A Sex Pheromone, Protoplast Release-inducing Protein (PR-IP) Inducer, Induces Sexual Cell Division and Production of PR-IP in Closterium

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The sex pheromone protoplast release-inducing protein (PR-IP) inducer and a sexual cell division-inducing pheromone-minus (SCD-IP-minus) that mediates the sexual reproduction of the heterothallic Closterium peracerosum-strigosum-littorale (C. psl) complex were investigated in this study. Recombinant PR-IP inducer produced by yeast cells was prepared and assayed for production of PR-IP and induction of SCD. Both biological activities were observed after treating mating-type plus (mt+) cells with the recombinant pheromone. SCD was induced by exposure to a lower concentration of the same pheromone and by a shorter treatment period with the pheromone than was production of PR-IP. This indicates that the previously characterized PR-IP inducer has both PR-IP-inducing and SCD-inducing activities with mt+ cells, although the inducing mechanisms of the two pheromones differ.

Keywords: Closterium — Conjugation — Pheromone — Sexual cell division — Sexual reproduction

Abbreviations: C. psl, Closterium peracerosum-strigosum-littorale; MI medium, mating-inducing medium; mt+, mating-type plus; mt−, mating-type minus; PR-IP, protoplast release-inducing protein; SCD, sexual cell division; SCD-IP, sexual cell division-inducing pheromone.

Introduction

Sexual reproduction is an essential process for almost all panmictic populations of organisms. Although each organism has its own manner of sexual reproduction, there is a common basic process in which two specialized, sexually competent cells recognize each other and fertilization or conjugation takes place between them. For these events to occur, one cell must have the ability to communicate with the other. Some specific substances, such as sex pheromones, are involved in the success of this communication.

In brown algae, chemotactic pheromones that are responsible for the attraction of the male gametes toward the female gametes are well characterized. These are volatile, low molecular weight hydrocarbons (Maier 1995). In the green alga Volvox carteri, a glycoproteinaceous pheromone, which is probably the most potent biological effector molecule known, acts as a sexual inducer (Hallmann 2003). In addition, chemotactic pheromones (lurlenic acid and lurlenol) have been reported in the heterogamous green alga Chlamydomonas allensworthii. These are derivatives of pentosylated hydroquinones, which are produced by the female gametes to attract the male gametes for mating (Starr et al. 1995, Jaenicke and Starr 1996).

Fig. 1 The effects of various doses of purified recombinant PR-IP inducer on the production of PR-IP and induction of SCD of mt+ cells. (A) Immunological detection of the 19 kDa subunit of PR-IP induced by recombinant PR-IP inducer. (B) The amount of 19 kDa subunit of PR-IP detected from each dose (filled circles), and the number of gametangial cells (open circles). The amounts were determined densitometrically from the bands on the blot. Both activities are expressed relative to the amount obtained with 3×10−7 M recombinant pheromone. The vertical bars indicate the SE (n = 3).

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The morphological features of vegetative cells and the modes of the sexual reproductive processes of Closterium (unicellular charophycean algae) have been well documented (Cook 1963, Lippert 1967). Recently, sequence tags expressed during the sexual reproduction of the Closterium peracrerosum-strigosum-littorale (C. psl) complex have also been obtained (Sekimoto et al. 2003).

It has been suggested that several sex pheromones mediate the sexual reproduction processes (Sekimoto 2000) of the C. psl complex. Two sex pheromones have been successfully isolated from the C. psl complex: protoplast release-inducing protein (PR-IP) and PR-IP inducer. PR-IP inducer is a glycoprotein with a mol. wt of 18,700 (Nojiri et al. 1995). It is released from mating-type minus (mt–) cells and induces the production of PR-IP from mating-type plus (mt+) cells (Sekimoto et al. 1993, Sekimoto et al. 1994, Sekimoto et al. 1998). PR-IP is a glycoprotein consisting of 19 and 42 kDa subunits, and it induces the release of gametic protoplasts of mt– cells (Sekimoto et al. 1990).

In Closterium ehrenbergii, the activating substances involved in sexual cell division (SCD) have been isolated (Fukumoto et al. 2002). The pheromone-inducing SCD of mt+ cells (an 18 kDa glycoprotein) was named SCD-inducing pheromone (SCD-IP). Its cDNA was cloned and revealed significant sequence similarity to the PR-IP inducer of the C. psl complex (Fukumoto et al. 2003). Recently, biologically active SCD-IPs specific for each mating type from the C. psl complex have also been detected (Tsuchikane et al. 2003). Partially purified SCD-IP-minus acting on mt+ cells and SCD-IP-plus acting on mt– cells have characteristics similar to the PR-IP inducer and PR-IP, respectively, in terms of their molecular weight, heat stability and the light dependency of their secretion. This indicates that SCD-IP-minus and the PR-IP inducer are related morphologically and physiologically.

Using a recombinant PR-IP inducer, we determined the dose dependency and incubation time required for inducing SCD- and PR-IP-inducing activities.

**Results**

When vegetative cells of the mt+ strain (mt+ cells) of the C. psl complex were incubated in nitrogen-depleted mating-inducing medium (MI medium; Ichimura 1971) containing various concentrations of the purified recombinant PR-IP inducer, greater amounts of PR-IP were produced (Fig. 1A). As shown in Fig. 1B, the number of gametangial cells increased with the dose of recombinant PR-IP inducer added to the MI medium.
To distinguish the two different activities of the pheromone, mt+ cells were incubated in MI medium with the recombinant PR-IP inducer for different times, and then transferred to pheromone-free medium. The number of gametangial cells and the amount of released PR-IP were quantified 24 and 48 h after beginning the treatment with recombinant pheromone. The production of PR-IP increased with the duration of incubation in MI medium with the recombinant PR-IP inducer (Fig. 2A, B). In contrast, the maximal number of gametangial cells was reached after a 2 h incubation with the pheromone (open circles). The SCD-inducing activity was expressed relative to the amount obtained for a 2 h incubation with 3×10^-7 M recombinant pheromone. The vertical bars indicate the SE (n = 3).

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When the mt+ cells were incubated in MI medium with various concentrations of purified recombinant PR-IP inducer for 2 h, SCD was initially induced by 1×10^-12 M recombinant PR-IP inducer, whereas production of PR-IP was not seen with 3×10^-7 M of the inducer (Fig. 3).

**Discussion**

In a previous study, we detected SCD-IPs (SCD-IP-minus and -plus), which were released from mt- and mt+ cells of the *C. psl* complex, and postulated that these were closely related to PR-IP inducer and PR-IP, respectively (Tsuchikane et al. 2003).

As shown in Fig. 1, the purified recombinant PR-IP inducer had PR-IP-inducing activity. The dose–response curve was similar to that reported for the native purified PR-IP inducer (Nojiri et al. 1995). Moreover, the recombinant PR-IP inducer also showed SCD-inducing activity, although the concentration required for induction was slightly lower than that for PR-IP induction. This indicates that the recombinant PR-IP inducer stimulates both production of PR-IP and induction of SCD in mt+ cells, and suggests that SCD-IP-minus and PR-IP inducer are identical.

As shown in Fig. 2, the amount of PR-IP produced was dependent on the duration of treatment with the PR-IP inducer. In contrast, SCD was induced with shorter treatments with the recombinant PR-IP inducer than required for the production of PR-IP. SCD was also induced by exposure to very low concentrations of the recombinant PR-IP inducer for short durations (Fig. 3). These results suggest that the modes of action for the two activities differ. The simplest explanation of the differences in the actions is the presence of different receptors. Using a FLAG-tagged recombinant pheromone would be a suitable way to elucidate these actions.

In the *C. psl* complex, sexual reproduction consists of the following processes: (i) a vegetative cell of each mating type produces two gametangial cells by SCD; (ii) a sexual pair is
formed between the different mating types of gametangial cells; (iii) each cell of the pair releases a naked cell (protoplast) of the gamete via the interaction of PR-IP and PR-IP inducer; and (iv) these two cells fuse to form a zygospore (Fig. 4). In addition, secretion of mucilage from each mating-type cell is induced soon after mixing the two mating types (Akatsuka et al. 2003). During these processes, SCD in mt+ cells is produced when the concentration of PR-IP inducer is still very low. The induction of PR-IP production in mt+ cells requires both the accumulation of PR-IP inducer released from mt+ cells and the action of the pheromone for a period of time.

As shown in Fig. 4, PR-IP and PR-IP inducer were also responsible for mucilage secretion in mt− and mt+ cells, respectively, although the dose dependency of this has not been examined (Akatsuka et al. 2003). Furthermore, the induction of SCD in mt+ cells by the action of purified PR-IP has been tentatively confirmed (S. Akatsuka et al. personal communication). The success of sexual reproduction in Closterium is largely dependent on exact intercellular communication mediated by sex pheromones, and this communication is very complex.

Materials and Methods

Strains and culture conditions

The strains NIES-67 (mt+) and NIES-68 (mt−) of heterothallic C. psl complex used in this study were obtained from the National Institute for Environmental Studies, Environmental Agency (Ibaraki, Japan). Clonal cultures were grown in 300 ml Erlenmeyer flasks containing 150 ml of nitrogen-supplemented medium (C medium; Ichimura 1971) at 24°C under a 16 h light/8 h dark regime. Light from fluorescent lamps (FL40SSD; Toshiba, Tokyo, Japan) was adjusted to 52 µmol m−2 s−1 at the surface of the culture medium.

Vegetative mt− and mt+ cells of the C. psl complex in the late logarithmic phase were centrifuged and washed three times with MI medium. The cells were incubated in MI medium for 24 h under continuous light and washed again with MI medium before use. These pre-cultured mt− cells were used to examine the production of PR-IP and SCD-inducing activity. To induce sexual reproduction, 10,000 pre-cultured cells of the mt− and mt+ strains were each mixed in 2 ml of MI medium and incubated for 72 h under continuous light (130 µmol m−2 s−1).

Expression and purification of recombinant proteins

Following the methods of Sekimoto (2002), transformed yeast cells that secreted FLAG-tagged recombinant PR-IP inducer were prepared. The recombinant yeast cells were cultured in liquid medium with minimal SD base (Clontech, Palo Alto, CA, USA) and with –Trp Dropout supplement (Clontech) (SDM minus Trp) at 30°C for 24 h. After a 48 h preliminary culture with shaking, the starter cultures were diluted 20-fold with YP4 high-stability expression media [YPHSM; 1% (w/v) glucose, 3% (w/v) glycerol, 1% (w/v) yeast extract, 8% (w/v) peptone, 20 mM CaCl2] and incubated at 30°C for 3 more days, with continued shaking of the flasks. The cultured cells were harvested by centrifugation for 30 min at 10,000 × g, and to remove all cells, the supernatant was filtered through a membrane filter (Durapore, GVWP, Millipore, Bedford, MA, USA). The filtrate was run through an ANTI-FLAG M2 affinity gel column (Sigma, St Louis, MO, USA), equilibrated with Tris-buffered saline. The recombinant PR-IP inducer was eluted with the same buffer containing 100 µg ml−1 FLAG peptide (Sigma) at a flow rate of 20 ml h−1. The eluate was subjected to ultrafiltration (Ultrafree-PF3, 5000 NMWL, Millipore) for desalting and removing the FLAG peptide, and the buffer was exchanged for MI medium. The protein content was determined using bovine serum albumin as the standard, according to the method of Bradford (1976).

Biological activity of recombinant PR-IP inducer in the production of PR-IP and the induction of SCD

To detect the production of PR-IP from mt+ cells, 50,000 cells, pre-cultured in MI medium, were incubated in 2 ml of MI medium containing various concentrations of the FLAG-tagged recombinant PR-IP inducer for 48 h under continuous light. The medium including released PR-IP was filtered through a membrane to remove the cells, and lyophilized. PR-IP was detected by immunoblotting after subjecting the samples to SDS–PAGE.

To detect SCD-inducing activity, 20,000 pre-cultured mt− cells were incubated in 2 ml of MI medium with various concentrations of FLAG-tagged recombinant PR-IP inducer under continuous light. After incubation for 24 h, the number of gametangial cells was counted under microscopy.

To examine the effect of the duration of incubation with the recombinant pheromone, mt− cells were incubated in MI medium containing 1 × 10−7 M or various concentrations of purified recombinant PR-IP inducer (first culture). After 0, 2, 4, 8, 24 and 48 h, the supernatant of each culture medium was collected and the resultants cells were incubated again in 2 ml of fresh MI medium (second culture). The number of gametangial cells was counted 24 h after beginning incubation with the recombinant pheromone. To detect PR-IP-inducing activity, a second culture medium was collected 48 h after beginning incubation with the recombinant pheromone, mixed with the respective first culture medium containing recombinant pheromone, lyophilized, and subjected to SDS–PAGE followed by immunoblotting.

Electrophoresis and immunological detection

SDS–PAGE was performed using the method of Laemmli (1967) on 15% polyacrylamide separation gels. Each sample was mixed with an equal volume of 120 mM Tris–HCl buffer (pH 6.8) that contained 4% (w/v) SDS (Wako Pure Chemical Industries, Osaka, Japan), 0.04% (w/v) bromophenol blue (Wako), 20% glycerol (v/v) and 10% (v/v) 2-mercaptoethanol, boiled for 5 min in a water bath, and then subjected to electrophoresis.

The proteins on the gel were transferred electrophoretically onto a polyvinylidene difluoride (PVDF) membrane filter (Millipore) in 25 mM Tris, 192 mM glycine and 20% (v/v) methanol in water at 60 V for 4 h (Gershoni and Palade 1982). The membrane was incubated with 10% (v/v) newborn calf serum (Dainippon Pharmaceutical, Osaka, Japan) in phosphate-buffered saline at room temperature for 1 h.

PR-IP was detected on an immunoblot using an anti-19 kDa subunit of PR-IP antiserum as the primary probe (Sekimoto 2002). The secondary probe was a goat anti-rabbit IgG alkaline phosphatase-conjugated antibody. The membrane was immersed for 5 min in ImmunoStar AP substrate (BIO-RAD, Hercules, CA, USA). Chemiluminescence on the blot was quantified using Light Capture (ATTO, Tokyo, Japan).

Acknowledgments

This work was supported by Grants-in-Aid (No. 1610982, 17370087 and 17405013) from the Japanese Society for the Promotion of Science to Y.T. and H.S.
References


(Received January 5, 2005; Accepted June 15, 2005)