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Biochemistry

p140/c-Abl that binds DNA is preferentially phosphorylated at tyrosine residues

(*EP* element/HBV enhancer/tyrosine kinases)

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ABSTRACT ***EP* is a DNA element found in the enhancer and promoter regions of several cellular and viral genes. Previously, we have identified the DNA binding p140/c-Abl protein that specifically recognizes this element. Here we show that phosphorylation is essential for the p140/c-Abl DNA binding activity and for the formation of DNA–protein complexes. Furthermore, by ^{32}P labeling of cells and protein purification, we demonstrate that *in vivo* the *EP*-DNA-associated p140/c-Abl is a tyrosine phosphoprotein. By employing two different c-Abl antibodies, we demonstrate the existence of two distinct c-Abl populations in cellular extracts. p140/c-Abl is quantitatively the minor population, is heavily phosphorylated at both serine and tyrosine residues, and is active in autophosphorylation reactions.**

c-Abl is a nonreceptor tyrosine kinase with four functional domains: the src homology (SH) domains 2 and 3, the kinase domain, and the C-terminal tail (1). These functional domains, with the exception of the C-terminal tail, are found in many key regulatory proteins and mediate protein–protein interactions (the SH2 and SH3 domains) (2, 3) and manifest catalytic activity (the kinase domain). In cells the kinase activity of c-Abl is highly regulated and can be only poorly demonstrated; consequently, protein substrates were hardly identified (4–7). In sharp contrast the transforming variants of this protein display a constitutive kinase activity that is accompanied by dramatic changes in cell behavior (4, 8, 9). One promising way to study the function of c-Abl is the genetic approach mainly through generation of animal null mutants. These studies have clearly shown that the C-terminal tail of c-Abl is crucial for its normal function (10), but these studies cannot clarify the responsible molecular mechanisms. Recently, it was demonstrated that the c-Abl C-terminal tail has a DNA binding activity (11). This is an important finding considering the nuclear localization of c-Abl; however, this binding is not sequence-specific, and to this end, no specific target gene has been identified. Previously, we have characterized a p140 sequence-specific DNA binding protein that by immunological techniques was characterized to be c-Abl and hence referred to as p140/c-Abl. p140/c-Abl is associated with nuclear protein complexes that specifically bind the *EP* DNA element of the hepatitis B virus enhancer (12). *EP* is a functional element of the enhancer of hepatitis B virus and several other viruses (13, 14) and displays a peculiar behavior in that it is important for the activity of several distinct viral enhancers but in isolation has no enhancer activity (13). The *EP* binding complex was detected in every cell line examined so far, suggesting that it is an essential cellular protein complex. More recently, a second protein named RFX1, the yeast homologue of which functions in controlling the cell cycle (15), was reported to be a component of the EP protein complex (16). In this report, we further investigate p140/c-Abl and show that this protein provides a minor fraction of the cellular c-Abl population. We show that p140/c-Abl is phosphorylated at serine and tyrosine residues and has kinase activity.

MATERIALS AND METHODS

Electrophoretic Mobility Shift Assay (EMSA), Immunoprecipitation, and Western and Southwestern Blot Analyses. Preparation of HeLa cell nuclear extracts, DNA binding assays (EMSAs), immunoprecipitation with both 8E9 and 543 antibodies, and Western and Southwestern blot analyses were conducted as reported (12). Preparation and determination of the specificity of the 543 antibody that was raised against the c-Abl C-terminal region (aa 631–981) were reported elsewhere (12, 17). SW, a new preparation of polyclonal antibody raised against the C-terminal domain of c-Abl by immunizing rabbits with the histidine-tagged c-Abl-(631–981) recombinant protein, behaves as 543.

Cell Maintenance and Labeling. HeLa and Hep1-SK cell lines were grown as reported (13). For labeling, cells were washed twice with phosphate-free DMEM, incubated for 1 hr in 2.5 ml of phosphate-free medium containing 2% (vol/vol) dialyzed fetal calf serum (GIBCO), subsequently supplemented with $^{32}P_i$ at 1 mCi/ml (1 Ci = 37 GBq), and incubated for 3 hr. Two hours before harvesting, sodium orthovanadate was added to a final concentration of 1 mM, to inhibit tyrosine phosphatases. Phosphoamino acid analysis was done as described (18). Phosphoamino acid standards were visualized by 0.25% ninhydrin staining in acetone.

DNA Affinity Columns and Protein Purification. HeLa and Hep1-SK nuclear extracts were prepared as described (19). All operations were performed in 4°C with buffer D containing 20 mM Hepes (pH 7.9), 100 mM KCl, 0.2 mM EDTA, 20% (vol/vol) glycerol, 0.5 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, aprotonin (1 μg/ml), and leupeptin (1 μg/ml). HeLa nuclear extract (75 mg) was subjected to *EP* purification by using the biotin-streptavidin-based affinity method essentially as described (20). Biotin-11-dUTP was incorporated by a fill-in reaction to multimerize either *EP* or *EP* mutant oligonucleotides, 15 μg of biotinylated *EP* mutated DNA and 600 μg of poly(dIdC) were added to the heparin-agarose active fraction, and then 30 μg of streptavidin was added and incubated for 5 min at 30°C. Next, pretreated biotin-agarose (BRL) was added and incubated for an additional 30 min at 4°C. After spinning, the supernatant was removed and bound in a similar manner to *EP* wild-type DNA and the pellet was washed twice with 1 ml of buffer D containing 0.5 M NaCl and bovine serum albumin (100 μg/ml). The proteins were eluted with 0.4-ml aliquots of buffer D containing 1 M NaCl.

Kinase Reactions. Kinase reactions were performed directly on the beads, either from the affinity column or immunopre-

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Abbreviations: SH, src homology domain; EMSA, electrophoretic mobility shift assay; PAP, potato acid phosphatase; CIP, alkaline phosphatase.

cipitated from the c-Abl-protein A-Sepharose column. The beads were washed and equilibrated with kinase buffer (20 mM Hepes, pH 7.6/4 mM $MgCl_2$/100 mM NaCl/20 mM $MnCl_2$/1 μM ATP). The reaction was performed in 50 μl by adding 30 μCi of [γ-^{32}P]ATP and incubating at 30°C for 30 min. In certain cases, the synthetic GAT substrate (6 μM) was added. The reaction was stopped by adding sample buffer, boiling for 5 min, and analyzing by SDS/PAGE.

RESULTS

The DNA Binding Activity of p140/c-Abl Is Regulated by Phosphorylation. Previously, Southwestern blot analyses have detected p140, a sequence-specific DNA binding protein that generated specific complexes with the *EP* element of the hepatitis B virus enhancer. This protein, by immunological tools, was identified as p140/c-Abl (12). To test whether protein phosphorylation is important for the generation of the *EP* DNA–protein complexes, dephosphorylation experiments were performed. This complex was immunoprecipitated by anti-Abl 543 antibodies, and the antibody-bound *EP* complexes were eluted by urea and subjected to EMSA (Fig. 1*A*). In agreement with our previous report the immunoprecipitated EP proteins could regenerate the *EP* DNA–protein complexes (Fig. 1*A*, lane 2). Interestingly, this activity was lost upon treatment with either potato acid phosphatase (PAP) or alkaline phosphatase (CIP, lanes 3 and 4), suggesting that protein phosphorylation is essential for the complex formation. Next, we investigated the behavior of p140/c-Abl under phosphatase treatment. Treated extracts were first examined by Southwestern blot analysis to detect the p140 and by subsequent immunoblot analysis with anti-Abl antibody to detect c-Abl (Fig. 1*B*). PAP or CIP significantly reduced the DNA binding activity of p140/c-Abl (Fig. 1*B Upper*), while the amount of c-Abl was not changed (Fig. 1*B Lower*). In an alternative approach, phosphatase treatment was done directly on the nitrocellulose filter prior to Southwestern and Western blot analyses. The capacity of p140/c-Abl to bind DNA was significantly reduced (Fig. 1*C Upper*), while the amount of c-Abl was not affected (Fig. 1*C Lower*). The obtained reduction in DNA binding activity is the direct result of phosphatase activity, since it was not seen when phosphatase inhibitors were added. Thus, these results strongly indicate that phosphorylation positively regulates the binding of p140/c-Abl to *EP*–DNA.

Cells Contain Two c-Abl Populations. Two specific antibodies, polyclonal 543 and anti-Abl monoclonal 8E9 antibodies (PharMingen), which were raised against C-terminal and N-terminal (SH2 domain) portions of c-Abl, respectively, were employed for immunoprecipitation experiments. HeLa nuclear extracts were first immunoprecipitated by 8E9, the unbound fraction was immunoprecipitated with either 543 or control antibodies, and the samples were examined by immunoblot analysis with 8E9. The majority, but not all, of the native c-Abl protein was immunoprecipitated with 8E9 (Fig. 2*A*). Interestingly, a minor fraction of c-Abl that was not recognized by 8E9 was efficiently immunoprecipitated by the 543 anti-Abl antibody (lane 543/8E9FT). A similar ratio between the two c-Abl fractions was seen when the 543 antibody was used for immunoblot analysis. Furthermore, SW, a second polyclonal anti-c-Abl C terminus antibody, showed similar results and behaved as 543 (data not shown). Southwestern blot analysis showed that the *EP* DNA binding p140/c-Abl was neither immunodepleted (Fig. 2*B*, lane 1) nor immunoprecipitated (lane 5) by the 8E9 antibody. However, when the 8E9 flow-through fraction was incubated with the 543 antibody, it was efficiently immunodepleted (lane 2) and immunoprecipitated (lane 4). Thus, cells contain two distinct c-Abl populations, one

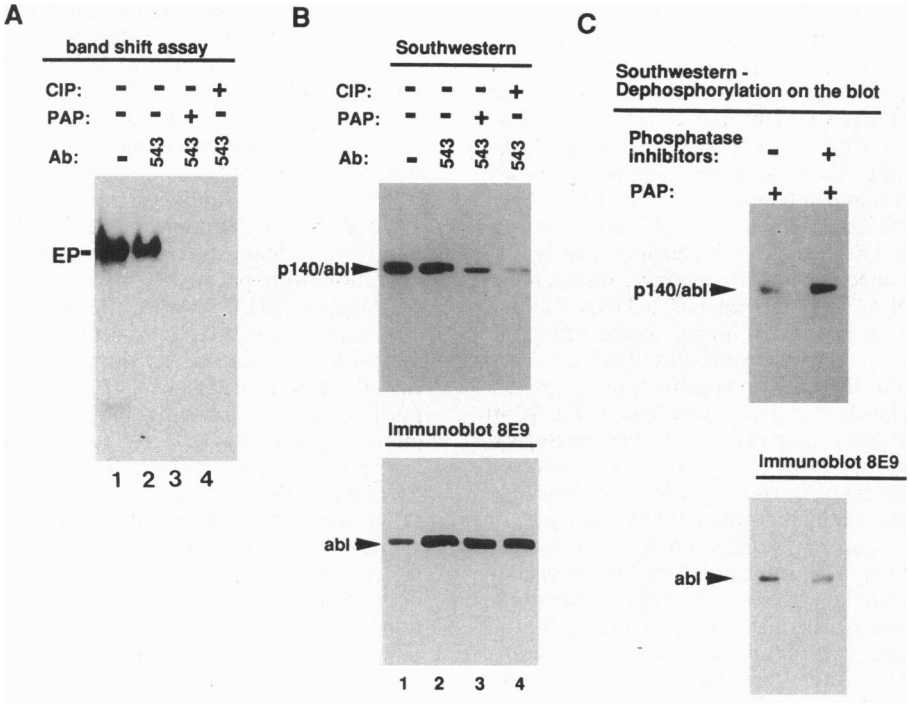

FIG. 1. Phosphorylation-dependent binding of EP protein. EP complex or p140 EP binding protein was immunoprecipitated from HeLa nuclear extract by 543 anti-Abl antibodies. The immune complexes were washed and then treated with PAP or CIP or untreated. (*A*) EMSA with ^{32}P-labeled EP oligonucleotide of phosphatase treated (lanes 3 and 4) or untreated (lane 2) immunopurified EP complex. A binding reaction of HeLa nuclear extract prior to immunoprecipitation is shown in lane 1. The EP complex was recovered from the protein A-Sepharose-bound immune complexes by using 5 M urea. (*B*) Southwestern blot analysis with *EP* DNA (*Upper*) and a subsequent immunoblot analysis with 8E9 monoclonal antibody (*Lower*) of 543 immune complexes, after treatment (lanes 3 and 4) with the indicated phosphatases. A control extract and untreated 543 immunoprecipitates are shown in lanes 1 and 2, respectively. (*C*) Southwestern blot analysis of HeLa nuclear extract (*Upper*) and immunoblot assay with 8E9 antibody (*Lower*) followed by PAP treatment in the presence (+) or absence (−) of phosphatase inhibitors (10 mM sodium phosphate/10 mM $PNPP_i$/50 mM NaF/2 mM $NaVO_3$) on the blot prior to the binding reaction. (The strips were washed twice prior to the binding reaction.)

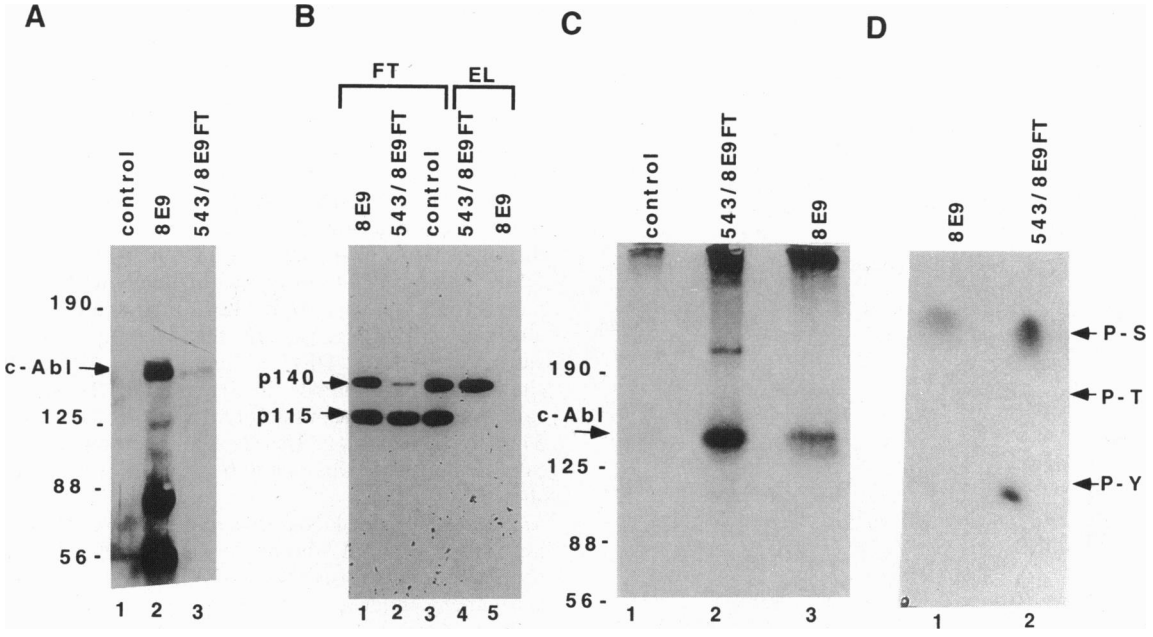

FIG. 2. Identification of two c-Abl populations. (*A*) HeLa nuclear extract was immunoprecipitated with either a nonrelevant antibody (anti-pX of hepatitis B virus) (lane control) or the c-Abl 8E9 monoclonal antibody (lane 8E9). The flow-through fraction was subjected to immunoprecipitation with 543 anti-C-terminal c-Abl antibody (lane 543/8E9FT). The immunoprecipitated proteins were subjected to SDS/PAGE, blotted, and immunoreacted with 8E9. The position of size marker molecular weight and c-Abl is shown. (*B*) As in *A* but was examined by Southwestern blot analysis with ^{32}P-labeled double-stranded oligonucleotides with the *EP* site sequence. The immunodepleted fractions (FT) and the eluted samples (EL) were analyzed as indicated. The position of p140 and the nonspecific p115 DNA binding protein (12) is shown. (*C*) SDS/PAGE of a HeLa extract that was metabolically ^{32}P-labeled and immunoprecipitated as in *A*. (*D*) Phosphoamino acid analysis of the c-Abl bands seen in *C*. The position of phosphoserine (P-S), phosphothreonine (P-T), and phosphotyrosine (P-Y) is shown.

of which is the p140/c-Abl population that is specifically reactive to 543 antibody. To determine whether these two c-Abl populations have distinct patterns of phosphorylation, similar experiments were done by using nuclear extracts of ^{32}P-labeled cells. Remarkably, p140/c-Abl was highly phosphorylated (Fig. 2*C*) compared with the major c-Abl population that was immunoprecipitated by 8E9 (compare Fig. 2 *A* with *C*). Furthermore, phosphoamino acid analysis revealed that the c-Abl monoclonal 8E9 antibody fraction is mostly phosphorylated on serine residues (ratio of phosphoserine to phosphotyrosine is 9 to 1) whereas the 543-specific fraction (p140/c-Abl) contains a higher amount of phosphotyrosine residues (ratio of 7 to 3) (Fig. 2*D*).

Phosphorylated p140/c-Abl Binds the *EP* DNA. The findings that p140/c-Abl is highly phosphorylated and has DNA binding activity suggest that the phosphorylated form of c-Abl is preferentially associated with DNA. To examine this possibility *in vivo* ^{32}P-labeled nuclear extracts were subjected to the following analysis. The extract was immunoprecipitated by the monoclonal 8E9 antibody (Fig. 3, lane 1), and the supernatant fraction, which contains p140/c-Abl, was divided into two parts; one was subjected to immunoprecipitation with 543 anti-Abl antibody and the second was purified on *EP* DNA affinity column. For the latter step, to eliminate any nonspecific DNA binding proteins, the extract was first passed through a column that contained mutated EP DNA and the unbound fraction was then purified on EP-specific affinity column. As expected, the majority of phosphorylated p140/c-Abl escaped the 8E9 monoclonal antibody but was immunoprecipitated by 543 antibody (lane 2) and retained on the *EP* DNA affinity column (lane 3). The 8E9 fraction contained ≈90% of c-Abl, as judged by Western blot analysis (data not shown). Interestingly, phosphoamino acid analysis shows that p140/c-Abl with EP binding activity is phosphorylated at tyrosine residues.

DNA-Associated p140/c-Abl Is Catalytically Active. Given the observation that p140/c-Abl is hyperphosphorylated and has DNA binding activity, we next investigated its kinase activity. HeLa extracts were first immunodepleted with the 8E9 c-Abl antibody and then subjected to either immunoprecipitation with the 543 antibody or DNA affinity column purification. The obtained samples were then subjected to kinase reactions. Both the 543 c-Abl population and the EP-DNA-associated p140/c-Abl were active in autophosphorylation, and a labeled band of the expected size was seen (Fig. 4, lanes 1 and 2). To confirm that the latter is indeed the c-Abl protein, the labeled DNA-associated proteins were subjected to immunoprecipitation with either 543 (lane 3) or a nonrelevant control antibody (lane 4). Out of few labeled bands, p140, the expected size of c-Abl, was specifically immunoprecipitated. Also, to substantiate the fact that the 543 antibody indeed recognizes only c-Abl, extracts that expressed the BCR–Abl fusion protein were subjected to similar immunoprecipitation and kinase reaction experiments, and as expected, only the fusion protein was detected (lane 5).

DISCUSSION

Previously we have shown that p140/c-Abl is associated with the *EP* DNA–protein complex and that in Southwestern blot analysis it has *EP*-DNA-specific binding activity (12). However, *in vitro*-synthesized Abl protein does not bind DNA, suggesting that either a post-translational modification or association with a second protein is critical for the EP-specific-binding activity. Herein we provide evidence in favor of the first possibility, namely, that the p140/c-Abl fraction that is associated with *EP* DNA is preferentially phosphorylated at serine and tyrosine residues. Furthermore, we show that protein phosphorylation is essential for the *EP* DNA binding activity. However, phosphorylation is required but is not sufficient and preliminary results tend to suggest the involvement of a second nuclear protein in this activity (unpublished observations). Previously, it has been reported that c-Abl is phosphorylated by cdc2 and the hyperphosphorylated c-Abl no

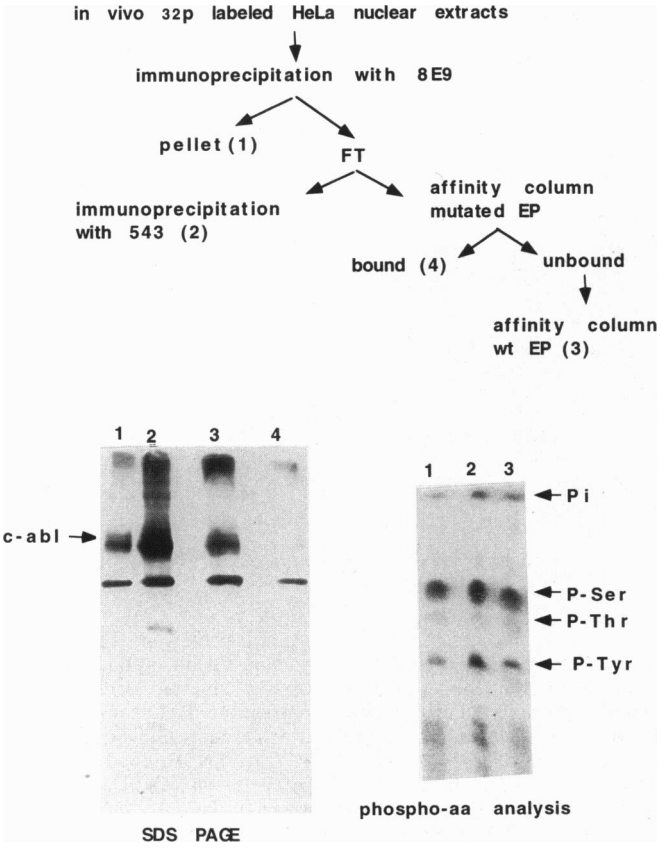

FIG. 3. *EP* DNA binding protein is tyrosine-phosphorylated. (*Upper*) Experimental strategy is shown. The numbers in parentheses correspond to the numbers of the lanes. (*Lower*) The c-Abl band seen in SDS/PAGE was analyzed for phosphoamino acids.

longer has DNA binding activity (11). Thus, it seems that phosphorylation at distinct residues of c-Abl regulates both its DNA association and dissociation.

By using two anti-Abl antibodies, we revealed two c-Abl populations in the cells. 8E9 is an anti-Abl monoclonal antibody that was specifically raised against the SH2 domain of c-Abl. This antibody is immunoreactive to >90% of the native

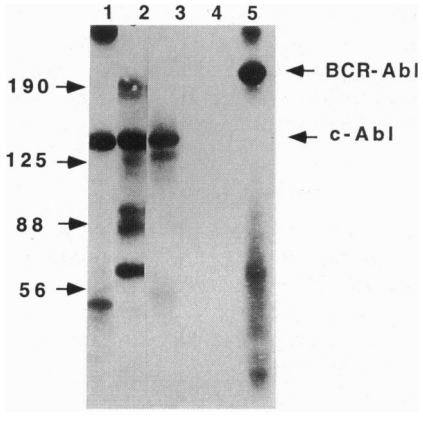

FIG. 4. Kinase activity of c-Abl. HeLa nuclear extracts were first immunodepleted by the 8E9 antibody and then either immunoprecipitated with the 543 antibody and subjected to a kinase reaction (lane 1) or subjected to *EP* DNA affinity column purification and then to the kinase reaction (lane 2). A fraction of the proteins separated in lane 2 was subjected to immunoprecipitation with the 543 antibody (lane 3) or nonrelevant control antibody (lane 4). In lane 5, the immunoprecipitation and kinase experiment was performed with extracts of the BV173 cell line that expresses the fusion BCR–Abl protein.

c-Abl protein, as judged by a combination of immunoprecipitation and Western blot analysis (Fig. 2*A*). The remaining minor population is immunoreactive to only the C-terminus-specific antibody. Interestingly, the latter contains the majority of the incorporated ^{32}P *in vivo*. Phosphoamino acid analysis revealed that phosphorylation occurred at both serine and tyrosine residues. p140/c-Abl is exclusively found in this c-Abl fraction, as demonstrated by Southwestern blot analysis. Furthermore, the p140/c-Abl that is 543-immunoreactive is indistinguishable from the protein that is retained on an *EP* DNA column and both are catalytically active in autophosphorylation reactions. It has been reported (21) that the 8E9 antibody did not recognize the fraction of c-Abl that is associated with Rb. This is despite the fact that the Rb binding site was localized outside the SH2 domain. These observations raise the interesting possibility that Rb is associated with the 543 c-Abl population. However, our attempts to experimentally demonstrate this possibility have so far failed (data not shown).

The finding that DNA-associated p140/c-Abl is not immunoreactive to the 8E9 monoclonal antibody, which recognizes the SH2 domain, raises the possibility that this domain of c-Abl is occupied. Indeed, it has been reported that the SH2 domain of c-Abl recognizes phosphotyrosyl residues in a specific protein sequence context (3, 22). The facts that p140/c-Abl fraction is active in tyrosine phosphorylation and is hyperphosphorylated favor this possibility and may suggest either an inter- or intramolecular interaction through the SH2 domain. We do not know which of these possibilities is correct, but preliminary data suggest association of a second DNA binding protein with p140/c-Abl (unpublished observations). In that case c-Abl seems to be like the STAT proteins, a family of latent cytoplasmic transcription factors that are activated by direct tyrosine phosphorylation to dimerize and to translocate to the nucleus to bind DNA (23, 24). Similar to STAT proteins, a fraction of c-Abl is cytoplasmatic and in direct interaction with actin and adaptor proteins (3, 7, 25). Furthermore, it has been reported that ionizing radiation potentiates the kinase activity of c-Abl (26), suggesting its involvement in signaling pathways. The diagnostic difference between the two signaling pathways is that unlike STAT proteins, the tyrosine kinase domain is an intrinsic part of c-Abl. Obviously this is an interesting possibility that should be experimentally pursued.

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