Recombinant human hyaluronan synthase 3 is phosphorylated in mammalian cells

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Hyaluronan is a ubiquitous component of vertebrate extracellular and cell-associated matrices that serves as a key structural component of skin, cartilage, eyes and joints, and plays important roles in dynamic cellular processes, including embryogenesis, inflammation, wound healing and metastasis. Hyaluronan is synthesized by three homologous hyaluronan synthases designated HAS1, HAS2 and HAS3 that differ in their tissue distribution, regulation and enzymatic characteristics. Some progress has been made in characterizing regulation of *HAS* transcripts and in distinguishing the enzymatic properties of the various HAS isoforms, but essentially nothing is known about their possible regulation by post-translational modification. Using [³²P]P_i radiolabelling of a recombinant FLAG (DYKDDDDK) epitope-tagged version of

human HAS3 expressed in COS-7 cells, we show that HAS3 is serine-phosphorylated and that this phosphorylation can be enhanced by a number of effectors – most significantly by a membrane-permeable analogue of cAMP. By employing a novel FLAG-tagged phosphorylated reference protein derived from EGFP (enhanced green fluorescent protein), we were able to estimate the stoichiometry of FLAG–HAS3 phosphorylation. It was approx. 0.11 in unstimulated cells and increased to as much as 0.32 in cells stimulated with 8-(4-chlorophenylthio)-cAMP.

Key words: cAMP, hyaluronan synthase (HAS), protein kinase, protein phosphorylation, signalling.

INTRODUCTION

HA (hyaluronan, hyaluronic acid) is a ubiquitous component of vertebrate extracellular and cell-associated matrices that is continuously synthesized and secreted by fibroblasts, keratinocytes, chondrocytes and other specialized cells throughout the body. HA is a linear unbranched polymer of alternating *N*-acetyl-D-glucosamine and D-glucuronic acid residues linked by $\beta(1 \rightarrow 4)$ and $\beta(1 \rightarrow 3)$ bonds. Native HA is typically larger than other glycosaminoglycans, reaching relative molecular masses of approx. 10^7 – and serves largely structural roles in skin, cartilage and the fluids of eyes and joints. HA also plays a dynamic role in many biological processes, including the modulation of cell migration and differentiation during embryogenesis, the regulation of metabolism and organization of the extracellular matrix, and the complex interactions underlying inflammation, wound healing, and metastasis [1–3].

In mammals HA is synthesized by HAS1, HAS2 and HAS3 isoforms of HAS (hyaluronan synthase; EC 2.4.1.212), which are the products of distinct genes that differ in tissue distribution, regulation and enzymatic properties [2,3]. These proteins are integral plasma membrane proteins which have been predicted, on the basis of studies of the homologous *Streptococcus pyogenes* HAS [4], to contain six membrane-spanning segments and two additional hydrophobic stretches that interact with the plasma membrane at its cytoplasmic face. A large cytoplasmic loop between the second and third predicted transmembrane segments contains the majority of residues conserved among all HAS family members and, presumably, contains the catalytic regions of the proteins [1,2].

HA biosynthesis is subject to control by a wide variety of growth factors and cytokines and, in many cases, stimulation of HA synthesis is paralleled by increases in *HAS2* transcripts (reviewed in [3]). There are also reports of cytokine-mediated regulation of *HAS1* transcripts in fibroblast-like synoviocytes [5] and of cytokine- and growth-factor-mediated regulation of *HAS3* transcripts in keratinocytes [6, 7]. *HAS3* transcripts are selectively elevated in lung fibroblasts in response to mechanical stretching [8]. Despite this abundant evidence for regulation of *HAS* transcript levels, striking changes in HA secretion are not always well correlated with changes in *HAS* mRNA levels (e.g. Recklies et al. [9]). Many explanations for these results are likely, including possible cytokine effects on the availability of the UDP-sugar precursors of HA [10] or cytokine effects on rates of translation or turnover of the HAS proteins. The abilities of these cytokines to modulate a variety of protein phosphorylation pathways [11–14], however, suggests the potentially important possibility that the activity of HAS isoforms may be regulated by protein phosphorylation. Phosphorylation provides a mechanism for rapid regulation of enzyme function to complement the slower regulation through changes in transcript levels. Because of technical difficulties in isolating the mammalian HAS proteins, no previous studies have directly investigated possible control of the HAS enzymes by post-translational modification. Nevertheless, there is indirect evidence for regulation of HAS activity by PKC (protein kinase C), PKA (protein kinase A) and/or by a calcium-dependent protein kinase [15,16]. For example, HAS activity in cell membranes could be decreased by phosphatase treatment and increased by incubation with ATP with or without a phorbol ester activator of PKC [15]. We report in the present paper, using ³²P-radiolabelling of FLAG

Abbreviations used: AEBF, 4-(2-aminoethyl)benzenesulphonyl fluoride; BAP, bacterial alkaline phosphatase; CPT-cAMP, 8-(4-chlorophenylthio)-cAMP; DMEM, Dulbecco's modified Eagle's medium; EGF, epidermal growth factor; EGFP, enhanced green fluorescent protein; HA, hyaluronic acid; HAS, hyaluronan synthase; IGF-1, insulin-like growth factor-1; LPA, lysophosphatidic acid; mRIPA buffer, modified radioimmunoprecipitation assay buffer; PrC tag, epitope tag (EDQVDPRLIDGK) from coagulation protein C; PKA, protein kinase A; PKC, protein kinase C; TBS-T, Tris-buffered saline with Tween 20.

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epitope-tagged protein expressed in mammalian cells, that human HAS3 is phosphorylated and that this phosphorylation can be enhanced by a variety of physiological effectors.

EXPERIMENTAL

Radiochemicals, chemicals and peptides

[32 P]P_i (carrier-free in water) was from PerkinElmer and MP Bio-medicals. LPA (lysophosphatidic acid) and ionomycin were from BIOMOL International L.P. Okadaic acid, calyculin A and PMA were from LC Laboratories. Recombinant human EGF (epidermal growth factor) was from Invitrogen. AEBSF [4-(2-aminoethyl)benzenesulphonyl fluoride] and E-64 [*trans*-epoxysuccinyl-L-leucylamido-(4-guanidino)butane] protease inhibitor were from Calbiochem/EMD Biosciences. CPT-cAMP [8-(4-chlorophenylthio)-cAMP], insulin (bovine), leupeptin, pepstatin A and sodium orthovanadate were from Sigma–Aldrich; the sodium orthovanadate was stored frozen at -80°C in small aliquots after activation as described by Gordon [17]. FLAG peptide (DYKDDDDK) and FLAG-tagged BAP (bacterial alkaline phosphatase) were from Sigma–Aldrich.

Expression constructs

The coding region for *HAS3* was amplified from cDNA from human MCF-10A cells by PCR using oligonucleotide primers that deleted the initiator Met codon, added an in-frame HindIII restriction site to the 5'-end and added two tandem stop codons and a NotI restriction site to the 3'-end. This product was then digested with HindIII and NotI restriction enzymes and subcloned into the vector pFLAG-CMV2 (Sigma–Aldrich), linearized using HindIII and NotI, to generate pFLAG-CMV2-hHAS3, an expression construct for HAS3 with an N-terminal FLAG epitope tag. The 3'-end of *HAS3* in this plasmid was then replaced with sequences that joined HAS3 to a C-terminal PrC tag [epitope tag (EDQVDPRLIDGK) from coagulation protein C] to generate an expression plasmid for the doubly tagged FLAG–HAS3–PrC. An expression plasmid for a FLAG-tagged version of EGFP (enhanced green fluorescent protein) was constructed by subcloning a HindIII/NotI fragment containing the coding region of EGFP from an EGFP expression plasmid (pcDNA3-GFP) into pFLAG-CMV2. The coding sequence for a consensus PKA phosphorylation site was then inserted into the HindIII site to generate an expression plasmid for FLAG–phos–EGFP, a chimaeric EGFP with an N-terminal FLAG tag followed by the consensus phosphorylation sequence (His-Arg-Arg-Ala-Ser-Ile-Ile-Phe-Gln) and a short sequence (Leu-Ala-Ala-Thr) immediately upstream of the EGFP coding sequence. The sequences for the inserts in these constructs were all verified in their entirety. Plasmid pME18S-FLAG-CaMKK α (1–434), an expression plasmid for a FLAG-tagged truncated form of calcium/calmodulin-dependent protein kinase [18] was a gift from Professor Thomas Soderling (Vollum Institute, Oregon Health and Science University, Oregon, OR, U.S.A.). An expression plasmid for the regulatory subunit of PKA with a C-terminal PrC tag was constructed in the pRevTRE vector (Clontech) using a combination of PCR to introduce new restriction sites and direct insertion of a synthetic duplex oligonucleotide.

Antibodies

The anti-FLAG monoclonal antibody M2 and agarose-linked anti-FLAG M2 were from Sigma–Aldrich. The anti-PrC monoclonal antibody HPC4 [19] was a gift from Dr C. Esmon (Oklahoma Medical Research Foundation, Oklahoma City, OK, U.S.A.).

Horseradish-peroxidase-conjugated anti-mouse IgG was product no. A2304 from Sigma–Aldrich.

Cell culture, transfection and radiolabelling

COS-7 cells were maintained in Advanced DMEM (Dulbecco's modified Eagle's medium; Invitrogen) supplemented with 5% fetal bovine serum at 37°C in a 5% CO₂ atmosphere. Transfections used Lipofectamine[®] 2000 (Invitrogen) and the above medium without serum as diluent for both DNA and transfection reagent, following the manufacturer's recommendations. Cells were radiolabelled and harvested 18–24 h after transfection. For most radiolabelling assays, cells were plated and transfected in wells of 24-well plates. The medium was aspirated and the cells were washed once with low-phosphate medium (phosphate-free DMEM supplemented with 1 mM sodium orthophosphate and 5 mg/ml protease-free fraction V BSA). After washing, the cells were then incubated for 3 or 3.5 h in 0.2 ml of low-phosphate medium plus 200 μCi of [32 P]P_i. Various drugs were added from 100-fold concentrated stocks in water or DMSO and incubation continued for a further 30 min, a time chosen to permit robust phosphorylation responses without significant changes in protein expression. For phosphoamino acid analysis, cells were plated in wells of a 12-well plate, and incubations used ~ 1.6 mCi of [32 P]P_i in 0.34 ml of low-phosphate medium.

Cell harvest and protein extraction

Medium was aspirated, and the cells were washed with cold PBS and then scraped into a small volume of PBS and transferred to microcentrifuge tubes. For most experiments the cells were then harvested by centrifugation for 2 s at 10 000 *g* and resuspended using 25 μl per well of a 24-well plate, in sucrose buffer (10 mM sodium phosphate, 10 mM sodium pyrophosphate, 10 mM 2-mercaptoethanol, 0.25 M sucrose, 1 mM EDTA, 1 mM EGTA, 100 mM NaF and 50 mM β -glycerophosphate, pH 7.0) freshly supplemented with 1 mM sodium orthovanadate, 400 μM AEBSF, 5 μM E-64 protease inhibitor, 2 μM leupeptin and 1 μM pepstatin A. Cells were then lysed by indirect sonication using a Fisher Model 500 Sonic Dismembrator with a 3 inch cup probe for 1 min of processor time at a power setting of eight, with cycles of 0.3 s on, 0.7 s off. Crude membranes were pelleted by centrifugation for 10 min at ~ 10000 *g*. FLAG–HAS3 was solubilized by incubating membranes for 10 min at 60°C in 1% SDS and 10 mM each of sodium phosphate and sodium pyrophosphate, pH 7.0, using 5 μl for membranes from ~ 25 μl of cell extract. After chilling on ice and centrifugation to collect condensate from the tube lid and sides, the solubilized membrane sample was diluted 10-fold with a solution to bring the composition to that of mRIPA buffer (modified radioimmunoprecipitation buffer): 10 mM sodium phosphate, 10 mM sodium pyrophosphate, 158 mM NaF, 1% (w/v) Triton X-100, 1% (w/v) sodium deoxycholate and 0.1% SDS, pH 7.0. After centrifugation for 10 min at ~ 10000 *g*, the supernatant fraction was applied to anti-FLAG affinity resin for purification. For the experiment of Figure 1 (below), pellets of harvested cells were extracted directly with a hot solution of 1% SDS as described previously [20].

FLAG affinity-purification

Solubilized membrane preparations in mRIPA buffer were mixed with 8 μl portions of agarose-linked anti-FLAG M2 antibody that had been diluted into mRIPA buffer, centrifuged at 10000 *g* for 10 s, and washed by resuspension with a 20-fold excess of mRIPA buffer. Some control samples of washed resin were pre-blocked by incubating with 0.5 mg/ml FLAG peptide in mRIPA buffer for

30 min at room temperature (24 °C) using 1.25 µg peptide/µl resin and then centrifuged before incubating with membrane extract. After 2 h on ice with occasional mixing, the resin was pelleted by centrifugation, and the supernatant fraction was removed. The resin was washed three times with a 20-fold excess of mRIPA buffer and the bound proteins were eluted by resuspension in 25 µl of SDS/PAGE sample buffer and heated for 10 min at 65 °C. The resin was then removed by centrifugation at 10 000 g for 10 s, before loading on a gel.

SDS/PAGE, Western immunoblots and phosphoamino acid analysis

Samples were subjected to SDS/PAGE (10 % gels) [21] and transferred to Immobilon-P membranes (Millipore) as described elsewhere [20]. For Western immunoblot analysis, membranes were blocked with 5 % (w/v) non-fat dried milk in TBS-T (Tris-buffered saline containing 0.3 % Tween 20) and incubated successively with either anti-FLAG or anti-PrC monoclonal antibodies and then a horseradish-peroxidase-conjugated goat anti-mouse IgG in blocking solution. Chemiluminescence detection used the SuperSignal West Pico substrate kit (Pierce Biotechnology). For [³²P]P_i incorporation experiments, membranes were washed three times with TBS-T containing 0.02 % NaN₃ to remove the chemiluminescent substrate and inactivate the peroxidase, and then exposed to X-ray film (X-omat AR or Biomax MS; Kodak) for 14 days at -80 °C with Lightning Plus intensifying screens (DuPont). Quantification of immunoblots and autoradiographs was determined by densitometry using a Molecular Dynamics model 300A computing densitometer. Silver-stain detection of unlabelled proteins used the method of Merrill et al. [22]. For phosphoamino acid analysis, radiolabelled bands were excised from gels that had been dried directly on paper backing (using tracings of autoradiograms as guides). Acid hydrolysis of the isolated phosphoproteins and analysis by electrophoresis on cellulose thin-layer plates followed the procedure described by Eckhart et al. [23] with modifications described elsewhere [24]. Unlabelled phosphoserine, phosphothreonine and phosphotyrosine standards were visualized by staining with ninhydrin.

RESULTS

Expression and immunodetection of full-length FLAG-HAS3

Following the success of Itano et al. [25] in demonstrating expression of active N-terminally FLAG-tagged versions of the mouse isoforms of HAS, we developed similar constructs for the three human HAS isoforms and transfected them into COS-7 cells for transient expression. As reported for the native HAS isoforms [26], the FLAG-tagged proteins migrated on SDS/PAGE as if they were approx. 16–18 kDa smaller than their calculated molecular masses, which are 66 kDa for HAS1, 64.7 kDa for HAS2 and 64.2 kDa for HAS3 (results not shown). Because expression of FLAG-HAS1 and FLAG-HAS3 was significantly more robust than that of FLAG-HAS2, we directed our initial studies on HAS phosphorylation to these two isoforms. FLAG-HAS3 exhibited both basal and effector-stimulated phosphorylation that is the focus of the present paper. FLAG-HAS1, on the other hand, exhibited little or no phosphorylation under basal conditions, and its phosphorylation was not enhanced by any of the effectors used in experiments described below (B. J. Goentzel, P. H. Weigel and R. A. Steinberg, unpublished work).

To be certain that the expressed FLAG-HAS3 protein was full-length, we added a C-terminal PrC tag [19] to the FLAG-HAS3 construct and tested whether the N-terminal FLAG and C-terminal PrC tags produced the same sized protein. Figure 1 shows Western immunoblot patterns of the expressed FLAG-

Figure 1 Recombinant epitope-tagged forms of HAS3 are expressed as full-length proteins in transfected COS-7 cells

Western-blot patterns are shown for whole-cell extracts of untransfected COS-7 cells or COS-7 cells transfected with expression constructs for positive control proteins, FLAG-tagged human HAS3 (FLAG-HAS3), or the doubly FLAG- and PrC-tagged human HAS3 (FLAG-HAS3-PrC). (A) Extracts were from cells expressing a FLAG-tagged fragment of Ca²⁺/calmodulin-dependent protein kinase α (lane a), untransfected cells (lane b), or cells expressing FLAG-HAS3 (lane c) or FLAG-HAS3-PrC (lane d); the primary antibody was the anti-FLAG M2 monoclonal antibody; the film exposure time was 10 s. (B) Extracts were from cells expressing a PrC-tagged regulatory subunit of PKA (lane a), untransfected cells (lane b), or cells expressing FLAG-HAS3 (lane c) or FLAG-HAS3-PrC (lane d); the primary antibody was the anti-PrC monoclonal HPC4 antibody; the film exposure time was 5 min. All patterns shown are from samples run on a single gel blotted on to one membrane that was cut in two for the different antibody incubations. For clarity and simplicity several lanes between lanes b and c in the original Western blots were electronically excised in preparing the Figure (at positions indicated by narrow vertical lines). Protein sizes are given in kDa (K).

HAS3 and FLAG-HAS3-PrC proteins with both anti-epitope antibodies (Figure 1, lanes c and d). Also shown are patterns from extracts of cells expressing soluble epitope-tagged proteins as positive controls (Figure 1, lanes a) and patterns from an extract of untransfected cells (Figure 1, lanes b). The difference in apparent molecular masses of the FLAG-HAS3 and FLAG-HAS3-PrC proteins in Figure 1(A) is consistent with the ~1.4 kDa PrC peptide, and both antibodies detected a single major species with an apparent size of ~51 kDa in the FLAG-HAS3-PrC sample. This result shows that the protein detected in the FLAG-HAS3-PrC sample has retained both the N-terminal FLAG tag and the C-terminal PrC tag and, therefore, is the full-length protein. The small difference in mobility between the FLAG-HAS3 and FLAG-HAS3-PrC species suggests that the slightly smaller FLAG-HAS3 protein is also full-length.

Extraction and affinity-purification of FLAG-HAS3

In order to use [³²P]P_i incorporation to detect phosphorylation of the FLAG-HAS proteins, we needed to extract the proteins from membranes and purify them with good yields. In contrast with the report of Yoshida et al. [27], we were unable to solubilize a significant portion of recombinant FLAG-HAS protein with CHAPS or other non-ionic or zwitterionic detergents. FLAG-HAS protein was solubilized with SDS and remained soluble when diluted with buffer containing non-ionic detergents and could then be affinity-purified using agarose-linked anti-FLAG M2 monoclonal antibody.

The immunoblot patterns of Figure 2(A) show that FLAG-HAS3 was adsorbed quantitatively by the anti-FLAG affinity resin (Figure 2A, compare lanes a and c), and essentially all of the bound FLAG-HAS3 was recovered by elution with SDS/PAGE sample buffer (Figure 2A, lane e). This selective binding of the FLAG-tagged protein was blocked by pre-incubation of the anti-FLAG resin with FLAG peptide (Figure 2A, lanes b and d).

Figure 2 FLAG-HAS3 protein can be affinity-purified after solubilization with SDS

Crude membranes from sonicated extracts of COS-7 cells expressing FLAG-HAS3 were isolated, solubilized with SDS, diluted into mRIPA buffer and purified with agarose-coupled anti-FLAG M2 monoclonal antibody. (A) Western immunoblot using the anti-FLAG monoclonal M2 antibody as the primary antibody. (B) Silver-stained SDS/PAGE gel. The sample for lane a was the solubilized membrane extract, those for lanes b and c were the unbound fractions after adsorption with the resin, and those for lanes d and e were the fractions eluted with SDS gel sample buffer after washing the resin with mRIPA buffer. The resin for lanes b and d was pre-blocked with a peptide containing the FLAG epitope. Sample volumes were adjusted to give equal portions of the various fractions (equivalent to $\sim 2 \mu\text{g}$ of protein in whole-cell extracts). Lanes f and g in (A) were loaded, respectively, with 10 and 20 ng of FLAG-BAP as a calibration standard. Protein sizes are given in kDa (K).

Lanes f and g of Figure 2(A) show samples of FLAG-BAP run as standards to estimate yields of the FLAG-HAS3 protein. Figure 2(B) shows silver-stained protein patterns from a parallel gel of the same samples used for Figure 2(A), lanes a–e. The nearly identical patterns and intensities of protein bands in the membrane extract (Figure 2B, lane a) and in fractions unbound by the affinity resin (Figure 2B, lanes b and c) indicate that very little of the extract protein bound non-specifically to the affinity resin. This conclusion is also supported by the absence of bands corresponding to the major membrane extract species in fractions bound to the resin and eluted with SDS/PAGE sample buffer (Figure 2B, lanes d and e).

Taken together with the immunoblotting result from Figure 2(A) showing nearly quantitative recovery of FLAG-HAS3 after affinity-purification, the paucity of contaminating membrane extract proteins in silver-stained patterns of the purified fractions suggests that the affinity-adsorption procedure provides substantial purification of FLAG-HAS3. Judging from the FLAG-BAP standards, the affinity-purified fractions contained approx. 10–15 ng of FLAG-HAS3 protein (Figure 2, lanes e), which is below the threshold for detection by silver staining. The strongly stained proteins with sizes of approx. 25 and 50 kDa in the eluted fractions (Figure 2B, lanes d and e) are Ig heavy and light chains released from the affinity resin by heating in SDS/PAGE sample buffer – they are also seen when resin that has not been exposed to extract proteins is ‘eluted’ in this manner (results not shown).

Phosphorylation of FLAG-HAS3 in COS-7 cells

Figure 3 shows the Western immunoblot and autoradiographic patterns of FLAG-HAS3 affinity-purified from extracts of cells radiolabelled with [^{32}P]P_i in the presence or absence of various

Figure 3 FLAG-HAS3 is phosphorylated

COS-7 cells transfected with an expression plasmid for FLAG-HAS3 (lanes a–h) or EGFP (lane i) were incubated for 3.5 h with [^{32}P]P_i in a serum-free low-phosphate medium. For the last 30 min of labelling the cells were exposed to 1 μM okadaic acid (lane b), 100 nM calyculin A (lane c), 100 μM sodium orthovanadate (lane d), 100 μM CPT-cAMP (lane e), 50 nM PMA (lane f), 20 ng/ml EGF (lane g) or 10 $\mu\text{g}/\text{ml}$ insulin (lane h). Control samples (lanes a and i) were exposed to 1% DMSO, which was the solvent for several of the drugs. The cells were then washed, harvested, and disrupted by sonication. Crude membrane fractions were isolated by centrifugation and solubilized with 1% SDS in 10 mM Tris/HCl, pH 7.4. The samples were diluted into mRIPA buffer, and FLAG-HAS3 was purified by anti-FLAG immunoaffinity adsorption, subjected to SDS/PAGE, and electroblotted on to a PVDF membrane. (A) Immunoblot detection with the anti-FLAG antibody. (B) Autoradiogram of the same membrane after washing in buffer containing 0.02% sodium azide and exposure to X-ray film for 14 days at -80°C in the presence of an intensifying screen. The three lanes to the left of the lettered lanes in (A) were loaded with 400, 200 or 100 fmol of FLAG-BAP as standards.

drugs that can enhance cellular protein phosphorylation, by either inhibiting protein phosphatases or stimulating protein kinases. Radiolabelled control cells were transfected with an expression plasmid for EGFP (Figure 3, lanes i) and no band was detected at the position of FLAG-HAS3, thus providing evidence that the radiolabelled band at this position reflected labelling of FLAG-HAS3 and not some contaminating cellular protein. Although ^{32}P -labelling intensities varied somewhat with drug treatments, the strongest labelling was observed in cells treated with CPT-cAMP, PMA or EGF. To quantify these differences, we used densitometry on the Western blot and autoradiographic patterns to integrate the signals in FLAG-HAS3. The Western-blot data were used to correct the ^{32}P -incorporation data for differences in FLAG-HAS3 expression and recovery, and the corrected incorporation values in the presence of drug were normalized to the drug-free controls in order to derive relative phosphorylation values.

Figure 4 summarizes the relative phosphorylation values for FLAG-HAS3 after treatment with numerous effectors from multiple independent determinations. Phosphorylation increases stimulated by these agents were generally small and variable, as indicated by the relatively large S.D. values. CPT-cAMP most reliably increased the phosphorylation of FLAG-HAS3, where increases of approx. 2-fold or greater were observed in four independent experiments. The calcium ionophore ionomycin and the serine/threonine phosphatase inhibitor calyculin A had no effect on FLAG-HAS3 phosphorylation in single experiments (Figure 3 and results not shown). Combinations of the protein serine/threonine-phosphatase inhibitor okadaic acid with ionomycin, LPA, CPT-cAMP or PMA, or of the protein-tyrosine phosphatase inhibitor sodium vanadate with EGF or insulin did not enhance phosphorylation over that observed with the single effectors (results not shown).

Stoichiometry of FLAG-HAS3 phosphorylation

Autoradiography provides data on relative rather than absolute levels of incorporated radioactivity, so we sought a suitable

Figure 4 Many effectors that stimulate protein kinases or inhibit protein phosphatases affect the phosphorylation of FLAG-HAS3

The effects of 1 μ M okadaic acid (OA), 1 μ M LPA, 50 nM PMA, 100 μ M CPT-cAMP (cAMP), 100 μ M sodium orthovanadate (VO₄), 20 ng/ml EGF and 10 μ g/ml insulin (Ins) on phosphorylation of FLAG-HAS3 were assessed in multiple experiments like that of Figure 3. Densitometry was used to integrate absorbances of the FLAG-HAS3 bands in the autoradiographs and Western-blot patterns, and the ratios of these integrated absorbances were used to correct the incorporation data for differences in FLAG-HAS3 protein recovery. For each experiment, the corrected incorporation values were then divided by the value for the control sample (from cells labelled without any added drug) to derive relative phosphorylation values. Bars show the means $\pm$ S.D. for the values determined in multiple experiments; the numbers of replicate determinations for each effector are in parentheses.

phosphoprotein to use as a reference standard to quantify the stoichiometry of FLAG-HAS3 phosphorylation. We made an expression construct encoding FLAG-EGFP and found, as expected, that the expressed FLAG-EGFP protein was not phosphorylated (results not shown). We then introduced a consensus substrate sequence for phosphorylation by PKA between the FLAG tag and the EGFP coding sequence of FLAG-EGFP in the hope that it would permit quantitative phosphorylation of the FLAG-EGFP at a single site. In contrast with the FLAG-EGFP protein, which gave a single band on SDS/PAGE and a single spot on high-resolution two-dimensional gel electrophoresis (results not shown), the FLAG-phos-EGFP protein gave two bands in SDS/PAGE (Figure 5A). Two-dimensional gel analysis revealed that the slower-migrating band contained a single protein species, but the faster-migrating band contained two major species separated by a distance consistent with a difference of a single charge unit (Figure 5B) [28]. (A negligibly small proportion of the protein is in a more basic charge variant, perhaps a deacetylated form, that is responsible for the very minor spots in Figures 5B and 5D.) Each of the slower- and faster-migrating forms of FLAG-phos-EGFP incorporated [³²P]P_i (Figure 5C); however, significant radiolabelling of only the more acidic of the two species resolved from the faster-migrating form of the protein suggests that these two species are non-phosphorylated and monophosphorylated versions of the protein (Figure 5D). The slower-migrating form of the protein also appears to be a monophosphorylated species, as it has a pI almost identical with that of the faster-migrating phosphorylated species. No forms of the protein were observed to have the more acidic pI values that would result from multiple phosphorylation events. As described below, the two phosphorylated forms of FLAG-phos-EGFP are phosphorylated on different residues – one a serine residue and the other a threonine residue – and the threonine phosphorylation apparently slowed the protein's mobility on SDS/PAGE as well as altering its charge. The results of Figure 5 indicate that the slower-migrating form of FLAG-phos-EGFP

Figure 5 FLAG-phos-EGFP provides a standard for quantifying phosphorylation of FLAG-HAS3

COS-7 cells were transfected with an expression construct for FLAG-phos-EGFP and radiolabelled with [³²P]P_i as described in the legend for Figure 3 with CPT-cAMP added for the last 30 min of labelling. Extracts were prepared by sonication, and the supernatant fractions after centrifugation for 10 min at $\sim$ 10 000 *g* were diluted with equal volumes of twice-concentrated mRIPA buffer and purified by adsorption to anti-FLAG-agarose and washing as described in the Experimental section. The sample for (A) and (C) was eluted from the affinity resin with SDS/PAGE sample buffer and subjected to SDS/PAGE, whereas the sample for (B) and (D) was eluted from the affinity resin with a urea-containing sample buffer [28] and subjected to high-resolution two-dimensional gel electrophoresis [29]. Relevant portions of the gels were electrophoretically transferred to membranes; the FLAG-phos-EGFP protein was visualized by immunodetection with anti-FLAG monoclonal antibody; and [³²P]P_i incorporation was detected by autoradiography as described in the legend to Figure 3. (A) Immunoblot pattern from one-dimensional gel; (B) immunoblot pattern from two-dimensional gel; (C) autoradiographic pattern from one-dimensional gel; (D) autoradiographic pattern from two-dimensional gel. The direction of migration in SDS/PAGE is shown to the right of the Figure, and arrows indicate the positions of the slower- and faster-migrating species of FLAG-phos-EGFP. Arrowheads in (B) and (D) indicate positions of the non-phosphorylated (left) and phosphorylated (right) forms of the faster-migrating species. For two-dimensional gel patterns (B and D), the orientation of the IEF (isoelectric focusing) dimension is indicated below (D).

is a pure singly phosphorylated species and therefore that the ratio of autoradiographic and immunoblot signals for this species corresponds to a stoichiometry of one phosphate group per protein chain. As long as the two proteins are labelled and analysed in parallel under identical conditions, the ratio of autoradiographic and (anti-FLAG) immunoblot signals for the slow-migrating form of FLAG-phos-EGFP can be used as a reference for assessing the stoichiometry of FLAG-HAS3 phosphorylation. (The faster-migrating band of FLAG-phos-EGFP can also be used as a phosphorylation reference standard, but only after correction, based on two-dimensional gel immunoblot patterns, for its lower stoichiometry of phosphorylation.) Using this standard we estimated that, under non-stimulating conditions, the FLAG-HAS3 is phosphorylated to a stoichiometry of approx. 0.11 in COS-7 cells and, following maximal stimulation with CPT-cAMP, phosphorylation was increased to approx. 0.32 mol of phosphate/mol of protein.

Identification of the amino acid(s) phosphorylated in FLAG-HAS3

To identify the phosphorylated residue(s) in FLAG-HAS3, we hydrolysed the affinity- and gel-purified protein isolated from cells radiolabelled in the presence of either CPT-cAMP or EGF and analysed the resulting products by electrophoresis on TLC plates. Figure 6 shows that the only ³²P-labelled amino acid released from FLAG-HAS3 was phosphoserine, whether the protein was labelled in the presence of CPT-cAMP or EGF. We also analysed hydrolysates of the slower- and faster-migrating forms of radiolabelled FLAG-phos-EGFP. The faster-migrating form had phosphoamino acid label only in phosphoserine, while the slower-migrating form had $\sim$ 75 % of its label in phosphothreonine and $\sim$ 25 % in phosphoserine. The phosphoserine label found in the

Figure 6 FLAG-HAS3 is serine phosphorylated

FLAG-HAS3 was labelled with [32 P]P_i in transfected COS-7 cells and purified from solubilized membrane fractions by immunoaffinity adsorption as for Figure 3, except that volumes and cell numbers were scaled up for wells of a 12-well plate and ~ 1.6 mCi of [32 P]P_i was used for each point. Samples were treated with either CPT-cAMP or EGF for the last 30 min of the 4 h labelling period. FLAG-phos-EGFP was labelled in a similar manner in cells treated with CPT-cAMP at the end of the labelling period. The FLAG-tagged proteins were affinity-purified as for Figures 3 and 5, subjected to SDS/PAGE, and localized by autoradiography of the fixed and dried gel. The phosphorylated FLAG-HAS3 and FLAG-phos-EGFP bands were excised, hydrolysed, and analysed by thin-layer electrophoresis as described in the Experimental section. Phosphoserine (P-Ser), phosphothreonine (P-Thr) and phosphotyrosine (P-Tyr) samples, run alone and in combination as standards, were visualized by ninhydrin staining, and the radiolabelled species were visualized by autoradiography. Lanes a and b are from FLAG-HAS3 labelled in the presence of CPT-cAMP and EGF. Lanes c and d are, respectively, from the slower- and faster-migrating forms of the FLAG-phos-EGFP protein.

slower-migrating form of FLAG-phos-EGFP probably reflects contamination with the faster-migrating form, because the two bands were not completely resolved.

DISCUSSION

Our results show for the first time that HAS3 is phosphorylated on one or more serine residues in live mammalian cells, and that HAS3 phosphorylation can be increased by a number of agents that activate protein kinases or inhibit protein phosphatases – including CPT-cAMP, PMA, LPA, EGF, insulin, okadaic acid and sodium orthovanadate. Mian [30] had reported both serine and tyrosine phosphorylation of components of a large membrane complex associated with synthesis of HA oligosaccharides, but the relationship, if any, of this complex to the now identified HAS isoforms remains unclear. CPT-cAMP and PMA were chosen for our studies on the basis of indirect studies that implicated PKA [15,31] and/or PKC [15,16,32,33] in the control of HAS activity. In our experiments the PKA-activator CPT-cAMP consistently elevated HAS3 phosphorylation approx. 2-fold or more and the PKC-activator PMA elevated phosphorylation approx. 50% on average. Almost as effective as PMA was LPA, which can activate a variety of protein kinases including Akt/protein kinase B, PKC, Rho-associated kinase and several members of the MAPK (mitogen-activated protein kinase) family through its interactions with G-protein-coupled receptors [34]. LPA is also an activator of PI3K, which has been implicated in HAS activation by an unknown Akt-independent mechanism [35].

Okadaic acid, an inhibitor of protein phosphatases 1 and 2A, gave only small enhancements of HAS3 phosphorylation and did not amplify the effects of the various protein serine kinase activators assayed. In a preliminary experiment, the PKA-selective-inhibitor H-89 elicited markedly greater inhibition of HAS3 phosphorylation stimulated by CPT-cAMP, than of basal phosphorylation or phosphorylation stimulated by PMA or LPA

(results not shown). This result suggests that HAS3 might be a substrate for both PKA and one or more other protein kinases that would probably act at distinct sites.

A number of agents that elevate protein tyrosine phosphorylation – including EGF, IGF-1 (insulin-like growth factor-1) and sodium orthovanadate – have been reported to enhance HA synthesis [9,32,36–38]. For EGF and IGF-1, the effects have been attributed, at least in part, to increases in the levels of HAS mRNAs [36,38]. In organotypic keratinocyte cultures, EGF induced 50–75% increases in both HAS2 and HAS3 mRNAs, while increasing HA concentrations approx. 4–5-fold; HAS1 expression was very low in these cells [36]. IGF-1 provoked approx. 2-fold increases in HA production in human skin fibroblasts [38] and in human articular chondrocytes [9]. Transcripts for all three HAS isoforms were increased approx. 2–3-fold in the IGF-1-treated fibroblasts [38], but no significant increases in mRNAs for any of the HAS isoforms were observed in the IGF-1-treated chondrocytes [9]. In several of our experiments EGF, insulin (at supra-physiological concentrations sufficient to activate IGF receptors) and sodium orthovanadate gave 70% or greater enhancements of HAS3 phosphorylation, but these effects were inconsistent; in approx. one-third of our experiments, the effects of these agents were not significant. The phosphorylation stimulated by either EGF or sodium orthovanadate was sensitive to H-89, suggesting that the effects of these agents might be through cross-talk of tyrosine kinase receptors with the cAMP pathway. Consistent with this notion, only radiolabelled phosphoserine was detected in HAS3, regardless of whether it was labelled in the presence of CPT-cAMP or EGF.

The physiological importance of HAS3 phosphorylation is impossible to judge at this time. Studies of transcripts for the three HAS isoforms in mouse embryos and in isolated or cultured cells generally reveal a predominance of HAS2 [3,22,36,38], although HAS1 and HAS3 are widely expressed in adult mouse tissues [22]. However, quantification of the relative amounts of the various HAS transcripts in these studies is problematic. When a quantitative PCR approach was used on three human cell types, HAS1 transcript levels were equivalent to those of HAS2, with HAS3 transcripts about an order of magnitude lower [9]. Not only has the lack of effective antibodies for immunoprecipitation of the three HAS proteins precluded direct studies on their phosphorylation, but also the relative expression of the three isoforms at the protein level remains unknown, and perhaps more importantly, so too does their relative concentrations at the plasma membrane. Furthermore, since HAS3 has a higher $V_{\max}$ than the other isoforms [3,21], it may contribute disproportionately to cellular HA production. Homozygous deletion of HAS2 in transgenic mice results in embryonic lethality [3], but HAS3 knockout mice are apparently viable and fertile [39]. The phenotype of HAS3 knockout mice has not been described in detail, but their resistance to inflammatory responses provoked by ventilator-induced lung injury supports a specific role of HAS3 up-regulation in generating low-molecular-mass HA in response to mechanical stretching in lung cells [8,39]. It is intriguing to consider the possibility that HAS3 phosphorylation might also play a role in this response.

Phosphorylation is the most prevalent post-translational modification of intracellular proteins, and kinase genes are estimated to account for $\sim 1.7\%$ of the human genome [40]. Approx. 30% of cellular proteins are phosphorylated *in vivo*, and, where it has been investigated, functional changes – including changes in enzymatic activities, metabolic stability, intracellular trafficking, and/or protein–protein interactions – are generally associated with the modification [41,42]. The activators we tested, which together can activate about a dozen protein kinases, barely scratch the

surface of testing for possible kinase regulators of HAS3. Analysis of the human HAS3 sequence for likely protein phosphorylation sites using NetPhos 2.0 [43] predicts 15 sites for serine phosphorylation, five for threonine phosphorylation and eight for tyrosine phosphorylation. A majority of these sites fall within the large central region of the protein predicted to be cytoplasmic and associated with catalysis [1,4,44]. Ser⁸⁶, near the N-terminus of this putative cytoplasmic loop, is within a consensus site for phosphorylation by PKA recognized by the motif scanner of Scansite [45]. This site is not conserved in either HAS1 or HAS2, and, although the serine residue and several residues downstream are conserved in the mouse Has3 homologue, the residues immediately upstream are different and do not constitute a recognizable PKA phosphorylation site. Further studies will be necessary to determine which serine residues are actually phosphorylated under basal conditions or in response to various stimuli.

A major limitation of most studies using radiolabelled phosphate to monitor protein phosphorylation is the difficulty of quantifying the extent of incorporation in order to assess its significance. For the present study we developed a phosphoprotein standard by introducing a phosphorylatable sequence into FLAG-tagged EGFP. Analysis by one- and two-dimensional gel electrophoresis and phosphoamino acid determination revealed that, in the design of the FLAG-phos-EGFP construct, we fortuitously introduced a threonine phosphorylation site as well as the intended serine phosphorylation site into the protein – presumably at a threonine residue immediately upstream of the normal EGFP coding region. (This threonine residue is also present in the FLAG-EGFP construct, but the different upstream context apparently precluded its phosphorylation.) As has been reported for several other proteins (e.g. the catalytic subunit of PKA [24]), the threonine phosphorylation reduced the SDS/PAGE mobility of the FLAG-phos-EGFP protein, effectively resolving it from the non-phosphorylated and serine-phosphorylated forms of the protein. Double-phosphorylated FLAG-phos-EGFP was not observed. The threonine-phosphorylated species therefore contained 1 mol of phosphate/mol of protein and could serve as a standard protein for estimating the molar phosphorylation of FLAG-HAS3 or other FLAG-tagged proteins labelled and analysed in parallel by Western immunoblotting and autoradiography. Application of this approach indicated that FLAG-HAS3 was phosphorylated to the extent of approx. one phosphate group per three protein molecules in COS-7 cells treated with CPT-cAMP. FLAG-HAS1, on the other hand, was phosphorylated to a stoichiometry of less than 0.08 in COS-7 cells treated with any of the agents used in our FLAG-HAS3 studies (B. J. Goentzel, P. H. Weigel and R. A. Steinberg, unpublished work).

The calculated stoichiometry of FLAG-HAS3 phosphorylation may be an underestimate for the functional protein, since much of the recombinant FLAG-HAS3 protein visualized by immunofluorescence was found in the perinuclear region of transfected cells (results not shown) – a localization that suggests aggregation and sequestration of misfolded protein molecules [46]. Alternatively, as suggested by a recent study of EGFP-tagged HAS2 and HAS3 expressed in keratinocytes [47], the perinuclear FLAG-HAS3 protein might reflect a reservoir of latent enzyme maintained in the endoplasmic reticulum and/or the Golgi complex. There was some cell-surface expression of active FLAG-HAS3, since, in contrast with untransfected COS-7 cells, the transfected cells formed an HA coat detectable by a particle exclusion assay (results not shown). If the intracellular pool of FLAG-HAS3 is sequestered from the phosphorylation machinery, the observed stoichiometry of FLAG-HAS3 in CPT-cAMP-treated cells might reflect complete – or even multi-site – phosphorylation of the active recombinant FLAG-HAS3 protein at the cell surface. Future

studies will address the phosphorylation status of the intracellular HAS.

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