Complete Sequences and Expression Kinetics of racG, racH, racI and racJ Genes in Dictyostelium discoideum

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We sequenced and characterized the expression patterns of the genes (racG, racH, racI and racJ) in the Rho-family. The nucleotide sequences of these genes suggest that racI would be a pseudogene, while the other genes are likely to encode typical Rac proteins which contain either GTP-binding domain or CAAX prenylation motif as observed in other members of the family. The Northern blot analyses show that the expression patterns of these genes are distinctively regulated during development. The racG gene is expressed at almost the same level from the vegetative to the slug stage, but the amount of its transcript gradually decreases after culmination. Expression of the racJ gene is undetectable at the vegetative stage, becomes observable at the mound stage, reaches a peak at the slug stage and then suddenly disappears in the culmination stage. The racH gene is expressed in two forms of transcripts, both of which are undetectable at the vegetatively growing stage but abruptly increase in amount after starvation. Southern blot hybridization analysis demonstrates that these transcripts were derived from a single copy of the gene. Such distinct kinetics of the expression patterns suggests that these genes would have unique roles in Dictyostelium development.

Key words Dictyostelium; small GTPase; rac; rho; prenylation

The cellular slime mold Dictyostelium discoideum is an excellent model organism for understanding the molecular mechanisms underlying cellular motility, development and other cellular functions essential for the higher eukaryotes. This is because the cellular architecture and behavior of this organism is similar to those of higher eukaryotes. D. discoideum cells multiply and develop as amoeboid cells until fruiting bodies are formed. In the vegetative stage, Dictyostelium cells multiply their number feeding on bacteria or taking nutrients by pinocytosis. When starved, these cells change their shape and aggregate into a multicellular structure called a slug, which eventually develops into a fruiting body consisting of a mass of spores at its top supported by a slender stalk.1,2)

We focused on Rac proteins, small GTPases in the Rho-family, in Dictyostelium since these proteins are known to regulate the actin-based cellular architecture, vesicular trafficking and secretion. It has been reported that D. discoideum has fourteen rac genes, rac1A, rac1B, rac1C, racA, racB, racC, racD, racE, racF1, racF2, racG, racH, racI and racJ.3–5) Among these Rac proteins, RacE and RacF1 are well characterized. RacE is essential for cytokinesis but not for phagocytosis, chemotaxis or multicellular development.4,6) By contrast, RacF1 is rather involved in the regulation of actin cytoskeleton rearrangement.5) The last four genes, racG, racH, racI and racJ, are identified in the Dictyostelium EST database (http://www.csm.biol.tsukuba.ac.jp/cDNAproject.html). In this study, we describe their characteristic structures deduced from nucleotide sequences and the expression profiles of these genes during development.

MATERIALS AND METHODS

Gene Screening The cDNAs encoding the rac genes were searched for in the EST database of the Dictyostelium cDNA project. To identify the initiation codon of racI, the 5’-region of the cDNA was amplified by polymerase chain reaction (PCR) from the cDNA library. The PCR machine used was a PCR Thermal Cycler MP (Takara Shuzo). The 1st PCR reaction mixture (20 μl) contained 10 ng template DNA (cDNAs), 2 μM reverse primer (5’-GGAAGCAGCTATGAC-CATG), 2 μM D371-3 primer (5’-CGCCAATGCTGATGAAATCC), 250 μM dNTP, 2.5 mM MgCl2, 0.25 units Ex Taq polymerase (Takara Shuzo) and reaction buffer. The PCR cycle consisted of 2 min at 94 °C followed by 30 cycles of 94 °C for 30 s, 50 °C for 30 s and 72 °C for 30 s. The PCR product was diluted and used as the template for the 2nd PCR. The 2nd PCR reaction mixture (20 μl) contained 1 ng template DNA, 2 μM T3 primer (5’-ATTAACCTCCTACTA-AAG), 2 μM D371-4 primer (5’-CCCCCGGATCTGCTATCATCATATG), 250 μM dNTP, 2.5 mM MgCl2, 0.25 units Ex Taq polymerase and reaction buffer. The reaction conditions of each cycle were 94 °C for 30 s, 50 °C for 30 s and 72 °C for 30 s. DNA fragments were amplified for 30 cycles. The PCR products were digested by Smal and cloned into pUC18. Three independent clones were sequenced.

DNA Sequencing The cDNA clones encoding the rac genes listed in the Table 1 were sequenced as described in the previous report.6) Among them, racI and racJ were sequenced again by an ABI PRISM 310 genetic Analyzer to fill the gaps. The sequencing reactions were performed with an ABI PRISM Cycle Sequencing Kit (PE Applied Biosystems). The RV primer (5’-CAGGAAAACAGCTATGAC), M4 primer (5’-GGTTTCCATGGCAGT), T3 primer, T7 primer (5’-ATAACCTCCTACTA-AAG) and racI specific primer (5’-TCATTGTGATCAGACAAATAATC) were used for the sequencing reaction.

Southern Hybridization Genomic DNA (5 μg) of Dictyostelium cells was digested with restriction enzymes, electrophoresed in 1.0% agarose gel, transferred to Hybond-N+ membrane (Amersham Pharmacia Biotech) and hybridized with the digoxigenin-labeled probe corresponding to the cod-
ing region of the racH gene. The probe preparation and the hybridization were performed as described in the instruction manual of the DIG DNA Labeling kit (Boehringer Mannheim).

Northern Blot Hybridization Total RNA was isolated from the Dictyostelium cells using the TRIZOL reagent (GIBCO BRL) following the manufacturer's instructions. Poly(A)⁺ RNA was purified with the Oligotex-dT30(superscript) (Takara Shuzo). Total RNA (5 μg) or Poly(A)⁺ RNA (3.5 μg) was loaded on a denaturing gel, blotted to Hybond-N+ membrane and hybridized with the 3²P-labeled probes corresponding to the coding region of the rac genes. The probe preparation and the hybridization were performed as described in Molecular Cloning.⁵)

RESULTS AND DISCUSSION

Sequence Analysis of the Rac Proteins The cDNA clones for rac genes identified in the Dictyostelium cDNA project are shown in the Table 1. Fourteen rac genes have been identified in D. discoideum at present but racF1 gene was not found in the EST database.

Since the cDNA clone SSD371 (racI) did not carry the 5'-region of the gene, we recovered the region from the cDNA library as described in Materials and Methods. The nucleotide sequence of the region is presented in Fig. 1 (underlined). The initiation codon (ATG) is not identified in this cDNA suggesting that racI does not encode a protein (Fig. 1). It would be a pseudogene. The N-terminal region of the open reading frame (ORF) of racJ was not published in the EST database. We sequenced cDNA clone SSK168 and the result is shown in the figure.

Figure 2 is an alignment of the amino acid sequences of the Rac family in D. discoideum. The alignment shows that Rac proteins conserve four regions involved in GTP-binding: the sequence GXXXXGKS/T that constitutes the phosphate binding loop L1, the sequence WDTAGQE that interacts with the gamma phosphate, and the N/TKXD and SAK/L sequences⁹) (indicated by thick bars).

Table 1. The cDNA Clones for Thirteen rac Genes Identified in the Dictyostelium cDNA Project

<table>
<thead>
<tr>
<th>Gene</th>
<th>cDNA clone</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>racA</td>
<td>SLB519</td>
<td>(3)</td>
</tr>
<tr>
<td>racB</td>
<td>SSH558</td>
<td>(3)</td>
</tr>
<tr>
<td>racC</td>
<td>SSK690</td>
<td>(3)</td>
</tr>
<tr>
<td>racD</td>
<td>SLK517</td>
<td>(3)</td>
</tr>
<tr>
<td>racE</td>
<td>SSK312</td>
<td>(3)</td>
</tr>
<tr>
<td>racF</td>
<td>SSS409</td>
<td>(3)</td>
</tr>
<tr>
<td>racG</td>
<td>SSS555</td>
<td>(3)</td>
</tr>
<tr>
<td>racH</td>
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<td>(4)</td>
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<tr>
<td>racI</td>
<td>SSB614</td>
<td>(5)</td>
</tr>
<tr>
<td>racJ</td>
<td>SSA135</td>
<td>(5)</td>
</tr>
<tr>
<td>racF2</td>
<td>SSA135</td>
<td>(5)</td>
</tr>
</tbody>
</table>

Fig. 1. The Nucleotide Sequence of racI and racJ

The cDNAs encoding the genes were sequenced as described in Materials and Methods. The regions identified in this work are underlined. The sequence of the ORF and its flanking region of each gene is presented. The nucleotide sequence of the untranslated regions is obtainable from the EST database if necessary.
The RacG, RacH and RacJ proteins ended in a CAAX motif that is found in Rac and many other Ras super family members (indicated by thin bar in the figure). This motif serves as a signal for attachment of lipid moieties that facilitate the association of the proteins with the membrane.

Expression of racG, racH, racI and racJ Genes during Growth and Development

Each of the cDNAs was used as the probe in Northern hybridization to examine their expression in the vegetatively growing cells (v) and developing cells (4—24 h). The transcripts of racH and racI were detected in the total RNA but those of racG and racJ were not because of the low level of expression, so that it was necessary to prepare poly(A)RNA to detect their signals. The result of the Northern blot hybridization is presented in Fig. 3.

The racG gene was expressed in the growing cells (v) and the cells of aggregation to slug stages (4—16 h) and decreased in subsequent stages (20—24 h). The RacG protein would not be important for the final development, the formation of the fruiting body. The size of the transcript (2.3 kb) is longer than that of any other rac genes. The untranslated region in the mRNA might be important for the translation control and/or mRNA stability.

The expression of racH was observed in the cells of the

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**Fig. 2.** Alignment of Rac Proteins Identified in *D. discoideum*

Asterisks indicate residues that are identical between the members of Rac proteins. The conserved four regions involved in GTP-binding and CAAX motif are indicated by thick and thin bars, respectively.

**Fig. 3.** Northern Blot Analysis of Four rac Genes

Total RNA was isolated from the vegetative growing cells (v) and developing cells every 4 h after starvation (4—24 h). Total RNA (racH and racJ) or poly(A)RNA (racG and racJ) was resolved by gel electrophoresis, blotted to membranes and hybridized with the probes corresponding to the coding region of rac genes. The ethidium bromide staining pattern of total RNA is shown (bottom).
aggregation stage (4—8 h) with maximum level and decreased in subsequent stages, thus this gene would be important for the migration of the cells to form the multicellular structure. Aggregation-competent cells are elongated and locomote by extension of pseudopods in the direction of the chemoattractant, a process that depends on reorganization of the cortical actin cytoskeleton; it therefore constitutes a potential target for regulation by RacH. Two signals were detected as shown in the figure. To confirm whether the transcripts were derived from a unique gene or two related genes, the cDNA was used to probe Southern blots of genomic DNA digested with restriction enzymes that did not cut within the cDNA (BclI, EcoRI and HincII). The probe for racH hybridized predominantly to single DNA fragments under high stringency hybridization conditions, suggesting that racH is a single copy gene (Fig. 4). Since the size of the cDNA SSF240 (970 b) is almost the same as that of the smaller signal, we would have cloned the cDNA derived from the smaller mRNA. The difference in the sequence and function of these two transcripts is not known.

The expression of racI was observed only in the vegetatively growing cells (v) but it would not encode protein as described above.

The racJ was expressed at a low level during the early developmental stages (4—12 h) and at a high level at the slug stage (16 h), but was not detected in the growing cells (v) and late developing cells (20—24 h). This gene product might function in the formation and migration of the multicellular structure but not for the endocytosis and cytokinesis.

These observations suggest that these rac genes are important for the growth or development of D. discoideum. To examine their functions, we are planning to establish the knockout and overexpressing strains.

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REFERENCES