A truncated *v-abl*-derived tyrosine-specific tyrosine kinase expressed in *Escherichia coli*

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Several biochemical properties of a 43 kDa *v-abl*-encoded tyrosine-specific protein kinase (p43^{v-abl}) expressed in *Escherichia coli* were examined. p43^{v-abl} is a fragment of a 60 kDa *v-abl*-encoded precursor, p60^{v-abl}, and could be generated by limited proteolysis of a purified p60^{v-abl} with trypsin. Tryptic cleavage of p60^{v-abl} was prevented in the presence of ATP. These results suggest that the catalytic kinase domain of *v-abl*-derived protein can be separated from other (regulatory) domains by limited proteolysis. p43^{v-abl} readily phosphorylated tyrosine residues on several different protein and peptide substrates, including peptides containing only two amino acid residues. However, the local sequence of the tyrosine-containing peptide substrate significantly affected its rate of phosphorylation. Thus the primary structure and local conformation at the tyrosine acceptor site can play an important role in determining the substrate specificity of *v-abl*-derived kinase. Phosphorylation by p43^{v-abl} requires Mn²⁺, Co²⁺ or Mg²⁺ and exhibits a strong preference for ATP as phosphate donor. Analogues of ATP and the thiol-reactive reagent *N*-ethylmaleimide inhibited p43^{v-abl} kinase activity. Purified p43^{v-abl} is intrinsically thermolabile ($t_{1/2} = 5$ min at 40 °C) and phosphorylates glycerol inefficiently ($K_m = 1.4$ M).

INTRODUCTION

Tyrosine-specific protein kinase activity is associated with the products of a number of retroviral oncogenes, including *v-abl*, *v-fes/fps*, *v-fgr*, *v-src*, *v-erbB*, *v-fms*, *v-ros* and *v-yes* (Bishop, 1985; Hunter & Cooper, 1985). Several growth-factor receptors, including those for insulin, platelet-derived growth factor, epidermal growth factor and somatomedin C, are also associated with tyrosine-specific kinase activity (Hunter & Cooper, 1985). The cellular homologues of two retroviral oncogenes (*v-erbB* and *v-fms*) have now been identified as genes encoding growth-factor receptors (epidermal-growth-factor receptor and macrophage-specific haemopoietic-growth-factor receptor respectively) (Downward *et al.*, 1984; Sherr *et al.*, 1985). In addition, oncogenic forms of distinct tyrosine-related cellular genes that lack known retroviral homologues have been identified (King *et al.*, 1985; Dean *et al.*, 1985; Coussens *et al.*, 1985). These findings suggest strongly that tyrosine-specific protein kinases function as important intracellular mediators in cell growth regulation in both normal and neoplastic cells. The cellular substrates for these enzymes have yet to be identified.

The transforming gene of the Abelson murine leukaemia virus (A-MuLV), *v-abl*, codes for a membrane-associated tyrosine-specific protein kinase (Wang *et al.*, 1982; Witte, 1983). The A-MuLV transforming protein comprises a segment of the Moloney MuLV *gag* coding sequence and a segment (*v-abl*) homologous to the mouse cellular *c-abl* gene (Witte, 1983). The resulting *gag-v-abl* fusion protein is typically a product of

90–160 kDa (Witte, 1983). The *v-abl*-gene product also differs from the predicted *c-abl*-gene product by the deletion of *N*- and *C*-terminal residues and by several amino acid substitutions (Wang *et al.*, 1984).

Analysis of the *v-abl* gene has shown that both the fibroblast-transforming and tyrosine-specific protein kinase activities reside within a minimal region encoding a protein of approx. 45 kDa (Prywes *et al.*, 1985). To obtain sufficient quantities of purified *v-abl* kinase for detailed analysis, the *v-abl* coding sequence encompassing this minimal domain was placed in an *Escherichia coli* plasmid expression vector (Ferguson *et al.*, 1985). With the use of this expression system, a 60 kDa *v-abl*-derived protein, designated p60^{v-abl}, accumulated in *E. coli* to the extent of about 10% of total cellular protein. Purified p60^{v-abl} efficiently transferred phosphate from ATP to tyrosine residues on exogenous substrates. In the present paper we show that the most active form of the tyrosine kinase isolated with the use of our procedure is a 43 kDa fragment of p60^{v-abl}, and we describe several properties of this protein.

EXPERIMENTAL

Materials

Carrier-free H₃³²PO₄ was from Amersham-Searle and 5'-*p*-fluorosulphonylbenzoyl[8-¹⁴C]adenosine ([¹⁴C]-FSBA) was from New England Nuclear. γ -³²P-labelled ATP, GTP, CTP and UTP were prepared by using the Gamma Prep Systems (Promega Biotec) and purity was confirmed by t.l.c. Octyl glucopyranoside was obtained

Abbreviations used: A-MuLV, Abelson murine leukaemia virus; PAGE, polyacrylamide-gel electrophoresis; p[NH]ppA, adenosine 5'-[β -imido]triphosphate; FSBA, 5'-*p*-fluorosulphonylbenzoyladenosine; Ap₄A, diadenosine tetraphosphate.

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from Calbiochem-Behring. Peptides were from Peninsula, Sigma Chemical Co., Bachem or Vega. Calmodulin, histone, phosvitin, casein, myosin light chain, glycerol kinase, bovine serum albumin, L-tosylphenylalanyl-chloromethane ('TPCK')-treated trypsin, ATP, GTP, CTP, UTP, cyclic AMP, cyclic GMP, diadenosine tetraphosphate (Ap_4A), adenosine 5'-[$\beta\gamma$ -imido]triphosphate ($\text{p}[\text{NH}]\text{ppA}$), 5'-*p*-fluorosulphonylbenzoyladenine (FSBA), *N*-ethylmaleimide and quercetin were from Sigma Chemical Co. Calmidazolium was obtained from Janssen. All other reagents were reagent grade or better.

Isolation of *v-abl*-derived tyrosine-specific kinase

Expression of *v-abl*-derived protein-tyrosine kinase in *E. coli* from the plasmid vector pAS1-Abl-60 (Ferguson *et al.*, 1985) was controlled by heat induction (Ferguson *et al.*, 1985) or by nalidixic acid induction with the use of defective bacteriophage λ lysogen host AR120 (Mott *et al.*, 1985). The *v-abl* product was extracted from *E. coli* and solubilized with 2 M-KSCN (Ferguson *et al.*, 1985). Soluble *v-abl* protein was dialysed extensively against buffer A (50 mM-Tris/HCl buffer, pH 7.5 at 25 °C, containing 1 mM-EDTA and 0.1 mM-dithiothreitol). The sample was applied to a MonoQ f.p.l.c. anion-exchange column system (Pharmacia) equilibrated with buffer A and eluted with a linear KCl gradient (0–0.5 M in buffer A). Fractions containing the peak of tyrosine-specific kinase activity were eluted at 100–150 mM-KCl and were pooled, made 50% (v/v) in glycerol, and stored at –20 °C. Protein concentrations were determined by the method of Bradford (1976), with bovine serum albumin as a standard. The relative concentrations and purities of $\text{p43}^{\text{v-abl}}$ and $\text{p60}^{\text{v-abl}}$ were determined by SDS/PAGE followed by Coomassie Blue staining and *v-abl*-derived protein-specific immunoblot analysis.

Limited tryptic proteolysis of $\text{p60}^{\text{v-abl}}$

Homogeneous $\text{p60}^{\text{v-abl}}$ (1 μg), purified by SDS/PAGE and electroelution, was incubated for 15 min at 32 °C in 50 μl of 0.1 M-Tris/HCl buffer, pH 7.5 at 25 °C, containing 0.2 mM-dithiothreitol, in the presence or in the absence of 2.0 μM -ATP and 10 mM- MgCl_2 . Proteolysis was initiated by the addition of L-tosylphenylalanylchloromethane-treated trypsin (5 ng). Similar reaction conditions were used for the limited proteolysis of $\text{p60}^{\text{v-abl}}$ with papain or subtilisin.

Assay of tyrosine-specific kinase activity

Tyrosine-specific kinase activity was measured with either casein or various synthetic peptides as phospho-acceptors. The standard reaction mixture for casein phosphorylation was 100 mM-Mops/NaOH buffer, pH 7.0, containing 10 mM- MgCl_2 , 20 μM -[γ - ^{32}P]ATP (6 Ci/mmol), 1 mg of casein/ml and 1–50 ng of *v-abl*-derived tyrosine kinase incubated in a total volume of 30 μl for 10 min at 25 °C. For inhibition studies 2 μM -ATP was used (Table 2). Casein phosphorylation was quantified by liquid-scintillation spectrophotometry following either SDS/PAGE isolation or the use of trichloroacetic acid precipitation and filtration of the total kinase reaction (Ferguson *et al.*, 1985). Phosphorylation of angiotensin II, [valine-5]angiotensin II (Wong & Goldberg, 1983a) and the modified autophosphorylation site of $\text{p60}^{\text{v-src}}$ (Casnellie *et al.*, 1982) were measured as described previously (Ferguson *et al.*, 1985). The phosphorylation of other tyrosine-containing peptides

was quantified by using a modification of the procedure of Braun *et al.* (1983). Reaction conditions were identical with those described above except that peptide was substituted for casein. The reaction was terminated by the addition of 100 μl of 10 mM-ATP and incubation in 1 M-HCl for 40 min at 90 °C. Inorganic phosphate was removed from the aqueous layer by organic extraction, and radioactivity in the aqueous layer was quantified by liquid-scintillation spectrometry. Phospho amino acid analyses of phosphorylated protein and peptides were carried out as described (Ferguson *et al.*, 1985).

Assay of glycerol phosphorylation

The phosphorylation of glycerol by *v-abl*-derived kinase was measured by incubating $\text{p43}^{\text{v-abl}}$ (50–200 ng) and glycerol (0.08–1.8 M) in 100 mM-Mops/NaOH buffer, pH 7.0, containing 10 mM- MgCl_2 and 20 μM -[γ - ^{32}P]ATP (6 Ci/mmol) in a total volume of 30 μl for 5–30 min at 25 °C. Samples were spotted on to Whatman 3MM paper and separated by high-voltage electrophoresis [2.5 h at 1500 V in water/acetic acid/pyridine (189:10:1, by vol.), pH 3.5]. Glycerol phosphate standard was prepared by incubating glycerol (1 mM) and glycerol kinase (5 units) for 5 min under the reaction conditions described above.

Affinity labelling of $\text{p43}^{\text{v-abl}}$ with FSBA

$\text{p43}^{\text{v-abl}}$ was labelled with [^{14}C]FSBA by incubating 2.0 μl of $\text{p43}^{\text{v-abl}}$ in 100 μl of 80 mM-Mops/NaOH buffer, pH 7.0, containing 10 mM- MgCl_2 , 0.58 mM-[^{14}C]FSBA (2.5 μCi , 43.2 mCi/mol) and 1% (v/v) dimethyl sulphoxide at 30 °C. Samples were taken at intervals and analysed by SDS/PAGE and autoradiography, and for tyrosine kinase activity. The non-hydrolysable ATP analogue $\text{p}[\text{NH}]\text{ppA}$ (10 mM) was used to block the incorporation of [^{14}C]FSBA into $\text{p43}^{\text{v-abl}}$.

RESULTS

Isolation of $\text{p43}^{\text{v-abl}}$

We previously reported the expression in *E. coli* of a 60 kDa *v-abl*-derived tyrosine kinase, designated $\text{p60}^{\text{v-abl}}$ (Ferguson *et al.*, 1985). With the use of this expression vector system $\text{p60}^{\text{v-abl}}$ accumulates in *E. coli* predominantly as an insoluble aggregate to the extent of 10% of the total cell protein. The *E. coli*-expressed *v-abl*-derived protein was obtained in a highly purified insoluble form by the use of an extraction procedure that solubilizes all of the host *E. coli* proteins (Ferguson *et al.*, 1985). Soluble and enzymically active *v-abl*-derived tyrosine kinase was isolated from the insoluble aggregate with the use of KSCN (Ferguson *et al.*, 1985). Homogeneous $\text{p60}^{\text{v-abl}}$ was then obtained by electroelution from preparative SDS/PAGE gels and used to immunize rabbits. The purity of the immunogen was confirmed by the failure of the $\text{p60}^{\text{v-abl}}$ -specific polyclonal rabbit antiserum to bind significantly to any *E. coli* proteins (Fig. 1a). In addition to the 60 kDa *v-abl*-derived protein, a major 43 kDa protein and several other *v-abl*-derived proteins of intermediate molecular size were detected by this antiserum in total cell extracts from induced *E. coli* containing pAS1-Abl-60 (Fig. 1a). The gel-purified $\text{p60}^{\text{v-abl}}$ antigen used for rabbit immunization did not contain detectable 43 kDa protein (Fig. 1a, lane 9). Thus the 43 kDa *v-abl*-derived protein, designated $\text{p43}^{\text{v-abl}}$, is clearly an immunologically related fragment of $\text{p60}^{\text{v-abl}}$.

Fig. 1. SDS/PAGE analysis of *v-abl*-derived proteins expressed in *E. coli*

(a) Immunoblot detection of the *v-abl*-derived proteins expressed in *E. coli*. *E. coli* AR120 cells containing pAS1-Abl-60 were grown until $A_{650} = 0.45$ and nalidixic acid was added. Immediately before nalidixic acid induction (lane 1) and 6 h after nalidixic acid induction (lanes 2 and 7) 0.1 μ l samples of the culture were analysed by SDS/PAGE and *v-abl*-derived-protein-specific immunoblot. *E. coli* cells containing the plasmid pAS1 were analysed before (lane 5) and after (lane 6) nalidixic acid induction. At 6 h after nalidixic acid induction, chloramphenicol and spectinomycin were added at final concentrations of 250 μ g/ml and 50 μ g/ml respectively. At 1 h (lane 3) and 3 h (lane 4) after addition of chloramphenicol and spectinomycin 0.1 μ l samples of the culture were analysed by immunoblot. Lanes 8 and 9 contained approx. 10 ng of purified p43^{v-abl} and p60^{v-abl} respectively. (b) Autophosphorylation of partially purified thiocyanate-solubilized p43^{v-abl} with [γ -³²P]ATP. (c) Coomassie Blue-stained p43^{v-abl} after purification by MonoQ column chromatography. (d) [14 C]FSBA incorporation into p43^{v-abl}. p43^{v-abl} was incubated for 20 min with [14 C]FSBA and analysed by SDS/PAGE and autoradiography. Samples in (b) and (d) contained approximately equal amounts of p60^{v-abl} and p43^{v-abl} as judged by immunoblot analysis or Coomassie Blue staining. The molecular sizes of p43^{v-abl} and p60^{v-abl} were estimated by reference to protein markers.

The KSCN-solubilized extract from *E. coli* expressing *v-abl* contained approximately equal amounts of p60^{v-abl} and p43^{v-abl}, as determined by immunoblot analysis (Fig. 1a).

The stability of the *v-abl*-derived polypeptides expressed in *E. coli* was measured by using a new procedure. *E. coli* cells containing pAS1-Abl-60 were induced with the use of nalidixic acid (Mott *et al.*, 1985) and incubated for 6 h, at which time the *v-abl*-derived polypeptides have accumulated to a steady-state concentration. Protein synthesis was then blocked by the addition of chloramphenicol and spectinomycin to the culture, and the *v-abl*-derived protein concentrations were analysed by SDS/PAGE and immunoblot at various times after the inhibition of protein synthesis. Addition of these protein-synthesis inhibitors before nalidixic acid induction showed that these drugs completely block expression of *v-abl*-derived proteins in *E. coli* (results not shown). With the use of this procedure the average stability of the total *v-abl*-gene product in the cell can be measured, in contrast with conventional pulse-chase methodologies that measure the stability of only the soluble nascent protein. As shown in Fig. 1(a), the 43 kDa and 60 kDa *v-abl*-derived proteins are both stable in *E. coli*; the half-life of each protein is greater than 3 h. About 95% of the *v-abl*-derived protein expressed in *E. coli* is present as an insoluble aggregate (Ferguson

et al., 1985). The present results show that the *v-abl*-derived protein is stable in *E. coli* in this aggregated state.

KSCN-solubilized *v-abl*-derived protein was chromatographed on a MonoQ f.p.l.c. anion-exchange column with a KCl gradient. The peak of highest tyrosine-specific kinase activity was eluted at 100–150 mM-KCl and contained primarily the 43 kDa fragment of p60^{v-abl} (Fig. 1a, lane 8, and Figs. 1b and 1c). p60^{v-abl} was eluted from the MonoQ column after p43^{v-abl} at about 200 mM-KCl. Comparison of the kinase activity profile and the *v-abl*-specific immunoblot profile of the MonoQ column eluate suggest that the specific activities for tyrosine-specific phosphorylation of casein and angiotensin II by p43^{v-abl} are greater than those exhibited by p60^{v-abl} (results not shown). Quantitative comparison of the specific kinase activities of p60^{v-abl} and p43^{v-abl} was not made since the p60^{v-abl}-containing column fractions also contained p43^{v-abl}. After MonoQ column chromatography p43^{v-abl} was approx. 90% pure on the basis of SDS/PAGE and Coomassie Blue staining (Fig. 1c). The specific tyrosine kinase activity determined for p43^{v-abl} (Table 1) is comparable with the activity exhibited by other preparations of tyrosine kinases (Foulkes *et al.*, 1985, and references cited therein).

Incubation of thiocyanate-stabilized *v-abl*-derived protein containing approximately equal amounts of 60 kDa and 43 kDa forms of *v-abl*-derived protein with

Table 1. Nucleoside triphosphate requirement for p43^{v-abl} tyrosine kinase activity

The phosphorylation of either casein or angiotensin II (2 mM) was assayed under standard conditions, as described in the Experimental section, except that ATP was replaced by various concentrations of the indicated nucleoside triphosphate. Kinetic parameters were determined by non-linear-regression analysis by using the equation:

$$v = (V_{\max} \cdot [S]) / (K_m + [S])$$

Substrate	K_m (μM)	$V_{\max}$ (nmol/min per mg of kinase)
ATP (casein)	18	200
ATP (angiotensin II)	14	220
GTP (casein)	1600	280
GTP (angiotensin II)	310	180
CTP (casein)	5500	180
UTP (casein)	6900	23

[γ -³²P]ATP resulted in the selective incorporation of phosphate by the 43 kDa form of *v-abl*-derived protein (Fig. 1*b*). Phospho amino acid analysis of phosphorylated p43^{v-abl} showed only phosphotyrosine (results not shown). p43^{v-abl} was also preferentially covalently labelled with the reactive analogue of ATP [¹⁴C]FSBA (Colman, 1983) in extracts containing approximately equal amounts of 60 kDa and 43 kDa forms of *v-abl*-derived protein (Fig. 1*d*). The incorporation of [¹⁴C]FSBA into p43^{v-abl} was blocked by 10 mM-p[NH]ppA, a non-hydrolysable analogue of ATP. Furthermore, the time-dependent incorporation of FSBA resulted in a corresponding loss in tyrosine kinase activity. Incorporation of approx. 0.9 mol of [¹⁴C]FSBA/mol of p43^{v-abl} resulted in loss of 95 % of the tyrosine kinase activity. Thus the 43 kDa fragment contains both the catalytic ATP-binding domain and a tyrosine autophosphorylation site. Moreover, the 43 kDa fragment appears to be a considerably more active tyrosine kinase than p60^{v-abl}. Indeed, highly purified p60^{v-abl} had relatively low kinase specific activity (2.4 pmol/min per μg , with casein as substrate) (Ferguson *et al.*, 1985) compared with purified p43^{v-abl}.

To test whether p43^{v-abl} arises from proteolytic cleavage of p60^{v-abl}, we examined whether purified p60^{v-abl} could be converted into p43^{v-abl} by incubation with limiting concentrations of trypsin. As shown in Fig. 2, trypsin digestion of p60^{v-abl} results in the formation of a 43 kDa fragment that is resistant to further degradation by trypsin. The SDS/PAGE mobility of this 43 kDa fragment is identical with that of purified p43^{v-abl}. Other proteinases, including papain and subtilisin, were also found similarly to cleave 60 kDa *v-abl*-derived protein to yield a 43 kDa fragment (results not shown). Thus it appears that p43^{v-abl} may be the product of cleavage of p60^{v-abl} at a site(s) that is relatively sensitive to proteolysis. Preincubation of p60^{v-abl} with ATP was found to inhibit the trypsin-mediated conversion of p60^{v-abl} into the 43 kDa fragment (Fig. 2). Electrophoretically purified p60^{v-abl}, however, lacked detectable autophosphorylation or kinase activity with casein as substrate.

Properties of p43^{v-abl}

p43^{v-abl} exhibits an absolute requirement for bivalent

Fig. 2. Conversion of p60^{v-abl} into a 43 kDa fragment by limited tryptic proteolysis

Purified p60^{v-abl} (lane 1) and p43^{v-abl} (lanes 2 and 11) were analysed by SDS/PAGE and *v-abl*-derived-protein-specific immunoblot. p60^{v-abl} was incubated with trypsin for 5 min (lanes 3 and 7), 15 min (lanes 4 and 8), 30 min (lanes 5 and 9) and 60 min (lanes 6 and 10) in the absence (lanes 3-6) or in the presence (lanes 7-10) of ATP, and analysed by SDS/PAGE and immunoblot. The position and size in kDa of protein markers are indicated.

Fig. 3. Bivalent metal cation requirements for casein phosphorylation by p43^{v-abl}

Phosphorylation was assayed under standard conditions except that 10 mM-MgCl₂ was replaced by the indicated concentration of MgCl₂ (○), CoCl₂ (◇) or MnCl₂ (□).

metal cations for kinase activity (Fig. 3). Mn²⁺, Co²⁺ and Mg²⁺ were approximately equivalent in the p43^{v-abl} kinase reaction when present at optimal concentrations of approx. 0.1, 1.0 and 10 mM respectively. We note that the optimal concentration for these bivalent cations will probably be a function of the concentration of ATP used

Fig. 4. Thermostability of p43^{v-abl} kinase

P43^{v-abl} (0.05 µg/ml) in 100 mM-Mops buffer, pH 7.0, was incubated for 5 min at the indicated temperature and assayed for casein kinase activity under standard conditions. p43^{v-abl} used in this experiment was only partially purified and represented approx. 25% of total protein in the sample.

Table 2. Inhibition of p43^{v-abl} tyrosine kinase activity

Casein phosphorylation by p43^{v-abl} was assayed as described in the Experimental section in the presence of various concentrations (0.1 µM–1 mM) of inhibitor. IC₅₀ is defined as the concentration of inhibitor that inhibited the phosphorylation of casein by 50% relative to control samples incubated without the inhibitor.

Inhibitor	IC ₅₀ (µM)
Ap ₄ A	36
p[NH]ppA	5.0
Quercetin	2.0
ADP	15
Cyclic AMP	150
Cyclic GMP	2000
Calmidazolium	1.9

in the reaction. High concentrations of these salts inhibited casein phosphorylation (Fig. 3). A similar pattern of kinase activity was obtained with chloride, acetate or sulphate salts of Mg²⁺ (results not shown), indicating that the cation, rather than the anion, is responsible for the inhibition of kinase activity observed at the higher salt concentrations. Phosphorylation of casein by p43^{v-abl} was not detected when Zn²⁺, Cu²⁺, Cd²⁺ and Ca²⁺ were tested at concentrations of 0.01–100 mM.

p43^{v-abl} kinase exhibits a strong preference for ATP as phosphate donor (Table 1). Although p43^{v-abl} can use GTP as a phosphate donor for both autophosphorylation (results not shown) and the phosphorylation of exogenous substrates, the *K_m* for GTP is at least 10-fold higher than the value for ATP. The pyrimidine nucleotides CTP and UTP were even less efficient phosphate donors.

Fig. 5. Phosphorylation of protein substrates by p43^{v-abl}

(a) p43^{v-abl} was incubated under the standard assay conditions except where casein was replaced by other proteins at a final concentration of 1 mg/ml. Lanes 1–5 contained calmodulin, histones, phosvitin, casein and myosin light chain respectively. The reaction products were analysed by SDS/PAGE and autoradiography. The position(s) of the major Coomassie Blue-staining component(s) of the reaction are indicated. Also shown is the position of autophosphorylated p43^{v-abl}. (b) p43^{v-abl}, p75 and p60^{v-src} were incubated at 30 °C in 100 mM-Mops buffer, pH 7.0, containing 10 mM-MgCl₂ and 20 µM-[α -³²P]ATP, in the presence and in the absence of 1 mg of casein/ml. The reaction products after 10 min incubation were analysed by SDS/PAGE and autoradiography. The incorporation of phosphate into each casein band was linear with time (0.5–30 min). The positions of the ³²P-labelled bands correspond to the two proteins of equal Coomassie Blue-staining intensity present in the casein sample. The p43^{v-abl} portion of the autoradiograph is not shown; autophosphorylation of p43^{v-abl} was not detected by use of the autoradiograph exposure shown.

The enzymic activity of p43^{v-abl} is relatively thermostable (Fig. 4). The rate of thermal denaturation/inactivation increased markedly above 35 °C, and at 40 °C the half-time for thermal inactivation was approx. 5 min. p43^{v-abl} activity also displays a broad pH optimum (6.0–7.2) (results not shown).

Table 3. Phosphorylation of peptide substrates by p43^{v-abl}

Tyrosine kinase activity was assayed as described in the Experimental section with either 5 mM-peptide or various concentrations of peptide. Kinetic parameters were determined by non-linear-regression analysis by using the equation:

$$v = (V_{\max} \cdot [S]) / (K_m + [S])$$

	K_m (mM)	$V_{\max}$ (nmol/min per mg of kinase)	Relative rate (at 5 mM-peptide)
D-R-V-Y-I-H-P-F*	4.7	120	100
D-R-V-Y-V-H-P-F†	6.2	340	230
R-R-L-I-E-D-A-E-Y-A-A-R-G‡	2.6	610	750
A-A-A-A-Y-A	11	190	100
A-A-A-Y-A	5.0	100	100
A-A-Y-A	15	210	120
A-Y-A	—	—	16
A-A-A-Y-A-A	—	—	11
A-A-Y-A-A	—	—	7
T-Y-K	—	—	62
K-Y-T	—	—	2
M-Y-K	13	200	120
S-Y-K	—	—	13
K-Y-S	—	—	11
K-Y-K	—	—	8
Y-Y-Y	—	—	96
Cyclo Y-Y	—	—	58
N-Acetyl-Y-Y	—	—	47
Y-Y-NH ₂	—	—	27
Y-Y	—	—	< 0.1
Y-NH ₂	—	—	< 0.1
Y	—	—	< 0.1

* Angiotensin II.

† [Valine-5]angiotensin.

‡ Modified autophosphorylation site of p60^{v-src} (Casnellie *et al.*, 1982).

Inhibitors of p43^{v-abl} kinase

p43^{v-abl}-mediated phosphorylation of casein was inhibited by the reactive ATP analogue FSBA (Table 2). Additional inhibitors of enzymes that utilize ATP, including Ap₄A, ADP, p[NH]ppA and quercetin, inhibited casein phosphorylation by p43^{v-abl} when present at concentrations of 1–10 μ M (Table 2). Cyclic AMP and cyclic GMP were less effective inhibitors. The adenosine analogue 5,6-dichloro-1-D-ribofuranosylbenzimidazole inhibits the serine/threonine-specific type-II casein kinase, but it had no effect on p43^{v-abl} (Zandomeni & Weinmann, 1984).

The thiol-reactive reagent *N*-ethylmaleimide inhibited p43^{v-abl}, indicating that reactive thiol groups are important for p43^{v-abl} kinase activity. Calmidazolium, a lipophilic cation and putative calmodulin-specific antagonist (Van Belle, 1981), also was a potent inhibitor of p43^{v-abl}. The mechanism of inhibition of p43^{v-abl} by this compound is not known, but it may reveal a new regulatory domain of the *v-abl*-derived kinase.

Substrates

p43^{v-abl} phosphorylated calmodulin, histones, phosphovitin and myosin light chain proteins (Fig. 5a). Phospho amino acid analysis of these products revealed exclusively phosphotyrosine (results not shown). The incorporation of phosphate per mg of protein into phosphovitin and the histone proteins (H2B and H4) was comparable with phosphate incorporation into casein, whereas the

phosphorylation of calmodulin and myosin light chain by p43^{v-abl} was less efficient. This variability presumably reflects differences in the number and/or local structure of accessible tyrosine residues on the surface of these proteins. However, the ability of p43^{v-abl} to phosphorylate different proteins suggests that the enzyme does not exhibit a strict sequence specificity for phosphorylation at tyrosine residues.

The products of casein phosphorylation by p43^{v-abl} can be resolved by SDS/PAGE into two proteins, which correspond to the two major Coomassie Blue-staining proteins in the casein sample (Fig. 5b; two casein bands are not resolved in Fig. 5a). Although p43^{v-abl} phosphorylated both casein proteins, *v-src*-derived and rat liver p75 tyrosine kinases (provided by A. Goldberg) phosphorylated only one of the casein proteins. Thus the substrate specificity exhibited by p43^{v-abl} can be distinguished from those of p75 and p60^{v-src}.

In an effort to examine the effect of the local primary structure of the tyrosine acceptor on its rate of phosphorylation by p43^{v-abl}, we examined the phosphorylation of a series of small tyrosine-containing synthetic peptides that differ in the number and sequence of amino acid residues and in the position of the tyrosine residue within the sequence. The peptide [valine-5]angiotensin (Wong & Goldberg, 1983a) and a peptide related to the autophosphorylation site of p60^{v-src} (Casnellie *et al.*, 1982) were phosphorylated on tyrosine by p43^{v-abl} (Table 3). As shown in Table 3, the rate of peptide phosphorylation by p43^{v-abl} varied con-

Fig. 6. Glycerol kinase activity of p43^{v-abl}

Glycerol kinase (lane 1) and p43^{v-abl} (lane 2) were incubated with [γ -³²P]ATP and glycerol and the reaction products were analysed by paper electrophoresis as described in the Experimental section. Samples of the glycerol kinase and p43^{v-abl} reactions were also mixed (lane 3). Lane 4 contained ³²P-labelled inorganic phosphate only. The positions of inorganic phosphate (P_i) and L-glycerol 3-phosphate (GP) are indicated.

siderably depending on the structure of the peptide. p43^{v-abl} phosphorylated tripeptides and dipeptides containing tyrosine, but phosphorylation of free tyrosine was not detected.

Previous reports have indicated that the v-src-derived tyrosine kinase is associated with weak glycerol kinase activity (Richert *et al.*, 1982; Graziani *et al.*, 1983a), and purified p43^{v-abl} was found to exhibit a similar property (Fig. 6). The K_m for glycerol in this reaction was 1.4 M. p43^{v-abl} did not exhibit detectable phosphatidylinositol kinase activity when assayed under conditions used by Sugimoto *et al.* (1984) and Macara *et al.* (1984) (results not shown).

DISCUSSION

Previously, we described the isolation of a 60 kDa v-abl-encoded tyrosine-specific kinase, designated p60^{v-abl}, expressed in *E. coli* (Ferguson *et al.*, 1985). Further purification of *E. coli*-expressed v-abl-derived protein by column chromatography revealed that the highest kinase activity is associated with a 43 kDa fragment of p60^{v-abl}, designated p43^{v-abl}. p43^{v-abl} exhibited tyrosine-specific kinase activity with exogenous peptide and protein substrates, was efficiently autophosphorylated on tyrosine residues, and could be labelled specifically with the reactive ATP analogue [¹⁴C]FSBA. Thus the 43 kDa fragment contains both the v-abl-derived catalytic ATP-binding domain and the tyrosine autophosphorylation site.

p43^{v-abl} is the smallest v-abl-derived protein shown to exhibit tyrosine-specific kinase activity. Deletion mapping within the v-abl gene has defined a minimum region required for both kinase activity and transformation of fibroblasts (Prywes *et al.*, 1983, 1985). This region of v-abl contains coding information for a protein of approx. 45 kDa. It is likely that p43^{v-abl} closely corresponds to

this previously defined minimal region of v-abl and is generated by the removal of a 17 kDa fragment from the C-terminus of p60^{v-abl}, since deletion from the N-terminus would result in loss of tyrosine kinase activity (Prywes *et al.*, 1985). A similar region of v-abl encoding a 40–45 kDa protein has also been expressed in *E. coli* as a translational fusion with 80 N-terminal amino acid residues from the simian-virus-40 small t-antigen and shown to exhibit tyrosine-specific kinase activity (Wang & Baltimore, 1985; Foulkes *et al.*, 1985).

The tyrosine kinase activity of p43^{v-abl} appears to be significantly greater than that of p60^{v-abl}. In the MonoQ chromatography profile, the peak of casein kinase activity was co-eluted with p43^{v-abl}. Moreover, in samples containing approximately equal amounts of p43^{v-abl} and p60^{v-abl}, as estimated by immunoblot analysis, p43^{v-abl} was more efficiently autophosphorylated and labelled with FSBA.

SDS/PAGE-purified p60^{v-abl} was converted into a 43 kDa product by limited proteolysis with trypsin, subtilisin or papain. The 43 kDa v-abl-derived protein was resistant to further cleavage by these proteinases. Apparently the 43 kDa tyrosine kinase domain of v-abl-derived protein is linked to other domains of v-abl-derived protein by a segment that is relatively sensitive to non-specific proteolytic cleavage. These results suggest that p43^{v-abl} is generated in *E. coli* by proteolytic cleavage of the p60^{v-abl} precursor. The proteolytic conversion of p60^{v-abl} into p43^{v-abl} is also consistent with the results obtained by Wang & Baltimore (1985), which indicated that when various v-abl-derived proteins were expressed in *E. coli* as translational fusions with simian-virus-40 small t-antigen the C-terminal regions of v-abl-derived protein located outside the minimal region defined for kinase activity were cleaved and degraded. It thus appears that the tyrosine kinase domain of v-abl-derived protein is resistant to limited proteolysis and that this domain in the full-length v-abl-gene product (p120^{gag-abl}) and c-abl-gene product (p150^{c-abl}) might be separated from other functional domains by means of limited proteolysis.

Preincubation of p60^{v-abl} with ATP completely blocked trypsin cleavage. Since the trypsin-cleavage site is located outside the ATP-binding site of the 43 kDa kinase domain, it appears that ATP indirectly protects p60^{v-abl} from trypsin cleavage by inducing a conformational change at the site(s) of trypsin cleavage. These results suggest that the C-terminal region of the full-length v-abl-derived protein outside the catalytic domain may influence or regulate the kinase activity of the protein. This conclusion is also supported by the finding that the specific activity of p43^{v-abl} is greater than that of p60^{v-abl}, as discussed above. Similar findings have been reported for the retrovirus-encoded tyrosine kinases p60^{v-src} and p90^{v-yes} (Brugge & Darrow, 1984; Levinson *et al.*, 1981). In both cases limited proteolysis generates a 30 kDa fragment that is resistant to proteolysis and exhibits greater tyrosine kinase activity than the precursor. Perhaps the products of other genes containing regions of sequence homology to the tyrosine kinase domain of abl or src (such as erbB, v-fms or c-abl) might also be activated by limited proteolytic cleavage. A 43 kDa fragment of the 170 kDa epidermal-growth-factor-receptor exhibiting tyrosine-specific kinase activity has also been obtained by limited proteolysis (Basu *et al.*, 1984). It appears that many tyrosine kinases share a similar structural organization with a kinase catalytic

domain of about 30–40 kDa that is resistant to limited proteolysis, linked to other (regulatory) domains of the molecule by a region that is sensitive to proteolytic cleavage. An active catalytic domain can also be dissociated from a regulatory domain in several serine/threonine-specific protein kinases, such as cyclic AMP- and cyclic GMP-dependent protein kinase (Nairn *et al.*, 1985) and protein kinase C (Kishimoto *et al.*, 1983).

Although the 43 kDa fragment of *v-abl*-derived protein is resistant to limited proteolysis, its kinase activity is intrinsically thermolabile. At 40 °C the half-life of $p43^{v-abl}$ activity was approx. 5 min. The *v-src*-derived (Sugimoto *et al.*, 1984) tyrosine kinase exhibits similar heat-instability.

ATP is the preferred phosphate donor for $p43^{v-abl}$. GTP also served as a phosphate donor, but the high K_m (at least 10-fold greater than that for ATP) suggests that ATP is the physiologically important phosphate donor for the *v-abl*-derived kinase. Similar results were reported for the *E. coli*-expressed *v-abl*-derived kinase ptab150, which is a fusion protein of simian-virus-40 small t-antigen and *v-abl*-derived protein (Foulkes *et al.*, 1985). It is not clear to what extent other tyrosine kinases can utilize GTP. There are conflicting reports as to whether the *v-src*-derived protein (Richert *et al.*, 1982; Graziani *et al.*, 1983b) and the epidermal-growth-factor receptor (Carpenter *et al.*, 1979; Avruch *et al.*, 1982; Erneux *et al.*, 1983; Pike *et al.*, 1984) tyrosine kinases are able to use GTP. The insulin receptor (Zick *et al.*, 1983; Yu & Czech, 1984) and rat liver p75 (Wong & Goldberg, 1983b) tyrosine kinases each have a strict requirement for ATP. As expected for an ATP-utilizing enzyme, a number of ATP analogues were shown to inhibit $p43^{v-abl}$ kinase activity effectively.

The thiol-reactive reagent *N*-ethylmaleimide inactivated $p43^{v-abl}$ kinase, indicating that at least one thiol group is important for $p43^{v-abl}$ activity. Like cyclic AMP-dependent protein kinase (Shalteil *et al.*, 1981), *v-abl*-derived kinase may contain a thiol group at its active site. *N*-Ethylmaleimide is known to inhibit other tyrosine kinases, including rat liver p75 (Wong & Goldberg, 1983b) and the insulin and epidermal-growth-factor receptors (Basu *et al.*, 1984; Pike *et al.*, 1984; Shia *et al.*, 1984).

The $p43^{v-abl}$ kinase, like *v-src*-derived kinase (Richert *et al.*, 1982; Graziani *et al.*, 1983a), phosphorylated glycerol. However, the high K_m for glycerol (1.4 M) indicates that $p43^{v-abl}$ would not be effective as a glycerol kinase in intact cells. The K_m for glycerol phosphorylation by *v-src*-derived kinase is similarly high (Richert *et al.*, 1982). However, the 43 kDa domain of *v-abl*-derived protein sufficient for tyrosine-specific kinase activity is not sufficient for the phosphorylation of phosphatidylinositol.

Our results show $p43^{v-abl}$ is capable of phosphorylating a variety of substrates on tyrosine residues and differs in specificity towards casein when compared with $p60^{v-src}$ and rat liver p75 tyrosine kinases. This finding is not surprising, since $p43^{v-abl}$ also phosphorylated peptides containing as few as two amino acid residues. Foulkes *et al.* (1985), have similarly shown that *E. coli*-expressed *v-abl*-derived ptab150 kinase can phosphorylate a dipeptide containing tyrosine. Free tyrosine or tyramine were not detectably phosphorylated by $p43^{v-abl}$. In addition, the dipeptide tyrosyltyrosine was not phosphorylated unless either the *N*-terminal or the *C*-terminal residue was

blocked (with either an acetyl or an amino group respectively). Apparently there is a minimal structural requirement that the tyrosine acceptor forms both an *N*-terminal and *C*-terminal peptide bond with at least one other residue.

The study examining the phosphorylation of tyrosine-containing peptides demonstrated that the local primary structure and conformation at the tyrosine acceptor site can play an important role in determining the specificity of $p43^{v-abl}$. The rate of phosphorylation by $p43^{v-abl}$ was found to vary considerably depending on the sequence of the peptide and the position of the tyrosine residue within the peptide. A systematic survey of synthetic peptide substrates will be necessary to attempt to define a consensus for the primary sequence determinants recognized by *v-abl*-derived kinase. The protein and synthetic peptide substrates shown to be phosphorylated by $p43^{v-abl}$ all displayed relatively high apparent K_m values (≥ 3 mM), and this probably reflects the lack of optimal primary sequence and/or higher-order protein structural determinants in these substrates. It should be of interest to test as substrate a peptide spanning the putative tyrosine autophosphorylation site of *v-abl*-derived protein. It remains to be established to what extent the primary, secondary and tertiary structures of the substrate define the specificity of phosphorylation by $p43^{v-abl}$, or other viral and cellular tyrosine kinases. Indeed, the nature of the cellular substrates for these enzymes is not well defined (for review see Hunter & Cooper, 1985).

The present experiments have characterized several properties of a derivative of the *v-abl*-gene product expressed in *E. coli*. This product corresponds closely to a minimum region of *v-abl* shown to be sufficient for both tyrosine kinase activity and the ability to transform fibroblasts (Prywes *et al.*, 1985). The *v-abl*-derived tyrosine kinase described in this study shares several similarities with cellular and viral tyrosine kinases isolated from eukaryotic sources. The experimental system developed for the expression and isolation of a functional *v-abl*-derived kinase offers a convenient and sensitive method for the analysis of other tyrosine kinases or putative tyrosine kinases. This system should be useful for screening the effects of random mutagenesis on tyrosine kinase function and in the evaluation of pharmacological agents that interact selectively with this important class of enzymes. In addition, *E. coli*-expressed $p43^{v-abl}$ provides a useful source of a protein-tyrosine kinase for X-ray-crystallographic studies.

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