Hormone-induced meiotic maturation in *Xenopus* oocytes occurs independently of p70S6k activation and is associated with enhanced initiation factor (eIF)-4F phosphorylation and complex formation

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SUMMARY

Hormone-induced meiotic maturation of the *Xenopus* oocyte is regulated by complex changes in protein phosphorylation. It is accompanied by a stimulation in the rate of translation, manifest at the level of polypeptide chain initiation. At later times in the maturation process, this reflects an increased ability for mRNA to interact with the 40 S ribosomal subunit. In mammalian cells there is growing evidence for the regulation of translation by phosphorylation of ribosomal protein S6 and of initiation factors responsible for the binding of mRNA to ribosomes. In this report, we show that although the 70 kDa form of S6 kinase is activated within 1.5 hours in response to progesterone or insulin, a time critical for protein synthesis, its activation is not required for hormone-induced stimulation of translation rates or maturation. In response to progesterone, activation of translation occurs in parallel with enhanced phosphate labelling of eIF-4α and eIF-4γ and eIF-4F complex formation, events which are thought to facilitate the interaction of eIF-4F with the mRNA cap structure. However, with insulin, activation of translation occurs prior to detectable de novo phosphorylation of eIF-4F, although a small enhancement of turnover of phosphate on eIF-4c may occur at this early time. With either hormone, enhanced phosphate labelling of eIF-4c is shown to reflect activation of eIF-4c kinase(s), which co-incides temporally with activation of p42 MAP and p90RSK kinases. The possible role of initiation factor modification on increased translation rates during meiotic maturation is discussed.

Key words: protein synthesis, initiation, phosphorylation, S6 kinase, maturation

INTRODUCTION

Fully grown *Xenopus* oocytes are physiologically arrested at the G2/M border of the first meiotic prophase. Treatment with progesterone, insulin, or insulin-like growth factor-1 (IGF-1) can induce progression through meiosis and the production of the mature unfertilised egg, by activating a number of protein kinases which initiate germinal vesicle breakdown (GVBD). The maturation process is associated with quantitative and qualitative changes in the translation of proteins, including the mitotic cyclins and the proto-oncogene c-mos (Maller, 1990; Jessus and Ozon, 1993). This encompasses a change in translation specificity from mRNAs encoding proteins that support growth to those which support the rapid division of cells, associated with a 2-3-fold increase in overall protein synthesis (Woodland, 1974; Laskey et al., 1977; Wasserman et al., 1982; Taylor and Smith, 1985; Patrick et al., 1989; Richter, 1994).

In unstimulated *Xenopus* oocytes only a small proportion of their ribosomes and mRNA are being utilised; even following maturation, only 1-2% of ribosomes are associated with polysomes, suggesting a severe limitation in protein synthesis initiation (Woodland, 1974; Laskey et al., 1977; Audet et al., 1987). Studies have also shown that, relative to the egg, the immature oocyte system exhibits an impairment of initiation of translation at or after the binding of mRNA to the 40 S ribosome (Laskey et al., 1977; Patrick et al., 1989).

In mammalian cells, there is evidence for the regulation of translation by phosphorylation of ribosomal protein S6 and of initiation factors involved in binding mRNA to the 40 S ribosomal subunit (reviewed by Hershey, 1989; Morley and Thomas, 1991; Merrick, 1992; Redpath and Proud, 1994; Morley, 1994). Although S6 phosphorylation may play a role in the selective binding of mRNA species to ribosomes (Jefferies et al., 1994a,b), the exact mechanism of this effect has not been resolved. The activity of certain initiation factors may influence the selection of mRNA from the cellular pool for translation, in a process which is believed to be a rate-limiting step in many systems (Morley, 1994). Briefly, the cap structure facilitates the binding of mRNA to the 40 S ribosomal subunit, a process mediated by at least three initiation factors (eIF-4A, eIF-4B, eIF-4F) and ATP hydrolysis. eIF-4F is a cap binding protein complex composed of three subunits; eIF-4α (eIF-4E) which specifically recognises the cap structure (Sonenberg, 1988), eIF-4β (eIF-4A) an ATP-dependent single-stranded-RNA-binding protein with helicase activity, which appears to recycle through the eIF-4F complex (Pause et al., 1994) and eIF-4γ (p220), whose integrity is required but whose
exact function is unclear (Rhoads, 1991; Morley, 1994). Although little is known about the protein-protein interactions within the eIF-4F complex (Tuzon et al., 1990), it has been recently shown that eIF-4F complex formation is increased following activation of T lymphocytes (Morley et al., 1993). It is believed that the eIF-4F complex functions to unwind secondary structure in the mRNA 5’ untranslated region to facilitate binding of the 40 S ribosome (reviewed by Morley, 1994).

In this report, we have analysed the possible role of the p70s6k family of S6 kinase and of the phosphorylation and assembly of the eIF-4F complex in the stimulation of translation rates during hormone-induced maturation of Xenopus oocytes. In contrast to the findings with mammalian cells, we show that activation of the p70s6k family of S6 kinase is not required for either increased translation rates or hormone-induced meiotic maturation in Xenopus oocytes. In response to progesterone, we show that enhanced phosphorylation of eIF-4α and eIF-4γ is concomitant with increased eIF-4F complex formation and increased rates of protein synthesis. However, in response to insulin, translation rates are substantially increased prior to time when we can detect the de novo phosphorylation of eIF-4α and eIF-4γ and enhanced eIF-4F complex formation. These data suggest that the activation of translational initiation factors can be modulated by multiple pathways during Xenopus oocyte maturation.

MATERIALS AND METHODS

Isolation of Xenopus oocytes

Xenopus oocytes were isolated from dissected ovaries treated with 1 mg/ml collagenase A (Boehringer Mannheim) in modified Barth’s medium (88 mM NaCl, 1 mM KCl, 2.4 mM NaHCO3, 0.3 mM Ca(NO3)2, 0.4 mM CaCl2, 0.82 mM MgCl2, 15 mM Hepes-KOH, pH 7.6) for 1-2 hours at room temperature, and then extensively washed with the same buffer. Stage V and VI oocytes were manually sorted and washed in modified Barth’s medium prior to incubation at 21°C.

Oocyte maturation

Oocytes were incubated in the presence of 5 µg/ml progesterone or 10 µM insulin for the times indicated. For the preparation of extracts to analyse eIF-4F or protein kinase activation, oocytes were washed and resuspended in ice-cold homogenisation buffer (50 mM Mops-KOH, pH 7.2, 50 mM NaCl, 10 mM sodium pyrophosphate, 50 mM NaF, 50 mM β-glycerophosphate, 5 mM EDTA, 5 mM EGTA, 2 mM benzamidine, 1 mM phenylmethylsulphonyl fluoride (PMSF), 14 mM β-mercaptoethanol, 100 µM GTP), lysed by pipetting and cell debris removed by centrifugation in a microfuge for 5 minutes at 4°C. Stage V and VI oocytes were manually sorted and washed in modified Barth’s medium prior to incubation at 21°C.

Preparation of cell-free extracts for protein synthesis assays

Following incubation in the presence of hormone as shown in individual figure legends, oocytes were lysed by centrifugation at 10,000 g in the presence of 20 mM Hepes-KOH, pH 7.5, 125 mM KCl, 2 mM magnesium acetate, 2 mM 2-mercaptoethanol, 3 µg/ml leupeptin, as described previously (Patrick et al., 1989). The cytoplasmic extracts were made 0.5 mg/ml in soybean trypsin inhibitor (Sigma) and used immediately. Protein synthesis assays contained 0.6 vol. extract and 0.4 vol. reaction mix, to give the following concentrations (in addition to those contributed by the extract): 50 mM KCl, 0.8 mM magnesium acetate, 40 mM creatine phosphate (+ creatine phosphokinase), 50 µg/ml calf liver tRNA, 1 mM dithiothreitol (DTT), 370 U/ml placental ribonuclease inhibitor, 50 µM unlabelled methionine and 1 µCi/ml [35S]methionine. Samples were incubated for the indicated times at 23°C, prior to precipitation of protein with trichloroacetic acid; total RNA concentration of the extracts was measured as described (Patrick et al., 1989).

Immunoblotting

After SDS-PAGE, proteins were transferred to polyvinylidene difluoride membrane (PVDF, Immobilon-P, Millipore), p90rsk, MAP kinase and p70s6k were decorated with specific anti-peptide polyclonal antisera kindly provided by Dr J. Blenis, Harvard University. eIF-4α and eIF-4γ were detected with specific rabbit anti-peptide antisera, raised against the following sequences, coupled to keyhole limpet haemocyanin using either glutaraldehyde or 1-ethyl-3-(3-dimethylamino propyl)-carbodiimide (EDC): eIF-4α, WALWFFKNDKSSKTWQANL, synthesised by O. Marin; eIF-4γ, KKEAVGDLLDAFKEVY, synthesised in-house. Antiseras were affinity purified using the respective peptide coupled to Affi-gel 10 (Bio-Rad), according to the manufacturers instructions. Immunoblots were visualised with goat anti-rabbit alkaline phosphatase coupled second antibody (Sigma).

Immunoprecipitation of p70s6k and eIF-4α

Oocyte extracts (20 µg in 20 µl homogenisation buffer) were diluted to 200 µl with Buffer B (50 mM Tris-HCl, pH 8.0, 120 mM NaCl, 20 mM NaF, 30 mM p-nitrophenyl phosphate (NPP), 1% (v/v) Nonidet P40 (NP40), 1 mM benzamidine, 0.1 mM PMSF), and incubated with antisera specific to p70s6k or eIF-4α for 1.5 hours on ice. Immunoglobulins were precipitated and recovered with Protein A-Sepharose (Pharmacia), as previously described (Lane et al., 1992). To assay for p70s6k activity in the immunocomplex, the beads were washed three times with Buffer B, once in assay dilution buffer (50 mM Mops-KOH, pH 7.2, 1 mM DTT, 10 mM MgCl2, 0.2% (v/v) Triton X-100, 5 mM pNPP, 2 mM benzamidine) and resuspended in 5 µl assay dilution buffer. Samples were assayed for their ability to phosphorylate 40 S ribosomal subunits as described (Lane et al., 1992). To analyse protein co-immunoprecipitated with eIF-4α, beads were washed as above, but bound proteins eluted directly with SDS-PAGE sample buffer.

Vertical slab iso-electric focussing (VSIEF) of eIF-4α

The isoelectric focussing method of O’Farrell (1975), modified for use in vertical slab gels, was used essentially as described by Jagus et al. (1993), with the following modifications: the IEF sample buffer contained 1 µM Microcysin LR in addition to the components listed; the anode buffer (upper tank) was 0.01 M glutamic acid and the cathode buffer (lower tank) was 0.05 M histidine. Following transfer of proteins to PVDF, eIF-4α was visualised by immunoblotting.

m⁷GTP-Sepharose affinity chromatography

For the isolation of eIF-4α, cell extracts were diluted to 300 µl with Buffer C (50 mM Mops-KOH, pH 7.2, 0.5 mM EDTA, 0.5 mM EGTA, 100 mM KCl, 14 mM 2-mercaptoethanol, 50 mM NaF, 80 mM β-glycerophosphate, 0.5 mM PMSF, 100 µM GTP) prior to the addition of 25 µl of a 50% (v/v) slurry of m⁷GTP-Sepharose (Pharmacia), equilibrated in Buffer C. Samples were shaken for 10 minutes at 4°C and the resin isolated by centrifugation in a microfuge. The beads were washed three times with Buffer C and bound protein eluted with either SDS-PAGE or VSIEF sample buffer, as indicated. For the isolation of the eIF-4F complex, m⁷GTP-Sepharose column chromatography was employed, as described previously (Morley and Traugh, 1990).

Calmodulin-Sepharose chromatography

Oocyte extracts (50 µl) were adjusted to 2 mM CaCl2 prior to passage through a 1 ml calmodulin-Sepharose column (Pharmacia) equilibrated in Buffer D (50 mM Tris-HCl, pH 7.4, 200 mM NaCl, 14 mM
2-mercaptoethanol, 0.2 mM EDTA, 2 mM benzamidine, 0.5 mM PMSF, 2 mM CaCl₂). Following an extensive wash, bound protein was eluted with Buffer D, but in the presence of 2 mM EDTA and 4 mM EGTA. Proteins were precipitated and analysed for the presence of eIF-4e and eIF-4γ by immunoblotting as described above.

RESULTS

Activation of the p70s6k family is not required for meiotic maturation of Xenopus oocytes

Previous studies with Xenopus oocytes have shown that progesterone-induced meiotic maturation results in a stimulation of the rate of translation on endogenous mRNA (Woodland, 1974; Laskey et al., 1977; Patrick et al., 1989). In many cell systems, activation of protein synthesis is associated with an increase in the phosphorylation of ribosomal protein S6 and translation initiation factors (reviewed by Morley, 1994). Whilst more than one family of S6 kinase is known to exist in Xenopus oocytes, their activation appears to be differentially regulated (Stefanovic and Maller, 1988; Erikson, 1991). Lane et al. (1992) have shown that progesterone induces the transient activation of the p70s6k family of S6 kinase within 1 hour of hormone treatment of non-primed oocytes, whilst others had shown that the major activation of the p90rsk family of S6 kinases occurs late during the maturation time course (Stefanovic and Maller, 1988; Erikson and Maller, 1989; Maller, 1990). We have utilised the macrolide immunosuppressant rapamycin, which specifically prevents activation of p70s6k in many cell systems (Chung et al., 1992; Downward, 1994) to investigate the potential role of this family of S6 kinases in the activation of translation. Fig. 1A shows that neither the rate nor the extent of progesterone- or insulin-induced meiotic maturation is affected by the presence of extracellular rapamycin (1 µM). To determine whether rapamycin was entering the oocytes and functioning as predicted, the activation of p42 MAP kinase, p90rsk and p70s6k kinases was visualised by polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting. All of these kinase families are themselves activated by phosphorylation (Erikson, 1991; Blenis, 1993), which results in a characteristic reduction in migration on SDS-PAGE, and hence in an apparent increase in molecular mass (Chung et al., 1992). As shown in Fig. 1B, this characteristic mobility shift of p90rsk and p42 MAP kinase induced by either progesterone or insulin occurs late.
during maturation and is unaffected by rapamycin. Immunoblot analysis of the activation of p70S6K by this method was inconclusive due to fact that p70S6K is down-regulated within 3 hours (Lane et al., 1992). Therefore, we directly assayed the activity of p70S6K in extracts prepared at 1.5 hours post-stimulation using immunoprecipitation and an immunocomplex assay (Chung et al., 1992). Fig. 1C shows that both progesterone and insulin stimulate the activity of p70S6K to a similar extent, as assayed by the increased ability of the immunocomplex to phosphorylate isolated 40 S ribosomal subunits. The low activity of p70S6K in the oocyte extract is consistent with the use of non-primed frogs in this work (Lane et al., 1992). However, in the presence of rapamycin, the activation of p70S6K is abrogated. These data have been confirmed utilising a peptide substrate for p70S6K designed after the known phosphorylation sites on S6 (data not shown). Therefore, we conclude that activation of p70S6K is not necessary for progesterone- or insulin-induced meiotic maturation in Xenopus oocytes.

Insulin stimulates protein synthesis at the level of initiation

We have also measured translation rates in cell extracts (Patrick et al., 1989), prepared at different times after hormonal stimulation, in the absence or presence of rapamycin. Table 1 shows that progesterone effects a 3-fold increase in translation rates on endogenous mRNA evident within 1.5 hours, with a further increase to maximal levels between 5-9 hours coincident with GVBD. In contrast, insulin causes a maximal increase in translation rate within 1.5 hours, prior to GVBD. In both cases, however, the decrease in protein synthesis rate observed at 5 hours or 3 hours post-hormone treatment, respectively, was not reproducible between experiments. The finding that increased translation rates were largely unaffected by rapamycin, suggests that activation of p70S6K is not required for the stimulation of protein synthesis observed at any stage during meiotic maturation. Fig. 2A-E shows [35S]methionine incorporation into protein in extracts prepared at different times following insulin treatment. Fig. 2A,B show that, in agreement with the data presented in Table 1, relative to the unstimulated oocyte system, insulin induces a substantial increase in the translation rate within 1.5 hours, which was not prevented by the presence of rapamycin. To determine whether increased translation rates reflect a stimulation of the initiation phase of protein synthesis, we have used the initiation inhibitor ecdine; by virtue of the increased sensitivity to ecdine, increased protein synthesis rates reflect, in part, an increased efficiency of reinitiation of translation on endogenous mRNA. This effect is further evident at later times of maturation (Fig. 2C,D) and illustrated in Fig. 2E, which shows the increase in ecdine-sensitive (i.e. initiation-dependent) incorporation in these extracts. To complement these studies, we also examined the translation products from endogenous mRNA in these extracts. Fig. 2F shows that in all cases, translated products encompass a wide range of molecular masses; at early times (lanes a-c), there was essentially no detectable qualitative changes in translation. These data suggest that the increased protein synthesis rates at these times reflects increased utilisation of mRNA already interacting with the translational apparatus. However, upon prolonged exposure to insulin (lanes e-g), a number of characteristic changes in the pattern of protein synthesis occurred, similar to that observed with progesterone (lane h). These included an increase in the synthesis of low molecular mass products, co-incident with an increase in initiation-dependent translation. A more detailed analysis involving two-dimensional gel electrophoresis would be required to characterise any early changes in individual translation products.

<table>
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<tr>
<th>Time after addition (hours)</th>
<th>Protein synthesis at 60 min (cpm/µg total RNA)</th>
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<tr>
<td></td>
<td>− rapamycin</td>
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<td>Progesterone</td>
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<td>0 (no hormone)</td>
<td>749</td>
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<tr>
<td>1.5</td>
<td>2101</td>
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<td>3.0</td>
<td>2160</td>
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<td>5.0</td>
<td>1277</td>
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<td>7.0</td>
<td>5840</td>
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<td>9.0</td>
<td>5221</td>
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<tr>
<td>Insulin</td>
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<tr>
<td>0 (no hormone)</td>
<td>901</td>
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<tr>
<td>1.5</td>
<td>4027</td>
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<tr>
<td>3.0</td>
<td>2418</td>
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n.d., not determined.

Progestosterone and insulin increase phosphate labelling of eIF-4α

To examine the potential role for increased phosphorylation of initiation factors in enhanced translation rates, we have analysed the phosphate labelling of eIF-4α and -4γ in intact oocytes during meiotic maturation. To this end, Xenopus oocytes were incubated with [32P]orthophosphate overnight and removed from the labelling medium prior to hormone treatment. At various times after hormone treatment, cell extracts were prepared and eIF-4α isoform isolated by m7GTP-Sepharose chromatography and equal amounts of eIF-4α protein visualised by SDS-PAGE. The autoradiogram presented in Fig. 3A shows that both progesterone and insulin increase the phosphate labelling of eIF-4α, evident typically at 30-40% GVBD. The increases in labelling are insensitive to rapamycin, and in the case of progesterone, co-incide roughly with the major increase in translational activity of the extracts (Table 1). In the case of insulin, protein synthesis activity had already increased substantially by 1.5 hours (Table 1, Fig. 2A-E), and therefore preceded the major increase in labelling of eIF-4α. However, a small but reproducible enhancement of labelling of eIF-4α after 1.5 hours of insulin treatment could be revealed by prolonged exposure of the gel, an effect not evident with progesterone (Fig. 3A). In order to assess whether increased phosphate labelling of eIF-4α reflects increased de novo phosphorylation or phosphate turnover, we have used one dimensional iso-electric focussing (VSIEF) and immunoblotting to examine the phosphorylation status of eIF-4α during maturation. Fig. 3B shows the results from one such experiment following isolation of eIF-4α from oocyte extracts by m7GTP-Sepharose affinity chromatography. In the unstimulated oocytes, immunoblot analysis of eIF-4α yields three bands; the lower band is the less-phosphorylated form, the middle band the more highly phosphorylated form and an
upper band, which is possibly an isoform of eIF-4α retained during the affinity chromatography step. Following hormonal stimulation for 5-7 hours, immunoblot analysis shows an increase in the level of eIF-4α protein migrating as the phosphorylated variant relative to the unstimulated oocyte. The identity of these species was confirmed by the use of potato acid phosphatase or following preparation of extracts in the absence of phosphatase inhibitors; under these conditions, all of the immunoreactive eIF-4α isolated from hormone-stimulated oocytes migrated largely as the less phosphorylated variant, as observed with the unstimulated oocyte (data not shown). These data suggest that progesterone and insulin either increase the activity of an eIF-4α-specific kinase or inactivate a specific phosphatase, or some combination of both. To resolve this, extracts were assayed for their ability to phosphorylate bacterially-expressed eIF-4α protein. The autoradiogram presented in Fig. 3C shows that progesterone treatment enhances the activity of eIF-4α kinase late during maturation, an event which can be correlated with the mobility shift of eIF-4α isolated from intact oocytes on VSIEF (Fig. 3B) and the activation of p90rsk and p42 MAP kinase (Fig. 1). Insulin induces a small, reproducible increase in eIF-4α kinase activity at 1.5 hours, reflected in the low level of phosphate labelling at this time (Fig. 3A) but prior to any detectable mobility shift of eIF-4α isolated from intact oocytes (Fig. 3B). A later phase of eIF-4α kinase activity is seen with similar characteristics to that observed with progesterone, suggesting that, at least in part, activation of eIF-4α kinase(s) is responsible for increased labelling of this protein during maturation.

**eIF-4γ phosphorylation and eIF-4F complex formation are enhanced during meiotic maturation**

There is growing evidence that phosphorylation of the eIF-4γ subunit of the eIF-4F complex plays an important role in translational control (reviewed by Morley, 1994). The formation of functional complexes between the individual subunits, the phosphorylation of the eIF-4F complex, and its interaction with other initiation factors plays a central role in the binding of mRNA to the 48 S initiation complex (Hershey, 1989; Merrick, 1992; Redpath and Proud, 1994; Morley, 1994). Therefore we have examined the phosphorylation status during meiotic maturation of eIF-4γ recovered on m7GTP-Sepharose by virtue of its affinity for eIF-4α (Morley and Traugh, 1990). Fig. 4A shows that in vivo phosphate-labelling of eIF-4γ is substantially increased in response to either progesterone or insulin, in a time course which parallels that of eIF-4α. Immunoblot
Fig. 3. Progesterone and insulin increase phosphate-labeling of eIF-4α and eIF-4γ. (A) Oocytes were incubated in the presence of 500 µCi/ml [γ-32P]orthophosphate for 16 hours at 21°C, washed and maintained in the same buffer. Oocytes were then incubated in the absence or presence of progesterone, insulin or rapamycin (1 µM), as indicated. Sets of 20 oocytes were removed at the time indicated, rinsed and lysed in an equal volume of lysis buffer. eIF-4α was purified by m7GTP-Sepharose affinity chromatography following extensive washing of the resin as described above, and visualised by SDS-PAGE and autoradiography. The right hand panel shows the phosphate labeling of eIF-4α following exposure of oocytes to progesterone (upper) or insulin (lower) for 0, 1.5 or 7 hours, as described, but with a 5-fold increase in exposure time to film over that shown in the left hand panel. (B) Oocyte maturation is associated with enhanced de novo phosphorylation of eIF-4α. Oocytes were prepared and incubated in the absence of phosphate-labeling, with or without hormone or rapamycin as indicated. At the times shown, 20 oocytes were removed, cell extracts prepared and incubated with 20 µl of a 50% (v/v) slurry of m7GTP-Sepharose for 10 minutes on ice. The resin was isolated by centrifugation, washed 3 times in buffer containing phosphatase and protease inhibitors, and bound protein eluted with IEF sample buffer (containing 1 µM Microcystin LR) and resolved by one-dimensional vertical slab isoelectric focussing (VSIEF). Following transfer to PVDF membrane, eIF-4α was visualised by immunoblotting using affinity-purified rabbit anti-peptide polyclonal antisera. Arrows indicate the migration of eIF-4α, with P indicating the more highly phosphorylated variant and P′ putative isoforms of eIF-4α. (C) Progesterone and insulin activate eIF-4α kinase(s). Oocyte extracts prepared as described above were assayed for their ability to phosphorylate bacterially expressed eIF-4α in vitro. Each assay (10 µl; containing 50 mM Mops-KOH, pH 7.2, 5 mM MgCl2, 200 µM (1320 cpm/pmol) ATP, 500 nM PKI, 2 mM DTT, Xenopus extract (50 µg protein), 2 µg recombinant eIF-4α protein, 5 mM ρ-NPP) was incubated for 20 mins at 23°C, prior to dilution to 200 µl with lysis buffer. eIF-4α was isolated from the reaction mixture by m7GTP-Sepharose chromatography as in Fig. 2B, and visualised by gel electrophoresis. The resulting autoradiogram is presented.

Analysis of eIF-4α and eIF-4γ in complexes isolated on m7GTP-Sepharose (Fig. 4A) shows that although the level of eIF-4α does not change during hormone-induced maturation, there is a large increase in the association of eIF-4γ with eIF-4α, suggesting that the level of eIF-4F complex in the oocyte increases during maturation. To look more directly at eIF-4F complex formation during meiotic maturation, eIF-4α was immunoprecipitated from cell extracts and the amount of associated eIF-4γ visualised by immunoblotting. Fig. 4B shows that following stimulation with either progesterone or insulin, there
was increased recovery of eIF-4γ associated with eIF-4α, coincident with their phosphorylation during maturation. Alternatively, eIF-4γ has been isolated from cell extracts by virtue of its affinity for calmodulin; in the presence of calcium, eIF-4γ is retained on the resin whilst eIF-4α not associated with eIF-4γ does not bind. Using this resin, the data presented in Fig. 4C indicates that following progesterone or insulin stimulation of oocytes, there is increased association between eIF-4α and eIF-4γ. This was confirmed using immunoprecipitation with serum specific to eIF-4γ (data not shown). Therefore, these data show that eIF-4F complex formation is enhanced during meiotic maturation, co-incident with its increased phosphorylation and the stimulation of protein synthesis initiation.

DISCUSSION

Hormone-induced meiotic maturation of the Xenopus oocyte is regulated by complex changes in protein phosphorylation (reviewed by Maller, 1990; Jessus and Ozon, 1993) and is accompanied by a 2-3-fold increase in the rate of protein synthesis (Woodland, 1974; Laskey et al., 1977; Wasserman and Smith, 1978; Wasserman et al., 1982; Taylor and Smith, 1985; Richter, 1994). This enhanced translation rate is associated with increased recruitment of mRNA into polysomes without a change in polypeptide elongation rates (Richter et al., 1982). This has been attributed to a number of concomitant events, such as the dissociation of masking proteins inhibiting the recruitment of mRNAs (Richter, 1986), regulation of the length of mRNA poly(A) tails, (where addition of poly(A) is correlated with activation of many maternal mRNAs (reviewed by Jackson and Standart, 1990; Sheets et al., 1994)), increased phosphorylation of ribosomal protein S6 (Cicirelli et al., 1988; Erikson and Maller, 1989; Maller, 1990) and the activation of initiation factor eIF-4A (Audet et al., 1987). We have used maturation of Xenopus oocytes as a model system to study the potential role of S6 and initiation factor phosphorylation in the activation of protein synthesis.

In Xenopus oocytes, S6 becomes multiply phosphorylated on serine residues in response to progesterone, insulin, phorbol esters and micro-injection of proteins encoded by transforming viruses (Maller, 1990; Morley and Thomas, 1991). We now show that insulin can activate the p70s6k family of kinase (Fig. 1), and that this effect co-occurs with a major increase in protein synthesis rate, which appears to reflect an increase in the efficiency of re-initiation of translation on endogenous mRNA (Fig. 2). To determine whether p70s6k plays an essential role in the increased rates of translation, we have used rapamycin, a macrolide immunosuppressant, which specifically prevents the activation of the p70s6k, but does not affect the activation of either p42 MAPK or p90rsk (Fig. 1). Pretreatment with rapamycin did not prevent either progesterone or insulin-induced maturation (Fig. 1), and had little effect on the activation of overall protein synthesis (Fig. 2 and Table 1). Our
data indicate that activation of p70S6K, which is believed to be the physiological S6 kinase in mammalian cells (Chung et al., 1992; Blenis, 1993; Morley, 1994; Downward, 1994) is not required for the overall activation of translation during progesterone or insulin-induced meiotic maturation. At this time, based on one-dimensional gel electrophoresis of labelled proteins (Fig. 2F), we do not know whether rapamycin affects the translation of specific classes of mRNA (Jefferies et al., 1994a,b). These data suggest either that S6 phosphorylation plays no role in the activation of protein synthesis in Xenopus oocytes or that p70S6K activation is sufficient to satisfy any requirement for S6 phosphorylation during this period. Indeed, stimulation of p70S6K activity can be correlated with increased translation rates, with p90RSK activated within 20-30 minutes in response to insulin (Cicirelli et al., 1988), an effect not observed with progesterone (Lane et al., 1992).

The activity of initiation factors can be regulated by phosphorylation in response to growth factors, mitogens, phorbol esters and transforming viruses. Recently, Xu et al. (1993) have shown that, co-incident with activation of translation, eIF-4 complex formation has increased during maturation of starfish oocytes, induced by 1-methyladenine. However, the authors have not determined whether this increased labelling reflects increased de novo phosphorylation or turnover of phosphate on eIF-4. In this study, we show that both progesterone and insulin-induced maturation of Xenopus oocytes are associated with increased phosphate labelling of eIF-4 (Fig. 3), which is clearly evident at 5 hours after addition of hormone, co-incident with activation of p42 MAP and p90RSK kinases. Prolonged exposure of the gel (Fig. 3A) revealed a small, reproducible increase in eIF-4 labelling within 1.5 hours of insulin treatment. Iso-electric focusing and immunoblotting indicate that this early phase of labelling probably reflects turnover of phosphate on eIF-4, since there is no detectable change in the distribution of the factor between phosphorylated and non-phosphorylated forms at this early time. However, the larger increase in labelling at later times of maturation primarily reflects de novo phosphorylation (Fig. 3A,B). In some ways, the increased phosphate labelling of eIF-4 co-incident with enhanced translation rates and the onset of GVBD is similar to that reported for starfish oocytes induced to mature with 1-methyladenine (Xu et al., 1993). However, in our study the major increase in phosphorylation of eIF-4 at 5 hours post-progesterone stimulation was partially inhibited by rapamycin; this was not a reproducible finding between different batches of oocytes. Preliminary data using 32P-labelled eIF-4F as a substrate has suggested that whilst phosphatase activity towards eIF-4 is increased in response to either hormone, the rate of dephosphorylation of eIF-4 is similar at 1.5 hours and 7 hours post-stimulation (S. J. Morley and V. M. Pain, unpublished data).

Therefore, the enhanced activity of eIF-4 at early times of insulin treatment are off-set by increased phosphatase activity, resulting in enhanced phosphate turnover on eIF-4, and no de novo phosphorylation (Fig. 2B,C); at later times, further kinase is activated with no change in phosphatase activity, resulting in de novo phosphorylation. We have also assayed extracts for their ability to phosphorylate a bacterially-expressed variant of eIF-4 protein, where the major site of phosphorylation (Ser53) has been mutated to Alanine (Ala53). Although phosphorylated with a lower stoichiometry, the Ala53 mutant protein is phosphorylated with a similar time course to that of the Ser53 protein (data not shown). This suggests that either the observed increase in eIF-4 kinase activity reflects, in part, activation of a non-specific kinase or else that eIF-4 can be phosphorylated at more than one site (Bu et al., 1993). Kaufman et al. (1993) have shown that the Ala53 mutant protein is efficiently phosphorylated in COS-1 cells, and that the phosphorylation of Ser53 is not required for its interaction with eIF-4. The physiological significance of phosphorylation sites additional to Ser53 has not as yet been determined.

Although the role of eIF-4 in translation is unclear, it is known to be required for the efficient translation of capped mRNA (reviewed by Sonenberg, 1988; Morley, 1994). It is now believed that eIF-4 coordinates the activity of eIF-4A and eIF-4 at the initiation of interaction with mRNA, in a sequence independent manner, prior to the specific interaction of eIF-4 with the cap structure (Pause et al., 1994). We now show that the phosphorylation of eIF-4 is increased during the later stages of both progesterone- and insulin-induced meiotic maturation, co-incident with enhanced interaction of this protein with the eIF-4 complex (Fig. 4). We have demonstrated this enhanced interaction using mGTP-Sepharose and by immunoprecipitation with anti-eIF-4 antibody and direct isolation of eIF-4 from the eIF-4 complex (data not shown). A possible relevance of increased levels of eIF-4 complex formation on rates of translation is shown by the finding that the eIF-4 complex has a 20-fold increased ability over free eIF-4 to unwind the secondary structure at the 5′ end of mRNA (Pause et al., 1994). Moreover, phosphorylation of eIF-4 in vitro stimulates its interaction with the mRNA cap structure (Morley et al., 1991). Enhanced eIF-4 complex formation has been reported following the mitogenic activation of protein synthesis in primary T cells (Morley et al., 1993) and treatment of HepG2 cells with okadaic acid (Bu et al., 1993), both of which result in increased phosphorylation of eIF-4.
uration through separate, but converging, signalling pathways. During the process of meiotic maturation, a dramatic increase in protein phosphorylation occurs due to activation of a cascade of protein kinases (Maller, 1990; Jessus and Ozon, 1993). These include the de novo synthesis and activation of the c-mos kinase, possibly regulated by increased polyadenylation of mos maternal mRNA (Sheets et al., 1994). This is the only protein whose synthesis is essential for the initiation of meiotic maturation (Matten et al., 1994). Completion of meiosis I and arrest in meiosis II is regulated by a progesterone-induced cascade of events including temporal changes in the activities of p70s6k (Lane et al., 1992), p34cdc2 kinase, cdc25, p42 MAP kinase, which lags about 2 hours behind the onset of mos protein synthesis, protein kinase A, and activation of the p90rsk family of S6 kinases (Erikson and Maller, 1989; Jessus and Ozon, 1993; Matten et al., 1994). A ras-dependent pathway of p42 MAP kinase activation also exists: Xenopus oocytes contain p21ras, which is required for insulin-stimulated maturation and is activated via the insulin receptor substrate 1 (IRS-1: Myers et al., 1994). By analogy with events known to occur in somatic cells (Grigorescu et al., 1994; Downward, 1994), insulin is believed to lead to the activation of p90rsk via MAP kinase kinase during maturation. Temporal correlations presented in Figs 1 and 3, suggest a possible link between the increased de novo phosphorylation of eIF4-α and eIF4-γ and the activation of the p42 MAP kinase pathway. In the light of the sensitivity to rapamycin, the above data also show that p70s6k itself, or any immediately downstream kinase activated by p70s6k is not responsible for increased phosphorylation of eIF4-α. Reddy et al. (1993) have suggested that one possible candidate for an eIF4-α kinase in a protamine kinase, activated via a MAP kinase pathway distinct to that of p42 MAP kinase. However, it is unlikely to be a candidate here as the level of protamine kinase activity did not increase during meiotic maturation (data not shown). At this time, the identity of any specific kinase(s) involved in phosphorylation of eIF4-α awaits determination.

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