Antisense RNA inactivation of gp138 gene expression results in repression of sexual cell fusion in *Dictyostelium discoideum*

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SUMMARY

A glycoprotein, gp138, is implicated in the sexual cell fusion of *Dictyostelium discoideum*. We previously cloned and sequenced the two genes encoding the gp138 protein, *GP138A* and *GP138B* (Fang et al. (1993) *Dev. Biol. 156, 201-208*). Here, we have constructed a vector producing antisense RNA for the gp138 genes and have transformed the vector into *Dictyostelium* cells. The transformed cells showed a reduction in the amounts of gp138 mRNA and protein and their sexual cell fusion activity was considerably repressed.

Key words: sexual cell fusion, *Dictyostelium*, cell surface glycoprotein

INTRODUCTION

Sexual fusion takes place between gametes in multicellular higher organisms. Eggs and sperm in a species recognize each other and fuse to form zygotes. In unicellular organisms such as cellular slime molds, however, sexual fusion takes place when cells are converted from a somatic-cell-like state into a gamete-like state.

In *Dictyostelium discoideum*, this conversion occurs under certain environmental conditions. Haploid cells of two opposite mating-type strains, HM1 and NC4, become fusion-competent when they are cultivated in liquid cultures in the dark for more than 6 hours, and fuse with each other to form zygotic diploid cells. During the development of zygotes into macrocysts, meiosis takes place (Blasckovics and Raper, 1957; Erdos et al., 1973; Okada et al., 1986; Szabo et al., 1982) and consequently a number of haploid recombinants emerge from each mature macrocyst (MacInnes and Francis, 1974; Okada et al., 1986).

The molecular mechanism of sexual cell fusion in *Dictyostelium* is presumed to be fundamentally similar to the mechanism of fertilization in higher organisms. Since little is known about the molecular mechanism of fertilization, the sexual fusion of *Dictyostelium* cells has been studied to understand a general mechanism of sexual cell fusion.

We have discovered two cell-surface glycoproteins, gp138 and gp70, which are relevant to sexual fusion between HM1 and NC4 cells (Suzuki and Yanagisawa, 1989; Urushihara et al., 1988). These glycoproteins only appear on the surface of fusion competent cells. Rabbit antisera against these glycoproteins completely inhibit their sexual fusion.

Recently, we cloned and sequenced two genes for gp138, *GP138A* and *GP138B*, which have 91.8% sequence identity (Fang et al., 1993). To examine directly the function of gp138 glycoprotein in the sexual fusion of *Dictyostelium* cells, we generated strains in which gp138 genes were inactivated by the presence of antisense RNA and tested whether the inactivation of gp138 genes would repress the sexual fusion.

MATERIALS AND METHODS

Strains and cell culture

HM1 and AX3 strains of *Dictyostelium discoideum* were employed. Axenic strain AX3 cells, derived from NC4, fuse sexually with HM1 cells as well as strain NC4. Cells of these strains were cultivated with *Klebsiella aerogenes* on nutrient SM agar plates for maintenance purposes (Sussman, 1987). For acquisition of sexual fusion competence, they were each cultivated in the dark for 15 hours in Bonner’s salt solution (BSS) (Bonner, 1947) containing bacteria. For transformation experiments, AX3 cells grown in HL5 medium were used.

Plasmid construction and transformation

An antisense RNA vector for gp138, pGP138as, was constructed from extrachromosomal vector pATANB43 of *Dictyostelium* (Dynes and Firtel, 1989), which includes a neomycin phosphotransferase gene. For expression in *D. discoideum* cells, an XbaI-ScaI fragment from *Dictyostelium* vector BS18 (Kumagai et al., 1989), which contains the actin 15 promoter and the 2H3 terminator, was inserted into the BamHI site of pATANB43 after blunt-ending the ends. This vector was referred to as p43BS18. The cDNA containing the entire coding region of *GP138A* or *GP138B* (Fang et al., 1993), was then inserted into the unique BgIII site between the actin 15 promoter and the 2H3 terminator. A clone, pGP138as, containing the genes in antisense orientation relative to the promoter, was selected by restriction cleavage analysis and used for antisense mutagenesis. A diagram of pGP138as is shown in Fig.
1. Vectors pGP138as and p43BS18, as a control, were introduced into AX3 cells by electroporation and neomycin resistant clones, the antisense-transformants, were isolated.

RNA preparation and analysis

Total RNA was isolated from fusion-competent AX3 cells using guanidine thiocyanate (Chirgwin et al., 1979) and treated with RNase-free DNase to eliminate contaminating DNA. The amounts of RNAs corresponding to the endogenous mRNA of gp138 genes and the antisense RNA from antisense transformation vector pGP138as were determined by the method of reverse transcription-polymerase chain reaction (RT-PCR) as described in our previous paper (Fang et al., 1993). The sequences of the primers used for RT-PCR are as follows:

- PRA1: 5′-GGTGATTCAAAAATTACCTTT1′-3′ (identical to the antisense strand of GP138A from nucleotide nt 551 to 531),
- PRA2: 5′-GTGAAATCTTTGTCTTTTAGG-3′ (identical to the sense strand of GP138A from nt 196 to 215),
- PRB1: 5′-TTTGATCCAAAAATTGCTATG-3′ (identical to the antisense strand of GP138B from nt 556 to 536), and
- PRB2: 5′-GTATATCTTTAATTTACC-3′ (identical to the sense strand of GP138B from nt 196 to 215) (Fang et al., 1993).

The radio-labeled products of RT-PCR were then separated on a 6% polyacrylamide gel and detected by autoradiography.

Gel electrophoresis and immunoblotting

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed by a modified method of Laemmli (1970). For immunoblotting, proteins separated in gels were transferred to nitrocellulose membranes (Schleicher and Schuell, Germany) and treated with EF11, a monoclonal antibody against gp138 glycoprotein (Aiba et al., 1993). These proteins were then visualized by the ECL western blotting analysis system (Amersham International, UK).

Assay for cell fusion activity

AX3 cells transformed by the vector p43BS18 or the antisense transformation vector pGP138as were cultivated in a liquid, BSS containing bacteria, in the dark to make them fusion-competent and mixed with fusion-competent HM1 cells (5×10⁶ cells/ml, each). The sexual fusion activity of the transformed cells was indicated by the percentage of fused cells, using the method of Saga et al. (1983).

RESULTS

In D. discoideum, antisense RNAs transcribed from transformation vector constructs are known to inhibit gene expression by destabilizing endogenous mRNAs (Crowley et al., 1985; Loomis and Fuller, 1991). We generated strains in which gp138 genes were inactivated by the presence of the antisense RNA and attempted to investigate directly the function of gp138 protein in the sexual fusion of Dictyostelium cells.

To examine whether the production of mRNA of gp138 genes is inhibited in the antisense-construct transformed cells, AX3 cells transformed by the vector p43BS18 or antisense-construct pGP138as were cultivated separately in liquid culture, BSS containing bacteria, in the dark for 15 hours. Total RNAs from these fusion-competent cells were prepared, reverse transcribed and amplified according to the method of Makino et al. (1990). For detection of mRNAs of endogenous GP138A and GP138B, the products of reverse transcription (RT) reactions primed by primer 1 (PRA1) were amplified with PRA1 and primer 2 (PRA2), and the products of RT reactions primed by primer 3 (PRB1) were amplified with PRB 1 and primer 4 (PRB2), respectively. To detect antisense RNAs of GP138A and GP138B, RT reactions were primed by PRA2 followed by amplification with PRA2 and PRA1, and RT reactions were primed by PRB2 followed by amplification with PRB2 and PRB1, respectively.

The production of GP138B mRNA in the vector-alone transformed cells and the pGP138Bas (the antisense construct for GP138B) transformed cells is shown in Fig. 2. The vector-alone transformed cells showed the expression of endogenous mRNAs from both GP138A and GP138B (Fig. 2, lanes 1 and 3), but did not show the antisense RNA expression (Fig. 2, lane 5). On the contrary, the antisense-construct transformed cells expressed only small amounts of the mRNAs from GP138A and GP138B (Fig. 2, lanes 2 and 4), but a large amount of the antisense RNA (Fig. 2, lane 6). The results also showed that the expression of both genes is repressed by the same antisense RNA. This was expected because the two genes, GP138A and GP138B, are very similar in the nucleotide sequence (Fang et al., 1993).

To detect the production of gp138 glycoprotein, total proteins of the vector-alone transformed and the antisense-construct pGP138Bas transformed cells cultivated in the dark liquid cultures were obtained and subjected to SDS-PAGE. A monoclonal antibody EF11, specific for gp138 glycoprotein (Aiba et al., 1993), was applied to detect gp138 gly-
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Fig. 2. Expression of the endogenous gp138 genes and the gp138 antisense construct. RNAs from the control (vector-alone transformed cells) (lanes 1, 3 and 5) or the antisense transformed cells (lanes 2, 4 and 6) were reverse-transcribed and amplified with the primers specific for *GP138A* (lanes 1 and 2) or *GP138B* (lanes 3 and 4) or the antisense RNA (lanes 5 and 6). The products were then subjected to electrophoresis and detected by autoradiography (indicated by an arrow).

Fig. 3. Expression of gp138 protein in the vector-transformed cells. Whole proteins of 1×10⁵ AX3 cells transformed with the parental vector p43BS18 (lane 1) or the antisense transformation vector pGP138Bas (lane 2) were separated by SDS-PAGE. The blot was immunostained with a monoclonal antibody to gp138 (EF11). The arrow indicates the position of gp138.

coprotein. The results are shown in Fig. 3. A clear single band was detected in the vector-alone transformed cells, but only a very faint band in the antisense-construct transformed cells. These results show that *GP138A* or *GP138B*, or both the genes together, are undoubtedly encoding gp138 glycoprotein.

The antisense-construct transformed cells allowed us to examine directly the function of gp138 in the sexual fusion of *D. discoideum* cells. The sexual fusion activity of the transformed cells was then examined. The antisense-construct pGP138Bas transformed cells were cultivated in the dark, taken at various times during cultivation, and mixed with fusion-competent HM1 cells to measure their sexual fusion activity. As controls, AX3 cells and the vector-alone transformed cells were cultivated under the same conditions and their sexual fusion activities were also examined. The results are shown in Fig. 4. The sexual fusion activities of both AX3 cells and the vector-alone transformed cells began to increase at 6 hours after the initiation of cultivation and reached their maximum by 12 hours, approximately 73% (data of AX3 not shown). On the contrary, the antisense-construct transformed cells showed a much poorer fusion activity, 25%. The experiments were repeated 3 times with similar results. Thus, the antisense-construct transformed cells clearly lower the level of sexual fusion activity. This fact directly supports our previous conclusion that gp138 is involved in the sexual fusion of *D. discoideum* cells (Suzuki and Yanagisawa, 1989; Fang et al., 1993), although the activity was not repressed completely. The sexual development of a mixed population of the antisense-construct transformed AX3 and HM1 cells was examined. Zygotic cells formed precysts and developed into morphologically normal mature macrocysts. Fruiting body formation of the antisense-construct transformed cells was also observed. Smaller fruiting bodies with shorter stalks were produced.

**DISCUSSION**

The results presented show that expression of gp138 genes is considerably repressed in antisense-construct transformed cells and that the transformed cells reduce their sexual fusion activity. This fact directly indicates that glycoprotein gp138 is involved in the sexual cell fusion of *D. discoideum*.

It is known that, in *Dictyostelium*, transformation using the actin promoter for antisense-constructs usually represses expression of selected genes, when cells are grown in axenic culture or developed on agar without bacteria. However, the transformation does not repress gene expression so well if they are grown with bacteria (Crowley et al., 1985; Knecht and Loomis, 1987; Klein et al., 1988; Jain et al., 1992). Antisense transformed cells express endogenous mRNA at a nearly equivalent level to that of normal cells and return to wild type during growth in a bacterial culture. This is assumed to be due to a decline of activity of the actin promoter in the antisense constructs (Knecht et al., 1986). In the present experiment, the antisense construct for gp138 genes with the actin promoter considerably repressed expression of the endogenous mRNA, but did not completely repress it. This could be due to insufficient production of the antisense RNA by the constructs and is consistent with the result mentioned above.

It was shown that the glycoprotein gp138 was present on the cell surface of both fusion-competent NC4 and HM1.
We examined a number of heterothallic and bisexual strains of *D. discoideum*: 7 strains of the former and 2 of the latter; those cells also expressed gp138 glycoprotein when they were fusion-competent. On the contrary, cells of homothallic and asxexual strains of *D. discoideum* and of other species or genera, such as *D. mucoroides* or *Polysphondylium pal-lidum*, expressed no gp138 (Aiba et al., 1993). Thus, only fusion-competent cells of *D. discoideum* strains, which have the cross-mating system, have gp138 on their surfaces. This fact also supports indirectly a view that glycoprotein gp138 is involved in the sexual cell fusion of *D. discoideum*.

The mechanism of membrane fusion has been studied in viruses, and genes encoding viral fusion proteins have been cloned (White, 1990). Many fusion proteins of viruses possess two characteristic structures, fusion peptides and a transmembrane domain. Recently, a gene encoding PH30, a sperm surface protein involved in sperm-egg fusion of the guinea pig, was cloned and sequenced. This protein was found to also contain the fusion peptides and a transmembrane domain, like viral fusion proteins (Blobel et al., 1992). Thus, the fusion protein of the mammalian gametes shares similarity in structure with viral fusion proteins. Blobel et al. (1992) propose the hypothesis that all proteins, because gp138 has neither fusion peptides nor a transmembrane domain might link with the cell membrane following modification with phospholipid. These results might therefore show that if gp138 is not a fusion protein, it might be involved in recognition for mating type or species and/or cell contact in the sexual cell fusion of *Dictyostelium*.

There seem to be several molecules participating in the sexual cell fusion of *D. discoideum*. We have detected at least 3 surface antigens involving in the sexual cell fusion of *D. discoideum*, GG6, DE1 and HH9, besides gp138 and gp70 (Aiba et al., 1992). The functions of these molecules should be investigated in further studies.

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REFERENCES


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