Site-specific anti-phosphopeptide antibodies: use in assessing insulin receptor serine/threonine phosphorylation state and identification of serine-1327 as a novel site of phorbol ester-induced phosphorylation

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Rabbit antisera were raised against synthetic phosphopeptides corresponding to defined or putative sites of insulin receptor serine/threonine phosphorylation (Ser-1305, Ser-1327, Thr-1348). All of these antibodies bound specifically to the immunogenic phosphopeptide but not to the non-phosphorylated form of the peptide or to other phosphopeptides, in a microtitre plate competition enzyme-linked immunosorbent assay. Anti-*P*S1327 antibody reacted well with native insulin receptor prepared from phorbol ester-treated transfected CHO.T cells, but showed little reaction with receptor from untreated cells. Anti-*P*T1348 antibody in crude form reacted substantially with receptor from both phorbol 12-myristate 13-acetate-treated and untreated cells, but displayed specificity for phosphoreceptor after adsorption to remove antibodies reactive with dephosphopeptide. The ability to discriminate between receptor from cells treated with or without phorbol ester was retained when these antibodies were used to probe denatured receptor on Western blots. Thus anti-*P*S1327 and anti-*P*T1348 react with insulin receptor in a site-specific and phosphorylation-state-dependent manner. Anti-*P*T1348, but not anti-*P*S1327, also showed increased reactivity with receptor prepared from insulin-treated cells. The third antibody, anti-*P*S1305, did not react with intact insulin receptor under any conditions. It is concluded that serine 1327 is a major, previously unrecognized, site of phorbol ester-induced receptor phosphorylation, and that anti-phosphopeptide antibodies will be valuable reagents with which to examine the serine/threonine phosphorylation state of receptor extracted from tissues.

INTRODUCTION

Activation of the insulin receptor tyrosine kinase, which is essential for the expression of biological responses to insulin, is dependent on receptor autophosphorylation on tyrosine residues (reviewed in [1,2]). However, insulin also induces increased phosphorylation of receptor serine and threonine residues [3,4], as does treatment of cells with phorbol ester [5,6]. The latter is presumed to activate protein kinase C, but the kinase responsible for the effect of insulin has not been identified. That the receptor is a substrate for more than one kinase is suggested by the observation that insulin and phorbol ester induce somewhat different patterns of serine/threonine phosphorylation [7,8]. Within the C-terminal domain, serines 1305/6 and threonine 1348 are major phosphorylation sites in response to insulin and phorbol ester respectively [9,10], and in the juxtamembrane region serines 967/8 are phosphorylated in response to insulin and, to a lesser extent, phorbol ester [11]. Other phosphorylation sites, which are revealed by phosphopeptide mapping, remain to be identified.

Several studies have suggested that increased receptor serine/threonine phosphorylation is associated with a reduced biological response to insulin. Phorbol esters have been reported to inhibit insulin-stimulated receptor autophosphorylation and tyrosine kinase activity in intact cells [5,12–14], although this has not been a consistent observation [6,8,15–18]. In addition, it has been reported that phosphorylation of receptor by protein kinase C *in vitro* reduces its tyrosine kinase activity [19].

Impairment of insulin-stimulated receptor tyrosine kinase activity has also been described in diabetic patients exhibiting insulin resistance (reviewed in [20]). It is not known whether elevated serine/threonine phosphorylation of the receptor contributes to the inhibitory mechanism in this situation. Attempts to address this issue have been hampered by lack of suitable methods for determining the phosphorylation state of receptor in tissue extracts. While anti-phosphotyrosine antibodies have been used extensively to detect and isolate tyrosine-phosphorylated proteins [21–23], antibodies specific for phosphoserine or phosphothreonine [24–26] have not so far proved generally useful. An alternative approach is the preparation of antibodies which react specifically with predefined phosphoproteins, by using synthetic phosphopeptides as immunogens [27,28]. The present study was therefore undertaken to produce antibodies to phosphopeptides containing known (Ser-1305, Thr-1348) or putative (Ser-1327) sites of phosphorylation of the insulin receptor, with a view to using these to study the phosphorylation state of receptor *in vivo*. A preliminary account of this work has been presented [29].

MATERIALS AND METHODS

Materials

Peptides and phosphopeptides were generously provided by Dr. D. D. Myles, Glaxo Group Research Ltd., Greenford, Middlesex, U.K. Phosphopeptides were synthesized using methods described previously [30], with sequences as follows: *P*S1305 (residues 1300–1313): V-P-L-D-R-(*P*)S-S-H-C-Q-R-E-E-A; *P*S1327 (residues 1323–1337): R-F-K-R-(*P*)S-Y-E-E-H-I-P-Y-

Abbreviations used: PMA, 4β-phorbol 12β-myristate 13α-acetate; PMSF, phenylmethanesulphonyl fluoride; NMA, *N*-ethylmorpholine acetate; TBS, Tris-buffered saline.

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T-H-M; and *P*T1348 (residues 1345–1355): R-I-L-(*P*)T-L-P-R-S-N-P-S. Corresponding non-phosphorylated peptides were synthesized by standard methods.

Reagents for SDS/PAGE were from Bio-Rad Laboratories Ltd., Hemel Hempstead, Herts. U.K., except acrylamide/bisacrylamide (30:0.8) which was from National Diagnostics, Hull, U.K. Cell culture reagents were from ICN-Flow, High Wycombe, Bucks., U.K. Unless otherwise indicated, other reagents were from Sigma Chemical Co. Ltd., Poole, Dorset, U.K.

Preparation of antibodies

Peptides were conjugated to keyhole limpet haemocyanin by their free amino groups, using glutaraldehyde [31], and rabbits were injected at monthly intervals with approx. 200 μg of conjugate. Most experiments employed crude serum, but for immunoprecipitation of phosphopeptides the immunoglobulin fraction was purified using Protein A–Sepharose [31]. For some experiments, anti-phosphopeptide sera were depleted of non-specific antibodies by incubation with non-phosphorylated peptide coupled to Reactigel HW-65F (Pierce Chemical Co.) according to the manufacturer's instructions (approx. 2 mg of immobilized peptide per ml of serum).

Sera were initially screened for the presence of antibodies by e.l.i.s.a. Microtitre plates were coated with phosphopeptide [5 μg/ml in 50 mM Tris, 150 mM NaCl (pH 7.5) (TBS), 100 μl per well, for 2 h at 20 °C], and then blocked with BSA (10 mg/ml, 200 μl per well, overnight at 4 °C). Incubations with primary antibody and alkaline phosphatase-conjugated secondary antibody were carried out as previously described [32].

Rabbit antiserum Ros-1, raised against a peptide corresponding to the C-terminal 15 amino acids of the insulin receptor, was as previously described [32]. Antibody IR.JM1 was a rabbit antiserum raised against a glutathione S-transferase fusion protein containing the juxtamembrane region (residues 953–1001) of the human insulin receptor [29].

Cell culture and receptor extraction

Transfected Chinese hamster ovary cell lines that stably overexpress human insulin receptor (CHO.T, [33]), insulin receptor and protein kinase Cα (CHO.T-PKCα, [18]) or C-terminally truncated insulin receptor (CHO.ΔCT69, [34]) were as previously described. Monolayer cultures were grown at 37 °C in a humidified atmosphere of air/CO_2 (95:5, v/v) in Ham's F12 medium supplemented with 10% (v/v) fetal-calf serum, 50 units/ml penicillin, 50 μl/ml streptomycin, 2 mM glutamine and 200 μg/ml G418. Medium for CHO.T-PKCα cells additionally included 250 μg/ml hygromycin.

Cells were serum-starved by incubation for 4 h in medium containing BSA (0.1% w/v) in place of fetal-calf serum. Cells were then treated with 1 μM 4β-phorbol 12β-myristate 13α-acetate (PMA), added from a 1 mM stock solution in dimethylsulphoxide, for 30 min, or with 1 μM insulin for 10 min. Insulin receptor was isolated from four 175 cm^2 flasks of confluent cells. Each flask was extracted into 2 ml of ice-cold buffer A (50 mM Hepes, pH 7.4, 1 mM EDTA, 1 mM Na_3VO_4, 30 mM NaF, 10 mM $Na_4P_2O_7$, 2.5 mM benzamidine, 1 μg/ml each of antipain, leupeptin and pepstatin) containing 1% (w/v) Triton X-100 and 0.5 mM phenylmethanesulphonyl fluoride (PMSF). The pooled lysate was centrifuged at 100000 *g* for 30 min at 4 °C and the supernatant tumbled end-over-end overnight at 4 °C with 0.5 ml of wheat germ agglutinin–Sepharose slurry that had been equilibrated with wash buffer [buffer A containing 0.1% (w/v) Triton X-100]. After washing three times with 5 ml of buffer, insulin receptor was eluted batchwise into 0.75 ml of ice-cold wash buffer containing 0.5 M *N*-acetylglucosamine, and stored frozen at −70 °C.

Two-dimensional phosphopeptide mapping

Confluent cells in 6-cm-diam. dishes were incubated for 3 h at 37 °C in 1 ml of phosphate- and serum-free Dulbecco's modified Eagle's medium supplemented with 10 mM Hepes, pH 7.5, and containing 1 mCi of [^{32}P]P_i (Amersham International). PMA (1.6 μM) was then added as required for 30 min. Cells were lysed and insulin receptors immunoprecipitated with antibody 83-14 as described previously [35]. Immunoprecipitates were resolved by SDS/PAGE on 7% acrylamide gels using a Bio-Rad Minigel apparatus, and ^{32}P-labelled receptor β-subunit was located by exposure to a phosphor screen and scanning with a Molecular Dynamics PhosphorImager. The β-subunit was isolated, digested with trypsin and analysed by two-dimensional phosphopeptide mapping as described previously [36].

When phosphopeptides were to be immunoprecipitated with anti-peptide antibodies before mapping, trypsin-digested receptor was resuspended in 100 μl of 100 mM *N*-ethylmorpholine acetate (NMA), pH 8.3. This was boiled for 5 min and 0.5 mM PMSF added to inactivate any remaining trypsin. The receptor digest was then incubated, by tumbling end-over-end for 2 h at 4 °C, with purified antiserum (5 μl) and Protein A–Sepharose (5 mg) in a final volume of 500 μl of NMA. Sepharose-bound antibody was recovered by centrifugation and washed twice with 1 ml of NMA. Phosphopeptide was eluted by resuspending the complex in 500 μl of 1 M acetic acid containing 10 μg of the appropriate carrier phosphopeptide, and tumbling end-over-end at 4 °C for 30 min. Acetic acid was removed by rotary evaporation and the phosphopeptide was washed extensively with water before analysis by two-dimensional mapping as described above.

Immunoprecipitation and immunoblotting of insulin receptor

Receptor which had been partially purified on wheat germ agglutinin–Sepharose was preincubated with ^{125}I-insulin for 16 h at 4 °C, so that 20% of the insulin was bound, as determined by precipitation with poly(ethyleneglycol). Immunoprecipitation of receptor–^{125}I-insulin complexes was performed as described previously [37]. In some experiments, precipitation was carried out in the presence of various concentrations of peptides.

For immunoblotting, partially purified receptor was electrophoresed on an SDS/7.5% polyacrylamide gel and semi-dry blotted on to poly(vinylidene difluoride) membranes (Imobilon P, Millipore) using Towbin transfer buffer [38]. Membranes were blocked with 5% (w/v) Marvel in TBS/0.1% (w/v) Tween 20 for 16 h at 4 °C. The blots were probed with primary antibody and immunocomplexes detected with horseradish peroxidase-conjugated secondary antibody (Sigma) by enhanced chemiluminescence (Amersham International) according to the manufacturer's instructions. Immunodetected proteins were visualized by exposure of Fuji RX Nif film to the blots.

RESULTS

Rabbits immunized with phosphopeptides (*P*S1305, *P*S1327, *P*T1348) all developed good titres of anti-peptide immunoreactivity as detected by e.l.i.s.a. For each phosphopeptide, at least one antiserum was obtained which discriminated between the phosphopeptide immunogen and the corresponding dephosphopeptide in a competition e.l.i.s.a. (Figure 1). Moreover the antibodies were also sequence-specific in that phospho- or dephospho-peptides unrelated to the immunogen, and phosphoserine, phosphothreonine or phosphotyrosine, did not inhibit

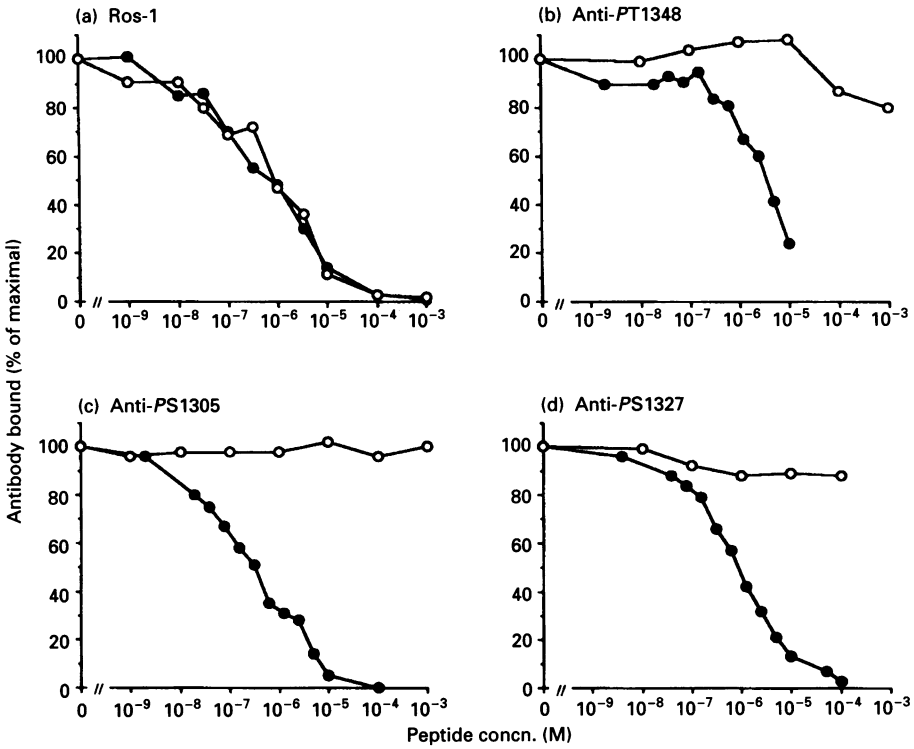

Figure 1 Reaction of antibodies with peptides in e.l.i.s.a.

Wells of a micotitre plate were coated with dephosphopeptide T1348 (**a**) or phosphopeptides *P*T1348 (**b**), *P*S1305 (**c**), and *P*S1327 (**d**), all at 5 μg/ml in 100 μl of TBS (150 mM NaCl, 50 mM Tris/HCl, pH 7.4), for 2 h at room temperature. The wells were then washed with TBS/0.1% Tween 20 (TBST), blocked by incubation overnight with BSA (10 mg/ml in 200 μl of TBST), and again washed. Phospho- or dephospho-peptides at the concentrations shown were incubated in the wells together with the antibody Ros-1 (**a**), anti-*P*T1348 (**b**), anti-*P*S1305 (**c**), or anti-*P*S1327 (**d**), all at a dilution of 1/1000 in 100 μl of TBST, for 2 h at room temperature. The wells were then washed and incubated for a further 1 h with alkaline phosphatase-conjugated sheep anti-(rabbit IgG) (Sigma), diluted 1/1000 in 100 μl of TBST. After further washing, bound antibody was quantified by using 10 mM *p*-nitrophenyl phosphate as substrate and measuring the absorbance at 405 nm. Maximum absorbances (in the absence of competing peptide) ranged from 0.6 to 1.0 and background absorbance (in the absence of antibody) was 0.08. Results are expressed as percentage of maximum binding after subtraction of background. Closed symbols represent competition of antibody binding using the immunogenic peptide and open symbols represent competition with the corresponding phosphopeptide (*P*T1348, **a**) or dephosphopeptide (T1348, S1305, S1327 in **b**, **c** and **d** respectively). Data points are mean values from duplicate determinations, and the plots are representative of at least two independent experiments.

binding of antibody in a competition e.l.i.s.a. at concentrations up to 1 mM (results not shown). However, a rabbit antiserum Ros-1, previously raised against a non-phosphorylated peptide corresponding to the C-terminal 15 amino acids of the insulin receptor and including Thr-1348 [32], reacted equally with dephospho- and phospho-forms of this peptide (Figure 1).

To determine whether the anti-phosphopeptide antibodies recognized native insulin receptor, their ability to precipitate receptor–^{125}I-insulin complexes was assessed. Cell lines that stably express high levels of the human insulin receptor (CHO.T), or insulin receptor together with the α isoform of protein kinase C (CHO.T-PKCα), or truncated insulin receptor with a 69-amino-acid C-terminal deletion (CHO.ΔCT69), were incubated with or without PMA. Insulin receptors from these cells were then partially purified on wheat germ agglutinin–Sepharose for use in ^{125}I-insulin-binding assays (Table 1).

Antisera raised against peptides *P*S1327 and *P*T1348 precipitated native receptor to varying extents, depending on the antibody and the cells from which the receptor was isolated. In contrast, antisera raised against peptide *P*S1305 were unable to precipitate native insulin receptor under any of the conditions tested (Table 1), as were antisera raised against the corresponding dephosphopeptide (results not shown). The anti-*P*T1348 serum precipitated a substantial amount of receptor even under basal conditions when cells had not been incubated with PMA, although the amount of receptor reacting in PMA-treated cells was somewhat greater. The specificity of the anti-*P*T1348 serum for phosphoreceptor was enhanced after the serum had been depleted of dephosphopeptide-binding activity by incubation with immobilized dephosphopeptide. This indicated that the reactivity of the crude serum with basal receptor was due primarily to the presence of antibodies recognizing dephospho-receptor, rather than a high level of phosphorylation of Thr-1348 under basal conditions.

The anti-*P*S1327 serum showed marked specificity for phosphoreceptor compared with dephosphoreceptor even without prior purification of the serum. This antibody precipitated 36% or 60% of receptor from PMA-treated CHO.T or CHO.T-PKCα cells respectively, but only 3% or 6% of receptor from untreated cells (Table 1). These results indicate that Ser-1327 is a previously unrecognized major site of receptor phosphorylation in response to activation of protein kinase C. Moreover, the relative levels of receptor reactivity in the different cell lines are consistent with the previously demonstrated enhancement of PMA-induced receptor phosphorylation in cells over-expressing protein kinase Cα in addition to insulin receptor [18].

In contrast with the specificity shown by the phosphopeptide antisera, the C-terminal-reactive antibody Ros-1, which failed to discriminate between phospho- and dephospho-peptides in the competition e.l.i.s.a., precipitated wild-type receptor equally

Table 1 Reaction of antibodies with native receptor

Partially purified insulin receptor, from the cell lines indicated, was pre-incubated with ^{125}I-insulin for 16 h at 4 °C, and the resulting receptor–^{125}I-insulin complex was then incubated with antibody in 200 μl of binding assay buffer for 2 h at 4 °C. Antibodies Ros 1 and anti-*P*S1327 were used at final dilutions of 1/1000. Crude and purified (adsorbed against dephosphopeptide) anti-*P*T1348 serum were used at final dilutions of 1/10000 and 1/1000 respectively, which contained equal titres of anti-phosphopeptide reactivity as assessed by e.l.i.s.a. Protein A–Sepharose (5 mg) was added for 1 h, antibody–receptor complex was collected by centrifugation and washed three times before determination of radioactivity. Results are expressed as the percentage of total receptor as determined by precipitation with anti-receptor monoclonal antibody IR 83-14 [37], which bound equally to receptor from PMA-treated and untreated cells (results not shown). Total receptor-bound radioactivity was 4500–5500 c.p.m., and non-specific binding in the presence of pre-immune serum was 50 c.p.m. Within each experiment incubations were carried out in duplicate, and values are shown are means (±S.E.M. where appropriate) for the number of independent experiments indicated in parentheses. Abbreviation: ND, not determined.

	Immunoreactive receptors (%)					
	CHO.T		CHO.ΔCT69		CHO.T-PKCα	
Antibody	Basal	PMA-treated	Basal	PMA-treated	Basal	PMA-treated
Ros-1	73 (2)	70 (2)	0 (1)	0 (1)	69±6 (3)	71±3 (3)
Anti-*P*S1327	3 (2)	36 (2)	0 (1)	0 (1)	6±1 (6)	60±2 (6)
Anti-*P*S1305	ND	ND	ND	ND	0 (1)	0 (1)
Anti-*P*T1348 (crude)	ND	ND	ND	ND	60 (2)	79 (2)
Anti-*P*T1348 (purified)	ND	ND	ND	ND	6±2 (3)	25±2 (3)

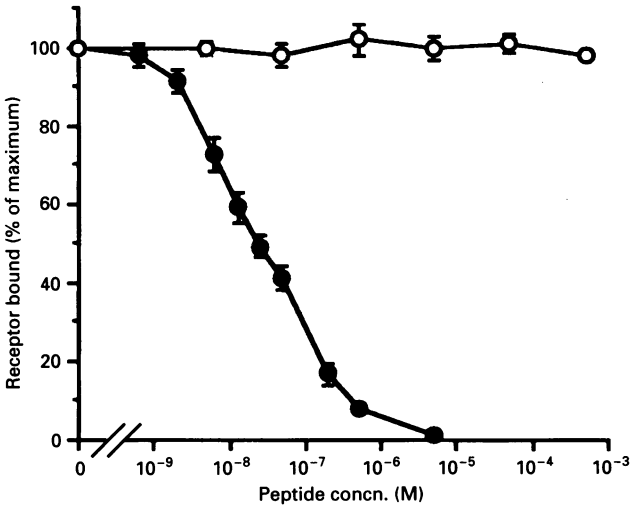

Figure 2 Inhibition of receptor binding to anti-*P*S1327 antibody by free peptide

Partially purified receptor from PMA-treated CHO.T-PKCα cells was pre-incubated with ^{125}I-insulin. Receptor–^{125}I-insulin complex was then incubated with anti-*P*S1327 antibody (1/1000 final dilution) and phospho- or dephosphopeptide in 200 μl of binding assay buffer for 2 h at 4 °C. Protein A–Sepharose was then added for 1 h. The immunocomplex was collected by centrifugation and washed three times before determination of radioactivity. Maximum antibody-bound radioactivity (in the absence of peptide) ranged from 2800 to 3300 c.p.m. and non-specific binding in the presence of pre-immune serum was 50 c.p.m. Each point represents the mean ± S.E.M. from three independent experiments, with duplicate determinations within each experiment. Open symbols represent competition with the dephosphopeptide and closed symbols competition using the phosphopeptide.

from both PMA-treated and untreated cells. As expected, none of the antibodies precipitated the C-terminally truncated form of the receptor, which lacks all the relevant epitopes (Table 1).

The basis of the specificity exhibited by anti-*P*S1327 serum towards native phosphoreceptor was investigated by attempting to compete out the immunoprecipitation with phospho- or dephospho-peptides. Peptide *P*S1327 inhibited precipitation of phosphoreceptor by anti-*P*S1327 with an IC_{50} of 25 nM, although the corresponding dephosphopeptide was without effect even at 0.5 mM (Figure 2). Other phosphopeptides (*P*S1305 and *P*T1348) and free phosphoserine or phosphothreonine, also at 0.5 mM, did not affect immunoprecipitation of phosphoreceptor by anti-*P*S1327 (results not shown).

The reactivity of the anti-phosphopeptide antibodies with denatured insulin receptor was studied by immunoblot analysis of partially purified receptor preparations. Equal amounts of purified insulin receptor were loaded from the different cell lines, as assessed by probing blots with antiserum Ros-1 [32] which reacts with the receptor C-terminus but does not discriminate phospho- and dephospho-receptor, and antiserum IR.JM1 [29], which is directed against the juxtamembrane domain of the receptor β-subunit.

The pattern of immunoreactivity of anti-*P*S1327 and anti-*P*T1348 sera observed with denatured receptor (Figure 3) was essentially the same as that seen with native receptor (Table 1). Thus anti-*P*S1327 reacted very effectively with receptor prepared from PMA-treated CHO.T-PKCα cells, and slightly less well with that from PMA-treated CHO.T cells. Receptor from untreated cells was detected only very weakly and was undetectable with some receptor preparations. Crude anti-*P*T1348 serum detected receptor from PMA-treated CHO.T-PKCα cells slightly more effectively than that from untreated cells. However, anti-*P*T1348 serum that had been purified by immunodepletion showed a much greater ability to discriminate between receptors from PMA-treated and untreated CHO.T-PKCα cells (Figure 3).

The anti-*P*S1327 serum failed to detect any increase in phosphorylation of receptor from CHO.T or CHO.T-PKCα cells following treatment with insulin alone (Figure 3). Moreover, addition of insulin before or after PMA reduced the magnitude of the PMA-induced response by approximately half, as detected by anti-*P*S1327 antibody on blots (results not shown). However, the purified form of anti-*P*T1348 demonstrated increased reactivity towards receptors from CHO.T and CHO.T-PKCα cells following insulin treatment (Figure 3).

Two-dimensional phosphopeptide mapping has previously been used to define sites of receptor phosphorylation, and the immunoreactivity of tryptic phosphopeptides resolved in this way was therefore investigated. The phosphopeptide containing Thr-1348 has been identified as a single discrete spot on two-dimensional maps of tryptic phosphopeptides. In CHO.T cells the stoichiometry of PMA-induced phosphorylation at this site appears to be approx. 0.6 mol of *P*/mol of receptor [35]. The

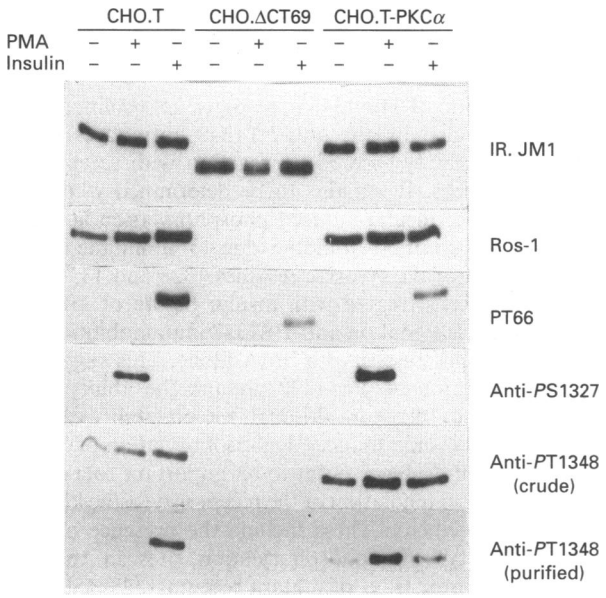

Figure 3 Reaction of antibodies with denatured receptor

Insulin receptors partially purified from the cell lines CHO.T, CHO.TΔ69 and CHO.T-PKCα, with or without prior exposure of cells to PMA (1 μM) or insulin (1 μM), were electrophoresed on SDS/7.5% polyacrylamide gels and transferred to poly(vinylidene difluoride) membranes as described in the Materials and methods section. The quantity of receptor loaded in each lane corresponded approximately to the yield from a 10 cm^2 area of confluent cells. After blocking with a solution of milk powder, blots were probed with the indicated primary antibodies at the following dilutions: anti-receptor IR.JM1 [29] and monoclonal anti-phosphotyrosine PT66 (Sigma), 1/500; anti-receptor Ros-1 [32], 1/2000; anti-*P*S1327 and crude anti-*P*T1348, 1/10000; purified anti-*P*T1348, 1/1000. Immunocomplexes were detected with the appropriate peroxidase-conjugated secondary antibody, at a dilution of 1/10000, and visualized as described in the Materials and methods section. Only the area of the blots containing the receptor β-subunit is shown, and no bands were visible at this exposure on other regions of the blots.

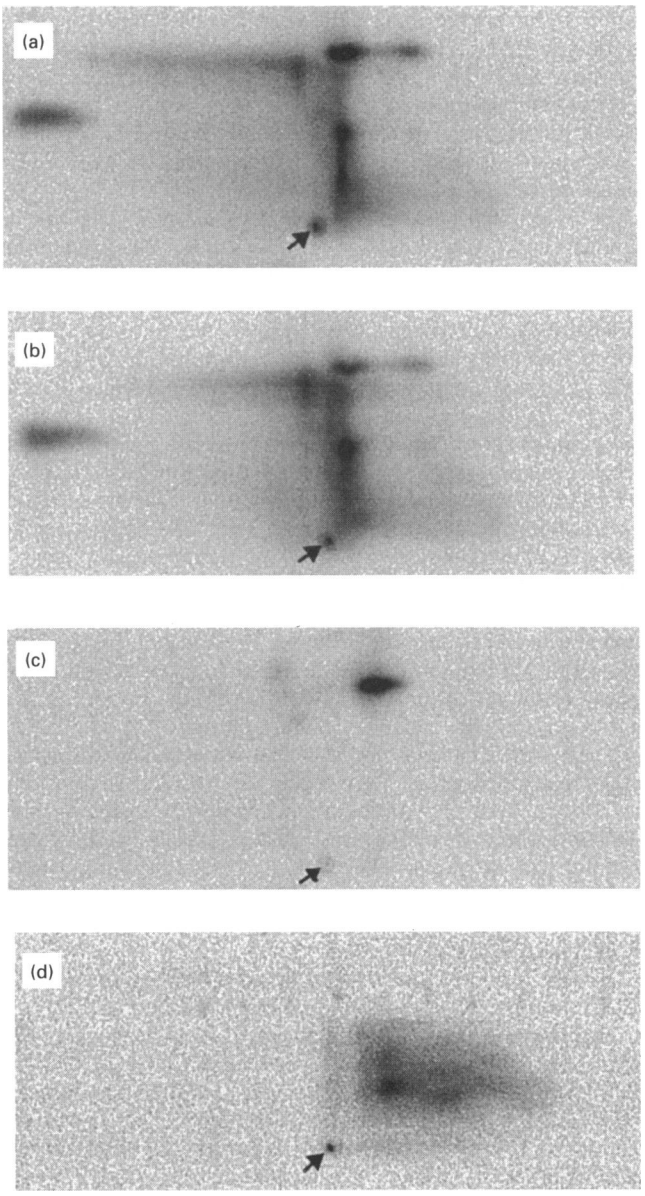

Figure 4 Identification of a tryptic peptide phosphorylated at serine 1327

A tryptic digest of insulin receptor was prepared from ^{32}P-labelled CHO.T-PKCα cells that had been treated with PMA (1.6 μM, 30 min). One portion of the receptor digest (8000 c.p.m.) was then incubated with pre-immune serum and the remainder (16000 c.p.m.) was incubated sequentially with protein A–Sepharose-purified anti-*P*S1327 and anti-*P*T1348, as described in the Materials and methods section. Two-dimensional phosphopeptide mapping was then performed on the supernatants from the control (**a**) and specific (**b**) immunodepletions of the receptor digest, and on material eluted from the anti-*P*T1348 (**c**) and anti-*P*S1327 (**d**) immunoprecipitates. Electrophoresis was carried out in the horizontal direction followed by chromatography in the vertical direction. The origin of sample application is indicated by an arrow. Loadings in (**a**–**d**) were 8000, 8000, 900 and 700 c.p.m. respectively.

results of the present study indicate that Ser-1327 is also a major site of PMA-induced receptor phosphorylation. To identify the tryptic phosphopeptide containing Ser-1327, a digest of ^{32}P-labelled receptor from PMA-treated CHO.T-PKCα cells was depleted of phosphopeptides by incubating with anti-*P*S1327 serum. The supernatant from the precipitation with anti-*P*S1327 serum was then re-precipitated with anti-*P*T1348 serum. The two antibodies bound similar amounts of radiolabelled peptide from the tryptic digest. A control depletion with pre-immune serum produced a phosphopeptide map that was identical with that for a receptor digest that had not been treated with any antibody (results not shown). Depletion of the digest with anti-phosphopeptide antibodies produced a map in which two phosphopeptides were greatly reduced in intensity (Figure 4). One of these corresponded to the peptide previously identified as containing phosphorylated Thr-1348, and the other was assumed to be the peptide containing phosphorylated Ser-1327. The peptides bound to the antibodies were eluted and also resolved by two-dimensional mapping. The eluate from anti-*P*T1348 antibody ran as a discrete spot in the expected position for the previously identified Thr-1348-containing phosphopeptide. The eluate from anti-*P*S1327 antibody ran as a more diffuse collection of peptides, the most prominent of which corresponded to the major peptide removed from the total digest by this antibody.

DISCUSSION

In the present study, we have produced two anti-phosphopeptide antibodies which react with the insulin receptor in a site-specific and phosphorylation state-dependent manner. One of these antibodies (anti-*P*T1348) is directed against a previously known phosphorylation site, while the other (anti-*P*S1327) identifies Ser-1327 as a novel major site of phorbol ester-induced phosphorylation. Antibodies were also obtained for both phospho- and dephospho-forms of a peptide containing another

previously known phosphorylation site, Ser-1305. However, these antibodies did not bind receptor from either PMA-treated or untreated cells. The unavailability of this epitope in native receptor was unexpected in view of the fact that the site is obviously accessible to a protein kinase. Moreover, antibody raised against a peptide corresponding to amino acids 1306–1329 does precipitate native receptor [39].

The C-terminal domain of the insulin receptor is subject to insulin- and autophosphorylation-induced conformational changes which are detected by anti-peptide antibodies directed against the sequences 1306–1329 or 1321–1338 [39,40]. The possibility that the specificity of anti-*P*S1327 antibody reflects exposure of a cryptic epitope due to a phosphorylation-induced conformational change, rather than direct dependence of antibody binding on phosphorylation, is ruled out by the fact that this antibody showed similar specificity for native and denatured receptor preparations. Moreover, non-phosphorylated receptor can be precipitated by an antibody raised against a peptide corresponding to residues 1321–1338 [39], indicating that this region is exposed in basal as well as phosphorylated receptor.

The specificity of anti-*P*S1327 serum for phosphorylated receptor was mirrored in its specificity for phosphopeptide in a competition e.l.i.s.a. However, anti-*P*T1348 serum reacted relatively well with receptor from untreated cells even though it appeared to be specific for phosphopeptide in an e.l.i.s.a. Indeed, antibodies reacting with non-phosphorylated receptor were removed from the crude anti-*P*T1348 serum by adsorption with dephosphopeptide. Presumably the e.l.i.s.a. detects only a subpopulation of antibodies capable of reacting with phosphopeptide adsorbed to plastic, which were indeed phosphorylation-specific. The anti-*P*T1348 serum must additionally contain a population of antibodies which recognize non-phosphorylated peptide and receptor, but are not detected by the phosphopeptide e.l.i.s.a.

The results of receptor–^{125}I-insulin co-precipitation assays, in which the fractions of receptor reacting with anti-*P*S1327 and purified anti-*P*T1348 could be compared directly, suggest that Ser-1327 may be a quantitatively more important site of PMA-induced phosphorylation than Thr-1348 (Table 1). The fact that anti-*P*S1327 precipitated 60% of receptors from PMA-treated CHO.T-PKCα cells indicates that at least 30% of the β-subunits were phosphorylated on Ser-1327, allowing for the fact that each receptor contains two β-subunits. The possibility cannot be ruled out that the smaller fraction of receptor reacting with anti-*P*T1348 serum was due in part to less efficient precipitation by this antibody, because of poorer titre or affinity, rather than a lower stoichiometry of phosphorylation at this site. Indeed, phosphopeptide mapping indicated that the peptide containing Thr-1348 was the most highly labelled peptide in the receptor from PMA-treated cells, and certainly more intense than the peptide depleted by anti-*P*S1327 antibody. It is possible, however, that several different peptides are generated containing Ser-1327, as a result of differential tryptic cleavage or chemical modification, and that not all of these retain an effective epitope for the anti-*P*S1327 antibody. Indeed, the presumed site of tryptic cleavage is immediately adjacent to Ser-1327 (sequence -Lys-Arg-Ser-). Moreover, while phosphopeptide eluted from anti-*P*T1348 migrated on two-dimensional maps as a discrete spot in the expected position, the phosphopeptide eluted from anti-*P*S1327 appeared to be heterogeneous. Thus the phosphopeptide depletion may underestimate the true extent of Ser-1327 phosphorylation in response to PMA. A low level of reactivity of anti-*P*S1327 antibody with receptor from cells which had not been treated with PMA most probably reflected basal phosphorylation of receptor rather than cross-reaction with unphosphorylated receptor, as this reactivity was still present in serum which had been adsorbed against dephosphopeptide (results not shown).

Phosphorylation of Thr-1348 in response to insulin, as well as PMA, was detected by the anti-*P*T1348 antibody. However, anti-*P*S1327 antibody showed no reaction with receptor from insulin-treated cells. It remains to be determined whether this reflects absence of insulin-induced phosphorylation at this site, or inhibition of antibody binding due to simultaneous autophosphorylation of the tyrosine residues 1328 and 1334 [41,42]. Receptor from cells treated with insulin before or after PMA gave a less intense signal on anti-*P*S1327 immunoblots than did receptor from cells treated with PMA alone. This suggests that autophosphorylation of Tyr-1328 impairs the ability of anti-*P*S1327 to bind to receptor, although the possibility cannot yet be excluded that insulin induces dephosphorylation of Ser-1327.

Several lines of evidence point to an important role of the C-terminal domain in regulation of the receptor tyrosine kinase and its signalling specificity. These include the presence of serine/threonine and tyrosine phosphorylation sites in the region [9,10,41,42], the properties of mutant receptors [43–45], and the effects of site-specific antibodies [46,47]. It has been reported that phosphorylation of receptor on serine/threonine residues, in response to PMA, has an inhibitory effect on tyrosine kinase activity [5,12–14], although this has not been consistently observed [6,8,15–18]. It may be that the effect of PMA on receptor function varies between different cell types, or that its manifestation depends on the substrate or other aspects of the method used to assay tyrosine kinase activity. Mutant receptors in which Ser-1305/6 or Thr-1348 have been substituted display properties indistinguishable from wild-type receptors [48,49]. It may be therefore that phosphorylation of other sites is of greater functional significance. In this context, the proximity of Ser-1327 to known sites of tyrosine autophosphorylation suggests that phosphorylation of this residue could significantly influence rates of tyrosine phosphorylation or dephosphorylation, or the interaction of phosphotyrosyl residues with other proteins involved in signalling [50]. These possibilities remain to be explored. It should be noted that the rat insulin receptor sequence has threonine rather than serine at the position corresponding to residue 1327 of the human sequence [51]. It is not yet known whether this threonine residue becomes phosphorylated in response to PMA in rat cells.

A further question which awaits resolution concerns the molecular mechanism underlying the impairment of insulin receptor tyrosine kinase activity in non-insulin-dependent diabetes [20]. The antibodies described in the present study will enable us to test the hypothesis that increased receptor serine/threonine phosphorylation is responsible for inhibition of the tyrosine kinase, by comparing the extent of serine/threonine phosphorylation of receptor extracted from tissues of normal and insulin-resistant subjects.

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