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IN THE ABSENCE OF ANTIBODY IgGSORB PRECIPITATES HUMAN PLACENTA PHOSPHOTYROSINE-CONTAINING PROTEINS

H. Joseph Goren and Donna Boland

Department of Medical Biochemistry
Faculty of Medicine
University of Calgary
Calgary, Alberta, Canada, T2N 4N1

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Lectin-purified human placenta plasma membrane proteins were phosphorylated in vitro. Mixing the reaction mixture with IgGsorb and incubation of the resultant pellet with p-nitrophenyl phosphate demonstrated the presence of phosphorylated-insulin receptor β-subunit and a phosphorylated-180 kDa protein in acrylamide gel electrophoresis. The same two proteins were detected in the electrophoretic analyses of anti-phosphotyrosine immunoprecipitated phosphorylation reaction mixtures. In the absence of antibody, the amount of phosphoprotein in the IgGsorb pellet was dependent on the amount of IgGsorb added. IgGsorb did not precipitate ^{125}I-labeled lectin-purified human placenta protein. Further, 10 mM O-phosphotyrosine completely inhibited the precipitation of phosphorylated human placenta proteins. These data suggest that IgGsorb specifically bound and precipitated phosphotyrosine-containing proteins in soluble human placenta plasma membranes. © 1989 Academic Press, Inc.

Protein A, a major component of most strains of Staphylococcal aureus, is commonly used to precipitate antigen-immunoglobulin G complexes (1). IgGsorb (The Enzyme Center, 30 Franklin Street, Malden, MA) is one commercial preparation of protein A. We have used both protein A and IgGsorb to precipitate phosphotyrosine-containing protein-antiphosphotyrosine antibody complexes. Surprisingly, IgGsorb in the absence of antibody precipitated phosphotyrosine-containing proteins.

MATERIALS AND METHODS

Crystalline pork insulin was a gift (Dr. D.A. Brodie, Novo Laboratories, Toronto, Canada). Electrophoresis reagents were purchased from Bio-Rad, [γ-^{32}P]ATP (5200 Ci/mmole) from ICN, sodium [^{125}I]iodide from Amersham, and X-OMAT (XR) autoradiographic film from Kodak. IgGsorb (Lot 10228) was resuspended to yield a 10% cell suspension (binding capacity 1.79 μg IgG/ml). Protein A was prepared, as previously described (1,2), from Staphylococcus aureus (kindly provided by Dr. P. Lee, University of Calgary). The final pellet was resuspended to 10% (w/v) in phosphate buffered saline-sodium azide

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(0.2 mg/ml), and stored at -70°C. Other reagents were of the highest grade commercially available and were purchased from Sigma Chemical Co. or Fisher Scientific Co.

Solubilized human placenta plasma membrane proteins were eluted off wheat germ agglutinin-agarose (Vector Laboratories) with N-acetyl glucosamine (3). This insulin receptor-enriched preparation (2 μg) was preincubated 10 min, 20°C, in the presence or absence of 10^{-7}M insulin in 50 mM Hepes - 0.1% Triton X-100, pH 7.4 (Buffer A) containing 5 mM $MnCl_2$. [γ-^{32}P]ATP (7 Ci/mmol), 20-50 μM (final concentration) was added and the reaction mixture (40 μl) was incubated a further 10 min, 20°C. Sodium orthovanadate (2mM), 5μl, was added followed by 5μl of 1/10 antiphosphotyrosine antiserum (4), or 5 μl of 1/10 non-immunized rabbit serum, or 5 μl Buffer A. After 1 h, 4°C, 20 μl IgGsorb or 20 μl protein A was added. Following 30 min, 0°C, phosphorylation reaction mixtures were centrifuged (Microfuge B, Beckman), supernatants were aspirated, and the pellets were washed twice with 100 μl 0.5 M NaCl, 0.1% SDS, 1% Triton X-100, 5 mM Tris, pH 7.4, and once with 100 μl Buffer A. Pellets were resuspended in 50 μl 10 mM p-nitrophenyl phosphate in Buffer A, incubated 15 min, 20°C, and then centrifuged. Supernatants (40-50 μl) were mixed with 10 μl 5X Laemmli solubilization buffer containing 75 mM dithiothreitol (5,6), heated 5 min 100°C, and applied to 7.5% (w/v) acrylamide gel electrophoresis (5). Gels were stained, destained, dried and exposed to x-ray film. Some phosphorylation reactions were termined with 10 μl 5X-Laemmli-dithiothreitol solubilization buffer following orthovanadate addition. Phosphoprotein radioactive content was estimated from densitometry tracings of autoradiograms (Bio-Rad Model 620 Densitometer, Bio-Rad 3392A Integrator).

Lectin-purified human placenta plasma membrane proteins were radiolabeled with chloramine T-Na^{125}I (7) and ^{125}I-labeled glycoproteins were isolated following passage over wheat germ agglutinin-agarose.

RESULTS AND DISCUSSION

Addition of [γ-^{32}P]ATP to wheat germ agglutinin-purified human placenta plasma membrane proteins results in phosphate incorporation into the insulin receptor β-subunit (95 kDa) and into a 180 kDa protein (4,8). Consistent with their phosphotyrosine content, these proteins were immunoprecipitated by an anti-phosphotyrosine antibody (Fig. 1). To precipitate the antigen-antibody complexes, either protein A (lanes 2, 3-Fig. 1A; lanes 1, 2 - Fig. 1B) or IgGsorb (Fig. 1C) was used. In the absence of anti-phosphotyrosine (lanes 3,4 - Fig. 1B) or in the presence of non-immunized rabbit serum (lane 4 - Fig. 1A), p-nitrophenyl phosphate released no phosphoproteins from protein A pellets. In contrast, both phospho-β-subunit and phospho-180 kDa protein were released from IgGsorb-pellets with control rabbit serum (not shown), or in the absence of serum (lane 5-Fig. 1B). Further, the amount of precipitated phosphoproteins was dependent on the amount of IgGsorb added and not on the size of the protein A pellet (lanes 5-8 - Fig. 1B; Fig. 2). These results suggested that IgGsorb bound phosphotyrosine containing proteins.

An alternative explanation was that IgGsorb non-specifically precipitated human placental glycoproteins; that is, since phosphoproteins were radiolabeled, they were detected autoradiographically. To test this hypothesis, ^{125}I-labeled human placenta glycoproteins (100 μl, 5.8 X 10^5 cpm)

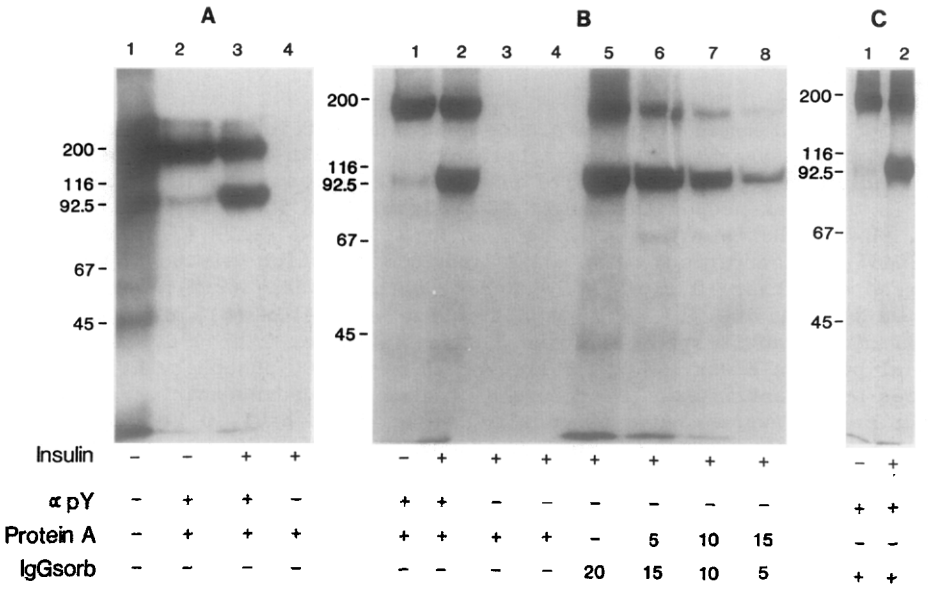

Fig. 1 Analyses of in vitro phosphorylated human placenta plasma membranes.

Wheat germ agglutinin-purified human placenta plasma membrane proteins were phosphorylated in the presence (+) or absence (-) of insulin.
A. Phosphorylation reactions were terminated with 5X Laemmli-dithiothreital solubilization buffer (lane 1), anti-phosphotyrosine antisera (αpY lanes 2, 3), or non-immunized rabbit serum (lane 4). Subsequent steps were as described in Methods, where the precipitant was protein A.
B. Phosphorylation reactions were terminated with αpY (lanes 1, 2), or buffer A (lanes 3-8). Protein A, 20 μl (lanes 1-4), 5 μl (lane 6), 10 μl (lane 7) or 15 μl (lane 8) were added and IgGsorb, 20 μl (lane 5), 15 μl (lane 6), 10 μl (lane 7), or 5 μl (lane 8) were added. Subsequent steps were as described in Methods.
C. Phosphorylation reactions were terminated with anti-phosphotyrosine and the precipitant was IgGsorb.

Mr X 10^{-3} of protein standards (Bio-Rad) are indicated to the left of each autoradiogram.

were incubated in the absence of serum with 40 μl IgGsorb under the above-described immunoprecipitation conditions. The pellets retained about 2 X 10^3 cpm (~0.3% of total radiolabel). Incubation of the pellets with 50 μl 10 mM p-nitrophenyl phosphate or 50 μl 0.1M acetic acid, 0.15 M NaCl released about 200 cpm ^{125}I-labeled protein into the supernatant. These results indicated that IgGsorb non-specific binding of human placental glycoproteins was not significant.

Specificity of IgGsorb for phosphotyrosine-containing proteins was supported when it was found that IgGsorb precipitation of phosphorylated protein could be inhibited completely by 10 mM O-phosphotyrosine. For example, the integral of densitometry tracings of phospho-β-subunit from IgGsorb precipitates of in vitro-phosphorylated human placenta membrane glycoproteins was 9.5 ± 0.5 (n=3) integral units in the absence of

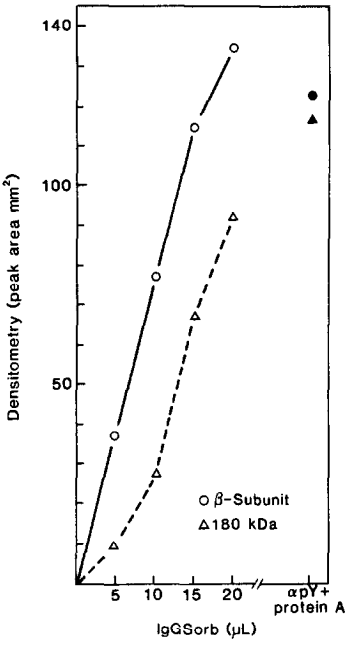

Fig. 2 **Relative amounts of phosphoproteins in IgGsorb precipitates.**

Densitometry tracings of lanes 2 - 8 (Fig. 1B) were performed. Areas under phospho-β-subunit (0), and phospho-180 kDa proteins (Δ) were calculated. The filled symbols represent data from the lane 2 tracing.

0-phosphotyrosine, and 0.1 integral units in the presence of 10 mM 0-phosphotyrosine.

Although both IgGsorb and anti-phosphotyrosine antibody bound phosphotyrosine-containing proteins, the specificity of the antibody was different. Thus, IgGsorb bound more phospho-β-subunit than phospho-180 kDa protein, whereas anti-phosphotyrosine immunoprecipitation demonstrated an equal amount of the two phosphoproteins (Fig. 2). Further, it is not known whether IgGsorb can bind phosphoproteins where phosphate is incorporated into amino acid residues other than tyrosine; that is, analyses of anti-phosphotyrosine immunoprecipitates and IgGsorb precipitates were of the p-nitrophenyl phosphate released material which will not contain phosphoserine- and phosphothreonine-containing proteins, also present in *in vitro* phosphorylated human placenta plasma membranes (lane 1 - Fig. 1A). Future investigations will examine IgGsorb binding specificity, as well as the identity of the phosphotyrosine binding species in IgGsorb.

In summary, it would appear that IgGsorb binds and precipitates phosphotyrosine-containing proteins in human placental plasma membranes, and it may be useful as a general method of precipitation of phosphotyrosine-containing protein.

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REFERENCES

1. Goding, J.W. (1978). J. Immunol. Met. 20, 241-253.
2. Lee, P.W.K., Hayes, E.C. and Joklik, W.K. (1981). Virology 108, 134-146.
3. Harrison, L.C. and Itin, A. (1980). J. Biol. Chem. 255, 12066-12072.
4. Neufeld, E., Goren, H.J. and Boland, D. (1989). Analyt. Biochem., in press.
5. Laemmli, U.K. (1970). Nature 227, 680-685.
6. Kasuga, M., White, M.F. and Kahn, C.R. (1985). in Met. Enzymol. (L. Birnbaumer and B.W. O'Malley, Eds.) Vol. 109, pp. 609-621, Academic Press, San Diego, CA.
7. Hunter, W.M. and Greenwood, F.C. (1962). Nature 194, 495-496.
8. Machicao, F., Haring, H., White, M.F., Carrascusa, J.M., Obermaier, B. and Wieland, O.H. (1987). Biochem. J. 243, 797-801.