PYK2 as a Mediator of Endothelin-1/Gα11 Signaling to GLUT4 Glucose Transporters*

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Endothelin-1 (ET-1) signaling through Gαq/11 stimulates translocation of intracellular GLUT4 glucose transporters to the plasma membrane of 3T3-L1 adipocytes by an unknown mechanism that requires protein tyrosine phosphorylation and ADP-ribosylation factor 6 (ARF6) but is independent of phosphatidylinositol 3-kinase (PI3-kinase)1 and the 3′,5′-cyclic adenylate cyclase (CADTK)-related non-kinase (CRNK) is a dominant negative form of PYK2 containing the C-terminal portion of PYK2 as a required signaling element in the regulation of GLUT4. Insulin, ET-1, and Gαq(q209L), the constitutively active mutant of Gαq, all trigger GLUT4 translocation as well as the formation of cortical F-actin (9), thought to be required for optimal GLUT4 recycling to the plasma membrane. However, insulin and ET-1 apparently regulate these processes via discrete signaling pathways. In addition to PI3-kinase independence, ET-1 and Gαq(q209L), but not insulin, stimulate cortical F-actin polymerization and GLUT4 translocation through a mechanism that requires ARF6 (9, 11).

Interestingly, the actions of both insulin and ET-1/ARF6 are blocked by microinjection of anti-phosphotyrosine antibody into 3T3-L1 adipocytes (12) or by treatment of cells with tyrosine kinase inhibitors (6). However, different sets of proteins are tyrosine-phosphorylated by ET-1 and insulin. In the case of insulin stimulation, many of these proteins have been identified, including the insulin receptor itself and insulin receptor substrate proteins (6), whereas the identity and the function of the tyrosine-phosphorylated proteins in ET-1-treated cells have not yet been characterized. We identify here two such proteins, the Ca2+-sensitive protein tyrosine kinase PYK2 and its substrate paxillin, and show that PYK2 functions selectively in the ET-1 signaling pathway that leads to GLUT4 glucose transporter translocation to the plasma membrane.

EXPERIMENTAL PROCEDURES

Materials—Endothelin-1 was purchased from the American Peptide Co. pcDNA-CADTK-WT-Myc, pcDNA-CADTK(K457A)-KD-Myc, and pcDNA-CRKNK-Flag were kindly provided by Dr. H. S. Earp at the University of North Carolina at Chapel Hill. The GLUT4 cDNA with a C-terminal EGFP and an exofacial Myc tag was cloned into pcDNA-CADTK-WT-Myc, pcDNA-CADTK(K457A)-KD-Myc, and pcDNA-CRKNK-Flag. After transfection into 3T3-L1 adipocytes, PYK2 was overexpressed in a Myc-tagged kinase-deficient PYK2 protein were acutely directed to F-actin-rich adhesion sites from the adiocyte cytoplasm in response to ET-1 but not insulin. CADTK-related non-kinase (CRNK) is a dominant negative form of PYK2 containing the C-terminal portion of the protein, which binds paxillin but lacks the PYK2 autophosphorylation site (Tyr402). CRNK expression in 3T3-L1 adipocytes inhibited ET-1-mediated F-actin polymerization and translocation of MYG-tagged GLUT4 enhanced green fluorescent protein (EGFP) to the plasma membrane without disrupting insulin action on these processes. These data reveal the tyrosine kinase PYK2 as a required signaling element in the regulation of GLUT4 recycling in 3T3-L1 adipocytes by ET-1, whereas insulin signaling is directed through a different pathway.

Insulin acts through a receptor tyrosine kinase to stimulate the uptake of glucose in fat and muscle cells by a mechanism that involves the translocation of specialized intracellular vesicles containing GLUT4 glucose transporters to the plasma membrane (1, 2). Although the signaling mechanism by which insulin mobilizes GLUT4 is not clear, it requires the p85/p110 type phosphatidylinositol 3-kinase (PI3-kinase) and the 3′,5′-cyclic adenosine monophosphate (cAMP) pathway.

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The abbreviations used are: PI3-kinase, phosphatidylinositol 3-kinase; PI, phosphatidylinositol; PtdIns(4,5)P2, phosphatidylinositol 4,5-bisphosphate; ET-1, endothelin-1; ARF6, ADP-ribosylation factor 6; CADTK, calcium-dependent tyrosine kinase; CRNK, CADTK-related non-kinase; WT, wild type; KD, kinase-deficient; FITC, fluorescein isothiocyanate; EGFP, enhanced green fluorescent protein; GTPγS, guanosine 5′-O-(thiotriphosphate); PYK2, proline-rich tyrosine kinase 2.
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**Fig. 1.** Endothelin-1 stimulates tyrosine phosphorylation of PYK2 and paxillin in 3T3-L1 adipocytes. Serum-starved 3T3-L1 adipocytes were left untreated (Basal) or treated with 10 nM ET-1 or 100 nM insulin for 10 min. The lysates were immunoprecipitated (IP) with 4G10 antibody and analyzed by silver staining (A) and immunoblotting (IB) with 4G10 antibody (B). The PYK2 peptide sequence was obtained by mass spectrometry. Asterisks indicate PYK2 (upper) and paxillin (lower).

The reduction signal of the silver-stained band at 70 kDa supports this idea (Fig. 1B, lower asterisk). Paxillin, a 70-kDa adapter protein and regulator of the actin cytoskeleton, has been reported to be tyrosine-phosphorylated by PYK2.

**RESULTS AND DISCUSSION**

To identify proteins that are tyrosine-phosphorylated in response to ET-1, 3T3-L1 adipocytes were left untreated or treated with either ET-1 or insulin, and then cell lysates were immunoprecipitated with the monoclonal anti-phosphotyrosine antibody, 4G10. As shown in Fig. 1B, insulin and ET-1 stimulated tyrosine phosphorylation of distinct sets of proteins. As expected, the major tyrosine-phosphorylated bands in the insulin-treated sample migrated at positions identical to those of insulin receptor substrates 1/2 (Fig. 1B, IRS-1/2) and the insulin receptor (IR), whereas two major tyrosine-phosphorylated proteins with apparent molecular sizes of 120 and 70 kDa were immunoprecipitated from the lysates of ET-1-treated cells. The 120-kDa protein was clearly visible when the 4G10 immunoprecipitates were analyzed by SDS-PAGE and silver staining (Fig. 1A). Upon tryptic digestion followed by mass spectrometry, the band was shown to contain a peptide (NLL-DAVQAK) corresponding to the calcium-dependent protein tyrosine kinase PYK2/CADTK/RAFTK/RAFTK/CAKp/PAK2 (15).

Fig. 2A shows that the PYK2 protein specifically immunoprecipitated with PYK2 antibody is indeed tyrosine-phosphorylated in 3T3-L1 adipocytes treated with ET-1 but not in control cells. Insulin had little or no effect on PYK2 tyrosine phosphorylation in several experiments. PYK2 is activated mainly by autophosphorylation at the tyrosine residue Tyr402, which can be induced by intracellular calcium (16, 17). Tyrosine phosphorylation of PYK2 in intact cells has been reported in response to various stimuli, including G protein-coupled receptor ligands (16), stress signals (18), and cell adhesion (19). Interestingly, this protein kinase was also identified as the major protein tyrosine phosphorylated upon incubation of permeabilized 3T3-L1 adipocytes with GTPγS (12), suggesting that GTPγS may also stimulate the PYK2 tyrosine phosphorylation via activation of Gα11.

The protein migrating at 70 kDa (Fig. 1B), selectively tyrosine-phosphorylated in response to ET-1 treatment of adipocytes, was not observed as a unique ET-1-specific band in the silver-stained gels (Fig. 1A). Western blotting suggests that this tyrosine-phosphorylated protein undergoes a significant mobility shift upon stimulation with ET-1, perhaps because of chemical modifications such as tyrosine and serine/threonine phosphorylation. The reduced signal of the silver-stained band at 70 kDa supports this idea (Fig. 1A, lower asterisk). Paxillin, a 70-kDa adapter protein and regulator of the actin cytoskeleton, has been reported to be tyrosine-phosphorylated by PYK2...
and displays a similar dispersed banding pattern on SDS-PAGE (20). Therefore, we examined whether paxillin is tyrosine-phosphorylated by ET-1 in 3T3-L1 adipocytes by immunoblotting paxillin precipitates with anti-phosphotyrosine antibody. Fig. 2B shows that ET-1 but not insulin stimulates the tyrosine phosphorylation of paxillin in cultured adipocytes as detected with this method. The same result was obtained by blotting 4G10 immunoprecipitates with anti-paxillin antibody (Fig. 2B). The similarly smeared banding patterns of this protein in Figs. 1B and 2B further imply that the 70-kDa protein is paxillin.

Based on these results, the localization of PYK2 and paxillin proteins in 3T3-L1 adipocytes were analyzed by immunofluorescence microscopy, and their responses to ET-1 and insulin were investigated. Fig. 2C shows that endogenous paxillin displays a rather diffuse distribution near the cell surface of unstimulated cultured adipocytes, and little F-actin is observed. ET-1 treatment of the adipocytes caused a marked recruitment of paxillin to focal adhesions, where paxillin decorated the tips of ET-1-induced cortical F-actin seen at the bottom surface of the cells. In contrast, insulin caused the formation of very thin and shorter actin fibers, with no change in paxillin localization. Endogenous PYK2 could not be detected by immunofluorescence microscopy in 3T3-L1 adipocytes using commercial antibodies. However, when the Myc epitope-tagged protein was expressed in adipocytes, PYK2 also dramatically localized to focal adhesions in response to ET-1 but not insulin (Fig. 3).

To test whether PYK2 is involved in F-actin polymerization downstream of ET-1, two dominant inhibitory mutants were utilized. The kinase-deficient PYK2 (PYK2-KD) bears a point mutation (K457A) in the kinase domain and displays minimal tyrosine kinase activity (17). A second mutant (CRNK for CADTK-related non-kinase) contains only the C-terminal portion (Met865–Glu1009) of PYK2, which spans the putative focal adhesion targeting domain. The mutant lacks the major tyrosine phosphorylation site (Tyr402) but retains the ability to bind paxillin in vitro. CRNK expression has also been shown to effectively abolish the autophosphorylation of PYK2 in intact cells (17). When electroporated into 3T3-L1 adipocytes, the wild type PYK2, PYK2-KD, and CRNK all showed similar diffuse cytoplasmic localization in the basal state (Fig. 3). In response to ET-1 stimulation, PYK2-KD, like PYK2-WT, localized to focal adhesions and did not inhibit cortical F-actin formation.

Recent work by our laboratory (9, 21) has demonstrated a requirement of intact F-actin for optimal GLUT4 recycling to the plasma membrane in response to either insulin or ET-1 in 3T3-L1 adipocytes. Because we observed that PYK2 is required for F-actin polymerization in response to ET-1 in the cultured adipocytes (Fig. 3), we tested the hypothesis that ET-1 signaling to GLUT4 requires PYK2-mediated F-actin formation. Adipocytes were electroporated with a GLUT4 construct tagged with both a C-terminal EGFP and a Myc epitope (15). The majority of cells expressing Myc-GLUT4-EGFP that displayed anti-Myc (red bars) and a similar dispersed banding pattern on SDS-PAGE (20). Therefore, we examined whether paxillin is tyrosine-phosphorylated by ET-1 in 3T3-L1 adipocytes by immunoblotting paxillin precipitates with anti-phosphotyrosine antibody. Fig. 2B shows that ET-1 but not insulin stimulates the tyrosine phosphorylation of paxillin in cultured adipocytes as detected with this method. The same result was obtained by blotting 4G10 immunoprecipitates with anti-paxillin antibody (Fig. 2B). The similarly smeared banding patterns of this protein in Figs. 1B and 2B further imply that the 70-kDa protein is paxillin.

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ablated cells (Fig. 4, blue bars). In the basal condition, Myc-GLUT4-EGFP was detected mostly in the perinuclear region in adipocytes, whereas the Myc staining was at a minimal background level. Expression of CRNK in the 3T3-L1 adipocytes had no effect on the intracellular distribution of Myc-GLUT4-EGFP in this condition. When cells were stimulated by insulin, Myc-GLUT4-EGFP rims at the plasma membrane were clearly visible in the majority of cells both by the EGFP signal and Myc staining (~80%, Fig. 4). ET-1 also stimulated the translocation of Myc-GLUT4-EGFP to the plasma membrane, yet to a lesser extent than insulin in about 50% of the Myc-GLUT4-EGFP transfected cells. In both basal and insulin-stimulated conditions, CRNK expression exerted no effect on Myc-GLUT4-EGFP distribution or the numbers of cells with signal intensity at cell rims. In contrast, CRNK expression almost completely blocked Myc-GLUT4-EGFP translocation in response to ET-1 but not insulin action and that cortical F-actin polymerized in response to insulin and ET-1 plays a crucial role in the membrane recycling of GLUT4.

The findings reported here indicating a requirement for PYK2 in ET-1 action on GLUT4 are consistent with previous reports that the inhibitory effects of CRNK on ET-1 signaling are not due to nonspecific interference of cellular functions. Rather, these data support the hypothesis that PYK2 is required selectively for ET-1 but not insulin action and that cortical F-actin polymerized in response to insulin and ET-1 plays a crucial role in the membrane recycling of GLUT4. PYK2 in ET-1-stimulated GLUT4 Translocation

ARF6 also activates phospholipase D, generating phosphatidic acid, another activator of inositol 4-phosphate 5-kinase α (35). PtdIns(4,5)P(2) in turn regulates multiple proteins involved in actin dynamics (36). Thus, ARF6 may be downstream of PYK2 and paxillin in the ET-1 signaling pathway, perhaps causing cortical actin filament formation, thought to be required for optimal GLUT4 exocytosis (9, 21). Further experiments will be required to test this important hypothesis and to clarify the molecular details related to the role of F-actin in GLUT4 exocytosis.

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