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The antibiotic azatyrosine suppresses progesterone or [Val^{12}]p21 Ha-*ras*/insulin-like growth factor I-induced germinal vesicle breakdown and tyrosine phosphorylation of *Xenopus* mitogen-activated protein kinase in oocytes

(*ras* oncogene/tyrosine kinase/myelin basic protein kinase/*Src* homology 2 domain)

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ABSTRACT **The antibiotic azatyrosine [DL-3-(5-hydroxy-2-pyridyl)alanine] suppressed meiotic maturation in oocytes induced by progesterone or the combination of [Val^{12}]p21$^{Ha\text{-}ras}$ microinjection and insulin-like growth factor I. The suppression was dose-dependent in the range of 20–250 μM azatyrosine. In addition, azatyrosine blocked the tyrosine phosphorylation of Xp42, a member of the mitogen-activated protein kinase family, after progesterone or [Val^{12}]p21$^{Ha\text{-}ras}$/insulin-like growth factor I stimulation. Activation of maturation-promoting factor, as shown by a decrease in the tyrosine phosphorylation of the *Xenopus* homolog of p34^{cdc2}, was also suppressed by azatyrosine. Azatyrosine had no effect *in vivo* or *in vitro* on the growth factor-induced autophosphorylation of the oocyte insulin-like growth factor I receptor. Azatyrosine has been shown by others [Shindo-Okada, N., Makabe, O., Nagahara, H. & Nishimura, S. (1989) *Mol. Carcinog.* 2, 159–167] to inhibit the growth of *ras*-transformed cells without affecting that of nontransformed cells. In oocytes, the antibiotic exerts an inhibitory action on both a *ras*-dependent and a *ras*-independent pathway. Lack of an effect of azatyrosine on germinal vesicle breakdown induced by the microinjection of an extract from mature oocytes, however, suggests that azatyrosine is acting upstream of maturation-promoting factor activation.**

The high incidence of mutated *ras* genes in human cancer (1, 2) has led many investigators to direct their research efforts toward *ras* as a potential target for cancer therapies. One approach has been to block the biosynthesis of the oncogene product by expression of antisense mRNA (3). This method was utilized in a human lung cancer cell line to block the production of the K-ras protein and has also met with some success in an *in vivo* tumor model. Another approach takes advantage of the ability of mutated ras proteins to cause transformation when expressed in cultured cells. By using this technique, large numbers of compounds can be screened on the basis of differential effects on the growth of the transformed vs. the parental cell line. This method was used by Shindo-Okada *et al.* (4) to identify a potential antitumor compound, azatyrosine. Azatyrosine, an antibiotic isolated from *Streptomyces chibanensis*, inhibited the growth of NIH 3T3 cells transformed by Ha-*ras* but had little effect on the growth of nontransformed cells. Moreover, azatyrosine caused the phenotypic reversion of transformed cells that persisted even after the antibiotic was removed from the culture medium.

Because the effect of azatyrosine appeared to be quite specific for the *ras*-transformed cells, we chose to investigate its action in a totally different *ras*-dependent system. Prophase-arrested oocytes of the African clawed frog *Xenopus laevis* can be induced to mature *in vitro* by progesterone, insulin, or insulin-like growth factor I (IGF-I). The pathway involving insulin or IGF-I requires endogenous ras protein (5) and can be activated in the absence of exogenous hormone or growth factor by the microinjection of oncogenic forms of *ras* (6). In contrast, the progesterone pathway is independent of the *ras* gene product. Use of the oocyte system, therefore, permits the investigation of the effects of azatyrosine on two separate pathways, both culminating in a common end, i.e., meiotic maturation.

In this paper, we show that azatyrosine suppressed maturation in oocytes induced by the combination of *ras* microinjection and IGF-I. The antibiotic also inhibited progesterone-induced maturation. Progesterone or *ras*/IGF-I treatment of oocytes induced tyrosine phosphorylation of Xp42, a member of the mitogen-activated protein (MAP) kinase family (7), prior to germinal vesicle breakdown (GVBD). This tyrosine phosphorylation of Xp42 was blocked by azatyrosine. The antibiotic had no detectable effect, however, on the autophosphorylation of the oocyte IGF-I receptor.

MATERIALS AND METHODS

Materials. Adult female *Xenopus laevis* were obtained from Xenopus I (Ann Arbor, MI). Receptor-quality IGF-I was purchased from Mallinckrodt. Azatyrosine [DL-3-(5-hydroxy-2-pyridyl)alanine] was generously provided by A. Hudson (Wellcome Research Laboratories, Beckenham, U.K.). Recombinant [Val^{12}]p21^{ras} was purified from *Escherichia coli* as described (8). [γ-^{32}P]ATP was from DuPont/NEN. L-[4,5-^{3}H]Leucine and L-[2,3,5,6-^{3}H]tyrosine were from Amersham. All other chemicals were purchased from standard suppliers.

The anti-phosphotyrosine and anti-p34^{cdc2} antibodies were both mouse monoclonal antibodies purchased from Upstate Biotechnology (Lake Placid, NY). The anti-p34^{cdc2} antibody was made using residues 30–57 (VAMKKIRLESEEEGVPSTAIREISLLKE) of the predicted amino acid sequence of human cdc2 as imunogen (9). The anti-Xp42 is a rabbit polyclonal antibody (no. 1913.2) and was kindly provided by J. Posada (Fred Hutchinson Cancer Research Center, Seattle).

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Abbreviations: IGF-I, insulin-like growth factor I; MAP, mitogen-activated protein; GVBD, germinal vesicle breakdown; MPF, maturation-promoting factor.

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Oocytes and Microinjection. Isolation and microinjection of oocytes as well as progesterone stimulation and scoring of GVBD were as described (8).

For *ras*/IGF-I-induced GVBD, oocytes were microinjected with 5–10 ng of [Val^{12}]p21$^{Ha\text{-}ras}$ and then placed into Barth's solution containing bovine serum albumin (Barth's/BSA) containing 2 nM IGF-I. For experiments involving azatyrosine treatment, the antibiotic was added to the oocyte incubation medium 3 h prior to stimulation with progesterone or *ras*/IGF-I.

Maturation-Promoting Factor (MPF)-Induced GVBD. Oocytes were induced to mature using 10 μM progesterone. Immediately after GVBD, 10 oocytes were rinsed twice with MPF buffer (80 mM β-glycerophosphate/20 mM Hepes, pH 7.5/20 mM EGTA/15 mM $MgCl_2$) and homogenized in a final volume of 20 μl of MPF buffer. The homogenate was centrifuged for 4 min at 4°C in a microcentrifuge and the supernatant fraction was used immediately for microinjection.

Oocyte Homogenization. At the indicated times after stimulation, oocytes were homogenized in 100 mM NaCl/20 mM Tris·HCl, pH 7.6/1% Triton X-100/50 mM NaF/1 mM Na_3VO_4/1 mM phenylmethylsulfonyl fluoride/10 μg of aprotinin per ml/10 μg of leupeptin per ml (oocyte homogenization buffer, OHB) using 10–20 μl of homogenization buffer per oocyte. Homogenates were cleared by centrifugation and the supernatants were used immediately or placed at −80°C until use.

Oocyte Protein Synthesis Assay. Protein synthesis in oocytes was assayed by the incorporation of radiolabeled amino acids into trichloroacetic acid-precipitable material. After the microinjection of L-[^{3}H]leucine or L-[^{3}H]tyrosine (15–35 Ci/mmol, 1 mCi/ml, 50 nl per oocyte; 1 Ci = 37 GBq), oocytes were incubated for various periods of time and homogenized as described above (20 μl of OHB per oocyte). Assays for total or trichloroacetic acid-precipitable radioactivity were performed in duplicate on 10 μl of the resulting supernatant fraction.

Electrophoresis and Immunoblot Analysis. Oocyte proteins were separated on 12% polyacrylamide gels in the presence of SDS (10); the protein from 1.5 to 3 oocytes per lane was used. The proteins in the gel were electrophoretically transferred to nitrocellulose in 25 mM Tris base/192 mM glycine/20% (vol/vol) methanol for 1.5–2 h at 200 mA.

For immunodetection of specific proteins, excess sites on the nitrocellulose were first blocked by incubation of the blots for 30 min at room temperature in buffer A [200 mM NaCl/50 mM Tris·HCl, pH 7.6/0.1% bovine serum albumin/0.1% polyethylene glycol (molecular mass, 15–20 kDa)/0.02% NaN_3] supplemented with 3% bovine serum albumin. The blots were then incubated for 1 h at room temperature with primary antibody diluted in buffer A. The blots were washed with buffer A for three 5-min periods and then incubated in diluted alkaline phosphatase-conjugated secondary antibody for 1 h. After washing as described above, immunoreactive proteins were visualized using nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate.

***In Vitro* Phosphorylation of Oocyte IGF-I Receptors.** One-hundred-sixty oocytes were homogenized in 1 ml of OHB and the homogenate centrifuged at 140,000 × *g* for 35 min in a fixed-angle rotor. This and all subsequent steps were carried out at 4°C. The supernatant fraction was diluted with an equal volume of column buffer (150 mM NaCl/50 mM Tris·HCl, pH 7.4/0.2% Triton X-100/1 mM phenylmethylsulfonyl fluoride/10 μg of aprotinin per ml/10 μg of leupeptin per ml) and applied to a 0.4-ml column of wheat germ agglutinin-agarose equilibrated with the same buffer. The flow-through was reapplied three times to the column and unbound material was washed through with 15 ml of column buffer. Bound proteins were eluted by applying 0.5 ml of elution buffer (0.3 M *N*-acetyl-D-glucosamine in column buffer) to the plugged column and incubating for 30 min. The eluate was collected and combined with an additional 0.6 ml of elution buffer wash of the column. *In vitro* autophosphorylation was performed using 50 μl of the eluate for each condition. To each tube was added azatyrosine (final concentration, 500 μM) or an equivalent amount of water followed by IGF-I (final concentration, 20 nM) or water. Incubation was carried out at room temperature for 30 min. Fifty microliters of 50 mM Tris·HCl, pH 7.5/40 mM $MgCl_2$/10 mM $MnCl_2$/[γ-^{32}P]ATP (0.2 μCi/ml; 30 Ci/mmol)/50 μM ATP was then added to each tube and the incubation was continued for an additional 10 min at room temperature. The reaction was terminated by the addition of 35 μl of 4× SDS sample buffer (10) and boiling for 4 min. Analysis was carried out by SDS/PAGE, preparation of an anti-phosphotyrosine immunoblot, and autoradiography. Forty microliters of each sample was used per lane of the gel.

RESULTS

Inhibition of GVBD by Azatyrosine. *Xenopus* oocytes can be induced to mature *in vitro* using progesterone, insulin, or IGF-I, or by the microinjection of oncogenic ras protein (6). We find that maturation can be triggered most effectively *in vitro* by the combination of [Val^{12}]p21$^{Ha\text{-}ras}$ microinjection (5–10 ng per oocyte) and externally applied IGF-I at 2 nM (unpublished data). The experiments described that involve *ras*/IGF-I-induced GVBD were carried out in this manner.

External application of azatyrosine suppressed *ras*/IGF-I-stimulated GVBD in a dose-dependent manner (Fig. 1). A slight but reproducible suppression was observed with 20 μM azatyrosine with much greater effect seen with the application of 100 or 250 μM antibiotic. Azatyrosine alone up to 2 mM had no observable effect on oocytes. L-Tyrosine at 2 mM had minimal effects on *ras*/IGF-I-induced GVBD. Thus the observed effect is specific to azatyrosine.

Given the apparent specificity of the growth-suppressing effect of azatyrosine for *ras*-transformed cultured cells (4), we determined whether the compound could block GVBD in oocytes induced by means other than [Val^{12}]p21$^{Ha\text{-}ras}$ and IGF-I. As shown in Fig. 1, azatyrosine also suppressed progesterone-induced GVBD. On the other hand, GVBD induced by microinjection of an extract from metaphase-arrested oocytes (eggs) was unaffected by azatyrosine. Pre-

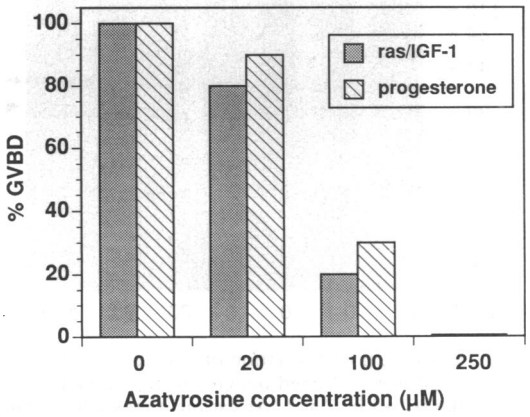

FIG. 1. Suppression of GVBD by azatyrosine. Primed oocytes were incubated in Barth's/BSA in the absence or presence of azatyrosine at 20, 100, or 250 μM for 3 h prior to stimulation. Oocytes were then stimulated with a combination of [Val^{12}]p21$^{Ha\text{-}ras}$ microinjection (5 ng in 20 mM Tris·HCl, pH 7.6/5 mM $MgCl_2$/1 mM 2-mercaptoethanol/10% glycerol) plus 2 nM IGF-I or with 10 μM progesterone. Azatyrosine was present throughout the remainder of the experiment. GVBD was scored 6 h after stimulation by the appearance of a circular unpigmented area at the animal pole.

incubation of oocytes in 500 μM azatyrosine for 3–6 h resulted in no change in either the rate or percentage of oocytes undergoing GVBD after microinjection of an extract containing active MPF (data not shown). Preincubation of oocytes for 15 min in 20 μg of cycloheximide per ml also had no effect on MPF-induced GVBD, indicating that MPF present in the extract was responsible for maturation.

In contrast to MPF-induced GVBD, progesterone-induced GVBD can be completely blocked by inhibition of protein synthesis (11). To determine whether the inhibition of progesterone- or *ras*/IGF-I-induced GVBD by azatyrosine was caused by a similar mechanism, we investigated the effects of azatyrosine on protein synthesis in oocytes. Coinjection of azatyrosine (500 μM) with L-[^{3}H]leucine or L-[^{3}H]tyrosine had no effect on the incorporation of either amino acid into trichloroacetic acid-precipitable material (data not shown). Moreover, preincubation of oocytes in 500 μM azatyrosine for up to 5 h had no effect on the incorporation of radiolabeled L-leucine or L-tyrosine into protein (data not shown).

Tyrosine Phosphorylation of Proteins in Oocytes Undergoing Meiotic Maturation. Given that tyrosine phosphorylation has been shown to be important for meiotic maturation (12, 13), we investigated the effect of azatyrosine on the pattern of protein tyrosine phosphorylation in oocytes after stimulation by progesterone or *ras*/IGF-I. Fig. 2 illustrates the alterations in protein tyrosine phosphorylation observed after progesterone treatment. The most obvious change is an increase with time after progesterone treatment in the tyrosine phosphorylation of a peptide migrating at ≈42 kDa. Enhanced phosphorylation on tyrosine is evident after only 30 min. By 2 h, 20% of the oocytes had undergone GVBD and the tyrosine phosphorylation of this protein had increased to a high level. Subsequent incubation of the oocytes until 100% had completed meiotic maturation resulted in further increases in the tyrosine phosphorylation of this protein. In contrast, a protein migrating at ≈34 kDa showed an apparent decrease in tyrosine phosphorylation with completion of GVBD.

The anti-phosphotyrosine antibody was shown to be specific for phosphotyrosine-containing proteins by preincubating the antibody with 5 mM phosphoamino acid. Interaction of the antibody with proteins on the blot was almost totally eliminated with phosphotyrosine preincubation whereas preincubation with phosphothreonine or phosphoserine was without effect (data not shown).

Recent work by Posada *et al.* (7) has shown that a *Xenopus* member of the MAP kinase family, Xp42, becomes increasingly tyrosine-phosphorylated as progesterone-induced GVBD progresses. We utilized Xp42 antiserum to identify the 42-kDa tyrosine-phosphorylated protein illustrated in Fig. 2. Immunoblots of oocyte proteins using this antiserum were compared to duplicate blots using the anti-phosphotyrosine monoclonal antibody. This analysis revealed identical migration patterns for the 42-kDa tyrosine phosphorylated oocyte protein and the immunoreactive peptide recognized by the anti-Xp42 antiserum (Fig. 3). The protein was detected in untreated oocytes and in oocytes induced to mature by progesterone or *ras*/IGF-I treatment (Fig. 3*C*, lanes 1–3). However, the protein lacking phosphotyrosine in unstimulated oocytes was not recognized by the anti-phosphotyrosine antibody (Fig. 3*B*, lane 1).

A monoclonal antibody directed against the human p34^{cdc2} kinase was used to tentatively identify the 34-kDa protein mentioned above (Fig. 3*A*). The upper band of the doublet migrating just above the 33-kDa marker in Fig. 3*A*, lane 1, represents the phosphorylated species. Dephosphorylation concomitant with maturation causes a shift in migration down into the heavily stained lower band of the doublet. The *Xenopus* homolog of the cdc2 kinase has been shown to be a component of MPF (14).

Effect of Azatyrosine on Tyrosine Phosphorylation of *Xenopus* Proteins. In an effort to decipher potential mechanisms by which azatyrosine exerts its suppressive effect on GVBD, progesterone- or *ras*/IGF-I-treated oocytes were challenged with 250 μM azatyrosine and the proteins were analyzed for changes in the pattern of tyrosine phosphorylation. In the absence of azatyrosine, oocytes stimulated with progesterone or *ras*/IGF-I matured normally and exhibited the characteristic increase in the tyrosine phosphorylation of Xp42

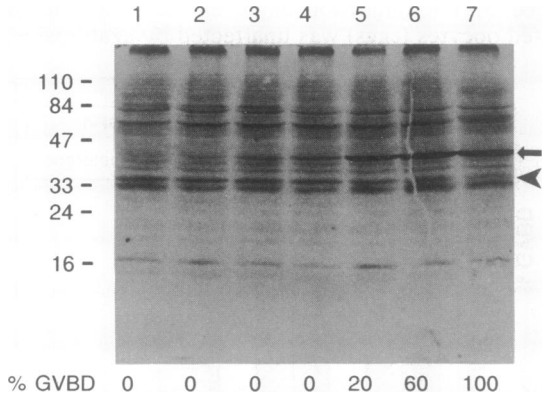

FIG. 2. Tyrosine phosphorylation of oocyte proteins after stimulation by progesterone. Primed oocytes were stimulated with progesterone as described in Fig. 1. At various times after stimulation, oocyte proteins were analyzed by SDS/PAGE and on an immunoblot with a monoclonal antibody directed against phosphotyrosine (final concentration, 1 μg/ml). The lanes correspond to oocytes homogenized before progesterone treatment (lane 1) or after 10, 30, 60, 120, 180, or 240 min of progesterone treatment (lanes 2–7, respectively). At the bottom of the figure are the percentages of oocytes that had completed maturation at the time of homogenization. The 42- and 34-kDa proteins described in the text are indicated by the arrow and arrowhead, respectively. Molecular masses, in kDa, are to the left of the figure.

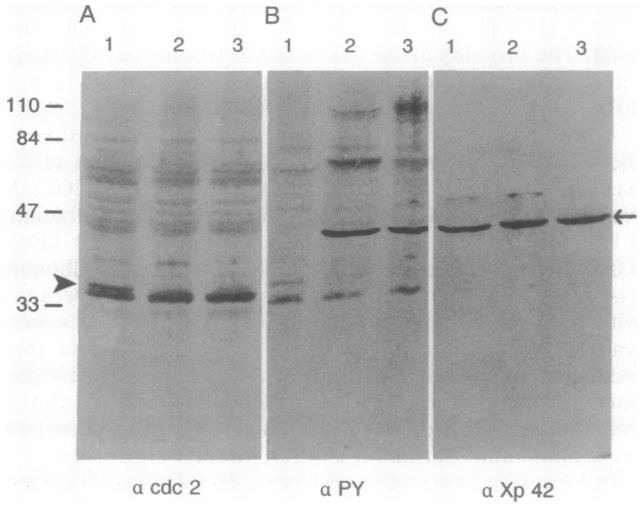

FIG. 3. Immunoblots of oocyte proteins with antibodies directed against p34^{cdc2}, Xp42, and phosphotyrosine. Primed oocytes were left untreated (lanes 1) or were induced to mature with progesterone (lanes 2) or *ras*/IGF-I (lanes 3). After completion of GVBD, oocytes were homogenized, and the proteins were separated by SDS/PAGE, blotted to nitrocellulose, and probed with antibodies specific for p34^{cdc2} (*A*), phosphotyrosine (*B*), or Xp42 (*C*). The anti-p34^{cdc2} and anti-phosphotyrosine antibodies were used at 5 and 1 μg/ml, respectively. The anti-Xp42 serum was used at a dilution of 1:2000. The arrow and arrowhead, respectively, denote the location of the 42- and 34-kDa proteins described in the text. Molecular masses, in kDa, are to the left of the figure.

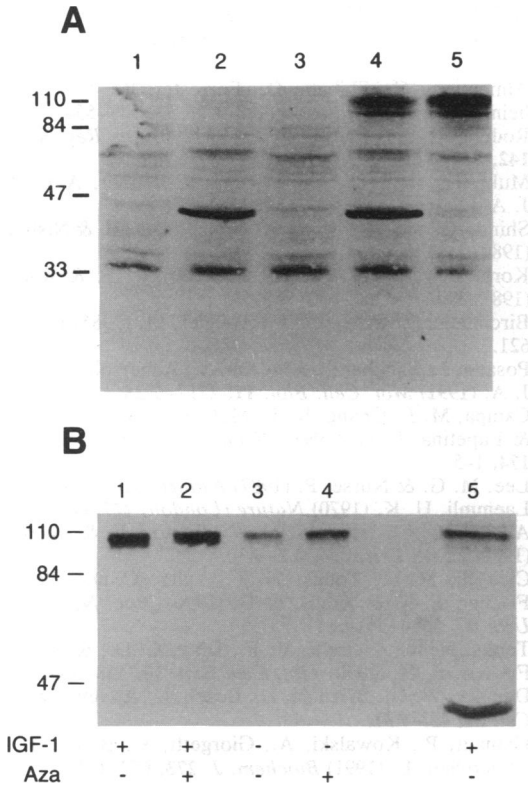

FIG. 4. (*A*) Effect of azatyrosine on tyrosine phosphorylation of Xp42 after stimulation of oocytes with progesterone or *ras*/IGF-I. Primed oocytes were left untreated (lane 1) or were treated with progesterone (lanes 2 and 3) or a combination of [Val^{12}]p21$^{Ha\text{-}ras}$ microinjection and IGF-I (lanes 4 and 5). In addition, some oocytes were preincubated in 250 μM azatyrosine for 3 h prior to the above treatments (lanes 3 and 5). Azatyrosine was present for the duration of the experiment. After oocytes treated with progesterone or *ras*/IGF-I in the absence of azatyrosine had completed GVBD, all oocytes were homogenized and the proteins were analyzed by SDS/PAGE and on an immunoblot with an anti-phosphotyrosine antibody as described in Fig. 2. Molecular masses, in kDa, are to the left of the figure. (*B*) Effect of azatyrosine on the *in vitro* autophosphorylation of the oocyte IGF-I receptor. Oocyte glycoproteins were concentrated by wheat germ agglutinin column chromatography. Fifty microliters of the column eluate was exposed to 20 nM IGF-I for 30 min (lanes 1 and 2) in the presence (lanes 2 and 4) or absence (lanes 1 and 3) of 500 μM azatyrosine (Aza). ATP (final concentration, 25 μM) supplemented with [γ-^{32}P]ATP was then added and the incubation was continued for an additional 10 min. Proteins were analyzed for the presence of phosphorylated tyrosine residues by SDS/PAGE and immunoblot analysis as described in Fig. 2. In addition, the blot was used to expose x-ray film (data not shown). Lane 5 contains proteins from oocytes that had been exposed *in vivo* to 20 nM IGF-I for 10 min and then homogenized. Molecular masses, in kDa, are to the left of the figure.

(Fig. 4*A*, lanes 2 and 4). Preincubation with azatyrosine, however, not only blocked GVBD but also prevented the Xp42 tyrosine phosphorylation as well (lanes 3 and 5). Close inspection of the blot reveals that the decrease in tyrosine phosphorylation of p34^{cdc2} observed upon GVBD is blocked as well. In contrast, azatyrosine had no effect on the tyrosine phosphorylation of a protein migrating at ≈110 kDa (lanes 4 and 5). The oocyte IGF-I receptor β subunit has been reported to migrate on SDS/polyacrylamide gels at the same approximate molecular mass (15). To show that these bands do indeed represent the oocyte IGF-I receptor β subunit, oocyte glycoproteins were concentrated by wheat germ agglutinin column chromatography and IGF-I-induced autophosphorylation was performed *in vitro* in the presence or absence of azatyrosine. As shown in Fig. 4*B*, IGF-I induced the tyrosine phosphorylation of a protein migrating at ≈110 kDa. This tyrosine phosphorylation was unaffected by the presence of 500 μM azatyrosine. The *in vivo*-phosphorylated IGF-I receptor β subunit is shown in lane 5, containing whole oocyte extract from IGF-I-treated oocytes.

DISCUSSION

The results presented above demonstrate the suppression of progesterone- or *ras*/IGF-I-stimulated meiotic maturation of oocytes by the antibiotic azatyrosine. Azatyrosine also inhibited the tyrosine phosphorylation of Xp42, a member of the MAP kinase family (7). Evidence has accumulated suggesting that MAP kinase plays an important role in the mitogenic response of cells to peptide growth factors (16, 17) and phorbol esters (18) and in the differentiation of PC12 cells after treatment with nerve growth factor (19, 20). Recently, two transcriptional factors, protooncogenes c-Myc and c-Jun, were shown to be substrates of MAP kinase (21, 22). Because full activation of MAP kinase, itself a serine/threonine protein kinase, requires phosphorylation on tyrosine (17, 23), the protein is an attractive candidate for integrating the tyrosine and serine/threonine phosphorylation events of various signal transduction cascades.

Even though azatyrosine suppresses GVBD and blocks the tyrosine phosphorylation of Xp42 in oocytes after stimulation by progesterone or *ras*/IGF-I, it cannot be assumed that Xp42 activation is required for meiotic maturation. The time course of oocyte protein tyrosine phosphorylation after progesterone treatment (Fig. 2) shows activation (i.e., tyrosine phosphorylation) of Xp42 prior to activation (i.e., tyrosine dephosphorylation) of p34^{cdc2}. The data, however, are insufficient to substantiate a causal relationship between the two events. Although others have reported oocyte p34^{cdc2} activation to be concurrent with (24) or slightly preceding MAP kinase activation (25), an unequivocal demonstration of the timing of the events *in vivo* has yet to be published. Likewise, the phosphorylation of oocyte MAP kinase after the microinjection of partially purified MPF (24) could be due to the presence of a MAP kinase kinase, not necessarily proving that MAP kinase activation is downstream of p34^{cdc2} activation. A kinase with the ability to reactivate MAP kinase after dephosphorylation of serine and threonine or tyrosine residues has been identified in PC12 cells (17). On the other hand, a recombinant MAP kinase was shown to undergo autophosphorylation on tyrosine residues (26), suggesting that the *in vivo* regulation of the kinase may be a result of a combination of auto- and transphosphorylation. Similar findings have been reported for the oocyte MAP kinase (23). To rule out the possibility of a contaminating MAP kinase kinase, microinjection experiments utilizing purified MPF are required before the temporal and causal relationships between the activation of p34^{cdc2} and MAP kinase can be ascertained.

Recent reports (19, 20) implicate p21$^{Ha\text{-}ras}$ in the sequence of events leading to MAP kinase activation subsequent to stimulation of PC12 cells with nerve growth factor. Moreover, the introduction of [Val^{12}]p21$^{Ha\text{-}ras}$ into 3T3 cells by scrape loading results in MAP kinase activation (27). In oocytes, MAP kinase tyrosine phosphorylation occurs after activation of a *ras*-dependent (i.e., *ras*/IGF-I) and a *ras*-independent (i.e., progesterone) pathway. Although the initial events after progesterone or IGF-I stimulation of oocytes are distinct, progesterone operating through trimeric guanine nucleotide binding proteins (28, 29) and IGF-I by receptor tyrosine autophosphorylation (30), later events appear to be common to both pathways. For example, the microinjection of specific phosphatases has shown that both modes of stimulation are dependent upon protein-tyrosine phosphorylation for their effects in oocytes (22, 23). In addition, activation of MPF prior to GVBD is a process common to

both the progesterone and IGF-I pathways (31). Because azatyrosine suppressed GVBD induced by either progesterone or *ras*/IGF-I, a plausible hypothesis, therefore, is that azatyrosine exerts its action at a point beyond the convergence of these two pathways.

In agreement with our results, Chung *et al.* (32) reported the inhibition of *ras*-induced GVBD by azatyrosine. The lack of effect of microinjected azatyrosine on progesterone-stimulated GVBD reported by Chung *et al.* (32) is in contrast to our data and may be the result of the timing of the microinjection with relation to exposure to the hormone. Microinjection of azatyrosine into oocytes immediately prior to progesterone treatment may not allow enough time for azatyrosine to diffuse sufficiently throughout the oocyte cytoplasm to block the effects of the hormone. However, the time required for the C-terminal processing of the microinjected ras protein may allow sufficient time for diffusion of the antibiotic. Moreover, Chung *et al.* (32) report that inhibition of *ras*-induced GVBD was only observed after microinjection of azatyrosine and not when the antibiotic was simply added to the oocyte incubation medium. These data are contrary to our results; the discrepancy possibly arising from differences in the experimental protocols. We routinely isolate oocytes manually, whereas Chung *et al.* (32) used collagenase. It is conceivable that the enzymatic treatment of the oocytes may have damaged the transporter responsible for the uptake of azatyrosine. We have found that azatyrosine inhibits the uptake by oocytes of L-[^{3}H]tyrosine, implying that both the native amino acid and the antibiotic share the same transport mechanism in oocytes (data not shown). Also, the preincubation of oocytes in azatyrosine employed by Chung *et al.* (32) may simply not have been of sufficient duration for the oocyte to accumulate sufficient antibiotic to inhibit GVBD.

In summary, we have shown that azatyrosine suppresses oocyte meiotic maturation induced by progesterone or by a combination of [Val^{12}]p21$^{Ha\text{-}ras}$ microinjection and IGF-I treatment. The antibiotic also inhibits the tyrosine phosphorylation of an oocyte MAP kinase, Xp42. Our demonstration that GVBD induced by MPF is unaffected by azatyrosine suggests that the antibiotic acts upstream of p34^{cdc2} activation though its precise mode of action is not known. Although we have no direct evidence that azatyrosine inhibits the action of protein-tyrosine kinases that may be involved in the activation of MAP kinase, it is apparent that the antibiotic has little or no effect on the autophosphorylation of the IGF-I receptor in oocytes (Fig. 4). Although in oocytes the immediate target of the IGF-I receptor is not known, an association of the activated receptor with phosphatidylinositol 3-kinase has been demonstrated in LISN C4 cells, a murine fibroblast cell line overexpressing the human IGF-I receptor (33, 34). Given that the 85-kDa subunit of phosphatidylinositol 3-kinase contains two SH2 (for *src* homology 2) domains (35), the association of phosphatidylinositol 3-kinase with activated IGF-I receptors is presumably mediated through a phosphotyrosine–SH2 domain interaction. In preliminary experiments using LISN C4 cells, azatyrosine had no effect on this association (data not shown). This result does not, however, preclude an interruption by azatyrosine of other phosphotyrosine–SH2 interactions that may be required for GVBD. To decipher the mechanism by which azatyrosine suppresses progesterone- or *ras*/IGF-I-induced meiotic maturation, it will be important to investigate its effects on other phosphotyrosine–SH2 interactions and its effects on the activation of MAP kinase by auto- or transphosphorylation.

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