Native rat kidney mineralocorticoid receptor is a phosphoprotein whose transformation to a DNA-binding form is induced by phosphatases

Mario D. GALIGNIANA¹

Departamento de Química Biológica, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, and PRHOM-CONICET, 1428 Buenos Aires, República Argentina

Addition of alkaline phosphatase to rat kidney cytosol diminishes the ability of the mineralocorticoid receptor (MR) to bind aldosterone in a time-, temperature- and concentration-dependent form. A variety of phosphatase inhibitors, including levamisole, are effective in preventing this inactivation. On the other hand, when the steroid–receptor complex is incubated in the presence of alkaline phosphatase, an increment in the rate of receptor transformation is evidenced by a change in the sedimentation coefficient from 8.8 S to 5.1 S, as well as increased DNA-binding capacity. The effects of alkaline phosphatase on activation and transformation can also be observed when the MR is incubated at 20 °C in the cytosolic medium, indicating

that the catalytic action of an endogenous phosphatase may be involved in the transformation process. The ability of phosphatase inhibitors such as levamisole for suppressing both alkaline phosphatase- and endogenous phosphatase-directed transformation does not correspond well between them. Evidence is presented to affirm that the endogenous phosphatase activity is not due to an alkaline phosphatase-type, but it may be due to a protein serine/threonine phosphatase, as evidenced by the inhibitory effects of okadaic acid. The experimental results also show direct evidence that the MR undergoes phosphorylation in a physiological milieu.

INTRODUCTION

The mineralocorticoid receptor (MR) is a member of the steroid/thyroid hormone receptor superfamily of ligand-activated transcription factors. All members of this family share a common set of domains, each domain having a conserved function [1]. An evolutionary tree shows that MR, glucocorticoid receptor (GR), progesterone receptor (PR) and androgen receptor (AR) belong to a subfamily with similar ligand- and DNA-binding specificities [2]. The MR-steroid-binding domain is located at the C-terminus of the protein and shares ~60% homology with GR. Compared with human (h) GR, the DNA-binding domain of hMR is the most conserved among all steroid receptors (~94%), and is located approximately in the central region of the receptor. On the other hand, the N-terminal immunogenic domain is the least conserved (less than 15%), and may be responsible for differences in target-gene specificity [3].

Native steroid receptors are not necessarily confined to one intracellular compartment, but are capable of passing freely into and out of the nuclei of hormone-free cells [4]. For example, under steady-state conditions, most MR and GR molecules are cytoplasmic, whereas most PR and oestrogen receptor (OR) molecules are nuclear. However, when receptors are expressed in some cells lines the distribution may be the opposite. In effect, GR is localized to the cytoplasm of mouse fibroblasts, whereas the same GR expressed in Chinese hamster ovary cells remains predominantly in the nucleus [5]. Similarly, the hAR is nuclear in HeLa cells, but cytoplasmic in COS-1 cells [6].

It is now generally accepted that the association of heat-shock proteins (hsps) with steroid receptors is important for the stabilization of the steroid receptors in a non-DNA-binding and

transcriptionally inactive state. However, in this state the receptors adopt a high steroid-binding affinity conformation. In the presence of hormone, the steroid receptors are transformed to a DNA-binding and transcriptionally active form [7]. These requirements have also been found for the MR [8]. Unlike other members of this superfamily, little is known about the mechanism of activation of the MR, which is unstable and sparse in tissues. Two major factors that have been shown to affect its stability are high sensitivity to both oxidative processes [9] and extensive proteolysis [10].

Phosphorylation plays a major role in the activation and transformation of the other steroid receptors. The pioneer report demonstrating the correlation between intracellular ATP levels and GR binding capacity was published in 1968 by Munck and Brinck-Johnsen [11]. Nielsen et al. [12] reported that incubation of fibroblast cytosol with alkaline phosphatase (AlkP) prevented the steroid-binding activity of GR. It was subsequently shown that almost all receptors of this superfamily are phosphoproteins [13], and the study of the steroid-receptor phosphorylation has become a subfield of nuclear-receptor research.

However, little is known about the MR. Alnemri et al. [14] overexpressed human MR in Sf9 insect cells, and reported that the *recombinant* MR also undergoes phosphorylation. Certain aspects of this phenomenon remain unclear, such as the amino acids involved, hormone dependence, whether or not *native* MR is present in a phosphorylated form and how this phosphorylation is regulated.

The present study shows that kidney MR is a phosphoprotein whose sedimentation and DNA-binding properties are altered by treatment with AlkP. Evidence also suggests that the endogenous phosphatase that appears to affect MR transformation belongs to the PP1/PP2A serine/threonine phosphatase subgroup.

Abbreviations used: MR, mineralocorticoid receptor; GR, glucocorticoid receptor; PR, progesterone receptor; AR, androgen receptor; h, human; OR, oestrogen receptor; hsp, heat shock protein; ALDO, aldosterone; AlkP, alkaline phosphatase; PNPP, *p*-nitrophenyl phosphate; G1P, α -(*p*)-glucose-1-phosphate; RU28362, 17- α -alkanyl-11 β ,17-dihydroxy-androsterone; 4-NPR, 4-[(*p*-nitrophenyl)azo] resorcinol; PAS, protein A-Sepharose.

¹ Present address to which correspondence should be sent, 1301 Medical Science Research Building III, Department of Pharmacology, The University of Michigan Medical School, Ann Arbor, Michigan, MI 48109-0623, U.S.A. (e-mail mgali@umich.edu).

MATERIALS AND METHODS

Chemicals

[1,2-³H]Aldosterone (specific radioactivity = 50.0 Ci/mmol) and ¹²⁵I-conjugated antibodies were purchased from New England Nuclear (Boston, MA, U.S.A.). Radioinert aldosterone (ALDO), bovine intestine AlkP (type VII-NT), AlkP from *Escherichia coli* (type III-L), potato acid phosphatase (fraction III), human acid phosphatase from semen, levamisole, leupeptin, aprotinin from bovine lung, trypsin/chymotrypsin inhibitor from soybean, PMSF, 4-[(*p*-nitrophenyl)azo] resorcinol (4-NPR), okadaic acid and double-stranded DNA–cellulose from calf thymus (7 mg of DNA/g) were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). 17- α -Alkanyl-11 β ,17-dihydroxy-androsterone (RU28362) was a kind gift from Roussel-Uclaf (Romainville, France). *p*-Nitrophenyl phosphate (PNPP) assay kit was purchased from Boehringer Mannheim (Buenos Aires, Argentina). BuGR2 monoclonal IgG antibody against GR was from Affinity Bioreagents (Golden, CO, U.S.A.); AC88 monoclonal IgG against hsp90 was from StressGen (Victoria, BC, Canada); the rabbit pAbhMR polyclonal antibody against MR was a kind gift of Dr. G. Litwack and Dr. N. Robertson (Jefferson Cancer Institute, Philadelphia, PA, U.S.A.); goat anti-mouse IgG–horseradish peroxidase conjugate was from Sigma; and donkey anti-rabbit IgG–horseradish peroxidase was from Pierce (Rockford, IL, U.S.A.). All reagents used for SDS/PAGE were purchased from National Diagnostics (Atlanta, GA, U.S.A.).

Binding assays

Rat kidney MR was obtained from 200 g male Sprague-Dawley rats, which were adrenalectomized 48 h previously, as described in detail in [15]. Kidneys blanching by profuse perfusion with cold 0.9 % NaCl were homogenized with two volumes of cold TEGI buffer [(25 mM Tris/1 mM EDTA/25 % glycerol/10 mM β -mercaptoethanol/0.1 mM PMSF/2 IU/ml aprotinin/30 μ g/ml trypsin/chymotrypsin inhibitor (pH 7.4)]. The homogenate was centrifuged at 67000 *g* for 45 min at 0 °C, and the supernatant from this centrifugation was referred to as the cytosol.

Kidney cytosol was incubated for 10–12 h at 0 °C in the presence of 10 nM [³H]ALDO and 1.0 μ M RU28362 as described before [16]. Under these conditions, no cross-reactions with kidney GR were observed [9,17]. A 1000-fold excess of radioinert ALDO was used to determine the non-specific binding (~25 % of the total). The standard reaction volume used in these studies was 0.5 ml. Bound steroid was separated from free by adding 1 volume of 2 % (w/v) charcoal AR coated with 0.2 % (w/v) dextran 15–20 in the above buffer; the mixture was then shaken twice for 5 s, maintained on ice for 5 min and centrifuged at 8160 *g* (10000 rev. per min) for 5 min at 4 °C. The radioactivity in 0.4 ml of supernatant was counted with 60 % efficiency for tritium.

Sucrose gradient ultracentrifugation

Kidney cytosol was radiolabelled with [³H]ALDO by incubating for 4 h at 0 °C, and the free steroid was then removed with charcoal:dextran. The supernatant was incubated at 20 °C for 20 min to allow hsp90 (and associated proteins) to dissociate, and was then centrifuged on a 5–20 % sucrose gradient in TEGI buffer (both in the presence or absence of 20 mM Na₂MoO₄ or 10 mM levamisole). This was accomplished using vertical-tube rotor sucrose gradients with a Beckman VTi80 rotor. The gradient was centrifuged at 463000 *g* (80000 rev. per min) to a present cumulative centrifugal effect (w^2t) of 3.0×10^{11} rad²/s in a Beckman VTi65.2 vertical rotor for 90 min at 0 °C. 250 μ l

fractions were collected by gravity flow. [¹⁴C]Ovalbumin (3.5 S) and rabbit muscle [¹⁴C]aldolase (7.9 S) were used as internal standards.

Binding of the steroid–receptor complexes to ionic interchange resins

[³H]ALDO-labelled receptors were applied to a column of Sephadex G25 (1 cm $\times$ 10 cm) equilibrated with TEG [10 mM Tris/1 mM EDTA/10 % glycerol/1 mM β -mercaptoethanol (pH 7.4)]. The peak-excluded fraction was applied immediately to a minicolumn of DEAE-cellulose (1 cm $\times$ 3 cm) pre-equilibrated in TEG and washed with 3 volumes of buffer. This eluted fraction was applied to another minicolumn of DNA-cellulose pre-equilibrated in TEG and washed with three volumes of buffer. The radioactivity bound to both columns was eluted in batch with elution buffer (TEG/0.5 M KCl/0.2 % Triton X-100) and the radioactivity was counted. The percentage of activation was calculated by dividing the c.p.m. bound to DNA-cellulose into the addition of c.p.m. bound to both resins.

Effect of phosphatase inhibitors on MR-transformation

Small slices of kidney (~0.2 cm $\times$ 0.2 cm) were suspended in a modified Krebs-Henseleit buffer medium containing: 118 mM NaCl, 4 mM KCl, 1 mM KH₂PO₄, 27.2 mM NaHCO₃, 1 mM CaCl₂, 1 mM MgCl₂, 5 mM glucose, 3 mM adenine, 0.5 mM pyruvate, 0.1 mM sodium ascorbate, 10 mM Hepes (pH 7.4). Incubations were performed in a shaking incubator (360 rev. per min) aerated with 95 % O₂/5 % CO₂ in the presence of 20 nM [³H]ALDO ($\pm$ 10 μ M radioinert ALDO) and 1.0 μ M RU28362 for 20 min at 35 °C. The slices were then washed with buffer, homogenized and the DNA-binding capacity of the resultant cytosol fractions was assayed as described above. When the inhibition of endogenous phosphatases was attempted, two approaches were used: (i) 20 mM sodium vanadate, 10 mM levamisole or 100 nM okadaic acid were added to the medium; (ii) Ca²⁺ or Mg²⁺ salts were omitted from the buffer and 10 mM EGTA or 5 mM 4-NPR were added, respectively, to chelate contaminant traces of the cations.

PNPP hydrolysis assay

The activity of phosphatases and the effects of inhibitors of the enzymes were analysed using the standard PNPP hydrolysis assay, with or without an appropriate concentration of inhibitors. Incubation medium (1 ml) was placed into a cuvette and 350 IU of enzyme was added. This sample was mixed immediately and placed into a Shimadzu UV-3000 dual-wavelength double-beam recording spectrophotometer. Reactions were carried out for 10 min at 20 °C, and readings were taken at an absorbance of 405 nm every 30 s ($\epsilon_{405}^{\text{mM}} = 18.5$). The initial rate of the reaction was calculated.

Western blotting

MR was immunoadsorbed with pAbhMR antibody from kidney cytosols as follows: rat kidneys were removed after 48 h of adrenalectomy, cortex and cortex-medulla interphases were minced in TEGiMo buffer (TEGI with 20 mM Na₂MoO₄), and cytosol was obtained as described above. Cytosolic extracts (200 μ l) were precleared with 60 μ l of 20 % (w/v) protein A-Sepharose (PAS) for 30 min at 4 °C. The supernatant was adsorbed with 10 μ l BuGR2 and 60 μ l PAS for 1.5 h at 4 °C, and pellets were discarded. This procedure, repeated twice, prevents the slight cross-reactivity of pAbhMR with GR. 5 μ l pAbhMR

was added to precleared supernatants and incubated for 3 h at 4 °C. 100 μ l PAS was added and incubated for 5 h. The immunopellets were washed five times with TEGIMo buffer, boiled in SDS sample buffer with 10 % β -mercaptoethanol, and resolved on 12 % SDS-polyacrylamide gel. Proteins were transferred to an Immobilon-P membrane and probed with pAbhMR (anti-MR), AC88 (anti-hsp90) and BuGR (anti-GR). The immunoblots were then incubated with the appropriate horseradish peroxidase-conjugated counter-antibody to visualize immunoreactive bands, reincubated with ^{125} I-conjugated counter-antibody and autoradiographed. In every immunoadsorption of MR, GR was immunoadsorbed with BuGR2 antibody in order to clear GR from kidney cytosol extracts. Control incubations with non-immune rabbit serum (for MR) or nonimmune mouse IgG (for GR) were also performed to evaluate the non-specific adsorption of other proteins.

Phosphorylation of rat kidney MR

2 g of kidney tissue (cortex and cortex-medulla interphase) was minced with scissors in two volumes of phosphate-free medium. 50 μ Ci of [^{32} P]-orthophosphate was added and incubated for 2 h with continuous shaking (360 rev. per min) under a 95 % O_2 /5 % CO_2 atmosphere. The tissue was washed three times with 10 ml of Earle's balanced saline buffer and cytosol was obtained in TEGIMo buffer supplemented with 20 mM Na_3VO_4 , 20 mM NaF, 1 mM EGTA and 1 μ M okadaic acid. GR was immunoadsorbed with BuGR2 and pellets were discarded. Both radioactive and non-radioactive supernatants were fractionated with solid ammonium sulphate. Precipitated proteins (10 % to 30 % of saturation) were dissolved in 200 μ l of supplemented buffer, immunoadsorbed with pAbhMR and washed with TEGIMo buffer. The immunopellets were then stripped for 1.5 h at 4 °C with 0.5 M NaCl, and washed three times with TEGIMo. Proteins were resolved by electrophoresis on a 12 % SDS/polyacrylamide gel, transferred to membranes and autoradiographed. When incubations with phosphatases were intended, molybdate was eliminated from the immunopellets by two washes with TEGI buffer. Samples were then incubated for 20 min at 20 °C with

150 μ l of cytosol in TEGI buffer, in the absence or presence of 400 μ g/ml AlkP.

RESULTS

AlkP promoted MR inactivation

The ability of bovine AlkP to inactivate kidney MR binding capacity at 20 °C is shown in Figure 1(A). The MR binding capacity is diminished in a concentration-dependent form by preincubating with AlkP, suggesting that a dephosphorylation may promote MR inactivation. It has been suggested that when GR from fibroblasts or liver cytosol is incubated with AlkP, the loss of GR steroid-binding capacity may be the result of changes in the redox potential due to the inactivation of NADPH [18]. This is not the case for kidney MR, since the binding capacity

Table 1 Effects of AlkP and phosphatase inhibitors on kidney MR binding capacity

Rat kidneys were homogenized in TEGI buffer with the supplements indicated. Cytosol (10.5 mg of protein/ml) was preincubated at 20 °C for 20 min. The specific binding was then measured in the presence of 10 nM [^{3} H]ALDO, 40 mM Na_2MoO_4 and 1.0 μ M RU28362 at 0 °C for 10 h. The specific binding measured in controls incubated at 0 °C for 10 h without treatment was 1275 ± 133 c.p.m./mg of protein. The data presented are from triplicate incubations and represent mean $\pm$ S.E.M.

Condition	Specific binding (%)
TEGI buffer	$100 \pm 4^*$
+ 400 μ g/ml AlkP	$68 \pm 4^\ddagger$
+ 40 mM phosphate	$121 \pm 4^\dagger$
+ 40 mM vanadate	$132 \pm 6^\dagger$
+ 40 mM fluoride	$127 \pm 5^\dagger$
+ 40 mM molybdate	$122 \pm 5^\dagger$
+ 10 mM levamisole	$140 \pm 9^\dagger$
+ 10 mM levamisole + 400 μ g/ml AlkP	96 ± 5

* Equal to 857 ± 35 c.p.m./mg of protein.

† Different from incubations performed with TEGI buffer, $P < 0.02$.

‡ Different from incubations performed with TEGI buffer, $P < 0.01$.

Figure 1 Effect of AlkP on MR binding capacity

(A) Inactivation of MR depends on AlkP concentration. Rat kidney cytosol (9 mg of protein/ml in TEGI buffer) was preincubated with different concentrations of bovine intestinal AlkP. After 20 min at 20 °C, 10 mM EDTA/20 mM Na_2MoO_4 (final concentration) were added to each sample, placed on ice and incubated for 10 h in the presence of 10 nM [^{3} H]ALDO and 1 μ M RU28362. A 1000-fold excess of radioinert ALDO was used to determine non-specific binding. Data represent mean $\pm$ S.E.M. ($n = 3$). (B) The effect of exogenous AlkP is time- and temperature-dependent. Cytosol was preincubated with 400 μ g/ml bovine AlkP at 0 °C, 20 °C and 32 °C. At indicated times, 0.5 ml aliquots were removed and the specific binding capacity was determined (mean $\pm$ S.E.M., $n = 3$) in accordance with the procedure described for panel A. Results are presented as the percentage of the specific binding measured in the presence of AlkP (B) with respect to controls incubated in the absence of AlkP (Bc).

Figure 2 MR sucrose-gradient ultracentrifugation properties

Kidney cytosol (10.8 mg of protein/ml) was labelled with 10 nM [^3H]ALDO at 0 °C for 4 h in the presence of 1.0 μM RU28362. After the removal of free hormone, the [^3H]steroid–MR complexes were transformed by incubating at 20 °C for 20 min (or kept at 0 °C) in a medium supplemented as is indicated below. Samples were centrifuged on 5–20% sucrose gradients using [^{14}C]ovalbumin (OA) and [^{14}C]aldolase (AL) as internal standards. (A) Samples were kept at 0 °C for 20 min in the presence of: buffer (□), 20 mM Na_2MoO_4 (■) or 10 mM levamisole (▲). (B) Samples were transformed at 20 °C for 20 min in the presence of: 400 $\mu\text{g}/\text{ml}$ bovine intestine AlkP (□), 400 $\mu\text{g}/\text{ml}$ AlkP plus 20 mM Na_2MoO_4 (■) and 400 $\mu\text{g}/\text{ml}$ AlkP plus 10 mM levamisole (▲). (C) Pooled fractions of 8.8 S peak were incubated for 20 min at 0 °C with 20 mM molybdate (■) or at 20 °C with 400 $\mu\text{g}/\text{ml}$ AlkP in the presence (▲) or absence (□) of 10 mM levamisole. Samples were then re-centrifuged on 5–20% sucrose gradient. (D) Samples were transformed at 20 °C for 20 min in the presence of 20 mM Na_2MoO_4 (■), 10 mM levamisole (▲) or buffer only (□).

remains unchanged when preincubations were performed in the presence of 5 mM NADPH (results not shown)

The control incubated without AlkP for 20 min at 20 °C shows a specific binding of 965 ± 89 c.p.m./mg, a value which is 27% lower ($P < 0.001$) than the specific binding measured when samples without AlkP were placed on ice for 20 min (1317 ± 101 c.p.m./mg). This observation shows that under these conditions, other temperature-dependent processes can also affect MR binding capacity besides the inactivation observed with exogenous AlkP.

Figure 1(B) shows the effect of time and temperature on MR inactivation caused by 400 $\mu\text{g}/\text{ml}$ AlkP. Results are referred as B/B_c ratio in order to normalize the different treatments, where B is the specific binding measured in the presence of AlkP, and B_c is the control incubated for the same period of time and at the same temperature, but in the absence of AlkP. This control evaluates the loss of binding capacity due to processes other than the dephosphorylating activity of added AlkP (i.e. due to thermal instability or proteolysis). As can be seen, MR is completely

inactivated after 10 min at 32 °C and partially inactivated at 20 °C for 20 min. On the other hand, there is no effect at 0 °C.

In this series of experiments, control samples were also preincubated in the presence of 40 mM orthovanadate and 10 mM levamisole in order to inhibit the phosphatase activities. Equivalent specific binding of [^3H]ALDO could be measured when cytosols were preincubated at 32 °C for 10 min (1197 ± 388 c.p.m./mg), 20 °C for 40 min (1278 ± 345 c.p.m./mg) and 0 °C for 40 min (1321 ± 277 c.p.m./mg). These observations confirm that the inactivation of MR observed in Figure 1(B) is due mainly to the phosphatase activity rather than proteolysis or thermal denaturation.

It should be noted that all reactions were performed at pH 7.4 to preserve MR stability since experiments performed at pH ≥ 8.0 exhibited significant loss of MR binding capacity. This may be due to the spontaneous dissociation of hsp90 from the receptor induced by the alkaline pH [18]. None the less, phosphatases were very active in the hydrolysis of PNPP (results not shown).

Figure 3 5.1 S is an hsp90-free form of MR that can bind to DNA

(A) The 5.1 S form of MR, but not the 8.8 S form, binds to DNA-cellulose. Pooled 8.8 S and 5.1 S fractions of MR were bound to DEAE-cellulose or DNA-cellulose. The radioactivity associated to each resin was counted and expressed as a percentage of the total radioactivity bound to both columns. (B) pAbhMR antibody exhibits slight cross-reactivity with immunoadsorbed GR. Pooled 5.1 S and 8.8 S peaks recovered from three sucrose gradients were concentrated by ultrafiltration, immunoadsorbed with BuGR antibody, resolved in 12% SDS/PAGE, transferred to membranes and blotted with BuGR2 (upper line) or pAbhMR (lower line). (C) The 5.1 S form of MR is not associated to hsp90. MR from three pooled 5.1 S and 8.8 S peaks was bound to DEAE-cellulose and DNA-cellulose, as shown in (A). The receptor associated with each resin was extracted by boiling in SDS sample buffer, electrophoresed in a 12% gel, and blotted with pAbhMR for MR and AC88 for hsp90. NI, non-immune antibody; I, immune antibody.

Zinc has been shown to be an essential cofactor for phosphatase activity [12]. However, Zn^{2+} was not added to the incubation media since its absence does not cause a significant decrease in enzymic activity. The presence of 1 mM EDTA also failed to alter AlkP activity, as measured by hydrolysis of PNPP and MR binding capacity, but a final concentration of 10 mM EDTA, which has been shown as optimal for preserving MR binding capacity [9], inhibited AlkP activity (~60%). Herein a concentration of 1 mM EDTA was used consistently.

The experiments shown in Table 1 were performed in order to determine the effect of compounds with known phosphatase

inhibitory activity on the MR steroid-binding capacity. Oxyanions such as molybdate, vanadate and phosphate, as well as sodium fluoride, were factors that not only preserved, but also increased significantly MR steroid-binding capacity over controls incubated in TEGI buffer. This observation may be associated with the ability of these compounds to inhibit endogenous phosphatase activity, as was also evidenced in experiments shown in Figure 1.

Levamisole, a specific inhibitor of AlkP, exhibited the highest degree of protection of MR binding capacity. This effect was observed not only when used to protect MR against exogenous AlkP, but also against the activity of endogenous AlkP, as 40% higher specific binding was observed over the controls incubated with buffer TEGI only.

The only phosphatases which promoted inactivation were those that belong to the AlkP family. Similar results to those shown here were obtained when AlkP from *E. coli* was used, but not when potato acid phosphatase or human semen acid phosphatase were assayed (results not shown), even when all phosphatases assayed were extremely active in the hydrolysis of PNPP.

AlkP promotes MR transformation

Experiments were performed to characterize the phosphatase-promoted receptor by using sucrose-gradient ultracentrifugation. Since sodium molybdate has been shown to stabilize untransformed steroid receptors, and levamisole was effective in protecting MR against exogenous phosphatases (Table 1), the effects of these reagents on hydrodynamic properties of the receptor were assayed.

Figure 2(A) shows that when homogenization and incubation are performed at 0 °C in a buffer without supplements, a typical pattern for steroid receptors is found. Under these conditions, MR shows a major peak of untransformed MR (8.8 S), which represents ~77% of the total, as well as a minor peak of transformed MR (5.1 S), which represents ~23% of the total. This second peak appears to fade in the presence of levamisole and disappears completely in the presence of molybdate. The addition of exogenous AlkP (Figure 2B) resulted in a large increase in the 5.1 S peak (78%), with a concomitant loss of the 8.8 S peak (22%). The presence of 20 mM molybdate inhibited the MR transformation induced by exogenous AlkP at 20 °C. However, this transformation was only partially inhibited in the presence of 10 mM levamisole. In effect, the 5.1 S peak represents 45% of the radioactivity whereas the 8.8 S peak accounts for the remaining 55%. This result suggests that MR transformation may be induced by another endogenous phosphatase that is not inhibited by levamisole. Figure 2(C) shows that pooled fractions of the 8.8 S peak reincubated with AlkP shifted to 5.1 S, whereas the presence of levamisole partially protected that transformation. The relative abundance of the 5.1 S peak observed in Figure 2(C) is lower than the one observed in Figure 2(B), suggesting a partial clarification of the putative endogenous phosphatase after the first gradient.

The experiment shown in Figure 2(D) was designed in order to confirm the afore-mentioned possibility that an endogenous phosphatase may be responsible for shifting the sedimentation coefficient of MR. The transformation of the receptor was observed when incubations of cytosol were performed at 20 °C in the absence of protecting agents. This transformation was totally abolished by the presence of 20 mM Na_2MoO_4 , but not by 10 mM levamisole, confirming that the endogenous phosphatase can be inhibited by molybdate and not by levamisole. These

Table 2 Effect of vanadate and levamisole on MR transformation

Kidney cytosol was labelled with [^{3} H]ALDO under optimal conditions (TEGI buffer with 20 mM molybdate, at 0 °C for 4 h). The samples were applied to a minicolumn of Sephadex G25 equilibrated in TEG buffer to remove free steroid and salts. The peak-excluded fraction was immediately concentrated by ultrafiltration and incubated at 20 °C for 20 min in the presence of levamisole, vanadate, bovine intestine AlkP or combinations of these treatments. After the incubation period, 20 mM Na₂MoO₄ was added to all samples to stabilize steroid–receptor complexes, which were placed on ice. The binding properties to DEAE-cellulose (MR activation) or DNA-cellulose (MR transformation) were assayed as described in the Materials and methods section. Results in parentheses are the percentage of the total binding shown in the first column.

Condition	Specific binding (c.p.m./mg of protein)		
	Total	DEAE-cellulose	DNA-cellulose
TEGI buffer	818	151 (18.5)	575 (70.3)
+ 10 mM levamisole	998	280 (28.1)	630 (63.1)
+ 40 mM VO ₄ ³⁻	1134	861 (75.9)	159 (14.0)
+ 400 μ g/ml AlkP	626	105 (16.8)	471 (75.2)
+ 400 μ g/ml AlkP + 10 mM levamisole	802	281 (35.0)	416 (51.9)
+ 400 μ g/ml AlkP + 40 mM VO ₄ ³⁻	1067	782 (73.3)	170 (15.9)
TEGI buffer incubated at 0 °C (control)	1227	921 (75.1)	218 (17.8)

results suggest that dephosphorylation may be an important factor required for MR transformation, but also that this dephosphorylation is not performed by an endogenous phosphatase belonging to the AlkP family.

The 5.1 S form of MR is dissociated from hsp90 and binds to DNA–cellulose columns

It is known that transformed MR possesses the ability to interact with cationic interchange resins such as DNA–cellulose, whereas untransformed oligomeric receptors bind to anionic interchange resins such as DEAE-cellulose [9]. To validate that correlation, [^{3} H]ALDO–MR complexes were incubated at 20 °C with AlkP or placed on ice in buffer containing molybdate. 5.1 S and 8.8 S forms were separated by centrifugation in a 5–20% sucrose gradient. Pooled peaks were applied to a minicolumn of DEAE-cellulose and washed, and the unbound fraction was immediately applied to a minicolumn of DNA–cellulose connected in series. The radioactivity associated with each column is shown in Figure 3(A). As expected, most of the 8.8 S form of MR bound to DEAE-cellulose while the 5.1 S bound to DNA–cellulose column. The bound receptor on each column was extracted by boiling the resin with SDS–sample buffer. Proteins were resolved by electrophoresis and Western blots with the antibodies pAbhMR (for MR), BuGR (for GR) and AC88 (for hsp90) were performed. Figure 2(B) shows the band of GR (~98 kDa) specifically immunoadsorbed with BuGR2 from either 5.1 S and 8.8 S pooled peaks. GR is mainly present in the 8.8 S fraction. The weak band of GR observed in 5.1 S fractions is related to the poor capacity of ALDO to transform GR at the concentration used in the incubations [17]. When immunoadsorptions were blotted with pAbhMR, a slight cross-reactivity can be observed with the GR present in the 8.8 S fraction. On the other hand, blots accomplished with pAbhMR show a band of ~105 kDa, which is attributed to MR in accordance with the calculated mass of the amino acid sequence of rat MR [19]. Figure 3(C) also demonstrates that hsp90 is co-immunoadsorbed with MR when the receptor is only in the 8.8 S form, confirming that 5.1 S forms lost hsp90 during the transformation process. It should be noted that the afore-described results were observed when the slight

Figure 4 Effects of fluoride on endogenous phosphatase activity

[^{3} H]ALDO-labelled cytosol was incubated for 20 min at 20 °C in the presence of the indicated concentrations of NaF. Samples were divided into two fractions: one was applied to a DNA–cellulose column to determine the percentage of MR transformation (■), and the other underwent ultracentrifugation on a 5–20% sucrose gradient, after which the 5.1 S peak was quantified (▲). Aliquots (50 μ l) of labelled cytosol were also assayed for PNPP hydrolysis ability (●).

Table 3 Effects of phosphatase inhibitors on kidney MR transformation

Kidney slices were incubated for 20 min at 20 °C with 20 nM [^{3} H]ALDO ($\pm$ 10 μ M radiolabelled ALDO) in a medium containing: (i) 10 mM EGTA without Ca²⁺ salts; (ii) 5 mM 4-NPR without Mg²⁺ salts; (iii) 100 nM okadaic acid [t_0]; and (iv) 20 mM sodium vanadate or 10 mM levamisole. The slices were washed and homogenized in TEGIMo buffer and the DNA–cellulose binding capacity was assayed on the resultant cytosol fractions. To evaluate the effects of okadaic acid on the conditions of incubation, slices were also preincubated for 45 min in the presence of inhibitor [t_{45}] followed by incubation with [^{3} H]ALDO as described below.

Condition	Specific binding (c.p.m./mg of protein)	DNA-binding capacity	
		c.p.m./mg of protein	% Total*
None	899	645	71
+ 20 mM VO ₄ ³⁻	1245	411	33
+ 10 mM Levamisole	1015	660	65
+ 10 mM EGTA/ – Ca ²⁺	923	618	68
+ 5 mM 4-NPR/ – Mg ²⁺	890	581	64
+ 100 nM Okadaic acid [t_0]	829	347	42
+ 100 nM Okadaic acid [t_{45}]	907	245	27

* Calculated as follows: [bound to DNA-cellulose/(bound to DNA-cellulose + unbound to DNA-cellulose)] $\times$ 100.

cross-reactivity of pAbhMR with GR was eliminated by previous immunoadsorption of GR with BuGR2 (see below).

Table 1 shows that vanadate and levamisole are the most effective agents in protecting MR binding capacity, whereas incubations with AlkP significantly inhibited it. In the experiments shown in Table 2 I used the differential ability of MR to

Figure 5 Phosphorylation of MR

Minced kidneys obtained from adrenalectomized rats were incubated for 2 h in phosphate-free medium with 50 μ Ci [^{32} P]orthophosphate. Cytosols were incubated with BuGR2 to adsorb out GR. The supernatants were immunoabsorbed with pAbhMR, stripped with 0.5 M NaCl, and resolved on 12% SDS/PAGE. (A) Labelled cytosol was immunoabsorbed with BuGR2 to eliminate the contamination with [^{32} P]GR. The resultant supernatant was fractionated with ammonium sulphate, dissolved in buffer and [^{32} P]MR was immunoabsorbed with non-immune rabbit serum (NI) or pAbhMR antibody (I). Immunopellets were then stripped with 0.5 M NaCl for 2 h, washed with TEGImo buffer, resolved in 12% SDS/PAGE and autoradiographed. Cyt = 5 μ l of crude kidney cytosol. (B) Kidney cytosol was clarified of [^{32} P]GR by three consecutive immunoabsorptions with BuGR2. Each immunopellet (1, 2 and 3) was washed, electrophoresed, transferred to Immobilon membranes and autoradiographed. The upper line shows ^{32} P-labelled GR obtained in each immunoabsorption with BuGR2. The lower line shows a Western blot developed with pAbhMR for each immunoabsorption of GR shown in the upper line. NI, adsorption with non-immune mouse IgG; I, adsorption with BuGR2 antibody. (C) [^{32} P]GR was adsorbed out with BuGR2. Supernatants were fractionated with ammonium sulphate, redissolved in buffer and immunoabsorbed with non-immune rabbit serum (lanes 1 and 2) or pAbhMR (lanes 3–5). Pellets were stripped with 0.5 M NaCl and washed with buffer lacking molybdate. Untreated kidney cytosol (150 μ l) in TEGImo buffer was added to each immunopellet and incubated for 20 min in the following conditions: Lanes 1 and 2, 0 $^{\circ}$ C; lane 3, 20 $^{\circ}$ C; lane 4, 20 $^{\circ}$ C with 100 nM okadaic acid; lanes 5 and 6, 20 $^{\circ}$ C with 400 μ g/ml bovine intestinal AlkP. Samples were washed twice with TEGImo, resolved in 12% SDS/PAGE and autoradiographed (upper line). The same incubations were performed in parallel with unlabelled receptor (lower line) and a Western blot was performed with pAbhMR antibody to evaluate the amount of protein after each treatment.

be bound by resins in order to study symmetrical effects of those compounds on MR transformation. Labelled receptors were incubated at 20 $^{\circ}$ C with levamisole, vanadate and/or AlkP. The products of these incubations were assayed by DEAE-cellulose (activation) and DNA-cellulose (transformation) binding assays. The radioactivity not bound by resins was considered to be the result of degradation products generated during the incubation at 20 $^{\circ}$ C ($\leq 15\%$).

Consistent with data of Table 1, controls performed with levamisole and vanadate show increased steroid binding, whereas AlkP reduced it in a levamisole- and vanadate-sensitive form. However, whereas VO_4^{3-} inhibits MR transformation, levamisole was shown to be unable to inhibit this process. These results show that dephosphorylation catalysed by bovine AlkP and endogenous phosphatases can inhibit steroid binding and cannot only change the hydrodynamic properties of the MR by increasing the 5.1 S form (Figure 2), but also that this shift confers DNA-binding properties to MR.

Table 2 shows again that vanadate is efficient in inhibiting the endogenous phosphatase as well as AlkP. In effect, at variance with the levamisole effect, the presence of vanadate inhibited the activity of both bovine AlkP (15.9% of binding to DNA) and the endogenous phosphatase (14% of binding to DNA). These values are similar to those observed in controls incubated at 0 $^{\circ}$ C.

AlkP treatment of preformed steroid-receptor complex led to a loss of the total binding capacity when compared with incubations performed at 20 $^{\circ}$ C with TEGImo buffer. On the other hand, the presence of vanadate led to the opposite effect. These observations may reflect the consequence of a different degree of MR phosphorylation on binding capacity; similarly with those results shown in Figure 1 and Table 1 for unoccupied receptors.

Effects of sodium fluoride on endogenous-receptor transformation

I endeavored to determine if NaF would be effective in suppressing both endogenous MR transformation and cytosolic phosphatase activity. In preliminary experiments (Table 1) it was found that, aside from the known effects of oxyanions on receptor binding capacity, NaF also exhibits a protective effect on MR steroid-binding ability. This may be due to their ability to inhibit endogenous phosphatases. As vanadium salts may also affect protease activity [20], my interest was focused on the effects of fluoride, since this compound is not known to be a protease inhibitor. Figure 4 shows that NaF inhibits receptor transformation in cytosols (tested as binding to DNA and shifting to 5.1 S form) and the endogenous phosphatase activity (measured as hydrolysis of PNPP) in an equivalent form. Similar results were also obtained when a full dose-response curve was achieved with vanadate (results not shown).

These experiments demonstrate that the 8.8 S $\rightarrow$ 5.1 S shift and DNA-binding capacity are both connected to the activity of the endogenous factor, which is inhibited by fluoride.

Endogenous phosphatase activity

The next goal was to identify which endogenous phosphatase may be involved in inhibiting MR transformation. Attention was focused on phosphoserine/threonine phosphatases, since NaF has been implicated as an efficient inhibitor of these enzymes [21]. The protein serine/threonine phosphatase family is divided into two groups, referred to as type 1 (PP1) and type 2 (PP2), depending on substrate specificities. In turn, the type 2 phosphatase group has been subdivided into three subgroups (PP2A,

PP2B and PP2C), based on their dependence on bivalent cations and sensitivity to certain inhibitors. Using these criteria, kidney slices were incubated in a Ca^{2+} - or Mg^{2+} -free medium, or in the presence of the PP1/PP2A specific inhibitor, okadaic acid. Inhibition of DNA-binding capacity by vanadate and levamisole were used as controls. Table 3 shows that the DNA binding ability of MR is not affected by the absence of either bivalent cation. This fact suggests the possibility that the endogenous phosphatase might not belong to the PP2B/PP2C type, since these enzymes exhibit an absolute requirement for bivalent cations. On the other hand, PP1/PP2A phosphatases exhibit activity in buffers lacking bivalent cations. Consistent with this observation, the inhibition of the MR DNA-binding capacity by the marine-sponge toxin okadaic acid argues in favour of PP1 and/or PP2A endogenous activities. Okadaic acid (100 nM) does not exhibit an inhibitory effect on either PP2B/PP2C phosphatases or AlkPs [22], once more supporting the notion that the endogenous phosphatase does not belong to this group of enzymes. It should be noted that okadaic acid does not affect MR steroid-binding capacity as AlkP does (Figure 1 and Table 1), suggesting the existence of different phosphate groups on the MR, which may be hydrolysed selectively by different kinds of phosphatases.

The studies shown in Figure 5 were accomplished in order to provide direct evidence for receptor phosphorylation and confirm the effect of the selective inhibitor for PP1/PP2A phosphatases. Minced kidney was incubated in a phosphate-free medium with [^{32}P]orthophosphate, while controls for Western blot were incubated without radioactivity. In order to prevent the slight cross-reaction of pAbhMR with labelled GR, cytosols were clarified previously by immunoadsorption with BuGR2. MR, labelled or not, was then purified partially, immunoadsorbed and blotted.

Figure 5(A) shows the comparative phosphorylation pattern of whole-kidney cytosol and partially purified and immunoadsorbed MR (same exposure time). A major 100–105 kDa phosphorylated band can be seen. Autoradiographs shown in Figure 5(B) demonstrate that when samples are clarified of labelled GR after three immunoadsorptions with BuGR2 antibody (efficiency 60 % per immunoadsorption), there is neither detectable radioactivity due to GR (Figure 5B, upper line), nor detectable GR with pAbhMR by Western blot (Figure 5B, lower line).

Figure 5(C) shows immunoadsorptions of radioactive MR (autoradiographs on upper line) and non-radioactive MR (immunoblots in lower line). Lane 2 shows the phosphorylation degree of MR in samples kept on ice. When the immunopellet was treated for 20 min at 20 °C with kidney cytosol as a source of endogenous phosphatase, the degree of phosphorylation of MR was diminished by 50 % (lane 3) in an okadaic acid-sensitive form (lane 4). Levamisole (10 mM) was added to all of these incubations in order to protect MR from endogenous AlkPs. Treatments with AlkP from bovine intestine (in the absence of levamisole) eliminated the phosphorylation of MR (lane 5). The amount of receptor was not changed in any treatment, as shown by immunoblots in the lower line. Lanes 1 and 6 of Figure 5(C) shows radioactivity non-specifically bound to the pellet (without and with treatment with AlkP, respectively).

DISCUSSION

The MR is the least-characterized receptor of the steroid/thyroid receptor superfamily. Compared with other members of this superfamily, little is known about the structure and mechanism of MR activation. The results shown here approach an almost

unknown field, such as the mechanism of transformation of MR by phosphorylation/dephosphorylation processes. Results argue strongly in favour of the need for a phosphorylated MR state to bind hormone and a dephosphorylated MR state to interact with DNA.

The evidence presented here in favour of the phosphorylated state of native rat kidney MR agree with the report of Alnemri et al. [14], who have shown that recombinant hMR expressed in *Spodoptera frugiperda* insect cells exhibits a high degree of ^{32}P incorporation when incubated in the presence of [^{32}P]orthophosphate.

Treatments with AlkP resulted in the loss of steroid-binding capacity and in dramatic changes of MR hydrodynamic properties by causing a shift from the 8.8 S form to the 5.1 S form. However, this 5.1 S form is not the exclusive result of the AlkP treatment because an endogenous phosphatase, which may belong to the protein serine/threonine phosphatase family, is responsible for generating a 5.1 S form that can also bind to DNA. On the other hand, incubations with AlkP generated a receptor that is unable to bind steroid, but is able to bind to DNA. These results suggest the existence of two different phosphate groups in MR, one of them would be essential for the steroid binding and the other for preventing receptor binding to DNA.

This indirect evidence should be confirmed. However, what is certain is that the dephosphorylation of some component in the cytosol may regulate the MR transformation process. This is supported by the fact that transformation is inhibited by well-documented phosphatase inhibitors.

One of the strongest arguments which can be brought against studies such as those presented here is that the transformation may be the consequence of proteolytic activity, even when the buffer contains protease inhibitors. However, Figure 5 shows that the molecular mass of MR estimated by blotting or by labelling with ^{32}P is not altered by treatments. Autoradiographs of incubations accomplished at 20 °C showed sometimes lower-molecular-mass fragments of ~ 40 kDa and ~ 15 kDa (results not shown), but the relative radioactivity, normalized as the ^{32}P -labelled fragment:[^{32}P]MR ratio, was constant for all the treatments.

As levamisole was ineffective as an inhibitor of the endogenous phosphatase activity and okadaic acid was able to inhibit such activity, it can be affirmed that a phosphoprotein phosphatase rather than an AlkP-type enzyme is the 'physiological' agent responsible for the transformation of MR. In this first attempt to identify that phosphatase, it was concluded that it may belong to the PP1/PP2A serine/threonine phosphatase subgroup. [Experiments with tautomycin were also accomplished while this manuscript was under revision. Inhibitory effects were observed *in vitro* and *in vivo* upon MR transformation in a concentration-dependent manner (Piwien-Pilipuk, G. and Galigniana, M. D., unpublished work), reinforcing the notion that the endogenous enzyme may be a protein serine/threonine phosphatase.] Recent publications by DeFranco et al. [23,24] also suggested that PP1/PP2A phosphatases may act on a specific subset of phosphorylation sites in rat GR and that the phosphorylation status is also associated with GR nucleocytoplasmic shuttling.

An important observation for the interpretation of these results must be noted: if MR is a multi-protein complex [8,14], features of MR may also reflect the phosphorylated status of some other component of the heterocomplex, but not exclusively the changes exhibited by the receptor. Although detailed studies of hsp90 binding to MR have not been performed, the available evidence is consistent with hsp90 binding to the MR hormone-binding domain [8]. As a consequence, most of the other proteins

which are associated with hsp90 should be present in the heterocomplex, as has been demonstrated for the other members of the steroid receptor family (see [18] for a recent review). Most of these proteins exhibit phosphoprotein properties and/or phosphatase activity. The disparity seen in MR sensitivity to different kinds of phosphatases may reflect this fact, since the degree of phosphorylation is essential for receptor activation and transformation.

In very recent work [25], a new protein has been described as a major component of GR-hsp90 complexes: protein phosphatase 5 (PP5). Interestingly, PP5 belongs to the phosphoserine/threonine phosphatase subgroup and is inhibited by okadaic acid [26]. To date, there is no evidence for a role of PP5 in GR and, of course, for its presence in MR.

This work is the first study performed on kidney cytosol to evaluate whether MR phosphorylation status may play a role in the mechanism of activation and transformation. The evidence favours the importance of the phosphorylation state in determining both MR events, regardless of which proteins in the heterocomplex may be phosphorylated. Future studies may help to elucidate the whole mechanism of transformation of the native MR, as well as the basis of the regulation by agonists and antagonists.

The author is most grateful to Dr. A. Aragonés for her generous financial support. Many thanks are due to Dr. G. Litwack and Dr. N. Robertson for the generous provision of the pAbhMR antibody. The author also wishes to thank Dr. C. P. Lantos and Dr. W. B. Pratt, who made this work possible by providing reagents and equipment, and Ms. M. E. Otero for her expert technical assistance.

REFERENCES

- 1 Evans, R. M. (1988) *Science* **240**, 889–895
- 2 Amero, S. A., Kretsinger, R. H., Moncrief, N. D., Yamamoto, K. R. and Pearson, W. R. (1992) *Mol. Endocrinol.* **6**, 3–7
- 3 Arriza, J. L., Weinberger, C., Cerelli, G., Glaser, T. M., Handelin, B. L., Housman, D. E. and Evans, R. M. (1987) *Science* **237**, 268–275
- 4 DeFranco, D. B., Madan, A. P., Tang, Y., Chandran, U. R., Xiao, N. and Yang, J. (1995) *Vitam. and Horm.* **51**, 315–338
- 5 Sanchez, E. R., Hirst, M., Scherrer, L. C., Tang, H. Y., Welsh, M. J., Harmon, J. M., Simons, S. S., Ringold, G. M. and Pratt, W. B. (1990) *J. Biol. Chem.* **265**, 20123–21130
- 6 Jenster, J., Trapman, J. and Brinkman, A. O. (1993) *Biochem. J.* **293**, 761–768
- 7 Pratt, W. B. (1997) *Annu. Rev. Pharmacol. Toxicol.* **37**, 297–326
- 8 Caamaño, C. A., Morano, M. I., Patel, P. D., Watson, S. J. and Akil, H. (1993) *Biochemistry* **32**, 8589–8595
- 9 Galigniana, M. D. (1996) *Life Sci.* **59**, 511–521
- 10 Trapp, T. and Holsboer, F. (1995) *Biochem. Biophys. Res. Commun.* **215**, 286–291
- 11 Munck, A. and Brinck-Johnsen, T. (1968) *J. Biol. Chem.* **243**, 5556–5565
- 12 Nielsen, C. J., Sando, J. J. and Pratt, W. B. (1977) *Proc. Natl. Acad. Sci. U.S.A.* **74**, 1398–1402
- 13 Orti, E., Bodwell, J. E. and Munck, A. (1992) *Endocr. Rev.* **13**, 105–128
- 14 Alnemri, E. S., Maksymowych, A. B., Robertson, N. M. and Litwack, G. (1991) *J. Biol. Chem.* **266**, 18072–18081
- 15 Galigniana, M. D., Vicent, G. P., Burton, G. and Lantos, C. P. (1997) *Steroids* **62**, 358–364
- 16 Burton, G., Galigniana, M. D., de Lavallaz, S., Brachet-Cota, A. L., Sproviero, E. M., Ghini, A., Lantos, C. P. and Damasco, M. C. (1995) *Mol. Pharmacol.* **47**, 535–543
- 17 Galigniana, M. D., Vicent, G. P., Lantos, C. P. and Burton, G. (1993) *An. Asoc. Quim. Argent.* **81**, 333–340
- 18 Pratt, W. B. and Toft, D. O. (1997) *Endocr. Rev.* **18**, 306–360
- 19 Patel, P. D., Sherman, T. G., Goldman, D. J. and Watson, S. J. (1989) *Mol. Endocrinol.* **3**, 1877–1885
- 20 Brautigan, D. L. and Shiner, C. L. (1988) *Methods Enzymol.* **159**, 339–346
- 21 Cohen, P. (1991) *Methods Enzymol.* **201**, 389–398
- 22 Bialojan, C. and Takai, A. (1988) *Biochem. J.* **256**, 283–290
- 23 DeFranco, D. B., Qi, M., Borror, K., Garabedian, M. J. and Brautigan, D. L. (1991) *Mol. Endocrinol.* **5**, 1215–1229
- 24 Somers, J. P. and DeFranco, D. B. (1992) *Mol. Endocrinol.* **6**, 26–34
- 25 Silverstein, A., Galigniana, M. D., Chen, M., Owens-Grillo, J., Chinkers, M. and Pratt, W. B. (1997) *J. Biol. Chem.* **272**, 16224–16230
- 26 Chen, M. X., McPartlin, A. E., Brown, L., Chen, Y. H., Barker, H. M. and Cohen, P. (1994) *EMBO J.* **13**, 4278–4290