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Gastrointestinal hormones/neurotransmitters and growth factors can activate P21 activated kinase 2 in pancreatic acinar cells by novel mechanisms ☆

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ABSTRACT

P-21-activated kinases (PAKs) are serine/threonine kinases comprising six isoforms divided in two groups, group-I (PAK1–3)/group-II (PAK4–6) which play important roles in cell cytoskeletal dynamics, survival, secretion and proliferation and are activated by diverse stimuli. However, little is known about PAKs ability to be activated by gastrointestinal (GI) hormones/neurotransmitters/growth-factors. We used rat pancreatic acini to explore the ability of GI-hormones/neurotransmitters/growth-factors to activate Group-I-PAKs and the signaling cascades involved. Only PAK2 was present in acini. PAK2 was activated by some pancreatic growth-factors [EGF, PDGF, bFGF], by secretagogues activating phospholipase-C (PLC) [CCK, carbachol, bombesin] and by post-receptor stimulants activating PKC [TPA], but not agents only mobilizing cellular calcium or increasing cyclic AMP. CCK-activation of PAK2 required both high- and low-affinity-CCK1-receptor-state activation. It was partially reduced by PKC- or Src-inhibition, but not with PI3K-inhibitors (wortmannin, LY294002) or thapsigargin. IPA-3, which prevents PAK2 binding to small-GTPases partially inhibited PAK2-activation, as well as reduced CCK-induced ERK1/2 activation and amylase release induced by CCK or bombesin. This study demonstrates pancreatic acini, possess only one Group-I-PAK, PAK2. CCK and other GI-hormones/neurotransmitters/growth-factors activate PAK2 via small GTPases (CDC42/Rac1), PKC and SFK but not cytosolic calcium or PI3K. CCK-activation of PAK2 showed several novel features being dependent on both receptor-activation states, having PLC- and PKC-dependent/independent components and small-GTPase-dependent/independent components. These results show that PAK2 is important in signaling cascades activated by numerous pancreatic stimuli which mediate their various physiological/pathophysiological responses and thus could be a promising target for the development of therapies in some pancreatic disorders such as pancreatitis.

1. Introduction

The p21-activated kinases (PAKs) are a family of serine threonine kinases that are one of the main effectors for the small Rho GTPases, Cdc42 and Rac [1–3]. The PAK family consists of six members that are segregated into two subgroups (Group I and Group II) based on sequence homology [1,3–6]. Group-I-PAKs (PAK 1–3) are the most extensively studied and play an important role in signaling for many cellular processes, such as regulation of cell survival, apoptosis, cell motility, tumorigenesis, protein synthesis, glucose homeostasis, secretion and cellular proliferation [1–5,7–14]. The Group-I-PAKs normal tissue expression varies greatly between the three isoforms [15,16]. PAK2 is expressed in a wide variety of different tissues and can be thought of as ubiquitously expressed. In contrast, PAK1 has a slightly more reserved distribution, with high levels of PAK1 in tissues such as muscle, spleen, heart and liver. PAK3 has a much more restricted expression, being predominantly expressed in the brain, although recent reports have identify it in enteroendocrine cells [17]. Studies demonstrate that PAKs play an important signaling role in various tissues for a wide

Abbreviations: CCK, COOH-terminal octapeptide of cholecystokinin; TPA, 12-O-tetradecanoylphorbol-13-acetate; PAKs, p21-activated kinases; SFK, Src family of kinases; PKC, protein kinase C; PYK2, proline-rich tyrosine kinase 2; FAK, focal adhesion kinase; PKD, protein kinase D; PI3K, phosphatidylinositol-3-kinase; HRP, horseradish peroxidase; HGF, hepatocyte growth factor; IGF-1, insulin-like growth factor 1; bFGF, basic fibroblast growth factor; VIP, vasoactive intestinal peptide; CCK-JMV, CCK-JMV-180; EGF, epidermal growth factor; PDGF, platelet-derived growth factor; 8-Br-cAMP, 8-bromo-cyclic adenosine monophosphate; PLC, phospholipase C; ET, endothelin; CDC42, cell control division protein; RAC, Ras-related C3 botulinum toxin substrate; GFX, GFX109203X, PKC inhibitor; MAPK/ERK, mitogen-activated protein kinase; PKA, protein kinase A; IPA-3, 1,1′-dithiodi-2-naphthtol; Pir 3,5, 6,6′-dithiodi-2-naphthtol; PP2, 4-amino-5-(4-chlorophenyl)-7-(*t*-butyl)pyrazolo[3,4-d]pyrimidine; PP3, 4-amino-7-phenylpyrazol[3,4-d]pyrimidine; LY294002, 2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one.

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range of cellular stimuli including bioactive lipids [18,19], oncogenes [20], chemokines [21], growth factors [22–29], cellular stress [9,10], radiation [30], and with activation of some G-protein-coupled receptors [26,31]. However, there is little information on the activation of Group-I-PAKs by gastrointestinal (GI) hormones/neurotransmitters and GI growth factors in normal GI tissues. Pancreatic acinar cells possess receptors for a large number of GI hormones/neurotransmitters and growth factors and are an excellent model to study their cellular basis of action, because the agents also alter cellular function activating numerous cellular signaling cascades [32–34]. At present it is unclear if any Group-I-PAKs occur in pancreatic acinar cells, although it is known that at least 2 types (PAK1, 3) occur in pancreatic islets [16,35,36]. It is also unknown whether activation of any of the GI hormones/neurotransmitter receptors or GI growth factor receptors in these cells activates acinar cell PAKs, or if present, any information on the cellular signaling mechanisms involved.

To address this question in the case of the PAKs, in the present study we sought to determine whether the three members of the Group-I-PAKs were present in rat pancreatic acinar cells and if they are involved in mediating the cellular signaling of gastrointestinal hormones/neurotransmitters and of various gastrointestinal growth factors, which are known to alter pancreatic acinar cell function. Particular attention was paid to the GI hormone/neurotransmitter, cholecystokinin (CCK), because this is a well-studied physiological regulator of pancreatic acinar cell function with effects on secretion, growth, and enzyme synthesis as well as playing a prominent role in a number of pancreatic acinar cell pathophysiological processes [37,38].

2. Materials and methods

2.1. Materials

Male Sprague–Dawley rats (150–250 g) were obtained from the Small Animals Section, Veterinary Resources Branch, National Institutes of Health (NIH), Bethesda, MD. PAK1 recombinant protein was from Abnova (Walnut, CA). Recombinant PAK2 protein active was from Active Motif (Carlsbad, CA). Pak 3 recombinant human protein was from Life Tech. (Grand Island, NY). Pak 4 recombinant human protein was from MyBIosource (San Diego, CA). Rabbit anti-PAK1, rabbit anti-phospho-PAK1/2 (Thr402/423), and nonfat dry milk were purchased from Cell Signaling Technology, Inc. (Beverly, MA). Stabilized goat anti-rabbit IgG peroxidase conjugated was from Pierce Biotechnology, Inc. (Rockford, IL). Goat PAK2 antibody, and anti-goat-HRP-conjugate antibodies were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Pak 3 antibody was from Abcam (Cambridge, MA). Tris/HCl pH 8.0 and 7.5 were from Mediatech, Inc. (Herndon, VA). 2-Mercaptoethanol, protein assay solution, sodium lauryl sulfate (SDS) and Tris/Glycine/SDS (10×) were from Bio-Rad Laboratories (Hercules, CA). $MgCl_2$, $CaCl_2$, Tris/HCl 1 M pH 7.5 and Tris/Glycine buffer (10×) were from Quality Biological, Inc. (Gaithersburg, MD). Pak6 recombinant protein, minimal essential media (MEM) vitamin solution, amino acids 100×, Dulbecco's phosphate buffered saline (DPBS), glutamine (200 mM), Tris–Glycine gels, L-glutamine, and fetal bovine serum (FBS) were from Invitrogen (Carlsbad, CA). COOH-terminal octapeptide of cholecystokinin (CCK), hepatocyte growth factor (HGF), bombesin, insulin-like growth factor 1 (IGF-1), basic fibroblast growth factor (bFGF), vasoactive intestinal peptide (VIP), endothelin and secretin were from Bachem Bioscience Inc. (King of Prussia, PA). CCK-JMV-180 (CCK-JMV) was obtained from Research Plus Inc. (Bayonne, NJ). Epidermal growth factor (EGF), thapsigargin, platelet-derived growth factor (PDGF), vascular endothelial growth factor (VEGF), PAK5 active protein, deoxycholic acid and P21-activated kinase inhibitor 3 (IPA-3) were from Calbiochem (La Jolla, CA). Carbachol, insulin, dimethyl sulfoxide (DMSO), 12-O-tetradecanoylphobol-13-acetate (TPA), L-glutamic acid, glucose, fumaric acid, pyruvic acid, trypsin inhibitor, HEPES, TWEEN® 20, Triton X-100, GFX (GFX109203X), phenylmethanesulfonylfluoride (PMSF), ethylenediaminetetraacetic acid (EDTA), ethylene glycol tetraacetic acid (EGTA), sucrose, sodium-orthovanadate, and sodium azide were from Sigma-Aldrich, Inc. (St. Louis, MO). Albumin standard and Super Signal West (Pico, Dura) chemiluminescent substrate were from Pierce (Rockford, IL). Phadebas Amylase test was from Magle Life Science (Lund, Sweden). Protease inhibitor tablets were from Roche (Basel, Switzerland). Purified collagenase (type CLSPA) was from Worthington Biochemicals (Freehold, NJ). Nitrocellulose membranes were from Schleicher and Schuell Bioscience, Inc. (Keene, NH). L-364,718 (3S(−)-N-(2,3-dihydro-1-methyl-2-oxo-5-phenyl-1H-1,4-benzodiazepine-3-yl-1H-indole-2-carboxamide)) was from Merck, Sharp and Dohme (West Point, PA). YM022 ((R)-1-[2,3-dihydro-1-(2′-methyl-phenacyl)-2-oxo-5-phenyl-1H-1,4-benzodiazepin-3-yl]-3-(3-methylphenyl)urea), SR27897 (1-[[2-(4-(2-chlorophenyl)-thiazol-2-yl)aminocarbonyl] indolyl]acetic acid) and PIR 3,5 (IPA-3 inactive control) were from Tocris Bioscience (Ellisville, MO). Albumin bovine fraction V was from MP Biomedical (Solon, OH). NaCl, KCl and NaH_2PO_4 were from Mallinckrodt (Paris, KY).

2.2. Methods

2.2.1. Pancreatic acini preparation

Pancreatic acini were obtained by collagenase digestion as previously described [34]. Standard incubation solution contained 25.5 mM HEPES (pH 7.45), 98 mM NaCl, 6 mM KCl, 2.5 mM NaH_2PO_4, 5 mM sodium pyruvate, 5 mM sodium glutamate, 5 mM sodium fumarate, 11.5 mM glucose, 0.5 mM $CaCl_2$, 1 mM $MgCl_2$, 1 mM glutamine, 1% (w/v) albumin, 0.01% (w/v) trypsin inhibitor, 1% (v/v) vitamin mixture and 1% (v/v) amino acid mixture.

2.2.2. Acini stimulation

After collagenase digestion, dispersed acini were pre-incubated in standard incubation solution for 2 h at 37 °C as described previously [34,39]. After pre-incubation 1 ml aliquots of dispersed acini were incubated at 37 °C with or without stimulants. Cells were lysed in lysis buffer (50 mM Tris/HCl pH 7.5, 150 mM NaCl, 1% Triton X-100, 1% deoxycholate, 0.1% sodium azide, 1 mM EGTA, 0.4 mM EDTA, 0.2 mM sodium orthovanadate, 1 mM PMSF, and one protease inhibitor tablet per 10 ml). After sonication, lysates were centrifuged at 10,000× *g* for 15 min at 4 °C and protein concentration was measured using the Bio-Rad protein assay reagent.

2.2.3. Inhibition experiments

We used small inhibitor molecules to identify upstream activators of the CCK/TPA-mediated activation of PAK2. Isolated acini were preincubated for 15 min with IPA-3 that prevents binding of CDC42 to group I PAKs and its inactive analog PIR 3,5 as a control; with the PI3K inhibitors wortmannin or LY294002, also for 15 min and with the Src inhibitor, PP2, and its inactive analog PP3 for 1 h. Cells were then treated for 3 min with physiological (0.3 nM) or supraphysiological (100 nM) concentrations of CCK and also with TPA (5 min, 1 μM), with untreated cells for each pretreatment used as controls. After incubation, cells were processed as stated above.

2.3. Western blotting

It was performed as described previously [40]. Whole cell lysates were subjected to SDS-PAGE using 4–20% Tris–Glycine gels. After electrophoresis, proteins were transferred to nitrocellulose membranes. Membranes were blocked in blocking buffer (50 mM Tris/HCl pH 8.0, 2 mM $CaCl_2$, 80 mM NaCl, 0.05% Tween® 20, 5% non fat dry milk) at room temperature for 1 h. Membranes were then incubated with primary antibody overnight at 4 °C under constant agitation at antibody dilutions suggested by the supplier. After primary antibody incubation membranes were washed twice in blocking buffer for 4 min and then incubated with HRP-conjugated secondary antibody (anti-mouse,

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