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Hormone-induced Hyperphosphorylation of Specific Phosphorylated Sites in the Mouse Glucocorticoid Receptor

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The glucocorticoid receptor (GR) is phosphorylated in its basal state, and rapidly undergoes hormone-induced hyperphosphorylation after binding glucocorticoids. Previously, we have identified seven phosphorylated sites in the mouse GR. Most of the sites are located in the regions of the N-terminal domain that are necessary for maximum transcriptional activity and reduce nonspecific binding to DNA. Using WCL2 cells, which overexpress mouse GRs, we now quantitate hormone-induced hyperphosphorylation at each of these sites. Addition of triamcinolone acetonide to WCL2 cells results in significant hyperphosphorylation at the majority of the sites. The hyperphosphorylation ratio, i.e. the ^{32}P incorporation into GRs from hormone-treated cells divided by ^{32}P incorporation into GRs from untreated cells, was above 1.0 for all sites but serine 150 and threonine 159. Serine 220 displays marked hormone dependence, with a ratio of 3. For most sites the ratio was about 1.5. Hormone-induced hyperphosphorylation not only increases the charge at selected phosphorylated sites but also provides a substantial increase in the overall negative charge around the region of the N-terminal domain that is involved in transactivation.

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INTRODUCTION

Phosphorylation regulates the activity of many transcription factors [1] and alters transcription efficiency of the estrogen receptor in several cells types [2, 3]. Steroid receptors are basally phosphorylated, and become hyperphosphorylated after binding ligand [4].

Previously we showed that GRs in the mouse thymoma cell line WEHI-7 contain an average of 2.6 phosphates/receptor [5]. Ortí *et al.* [6, 7] demonstrated that agonists induce hyperphosphorylation of the GR in WEHI-7 mouse thymoma cells and WCL2 cells (Chinese hamster ovary cells which overexpress the mouse GR [8]). The antagonist RU486 fails to elicit hyperphosphorylation and antagonizes the effect of cortisol on GR hyperphosphorylation. Hormone-induced hyperphosphorylation has also been seen with the mouse GR in NIH 3T3 cells [9, 10] and with the rat GR in FTO 2B hepatoma cells [11].

The seven phosphorylated sites that we have identified in the mouse GR [12] are located in the N-terminal domain. Most of them lie within regions that are necessary for maximum transcriptional activity of the mouse GR and reduce nonspecific binding to DNA [13].

Neither the kinases involved nor the cellular site of hormone-induced hyperphosphorylation are known, but five of the seven sites are in consensus sequences for cell cycle-dependent kinases [12]. Kinetic analysis of GR phosphorylation in WEHI-7 and WCL2 cells has demonstrated that hyperphosphorylation occurs after the GR has become activated and acquired the ability to bind to DNA [7].

Using WCL2 cells, we have looked at specific phosphorylated sites that are affected by hormone-induced hyperphosphorylation. WCL2 cells were chosen for this study because their large number of GRs greatly facilitates measurement of phosphorylation. Their GRs have an identical phosphopeptide map to those in WEHI-7 cells [12] and exhibit similar hormone-induced hyperphosphorylation [7]. This study identifies the phosphorylated sites involved in hormone-induced

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Abbreviations: DMEM, Dulbecco's modified Eagle's medium; EDTA, disodium ethylenediamine tetraacetate; EGTA, [ethylenebis (oxyethylenenitrilo)]tetraacetic acid; GR, glucocorticoid receptor; HEPES, *N*-2-hydroxyethylpiperazine-*N*'-2-ethanesulfonic acid; Hsp90, ~90 kDa heat shock protein; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate; TA, triamcinolone acetonide, 9α-fluoro-11β,16α,17α,21-tetrahydroxypregna-1,4,diene-3,20-dione-16,17-acetonide.

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hyperphosphorylation and quantitates the relative change in these sites after addition of hormone.

MATERIALS AND METHODS

Materials

Sources for materials used in this study have been listed previously [12]. TA was obtained from Steraloids (Wilton, NH).

Buffers

The following buffers were used: FT, 25 mM TES (pH 8.2 at 3°C) 2 mM EDTA, 2 mM EGTA, 50 mM sodium fluoride, 20 mM sodium molybdate and 10% glycerol (v/v); FTT, FT buffer plus 0.2% Triton X-100; FTN, FT buffer plus 0.4 M sodium chloride; FTS buffer, FT buffer plus 1.6% SDS; FTNT, FTN buffer plus 0.2% Triton X-100; HBS buffer, 10 mM HEPES (pH 7.35) and 150 mM sodium chloride; KRBGH, Krebs–Ringer bicarbonate buffer supplemented with 5.5 mM glucose, 25 mM HEPES (pH 7.35) and 1 mg bovine serum albumin/ml; phosphate-free KRBGH, KRBGH where all phosphate has been replaced by an equimolar amount of saline; TSTG, 40 mM TRIS (pH 7.8) 4% SDS, 10% glycerol (v/v) and 20 mM dithiothreitol.

Cell culture and whole cell metabolic labeling

WCL2 cells were generously provided by Dr Margaret Hirst [8]. Cells were cultured in DMEM supplemented with 10% calf serum fortified with transferrin (Sigma Chemical Co., St Louis, MO, U.S.A.), 39.5 μg proline/ml, and 3×10^{-6} M methotrexate. Cells were maintained at 37°C with 5% CO_2.

For these studies we have used cells attached to microcarrier beads to ensure uniform metabolic labeling of the GR with [^{35}S]methionine and [^{32}P]orthophosphoric acid. Prior to each experiment, cells at ~50% confluency in four or five 162 cm^2 flasks (Costar, Cambridge, MA) were collected by trypsinization (2 ml, 0.05% trypsin, Sigma). The cells, resuspended in 50 ml of culture medium, were then incubated with 3 ml of Cytodex 3 microcarrier beads (Pharmacia, Piscataway, NJ) in a 162 cm^2 flask for 6–8 h. Most of the cells become attached to the beads during this incubation. Cells/beads were washed in fresh medium to remove non-adherent cells and then incubated overnight to allow the cells to fully attach. The next morning the cells/beads were washed and incubated for 24–36 h in 75 ml of fresh medium containing 6 mg/l methionine and 7.5 mCi of [^{35}S]methionine. Cells/beads were transfered to a 50 ml conical tube and allowed to settle. The supernatant was removed and the tube filled with phosphate-free DMEM containing 2.5% dialyzed fetal bovine serum. The tube was then laid horizontally in the cell culture incubator for 5 min, and vertically for another 5 min to sediment cells. The supernatant was removed, and the whole washing procedure repeated ~7 more times to remove phosphate. Cells/beads were transferred to a 162 cm^2 flask. The total volume of beads and phosphate-free medium was 75 ml. [^{32}P]orthophosphoric acid (12.5 mCi) was added to the flask and incubated at 37°C for 30 min. The cells/beads were mixed thoroughly and 35 ml aliquots were removed to two new flasks. TA (~4.5×10^{-5} M in HBS) was added to a final concentration of 5×10^{-7} M in one flask and HBS was added to the other flask. After 45 min to 2 h at 37°C the cells/beads were transferred to a 15 ml conical tube and centrifuged (500 ***g***, 20 s) to pellet the cells/beads. After removing the supernatant and washing with 10 ml of phosphate-free KRBGH, the cells/beads were centrifuged and the supernatant removed.

To extract all forms of the GR, cells were lysed by adding 5 ml of TFS, vortexing for 5 s and placing the tube in boiling water for 3 min. Once the sample had cooled to room temperature, 2.7 ml of FT plus 20% triton X-100 was added to scavenge the SDS. The sample was vortexed to shear the extracted DNA, and the supernatant was removed after centrifuging for 5 min at ~1000 ***g***.

Immunopurification of receptor

This procedure has been described in detail [12]. Briefly, the extract prepared above was cooled on ice for 15 min. FIGR antibody, a monoclonal antibody that reacts against both rat and mouse GR, was added to a 5-fold molar excess over the amount of receptor in the preparation (estimated from the number of cells times the number of receptors per cell). After ~14 h, Protein A-Sepharose was added to a 5-fold molar excess of binding capacity over the amount of FIGR antibody and the tube was slowly twirled for ~4 h. Protein A-Sepharose was collected by centrifugation (3 min at 200 ***g***, supernatant was removed and the Protein A-Sepharose was washed 6 times with 10 ml of FTN buffer. Protein A-Sepharose was transfered to a 1 ml column and washed with 7 ml of FTNT and 3 ml of FT buffer. Excess liquid was removed, the Protein A-Sepharose was expelled into 180 μl of TSTG buffer and the mixture placed in boiling water for 2 min. After cooling to room temperature the liquid was separated from the Protein A-Sepharose by centrifugation and the supernatant alkylated with iodoacetamide.

SDS–PAGE and generation of phosphopeptides

SDS–PAGE of the alkylated receptor on 7% gels, transfer to a PVDF membrane, elution of the GR from the membrane and generation of phosphopeptides by the use of methylated trypsin, were carried out as described previously [12].

Purification and analysis of phosphopeptides

HPLC analysis was performed on a Waters 840 HPLC work station equipped with two model 510 pumps, a U6K injector and a Beckman model 165 multi-wavelength detector. Samples were chromatographed on a

Vydac C4 column (2.1×250 mm, 5 μ beads with 300 Å pores) using 0.06% TFA (solvent A) as the ion pairing agent and 75% acetonitrile in 0.056% TFA (solvent B) as the organic modifier [14]. The flow rate was 0.15 ml/min and the peptides were eluted with a linear gradient from 0 to 48% solvent B at a rate of increase of 0.25% solvent B per min. Fractions were collected every 0.7 min and radioactivity was determined by liquid scintillation counting after addition of 2 ml of Hydrofluor (Research Diagnostics, Atlanta, GA). Corrections were made for spillover of ^{32}P into the ^{35}S spectrum. The amount of ^{32}P associated with each phosphopeptide was then determined by summing the radioactivity in all of the fractions under each peak after subtraction of background. Assignment of ^{32}P to specific phosphorylated sites was made by summing the radioactivity attributed to a site from all the phosphopeptides containing that site [12].

Calculation of hyperphosphorylation ratios

The sum of all ^{35}S in the chromatogram (typically 4000–7000 cpm) served to indicate how much GR was actually analyzed from each treatment. This number was used to normalize the ^{32}P in phosphorylated sites to the control GR concentration. After normalization the hyperphosphorylation ratio, defined as the ratio of TA treated to control ^{32}P, was determined for each phosphorylated site. The relative hyperphosphorylation ratio, which is a measure of each site's contribution to the total GR hyperphosphorylation, was determined by dividing the increase in ^{32}P incorporation of a particular site by the overall increase in incorporation for the entire GR (determined by summing all the individual sites).

RESULTS AND DISCUSSION

The bottom section of Fig. 1 shows typical HPLC phosphopeptide maps of the mouse GR from TA-treated

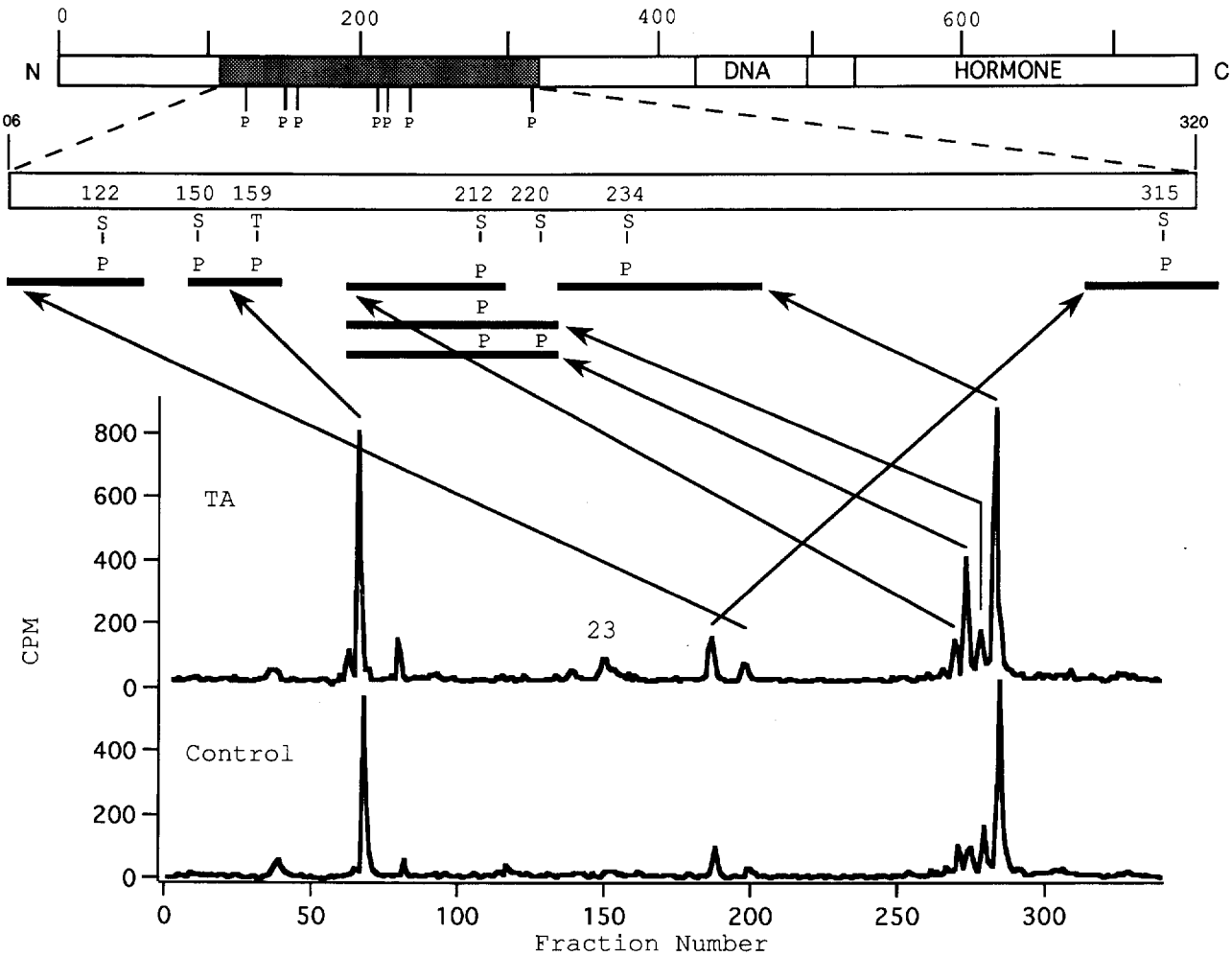

Fig. 1. Relationship of phosphorylated sites in the GR to the tryptic phosphopeptide map. At the top is a diagram of the mouse GR, with numbered residues. The shaded region, which is expanded below, contains all seven phosphorylated sites. Positions of individual phosphopeptides are shown by the solid black bars. The letters S, T and P designate serine, threonine and phosphate, respectively. At the bottom are representative HPLC phosphopeptide maps of GRs from TA-treated (1 h) and control WCL2 cells. Peaks and corresponding phosphopeptides previously identified [12] are indicated by arrows.

and control WCL2 cells. The arrows indicate the relationship of peaks to phosphopeptides containing known phosphorylated sites [12]. The phosphorylated sites at serines 212 and 220 are associated with more than one phosphopeptide because of incomplete digestion of the GR. Incomplete digestion appears to be a consequence of the acidic side chain of glutamic acid 216 adjacent to the tryptic site at lysine 215 [12]. Thus the four-peak complex around fraction 275 represents three of the seven phosphorylated sites. The left-most peak is the phosphopeptide containing phosphoserine 212. The next peak is an incompletely digested peptide containing phosphoserines 212 and 220. The third peak is the same incompletely digested peptide, phosphorylated only on serine 212. The last peak is the peptide containing phosphoserine 234. Fortunately there is no ambiguity and each of the sites can be quantitated by summing counts in the individual peaks. In the phosphopeptide containing both 212 and 220 one half of the radioactivity is allocated to each site. A single phosphopeptide contains both phosphoserine 150 and phosphothreonine 159. The phosphorylated site or sites associated with phosphopeptide 23 have not been definitively identified.

Kinetic analysis [7] of hormone-induced hyperphosphorylation has demonstrated that hyperphosphorylation is seen in cytosolic activated GRs (DNA binding forms) within 30 min of adding TA to WCL2 cells. By $\sim$2 h these hyperphosphorylated GRs are recycled back to unliganded GRs in the hyperphosphorylated state. Thus we have designed our experiments to study hormone-induced hyperphosphorylation of specific sites between 45 min and 2 h of hormone treatment.

In the basal state the mouse GR in WEHI-7 cells is phosphorylated at seven specific sites [12] yet contains an average of 2.6 mol of phosphate [5]. At this level of phosphorylation only $\sim$35% of the seven possible sites contain phosphate, while the remaining $\sim$65% are not phosphorylated. Steady state labeling experiments [12] show marked differences in the extent of phosphorylation between individual sites. Therefore there must be subpopulations of GRs having different combinations of phosphorylated and nonphosphorylated residues. An increase in the level of GR phosphorylation, such as in hormone-induced hyperphosphorylation, will alter the distribution of these subpopulations and must involve the phosphorylation of those GRs with nonphosphorylated sites.

We have evaluated the effect of hormone-induced hyperphosphorylation at individual sites by using the hyperphosphorylation ratio, defined as the ratio of ^{32}P associated with individual phosphorylated sites from TA treated cells compared to control cells. This ratio is a measure of the number of GRs that become phosphorylated at a site after hormone administration.

Hyperphosphorylation ratios from four experiments, with TA treatment varying from 45 min to 2 h, are presented in Fig. 2(A). The first point of note is that the time of TA treatment from 45 min to 2 h has little if any influence on the degree of hyperphosphorylation. The results clearly show that the ratio is substantially greater than 1.0 for the majority of sites. Serine 150 and threonine 159 had the lowest ratios, with ratios below 1.0 in two of the experiments and above 1.0 in the other two. Phosphoserine 220 and the unidentified site(s) associated with phosphopeptide 23 had ratios of nearly three, considerably more than other phosphorylated sites. Peptide 23, which accounts for a very minor fraction of total phosphorylation, elutes from the HPLC as a broad peak suggesting the presence of more than one phosphopeptide, or of heterogeneous post-translational modifications. The small peaks on either side of the peak representing phosphorylated sites 150 and 159 (Fig. 1) appear more phosphorylated after hormone treatment but these changes were not consistent between experiments.

To a large degree, the hyperphosphorylation ratio of individual sites depends on the initial number of GRs that were phosphorylated at that site. Relative hyperphosphorylation for each site from the pooled experiments in Fig. 2(A) is displayed in Fig. 2(B). It is the change in ^{32}P associated with an individual site after hormone treatment, expressed as a fraction of the sum of the change in phosphorylation of all the sites, and shows the relative contribution of each site to the average increase in charge of the GR. The bulk of the charge added to the GR during hormone-induced hyperphosphorylation occurs on serines 212 and 234. These two sites account for nearly half of the total hyperphosphorylation. Serine 220 and phosphopeptide 23, though having the highest hyperphosphorylation ratios, account together for only a third of the total increase in charge. Serines 315 and 122 have little effect on the overall charge, while serine 150 and threonine 159 showed both positive and negative effects depending on the experiment.

This is the first report that identifies and quantitates hormone-induced hyperphosphorylation of individual phosphorylated sites in GRs. In a previous study, using two-dimensional phosphopeptide mapping, DeFranco *et al.* [15] looked at the effect of dexamethasone on tryptic phosphopeptides from the rat GR in 6M2 cells. They showed that, relative to a constitutive phosphopeptide, hormone treatment increased four phosphopeptides by 1.3–13-fold, but they did not identify the phosphorylated sites.

Our results suggests two possible mechanisms that phosphorylation might influence receptor action. One is through large increases at specific sites such as seen at serine 220 or the site(s) contained in peptide 23, which will be reflected in increases in the GR subpopulations that are phosphorylated at those sites [4]. The second is through a general increase in the overall average negative charge in the N-terminal domain of the GRs. Additional

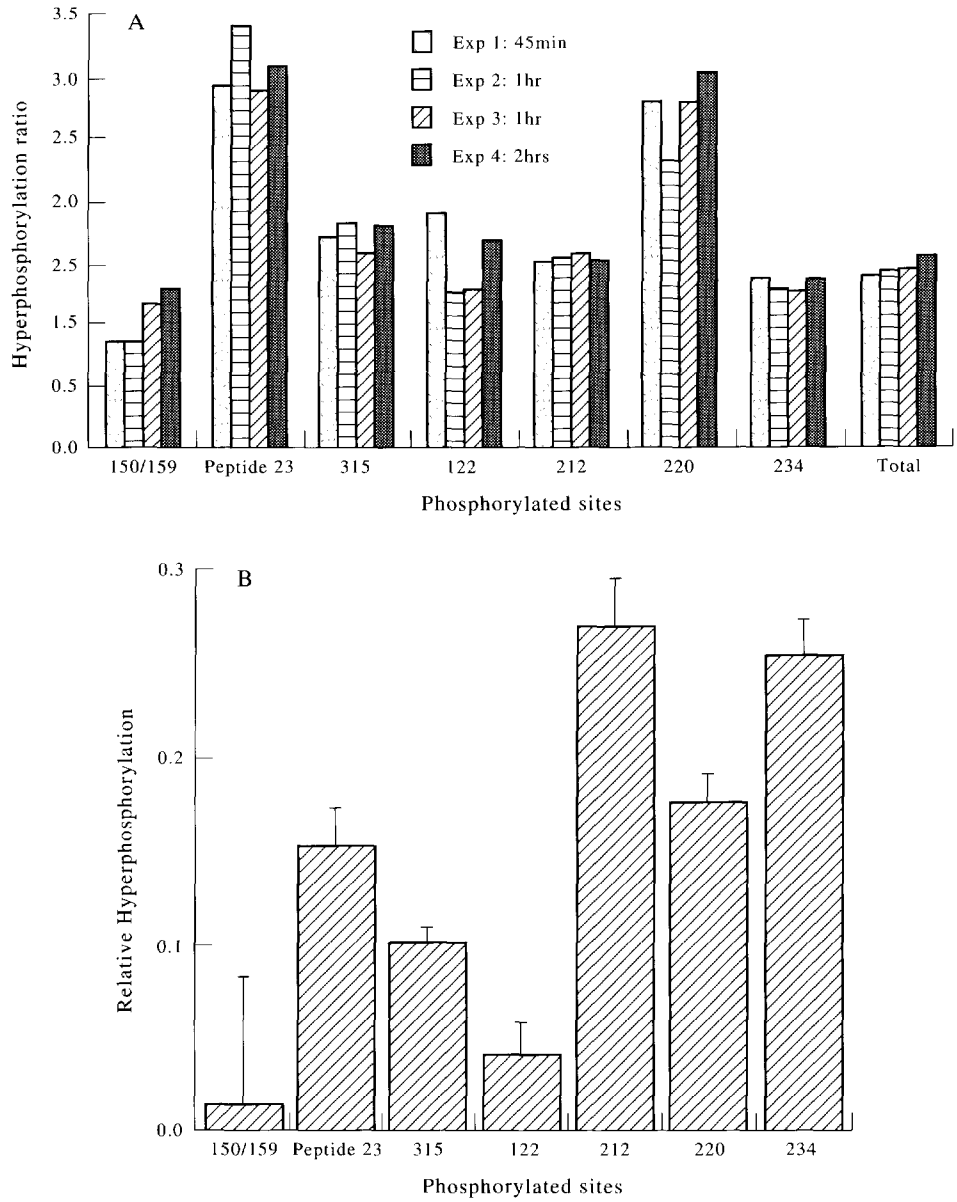

Fig. 2. Hormone-induced hyperphosphorylation of specific phosphorylated sites in the mouse GR. WCL2 cells attached to cytodex carrier beads were metabolically labeled for 24–36 h with [^{35}S]methionine. Cells/beads were incubated with [^{32}P]orthophosphoric acid for 30 min prior to the addition of TA or buffer for the indicated times. GR phosphopeptides were prepared and analyzed on HPLC. Typical chromatograms are shown in Fig. 1. The ^{32}P associated with each phosphopeptide was determined and assigned to specific phosphorylation sites. These values were corrected to a constant GR level using the total ^{35}S as a measure of GR content. (A) The hyperphosphorylation ratio, the ratio of ^{32}P in each individual site from TA treated cells to control cells. B. Relative hyperphosphorylation (see text), the relative contribution of each site to the overall average increase in charge of the GR. Bars represent the average (±SEM) of the four experiments shown in panel A.

studies will be needed to determine the relative importance of these two alternatives.

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