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# Insulin and rabbit anti-insulin receptor antibodies stimulate additively the intrinsic receptor kinase activity

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**This paper describes the properties of rabbit polyclonal antibodies directed against purified human insulin receptor which strongly stimulate the intrinsic tyrosine kinase activity. The stimulatory effect of the antibodies on the kinase activity was obtained on the insulin receptor autophosphorylation as well as on the kinase activity towards a synthetic substrate. This stimulation is additive to that induced by insulin. Moreover, rabbit antibodies do not impair insulin binding. These data strongly suggest that antibodies and insulin act through separate pathways. This conclusion is reinforced by the differences observed on the phosphopeptide maps of the receptor's β subunit whose phosphorylation was performed either in the presence of insulin or rabbit antibodies. Interestingly, these polyclonal antibodies can also induce an activation of the receptor autophosphorylation by interacting only with extracellular determinants. The anti-insulin receptor antibodies mimic insulin in their stimulatory effect on amino acid (AIB) uptake, but they have a different effect to that found on the kinase activity; the simultaneous addition of the antiserum and insulin failed to stimulate this amino acid transport over the level induced by a saturating concentration of hormone.**

*Key words:* polyclonal antibodies/tyrosine kinase stimulation/insulin receptor/phosphopeptide mapping/phosphorylation stimulation

## Introduction

Insulin initiates diverse biological responses after its interaction with a specific receptor protein (Kahn *et al.*, 1981). This receptor protein inserted in the plasma membrane is composed of two glycopolypeptides: the α subunit ($M_r$ = 130 000-135 000) and the β subunit ($M_r$ = 94 000-97 000) linked together via disulfide bonds (Jacobs *et al.*, 1983). Recently, it has been demonstrated that the binding of insulin activates an intrinsic tyrosine kinase activity resulting in the autophosphorylation of the receptor on the β subunit (Kasuga *et al.*, 1982; Van Obberghen and Kowalski, 1982; Roth *et al.*, 1983; Shia and Pilch, 1983; Van Obberghen *et al.*, 1983). It has been proposed that this enzymatic activity, which phosphorylates the β subunit of the receptor as well as exogeneous substrates (Gazzano *et al.*, 1983; Braun *et al.*, 1984; Stadtmauer and Rosen, 1985), is implicated in the mediation of insulin biological effects. This hypothesis is supported by the fact that agents such as phorbol esters which antagonize the effects of the hormone on glycogen synthase (Takayama *et al.*, 1984) similarly decrease the level of the insulin-stimulated tyrosine kinase activity of the receptor. Conversely, lectins (Roth *et al.*, 1983) and trypsin (Tamura *et al.*, 1983) which mimic insulin's effects stimulate at the same time the intrinsic tyrosine kinase activity. Moreover, in certain physiological situations such as severe insulin-resistant states in humans (Grigorescu *et al.*, 1984; Grunberger *et al.*, 1984) or diabetes (Burant *et al.*, 1986) and obesity (Le Marchand-Brustel *et al.*, 1985) in animal models, insulin resistance has been described to parallel a decrease in the tyrosine kinase activity.

Nevertheless, the putative role of tyrosine kinase activity in the mediation of insulin action is questioned by recent reports stating that some polyclonal anti-receptor antisera from patients which have been demonstrated to mimic insulin biological effects have no effect on the autophosphorylation level of the insulin receptor (Simpson and Hedo, 1984; Zick *et al.*, 1984). These data highlight the importance of antibodies as tools to define further the role that receptor kinase might play in expressing biological effects of insulin. Most of the polyclonal antibodies directed against insulin receptor described so far have been obtained from patients. Problems of interpretation may arise as these sera may also contain antibodies directed against several proteins which may interfere in the expression of insulinic biological effects. Furthermore, among the multiple monoclonal antibodies directed against insulin receptor, none has so far been described to stimulate *per se* the tyrosine kinase activity. In the present paper we describe a rabbit anti-insulin receptor antiserum obtained under defined conditions by injecting a rabbit with a highly purified insulin receptor preparation. This antiserum strongly stimulates the receptor autophosphorylation as well as the tyrosine kinase activity in an additive fashion with insulin, suggesting that this antibody acts via its own mechanistic pathway.

## Results

### *Immunoreactivity of the rabbit anti-insulin receptor antiserum*

Polyclonal antibodies were obtained by immunizing a rabbit with a highly purified insulin receptor fraction (insulin binding capacity: 3 nmol/mg of protein) following the procedure described in Materials and methods. The immunospecificity of our rabbit anti-insulin receptor (RAIR) antiserum was first tested on its ability to recognize intact insulin receptors. In these experiments we used receptor molecules labeled either on the α subunit using the photoreactive probe [$^{125}$I]NAPA insulin (Figure 1 lanes A and B), or on the phosphorylation sites carried by the β subunit using [γ-$^{32}$P]ATP (Figure 1 lanes C-F). In both cases RAIR antibodies immunoprecipitated the labeled receptor molecules indicating that they interact with a native form of the insulin receptor where binding capacity and tyrosine kinase were fully active. The specificity of the polyclonal antiserum towards the insulin receptor was demonstrated in experiments where the antiserum was incubated with a bulk of glycoproteins previously solubilized from [$^{35}$S]methionine-labeled IM9 cells and partially purified on agarose-bound wheat germ agglutinin (WGA). In these conditions only two polypeptides were immunoprecipitated, as shown in Figure 1 lanes G and H, which corresponded respectively to the α subunit ($M_r$ = 130 kd) and the β subunit ($M_r$ =

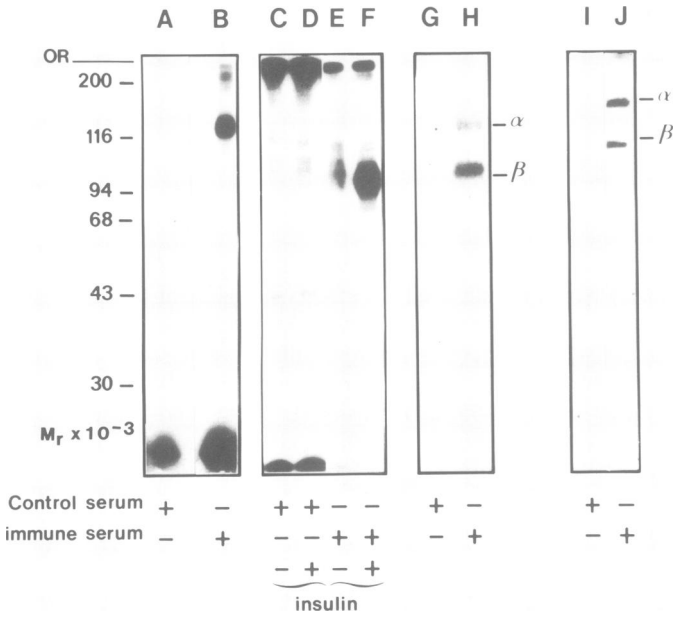


**Fig. 1.** Characterization of RAIR antiserum specificity. **First box:** immunoprecipitation of insulin receptor labeled on the α subunit. Solubilized receptor from IM9 cells **(lanes A,B)** was labeled with [$^{125}$I]$B_{29}$-NAPA insulin and immunoprecipitated with a 1/50 dilution of non immune serum **(lane A)** or RAIR antiserum **(lane B). Second box:** immunoprecipitation of insulin receptor labeled on the β subunit **(lanes C–F).** Solubilized receptor from IM9 cells was incubated in the absence **(lanes C,E)** and presence **(lanes D,F)** of 100 nM insulin before addition of [γ-$^{32}$P]ATP (15 mM), $MnCl_2$ (4 mM), $MgCl_2$ (8 mM). Receptor molecules were immunoprecipitated afterwards using a 1/50 dilution of non immune serum **(lanes C,D)** or RAIR antiserum **(lanes E,F). Third box:** immunoprecipitation of [$^{35}$S]methionine-labeled glycoproteins **(lanes G,H).** Cells were incubated with [$^{35}$S]methionine for 14 h as described in Materials and methods before solubilization using 1% Triton X-100. Labeled proteins were prepurified on wheat germ agglutinin agarose prior to incubation overnight with a 1/50 dilution of non-immune serum **(lane G)** or RAIR antiserum **(lane H). Fourth box:** immunoblotting. Highly purified insulin receptor (1 μg) was subjected to SDS–PAGE and the polypeptides transferred to nitrocellulose. The replicate was incubated with a 1/50 dilution of non-immune serum **(lane I)** and RAIR antiserum **(lane J)** before immunodetection using anti-rabbit IgG coupled to peroxidase.

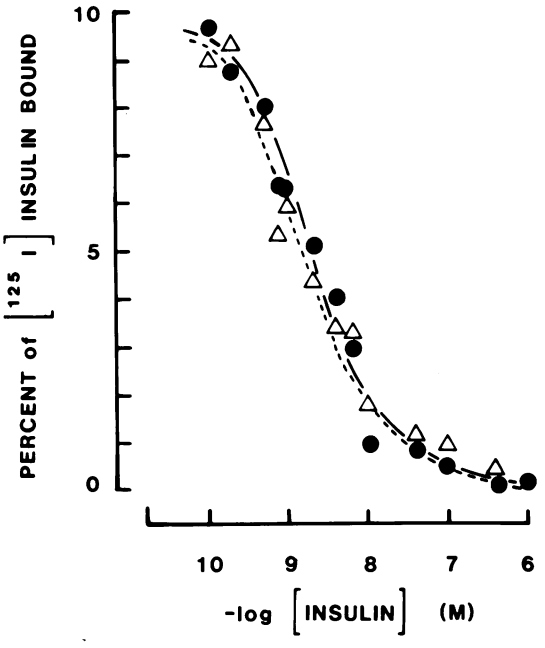


**Fig. 2.** Insulin binding on IM9 cells in the presence or absence of RAIR antiserum. IM9 cells ( 6 × $10^5$) were incubated with a 1/100 dilution of non-immune serum (●) or RAIR antiserum (Δ) for 20 min at 37°C. Binding experiments were initiated by the simultaneous addition of [$^{125}$I]insulin ($10^5$ c.p.m.) and the indicated concentration of unlabeled hormone. The results are expressed as the percent of hormone specifically bound to the cell surface.

97 kd) of the insulin receptor. To characterize further our polyclonal anti-receptor antiserum we used an immunoblotting technique. As shown in Figure 1 lanes I and J, RAIR antiserum at a 1/150 dilution reacted with both the α and β subunits of a highly purified insulin receptor fraction, previously separated by SDS–PAGE and blotted on nitrocellulose. This result indicates that RAIR antibodies can also recognize the denatured form of the receptor polypeptide components.

*Lack of effect of RAIR antiserum on insulin binding*

[$^{125}$I]insulin binding has been measured on intact IM9 cells or partially purified solubilized receptor from the same cell line. Intact cells or solubilized receptor were respectively preincubated for 30 min at 37°C and 14 h at 4°C with RAIR antiserum (1/100) or an equivalent amount of non-immune serum prior to addition of [$^{125}$I]insulin. As shown in Figure 2 no difference in the insulin-binding parameters was observed when intact IM9 cells were incubated with a 1/100 dilution of RAIR antiserum compared to an equivalent amount of non-immune serum. These data demonstrate that RAIR antibodies do not impair insulin binding on living cells.

The titer of antibodies raised against insulin receptor was estimated by measuring the percentage of immunoprecipitation of a constant amount of partially purified insulin receptor covalently labeled with [$^{125}$I]NAPA insulin at various concentrations of purified RAIR immunoglobulins. The dose–response presented in Table I shows that almost 100% of a partially purified preparation of the receptor solubilized from human lymphocytes was precipitated at a concentration of 140 μg/ml of RAIR IgG. Binding experiments performed at the same concentration of IgG showed that only 15% of the binding capacity was decreased, demonstrating that RAIR antibodies did not significantly modify the interaction of the hormone with the solubilized receptor.

**Table I.** Dose-dependent effect of RAIR antiserum on the immunoprecipitation efficiency and on the insulin binding measured to solubilized human lymphocyte receptor

| RAIR IgG concentration (μg/ml) | Per cent of insulin receptor immunoprecipitated[a] | Per cent of insulin binding inhibition[b] |
|---|---|---|
| 7 | 12.5 | 0 |
| 14 | 26 | 5 |
| 24 | 31 | 9 |
| 70 | 85 | 13 |
| 140 | 100 | 15 |

[a]Values were calculated by counting after PAGE analysis the percent of radioactivity incorporated in the α subunit labeled with [$^{125}$I]NAPA insulin after immunoprecipitation.
[b]Values were calculated by substracting the contribution of equivalent amounts of control serum to the insulin-binding inhibition obtained at the indicated antiserum dilutions.

*Antiserum induces tyrosine kinase stimulation on intact cells*

We wished to know whether RAIR antiserum could trigger intrinsic tyrosine kinase stimulation when it was applied at the external face of living cells. Experiments were carried out on intact

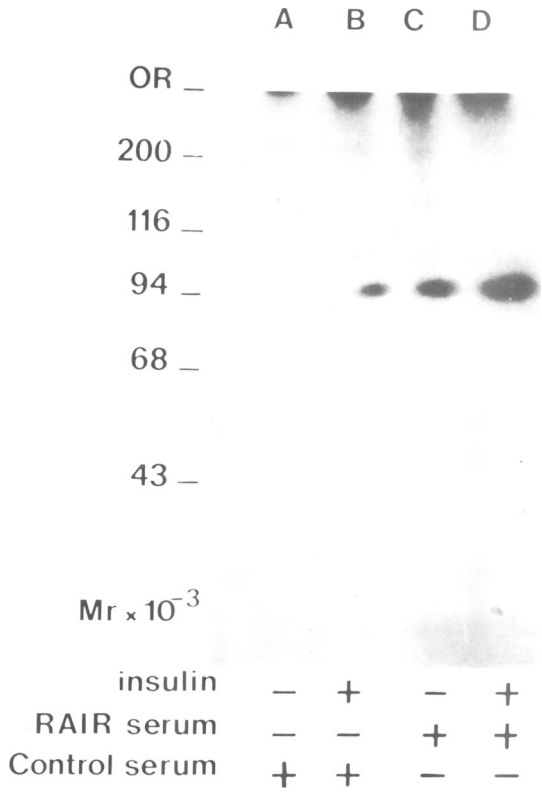


**Fig. 3.** Stimulation of receptor autophosphorylation by RAIR antiserum on intact IM9 cells. IM9 cells were incubated with [$^{32}$P]inorganic phosphate (8 mCi) for 2 h. Samples containing 2 × $10^8$ cells were incubated with: 1/100 dilution of non-immune serum **(lane A)**; 100 nM insulin **(lane B)**; 1/100 dilution of RAIR antiserum **(lane C)**; a mixture of 100 nM insulin and RAIR antibodies (1/100) **(lane D)** for 20 min at 37°C. The insulin receptors were then solubilized with 1% Triton X-100, partially purified on wheat germ agglutinin agarose and submitted to immunoprecipitation with an excess of RAIR antibodies. The immunoprecipitated polypeptides were separated by SDS−PAGE and the β subunit localized by autoradiography.

**Table II.** Dose-dependent stimulation of the intrinsic kinase activity by RAIR antibodies

| IgG concentration (μg/ml) | Radioactivity incorporated into polyglutamate/tyrosine (nmol/30 min/mg)[a] | |
|---|---|---|
| | RAIR IgG | Control IgG |
| 7 | 0.54 | 0.37 |
| 21 | 0.7 | 0.36 |
| 70 | 0.99 | 0.34 |
| 140 | 1.02 | 0.37 |

[a]Solubilized insulin receptor from IM9 cells was incubated with various IgG concentrations before being tested for kinase activity towards an exogenous substrate.

IM9 cells whose ATP pool had been previously labeled by a 2-h incubation with $^{32}$P-labeled inorganic phosphate. Cells were then exposed to either 100 nM insulin or a 1/100 dilution of RAIR antiserum or a mixture of both. As shown in Figure 3 lane C, the phosphorylation level of the β subunit of the insulin receptor extracted from cells previously incubated with RAIR antiserum was clearly stimulated compared to the same sample incubated with control serum (Figure 3 lane A). A similar extent of stimulation was obtained when cells were incubated with 100 nM insulin (Figure 3 lane B). When insulin (100 nM) and RAIR antiserum (1/100) were added at the same time to the cell suspension (Figure 3 lane D) the incorporation of $^{32}$P in the β subunit reached a value higher than that obtained in the presence of either the two effectors applied separately.

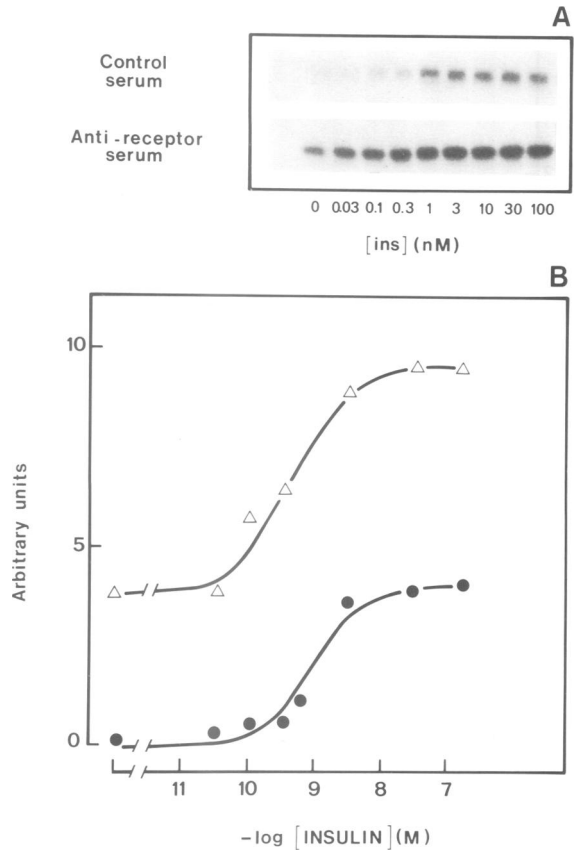


**Fig. 4.** Stimulation of the solubilized insulin receptor autophosphorylation by insulin and RAIR antibodies. Solubilized receptor from IM9 cells was incubated overnight at 4°C with a 1/100 dilution of RAIR antiserum or control serum prior to addition of various insulin concentrations for 3 h at 15°C. The phosphorylation was initiated by addition of 15 μM [γ-$^{32}$P]ATP, 4 mM $MnCl_2$, 8 mM $MgCl_2$ pH 7.6 and stopped after 15 min at 15°C by addition of 100 mM NaF, 20 mM EDTA. Immunoprecipitated polypeptides were separated on polyacrylamide gel and autoradiographed. **(A)** Autoradiograms of the β subunit of insulin receptor phosphorylated in the presence of various concentrations of insulin and a 1/100 dilution of control serum (upper row) or RAIR antiserum (lower row). **(B)** Densitometric analysis of the autoradiograms presented above; control serum (●), RAIR antiserum (Δ).

*RAIR antiserum stimulates the intrinsic tyrosine kinase activity on solubilized receptor*

To characterize further the mode of action of RAIR antibodies at the molecular level we have tested the ability of RAIR immunoglobulins to stimulate the intrinsic receptor kinase activity on a solubilized receptor fraction.

As shown in Table II, in the absence of insulin, RAIR immunoglobulins stimulated *per se* the intrinsic tyrosine kinase activity. This stimulation was dose dependent, the maximal effect being reached at a final concentration of immunoglobulin of 70 μg/ml.

Moreover, when various insulin concentrations were added to a receptor fraction previously incubated with RAIR immunoglobulins (70 μg/ml) the autophosphorylation level of the β subunit was clearly overstimulated compared to that obtained in the presence of insulin alone (Figure 4A). The densitometric scanning analysis of the autoradiograms indicates that no major change in the apparent affinity for insulin occurred when the receptor was complexed with RAIR antibodies (Figure 4B).

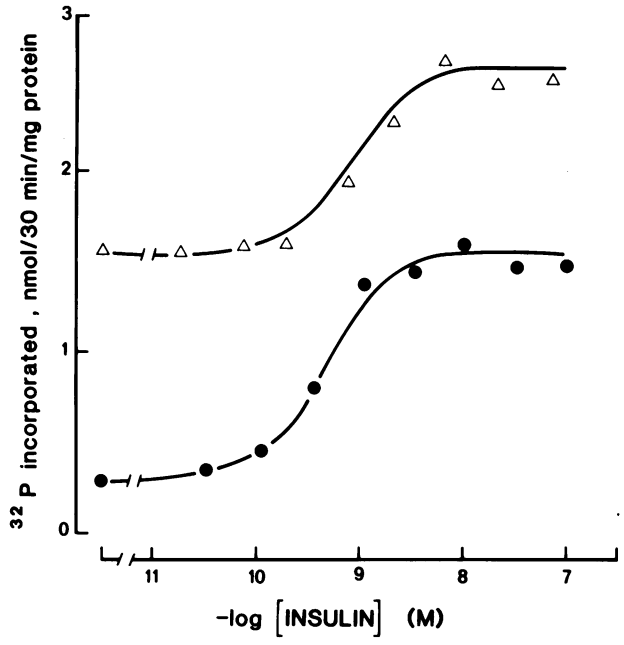


**Fig. 5.** Additive effects of insulin and RAIR antibodies on the tyrosine kinase activity towards a synthetic substrate. Wheat germ eluates derived from IM9 cells were incubated overnight at 4°C with a 1/100 dilution of control serum (●) or RAIR antiserum (Δ) prior to addition of various concentrations of insulin for 3 h at 15°C. Samples were then tested for their ability to transfer the terminal phosphate group of [$\gamma$-$^{32}$P]ATP to polyglutamate/tyrosine (4:1). The phosphorylation reaction was performed in 0.18 ml of a medium containing 50 mM Hepes, 4 mM $MnCl_2$, 8 mM $MgCl_2$, 20 $\mu$M [$\gamma$-$^{32}$P]ATP (0.25 Ci/mmol), 0.25 mg/ml polyglutamate/tyrosine. The reaction was stopped by TCA precipitation of an aliquot fraction blotted on filter paper.

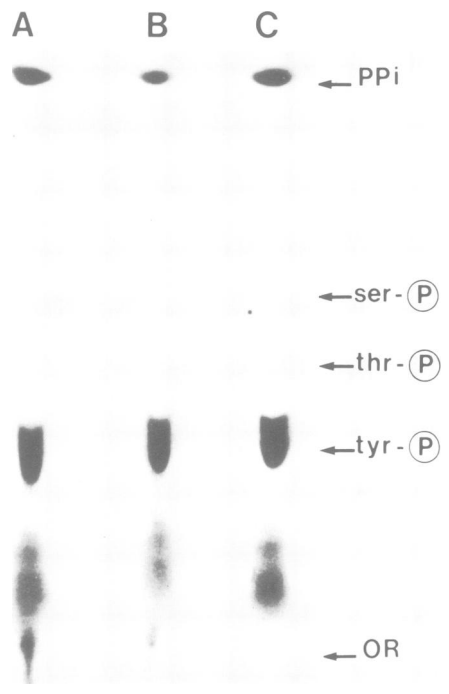


**Fig. 6.** Phosphoamino acid analysis of insulin receptor phosphorylated under stimulation by insulin or RAIR antibodies. Insulin receptor partially purified on wheat germ agglutinin agarose was phosphorylated as described in Materials and methods after incubation with: 100 nM insulin **(lane A)**, a 1/100 dilution RAIR antiserum **(lane B)**, a mixture of insulin and RAIR antibodies at the same concentrations as indicated above **(lane C)**. Immunoprecipitated polypeptides were separated by SDS–PAGE, the $\beta$ subunit localized by autoradiography, excised and eluted from the gel. After hydrolysis in 6 N HCl for 2 h at 110°C, the samples were analyzed by electrophoresis at pH 3.5, 500 V for 2 h. The same amount of radioactivity was applied on the plate for the three conditions.

When the kinase activity was followed by measuring the phosphorylation of a glutamate and tyrosine containing copolymer (Figure 5) we observed the same kind of phenomena as described above: (i) insulin and RAIR antibodies stimulate additively the tyrosine kinase activity of the insulin receptor; (ii) the increment of tyrosine activity promoted by RAIR antibodies was identical to that obtained in the sole presence of a saturating concentration of insulin (100 nM); (iii) the apparent affinity for insulin is not modified by interaction of RAIR antibodies on the receptor molecules.

To rule out the possibility of a contaminating tyrosine kinase activity, we checked that RAIR antiserum in the absence of insulin receptor was devoid of any phosphorylating effect on polyglutamate/tyrosine. These data (not shown) strongly suggest that the tyrosine kinase stimulation is due to a direct interaction of antibodies with the insulin receptor molecules.

*Phosphoamino acid determination*

Solubilized insulin receptor is mainly phosphorylated on tyrosine residues when exposed to insulin (Kasuga *et al.*, 1982; Gazzano *et al.*, 1983). We investigated whether the nature of the phosphoamino acids involved during the autophosphorylation induced by RAIR antibodies was the same as that observed under insulin stimulation. The data presented in Figure 6 clearly indicate that in the presence of either RAIR antiserum (1/100) (lane B) or 100 nM insulin (lane A) or both (lane C) the insulin receptor was only phosphorylated on tyrosine residues.

*Phosphopeptide mapping*

To gain information on the nature of the phosphorylation sites involved during the tyrosine kinase stimulation specifically induced by RAIR antibodies, we carried out a two-dimensional separation of the phosphopeptides obtained after extensive proteolysis of the phosphorylated $\beta$ subunit (Figure 7). In these experiments receptor molecules were previously phosphorylated in the presence of RAIR antibodies (panel B), insulin (panel C) or addition of both (panel D).

Although most of the phosphopeptides were common among the three patterns, two polypeptides, namely $I_1$ and $I_2$, were only present on the two-dimensional pattern obtained when the receptor phosphorylation was performed in the presence of insulin. On the other hand, three other spots $R_1$, $R_2$ and $R_3$, were only visible on the fingerprint pattern of the receptor phosphorylated under stimulation by RAIR antibodies. Insulin receptor molecules incubated in the simultaneous presence of insulin and RAIR antiserum exhibited a peptide map that was a combination of the phosphopeptides obtained from receptor molecules treated separately with insulin and RAIR antibodies.

*Effect of anti-receptor antibodies on amino acid uptake*

Most of the biochemical characterization of our rabbit antiserum has been performed on IM9 extracts, however this cell line did not respond to insulin for any of the biological effects we have tested. For this reason we chose to investigate a putative effect of RAIR antibodies on a human promyelocyte cell line (HL60), and on isolated rat hepatocytes. The data presented in Table III show that RAIR antiserum could mimic insulin in its ability to stimulate amino-isobutyric acid (AIB) uptake in both systems. Nevertheless, incubation of both types of cell in the simultaneous

**Table III.** Comparative effects of RAIR antiserum on AIB uptake measured on rat hepatocytes and human promyelocytes and on tyrosine kinase activity measured on WGA extracts from the same species

| | Phosphate incorporation using poly(Glu-Tyr) as substrate (pmol/min/mg) | AIB uptake (nmol/min/mg) |
|---|---|---|
| | Solubilized human receptor (IM9 cells) | Human promyelocytes (HL60 cells) |
| Control serum (dilution 1:50) | 6.6 | 0.44 ± 0.006 |
| Control serum + 100 nM insulin | 53.3 | 1.35 ± 0.009 |
| RAIR antiserum (dilution 1:50) | 50.3 | 1.22 ± 0.11 |
| RAIR antiserum + 100 nM insulin | 91.5 | 1.24 ± 0.13 |
| | Solubilized rat receptor (liver) | Isolated rat hepatocytes |
| Control serum (1:20 dilution) | 1.55 | 0.07 ± 0.006 |
| Control serum + 100 nM insulin | 5.2 | 0.12 ± 0.011 |
| RAIR antiserum (1:20 dilution) | 3.13 | 0.105 ± 0.01 |
| RAIR antiserum + 100 nM insulin | 7.8 | 0.117 ± 0.009 |

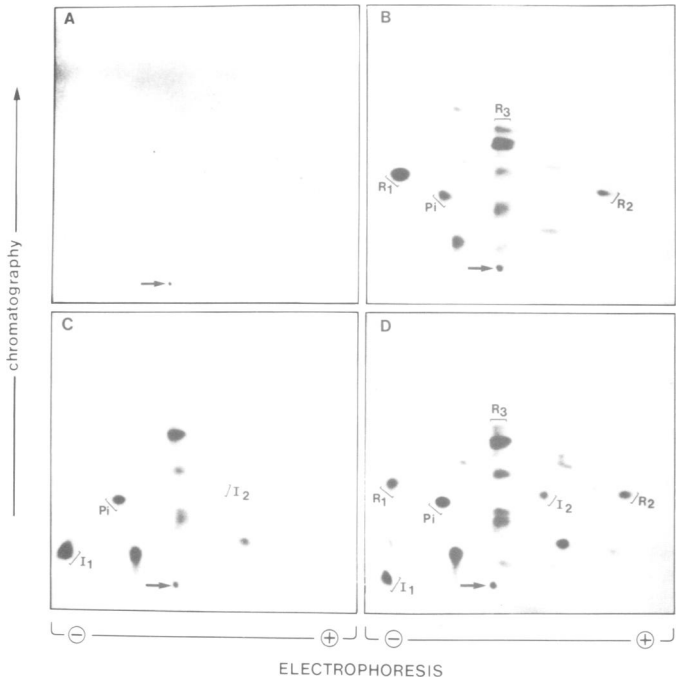


**Fig. 7.** Two-dimensional phosphopeptide mapping of the β subunit of insulin receptors phosphorylated under stimulation by insulin or RAIR antiserum. Solubilized insulin receptor was autophosphorylated in the presence of: control serum (1/100) **(A)**; RAIR antiserum (1/100) **(B)**; 100 nM insulin **(C)**; RAIR antiserum and 100 nM insulin **(D)**. The receptor was immunoprecipitated with an excess of RAIR antiserum. The polypeptides were separated by SDS–PAGE, the β subunit localized by autoradiography, excised and incubated with 0.1 mg/ml of TPCK-treated trypsin for 48 h at 37°C. Phosphopeptides were separated on cellulose thin-layer plates by electrophoresis (pH 1.9, 500 V for 30 min) and ascending chromatography.

presence of anti-insulin receptor antibodies and insulin, failed to stimulate the AIB uptake over the level induced by a saturating concentration of the hormone. Conversely we obtained under the same conditions a neat cumulative effect of the two effectors on the kinase activity in intact cells (Figure 3) as well as in acellular systems (Table III).

## Discussion

Although the intrinsic tyrosine kinase activity is likely to be involved in the expression of insulin's biological effects, no direct proof has been given yet that it is a necessary step in the mechanism of action of the hormone. Antisera raised against insulin receptor which have been very useful in studying the structure (Jacobs and Cautrecasas, 1981) and biosynthesis of the receptor (Van Obberghen *et al.*, 1981; Hedo *et al.*, 1983; Jacobs *et al.*, 1983), as well as the insulin-dependent activation of the tyrosine kinase activity (Kasuga *et al.*, 1982; Van Obberghen and Kowalski, 1982) appear also to be excellent probes with which to eluciddate the putative role of this enzymatic activity in insulin action. We present here the characterization of a rabbit polyclonal antiserum raised against purified insulin receptor which acts as a powerful activator of the tyrosine kinase activity. We found that RAIR antibodies specifically recognized not only the two subunits of the receptor on their active form but also their denatured form when transferred to nitrocellulose.

When tested on living cells RAIR antibodies stimulate the autophosphorylation level of the insulin receptor's β subunit. This effect is additive to that obtained in the presence of insulin (Figure 3). Several of the polyclonal antibodies described so far (Simpson and Hedo, 1984; Zick *et al.*, 1984), as well as lectins (Roth and Cassell, 1983) have been reported to activate the intrinsic tyrosine kinase activity, however, their stimulatory effect most probably implies a direct interaction with the insulin-bindign site (Katzen *et al.*, 1981; Kasuga *et al.*, 1983) RAIR antibodies differ from lectins and other antibodies by their ability to mimic insulin action on tyrosine kinase activity through interactions with antigenic sites distinct from the insulin-binding domain. The only antibody which presents similarities with RAIR antibody is a rabbit polyclonal antiserum raised against insulin receptor which promotes biological effects without affecting insulin binding (Jacobs and Cuatrecasas, 1978). However no information has been reported on the effects of this antiserum on the tyrosine kinase activity.

To characterize further the RAIR effect at the molecular level we investigated the effect of these antibodies to human receptor solubilized from IM9 cells. In this system polyclonal rabbit antibodies were able to induce by themselves the same extent of stimulation of receptor autophosphorylation as that produced by a saturating insulin concentration. Besides, the receptor kinase activity towards a synthetic substrate was also increased under the same conditions. In both cases, the stimulatory effects of antibodies and insulin were completely additive, suggesting the two effectors act through separate mechanistic pathways. This hypothesis is supported by: (i) the lack of competition between

RAIR antibodies and insulin at the hormone-binding site; and (ii) the differences observed on the phosphopeptide patterns obtained after extensive proteolysis of the solubilized receptor phosphorylated under insulin stimulation versus that obtained under RAIR antiserum stimulation. However, the nature of the amino acids phosphorylated under stimulation by RAIR antibodies is the same as that observed under insulin stimulation, e.g. tyrosine residues.

At this point three hypotheses, exclusive of a direct interaction of RAIR antibodies with insulin receptors, could account for the stimulatory effect of RAIR antibodies on tyrosine kinase activity: (i) opposite to non-immune serum, anti-insulin receptor antiserum or the corresponding immunoglobulin fraction contains a contaminating tyrosine kinase activity; (ii) RAIR antibodies inhibit a protein phosphatase present in the receptor fraction; (iii) RAIR antibodies interact with IGF receptors and stimulate specifically the tyrosine kinase activity of these receptors. First, we have found that RAIR antiserum was devoid of any contaminating tyrosine kinase activity. Second, if one considers that RAIR antibodies increase the autophosphorylation level through inhibition of a protein phosphatase there is no reason to expect a modification of the phosphopeptide map of the insulin receptor phosphorylated in the presence of insulin versus that obtained under RAIR stimulation. Third, we checked that IGF at 1 nM was unable to stimulate any phosphate incorporation in the region of 95 kd using a lectin purified insulin receptor fraction from IM9 cells.

In the view of these data, RAIR antiserum most probably acts by stabilizing a conformation or an aggregation state where the intrinsic kinase activity of the insulin receptor is dramatically increased compared to the basal activity expressed on the free receptor molecules.

Since the recent elucidation of the amino acid sequence of the insulin receptor (Ullrich *et al.*, 1986; Ebina *et al.*, 1985b) it has been proposed that the $\alpha$ subunit protrudes at the extracellular side of the membrane, while the $\beta$ subunit is mainly located at the intracellular side. In this view, RAIR immunoglobulins which trigger the tyrosine kinase activity by interacting with extracellular epitopes of the insulin receptor, as distinct from the insulin-binding domain, appear to be excellent tools to understand better the mechanism by which the hormonal signal is transduced from the $\alpha$ to the $\beta$ subunit.

Since RAIR antiserum is the first antibody, to our knowledge, reported to stimulate the insulin receptor autophosphorylation when incubated with living cells, we wished to know whether it could mimic the hormone for biological effects. We found that even if it activates the tyrosine kinase activity through its own pathway our anti-insulin receptor antiserum can mimic insulin for the stimulation of AIB uptake on human HL60 cells or rat hepatocytes. However, the overstimulation we observed on the tyrosine kinase activity when RAIR antiserum and insulin were added simultaneously to intact cells or lectin purified receptor was not visible on AIB uptake under the same conditions. These data suggest that in the cascade of events leading to the insulin-controlled increase of AIB uptake, the tyrosine kinase activity level is not the limiting step. A similar result has been reported by Ebina *et al.* (1985a) who claimed that in transfected CHO cells expressing 100-fold more receptors than wild-type cells there was no visible increase in glucose uptake but a shift in insulin response. In contrast, in adipose cells from young, obese Zucker rats the increase of tyrosine kinase activity per mole of receptor (Debant *et al.*, 1986) parallels a 2-fold level of glucose transport as compared to lean animals (Guerre-Millo *et al.*, 1985).

In conclusion, RAIR antiserum is a powerful mimic of insulin for the stimulation it induces on the intrinsic kinase activity as well as AIB uptake. Interestingly it acts on the kinase activity without competing for insulin and without altering the proper stimulatory effect of the hormone. These features imply that our polyclonal antiserum may be a promising tool to investigate the putative role that tyrosine kinase activity might play in the mechanism of insulin action.

## Materials and methods

*Chemicals*

The photoreactive insulin analog $B_{29}$ (2-nitro, 4-azido phenylacetyl)des-Phe insulin (NAPA insulin) was kindly supplied by Dr Brandenburg. This reagent was iodinated in the dark to a specific activity of 200–250 $\mu$Ci/$\mu$g using a modification of the chloramine T method (Freychet, 1976). Porcine monocomponent insulin was a gift from Novo Research Institute, Copenhagen, Denmark. $Na^{125}I$, [$\gamma$-$^{32}$P]ATP, [$^{32}$P]inorganic phosphate, [$^{35}$S]methionine were purchased from the Radiochemical Centre, Amersham, UK. Bovine serum albumin, bovine insulin, bacitracin, phenylmethylsulphonyl fluoride (PMSF), *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (Hepes), wheat germ agglutin agarose (WGA) and polyglutamate/tyrosine (4:1) were from Sigma, St. Louis, MO, USA. RPMI medium and calf serum were obtained from Boehringer, Mannheim, FRG. Sephacryl S-400 and cyanogen promide-treated Sepharose were purchased from Pharmacia, Uppsala, Sweden. All other reagents were of the best grade commercially available.

*Receptor purification*

Human placenta membranes were prepared as described by Harrison and Itin (1981) and solubilized with 1.5% Triton X-100 overnight at 4°C, prior to centrifugation at 100 000 *g* for 60 min. The supernatant was loaded on Sephacryl S-400 (70 cm × 5 cm) and eluted with 50 mM Tris, 0.1% Triton X-100, 100 $\mu$M PMSF, pH 7.5. The head fractions containing insulin receptor were collected and tested for insulin binding. At this step the binding capacity was stable for 2 weeks. The pooled fractions were then passed through an insulin-bound Sepharose column synthesized according to Cuatrecasas (1972) and recycled overnight at 4°C. The column was extensively washed with 50 mM Tris, 1 M NaCl, 0.1% Triton X-100 pH 7.6 and elution carried out with 50 mM Na acetate, 0.1% Triton X-100, 1 M NaCl pH 5.0. Fractions were neutralized and tested for insulin binding. The insulin-binding fractions were pooled and dialyzed overnight against 50 mM Tris, 0.1% Triton X-100 pH 7.5. In order to concentrate the purified insulin receptor the dialysate was passed through a 1 ml DEAE Trisacryl column and eluted with 300 mM ammonium acetate pH 6.3. The peak protein was collected and the pooled fractions frozen in liquid nitrogen before storage at −80°C for several months.

Insulin receptor was extracted from IM9 cells and partially purified as previously described (Van Obberghen *et al.*, 1981).

*Production of polyclonal anti-insulin receptor antibodies*

A rabbit was injected with 20 $\mu$g of purified receptor in the lymph node. The rabbit was boosted with the same amount of receptor three times at 20, 27 and 28 days respectively, following the first injection. Bleedings were collected after 7–10 days following the last boost injection and every week for 1 month as described in detail by Louvard *et al.* (1982).

*IgG purification*

IgG were isolated from rabbit serum with the aid of caprylic acid according to the technique described by Steinbuch and Audran (1969).

*Cell culture*

Human lymphocytes were grown in RPMI containing 10% fetal calf serum supplemented with 2 mM glutamine and 50 U/ml penicillin and 50 $\mu$g/ml streptomycin. Rat hepatocytes were isolated following the procedure previously described by Le Cam *et al.* (1976).

HL60 cells were grown in RPMI 1640 medium supplemented in 10 mM Hepes, 2 mM glutamine, 20% fetal calf serum, penicillin (50 U/ml) and streptomycin (50 $\mu$g/ml) pH 7.3. Cells were divided every 3 days to maintain a density of 6–10 × $10^5$ cells/ml. Cell viability was greater than 95% as assessed by Trypan blue exclusion.

*Insulin binding*

IM9 cells (2 × $10^5$) were incubated with $^{125}$I-labeled insulin ($10^5$ c.p.m.) and various concentrations of the unlabeled hormone in 0.25 ml of 50 mM Hepes, 120 mM NaCl, 1.2 mM $MgSO_4$, 1 mM EDTA, 10 mM glucose, 15 mM Na acetate, 1% bovine serum albumin pH 7.6 for 3 h at 15°C. Ice cold medium (1 ml) was then added and the cells spun down. The supernatant was discarded, and the radioactivity associated to the cell pellet counted. Insulin-binding ex-

periments on solubilized receptor were carried out using the polyethylene glycol precipitation procedure as described by Desbuquois and Aurbach (1971).

*Amino acid transport*

Freshly isolated rat hepatocytes ($2 \times 10^6$ cells/ml) were incubated for 2 h with RAIR antiserum or non-immune serum (1/20 dilution) in Krebs–Ringer bicarbonate buffer pH 7.4 supplemented with 0.1% BSA in the absence or presence of insulin (100 nM).

The transport assay was initiated by the addition of a solution containing [$^{14}$C]AIB at a final concentration of 0.1 mM (0.20 $\mu$Ci). After 4 min at 37°C the cells were diluted 10-fold with cold PSB and washed twice before counting.

*Immunoprecipitation procedure*

Solubilized receptor was incubated overnight at 4°C with various dilutions of anti-receptor antisera or with equivalent dilutions of a non-immune serum. Pansorbin suspension (50 $\mu$l) was added and IgG allowed to interact for 30 min at 4°C. The immune complexes were sedimentated by centrifugation at 8000 *g* for 5 min and washed several times with 50 mM Hepes, 150 mM NaCl, 0.1% Triton X-100, pH 7.6.

*Affinity labeling of the insulin site*

Insulin-binding sites were specifically labeled as described by Le Marchand-Brustel *et al.* (1985). Typically, 10 $\mu$g of solubilized wheat germ agglutinin purified insulin receptor from IM9 cells were incubated in the presence of $10^6$ c.p.m. of [$^{125}$I]$B_{29}$ NAPA-insulin (1500 Ci/mmol) in 100 $\mu$l of a medium containing 50 mM Hepes, 150 mM NaCl, pH 7.6 for 60 min at room temperature, in the dark. The solution was then irradiated for 5 min at 4°C under a mercury lamp equipped with selective filters. Samples were finally submitted to the immunoprecipitation procedure.

*Tyrosine kinase activity and receptor autophosphorylation*

The specific effect of insulin or RAIR antibodies on the autophosphorylation level was measured by incubating partially purified receptor (20 $\mu$g) in a reaction mixture containing 50 mM Hepes, 150 mM NaCl pH 7.6 with various concentrations of insulin for 3 h at 15°C or with a 1/100 dilution of either rabbit anti-insulin receptor antiserum (RAIR) or non-immune serum at 4°C overnight. Thereafter the insulin receptor autophosphorylation was initiated adding: 15 $\mu$M [$\gamma$-$^{32}$P]ATP (2.5 Ci/mmol), 4 mM $MnCl_2$, 8 mM $MgCl_2$. After 30 min at 15°C, the reaction was stopped by adjusting the final concentration to 100 mM NaF and 20 mM EDTA. To immunoprecipitate insulin receptors, RAIR antiserum (1:50 dilution) was added to each sample and the IgG allowed to interact with insulin receptors overnight at 4°C. Insulin receptor IgG complexes were precipitated by addition of Pansorbin (50 $\mu$l). The immunoprecipitates were boiled for 5 min in a solution containing SDS (3%, w/v), glycerol (10% v/v), 10 mM Na phosphate, 3% 2-mercaptoethanol and 0.05% bromophenol blue and subjected to SDS–PAGE.

The tyrosine kinase activity towards an exogenous substrate was measured after solubilized insulin receptor was incubated with insulin or antibodies as described above. Incoporation of radioactivity in the synthetic substrate polyglutamate/tyrosine (4:1) was followed as previously described (Braun *et al.*, 1984).

When the additive effect of insulin and antibodies were tested, insulin receptors were first incubated overnight at 4°C with appropriate dilutions of immune or non-immune sera before addition of various concentrations of insulin. Incubations were pursued for 3 h at 15°C before intrinsic kinase activity was measured following incorporation of radioactivity in the receptor's $\beta$ subunit or in the synthetic substrate polyglutamate/tyrosine as described above.

*[$^{35}$S]methionine metabolic labeling of IM9 cells proteins*

IM9 cells were biosynthetically labeled according to Van Obberghen *et al.* (1981). Cells were washed and resuspended in 50 ml of methionine-free Eagle's MEM containing 10% dialyzed fetal calf serum, at a density of $20 \times 10^6$ cells/ml; protein labeling was started by addition of 2.5 mCi of [$^{35}$S]methionine to the cell suspension. After an incubation of 14 h at 37°C, cells were centrifuged at 600 *g* and washed twice in Eagle's MEM. Solubilization of membrane proteins was performed by maintaining the cellular suspension in 10 ml of 50 mM Hepes, 150 mM NaCl and 1% Triton X-100, 1 mM PMSF, 100 U/ml bacitracin and 1000 U/ml aprotinin under gentle stirring for 90 min at 4°C. The solubilized material was centrifuged at 100 000 *g* for 60 min at 4°C and the supernatant finally applied to a wheat germ agglutinin–Sepharose column. The column was extensively washed with a buffer containing 30 mM Hepes, 30 mM NaCl, 0.1% Triton X-100 pH 7.5. Glycoproteins were then eluted in the same buffer containing 0.5 M *N*-acetylglucosamine. This fraction, referred to as partially purified insulin receptor, was used for immunoprecipitation studies. Previous addition of anti-insulin receptor antibodies, the solubilizate was precleared by addition of 50 $\mu$l of Pansorbin suspension for 1 h at 4°C.

*Trypic fragments of phosphorylated insulin receptor*

Autophosphorylation of the insulin receptor was done as described above. $^{32}$P-labeled insulin receptor (200 $\mu$g of protein) was immunoprecipited and the subunits separated by PAGE. The $\beta$ subunit was localized by autoradiography, excised and incubated in 1 ml of 50 mM $NH_4HCO_3$ pH 8.0 containing 80 $\mu$g of p-tosylphenylalanine chloromethyl ketone (TPCK)-treated trypsin for 48 h at 37°C. Gel fragments were removed by centrifugation. Samples were lyophilized and residual $NH_4HCO_3$ removed by resuspending the residue in water before subjecting it to a second lyophilization. This cycle was repeated three times. Finally, peptides were taken up in 6 $\mu$l of 15 mM $NH_4OH$. Phosphopeptides were separated as described by Ellis *et al.* (1981). The first dimension was performed on cellulose-coated thin-layer plates using electrophoresis in 30% formic acid, pH 1.9, 500 V for 90 min at 4°C. Chromatography was performed in a second dimension using L-butanol/acetic acid/pyridine/water (60/12/40/48) (pH 3.5) for 6 h at room temperature. Two dimensional peptide fingerprints were then exposed to Kodak X-AR films at −80°C.

*Phosphorylation of the insulin receptor in intact IM9 cells*

IM9 cells were washed twice in a buffer containing 50 mM Hepes, 150 mM NaCl pH 7.6. Cells were resuspended in 8.5 ml of this buffer ($16 \times 10^6$ cells/ml) and incubated for 1 h at 37°C to clear the hormone-bound insulin-receptor molecules from the cell surface. Labeling of the intracellular ATP pool was carried out by addition of 8 mCi of [$^{32}$P]inorganic phosphate for 2 h at 37°C. Cells were then divided into four aliquots and incubated for 20 min at 37°C with either the rabbit antireceptor antiserum or the non-immune serum (1:100 dilution) in the absence or in the presence of 100 nM insulin. The cells were washed once at 4°C in 50 mM Hepes, 150 mM NaCl, plus protease inhibitors, pH 7.6 and resuspended in the same buffer prior to addition of 1% Triton X-100. Solubilization of insulin receptor was performed in that medium under gentle stirring for 90 min at 4°C. The solubilized material was then submitted to a 100 000 *g* centrifugation for 60 min and partially purified on a wheat germ agglutinin column as described above.

*Immunoblotting*

The immunoreactivity of the anti-receptor antiserum towards denatured subunits of the insulin receptor blotted on nitrocellulose was tested as described by Towbin *et al.* (1979). Typically, 1 $\mu$g of highly purified human insulin receptor was applied on SDS–PAGE and the subunits transferred (5 h, 50 V) to nitrocellulose. The replicate blotted on nitrocellulose was then incubated for 90 min at room temperature with either a non-immune serum or RAIR antibodies (1/150) dilution). The IgG bound to the transferred peptides were finally visualized by the use of goat anti-rabbit immunoglobulins coupled to peroxidase.

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