The EMBO Journal vol.8 no.9 pp.2503–2507, 1989

A leucine-to-proline mutation in the insulin receptor in a family with insulin resistance

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Communicated by W.Möller

We have determined the primary structure of a mutant insulin receptor of a leprechaun patient born from a consanguineous marriage. A characteristic feature of leprechaunism is an extreme resistance to insulin. In this patient the insulin resistance seems to result from an observed lack of insulin binding to intact cells. Solubilization of cells in non-ionic detergents leads to the appearance of insulin receptors which can bind insulin. However, the insulin-stimulated autophosphorylation of the receptor's β subunit is markedly reduced. Cloning and sequencing of cDNA derived from insulin receptor mRNA of this patient revealed a leucine-to-proline mutation at position 233 in the α subunit. By means of DNA amplification we found that the patient is homozygous for this mutation and that the parents and two grandparents from the consanguineous line are heterozygous. The heterozygous individuals all show decreased insulin binding to cultured fibroblasts. In addition, they are mildly insulin resistant *in vivo*. These observations show a linkage between the leucine-to-proline mutation and the observed insulin resistance in this family. We therefore conclude that the mutation in the homozygous form is responsible for the extreme insulin resistance in the leprechaun patient. The mutation for the first time characterizes a region in the insulin receptor which seems to be involved in transmitting the insulin binding signal to the tyrosine kinase domain.

Key words: binding defect/insulin receptor/insulin resistance/leprechaunism/signal transduction

Introduction

Insulin resistance has been implicated in a variety of disorders, e.g. type 2 diabetes mellitus and leprechaunism (Rosenberg *et al.*, 1980), and can lead to elevated insulin levels and disturbed glucose homeostasis. The underlying molecular mechanism is not clear, although recent findings suggest the involvement of the insulin receptor (Kadowaki *et al.*, 1988; Yoshimasa *et al.*, 1988). This receptor is a transmembrane protein of $\alpha_2\beta_2$ subunit composition. The α and β subunits are encoded by a single open reading frame (Ebina *et al.*, 1985a; Ullrich *et al.*, 1985). Insulin binds to the extracellularly localized α subunit, probably interacting with a phenylalanine residue at position 89 (De Meyts *et al.*, 1988; Whittaker *et al.*, 1988) and a region between amino acids 243–251 (Yip *et al.*, 1988). Signal transduction is reflected by subsequent autophosphorylation of the intracellular part of the β subunit and increased tyrosine kinase activity (White and Kahn, 1986). How the binding signal is transduced to the tyrosine kinase domain is not known.

Recently we described a leprechaun patient named Geldermalsen after the town of origin (Maassen *et al.*, 1988a). This patient was born from a consanguineous marriage. Cultured primary fibroblasts of this patient remained insulin resistant as reflected by a decreased insulin-stimulated 2-deoxyglucose uptake. Insulin binding to intact cells was almost completely abolished, though the cells synthesize insulin receptors in normal amounts, as shown by the appearance of insulin receptors after solubilization of the cells in Triton X-100. The solubilized receptors did bind insulin but showed a diminished degree of insulin-stimulated autophosphorylation. These findings indicate that the defect leading to insulin

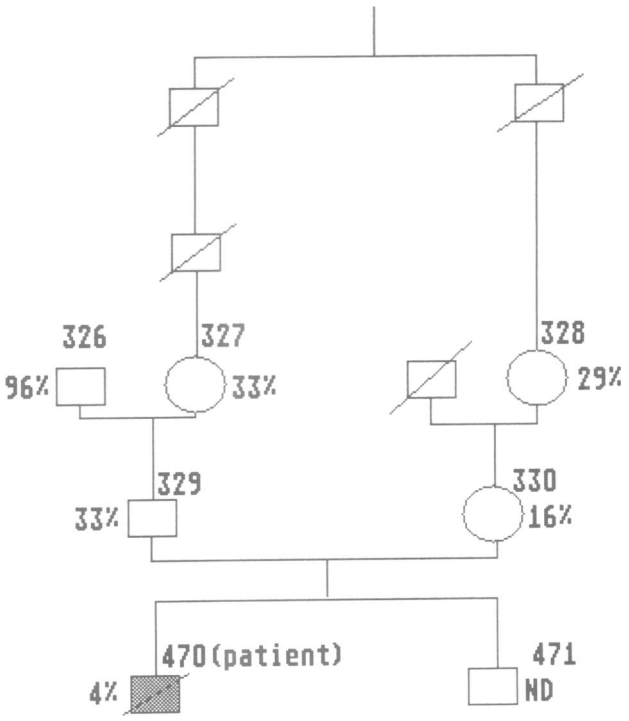

Fig. 1. Simplified pedigree of the Geldermalsen family together with specific [^{125}I]insulin binding to cultured primary fibroblasts as a percentage of control values. 326, supposed non carrier grandfather; 327 and 328, supposed carrier grandmothers; 329 and 330, obligate carrier father and mother; 470, leprechaun patient; 471, newborn baby; ND, not determined.

Table I Comparison of leprechaun Geldermalsen and two wild-type insulin receptor cDNAs and protein sequences

Leprechaun Geldermalsen nucleotide (amino acid)	Ullrich *et al.*	Ebina *et al.*
α chain:		
T-559 (Tyr)	+ (Tyr)	− (His)
C-827 (Pro)	− (Leu)	− (Leu)
G-957 (Gln)	+ (Gln)	− (Gln)
T1391 (Ile)	+ (Ile)	− (Thr)
C-1522 (Gln)	+ (Gln)	− (Lys)
C-1681 (Asp)	+ (Asp)	− (Asp)
A-1698 (Ala)	+ (Ala)	− (Ala)
Exon 11, 36 nucleotides between 2277 and 2278	−	+
β chain:		
T-2711 (Val)	− (Asp)	+ (Val)
T-2713 (Ser)	− (Thr)	+ (Ser)
G-3846 (Lys)	− (Asn)	+ (Lys)

Nucleotide numbering is according to Ullrich *et al.* (1985). A plus sign indicates that the nucleotide sequence is present in the wild-type sequence obtained by Ullrich *et al.* (1985) or Ebina *et al.* (1985). A minus sign indicates a change. Predicted amino acids are indicated.

resistance in this patient resides within the insulin receptor and is expressed as a decreased insulin binding to intact cells because of improper expression of the receptor at the cell surface. In addition the defect results in an impaired transduction of the insulin binding signal to the tyrosine kinase domain.

To investigate the mode of inheritance of the receptor defect within the consanguineous family we performed insulin binding experiments on cultured primary fibroblasts of the patient's relatives. Obligate and supposed carriers showed diminished binding values though still higher than that of the patient. Oral glucose tolerance tests on these individuals showed a tendency for hyperinsulinaemia indicating a mild form of insulin resistance (Lekanne Deprez *et al.*, 1989). Cloning and sequencing of the patient's insulin receptor cDNA revealed a leucine-to-proline mutation at position 233 in the α subunit. Obligate and supposed carriers were found to be heterozygous for this mutation whereas the proband is homozygous. Because the pattern of inheritance of the leucine-to-proline mutation matches with the consanguineous line within the pedigree and the insulin binding defects ($P < 0.02$), we conclude that this mutation in the homozygous form causes leprechaunism. Since one of the defects of insulin receptors from the leprechaun patient is an imparied insulin-stimulated autophosphorylation we further conclude that the mutation is in a region which functionally links the insulin binding site on the α subunit with the tyrosine kinase domain on the β subunit.

Results

Binding experiments with [^{125}I]insulin

Leprechaun Geldermalsen was born from a consanguineous marriage with an inbreeding coefficient of 1/128 (for pedigree see Figure 1). Since leprechaunism is an autosomal recessive disease (Rosenberg *et al.*, 1980) the carriers in the Geldermalsen pedigree may exhibit one of the insulin receptor abnormalities observed in the patient's fibroblasts (Maassen *et al.*, 1988a) with a decreased intensity. We tested this assumption using [^{125}I]insulin binding assays. Cultured primary fibroblasts of the supposed carriers (Figure 1, nos. 327 and 328) and the obligate carriers (Figure 1, nos 329 and 330) showed decreased [^{125}I]insulin binding with values that are intermediate between that in the patient (Figure 1, no. 470) and those obtained in controls. Insulin binding to control fibroblasts was independent of age and sex. The individuals with decreased insulin binding to cultured fibroblasts showed increased blood insulin concentrations during oral glucose load indicating mild insulin resistance (Lekanne Deprez *et al.*, 1989). The grandfather who maps outside the consanguineous line had normal [^{125}I]insulin binding to cultured fibroblasts (Figure 1, no. 326). Fibroblasts of the newborn baby (Figure 1, no. 471), who does not have any clinical features of leprechaunism, were not available so insulin binding experiments could not be performed. Taken together these data indicate that both insulin receptor alleles of leprechaun Geldermalsen are affected and that heterozygous individuals are characterized by a decreased insulin binding to fibroblasts in conjunction with a mild insulin resistance.

Isolation and characterization of cDNA clones for the leprechaun insulin receptor

To characterize the molecular nature of the genetic defect we constructed several cDNA libraries of poly(A)$^+$ RNA from leprechaun Geldermalsen fibroblasts. Because of the low concentration of insulin receptor mRNA in fibroblasts, a specific primer downstream of the stop codon was used in addition to oligo(dT) for first strand cDNA synthesis. When 6×10^6 independent clones were screened with human insulin receptor cDNA (Ullrich *et al.*, 1985) or synthetic oligonucleotides, nine positive clones were identified. These clones varied in size from 900−2950 nucleotides and covered the total coding sequence for the insulin receptor. Inspection of the sequence (Table I) reveals only one nucleotide (at position 827) that differs from the two published wild-type sequences reported by Ebina *et al.* (1985a) and Ullrich *et al.* (1985). This mutation, which is present in two independently obtained clones, thereby making a cloning artefact unlikely, causes a substitution of leucine to proline at position 233 in the α subunit. At all other positions the leprechaun Geldermalsen sequence matches with at least one of the two wild-type sequences. The observed variations between the two published wild-type sequences are thought to reflect functionally neutral polymorphisms since both wild-type insulin receptors, when expressed in transfected cells, function normally (Ebina *et al.*, 1985b; Chou *et al.*, 1987; Whittaker *et al.*, 1987). Exactly the same set of polymorphic amino acids were subsequently found by three other groups (Whittaker *et al.*, 1987; Kadowaki *et al.*, 1988; Seino *et al.*, 1989) thereby limiting the polymorphic sites in the insulin receptor to six. Exon 11, which consists of 36 nucleotides, is alternatively spliced depending on the cell type (Seino *et al.*, 1989). Therefore the only possible candidate responsible for the defective insulin binding and autophosphorylation of the patient's receptor is the mutation at nucleotide position 827.

Inheritance of the leucine-to-proline mutation

The leucine-to-proline mutation at nucleotide position 827 converts the CTGG sequence at position 826−829 into

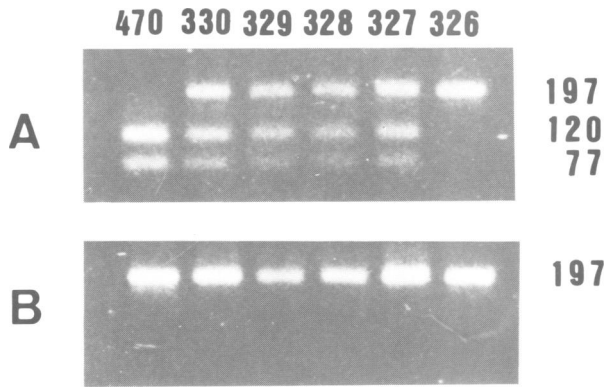

Fig. 2. Amplification by the polymerase chain reaction of genomic DNA between insulin receptor cDNA positions 707 and 903. *Msp*I digested (**A**) and undigested DNA (**B**) was analysed by electrophoresis on a 2% agarose gel. 326, 327, 328 (grandparents), 329, 330 (parents) and 470 (patient); see also pedigree Figure 1. Fragment sizes are indicated in bp.

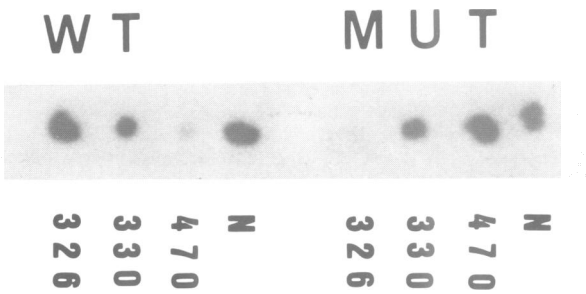

Fig. 3. Amplification by the polymerase chain reaction of genomic DNA between insulin receptor cDNA positions 707–903. Amplified DNA was spotted on Nylon membranes and hybridized with a wild-type (WT) or a mutant oligonucleotide probe (MUT). 326 (non carrier grandfather), 330 (mother) and 470 (patient); see also pedigree Figure 1. N, newborn baby, 471 in Figure 1.

CCGG thereby creating an *Msp*I restriction site. This implies that in the mutant insulin receptor gene there should be an *Msp*I restriction site at this position and not in the wild-type gene. We made use of this property to determine whether in this pedigree the residue 827 mutation coincides with the consanguineous line and is linked to the observed inheritance of the insulin binding defect (Figure 1). For this purpose we amplified genomic DNA covering the cDNA sequence between nucleotides 707–903 of the patient and his relatives and subjected it to *Msp*I restriction analysis (Figure 2). *Msp*I degraded all of the amplified DNA in the case of leprechaun Geldermalsen (470), whereas cleavage of amplified DNA from the individuals 327, 328, 329 and 330 was only ~50%. This finding implies that the patient is homozygous for the leucine-to-proline mutation and that the obligate and supposed carriers are heterozygous. Amplified DNA from the available non carrier grandfather (Figure 1, no. 326) and 20 controls (data not shown) could not be cleaved by *Msp*I. The lack of cleavage was not related to the quality of the amplified DNA since all DNA samples were 100% sensitive to *Rsa*I which cleaves at position 861 (data not shown). These findings imply that the 95% confidence interval of the gene frequency in the general population is between 0 and $1-0.05^{1/40} = 0.07$. For further calculations we used the upper limit of the estimated gene frequency of the mutation. Likelihood evaluation yielded a p value <0.02 emphasizing the relationship between the leucine-to-proline mutation and the disease.

Genotype of individual 471

At the time of the experiments only minute amounts of chromosomal DNA derived from cultured amniotic fluid cells of individual 471 (Figure 1) was available. Amplification of this DNA did not result in a distinct 197 bp band on agarose gel. Restriction analysis with *Msp*I was therefore not possible. However to investigate the occurrence of the leucine-to-proline mutation we spotted amplified DNA of 471 and three known genotypes (326, 330 and 470, Figure 1) on Nylon filters and hybridized the filters to either a wild-type or a mutant probe. Using critical washing conditions we determined that 471 is heterozygous for the mutation at position 827 (Figure 3) because both probes hybridized to his amplified DNA. After birth the child was found not to be affected by leprechaunism.

Discussion

We have demonstrated that in a family with leprechaunism an insulin binding defect is inherited in a Mendelian way. Individuals with the binding defect also showed increased blood insulin concentrations during an oral glucose load (Lekanne Deprez *et al.*, 1989). This implies that a diminished insulin binding to cultured fibroblasts results *in vivo* in a moderate insulin resistance. In the case of leprechaun Geldermalsen, insulin binding to fibroblasts is almost completely abolished although these cells synthesize insulin receptors (Maassen *et al.*, 1988a). We do not know yet whether this abnormality results from a defective transport to the cell membrane or an incorrect folding of the receptor on the cell surface. An additional defect is that insulin receptors of the patient are defective in insulin stimulated autophosphorylation. As the receptors were found to bind insulin under the assay conditions, i.e. in the presence of Triton X-100, we concluded that insulin binding did not activate the tyrosine kinase on the β subunit of the receptor. To understand the molecular nature of these defects we cloned the cDNA for the insulin receptor of this patient and analysed its sequence. The published wild-type insulin receptor sequences differ at only six specific positions, but these differences are thought to be functionally insignificant since expression in transfected cells results in normal insulin receptors (Ebina *et al.*, 1985b; Chou *et al.*, 1987; Whittaker *et al.*, 1987). The sequence of the patient's receptor contains a new variation resulting in the conversion of leucine into proline at position 233 in the α subunit. The fact that the consanguineous line, the mode of inheritance of the insulin binding defect and the genotype for the leucine-to-proline mutation in the Geldermalsen family all match with each other has led us to the conclusion that the characterized mutation in the homozygous form results in severe insulin receptor malfunctioning and leprechaunism. To get a more objective impression of the significance of the leucine-to-proline mutation being linked to the disease, we performed statistical analysis which yielded a p value <0.02 for such a linkage. The finding that a newborn baby with no features of leprechaunism is heterozygous for this mutation strengthens this conclusion. In the heterozygous form a normal phenotype with mild insulin resistance is observed.

As the predominant appearance of the insulin receptor has an $\alpha_2\beta_2$ subunit composition, the situation may occur that chimeric insulin receptors consisting of a mutant $\alpha\beta$ and a wild-type $\alpha\beta$ are formed. These chimeras may be unable to bind insulin. Such a situation can explain the observation that insulin binding to fibroblasts of the heterozygotes is only 16–33% of control values (instead of the expected 50%).

Until now the insulin receptor structure of one leprechaun patient, a compound heterozygote, has been determined (Kadowaki *et al.*, 1988). In this patient one allele yields a truncated receptor as a result of a premature stop codon in the α chain DNA. The other allele codes for a receptor with a lysine-to-glutamic acid mutation at position 460 in the α chain. This mutant receptor has normal signal transduction but seems to have lost the ability of positive co-operativity (Kadowaki *et al.*, 1989). We recently described amplified DNA from another leprechaun patient (Maassen *et al.*, 1988b) which was not cut by *Msp*I at position 827 (data not shown). Since this patient also has an insulin receptor with a binding defect, the absence of the leucine-to-proline mutation emphasizes the heterogeneous nature of the genesis of insulin resistance in leprechaunism.

The mutation in the insulin receptor of leprechaun Geldermalsen maps outside the domains which are reported to be involved in insulin binding (De Meyts *et al.*, 1988; Whittaker *et al.*, 1988; Yip *et al.*, 1988) and tyrosine kinase activity (Ebina *et al.*, 1985a; Ullrich *et al.*, 1985). Since some deletions in the insulin receptor α subunit result in a receptor with constitutively elevated autophosphorylation and tyrosine kinase activity (Ellis *et al.*, 1987) and since tryptic digestion of intact cells mimics insulin action (Shoelson *et al.*, 1988) it has been proposed that the α chain exerts a negative constraint on the tyrosine kinase activity of the β chain (reviewed by Andersen, 1989). Insulin binding releases this constraint. In the leprechaun insulin receptor the negative influence of the α subunit seems permanent however, because detergent-solubilized receptors bind insulin without being fully autophosphorylated. Secondary structure analysis according to Chou and Fasman (1978) predicts the introduction of a β-turn as a result of the conversion of leucine into proline (data not shown). Though it is unlikely that such a structural change will affect the overall folding of the protein, it may introduce a steric hindrance in the α-chain which prevents correct folding of the insulin binding site. An incorrect partial folding of the receptor at the cell surface may explain the absence of insulin binding to fibroblasts. Our observation that Triton X-100-solubilized receptors bind insulin (Maassen *et al.*, 1988a) may be related to an induced loosening of the folding of the polypeptide chain. Another possibility is that the mutation interferes with the translocation of the insulin receptor to the cell membrane and that solubilization unveils intracellular receptors. However, as the insulin stimulated autophosphorylation of the β subunit is not restored by solubilization, we conclude that the mutation may be localized in a new region required for transmitting the insulin binding signal to the tyrosine kinase region.

Materials and methods

Fibroblast culture

Human fibroblasts were developed from explants of skin biopsies from the leprechaun patient, his relatives and twelve controls. Stock cultures were harvested around the third passage and stored in liquid nitrogen. Cells were grown in F10 medium supplemented with 10% heat inactivated fetal calf serum in a 2.5% CO_2 atmosphere. Prior to RNA extraction from leprechaun Geldermalsen fibroblasts, cells were grown overnight in F10 medium supplemented with 1 μM dexamethasone to increase insulin receptor mRNA levels (Maassen *et al.*, 1987).

[^{125}I]Insulin binding assays

[^{125}I]Insulin binding to cultured primary fibroblasts was as described previously (Maassen *et al.*, 1988a). Averaged insulin binding data from twelve controls were set at 100%. Standard deviations in the insulin binding assays were $<10\%$.

cDNA cloning and sequencing

Poly(A)$^+$ mRNA of leprechaun Geldermalsen fibroblasts was purified by the LiCl/ureum method (Auffrey and Rougeon, 1980) and oligo(dT)–cellulose chromatography. It was copied into cDNA (Gubler and Hoffman, 1983) with as first-strand primers, oligo(dT) and an insulin receptor specific oligonucleotide (5′-TCCTCTGCAGGACTAGTTAAATTGGTA-3′). λgt10 libraries were constructed according to Huynh *et al.* (1985). Libraries were screened with insulin receptor cDNA probes (Ullrich *et al.*, 1985) or insulin receptor oligonucleotides. Positive clones were subcloned into M13mp18 or M13mp19 for sequence analysis by the dideoxy chain determination method (Sanger *et al.*, 1977) with Sequenase (United States Biochemicals). Ambiguities were resolved with the chemical degradation method (Maxam and Gilbert, 1977).

DNA amplification and mutation detection

DNA was isolated from cultured primary fibroblasts or cultured amniotic fluid cells according to Maniatis *et al.* (1982). 1 μg DNA was amplified by 32 cycles of the chain reaction (Saiki *et al.*, 1988) catalysed by Taq polymerase (Perkin-Elmer-Cetus) with two insulin receptor specific oligonucleotides: 5′-CGACCATCTGTAAGTCACAC-3′ and 5′-GAAGCTGAAGTTCACACAGC-3′. Amplified DNA from fibroblasts was digested with *Msp*I according to the manufacturer (Boehringer, Mannheim) and analysed by electrophoresis on a 2% agarose gel. The fragment sizes were calculated by comparison with the mobilities of known restriction fragments. Amplified DNA from cultured amniotic fluid cells was spotted on Nylon filters and hybridized to a wild-type oligonucleotide (5′-AACTTCTACCTGGACGGCAG-3′) or to a mutant oligonucleotide (5′-AACTTCTACCCGGACGGCAG-3′) (Verlaan-de Vries *et al.*, 1986).

Statistical analysis

The likelihood of the observed data was calculated for two situations: (i) the mutation is completely linked to the insulin receptor gene but there is no relationship between the leucine-to-proline mutation and the disease and (ii) the mutation is completely linked to the insulin receptor gene and the disease does not occur without the leucine-to-proline mutation and vice versa (this is equivalent to a situation with complete linkage disequilibrium). Likelihoods were calculated with the computer program MLINK version 5.03 (Lathrop *et al.*, 1984). Likelihoods were compared in a likelihood-ratio test yielding a χ^2 statistic with one degree of freedom.

Acknowledgements

We thank Dr W.J.Kleijer for providing the fibroblasts from the Geldermalsen family. Dr A.Ullrich for the generous gift of the wild-type insulin receptor cDNA, Dr H.van Ormondt for assistance in computer-aided sequence analysis and Drs H.Vrieling and A.Pastink for their help with the polymerase chain reaction. This work was supported by the Netherlands Organization for Advancement of Pure Research (NWO) through the Foundation for Medical Research (MEDIGON) and the Diabetes Fonds Nederland.

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Received on March 28, 1989; revised on May 24, 1989