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Cell-Specific Phosphorylation of Zfh1a Transcription Factor

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Abstract

Zinc finger homeodomain enhancer-binding protein (Zfh1a/Zfh1a) is a transcription factor essential for immune system development, skeletal patterning, and life. Regulation of the interleukin 2 gene in T cells has been suggested to depend on post-translational processing of Zfh1a, however, no modifications of Zfh1a are known. Here we demonstrate that Zfh1a is present in both hyperphosphorylated and hypophosphorylated forms. Western analysis demonstrates two forms of Zfh1a with different mobilities. Differences in phosphorylation are sufficient to explain the difference in mobilities. Zfh1a is primarily phosphorylated on Ser and Thr residues since PP2A dephosphorylates the slower mobility band. Treatment of nuclear extract with O-GlcNAcase did not detect O-linked sugar. Importantly, post-translational processing is cell-specific. Doublets of Zfh1a were detected in five cell lines, whereas 6 cell lines contain only, or predominantly, non-phosphorylated Zfh1a, and Saos-2 cells contain predominantly the phosphorylated form. These data provide the first demonstration that Zfh1a is post-translationally modified.

Keywords

ZEB; deltaEF1; AREB6; Zfh1a; phosphatase 2A; vomitoxin; COS; C2C12; CHO

INTRODUCTION

The activity of many transcription factors is physiologically regulated by post-transcriptional modifications, including phosphorylation and O-linked glycosylation. For example, phosphorylation of CREB is essential for transcriptional activation (1). Even nuclear receptors, which are ligand-dependent transcription factors, are ligand-independently activated by phosphorylation. In many cases, extracellular signals regulate the activity of kinases and phosphatases that modify transcription factors (2). Phosphorylation of transcription factors can regulate various activities, including DNA-binding, as observed with Dlx3 (3), CREB (4), GATA-4 (5, 6), Nur-77 (7), and c-Myb (8). Transcription factors can also be dynamically glycosylated with O-linked N-acetylglucosamine (O-GlcNAc) on Ser or Thr residues. GlcNAcylation can occur in the nucleus, and regulate the activity of transcription factors, such as Sp1 and c-Myc (9–11).

Zfh1a (Zfh1a/Nil-2a/AREB6/ZEB/deltaEF-1/BZP) is a member of an unusual family of transcription factors having multiple zinc fingers and also having one or more homeodomains (12–21). Zfh1a is essential for differentiation or proliferation of a very early

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T cell precursor in the embryo (22), as well as craniofacial development, and skeletal patterning (23). Zfh₁ has been suggested to be a repressor of myogenesis (24) since cotransfection of myoD and Zfh₁ cDNAs shows that Zfh₁ effectively blocks myoD-induced myogenesis or transcriptional activation *in vitro* (25, 26). We recently demonstrated that the expression pattern of Zfh₁ is consistent with a role in development of the brain and proliferation of brain progenitor cells (27).

In addition to its role in early differentiation, Zfh₁ participates in regulation of interleukin-2 (IL-2) in mature T cells. Prior to T cell activation, Zfh₁ binds a negative regulatory element (NRE-A) in the IL-2 gene and represses transcription (21). Activation of T cells increases the binding of transcriptional activators (such as NF- κ B and AP1) to the IL-2 gene, and decreases Zfh₁ binding to NRE-A (28). The mycotoxin deoxynivalenol induces the IL-2 gene in part by inhibition of I- κ B (28), and inhibition of Zfh₁/ZEB binding to NRE-A (29). Deoxynivalenol decreases binding to NRE-A without blocking expression of Zfh₁, indicating a post-translational mechanism. However, no modifications of Zfh₁ have been described to date.

While transfection of exogenous Zfh₁ has been extensively used to study the role of Zfh₁, nothing is known about the regulation of Zfh₁ protein activity. Zfh₁ is a repressor of transcription at certain target genes (17, 19, 20, 25, 26, 30–33), and yet is reported to activate transcription of other target genes (34–36). Activation of the vitamin D receptor gene by Zfh₁/ZEB is cell-specific (35). The molecular mechanisms underlying the choice between activation or repression by Zfh₁ are unknown, but may include cell-specific differences in post-translational modification. We demonstrate that the Zfh₁ protein is expressed in both a hyperphosphorylated, and a hypophosphorylated form. These two forms are differentially expressed in different cell types.

MATERIALS AND METHODS

Cell lines

COS-7 and CV-1 (green monkey kidney lines), U138 MG (human glioblastoma), JEG-3 (human choriocarcinoma), C₂C₁₂ (mouse myoblast), Saos-2 (human osteosarcoma), Neuro2a (mouse neuroblastoma), F9 (mouse embryonal carcinoma), SK-N-SH (human neuroblastoma), GH3, GH₄C₁, and α T3-1 (rat pituitary tumor lines), PC12 (pheochromocytoma), CHO (Chinese hamster ovary), and HEPM (human embryonic palatal mesenchyme) cells were obtained from American Type Culture Collection (ATCC, Rockville, MD), Dr. William Young (CHO), or Dr. P. L. Mellon (α T3-1). Cells were propagated at 37°C in 5% CO₂ with Dulbecco's modified Eagle's medium (DMEM, Invitrogen, Carlsbad, CA) supplemented with 10% fetal calf serum, 100 U/ml penicillin and 100 μ g/ml streptomycin, or alpha minimum essential medium (α MEM, Invitrogen) containing 10 μ g/ml ribonucleosides and deoxyribonucleosides and antibiotics.

Nuclear extracts

Nuclear extracts were prepared by the method of Schrieber et al. (37) with modifications. Cells grown in T150 flasks to 90–100% confluency were washed with 10 ml ice-cold 10 mM Tris pH 7.4, 150 mM NaCl (TBS) and scraped in TBS with 1 mM PMSF. Cells were pelleted by centrifuging 1500 \times g at 4°C for 5 minutes and washed in ice cold TBS. The cells were resuspended in 800 μ l ice cold Buffer A (10 mM HEPES pH 7.9, 10 mM KCl, 0.1 mM EGTA, 0.1 mM EDTA, 1 mM DTT, 0.5 mM PMSF, 1 \times protease inhibitors having 10 μ g/ml each of the following: antipain, aprotinin, chymostatin, leupeptin and pepstatin A (ICN Pharmaceuticals, Costa Mesa, CA)) and allowed to swell on ice for 15 minutes then lysed with 0.625% NP-40. After vortexing vigorously for 10 seconds, the lysate was

centrifuged for 45 seconds at 4°C and 16,000 × g. To extract the nuclear protein, the nuclear pellet was resuspended in 200 µl ice-cold Buffer C (20 mM Hepes pH 7.9, 400 mM NaCl, 1 mM EGTA, 1 mM EDTA, 1 mM DTT, 1 mM PMSF, 1 × protease inhibitors). The tubes were mixed on a rotator 30 minutes at 4°C, then centrifuged for 15 minutes at 4°C and 16,000 × g. The supernatant was dialyzed against 250 volumes dialysis buffer (20 mM Hepes, 50 mM KCl, 20% glycerol, 0.5 mM DTT, 0.5 mM PMSF) overnight. The dialyzed extract was centrifuged for 30 minutes at 4°C and 16,000 × g. The soluble supernatant was either used immediately or frozen in aliquots at -80°C.

Enzymatic treatment of Zfh_{ep}

Zfh_{ep} was dephosphorylated to determine whether it was post-translationally modified by phosphorylation in certain cell types (38). U138MG nuclear extract (50 µg) was brought to 50 mM Hepes pH 7.9, 1 mM MgCl₂, and 1 × protease inhibitor mix and pre-incubated for 10 minutes at 30°C. 20 U of Calf Intestinal alkaline Phosphatase (CIP, Roche Molecular Biochemicals, Indianapolis, IN), or 0.3 U of human purified protein phosphatase 2A (PP2A, Upstate Biotechnology, Inc., Lake Placid, NY) was then added and the sample incubated an additional 15 minutes at 30°C. To demonstrate that the changes in electrophoretic mobility of Zfh_{ep} were due to the dephosphorylating activity of the CIP enzyme, and not to contaminating proteases or glycosidases, samples were incubated with CIP in the presence of 100 mM sodium phosphate, an inhibitor of CIP activity. Dephosphorylation reactions were terminated by the addition of 20 µl of 4 × SDS sample buffer followed by boiling for 5 minutes. Changes in the electrophoretic migration of Zfh_{ep} in mock-treated and phosphatase-treated samples were visualized by immunoblotting. The presence of O-linked glycosylation was tested by incubation for 18 hours at 37°C with N-acetyl-D glucosaminidase (O-GlcNAcase; Roche) in 0.1% SDS, 50 mM citric phosphate pH 4.

Immunoblotting

Codons for amino acids 557–663 encompassing the Zfh_{ep} homeodomain (HD) were cloned into pGEX-2TK (Amersham Pharmacia, Piscataway, NJ). Bacterially expressed peptide was purified and injected into rabbits to raise antibodies to rat Zfh_{ep} protein (27, 39). Zfh_{ep} protein was visualized by western blotting. Protein samples were separated by SDS-PAGE and transferred to polyvinylidene difluoride membrane. The blot was blocked in 0.5% Tween 20 and 5% non-fat dried milk in TTBS (20 mM Tris pH 7.5, 500 mM NaCl, 0.05% Tween 20) and washed in TTBS. Primary antibody was diluted 1:6,000 in TTBS and incubated with the blot overnight. The blot was then washed extensively in TTBS. Goat anti-rabbit horseradish peroxidase conjugated secondary antibody was diluted 1:10,000 in TTBS and incubated with the blot for 1 hour. The blot was then rinsed twice and washed for 30 minutes, 15 minutes, and four times at 5 minutes in TTBS. The blot was reacted with the chemiluminescent substrate Luminol (Pierce, Rockford, IL) for 10 minutes and exposed to Biomax MR film (Kodak, New Haven, CT).

RESULTS

Western blot of nuclear proteins separated on an 8% polyacrylamide gel decorated protein that is present in COS-7 and CHO (Figure 1a), with a molecular mass of approximately 180 kDa. This is consistent with earlier reports with the chicken (18, 26), mouse and hamster (17) orthologues of Zfh_{ep}, and is significantly higher than the calculated molecular mass (124 kDa). Preimmune serum did not label protein in nuclear extracts (Figure 1a). We used a lower percentage acrylamide SDS-PAGE to further define the size of Zfh_{ep}. Analysis of COS-7 cell nuclear extracts on 4% SDS-PAGE demonstrated the presence of two separate immunoreactive proteins. The upper band has a relative mobility of 185 kDa whereas the lower band is 180 kDa (Figure 1b). The doublet was observed in multiple, independent

COS-7 nuclear extracts, including different preparations of protease inhibitors. The doublet was present in COS-7 cells from either low density or confluent cultures, and was also observed in nuclear extracts from U138 cells.

To investigate the cause of the Zfh_{ep} doublet, we determined whether dephosphorylation or deglycosylation alters the mobility of the bands. Nuclear extract from U138MG cells was incubated with, or without, calf intestinal phosphatase to remove phosphate groups. Mock incubation did not alter the ratio of the two bands in the Zfh_{ep} doublet, however, addition of CIP caused a complete disappearance of the upper band, and an increased intensity of the lower band (Figure 2a). Incubation with CIP in the presence of phosphate buffer (to block phosphatase activity) demonstrated that the loss of the upper band was specifically due to the phosphatase activity of CIP, not any contaminating protease (Figure 2a). CIP treatment of COS-7 nuclear extracts also collapsed the Zfh_{ep} doublet into a single band (Figure 2b). CIP treatment of CHO nuclear extract does not change the alignment of the single band on the immunoblots (not shown). In an independent set of experiments, treatment of COS nuclear extract with protein phosphatase 2A also diminished the upper band (Figure 3). PP2A is active at phospho-Ser and phospho-Thr, but not phospho-Tyr. These data demonstrate that the slower mobility anti-Zfh_{ep}-reactive protein is phosphorylated, whereas the higher mobility form of Zfh_{ep} is not. Therefore, we tested whether the presence of the hypophosphorylated band was an artifact of dephosphorylation by endogenous phosphatases during cell lysis. To address this question, nuclear extracts were isolated in the presence of 100 mM phosphate buffer to block endogenous phosphatase activity, however, this did not shift the ratio of the two bands (not shown). Many nuclear proteins have O-linked GlcNAc moieties, therefore, we tested whether deglycosylation also altered the mobility of the Zfh_{ep} bands. Treatment of COS-7 nuclear extract with O-GlcNAcase had no effect on either the mobility or the ratio of the two Zfh_{ep} bands (Figure 4). In summary, differences in phosphorylation are sufficient to explain the different mobilities of the two forms of Zfh_{ep}.

We investigated whether Zfh_{ep} is present as a doublet in various cell types. Nuclear extracts were prepared from cultures of 15 cell types. We found a doublet of Zfh_{ep} immuno-reactive bands in HEPM, COS-7, U138 MG, CV1, and SK-N-SH cells, but no expression in GH₃, JEG3, and PC12 cells (Figure 5). Only, or predominantly, the hypophosphorylated band was expressed in F9, GH₄C₁, Neuro2a, αT3-1, CHO, and C₂C₁₂ cells, as opposed to Saos-2 cells which produce predominantly the upper band. Very long exposures of westerns with CHO nuclear extracts failed to show any evidence of phosphorylated Zfh_{ep}. The absence of the slower mobility phosphorylated Zfh_{ep} protein in certain cell types indicates that the post-translational processing of Zfh_{ep} is cell-specific.

We also investigated whether nuclear localization was controlled by phosphorylation of Zfh_{ep}. Given the presence of both forms of Zfh_{ep} in COS-7 cells, immunofluorescence with the anti-Zfh_{ep} primary antiserum and FITC-conjugated anti-rabbit IgG secondary antibody (Chemicon, Temecula, CA) was used to determine if either form was localized outside the nucleus. Immunocytochemical assay of COS-7 cells detected Zfh_{ep} only in the nucleus, indicating that both isoforms are nuclear (not shown). In support of this, no Zfh_{ep} was detected in western analysis of cytosol from COS-7, CHO, or GH3 cells (not shown). Also, immunocytochemistry did not detect cytosolic or nuclear Zfh_{ep} in JEG-3 cells, consistent with the western analysis in Figure 5.

DISCUSSION

We have made the novel observation that Zfh_{ep} is present as two forms. The increased acuity provided by a 4% acrylamide gel in western analysis shows that Zfh_{ep} is expressed as a doublet of proteins with relative mobilities of approximately 180 kDa and 185 kDa in

several cell types. The presence of two separate forms of Zfh₁ on western analysis allows direct assay of the modifications that are responsible for the different mobilities. We have shown that the 185 kDa form of Zfh₁ is phosphorylated, whereas the 180 kDa Zfh₁ is hypophosphorylated, if at all. The lower Zfh₁ band apparently contains few or no phosphate groups since CIP caused no change of mobility in these experiments. The difference of mobility of 5,000 Da is similar to that seen with phosphorylation of protein kinase C epsilon (40). The shift to a slower mobility likely reflects decreased binding of SDS anions due to the negatively charged phosphates.

The presence of the phosphorylated form of Zfh₁ is cell-specific, which may contribute to different activities of Zfh₁ in different cell types. For example, transcriptional activation of the VDR gene by Zfh₁ is known to be cell-type specific (35). The number of potential Ser/Thr phosphorylation sites in rat Zfh₁ was determined by analysis with PhosphoBase (<http://www.cbs.dtu.dk/databases/PhosphoBase/>) (41). PhosphoBase identified 61 consensus phosphorylation sequences conserved in rat and human Zfh₁, representing 8 different kinases. The locations of potential phosphorylation sites suggest possible roles for phosphorylation. Zfh₁ contains three PL^D/_NLS repeats that are binding sites for the corepressor CtBP (30, 32). These residues are not phosphorylated, however, clusters of phosphorylation sequences are located around and between the PL^D/_NLS sequences. Phosphorylation at these sites may modify the ability of CtBP to bind Zfh₁. Similarly, clusters of phosphorylation sites flanking the acidic activation domain may influence the activity of this domain, providing a mechanism for regulating transcriptional activation by Zfh₁.

Zfh₁/ZEB is a physiologically important repressor of the interleukin 2 gene in T cells. Yang et al. (2002) recently found that superinduction of IL-2 by deoxynivalenol is due in part to loss of the DNA-binding activity of Zfh₁, apparently due to post-translational modification of Zfh₁ (29). Our data provide the first demonstration that Zfh₁ is post-translationally modified, suggesting the possibility that changes in phosphorylation in response to deoxynivalenol are responsible for the loss of DNA-binding activity of Zfh₁.

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Abbreviations used

Zfh₁	zinc finger homeodomain enhancer-binding protein
IL-2	interleukin 2
PP2A	protein phosphatase 2A
O-GlcNAc	O-linked N-acetylglucosamine
CIP	calf intestinal phosphatase
O-GlcNAcase	N-acetyl-D glucosaminidase
CtBP	C-terminal binding protein
NRE-A	negative regulatory element-A
NF-κB	nuclear factor kappa B
I-κB	inhibitor of κB

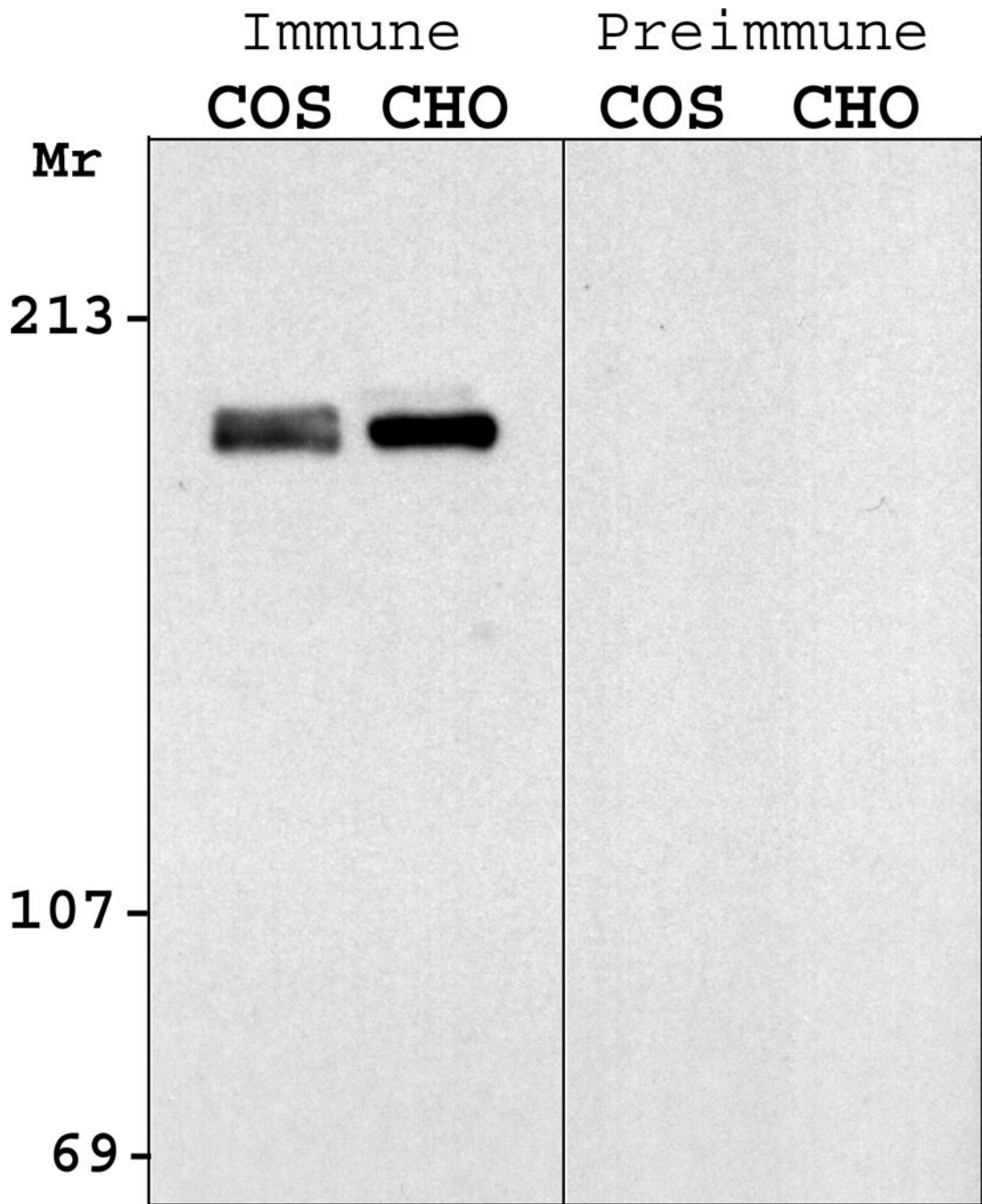
DTT	dithiothreitol
GST	glutathione-S-transferase
NP-40	Nonidet-P40

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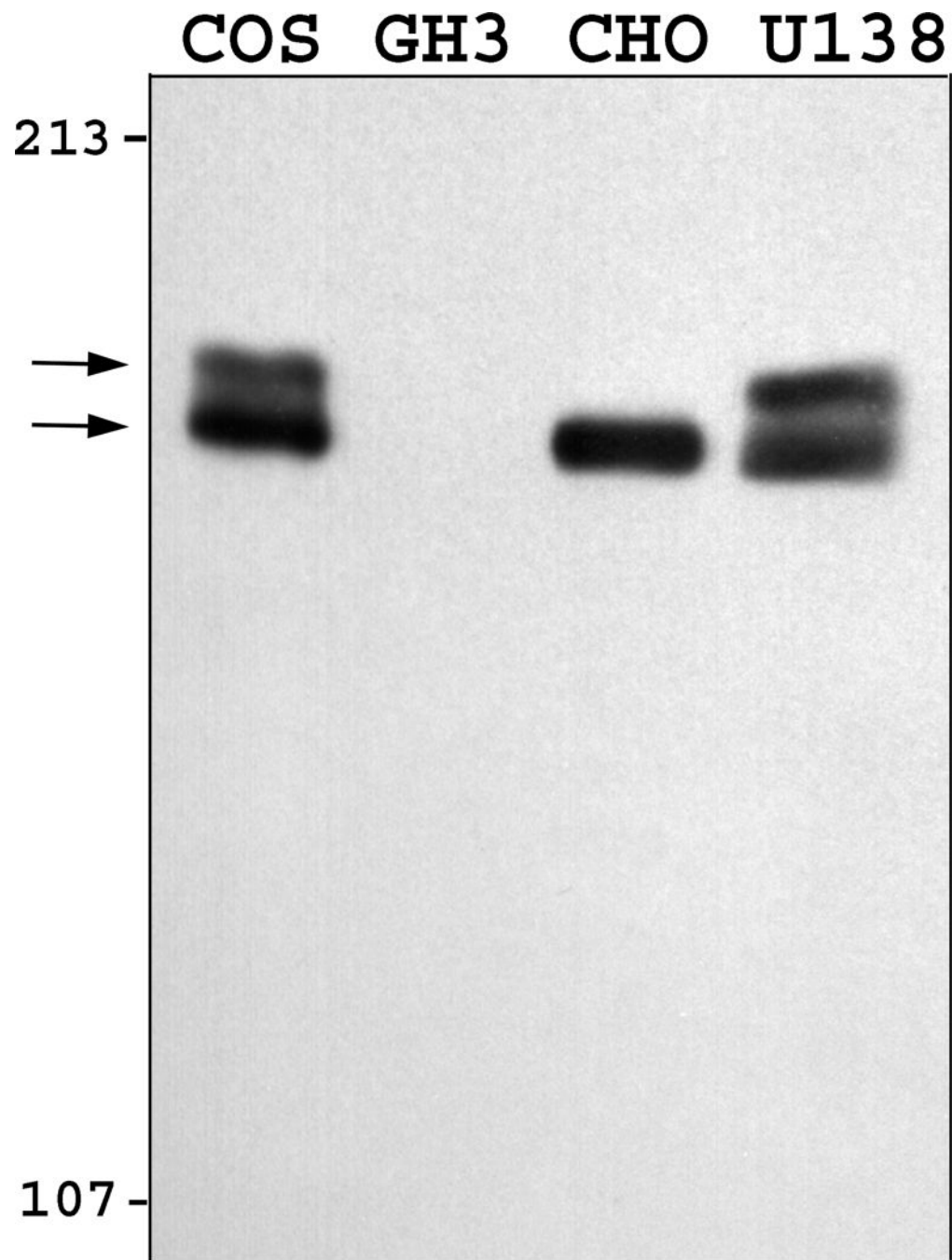
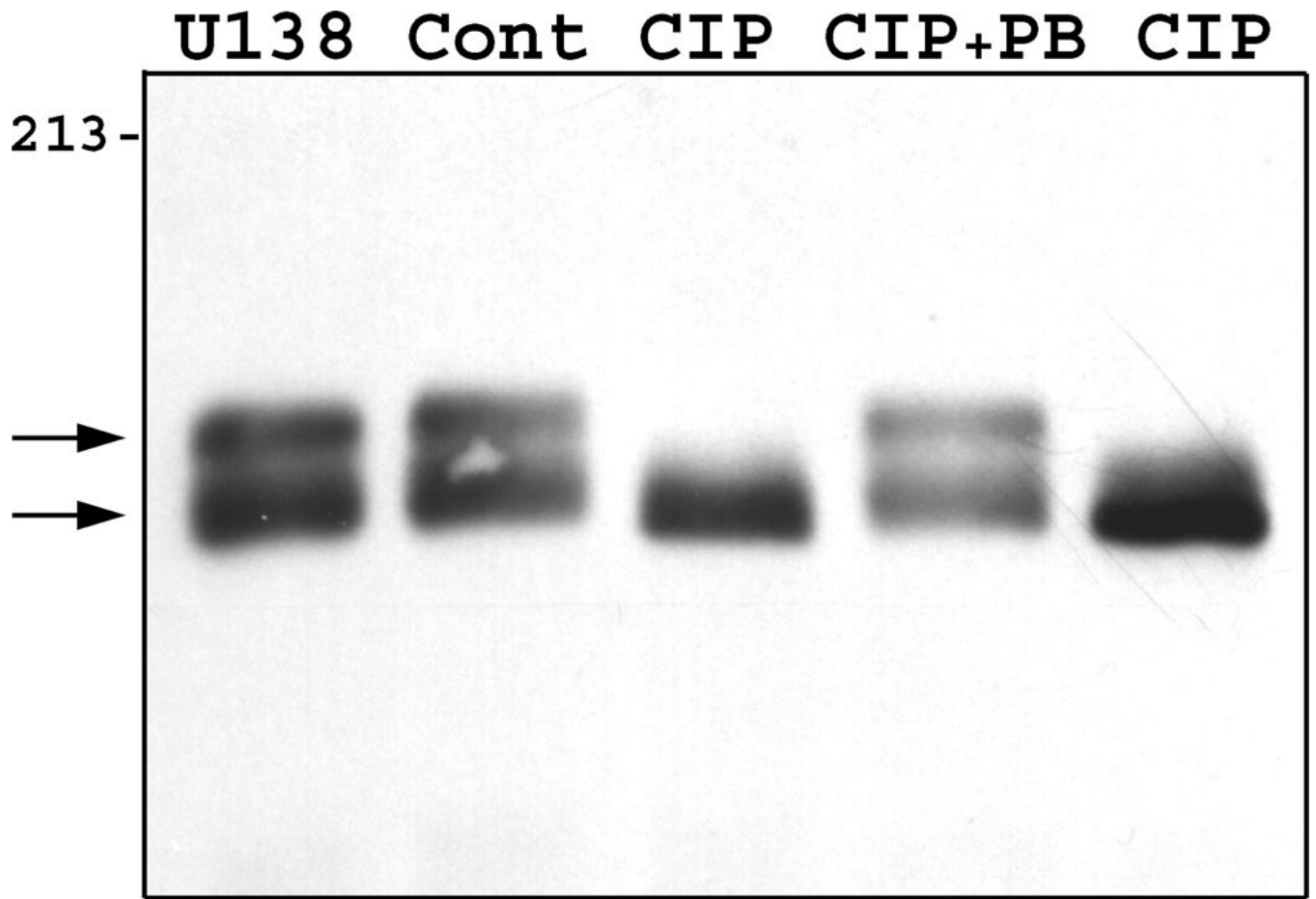


Figure 1. Zfh1 is expressed as a doublet. Nuclear extracts were separated on SDS-PAGE with either (A) 10% polyacrylamide, or (B) 4% polyacrylamide gels. Western analysis was done with either the anti-Zfh1 antiserum, or preimmune serum. Zfh1 protein appears as a doublet in COS-7 and U138MG cells (but not CHO cells) only with the 4% gel.



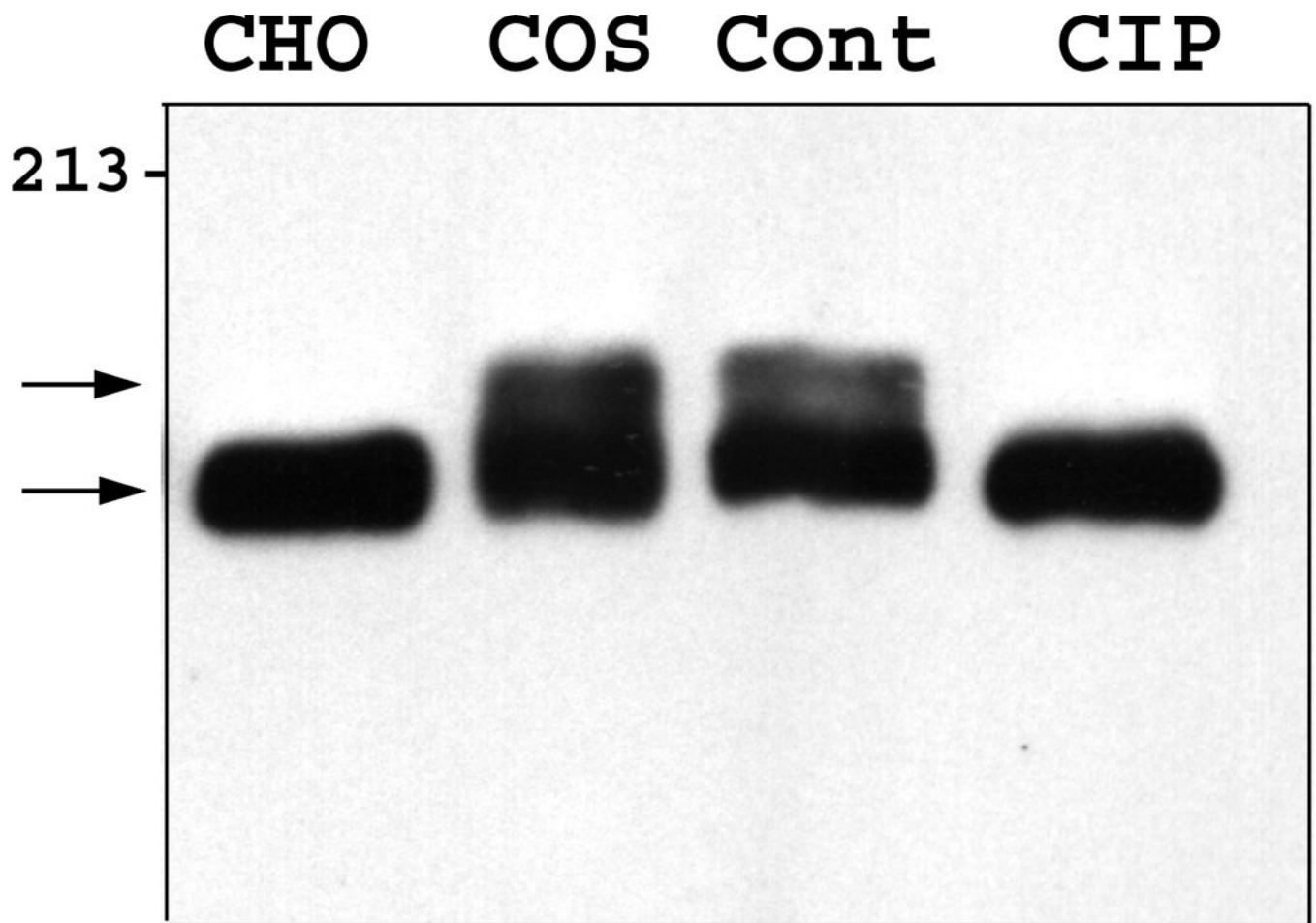


Figure 2.

The difference in mobility of the two Zfh1 bands is caused by phosphorylation. A.) U138MG nuclear extract was incubated with or without CIP prior to western analysis with anti-Zfh1 antiserum. Lane 1: untreated U138 proteins, Lane 2: control incubation, Lane 3: CIP, Lane 4: CIP in 100 mM phosphate buffer, Lane 5: CIP in HEPES buffer. B.) Nuclear extract from COS-7 cells was pretreated as described prior to immunoblotting with anti-Zfh1. Lane 1: untreated CHO nuclear extract, Lane 2: untreated COS-7 nuclear extract, Lane 3: control incubation of COS extract, Lane 4: CIP incubation of COS extract.

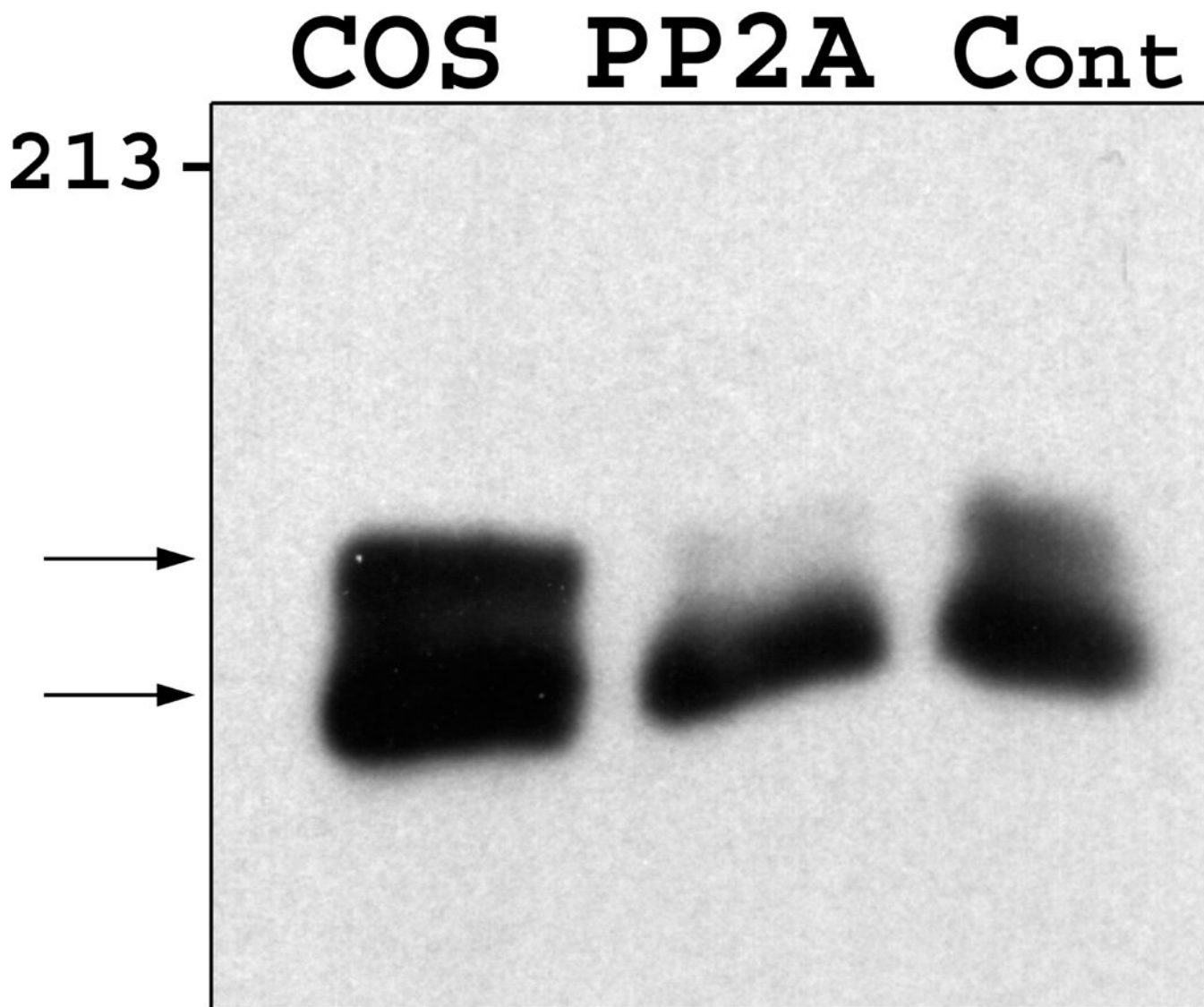


Figure 3. Zfhep is phosphorylated on Ser/Thr residues. Nuclear extract from COS-7 was incubated with or without PP2A prior to immunoblotting. PP2A dephosphorylates pSer or pThr residues, but not pTyr. Lane 1: untreated COS nuclear extract, Lane 2: PP2A incubation, Lane 3: control incubation.

Cont GNCase

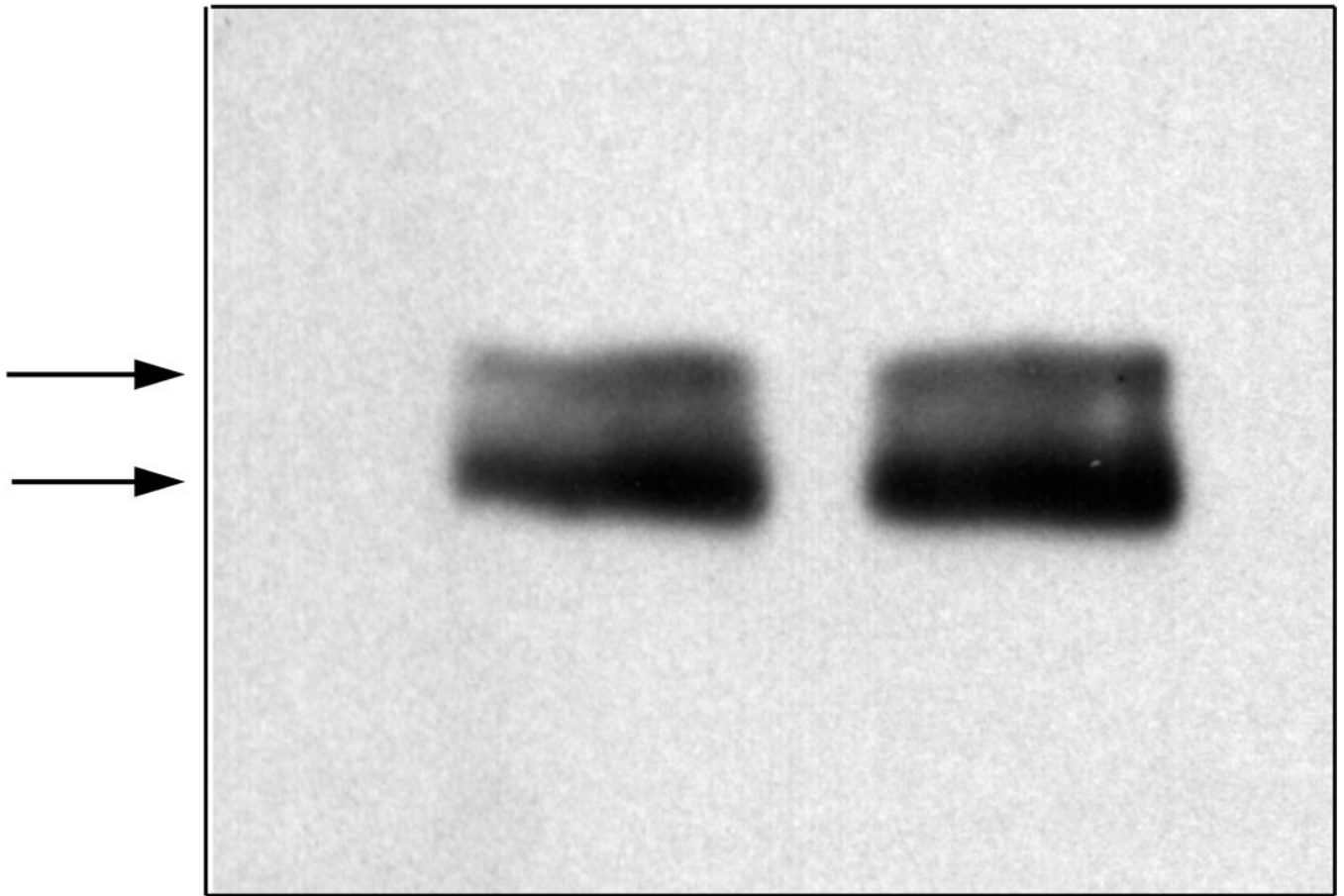


Figure 4. O-GlcNAcylation does not alter the mobility of Zfh1p. Nuclear extract from COS-7 were incubated with or without O-GlcNAcase (Gncase) prior to western analysis for Zfh1p.

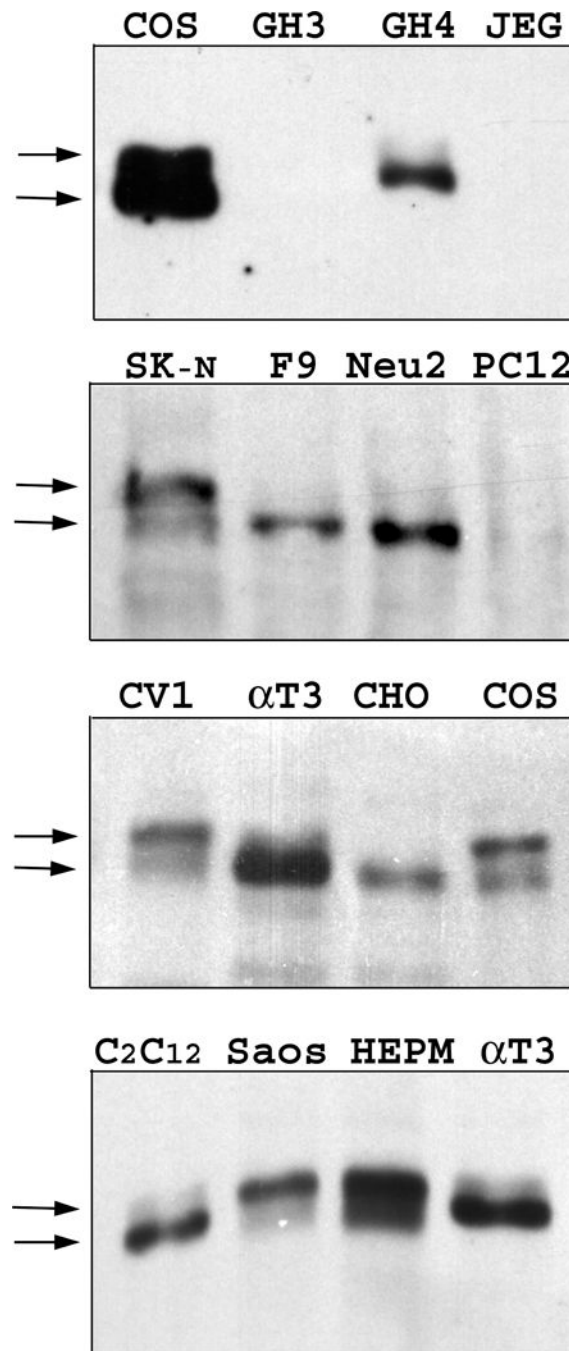


Figure 5. Phosphorylation of Zfh is cell-specific. Expression of Zfh in untreated nuclear extracts from different cell types was analyzed by western blot of 4% acrylamide gels. The full names of the cell lines are given in the Results section.