Vol. 196, No. 1, 1993 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
October 15, 1993 Pages 216-222

ANTI-PHOSPHOSERINE AND ANTI-PHOSPHOTHREONINE ANTIBODIES MODULATE AUTOPHOSPHORYLATION OF THE INSULIN RECEPTOR BUT NOT EGF RECEPTOR

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Received September 1, 1993

Summary: We examined the effect of anti-phosphothreonine and anti-phosphoserine antibodies on insulin receptor autophosphorylation. These antibodies did not affect insulin binding activity of the receptor. These antibodies, however, inhibited insulin-stimulated autophosphorylation of insulin receptor, while did not affect EGF-stimulated autophosphorylation of EGF receptor. The inhibition was reversed by adding large amounts of phosphoserine or phosphothreonine. These data suggest that phosphoserine and phosphothreonine on insulin receptor play an important role in insulin-induced conformational change of the receptor.

Insulin receptor is a heterotetrameric glycoprotein consisting of two α- and two β-subunits. Insulin binds to the α-subunit which is external to the cell membrane. This interaction induces the conformational change of the receptor and stimulates a tyrosine kinase activity present at the cytoplasmic portion of the β-subunit, leading to autophosphorylation of the receptor on tyrosine residues in intact cells and in cell-free preparations of the receptor(1,2).

A number of polyclonal and monoclonal antibodies against the insulin receptor as well as anti-phosphotyrosine antibodies(αP-Tyr) have been shown to be useful to study the structure-function relationship of the receptor(3-8). It has been demonstrated that anti-phosphotyrosine antibodies modulate (increase or decrease) insulin receptor kinase activity, probably through affecting a conformational change of the receptor(4-6).

Serine and threonine residues of the β-subunit are phosphorylated at the basal state and enhanced when intact cells are stimulated with insulin(2,9). It has been reported that the phosphorylation on serine and threonine residues may modulate the receptor kinase activity through unknown mechanisms(10). Recently we have produced anti-phosphoserine(αP-Ser) and anti-phosphothreonine(αP-Thr) antibodies which are able to recognize various proteins phosphorylated on serine and threonine residues including insulin and EGF receptors(11).

0006-291X/93 $4.00

In the present study we examined whether the anti-phosphoserine and anti-phosphothreonine antibodies modulate the insulin receptor function.

Materials & Methods

Cells and cell culture

CHO-HIR cells which express a large amount of human insulin receptors in Chinese hamster ovary cells were prepared by using vectors containing the mouse dihydrofolate reductase gene as described previously(12). CHO-HIR cells were harvested with 1 μM methotrexate in α-minimum essential medium(αMEM)(without deoxynucleosides) supplemented with 10% dialyzed fetal bovine serum. Human epidermoid carcinoma A431 cells were grown in Dulbecco's modified Eagle's medium.

Preparation of antibodies toward phosphoserine(P-Ser) and phosphothreonine(P-Thr)

Antibodies toward P-Ser and P-Thr(αP-Ser and αP-Thr)were prepared as described previously(11).

Effects of αP-Ser and αP-Thr on insulin receptor autophosphorylation

Insulin receptor was partially purified from rat liver and CHO-HIR cells on a wheat germ agglutinin(WGA) column by the methods as described previously(13). Briefly, CHO-HIR cells and rat liver were homogenized in 50 mM Hepes(pH 7.4), 1% Triton X-100, 5 mM EDTA, 2mM phenylmethylsulfonyl fluoride(PMSF) and 0.1mg/ml aprotinin (buffer A) at 4°C and centrifuged at 150,000g for 60min. The supernatant was applied to WGA affinity column and the column was eluted with 0.3M N-acetyl glucosamine in buffer B(50mM Hepes(pH 7.4), 0.1% TritonX-100, 2mM PMSF, 0.1mg/ml aprotinin). The insulin receptor was further purified with insulin-agarose by the methods of Lewis et al(9,14).

Partially purified insulin receptor was incubated with various concentrations of αP-Ser, αP-Thr or αP-Tyr(15) at 22°C for 2h. After insulin(final concentration,10^{-7}M) was added at 22°C for 30min, the mixture was phosphorylated for 5min in 50mM Hepes(pH 7.4),100μM[γ-^{32}P]ATP, 5mM $MnCl_2$, and 0.1% Triton X-100. The reaction was terminated by boiling in Laemmli's sample buffer. The mixture was then subjected to 7.5% sodium-dodecyl-sulfate polyacrylamide gel electrophoresis(SDS-PAGE), followed by autoradiography.

Effect of αP-Ser and αP-Thr on EGF receptor autophosphorylation

EGF receptor was partially purified from A431 cells with WGA column. A431 cells were homogenized in buffer A and centrifuged at 150,000g for 60 min. The supernatant was applied to WGA column and the column was eluted with 0.3M N-acetyl glucosamine in buffer B. The effect of αP-Ser and αP-Thr on EGF receptor autophosphorylation was examined as described above.

Results & Discussion

Effect of αP-Ser and αP-Thr on insulin receptor autophosphorylation

αP-Ser and αP-Thr were studied to see if they affect the insulin receptor autophosphorylation. After incubation of the WGA-purified insulin receptor from CHO-HIR cells with 10μg/ml of αP-Ser, αP-Thr or αP-Tyr, insulin was added to autophosphorylate the receptor. As shown in Fig.1, both αP-Ser and αP-Tyr considerably inhibited the autophosphorylation of the receptor, while αP-Thr partially inhibited at the protein concentration of 10μg/ml. Insulin-stimulated phosphorylation of 220-kDa and other minor proteins was also inhibited by these antibodies. In order to examine whether the inhibition of the autophosphorylation is due to a direct binding of

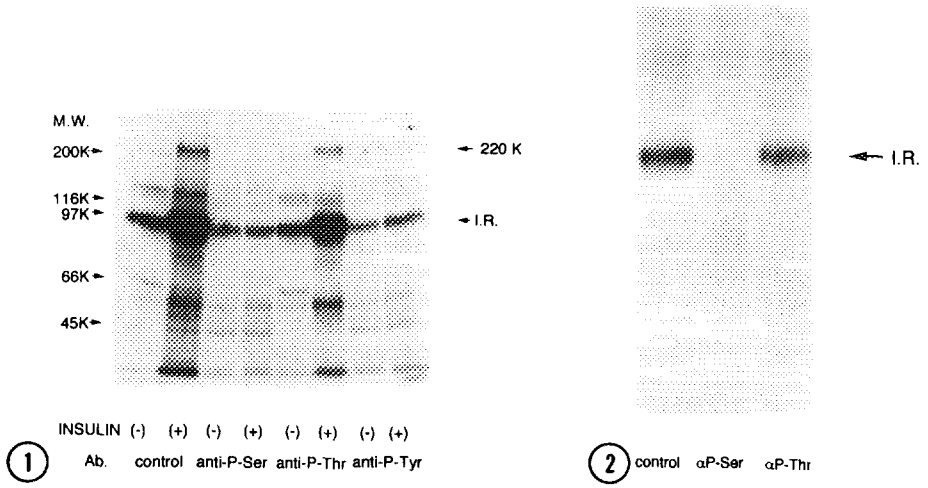


Fig.1. Inhibition of WGA-purified insulin receptor autophosphorylation by αP-Ser,αP-Thr and αP-Tyr; WGA-purified insulin receptor from CHO-HIR cells was incubated with 10 μg/ml of control IgG, αP-Ser, αP-Thr or αP-Tyr, stimulated with 10^{-7}M insulin and then phosphorylated by adding [γ-^{32}P]ATP. Phosphorylated insulin receptor (95-kDa) was separated on 7.5% SDS-PAGE and visualized by autoradiography.

Fig.2. Inhibition of insulin-agarose-purified insulin receptor autophosphorylation by αP-Ser and αP-Thr; WGA-purified insulin receptor was further purified with insulin-agarose.The effect of 10 μg/ml of control IgG, αP-Ser or αP-Thr on highly-purified insulin receptor autophosphorylation was examined as described in Fig.1 and "Materials & Methods".

the antibodies to the receptor, WGA-purified insulin receptor was further purified with insulin-agarose. The autophosphorylation of the highly-purified insulin receptor was also inhibited completely by αP-Ser and partially by αP-Thr at the protein concentration of 10μg/ml (Fig.2). Dose dependency of the inhibition was next examined in Fig.3. Ten μg/ml of αP-Ser inhibited insulin-stimulated autophosphorylation of the receptor to the

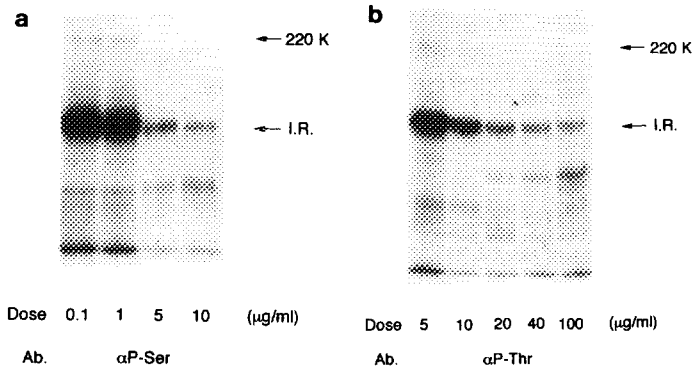


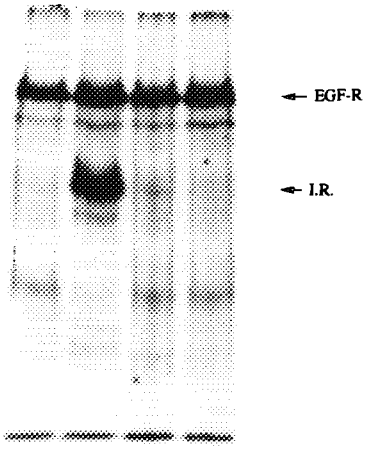
Fig.3. Inhibition of insulin receptor autophosphorylation by various concentrations of αP-Ser and αP-Thr; WGA-purified insulin receptor was incubated with the indicated concentrations of αP-Ser(a) and αP-Thr(b). Insulin receptor autophosphorylation was examined as described in "Materials & Methods".

basal level (before insulin stimulation) while a protein concentration of more than 20μg/ml was necessary to give rise to this degree of the inhibition in the case of αP-Thr. Insulin-stimulated phosphorylation of 220-kDa protein was also affected with a similar dose-dependency. Both antibodies did not affect insulin binding activity of the receptor(data not shown).

Effect of αP-Thr and αP-Ser on EGF receptor autophosphorylation

WGA eluate from rat liver, which contains both insulin and EGF receptors, was incubated with 20 μg/ml of αP-Thr. Insulin was added and then the proteins were phosphorylated. Phosphoproteins were analyzed by SDS-PAGE and autoradiography(Fig.4). αP-Thr completely inhibited insulin receptor autophosphorylation. In contrast, αP-Thr did not affect the phosphorylation of EGF receptor which is known to be partially activated *in vitro* after cell solubilization.

EGF receptor was purified from human epidermoid carcinoma cell(A431 cells) with the WGA column. After incubation with 20 μg/ml of αP-Thr, the EGF receptor was stimulated by EGF and phosphorylated(Fig.5). Autophosphorylation of the receptor was not affected by αP-Thr. Even at a higher concentration of αP-Thr, both the binding



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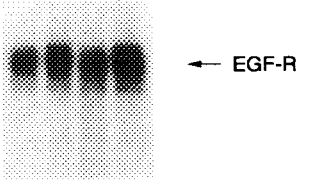

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Fig.4. Effect of αP-Thr on phosphorylation of the insulin and EGF receptor; Insulin and EGF receptor were purified from rat liver with WGA column. They were preincubated with 20 μg/ml of control IgG or αP-Thr, stimulated with 10^{-7}M insulin, and then phosphorylated by adding [γ-^{32}P]ATP. Phosphorylated insulin receptor (95-kDa) and EGF receptor (170-kDa) were separated on 7.5% SDS-PAGE and visualized by autoradiography.

Fig.5. Effect of αP-Thr on EGF receptor autophosphorylation; EGF receptor was purified from A431 cells with WGA column. EGF receptor was preincubated with 20 μg/ml of control IgG or anti-P-Thr, stimulated by 200 ng/ml EGF, and then phosphorylated by adding [γ-^{32}P]ATP. Phosphorylated EGF receptor was separated on 7.5% SDS-PAGE, followed by autoradiography.

activity and autophosphorylation of EGF receptor were not inhibited(data not shown). αP-Ser did not cause any significant changes in EGF receptor autophosphorylation either(data not shown). Furthermore, EGF-induced autophosphorylation of EGF receptor in the membrane preparation was not inhibited by these antibodies(data not shown).

Reversibility of the effect of αP-Thr and αP-Ser on insulin receptor autophosphorylation

To see whether or not the inhibition is reversible, 20 mM of P-Thr was added to the WGA-purified insulin receptor preincubated with 10 μg/ml of αP-Thr. The receptor was then further incubated with insulin, and phosphorylated(Fig.6). Inhibition of the autophosphorylation was reversed by a large amount of P-Thr. However, phosphorylation of EGF receptor was not affected by these procedures. Inhibition of insulin receptor autophosphorylation by αP-Ser was also reversible(data not shown). These results suggest that specific binding of the antibody to P-Thr or P-Ser on insulin receptor results in the inhibition of insulin-induced autophosphorylation of the receptor.

αP-Tyr has also been shown to decrease or increase the insulin receptor kinase activity(4-6). Although the reason for such contradictory results is unknown, it has been proposed that αP-Tyr locks the conformation of the insulin receptor in tyrosine kinase active or inactive form(6). In the present study, we examined whether αP-Ser and αP-

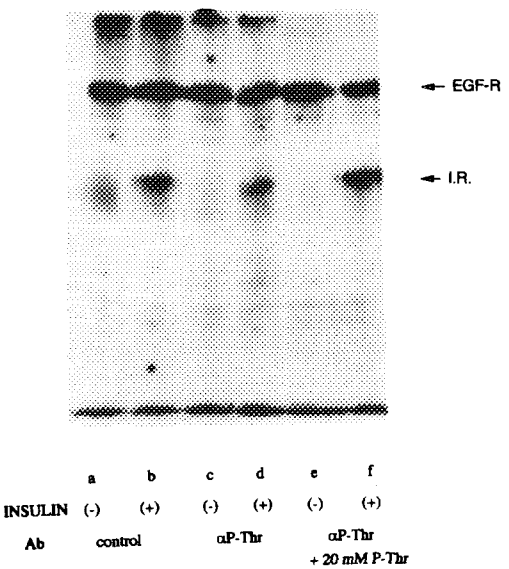

Fig.6. Reversal of the antibody-induced inhibition of WGA-purified insulin receptor autophosphorylation; WGA-purified insulin receptor from rat liver was incubated with 10 μg/ml of control IgG (lanes a and b) or αP-Thr (lanes c, d,e and f). The receptors were then stimulated with (lanes b,d and f) or without 10^{-7}M insulin (lanes a,c and e) and phosphorylated by adding [γ-^{32}P]ATP. In lanes e and f, 20 mM of P-Thr was added before the insulin stimulation. Phosphorylated insulin receptor (95-kDa) was separated on 7.5% SDS-PAGE, followed by autoradiography.

Thr also modulate the insulin and EGF receptor function. We found that the antibodies inhibited autophosphorylation of the insulin receptor but not EGF receptor. When the antibodies and [γ-^{32}P]ATP were incubated simultaneously with the receptor for 5 min, receptor autophosphorylation was not inhibited at all. Thus it is unlikely that these antibodies bind [γ-^{32}P]ATP in a non-specific manner, making ATP less available for the reaction. It seems also unlikely that these antibodies activated phosphotyrosine phosphatase activities present in the WGA eluate, since the effect of the antibodies was also seen using insulin-agarose-purified insulin receptor in the presence of phosphatase inhibitors(data not shown). Insulin receptor is phosphorylated on a number of different serine residues(16), as well as threonine residues(2,9)without insulin stimulation in intact cells. Serines 1293 and 1294 and threonine 1336 have been proposed as sites of insulin- and phorbol ester-stimulated insulin receptor phosphorylation(9,17). It was recently reported that serine in the juxtamembrane region of the insulin receptor was phosphorylated before insulin stimulation and insulin enhanced its phosphorylation(18). Although the antibody recognition sites are not known, our data favor the possibility that these antibodies react with some phosphothreonine and phosphoserine residues on the insulin receptor before insulin stimulation and lock the receptor into the kinase inactive form. Our data suggest that the phosphorylation on serine and threonine residues of the insulin receptor play an important role in inducing conformational change of the receptor upon insulin binding.

Acknowledgment

This study was supported by a grant for diabetes from Otsuka Pharmaceutical Co.,Ltd,Japan.

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