Role of proline 193 in the insulin receptor post-translational processing

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Abstract

Aims/hypothesis. A point mutation, P193L, in the insulin receptor α subunit, has been previously identified in a patient affected by an extreme form of insulin resistance due to reduced insulin binding. In our study we investigated the cellular mechanisms by which P193L substitution causes a reduction of insulin receptor numbers on the cell surface.

Methods. Mutated insulin receptors have been generated and expressed in COS1 cells. Transcription as well as translation of P193L insulin receptor have been measured and compared with wild type insulin receptor.

Results. P193L insulin receptor is normally transcribed and progresses to the step of insulin proreceptor, which does not proceed to dimerization, resulting in the accumulation of the 210 kDa form. These findings suggest that the P193L insulin proreceptor is retained in the endoplasmic reticulum, where several

molecular chaperones drive the folding of protein precursors. Therefore the interaction between mutated insulin receptor precursor and two endoplasmic reticulum resident chaperones (GRP78 and calnexin) were investigated. P193L insulin proreceptor co-immunoprecipitates with greater amounts of GRP78 and its interaction with calnexin is greatly delayed compared with wild type insulin receptor precursor. Co-transfection of wild type and mutated insulin receptors causes a considerable reduction of cell surface wild type insulin receptors.

Conclusion/interpretation. P193 is critical for insulin proreceptor folding. The monomeric form of P193L insulin proreceptor is retained in the endoplasmic reticulum by a calnexin and GRP78 mediated mechanism that reduces mature insulin receptor expression on the cell surface. [Diabetologia (1999) 42: 435–442]

Keywords P193L mutation, insulin resistance, insulin receptor processing, GRP78, calnexin.

The mature insulin receptor (IR) is an $\alpha 2$ - $\beta 2$ oligomeric transmembrane glycoprotein with tyrosine ki-

nase activity [1]. The native insulin proreceptor progresses through a complex intracellular pathway to acquire the definitive structure, function and localization on the cell surface. The IR precursor, a single chain high mannose polypeptide (210 kDa), enters endoplasmic reticulum (ER) lumen where early maturation steps occur. Here, the polypeptide acquires dimeric structure and insulin binding capacity by rearrangement of disulphide bonds. Then proteolytic cleavage and O-glycosylation of the insulin proreceptor dimer (420 kDa) takes place in the Golgi apparatus. Finally, the mature IR $\alpha 2$ - $\beta 2$ tetramer localizes on the cell surface as a transmembrane glycoprotein able to bind insulin and to transduce hormone signalling [2]. In the early steps of

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Abbreviations: IR^{WT}, Wild type insulin receptor; IR^{P193L}, insulin receptor carrying P193L mutation; ER, endoplasmic reticulum; Hsp70, 70 kDa heat shock protein; GRP78, 78 kDa binding protein glucose-regulated protein; BiP, immunoglobulin heavy chain binding protein; DMEM, Dulbecco's modified Eagle's medium; RT-PCR, reverse transcription-polymerase chain reaction; IGF-1-R, IGF-1 receptor; IRR, insulin related

biosynthesis at least three molecular chaperones: Hsp70, calnexin and GRP78 [3–5] catalyse insulin proreceptor folding. Hsp70 is a cytosolic protein with antifolding function [6], while calnexin associates selectively with newly synthesized glycoprotein in the ER [7]. GRP78, also known as BiP, catalyses the assembly of subunits of a number of proteins which are co-translationally translocated into the ER. In particular, GRP78 retains misfolded proteins acting as a quality control mechanism of newly synthesized polypeptides at an early step in the folding process [6].

It has been suggested that mutations in the extracellular domain of IR reduce the number of insulin binding sites on the cell surface impairing post translational processing of insulin proreceptor [8, 9]. Recently, two patients with extreme insulin resistance, carrying a homozygous Leu for Pro 193 substitution have been described independently [10, 11]. In both patients a severely reduced insulin binding capacity was reported. To characterize the cellular mechanism underlying the defect induced by P193L mutation, we overexpressed wild type and mutated IR in COS1 cells and investigated different steps of the IR biosynthetic pathway.

In this paper we show that 1) P193L mutation reduces insulin binding through a post translational impairment in mutant proreceptor processing, 2) the limiting step might be localized at the level of insulin proreceptor interaction with GRP78 and calnexin and 3) IRP^{193L} precursor expression in COS1 cells impairs wild type insulin proreceptor biosynthesis, as observed in co-transfection experiments.

Materials and methods

Materials. Oligonucleotides were purchased from TIB MOL-BIOL (Genoa, Italy). COS1 cells [12] were obtained from ATCC (American Types Culture Collection, Rockville, Md., USA). Dulbecco's modified Eagle's medium (DMEM), FCS and PBS were purchased from Sigma Chemicals (St. Louis, Mo., USA). Glutamine, penicillin G, streptomycin sulphate; DMEM without cystine, methionine, cysteine and Trans ³⁵S-label were from ICN Flow (Costa Mesa, Calif., USA). NHS-LC-biotin and horseradish peroxidase-linked streptavidin were purchased from Pierce (Rockford, Ill., USA). Human recombinant insulin, ¹²⁵I insulin, ¹²⁵I IGF-1, Hybond-C, anti rabbit Ig and ECL reagents were obtained from Amersham (Cleveland, Ohio, USA). Sac2 and AMV reverse transcriptase were from Promega (Madison, Wis., USA); Taq polymerase, High Pure RNA isolation kit and hexokinase from Boehringer (Mannheim, Germany); Bsm1 from New England Biolabs (Beverly, Mass., USA). Sequenase 2.0 kit was obtained from USB (Cleveland, Ohio, USA). Protein A Sepharose was purchased from Pharmacia LKB Biotechnology (Uppsala, Sweden). Anti GRP78 antibody was obtained from Santa Cruz Biotechnology (Santa Cruz, Calif., USA) while anti calnexin antibody was purchased from StressGen Biotechnology (Victoria, Canada). A polyclonal anti IR β -subunit antibody was kindly provided by G. Sesti (Università Tor Vergata, Rome, Italy).

Site-directed mutagenesis and construction of expression vectors. Site-directed mutagenesis was done by overlapping extension technique. Two separate reactions were carried out, using pCMVHIR as template, to obtain fragments with the desired mutation at their end. The first product, 614 bp, was generated using the oligonucleotides 5'-CCGCGCTGCTACTGGGCGGCGGC and 3'-ATGTCTACCAGCCCCGTTTGAAAG; the second, 1529 bp, using the oligonucleotides 5'-GAAAGTTTGCCCGACCATCTGTA and 3'-CAGGTCGAGGAAGTCCTAGACTC. PCR products were then hybridized together and used as template for further amplification using flanking oligonucleotides 5'-CCGCGCTGCTACTGGGCGGCGGC and 3'-CAGGTCGAGGAAGTCCTAGACTC. Resulting fragment was digested with Bsm1 and Sac2 and ligated in pCMV-HIR, replacing the corresponding wild type fragment. Product derived from PCR mutagenesis was sequenced by dideoxy method.

Cell culture and transfection. COS1 cells were grown in DMEM supplemented with 2 mmol/l glutamine, 10% FCS, 100 units/ml penicillin G, 100 μ g/ml streptomycin sulphate. Subconfluent cells were transfected with wild type or mutant expression vector by calcium phosphate precipitation. A glycerol shock was done 4 h after transfection. Experiments were carried out after 48 h.

Insulin and IGF-1 binding studies. Confluent cells, grown in six well plates, were washed twice with PBS and incubated for 3 h at 16°C in HEPES buffer (25 mmol/l HEPES, 120 mmol/l NaCl, 5 mmol/l KCl, 1 mmol/l MgSO₄, 1 mmol/l CaCl₂, 1% BSA pH 7.8) in the presence of 100 pmol of ¹²⁵I insulin or IGF-1. The non-specific binding, determined in the presence of 1 μ g insulin or IGF-1, was subtracted.

Reverse transcription-polymerase chain reaction (RT-PCR). Total mRNA was extracted from subconfluent cells by High Pure RNA Isolation Kit (Boehringer, Mannheim, Germany) and 1 μ g was used to synthesize first strand cDNA in the presence of DNase I, as described previously [13]. Primers were deduced from cDNA sequences [14–16]. For human IR the 5' primer was CTGGTCACCTTTTCGGATGAAC (nucleotides 1820–1841) and the 3' primer was GACTCCTCCAGTCTCCTTCAGGAT (nucleotides 2189–2211) that generated a fragment of 391 bp. For human β_2 microglobulin the 5' primer was TTCAGCAAGGACTGGTCT (nucleotides 1498–1516) and the 3' primer was CCTGCTTACATGTCTCGAT (nucleotides 2235–2252) that generated a fragment of 118 bp. PCR amplification was done as described [13] in the presence of ³²P ATP end labelled antisenses. PCR products were separated on 12% SDS-PAGE and exposed at –80°C. The bands of interest were cut under radiographic guide and counted in a β -scintillation counter. To test the presence of contaminating DNA, reverse transcriptase and PCR amplification were also done by omitting AMV reverse transcriptase.

Metabolic labelling. Subconfluent cells in 10 cm petri dishes were washed twice in PBS and incubated in methionine and cysteine free DMEM for 30 min at 37°C in a CO₂ incubator. The labelling reaction followed by addition of 300 μ Ci/petri of Trans ³⁵S-label for 30 min. Then cells were washed twice with PBS and incubated in chase medium (DMEM 10% FCS containing 2 mmol/l methionine and 0.5 mmol/l cysteine) for the indicated times. Finally, monolayers were washed twice with ice-cold PBS and lysed as described previously [13]. For GRP78 immunoprecipitation experiments, a modified lysis buffer, supplemented with 5 U/ml hexokinase plus 10 mmol/l

glucose, was used to deplete intracellular ATP [3]. Total cellular lysates were centrifuged at 15000 *g* for 30 min at 4°C and supernatants proceeded to immunoprecipitation.

Biotinylation of cell surface insulin receptors. Subconfluent cells were washed twice with PBS 0.1 mmol/l CaCl₂ 1 mmol/l MgCl₂. Cell surface proteins were biotinylated by incubation with 0.5 mg/ml N-hydroxysuccinimide-long chain-biotin diluted in PBS 0.1 mmol/l CaCl₂ 1 mmol/l MgCl₂ for 30 min at 4°C. Finally, cells were washed twice with PBS 0.1 mmol/l CaCl₂ 1 mmol/l MgCl₂ and lysed. Immunoprecipitated IRs were resolved on 8% SDS-PAGE. After electroblotting, nitrocellulose filters were blocked in PBS Tween 20 0.1% and 10% dry milk for 1 h at room temperature. Filters were then incubated with horseradish peroxidase-linked streptavidin for 1 h at room temperature. Following a final washing in PBS tween 20 0.1%, proteins were detected using ECL reagents. Blots were subsequently stripped and reprobed with anti IR β -subunit antibody.

Immunoprecipitation and immunoblotting. Immunoprecipitations were carried out with the indicated antibody for 16 h at 4°C by rocking the substances. Protein A sepharose was then added for an additional incubation of 90 min. Sepharose bound immunocomplexes were washed extensively in lysis buffer and incubated for 10 min at 80°C in Laemmli sample buffer. For experiments done under reducing conditions, 1,4-Dithiothreitol (DTT), at a final concentration of 100 mmol/l, was added to sample buffer. Finally, supernatants were resolved on 8% (reducing conditions) or 5% (nonreducing conditions) SDS-PAGE. The gels dried and were exposed for autoradiography. Radioactivity of the bands of interest, excised under autoradiographic guide, was also assessed in a β -scintillation counter.

Electrotransfer of proteins to nitrocellulose was done in Towbin buffer (20 mmol/l Tris, 150 mmol/l glycine, 20% methanol, 0.02% SDS) for 1 h at 150 mA. Nitrocellulose filters were blocked in PBS Tween 20 0.1% and 3% dry milk for 1 h at room temperature. Filters were incubated with primary antibody for 1 h at room temperature on a rocking platform, washed extensively in PBS Tween 20 0.1% and anti rabbit Ig secondary antibody was added for 1 h at room temperature. Following a final washing in PBS Tween 20 0.1%, bound antibodies were detected using ECL reagents. All procedures were carried out according to the manufacturer's instructions. Bands of interest were quantitated by densitometry using the National Institute of Health Image software (Bethesda, Md., USA).

Statistical analysis. Statistical analysis was done using paired Student's *t*-test. *P* less than 0.05 was considered to be significant.

Results

Insulin binding. Preliminary experiments were carried out to determine how increasing amounts of transfected cDNA relates to the insulin receptor binding activity on the cell surface. A linear increase in ¹²⁵I insulin binding up to 5 μ g of cDNA transfected was observed in COS-IR^{WT}, while no increase was measured in COS-IR^{P193L} (data not shown). When compared with COS-IR^{WT} alone (2.5 μ g), co-transfecting native COS1 cells with 2.5 μ g each of IR^{WT}

and IR^{P193L} cDNA (COS-IR^{WT+P193L}), showed a 50% reduction of ¹²⁵I insulin binding on the cell surface. COS1 cells transfected with the IR^{WT} express $3 \pm 0.5 \times 10^5$ high affinity insulin binding sites while mock transfected COS1 and COS-IR^{P193L} express $3\text{--}5 \times 10^3$ insulin binding sites. Co-transfected COS-IR^{WT+P193L} express $1.3 \pm 0.3 \times 10^5$ insulin binding sites (Fig. 1).

IR mRNA expression. The amount of IR mRNA expressed by mock transfected COS1 cells, COS-IR^{WT}, COS-IR^{P193L} and co-transfected COS-IR^{WT+P193L} was determined by semi-quantitative RT-PCR using β_2 -microglobulin as invariant control gene. The mRNA coding for IR^{WT} and IR^{P193L} is expressed in a similar amount. In COS-IR^{WT+P193L}, the amount of IR mRNA is, as expected, doubled, indicating that co-transfection of these two cDNAs does not affect each other's transcription (Fig. 2).

IR processing. Kinetics of IR biosynthesis, assessed by ³⁵S methionine labelling, showed equal amounts of wild type and mutated insulin proreceptors at an early time of chase (Fig. 3). After 4 and 8 h of chase, radioactivity associated to the IR^{WT} precursor declined and mature α and β subunits appeared. In contrast, in COS-IR^{P193L} cells radioactivity associated prevalently with the mutated proreceptor band (Fig. 3). The prolonged *t*_{1/2} of IR^{P193L} precursor (longer than 10 h compared with 3.5 h of WT insulin proreceptor) indicates that it is retained in a subcellular compartment. In nonreducing conditions (Fig. 4), in COS-IR^{WT}, the IR precursor acquires a tetrameric (420 kDa) structure while, in COS-IR^{P193L}, high molecular weight products were not detected. In co-transfection experiments the amount of dimers and tetramers is about 50% less than that observed in COS-IR^{WT}.

Biotinylation of cell surface insulin receptors and immunoblotting. Biotinylated cell surface proteins were immunoprecipitated with an anti IR β subunit antibody. In COS-IR^{WT} a 135 kDa band and a 95 kDa band, corresponding respectively to an α and β subunit, were observed. In mock transfected COS1 and COS-IR^{P193L} cells, α and β subunits were absent, while in COS-IR^{WT+P193L} a 54% reduction of intensity was observed after densitometric analysis (Fig. 5).

Blots were stripped and reprobed with an anti IR β subunit antibody. A 210 kDa band, corresponding to the insulin proreceptor, and a 95 kDa band, the IR β subunit, were observed in the COS-IR^{WT} cells, while only the 210 kDa uncleaved proreceptor was detected in COS-IR^{P193L} lysates (Fig. 5). Intensity of 210 kDa band in COS-IR^{P193L} was higher than the one measured in COS-IR^{WT} after densitometric analysis. In agreement with the results obtained in biotinylated cells, co-transfected COS-IR^{WT+P193L} showed an

Fig. 1. Determination of specific insulin binding to intact cells. Confluent mock transfected COS1 (A), COS-IR^{WT} (B), COS-IR^{P193L} cells (C) and COS-IR^{WT+P193L} (D), in six well plates, were incubated in the presence of 100 pmol of ¹²⁵I insulin. The non-specific binding, determined in the presence of 1 µg insulin, was subtracted. After 3 h incubation at 16 °C, cell monolayers were washed, lysed in 0.5 mmol/l NaOH and radioactivity was counted in a γ counter. Binding results, expressed as B/F, are the mean ± SEM of three different experiments in triplicate: mock transfected COS1 0.046 ± 0.004, COS-IR^{WT} 0.84 ± 0.03, COS-IR^{P193L} 0.05 ± 0.004, COS-IR^{WT+P193L} 0.37 ± 0.001. **, ** *p* < 0.01 vs COS-IR^{WT}.

increase in insulin proreceptor compared with wild type, suggesting that biosynthesis is unaffected by co-transfection up to this step while the appearance of α and β subunits is greatly reduced. These results suggest that P193L insulin proreceptor processing is impaired leading to an accumulation of the mutated IR precursor that does not reach the plasma membrane.

GRP78 and calnexin interaction. Since insulin proreceptor folding and maturation are driven by the ER chaperones GRP78 and calnexin [3–5], we tested the hypothesis that the P193L mutation could affect IR precursor interaction with these proteins. Cells were pulsed with ³⁵S methionine for 30 min and lysed after 1 h of chase. Cell lysates were immunoprecipitated with an antibody directed against GRP78. As shown in Figure 5, anti GRP78 antibody immunoprecipitated a greater amount of P193L insulin proreceptor than IR^{WT} precursor. When supernatants from this experiment were probed with an anti-insulin receptor antibody (Fig. 6), a lower amount of IR^{P193L} precursor was immunoprecipitated, compared with WT insulin proreceptor. These findings suggest that the P193L mutation increases mutant IR precursor association with GRP78. GRP78 and calnexin can act sequentially in protein folding [17, 18]. Therefore, the co-immunoprecipitation of insulin proreceptors with calnexin

Fig. 2. Insulin receptor mRNA expression. Total RNA was extracted from mock transfected COS1 (A), COS-IR^{WT} (B), COS-IR^{P193L} (C), COS-IR^{WT+P193L} cells (D) and reverse transcribed cDNA was amplified as detailed in Materials and methods. In the upper panel, insulin receptor and β₂ microglobulin expression was presented. Bands of interest were quantitated in a β scintillation counter and presented in the lower panel, as IR/β₂ microglobulin ratio. The actual dpm values were: COS-IR^{WT} 1423 ± 143, COS-IR^{P193L} 1387 ± 192, COS-IR^{WT+P193L} 2712 ± 107. Results presented are the mean ± SEM of three independent experiments done in duplicate.

was analysed. As shown in Figure 7, the amount of IR precursors detected in anti calnexin immunoprecipitates from ³⁵S labelled COS-IR^{P193L} was lower compared with that observed in COS-IR^{WT}. Surprisingly, when transfected unlabelled cells were examined, we found that a higher amount of calnexin co-immunoprecipitated with IR^{P193L} precursor, compared with wild type (Fig. 7). Experiments done at different times of chase in ³⁵S labelled cells have shown that the IR precursor-calnexin association reached the maximum between 15 and 30 min COS-IR^{WT} while P193L insulin proreceptor reached the maximum only after 120 min (data not shown). These findings suggest that progression of IR mutated precursor to calnexin is delayed.

Since calnexin binds exclusively the monomeric form of IR [5] in the ER, the higher amount of calnexin co-immunoprecipitated with anti IR antibody in COS-IR^{P193L} confirms that the mutated IR precursor is retained in the ER compartment.

To test the specificity of the P193L induced defect in IR^{WT} precursor processing, additional experiments

Fig. 3. Biosynthesis of insulin receptor in COS-IR^{WT} and COS-IR^{P193L}. Cells were pulsed for 30 min in the presence of ³⁵S methionine and chased for indicated times. Cell lysates were immunoprecipitated with an anti IR β antibody, resolved on 8% SDS-PAGE. Gels were dried and exposed at -80°C

Fig. 4. Effect of P193L mutation on dimers and tetramers synthesis in COS-IR^{WT}, COS-IR^{P193L} cells and COS-IR^{WT+P193L}. Experiments were carried out exactly as in Figure 3. Cell lysates were immunoprecipitated with an anti IR β subunit antibody, resolved on 5% SDS-PAGE under non-reducing conditions

co-transfecting IR^{WT} and two other mutated IRs were done: IR^{C860S}, which is normally synthesized [13] and IR^{C786S}, which does not undergo dimerization, but interacts normally with GRP78 and calnexin [19]. In both cases, co-transfections did not affect the expression of IR^{WT}, further supporting the specificity of P193L mutation in the inhibition of IR^{WT} production (data not shown).

In line with these observations, it is noteworthy that also the amount of IGF-1 binding was comparable in COS-IR^{P193L} and COS-IR^{WT} cells (IGF-1 binding results, expressed as B/F, were: mock transfected

Fig. 5 A, B. Biotinylation of cell surface insulin receptors and immunoblot. (A) Total cell lysates from biotinylated mock transfected COS1 (A), COS-IR^{WT} (B), COS-IR^{P193L} cells (C) and COS-IR^{WT+P193L} (D) were solubilized and proteins were separated on 8% SDS-PAGE, electrophoretically transferred to nitrocellulose and detected by incubation with streptavidin, as described in Materials and methods. (B) Blots were stripped and reprobed with an anti IR β subunit antibody. Immunoblots presented are representative of three independent experiments

COS1 cells 0.29 ± 0.03 , COS-IR^{WT} 0.26 ± 0.02 , COS-IR^{P193L} 0.27 ± 0.004).

Discussion

Mutant gene products, with impaired biosynthetic pathway, were shown in several human diseases [20]. Misfolded polypeptides are retained in ER by a mechanism of increased affinity for folding catalyst proteins, such as GRP78 and calnexin, which act as quality controllers of newly synthesized polypeptides

Fig. 6. Sequential immunoprecipitation of insulin proreceptors by anti GRP78 and anti IR antibodies. (Left panel) Confluent monolayers of COS-IR^{WT} (lanes A and C) and COS-IR^{P193L} (lanes B and D) were ³⁵S methionine labelled for 30 min and chased for 1 h, as detailed in Materials and methods. Cell lysates were immunoprecipitated with anti GRP78 antibody and resolved on 8% SDS-PAGE (lanes A and B). Supernatants from the previous experiments were then immunoprecipitated with anti IR β subunit antibody (lanes C and D). The bands at 78 kDa correspond to GRP78 and those at 210 kDa correspond to insulin proreceptor.

(Right panel) Quantitative results from experiments shown in the left panel were summarized and expressed as GRP78-insulin proreceptor bound/GRP78-insulin proreceptor unbound. * $p < 0.05$ vs COS-IR^{WT}

Fig. 7. Co-immunoprecipitation of insulin proreceptor and calnexin in COS-IR^{WT} (A), COS-IR^{P193L} cells (B). Experiments in ³⁵S labelled cells were carried out exactly as described in Figure 5. Cell lysates were immunoprecipitated with anti calnexin antibody and resolved on 8% SDS-PAGE (left panel). Immunoblot of calnexin in anti IR immunoprecipitates (right panel) was done as described in Materials and methods. Bands at 90 kDa correspond to calnexin

[21–24]. Similarly, it has been shown that mutations impairing insulin proreceptor processing cause severe forms of insulin resistance by reducing the IR number on the cell surface [8, 9]. Mutations in the extracellular domain of the IR, which localizes inside the ER in the early steps of post translational processing, are supposed to affect proper folding of IR. In this regard, it has been reported that an anti GRP78 antibody is more efficient to co-immunoprecipitate the naturally occurring H209R and F382V mutant IR, as well as the $\Delta 43$ IR, compared with IR^{WT} [3, 4]. It has been suggested that GRP78 and calnexin could act sequentially in protein folding [17, 18] and recently a cooperative action of these two chaperones in IR biosynthesis has been observed [5]. Here we have shown that the P193L mutation affects the early steps of IR precursor processing, driven by interaction with GRP78 and calnexin. The mutated proreceptor does not proceed to dimerization and does not reach the cell surface. The presence of a limiting step in IR biosynthesis at the GRP78 and calnexin level is further supported by the results obtained in co-transfection experiments. We have in fact observed that in COS-IR^{WT+P193L} cells also the biosynthesis of WT insulin proreceptors is reduced. This effect cannot be explained by the production of heterodimers between wild type and mutated insulin proreceptor since i. IR^{P193L} precursor does not reach the step of dimerization; ii. in co-transfection experiments, the amount of dimers is about 50% lower compared with the amount of monomers; iii. calnexin binds exclusively the IR monomeric form [5]. We propose that the reduced amount of IR^{WT} in co-transfection experiments could be explained by repeated binding-release cycles of the IR^{P193L} precursor with GRP78 and calnexin, which makes these chaperones unavailable for the IR^{WT} precursor. A similar mechanism has been recently proposed for mutations in the extracellular region of rhodopsin: co-expression of mutant rhodopsin (Rh1) interferes with wild type protein processing

leading to reduction in Rh1 concentrations [25]. It has to be stated, however, that Pat-Mil-1's heterozygous parents [10] did not show signs of insulin resistance and it is likely that, while the GRP78 system is saturated in our transfected COS1 cells, this is not the case in the entire human body.

To explain the tight interaction of IR^{P193L} precursor with GRP78 and calnexin, different hypotheses could be proposed. GRP78 binds many proteins carrying mutations that cause misfolding. The P193L mutation is predicted to cause an α sheet $\rightarrow$ β turn transition in the secondary structure of the insulin receptor that could modify the exposure of peptide segments recognized by molecular chaperones. We have previously observed that MA10, an antibody recognizing conformation dependent epitopes of the α subunit, failed to immunoprecipitate IR^{P193L} [10]. These findings suggest that the P193L mutation could modify overall folding of the α subunit, as previously observed for the other IR mutations impairing insulin proreceptor processing [26–28].

Pro193 resides in the cysteine-rich region of insulin receptor and it has been previously suggested that mutations in cysteine rich or cysteine flanking regions can impair protein processing probably altering intrachain disulphide bonds formation, a mechanism that might be operating in the mutated insulin receptor [3, 26, 27], LDL receptor [29, 30], thyroglobulin [31], haemagglutinin HA0 precursors [32] and immunoglobulin light chain [33].

It is also of interest that IR^{P193L} precursor expression does not affect the post translational processing of the IGF-1 receptor, suggesting a difference in the biosynthetic pathway between these highly related receptors.

The relevance of P193 in IR function is also suggested by its high degree of conservation in the IR superfamily members (IR, IRR, IGF-1-R) and in the insulin-like peptide receptor from amphioxus, the common progenitor of insulin and IGF-1 receptors [34]. Note that this proline residue is conserved in the cysteine repeats motif identified in the tumor necrosis factor receptor and in the cysteine rich regions of insulin and epidermal growth factor receptors [35]. This notion suggests that the folding pattern of this region is critical for insulin signal transduction and it could mediate similar types of interactions in different transmembrane receptors.

Little is known of the mechanisms of degradation of misfolded protein precursors, retained in the ER to prevent the toxic effects of their intracellular accumulation [20]. It is relevant to note that the longer $t_{1/2}$ we have observed in COS-IR^{P193L} suggests that the mutated insulin receptor precursors could not readily reach the lysosome and probably they were subjected to ER-associated degradation.

In conclusion, our results indicate that P193 in the IR α subunit is critical for insulin proreceptor folding. Its substitution with a Leu causes the retention of

IR^{P193L} precursor in the ER by binding to GRP78 and calnexin. Further investigation is required to establish the relative importance of GRP78 and calnexin in this process.

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