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CD34 antigen: Determination of specific sites of phosphorylation *in vitro* and *in vivo*

Leesa J. Deterding[a,*], Jason G. Williams[a], Margaret M. Humble[b], Robert M. Petrovich[a], Sung-Jen Wei[b,1], Carol S. Trempus[b], Matthew B. Gates[a], Feng Zhu[c,2], Robert C. Smart[c], Raymond W. Tennant[b], Kenneth B. Tomer[a]

[a] *Laboratory of Structural Biology, National Institute of Environmental Health Sciences, National Institutes of Health, PO Box 12233, Research Triangle Park, NC 27709, United States*
[b] *Laboratory of Pharmacology and Toxicology, National Institute of Environmental Health Sciences, National Institutes of Health, PO Box 12233, Research Triangle Park, NC 27709, United States*
[c] *Cell Signaling and Cancer Group, Department of Environmental and Molecular Toxicology, North Carolina State University, Box 7633, Raleigh, NC 27695, United States*

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ABSTRACT

CD34, a type I transmembrane glycoprotein, is a surface antigen which is expressed on several cell types, including hematopoietic progenitors, endothelial cells, as well as mast cells. Recently, CD34 has been described as a marker for epidermal stem cells in mouse hair follicles, and is expressed in outer root sheath cells of the human hair follicle. Although the biological function and regulation of CD34 is not well understood, it is thought to be involved in cell adhesion as well as possibly having a role in signal transduction. In addition, CD34 was shown to be critical for skin tumor development in mice, although the exact mechanism remains unknown.

Many proteins' functions and biological activities are regulated through post-translational modifications. The extracellular domain of CD34 is heavily glycosylated but the role of these glycans in CD34 function is unknown. Additionally, two sites of tyrosine phosphorylation have been reported on human CD34 and it is known that CD34 is phosphorylated, at least in part, by protein kinase C; however, the precise location of the sites of phosphorylation has not been reported. In an effort to identify specific phosphorylation sites in CD34 and delineate the possible role of protein kinase C, we undertook the identification of the *in vitro* sites of phosphorylation on the intracellular domain of mouse CD34 (aa 309–382) following PKC treatment. For this work, we are using a combination of enzymatic proteolysis and peptide sequencing by mass spectrometry. After which the *in vivo* sites of phosphorylation of full-length mouse CD34 expressed from HEK293F cells were determined. The observed *in vivo* sites of phosphorylation, however, are not consensus PKC sites, but our data indicate that one of these sites may possibly be phosphorylated by AKT2. These results suggest that other kinases, as well as PKC, may have important signaling functions in CD34.

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1. Introduction

CD34, a type I transmembrane glycoprotein, is a surface antigen which is expressed on several cell types, including hematopoietic progenitors, endothelial cells [1], and mast cells [2]. Recently, CD34 has been described as a marker for epidermal stem cells in mouse hair follicles [3,4], and is expressed in outer root sheath cells of the human hair follicle [5]. Although the biological function and regulation of CD34 is not well understood, it is thought to be involved in cell adhesion, depending upon the cellular context. For example, CD34 acts as an adhesion molecule through interaction with L-selectin, facilitating homing of lymphocytes to peripheral lymph nodes [6]. Conversely, CD34 has an anti-adhesion function in mast cells, preventing aggregation by virtue of the surface glycosylation [7]. A potential interaction with the adaptor protein CrkL has also

Abbreviations: PKC, protein kinase C; α-cyano, α-cyano-4-hydroxycinnamic acid; DHB, 2,5-dihydroxybenzoic acid; CRC, compact reaction column; TiO_2, titanium dioxide.

* Corresponding author at: National Institute of Environmental Health Sciences, PO Box 12233, MD F0-03, Research Triangle Park, NC 27709, United States. Tel.: +1 919 541 3009; fax: +1 919 541 0220.
E-mail address: deterdi2@niehs.nih.gov (L.J. Deterding).
[1] Current address: Department of Pharmacology, Medical Research Division, Edinburg-Regional Academic Health Center, UT Health Science Center at San Antonio, 1214W. Schunior St., Edinburg, TX 78541, United States.
[2] Current address: Hormel Institute, University of Minnesota, Austin, MN 55512, United States.

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been described, suggesting a role in signal transduction [8]. Finally, CD34 was shown to be critical for skin tumor development in mice [9], although the exact mechanism remains unknown.

Two forms of CD34 mRNA have been reported and occur as the result of an alternative splicing mechanism [10,11]. One of the full-length mouse forms is composed of 382 amino acids with a 73 amino acid intracellular domain whereas the other form contains a shorter 16 amino acid cytoplasmic domain. The extracellular domain of CD34 is heavily glycosylated but the role of these glycans in CD34 function is unknown [6,12,13]. The cytoplasmic region of CD34 is found to be highly conserved across several species (e.g., >90% amino acid identity between human and mouse). Such a high degree of sequence conservation suggests that this region of the protein may play an important functional role *in vivo*. Many proteins' functions and biological activities are regulated through post-translational modifications. These modifications include phosphorylation, methylation, nitrosylation, ubiquitination, as well as others. For mouse CD34, two sites of tyrosine phosphorylation have been reported: tyrosine-326 [14,15] and tyrosine-336 [15]. In addition, the C-terminal domain of CD34 contains several consensus protein kinase phosphorylation target sites. The mouse intracellular domain has two protein kinase C (PKC) sites [16] based on the consensus sequence S/T-X-R/K [17] at threonine-365 and serine-371. It has been reported previously that CD34 is phosphorylated, at least in part, by PKC [18], however, the precise location of the sites of phosphorylation were not reported. In addition, Fackler et al. [19] reported that when cells were treated with PKC, the expression of CD34 was up regulated.

To identify specific phosphorylation sites in CD34 and delineate the possible role of PKC, we investigated the sites of phosphorylation on the 73 amino acid intracellular domain of mouse CD34 (aa 309–382) following PKC treatment. For this work, we used a combination of enzymatic proteolysis and peptide sequencing by mass spectrometry. Because mass spectrometric-based analyses of phosphopeptides can suffer from suppression effects and/or low ionization efficiency, a metal oxide resin (titanium dioxide) for phosphopeptide enrichment was also employed. Recently, various metal oxides have been shown to be useful for the enrichment of phosphorylated peptides prior to MS analyses [20–24]. By selectively enriching phosphopeptides, the suppression effects mentioned above can be greatly reduced, thereby increasing the probability of detecting the phosphopeptides. Using electrospray ionization mass spectrometry, we show that the intracellular domain of CD34 can be phosphorylated by *in vitro* treatment with PKC. Tandem mass spectrometry was used to identify and sequence the phosphopeptides in order to assign phosphorylation sites to specific amino acids. From these analyses, five different sites of phosphorylation were identified; one of which is a consensus PKC phosphorylation site.

To determine if the sites of phosphorylation identified *in vitro* are consistent with *in vivo* phosphorylation, full-length mouse CD34 (amino acids 1–382) was transiently transfected and expressed in HEK293F cells. The full-length protein was then gel purified, digested, and analyzed by mass spectrometry to determine the *in vivo* sites of phosphorylation. From these analyses, two of the five sites of phosphorylation which were observed in the *in vitro* kinase assay experiments were also observed in the *in vivo* experiments. The observed *in vivo* sites of phosphorylation, however, are not consensus PKC sites. This implies that other kinases, as well as PKC, may have important signaling functions in CD34 *in vivo*. Based on the phosphorylation sites found *in vivo* and the knowledge-base of known consensus phosphorylation sites of kinases, three candidate kinases, AKT2, JNK2, and IKKβ, were employed for further *in vitro* phosphorylation experiments. While there were ambiguities as to the site(s) of phosphorylation for JNK2 and IKKβ, AKT2 was found to phosphorylate CD34 *in vitro* at one of the same phosphorylation sites identified *in vivo*.

2. Experimental

2.1. Materials

Ammonium bicarbonate, 2,5-dihydroxybenzoic acid (DHB), α-cyano-4-hydroxycinnamic acid (α-cyano), and formic acid (FA) were purchased from Sigma–Aldrich Chemical Company (St. Louis, MO). Acetonitrile (ACN) was purchased from Caledon (Georgetown, Ontario, Canada), trifluoroacetic acid (TFA) was acquired from Pierce (Rockford, IL), and phosphoric acid (PA) was from EMD Chemicals (Gibbstown, NJ). All solvents were HPLC grade. Sequencing-grade modified porcine trypsin was purchased from Promega Corporation (Madison, WI). All solutions and buffers were prepared using deionized water (18 MΩ) from an in-house Hydro Services (Durham, NC) Picopure 2 water system. Compact reaction columns (CRC) and filters (10 μm pore size) were obtained from USB Corporation (Cleveland, OH). Titansphere® TiO_2 resin was a gift from GL Sciences (Torrance, CA).

2.2. Cloning and expression of the intracellular domain of mouse CD34

The intracellular domain (amino acids 309–382) of mouse CD34 (AAH06607) was amplified from mouse keratinocytes isolated from CD-1 mice as described previously [3] then expressed using the Gateway system from Invitrogen (Carlsbad, CA). The fragment was PCR cloned in 2 steps. The first round of PCR introduced a stop codon and the attB2 recombination site with the following primers: 5′CTGGTTCCGCGTGGATCCATGAACCGTCGCAGTTGGAGC3′ and 5′GACCCAGCTTTCTTGTACAAAGTGGACCCC3′. The product from the first round of PCR was gel purified and used as a template for the second round. The second round of PCR introduced the attB1 site using the forward primer 5′GGGGACAAGTTTGTACAAAAAAGCAGGCTTCCTGGTTCCGCGTGGATCC3′ and the same reverse primer from round 1. The PCR product from the second round was gel purified and recombined into pDONR221. The gene fragment was then migrated to pDEST17 for expression with an N-terminal His tag. The His-CD34 fragment was expressed using the *Escherichia coli* strain Rosetta(DE3)pLacI (Novagen, EMD Chemicals, Inc., Gibbstown, NJ). The cells were grown in LB media and induced with 0.2 mM IPTG at 30 °C. Four hours after induction the cells were harvested and stored at −80 °C until used.

2.3. Purification of the His-CD34 domain fusion

The cell pellet was suspended in B-PER (Pierce, Rockford, IL) containing EDTA free complete protease inhibitor cocktail (Roche, Indianapolis, IN). After a 10 min incubation at room temperature, the sample was cooled on ice and sonicated 3 times for 30 s using continuous bursts at max power with a Branson Sonifier 450 (Danbury, CT). Insoluble material was removed by centrifugation at 27,000 × *g* for 30 min. The soluble protein was then flow loaded onto a Ni-NTA (QIAGEN, Inc., Valencia, CA) column pre-equilibrated with 50 mM HEPES (pH 7.5), 200 mM NaCl, and 10 mM imidazole. Bound protein was eluted with a linear gradient from 10 mM to 500 mM imidazole. The His-CD34 fusion protein eluted as a single peak centered at ∼175 mM imidazole. The peak fractions were pooled and dialyzed overnight at 4 °C in 20 mM MOPS (pH 7.2) prior to use in the PKC assay to ensure buffer compatibility.

2.4. Protein kinase C assays

In vitro PKC phosphorylation of the purified CD34 fusion protein (20 mM MOPS, pH 7.2) was performed using a PKC Isoform Panel Miniature Set (Millipore/Upstate Biotechnology, Inc., Lake Placid, NY) per the manufacture's recommendations. Briefly, just prior to each assay, the isoforms were diluted to a concentration of 10–20 ng/μl in 20 mM HEPES (pH 7.4), 2 mM EDTA, 5 mM DTT, 100 mM NaCl, 0.05% Triton X-100, and 50% glycerol. Depending upon the isoform, CD34 was diluted in the following buffers (supplied in the kit): 20 mM MOPS (pH 7.2), 25 mM β-glycerophosphate, 1 mM sodium orthovanadate, 1 mM DTT, and 1 mM $CaCl_2$ for PKC α, βI, βII, and γ, and 20 mM MOPS (pH 7.2), 25 mM β-glycerophosphate, 1 mM sodium orthovanadate, and 1 mM DTT for PKC δ, ε, ζ, and η. Each individual kinase reaction consisted of 500 ng CD34 substrate, 25 ng of each PKC isoform, 1 μCi of [γ-^{32}P]-ATP (3000 Ci/mmol) (DuPont-NEN), 5 μg phosphatidylserine, and 0.5 μg diglycerides. The kinase reactions were incubated at 30 °C for 15 min. To verify the specificity of PKC, 1 μM of inhibitor residues 19–31 pseudosubstrate was added 1 h prior to reaction. Following the kinase reactions, SDS-PAGE was performed using 4–12% Bis–Tris NuPAGE SDS gradient gels (Invitrogen, Carlsbad, CA). Following electrophoresis, the gels were stained with Coomassie brilliant blue R250 (CBB) R-250 and subjected to autoradiography using Typhoon 8600 phosphorimager (GE Healthcare Biosciences).

2.5. Other kinase assays

The CD34 fusion protein (0.5 μg) was mixed together with active AKT2, JNK2, or IKKβ kinase (Millipore/Upstate Biotechnology, Inc., Lake Placid, NY), 1 μCi of [γ-^{32}P]-ATP (3000 Ci/mmol) and 1× kinase buffer. Kinase reactions were performed according to the manufacturer's protocol using the supplied reagents with the assay. The kinase reaction mixtures were incubated at 30 °C for 15 min followed by separation using a 15% NuPAGE gel (Invitrogen, Carlsbad, CA) and exposed to X-ray film for autoradiography.

2.6. Solution digestion conditions

Following the protein kinase assay, the protein samples were subjected to tryptic digestion by adding sequencing-grade modified porcine trypsin at a protein:enzyme ratio of 20:1. The reactions were allowed to proceed overnight at 37 °C.

2.7. Cloning, expression, and purification of full-length mouse CD34

Using mouse integrin $\alpha6^+CD34^+$ keratinocyte stem cells as a source of total RNA, the full-length CD34 was amplified from cDNA (by RT-PCR using a pair of gene specific primers containing a Kozak sequence) and was cloned directionally into the vector pcDNA3.1D/V5-His (Invitrogen, Carlsbad, CA). The mouse *CD34* full-length cDNAs were then subcloned and ligated into the protein expression vector pFLAG-CMV-5c (Sigma–Aldrich, St. Louis, MO) using Hind III/Not I restriction enzyme sites. All DNA sequences were verified by the NIEHS DNA Sequencing Core Facility using a BigDye Terminator kit and automated sequencer (Applied Biosystems, Foster City, CA).

For protein expression studies, the HEK293F cells were transiently transfected either with empty vector (pFLAG-CMV-5c) or plasmid containing wild-type CD34 cDNA using Lipofectamine PLUS™ reagents (Invitrogen, Carlsbad, CA). After 48 h of incubation, total protein lysates were obtained using the radioimmunoprecipitation assay (RIPA) buffer including protease inhibitors (aprotinin, leupeptin, pepstatin and PMSF) and phosphatase inhibitors (sodium orthovanadate and NaF), followed by centrifugation at 14,000 × *g* for 15 min at 4 °C. Protein concentration was determined by BCA protein assay (Pierce, Rockford, IL).

CD34 protein was immunoprecipitated at 4 °C overnight with EZ View Red resin-bound anti-FLAG M2 monoclonal antibody (Sigma–Aldrich). Immunoprecipitates were centrifuged at 2500 rpm for 3 min at 4 °C and washed 4 times with RIPA buffer. The CD34-FLAG bound fractions were eluted with an excess of FLAG3 peptide (Sigma–Aldrich) (100 μg/mL), concentrated with acetone (1/5 = v/v), subjected to 4–12% Bis–Tris NuPAGE SDS gradient gel (Invitrogen, Carlsbad, CA), and stained with CBB R250.

2.8. In-gel digestion

The protein band corresponding to full-length CD34 was manually excised from the gel, cut into small pieces, and transferred into a 96-well microtiter plate. Gel pieces were subjected to automatic tryptic digestion using an Investigator™ Progest protein digestion station (Genomic Solutions, Ann Arbor, MI). Briefly, gel bands were sequentially washed twice with 25 mM ammonium bicarbonate buffer (pH 7) and acetonitrile, dehydrated, rehydrated with 25 μL of the enzyme solution, and digested at 37 °C for 8 h. The enzyme solution used was sequencing-grade modified trypsin at a concentration of 0.01 mg/mL in 25 mM ammonium bicarbonate buffer (pH 7). Resulting tryptic peptides were extracted from the gel, lyophilized, and stored at −80 °C. Prior to mass spectrometric analysis, the peptides were reconstituted in 40 μL of a 97:3 solution of water:acetonitrile (0.1% formic acid).

2.9. Titanium dioxide enrichment

The titanium dioxide (TiO_2) phosphopeptide enrichment procedure was adopted from Larsen et al. [21]. Briefly, 1 mg of Titansphere TiO_2 resin was added to 10 μL bead buffer (80% ACN, 19.9% H_2O, 0.1% TFA) and applied to a CRC tube. Binding of phosphopeptides to the TiO_2 column was achieved by first draining the bead buffer, then loading the protein digestion mixture with 2 μL protein buffer (100 mg/mL DHB in 80% ACN, 19.9% H_2O, 0.1% TFA). The mixture was allowed to incubate at room temperature for 10 min, after which, the column was drained and washed 1 × 30 μL protein buffer and 1 × 30 μL bead buffer. Elution of the bound peptides was accomplished by adding 5 μL of 200 μM ammonium bicarbonate buffer (pH 10.5) to the column and incubating for 5 min at room temperature. The 5 μL elution solution was then drained and diluted with 5 μL water. 0.5 μL of the eluent solution was spotted on a MALDI target with 0.5 μL of DHB matrix (20 μg/mL DHB in 50% ACN, 49% H_2O, 1% PA).

2.10. MALDI mass spectrometry

MALDI analyses were performed using either an ABI Voyager Super DE-STR or an ABI 4700 Proteomics Analyzer (Applied Biosystems, Inc., Framingham, MA) in the positive ion reflector mode. For sample analyses not involving titanium dioxide enrichment, the MALDI matrix was prepared initially as a saturated solution of α-cyano-4-hydroxycinnamic acid in 50:50 acetonitrile:water containing 0.1% FA (v/v). This saturated solution of α-cyano was then diluted 1:2 (v/v) with 50:50 acetonitrile:water containing 0.1% FA, of which 0.3 μL is mixed with 0.3 μL of the peptide digestion solution on a 100-well MALDI sample target. All MALDI spectra were obtained over the mass range of 700–4000 Da with 100–1000 laser shots per spectrum. For each sample analysis on the ABI 4700 Proteomics Analyzer, data dependent acquisitions were acquired in a fully automated mode such that a MALDI mass spectrum is acquired followed by MS/MS of the five most abundant ions in the spectrum (excluding ions from matrix and trypsin autolysis products). Additionally, ions that matched in nominal mass to putatively phos-

phorylated tryptic peptides of CD34 were also further interrogated manually by MS/MS. In the MS/MS mode, 1 kV was used for the fragmentation energy. Ions corresponding in mass to trypsin autolysis products were used to internally calibrate in the MS mode; thereby, allowing a routine mass accuracy of 10 ppm or less, while in the MS/MS mode an external calibration was employed using the fragment ions of Angiotensin 1 (theoretical m/z 1296.6853). Following the analyses, mass spectra were processed manually or analyzed using a Global Proteome Server Explorer™ workstation and software (Applied Biosystems, Framingham, MA).

2.11. Electrospray mass spectrometry

For the intact protein analyses, a Waters Micromass Q-Tof Ultima Global (Milford, MA) hybrid tandem mass spectrometer was used. This instrument is equipped with a nanoflow electrospray source and consists of a quadrupole mass filter and an orthogonal acceleration time-of-flight mass spectrometer. The needle voltage was ~3500 V, collision energy was 10 eV, the scan range was 300–3000, and the scan time for the MS analyses was 1.9 s/scan. For the LC analyses, a Waters CapLC HPLC system (Milford, MA) consisting of a micro-autosampler and binary pumps (solvent A = water, 0.1% formic acid and solvent B = acetonitrile, 0.1% formic acid) and a micro-autosampler were used to deliver the gradients. Injections of 5 pmol of CD34 were made onto the column. For the chromatographic separations, the gradients were as follows: 5% B for 30 min, then a linear gradient of 5–95% B over 60 min followed by 10 min at 95% B. The column used was a 5 cm × 75 μm C4 PepMap300 column (LC Packings, San Francisco, CA) at a flow rate of 500 nL/min.

For the LC/MS/MS analyses, an Agilent Technologies XCT Ultra ion trap (Santa Clara, CA) was used. The Agilent XCT Ultra ion trap is equipped with an HPLC-Chip Cube MS interface and an Agilent 1100 nanoLC system. Injections of 30 μL from a 1:10 dilution of the peptide digests were made onto a 40 nL enrichment column followed by a 43 mm × 75 μm analytical column packed with ZORBAX 300SB C18 particles. Peptides were separated and eluted using a linear gradient of 3–50% acetonitrile (0.1% formic acid) over 40 min, followed by a linear gradient of 50–95% acetonitrile over 7 min at a flow rate of 500 nL/min. The ion trap mass spectrometer was operated in the positive ion mode, standard enhanced mode using the following settings: capillary voltage, −2150 V; mass range, 300–1500; ICC smart target (number of ions in the trap prior to scan out), 100,000 or 200 ms of accumulation; and MS/MS fragmentation amplitude, 1.0 V. During the LC/MS/MS analyses, automated data dependent acquisition software was employed with the six most abundant ions (threshold requirement of 10,000 counts) from each spectrum selected for MS/MS analysis. Following the analyses, the MS/MS data were extracted and analyzed using Spectrum Mill MS Proteomics software (Agilent Technologies, Inc.). To generate peak lists, the raw data files were processed using the Data Extractor function with the following parameters: deconvoluted ions of 300–6000 Da and a retention time of 10–60 min. MS scans with the same precursor m/z were merged based on a ±1.4 m/z window and a ±15 s retention time window. Using the extracted data, searches were performed against an in-house database using the MS/MS search function. All sites of phosphorylation were manually validated.

3. Results

The amino acid sequence for the full-length mouse CD34 is shown in Fig. 1A. The C-terminal intracellular domain of the CD34 protein (amino acids 309–382) was expressed, His-tagged, and purified (Fig. 1B). As previously mentioned, PKC has been shown to phosphorylate CD34 [19], however, the precise sites of phosphorylation have not been identified. Because PKC isoforms differ in primary structure, cofactor requirements, and mode of action *in vitro*, a number of isoforms were used to investigate the *in vitro* phosphorylation of C-terminal intracellular domain of CD34, including PKC α, β, γ, δ, ε, ζ, and η. Phosphorylation of the C-terminal subdomain of CD34 was evident for all the PKC isoforms investigated (Fig. 2A and Supplemental Fig. 1), while the specific inhibitor was added to block the activity of PKC isoforms. However, under these assay conditions, PKCα incorporated the highest level of phosphorylation in CD34 (Fig. 2A) relative to the other PKC isoforms (Supplemental Fig. 1). Although the substrate specificities of PKC isozymes for CD34 protein have not previously been reported, other proteins have been reported to be differentially phosphorylated by different PKC isozymes. These data suggest that the intracellular domain of CD34 is a good substrate for PKCα. Therefore, for the subsequent mass spectrometric analyses of the subdomain of CD34, PKCα and nonradiolabeled ATP were used.

(A) 1MQVHRDTRAG LLLPWRWVAL CLMSLLHLNN LTSATTETST
QGISPSVPTN ESVEENITSS IPGSTSHYLI YQDSSKTTPA
ISETMVNFTV TSGIPSGSGT PHTFSQPQTS PTGILPTTSD
SISTSEMTWK SSLPSINVSD YSPNNSSFEM TSPTEPYAYT
SSSAPSAIKG EIKCSGIREV RLAQGICLEL SEASSCEEFK
KEKGEDLIQI LCEKEEAEAD AGASVCSLLL AQSEVRPECL
LMVLANSTEL PSKLQLMEKH QSDLRKLGIQ SFNKQDIGSH
QSYSRKTLIA LVTSGVLLAI LGTTGYFL309MN RRSWSPTGER
LGEDPYYTEN GGGQGYSSGP GASPETQGKA NVTRGAQENG
TGQAT*SRNGH *SARQHVVADT EL382

(B) SYYHHHHHH LESTSLYKKA GFLVPRGSMN309 RRSWSPTGER
LGEDPYYTEN GGGQGYSSGP GASPETQGKA NVTRGAQENG
TGQATSRNGH SARQHVVADT EL382

Fig. 1. (A) Amino acid sequence of full-length mouse CD34. Underlined residues correspond to the intracellular subdomain (amino acids 309–382) of mouse CD34. Residues labeled with an asterisk (*) denote consensus PKC phosphorylation sites. (B) Amino acid sequence of the expressed His-tagged CD34 protein.

3.1. In vitro sites of phosphorylation from PKCα treatment

To determine the extent of *in vitro* phosphorylation, the expressed CD34 subdomain was analyzed by liquid chromatography/mass spectrometry (LC/MS) before and after PKCα treatment. The resulting deconvoluted ESI spectra are shown in Fig. 2B and C. In the deconvoluted ESI mass spectrum of untreated CD34 (Fig. 2B), a protonated molecule of average molecular weight (M_r) of 10,950 is observed. Based on the amino acid sequence, the calculated M_r is 11,080.93 Da. These results indicate that the N-terminal methionine was lost during expression (calculated M_r minus methionine is 10,949.73). In comparison, the deconvoluted ESI mass spectrum of the PKCα-treated CD34 (Fig. 2C) shows a low abundance ion of average molecular weight of 11,189 which corresponds in mass to the addition of three phosphates (HPO_3) to the protein (calculated M_r is 11,189.67). The two most abundant protonated ions observed, however, are of average molecular weight of 11,205 Da and 11,285 Da. These ions correspond in mass to the oxidized form of the protein plus the addition of three and four phosphates (calculated M_r is 11,205.67 and 11,285.65, respectively). According to these data, at least four different sites within the amino acid sequence of the C-terminal domain of CD34 are phosphorylated

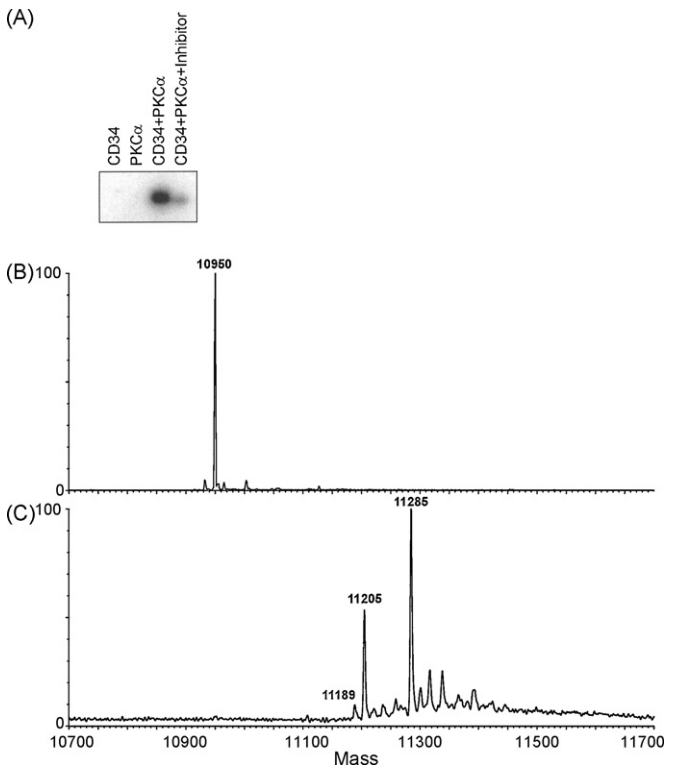

Fig. 2. (A) *In vitro* phosphorylation of the intracellular C-terminal subdomain of CD34 by PKCα. Kinase assay was performed as described in Section 2. (B) Deconvoluted electrospray mass spectrum of the intact intracellular subdomain of native CD34. (C) Deconvoluted electrospray mass spectrum of the intact intracellular subdomain of CD34 following treatment with PKCα.

under these *in vitro* conditions. The occupancy at any one specific phosphorylation site, however, cannot be addressed based on these data alone.

The location of the specific sites of phosphorylation in CD34 was determined by using a combination of tryptic peptide mapping, titanium dioxide enrichment, and MS sequencing analyses by both MALDI/MS/MS and LC/MS/MS. Metal oxides have been reported to show improved enrichment of phosphopeptides over traditional IMAC resins. The MALDI spectra of the CD34 tryptic digest before and after TiO_2 enrichment were acquired (Supplemental Fig. 2). Several ions are observed before enrichment (Supplemental Fig. 2A) which correspond in mass to predicted tryptic peptides (i.e., tryptic peptides T3, T6, T7, and T9) of the intracellular domain of CD34. After TiO_2 enrichment (Supplemental Fig. 2B), several ions are observed which were not observed in the tryptic digest without enrichment. These peptides correspond in mass to tryptic peptide T10 plus 80 Da, tryptic peptide T4-5 plus 80 Da, tryptic peptide T6 plus 80 Da, and tryptic peptide T9 plus 80 Da. In addition, the oxidized form of tryptic peptide T4-5 plus 80 Da is observed.

To identify the specific residues containing the phosphogroups, both the TiO_2 enriched eluent and the nonenriched CD34 tryptic digest were analyzed by LC/MS/MS. The resulting MS/MS data were then searched for tryptic peptides modified by the addition of multiples of 80 Da. Fig. 3A shows the MS/MS spectrum of the $(M+2H)^{2+}$ ion of m/z 500.3 which corresponds in mass to the tryptic peptide T6 (amino acids 313–320) plus a phosphate group. The amino acid numbers refer to the full-length mouse CD34 residues. The most abundant fragment ion observed corresponds to the loss of H_3PO_4 from the protonated molecule. The observation of this fragment ion indicates the presence of a phosphate group within the peptide. In addition, the b_2, y_5, and y_6 fragment ions are observed which allows the assignment of the site of phosphorylation in this peptide to Ser-313.

The MS/MS spectrum of the $(M+3H)^{3+}$ ion of m/z 572.3 is shown in Fig. 3B. This ion corresponds in mass to the monophosphorylated tryptic peptide T10-11 (aa 368–382) containing the amino acid sequence NGHSARQHVVADTEL. Although the S:N ratio in this spectrum is low, a series of b ions (b_9–b_{14}) are observed as well as the loss of H_2O or H_3PO_4 from some of these ions. These structurally diagnostic ions allow for the assignment of the phosphorylation site to the Ser-371 residue (as the cleavages around Thr-380 are observed without the addition of the phosphate group). Additionally, the MALDI/MS/MS spectrum of the $(M+H)^+$ ion of m/z 721.3 (corresponding in mass to the monophosphorylated tryptic peptide T10 NGHSAR, aa 368–373) was also obtained (Supplemental Fig. 3). Fragment ions corresponding to the loss of HPO_3 and H_3PO_4 were observed as well as the fragment ions of y_1, y_2, y_5, and b_5 which allowed for further confirmation of the site of phosphorylation as Ser-371.

Ions corresponding in mass to the tryptic peptide containing residues 321–349 (tryptic peptide T7) plus the addition of one and two phosphate groups were observed. The MS/MS spectrum of the $(M+3H)^{3+}$ ion of m/z 995.1 which corresponds in mass to the monophosphorylated tryptic peptide T7 (amino acids 321–349) is shown in Fig. 4A. A series of backbone cleavages (b and y ions) are observed which correspond in mass to the addition of a phosphate group (e.g., b_{25}, y_{13}–y_{15}, and y_{19}–y_{25}). Fragment ions which do not contain the addition of the phosphate group are observed for the y_4, y_6, b_4, b_6–b_8, b_{10}, and b_{14} ions. These data allow the assignment of the phosphate group within residues 335–343 (GYSSGPGAS). Upon further interrogation of these data, the y_7 and y_{10} fragment ions are observed both with (labeled as y_7 and y_{10}) and without the phosphate group (labeled as $y_7{}^*$ and $y_{10}{}^*$) and, thus, are observed as having a mass difference of 80 Da. It has been shown previously [25] that phosphoserine-containing peptides predominately lose a mass of 98 Da rather than 80 Da upon CID in an ion trap mass spectrometer. Therefore, the ions labeled as $y_7{}^*$ and $y_{10}{}^*$ are most likely the result of a nonphosphorylated serine and not the loss of 80 Da from the y_7 and y_{10} ions. These results suggest that this monophosphorylated peptide contains a phosphate group at two different sites—neither of which are 100% occupied. One of the sites of phosphorylation is Ser-343 based on the observation of the y_7 ions both with and without phosphate. The second site of phosphorylation within this peptide is either the Ser-337 or Ser-338 residue. Because backbone fragmentation between the neighboring serine residues is not observed, one cannot definitely say which specific serine residue contains the phosphate group based on these data.

The MS/MS spectrum of the $(M+3H)^{3+}$ ion of m/z 1021.7 is shown in Fig. 4B. This ion corresponds in mass to amino acid residues 321–349 (tryptic peptide T7) plus two phosphate groups. The most abundant fragment ion observed corresponds in mass to the loss of H_3PO_4 from the molecular ion. Additionally, ions corresponding in mass to the loss of a phosphate and water ($-H_3PO_4-H_2O$) and the loss of two phosphate groups ($-2H_3PO_4$) are observed. Aside from the loss of phosphate groups, a series of backbone cleavages (b and y ions) are also observed. Structurally diagnostic ions observed which allow for the assignment of the two sites of phosphorylation within this peptide include the y_6 ion which is observed without the addition of a phosphate group. This indicates that residues 346–349 do not contain a phosphate group. Additionally, the y_7 and y_{10} ions are observed with the addition of a phosphate group and a very low abundance ion corresponding in mass to y_{13} plus two phosphate groups is observed. These y_7, y_{10}, and y_{13} ions are only observed with the addition of the phosphate groups (i.e., the nonphosphorylated forms are not observed); thereby, allowing assignment of the phosphates to serine-343 and either serine-337 or serine-338. Similar to the MS/MS spectrum of the monophosphorylated form of this peptide (Fig. 4A), fragmentation between the serine-337 and serine-338 residues is not observed. Consequently, the unequivo-

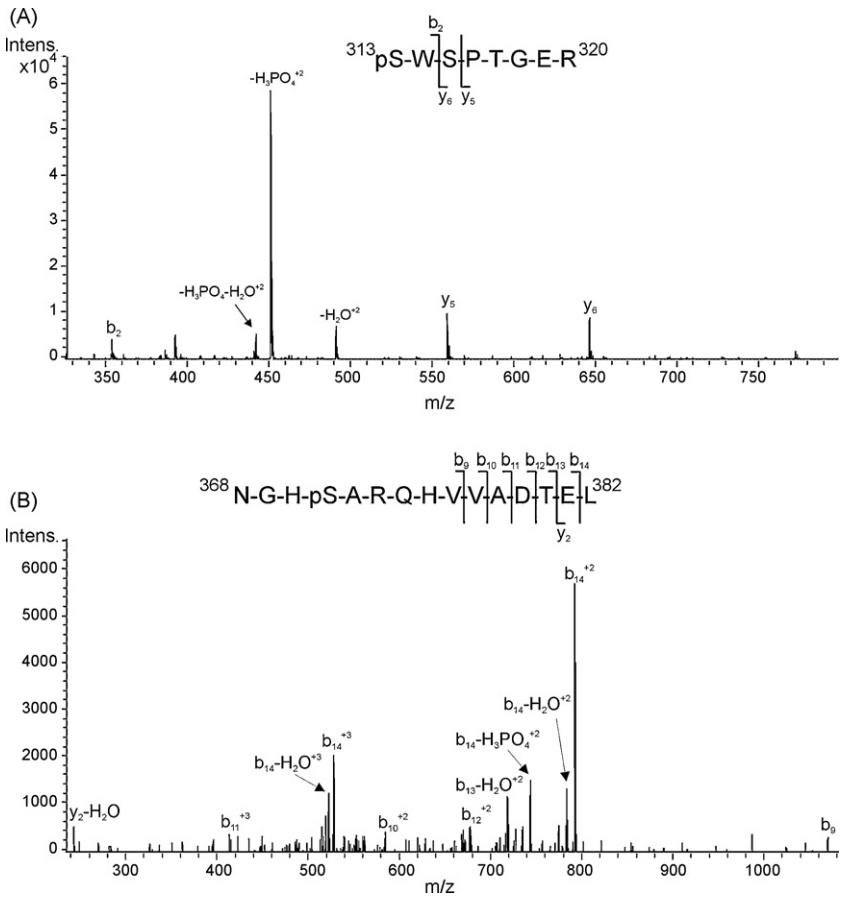

Fig. 3. Tandem mass spectra of phosphorylated tryptic peptides derived from the *in vitro* PKCα treatment of the intracellular C-terminal subdomain of mouse CD34. (A) MS/MS data from the $(M+2H)^{2+}$ ion of *m*/*z* 500.3 which corresponds in mass to the tryptic peptide T6 (amino acids 313–320) plus a phosphate group. (B) MS/MS data of the $(M+3H)^{3+}$ ion of *m*/*z* 572.3 which corresponds in mass to the monophosphorylated tryptic peptide T10-11 (aa 368–382). The amino acid numbers refer to the full-length mouse CD34 residues.

cal assignment of the second phosphate to one of these two serine residues is not possible based on these data.

One additional *in vitro* site of phosphorylation was determined based on the MS/MS data (Supplemental Fig. 4) for the serine residue in the amino acid sequence GSMNRR (tryptic peptide T4-5 observed in the MALDI mass spectrum shown in Supplemental Fig. 2B). This serine residue, however, is part of the sequence tag implemented for expression and purification of the subdomain of CD34 and, therefore, is not biologically relevant. Collectively, five sites of phosphorylation were determined for the *in vitro* PKC treatment of the expressed form of the CD34 subdomain even though only one of the observed sites contains a PKC consensus sites. These sites are Ser-313, Ser-343, Ser-337/338, and Ser-371 in CD34, and a serine residue in the N-terminal expression tag. These results are not surprising as it has been reported previously that protein kinase C exhibits broad substrate specificity *in vitro* [17].

3.2. *In vivo* sites of phosphorylation

To determine *in vivo* sites of phosphorylation in full-length CD34, the full-length gene in vector pFLAG-CMV-5c was transiently transfected into HEK293F cells. After 48 h of incubation, total protein lysates were isolated and digested as detailed in Section 2. The resulting digest was then analyzed by LC/MS/MS. From these analyses, the tryptic peptide containing amino acids 321–349 was observed with the addition of one and two phosphate groups (triply charged ions of *m*/*z* 995.1 and 1021.7, respectively). To identify the sites of *in vivo* phosphorylation, the corresponding MS/MS data of these ions were acquired. The MS/MS spectrum of the $(M+3H)^{3+}$ ion of *m*/*z* 995.1 corresponding in mass to the monophosphorylated tryptic peptide is shown in Fig. 5A. Many structurally significant fragment ions corresponding to cleavages along the peptide backbone are observed. Most importantly, the observation of the y_7 ion (which includes the mass of a phosphate) and the lack of evidence for the y_7* ion (nonphosphorylated y_7) allows for the determination that serine-343 as the phosphorylation site. Additionally, unlike the MS/MS spectrum of the *in vitro* monophosphorylated T7 (Fig. 4A), there is no evidence for a second phosphate group in this peptide.

Fig. 5B shows the MS/MS spectrum of the triply charged ion of *m*/*z* 1021.7 which corresponds in mass to the doubly phosphorylated form of the tryptic peptide comprising residues 321–349. A series of *b* ions corresponding in mass to cleavages at the N-terminus of the peptide are observed without the addition of a phosphate group (i.e., b_4, b_6, b_7, b_9, b_{11}, and b_{14}). Moreover, several y ions are observed; of note, the ions which correspond in mass to the y_7 and y_9 fragmentations plus one phosphate and the ion corresponding in mass to the y_{13} fragment with the addition of two phosphate groups. These data allow for the assignment of the sites of phosphorylation within this peptide to serine-343 and serine-337/338. In summary, two sites of *in vivo* phosphorylation were determined for full-length mouse CD34, Ser-343 and Ser-337/338. Both of these sites are located in peptides which do not contain consensus PKC sites. The same tryptic peptides which contain the Ser-343 and Ser-337/338 also contain the previously reported sites of phosphorylation, Tyr-326 and Tyr-336 for mouse CD34. We did not, however, observe any fragment ions that indicate the presence of phosphorylation at either of these residues in our analyses.

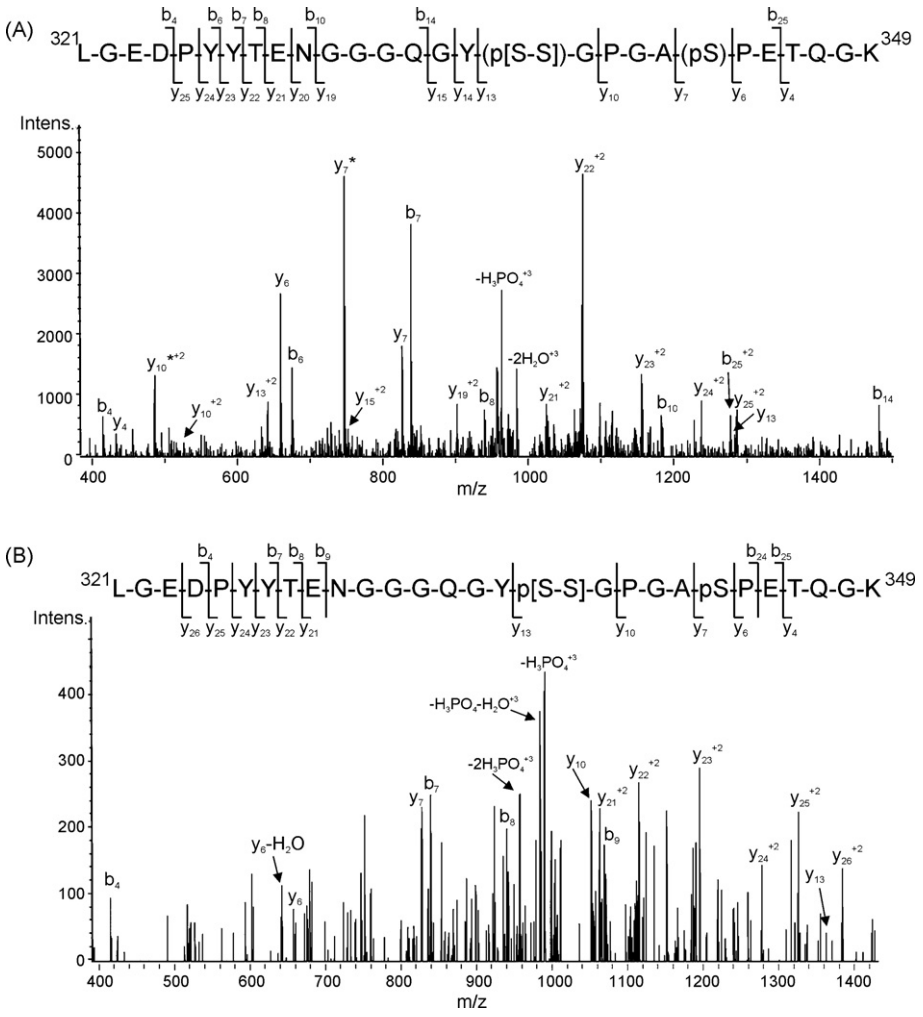

Fig. 4. Tandem mass spectra of phosphorylated tryptic peptides derived from the *in vitro* PKCα treatment of the intracellular C-terminal subdomain of mouse CD34. (A) MS/MS data of the $(M+3H)^{3+}$ ion of m/z 995.1 which corresponds in mass to the monophosphorylated tryptic peptide T7 (amino acids 321–349). (B) MS/MS data of the $(M+3H)^{3+}$ ion of m/z 1021.7 which corresponds in mass to amino acid residues 321–349 (tryptic peptide T7) plus two phosphate groups. The amino acid numbers refer to the full-length mouse CD34 residues.

3.3. *In vitro* sites of phosphorylation from other kinase treatments

To investigate the possible biological relevance of the two sites of phosphorylation identified *in vivo*, the amino acid sequence for the C-terminal subdomain of CD34 was submitted to a kinase-specific prediction program. The GPS (Group-based Prediction System) 2.1 software tool [26] is available on-line and was used to predict which kinases may be involved in the phosphorylation of Ser-343 and Ser-337/338. Using this prediction tool, several kinases, including AKT2, JNK, and IKKβ, were predicted to phosphorylate these two serine residues. *In vitro* assays were performed with these enzymes to determine whether these kinases would phosphorylate the CD34 subdomain (Fig. 6A). Lanes 3, 6, and 9 correspond to the complete reaction mixtures which consist of CD34 and the kinase AKT2, JNK2, and IKKβ, respectively. The remaining lanes correspond to the controls of either CD34 only (Lanes 1, 4, and 7) or kinase only (Lanes 2, 5, and 8). These data suggest that JNK2 and IKKβ phosphorylate CD34 to a greater extent than AKT2.

To determine whether sites of phosphorylation could be identified in CD34 following the AKT2, JNK2, and IKKβ treatments, the CD34 was digested with trypsin and analyzed by mass spectrometry. In the LC/MS analyses of all three kinase treatments, a triply charged ion of m/z 995.0 was observed which corresponds in mass to the tryptic peptide containing amino acids 321–349. The MS/MS data of the $(M+3H)^{3+}$ ion of m/z 995.0 resulting from the tryptic digest following AKT2 treatment of CD34 is shown in Fig. 6B. The observation of the singly charged fragment ions of m/z 659.4 and 826.3 correspond to y_6 and y_7 fragment ions of this peptide. The mass difference between these two ions corresponds to a phosphoserine residue; thereby allowing the assignment of the phosphorylation site in this peptide to serine-343. From the MS/MS data obtained from this ion resulting from tryptic digests of CD34 following the IKKβ and JNK2 treatments, the S/N level was such that unequivocal assignment for the precise location of the phosphorylation site could not be made.

4. Discussion

Because protein kinase C is thought to play roles in a variety of cellular processes, the *in vitro* PKC treatment of mouse CD34 was investigated. Although the PKC treatment of human CD34 has been investigated [14,15], the exact location of the sites of phosphorylation have not been reported nor has the PKC treatment of mouse CD34. A multifaceted strategy was undertaken to identify the potential *in vitro* PKC and *in vivo* phosphorylation sites in CD34. These strategies include: (1) cloning, expression, and *in vitro* PKC kinase treatment of the intracellular subdomain of CD34; (2) an immunoprecipitation approach to isolate full-length CD34 from transfected HEK293F cells for *in vivo* phos-

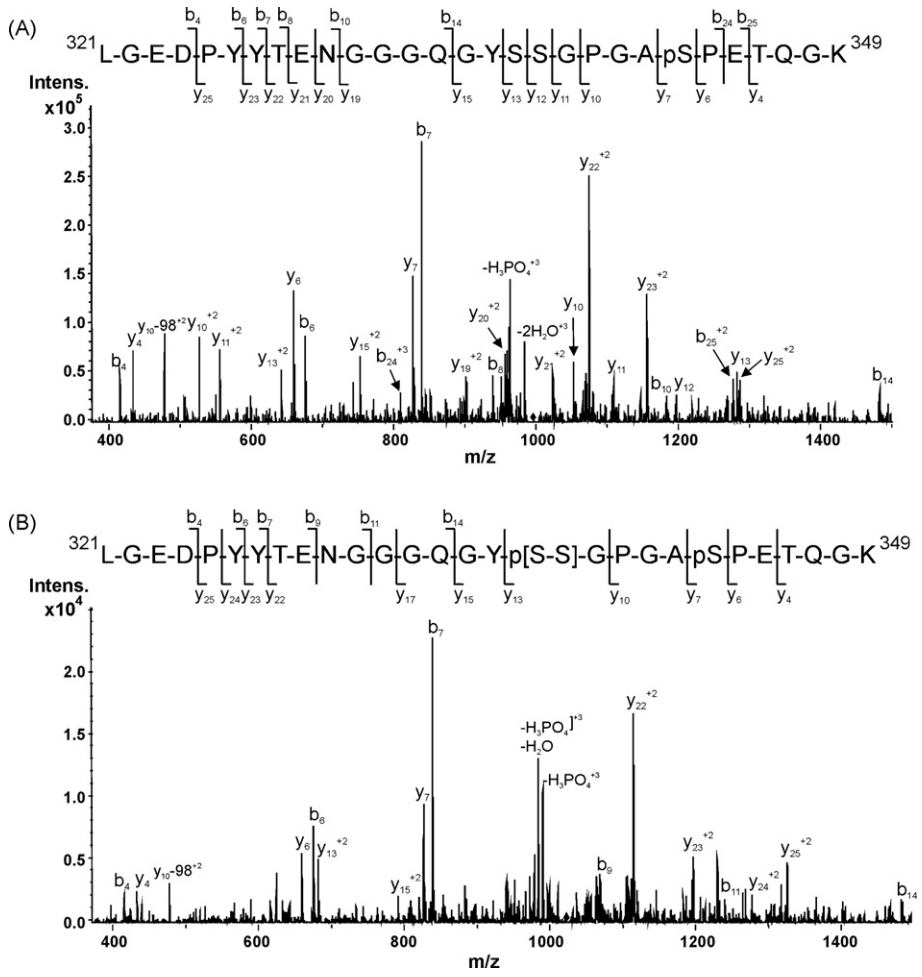

Fig. 5. Tandem mass spectra of phosphorylated tryptic peptides derived *in vivo* from full-length mouse CD34. (A) MS/MS data of the $(M+3H)^{3+}$ ion of *m*/*z* 995.1 which corresponds in mass to the monophosphorylated peptide comprising residues 321–349. (B) MS/MS data of the $(M+3H)^{3+}$ ion of *m*/*z* 1021.7 which corresponds in mass to the tryptic peptide containing residues 321–349 plus two phosphate groups.

phorylation analysis; and (3) proteolysis, TiO_2 enrichment, and tandem mass spectrometry to determine the specific sites of phosphorylation. From the *in vitro* PKC kinase assays, it was determined that the highest level of phosphorylation of the intracellular domain of CD34 was observed for the PKCα isoform, although other PKC isoforms phosphorylated CD34 to a lower extent.

The identification of the sites of phosphorylation of CD34 may be important in defining their biological roles. Initially, we investigated the sites of phosphorylation that resulted from the *in vitro* PKCα treatment of the intracellular domain of CD34. For this work, a combination of proteolysis, phosphopeptide enrichment via metal oxide resin, and mass spectrometry were employed. Tryptic digests of the PKCα-treated CD34 resulted in the identification of a phosphorylated peptide corresponding to a PKC consensus site. MS/MS of this peptide allowed the unequivocal assignment of a site of phosphorylation at Ser-371. In addition, several other sites of serine phosphorylation in CD34 were identified within peptides which do not contain strict PKC consensus sites. These sites include Ser-313, Ser-343, and Ser-337/338. These results are not unexpected as it is known that the specificity of *in vitro* kinase experiments can vary and also depend on the primary sequence surrounding the phosphorylation site [17].

To explore the biological relevance of and to determine the *in vivo* occurrence of the sites of phosphorylation identified in the *in vitro* PKCα kinase assays, the full-length mouse CD34 protein was expressed in HEK293F, a transformed cell line derived from human embryonic kidney. Following immunoprecipitation, SDS-PAGE separation, in-gel digestion, and MS/MS analyses, two sites of phosphorylation were identified in full-length CD34. Although both of these sites were identified in the *in vitro* kinase experiments, both sites are located within peptides which do not contain PKC consensus sites. These results imply that phosphorylation by other kinases may occur *in vivo*.

Based on these phosphorylation sites found *in vivo* and the knowledge-base of known consensus phosphorylation sites of kinases, three candidate kinases, AKT2, JNK2, and IKKβ, were employed for further *in vitro* phosphorylation experiments. While there were ambiguities as to the site(s) of phosphorylation for JNK2 and IKKβ, AKT2 was found to phosphorylate CD34 *in vitro* at the same phosphorylation site identified *in vivo*. The fact that AKT2 can phosphorylate CD34 at a biologically relevant site is intriguing for a variety of reasons. AKT family members are known regulators of several cellular processes including migration, proliferation, and terminal differentiation [for review see Refs. 27,28] and AKT2, specifically, has been identified as a determinant of hair follicle development and is highly expressed in the outer root sheath [29], the same region of the follicle where CD34 is expressed [5]. Moreover, while CD34 has not yet been identified as a *bona fide* signaling molecule, other adhesion proteins, integrins and cadherins, have well-characterized involvement in keratinocyte growth and differentiation [for review see Ref. 30]. Hence, on account of the findings detailed in this manuscript and on CD34's characterization as an epidermal stem cell marker [3,4] and its requirement for skin

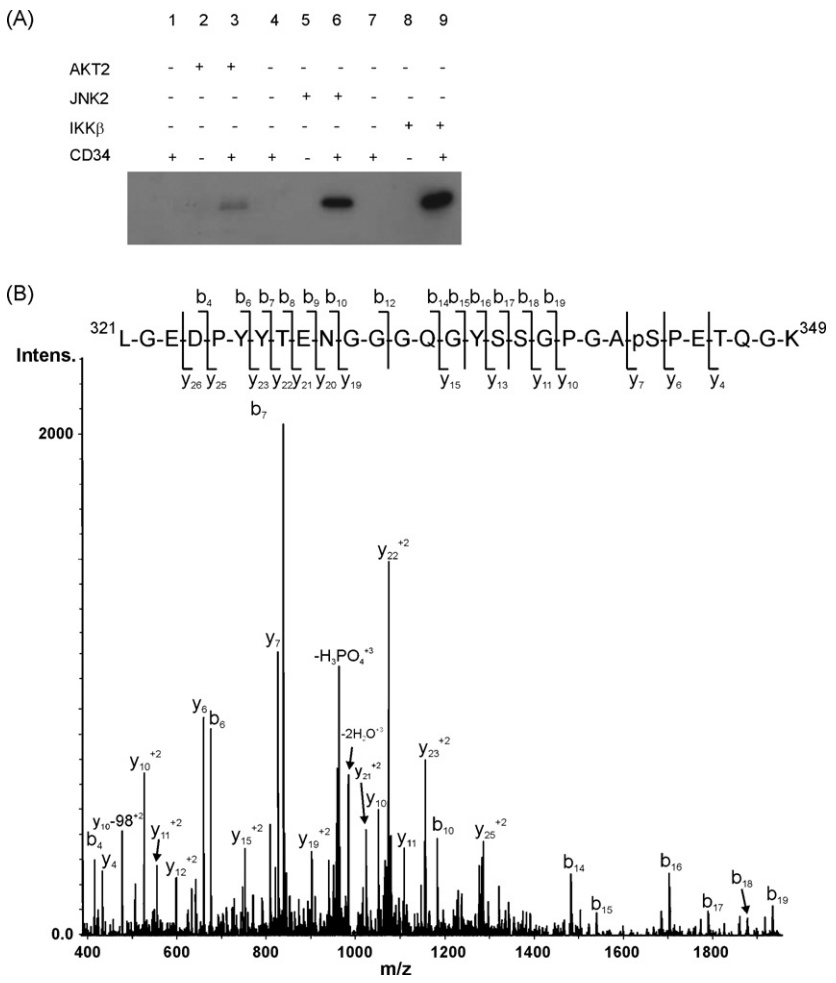

Fig. 6. (A) *In vitro* phosphorylation of the intracellular C-terminal subdomain of CD34 by AKT2, JNK2, and IKKβ kinases. Kinase assays were performed as described in Section 2. (B) Tandem mass spectrum of a phosphorylated tryptic peptide derived from the *in vitro* AKT2 treatment of the intracellular C-terminal subdomain of mouse CD34. MS/MS data of the $(M+3H)^{3+}$ ion of *m*/*z* 995.0 which corresponds in mass to the monophosphorylated tryptic peptide comprising residues 321–349.

tumorigenesis [9], it is reasonable to hypothesize that CD34 may lie somewhere in an AKT-dependent signaling pathway that controls keratinocyte growth and differentiation. Furthermore, in addition to this work and the reports of tyrosine phosphorylation in CD34 [14,15], several studies have alluded to the important influence of CD34 phosphorylation in its biological regulation. Therefore, we hypothesize that different phosphorylation sites are utilized differentially by CD34 and are unique to particular kinases with functional consequences. Further functional studies on these sites of phosphorylation should increase our understanding of their impact on CD34 structure and the role these specific sites play in its biological function. Moreover, the continued developments in the area of mass spectrometry should provide new insights, not only into the function of proteins, but also into the basic regulatory mechanisms that control cellular functions.

5. Conclusions

In summary, we have characterized both the *in vitro* PKCα and *in vivo* sites of phosphorylation in mouse CD34 using mass spectrometry. For the *in vitro* work, the intracellular domain of CD34 and PKC assays were employed. For the *in vivo* analyses, full-length mouse CD34 was expressed and isolated from HEK293F cells. Using a combination of proteolysis, metal oxide enrichment, peptide mapping, and MS sequencing methodologies, five sites of phosphorylation in CD34 from the *in vitro* experiments and two sites of phosphorylation from the *in vivo* analyses have been identified. Of the five sites identified *in vitro* (one on a serine residue in the N-terminal tag, Ser-313, Ser-371, Ser-343, and Ser-337/338), only Ser-371 is a strict PKC consensus site. Neither of the two sites identified *in vivo* (Ser-343 and Ser-337/338) contain a strict consensus PKC site; thereby, implying that other kinases are involved in the regulatory control of CD34. While there were ambiguities as to the site(s) of phosphorylation for JNK2 and IKKβ, AKT2 was found to phosphorylate CD34 *in vitro* at the same phosphorylation site identified *in vivo* (i.e., Ser-343).

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ijms.2010.05.027.

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