Ca²⁺/calmodulin-dependent protein kinase mediates the phosphorylation of CD44 required for cell migration on hyaluronan

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CD44 is the principal cell surface receptor for the extracellular matrix glycosaminoglycan hyaluronan, and binding to this ligand underlies CD44-mediated cell attachment and migration. As would be expected for a widely expressed adhesion receptor, CD44 is subject to complex regulatory events, and mis-regulation of the receptor has been associated with a number of disease pathologies, including chronic inflammatory conditions and the progression of metastatic tumours. In previous studies we have demonstrated that a key control point for this receptor is the phosphorylation of CD44 on a conserved cytoplasmic serine residue, Ser³²⁵. This modification is not required for efficient ligand binding, but is an essential component of CD44-dependent

cell migration on a hyaluronan substratum. To understand better the mechanism regulating CD44 phosphorylation on Ser³²⁵, we have generated a monoclonal antibody that specifically recognizes CD44 phosphorylated on Ser³²⁵, and have developed assays to identify the Ser³²⁵ kinase. We demonstrate here that CD44 is phosphorylated to high stoichiometry in resting cells and that Ca²⁺/calmodulin-dependent protein kinase II is a CD44 Ser³²⁵ kinase.

Key words: adhesion, extracellular matrix, glycosaminoglycan, receptor.

INTRODUCTION

CD44 is an abundant cell surface receptor that can mediate both cell–cell and cell–matrix interactions via binding to its extracellular matrix glycosaminoglycan ligand, hyaluronan. As with the majority of adhesion receptors, these interactions are required for a variety of normal physiological processes, but mis-regulation can be contributory to a number of disease pathologies (for reviews see [1–3]). CD44 is subject to strict control mechanisms, and studies from different laboratories have demonstrated that receptor activity can be regulated by changes in glycosylation and the insertion of different combinations of 10 variant exons into the extracellular domain [1,2]. In addition, CD44 function can be modulated by intracellular events, and these require the presence of the receptor cytoplasmic domain. For example, deletion of this domain results in a receptor which is expressed at the cell surface, but which is incorrectly localized, is deficient in its ability to bind hyaluronan, cannot support hyaluronan-dependent cell migration and has an impaired ability to support tumour formation *in vivo* [4–9]. This 72-amino-acid cytoplasmic domain shows strong conservation between species [10], and molecular dissection has revealed that it contains spatially discrete motifs within it. In a juxtamembrane position the sequence Arg²⁹²–Lys³⁰⁰ (Figure 1A) contains two clusters of basic amino acids that mediate the interaction between CD44 and the ERM (ezrin/radixin/moesin) membrane–cytoskeletal linker proteins [11,12]. In addition, an ankyrin-binding region has been identified at Asn³⁰⁴–Leu³¹⁸ [7], and the dihydrophobic motif Leu³³¹–Val³³² has been shown to be required for the correct trafficking of CD44 in polarized epithelial cells [13].

In order to understand how CD44 function may be modulated by intracellular events, we have investigated the role of receptor

phosphorylation. In all cells examined, CD44 is found to be constitutively phosphorylated [14–16], with phosphorylation being restricted to serine residues [17]. Initial mutagenesis studies demonstrated that, of the seven serine residues within the cytoplasmic domain, substitution of either Ser³²³ or Ser³²⁵ with neutral amino acids resulted in the abolition of CD44 phosphorylation [17,18]. However, further analyses in which both of these residues were substituted with threonines demonstrated that Ser³²⁵ is the principal site of constitutive CD44 phosphorylation, and that Ser³²³ is required for kinase binding, but is not a kinase substrate [19]. Expression of Ser³²⁵ mutants in cultured cells has demonstrated that phosphorylation at this site is not required for efficient hyaluronan binding; however, it is essential for CD44-dependent migration on a hyaluronan substratum [9,20]. Moreover, the hyaluronan-dependent migration of cells expressing wild-type (WT) CD44 can be blocked using a CD44 peptide phosphorylated on Ser³²⁵ [19]. Together, these data establish a role for CD44 phosphorylation in modulating receptor function. In addition, they suggest that CD44-dependent migration requires a specific interaction of intracellular component(s) with Ser³²⁵-phosphorylated receptor, and that phosphorylation of Ser³²⁵ must be subject to regulatory mechanisms.

To unravel these regulatory mechanisms, it is necessary to identify the Ser³²⁵ kinase and to develop reagents that will allow the quantification and monitoring of this phosphorylation event. Examination of the sequence surrounding a phosphorylation residue and comparison with known kinase consensus sites can provide clues to the identity of the kinase [21]. However, in the case of CD44, the sequence surrounding Ser³²⁵ does not bear any strong similarity to known kinase motifs. To address these issues, we have taken the approach of generating a monoclonal antibody

Abbreviations used: CaMKII, Ca²⁺/calmodulin-dependent protein kinase II; FCS, fetal calf serum; GST, glutathione S-transferase; HRP, horseradish peroxidase; mAb, monoclonal antibody; WT, wild type.

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Figure 1 CD44 is constitutively phosphorylated on Ser³²⁵

(A) Amino acid sequence of the human CD44 cytoplasmic domain. Ser³²⁵ is indicated in bold, and the underlined sequence indicates the peptide used to generate the Ser³²⁵-phosphorylation-specific mAb. The non-phosphorylated (NP) and Ser³²⁵-phosphorylated (SP) Penetratin peptide sequences are indicated below, with the Penetratin sequence in italics. (B) RPM-MC cells expressing WT CD44 or CD44(Thr³²⁵) were lysed and immunoprecipitated with either the anti-CD44 mAb E1/2 or the CD44 Ser³²⁵-phosphorylation-specific mAb 14E4. Immunoprecipitates were resolved by SDS/10%-PAGE and subjected to immunoblotting with mAb IM7 followed by HRP-conjugated anti-(rat Ig). The blots were developed with ECL reagent and exposed to X-ray film for 30 s. Molecular mass markers are in kDa.

(mAb) that specifically recognizes CD44 phosphorylated on Ser³²⁵, but not the non-phosphorylated receptor. Together with the establishment of suitable kinase substrates, we demonstrate that CD44 is phosphorylated to high stoichiometry, and have identified Ca²⁺/calmodulin-dependent protein kinase II (CaMKII) as a Ser³²⁵ kinase.

EXPERIMENTAL

Cells and antibodies

All cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) fetal calf serum (FCS). E1/2 [14] and IM7 [4] are mouse and rat mAbs respectively that recognize extracellular epitopes in human CD44. An antibody against glutathione S-transferase (GST) was purchased from Pharmacia, a mouse mAb specific for the α -subunit of CaMKII was purchased from Zymed (#13-7300), and horseradish peroxidase (HRP)-conjugated second-layer antibodies were purchased from Jackson ImmunoResearch. The generation of mAb 14E4, which specifically recognizes CD44 phosphorylated at Ser³²⁵, will be described in detail elsewhere (C. A. Lewis, J. W. Legg and C. M. Isacke, unpublished work; details available on application to the authors). Briefly, a peptide corresponding to Leu³¹⁸–His³³⁰ of the CD44 cytoplasmic domain containing a phosphoserine at Ser³²⁵ (Figure 1A) was conjugated via a C-terminal cysteine residue to keyhole-limpet haemocyanin. Spleens from BALB/c mice immunized with the CD44–(keyhole-limpet haemocyanin) peptide were fused with NSO1 myeloma cells, and hybridoma 14E4 was selected for its ability to recognize a CD44 peptide phosphorylated at Ser³²⁵, but not the non-phosphorylated peptide or non-CD44 phosphoserine-containing peptides.

Immunoblotting

Immunoblot analysis was as described previously [6]. Briefly, cells were lysed in SDS/PAGE sample buffer and sonicated, and equal amounts were loaded on to parallel gels for blotting with either mAb E1/2 or mAb 14E4. Chlorpromazine and verapamil (Sigma) were made up as stock solutions of 100 mM in water, KN-93 and KN-92 (Calbiochem) were made as stock solutions of 10 mM in water, and KN-62 (Sigma) was made up as a stock solution of 10 mM in DMSO. As indicated, blots were scanned and quantified using MacBas software.

Generation and expression of constructs

The pJPA7 plasmid containing the H282R activated CaMKII construct [22,23] was kindly donated by Dr Thomas Soderling. Bacterial and mammalian GST fusion proteins were generated using the pGEX-KG and pEBG-3X vectors respectively. The CD44 cytoplasmic domain (amino acids 290–361; Figure 1A) was PCR-amplified from pSR α constructs containing full-length WT CD44 or Ala³²⁵ or Thr³²⁵ mutant CD44 [17,19] using forward (5' AACAGTCGAAGAAGGTGTGG 3') and reverse (5' GTCCAGCTCCCTGTAATGG 3') oligonucleotides. Amplified products were blunt-end-ligated into *Eco*RI-digested pGEX-KG vector or *Not*I-digested pEBG-3X vector that had been end-filled using Klenow polymerase. All plasmids were sequenced to confirm the presence of the desired mutation and that the insertion was in-frame. pGEX-KG constructs were transfected into BL21(DE3)pLysS bacteria, and 100 ml overnight cultures were induced with 0.4 mM isopropyl β -D-thiogalactoside for 1 h at 37 °C. Bacteria were resuspended in 5 ml of lysis buffer (150 mM EDTA, 1 μ g/ml antipain, 1 μ g/ml aprotinin, 1 μ g/ml

leupeptin, 1 mM PMSF and 1 mg/ml lysozyme, pH 8.0) and sonicated for 45 s. Triton X-100 was then added to 1% (v/v), and the lysate was incubated on ice for 15 min and then subjected to centrifugation at 12000 *g* for 10 min. To extract the fusion proteins, glutathione-agarose beads (Sigma) were incubated with bacterial lysate for 1 h at 4 °C and then washed five times in lysis buffer containing 1% Triton X-100. Mammalian expression vectors were transiently transfected into COS cells by electroporation as described previously [17].

Penetratin peptides

N-terminal biotinylated and non-biotinylated peptides comprising the 16-amino-acid Penetratin sequence (RQIKIWFQNRR-MKWKK) followed by amino acids 318–330 from the CD44 cytoplasmic domain (LNGEASKSQEMVH; Figure 1A) with a serine or phosphoserine present at position 325 (underlined) have been described previously [19].

In vivo phosphorylation assays

For ³²P labelling, cells were cultured overnight in phosphate-free Dulbecco's modified Eagle's medium containing 1% (v/v) FCS and 1 mCi/ml [³²P]P_i. ³²P-labelled cells were lysed in Triton X-100 lysis buffer (15 mM Tris, pH 7.4, 150 mM NaCl, 0.5% Triton X-100, 0.2 mM sodium orthovanadate, 25 mM NaF, 10 mM sodium pyrophosphate, 1 µg/ml aprotinin, 1 µg/ml antipain, 1 µg/ml leupeptin and 1 mM PMSF), clarified by centrifugation (13000 *g* for 15 min) and then incubated with mAb E1/2 or mAb 14E4 bound to Protein G-agarose or with glutathione-agarose for 1 h at 4 °C. Beads were washed in RIPA buffer (Triton X-100 lysis buffer containing 1% sodium deoxycholate, 0.3% SDS and 2 mM EDTA). Bound proteins were then eluted by heating at 90 °C for 3 min with sample buffer and resolved by SDS/10%-PAGE. Gels were dried and exposed to X-ray film at –70 °C with an intensifying screen.

For the *in vivo* phosphorylation of biotinylated Penetratin peptides, ³²P-labelled COS cells were incubated with a concentration of 100 µg/ml of the biotinylated form of either non-phosphorylated or Ser³²⁵-phosphorylated Penetratin-CD44 peptide (Figure 1A) at 37 °C for 4 h. The cells were lysed as described above and incubated for 1 h at 4 °C with 50 µl of streptavidin-agarose (Sigma). Beads were washed extensively in RIPA buffer, added to 3 ml of Ecosint H (National Diagnostics) and counted for radioactivity in a 1214 RackBeta liquid scintillation counter (LKB Wallac).

CaMKII assays

An aliquot of 0.5 µl of CaMKII (500000 units/ml; New England Biolabs; #6060) in 30 µl of buffer containing 20 mM Tris, pH 7.5, 10 mM MgCl₂, 0.5 mM dithiothreitol, 0.1 mM EDTA, 2 mM CaCl₂, 24 µM calmodulin, 100 µM ATP and 2 µCi of [³²P]ATP was mixed with either GST-(CD44 cytoplasmic domain) fusion proteins bound to glutathione-agarose beads or with 20 µg of Penetratin-CD44 peptides and incubated at 30 °C for 30 min. Glutathione-agarose beads were washed and fusion proteins were resolved by SDS/12%-PAGE as described above. For analysis of Penetratin peptide phosphorylation, 10 µl of the reaction mixture was dried on to P81 paper (Whatmann). The paper was washed extensively in 75 mM phosphoric acid, added to 3 ml of Ecosint and subjected to scintillation counting as described above.

RESULTS AND DISCUSSION

CD44 is constitutively phosphorylated to high stoichiometry at Ser³²⁵

Mutagenesis studies have identified Ser³²⁵ as the principal site of CD44 phosphorylation [17–19]. In order to confirm by independent means that Ser³²⁵ represents the true *in vivo* phosphorylation site and to investigate the regulation of this phosphorylation, mice were immunized with a CD44 Ser³²⁵ phosphopeptide and the resulting hybridomas were screened for their ability to bind to this peptide, but not to an equivalent non-phosphorylated peptide or to non-CD44 phosphoserine-containing peptides (see the Experimental section). Further analysis of CD44 expressed in mammalian cells demonstrated that mAb 14E4 specifically recognized CD44 phosphorylated on Ser³²⁵ and showed no reactivity against non-Ser³²⁵-phosphorylated CD44 (C. A. Lewis, J. W. Legg and C. M. Isacke, unpublished work). This specificity is illustrated in Figure 1(B), where cells expressing

Figure 2 CD44 is phosphorylated on Ser³²⁵ to high stoichiometry

(A) Flow2000 and HT1080 cells were labelled overnight with [³²P]P_i and then immunoprecipitated serially with two rounds of mAb 14E4 [14E4(1) and 14E4(2)] followed by two rounds of mAb E1/2 [E1/2(1) and E1/2(2)]. Immunoprecipitates were resolved by SDS/10%-PAGE and gels were exposed to X-ray film overnight. (B) Immunoprecipitates from parallel dishes of unlabelled cells were immunoblotted with mAb IM7 followed by HRP-conjugated anti-(rat Ig), and the blots were developed with ECL reagent and exposed to X-ray film for 30 s. The proportion of CD44 immunoprecipitated with mAb 14E4 compared with the amount of total receptor (CD44 immunoprecipitated with mAb 14E4 plus CD44 immunoprecipitated with mAb E1/2) was assessed. Molecular mass markers are in kDa. Equivalent results were obtained in three separate experiments.

WT CD44 or a phosphorylation-incompetent form of CD44 {CD44(Thr³²⁵), in which Ser³²⁵ had been mutated to a threonine residue [19]} were immunoprecipitated with either mAb E1/2 (which recognizes an extracellular CD44 epitope) or mAb 14E4. It can be seen that mAb E1/2 efficiently immunoprecipitated both WT CD44 and CD44(Thr³²⁵), whereas mAb 14E4 recognized WT CD44, but not the phosphorylation-incompetent CD44(Thr³²⁵) (Figure 1B).

Phosphorylation-specific mAbs provide a powerful tool for the analysis of kinase activity *in vivo*, as they allow a direct assessment of phosphorylation at an individual site and quantification of this event. To investigate the stoichiometry of Ser³²⁵ phosphorylation, [³²P]_i-labelled Flow2000 human fibroblasts or HT1080 human fibrosarcoma cells were subjected to serial immunoprecipitation with two rounds of mAb 14E4 followed by two rounds of mAb E1/2 (Figure 2A). We found that > 90 % of the phosphorylated CD44 was immunoprecipitated by mAb 14E4 in the first round, with only negligible amounts of phosphorylated CD44 being extracted in the subsequent mAb 14E4 immunoprecipitation. Immunoprecipitation of the mAb-14E4-depleted lysate with the pan-anti-CD44 mAb E1/2 resulted in the isolation of only minor amounts of phosphorylated CD44, thus providing independent evidence that Ser³²⁵ is indeed the principal site of constitutive CD44 phosphorylation *in vivo*. Parallel immunoprecipitations from unlabelled cell lysates were immunoblotted with the cross-species-reactive pan-CD44 mAb IM7. As shown in Figure 2(B), 40–50 % of the Flow2000 and 25–35 % of the HT1080 CD44 is phosphorylated at Ser³²⁵. Immunofluorescence analysis of these cells with mAb 14E4 suggests that these values represent a balance of phosphorylated and non-phosphorylated CD44 within individual cells, rather than reflecting heterogeneity between cells (results not shown). As discussed below, these findings have implications for the mechanism by which CD44 phosphorylation might regulate cell migration.

CD44 substrates are constitutively phosphorylated at Ser³²⁵

Having established that Ser³²⁵ is the true constitutive site of CD44 phosphorylation, and that this phosphorylation occurs at high stoichiometry, a series of experiments was undertaken to identify the CD44 Ser³²⁵ kinase. Two types of CD44 substrate were tested in initial experiments: GST–(CD44 cytoplasmic domain) fusion proteins and Penetratin–CD44 peptides. There are numerous examples where substrate proteins can be phosphorylated by purified kinases *in vitro* but do not appear to act as substrates *in vivo*. Consequently it is important to develop assays that allow an unambiguous demonstration that the amino acid(s) phosphorylated are the same *in vitro* and *in vivo*. The latter is of particular importance for molecules such as CD44, in which the substrate amino acid (Ser³²⁵) lies in close proximity to other potential kinase targets (Ser³²³). GST–(CD44 cytoplasmic domain) (amino acids 290–361; Figure 1A) constructs were generated in the pEBG-3X vector to allow expression of the fusion proteins in mammalian cells. COS cells transfected with full-length WT CD44 or with the pEBG-3X constructs were labelled overnight with [³²P]_i, then WT CD44 was immunoprecipitated with the pan-CD44 mAb E1/2 and the GST proteins were purified on to glutathione–agarose. Full-length WT receptor and fusion proteins containing WT CD44 cytoplasmic domain (GST–CD44) were both shown to be highly phosphorylated. By contrast, there was negligible labelling of either the GST protein alone or of fusion proteins in which Ser³²⁵ had been mutated to alanine [GST–CD44(Ala³²⁵)] or threonine [GST–CD44(Thr³²⁵)]. This lack of phosphorylation did not result from impaired

Figure 3 GST–CD44 fusion proteins are phosphorylated on Ser³²⁵

(A) COS cells transiently transfected with pEBG-3X alone, pEBG-3X containing WT CD44, CD44(Ala³²⁵) or CD44(Thr³²⁵) cytoplasmic tails, or pSRα containing full-length WT CD44 were labelled overnight with [³²P]_i. The GST fusion proteins were purified from the cell lysates on to glutathione–agarose beads and the WT CD44 was immunoprecipitated from the cell lysates with mAb E1/2. The purified proteins were then resolved by SDS/12%–PAGE, and the gels were exposed to X-ray film overnight. In parallel, GST fusion proteins were purified from unlabelled cell lysates and levels of expression were assessed by immunoblotting with anti-GST antiserum followed by HRP-conjugated anti-(goat Ig). The blots were developed using ECL reagent and exposed to X-ray film for 30 s. Molecular mass markers are in kDa. Equivalent results were obtained in three separate experiments.

expression, as parallel immunoblots demonstrated equivalent levels of GST protein expression (Figure 3). These results demonstrate that a GST–CD44 fusion protein, like full-length CD44 [17,19], is phosphorylated principally at Ser³²⁵, and that the kinase responsible cannot tolerate a threonine substitution at this position. Thus, although the GST fusion proteins are soluble in the cytoplasm, their phosphorylation profile is the same as that of the transmembrane receptor.

To investigate further the requirement for substrate localization within the cell and to determine whether discrete peptides could be phosphorylated *in vivo*, use was made of the Penetratin system. It had been demonstrated previously that Leu³¹⁸–His³³⁰ CD44 peptides linked to the 16-amino-acid Antennapedia homeodomain peptide (Penetratin) (see [24] for a review) could be internalized efficiently by cultured cells [19]. COS cells labelled overnight with [³²P]_i were incubated for 4 h with no peptide, non-phosphorylated Penetratin–CD44 peptide or Penetratin–CD44 peptide containing a phosphoserine at amino acid 325 (Figure 1A). The presence of a biotin group at the N-terminus allowed staining of the cells with streptavidin/rhodamine to confirm the efficiency of internalization (results not shown) and isolation of the peptides on streptavidin–agarose. When streptavidin–agarose beads were washed and subjected to scintillation counting, the following values were obtained: no peptide added, $(120 \pm 5) \times 10^3$ c.p.m.; Ser³²⁵-phosphorylated peptide, $(98 \pm 0.4) \times 10^3$ c.p.m.; non-phosphorylated peptide, $(340 \pm 45) \times 10^3$ c.p.m. (values represent mean $\pm$ S.E.M. of at least three determinations). These results demonstrate that the non-phosphorylated peptide could be phosphorylated during the course of the incubation, indicating that the Penetratin–CD44 peptides are suitable kinase assay substrates and that non-membrane-associated substrates can be phosphorylated *in vivo*. In addition, as peptides can be synthesized with phosphorylated residues, questions concerning secondary phosphorylation events can be addressed. There are many cases where substrate phosphorylation by one kinase is a prerequisite for subsequent phosphorylation by a second kinase(s) at neigh-

Figure 4 *In vitro* phosphorylation of CD44 on Ser³²⁵ by CaMKII

GST-CD44 fusion proteins bound to glutathione-agarose were incubated with 30 μL of CaMKII kinase buffer supplemented with 2 mM CaCl_2 , 2.4 μM calmodulin, 250 units of CaMKII and 2 μCi of [γ -³²P]ATP at 30 °C for 30 min. The beads were then washed extensively, the fusion proteins were eluted in sample buffer and resolved by SDS/12%-PAGE, and the gels were exposed to X-ray film overnight. The amount of fusion protein in the assay was assessed by immunoblotting of parallel samples with an anti-GST antiserum followed by HRP-conjugated anti-goat Ig. The blots were developed with ECL and exposed to X-ray film for 10 s. Molecular mass markers are in kDa. Equivalent results were obtained in three separate experiments.

bouring residues. Ser³²⁵ lies in close proximity to Ser³²³, and it has not been possible to date to discount the possibility that there is phosphorylation at Ser³²³, but only subsequent to Ser³²⁵ phosphorylation; if this were the case, mutation of Ser³²⁵ would result in the loss of phosphorylation at both sites. It is demonstrated here that Penetratin-CD44 peptides containing a phosphorylated serine at position 325 showed no incorporation of ³²P when introduced into cells. This strongly suggests that Ser³²³ is not phosphorylated as a consequence of Ser³²⁵ phosphorylation, and confirms Ser³²⁵ as the principal site of constitutive CD44 phosphorylation.

Ser³²⁵ of CD44 is a substrate for CaMKII

As stated above, the surrounding amino acid sequence provides few clues as to the identity of the Ser³²⁵ kinase. Therefore, using the two types of CD44 substrate validated as described above and the phosphorylation-specific 14E4 antibody, a number of known serine/threonine kinases were tested for their ability to phosphorylate CD44 on Ser³²⁵. Initial candidates were protein kinase C, casein kinase II and CaMKII, as these kinases are known to be both multifunctional and ubiquitous in their tissue distribution. No phosphorylation of Ser³²⁵ was detected with either protein kinase C or casein kinase II, although effective phosphorylation of control substrates was observed (results not shown). In contrast, *in vitro* assays with CaMKII using the Penetratin-CD44 peptides as substrates gave the following results: samples with no peptide added, $(19932 \pm 996) \times 10^3$ c.p.m. (presumably reflecting autophosphorylation of CaMKII); samples containing non-phosphorylated peptide, $(113679 \pm 10188) \times 10^3$ c.p.m.; samples containing Ser³²⁵-phosphorylated peptide, $(27606 \pm 4428) \times 10^3$ c.p.m. (values represent means $\pm$ S.E.M. of at least three determinations). Equivalent results were obtained in three separate experiments. These data indicated that CaMKII could act as a CD44 Ser³²⁵ kinase. To investigate this further, bacterially expressed GST fusion proteins were subjected to phosphorylation by CaMKII *in vitro*. The WT CD44 cytoplasmic domain expressed as a GST fusion protein in bacteria underwent a high level of CaMKII-mediated

Chlorpromazine

Verapamil

Figure 5 Phosphorylation of CD44 on Ser³²⁵ *in vivo* is inhibited by calmodulin and calcium antagonists

Flow2000 fibroblasts cultured in 24-well plates at 37 °C for 48 h were stimulated for 0–60 min with either 50 μM chlorpromazine or 50 μM verapamil. Cell lysates were resolved by SDS/10%-PAGE and immunoblotted with either mAb 14E4 or mAb E1/2 followed by HRP-conjugated anti-mouse Ig. The blots were developed with ECL reagent and exposed to X-ray film for 30 s. Molecular mass markers are in kDa. Equivalent results were obtained in three separate experiments.

phosphorylation, whereas GST alone or GST-CD44(Ala³²⁵) remain unphosphorylated (Figure 4). Further evidence that CaMKII may function as a true CD44 Ser³²⁵ kinase came from experiments with the CD44(Thr³²⁵) mutant. It was originally expected that substitution of Ser³²⁵ with a threonine would result in CD44 phosphorylation on Thr³²⁵, as most serine/threonine kinases, including CaMKII [25], can modify both amino acids. However, in [³²P]P_i labelling experiments it was demonstrated that Thr³²⁵ of the mutant CD44 is not phosphorylated *in vivo* (Figure 3 and [19]). Consequently it is a criterion that a candidate CD44 Ser³²⁵ kinase cannot tolerate a threonine substitution in this position. In accordance with this, it was demonstrated that a GST-CD44 fusion protein containing a Thr³²⁵ substitution could not act as a substrate for CaMKII (Figure 4).

CD44 is phosphorylated by CaMKII *in vivo*

To investigate whether CD44 is subject to phosphorylation by CaMKII *in vivo*, a number of experiments were undertaken. First, cells were treated with the calmodulin antagonist chlorpromazine or the calcium antagonist verapamil (Figure 5). Immunoblotting of the samples with mAb E1/2 demonstrated that these treatments resulted in small decreases in total CD44 levels (13.7% and 8.9% with chlorpromazine and verapamil respectively). In contrast, immunoblotting either sample with mAb 14E4 revealed a significant decrease in CD44 phosphorylation on Ser³²⁵ (78% and 63% decreases with chlorpromazine and verapamil respectively). Although it would be expected that calcium and calmodulin antagonists would reduce CaMKII

Figure 6 The CaMKII inhibitors KN-93 and KN-62 inhibit phosphorylation of CD44 on Ser³²⁵

F1084 (**A**) or Flow2000 (**B**) cells were cultured for 24 h and then incubated with 50 μ M KN-93 (CaMKII inhibitor) or 50 μ M KN-92 (inactive analogue). After 16 h, cell lysates were resolved by SDS/10%-PAGE and immunoblotted with mAb 14E4 or mAb E1/2 followed by HRP-conjugated anti-(mouse Ig). Blots were developed with ECL reagent and exposed to X-ray film for 20 s. Molecular mass markers are in kDa. Equivalent results were obtained in four separate experiments. (**C**) Flow2000 cells were cultured for 48 h and then treated with 10 μ M KN-62 for 0–60 min prior to lysis and blotting as described above. Equivalent results were obtained in two separate experiments.

activity, many other cellular kinases are known to be dependent on these components. To examine more directly the role of CaMKII, cells were initially treated with the cell-permeable CaMKII inhibitor KN-93, or with an inactive analogue, KN-92. Blotting of cell lysates with mAb 14E4 demonstrated that, in the presence of KN-93, there was a significant decrease in Ser³²⁵ phosphorylation (55.4% and 46.6% decreases in F0184 and Flow2000 cells respectively; Figures 6A and 6B). To gain further information with regard to the time course of this inhibition and to assess further the inhibitor specificity, cells were treated for up to 1 h with the cell-permeable CaMKII inhibitor KN-62. Again, in the presence of the CaMKII inhibitor, there was a significant decrease in Ser³²⁵ phosphorylation (50.4% decrease at 30 min; Figure 6C).

As an alternative approach to examining the potential of CaMKII as a CD44 Ser³²⁵ kinase, COS cells were transfected with WT CD44 with or without a constitutively active form of

Figure 7 Phosphorylation of CD44 Ser³²⁵ *in vivo* by activated CaMKII

COS cells were transiently transfected with WT CD44, WT CD44 plus activated CaMKII (aCaMKII) or vector alone. After 24 h cell lysates were resolved by SDS/10%-PAGE and immunoblotted with mAb E1/2, anti-CaMKII or mAb 14E4, followed by HRP-conjugated anti-(mouse Ig). Blots were developed with ECL reagent and exposed to X-ray film for 30 s. Molecular mass markers are in kDa. Equivalent results were obtained in two separate experiments.

CaMKII containing a His²⁸² $\rightarrow$ Arg²⁸² mutation [22,23]. His²⁸² lies in the CaMKII autoinhibitory domain [26,27], which occupies the ATP-binding motif in the catalytic domain, causing inactivation of the kinase. Substitution of His²⁸² with an arginine residue prevents this interaction and allows significant kinase activity in the absence of Ca²⁺/calmodulin. Phosphorylation of Ser³²⁵ of the transfected WT CD44, as monitored by mAb 14E4 reactivity, was shown to increase 2.8-fold in the presence of activated CaMKII (Figure 7).

Together, these data indicate that CaMKII has a role in the *in vivo* phosphorylation of CD44 at Ser³²⁵. However, it should be noted that neither the calcium/calmodulin antagonists nor the CaMKII inhibitors lowered CD44 Ser³²⁵ phosphorylation to background levels. Although it is frequently difficult to obtain complete inhibition of kinases in live cells, the possibility that another kinase(s) could target this site cannot be discounted.

CaMKII is expressed in most tissues, and due to its ability to phosphorylate a wide variety of substrates is often referred to as a multifunctional kinase [27]. This multisubstrate, multifunctional role includes its activities in fibroblasts. For example, in these cells CaMKII has been shown to phosphorylate the epidermal growth factor receptor [28], to regulate the exchange factor activity of Tiam1 [29] and to control the affinity state of the α 5 β 1 integrin [30]. In this respect, CaMKII differs from many members of the Ca²⁺/calmodulin-dependent protein kinase family, such as myosin light chain kinase and CaMKIII, which exclusively phosphorylate one target substrate. Early studies defined a CaMKII minimal recognition motif of Arg-Xaa-Xaa-Ser(Thr) [31], which has been refined recently to give the preferential substrate sequence of Hyd-Xaa-Arg-NB-Xaa-Ser(Thr), where Hyd indicates a hydrophobic amino acid and NB indicates a non-basic residue [25]. The amino acid sequence upstream of CD44 Ser³²⁵ does not conform to this consensus, but CD44 is not unique in this respect. A variety of CaMKII substrates, including cAMP response element-binding protein (CREB), vimentin and synapsin I, have been identified that lack the arginine residue in the –3 position and/or the hydrophobic residue in the –5 position [25]. What remains to be determined

is whether the non-optimal recognition sequence in CD44 reflects a non-optimal rate of CaMKII-mediated phosphorylation and/or whether the secondary structure of CD44 has a role in enhancing substrate ability.

The identification of CaMKII as a CD44 Ser³²⁵ kinase raises the question of how phosphorylation of CD44 by CaMKII is regulated. It is known that a large number of different signals can stimulate the levels of intracellular Ca^{2+} by increasing the influx of Ca^{2+} and/or the phosphoinositide-mediated release of Ca^{2+} from intracellular stores. Once elevated, this ubiquitous second messenger can bind calmodulin, leading to the activation of Ca^{2+} /calmodulin effectors such as CaMKII. With respect to CD44, it has been reported that both antibody-mediated receptor ligation [32] and hyaluronan binding [33] can result in elevated intracellular Ca^{2+} levels. It is anticipated that one consequence of this would be enhanced CaMKII-mediated CD44 Ser³²⁵ phosphorylation, thereby providing a potential link between ligand binding and cell migration. However, it is likely that the control of Ser³²⁵ phosphorylation is more complex. We demonstrate here that a substantial proportion of CD44 is phosphorylated on Ser³²⁵ in fibroblasts cultured for 24–48 h in 10% FCS, which raises the question of whether CaMKII is constitutively active in these cells. Two potential mechanisms may exist to maintain CaMKII in an active state. First, FCS will result in elevated intracellular Ca^{2+} levels and subsequent kinase activation. The latter is accompanied by autophosphorylation, which increases by several hundredfold the affinity of the kinase for calmodulin by reducing the rate of dissociation of the kinase–calmodulin complex. This effectively results in ‘trapping’ of the calmodulin [34], so that even when Ca^{2+} levels are reduced the kinase may remain active. Secondly, it is possible that, in these cells, reduced CaMKII activation is balanced by reduced phosphatase activity, which would result in the maintenance of phosphorylated, and hence active, CaMKII. However, it is important to note that, as our assays used a phosphorylation-specific mAb to monitor CD44 phosphorylation directly, the level of phosphorylation observed in fibroblast cultures could result from the inactivity of an as yet unidentified Ser³²⁵ phosphatase rather than, or in combination with, the sustained activity of CaMKII. Certainly the future challenge will be to understand how this balance between Ser³²⁵ phosphorylation and dephosphorylation is controlled and the mechanism by which phosphorylation/dephosphorylation of CD44 Ser³²⁵ regulates cell migration.

We thank Dr Thomas Soderling (Vollum Institute, Oregon Health Science University, Portland, OR, U.S.A.) for the H282R activated CaMKII construct, Dr Sibylle Mitnacht (Institute of Cancer Research, London, U.K.) for advice on the generation of the phosphorylation-specific mAb, and members of our laboratory for their help during this project. C.A.L. was funded by a Cancer Research Campaign studentship. This work was supported by a grant from the Cancer Research Campaign.

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Received 26 February 2001/23 April 2001; accepted 30 May 2001