Although Ras-related small GTPases are believed to control cell proliferation and motility through activation of protein kinase cascades, little is known about the intracellular protein targets of activated kinases. Here we show that the p90 ribosomal S6 kinase 2 (RSK2) phosphorylates actin-binding protein (ABP-280) in intact rat 3Y1 fibroblasts. Growth factors such as fetal calf serum, epidermal growth factor, phorbol 12-myristate 13-acetate, and lysophosphatidic acid stimulate the phosphorylation of serine residues in ABP-280 in quiescent 3Y1 cells. Extracts from 3Y1 cells prepared after stimulation by lysophosphatidic acid, fetal calf serum, and epidermal growth factor retain activated protein kinase activity toward ABP-280 in vitro. ABP kinase activities in lysates from lysophosphatidic acid-stimulated 3Y1 cells can be fractionated by MonoQ anion exchange chromatography into three peaks having ABP kinase activities. One (ABP kinase peak 1) coelutes with the peak of RSK2 as judged by immunoblotting and S6 peptide kinase assays. Two-dimensional phosphopeptide maps show RSK2 phosphorylated ABP-280 to be phosphorylated at the same site(s) as those stimulated by growth factors in vivo. Incubation of ABP kinase peak 1 fractionated from unstimulated cells with activated ERK2 activates latent ABP kinase activity. These results show RSK2 to phosphorylate ABP-280 in vivo.

Protein phosphorylation cascades are essential for many processes in mammalian cells. In particular, transmission of mitogenic signals to intracellular targets is mediated by the activation of protein kinases. One pathway is the Ras-dependent or mitogen-activated protein kinase (MAPK) pathway signaling cascade (1–5). In this pathway, binding of growth factors to their receptors activates the small GTPase Ras through the GRB2 adapter protein and guanine nucleotide exchange factor (SOS). This is followed by a sequential activation of multiple protein kinases (raf-1, MEK, MAPK, and RSK). This protein kinase cascade leads to the phosphorylation of factors which initiate gene expression and modify metabolic and cytoskeletal processes to sustain or promote cell growth and differentiation.

Hence, activation of the serine/threonine-specific protein kinases (i.e. MAPKs and RSKs) is believed to effect the phosphorylation of targets that play important regulatory roles in a variety of cellular processes (6, 7). However, the identification of intracellular protein targets and the effect of phosphorylation on the function of these proteins are poorly understood.

ABP-280 is a member of a large protein family that shares the ability to cross-link actin filaments into orthogonal networks (8). ABP-280 is an elongated dimer composed of identical 28-kDa subunits, each composed of long rod domains of repetitive α-sheet motifs that connect an amino-terminal actin-binding domain to a carboxyl-terminal self-association site (9, 10).

ABP-280 and its muscle isoform (filamin) are phosphorylated in intact cells (11, 12), and cAMP-dependent protein kinase (cAMP kinase), protein kinase C, and Ca2+/calmodulin-dependent protein kinase II (CaM kinase II) phosphorylate ABP-280 and/or filamin in vitro (13–16). Some effects of in vitro phosphorylation have been identified. ABP-280 phosphorylated by cAMP kinase has increased resistance to the calcium-activated protease calpain (14) and altered interaction with GTP and/or unidentified small GTP-binding protein(s) (17, 18). ABP-280 in platelets is a substrate for CaM kinase in vivo. Compounds, such as prostaglandins I2 and E1 or forskolin, which increase the intracellular CaMP levels, stimulate ABP-280 phosphorylation in platelets (14). It is unclear, however, if protein kinases other than cAMP kinase phosphorylate ABP-280 in intact cells. Phosphorylation of filamin by CaM kinase II decreases filamin’s actin filament cross-linking activity (16).

In this study, we find that stimulation of quiescent 3Y1 rat fibroblasts by growth factors such as epidermal growth factor (EGF) and lysophosphatidic acid (LPA) results in a rapid increase in the phosphorylation of ABP-280 through serine/threonine-specific protein kinases. We identified one of these protein kinases to be the p90 ribosomal S6 protein kinases (RSK2).

**EXPERIMENTAL PROCEDURES**

Reagents—[γ-32P]ATP (3000 Ci/mmol) and [32P]orthophosphate (8500–9120 Ci/mmol) were purchased from DuPont NEN. Trypsin, chymotrypsin, calpain, phenylmethylsulfonyl fluoride, catalytic subunit of cAMP-dependent protein kinase, a peptide inhibitor of cAMP-dependent protein kinase and lysophosphatidic acid were purchased from Sigma. Mouse EGF was from Collaborative Research. Protein kinase C was from Lipidex. MAP kinase (ERK2), protein phosphatase 2A, p90 RSK2, anti-RSK1 kinase antibody, anti-RSK2 antibody, anti-p70 S6 kinase antibody, and S6 peptide (RRRSLSSRA) were from Upstate Biotechnology, Inc. Raf kinase, MEK, cdc2 kinase, cascin kinase II, Cdc25 protein, and Rsk kinases were purified from HeLa cells by our laboratory (8). Phosphate-free RPMI 1640 medium was from Mediatech, Inc. XAR-5 x-ray film was from Eastman Kodak Co.
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purified from human uterine as described previously (19).

Cells—3Y1 cells derived from Fischer rat embryo fibroblasts and Cos cells were grown at 37°C in tissue culture dishes in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum.

Labeling of Cells with [32P]Orthophosphate and Immunoprecipitation—Cells were grown to confluence in 3.5 cm diameter plastic tissue culture dishes containing 10 ml of Dulbecco’s modified Eagle’s medium supplemented with 10% FCS. Confluent monolayers of cells were rendered quiescent by incubation in serum-free medium for 24 h. Quiescent cells were labeled for 16 h in 0.9 ml of phosphate- and serum-free RPMI 1640 medium containing carrier-free [32P]orthophosphate (0.2 mCi/ml). Labeled cells were then treated essentially as described (19). The indicated concentrations of agonists such as FCS, LPA, EGF, 12-O-tetradecanoylphorbol-13-acetate, dibutyryl cAMP, or ionomycin at 37°C for various time periods. Activated cells were washed in saline, freeze-thawed in liquid nitrogen, then solubilized with 0.15 ml of a solution containing 50 mM Tris/HCl, pH 7.4, 0.5 mM NaCl, 0.5% Triton X-100, 0.5% deoxycholate, 0.1% SDS, 1 mM EDTA, 1 mM sodium orthovanadate, 30 mM sodium pyrophosphate, 50 mM NaF, 1 mM phenylmethylsulfonyl fluoride, and 2% aprotinin at 0°C. Cells were scraped from Petri dishes and insoluble materials were removed by centrifugation at 15,000 × g for 20 min at 4°C. ABP-280 in the soluble phase was immunoprecipitated with monoclonal anti-ABP-280 using pH 3.5 buffer of pyridine/glacial acetic acid/H2O (1:10:189, v/v). To plates without fluorescent indicators, E. M. Science, Cherry HIll, NJ) Eagle’s medium supplemented with 10% fetal calf serum.

M NaCl, 0.5% Triton X-100, 0.5% deoxycholate, 0.1% SDS, 1 mM EDTA, 1 mM NaCl, 0.5% Triton X-100, 0.5% deoxycholate, 0.1% SDS, 1 mM EDTA, 1 mM sodium orthovanadate, 30 mM sodium pyrophosphate, 50 mM NaF, 1 mM phenylmethylsulfonyl fluoride, and 2% aprotinin at 0°C. Samples were dissolved in 200 μl of reaction buffer containing 50 mM Tris/HCl, pH 7.4, 0.5 mM NaCl, 0.5% Triton X-100, 0.5% deoxycholate, 0.1% SDS, 1 mM EDTA, 1 mM sodium orthovanadate, 30 mM sodium pyrophosphate, and 50 mM NaF. Bound protein was released from the particle by boiling for 2 min in 60 μl of SDS-sample buffer, analyzed by SDS-PAGE and autoradiography. The relative intensity of the 32P-labeled band was determined by Phosphorimager (Molecular Dynamics). In some experiments, the Coomassie-stained ABP-280 subunit was excised, and its 32P content was determined by liquid scintillation counting.

Phosphoamino Acid Analysis—The phosphoamino acid composition was analyzed as described previously (20) with some modifications. Polyacrylamide fragments containing 32P-labeled ABP-280 were excised, washed extensively with 25% (v/v) isopropanol alcohol, then 10% (v/v) methanol, and lyophilized. The dried gel slices were incubated at 37°C for 24 h in 4 ml of 50 mM NH4HCO3, pH 8.0, containing 100 μg/ml trypsin and 50 μg/ml chymotrypsin, centrifuged at 1000 × g for 5 min. The supernatants were collected and lyophilized. Dried supernatant samples were dissolved in 200 μl of 6.0 N HCl and hydrolyzed at 110°C. After acid hydrolysis, the samples were dried and dissolved in 20 μl of a solution containing phosphoserine, phosphothreonine, and phosphoserine (1 mg/ml) each as markers. Phosphoamino acids were separated by high voltage electrophoresis at 1.5 kV for 20 min on TLC plates (20 cm microcrystalline cellulose containing 15% by weight of phosphoric acid) at 37°C for 24 min in 4 ml of 50 mM NH4HCO3, pH 8.0, containing 100 μg/ml trypsin and 50 μg/ml chymotrypsin, centrifuged at 1000 × g for 5 min. The supernatants were collected and lyophilized. Dried supernatant samples were dissolved in 200 μl of 6.0 N HCl and hydrolyzed at 110°C. After acid hydrolysis, the samples were dried and dissolved in 20 μl of a solution containing phosphoserine, phosphothreonine, and phosphoserine (1 mg/ml) each as markers. Phosphoamino acids were separated by high voltage electrophoresis at 1.5 kV for 20 min on TLC plates (20 cm microcrystalline cellulose containing 15% by weight of phosphoric acid) at 37°C for 24 min in 4 ml of 50 mM NH4HCO3, pH 8.0, containing 100 μg/ml trypsin and 50 μg/ml chymotrypsin, centrifuged at 1000 × g for 5 min. The supernatants were collected and lyophilized.

RESULTS

LPA and Growth Factors Induce Phosphorylation of ABP-280 in Intact Rat Fibroblastic Cells—Fig. 1A shows that treatment of confluent serum-deprived quiescent rat 3Y1 cells with LPA and EGF markedly increased the phosphorylation of ABP-280 in 3Y1 cells. Addition of dBcAMP, PMA, and FCS to cells also stimulated the phosphorylation of ABP-280. The time course of
ABP-280 phosphorylation in fibroblasts exposed to FCS or LPA is shown in Fig. 1B. Incorporation of phosphate into ABP-280 was maximal after ~10 min for both FCS and LPA. Phosphorylation remained constant for 60 min after the treatment of cells with LPA but the phosphorylation induced by FCS was transient. LPA stimulated the phosphorylation of ABP-280 in a dose-dependent manner. Maximal phosphorylation of ABP-280 was attained between 0.2 and 10 μg/ml LPA (data not shown).

Phosphoamino acid analysis of ABP-280 immunoprecipitated from lysates of LPA-treated cells reveals serine as the major phosphate-containing residue (Fig. 2A), demonstrating that growth factors activate similar protein kinases which phosphorylate specific serine residues in the carboxy-terminal tail of ABP-280.

LPA and Growth Factors Stimulate ABP Protein Kinase Activities—To characterize the protein kinase(s) which phosphorylates ABP when 3Y1 cells are treated with growth factors, quiescent cells or cells stimulated for 5 min by LPA (2 μg/ml), FCS (10%), or EGF (100 ng/ml) were lysed and tested for their phosphorylation.

Fig. 1. Treatment of quiescent rat 3Y1 fibroblasts with growth factors induces ABP-280 phosphorylation. A, quantitation of the effects of various agents on the phosphorylation of ABP-280 in 3Y1 cells. 3Y1 fibroblasts were labeled for 18 h in phosphate-free RPMI 1640 medium containing [32P]orthophosphate (0.2 mCi/ml). [32P]-Labeled cells were treated at 37 °C for 10 min with Tris-buffered saline (control), 100 ng/ml PMA, 1.0 mM dibutyryl cAMP, 100 ng/ml EGF, 0.5 mM iodonitrotetrazolium, 10% FCS, or 10 μg/ml LPA. Cell extracts were prepared and ABP-280 immunoprecipitated with monoclonal anti-ABP-280 antibody. Phosphoproteins were analyzed by SDS-PAGE followed by autoradiography. The extent of phosphate incorporation into ABP-280 band is expressed as the percentage relative to the control value. The number of experiments performed are indicated. Each value represents the mean ± S.E. The inset shows the autoradiograph of [32P]-labeled ABP-280 immunoprecipitated from 3Y1 cells stimulated by various agents. The [32P] contents of the excised ABP-280 polypeptide from the SDS-PAGE gel were determined by scintillation counting (Cerenkov). They were 72 cpm (control), 157 cpm (LPA), 154 cpm (EGF), and 118 cpm (dBcAMP), respectively. The amount of ABP-280 immunoprecipitated from each cell extract was identical between control and stimulated cells as judged by Coomasie Blue protein staining of the 280-kDa subunit of immunoprecipitated ABP-280. B, time course for the phosphorylation of ABP-280 after exposure of quiescent 3Y1 cells to 10% FCS (●) or 10 μg/ml LPA (○). [32P] Incorporation is plotted relative to the amount of phosphate incorporation into untreated cells. The [32P] content was determined by scintillation counting of excised ABP-280 from SDS-PAGE gels. Each value represents the mean ± S.E. (n = 3)

Fig. 2. Phosphoamino acid analysis and phosphopeptide map of ABP-280 isolated from [32P]-labeled 3Y1 cells. A, two-dimensional phosphoamino acid analysis of ABP-280. [32P]-Labeled 3Y1 cells were incubated with 10% FCS for 10 min at 37 °C. Cell extracts were prepared and ABP-280 immunoprecipitated with anti-ABP-280 antibody. After SDS-PAGE and autoradiography, the phospho-ABP-280 subunits were excised and incubated with trypsin (100 μg/ml) and α-chymotrypsin (50 μg/ml) for 24 h at 37 °C. The phosphoamino acids of the eluted peptides were determined as described under “Experimental Procedures.” The directions of electrophoresis are indicated. P-Ser, phosphoserine; P-Thr, phosphothreonine; P-Tyr, phosphotyrosine. B, one-dimensional phosphopeptide map of ABP-280 after calpain digestion. [32P]-Labeled 3Y1 cells were treated at 37 °C for 10 min with Tris-buffered saline (control), 10 μg/ml LPA, 10% FCS, 100 ng/ml EGF, or 100 ng/ml PMA. ABP-280 was immunoprecipitated from cell lysate, the immunoprecipitates were washed and incubated with 0.01 unit/ml calpain in the presence of 5 mM CaCl2, as described under “Experimental Procedures.” Phosphopeptides were analyzed by SDS-PAGE followed by autoradiography. The position of the well defined cleavage fragments of 190, 100, and 90 kDa are indicated.

serinethreonine-specific protein kinase(s) to phosphorylate ABP-280 in intact cells. The phosphorylation site(s) are in the carboxy-terminal third of the ABP-280 polypeptide (Fig. 2B), which is easily dissected from the amino end of ABP-280 using the calcium-activated enzyme calpain. There are only two calpain sites in ABP-280, and the most rapidly cleaved site generates 190- and 100-kDa subfragments. The 190-kDa fragment contains the amino termini F-actin binding domain of ABP-280. The 100-kDa carboxy-terminal piece contains the second calpain site, and upon extended digestion, is cleaved into 90- and 10-kDa subfragments. This 90-kDa calpain fragment was phosphorylated when cells were incubated with FCS, EGF, and PMA (Fig. 2B). Two-dimensional phosphopeptide mapping analysis reveals that treatment with LPA (Fig. 5B) and other agents (data not shown) increases the phosphorylation of two major peptides of ABP-280 in intact cells. These results suggest that growth factors activate similar protein kinases which phosphorylate specific serine residues in the carboxy-terminal tail of ABP-280.
ability to phosphorylate human ABP-280 in vitro. Fig. 3 shows that a clarified extract isolated from cells treated with LPA has potentiated ABP protein kinase activity compared to an identical extract prepared from quiescent cells. Phosphorylation of the endogenous ABP-280 without exogenously added ABP-280 was low in extracts from quiescent and as well as LPA-treated cells (Fig. 3, inset). Extracts prepared from FCS- or EGF-stimulated cells also contain increased protein kinase activity for human ABP-280 (Fig. 3). ABP kinase activity was 5–8-fold greater in extracts from stimulated cells than those from quiescent cells.

Fig. 4A shows the relationship between time of exposure of fibroblasts to LPA and the amount of ABP kinase activity present in extracts from lysates of these cells. The half-maximal activation of protein kinase activity occurred 2 min after the addition of 2 μg/ml of LPA to the cells; maximal stimulation (~5-fold relative to untreated cell extracts) was reached in ~5 min. ABP kinase activity declined thereafter and returned to near basal activity after 30 min. ABP kinase activity was not affected by the addition of specific inhibitors to protein kinase C or cAMP kinase (Table I) and was reduced about 50% in the presence of 1 mM CaCl2 or 50 mM NaF. Direct addition of LPA affected by the addition of specific inhibitors to protein kinase activity depends on the receptor-mediated signaling (data not shown).

ABP-280 is phosphorylated in vitro at the same sites as it is in vivo after growth factor treatment of cells. Phosphoamino acid analysis of ABP-280 phosphorylation using clarified extract from LPA-treated cells shows serine to be the major residue phosphorylated (Fig. 4B). Extracts isolated from cells treated with FCS or EGF also phosphorylate serine residues in ABP-280 (data not shown). These data demonstrate that serine/threonine-specific protein kinases present in cell extracts are activated by growth factors and are responsible for the phosphorylation of ABP-280 in vitro. ABP-280 was phosphorylated using LPA-treated cell extracts and immunoprecipitated with anti-ABP-280 antibody. The immune complex was washed extensively, and bound ABP-280 was cleaved using calpain. The carboxyl-terminal tail of ABP-280 is phosphorylated in vitro as is ABP-280 phosphorylated in vivo (Fig. 4C). A two-dimensional phosphopeptide mapping analysis of ABP-280 phosphorylated by extracts from LPA-treated cells in vitro also showed two major phosphopeptides whose positions were identical to those generated from ABP-280 phosphorylated in vivo in response to LPA (data not shown). These results strongly suggest that the ABP kinase activity measured in vitro is also responsible for the phosphorylation of ABP-280 in intact cells.

p90 RSK2 Phosphorylates ABP-280 In Vitro—Several serine/threonine-specific protein kinases are activated when quiescent fibroblasts are treated with growth factors. We determined whether known protein kinases can phosphorylate purified human ABP-280 in vitro. Casein kinase II, cdc2 ki-
Fig. 5. Phosphorylation of ABP-280 by RSK2. A, purified ABP-280 (a) or 40 S ribosomal protein (b) were incubated with purified RSK2 in the presence of \([\gamma-32P]ATP\). Proteins were separated and analyzed by SDS-PAGE followed by autoradiography. The autoradiogram is shown. The positions of the ABP-280 subunit and 40 S ribosomal polypeptide are indicated by arrows. B, comparison of two-dimensional phosphopeptide map of ABP-280 phosphorylated in quiescent, LPA-treated cells or by RSK2 in vitro. \([\gamma-32P]ATP\) was added to the cells. The autoradiogram is shown.

Table 1: Effects of protein kinase inhibitors and other agents on ABP kinase activity.

| Treatment          | Mean S.E. Relative ABP kinase activity |
|--------------------|-----------------|
| LPA                | 100              |
| PKC inhibitor      | 98.1             | 5.0 |
| PKI-tide           | 97.3             | 4.6 |
| Ca\(^{2+}\)        | 48.3             | 13.6 |
| NaF                | 42.1             | 1.4 |

Fig. 6. Fractionation of ABP kinase activities by MonoQ FPLC. Cytosolic extracts from untreated (○) or 2 µg/ml LPA treated (●) 3Y1 cells for 5 min were fractionated by MonoQ FPLC as described under “Experimental Procedures.” Eluted fractions were assayed for ABP kinase activity (A) and S6 peptide kinase activity (B). C, aliquots of eluted fractions (shown in A) were immunoblotted with affinity-purified anti-RSK1, anti-RSK2, and anti-p70 S6 kinase antibodies following SDS-PAGE and transferred to polyvinylidene difluoride. The fraction number is indicated. D, cytosolic extracts from unstimulated 3Y1 cells were fractionated by MonoQ FPLC. Fractions were probed with (●) or without (○) RSK2 in the presence of MgCl\(_2\) and ATP for 20 min at 30°C. They were then incubated with ABP-280 and \([\gamma-32P]ATP\) (0.25 µCi/tube) for 20 min at 30°C. The reaction was stopped by the addition of 10 µl of SDS-sample buffer, boiled for 2 min, and analyzed by SDS-PAGE followed by autoradiography. The autoradiogram was digitized using the NIH Image analysis program. Phosphorylation of ABP-280 by ERK-GST alone was negligible in this experiment. The extent of ABP-280 phosphorylation mediated by ABP kinase peak 1 (fraction 12) in the presence of GST-beads was defined as 100%, and from this, the relative intensity of phosphorylated ABP-280 was compared.

Phosphorylation of ABP-280 by RSK2. A, purified ABP-280 (a) or 40 S ribosomal protein (b) were incubated with purified RSK2 in the presence of \([\gamma-32P]ATP\). Proteins were separated and analyzed by SDS-PAGE followed by autoradiography. The autoradiogram is shown. The positions of the ABP-280 subunit and 40 S ribosomal polypeptide are indicated by arrows. B, comparison of two-dimensional phosphopeptide map of ABP-280 phosphorylated in quiescent, LPA-treated cells or by RSK2 in vitro. \([\gamma-32P]ATP\) was added to the cells. The autoradiogram is shown.
Phosphorylation of ABP-280 by RSK2

chromatographed by MonoQ column and eluted fractions incubated in the presence or absence of active ERK2 before addition of the ABP-280 substrate. Fig. 6D shows that only ABP kinase activity in peak 1 was activated by preincubation with ERK2.

**DISCUSSION**

ABP-280 is phosphorylated in vivo in various cells (11–14). In normal and Rous sarcoma virus-transformed chick fibroblasts, 5–10% of total ABP-280 is phosphorylated at serine residues suggesting serine-specific protein kinases to be responsible for its phosphorylation in vivo, but the identities of the protein kinases were undefined (11). We have now demonstrated that growth factors such as LPA, FCS, and EGF increase the phosphorylation of ABP-280 in quiescent rat 3Y1 fibroblastic cells through the activation of serine/threonine-specific protein kinases.

ABP-280 phosphorylating activity was fractionated by MonoQ column chromatography from LPA-stimulated 3Y1 cell extracts. Several lines of evidence suggest that one of the ABP protein kinases (ABP kinase peak 1) is p90 RSK2. First, RSK2 coelutes with the peak of ABP kinase activity in peak 1 in MonoQ column as judged by S6 peptide kinase activity and immunoblotting (Fig. 6). Second, incubation of the MonoQ fractions from extracts from quiescent cells eluting at the same salt concentration as ABP kinase activity in peak 1 with activated MAP kinase (ERK2) activates ABP kinase activity in peak 1 (Fig. 6D). This indicates the ABP kinase in peak 1 is activated by ERK2 and suggests it is downstream of ERK. Third, purified RSK2 phosphorylates purified ABP-280 in vitro at the same serine residues that are phosphorylated in vivo as shown by two-dimensional phosphopeptide mapping (Fig. 5B). Phosphorylation sites are located at the 90-kDa carboxyl-terminal tail domain of ABP-280 which contains four consensus phosphorylation sequences for RSK2 (i.e. R)(R)X(S/X) (25). Although RSK2 derives its name from its ability to phosphorylate ribosomal protein S6 in vitro, S6 is not the physiological substrate for RSK2 in vivo, and only a limited number of substrates for RSK2 have been identified (6, 26). Our present study demonstrates that ABP-280 is a physiological substrate for RSK2 and suggests a possible linkage between MAP/RSK signaling pathway and actin cytoskeleton.

At present, the identities of the two other ABP kinase activities (i.e. ABP kinase activity 2 and 3) revealed by MonoQ fractionation are unknown. Although ABP kinase peak 3 coelutes with RSK1 as shown by S6 peptide kinase activity assay and immunoblotting (Fig. 6), we cannot conclude that RSK1 is the only kinase in ABP kinase peak 3. First, ABP kinase peak 3 from quiescent cells is not activated by preincubation with ERK2 in vitro (Fig. 6D). Second, recombiant RSK1 purified from Cos cells transfected with epitope-tagged RSK1 gene (27) failed to phosphorylate ABP-280 in vitro although LPA did stimulate the S6 peptide kinase activity of RSK1 in the Cos cells, as determined by immune complex kinase assay, but did not stimulate ABP-280 phosphorylation (data not shown).

MAP kinase (HOG-1) is also known to activate MAPK-activated protein kinase-2 (MAPKAP kinase-2) which phosphorylates heat shock protein 25 (28, 29). However, MAPKAP kinase-2 is unlikely to correspond to ABP kinase peaks 2 or 3. First, growth factors do not stimulate the HOG-1/MAPKAP kinase-2 pathway. Second, MAPKAP kinase-2 activity is stimulated in vitro by preincubation with ERK while ABP kinase peaks 2 and 3 are not. Lastly, human ABP-280 does not contain a consensus sequence for phosphorylation by MAPKAP kinase-2 (XX-Hyd-XRXXS, where Hyd is a bulky hydrophobic residue (30)).

Although LPA is known to activate protein kinase C (31) and protein kinase C phosphorylates ABP-280 in vitro (15), protein kinase C is unlikely to correspond to one of the identified ABP kinase fractions. First, ABP kinase activity isolated from LPA-stimulated cell extracts is insensitive to specific inhibitors of protein kinase C (Table I). Second, phosphorylation of ABP-280 elicited by LPA occurs in the 90-kDa carboxyl-terminal tail domain (Fig. 2B), while protein kinase C phosphorylates ABP-280 mainly in the 190-kDa amino-terminal domain in vitro (data not shown). It is also not likely that LPA-stimulated ABP kinases correspond to cAMP kinase, although a number of studies have suggested that cAMP kinase phosphorylates ABP-280 in vitro and in vivo (13, 14, 32, 33) and treatment with dbcAMP increases the phosphorylation of ABP-280 in intact 3Y1 cells (Fig. 1A). LPA has been shown to inhibit adenylyl cyclase via pertussis-toxin-sensitive G, and reduce cellular cAMP levels in Rat 1 cells and human foreskin fibroblasts (31), and ABP kinase activity in LPA-stimulated cell extracts is insensitive to a specific inhibitor of cAMP kinase (Table I).

Since the phosphorylation sites in ABP-280 phosphorylated by ABP kinase peaks 2 and 3 are similar or identical to those by RSK2, it is likely that ABP kinase(s) in these peaks are unidentified serine/threonine-specific protein kinases which locate downstream of MAP kinase like protein kinases. Further studies are necessary to determine the identities of ABP kinase activity 2 and 3.

The specific physiological function of the phosphorylation of ABP-280 by RSK2 is not known. Studies suggest that the MAPK/RSK pathway is involved in the control of the cellular actin cytoskeleton. ERK2 and p90 S6 kinase activate rapidly through thrombin-induced platelet activation and aggregation (34). Overexpression of dominant negative MEK in NIH 3T3 cells suppresses p90 S6 kinase activity and alters actin cytoskeleton and cell morphology (35). We have, however, failed to detect any changes of the affinity of ABP-280 to actin filaments in vitro (data not shown). This indicates that regulation of the ABP-280 function by phosphorylation occurs at a different level. One possibility is that phosphorylation of ABP-280 could regulate the interaction of ABP-280 with other molecules such as small GTP binding proteins (17, 18) and membrane receptors (36, 37). Further study is necessary to define the role of phosphorylation of ABP-280 in intact cells.

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