The Carboxyl-Terminal Domain of the Insulin Receptor: Its Potential Role in Growth-Promoting Effects†

Véronique Baron, Nadine Gautier, Perla Kaliman, Jacqueline Dolais-Kitabgi, and Emmanuel Van Obberghen*
Laboratoire de Recherche sur les Hormones Polypeptidiques et la Physiopathologie Endocrinienne, INSERM U 145, Faculté de Médecine, Avenue de Valombrose, 06034 Nice Cédex, France
Received April 8, 1991; Revised Manuscript Received July 9, 1991

ABSTRACT: The role of the insulin receptor carboxyl-terminal domain in regulation of insulin signal transduction was studied with antipeptide antibodies against the sequence 1321–1338, which contains two autophosphorylation sites, tyrosine 1328 and tyrosine 1334. The antibodies were introduced by electroporation in murine fibroblasts transfected with an expression plasmid encoding the human insulin receptor. We found that introduction of these antipeptides into cells stimulated cellular proliferation, compared to cells loaded with nonimmune Ig. In contrast, neither glucose transport nor amino acid transport was stimulated by the antibodies. Despite its stimulatory effect on cell growth, the injected antipeptide did not enhance phosphorylation of ribosomal protein S6. In vitro, anti-C1 antipeptide stimulated insulin receptor autophosphorylation but did not increase receptor-mediated phosphorylation of the copolymer (glutamate/tyrosine, 4/1), while histone phosphorylation was increased. We interpret our results to mean that perturbation of the receptor C-terminus could lead to phosphorylation of selected substrates, which may be involved in cell growth regulation. Taken together, our data suggest that (i) insulin receptor mediated stimulation of cell growth and stimulation of ribosomal protein S6 phosphorylation result from divergent signaling pathways and (ii) the insulin receptor C-terminal domain exerts an inhibition on the growth signal mediated by the receptor. This inhibition appears to be released upon insulin binding to receptor or by interaction of the antipeptide with the receptor.

Insulin generates its biological effects through interaction with its specific cell surface receptor. This receptor is a tetrameric glycoprotein consisting of two extracellular α subunits and two transmembrane β subunits, linked together by disulfide bonds. The α subunit binds insulin, whereas the β subunit cytoplasmic domain contains an insulin-sensitive tyrosine kinase activity (Gammeltoft & Van Obberghen, 1986; Kahn & White, 1988; Rosen, 1987). Studies of the mechanism of receptor activation have led to the proposal of the following scenario. Insulin binding results in a conformational change in the receptor molecule, which is transmitted to the β subunit down to the C-terminal domain (Baron et al., 1990). The receptor becomes then competent for autophosphorylation, and this phosphorylation not only enhances the receptor tyrosine kinase activity toward substrates (Klein et al., 1986; Rosen et al., 1983; Yu & Czech, 1984) but also promotes another conformational change unmasking epitopes recognized by certain antipeptides (Herrera & Rosen, 1986; Perlman et al., 1989). Despite extensive research, the necessary links between insulin receptor activation and the final cellular responses are still missing. While putative substrates for the insulin receptor tyrosine kinase have been identified in intact cells, their role in insulin signaling has not been documented (Sadoul et al., 1985; Rees-Jones & Taylor, 1985; White et al., 1985). Studies using mutated receptors suggest that divergent signal transduction pathways may be involved in the generation of the pleiotropic cellular program evoked by insulin. Thus, a number of mutations in the insulin receptor β subunit affect differently the growth-promoting effects of insulin compared to the metabolic ones (Thies et al., 1989; Wilden et al., 1990).

† V.B. is a recipient of a fellowship from the Association pour la Recherche sur le Cancer (ARC) and P.K. a recipient of a Poste Vert INSERM. This work was supported by INSERM, Bayer-Pharma Industry, ARC (Grant 6432), Ligue Départementale contre le Cancer, and University of Nice-Sophia Antipolis.

The β-subunit C-terminal region contains two autophosphorylation sites, tyrosine 1328 and tyrosine 1334, which are probably not playing a determining role in receptor tyrosine kinase activation (Tornqvist & Avruch, 1988; White et al., 1988). Moreover, this domain is the place of conformational changes during the process of receptor activation and autophosphorylation (Baron et al., 1990). Thus, an intriguing question arises as to its role in signal transduction. To define the function of the C-terminus including the autophosphorylation sites, tyrosine 1328 and tyrosine 1334, we took advantage of an antipeptide antibody to this region which we previously reported to detect conformational changes in the receptor. We show here that our antibody stimulates cell growth without affecting metabolic responses, such as glucose and amino acid transport. Interestingly, the antipeptide does not enhance phosphorylation of ribosomal protein S6, which is a rapid effect of insulin (occurring within minutes) but which is commonly considered as part of the growth response (Kozma et al., 1989). Our results emphasize the notion that insulin- and/or phosphorylation-induced conformational changes allow removal of a constraint imposed by the C-terminus on receptor-mediated induction of cell growth.

Materials and Methods

(*1*) *Cell Culture.* NHIR cells, mouse embryo fibroblasts transfected with an expression plasmid encoding the human insulin receptor, were a gift from Jonathan Whittaker (Stony Brook, NY) (Whittaker et al., 1987). They were grown in DMEM (GIBCO) supplemented with 10% fetal calf serum (FCS).

(*2*) *Production and Purification of the Antipeptide.* Peptide C1 corresponds to the insulin receptor sequence 1321–1338 according to the sequence published by Ebina et al. (1985). Antibodies were produced and partially purified on protein A–Sepharose as described previously (Baron et al., 1990).

0006-2960/91/0430-9365$02.50/0

Affinity purification was performed with a column of peptide bound to Sepharose. The elution procedure consisted of using the following buffers sequentially: (i) buffer 1 containing 0.1 M glycine and 0.5 M NaCl, pH 2, followed by a wash with PBS (phosphate-buffered saline), pH 7.5, and (ii) buffer 2 containing 3.5 M NaSCN in PBS, pH 7.5. The antibodies were then dialyzed against PBS and concentrated to 2 mg/mL.

(*3*) *Cell-Free Insulin Receptor Autophosphorylation and Phosphorylation of Substrates.* Insulin receptors from NHIR cells were partially purified as previously described by chromatography on wheat germ agglutinin (Van Obberghen et al., 1981). These receptors (300 fmol/sample) were incubated with partially purified antipeptide antibodies or preimmune immunoglobulins for 2 h at 22 °C, and when indicated insulin (10^{-7} M) was added for 1 h. Receptor autophosphorylation was initiated by addition of 15 μM [γ-^{32}P]ATP (2.5 mCi/mmol), 4 mM $MnCl_2$, and 8 mM $MgCl_2$, for the periods mentioned in the figure legends. For measurement of substrate phosphorylation, the receptor was preincubated with either the copolymer (Glu/Tyr, 4/1) (0.2 mg/mL) or histone H2b (50 μg/sample), the phosphorylation mixture was added, and phosphorylation was measured during 30 min for the copolymer (Glu/Tyr) and during 15 min for histone. Phosphate incorporation into the copolymer (Glu/Tyr) was determined as previously described with a filter paper assay (Le Marchand-Brustel et al., 1985). Histone phosphorylation and receptor autophosphorylation were stopped by addition of Laemmli sample buffer containing 5% β-mercaptoethanol, and the phosphoproteins were analyzed by one-dimensional SDS–polyacrylamide gel electrophoresis (Laemmli, 1977).

(*4*) *Transfer of Antipeptide Antibodies into Cells.* Transfer was performed by electroporation as described by Chakrabarti et al. (1989) using the Electropulsing Unit Apelex, Paris, France. Exponentially growing cells were used to optimize the efficiency of electroporation. Briefly, cells were trypsinized, washed once with DMEM/10% FCS and once with PBS, and resuspended in PBS at 2.5 × 10^6 cells/mL containing 250 μg/mL purified anti-C1 or nonimmune Ig. Cells were transferred to the sterile electroporation chambers and exposed to a single electric pulse of 750 V/cm for 0.93 ms at 23 °C. They were maintained for 10 min at 4 °C. Then, they were resuspended into DMEM/10% FCS and plated. When fully attached (approximately 3 h after electroporation), cells were washed three times with PBS and placed in DMEM containing 10% FCS or 0.2% BSA.

Incorporation into cells was quantitated by performing the electroporation protocol described above in the presence of immunoglobulins labeled with ^{125}I. After 4 h the attached cells were washed with PBS and placed in DMEM/10% FCS. The next day they were washed three times with PBS, membranes were disrupted, and cell lysates were centrifuged at 15000*g* for 20 min. Radioactivity accumulated in the cytosols of electroporated and nonelectroporated cells was counted. Under these experimental conditions, approximately 6 μg of Ig/mL of cell volume was transferred into cells.

(*5*) *Glucose and Amino Acid Transport.* Cells were loaded with anti-C1 or nonimmune Ig and plated in 12-well dishes with DMEM containing 10% serum. Fifteen hours after electroporation, they were washed twice with PBS and incubated for 3 h in DMEM supplemented with 0.2% BSA and then for an additional 3-h period without or with insulin (10^{-7} M). Glucose transport and amino acid transport were measured as described by Le Marchand-Brustel et al. (1978) and Fehlmann et al. (1983), respectively. Briefly, cells were washed with KRP/HEPES buffer (glucose transport) or KRB buffer (amino acid uptake) and incubated for 15 min in the corresponding buffers containing 0.2% BSA in the absence or presence of insulin. Thereafter, 0.1 mM [^{3}H]deoxyglucose (DOG; 0.2 μCi/well) or 0.1 mM [^{14}C]aminoisobutyric acid (AIB; 0.1 μCi/well) was added for 5 and 30 min, respectively. After three washes with cold PBS, cells were solubilized in NaOH for scintillation counting.

Table I: Effect of Anti-C1 on Cell Growth[a]

Ig	cell number (% of cell number at day 0)			
	0[b]	1[b]	2[b]	3[b]
nonimmune	100	96 ± 0.7	100 ± 5.4	107 ± 7.8
anti-C1	100	126 ± 7.8	148 ± 13	180

[a] Cells were loaded with nonimmune or anti-C1 Ig and placed in DMEM/0.2% BSA, as described under Materials and Methods. Cell number was determined at 1, 2, and 3 days after electroporation. Results are means ± SEM of three wells. [b] Days after cell loading.

(*6*) *Cell Proliferation.* Nonimmune Ig and purified antipeptides were transferred into cells, seeded in six-well culture plates. Four hours after electroporation, attached cells were washed three times in PBS and incubated for 2 days in DMEM/0.2% BSA supplemented with buffer, insulin (10^{-7} M), or serum (10%). The medium was removed and fresh DMEM/BSA without or with effectors was added. The next day cells were trypsinized and resuspended in 1 mL of DMEM/10% FCS, and the cell number was determined with a Coulter counter (Coultronics, Paris, France).

(*7*) *Phosphorylation of Ribosomal Protein S6.* Fifteen hours after electroporation, injected cells (12 × 10^6 cells/dish) were incubated in DMEM/0.2% BSA for 4 h and then with [^{32}P]orthophosphate (0.5 mCi/mL) in a phosphate-free buffer containing 0.2% BSA for 3 h at 37 °C and, finally, exposed to buffer, insulin (10^{-7} M), or serum (10%) for 15 min. Cells were solubilized in 50 mM Tris-HCl, pH 7.4, 1.5 mM KCl, 5 mM $MgCl_2$, 1% Triton X-100, 1% deoxycholic acid, 100 mM sodium fluoride, 200 μM sodium vanadate, 100 mM sodium tetraphosphate, and protease inhibitors. Samples were subjected to a first centrifugation during 30 min at 15000*g*. Supernatants were then centrifuged on a sucrose cushion for 15 h at 170000*g*. Pellets containing total ribosomal proteins were analyzed by SDS–PAGE in reducing conditions on a 10% acrylamide gel (Nielsen et al., 1982).

Results

To investigate the role of the insulin receptor C-terminus in regulation of receptor-mediated responses, we transferred the antipeptide, anti-C1, into murine fibroblasts expressing human insulin receptors and studied its effects on a series of biological responses including glucose and amino acid uptake, cellular growth, and ribosomal protein S6 phosphorylation.

(*1*) *Effect of Anti-C1 Antipeptide on Cell Growth.* We first tested the effect of anti-C1 antipeptide at different times after cell injection. Cells were loaded with nonimmune or anti-C1 Ig and incubated in DMEM/0.2% BSA. The cell number was determined at 1, 2, and 3 days after electroporation. As shown in Table I, anti-C1 stimulates the cell growth, this effect being observed as early as 1 day after cell loading and increasing until day 3. We could not prolong the experiment further, since after 4 days in serum-deprived medium the cell number sharply decreased in all conditions. Therefore, other experiments concerning cell growth measurement were performed 3 days after electroporation.

In these experiments, cells were loaded with nonimmune Ig or purified anti-C1 and incubated in DMEM/0.2% BSA or in the same medium enriched with insulin (10^{-7} M) or FCS (10%). The cell number was determined after 3 days. As

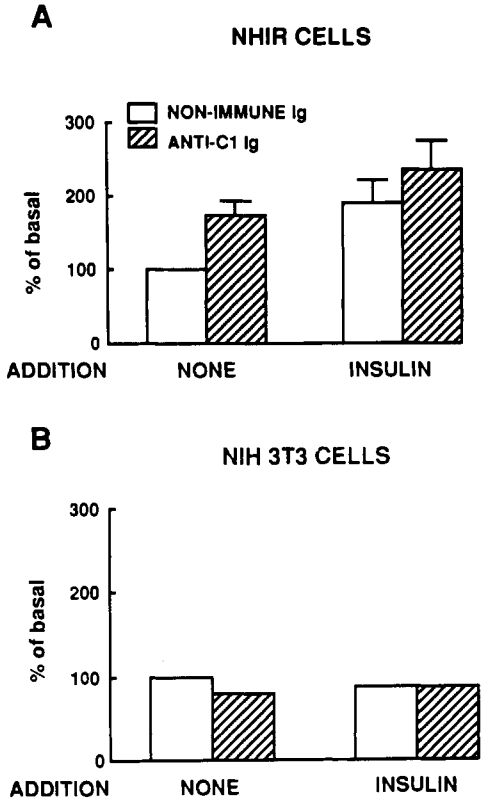

FIGURE 1: Effect of anti-C1 antipeptide on cellular growth. Cells injected with nonimmune or anti-C1 Ig were placed in DMEM/0.2% BSA, without or with insulin (10^{-7} M). After 2 days, the medium was changed, and after an additional day, cell numbers were determined with a Coulter counter. (A) NHIR cells, mouse fibroblasts transfected with an expression plasmid encoding the human insulin receptor, were used. Results are the means ± SEM of five experiments, each run in triplicate. (B) Untransfected NIH 3T3 cells were used. This is the mean of two experiments run in triplicate.

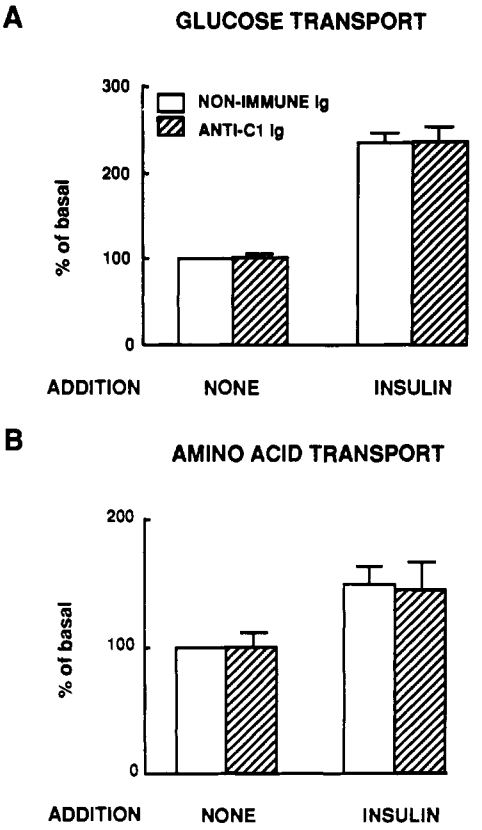

FIGURE 2: Effect of anti-C1 antibodies on glucose and amino acid transport. Cells loaded with nonimmune or anti-C1 Ig were incubated without or with insulin (10^{-7} M) for 3 h. After measurement of transport, cells were solubilized in NaOH and the incorporated radioactivity was counted after addition of scintillation fluid. (A) [^{3}H]Deoxyglucose was added for 5 min. Results are the means ± SEM of five experiments. (B) [^{14}C]Aminoisobutyric acid was added for 30 min. The means ± SEM of three experiments are shown.

shown in Figure 1A, electroporation does not impair the capacity of cells to respond to insulin. Indeed, the hormone stimulates NHIR cell growth by a factor of 2 approximately as compared to unstimulated cells. Cells loaded with anti-C1 have a proliferation rate 2-fold higher than cells loaded with nonimmune Ig. Thus, the antibody is able to induce cell proliferation, and the anti-C1 stimulation of cell growth is of the same magnitude as the hormone-induced one. Further, the effects of the antipeptide and insulin are not additive.

Anti-C1 does not appear to modify the level of serum stimulation, since maximal growth stimulation, obtained with 10% FCS, was 502 ± 119% of basal levels in control cells and 708 ± 254% in cells loaded with anti-C1 (data not shown).

To ensure that the anti-C1 effect on cell growth was due to a direct interaction with human insulin receptors, the antibody was injected into NIH 3T3 cells, which were not transfected with human insulin receptor cDNA and possess few endogenous murine insulin receptors. Figure 1B shows that, in these cells, injection of anti-C1 antipeptide does not result in increased proliferation compared to cells loaded with nonimmune Ig. Likewise, insulin does not stimulate NIH 3T3 cell growth. Taken together, our results indicate that the increase in cellular proliferation seen with anti-C1 antibodies is due to their interaction with human insulin receptors.

(*2*) *Effect of Anti-C1 on Glucose and Amino Acid Transport.* The effect of anti-C1 on glucose and amino acid transport, which are two metabolic responses stimulated by insulin, was measured 15 h after electroporation. As illustrated in Figure 2, insulin enhances both uptake of glucose and of amino acid in cells injected with nonimmune Ig. In contrast to its stimulatory action on cell growth, anti-C1 antipeptide is without effect on basal and on insulin-induced transport of glucose and amino acid.

(*3*) *Effect of Anti-C1 on Ribosomal Protein S6 Phosphorylation.* Since anti-C1 was shown to stimulate cell growth, it was of interest to investigate whether phosphorylation of ribosomal protein S6 was also increased by the antibody. Figure 3A represents the results observed 24 h after electroporation of cells with antibody. Injected cells were labeled with [^{32}P]orthophosphate and incubated for 15 min with buffer, insulin, or serum before solubilization. As expected, both insulin and serum stimulated protein S6 phosphorylation. However, no effect was observed in cells electroporated with anti-C1 alone. Several lengths of insulin exposure as well as different postelectroporation periods were tested. In the experiment shown in Figure 3B, insulin was added after cell recovery and maintained during 24 h. Thus, cells were exposed to anti-C1 or insulin during the same period. Insulin stimulation of protein S6 phosphorylation was still observed after this period, but no stimulation by the antipeptide anti-C1 could be detected. The anti-C1 lack of effect on S6 phosphorylation was still observed 3 days after its injection into cells, the time at which we measured cellular growth (data not shown). In conclusion, antibodies to the receptor C-terminus are able to stimulate cell multiplication, but they do not induce detectable phosphorylation of ribosomal protein S6.

(*4*) *Effect of Anti-C1 on in Vitro Insulin Receptor Kinase Activity.* The electroporation technique used in this study requires a recovery period between cell loading and measurement of biological responses. As insulin-induced receptor

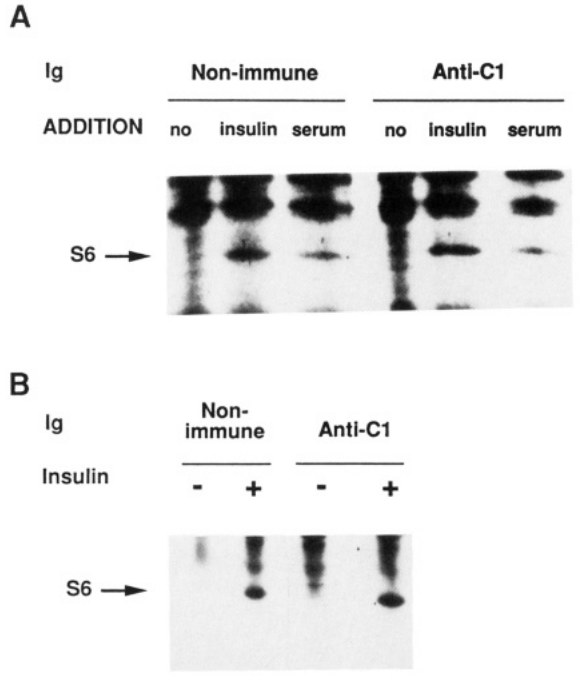

FIGURE 3: Effect of anti-C1 on ribosomal protein S6 phosphorylation. (A) Cells loaded with nonimmune or anti-C1 Ig were placed in a phosphate-free buffer containing 0.2% BSA and [^{32}P]orthophosphate (0.5 mCi/mL) during 3 h at 37 °C. Then buffer, insulin (10^{-7} M), or fetal calf serum (10% v/v) was added for 15 min. Ribosomal proteins were extracted and analyzed by SDS–PAGE chromatography. (B) The experiment was performed as described above, except that insulin was added at the time of electroporation and maintained during the experiment.

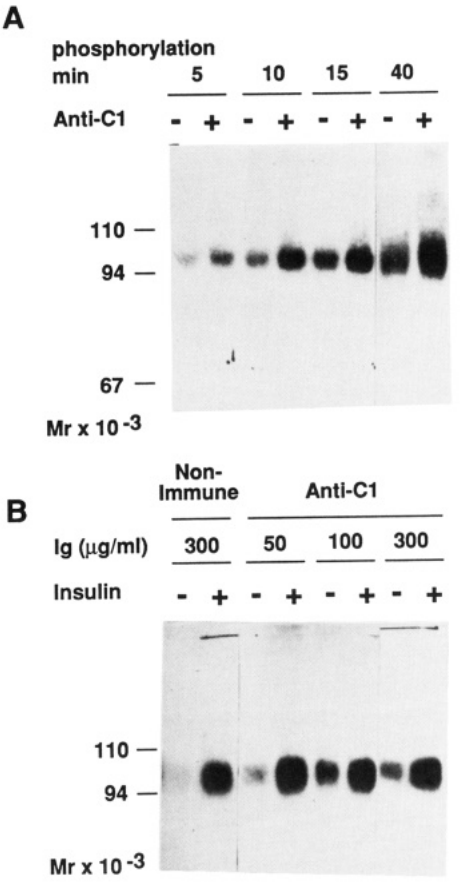

FIGURE 4: Effect of anti-C1 on cell-free insulin receptor autophosphorylation. (A) Partially purified receptors were incubated with anti-C1 antibodies or nonimmune Ig for 2 h at 22 °C and then with [γ-^{32}P]ATP during 5, 10, 15, or 40 min. The phosphorylation reaction was stopped by addition of SDS (3% v/v), and samples were analyzed by SDS–PAGE under reducing conditions. (B) Insulin receptors were incubated with anti-C1 or nonimmune Ig for 2 h at 22 °C and then without or with insulin (10^{-7} M) for 60 min. Finally, [γ-^{32}P]ATP was added for 15 min and phosphorylation was stopped as described in (A).

autophosphorylation and phosphorylation of endogenous substrates occur within minutes, we could not investigate the effect of the antipeptide in intact cells. Therefore, we used receptor cell-free phosphorylation.

Figure 4 shows the effect of the antipeptide on receptor autophosphorylation in the absence of insulin and at various phosphorylation times. Anti-C1 increased receptor autophosphorylation compared to nonimmune Ig (Figure 4A). However, when compared to the insulin-induced receptor phosphorylation illustrated in Figure 4B, the magnitude of the antipeptide stimulation was smaller than the one obtained with insulin, even at optimal antibody concentration.

We next examined the effect of anti-C1 antipeptide on receptor-mediated phosphorylation of two exogenous substrates. The antibody had a weak effect on phosphorylation of poly(Glu/Tyr) (Figure 5A). Indeed, at a concentration of anti-C1 which was found to be optimal for stimulation of receptor autophosphorylation, phosphate incorporation into poly(Glu/Tyr) amounted to 171 ± 15% of basal. For comparison, insulin produced a 480% increase in phosphorylation of this substrate.

In contrast, our antipeptide was able to stimulate phosphate incorporation into histone (Figure 5B). The increase amounted to 1361% of basal with 100 μg/mL anti-C1 and to 3265% of basal with insulin (10^{-7} M). Note that the magnitude of antipeptide stimulation of histone phosphorylation correlated well with its effect on receptor autophosphorylation.

Discussion

In a previous work we demonstrated that the insulin receptor C-terminal domain participates in conformational changes which occur after insulin binding (Baron et al., 1990). This observation led us to believe that this receptor domain could be implicated in the generation and/or regulation of insulin's biological effects. To test our views, antipeptide antibodies against the receptor C-terminal domain comprising amino acids 1321–1338 were introduced into cells.

The use of the electroporation technique allows accumulation of macromolecules into cells with a low cell mortality (<10%). The alternative approach to define the role of certain receptor domains in biological signaling—i.e., expression of mutated receptors—has a major drawback as basal activities and sensitivities to ligands generally differ among the transfected cell lines. The method based on the microinjection of antipeptides into cells to study receptor domains does not suffer from this problem as the receptor is the same in all conditions studied.

We show here that glucose transport and amino acid transport were not modified in cells injected with anti-C1 antipeptide compared to cells injected with nonimmune Ig, independently of the presence or absence of insulin. By contrast, an increased cell growth was found in cells loaded with anti-C1 in the absence of insulin. This stimulatory action was mediated through the transfected human insulin receptors, since the antibodies had no effect in cells which were not transfected with human insulin receptor cDNA.

The results showing an increase in cellular proliferation after anti-C1 injection are in agreement with studies using C-terminal deleted insulin receptor mutants, since expression of this mutant receptor into cells led to increased growth compared

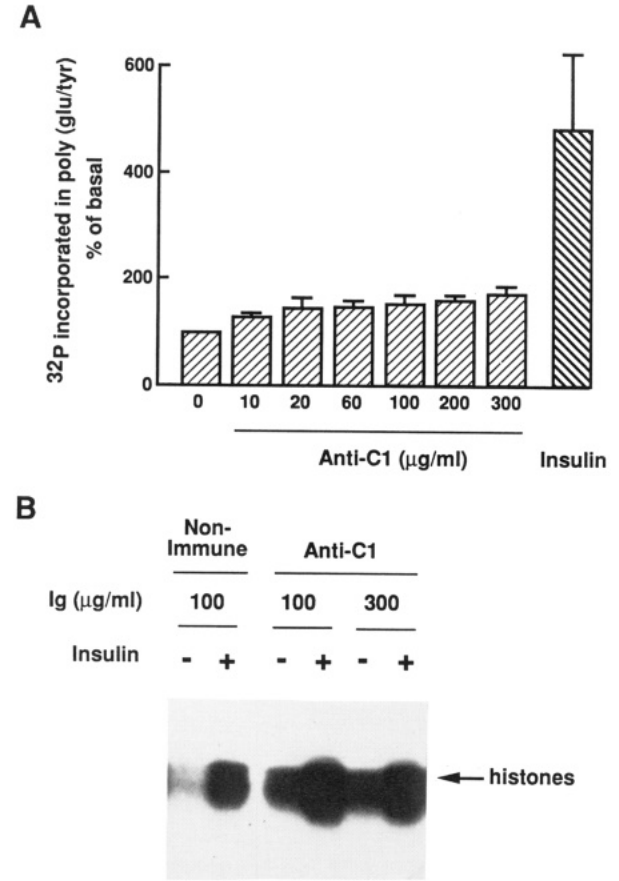

FIGURE 5: Effect of anti-C1 on receptor tyrosine kinase activity toward substrates. Partially purified insulin receptors were incubated with anti-C1 or nonimmune Ig for 2 h at 22 °C, prior to addition of buffer or insulin (10^{-7} M) for 60 min. (A) Poly(Glu/Tyr) at a final concentration of 0.2 mg/mL was added with the phosphorylation mix. After 30 min at 22 °C, the reaction was terminated by precipitation on filter papers following addition of TCA (10% v/v). Phosphate incorporation was determined by Cherenkov counting. Data are the means ± SEM of five separate experiments. (B) Histone H2b (50 μg/sample) was added with [γ-^{32}P]ATP for 15 min at 22 °C, and the reaction was stopped as described in the legend to Figure 4A. Samples were analyzed by SDS–PAGE electrophoresis. An autoradiogram of the gel is shown.

to cells carrying native receptors (Thies et al., 1989). However, these truncated receptors display in addition a low activity in mediating insulin-stimulated "metabolic" responses such as glucose transport and glycogen synthase (Maegawa et al., 1988; McClain et al., 1988). At variance with these observations, we did not observe any change in glucose transport or amino acid uptake in anti-C1 electroporated cells. This interesting difference may be due to the fact that the receptors we use possess the native receptor C-terminus.

Taken together, our data indicate that the insulin receptor C-terminal domain is likely involved in signaling. This role could be operating at least at two levels, namely, (i) recognition of metabolic substrates and (ii) imposition of a constraint on some cellular growth signal, which is released upon insulin binding.

An intriguing observation of the present work is the lack of anti-C1 effect on ribosomal protein S6 phosphorylation. Although we did observe that insulin stimulation of protein S6 phosphorylation persists over 72 h, the possibility exists that anti-C1 antipeptide stimulates S6 phosphorylation in a transient way or with a rapid dephosphorylation. The most simple explanation of our findings could be that the kinases, which are thought to be links between the insulin receptor tyrosine kinase and the S6 kinase, are not good substrates for the anti-C1-activated receptor. This view is supported by our findings that, at least in a cell-free system, the insulin receptor activated by anti-C1 appears to have a more limited substrate choice compared to receptors stimulated by insulin. Hence, anti-C1 could favor receptor association with cellular proteins, which are thought to be involved in generation of mitogenic signals and which appear to be independent of protein S6 phosphorylation.

Usually insulin and growth factors modify the phosphorylation of S6 protein and cellular growth in a parallel fashion. Our data could indicate that the pathways involved in insulin stimulation of protein S6 phosphorylation and of cellular growth may be differentially regulated and that the insulin receptor C-terminal domain may play a role in generation of this divergency. To our knowledge, this is the first observation that phosphorylation of S6 protein seems to be uncoupled from cellular proliferation in the case of a receptor tyrosine kinase.

To explain the biological effects induced by our antipeptide, we propose that anti-C1 antibodies induce or stabilize a receptor conformation, which leads to a modification in receptor interaction with a particular set of substrates directly involved in growth control. Alternatively, our antipeptides could interfere with receptor autophosphorylation, especially in the C-terminal domain. Indeed, we observed that, in a cell-free system, anti-C1 increases receptor autophosphorylation. Interestingly, a previous report has shown that antipeptides against the sequence 1340–1355 in the C-terminal domain have no effect on receptor autophosphorylation or on kinase activity toward substrates (Herrera et al., 1985). This difference could be due to the fact that our antipeptide is directed against the autophosphorylation sites 1328 and 1334. The phosphorylation state of tyrosine kinase receptors appears to be crucial for association with regulatory proteins such as the raf1 kinase or phosphatidylinositol 3-kinase (PI3-kinase) (Cantley et al., 1991; Ullrich & Schlessinger, 1990). Furthermore, it has recently been shown that the tyrosine phosphorylated C-terminal domain of the EGF receptor is the binding site for PLC-γ (Margolis et al., 1990; Skolnik et al., 1991). Incidentally, the insulin receptor domain which is recognized by our antipeptide resembles the consensus sequence for receptor binding to PI3-kinase (Cantley et al., 1991).

After this paper was submitted, it was reported by Takata et al. (1991) that a receptor mutated at the two autophosphorylation sites, 1328 and 1334, has an unaltered capacity to transmit metabolic signals, whereas this mutated receptor displays enhanced insulin-induced mitogenic signals. This is in total agreement with our study and further documents the specific function of the receptor C-terminal part in cell growth regulation.

ACKNOWLEDGMENTS

We thank Drs. Y. Le Marchand-Brustel and Ellen Van Obberghen-Schilling for careful reading of the manuscript and for fruitful discussions. We acknowledge Dr. Drobecq and Dr. Lavielle (France) for peptide C1 synthesis.

Registry No. Tyr, 60-18-4; insulin, 9004-10-8; insulin receptor tyrosine kinase, 88201-45-0; D-glucose, 50-99-7.

REFERENCES

Baron, V., Gautier, N., Komoriya, A., Hainaut, P., Scimeca, J. C., Mervic, M., Lavielle, S., Dolais-Kitabgi, J., & Van Obberghen, E. (1990) *Biochemistry 29*, 4634–4641.

Cantley, L. C., Auger, K. R., Carpenter, C., Duckworth, B., Graziani, A., Kapeller, R., & Soltoff, S. (1991) *Cell 64*, 281–302.

Chakrabarti, R., Wylie, D. E., & Schuster, S. M. (1989) *J. Biol. Chem. 264*, 15494-15500.
Ebina, Y., Ellis, L., Jarnagin, K., Edery, M., Graf, L., Clauser, E., Ou, J.-H., Masiarz, F., Kan, Y. W., Goldfine, I. D., Roth, R. A., & Rutter, W. J. (1985) *Cell 40*, 747-758.
Fehlmann, M., Crettaz, M., & Kahn, R. (1983) *Biochem. J. 214*, 845-850.
Gammeltoft, S., & Van Obberghen, E. (1986) *Biochem. J. 235*, 1-11.
Herrera, R., & Rosen, O. M. (1986) *J. Biol. Chem. 261*, 11980-11985.
Herrera, R., Petruzzelli, L., Thomas, N., Bramson, H. N., Kaiser, E. T., & Rosen, O. M. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 7899-7903.
Kahn, C. R., & White, M. F. (1988) *J. Clin. Invest. 82*, 1151-1156.
Klein, H. H., Freidenberg, G. R., Kladde, M., & Olefsky, J. M. (1986) *J. Biol. Chem. 261*, 4691-4697.
Kozma, S. C., Ferrari, S., & Thomas, G. (1989) *Cell. Signalling 1*, 219-225.
Laemmli, U. K. (1977) *Nature 277*, 680-685.
Le Marchand-Brustel, Y., Jeanrenaud, B., & Freychet, P. (1978) *Am. J. Physiol. 234*, E348-E358.
Le Marchand-Brustel, Y., Grêmeaux, T., Ballotti, R., & Van Obberghen, E. (1985) *Nature 315*, 676-679.
Maegawa, H., McClain, D. A., Freidenberg, G., Olefsky, J. M., Napier, M., Lipari, T., Dull, T. J., Lee, J., & Ullrich, A. (1988) *J. Biol. Chem. 263*, 8912-8917.
Margolis, B., Li, N., Koch, A., Mohammadi, M., Hurwitz, D. R., Zilberstein, A., Ullrich, A., Pawson, T., & Schlessinger, J. (1990) *EMBO J. 9*, 4375-4380.
McClain, D. A., Maegawa, H., Levy, J., Hueckstead, T., Dull, T. J., Lee, J., Ullrich, A., & Olefsky, J. M. (1988) *J. Biol. Chem. 263*, 8904-8911.
Nielsen, P. J., Thomas, G., & Maller, J. L. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 2937-2941.
Perlman, R., Bottaro, D. P., White, M. F., & Kahn, C. R. (1989) *J. Biol. Chem. 264*, 8946-8950.
Rees-Jones, R. W., & Taylor, S. I. (1985) *J. Biol. Chem. 260*, 4461-4467.
Rosen, O. M. (1987) *Science 237*, 1452-1458.
Rosen, O. M., Herrera, R., Olowe, Y., Petruzzelli, L. M., & Cobb, M. H. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 3237-3240.
Sadoul, J. L., Peyron, J. F., Ballotti, R., Debant, A., Fehlmann, M., & Van Obberghen, E. (1985) *Biochem. J. 227*, 887-892.
Skolnik, E. Y., Margolis, B., Mohammadi, M., Lowenstein, E., Fischer, Drepps, A., Ullrich, A., & Schlessinger, J. (1991) *Cell 65*, 83-90.
Takata, Y., Webster, N. J. G., & Olefsky, J. M. (1991) *J. Biol. Chem. 266*, 9135-9138.
Thies, R. S., Ullrich, A., & McClain, D. A. (1989) *J. Biol. Chem. 264*, 12820-12825.
Tornqvist, H. E., & Avruch, J. (1988) *J. Biol. Chem. 263*, 4593-4601.
Ullrich, A., & Schlessinger, J. (1990) *Cell 61*, 203-212.
Van Obberghen, E., Kasuga, M., Le Cam, A., Hedo, J. A., Itin, A., & Harrison, L. C. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 1052-1056.
White, M. F., Maron, R., & Kahn, C. R. (1985) *Nature 318*, 183-186.
White, M. F., Shoelson, S. E., Keutmann, H., & Kahn, C. R. (1988) *J. Biol. Chem. 263*, 2969-2980.
Whittaker, J., Okamoto, A. K., Thys, R., Bell, G. I., Steiner, D. F., & Hofmann, C. A. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 5237-5241.
Wilden, P. A., Backer, J. M., Kahn, C. R., Cahill, D. A., Schroeder, G. J., & White, M. F. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 3358-3362.
Yu, K-T., & Czech, M. P. (1984) *J. Biol. Chem. 259*, 5277-5286.

α-Conotoxins, Small Peptide Probes of Nicotinic Acetylcholine Receptors†

Richard A. Myers, Glenn C. Zafaralla, William R. Gray, Jeff Abbott, Lourdes J. Cruz, and Baldomero M. Olivera*
Department of Biology, University of Utah, Salt Lake City, Utah 84112
Received March 11, 1991; Revised Manuscript Received June 4, 1991

ABSTRACT: α-Conotoxins, a family of small peptides from the venoms of the *Conus* marine moluscs, are selective, snake α-neurotoxin-competitive antagonists of the nicotinic acetylcholine receptor. A new α-conotoxin, SIA, has been purified, sequenced, and synthesized. Cross-linking with bivalent reagents and photoaffinity labeling of the acetylcholine receptor with α-conotoxin yield covalent adducts. Surprisingly, cross-linking to other subunits is considerably more efficient than to the α subunit. The relative efficiency of photoactivatable cross-linking to different subunits of the receptor is a function of placement of the photoactivatable group on the toxin. Since the structures of α-conotoxins can be solved by 2D NMR [see Pardi et al. (1989) *Biochemistry 28*, 5494-5508; Kobayashi et al. (1989) *Biochemistry 28*, 4853-4860], this family of toxins should provide a set of new ligands for probing the acetylcholine receptor with considerable precision.

The fish-hunting cone snails (*Conus*) use venoms to paralyze their faster moving prey. These venoms are complex mixtures of peptides, each targeted to a particular macromolecular receptor (Olivera et al., 1989, 1990). The α-conotoxins, the subject of this report, are paralytic peptides found in the venoms which target to the nicotinic acetylcholine receptor (Gray et al., 1981; Olivera et al., 1990). Other characterized peptides in *Conus* venoms target to presynaptic calcium channels (the ω-conotoxins; Olivera et al., 1984), muscle sodium channels (the μ-conotoxins; Cruz et al., 1987), and

† This work was supported by Grants GM22737 and 34913 from the U.S. Public Health Service.
* To whom correspondence should be addressed.

0006-2960/91/0430-9370$02.50/0