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Use of an anti-insulin receptor antibody to discriminate between metabolic and mitogenic effects of insulin: correlation with receptor autophosphorylation

Gilles Ponzio, Jean-Olivier Contreres, Anne Debant, Véronique Baron, Nadine Gautier, Jacqueline Dolais-Kitabgi and Bernard Rossi

Unité INSERM 145, Faculté de Medecine, Avenue de Valombrose, 06034 Nice Cédex, France

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In a previous report we described the properties of a rabbit anti-insulin receptor antibody (RAIR-IgG) and its effects on the autophosphorylation and kinase activity of human insulin receptors. The present study was carried out on the hepatoma cell line Fao. We tested the mimetic effects of RAIR-IgG on different biological parameters known to be stimulated by insulin, receptor autophosphorylation and kinase activity. RAIR-IgG stimulated the metabolic effects (glucose and amino acid transport) but, unlike insulin, was unable to promote cell proliferation. These data clearly demonstrated the existence of two distinctly controlled pathways in the mediation of the hormonal response. When we investigated the effects of this antibody at the molecular level we found that in a cell-free system RAIR-IgG weakly stimulated receptor autophosphorylation on non-regulatory sites and failed to stimulate tyrosine kinase activity toward exogenous substrates. Accordingly, RAIR-IgG did not stimulate receptor autophosphorylation in ^{32}P-labelled intact cells. Interestingly, under similar conditions RAIR-IgG elicited ribosomal S6 protein phosphorylation, as did insulin. The possibility that RAIR-IgG activated a cryptic tyrosine kinase activity is discussed.

Key words: antibodies/autophosphorylation/metabolic effects/mitogenesis/signalling pathways

Introduction

The involvement of tyrosine kinase activity in the mediation of the effects of insulin is now well established by several observations showing that expression of insulin effects on cell metabolism are impaired when tyrosine kinase activity is altered, by physiological changes such as those occurring in obesity (Le Marchand Brustel *et al.*, 1985), or when this activity is intentionally altered by microinjection of inhibiting antibodies (Morgan and Roth, 1987) or by site-directed mutagenesis (Chou *et al.*, 1987; Ebina *et al.*, 1987). In this respect, Ellis *et al.* (1986) have demonstrated the pivotal role that tyrosine residues located in position 1162-1163 play in the control of both insulin-stimulated protein kinase activity toward exogenous substrates and glucose transport. However, several studies using polyclonal anti-insulin receptor antibodies (Zick *et al.*, 1984; Simpson and Hedo, 1984) have shown an uncoupling between the receptor phosphorylation and the stimulation of biological effects, although this has been recently reevaluated (Gherzi *et al.*, 1987). Clearer information was obtained by the use of monoclonal antibody (Forsayeth *et al.*, 1987), providing evidence that the autophosphorylation step might not be closely correlated to the expression of the effects of insulin. In an attempt to further define the involvement of autophosphorylation in the control of insulin action, we made use of a rabbit polyclonal antibody, that has the property of mimicking the action of insulin without interacting with the insulin binding domain (Ponzio *et al.*, 1987). Using a rat hepatoma cell line which expresses several effects of insulin, we observed that our rabbit polyclonal antibody can selectively stimulate the metabolic effects without affecting cell growth, indicating that these two types of insulin-mediated effects use distinct pathways. Additionally, the data confirm the fact that an anti-insulin receptor antibody can trigger metabolic effects, without stimulating receptor autophosphorylation. The possibility exists that insulin receptors display a cryptic tyrosine kinase activity which is not coupled to autophosphorylation of the receptor.

Results

Effect of RAIR-IgG on amino acid and glucose transport

The rat hepatoma Fao cell is a convenient model to study the insulin mimetic effects of antibodies because it exhibits a large spectrum of insulin effects. Initially we looked to see if purified immunoglobulins from our rabbit anti-insulin receptor antiserum were able to stimulate amino acid and deoxyglucose transport. The data shown in Figure 1 (open bars) show that insulin, at a concentration of 10 nM in the presence of non-immune IgG, increased α-aminoisobutyric acid (AIB) uptake by a factor of 2.7. This effect was concentration-dependent with an EC_{50} = 0.3 nM (data not shown). Rabbit anti-insulin receptor antibody (RAIR-IgG) at a concentration of 300 μg/ml also stimulated AIB uptake to an extent corresponding to 60% of the effect of insulin. The maximal level was obtained after an incubation time of 6 h, regardless of the nature of the effector. At the same concentration, control IgG was without effect on AIB uptake (results not shown).

RAIR-IgG (300 μg/ml), after 15 min of incubation, stimulated deoxyglucose transport in Fao cells (Figure 1, hatched bars) to a value corresponding to 55% of that obtained at saturating insulin concentrations (maximal insulin stimulation factor = 2).

Effect of RAIR-IgG on S6 protein phosphorylation

Phosphorylation of ribosomal S6 protein is one of the first molecular events leading to protein synthesis. This step, which needs the activation of a specific S6 kinase activity, has been shown to be regulated by insulin, probably through a cascade of phosphorylation events (Nilsen-Hamilton *et al.*, 1982; Pierre *et al.*, 1986). It was of interest to find out if

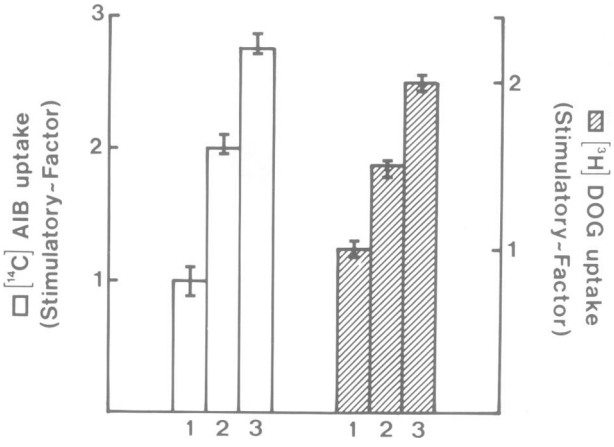

Fig. 1. Effect of insulin and RAIR-IgG on AIB uptake and 2-deoxyglucose transport in Fao cells. α-Aminoisobutyric acid (AIB) transport (open bars). Exponentially growing Fao cells were deprived of serum for 48 h before being incubated at 37°C for 15 h with 300 μg/ml RAIR-IgG (bar 2) or non-immune IgG (300 μg/ml) plus 100 nM insulin (bar 3) then pulsed for 15 min at the same temperature with radiolabelled AIB. Bar 1 represents the basal transport of AIB, carried out in presence of non-immune IgG (300 μg/ml). 2-Deoxyglucose (DOG) uptake (hatched bars). DOG transport was measured for 10 min after a 30-min pre-incubation at 37°C with control IgG (300 μg/ml) (bar 1), RAIR-IgG (300 μg/ml) (bar 2) and control IgG (300 μg/ml) plus 100 nM insulin (bar 3). For both AIB and DOG transport all experiments were assayed in triplicate and the results expressed as the fold change or 'stimulatory factor' compared to the basal value measured in the presence of control IgG.

our anti-insulin receptor antibody was able to cause this short-term effect. For this purpose, we exposed, for a 15 min period, Fao cells, previously loaded for 2 h with inorganic $^{32}PO_4^{3-}$, to a non-relevant IgG fraction (Figure 2A), to 300 μg/ml RAIR-IgG (Figure 2B) and to 100 nM insulin (Figure 2C). Ribosomal proteins were extracted at pH 4.0 and analysed by two-dimensional electrophoresis. As shown in Figure 2, both insulin and RAIR-IgG induced a marked increase of protein S6 phosphorylation (M_r = 32 kd). The phosphorylation induced by RAIR-IgG was quantitated by measuring the radioactivity incorporated into the 32-kd band and corresponded to 50–60% of the effect of insulin. It has been demonstrated that S6 kinase activation depends upon insulin receptor tyrosine kinase stimulation (Chou *et al.*, 1987), and our data strongly suggest that RAIR-IgG, was able to activate the tyrosine kinase associated with the insulin receptor of intact Fao cells.

Lack of effect of RAIR-IgG on DNA synthesis

It was of critical importance to determine whether RAIR-IgG was able to stimulate DNA synthesis when applied to insulin-responsive cells. It has recently been demonstrated that insulin induces cell proliferation by interacting with its own receptors in H35 rat hepatoma cells (Koontz and Iwahashi, 1981; Massagué and Czech, 1982). We have found that in the Fao cell, which is well-differentiated H35 hepatoma variant cell line, insulin stimulated thymidine incorporation into DNA with the same efficiency as 10% fetal calf serum (FCS). As shown in Figure 3, insulin stimulated [^{3}H]thymidine incorporation over the concentration range of 1 pM to 100 nM and one can reasonably assume that insulin promotes Fao cell proliferation by an action on its own receptors on the basis of the high sensitivity to the hormone (EC_{50} = 0.1 nM). It can also be seen from the results shown in Figure 3 that when quiescent Fao cells were incubated for 15 h with RAIR-IgG (300 μg/ml) they failed to stimulate [^{3}H]thymidine incorporation, while under similar conditions insulin induced a 10-fold stimulation of DNA synthesis. In a parallel experiment we verified that after the same incubation time RAIR-IgG was able to stimulate maximally AIB transport, suggesting that the lack of effect of the antibody on DNA synthesis could not be attributed to any inactivation of RAIR-IgG during the long-term incubations.

Lack of effect of RAIR-IgG on receptor autophosphorylation in intact cells

Previous studies have demonstrated that activation of the tyrosine-specific protein kinase activity was correlated with insulin receptor autophosphorylation (Kohanski and Lane, 1986; Tornqvist and Avruch, 1988; Yu and Czech, 1984). By the use of human receptors mutated at the tyrosine kinase domain, Ellis *et al.* (1986) have shown the importance of the kinase activity in the mediation of insulin action. Mutation of the twin tyrosine domain resulted in complete inhibition of both the tyrosine-specific protein kinase activity and the effect of insulin on glucose uptake. However, the extent of the correlation of receptor autophosphorylation with this particular biological effect is not clear, and it appeared that anti-insulin receptor antibodies, which interact with the receptor at sites distinct from the hormone binding domain, could be helpful in gaining information on this specific point. In fact, as shown in Figure 4 (panel C) RAIR-IgG, which is able to trigger several biological effects, failed to promote receptor autophosphorylation under conditions where insulin produced a dramatic effect (Figure 4, panel B). This behaviour was reproducibly observed in several additional experiments. However, in contrast to other anti-receptor antibodies (Forsayeth *et al.*, 1987; Morgan *et al.*, 1986), RAIR-IgG did not alter the insulin effect when the two effectors were used simultaneously (Figure 4, panel D).

Effect of RAIR-IgG on autophosphorylation in a cell-free system

We first examined the ability of RAIR-IgG to cause autophosphorylation of the β subunit of partially purified receptors from Fao cells. The autoradiogram shown in Figure 5 corresponds to the concentration-dependent stimulation by insulin of phosphorylation of the β subunit in the presence or absence of RAIR-IgG. The half-maximum effect of insulin, as assessed by the radioactivity incorporated in the 95-kd subunit, occurred in the range of 3 nM, regardless of the presence of RAIR-IgG. These data confirm the fact that RAIR-IgG did not interact with the insulin-binding site. We found, in a parallel experiment, that no incorporation of radioactivity occurred in the region of 95 kd when IGF-I was used in place of insulin, suggesting the absence of IGF-I receptors in Fao cells with which RAIR-IgG could have interacted (data not shown).

The stimulatory effect of RAIR-IgG on the autophosphorylation of the insulin receptor was estimated to be ~5% of that of insulin (Figure 5).

Lack of effect of RAIR-IgG on the receptor protein kinase

Several tyrosine-containing proteins can serve as exogenous

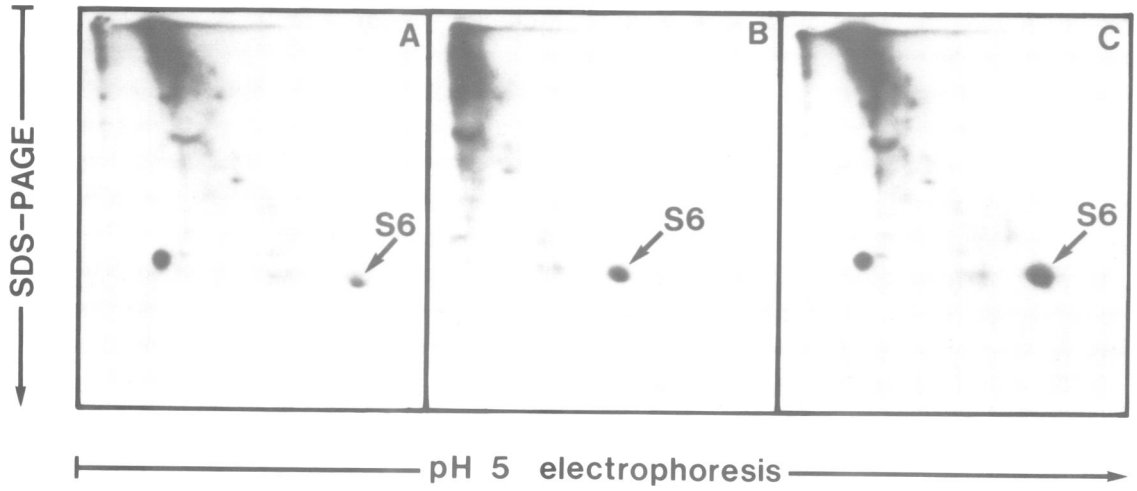

Fig. 2. Phosphorylation of S6 ribosomal protein by RAIR-IgG and insulin in Fao cells. ^{32}P-Labelled Fao cells were stimulated for 15 min at 37°C with 300 μg/ml control IgG (**panel A**), 300 μg/ml RAIR-IgG (**panel B**), 300 μg/ml control IgG plus 100 nM insulin (**panel C**). The ribosomes were isolated and the ribosomal proteins separated as described in Materials and methods. The arrows indicate the position of the phosphorylated S6 ribosomal protein.

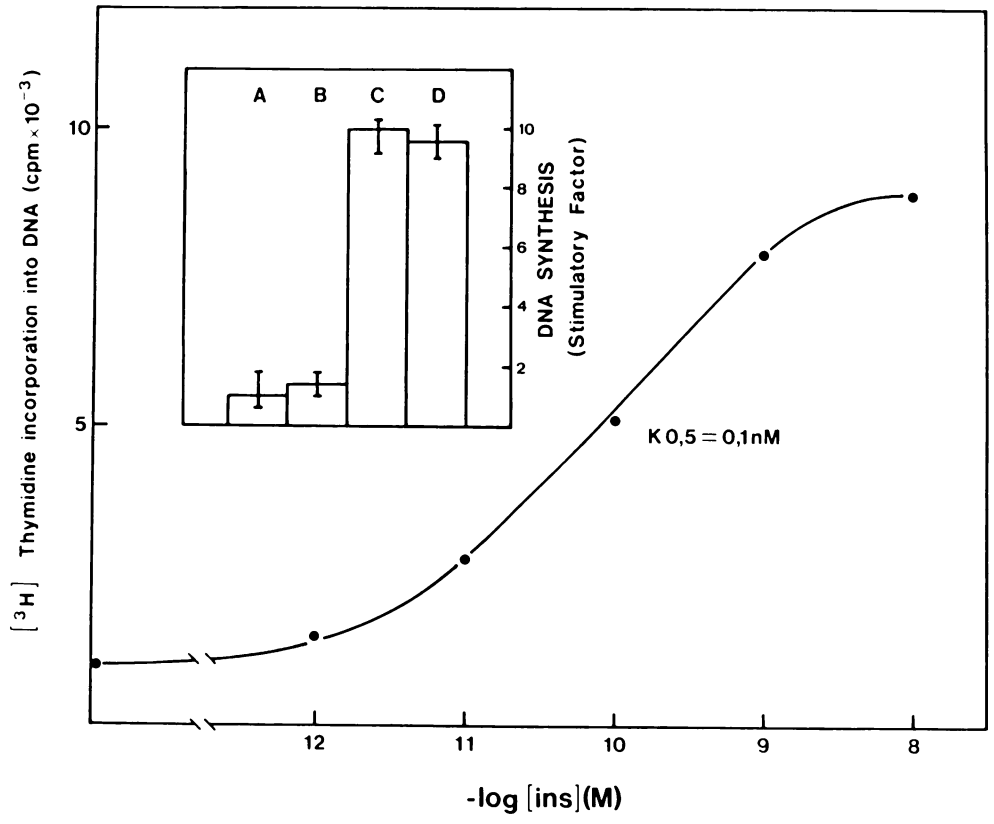

Fig. 3. Effect of RAIR-IgG and insulin on DNA synthesis in Fao cells. Fao cells were incubated for 48 h in serum-free medium and then incubated with insulin concentrations of 1 pM to 10 nM (●) for a further 15 h. This incubation was followed by a 1-h pulse of [^{3}H]thymidine (1 μCi) and the labelled DNA was isolated as described in Materials and methods. Each experiment was carried out in triplicate, and the results are expressed in c.p.m. per 10^6 cells. In the insert is shown the level of [^{3}H]thymidine incorporated into the DNA of Fao cells pre-treated for 15 h with: 300 μg/ml control IgG (**panel A**), 300 μg/ml RAIR-IgG (**panel B**), 300 μg/ml control IgG plus 100 nM insulin (**panel C**) and 300 μg/ml RAIR-IgG plus 100 nM insulin (**panel D**). All the experimental values are expressed as the fold change or stimulatory factor compared to the basal value and represent the mean SEM of three different experiments.

substrates for the insulin receptor kinase (Kasuga *et al.*, 1983; Nemenoff *et al.*, 1984). We have examined the ability of insulin (100 nM) and RAIR-IgG (300 μg/ml) to stimulate the kinase activity of a partially purified insulin receptor fraction, extracted from Fao cells, toward exogenous substrates. The data presented in Figure 6 show that RAIR-IgG did not significantly stimulate the phosphorylation of casein (panel A), histone (panel B) or poly(Glu/Tyr) (panel C), whereas it elicited slight phosphorylation of the β subunit (panel A/B).

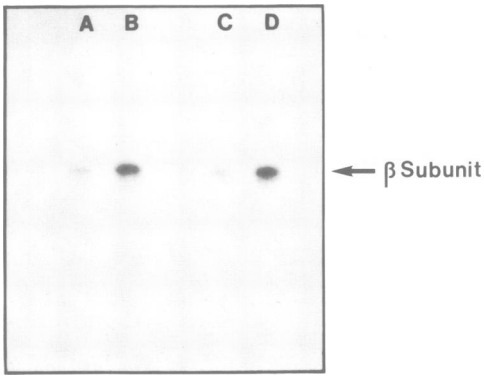

Fig. 4. Effect of RAIR-IgG and insulin on β subunit phosphorylation in intact Fao cells. Fao cells (2×10^6 cells) were loaded for 2 h with [^{32}P]orthophosphate and then exposed for 30 min to 300 μg/ml control IgG (**lane A**), 300 μg/ml control IgG plus 100 nM insulin (**lane B**), 300 μg/ml RAIR-IgG (**lane C**) and 300 μg/ml RAIR-IgG plus 100 nM insulin (**lane D**). The solubilized glycoproteins were enriched on wheat germ agglutinin agarose and the insulin receptor purified as described in Materials and methods. The autoradiogram shows the level of phosphate incorporated into the β subunit of the receptor after stimulation of the Fao cells under the conditions described.

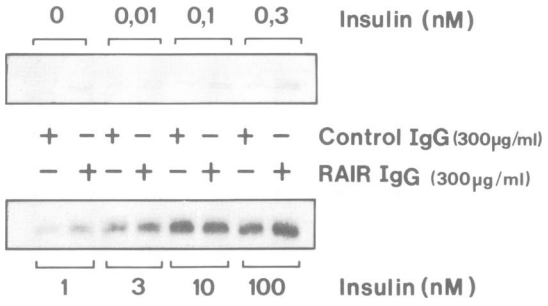

Fig. 5. Phosphorylation of the β subunit by RAIR-IgG and insulin in a cell-free system. Wheat-germ agarose-purified insulin receptor from Fao cells was phosphorylated as described in Materials and methods, after an overnight incubation at 4°C with various concentrations of insulin in the presence of either control IgG or RAIR-IgG (300 μg/ml). For each of the insulin concentrations indicated (0−100 nM) the autoradiogram represented alternately the region of the SDS−PAGE corresponding to the β subunit of the receptor stimulated by control IgG plus insulin and by RAIR-IgG plus insulin.

Comparative analysis of the phosphopeptide maps obtained under insulin and RAIR-IgG stimulation

In an attempt to understand better the molecular basis of the slight effect that RAIR-IgG exerts on the autophosphorylation of solubilized receptors, we compared the tryptic phosphopeptide maps of the β subunit, phosphorylated in the presence of insulin and in the presence of RAIR-IgG. Partially purified insulin receptors were incubated with 100 nM insulin or 300 μg/ml RAIR-IgG prior to addition of the labelled ATP mixture. After a selective immunoprecipitation, the two insulin receptor subunits were separated by PAGE and the β subunit subjected to extensive trypsinolysis (100 μg/ml) for 48 h before analysis by two-dimensional separation. Due to the multiple steps involved in this technique the data can only be interpreted qualitatively. As shown in Figure 7 (panels A and B) the two effectors increased the phosphorylation level of zwitterionic peptides that do not migrate in the electrophoretic dimension. However, the positively charged peptides named 1−7 were not present on the map obtained under RAIR-IgG stimulation (Figure 7B). The absence of phosphorylation appears to represent the lack of effect of the antibody on the tyrosine kinase activity. In order to clarify this point we looked carefully at those parts of the sequence which carry tyrosyl residues and which are known to be phosphorylated upon tyrosine kinase activation. Previous reports have established that the major sites involved during the *in vitro* and *in vivo* autophosphorylation processes are located at tyrosine residues 1158, 1162 and 1163 carried by the tryptic peptide P5 and tyrosine residues 1328, 1334 carried by peptide P1 (Tornqvist *et al.*, 1987, 1988). The sequences are shown in Table I. In order to identify which of the spots present on the two-dimensional pattern correspond to these important domains, we subjected phosphorylated synthetic peptides, corresponding to the P1 and P5 regions, to the same separation procedure as used for the receptor subunits. In a separate experiment we determined that tryptic fragments from the phosphorylated P1 peptide (Figure 7C) co-migrated with fragments 5, 6 and 7 of panel A, suggesting that they correspond to the same sequence situated at the C terminus end of the insulin receptor. Similarly, phosphopeptides resulting from the trypsinolysis of P5 (panel C) co-migrated with the peptide fragments 1, 2, 3 and 4 suggesting that they belong to the twin tyrosine region of the kinase domain (Tyr 1162−1163). The autoradiogram shown in panel C represented the migration of the mixture of phosphopeptides resulting from trypsinolysis of the P1 and P5 peptides. Clearly, none of these peptides were phosphorylated under RAIR-IgG stimulation, allowing us to conclude that the slight autophosphorylation observed in the cell-free condition in the presence of RAIR-IgG mainly occurred on sites which are exclusive of the two domains controlling the activation of the tyrosine-specific protein kinase activity. These data support the idea that the autophosphorylation induced by RAIR-IgG was artefactual and does not correlate with the exogenous tyrosine kinase activity of the receptor.

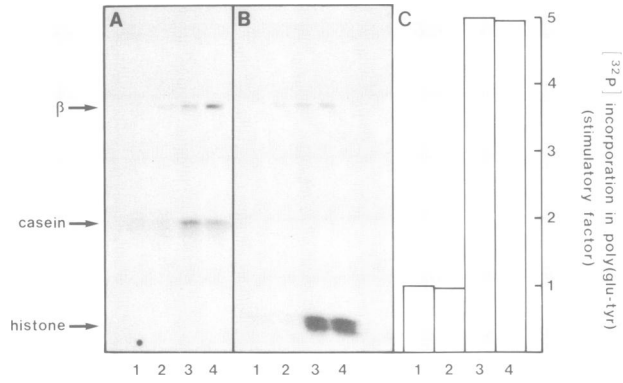

Fig. 6. Effect of RAIR-IgG and insulin on insulin receptor kinase activity toward exogenous substrates. The partially purified receptor, incubated overnight at 4°C with 300 μg/ml control IgG (**lane 1**), 300 μg/ml RAIR-IgG (**lane 2**), 300 μg/ml control IgG plus 100 nM insulin (**lane 3**) and 300 μg/ml RAIR-IgG plus 100 nM insulin (**lane 4**) was used to phosphorylate exogenous substrates such as, 1 mg/ml casein (**A**), 1 mg/ml histone H2b (**B**), and 0.2 mg/ml poly(Glu-Tyr) (**C**). The experiments were repeated four times. Only one of them is shown in the case of the kinase activity measured toward histone and casein (autoradiogram). The average of the four experiments is presented in the histogram of **C**.

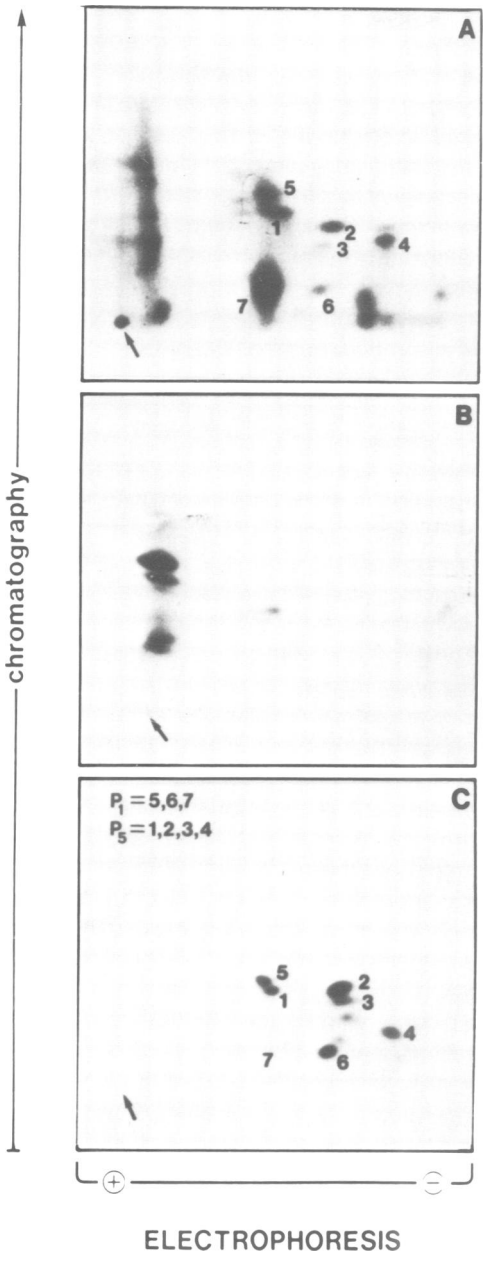

Fig. 7. Qualitative analysis of the phosphopeptides from the tryptic digestion of the β subunit of the insulin receptor obtained under insulin and RAIR-IgG stimulation. Partially purified insulin receptors were phosphorylated after a 15-h incubation with, respectively, 100 nM insulin (**panel A**) or 300 μg/ml RAIR-IgG (**panel B**), submitted to an extensive trypsinolysis and ultimately separated by two-dimensional electrophoresis/chromatography as indicated in Materials and methods. In **panel C** is represented the two-dimensional migration pattern of both P5 and P1 tryptic phosphopeptides (for sequences see Table I) respectively named 1,2,3,4 and 5,6,7. Consequently, these were designated 1–7 in the β subunit's phosphopeptide map by reference to the pattern obtained with the phosphorylated synthetic peptides.

Discussion

It is generally considered that autophosphorylation of the insulin receptor on its β subunit is a prerequisite for stimulation of its exogenous protein kinase activity (Kohanski and Lane, 1986; Tornqvist and Avruch, 1988; Yu and Czech, 1984; Kasuga *et al.*, 1983; Nemenoff *et al.*, 1984).

However, the linkage between these two events (autophosphorylation and protein kinase activation) has been recently questioned by evidence that the substrate itself can modulate the level of the insulin receptor phosphorylation. Indeed, at saturating concentrations of polyGlu/Tyr, insulin receptors exhibit a fully active protein kinase activity without any detectable autophosphorylation of the β subunit (Morrison and Pessin, 1987). Also, use of anti-receptor monoclonal antibodies has shown that, in some conditions, the biological effects of insulin were not correlated with autophosphorylation of the receptor (Forsayeth *et al.*, 1987; O'Brien *et al.*, 1987). Clearly, better definition of the roles that autophosphorylation and/or protein kinase activity play in the mediation of the action of insulin is required, and studies need to be performed, in the same biological system, on the connection between these molecular events and the expression of the different effects on insulin. On the other hand, exploration of the different pathways intervening in the mediation of insulin action requires the use of effectors capable of inducing only part of the hormonal response. Along this line, King *et al.* (1980) reported that human polyclonal antibodies were able to mimic metabolic effects on rat adipocytes but failed to promote cell growth on human fibroblasts. Nevertheless, these authors concluded from their study that the mediation of mitogenic effects occurs through IGF-1 receptors. A biological system well-adapted for this kind of study is the rat hepatoma cell line (Fao), derived from Reuber H35 cell line. This H35 subline, which is devoid of IGF-1 receptors (Massagué *et al.*, 1982), has been proven to express the mitogenic effect through insulin receptors, in addition to several metabolic effects (Koontz and Iwahashi, 1981). Our study aims to define the different pathways involved in this system to mediate the insulin action. For that purpose we made use of a rabbit polyclonal antibody which has been shown to interact with insulin receptors on epitopes distinct from the insulin binding site (Ponzio *et al.*, 1987). When exposed to Fao cells, RAIR-IgG exhibited effects on both glucose and amino acid transport, to an extent approximating 50–60% of the maximal effect of insulin (Figure 1). Insulin has been shown to promote the proliferation of Reuber H35 cells at physiological concentrations, suggesting that the hormone acts via its own receptors. Our data confirm that result, using Fao cells, a well-differentiated cell line derived from Reuber H35 hepatoma. As shown in Figure 3 the concentration of insulin which produced a half-maximal response on DNA replication was 0.1 nM. However, RAIR-IgG was without any effect on DNA synthesis indicating that this antibody was able to discriminate among the pleiotropic effects of the hormone. We can conclude therefore, from the selective effect of our antibody, that the mitogenic effect and the metabolic effects (glucose and amino acid transport) of insulin are conveyed through distinct pathways. This dichotomy was undetectable using insulin since both types of bioeffects (mitogenic and metabolic) are equally hormone sensitive. Using a genetic

Table I. Sequences of the peptides containing the major autophosphorylation sites within the β subunit of the insulin receptor

Peptide	Position in the receptor sequence	Sequence
P1	1321–1338	SLGFKRS<u>Y</u>EEHIP<u>Y</u>THMN
P5	1154–1170	TRDI<u>Y</u>ETD<u>YY</u>RKGGKGL

approach, Shimizu and Shimizu (1986) also concluded that the mediation of mitogenic and metabolic insulin responses was via separate pathways. Accordingly, we have recently shown that insulin receptors mutated on Tyr 1162–1163 were unable to mediate the metabolic effects (glucose transport and glycogen synthesis) while they displayed a full mitogenic response, confirming by a different approach the presence of at least two distinct signalling pathways (Debant *et al.*, 1988).

In a previous study (Ponzio *et al.*, 1987) we reported that RAIR-IgG was able to activate, additively with insulin, both tyrosine kinase activity toward exogenous substrates and the autophosphorylation of the insulin receptor's β subunit from human origin (IM9 cells). Conversely, the antibody was unable to stimulate the autophosphorylation of the β subunit when applied to intact Fao cells (Figure 4), indicating that a given antibody can behave differently depending on the biological system used. Similarly, it has been reported that polyclonal antibodies from patients can elicit biological effects without stimulating receptor phosphorylation (Zick *et al.*, 1984; Simpson and Hedo, 1984). Aware of contradictory results obtained using polyclonal antibodies (Zick *et al.*, 1984; Simpson and Hedo, 1984; Gherzi *et al.*, 1987) we decided to investigate autophosphorylating ability of our antibody on solubilized receptors. We did find a small increase in autophosphorylation (Figure 6). However, this increase in autophosphorylation was not accompanied by stimulation of the tyrosine-specific protein kinase activity (Figure 6). Furthermore, when we analysed the phosphopeptide map of the β subunit, phosphorylated in the presence of RAIR-IgG, we were unable to visualize any phosphorylation on either the twin-tyrosine region (1162–1163) or on the C terminus domain (1328,1334) which are known to be phosphorylated concomitantly with the increase of tyrosine kinase activity (Herrera and Rosen, 1986). These data suggest that the slight autophosphorylation induced by RAIR-IgG interaction with receptors, occurred on non-regulatory sites. Furthermore, we were unable to detect any autophosphorylation of insulin receptor when RAIR-IgG was applied to intact cells, even though it induced the expression of metabolic effects. These data suggest that the slight autophosphorylation observed in the cell-free system was irrelevant to the expression of the insulin-mimetic effects of the antibody.

In order to shed some light on this puzzling problem, we measured, in intact Fao cells, the phosphorylation of the ribosomal protein S6. This intracytoplasmic event requires the activation of a highly specific 63-kd serine kinase whose activity has been shown to be regulated by the tyrosine kinase activity associated with several oncogene-encoded proteins and/or growth factor receptors (Blenis *et al.*, 1987) including the insulin receptor (Nilsen-Hamilton *et al.*, 1982; Pierre *et al.*, 1986). As presented in Figure 2, RAIR-IgG stimulated the phosphorylation of the ribosomal S6 protein, whereas it was unable to activate the tyrosine kinase of solubilized insulin receptors. This result is indicative of the expression of an insulin-dependent tyrosine kinase activity which, in intact cells, induced the activation of S6 kinase. Surprisingly, this activity was not measurable in the cell-free assay, using classical exogenous substrates.

In conclusion, two hypotheses can be considered: (i) the insulin-mimetic metabolic effects of RAIR-IgG are independent of the activation of tyrosine kinase and/or phosphorylation of an endogenous substrate and therefore must be mediated through another pathway; (ii) mediation of these biological effects necessitate the phosphorylation of endogenous substrate(s) by a receptor-mediated tyrosine kinase that was not detectable in cell-free assay. Characterization of the different endogenous substrates involved in the multiple actions of insulin will be helpful in understanding the events involved in the fine tuning of the receptor kinase activity, thus allowing discrimination between these two possibilities.

Materials and methods

[^{14}C]α-Amino isobutyric acid (AIB) transport

Cells grown in 35 mm dishes, were depleted for 48 h in Ham's F12/Dulbecco's-modified Eagle medium (50/50%, v/v) supplemented with 1% bovine serum albumin (BSA). Insulin (100 nM) and/or RAIR-IgG (300 μg/ml) were added in the same incubation medium and the incubation was resumed for 15 h at 37°C. Cells were rinsed twice with 1.5 ml of Krebs' Ringer buffer and pulsed with [^{14}C]AIB (0.2 μCi/ml, 0.1 mM). After 15 min at 37°C the medium was removed, cells were washed in ice-cold phosphate-buffered saline (PBS) and then lysed in 1 M NaOH before measuring the radioactivity.

[^{3}H]2-Deoxyglucose uptake

The cells, in 35 mm dishes were incubated for 30 min with 100 nM insulin and/or 300 μg/ml RAIR-IgG at 37°C in PBS before 0.1 mM [^{3}H]2-deoxyglucose (0.4 μCi/ml) was added for 10 min at 37°C. The incubation medium was then aspirated and the cells were rinsed twice with the ice-cold PBS. They were finally lysed with 1 M NaOH and the radioactivity measured.

Phosphorylation of the S6 ribosomal protein

The ribosomal protein S6 was isolated following the procedure previously described by Nilsen-Hamilton *et al.* (1981) after slight modifications. In brief, cells grown in 35 mm wells were loaded with [^{32}P]orthophosphate (0.2 mCi/35 mm dish) for 90 min at 37°C, before 100 nM insulin and/or 300 μg/ml RAIR-IgG were added for 15 min at 37°C. Cells were then solubilized in the following buffer: 7 mM Tris pH 7.4/1 mM phenylmethylsulphonyl fluoride (PMSF)/100 mM NaF/1 mM $MgCl_2$/7 mM NaCl/1% NP-40/1% deoxycholate. The samples were subjected to a first centrifugation (3000 *g*, 10 min). The supernatants obtained were then centrifuged at 120 000 *g* for 10 min at 4°C. The pellets were subjected to an acid extraction as previously described (Goreinstein and Warnar, 1976). The ribosomal proteins in the supernatant were then precipitated with acetone, in the presence of 30 μg of BSA as carrier. The pellets were resuspended in a buffer containing urea, before being subjected to a two-dimensional electrophoresis (Goreinstein and Warnar, 1976).

Incorporation of [^{3}H]thymidine into DNA

Cells were depleted for 48 h in serum-free Ham's F-12/Dulbecco's-modified Eagle's medium H-21 (50/50%, v/v) containing 1% BSA. RAIR-IgG (300 μg/ml) and/or insulin at various concentrations were then added for 15 h. Cells were pulsed with 1 μCi/ml of [^{3}H]thymidine for 1 h in the same medium at 37°C. DNA was then precipitated by 10% trichloracetic acid (TCA) for 30 min at 4°C. The resulting precipitates were washed twice with 5% TCA, solubilized in 1 M NaOH, neutralized with an equivalent amount of 1 M HCl and the radioactivity measured.

Phosphorylation of insulin receptors in intact Fao cells

Cells (2×10^6) grown in 35 mm dishes were depleted for 48 h at 37°C in serum-free Ham's F-12/Dulbecco's-modified Eagle's medium (50–50%) supplemented with 1% BSA. Cells were loaded with [^{32}P]orthophosphate (1 mCi/dish) for 2 h at 37°C. At the end of the incubation time, cells were exposed to insulin (100 nM) and/or RAIR-IgG (300 μg/ml) at 37°C. The ^{32}P-labelled cells were then solubilized in 30 mM Hepes pH 7.6/1% Triton X-100/30 mM NaCl/3 mM VO_4/10 mM sodium pyrophosphate/100 mM NaF/20 mM EDTA/2 mg/ml bacitracin/0.2 mg/ml PMSF/100 U/ml aprotinin, for 90 min at 4°C under gentle stirring. Insulin receptor purification was then carried out on wheat-germ agglutinin agarose as described previously (Van Obberghen *et al.*, 1981). Finally, an identical amount of receptor, as estimated by [^{125}I]insulin binding, was subjected to the immunoprecipitation procedure using RAIR-IgG. Polypeptides were separated by SDS–PAGE and the gel dried before autoradiography.

Autophosphorylation and kinase activity of partially purified insulin receptor

The insulin effect on the receptor autophosphorylation and the kinase activity toward exogenous substrates were measured by incubating the same amount of solubilized receptors with 100 nM insulin and/or 300 μg/ml RAIR-IgG overnight at 4°C. Both activities were initiated by adding the following reaction mixture: 15 μM [γ-^{32}P]ATP/4 mM $MnCl_2$/8 mM $MgCl_2$. Kinase activity was measured in the presence of the following substrates: 1 mg/ml histone H2B, 1 mg/ml casein or 0.2 mg/ml poly(Glu/Tyr). When insulin receptor kinase activity was measured toward the co-polymer poly(Glu/Tyr) the reaction was stopped after a 30 min incubation at 25°C, as previously described (Braun *et al.*, 1984). For other conditions the reaction was stopped after 15 min at 25°C by addition of a solution containing 3% w/v SDS/3% 2-mercaptoethanol/10% glycerol/10 mM Na_2HPO_4/0.05% bromophenol blue and samples subjected to SDS–PAGE.

Tryptic fragments of phosphorylated insulin receptor

Solubilized Fao receptor phosphorylated under the conditions described above was submitted to SDS–PAGE. Once localized by autoradiography, the bands corresponding to the β subunit were excised and incubated for 24–48 h at 37°C in 1 ml of 50 mM NH_4HCO_3 pH 8.5 containing 100 μg of tosylphenylalanine chloromethyl ketone (TPCK)-treated trypsin. Gel fragments were removed by centrifugation. Samples were then lyophilized and taken up in 5 μl of 15 mM NH_4OH. Phosphopeptides were subjected to electrophoretic separation on cellulose thin layer plates in 30% formic acid, pH 1.9, 500 V for 90 min at 4°C. Peptides were finally separated by second dimensional chromatography using 1-butanol/acetic acid/pyridine/water (60/12/40/48) pH 3.5 for 6 h at room temperature. The cellulose plates were ultimately autoradiographed at −80°C.

Phosphorylation of synthetic peptides with the insulin-dependent protein kinase

Peptides containing Tyr-1162, 1163 (P5) and 1324 (P1) were phosphorylated by insulin receptor purified from transfected rat fibroblasts (McClain *et al.*, 1987). Briefly, insulin (100 nM) was pre-incubated with the receptor (50 fmol) at 25°C for 15 min prior to addition of substrates (200 μM). The phophorylation reaction was initiated by adding 4 mM $MnCl_2$/8 mM $MgCl_2$ and [γ-^{32}P]ATP (50 μM/25 μCi) and stopped after 45 min incubation at 25°C, by TCA precipitation on phosphocellulose papers (Stadtmauer and Rosen, 1983). The phosphorylated peptides were then eluted in 50 mM NH_4HCO_3 pH 8.5 and incubated at 37°C for a 48-h trypsinolysis (100 μg/ml). The tryptic fragments were ultimately subjected to a two-dimensional separation under conditions as described above.

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