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Deletion of an N-terminal regulatory domain of the c-*abl* tyrosine kinase activates its oncogenic potential

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The requirements for the oncogenic conversion of the c-*abl* proto-oncogene have been determined by the expression of N-terminal deleted forms and viral *gag*-fused forms of the c-*abl* proteins from a selectable retroviral vector. To activate the transforming potential of c-*abl*, it is necessary that (i) specific N-terminal amino acids are deleted to release the kinase from negative regulation *in vivo*; (ii) an N-terminal myristylation site is part of the activated kinase; (iii) the fatty-acylated, activated kinase is overproduced. The N-terminal amino acids found to be necessary for the cellular inhibition of c-*abl* tyrosine phosphorylation are part of a homologous region present in many non-receptor tyrosine kinases, the v-*crk* oncogene and phospholipase C-II. Overproduction of a deregulated and myristylated c-*abl* tyrosine kinase induces the transformation of NIH 3T3 cells.

Key words: Abelson murine leukemia virus/myristylation/overproduction/phospholipase C-II/v-*crk* homology domain

Introduction

The proto-oncogene c-*abl* is the normal cellular homolog of the v-*abl* oncogene, which was first isolated from Abelson murine leukemia virus (A-MuLV; Abelson and Rabstein, 1970; Goff *et al.*, 1980). Like most mammalian proto-oncogenes, the c-*abl* gene is present in all vertebrate genomes and homologs of c-*abl* have been isolated from the genomes of *Drosophila melanogaster* and *Caenorhabditis elegans* (Henkemeyer *et al.*, 1988; Goddard *et al.*, 1986). In addition to the transduction of mouse c-*abl* by A-MuLV, the cat c-*abl* gene has also been transduced by a feline retrovirus to generate the Hardy-Zuckerman-2 feline sarcoma virus (HZ2-FeSV; Besmer *et al.*, 1983). The human c-*abl* gene, located on chromosome 9, is found to be consistently translocated onto chromosome 22 to form the Philadelphia chromosome (Ph^+) in chronic myelogenous leukemia (CML; de Klein *et al.*, 1982; Heisterkamp *et al.*, 1983). Recently, translocation of the c-*abl* gene has also been demonstrated to occur in Ph^+-positive acute lymphocytic leukemia (ALL; Hermans *et al.*, 1987; Chan *et al.*, 1987). Thus, the normal c-*abl* genes of three mammalian species have been correlated with oncogenic processes through retroviral transduction or chromosomal translocation.

The mammalian c-*abl* gene generates two transcripts differing on their 5'-ends (Ben-Neriah *et al.*, 1986; Shtivelman *et al.*, 1986; Bernards *et al.*, 1988). The 5'-heterogeneity is the result of alternative splicing of variable 5'-exons to a set of common exons in the c-*abl* locus (Ben-Neriah *et al.*, 1986; Shtivelman *et al.*, 1986). The variable 5'-exons contain protein coding sequences: the longer 5'-exon (human Ib or mouse type IV) encodes 45 amino acids, whereas the shorter 5'-exon (human Ia or mouse type I) encodes 26 amino acids. The v-*abl* oncogene of A-MuLV is a truncated version of the c-*abl* cDNA lacking both 5' and 3' c-*abl* sequences (Wang and Baltimore, 1983; Wang *et al.*, 1984). The 5'-variable exons plus the first 264 bp of the c-*abl* common exons as well as 1.2 kb of the 3'-untranslated region are deleted in the v-*abl* oncogene (Wang *et al.*, 1984; Ben-Neriah *et al.*, 1986). The 5'-deletion eliminates the variable amino acids plus 88 amino acids of the common region. The oncogene of HZ2-FeSV is also a deletion mutant of the cat c-*abl* with truncations of both the 5'- and 3'-ends (Bergold *et al.*, 1987). Translocation of the c-*abl* gene in the Philadelphia chromosome results in the production of a fused mRNA which links the *bcr* coding sequence in frame with the c-*abl* common region (Shtivelman *et al.*, 1985 and 1986). Thus, the *bcr/abl* fusion protein lacks only the c-*abl* variable amino acids.

The common region of the c-*abl* gene contains a tyrosine kinase domain homologous with other tyrosine kinases (Wang and Baltimore, 1985; Hank *et al.*, 1988). Tyrosine kinase activity has been shown to be essential to the transforming activity of the v-*abl* oncogene (Prywes *et al.*, 1985). Nucleotide sequence comparison shows no mutation in the tyrosine kinase domain between the c-*abl* and the v-*abl* genes (Oppi *et al.*, 1987; M.Trepanier and J.Y.J.Wang, unpublished data). It has been proposed that the addition of viral *gag* or cellular *bcr* amino acids to the c-*abl* protein activates its tyrosine kinase activity, leading to oncogenic conversion (Davis *et al.*, 1985). However, there has not been a systematic study on the role of the different 5'-deletions in the activation of the c-*abl* oncogenic potential. In this report, we describe results that demonstrate the importance of specific 5'-deletions in the activation of the c-*abl* proto-oncogene. We have also investigated the role of the viral *gag* sequence and protein overproduction in the transforming activity of c-*abl*-derived oncogenes.

Results

Strategy for expression of normal c-abl proteins and their derivatives

To examine the effect of N-terminal amino acids on the c-*abl* transforming activity, a set of specific 5'-deletions was generated (Figure 1, panels A and B). Truncations were made at three restriction enzyme sites, *Bgl*II, *Pst*I and *Hin*cII, in the type I c-*abl* cDNA (Figure 1A). Cutting at *Bgl*II eliminates the first three codons of the type I variable exon. Truncation at *Pst*I deletes the variable exon and four codons of the common region, resembling the deletion found in the

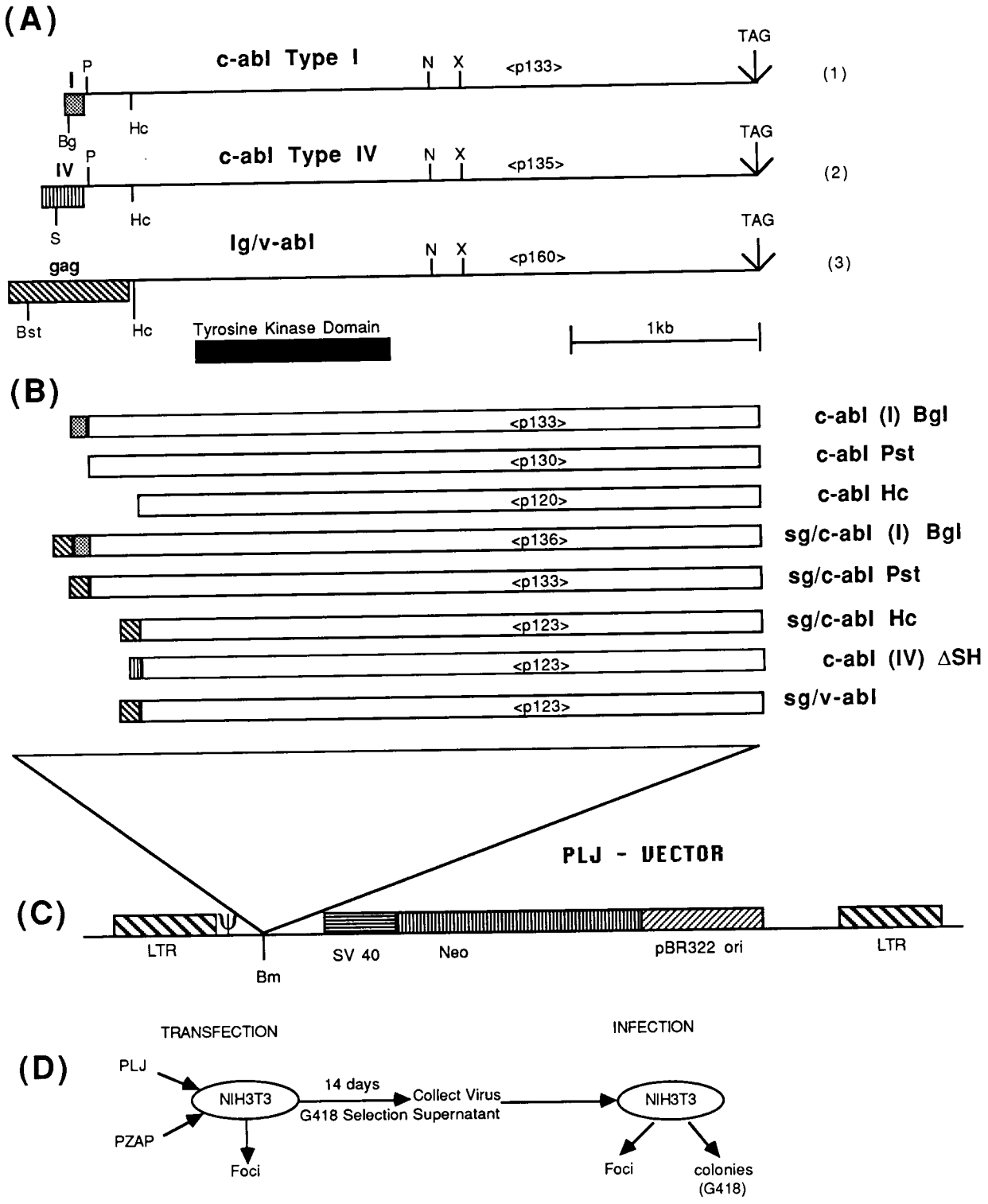

Fig. 1. Restriction maps, constructs, expression vector and scheme. (**A**) Restriction maps of the protein coding region of the c-*abl* type I (1) and type IV (2) cDNAs and the lg/v-*abl* oncogene of A-MuLV p160 (3). Two forms of c-*abl* proteins are distinguished by their variable 5′ exons, encoding 26aa (type I) or 45aa (type IV). In A-MuLV, c-*abl* sequences 5′ of the *Hin*cII site are substituted by the M-MuLV *gag* sequence (lg = large *gag*). The indicated mol. wt (p plus number of kd) were those observed for each protein on 7% SDS−polyacrylamide gels. Important restriction sites for subcloning are abbreviated as follows: Bgl = *Bgl*II, Bm = *Bam*HI, Bst = *Bst*EII, Hc = *Hin*cII, N = *Nar*I, P = *Pst*I, S = *Stu*I, X = *Xho*I. Further abbreviations: kb = kilobase, TAG = three letter translation stop codon. (**B**) Deletion- and fusion-protein coding sequences generated for this study. Each construct is named by the origin (c- or v-*abl*) of DNA and the restriction site used for subcloning. The abbreviation sg (small *gag*) corresponds to the first 35 amino acids of Moloney p15 *gag* protein, Δ stands for an internal deletion of DNA sequence, / is symbolizing a fusion construct, () indicates the source of c-*abl* cDNA. (**C**) Diagram of the retroviral PLJ vector (Korman *et al.*, 1987); c-*abl* and v-*abl* constructs are inserted at the unique Bm site. Used abbreviations are: LTR = long terminal repeat promoter/enhancer sequence of M-MuLV; ψ, *cis* acting retroviral packaging signal; SV40, large T promoter/enhancer sequence of simian virus 40; Neo, neomycin/G418 resistance gene; pBR322 ori, origin of replication from the plasmid pBR322. (**D**) PLJ-constructs together with M-MuLV helper virus clone, pZAP, were transfected into NIH 3T3 fibroblasts. The supernatants were used for infection.

bcr/abl fused mRNA of Ph$^+$ CML or ALL (Shtivelman *et al.*, 1985; Hermans *et al.*, 1987). Digestion with *Hin*cII deletes the variable exon and a total of 93 common region codons, resembling the deletion found in A-MuLV (Wang *et al.*, 1984; Ben-Neriah *et al.*, 1986). An ATG codon in the context of an *Nco*I recognition sequence (CCATGG) for translational initiation (Kozak, 1986), and an alanine codon for protein stability (Bachmair *et al.*, 1986), were added to the deleted c-*abl* coding sequences to express the truncated proteins (Wang, 1988).

A second set of viral *gag* fusion proteins was generated by ligating the coding sequence for the first 35 amino acids of the Moloney-MuLV (M-MuLV) p15 *gag* protein to each of the c-*abl* deletion constructs. These 'small' *gag* (sg) fusion proteins lack 90% of the *gag* sequence found in the wild-type A-MuLV (Figure 1, panels A and B). A sg/v-*abl* fusion protein, serving as a positive control for our experiments, has previously been demonstrated as retaining full transforming activity in NIH 3T3 cells (Prywes *et al.*, 1983). An internal in-frame deletion mutant was generated in the type IV cDNA by excising sequences between the *Stu*I site in the type IV variable exon and the *Hin*cII site in the common region. This c-*abl* (IV) ΔSH construct lacks 31 of the type IV amino acids and the first 93 common amino acids. The various c-*abl* deletion and fusion sequences were inserted into the *Bam*HI site of the retroviral PLJ-vector (Korman *et al.*, 1987). The c-*abl* and v-*abl* genes are expressed from the LTR of M-MuLV and the selectable neomycin resistance gene is expressed from the internal SV40 early promoter (Figure 1C). NIH 3T3 cells were co-transfected with PLJ-plasmids and a cloned DNA of replication-competent M-MuLV, pZAP (Goff *et al.*, 1982). The number of transformed foci and G418 resistant colonies were determined for each PLJ construct (Figure 1D).

Transforming activity of c-abl proteins

The transforming protein of A-MuLV, lg/v-*abl* (p160), or its derivative sg/v-*abl* (p123), produced 1600 foci/μg DNA when expressed from the PLJ vector (Table I). In the same transfections, the number of neomycin resistant colonies was on average four-fold higher, at 6000 colonies per μg DNA (Table I). Neither of the two normal c-*abl* constructs nor the N-terminal-deleted Bgl, Pst or Hc constructs produced any foci, although these plasmids all gave rise to G418 resistant colonies (Table I). Addition of the first 35 viral *gag* amino acids to the Bgl or Pst truncated c-*abl* proteins did not convert them into oncogenic proteins. However, addition of the same *gag* sequence to the Hc truncated c-*abl* protein did result in the production of foci (Table I). Interestingly, the Stu-Hc internal deletion of the type IV protein also led to activation of the transforming potential of the c-*abl* gene (Table I). The efficiency of foci formation of the two activated c-*abl* genes, defined as the ratio of foci/colonies, was found to be much lower than that of the lg/v-*abl* or sg/v-*abl* oncogenes in repeated experiments (Table I). These results show that the normal or N-terminal truncated c-*abl* proteins have no transforming activity when expressed from the LTR promoter. Since the addition of viral *gag* amino acids to the Bgl or Pst truncated c-*abl* proteins does not lead to oncogenic conversion, the viral *gag* sequence alone is insufficient for activating the c-*abl* oncogenic potential. The combination of viral *gag* sequence with a deletion of the first 93 common region amino acids is required to convert c-*abl* into a transforming gene. The viral *gag* sequence can be replaced with the 14 N-terminal amino acids of type IV to achieve oncogenic conversion of c-*abl*, as demonstrated with the c-*abl* (IV) ΔSH construct. However, the converted c-*abl* oncogenes have lower transforming efficiency than the sg/v-*abl* oncogene.

Table I. Transforming activity of c-*abl*-derived proteins

Expressed sequence	Transfection			Infection		
	foci/μg	col./μg	Ratio (%)	f.f.u./ml	c.f.u./ml	Ratio (%)
PLJ-Vector	0	8200	0	0	6.7×10^5	0
c-*abl* type I	0	7860	0	0	4.9×10^5	0
c-*abl* type IV	0	4300	0	0	1.7×10^5	0
c-*abl* (I) Bgl	0	2640	0	0	5.8×10^5	0
sg/c-*abl* (I) Bgl	0	4320	0	0	4.4×10^5	0
c-*abl* Pst	0	3120	0	0	3.5×10^5	0
sg/c-*abl* Pst	0	3480	0	0	4.0×10^5	0
c-*abl* Hc	0	1920	0	0	6.5×10^5	0
sg/c-*abl* Hc	250	5730	4.4	4.0×10^3	2.0×10^5	2.0
c-*abl* (IV) ΔSH	14	4100	0.3	3.2×10^2	4.6×10^5	0.1
lg/v-*abl*	1600	6100	26.0	8.0×10^4	2.0×10^5	40.0
sg/v-*abl*	1600	6300	25.0	6.0×10^4	2.0×10^5	30.0
PZAP	0	0	–	0	0	–

Transforming assays were performed by DNA transfection and by viral infection. The ratio of foci to colonies or f.f.u. to c.f.u. is used to compare the efficiency of transformation of each construct. Each value represents the average of three independent determinations.

It has previously been shown that transfection of NIH 3T3 cells with the A-MuLV DNA is lethal, but that infection of NIH 3T3 cells with the virus is not (Goff *et al.*, 1982). To avoid the possible lethal effect associated with the transfection assay, viral stocks were prepared from G418 selected cells and then applied to fresh NIH 3T3 cells. The lg/v-*abl* and sg/v-*abl* stocks contained $6-8\times10^4$ foci forming units (f.f.u.)/ml and 2×10^5 colony forming units (c.f.u.)/ml (Table I). Thus, the infection assay was 30–50 times more sensitive than the transfection assay. The transformation efficiencies, i.e. f.f.u./c.f.u., obtained with the infection assay were comparable to those obtained with the transfection assay (Table I). This result indicated that secondary activating mutations did not occur during viral passage. The infection assays showed, in support of the transfection results, that only two c-*abl*-derived viruses, sg/Hc and ΔSH, transformed fibroblasts, and with lower transforming efficiencies than the sg/v-*abl* virus (Table I).

The reduced transforming activity of the activated c-*abl* genes was also manifested in the size of foci obtained in the transfection; foci induced by the c-*abl* constructs (sg/Hc or ΔSH) appeared after a 3–5 day latency period and grew to a smaller size than those induced by the sg/v-*abl* gene (Figure 2A and B). Transforming activity of the activated c-*abl* genes was further assessed by anchorage independent growth in soft agar. The sg/Hc- and ΔSH-derived transformed cell lines formed smaller colonies in soft agar than the sg- or lg/v-*abl* transformed cells. These observations confirm the reduced transforming activity of the activated c-*abl* genes. Approximately 20% of the G418 resistant colonies obtained by transfection with PLJ-sg/v-*abl* were transformed (Figure 2D), whereas only 3% of the sg/Hc-derived G418 resistant colonies contained transformed cells. None of the ΔSH-derived G418 resistant colonies showed

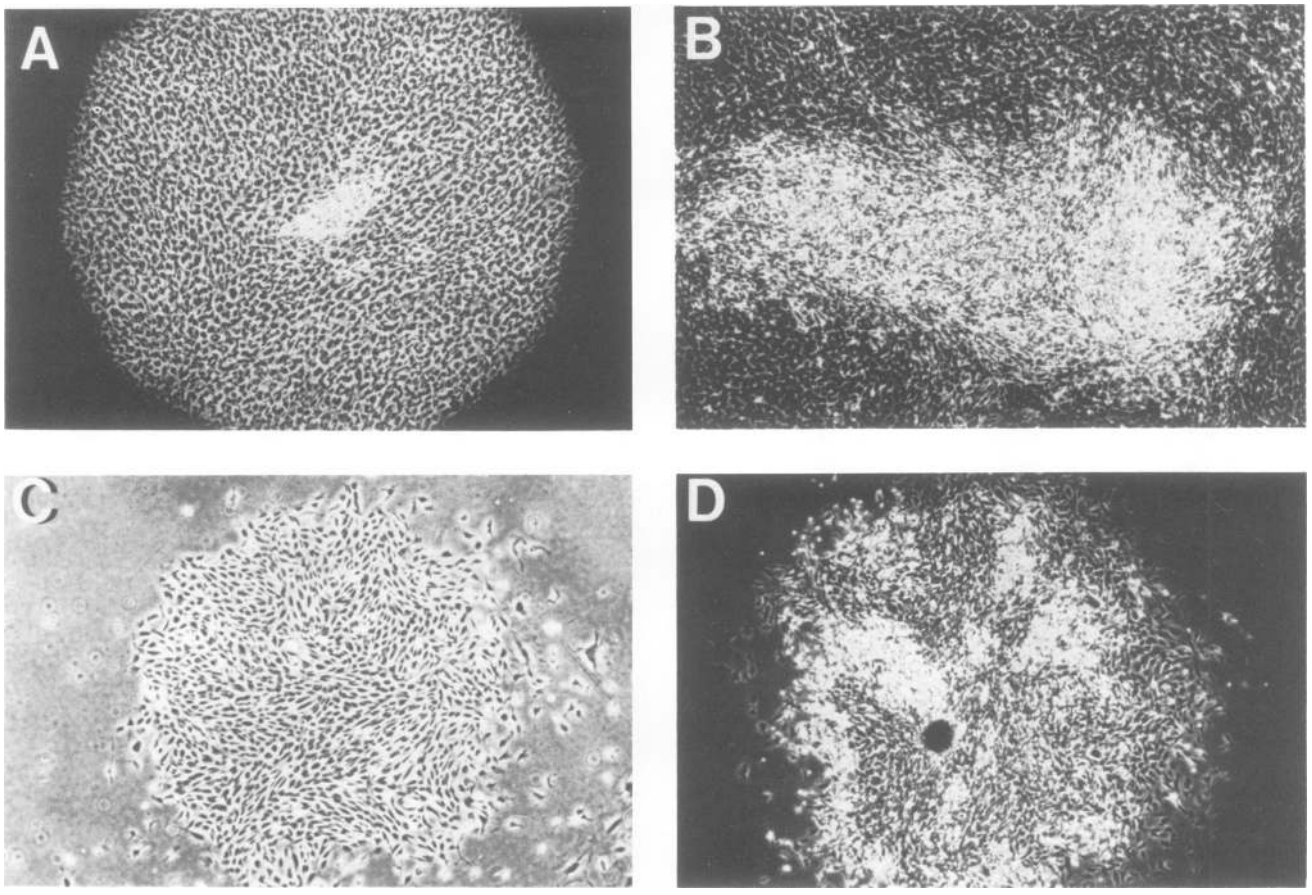

Fig. 2. Comparison of v-*abl* and c-*abl* induced foci and colonies. (**A**) Representative focus from c-*abl* (IV) ΔSH transfection. (**B**) Representative focus from sg/v-*abl* transfection; pictures were taken 19 days after transfection with a magnification of ×37. (**C**) G418 selected colony from c-*abl* (IV) ΔSH transfection with normal morphology. (**D**) G418 selected colony from sg/v-*abl* transfection with transformed morphology; pictures were taken 14 days after transfection with a magnification of ×37.

any transformed morphology (Figure 2C). These values were consistent with the f.f.u./c.f.u. ratio determined by the focus forming assay (Table I).

Expression of the recombinant c-abl proteins in transfected fibroblasts

To examine the expression of the non-transforming c-*abl* proteins, lysates from polyclonal populations of G418 resistant cells were analyzed by immunoblotting with a monoclonal anti-*abl* antibody (Kipreos *et al.*, 1987; Richardson *et al.*, 1987). Although these recombinant c-*abl* proteins were expressed from the same retroviral vector, the average levels of their expression were not identical, probably due to differences in their stability (Figure 3, panel A). The type IV (3.7-fold, lane 3) and the Pst truncated (4.8-fold, lane 6) proteins were 3- to 4-fold the level of the endogenous c-*abl* protein (1-fold, lane 1). The type I (2.2-fold, lane 2), Bgl (1.6-fold, lane 4) and Hc truncated (1.4-fold, lane 8) proteins were expressed at levels equivalent to the endogenous c-*abl* protein (1-fold, lane 1) in these polyclonal cells.

The increase in mol. wt from the addition of the 35 *gag* amino acids could be discerned on our immunoblots (Figure 3A, lanes 4 and 7). The presence of the p15 *gag* sequence in the fusion recombinant proteins was also confirmed by immunoprecipitation with anti-*gag* antisera (not shown). The addition of the small *gag* sequence increased the expression of the Pst or the Bgl truncated c-*abl* proteins 2-fold (11.6-fold, lane 7 and 3.0-fold, lane 5), indicating that the sg fusion proteins may be more stable. The average levels of expression of the two transforming c-*abl* proteins, sg/Hc or ΔSH, in polyclonal G418 resistant cells were found to be about twice that of the endogenous c-*abl* level (not shown). These results show that all recombinant c-*abl* proteins were expressed in transfected NIH 3T3 cells and that the average level of expression in the polyclonal populations did not correlate with transforming activity.

To compare the level of expression of the transforming c-*abl* and v-*abl* proteins in transformed cells, foci were picked and grown in G418 containing media. All of the morphologically transformed cell lines expressed the transforming proteins at elevated levels, between 7- and 12-fold higher than the endogenous c-*abl* protein level. No apparent difference in the amount of the c-*abl* (sg/Hc or ΔSH) or v-*abl* (sg or lg/v-*abl*) transforming proteins was found in transformed cells (Figure 3, panel B). The observation that the transforming c-*abl* proteins were expressed at high levels in cells derived from foci and low levels in cells derived from non-transformed G418 colonies, suggested that manifestation of the transforming activity required overexpression of the transforming proteins.

To estimate the level of overexpression necessary for transformation, G418 resistant colonies with normal morphology were picked from cells transfected with the

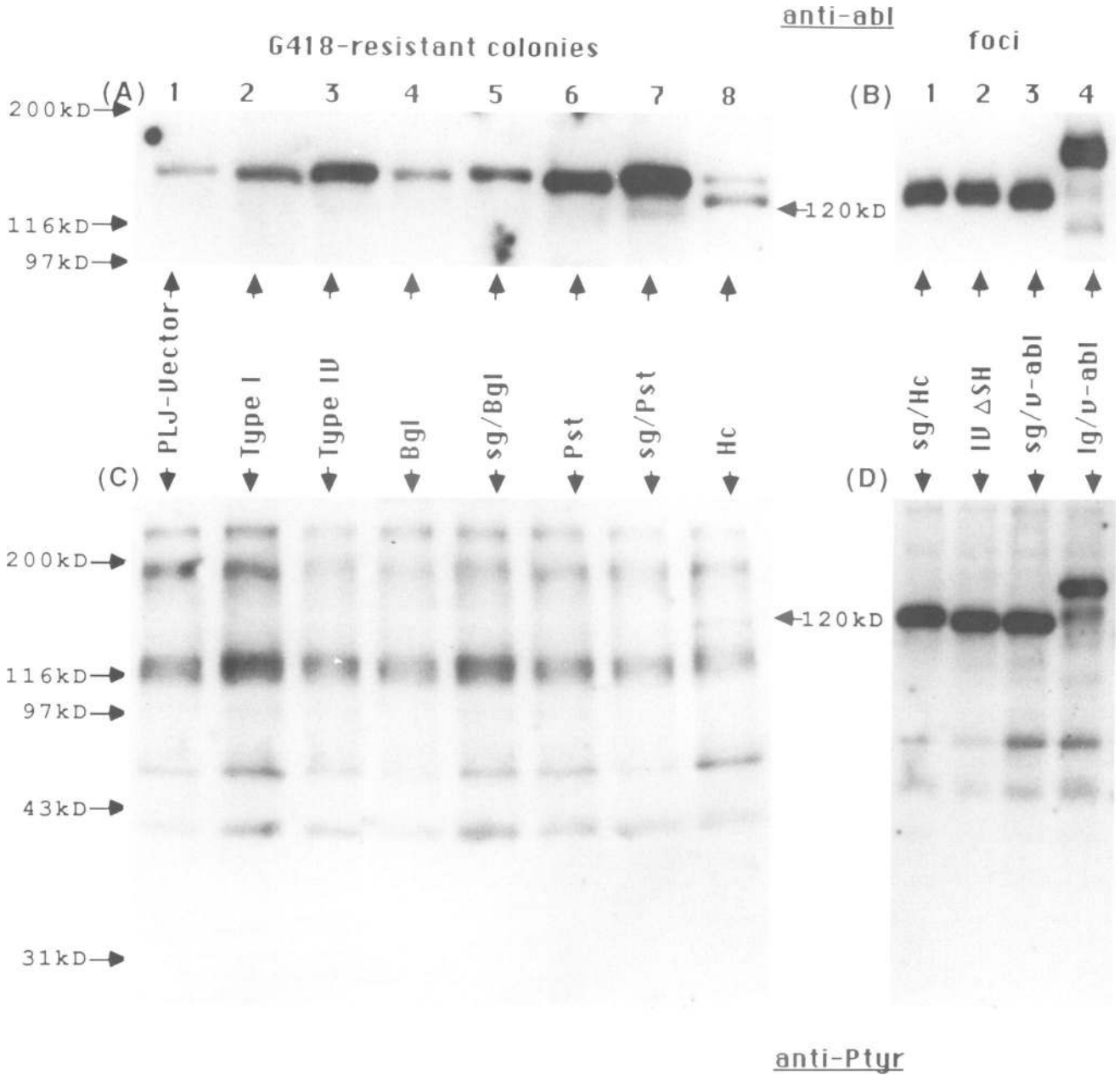

Fig. 3. Expression of *abl* proteins and cellular Ptyr levels in G418 resistant colonies or foci. **Panels A** and **B** are immunoblots with monoclonal anti-*abl* antibody to assay for c-*abl* or v-*abl* proteins from polyclonal G418 colonies obtained with the non-transforming constructs (A) or from foci obtained with the transforming constructs (B). **Panels C** and **D** are immunoblots with anti-Ptyr antibody to assay for Ptyr proteins from untransformed G418 resistant cells (C) or from transformed cells (D). Sodium orthovanadate (50 μM) was added to the untransformed G418 resistant cells for 12 h prior to the collection of cell lysates. Transformed cells were not treated with vanadate. Equal amounts of cellular protein (50 μg) were loaded in each lane of the blots. Positions of mol. wt standards (Bio Rad) are indicated on the left sides of panels A and C. Room temperature exposures of the anti-*abl* blots were scanned with a laser densitometer (LKB). The level of expression of each recombinant protein was compared to that of the endogenous c-*abl* proteins in NIH 3T3 cells as indicated in the text.

lg/v-*abl*, sg/v-*abl* and sg/Hc constructs. About 20−30% of these G418 selected colonies expressed the P160 lg/v-*abl* or P123 sg/v-*abl* or sg/Hc c-*abl* protein and the level of expression varied between 0.3- to 4-fold that of the endogenous c-*abl* protein. Two representative G418 resistant lines with normal morphology (Figure 4, panel A, lanes 2 and 3) showed a 1- or 4-fold expression of the lg/v-*abl* protein over the endogenous c-*abl* level (lane 1). However all transformed lines contained between 6- and 12-fold over-production of the lg/v-*abl* protein (lanes 4−6). A similar 6- to 12-fold overproduction of the sg/Hc c-*abl* protein in its transformed cells was also observed. Thus at least a 6-fold overproduction is required for these oncogenic proteins to transform the NIH 3T3 cells. As the level of expression is crucial to the transforming function of these proteins, it is necessary that the levels of the non-transforming c-*abl* proteins be measured in clonal cell lines to rigorously rule out the possibility that the inability to transform was due to insufficient levels of expression. Twenty to thirty colonies from each transfection were screened to isolate clones which expressed high levels of the non-transforming c-*abl* proteins. High producers of type IV (not shown), Pst, sg/Pst and Hc

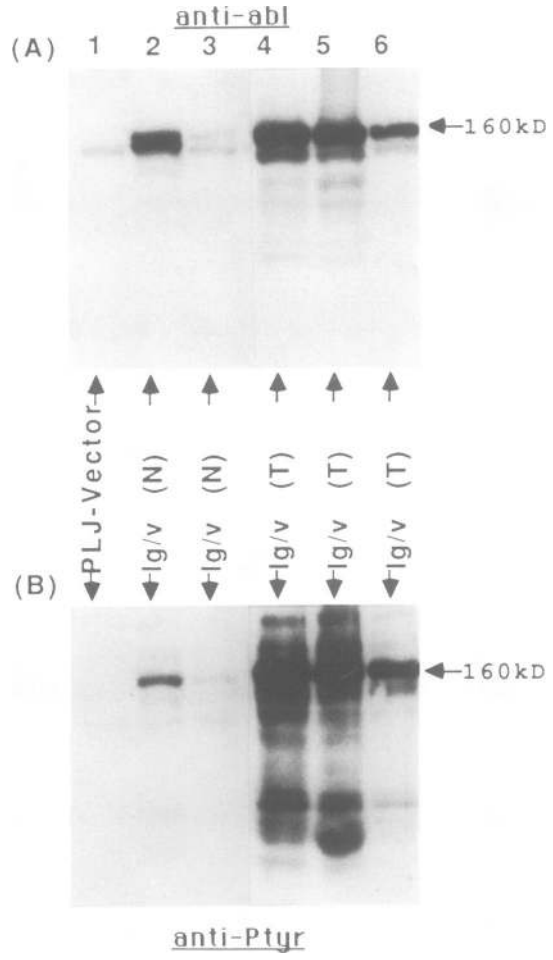

Fig. 4. Comparison of lg/v-*abl* (p160) protein level in transformed and untransformed lines. PLJ-lg/v-*abl* transfected cell lines with normal morphology, [lg/v(N)] were established from G418 resistant colonies and compared to transformed cell lines [lg/v(T)] derived from foci. Immunoblots were probed with either anti-*abl* antibodies (**panel A**) or anti-Pytr antibodies (**panel B**). Cells were not treated with vanadate in this experiment. Equal amounts of total cellular protein were loaded in each lane of the blot.

proteins at levels 10-fold that of the endogenous c-*abl* were obtained (Figure 5, panel A). In these clones, expression of proteins was as high as the sg/Hc or the sg/v-*abl* proteins in transformed foci, yet they showed no transformed phenotype. These results demonstrate that the inability of the type IV, Pst, sg/Pst or Hc c-*abl* proteins to transform cells is not due to insufficient expression and must be due to intrinsic non-transforming properties of these c-*abl* proteins. We could not determine whether overproduction of the type I, Bgl and sg/Bgl protein could lead to cell transformation, because only moderate levels (2- to 6-fold over endogenous c-*abl* level) of expression could be achieved in clones transfected with these constructs. It appears that c-*abl* proteins with the type I-variable amino acids could not be overproduced in NIH 3T3 cells, which contained more type I than type IV c-*abl* mRNA (Renshaw *et al.*, 1988).

Protein tyrosine phosphorylation in transfected cells

Because the tyrosine kinase activity of the v-*abl* oncogene is essential to transformation, we tested whether the c-*abl* transforming activity was correlated with its ability to phosphorylate tyrosine on cellular proteins. The steady-state

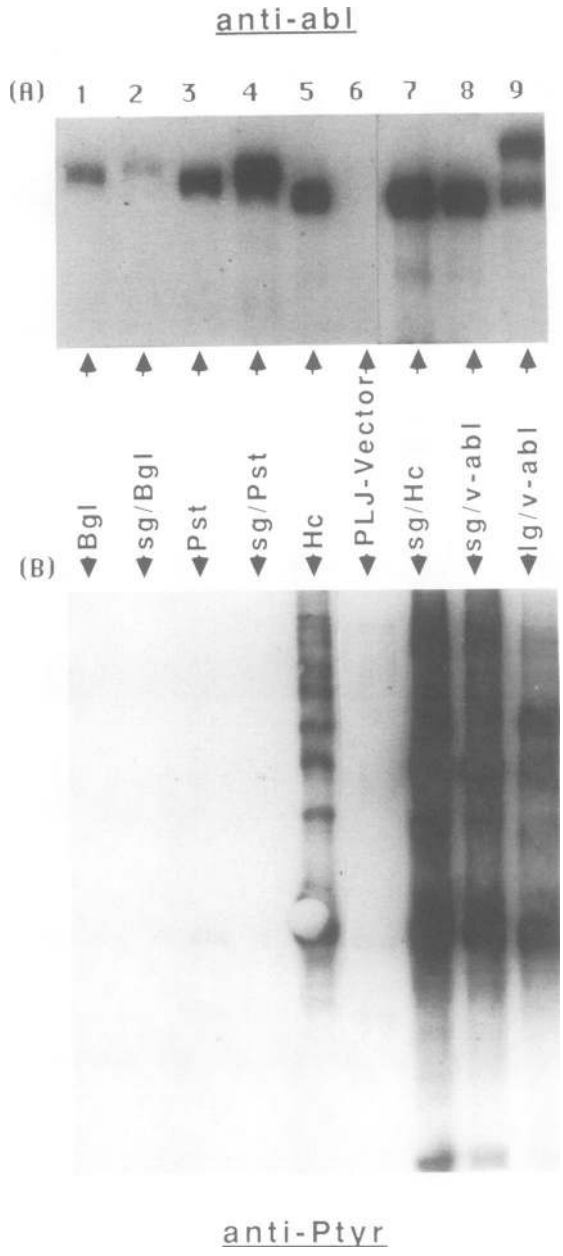

Fig. 5. Detection of *abl* proteins and cellular Ptyr proteins in high producer clonal cell lines. (**A**) anti-*abl* and (**B**) anti-Ptyr immunoblots. The endogenous c-*abl* protein in the control PLJ-transfected cell lysates was not visible at this level of exposure (panel A, lane 6). All cell lines were treated with vanadate to increase the Ptyr level (panel B). Lanes 7–9 contain lysates from transformed cells, whereas lanes 1–6 contain lysates from G418 selected cells with normal morphology.

levels of tyrosine-phosphorylated proteins in cells were examined by immunoblotting of total cellular lysates with affinity-purified antibodies for phosphotyrosine (Wang, 1985; Morla and Wang, 1986). To enhance the Ptyr signal in the G418 selected untransformed cells, a phosphatase inhibitor, sodium orthovanadate, was added to the culture media 12 h prior to cell lysis (Figure 3, panel C). Vanadate treated NIH 3T3 cells contained high levels of Ptyr proteins as shown in the PLJ-vector transfected cells (Figure 3C, lane 1). The same Ptyr banding pattern was found in cells expressing the non-transforming c-*abl* proteins (Figure 3C, lanes 1–7). Only in cells transfected with the Hc truncated construct was an additional Ptyr band of 120 kd observed

(Figure 3C, lane 8, arrow). This 120 kd Ptyr band is the Hc truncated c-*abl* protein, demonstrated by immunoprecipitation of this Ptyr protein with anti-*abl* antibody (not shown). It was interesting to find that the p120 Hc protein expressed at low level (1.4-fold) contained phosphotyrosine, whereas the Pst (4.8-fold, Figure 3, lane 6) and sg/Pst (11.8-fold, Figure 3, lane 7) proteins expressed at higher levels were not tyrosine phosphorylated. The lack of Ptyr in the non-transforming recombinant c-*abl* proteins other than the Hc protein was confirmed by immunoprecipitating each of the c-*abl* proteins with anti-*abl* antibody followed by immunoblotting with anti-Ptyr antibody (not shown). These results showed that *in vivo* tyrosine phosphorylation of the c-*abl* protein could be activated by truncating the protein at the *Hinc*II site but not by truncation at the *Bgl*II or the *Pst*I site. Addition of the 35 *gag* amino acids could not activate the tyrosine phosphorylation of the Bgl or Pst truncated c-*abl* proteins.

Since all the transforming c-*abl* and v-*abl* constructs contain deletions similar to the Hc truncation, it is expected that these transforming proteins are tyrosine phosphorylated *in vivo*. The lg/v-*abl* protein was tyrosine phosphorylated either at low or high levels of expression and the Ptyr increase in other cellular proteins correlated with the level of the p160 protein (Figure 4B, lanes 2–6). This was also true with the sg/v-*abl* or the sg/Hc proteins (not shown). In cells expressing low levels of the Hc truncated c-*abl* protein, only that protein was found to contain phosphotyrosine (Figure 3C, lane 8). In high producer clones (10-fold that of endogenous c-*abl*), Ptyr increase in other cellular proteins was observed (Figure 5B, lane 5). This result showed that the Hc truncated c-*abl* protein when overproduced was capable of causing an increase in cellular Ptyr levels.

Although overproduction of the Hc c-*abl* protein caused an increase in the cellular Ptyr protein levels, these clones showed no transformed properties and the Hc construct generated no foci in our assays. Thus, activation of the c-*abl* kinase activity alone was not sufficient to induce cell transformation. Since overproducers of the sg/Hc protein formed foci, addition of the 35 *gag* amino acids to an activated tyrosine kinase was necessary to complete the oncogenic conversion of c-*abl*. The 35 *gag* amino acids contain an N-terminal glycine which has been shown to be myristylated (Schultz and Oroszlan, 1984). It is likely that the activated c-*abl* tyrosine kinase, analogous to the v-*src* protein, needs an N-terminal fatty acid to become transforming (Kamps *et al.*, 1985; Cross *et al.*, 1984). This was supported by the finding that the c-*abl* (IV) ΔSH protein had transforming activity in NIH 3T3 cells. The c-*abl* type IV N-terminal amino acids contain a consensus sequence for myristylation (Ben-Neriah *et al.*, 1986; Kaplan *et al.*, 1988). Although the normal type IV protein was not transforming, an internal deletion of 31 variable and 93 common codons resulted in the activation of *in vivo* kinase activity (Figure 3D, lane 2) and oncogenic conversion (Table I). The ΔSH protein was also an active tyrosine kinase *in vivo* (Figure 3D, lane 2).

To demonstrate that tyrosine kinase activity differs between Pst and Hc truncated c-*abl* proteins, Ptyr levels in the overproducers of the Pst or the sg/Pst proteins were examined. No Ptyr increase was detected in these overproducers even after a 12 h treatment with orthovanadate (Figure 5B, lanes 3 and 4). These results clearly showed

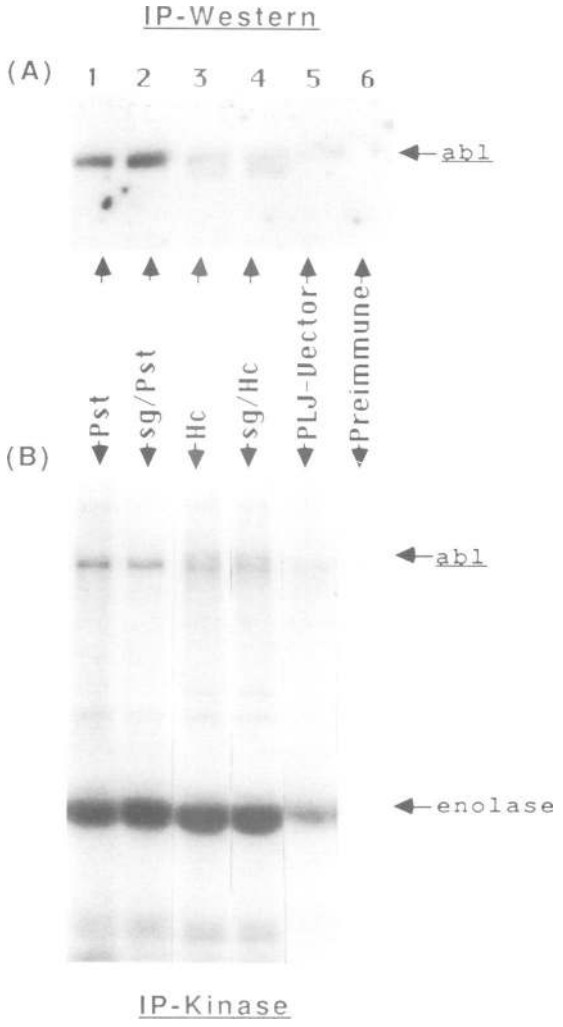

Fig. 6. Immune complex kinase assays. Lysates were prepared from G418 selected polyclonal cells obtained from transfections with PLJ or PLJ c-*abl* constructs as indicated in the figure. The c-*abl* proteins were immunoprecipitated with anti-*abl* monoclonal antibody. Half of the immunoprecipitates were immunoblotted to determine the level of c-*abl* protein (**panel A**). The other half was added to the kinase reaction with acid-denatured enolase (**panel B**) as described in Materials and methods.

that the Pst truncation did not activate the c-*abl* tyrosine kinase even when the protein was expressed at 6- to 10-fold over the level of the endogenous c-*abl* protein. Thus, deletion of the variable exon sequences in itself is not sufficient to activate c-*abl* kinase; additional amino acids between the *Pst*I and *Hinc*II sites must also be removed to activate the c-*abl* kinase *in vivo*.

In vitro kinase activity of the c-abl proteins

Although normal c-*abl* proteins are not tyrosine phosphorylated *in vivo*, their activity can be measured *in vitro* in an immune complex autokinase or enolase-kinase assay (Konopka and Witte, 1985). This assay was used to demonstrate that the recombinant Pst and sg/Pst proteins, which had no *in vivo* autokinase activity, were capable of enzymic activity. The amount of c-*abl* protein present in each immune complex was determined by immunoblotting with anti-*abl* monoclonal antibody as shown in Figure 6A. *In vitro*

kinase assays showed autophosphorylation and enolase phosphorylation activities for all c-*abl* proteins (Figure 6B, lanes 1–5) and no activity when monoclonal anti-*abl* antibody was omitted from the immunoprecipitation (Figure 6B, lane 6). Because immune complexes containing the Pst, sg/Pst, Hc and sg/Hc (lanes 1–4) proteins gave higher enolase phosphorylation than the complex containing only the endogenous c-*abl* protein (lane 5), these assays showed that the Pst and sg/Pst c-*abl* proteins were active enzymes *in vitro*. The average *in vitro* kinase activity of the Pst truncated protein was 3- to 5-fold higher than the normal c-*abl* protein and 2- to 3-fold lower than the lg or sg/v-*abl* protein, as has been reported previously (Wang, 1988). The *in vitro* kinase activity of the Pst or sg/Pst proteins was found to be comparable, which again showed that the *gag* amino acids did not activate the c-*abl* tyrosine kinase. In Figure 6 the Hc or sg/Hc proteins appeared to have higher enolase kinase activity than the Pst or sg/Pst proteins, although this was not always true in our assays. The average activity of Pst and Hc truncated proteins appeared to be comparable in repeated immune complex assays. Therefore, deletion of the amino acids between the *Pst*I and *Hinc*II sites does not significantly alter the c-*abl* kinase activity measured *in vitro*. This result shows that the first 93 common amino acids do not have intrinsic inhibitory activity towards the c-*abl* tyrosine kinase.

Discussion

The results described in this paper establish three requirements for the oncogenic conversion of c-*abl*: (i) activation of the c-*abl* tyrosine kinase activity by the deletion of N-terminal amino acids between the Pst and Hc sites; (ii) appendage of a myristylation site to the N-terminus of this activated kinase; and (iii) overproduction of the fatty-acylated active kinase at least 6-fold above the endogenous c-*abl* level. The mechanism of activation of the c-*abl* gene therefore involves both qualitative and quantitative alterations.

Kinase regulatory domain

The normal c-*abl* proteins are not tyrosine phosphorylated even when they are produced at 10-fold the normal level, as shown in this study. We have proven, by the mutation of the ATP binding site, that the *in vivo* tyrosine phosphorylation of the c-*abl* protein is due to autokinase activity (J.Y.J.Wang and S.M.Seaton, in preparation). Therefore, the lack of Ptyr in the c-*abl* proteins *in vivo* is indicative of inhibition of autokinase activity. Our results demonstrate that to activate the c-*abl* oncogenic potential, it is necessary to release the c-*abl* autokinase from cellular inhibitory mechanism(s). This can be achieved by the deletion of the 89 common amino acids, between the *Pst*I and *Hinc*II sites (Figure 7). Mutant proteins lacking these amino acids contain Ptyr *in vivo* even when they are not overproduced. Thus, the *in vivo* inhibition of c-*abl* autokinase activity requires the presence of specific N-terminal amino acids common to both types of c-*abl* proteins. Oncogenic activation of several tyrosine kinases has been shown to involve deregulation of that enzyme activity. Mutations that prevent the phosphorylation of a C-terminal tyrosine (527) of c-*src* have been shown to activate that enzyme and the c-*src* transforming activity (reviewed by Jove and Hanafusa, 1987). Although the c-*abl* tyrosine kinase is ~50% homologous with the c-*src* kinase, it does not contain a tyrosine equivalent to the c-*src* tyr-527. A regulatory region of the c-*abl* kinase, as shown in this study, is found in the N-terminal portion of the c-*abl* protein.

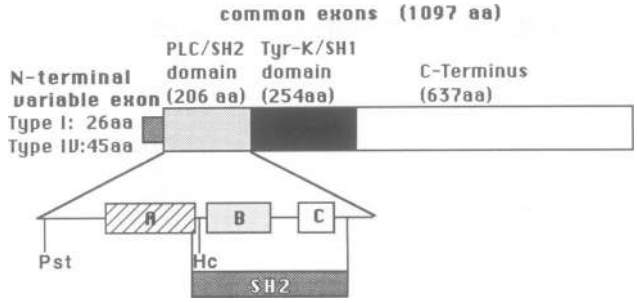

Fig. 7. Negative regulatory domain of c-*abl* tyrosine kinase shares homology with the A region of phospholipase C-II and v-*crk*. The two c-*abl* proteins contain different N-terminal amino acids but share 1097 common amino acids. The N-terminal kinase regulatory domain shares homology with other non-receptor tyrosine kinases, the v-*crk* oncogene and phospholipase C-II (PLC II; Mayer *et al.*, 1988; Stahl *et al.*, 1988). Deletion of the A region in this domain between the Pst and Hc sites activates c-*abl* autokinase activity *in vivo*. The remaining B and C regions of the PLC II domain span the *src* homologous region 2 (SH2), followed by the tyrosine kinase domain, *src* homologous region 1 (SH1).

The N-terminal region of non-receptor tyrosine kinases, such as *src*, *abl* and *fps*, contain conserved amino acids also found in proteins which are not kinases, e.g. the v-*crk* oncoprotein and phospholipase C-II (Mayer *et al.*, 1988; Stahl *et al.*, 1988). These conserved amino acids can be subdivided into three regions: A, B and C, as described in Stahl *et al.* (1988; see Figure 7). The subdivisions are meaningful because the GTPase activating protein (GAP) of the *ras* oncogene-encoded GTPase contains only the B/C amino acids but no A region (Vogel *et al.*, 1988). The activating deletion (Pst–Hc), defined in this study, eliminates the A region amino acids of the c-*abl* protein. These deleted amino acids do not have an intrinsic inhibitory effect on the c-*abl* tyrosine kinase because the Pst-truncated protein, which contains these amino acids, has an *in vitro* kinase activity comparable to that of the Hc-truncated protein. These results indicate that the 89 amino acids encoded by the Pst–Hc segment are necessary for the inhibition of the c-*abl* kinase activity in intact cells, and they suggest that the A region amino acids may play a negative role in the regulation of c-*abl* tyrosine kinase. On the contrary, the B/C region, also called the SH2 domain has a positive role in the regulation of tyrosine kinases (Sadowski *et al.*, 1986). Mutations in the B/C regions of the v-*fps* and the v-*abl* oncogenes can cause a reduction in the kinase activity and affect the transforming functions of these oncogenes (Prywes *et al.*, 1985; Sadowski *et al.*, 1986).

We have found four ways to activate the c-*abl* tyrosine kinase activity: (i) deletion of Pst–Hc region amino acids without overproduction; (ii) 50- to 200-fold overproduction of normal c-*abl* proteins (J.Y.J.Wang and S.M.Seaton, in preparation); (iii) immunoprecipitation of the c-*abl* protein from cell lysates; and (iv) expression of c-*abl* proteins in *Escherichia coli* (Wang, 1988). These observations can be explained by a hypothesis that a certain negative regulator of the c-*abl* tyrosine kinase exists in cells, but not in *E.coli*, to inhibit that enzyme, and the negative regulator interacts with the c-*abl* protein through amino acids in the Pst–Hc region. Production of the normal c-*abl* protein in excess of

the negative regulator or deletion of the Pst−Hc region releases the c-*abl* kinase from inhibition *in vivo*. The activation of c-*abl* tyrosine kinase *in vitro* could be due to dissociation of the negative regulator during cell lysis and immunoprecipitation.

The requirements for oncogenic conversion established by our studies predict that the *bcr/abl* fusion protein will not have transforming activity in NIH 3T3 cells, because that protein contains the Pst−Hc region amino acids and lacks a myristylation site. In fact, *bcr/abl* has been shown to lack transforming activity (Daley *et al.*, 1987). It is interesting, however, that the *bcr/abl* protein is an active tyrosine kinase *in vivo* despite the presence of the intact Pst−Hc region. We have recently demonstrated that the *bcr* protein sequence is directly responsible for the activation of the c-*abl* tyrosine kinase in the *bcr/abl* fusion protein (J.R.McWhirter and J.Y.J.Wang, in preparation). The small viral *gag* sequence, on the other hand, does not activate c-*abl* tyrosine kinase. Therefore, the cellular *bcr* protein and the small viral *gag* protein make different contributions to the activation of the c-*abl* oncogenic potential. This may reflect the different mechanisms in the *in vitro* transformation of established cells and the *in vivo* tumorigenic process in human leukemias.

Myristylation site

The activated c-*abl* tyrosine kinase requires an N-terminal myristylation site, provided either by the *gag* or the c-*abl* type IV amino acids, to become transforming. Recently, the recognition sequence for myristylation has been determined for the v-*src* protein (Kaplan *et al.*, 1988). This recognition sequence is present in the c-*abl* type IV N-terminal sequences, supporting the notion that only a fatty-acylated c-*abl* tyrosine kinase has transforming activity. Addition of the *gag* myristylation sequence to the *bcr/abl* protein has been shown to convert it into an oncogene (Daley *et al.*, 1987). These findings are analogous to the v-*src* oncogene which looses transforming activity when the N-terminal glycine is mutated (Kamps *et al.*, 1985; Cross *et al.*, 1984).

It is interesting to note that both myristylated and non-myristylated forms of the normal c-*abl* proteins exist in mammalian cells: the type IV (Ib) protein can be modified with fatty acid while the type I (Ia) protein contains no myristylation site (Ben-Neriah *et al.*, 1986; Shtivelman *et al.*, 1986). We have shown that the type IV mRNA is present in all mouse tissues and cell types at a constant level whereas the type I mRNA level is regulated in a tissue- and cell type-specific manner, indicating that these two proteins may serve different cellular functions (Renshaw *et al.*, 1988). It is reasonable to assume that the two forms of c-*abl* proteins phosphorylate different substrates, dictated by their separate subcellular locations. Our results suggest that the substrates available for the ubiquitous type IV c-*abl* tyrosine kinase may be important for the process of cell transformation.

Overproduction

Using a selectable PLJ retroviral vector, we have demonstrated that transformation with v-*abl* or c-*abl*-derived oncogenes requires overproduction of the transforming proteins. A 6- to 12-fold overproduction of lg/v-*abl* above the endogenous c-*abl* level was found in transformed foci, whereas a 4-fold expression of the same protein in G418 resistant colonies did not cause morphological alterations. Our results suggest that production of the v-*abl* or c-*abl* transforming proteins beyond a minimal level of 6-fold over the endogenous c-*abl* is required for the induction of cell transformation. This finding is again similar to the v-*src* oncogene, which requires at least a 4-fold overproduction of pp60 v-*src* (Jakobovits *et al.*, 1984). Overexpression of normal c-*abl* type IV or the Pst and Hc truncated proteins, however, did not lead to focus formation or morphological alteration.

The role of overproduction in transformation is not yet well defined. We found that untransformed cells with lower levels of the lg/v-*abl* protein contained reduced levels of cellular Ptyr proteins. Overproduction of the kinase may be necessary to sustain higher steady-state levels of Ptyr in critical substrate proteins. It is also possible that high concentrations of the oncogenic kinase allow the phosphorylation of low affinity substrates. In other words, overproduction of an activated kinase leads to the phosphorylation of proteins other than or in addition to the usual substrates and this unnatural combination of substrate phosphorylation may be responsible for the induction of cell transformation.

Although the three defined alterations lead to the activation of the c-*abl* oncogenic potential, the transforming activity of the sg/Hc c-*abl* protein is much lower than that of the sg/v-*abl* protein in our transformation assays. This reduced activity must be due to differences between the v-*abl* and c-*abl* genes. We have sequenced our c-*abl* cDNA clones and found no mutations in the v-*abl* tyrosine kinase domain (M.Trepanier and J.Y.J.Wang, unpublished data). The major difference between c-*abl* and v-*abl* is a 23 amino acid frameshift downstream from the tyrosine kinase domain between the *Nar*I and *Xho*I sites (see Figure 1A) (Oppi *et al.*, 1987). This region has previously been shown to be dispensable for the A-MuLV transforming function and it is not present in the v-*abl* of HZ2-FeSV (Prywes *et al.*, 1983; Bergold *et al.*, 1987). However, it is possible that this frameshift mutation has an effect on the kinase activity, because we and others have found that the v-*abl* kinase has at least twice the c-*abl* kinase activity in immune complex kinase assays (Wang, 1988; Konopka and Witte, 1985). It remains to be determined if this frameshift in the v-*abl* oncogene contributes to its transforming activity.

The transforming activity of the c-*abl* (IV) ΔSH protein was even lower than the c-*abl* sg/Hc protein. The sg/Hc and the ΔSH proteins only differ at the N-terminal end; sg/Hc contains 35 *gag* amino acids whereas ΔSH contains 14 type IV amino acids. It has been shown that the variable exon-encoded amino acids have an intrinsic inhibitory activity on the c-*abl* tyrosine kinase (Wang, 1988). The 14 N-terminal type IV amino acids might cause a reduction in the kinase activity and the transforming activity of the ΔSH protein. Alternatively, the 35 *gag* amino acids might add to the transforming activity by mechanisms other than the provision of a myristylation site. For example, we found the 35 *gag* amino acids could increase the steady-state levels of the c-*abl* proteins. Since the level of the transforming protein is important for the induction of transformation, the stability factor may explain the higher transforming activity of the sg/Hc protein. The 35 *gag* amino acids may also provide a different affinity of the activated c-*abl* kinase for membranes and so increase the transforming activity. It appears that multiple regulatory mechanisms exist in cells

to modulate the function of the c-*abl* tyrosine kinase. Qualitative and quantitative alterations in the regulatory mechanisms are the basis for the oncogenic conversion of the c-*abl* proto-oncogene.

Materials and methods

Plasmids

The A-MuLV sequence (lg/v-*abl*) was derived from pAB160 (Latt *et al.*, 1983) by digestions with *Pvu*I and *Bam*HI which excised a 4.9 kb fragment containing the entire coding sequence of A-MuLV (p160). This lg/v-*abl* coding fragment was inserted into the PLJ plasmid at its unique *Bam*HI site (see Figure 1; Korman *et al.*, 1987). Murine c-*abl* types I and II cDNAs were prepared using RNA from SCRF-60A cells (J.Y.J.Wang, unpublished data). Clones containing full length type I or IV coding sequences were also inserted into PLJ, generating PLJ c-*abl* types I and IV. Three N-terminal deletions of PLJ c-*abl* type I (see restriction map Figure 1A) were generated by using the designated *Bgl*II, *Pst*I and *Hin*cII sites. After partial digestion, an *Nco*I restriction site containing an ATG codon and an alanine codon were added in the reading frame to provide translation initiation (Wang, 1988). Excision of a *Stu*I–*Hin*cII fragment in PLJ c-*abl* type IV (Figure 1a) generated an in-frame internal deletion construct: c-*abl* (IV) ΔSH. Fusion constructs with the small *gag* sequence were made by the in-frame ligation of the 5′ *gag* region, from the *Pvu*I site to the *Bst*EII of the M-MuLV sequence in A-MuLV, to each of the truncated c-*abl* coding sequences.

Cell culture transfection

NIH 3T3 fibroblasts were maintained in Dulbecco's modified Eagle's medium, 10% calf serum (Hyclone) and antibiotics (50 U/ml of penicillin and 50 μg/ml of streptomycin). Transfections were performed using a modified calcium phosphate co-precipitation technique (Graham and Van der Eb, 1973; Spandidos and Wilkie, 1984). One microgram of the different PLJ constructs was co-transfected with 10 μg of a replication competent Moloney virus clone, pZAP, as described previously (Goff *et al.*, 1982). Two days after transfection, cells were split 1:10 for G418 selection and 1:2 for the focus forming assay. Colonies were counted 14 days and foci 19 days after transfection. G418 resistant colonies and foci were isolated with glass cylinders.

Virus infection

The culture media from multiple G418 resistant colonies were collected, filtered (0.45 μm filter, Nalgene), and stored at −70°C. Recipient NIH 3T3 fibroblasts, seeded 24 h before at 3×10^5 cells/6 cm plate, were infected with the virus-containing medium in the presence of 8 μg/ml polybrene for 2 h. After 48 h the cells were trypsinized and replated 1:10 for G418 selection and 1:2 for focus formation assay. Foci and colonies were counted 14 days after infection.

Immunoblotting and immunoprecipitation

Immunoblottings with either monoclonal anti-*abl* antibody, 8E9 (Richardson *et al.*, 1987), or affinity purified anti-Ptyr antibodies (Wang, 1985) were performed as described previously (Morla and Wang, 1986; Wang, 1985). Identical amounts of total cellular proteins were loaded in each lane of the gel for immunoblotting, and the protein concentrations of the cell lysates were determined by the method of Lowry after precipitation of proteins with trichloroacetic acid (Lowry *et al.*, 1951). Polyclonal anti-*gag* p15 antibodies were obtained from Microbiological Associates Inc. Immunoprecipitations were carried out by the standard method using formaldehyde fixed *Staphylococcus aureus* (Wang, 1985).

Immune complex kinase assays were carried out essentially according to the method of Konopka and Witte (1985) except that cells were extracted in buffer containing no SDS, as we found that SDS, even at 0.5%, reduced the kinase activity (J.Y.J.Wang, unpublished data). Briefly, cells were lysed in buffer containing 10 mM Tris–HCl (pH 8.0), 5 mM EDTA, 130 mM NaCl, 30 mM NaPi (pH 7.0), 1% Triton X-100, 1 mg/ml BSA and 1 mM PMSF, at 2×10^6 cells/ml. Insoluble materials were removed by centrifugation at 100 000 *g* for 30 min. Lysates were incubated with 10 μg/ml monoclonal anti-*abl* Ab 8E9 plus 20 μg/ml rabbit anti-mouse IgG overnight at 4°C. The immune complex was adsorbed onto protein-A-Sepharose (Pharmacia) and half of it was incubated for 15 min at 30°C in 20 μl of kinase reaction buffer containing 20 mM Tris–HCl (pH 7.4), 20 mM $MnCl_2$, 2 μM ATP, 10 μCi [γ-^{32}P]ATP and 12 μg acid-denatured enolase. Reactions were terminated by boiling samples in SDS buffer (Wang and Baltimore, 1985) and then loaded on 7% SDS–PAGE gels. Fixed gels were treated in 1 N KOH at 55°C for 2 h. The other half of the precipitated immune complex was immunoblotted and autoradiographed with monoclonal anti-*abl* antibody to determine the amount of *abl* protein present in the kinase assay reaction.

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