antigen binding (to be submitted for publication). This line of analyses using the ^{13}C chemical shift data of the unambiguously assigned carbonyl carbon resonances would be useful in mapping the antigen binding site. It should also be of interest to use, for example, the Met-314H resonance as a probe for the binding of staphylococcal protein A to the Fc region (Deisenhofer, 1981).

In order to proceed further, it is obviously necessary to look at different parts of the antibody molecule by using different types of ^{13}C labeling. Further analyses are under way in our laboratory using switch variant antibodies labeled with [1-^{13}C]Trp, [1-^{13}C]Tyr, and [1-^{13}C]Cys.

ACKNOWLEDGMENTS

We are grateful to Professor L. A. Herzenberg, Stanford University, and Dr. V. T. Oi, Becton Dickinson Immunocytometry Systems, for generously providing us with the switch variant cell lines used in the present work and for making sequence data of the V_L region available to us prior to publication. We also thank Professor M. Kainosho, Tokyo Metropolitan University, for his helpful discussion. Thanks are due to M. Waelchli, Bruker Japan, for his help in measuring some of the spectra given in the present paper.

SUPPLEMENTARY MATERIAL AVAILABLE

Eight figures showing additional ^{13}C NMR spectral data for singly and doubly labeled Fv, Fab, and Fc fragments used for the assignments in this work (10 pages). Ordering information is given on any current masthead page.

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Purification and Properties of Extracellular Signal-Regulated Kinase 1, an Insulin-Stimulated Microtubule-Associated Protein 2 Kinase†

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ABSTRACT: In rat 1 fibroblasts, insulin has little or no stimulatory effect on the activities of either MAP2 protein kinase or ribosomal protein S6 kinase. In contrast, in rat 1 cells that overexpress the normal human insulin receptor (rat 1 HIRc B; McClain et al. (1987) *J. Biol. Chem. 262*, 14663-14671), insulin activates both MAP2 and S6 kinase activities close to 5-fold. A MAP2 kinase has been purified from insulin-treated rat 1 HIRc B cells over 6300-fold by chromatography on Q-Sepharose, phenyl-Sepharose, S-Sepharose, phosphocellulose, QAE-Sepharose, UltrogelAcA54, DEAE-cellulose, and a second Q-Sepharose. Its specific activity is approximately 0.8-1 μmol·min^{-1}·mg^{-1} with MAP2 and 3 μmol·min^{-1}·mg^{-1} with myelin basic protein. The enzyme preparation contains one major band of M_r = 43 000 upon SDS-polyacrylamide gel electrophoresis, which is immunoblotted by antibodies to phosphotyrosine. A sequence from the 43-kDa band led to the isolation of a cDNA encoding the enzyme, which we have named ERK1 for extracellular signal-regulated kinase (Boulton et al. (1990) *Science 249*, 64-67).

Insulin exerts its effects on many cellular processes by regulating the phosphorylation state of serine and threonine residues of proteins. The phosphorylation of ribosomal protein S6 on serine residues is rapidly increased in many cell types by insulin (Smith et al., 1980), growth factors (Martin-Pérez et al., 1984), and transformation by viruses (Decker, 1981), largely as a result of the stimulation of ribosomal protein S6 kinases (Rosen, 1987; Erikson & Maller, 1986; Cobb, 1986; Gregory et al., 1989; Blenis & Erikson 1985; Jenö et al., 1989).

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Because the insulin receptor has intrinsic tyrosine-specific protein kinase activity, it is logical to postulate that the enzymatic activity confers upon the receptor the ability to signal to the internal milieu. However, although S6 kinases are insulin-sensitive, they are not substrates of the insulin receptor. Regulation of these S6 kinases occurs by phosphorylation not of tyrosine but of serine and/or threonine residues on the enzymes (Ballou et al., 1988; Erikson & Maller, 1989). Therefore, one or more other insulin-stimulated protein kinases must be intermediates in the S6 phosphorylation cascade. These intermediates, perhaps themselves insulin receptor substrates, catalyze the serine/threonine phosphorylations of the S6 kinases that result in their activation.

Ray and Sturgill (1987, 1988a,b) have suggested that a partially purified insulin-sensitive MAP2[1] kinase from 3T3-L1 cells is such an intermediate. This enzyme phosphorylates the *Xenopus* S6 kinase II causing an increase in its activity (Sturgill et al., 1988). We have corroborated this observation from studies in vitro of a rabbit liver S6 kinase and an insulin-dependent MAP2 kinase from rat 1 HIRc B and CHO cells (Gregory et al., 1989). The purification of an insulin-stimulated MAP2 kinase to near homogeneity has not previously been reported nor has its specific activity been estimated. The present work describes the purification of ERK1, an insulin-stimulated MAP2 kinase, over 6300-fold from rat 1 HIRc B cells. Sequences obtained from enzyme purified as described here led to the isolation of a cDNA encoding this enzyme (Boulton et al., 1990a).

Materials and Methods

Materials. Rat 1 HIRc B cells (McClain et al., 1987), obtained from Dr. Don McClain (Veterans Administration Medical Center, San Diego, CA), were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and 10^{-8} M methotrexate. Porcine insulin was a gift from Dr. Mary Root (Eli Lilly). MAP2 was prepared according to Kim et al. (1979). Antibodies to phosphotyrosine were prepared as described (Ek & Heldin, 1984) and were affinity purified on phosphotyrosyldiaminopentane–Sepharose. *Artemia salina* 40S ribosomal subunits, prepared by the method of Zasloff & Ochoa (1974), were resuspended and diluted to an optical density of 40–50 at 260 nm. [γ-^{32}P]ATP was prepared by the method of Johnson and Walseth (1979). Histones were prepared by the method of Johns (1964). Cardiac myosin light chains were obtained from Dr. James Stull, and ATP–citrate lyase was a gift of Dr. Paul Srere. Bovine adrenal kinesin, hsc 90, hsc 70, and *Dictyostelium discoideum* myosin I were obtained from Dr. Joseph Albanesi. All other chemicals were from standard sources except as noted (Cobb, 1986). Protein was measured by the method of Lowry (Lowry et al., 1951) or with Amido Black (Shaffner & Weissman, 1973) with bovine serum albumin as a standard. MAP2 and myelin basic protein were excised from SDS–PA gels and hydrolyzed in 6 N HCl. Phosphoamino acid analyses were performed as described (Cobb, 1986).

Preparation of Cell Extracts. Confluent rat 1 HIRc B cells were incubated 12–18 h in serum-free medium. After exposure of the cells to insulin for 5 (MAP2 kinase) or 20 (S6 kinase) min, the medium was removed and the cells were rinsed and scraped into iced homogenization solution that contained 20 mM *p*-nitrophenyl phosphate, 20 mM Tris-HCl, pH 7.5, 1 mM EGTA, 50 mM sodium fluoride, 50 μM sodium orthovanadate, and 5 mM benzamidine (MAP2 kinase) or 80 mM β-glycerophosphate, pH 7.3, 20 mM EGTA, 15 mM $MgCl_2$, and 1 mM benzamidine (S6 kinase). As indicated equal numbers of dishes of untreated cells were harvested as controls. All further steps were performed at 4 °C. Cells were broken with 30–50 strokes of a Dounce homogenizer and homogenates were centrifuged at 4000*g* for 5 min. The supernatants were then centrifuged at 97000*g* for 60 min. The resulting supernatants were assayed immediately, then frozen in liquid nitrogen, and stored at −80 °C.

Phosphotransferase Assays. The assay for phosphorylation of MAP2 contained 30 mM Hepes, pH 8, 50 μM ATP (1–50 cpm/fmol), 1 mM dithiothreitol, 1 mM benzamidine, 10 mM $MgCl_2$, 100 μg/mL bovine serum albumin, 3 μg of MAP2, and no more than 10 μg of sample protein in a final volume of 30 μL for 10 min at 30 °C. The amount of MAP2 in the assay (100 μg/mL) was chosen for convenience of analysis both by SDS–PAGE and by precipitation (see below). The enzyme is not saturated with substrate even at 1.36 mg of MAP2/mL. However, with 100 μg of MAP2/mL of enzyme activity was linear with time for at least 30 min. All samples except for unfractionated supernatants were routinely assayed as above in the presence of 1 mg of bovine serum albumin. Assays were terminated by the addition of 10% trichloroacetic acid and precipitates were collected on glass filters. The assay for phosphorylation of S6 contained 30 mM Hepes, pH 8, 100 μg/mL albumin, 5 mM $MgCl_2$, 0.5 mM dithiothreitol, 10 μM ATP (specific activity 3–100 cpm/fmol), 3 μL of 40S subunits, and sample in a final volume of 30 μL, for 20 min at 30 °C. All assays except as noted above were terminated by the addition of 0.25 volume of 0.3 M Tris-HCl, pH 6.9, containing 2 M mercaptoethanol, 50% glycerol, and 10% SDS and analyzed by electrophoresis in SDS by using either 15% (S6) or 5% (MAP2) polyacrylamide gels. The gels were stained with Coomassie blue, destained in 10% methanol and 10% acetic acid, dried, and subjected to autoradiography at −80 °C by using Kodak XS-5 or BB-5 film with Du Pont Quanta III intensifying screens. Substrate bands were excised from gels and ^{32}P was quantitated using Liquiscint (National Diagnostics) in a Beckman LS-3801 liquid scintillation spectrometer.

Purification of ERK1. Because enzyme activity increases with increasing MAP2 concentration (see above), each purification has been quantitated by assays with a single preparation of MAP2 to ensure uniformity of substrate. Enzyme activity has been calculated by using 3 μg of MAP2 per assay. Soluble fractions (225–300 mL) combined from 150 to 200 150-cm^2 dishes of insulin-treated rat 1 HIRc B cells were adjusted to a conductivity of 3.5 mS (with water) and to concentrations of 40 μM cAMP, 0.5 mM phenylmethanesulfonyl fluoride, and 0.1 μM pepstatin prior to chromatography on a Q-Sepharose column (1.5 × 19 cm). The column was washed with 4–5 volumes of buffer A (10% glycerol, 25 mM Tris-HCl, pH 7.5, 50 μM sodium orthovanadate, 1 mM dithiothreitol, 50 mM NaF, 20 mM β-glycerol phosphate, 1 mM EGTA, 10 mM benzamidine, 10 mM *p*-nitrophenyl phosphate, 0.5 mM phenylmethanesulfonyl fluoride, and 0.1 μM pepstatin) containing 40 μM cAMP. Protein was then eluted by a gradient of 0–0.4 M NaCl in buffer A. Fractions containing stimulated MAP2 kinase activity were pooled and applied to a phenyl-Sepharose column (1.5 × 18 cm). The column was washed with 5 column volumes of buffer A containing 0.25 M NaCl and protein was eluted with a descending gradient of 0.25–0.025 M NaCl plus an ascending gradient of 0–65% ethylene glycol in buffer A without glycerol. Kinase activity pooled from the phenyl-Sepharose column was then

[1] Abbreviations: MAP2, microtubule-associated protein 2; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; TBS, 20 mM Tris-HCl, pH 7.6, and 0.5 M NaCl.

applied directly to a 5-mL column (1.5 × 3 cm) of S-Sepharose followed by a 5-mL column of phosphocellulose (1.5 × 3 cm). In both cases, unadsorbed material containing MAP2 kinase activity and 2 column volumes of wash was collected. The MAP2 kinase activity from the phosphocellulose column was applied directly to a QAE-Sepharose column (1 × 24 cm). The column was washed with 5 volumes of buffer A and protein was eluted with a gradient of 0–0.4 M NaCl in buffer A. The fractions containing MAP2 kinase activity were pooled, Brij-58 was added to give a final concentration of 0.01% (included in all subsequent steps), and the sample was concentrated by ultrafiltration to 1.5–2 mL in order to load onto an Ultrogel AcA54 column (1 × 112 cm) equilibrated in buffer A containing 0.2 M NaCl and 0.01% Brij-58. Fractions from the gel filtration column were collected into tubes containing 2.4 mM leupeptin. The fractions containing activity were concentrated and diluted with 25 mM Tris, pH 7.5, 1 mM DTT, 10 mM sodium phosphate, 0.1 μM pepstatin, and 0.5 mM phenylmethanesulfonyl fluoride containing 0.01% Brij-58 until the conductivity was reduced to 3 mS and then applied to DEAE-cellulose (0.7 × 18 cm). The activity was eluted with a gradient of 0–0.25 M NaCl in buffer A. Fractions containing activity were pooled and, as necessary, concentrated and diluted as above to apply to either a Mono Q HR 5/5 or a Q-Sepharose (0.5 × 9 cm) column. The MAP2 kinase activity was eluted with a gradient of 0–0.25 M NaCl (Mono Q) or 0–0.35 M NaCl (Q-Sepharose) in buffer A. Fractions were assayed and then immediately frozen in liquid nitrogen.

Purification and Sequencing of Tryptic Peptides from ERK1. Fractions containing the enzyme were pooled and final concentrations of 0.05% Lubrol and 8.5% trichloroacetic acid (w/w) were added to precipitate the protein. After washing with acetone, the protein was dissolved in electrophoresis buffer and a 250-pmol sample was loaded onto a 10% polyacrylamide gel in SDS. Protein was electrophoretically transferred to nitrocellulose (Schleicher and Schuell, Keane, NH). The 43-kDa band was excised for in situ digestion with trypsin (Abersold et al., 1987), leaving the minor component, which migrates only slightly faster, on the nitrocellulose. Peptides released from the excised piece of nitrocellulose were subjected to HPLC on a Model 130A chromatography system (Applied Biosystems, Inc., Foster City, CA) equipped with a 2.1 × 100 mm Applied Biosystems RP-300 column. Separations were performed in 0.1% trifluoroacetic acid at a flow rate of 50 μL/min by using a gradient of 0–70% (v/v) acetonitrile of 100-min duration. Absorbency of the eluate was monitored at 214 nm and the components that eluted were collected manually. Peptides were dried onto 1-cm disks of Whatman GF/C paper and sequenced by using an Applied Biosystems, Inc., Model 470A amino acid sequencer equipped with a Model 120A phenylthiohydantoin analyzer, according to manufacturer's specifications.

Immunoblotting with Antiphosphotyrosine Antibodies. Samples to be probed with antibody were separated on 10% PA gels in SDS and electrophoretically transferred to nitrocellulose in 25 mM Tris, 0.19 M glycine, and 20% methanol. The nitrocellulose was incubated in TBS containing 0.05% Tween-20 and 0.1% bovine serum albumin for 1 h at room temperature. Affinity-purified antibodies to phosphotyrosine were incubated with the nitrocellulose at room temperature overnight. After washing 3 times for 10 min with TBS plus 0.05% Tween-20, the nitrocellulose was incubated with horse radish peroxidase conjugated to goat antirabbit IgG (Cappel) for 2 h. After washing twice as above and a third time in TBS, the nitrocellulose was developed with 4-chloro-1-naphthol. The specificity of the antiphosphotyrosine antibody was tested with samples containing other phosphoamino acids. The antibody did not cross-react with phosphohistidine in ATP–citrate lyase. Samples of myelin basic protein, one of which contained greater than 10 pmol of phosphoserine (stoichiometry 1 mol/mol) and the other of which contained greater than 10 pmol of phosphothreonine (0.3 mol/mol), were also not recognized by the antibody.

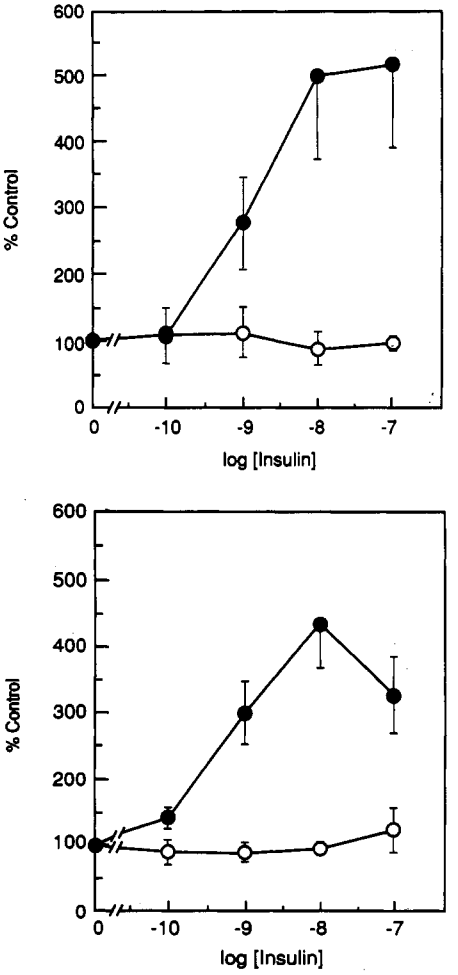

FIGURE 1: Effect of insulin concentration on MAP2 and S6 protein kinase activity in rat 1 parent and rat 1 HIRc B cell lines. (A, Top) Confluent cultures of rat 1 parent (○) and rat 1 HIRc B (●) cells were treated with the indicated concentrations of insulin for 5 min. MAP2 protein kinase activity in supernatants was assayed as described under Materials and Methods and is expressed as the percent of the value measured without insulin treatment (% control). The means of three or more experiments are shown with standard errors of the mean. (B, Bottom) Cultures were treated with the indicated concentrations of insulin for 20 min. S6 protein kinase activities from supernatants of rat 1 parent (○) and rat 1 HIRc B (●) cells were measured as described under Materials and Methods and are means of three experiments. Standard errors of the mean are shown.

RESULTS

In an effort to identify a cell line in which stimulation of MAP2 kinase activity by insulin could easily be detected, we examined the effects of insulin on MAP2 kinase activity in rat 1 cells and in rat 1 cells that overexpress the human insulin receptor [rat 1 HIRc B cells generated by McClain et al. (1987)]. In the rat 1 parent cell, insulin from 10^{-10} up to 10^{-7} M has no effect on MAP2 kinase activity. In rat 1 HIRc B cells insulin stimulates MAP2 kinase activity 5-fold (Figure 1A). The maximum response is generated at approximately 10^{-8} M insulin, with a 2.8-fold response at 10^{-9} M insulin.

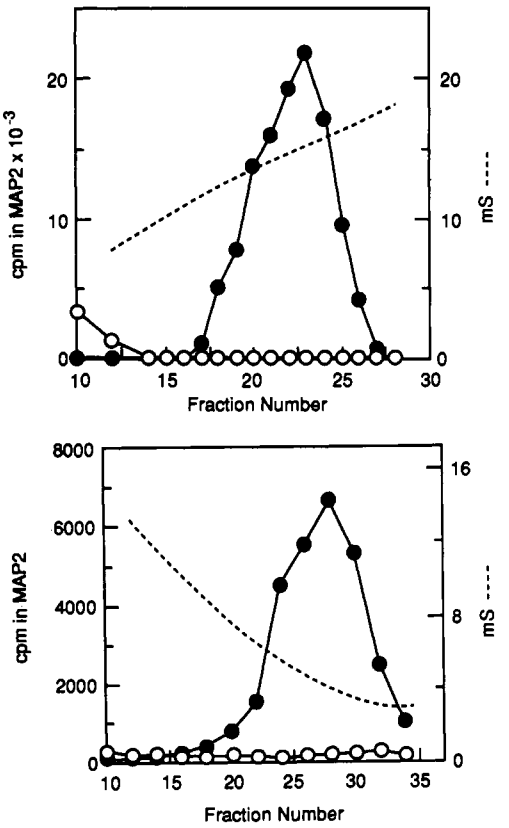

FIGURE 2: Chromatography of ERK1 from insulin-treated and untreated cells. (A, Top) Supernatants containing 10 mg of protein from treated (●) or nontreated (○) cells were applied to Q-Sepharose (1 × 4 cm) and eluted with a gradient of 0–0.4 M NaCl. (B, Bottom) Fractions pooled from the Q-Sepharose in (A) were applied to phenyl-Sepharose (0.7 × 10 cm) and eluted as described under Materials and Methods. Activity is expressed as cpm in MAP2 and conductivity is plotted in mS.

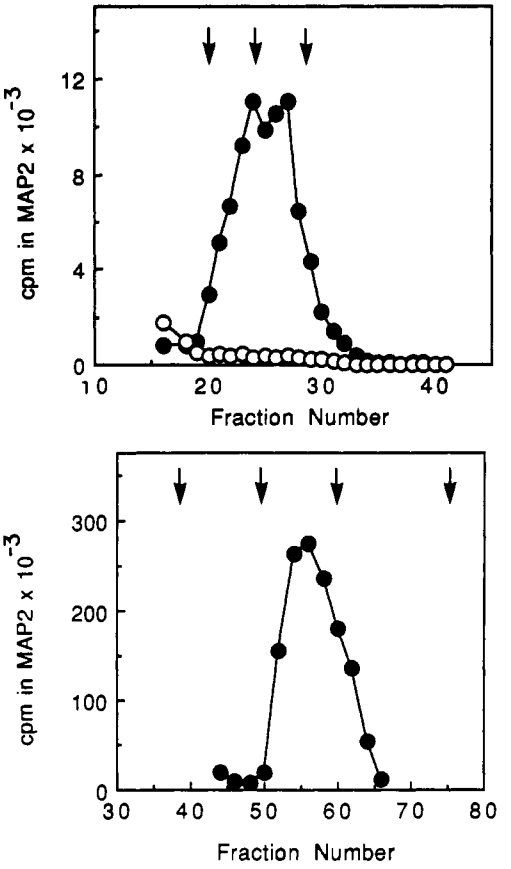

FIGURE 3: Chromatography of ERK1 on Ultrogel AcA44 and Ultrogel AcA54. (A, Top) Fractions containing MAP2 kinase activity eluted from phenyl-Sepharose were pooled and applied to AcA44 columns. The elution of standard proteins, alkaline phosphatase (87 000), cyclic AMP dependent protein kinase catalytic subunit (39 000), and cytochrome *c* (13 400), corresponds to the arrows. MAP2 kinase activity, plotted as cpm in MAP2, was determined as described under Materials and Methods: (●) insulin; (○) control. (B, Bottom) MAP2 kinase activity from a large-scale purification was chromatographed on AcA54. The arrows indicate the fractions in which blue dextran (leftmost arrow) and the standards listed above elute. MAP2 kinase activity is plotted as cpm in MAP2.

Thus, in cells expressing large numbers of functional receptors, a response to insulin, not detectable in the parental line, is now observed. Similar results are obtained for stimulation of S6 kinase by insulin (Figure 1B). There is little or no effect of insulin on S6 kinase in the rat 1 parent, whereas in rat 1 HIRc B cells insulin enhances S6 kinase activity over 4-fold. The concentration dependences for stimulation of MAP2 and S6 kinases by insulin are nearly identical.

The comparison of kinase activities in untreated and hormone-stimulated cultures has been the major method of identifying insulin-regulated enzymes. Hormone-sensitive kinases can thus be distinguished from numerous other protein kinases not regulated by insulin. An insulin-dependent MAP2 kinase activity was identified following chromatography of high-speed supernatants prepared from 10–20 150-cm^2 dishes of rat 1 HIRc B cells, either untreated or treated with insulin (8.8×10^{-8} M). Samples were applied to columns of Q-Sepharose (1 × 4 cm). The columns were washed with 40 μM cAMP to remove the catalytic subunit of cyclic AMP dependent protein kinase and then proteins were eluted with increasing ionic strength. One large peak of insulin-stimulated MAP2 kinase activity began to elute at a conductivity of 11 mS (Figure 2A). The majority of protein kinase C activity eluted before the insulin-stimulated MAP2 kinase [not shown; see Cobb (1986)]. The comparable fractions from untreated cells contained very little MAP2 kinase activity; fractions of the same conductivity as those containing the insulin-enhanced activity were pooled. To characterize the activity further and compare it with that previously described (Ray & Sturgill, 1988b), the pooled Q-Sepharose fractions were applied to columns of phenyl-Sepharose (0.7 × 10 cm). Again there was one resulting peak of MAP2 kinase activity (Figure 2B) eluting between conductivities of 5.5 and 3 mS and at the highest concentrations of ethylene glycol. The kinase activity was not completely eluted from the column unless 25 mM sodium chloride was included in the buffer containing ethylene glycol. Phenyl-Sepharose fractions having MAP2 kinase activity were applied to S-Sepharose (1 × 2.5 cm). Unadsorbed material plus material in 4 mL of buffer A used to wash the column were combined, concentrated, and applied to Ultrogel AcA44 (1 × 40 cm) (Figure 3A). The activity eluted between the standards alkaline phosphatase (87 000) and cytochrome *c* (134 000). At this stage of the purification, there was no activity detectable in the control profile. Frequently the insulin-dependent activity eluted from the gel filtration column in two poorly resolved peaks. Based on results from large scale purifications described below, this elution behavior may have been caused by proteolysis.

Supernatants from insulin-treated and untreated cells were used in the identification of an insulin-activated MAP2 kinase. Because no MAP2 kinase activity appeared in the control fractions as described above, only material from insulin-treated cells has been subjected to large-scale purification. To scale up the purification of the enzyme, supernatants prepared from 150–200 150-cm^2 dishes of insulin-treated cells were combined

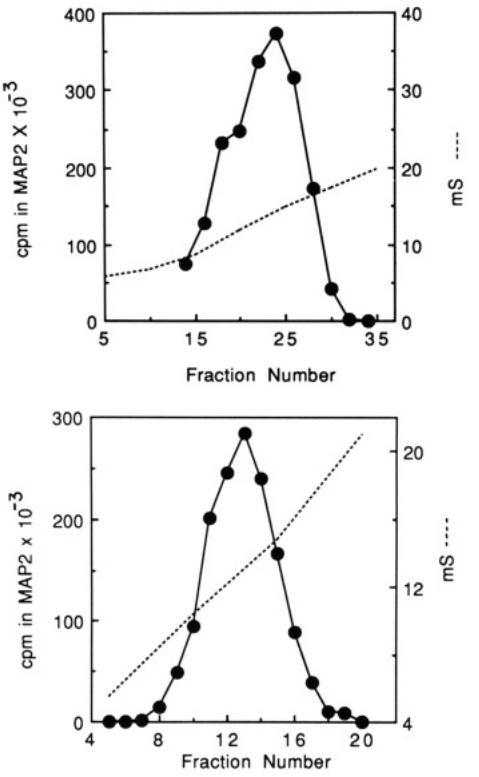

FIGURE 4: Chromatography of ERK1 on QAE-Sepharose and Q-Sepharose. Fractions containing MAP2 kinase activity from the Ultrogel AcA54 column were pooled and applied to (A, top) QAE-Sepharose and eluted with a gradient of 0–0.4 M NaCl. Fractions pooled from QAE-Sepharose were applied to DEAE-cellulose and eluted with a gradient of 0–0.25 M NaCl. The peak of MAP2 kinase activity was pooled from the DEAE-cellulose column and applied to (B, bottom) Q-Sepharose. Protein was eluted with a gradient of 0–0.35 M NaCl. Activity (cpm in MAP2) and conductivity (mS) of the eluted fractions are shown.

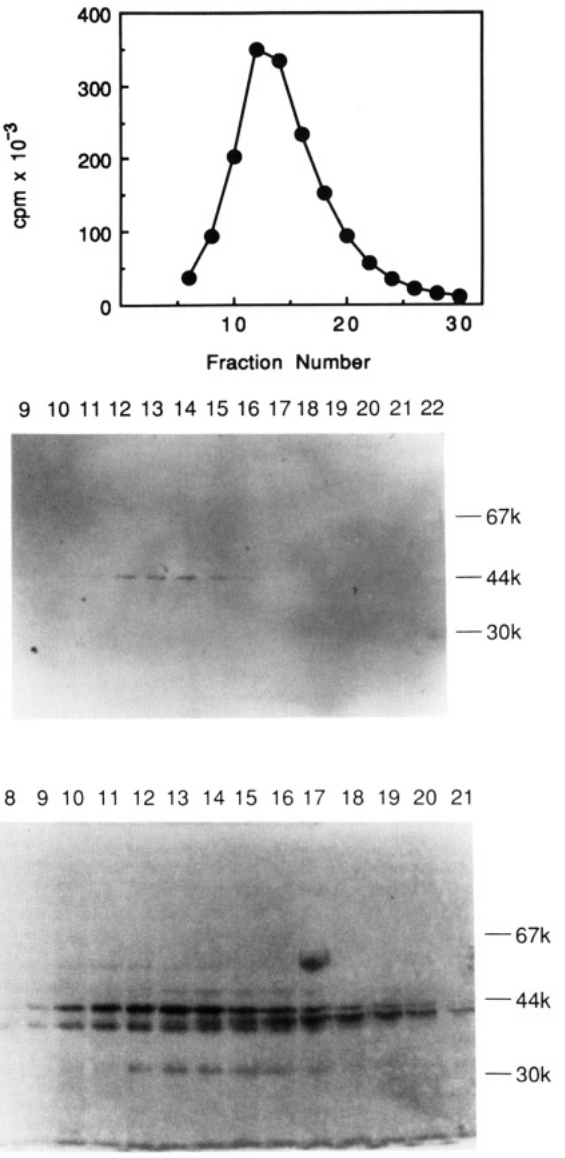

FIGURE 5: Comparison of activity, immunoblotting, and protein patterns of ERK1 following chromatography on DEAE-cellulose. ERK1 was purified through step 6 and chromatographed on DEAE-cellulose as described in Figure 4. (A, Top) Activity (cpm in MAP2) of the eluted fractions. (B, Middle) The immunoblot of the indicated fractions with antibodies to phosphotyrosine. (C, Bottom) The silver-stained SDS–PA gel of the indicated fractions. The arrow denotes the 43-kDa band that contains phosphotyrosine.

and applied to Q-Sepharose and phenyl-Sepharose as above. Q-Sepharose fractions containing MAP2 kinase activity (pooled between conductivities of 10.5 and 16 mS) contained approximately 6–10% of the applied protein and consistently more than the units of activity applied. The units applied were measured by excising labeled MAP2 from SDS–PA gels. In this manner phosphorylation of endogenous substrates could be distinguished from phosphorylation of MAP2. Subsequent assays were quantitated by acid precipitation, which recovers labeled proteolytic fragments of MAP2 and other trace amounts of kinase substrates in the MAP2 preparation. The former method of assay may underestimate the units of enzyme activity compared with the latter method of assay. Thus, the apparent increase in units upon Q-Sepharose chromatography may result in part from the difference in the two assay procedures; however, for purified preparations, enzyme activities measured by the two methods were within 10%.

The majority of the kinase activity from the pooled Q-Sepharose fractions was adsorbed to phenyl-Sepharose; protein was eluted as described above. The phenyl-Sepharose fractions containing kinase activity that phosphorylated MAP2 were applied sequentially to a 5-mL column of S-Sepharose and then to a 5-mL phosphocellulose column. The material not adsorbed to the resin was collected in both cases. These two steps were important in order to remove trace quantities of other kinases that phosphorylate MAP2 that were not completely resolved earlier in the purification (e.g., the S6 kinases and casein kinases I and II). This material was then applied to QAE-Sepharose. MAP2 kinase activity eluted between conductivities of 9 and 17 mS; the active fractions contained about 40% of the applied protein (Figure 4A). Fractions from the QAE-Sepharose column were further fractionated on Ultrogel AcA54 (Figure 3B) because it removed some high molecular weight contaminants more effectively than AcA44, which was used in the comparison of material from insulin-treated and untreated cells. If the concentrations of protease inhibitors in the gel filtration buffer were lower than noted below, MAP2 kinase activity eluted as two poorly resolved peaks. In the presence of 10 mM benzamidine, 1 μM pepstatin, and 0.5 mM phenylmethanesulfonyl fluoride, there was a single peak of MAP2 kinase activity. Approximately 20–25% of the total protein applied to the Ultrogel AcA54 column was recovered in the pooled fractions.

The activity was further chromatographed on DEAE-cellulose (Figure 5). Figure 5 shows one of the least pure preparations to demonstrate clearly the number of bands that may still be present after this many chromatographic steps. The fractions with the greatest amount of activity also contained a band of 43 kDa that cross-reacted with antibodies

Table I: Purification of Insulin-Stimulated ERK1

step	protein (mg)	specific activity (nmol·min^{-1}·mg^{-1})	units (nmol/min)	recovery (%)	purification (x-fold)
supernatant	660	0.12	82	100	1
Q-Sepharose	60	2.8	170	202	22
phenyl-Sepharose	7.7	15	120	142	123
S-Sepharose	7.7	18	140	170	145
phosphocellulose	5.1	20	100	123	159
QAE-Sepharose	1.8	44	77	93	350
AcA54	0.40	190	75	91	1500
DEAE-cellulose	0.11	260	28	34	2040
Q-Sepharose	0.004	800	3.1	3.8	6380

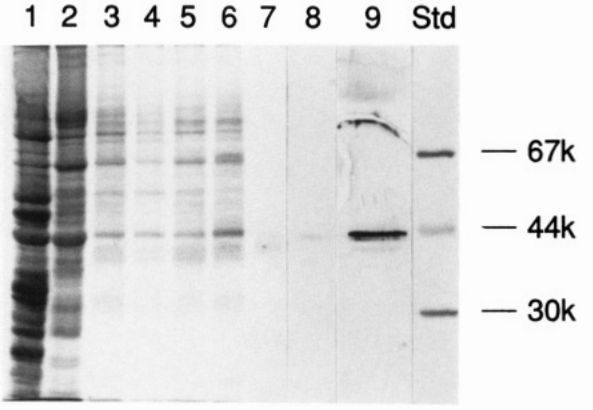

FIGURE 6: Silver-stained SDS–PA gel of fractions from the purification of ERK1. Aliquots from the following pools were analyzed on a 10% PA gel in SDS. Each lane contained 0.04 unit of MAP2 kinase activity from the specified purification step, except the supernatant, which contained 0.0055 unit, DEAE-cellulose fraction 14 from this purification, which contained 0.125 unit (lane 8), and the DEAE-cellulose pool from another purification, which contained 0.8 unit (lane 9). Lane 1, supernatant; lane 2, Q-Sepharose; lane 3, phenyl-Sepharose; lane 4, S-Sepharose; lane 5, phosphocellulose; lane 6, QAE-Sepharose; lane 7, AcA54; lane 8, DEAE-cellulose fraction 14; lane 9, DEAE-cellulose. Lane 9 was torn during the staining process.

to phosphotyrosine (Figure 5B). After this extent of purification, there were several protein species, whose sizes on gels are in the range 35–43 kDa (Figure 5C), in the fractions with activity. However, the 43-kDa protein was the only one that cross-reacted with the antibodies and coeluted with kinase activity. From 30 to 40% of the applied protein was pooled along with the activity in the fractions between conductivities of 8 and 9.5 mS. As necessary, the enzyme was further purified on a Mono Q HR 5/5 or a Q-Sepharose column (Figure 4B). Enzyme recovered from Mono Q and from Q-Sepharose was similar both in purity and in yield (Table I). If only the most active fractions (not including the leading and trailing edges of the peak) were pooled from each chromatographic step, the DEAE-cellulose fractions containing MAP2 kinase activity were largely resolved from the major impurities, all of which were similar in size to MAP2 kinase (Figure 6, lane 8). This circumvented the loss of activity (nearly 90%) and protein that occurred in reducing the conductivity of the DEAE-cellulose pool and fractionating on Q-Sepharose or on Mono Q.

The silver-stained gel of the purified enzyme contains one major band of $M_r \simeq 43$K (Figure 6, lanes 8 and 9). Prior to gel filtration this band is obscured by another darkly staining band of approximately the same size (lane 6). After gel filtration the enzyme band is not easily visible, until after DEAE-cellulose chromatography. This step concentrates the enzyme and eliminates some of the bands of slightly smaller size that coeluted with the activity at earlier steps. In some preparations a band of 41 kDa (see Figure 5C) and a small amount of a 42-kDa band (Figure 6, lane 9) are also present.

Table II: Amino Acid Sequence Analysis of Two Tryptic Peptides of ERK1[a]

	A			B		
cycle	amino acid	yield (pmol)	cDNA[b]	amino acid	yield (pmol)	cDNA[b]
1	Tyr	5.8	Tyr	Asp	8.5	Asp
2	Thr	2.0	Thr	Leu	3.6	Leu
3	Gln	4.0	Gln	Lys	2.0	Lys
4	Leu	2.5	Leu	Pro	7.0	Pro
5	Gln	4.0	Gln	Ser	2.3	Ser
6	Tyr	3.0	Tyr	Asn	4.8	Asn
7	Ile	3.2	Ile	Leu	4.0	Leu
8	Gly	4.4	Gly	Leu	4.0	Leu
9	Glu	3.0	Glu	Ile	2.6	Ile
10	Gly	3.0	Gly	Asn	3.5	Asn
11	Ala	3.0	Ala	Thr	1.5	Thr
12	Tyr	2.0	Tyr	Thr	1.5	Thr
13	Gly	2.3	Gly			Cys
14	Met	0.8	Met	Asp	1.0	Asp
15	Val	1.2	Val	Leu	2.0	Leu
16	Ser	1.1	Ser	Lys	0.5	Lys
17	Ser	1.1	Ser	Ile	1.3	Ile
18	Ala	1.6	Ala			Cys
19	Tyr	1.2	Tyr	Asp	0.3	Asp
20	Asp	0.9	Asp			Phe
21	His	0.4	His			
22	Val	0.4	Val			
23			Arg			

[a] Yields from successive cycles of automated Edman degradation of the phenylthiohydantoin derivatives are listed next to the corresponding amino acid described at each position in peptide 15 (A) and 20 (B). Yields were calculated by comparison of the heights of chromatographic peaks for each residue, measured on the basis of absorption at 269 nm, with the corresponding peak heights for quantitative external standards, following background subtraction. [b] Predicted by the cDNA in Boulton et al. (1990a).

In addition to the fact that the activity migrates with the 43-kDa band, further evidence that this band is the kinase comes from the sequence of two peptides recovered following tryptic digestion of the 43-kDa band from the same preparation as shown in lane 9 of Figure 6. Peptide A (Table II) contains a consensus sequence for nucleotide binding proteins typical of protein kinases (GEGAYG). Peptide B contains a sequence characteristic of serine/threonine protein kinases (DLKPSN). The presence of both of these peptides in the purified protein is strong evidence that it is a protein kinase. Degenerate oligonucleotides to these sequences were used in a polymerase chain reaction based strategy to clone the enzyme ERK1 (Boulton et al., 1990a). The ERK1 cDNA predicts a protein of at least 42 kDa with all of the conserved residues expected for a serine/threonine kinase. The sequences predicted by the cDNA are compared to the tryptic peptide sequence in Table II.

The specific activity of the kinase with MAP2 as substrate increased from 125 pmol·min^{-1}·mg^{-1} in supernatants to 800 nmol·min^{-1}·mg^{-1} for the purified enzyme, corresponding to a purification of over 6300-fold (Table I). Both serine and threonine were phosphorylated in MAP2 (P-Ser, 85%; P-Thr,

Table III: Phosphorylation of Substrates by ERK1 and Effects of Various Agents[a]

substrate	% MAP2 phosphorylation
MAP2	100
+ PKI	91
+ 0.1 μM Ca^{2+}	101
+ 1 μM Ca^{2+}	96
+ 10 μM Ca^{2+}	100
+ 100 μM Ca^{2+}	98
+ 0.5 mM EDTA	96
+ 1 μg/mL heparin	102
+ 10 μg/mL heparin	101
+ 100 μg/mL heparin	95
+ 10 μM GTP	105
+ 100 μM GTP	108
+ 30 μg/mL protamine	152
+ 200 μg/mL protamine	133
+ 100 μM quercetin	69
+ 1 mM quercetin	10
+ 1 μM isobutylmethylxanthine	115
+ 50 μM isobutylmethylxanthine	89
+ 10 μM 2-amino-6-mercaptopurine	140
+ 200 μM 2-amino-6-mercaptopurine	105
+ 1 mM 2-aminopurine	92
+ 10 mM 2-aminopurine	42
+ 100 μM chloroquine	66
+ 0.1 μM staurosporine	77
+ 1 μM staurosporine	33
histone	
2AS	0.6
5S	5.5
7S	1.3
6S	0.4
8S	3.5
7S + PKI	1.7
casein	2.1
phosvitin	0.5
cardiac myosin light chains	7.0
ATP–citrate lyase	0
myelin basic protein	357
protamine	0
S6	0.07

[a] Fraction 12 from the final Q-Sepharose was assayed as under Materials and Methods in the presence of 0.3 mg/mL of each substrate with the exception of 40S subunits (S6), ATP–citrate lyase, and cardiac myosin light chains. S6 was assayed as under Materials and Methods. ATP–citrate lyase and cardiac light chains were present at approximately 0.6 mg/mL. Histone nomenclature is that of Johns (1964), 5S is type 1, 7S is type 2b, 6S is type 2a, and 8S is type 3. PKI is the protein kinase inhibitor protein.

15%). With myelin basic protein as substrate the specific activity was 3 $\mu mol \cdot min^{-1} \cdot mg^{-1}$, corresponding to a turnover number of 130 min^{-1}. The enzyme was saturated with myelin basic protein under these assay conditions. The amino acid phosphorylated in myelin basic protein was threonine (P-Thr ≥ 96%).

Unlike many enzymes, ERK1 is nearly as active with Mn^{2+} as with Mg^{2+}. The activity with 1 mM Mn^{2+} is 70% and with 10 mM Mn^{2+} it is 35% of the activity with 10 mM Mg^{2+}. The kinase shows a marked preference for ATP over GTP (Table III). Interestingly, 1 μM isobutylmethylxanthine weakly stimulated MAP2 kinase activity (Table III). In addition, 10 μM 2-amino-6-mercaptopurine also stimulated activity by 40%. This distinguishes ERK1 from a nerve growth factor stimulated kinase described earlier (Volonté et al., 1989).

Other proteins, including ribosomal protein S6, ATP–citrate lyase, histones, casein, phosvitin, and protamine, which serve as substrates in vitro for a number of protein kinases, were phosphorylated very slowly or not at all by ERK1 (Table III). Kinesin, myosin I, hsc 90, and hsc 70 were also not substrates. Cardiac myosin light chains were phosphorylated at 7% of the rate of phosphorylation of MAP2. Neither the protein kinase inhibitor of the cAMP-dependent protein kinase nor Ca^{2+} (0.1–100 μM) had a significant effect on the kinase activity of ERK1. The latter result distinguishes this enzyme from a MAP kinase described by Hoshi et al. (1988) that is inhibited by low concentrations of calcium. The polycation, protamine, has a small stimulatory effect on the phosphorylation of MAP2 by the enzyme, but the polyanion, heparin, has no effect on the phosphorylation of MAP2. To facilitate the comparison of this purified MAP2 kinase to other enzymes, several compounds known to inhibit protein kinases were also tested. Quercetin (Cochet et al., 1982) was required at high concentrations (100 μM–1 mM) to inhibit activity, as was staurosporine (1 μM), a very potent inhibitor of cAMP-dependent protein kinase, protein kinase C, and myosin light chain kinase (K_is, 0.7–7 nM; Tamaoki et al., 1986).

Discussion

We have purified ERK1, an insulin-stimulated MAP2 kinase, from high-speed supernatants of insulin-treated rat 1 HIRc B cells by over 6300-fold with a recovery of 3.8% to a final specific activity of 800 $nmol \cdot min^{-1} \cdot mg^{-1}$ with MAP2 and 3 $\mu mol \cdot min^{-1} \cdot mg^{-1}$ with myelin basic protein. The turnover number of 130 min^{-1} compares well with turnover numbers of other purified protein kinases; for example, the turnover number of the purified catalytic subunit of the cAMP-dependent protein kinase ranges from 320 to 800 min^{-1} (Edelman et al., 1987), that of a purified S6 kinase is approximately 42 min^{-1} (Jenö et al., 1989), and that of the insulin receptor kinase is ~3–10 min^{-1} (Cobb et al., 1989). The conclusion that the 43-kDa band that we have identified is the insulin-responsive kinase is also supported by the observation that the enzyme contains phosphotyrosine and that it is inactivated >90% by phosphatase 2a and >50% by CD45.[2] These properties have been reported for stimulated MAP2 kinases from other sources (Ray & Sturgill, 1988a; Sturgill et al., 1988; Ahn et al., 1990). The major protein in our preparation is a protein kinase as shown by its partial amino acid sequence. The amino acid sequences of the tryptic peptides derived from the 43-kDa band contain consensus sequences highly conserved among serine/threonine protein kinases (Hanks et al., 1988). These sequences encompass a part of the nucleotide binding domain, including the GEGAYG sequence, and another region with the sequence DLKPSN, corresponding to residues 166–171 in cAMP-dependent protein kinase. On the other hand, none of the sequences are identical with protein kinases or other proteins already in the GenBank data base. A cDNA encoding this MAP2 kinase has recently been isolated and sequenced (Boulton et al., 1990a). All of the tryptic peptides derived from the 43-kDa band are contained within the predicted translation product of the cDNA (Boulton et al., 1990a). The tryptic peptides account for ~30% of a 43-kDa protein and are identical in the protein encoded by the cDNA.

At least three other names have been proposed for similar enzymes. We named the cloned enzyme ERK1 (extracellular signal-regulated kinase 1), instead of using one of the other names, for the following reasons. ERK1 may be one of a family of four or more closely related kinases, three of which we have already sequenced (Boulton et al., 1990a).[2] The substrate specificity for the other members of this group may not be the same as that of ERK1. We expect to be able to identify more of these enzymes prior to knowing what they do. Thus, the names MAP2 or myelin basic protein kinase, or even S6 kinase kinase, may be misleading. We did not choose another proposed name, mitogen-activated protein

[2] Unpublished data.

kinase, because extracellular signals other than mitogens (i.e., nerve growth factor and insulin) also activate ERK1. Thus, we named the enzymes of this family ERKs to reflect a general mechanism for their activation.

The specific activity of insulin-stimulated ERK1 is nearly 4-fold higher than that of a myelin basic protein kinase recently purified from sea star oocytes by Sanghera et al. (1990). The sea star enzyme has some similarities to the insulin-stimulated enzyme, including molecular weight and chromatographic properties. However, there are modest differences in substrate specificity. There have also been reports on a partially purified preparation of MAP2 kinase from 3T3-L1 cells (Ray & Sturgill, 1987, 1988a,b). The insulin-stimulated kinase purified here has many properties, including chromatographic behavior, substrate specificity, reactivity with antibodies to phosphotyrosine, and ability to activate an S6 kinase, quite similar to the partially purified 3T3-L1 enzyme. Thus, we tentatively conclude that these activities arise from the same enzyme. Activities with similar properties have also been identified in NGF-treated PC12 cells (Tsao et al., 1990; Miyasaka et al., 1990)[2] and *Xenopus* eggs (Ferrell & Martin, 1990). To the best of our knowledge this report describes for the first time the purification of this enzyme to near homogeneity.

With respect to substrate specificity, a phosphorylatable sequence, Pro-(Arg/Lys)-(Ser/Thr)-Pro, occurs in both myelin basic protein (Chan et al., 1987) and in MAP2 (Lewis et al., 1988). In myelin basic protein the only sequence of this type contains threonine whereas in MAP2 both serine and threonine occur in this motif. Phosphorylation of this type of site would be consistent with the phosphoamino acid analyses of the two proteins. Thus, we suggest the possibility that ERK1 may phosphorylate this sequence.

It has been proposed that the insulin-dependent activation of an enzyme originally identified as a MAP2 protein kinase enables this enzyme to phosphorylate and thereby activate an S6 protein kinase (Sturgill et al., 1988). Consistent with this hypothesis, we have observed the following: (1) The insulin-regulated ERK1 has the ability to activate a rabbit liver S6 protein kinase in vitro (Gregory et al., 1989). (2) In this study we have determined that the rat 1 cell overexpressing the human insulin receptor acquires the ability, which it lacked prior to transfection, to respond to insulin with an increase in both S6 and MAP2 protein kinase activities. (3) An insulin receptor hybrid containing the transmembrane and cytoplasmic domains of the $PP68^{gagros}$ transforming protein also elicits increases in both S6 and MAP2 kinase activity in response to insulin (Boulton et al., 1990b). In contrast, an earlier study concluded that this chimeric receptor was unable to enhance either glucose uptake or thymidine incorporation in response to insulin (Ellis et al., 1987). Taken together these data are in agreement with the model of Sturgill et al. (1988) that stimulation of MAP2 kinase activity by insulin or by other agents (Rossomando et al., 1989; Boulton et al., 1990b) precedes and is required for the activation of an S6 kinase in the cascade of reactions leading to increased S6 phosphorylation. If this model is correct, elucidating the mechanisms of regulation of the MAP2 kinase, ERK1, will be important in order to delineate the pathway of activation of S6 phosphorylation by insulin. The purified ERK1 will be an instrumental reagent for use in identifying the kinase(s) upstream in this regulatory pathway.

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Catalytic Cooperativity Induced by SH_1 Labeling of Myosin Filaments†

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ABSTRACT: Modifications of SH_1 groups on isolated myosin subfragment 1 (S-1) and myosin in muscle fibers affect differently the acto-S-1 ATPase and the fiber properties. Consistent with the findings of earlier work on fibers, the modification of SH_1 groups in relaxed myofibrils with phenylmaleimide caused a loss of their shortening. This loss paralleled the decrease in the V_{max} of extracted myosin but was not linear with the extent of SH_1 labeling. Strikingly, the decrease in V_{max} of S-1 prepared from the modified myofibrils was directly proportional to the extent of SH_1 labeling. The specificity of SH_1 labeling in myofibrils was verified by ATPase activities, thiol titrations, radiolabeling experiments, and comparisons to myosin labeled on SH_1 in solution. To test for intermolecular interactions in the myosin filaments and their contribution to the differences between S-1 and myosin, the catalytic properties of copolymers of myosin were examined. Copolymers of myosin and rod minifilaments were formed in 5 mM citrate-Tris (pH 8.0) buffer, and their homogeneity was verified by sedimentation velocity analysis. The inhibition of actomyosin ATPase by rod particles was related to the decrease in the K_m value. When rod particles were replaced in these minifilaments by SH_1-modified myosin, the ATPase of the copolymers was increased over that of the combined ATPases of the individual filaments. The actomyosin ATP turnover rates on the unmodified heads were increased severalfold by the modified heads. These results demonstrated that the catalytic function of myosin heads in the filaments is affected by the interactions of vicinal myosin molecules with actin. The results also suggest that SH_1 modification of myosin may impede its ability to undergo a normal crossbridge cycle.

Muscles produce force by cyclic crossbridge interactions between actin and myosin which are coupled to the hydrolysis of ATP. Understanding these processes depends on the coordination of studies done on muscle fibers and the work on solubilized muscle proteins and protein complexes. Mechanisms of ATP hydrolysis have been investigated extensively in solution (Sleep et al., 1981), and measurements in fibers and myofibrils are consistent with these mechanisms (Sleep, 1981; Glyn & Sleep, 1985). Foundations for the understanding of actomyosin interaction in fibers were laid by detailed solution investigations on actin and myosin [for a review, see Cooke (1986)]. Although the elucidation of the possible changes in crossbridge structure and orientation during contraction depends on the analysis of fiber experiments, these studies are linked to solution work as well. Understanding the effects of introducing probes to monitor changes in crossbridges must rely on characterization in solution of the chemical reaction and the labeled proteins it generates.

The most popular probe site on myosin has been the reactive SH_1 group on the myosin head. The effects of modifications of sulfhydryl groups on myosin subfragment 1 (S-1)[1] are well characterized (Sekine & Kielley, 1964; Silverman et al., 1972; Mulhern et al., 1975; Mulhern & Eisenberg, 1976, 1978). When the most reactive thiol, SH_1, is specifically labeled, the K^+ (EDTA) ATPase and actin-activated ATPase are abolished, while the basal Mg^{2+} and Ca^{2+} ATPases are elevated (Sekine & Kielley, 1964; Mulhern & Eisenberg, 1976). The inhibition of acto-S-1 ATPase activity by labeling SH_1 is

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[1] Abbreviations: DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; HMM, heavy meromyosin; kDa, kilodalton(s); NEM, *N*-ethylmaleimide; OD, optical density; pPDM, *p*-phenylenedimaleimide; PM, phenylmaleimide; S-1, myosin subfragment 1; SDS, sodium dodecyl sulfate.

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