Characterization of OSR1, a Member of the Mammalian Ste20p/Germinal Center Subfamily

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In examining the protein kinase components of mitogen-activated protein (MAP) kinase (MAPK) cascades that regulate the c-Jun N-terminal kinase (JNK) in Drosophila S2 cells, we previously found that distinct upstream kinases were involved in responses to sorbitol and lipopolysaccharide. Here we have extended our analysis to the possible MAPK kinase kinases (MAP4Ks) in the JNK pathway. Fray, a putative Drosophila MAP4K, provided a major contribution to JNK activation by sorbitol. To explore the possible link to JNK in mammalian cells, we isolated and characterized OSR1 (oxidative stress-responsive 1), one of two human Fray homologs. OSR1 is a 58-kDa protein of 527 amino acids that is widely expressed in mammalian tissues and cell lines. Of potential regulators surveyed, endogenous OSR1 is activated only by osmotic stresses, notably sorbitol and to a lesser extent NaCl. However, OSR1 did not increase the activity of coexpressed JNK, nor did it activate three other MAPKs, p38, ERK2, and ERK5. A two-hybrid screen implicated another Ste20p family member, the p21-activated protein kinase PAK1, as an OSR1 target. OSR1 phosphorylated threonine 84 in the N-terminal regulatory domain of PAK1. Replacement of threonine 84 with glutamate reduced the activation of PAK1 by an active form of the small G protein Cdc42, suggesting that phosphorylation by OSR1 modulates the G protein sensitivity of PAK isoforms.

Cell growth and differentiation are precisely regulated by complex systems involving protein kinase cascades. A family of these cascades contain the pleiotropic mitogen-activated protein kinases (MAPKs). These enzymes play critical roles in transducing signals from extracellular stimuli, including hormones, growth factors, and environmental stresses, throughout the cell (1–4). The core modules of MAPK cascades are composed of three sequentially acting protein kinases, a MAPK activated by a MAP kinase (MAP2K), which is activated by a MAPK kinase (MAP3K). In mammals, the most studied MAPKs are ERK1/2, the c-Jun N-terminal kinase (JNK), p38, and ERK5.

Ste20p is the yeast MAP4K protein that activates the MAP3K in the pheromone-responsive MAPK cascade of the budding yeast mating pathway (5, 6). In the past several years, numerous protein kinases with catalytic domains closely related to that of Ste20p have been identified and constitute the Ste20p family. Based on structure and regulation, two subfamilies have been defined, the p21-activated kinase (PAK) subfamily and the larger germline center kinase (GCK) subfamily (7). The PAKs include the six enzymes nearest in characteristics to Ste20p itself; each contains a C-terminal catalytic domain and an N-terminal regulatory domain with a small G protein binding motif. PAKs have been shown not only to activate MAPKs (primarily JNK and p38) but also to influence disassembly of the actin cytoskeleton and apoptosis (8–14). The GCKs are distinct from PAKs in that they have N-terminal catalytic domains followed by C-terminal putative regulatory regions without conserved G protein binding sites. The 28 known human GCK-related kinases are classified in eight subdivisions and have diverse and much less well characterized functions (7). Some, like Ste20p and PAKs, regulate the JNK and p38 MAPK pathways. Those reported to activate JNK include the GCK-IV subfamily members, MINK, NIK, HIK, TNK; GCK-I subfamily members, GCK, HPK1, GLK; and the GCK-V subfamily member, SLK (15–24). Those reported to activate p38 include the GCK-VI subfamily member SPAK and the GCK-VII subfamily members TA01 and TA02 (25–27). However, some have no apparent connection to known MAPK pathways. These include the GCK-II subfamily member MST1, the GCK-III subfamily members MST3 and MST4, the GCK-V subfamily member LOK, and the GCK-III subfamily member SOK-1 (28–32). Meanwhile, some GCKs have been reported to regulate F-actin structure, cell spreading, and apoptosis (21, 24, 33–36). Among novel functions that have been found, SPAK is reported to regulate the Na-K-Cl cotransporter (NKCC1), and a role in cell cycle control has been inferred for Stk10, which is a novel polo-like kinase (PLK) kinase (37–39).

We previously examined the MAPK cascade components used by two agents to stimulate JNK in Drosophila S2 cells (40). To extend these studies here, we have examined the potential involvement of putative MAP4Ks in regulating Drosophila JNK. We found that when the Ste20p relative Fray was knocked down using RNA interference, JNK activity stimulated by sorbitol decreased markedly, whereas ablation of other putative MAP4Ks decreased JNK activity little (CG4527) or not at all. These results suggested that Fray was the major MAP4K regulating the JNK pathway in response to sorbitol in S2 cells. We then wished to determine whether mammalian Fray homologs were MAP4Ks upstream of JNK. The kinases

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1 The abbreviations used are: MAP, mitogen-activated protein; MAPK, mitogen-activated protein kinase; MAP4K, MAPK kinase kinase; ERK, extracellular signal-regulated kinase; MEKK, MAPK/ERK kinase; JNK, c-Jun N-terminal kinase; PAK, p21-activated protein kinase; GCK, germinal center kinase; GST, glutathione S-transferase; HA, hemagglutinin; DMEM, Dulbecco’s modified Eagle’s medium; FBS, fetal bovine serum; dsRNA, double-stranded RNA; MBP, myelin basic protein; RT, reverse transcriptase.

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most closely related to Fray in the human genome are OSR1 (oxidant stress-responsive protein 1) and SPAK (Ste20/SPS1-related, proline alanine-rich kinase). SPAK has been reported to activate p38 but not JNK (25). Human OSR1 had been isolated but not characterized. Here, we report the characterization of human OSR1 and an initial analysis of its biochemical functions.

MATERIALS AND METHODS

Cloning, Subcloning, Mutagenesis, and Plasmids—Total RNA prepared from HeLa cells was subjected to RT-PCR with a pair of primers spanning the complete human OSR1 cDNA synthesized based on the NCBI data base. These primers were cloned into the GST-tagged bacterial expression vector pGEX-KG. This plasmid was used as the template for subsequent subcloning. Full-length OSR1 cDNA was also subcloned into pEFXFLAG-CMV and pCMV5-Myc. Fragments encoding OSR1-(1–433), OSR1-(1–344), OSR1-(1–291), and OSR1-(345–527) were amplified by PCR and subcloned into pGEX-KG, pPET7c (His$_6$ tag), pCMV5-Myc, and pVL11 as indicated. Kinase-dead mutants of OSR1 (OSR1KR) and fragments were generated by mutating lysine 46 in the ATP binding pocket to arginine. All constructs were transformed into the bacterial strain TG-1 and grown at 30 °C to reduce the frequency of mutations. All clones were sequenced to confirm correct amplification.

The plasmids pCEP4-HA-ERK2, pCEP-HA-JNK1, pCEP-HA-p38, and pCEP4-HA-ERK5 were described previously (41). Constructs encoding pCMV-Myc-Y59Cdc42, pCMV-Myc-PAK1 (rat sequence), pCMV-Myc-PAK1 H83L/H86L, pCMV-Myc-PAK1 L107F, and GST-PAK1-(1–231), (1–231) H85L/H86L, (1–231) L107F, (1–231) D532A (kinase-dead), (1–122), (1–231), (1–231) D532A (kinase-dead), (1–544) K298A (kinase-dead) were described previously (10). Plasmids encoding GST-PAK1-(1–92), (1–86–231), (1–100), and (1–231) were constructed by inserting the appropriate PCR products into pGEX-KG. Mutations, including T82A, T82E, and T109A/T113A of PAK1, were generated with the QuikChange site-directed mutagenesis kit (Stratagene).

Proteins and Antibodies—All GST and His$_6$ epitope tagged fusion proteins were expressed in the bacterial strain BRL(DE3)pLys (Novagen). Cells were grown at 30 °C to A$_{600}$ of 0.5–0.6, and protein expression was induced with 0.5 mM isopropyl-1-thio-D-galacto-pyranoside at 30 °C for 4–6 h before harvest. Proteins were purified on glutathione-agarose or Ni$_2$-nitrilotriacetic acid-agarose, respectively, as described by the manufacturers. Myelin basic protein (MBP) was purchased from Sigma, GST-c-Jun, GST-MEF2C, GST-MEK1-(30–291), and the GST-MEK1 proline-rich insert (residues 265–301) were described as indicated (42–44).

The HA antibody (12CA5) was from Berkeley Antibody, and the anti-Myc antibody (9E10) was from the National Cell Culture Center, and both were used at a dilution of 1:1000 for immunoblotting. The monoclonal anti-FLAG antibody was from Sigma and was used at 1:5000 dilution. The anti-AAC antibody was from Santa Cruz Biotechnology and was used at 1:1000. The polyclonal anti-OSR1 serum (U5438) was raised against His$_6$-OSR1 (345–527) and was used at 1:8000.

Cell Culture and Transfection—HEK 293 and HeLa cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 1 mM l-glutamine, and 100 units/ml penicillin/streptomycin at 37 °C under 10% CO$_2$. HEK 293 cells were transfected using calcium phosphate as described (45). HeLa cells were transfected using FuGENE 6 following the manufacturer's protocol (Roche Applied Science). Drosophila S2 cells were cultured, and RNA interference was as described previously (40).

Fractionation and Immunofluorescence—Cell fractionation was as described (41). Immunofluorescence was as described (46) with the following changes. HeLa cells were grown to confluence and starved in medium with 0.5% FBS overnight. Cells on coverslips were fixed in 2% paraformaldehyde for 10 min, permeabilized in cold methanol at −20 °C for 10 min, and then incubated with anti-OSR1 U5438 antibody (1:800). After incubation with anti-rabbit secondary antibody (Alexa, 1:3000), the OSR1 localization was observed using a Zeiss AxioScope 2 plus fluorescence microscope.

Preparation of Tissue and Cell Lysates and Immunoblotting— Cultured cells or tissues from a 13-month-old mouse (provided by David Russell, Department of Molecular Genetics) were homogenized and lysed in Triton X-100 lysis buffer (20 mM Tris, pH 7.5, 150 mM NaCl, 0.5% Triton X-100, 0.1% sodium deoxycholate, 20 μg/ml aprotinin, 10 μg/ml pepstatin A, 10 μg/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride). Insoluble material was sedimented in a microcentrifuge for 15 min at 4 °C. Protein concentration was measured by Bradford assay using bovine serum albumin as standard. Thirty μg of soluble protein from each sample was resolved by SDS-PAGE and transferred to nitrocellulose membranes. Membranes were blocked with 5% non-fat milk (40) for 1 h at room temperature and then incubated with the appropriate antibody.

Immunoprecipitation, in Vitro Kinase Assays, and Phosphoamino Acid Analysis—Lysate protein (300 μg) was incubated with the indicated antibody for 1 h at 4 °C and then with 30 μl of a 50% slurry of protein A-Sepharose beads for 1 h. After three washes with detergent buffer (0.25 M Tris, pH 7.4, 1 mM NaCl, 0.1% Triton X-100, 0.1% sodium deoxycholate) and one with 10 mM HEPES (pH 7.6), beads were incubated with labeled substrates in 50 μl of 1× kinase buffer (20 mM HEPES, pH 7.6, 5 μM ATP (5 μCi of [γ-32P]ATP), 10 mM MgCl$_2$, 10 mM β-glycerol phosphate) at 30 °C for 30 min for kinase assays. Purified proteins were incubated with indicated substrates in 30 μl of 1× kinase buffer at 30 °C for 30 min. One-dimensional phosphoamino acid analysis was performed as described (47).

Yeast Two-hybrid Analysis—A neonatal mouse brain cDNA library (gift from Mark Henkemeyer, Center for Developmental Biology) in plasmid pGADG1 was screened as described.

RESULTS AND DISCUSSION

MAP4Ks and Activation of JNK in S2 Cells—In an earlier study, we examined the components of the protein kinase cascades that control JNK activity in response to lipopolysaccharide and sorbitol in Drosophila S2 cells (40). Both agents used both of the MAP2Ks MEK4 and MEK7 to activate JNK. Although lipopolysaccharide required a single MAP3K (DTAK), sorbitol employed four MAP3Ks to stimulate JNK activity. We have completed the examination of the likely kinase components of the MAPK module by testing the potential involvement of putative MAP4Ks in regulating Drosophila JNK. Based on sequence alignments and a consideration of the published classification of the fly protein kinases (48), six putative MAP4Ks, CG11228, DPAK, DPAK3, DMSN, CG4527, and Fray, were found to be expressed in S2 cells as determined by PCR analysis (data not shown). The expression of each of these was reduced using RNA interference, and the effect of the loss of each singly on activation of JNK by sorbitol was then examined (Fig. 1, A and B; data not shown). When expression of Fray, a Drosophila GCK-VI kinase family member, was knocked down, JNK activity stimulated by sorbitol decreased significantly. Reduction in expression of one of the other putative MAP4Ks, CG4527, decreased sorbitol-stimulated JNK activation to a small but reproducible extent. Reducing expression of CG4527 caused a further reduction in the residual JNK activation remaining upon suppression of Fray (Fig. 1C). These results suggested that Fray was the major MAP4K regulating the JNK pathway in response to osmotic stress in S2 cells. Interestingly, Fray is required for normal axonal ensheathment during fly development (49).

Structure and Expression of OSR1—We wished to learn whether mammalian homologs of Fray, SPAK and OSR1, also regulate the JNK pathway. SPAK has been reported to activate p38 but not JNK (25). Thus, we focused on analyzing OSR1, which has not been characterized previously as a protein. Human OSR1 contains 527 amino acids with a predicted molecular mass of 58 kDa. We isolated a cDNA encoding OSR1 by RT-PCR using HeLa RNA. It has a conserved Ste20p-like protein-serine/threonine kinase domain at its N terminus with a C-terminal region of undefined function. The OSR1 kinase domain has the highest identity to the kinase domain of SPAK (89%), Drosophila Fray (74%), and the Caenorhabditis elegans Y59A8B.23 gene product (71%). These four enzymes comprise the GCK-VI subfamily of Ste20p protein kinases. Two small regions of similarity were found in the C-terminal regions of GCK-VI subfamily members, which were named PF1 and PF2.

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domains. They may represent regulatory or targeting elements (25). A putative caspase 3 cleavage site (DEFD) is present at the end of the PF1 domain of OSR1 (Fig. 2).

To detect the expression of OSR1, a rabbit polyclonal antibody was generated against the C-terminal, poorly conserved region. The specificity of the anti-OSR1 antibody was confirmed by immunoblotting and immunoprecipitating overexpressed Myc-OSR1 in HEK-293 cells (data not shown). With this antiserum, a 58-kDa protein was recognized in all mouse tissues examined except thymus, including heart, spleen, liver, kidney, lung, testis, large intestine, small intestine, and stomach. OSR1 was also detected in mammalian cell lines including HEK 293, HeLa, cervix; H29, kidney; C2C12 and 3T3, mouse embryo fibroblasts; Cos-1, monkey kidney. B, subcellular localization of OSR1. Subcellular fractions from HeLa cells (from a cervical adenocarcinoma) were immunoblotted (IB) with OSR1 antibody U5438 or anti-Lamin A/C to identify the nuclear marker. C, immunofluorescence of OSR1. Left panel, OSR1; right panel, 4′,6-diamidino-2-phenylindole (DAPI) staining. Consistent with the broad transcription of OSR1 mRNA as deduced from Northern blotting (50).

To detect the subcellular localization of endogenous OSR1, proteins from soluble, particulate, and nuclear fractions derived from HeLa cells were immunoblotted with anti-OSR1 and anti-lamin A/C antibodies. OSR1 was detected in all three fractions, whereas lamin A/C was detected only in the nuclear fraction (Fig. 3B). Endogenous OSR1 detected by immunofluo-
rescence was distributed throughout HeLa cells, consistent with the fractionation data (Fig. 3C).

Protein Kinase Activity of OSR1—To verify that OSR1 is a serine/threonine protein kinase, recombinant wild type GST-OSR1 and the kinase inactive mutant GST-OSR1KR were expressed in bacteria and assayed with MBP as substrate. Wild type GST-OSR1 phosphorylated both MBP and itself, but GST-OSR1KR showed no detectable activity (Fig. 4A). Phosphoamino acid analysis of autophosphorylated GST-OSR1 revealed primarily phosphothreonine (Fig. 4B). Equal amounts of Myc-tagged OSR1 and OSR1KR expressed in HEK 293 cells were immunoprecipitated with the anti-Myc antibody and assayed with MBP as substrate (data not shown). A phosphorylated band at 58 kDa, representing autophosphorylated Myc-OSR1, and phosphorylated MBP were detected in assays with the wild type OSR1 immunoprecipitate. However, the same bands were also detected in assays with OSR1KR. Similar experiments performed with different epitope tags (3XFLAG) and different cell types (Cos-1 and HeLa) yielded similar results (data not shown). Because OSR1KR had no activity when expressed in bacteria and because MBP is phosphorylated by many abundant protein kinases, we conclude that the phosphorylation comes from contaminating proteins co-purifying with or non-specifically trapped in OSR1 in immune complexes; some of these may be kinases that normally phosphorylate OSR1. As a consequence, MBP was not a suitable substrate to measure OSR1 activity in cell lysates or immunoprecipitates, although it was useful to characterize the activity of the protein expressed in bacteria.

The PF1 Domain Is Necessary for OSR1 Kinase Activity—To examine the contribution of the C-terminal region to the kinase activity of OSR1, truncated forms of OSR1 were expressed as GST fusions in bacteria, and the purified proteins were assayed with MBP as substrate. The fainter bands migrating between MBP and the OSR1 fusion proteins are most likely partially degraded forms of the OSR1 fusion proteins.

Endogenous OSR1 is stimulated by sorbitol. OSR1 was immunoprecipitated with U5438 from lysates of HeLa cells treated as indicated. Autophosphorylation was examined. As shown in A, cells were treated with 0.5, 0.7, or 1 M sorbitol for 15, 30, 45, or 60 min. As shown in B, cells were treated with 0.5 M NaCl or sorbitol for 2, 5, 10, and 15 min.

OSR1 does not activate MAPK pathways. Empty vector (V), Myc-OSR1, OSR1KR, OSR1(1–433), or OSR1(1–344) (all 5 μg) were cotransfected with 1.5 μg of HA-ERK2, ERK5, or p38 or 5 μg of JNK1 in HEK 293 cells. After 24 h, cells were starved in DMEM with 0.5% FBS overnight. ERK2, ERK5, p38, and JNK1 were immunoprecipitated from lysates with anti-HA antibody and assayed with 1 μg of MBP, 1 μg of GST-MEF2C (ERK5 and p38), or 1 μg of GST-c-Jun as substrates, respectively. The amount of each MAPK in immune complexes was measured by immunoblotting with anti-HA antibody. The panel showing the MEF2C autoradiogram is from a long exposure so that weak bands might have been detected.
with MBP as substrate. Full-length OSR1, OSR1-(1–433) and OSR1-(1–344) have nearly identical kinase activity toward MBP or themselves. In contrast, OSR1-(1–291), a truncated protein with intact, conserved kinase domain but without the PF1 domain, has no detectable activity toward MBP or itself (Fig. 5). Thus, the PF1 domain is essential for OSR1 kinase activity. Many Ste20p-related kinases contain autoinhibitory domains; removal of the regulatory domains results in a significant increase in kinase activity due to loss of autoinhibition. This has been shown for PAKs, MST1, MST2, TAOs, and SOK1, for example (10, 26, 32, 51, 52). In the case of OSR1 and other GCK-VI kinases, the PF1 domain may comprise an essential part of the catalytic domain. Because there is no significant difference in the kinase activity of full-length OSR1 and OSR1-(1–433), the PF2 domain is apparently not involved in regulating catalytic activity.

**Cellular Stimuli That Activate OSR1**—To identify possible regulators of OSR1, a number of stimuli were tested on HeLa cells. Autophosphorylation was used to assess activity of endogenous, immunoprecipitated OSR1. Treatment with 0.5 M sorbitol for 30 min caused an obvious increase in OSR1 autophosphorylation. OSR1 was activated in the range of 0.5–0.7 M, with activation detectable from 15 to 60 min of treatment. Higher concentrations of sorbitol resulted in reduced activation (Fig. 6A). In contrast to strong activation of OSR1 by sorbitol, modest or no activation was caused by NaCl, anisomycin, okadaic acid, serum, nocodazole, Taxol, H2O2, phorbol ester, and epidermal growth factor (see below and data not shown). A time course of activation by NaCl showed that the greatest effect was at 15 min of NaCl treatment (Fig. 6B); weaker activation was detected at 2, 5, 10, and 30 min. The effect of sorbitol on OSR1 activity was consistently greater than that of NaCl under all conditions examined.

**OSR1 Does Not Activate Four Known MAPKs**—To determine whether OSR1, like Fray, is an upstream regulator of JNK or other MAPKs, Myc-OSR1, OSR1KR, or the fragments OSR1-
As shown in C, 3 μg of GST-OSR1 or GST-OSR1-(1–344) was assayed with 2 μg of GST-PAK1 truncations as substrates. Asterisks indicate the sizes of the substrates. As shown in B, 3 μg of GST-OSR1 or GST-OSR1-(1–344) was assayed with 2 μg of GST-PAK1-(1–231) or -(1–344) were co-expressed with HA-MAPKs, JNK, p38, ERK5, or ERK2 in HEK 293 cells. The HA-MAPKs were immunoprecipitated and assayed with GST-c-Jun, GST-MEF2C, or MBP as indicated. Neither JNK nor any of the other MAPKs were activated by coexpression with wild type or truncated OSR1 (Fig. 7). In addition, reduction of OSR1 expression by more than 75% with dsRNA oligonucleotides did not decrease JNK activation by sorbitol in HeLa cells (data not shown). Thus, it appears that OSR1 participates in distinct stress pathways from those controlling MAPKs. Alternatively, OSR1 may activate JNK or other MAPK pathways under conditions or in the presence of accessory proteins that we did not identify here.

OSR1 phosphorylates the N terminus of PAK1—To identify possible substrates of OSR1, a two-hybrid screen was performed using OSR1-(1–344) and the kinase-dead mutant as baits. The catalytic domain of PAK1 was identified as an interactor from both screens (data not shown). PAK1 is also a Ste20p-related kinase (Fig. 8A); the activity of the C-terminal kinase domain of PAK1 (residues 232–544) is inhibited by its N-terminal regulatory domain (residues 1–231) (10). However, PAK1 did not co-immunoprecipitate with OSR1 when both were overexpressed in 293 cells (data not shown). GST-OSR1 and OSR1 KR were assayed with a group of potential substrates including PAK1 fragments. Only MBP and GST-PAK1-(1–231) were phosphorylated of the proteins tested, which included histone H1, His6-OSR1-(345–527), PAK1 K298A, PAK1-(232–544) D406A, GST-c-Jun, GST-MEF2-C, MEK1KM, MEK3KM, MEK4KM, and an MEKK1 N-terminal fragment (30–220) (Fig. 8B and data not shown). Interestingly, full-length PAK1 K298A was not phosphorylated. The N-terminal domain of full-length PAK1 is protected through its interaction with the catalytic domain until inhibition is released by binding to GTP-ligated Cdc42 or Rac small G proteins, most likely accounting for the failure of full-length PAK1 to be phosphorylated (53). Because neither full-length OSR1 nor fragments were substrates of PAK1-(232–544) (Fig. 8C), PAK1 is apparently downstream not upstream of OSR1. The N terminus of PAK1 has several subdomains with regulatory functions; therefore, shorter truncated proteins were used as substrates to narrow the region that was phosphorylated. As shown in Fig. 8, A and B, fragments containing residues 75–132 were phosphorylated. Endogenous OSR1 activated by sorbitol was immunoprecipitated from HeLa cells and assayed using GST-PAK1-(1–132) as substrate. Phosphorylation of GST-PAK1-(1–132) by immunoprecipitated OSR1 correlated well with OSR1 autophosphorylation (Fig. 9D). GST-PAK1-(1–231) and -(75–132) were also phosphorylated by activated endogenous OSR1 (data not shown).

PAK1 is phosphorylated on Thr-84 by OSR1—Phosphoamino acid analysis of GST-PAK1-(1–231) revealed that threonine was phosphorylated by OSR1. Serine was only weakly phosphorylated in comparison (Fig. 9A). Five threonine residues, Thr-84, Thr-93, Thr-97, Thr-107, and Thr-109, lie in the fragment 75–132 (Fig. 8A). To determine which of these residues are phosphorylated, different PAK1 truncations were tested as substrates (Fig. 9B). GST-PAK1-(1–92), -(86–231), with 2 μg of GST-PAK1-(1–231), -(1–132), -(75–132), or T84A mutants as substrates. As shown in D, HeLa cells were untreated or treated with 0.5 mM sorbitol, 0.5 mM NaCl, 1 mM okadaic acid, 10 mM/μl anisomycin, DMEM with 10% FBS, DMEM with 30% FBS for 30 min, or 5 mM taxol or 2 mM nocodazole for 1 h. Endogenous OSR1 was immunoprecipitated from lysates and assayed with 2 μg of GST-PAK1-(1–132) or GST-PAK1-(1–132)T84A as substrates as indicated. Phosphorylation of GST-PAK1-(1–132) or GST-PAK1-(1–132) T84A by GST-OSR1-(1–344) is shown as a control.
-1 to 100) were phosphorylated. PAK1-(1–92) was phosphorylated within the first 100 residues of PAK1. However, as compared with the phosphorylation of wild type truncated protein (101–231), no decrease was detected in the phosphorylation of double mutant protein (101–231) T107A/T109A, suggesting that the trace serine phosphorylation occurs in this fragment. Consistent with the identification of Thr-84 as the PAK1 phosphorylation site, phosphorylation of several PAK1 fragments by recombinant and endogenous OSR1 was largely eliminated by mutating Thr-84 to Ala (Fig. 9, C and D).

The isolated N terminus of PAK1-(1–231) inhibits the activity of the PAK1 catalytic domain in vitro. Known activating mutations of PAK1, H83L/H86L and L107F, are located in the autoinhibitory domain. The inhibitory activity of GST-PAK1-(1–231) H83L/H86L and L107F mutants decreased markedly as compared with the activity of the wild type fragment (10, 54, 55). Because Thr-84 is also located in this region, we tested the idea that phosphorylation on this site may influence the autoinhibitory activity of the PAK1 N terminus. The inhibitory activity of GST-PAK1-(1–231) T84E, a possible phosphomimic of phosphorylated Thr-84, was measured. Results showed no difference in the inhibitory activity of wild type and T84E PAK1-(1–231) (Fig. 10A). PAK1-(1–231) previously phosphorylated by OSR1 to a stoichiometry of 0.4 mol of phosphate/mol inhibited the activity of PAK1 catalytic domain, as well as did unphosphorylated PAK1-(1–231) (data not shown). To determine whether the activity of PAK1 was influenced by OSR1 (or other MAPKs). It is, nevertheless, in a stress-sensitive pathway, because it is activated by sorbitol and NaCl. Interestingly, OSR1 phosphorylates another Ste20p family member (Ste20p family members). In contrast to our expectations based on studies in S2 cells, OSR1 did not activate mammalian JNK or other MAPKs. It is, nevertheless, in a stress-sensitive pathway, because it is activated by sorbitol and NaCl. Interestingly, OSR1 phosphorylates another Ste20p family member (Ste20p family members). In contrast to our expectations based on studies in S2 cells, OSR1 did not activate mammalian JNK or other MAPKs. It is, nevertheless, in a stress-sensitive pathway, because it is activated by sorbitol and NaCl. Interestingly, OSR1 phosphorylates another Ste20p family member (Ste20p family members). In contrast to our expectations based on studies in S2 cells, OSR1 did not activate mammalian JNK or other MAPKs. 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PAKs 1, -2, and -3, the three PAK family members that contain conserved small G protein binding domains, suggesting that OSR1 has the capacity to modulate the activation of all three of these PAKs. We have found that the C terminus of OSR1 binds to PAKs 1, -2, and -3, the three PAK family members that contain and comment about this work, Lisa Lenertz and Angelique Whitehurst regulating the actin cytoskeleton. Because OSR1 can phosphorylate PAK1 and bind to actin filaments, nucleates actin assembly, and severs actin filaments. Because OSR1 can phosphorylate PAK1 and bind to gelosin, we suggest that OSR1 may also have a function in regulating the actin cytoskeleton.

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