Activated Protein Kinase C Directly Phosphorylates the CD34 Antigen on Hematopoietic Cells*

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The CD34 antigen is a human leukocyte membrane protein expressed specifically by lymphohematopoietic progenitor cells. We found that CD34 is a phosphoprotein and therefore examined the regulation of its phosphorylation. Activation of protein kinase C (PKC) enhanced CD34 phosphorylation. The PKC activators, 12-O-tetradecanoylphorbol-13-acetate and bryostatin-1, induced rapid, stoichiometric hyperphosphorylation of CD34 protein in cells, resulting in a 5-fold increase in CD34 phosphorylation. In vitro kinase studies revealed that purified PKC could directly phosphorylate purified CD34. Only serine phosphorylation was detected in the CD34 molecule. Two-dimensional phosphopeptide mapping experiments indicated that PKC induces the phosphorylation of identical serine residue(s) in vitro and in vivo (in KG1 cells). These are newly phosphorylated serine residue(s), which are not detectably phosphorylated in CD34 from exponentially growing KG1 cells. These data indicate that the developmental stage-specific molecule, CD34, is a phosphorylation target for activated PKC. Furthermore, these findings raise the possibility that PKC activation and phosphorylation of the CD34 molecule may play a role in signal transduction during early lymphohematopoiesis.

CD34 is a membrane-associated glycoprotein whose surface expression is confined to lymphohematopoietic (1-4) and some endothelial cells (5, 6). Since CD34 binds to its ligand, the corresponding protein may be a receptor for the CD34 molecule and therefore examined the regulation of its phosphorylation. Activation of protein kinase C (PKC) enhanced CD34 phosphorylation. The PKC activators, 12-O-tetradecanoylphorbol-13-acetate and bryostatin-1, induced rapid, stoichiometric hyperphosphorylation of CD34 protein in cells, resulting in a 5-fold increase in CD34 phosphorylation. In vitro kinase studies revealed that purified PKC could directly phosphorylate purified CD34. Only serine phosphorylation was detected in the CD34 molecule. Two-dimensional phosphopeptide mapping experiments indicated that PKC induces the phosphorylation of identical serine residue(s) in vitro and in vivo (in KG1 cells). These are newly phosphorylated serine residue(s), which are not detectably phosphorylated in CD34 from exponentially growing KG1 cells. These data indicate that the developmental stage-specific molecule, CD34, is a phosphorylation target for activated PKC. Furthermore, these findings raise the possibility that PKC activation and phosphorylation of the CD34 molecule may play a role in signal transduction during early lymphohematopoiesis.

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EXPERIMENTAL PROCEDURES

Materials—Murine monoclonal My10 (IgG, directed against CD34 antigen) and MOPC 21 (IgG, irrelevant antibody) ascites were obtained as described previously (1). TUK3 (IgG, directed against CD34 antigen) ascites was kindly provided by Dr. B. Uchanska-Ziegler (Institute for Experimental Immunology, Marburg, Germany) (4). Bryostatin-1 was generously provided by Dr. G. Robert Pettit (Arizona State University, Tempe, AZ). 12-O-tetradecanoylphorbol-13-acetate (TPA) and 4-a-phorbol were purchased from Sigma. All other reagents were purchased from commercial sources.

Cell Culture—Human leukemic cell lines were cultured as described (1). KG1 and KG1A (an undifferentiated subline of KG1) myelogeneous leukemia cells (14, 15) were kindly provided by Dr. D. Golde (UCLA). The T-lymphoblastic cell lines RPMI 8402 (16) and MOLT 13 (derived by Dr. J. Minowada, Hayashibara Biochemical Laboratories, Okayama, Japan) were obtained from the Roswell Park Cell Repository (Buffalo, NY) and Dr. Michael Brenner (Dana Farber Cancer Institute, Boston, MA), respectively.

Metabolic Labeling and Treatment of Cells—Cells in the exponential phase of growth were washed with phosphate-free RPMI 1640 media (GIBCO) and equilibrated with carrier-free [32P]orthophosphate (Amersham Corp.; 100 μCi/ml, 60-75 min, 37°C). Cells (4 × 10⁷) were treated for 30 min with PKC activators or growth factors. Following treatment, cells were washed in phosphate-buffered saline at 4°C. Subsequent manipulations were carried out at 4°C. The cell pellet was lysed in 250 μl of detergent disruption buffer (10 mM Tris-HCl, pH 7.5, containing 1% Nonidet P-40, 200 units/ml of Trasylol (Moby Chemical, New York), 0.005% leupeptin, and 10 mM EDTA), and vortexed vigorously every 5 min, for 30 min. The lysate was centrifuged at 15,000 × g for 10 min in a Beckman microcentrifuge, quenching and determination of the amino acid and carbohydrate compositions, have revealed that the CD34 antigen is a highly acidic (pI < 4.0), heavily glycosylated cell surface molecule of 110,000 daltons (6, 7, 48). In the course of these studies, our experiments indicated that CD34 is a phosphoprotein. Since phosphorylation is often involved as a mechanism for functional regulation of surface glycoproteins, such as growth factor receptors, we have further investigated this property of CD34 reasoning that it may provide some clue(s) concerning the regulation and function of CD34 on hematopoietic cells. The findings reported here indicate that CD34 can be stoichiometrically phosphorylated by protein kinase C (PKC), when the latter is activated by tumor-promoting phorbol esters or bryostatin-1 (8). Since PKC, a Ca²⁺/phospholipid-dependent serine/threonine protein kinase, appears to mediate hematopoietic growth and differentiation (9, 10) and can phosphorlyate specific membrane-bound growth factor receptors (e.g. epidermal growth factor (11)), as well as certain leukocyte differentiation antigens (e.g. CD3, CD4, CD8, CD43, CD45 (12, 13)), we sought to determine the mechanism(s) whereby CD34 was phosphorylated and the consequences, if any, of this covalent modification.
Protein Kinase C Phosphorylates the CD34 Antigen

and CD34 protein was immunoprecipitated from the resulting supernatant. Immunoprecipitation of CD34—To immunoprecipitate the CD34 antigen, the cellular lysates (the equivalent of 2–4 × 10^7 cells) were precleared by adding 100 μl of phosphate-buffered saline containing 2% bovine serum albumin, 200 μl of Sansorbin (Calbiochem), and 40 μl of Protein A-Sepharose (Sigma). The resulting suspension was mixed by rocking for 30 min and then centrifuged at 15,000 × g for 10 min. The precleared lysate was incubated for 2 h with monoclonal My10 (± TUK3) antibody-coated Sepharose (40 μl; prepared by preincubating Protein A-Sepharose with rabbit anti-mouse IgG (Southern Biotechnology, Birmingham, AL), washing, then incubating with an equal mixture of these two monoclonals). Immune complexes were then washed extensively in phosphate-buffered saline, then boiled for 3 min in Laemmli sample buffer (17) before electrophoresis. In some cases, the CD34 contained in the complexes was desialylated by treatment with neuraminidase (1 unit/ml in 50 μl 2% bovine serum albumin, 200 μl of Sansorbin (Calbiochem), and 40 μl of 100 mM sodium acetate, 2% 30:9:36) until the leading edge of the solvent had reached 1–2 cm from the top (~45 min).

Phosphoamino Acid Analysis—A portion of the resulting tryptic digests from 32P-labeled CD34 antigen was incubated in 500 μl of 6 N HCl at 105°C for 60 min as described (21). The standards were identified using ninhydrin spray (Sigma). 32P-labeled phosphoamino acids were visualized by autoradiography.

RESULTS

Activators of Protein Kinase C Mediate Hyperphosphorylation of the CD34 Antigen in Cells—The CD34+ cell lines, KG1 and KG1a myelogenous leukemia cells, and RPMI 8402 and MOLT 13 T-lymphoblastic leukemia cells were equilibrated in [32P]orthophosphate, and the CD34 antigen was immunoprecipitated from cellular lysates by using specific antibodies. Low level incorporation of [32P]-labeled phosphate into the 110-kDa CD34 molecule was detected in exponentially growing CD34+ cells (Fig. 1A, lane 1). To confirm that the immunoprecipitated, [32P]-labeled 110-kDa protein identified was the CD34 molecule, we treated the resulting immune complex with neuraminidase to release terminal sialic acid residues before SDS-PAGE. The apparent molecular weight of the CD34 molecule shifted up from M_0 = 110,000 to M_0 = 150,000 after this treatment, a characteristic previously described for the asialo-CD34 molecule (Fig. 1B) (6, 7).

Since PKC activators such as the phorbol ester, TPA, and the macrocyclic lactone, bryostatin-1, have been reported to stimulate hematopoietic progenitor cells in culture (22–24), we wished to determine whether the CD34 antigen could be phosphorylated by activated PKC. As a first approach, after addition of a biologically active concentration of TPA (23, 24), hyperphosphorylation of the CD34 antigen was detected in all CD34+ cell lines tested (Fig. 1A, lanes 2). TPA and bryostatin-1 (as well as 4-β-phorbol 12,13-didecanoate, in experiments not shown) induced similar hyperphosphorylation of the CD34 antigen in KG1 cells (Fig. 2). In contrast, the biologically inactive 4-α-phorbol or the solvent dimethyl sulfoxide, failed to increase CD34 phosphorylation (Fig. 2). Hyperphosphorylation was rapid and could be detected within 1 min following TPA addition (results not shown). The irrelevant isotype-matched antibody, MOPC 21, did not immunoprecipitate this phosphoprotein (Fig. 1A). These results suggest that activated PKC may directly phosphorylate CD34.

Since PKC activators can mimic certain effects of hematopoietic growth factors (23, 25, 26), we assessed whether hematopoietic growth factors could mediate CD34 phosphorylation. KG1 cells are known to bind and metabolize IL-3 and GM-CSF (27), and we would expect these growth factors to mediate a biological response in these cells. Neither IL-3, GM-CSF, G-CSF, M-CSF, nor IL-1α, added at saturating concentrations for 30 min at 37°C, altered the phosphorylation status of the CD34 molecule in KG1 cells (data not shown). The lack of effect of these growth factors on CD34 phosphorylation is difficult to interpret since we cannot rule out the possibility that this is a KG1-specific phenomenon. However, neither TPA nor the hematopoietic growth factors changed the level of CD34 expression on the KG1 cell surface, as assessed by flow cytometry and Western blotting (results not shown). Also, no change in CD34 expression was observed when cells were cultured with 100 nM TPA for up to 3 days (results not shown). These results suggest that while PKC might directly phosphorylate the CD34 antigen, hyperphosphorylation of CD34 by PKC does not regulate its level of phosphorylation.
PKC Directly Phosphorylates the CD34 Antigen in Vitro—

To determine whether PKC directly phosphorylates CD34, purified CD34 protein was mixed with purified rat brain PKC in a cell-free phosphorylation system (Fig. 3, lane 1) (28). The results indicate that the CD34 antigen can be phosphorylated directly by the activated PKC (Fig. 3, lane 1). CD34 phosphorylation did not occur in the absence of calcium, phosphatidylserine, and TPA, indicating that CD34 phosphorylation is a Ca**+/phospholipid-dependent reaction, consistent with the known requirements for PKC activation (Fig. 3, lane 4). In the absence of PKC, no CD34 phosphorylation was observed, indicating that CD34 does not contain intrinsic autokinase activity under these assay conditions (Fig. 3, lane 2). These results indicate that the CD34 antigen can be phosphorylated directly by activated PKC. As can be seen in Fig. 3, autophosphorylation of PKC (Mr = 80,000) was detected whether or not CD34 antigen was present (lane 1 and lane 3). We also noted that purified CD34, when added at higher concentrations (up to 4 µg), could enhance the autophosphorylation of PKC; however, the significance of this finding is unknown (data not shown).

CD34 Antigen Is Stoichiometrically Phosphorylated—Since PKC is a Ser/Thr protein kinase, we determined which amino acid(s) of the CD34 protein were phosphorylated in response to TPA. Phosphoamino acid analysis was performed on acid-hydrolyzed, 32P-labeled immunoprecipitated CD34 antigen. Serine was the only amino acid found to be phosphorylated in CD34, isolated from untreated (Fig. 4, left lane) or TPA-stimulated (middle lane) cells or from in vitrol phosphorylated CD34 (right lane). These findings are compatible with hyperphosphorylation of CD34 being mediated by activated PKC.

CD34 Antigen Is Stoichiometrically Phosphorylated—If the hyperphosphorylation of CD34 in cells is a physiologically relevant effect, the CD34 phosphorylation would be stoichiometric. In contrast, if the observed incorporation of phosphate represented only a slight (i.e. nonstoichiometric) increase in the total phosphate incorporated per mol of CD34, then it could be reasonably argued that such a covalent modification
FIG. 4. Phosphoamino acid analysis of CD34 antigen. Phosphoamino acid analysis was performed on $^{32}$P-labeled CD34 antigen, immunoprecipitated from whole cells (treated ± TPA, left and middle panel), or phosphorylated in vitro (right panel), as described under "Experimental Procedures." $^{32}$P-labeled phosphoserine was detected, after exposure at $-80^\circ$C for 1 week. Samples were spotted at the bottom. The mobility of free phosphate (PO$_4^-$), phosphotyrosine (P-TYR), phosphothreonine (P-THR) and phosphoserine (P-SER) amino acid standards is indicated at the far left and the far right. Incompletely hydrolyzed $^{32}$P-labeled peptides migrated between the point of application of the sample and the phosphotyrosine standard.

TABLE I

Effect of TPA on intracellular ATP levels

Results represent the average of three separate experiments. From these values, the stoichiometry of phosphorylation of CD34 antigen in control (untreated) and TPA-stimulated KG1 cells was 0.20 and 0.90 mol of $^{32}$P incorporated/mol of CD34 antigen, respectively.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>$\text{pmol} \cdot 10^{-6} \text{ cell}$</th>
<th>$\text{Ci} \cdot \text{mol}^{-1}$</th>
</tr>
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<tbody>
<tr>
<td>Control</td>
<td>2062 ± 67</td>
<td>1322 ± 10</td>
</tr>
<tr>
<td>TPA</td>
<td>1970 ± 60</td>
<td>1343 ± 9</td>
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may have no physiological consequence. Accordingly, the stoichiometry of incorporation of $^{32}$P into the CD34 molecule was determined in KG1 cells. We estimate that following TPA stimulation, at least 1 mol of ($^{32}$P)ATP is incorporated for every mole of CD34 (Table I). Since only $\sim 20\%$ of the CD34 molecules were found to contain $^{32}$P in the untreated cells, this indicates that the addition of TPA induced a substantial increase in total phosphorylation of the CD34 antigen (i.e. $\sim 5$-fold), which resulted in stoichiometric phosphorylation.

PKC Mediates Phosphorylation of Distinct Sites on the CD34 Antigen—In order to determine whether activated PKC phosphorylates the same or unique serine site(s), peptide mapping studies were performed. Comparison of the site(s) of phosphorylation of CD34 was performed by two-dimensional separation of tryptic fragments of immunoprecipitated $^{32}$P-labeled CD34 antigen, which was purified from TPA-treated or untreated KG1 cells or from in vitro studies. Two new CD34 phosphopeptides were identified when KG1 cells were stimulated with TPA (peptides 1 and 2, Fig. 5, middle panel) which were not detected in untreated cells (top panel). The same two new phosphopeptides were present when purified CD34 antigen was phosphorylated directly by activated PKC in a cell-free system (Fig. 5, bottom panel). Furthermore, several small CD34 phosphoserine-containing peptides were identified in untreated KG1 cells (Fig. 5, top panel, arrowheads). The amplitude of phosphorylation of these smaller phosphopeptides was not altered by incubation of the cells with TPA (middle panel, arrowheads), indicating that they may not be sites of PKC phosphorylation.

DISCUSSION

The CD34 antigen is a developmentally restricted lymphohematopoietic progenitor cell surface molecule (1-4). While little is known about the regulation of hematopoietic cell growth/differentiation, evidence suggests that this may involve the biochemical transduction of cellular signals, mediated at least in part by protein kinase C. For example, activators of PKC can induce differentiation of human leukemic HL60 and KG1 cells (29, 30). Furthermore, the PKC activators TPA and bryostatin-1 stimulate growth of human bone marrow progenitor cells (22, 23, 31), while PKC inhibitors (e.g. staurosporine or H-7) block proliferation of both these normal cells (9, 10) and HL60 leukemia cells (32). Since activated PKC can regulate hematopoietic progenitor cells, and since CD34 is a progenitor cell-specific molecule, we were interested in whether CD34 is phosphorylated and if it is a physiologic substrate for PKC.

Our findings raise the possibility that CD34 antigen phosphorylation may be important for signal transduction in human lymphohematopoietic progenitor cells. Although earlier studies failed to demonstrate that the CD34 structure was phosphorylated in vivo (7), the more recent availability of higher affinity antibodies led us to retest this possibility. We found that the CD34 antigen is a phosphoprotein and represents a membrane target for activated PKC. Based on two-dimensional phosphopeptide maps, we found that hyperphosphorylation of CD34 is directly mediated by PKC (Fig. 5). Activated PKC selectively phosphorylates at least one unique...
site in the CD34 molecule which is not identified in growing cells (e.g. in the steady state). Furthermore, PKC activation is stoichiometric and results in a ~5-fold increase in 32P incorporation into CD34, and, thus, phosphorylation of CD34 may represent a physiologically significant post-translational modification.

Analysis of the human CD34 cDNA sequence reveals that the protein appears to be a type I transmembrane molecule. The predicted internal portion of the protein contains basic amino acid residues adjacent to Ser residues which represent at least two potential target sites for PKC phosphorylation (Arg × Ser and Ser × Arg) (33, 34; serine protein kinase target motifs reviewed by Traugh (35)). Furthermore, the serine from the latter of the two PKC sites may also serve as the distal phosphoserine in a consensus motif potentially recognized by glycogen synthase kinase-3 (Fa [Ser(X), Ser(P)] (36)). In addition, there are two other consensus motifs that correspond to potential target sites for a Ca2+/calmodulin-dependent kinase and/or protease-activated kinase (Arg × Ser) (37, 38). Interestingly, in exponentially growing KG1 cells there are additional phosphopeptides detected by two-dimensional phosphopeptide mapping which are not hyperphosphorylated following activation of PKC (Fig. 5). Thus, it is possible that these may be additional target sites in the CD34 molecule for one or more serine protein kinases other than PKC. This possibility is currently under investigation.

The functional significance of phosphorylation of the CD34 molecule is as yet unknown, but it might be comparable to the role that PKC plays in the activation of certain other molecules. PKC is known to phosphorylate leukocyte-associated differentiation antigens (12). PKC-mediated phosphorylation of certain of these surface proteins induces or is associated with surface receptor modulation, either down-regulation (e.g. CD4, CD3ϒ, CD71 (transferrin receptor), and the M-CSF receptor (c-fms)) or up-regulation (e.g. fibronectin receptor (39)). However, CD34 phosphorylation is apparently not associated with altered levels of surface expression. On the other hand, functional activation of surface proteins as a consequence of PKC-mediated phosphorylation may occur in the absence of surface modulation. This is apparently the case for CD8, which is linked to signal transduction via the CD2 and the CD3-CD4-CD8 pathways (40–43). Moreover, the sialophorin/leukosialin glycoprotein (CD43), another lymphohematopoietic cell-specific molecule, shares many biochemical/structural properties with the CD34 antigen (44, 45), and can even be phosphorylated by activators of PKC (12). While the physiological effect of CD43 phosphorylation is not known, it has been suggested that CD43 may be important for monocyte activation and possibly cellular adhesion (46, 47).

Thus, like the antigens described above, the CD34 molecule may transduce signal(s) which ultimately lead to cellular activation events including proliferation, differentiation, and/or cellular adhesion, even in the absence of surface modulation. While highly speculative, in the case of cellular adhesion, it is possible that phosphorylation of CD34 may induce conformational changes which modify its function.

Since CD34 is uniquely distributed on lymphohematopoietic progenitor cells and is phosphorylated by PKC, the CD34 antigen is a candidate molecule for participation in signal transduction in lymphohematopoietic progenitor cells of all lineages.

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