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Insulin receptor autophosphorylation and exogenous substrate phosphorylation: Role of receptor C-terminus and effects of mild reduction

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Summary: A mutant insulin receptor lacking the final 69 amino acids of the β-subunit (Δ69) was used to examine the role of the receptor C-terminal domain in kinase activation. With increasing deletion of the C-terminus from 43 to 69 amino acids we show that exogenous peptide kinase activity is lost before autokinase activity. Despite this, phosphorylation of an in vivo insulin receptor substrate, IRS-1, and insulin bioeffects are similar to wild-type. In addition, with the exception of insulin-stimulated peptide phosphorylation, the reductant glutathione modified kinase activity in a similar manner for both wild-type and mutant Δ69 receptors. These results suggest that conformational changes proposed to occur within the receptor C-terminus upon insulin binding may not be necessary for kinase activation under a variety of conditions.

The tyrosine kinase activity of the insulin receptor, essential for normal insulin signalling, is located within the cytoplasmic domain of the insulin receptor β-subunit, between a "juxtamembrane domain" and a "C-terminal domain" (reviewed in 1,2). These three domains have different functional properties. The juxtamembrane region has been shown to be important in receptor signalling via the associated protein IRS-1, and also plays a role in receptor endocytosis. The kinase domain, activated following insulin binding, is the initial site of signal generation. The function of the C-terminal tail is less clear, whilst some studies have proposed a role for this domain in insulin signalling this has not been a universal finding (2). Although the C-terminal domain is not involved directly in the enzymatic activity of the receptor it may still play a role in modulating kinase function indirectly, perhaps via steric means. A conformational change in the C-terminus of the receptor can be detected using antibodies directed against receptor epitopes in the region 1306-1338[1] (3,4). This change, apparent following insulin binding, is distinct from the

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

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global conformational changes accompanying kinase activation which can also be detected in the juxtamembrane and kinase domains in addition to the C-terminal domain (4). It has been proposed that the change normally induced by insulin binding produces a short-lived receptor species (transition state) that becomes competent to bind ATP leading to full receptor activation (4). This hypothesis is supported by a receptor mutation, (382F→V)[2] in the extracellular domain of the α-subunit, which is locked in the basal (inactive) conformation in the presence of insulin and has impaired kinase activity (5). We have recently shown differential effects of the physiological reductant glutathione (GSH) on insulin receptor kinase activity and postulated that this may be due to GSH interacting with the different conformational states of the receptor in basal and insulin-activated conditions (6).

Studies with deletion mutants of the C-terminal domain have also revealed possible contributions of this region to overall receptor function. Whilst a Δ43 (terminates at amino acid 1303) mutant receptor appears fully kinase active (7), a Δ82 (1274) mutant receptor is kinase negative in vitro (8). A Δ69 (1287) mutant receptor, transiently expressed in COS cells, has kinase activity but this may be impaired (9). Using the Δ69 mutant receptor stably expressed in CHO cells, with or without GSH treatment, we now show that (i) insulin-stimulated peptide phosphorylation in vitro is lost before insulin-stimulated autophosphorylation and (ii) GSH treatment differentially affects insulin-stimulated peptide phosphorylation by wild-type and Δ69 receptors although autophosphorylation is identical for the two receptors. These results are discussed in relation to the conformational changes believed to occur within the C-terminal domain.

MATERIALS AND METHODS

Cells: CHOT cells, overexpressing human insulin receptors (gift from Dr L. Ellis, Houston, Texas), and Δ69 cells, CHO cells expressing a mutant insulin receptor lacking the last 69 amino acids from the C-terminus of the β-subunit (gift from Dr J. Tavaré, Bristol, UK) were maintained in α-MEM containing 10% (w/v) fetal calf serum.
[^{125}I]Insulin Binding: Cells, plated in 24-well plates, were incubated in buffer containing 20,000 cpm [^{125}I]insulin (140μCi/μg) and increasing concentrations of unlabelled insulin (1-100ng/ml) for 4h at 8°C. Cells were washed and bound radioactivity measured as described previously (6). Non-specific binding was measured in the presence of 10μg/ml unlabelled insulin and was always less than 2% of total binding. Data was analysed by the method of Scatchard (10).
Phosphorylation Assays: CHOT and Δ69 cells were treated with insulin (1μg/ml), lysed and insulin receptors immunoprecipitated with antibody 83-7 (gift from Prof. K. Siddle,

[2] Single letter amino acid code used.

Cambridge, UK.) as described previously (6). Where GSH treatment was included the immunoprecipitates were incubated with 1mM GSH for 15min at 22°C. Autophosphorylation assays were carried out as descibed previously (6). To measure peptide phosphorylation, receptor immunoprecipitates were incubated in 40μl phosphorylation buffer (6) containing 200μM FYF peptide (RRDIFETDYFRK) (Auspep) and 0.4μCi [^{32}P]ATP for 5min at 30°C. At this time duplicate 10μl aliquots were spotted onto P81 Whatman filters, washed 3 times in 30% w/v acetic acid/0.05% w/v phosphoric acid, dried and radioactivity measured in a liquid scintillation spectrometer. Blank values, in the absence of peptide, were subtracted from each result.

In Vivo IRS-1 Phosphorylation: CHOT and Δ69 cells were treated with or without insulin (10^{-9}, 10^{-7}M) lysed and tyrosine phosphorylated proteins immunoprecipitated with antibody PY-20 (ICN). After separation of proteins by reducing SDS-PAGE and transfer to nitrocellulose membranes, tyrosine phosphorylated proteins were detected with PY-20 and visualised by ECL (Amersham) and autoradiography.

Glucose Utilisation: Glucose utilisation was determined as the conversion of [5-^{3}H]glucose to $^{3}H_2O$ as described previously (11).

RESULTS

Table 1 shows that the insulin binding characteristics of CHOT cells (expressing wild-type human insulin receptors) and Δ69 cells (expressing a 69 amino acid C-terminal deletion of the human insulin receptor) were similar.

Basal Δ69 receptor autophosphorylation was two fold higher than that of wild-type receptors when measured in vitro, however, the fold stimulation by insulin (approx. 7-fold) remained the same for the two receptors (Figure 1A). In concurrent experiments, exogenous peptide phosphorylation by Δ69 receptors was significantly lower than phosphorylation by wild-type receptors both in the presence (50% of wild-type) and absence (80% of wild-type) of insulin (Figure 1B). However, as has been observed previously with both Δ43 (12) and Δ82 (8) mutant receptors, phosphorylation of a major in vivo insulin receptor substrate IRS-1 (pp185) in Δ69 cells was similar to CHOT cells

Table 1 Insulin Receptor Binding Characteristics for CHOT and Δ69 Cells

	Receptor number/cell	Kd_1	Kd_2
CHOT	7.7×10^5	6.1×10^{-10}M	2.1×10^{-8}M
Δ69	6.7×10^5	6.1×10^{-10}M	1.6×10^{-8}M

Data, derived from Scatchard plot analysis (10), are the mean of at least two separate experiments with triplicate measurements at each point.

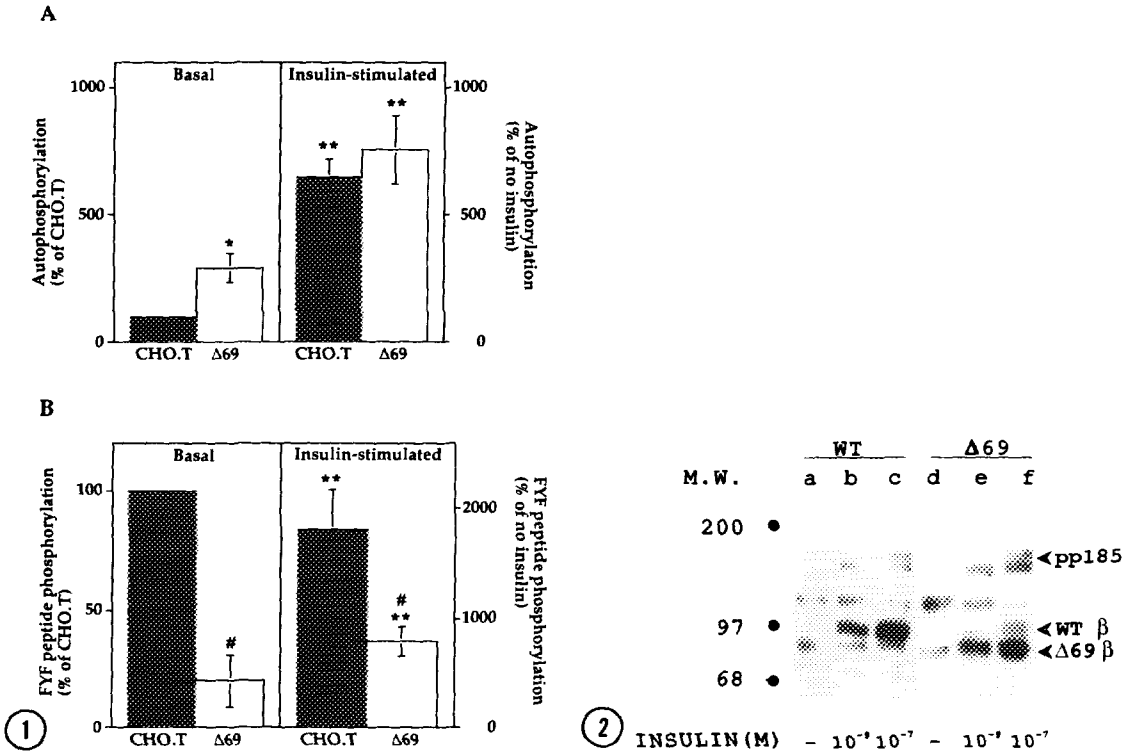

Figure 1. Autophosphorylation (panel A) and peptide phosphorylation (panel B) by insulin receptors isolated from CHOT and Δ69 cells.
Cells were stimulated with or without 1μg/ml insulin before immunoprecipitation of receptors and assay of either autophosphorylation or peptide phosphorylation as described in Methods. Results are expressed as a percentage of those obtained for CHOT receptors (basal, set at 100%) or as a percentage of no insulin addition (insulin-stimulated) and are the mean ± SEM of at least four separate determinations. Note the different scale for basal peptide phosphorylation (panel B). * $p < 0.05$; **$p < 0.01$ compared with relevant basal value; # $p < 0.05$ compared with relevant CHOT value.

Figure 2. In vivo phosphorylation of IRS-1 and insulin receptors in CHOT and Δ69 cells.
CHOT (lanes a-c) and Δ69 (lanes d-f) cells were stimulated with (lanes b,c,e,f) or without (lanes a,d) insulin before immunoprecipitation of tyrosine phosphorylated proteins. After SDS-PAGE and transfer of separated proteins the membranes were probed with antiphosphotyrosine antibodies. The positions of phosphorylated wild-type and mutant Δ69 receptor β-subunits and the IRS-1/pp185 protein are indicated.

(Figure 2). Insulin-stimulated glucose utilisation was also similar between CHOT and Δ69 cells (CHOT, basal 21 ± 2 pmol/10^5 cells, insulin-stimulated 54 ± 6 pmol/10^5 cells; Δ69, basal 21 ± 6 pmol/10^5 cells, insulin-stimulated 61 ± 4 pmol/10^5 cells), further supporting a contributory role for IRS-1 phosphorylation in subsequent signal transduction events.

Studies adding GSH (1mM) into the incubation mixtures revealed a more complex picture. In cells incubated in the absence of insulin (basal) both wild-type and Δ69

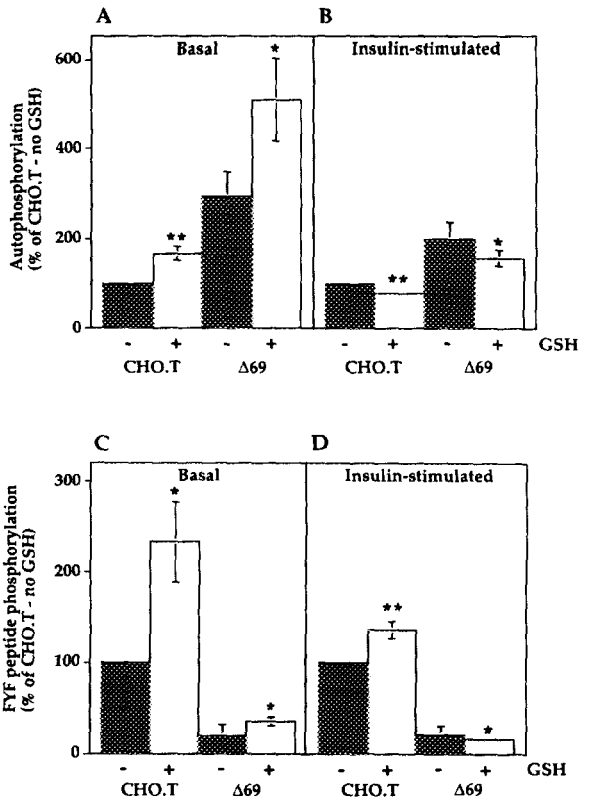

Figure 3. Autophosphorylation (panels A and B) and peptide phosphorylation (panels C and D) by insulin receptors isolated from CHOT and Δ69 cells treated with or without GSH.

As for Figure 1 except that immunoprecipitates were treated with or without 1mM GSH before the phosphorylation assays. Results are expressed as a percentage of those obtained for CHOT receptors in the absence of GSH and are the mean ± SEM of at least four determinations. * $p < 0.05$; ** $p < 0.01$ compared with no GSH addition.

receptors showed enhanced autophosphorylation and peptide phosphorylation in the presence of GSH (Figure 3A & 3C). When cells were stimulated with insulin prior to GSH treatment autophosphorylation was inhibited for both receptors (Figure 3B & reference (6)). However, whilst peptide phosphorylation by Δ69 receptors was slightly inhibited (74 ± 6% of control), surprisingly, wild-type receptors showed a small (144 ± 12% of control) but significant stimulation (Figure 3D). These results were not due to differential interaction of GSH with C1308 the sole cysteine residue deleted in Δ69 mutant receptors, as a point mutation of this residue (1308C→A) when expressed in CHO cells gave identical results to wild-type receptor (not shown). The differences observed in Figure 3D could be due to differential phosphorylation of the core tyrosines (Y1158, Y1163, Y1164) in wild-type versus Δ69 receptors with or without GSH. However,

phosphopeptide mapping (9) of [^{32}P]labelled β-subunits showed no significant differences between the cell-lines (not shown).

DISCUSSION

Combined with previous results on kinase activity of insulin receptors with either 43 (7) or 82 (8) amino acid deletions, our results demonstrate that, with progressive deletion of the C-terminal domain, the ability of the insulin receptor to phosphorylate a peptide is lost before autophosphorylation capability. However, as with the other deletion mutants (Δ43 and Δ82) in vivo phosphorylation of IRS-1 is unaffected. IRS-1 signalling is dependent upon autophosphorylation of residue Y972 in the juxtamembrane region of the insulin receptor (13) which is presumably unaltered in Δ69 receptors. Phosphorylation of IRS-1 is linked with insulin bioactivity (13) which probably explains why all the deletion mutants in CHO cells demonstrate normal insulin signalling.

In contrast with our results, previous studies with a Δ69 mutant receptor (9) transiently expressed in COS cells showed no apparent differences in autophosphorylation compared with wild-type receptors, when measured in vivo. In addition, Tavare et al (9) showed a 5-fold elevation in basal kinase activity towards a peptide although insulin-stimulated peptide phosphorylation was less than that of wild-type receptors, whereas we show impaired peptide phosphorylation by both basal and insulin stimulated Δ69 receptors. The reason for these differences is unclear but may reflect the different cell backgrounds (COS versus CHO cells). Variable findings have been reported with Δ43 deletion mutants expressed in different cell backgrounds (7,12,14,).

The effects we observed with GSH are not due to direct interaction of GSH with the extreme C-terminus of the receptor (residues 1287-1355) as they were seen with both wild-type and Δ69 receptors. Insulin-stimulated peptide phosphorylation, already severely impaired in Δ69 receptors, is further inhibited by GSH as might be predicted from the inhibition of autophosphorylation by GSH. However, peptide phosphorylation by insulin-stimulated wild-type receptors increases. The mechanism whereby GSH decreases insulin-stimulated autophosphorylation but increases insulin-stimulated peptide phosphorylation by wild-type receptors is unclear but may be linked to the finding that the capacity of the receptor to phosphorylate a peptide is much greater than that for autophosphorylation. The different magnitude of insulin stimulation for the two reactions (7 fold for autophosphorylation but up to 19 fold for peptide phosphorylation) would support this. Inhibition of autophosphorylation by GSH is small (20%), perhaps kinase

activation per se is only minimally impaired and not severely enough to inhibit peptide phosphorylation. The stimulation by GSH, above that with insulin alone suggests an alteration in the interaction of the peptide with the receptor under these conditions.

The extent of the conformational changes reported to occur upon insulin binding are unknown as the antibodies used to detect them have limited epitopes (3,4). However, removal of these epitopes in the Δ69 mutant receptor does not prevent insulin activation of this receptor kinase. Nor, in the deletion mutant Δ82 where in vitro phosphorylation is completely absent is the phosphorylation of IRS-1 altered or the bioeffects of insulin ablated (8). Our results with GSH further suggest that if conformational changes are relevant they may be more widespread than just the C-terminus and may alter depending upon the actual receptor structure under investigation.

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