Identification of in Vivo Phosphorylation Sites of CD45 Protein-tyrosine Phosphatase in 70Z/3.12 Cells*

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Phosphorylation of CD45, a transmembrane protein-tyrosine phosphatase (PTPase), has been proposed to mediate docking of signaling proteins and to modulate PTPase activity. To study the role of phosphorylation in CD45, in vivo phosphorylation sites of CD45 from 70Z/3.12 cells were identified using 32P labeling, trypsin digestion, two-dimensional peptide mapping, high performance liquid chromatography, phosphoamino acid analysis, matrix-assisted laser desorption/ionization mass spectrometry, and specific enzymatic degradation. Eight phosphopeptides, a through h, were isolated and four phosphorylation sites were identified. All four phosphorylation sites were in the membrane-distant PTPase domain (D2) and the C-terminal tail and none were in the membrane-proximal PTPase domain (D1). One site, Ser(P)1246, peptide h, was in the D2 domain and, by comparison to the three-dimensional structure of PTP1B, is predicted to lie at the apex of the substrate binding loop. Ser939 was the only in vitro phosphorylation site for protein kinase C among the phosphorylation sites identified. Four of the C-terminal peptides identified (d, e, f, and g) spanned the same sequence and were derived from the same phosphorylation site in the C-terminal tail, Ser1204. Peptide a was derived from the intact C terminus and comprised a mixture of mono-phosphorylated peptides containing either Ser(P)1248 or Thr(P)1248. Knowledge of the precise phosphorylation sites of CD45 will lead to the design of experiments to define the role of phosphorylation in PTPase activity and in signaling.

Protein-tyrosine phosphatase and dephosphorylation play an important role in regulating cellular differentiation, proliferation, and activation. The role of dephosphorylation by CD45 protein-tyrosine phosphatase (PTPase) in lymphocyte signaling has been the subject of intense investigation (1–4). CD45 (T200, B220, L-CA) is a transmembrane PTPase of hematopoietic cells of 1268 total amino acids, with a cytoplasmic domain of 702 amino acids containing tandem repeated PTPase homology domains (designated D1 and D2). The membrane-proximal PTPase domain (D1) is considered to be constitutively active, and the second PTPase domain (D2) is usually considered to be inactive. CD45 has been intensely studied because of its well documented role in the antigen-specific activation of B and T cells (1–3). T cells lacking CD45 fail to respond to stimulation of the T cell antigen receptor (5, 6), and the catalytic activity of the CD45 D1 domain is required for T cell receptor activation (7). Chimeric proteins, in which the extracellular domain of CD45 was replaced, restored normal T cell receptor activation (8–10). Similarly, CD45-deficient B cells do not respond to stimulation of the IgM receptor (11). It is believed that CD45 activates the Src family PTKs by dephosphorylating the regulatory Tyr(P) near the C terminus of T cell receptor or B cell receptor-associated Src family kinases (12–15). Since CD45 is abundantly expressed on all nonerythroid hematopoietic cells, it has also been hypothesized that CD45 may be involved in the regulation of other fundamental cell processes such as cell growth and cell cycle (1, 16, 17).

Phosphorylation of the cytoplasmic domain of CD45 has been described, and this phosphorylation has been proposed to play a role in the regulation of biological function by providing docking sites (18) or by altering PTPase activity (18–20). The modulation of CD45 PTPase activity has been found to correlate with phosphorylation of the cytoplasmic domain in several studies. 1) Treatment of T cell clones with a Ca2+ ionophore decreased the Ser phosphorylation of CD45 and simultaneously decreased the PTPase activity of the molecule (20). 2) Phosphorylation of human CD45 (by overexpression of CD45 in Cos cells with p50cbl PTK) increased the PTPase activity of CD45 and increased the association of CD45 with a putative substrate, p561ck (18). The p50cbl PTK phosphorylation site was identified as Tyr1100. 3) The sequential in vitro phosphorylation of human CD45 by Abl PTK and casein kinase II increased the activity of CD45 for certain substrates (19). 4) The increase in CD45 phosphorylation due to phorbol ester treatment of cells was associated with a decrease in PTPase activity (21). Other reports indicate that the phosphorylation of CD45 increased after stimulation by phorbol esters (22) or interleukin-2 (23) without apparent change in CD45 activity.

The elucidation of the role of CD45 phosphorylation in PTPase activity and in signaling has been hampered by the lack of precise knowledge of naturally occurring CD45 phosphorylation sites. The large number of potential phosphorylation sites in the CD45 cytoplasmic domain make identification by sequencing or mutagenesis extremely difficult. To resolve this problem, we have identified the in vivo phosphorylation sites of CD45 in 70Z/3.12 cells (a mouse pre-B cell line) by two-dimensional phosphopeptide mapping, reverse-phase HPLC, phosphoamino acid analysis, enzymatic degradation,
and matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS).

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—The mouse pre-B lymphocyte cell line 70Z/3.12 was obtained from the American Type Culture Collection (ATCC) and maintained at 37 °C in 90% RPMI 1640 medium (Life Technologies, Inc.) containing 10% heat inactivated fetal bovine serum (FBS) (Life Technologies), 25 mM HEPES, 50 μM 2-mercaptoethanol, 100 units/ml penicillin, 100 units/ml streptomycin (complete RPMI 1640 medium) in 5% CO2. Cells were counted using a Coulter Cell Counter (Coulter Electronics, Hialeah, FL) and maintained in an exponential growth state (0.1–4.0 × 106 cells/ml). Cultures were harvested at 8 × 106 cells/ml with viability in excess of 97%.

**CD45 Trypsin Phosphopeptide Mapping**—In an effort to map phosphorylation sites of CD45, we first prepared a large scale, unlabeled CD45 immunoprecipitate from cells grown at the same density as described above. After SDS-PAGE, Western transfer, and trypsin digestion, the digested mixture was fractionated using a microbore reverse-phase HPLC system (Microm BioResources, Inc.) with a 0–86% gradient of acetonitrile in 0.1% aqueous trifluoroacetic acid and a flow rate of 50 μl/min on a Reliasil C18 column (5 mm, 300 A, 1.0 × 150 mm, Microm BioResources). The solvent gradient started at 0% solvent B, then increased to 20% solvent B at 15 min, 60% solvent B at 20 min, and 95% solvent B at 21 min, where solvent A was 0.1% trifluoroacetic acid in water and solvent B was a mixture of acetonitrile/0.1% trifluoroacetic acid in water (9:1). The eluant was monitored by a UV absorbance at 214 nm, and fractions were collected every 30 s. A 1-μl sample of each HPLC fraction was spotted on a TLC plate, followed by detection of 32P radioactivity by Betascope (Betagen) analysis.

**RESULTS**

**CD45 Trypsin Phosphopeptide Mapping**—In an effort to map the in vivo phosphorylation sites of CD45, we first prepared a large scale, unlabeled CD45 immunoprecipitate from cells grown at the same density as described above. After SDS-PAGE, Western transfer, and trypsin digestion, the digested mixture was fractionated using a microbore reverse-phase HPLC system (Microm BioResources, Inc.) with a 0–86% gradient of acetonitrile in 0.1% aqueous trifluoroacetic acid and a flow rate of 50 μl/min on a Reliasil C18 column (5 mm, 300 A, 1.0 × 150 mm, Microm BioResources). The solvent gradient started at 0% solvent B, then increased to 20% solvent B at 15 min, 60% solvent B at 20 min, and 95% solvent B at 21 min, where solvent A was 0.1% trifluoroacetic acid in water and solvent B was a mixture of acetonitrile/0.1% trifluoroacetic acid in water (9:1). The eluant was monitored by a UV absorbance at 214 nm, and fractions were collected every 30 s. A 1-μl sample of each HPLC fraction was spotted on a TLC plate, followed by detection of 32P radioactivity by Betascope (Betagen) analysis.

**Mass Spectrometry**—All MALDI-MS spectra were obtained on a Voyager mass spectrometer (PerSeptive Biosystems, Framingham, MA), used in linear mode, equipped with a nitrogen laser (337 nm, 3-ns pulse). The accelerating voltage in the ion source was 26 kV. Data were acquired with a transient recorder with 2-ns resolution. The matrix used in this work was a-cyano-4-hydroxyquinamic acid, dissolved in water/acetonitrile (1:1, v/v) to give a saturated solution at room temperature. To prepare the sample for analysis, 1 μl of the peptide solution (1–10 pmol/0.1% trifluoroacetic acid) was added to 1 μl of the matrix solution and applied to a stainless steel sample plate. The mixture was then allowed to dry on the sample plate before being introduced into the mass spectrometer. Each spectrum was produced by accumulating data from 50–256 laser pulses. Time-to-mass conversion was achieved by external or internal calibration using bradykinin (MH+ 1061.2) and insulin (MH+ 5739.6). The accuracy of mass assignments was approximately ± 0.1% (< 1 Da/10,000 Da). A computer program, MSU MassMap (26) was used to calculate the average masses of all possible peptide and phosphopeptide fragments from CD45, and the m/z value of the mass spectral peak for the corresponding MH+ ion. Dephosphorylation of 5 μl of the HPLC fractions containing phosphopeptide was achieved by incubation of the peptide with 1–2 units of porcine intestinal phosphatase in 50 mM NH4HCO3, pH 8.0 at 37 °C for 4 h.

**Hydroxylamine and Glu-C Digestion**—Digestion of TLC isolated peptide a with hydroxylamine was performed in 2 μM guanidine-HCl, 2 μM NH4OH-HCl, 0.2 μM K2CO3 (Sigma), pH 9, for 4 h at 45 °C (24). After NH4OH digestion the peptide was purified by HPLC using conditions described above to remove salts (yielding a single radioactive peak at 21 min, retention time) and was analyzed by one-dimensional TLC using the conditions described above. The NH4OH-digested peptide was then digested with 1 μg of endoproteinase Glu-C (Boehringer Mannheim) in 50 mM NH4HCO3, pH 7.8, 18 h at 25 °C. The NH4OH- and endopeptidase Glu-C-digested peptide a was then analyzed by two-dimensional TLC as described above. Radioactivity was detected using a Molecular Dynamics PhosphorImager.

**In Vivo Phosphorylation of CD45 by Protein Kinase C (PKC)—Purified rat brain PKC was provided by Dr. A. Nairn (Rockefeller University, New York, NY), and the purified, bacterially expressed cytoplasmic domain of murine CD45 was provided by Dr. P. Johnson (University of British Columbia, Canada) (27). The phosphorylation of 1 μg of cytoplasmic CD45 was carried out with 1 μg of PKC at 30 °C for 30 min in 20 mM Tris-HCl (pH 7.4), 5 mM MgCl2, 1 mM dithiothreitol, 50 μg/ml phosphatidylinerine, 100 μM PMA, 2 μM CaCl2, and 0.01 μl of 1,5′-dIPAP (3000 C/mmol, DuPont NEN). The reaction was terminated by the addition of SDS-PAGE sample buffer. The sample was then boiled and resolved on a 4–15% SDS-PAGE gradient gel and transferred to a PVDF membrane, followed by autoradiography.

The CD45 band was excised, digested with trypsin, and analyzed as described above.
two-dimensional map of tryptic phosphopeptides from 70Z/3.12 cells cultured at high density (8 \times 10^5 cells/ml). Cells were labeled with \([\text{32P}]\)orthophosphate for 4 h and immunoprecipitated CD45 was separated by SDS-PAGE and transferred to PVDF for autoradiography (Fig. 1A). The CD45 band was excised, digested with trypsin, and subjected to two-dimensional phosphopeptide mapping (Fig. 1B). Tryptic phosphopeptides were designated a–h (Fig. 1B). Phosphoamino acid analysis of each of the radioactive peptides eluted from the TLC plate (Fig. 2) revealed that each contained only Ser phosphorylation, except for phosphopeptide a, which contained both Ser and Thr phosphorylation in about a 1:1 ratio (Fig. 2, lane 2). No tyrosine phosphorylation of CD45 was detected. Betascope and phosphorimage analysis was used in this work, because it was quantitative and it allowed the detection of very low levels of radioactivity.

Separation of Tryptic Peptides of CD45 by Reverse-phase HPLC—Reverse-phase HPLC was performed to isolate larger quantities of the tryptic phosphopeptides for detailed analysis of the phosphorylation sites of CD45. Approximately 40 μg of CD45 (about 200 pmol) was purified from 5 \times 10^9 70Z/3.12 cells (8 \times 10^5 cells/ml) by immunoprecipitation with M1/9.3.4 monoclonal antibody. A radioactive tracer was prepared by immunoprecipitating CD45 from 4 \times 10^7 70Z/3.12 cells (8 \times 10^5 cells/ml) labeled with 4 mCi of \([32P]\) for 4 h, and added to the nonradioactive preparation. After tryptic digestion, the CD45 peptide mixture was subjected to fractionation by HPLC. Fractions were collected every 30 s and numbered according to HPLC retention time (Fig. 3A). The radioactive fractions were identified by application of 1 μl of each fraction to a TLC plate, followed by Betascope detection (Fig. 3B). Fractions at the following times were found to be radioactive and are hereafter designated by their retention time: 4, 18, 18.5, 20.5, 21, 21.5, 22, and 23.

Each radioactive HPLC fraction was aligned with each phosphopeptide from two-dimensional phosphopeptide map by subjecting each HPLC fraction to one-dimensional TLE and one-dimensional TLC (Fig. 4, A and B). The unique mobility in one dimension of each spot allowed us to correlate the HPLC fraction with the two-dimensional phosphopeptide map. TLE analysis indicated that each HPLC fraction contained primarily one radioactive peptide, except for fraction 4, which contained two peptides (designated peptide b/c on the two-dimensional phosphopeptide map). TLC analysis indicated that each HPLC fraction contained primarily one radioactive peptide, except for fraction 21.5 which was separated into two phosphopeptides on TLC (designated peptide e,f). The TLE and TLC results
matched the HPLC results in which both fraction 4 and 21.5 eluted as doublets. The HPLC chromatographic pattern was consistent with TLE (each consecutive fraction (except part of 4 and 21.5) generally became less charged; Fig. 4A) and TLC (each consecutive fraction became more hydrophobic; Fig. 4B). The results from one dimensional analysis allowed the correlation of each radioactive HPLC peptide fraction with each two-dimensional phosphopeptide spot (peptide designation shown at the bottom of Fig. 4). The position of each fraction is indicated by a dotted line on the right of each panel. Each lane is designated with HPLC retention time and peptide letter.

**MALDI-MS Identification of Peptides**—MALDI-MS analysis was performed on each radioactive HPLC fraction to determine the identity of the CD45 phosphopeptides. The observed mass values obtained for each radioactive HPLC fraction were compared with a table of predicted masses of all possible tryptic peptides of CD45 cytoplasmic domain below 3000 Da (including partially digested, unphosphorylated, and phosphorylated peptides). Each sample was then treated with alkaline phosphatase and subjected to re-analysis by MALDI-MS to observe loss of phosphate (~80 Da or multiples of ~80 Da shifts in the spectra). The results of MALDI-MS analysis for each radioactive HPLC fraction are described below.

**Peptide a, Fraction 18.5**—The MALDI-MS spectrum of HPLC fraction 18.5 contained a single peak at m/z 2960.2 (Fig. 5A). Comparison with a table of calculated mass values of all possible tryptic peptides of CD45 cytoplasmic domain (724 possible peptides) indicated a close match to the predicted peak at 2958.9, corresponding to the monophosphorylated peptide 1239–1268 (Tables I and II). Dephosphorylation of the sample followed by MALDI-MS clearly showed a loss of only one phosphate (~80 Da) to m/z 2880.0. Because phosphoamino acid analysis showed peptide a was phosphorylated equally on Ser and Thr, it must therefore consist of a mixture of phosphopeptides with the same sequence: one with a single Ser(P) and one with a single Thr(P). The Ser(P) and Thr(P) sites were localized using the following strategy. Digestion of peptide a with NH$_2$OH at Asn-Gly resulted in a single $^{32}$P-phosphopeptide, which eluted at 21 min from HPLC, compared with undigested peptide a, which eluted at 18.5 min (data not shown). This HPLC-purified peptide contained both Ser(P) and Thr(P) (data not shown) and had a slightly slower mobility by TLC than peptide a (Fig. 5B, lanes 2 and 3). (HPLC purification was required to remove salts before TLC or enzymatic digestion.). Thorough digestion of the HPLC purified radioactive peptide with endoproteinase Glu-C resulted in only one $^{32}$P-phosphopeptide with altered TLC mobility (Fig. 5B, lane 4), which contained both Ser(P) and Thr(P) (data not shown). Thus we conclude that the phosphorylation sites of peptide a were Thr$^{1246}$ and Ser$^{1244}$. This conclusion is based on the observation that only the N-terminal portion of peptide a, after digestion at Asn-Gly, contains Glu. Furthermore, extensive digestion of this peptide with endoproteinase Glu-C results in only one product with a single Ser and a single Thr.

**Peptides b and c, Fraction 4**—Fraction 4 contained two extremely hydrophilic Ser(P)-containing phosphopeptides, b and c, which were found in the non-retained fraction from the HPLC column. Mass spectral signals for this fraction were not obtained. Their signals were probably suppressed by co-eluting salt contaminants, and efforts to remove the peptides from these contaminants were not successful.

**Peptide d, Fraction 21**—MALDI-MS analysis of fraction 21, peptide d (Fig. 6A), exhibited one phosphorylated peak and one non-phosphorylated CD45 peptide. The mass spectral peak at m/z 2127.4 shifted to m/z 2048.3 (~80 Da) after treatment with alkaline phosphatase (Table I). Ser$^{1240}$ was identified as the phosphorylation site, since there is only one Ser in this peptide and Thr(P) was not detected by phosphoamino acid analysis. The other major peak at m/z 1548.0 did not move after phosphatase treatment and was identified as an unphosphorylated CD45 peptide (573–585) from the membrane-proximal region of the cytoplasmic domain.

**Peptide e/f, Fraction 21.5**—MALDI-MS analysis of fraction 21.5 showed three major peaks that correspond to CD45 peptides in the fraction (Fig. 6B). One at m/z 1548.0 represented the same unphosphorylated fragment identified in the previous fraction. The other two peaks, m/z 1886.2 and 1999.6, were found to shift by ~80 Da after dephosphorylation: m/z 1999.6 to m/z 1921.6 and m/z 1886.2 to m/z 1807.5 (Fig. 6B). Two phosphopeptides were also found in this fraction by one-dimensional TLC (Fig. 4B). The peak at m/z 1999.6 was compared with the calculated mass values and identified as peptide e with a calculated m/z of 1999.0 (Table I). The only possible phosphorylation site, Ser$^{1244}$, was the same phosphorylation site of peptide d, and the difference between phosphopeptide d and e is one N-terminal Lys.

The observed mass of peptide f, m/z 1886.2 (Fig. 6B), did not match any tryptic phosphopeptide of CD45 but instead matched a product of peptide d involving loss of the C-terminal...
GGK from peptide \( d \) (loss of 242.4 Da (calculated)). Instability of the \( \text{Asp} - \text{X} \) (D-X) peptide bond at low pH is well known and likely resulted in backbone cleavage of the D-G bond in our peptides (28–31), and the procedure used involved dissolving the peptides in pH 1.9 buffer before electrophoresis or HPLC. Peptide \( e \) was phosphorylated since 80 Da was lost after phosphatase treatment. Peptide \( f \), resulting from loss of GGK, was found in the appropriate position in HPLC (more hydrophobic), TLC (more hydrophobic) and TLE (more negatively charged) compared with peptide \( d \). The TLC and TLE positions of peptides \( e \) and \( f \) were distinguished because a portion of peptide \( e \) was found in the preceding HPLC fraction 21 (Fig. 4B), which resulted in a weak signal of \( m/z \) 1999.6 in the mass spectra of fraction 21. Despite the fact that these peptides resulted from

**FIG. 5.** Analysis of HPLC fraction 18.5 from a tryptic digest of \textit{in vivo} labeled CD45. A, HPLC fraction 18.5 was analyzed by MALDI-MS before (top panel) and after phosphatase treatment (bottom panel). Results showing observed and calculated peptide masses are shown in Table I. The \( m/z \) value of peptide \( a \) is indicated in the top panel, and the \( m/z \) value of the dephosphorylated peptide (~80 Da) is shown in the bottom panel. B, one-dimensional TLC of peptide \( a \) after chemical and enzymatic digestion. Lane 1, total CD45 tryptic \( 32^{\text{P}} \)-phosphopeptides; lane 2, peptide \( a \) isolated from two-dimensional phosphopeptide map; lane 3, the product of hydroxylamine cleavage (HPLC-purified) of peptide \( a \); lane 4, endoproteinase Glu-C treatment of the peptide in lane 3.

**TABLE I**

| HPLC fraction | Peptide | Peptide site | No. of P\(^\circ\) | MH\(^\circ\) | MH\(^\circ\) after phosphatase |
|---------------|---------|--------------|-----------------|---------|-----------------------------|
| 18.5          | a\(^b\) | GVGTPEP\(124^\text{TS}\)Nps\(124^\text{PS}\)AEEPEHAANGSPAPTQSS\(^b\) | 1239–1268 | 1 | 2960.2 | 2958.9 | 2880.0 | 2878.9 |
| 21            | d       | KTnps\(120^\text{DS}\)QDKIEFHNEVDGGK | 1201–1218 | 1 | 2127.4 | 2127.2 | 2048.3 | 2047.2 |
|               |         | (np) \(^c\) | 573–585 | 0 | 1548.0 | 1547.6 | 1547.9 | 1547.6 |
| 21.5          | e       | TNps\(120^\text{DS}\)QDKIEFHNEVDGGK | 1202–1218 | 1 | 1999.6 | 1999.0 | 1921.6 | 1919.0 |
|               | f       | KTnps\(120^\text{DS}\)QDKIEFHNEVD | 1202–1215 | 1 | 1886.2 | 1884.8 | 1807.5 | 1804.8 |
|               |         | (np) \(^c\) | 573–585 | 0 | 1548.0 | 1547.6 | 1549.2 | 1547.6 |
| 22            | g       | TNps\(120^\text{DS}\)QDKIEFHNEVD | 1202–1215 | 1 | 1757.4 | 1756.6 | 1677.1 | 1676.6 |
| 23            | h       | NRnps\(13^\text{DS}\)NVVPYDFNR | 936–948 | 1 | 1676.1 | 1675.7 | 1595.4 | 1595.7 |
|               |         | NSNVVPYDFNR (np) \(^c\) | 938–948 | 0 | 1325.6 | 1325.4 | 1324.4 | 1325.4 |

\(^a\) No. of phosphates in each peptide determined from phosphatase treatment.

\(^b\) Peptide \( a \) consists of a monophosphorylated form containing either phosphothreonine or phosphoserine (pS) as indicated.

\(^c\) (np), a non-phosphorylated CD45 peptide.
chemical cleavage, the peptide maps were remarkably reproducible.

Peptide **g**, Fraction 22—The MALDI-MS spectrum of fraction 22 displayed a number of signals. Only one peptide, m/z 1757.4 (Fig. 6C, Table I), shifted by 280 Da to m/z 1677.1 after phosphatase treatment. No putative tryptic phosphopeptide has a m/z value close to 1757.4, but this number matched that expected from the loss of GGK from peptide **e**. Thus the four peptides, **d**, **e**, **f**, and **g**, were all derived from the same sequence region and therefore represent a single phosphorylation site, Ser1204.

Peptide **h**, Fraction 23—MALDI-mass spectrometry of HPLC fraction 23, peptide **h**, showed two mass spectral peaks at m/z 1325.6 and m/z 1676.1 (Fig. 7, Table I). After dephosphorylation with alkaline phosphatase, the peak at m/z 1325.6 remained unchanged (m/z 1324.4) and the peak at m/z 1676.1 shifted to m/z 1596.4 (-80 Da, loss of one phosphate). This mass matched a tryptic phosphopeptide with a calculated mass of 1675.7 designated **h** (Table I). Since there is only one Ser residue in this peptide, the phosphorylation site is Ser939. This phosphopeptide includes one site (Arg937) that is not cleaved by trypsin when the sequence -RXpS- is present (24). The second peak in this fraction, at m/z 1325.6, matched unphosphorylated form of the same peptide with a slightly shorter N terminus. Thus the unphosphorylated and phosphorylated Ser939 peptides were present in the same mass spectrum.

**In Vitro PKC Phosphorylation of CD45**—Since CD45 phosphorylation increases after PMA treatment of cells (21, 22), we wished to determine if any of the sites that we identified could serve as substrates for PKC phosphorylation. The phosphorylation sites at Ser939 (**h**) and Ser1204 (d-g) conform to the consensus sequence described for PKC (see “Discussion”). Activation of 70Z/3.12 cells with PMA resulted in increased phosphorylation on both Ser939 and Ser1204 (data not shown), but it is difficult to conclude that this phosphorylation was a direct result of PKC activity. To resolve this question, bacterially
expressed, purified murine CD45 cytoplasmic domain was phosphorylated by a purified preparation of rat brain PKC (Fig. 8). Phosphorylated CD45 was gel-purified to separate it from the lightly autophosphorylated PKC present after the in vitro kinase reaction (Fig. 8A). Tryptic phosphopeptide mapping of CD45 indicated that there were several CD45 peptides phosphorylated. However, only one peptide matched the mobility of an in vivo phosphorylated peptide, i.e. peptide h. That peptide h is an in vitro target of PKC was confirmed by mixing the eluted PKC peptide labeled h (from Fig. 8B) with in vivo labeled CD45 peptides (Fig. 8D). PKC-labeled peptide h contained only Ser(P) (data not shown), and its position was precisely coincident with in vivo labeled peptide h.

**DISCUSSION**

The identification of CD45 in vivo phosphorylation sites is essential to determine which of the many possible sites may be functionally relevant to the modulation of activity and to the docking of CD45 associated proteins. The current study was designed to characterize the in vivo phosphorylation sites of CD45. Four CD45 in vivo phosphorylation sites were identified using a combination of two-dimensional phosphopeptide mapping, reverse-phase HPLC, phosphoamino acid analysis, MALDI-MS, and specific enzymatic degradation. The method used is advantageous because phosphopeptides can be detected in the picomole range, because phosphorylation can be confirmed by observation of a loss of 80 Da after dephosphorylation, and because the target phosphopeptide does not need to be absolutely pure (26). The major type of phosphorylated amino acid observed in CD45 was Ser(P) with a minor amount of Thr(P) in peptide a. This is the first time that Thr(P) has been observed in CD45. Three phosphorylation sites were identified in the C-terminal tail, Ser1204, Thr1246, and Ser1248 (summarized in Fig. 9) and one phosphorylation site, Ser939, was in the D2 domain (peptide h). No phosphorylation sites were observed in the D1 domain or in the connector between PTPase domains.

The phosphorylation site at Ser939 (peptide h) is conserved in mouse, rat, and human CD45; instead of Ser939, chicken and shark CD45 have a Ser in an adjacent residue. In addition, the sequence around the Ser939 is highly conserved among PTPases. The three-dimensional structure of the phosphatases has been found to be remarkably conserved (32), and comparison of CD45 and PTP1B predicts that Ser939 is located at the apex of the substrate binding loop at a position homologous to Arg47 in PTP1B (33). This suggests that phosphorylation of Ser939 may be important in the regulation of binding of substrates in the D2 domain or in the binding of signaling proteins which may interact with the D2 active site. Ser939 also falls in
FIG. 9. Location of in vivo phosphorylation sites in CD45 cytoplasmic domain. The CD45 cytoplasmic domain from amino acid 567 to 1268 (C-terminal) is depicted including the PTPase homology domains (D1 and D2), the catalytic Cys817 in the PTPase I domain (C) and the homologous Cys132 in PTPase II domain (C). In vivo phosphorylation sites are indicated by the amino acid number.

the homologous interaction region resulting in dimer formation of RPTPα D1, which blocks the active site (34). Potential dimerization of CD45 (D1-D1 or D1-D2) may modulate activity since the PTPase activity of a CD45-epidermal growth factor receptor chimeric molecule was functionally inactivated upon in vitro PTPase activity of a CD45-epidermal growth factor receptor chimeric molecule was functionally inactivated upon inhibition of epidermal growth factor receptor.

Peptide h is also of interest because it contains the target for Abl kinase, which is proposed to modulate the PTPase activity of CD45 cytoplasmic domain from amino acid 567 to 1268 (C-terminal) as described previously (35, 36). Phosphotyrosine was not detected in our in vivo study, even when the PTPase inhibitor (phenylarsine oxide) was included in the isolation. It is possible that CD45 tyrosine phosphorylation occurs transiently or at very low stoichiometry. Peptide h also overlaps with a fodrin-binding site (E930ENKKKNRNS939) of CD45, and fodrin binding increases the PTPase activity of CD45 (35, 36). Phosphorylation at Ser939 may serve to regulate the binding of CD45 to the cytoskeleton. Other studies have suggested that CD45 interacts with the cytoskeleton and has a role in the coordination of cytoskeletal remodeling (37, 38).

In the current report, peptide h (Ser939) was not the major phosphorylated peptide observed. However, it is unlikely that phosphorylation reached equilibration during the labeling since CD45 is expressed at high levels and the turnover of CD45 protein is very slow (39). Thus, the phosphorylation of CD45 protein is very slow (39). Thus, the phosphorylation of CD45 in our study probably resulted primarily from turnover of phosphate at individual phosphorylation sites. It is difficult to reach conclusions about stoichiometry from our 32P incorporation experiments, since, for example, a very stable phosphorylation site would only become weakly phosphorylated. In the current study the stoichiometry of phosphorylation at Ser939 was more directly estimated by comparison of the MALDI-MS signals for peptide h and an almost identical non-phosphorylated peptide containing Ser939 in the same HPLC fraction (Table I and Fig. 7). Assuming comparable trypsin cleavage, the intensity of the MALDI-MS signal of the dephosphorylated form of peptide h was of the same magnitude as the equivalent non-phosphorylated peptide in the same mass spectrum, suggesting equal abundance.

The phosphorylation site at Ser1204 is conserved in mouse and rat but not in human, chicken, or shark CD45. The other sites in the C-terminal tail, Thr1246 and Ser1248, are not precisely conserved among these species, but this region (within 1–2 residues) contains multiple Ser and/or Thr residues suggesting that this may also be a phosphorylation site common to all species. The MALDI-MS signals of peptides b and c (HPLC fraction 4) were not observed because of the presence of salts in this fraction. It is possible that phosphopeptides in this fraction are homologous to a peptide obtained from a tryptic digest of in vivo 32P-labeled, human Jurkat cell CD45 (40). This sequence, which is highly conserved in mouse CD45, contains casein kinase II phosphorylation sites and in vitro phosphorylation of the cytoplasmic domain of CD45 by casein kinase II revealed one major phosphopeptide which migrated very close to peptides b and c in two-dimensional mapping (data not shown).

The phosphorylation sites Ser1204 and Ser1204 conform to the consensus sequence described for PKC (41). We have confirmed that, of these potential in vivo phosphorylation sites, only Ser1204 is phosphorylated in vivo. Several other peptides were also labeled in the in vitro PKC reaction, which did not appear in vivo labeled CD45. Increased phosphorylation at Ser939 and Ser1204 was observed after PMA treatment of 32P-labeled, human Jurkat cell CD45 (40). It has been reported that other PTPases such as PTP1B, RPTPα, and PTP-PEST are also substrates of PKC (43–45), and in vitro PKC phosphorylation of PTP-PEST resulted in decreased PTPase activity (45).

Our results suggest that Ser939 may be an important phosphorylation site, which is phosphorylated in vivo by PKC. This phosphorylation may play a role in the regulation of D2 domain activity, in potential dimerization involving the D2 domain, or in the interactions of other molecules with the active site of the D2 domain. The C-terminal phosphorylation sites may also have a regulatory role in CD45 activity. Direct confirmation of the role of these phosphorylation sites in the function of CD45 is currently under investigation by site-directed mutagenesis.

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REFERENCES

1. Trowbridge, I. S., and Thomas, M. L. (1994) Annu. Rev. Immunol. 12, 85–116
2. Justement, L. B., Brown, V. K., and Lin, J. (1994) Immunol. Today 15, 399–406
3. Woodward-Thomas, T., and Thomas, M. L. (1993) Semin. Cell Biol. 4, 409–418
4. Donovan, J. A., and Koretzky, G. A. (1993) J. Am. Soc. Nephrol. 4, 976–985
5. Koretzky, G. A., Fiers, J., Thomas, M. L., and Weiss, A. (1990) Nature 346, 66–68
6. Pinel, J. T., and Thomas, M. L. (1989) Cell 58, 1055–1065
7. Desai, D. M., Sap, J., Silvennoinen, O., Schlessinger, J., and Weiss, A. (1994) EMBO J. 13, 4009–4010
8. Hovis, R. R., Donovan, J. A., Musci, A. M., Mooto, D. G., Goldman, F. D., Ross, S. E., and Koretzky, G. A. (1993) Science 260, 544–546
9. Desai, D. M., Sap, J., Schlessinger, J., and Weiss, A. (1993) Cell 73, 541–554
10. Vogler, S., Naidinka, B. B., Burns, C. M., Fieker, P. M., and Weiss, A. (1992) EMBO J. 11, 3041–3046
11. Justement, L. B., Campbell, K. S., Chien, N. C., and Cambier, J. C. (1991) Science 253, 1839–1842
12. Ostergaard, H. L., Shackelford, D. A., Hurley, T. R., Johnson, P., Hyman, R., Selton, B. M., and Trowbridge, I. S. (1988) Proc. Natl. Acad. Sci. U. S. A. 86, 8959–8963
13. Hurley, T. R., Hyman, R., and Selton, B. M. (1993) Mol. Cell. Biol. 13, 1651–1656
14. Mustelin, T., Pessa-Morikawa, T., Autero, M., Gassmann, M., Andersson, L. C., Gahlenberg, C. G., and Burn, P. (1990) Eur. J. Immunol. 20, 1173–1178
15. Fisch, M., Bolen, J. B., and Weissman, A. M. (1992) Eur. J. Immunol. 22, 1173–1178
16. Fischer, E. H., Charbonneau, H., and Tonks, N. K. (1991) Science 253, 401–406
17. Melkerson-Watson, L. J., Waldmann, M. E., Gunter, A. D., Zaroukian, M. H., and Essef, W. J. (1994) J. Immunol. 153, 2004–2013
18. Autero, M., Saharinen, J., Pessa-Morikawa, T., Soula-Rothhut, M., Oetken, C., Gassmann, M., Bergman, M., Alitalo, K., Burn, P., Gahlenberg, C. G., and Mustelin, T. (1994) Mol. Cell. Biol. 14, 1308–1321
19. Stover, D. R., and Walsh, K. A. (1994) Mol. Cell. Biol. 14, 5525–5532
20. Ostergaard, H. L., and Trowbridge, I. S. (1991) Science 253, 1423–1425
21. Yamada, A., Streuli, M., Saito, H., Rothstein, D. M., Schlossman, S. F., and Morimoto, C. (1990) Eur. J. Immunol. 20, 1655–1660
22. Autero, M., and Gahmberg, C. G. (1987) Eur. J. Immunol. 17, 1503–1506
23. Valentine, M. A., Widmer, M. B., Ledbetter, J. A., Pinauld, F., Voice, R., Clark, E. A., Gallis, B., and Brautigan, D. L. (1991) Eur. J. Immunol. 21, 913–919
24. van der Geer, P., Lou, K., Sefton, B. W., and Hunter, T. (1994) in Cell Biology: A Laboratory Handbook (Celis, J. E., ed) Vol. 3, pp. 422–447, Academic Press, Inc., San Diego
25. Hunter, T., and Sefton, B. M. (1985) Proc. Natl. Acad. Sci. U. S. A. 77, 1311–1315
26. Liao, P. C., Leykam, J., Andrews, P. C., Gage, D. A., and Allison, J. (1994) Anal. Biochem. 219, 9–20
27. Ng, D. H., Maiti, A., and Johnson, P. (1995) Biochem. Biophys. Res. Commun. 206, 302–309
28. Oliyai, C., and Borchardt, R. T. (1993) Pharm. Res. 10, 95–102
29. Tsuda, T., Uchiyama, M., Sato, T., Yoshino, H., Tsuichya, Y., Ishikawa, S., Ohmae, M., Watanabe, S., and Miyake, Y. (1995) J. Pharm. Sci. 79, 223–227
30. Brennan, T. V., and Clarke, S. (1993) Protein Sci. 2, 331–338
31. Oliyai, C., and Borchardt, R. T. (1994) Pharm. Res. 11, 751–756
32. Fauman, E. B., and Saper, M. A. (1996) Trends Biochem. Sci. 21, 413–417
33. Jia, Z., Barford, D., Flint, A. J., and Tonks, N. K. (1995) Science 268, 1754–1758
34. Bilwes, A. M., den Hertog, J., Hunter, T., and Noel, J. P. (1996) Nature 382, 555–559
35. Lokeshwar, V. B., and Bourguignon, L. Y. W. (1992) J. Biol. Chem. 267, 21551–21557
36. Iida, N., Lokeshwar, V. B., and Bourguignon, L. Y. W. (1994) J. Biol. Chem. 269, 28576–28583
37. Arendt, C. W., Hsi, G., and Ostergaard, H. L. (1995) J. Immunol. 155, 5095–5103
38. Klaus, S. J., Sidorenko, S. P., and Clark, E. A. (1996) J. Immunol. 156, 2743–2753
39. Deans, J. P., Boyd, A. W., and Pilarski, L. M. (1989) J. Immunol. 142, 1239–1238
40. Stover, D. R., and Walsh, K. A. (1993) in Techniques in Protein Chemistry (Angeletti, R. H., ed) Vol. IV, pp. 193–204, Academic Press, San Diego
41. Kennelly, P. J., and Krebs, E. G. (1991) J. Biol. Chem. 266, 15555–15558
42. Shackelford, D. A., and Trowbridge, I. S. (1986) J. Biol. Chem. 261, 8334–8341
43. Flint, A. J., Gehbink, M. F., Franza, B., Jr., Hill, D. E., and Tenks, N. K. (1993) EMBO J. 12, 1937–1946
44. Tracy, S., van der Geer, P., and Hunter, T. (1995) J. Biol. Chem. 270, 10587–10594
45. Garton, A. J., and Tenks, N. K. (1994) EMBO J. 13, 3763–3771