirmed by Southern blotting (data not shown).

2.3. Nucleic acid isolation and manipulation

Fungal mycelia were harvested by filtration, briefly washed with tap water, frozen and ground in liquid nitrogen. Extraction of genomic DNA was performed as described previously (Schmoll et al., 2004). Total RNA for Northern blots was isolated by the guanidinium thiocyanate method (Chomczynski and Sacchi, 1987). Standard methods (Sambrook et al., 1989) were used for electrophoresis, blotting and hybridization of nucleic acids. Northern blot analysis was performed as described previously (Sambrook et al., 1989; Schmoll et al., 2004). Twenty micrograms of total RNA were loaded onto the gel and blotted. The following probes were used: for *18S rRNA* hybridization a probe using primers 18SRF and 18SRR (for all primer sequences see Table 1), amplifying a 297 bp fragment and a 1264 bp *cbh1* fragment, generated by PCR amplification with primers cbh1S1F and cbh1S1R.

2.4. RNA preparation and reverse transcription for quantitative RT-PCR

Fungal mycelia were harvested by filtration, briefly washed with tap water and frozen in liquid nitrogen. For extraction of total RNA, buffers and columns of the RNeasy Plant Mini Kit (QIAGEN,