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DIF-1, an anti-tumor substance found in *Dictyostelium discoideum*, inhibits progesterone-induced oocyte maturation in *Xenopus laevis*

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Abstract

Differentiation-inducing factor-1 (DIF-1; 1-(3,5-dichloro-2,6-dihydroxy-4-methoxyphenyl)hexan-1-one) is a putative morphogen that induces stalk-cell formation in the cellular slime mold *Dictyostelium discoideum*. DIF-1 has previously been shown to suppress cell growth in mammalian cells. In this study, we examined the effects of DIF-1 on the progesterone-induced germinal vesicle breakdown in *Xenopus laevis*, which is thought to be mediated by a decrease in intracellular cAMP and the subsequent activation of mitogen-activated protein kinase (MAPK) and maturation-promoting factor, a complex of cdc2 and cyclin B, which regulates germinal vesicle breakdown. DIF-1 at 10–40 μM inhibited progesterone-induced germinal vesicle breakdown in de-folliculated oocytes in a dose-dependent manner. Progesterone-induced cdc2 activation, MAPK activation, and c-Mos accumulation were inhibited by DIF-1. Furthermore, DIF-1 was found to inhibit the progesterone-induced cAMP decrease in the oocytes. These results indicate that DIF-1 inhibits progesterone-induced germinal vesicle breakdown possibly by blocking the progesterone-induced decrease in $[cAMP]_i$ and the subsequent events in *Xenopus* oocytes.

Keywords: DIF-1; MAP (mitogen-activated protein) kinase; Germinal vesicle breakdown; cAMP

1. Introduction

Differentiation-inducing factor-1 (DIF-1; 1-(3,5-dichloro-2,6-dihydroxy-4-methoxyphenyl)hexan-1-one) (Fig. 1) is a chlorinated alkylphenone that induces stalk cell differentiation in the cellular slime mould *Dictyostelium discoideum* (Morris et al., 1987; Kay et al., 1989). DIF-1 is thought to function at least in part by raising intracellular Ca^{2+} concentration (Kubohara and Okamoto, 1994; Schaap et al., 1996; Azhar et al., 1997), but the precise signal transduction system of DIF-1, including the target molecule(s) of DIF-1, is still unknown.

Recently, it has been shown that DIF-1 exhibits anti-tumor activities: inhibition of cell growth (G1/0 arrest), induction and promotion of cell differentiation, and induction of apoptosis depending on concentrations of DIF-1 (Asahi et al., 1995; Kubohara et al., 1995a,b; Kubohara, 1997, 1999; Kubohara and Hosaka, 1999). As to the mechanism of the actions of DIF-1 in tumor cells, we have shown that DIF-1 increases cytosolic Ca^{2+} concentrations and activates protein kinase B (PKB or Akt), a protein kinase that has crucial roles in cell survival, insulin action, and cell differentiation (Kubohara et al., 1995a,b; Kubohara and Hosaka, 1999). Furthermore, it was demonstrated with vascular smooth muscle cells that DIF-1 inhibits the expression of cyclins D/E and the phosphorylation of the Rb protein, thereby arresting the cell cycle at G1/0 and inducing muscle cell differentiation in vitro (Miwa et al., 2000). Yet, the mechanisms underlying the action of DIF-1 remain to be elucidated in mammalian cells.

Xenopus oocyte is one of the best models to investigate the mechanisms of meiosis and its signal transduction system. When oocytes are stimulated with progesterone, germinal vesicle breakdown, the apparent phenomenon of

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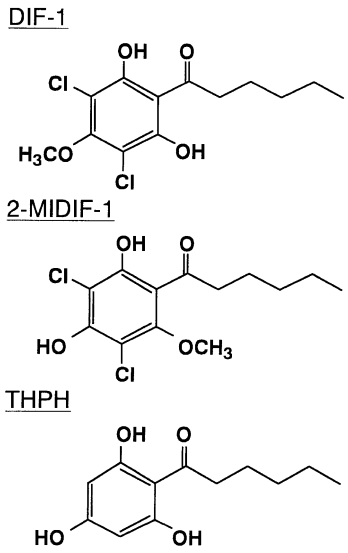

Fig. 1. Chemical structure of DIF-1 and its analogues. DIF-1; 1-(3,5-dichloro-2,6-dihydroxy-4-methoxyphenyl)hexan-1-one. 2-MIDIF-1; 2-methoxy isomer of DIF-1. THPH; 1-(2,4,6-trihydroxyphenyl)hexan-1-one.

oocyte maturation (meiosis), occurs. It is known that MPF (maturation-promoting factor), a complex of the serine/threonine protein kinase cdc2 and cyclin B, plays a key role in the induction of germinal vesicle breakdown (Nebreda et al., 1995; Nebreda and Ferby, 2000). Progesterone is thought to activate MPF and induce germinal vesicle breakdown via induction of a transient decrease in intracellular cAMP, thereby affecting subsequent events including the activation of a MAPK (mitogen-activated protein kinase) cascade in oocytes, although the presence of a MAPK-independent pathway for the activation of MPF has been postulated (Finidori-Lepicard et al., 1981; Sadler and Maller, 1981; Finidori et al., 1982; Schorderet-Slatkine et al., 1982; Cicirelli and Smith, 1985; Sagata et al., 1988; Ferrell et al., 1991; Gotoh et al., 1991; Posada et al., 1991; Matsuda et al., 1992; Sheets et al., 1995; Frank-Vaillant et al., 1999; Nebreda and Ferby, 2000).

In the present study, in order to assess whether DIF-1 inhibits meiosis as well as mitosis and whether DIF-1 affects the MAPK-MPF signaling system, we examined the effects of DIF-1 on the progesterone-induced oocyte maturation in *Xenopus laevis*. We here show that DIF-1 inhibits progesterone-induced germinal vesicle breakdown, possibly by blocking the progesterone-induced cAMP decrease and subsequent events of c-Mos accumulation and MAPK/MPF activation.

2. Materials and methods

2.1. Chemicals

1-(3,5-Dichloro-2,6-dihydroxy-4-methoxyphenyl)hexan-1-one (DIF-1) was purchased from Affiniti Research Products (UK) and progesterone was from Wako Pure Chemical Industries (Japan). 1-(2,4,6-Trihydroxyphenyl)hexan-1-one (THPH) and 2-methoxy isomer of DIF-1 (2-MIDIF-1) (Fig. 1) were synthesized by a chemical company following the method of Masento et al. (1988). DIF analogues and progesterone were stored as 10- and 1-mM solutions in ethanol, respectively. Rabbit anti-Erk1/2 antibody and anti-active MAPK antibody were obtained from Promega (USA), rabbit anti-Mos^{xe} (C237)-G antibody was from Santa Cruz Biotechnology (USA), anti-cdc2 monoclonal antibody was from Oncogene Research Products (USA), and rabbit anti-phospho-cdc2 (Tyr15) was from Cell Signaling Technology (USA).

2.2. Oocyte preparation and de-folliculation

Adult female *X. laevis* were fed as described previously (Kato et al., 1997) and used in this study. Frogs were killed by decapitation and their ovaries were removed. The ovarian fragments (the oocytes covered with follicle cells) were used in some experiments (Fig. 2). De-folliculated oocytes were prepared by removing the follicular cell layer manually after treating the ovarian fragments with 5 mM dithiothreitol in 2 × Steinberg's solution (Masui, 1967) at 20 °C.

2.3. Observation of ovulation and germinal vesicle breakdown

Prepared ovarian fragments or de-folliculated oocytes were incubated in a multi (24)-well plate (usually 15–30 oocytes/well), each well containing 1 ml of Steinberg's solution containing 0.1% (w/v) bovine serum albumin (designated SB solution) with or without human chorionic gonadotropin (50 IU/ml), progesterone (5 μM), and/or DIF-1 (10–40 μM) for 6–16 h at 22 °C. Ovulation was observed by checking that the oocytes have become detached from the ovarian fragments. For the observation of germinal vesicle breakdown, the oocytes were fixed by boiling for 10 min and then dissected. The rate of ovulation and germinal vesicle breakdown is expressed as a percentage of the number of such oocytes/total oocytes incubated (usually 30–60 oocytes were assessed for each condition in an experiment).

2.4. Immunoblotting

De-folliculated oocytes were incubated in a multi (24)-well plate with or without human chorionic gonadotropin, progesterone, and/or DIF-1 as described above. The oocytes were suspended in a sodium dodecyl sulfate (SDS) sample buffer (1 oocyte: 50 μl SDS sample buffer) and lysed by sonication, and insoluble materials were removed by centrifugation (10,000 × *g*, 5 min). The oocyte proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE; 4–20% polyacrylamide gradient gels were used) and transferred onto a nitrocellulose membrane as described earlier (Kubohara and Okamoto,

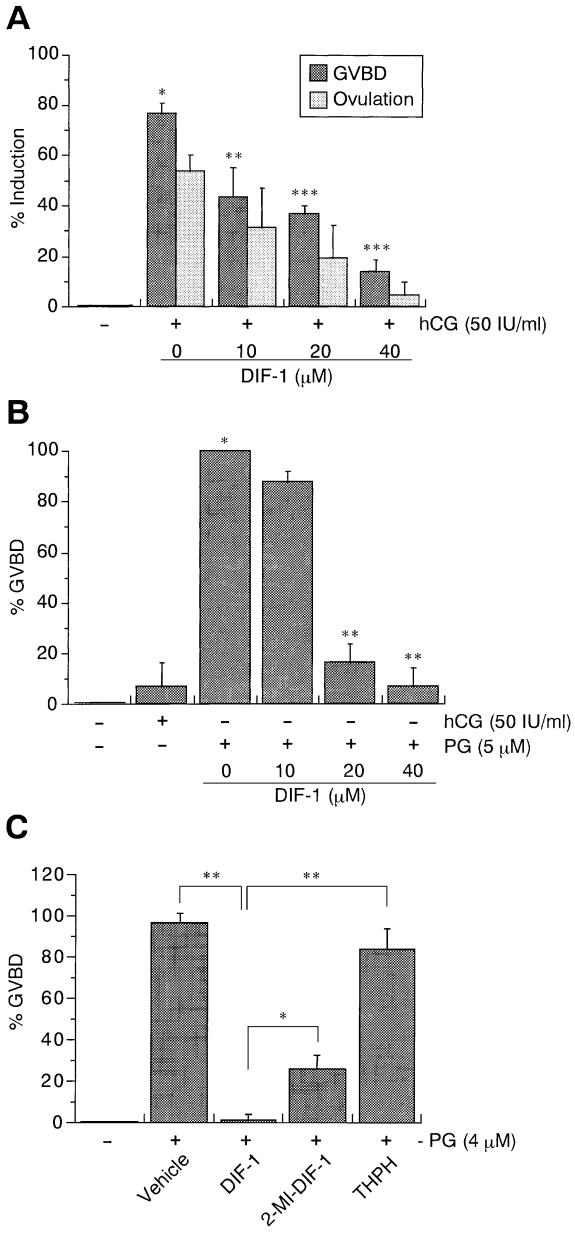

Fig. 2. (A) Effects of DIF-1 on hCG-induced ovulation and germinal vesicle breakdown. Ovarian fragments (oocytes with follicle cells) were incubated in vitro with or without DIF-1 (10–40 μM) and/or human chorionic gonadotropin (hCG; 50 IU/ml) for 12–16 h, and ovulation and germinal vesicle breakdown (GVBD) were observed. Data are given as mean ± S.D. of three independent experiments ($n=3$). **$P<0.01$; ***$P<0.001$ vs. *Control (calculated by ANOVA: post hoc Fisher's PLSD). (B) Effects of DIF-1 on progesterone-induced germinal vesicle breakdown. De-folliculated oocytes were incubated in vitro with or without DIF-1 (10–40 μM), hCG (50 IU/ml), and/or progesterone (PG; 5 μM) for 6 h, and germinal vesicle breakdown was observed. Data are given as the mean ± S.D. of three independent experiments ($n=3$). **$P<0.001$ vs. *Control (by ANOVA). It should be noted that germinal vesicle breakdown was seldom induced by hCG, which indicates that follicle cells were removed well. (C) Effects of DIF analogues on progesterone-induced germinal vesicle breakdown. De-folliculated oocytes were incubated in vitro with or without DIF-1, 2-MIDIF-1, or THPH (15 μM) and/or progesterone (PG; 4 μM) for 6 h, and germinal vesicle breakdown was observed. The mean ± S.D. of two independent experiments ($n=2$). *$P<0.05$; **$P<0.001$ (by ANOVA).

1990). The membrane was incubated for 1 h with a primary antibody in a Tris–HCl buffer (10 mM Tris–HCl, pH 7.5, 137 mM NaCl, 0.1%(w/v) Tween 20) (designated TBS–Tween) containing 5% non-fat dry-milk powder (for a rabbit anti-Erk1/2 antibody (1:2500 diluted) or a rabbit anti-Mos^{xe} antibody; 1:500 diluted), or 1% bovine serum albumin (for a rabbit anti-active MAPK antibody; 1:2500 diluted). After being washed with TBS–Tween several times, the membrane was incubated for 1 h with horseradish peroxidase-conjugated goat anti-rabbit antibody (1:1000 diluted) in TBS–Tween containing 5% non-fat dry-milk powder. After being washed with TBS–Tween several times, the membrane was processed for visualization using an enhanced chemiluminescence (ECL) kit according to the manufacturer's instructions (Amersham) and exposed to Hyperfilm for ECL (Amersham, UK). The visualized bands in the ECL image were digitized and quantified using the Adobe Photoshop and NIH Image softwares.

2.5. Assay for cAMP

De-folliculated oocytes were incubated in Eppendorf tubes (5 oocytes/tube), each containing 800 μl of SB solution with or without DIF-1 (30 μM) and/or progesterone (5 μM), for 10 min at room temperature (approximately 20 °C) on a twin mixer. Two hundred microliters of 1 N HCl was added into the tubes and the oocytes were disrupted by sonication. After centrifugation (10,000 × g, 5 min), aliquots of the supernatants were used for the assay for cAMP contents using a radioimmunoassay (RIA) method (Hattori et al., 1985) where triplicate determination was performed for each sample.

3. Results

3.1. Effects of DIF-1 on germinal vesicle breakdown in Xenopus oocytes

It has been shown previously that DIF-1 inhibits cell growth (mitosis) in mammalian cells (Asahi et al., 1995; Kubohara et al., 1995a,b, 1998; Kubohara, 1997, 1999; Kubohara and Hosaka, 1999; Miwa et al., 2000). Here, we first examined the effect of DIF-1 on germinal vesicle breakdown (an index of oocyte maturation; meiosis) induced by human chorionic gonadotropin (hCG) in *Xenopus* follicular oocytes (Fig. 2A). hCG is thought to stimulate the follicular cells surrounding an oocyte to produce progesterone, which eventually induces germinal vesicle breakdown in the oocytes. hCG induced both germinal vesicle breakdown and ovulation in oocytes with follicular cells (Fig. 2A). DIF-1 at 10–40 μM inhibited hCG-induced germinal vesicle breakdown and ovulation in a dose-dependent manner (Fig. 2A). It should be noted that DIF-1 alone at 10–40 μM showed no marked effect on the oocytes (data not shown). Since the aim of this study was to

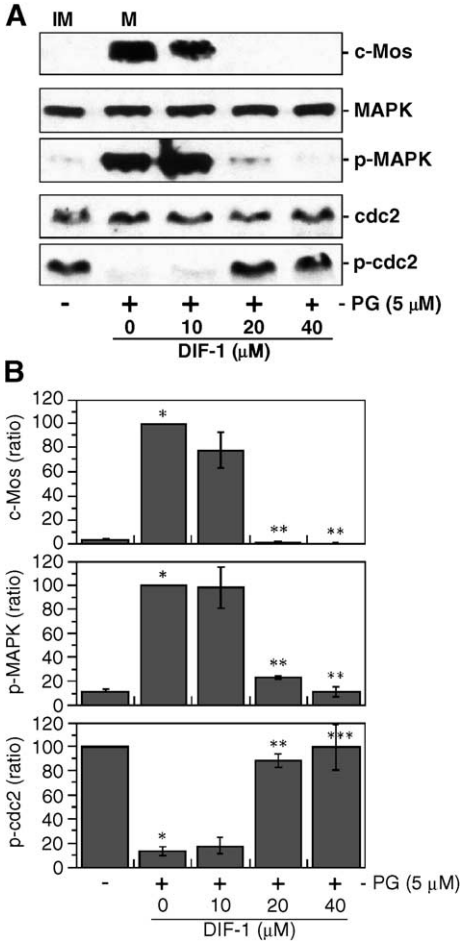

Fig. 3. Effects of DIF-1 and progesterone on the activity of cdc2 and MAP kinase and the accumulation of c-Mos. (A) De-folliculated oocytes were incubated in vitro with or without DIF-1 (10–40 μM) and/or progesterone (PG; 5 μM) for 6 h, and the oocyte proteins were analyzed by Immunoblotting (1/5 oocyte proteins/lane) for cdc2, phospho-cdc2 (Tyr15) (p-cdc2), MAPK, phospho-MAP kinase (p-MAPK), and c-Mos. Note that the phosphorylated form of MAPK was active, while the phosphorylated form of cdc2 was inactive in the oocytes. IM and M in the panel indicate immature and mature oocytes, respectively. (B) The bands in the blots were quantified using NIH Image software. The quantified values of c-Mos are presented as a ratio to the positive control (lane M in panel (A)). The quantified values of p-MAPK were normalized with the total amounts of MAPK and are presented as a ratio to the control (lane M in panel (A)). The quantified values of p-cdc2 were normalized with the total amounts of cdc2 and are presented as a ratio to the control (lane IM in A). Data are given with the mean ± S.D. of two independent experiments ($n=2$). $^{**}P<0.01$; $^{***}P<0.05$ vs. *Control (by ANOVA).

examine the effects of DIF-1 on meiosis and to analyze the mechanism of the action of DIF-1, we focused on the inhibitory effect of DIF-1 on germinal vesicle breakdown in the experiments hereafter.

We then examined the effect of DIF-1 and its analogues on progesterone-induced germinal vesicle breakdown in de-folliculated oocytes (Fig. 2B,C). DIF-1 at 10–40 μM inhibited progesterone-induced germinal vesicle breakdown (Fig. 2B,C), while 1-(2,4,6-trihydroxyphenyl)hexan-1-one (THPH) did not greatly affect progesterone-induced germinal vesicle breakdown (Fig. 2C). 2-Methoxy isomer of DIF-1 (2-MIDIF-1) also inhibited progesterone-induced germinal vesicle breakdown but its effect was not as strong as that of DIF-1 (Fig. 2C). These results indicate that the effect of DIF-1 is structure-specific. It is of importance to note here that the inhibitory effect of DIF-1 on progesterone-induced germinal vesicle breakdown was reversible since withdrawal of DIF-1 from the media triggered germinal vesicle breakdown in the presence of progesterone (data not shown).

3.2. Effects of DIF-1 on progesterone-induced MPF/MAPK activation, c-Mos accumulation, and cAMP concentration

Progesterone is thought to induce germinal vesicle breakdown via: (1) a decrease in cAMP concentration (Finidori-Lepicard et al., 1981; Sadler and Maller, 1981; Finidori et al., 1982; Schorderet-Slatkine et al., 1982; Cicirelli and Smith, 1985), (2) the subsequent induction of c-mos-mRNA translation, (3) the activation of mitogen-activated protein kinase (MAPK), and (4) the activation of maturation-promoting factor (MPF) (Sagata et al., 1998; Ferrell et al., 1991; Gotoh et al., 1991; Posada et al., 1991; Matsuda et al., 1992; Sheets et al., 1995) (Fig. 5). In order to elucidate the mechanism of the inhibitory effect of DIF-1 on progesterone-induced germinal vesicle breakdown, we examined the effects of DIF-1 on MPF activation (Fig. 3), MAPK activation (Fig. 3), c-Mos accumulation (Fig. 3), and cAMP concentration (Fig. 4) using de-folliculated oocytes.

MPF, a complex of cdc2 kinase and cyclin B, is a key factor that controls germinal vesicle breakdown (Nebreda et al., 1995; Nebreda and Ferby, 2000). In immature oocytes, cdc2 was phosphorylated (inactive) and was de-phosphorylated (activated) in response to progesterone (Fig. 3). However, when DIF-1 was present, the progesterone-induced dephosphorylation of cdc2 was inhibited (Fig. 3). In addition, DIF-1 inhibited the progesterone-induced activation of MAPK, but did not suppress the expression of the protein

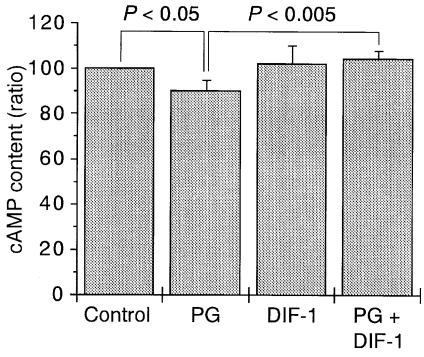

Fig. 4. Effects of DIF-1 and progesterone on $[cAMP]_i$. De-folliculated oocytes were incubated in vitro with or without DIF-1 (30 μM) and/or progesterone (PG; 5 μM) for 10 min and assayed for $[cAMP]_i$. Data were converted into relative values (% of control) and are given with the mean ± S.D. of four independent experiments ($n=4$). Actual value of the control was 4.46 ± 0.37 (pmol/oocyte). The statistical significance was calculated by ANOVA.

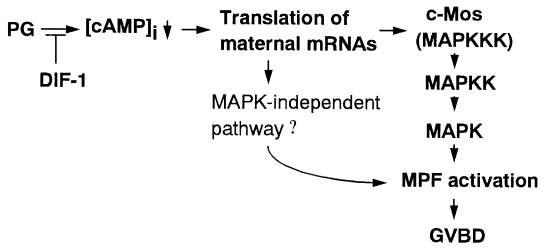

Fig. 5. Scheme for progesterone-induced germinal vesicle breakdown and the action point of DIF-1. Progesterone (PG) decreases $[cAMP]_i$, which triggers the accumulation of c-Mos (MAPKKK) and the subsequent activation of a MAPK cascade. The activation of MAPK induces the activation of MPF and eventually germinal vesicle breakdown (GVBD). There may be a MAPK-independent pathway for the activation of MPF. DIF-1 inhibits germinal vesicle breakdown by blocking the progesterone-induced decrease in $[cAMP]_i$ and/or the progesterone-induced translation of maternal mRNAs including c-mos in some way.

(Fig. 3). DIF-1 in the same concentration range also inhibited the progesterone-induced accumulation of c-Mos in a dose-dependent manner (Fig. 3). The levels of MPF/MAPK activity and c-Mos accumulation (Fig. 3) seemed to correspond well with the percentage of germinal vesicle breakdown under the same conditions (Fig. 2B).

The effects of progesterone and DIF-1 on intracellular cAMP concentration ($[cAMP]_i$) were then examined in *Xenopus* oocytes (Fig. 4). Treatment with progesterone slightly reduced $[cAMP]_i$ as previously shown (Cicirelli and Smith, 1985), but in the presence of DIF-1, progesterone did not reduce $[cAMP]_i$ (Fig. 4). Although it was very difficult to evaluate the small changes in cAMP in the oocytes, the effect of DIF-1 on the progesterone-induced decrease in $[cAMP]_i$ was statistically significant (Fig. 4). These results indicate that DIF-1 inhibits progesterone-induced germinal vesicle breakdown possibly by blocking the progesterone-induced decrease in $[cAMP]_i$ and the subsequent activation of a MAPK cascade in *Xenopus* oocytes (Fig. 5).

4. Discussion

We have previously shown that DIF-1 suppresses cell growth (mitosis) and induces/promotes cell differentiation in mammalian cells (Asahi et al., 1995; Kubohara et al., 1995a,b; Kubohara, 1997, 1999; Miwa et al., 2000). In order to assess whether DIF-1 inhibits meiosis and, if so, to analyze the mechanism of meiosis inhibition by DIF-1, we examined the effects of DIF-1 on progesterone-induced oocyte maturation in *X. laevis*, which is an excellent and well-studied model system for analyzing such topics.

Progesterone is thought to induce oocyte maturation in *Xenopus* by decreasing $[cAMP]_i$ (Finidori-Lepicard et al., 1981; Sadler and Maller, 1981; Finidori et al., 1982; Schorderet-Slatkine et al., 1982; Cicirelli and Smith, 1985), inducing translation of maternal c-mos mRNA (c-Mos = MAPKKK), and activating MAPK and MPF (Sagata et al., 1998; Ferrell et al., 1991; Gotoh et al., 1991; Posada et al., 1991; Matsuda et al., 1992; Sheets et al., 1995; Frank-Vaillant et al., 1999; Nebreda and Ferby, 2000) (Fig. 5). It has recently been suggested that the activation of MAPK is not necessarily required for germinal vesicle breakdown (Fisher et al., 1999; Gross et al., 2000; Nebreda and Ferby, 2000). However, it is likely that MAPK normally plays a major role in oocyte maturation and there are parallel or redundant MAPK-independent pathways during oocyte maturation (Nebreda and Ferby, 2000) (Fig. 5). We have shown here for the first time that DIF-1, an anti-tumor (anti-mitotic) agent in mammalian cells, suppresses the progesterone-induced germinal vesicle breakdown (meiosis) in *Xenopus* oocytes (Fig. 2), possibly by inhibiting the progesterone-induced decrease in $[cAMP]_i$ (Fig. 4) and subsequent signal cascade (Fig. 3). However, since it is very difficult to evaluate the small changes in cAMP in the oocytes (Fig. 4), we cannot exclude the possibility that DIF-1 affects another point of the signal cascade triggered by progesterone.

It has been shown previously that DIF-1 may function at least in part via an increase in $[Ca^{2+}]_i$ both in *D. discoideum* (Kubohara and Okamoto, 1994; Schaap et al., 1996; Azhar et al., 1997) and mammalian cells (Kubohara et al., 1995a,b; Kubohara, 1997, 1999). Quite interestingly, however, in *Xenopus* oocytes, DIF-1 is unlikely to function via an increase in $[Ca^{2+}]_i$ since DIF-1 did not induce elctrophysiological changes in oocytes in preliminary experiments (unpublished observations).

It has been shown that phophoinositide 3-kinase (PI3-kinase), Akt/PKB and glycogen synthase kinase-3 (GSK-3) are involved in the oocyte maturation induced by insulin or IGF-1 (insulin-like growth factor-1) (Andersen et al., 1998; Fisher et al., 1999; Lopez-Hernandez and Santos, 1999) and constitutively active Akt/PKB can induce resumption of meiosis (Andersen et al., 1998). Since DIF-1 has been shown to activate Akt/PKB in K562 leukemia cells (Kubohara and Hosaka, 1999), it seemed possible that DIF-1 itself might induce germinal vesicle breakdown by activating Akt/PKB in *Xenopus* oocytes. However, DIF-1 alone did not induce germinal vesicle breakdown (data not shown), suggesting that DIF-1 does not activate Akt/PKB and its downstream pathway in the oocytes.

In spite of the efforts by researchers (Insall and Kay, 1990), the target(s) of DIF-1 has not been identified even in *Dictyostelium*. The present information that DIF-1 might block the progesterone-induced decrease in $[cAMP]_i$ in *Xenopus* oocytes could provide a starting point and an excellent system for the identification of the target(s) of DIF-1 from another angle.

Acknowledgements

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