Vol. 186, No. 1, 1992 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
July 15, 1992 Pages 244-250

Analysis of the order of autophosphorylation of human insulin receptor tyrosines 1158, 1162 and 1163

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Received May 4, 1992

SUMMARY Insulin receptor tyrosines 1158, 1162 and 1163 are the most rapidly autophosphorylated residues following insulin binding. Although progression of these tyrosines from a bis- to tris-phosphorylated state leads to activation of the receptor tyrosine kinase towards added substrates, rather paradoxically, a receptor with a Y1158F mutation has been reported to be capable of normal activation. In the present study we demonstrate that autophosphorylation of the insulin receptor probably initiates on either of tyrosines 1158 and 1162 while autophosphorylation of tyrosine 1163 occurs predominantly late in the autophosphorylation cascade. Our results are compatible with tyrosines 1162 and 1163 being the major determinants of kinase activity and explain why wild-type insulin receptors only become active after all three of tyrosines 1158, 1162 and 1163 have been phosphorylated.
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An obligatory requirement for signalling by most, if not all, receptor-tyrosine kinases is the ability to catalyse the phosphorylation of tyrosine residues on proteins; replacement of residues involved in ATP binding by site-directed mutagenesis severely compromises normal signalling. The role of the protein-tyrosine kinase activity of these receptors has remained elusive although it is clear that they phosphorylate a number of protein substrates within cells and that, at least in some cases (e.g., phospholipase C_γ), this phosphorylation appears to be accompanied by a change in the activity of the specific substrate (1,2). In the case of the insulin receptor tyrosine autophosphorylation also stimulates the catalytic activity of the receptor towards exogenous substrates (3,4).

Autophosphorylation of the insulin receptor is rapid and occurs at tyrosines 1158, 1162 and 1163 within the kinase homology region and tyrosines 1328 and 1334 at the carboxyl-terminus (5-8). The importance of the region surrounding tyrosines 1158, 1162 and 1163 is exemplified by three general observations: (i) Mutagenesis of tyrosines 1162 and 1163 compromises insulin-stimulation of glucose transport, glycogen synthesis, insulin receptor threonine 1348 phosphorylation, mitogen-activated protein kinase and a 90 kDa ribosomal protein S6 kinase (9-13). (ii) Autophosphorylation of the insulin receptor appears to induce a conformational change in the β-subunit, such that the region surrounding

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0006-291X/92 $4.00
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tyrosines 1158, 1162 and 1163 becomes accessible to anti-peptide antibodies (14,15). (iii) Progression of the insulin receptor tyrosines 1158, 1162 and 1163 from the bis- to tris-phosphorylated state correlates with activation of the receptor tyrosine kinase towards exogenous substrates *in vitro* (7,8). The tyrosine kinase is poorly activated if the receptor is autophosphorylated in the presence anti-phosphotyrosine antibodies which trap the receptor tyrosines 1158, 1162 and 1163 in a predominantly bis-phosphorylated state (7).

Several lines of evidence, however, suggest that a mechanism more complex than simple tris-phosphorylation may underlie kinase activation: (i) Tornqvist *et al.* (16) reported that less than complete phosphorylation of tyrosines 1158, 1162 and 1163 appeared to be adequate for full tyrosine kinase activation. (ii) Under conditions where the replacement of tyrosines 1162 and 1163 with phenylalanines blocked tyrosine kinase activition, substitution of tyrosine 1158 with phenylalanine had no effect and bis-phosphorylation of tyrosines 1162 and 1163 still occurred (17). This suggests that bis-phosphorylation of tyrosines 1162 and 1163 could be the most important determinant of kinase activity with the phosphorylation of tyrosine 1158 having little contribution. In contrast, however, Wilden *et al.* have reported that autophosphorylation and exogenous tyrosine kinase activity were markedly reduced in a Y1158F mutant (18).

In light of these apparent discrepancies we have further assessed the role of these tyrosine residues in kinase activation by examining their potential order of autophosphorylation using wild-type and mutant human insulin receptors in which tyrosine 1162 has been substituted with phenylalanine (Y1162F mutant; see ref 9).

METHODS

Materials and cell lines. All reagents and cell lines were as described previously (12). In brief, CHO.T cells are transfected with the human insulin receptor cDNA, and CHO.YF1 cells express mutant human insulin receptors in which tyrosine 1162 has been replaced with phenylalanine (9).

Partial purification and autophosphorylation of insulin receptors form CHO.T and CHO.YF1 cells. CHO.T or CHO.YF1 cells were grown to confluence in 40 x 7.5 cm Petri dishes and insulin receptors purified and concentrated as described (12). Partially purified wild-type or Y1162F mutant insulin receptor preparations (20 µl) were incubated with 1µM insulin for 15 min at 0 °C in a final volume of 60 µl containing 20 mM MOPS, pH 7.4, 0.25 mM dithiothreitol, 12 mM $MgCl_2$, 2 mM $MnCl_2$, 1 mM Na_3VO_4. Autophosphorylation was initiated by the addition of [γ-^{32}P]ATP (1500-2000 cpm/pmol) to 100 µM and incubation continued at 0 °C for the times indicated in the figure legends. Reactions were terminated by the addition of SDS PAGE sample buffer and proteins separated on SDS polyacrylamide gels (7 % acrylamide).

Two-dimensional tryptic phosphopeptide mapping. ^{32}P-labelled insulin receptor β-subunits were located within the polyacrylamide gels by autoradiography and were eluted from the excised gel chip as described by Tavaré & Denton, (6). Eluted proteins were precipitated, digested with TPCK-trypsin (Worthington Diagnostic Systems) and tryptic peptides separated by two-dimensional thin layer cellulose chromatography (electrophoresis at pH 3.5 followed by ascending chromatography) as described (6). Plates were dried and

phosphopeptides revealed by autoradiography with pre-flashed Kodak X-Omat S film in cassettes with intensifying screens at -70 °C.

V8 protease analysis. The area of cellulose containing peptide C1 was recovered from thin-layer plates and the peptide eluted by suspension of the cellulose in 50% (v/v) acetonitrile. Cellulose was removed by centrifugation and the supernatant lyophilised. The peptide was resuspended in 50µl of 50 mM ammonium bicarbonate, pH 7.8 with 1 µg of *S. aureus* protease V8 and incubated at 30 °C for 16 h.. A further 1 µg of protease was added and incubation continued for 5 h. The reaction was terminated by boiling for 5 min prior to lyophilisation. Phosphopeptides were separated by thin layer electrophoresis on cellulose chromatography plates at 400 V for 2 h at pH 3.5 (pyridine/acetic acid/water (1:10:189 v/v)). Phosphopeptides were detected by autoradiography.

RESULTS AND DISCUSSION

Investigations into the potential order of autophosphorylation of tyrosines 1158, 1162 and 1163

The earliest detectable event after the binding of insulin to its receptor is the autophosphorylation of the domain containing tyrosines 1158, 1162 and 1163 (5-8). These tyrosines are detected in tryptic digests of ^{32}P-labelled insulin receptors as a series of related mono-, bis- and tris-phosphorylated peptides with the general sequence DIYETDYYRK (aspartate 1156 to lysine 1164). Our previous studies have demonstrated that ^{32}P-label in mono-phosphopeptides becomes associated with the tyrosines on both sides of the V8 cleavage site (glutamate 1159) shortly after initiation of autophosphorylation with insulin and [γ-^{32}P]ATP (6). Bis-phosphopeptides, however, were predominantly phosphorylated on tyrosine 1158 and either tyrosine 1162 or 1163, with relatively few (<15 %) phosphorylated on tyrosines 1162 *and* 1163 (6-8).

These results suggest that the phosphorylation of tyrosine 1158 is an early event in the cascade of phosphorylation of tyrosines 1158, 1162 and 1163. However, using radiosequencing of tryptic phosphopeptides, it is difficult to distinguish confidently between the phosphorylation of tyrosines 1162 and 1163 due to their close proximity. It has been well documented that phosphorylation of residues can interfere with tryptic cleavage and indeed this appears to be the case for the human insulin receptor (5-8). Thus we investigated how the specific phosphorylation of tyrosine 1163 might influence tryptic cleavage at arginine 1164.

We examined two dimensional tryptic phosphopeptide maps of wild-type and Y1162F mutant human insulin receptors incubated with insulin and [γ-^{32}P]ATP (Fig. 1). The Y1162F mutant insulin receptor is rapidly autophosphorylated *in vitro* on both tyrosines 1158 and 1163 (giving bis-phosphopeptides B2 and B3; Fig 1). The ratio of bis-phosphopeptides B2 and B3 appears to reflect the efficiency of tryptic cleavage at arginine 1164 versus lysine 1165. A high B2/B3 ratio would suggest more efficient cleavage at lysine 1165 and a low ratio to suggest cleavage predominantly at arginine 1164. Analysis of several maps of wild-type insulin receptors similar to those illustrated in Fig. 1, reveals that the progressive phosphorylation of tyrosines 1158, 1162 and 1163 in the wild-type insulin receptor promotes

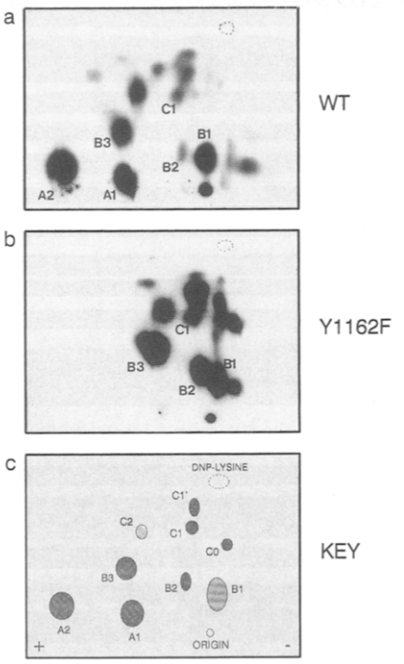

Fig.1. Tryptic phosphopeptide mapping of wild-type and Y1162F mutant human insulin receptors autophosphorylated *in vitro*

Partially purified wild-type (*panel a*) or Y1162F mutant (*panel b*) human insulin receptor preparations were incubated at 0 °C in the presence of 1 μM insulin and [γ-^{32}P]ATP for 30 min. and 60 min., respectively. ^{32}P-labelled β-subunits were then isolated by SDS PAGE and subjected to tryptic phosphopeptide mapping as described in the Methods section. The resulting autoradiographs are representative of maps obtained in three separate experiments. *Panel c* is a key identifying the major phosphopeptides observed. Peptides A1, A2, B2, B3 and C1 are related peptides containing tyrosines 1158, 1162 and 1163 phosphorylated on one (C1), two (B2 and B3) or all three (A1 and A2) tyrosines (the general sequence around this region is RDIYETDYYRKGGK). Peptide B1 contains carboxyl-terminal tyrosines 1328 and 1334. It should be noted that we previously concluded that tryptic cleavage of the RDIYETDYYRKGGK sequence occured at arginine 1155, and either lysine 1165 or lysine 1168, based on their relative charge before and after cleavage with *S. aureus* V8 protease (6). However, when synthetic phosphopeptides of sequence DIYETDY(P)YRK and DIYETDY(P)YR are separated by two-dimensional thin layer chromatography, they migrate at positions C0 and C1, respectively (see key; data not shown). As the predicted net charge of DIYETDYYRK at pH 3.5 is -0.2 but it migrates towards the cathode we have concluded that the peptide migrates abberantly due to electroendosmosis. Accordingly, we have modified our assignment of the sequence of this series of phosphopeptides. This does not in any way, however, affect our original conlcusions concerning the identity or characteristics of any insulin receptor tryptic phosphopeptide (as given in ref. 6).

cleavage predominantly at lysine 1165 rather than arginine 1164 (i.e., the ratio of tris-phosphopeptides A1/A2 (0.70 ± 0.06; mean ± S.E.M. for six experiments) is significantly higher than the ratio of the bis-phosphopeptides B2/B3 (0.26 ± 0.05 for six experiments; $P < 0.001$).

Further analysis of bis-phosphopeptides B2 and B3 reveals a consistently higher B2/B3 ratio in the Y1162F mutant receptor (0.47 ± 0.04 in three experiments; $P < 0.05$) than

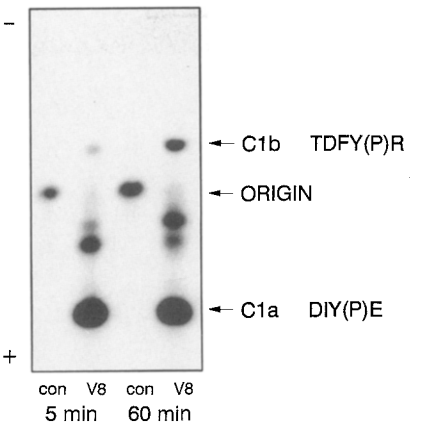

Fig.2. V8 protease analysis of phosphopeptide C1 from Y1162F mutant human insulin receptors autophosphorylated *in vitro*

Phosphopeptide C1 was isolated from tryptic phosphopeptide maps of Y1162F mutant human insulin receptors previously incubated in the presence of 1 μM insulin and [γ-^{32}P]ATP for 5 or 60 min. The sample was lyophilised and incubated with (V8) or without (con) 1μg of *S.aureus* V8 protease at 30 ^{o}C for 16 h. The phosphopeptides produced were resolved by thin layer electrophoresis at pH 3.5 as described in Methods. The figure shows the resulting autoradiograph (5 days).

in wild-type receptors (see above). Thus in the Y1162F mutant, tryptic cleavage of bis-phosphopeptides favours lysine 1165 and arginine 1164 approximately equally due to the presence of phosphate on tyrosine 1163. Cleavage of bis-phosphopeptides from the wild-type receptor, however, occurs predominantly at arginine 1164. This suggests, that bis-phosphopeptides from the wild-type insulin receptor are phosphorylated predominantly on tyrosines 1158 and 1162 and that phosphorylation of tyrosine 1163 occurs late in the cascade of autophosphorylation of the kinase domain tyrosines.

We further examined the order of phosphorylation of tyrosines 1158 and 1163 in the Y1162F mutant insulin receptor using the *S. aureus* V8 protease cleavage site (glutamate 1159) between the two tyrosines present in the tryptic peptide generated from this mutant. Monophosphopeptides (C1; amino acid sequence DIYETDFYR) generated at both early (5 min; not shown) and late (60 min; Fig. 1) time points were recovered from the thin-layer plate, digested with V8 protease and the products separated on thin-layer plates by electrophoresis at pH 3.5 (Fig. 2). The predominant product of V8 digestion at both time points is a peptide with a net negative charge (peptide C1a; Fig. 2). Only at the later time point is a small amount of peptide migrating with net positive charge apparent (C1b; Fig. 2). (It should be noted that V8 peptides running at positions intermediate between C1a and C1b originate from tryptic phosphopeptides derived from the domain containing tyrosines 965, 972 and 984 which contaminate tryptic peptide C1; see ref 16.) Peptide C1a corresponds to the V8 product DIY(P)E which carries a predicted net charge of -1.7 at pH 3.5. Peptide C1b is the cognate V8 fragment of sequence TDFY(P)R which has a net charge of -0.2 at pH 3.5 but runs with apparent net positive charge probably because of electroendosmosis (see legend to Fig. 1).

These results demonstrate that tyrosine 1158 is phosphorylated before tyrosine 1163 in the Y1162F mutant insulin receptor. Interestingly, the same order of phosphorylation is observed in the synthetic peptide substrate RRDIYETD*F*YRK incubated in the presence of Mg[γ-^{32}P]ATP and a recombinant soluble insulin receptor protein-tyrosine kinase (19). This contrasts, however, with the highly ordered phosphorylation of the synthetic peptide of wild-type sequence (RRDIYETDYYRK) by the recombinant kinase (initiates at tyrosine 9 [equivalent to tyrosine 1162 of insulin receptor] followed by tyrosine 10 and finally by tyrosine 5; ref 19). However, it should be noted that this sequence of amino acids probably has a quite different conformation as a free peptide compared to that in the more contrained environment within the intact receptor.

Taken together, our results suggest that autophosphorylation of tyrosine 1158 occurs early and that of tyrosine 1163 late in the cascade of autophosphorylation of tyrosines 1158, 1162 and 1163 in the wild-type insulin receptor. As bis-phosphorylation of tyrosines 1162 and 1163 in a Y1158F mutant insulin receptor can fully stimulate exogenous kinase activity (17) it is likely that full kinase activation in the wild-type insulin receptor will occur only once all three tyrosines are phosphorylated. This would be consistent with previous observations that tris-phosphorylation of tyrosines 1158, 1162 and 1163 correlates with kinase activation.

ACKNOWLEDGMENTS

We thank the Medical Research Council and British Diabetic Association for financial assistance. M.D. was supported by a Medical Research Council studentship. We are grateful to Prof. R.M. Denton and Dr. L. Ellis for many useful discussions.

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