Targeting of PED/PEA-15 Molecular Interaction with Phospholipase D1 Enhances Insulin Sensitivity in Skeletal Muscle Cells*

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Phosphoprotein enriched in diabetes/phosphoprotein enriched in astrocytes (PED/PEA-15) is overexpressed in several tissues of individuals affected by type 2 diabetes. In intact cells and in transgenic animal models, PED/PEA-15 overexpression impairs insulin regulation of glucose transport, and this is mediated by its interaction with the C-terminal D4 domain of phospholipase D1 (PLD1) and the consequent increase of protein kinase C- activity. Here we show that interfering with the interaction of PED/PEA-15 with PLD1 in L6 skeletal muscle cells overexpressing PED/PEA-15 (L6_PED/PEA-15) restores insulin sensitivity. Surface plasmon resonance and ELISA-like assays show that PED/PEA-15 binds in vitro the D4 domain with high affinity (KD = 0.37 ± 0.13 μM), and a PED/PEA-15 peptide, spanning residues 1–24, PED-(1–24), is able to compete with the PED/PEA-15-D4 recognition. When loaded into L6_PED/PEA-15 cells and in myocytes derived from PED/PEA-15-overexpressing transgenic mice, PED-(1–24) abrogates the PED/PEA-15-PLD1 interaction and reduces protein kinase C- activity to levels similar to controls. Importantly, the peptide restores insulin-stimulated glucose uptake by ~70%. Similar results are obtained by expression of D4 in L6_PED/PEA-15. All these findings suggest that disruption of the PED/PEA-15-PLD1 molecular interaction enhances insulin sensitivity in skeletal muscle cells and indicate that PED/PEA-15 as an important target for type 2 diabetes.

PED/PEA-15 (1) is a ubiquitously expressed protein that controls cell proliferation and death (2–6). It has been found that PED/PEA-15 is overexpressed 2- to 3-fold in skeletal muscle, adipose tissue, fibroblasts, and white blood cells from a large population of type 2 diabetic individuals and their first degree relatives (7, 8). In cellular and animal models, PED/PEA-15 overexpression affects both insulin-stimulated glucose transport and glucose-stimulated insulin secretion (7, 9–11). In particular, forced expression of PED/PEA-15 in muscle and adipose cells to levels comparable to those occurring in type 2 diabetes severely impairs insulin-stimulated glucose transport (11) and cell-surface recruitment of GLUT4, a major insulin-sensitive glucose transporter (7). Furthermore, transgenic mice for PED/PEA-15 display impaired glucose tolerance and develop diabetes, if fed a high fat diet (9). All these observations suggest that PED/PEA-15 overexpression could be involved in the complex series of events ultimately leading to type 2 diabetes, one of the most common disorders in the world associated with impaired insulin action and secretion and for which no single defect has been so far unequivocally determined (12–14).

PED/PEA-15 is a 130-amino acid protein containing an α-helical-rich DED domain at its N terminus, whereas the C-terminal 40 residues appear largely unstructured (15). Two consensus serine phosphorylation sites have been identified at the C terminus of the protein (Ser105 and Ser110), and phosphorylation by protein kinase C (PKC)3 calmodulin kinase II, and AKT/protein kinase B has been shown to occur in different cells types and to contribute to the regulation of PED/PEA-15 protein stability (16–18). However, mutation experiments show that PED/PEA-15 phosphorylation, which is responsible for PED/PEA-15 antiapoptotic function (17, 18), is not directly involved in changes to its gluco-regulatory functions (7, 11), suggesting the implication of a different mechanism. PED/PEA-15 has been found to be an interactor of the human phospholipase D1 (PLD1), an interaction which promotes PLD1 activity. Although the mechanism by which this occurs is still unknown, it has been observed that increasing PED/PEA-15

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3 The abbreviations used are: PKC, protein kinase C; PLD1, phospholipase D1; HA, hemagglutinin; DMEM, Dulbecco’s modified Eagle’s medium; FITC, fluorescein isothiocyanate; PBS, phosphate-buffered saline; Trx, thioredoxin; TCEP, tris(carboxymethyl)phosphine; TEV, tobacco etch virus; LC-MS, liquid chromatography-mass spectrometry; MS/MS, tandem MS; RP-HPLC, reversed phase-high performance liquid chromatography; Fmoc, N-(9-fluorenyl)methoxycarbonyl; RU, relative unit(s); ELISA, enzyme-linked immunosorbent assay; Tg, transgenic; Wt, wild type.
Targeting of the PED/PEA-15-PLD1 Interaction

cellular abundance lengthens PLD1 persistence in the cell rather than increasing its enzymatic activity (9, 19).

PLD1 is widely distributed in animals, plants, fungi, and bacteria and is implicated in several cellular processes, including receptor signaling, control of vesicular trafficking, and glucose transport (20). Furthermore, phospholipase D catalyzes the hydrolysis of the phosphodiester bond of glycerophospholipids to generate phosphatidic acid, an intracellular messenger implicated in a wide range of cellular processes. Phosphatidic acid can also be converted to other mediators, such as lysophosphatidic acid and diacylglycerol (21), with the latter being a major activator of the conventional PKC isoforms. Indeed, in PED/PEA-15 overexpressors, both diacylglycerol concentration (9) and PKC-α activity (11) are constitutively increased. It has also been shown that overactivation of PKC-α negatively regulates the activity of PKC-ζ (11), a major controller of insulin-stimulated glucose transport. Therefore, disrupting the interaction between PED/PEA-15 and PLD1 by a cell-penetrating compound may represent a novel strategy for improving insulin sensitivity in target cells.

Using a two-hybrid screen, the shortest PED/PEA-15-interacting region of PLD1 (residues 712–1074) has been identified and named D4 (19). Random mutagenesis studies have also revealed that PED/PEA-15 contacting residues are spread over the entire D4 sequence (19). Nevertheless, to date, very little is known about the PED/PEA-15-PLD1 interaction. The impact on insulin sensitivity, for instance, has been only indirectly validated by blocking PED/PEA-15 or PLD1 expression using antisense approaches or PLD1 and PKC-α activities with pharmacological inhibitors (7–11).

As a proof of principle, using L6 skeletal muscle cells stably overexpressing PED/PEA-15 (L6PED/PEA-15), we show here that, by disrupting PED/PEA-15-PLD1 binding with protein fragments involved in the protein–protein interaction, the PED/PEA-15 downstream signaling and the negative effects on glucose uptake are largely reversed. Indeed, D4 expression in L6PED/PEA-15 reduces the interaction between PLD1 and PED/PEA-15, lowers PKC-α activation, and restores insulin effect on glucose uptake. Consistently, the PED/PEA-15 region entailed in PLD1 recognition and identified following an approach of protein fragmentation and fractionation, when incorporated into L6PED/PEA-15 cells, is capable of displacing the PED/PEA-15-PLD1 interaction and increasing insulin-stimulated glucose transport.

EXPERIMENTAL PROCEDURES

pcDNA3 Vector Preparation—Cloning of PED/PEA-15 cDNA in pcDNA3 was previously described (22). The D4 cDNA was amplified by PCR from HeLa cells in a reaction mixture with the Taq-Long Expand PCR System (Roche Diagnostics, Mannheim, Germany) and with 5'-CGATCAAGTTCGCTGAGGAA-TCTTCCAAATAAAT-3' and 5'-CGATCTCTAGAGGATAGCG-AGAATGCGTCAGG-3' primers. The PCR product (1.1 kb) was cloned into pcDNA3-HA vector carrying HindIII-XbaI restriction sites and sequenced (Primm, San Raffaele Biomedical Science Park, Milan, Italy).

A deletion mutant of D4 (named dmD4), spanning residues 929–1030 and with the two cysteines replaced by serines, was used as negative control in all experiments. Site-directed mutagenesis was performed using the QuickChange site-directed mutagenesis kit from Stratagene, according to the producer’s protocol. Mutant oligonucleotides complementary to the single-stranded DNA and encoding the specific mutation were as follows: 5'-GGACTTCTGGCTCACATGCTTTAGGGTGTC-3' and 5'-GGAACAACCTAAGAGCTGTAGCCGAAAGTCC-3', and 5'-GACAAGGTTTCCGTTCCCTCCACTGATGAAAG-3' and 5'-CTCTCATCATTGGGAGGACCGGAAACCTTTGTC-3'. Mutations were confirmed by nucleotide sequencing. dmD4 was then subcloned into the pcDNA3-HA expression vector.

Cell Culture, Transfections, Western Blot Analysis, Co-immunoprecipitation Assay, and 2-Deoxy-D-glucose Uptake—L6 rat skeletal muscle cells were grown in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal calf serum, 10,000 units/ml penicillin, 10 mg/ml streptomycin, and 2% l-glutamine (Invitrogen), in a 5% humidified CO2 incubator. Stable transfections were performed by selecting positive clones with G418 (Calbiochem) at the effective dose of 0.8 mg/ml. Cellular loading of FITC-conjugated peptides was performed with cationic lipid mixture Pro-Ject™ Protein Transfection Reagent kit according to the manufacturer’s instructions (Invitrogen). Transient transfections were performed using the Lipofectamine Plus according to the manufacturer’s instructions (Pierce). Production of rabbit polyclonal PED/PEA-15 antiserum, Western blot and co-immunoprecipitation analyses, and 2-deoxy-D-glucose uptake measurement were performed as previously reported (7, 23, 24, 11).

Mouse Primary Fibroskeletal Muscle Cell Culture—Skeletal muscle biopsies were obtained after WT and Tq PED/PEA-15 mice were sacrificed by pentobarbitone overdose, as previously described (25). The biopsies were collected in cold phosphate-buffered saline (PBS) supplemented with 1% PenSt (100 units/ml penicillin and 100 μg/ml streptomycin), dissected, finely minced, and transferred to a digestion solution (0.015 g of Collagenase IV, 8% 10× trypsin, 0.015 g of bovine serum albumin, 1% PenSt, in DMEM supplemented with 10% fetal calf serum, 10,000 units/ml penicillin, 10 mg/ml streptomycin, and 2% l-glutamine), then and incubated with gentle agitation at 37 °C for 15–20 min. Thereafter, undigested tissue was allowed to settle, and supernatant was collected and mixed with DMEM supplemented with 20% fetal calf serum and 1% PenSt. The remaining tissue was digested for a further 15–20 min at 37 °C with fresh digestion solution. The resultant supernatant was then pooled with the previous cells and centrifuged for 10 at 350 × g. The cell pellet was resuspended in DMEM supplemented with 20% fetal calf serum and 1% PenSt and was then seeded and grown in culture flask. After this, medium was again changed to DMEM supplemented with 10% fetal calf serum and 1% PenSt. Before any experiment, cells were serum-starved for 16 h and stimulated with insulin at specific times and concentrations as indicated.

PKC-α Activity Assay—PKC-α activation was measured by evaluating PKC-α Ser-657 phosphorylation. Western blot assay was performed with a specific pPKC-α antibody purchased from Upstate Biotechnology Inc. (Lake Placid, NY), and the intensity of the spots was evaluated by densitometric analysis, using Scion Image Analyzer software. All data were expressed as mean ± S.D.
Statistical Analysis—Data were analyzed with StatView software (Abacus Concepts) by one-factor analysis of variance. *p* values of <0.05 were considered statistically significant.

PLD Assay in Intact Cells—PLD activity was evaluated by measuring phosphatidic acid and phosphatidylbutanol levels (26). Cells were labeled with $5 \mu$Ci of $[^{14}C]$palmitic acid for a 60-mm dish and incubated for 16 h at 37 °C in a 5% CO$_2$-enriched, humidified atmosphere. Cells were then washed twice in HHBG buffer (10 mm HEPES, 1.26 mm CaCl$_2$, 0.5 mm MgCl$_2$, 0.4 mm MgSO$_4$, 5.37 mm KCl, 137 mm NaCl, 4.2 mm Na$_2$HPO$_4$, 1% (v/v) bovine serum albumin, 10 mm glucose, pH 7.4) and incubated in 0.3% (v/v) butan-1-ol in HHBG buffer for 20 min at 37 °C. Subsequently, cells were treated with or without 100 nm phorbol myristate acetate for 30 min. After incubation, buffer was removed and 0.5 ml of ice-cold methanol was added to each cell. Cell debris was scraped into a glass vial and kept on ice. Cellular lipids were obtained using the Bligh and Dyer procedure and spotted onto Whatman TLC plates. Labeled products were separated by TLC using the upper phase of a mixture of ethyl acetate/2,2,4-trimethylpentane/acetic acid/H$_2$O in the ratio of 13:2:3:10 (v/v). Positions of the spots corresponding to $[^{14}C]$phosphatidylbutanol were determined by autoradiography. The area containing phosphatidylbutanol was scraped, and radioactivity was counted.

Fluorescence Microscopy—Cells were grown onto glass cover-slips in 6-well multidishes and allowed to adhere for 24 h. Cells were washed with PBS (137 mm NaCl, 2.7 KCl mm, 10 mm Na$_2$HPO$_4$, 2 mm KH$_2$PO$_4$, pH 7.4) and fixed with 3% paraformaldehyde. After additional washes with PBS, the cover-slips were stained with 4,6-diamidino-2-phenylindole to identify nuclei and the area of interest was imaged using a fluorescence microscope (Axiovert, Carl Zeiss).

**PED/PEA-15 Expression and Purification—**PED/PEA-15 cDNA, cloned into pETM-30 (EMBL, Heidelberg, Germany), was transformed into BL21 (DE3) cells (Novagen). Expression of GST-His$_6$-PED/PEA-15 was induced in 100 ml of LB with 1 mm isopropyl 1-thio-β-δ-galactopyranoside (IPLP), for 3 h at 37 °C. Using a 1-ml His-trap column (GE Healthcare), the fusion protein was eluted and subsequently treated for 16 h at 37 °C in the presence of tobacco etch virus (TEV) protease (1:100 enzyme:substrate ratio in 50 mM Tris-HCl, pH 8, buffer). The digested protein was loaded again on the His-trap column, and PED/PEA-15 was recovered in the flow-through. PED/PEA-15 was further purified on a 1-ml Mono Q HR 5/5 column in the presence of 1 mm isopropyl 1-thio-β-δ-galactopyranoside for 3 h at 37 °C. The entire fusion protein was purified on 1 ml of Chelating Sepharose Fast Flow resin (GE Healthcare), and PED/PEA-15 was then separated from the stuffer protein after digestion with TEV protease. A further passage on Chelating Sepharose Fast Flow resin allowed us to obtain PED/PEA-15 as the only protein unbound to the resin.

**PED/PEA-15 Trypsin Digestion—**PED/PEA-15 proteolysis was performed by digestion with 1:1-tosylamido-2-phenylthyl chloromethyl ketone-treated trypsin (Sigma-Aldrich) at an enzyme:substrate ratio of 1:100 (w/w) in 50 mm Tris-HCl, pH 7.5, for 16 h. Tryptic digests were separated into seven fractions on a Phenomenex Jupiter C18 column ($1 \times 25$ cm, 10 μm) and analyzed by using a 50-μm inner diameter C18 BioBasic column (ThermoFisher). LC-MS was performed using a LCQ DCA XP Ion Trap spectrometer (ThermoElectron). This was equipped with an Opton electrospray ionization source (operating at a needle voltage of 4.2 kV and at a temperature of 320 °C) and a complete Surveyor HPLC system (including an MS pump, an autosampler, and a photo diode array). Mass spectra were recorded continuously between the mass interval 400–2000 atomic mass units in positive mode and data-dependent analysis to fragment the eluted peptides and to obtain...
Targeting of the PED/PEA-15-PLD1 Interaction

**Figure 2. Effect of D4 transfection in L6 and L6 PED/PEA-15 cells.**

A. pcDNA3-HA-D4, pcDNA3-HA-dmD4, and the control plasmid pcDNA3-HA were transfected in both L6 and L6 PED/PEA-15 cells, and protein lysates were analyzed by Western blot with anti-HA (Upstate Biotechnology) and anti-PED/PEA-15 antibodies. B. 300 μg of protein lysates from cells transfected with pcDNA3-HA-D4, pcDNA3-HA-dmD4, or pcDNA3-HA was immunoprecipitated with anti-PED/PEA-15 antibodies and probed with anti-PLD1 or anti-PED/PEA-15 antibodies. The shown autoradiographs are representative of five independent experiments. C. PKC-α activation was assessed by immunodetection of phosphorylated PKC-α in protein lysates from L6 and L6 PED/PEA-15 cells, transfected with either pcDNA3-HA or with pcDNA3-HA-D4 or pcDNA3-HA-dmD4, as indicated. *Bar graphs* represent mean ± S.D. of data obtained from the densitometric analysis of three independent experiments in triplicate. Asterisks denote statistically significant differences (***, p < 0.001). D. L6 and L6 PED/PEA-15 cells, transfected with either pcDNA3-HA or with pcDNA3-HA-D4 or pcDNA3-HA-dmD4, as indicated, were incubated with 0 and 100 nM insulin and then assayed for 2-deoxyglucose uptake. *Bars* represent the means ± S.D. of triplicate determinations in four independent experiments. Asterisks denote statistically significant differences (**, p < 0.01).

Sequence information. Fragmentation was induced on selected ions from 400 to 1600 atomic mass units, with a fixed 35% of total energy. Multicharge spectra were then deconvoluted by means of selected multiple charged ions from 400 to 1600 atomic mass units, with a fixed 35% of total energy. Multicharge spectra were then deconvoluted using a computer program (Phylogram). All masses were reported as average values.

**Peptide Synthesis**—Peptides were synthesized using solid phase chemistry on a fully automated peptide synthesizer AAPTECH 348 W (Advanced Chemtech), following standard Fmoc methodology. Protected N-α-Fmoc-amino acid derivatives and coupling reagents for peptide synthesis were from Inbios (Pozzuoli, Italy). All syntheses were carried out on a 50-μmol scale, using a RINK AMIDE resin (substitution, 1.1 mmol/g). After on-resin assembly, peptides were cleaved with a trifluoroacetic acid-H2O-triisopropylsilane mixture (500 μl/100 mg of resin) and purified by RP-HPLC. All peptides were characterized by LC-MS to assess purity and identity. PED-(1–24), -(36–54), and -(72–83) were produced with free acid C termini and obtained by tryptic treatment of the synthetic PED-(1–30), -(33–56), and -(71–92) fragments, respectively. The hydrolyzed peptides were then separated by semi-preparative RP-HPLC. PED-(1–24) and -(79–112) were also N-terminally fluoresceinated synthesized for cellular experiments, utilizing FITC linked to a β-alanine residue. Semi-preparative RP-HPLC was used to obtain highly pure molecules (>95%) by RP-HPLC analysis.

**SPR Analysis**—PED/PEA-15 was immobilized on a CM5 sensor chip using standard amine coupling procedures, as described by the manufacturer’s instructions (Pharmacia Biosensor AB), to obtain a final 11,000 RU immobilization level. All assays were carried out at 25 °C, at a flow rate of 30 μl/min in 50 mM sodium phosphate, 150 mM NaCl, 1 mM TCEP, pH 7.4, buffer. Data were processed using BIAevaluation software, version 4.1 (BIACore Technologies, Inc.).

For competition experiments with fractions derived from trypsin-treated PED/PEA-15, 20 μl of each fraction was preincubated with 1 μM Trx-His6-D4 in a final volume of 100 μl (assuming that 0.13 μmol of each peptide was recovered after fractionation, the theoretical peptide concentration for these experiments was 260 μM) at 25 °C for 30 min and then injected. In the co-injection method, 15 μl of each pool (diluted 1:1 in running buffer) was injected after injection of 60 μl of 1 μM Trx-His6-D4. For competition experiments with the synthetic PED/PEA-15 peptides, 1 μM Trx-His6-D4 was preincubated with 30 μM of each peptide at 25 °C for 30 min before injection.

**Enzyme-linked Immunosororbent Assays**—All assays were performed using an integrated platform for High-Throughput Screening (Hamilton Robotics) comprising a fully equipped Starlet 8 channel liquid handler, a robotic arm, a washer, and a multiwavelength plate reader. PED/PEA-15 was biotinylated using EZ-Link NHS-Biotin reagents, following the manufacturer’s protocol (Pierce); LC-MS confirmed biotinylation. 100 μl of a 0.5 μM solution of Trx-His6-D4 was coated onto the wells for 16 h at 4 °C; wells filled with 100 μl of 0.5 μM Trx-His6 were used as blank control. Each data point was in triplicate. After washing with PBS-NaCl buffer (300 mM NaCl, 2.7 mM KCl, 10 mM Na2HPO4, 2 mM KH2PO4, 0.004% Tween 20, pH 7.4), wells
were blocked with a 1% w/v solution of bovine serum albumin in PBS. Biotinylated-PED/PEA-15 at a fixed concentration of 1.5 μM was preincubated at 25 °C with 7.5 μM peptide competitors for 30 min in 50 mM sodium phosphate, NaCl 300 mM, TCEP 1 mM, 0.004% Tween 20, pH 7.5, buffer and added to the washed wells. Following 1-h incubation in the dark at 37 °C, solutions were removed, and wells were again washed with PBS-NaCl buffer. 100 μl of a 1 mg/ml solution of streptavidin-horseradish peroxidase (Sigma-Aldrich) was added to each well, and the plate was left to incubate for 1 h at 37 °C in the dark. After removal of the enzyme solution, Sigma-fast o-phenylenediamine dihydrochloride Tablet Set (Sigma-Aldrich) was used, and absorbance at 490 nm was monitored. Values were properly averaged, subtracting the corresponding blank lanes.

RESULTS

Effects of PED/PEA-15 Overexpression on Phospholipase D1 in L6 Cells—Preparation of L6 skeletal muscle cells (L6) stably transfected with PED/PEA-15 cDNA (L6PED/PEA-15) was previously described (7). In these cells, overexpression of PED/PEA-15 was paralleled by a consistent increase of PLD1 cellular content (Fig. 1A). Similarly, detection of PLD1 in PED/PEA-15 immunoprecipitates was increased more than 3-fold in L6PED/PEA-15, as compared with untransfected L6 controls (Fig. 1B). To further address if the larger amount of PLD1 was accompanied by an increase in its enzymatic activity, we measured the levels of transphosphatidylbutanol generated by the PLD1-mediated reaction. As shown by TLC analysis (Fig. 1C), both basal and phorbol myristate acetate-stimulated PLD1 activity was increased in L6PED/PEA-15 compared with L6 cells (Fig. 1D).

Effects of D4 Expression on PED/PEA-15 Interaction with PLD1 and on Glucose Uptake—Following reverse transcription-PCR amplification of D4 from HeLa cells, the cDNA was inserted into the pcDNA3 vector in-frame with the HA epitope, and the entire construct (pcDNA3-HA-D4) was transfected in L6 and in L6PED/PEA-15 cells. The HA-tagged D4 domain was expressed at comparable levels both in L6 and L6PED/PEA-15 cells (Fig. 2A). Interestingly, HA-D4 expression significantly reduced the amount of PLD1 co-precipitated with PED/PEA-15, as compared with untransfected L6 controls (Fig. 2B). In addition, HA-D4, but not the control vector, reduced the activity of PKC-α in L6PED/PEA-15 by ~60% (Fig. 2C). The expression of HA-D4 in L6PED/PEA-15 cells was also accompanied by a recovery of the effect of insulin on glucose uptake. Indeed, insulin treatment failed to increase glucose uptake in L6PED/PEA-15 cells, which instead was largely rescued in cells expressing the HA-D4 construct, at levels comparable to those observed in L6 control cells (Fig. 2D). Transfection of a deletion mutant of D4 (dmD4, see also

TABLE 1
PED/PEA-15 peptides identified by LC-MS/MS analysis of HPLC fractions derived from tryptic digestion of PED/PEA-15

The peptide in fraction 6 contains the extra dipeptide Gly-Ala at its N terminus derived from cloning. The relative amount within the fractions was calculated by comparing peak areas from the corresponding extracted ions.

| Fraction number | PED/PEA15 peptides | Relative amount |
|-----------------|--------------------|----------------|
| 0               |                    |                |
| 1               |                    |                |
| 2               | 123LAPPPKKA<sup>120</sup> | 92% |
| 3               | 12<sup>1</sup>EDIPSEK<sup>29</sup> | 18% |
| 4               | 89<sup>1</sup>SEEDLDYT<sup>98</sup> | 75% |
| 5               | 10<sup>1</sup>IKDIIR<sup>113</sup> | 25% |
| 6               | 72<sup>1</sup>RDPLLTVMVYDR<sup>123</sup> | 38% |
| 7               | 26<sup>1</sup>SEETTDGSAWSFleshnk<sup>36</sup> | 26% |
| 8               | 56<sup>1</sup>LSDKNLSYIEHIFIER<sup>71</sup> | 18% |
| 9               | 36<sup>1</sup>DNLSYIEHIFIER<sup>71</sup> | 14% |
| 10              | 36<sup>1</sup>SEETTDGSAWSFleshnkldk<sup>36</sup> | 4% |
| 11              | 2<sup>1</sup>GAMAEPYGGTLQDPLNNTLDLQLK<sup>54</sup> | 100% |

FIGURE 3. SPR analysis of the interaction between PED/PEA-15 and Trx-His<sub>6</sub>-D4. Increasing concentrations of Trx-His<sub>6</sub>-D4 (2.6, 1.7, 1.3, and 0.65 μM) were injected over the PED/PEA-15-derivatized CM5 sensor chip in 50 mM sodium phosphate, 150 mM NaCl, 1 mM TCEP, pH 7.4, buffer at 25 °C, at a flow rate of 30 μl/min. A 5 μM solution of Trx-His<sub>6</sub>-dmD4 was also injected and no binding was detected.

FIGURE 4. SPR-based competition experiments using HPLC fractions derived from trypsin-generated PED/PEA-15 peptides. Results were reported as (RU/RU<sub>0</sub>)<sup>*</sup>100, where RU is the maximum response (RU<sub>max</sub>) for a given competitor and RU<sub>0</sub> is the RU<sub>max</sub> without competitors.
Targeting of the PED/PEA-15-PLD1 Interaction

“Experimental Procedures”) in L6PED/PEA-15 cells (Fig. 2A) did not affect either the interaction between PED/PEA-15 and PLD1 (Fig. 2B) or the constitutive activation of PKC-α (Fig. 2C) and the uptake of 2-deoxyglucose (Fig. 2D). It should be also noted that L6PED/PEA-15 cells displayed a higher basal glucose uptake (7, 11), which is decreased by the expression of HA-D4 (Fig. 2D). These findings led us to hypothesize that the D4 fragment could bind PED/PEA-15 preventing its interaction with the full-length PLD1 and restoring insulin action.

TABLE 2
List of synthesized peptides

| Synthetic peptides | Secondary structure |
|--------------------|---------------------|
| 1MAEYGTLLQDLTNN15 | α plus part of α2  |
| 1MAEYGTLLQDLTNN15 | α1                  |
| TLEDLEQKSAED24    | α2                  |
| RPDLLTMVVDYR29    | Part of α6         |
| 71RPDLLTMVVDYR29  | α6                  |
| 34SEEITGSAWFSEHSN15 | Part of α3 plus α4 |
| 33SEEITGSAWFSEHSN15 | α3 plus α4      |
| 40TTGSANWFSEHSNK24 | α4                  |
| 53NKLKDNLNISYEHFESIRPDRLL17 | α5 plus part of α6 |
| 79VVYTRTVKLISSEDDELTKLTRIPSAKKYDII122 | Part of α6 |
| 116QPSEEIIK22     | Unstructured       |

PED/PEA-15-D4 in Vitro Binding Assay—The in vitro interaction between PED/PEA-15 and D4 was analyzed by SPR using a BLACore 3000 system. To set up this assay, both PED/PEA-15 and D4 were recombinantly expressed in an E. coli expression system. PED/PEA-15 was preliminarily expressed using the pETM30 vector; following purification and digestion with TEV protease to remove the fusion partners, ~20 mg of PED/PEA-15 per liter of initial culture was purified. Instead, the D4 domain of PLD1 was expressed and fused at its N terminus to a thioredoxin A and a histidine tag for affinity purification (Trx-His); yield was low (~1 mg of purified protein per liter of culture), but it was sufficient for our experiments. Notably, any attempt to cleave D4 from its fusion partner failed or led to a highly unstable product, so the whole protein (named Trx-His-D4) was used in all subsequent experiments. Due to the high number of cysteines, purifications and subsequent manipulations were all performed in 1 mM TCEP-containing buffers. The protein was purified to homogeneity by two subsequent steps of affinity purification and ion-exchange chromatography.

PED/PEA-15 was thus immobilized on the surface of a CM5 sensor and, to exclude any interaction between PED/PEA-15 and the fusion partner of D4, solutions of Trx-His6 (expressed and purified independently) at concentrations up to 3.6 μM, were firstly injected on the PED/PEA-15-derivatized microchip. Under these conditions, no interaction was detected between PED/PEA-15 and Trx-His6. Then increasing amounts of Trx-His6-D4 were analyzed using a TCEP-containing buffer. Trx-His6-D4 exhibited dose-dependent association curves (Fig. 3), characterized by slow association and dissociation rates (dissociation constant of 0.37 ± 0.13 μM). As a further control, the deletion mutant dmD4, also fused to Trx-His6, was not able to associate to immobilized PED/PEA-15. Thus, in vitro the D4 domain of PLD1 selectively interacts with PED/PEA-15.

Identification of D4-binding PED/PEA-15 Region—To delineate PED/PEA-15 sub-domains involved in D4 binding, we extensively hydrolyzed PED/PEA-15 with trypsin to obtain short peptide fragments of known size and sequence. To this aim, a protein aliquot (0.13 μmol, 2.0 mg) was treated with the enzyme at a 1:100 ratio for 16 h at 37 °C. A small aliquot (500 ng) was analyzed by LC-MS/MS to assess the completion of the reaction and to identify fragments, before adding trifluoroacetic acid to stop the hydrolysis reac-

**FIGURE 5.** A, ELISA-based competition assay. Competitors were used at a fixed concentration corresponding to a 5-fold molar excess over the soluble biotin-PED. Results were reported as (A/A0)*100, where A is the average optical density from the triplicate data for a given competitor and A0 is the average optical density determined without competitors. Competition by ELISA assay was performed twice, and the average results are reported. B, dose-dependent inhibition by peptides PED-(1–24) and PED-(79–112) (used as negative control) are shown by plotting (A/A0)*100 versus the logarithm of molar concentration of added peptide.
Tryptic fragments were then fractionated by RP-HPLC recovering six separate aliquots that were subsequently lyophilized, dissolved in 100 \( \mu \)l of \( \text{H}_2\text{O} \), and characterized by LC-MS/MS (Table 1); hence PED/PEA-15 peptide fragments will be indicated as PED (fragment numeration). PED tryptic fragments PED-(25–28), -(84–88), -(99–107), and -(114–122) were not recovered within the HPLC fractions, whereas fractions 0, 1, and 4 contained no peptides.

All fractions were subsequently used in competition experiments of PED/PEA-15-D4 binding on the BIAcore 3000 system. At first, they were tested for their capacity to bind the immobilized protein to exclude possible false negative results originating from signal compensation effects. No evident interactions were recorded with all tested samples (data not shown).

Competition was carried out using two different approaches. Initially, peptide competitors were preincubated with Trx-His\(_6\)-D4 and injected on the chip (incubation method). As shown in Fig. 4, Fraction 6, containing the fragment Gly-Ala-PED-(1–24) with the dipeptide Gly-Ala on the N terminus derived from the vector linker, reduced binding by 44%, suggesting an active role of this peptide in preventing protein-protein contact. Fraction 5, containing peptides spanning the region 36–83, reduced the binding of Trx-His\(_6\)-D4 to immobilized PED/PEA-15 by 30%. Fractions 2 and 3, containing peptides from the C-terminal region and the peptide 29–35, had no significant effects on protein-protein interaction. In a second approach, the six fractions were instead injected at the end of the association phase with D4, and their relative capacity to displace the bound ligand were evaluated (co-injection method). Again, peptide Gly-Ala-PED-(1–24) confirmed its capacity to interfere with the PED/PEA-15-D4 recognition (Fig. 4), although this method appeared generally less sensitive than the previous one.

To further investigate the ability of these fragments to compete with PED/PEA-15 binding to D4, we prepared synthetic peptides corresponding to some of the tryptic fractions. These peptides are listed in Table 2 along with the secondary structure they correspond to (15). In particular, we synthesized peptide PED-(1–24), found in Pool 6 (without the N-terminal dipeptide Gly-Ala), and peptides PED-(72–83) and PED-(36–54), corresponding to the major components of Pool 5. Furthermore, we synthesized a panel of new peptides corresponding to most PED/PEA-15 helices that were only partially represented by the tryptic fragments (PED-(1–15), PED-(16–30), PED-(71–92), PED-(33–56), and PED-(40–56)).

We also prepared peptides PED-(53–77) and PED-(79–112), which cover the PED/PEA-15 region 53–112 previously hypothesized (21) as the shortest sequence interacting with D4, and PED-(114–122), which is the largest fragment not recovered after PED/PEA-15 digestion. Again, to exclude possible false negative results, these peptides were preliminarily assayed for their capacity to associate with the on-chip immobilized protein. For this purpose, 30 \( \mu \)M solutions of each peptide were injected on the chip and the binding signal recorded. Of note, peptide PED-(71–92) was able to interact with the full-length PED/PEA-15 protein; therefore, this peptide was not considered in subsequent experiments. The remaining peptides were instead utilized in the SPR-based competition assay using the incubation method. Data obtained by this approach confirmed the ability of peptide PED-(1–24) to interfere with PED/PEA-15-D4 recognition.

To confirm these data, we set up a competition experiment based on an ELISA-like assay whereby Trx-His\(_6\)-D4 was immobilized on the microwell surface, and biotinylated PED/PEA-15 (biotin-PED) was instead used as the soluble binder. Initially dose-dependent binding of PED/PEA-15 to immobilized Trx-His\(_6\)-D4 (0.5 \( \mu \)M) was assessed, observing signal saturation for concentrations of biotin-PED higher than 2 \( \mu \)M (not shown). Peptides at a 5-fold molar excess over biotin-PED were then used to disrupt the binding. As shown in Fig. 5A, synthetic PED-(1–24) was again the most efficacious competitor, whereas PED-(1–15) and PED-(16–30) were only slightly active and were not further investigated. To further assess the capacity of PED-(1–24) to interfere with the interaction of the
Targeting of the PED/PEA-15-PLD1 Interaction

As a control, treatment with FITC-conjugated PED-(79–112) had no effect. To further investigate the functional effects of PED-(1–24) on PED/PEA-15 downstream signaling, we measured PKC-α activity and glucose uptake. Basal activity of PKC-α in L6 cells was not significantly modified by the presence of PED-(1–24) peptide; by contrast, in the L6PED/PEA-15 cells, the same peptide reduced PKC-α activity to levels comparable to those measured in control L6 cells (Fig. 7B). At variance, the control PED-(79–112) peptide had no effect on PKC-α. Furthermore, treatment of L6PED/PEA-15 cells with PED-(1–24) restored insulin-induced activation of PKC-ζ (Fig. 7C). Activation of Akt, instead, was not affected by the presence of PED-(1–24) (Fig. 7D). This is consistent with previous observations that PED overexpression does not impinge on insulin-induced PI3K and Akt activities (7, 11).

Effect of PED-(1–24) on Primary Myocytes from PED/PEA-15 Transgenic Mice—The effects of the PED-(1–24) peptide were finally tested in primary myocytes derived from quadriceps muscles of transgenic (Tg) mice overexpressing PED/PEA-15 (9) and their wild-type littermates. Interestingly, peptide delivery in Tg myocytes led to a drastic reduction of PKC-α activation. No significant reduction of PKC-α activity was observed in WT myocytes. This is consistent with previous observations that PED overexpression does not impinge on insulin-induced PI3K and Akt activities (7, 11).

DISCUSSION

Diabetes is a widespread disease with more than 150 million individuals affected worldwide. Of these, most are affected by the type 2 form (source: American Diabetes association, see www.diabetes.org), which is characterized by resistance to insulin action on glucose metabolism. No single defects have been identified for this disease, rather a multitude of concurrent alterations contribute to disease onset and progression...
Either D4 transfection or PED-(1–24) loading into intact L6PED/PEA-15 cells and in myocytes from PED/PEA-15 Tg mice, disrupt PED/PEA-15-PLD1 interaction. Noticeably, PED/PEA-15 Tg mice are insulin-resistant and highly susceptible to diabetes (9). In addition, as previously reported, overexpression of PED/PEA-15 in skeletal muscle cells, impairs insulin action on glucose uptake without affecting the early steps of insulin signaling (7, 11). We now provide the first experimental evidence that blocking the protein-protein interaction between PED/PEA-15 and PLD1 is sufficient to impair the molecular mechanisms triggered and maintained by PED/PEA-15 overexpression. Indeed, ectopic expression of the PLD1 D4 domain abolishes binding of the full-length parental protein to PED/PEA-15 and restores basal PKC-α activity and normal insulin-stimulated glucose uptake. Consistently, loading of the L6PED/PEA-15 cells with the PED/PEA-15 N-terminal peptide responsible of the D4 (PLD1) recognition also rescues insulin action. It must be underscored that, although targeting a protein-protein interaction might generally be largely more difficult than finding kinase or phospholipase inhibitors, directly blocking these other players can provide several deleterious outcomes. Indeed, PKC-α plays important roles in many different cellular processes, including cell proliferation, cell cycle checkpoint, cell adhesion, and cell volume control (28). For example, impairment of PKC-α activity can affect cardiac function, because it has been identified as a major regulator of cardiac contractility and calcium handling in myocytes (29). Likewise, PLD1 has pleiotropic roles in many aspects of cell regulation, including proliferation, survival, and vesicular transport exocytosis (20). Thus, by disrupting the interaction with PED/PEA-15, one might expect to reduce only the pathologically high cellular PLD1 and PKC-α activities, without affecting regulation by other factors. To support this hypothesis, no significant change of PLD1 and PKC-α activity has been detected following D4 transfection or peptide loading of wild-type L6 cells and primary myocytes. These observations indicate that disruption of the PED/PEA-15-PLD1 interaction selectively ameliorates insulin signaling in cells bearing high levels of PED/PEA-15. In addition, forced D4 expression induced no variation of growth profiles in both L6 and L6PED/PEA-15 cells (data not shown), suggesting that no gross impairment of the cell cycle was induced by blocking of PED/PEA-15-PLD1 interaction. Because PLD1 and PLD2 isoforms display a very high sequence homology in the D4 region (~90%), one would expect that PLD2 may contribute as well to the effects mediated by the D4 or by the antagonist peptide.

In conclusion, we show that the PED/PEA-15-PLD1 interaction may represent a privileged molecular target for insulin resistance, particularly in those individuals with high PED/PEA-15 levels. Indeed, selective disruption of this interaction with cell-penetrating agents does not affect constitutive PKC-α and PLD1 functions, which may turn out to be of great physiological relevance, but rescues insulin action on glucose uptake in skeletal muscle cells.

REFERENCES

1. Araujo, H., Danziger, N., Cordier, J., Glowinski, J., and Chneiweiss, H. (1993) J. Biol. Chem. 268, 5911–5920
Targeting of the PED/PEA-15-PLD1 Interaction

2. Condorelli, G., Vigliotta, G., Cafieri, A., Trencia, A., Andalo, P., Oriente, F., Miele, C., Caruso, M., Formisano, P., and Beguinot, F. (1999) Oncogene 18, 4409–4415
3. Estelles, A., Charlton, C. A., and Blau, H. M. (1999) Dev. Biol. 216, 16–28
4. Trencia, A., Fiory, F., Maitan, M. A., Vito, P., Barbagallo, A. P., Perfetti, A., Miele, C., Ungaro, P., Oriente, F., Cilenti, L., Zervos, A. S., Formisano, P., and Beguinot, F. (2004) J. Biol. Chem. 279, 46566–46572
5. Xiao, C., Yang, B. F., Asadi, N., Beguinot, F., and Hao, C. (2002) J. Biol. Chem. 277, 25020–25025
6. Formisano, P., Perruolo, G., Libertini, S., Santopietro, S., Troncone, G., Raciti, G. A., Oriente, F., Portella, G., Miele, C., and Beguinot, F. (2005) Oncogene 24, 7012–7021
7. Condorelli, G., Vigliotta, G., Iavarone, C., Caruso, M., Tocchetti, C. G., Formisano, P., Cafieri, A., Beguinot, L., and Beguinot, F. (1998) EMBO J. 17, 3858–3866
8. Valentino, R., Lupoli, G. A., Raciti, G. A., Oriente, F., Farinaro, E., Della Valle, E., Salemone, M., Riccardi, G., Vaccaro, O., Donnarumma, G., Sesti, G., Hrihal, M. L., Cardellini, M., Miele, C., Formisano, P., and Beguinot, F. (2006) Diabetologia 49, 3058–3066
9. Vigliotta, G., Miele, C., Santopietro, S., Portella, G., Perfetti, A., Maitan, M. A., Cassese, A., Oriente, F., Trencia, A., Fiory, F., Romano, C., Tiveron, C., Tatangelo, L., Troncone, G., Formisano, P., and Beguinot, F. (2004) Mol. Cell. Biol. 24, 5005–5015
10. Miele, C., Raciti, G. A., Cassese, A., Romano, C., Giacco, F., Oriente, F., Paturzo, F., Andreozzi, F., Zabatta, A., Troncone, G., Bosch, F., Pujol, A., Chneiweiss, H., Formisano, P., and Beguinot, F. (2007) Diabetes 56, 622–633
11. Condorelli, G., Vigliotta, G., Treancia, A., Maitan, M. A., Caruso, M., Oriente, F., Santopietro, S., Formisano, P., and Beguinot, F. (2001) Diabetes 50, 1244–1252
12. Mercado, M. M., McLenithan, J. C., Silver, K. D., and Shuldiner, A. R. (2002) Curr. Diab. Rep. 2, 83–95
13. Bonadonna, R. C., and De Fronzo, R. A. (1991) Diabetes Metab. 17, 112–135
14. Owen, K. R., and McCarthy, M. I. (2007) Curr. Opin. Genet. Dev. 17, 239–244
15. Hill, J. M., Vaidyanathan, H., Ramos, J. W., Ginsberg, M. H., and Werner, M. H. (2002) EMBO J. 21, 6494–6504
16. Kubes, M., Cordier, J., Glowinski, I., Girault, J. A., and Chneiweiss, H. (1998) J. Neurosci. 21, 1307–1314
17. Trencia, A., Perfetti, A., Cassese, A., Vigliotta, G., Miele, C., Oriente, F., Santopietro, S., Giacco, F., Condorelli, G., Formisano, P., and Beguinot, F. (2003) Mol. Cell. Biol. 23, 4511–4521
18. Krueger, J., Chou, F. L., Glading, A., Schafer, E., and Ginsberg, M. H. (2005) Mol. Cell. Biol. 16, 3552–3561
19. Zhang, Y., Redina, O., Alshuller, Y. M., Yamazaki, M., Ramos, J., Chneiweiss, H., Kanaho, Y., and Frohman, M. A. (2000) J. Biol. Chem. 275, 35224–35232
20. McDermott, M., Wakeham, M. J., and Morris, A. J. (2004) Biochem. Cell Biol. 82, 225–253
21. Besterman, J. M., Duronio, V., and Cuatrecasas, P. (1986) Proc. Natl. Acad. Sci. U. S. A. 83, 6785–6789
22. Condorelli, G., Trencia, A., Vigliotta, G., Perfetti, A., Goglia, U., Cassese, A., Musti, A. M., Miele, C., Santopietro, S., Formisano, P., and Beguinot, F. (2002) J. Biol. Chem. 277, 11013–11018
23. Fiory, F., Alberobello, A. T., Miele, C., Oriente, F., Esposito, I., Corbo, V., Ruvo, M., Tizzano, B., Rasmussen, T. E., Gammeltoft, S., Formisano, P., and Beguinot, F. (2005) Mol. Cell. Biol. 25, 10803–10814
24. Fiory, F., Oriente, F., Miele, C., Romano, C., Treancia, A., Alberobello, A. T., Esposito, I., Valentino, R., Beguinot, F., and Formisano, P. (2004) J. Biol. Chem. 279, 11137–11145
25. Krämer, D. K., Al-Khalili, L., Guigas, B., Leng, Y., Garcia-Roves, P. M., and Krook, A. (2007) J. Biol. Chem. 282, 19313–19320
26. Slaaby, R., Du, G., Alshuller, Y. M., Frohman, M. A., and Seedorf, K. (2000) Biochem. J. 351, 613–619
27. Viparelli, F., Dotti, N., Sandomenico, A., Marasco, D., Dathan, N. A., Miele, C., Formisano, P., and Monti, S. M., and Ruvo, M. (2008) Protein Expr. Purif. 59, 302–308
28. Coussens, L., Parker, P. J., Rhee, I., Yang-Feng, T. L., Chen, E., Waterfield, M. D., Francke, U., and Ullrich, A. (1986) Science 233, 859–859
29. Braz, J. C., Gregory, K., Pathak, A., Zhao, W., Sahin, B., Klevitsky, R., Kimball, T. F., Lorenz, J. N., Nairn, A. C., Liggett, S. B., Bodi, I., Wang, S., Schwartz, A., Lakatta, E. G., De Paoli-Roach, A. A., Robbins, J., Hewett, T. E., Bibb, J. A., Westfall, M. V., Kranias, E. G., and Moljkentin, J. D. (2004) Nat. Med. 10, 248–254