Smooth-muscle mitogen-activated protein (MAP) kinase: purification and characterization, and the phosphorylation of caldesmon

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A single 42 kDa isoform of mitogen-activated protein (MAP) kinase is expressed in both embryonic and adult chicken gizzard. The gizzard MAP kinase, which cross-reacts with anti-p44^{mpk} antibody, has been purified from adult chicken gizzard and partially characterized. The purification protocol employs phenyl-Sepharose, polylysine-agarose, hydroxyapatite, Mono-Q and phenyl-Superose column chromatography. The purified enzyme phosphorylates myelin basic protein and gizzard high-molecular-mass (*h*-)caldesmon. Sea-star p44^{mpk} and gizzard MAP kinase phosphorylate *h*-caldesmon at identical sites at the C-

terminal domain, as revealed by tryptic-peptide mapping of the phosphorylated protein. Phosphorylation of *h*-caldesmon by gizzard MAP kinase abolishes its interaction with polymerized tubulin. The specific activity of the purified gizzard kinase toward myelin basic protein is similar to that of brain tau kinase, but is only a fraction of that of activated sea-star p44^{mpk}. This suggests that, although a large amount of MAP kinase is present in the gizzard, only a small percentage of the enzyme is activated normally. Autophosphorylation of the gizzard kinase, at least in part on tyrosine residues, activates its kinase activity.

INTRODUCTION

Smooth-muscle high-molecular-mass (*h*-)caldesmon can be phosphorylated *in vitro* by a number of protein kinases, including protein kinase A and C, Ca²⁺-calmodulin-dependent protein kinase II, casein kinase II (Sobue and Sellers, 1991; Marston and Redwood, 1991), p34^{cdc2} (Mak et al., 1991) and p44^{mpk} (Childs et al., 1992). However, the physiological significance of phosphorylation of *h*-caldesmon by any of these kinases remains unclear. Adam et al. (1992) showed that phorbol ester stimulated the phosphorylation of *h*-caldesmon in dog aorta at two Ser-Pro sites, SPAPK and VTSPTKV, suggesting that phosphorylation of *h*-caldesmon *in vivo* may involve a Pro-directed kinase. Consistent with this hypothesis is our finding, *in vitro*, that p34^{cdc2} and sea-star p44^{mpk} phosphorylate chicken gizzard *h*-caldesmon (Childs et al., 1992). Although both of these kinases phosphorylate Ser/Thr-Pro sites in the C-terminal domain of gizzard *h*-caldesmon, the preferred phosphorylation sites are different, and one of the p44^{mpk} sites appears to correspond to one of the sites observed *in vivo* by Adam et al. (1992). Phosphorylation of *h*-caldesmon by p34^{cdc2} inhibits the binding of caldesmon to actin (Mak et al., 1991). In contrast, phosphorylation of caldesmon by p44^{mpk} has only a slight effect on the binding of caldesmon to actin and does not affect its binding to tropomyosin and calmodulin (Childs et al., 1992). Recent reports suggest that caldesmon may also be involved in cross-linking microtubules and microfilaments and that p34^{cdc2} may play a regulatory role in this interaction (Ishikawa et al., 1992a,b). Taken together, these findings indicate that different members of the Pro-directed kinase family phosphorylate caldesmon, but prefer different subsets of the six potential sites. Such a differential phosphorylation process may induce subtle changes in the structure and function of caldesmon in differentiated as well as in dividing smooth-muscle cells.

The main objective of the present study is to characterize further the role of mitogen-activated protein (MAP) kinase in differentiated smooth-muscle cells, using chicken gizzard as a study model. We have previously demonstrated that a 42 kDa MAP kinase isoform is present in chicken gizzard, but it was not clear whether the smooth-muscle kinase phosphorylated *h*-caldesmon at the same sites as did sea-star p44^{mpk}. In the present study, we have: (1) purified the 42 kDa MAP kinase from chicken gizzard to homogeneity and determined substrate specificity of the purified enzyme; (2) compared the sea-star p44^{mpk} and smooth-muscle MAP kinase phosphorylation sites on caldesmon; (3) investigated the effect of phosphorylation of *h*-caldesmon by the gizzard MAP kinase on its binding to tubulin; and (4) studied activation by autophosphorylation and probed for a MAP kinase activator with antibodies raised against *Saccharomyces cerevisiae* STE7.

MATERIALS AND METHODS

Materials

Mono Q, phenyl-Superose and phenyl-Sepharose were obtained from Pharmacia LKB Biotechnology Inc. Hydroxyapatite, molecular-mass standards and Bradford reagent came from Bio-Rad. Immobilon was purchased from Millipore. The ECL (enhanced chemiluminescence) Western blotting kit was purchased from Amersham. [γ -³²P]ATP (4500 Ci/mmol), acrylamide and Tris/HCl were obtained from ICN. P81 phosphocellulose and 3 MM paper were purchased from Whatman. EGTA came from Fisher. SDS, *NN'*-methylenebisacrylamide, ethylene glycol and dithiothreitol (DTT) were obtained from BDH. Histone H1 was from Boehringer. Anti-p44^{mpk} is an affinity-purified rabbit polyclonal antibody against purified sea-star p44^{mpk}. Anti-STE7-VIII is an affinity-purified rabbit polyclonal antibody against a synthetic peptide (FVGTSTYMSPERIC) based on residues

Abbreviations used: p44^{mpk}, meiosis-activated protein kinase purified from sea-star oocytes; STE7, kinase encoded by *Saccharomyces cerevisiae* STE7 gene with homology to eukaryotic MAP kinase kinase; *h*-caldesmon, high-molecular-mass isoform of caldesmon; *l*-caldesmon, low-molecular-mass isoform of caldesmon; MAP, mitogen-activated protein; MBP, myelin basic protein; ECL, enhanced chemiluminescence; DTT, dithiothreitol; TBS, Tris-buffered saline; MEK, MAP kinase (or ERK) kinase.

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434-446 of *S. cerevisiae* STE7 (Teague et al., 1986) in the kinase subdomain VIII region. Caldesmon was prepared from fresh chicken gizzard as described by Bretscher (1984). Tubulin was prepared from rat brain as described by Vallee (1986). All other chemicals and reagents were purchased from Sigma.

Assay for MAP kinase activity

MAP kinase activity was determined by measuring the incorporation of PO_4^{2-} into myelin basic protein (MBP). Unless otherwise stated, assays contained, in a final volume of 25 μl : 1 mg/ml MBP, 1 mM [γ - ^{32}P]ATP (500 c.p.m./pmol), a portion of enzyme sample, and kinase assay buffer (20 mM Mops, pH 7.2, 30 mM β -glycerophosphate, 20 mM MgCl_2 , 5 mM EGTA, 1 mM DTT). Other substrates, substituted in place of MBP, included MBP peptide (SSAPRTPGGRR), histone H1, H1 peptide (KTPKKAKKPKTPKKAKKL) and caldesmon, all at a concentration of 100 μM . After incubation at 30 °C, reactions were terminated by spotting 5 μl portions on a 1.5 cm² piece of Whatman P81 phosphocellulose paper or by addition of SDS/PAGE sample buffer. The P81 paper squares were washed for 4 $\times$ 5 min in 75 mM H_3PO_4 , then rinsed in 95 % ethanol, and finally in acetone. They were then transferred to scintillation vials, and 3 ml of scintillation fluid was added, followed by radioactivity counting.

Gel electrophoresis and autoradiography

SDS/PAGE was performed on 0.75 mm-thick gels by the method of Laemmli (1970). Gels were stained with Coomassie Blue or silver. Autoradiography was performed at -70 °C by using Kodak AR-5 film in a cassette with intensifying screens.

Western blotting

Samples were subjected to SDS/PAGE (12 % gels) and subsequently equilibrated for 15 min in transfer buffer, containing 25 mM Tris, 192 mM glycine and 0.02 % SDS. Proteins were transferred to Immobilon (Millipore) for 1 h at 100 V. The membrane was then blocked with TBS (Tris-buffered saline: 20 mM Tris, pH 7.6, 137 mM NaCl) containing 5 % skim milk for 2 h at room temperature. The membrane was then incubated in TBS containing 0.05 % Tween 20 (TTBS) and 1 % skim milk, and the relevant primary antibody (1:1000 dilution) overnight at room temperature. The next day the membrane was washed three times (10 min each time) in TTBS before incubating the blot in 1 % skim milk/TTBS containing either a goat anti-rabbit IgG conjugated to horseradish peroxidase for anti-p44^{mapk} and anti-STE7-VIII, or goat anti-mouse IgG conjugated to alkaline phosphatase for PY20, both at 1:1000 dilution for 2 h. After this incubation, the membrane was subjected to three 10 min washes in TTBS. Blots using the anti-p44^{mapk} and anti-STE7-VIII antibodies were developed by the ECL method (Amersham). Blots which used the PY20 antibody were subjected to a further wash in 100 mM Tris (pH 9.5)/100 mM NaCl/5 mM MgCl_2 with final colour development in 20 ml of the same buffer containing 0.33 mg/ml Nitro Blue Tetrazolium and 0.16 mg/ml 5-bromo-4-chloro-3-indolyl phosphate.

Purification of MAP kinase

Approx. 100 g of fresh chicken gizzard was homogenized in 200 ml of buffer A (25 mM β -glycerophosphate, 10 mM Mops, pH 7.2, 2 mM EDTA, 5 mM EGTA, 2 mM sodium vanadate, 1 mM DTT) containing 0.5 mM phenylmethanesulphonyl

fluoride. The homogenate was clarified first by centrifugation at 12000 g for 10 min and then by centrifugation at 100000 g for 30 min. The supernatant (125 ml) was diluted 1:1 in buffer B (12.5 mM Mops, pH 7.2, 12.5 mM β -glycerophosphate, 7.5 mM MgCl_2 , 0.5 mM EGTA, 0.05 mM NaF, 1 mM DTT) and applied to a phenyl-Sepharose column (2.5 cm $\times$ 5.0 cm) equilibrated in buffer B at a flow rate of 1 ml/min. After sample application, the column was washed with 1 bed vol. of buffer B and then developed with a 300 ml linear gradient of 0-6 % Brij 35 in buffer B. The column was then washed with 70 % (v/v) ethylene glycol in buffer B. The 42 kDa isoform was eluted in the ethylene glycol wash. Fractions were pooled on the basis of antibody reactivity, by using polyclonal antibodies raised against sea-star p44^{mapk}, and kinase activity towards MBP.

The sample of 42 kDa isoform was dialysed against 1 litre of buffer B for 4 h and loaded at 1 ml/min on a polylysine-agarose column (2.5 cm $\times$ 5.0 cm) pre-equilibrated in buffer B. The column was washed with 1 column vol. of buffer B, and the MAP kinase was eluted with a 300 ml linear gradient of 0-0.8 M NaCl in buffer B. Most of the antibody reactivity and MBP activity were eluted at approx. 400 mM NaCl. The peaks were pooled, dialysed against 1 litre of buffer B for 4 h, and loaded on an hydroxyapatite column (2.5 cm $\times$ 5.0 cm) pre-equilibrated in buffer B at 1 ml/min. The column was washed with the starting buffer, followed by a 300 ml linear gradient of 0-0.35 M K_2HPO_4 in buffer B. The fractions containing MBP kinase activity and proteins which were cross-reactive with anti-p44^{mapk} were eluted at approx. 0.17 M K_2HPO_4 . These fractions were pooled and dialysed in 1 litre of buffer B for 4 h, and then loaded on a Mono Q HR5/5 column pre-equilibrated in buffer B at 1 ml/min. The column was washed and then eluted with a 20 ml linear gradient of 0-0.8 M NaCl in buffer B. The fractions which still contained antibody cross-reactivity and MBP kinase activity were eluted at about 180 mM NaCl. These fractions were pooled and dialysed in 1 litre of buffer B containing 250 mM NaCl for 4 h, and then loaded on a phenyl-Superose column pre-equilibrated in buffer B containing 250 mM NaCl. The column was washed and then eluted with a decreasing NaCl gradient from 250 to 10 mM and a simultaneously increasing Brij 35 gradient from 0 to 70 %. The pure kinase was eluted as a single peak at approx. 90 mM NaCl and 46 % Brij 35. Protein was estimated by the Bio-Rad protein assay, based on the method of Bradford. BSA was used as the standard.

Identification of MAP kinase in a polyacrylamide gel after electrophoretic isolation

To identify the presence of the MAP kinase in a polyacrylamide gel after electrophoresis (in-the-gel assay), the method of Kame-shita and Fujisawa (1989) was used. Briefly, MBP was added to an SDS/PAGE (12 %-acrylamide) separating gel, to a final concentration of 0.5 mg/ml, just before polymerization. Samples from phenyl-Superose chromatography were then electrophoresed on this gel at 150 V for 1 h. SDS was removed by washing the gel in two changes (1 h and 100 ml each time) of 50 mM Tris (pH 8.0)/20 % (v/v) propan-2-ol at 22 °C. This was followed by a 1 h wash in 100 ml of 50 mM Tris (pH 8.0)/5 mM β -mercaptoethanol (buffer A) at 22 °C. The protein was denatured in two 50 ml washes (each 1 h) with 6 M guanidine/HCl in buffer A at 22 °C. Renaturation was achieved with five 250 ml washes of buffer A + 0.04 % Tween 20 over 16 h at 4 °C. The gel was then equilibrated in 5 ml of kinase assay buffer in a plastic bag for 30 min at 22 °C. Then the buffer was replaced, and approx. 25 μCi of [γ - ^{32}P]ATP (50 μM ATP) was added and incubation was carried out at the same temperature for an

additional 1 h. The gel was then treated with five 500 ml washes (each 1 h) in 5% trichloroacetic acid/1% pyrophosphate, dried and autoradiographed.

Phosphorylation of caldesmon

Caldesmon (1.5 mg/ml) in 20 mM Mops (pH 7.2) / 1 mM EGTA / 30 mM β -glycerophosphate / 20 mM MgCl_2 / 1 mM [γ - ^{32}P]ATP (5×10^6 c.p.m./nmol) was incubated at 30 °C with p44^{mapk} for 2 h or with gizzard p42^{mapk} for 24 h. Samples (10 μ l) were counted for radioactivity by the Whatman P81 filter-paper method described above, and 5 μ l samples were analysed by SDS/PAGE followed by autoradiography.

Tryptic-peptide mapping

^{32}P -labelled caldesmon (0.25 mg) was digested with trypsin (treated with tosylphenylalanylchloromethane; from Sigma) at an enzyme/substrate weight ratio of 1:50 for 20 h at 37 °C in 50 mM NH_4HCO_3 , pH 8.0. The digested sample was freeze-dried and suspended in 20 μ l of pH 3.5 thin-layer electrophoresis buffer (water/acetic acid/pyridine, 89:10:1, by vol). The tryptic digest (5 μ l) was subjected to two-dimensional mapping on Kodak thin-layer cellulose plates (20 cm $\times$ 20 cm). The first dimension involved electrophoresis at 400 V for 2 h in the pH 3.5 buffer, and the second dimension involved ascending chromatography in butan-1-ol/pyridine/acetic acid/water (4:5:1:4, by vol.).

Assay for caldesmon binding to microtubules

Tubulin was polymerized in a solution containing 100 mM Mops (pH 6.8), 1 mM GTP, 1 mM DTT, 1 mM MgCl_2 , 15 mM KCl and 20 $\mu\text{g}/\text{ml}$ taxol for 30 min at 37 °C. The final concentration of microtubules was altered to 10 μM . Various concentrations of caldesmon (1–10 μM), made up in the same buffer lacking taxol, were added to the microtubules and allowed to incubate for 30 min at room temperature. All solutions were then centrifuged at 140000 g for 20 min, and the supernatant and pellet were separated and resuspended in SDS/PAGE sample buffer to the same final volume. The amounts of each protein were then determined by laser densitometry of Coomassie-Blue stained SDS/12% polyacrylamide gels.

Autophosphorylation and autoactivation of MAP kinase

Kinase (0.5–1.0 μg) in a total volume of 22.2 μ l of assay buffer was incubated in the presence of 1 mM [γ - ^{32}P]ATP (500 c.p.m./pmol) for 30 min at 30 °C. To assay for autophosphorylation, 8 μ l of the reaction mixture was added to 2 μ l of SDS/PAGE sample buffer, electrophoresed on 12% gels, dried, and analysed by autoradiography. To assay for activation of the MAP kinase toward MBP by autophosphorylation, MBP was added to the ATP-treated kinase solution to a final concentration of 1 mg/ml and incubated at 30 °C for 30 min. The reaction was terminated by addition of SDS/PAGE sample buffer, and phosphorylation of MBP was analysed by SDS/PAGE followed by autoradiography. A control experiment was carried out simultaneously and under identical conditions as described above, except that the kinase was not preincubated with [γ - ^{32}P]ATP.

Detection of phosphotyrosine in autophosphorylated MAP kinase

Approx. 0.5–1.0 μg of kinase was added to 20 μ l of assay buffer containing 1 mM ATP. The reaction mixture was incubated for

60 min at 30 °C, and reaction was terminated by addition of SDS/PAGE sample buffer. Western blotting with PY20 antibody was then employed to detect phosphotyrosine.

RESULTS

Purification of chicken gizzard MAP kinase

We previously detected only a single 42 kDa MAP kinase (gizzard p42^{mapk}) in adult chicken gizzard, using anti-p44^{mapk} antibodies (Childs et al., 1992), although both 42 and 44 kDa isoforms have been identified in bovine aortic smooth-muscle cells (Adam and Hathaway, 1993). To determine whether other isoforms of gizzard MAP kinase may be under developmental control, soluble extracts of embryonic-chicken gizzard were assayed for the presence of MAP kinase with anti-p44^{mapk}. As shown in Figure 1, only the 42 kDa MAP kinase was detected in all the embryonic and adult gizzard samples.

Figure 1 Developmental expression of MAP kinase in chicken gizzard smooth muscle

Samples of chicken gizzard from 11, 13, 14, 15, 17, 19, and 21 days of development within the egg, as well as a sample of adult gizzard (A), were homogenized in buffer A (see the Materials and methods section). These samples were run on SDS/PAGE (12% gels), transferred to Immobilon, and the resulting Western blot, using anti-p44^{mapk} antibody, is shown. 'Days' refers to days of development after fertilization. Hatching occurred at roughly 21 days. The number to the right indicates the position of the 42 kDa kinase.

Figure 2 Purification of gizzard MAP kinase at different chromatographic steps

(a) shows a silver-stained SDS/12% polyacrylamide gel of samples from each stage of the purification. Lanes: 1, crude homogenate; 2, pooled fractions from phenyl-Sepharose; 3, pooled fractions from polylysine-agarose; 4, pooled fractions from hydroxyapatite; 5, pooled fractions from Mono Q; 6, pooled fractions from phenyl-Superose; 7, molecular-mass standards (kDa). (b) Western blot of the final purified kinase probed with anti-p44^{mapk}.

Figure 3 Phenyl-Superose chromatography

The pooled fractions from the Mono Q chromatography were loaded on a phenyl-Superose column equilibrated in buffer B. The bound proteins were then eluted with a decreasing gradient of 250–10 mM NaCl and a simultaneously increasing gradient of 0–70% ethylene glycol, all in buffer B; 0.5 ml fractions were collected. (a) Elution profile; (b) Coomassie-Blue-stained proteins in elution fractions after separation by SDS/PAGE; (c) Western blot using anti-p44^{mapk} antibody; (d) is the MBP kinase assay (for conditions see the Materials and methods section); (e) an in-the-gel assay using MBP as the substrate.

Table 1 Relative phosphorylation of *in vitro* substrates by MAP kinase

For gizzard p42^{mapk}, 100% equals 0.5 nmol/min per mg, and for sea-star p44^{mapk}, 100% equals 0.49 μ mol/min per mg.

	Gizzard p42 ^{mapk}	Sea-star p44 ^{mapk}
MBP	100	100
MBP peptide	14	60
H1	13	16
H1 peptide	12	23
Caldesmon	14	82

The purification scheme for gizzard p42^{mapk} employed five column-chromatographic steps (see the Materials and methods section for details). The pooled fractions of the various chromatographic steps, containing both MBP kinase activities as well as the gizzard p42^{mapk} detected by anti-p44^{mapk}, were analysed on

SDS/PAGE and stained with Coomassie Blue (Figure 2a). As shown in lane 6, the purified gizzard p42^{mapk} obtained from the last column (phenyl-Superose) appeared as a single band with an apparent molecular mass of 42 kDa which cross-reacted with anti-p44^{mapk} (Figure 1b). Although MBP has been commonly used as a substrate *in vitro* for MAP kinase, many other kinases present in the gizzard extracts at the early stages of purification are capable of phosphorylating MBP; thus constructing a purification Table based on MBP activities would be misleading. However, from 100 g of chicken gizzard, a yield of about 320 μ g of pure gizzard p42^{mapk} was obtained with a specific activity of 570 pmol/min per mg towards MBP.

The profile of the gizzard p42^{mapk} eluted from the phenyl-Superose column is shown in Figure 3. The single A_{280} peak corresponding to fractions 28–31 coincides with the Coomassie-Blue-stained gizzard p42^{mapk} (Figure 3b), which was also cross-reactive with anti-p44^{mapk} antibody (Figure 3c). However, the peak of MBP kinase activity, fractions 27–30, was eluted on the leading edge of the protein peak (Figure 3d). This indicates that fractions 27–31 contain two populations of gizzard p42^{mapk}. The more active one is presumably phosphorylated at tyrosine and threonine residues, and would be eluted slightly before the inactive and dephosphorylated one. This is consistent with the assay for MBP kinase activity using gizzard p42^{mapk} immobilized in an SDS/polyacrylamide gel as described by Kameshita and Fujisawa (1989) (Figure 3e), which shows that an active 42 kDa kinase is present in fractions 28–29.

Substrate specificity of gizzard p42^{mapk} and phosphorylation of *h*-caldesmon

The minimal recognizable phosphorylation motifs for p34^{cdc2} and MAP kinase share the sequence Ser/Thr-Pro (Clark-Lewis et al., 1991; Maller, 1990). It is clear from several reports, using either protein or peptide substrates, that these Pro-directed kinases have subtle differences in their preferred phosphorylation sites, depending on the primary structure and the conformation of these sites. We have compared the kinase activities of both activated sea-star p44^{mapk} and gizzard p42^{mapk} toward MBP, histone H1 and *h*-caldesmon, all of which have been shown to be substrates *in vitro* for MAP kinase and p34^{cdc2}. Table 1 shows that MBP and *h*-caldesmon are excellent substrates for the activated sea-star p44^{mapk}, but histone H1, which is a good substrate for p34^{cdc2} (Félix et al., 1990), is a poor substrate for this enzyme. MBP is also a good substrate for gizzard p42^{mapk}, but *h*-caldesmon is phosphorylated to only 14% of the level achieved with MBP (Table 1). However, gizzard p42^{mapk} has a specific activity (about 0.5 nmol/min per mg towards MBP) which is approx. 0.1% of the activity of the activated sea-star p44^{mapk} (0.49 μ mol/min per mg towards MBP).

Gizzard p42^{mapk} phosphorylates a synthetic peptide (SSAPRTPGGRR), which contains the Thr-Pro phosphorylation site of MBP. Partial acid hydrolysis of the phosphorylated MBP peptide yielded phospho-Thr but not phospho-Ser (results not shown), confirming that gizzard p42^{mapk} is Pro-directed. Thus, in spite of the presence of a large amount of gizzard p42^{mapk} in the adult tissue, it appears that either only a small fraction of the kinase is activated normally or most of the kinase was deactivated via dephosphorylation during the early stages of purification, even though phosphatase inhibitors have been included in all the buffers.

Due to the relatively low activity of gizzard p42^{mapk} towards *h*-caldesmon, the phosphorylation reaction was allowed to proceed for 24 h to incorporate enough phosphate for subsequent analyses. Within 2 h, sea-star p44^{mapk} incorporates up to 2 mol of

Figure 4 Tryptic maps of caldesmon phosphorylated by p44^{mpk} and gizzard p42^{mapk}

Caldesmon phosphorylated by sea-star p44^{mpk} (a) or gizzard p42^{mapk} (b) was digested with trypsin (enzyme/substrate, 1:50, w:w) for 20 h at 37 °C in 50 mM NH₄HCO₃, freeze-dried, and then resuspended in pH 3.5 buffer. It was then subjected to electrophoresis on thin-layer cellulose (Kodak) at 400 V for 2 h and finally to ascending chromatography in butan-1-ol/pyridine/acetic acid/water (4:5:1:4, by vol.). Electrophoresis (E) is from left to right and chromatography (C) is vertical: O, origin.

Figure 5 Binding of smooth-muscle caldesmon to microtubules

Microtubules (10 μM) were mixed with phosphorylated or non-phosphorylated caldesmon (1–10 μM) in 100 mM Mops (pH 6.8)/1 mM GTP/1 mM DTT/1 mM MgCl₂/15 mM KCl for 30 min at room temperature, and then spun at 140 000 *g* for 20 min. Supernatants and pellets were subjected to SDS/PAGE (12% gels), and the amounts of caldesmon and tubulin were determined by laser densitometry.

phosphate per mol of *h*-caldesmon (87 kDa), with little effect on its binding to actin, tropomyosin or calmodulin (Childs et al., 1992). Gizzard p42^{mapk} incorporated 0.9 mol of phosphate after

Figure 6 Characterization of smooth-muscle MAP kinase

(a) A sample of the purified kinase was incubated in the presence of 1 mM ATP at 30 °C for 60 min. Subsequently, the reaction was terminated by addition of SDS/PAGE sample buffer and then Western-blotted as described in the Materials and methods sector using a PY20 anti-phosphotyrosine antibody. Lane 1, autophosphorylated p42; lane 2, p42 immediately after purification (control). (b) The kinase was preincubated in the absence (lane 1) or presence (lane 2) of 1 mM [γ -³²P]ATP (500 c.p.m./pmol) for 30 min at 30 °C. After this preincubation, 8 μl from each of the four reaction mixtures was removed and terminated by addition of SDS/PAGE sample buffer. In the remaining volume, the concentration of [γ -³²P]ATP was made equal and then the substrate (MBP, final concn. 1 mg/ml) was added. The samples were then incubated at 30 °C for a further 30 min (lanes 3 and 4). The reactions were terminated, again by addition of SDS/PAGE sample buffer, and all samples were analysed by SDS/PAGE (12% gel) followed by autoradiography. (c) Crude extracts of bovine brain (lane 1) and chicken gizzard (lane 2) were run on SDS/PAGE (12% gel) and Western-blotted using anti-STE7-VIII, which reacts with proteins similar to MAP kinase kinase. Numbers on the right indicate the position of the 42 kDa kinase.

24 h. Phosphorylation of *h*-caldesmon by the gizzard and sea-star MAP kinase yields identical tryptic maps revealing two major phosphorylated spots as shown in Figure 4. This indicates that both kinases phosphorylate *h*-caldesmon at identical sites and must affect the function of caldesmon similarly. This finding validates the use of activated sea-star p44^{mpk} instead of the less active gizzard p42^{mapk} in the phosphorylation of *h*-caldesmon for further studies.

It has been shown that both *h*- and *l*-caldesmon bind to microtubules and that phosphorylation of *l*-caldesmon by p34^{cdc2} inhibits this interaction (Ishikawa et al., 1992a,b). As shown in Figure 5, *h*-caldesmon interacts with microtubules, giving a *K_a* of 3.4×10^6 M⁻¹, which is similar to the *K_a* reported by Ishikawa et al. (1992a). Phosphorylation by sea-star p44^{mpk} decreases the caldesmon-microtubule interaction by over 30-fold (*K_a* 1.1×10^5 M⁻¹).

Activation of gizzard p42^{mapk}

Incubation of gizzard p42^{mapk} in the presence of ATP for 60 min results in autophosphorylation of at least one tyrosine residue, as detected by an anti-phosphotyrosine antibody (Figure 6a). Similarly to MAP kinases from other sources (Wu et al., 1991; Seger et al., 1991) autophosphorylation activates gizzard p42^{mapk} phosphorylation of MBP, as shown in Figure 6(b).

In mammalian cells, MAP kinase is activated by a MAP kinase activator, MEK (Crews et al., 1992; Seger et al., 1992; Matsuda et al., 1992; Nakielnny et al., 1992), which bears strong similarity to STE7 in budding yeast. Using an anti-STE7-VIII antibody raised against a peptide corresponding to residues 434–446 of STE7, we were able to detect a strong cross-reactive 46 kDa protein in the brain extract, which probably represents

the MEK homologue, as shown in Figure 6(c). However, a much more weakly cross-reactive 46 kDa protein is detected in adult chicken gizzard. If MAP kinase is present predominantly in the active form in differentiated chicken gizzard, activation of gizzard $p42^{\text{mapk}}$ must occur in response to physiological demands via the Ras/Raf and/or G-protein pathways (Lange-Carter et al., 1993). Thus it is likely that a MEK-like gizzard $p42^{\text{mapk}}$ activator does exist in gizzard, but the region around residues 434–446 in STE7 bears a weak sequence homology to the same region in the gizzard $p42^{\text{mapk}}$ activator.

DISCUSSION

We have previously shown the presence of a single 42 kDa MAP kinase homologue in chicken gizzard smooth muscle (Childs et al., 1992). To further our examination of the role of MAP kinase phosphorylation of smooth-muscle caldesmon, we have purified the gizzard $p42^{\text{mapk}}$ and have shown that it phosphorylated *h*-caldesmon at sites identical with those phosphorylated by sea-star $p44^{\text{mpk}}$. This finding justifies the use of the much more active $p44^{\text{mpk}}$ for future work on caldesmon phosphorylation and substantiates the results derived from the use of $p44^{\text{mpk}}$ in our previous work (Childs et al., 1992).

It is interesting that there is only one isoform present in this muscle, since it is known that both 42 and 44 kDa isoforms are present in aorta (Adam and Hathaway, 1993). A developmental time course of chicken gizzard shows that, at all stages, only the 42 kDa isoform is expressed. Although it is possible that another isoform is present in chicken gizzard smooth muscle which has diverged substantially in its antigenic epitopes, and is therefore undetectable with our polyclonal antibody, it seems more likely that gizzard smooth muscle functions with only one MAP kinase isoform. This suggests that in other tissues, especially other types of smooth muscle, which have two MAP kinase isoforms there may be functional redundancy among these MAP kinase isoforms. Alternatively, chicken gizzard smooth-muscle MAP kinase may be capable of performing all of the roles fulfilled by the 42 and 44 kDa MAP kinases in other tissues.

In spite of many reports on the phosphorylation of caldesmon by protein kinases A and C, Ca^{2+} -calmodulin-dependent protein kinase II, and casein kinase II (Sobue and Sellers, 1991; Marston and Redwood, 1991), none of these kinases has been shown to phosphorylate caldesmon *in vivo* in muscle. On the other hand, phosphorylation of *h*-caldesmon by Pro-directed kinases has been demonstrated to occur *in vivo* in muscle by Adam et al. (1992) at two sites, SPAPK and VTSPK. They have recently confirmed that MAP kinase phosphorylates these two sites *in vitro* (Adam and Hathaway, 1993). The former site is identical with one of the predicted $p44^{\text{mpk}}$ phosphorylation sites at the C-terminal end of gizzard caldesmon (Childs et al., 1992), whereas the latter site is located at the very C-terminal of the mammalian caldesmon molecule and is not present in chicken gizzard (Bryan et al., 1989). Although caldesmon phosphorylation by MAP kinase is likely to be physiologically relevant, its role *in vivo* with respect to smooth-muscle contractility and cytoskeletal structure is not clear. Pinter and Marston (1992) have demonstrated that phosphorylation of caldesmon by an endogenous kinase does not inhibit actomyosin ATPase activity. Recently, Redwood et al. (1993) reported that replacing Ser-702 with Asp at the SPAPK site of caldesmon impairs its inhibition of actomyosin ATPase activity and its binding to actin at the high-affinity site, but not at the low-affinity site. This is consistent with our previous studies that phosphorylation of *h*-caldesmon by $p44^{\text{mpk}}$ has a small effect on its interaction with actin using low concentrations

of caldesmon, where only the high-affinity sites would be occupied (Childs et al., 1992). It is therefore possible that phosphorylation of caldesmon by MAP kinase may play a role in the regulation of myosin ATPase activity in smooth-muscle cells.

Recent studies suggest that caldesmon may also be involved in the regulation of microtubule structure (Ishikawa et al., 1992a,b). Phosphorylation of caldesmon by $p34^{\text{cdc2}}$ abolishes the caldesmon-microtubule interaction, which suggests that caldesmon phosphorylation by Pro-directed kinases is involved in the modulation of cytoskeletal architecture in smooth muscle. In support of this suggestion, we found that MAP kinase is able to decrease dramatically the affinity of caldesmon for tubulin. The functional significance of the caldesmon-tubulin interaction may involve caldesmon's ability to enhance tubulin polymerization (Ishikawa et al., 1992a). Interestingly, caldesmon has been shown to co-purify with microtubules from brain (Ishikawa et al., 1992b). It has also been demonstrated that caldesmon can inhibit the attachment and sliding of microtubules on dynein, and therefore it has been speculated that caldesmon may regulate microtubule-transport systems (Ishikawa et al., 1992a). In addition, there have been suggestions that actin filaments and microtubules may interact *in vivo* (Nishida et al., 1981; Sattilaro et al., 1981).

Finally, we were able to detect a 46 kDa protein in chicken gizzard smooth muscle which cross-reacts weakly with anti-STE7-VIII antibody. In view of the report by Adam and Hathaway (1993), who have detected MEK and its upstream activators, MEKK and Raf, in bovine aorta, we expect that the same signal-transduction apparatus is also present in gizzard. It will be interesting to discover the basis for the weak anti-STE7-VIII cross-reactivity in chicken gizzard, and we are continuing to examine the MAP kinase signal-transduction pathway in chicken gizzard smooth muscle.

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