Tyrosine Phosphorylation of RNA Polymerase II Carboxyl-terminal Domain by the Abl-related Gene Product*

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The largest subunit of RNA polymerase II contains a C-terminal repeated domain (CTD) that is the site of phosphorylation by serine (threonine) and tyrosine kinases. Phosphorylation of the CTD is correlated with transcription elongation. A number of different kinases have previously been shown to phosphorylate the CTD; among them is a nuclear tyrosine kinase encoded by the c-abl proto-oncogene. The processive and high stoichiometric phosphorylation of RNA polymerase II by c-Abl requires the tyrosine kinase, the SH2 domain, and a CTD-interacting domain (CTD-ID) in the Abi protein. The physiological tyrosine phosphorylation of RNA polymerase II, however, is observed in cells derived from abl-deficient mice, indicating the existence of other CTD tyrosine kinases. In this report, we show that the tyrosine kinase encoded by an Abl-related gene (Arg) also phosphorylates the CTD in vitro and in transfected cells. The SH2 and kinase domain of Arg are 95% identical to that of c-Abl. However, these two proteins share only 29% identity in the large C-terminal region. Interestingly, a CTD-ID is also found in the C-terminal region of Arg. Mapping studies and sequence analysis have led to the identification of the CTD-ID that is highly conserved among the divergent C-terminal regions of Abl and Arg. These results indicate that tyrosine phosphorylation of RNA polymerase II CTD could be catalyzed by either c-Abl or Arg kinase.

The product of proto-oncogene c-abl and its related gene, arg, encode nonreceptor tyrosine kinases that are ubiquitously expressed in mouse and human cells (1–3). The two proteins are 95% identical in the N-terminal SH3, SH2, and tyrosine kinase domains. The C-terminal regions that constitute more than one-half of both proteins exhibit an overall identity of only 29% (2). This large C-terminal region that distinguishes c-Abl from the other family of nonreceptor kinases is required for the proper biological function of c-Abl. Truncation of the C terminus causes neonatal lethality in mice, a phenotype similar to the one observed with mice carrying homozygous null mutation for c-abl (4, 5). Several functional domains have been identified in the C-terminal region of c-Abl, including three nuclear localization signals (6), a DNA binding domain composed of three high mobility group-like boxes (7, 8), and binding domains for G- and F-actin (9, 10). Two physiological substrates for c-Abl have been identified for which substrate binding sites have also been identified in the C-terminal region. They are the CRK family of SH2/SH3 adapter proteins (11, 12) and RNA polymerase II (13–15).

The largest subunit of RNA polymerase contains a unique C-terminal domain that is composed of a seven-amino acid repeat with the consensus sequence YSPTSPS (16, 17). The heptad sequence is repeated 52 times in mammals, 44 times in Drosophila melanogaster, and 26 times in Saccharomyces cerevisiae (17). The CTD of RNAP is essential for cell growth because truncation of more than half of the repeats in yeast causes cold sensitivity and inability to induce specific gene expression such as INO 1 and GAL 10 (18). In mouse, a similar truncation in an α-amanitin-resistant RNA polymerase caused inability of the polymerase to confer α-amanitin resistance (19).

Because the CTD is rich in serine, threonine, and tyrosine, it serves as a substrate for both serine (threonine) and tyrosine kinases. Several CTD kinases have been identified in yeast and mammals. In yeast, the cyclin-dependent kinase Kin 28, a component of the holo-TFIH, has been shown to phosphorylate the CTD (20, 21). The mammalian homologue of TFIH-associated CTD kinase has been shown to be the cdk7/cyclin H kinase pair (22). Another yeast CTD kinase, SRB10/11 kinase-cyclin pair, has been identified as the mammalian cdk8/cyclin C (23). Thus far, the only kinase known to phosphorylate RNAP-CTD on tyrosines is c-Abl (13–15). c-Abl can phosphorylate the CTD to high stoichiometry with the incorporation of >30 mol of phosphate/mol of CTD. Such a high stoichiometric phosphorylation by Abl requires binding of the SH2 domain of Abl to partially tyrosine-phosphorylated CTD (13). Furthermore, CTD phosphorylation by Abl both in vivo and in vitro requires a CTD-interacting domain (CTD-ID) present at the C terminus of Abl (15).

The strongest evidence that c-Abl phosphorylates RNA polymerase II in vivo came from the study of cellular response to DNA damaging agents such as methyl methanesulfonate (MMS). We have found that MMS activates c-Abl tyrosine kinase in S phase cells (24). We have also shown that MMS can cause an increase in the phosphoryrosine content of RNA polymerase II, but only when c-Abl is present (24). In Abl-null cells or in cells reconstituted with a kinase-defective c-Abl, MMS did not induce a significant increase in the tyrosine phosphorylation of the largest subunit of RNA polymerase II

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*The abbreviations used are: CTD, C-terminal repeated domain; CTD-ID, CTD-interacting domain; RNAP, RNA polymerase; MMS, methyl methanesulfonate; GST, glutathione S-transferase; Arg, Abl-related gene.
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(24). However, Abl-null cells or cells expressing only the kinase-defective Abl contain basal levels of phosphotyrosine in the largest subunit of RNA polymerase II (14, 24). This suggested that basal tyrosine phosphorylation could be catalyzed by another tyrosine kinase. To identify the alternative CTD- tyrosine kinase, we tested the Arg gene product and showed that this tyrosine kinase can indeed phosphorylate the CTD.

MATERIALS AND METHODS

Cell Culture and Transfection—COS cells were cultured at 37°C in Dulbecco’s modified Eagle’s medium containing 10% supplemented calf serum (HyClone Laboratories). Cells were transfected using LipofectAMINE (Life Technologies, Inc.) according to manufacturer protocol or using the DEAE-dextran method. Two days after transfection, the cells were incubated for an additional 24 h with 1 μM ouabain before harvesting (15).

Plasmids—The wild type and mutant Arg cDNA (human type IB) were cloned in pBS (Bluescript, Stratagene) vector. For transient co- transfection studies, wild type and mutants of Arg were expressed from a cytomegalovirus promoter-based vector CB6 (25). The ΔSma construct removed amino acids 1128–1182, whereas ΔAcc deleted 1043–1182, ΔCla-Pflm1 removed 547–913, ΔPflm1 deleted 913–1182, and ΔPflm1-Acc removed amino acids 913–1043. The cDNA of murine CTD was subcloned into pEBG to obtain pEBG-CTD, which expresses the CTD of RNAP that is the target of Arg tyrosine kinase, the control immunoprecipitates (25, 27). Monoclonal anti-Tyr(P) antibodies—Monoclonal (8WG16) and polyclonal antibodies against RNAP and c-Abl have been described previously (15, 26). Polyclonal anti-Abl or Arg proteins were obtained by in vitro translation using TNT reticulocyte lysate (Promega) with either T3 or T7 RNA polymerase.

Antibodies—Monoclonal (8WG16) and polyclonal antibodies against RNAP and c-Abl have been described previously (15, 26). Polyclonal anti-Arg antibody has been described (25, 27). Monoclonal anti-Tyr(P) was purchased from ICN Pharmaceuticals. Horseradish peroxidase-conjugated secondary antibody was obtained from Life Technologies, Inc.

Immunoprecipitation and Kinase Reaction—Wild type and mutant Abl or Arg proteins were obtained by in vitro translation using TNT reticulocyte lysate (Promega) with either T3 or T7 RNA polymerase. The translation mixture was diluted to give a final concentration of 10 mM Tris-HCl (pH 7.4), 5 mM EDTA, 130 mM NaCl, 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, and 10 μg each of phenanthroline, aprotinin, leupeptin, and pepstatin. Immunoprecipitation with anti-Arg or anti-Abl antibody was carried out as described previously (15). The mixture was incubated for 2 h at 4°C, after which Protein A/G-Sepharose beads were added and incubated for an additional 1 h. The immunoprecipitates were washed twice with Buffer A containing 500 mM NaCl, twice with Buffer A containing 100 mM NaCl, and twice with Buffer A alone. The immune complexes containing similar amounts of wild type and mutant Arg or Abl proteins were rinsed and suspended in 20 μl of kinase buffer (20 mM Tris-HCl (pH 7.4), 10 mM MgCl2, and 1 mM dithiothreitol) containing 0.2 μg of GST-CTD or 0.05 μg of purified HeLa RNAP IIA or IIB. Kinase reactions were initiated by the addition of 10 μM ATP plus 25 μCi of [γ-32P]ATP (7000 Ci/mmol; ICN). Reaction mixtures were incubated at room temperature for 10 min (RNAP II) and 30 min (GST-CTD substrates) before the addition of SDS sample buffer. The samples were resolved on 8% SDS-polyacrylamide gel electrophoresis, transferred to Immobilon-P, and exposed for autoradiography. Quantitation was done using PhosphorImager (Molecular Dynamics).

RESULTS

Arg Tyrosine Kinase Phosphorylates the CTD of RNA Pol II—The identification of tyrosine-phosphorylated RNA polymerase II in Abl-null cells indicated the presence of other tyrosine kinases in HeLa cells, a c-Abl-related gene product, Arg, has been identified in mouse, a c-Abl-related gene product, Arg, has been identified in HeLa cells, a c-Abl-related gene product, Arg, has been identified in HeLa cells, a c-Abl-related gene product, Arg, has been identified in HeLa cells, a c-Abl-related gene product, Arg, has been identified in HeLa cells, a c-Abl-related gene product, Arg, has been identified in HeLa cells, a c-Abl-related gene product, Arg, has been identified in HeLa cells, a c-Abl-related gene product, Arg, has been identified in HeLa cells, a c-Abl-related gene product, Arg, has been identified in HeLa cells, a c-Abl-related gene product, Arg, has been identified in HeLa cells, a c-Abl-related gene product, Arg, has been identified in HeLa cells. This suggested that basal tyrosine phosphorylation could be catalyzed by another tyrosine kinase. To identify the alternative CTD- tyrosine kinase, we tested the Arg gene product and showed that this tyrosine kinase can indeed phosphorylate the CTD. Under similar reaction conditions, only IIA, but not the C-terminal truncated IIB, could be phosphorylated by the Arg kinase (Fig. 2A, compare lane 2 with lane 1). The inability to phosphorylate subunit IIB, which lacks the CTD but contains 32 tyrosines, strongly suggests that Arg specifically phosphorylates tyrosine residues within the CTD. To test this directly, a GST-CTD fusion protein expressed and purified from bacteria was also tested. The GST-CTD was found to be an effective substrate for the Arg tyrosine kinase since, under the condition assayed, a smearable band was observed upon incubation of GST-CTD with Arg (Fig. 2B). The observation that the GST alone was not phosphorylated indicates that phosphorylation occurs within the CTD portion of the fusion protein. Taken together, these results show that the CTD of RNA polymerase II is a substrate for the Arg tyrosine kinase.

FIG. 1. Schematic diagram showing different functional domains of c-Abl and Arg tyrosine kinase. NLS, nuclear localization domain; SH3 and SH2, Src homology domains 3 and 2, respectively; COOH, carboxyl-terminal domain.
Both c-Abl and Arg Phosphorylate the CTD of RNAP with Equal Efficiency—In an effort to compare the ability of Abl and Arg to phosphorylate the CTD, both the kinases were immunoprecipitated from in vitro translated wild type c-Abl (lanes 1–5) or wild type (wt) Arg (lanes 6–10) were immunoprecipitated with anti-Abl or anti-Arg antibody, and the kinase activity was measured as a function of GST-CTD concentrations. The amounts of GST-CTD added were 0.002 (wt) μg of antibody, and the kinase activity was measured as a function of GST-CTD kinase activity of wt-Abl is set at 1 and given a + sign. Mutants that exhibited 3–5-fold reduction in CTD-specific phosphorylation when compared with wt-Arg were assigned a − sign.

CTD-specific kinase activity for each of these mutants was determined by normalizing the kinase activity for each of these mutants with a nonspecific substrate, enolase. Although specific mutations in the C terminus affected CTD phosphorylation, they did not affect enolase phosphorylation. A compilation of the results obtained with various mutants of Arg after normalizing for equal protein input is given in Fig. 4. As expected, C-terminal deletion mutants, ∆Plm1-1 (not shown) and ∆Acc (Fig. 5, lanes 5–7) in which 269 and 139 amino acids were deleted could not phosphorylate the CTD efficiently. However, deletion of 54 amino acids from the C terminus did not affect CTD phosphorylation (Fig. 5, lanes 9–11). This indicated that the region between Acc and Sma is necessary for efficient CTD phosphorylation. An internal deletion mutant ∆Plm1-1-Acc in which amino acids 547–913 were removed exhibited CTD kinase activity similar to that of full-length Arg (not shown). Taken together, these results indicate that a specific region in the C terminus of Arg kinase (between Acc and Sma amino acids 1043–1128) is required for efficient phosphorylation of the CTD.

The CTD-ID Is Required for in Vivo Phosphorylation of the GST-CTD—Because Arg phosphorylates the CTD efficiently in vitro, we tested whether Arg can phosphorylate RNAP-CTD in vivo. For this purpose, Arg was cotransfected with GST-CTD construct in COS cells, and the phosphotyrosine content of the CTD was analyzed by reacting to anti-Tyr(P) antibodies. Anti-GST immunoprecipitates prepared from cells transfected with GST-CTD in the presence and absence of Arg kinase were probed with anti-CTD and anti-Tyr(P) antibody. Cells transfected with GST-CTD alone contained a protein band of the expected molecular weight for GST-CTD (Fig. 6A, lane 1). However, in the presence of wild type Arg, a smeary shifted band was observed (lane 2). Anti-Tyr(P) immunoblotting of a similar blot indicated that the mobility-shifted band contained phosphotyrosine (panel C, compare lane 1 with lane 2). The anti-Tyr(P) signal was completely eliminated when phosphotyrosine was included in the immunoblotting reaction, indicating specificity of the signals generated by the Tyr(P) antibodies (panel B). Thus, Arg kinase phosphorylates the CTD in vivo.

To determine if the phosphorylation of the RNAP II CTD by Arg kinase requires the region between Acc and Sma, the assay was applied to relevant Arg mutant proteins overproduced in COS cells. Mutants of Arg such as ∆Sma and ∆Plm1-1-Acc, which phosphorylated the CTD efficiently in vitro, scored positive in this assay (Fig. 6A, lanes 4 and 5). However, ∆Plm1-1 and ∆Acc could not phosphorylate the CTD (lanes 3 and 6) despite equal levels of expression in these transfected cells (Fig. 6D). The low molecular weight bands are most likely due to degradation. Since the autophosphorylation ability of these mutants was not affected (not shown), it can be inferred that it...
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The CTD-ID Is Highly Conserved both in Abl and in Arg—A comparison of the amino acid sequences between Acc and Sma in Arg kinase to that of previously mapped CTD-ID (Sal-Bgl) in Abl kinase indicated the presence of a highly conserved amino acid stretch (see Fig. 8A). Previously, we mapped the amino acids 935–1065 (Sal-Bgl) of Abl kinase C terminus to be the CTD-ID (Fig. 1). The results obtained from Arg CTD-ID suggested that the CTD-ID in Arg is between amino acids 1002 and 1065 (Sca-Bgl). To determine if this was the case, an internal deletion mutant of Abl, Sca-Bgl, was constructed and tested for its ability to phosphorylate the CTD in vivo. Anti-GST immunoprecipitates from wild type Abl as well as ΔBgl, but not ΔSal-Bgl and ΔSca-Bgl-transfected cells, contained the mobility-shifted CTD band (Fig. 7A, compare lanes 2 and 5 with 3 and 4). The presence of phosphotyrosine in mobility-shifted GST-CTD was confirmed by anti-Tyr(P) immunoblotting (panel C). The expression levels of various Abl mutants shown in Fig. 7 correspond to an 18-amino acid stretch in the CTD-ID (Fig. 2 and 3). The presence of phosphotyrosine in mobility-shifted GST-CTD was confirmed by anti-Tyr(P) immunoblotting (panel C). The expression of the Arg proteins was detected by immunoblotting with anti-Abl antibody. The expression of Arg proteins was detected by immunoblotting with anti-Abl antibody.

is the lack of CTD-ID that prevented these mutants from phosphorylating the CTD in vivo.

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A CTD-ID Peptide Can Inhibit both Abl and Arg CTK Kinase Activity—If the highly conserved region in Arg and Abl C termini constitutes the CTD-ID, then addition of exogenous CTD-ID should compete for the CTD and, thus, inhibit Abl as well as Arg phosphorylation of the CTD. A synthetic peptide corresponding to an 18-amino acid stretch in the CTD-ID (Fig. 8A) was synthesized and added to in vitro Abl kinase. A nonspecific peptide that contained an identical amino acid composition, but scrambled sequence, was also used as a control. The nonspecific peptide had little effect on the ability of Abl or Arg to phosphorylate CTD (Fig. 8B, lanes 1–3 and 7–9). A small inhibition observed in the CTD kinase activity of Abl at 1:50 molar ratio could be a nonspecific effect. Nevertheless, both Abl and Arg CTK kinase activity was affected at each of the specific peptide concentrations tested (Fig. 8, compare lanes 4–6 with 10–12). These results provide additional support that the conserved C-terminal region between Abl and Arg kinase contains the CTD-interacting domain.

DISCUSSION

The CTD of RNA polymerase II is found to be a substrate for the Abl-related gene (arg) product. Arg. Full-length RNA polymerase II large subunit purified from HeLa cells and a bacterially expressed GST-CTD could be efficiently phosphorylated by the Arg kinase. On comparison, the CTD kinase activity of Arg was equivalent to that of c-Abl kinase with a similar value of in 0.4–1.0 μM range. In addition, CTD phosphorylation by Arg is shown to require a CTD-interacting domain that has been previously identified in the C terminus of c-Abl kinase. Mapping studies and sequence comparison led to the assignment of the CTD-ID to a highly conserved sequence in the otherwise divergent C-terminal region of Abl and Arg. The identification of the CTD-ID was further confirmed by the
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FIG. 8A, comparison of the C-terminal region of Abl and Arg amino acid sequence. The CTD-ID has been previously mapped between amino acids 935 and 1002 (Sal-Bgl) of mouse c-Abl. In Arg, the corresponding CTD-ID region is located between amino acids 1043 and 1182 (Acc-Sma) of human Arg. The overlapping and highly conserved CTD-ID region sequences of mouse, human c-Abl, and Arg are aligned. The mouse Arg sequence was determined from a genomic fragment (J. Y. J. Wang, unpublished data). The specific and the nonspecific peptide sequences used in the CTD kinase inhibition assays are given. B, inhibition of CTD phosphorylation by peptides containing the conserved CTD-ID sequence. Full-length Abl as well as Arg kinase was immunoprecipitated from in vitro translation products and incubated with GST-CTD in the presence of increasing concentrations of a nonspecific (lanes 1–3 and 7–9) or a specific peptide (lanes 4–6 and 10–12). Each of the kinase reactions contained Abl or Arg and 0.2 µg of GST-CTD. Lanes 1–3 and 7–9 contained 5, 50, and 500 ng of nonspecific peptide containing the sequence AKFQQRKQLNSMRAF. Lanes 4–6 and 9–12 contained similar amounts of specific peptide sequence SIQMQRRNKFAFREAINKL. The reaction was carried for 30 min, and products were analyzed by autoradiography and by electrophoresis and by autoradiography, act, wild type.

inhibition of CTD kinase with a synthetic peptide containing the identified CTD-ID sequence.

The high degree of conservation of amino acid sequence in the SH2, kinase, and the CTD-ID region between Arg and Abl kinases indicates that these two kinases share substrate specificity for RNAPII phosphorylation. The c-Abl kinase has been shown to be present both in the nucleus and in the cytoplasm (9, 10). Wang and Kruh (25) recently reported that when overexpressed in COS cells, Arg is mostly localized in cytoplasm, with minor fractions of the protein present in the nucleus. Due to the lack of specific antibodies that can recognize endogenous Arg, it has not been possible to determine the subcellular localization of Arg in Abl-null cells. In any case, it is possible that Arg may substitute for Abl kinase in these cells in order to catalyze the basal tyrosine phosphorylation of RNA polymerase II. However, we cannot rule out that the basal tyrosine phosphorylation of RNA polymerase II is catalyzed by other tyrosine kinases that are unrelated to either Abl or Arg.

Recent reports have shown that several DNA damaging agents such as cisplatin, IR (γ-irradiation), MMS, and mitomycin C can activate Abl kinase activity, and this activation is correlated with an increase in the phosphotyrosine content of RNAPII (24). When Abl-null cells were exposed to DNA-damaging agents, no increase in the phosphotyrosine content of RNA polymerase II was observed (24). We have also shown that the IR activation of Abl is dependent on the function of the actin-teliangiectasia mutated gene product. In AT patient cells, IR does not activate Abl and does not induce tyrosine phosphorylation of RNAPII (29). If Arg is involved in maintaining the basal tyrosine phosphorylation of RNA polymerase II, this result would suggest that Arg may not be activated by DNA damage-induced signaling event. Given the fact that Arg and Abl are highly divergent in the C-terminal region except for the CTD-interacting domain, it is possible that these two kinases may transduce different signals to mediate the tyrosine phosphorylation of RNA polymerase II.

Tyrosine phosphorylation of the CTD has been correlated with the stimulation of promoter activity (15). The c-Abl tyrosine kinase is regulated by at least three signals. Integrin receptor-mediated adhesion to the extracellular matrix is required to activate c-Abl (30). In the nucleus, c-Abl activity is further regulated by retinoblastoma in the cell cycle (31, 32) and by DNA damage (24, 29). At present, it is not known what signals regulate Arg. Because c-Abl and Arg can phosphorylate RNAPII-CTD, these tyrosine kinases can transduce signals directly to RNA polymerase II to regulate transcription.

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