Vol. 190, No. 1, 1993 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
January 15, 1993 Pages 283-288

PREPARATION OF ANTI-PHOSPHOSERINE AND ANTI-PHOSPHOTHREONINE ANTIBODIES AND THEIR APPLICATION IN THE STUDY OF INSULIN- AND EGF- INDUCED PHOSPHORYLATION

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Received December 7, 1992

Summary: We prepared antibodies against phosphoserine (P-Ser) and phosphothreonine (P-Thr) by immunizing rabbits with P-Ser or P-Thr conjugated to bovine serum albumin. The antibodies (anti-P-Ser and anti-P-Thr) were purified using P-Ser or P-Thr affinity columns. Anti-P-Thr was highly specific for P-Thr, while anti-P-Ser showed weak cross-reacitivity with P-Thr. We showed that these antibodies can immunodetect serine/threonine phosphorylated insulin and epidermal growth factor (EGF) receptors and several proteins which are phosphorylated on serine/threonine residues in response to insulin or EGF stimulation. The antibodies will certainly provide a good tool for discovering novel kinases and substrates involved in signal transduction.

The interaction of insulin with its specific receptors induces a series of events that lead to signal transduction to its intracellular effectors. Insulin stimulates a tyrosine kinase activity of its receptor (1,2). Insulin also enhances phosphorylation on serine and threonine residues of intracellular molecules far more predominantly than on tyrosine residues, which may play important roles in the signal transduction (3-8). However, the total picture of insulin-stimulated serine/threonine phosphorylation remains to be elucidated. In the present study, we attempted to prepare anti-phosphoserine and anti-phosphothreonine antibodies with a high specificity and affinity in order to study insulin-induced serine, threonine phosphorylation in cells.

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(9). CHO-HIR cells were harvested with 1mM methotrexate in α-minimum essential medium(αMEM)(without deoxynucleosides)supplemented with 10% dialyzed fetal bovine serum.

0006-291X/93 $4.00

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

P-Ser or P-Thr (20mg each, purchased from Sigma) was coupled to 20mg of bovine serum albumin (BSA) or keyhole limpet hemocyanin (KLH) with 80mg of 1-ethyl-3- (3-dimethylaminopropyl) carbodiimide in 10ml reaction mixture (pH 8.5) at 4°C overnight. The conjugate was dialyzed against phosphate-buffered saline (PBS), pH 7.5. Rabbits were immunized with 1mg of P-Ser- or P-Thr-BSA conjugate. Booster injections of the same amount of the conjugate were given at 3, 5, and 7 weeks after the first injection. Serum obtained from rabbits immunized with P-Ser-BSA was passed through a column of CN-Br Sepharose 4B coupled with P-Ser. After extensive washing with PBS, the column was first eluted with 40mM P-Thr (αP-Ser/P-Thr), washed, and then eluted with 40mM P-Ser (αP-Ser). Serum from P-Thr-BSA immunized rabbits was passed through the P-Thr column. Antibodies were collected with 40mM P-Ser (αP-Thr/P-Ser), and then with P-Thr (αP-Thr). Each antibody was dialyzed extensively against PBS.

Immunoprecipitation of phosphoprotein in CHO-HIR cells

Subconfluent CHO-HIR cells were harvested in phosphate -free αMEM for 12h, then [^{32}P]-orthophosphate was added. After 2h, the cells were incubated in the presence or absence of 10^{-7}M insulin for 5min. They were frozen in liquid nitrogen, homogenized in buffer A (50mM Hepes, 20mM NaF, 10mM sodium pyrophosphate, 2mM $NaVO_3$, 5mM EDTA, 2mM phenylmethylsulfonyl fluoride and 0.1mg/ml aprotinin, pH 7.4) including 1.5% Triton X-100 and centrifuged at 150,000g for 60min. The supernatant was applied to wheat-germ-agglutinin (WGA) affinity column and the column was eluted with 0.3M N-acetyl glucosamine in buffer A including 0.1% Triton X-100. The WGA eluate was incubated overnight with αP-Thr, αP-Ser, or αP-Tyr, and then with protein A -Sepharose for an additional 2h at 4°C. The immunoabsorbed phosphoprotein was washed three times with buffer A including 0.1% Triton X-100, boiled in Laemmli's sample buffer, and subjected to 7.5% sodium-dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

Immunoblotting of insulin-stimulated phosphoprotein

CHO-HIR cells, stimulated with or without insulin (10^{-7}M), were homogenized and processed as described above without using [^{32}P]-orthophosphate. Fifty μg of solubilized protein was boiled in Laemmli's sample buffer, run on SDS-PAGE and electric-transferred to nitrocellulose membranes. Phosphoproteins on the membranes were immunodetected with αP-Ser, αP-Thr or αP-Tyr(17) as described above.

Stimulation of A431 cells with 12-O-tetradecanoylphorbol-13-acetate (TPA)

A431 cells were washed three times with Dulbecco's PBS and incubated with or without 10^{-8}M TPA for the indicated time. After incubation the cells were solubilized in Laemmli's sample buffer. The phosphoproteins were detected as described above.

Results & Discussion

Characterization of antibodies

The specificity of the antibodies was examined by immunoblotting using P-Ser, P-Thr and P-Tyr coupled to BSA or KLH as antigens (Fig 1). αP-Thr/P-Ser and αP-Thr reacted mainly with P-Thr/BSA. αP-Thr/P-Ser cross-reacted weakly with P-Ser/BSA, when the quantity of antigen was more than 100μg (data not shown). αP-Ser and αP-Ser/P-Thr recognized P-Ser/BSA and weakly P-Thr/BSA. The cross-reactivity with P-Thr/BSA was greater in αP-Ser/P-Thr. These four antibodies cross-reacted very weakly with P-Tyr/KLH, when the quantity of P-Tyr/KLH was more than 10μg. None of the antibodies reacted with BSA (data not shown). Binding of αP-Thr/P-Ser to P-Thr/BSA conjugate, dot-blotted on the nitrocellulose paper, was inhibited by 20mM of both P-Thr and P-Ser but not by 20mM of P-Tyr, Thr or Ser, while that of αP-Thr was inhibited only by P-Thr (data not shown). Binding of αP-Ser to P-Ser/BSA conjugate was also inhibited by 20mM of P-Thr and P-Ser but not by 20mM of P-Tyr, Ser or Thr. These

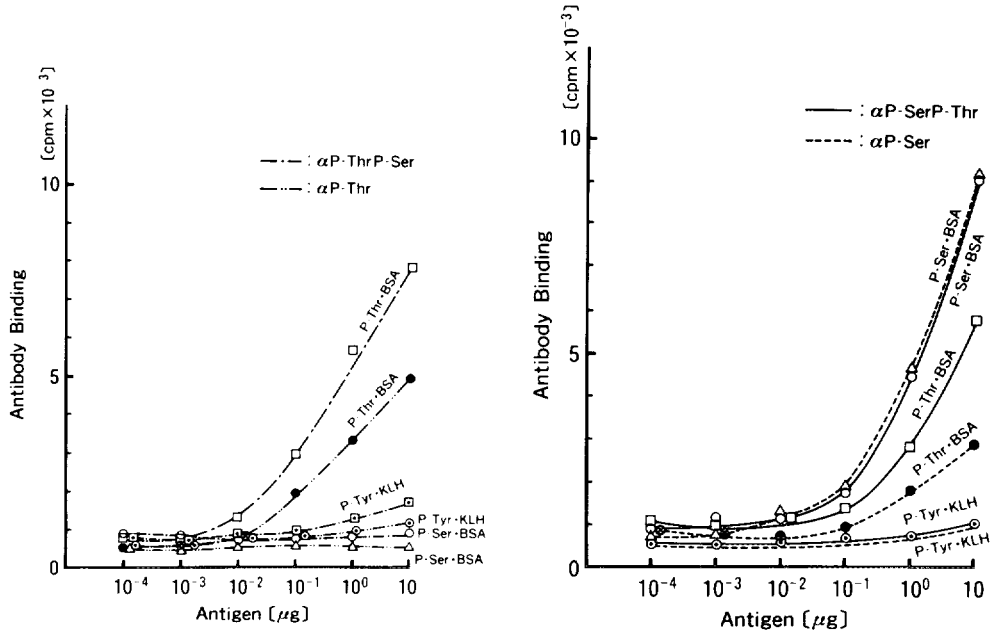

Fig.1. Sensitivity of the antibodies;Various doses of antigens (P-Ser/BSA, P-Thr/BSA and P-Tyr/KLH) on the membrane were reacted with each antibody(5μg/ml) and then [^{125}I]-protein A.

results indicated that the antibodies are produced against phosphoamino acids (P-Ser and P-Thr), and that αP-Thr is highly specific for P-Thr.

Immunodetection of insulin-induced phosphoproteins in the CHO-HIR cells

We checked if these antibodies can immunodetect phosphoproteins, especially insulin receptor, in CHO-HIR cells by both immunoblotting and immunoprecipitation. In the WGA eluate from [^{32}P]-labelled CHO-HIR cells, both αP-Ser and αP-Thr immunoprecipitated insulin receptor(95-kDa phosphoprotein)(Fig 2a). Since the cross-reactivity of these antibodies with P-Tyr is very low, these results indicate that the antibodies detect P-Ser and/or P-Thr on the receptors. αP-Thr immunoprecipitated various phosphoproteins, which were not recognized by αP-Tyr and not stimulated by insulin. As shown in Fig 2b, in the crude Triton X-100 solubilized proteins, αP-Ser immunoblotted 170-kDa phosphoprotein and insulin receptor, which were both stimulated by insulin, while αP-Thr did mainly insulin receptor. On the other hand, αP-Tyr recognized several insulin-induced phosphoproteins including insulin receptor and 185-kDa phosphoprotein (IRS-1) (4,16). Phosphoamino acid analysis of [^{32}P]-labelled insulin receptor revealed that it was mainly phosphorylated on serine residues at the basal condition, while on serine, threonine, and tyrosine residues after insulin stimulation (data not shown). But αP-Ser barely immunodetected insulin receptor at the basal condition. Moreover, this αP-Ser detected fewer phosphoproteins than αP-Thr by both immunoprecipitation and immunoblotting. Differnces in the affinity of the antibody as

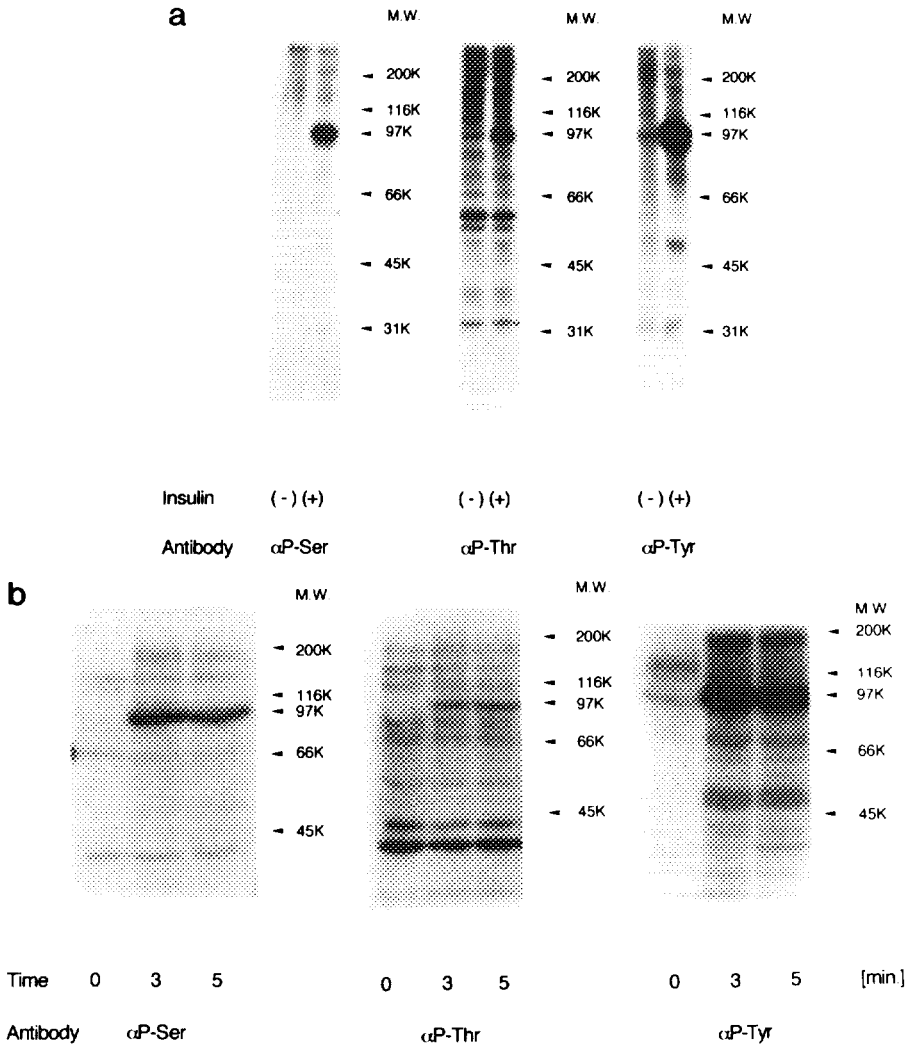

Fig.2 a) Immunoprecipitation of WGA-purified phosphoproteins in [^{32}P]-labelled CHO-HIR cells:CHO-HIR cells, labelled with [^{32}P]-orthophosphate, were stimulated with or without 10^{-7}M insulin for 5 min. WGA-purified protein from the cells was immunoprecipitated with αP-Ser, αP-Thr, or αP-Tyr and subjected to SDS-PAGE. b) Immunoblotting of insulin-stimulated phosphoproteins in CHO-HIR cells:Triton X-100 solubilized proteins (50μg) from insulin-stimulated CHO-HIR cells were run on SDS-PAGE and immunoblotted with αP-Ser, αP-Thr, or αP-Tyr.

well as the accessibility of the target residues on the molecule by the antibody may account for the findings.

Immunodetection of EGF-stimulated phosphoproteins in A431 cells

Next we examined if these antibodies recognized EGF receptor. The A431 cells were stimulated with EGF for 5 min, solubilized in Triton X-100. The phosphorylated EGF receptor (170 kDa) was immunodetected with αP-Thr and αP-Ser(Fig.3). αP-Thr recognized EGF-stimulated 240-kDa phosphoprotein.

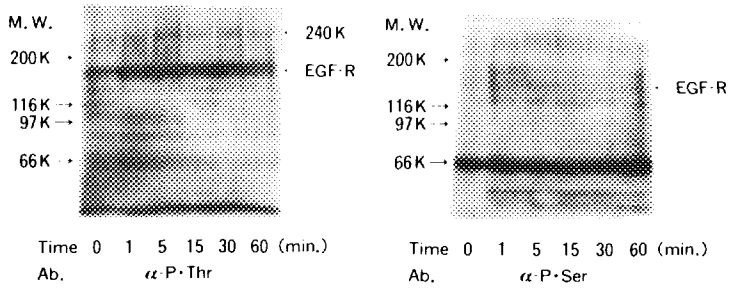

Fig.3 Immunoblotting of EGF-stimulated phosphoproteins in A431 cells:A431 cells were stimulated with 200ng/ml EGF for the indicated time and boiled in Laemmli's sample buffer. The protein was run on SDS-PAGE and immunoblotted with αP-Thr or αP-Ser.

Effect of treatment of A431 cells with phorbol ester

Tumor-promoting phorbol ester, TPA, is reported to activate protein kinase C, which results in phosphorylation of threonine 654 of the EGF receptor and thus inhibits its tyrosine kinase activity (10-13). We investigated if these events could be demonstrated by immunoblotting with αP-Tyr and αP-Thr (Fig.4). Treatment of the cells with TPA resulted in an increase in phosphothreonine (occurring between 1 and 15 min)(Fig.4a) Pretreatment with TPA (15 min) caused dramatic decreases of EGF-stimulated tyrosine phosphorylation of the receptor, 100- and 85-kDa proteins, while it produced increases of EGF-stimulated threonine phosphorylation of the 240- and 40kDa proteins (Fig.4b). Pretreatment with TPA did not change appreciably the phosphothreonine content of the receptor. This may reflect that EGF receptor tyrosine kinase-dependent threonine kinase, which phosphorylates threonine residues on the receptor, is partialy suppressed by protein kinase C. These results provide additional evidence that αP-Thr recognized EGF receptor, not due to the cross-reactivity with P-Tyr, but due to the reactivity with P-Thr.

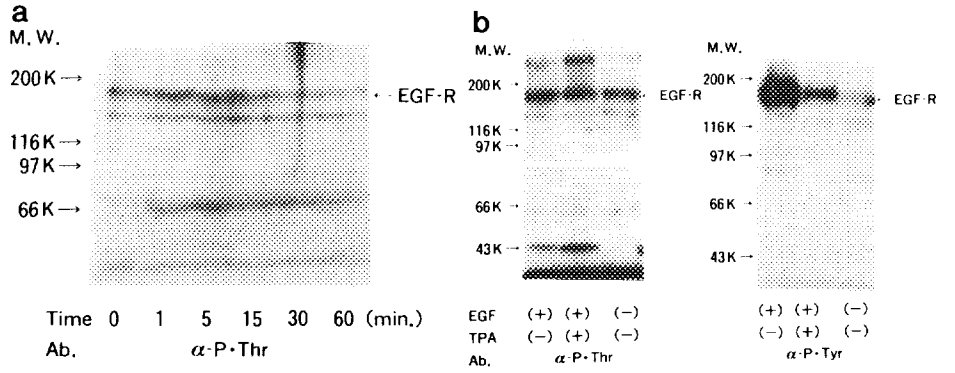

Fig.4a) Immunoblotting of TPA-stimulated phosphoproteins in A431 cells:A431 cells were stimulated with or without 10^{-8}M TPA for the indicated time and boiled in Laemmli's sample buffer. The proteins were run on SDS-PAGE and immunoblotted with αP-Thr.

b) Immunoblotting of EGF-induced phosphoproteins in A431 cells pretreated with TPA A431 cells, pretreated with or without 10^{-8}M TPA for 15 min at 37°C, were stimulated with 20 ng/ml EGF for 5 min and boiled in Laemmli's sample buffer. The proteins were run on SDS-PAGE and immunoblotted with αP-Thr or αP-Tyr.

Recently, a few papers have been published on the preparation of antibodies toward phosphoserine and phosphothreonine (14,15). Unlike these previously reported methods, we used BSA conjugates of phosphothreonine or phosphoserine as antigens, and purified the antibodies with phosphothreonine or phosphoserine affinity column. To obtain αP-Thr, the phosphothreonine column was eluted first with phosphoserine and then with phosphothreonine, while for αP-Ser, the phosphoserine column was eluted with phosphothreonine and then with phosphoserine. These procedures may have enabled us to obtain antibodies with high specificity. These antibodies were capable to immunodetect some, but not all, proteins which were phosphorylated on serine or threonine residues. Some phosphoproteins were immunoblotted by the antibodies but not immunoprecipitated. These data suggest that some proteins were recognized by the antibodies only when they exist either as intact molecules or as denatured molecules.

To our knowledge, this is the first report on antibodies which recognize insulin- or EGF-stimulated serine or threonine phosphoproteins, in particular, insulin receptors. The antibodies will certainly provide a good tool in the evaluation of insulin and EGF receptor's phosphorylation and the detection of novel kinases and substrates in the signal transduction as the antibody against phosphotyrosine facilitated the discovery of IRS-1 (16).

Acknowledgment

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

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