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Isoforms of gp138, a Cell-Fusion Related Protein in *Dictyostelium discoideum*[1]

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Sexual development of *Dictyostelium discoideum* is a unique and useful system for the study of sexual phenomena. We have been studying molecular mechanisms of sexual cell fusion in *D. discoideum* and have identified several relevant cell-surface proteins. One of the proteins, gp138, was identified as a target molecule for fusion-blocking antibodies, and two genes for gp138, *GP138A* and *GP138B*, were cloned. The participation of gp138 in the sexual cell fusion was confirmed by antisense RNA mutagenesis, but it is unclear which of the genes encodes gp138. Moreover, the presence of a third gene for gp138 was indicated by gene disruption. In the present study, we generated strains of *D. discoideum* overexpressing either *GP138A* or *GP138B* to investigate the products of these genes. The transformants overexpressing *GP138A* and *GP138B* overproduced glycoproteins with molecular masses of 135 and 130 kDa, respectively. Although their molecular masses were different from that of gp138, the results of peptide mapping and amino acid sequencing showed that they are related proteins, suggesting that the proteins encoded by *GP138A* and *GP138B* are isoforms of gp138 protein.

Key words: cell-surface glycoprotein, cell-fusion related protein, *Dictyostelium discoideum*, sexual cell fusion.

Dictyostelium discoideum has two alternative developmental modes, asexual and sexual, and each is determined by environmental conditions (*1, 2*). In the sexual cycle, called macrocyst formation, *D. discoideum* cells acquire cell-fusion competence under conditions such as darkness and excess water (*3, 4*), and then undergo sexual cell fusion (*3, 5*). The resultant zygotes, called giant cells, phagocytose surrounding cells and develop into macrocysts (*6-8*).

We have identified several cell-surface proteins responsible for sexual cell fusion using heterothallic strains of *D. discoideum* (*9-11*). One of these proteins gp138, was identified as a cell-surface antigen for cell-fusion blocking antibodies (*10*), and partially purified and characterized (*12*). We recently cloned two candidate genes for gp138, *GP138A* and *GP138B*, whose nucleotide sequences are very similar to each other (*13*). The expression of antisense RNA for *GP138B* cDNA resulted in the repression of gp138 production and of the sexual cell fusion (*14*), indicating that gp138 is indispensable for the sexual cell fusion of *D. discoideum*. To investigate whether *GP138A* or *GP138B*, or both, are responsible for the sexual cell fusion, *GP138A* and *GP138B* were each disrupted by homologous recombination (*15*). The results, however, showed that sexual cell fusion occurred normally and that the level of gp138 protein was unchanged even in a double knockout mutant. Analysis of the double knockout mutant indicated the presence of a third gene for gp138 (*15*). Gene mapping analysis carried out by Kuspa and Loomis (*16*) also indicated that the *Dictyostelium* genome has other possible gp138 genes besides *GP138A* and *GP138B*.

However, it is unknown whether the gp138 protein is encoded by all gp138 genes. If *GP138A* and *GP138B* encode gp138, the third gp138 gene, which is predicted from the results of gene disruption by Yamaguchi *et al.* (*15*), will need to be expressed at a higher-than-ordinary level to maintain the gp138 level in the double knockout mutants, since the gp138 level is normal even in them. In the present study, we generated strains of *D. discoideum* overexpressing *GP138A* or *GP138B* to analyze whether these genes encode gp138. Our results show that the products of *GP138A* and *GP138B* are not gp138 itself, but its isoforms.

MATERIALS AND METHODS

Strains and Culture Conditions—Heterothallic strains of *D. discoideum*, KAx3 (mating type; *mat A*) and KAx3-derived *GP138A*- (A82), *GP138B*- (B4), and a double-deficient strain (AB82-9) (*15*) were used. Cells of these strains were cultured in Bonner's salt solution (BSS) (*17*) with *Klebsiella aerogenes* or in HL5 medium. For transformation experiments, KAx3 cells grown in HL5 medium were used.

Vector Construction and Transformation—The full-length cDNA for *GP138A* and *GP138B* (*13*) were inserted

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into the *Bgl*II site between the actin 15 promoter and 2H3 terminator of the vector BS18·2H3 (*18*) (Fig. 1). The orientation of the insert relative to the promoter was identified by restriction cleavage analysis. The transformation vectors thus constructed were introduced into KAx3 cells by electroporation, and G418-resistant clones were isolated as described by Fang *et al.* (*14*).

Gel Electrophoresis and Immunoblotting—Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed by the method of Laemmli (*19*) with minor modifications. For immunoblotting, proteins in the gel were transferred to a nitrocellulose membrane (0.45 μm, Schleicher & Schuell, Germany), which was then incubated with EF11, an anti-gp138 monoclonal antibody (*20*). The antibody-bound proteins were visualized by horseradish peroxidase (HRP)-conjugated anti-mouse IgG (Jackson) and an ECL-Western blotting detection system (Amersham, UK).

Labeling Cell-Surface Protein and Immunoprecipitation—Cell-surface proteins were labeled with biotin according to the procedure of Ingalls *et al.* (*21*) with minor modifications. *Dictyostelium* cells were suspended at 4×10^7 cells/ml in Sorensen's buffer, pH 8.1 (Sorensen's buffer, pH 6.1, raised to pH 8.1 with 6 N NaOH) and incubated with 0.4 mg/ml NHS-LC-biotin (Pierce) for 30 min on ice. After labeling, the cells were washed with Sorensen's buffer (pH 6.1) and were solubilized with a lysis buffer (1% Nonidet P-40, 0.5% sodium deoxycholate, 1 mM phenylmethylsulfonyl fluoride, 0.15 M NaCl, 50 mM Tris-HCl, pH 7.5). Immunoprecipitation was performed as described by Sambrook *et al.* (*22*). Nonspecifically binding molecules in the lysate were absorbed using mouse preimmune serum and fixed *Staphylococcus aureus* cells (Hoechst), and then a target protein in the lysate was precipitated with protein A-Sepharose (Sigma) and EF11. The precipitated proteins were subjected to SDS-PAGE, and biotin-labeled proteins were detected with HRP-conjugated streptoavidin (Amersham) and the ECL-Western blotting detection system.

Purification of gp138—gp138 was partially purified using a wheat germ agglutinin (WGA) affinity column as described previously (*20*). For further purification, an immunoaffinity chromatography was applied. The partially purified WGA-bound fraction containing gp138 was preabsorbed by loading onto a mouse IgG-protein A-Sepharose matrix to remove nonspecifically binding components, and then loaded onto an immunoaffinity column of EF11-protein A-Sepharose matrix that had been cross-linked with dimethyl pimelimidate dihydrochloride (Pierce) (*23*). Antigens specifically bound to the matrix were eluted with 50 mM diethylamine (pH 11.2). The eluted material was immediately brought to near neutrality by the addition of 1/10 volume of 0.5 M NaH_2PO_4.

Peptide Mapping—Protein digestion for peptide mapping was done by the method of Cleveland *et al.* (*24*). The purified proteins were separated in a gel for SDS-PAGE and were stained with Coomassie Blue. The individual bands were cut out and digested with a given amount of the *S. aureus* V8 protease (Wako) in a stacking gel at 37°C for 60 min. If the samples were to be sequenced, 0.002% thioglycolic acid (Sigma) was added to the upper electrophoresis buffer, and sequence-grade V8 protease (Boehringer Mannheim, Germany) was used. The digestion products were electrophoresed and visualized by a modified silver staining procedure (*25*).

Amino Acid Sequencing—The amino acid sequencing of the immunopurified proteins and V8-digested peptides of gp138 were determined. After electrophoresis, proteins in the gel were transferred to a PVDF membrane (Immobilon-P, Millipore) and stained with Coomassie Blue according to the method of Matsudaira (*26*). Sequence analysis was carried out on an Applied Biosystems Procise sequencer.

RESULTS

Protein Products from GP138A and GP138B—The transformation vector BS18·2H3 with or without cDNA insertion was transformed into KAx3 cells, and G418-re-

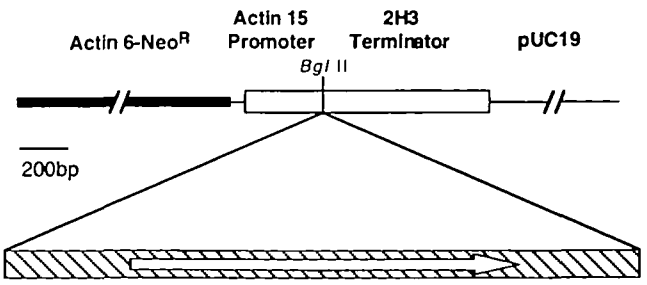

Fig. 1. **Partial map of the vector used for overexpression of gp138 genes.** *GP138A* cDNA or *GP138B* cDNA was inserted into the unique *Bgl*II site of the vector BS18·2H3, between the actin 15 promoter and the 2H3 terminator. The orientation of the insert relative to the promoter is shown by the arrow. Actin 6-Neo^R cassette encodes G418 resistance, the selectable marker for DNA-mediated transformation.

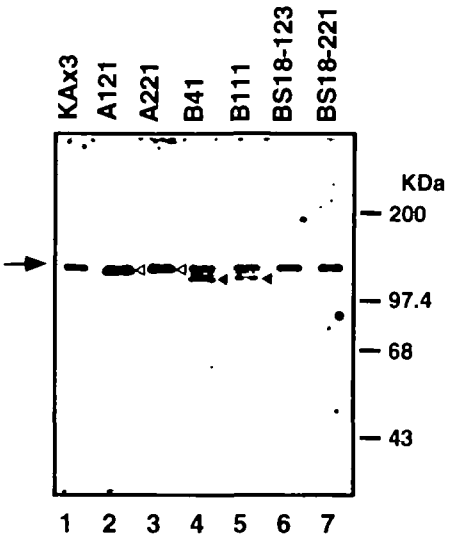

Fig. 2. **Immunoblot analysis of overexpression of *GP138A* and *GP138B*.** Parent cells (KAx3), *GP138A*-overexpressers (A121 and A221), *GP138B*-overexpressers (B41 and B111) and vector-alone transformants (BS18-123 and BS18-221) were cultured in a liquid medium with bacteria. Whole proteins obtained from 1×10^5 cells were subjected to SDS-PAGE and transferred to a nitrocellulose membrane for immunostaining with EF11. The arrow indicates the position of gp138. The open and solid triangles indicate the 135 kDa protein and the 130 kDa protein, respectively.

sistant clones were isolated. Among them, the *GP138A*-overexpressers A121 and A211, the *GP138B*-overexpressers B41 and B111, and the vector-alone transformants BS18-123 and BS18-221 were used for further studies.

To detect the overexpressed products of *GP138A* and *GP138B*, the total proteins of bacterially grown cells were separated by SDS-PAGE, transferred to a nitrocellulose membrane, and then reacted with EF11, the anti-gp138 monoclonal antibody (Fig. 2). While gp138 was detected as a single band in parental KAx3 and control transformants (lanes 1, 6, and 7), a 135 kDa protein in the *GP138A*-overexpressers and a 130 kDa protein were detected in the *GP138B*-overexpressers, along with gp138, at comparable

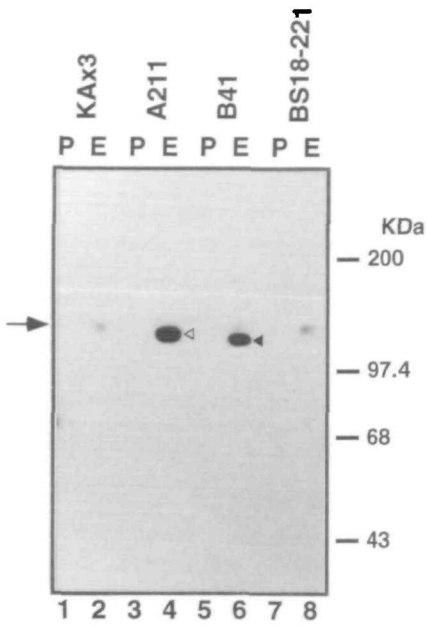

Fig. 3. **Cell-surface expression of *GP138A* and *GP138B* products.** Bacterially grown cells of KAx3, A121, B41, and BS18-221 were surface-labeled with NHS-LC-biotin. The precipitated proteins from 2.5×10^6 cells with mouse preimmune serum (P) or EF11 (E) were subjected to SDS-PAGE. They were transferred to a membrane and detected with HRP-conjugated streptoavidin. The arrow indicates the position of gp138. The open and solid triangles indicate the 135 kDa protein and the 130 kDa protein, respectively.

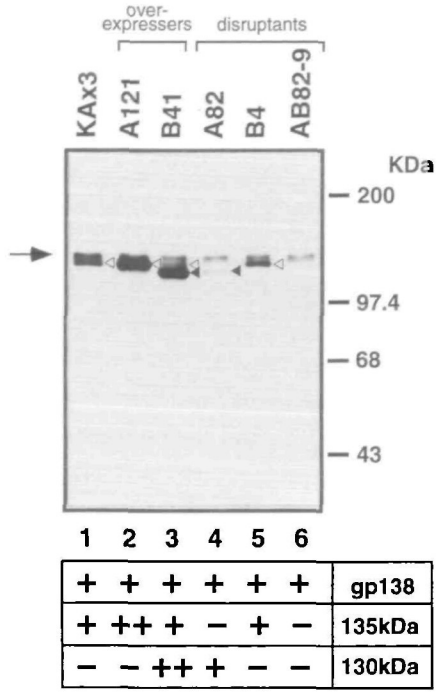

Fig. 4. **Detection of *GP138A* and *GP138B* products in axenically grown cells.** Whole proteins obtained from axenically grown cells (1×10^5 cells) of KAx3, transformants overexpressing gp138 genes (A121 and B41), and transformants with disrupted gp138 genes (A82 as *GP138A*-minus, B4 as *GP138B*-minus, and AB82-9 as both-minus) were subjected to SDS-PAGE. They were transferred to a membrane and immunostained with EF11. The arrow indicates the position of gp138. The open and solid triangles indicate the 135 kDa protein and the 130 kDa protein, respectively.

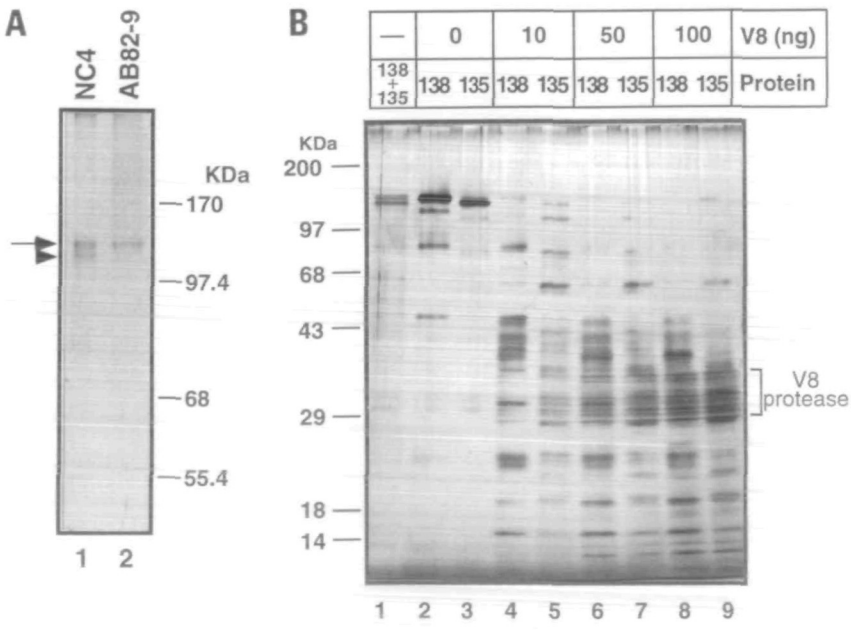

Fig. 5. **Immunopurification and peptide maps of gp138 and the 135 kDa protein.** (A) Antigens were purified from $4\text{-}7\times10^9$ cells of NC4 (a parent strain of KAx3) or AB82-9 (both *GP138A* and *GP138B*-deficient cells) as described in "MATERIALS AND METHODS." They were subjected to SDS-PAGE and stained with silver. The arrow and arrowhead indicate the positions of gp138 and the 135 kDa protein, respectively. (B) gp138 (138) and 135 kDa protein (135) purified with an EF11-immunoaffinity column were digested with 0, 10, 50, or 100 ng of *S. aureus* V8 protease in a stacking gel and separated in a separating gel. The digested products were stained with silver. The bands of V8 protease are shown in brackets.

intensity (lanes 2-5). Although the separation of the 135 kDa protein and gp138 is difficult to discern in Fig. 2 because of the overexpression of the 135 kDa protein (lanes 2 and 3), the faster mobility of the band is evident. The amount of gp138 in the overexpressers appeared to be at the same level as that of controls. Thus, the overexpression products of *GP138A* and *GP138B* were smaller in size than gp138. There are two possible causes of the difference in size. The first is that the overexpression products are premature forms of gp138, and the second is that those genes encode distinct proteins from gp138.

The first possible cause was investigated as follows. Most cell-surface proteins are known to be processed by several modifications, such as glycosylation, prior to expression on the cell surface as mature proteins. Therefore, the overexpression products were examined as to whether they would be expressed on the cell surface. After cell-surface labeling with biotin, immunoprecipitation was performed (Fig. 3). While biotin-labeled gp138 alone was detected in the controls KAx3 and BS18-221 (lanes 2 and 8), massive amounts of the 135 kDa protein and the 130 kDa protein were detected in a *GP138A*-overexpresser A221 and a *GP138B*-overexpresser B41, respectively, in addition to a small amount of gp138 (lanes 4 and 6). These bands were not nonspecific, because no signal was detected in the sample precipitated by mouse preimmune serum (lanes 1, 3, 5, and 7). Each immunoprecipitate was recognized with a lectin, WGA, indicating that they were glycoproteins (data not shown). These results suggested that the overexpression products might be mature forms.

To investigate the second possible cause of the size difference, *GP138A*- or *GP138B*-overexpressers and -disruptants were cultured in HL5 axenical medium and their total proteins were subjected to SDS-PAGE followed by immunoblotting (Fig. 4). We found that two EF11-reactive proteins, gp138 and a protein with a molecular mass of 135 kDa, were detected in KAx3 cells cultured in HL5 medium (Fig. 4, lane 1), whereas gp138 alone was detected in KAx3 cells cultured in BSS medium containing bacteria (Fig. 2, lane 1). The mobility of the overproduct in a *GP138A*-overexpresser, A121 in SDS-PAGE coincided with that of the 135 kDa protein in KAx3 cells (Fig. 4, lanes 1 and 2). In contrast, an axenically grown *GP138A*-disruptant, A82, possessed two EF11-reactive proteins, gp138 and 130 kDa protein, but not the 135 kDa protein, which was detected in KAx3 cells and a *GP138A*-overexpresser (Fig. 4, lane 4). These results demonstrated that *GP138A* undoubtedly encodes the 135 kDa protein. In a *GP138B*-overexpresser, B41, EF11 detected three bands, gp138, a 135 kDa protein and a predominant 130 kDa protein (Fig. 4, lane 3). The same bands as those observed in the KAx3 cells were detected in a *GP138B*-disruptant, B4 (Fig. 4, lane 5). In a double knockout strain, AB82-9, gp138 was detected by EF11 as the only band (Fig. 4, lane 6). These results demonstrated that the *GP138B* product is certainly the 130 kDa protein, and it appears to be complementarily expressed in a *GP138A*-disruptant in place of the *GP138A* product (Fig. 4, lane 3).

Structural Similarity of gp138, 135 kDa Protein and 130 kDa Protein—Since the 135 kDa protein and the 130 kDa protein were reactive to EF11, an anti-gp138 monoclonal antibody, the three proteins seem to be similar in structure. This was confirmed by the following two series of experiments, *i.e.*, peptide mapping and amino acid sequence analysis.

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Intact          VIDPSQKDVMSDLLFNXYGY
pGP138A    21   ******NE********L****
pGP138B    21   ******NE********L****

F-1             XXFYXFNXIQITEXKXVLPN
pGP138A   587   VT**Y**S*N****E*L**N*
pGP138B   587   VT**Y**S*N****E*L**N*

F-2             KYIXYNXXXXDIPEXYF
pGP138A   363   VD*YQSVVVG****S*C
pGP138B   363   IY*SESVVVG****S*C

F-3             LDGTVPSXIQXLXGVK
pGP138A   391   *N**V**C*QC*G*VK
pGP138B   391   *N**A**C*LC*G*NR

F-4             NYTNLLXTDGTG
pGP138A   435   ***H*VA***TE
pGP138B   435   ***N*VA***IG

F-5             RFTIYNPSVPLG
pGP138A   139   T*T**K*****S
pGP138B   139   T*S**K*****S

F-6             IEFTDSDNPQEFSIHSFLSN
pGP138A   241   ******D********H******
pGP138B   241   ******Y********N******

F-7             IDSXSILNNQKGTXIKFQKY
pGP138A   298   *QQT*L***C**IIL*SP*K
pGP138B   298   *QES*L***C**KSF*SP*K

F-8             YVITQDLSPL
pGP138A    79   *******T**
pGP138B    79   *******T**
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Fig. 6. **Comparison of amino acid sequences among gp138, *GP138A* product, and *GP138B* product.** The N-terminal and internal amino acid sequences of gp138 were determined as described in "MATERIALS AND METHODS." Intact and F indicate undigested gp138 and the V8-proteolytic fragments derived from gp138, respectively. An X indicates that a determination could not be made at a given position. These sequences, determined by amino acid sequencing, were compared with the sequences predicted from cDNAs of *GP138A* and *GP138B*. p*GP138A* and p*GP138B* indicate *GP138A* product and *GP138B* product, respectively. The numbers at p*GP138A* and p*GP138B* are the beginning numbers in the predicted amino acid sequences. Asterisks show identical amino acid residues among the three proteins.

When EF11-reactive proteins were recovered from bacterially grown NC4 cells by EF11-immunoaffinity chromatography, gp138 and the 135 kDa protein were copurified (Fig. 5A, lane 1). Using NC4-derived KAx3 cells, the same result was obtained as with NC4 cells (data not shown). In contrast, gp138 alone was purified from the double knockout cells, AB82-9 (lane 2). These results

TABLE I. **Homology between gp138 and 135 and 130 kDa protein.**

gp138	135 kDa protein		130 kDa protein	
	Identity (%)	Similarity (%)	Identity (%)	Similarity (%)
Intact	85	90	85	90
F-1	60	60	60	60
F-2	35	41	41	47
F-3	75	81	50	63
F-4	67	83	75	92
F-5	75	92	75	92
F-6	100	100	90	95
F-7	45	70	45	75
F-8	90	100	90	100

indicated that the copurified 135 kDa protein was a *GP138A* product, but not a degradation product of gp138. The bands corresponding to gp138 and the 135 kDa protein were cut from the first gel of SDS-PAGE, and subjected to partial proteolysis with 0, 10, 50, or 100 ng of *S. aureus* V8 protease with high specificity in a second gel, as described in "MATERIALS AND METHODS" (Fig. 5B). Although there were a few nonspecifically degraded fragments, the cutting of two bonds was accomplished without mutual contamination (lanes 1-3). As the amount of V8 protease increased, peptide fragments with identical mobility below 24 kDa arose from gp138 and the 135 kDa protein. This result demonstrated that there is considerable similarity in structure between gp138 and the 135 kDa protein.

The N-terminal amino acid sequences of intact gp138 and its V8-proteolytic fragments were determined and compared to those of the 135 and 130 kDa proteins deduced from the nucleotide sequences of *GP138A* and *GP138B*, respectively (Fig. 6 and Table I). All the obtained sequences were highly homologous to the 135 and 130 kDa proteins, except for the fragment F-2, where many amino acids were left undetermined. The above results demonstrate that the three proteins are very similar to each other and can be regarded as isoforms.

DISCUSSION

The present results clarified the relationships among *GP138A*, *GP138B*, and gp138. That is, *GP138A* and *GP138B* code for the 135 kDa protein and 130 kDa protein, respectively, and gp138 is encoded by a distinct gene. These three proteins are structurally very similar and immunologically cross-reactive, suggesting their functional relatedness. Thus, we may refer to these isoforms as DdFRPs (*D. discoideum* Fusion-Related Proteins); gp138, the 135 kDa protein and the 130 kDa protein being renamed DdFRP1, DdFRP2, and DdFRP3, respectively. The existence of these DdFRP isoforms accounts for all of the previous results, especially those reported by Yamaguchi *et al.* (*15*), in which the level of gp138 (DdFRP1) was not altered in double knockout mutants. The finding that the expression of antisense RNA for *GP138B* repressed the gp138 level and cell fusion is also clarified, since the genes for DdFRPs are mutually similar and would be expected to be repressed by the same antisense RNA as in the case of discoidin I (*27*).

The *GP138A* product, DdFRP2, was virtually undetected in bacterially grown cells by an anti-gp138 monoclonal antibody, although *GP138A* mRNA is present in them (*13*), whereas it was detected in axenically grown cells in the present study. The reason for this differential expression is unclear at present, but it is possible that partial starvation in the axenic medium may enhance the expression, as in the case of discoidin I genes, which are expressed in axenically grown cells, but not in bacterially grown cells (*28, 29*). Conversely, the *GP138B* product, DdFRP3, was not detected in either bacterially or axenically grown cells. It could only be detected in axenically grown *GP138A*-deficient cells, except for the *GP138B*-overexpressers, probably reflecting the low level of its expression.

Browning *et al.* recently identified gp130, a WGA-binding glycoprotein that was detected in association with the sexual development of *D. discoideum* (*30, 31*). Although this protein and DdFRP3 are similar in that both have WGA-binding sugar chains and the same molecular mass, we do not consider them to be identical, for the following reasons. First, their expression levels are different; the former is detected while the latter is not detected by the lectin staining. Second, their expression period is different; *GP138B* is expressed in vegetative cells (*13*), while the gp130 identified by Browning *et al.* is expressed after cell fusion (*30, 31*).

What are the structures of DdFRPs and what roles do they play in cell fusion? Gerisch's group recently determined the nucleotide sequence of a gene encoding a 130 kDa protein, a putative adhesion molecule (*32, 33*), and the gene was found to be identical to *GP138B* (Gerisch, G., personal communication). Hence, the other DdFRPs may be adhesion molecules. In the light of the DdFRPs' role in cell fusion, it is worth noting that they seem to exist only in cross-mateable *D. discoideum* strains, *i.e.*, heterothallic and bisexual strains (*20, 34*, Higuchi *et al.*, unpublished data). The functional relationship among DdFRPs is interesting, since they are similar to one another, even though the overexpression of DdFRP2 or DdFRP3 in KAx3 cells did not affect sexual cell fusion (data not shown). Their relationship should be clarified by further studies, such as cloning and analysis of the gene encoding DdFRP1. Although the strong similarity of DdFRP genes has hampered the cloning of DdFRP1 gene from KAx3, use of the double knockout strain should make it feasible.

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