Protein Kinase C-ζ and Phosphoinositide-dependent Protein Kinase-1 Are Required for Insulin-induced Activation of ERK in Rat Adipocytes* 

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The mechanisms used by insulin to activate the multifunctional intracellular effectors, extracellular signal-regulated kinases 1 and 2 (ERK1/2), are only partly understood and appear to vary in different cell types. Presently, in rat adipocytes, we found that insulin-induced activation of ERK was blocked (a) by chemical inhibitors of both phosphatidylinositol 3-kinase (PI3K) and protein kinase C (PKC)-ζ and (b) by transient expression of both dominant-negative Δp85 PI3K subunit and kinase-inactive PKC-ζ. Further, insulin effects on ERK were inhibited by kinase-inactive 3-phosphoinositide-dependent protein kinase-1 (PDK-1), and by mutation of Thr-410 in the activation loop of PKC-ζ, which is the target of PDK-1 and is essential for PI3K/PDK-1-dependent activation of PKC-ζ. In addition to requirements for PI3K, PKD-1, and PKC-ζ, we found that a tyrosine kinase (presumably the insulin receptor), the SH2 domain of GRB2, SOS, RAS, RAF, and MEK1 were required for insulin effects on ERK in the rat adipocyte. Our findings therefore suggested that PDK-1 and PKC-ζ serve as a downstream effectors of PI3K, and act in conjunction with GRB2, SOS, RAS, and RAF, to activate MEK and ERK during insulin action in rat adipocytes.

Mitogen-activated protein kinases, extracellular signal-regulated kinases (ERKs)1 and 2, are activated by insulin through a mechanism involving tyrosine phosphorylation of insulin receptor substrate (IRS) family members or SHC, followed by sequential activation of GRB2, SOS, RAS, RAF, and MEK, which phosphorylates threonine and tyrosine residues on ERK1/2 (1). Although ERK1/2 activation may occur independently of phosphatidylinositol 3-kinase (PI3K) in some cell types (2), inhibitors of PI3K have been reported to inhibit insulin-induced increases in ERK1/2 activity in a number of important cell types, including L6 myotubes (3), Chinese hamster ovary cells (4), rat adipocytes (5), 3T3/L1 adipocytes (6), rat brown fat cells (7), human hepatoma Hep3B cells (8), and rat hepatocytes (9). This apparent dependence of insulin-stimulated ERK1/2 activation on PI3K has been neither confirmed by other experimental approaches nor satisfactorily explained in relationship to other signaling factors. In this regard, PI3K has been suggested to function downstream (10, 11) or upstream (12) of RAS, but insulin does not appear to activate PI3K via RAS (13). Presently, in rat adipocytes, we confirmed that PI3K was required, along with GRB2, SOS, RAS, RAF, and MEK1, for insulin-induced activation of ERK2; moreover, we found that downstream effectors of PI3K, viz., 3-phosphoinositide-dependent protein kinase-1 (PDK-1) and protein kinase C (PKC)-ζ were also required for insulin-induced activation of ERK2.

EXPERIMENTAL PROCEDURES

Cell Incubations—As described (5, 14, 15), adipocytes were isolated by collagenase digestion of epididymal fat pads of 250-g male Harlan Sprague-Dawley rats, and suspended in glucose-free Krebs-Ringer phosphate (KRP) medium containing 1% bovine serum albumin. In some experiments, where indicated, the cells were equilibrated with 100 nM wortmannin (Sigma), 100 μM LY294002 (Alexis), or for 180 min with myristoylated PKC-ζ pseudosubstrate (see Ref. 14), (Quality Controlled Biochemicals Inc., Hopkinton, MA), or for 180 min with a GRB2 SH2 domain inhibitor, a phosphotyrosine pY mimetic, compound t-20d, an Nα-oxaryl-tripeptide containing a phosphomethylphenylalanine residue (see Ref. 16), and then treated with or without 10 nM insulin for 10 min (this time was optimal for observing changes in ERK).

Assay of Immunoprecipitable ERK—As described in previous studies (5, 15) of total mitogen-activated protein kinase activation, after incubation, adipocytes were sonicated in buffer containing 40 mM β-glycerophosphate (pH, 7.3), 0.5 mM dithiothreitol, 0.75 mM EGTA, 0.15 mM Na3VO4, 5 μg/ml leupeptin, 5 μg/ml aprotinin, 0.1 mM phenylmethylsulfonyl fluoride, and 5 μg/ml trypsin inhibitor. The resulting homogenates were centrifuged at 700 × g for 10 min to remove fat, cell debris, and nuclei. Post-nuclear supernatants were then supplemented with 0.154 mM NaCl, 1% Triton X-100, and 0.5% Nonidet, and equal amounts of lysate protein in each experiment (varying from 200 to 500 μg between experiments) were subjected to overnight immunoprecipitation at 4 °C with mouse monoclonal anti-ERK2 antibodies (Santa Cruz Biotechnologies, Inc., Santa Cruz, CA), which, as shown below, immunoprecipitated ERK1, as well as ERK2, despite the fact that these antibodies reacted only with ERK2 in Western analyses. Precipitates were collected on Protein-AG-agarose beads, washed and incubated for 10 min at 30 °C in 50 μl of buffer containing 25 mM β-glycerophosphate (pH, 7.3), 0.5 mM dithiothreitol, 1.25 mM EGTA, 0.5 mM Na3VO4, 10 μM MgCl2, 1 mg/ml bovine serum albumin, 1 μM okadaic acid, 0.1 mM [γ-32P]ATP (NEN Life Science Products; approximate specific activity, 1,500,000 dpm/mmol), and 50 μg of myelin basic protein (Sigma). After incubation, an aliquot of the reaction mixture was spotted on p81 filter paper, which was washed and counted for 32P radioactivity (5, 15).

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Adipocytes were treated first without inhibitors or with 100 nm wortmannin for 15 min, or with 50 μM myristoylated (MYR) PKC-ζ pseudosubstrate (PS) for 60 min, and second with or without 10 nx insulin for 10 min. ERK was immunoprecipitated with anti-ERK2 mouse monoclonal antibody, and precipitates resolved by SDS-polyacrylamide gel electrophoresis and blotted with a rabbit polyclonal anti-ERK antiserum that recognizes both ERK1 and ERK2. After chemiluminescence development, p44 ERK1 and p42 ERK2 bands were quantitated with Bio-Rad molecular analyst chemiluminescence/32P imaging system. Values are mean ± S.E. of four determinations. Note that the mean control value was set at a relative value of 1.00, and four blots were compared simultaneously on the same Bio-Rad molecular analyst chemiluminescence imaging screen, thus allowing the direct comparison of 4 sets of immunoprecipitations, with each set containing each treatment. See Fig. 1 for representative blots.

### Results and Discussion

Initially, we used inhibitors to identify factors required for insulin-induced activation of immunoprecipitable ERK2 in rat adipocytes. As seen in Fig. 2, PI3K inhibitors, wortmannin (100 nm) and LY294002 (100 μM) (i.e. in concentrations required to largely inhibit insulin-stimulated glucose transport in the rat adipocyte), and the MEK1 inhibitor, PD908059 (10 μM, which did not inhibit insulin-stimulated glucose transport), inhibited insulin-stimulated increases in immunoprecipitable ERK. Of particular interest, the cell-permeable myristoylated PKC-ζ pseudosubstrate inhibited insulin-induced increases in immunoprecipitable ERK activity over a concentration range comparable with that which is effective in inhibiting PKC-ζ (14). In this regard, diacylglycerol-dependent PKCs are not required for insulin-induced activation of ERK in rat adipocytes (see Ref. 15; presently, we also confirmed that phorbol ester-induced PKC down-regulation inhibited the acute effects of phorbol esters but did not inhibit insulin-induced activation of immunoprecipitable ERK; data not shown), and it is therefore clear that the PKC-ζ pseudosubstrate did not exert its effects through inhibition of diacylglycerol-dependent PKCs. These findings with inhibitors suggested that PI3K and PKC-ζ (or PKC-λ, which is 72% homologous to PKC-ζ and has an identical pseudosubstrate sequence), as well as MEK1, are required for insulin-induced activation of ERK in rat adipocytes.

Further evidence implicating PI3K in insulin-induced activation of ERK was obtained in experiments in which rat adipocytes were transiently co-transfected with plasmids encoding MYC-tagged ERK2 and a dominant-negative mutant form of the p85 subunit of PI3K, Δp85 P13K, and various forms of PKC-ζ and PKD-1 had relatively little or no significant effect on the levels of immunoprecipitable epitope-tagged ERK2. Also noted that only ERK2 was recovered in immunoprecipitates obtained with anti-HA and anti-MYC antibodies (Fig. 1).
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Fig. 1. Panel A, effects of insulin, wortmannin, and myristoylated PKC-ζ pseudosubstrate on immunoprecipitable ERK1 and ERK2. Panels B and C, effects of co-transfection of dominant negative forms of RAS, RAF, MEK1, Δp85 PI3K, SOS, and various forms of PKC-ζ and PKD-1 on immunoprecipitable epitope-tagged ERK2. In panel A, adipocytes were treated with or without 100 nM wortmannin (WM) for 15 min or 30 μM myristoylated PKC-ζ pseudosubstrate (ζ-PS) for 60 min before treatment with or without 10 ng insulin (INS) as indicated. In panels B and C, adipocytes were co-transfected with plasmids encoding WT, constitutive (Constit), dominant-negative (DN), KI, or other mutant (e.g. activation-resistant T410A PKC-ζ) signaling proteins, along with plasmid encoding HA- or MYC-tagged ERK2. After incubation, immunoprecipitates were prepared as described under “Experimental Procedures,” and all were blotted with a rabbit polyclonal antiserum that recognizes both ERK1 and ERK2. Note that both ERK1 and ERK2 were recovered in precipitates brought down by mouse monoclonal anti-ERK2 antibodies (A), whereas only ERK2 was recovered in precipitates brought down with rabbit polyclonal anti-MYC antiserum (B) and mouse monoclonal anti-HA antibody (C). Shown here are representative immunoblots, repeated at least 4 times with similar results. Levels of ERK1 and ERK2, as determined by quantitation of chemiluminescence in a Bio-Rad molecular analyst chemiluminescence/32P-imaging system, are given in Table 1.

In addition to PI3K, we found that SOS, RAS, and RAF were required for insulin-induced activation of ERK2 in rat adipocytes. As seen in Fig. 3, transient transfection of dominant-negative mutant forms of SOS, RAS, and c-RAF-1 inhibited the activation of co-transfected HA- or MYC-tagged ERK2 by insulin; in addition, constitutively active RAS markedly stimulated HA-ERK2 activity. In contrast to the c-RAF-1 mutant, transfection of a dominant-negative kinase-inactive mutant form of MEK1, which like RAF and PI3K (21) can interact with RAS (22), had no effect on basal or insulin-stimulated MYC-ERK2 (Fig. 3). Also, in contrast to inhibitory effects of dominant-negative RAS on insulin-induced activation of HA-ERK2, this RAS mutant did not inhibit the acute activating effects of phorbol esters on HA-ERK2 (data not shown), which may in some cell types occur independently of RAS (23). Thus, the inhibitory effects of both dominant-negative RAS and c-RAF-1 on insulin-induced activation of HA-ERK2 appeared to be specific.

The above findings suggested that PI3K along with SOS, RAS, c-RAF-1, and MEK1 was required for insulin-induced activation of ERK2 in the rat adipocyte. Because PKC-ζ (and/or λ) is known to serve as an effector of PI3K during insulin action in rat adipocytes (14) and other cells (24–27), and in view of the above-described inhibitor studies, we tested the possibility that PKC-ζ may function downstream of PI3K during ERK activation by transiently co-transfecting rat adipocytes with plasmids encoding HA-ERK2 and various forms of PKC-ζ. As seen in Fig. 4, whereas WT PKC-ζ had no effect on HA-ERK2 activity, both a KI form of PKC-ζ (K271N mutant) and an activation-resistant form of PKC-ζ (T410A mutant that cannot be activated by PDK-1; see Refs. 19 and 28) markedly inhibited insulin-stimulated HA-ERK2 activity but had little effect on basal or phorbol ester-stimulated HA-ERK2 activity. Moreover, the inhibitory effect of KI-PKC-ζ could be reversed (or prevented) by co-transfecting plasmid encoding WT-PKC-ζ, which alone had no effect on basal or insulin-stimulated ERK2. These findings suggested that the point-mutation per se in KI-PKC-ζ was responsible for its inhibitory effects on insulin-induced activation of ERK2 and further implied that the kinase activity of PKC-ζ is specifically required, presumably to phosphorylate a presently undefined substrate that is required for subsequent ERK2 activation in rat adipocytes.

Whereas mutant forms of PKC-ζ inhibited insulin-induced activation of HA-ERK2, constitutive PKC-ζ provoked insulin-like increases in HA-ERK2 activity, even in the absence of
insulin (Fig. 4). Further stimulatory effects of insulin on ERK2 activity in cells expressing constitutive PKC-z (Fig. 4) were also observed, and this may reflect the activation of endogenous PKC-z, or the fact that insulin can provoke further increases in activity of “constitutive” PKC-z.2

Because PDK-1, in conjunction with PI3K-dependent increases in PI-3,4,5-(PO4)3, has been reported to transmit activating signals from PI3K to PKC-z (19, 28) (note that we have confirmed that PDK-1 and its target in PKC-z, threonine-410, are required for PKC-z activation by insulin in rat adipocytes; see Ref. 29), we examined the role of PDK-1 in insulin-induced activation of ERK2 in transiently transfected rat adipocytes. As seen in Fig. 5, WT-PDK-1 enhanced basal HA-ERK2 activity, and KI-PDK-1 inhibited insulin-stimulated HA-ERK2 activity. Further, the inhibitory effect of KI-PDK-1 on insulin-stimulated ERK2 activity was reversed by co-transfection of WT-PDK-1 (Fig. 5), indicating that its kinase activity (presumably to phosphorylate Thr-410 in PKC-z, like that of PKC-z, is specifically required for insulin-induced activation of ERK2.

Our findings suggested that PI3K, PDK-1, and PKC-z, along with SOS, RAS, c-RAF-1, and MEK1 were required for ERK2 activation during insulin stimulation of rat adipocytes. In this regard, RAS is known to bind to the p110 subunit of PI3K (10, 11), and we presently found that both the p110 and p85 subunits of PI3K were recovered in RAS immunoprecipitates prepared from lysates of rat adipocytes (Fig. 6). However, we did not observe any effects of insulin on (a) p85 or p110 PI3K subunit levels in RAS immunoprecipitates or (b) PI3K enzyme activity recovered in RAS immunoprecipitates (Fig. 6). Moreover, as discussed above, the inhibitory effects of dominant-negative Δp85 on insulin-induced activation of ERK2 suggested that the activation of PI3K that is relevant to ERK activation requires input from a factor that is capable of activating the p85 subunit of PI3K, e.g. an IRS family member. Collectively, these findings suggested that RAS may serve in the localization but not in the activation of PI3K.

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Finally, we found that inhibitors of tyrosine kinase and the GRB2 SH2 domain, viz., genistein and a N-oxalyl-tripeptide-pY mimetic (see above and Ref. 16), respectively, also inhibited insulin-induced activation of immunoprecipitable ERK in the rat adipocyte (Fig. 7). These findings therefore suggested that a tyrosine kinase-dependent substrate, i.e. IRS and/or SHC, along with GRB2 and SOS, operate upstream of RAS in insulin-induced activation of ERK2 in rat adipocytes.

To summarize, our findings suggested that factors in two signaling pathways that are frequently considered to be functionally separate during insulin action, viz., the GRB2/SOS/RAS/RAF pathway and the PI3K/PDK-1/PKC-ζ pathway, are jointly required for insulin-induced activation of ERK in rat adipocytes. Although there are still gaps in our understanding of how these pathways are activated and interact with each other, it seems likely that one or more activated forms of IRS family members acts upon a specific pool of PI3K that operates in conjunction with GRB2/SOS and RAS. This PI3K apparently contains or subsequently recruits RAS, PI3K, PDK-1, PKC-ζ and RAF. Further studies are needed to more precisely define (a) how PI3K and RAF operate with respect to each other and (b) the role of PKC-ζ in insulin-induced activation of ERK.

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