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Related Proteins Are Phosphorylated at Tyrosine in Response to Mitogenic Stimuli and at Meiosis

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Forty-two-kilodalton proteins that contain phosphotyrosine in metaphase-arrested *Xenopus laevis* eggs are closely related to p42, a protein that is phosphorylated at tyrosine when somatic cells are exposed to mitogenic stimuli.

Progesterone or other stimuli cause *Xenopus* oocytes, blocked naturally in first meiotic prophase, to complete the first (reduction) division of meiosis and to arrest as eggs in the second meiotic metaphase (33, 35, 36, 46). After fertilization, meiosis is completed, and rounds of synchronous mitotic divisions ensue (25). Nuclear events in meiosis and mitosis are triggered by activation of maturation promoting factor (MPF), a multiprotein complex with protein-serine kinase activity (2, 15, 17, 18, 28, 29). The block to metaphase II in unfertilized eggs appears to be due to stabilization of MPF in its active form by a Ca^{2+}-sensitive factor known as cytostatic factor (31, 38, 40).

Analysis of oocytes labeled by microinjection of $^{32}P_i$ or [γ-^{32}P]ATP has revealed a three- to fivefold increase in total protein phosphorylation during maturation, starting when MPF is activated about 60 min before germinal vesicle breakdown (32). Several new phosphoproteins are labeled during this period (34). Some of the predominant phosphoproteins disappear following fertilization and reappear at subsequent mitoses (23, 30). These phosphoproteins are candidate components of the mitotic machinery and may be substrates for MPF. Another set of egg phosphoproteins also disappears after fertilization but does not reappear subsequently. These phosphoproteins could be involved with cytostatic factor action (14).

Among the proteins phosphorylated in mature eggs is a group of two or three proteins of about 42 kilodaltons (kDa) and neutral pI. These proteins can be labeled with [γ-^{32}P]ATP in preparations of crushed eggs (30). Their phosphorylation in vitro is abolished by Ca^{2+} but can be rescued by exogenous MPF (30). These 42-kDa phosphoproteins differ from other phosphoproteins examined in that tyrosine residues, as well the usual serine and threonine residues, are phosphorylated in vitro and in vivo (30).

Phosphotyrosine constitutes only 1% of the total protein phosphate in oocytes (47). In somatic cells, tyrosine phosphorylation is even more uncommon; phosphotyrosine is only 0.02% of the protein phosphate in fibroblasts (45). This is temporarily increased severalfold when resting (G_0 phase) cells are treated with growth factors (8, 39). Two-dimensional gel electrophoresis of stimulated fibroblasts or epithelial cells shows a pair of 42-kDa, neutral-pI, phosphotyrosine-containing phosphoproteins (pp42A and pp42B) that lack phosphate in unstimulated cells (7, 8, 26). pp42A and pp42B are structurally similar to each other and to two other growth factor-induced phosphotyrosine-containing proteins of about 45 kDa (13, 26).

A nonphosphorylated precursor of pp42A and pp42B, p42, has been identified on two-dimensional gels of proteins from unstimulated cells (11). p42 is present at about 10^5 molecules per fibroblast, and growth factors cause about one-half of the p42 molecules to be transiently phosphorylated at tyrosine, serine, and threonine residues (11). During the normal cell cycle, p42 phosphorylation is uniformly low, but it can be increased by adding growth factors, suggesting that the substrate protein and the requisite phosphorylation machinery are present continuously (J. A. Cooper and T. Hunter, unpublished results). Indeed, p42 is constitutively phosphorylated in certain growing fibroblasts transformed by retroviruses (9, 10, 13, 19). About 25% of total protein phosphotyrosine in mitogen-stimulated cells is contained in 42-kDa proteins (39). Since p42 tyrosine phosphorylation can be stimulated by factors whose receptors lack intrinsic protein-tyrosine kinase activity, tyrosine phosphorylation must be activated indirectly (3, 7, 13, 20, 27). Indeed, it appears that tyrosine phosphorylation of p42 requires activation of protein kinase C (PKC), a protein-serine/threonine kinase that is stimulated indirectly by many mitogens (24, 49). How PKC stimulates p42 tyrosine phosphorylation is not clear. The full range of cell types that expresses p42 is also unknown, but the fact that p42 phosphorylation increases with many different stimuli suggests that it plays a fundamental role in cell proliferation.

In view of the similar gel mobilities of the egg phosphotyrosine-containing protein and p42, I set out to test whether they were related. *Xenopus* eggs were crushed in the presence of a calcium chelator, and a supernatant fraction was prepared as described elsewhere (30). Extracts were incubated with [γ-^{32}P]ATP, and labeled proteins were resolved by two-dimensional gel electrophoresis (Fig. 1A). Alkali treatment of gels removes phosphate from many but not all proteins phosphorylated at serine and threonine (9); two 42-kDa spots labeled in crushed eggs were alkali stable (Fig. 1C, Xa and Xb). Partial acid hydrolysis of Xa and Xb released phosphotyrosine and lesser amounts of phosphoserine (Fig. 2).

Three arguments indicate that Xa and Xb are the same as the 42-kDa phosphoproteins detected by Lohka et al. in egg extracts (30). (i) The two-dimensional-gel mobilities are similar, referring to a major 20-kDa phosphoprotein (Fig. 1C) and nucleoplasmin as internal markers for pI and molecular mass. (ii) Xa and Xb are phosphorylated in vitro at tyrosine, as are the proteins of Lohka et al. (30). (iii) ^{32}P labeling of living eggs showed that the 42-kDa phosphotyrosine-containing proteins were phosphorylated at tyrosine in vivo (30), which is consistent with the detection of Xa and Xb with

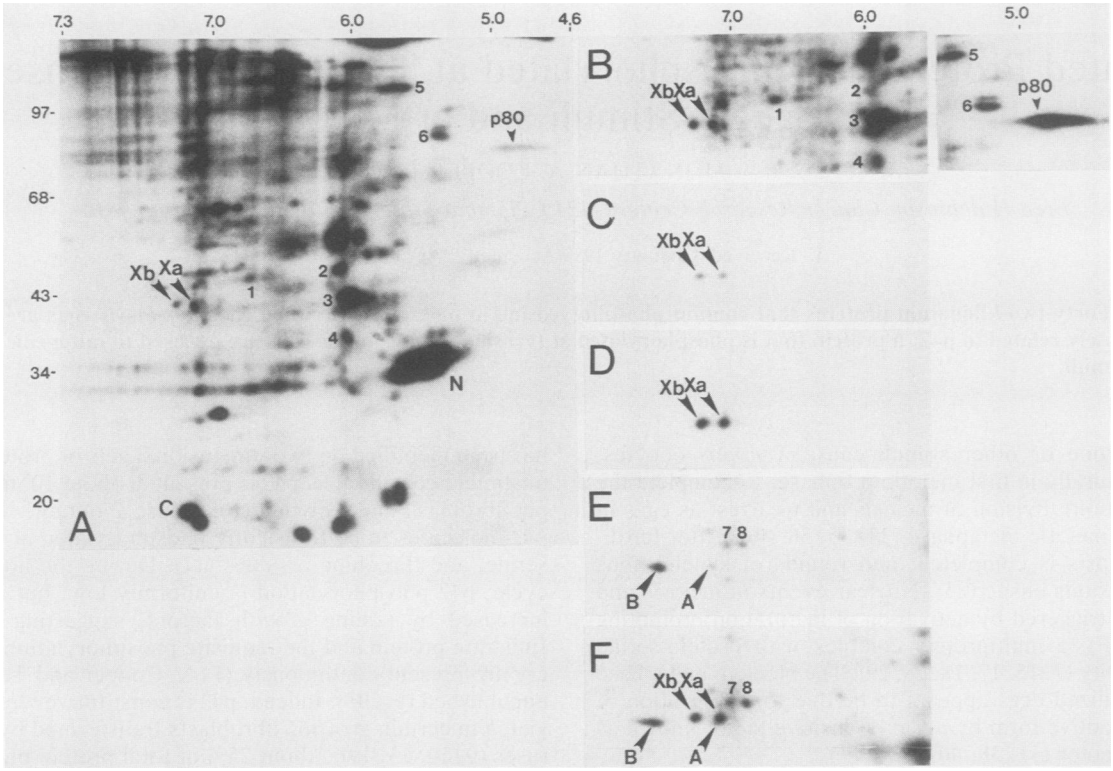

FIG. 1. Two-dimensional-gel analysis of *Xenopus* egg phosphoproteins. *X. laevis* eggs were crushed by centrifugation, and a low-speed supernatant fraction was obtained as described previously (30). Twenty microliters of crushed eggs was incubated with 100 μCi of [γ-^{32}P]ATP for 10 min at 20°C in the absence (A and C) or presence (B and D) of a mixture containing 2 U of PKC (purified from rat brain [1]), 2 mM $CaCl_2$, 10 μg of TPA per ml, 100 μg of phosphatidyl serine per ml, and 10 μg of dioctanoyl-sn-glycerol per ml. One-fifth of each reaction was analyzed by two-dimensional gel electrophoresis with ampholytes at pH 3.5 to 10 (24). Dried gels were exposed to film with a fluorescent screen at −70°C for 18 h (A and B). The gels were then incubated in alkali (9) and reexposed for 44 h (C and D). As markers for pp42A and pp42B, Swiss 3T3 cells were labeled for 4 h with $^{32}P_i$, exposed to platelet-derived growth factor for 10 min, and prepared for two-dimensional gel electrophoresis. The 3T3 cell sample was analyzed alone (E) and after being mixed with the crushed-egg reaction products (F), and the gels were incubated in alkali prior to autoradiography. Approximate molecular masses (in kilodaltons) are given on the left, and isoelectric points are shown across the top. Xa and Xb, Major alkali-stable phosphoproteins labeled in crushed egg; A and B, 3T3 cell phosphoproteins pp42A and pp42B; p80, protein whose phosphorylation is increased by PKC activators (44); C, 20-kDa phosphoprotein; N, nucleoplasmin; numbers, other phosphoproteins indicated to allow comparison of different gels.

antiphosphotyrosine antibody on immunoblots of two-dimensional gels of egg proteins (data not shown).

To compare Xa and Xb with fibroblast phosphoproteins, pp42A and pp42B were identified on a two-dimensional gel of platelet-derived growth factor-stimulated, $^{32}P_i$-labeled Swiss 3T3 cells (Fig. 1E) (8). Mixing of the Swiss 3T3 and *Xenopus* egg samples showed that the Xa was very similar in molecular size and pI to pp42A (Fig. 1F). Xb was intermediate in pI between pp42A and pp42B. Portions of two-dimensional gels containing Xa, Xb, pp42A, and pp42B were cut out and subjected to partial proteolysis (6). When digested with either high (Fig. 3A) or low (Fig. 3C) doses of *Staphylococcus aureus* V8 protease, with chymotrypsin (Fig. 3B) or with papain (Fig. 3D), Xa and Xb gave very similar patterns. The fragments of Xa and Xb also closely resembled those from pp42A and pp42B, except for low-molecular-mass fragments of pp42A that were produced by high doses of V8 protease or chymotrypsin. The sizes of small fragments could differ if there were distinct phosphorylation sites within an identical p42 sequence, if there were sequence or modification differences between species, or if p42 sequences were polymorphic. The strong similarities between Xa, Xb, and pp42B suggest that Xa and Xb are the phosphorylated forms of a *Xenopus* protein equivalent to mouse fibroblast p42. To distinguish between mouse and *Xenopus* p42, I will call them $p42^m$ and $p42^x$, respectively.

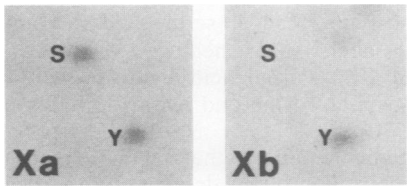

FIG. 2. Phosphoamino acid content of Xa and Xb. Crushed *Xenopus* egg was labeled with 1 mCi of $^{32}P_i$ in the presence of 0.5 mM sodium pyruvate, 0.5 mM NAD^+, and 2 mM fructose 1,6-bisphosphate (21). Xa and Xb were extracted from triplicate two-dimensional gels of the products and subjected to partial acid hydrolysis (12). Phosphoamino acids were separated by two dimensional thin-layer electrophoresis (12). Xa (70 cpm) and Xb (60 cpm) were analyzed, and the plate was exposed for 6 days. S, Phosphoserine; Y, phosphotyrosine.

In fibroblasts, $p42^m$ phosphorylation is invariably associated with PKC activation (24, 49), evident from increased phosphorylation of an acidic 80-kDa protein (p80) (44, 51). Is PKC involved in $p42^x$ phosphorylation? Little p80 phosphorylation was detected in crushed eggs under conditions in

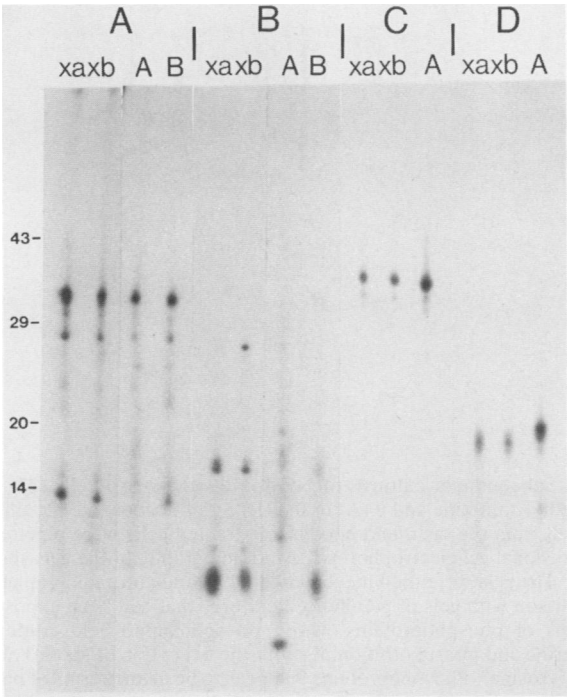

FIG. 3. Partial proteolytic digestion of Xa, Xb, pp42A, and pp42B. Portions of non-alkali-treated, two-dimensional gels containing Xa, Xb, pp42A, and pp42B were excised and subjected to partial proteolysis during gel electrophoresis (6) using 50 (A) or 5 (C) ng of *S. aureus* V8 protease, 1 μg of chymotrypsin (B), or 1 ng of papain (D). The figure is a composite of different-length autoradiographic exposures of two gels (parts A and B from one gel and parts C and D from the other). Approximate molecular masses (in kilodaltons) are shown. The undigested phosphoproteins migrated slightly ahead of the 43-kDa mobility marker.

which $p42^x$ phosphorylation was readily detectable (Fig. 1A). Adding PKC activators to the reaction increased phosphorylation of p80 10- to 20-fold and of Xa and Xb 2- to 4-fold (Fig. 1A through D; exogenous PKC was not necessary; data not shown). Stimulation of phosphorylation of $p42^x$ by PKC activators may be restricted by Ca^{2+} stimulation of $pp42^x$ dephosphorylation (30). These experiments suggest that PKC is present in the egg extracts, although it is relatively inactive under conditions in which $p42^x$ phosphorylation can be detected.

It is not clear whether PKC is active during egg maturation. Tetradecanoyl phorbol 13 acetate (TPA) is able to trigger oocyte maturation, but PKC is not rate limiting for normal maturation initiated by progesterone (48). My data show that eggs contain the components required for the PKC-regulated $p42^x$ phosphorylation that occurs in mitogen-stimulated fibroblasts (24, 49) but suggest that tyrosine phosphorylation of $p42^x$ in eggs may normally occur in a PKC-independent fashion.

The properties of $pp42^x$ suggest that it is the U46 phosphoprotein of Karsenti and colleagues (14, 23). U46 is phosphorylated in intact eggs and has two-dimensional-gel mobilities similar to those of $pp42^x$, referring to phosphoprotein C and nucleoplasmin as internal markers. Lohka et al. showed that phosphate was lost from $pp42^x$ upon parthenogenetic activation of intact eggs (30). Similarly, U46 is dephosphorylated upon fertilization or parthenogenetic activation (23). U46 is not phosphorylated in stage VI oocytes (14). U46 is phosphorylated in eggs derived from oocytes that were enucleated prior to maturation, suggesting that U46 and its kinase are not confined to the oocyte nucleus (14); $p42^m$ is known to be cytoplasmic in fibroblasts (11).

U46 (presumptive $pp42^x$) is not detected when fertilized eggs go through zygotic mitosis (23). To test directly whether $p42^m$ phosphorylation occurs in mitosis, I analyzed the phosphoproteins of fibroblasts arrested in mitotic prophase with nocodazole (52). Subconfluent cultures of NIH[pMc-*src*/focus]B cells (a NIH 3T3 derivative expressing high levels of exogenous $p60^{c\text{-}src}$; 22) were incubated with 0 or 0.4 μg of nocodazole per ml for 20 h, with $^{32}P_i$ added for the final 3 h. Two-dimensional-gel analysis showed that $p42^m$ was not significantly phosphorylated in nocodazole-arrested cells relative to growing sister cells (Fig. 4) and that much greater phosphorylation could be stimulated by platelet-derived growth factor treatment of a control culture (data not shown). The lack of $p42^m$ phosphorylation in nocodazole-arrested fibroblasts is consistent with the absence of $pp42^x$ (U46) from mitotic fertilized eggs (23) but rather surprising in view of the enzymatic activation of the protein-tyrosine kinase $p60^{c\text{-}src}$ in mitotic cells (5). Since nocodazole induced the phosphorylation of a number of proteins that were not phosphorylated in control cultures (Fig. 4), several phosphoproteins were examined for the presence of phosphotyrosine to see if they might have been phosphorylated by $p60^{c\text{-}src}$. The phosphoamino acid contents of the 12 alkali-stable phosphoproteins or clusters thereof (numbered on Fig. 4B) were determined. None contained phosphotyrosine except spots 9. Immunoprecipitation and peptide mapping suggested that spots 9 are acidic isoforms of $p60^{c\text{-}src}$ that accumulate with mitotic arrest (data not shown) (5). Nocodazole arrest of Swiss 3T3 cells caused changes in the pattern of phosphoproteins very similar to those seen in NIH[pMc-*src*/focus]B cells, except that these acidic forms of $p60^{c\text{-}src}$ were not detected. Phosphotyrosine-containing proteins may be present in nocodazole-arrested cells but may have escaped detection by this approach if they did not focus in the pH range studied or were of low abundance.

It may seem odd that p42 is phosphorylated at tyrosine in both mature, meiotic eggs and in mitogen-stimulated G_0 somatic cells. Phosphorylation of p42 could direct metabolic events common to oocyte maturation and to progression through G_0, or it could act via different pathways, depending on the stage of the cell cycle. The two systems are alike in that there is an acute change from a quiescent to a metabolically active state but are different in the duration of p42 phosphorylation, which is sustained during metaphase arrest of eggs but transient in stimulated somatic cells. It has been suggested that proteins such as p42 that are phosphorylated at meiosis but not at mitosis could be involved with MPF stabilization by cytostatic factor (14). Cytostatic factor may be very short-lived in the normal somatic cell cycle and difficult to detect by the labeling techniques used. It is interesting that some purified preparations of MPF contain a 42-kDa protein (17) and that another component of MPF (a 34-kDa protein encoded by *CDC28* of *Saccharomyces cerevisiae* and by *cdc2* of *Schizosaccharomyces pombe*; 2, 16–18, 37, 50) is involved in G_1, meiosis, and mitosis (4, 41–43). Purification of $p42^x$ is under way, and the properties of isolated $p42^x$ may shed light on its functions.

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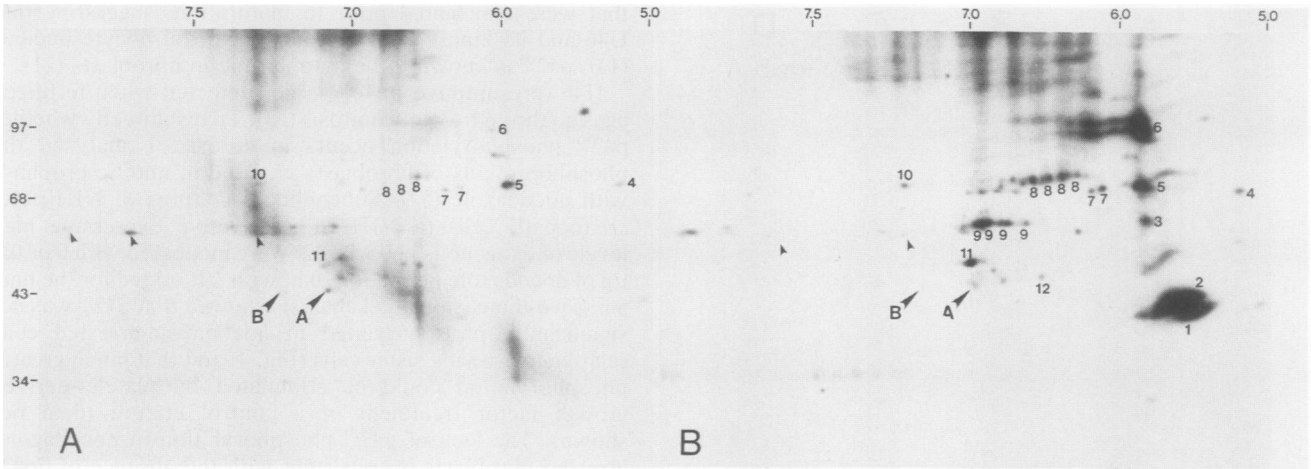

FIG. 4. Alkali-stable phosphoproteins of fibroblasts arrested in mitosis. Subconfluent cultures of NIH[pMc-*src*/focus]B cells (22) were incubated for a total of 20 h in low-phosphate medium containing 0.1% dimethyl sulfoxide and 0 (A) or 0.4 (B) μg of nocodazole per ml. For the final 3 h, 1 mCi of $^{32}P_i$ per ml was also added. Control cells were scraped from the dish, and nocodazole-treated cells were pipetted off the dish. Samples corresponding to 2×10^4 cells were analyzed by two-dimensional gel electrophoresis. Autoradiography of the gels showed that approximately equal amounts of radioactivity were present (not shown). The gels were then incubated in alkali and autoradiographed for 20 h. Large arrowheads, Positions of pp42A and pp42B, identified by comparison with gels of parallel cell cultures that had been exposed to platelet-derived growth factor for 10 min; numbers, individual or groups of phosphoproteins whose phosphoamino acid content in nocodazole-treated cells was analyzed. Each spot contained both phosphoserine and phosphothreonine, with the exception of spots 3 and 12 (phosphoserine only) and 9 (phosphoserine, phosphothreonine, and phosphotyrosine). Phosphoproteins 9 appear to be acidic forms of $p60^{c\text{-}src}$ (data not shown). Small arrowheads, Additional forms of $p60^{c\text{-}src}$ (data not shown). Apparent molecular masses (in kilodaltons) are on the left, and isoelectric points are given across the top.

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