Characterization of protein kinases in mitotic and meiotic cell extracts

Margaret S. Halleck, Katherine Lumley-Sapanski, Jon A. Reed, Anand P. Iyer, Andrea M. Mastro and Robert A. Schlegel*

Department of Biochemistry, Microbiology, Molecular and Cell Biology, The Pennsylvania State University, University Park, PA 16802, USA

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A number of protein kinases have been separated and identified in extracts from mitotic and interphase culture cells and from mature and immature amphibian oocytes using nondenaturing polyacrylamide gel electrophoresis followed by in situ phosphorylation assays. Certain of these protein kinase activities appear to correlate with the biological activity of extracts, assayed by their ability to induce meiotic maturation following injection into *Xenopus* oocytes. These results are consistent with the notion that protein phosphorylation/dephosphorylation may be integral to the mechanisms of both nuclear membrane breakdown and chromosome condensation, events common and distinctive to mitosis and meiosis.

In situ protein kinase assay *Mitosis* *Meiosis* *Nuclear membrane breakdown* *Chromosome condensation* Xenopus *oocyte*

1. INTRODUCTION

Somatic cells entering mitosis, and maturing oocytes, each undergo two distinctive cytological events, breakdown of the nuclear membrane and condensation of chromatin into distinct chromosomes. Injection of extracts from mammalian mitotic cells into immature amphibian oocytes can induce these late maturational events [1–3], suggesting that a common mechanism may operate in both mitosis and meiosis. In both cases, a variety of evidence implicates protein phosphorylation/dephosphorylation in these distinctive events [4–8]. Although the phosphoproteins of mitotic cells are being characterized using monoclonal antibodies [9], a detailed accounting of mitotic- or meiotic-specific protein kinases is lacking. Here, we have characterized the protein kinases of extracts from mitotic and meiotic cells, employing an in situ method to localize these activities on nondenaturing gels [10]. These same extracts were then injected into oocytes to test for biological activity, to determine whether particular protein kinases might be present in all biologically active extracts but absent in those lacking such activity.

* To whom correspondence should be addressed

Abbreviation: PMSF, phenylmethylsulfonyl fluoride

2. MATERIALS AND METHODS

2.1. *Cell culture and synchronization*

Human D98/AH2 cells, a HeLa derivative, were grown in Eagle's minimum essential medium (MEM) supplemented with 10% calf serum (CS). Mitotic cells (75–95%) were collected by shakeoff from cultures grown for 10–11 h in the presence of 0.02 μg/ml of colcemid. Cells were synchronized in interphase by growth for 44 h in isoleucine-deficient MEM supplemented with 10% dialyzed CS [11]. Release of these G1-arrested populations into complete growth medium for 4–6 h produced G1-traversing populations.

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2.2. *Preparation of somatic cell extracts*

Interphase or mitotic cell preparations were suspended in hypotonic buffer A [12] minus PMSF and broken open by repeated passages through a 23-gauge needle. Nuclei (interphase) or chromosomes (mitotic) and debris were removed by centrifugation [12] and the resulting supernatants were designated 'crude extracts'. 'Precipitated mitotic extracts' were prepared by salting out with ammonium sulfate, retaining the 20–40% cut [2], and were dialyzed against 10 mM Tris–HCl, 5 mM β-glycerol phosphate, 1 mM ATP, 10 mM $MgCl_2$ (pH 7), to remove residual salt.

2.3. *Preparation of oocyte extracts*

Large stage VI [13] oocytes were obtained surgically from *Xenopus laevis* frogs primed by injection with pregnant mare's serum gonadotropin, as in [14]. Either these immature oocytes or mature oocytes produced by in vitro exposure to progesterone (10 μg/ml) were broken open by ultracentrifugation (160000 × g, 35 min, 4°C) in extraction buffer [8] plus 1 mM ATP. The resulting clear infranatant between cellular debris and yolk was designated 'crude extract'. Ammonium sulfate precipitation (20–40% cut) produced 'precipitated oocyte extract'.

2.4. *Nondenaturing polyacrylamide gel electrophoresis and in situ assay for protein kinase activity*

The procedure followed was that in [10]. In brief, clarified samples (150000 × g) were run on polyacrylamide slab gels prepared as in [15] except that SDS was excluded. Gels were phosphorylated using [γ-^{32}P]ATP in the presence or absence of exogenous histone substrate, stained for protein (if histone was excluded), dried and autoradiography performed.

2.5. *Oocyte assay*

Individual stage VI immature oocytes were injected with 50–80 nl of extract using glass needles.

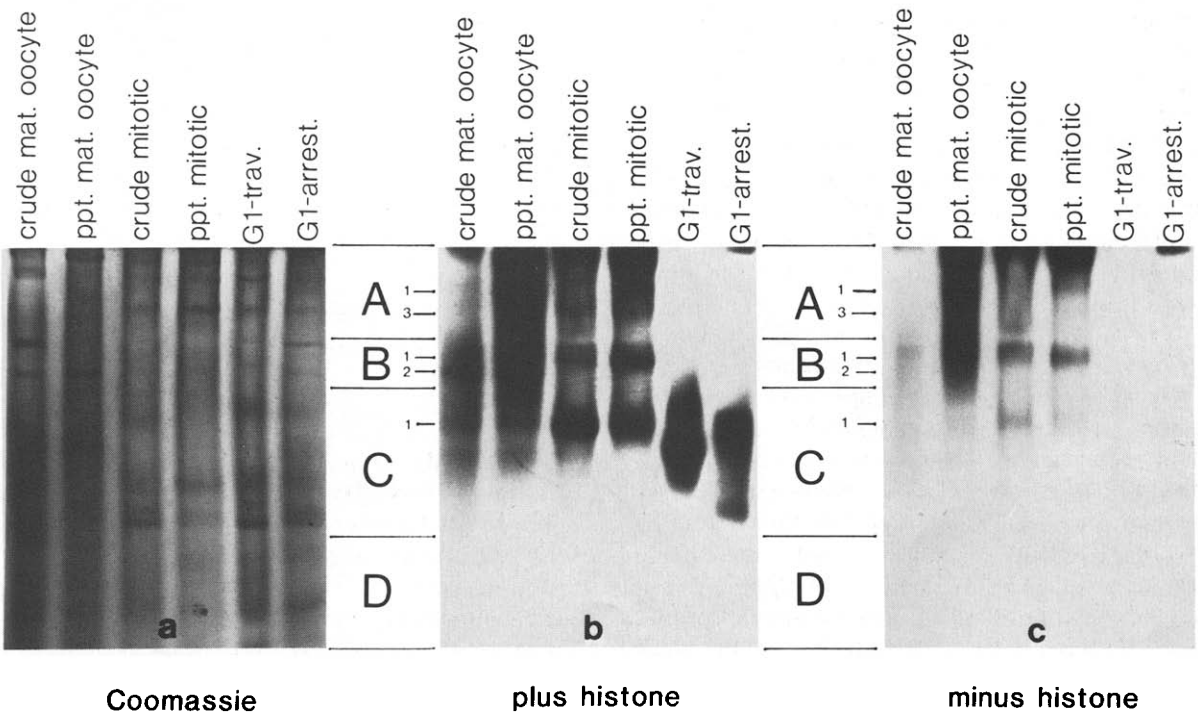

Fig.1. In situ assay for protein kinase activity of meiotic, mitotic and interphase extracts separated on nondenaturing polyacrylamide gels. (a) Coomassie blue staining of one half of a gel. (b) Autoradiogram of the other, duplicate half of the gel phosphorylated in the presence of exogenous histone substrate. (c) Autoradiogram of the same stained half of the gel seen in (a), phosphorylated in the absence of exogenous substrate. Equivalent amounts of protein were loaded into each well.

Meiotic maturation was identified cytologically by the appearance of a characteristic 'white spot' in the polar region of the pigmented animal hemisphere, indicative of germinal vesicle breakdown and chromosome condensation. Oocytes matured by progesterone in vitro served as positive controls; negative controls were pricked oocytes or oocytes injected with buffer only.

3. RESULTS AND DISCUSSION

When crude cytoplasmic extracts prepared from a population of mammalian culture cells arrested in mitosis were electrophoresed in nondenaturing polyacrylamide gels, approx. 10–12 major protein bands were visualized by staining with Coomassie blue (fig.1a). When phosphorylation reactions were performed in situ, 4–6 of these protein bands demonstrated kinase activity, revealed by autoradiography, by their ability to transfer ^{32}P from ATP to histone substrate (fig.1b). Because the maturation promoting activity (MPA) of extracts, i.e., their ability to induce germinal vesicle breakdown and chromosome condensation when injected into immature oocytes, has been shown to be autocatalytic [11,16,17], and since one possible mechanism for this autoactivation might be phosphorylation, protein kinase assays were also carried out in the absence of exogenously added substrate. Fig.1c is an autoradiogram of the other, duplicate half of the gel shown in fig.1b, with no substrate added during phosphorylation. A few of the bands identified by phosphorylation in the presence of exogenous substrate are also detected in its absence (band B1, in particular). Although these bands were likely produced by autophosphorylation, the possibility that an endogenous substrate might comigrate with a protein kinase cannot be excluded. Ammonium sulfate precipitation of mitotic extract resulted in an increase in intensity of protein kinase bands relative to those of their crude counterparts, when equivalent amounts of protein were separated and assayed on the same gel (fig.1b,c). A better example of this enrichment with two other extracts is seen in fig.2.

When crude cytoplasmic extracts from populations of interphase culture cells were examined on the same gel, the pattern of bands seen upon Coomassie staining was similar to that of mitotic

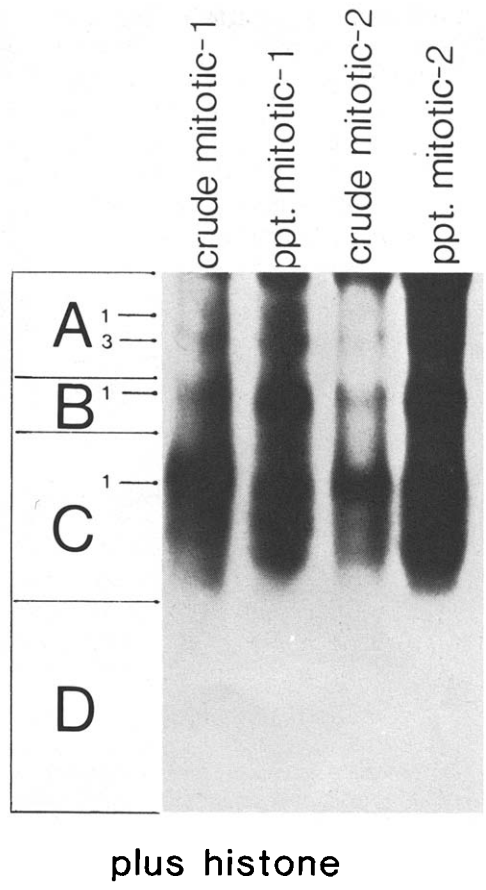

Fig.2. Comparison of crude and ammonium sulfate precipitated mitotic extracts. Autoradiogram of a gel containing two different mitotic extracts (each unfractionated or precipitated) phosphorylated in the presence of histone. Equivalent amounts of protein were loaded into each well.

extracts, whether extracts from G1-traversing or G1-arrested cells were examined (fig.1a). However, only a few bands of protein kinase activity resulted when assays were performed using histone substrate (fig.1b). Although some differences between the two types of interphase extract were apparent, in both cases protein kinase activity was mainly confined to the C region of gels, in marked contrast to mitotic extracts, where considerable protein kinase activity was found in the A and B regions as well. Interphase extracts phosphorylated in the absence of added substrate produced some faintly visible bands (fig.1c). However, phosphorylation of band B1, the

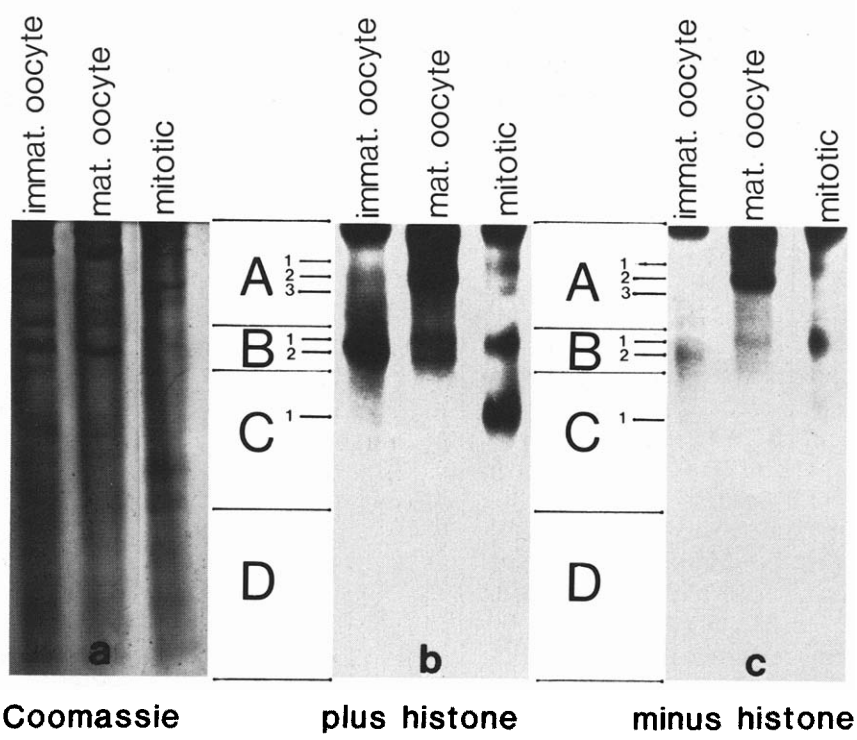

Fig.3. Comparison of immature and mature oocyte extracts. (a–c) As in fig.1. All extracts, including the mitotic extract used for comparison, were ammonium sulfate precipitated. Equivalent amounts of protein were loaded into each well.

predominant (auto)phosphorylated species in mitotic extrats, was not detectable in interphase extracts, either in the presence or absence of exogenous substrate.

Not surprisingly, the pattern of Coomassie-stained protein bands produced by a cytoplasmic extract prepared from frog oocytes was quite different from that of somatic culture cell extracts (fig.1a). Yet, certain similarities were seen when the protein kinase activities of extracts from mature oocytes were compared with those from mitotic cells (fig.1b). Prominent activity was again seen in the B region, although mature oocyte extracts produced two bands (B1 and B2), compared to only one in mitotic extracts (B1). Further, fractionation of extracts by ammonium sulfate precipitation enriched for protein kinase activity in the A and B regions (fig.1b,3b), as was seen with mitotic extracts. (Auto)phosphorylation in the A and B regions in the absence of exogenous substrate was also greatly enriched by this fractionation procedure (fig.1c,3c).

Extracts from immature oocytes were also examined. The pattern of Coomassie-stained protein bands produced by these extracts after precipitation by ammonium sulfate was similar to comparable samples from mature oocytes (fig.3a). However, in contrast to mature oocytes, only a few bands of kinase activity were seen in immature extracts assayed in the presence of histone substrate (fig.3b). Bands in the A region and band B1, prominent in mature oocyte extracts and mitotic extracts, were not seen in immature oocyte extracts, although both mature and immature extracts demonstrated activity at band B2 (fig.3b). Only slight (auto)phosphorylation was seen when kinase activity was assayed in the absence of exogenous substrate (fig.3c), in marked contrast to extracts from mature oocytes and mitotic cells, which produced prominent (auto)phosphorylation at bands A1 and B1, and A2 in mature oocytes.

The protein kinase activity profiles of extracts were then compared with their biological activity, i.e., their MPA as judged by their ability to induce

germinal vesicle breakdown and chromosome condensation when injected into immature oocytes. These results are presented in fig.4, where protein kinase activities of each type of extract are compared side-by-side, assayed in the presence of histone substrate, and the biological activity of each extract is indicated at the bottom of the figure. This comparison reveals that active extracts (mitotic and mature oocyte) [1–4,8,18] exhibit certain similarities in their kinase profiles. In particular, active extracts share band B1, not found in any of the biologically inactive extracts (interphase cells and immature oocytes). Active extracts also share band A1 (again absent in biologically inactive extracts), obscured by other bands in maturing oocyte extracts except in the autophosphorylation panel of fig.3. It is pertinent that these shared bands are revealed even in the absence of exogenous substrate, in light of the demonstration that the biological activity of these extracts is autocatalytic.

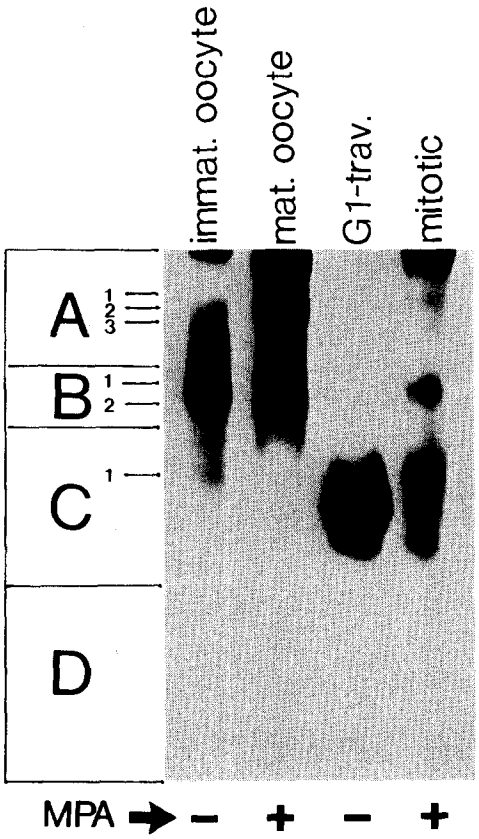

Fig.4. Comparison of protein kinase activities and biological activity of extracts. Autoradiogram of a gel containing immature and mature oocyte extracts, interphase and mitotic somatic cell extracts, phosphorylated in the presence of histone. The MPA of each extract, assayed by injection into immature oocytes, is indicated underneath each lane. Equivalent amounts of protein were loaded into each well.

In summary, these experiments demonstrate that the presence of particular protein kinase activities in cellular extracts appears to correlate with the biological activity of the extracts. In addition, enrichment of biological activity by ammonium sulfate precipitation [2,4,8] also enriches for these protein kinases. It will be interesting to determine whether the protein kinase activities identified here continue to copurify with biological activity upon further fractionation. Considering the possible involvement of phosphorylation/dephosphorylation in nuclear membrane breakdown and chromosome condensation, these protein kinase species are possible candidates for mediators of these events.

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