Normal Cellular and Transformation-Associated abl Proteins Share Common Sites for Protein Kinase C Phosphorylation

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Viral transduction and chromosomal translocations of the c-abl gene result in the synthesis of abl proteins with structurally altered amino termini. These altered forms of the c-abl protein, but not the c-abl protein, are detectably phosphorylated on tyrosine in vivo. In contrast, all forms of the c-abl protein are phosphorylated on serine following in vivo labeling with P32. Treatment of NIH-3T3 cells with protein kinase C activators resulted in a four- to eightfold increase in the phosphorylation of murine c-abl due to modification of two serines on the c-abl protein. Purified protein kinase C phosphorylated all abl proteins at the same two sites. Both sites are precisely conserved in murine and human abl proteins. The sites on the abl proteins were found near the carboxy terminus. In contrast, for the epidermal growth factor receptor (T. Hunter, N. Ling, and J. A. Cooper, Nature [London] 311:480-483, 1984) and pp60V-src (K. L. Gould, J. R. Woodgett, J. A. Cooper, J. E. Buss, D. Shalloway, and T. Hunter, Cell 42:849-857, 1985), the sites of protein kinase C phosphorylation are amino-terminal to the kinase domain. The abl carboxy-terminal region is not necessary for the tyrosine kinase activity or transformation potential of the viral abl protein and may represent a regulatory domain. Using an in vitro immune complex kinase assay, we were not able to correlate reproducible changes in c-abl activity with phosphorylation by protein kinase C. However, the high degree of conservation of the phosphorylation sites for protein kinase C between human and mouse abl proteins suggests an important functional role.

The c-abl proto-oncogene is the normal cellular homolog of the v-abl transforming gene of Abelson murine leukemia virus (A-MuLV) (23, 55, 60). A-MuLV encodes a 160-kilodalton protein that contains N-terminal sequences derived from Moloney murine leukemia virus gag sequences fused to c-abl sequences (20). P160v-abl is a tyrosine-specific protein kinase phosphorylated on tyrosine in vivo and in vitro (40, 47, 56, 58). The kinase activity of P160v-abl mediates the transformation of fibroblast and lymphoid and lymphoid cells in vitro (2, 41-43, 46, 59, 61) and induction of B-cell lymphomas in vivo (1).

Activation of the oncogenic potential of c-abl can also occur by chromosomal translocations. The human c-abl is altered by translocation from chromosome 9 to 22 in Philadelphia chromosome (Ph1)-positive chronic myelogenous leukemia cells (3, 32). This results in the expression of a chimeric P210Bcr-abl protein with associated tyrosine kinase activity (14, 29, 31). Recently, a new c-abl-derived protein (P185abl-abl) has been identified in Ph1-positive acute lymphocytic leukemia cells (9).

The c-abl gene is highly conserved (24, 37, 54) and is expressed in all vertebrate tissues examined (4, 37, 54) as two mRNA size classes of 6.7 and 7 kilobases (kb) in humans and 5.3 and 6.5 kb in the mouse. Examination of cDNA clones reveals that they contain different 3' exons which are alternatively spliced onto a set of common exons (4, 51). From analysis of the corresponding sequences, it is predicted that one of the c-abl proteins is cytoplasmic (type I), while another may be membrane bound (type IV) through a myristylated glycine residue, similar to v-abl (48, 49), v-src (7, 50), and c-src (7).

The c-abl proteins (31, 60) have been shown to have associated tyrosine kinase activity in vitro (33) but are not detectably phosphorylated on tyrosine in vivo (31, 40). In contrast, all normal and altered abl proteins are phosphorylated on serine following in vivo labeling with P32 (31, 40).

Considerable attention has focused in recent years on the role that the Ca2+- and phospholipid-dependent protein kinase, or protein kinase C, may play in the transmission of mitogenic signals generated by compounds such as tumor-promoting phorbol esters and polypeptide growth factors such as the platelet-derived growth factor (for reviews, see references 38 and 45). Treatment of cells with the protein kinase C activator 12-O-tetradecanoylphorbol-13-acetate (TPA) (8) has been shown to elevate the levels of phosphotyrosine in specific cellular proteins (5, 12, 19). Activation of protein kinase C may be involved in activation of one or many tyrosine-specific protein kinases or inhibition of phosphotyrosine phosphatases.

Protein kinase C has also been shown to decrease the activity of the receptor-associated tyrosine protein kinases for epidermal growth factor (EGF) (10, 13, 25) and insulin (27, 52) in vivo and in vitro. In both cases, phosphorylation by protein kinase C results in a reduction of the receptor-associated tyrosine kinase activities (6, 10, 18, 26). Protein kinase C also phosphorylates the transforming protein of Rous sarcoma virus, pp60src, and its normal cellular homolog, pp60v-src (22). No change in pp60v-src tyrosine kinase activity was observed following phosphorylation by protein kinase C (22).

We present evidence that protein kinase C phosphorylates abl proteins in vivo and in vitro. The sites of protein kinase C phosphorylation on all abl molecules are identical and are precisely conserved in mouse and human cells.

MATERIALS AND METHODS

Cells and antisera. SCRF 60A is a murine thymoma cell line (35). Human leukemia cell lines included Ph1-negative

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HL-60 (11) and Ph1-positive K562 (36). The NIH-3T3 cell line and A-MuLV strains P160, P120, P87, P84, P80, P92td, P100, and P90 have been described previously (43, 44, 57, 59, 62). Site-directed, abl-specific antisera were prepared as described previously (30).

Cell labeling and immunoprecipitation. Exponentially growing NIH-3T3 cells (1 × 10^6 to 2 × 10^6 per 100-mm dish) were maintained in Dulbecco modified Eagle medium with 0.5% calf serum for 16 h prior to labeling. The cells were labeled with ^32P or [35S]methionine as described (31) for 4 h. TPA (Sigma Chemical Co.) dissolved in dimethyl sulfoxide was added at a final concentration of 100 ng/ml for 45 min at the end of the labeling period. Labeling was terminated by washing the cells twice in phosphate-buffered saline. Cell extraction and immunoprecipitation of abl proteins were performed as described earlier (33).

In vitro phosphorylation reactions. Exponentially growing hematopoietic cells (1 × 10^5 to 3 × 10^5) or AMLV-transformed NIH-3T3 fibroblasts (2 × 10^5 to 4 × 10^6) were washed twice in phosphate-buffered saline and lysed as described previously (33). Immunoprecipitation and auto-phosphorylation of the abl proteins were performed as described (33).

For phosphorylation of the abl proteins by protein kinase C, the immunoprecipitates were suspended in a 40-μl reaction mixture containing 50 mM Tris hydrochloride, pH 7.4 (at 30°C), 10 mM MgCl2, 30 mM 2-mercaptoethanol, 0.3 mM EGTA [ethylene glycol-bis(β-aminoethyl) ether]-N,N,N',N'-tetraacetic acid], and, where indicated, 0.5 mM CaCl2, phosphatidylserine (10 μg/ml), phosphatidylinositol (10 μg/ml), and diolein (0.8 μg/ml). To the reaction mixture, 50 μl of protein kinase C was added. One unit of protein kinase C activity is defined as the amount of enzyme which catalyzes the incorporation of 1 pmol of P_i from [γ-32P]ATP into histone 1 per min at 30°C. Phosphorylation was initiated by addition of 10 to 15 μCi of [γ-32P]ATP (4,500 Ci/mmol) (ICN). Incubation was for 30 min at 30°C, and the reactions were terminated and analyzed as described for the auto-phosphorylation reactions (33).

Phosphorylation of angiotensin II (Sigma) by the abl proteins was carried out in a 40-μl reaction mixture containing 25 mM Pipes (1,4-piperazinediethanesulfonate), pH 7.0, 10 mM MgCl2, 30 mM 2-mercaptoethanol, 0.3 mM EGTA, 0.5 mM CaCl2, phosphatidylserine (10 μg/ml), phosphatidylinositol (10 μg/ml), diolein (0.8 μg/ml), 2 μM angiotensin II, and, where indicated, 50 μl of protein kinase C or buffer. The reactions were initiated by the addition of increasing concentrations (8 to 64 μM) of [γ-32P]ATP (5 to 150 Ci/mmol). Incubation was for 17 min at 30°C. The reactions were terminated and analyzed as described previously (14).

Alternatively, phosphorylation of angiotensin II was carried out in the presence of MnCl2 in place of MgCl2. Prior to phosphorylation of angiotensin II, the immunoprecipitated abl proteins were incubated with protein kinase C (30 U) or buffer in the presence of 10 mM MgCl2, 0.2 mM ATP, and the protein kinase C activators Ca^{2+}, phospholipids, and diolein. Following incubation for 10 min at 30°C, the immunoprecipitates were washed with 1 ml of 50 mM Tris hydrochloride, pH 7.0, and then used to phosphorylate angiotensin II. Phosphorylations were carried out in 40 μl of a solution containing 25 mM Pipes, pH 7.0, 20 mM MnCl2, 2 mM angiotensin II, and increasing concentrations (0.4 to 10.0 μM) of [γ-32P]ATP (10 to 400 Ci/mmol). Incubation was at 30°C for 17 min.

Two-dimensional phosphopeptide mapping and phosphoamino acid analysis. Proteins were localized by autoradiography and cut out of the gel. The phosphoproteins were extracted from the gel and subjected to phosphoamino acid analysis or phosphopeptide mapping as described previously (31). For secondary digestions, the tryptic phosphopeptides were eluted from the cellulose thin-layer plates and then incubated with chymotrypsin or thermolysin. Tryptic phosphopeptides obtained after phosphorylation of abl proteins with protein kinase C were scraped off the cellulose plates and eluted into a buffer containing acetic acid-formic acid-water (78:25:87), pH 1.9. The buffer was removed by lyophilization, and the phosphopeptides were incubated overnight with 80 μg of chymotrypsin or thermolysin. Digests were analyzed by electrophoresis at 400 V for 1 h or 40 min in 1% (NH4)2CO3, pH 8.9.

Purification of protein kinase C. Protein kinase C was purified from 600 g of frozen bovine liver. Liver was homogenized in 2 volumes of buffer A (20 mM Tris hydrochloride, pH 7.8, at 4°C, 10 mM EDTA, 10 mM EGTA, 10 mM 2-mercaptoethanol, 0.02% NaN3, 50 μM cyclic AMP, leupeptin [4 μg/ml], pepstatin [4 μg/ml]) with a polytron homogenizer. Phenylmethylsulfonyl fluoride was added to the homogenate to 0.5 mM and after each chromatography step to 0.1 mM. The homogenate was centrifuged at 9,000 × g for 30 min, and the resulting supernatant was centrifuged at 37,000 rpm in a Beckman Ti65 rotor for 2 h. The supernatant was removed and diluted to the conductivity of buffer B (20 mM Tris hydrochloride, pH 7.8, 50 mM NaCl, 1 mM EDTA, 1 mM EGTA, 10 mM 2-mercaptoethanol, 0.02% NaN3), and the pH was brought to 7.8 at 4°C. The sample was batch adsorbed to 1 liter of DEAE-cellulose for 1 h at 4°C and washed with 3 liters of buffer B; a 3.5-liter gradient from 0 to 0.4 M KCl was applied. Fractions from 0.05 to 0.1 M KCl containing protein kinase C were pooled and applied to a 75-ml phosphocellulose column equilibrated in buffer B. Protein kinase C did not adhere to the resin, and the eluate was applied to a 20-ml protamine-agarose column (lot no. 125F-9670; Sigma) equilibrated in buffer C (20 mM Tris hydrochloride, pH 7.8, 100 mM NaCl, 7.5 mM MgCl2, 1 mM EDTA, 1 mM EGTA, 10 mM 2-mercaptoethanol, 0.02% NaN3). The column was washed with buffer C, and protein kinase was eluted with a 1-liter gradient from 0.1 to 1.3 M NaCl. Protein kinase C eluted between 0.6 and 0.8 M NaCl.

RESULTS

Phorbol esters modulate the phosphorylation of c-abl proteins on serine in cultured cells. Treatment of NIH-3T3 cells with the protein kinase C activator TPA (8) resulted in a four- to eightfold increase of murine P150-cabl phosphorylation (Fig. 1B, lane 4) compared with control cells (Fig. 1B, lane 3). Labeling of the cells with [35S]methionine indicated that the amount of P150-cabl isolated from control and TPA-treated cells was indistinguishable over a number of experiments (Fig. 1A, lanes 3 and 4). Increased phosphorylation correlated with retarded mobility of P150-cabl in the gel (Fig. 1B, lane 4). The slightly higher-molecular-weight form of the c-abl protein was also observed when P150-cabl was isolated from [35S]methionine-labeled cells following treatment of the cells with TPA (Fig. 1A, lane 4). This indicated that most of the population of P150-cabl molecules was shifted to the slowly migrating species in the TPA-treated cells.

Since the antibody used to immunoprecipitate P150-cabl (αEX-4) recognizes the carboxy-terminal region present in all forms of the c-abl proteins, the results in Fig. 1 did not
NIH-3T3 cells. Following serum starvation for 16 h, NIH-3T3 cells were metabolically labeled with [35S]methionine (A) or [32P] (B) for 4 h. TPA (dissolved in dimethyl sulfoxide) was added to a final concentration of 100 ng/ml for 45 min at the end of the labeling period (lanes 2 and 4). Control cells (lanes 1 and 3) received the corresponding volume of dimethyl sulfoxide. P150c-abl was immunoprecipitated with normal rabbit serum (lanes 1 and 2) or opEX-4 (lanes 3 and 4). The immunoprecipitates were analyzed by electrophoresis on 7% SDS-polyacrylamide gels. [32P]-labeled proteins were detected by autoradiography with an intensifying screen for 2 days. [35S]methionine-labeled samples were detected by fluorography for 2 days.

**FIG. 1.** Effect of TPA treatment on the phosphorylation of c- abl from NIH-3T3 cells. Following serum starvation for 16 h, NIH-3T3 cells were metabolically labeled with [35S]methionine (A) or [32P] (B) for 4 h. TPA (dissolved in dimethyl sulfoxide) was added to a final concentration of 100 ng/ml for 45 min at the end of the labeling period (lanes 2 and 4). Control cells (lanes 1 and 3) received the corresponding volume of dimethyl sulfoxide. P150c-abl was immunoprecipitated with normal rabbit serum (lanes 1 and 2) or opEX-4 (lanes 3 and 4). The immunoprecipitates were analyzed by electrophoresis on 7% SDS-polyacrylamide gels. [32P]-labeled proteins were detected by autoradiography with an intensifying screen for 2 days. [35S]methionine-labeled samples were detected by fluorography for 2 days.

**FIG. 2.** Phosphorylation of c- abl by purified protein kinase C. P150c-abl was immunoprecipitated with normal rabbit serum (lanes 1) or opEX-4 (lanes 2) from the murine thymoma cell line SCRF 60A. In vitro kinase assays were carried out in the presence of Mg2+ (lanes A, B, C, D, and F) or Mn2+ (lanes E and G). The immunoprecipitates in lanes F and G were heated at 55°C for 10 min prior to assay. Lanes B, C, D, and F contain 50 U of protein kinase C (PKC). Lanes C, D, and F also contained phospholipids and diolien (PL/DL) (see text). Lanes D and F also contain 0.5 mM CaCl2. Reactions for lanes E and G were carried out in 20 mM PIPES (pH 7.0)–10 mM MnCl2. All reactions were initiated by addition of 15 μCi of γ[32P]ATP, and incubation was for 30 min at 30°C. Reactions were terminated by addition of 1 ml of cold phosphate lysis buffer plus 5 mM EDTA. The phosphorylated proteins were analyzed by electrophoresis on 8% SDS–polyacrylamide gels and autoradiographed for 5 h with an intensifying screen.

Protein kinase C phosphorylates P150c-abl in vivo and in vitro at two major sites. Two-dimensional tryptic peptide maps showed that protein kinase C phosphorylated P150c-abl at two major sites in vitro which were distinct from the major autophosphorylation site obtained when P150c-abl was incubated with Mn2+-ATP (Fig. 4A and C). Phosphopeptide maps of P150c-abl isolated from serum-starved cells following labeling with [32P] indicated that phosphorylation occurred mainly at one site, designated site 3 (Fig. 4B). Maps of P150c-abl from TPA-treated NIH-3T3 cells showed the appearance of two new phosphopeptides, designated 1 and 2, with no change in the phosphorylation of site 3 which was observed in control cells (Fig. 4D). Sites 1 and 2, phosphorylated in vivo in TPA-treated cells, corresponded to sites 1 and 2 obtained following the phosphorylation of P150c-abl by protein kinase C in vitro. Analysis of mixtures of in vivo and in vitro phosphorylated P150c-abl confirmed the identity of the phosphopeptides (data not shown). We conclude that sites 1 and 2 are not detectably phosphorylated in vivo by any protein kinase in the absence of protein kinase C activation.

**FIG. 3.** The predominance of murine P150c-abl is phosphorylated with serine residues (Fig. 3, lane D). Thus, increased phosphorylation of P150c-abl on serine via a TPA-activated serine-specific protein kinase did not induce stimulation of the tyrosine protein kinase autophosphorylation activity of P150c-abl in vivo.

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A recent study examined the phosphorylation of c-abl protein kinase at various sites. The investigation revealed that c-abl, when phosphorylated in vitro (lanes A and B) or in vivo (lanes C and D), exhibited distinct phosphorylation patterns. The in vitro kinase reactions were conducted in the presence of Mn++, Mg++, protein kinase C, phospholipids, diolein, and Ca++ (B). In vivo labeling of NIH-3T3 cells was performed as described in the legend to Fig. 1 for control (C) or TPA-treated (D) cells. Following electrophoresis on SDS-polyacrylamide gels, the phosphorylated proteins were eluted from the gel and subjected to acid hydrolysis (31).

**Identification of protein kinase C phosphorylation sites on c-abl.** Two different approaches were taken to identify the sites of protein kinase C phosphorylation on c-abl. First, we examined the ability of a number of anti-abl-specific sera to recognize phosphorylation by protein kinase C at one or both sites. When P145c-abl was immunoprecipitated with apEX-2 (directed against sequences close to the kinase domain) (30) and then phosphorylated by protein kinase C, the two sites were phosphorylated to the same extent (Fig. 5A). However, phosphorylation of P145c-abl by protein kinase C following immunoprecipitation with the carboxyl terminus-directed antisera apEX-5 (30) resulted in selective block of site 2 phosphorylation, with no change in the phosphorylation of site 1 (Fig. 5B). These results suggest that site 2 may be located near the carboxy terminus of the abl protein, near or within sequences recognized by the apEX-5 antibody.

A second approach to map the sites of protein kinase C phosphorylation on c-abl more precisely was to examine whether the wild-type strains of A-MuLV, P160 and P120, and a number of A-MuLV-derived mutant proteins of smaller size were phosphorylated by protein kinase C at sites 1 and 2 identified above. The majority of the mutants expressed the kinase activity but carried deletions in the carboxy terminus of the Abelson protein (21, 57, 62). In addition, a transformation-defective mutant protein of A-MuLV called P92td (59) was tested as a substrate for protein kinase C. This mutant strain lacks in vitro tyrosine kinase activity (59) as a result of a deletion in the kinase domain (21).

Protein kinase C phosphorylated the heat-inactivated P160y-abl at the same two major sites phosphorylated in P150c-abl (Fig. 6B-3). In contrast, heat-inactivated P120y-abl was phosphorylated only at site 2 by protein kinase C (Fig. 6B-4). DNA sequence analysis showed that the P160y-abl and P120y-abl genomes were homologous, with the exception of a single 800-base-pair deletion in P120y-abl (21). The approximate location of the deletion is diagrammed in Fig. 6A. Other P120y-abl-derived mutant proteins such as the transformation-defective P92td (Fig. 6A and B-6) and P100 (Fig. 6A) also lacked site 1 when phosphorylated by protein kinase C. These results suggest that site 1 is located within the region deleted in P120y-abl and other P120y-abl-derived proteins.

Three P160y-abl-derived mutant proteins, P87 (Fig. 6A and B-5), P84 (Fig. 6A), and P80 (Fig. 6A), not only lacked phosphorylation by protein kinase C at site 1 but also at site 2. All three P160y-abl-derived mutants contained large carboxyl-terminal deletions which extended into the region deleted in P120y-abl. These results are in agreement with the observation that immunoprecipitation with the carboxy terminus-directed antibody, apEX-5, resulted in selective block of site 2 phosphorylation by protein kinase C and suggest that site 2 is located within the carboxy-terminal region of the abl proteins.

Heat inactivation of P160y-abl and P120y-abl as well as P100, P87, P84, and P80 was required to completely abolish the

**Fig. 3.** Phosphoamino acid analysis of in vitro- and in vivo-phosphorylated P150c-abl, P150c-abl from NIH-3T3 cells was phosphorylated in vitro (lanes A and B) or in vivo (lanes C and D). The in vitro kinase reactions were carried out in the presence of Mn++ (A) or Mg++, protein kinase C, phospholipids, diolein, and Ca++ (B). In vivo labeling of NIH-3T3 cells was performed as described in the legend to Fig. 1 for control (C) or TPA-treated (D) cells. Following electrophoresis on SDS-polyacrylamide gels, the phosphorylated proteins were eluted from the gel and subjected to acid hydrolysis (31).

**Fig. 4.** Two-dimensional phosphopeptide maps of P150c-abl phosphorylated in vitro and in vivo. P150c-abl was autophosphorylated in vitro in the presence of Mn++ (A) or phosphorylated by protein kinase C in the presence of Mg++, Ca++, phospholipids, and diolein (C). In vivo labeling of P150c-abl was carried out as described in the legend to Fig. 1 for control (B) or TPA-treated (D) cells. Proteins were analyzed by SDS-polyacrylamide gel electrophoresis and autoradiography. The phosphorylated proteins were eluted from gel slices, oxidized, and subjected to trypsin digestion (31). Analysis in the first dimension was carried out by electrophoresis for 1 h at 400 V in 1% ammonium carbonate (pH 8.9) on thin-layer cellulose plates. The origin is at the center of the baseline (indicated by the arrow), and the cathode is to the right. Ascending chromatography in the second dimension was performed in n-butanol-pyridine-acetic acid-H2O (15:10:3:12). Plates were autoradiographed for 2 days (A and C) or 20 days (B and D).
tyrosine kinase activity of these proteins which would otherwise obscure mapping of the protein kinase C phosphorylation sites. In contrast to the c-abl proteins, all v-abl proteins showed significant levels of autophosphorylation activity in the presence of Mg\(^{2+}\) ions, which are required for the phosphorylation of abl proteins by protein kinase C. When P87, P84, and P80 were heat inactivated, a new phosphoserine residue (Fig. 6B-5), which migrated close to the origin and was not present in the untreated proteins, was observed. Phosphorylation at this site may be due to unfolding of the proteins and exposure of a serine upon heat treatment.

Although the amino-terminal sequences for the different exon I types of the c-abl protein contain potential serine targets for protein kinase C phosphorylation, none of these amino-terminal serines corresponded to sites 1 and 2. Both sites have been shown to be present in altered abl proteins in which the 5' first exon of c-abl has been replaced with new sequences. The data presented here localize the two sites in sequences common to all abl proteins.

Comparison of human and mouse abl sequences to identify common targets for protein kinase C. The human abl proteins P145c-abl and the altered P210BCR-abl were also substrates for protein kinase C (Fig. 6B-2 and 6B-1). Both proteins were phosphorylated at the same two sites as their murine counterparts. The human c-abl sequence (51) was compared with the mouse c-abl sequence (4, 34) (Fig. 7). The sequences compared correspond to the carboxy-terminal half of each protein, including the region deleted in P120c-abl (corresponding to residues 543 to 808 in the human sequence) and also the region used to prepare the phospho-abl antiserum (residues 967 to 1,130 in the human sequence).

Studies on the structural requirements that determine the substrate specificity of protein kinase C have shown that all sites phosphorylated by this protein kinase are next to or in proximity to (one or two amino acids) arginine or lysine residues (16, 28, 53). Within the sequence deleted in P120c-abl, there were 13 serines located next to or within one or two amino acids of arginine or lysine residues that were present in both mouse and human c-abl (Fig. 7). To confirm the identity of the site phosphorylated by protein kinase C in this region, the tryptic phosphopeptide designated site 1 was eluted from the cellulose plate and subjected to secondary digestions with chymotrypsin or thermolysin. The digests were then analyzed by two-dimensional phosphopeptide mapping. The phosphopeptide containing site 1 was cleaved by both chymotrypsin (Fig. 8A) and thermolysin (Fig. 8B). In both cases, the phosphopeptides generated were negatively charged and migrated readily towards the anode. Chymotrypsin digestion of site 1 was incomplete, and some residual, uncleaved peptide was observed (Fig. 8A) in addition to the negatively charged cleavage product. Chymotrypsin specifically cleaves after Phe, Tyr, and Trp, while thermolysin cleaves before amino acids with hydrophobic groups such as Leu, Ile, Val, Phe, and Met. Among the various potential targets for protein kinase C in this region, the one that best fit the data obtained here was the site containing Ser-618 to Ser-620 in the sequence Lys-Arg-Ser-Ser-Ser-Phe-Arg in both the human and mouse abl proteins. Within this region, there was an additional potential site, Ser-601, which was flanked by basic amino acids on the N- and C-terminal sides and which also could be cleaved by both chymotrypsin and thermolysin. This serine, however, would be a less likely target for protein kinase C, as the basic residues were four amino acids away at the N terminus and three amino acids away at the C terminus of the potential serine target. We propose that the most likely candidate for protein kinase C site 1 is the sequence containing serines 618 to 620.

To establish the location of protein kinase C site 2, we examined the sequences carboxy-terminal to the region deleted in P120c-abl. Within the sequence against which the opEX-5 antibody is directed, serine 1,102 in the human sequence (or serine 1,004 in the mouse) was readily identified as a protein kinase C target. This serine was flanked by several basic amino acids in the sequence Arg-Val-Ser-Leu-Arg-Lys-Thr-Arg. There were two other serines within this region (1,119 and 1,120 in human c-abl or 1,112 and 1,113 in mouse c-abl) which were also flanked by basic amino acids, in the sequence Lys-Leu-Ser-Val-Ser-Val-Lys. From comparison with other known substrates for protein kinase C, the former site, containing serine 1,012 in human c-abl, would be the preferred substrate over the latter, as it was located in a sequence containing four basic amino acids, as opposed to two basic amino acids for serines 1,119 and 1,120, and the basic residues were in greater proximity. To assign the protein kinase C target in this region more precisely, we subjected the phosphopeptide designated site 2 to secondary digestions. The phosphopeptide was not cleaved by chymotrypsin (Fig. 8C), but it was readily cleaved by thermolysin (Fig. 8D). Both the tryptic peptide containing Ser-1012 and that containing serines 1,119 and 1,120 could be cleaved by thermolysin but not by chymotrypsin. From the secondary-digestion data and amino acid sequence, we could not distinguish between these two sites as site 2. However, from comparison with sequences found in other known substrates for protein kinase C, the preferred target would most likely be serine 1,012.
activity. To assess the effect of protein kinase C phosphorylation on the tyrosine kinase activity of the abl proteins, we used an immune complex kinase assay with angiotensin as the substrate. Summarized in Table 1 are the kinetic constants for ATP with angiotensin II as the phosphate acceptor. Values are indicated for reactions performed with MgCl₂ or MnCl₂. All abl proteins had a higher affinity for Mn²⁺-ATP than Mg²⁺-ATP (33). The $K_m$ values for ATP in the presence of MgCl₂ were 10- to 35-fold higher than those obtained with MnCl₂. These values for cellular, viral, and translocated forms of the abl proteins expressed in mammalian cells are comparable to those obtained by Ferguson et al. (15) ($K_m\text{ATP} = 14 \mu M$) and Foulkes et al. (17) ($K_m\text{ATP} = 39 \mu M$) for truncated forms of v-abl expressed in Escherichia coli under similar reaction conditions. Although the affinity of the abl proteins for ATP was higher in the presence of Mn²⁺ than Mg²⁺ ions, the maximal velocities of the reactions were 5- to 8-fold lower with Mn²⁺ than with Mg²⁺ ions. These results are similar to those obtained by Foulkes et al. (17) for the truncated form of v-abl, pt abl 50. In the presence of Mn²⁺ ions, the purified pt abl 50 kinase showed less than 20% of the activity obtained with Mg²⁺ ions (17).

As shown in Table 1, no changes in the activities of P160v-abl and P210 bcr-abl were observed following phosphorylation by protein kinase C. These results were not unexpected, as both P160v-abl and P210 bcr-abl are altered proteins with deregulated tyrosine kinase activities. In contrast, phosphorylation of P150c-abl with protein kinase C resulted in variable responses, from no change to a 2.4-fold increase in activity due to a corresponding increase in the velocity of the reaction when MgCl₂ but not MnCl₂ was used in the reaction (Table 1). The higher affinity of the enzyme for Mn²⁺ ions may preclude observation of subtle changes in activity due to phosphorylation by protein kinase C. These changes may only be detected with Mg²⁺ ions, which constitute the principal divalent cation in the cell. The effect of protein kinase C phosphorylation is on $V_m$ with no change in the $K_m$. A 2.0- to 2.4-fold increase in $V_m$ was observed in three experiments, while in two other experiments only a 1.1- and 1.4-fold increase in $V_m$ was measured following phosphorylation by protein kinase C (Table 1). The lack of reproducible activation of P150c-abl activity by protein kinase C may reflect variations in the phosphorylation state of P150c-abl as extracted from the cells from experiment to experiment. From the data presented in Fig. 4, we can assume that sites 1 and 2 are found in the unphosphorylated state of P150c-abl immunoprecipitated from non-TPA-treated cells. However, the variability in the degree of phosphorylation at other sites on the c-abl protein may affect the efficiency of protein kinase C phosphorylation at sites 1 and 2. Interaction among sites in multiply phosphorylated proteins has been best documented for glycogen synthase. Phosphorylation of glycogen
FIG. 7. Comparison of the amino acid sequences of human and mouse abl proteins. The carboxy-terminal half of the human abl (51) amino acid sequence (upper line) is compared with the corresponding murine sequence of v-abl (34) (lower line). The sequence includes all the amino acids from amino acid 501 (in the human and mouse proteins) to the last amino acid. Homologous amino acids are indicated with asterisks. The numbers correspond to amino acids in the human sequence. The location of the sequences deleted in P120\(^{v-abl}\) (residues 543 to 808) and the region against which opEX-5 is directed (residues 967 to 1.130) are indicated by the solid vertical lines, in the direction of the arrows. Suggested protein kinase C sites 1 and 2 are underlined.

FIG. 8. Secondary digestion of protein kinase C phosphorylation sites on the abl proteins. Tryptic phosphopeptides were obtained as described in the text and eluted off the cellulose plates. The phosphopeptides were then subjected to secondary digestions with chymotrypsin (A and C) or thermolysin (B and D). Ascending chromatography was performed as described in the legend to Fig. 4 for the tryptic phosphopeptides. (A and B) Site 1 digested with chymotrypsin and thermolysin, respectively; (C and D) site 2 incubated with chymotrypsin (C) or thermolysin (D). The origin is indicated by the arrow, and the cathode is to the right. Autoradiography was for 5 days at \(-70^\circ\text{C}\."

synthase at site 5 by casein kinase II is necessary for the phosphorylation of sites 3a, 3b, and 3c by glycogen synthase kinase 3 (39). It is important to indicate that no increase in \(V_m\) was observed in the presence of protein kinase C, but in the absence of protein kinase C activators or with activators alone (data not shown). Phosphorylation by protein kinase C did not appear to affect the \(K_m\) or \(V_m\) for angiotensin II (data not shown).

**TABLE 1.** Kinetic constants for normal cellular and transformation-associated abl tyrosine kinase activity in the absence or presence of protein kinase C

<table>
<thead>
<tr>
<th>abl protein</th>
<th>Cation</th>
<th>Protein kinase C</th>
<th>(K_m^{\text{ATP}}) (mM)</th>
<th>(V_m^{*\text{ATP}}) (pmol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>P150(^{v-abl})</td>
<td>Mg(^{2+})</td>
<td>–</td>
<td>58 (49-74)</td>
<td>0.148 (0.11-0.19)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>+</td>
<td>61 (53-67)</td>
<td>0.260 (0.21-0.36)</td>
</tr>
<tr>
<td>P160(^{v-abl})</td>
<td>Mg(^{2+})</td>
<td>–</td>
<td>13</td>
<td>8.4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>+</td>
<td>15</td>
<td>9.3</td>
</tr>
<tr>
<td>P210(^{cr-abl})</td>
<td>Mg(^{2+})</td>
<td>–</td>
<td>45</td>
<td>1.25</td>
</tr>
<tr>
<td></td>
<td></td>
<td>+</td>
<td>38</td>
<td>1.0</td>
</tr>
<tr>
<td>P150(^{v-c-abl})</td>
<td>Mn(^{2+})</td>
<td>–</td>
<td>1.3</td>
<td>0.30</td>
</tr>
<tr>
<td></td>
<td></td>
<td>+</td>
<td>1.1</td>
<td>0.33</td>
</tr>
<tr>
<td>P160(^{v-c-abl})</td>
<td>Mn(^{2+})</td>
<td>–</td>
<td>1.3</td>
<td>0.25</td>
</tr>
<tr>
<td></td>
<td></td>
<td>+</td>
<td>1.4</td>
<td>0.25</td>
</tr>
</tbody>
</table>

\(^{a}\) abl kinase activity was measured by the incorporation of ^3P from [\(\gamma-\text{TP}\)]ATP into angiotensin II as described in Materials and Methods.

\(^{b}\) Kinetic parameters were determined from initial velocity measurements by establishing linearity with respect to both time and enzyme concentration. Reactions were linear for 60 min at 30°C. Routine phosphorylations were done for 17 min at 30°C. Each data point was performed in duplicate. Data were analyzed by a computerized linear regression analysis of plots of V versus substrate concentration.

\(^{c}\) Units of \(V_m\) are picomoles per 17 minutes per 40-\(\mu\)l reaction mixture.

\(^{d}\) The values of \(K_m\) and \(V_m\) for P150\(^{v-c-abl}\) in the presence of Mg\(^{2+}\) represent the average of five independent experiments. The range of values is indicated in parentheses.
not shown). In addition, the autophosphorylation activity of the abl proteins measured in vitro in an immune complex kinase assay was not affected by phosphorylation with protein kinase C (data not shown).

DISCUSSION

All abl proteins are phosphorylated in vivo at multiple sites (31, 40). The majority of these phosphorylation events occur on serine residues. Phosphotyrosine is also detected on viral abl proteins and the altered P210<sup>pp60<sub>cr</sub></sup> protein found in Ph<sup>1</sup> chronic myelogenous leukemia but not on normal human P145<sup>c-abl</sup> or murine P150<sup>c-abl</sup> (31, 40). We have demonstrated that protein kinase C mediates the phosphorylation on serine at two major sites which are common to human and murine abl proteins.

Two-dimensional phosphopeptide mapping of normal and transformation-associated abl proteins from mouse and human cells indicated that phosphorylation by protein kinase C occurred at two sites in approximately equal amounts in the abl proteins. The two sites are localized in the carboxy-terminal half of the abl proteins. By using a number of A-MuLV-derived mutant proteins with specific known deletions, it was possible to determine that one of the sites (site 1) was located within the sequences deleted in the viral P120 protein. The second site was found to be localized in the carboxy-terminal regions of the abl proteins, which is recognized by the αpEX-5 antibody. It is noteworthy that neither site was inhibited by the αpEX-4 antibody, which is directed against sequences between amino acids 674 and 849 (Fig. 7). These sequences are localized between the two proposed sites and do not overlap them. Thus, neither site should be inhibited by αpEX-4, in agreement with the data in Fig. 2 and 4.

The carboxy-terminal 501 amino acids of the c-abl proteins are encoded by a single, large exon of 3.5 kb, which is preceded by 12 to 14 small exons encoding the remainder of the protein. Mouse and human abl proteins are almost identical in the amino-terminal and kinase domains (99% homology); however, the carboxy-terminal region is only 74% homologous (51). It is interesting that even though the carboxy-terminal sequences show the most divergence between mouse and human abl proteins, the sequences immediately surrounding the two suggested protein kinase C sites are precisely conserved. The prevalence of these sites between species suggests an important physiological role.

Comparison of the protein kinase C phosphorylation sites on the abl proteins with those found in other tyrosine-specific protein kinases reveals some common characteristics among the sites. Similar to the abl proteins, the sites phosphorylated by protein kinase C on the EGF receptor (13) and on pp60<sup>src</sup> (22) are surrounded by basic amino acids. There is, however, an important difference. For both pp60<sup>src</sup> and the EGF receptor, the phosphorylation sites are located amino-terminal to the kinase domain, with the phosphorylated residue localized about 10 amino acids away from the cytoplasmic side of the plasma membrane (13, 22, 25). In contrast, the two sites of protein kinase C phosphorylation on the abl proteins are found at the carboxy-terminus.

Sequence data predict (4, 51) and results obtained in our laboratory indicate (Pendergast et al., in preparation) that one form of the c-abl protein (mouse type IV or human Ib) is myristylated and may be associated with the plasma membrane. In vivo, the membrane- associated form of the c-abl protein must be folded in such a manner as to make the carboxy-terminal sites available to phosphorylation by protein kinase C.

Phosphorylation of the altered abl proteins P160<sup>c-abl</sup> and P210<sup>pp60<sub>cr</sub></sup> by protein kinase C did not dramatically alter their kinase activities. Examination of the effect of protein kinase C phosphorylation on P150<sup>c-abl</sup> activity gave variable results. While in some experiments we observed up to a twofold increase in kinase activity following phosphorylation by protein kinase C, only slight or undetectable changes were seen in other experiments. The variability of these results may reflect different physiological states of P150<sup>c-abl</sup> as isolated from the cells in the various experiments. It is also possible that the immune complex kinase assay does not effectively measure the effect of protein kinase C phosphorylation on c-abl activity, as the c-abl protein is not free in solution but immobilized to a solid phase.

The high efficiency of phosphorylation at sites 1 and 2 on c-abl by protein kinase C as well as the highly conserved nature of both sites suggest that phosphorylation of c-abl at these sites may be important to modulate c-abl functions in vivo. Although the carboxy-terminal sequences are not necessary for enzyme activity, these sequences appear to mediate the lethal effect of P160<sup>pp60<sub>cr</sub></sup> on fibroblasts (57, 62) and also enhance lymphoid transformation (44). It would be of interest to test whether phosphorylation by protein kinase C affects these functions. The carboxy-terminal sequences may also affect the interaction of c-abl with specific cellular proteins, which could then be phosphorylated on tyrosine. In addition to substrate selectivity, the carboxy-terminal sequences may function in cis to regulate the kinase activity of c-abl. The existence of protein kinase C phosphorylation sites in the carboxy-terminus of c-abl requires that all of the above interesting possibilities be tested further.

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