PED/PEA-15 gene controls glucose transport and is overexpressed in type 2 diabetes mellitus

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We have used differential display to identify genes whose expression is altered in type 2 diabetes thus contributing to its pathogenesis. One mRNA is overexpressed in fibroblasts from type 2 diabetics compared with non-diabetic individuals, as well as in skeletal muscle and adipose tissues, two major sites of insulin resistance in type 2 diabetes. The levels of the protein encoded by this mRNA are also elevated in type 2 diabetic tissues; thus, we named it PED for phosphoprotein enriched in diabetes. PED cloning shows that it encodes a 15 kDa phosphoprotein identical to the protein kinase C (PKC) substrate PEA-15. The PED gene maps on human chromosome 1q21–22. Transfection of PED/PEA-15 in differentiating L6 skeletal muscle cells increases the content of Glut1 transporters on the plasma membrane and inhibits insulin-stimulated glucose transport and cell-surface recruitment of Glut4, the major insulin-sensitive glucose transporter. These effects of PED overexpression are reversed by blocking PKC activity. Overexpression of the PED/PEA-15 gene may contribute to insulin resistance in glucose uptake in type 2 diabetes.

Keywords: genetics/glucose/glucose transporter/insulin resistance/metabolism

Introduction

Glucose transport down its concentration gradient is mediated by members of the facilitative glucose transporter (Glut) family expressed on the plasma membrane of all cells (Bell *et al.*, 1993; Thorens, 1993). Muscle and adipose cells express two major glucose transporter isoforms, Glut1 and Glut4 (Bell *et al.*, 1993; Thorens, 1993). Glut1 is believed to reside constitutively on the plasma membrane and to be responsible for basal transport in these tissues (Burant *et al.*, 1991; Lienhard *et al.*, 1992). Glut4 is instead largely responsible for the insulin-stimulated transport (Burant *et al.*, 1991; Lienhard *et al.*, 1992). However,

the molecular mechanisms underlying Glut1 and Glut4 functions and their regulation, are not yet well defined.

Reduced insulin action on glucose transport and metabolism, i.e. insulin resistance, is a prominent feature of a number of human diseases including type 2 diabetes mellitus (Stern, 1997), and is contributed by genetic and environmental factors (DeFronzo, 1997). These factors might affect either the process of insulin signal transmission across the plasma membrane and/or the biochemical pathways allowing glucose uptake and metabolism by the cells (Kahn, 1994). While several environmental factors have been identified (De Meyts, 1993; Kahn, 1994), the precise nature of the gene(s) involved in insulin resistance is still unknown. Attempts to locate these genes benefited from random genome-wide screening procedures. Using this approach, some susceptibility loci for type 2 diabetes have been identified in different groups of individuals (Hanis *et al.*, 1996; Mahtani *et al.*, 1996). The molecular identity of type 2 diabetes genes has also been addressed through candidate gene studies (Kahn, 1994) and, in one case, through a subtraction cloning strategy (Reynet and Kahn, 1993). These studies led to the identification of several mutated genes which may contribute to impaired insulin action in diabetes (Kahn, 1994; DeFronzo, 1997). Thus, in late-onset type 2 diabetes, mutations have been detected in insulin (Steiner *et al.*, 1990; Kishimoto *et al.*, 1992), in many different proteins involved in the insulin signalling system (De Meyts, 1993; Kahn, 1994) and in mitochondrial genes (Gerbitz, 1992; Reardon *et al.*, 1992; Kahn, 1994). Collectively, however, these abnormalities only account for a small percentage of the total type 2 diabetic patients. New candidate gene cloning approaches complementary to formal genetic studies have to be adopted to identify genes which may contribute to its pathogenesis.

In the present work, we have used differential display to identify genes whose expression is altered in type 2 diabetes. We describe the cloning and the function of a protein which controls the level of the Glut1 on the cell membrane and which is overexpressed in type 2 diabetics. Overexpression of this protein impairs insulin-stimulated glucose transport and Glut4 translocation in skeletal muscle, a major site of insulin resistance in diabetes.

Results

Identification and cloning of differentially expressed mRNAs from fibroblasts of type 2 diabetic and control individuals

To identify primary abnormalities in mRNA expression in type 2 diabetes, we first analysed, by differential display, pools of mRNA extracted from fibroblasts derived from six type 2 diabetics and six non-diabetic individuals. The clinical characteristics of these subjects have been

Fig. 1. Expression of the 10.1 mRNA in control and type 2 diabetic fibroblasts and tissues. Total RNA preparations from (A) primary fibroblasts or (B) skeletal muscle and adipose tissues from individual subjects were dot-blotted and probed with the 10.1 cDNA as described in Materials and methods. Blots were rehybridized with a β -actin probe and normalized to β -actin expression. Quantitation was performed by laser densitometry of the autoradiographs. Filled symbols represent diabetic individuals, open symbols represent the non-diabetics; in each of these two groups, the triangles represent the lean, while the circles represent obese subjects. Horizontal bars represent the mean values of the intensities in each group of individuals. By *t*-test analysis, the differences between the means from type 2 diabetic and control subjects were significant at the $P < 0.01$ (fibroblasts) and $P < 0.05$ (muscle and fat) levels.

described previously (Miele *et al.*, 1997). Forty-five bands appeared differentially expressed in the two groups of fibroblasts. Out of these, eight were confirmed to be overexpressed and 12 were expressed at lower levels in type 2 diabetic fibroblasts compared with those from the non-diabetic individuals, and were cloned further in the pT7Blue vector. One of these clones, termed 10.1, is reported herein.

By dot-blot analysis, the 10.1 probe hybridized 2-fold more intensely to RNA preparations from fibroblasts derived from 12 type 2 diabetic subjects compared with those from 13 controls (Figure 1A). This difference persisted whether obese or lean individuals were compared, indicating that it was not associated with obesity *per se*. A similar increase in the 10.1 mRNA expression was also measured in skeletal muscle and adipose tissues from the type 2 diabetics compared with the non-diabetics

Fig. 2. Northern blot analysis and tissue distribution of the 10.1 mRNA. (A) RNAs were prepared from primary fibroblasts of type 2 diabetic and control individuals, blotted and probed with the 10.1 cDNA. The negative image of the ethidium bromide-stained RNA loaded in each lane is also shown. (B) Human mRNAs from different normal tissues (Clontech, Palo Alto, CA) were probed with the 10.1 cDNA. The 2.8 kb mRNA is also expressed in adipose tissue at 2-fold higher levels than in skeletal muscle (not shown).

(Figure 1B). However, no difference from non-diabetic subjects was detected in cells from type 1 diabetics. Northern blot analysis with fibroblast RNAs showed that the 10.1 probe hybridized to a major 2.8 kb band. As in the dot-blot assays, this mRNA appeared 2-fold more abundant in type 2 diabetic tissues (Figure 2A). The higher levels of the 2.8 kb mRNA in diabetic cells resulted from increased expression as determined by run on experiments rather than changes in the mRNA half-life, as determined by actinomycin D treatment (data not shown). The 2.8 kb band is expressed ubiquitously in human tissues (Figure 2B), but it is more abundant in those tissues which largely utilize glucose as the energy source, such as heart, brain, muscle and adipose tissue, and is almost absent in the glucose-producing tissues, liver and kidney. In human heart and skeletal muscle, the 10.1 probe also identified a fainter 1.7 kb mRNA species.

10.1 cDNA cloning and chromosomal localization

To obtain the full-length cDNA, we have screened a human heart library with the 10.1 clone. Nine positive clones were isolated, and two of them, termed clones 1 and 4, encoding two cDNAs of 2.8 and 1.7 kb, respectively, were characterized further. Clones 1 and 4 contain the

same 390 bp open reading frame (ORF) and 5'-untranslated region (5'-UTR), and exhibit variability in the 3'-UTR. Clone 1 was sequenced fully and entered in the EMBL database (EMBL locus HPED, accession No. Y13736). Matching clone 1 with the EMBL database revealed >99% identity with two previously reported cDNAs. One of these cDNAs corresponds to the protein kinase C (PKC) substrate PEA-15 (phosphoprotein enriched in astrocytes, EMBL locus HSPEA-15, Araujo *et al.*, 1993). Current evidence indicates that *PEA-15* mRNA is present in cells in two differentially spliced forms of ~2.8 and 1.7 kb (Estelles *et al.*, 1996), which correspond to our clones 1 and 4, respectively. The ORF of *PED* compared with *PEA-15* showed four codon differences. Three are conservative amino acid substitutions (Ala→Val2, Leu→Phe8 and Ala→Gly124). The fourth difference in *PED* causes substitution of Tyr→Ile62, present in PEA-15. Other differences are also present in the 5'- and 3'-UTRs of *PED* and *PEA-15*. The 3'-UTRs of clones 1 and 4 cDNAs are also homologous to an oncogenic sequence from a chemically induced mammary tumor [*MAT-1* (Bera *et al.*, 1994), EMBL locus HSMAT-1H]. *MAT-1* protein has never been identified in cells. Further studies have shown that the *MAT-1* sequence is included within the 3' UTR of *PEA-15* cDNA, suggesting that *MAT-1* is not translated into a distinct protein *in vivo* (Estelles *et al.*, 1996).

An expressed sequence tag (EST) database search (Boguski *et al.*, 1993) revealed the presence of several ESTs identical to *PED* cDNA. Sequence tagged sites (STSs) generated from these ESTs were used for radiation hybrid mapping enabling localization of *PED* on chromosome 1q21–22, between markers *DIS2635* and *DIS484*.

Expression of *PED/PEA-15* protein in type 2 diabetes

To define the physiological role of *PED/PEA-15*, we have first raised antisera against *PED* (α -*PED* Ab) and against the putative *MAT-1* (α -*MAT* Ab). Consistent with the view that *MAT-1* sequence is not translated *in vivo* (Estelles *et al.*, 1996), a specific *MAT-1* Ab did not recognize any protein in all cells analysed (data not shown). In contrast to this finding, immunoblots from lysates of several different human cell types with α -*PED* Ab revealed a single protein with the predicted 15 kDa mol. wt (Figure 3A). As in the case of the mRNA levels, *PED* protein expression is ~2-fold increased in the type 2 diabetic compared with control fibroblasts (Figure 3B). Therefore, we named this protein *PED* for phosphoprotein enriched in diabetes. *PED* overexpression did not occur in fibroblasts from type 1 diabetic individuals, strongly suggesting that its increased expression in type 2 diabetes is not the effect of metabolic dysregulation which also occurs in other diabetic states.

To address the relevance of a *PED/PEA-15* defect in type 2 diabetes, *PED* full-length cDNA was stably transfected in the differentiating L6 skeletal muscle cells. Immunoblotting of lysates from eight individual clones and from the cell pool indicated that *PED* expression was increased by 2- to 3-fold in these cells (L6_{PED}) with respect to control cells, either transfected with vector alone (L6_{PC}, six control clones) or untransfected (L6_{WT}) (Figure 4A). Therefore, overexpression is comparable with that found in type 2 diabetic tissues. *PED*-overexpressing

Fig. 3. Expression of *PED* protein in human cell lines. (A) Total cell lysates (identical amounts of proteins) were immunoblotted with α -*PED* Ab as described in Materials and methods. *PED* detection was performed by enhanced chemiluminescence and quantitation achieved by laser densitometry of the autoradiographs. 1, NIH-3T3 mouse fibroblasts; 2, L6 rat myotubes; 3, HepG2 human liver cells; 4, TAD human thyroid cells; 5, Kb human larynx carcinoma cells; 6, RL-95 human cervix carcinoma cells; 7, MCF7 human breast carcinoma cells; 8, human monocytes; 9, A2058 human melanoma cells; 10, human skin fibroblasts. (B) Detection and quantitation of *PED* levels in primary fibroblasts from type 2 and type 1 diabetic and non-diabetic (control) individuals was performed as described above. In each individual subject, these protein levels closely correlated with *PED* mRNA levels.

L6 cells did not differ from controls in gross morphology or in their ability to differentiate into mature myotubes based on morphological and biochemical indexes of differentiation (Table I). Likewise, overexpression of *PED* had no effect on [³H]thymidine incorporation by the L6 myoblasts following exposure to insulin or 10% serum (Figure 4B). To determine which insulin-regulated function is affected by *PED*, L6_{PED} cells were assayed for glucose uptake in the absence or presence of 100 nM insulin. Basal 2-deoxy-D-glucose (2-DG) uptake was slightly increased in L6_{PED} compared with control cells (Figure 5). The difference in the basal uptake was more evident at the myoblast than at the myotube stage. Most importantly, however, both in blasts and in myotubes, *PED* overexpression determined >90% inhibition of the insulin-stimulated uptake. This inhibition was not caused by changes in insulin binding or receptor phosphorylation in *PED* overexpressors (Table II). In addition, uptake of the amino acid analogue α -aminoisobutyric acid (AIB) was identical in L6_{PC} and L6_{PED} cells, both in the absence and the presence of insulin, indicating that changes in glucose uptake are specific and not generalized to other membrane transport systems (Figure 4C). We then sought to investigate whether the effect of *PED* overexpression on glucose uptake also occurred in other insulin-sensitive cell types. To this end, *PED* full-length cDNA was stably transfected in 3T3-L1 cells. Three clones of transfected cells were chosen for further studies overexpressing *PED* by 2- to 5 fold above the levels of control cells (Figure 6A). As was

Fig. 4. PED overexpression in L6 cells. (A) PED levels were analysed in L6 myotubes untransfected (L6_{WT}) or transfected with the pcDNA3 vector alone (L6_{PC}) or enclosing the cDNA coding region of clone 1 (L6_{PED}). Immunoblot detection and quantitation were achieved as described in the legend to Figure 3. To ensure accurate normalization, the same blot subsequently was probed with β-actin Abs (lower inset). (B and C) L6 cells were maintained in the absence or the presence of 100 nM insulin and then assayed for thymidine incorporation into DNA (B) or α-aminoisobutyric acid (AIB) uptake (C) as described in Materials and methods. Each bar represents the mean ± SD of triplicate determinations in three (AIB uptake) or four (thymidine incorporation) independent experiments.

the case for the L6 cells, PED overexpression did not impair differentiation of the 3T3-L1 adipocytes, insulin binding or receptor phosphorylation in these cells. Interestingly, as in the L6 myoblasts, PED overexpression was accompanied by a 70% increase in basal 2-DG uptake but almost completely blocked the insulin-stimulated uptake in the adipocytes (Figure 6B).

Glut4 is the major insulin-stimulated glucose transporter in muscle and adipose tissues, and undergoes translocation from internal membrane compartments to the plasma membrane following insulin stimulation. In contrast, Glut1 largely localizes at the cell surface in the basal state and translocates to a smaller extent in response to insulin (Bell *et al.*, 1993). Immunoblotting of differentiated L6 myotubes revealed no detectable differences in the total content of either Glut4 or Glut1 between control cells and

Fig. 5. 2-DG uptake in L6_{PED} cells. PED-overexpressing L6 myotubes (A), myoblasts (B) and control cells (L6_{PC}) were incubated in the absence or presence of 100 nM insulin as indicated and then assayed for 2-deoxyglucose (2-DG) uptake as described in Materials and methods. Each value represents the mean ± SD of triplicate determinations in four independent experiments. By *t*-test analysis, the increased basal uptake in PED-overexpressing compared with control myoblasts was significant at the *P* < 0.01 level.

Table 1. Time course of cell fusion and creatine kinase accumulation in L6_{PED} cells

Time after plating (days)	Nuclei in myotubes (%)			Creatine kinase activity (MIU/mg cell protein)		
	L6 _{WT}	L6 _{PC}	L6 _{PED}	L6 _{WT}	L6 _{PC}	L6 _{PED}
0	0	0	0	6 ± 0.3	5 ± 0.2	6 ± 0.5
3	5 ± 4	6 ± 3	5 ± 3	34 ± 5	39 ± 7	35 ± 6
6	37 ± 5	34 ± 4	38 ± 6	101 ± 5	98 ± 7	105 ± 5
12	87 ± 5	84 ± 9	83 ± 6	165 ± 4	166 ± 7	161 ± 2

Data represent the means ± SD of determinations with parental (L6_{WT}) and each of the L6_{PC} and L6_{PED} cell clones described in the present work in four independent experiments. At no time point did the differences between any of the cell clones reach statistical significance.

Table II. Insulin receptor functions in L6_{PED} cells

	L6 _{WT}	L6 _{PC}	L6 _{PED} (clone D)	L6 _{PED} (clone T)
[¹²⁵ I]Insulin binding (% specific binding/mg cell protein)	0.9 ± 0.3	0.8 ± 0.2	0.9 ± 0.3	0.9 ± 0.2
Insulin-induced auto-phosphorylation (densitometry; % of L6 _{WT})	100	98 ± 5	104 ± 7	100 ± 4

Data represent the means ± SD of determinations with parental (L6_{WT}) and the L6_{PC} and L6_{PED} cell clones described in the present work in three independent experiments. At no time point did the differences between any of the L6 cell clones and the parental cells reach statistical significance.

PED overexpressors (Figure 7A and B). In the basal state, the levels of Glut4 located in the plasma membrane were also very similar in L6_{PED} and L6_{PC} cells. After insulin exposure, however, the L6_{PC} plasma membrane content of Glut4 increased ~6-fold, while exhibiting very little change in the L6_{PED} cells. Plasma membrane content of Glut1 also exhibited little change after insulin exposure of L6_{PED} cells, but, in contrast to Glut4, the basal plasma membrane content of Glut1 was 4-fold higher in L6_{PED} compared with L6_{PC} cells. Similar findings were observed with the 3T3-L1 cells upon PED transfection (data not shown). Thus, in muscle and adipose cells, PED expression appears specifically to control the plasma membrane content of Glut1 and the insulin-dependent trafficking of Glut4 from the cell interior to the surface.

It has been well established that insulin-stimulated translocation of Glut4 requires phosphatidylinositol (PI)-3-kinase activity (reviewed by Kahn, 1994), raising the possibility that PED overexpression may impair this key step in insulin action. However, in all of the cell clones analysed, IRS-1-associated PI-3-kinase was stimulated normally by insulin (Figure 8), suggesting that PED does not interact with the PI-3-kinase-dependent pathway. As expected based on the presence of three consensus phosphorylation sites for PKC (amino acids 33 SEK, 70 SRR and 104 SAK), PED was basally phosphorylated in L6 cells and exhibited a 2-fold increase in phosphorylation upon PKC activation by 12-*o*-tetradecanoyl-13-acetate (TPA) (Figure 9A). In L6_{PED} cells, basal and TPA-stimulated phosphorylations of PED were higher than in control cells. In addition, the basal and TPA-stimulated phosphorylations were inhibited by the PKC inhibitors bis-indolylmaleimide and staurosporine in both cell lines. In parallel, block of PKC activity by both inhibitors returned the basal and the insulin-stimulated glucose uptake in L6_{PED} cells to levels similar to those of control cells (Figure 9B), allowing an almost normal insulin response in L6_{PED} cells. These data indicate that the effect of PED overexpression in type 2 diabetic cells may be controlled by PKC phosphorylation.

Discussion

In this work, we report the cloning of a novel gene, PED, which is overexpressed in type 2 diabetic tissues and cells. In addition, we describe the consequences of this abnormality on glucose uptake in skeletal muscle cells, a major site of insulin resistance in diabetes (Kahn, 1994; De Fronzo, 1997). PED/PEA-15 is a highly conserved protein (Danziger *et al.*, 1995), originally identified as a PKC substrate (Araujo *et al.*, 1993). However, very little of its physiological functions were known until now. In the present report, we show that the expression of PED/

Fig. 6. Overexpression of PED in 3T3-L1 cells. (A) PED levels were analysed in 3T3-L1 untransfected adipocytes (L1_{WT}), transfected with the pcDNA3 vector alone (L1_{PC}) or encoding PED full-length cDNA (L1_{PED}). Immunoblot detection and quantitation were achieved as described in Materials and methods. To ensure accurate normalization, the same blot was probed subsequently with β-actin Abs.

(B) 2-Deoxyglucose uptake was measured in PED-overexpressing 3T3-L1 adipocytes and in control cells in the absence or presence of 100 nM insulin as indicated. Each value represents the mean ± SD of triplicate determinations in three independent experiments. Based on *t*-test analysis, the difference in 2-DG uptake in the absence or the presence of insulin was not significant in any of the L1_{PED} clones but was significant at the *P* < 0.001 level in control cells.

PEA-15 is almost ubiquitous in human tissues, suggesting that it may be involved in important cellular functions. We found that a 2-fold overexpression of PED/PEA-15 above the basal level is sufficient to expand the Glut1 plasma membrane pool while not affecting the total Glut1 content of the cells. This change prevents further membrane translocation of Glut1 following insulin stimulation and may account for the lack of insulin-stimulated glucose transport in cells which do not possess the major insulin-sensitive transporter, Glut4, like the PED-overexpressing MCF-7 carcinoma cells (data not shown). Most importantly, however, we show that in cells which possess both Glut1 and Glut4, such as the L6 skeletal muscle cells and the 3T3-L1 adipocytes, the increased Glut1 presence at the cell surface caused by PED overexpression is responsible for the small increase in basal glucose flux into the cells. In addition, a marked inhibition of insulin-regulated glucose transport is observed upon insulin stimulation of these cells and is due to the block in Glut4 translocation to the plasma membrane. Increased

Fig. 7. Insulin effect on glucose transporters in L6_{PED} cells. To analyse plasma membrane (P.M.) transporters, cell surface proteins were biotinylated at 4°C, followed by precipitation with (A) Glut4- or (B) Glut1-specific Abs, Western blotting and identification by chemiluminescence and autoradiography as described in Materials and methods. The total transporters of the cells were quantified by directly immunoblotting cell lysates. Equal protein loading and transfer were ensured as described in the legend to Figure 4.

Fig. 8. Insulin effect on PI-3-kinase activity in L6_{PED} cells. The cells were stimulated with 100 nM insulin as indicated. IRS-1-associated PI-3-kinase activity was quantitated as described in Materials and methods and the density of the spots quantified by laser densitometry. The bar graph shows the mean values $\pm$ SD of duplicate determinations in three independent experiments (a representative one is shown in the inset).

presence of Glut1 at the cell surface has also been reported to be accompanied by inhibition of insulin-stimulated glucose transport and Glut4 translocation in other cell and animal models (Czech *et al.*, 1992; Marshall, 1993). The mechanism responsible for this inhibition in PED-overexpressing L6 cells does not appear to be caused by changes in the PI-3-kinase activity and its dependent pathway which allows translocation of glucose transporters. It is also unlikely to be caused by a generalized alteration in the endocytotic and recycling pathways since internalization and recycling of the low density lipoprotein (LDL) receptor occur normally in these cells (data not shown). At variance with this finding, the inhibition of insulin-dependent Glut4 translocation and glucose transport may depend on the fact that the plasma membrane has a maximal capacity to accommodate glucose transporters, independently of the particular isoforms. In addition, the increased basal flux of glucose into the cells may impair subsequent insulin response. Thus, we propose that PED plays a physiological role in controlling the size of the

Fig. 9. Effect of staurosporine on PED phosphorylation by PKC and on glucose uptake in L6_{PED} cells. (A) L6_{PC} (clone A) and L6_{PED} (clone T) cells were metabolically labelled with [³²P]orthophosphate, pre-incubated with 100 nM bisindolylmaleimide or 6 μ M staurosporine for 45 min, and then stimulated with 1 μ M TPA for an additional 45 min as described in Materials and methods. Cell lysates (equal amounts of proteins) were precipitated with α -PED Ab, separated by SDS-PAGE and autoradiographed. A representative experiment is shown. (B) PED overexpressors (L6_{PED}, clone T) and control cells (L6_{PC}) were pre-incubated with 100 nM bisindolylmaleimide or 6 μ M staurosporine for 45 min before insulin exposure (100 nM). 2-DG uptake was determined as described above. Each bar represents the mean $\pm$ SD of triplicate determinations in three independent experiments.

constitutive Glut1 pool at the cell surface and, through this function, it may serve as a negative regulator of insulin-stimulated glucose transport. We have not yet defined the molecular mechanisms and pathway through which PED controls the content of glucose transporters in the plasma membrane. However, this is also the case for the PI-3-kinase-dependent pathway which is considered the major player regulating the cellular localization of glucose transporters (Holman and Kasuga, 1997). Further studies on PED functions are ongoing in the laboratory. In keeping with this positive role in regulating Glut1 on the cell membrane, PED/PEA-15 mRNA and protein are particularly abundant in organs that largely utilize glucose as the energy source such as human skeletal muscle, heart and brain, but are also expressed at high levels in cells which contribute little to total glucose disposal such as fibroblasts (this study) and astrocytes (Araujo *et al.*, 1993). The ability of PED to expand the number of Glut1 transporters at the cell surface is consistent with an anti-apoptotic function of the protein since apoptosis may be suppressed by regulating the rate of glucose flux into the cells (Kan *et al.*, 1994). In fact, in our own work, we have shown that PED possesses a death effector domain and disrupts the assembly of FAS and TNF- α receptor signalling complexes preventing apoptosis in a variety of cell types (G. Condorelli and F. Beguinot, in preparation).

It has also been demonstrated by us and others that PED/PEA-15 possesses the structural requirements to serve as a PKC substrate and undergoes phosphorylation *in vitro* and in intact cells (Danzigen *et al.*, 1995; this study). In PED-overexpressing L6 muscle cells, a block of PKC activity by staurosporine or bisindolylmaleimide reduced PED basal phosphorylation to levels similar to those detected in control cells. This is paralleled by a decrease in the high basal glucose flux caused by PED overexpression in L6_{PED} cells and by the reappearance of insulin-stimulated glucose transport. These data may suggest that the functions of PED in cells are in turn regulated by PKC.

PED/PEA-15 expression is not altered in type 1 diabetic fibroblasts. However, in type 2 diabetic fibroblasts, PED/PEA-15 overexpression persists through at least 20 generations in culture, indicating that it is not a secondary alteration due to the internal milieu of the patients. Overexpression of PED/PEA-15 in the diabetics may be caused by its transcriptional induction since no *PED* mutations were detected by denaturing gradient gel electrophoresis (DGGE) in the coding region of the gene in 23 type 2 diabetic individuals analysed (data not shown). Further studies will be necessary to isolate the regulatory region of the *PED* gene in order to address its regulation in normal and diabetic states. No known genetic locus in linkage with late-onset type 2 diabetes is as common as PED overexpression in the diabetics described in this work. It is formally possible that we have selected a very homogeneous group of individuals, but it is more likely that PED/PEA-15 overexpression represents a molecular abnormality downstream of several distinct diabetes susceptibility genes which may cause a modification of the expression of PED/PEA-15. As such, PED/PEA-15 may represent a major effector of genes responsible for insulin resistance of glucose uptake.

Proteins whose increased expression may contribute to insulin resistance in non-insulin-dependent diabetes mellitus (NIDDM) include the IR kinase inhibitor PC1 (Maddux *et al.*, 1995) and Rad, a Ras-like GTPase which inhibits glucose transport in muscle and fat cells (Moyers *et al.*, 1996). Rad levels in human muscle tissue positively correlate with obesity (Garvey *et al.*, 1997) and with type 2 diabetes, though in some groups of individuals (Reynet and Kahn, 1993) and not in others (Garvey *et al.*, 1997). In contrast, PED/PEA-15 overexpression is not correlated with obesity but appears to be independent, while it is directly correlated with type 2 diabetes. At variance with PED/PEA-15, it appears that the inhibitory effect of Rad on glucose uptake is determined through a decrease in the intrinsic activity of the individual transporters rather than an effect on Glut4 translocation (Moyers *et al.*, 1996). Similarly to PED/PEA-15, however, Rad is also a cytoskeleton-associated protein (Zhu *et al.*, 1996). It is intriguing to speculate that both Rad and PED/PEA-15 have a role in cytoskeletal rearrangements and therefore abnormalities in the expression of both of these proteins may impair glucose uptake by affecting transporter function. In human obesity and diabetes, therefore, distinct defects seem to impair regulated glucose transport by converging on cytoskeleton functions.

In conclusion, we have cloned a gene which appears to control both the constitutive and regulated glucose trans-

port in cells. Overexpression of this protein in major insulin target tissues appears to generate insulin resistance in type 2 diabetic individuals.

Materials and methods

General

Preparation of plasmid DNA, agarose gel electrophoresis, restriction enzyme digestion and DNA sequencing were performed by standard methods (Sambrook *et al.*, 1989). Plasmid pT7Blue T-vector was obtained from Novagen (Cleveland, OH) and used according to the manufacturer's instructions. Random priming labelling was performed with the random priming labelling kit from Boehringer Mannheim (Kvistegard, Denmark). Sequencing was performed with the Sequenase kit (USB, Cleveland, OH). Total cellular RNAs were extracted as previously described (Chomczynski and Sacchi, 1987), and poly(A) RNAs were purified directly from cultured cells by the Quickprep kit (Pharmacia-LKB, Milan, Italy). Enzymes were from Boehringer Mannheim (Kvistegard, Denmark) and radiochemicals from Amersham (Milan, Italy). Media, sera for tissue culture and G-418 were from Gibco (Grand Island, NY), electrophoresis and Western blot reagents from Bio-Rad (Richmond, CA) and sulfo-NHS-biotin from Pierce (Rockford, IL). Bisindolylmaleimide (GF-109203X) was purchased from LC Laboratories (Läufelfingen, Switzerland). All other chemicals were from Sigma (St Louis, MO).

Subject selection, fibroblast cultures and skeletal muscle sampling

The characterization of all of the diabetic and the control subjects analysed in the present study has been reported previously (Miele *et al.*, 1997). Fibroblast cultures and skeletal muscle sampling from these patients have also been described (Miele *et al.*, 1997). Subcutaneous adipose tissue and muscle biopsies were obtained simultaneously at the time of surgery.

Primer syntheses, differential display, recovery and reamplification of cDNA probes

Degenerate oligonucleotide primers T₁₂XG, T₁₂XA, T₁₂XT and T₁₂XG, and three arbitrary primers Ldd1, Ldd2 and Ltk3 (Liang and Pardee, 1992), were synthesized by Pharmacia (Milan, Italy). The arbitrary primers OPA1–20 were purchased from Operon Biotechnology Inc. (Alameda, CA). PCR amplification and differential display were performed as in Liang and Pardee (1992) using the four degenerate T₁₂XN primers in combination with 10 arbitrary 10mers. A total of 3500 bands were revealed on autoradiographs from both the NIDDM and the control individuals. Assuming that each band corresponds to an individual RNA species, we estimated that this analysis included approximately one-fifth of the total mRNAs of the cell. In order to avoid the otherwise possible selection of Y-chromosome sequences, in this phase of the work, only cells from female subjects were used. Recovery and reamplification of cDNA from the differential display bands were achieved as in Liang and Pardee (1992). The 10.1 species described here was obtained by PCR amplification of the differential cDNA band with the T₁₂XC–LTK3 primers and subsequently cloned in the *Hind*III–*Bam*HI sites of the pT7Blue vector.

Dot and Northern blot analysis

In contrast to the above-mentioned RNAs, in this phase of the work, cells from both male and female individuals were used to confirm that differential expression was not dependent on gender. Dot and Northern blot assays were performed as previously described (Sambrook *et al.*, 1989), using 12.5 and 25 µgRNA/sample, respectively. In both assays, equal amounts of RNA were loaded for each sample. The density of the spots/bands on the autoradiographs was quantitated by laser densitometry using a Pharmacia LKB densitometer. Densitometric values were normalized for β-actin and ethidium bromide staining and expressed as arbitrary units.

Cloning and chromosomal mapping of PED cDNA

The 10.1 cDNA was used as a probe to screen a human heart cDNA library (Clontech, Palo Alto, CA) according to Sambrook *et al.* (1989). Thirteen positive colonies were identified by the first screening, and nine of them were investigated further through secondary and tertiary screenings. The cDNAs from clones 1 and 4 were subcloned in the *Eco*RI cloning site of the pGem3Z vector. By *Eco*RI restriction, clones 1 and 4 were found to encode two cDNAs of 2400 and 1700 bp,

respectively. *PED* chromosomal localization was performed as in Jackson *et al.* (1997)

Antisera generation and detection of PED/PEA-15 in cells

Antisera against PED and MAT (α PED and α MAT Abs) were prepared in rabbits using PED/PEA-15- and MAT-specific peptides (PED amino acids 108–122 YKDIIRQPSEEEIHK; MAT amino acids 20–34 LLSRPERGWEGNATV) according to standard methods (Sambrook *et al.*, 1989). Determination of PED/PEA-15 levels in cells was achieved by immunoblotting. To this end, cells were solubilized in 1% SDS, 1 mM Na_3VO_4 , 2 mM phenylmethylsulfonyl fluoride (PMSF). Equal amounts of protein extracts were separated on reducing tricine gels (Liang and Pardee, 1992), transferred to Hybond nitrocellulose membranes (Amersham, Milan, Italy) and incubated with 1:2000 α PED Ab for 16 h at 4°C. Membranes were washed three times with 0.05% Tween-TBS, further incubated with peroxidized anti-IgG antibodies for 1 h at room temperature, and blotted proteins were revealed by detection of enhanced chemiluminescence (ECL detection system, Amersham, Milan, Italy), according to the manufacturer's instructions.

Expression studies

The *PED* ORF (bp 71–462) was amplified from clone 1 cDNA by PCR using the primers 5'-GGCCGGTACCGCCAGAGCGCGGGGGCAG-TGTG-3' and 5'-ATATATGCGCCGCGGTCTGATGAAGGTCCA-ACC-3'. The amplified fragment was subcloned in the *Kpn*I and *Not*I sites of the pcDNA3 expression vector containing the neo^r selectable marker and re-sequenced. The construct was transfected into the L6 skeletal muscle cells using the lipofectamine method, as described in Caruso *et al.* (1997). G-418 (Life Technologies Inc.) was used at the effective dose of 0.8 mg/ml. Individual G-418-resistant clones were selected by the limiting dilution technique (Caruso *et al.*, 1997). PED expression by the individual clones was evaluated by Western blotting as described above, showing no change with differentiation from the myoblast to the myotube stage. Clones of L6 cells transfected with the pcDNA3 vector alone were also selected by G-418 resistance and used for controls. The L6 cells were grown and maintained as described in Caruso *et al.* (1997). Insulin binding, ³²P-metabolic labelling, receptor autophosphorylation, fusion index and creatine kinase activities in these cells were also determined as reported in Caruso *et al.* (1997). Determinations of 2-DG and AIB uptake, thymidine incorporation and Glut4 and Glut1 levels in PED/PEA-15 overexpressors were performed as reported in Caruso *et al.* (1997) and Formisano *et al.* (1993) for the L6 cells and in Clancy *et al.* (1990) for the 3T3-L1 adipocytes. IRS-1-associated PI-3-kinase activity was assayed as described by Tsakiridis *et al.* (1995).

The 3T3-L1 fibroblasts used in this study were a generous gift of Professor A.Fusco (Catanzaro University Medical School, Italy). The cells were cultured and induced to differentiate into adipocytes as previously described (Clancy *et al.*, 1990). The adipocytes were used at least 10 days after the induction of differentiation, at which time >90% of the cells expressed an apparent adipocyte phenotype as determined by visual inspection of the cell monolayers, and were expressing Glut4 as measured by Western blotting.

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