Rabbit antibodies with specificity for tyrosine phosphate are not reactive with tyrosine sulphate

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Tyrosine phosphate is a frequently observed modification in a limited number of protein molecules, principally the products of viral oncogenes and membrane receptors for mitogenic growth factors. Such a group is therefore an attractive candidate for affinity selection of such phosphoproteins by antibodies with specificity for tyrosine phosphate. This report demonstrates that antisera raised against 4-aminobenzylphosphonic acid have specificity for tyrosine phosphate, but are not inhibited by tyrosine sulphate. This observation has important ramifications for the detection and isolation of phosphotyrosine-containing proteins without copurification or detection of tyrosine sulphate-containing proteins, which are present in cells of all lineages. Furthermore, we demonstrate that antisera raised against the sulphanilate moiety are not reactive with tyrosine sulphate.

Tyrosine phosphorylation appears to play a role in the mechanism of action of the products of certain oncogenic viruses, and in the function of cellular receptors for mitogenic growth factors (1-5). The overall level of tyrosine phosphate in cells is in the range 0.5-2% of total cellular protein-associated phosphate, and seems to be restricted to those classes of molecules listed above. The limited distribution of protein-associated tyrosine phosphate makes this modified amino acid a useful target for affinity purification strategies and, to this end, several laboratories have developed antibodies with specificity for tyrosine phosphate. Such reagents may be prepared using tyrosine phosphate itself as a hapten (6) or by employing a biologically inert analogue, such as 4-aminobenzylphosphonic acid (7-9). Antibodies raised by either regimen, and prepared as polyclonal sera or as monoclonal reagents, have been used successfully in the isolation of oncogene products and cellular growth factor receptor molecules (6,8).

Recent experiments have illustrated the ubiquity of tyrosine sulphate in cells of all lineages (10). Clearly, the phosphate and sulphate esters of tyrosine are closely related in size and charge, and there is therefore a possibility that tyrosine sulphate may act as an

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antigenic epitope for antibodies designed to have exclusive specificity for the phosphate ester of tyrosine. This possibility has been evaluated, and this report presents data to suggest that tyrosine sulphate does not act as an inhibitor of an antiserum with specificity for tyrosine phosphate.

**Materials and Methods**

**Reagents**

All chemicals were obtained from Sigma, with the exception of tyrosine sulphate, which was synthesized according to the method of Reitz et al. (11). The antisera were prepared by immunizing rabbits with keyhole limpet haemocyanin (KLH) derivatized with the azo form of 4-aminobenzyl phosphonic acid (ABP) or with the azo form of sulphanilic acid (i.e. 4-azobenzensulphonic acid, ABS).

The antisera raised were numbered as RD 120 (anti-ABP) and RD 177 (anti-ABS). Peroxidase-conjugated goat anti-rabbit IgG (heavy and light chain reactive) was purchased from Miles Laboratories.

**ELISA**

ELISA was performed according to the method of Campbell (12). Briefly, 96-well microELISA trays were coated with 100 µl of antigen solution (100 µg/ml in PBS) for 1 h at room temperature, and then excess protein binding sites were blocked by addition of 5% (v/v) normal goat serum / 3% (w/v) bovine serum albumin in PBS for 1 h. The plates were then exhaustively washed with distilled water. Dilutions of heat-inactivated antisera were made in 200 mM Tris/HCl, pH 7.5 / 0.9% (w/v) NaCl and added to wells in 100 µl volumes. After incubation at room temperature for 2 h, the plates were washed and a 1:1000 dilution in 0.9% (w/v) saline / 0.5% (w/v) bovine serum albumin of a peroxidase conjugated goat anti-rabbit IgG was added. After further incubation for 1 h and washing, substrate (0.4 mg/ml o-phenylene diamine / 0.01% (v/v) H₂O₂ in 0.05 M sodium citrate, 0.1 M sodium phosphate buffer, pH 6.0) was added. The reaction was terminated after 10 min by addition of 50 µl of 4 N H₂SO₄, and the absorbance at 492 nm measured on a Titertek 'Multiskan' instrument. In experiments where inhibitors were employed, these were present in the wells prior to the addition of 50 µl of antisera, and the final concentration of the inhibitors was 5 mM.

In all experiments, assays of binding of normal rabbit serum were included to control for non-specific binding of rabbit immunoglobulin to the plates. Furthermore, the A₄₉₂ values obtained for all antisera on the different plates were adjusted to take account of non-specific binding of the peroxidase conjugate, and of background substrate conversion in the absence of enzyme.

**Results**

*Antibody reactivity*

The RD 120 antiserum was tested for its ability to react with the haptenic group. Figs. 1a and 1b illustrate that the serum binds to both the immunogen (KLH-ABP), and to the carrier (KLH) alone,