Antibodies to insulin receptor tyrosine kinase stimulate its activity towards exogenous substrates without inducing receptor autophosphorylation

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Anti-peptide antibodies directed against a highly-conserved sequence of the insulin receptor tyrosine kinase domain have been used to study the relationship between this specific region and kinase activation. Antibodies have been prepared by the injection into a rabbit of a synthetic peptide (P2) corresponding to residues 1110–1125 of the proreceptor. The peptide exhibits 88–95% sequence similarity with the corresponding sequence in the *v-ros* protein and in receptors for epidermal growth factor and for insulin-like growth factor 1. Two antibodies with different specificities could be separated from total antiserum obtained after immunization with P2. One antibody [anti-(P-Tyr)] cross-reacted with phosphotyrosine and immunoprecipitated solely autophosphorylated receptors. This antibody was shown to increase or decrease the receptor tyrosine kinase activity depending on its concentration. In all circumstances receptor autophosphorylation and substrate phosphorylation were modulated in a parallel fashion. The second antibody (anti-P2) failed to immunoprecipitate the insulin receptor, but was found to interact with both the peptide and the receptor by e.l.i.s.a. assay. Using a tyrosine co-polymer we found that anti-P2 activated the insulin receptor kinase leading to substrate phosphorylation at a level similar to that observed with insulin. This effect was additive to the hormonal effect. In contrast, receptor autophosphorylation was not modified by the anti-peptide. The differential effect of this anti-peptide further supports the idea that receptor autophosphorylation and kinase activity towards exogenous substrates might be independently regulated. Finally, our data suggest that conformational changes in the receptor tyrosine kinase domain may be sufficient for activation of its enzymic activity.

INTRODUCTION

The first event so far identified upon interaction of insulin with its specific receptor is an increased receptor autophosphorylation on tyrosine residues (Avruch *et al.*, 1982; Van Obberghen & Kowalski, 1982; Kasuga *et al.*, 1982). The insulin-sensitive kinase is intrinsic to the insulin receptor (Petrucelli *et al.*, 1982; Van Obberghen *et al.*, 1983), and the activated receptor is able to catalyse the phosphorylation of exogenous and endogenous substrates. A pronounced sequence similarity exists between the amino acid sequence of the receptor kinase domain and other known tyrosine kinases such as receptors for epidermal growth factor (EGF), insulin-like growth factor 1 (IGF-1) and platelet-derived growth factor (PDGF), and proteins encoded by some retroviral oncogenes (Ullrich *et al.*, 1984, 1985, 1986; Ebina *et al.*, 1985; Martin-Zanca *et al.*, 1986; Yarden *et al.*, 1986). The precise mechanism of action of these tyrosine kinases in mediating cellular responses is unknown. However, for the insulin receptor a general consensus has been reached indicating that tyrosine kinase activity is essential for hormonal action. Thus, alterations in insulin responses are parallel to alterations in kinase activity (Grigorescu *et al.*, 1984; Grunberger *et al.*, 1984; Le Marchand-

Brustel *et al.*, 1985; Morgan & Roth, 1987), and experiments with mutated receptors show that cells expressing kinase-deficient receptors fail to mediate hormonal effects (Chou *et al.*, 1987; Ebina *et al.*, 1987; MacClain *et al.*, 1987). However, little is known about the regulation of the tyrosine kinase activity or about the relationship between receptor phosphorylation and receptor kinase activity towards substrates.

Knowledge of the amino acid sequence of the insulin proreceptor (Ebina *et al.*, 1985; Ullrich *et al.*, 1985) makes it now possible to investigate particular receptor domains using antibodies against synthetic peptides corresponding to these domains. Thus, the importance of the regions containing residues Tyr-960 and Tyr-1150 was first demonstrated by the use of anti-peptide antibodies (Herrera *et al.*, 1985, 1986). Here we report data obtained with an antibody directed against a peptide corresponding to residues 1110–1125 of the insulin proreceptor sequence (Ullrich *et al.*, 1985). This sequence is located in the receptor kinase domain and presents a particularly high degree of sequence similarity to other tyrosine kinases. Thus, the peptide contains a sequence of nine residues entirely conserved in receptors for growth factors such as EGF (Ullrich *et al.*, 1984), IGF-1 (Ullrich *et al.*, 1986) and PDGF (Yarden *et al.*, 1986). This

Abbreviations used: EGF, epidermal growth factor; IGF-1, insulin-like growth factor; PDGF, platelet-derived growth factor; PAGE, polyacrylamide-gel electrophoresis; PBS, phosphate-buffered saline (137 mM-NaCl/2.6 mM-KCl/6 mM-Na₂HPO₄/1.47 mM-KH₂PO₄, pH 7.4).

conserved sequence includes a triplet, Arg-Asp-Leu, which is usually present in the catalytic domain of protein kinases at a distance of about 100 residues from the ATP-binding site (Hunter, 1987).

MATERIALS AND METHODS

Materials

Triton X-100, *N*-acetyl-D-glucosamine, bovine serum albumin, bacitracin, phenylmethanesulphonyl fluoride and poly[Glu-Na/Tyr (4:1)], were from Sigma, St. Louis, MO, U.S.A. Wheat-germ-agglutinin agarose came from Miles Laboratories. Protein A-Sepharose was from Calbiochem. Reagents for SDS/polyacrylamide gel electrophoresis (SDS/PAGE) were from Bio-Rad, Richmond, CA, U.S.A. [γ - 32 P]ATP was purchased from Amersham International, Amersham, Bucks., U.K. Na 125 I was from CEA, Saclay, France. Pig insulin was a gift from Novo Research Institute, Copenhagen, Denmark. Antiserum to insulin receptor (B7) was a gift from Dr. P. Gorden (NIH, Bethesda, MD, U.S.A.). The photoreactive insulin analogue [NAPA B29]-insulin was kindly supplied by Dr. D. Brandenburg (Aachen, Germany). All reagents were of the purest grade available.

Peptide synthesis

The peptide Tyr-Leu-Asn-Ala-Lys-Lys-Phe-Val-His-Arg-Asp-Leu-Ala-Ala-Arg-Asn (P2), corresponding to residues 1110–1125 of the insulin proreceptor sequence (Ullrich *et al.*, 1985), was synthesized according to Merrifield (1969). Peptide purity was assessed by h.p.l.c., and the sequence was confirmed by quantitative amino acid analysis.

Coupling of the peptide and production of anti-peptide antibodies

The peptide was coupled to keyhole limpet haemocyanin using the heterofunctional reagent *N*-succinimidyl-3-(2-pyridyldithio)propionate (Carlson *et al.*, 1978); 400 μ g of antigen was injected into a rabbit. After an initial intradermal injection in complete Freund's adjuvant, animals were given 6–8 boosts of peptide antigen in incomplete Freund's adjuvant. Blood was collected 10 days after each injection and preimmune serum served as a negative control. Interactions of antisera with P2 were determined by immunoprecipitation of 125 I-labelled peptide (300 mCi/ μ mol) with Protein A-Sepharose. The peptide was labelled using the chloramine-T method (Freychet, 1976). Immunoglobulins were purified by passing the immune serum over a column of protein A-Sepharose. After elution with 100 mM-citrate buffer, pH 3.5, and immediate neutralization, the eluate was dialysed against 20 mM-Hepes, pH 7.6, and stored in small portions. In some experiments, anti-P2 serum was applied to a phosphotyrosine-agarose column and the immunoglobulins collected in the flow-through were purified on Protein A-Sepharose as described above. The antibodies bound to the phosphotyrosine column were eluted and dialysed in a similar fashion. Where indicated, anti-P2 was affinity-purified using a Sepharose-bound peptide column (Herrera *et al.*, 1985). After elution with 0.1 M-glycine/0.5 M-NaCl, pH 2, antibody to P2 was dialysed and concentrated.

E.I.s.a. with insulin receptor

Assays were performed using a method developed in our laboratory (N. Rochet, B. Ferrua & V. Baron, unpublished work). Briefly, microtiter wells were incubated overnight at 4 °C with solubilized receptor (about 2 fmol of insulin-binding activity/ μ l) in 50 mM-carbonate buffer, pH 9.6, before saturation with phosphate-buffered saline (PBS) supplemented with 0.05% Tween and 1% bovine serum albumin. Various concentrations of anti-P2 serum were incubated on the coated plates for 2 h at 22 °C. Thereafter, horseradish-peroxidase-conjugated goat anti-rabbit Ig were added for 30 min at 22 °C, followed by a 15 min incubation with the substrate *o*-phenylenediamine dihydrochloride. Finally, the reaction was stopped with 2 M-sulphuric acid; and absorbance was measured at 492 nm. Between each step of the assay, the plates were washed three times with PBS/0.05% Tween. The procedure was similar when plates were coated with peptide P2 (10^{-7} M).

Cell culture

Rat embryo fibroblasts transfected with human insulin receptor (HIRc) were a gift from Dr. Axel Ullrich (Genentech, San Francisco, U.S.A.). They were cultured in H21/F12 medium (1:1 v/v) supplemented with 7% dialysed fetal calf serum and 5×10^{-7} M-methotrexate.

Partial purification of the insulin receptor

Insulin receptors from HIR cells were partially purified by using wheat-germ-agglutinin chromatography as previously described (Van Obberghen & Kowalski, 1982). All of the procedure was performed in presence of the protease inhibitors aprotinin (1000 units/ml), bacitracin (1 mM), and phenylmethanesulphonyl fluoride (1 mM). The preparations obtained bound 50–100 fmol of insulin/ μ l.

Immunoprecipitation of the insulin receptor

The partially-purified receptor (10–20 fmol of insulin-binding activity/ μ l) was incubated overnight at 4 °C with anti-P2 antibodies in presence of protease inhibitors; phosphatase inhibitors were added when the receptor had been previously phosphorylated. The immune complex was precipitated by addition of Protein A-Sepharose. The ability of anti-peptide antibodies to immunoprecipitate the receptor was tested using different experimental conditions. In some experiments the insulin receptor was labelled with 125 I-[NAPA B29]-insulin as previously described (Fehlmann *et al.*, 1982; Le Marchand-Brustel *et al.*, 1985) before immunoprecipitation. In other experiments the receptor β -subunit was labelled with 32 P in presence of insulin before precipitation of the immune complexes. The precipitates were analysed by SDS/PAGE (Laemmli, 1970).

Receptor autophosphorylation and tyrosine kinase activity towards exogenous substrates

The effect of antibodies on receptor autophosphorylation was studied by preincubating the solubilized receptor (10–20 fmol of insulin-binding activity/ μ l) for 2 h at 20 °C with antibodies or preimmune serum before addition of insulin (10^{-7} M) for 90 min at 15 °C. Receptor phosphorylation was then initiated by adding 30 μ M- $[\gamma$ - 32 P]ATP (2.5 Ci/mmol)/4 mM-MnCl $_2$ /8 mM-MgCl $_2$, and after 15 min at 15 °C the reaction was stopped by addition

of Laemmli sample buffer containing β -mercaptoethanol (5%). The samples were analysed by one-dimensional SDS/PAGE under reducing conditions. The tyrosine kinase activity towards exogenous substrates was measured after incubation of solubilized receptor with antibodies in absence or presence of insulin as described above, except that synthetic substrate poly(Glu/Tyr) was added before the phosphorylation assay. Incorporation of radioactive phosphate into the substrate was measured as previously described using a filter paper assay (Braun *et al.*, 1984; Le Marchand-Brustel *et al.*, 1985). Phosphorylation of peptide (5×10^{-4} M) was carried out using the method described by Stadtmauer & Rosen (1986) for phosphorylation of peptides by the insulin receptor kinase.

RESULTS

Immunoprecipitation of autophosphorylated insulin receptor

The ability of the total anti-P2 serum to immunoprecipitate solubilized insulin receptor was tested by incubating the anti-peptide with either 125 I-labelled insulin receptor or 32 P-labelled receptors. Anti-P2 antiserum was found to precipitate insulin receptor only in experiments performed with phosphorylated receptor (Fig. 1). Solubilized insulin receptor was covalently bound with 125 I-labelled [NAPA^{B29}]insulin, then incubated with anti-P2 total Ig, anti-(insulin receptor) serum B7 or non-immune immunoglobulins (Fig. 1a). No labelled band corresponding to the receptor could be detected after incubation with anti-P2 total Ig (lane 3)

indicating that the anti-peptide was unable to immunoprecipitate the receptor under these conditions. In contrast, the antibody immunoprecipitated insulin receptor that had been previously phosphorylated in presence of insulin (Fig. 1b, lane 6). When receptor autophosphorylation was inhibited by addition of EDTA, receptor immunoprecipitation was abolished as demonstrated by the lack of precipitated 125 I-labelled receptors, and the absence of precipitated insulin-binding activity (results not shown).

The reactivity of the antiserum with the phosphorylated receptor could be due either to a conformational change induced by phosphorylation or to a cross-reaction with a phospho amino acid. To approach this question the antiserum specificity was further characterized. In Fig. 2(a) (lanes 1–4) it can be seen that immunoprecipitation of the phosphorylated receptor was inhibited by prior addition of the peptide P2 only when the peptide had been phosphorylated (lane 4). Moreover, phosphotyrosine at 1 mM prevented immunoprecipitation of the receptor (Fig. 2b, lane 7), whereas tyrosine did not (Fig. 2b, lane 8). Finally it was found that phosphoserine and phosphothreonine at 1 mM failed to inhibit the precipitation of the phosphoreceptor. These data indicate that our anti-peptide antibody reacted with phosphotyrosine. However, the same antiserum was also able to precipitate the non-phosphorylated peptide P2 (results not shown). Therefore, we concluded that anti-P2 antiserum contains two antibodies with different specificities, and their separation was undertaken. Using a phosphotyrosine agarose column we were able to adsorb antibodies that cross-react with phosphotyrosine;

Fig. 1. Total anti-P2 immunoprecipitates only the phosphorylated receptor

(a) Partially-purified insulin receptor was labelled with photoreactive insulin, incubated overnight at 4 °C with 0.6 mg/ml of either non-immune immunoglobulins (lane 2) or total anti-P2 Ig (lane 3). After addition of Protein A, the precipitates were analysed by SDS/PAGE under reducing conditions and autoradiography. In lane 1, the photo-labelled receptor was incubated with a 1:100 dilution of serum B7 containing autoantibodies against the insulin receptor. (b) Partially-purified insulin receptor was autophosphorylated in presence of insulin (10^{-7} M) with $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ as described in the Materials and methods section. The phosphorylated receptor was then incubated as above either with serum B7 (lane 4), non-immune Ig (lane 5), or total anti-P2 Ig (lane 6). The immune complexes were precipitated with Protein A-Sepharose and analysed by SDS/PAGE under reducing conditions followed by autoradiography. Or., origin.

Fig. 2. Specificity of immunoprecipitation of the phosphorylated insulin receptor by total anti-P2

Solubilized insulin receptor was autophosphorylated in presence of insulin at 10^{-7} M as described in the Materials and methods section before incubation with 0.6 mg/ml of either non-immune Ig (lanes 1 and 5) or total anti-P2 Ig (lanes 2–4 and 6–8). Where indicated, the incubation was performed in presence of peptide P2 or amino acids. Two autoradiographs are shown. In (a) the phosphorylated insulin receptor was incubated with anti-P2 alone (lane 2), or with anti-P2 and an excess of either peptide P2 (lane 3) or peptide P2 that had been phosphorylated (lane 4). In (b), autophosphorylated insulin receptor was incubated with anti-P2 alone (lane 6), or with anti-P2 in presence of phosphotyrosine (lane 7) or tyrosine (lane 8).

Fig. 3. Anti-(*P*-Tyr) immunoprecipitates the phosphorylated insulin receptor whereas anti-P2 does not

Autophosphorylated insulin receptor was incubated with antibodies, immunoprecipitated and the precipitates analysed as described in the legends to Figs. 1 and 2. Total anti-P2 (lanes 1–3) corresponds to the total fraction of Ig purified from anti-P2 serum. Anti-(*P*-Tyr) (lanes 4–6) are antibodies eluted from the phosphotyrosine-agarose column (see the Materials and methods section). Anti-P2 (lanes 7–9) are Ig that had been purified from the flow-through of the phosphotyrosine column.

these are called anti-(*P*-Tyr). The flow-through immunoglobulins depleted of anti-(*P*-Tyr), were further purified on Protein A-Sephrose, and are called anti-P2. These two fractions were tested for their ability to precipitate the phosphorylated receptor. Anti-(*P*-Tyr) was able to immunoprecipitate the phosphoreceptor (Fig. 3, lanes 4–6) as did anti-P2 total immunoglobulins (lanes 1–3); on the contrary, anti-P2 could not do so (lanes 7–9). Since in additional experiments we were unable to detect any immunoprecipitation of the receptor when a higher (1 mg/ml) antibody concentration was used, we conclude that anti-P2 fails to precipitate the insulin receptor.

We then examined the ability of the anti-peptide to react with the receptor by using the e.l.i.s.a. technique. In these experiments both anti-P2 and affinity-purified anti-peptide were tested. Solubilized insulin receptor coated to microtiterplates was incubated with either anti-P2 antibody [total immunoglobulins depleted of anti-(*P*-Tyr) or affinity-purified anti-peptide], or non-immune immunoglobulins at various concentrations. As illustrated in Fig. 4 (*a,b*), absorbance values were markedly enhanced in wells that had been treated with the antibody, indicating that anti-P2 antibody was bound to insulin receptor. In other experiments, wells coated with peptide P2 were incubated with anti-P2 that had or had not been preincubated with solubilized insulin receptor. The results depicted in Fig. 4 (*c,d*) show that addition of insulin receptor did inhibit the fixation of the antibody to the peptide. This indicates that anti-P2 antibody recognizes both the peptide and the receptor.

Fig. 4. Anti-P2 binding to insulin receptor shown by e.l.i.s.a.

(*a*), (*b*) Increasing concentrations of anti-P2 immunoglobulins were incubated for 2 h at 22 °C in microtiter plates coated with insulin receptor. Then, goat anti-rabbit Ig conjugated to horseradish peroxidase were added for an additional 30 min and the plates were washed three times. After addition of the substrate *o*-phenylenediamine dihydrochloride, the incubation was prolonged for 15 min and stopped using 2 M-H₂SO₄. Results are absorbance values measured at 492 nm. (*c*), (*d*) The procedure was similar except that wells were coated with peptide P2 and that anti-P2 immunoglobulins were preincubated for 2 h at 20 °C in the absence or in the presence of solubilized insulin receptor (about 50 fmol of insulin-binding activity/µl) before addition to microtiter plates.

Fig. 5. Effect of anti-(*P*-Tyr) on insulin receptor autophosphorylation

Solubilized insulin receptor was incubated for 2 h at 22 °C with either non-immune Ig (90 µg/ml) or anti-(*P*-Tyr) at concentrations varying from 20 to 90 µg/ml, in the absence (lanes 1, 3, 5, 7 and 9) or presence of insulin (10^{-7} M) (lanes 2, 4, 6, 8 and 10). The phosphorylation reaction was then initiated as described in the Materials and methods section. After 15 min at 15 °C, Laemmli sample buffer was added and the samples were analysed by SDS/PAGE under reducing conditions followed by autoradiography. Non-immune Ig (lanes 1 and 2), anti-(*P*-Tyr) at 20 µg/ml (lanes 3 and 4), 40 µg/ml (lanes 5 and 6), 70 µg/ml (lanes 7 and 8), 90 µg/ml (lanes 9 and 10).

Effect of anti-(*P*-Tyr) on insulin kinase activity

The effect of anti-(*P*-Tyr) on insulin receptor autophosphorylation was measured in a cell-free system after preincubation of the antibody or preimmune immunoglobulins with the solubilized insulin receptor. Incorporation of 32 P into insulin receptor was analysed by SDS/PAGE under reducing conditions followed by autoradiography (Fig. 5). As expected, insulin enhanced the phosphorylation of its receptor β -subunit (lane 2). In the absence of insulin, anti-(*P*-Tyr) either increased or decreased receptor autophosphorylation, depending on the Ig concentration. Thus, low concentrations of anti-(*P*-Tyr) immunoglobulins (lanes 3 and 5) increased the receptor β -subunit phosphorylation level, compared with that observed with non-immune Ig (lane 1). In addition, anti-(*P*-Tyr) was able to markedly enhance the receptor autophosphorylation in presence of insulin (compare lanes 4 and 6 with lane 2). Different results were obtained when insulin receptors were incubated with higher concentrations of anti-(*P*-Tyr) Ig (lanes 7–10). At 70 and 90 µg/ml, anti-(*P*-Tyr) decreased both basal and insulin-stimulated receptor autophosphorylation.

The ability of anti-(*P*-Tyr) to modulate receptor kinase activity was also examined by studying its effect on the substrate poly(Glu/Tyr) phosphorylation. Here, incorporation of 32 P into poly(Glu/Tyr) was measured after preincubation of solubilized insulin receptor with anti-(*P*-Tyr) at concentrations varying from 10 to 70 µg/ml.

Fig. 6. Effect of anti-(*P*-Tyr) on insulin receptor kinase activity towards poly(Glu/Tyr)

Partially-purified receptor was incubated for 2 h at 22 °C with non-immune Ig and various concentrations of anti-(*P*-Tyr) prior to addition or not of insulin (10^{-7} M) for 90 min at 15 °C. Poly(Glu/Tyr) was then added to a final concentration of 0.2 mg/ml and the phosphorylation assay performed as described in the Materials and methods section. After 45 min at 15 °C the reaction was stopped and the samples were analysed using a filter paper assay. Results illustrate one typical experiment where each point was run in duplicate.

As for receptor autophosphorylation, depending on the anti-(*P*-Tyr) concentration tested, opposite effects were observed (Fig. 6). Thus, at 10 µg/ml, the antibody increased basal and insulin-stimulated substrate phosphorylation by 75 and 65 % respectively, whereas a higher concentration (70 µg/ml) caused a 100 % inhibition of the insulin-stimulated kinase activity and a 35 % inhibition of basal phosphorylation.

Effect of anti-P2 on insulin receptor tyrosine kinase activity

To investigate whether anti-P2 could modulate insulin receptor autophosphorylation, the antibody was preincubated with the receptor before phosphorylation in absence or presence of insulin (10^{-7} M). As illustrated in Fig. 7 (lanes 1 and 2), insulin increased the labelling of the receptor β -subunit (95 kDa). Preincubation with anti-P2 had virtually no effect on the insulin receptor autophosphorylation (lanes 3–10). In absence of insulin, autophosphorylation was unchanged by anti-P2 (lanes 3, 5, 7 and 9). Similarly, the anti-peptide did not significantly modify the level of autophosphorylation in presence of hormone (lanes 4, 6, 8 and 10).

To investigate the possible effect of the anti-peptide on the receptor kinase activity towards exogenous substrates, anti-P2 antibody was preincubated with the solubilized receptor before addition of insulin and phosphorylation of the synthetic substrate poly(Glu/Tyr). In the absence of insulin, anti-P2 stimulated the receptor kinase activity in a dose-dependent fashion (Fig. 8). Maximal stimulation was observed at a concentration of 600 µg/ml and reached 100 % of the activation observed with insulin alone (10^{-7} M). The effect of anti-P2 was also tested in presence of insulin at saturating concentrations. Under these conditions, anti-P2 stimulated phosphate

Fig. 7. Lack of effect of anti-P2 on insulin receptor phosphorylation

Partially-purified insulin receptor was preincubated for 2 h at 22 °C with either 600 µg/ml of non-immune Ig, or anti-P2 antibodies at various concentrations. The incubation was prolonged for 90 min at 15 °C in absence (lanes 1, 3, 5, 7 and 9) or presence of insulin (10^{-7} M) (lanes 2, 4, 6, 8 and 10), and the phosphorylation reaction was initiated as described in the Materials and methods section. After 15 min at 15 °C, the reaction was stopped by adding directly Laemmli sample buffer, and the samples were analysed by SDS/PAGE followed by autoradiography. Non-immune Ig (lanes 1 and 2), anti-P2 at 300 µg/ml (lanes 3 and 4), 400 µg/ml (lanes 5 and 6), 500 µg/ml (lanes 7 and 8), 600 µg/ml (lanes 9 and 10).

incorporation into the substrate compared with the activation observed in the sole presence of hormone. At each anti-P2 concentration tested, stimulation of tyrosine kinase activity due to insulin and to the anti-peptide was at least additive. At optimal concentration of both effectors, the total effect was 20% higher than that expected from a strict additivity, indicating that the antibody can slightly potentiate insulin's effect. Such an effect was confirmed in experiments performed with insulin and anti-P2 at submaximal concentrations, where antibody at 0.4 mg/ml and insulin at 10^{-10} M increased the kinase activity by 28 and 13% respectively. The kinase activation obtained with the two effectors was 63%, a value significantly higher than the 41% that would be obtained with additivity.

To further assess the specificity of the antibody effect, peptide P2 was preincubated with anti-P2 before addition of insulin receptor and measurement of its kinase activity. Additionally, these experiments were performed using affinity-purified anti-P2 (Table 1). Under these conditions, anti-P2 at a concentration of 5 µg/ml increased by 64% the phosphorylation of poly(Glu/Tyr), and free P2 (10^{-7} M) completely prevented the antibody-induced stimulation of the receptor kinase activity towards exogenous substrates. In parallel experiments, it was confirmed that affinity-purified anti-P2 did not affect receptor autophosphorylation.

Fig. 8. Anti-P2-induced stimulation of the insulin receptor tyrosine kinase towards poly(Glu/Tyr)

Solubilized insulin receptor was preincubated 2 h at 22 °C with non-immune Ig and increasing concentrations of anti-P2 before addition of insulin (10^{-7} M) for 90 min at 15 °C. Measurement of substrate phosphorylation was carried out as described in legend to Fig. 6. Data shown illustrate a representative experiment where each concentration was run in duplicate.

Table 1. Inhibition by peptide P2 of anti-P2 induced stimulation of the insulin receptor tyrosine kinase

Affinity-purified anti-P2 (5 µg/ml) was preincubated for 2 h at 15 °C with or without peptide P2 (10^{-7} M) before incubation with solubilized insulin receptor for 2 h at 22 °C. Then, phosphorylation of poly(Glu/Tyr) was measured as described in the Materials and methods section. Data are the means $\pm$ S.E.M. for three separate experiments.

Addition	Receptor kinase activity (% of control)
Control	100
Anti-P2	161 $\pm$ 11
Anti-P2 + P2	99 $\pm$ 6

DISCUSSION

Antibodies directed against known sequences of the insulin receptor β -subunit are useful probes with which to investigate the role of specific tyrosine kinase domains in the expression and regulation of the receptor enzymic activity. In this work we investigated a 16 amino acid long sequence of the insulin receptor, that exhibits extensive (88–95%) similarity with the corresponding sequence in the *v-ros* protein and in the receptors for EGF and for IGF-I. Moreover, this domain contains a sequence of nine residues shared by tyrosine kinase receptors such as those for EGF, IGF-I and PDGF (Ebina

et al., 1985; Ullrich *et al.*, 1984, 1985, 1986; Yarden *et al.*, 1986) and proteins encoded for by several retroviral oncogenes (Martin-Zanca *et al.*, 1986). Finally, the region involved includes a short sequence of three residues (Arg-Asp-Leu) common to sequences of various proteins with tyrosine kinase activity and which is located in the catalytic domain of the kinase approx. 100 residues away from the ATP-binding site (Hunter, 1987). Polyclonal antibodies specific to this domain were developed using a synthetic peptide (P2) corresponding to residues 1110–1125 of the insulin proreceptor (Ullrich *et al.*, 1985). The crude total antiserum was found to contain two antibodies with different specificities. The first antibody, anti-(P-Tyr), immunoprecipitates only the phosphorylated receptor, an observation similar to that reported by Herrera & Rosen (1986) using antibodies directed against residues 1143–1162. Further investigation led to the conclusion that this antibody cross-reacts with phosphorylated tyrosine residues. It may have been induced after antigen phosphorylation *in vivo*. The second antibody, anti-P2, recognizes both the peptide and the insulin receptor, and is thus specific for the amino acid sequence 1110–1125 of the receptor.

The ability of both antibodies to modulate the insulin receptor tyrosine kinase was investigated using a cell-free phosphorylation system. We demonstrated here that antibodies to phosphotyrosine residues [anti-(P-Tyr)] may either stimulate or inhibit insulin receptor kinase depending on the antibody concentration tested. This could suggest that differences reported in the literature may only be due to the experimental conditions used. Indeed, inhibition of insulin-stimulated kinase activity by anti-phosphotyrosine has been reported by White *et al.* (1988), whereas we recently found that another antibody to phosphotyrosine residues increases the insulin receptor tyrosine kinase activity (Ballotti *et al.*, 1989). Our present results illustrate that the same antibody may have dual effects on the receptor kinase activity. Our study also stresses that anti-peptide antibodies against domains with tyrosine residue(s) could contain, in addition to the specific anti-peptides, anti-phosphotyrosine antibodies. This possibility has to be taken into consideration, especially when antibodies are found only to react with the phosphorylated form of the protein. The mechanism by which anti-phosphotyrosine antibodies affect the receptor tyrosine function is not established, but our results showing that one antibody may modulate the receptor kinase activity is in agreement with the idea that phosphorylation of specific sites regulates the phosphotransferase activity of the receptor. Consistent with the hypothesis of a cascade of autophosphorylation reactions on receptor sites with different regulatory functions (White *et al.*, 1988), inhibition of the insulin effect could be due to antibody blockade of the first autophosphorylation sites necessary for further activation. In contrast, incomplete saturation of these sites could facilitate further activation by locking the receptor in a more active conformation. Another possibility is that anti-phosphotyrosine antibodies exhibit different affinities for the phosphorylated sites. According to this hypothesis, binding of antibodies to distinct autophosphorylation sites would vary with the antibody concentration and result in different effects on kinase activity depending on the particular phosphotyrosine residues involved. This is further supported by the observation that interaction of our anti-phosphotyrosine

antibody with other synthetic insulin receptor peptides that have been phosphorylated varies with the peptide used (V. Baron, N. Gautier & J. Dolais-Kitabgi, unpublished work).

We also found that an antibody directed against a highly-conserved domain of the β -subunit (anti-P2) was capable of activating the insulin receptor tyrosine kinase activity towards exogenous substrates without significant modification of receptor autophosphorylation. This characteristic effect of the anti-peptide was observed both in the absence and the presence of insulin. The effects of anti-peptide and insulin on the enzymic activity are different, since the stimulating hormonal effect generally correlates with receptor autophosphorylation. Moreover, as stimulation by insulin and by anti-P2 are additive, we may conclude that they probably act through different mechanisms.

Antibodies directed against the insulin receptor have been reported to modulate the receptor kinase (Zick *et al.*, 1984; Herrera *et al.*, 1985, 1986; Morgan & Roth, 1987; Ponzio *et al.*, 1987), but the effects of our anti-peptide appear to be different from those previously published. Thus, anti-P2 is capable of regulating the receptor kinase activity without similar modification of the receptor phosphorylation, adding further weight to the idea that regulation of receptor phosphorylation and of substrate phosphorylation might be different.

The mechanism by which the anti-peptide antibody exerts its effects is unknown. One explanation may be that our antibody acts by inducing conformational changes in the kinase domains that facilitate association with exogenous substrates. Thus, a better interaction between the substrate-binding region of the β -subunit and the substrate itself would result in an increased phosphorylation of exogenous substrate. This is in agreement with the hypothesis that phosphorylation-induced conformational changes are involved in activation of the kinase towards exogenous substrates. Another effect of the anti-peptide could be to maintain the receptor in a state that is particularly active for phosphorylation of exogenous substrates. According to this hypothesis, the antibody would lock the receptor in a conformation that activates the receptor-catalysed transfer of phosphate to the substrate. The nature of these conformational changes is a matter of speculation. They could occur at the level of single β -subunits; alternatively, they could involve cross-linking of β -subunits within one $\alpha_2\beta_2$ receptor monomer, or cross-linking of β -subunits of adjacent receptors.

To our knowledge, the particular receptor domain corresponding to P2 has not previously been investigated, and the specific properties of our anti-peptide antibody may be unique to the region studied. This region represents a domain which is highly conserved in proteins that possess tyrosine kinase activity, and which is likely to play a crucial role in regulation of the enzymic activity. It will be of interest to investigate the effect of the antibody on other receptors with tyrosine kinase activity.

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