Characterization of a REG/PA28 proteosome activator homolog in *Dictyostelium discoideum* indicates that the ubiquitin- and ATP-independent REGgamma proteosome is an ancient nuclear protease.

Patrick Masson¹, Daniel Lundin², Fredrik Söderbom³, and Patrick Young⁴

¹Swiss Institute of Bioinformatics Swiss-Prot Group CMU - 1, rue Michel Servet CH-1211 Geneva 4 Switzerland; ²Department of Molecular Biology and Functional Genomics, Stockholm University, S-10691 Stockholm, Sweden; ³Department of Molecular Biology, Swedish University of Agricultural Sciences (SLU), Uppsala Biomedical Center (BMC), Box 590, S-75124; ⁴Department of Genetics Microbiology and Toxicology, Stockholm University, S-10691 Stockholm, Sweden.

**Running Title:** *Dictyostelium* Proteasome Regulator

**Key words:** proteasome, 11 S REG, PA28, *Dictyostelium*

This work was supported by grants from the Swedish Research Council and the Swedish Cancer fund.

⁴Corresponding Author Tel: 46-8-163107

E-mail patrick.young@gmt.su.se
ABSTRACT
The nuclear proteasome activator REG/PA28 is an ATP-ubiquitin independent activator of the 20 S proteasome and has been proposed to degrade and thereby regulate both a key human oncogene, SRC-3/AIB1 coactivator and the cyclin-dependent kinase inhibitor, p21(Waf/Cip1). We report the identification and characterization of a PA28/REG proteasome activator homolog in Dictyostelium. Association of recombinant Dictyostelium REG activator with purified Dictyostelium 20 S proteasome led to the preferentially stimulation of the trypsin-like proteasome peptidase activity. Immuno-localization studies demonstrate the proteasome activator is localized to the nucleus and is present in growing as well as starving Dictyostelium cells. Our results indicate that the Dictyostelium PA28/REG activator can stimulate both the trypsin-like and chymotrypsin-like activities of the 20 S proteasome and supports the idea that the REG-20 S proteasome represents an early unique nuclear degradation pathway for eukaryotic cells.

INTRODUCTION
The proteasome is a large multicatalytic enzyme involved in non-lysosomal regulated protein degradation (7), and has been shown to be an essential factor in controlling various cellular processes such as cell cycle progression, transcriptional regulation and metabolic pathways through regulated proteolysis (3). The 20 S proteasome is a barrel shaped cylinder made up four stacked rings of seven subunits each. In isolation, the 20 S proteasome active sites are sequestered behind closed gates that are formed by the outer alpha subunit rings of the 20 S proteasome (9). It is currently thought that the vast majority of proteins are degraded by the proteasome system only when the 20 S proteasome is associated with an activating sub-complex, such as the 19 S regulator complex to form the 26 S proteasome (22, 27). Several additional activating complexes have also been found to associate with the 20S proteasome such as the PA200 (26), and the REG/PA28 family, which is the central focus of the following work (8, 10, 23).

While the composition and structure varies considerably between the proteasome activator complexes, a common feature that is shared between the divergent complexes is the ability to associate and change the conformational position of the alpha rings to open the closed gate on either end of the 20 S proteasome (28).
The human REG family constitutes a distinct class of proteasome regulatory complexes. Three subunits, $\alpha$, $\beta$ and $\gamma$, are able to assemble two distinct heptameric rings: REG$\alpha\beta$ can associate as a three $\alpha$-subunits, four $\beta$-subunit heptameric ring (31) while the REG$\gamma$ complex is proposed to form a homopolymer of seven identical $\gamma$-subunits. Unlike the 19 S proteasome, the REG activator has been previously characterized only in metazoans and is apparently absent in plants and yeast. A similar or distantly related activator to the REG has been characterized in Trypanosoma brucei and termed PA26 but demonstrates little sequence identity or similarity with the three isoforms of mammalian proteasome REG $\alpha$, $\beta$ and $\gamma$ (29). Surprisingly with the lack of sequence homology the PA26 is capable of forming a heptamer ring structure like REG and activates the 20S proteasome in a similar manner (5).

While the REG activators have been well characterized in their ability to promote the degradation of small peptides, evidence for their role in promoting degradation of full-length proteins has only recently been obtained. The first proposed protein target for the nuclear REG$\gamma$ proteasome complex has been identified and corresponds to the steroid receptor SRC-3/AIB1 coactivator, an important oncogene that is commonly present at high concentrations in human breast cancers (14). The SRC-3/AIB1 coactivator is proposed to be degraded in a ubiquitin- and ATP-independent manner by the REG$\gamma$ proteasome. Recently, two groups have reported that the key central cyclin-dependent kinase inhibitor, p21(Waf/Cip1) is another endogenous target. RNAi knockdown, gain-of-function analysis, and pulse-chase experiments substantiate that REG$\gamma$ promotes degradation of unbound p21 (2, 13). In vitro assays using purified REG$\gamma$, p21, and the 20S proteasome confirm that REG$\gamma$ directly mediates degradation of free p21 in an ATP- and ubiquitin-independent manner. These two recent examples suggest that further studies using various model systems and assays will likely identify additional protein substrates that are degraded by the REG$\gamma$ proteasome complex.

In this report, we have cloned a Dictyostelium gene that has clear sequence similarity to the human REG$\gamma$. Expression and purification of the Dictyostelium gene product in Escherichia coli generates a PA28/REG complex that can associate and activate the 20 S proteasome and has allowed us to identify conserved and divergent properties between the human and Dictyostelium forms of this proteasome activator.
Materials and Methods

Chemical Reagents and Antibodies–Rabbit polyclonal antibodies against a SDS-PAGE purified recombinant *Dictyostelium* REG were raised in rabbits by Agrisera. Initial Western immunoblot tests confirmed the production of an anti-REG antibody that revealed a single band with the expected molecular weight migration value that matched the *Dictyostelium* REG migration value. The specificity of the anti-REG antibody was confirmed by immunoblotting against purified REG protein and by depleting the anti-REG antibody against PVDF bound purified REG protein. For specificity testing an excess of purified REG protein was incubated 1 hr in TBS, with a 1 cm² of PVDF transfer membrane and washed 3 times and then blocked with 5 % milk in TBS. A total of 10 µl of anti-REG antibody was then incubated for 1 hr at 4°C in 1 ml TBS buffer with the immobilized REG protein. The 1 ml solution was then used to probe an immunoblot of purified REG protein and crude *Dictyostelium* extract and compared to the results of anti-REG sera that was not pre-absorbed against REG protein. TRITC secondary antibody was purchased from Sigma. Boc-Leu-Arg-Arg-MCA, Suc-Leu-Leu-Val-Tyr-MCA, and benzoxycarbonyl-Gly-Gly-Leu-MCA, were purchased from AFFINITI Research Products. The peptide Ac-Asp-Glu-Val-Asp-MCA was purchased from Peninsula Laboratories Europe.

Purification of Dicytostelium 20 S proteasome–*Dictyostelium* was grown in HL5 medium according to Sussman (24). The *Dictyostelium* cells were collected by centrifugation followed by freezing at ~80°C. A total of 20 g (wet weight) were French pressed three times and resuspended in 3 volumes, 60 ml, MVB buffer, 20mM MOPS pH7.5, 20mM NaAc, 20mM KCl, 10mM NaCl, 2mM MgCl₂, 1mM DTT with 10% glycerol. The crude lysate was centrifuged for 1 hr at 20,000 x g at 4°C. The crude supernatant was incubated for 1 h at 4°C with 10% streptomycin sulfate, centrifuged at 20,000 x g for 15 minutes, and then dialyzed overnight against 2 L of MVB buffer 10% glycerol at 4°C. The dialyzed extract was centrifuged an additional time for 5 min at 10,000 x g. The extract of 90 ml, 6 mg/ml, was passed over a DEAE cellulose column that had been equilibrated in TBS buffer and 10% glycerol. The 20 S proteasome was eluted with one liter KCl gradient, 0 to 1 M, and the fractions were assayed for 20 S proteasome LLVY-MCA activity in TS buffer, 10
mM Tris-HCl, pH 8.8, 25 mM KCl, 10 mM NaCl, 1.1 mM MgCl2, and 0.1 mM EDTA, containing 0.035% SDS. Fractions containing proteasome activity were concentrated using Amicon Centricons. The *Dictyostelium* 20 S proteasome extract was passed over a Superose 6 gel-filtration column equilibrated in MVB buffer and 10% glycerol. The fractions were assayed for 20 S proteasome activity and stored frozen at –80°C. The purity of the 20 S proteasome was analyzed by electrophoresis on a 10-15% SDS-polyacrylamide gel.

**Purification of the *Dictyostelium* proteasome activator—**The *Dictyostelium* REG cDNA, clone series SSH1-D, clone SSH185 (19) was PCR amplified and inserted into PET26b between restriction sites NdeI and EcoRI. BL21 (DE3) *Escherichia coli* transformed with pET26b containing the *Dictyostelium* REG was grown at 30 °C in LB medium until an A600 of 0.3 was reached. The bacteria were induced with a final concentration of 300 μM isopropyl-1-thio-D-galactopyranoside and harvested after a 2 hr induction. The soluble protein fraction was obtained from induced recombinant cells using a french press and resuspending in two pellet volumes of TS buffer (10 mM Tris-HCl, pH 8.8, 25 mM KCl, 10 mM NaCl, 1.1 mM MgCl2, and 0.1 mM EDTA), followed by centrifugation at 39,000 x g for 30 min at 4 °C. The soluble protein extract was treated with 10% streptomycin sulfate and centrifuged, 20,000 x g, and the supernatant was dialyzed overnight in TS buffer at 4 °C. The extract was initially passed over a 50-ml DEAE cellulose column followed by elution with a 1-liter 0-1 M KCl gradient in TS buffer, pH 8.8. Fractions containing *Dictyostelium* REG were identified by SDS-PAGE and Coomassie staining. Fractions with the recombinant *Dictyostelium* REG were further purified by gel filtration using a Superdex 200 column equilibrated with TS buffer and 1 mM dithiothreitol. The *Dictyostelium* REG eluted as a complex of expected size.

**Gel filtration of *Dictyostelium* proteasome and REG—** To identify a physical interaction between the REG complex and the proteasome the two purified complexes, 1 μg of purified *Dictyostelium* proteasome, and 7.5 μg of purified recombinant *Dictyostelium* REG were pre-incubated for 15 minutes and passed over a Superose 6 FPLC column in 20 mM MOPS pH7.5, 20 mM Na acetate, 20 mM KCl, 100 mM NaCl, 2 mM MgCl2, 1 mM DTT at room temperature. Individual runs of 20
S proteasomes or REG at the same protein concentrations were also performed under identical conditions.

**Fluorometric Assays of Proteasome Activities**—Spectrofluorometric assays were performed in the presence of fluorogenic peptides, 1 µg of purified *Dictyostelium* proteasome, and 7.5 µg of purified recombinant *Dictyostelium* REG, in 20 mM MOPS pH7.5, 20 mM Na acetate, 20 mM KCl, 10 mM NaCl, 2 mM MgCl₂, 1mM DTT. Proteasome and *Dictyostelium* REG were incubated together for 15 min at room temperature to allow association, prior to the addition of the fluorogenic peptide substrates. Reactions were performed at room temperature for 30 minutes and terminated by the addition of 200 µl of ice-cold ethanol. Fluorescence was measured with a Bio-Rad fluorometer using an excitation wavelength of 380 nm and an emission wavelength of 440 nm. All substrate peptides contained the MCA fluorogenic reporter group. Fluorogenic peptides, Boc-Leu-Arg-Arg-MCA, Suc-Leu-Leu-Val-Tyr-MCA, and benzoxycarbonyl-Gly-Gly-Leu-MCA, were purchased from AFFINITI Research Products. The peptide Ac-Asp-Glu-Val-Asp-MCA was purchased from Peninsula Laboratories Europe.

**Immunostaining of Dictyostelium cells**—Dictyostelium cells were cultured either in growth media (HL-5) or subjected to starving conditions in PDF buffer, 20 mM KCl, 5 mM MgCl₂, 20 mM KPO₄ pH 6.4. Cells were placed on plastic cover slips, and allowed to attach for 30 minutes. Cells were fixed with a solution of 3.7% formaldehyde in 15 mM Na/K-PO₄ buffer, pH 6.5 for 10 minutes and permeabilized for 5 min in −20°C methanol containing 1% formaldehyde. Cells were incubated with *Dictyostelium* REG polyclonal antibody 1/500 for 1 hour at room temperature in 0.1% BSA in PBS, washed three times with PBS containing 0.05% Tween 20, then incubated with TRITC secondary antibody to a dilution of 1/100 at room temperature in 0.1% BSA, 0.05% Tween 20 in PBS. Finally three additional washes of 5 minutes were carried out in PBS Tween, and Hoescht dye was added during the final wash to a 5 µg/ml concentration. Finally, coverslips were mounted in a 50% glycerol/50% PBS solution. The immunostaining protocol was repeated with only the secondary antibody in order to rule out its hybridization to the nucleus.
RESULTS

Sequence similarities of the REG gene family

Initial BLAST searches revealed a number of partial sequences from the Dictyostelium development cDNA database (19) that showed high similarity to the REG proteasome activator present in metazoans. The cDNA clone SSH185 appeared to contain the longest cDNA sequence of the matching sequences but was originally sequenced to code for only a partial open reading frame that matched part of the REG proteasome activator sequence. However, sequencing of this clone revealed instead a full-length open reading frame that contained high similarity to the REG family throughout the sequence. The open reading frame translated into a corresponding protein of 26 kDa and the gene to psmE3, denoted DDB0191141 in dictyBase (http://dictybase.org/). Alignment of metazoan REG sequences with the Dictyostelium protein reveals high conservation of the activation domain (Figure 1).

For metazoan REG subunits the three different classes, α β and γ, can be identified directly from their primary sequences within two specific regions, the conserved region that codes for the single long alpha helix that forms the seven-member channel and the homolog specific region. The homolog specific region codes for a flexible loop region that is present on top of the REG-proteasome and has been shown to contain a conserved nuclear localization signal (17). For the homolog specific region the Dictyostelium REG sequence shows by far the shortest loop sequence of all identified REG sequences, with the loop region almost absent except for a small region that shows strong similarity to the proposed REG γ nuclear localization signal.

For the alpha helix channel the Dictyostelium REG sequence shows only limited sequence similarity to any of the metazoan classes. A limited number of residues are conserved both in the gamma class and the Dictyostelium REG. For the channel alpha helix region the Dictyostelium sequence shows 34% similarity with the human gamma sequence while only 23% and 21% for the human alpha, and beta sequences, respectively.

Genomic sequence of the Dictyostelium REG gene confirms the sequence of our cDNA clone and reveals the position of the introns within the Dictyostelium REG gene on chromosome 4. The gene contains 3 small introns, which is well above average for such a small gene. The average number of introns in Dictyostelium is 1.9 with 30% of genes containing no introns (4). Genomic searches yield a single copy of
the REG gene without any apparent duplications or related sequences with significant similarity scores. In order to address whether the *Dictyostelium* REG gene was a recent acquisition, we compared the GC content of the REG sequence versus other *Dictyostelium* genes. The A-T content is 74% for the REG ORF. This extremely high A-T content matches the overall ratio found in *Dictyostelium* ORFs, which is 72% (4) and does not implicate the REG gene as being recently acquired by horizontal transfer from a metazoan.

**Proteasome activation by *Dictyostelium* REG**

The open reading frame for *Dictyostelium* REG was cloned into an expression vector and the corresponding *Dictyostelium* protein was over expressed in *E. coli*. The purified recombinant *Dictyostelium* REG protein was analyzed for its ability to stimulate peptide degradation by the 20 S proteasome. Initially, purified *Dictyostelium* 20 S proteasome was obtained using standard DEAE and gel-filtration chromatography. The purification was analyzed by SDS-PAGE (Figure 2A) as well as by a simple protease fluorogenic peptide assay. To confirm that that the purified 20 S proteasome was functional and in a closed gated state, LLVY-MCA assays were carried out with and without the presence of low concentrations of SDS, data not shown. The purified *Dictyostelium* 20 S proteasome as expected was active as shown by its activation by low concentrations of SDS, 0.035%.

The recombinant *Dictyostelium* REG was also purified using DEAE ion exchange and gel-filtration chromatography. The location of the *Dictyostelium* REG from the DEAE eluated fractions was determined by SDS-PAGE (Figure 2A). The presence of *Dictyostelium* proteasome activator was also followed by a simple proteasome fluorogenic peptide assay. For the assay partially purified fractions from the *Dictyostelium* REG purification were mixed with 20 S proteasome and LLVY-MCA fluorogenic peptide solution. Fractions that stimulated LLVY-MCA proteolytic activity were found to co-localize with a band that matched the *Dictyostelium* REG molecular weight when ran out on denaturing SDS polyacrylamide gels. Control *E. coli* extracts that lacked the *Dictyostelium* REG plasmid did not show this stimulatory activity or the presence of the band on gels (data not shown). Finally gel filtration of the REG-20S proteasome complex was carried out. An excess of REG complex was pre-incubated with 20 S proteasome and passed over a Superose 6 column at room
temperature (Figure 2B). Comparisons of chromatographic runs of the individual complexes versus pre-incubated REG and 20 S reveal the formation of a high molecular weight co-complex. Immunoblots of specific fractions using a REG antibody confirmed the presence of REG in the higher molecular weight complex, data not shown.

To determine relative fold increases in peptidase activation, purified proteasomes from Dictyostelium cells were mixed with purified Dictyostelium REG and the C-terminal cleavage of these fluorogenic tri or tetra peptides were monitored for increase production of cleaved fluorogenic MCA, 7-amino-4-methyl-coumarin. The assays were performed with a variety of fluorogenic peptides, LLR-MCA, LLVY-MCA, and either DEVD-MCA or LLE-MCA to measure the trypsin, chymotrypsin, and acidic residues activity respectively. As shown in Figure 3, the Dictyostelium REG was able to enhance peptide cleavage in a broad spectrum for different peptide substrates. However, the relative fold stimulation showed significant differences. The chymotrypsin-like activity monitored by the cleavage of LLVY-MCA peptide was enhanced six fold in the presence of Dictyostelium REG, whereas the trypsin-like activity evaluated by LRR-MCA was increased 14-fold when the 20 S proteasome was incubated and allowed to associate with the Dictyostelium REG. As expected the proteasome activation did not require the addition of ATP, data not shown.

**Dictyostelium REG is a nuclear protein in growing and starving cells**

In order to investigate the localization of the REG complex in Dictyostelium cells, we overexpressed the recombinant protein in E. coli, extracted the corresponding band from polyacrylamide gels and generated several polyclonal antibodies. For cellular staining studies a high titer antibody was selected that had minimum cross-reactivity with E. coli or other Dictyostelium proteins (Figure 4A). This polyclonal antibody was used for immunofluorescence studies on fixed Dictyostelium cells (Figure 4B-E). Dictyostelium cells were harvested both before and after media starvation. To visualize the localization of the nucleus, Hoechst fluorochrome staining of the DNA was carried out after immunostaining of the fixed cells with Dictyostelium REG antibody and fluorescent secondary antibody, (Figure 4 C and E). The commercial secondary antibody does not itself stain the nucleus of
fixed *Dictyostelium* cells, data not shown. The Hoechst stain did not affect REG immunostaining and cells stained only with anti-REG antibody showed the same pattern as the dual stained cells, data not shown. For *Dictyostelium* REG a strong nuclear staining in the nucleus was observed. The cytoplasm contained very little or no staining suggesting the *Dictyostelium* REG complex is a nuclear protein as has been seen for REGγ class of metazoan proteasome activators. Initial comparisons between growing and starving *Dictyostelium* cells showed no differences in the amount of *Dictyostelium* REG present within the nuclei of the two stages with both showing strong nuclear localization signals.

**Gene disrupted REG *Dictyostelium* strains were not isolated.**

To determine the role of PA28/REG proteasome activating complex homologous recombination was attempted to knockout the PA28/REG gene by transformation of a plasmid containing the *Dictyostelium* REG genomic sequence disrupted with selectable markers. Multiple attempts were carried out to generate a REG knockout *Dictyostelium*. However, Western blot analysis revealed that all isolated colonies still expressed the *Dictyostelium* REG proteasome activator at wild type levels, data not shown.

**DISCUSSION**

It is currently unclear why a small subset of nuclear proteins bypasses the requirement for ubiquitination and degradation by the 26 S proteasome and instead is apparently degraded by the REGγ 20 S proteasome. Is this type of degradation a recent adaptation in animal cell nuclei or is it an ancient pathway that precedes the ubiquitin proteasome system? A key feature for *Dictyosteliums* are their ability to grow as single cells and then differentiate to multicellular stalked fruiting bodies that contain haploid spores. The proteasome is required for *Dictyostelium* developing from the proliferative to the differentiated state. Using differential display, a *Dictyostelium* 20 S proteasome subunit gene was isolated as one preferentially expressed during the transition from growth to differentiation (1). The UbpA deubiquitinating enzyme has also been identified as being essential for development but not growth for *Dictyostelium* (15).
In general, the metazoan REG activators are present both in the cytoplasm and nucleus. The REGαβ is found in the cytoplasm of animals possessing an adaptive immune system, and the presence of this complex in zebrafish suggests that the origin of these genes preceded the divergence of bony fishes and tetrapods (20). The REGγ gene appears to have a more ancient origin since it has been characterized in invertebrates such as Drosophila, Rhipicephalus appendiculatus, and Caenorhabditis elegans. The nuclear localization signal for REGγ has been identified in Drosophila and resembles the c-myc monopartite NLS (16, 17). The REGγ NLS is present in the middle of a flexible loop region that is disordered in the crystal structure for REGα and was termed the homology insert region (30) (Figure 5A). Both Dictyostelium REG and metazoan gamma activators are present in the nucleus but not obviously attached to structural elements of the nuclei.

The activity characterization of Dictyostelium REG suggests that the Dictyostelium REG protein is similar in nuclear localization to the gamma class of REG proteasome activator in metazoans with some differences in activity towards peptide cleavage activation. In the past it has been observed that treatment of metazoan REG activators with ammonium sulfate resulted in changes in activation. Previous studies have shown that ammonium sulfate broadens the substrate specificity for mammalian REGγ (6). During the purification of the Dictyostelium REG complex ammonium sulfate precipitation was not carried out on the Dictyostelium activator. For Dictyostelium REG the trypsin-like proteasome activity was enhanced over other tested protease activities and this preference to stimulate cleavage after basic amino acid residues is similar to the gamma class. However, the proposed inner channel residues of the Dictyostelium REG are significantly different from that of any of the three classes found in metazoans. Furthermore, Dictyostelium REG showed a broader range of proteasome activation than the metazoan nuclear activators. The Dictyostelium protein was able to stimulate the proteolysis of a chymotrypsin model substrate while metazoan REG γ complexes inhibit this proteasome activity (17).

A number of close relationships have been identified between Dictyostelium and metazoans at the protein level, and the recent ability to compare complete proteomes has greatly helped in the understanding of the lineage relationships (4). It is currently accepted that Dictyostelium diverged from the animal line shortly after the plants and shortly before fungi and yeasts. A search for genomes that contain
sequences with high similarity to REG proteasome activators reveals a number of new candidates. Recent finished fungi genomes reveals the presence of genes with high similarity to REGγ and conservation in both location and sequence of a potential monopartite NLS signal for a number of sequences, (Figure 5B). Alignment search of newly sequenced genomes indicate that a wide range of Eukaryotes contain REG proteasome activators and that the complex has been lost both in yeast and modern plants. For the phytoplankton *Emiliania huxleyi* an initial low overall similarity was found between a candidate gene and the human REGγ protein sequence. The low initial score was due to the lack of a homolog insert specific domain in the phytoplankton sequence and instead a large insertion is present C-terminal of the activation domain region, (Supplementary Figure 1). Interestingly, mapping the *Emiliania huxleyi* predicted REG onto the structure of the human REGα revealed that the extra sequence is inserted into the neighboring conserved turn domain atop the REG structure (Figure 5C). This suggests that the observed large differences in the primary sequences between the human and phytoplankton proteins may reflect only small structural tertiary differences. The mapping evidence indicates that the homolog specific inserts are evolutionarily conserved at the opening of the REG complex and function as localization domains and possibly contribute to acquisitions of protein substrates. Mapping the presence of REGγ on the tree of Eukaryotes indicates that the nuclear REGγ proteasome degradation pathway is ancient and was likely lost or replaced in plants and yeast (Figure 5D). Cluster analysis based on sequence similarity of the newly obtained REG sequences, supports three classes with the non-metazoan REG sequences being within the REGγ class, manuscript in preparation. Mapping the presence of REGγ on the tree of Eukaryotes indicates that the REGγ proteasome degradation pathway is ancient and was likely lost or replaced in plants and yeast (Figure 5D). The apparent conservation of a nuclear localization signal in the majority of REGγ sequences suggests that a REGγ-proteasome was a nuclear complex in ancestral Eukaryotes.

A role for REGγ in cell cycle progression has been suggested after the observation that REGγ is abnormally overexpressed in thyroid cancer cells, especially in cells at the peripheral region of the cancer (21). In *Drosophila*, study of the complex promoter region revealed that transcription of REGγ is under the control of DREF, a transcription factor typically found to activate *Drosophila* genes involved in
cell cycle progression and DNA replication (18). Currently the oncogene SRC-3/AIB1 and cyclin-dependent kinase inhibitor, p21 (Waf/Cip1) are the only demonstrated protein substrates known to be degraded by the REG-proteasome complex in mammalian cells (2, 13, 14). From the above studies the nuclear REG complex appears to function in processes that promote or repress cell cycle progression, suggesting that this conserved degradation complex may degrade a number of nuclear targets that have not yet been identified. The Dictyostelium system should serve as a useful model to discover additional proteins that are degraded in a ubiquitin and ATP independent manner by the proteasome. The properties and cellular location of the Dictyostelium REG suggests an ancient lineage for the REG gamma class of proteasome activators.

Acknowledgments

We thank Anthony Poole for discussions and comments. Dr. Takahiro Morio from the University Tsukuba, Tsukuba, Ibaraki Japan, for providing the original cDNA clone from the Dictyostelium cDNA project that was originally supported by the Japan Society for the Promotion of Science (RFTF96L00105) and Ministry of Education, Science Sports and Culture of Japan (08283107).

REFERENCES


FIGURE LEGENDS

Figure 1. Sequence alignment of the Dictyostelium REG and other 11S REG members. A, comparison of the deduced amino acid sequence of Dictyostelium REG with homologues including Drosophila REG, C. elegans REG, Zebrafish REGα, β and γ as well as human REGα, β and γ. The sequence alignment was obtained using MegAlign program from DNASTAR. Specifically, the alignment of REG protein sequences was carried out using ClustalW and a PAM 250 scoring matrix. Residues that are identical are shaded in black. The homolog-specific insert region is boxed. The proposed residues for the α-helix forming the inner channel are marked with a thin line. Another thin black line notes the activation region that interacts with the 20S proteasome. The asterisk marks the residue corresponding to the human REGγ Lys188 that converts the activation pattern from REGγ to REGα in human when mutated to a Asp or Glu residue (12). B, Comparison of sequence similarities in percent scores for the different members of the REG family.

Figure 2. Purification of the Dictyostelium 20 S proteasome and Dictyostelium REG.
A. Coomassie stained SDS-page after the various purification steps for the 20 S proteasome and for purification of the recombinant Dictyostelium REG expressed in E.coli. Equivalent amount of total proteins were loaded on a 10-15% SDS-page gel. Line represents expected migration area for various 20 S proteasome subunits B. To identify a physical interaction between the REG complex and the proteasome the two purified complexes, 1 µg of purified Dictyostelium 20 S proteasome, and 7.5 µg of purified recombinant Dictyostelium REG were pre-incubated for 15 minutes and
passed over a Superose 6 FPLC column and compared to chromatographic runs of the individual complexes.

**Figure 3. Activation of purified Dictyostelium 20 S proteasome by Dictyostelium recombinant REG.** A, Fluorescence intensity representing the fluorogenic peptide cleavage by the 20 S proteasome purified from Dictyostelium cell extract without (black squares) or with (grey squares) Dictyostelium REG. Dictyostelium REG and proteasome were preincubated 10 minutes at room temperature and then incubated with a specific fluorogenic peptide. B, Comparison of the ability of Dictyostelium REG to stimulate fluorogenic peptide degradation by the 20 S proteasome. The results are represented as 20 S proteasome fold stimulation for each tested fluorogenic peptide.

**Figure 4. The Dictyostelium REG is predominantly nuclear, both in growing and starving cells.** (A) An immunoblot were prepared and transferred with E. coli expressed recombinant REG or crude protein extract from Dictyostelium cells in duplicate and divided into two (left panel and middle panel). Both halves of the immunoblot were incubated with an equal volume of anti-Dicty REG polyclonal sera. The middle panel sera was pre-adsorbed with recombinant REG, before incubation with the membrane (middle panel). Right panel shows Coomassie staining of the transferred proteins. (B-E) Dictyostelium cells were stained with polyclonal anti-Dicty REG polyclonal antibody followed by rhodamine secondary antibody, B and D. Cells show nuclear localization of the proteasome regulator when compared to DNA staining with Hoechst 33258, C and E. Panels B and C show individual Dictyostelium cells and panels D and E represent Dictyostelium cells under starvation.

**Figure 5. Nuclear REG-proteasomes are an ancient Eukaryotic pathway.** An illustration of the proposed REG complex based on the known crystal structures (10, 11). The main difference between the Dictyostelium and human REG complexes is the length of the homolog insert regions. However, both contain mono-partite NLS sequences in this unstructured region. B, Alignment of monopartite NLSs found
within the homolog insert regions from various organisms. The overall similarities of predicted proteins to the entire length of human REGγ were: *Coprinopsis cinerea*, XP_001829111, 35% identical, expect = 2e-34; *Ustilago maydis*, XP_756858, 27% identical, expect = 7e-24; *Nematostella vectensis*, XP_001638241, 48% identical, expect = 8e-63; *Emiliania huxleyi*, DQ658283, 19% identical, expect = 1e-07. The c-myc NLS sequence motif is from human (16). C, Comparison of the insert position to the known human REGγ (11) crystal structure reveals that the insert aligns with the turn of the adjacent long alpha helical pair of the REG monomer. D, The position of *Dictyostelium* in eukaryotic phylogeny and mapping the absence or presence of the REG activators on the eukaryotic tree. Red lines represent groups that have REG proteasome activators sequence present in their genomes. Whole-proteome comparisons of *Dictyostelium* and representatives of key groups, rooted on archaeal species, were used to generate this phylogenetic tree modified from original data from (4).

Masson et al. Figure 1

A

Homolog-specific insert Region

inner channel

Activation domain

B

<table>
<thead>
<tr>
<th></th>
<th>Human REG</th>
<th>Drosophila REG</th>
<th>C. elegans REG</th>
<th>Human REG</th>
<th>Human REG</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dicty-REG</td>
<td>29.8</td>
<td>27.6</td>
<td>25.8</td>
<td>30.2</td>
<td>20</td>
</tr>
<tr>
<td>Human REG</td>
<td></td>
<td>46.9</td>
<td>47.2</td>
<td>39.4</td>
<td>34.3</td>
</tr>
<tr>
<td>Drosophila REG</td>
<td></td>
<td>45.3</td>
<td>36.7</td>
<td>30.5</td>
<td></td>
</tr>
<tr>
<td>C. elegans REG</td>
<td></td>
<td>34.3</td>
<td>31.4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Human REG</td>
<td></td>
<td>48.5</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Masson et al. Figure 3

A

![Graph A](image)

B

![Graph B](image)
Masson et al. Figure 4

A) Western blot analysis showing different protein bands under various conditions:
- Control
- Pre-absorbed against REG
- Coomassie stained control membrane

B) Fluorescence microscopy image showing red fluorescent spots in a sample.

C) Fluorescence microscopy image showing blue fluorescent spots in a sample.

D) Fluorescence microscopy image showing a complex pattern of red fluorescent spots.

E) Fluorescence microscopy image showing a complex pattern of blue fluorescent spots.
Masson et al. Figure 5

A. Homolog specific insert region

B. NLS Homolog Specific insert region

C. All other REGs

D. Phylogenetic tree

Key:
- **Blue**: c-myc nuclear localization signal
- **Red**: Dictyostelium
- **Green**: Pathogenic plant fungus, *Ustilago maydis*
- **Yellow**: Sea Anemone, *Nematostella vectensis*
- **Black**: *Drosophila melanogaster*
- **Orange**: *C. elegans*
- **Red**: Emiliania huxleyi (unique insert region)
- **Green**: Giardia lamblia

**D**
- Yeast
- Fungi
- Animals
- Plasmodium
- Chromista (Haptophyta) phytoplankton
- Plants and Green Algae