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Mutagenesis of Phe[381] and Phe[382] in the extracellular domain of the insulin receptor: effects on receptor biosynthesis, processing, and ligand-dependent internalization

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Abstract

Mutations of the extracellular domain of the insulin receptor impair processing and transport of receptors to the plasma membrane. We have previously reported that a mutation substituting Val for Phe[382] in the α-subunit of the insulin receptor impairs intracellular processing and insulin-induced autophosphorylation of the mutant receptor. In this investigation, we have generated two independent mutations of amino acids Phe[381] and Phe[382] of the insulin receptor: Val for Phe[381] and Leu for Phe[382]. These substitutions cause a slight impairment of intracellular processing and transport of the mutant receptors. Furthermore, insulin-dependent internalization of the mutant receptors is unaffected by these mutations. Thus, of the three substitutions studied to date, Val for Phe[382] is the only mutation of the Phe[381]–Phe[382] sequence that causes a major defect in post-translational processing of the receptor.

Key words: Insulin receptor; Mutagenesis; Intracellular transport; Genetic disease; Insulin resistance

1. Introduction

The insulin receptor belongs to a family of hormone receptors with tyrosine kinase activity [1]. Binding of insulin to the extracellular domain of the insulin receptor α-subunit triggers tyrosine phosphorylation of the intracellular domain of the β-subunit. Phosphorylation of other protein substrates by the receptor tyrosine kinase is thought to represent an important mechanism by which insulin exerts its actions on target cells [1].

Studies of mutations of the insulin receptor gene in patients with genetic forms of insulin resistance have provided valuable insights into structure–function relationships of the insulin receptor. Several of the point mutations identified in the N-terminal half of the α-subunit are associated with defective processing of the insulin receptor precursor, and with an impaired rate of transport of mutant receptors to the plasma membrane [2–8]. Defective transport is probably caused by a conformational change in the secondary structure of the receptor that causes mutant receptors to be retained in the rough endoplasmic reticulum, where they are bound to the immunoglobulin binding protein BiP [4].

Furthermore, the conformational changes caused by these point mutations can affect the function of the receptor in other ways. For example, substitution of Lys for Asn[15] is associated with a reduced affinity of the receptor to bind insulin [5], and substitution of Val for Phe[382] is associated with reduced insulin-induced phosphorylation of the β-subunit [9,10].

Substitution of Val for Phe[382] was the first mutation reported to interfere with transport of the insulin receptor to the cell surface [3]. In the present study, we have used site-directed mutagenesis to further analyze the effect of replacing amino acids Phe[381] and Phe[382] in the primary sequence of the insulin receptor. We have replaced Phe[382] with Leu, the corresponding amino acid found in the sequence of the highly homologous receptor for insulin-like growth factor-I (IGF-I) [11]. Furthermore, we have changed the preceding amino acid in the primary sequence of the insulin receptor, Phe[381], to Val. Unlike the Val[382] mutation, the Phe[381] and the Leu[382] substitutions caused little if any defect in processing and transport of mutant receptors to the plasma membrane.

2. Materials and methods

2.1. Cell lines

NIH-3T3 fibroblasts transfected with expression plasmids encoding the wild-type human insulin receptor (WT) or the Val[382] mutant receptor (Val[382]) have been described in previous studies [3,4,9]. Expression vectors for the Val[381]- or Leu[382]-mutant insulin receptors were generated

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by oligonucleotide-directed mutagenesis of the human insulin receptor cDNA [12]. In the Val^{381} construct, codon 381 (TTC^{Phe}) was replaced by GTC^{Val} using a mutagenic oligonucleotide corresponding to nucleotides 1,263–1,284 of the human insulin receptor cDNA; in the Leu^{382} construct, codon 382 (TTC^{Phe}) was replaced by CTC^{Leu} using a mutagenic oligonucleotide corresponding to the same cDNA sequence indicated above. Mutant cDNAs were then cloned in a SV40-based expression plasmid and transfected in NIH 3T3 cells as described before [3]. The presence of the point mutations at codons 381 and 382 was confirmed using a reverse transcriptase/polymerase chain reaction assay (RT/PCR) to amplify a fragment of human insulin receptor cDNA encompassing nucleotides 1,199–1,308 [12] as described previously [3]. Direct sequencing of PCR-amplified cDNA was performed as described previously [13].

2.2. [^{125}I]Insulin binding assay

[^{125}I]Insulin binding assays were performed on confluent monolayers of intact cells as described previously [3]. To determine the internalization rates, binding assays were performed overnight at 4°C and unbound insulin was removed by washing with ice-cold PBS. Thereafter, binding assay medium was added, and the cells were transferred to a 37°C incubator for varying periods of time. At each time point, insulin that remained at the cell surface was removed by an acid wash with a binding assay solution at pH 4.5, and internalized insulin was determined separately by lysing cells in NaOH [5].

2.3. Biosynthetic labeling

Confluent monolayers of transfected cells were incubated for 1 h in methionine-free DMEM. Thereafter, cells were incubated in complete medium containing 0.1 mCi/ml of [^{35}S]methionine (1,100 Ci/mmol; Dupont-NEN, Wilmington, DE) for 1 h at 37°C, followed by a chase period of up to 24 h in complete medium. At each time point during the chase period, cells were harvested and processed for immunoprecipitation and gel electrophoresis as described previously [3].

3. Results

NIH 3T3 cells transfected with expression vectors encoding wild-type or Val^{382}-, Val^{381}-, or Leu^{382}-mutant insulin receptor cDNA were assayed for [^{125}I]insulin binding to cell surface receptors (Fig. 1). One clone expressing each type of receptor was selected for further studies. Sequence analysis of amplified cDNA derived from transfected cell lines confirmed the presence of the point mutations introduced at positions 381 and 382 (data not shown). Scatchard analysis of binding data indicated that insulin binding affinity was unaffected by mutations substituting Val for Phe^{381}, Val for Phe^{382} and Leu for Phe^{382} (not shown).

3.1. Metabolic labeling experiments

Substitution of Val for Phe^{382} impairs processing of the insulin receptor and its transport to the plasma membrane [3,4]. To study the effect of substituting Val for Phe^{381} or Leu for Phe^{382} on receptor processing, pulse-chase labeling experiments with [^{35}S]methionine were performed on transfected cells (Fig. 2). After labeling the cells, receptors were solubilized with Triton X-100 and immunoprecipitated with anti-receptor antibody. The wild-type insulin receptor is synthesized as a single-chain glycoprotein precursor with $M_r \approx 190{,}000$. This product undergoes several post-translational modifications, including proteolytic cleavage into α- and β-subunits and

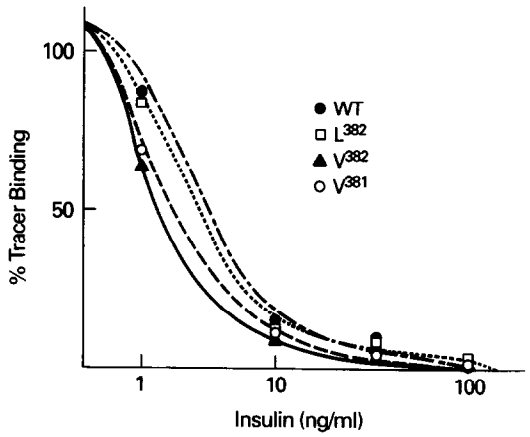

Fig.1. [^{125}I]Insulin binding to cells transfected with wild-type and mutant insulin receptors. Monolayer cultures of NIH 3T3 cells transfected with wild-type, Val^{382}-, Leu^{382}-, or Val^{381}-mutant insulin receptor cDNA were incubated overnight with [^{125}I]insulin in the presence of increasing concentrations of unlabeled insulin. Unbound insulin was removed by washing monolayers with ice-cold PBS, and radioactivity was determined on total cellular lysates in a gamma counter. The amount of tracer bound in the absence of unlabeled ligand was normalized to 100%.

N-linked glycosylation [14–16]. Cleavage of the precursor into separate subunits and maturation of the N-linked carbohydrate side chains occur virtually simultaneously in cells transfected with wild-type receptors, so that α- and β-subunits are visualized as single bands of M_r 135,000 and 95,000, respectively (Fig. 2, lanes 1–6). As shown previously [3,4], post-translational processing of the insulin receptor is substantially retarded by substitution of Val for Phe^{382}. This retarded processing is reflected by the appearance of a characteristic doublet of bands in the region of the gel corresponding to α- and β-subunits, due to the presence of incompletely processed pre-α and pre-β-subunits (Fig. 2, lanes 7–12) [3–6]. Interestingly, the Val^{381}- and the Leu^{382}-mutant receptors showed only a slight retardation of the processing rate. In cells transfected with the Val^{381} receptor, mature α- and β-subunits could only be visualized at 4 h and not at 1 h of chase (compare Fig. 2, lanes 1,2 and 13–15). Likewise, processing of the Leu^{382} receptor was retarded when compared to the wild-type receptor, so that at 1 h of chase only pre-α- and pre-β-subunits could be seen (Fig. 2, lanes 19–21). In contrast to the Val^{382} receptor, pre-α- and pre-β-subunits disappeared after 1 h, whereas they persisted for up to 12 h in Val^{382}-transfected cells (Fig. 2, lanes 7–11). Similar data were obtained when processing of the carbohydrate side chains was studied with [^{3}H]mannose labeling followed by endoglycosidase H digestion (data not shown).

3.2. Internalization of the insulin–receptor complex

Following insulin binding, the insulin-receptor complex is internalized through clathrin-coated pits into endocytic vesicles [17]. We wanted to determine whether

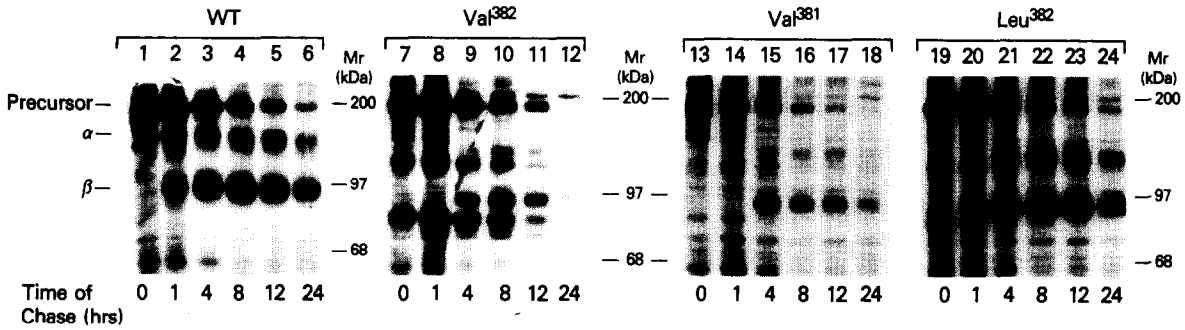

Fig. 2. Biosynthetic labeling of insulin receptors with [^{35}S]methionine. Transfected cells were incubated for 1 h in methionine-free DMEM and then pulse-labeled for 1 h in the presence of 0.1 mCi/ml of [^{35}S]methionine. Thereafter, the medium was changed to complete DMEM and the incubation was continued for 1 to 24 h. At the end of each time point, cells were removed from the dishes by lysis in a buffer containing 1% Triton X-100. Lysates were immunoprecipitated using an anti-insulin receptor antibody or non-immune rabbit serum as described in section 2. Lanes 1–6, wild-type insulin receptor; lanes 7–12, Val^{382}-mutant; lanes 13–18, Leu^{382}-mutant; lanes 19–24, Val^{381}-mutant.

mutations at positions 381 and 382 in the extracellular domain would interfere with the endocytic pathway. [^{125}I]Insulin was allowed to bind to cell surface receptors for 16 h at 4°C. Under these conditions, no significant internalization occurs. Thereafter, cells were incubated at 37°C to allow for endocytosis of the hormone–receptor complex. [^{125}I]Insulin that remained bound to the cell surface was removed by an acid wash (pH 4.5). [^{125}I]Insulin disappeared from the cell surface at approximately the same rate in cells expressing all four types of receptors (Fig. 3A). After incubation for 5 min at 37°C, cell surface radioactivity had declined to approximately 40% of its initial value. Internalized insulin was measured as radioactivity that remained cell-associated after the acid wash (Fig. 3B) [5]. Within 20 min, approximately 40% of [^{125}I]insulin was internalized into cells expressing the wild-type insulin receptor (Fig. 2B). Interestingly, the three mutant receptors (Val^{382}, Leu^{382}, and Val^{381}) had internalization kinetics that were nearly identical to the wild-type receptor (Fig. 3A,B). The Val^{382} and Leu^{382} receptors were virtually indistinguishable from the wild-type receptor, while a small difference was observed in the amount of insulin internalized into cells transfected with the Val^{381} receptor (Fig. 3B). The rate of dissappearance of the tracer from the cell surface was, however, similar to that observed in the other three cell lines (Fig. 3A).

4. Discussion

Mutations of the extracellular domain of the insulin receptor have been identified in patients with genetic forms of insulin resistance [2]. These mutations localize to the N-terminal half of the α-subunit of the insulin receptor and interfere with multiple aspects of insulin receptor function: processing of the proreceptor, transport to the plasma membrane, insulin binding, and recycling of the insulin–receptor complex. Transport-defective mutants of the insulin receptor are generally the result of single amino acid substitutions in the receptor's extracellular domain. It has been proposed that the presence of point mutations in this region of the receptor may affect folding of the receptor molecule and prevent intracellular processing and transport to the plasma mem-

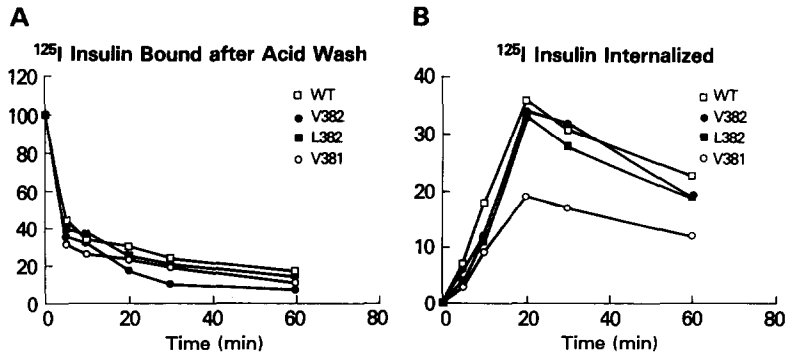

Fig. 3. Insulin-induced internalization of the hormone–receptor complex. Transfected cells were incubated overnight with [^{125}I]insulin at 4°C. Thereafter, unbound insulin was removed by washing twice with ice-cold PBS, and 2 ml of a binding assay solution [8] (pH 7.4) were added. Cells were transferred to a 37°C incubator, and the incubation was continued for up to 1 h. At each time point, cells were washed again and then incubated in binding assay solution at pH 4.5. After 30 min, the solution was removed and counted in a gamma counter (A). Cell- associated radioactivity was also measured by solubilizing monolayers in 1 N NaOH (B) [5].

brane of the mutant receptors. In some instances it has been possible to show that mutant receptors are associated with the immunoglobulin binding protein BiP in the rough endoplasmic reticulum [4]. Because BiP has important functions, including to catalyze folding and multimerization of nascent polypeptide chains and also to retain misfolded mutant proteins in the endoplasmic reticulum, this finding has led to the suggestion that binding to BiP may represent the mechanism that inhibits transport of mutant insulin receptors to the cell surface [4]. Similar findings have now been reported for other mutations associated with human diseases, like cystic fibrosis [18] and osteogenesis imperfecta [19].

In the present study, we have extended our characterization of mutations of the extracellular domain of the insulin receptor by generating two site-directed mutants of the sequence Phe^{381}–Phe^{382} : Val^{381} and Leu^{382}. This double phenylalanine motif is highly conserved in the family of receptors homologous to the insulin and EGF receptor: human and guinea pig insulin receptor-related receptor [20], human and *Drosophila* EGF receptor [21,22], and c-erb-B2 protein [23]. However, the closely related IGF-1 receptor differs at this position within an otherwise highly conserved sequence [11]. In the sequence of the IGF-1 receptor, leucine is found at the homologous amino acid residue (Leu^{372}). In the insulin receptor, replacement of Phe^{382} by Leu exerts a minimal effect on intracellular processing and transport of the insulin receptor. Thus, the impairment in receptor function is specifically associated with the presence of the amino acid residue Val at position 382. Our observation that substitution of Val for Phe^{381} also does not affect receptor function in a major way suggests that Phe^{382} plays a more important role in receptor folding and stabilizing the native conformation of the receptor. Interestingly, Val is found at the amino acid residue homologous to Phe^{381} of the c-erb-B2 receptor (Val^{424}) [23]. These observations are also consistent with the conclusion that there is not a requirement for two adjacent aromatic amino acid residues at this location to stabilize the conformation of the insulin receptor and other homologous receptors.

It is not clear why there is a difference between the Val^{382}- and the Val^{381}- and Leu^{382}-mutant receptors. The presence of Val at amino acid residue 382 might cause the mutant receptor to bind more tightly to the immunoglobulin binding protein BiP so that it would be impaired in its ability to be transported from the endoplasmic reticulum through the Golgi to the plasma membrane [4]. Available data are not adequate to answer this question. However, studies of the interaction between small peptides and BiP suggest that the presence of leucine residues in random peptides (composed of up to seven amino acids) tends to increase the potency of the peptides in stimulating the ATPase activity of the BiP protein. This suggests that the presence of leucine tends to enhance the ability of a peptide to interact productively with the BiP protein. While other hydrophobic amino acids are also found with increased frequency in the binding site of BiP, the affinity of a random peptide containing Leu at any given position is higher than that of a similar peptide containing Val or Phe in the same position [24].

4.1. Val^{382}-mutant receptor undergoes normal ligand-induced endocytosis

Previous studies with site-directed mutants of the insulin receptor have suggested that receptor tyrosine kinase activity is required for ligand-stimulated internalization of receptors [16,25–30]. Because we had previously shown that the Val^{382}-mutation impaired receptor autophosphorylation [9,10] we wished to investigate receptor-mediated endocytosis of Val^{381}-, Val^{382}-, and Leu^{382}-mutant receptors. We did not detect major differences between the internalization rates of wild-type and mutant receptors. It is not clear why the Val^{382} mutation differs in this respect from other mutations that impair receptor autophosphorylation. However, it is intriguing that the other mutations mapped to the intracellular domain of the receptor while the Val^{382} mutation is located in the extracellular domain. Our observations raise the possibility that the inhibition of endocytosis is not due to the defect in receptor autophosphorylation per se, but to an associated abnormality in the conformation of the intracellular domain.

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