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A novel site of AKT-mediated phosphorylation in the human MDM2 onco-protein

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Abstract MDM2 is an E3 ubiquitin ligase which mediates ubiquitylation and proteasome-dependent degradation of the p53 tumor suppressor protein. Phosphorylation of MDM2 by the protein kinase AKT is thought to regulate MDM2 function in response to survival signals, but there has been uncertainty concerning the identity of the sites phosphorylated by AKT. In the present study, we identify Ser-166, a site previously reported as an AKT target, and Ser-188, a novel site which is the major site of phosphorylation of MDM2 by AKT in vitro. Analysis of MDM2 in cultured cells confirms that Ser-166 and Ser-188 are phosphorylated by AKT in a physiological context.
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Keywords: MDM2; AKT; Phosphorylation; p53; Survival signaling

1. Introduction

The p53 tumor suppressor protein is a highly labile transcription factor which promotes growth arrest or apoptosis in response to a host of environmental stresses [1–3]. p53 is controlled mainly through its interaction with MDM2, an E3 ubiquitin ligase which mediates ubiquitylation and rapid proteasome-dependent degradation of p53. Cellular stresses which induce p53 disrupt the p53–MDM2 interaction, or inactivate MDM2, leading to p53 stabilization (reviewed in [1,4,5]).

Survival signals significantly influence both basal p53 levels and the ability to induce p53 in response to DNA damage-inducing agents [6–11]. These signals are mediated through the PI3-kinase (phosphatidylinositol 3-kinase) pathway leading to the activation of protein kinase B (also known as AKT [hereafter AKT]; reviewed in [12,13]). AKT, in turn, has been reported to phosphorylate MDM2 at two sites, serines 166 and 186 [8,11]. Recent studies, however, have raised uncertainty concerning the identities of these sites [6,7,14] prompting efforts to clarify identification.

In exploring the regulatory mechanisms that govern MDM2 action, we have screened cell extracts for protein kinase activities which phosphorylate sites within amino acids 108–200 of human MDM2. In normally growing, unstressed cells we detect several activities which phosphorylate this region and confirm that one of these protein kinases is, indeed, AKT. Strikingly, we find that Ser-188 is the major site in human MDM2 that is phosphorylated by AKT in vitro and is a novel site of AKT-mediated phosphorylation in vivo.

2. Materials and methods

2.1. Specialized reagents

Purified, recombinant enzymes (activated where appropriate) were obtained as follows: AKT1/PKBα, p90RSK1 (MAPKAPK-1a), and p70S6K-1 (ribosomal protein S6 kinase-1) were from Prof. D. Alessi, MRC Protein Phosphorylation Unit, University of Dundee; p70S6K-2 (ribosomal protein S6 kinase-2) was a gift from Dr. Ivan Gout (Ludwig Institute, University College, London); and Chk1 was a gift from Dr. Jim Hutchins (Biomedical Research Centre, Dundee).

2.2. Construction of plasmids expressing GST-MDM2 fusion proteins

Expression and purification of a series of fusion proteins comprising glutathione S-transferase (GST) linked to the N-termini of full length MDM2 or overlapping domains of the MDM2 protein (termed MP1 [MDM2 aa 1–110], MP2 [108–200], MP3 [196–282] and MP4 [276–491]) have been described elsewhere [15,16].

2.3. Mutagenesis and oligonucleotides

Site-directed mutagenesis was performed using the Stratagene Quick-Change system according to the manufacturer's protocol. Following mutagenesis, the DNAs were sequenced to ensure that only the desired mutations were present.

2.4. Antibodies

The following antibodies were used: MDM2 (D12 and SMP-14, both from Santa Cruz Biotechnology Inc.); AKT (polyclonal antibody from Prof. D. Alessi, University of Dundee), Ran (from Dr. B. McStay, University of Dundee), and Myc 9E10 (CRUK).

2.5. Generation and purification of phospho-specific antibodies

Rabbit polyclonal phospho-specific antibodies were raised against the phosphopeptides: SRRRAIS(phos)ETEEN (phospho-serine 166) and KSDS(phos)ISLSFDES (phospho-serine 188), coupled to keyhole limpet hemocyanin, and a mouse monoclonal antibody was raised against the phosphopeptide: QRKRHKS(phos)DSIS (phospho-serine 186), coupled to keyhole limpet hemocyanin. Antibodies were affinity-purified on sepharose 4B resin to which the appropriate phosphorylated

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Abbreviations: ERK, extracellular ligand-activated kinase; GST, glutathione S-transferase; MEK, MAP and ERK kinase; MP2, mini-protein 2 comprising glutathione S-transferase fused through its C-terminus to amino acids 108–200 of human MDM2; p90RSK, 90K ribosomal S6 kinase; p70S6K1,2, 70K ribosomal S6 kinase 1,2

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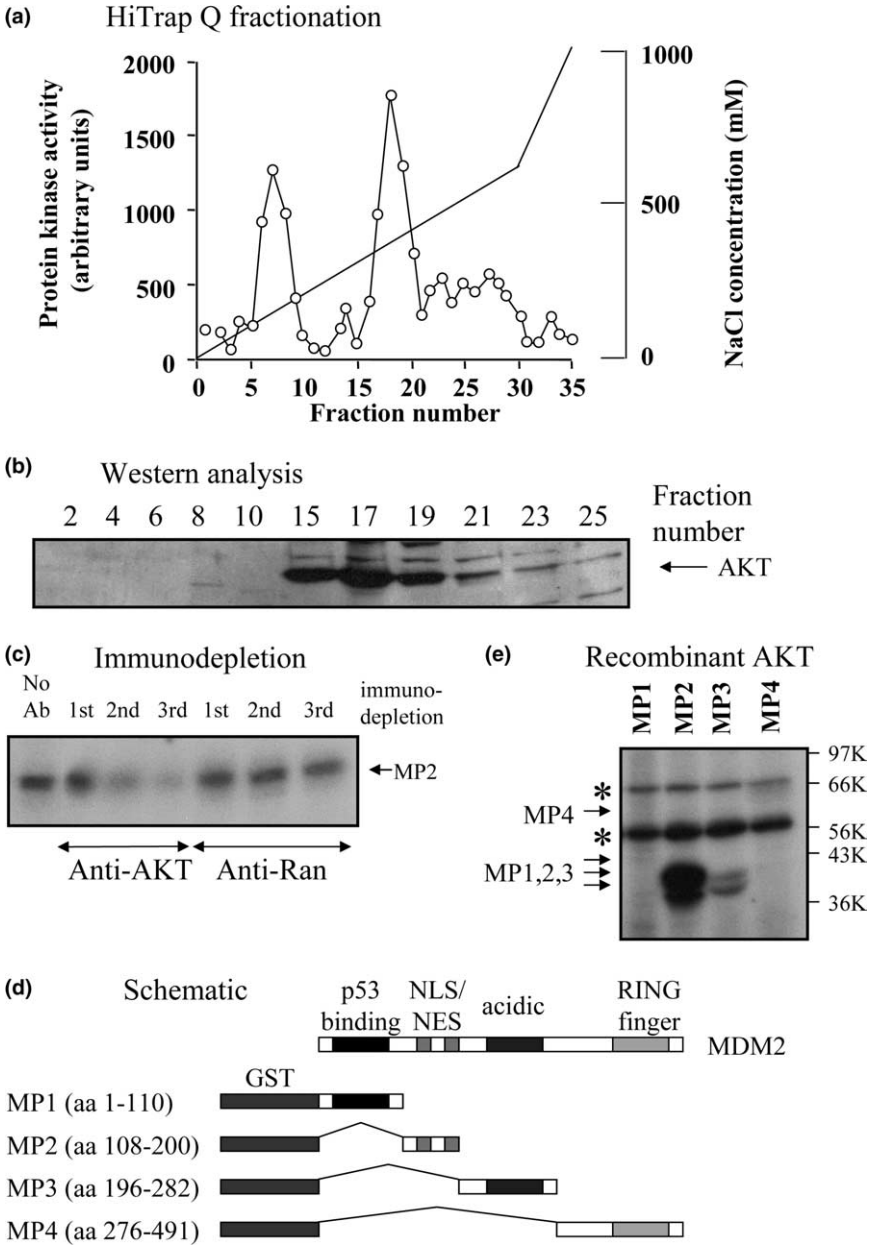

Fig. 1. Cell extracts contain protein kinase activities that phosphorylate the region of human MDM2 comprising amino acids 108–200. (a) MCF-7 cell extracts were fractionated on a HiTrap Q column and eluted with a gradient of NaCl. Kinase activities were measured using the MP2 mini-protein. (b) Western blot showing the presence of the AKT in the fractions. (c) MDM2 kinase activity following immuno-depletion of AKT with anti-AKT and anti-Ran (control) antibodies. (d) Schematic representation showing the MDM2 protein and the four GST-MDM2 fusion proteins (MP1-4) used as substrates. (e) Phosphorylation of equal amounts of each of the four mini-proteins, MP1-MP4, by AKT. The positions of the mini-proteins are indicated.

peptide had been covalently coupled and passed through a second column containing the unphosphorylated peptide to remove any antibodies that did not recognize the phosphorylated epitope.

2.6. Cell lines and transfection

MCF-7 (breast carcinoma) cells, U2OS (osteosarcoma) and OSA (SJSA; osteosarcoma) cells, all expressing wild type p53, were routinely grown in Dulbecco's modified Eagle's medium containing 10% (v/v) bovine fetal serum. Cells were transfected with plasmid DNAs using Lipofectamine (Life Technologies).

2.7. Immunoprecipitation and Western blotting

Immunoprecipitation and Western blotting were carried out using standard procedures as described previously [17]. For the detection of phosphorylated species of MDM2, the phospho-specific antibodies described above were used at a concentration that was optimized empirically and in the presence of unphosphorylated peptide, again at an empirically optimized concentration, to minimize any cross reactivity with the unphosphorylated protein. Blocking and subsequent procedures were carried out in Tris-buffered saline containing 5% (w/v) bovine serum albumin and 0.1% (v/v) Tween 20.

2.8. Preparation of cell extracts for protein kinase assays and ion exchange chromatography

Cell extracts were prepared and ion exchange chromatography carried out as described previously [18] with the exception that a HiTrap Q column (Amersham Pharmacia Biotech) was used. Column fractions and the unbound "flow-through" material were screened for protein

kinase activity using the MP2 proteins (GST fused to MDM2 amino acids 108–200) as substrates.

2.9. Protein kinase assays

MDM2 kinase assays were performed in 50 mM Tris, pH 7.5, 10 mM $MgCl_2$ and 20 μM [γ-^{32}P]ATP (Amersham Pharmacia Biotech, at a specific activity of 12.5 Ci/mmol) in a volume of 20 μl. Assays were initiated by the addition of protein kinase (generally 0.5–1 unit [nmol/min] of activity) and incubated at 30 °C for 30 min. Reactions were terminated by adding SDS sample buffer and heating at 100 °C for 2 min. Phosphorylated proteins were detected following SDS–PAGE and autoradiography. Alternatively, a non-radioactive assay was used in which the ATP concentration was 100 μM and the phosphorylated proteins were detected by Western blotting using phospho-specific antibodies.

2.10. Tryptic phosphopeptide mapping

Two-dimensional thin layer phosphopeptide analysis of MDM2 was carried out using essentially the same procedure as described for p53 [19] with the exception that the solvent used for the chromatographic separation comprised isobutyric acid (62.5%, v/v), *n*-butanol (1.9%, v/v), pyridine (4.8%, v/v) and glacial acetic acid (2.9%, v/v) in deionized water.

3. Results and discussion

3.1. MDM2 is phosphorylated in vitro by the protein kinase AKT (PKB)

Ion exchange chromatography on a HiTrap Q column revealed two sharp peaks of protein kinase activity that phosphorylated the MP2 protein (GST linked to amino acids 108–200 of MDM2; Fig. 1(a)). A third MP2 kinase activity(ies) was present in the flow-through fraction and did not arise from over-loading the column (data not shown). Western blotting analysis of the fractions with an anti-AKT polyclonal antibody indicated that AKT co-elutes with the second of the two MDM2 kinase activities (Fig. 1(b)). Immuno-depletion of AKT from the peak fraction (fraction 17) led to loss of the MDM2 protein kinase activity, while an irrelevant antibody (anti-Ran) failed to deplete the MDM2 kinase (Fig. 1(c)). When phosphorylation reactions were carried out in the presence of recombinant, activated AKT using a series of MDM2 mini-proteins as substrates (Fig. 1(d)), the MP2 protein became highly phosphorylated (Fig. 1(e)). In contrast, neither the MP1 nor MP4 proteins were phosphorylated. A low level of phosphorylation was observed with the MP3 protein, but this was significantly less than that observed with MP2 (Fig. 1(e)). GST alone was not a substrate for recombinant AKT (data not shown). These data are consistent with the idea that MDM2 is a target for phosphorylation by AKT in vitro and that the site(s) or phosphorylation lie between amino acids 108 and 200.

3.2. MDM2 is phosphorylated by AKT in vitro at Ser-166 and Ser-188, a novel target residue for AKT

To identify the site(s) of AKT phosphorylation in MDM2, in vitro phosphorylation reactions were carried out in the presence of activated, recombinant AKT using the MP2 mini-protein, or a series of MP2 derivatives encompassing serine to alanine substitutions at potential sites of phosphorylation, as substrates. While wild type [WT] MP2, S157A, S166A and S186A mutants, and a S166A/S186A double mutant were all phosphorylated by AKT to a similar extent, phosphorylation of an S188A mutant was significantly reduced, but not completely abolished (Fig. 2(a)). Similarly, when the WT or S188A MP2 proteins were phosphorylated in the presence of the peak HiTrap Q fractions containing AKT, phosphorylation of the S188A mutant was greatly reduced (Fig. 2(b)). Taken together, these data suggest that Ser-188 is the principal, but not the sole, target of AKT in vitro. To explore further the identity of the sites phosphorylated by AKT, the phosphorylation site-mutant proteins used in panel (a) were phosphorylated by recombinant Akt in vitro and analyzed by Western blotting using phospho-specific antibodies recognizing phosphoserines 166, 186 and 188, respectively. The data (Fig. 2(c)) confirmed that Ser-188 was phosphorylated by AKT and indicated that Ser-166 could also be modified. Phosphorylation of ser186 was not detected using this approach (Fig. 2(c)). As an additional means of confirming the sites of phosphorylation, the MP2 protein was phosphorylated by AKT over a 2 h time period.

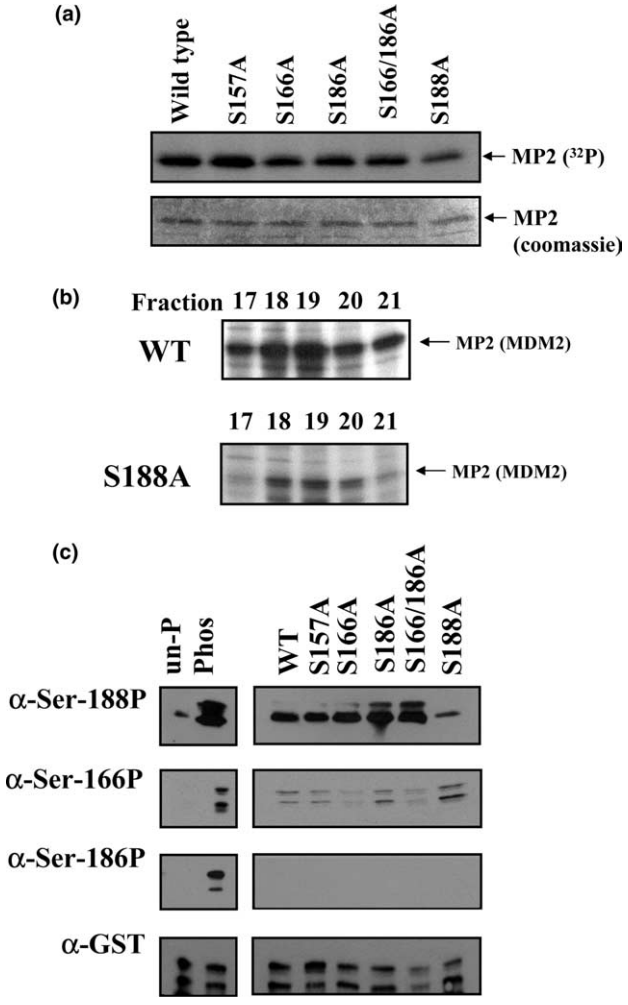

Fig. 2. AKT phosphorylates Ser-166 and Ser-188 in MDM2. (a) Phosphorylation of GST-MDM2 fusion protein MP2, or a series of Ser-Ala substitution mutants, in vitro by recombinant AKT. Approximately equal amounts of substrate proteins were used in each assay (lower panel). (b) Kinase activity of HiTrap Q column fractions towards WT GST-MDM2 (MP2) and a S188A mutant. (c) Western blotting of in vitro-phosphorylated MP2, or alanine substitution mutants, using phospho-specific antibodies. Each of the small left hand panels shows positive (MP2 phosphorylated by the Chk1 kinase) and negative (unphosphorylated MP2) controls for each phospho-specific antibody. Approximately equal amounts of substrate proteins were used in each assay (lower panel).

Tryptic phosphopeptides were generated, purified by HPLC and analyzed by mass spectrometry and solid phase sequencing. This approach confirmed that Ser-166 and Ser188 were targets of AKT (data not shown).

To eliminate the possibility that Ser-188 was a cryptic phosphorylation site revealed in the MP2 protein, GST-full length MDM2 was phosphorylated by recombinant AKT and analyzed by phosphopeptide mapping. The phosphopeptide maps obtained from full length MDM2 and MP2 were identical (Fig. 3(a) and (b)). Moreover, when the phosphopeptides from each reaction were mixed prior to loading on the thin layer plate, the phosphopeptides co-migrated (panel c). When the S188A mutation was introduced into the full length protein, the major phosphopeptide disappeared (panel d). These data confirm that, in the context of full length MDM2, Ser-188 is the major site of phosphorylation by AKT in vitro.

3.3. *Ser-166 and Ser-188 are phosphorylated in vitro and in cultured cells in response to serum stimulation*

To provide further support that the protein kinase detected in cell extracts was indeed AKT, the PI3-kinase pathway was stimulated in serum-starved cells by the re-addition of serum. Protein kinase assays, in which phosphorylation of the MP2 protein was measured by the incorporation of radioactive phosphate from [γ-^{32}P]ATP, showed that MP2 kinase activity was rapidly stimulated following the addition of serum to the cells (Fig. 4(a)). When the S188A mutant was used as substrate in this assay, stimulation of MP2 kinase activity was still evident but the level of phosphorylation was much less, indicating that Ser-188 was a target of the stimulated kinase. Significantly, a low level of Ser-188 kinase activity was already present in the unstimulated cell extracts, suggesting that an additional Ser-188 kinase activity was present.

To determine whether Ser-166 kinase activity was also stimulated, protein kinase assays were carried out in the presence of unlabeled ATP followed by Western blotting using the Ser-166(P) phospho-specific antibody as probe. Using this approach, Ser-166 kinase activity was also observed within 30 min following the re-addition of serum (Fig. 4(b)). Significantly, and consistent with the data in panel (a) obtained from using the S188A substrate, no Ser-166 activity could be detected in the unstimulated cells using this approach. Taken together, these data are consistent with the idea that growth/survival factor stimulation of the PI3-kinase pathways leads to AKT-dependent phosphorylation of MDM2.

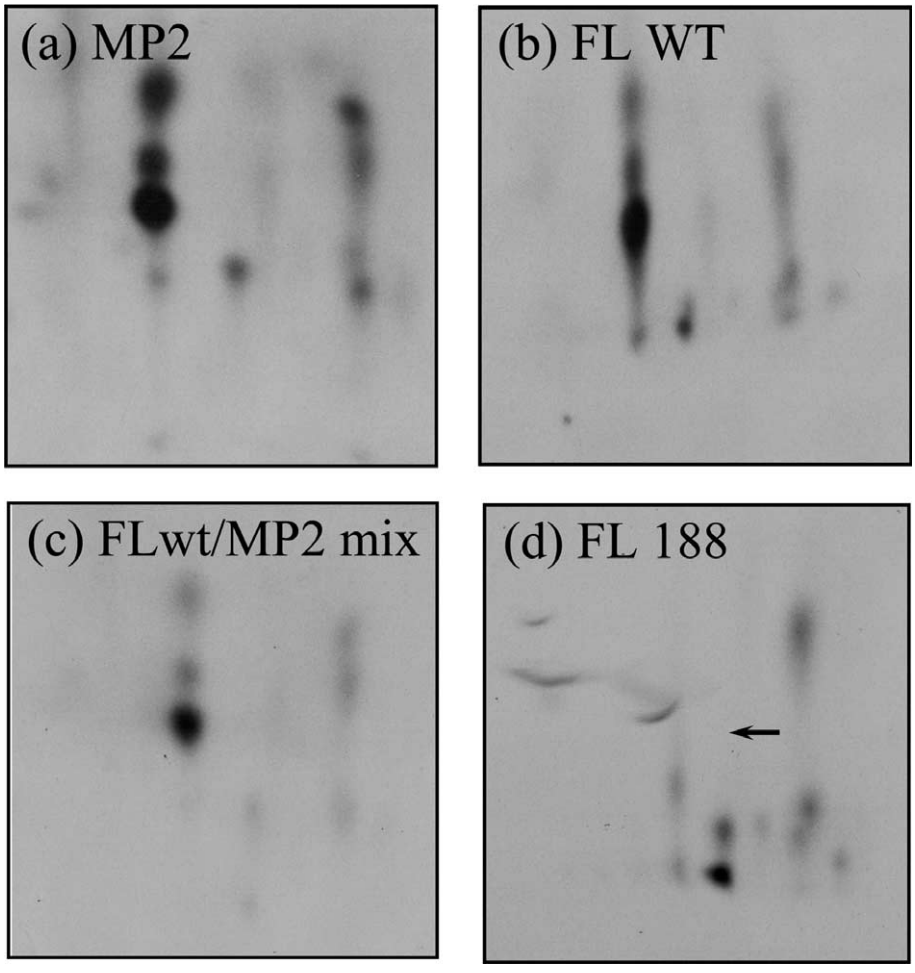

Fig. 3. AKT phosphorylates full length MDM2. Tryptic phosphopeptide mapping of MP2 (a), GST-tagged MDM2 (FL WT (b)), and GST-tagged MDM2 in which Ser-188 is substituted by alanine (FL 188, panel (d)), phosphorylated in vitro by AKT. In panel (c), equal amounts of radioactive phosphopeptides from the MP2 and the full length MDM2 phosphorylation reactions were mixed prior to loading onto the thin layer plate (FLwt/MP2 mix, panel (c)). The arrow in panel (d) indicates the absence of the major phosphopeptide in the map obtained from the FL188 mutant protein.

To determine whether Ser-166 was phosphorylated in an AKT-dependent manner in cultured cells, phosphorylation of endogenous MDM2 was determined following Western blotting of stimulated cell extracts using the Ser-166(P) phospho-specific antibody. The data indicate that MDM2 underwent serum-dependent phosphorylation of Ser-166 (Fig. 4(c)) with kinetics that match the in vitro measurements of Ser-166 protein kinase activity in panel (Fig. 4(b)). When the cells were pretreated with LY294002, a specific inhibitor of PI3-kinase (and consequently of AKT activation), the serum-dependent phosphorylation of Ser-166 in MDM2 was abolished, suggesting that phosphorylation of MDM2 occurs through the PI3-kinase pathway. A similar set of data were obtained using MCF-7 cells (data not shown).

PI3-kinase also lies upstream of p70S6K1, p70S6K2 and p90RSK. However, none of these kinases was able to phosphorylate serine 188 in vitro and only p90RSK phosphorylates Ser-166 (data not shown). Additionally, however, p90RSK requires the extracellular ligand-activated kinase (ERK) pathway for activity [20]. Treatment of MCF-7 cells with U0126, an inhibitor of MEK (the protein kinase which activates ERK), did not block MDM2 phosphorylation of Ser-166 (data not shown). Taken together, the data are consistent with the idea that AKT is the serum-stimulated MDM2 Ser-166 kinase and confirm previously published findings [7–9,11,14].

To determine initially whether Ser-188 was a physiological site of phosphorylation, WT MDM2 and an S188A mutant were expressed in MCF-7 cells, following which the cells were pulse-labeled with ^{32}P-orthophosphate. Two-dimensional tryptic phosphopeptide mapping (Fig. 5(a)) showed that a major phosphopeptide present in WT MDM2 (indicated by a black arrow) was significantly depleted in the S188A mutant protein. In vitro analysis established that this peptide contained Ser-188 [data not shown]. These data support the idea that Ser-188 is phosphorylated physiologically. The residual

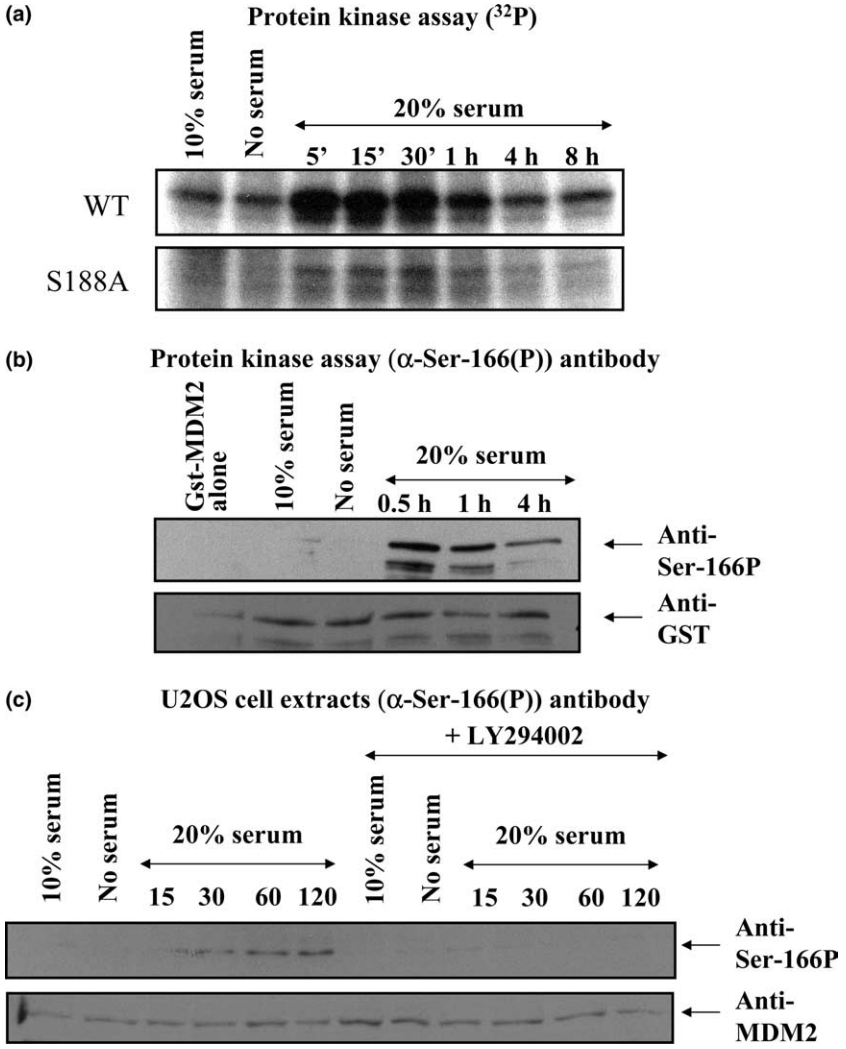

Fig. 4. MDM2 is phosphorylated at Ser-166 in response to serum stimulation. U2OS cells were maintained in 10% FBS or in the absence of serum for 24 h. Cells deprived of serum were stimulated in the presence of 20% FBS for up to 8 h. (a, b) Cell extracts were prepared and MDM2 phosphorylation reactions were carried out in vitro on GST-MDM2. (a) Detection by autoradiography when [γ-^{32}P]ATP was used as the phosphate donor. (b) Western blotting was performed using the anti-phospho-serine 166, or anti-GST antibodies. (c) Phosphorylation of endogenous MDM2 in response to serum and in the presence or absence of the PI3-kinase inhibitor, LY294002. MDM2 was immunoprecipitated using the monoclonal antibodies SMP14 and D12. Immunoprecipitated proteins were Western blotted for the presence of phospho-serine 166 and MDM2 (SMP14/D12).

signal is likely to occur through phosphorylation of one or more of the remaining four serine residues within this peptide.

Attempts to detect serum-induced Ser-188 phosphorylation of MDM2 in U2OS or MCF-7 cells using the Ser-188(P) phospho-specific antibody were limited by the low levels of MDM2 in these cells and low sensitivity of the antibody. To overcome this difficulty, the experiments were carried out using SJSA cells which express high levels of endogenous MDM2. As with the U2OS cells, re-addition of serum to serum-starved SJSA cells led to stimulation of Ser-188 kinase activity (Fig. 5(b)). Activation of this kinase was abolished when the cells had been pretreated with LY294002. As with the U2OS cells, there appeared to be a basal level of Ser-188 kinase activity in the unstimulated cells, suggesting that either the Ser-188 antibody could detect a low level of unphosphorylated substrate or, more likely, that another Ser-188 kinase was present in the extracts. Stimulation of Ser-166 kinase activity was also detected in SJSA cells with identical kinetics to the induction of the S188 activity (data not shown).

Western blotting analysis of the endogenous MDM2, using the Ser-188(P) phospho-specific antibody, indicated that Ser-188 phosphorylation was indeed serum-dependent and could be inhibited by pretreating the cells with LY294002 (Fig. 5(c)). Again, the kinetics of Ser-188 phosphorylation reflected activation of the protein kinase (compare with panel b). These data support the idea that Ser-188 is phosphorylated physiologically by AKT. Additionally, the data suggest a low level of serum-independent Ser-188 phosphorylation consistent with the conclusions reached from the in vitro kinase assays (Figs. 4(a) and 5(b)).

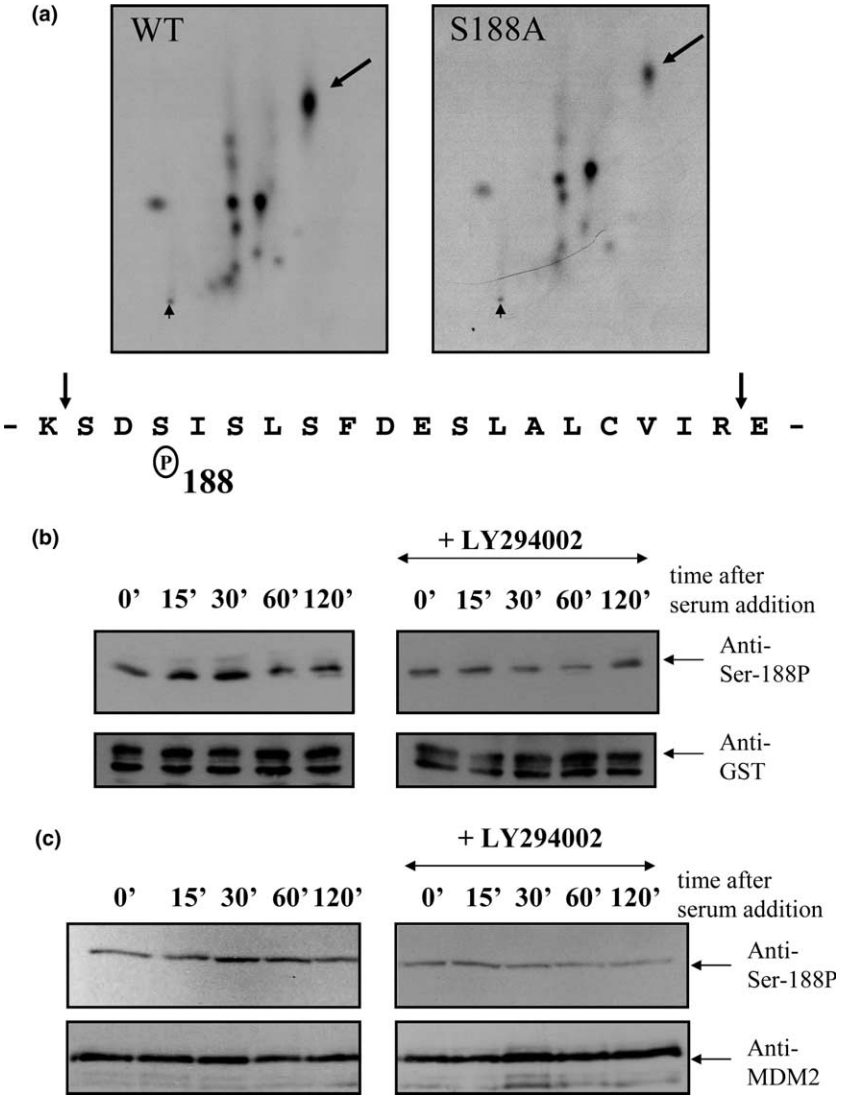

Fig. 5. MDM2 is phosphorylated physiologically at Ser-188. (a) Tryptic phosphopeptide mapping of MCF-7 cells transfected with plasmids encoding Myc-tagged WT or S188A MDM2 and pulse-labeled with ^{32}P-orthophosphate. Arrowheads mark the origins. Black arrows show the Ser-188 phosphopeptides. The sequence shown at the base of the plates represents amino acids 185-204 of MDM2 and indicates the position of phosphoserine 188. The vertical arrows indicate the sites of trypsin digestion. (b) SJSA cells deprived of serum were stimulated in the presence of 20% FBS for up to 2 h. Cell extracts were prepared and MDM2 phosphorylation reactions were carried out in vitro on GST-MDM2. Western blotting was performed using the anti-phospho-serine 188, or anti-GST antibody. (c) Phosphorylation of endogenous MDM2 in response to serum and in the presence or absence of the PI3-kinase inhibitor, LY294002. Immunoprecipitated MDM2 proteins were Western blotted for the presence of phosphoserine 188 and MDM2 (SMP14/D12).

3.4. MDM2-Ser-188 as a novel target for phosphorylation by AKT

While there is general agreement that AKT regulates MDM2 physiologically [6–11,14], several groups have not been able to detect AKT-mediated phosphorylation of Ser-186 [6,7,14], one of the sites originally reported as an AKT target [8,11]. In the present study, we confirm that Ser-186 is not a major target of AKT in vitro but verify that Ser-166 is an AKT target both in vitro and in vivo. We have not been able to detect AKT-mediated Ser-186 phosphorylation of endogenous MDM2 using the Ser-186(p) phospho-specific antibody (data not shown). In contrast, we have identified a novel AKT phosphorylation site, Ser-188, which is the major site modified by AKT in vitro (Figs. 2 and 3). Like Ser-166, Ser-188 kinase activity is induced by serum stimulation (Figs. 4(a) and 5(b)). Moreover, serum-induced phosphorylation of Ser-188 in endogenous MDM2 occurs in a LY294002-sensitive manner, consistent with this residue being a cellular target of AKT (Fig. 5(c)).

While the present manuscript was in preparation, Feng et al. [14] published a study indicating that Ser-188 of MDM2 is a major target of AKT in vitro and in vivo. The data presented in the present study are therefore supportive of the conclusions of Feng et al. [14] and highlight the physiological relevance of Ser-188 as a novel AKT target.

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