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Benchmark

Stoichiometry of Site-Specific Protein Phosphorylation Estimated with Phosphopeptide-Specific Antibodies

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In recent years, antibodies specific for phosphopeptides have become a major tool for assessing the phosphorylation of specific serine, threonine, and tyrosine sites on proteins (1,2). Typically, a phosphopeptide-specific antibody is used for the qualitative detection of phosphorylation at a site through immunoblotting the protein of interest. Here we describe a relatively simple method by which a phosphopeptide-specific antibody can be used to estimate the stoichiometry of phosphorylation at its corresponding site. By contrast, other methods for the estimation of the stoichiometry of phosphorylation at a site are considerably more demanding and complicated (3).

The stoichiometry of phosphorylation at a particular site is defined as Pt*/Pt, where Pt* is the total moles of protein phosphorylated on the site and Pt is the total moles of protein. To determine this ratio, the phosphopeptide-specific antibody is used to immunoprecipitate only the phosphorylated form of the protein. The overall efficiency of immunoprecipitation of the protein is measured by immunoblotting aliquots of the starting solution and the immunoprecipitate with an antibody against a nonphosphorylated epitope in the protein and comparing the signals of the latter with those of the former. This efficiency is P*p/Pt, where P*p is the moles of immunoprecipitated phosphorylated protein. If all the phosphorylated form of the protein were immunoprecipitated, then P*p would equal P*t, and the stoichiometry of phosphorylation would be equal to the overall efficiency of immunoprecipitation. However, usually only a fraction of the phosphorylated form is immunoprecipitated, and, consequently, a correction must be made. To do so, the efficiency of immunoprecipitation of only the phosphorylated protein is measured by immunoblotting aliquots of the starting solution and the immunoprecipitate with the phosphopeptide-specific antibody and comparing the signals of the latter with those of the former. This efficiency is P*p/P*t. Thus, the stoichiometry of phosphorylation (P*t/Pt) is equal to the ratio of the overall efficiency of immunoprecipitation (P*p/Pt) to the efficiency of immunoprecipitation of the phosphorylated form (P*p/P*t).

The protein is prepared for the immunoprecipitation with the phosphopeptide-specific antibody by denaturation with SDS and reduction with dithiothreitol. This procedure should expose the phosphopeptide site in the protein for reaction with the antibody. In addition, if the native protein exists as an oligomer, it converts it into the individual subunits, and thus the nonphosphorylated subunits do not coimmunoprecipitate with phosphorylated ones. The immunoprecipitation is performed after addition of sufficient nonionic detergent to incorporate the SDS into micelles so that the antibody is not denatured. A useful modification of the method would be to cap the sulfhydryl groups with N-ethylmaleimide to ensure that no disulfide bonds reform. This was not done in the present study because the protein examined contains a cysteine two residues from a phosphorylation site.

As an example of this method, we examined the phosphorylation of the serine kinase Akt1 on two sites, Thr 308 and Ser 473. Phosphorylation of these sites results in activation of this kinase (4). The required reagents were purchased as follows: active and inactive recombinant Akt1 (Upstate, Charlottesville, VA, USA); antibodies against the Akt1 phospho Thr 308 and phospho Ser 473 peptides (Cell Signaling Technology, Beverly, MA, USA); and a monoclonal antibody against the nonphosphorylated PH domain of Akt1 (Upstate). Stock solutions of 500 ng/mL Akt1 were prepared in 2% SDS, 10 mM dithiothreitol, 150 mM NaCl, 50 mM HEPES, pH 7.4, with 1 mg/mL ovalbumin as carrier, and held at 100°C for 5 min. For immunoprecipitations, 200 µL of this solution were mixed with 1.125 mL 1.7% nonaethyleneglycol dodecyl ether (Sigma, St. Louis, MO, USA), 150 mM NaCl, 50 mM HEPES, pH 7.4 (buffer A), and either 1 µg antibody against phospho Thr 308 or 200 ng antibody against phospho Ser 473 were added. After 2 h at 4°C, the immune complexes were collected by mixing for a further 2 h with 20 µL protein A-Sepharose™. The beads were then washed four times with buffer A, and

Table 1. Outline of the Protocol for Estimating the Stoichiometry of Site-Specific Phosphorylation

1. Denature and reduce the isolated protein in 2% SDS/10 mM dithiothreitol at 100°C for 5 min. Cool and cap the thiol groups by the addition of 2.5 moles of N-ethylmaleimide per mole dithiothreitol. Save an aliquot of this solution (the input solution) to use for immunoblotting.
2. To the remainder, add a sufficient volume of 1.7% nonaethyleneglycol dodecyl ether, 150 mM NaCl, 50 mM HEPES, pH 7.4 (buffer A), so that the ratio of this detergent to SDS is at least 3.5 by weight.
3. Immunoprecipitate the protein in buffer A with the phosphospecific antibody. Adsorb the immune complexes on protein A-Sepharose.
4. Elute the immunoprecipitated protein by suspending the protein A-Sepharose beads in SDS sample buffer and holding at 100°C for 5 min.
5. Immunoblot samples of the input solution and of the immunoprecipitate with an antibody against a nonphosphorylated epitope on the protein. Calculate the total efficiency of immunoprecipitation from the relative intensities on the immunoblot and the sample sizes as fractions of the input solution from which they were derived. Perform a second immunoblot of this type with the phosphopeptide-specific antibody, and calculate the efficiency of immunoprecipitation of the phosphorylated form in the same way.
6. The estimated stoichiometry of phosphorylation is the ratio of the total efficiency of immunoprecipitation to the efficiency of immunoprecipitation of the phosphorylated form.

the immunoprecipitate was released by heating with 150 µL SDS sample buffer (4% SDS, 10% glycerol, 1 mM EDTA, 0.004% bromophenol blue, 90 mM Tris, pH 6.8) containing 10 mM dithiothreitol at 100°C for 5 min. Ovalbumin at 100 µg/mL was included as carrier in the SDS sample buffer used for dilutions.

Samples of the starting solution and the immunoprecipitates were separated by SDS-PAGE and transferred electrophoretically to an Immobilon™ membrane (Millipore, Bedford, MA, USA). The membrane was blocked with 1% BSA in TBST (20 mM Tris, pH 7.6, 150 mM NaCl, 0.3% Tween® 20) treated with primary antibodies in 0.5% BSA, TBST for at least 1 h, and washed with TBST. Primary antibodies were used at the manufacturer's recommended concentrations. The membrane was then treated with HRP-protein A conjugate in the case of the rabbit phosphopeptide antibodies or HRP-goat anti-mouse immunoglobulin conjugate in the case of the mouse antibody against the PH domain (Bio-Rad Laboratories, Hercules, CA, USA). Detection was with the SuperSignal® chemiluminescence reagent (Pierce Chemical, Rockford, IL, USA) and autoradiography. Each immunoblot contained four amounts (1×, 1/2×, 1/4×, and 1/8×) of the starting solution of Akt1, which were chosen so that their signals bracketed one or more signals given by aliquots of the immunoprecipitate. The amount of Akt1 in each immunoprecipitate sample was estimated by visual comparison of its signal with those given by these standards. In our hands, this method gives values that are the same as those given by densitometry of the autoradiograms. The efficiency of immunoprecipitation was then calculated from the amount of Akt1 in the immunoprecipitate and the amount of Akt1 in the solution from which the immunoprecipitate was derived. We estimate that the error in the efficiency measured in this way is no more than ± 25%. If greater accuracy is desired, it might be achieved by the use of a range of more closely spaced input standards and instrumental quantitation of the blots.

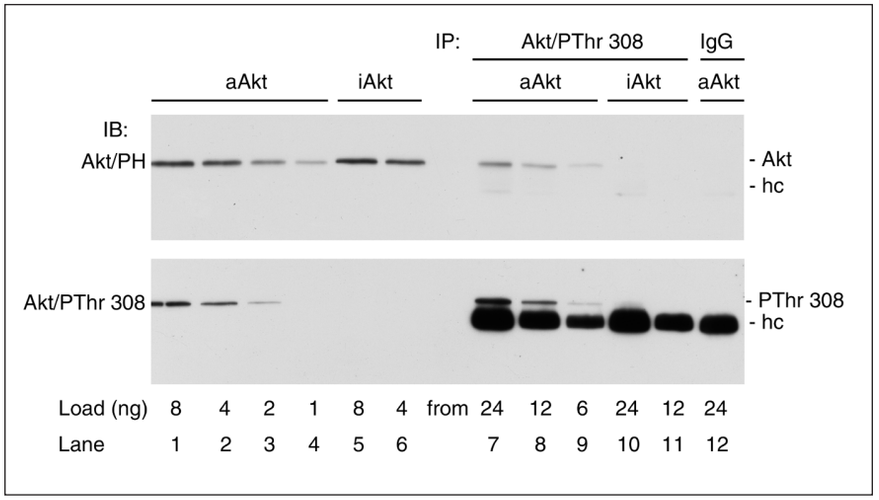

Figure 1. Immunoprecipitation of Akt1 with the antibody against phospho Thr 308. Aliquots of the starting solutions of active Akt1 (aAkt) (lanes 1–4) and inactive Akt1 (iAkt) (lanes 5 and 6) and of the immunoprecipitates of active Akt1 (lanes 7–9) and inactive Akt1 (lanes 10 and 11) with anti-phospho Thr 308 were immunoblotted with either the antibody against the Akt1 PH domain (upper panel) or the antibody against phospho Thr 308 (lower panel). Lane 12 contained a control immunoprecipitate in which active Akt1 was immunoprecipitated with irrelevant rabbit immunoglobulin (IgG). The band due to the heavy chain of the precipitating antibody is designated hc. For lanes 1–6, the loads of Akt1 in nanograms are given. For lanes 7–12, in each case, the nanogram given is the amount of Akt1 in the starting solution from which the load of immunoprecipitate was derived. Two repetitions of this experiment gave values similar to this one for the fraction of active Akt1 phosphorylated on Thr 308.

Figure 1 presents the results with the antibody specific for phospho Thr 308. The overall efficiency of immunoprecipitation of active Akt1 was approximately 8% (upper panel, compare lanes 7–9 with lanes 1–4 and see the figure legend), whereas the efficiency of immunoprecipitation of the phosphorylated active Akt1 was approximately 33% (lower panel, compare lanes 7–9 with lanes 1–4). Thus, approximately 8/33 or 24% of Thr 308 is phosphorylated in this preparation of active Akt1. Con-

trols showed that inactive Akt1 did not react with the antibody against phospho Thr 308, whereas it reacted equally as well with the antibody against the PH domain of Akt1 (lanes 5 and 6 of lower and upper panels, respectively). Also, the antibody against phospho Thr 308 did not immunoprecipitate inactive Akt1 (lanes 10 and 11 in both panels), nor did an irrelevant antibody immunoprecipitate active Akt1 (lane 12).

The same analysis was performed to determine the stoichiometry of phosphorylation on Ser 473 in Akt1, with the antibody against the phospho Ser 473 peptide. In this case, with active Akt1 the overall efficiency of immunoprecipitation and the efficiency of immunoprecipitation of the phosphorylated form were each approximately 33%, whereas no inactive Akt1 was immunoprecipitated (data not shown). Consequently, approximately 100% of the Akt1 in this preparation was phosphorylated on Ser 473.

The active Akt1 used here was prepared by phosphorylation of Thr 308 with the kinase PDK1 and Ser 473 with the kinase MAPKAP2 according to methods developed by Dr. Dario Alessi at the University of Dundee and transferred to Upstate. The stoichiometry of phosphorylation at each site for such preparations has been estimated from the incorporation of ^{32}P phosphate from $\gamma[^{32}P]$ATP into known amounts of Akt, and the values are in the range of 30%–70% (private communication, Dr. Alessi and Reference 5). Thus, the values here are in reasonable agreement with the estimates from this alternative method, when one considers that our method has the potential for some error due to some imprecision in the values for the efficiencies of immunoprecipitation and that the other method relies on estimates of protein amount, which can be inaccurate.

In assessing the significance of the phosphorylation of a protein in vivo at a particular site, it is useful to have an estimate of the stoichiometry. This method should be applicable to a phosphorylated protein in a cell lysate, by first isolating a representative mixture of phosphorylated and nonphosphorylated protein. For example, cells could be lysed in SDS/dithiothreitol and then the lysate prepared for immunoprecipitation with excess nonionic detergent and N-ethylmaleimide. The mixture of phosphorylated and non-phosphorylated protein could then be immunoprecipitated with the antibody against the non-phosphorylated epitope. This immunoprecipitate would then be solubilized in SDS and analyzed as described in the text and in Table 1, which summarizes the method.

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