Vol. 117, No. 3, 1983 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
December 28, 1983 Pages 885-893

DEPHOSPHORYLATION OF THE HEPATIC INSULIN RECEPTOR: ABSENCE OF INTRINSIC PHOSPHATASE ACTIVITY IN PURIFIED RECEPTORS

Aline Kowalski, Hélène Gazzano, Max Fehlmann and Emmanuel Van Obberghen*

Groupe de Recherches sur les Hormones Polypeptidiques et la Physiopathologie Endocrinienne (I.N.S.E.R.M. U 145)
Faculté de Médecine, Avenue de Vallombrose, 06034 Nice Cédex, France

Received November 7, 1983

ABSTRACT: We have compared here the reversibility of phosphorylation of insulin receptors either partially purified by lectin chromatography, or highly purified by specific immunoprecipitation with anti-receptor antibodies. We found that the β subunit of partially purified insulin receptors was rapidly dephosphorylated (t ½= 15 min). In contrast, the level of phosphorylation of immunoprecipitated receptors remained unchanged for up to 4 hours at 37°C. However, cytosolic phosphatases, which are inhibited by vanadate, were able to induce a complete dephosphorylation of immunoprecipitated receptors. These results show that 1. phosphorylation of insulin receptors is reversible; and 2. no phosphatase activity is contained in the insulin receptor structure itself.

The native insulin receptor is a glycoprotein complex composed of two M_r 130,000 (α) and two M_r 95,000 (β) subunits linked together by disulphide bonds. Binding of insulin to its receptors located on the plasma membrane of target cells triggers an array of biochemical events culminating in the final biological effects of the hormone (1). In search of initial events in the mechanism of insulin action, different laboratories, including our own, have reported that insulin enhances the phosphorylation of the β subunit of its receptor both in intact cells (2, 3) and cell-free systems (3-6). Further, the identification of an ATP binding site on the β receptor subunit (6-8), and the demonstration that

* To whom all correspondence should be addressed.

0006-291X/83 $1.50

partially purified (9) and immunoprecipitated receptors (10) can catalyse the phosphorylation of exogenous substrates strongly indicate that the insulin receptor in an insulin-activated protein kinase. It has become also clear that the reversible covalent modification of enzymes and regulatory proteins through phosphorylation and dephosphorylation represents a critical post-translational mechanism of control for the regulation of various biological processes (11). Likewise, for the biological relevance of the concept of insulin receptor phosphorylation as crucial early step in insulin action, the covalent modification of the receptor must be reversible, and regulated by cellular protein phosphatases. In the present work, we have addressed the following questions of (i) the reversibility of the insulin receptor phosphorylation, and (ii) the existence of a putative protein phosphatase activity associated with the insulin receptor.

MATERIALS AND METHODS

Materials Porcine insulin (monocomponent) was purchased from Novo (Copenhagen, Denmark); collagenase (type II) was from Worthington; Nonidet P-40, adenosine 5'-triphosphate, N-acetyl D-glucosamine, wheat germ agglutinin-agarose, bovine serum albumin (fraction V), and bacitracin were from Sigma. Adenosine 5'-(γ-^{32}P)triphosphate, triethylammonium salt (aqueous solution, 5,000 Ci/mmol) was from the Radiochemical Centre Amersham, (U.K.); Staphylococcus aureus cells (Pansorbin) from Calbiochem-Behring. All reagents for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) were purchased from Biorad. Serum from patient B_2 with autoantibodies to the insulin receptor was a generous gift from C.R. Kahn (Joslin Research Laboratory, Boston, MA, USA) (1).

Methods Insulin receptors were partially purified from male Wistar rat livers (3, 6) and immunoprecipitated with specific anti-receptor antibodies and protein A (Pansorbin) as previously described (3, 6, 12).

Phosphorylation and dephosphorylation of insulin receptors: partially purified or immunoprecipitated insulin receptors were incubated in a reaction mixture containing Hepes (20 mM; pH 7.6), NaCl (30 mM), $MnCl_2$ (4 mM), $MgCl_2$ (8 mM), and, if present, insulin (0.1 μM). To allow for insulin binding, the tubes were incubated for 2 hr at 20°C. Thereafter, the phosphorylation reaction was initiated by the addition of (γ-^{32}P) ATP (20 μM). The reaction tubes were incubated for 15

min at 24°C. The reaction was terminated by addition of unlabeled ATP at a final concentration of 20mM. To study dephosphorylation, the phosphorylated, partially purified or immunoprecipitated, receptors were incubated in the presence of unlabeled ATP, $MnCl_2$, and $MgCl_2$ at final concentrations of 5 mM, 4 mM, and 8 mM, respectively. At different time-intervals aliquots were taken and the reaction stopped by addition of boiling SDS solution.

Polyacrylamide Gel Electrophoresis and Autoradiography: aliquots of the phosphorylated partially purified or immunoprecipitated insulin receptors were boiled for 5 min in a solution containing 3% (w/v) SDS, 10% (v/v) glycerol, 10 mM sodium phosphate, 2% (v/v) 2-mercaptoethanol, and 0.01% bromophenol blue. The aliquots were analyzed by one-dimensional SDS-PAGE as described by Laemmli (13), with a 5-15% linear gradient of acrylamide or a 7.5% acrylamide gel as the resolving gel. The M_{rs} of the standards used were: myosin, 200,000; β-galactosidase, 116,000; phosphorylase B, 94,000; bovine serum albumin, 67,000; ovalbumin, 43,000; carbonic anhydrase, 30,000; soybean trypsin inhibitor, 20,000; and lysozyme, 14,400. The gels were processed as described (3, 6). The autoradiograms were scanned in a microdensitometer (Gelman) for quantitative analysis.

RESULTS

1. Partially purified insulin receptors.

We first investigated whether partially purified insulin receptors obtained by wheat germ lectin chromatography could be dephosphorylated. To this end partially purified receptors were first incubated with (γ-^{32}P)ATP together with insulin to allow for maximal phosphorylation of the insulin receptor. As we have previously demonstrated (3, 6) and as shown in figure 1, lane A, the β-subunit of the insulin receptor with M_r 94,000 is found to be heavily phosphorylated under these conditions. Evidence that this polypeptide with M_r 94,000 is indeed the β subunit of the insulin receptor is shown in fig. 2, where the phosphorylated partially purified receptors were immunoprecipitated with highly specific autoantibodies to the insulin receptor. When the phosphorylated partially purified insulin receptors were incubated at 24°C in the presence of an excess of unlabeled ATP, a rapid decrease in the labeling of the β insulin receptor subunit was observed (fig. 1 and 2). Thus, quantitative scanning revealed a loss in ^{32}P from the β subunit

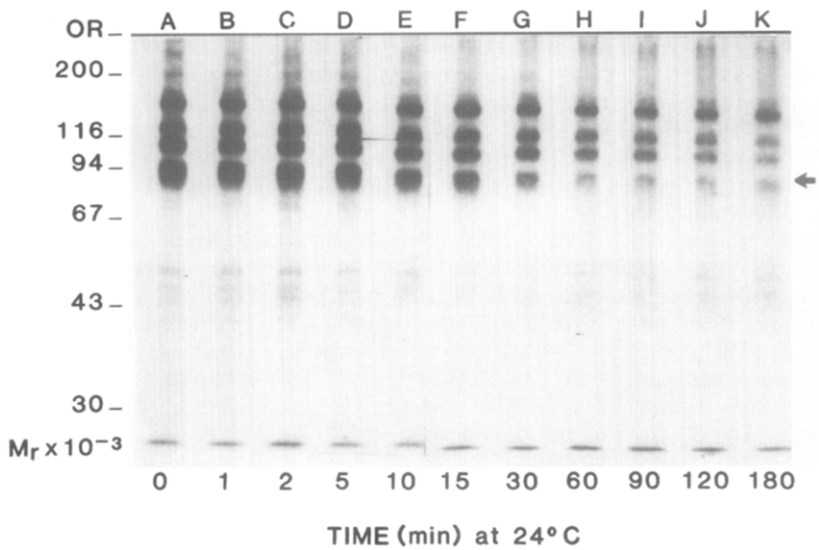

FIG. 1 Dephosphorylation of partially purified insulin receptors. Partially purified insulin receptors were first phosphorylated and then incubated with a large quantity of unlabeled ATP to study dephosphorylation as described under Materials and Methods. At different time-intervals aliquots were taken, the reaction was stopped with boiling SDS solution and the phosphoproteins were analysed by one-dimensional SDS-PAGE under reducing conditions. An autoradiogram of the gel is shown.

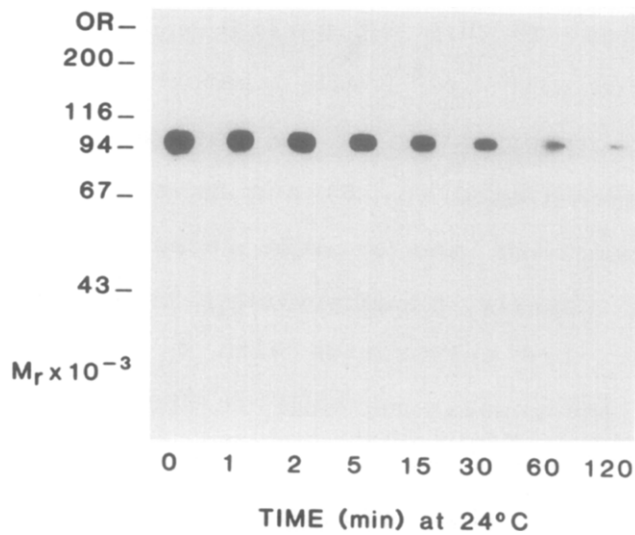

FIG. 2 Immunoprecipitation of partially purified insulin receptors after dephosphorylation. Partially purified insulin receptors were phosphorylated and dephosphorylated as described in the legend to fig. 1. At different time-intervals during the dephosphorylation the reaction was stopped by addition of EDTA and NaF at final concentrations of 30 mM and 100 mM, respectively. The insulin receptors were then specifically immunoprecipitated with anti-receptor antibodies as previously described (3, 6), and the immunoprecipitates were analysed by SDS-PAGE. An autoradiogram of the gel is shown.

noticeable after one min, and a t½ of dephosphorylation of approximately 15 minutes. This dephosphorylation was unaltered in the presence of insulin (data not shown).

2. Immunoprecipitated insulin receptors.

In this experiment, partially purified receptors were first specifically precipitated with anti-receptor antibodies, and then labeled with (γ-^{32}P)ATP. After addition of an excess of unlabeled ATP to study putative dephosphorylation, the immunoprecipitated receptors were incubated either at 24° or 37°C. As shown in figure 3 and 4, the level of phosphorylation of the immunoprecipitated β insulin receptor subunit remained stable for a period up to 4 hours at 37°C. Similar results were

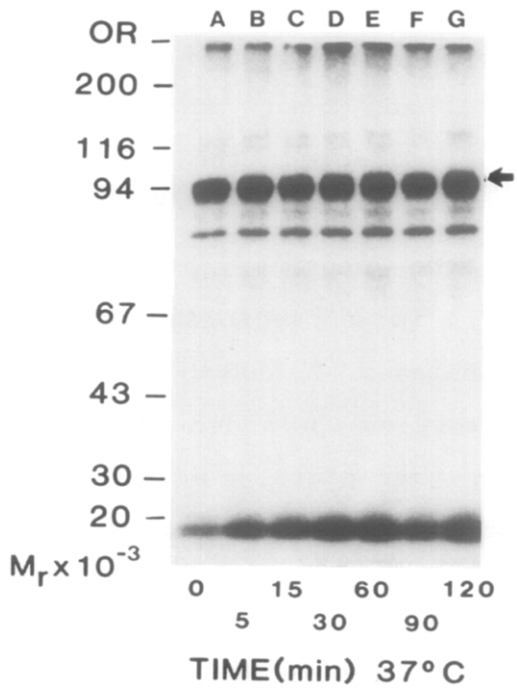

FIG. 3 Dephosphorylation of immunoprecipitated insulin receptors. Partially purified insulin receptors were immunoprecipitated with anti-receptor antibodies as described (3, 6). After phosphorylation the immunoprecipitated insulin receptors were incubated with unlabeled ATP to study putative dephosphorylation. At different time intervals aliquots were taken, the reaction was stopped with a boiling SDS solution, and the immunoprecipitated receptors were analysed by SDS-PAGE. An autoradiogram of the gel is shown.

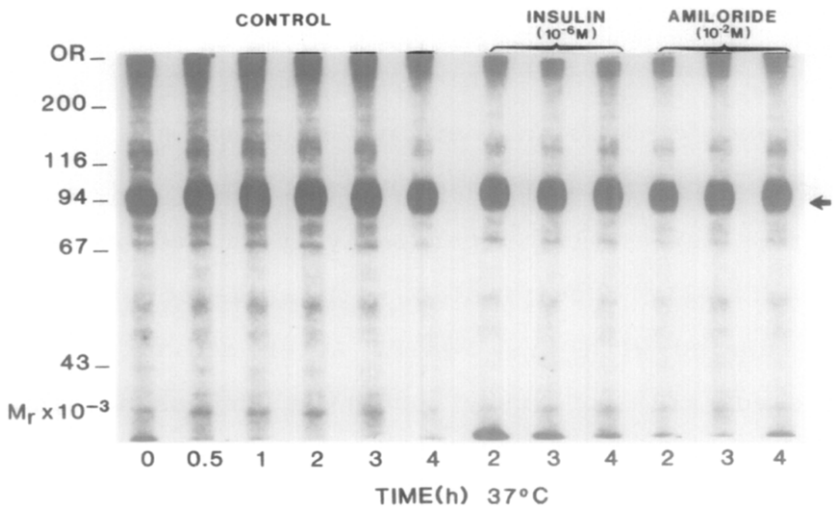

FIG. 4 Dephosphorylation of immunoprecipitated receptors in the presence of insulin or amiloride. The experiment is identical to the one described in the legend to fig. 3, except that during the chase experiment buffer (left panel), or insulin (10^{-6}M) (middle panel), or amiloride (10^{-2}M) (right panel) were present.

found at 24°C (data not shown). Further, neither insulin nor amiloride, which has been shown to activate hepatic phosphatases (14), affected the dephosphorylation of the insulin receptor (fig. 4). To address the question of the reversibility of the phosphorylation of insulin receptors purified by specific immunoprecipitation, we studied the effect of cytosolic phosphatases. As shown in figure 5, when a cytosol prepared from hepatocytes was added to phosphorylated immunoprecipitated insulin receptors, a virtually total dephosphorylation of the β receptor subunit was observed. Further, the action of the cytosolic phosphatases was completely blocked by the addition of vanadate (fig. 5, lane 8).

DISCUSSION

Insulin binds to its specific receptor and activates a protein kinase that catalyses the phosphorylation of its own receptor both in intact cells (2, 3), and cell-free systems consisting of partially purified (3-9) or highly purified

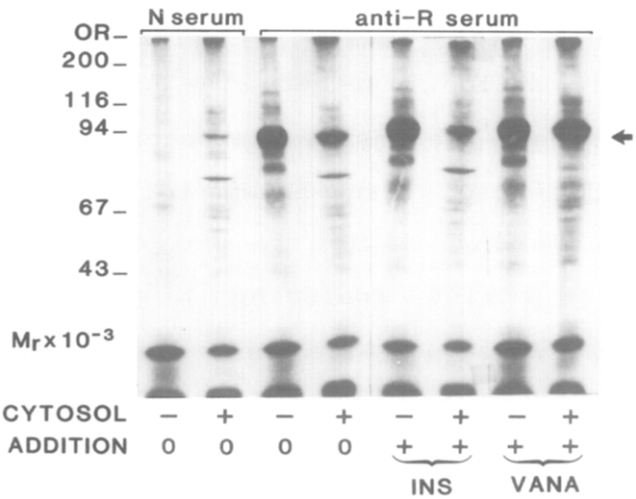

FIG. 5 Effect of cytosolic phosphatases on dephosphorylation of immunoprecipitated insulin receptors. Partially purified receptors were exposed to normal serum (N serum) or anti-receptor serum (anti-R). The immunoprecipitates obtained after addition of protein A were washed, and thereafter incubated with [γ-^{32}P]ATP. After 60 min at 4°C we added a cytosol derived from rat hepatocytes and supplemented with buffer, or insulin (10^{-6}M), or vanadate (10^{-3}M) as indicated. After 30 min at 4°C the reaction was stopped by addition of a boiling SDS solution and the phosphoproteins were analysed by SDS-PAGE. An autoradiogram of the gel is shown.

insulin receptors (5, 6). Further, using covalent affinity labeling techniques we and others have identified an ATP binding site on the β subunit of the insulin receptor, indicating that the insulin receptor itself is a protein kinase capable of self-phosphorylation (6-8).

To approach the possible regulatory role of insulin receptor phosphorylation in insulin action, we have studied here the reversibility of this covalent modification. We show that a phosphatase activity co-purifies with the insulin receptor on lectin chromatography, but this enzymatic activity is no longer detected in highly purified receptors indicating that it is not an integral constituent of the receptor molecule. However, the highly purified insulin receptor remains very sensitive to phosphatases present in cytosols derived from hepatocytes. Insulin receptor phosphorylation is thus endowed

with the necessary reversibility requirements for a regulatory event in insulin action. Further, the cytosolic phosphatases, which dephosphorylate the insulin receptor, can be inhibited by vanadate. Vanadate has been shown to oppose a variety of enzymatic reactions in relation to phosphorylation and dephosphorylation of proteins including Na^+, K^+ ATPase, myosin ATPase, dynein ATPase, alcaline and acid phosphatases, adenylate kinase, and phosphofructokinase (15, 16). Almost all of these enzymes are phosphohydrolases, and a phosphoenzyme intermediate plays often a role in the mechanism of action of these enzymes. The precise mode of action of vanadate is not established, but current evidence indicates that vanadate competes with phosphate for the enzyme-binding site (15). More recently, vanadate has also been shown to inhibit membrane phosphotyrosyl-protein phosphatase activity of A-431 cell membranes (17), and to increase the net phosphorylation of the insulin receptor in adipocytes (18). In the present study we have confirmed and extended this last observation, which is particularly intriguing in the light of the insulin-like effects of vanadate as shown by Dubyak and Kleinzeller (19), and Shechter and Karlish (20). Indeed, our data indicate that vanadate is able to inhibit cytosolic phosphatases resulting in an apparent increased phosphorylation of the insulin receptor. The evidence presented here together with our previously published data (3, 6, 10) clearly indicate that insulin and the insulin-like agent, vanadate, affect insulin receptor phosphorylation by totally opposite mechanisms. Indeed, insulin activites a protein kinase contained in the receptor itself leading to self-phosphorylation, whereas vanadate inhibits a phosphatase activity which is not a constituent of the insulin receptor. The fact that both, insulin and the insulin-like

agent, vanadate, lead to increased receptor phosphorylation and insulin's biological effects, makes it tempting to envisage that receptor phosphorylation is indeed a necessary early step in insulin action.

ACKNOWLEDGEMENTS. We thank G. Visciano for illustration work and A. Rainaud for secretarial assistance.

REFERENCES

1. Kahn, C.R., Baird, K.L., Flier, S., Grunfeld, C., Harmon, J.T., Harrison, L.C., Karlsson, F.A., Kasuga, M., King, G.L., Lang, U.C., Podskalny, J.M., and Van Obberghen, E. (1981) Recent Progress in Hormone Research 37, 477-533.
2. Kasuga, M., Karlsson, F.A., and Kahn, C.R. (1982) Science 215, 185-187.
3. Van Obberghen, E., and Kowalski, A. (1982) FEBS Lett. 143, 179-182.
4. Kasuga, M., Zick, Y., Blithe, D.L., Crettaz, M., and Kahn, C.R. (1982) Nature (London) 298, 667-669.
5. Kasuga, M., Fujita-Yamaguchi, Y., Blithe, D.L., and Kahn, C.R. (1983) Proc. Natl. Acad. Sci. USA 80, 2137-2141.
6. Van Obberghen, E., Rossi, B., Kowalski, A., Gazzano, H., and Ponzio, G. (1983) Proc. Natl. Acad. Sci. USA 80, 945-949.
7. Shia, M.A., and Pilch, P.F. (1983) Biochemistry 22, 717-721.
8. Roth, R.A., and Cassel, D.J. (1983) Science 219, 299-301.
9. Petruzzelli, L.M., Ganguly, S., Smith, C.J., Cobb, M.H., Rubin, C.S., and Rosen, O. (1982) Proc. Natl. Acad. Sci. USA 79, 6792-6796.
10. Gazzano, H., Kowalski, A., Fehlmann, M., and Van Obberghen, E. (1983) Biochem. J. (in press).
11. Cohen, P. (1982) Nature (London) 296, 613-620.
12. Van Obberghen, E., Kasuga, M., Le Cam, A., Hedo, J.A., Itin, A., and Harrison, L.C. (1981) Proc. Natl. Acad. Sci. USA 78, 1052-1056.
13. Laemmli, U.K. (1970) Nature (London) 227, 680-685.
14. Le Cam, A., Auberger, P., and Samson, M. (1982) Biochem. Biophys. Res. Commun. 106, 1062-1070.
15. Macara, I.G. (1980) Trends Biochem. Sci. 5, 92-94.
16. Simons, T.J.B. (1979) Nature (London) 281, 337-338.
17. Swarup, G., Cohen, S., and Garbers, D.L. (1982) Biochem. Biophys. Res. Commun. 107, 1104-1109.
18. Tamura, S., Brown, T.A., Dubler, R.E., and Larner, J. (1983) Biochem. Biophys. Res. Commun. 113, 80-86.
19. Dubyak, G.R., and Kleinzeller, A. (1980) J. Biol. Chem. 255, 5306-5312.
20. Shechter, Y. and Karlish, S.J.D. (1980) Nature (London) 284, 556-558.