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An assessment of human insulin receptor phosphorylation and exogenous kinase activity following deletion of 69 residues from the carboxyl-terminus of the receptor β-subunit

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Summary: A mutant human insulin receptor with a carboxyl-terminal deletion of 69 amino acids (proreceptor residues 1287-1355) is expressed as a stable protein in transiently transfected COS cells. We find that in intact cells this mutant is phosphorylated in an insulin-dependent manner on core tyrosines 1158, 1163 and 1163. As expected, the carboxyl-terminal β-subunit phosphorylation sites (serines 1305/6, tyrosines 1328/34 and threonine 1348) are absent from this mutant. However, the two major insulin-stimulated serine phosphopeptides remain. In intact cells, insulin stimulates exogenous substrate phosphorylation by the truncated receptor only ~1.9-fold (cf. ~9-fold for the wild-type receptor in these cells), a consequence of a ~4.8-fold elevation in basal insulin-independent kinase activity.

The initial step in the insulin response is the binding of the hormone to the insulin receptor, a transmembrane heterotetrameric $(\alpha\beta)_2$ glycoprotein comprised of 1355 amino acids per αβ half-receptor (1-2). The binding of insulin to the extracellular α-subunits activates a protein-tyrosine kinase intrinsic to the cytoplasmic ~2/3s of the β-subunit. As a consequence, the receptor β-subunit is rapidly autophosphorylated on at least five tyrosines (residues 1158, 1162, 1163, 1328 and 1334: 3-7)[1]. Autophosphorylation of tyrosines 1158, 1162 and 1163 in the kinase homology region of the receptor results in further activation of the protein-tyrosine kinase towards exogenous substrates (7-12).

The carboxyl-terminal ~1/4 of the β-subunit cytoplasmic domain possesses little homology with other soluble or growth factor receptor protein-tyrosine kinases (including the related insulin-like growth factor-I receptor), but is highly conserved between species, as the mouse and rat insulin receptors share 85/92 and 84/92 amino acid identity, respectively, with the equivalent sequence of the human receptor.

[1] The numbering of insulin receptor residues is that according to reference (1).

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While this suggests that the carboxyl-terminus is likely to subserve a critical function in insulin receptor function and/or signalling, its role is at present obscure.

Leucine 1263 demarcates the approximate carboxyl-terminal boundary of the protein-tyrosine kinase homology region of the human insulin receptor (hIR). Truncation amino-terminal to the analogous residue of $pp60^{v\text{-}src}$ (leucine 516) results in an unstable protein with reduced protein-tyrosine kinase activity (13). Similarly, an insulin receptor truncated following arginine 1243 (the T-t mutant of ref. 14) is unstable and apparently devoid of protein-tyrosine kinase activity. As we wish to examine the functional consequences of large carboxyl-terminal deletions in the context of a receptor with protein-tyrosine kinase activity, we have engineered a deletion of the carboxyl-terminal 69 amino acids of the receptor (residues 1287 to 1355). We demonstrate that the β-subunit of this mutant receptor, designated hIRΔCT69, is a stable active protein-tyrosine kinase. Furthermore, we have used this mutant receptor to characterize the approximate location of some of the many insulin-stimulated β-subunit serine phosphorylations sites.

Methods

Construction of plasmid hIRΔCT69 -- Plasmid pehIRΔCT69 is comprised of a cDNA encoding the human insulin proreceptor with a deletion of bp 4079 to 4443 (i.e., amino acids 1287 to 1355) cloned into the ~2.9 kb SV40 expression plasmid pECE (14). The starting construct was the plasmid pespBamYF3, which lacks proreceptor residues 13 to 599, and has the Y1162/3F mutation (14). This plasmid was digested to completion with Hind III (in the 5' polylinker) and Xho I (bp 3195 of the receptor), and the resulting ~4.1 kb fragment (bp 3195 to 4443 of receptor coding sequence plus the pECE vector) was ligated to a double-stranded synthetic oligonucleotide (in upper case letters below; complementary vector sequence is in lower case letters) which includes 5' Hind III (AGCT, top strand) and Xho I (TCGA, bottom strand) overhangs, a consensus sequence for the initiation of translation ($[GCC]_3$; 15) and a mutation within the Xho I site, viz.,

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      Hind III                          Met  Val  Ser  arg
5'  a A G C T T G C C G C C G C C A T G G T G A G t c g a  3'
3'  t t c g a A C G G C G G C G G T A C C A C T C A G C T  5'
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The latter modification results in the removal of the Xho I site (bp 3195), and thus one of the two Ava I sites (at bp 3195, leaving the 3' Ava I site at bp 4073) of the kinase domain, by changing the codon for serine 992 from TCt to AGt. The predicted amino-terminal sequence of the resulting protein, a soluble cytoplasmic protein-tyrosine kinase comprised of receptor residues 991 to 1355, is Met-Val-Ser-arg. This plasmid was then digested to completion with Ava I (at bp 4073) and Xba I (in the 3' polylinker) and ligated with a double-stranded synthetic oligonucleotide which retains both the Ava I (cCCGAG) and Xba I (Tctaga) sites, introduces a new BamH I site (GGATCC) and has a stop codon (TAA) in place of Ser 1287, to yield the plasmid pIRKΔNT38(Y1162/3F)ΔCT69 (which now encodes a soluble cytoplasmic protein-tyrosine kinase which includes receptor residues amino 991 to 1286), viz.,

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      Pro  Glu  stop  BamH I      Xba I
5'  c C C G A G T A A G G A T C C T c t a g a  3'
3'  g g g c t C A T T C C T A G G A G A T C t  5'
```

To provide the carboxyl-terminal deletion of 69 amino acids in the context of the native membrane-associated receptor (with tyrosines at positions 1162 and 1163), a restriction fragment of pIRKΔNT38(Y1162/3F)ΔCT69 including the 3' deletion was obtained by digesting pIRKΔNT38(Y1162/3F)ΔCT69 with Hind III and partially with Fsp I (bp 3947 of the receptor; New England Biolabs). The resulting ~3.1 kb fragment (bp 3947 to 4078 of receptor coding sequence plus the pECE plasmid) was ligated to a ~3.9 kb fragment (bp 64 to 3946) derived from digestion of the plasmid peT3 (which contains the entire coding sequence of the wild-type hIR cDNA) with Hind III and Fsp I, to yield plasmid pehIRΔCT69. The sequence of this new 3' terminus of the receptor (introduced by the synthetic oligonucleotide) was confirmed by sequencing from bp 4000 of the receptor to a 3' site in pehIRΔCT69.

Expression of the receptor in COS cells, and analysis of insulin receptor phosphorylation sites and tyrosine kinase activity-- All materials and antisera were as described (16). The methods for transient transfection of COS cells with insulin receptor cDNA, ^{32}P-labelling of insulin receptors followed by phosphopeptide mapping/phosphoamino acid analysis and measurement of exogenous tyrosine kinase activity of immunopurified insulin receptors have been described elsewhere (16).

Results and Discussion

In transfected COS cells, the wild-type and mutant hIRΔCT69 receptors are synthesized as proreceptors of ~180-kDa and ~170-kDa, respectively (Fig. 1). These are subsequently cleaved to generate mature α- and β-subunits with essentially indistinguishable rates of synthesis and degradation (Fig. 1). The molecular weight of the hIRΔCT69 β-subunit (~85-kDa) is ~10-kDa less than that of the wild type receptor (95-kDa; Fig. 1), consistent with the M_r (7796) of the 69 amino acids deleted from the β-subunit carboxyl-terminus. We confirmed deletion of the carboxyl-terminus by demonstrating that anti-peptide antibodies raised against carboxyl-terminal epitopes of the wild-type receptor (proreceptor sequences 1298-1317, 1325-1341 and 1341-1355) do not cross-react with the hIR–ΔCT69 β-subunit (not shown).

Transiently transfected COS cells were metabolically labelled with [^{32}P]-P_i, followed by incubation in the presence or absence of insulin. As shown in Fig. 2 there is a small reduction in the level of ^{32}P incorporated into the truncated receptor in both the basal (~26%; cf. lanes a and c) and insulin-stimulated (~12%; cf. lanes b and d) states. Analysis of the phosphoamino acid content of ^{32}P-labelled receptors demonstrates that the truncated receptor is phosphorylated in an insulin-dependent manner on both tyrosine and serine residues, while phosphorylation on threonine is compromised (not shown).

We next compared two-dimensional tryptic phosphopeptide maps prepared from equivalent amounts of β-subunit of wild-type (Fig. 3, panels c and d) and truncated (Fig. 3, panels e and f) receptors. As illustrated in panels c and d of Fig. 3, the wild-type receptor is phosphorylated in an insulin-dependent manner on tyrosines 1158, 1162 and 1163, which is in agreement with maps observed for both transfected CHO cells (6, 17) and rat hepatocytes (18). These three tyrosines reside on a common tryp-

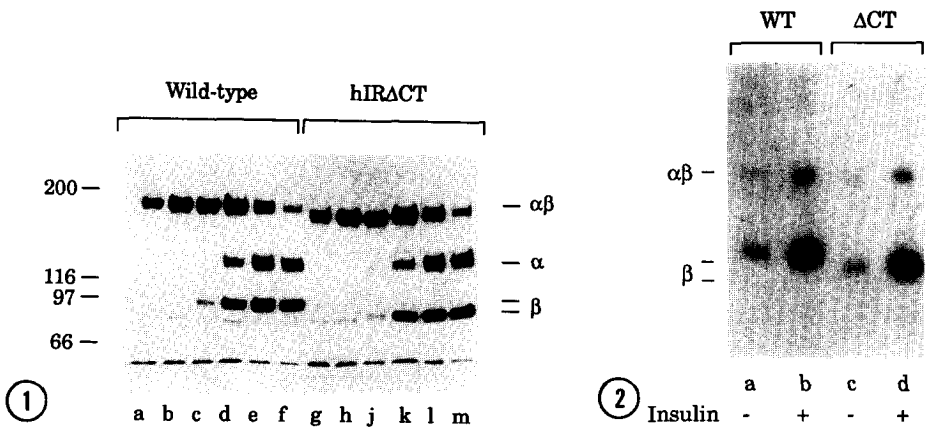

Figure 1. Pulse-chase analysis of metabolically labelled receptors. Transfected COS cells that express either wild-type (lanes a to f) or hIRΔCT69 mutant (lanes g to m) receptors were incubated with [^{35}S]-methionine and [^{35}S]-cysteine for 15 min at 37 °C. The pulse medium was removed and incubation was continued with 5 ml of DMEM containing 10% dialyzed fetal calf serum for 0 (lanes a and g), 0.5 (lanes b and h), 1 (lanes c and j), 2 (lanes d and k), 8 (lanes e and l) or 24 (lanes f and m) hours, at which time the cells were extracted and receptors were isolated by immunoprecipitation with monoclonal antibody 83.14. The figure is an autoradiograph (24 h exposure at -80 °C) of the resulting SDS-polyacrylamide gel. The positions of molecular weight markers (in kDa) are shown. The proreceptor (αβ) and the mature receptor α- and β-subunits are also indicated.

Figure 2. Phosphorylation of insulin receptors in intact COS cells. Transfected COS cells that express either wild-type (WT) or hIRΔCT69 mutant (ΔCT) receptors were incubated with [^{32}P]-P_i and then without (lanes a and c) or with (lanes b and d) insulin for 5 min. Cells were extracted and insulin receptors were isolated by immunoprecipitation with monoclonal antibody 83.14. The figure illustrates an autoradiograph (2 h exposure at room temperature) of the resulting SDS polyacrylamide gel. The proreceptor (αβ) and the mature receptor β-subunits are indicated.

tic peptide (DIYETDYYR[K]) which migrates as peptides A1 and A2 (tris-phosphorylated), B2 and B3 (bis-phosphorylated) and C1 (mono-phosphorylated; cf. the cartoon of Fig. 3, panel b). Insulin further stimulates wild-type receptor phosphorylation on tyrosines 1328 and 1334 (peptide B1), threonine 1348 (peptide T) and on five phosphoserine containing peptides (S1-S5), one of which is phosphorylated on one or both of serines 1305 and 1306 (peptide S5; see ref. 16).

Following truncation of the carboxyl-terminal 69 residues of the receptor, the loss of peptide B1, peptide T and three serine peptides (S3-S5) are apparent. The loss of B1, T and S5 are consistent with the extent of the deletion. However, the exact location of peptides S3 and S4 are not known at present. They may reside in the carboxyl-terminal 69 amino acids of the receptor (i.e., one or more of serines 1287, 1320, 1321, 1327, 1352 or 1355), and hence be deleted by the 69 residue truncation, or they may reside within the enzymatic core of the kinase, but their phosphorylation is somehow perturbed as a result of the truncation. Peptides S1 and S2 must clearly

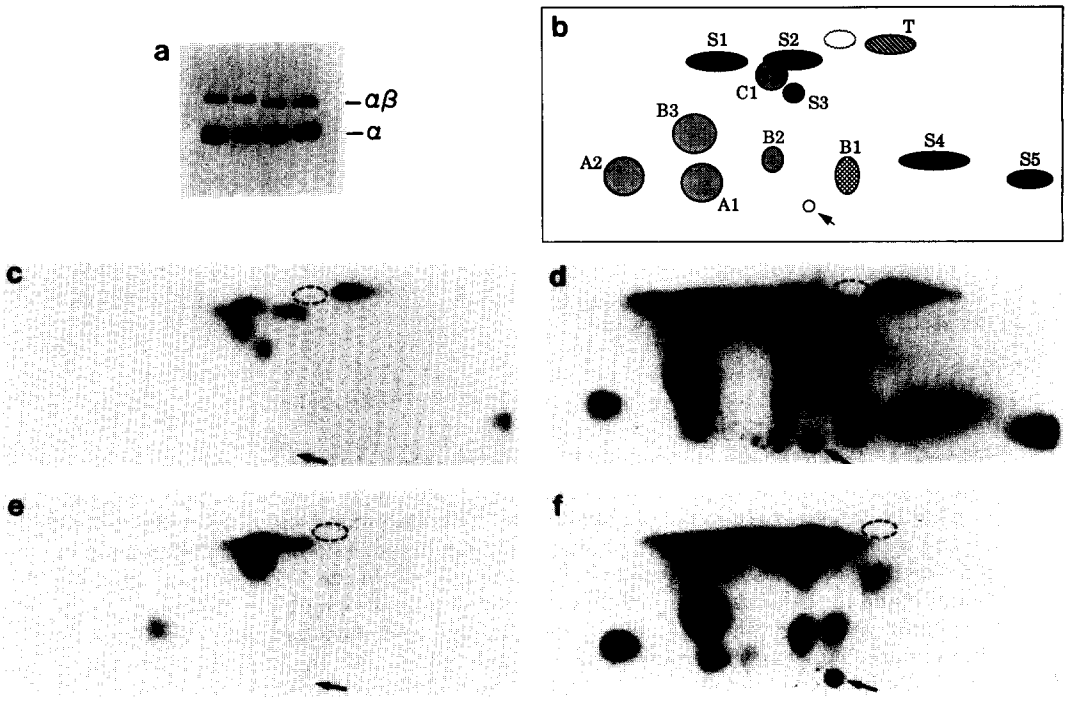

Figure 3. Analysis of insulin receptor phosphorylation in intact cells by two-dimensional phosphopeptide mapping. Insulin receptor β-subunits were cut out and electroeluted from the gel of Fig. 2, and digested with trypsin. An immunoblot of the remaining gel was incubated with affinity purified anti-peptide antibody anti-1-12 (specific for residues 1 to 12 of the receptor α-subunit) followed by ECL-chemiluminescent detection (panel a). This allows direct quantitation of the amount of receptor α-subunit in the same immunoprecipitate; thus, equal amounts of peptides from receptor β-subunits are subjected to two-dimensional separation. Panels c to f are autoradiographs of maps from wild-type (panel c, - insulin; panel d, + insulin) and truncated (panel e, - insulin; panel f, + insulin) receptors. The arrow denotes the origin of sample application and the dotted oval represents the position of a marker dye (dintitrophenyl lysine). Panel b is a key to the identification of tryptic phosphopeptides derived from the wild-type insulin receptor β-subunit (see text for description).

reside within the enzymatic core of the receptor, as they remain in the maps of the truncated receptor (Fig. 3, panels e and f). Thus, the peptide maps of the hIRΔCT69 mutant receptor reveal multiple domains of β-subunit serine phosphorylation. Given that the major, if not only, site of threonine phosphorylation is proximal to the carboxyl-terminus of the receptor (residue 1348; see ref. 16), it is difficult to explain how an insulin receptor with a 43 amino acid carboxyl-terminal deletion has been reported by others to still exhibit insulin-stimulated phosphorylation on a threonine residue(s) (19). However, as these latter results derive from phosphoamino acid analysis, and not peptide mapping, it is not clear whether the phosphothreonine observed in this study is equivalent to that observed in the wild-type receptor, or whether a novel cryptic site(s) has been exposed as a consequence of the truncation.

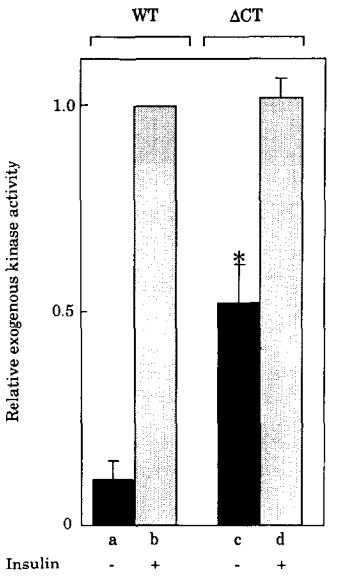

Figure 4. Exogenous protein-tyrosine kinase activity of insulin receptors isolated from intact COS cells. Transfected COS cells expressing either wild-type (WT, lanes a and b) or hIRΔCT69 mutant (ΔCT, lanes c and d) receptors were incubated in the absence (lanes a and c) or presence (lanes b and d) of insulin for 5 min at 37 °C, and cells were extracted with a buffer containing phosphatase inhibitors. Receptors were isolated by immunoprecipitation with monoclonal antibody 83.14 and assayed for protein-tyrosine kinase activity towards peptide RRDIFETDYFRK. Relative activities were normalized by immunoblotting samples of cell extracts or immunoprecipitates with affinity-purified anti-peptide antibody anti-1-12. Results are expressed as means +/- S.E.M. of three separate experiments each performed in duplicate. At the asterisk (*), $P < 0.05$ when compared to the activity in lane a.

To assess the exogenous kinase activity of truncated versus wild-type receptors, intact cells were treated with or without insulin and receptors immunopurified in the presence of phosphatase inhibitors. For these comparisons, the amount of receptor in each individual immune complex was directly determined by immunoblotting with anti-peptide antibody anti-1-12 (see the legend to Fig. 4). As illustrated in Fig. 4, insulin stimulates exogenous kinase activity ~9- and ~1.9-fold for the wild-type and truncated receptors, respectively. As the level of maximally observed activity of both receptors is comparable, the reduction in insulin's effect on the activity of the mutant receptor is a consequence of an ~4.8-fold elevation of basal exogenous kinase activity (Fig. 4, cf. lanes a and c).

Close inspection of the maps in Fig. 3 shows that the truncation promotes a small but significant increase in the level of mono- (peptide C1; 2.0- and 3.4-fold in two separate experiments) and bis- (peptides B2 and B3; 6.0- and 8.5-fold in peptide B3 in two separate experiments) phosphorylation of tyrosines 1158, 1162 and 1163 in the absence of insulin (cf. Figs. 3, panels e versus c). Tris-phosphopeptides A1 and A2 are not detectable in phosphopeptide maps from hIRΔCT69 cells in the absence of insulin (Fig. 3, panel e). These results suggest that perhaps the elevated basal exogenous kinase activity of the truncated receptor is a consequence of the observed increase in

mono- and bis-phosphorylation of tyrosines 1158, 1162 and 1163. However, this would be inconsistent with previous studies showing that activation requires tris-phosphorylation of these residues (7, 12, 20). Alternatively, perhaps, truncation of the receptor may have removed a steric constraint, or resulted in a subtle conformational perturbation, which results in the observed difference in basal kinase activity independently of phosphorylation of tyrosines 1158, 1162 and 1163.

In contrast to our study, but using similar methodology, a mutant human insulin receptor with a truncation of the last 43 carboxyl-terminal residues exhibits basal and insulin-stimulated exogenous kinase activity towards histone-2B comparable to that of the wild-type receptor (21). Whether these differences reflect functional distinctions related to the degree of receptor truncation, substrate specific effects or differences with respect to the cell type used for expression, will require further study. When the hIRΔCT69 is expressed in stably transfected CHO cells, we find that the sensitivity of mitogen activated protein kinase(s) to insulin is enhanced with respect to that observed in cells expressing wild-type human receptors (the CHO.T cells of ref. 14; see ref. 22). Whether this observed functional change found in CHO cells is related to the enhanced exogenous protein-tyrosine kinase activity found in COS cells remains to be established. However, it should be noted that the 43 amino acid deletion has been reported to result in augmented insulin-stimulated thymidine uptake in Rat1 cells (23) but not in CHO cells (19).

In summary, the hIRΔCT69 mutant described herein exhibits the largest carboxyl-terminal deletion yet reported for the human insulin receptor that does not result in the loss of β-subunit stability, autophosphorylation and protein-tyrosine kinase activity. Furthermore, these results support the concept that the cytoplasmic portion of the β-subunit is comprised of two distinct (globular) domains, a core enzymatic domain and a carboxyl-terminal tail, each with its distinct complement of insulin-stimulated phosphorylation sites.

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