Characterization of Fodrin Phosphorylation by Spleen Protein Tyrosine Kinase[†]

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ABSTRACT: Fodrin, an actin and calmodulin binding and spectrin-like protein present in many nonerythrocyte tissues, could be phosphorylated up to more than 1.5 mol of phosphate/mol of protein by a highly purified non-receptor-associated protein tyrosine kinase from bovine spleen. The protein phosphorylation was not affected by Ca^{2+}/calmodulin or by F-actin. K_m and V_{max} values of the reaction were 91 nM and 0.35 nmol of P_2 min^{-1} (mg of kinase)$^{-1}$, respectively. Both subunits A and B of fodrin were phosphorylated, with the rate of subunit A phosphorylation much greater than that of subunit B phosphorylation. Tryptic phosphopeptide mapping of the phosphorylated subunits suggested that there were three major phosphorylation sites in subunit A and one in subunit B. Phosphotyrosylfodrin could be dephosphorylated by the calmodulin-stimulated phosphatase (calcineurin) in the presence of activating metal ions; Ni^{2+} was a much more effective activator than Mn^{2+} for this reaction. Fodrin phosphorylation by the spleen protein tyrosine kinase did not appear to alter the actin and calmodulin binding properties of the protein. On the other hand, the calmodulin-dependent stimulation of smooth muscle actomyosin Mg^{2+}-ATPase by fodrin was enhanced by 101% ± 3% ($n = 3$) upon fodrin phosphorylation. Ni^{2+}–calcineurin, which was shown to effectively dephosphorylate the phosphotyrosyl residues on fodrin, could reverse the phosphorylation-enhanced Mg^{2+}-ATPase stimulatory activity of fodrin.

Oncogene products of certain retroviruses (Cooper & Hunter, 1983) or membrane receptors of many growth factors (Ek et al., 1982; Ushiro & Cohen, 1980; Roth & Cassell, 1983) or growth-related hormones (Rubin et al., 1983) possess protein tyrosine kinase activities. The observation suggests that protein tyrosine kinase phosphorylation may play important roles in the control of cell growth. Normal uninfected vertebrate cells also contain protein tyrosine kinases which do not appear to be associated with membrane receptors. These protein kinases may also be, at least in part, involved in the regulation of cell growth. The question of how protein tyrosine kinases exert their regulatory actions toward cell proliferation has been under intensive study, but the answer is far from clear [for a review, see Sefton and Hunter (1984)]. A number of cellular proteins including certain membrane-associated proteins (Gallis et al., 1981), cytoskeletal proteins (Sefton et al., 1981; Akiyama et al., 1986), and glycolytic enzymes (Cooper et al., 1983) have been identified as substrates of viral and receptor-associated protein tyrosine kinases. However, it is not known how tyrosine phosphorylation affects the activities and functions of these proteins. In fact, the functions of many of these substrate proteins are not well established.

Due to their low abundancies, non-receptor-associated protein tyrosine kinases of uninfected cells are much less studied than the other protein tyrosine kinases. In addition, cellular protein substrates of these protein kinases are not known. Swarup et al. (1983) have surveyed many rat tissues and found that spleen has the highest protein tyrosine kinase activity. We have recently purified a protein tyrosine kinase from bovine spleen to about 40% purity (Kong & Wang,

1987). The preparation contains no detectable protein serine/threonine kinase and protein tyrosine phosphatase activities and therefore appears sufficiently pure for enzymological characterizations. During the purification, the protein tyrosine kinase activity was assayed by using the peptide substrate angiotensin II.

In an attempt to identify cellular protein substrates of this protein tyrosine kinase, membrane fractions of bovine spleen and brain have been phosphorylated by the purified kinase, and alkaline-resistant phosphopeptides are identified on the autoradiograms of an alkaline-treated sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE)[1] gel. The result shows that fodrin may be one of the proteins phosphorylated by the spleen protein tyrosine kinase. This phosphorylation reaction has been characterized by using purified bovine brain fodrin. Very recently, fodrin has been shown to stimulate smooth muscle actomyosin Mg^{2+}-ATPase in a Ca^{2+}- and calmodulin-dependent manner (Wang et al., 1987a). Results of the present study show that phosphorylation of fodrin by the spleen protein tyrosine kinase enhances the actomyosin Mg^{2+}-ATPase stimulating activity of fodrin.

EXPERIMENTAL PROCEDURES

[γ-^{32}P]ATP (10 Ci/mmol) was purchased from Amersham Corp. Bovine brain fodrin, calmodulin, and calcineurin were purified as described previously (Wang et al., 1987a; Sharma & Wang, 1979; Sharma et al., 1983). Bovine spleen protein tyrosine kinase was purified to about 40% purity by the procedure of Kong and Wang (1987). Rabbit skeletal muscle

[1] Abbreviations: ATPγS, adenosine 5'-O-(3-thiotriphosphate); PMSF, phenylmethanesulfonyl fluoride; SDS, sodium dodecyl sulfate; DTT, DL-dithiothreitol; βME, β-mercaptoethanol; PAGE, polyacrylamide gel electrophoresis; EGTA, ethylene glycol bis(β-aminoethyl ether)-N,N,-N',N'-tetraacetic acid; MLCK, myosin light-chain kinase; TPK, tyrosine protein kinase; CaN, calcineurin; PNPP, p-nitrophenyl phosphate; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; kDa, kilodalton(s).

actin, chicken gizzard myosin, tropomyosin, and myosin light-chain kinase were purified following established procedures (Zot & Potter, 1981; Persechini & Hartshorne, 1981; Bertscher, 1984; Ngai et al., 1984). Myosin was stored on ice and not used beyond 3 weeks of preparation. The actin-activated ATPase activity of gizzard myosin undergoes a slow loss of activity over this period, so the absolute ATPase rates obtained in experiments conducted at different times cannot be compared directly. The preparation of thiophosphorylated myosin was performed as described by Wang et al. (1987a).

Preparation of Membrane-Associated Proteins. Bovine brain was homogenized in buffer (15 × v) containing 20 mM Tris-HCl, pH 7.5, 1 mM PMSF, 5 mM EGTA, 15 mM βME, and 1 mM o-phenanthroline and sedimented at 150000g for 1 h at 4 °C. In order to obtain most peripheral membrane-associated proteins, the pellet was first extracted in low ionic strength buffer (15 × v) containing 20 mM Tris-HCl, pH 7.5, 1 mM PMSF, 15 mM βME, and 0.1 M KCl and centrifuged at 150000g for 1 h at 4 °C. The resulting pellet was then reextracted in high ionic strength buffer (15 × v) containing 20 mM Tris-HCl, pH 7.5, 1 mM PMSF, 15 mM βME, and 0.6 M KCl and centrifuged at 150000g for 1 h at 4 °C. Supernatants from both extractions were combined, concentrated, and dialyzed against buffer containing 20 mM Tris-HCl, pH 7.5, 1 mM PMSF, 15 mM βME, and 0.3 M KCl and used in tyrosine phosphorylation experiments.

Electrophoresis and Autoradiography. Electrophoresis was performed in 5–20% polyacrylamide gradient slab gels (1.5 mm thick) with 4.5% acrylamide stacking gels, in the presence of 0.1% SDS using the buffer system of Laemmli (1970). Electrophoresis through the stacking gel was performed at 25 mA and the separating gel at 50 mA. Gels were stained in 40% (v/v) ethanol and 10% (v/v) acetic acid containing 0.06% Coomassie Brilliant Blue R-250 and destained in 20% (v/v) methanol and 10% (v/v) acetic acid. For autoradiography, gels were dried on an LKB-Bromma Gel drier at 60 °C for 1 h and then exposed to Kodak X-Omat AR film in Kodak X-Omatic cassettes at −70 °C.

Phosphorylation Assays. The standard phosphorylation mixture (45 μL) contained fodrin and protein tyrosine kinase (at concentrations as indicated in the figure legends) in 50 mM Tris-HCl, pH 7.5, 50 mM MgCl$_2$, 1 mM DTT, 0.1% NP-40, and 1.5% glycerol. Phosphorylation was initiated by adding 5 μL of 1 mM [γ-^{32}P]ATP (500–2000 cpm/pmol) to a final concentration of 0.1 mM and incubated at 30 °C for the desired length of time. The reaction was terminated with the addition of 50 μL of 2-fold-concentrated Laemmli sample buffer (62.5 mM Tris-HCl, pH 6.8, 20% glycerol), 2% SDS, 4% βME, and 0.002% Bromophenol Blue). The samples were boiled for 5 min and then analyzed on SDS–PAGE gel followed by autoradiography. Quantitation of the phosphate incorporation was determined by counting the radioactivities of phosphoprotein gel slices.

Calcineurin–Phosphatase Assays. Fodrin (200 μg/mL) was phosphorylated by spleen protein tyrosine kinase (10 μg/mL) at 30 °C for 2.5 h. Unlabeled ATP (5 mM) and bovine serum albumin (0.5 mg/mL) were added to the phosphorylation mixture immediately before it was applied on a Sephadex G-50 (fine, 0.8 × 26 cm) column for the removal of free ATP. Calcineurin (150 μg/mL) used for the dephosphorylation reaction was activated by incubating with 1 mM Ni^{2+} or Mn^{2+} plus 100 μg/mL calmodulin at 30 °C for 1 h prior to its addition to initiate the protein dephosphorylation (Pallen et al., 1985). Dephosphorylation reactions contained proteins (500–2000 cpm), 20 mM Tris-HCl, pH 7.5, 250 μM Ni^{2+} or

Mn^{2+}, 0.1 mM Ca^{2+}, and 25 μg/mL calmodulin. The dephosphorylation reactions were initiated by the addition of 20 μg/mL activated calcineurin and terminated by the addition of an equal volume of 2-fold-concentrated Laemmli sample buffer. The mixtures were boiled for 5 min and analyzed on a 5–20% SDS–PAGE gel. The ^{32}P remaining bound to proteins was determined by counting the radioactivities of excised gel slices.

Phosphoamino Acid Analysis and Phosphopeptide Mapping. Fodrin was phosphorylated for 2 h at 30 °C under the conditions described previously and then subjected to SDS–PAGE. The polypeptide band corresponding to fodrin was excised, washed thoroughly with 50% methanol, and treated with 1–2 mL of a 50 μg/mL trypsin solution in 50 mM ammonium bicarbonate, pH 8.0, for 24 h at 30 °C. The gel pieces were removed by centrifugation at 5000g for 5 min. For phosphoamino acid analysis, half of the supernatant was heated in the presence of 6 N HCl and phosphoserine, phosphothreonine, and phosphotyrosine standards for 2 h at 110 °C. The sample was then lyophilized, dissolved in 20 μL of buffer (acetic acid/pyridine/H$_2$O, 10:1:189), and run on a thin-layer phosphocellulose electrophoresis plate at 1000 V for 45 min in the same buffer. For phosphopeptide mapping, the remaining supernatant was lyophilized and dissolved in 30 μL of buffer (acetic acid/pyridine/H$_2$O, 10:1:89). The first-dimension analysis was performed by running the sample on a thin-layer electrophoresis plate in the same buffer at 1000 V for 1.5 h; the second-dimension analysis was performed by subjecting the plate to ascending chromatography in buffer containing pyridine/1-butanol/acetic acid/H$_2$O (15:10:3:12) for 4 h. For both phosphoamino acid and phosphopeptide analyses, the plates were dried and autoradiographed.

Fodrin Stimulation of Smooth Muscle Actomyosin Mg^{2+}-ATPase. The basic actomyosin Mg^{2+}-ATPase assay was carried out in a reaction mixture containing actin, thiophosphorylated myosin, tropomyosin, and calmodulin at final concentrations of 0.25 mg/mL, 0.44 mg/mL, 50 μg/mL, and 10 μg/mL, respectively. Reaction mixtures were preincubated at 30 °C for 2 min in buffer (20 mM Tris-HCl, pH 7.5, 60 mM KCl, 1 mM DTT, 10 mM MgCl$_2$, and 0.1 mM Ca^{2+}) before initiation of the reaction by the addition of [γ-^{32}P]ATP to a final concentration of 1.0 mM. Aliquots were withdrawn at selected times for quantitation of [^{32}P]P$_i$ release as previously described (Ikebe & Hartshorne, 1985). In order to demonstrate the fodrin stimulation of smooth muscle actomyosin Mg^{2+}-ATPase, fodrin (3 μg/mL) was added to the basic reaction mixture and preincubated for 10 min at 30 °C before the addition of [γ-^{32}P]ATP (Wang et al., 1987). Under these assay conditions, the phosphate production showed an initial burst which was followed by a linear time course lasting for at least 10 min. The rate of ATPase reaction was calculated from the slope of the linear time course assay.

RESULTS

Phosphorylation of Fodrin by Spleen Protein Tyrosine Kinase. The membrane-associated proteins obtained by extracting a bovine brain membrane fraction with both low and high ionic strength buffers (see Experimental Procedures) were phosphorylated by the purified spleen protein tyrosine kinase (Kong & Wang, 1987) in the presence of [γ-^{32}P]ATP. Similar to the phosphorylation of angiotensin II (Kong & Wang, 1987), the phosphorylation of protein substrates by the spleen protein tyrosine kinase also depends on high concentration of MgCl$_2$; the maximal enzyme activity was observed at close to 50 mM (data not shown). The phosphorylated sample was subjected to 5–20% SDS–PAGE followed by incubating the
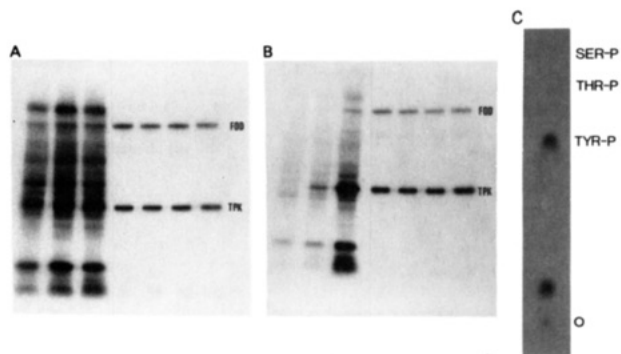
FIGURE 1: Phosphorylation of fodrin by spleen protein tyrosine kinase. (A) Autoradiogram of phosphorylated membrane-associated protein fraction and purified fodrin from bovine brain. Membrane-associated protein fraction (171 μg/mL) was phosphorylated in the absence (lane 1) or presence of 2.9 μg/mL (lane 2) or 8.7 μg/mL (lane 3) exogenously added spleen protein tyrosine kinase for 30 min at 30 °C. The phosphorylation was carried out in a buffer system containing 50 mM Tris-HCl, pH 7.5, 50 mM MgCl$_2$, 0.1% NP-40, 1.5% glycerol, 7 mg/mL PNPP, 2.5 mM VO$_3^{4-}$, and 1 mM NaF. The reaction was terminated by the addition of 2-fold-concentrated Laemmli sample buffer and analyzed on a 5–20% SDS–PAGE gel. In addition, purified fodrin (30 μg/mL) was phosphorylated by the kinase (2.9 μg/mL) in the presence of 0.1 mM Ca^{2+} (lane 4), 0.1 mM Ca^{2+} and 10 μg/mL calmodulin (lane 5), 1 mM EGTA (lane 6), and 1 mM EGTA and 10 μg/mL calmodulin (lane 7). (B) Autoradiogram of alkaline-treated (A). Duplicate SDS–PAGE gel of (A) was treated with 1 N KOH at 55 °C for 2 h to remove alkaline-sensitive phosphopeptides. (C) Autoradiogram of acid-hydrolyzed phosphorylated fodrin on thin-layer electrophoresis. Abbreviations: SER-P, phosphoserine; THR-P, phosphothreonine; TYR-P, phosphotyrosine; O, origin; TPK, tyrosine protein kinase; FOD, fodrin.

SDS–PAGE gel in 1 N KOH at 55 °C for 1 h to hydrolyze most of the phosphoserine and phosphothreonine residues. The KOH-treated gel was then autoradiographed to reveal the alkaline-resistant phosphopeptides which were suggested to contain mainly phosphotyrosyl proteins (Cooper & Hunter, 1981). Figure 1B shows that there are several alkaline-resistant phosphopeptides in this sample in addition to the 50-kDa phosphopeptide which corresponds to the spleen protein tyrosine kinase. One of these phosphopeptides appears to have electrophoretic characteristics identical with those of fodrin (Figure 1B, lanes 3 and 4). Fodrin purified from both bovine brain and spleen tissues can be phosphorylated by spleen protein tyrosine kinase. Due to the low abundancy of fodrin in bovine spleen (data not shown), brain fodrin was used for the further characterization of the phosphorylation reaction. Phosphoamino acid analysis of fodrin phosphorylation by the spleen protein tyrosine kinase showed that the phosphorylation was confined exclusively to tyrosine residues (Figure 1C). Although fodrin is a calmodulin binding protein, the phosphorylation of fodrin was not altered by binding to calmodulin (Figure 1A,B, lanes 4–7). Similarly, when fodrin was subjected to conditions which favor F-actin binding, the phosphorylation by the spleen protein tyrosine kinase was found to be unaffected (Figure 2). Since the protein tyrosine kinase was isolated from the particulate fraction of bovine spleen, we have also considered the possibility that the kinase might bind to F-actin to result in a change in the enzyme activity. When casein, an in vitro substrate of the kinase known not to specifically interact with actin, was included in the reaction mixture, the phosphorylation of casein by the protein tyrosine kinase was not affected by the presence of F-actin (Figure 2). In addition to fodrin and casein, actin was also phosphorylated by the spleen protein tyrosine kinase. However, the stoichiometry of actin phosphorylation under this condition was very low.
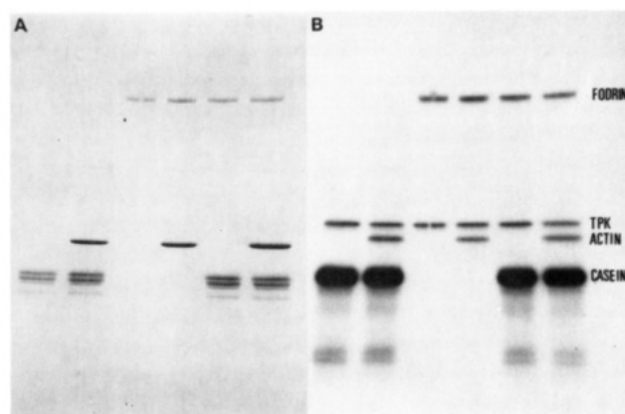

FIGURE 2: Effect of F-actin on phosphorylation of fodrin by spleen protein tyrosine kinase. Fodrin (80 μg/mL) was preincubated at 30 °C for 30 min with (lanes 4 and 6) or without (lanes 3 and 5) 400 μg/mL actin in the actin binding buffer (20 mM Tris-HCl, pH 7.5, 1 mM DTT, 5 mM MgCl$_2$, and 0.1 M KCl). The concentration of MgCl$_2$ for the reaction mixture was adjusted to 50 mM immediately before the phosphorylation reaction was initiated by the addition of tyrosine kinase and 0.1 mM [γ-^{32}P]ATP and carried on for 30 min at 30 °C. Casein (800 μg/mL), a non-actin-binding protein, was added as control (lanes 1, 2, 5, and 6). The reaction products were analyzed by 5–20% SDS–PAGE. (A) Coomassie Blue stained gel; (B) corresponding autoradiogram.

Stoichiometry of Fodrin Subunit Phosphorylation. To determine the stoichiometry of fodrin phosphorylation by spleen protein tyrosine kinase, complete time courses of fodrin phosphorylation were carried out at two different protein tyrosine kinase concentrations (data not shown). At low protein tyrosine kinase concentration (2.9 μg/mL), the phosphorylation of fodrin increased slowly to 0.8 mol of P$_i$/mol of fodrin dimer at 150 min. When 8.7 μg/mL protein tyrosine kinase was used in the assay system, there was an initial rapid phase of phosphorylation followed by a more gradual phosphate incorporation to a level of 1.6 mol of P$_i$/mol of fodrin dimer. In both cases, the time courses for fodrin phosphorylation revealed that there was still slow, ongoing phosphate incorporation after 150 min.

Since fodrin is composed of two subunits with slightly different molecular weights (A = 240 000 and B = 235 000), a 5% SDS–PAGE gel was used to resolve the two subunits in order to determine the phosphorylations of the individual subunits. Figure 3 shows the time courses of the subunit phosphorylation. The A subunit was phosphorylated much more rapidly than the B subunit, and the phosphate incorporation into the A subunit was more than 3-fold higher than that of the B subunit at 150 min. When the sites of phosphorylation in each subunit were examined by two-dimensional mapping of the phosphopeptides generated by tryptic digestion, multiple phosphorylation sites were observed for both subunits. The A subunit contained three major phosphopeptides while the B subunit contained only one major and a few minor phosphopeptides (Figure 4). The multiple phosphopeptides generated by tryptic digestion suggested that these sites were only partly phosphorylated. The possibility that purified fodrin may be partially phosphorylated was considered and tested by pretreating fodrin with phosphatase (see next section).

Kinetic studies were carried out by phosphorylating purified fodrin at concentrations ranging from 126 to 735 nM with 23 nM protein tyrosine kinase. The phosphorylation reactions were carried on for 30 min at 30 °C, and phosphate incorporations were calculated from counting SDS–PAGE gel slices containing ^{32}P-bound fodrin. Lineweaver–Burk plot of this experiment revealed that the K_m of fodrin phosphorylation was
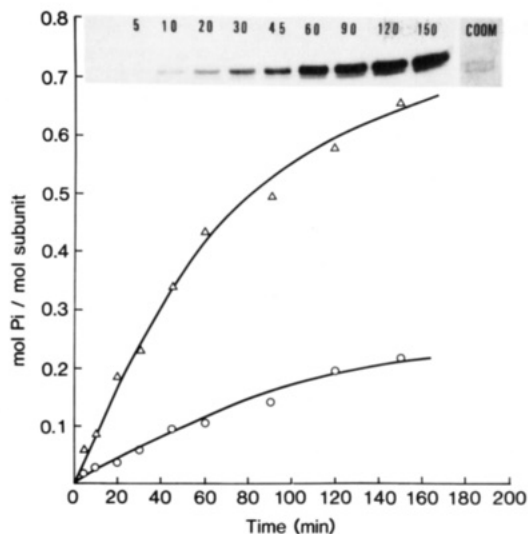
FIGURE 3: Time courses of phosphorylation of fodrin subunits. Purified fodrin (30 μg/mL) was subjected to phosphorylation with 2.9 μg/mL spleen protein tyrosine kinase under the conditions described under Experimental Procedures. At indicated times, the reaction was terminated and analyzed by SDS–PAGE followed by autoradiography (see inset). A and B subunits were excised separately according to their Coomassie Blue stained positions and counted in the scintillation counter. Phosphate incorporations into the A and B subunits were expressed as moles of phosphate per mole of subunit. (△) A subunit of fodrin; (○) B subunit of fodrin. Coom, Coomassie Blue stained gel pattern.

91 nM (data not shown). This K_m value is much lower than those reported previously for the phosphorylation of fodrin by other protein tyrosine kinases (Kadowaki et al., 1985; Akiyama et al., 1986). The V_{max} was calculated to be 0.35 nmol of P_i min^{-1} (mg of kinase)$^{-1}$. Although this value is much lower than the V_{max} of angiotensin II phosphorylated by this kinase, it is similar to the V_{max}'s reported for protein substrates phosphorylated by a number of other protein tyrosine kinases (Akiyama et al., 1986).

Dephosphorylation of Phosphotyrosylfodrin by Calcineurin. Fodrin, phosphorylated by the spleen protein tyrosine kinase, could be dephosphorylated by the calmodulin-dependent phosphatase (calcineurin). As shown in Figure 5, the protein was preferentially dephosphorylated by the Ni^{2+}-activated form of calcineurin; more than 75% of the phosphate was removed by Ni^{2+}–calcineurin within 20 min whereas only 30% of the phosphate was removed by Mn^{2+}–calcineurin. The auto-phosphorylated protein tyrosine kinase was only slightly de-phosphorylated by either form of calcineurin with a maximal dephosphorylation of 25%. The possibility that fodrin may

Table I: Effects of Phosphorylation on Smooth Muscle Actomyosin Mg^{2+}-ATPase Stimulatory Activity of Fodrin

reaction mixtures	ATPase rate[d] [nmol of P_i (mg of myosin)$^{-1}$ min^{-1}]
control[a]	57.8
control + tyrosine kinase[b]	55.0
control + fodrin (3 μg/mL)	84.2
control + fodrin[c]	81.1
control + phosphorylated fodrin	113.3

[a] Actomyosin Mg^{2+}-ATPase mixture containing actin, thio-phosphorylated myosin, tropomyosin, and calmodulin at final concentrations and conditions as described under Experimental Procedures, was preincubated for 10 min at 30 °C before the ATPase reaction was initiated by the addition of 1.0 mM [γ-^{32}P]ATP. [b] Tyrosine kinase (2.9 μg/mL) was autophosphorylated for 2 h at 30 °C prior to its addition to the ATPase mixture to a final concentration of 0.29 μg/mL. [c] Fodrin (30 μg/mL) was preincubated under phosphorylation conditions in the absence of tyrosine kinase for 2 h at 30 °C prior to its addition to the ATPase mixture to a final concentration of 3 μg/mL. [d] The actomyosin Mg^{2+}-ATPase rates were calculated from the linear time course assays with linear regression values (r) ranging from 0.993 to 1.000.

be partially phosphorylated at tyrosine residues upon isolation could not be examined by pretreating fodrin with Ni^{2+}–calcineurin prior to protein tyrosine kinase phosphorylation. It was found that the level of phosphate incorporated for pre-treated fodrin was the same as that for nontreated fodrin, suggesting that purified fodrin was not partially phosphorylated at tyrosine residues (data not shown).

Effects of Phosphorylation on Fodrin Activities. Fodrin possesses a number of biological activities which can be monitored readily in vitro. We have examined the effects of fodrin phosphorylation by the spleen protein tyrosine kinase on its binding to F-actin and calmodulin. Both un-phosphorylated and phosphorylated forms of fodrin bind equally well to F-actin as examined by means of the centri-fugation method (Kakiuchi et al., 1982) and to calmodulin as examined by means of calmodulin affinity column chromatography (data not shown).

In addition, the effect of phosphorylation on fodrin stimu-lation of smooth muscle actomyosin Mg^{2+}-ATPase was ex-amined. Under a set of restricted conditions including optical fodrin concentration (3 μg/mL), optimal incubation time of actin and myosin (10 min at 30 °C), and the presence of tropomyosin, myosin light-chain kinase, and Ca^{2+}/calmodulin, fodrin is capable of stimulating the smooth muscle actomyosin Mg^{2+}-ATPase by 50–70% (Wang et al., 1987a). Table I shows that when fodrin was preincubated with the spleen protein tyrosine kinase under the phosphorylation conditions for 2 h (approximately 1 mol of phosphate/mol of fodrin
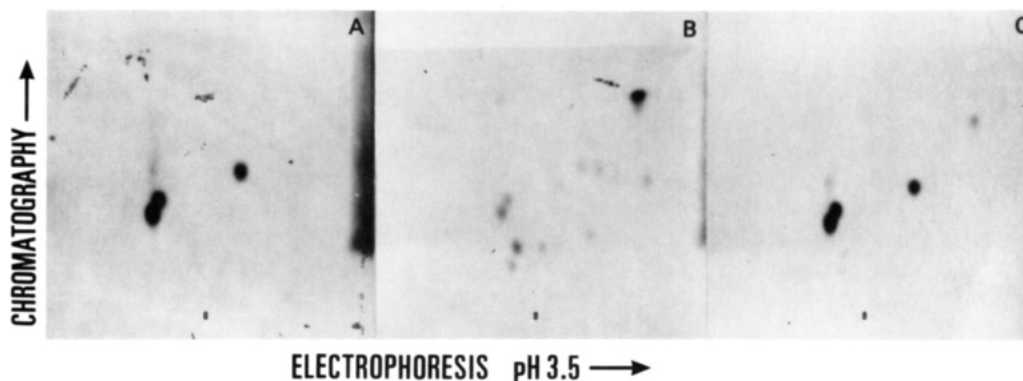

FIGURE 4: Autoradiography of two-dimensional proteolytic peptide maps of phosphorylated fodrin. Following SDS–PAGE, the phosphorylated fodrin band was excised and extensively digested with trypsin as described under Experimental Procedures. The peptides were applied at the origin and subjected to electrophoresis (horizontal dimension with cathode at right) at pH 3.5 before ascending chromatography (vertical dimension). (A) A subunit of fodrin; (B) B subunit of fodrin; (C) A and B subunits of fodrin.
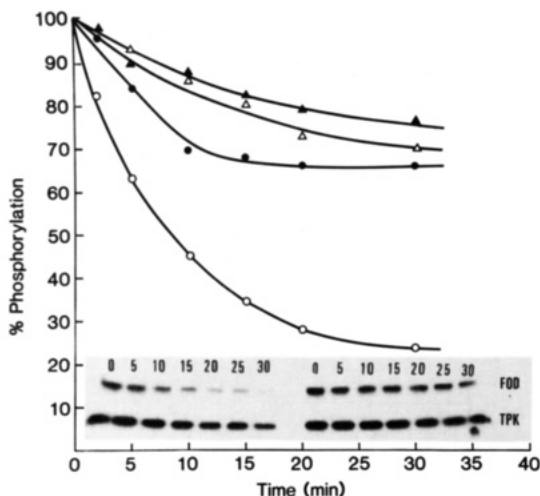
FIGURE 5: Time courses of fodrin and protein tyrosine kinase dephosphorylation by calcineurin. Calcineurin (150 µg/mL) was preincubated with 1 mM Ni^{2+} (△, ○) or 1 mM Mn^{2+} (▲, ●) in the presence of 100 µg/mL calmodulin at 30 °C for 1 h and then added to a sample of phosphorylated fodrin and protein tyrosine kinase to final concentrations of 20 µg/mL to start the reaction. At indicated times, the reaction was terminated and analyzed by 5–20% SDS–PAGE and autoradiography. Inset: (right panel) dephosphorylation by Ni^{2+}–calcineurin; (left panel) dephosphorylation by Mn^{2+}–calcineurin. The rates of dephosphorylation were determined from the relative radioactivities of excised protein bands wherein the radioactivities of control samples (minus calcineurin) were assigned a value of 100%. (○, ●) Dephosphorylation of fodrin; (△, ▲) dephosphorylation of protein tyrosine kinase.

incorporated), the fodrin stimulation of the actomyosin Mg^{2+}-ATPase was almost doubled (from 53% to 105%). The notion of increased Mg^{2+}-ATPase stimulatory activity being a direct result of fodrin phosphorylation was supported by the observation that preincubation of fodrin in the absence of protein tyrosine kinase under the phosphorylation conditions for 2 h did not result in an altered activity. The possibility of protein tyrosine kinase exerting a direct effect on other proteins (such as actin, see Figure 2) in the reconstituted Mg^{2+}-ATPase system was also ruled out by the observation that there were no detectable changes in the Mg^{2+}-ATPase activity when protein tyrosine kinase alone was added (Table I). Similar results were obtained from two other experiments using different preparations of phosphorylated fodrin and gizzard myosin. The average value of the enhancement of the ATPase stimulatory activity by phosphorylated fodrin calculated from results of three experiments was 101% ± 3%.

Since the Ni^{2+}-activated calcineurin can effectively dephosphorylate the phosphotyrosylfodrin (Figure 5), we have examined its effect on the phosphorylated fodrin stimulated smooth muscle actomyosin Mg^{2+}-ATPase activity. Figure 6 shows that when Ni^{2+}–calcineurin was added to an actomyosin Mg^{2+}-ATPase reaction which was prestimulated by the tyrosine kinase phosphorylated fodrin, the rate of the reaction declined gradually to that of the reaction stimulated by nonphosphorylated fodrin (Figure 6). It should be noted that the initial linear time course lasted for about 10–15 min for all reactions (see inset of Figure 6). The ATPase activities calculated from the slopes of these linear regions showed that nonphosphorylated and phosphorylated fodrin could stimulate the ATPase by 55.8% and 114.9%, respectively. These values are in agreement with the results indicated in Table I.

Although the reversal of the tyrosyl phosphorylation induced enhancement in fodrin activity was demonstrated by the addition of calcineurin (Figure 6), the time course of this reversal could not be readily estimated. This is due to, at least in part,
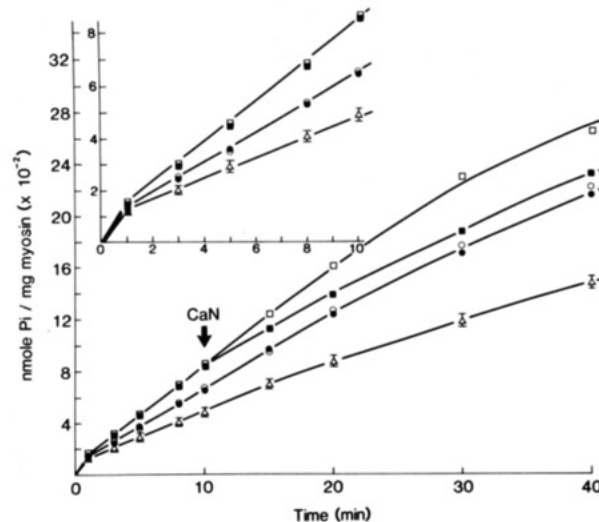

FIGURE 6: Effect of calcineurin on the phosphorylation-induced enhancement of Mg^{2+}-ATPase stimulatory activity of fodrin. The actomyosin Mg^{2+}-ATPase reactions were carried out with the following conditions: (△̄) This symbol represents the average value of four independent experiments with similar ATPase activities. (Experiment 1) Actomyosin mixture was preincubated for 10 min at 30 °C in a buffer system described under Experimental Procedures. The ATPase reaction was initiated by the addition of 1.0 mM [γ-^{32}P]ATP. (Experiment 2) Same as experiment 1 except 20 µg/mL Ni^{2+}–calcineurin was added at 10 min during the ATPase assay. (Experiment 3) Same as experiment 1 except protein tyrosine kinase was autophosphorylated under the phosphorylation conditions for 2 h at 30 °C and then added to the ATPase reaction to a final concentration of 0.29 µg/mL. (Experiment 4) Same as experiment 3 except calcineurin was included at 10 min. (○) Same as experiment 1 except fodrin (3 µg/mL) was added along with ATP to initiate the ATPase reaction. (●) Same as (○) except calcineurin was added at 10 min. (□) Same as (○) except fodrin (30 µg/mL) was phosphorylated by 2.9 g/mL protein tyrosine kinase for 2 h at 30 °C and then added to the ATPase reaction to a final concentration of 3 µg/mL fodrin. (■) Same as (□) except calcineurin was added at 10 min. Inset: Results for the first 10 min were plotted on an expanded scale.

the nonlinear time course of the ATPase reactions (beyond 10 min). It should be stressed, however, that the ATPase reactions tested under other conditions were not affected by calcineurin (Figure 6), strongly supporting the suggestion that the action of calcineurin in the ATPase reactions is on dephosphorylating phosphotyrosylfodrin. The possibility that calcineurin affects the ATPase activities by competing with fodrin for calmodulin binding may be ruled out on the basis of two criteria: (i) the concentration of calmodulin is in excess over that of calcineurin; (ii) the addition of calcineurin to the nonphosphorylated fodrin-stimulated actomyosin Mg^{2+}-ATPase did not result in significant changes in the rate of the Mg^{2+}-ATPase reaction.

DISCUSSION

A large number of cellular protein substrates of viral and receptor-associated protein tyrosine kinases have been identified. Some have been discovered in vitro by using isolated protein tyrosine kinases whereas others were identified as phosphotyrosyl proteins in cells stimulated by appropriate factors or were transformed by certain retroviruses. Many investigators have examined the cytoskeletal proteins as potential protein tyrosine kinase substrates (Sefton et al., 1981; Akiyama et al., 1986), since some of the characteristic cellular changes associated with virus-induced cell transformation such as alterations in cell shape and contact inhibition appear to involve cytoskeletal systems. A number of cytoskeletal proteins including vinculin, actin, tubulin, microtubule-associated proteins, and fodrin have been shown to be in vitro substrates

of either viral or receptor-associated protein tyrosine kinases. However, little is known about how tyrosine phosphorylation affects the functions of these proteins.

Only limited efforts have been directed toward the search of substrates for the non-receptor-associated protein tyrosine kinase of normal tissues. In the present study, we have demonstrated that fodrin and a number of other membrane-associated proteins are in vitro substrates of a highly purified bovine spleen non-receptor-associated protein tyrosine kinase. The phosphorylation of fodrin was chosen for further characterization on the basis of several considerations. First, fodrin is an important member of the cytoskeleton, and its localization at the inner lining of cell membranes is suggestive of a role of fodrin in cell shape maintenance. Second, the spleen protein tyrosine kinase is also membrane-associated and therefore may be colocalized with fodrin in vivo. Third, fodrin has been well characterized in terms of its physical properties and subunit structure, and reasonable quantities of the pure protein can be obtained with relative ease from bovine brain. Lastly, although the physiological functions of fodrin are not entirely clear, certain biological activities such as calmodulin and actin binding activities may be readily measured. Thus, possible effects of protein tyrosine phosphorylation on some of its biological activities can be examined.

Fodrin was reported previously to be an in vitro substrate of the insulin receptor tyrosine kinase (Kadowaki et al., 1985), src tyrosine kinase, and EGF tyrosine kinase (Akiyama et al., 1986). The levels of phosphorylation by these kinases were relatively low, ranging from 0.1 to 0.3 mol of P_i/mol of protein. A significantly higher level of phosphorylation of fodrin at tyrosine residues was observed in this study using the purified spleen non-receptor-associated protein tyrosine kinase. Judging from the kinetics of the phosphorylation reaction, fodrin appears to be a good substrate in terms of the enzyme affinity (K_m) but a poor substrate in terms of the V_{max} of the phosphorylation reaction, which is less than 1% of the V_{max} for angiotensin II phosphorylation by the same kinase. However, the V_{max} reported in this study is comparable to the V_{max} values for protein substrates phosphorylated in vitro by a number of protein tyrosine kinases (Akiyama et al., 1986). The physiological significance of the tyrosine phosphorylation of fodrin is not known at present. A number of observations, such as a slow rate of phosphorylation and a requirement of high $MgCl_2$ concentrations, seem to argue against the notion that fodrin is the physiological substrate of this kinase. However, since both fodrin and protein tyrosine kinase exist in association with membranes and/or the cytoskeleton in the cell, it is possible that the phosphorylation efficiency and the metal ion requirement established in this study are very different from those in intact cells. The potential that fodrin phosphorylation by this tyrosine kinase can be stimulated by specific cellular factors cannot be overlooked. A documented example is the phosphorylation of vinculin, a cytoskeletal protein concentrated on the adhesion plaques of the culture cells, by the $pp^{60v\text{-}src}$ tyrosine kinase. Vinculin was found to be a poor in vitro substrate of the kinase, but its phosphorylation can be stimulated many fold by the presence of acidic phospholipids (Ito et al., 1982).

In this study, we have examined the effect of tyrosine phosphorylation on actin binding, calmodulin binding, and smooth muscle actomyosin Mg^{2+}-ATPase stimulating activities of fodrin, and we have found that only the last activity is affected by the phosphorylation. A clear demonstration of the Mg^{2+}-ATPase stimulatory activity of fodrin depends on a set of restricted conditions such as the optimal concentration of

fodrin (3 μg/mL), the optimal preincubation time between actin and myosin (10 min), phosphorylation of the myosin regulatory light chains, and the presence of tropomyosin and Ca^{2+}/calmodulin (Wang et al., 1987a). It is not known if the same set of conditions also governs the optimal stimulation by the tyrosine-phosphorylated fodrin. Systematic studies on these conditions and a close examination of the correlation between the extent of phosphorylation and the subsequent activity changes of fodrin are now in progress. Since among the various in vitro activities of fodrin only the stimulation of smooth muscle actomyosin Mg^{2+}-ATPase by fodrin is shown to be Ca^{2+} and calmodulin dependent, it has been suggested that this may be one of the physiological functions of fodrin (Wang et al., 1987a). The observation that this stimulatory activity of fodrin can be fine-tuned by tyrosine phosphorylation of fodrin strengthens this suggestion. In addition, we have recently reported that serine-phosphorylated fodrin (by the catalytic subunit of cyclic AMP dependent protein kinase) can no longer stimulate the smooth muscle actomyosin Mg^{2+}-ATPase (Wang et al., 1987b).

In conclusion, the present study provides the first detailed characterization of the spleen non-receptor-associated protein tyrosine kinase reaction using a cellular substrate. In addition, it represents the first example of such a tyrosine phosphorylation which results in a change in the in vitro biological activities of the substrate. However, the occurrence of such a phosphorylation in vivo and its possible physiological functions require further studies.

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Methionine-90-Spin-Labeled Bovine α-Lactalbumin: Electron Spin Resonance and NMR Distance Measurements[†]

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ABSTRACT: The unique methionine residue of bovine α-lactalbumin was modified by irreversible alkylation with the bromoacetamido nitroxide spin-label 4-(2-bromoacetamido)-2,2,6,6-tetramethylpiperidine-*N*-oxyl. The line shape of the electron spin resonance (ESR) spectrum was indicative of a fairly mobile spin-label and was sensitive to the calcium-induced conformational change. Paramagnetic broadening of the spin-label ESR lines by a Gd(III) ion substituted at the high-affinity calcium site of the protein yielded a distance between the spin-label and the metal-binding site of 8.0 ± 1.0 Å. The extent of the paramagnetic line broadening by the covalently attached nitroxide spin-label on the proton resonances of several amino acid residues of the protein at 500 MHz allowed estimation of intramolecular distances between the methionine-90 residue and several resolvable protons.

α-Lactalbumin (α-LA)[1] is the regulatory subunit of the "lactose synthase" complex. Upon binding to the enzyme galactosyltransferase (EC 2.4.1.22), lactose is efficiently synthesized from UDP-galactose and glucose. A putative, energy-minimized, three-dimensional structure was proposed by Warme et al. (1974) which was based, in part, on the high degree of primary structure homology between α-LA and lysozyme (Brew et al., 1970; Shewale et al., 1985). More recently, Smith et al. (1987) have reported a low-resolution crystallographic analysis of baboon α-LA, which, while not at the precise atomic resolution of most protein structures, confirmed that α-LA probably evolved from a lysozyme precursor. In spite of their structural similarities, the latter two proteins are quite functionally and physicochemically different. In particular, α-lactalbumin is a metalloprotein, with high affinities for Ca(II) (Permyakov et al., 1981, 1985; Murakami et al., 1982) and Zn(II) (Murakami et al., 1982; Musci &

Berliner, 1985a). Although some controversy existed over the classification of α-LA as a strong calcium binding protein, it has been clearly confirmed that the dissociation constant lies in the low nanomolar range (Permyakov et al., 1987; Berliner & Johnson, 1987). Several lanthanides bind specifically to the calcium site (site I), most with higher affinity than that for Ca(II) (Murakami et al., 1982; Berliner et al., 1986).

One of our goals has been to structurally map the α-LA molecule in solution by several spectroscopic methods. A previous report documents several intramolecular distances by fluorescence measurements (Musci & Berliner, 1986). The precise location of the calcium binding site has recently been found definitively by X-ray crystallography (Stuart et al., 1986) to encompass Asp residues 82, 87, and 88 and the carbonyl oxygens of residues 79 and 84. Bovine α-LA contains the same calcium binding loop sequence as corrected by Shewale et al. (1985). A single methionine residue at position

[1] Abbreviations: α-LA, α-lactalbumin; bromoacetamido spin-label, 4-(2-bromoacetamido)-2,2,6,6-tetramethylpiperidine-*N*-oxyl; 2D NMR, two-dimensional nuclear magnetic resonance; ESR, electron spin resonance; FID, free induction decay; NMR, nuclear magnetic resonance; NOE, nuclear Overhauser effect; Met-90-spin-labeled α-LA, α-LA labeled at Met-90 with the bromoacetamido spin-label 4-(2-bromoacetamido)-2,2,6,6-tetramethylpiperidine-*N*-oxyl; Tris, tris(hydroxymethyl)aminomethane.