The EMBO Journal vol.6 no.13 pp.4003–4010, 1987

Monoclonal antibodies to the insulin receptor stimulate the intrinsic tyrosine kinase activity by cross-linking receptor molecules

R.M.O'Brien, M.A.Soos and K.Siddle

Department of Clinical Biochemistry, University of Cambridge, Addenbrooke's Hospital, Hills Road, Cambridge CB2 2QR, UK

Communicated by C.Milstein

The effect of monoclonal anti-insulin receptor antibodies on the intrinsic kinase activity of solubilized receptor was investigated. Antibodies for six distinct epitopes stimulated receptor autophosphorylation and kinase activity towards exogenous substrates. This effect of antibodies was seen only within a narrow concentration range and monovalent antibody fragments were ineffective. Evidence was obtained by sucrose density-gradient centrifugation for the formation of antibody–receptor complexes which involved both inter- and intra-molecular cross-linking, although stimulation of autophosphorylation appeared to be preferentially associated with the latter. There was partial additivity between the effects of insulin and antibodies in stimulating autophosphorylation, although the sites of phosphorylation appeared identical on two-dimensional peptide maps. Antibodies for two further epitopes failed to activate receptor kinase, but inhibited its stimulation by insulin. The effects of antibodies on kinase activity paralleled their metabolic effects on adipocytes, except for one antibody which was potently insulin-like in its metabolic effects, but which antagonized insulin stimulation of kinase activity. It is concluded that antibodies activate the receptor by cross-linking subunits rather than by reacting at specific epitopes. The ability of some antibodies to activate receptor may depend on receptor environment as well as the disposition of epitopes.

Key words: monoclonal antibodies/kinase/phosphorylation/insulin receptor

Introduction

The insulin receptor is a transmembrane glycoprotein composed of two α-(M_r ~135 kd) and two β-(M_r ~95 kd) subunits linked through disulphide bridges (reviewed by Jacobs and Cuatrecasas, 1983; Czech, 1985). Binding of insulin to the α-subunit on the extracellular domain of the receptor is transformed into a transmembrane signal which activates an intrinsic tyrosine-specific protein kinase associated with the cytosolic domain of the β-subunit (reviewed by Cobb and Rosen, 1984; Gammeltoft and Van Obberghen, 1986). Several lines of evidence have implicated the tyrosyl kinase activity in the mechanism of insulin action: metabolic effects of insulin are inhibited by the introduction of kinase-inhibitory antibodies into cells (Morgan *et al.*, 1986; Morgan and Roth, 1987); cells transfected with mutant insulin receptors which are defective in kinase activity cannot respond normally to insulin (Ellis *et al.*, 1986; Ebina *et al.*, 1987; Chou *et al.*, 1987); insulin resistance in man and animals has been associated with decreased kinase activity of receptors (Le Marchand-Brustel *et al.*, 1985; Burant *et al.*, 1986; Caro *et al.*, 1986; Grigorescu *et al.*, 1987).

Several different polypeptides have been described which show increased tyrosine phosphorylation in intact cells in response to insulin (White *et al.*, 1985; Haring *et al.*, 1987; Machicao *et al.*, 1987; Perrotti *et al.*, 1987; Bernier *et al.*, 1987). The identity of these polypeptides and their significance in terms of the mediation of insulin action is unclear. Guanine-nucleotide binding regulatory proteins are substrates for the insulin receptor kinase *in vitro* (Zick *et al.*, 1986; O'Brien *et al.*, 1987; Korn *et al.*, 1987) but again the physiological importance of these reactions remains to be established. Autophosphorylation of the insulin-receptor β-subunit is readily demonstrable both with purified receptor and in intact cells, and is stimulated by insulin (Gammeltoft and Van Obberghen, 1986). This autophosphorylation leads to activation of the insulin receptor kinase so that it becomes independent of bound insulin (Yu and Czech, 1984). Whether there are other consequences of autophosphorylation, perhaps modulating interaction of the receptor with other proteins, is unknown.

The amino acid sequence of the insulin receptor, deduced from the sequence of cloned cDNA (Ullrich *et al.*, 1985; Ebina *et al.*, 1985) has suggested a structural model in which only a single 23-amino acid helical stretch of the β-subunit traverses the membrane. This raises the question of how the interaction of insulin with the extracellular binding domain transmits a signal to the intracellular kinase domain. A tetrameric holoreceptor appears to be a prerequisite for such signalling (Boni-Schnetzler *et al.*, 1986). Studies with polyclonal antibodies have suggested that cross-linking of receptor subunits perhaps within $\alpha_2\beta_2$ hetero-tetramers, can activate the kinase (Heffetz and Zick, 1986) although it may be that the consequences of receptor activation by antibodies and by insulin differ in some respects (Ponzio *et al.*, 1987). It has also been reported that antibodies can mimic insulin action in intact cells although unable to activate the receptor kinase (Zick *et al.*, 1984; Simpson and Hedo, 1984; Forsayeth *et al.*, 1987). In order to avoid problems of interpretation inherent in the use of polyclonal antisera, we have made use of a panel of monoclonal antibodies to multiple distinct epitopes on the insulin receptor (Soos *et al.*, 1986; Taylor *et al.*, 1987) to investigate the mechanism of kinase activation and its relationship to metabolic stimulation. A preliminary account of some of this work has been presented (O'Brien *et al.*, 1986b).

Results

Initial screening of monoclonal antibodies

Insulin receptor solubilized from placental microsomal membranes was used to screen antibodies for effects on tyrosyl kinase activity. In this crude system a variety of components become phosphorylated on incubation with [^{32}P]ATP and these can be resolved by SDS–PAGE. The β-subunit of the insulin receptor is prominent as a phosphorylated band of ~M_r 95 kd, which shows a marked increase in phosphorylation in response to insulin, this phosphorylation being shown to be on tyrosine residues by its stability to alkaline hydrolysis and by phosphoamino acid

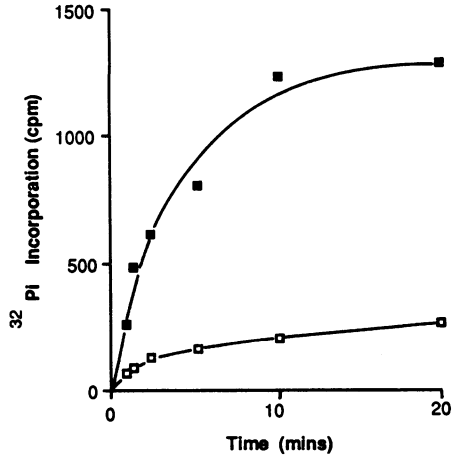


Fig. 1. Time course of receptor autophosphorylation. Crude placental extract was pre-incubated in the presence (—■—) or absence (—□—) of insulin (10^{-7} M) before carrying out the phosphorylation reaction as described in Materials and methods. Phosphorylation of the β-subunit was quantified by radioactive counting.

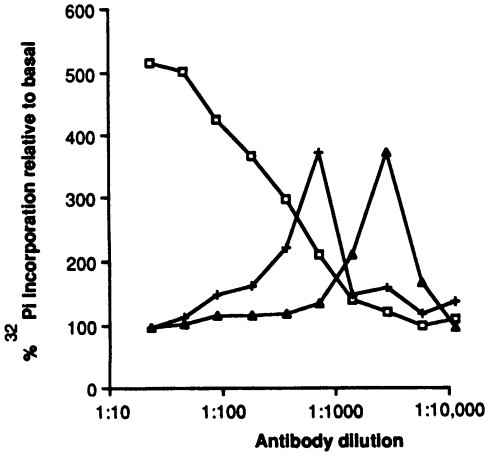


Fig. 2. Stimulation of receptor autophosphorylation by monoclonal and polyclonal antibodies. Crude placental extract was pre-incubated with various dilutions of monoclonal antibody 83−7 (—▲—) or 83−14 (+) or with a rabbit polyclonal anti-receptor antiserum (—□—) before carrying out the phosphorylation reaction as described in Materials and methods. Phosphorylation of the β-subunit was quantified by radioactive counting and expressed as a percentage of basal for each experiment. Basal phosphorylation was ~100 c.p.m.

analysis (results not shown). The extent of receptor autophosphorylation was quantified by densitometric scanning of autoradiograms or by direct counting of radioactivity in the appropriate band excised from the gel. Phosphorylation assays were generally carried out for 10 min at 4°C, under which conditions insulin maximally stimulated phosphorylation ~5- to 7-fold (Figure 1).

A panel of 28 monoclonal antibodies was available for study, which were the product of four independent fusions and recognized at least nine distinct epitopes on extracellular domains of the insulin receptor (Soos *et al.*, 1986). Antibodies representative of six different epitopes stimulated the receptor autophosphorylation reaction at appropriate concentrations (Figure 2), namely antibody 83−14 (epitope 3); 18−26 (epitope 4); 18−34 (epitope 5); 18−43 (epitope 6); 18−41, 18−44 and 18−45 (epitope 7); 83−7 and 83−12 (epitope 8). This list includes antibodies reacting with α- and β-subunits, antibodies which were inhibitory or non-inhibitory for insulin binding and a variety of different

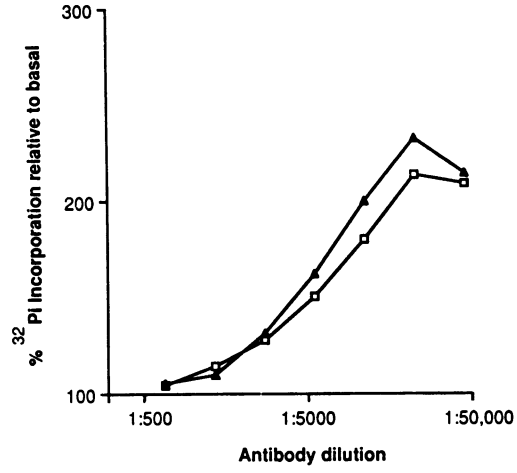


Fig. 3. Stimulation of insulin receptor phosphorylation of exogenous substrate by monoclonal antibody. Purified insulin receptor was pre-incubated with various dilutions of monoclonal antibody 83−7, and then histone H2B (10 μM) was added before carrying out the phosphorylation reaction as described in Materials and methods. Phosphorylation of the β-subunit (—▲—) and histone H2B (—□—) were quantified by radioactive counting and expressed as a percentage of basal. Basal insulin receptor β-subunit and histone phosphorylation were 60 and 90 c.p.m. respectively.

immunoglobulin isotypes. In all cases the stimulation was observed only within a narrow antibody concentration range, high concentrations of antibody being ineffective. In contrast a rabbit polyclonal anti-receptor serum stimulated autophosphorylation over a broad concentration range, reaching a plateau at high concentrations (Figure 2).

It was confirmed that highly purified receptor, obtained by an immunoaffinity technique (O'Brien *et al.*, 1986a) behaved similarly to that in crude extracts in terms of stimulation by insulin and antibodies. Thus purified receptor showed up to a 12-fold increase in autophosphorylation with antibodies 83−7, 18−41 and 18−44, but only within a narrow concentration range, with a sharp optimum, for each antibody (results not shown).

It was also shown that the phosphorylation of exogenous substrates such as histone and casein was stimulated by the antibodies within the same concentration range effective in the autophosphorylation reaction (Figure 3). This observation confirms that the effect of antibodies on the autophosphorylation reaction reflects a stimulation of the kinase activity *per se* rather than enhancement of the ability of the β-subunit to act as a substrate.

Characterization of the biphasic response to antibodies

The lack of stimulation of receptor kinase at high antibody concentrations was not due to non-specific effects on antibody binding or kinase activity. Highly purified antibodies behaved similarly to crude ascites. Receptor autophosphorylation was stimulated by insulin even in the presence of supramaximal antibody concentrations which were not themselves effective and such supramaximal antibody concentrations still bound iodinated, purified receptor under the conditions of the kinase assay (results not shown).

The narrow effective antibody concentration range suggested that kinase activation might reflect cross-linking of αβ dimers within $\alpha_2\beta_2$ receptors by bivalent antibodies, as it would be expected that the formation of cross-linked species would depend critically on antibody/receptor ratio and would not occur with antibody excess. The fact that many different antibodies stimulated the kinase was also consistent with an activation mechanism dependent primarily on a general property of antibodies, rather than reaction at particular sensitive epitopes.

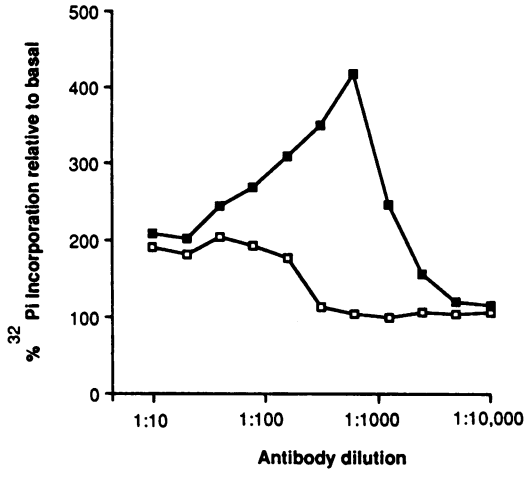


Fig. 4. Stimulation of receptor autophosphorylation by monoclonal antibody 18–41 and its Fab′ fragment. Crude placental extract was pre-incubated with 18–41 IgG (—■—) or 18–41 Fab′ (—□—) before carrying out the phosphorylation reaction as described in Materials and methods. Phosphorylation of the β-subunit was quantified by radioactive counting and expressed as a percentage of basal for each experiment. Basal phosphorylation was ~30 c.p.m.

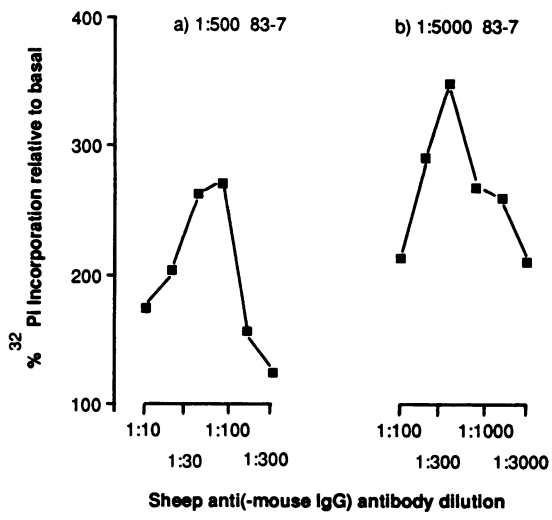


Fig. 5. Potentiation of the effect of monoclonal antibody 83–7 by sheep anti-mouse IgG. Crude placental extract was pre-incubated with submaximal (1/5000) and supramaximal (1/500) concentrations of 83–7 and various dilutions of sheep anti-mouse IgG before carrying out the phosphorylation reaction as described in Materials and methods. Phosphorylation of the β-subunit was quantified by radioactive counting and expressed as a percentage of basal for each experiment. Basal phosphorylation in the absence of 83–7 was 64 c.p.m., while phosphorylation in the presence of submaximal and supramaximal 83–7 but without sheep anti-mouse IgG was 110 and 89 c.p.m. respectively.

In support of this hypothesis, it was shown that monovalent Fab′ antibody fragments produced much diminished stimulation of autophosphorylation relative to bivalent antibodies, although such Fab′ fragments retained full activity in receptor binding assays and antagonized the kinase-stimulatory effects of the corresponding bivalent antibody when both were added together. The Fab′ fragment of antibody 83–7, across a broad concentration range, had no effect on receptor autophosphorylation, consistent with the complete lack of stimulation by excess bivalent antibody (data not shown). Results with antibody 18–41 were somewhat more complex (Figure 4). Whereas the bivalent antibody and $F(ab')_2$ fragments produced a maximum 4-fold effect with a sharp concentration optimum, the monovalent Fab′ fragment produced only a 2-fold stimulation which reached a plateau at high concentrations. Interestingly, this corresponded to the level of stimulation reached at high concentrations of bivalent 18–41 and other antibodies reacting with the same epitope.

Further evidence for the importance of cross-linking in the mechanism of kinase activation was provided by the potentiating effects of sheep anti-(mouse IgG) antibody (Figure 5). Thus stimulations of autophosphorylation of 1.7-fold and 1.4-fold by submaximal and supramaximal concentrations of antibody 83–7 were increased to 2.4-fold and 2.7-fold respectively by appropriate concentrations of second antibody. The response to second antibody again showed a marked concentration-dependence, with a sharp optimum which depended on monoclonal antibody concentrations.

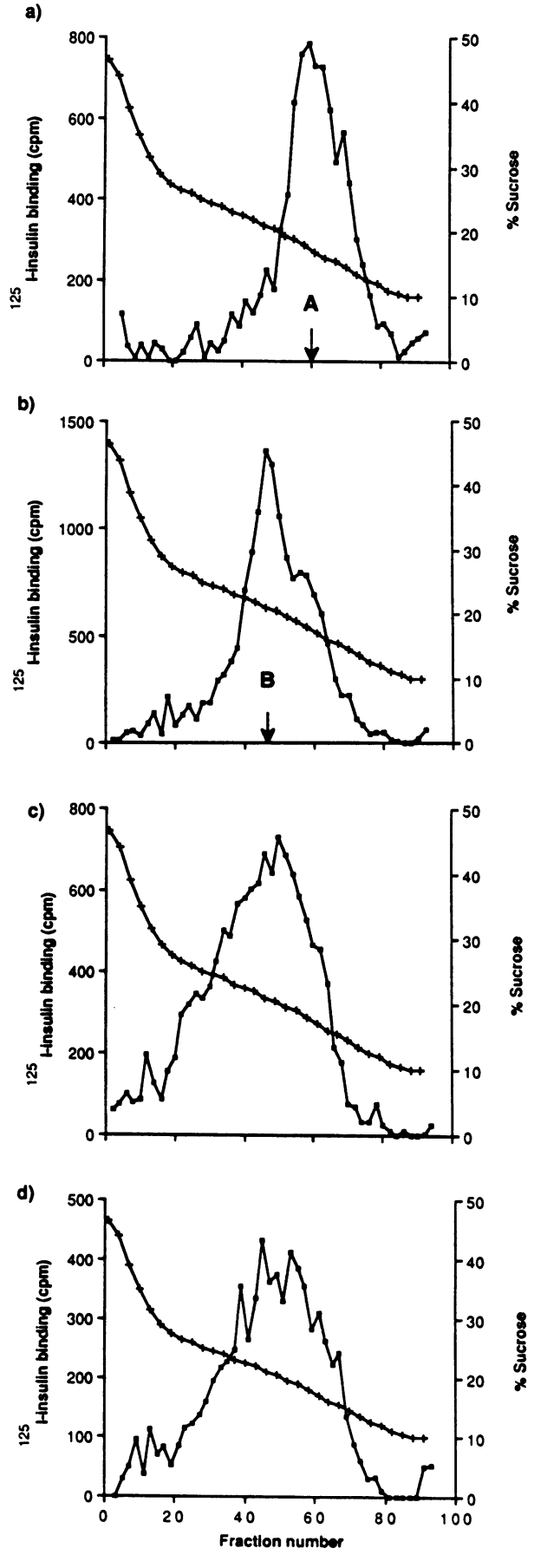
Characterization of antibody–receptor complexes

Sucrose density gradient centrifugation was utilized to investigate the nature of the antibody–receptor complexes formed at different antibody concentrations. Receptor was incubated under conditions of the autophosphorylation assay either in the absence of antibody or with a large excess of antibody, or with the optimal antibody concentration for kinase stimulation. The incubation mixtures were then layered on sucrose gradients and following centrifugation the distribution of receptor was determined by measurement of insulin binding activity, the antibodies used in these experiments being ones which did not inhibit insulin binding (Figure 6). In the presence of excess antibody, the receptor was localized as a fairly sharp band (B) which sedimented more rapidly than the free receptor (A) and which presumably represents $\alpha_2\beta_2$ receptor with two antibody molecules bound. However, in the presence of a lower antibody concentration previously shown to be optimal for kinase stimulation, a much broader distribution of receptor was observed. This included peaks of insulin binding, in the position of bands A and B, a third peak C in an intermediate position which was therefore compatible with $\alpha_2\beta_2$ receptor with a single antibody bound and a fraction sedimenting more rapidly than band B, which presumably represents aggregated receptor molecules cross-linked by antibody. It should be noted that the profile of insulin binding activity observed in the presence of antibody may not be a precisely quantitative measure of the relative amounts of receptor at different points on the gradient, because of the possibility that the antibodies used themselves stimulate insulin binding (Soos *et al.*, 1986).

When radioactively labelled receptor from autophosphorylation reaction mixtures was similarly fractionated, the ratio of labelled material to insulin binding activity was greatest for the fractions sedimenting in the position of band C, when measured in the presence of an optimally stimulating antibody concentration (Figure 7). This suggests that the receptor with stimulated kinase activity was predominantly associated with a complex containing one molecule of antibody and one molecule of receptor. Given the previously demonstrated requirement for antibody bivalency for kinase activation, it appears that this complex must involve intramolecular cross-linking within the $\alpha_2\beta_2$ holomeric receptor, via epitopes on either the α- or the β-subunit.

Additivity of effects of antibodies and insulin

The effect of antibodies on receptor autophosphorylation was tested in the presence of a maximally effective concentration of insulin (Figure 8). Surprisingly the effects of insulin and antibody together were greater than either alone, if somewhat less than additive, even though the incubation time was chosen such that



autophosphorylation in the presence of insulin or antibody alone had reached a plateau. Similar results were obtained by using highly purified receptor or crude detergent solubilized placental membranes.

To compare the sites of autophosphorylation in the presence of antibodies and insulin, a two-dimensional separation of the phosphopeptides obtained after tryptic digestion of the phosphorylated β-subunit was performed. Identical phosphopeptide maps were obtained whether receptor had been phosphorylated in the presence of insulin or antibody (Figure 9) or both (result not shown).

Characterization of inhibitory monoclonal antibodies

Two antibodies, namely 47–9 (epitope 1) and 25–49 (epitope 2) had little or no effect on receptor autophosphorylation on their own. Both these antibodies potently inhibited insulin binding to the receptor (Soos *et al.*, 1986) and as expected therefore antagonized the stimulatory effect of insulin on receptor autophosphorylation and on the phosphorylation of exogenous substrates (Figure 10). Not all binding-inhibitory antibodies behaved in this way however. Antibody 83–14 itself stimulated kinase at an appropriate concentration (Figure 2), although excess of this antibody antagonized the stimulatory effect of insulin.

Discussion

The aims of this study were 2-fold: firstly to compare the effects of anti-receptor antibodies on receptor tyrosyl kinase activity with their effects on insulin binding and cellular metabolism; secondly to investigate the mechanism of antibody-induced receptor activation. In pursuit of these aims the effects of antibodies on the autophosphorylation of receptor in detergent extracts of placental microsomal membranes were tested. Although the receptor is very impure in this system, it has been subjected only to minimal processing. Moreover it was confirmed using selected antibodies that results obtained by studying autophosphorylation in such receptor preparations were not qualitatively different from those obtained for autophosphorylation of highly purified receptor, nor for phosphorylation of exogenous substrates. It is assumed therefore that the autophosphorylation reaction studied provides a valid measure of the kinase activity of the receptor *per se*, rather than potential secondary effects of antibodies on the ability of the receptor β-subunit to act as a substrate for the kinase or for phosphatases.

In almost all cases, the ability of antibodies to activate the receptor tyrosyl kinase paralleled their ability to mimic metabolic effects of insulin. Thus antibodies 83–7, 83–14 and 18–44 which were previously shown to stimulate lipogenesis and inhibit lipolysis in isolated adipocytes (Taylor *et al.*, 1987) all activated receptor kinase at appropriate concentrations (Figure 2), while antibody 47–9, which blocked the binding and action of insulin in adipocytes, also antagonized the stimulatory effect of insulin on kinase activity (Figure 10). These results confirm that there is no simple relationship between the binding sites of antibodies and their biological effects. Antibodies which inhibit insulin bin-

Fig. 6. Characterization of antibody–receptor complexes by sucrose density gradient centrifugation. Insulin receptor preparations comprising free receptor (**a**), receptor with excess 18–44 (1:200 dilution) (**b**), receptor with peak kinase stimulatory dilution of 18–44 (1:12 800) (**c**), receptor with peak kinase stimulatory dilution of 83–7 (1:51 200) (**d**), were layered on sucrose gradients and following centrifugation fractions were collected from the bottom and analysed for insulin binding activity as described in Materials and methods.

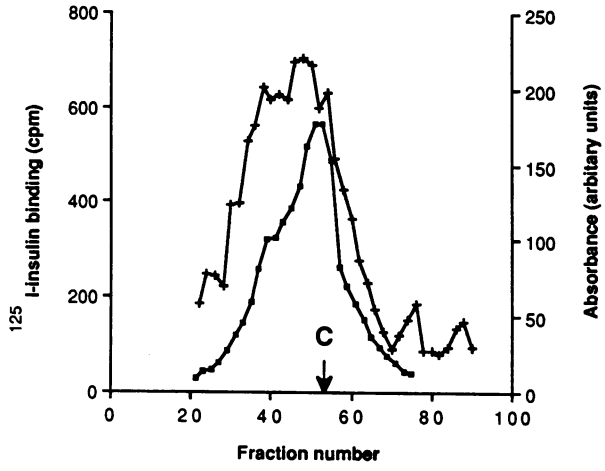


Fig. 7. Characterization of phosphorylated receptor–antibody complexes by sucrose density gradient centrifugation. Insulin receptor was pre-incubated with the peak kinase stimulatory dilution of 18–44 (1:12 800) before initiating the phosphorylation reaction as described in Materials and methods. The preparation was layered on the sucrose gradient immediately following addition of [^{32}P]ATP and following centrifugation fractions were collected from the bottom, solubilized in sample buffer and separated on SDS–polyacrylamide gels. Phosphorylated receptor distribution (—■—) was analysed by densitometric scanning of autoradiographs and compared with the distribution of insulin binding activity (+).

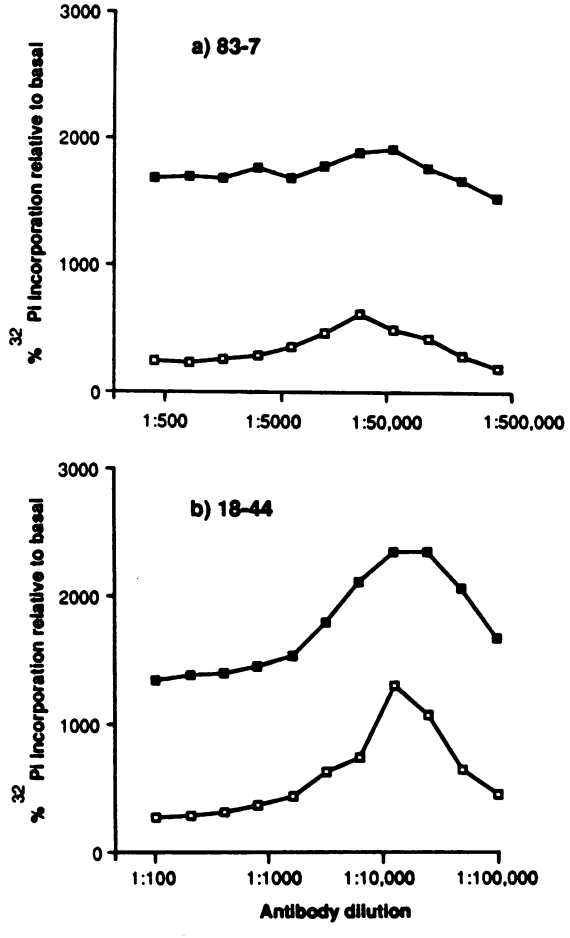


Fig. 8. Additive stimulation of autophosphorylation by antibody and insulin. Insulin receptor was pre-incubated with various dilutions of 83–7 (**a**) or 18–44 (**b**) in the presence (—■—) or absence (—□—) of 10^{-7} M insulin before carrying out the phosphorylation reaction as described in Materials and methods. Phosphorylation of the β-subunit was quantified by densitometric scanning of autoradiographs and expressed as a percentage of basal from each experiment.

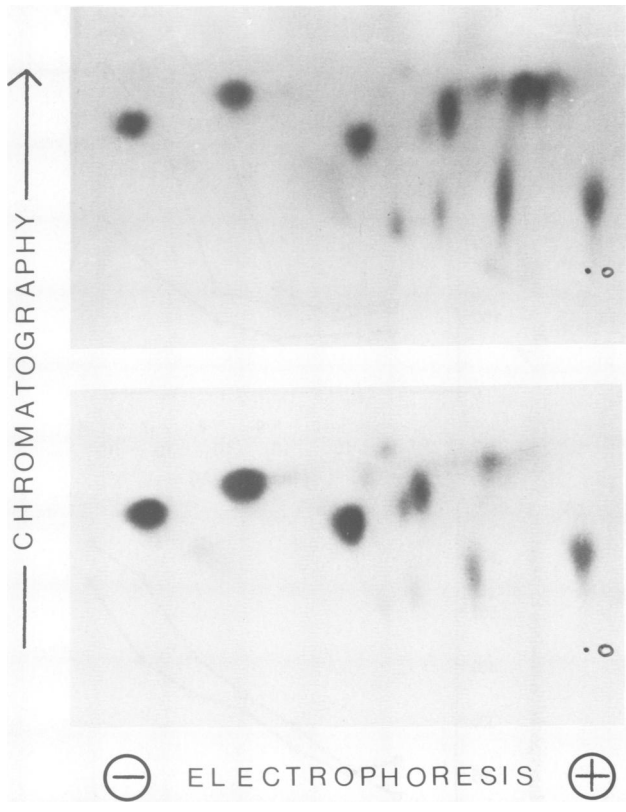


Fig. 9. Phosphopeptide mapping of insulin receptor tryptic peptides. Insulin receptor was pre-incubated with 10^{-7} M insulin (**a**) or the peak kinase stimulatory concentration of 83–7 (**b**) before carrying out the phosphorylation reaction as described in Materials and methods. Following immunoprecipitation and SDS–PAGE the region of gel containing the 95 kd phosphoprotein was excised, digested with trypsin and the peptides separated on cellulose thin layer plates as described in Materials and methods.

ding may either inhibit (47–9) or mimic (83–14) insulin action and insulin-like effects can also be initiated by antibodies reacting well away from the insulin binding site, involving even epitopes on the β-subunit (18–44).

However, one antibody, 25–49, which was a potent mimic of insulin action on adipocytes, failed reproducibly to stimulate receptor kinase activity, although a very small enhancement of receptor autophosphorylation (up to 50% increase above basal) was seen in some experiments. This contrasted with the 2.5- to 5-fold stimulation induced by other insulin-mimetic antibodies. In fact far from itself increasing receptor phosphorylation, antibody 25–49 inhibited the stimulatory effect of insulin, in line with its known ability to inhibit insulin binding (Soos *et al.*, 1986). In this respect antibody 25–49 is similar to certain other polyclonal antisera (Simpson and Hedo, 1984; Zick *et al.*, 1984) and monoclonal antibodies (Forsayeth *et al.*, 1987) which have been reported to produce insulin-like metabolic effects, although unable to stimulate receptor phosphorylation either in intact cells or in cell-free systems. However, it may be important to document receptor phosphorylation and metabolic effects in parallel in the same cell system before making too much of such observations and even then it must be borne in mind that in many cell types maximal metabolic effects may be achieved by activating only a small fraction of cell surface receptors, at which level of occupancy a significant increase in total receptor phosphorylation may be difficult to detect. Further the ability of antibodies to activate the receptor may depend on aspects of the membrane

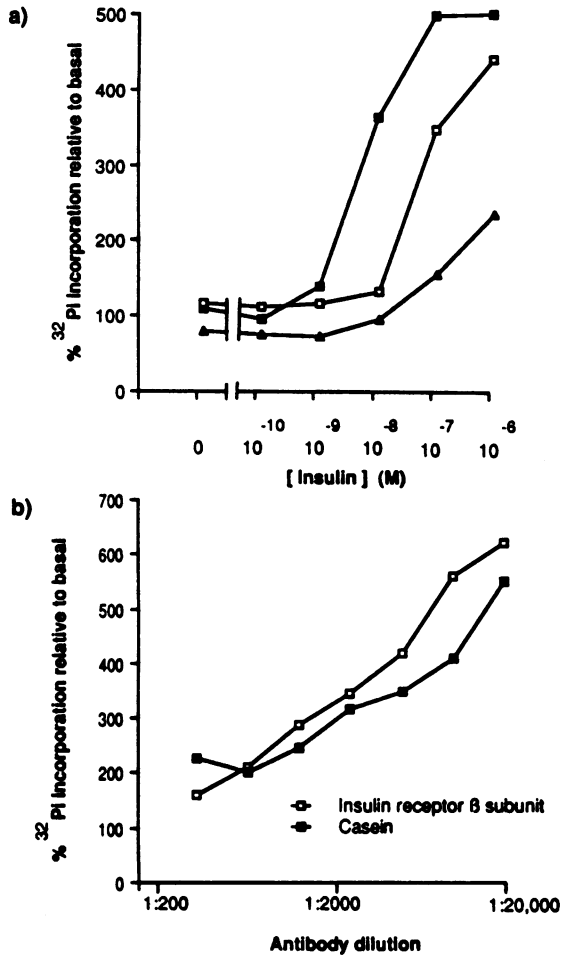


Fig. 10. Inhibition of insulin stimulated autophosphorylation by antibodies. Insulin receptor was pre-incubated either with 1:100 47−9 (—▲—) or 1:100 25−49 (—□—) or buffer (—■—) and various concentrations of insulin (**a**) or with 10^{-7} M insulin, 0.5 mg/ml casein and various dilutions of antibody 25−49 (**b**) before carrying out phosphorylation reactions as described in Materials and methods. Phosphorylation of the β-subunit [and casein in (**b**)] was quantified by radioactive counting and expressed as a percentage of basal for each experiment.

environment which are lost on solubilization.

With regard to the mechanism of signal transduction between extracellular and cytosolic domains of the insulin receptor, several lines of evidence indicated the importance of antibody bivalency and receptor cross-linking in the receptor activation process. Antibodies for mulitple epitopes all stimulated kinase activity in a markedly concentration-dependent manner (Figure 2), while monovalent antibody fragments did not generally stimulate kinase activity but antagonized the effect of their bivalent counterparts (Figure 4). The stimulatory effect of both submaximal and supramaximal antibody concentrations was potentiated by addition of sheep anti-(mouse IgG) antiserum to cross-link anti-receptor antibody molecules (Figure 5).

The dependence of insulin-like activity on antibody bivalency and receptor cross-linking was first demonstrated by Kahn *et al.* (1978) in studies of the metabolic effects of human anti-receptor autoantibodies. In extending this work to study activation of tyrosine kinase in solubilized receptor preparations, Heffetz and Zick (1986) reached a similar conclusion but further proposed that the cross-linking of αβ-units occurred intramolecularly in the $\alpha_2\beta_2$ receptor. In the present work, sucrose gradient centrifugation provided evidence for the formation of antibody−receptor complexes involving both intramolecular cross-links as well as aggregates cross-linked intermolecularly (Figure 6). In the case of antibody 18−44, stimulation of autophosphorylation was shown to occur predominantly in the putative intramolecularly cross-linked species containing one antibody and one receptor molecule (Figure 7). It would be surprising if as many as six different epitopes on both α- and β-subunits could be so disposed as to permit intramolecular cross-linking, while also allowing displacement of this reaction in favour of binding of two separate antibody molecules under conditions of antibody excess (Conti-Tronconi *et al.*, 1981). It is also unclear how the addition of sheep anti-(mouse IgG) antibody could potentiate stimulation which depended solely on intramolecular cross-links. A role for intermolecular aggregation in kinase stimulation cannot therefore be completely ruled out.

Interestingly, the biological effects of various other anti-receptor antibodies have been shown to require bivalency or cross-linking. In the case of the epidermal growth factor (EGF) receptor, the mitogenic activity of antibodies depended absolutely on bivalency, although their ability to stimulate receptor tyrosyl kinase did not (Schreiber *et al.*, 1983) and in other studies antibody-induced receptor clustering was found not always to be a sufficient signal for induction of EGF-like effects (Defize *et al.*, 1986). The activation of receptors by antibody-induced clustering is not confined to receptors with intrinsic tyrosine kinase activity, having been demonstrated also for lutropin receptors (Podesta *et al.*, 1983) and prolactin receptors (Djiane *et al.*, 1985).

No antibody was identified which appeared to mimic insulin action by simple bimolecular reaction with an epitope at or near the insulin binding site. However, there were some effects which were related to specific epitopes, superimposed on the more general stimulation attributable to cross-linking. Thus an excess of bivalent antibodies for a site on the β-subunit (18−41, 18−44 and 18−45; epitope 7) as well as their monovalent counterparts, did induce some increase in receptor autophosphorylation which was not evident with antibodies for other epitopes under similar conditions (Figures 2 and 4).

The question arises as to the relevance of observations on antibody-induced receptor activation to the mechanism of action of insulin. It is clear that insulin itself could not physically cross-link receptor subunits, although interaction between αβ dimers is critical for the insulin-dependent activation of the kinase, if not for maintenance of activity once autophosphorylation has occurred (Boni-Schnetzler *et al.*, 1986). The extent of stimulation of receptor kinase and autophosphorylation by antibodies was generally not as great as that obtained with a maximally effective insulin concentration, presumably because not all receptor molecules were cross-linked into active species (Figures 6 and 7). Surprisingly the combination of antibody plus insulin produced greater stimulation than either agent alone (Figure 8). It is possible that this additivity reflects the influence of a contaminating phosphatase activity on the steady-state level of autophosphorylation rather than a true difference in the extent of reaction at completion, although this appears unlikely as the same phenomenon was seen with highly purified receptors as with crude detergent extracts of membranes. A further possibility is that insulin and antibody differentially activate separate populations of receptor molecules differing in their state of aggregation or thiol oxidation (Yip and Moule, 1983; Koch *et al.*, 1986). Heffetz and Zick (1986) found that a polyclonal autoantibody stimulated receptor kinase submaximally relative to insulin, but insulin had no additional effect even though its binding was only

partially inhibited by antibody. In contrast Ponzio *et al.* (1987) reported that the stimulation of solubilized insulin receptor kinase by a polyclonal antiserum was fully additive with the effect of insulin and that there were differences as well as similarities in the respective autophosphorylation sites as analysed by two-dimensional phosphopeptide mapping. However, in the present studies such phosphopeptide maps were indistinguishable, whether autophosphorylation was induced by insulin, antibody or both together (Figure 9), suggesting that the two agents induced essentially similar active conformations of the kinase domain.

In summary, we have shown that insulin receptor activation may be triggered by antibodies binding at multiple different sites, such activation depending primarily on the intramolecular cross-linking of receptor molecules and resulting in an autophosphorylation reaction essentially indistinguishable from that induced by insulin. These results provide further evidence that the aggregation state of insulin receptors and interactions between subunits are important determinants of their conformation and activity.

Materials and methods

Reagents

Porcine monocomponent insulin was a generous gift from Novo Research Institute, Copenhagen, Denmark. [^{125}I]Na (IMS 30) and [^{32}P]phosphate (PBS-11) were from Amersham International, Amersham, Bucks, UK and [γ-^{32}P]ATP was synthesized as described (Glynn and Chappell, 1964). Bovine serum albumin (BSA), pepstatin A, leupeptin, antipain, benzamidine, phenylmethylsulphonyl fluoride (PMSF), dithiothreitol, iodoacetamide, Tris base, casein, histone H2B, pepsin and *N*-2-hydroxyethyl piperazine-*N'*-2-ethanesulphonic acid (Hepes) were from Sigma Chemical Co., Poole, Dorset, UK. Protein A–Sepharose was from Pharmacia, Milton Keynes, Bucks, UK. Ultrogel AcA 34 was from LKB Instruments Ltd, Selsdon, Surrey, UK. Triton X-100 (scintillation grade) was from Koch-Light Ltd, Haverhill, Suffolk, UK. Trypsin (Worthington, TPCK-treated) was from Lorne Diagnostics Ltd, Bury St Edmunds, Suffolk, UK. Nycodenz was from Nycomed, Birmingham, UK. Cellulose thin layer plates were from Eastman-Kodak, Liverpool, UK. All other reagents were of the best grade commercially available. Anti-insulin receptor antibodies and sheep anti-(mouse IgG) antiserum were as described previously (Soos *et al.*, 1986).

Receptor purification

Membranes from human placenta were prepared essentially as described by Fujita-Yamaguchi *et al.* (1983), except that 50 mM Hepes, pH 7.6, was used in place of Tris buffer. The protease inhibitors pepstatin A, leupeptin and antipain (1 μg/ml), benzamidine (2.5 mM) and PMSF (0.1 mM) were added to the membranes (20 mg/ml) which were then solubilized with 1% Triton X-100 for 1 h at 4°C, prior to centrifugation at 150 000 *g* for 45 min. The supernatant is referred to in the text as 'crude placental extract'. Where indicated the receptor was purified using an immunoadsorption technique employing a monoclonal anti-receptor antibody (O'Brien *et al.*, 1986a).

Monoclonal antibody purification

Antibody was used as either (i) ascites fluid or (ii) partially purified from ascites fluid by precipitation with 40% saturated (v/v) $(NH_4)_2SO_4$ followed by reconstitution in phosphate-buffered saline to the original ascites volume or (iii) highly purified by hydroxylapatite chromatography (Stanker *et al.*, 1985).

Preparation of Fab' fragments

Highly purified antibody was digested overnight at 37°C with pepsin in 0.1 M sodium acetate, pH 4, at an enzyme:substrate ratio of 1:40 (w/w) (Lamoyi and Nisonoff, 1983). The reaction was stopped by adjusting the pH to 7.5 with NaOH, followed by dialysis against 0.1 M Tris–HCl, 0.1 M NaCl, pH 7.5. Undigested IgG was removed from F(ab')$_2$ by protein A–Sepharose chromatography (Goding, 1986). Fab' fragments were produced by reduction of F(ab')$_2$ fragments in 10 mM cysteine for 2 h at 37°C. The reaction was stopped by addition of 30 mM iodoacetamide for 1 h at room temperature in the dark (Goding, 1986). Fab' fragments were purified by gel filtration chromatography on a 1.6 × 85 cm column of Ultrogel AcA 34.

Autophosphorylation of insulin receptor

Receptor (crude placental extract or purified material) was pre-incubated with insulin or antibody before initiating the kinase reaction essentially as described by Tavare *et al.* (1985). Pre-incubation mixtures (total volume 30 μl) contained ~20–200 ng insulin receptor in 20 μl buffer consisting of 25 mM Hepes, pH 7.6, 0.1% Triton X-100, 24 mM $MgCl_2$, 4 mM $MnCl_2$, 0.2 mM $NaVO_3$, 0.5 mM dithiothreitol, together with antibody or insulin added in 10 μl of 25 mM Hepes, 0.1% Triton X-100. After incubation for 15 min at 23°C, samples were placed on ice for 10 min prior to mixing with 10 μl of 0.4 mM [^{32}P]ATP (2 Ci/mmol) in 25 mM Hepes. Except where indicated, the autophosphorylation reaction was carried out for 10 min at 0°C. Reactions were terminated by addition of 3× concentrated electrophoresis sample buffer (20 μl) containing 0.19 M Tris–HCl. pH 6.8, 6% (w/v) SDS, 30% (v/v) glycerol, 15 mM EDTA, 300 mM dithiothreitol and 0.02% (w/v) bromophenol blue. After heating for 5 min at 100°C samples were analysed by electrophoresis on SDS–polyacrylamide gels (Laemmli, 1970). Stained and dried gels were subjected to autoradiography using Dupont Cronex 4 film. Results were quantified by excision of the appropriate regions from the gel and determination of ^{32}P by counting Cerenkov radiation or by densitometric scanning of autoradiograms using a Joyce-Loebel Chromoscan 3.

Phosphorylation of exogenous substrates

Receptor was pre-incubated with insulin or antibody as described for the autophosphorylation reaction. After 15 min at 23°C, casein (final concentration 0.5 mg/ml) or histone H2B (final concentration 10 μM) was added as 10 μl of solution in 25 mM Hepes, pH 7.6, 0.1% Triton X-100 and incubation continued for a further 10 min at 23°C before addition of 10 μl of 0.4 mM [^{32}P]ATP to initiate the phosphorylation reaction. This reaction with exogenous substrates was carried out for 10 min at 23°C and samples were then processed for electrophoresis and autoradiography as described for the autophosphorylation reaction.

Velocity sedimentation

Velocity sedimentation was performed in a linear 10–30% sucrose density gradient containing 30 mM Hepes, pH 7.6, 0.1% Trixon X-100 and 0.02% azide, on a 0.75 ml Nycodenz cushion. Receptor preparations (500 μl) were layered onto 4 ml gradients and centrifuged for 1 h at 219 000 *g* at 4°C in a vertical rotor (Sorvall TV-856) (Branch *et al.*, 1987). Fractions (50 μl) were collected from the bottom and assayed for insulin binding activity or mixed with electrophoresis sample buffer and analysed by SDS–PAGE and autoradiography.

Insulin binding assay

Insulin was radioiodinated as described by Linde *et al.* (1981) to a specific activity of ~200 μCi/μg. Receptor in crude placental extracts or fractions from sucrose gradients (50 μl) was incubated for 18 h at 4°C with [^{125}I]insulin (30 000 d.p.m., final concentration ~0.1 nM) added in 150 μl of buffer containing 75 mM Tris–HCl, pH 7.3 at 23°C, 30 mM NaCl, 10 mM glucose, 0.5 mM EDTA, 0.1 mM PMSF, 0.1% BSA and 0.05% Triton X-100. Receptor-bound [^{125}I]insulin was determined by precipitation with poly(ethylene glycol) 6000 as previously described (Baron and Sonksen, 1982).

Two-dimensional tryptic peptide mapping of phosphorylated insulin receptor

Insulin receptor was phosphorylated in the presence of insulin, antibody or both as described above. The reaction was stopped by addition of a 4× concentrated solution to achieve final concentrations of 5 mM EDTA, 1 mM PMSF, 100 mM NaF, 10 mM $Na_4P_2O_7$, 5% glycerol. Immunoadsorbent consisting of anti-receptor monoclonal antibody 18–42 covalently coupled to aminocellulose (O'Brien *et al.*, 1986a) was added for 60 min at 4°C before centrifugation and solubilization of bound protein in electrophoresis sample buffer. The ^{32}P-labelled subunit of the receptor was identified by autoradiography following SDS–PAGE. The appropriate region was excised from the gel and processed essentially as described by Stadtmauer and Rosen (1986). Gel slices were fixed and then washed for 6 × 1 h in 20% isopropanol. They were then cut into 1 mm squares, dried for 2 h at 80°C and incubated with shaking for 24 h at 37°C with 1 ml 50 mM NH_4HCO_3 buffer, pH 8.0 containing 100 μg TPCK-treated trypsin. This solution was removed and the gel pieces were incubated again for 2 h at 37°C with 1 ml H_2O. The two supernatant fluids were combined (recovery of ^{32}P was approximately 80%). Samples were lyophilized and residual NH_4HCO_3 removed by resuspending the residue in water before re-lyophilizing. This cycle was repeated three times. The pellet was dissolved in 10 μl H_2O and spotted on a cellulose thin layer plate. Phosphopeptides were separated essentially as described by Ellis *et al.* (1981). Electrophoresis was performed in the first dimension on a flat bed apparatus in 30% formic acid (pH 1.9) for 1 h at 1000 V. The plates were dried overnight and chromatography was performed in the second dimension using 1-butanol:pyridine:acetic acid:water (90:60:18:72, v/v) (pH 3.5).

Acknowledgements

We thank Mr W.Branch and Dr B.Mullock for help with density gradient centrifugation experiments. We are grateful to the MRC, the Wellcome Trust and Serono Diagnostics for financial support. R.M.O'B. was the recipient of an MRC research studentship and K.S. is a Wellcome Lecturer.

References

Baron,M.D. and Sonksen,P.H. (1982) *Biosci. Rep.*, **2**, 785–793.
Bernier,M., Laird,D.M. and Lane,M.D. (1987) *Proc. Natl. Acad. Sci. USA*, **84**, 1844–1848.

Boni-Schnetzler,M., Rubin,J.B. and Pilch,P.F. (1986) *J. Biol. Chem.*, **261**, 15281–15287.
Branch,W.J., Mullock,B.M. and Luzio,J.P. (1987) *Biochem. J*, **244**, 311–315.
Burant,C.F., Treutelaar,M.K. and Buse,M.G. (1986) *J. Clin. Invest.*, **77**, 260–270.
Caro,J.F., Ittoop,O., Pories,W.J., Meelheim,D., Flickinger,E.G., Thomas,F., Jenquin,M., Silverman,J.F., Khazanie,P.G. and Sinha,M.K. (1986) *J. Clin. Invest.*, **78**, 249–258.
Chou,C.K., Dull,T.J., Russell,D.S., Gherzi,R., Lebwohl,D., Ullrich,A. and Rosen,O.M. (1987) *J. Biol. Chem.*, **262**, 1842–1847.
Cobb,M.H. and Rosen,O.M. (1984) *Biochim. Biophys. Acta*, **738**, 1–8.
Conti-Tronconi,B., Tzartos,S. and Lindstrom,J. (1981) *Biochemistry*, **20**, 2181–2191.
Czech,M.P. (1985) *Annu. Rev. Physiol.*, **47**, 357–381.
Defize,L.H.K., Mollenaar,W.H., van der Saag,P.T. and de Laat,S.W. (1986) *EMBO J.*, **5**, 1187–1192.
Djiane,J., Dusanter-Fourt,I., Katoh,M. and Kelly,P.A. (1985) *J. Biol. Chem.*, **260**, 11430–11435.
Ebina,Y,. Ellis,L., Jarnagin,K., Edery,M., Graf,L., Clauser,E., Ou,J., Masiarz,F., Kan,Y.W., Goldfine,I.D., Roth,R.A. and Rutter,W.J. (1985) *Cell*, **40**, 747–758.
Ebina,Y., Araki,E., Taira,M., Shimada,F., Mori,M., Craik,C.S., Siddle,K., Pierce,S.B., Roth,R.A. and Rutter,W.J. (1987) *Proc. Natl. Acad. Sci. USA*, **84**, 704–708.
Ellis,R.W., DeFeo,D., Shih,T.Y., Gonda,M.A., Young,H.A., Tsuchida,N., Lowy,D.R. and Scolnick,E.M. (1981) *Nature*, **292**, 506–511.
Ellis,L., Clauser,E., Morgan,D.O., Edery,M., Roth,R.A. and Rutter,W.J. (1986) *Cell*, **45**, 721–732.
Forsayeth,J.R., Caro,J.F., Sinha,M.K., Maddux,B.A. and Goldfine,I.D. (1987) *Proc. Natl. Acad. Sci. USA*, **84**, 3448–3451.
Fujita-Yamaguchi,Y., Choi,S., Sakamoto,Y. and Itakura,K. (1983) *J. Biol. Chem.*, **258**, 5045–5049.
Gammeltoft,S. and Van Obberghen,E. (1986) *Biochem. J.*, **235**, 1–11.
Glynn,I.M. and Chappell,J.B. (1964) *Biochem. J.*, **90**, 147–149.
Goding,J.W. (1986) *Monoclonal Antibodies: Principles and Practice*, 2nd edn. Academic Press, London.
Grigorescu,F., Herzberg,V., King,G., Meistas,M., Elders,J., Frazer,T. and Kahn,C.R. (1987) *J. Clin. Endoc. Metab.*, **64**, 549–556.
Haring,H.U., White,M.F., Machicao,F., Ermel,B., Schleicher,E. and Obermaier,B. (1987) *Proc. Natl. Acad. Sci. USA*, **84**, 113–117.
Hefferz,D. and Zick,Y. (1986) *J. Biol. Chem.*, **261**, 889–894.
Jacobs,S. and Cuatrecasas,P. (1983) *Annu. Rev. Pharmacol. Toxicol.*, **23**, 461–479.
Kahn,C.R., Baird,K.L., Jarrett,D.B. and Flier,J.S. (1978) *Proc. Natl. Acad. Sci. USA*, **75**, 4209–4213.
Koch,R., Deger,A., Jack,H.M., Klotz,K.N., Schenzle,D., Kramer,H., Kelm,S., Muller,G., Rapp,R. and Weber,U. (1986) *Eur. J. Biochem.*, **154**, 281–287.
Korn,L.J., Siebel,C.W., McCormick,F. and Roth,R.A. (1987) *Science*, **236**, 840–843.
Laemmli,U.K. (1970) *Nature*, **227**, 680–685.
Lamoyi,E. and Nisonoff,A. (1983) *J. Immunol. Methods*, **56**, 235–243.
Le Marchand-Brustel,Y., Gremeaux,T., Ballotti,R. and Van Obberghen,E. (1985) *Nature*, **315**, 676–679.
Linde,S., Hansen,B., Sonne,O., Holst,J.J. and Gliemann,J. (1981) *Diabetes*, **30**, 1–8.
Machicao,F., Haring,H., White,M.F., Carrascosa,J.M., Obermaier,B. and Wieland,O.H. (1987) *Biochem. J.*, **243**, 797–801.
Morgan,D.O. and Roth,R.A. (1987) *Proc. Natl. Acad. Sci. USA*, **84**, 41–45.
Morgan,D.O., Ho,L., Korn,L.J. and Roth,R.A. (1986) *Proc. Natl. Acad. Sci. USA*, **83**, 328–332.
O'Brien,R.M., Soos,M.A. and Siddle,K. (1986a) *Biochem. Soc. Trans.*, **14**, 316–317.
O'Brien,R.M., Soos,M.A. and Siddle,K. (1986b) *Biochem. Soc. Trans.*, **14**, 1021–1023.
O'Brien,R.M., Houslay,M.D., Milligan,G. and Siddle,K. (1987) *FEBS Lett.*, **212**, 281–288.
Perrotti,N., Accili,D., Marcus-Samuels,B., Rees-Jones,R.W. and Taylor,S.I. (1987) *Proc. Natl. Acad. Sci. USA*, **84**, 3137–3140.
Podesta,E.J., Solano,A.R., Attar,R., Sanchez,M.L. and Molina y Vedia,L. (1983) *Proc. Natl. Acad. Sci. USA*, **80**, 3986–3990.
Ponzio,G., Dolais-Kitabgi,J., Louvard,D., Gautier,N. and Rossi,B. (1987) *EMBO J.*, **6**, 333–340.
Schreiber,A.B., Libermann,T.A., Lax,I., Yarden,Y. and Schlessinger,J. (1983) *J. Biol. Chem.*, **258**, 846–853.
Simpson,I.A. and Hedo,J.A. (1984) *Science*, **223**, 1301–1304.
Soos,M.A., Siddle,K., Baron,M.D., Heward,J.M., Luzio,J.P., Bellatin,J. and Lennox,E.S. (1986) *Biochem. J.*, **235**, 199–208.
Stadtmauer,L. and Rosen,O.M. (1986) *J. Biol. Chem.*, **261**, 10000–10005.
Stanker,L.H., Vanderlaan,M. and Juarez-Salinas,H. (1985) *J. Immunol. Methods*, **76**, 157–169.
Tavare,J.M., Smyth,J.E., Borthwick,A.C., Brownsey,R.W. and Denton,R.M. (1985) *Biochem. Soc. Trans.*, **13**, 734–735.
Taylor,R., Soos,M.A., Wells,A., Argyraki,M. and Siddle,K. (1987) *Biochem. J.*, **242**, 123–129.
Ullrich,A., Bell,J.R., Chen,E.Y., Herrera,R., Petruzelli,L.M., Dull,T.J., Gray,A., Coussens,L., Liao,Y.C., Tsubokawa,M., Mason,A., Seeburg,P.H., Grunfeld,C., Rosen,O.M. and Ramachandran,J. (1985) *Nature*, **313**, 758–761.
White,M.F., Maron,R. and Kahn,C.R. (1985) *Nature*, **318**, 183–186.
Yip,C.C. and Moule,M.L. (1983) *Diabetes*, **32**, 760–767.
Yu,K.T. and Czech,M.P. (1984) *J. Biol. Chem.*, **259**, 5277–5286.
Zick,Y., Rees-Jones,R.W., Taylor,S.I., Gorden,P. and Roth,J. (1984) *J. Biol. Chem.*, **259**, 4396–4400.
Zick,Y., Sagi-Eisenberg,R., Pines,M., Gierschik,P. and Spiegel,A.M. (1986) *Proc. Natl. Acad. Sci. USA*, **83**, 9294–9297.

Received on August 31, 1987