CELL STRUCTURE AND FUNCTION 24: 123–129 (1999)

A New Member of the *GP138* Multigene Family Implicated in Cell Interactions in *Dictyostelium discoideum*

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ABSTRACT. **The cellular slime mold *Dictyostelium discoideum* reproduces sexually under submerged and dark conditions. Its mating system is polymorphic and particularly interesting with respect to mechanisms of cell recognition. The cell-surface glycoprotein gp138 has been implicated in sexual cell interactions, as it was identified as a target molecule for the antibodies that block sexual cell fusion in *D. discoideum*. Two mutually homologous genes, *GP138A* and *GP138B*, have been cloned, but gene disruption experiments to clarify their functional relationships suggested that there is at least one more gene for gp138. Further protein analysis including peptide mapping also revealed that gp138 exists as three isoforms, DdFRP1, DdFRP2, and DdFRP3. *GP138A* encodes DdFRP2 and *GP138B*, DdFRP3, and the presence of a third gp138 gene encoding DdFRP1 was suggested.**

Here, we isolated and characterized a third *GP138* gene, *GP138C*. Although the deduced amino acid sequences of *GP138C* matched completely with those of peptide fragments of DdFRP1 in the N-terminal half, the rest did not give complete matches. Overexpression of *GP138C* caused an increase in the intensity of DdFRP1, but disruption of this gene did not diminish DdFRP1. Our results indicate that *GP138C* encodes a protein very similar to but distinct from DdFRP1. The *GP138* multigene family is thus composed of more members than previously expected, and their functional relationships are of special interest.

Key words: *Dictyostelium discoideum*/cell fusion/cell recognition/multigene family

Construction of multicellular structures requires elaborate systems for cell-cell interactions and intracellular signal transduction. The cellular slime mold *Dictyostelium discoideum* is an excellent experimental organism to examine the molecular basis of multicellular construction as it has temporally separated unicellular and multicellular stages in its life cycle (14). In addition, it also reproduces sexually under submerged and dark conditions (6, 17, 18). The mating system of *D. discoideum* includes homothallism and bisexuality (20) as well as widely observed heterothallism (9), making cell recognition mechanisms governing conjugation in this organism particularly interesting.

A cell-surface glycoprotein, gp138, has been implicated in sexual cell interactions as it was identified as a target molecule for antibodies that block sexual cell fusion of *D. discoideum* (24, 25). Two very similar genes, *GP138A* and *GP138B*, have been cloned using oligonucleotide probes corresponding to the N-terminal amino acid sequence of gp138 (10). Expression of antisense RNA for *GP138B* reduced the sexual potency of *Dictyostelium* cells, indicating that one or both of these genes are indispensable for sexual cell fusion in this organism (11). However, gene disruption experiments to clarify their functional relationships suggested that there is at least one more gene for gp138 (29). Further protein analysis including peptide mapping also revealed that gp138 exists as three isoforms with molecular masses of 138, 135, and 130 kD (2). These have been named DdFRP (*D. discoideum* Fusion-Related Protein) 1, DdFRP2, and DdFRP3, respectively. *GP138A* encodes DdFRP2 and *GP138B* encodes DdFRP3. Here,

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Accession number for *GPI38C* is AB016609.

Abbreviations: CRT, competitive reverse transcription; DdFRP, *Dictyostelium discoideum* Fusion-Related Protein; HRP, horseradish peroxidase; MCS, multiple cloning site; ORF, open reading frame; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; RT, reverse transcription.

we isolated a third *GP138* gene, *GP138C*, and examined the possibility that it encodes DdFRP1.

Materials and Methods

Organisms and culture conditions

Heterothallic strains of KAX3 and AX3-ORF$^+$ (16) were used as the wild type parental strains. The derived transformants used in the present study are listed in Table I. All the cells were maintained as fruiting bodies on nutrient SM-agar plates (23) in association with *Klebsiella aerogenes* as a food source, and grown for experiments in HL5 medium (8) containing 50 μg/ml streptomycin sulfate. Transformants were cultured in the presence of G418 (10 μg/ml) and/or blasticidin S (10 μg/ml) according to their drug resistance properties. To induce sexual maturation, cells were suspended in Bonner's salt solution (BSS) (7) containing condensed *K. aerogenes* and cultured at 120 strokes/min in the dark. Sexual fusion competency was determined by incubating the cells with fusion-competent HM1, cells of the opposite mating-type, for 30 min at 22°C (22). The extent of cell fusion was described by fusion index, which corresponded to a percentage of cells fused during this incubation.

Vector constructions

The overexpression vector for *GP138C* was constructed by inserting the full-length *GP138C* cDNA obtained by RT-PCR downstream of the actin 15 promoter of pDXA-HY carrying the *Neo*^{r} gene (16) in the sense orientation (Fig. 3A). The *GP138C* disruption vector (Fig. 4A) was designed to substitute most of the coding region of *GP138C* with the blasticidin S-resistance sequence from pUCBsrΔBam (3). To do this, pBGP138-K4.3R containing the entire coding and 5′ franking regions of *GP138C* was digested with *Spe* I and *Not* I to remove the *Xba* I site in the MCS, and then the 2.0 kb *Xba* I-*Hin* dIII fragment was replaced with the 1.3 kb Blasticidin S-resistance sequence. Next, the 1.5 kb *Eco* RI fragment from pBGP138-Hf2.7 was inserted into the *Eco* RI site within the MCS.

Transformation of D. discoideum cells

Transformation of *D. discoideum* was performed as described previously (29). Briefly, cells at an early growth phase (2×10^7 cells) were mixed with vector DNA (20 μg) in 0.4 ml of an electroporation buffer (5 mM KH_2PO_4, 1.7 mM Na_2HPO_4, 50 mM sucrose) and pulsed with an electric pulser (Biotechnologies & Experimental Research, Inc., San Diego, USA). Transformants were selected in HL5 medium containing G418 (10 μg/ml) (for overexpression) or blasticidin S (for disruption) for 7 to 10 days or until colonies were visible, and then harvested and plaque cloned on a lawn of *K. aerogenes*.

SDS-PAGE and immunoblotting

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed by the method of Laemmli (13) with minor modifications. The proteins separated in the gel were transferred onto nitrocellulose membranes (0.45 μm, Schleicher & Schuell, Dassel, Germany), incubated with EF11, an anti-gp138 monoclonal antibody (1), followed by treatment with horseradish peroxidase (HRP)-conjugated anti-mouse IgG (Jackson Immuno Research Laboratories, Inc., West Grove, USA). The EF11-bound proteins were visualized by the ECL-Western blotting analysis system (Amersham, Buckinghamshire, UK).

Competitive RT-PCR

Competitive RT-PCR (CRT-PCR) was performed by the method of Becker-André and Hahlbrock (4). The RNA competitor was generated by *in vitro* transcription of the DNA competitor, a portion of *GP138C* cDNA with an internal

Table I. *Dictyostelium discoideum* STRAINS USED IN THE PRESENT STUDY

Strain	Genotype[a]	Drug resistance	Reference
KAX3	(Parent)	None	
AX3-ORF$^+$	(Parent)	None	Manstein *et al.*, 1995
A121	*GP138A*$^{++}$	*Neo*^{r}	Aiba *et al.*, 1997
B41	*GP138B*$^{++}$	*Neo*^{r}	Aiba *et al.*, 1997
AB82-9	*GP138A*$^-$, *GP138B*$^-$	*Neo*^{r}, *bsr*^{r}	Yamaguchi *et al.*, 1996
C6	*GP138C*$^-$	*bsr*^{r}	This study
HY3	(pDXA-HY)[b]	*Neo*^{r}	This study
HY4	(pDXA-HY)[b]	*Neo*^{r}	This study
C201	*GP138C*$^{+-}$	*Neo*^{r}	This study
C202	*GP138C*$^{-+}$	*Neo*^{r}	This study
C205	*GP138C*$^{++}$	*Neo*^{r}	This study

[a] ++ and − indicate overexpression and disruption, respectively.
[b] Vector alone transformants.

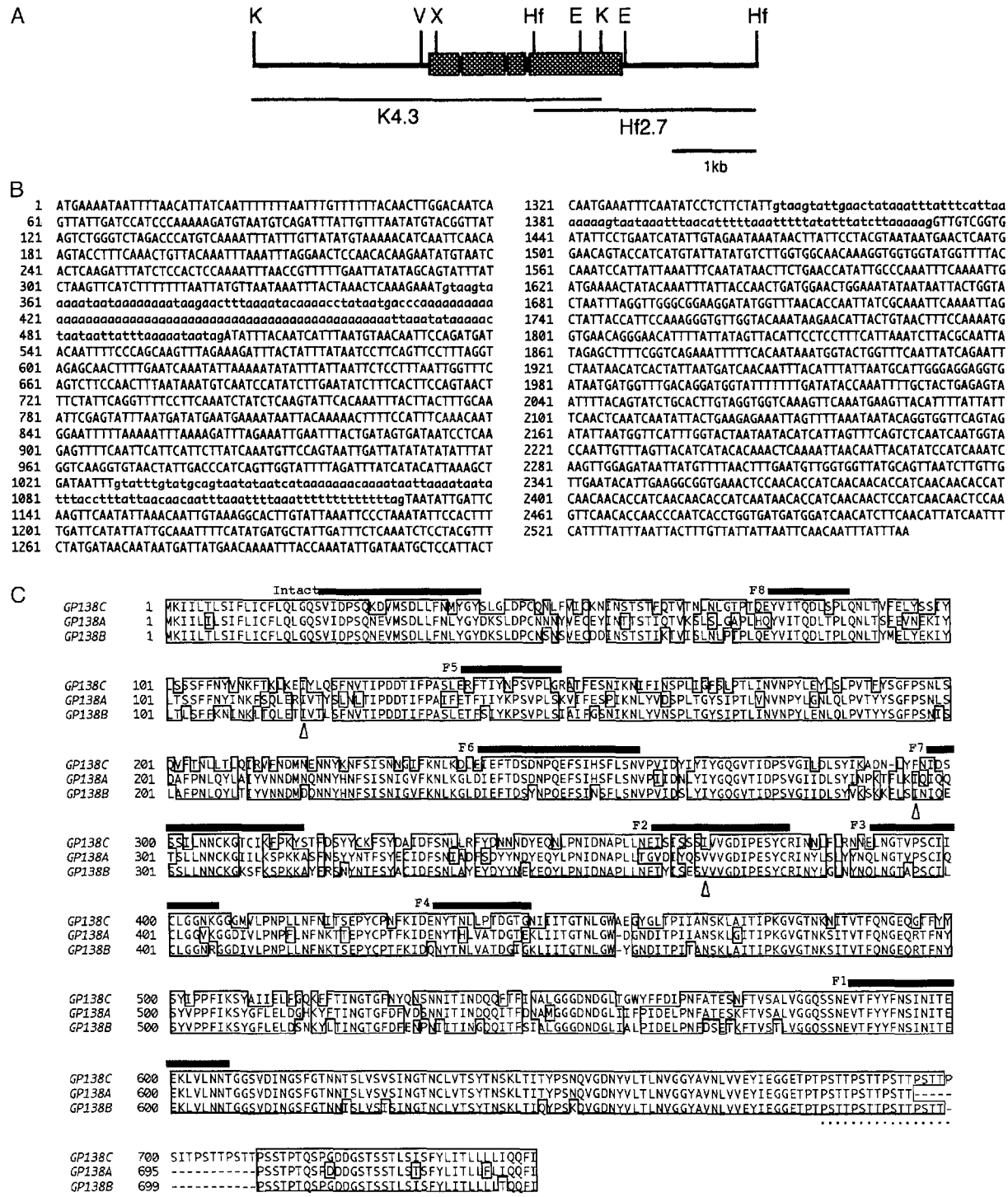

Fig. 1. Structure of *GP138C*. (A) Alignment of K4.3 and Hf2.7 in relation to the genomic structure of *GP138C*. The restriction sites are; E; *Eco* RI, Hf; *Hin* fI, K; *Kpn* I, X; *Xba* I, V; *Eco* RV. (B) The nucleotide sequence of *GP138C*. The sequence between initiation to termination codons is shown. Lowercase letters indicate introns, which were confirmed by sequencing the RT-PCR products. (C) The deduced amino acid sequence of *GP138C* in comparison with those of *GP138A* and *GP138B*. Identical amino acid residues are boxed. The proline-rich repeats are marked with dots. The triangles indicate the positions of introns, and the bars over the sequence show alignment of the peptide fragments derived from DdFRP1.

deletion of 131 bp inserted into the *Eco* RV site of pBluescriptII SK$^+$ (Fig. 5A), with T3 RNA polymerase (RNA Transcription Kit, STRATAGENE, San Diego, USA) according to the instructions of the manufacturer. Total cellular RNA (1.5 μg) and the RNA competitor (ranging from 1.0×10^5 to 1.0×10^9 copies) were mixed and treated with DNase I, and then amplified by PCR using the primers PRCOMP and PROC.

The PCR products were electrophoresed in a 3.6% polyacrylamide gel, stained with ethidium bromide, and photographed. The DNA bands were quantified using the public domain software NIH Image (developed at the U.S. National Institutes of Health and available from the Internet by anonymous FTP from zippy.nimh.nih.gov or on floppy disk from the National Technical Information Service, Springfield, Virginia, part number PB95-500195GEI). The logarithm of density ratio of the competitor band to the target band was plotted versus the logarithm of copies of RNA competitor added to reverse transcription reaction, and the competition equivalence point (log ratio = 0) was obtained to determine the amount of target mRNA.

Results

Molecular cloning and structural analysis of a new GP138 gene

The existence of a third gene for gp138 was suggested by the presence of several bands that hybridized with a *GP138A* sequence in *GP138A* and *GP138B* double knockout mutants (29). Thus, the 2.7 kb *Hin* fI fragment (Hf2.7) which hybridized with the internal *Hin* dIII-*Kpn* I fragment (H-K) of *GP138A* was cloned by screening a *Hin* fI 2.7 kb subgenomic library constructed from a double knockout line, AB82-9. The nucleotide sequence of Hf2.7 contained the region homologous to the 3′ portion of *GP138A* (and *GP138B*). To obtain the upstream region of Hf2.7, an overlapping 4.3 kb *Kpn* I fragment (K4.3) was cloned by chromosome walking (Fig. 1). The nucleotide sequences of Hf2.7 and K4.3 revealed an ORF with a high degree of homology to *GP138A* and *GP138B* (84.1% and 85.0% identity, respectively). This ORF appeared to represent the predicted third gene for gp138, and we named it *GP138C*. The high degree of sequence homology to *GP138A* and *GP138B* was maintained throughout the ORF, except for the C-terminal sequence containing the PSTT repeat; the number of repeats in *GP138C* was 7, whereas *GP138A* and *GP138B* contained 3 and 4 repeats, respectively.

Identification of GP138C product

When the N-terminal amino acid sequences of intact and V8-digested fragments of DdFRP1 determined by Aiba *et al.* (1997) were compared with the deduced amino acid sequence of *GP138C*. Five of 9 N-terminal sequences (Intact, F-4, F-5, F-6 and F-8) showed 100% identities as expected (Fig. 2). However, the rest did not give complete matches, although the levels of identity were still very high. Since *GP138C* did not appear to encode DdFRP1, the overexpression vector pDXA-138C was constructed and introduced into AX3-ORF$^+$ cells to identify its product. Total proteins of resultant transformants were analyzed by SDS-PAGE followed by Western immunoblotting. As can be seen in Figure 3, all of the *GP138C* overexpressers showed an increase in the intensity of the band corresponding to DdFRP1. Nevertheless, the *GP138C* knockout strain C6 still seemed to possess DdFRP1 as determined by immunoblotting (Fig. 4). All these results indicate that *GP138C* encodes a protein very similar in both size and sequence to, but distinct from DdFRP1.

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Intact          VIDPSQKDVMSDLLFNXYGY          100 %
GP138C    21 ***************.***

F-1             XXFYXFNXIQITEXKXVLPN          85.7 %
GP138C   587 ..**.**.*N***.*.**N*

F-2             KYIXYNXXXXDIPEXYF             54.5 %
GP138C   362 IS*.SS....****.*C

F-3             LDGTVPSXIQXLXGVK              76.9 %
GP138C   390 *N*****.*I.*.*N*

F-4             NYTNLLXTDGTG                  100 %
GP138C   434 ******.*****

F-5             RFTIYNPSVPLG                  100 %
GP138C   139 ***********

F-6             IEFTDSDNPQEFSIHSFLSN          100 %
GP138C   241 ********************

F-7             IDSXSILNNQKGTXIKFQKY          88.9 %
GP138C   297 ***.*****C***.***P**

F-8             YVITQDLSPL                    100 %
GP138C    78 **********
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Fig. 2. Alignment of the DdFRP1-derived peptide fragments to the deduced amino acid sequence of *GP138C*. X indicates an unidentified amino acid, and these were omitted from the calculation of percent identity shown on the right of alignments. Asterisks indicate identical amino acids, and dots mark amino acids corresponding to X. Numbers on the left of amino acid sequences of *GP138C* indicate the positions of the first amino acids.

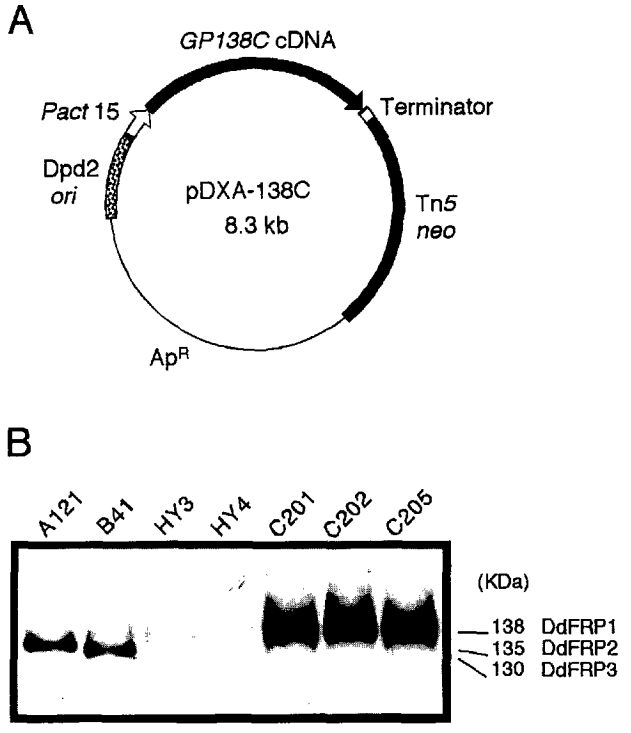

Fig. 3. Overexpression of *GP138C*. (A) The *GP138C* overexpression vector constructed from pDXA-HY. (B) Total proteins obtained from 2×10^5 cells were loaded in each lane of the gel, electrophoresed, and processed for immunoblotting. For explanation of strain names, see Table I.

Relevance of GP138C to sexual cell interactions

To investigate the possible role of *GP138C* in gamete interactions, we determined its expression pattern during sexual maturation of KAX3. Since the members of the *GP138* multigene family were very similar in sequence and size, Northern blotting analysis was not performed, but the CRT-PCR was performed instead (Fig. 5). The mean amount of *GP138C* mRNA was estimated to be about 5×10^7 copies in 1.5 μg of total RNA obtained from 1×10^7 asexually growing KAX3 cells. Although level of *GP138C* transcript was not increased in fusion-competent as compared to fusion-incompetent cells, it showed an interesting temporal change. That is, the level of *GP138C* mRNA decreased to 1/5 during the first 3 h of culture for sexual maturation, increased by 3-4-fold in accordance with the increase in the sexual fusion competence, and then reached a plateau.

The sexual potency of all overexpressers and a disruptant did not show remarkable changes, forming normal zygotes and macrocysts (data not shown). Thus, the critical importance of *GP138C* in sexual cell interactions is currently uncertain.

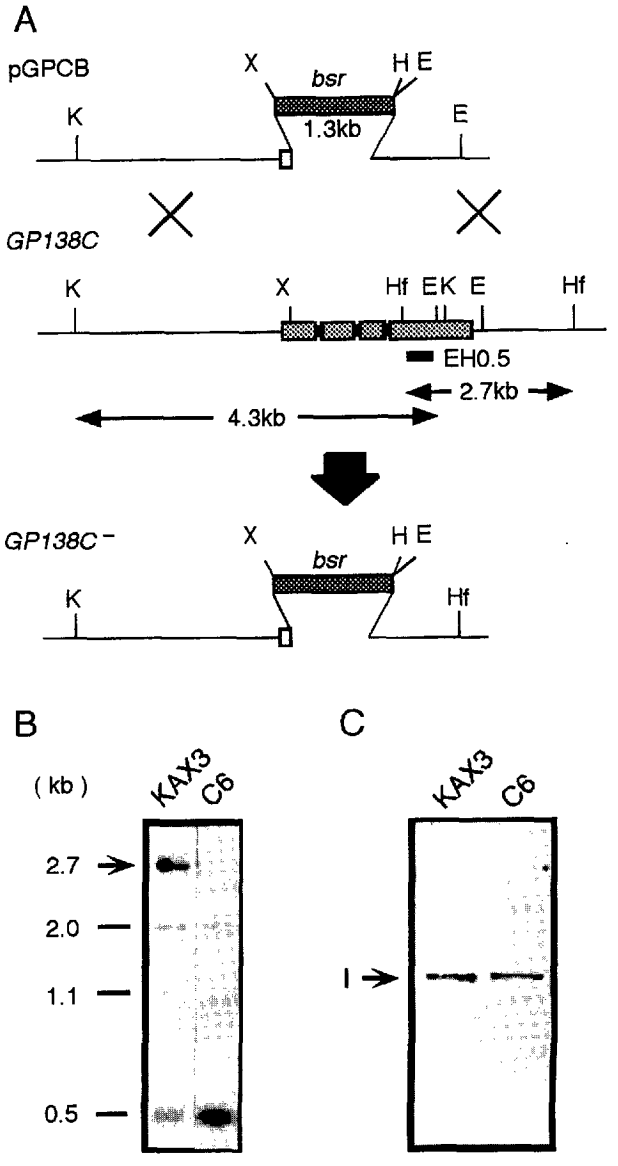

Fig. 4. Disruption of *GP138C*. (A) Homologous recombination between *GP138C* and the disruption vector, pGPCB, is shown. After recombination, *Hin* fI site in the exon 4 of *GP138C* is lost and Hf2.7 disappears from the chromosome sequence. EH0.5 shows the position of an *Eco* RI-*Hin* fI 0.5 kb fragment used as a probe for hybridization. The restriction sites are: E; *Eco* RI, H; *Hin* dIII, Hf; *Hin* fI, K; *Kpn* I, X; *Xba* I. (B) Southern hybridization analysis to confirm disruption of *GP138C*. Genomic DNA from KAX3 and the disruptant strain C6 was digested with *Hin* fI, electrophoresed, and processed for Southern blotting. The membrane was probed with EH0.5. The 2.7 kb band corresponding to the 3′ portion of *GP138C* was missing in C6. (C) Western analysis to detect gp138. Total cellular proteins (1×10^5 cells/lane) were electrophoresed and processed for immunoblotting. The arrowhead indicates the position of DdFRP1.

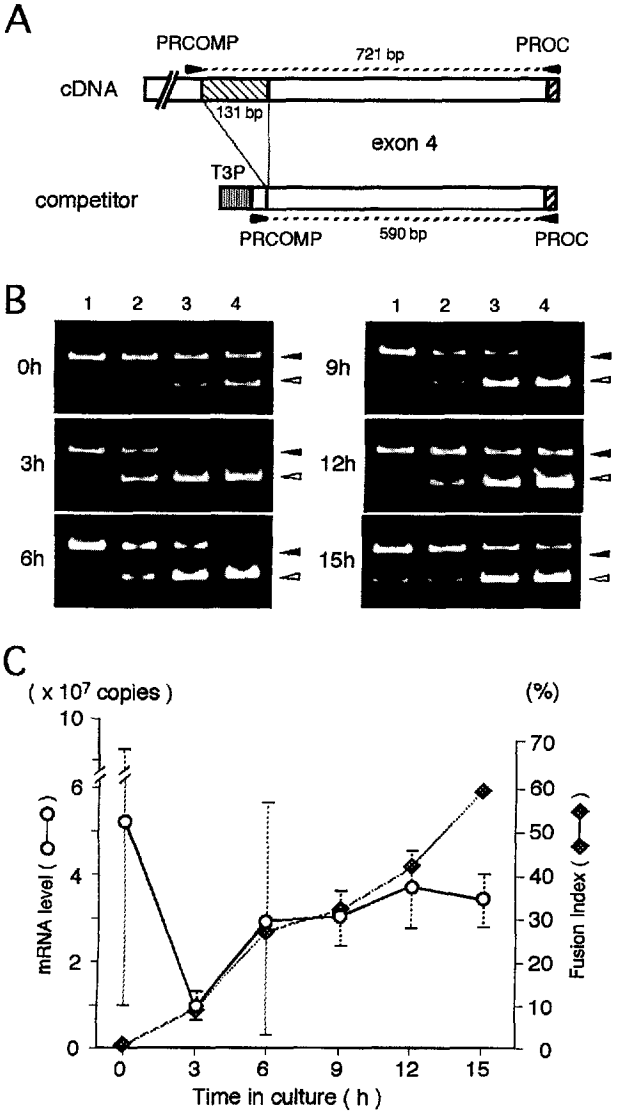

Fig. 5. Expression pattern of *GP138C* determined by CRT-PCR. (A) The DNA competitor was made by inserting truncated exon 4 of *GP138C* generated using PRCOMP downstream of the T3 promoter. Competitor RNA was produced by in vitro transcription. RT-PCR using PROC and PRCOMP yielded 721 bp and 590 bp DNA for *GP138* mRNA and competitor RNA, respectively. (B) KAX3 cells were cultured in liquid medium in the dark to induce sexual maturation. At the indicated time points, small samples were taken to obtain the fusion indices and RNA was prepared from the rest of the culture. The reaction products of CRT-PCR were analyzed on 3.6% polyacrylamide gels. Closed and open triangles indicate the positions of *GP138C* mRNA-derived and competitor RNA-derived products, respectively. Amounts of competitor RNA in lanes 1 to 4 were 5×10^6, 1×10^7, 5×10^7 and 1×10^8 copies, respectively. (C) Fusion index and calculated *GP138C* mRNA level in 1.5 μg total RNA are plotted vs. time in maturation culture.

Discussion

In the present study, we cloned the third *GP138* gene. Since gp138 consists of three isoforms, DdFRP1, DdFRP2 and DdFRP3, and *GP138A* and *GP138B* encode DdFRP2 and DdFRP3, respectively (2), this gene, *GP138C*, was first expected to encode DdFRP1, but turned out to encode a protein very similar to, but distinct from DdFRP1. Since DdFRP1, which was originally regarded as a single protein now appears to include at least two distinct proteins, we renamed the original DdFRP1α to distinguish it from the new protein encoded by *GP138C*; the latter was named DdFRP1β.

GP138C shows two unique properties. First, the amino acid sequence of its product, DdFRP1β, within the exons 1 and 2 shows 100% matches with those of "DdFRP1" fragments, while those within exons 3 and 4 showed less perfect matches. Since the N-terminal portion of gp138 is assumed to interact with other cells (10), conservation in this region suggests that DdFRP1β interacts with the same molecules as DdFRP1α. Although it is also possible that the peptide fragments of "DdFRP1" used for determination of the amino acid sequence were mixtures of DdFRP1α and DdFRP1β, the clustered distribution of 100%-match fragments in the N-terminal half of the ORF suggested that this was unlikely.

Second, the expression pattern of *GP138C* determined by CRT-PCR was very different from those of *GP138A* and *GP138B*. While the latter two genes are simply induced or repressed by cellular sexual maturation (10), the expression of *GP138C* was first decreased and then increased with the sexual maturation of cells. Several genes in *D. discoideum* have been shown to increase or decrease in expression level by transferring the cells into submerged conditions (Iijima and Yamamoto, personal communication). *GP138C* seems to be involved in such a gene group, responding to the aqueous environment. The above differences in expression pattern among *GP138* genes may have some implications in the control of sexual maturation.

The present study revealed that the *GP138* multigene family has at least 4 members, *GP138A*, *GP138B*, *GP138C* and the gene encoding DdFRP1α. Several other multigene families have been reported in *D. discoideum*. These include those encoding cytoskeletal components such as actin (21) and myosin (26, 27), and signaling elements such as protein kinases (12). In addition to these relatively large families, small families such as gp24 contact site B (15), thioredoxins (28), and cytochrome c oxidase subunits (5) have been reported. The latter group may have arisen by accidental duplication of chromosome regions. The former large families may involve further functional differentiation as well as

a backup system. There are an increasing number of reports concerning gene disruption in *D. discoideum* without notable effects on phenotypes. This may be partly due to the sensitivity of detection system as is shown in the case of csA (19). Further and careful examinations are required to determine the structural and functional relationships among members of the *GP138* multigene family.

Acknowledgments. We are grateful to Dr. D.J. Manstein at Max Planck Institute for kindly sending us pDXA-HY. This work was supported by a grant in aid from the Ministry of Education, Science, Sport and Culture of Japan to H. Urushihara (#09264203).

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(Received for publication, April 2, 1999
and accepted, April 27, 1999)