

Expression of *ras*-family genes in the cell cycle and during differentiation of the lower eukaryote *Physarum polycephalum**

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Messenger RNA levels of three *ras*-family genes (*Ppras1*, *Ppras2*, and *Pprap1*) were measured in different life forms and throughout the cell cycle of the slime mold *Physarum polycephalum*. All three genes are expressed at constant rates in the unicellate amoebae and flagellates, regardless of the culture conditions (solid or liquid medium, particulate or dissolved nutrients). In the multinucleate stages (micro- and macroplasmodia) *Ppras1* and *Pprap1* mRNAs are somewhat less abundant, while *Ppras2* is not expressed at all. The early stages of the amoeba-plasmodium transition proceed without any drop in *Ppras2* expression. During the synchronous cell cycle in macroplasmodia *Ppras1* and *Pprap1* are expressed at a constant level.

Ras and related small GTPases form a large, highly conserved, ubiquitous superfamily of signal-transducing proteins [1]. Individual families within the superfamily specialize in different cellular events, e.g., Rab proteins are involved in intracellular vesicle trafficking [2], Rho control the cytoskeleton [3], and the Ras family members regulate cell proliferation and differentiation [4]. It is the latter proteins which attract particular attention, initially fuelled by their oncogenic properties [5], but now justified chiefly by their involvement in normal development. Their principal mode of action in mammalian cells has been

thoroughly studied and consists in linking cell membrane receptor or non-receptor tyrosine kinases to a serine/threonine protein kinase cascade culminating in the phosphorylation of nuclear factors [6, 7]. However, novel data concerning their downstream effectors and cross-talk with other signal transduction pathways are accumulating at an amazing pace [8].

Significantly less is known regarding Ras-family proteins in most other organisms. Only in a number of model cases has their role in highly specific differentiation events been elucidated to a level comparable to that of the mammalian cells. These include, i.a.,

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Abbreviation: SDS, sodium dodecyl sulfate.

the differentiation of eye cells in *Drosophila melanogaster* [9] and vulva formation in *Caenorhabditis elegans* [10]. In the most thoroughly studied lower eukaryote, the yeast *Saccharomyces cerevisiae*, Ras proteins seem to be unique both in their structure (being considerably larger than in other organisms) as well as in their mode of action (*via* adenylate cyclase) [11].

In order to broaden the base of *ras* studies we chose to investigate the myxomycete (slime mold) *Physarum polycephalum*. This "simple" organism is easy to study, yet it has several features making it particularly attractive as a model [12]. These include a natural mitotic synchrony of a billion or so nuclei in a macroplasmidium and a complex life cycle with several alternative developmental transitions. Previous work from this laboratory has identified in *P. polycephalum* two *ras* genes [13, 14] and a closely related *rap* gene [15], and shown that their expression varies with the developmental stage [16]. The current report addresses the expression pattern of the *ras*-family genes throughout the mitotic cycle of *P. polycephalum* and during developmental transitions not investigated in the previous study.

MATERIALS AND METHODS

P. polycephalum strains LU353 (amoebae), LU352 (amoebae and flagellates) and CL (amoebae, microplasmodia and macroplasmodia), obtained from Dr. J. Dee, were propagated and induced to differentiate using standard procedures [17]. Macroplasmodia were formed by spotting 100 μ l of a dense microplasmodial suspension on Whatman 4 filters and allowing 20 min for fusion. Afterwards the filters were underlaid with liquid medium. The progression of macroplasmodia through the mitotic cycle was determined by microscopic examination of nuclear morphology in fixed smears and was independently verified by measuring *in vivo* incorporation of [3 H]-

thymidine pulses into acid-precipitable counts. For RNA analysis individual, intact macroplasmodia (one plasmodium per sample) were collected between second and third post-fusion metaphase (M_{II}-M_{III}).

Total RNA was isolated by a modification of the guanidine thiocyanate-acidic phenol method [18]; to remove the contaminating polysaccharide final preparations were washed, as required, with 4 M LiCl. RNA was then applied to Hybond N (Amersham) either by slot-blotting (3 μ g or less per slot) or by Northern blotting after electrophoresis in a formaldehyde/agarose gel (3 μ g per lane). Hybridization with 32 P-labeled probes was conducted overnight in 0.5 M NaP_i, 7% SDS, 1 mM EDTA, pH 7.5 at 65°C, followed by successive washes at the same temperature with 0.2 M NaP_i, 1% SDS; 0.1 M NaP_i, 1% SDS; 0.04 M NaP_i, 0.1% SDS. Filters were exposed against Kodak XAR or Foton XS-1 film at room temperature without screens (slot-blots and Northern) or with an X-Omat intensifying screen at -70°C (Northern). For quantitation, autoradiograms were scanned in a laser densitometer; care was taken to work within the linear response range of the films.

The following cDNA probes, donated by Dr. P. Kozłowski, were used for hybridization: *Ppras1* [13], *Ppras2* [14] and *Pprap1* [15]. *P. polycephalum* actin (*ardC*) cDNA ([19], a generous gift from Dr. D. Pallotta) was used as a control for technical accuracy of the procedures, and *P. polycephalum* H4.1 histone gene probe ([20], made available by Dr. F. Wilhelm) was used to verify the synchrony of the macroplasmodia used for the cell-cycle analysis; this gene has a well-defined cell-cycle-dependent expression profile [21]. Probes were labeled with [32 P]dATP using Mega-Prime kit (Amersham).

RESULTS AND DISCUSSION

To study the expression patterns of the three *ras*-family genes (*Ppras1*, *Ppras2* and

Pprap1) I made use of the natural mitotic synchrony of the *P. polycephalum* macroplasmidia. These are macroscopic (several centimeters in diameter, or larger) syncytia containing $\geq 10^9$ nuclei undergoing concerted DNA replication and endomitotic karyogamy. Under standard conditions the cell cycle lasts approx. 8–10 h, with about 0.5 h-long mitosis, followed by about 2 h long S-phase, the remainder being the G2 phase (there is no measurable G1). Beginning with the second post-fusion metaphase (M_{II} , see Methods) individual macroplasmidia were collected at one-hour intervals and total RNA was isolated. A series of 9–10 consecutive preparations corresponding to the full mitotic cycle (from M_{II} through M_{III}) was analyzed by slott-blotting, hybridization with appropriate cDNA probes, autoradiography and densitometry. Results (averages of 5 independent experiments) are presented in Table 1.

While no *Ppras2* mRNA could be detected in shaker (asynchronous) plasmodial cultures [16], one could argue that its expression is only limited to a particular, short period during the cell cycle and the transcript would be highly diluted and consequently undetectable when analyzing the unsynchronized bulk plasmodial population. To accommodate this possibility I included the *Ppras2* probe in this study. However, no *Ppras2*-specific signal could be detected at any of the mitotic stages sampled in the synchronous macroplasmidia. It is thus safe to say that this gene is not transcribed to any significant level throughout the plasmodial cell cycle.

Transcripts of the two other genes, *Ppras1* and *Pprap1*, were fairly abundant (as esti-

mated by comparing the strength of the signal against actin and H4 signals, not shown) and remained fairly constant throughout the mitotic cycle. The fluctuations observed were not statistically significant and probably reflected random physiological variations as well as technical imperfections. Normalizing the data against the actin signal rather than the amount of total RNA loaded did not change this conclusion (data not shown).

It ought to be stressed here that particular care was taken to ensure that the plasmodia used for the experiment were indeed synchronous. This concerns both the synchrony of all the nuclei within a macroplasmidium, as well as the synchrony among plasmodia in a series. By all three criteria (microscopic observation of the nuclear morphology, *in vivo* incorporation of radiolabeled thymidine, and the pattern of H4 histone mRNA, see Methods) this was indeed the case.

Thus, one may conclude that the *Ppras 1* and *Pprap1* genes are expressed in *P. polycephalum* macroplasmidia at a constant level throughout the mitotic cycle. While transcriptional modulation is by no means the only way of regulating the abundance of functional Ras and Rap proteins, these data suggest that they are needed at fairly constant amounts during all stages of the cell cycle of the non-differentiating plasmodium.

The constant expression of the *ras*-family genes throughout the *P. polycephalum* cell cycle is in sharp contrast with the data obtained for cells undergoing differentiation both in this [16] and in other (e.g., [22]) organisms. Of the several *ras*-family genes identified in most of the species studied at least some show

Table 1. Cell-cycle levels of *ras*-family transcripts in *P. polycephalum* macroplasmidia

Gene	Point in the mitotic cycle (hours after the second metaphase, M_{II})								
	M_{II}	+1	+2	+3	+4	+5	+6	+7	M_{III}
<i>Ppras1</i>	93 ± 22	105 ± 21	102 ± 18	88 ± 27	110 ± 29	98 ± 20	96 ± 23	106 ± 19	102 ± 26
<i>Pprap1</i>	103 ± 24	105 ± 27	94 ± 19	97 ± 21	101 ± 27	104 ± 20	89 ± 28	96 ± 23	109 ± 26

Numbers, expressed in arbitrary units, represent averages ± standard deviations of five independent experiments. One hundred units correspond to the averaged reading for all experimental points for a particular gene. Signal for *Ppras2* was undetectable. See text for details.

pronounced differentiation-dependent expression. In *P. polycephalum* this is most clearly seen for the *Ppras2* gene, which seems to be dormant in plasmodia (see above) and becomes transcriptionally activated only upon formation of fruiting body; uninucleate amoebae which emerge from the germinating spores express the gene at a level comparable to that of the two other members of the family [16]. One may thus reason that the expression of *Ppras2* is necessary for the transition from the multinucleate plasmodium to the uninucleate amoeba.

What about the reciprocal transition? Does the expression of *Ppras2* preclude the differentiation of amoebae into plasmodia? It is worth mentioning that these two cell types differ markedly in their physiology [23], perhaps the crucial difference being in the mode of nuclear division: open mitosis in amoebae and closed mitosis in plasmodia. Substantial differences concern also the organization and composition of the microtubular apparatus. In certain laboratory strains of *P. polycephalum*, e.g. the CL strain, such transition may be achieved apogamously: individual haploid amoebae change their mode of nuclear division from an open to a closed one, no longer followed by cytokinesis, which eventually leads to clonal formation of a plasmodium. The early stage of this transition, before the first closed karyogamy, is known as the commitment and is induced by some extracellular stimulus, possibly related to cell density (see [23] for references). Consequently, I asked whether the cessation of *Ppras2* expression takes place in committed amoebae.

I assayed the level of the *ras*-family mRNAs during the early stages of the amoeba-plasmodium transition by Northern blotting. As shown in Fig. 1., lanes 4–6, no drop in the level of *Ppras2* mRNA (nor of the other genes studied) is evident in committed cells. For comparison some other stages of the *P. polycephalum* life cycle were included in the analysis. Since Ras proteins are engaged in a variety of cellular responses to extracellular

stimuli, including endocytosis [24], one could expect adjustments in the expression of *ras*-family genes depending on the culturing conditions of an organism. Thus I analyzed surface grown amoebae (feeding on bacteria) and axenically liquid-grown ones, as well as non-feeding flagellates. However, except for the previously noted dramatic difference in the level of *Ppras2* mRNA between amoebae and plasmodia, there were no other changes in expression of the *ras*-family genes. This concerns also the transition from metabolically active, dividing amoebae to non-dividing flagellates. The differences in the content of *Ppras1* and *Pprap1* mRNAs between amoebae

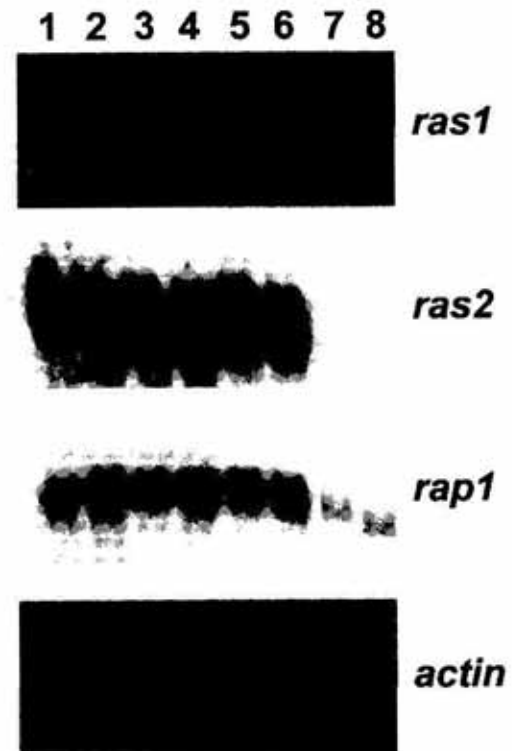


Figure 1. Expression levels of *ras*-family genes in different life-forms of *P. polycephalum*.

Total RNA was prepared from: 1) surface-grown LU353 amoebae; 2) LU352 flagellates; 3) liquid-grown LU352 amoebae; 4) surface-grown CL amoebae; 5) differentiating CL amoebae, 36% committed; 6) differentiating CL amoebae, 60% committed; 7) CL macroplasmodia; 8) CL microplasmodia. Northern blots were probed with the indicated cDNA probes. For *Ppras2* an overexposed autoradiogram is shown to highlight the complete absence of the signal in plasmodia. See text for details.

and plasmodia are much less pronounced than for *Ppras2*. Since Ras are membrane-associated proteins some difference in the abundance of their mRNAs should be expected when the surface-to-volume ratios of the large plasmodium and the microscopic amoeba are taken into account. In addition, the observed differences in the strength of the *Ppras1* and *Pprap1* signals are to a large extent due to the lowered proportion of mRNA in the total RNA preparation from plasmodia, as evidenced by the actin signal. This is most likely due to a higher biosynthetic rate in plasmodia compared with amoebae, reflecting their several-fold shorter cell cycle. A higher relative content of ribosomes (and, hence, of rRNAs) is needed in plasmodia to enable the rapid synthesis of cellular proteins.

Summing up the data presented in this paper, one may conclude that there is no indication for transcriptional regulation of *Ppras1* and *Pprap1* genes during the cell cycle in *P. polycephalum* plasmodia. In contrast, *Ppras2* is expressed selectively in the life cycle of the organism, being inactive in the plasmodia. However, there is no drop in *Ppras2* expression at the early stages of the amoeba→plasmodium transition. All three genes are expressed at a constant level regardless of the culturing conditions.

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