The EMBO Journal vol.13 no.10 pp.2341 – 2351, 1994

c-Abl kinase regulates the protein binding activity of c-Crk

Stephan M.Feller, Beatrice Knudsen and Hidesaburo Hanafusa

Laboratory of Molecular Oncology, The Rockefeller University, New York, NY 10021, USA

Communicated by P.Comoglio

c-Crk is a proto-oncogene product composed largely of Src homology (SH) 2 and 3 domains. We have identified a kinase activity, which binds to the first Crk SH3 domain and phosphorylates c-Crk on tyrosine 221 (Y221), as c-Abl. c-Abl has a strong preference for c-Crk, when compared with common tyrosine kinase substrates. The phosphorylation of c-Crk Y221 creates a binding site for the Crk SH2 domain. Bacterially expressed c-Crk protein lacks phosphorylation on Y221 and can bind specifically to several proteins, while mammalian c-Crk, which is phosphorylated on tyrosine, remains uncomplexed. The protein binding activity of c-Crk is therefore likely regulated by a mechanism similar to that of the Src family kinases. v-Crk is truncated before c-Crk Y221 and forms constitutive complexes with c-Abl and other proteins. Our results suggest that c-Abl regulates c-Crk function and that it could be involved in v-Crk transformation.

Key words: c-Abl/c-Crk/phosphorylation/tyrosine kinase

Introduction

The strictly controlled processes of cell division and cell differentiation in higher eukaryotes can be deregulated through cellular and viral oncogenes (Bishop, 1991; Cantley *et al.*, 1991). These oncogenes mediate their often fatal actions through interference with normal cellular signal transduction processes. In 1988, v-*crk*, the first representative of a new group of oncogenes, was discovered in the avian retrovirus CT10 (Mayer *et al.*, 1988), and shortly thereafter in another avian retroviral isolate named ASV-1 (Tsuchie *et al.*, 1989). Despite the fact that v-Crk lacks an apparent protein kinase domain, several proteins in v-Crk-transformed cells are heavily phosphorylated on tyrosyl residues. It has therefore been suggested that the v-Crk protein may induce cell transformation by deregulating a cellular protein tyrosine kinase (Mayer *et al.*, 1988; Mayer and Hanafusa, 1990b). A large proportion of the v-Crk protein sequence originating from cellular DNA has homology to conserved protein domains termed SH2 and SH3 (for Src homology regions 2 and 3). These domains were initially discovered in non-receptor-type protein tyrosine kinases, outside the catalytic domain, and are important for the activity of protein kinases and other signal transduction proteins (Koch *et al.*, 1991; Musacchio *et al.*, 1992; Mayer and Baltimore, 1993). The widely expressed c-Crk protein contains an N-terminal SH2 domain followed by two SH3 domains (Matsuda *et al.*, 1992b; Reichman *et al.*, 1992). The viral Crk protein is truncated after the first SH3 domain, similar to another cellular Crk protein detected in primary human embryonic lung cells (Matsuda *et al.*, 1992b). Truncation, together with point mutations in the SH2 and SH3 domains and fusion with the viral Gag protein, appears to mediate the oncogene potential of v-Crk (Reichman, 1992). While the Crk SH2 domain was shown to be required and sufficient to bind specific phosphotyrosine-containing proteins (Matsuda *et al.*, 1991), both SH2 and SH3 domains in v-Crk are required for the transformation of chicken embryo fibroblasts (Mayer and Hanafusa, 1990a). Indirect evidence for the functional importance of SH3 domains was initially derived from mutational studies with protein kinases and other proteins, including v-Crk, Ras-GAP and Sem-5 (Matsuda *et al.*, 1991; Clark *et al.*, 1992; Musacchio *et al.*, 1992; Duchesne *et al.*, 1993; and references therein). Based on genetic observations, recently manifold evidence for SH3-mediated binding of the SH2/SH3-containing protein Ash/Grb2 and its homologues Sem-5 and Drk to the Sos GDP/GTP exchange factors has been presented (Buday and Downward, 1993; Chardin *et al.*, 1993; Egan *et al.*, 1993; Gale *et al.*, 1993; Li *et al.*, 1993; Olivier *et al.*, 1993; Rozakis-Adcock *et al.*, 1993; Simon *et al.*, 1993). Other groups have reported intermolecular protein–protein interactions for Abl and Src family kinase SH3 domains (Cicchetti *et al.*, 1992; Prasad *et al.*, 1993; Weng *et al.*, 1993), as well as protein targeting and enzyme regulation through different SH3 domains (Bar-Sagi *et al.*, 1993; Gout *et al.*, 1993). Similar to the results of binding studies with SH2 domains (Waksman *et al.*, 1992; Songyang *et al.*, 1993), specificity and high affinity binding of SH3 domains also appear to depend on the recognition of several amino acid residues within a short stretch of a protein. Prolyl residues are possibly a common feature of sequences binding to the SH3 domains of Abl, Ash/Grb2, Crk and other proteins (Chardin *et al.*, 1993; Egan *et al.*, 1993; Li *et al.*, 1993; Ren *et al.*, 1993; Rozakis-Adcock *et al.*, 1993; B.Knudsen *et al.*, unpublished data), comparable to the importance of phosphotyrosyl residues in the binding of SH2 domains.

Little is yet known about the function of the c-Crk protein, although the existence of an oncogenic derivative and its composition of SH2 and SH3 domains is suggestive of its importance for signal transduction. The binding of c-Crk to Sos proteins (Gout *et al.*, 1993; S.M.Feller *et al.*, unpublished data) and a new protein with CDC25 homology (B.Knudsen *et al.*, unpublished data; Tanaka *et al.*, 1994), also supports this notion. Together with the Crk-like protein (CRKL), Nck, Ash/Grb2 and the Ash/Grb2 homologues Drk and Sem-5 (Lehmann *et al.*, 1990; Clark *et al.*, 1992; Lowenstein *et al.*, 1992; Matuoka *et al.*, 1992; Olivier *et al.*, 1993; Simon *et al.*, 1993; ten Hoeve *et al.*, 1993) it forms a growing new class of signal transduction proteins. This class

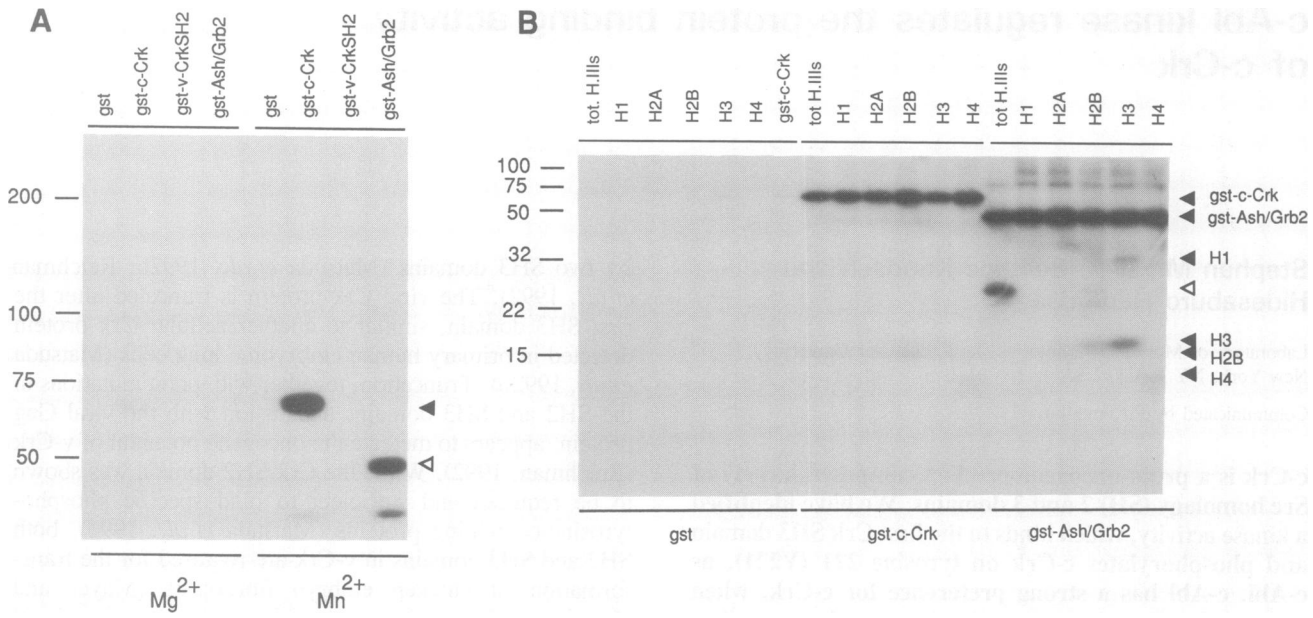


Fig. 1. c-Crk and Ash/Grb2 bind and are phosphorylated by distinct kinase activities. **(A)** *In vitro* kinase assays of HeLa S100 proteins precipitated by glutathione-S-transferase (gst) or gst fusion proteins immobilized on glutathione−Sepharose. The alkaline-stable phosphorylation of gst-c-Crk (filled arrowhead) and gst-Ash/Grb2 (open arrowhead) is indicated. The kinase reaction was done with 5 mM $MgCl_2$ or $MnCl_2$, without exogenous substrate for 30 min. Proteins were separated by SDS−PAGE (7%). An exposure of the gel after sodium hydroxide treatment is shown. Molecular weight standards are indicated in kDa. **(b)** Alkali-stable substrate phosphorylation by the Crk and Ash/Grb2 binding kinase activities precipitated from HeLa S100, using different histones as exogenous substrates (indicated on top). The kinase reaction was for 1 h with 5 mM $MnCl_2$. Partially purified histones or crude histones type IIIs (tot H. IIIs) were tested. Proteins used for the precipitation of HeLa S100 proteins are indicated at the bottom. Proteins were separated by SDS−PAGE (15%). Treatment of the gel with alkali enhanced the detection of specific signals, since significant serine/threonine kinase activity precipitates with gst and gst-containing proteins. The open arrowhead indicates an unidentified substrate of the Ash/Grb2 binding kinase in the crude histone preparation.

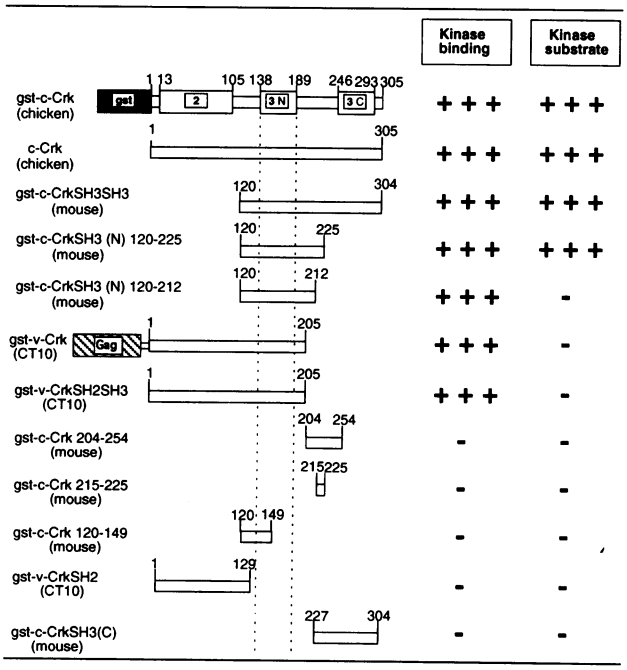
of small SH2/SH3-containing proteins lacks an apparent catalytic domain and may serve as regulatory subunits of enzymes, and as adaptors, coupling different proteins of a signal transduction cascade. Since Crk is present in virtually all tissues and cells analysed so far (Matsuda *et al.*, 1992b; Reichman *et al.*, 1992; S.M.Feller, unpublished data), it may be of general importance in the signal transduction of cells. Our results show that c-Abl binds to the first Crk SH3 domain and efficiently phosphorylates a tyrosyl residue in the spacer region between the Crk SH3 domains. This phosphorylation creates a binding site for the Crk SH2 domain. We present evidence consistent with a regulatory role of this phosphorylation. Furthermore, we show that c-Abl can stably bind to v-Crk, making it a strong candidate for the tyrosine kinase(s) involved in v-Crk transformation.

Results

Crk and Ash/Grb bind two distinct, cytosolic tyrosine kinase activities

In initial studies, different mammalian cell lines were analysed for c-Crk specific binding proteins. Several c-Crk-specific binding proteins (of 185, 170, 155−145, 115−125 and 85 kDa), which bound to the first Crk SH3 domain, were detected. The 170 kDa protein, which bound also to Nck and Ash/Grb2, was identified as a member of the GDP/GTP exchange protein family Sos (S.M.Feller *et al.*, manuscript in preparation). Most of the Crk binding proteins were enriched in a cytosolic fraction (S100) of HeLa cells, which was used for most further analyses. We assayed the Crk binding proteins for kinase activity, since v-Crk transformation requires a functional SH3 domain and is possibly linked to the activation of a cellular protein tyrosine kinase. As a control for specificity we also investigated Ash/Grb2.

Table I. Mapping of the kinase binding region and the kinase substrate site in c-Crk and v-Crk



Schematic summary of results from *in vitro* kinase assays. For experimental details see Figure 2. Amino acid boundaries are indicated for all fusion proteins.

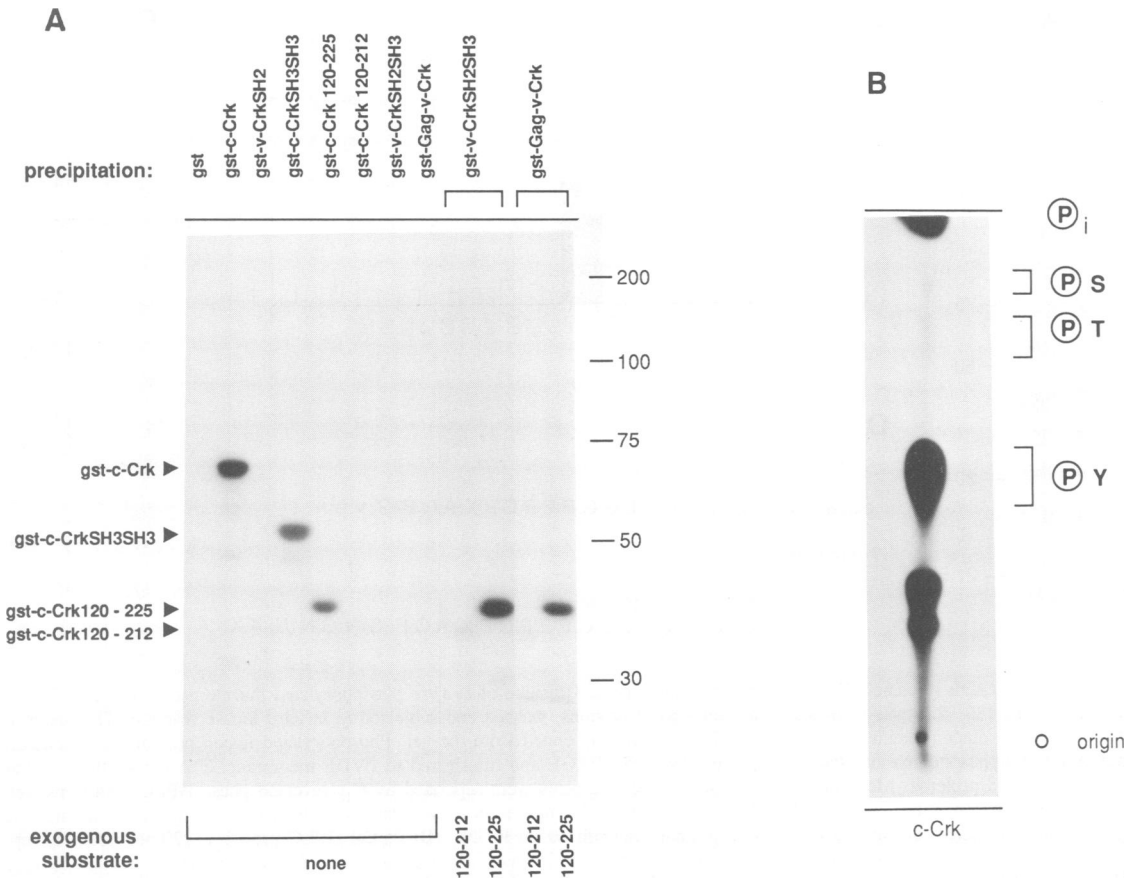


Fig. 2. c-Crk and v-Crk bind a HeLa S100 tyrosine kinase activity, but only c-Crk contains the major kinase substrate site. (**A**) *In vitro* kinase assay of different c-Crk and v-Crk fusion proteins (see also Table I). Kinase reactions were done as described in Figure 1B. Proteins were separated by SDS−PAGE (9%). Fusion proteins used for precipitation (top) and as substrates (bottom) are indicated. (**B**) Phosphoamino acid analysis of *in vitro* phosphorylated c-Crk by one-dimensional thin layer chromatography. c-Crk was cleaved from gst, bound with Crk antiserum to protein A−Sepharose and used for precipitation of HeLa S100 proteins. Washed precipitates were subjected to *in vitro* kinase assay and SDS−PAGE. The eluted c-Crk band was digested with protease, then hydrolysed with hydrochloric acid and analysed for phosphoamino acids. Sample origin, migration of the phosphoamino acid standards and inorganic phosphate (P_i) are indicated.

Both c-Crk and Ash/Grb2 bound to protein kinases in the HeLa S100 fraction and were phosphorylated by these kinases. The kinases failed to autophosphorylate (Figure 1A), but were highly active towards c-Crk or Ash/Grb2 respectively. The bands seen at the bottom of the gel are proteolytic fragments of the fusion proteins. The phosphorylation was alkali-stable in both cases, suggestive of phosphorylated tyrosyl residues. The kinase activities associated with c-Crk or Ash/Grb2 had a strong substrate preference for the protein to which they bound. In other words, little or no phosphorylation was seen when Ash/Grb2 was added as an exogenous substrate into a Crk precipitate and vice versa. Furthermore, they had poor activity towards common tyrosine kinase substrates such as enolase, poly(Glu/Tyr), Raytide, ^{5}Val-angiotensin II and a Src (Y416) derived peptide, and did not phosphorylate glutathione-S-transferase (gst; data not shown). Further analysis of other known kinase substrates revealed that the Ash/Grb2 binding kinase phosphorylated histones H3 and H4 and an unidentified protein in crude histone type IIIs, while the Crk binding kinase had some activity towards histones H2 B and H4 (Figure 1B). While HeLa S100 was used in most of the studies, the Crk kinase activity was also detected in a crude membrane fraction (P100) of HeLa cells and in total lysates, S100 and P100 of several other cell lines (not shown).

Crk proteins bind a tyrosine kinase via the first SH3 domain

The further analysis focused on the Crk binding kinase. Various deletion mutants of c-Crk and v-Crk were constructed and examined for their ability to bind kinase activity and to serve as kinase substrates (summarized in Table I). Both v-Crk and c-Crk-derived proteins containing the first SH3 domain [SH3(N)] could precipitate the kinase from HeLa S100. Selected results are shown in Figure 2A. The murine c-Crk protein gst-c-Crk 120−125, which contains tyrosine 221 (Y221, homologous to Y222 of chicken c-Crk) was the shortest good kinase substrate. A slightly truncated protein (gst-c-Crk 120−212), lacking this residue, was barely phosphorylated, suggesting that Y221 could be the primary phosphate acceptor residue. Subsequently, c-Crk was cleaved with Factor Xa from its expression tag, immobilized on protein A−Sepharose with a Crk antiserum, incubated with HeLa S100 and subjected to *in vitro* kinase assay. Phosphoamino acid analysis showed that the phosphorylation was almost exclusively on tyrosyl residues (Figure 2B).

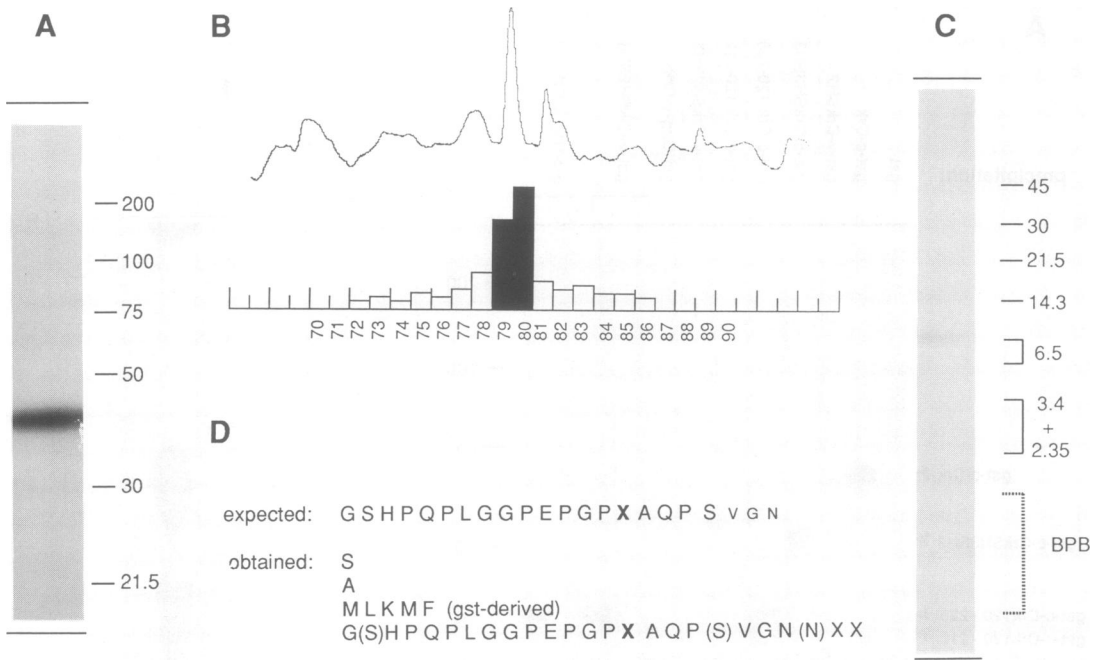


Fig. 3. Identification of c-Crk tyrosine 221 as the phosphorylated tyrosyl residue of *in vitro* phosphorylated murine gst-c-Crk 120−225. Immobilized fusion protein gst-c-Crk 120−225 was incubated with HeLa S100 proteins, washed and subjected to *in vitro* kinase reaction. The fusion protein was separated from ATP and other proteins by preparative SDS−PAGE and eluted from the gel. Phosphorylated fusion protein was isolated by affinity chromatography on anti-phosphotyrosine agarose, separated by SDS−PAGE and transferred to PVDF membrane. The immobilized protein was cleaved with *S.aureus* V8 protease. Membrane-released proteolytic fragments were separated by C_{18} reversed phase HPLC. Fractions were collected and analysed by Cerenkov counting and SDS−PAGE (20%, pH 9.3). A single radioactive peak was obtained and subjected to amino acid sequencing. (**A**) Phosphorylated, affinity purified fusion protein immobilized on PVDF. (**B**) Partial HPLC profile at 220 nm from the separation of the proteolytic fragments. The single radioactive peak detected in fractions 79 and 80 is indicated. (**C**) Analysis of HPLC fraction 79 (5% of total) by SDS−PAGE. Migration standards are 'Rainbow Markers' (Amersham) and bromophenol blue (BPB). (**D**) Amino acid sequence expected from prior mapping experiments, and actual result. The letter X indicates a modified tyrosyl residue which, in contrast to unmodified tyrosyl residues, cannot be recovered with the standard amino acid sequencing technique used here. Amino acids indicated in smaller letters (top row) result from the expression vector used. A copurifying, contaminating peptide from the gst region of the fusion protein, which lacks putative phosphate acceptor residues, was also identified.

c-Crk Y221 is the major substrate site for the Crk binding kinase

In our experiments with the deletion mutant constructs (Table I and Figure 2A) we detected one likely target site for the Crk binding kinase, c-Crk Y221, which is conserved in all species analysed so far. However, gst fusion proteins containing c-Crk amino acids 204−254 or 215−225 were not phosphorylated by the Crk binding kinase, despite the presence of Y221 in these peptides. These results could be explained by incorrect folding of the short c-Crk fragments. To determine unambiguously the phosphorylation site, gst-c-Crk 120−225 was phosphorylated and purified as outlined in the legend of Figure 3. The purified phosphoprotein (Figure 3A) was subjected to proteolytic digestion and phosphopeptides were analysed by HPLC (Figure 3B). A single proteolytic phosphopeptide was obtained (Figure 3B and C). The amino acid sequence of this peptide (Figure 3D) identified c-Crk Y221 as the modified tyrosyl residue. Thus, Y221 is indeed the phosphorylation site of a specific Crk binding kinase.

This site is particularly interesting, because Y221 is the only c-Crk tyrosine with a proline in position +3 and may become a binding site for the Crk SH2 domain when phosphorylated. Synthetic phosphotyrosyl peptides with a proline in position +3 are enriched from a peptide library by Crk SH2 affinity chromatography (Songyang *et al.*, 1993), and compete with paxillin for Crk SH2 binding (Birge *et al.*, 1993). In addition, the CRKL protein (ten Hoeve *et al.*, 1993), a distinct gene product with high homology to Crk in the SH2 and SH3 domains, contains in the spacer between the SH3 domains a region around Y207 with significant homology to the amino acid sequence in the Crk Y221 region.

c-Abl and Arg kinases bind to c-Crk and phosphorylate Y221

The unique substrate specificity of the Crk binding kinase prompted us to determine its identity. The few kinases initially tested (Src, Csk, Fer and EGF receptor) showed poor kinase activity towards the c-Crk Y221 site (data not shown). The similarity of the amino acids around c-Crk Y221 to a newly found substrate of c-Abl (Baskaran *et al.* 1993), prompted us to analyse the Abl family kinases c-Abl and Arg (Kruh *et al.*, 1990; Wang, 1993) for their ability to bind the first SH3 domain of Crk and to phosphorylate Y221. Western blots with Abl- and Arg-specific antibodies revealed the binding of both kinases to fusion proteins containing the Crk SH3(N) domain (Figure 4A). Some binding was also detected with gst-Ash/Grb2. While this manuscript was in preparation, Pendergast and colleagues reported the complex formation of Ash/Grb2 with c-Abl *in vitro* (Pendergast *et al.*, 1993), consistent with our finding. Weak binding of c-Abl was also seen in experiments with a gst-Nck protein lacking the first SH3 domain, and

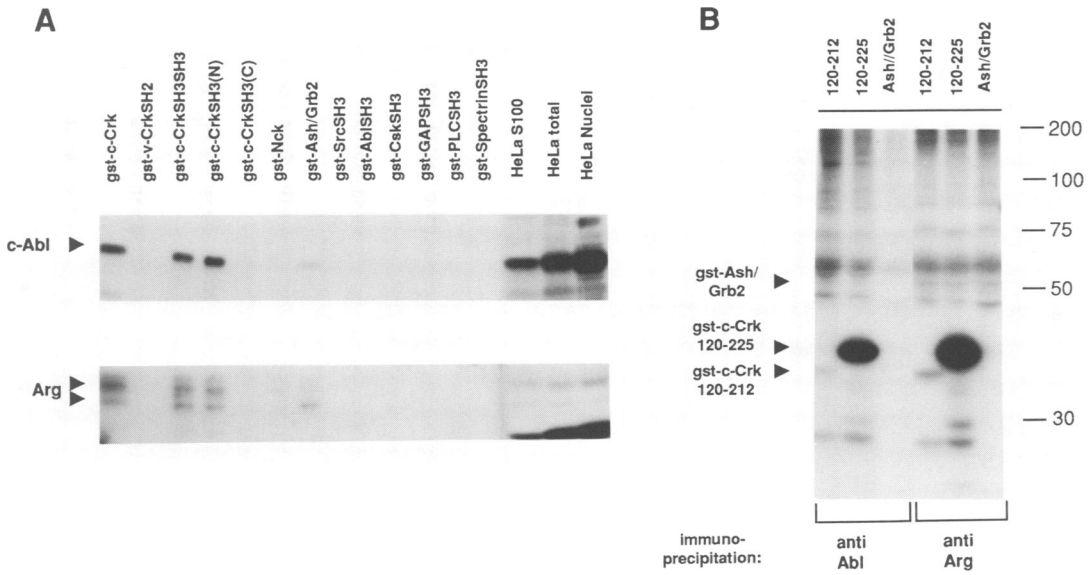


Fig. 4. c-Abl and Arg can bind to the first SH3 domain of c-Crk and phosphorylate c-Crk *in vitro*. (**A**) Equimolar amounts of immobilized gst fusion proteins, containing SH2 and SH3 domains of different cellular proteins, were incubated with HeLa S100, washed, separated by SDS−PAGE (7%) and analysed by Western blot with a monoclonal antibody specific for Abl (top) or a polyclonal antiserum specific for Arg (bottom). The three right lanes contain HeLa protein extracts as indicated. (**B**) *In vitro* kinase assay with c-Abl kinase or Arg-kinase immunoprecipitated from HeLa S100. Equimolar amounts of gst fusion proteins containing c-Crk amino acids 120−212 or 120−225, or gst-Ash/Grb2, were added as exogenous substrates as indicated on top.

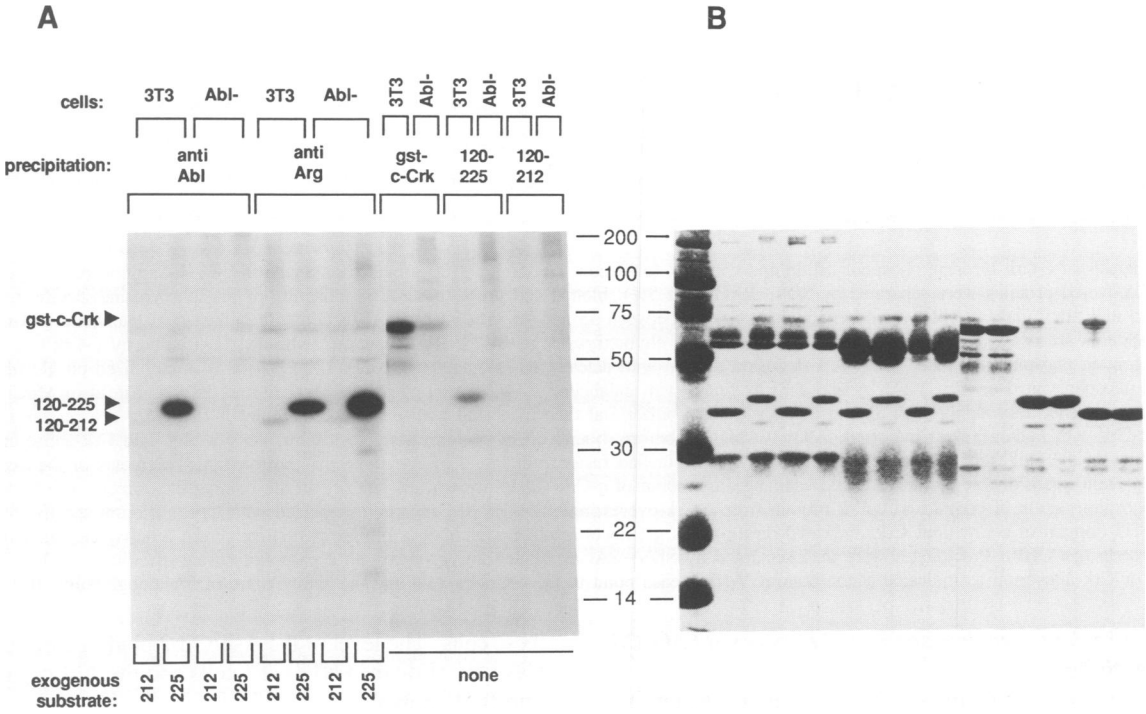


Fig. 5. Crk binding kinase activity is drastically reduced in fibroblasts derived from mice with c-*abl* gene knockout. (**A**) Total cell lysates from NIH-3T3 mouse fibroblasts (3T3) or a fibroblast cell line derived from mice lacking a functional c-Abl kinase (Abl−) were precipitated with immobilized antibodies or gst fusion proteins as indicated on top and subjected to *in vitro* kinase reactions. gst-c-Crk 120−212 (212) or 120−225 (225) was added as exogenous substrate into immunoprecipitates as indicated at the bottom. Proteins were separated by SDS−PAGE (11%). (**B**) Coomassie Blue staining of the gel from (A).

with the Abl SH3 domain, upon prolonged exposure (not shown). In addition, immunoprecipitates of c-Abl and Arg both efficiently phosphorylated Crk Y221, but failed to phosphorylate gst-Ash/Grb2 (Figure 4B). Limited phosphorylation was detected in gst-c-Crk 120−212. This may be due to the phosphorylation of additional Crk tyrosine residues by c-Abl and Arg. Both the binding of c-Abl and Arg to the first SH3 domain of Crk and the efficient phosphorylation of c-Crk Y221 suggested that these kinases could account for the Crk binding kinase activity.

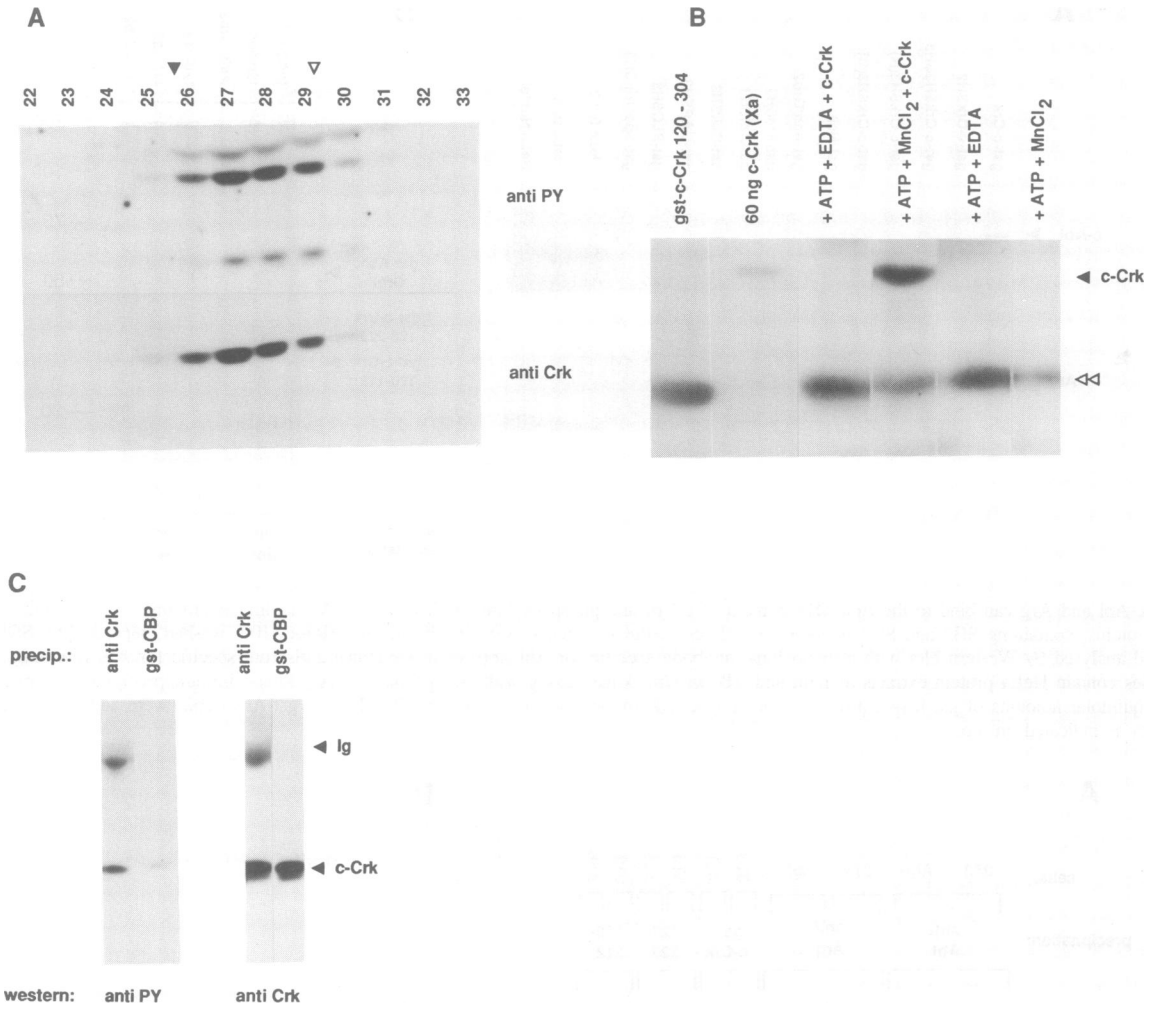


Fig. 6. Analysis of c-Crk tyrosine phosphorylation and complex formation. (**A**) HeLa S100 protein was analysed by gel filtration on a Superose 12 column. Recovered proteins were separated by SDS−PAGE (11.5%), blotted and probed with Crk antiserum or phosphotyrosine-specific monoclonal antibody as indicated. Only the blot region containing proteins between 35 and 45 kDa is shown. Fractions are indicated by numbers. Gel filtration protein standards are ovalbumin (43 kDa, filled arrowhead) and chymotrypsinogen A (25 kDa, open arrowhead). (**B**) Binding of c-Crk to a truncated gst-c-Crk protein phosphorylated on Y221. A Crk fusion protein with deleted SH2 domain (gst-c-Crk 120−304) was immobilized on glutathione-beads and phosphorylated on Crk Y221 by HeLa S100 kinase with unlabelled ATP, or mock-incubated with 5 mM EDTA instead of $MnCl_2$. Samples were washed thoroughly with high stringency (RIPA) buffer and then incubated with or without soluble, full-length c-Crk protein (cleaved from gst-c-Crk with Factor Xa) as indicated. After washing as before, bound c-Crk protein was detected by Western blot with Crk antiserum. The two left lanes of the blot were loaded with unphosphorylated, purified fusion proteins as indicated, to detect proteolytic fragments of the expressed gst-c-Crk protein (open double arrowhead) and estimate the amount of c-Crk (filled arrowhead) retained in the binding experiment. (**C**) Reduced tyrosine phosphorylation of complexed c-Crk protein from c-Crk-overexpressing CEF. c-Crk was precipitated with Crk antiserum specific for the C-terminal SH3 domain of c-Crk (anti Crk; indicated on top) or a gst fusion protein containing a proline-rich stretch from a newly-cloned, Crk-specific binding protein (gst-CBP). Precipitates were then separated by SDS−PAGE, blotted and probed with a phosphotyrosine-specific monoclonal antibody (anti PY) or Crk antiserum as indicated at the bottom. Arrowheads point to the c-Crk protein and the heavy chain of immunoglobulins (Ig).

Mouse fibroblasts lacking active c-Abl contain little Crk kinase activity

To determine whether c-Abl and Arg bind and phosphorylate c-Crk equally well, we compared NIH-3T3 cells and a fibroblast cell line established from mice deficient in active Abl kinase after targeted gene disruption (Tybulewicz *et al.*, 1991). Possibly due to cellular compensation mechanisms, fibroblasts lacking c-Abl had higher Arg *in vitro* kinase activity, when compared with NIH-3T3 cells (Figure 5A). However, only a low amount of Crk kinase was detected after incubation of lysates from c-Abl-deficient cells with gst-c-Crk or gst-c-Crk 120−225, suggesting that c-Abl may be primarily responsible for c-Crk Y221 phosphorylation in normal cells. These data also argue against the presence of additional Crk binding and phosphorylating kinases in these cells. The level of the Ash/Grb2 binding kinase activity was very similar in NIH-3T3 cells and the Abl-negative cell line (not shown).

c-Crk is tyrosine phosphorylated and not complexed in vivo

Subcellular fractionation was performed to study the distribution, native molecular weight and the phosphorylation status of endogenous c-Crk protein. c-Crk was enriched in the S100 fraction of HeLa cells, but not detectable in a crude mitochondrial fraction (P10), crude membrane fraction (P100), purified nuclei, or a Triton X-100-insoluble protein fraction. Gel filtration chromatography of HeLa S100 showed c-Crk exclusively in fractions expected for a non-complexed, monomeric Crk protein (Figure 6A). The Crk

protein comigrated perfectly with the major phosphotyrosyl protein detected in this size range. Although several Crk binding proteins, including c-Abl and Arg, were also present in HeLa S100, the endogenous c-Crk protein did not bind to them in detectable amounts. This result indicates that both endogenous c-Crk protein and the Crk binding proteins, are present in the S100 fraction of HeLa cells, but that they do not form constitutive complexes. The interaction of c-Crk with other proteins appears therefore to be transient in HeLa cells, unlike the stable association between Ash/Grb2 and Sos proteins seen in various cell lines.

Phosphorylated Crk Y221 binds the Crk SH2 domain with high efficiency

The previous results suggested that c-Crk Y221 could indeed be the *in vivo* site of phosphorylation by c-Abl. Since c-Crk contains a proline in position 224, phosphorylation of Y221 should generate a preferred binding site for the Crk SH2 domain (Songyang *et al.*, 1993) and may therefore promote an intramolecular interaction within the c-Crk protein. This situation is somewhat analogous to a regulatory mechanism previously proposed for the Src kinase family (Matsuda *et al.*, 1990; Roussel *et al.*, 1991; Gervais *et al.*, 1993; Liu *et al.*, 1993), although stronger evidence to support this model has yet to be obtained from the structural analysis of active and inactive Src family kinase proteins.

To examine whether stable interaction of phosphorylated Y221 with the Crk SH2 domain can occur, immobilized c-Crk fusion protein with a deleted SH2 domain (gst-c-CrkSH3SH3, amino acids 120–304) was phosphorylated on Y221 and tested for binding to soluble, full-length c-Crk, cleaved from gst. As shown in Figure 6B, phosphorylated gst-c-CrkSH3SH3 bound soluble c-Crk under conditions of high stringency, whereas very little binding was seen with the unphosphorylated gst-c-CrkSH3SH3 protein. Calculations of the phosphorylation efficiency for the gst-c-Crk-SH3SH3 protein and estimation of the amount of soluble c-Crk protein bound after washing with high stringency buffer allow us to conclude that the phosphorylated gst-c-CrkSH3SH3 protein bound full-length c-Crk very efficiently, at least 150-fold better than the non-phosphorylated protein.

If phosphorylation of Y221 results in the intramolecular interaction with the Crk SH2 domain, and thus the inhibition of interactions between the first Crk SH3 domain and other proteins, c-Crk may form complexes when it is dephosphorylated. To overcome our previous failure to detect c-Crk in complexes with other proteins, we overexpressed c-Crk in chicken embryo fibroblasts (CEF) by infection with recombinant retrovirus (Reichman, 1992) and analysed these cells by subcellular fractionation and precipitation experiments. A significant amount of c-Crk protein in S100 of these cells was recovered in fractions expected for a complexed Crk protein after gel filtration chromatography. The majority of this Crk protein formed complexes comigrating with proteins of 200–250 kDa native molecular weight. A small Crk protein fraction, detected as a distinct peak, migrated at ~500 kDa (data not shown). Complex formation in these cells was further confirmed by coprecipitation of c-Crk with antiserum against a newly cloned Crk binding protein (CBP; B.Knudsen *et al.*, manuscript in preparation). It is presently unclear whether this complex formation is primarily a consequence of the c-Crk overexpression or results also from the cell type (CEF) used.

To analyse the degree of tyrosine phosphorylation in c-Crk protein capable of forming complexes, lysates of c-Crk overexpressing CEF were either immunoprecipitated with Crk antiserum, raised against the second SH3 domain of c-Crk, or precipitated with a gst fusion protein containing a 113 amino acid region with a proline-rich stretch, which was derived from CBP. These 113 amino acids can stably bind to the first SH3 domain of bacterially expressed c-Crk protein. The precipitations were carried out in the presence of high concentrations of phosphatase inhibitors. Precipitates were then analysed by Western blot with Crk antiserum or phosphotyrosine-specific monoclonal antibody (Figure 6C). In order to normalize carefully the amount of Crk protein precipitated with the different affinity reagents, various amounts of proteins from cell lysates were used for the precipitations and the bound Crk protein was quantified by densitometric laser scanning of the Western blots (not shown). Comparison of c-Crk protein bound with antiserum or the 113 amino acid polypeptide showed clearly that c-Crk protein recovered by binding to the gst fusion protein is less phosphorylated on tyrosine. The presence of some phosphotyrosine in these precipitates could be explained by multiple *in vivo* phosphorylation sites. The result was not unexpected, since v-Crk, which is truncated before c-Crk Y221, was previously shown to be phosphorylated on tyrosine *in vivo* (Mayer and Hanafusa, 1990b). The binding of specific cellular proteins to bacterially expressed c-Crk protein, which lacks phosphotyrosine, as well as this result indicates that dephosphorylated c-Crk forms complexes with other cellular proteins *in vivo*.

Complex formation of c-Abl with v-Crk

Cell transformation by v-Crk correlates closely with the elevation of phosphotyrosyl proteins (Matsuda *et al.*, 1992a). It has been suggested that activation of tyrosine kinase(s) may be important (Mayer *et al.*, 1988; Mayer and Hanafusa, 1990b; Sabe *et al.*, 1992b). However, no conclusive evidence for the activation of a tyrosine kinase crucial for v-Crk transformation has yet been presented. From the findings of the present study, one possible mechanism for such a kinase activation in v-Crk-transformed cells would be the formation of stable complexes between v-Crk and the Abl family kinases. Kinase assays (Table I and Figure 2A) and Western blots (data not shown) demonstrated the binding of gst-v-Crk and gst-v-CrkSH2SH3 to c-Abl *in vitro*. The formation of complexes between v-Crk and c-Abl or Arg *in vivo* could result in a subcellular redistribution of the kinases, making new substrates available to them. It is also conceivable that constitutive activation of the c-Abl and Arg kinases could result from binding to v-Crk. Therefore, we investigated the subcellular localization of c-Abl and Arg in v-Crk-transformed cells, as well as possible complexes with v-Crk. v-Crk-transformed 3Y1 rat fibroblasts and parental 3Y1 cells were lysed or subjected to cell fractionation, followed by Western blot analysis with monoclonal antibodies against c-Abl or Gag [for detection of v-Crk ($p47^{gag\text{-}crk}$)]; Figure 7A). The amount of c-Abl was slightly increased in total cell lysates of v-Crk 3Y1 cells, significantly increased in a crude membrane fraction (P100) and decreased in the nuclear fraction when compared with the parental 3Y1 cells. Similar results were obtained for Arg (data not shown). Western blot analysis detected a coprecipitation of v-Crk and

c-Abl in Abl immunoprecipitates (Figure 7B). Cell lysates from CT10 virus-infected CEF, and other v-Crk-expressing mammalian cell lines, also showed coprecipitation of v-Crk with c-Abl and Arg (S.M.Feller, unpublished data). Further analysis of the Abl family kinases in v-Crk transformation could therefore provide interesting insights into transformation mechanisms.

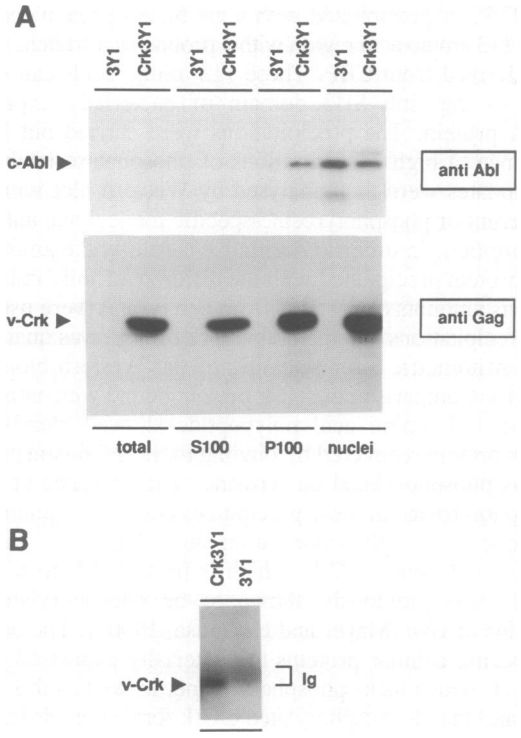


Fig. 7. c-Abl is redistributed in v-Crk-expressing 3Y1 rat fibroblasts and coprecipitates with v-Crk. (**A**) Protein lysates from total cells, cell fractions of parental 3Y1 cells or 3Y1 cells expressing v-Crk (Crk3Y1) were separated by 7% SDS–PAGE and analysed by Western blot with monoclonal antibodies specific for Abl or Gag protein. Cell fractions analysed are S100 proteins, crude membrane fraction (P100), or purified nuclei. (**B**) Coprecipitation of v-Crk and c-Abl in v-Crk-transformed 3Y1 cells. Total lysates of 3Y1 cells or Crk3Y1 cells were precipitated with monoclonal antibody against c-Abl. Precipitated proteins were separated by SDS–PAGE (10%) and probed with monoclonal antibody against the viral Gag protein.

Discussion

We have shown that both c-Crk and Ash/Grb2 bind distinct cytosolic kinase activities which do not autophosphorylate significantly (Figure 1). c-Crk was an excellent substrate for the Crk binding kinase, but all common tyrosine kinase substrate tested were poorly phosphorylated, when compared with c-Crk. The specific binding of the kinase activity to Crk, and its apparently very narrow substrate range, together with its strong substrate preference for Crk, suggested to us that the Crk phosphorylation could be functionally significant. *In vitro* kinase assays with Crk deletion mutants, combined with amino acid sequencing of phosphopeptides (Table I; Figures 2 and 3), identified c-Crk Y221 as a target site of the Crk binding kinase. In the same set of experiments we also showed that the first SH3 domain of c-Crk can bind the kinase under high stringency conditions. This demonstrates a new function for an SH3 domain, namely the targeting of a kinase to its substrate protein. Whether this binding results also in a direct activation of the SH3 binding kinase is presently unclear, although some preliminary evidence supports this idea. While this manuscript was in preparation, it was shown that some SH3 domains can activate the GTPase activity of dynamin (Gout *et al.*, 1993) *in vitro*. Therefore, SH3 domains probably have functions beyond the formation of specific protein complexes *in vivo*, including the regulation of enzymatic activities.

Western blot analysis and immune complex kinase assays, showed that both c-Abl and Arg can bind to the first SH3 domain of c-Crk and that the immunoprecipitated kinases can phosphorylate Crk Y221 efficiently *in vitro* (Figure 4). Analysis of kinase activity precipitated with Crk fusion proteins from mouse fibroblasts lacking c-Abl (Figure 5) showed that Arg, while elevated in these cells, is not capable of compensating for the c-Abl deficiency *in vitro*. Since the Crk binding kinase activity was greatly decreased in c-Abl-

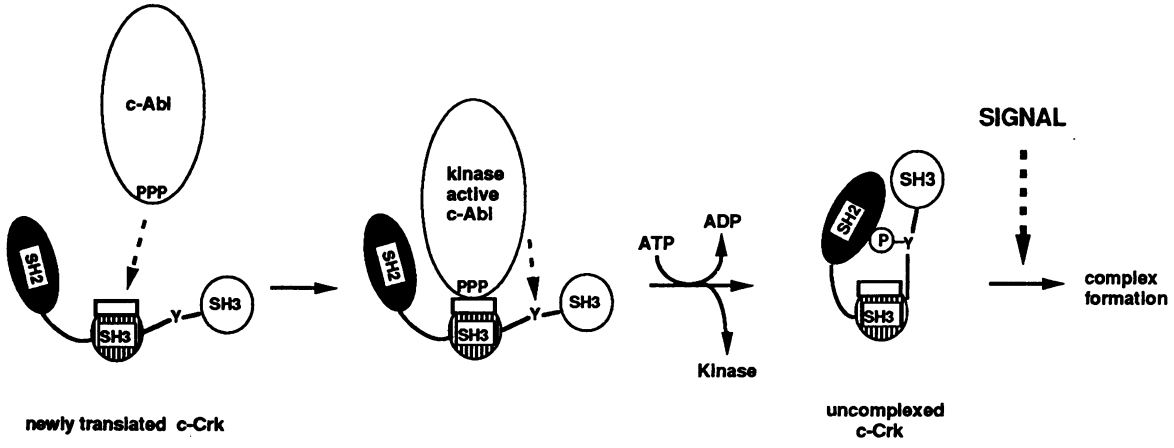


Fig. 8. Model for the regulation of c-Crk protein binding activity by c-Abl. Newly translated c-Crk protein binds c-Abl via its first SH3 domain. c-Crk may function as a positive regulator of c-Abl kinase activity and is subsequently phosphorylated on Y221. The binding of c-Abl to c-Crk is transient. Intramolecular binding of phosphorylated Y221 to the SH2 domain blocks SH3(1)-dependent protein binding. Activation of c-Crk signal transduction is possibly linked to dephosphorylation of Crk Y221 by a cellular phosphatase. Unmasking of the first Crk SH3 domain results in complex formation with specific proteins. It is presently unclear whether the c-Crk SH2 domain has more than a negative regulatory function *in vivo* and what the function of the second SH3 domain is.

negative cells, it is also unlikely that additional kinases which can bind and phosphorylate Crk are present in these mouse fibroblasts. c-Abl is therefore probably responsible for the Crk binding kinase activity. c-Abl kinase activity is not easily detectable *in vivo* and, contrary to v-Abl and Bcr-Abl (for reviews see Rosenberg and Witte, 1988; Daley and Ben-Neriah, 1991) little is yet documented about its *in vivo* substrates. Histones H2B and H4 were the best 'non-Crk' substrate proteins detected in our studies (Figure 1B). This is in agreement with an earlier report showing that v-Abl can phosphorylate these histones (Wang and Baltimore, 1985). The Arg kinase also binds to the first c-Crk SH3 domain, but has apparently little kinase activity. The phosphorylation of Crk Y221 by Arg in immunoprecipitates (Figure 4B) may be due to its activation by binding of the Arg-specific antibodies. Therefore, c-Crk may activate c-Abl, but fails to activate the Arg kinase efficiently. The explanation for this difference could lie in the sequence diversity of the Abl-family kinases. It is likely that the Crk SH3 domain binds to one or more of the short proline-rich clusters scattered throughout the C-terminal region of c-Abl and Arg. These proline clusters are only partially conserved between the Abl kinase family members and c-Abl and Arg binding may occur via non-conserved sites.

The functional significance of the interaction between c-Abl and c-Crk is presently hypothetical. The kinase binding site and the substrate site in c-Crk, together with previously published characteristics of the Crk proteins, suggested to us an attractive hypothesis which can account for many of the biological features of c-Crk and v-Crk. According to this model, c-Abl binds to c-Crk and phosphorylates Y221, in the spacer region between the SH3 domains. The resulting pY-X-X-P motif creates an intramolecular binding site for the Crk SH2 domain (Figure 8). A similar type of intramolecular interaction has been previously proposed to regulate the activity of Src family kinases (Matsuda *et al.*, 1990; Roussel *et al.*, 1991). c-Abl would thus function as a negative regulator of c-Crk. If this model is correct, uncomplexed c-Crk should be a phosphotyrosyl protein *in vivo*. Our subsequent analysis showed that, consistent with our hypothesis, endogenous c-Crk migrates like a monomeric, non-complexed protein and is tyrosine phosphorylated (Figure 6A and C). Cell fractionation experiments showed that c-Crk colocalized with several, apparently Crk-specific, binding proteins in the S100 fraction, but it was obviously incapable of forming stable complexes with these proteins. On the other hand, bacterially expressed c-Crk, which is not phosphorylated on tyrosine, can form highly stable complexes with these cytosolic proteins through the first SH3 domain. Furthermore, phosphorylation of Y221 by c-Abl in a Crk protein with deleted SH2 domain, increased its binding affinity towards full-length c-Crk at least 150-fold (Figure 6B). c-Crk may be rapidly tyrosine phosphorylated after its translation in cells, inducing intramolecular binding and rendering it inactive to form constitutive signal transduction complexes (Figure 8). Microinjection studies show indeed rapid tyrosine phosphorylation of Crk fusion proteins containing Y221 (unpublished data), supporting this idea. The subsequent formation of Crk complexes with specific cellular proteins during the transduction of cellular signals could result from dephosphorylation of Y221. The Crk-like protein (CRKL; ten Hoeve *et al.*, 1993) could be regulated by the same mechanism proposed for c-Crk, via phosphorylation of CRKL Y207.

The identity and the function of the Ash/Grb2 binding kinase is presently unclear. The binding preference of the Sem-5 SH2 domain for a pY-X-N-X motif (Songyang *et al.*, 1993), which cannot be generated by phosphorylation of Sem-5/Ash/Grb2, as well as the formation of constitutive complexes between Ash/Grb2 and Sos proteins, argues against a regulatory phosphorylation of Ash/Grb2 similar to the one proposed here for c-Crk. Despite the binding of c-Abl and Arg to Ash/Grb2 *in vitro* (Figure 4A), it is unlikely that c-Abl or Arg account for the Ash/Grb2 binding kinase activity, since kinase active immunoprecipitates of c-Abl and Arg show no phosphorylation of Ash/Grb2 (Figure 4B).

v-Crk transforms cells and elevates the tyrosine phosphorylation of specific cellular proteins, while overexpression of c-Crk fails to do so (Reichman, 1992). v-Crk is truncated and lacks c-Crk Y221. It may mimic a constitutively activated c-Crk with the capacity to form stable complexes. This is again reminiscent of the constitutive kinase activation by elimination of the regulatory Y527 in viral Src proteins. v-Crk can stably bind several phosphotyrosine containing proteins via its SH2 domain (Matsuda *et al.*, 1990; Mayer and Hanafusa, 1990b). We report here that the c-Abl kinase also coprecipitates with v-Crk (Figure 7B). c-Abl and/or Arg may play a direct role in the elevation of phosphotyrosyl proteins of v-Crk-transformed cells. Even if c-Abl and Arg are not responsible for the hyperphosphorylation of proteins in v-Crk-transformed cells, they may still play a role in v-Crk transformation. For example, binding of v-Crk could deplete c-Abl and Arg from their normal cellular localization. Depletion of c-Abl from nuclei has been consistently observed in association with transformation by Abl-derived oncoproteins (Van Etten *et al.*, 1989; Wang, 1993). Our cell fractionation analysis of 3Y1 fibroblasts and v-Crk-overexpressing 3Y1 cells shows a clear increase of c-Abl in the crude membrane (P100) fraction of the Crk3Y1 cells, which is probably a consequence of its association with the highly abundant v-Crk protein. Crk3Y1 nuclei have a reduced content of c-Abl, despite the fact that the total c-Abl content in the cells is increased and that significant amounts of v-Crk purify with the nuclear fraction. The c-Abl protein detected in the nuclear fraction of Crk3Y1 cells may be localized outside the nucleus itself, and the intranuclear content of c-Abl may actually be very low.

The interaction of c-Abl with the Crk proteins is intriguing for many reasons. Our data suggest that different classes of signal transduction proteins are negatively regulated by the same principle: phosphorylation dependent, intramolecular binding of SH2 domains. Future experiments will aim to analyse this interaction in more detail and to unravel the signals and processes that lead to the formation of complexes between c-Crk and other signal transduction proteins.

Materials and methods

Cell culture, fractionation and lysis

CEF and c-Crk-overexpressing CEF were prepared as described (Reichman, 1992). 3Y1 rat fibroblasts, v-Crk-transformed 3Y1 cells (Chou *et al.*, 1992), NIH-3T3 mouse fibroblasts, and c-Abl-deficient mouse fibroblasts (clone 1018.3, gift of J.Y.J.Wang) were cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum and antibiotics and

antimycotics (ABAM). Adherent growing HeLaS3 cells were cultured in DMEM with 5% calf serum and ABAM. HeLaS3 spinner culture and preparation of HeLa S100 from spinner culture cells were performed essentially as described (Feller and Wong, 1992). Concentrated HeLa S100 for gel filtration analysis was obtained as described below. For protein extraction from adherent cells, cells were washed three times with chilled cell wash buffer (25 mM Tris−HCl pH 7.5, 135 mM NaCl, 5 mM KCl, 0.7 mM Na_2HPO_4, 5 mM glucose and 1 mM EDTA) and scraped on ice from the plate after addition of chilled radioimmunoprecipitation assay buffer (RIPA buffer) containing 50 mM Tris−HCl pH 7.5, 100 mM NaCl, 5% glycerol (v/v), 2.5 mM EDTA, 1 mM dithiothreitol (DTT), 1% (v/v) Triton X-100, 0.5% (w/v) deoxycholate, 0.1% (w/v) SDS, protease inhibitors (100 kIU/ml aprotonin, 0.5 μg/ml leupeptin, 5 μg/ml antipain-dihydrochloride, 0.7 μg/ml pepstatin) and phosphatase inhibitors (1 mM sodium orthovanadate, 1 mM sodium molybdate and 10 mM sodium fluoride). HeLa spinner cells were pelleted by centrifugation for 10 min at 500 *g*, resuspended in cell wash buffer, pelleted again and homogenized through pipetting with 2 cell volumes of RIPA buffer. Insoluble material was removed by centrifugation at 10 000 *g* for 15 min and supernatants were frozen in aliquots. Cell fractionation of CEF and adherent growing HeLa for analysis of protein phosphorylation and gel filtration chromatography were done as essentially described (Chen *et al.*, 1992), with some modifications. Cells were washed twice with cell wash buffer and twice with hypotonic lysis buffer (HLB) pH 7.5 containing 10 mM Tris−HCl, 1 mM EGTA, 1 mM EDTA, 10 mM KCl, 2 mM $MgCl_2$, 1 mM DTT, and the same protease and phosphatase inhibitors contained in RIPA buffer. Excess buffer was removed, cells were scraped, incubated on ice for 20 min and Dounce homogenized 20 times with a small clearance pestle. For purification of HeLa and rat fibroblast nuclei, the homogenate was diluted with 10 vol of HLB, layered on a cushion of HLB with 1 M sucrose, and centrifuged at 1500 *g* for 10 min. Pelleted nuclei were recovered and lysed in RIPA buffer. CEF nuclei were purified using HLB with 0.5 M sucrose. To isolate concentrated cytosolic proteins (S100) for gel filtration chromatography, the undiluted homogenate was centrifuged for 30 min at 10 000 *g*. The supernatant (S10) was recentrifuged at 100 000 *g* and the S100 recovered. The crude membrane fraction (P100) was obtained in the same way, except for the use of the diluted supernatant from the purification of nuclei.

Expression and purification of gst fusion proteins

The expression vectors for gst, full-length gst-c-Crk, gst-v-Crk SH2, gst-v-Crk SH2SH3, gst-Gag-Crk (gst-v-Crk), gst-Nck ΔSH3(1), gst-Ash/Grb2, gst-Src SH3 and gst-Abl SH3 proteins have been described elsewhere (Birge *et al.*, 1992, 1993; Chou *et al.*, 1992; Cicchetti *et al.*, 1992; Matuoka *et al.*, 1992). The murine c-*crk* cDNA used for the murine gst-c-Crk fusion protein was a gift of Hisamura Hira (University of Tokyo, Tokyo, Japan). Gst-c-Crk SH3SH3 was obtained by cloning an *Msc*I−PvuII fragment (amino acids 120−304) into pGEX-3X. Gst-c-Crk SH3(N) was made by cloning an *Msc*I−*Hinc*II fragment (amino acids 120−225) into pGEX-1N. A *Hinc*II−*Hinc*II fragment (amino acids 227−304) was cloned into pGEX-3X for the gst-c-Crk SH3(C) protein. The expression vectors for gst fusion proteins of Ash/Grb2 and bovine PLC-γ SH3 were gifts from Tadaomi Takenawa (University of Tokyo, Tokyo, Japan), and the vectors for gst-Src SH3, gst-Abl SH3 and gst-GAP SH3 gifts of David Baltimore (Rockefeller University). Gst-Csk SH3, containing amino acids 14−68 of chicken Csk (Sabe *et al.*, 1992a), was kindly provided by Hisataka Sabe from our laboratory. The expression vector for gst-spectrin SH3 (Wasenius *et al.*, 1989) was a gift of Veli-Pekka Lehto (University of Oulu, Oulu, Finland). Purification of the gst fusion proteins was done as described (Smith and Johnson, 1988), and glutathione was removed by dialysis against 10 mM Tris−HCl pH 7.5.

Protein and peptide analysis

The purity and integrity of bacterially expressed gst fusion proteins was assessed by SDS−PAGE (Laemmli, 1970) and Coomassie Blue staining of the proteins. Protein purity was mostly >95%. Exceptions were the gst-c-Crk SH3 (C) protein, which was always seen as a protein doublet, and gst-PLC-γ SH3 and gst-Gag-V-Crk, which contained significant amounts of truncated fragments. Protein quantification was done as described (Bradford, 1976), using bovine serum albumin as standard. Peptides were analysed with modified SDS−PAGE (20% separating gel containing 10% glycerol and Tris−HCl pH 9.3). 'Rainbow Markers' (Amersham) and bromophenol blue were used as migration standards. Alkaline treatment of gels was done with 1 M NaOH at 55°C for 2 h. Gel filtration chromatography was done with gravity flow (2 ml/h) on a column packed with 20 ml Superose 12 (Pharmacia). Column buffer was 20 mM Tris−HCl pH 7.5, 100 mM NaCl, 1 mM EDTA, 1 mM DTT, phosphatase inhibitors as in RIPA buffer, and protease inhibitors at one-tenth of the concentration used in RIPA buffer. Gel filtration standards were from Pharmacia.

Proteins precipitations, kinase assays, phosphoamino acid analysis and Western blots

For precipitations with gst fusion proteins 3 μg of gst, or equimolar amounts of other fusion proteins, and 15 μl glutathione−Sepharose beads were used unless indicated differently. 500 μg of total proteins, or proteins from cell fractions, were used unless indicated otherwise. Proteins were incubated with 0.5 ml precipitation buffer containing 50 mM Tris−HCl pH 7.5, 100 mM NaCl, 1 mM EDTA, 0.1% Tween 20, 1% (w/v) ovalbumin, 1 mM DTT, 10 μM sodium molybdate and protease inhibitors, in a total volume of 0.6−0.8 ml. After precipitation overnight at 4°C, samples were washed three times with 1 ml RIPA buffer (without inhibitors) and once with 1 ml kinase buffer containing 50 mM Tris−HCl pH 7.5, 100 mM NaCl, 0.1% (v/v) β-mercaptoethanol, 0.1% Tween 20, 5 mM $MnCl_2$ and 10 μM sodium molybdate. Kinase reactions were usually done at 25°C for 30 min with 2−5 μCi [γ-^{32}P]ATP (3000 Ci/mmol). 5 μg of purified histones (Boehringer), 10 μg of histone type IIIs (Sigma), 5 μg of gst-c-Crk 120−225, or equimolar concentrations of other gst fusion proteins, were added as exogenous substrates as indicated. Autoradiography of kinase assays was for 1−2 h. Phosphoamino acid analysis was done as described (Feller and Wong, 1992). For precipitation of c-Crk with Y221-phosphorylated gst-c-CrkSH3SH3, 10 μg of gst-c-Crk 120−304 was incubated with 2 mg of HeLa S100 as before and phosphorylated with 25 μM unlabelled ATP for 5 h. Controls were incubated with 5 mM EDTA instead of 5 mM $MnCl_2$. Samples were then washed three times with RIPA buffer and twice with PBS and incubated with 1 μg of c-Crk which had been cleaved from gst with Factor Xa (Boehringer), at 4°C overnight in precipitation buffer containing 1 mg/ml heat inactivated, dialysed total *E.coli* proteins and phosphatase inhibitors (100 μM). Precipitates were washed again with RIPA buffer as before and then subjected to SDS−PAGE and Western blot. Polyclonal antisera made against v-Crk (Mayer and Hanafusa, 1990), the second SH3 domain of murine c-Crk, or Arg (gift of Stuart A.Aaronson) were immobilized on protein A−Sepharose, monoclonal antibody for Abl (Ab-3; Oncogene Science) was immobilized on protein G−Sepharose. The same immunoreagents were also used for Western blots. The monoclonal antibody against Gag has been described (Potts *et al.*, 1987). Immunoprecipitations were done from 400 μg of proteins unless indicated otherwise. For Western blot detection of the primary antibodies ^{125}I-labelled protein A, or an ^{125}I-labelled antiserum specific for mouse immunoglobulins (Amersham), was used.

Determination of protein phosphorylation sites

1 mg of purified, immobilized fusion protein gst-c-Crk 120−225 was incubated with 50 mg of HeLa S100 proteins, washed and subjected to an *in vitro* kinase reaction with 25 μM ATP (^{31}P:^{32}P = 400:1) for 5 h at 25°C. The fusion protein was separated from ATP and other proteins by preparative SDS−PAGE (10%). All of the purification steps were controlled by following the radioactive tracer phosphate. The radioactive band was eluted from the gel for 4 × 12 h with 10 ml each of 10 μM sodium molybdate on a shaker. Eluates were pooled and the volume was reduced by lyophilization to 5 ml. Non-denaturing conditions were achieved by adding 3 ml of RIPA buffer without SDS or inhibitors and pH was adjusted to 7 with 1 M HCl. The phosphorylated fusion protein was isolated by affinity chromatography on anti-phosphotyrosine agarose (monoclonal antibody PY20; ICN, Irvine, CA). 100 μl affinity resin was incubated with the eluted protein overnight at 4°C, then washed thoroughly with RIPA buffer and PBS. Elution was for 3 × 12 h at 4°C with 1 mM *o*-phospho-L-tyrosine (Sigma) in 20 mM Tris pH 8.3. The eluate was lyophilized, subjected to SDS−PAGE and electrotransferred to PVDF membrane. The immobilized protein was cleaved with *Staphyloccocus aureus* V8 protease in the presence of hydrogenated Triton X-100 as essentially described (Fernandez *et al.*, 1992). The inclusion of 100 μM sodium orthovanadate and sodium molybdate was sufficient to overcome initial dephosphorylation problems during proteolysis. Membrane-released proteolytic fragments were separated by C_{18} reversed phase HPLC with a 2−60% acetonitrile gradient. 200 fractions were collected and analysed by Cerenkov counting and SDS−PAGE (20%, pH 9.3). A single radioactive peak was obtained and subjected to amino acid sequencing as described (Fernandez *et al.*, 1992).

Acknowledgements

We thank Stuart A.Aaronson, David Baltimore, Hisamaru Hirai, Veli-Pekka Lehto, Hisataka Sabe, Tadaomi Takenawa, Victor L.J.Tybulewicz and Jean Y.J.Wang for their generous gifts of reagents. We are also grateful for discus-

sions with Tai Wai Wong, to Jean Y.J.Wang for providing information prior to publication, and to the Protein Sequence Facility of the Rockefeller University for help in the determination of phosphorylation sites. This study was supported by grant VM2 from the American Cancer Society, grant CA44356 and training grant CA09673-17 from the National Cancer Institute.

References

Bar-Sagi,D., Rotin,D., Batzer,A., Mandiyan,V. and Schlessinger,J. (1993) *Cell*, **74**, 83–91.

Baskaran,R., Dahmus,M.E. and Wang,J.Y.J. (1993) *Proc. Natl Acad. Sci. USA*, **90**, 11167–11171.

Birge,R.B., Fajardo,J.E., Mayer,B.J. and Hanafusa,H. (1992) *J. Biol. Chem.*, **267**, 10588–10595.

Birge,R.B., Fajardo,J.E., Reichman,C., Shoelson,S.E., Songyang,Z., Cantley,L. and Hanafusa,H. (1993) *Mol. Cell. Biol.*, **13**, 4648–4656.

Bishop,J.M. (1991) *Cell*, **64**, 235–248.

Bradford,M.M. (1976) *Anal. Biochem.*, **72**, 248–254.

Buday,L. and Downward,J. (1993) *Cell*, **73**, 611–620.

Cantley,L.C., Auger,K.R., Carpenter,C., Duckworth,B., Graziani,A., Kapeller,R. and Soltoff,S. (1991) *Cell*, **64**, 281–302.

Chardin,P., Camonis,J.H., Gale,N.W., Van Aelst,L., Schlessinger,J., Wigler,M.H. and Bar-Sagi,D. (1993) *Science*, **260**, 1338–1343.

Chen,R.-H., Sarnecki,C. and Blenis,J. (1992) *Mol. Cell. Biol.*, **12**, 915–927.

Chou,M.M., Fajardo,J.E. and Hanafusa,H. (1992) *Mol. Cell. Biol.*, **12**, 5834–5842.

Cicchetti,P., Mayer,B.J., Thiel,G. and Baltimore,D. (1992) *Science*, **257**, 803–806.

Clark,S., Stern,M.J. and Horvitz,H.R. (1992) *Nature*, **356**, 340–344.

Daley,G.Q. and Ben-Neriah,Y. (1991) *Adv. Cancer Res.*, **57**, 151–184.

Duchesne,M., Schweighoffer,F., Parker,F., Clerk,F., Frobert,Y., Thang,M.N. and Tocque,B. (1993) *Science*, **259**, 525–528.

Egan,S.E., Giddings,B.W., Brooks,M.W., Buday,L., Sizeland,A.M. and Weinberg,R.A. (1993) *Nature*, **363**, 45–51.

Feller,S.M. and Wong,T.W. (1992) *Biochemistry*, **31**, 3044–3051.

Fernandez,J., DeMott,M., Atherton,D. and Mische,S.M. (1992) *Anal. Biochem.*, **201**, 255–264.

Gale,N.W., Kaplan,S., Lowenstein,E.J., Schlessinger,J. and Bar-Sagi,D. (1993) *Nature*, **363**, 88–92.

Gervais,F.G., Chow,L.M.L., Lee,J.M., Branton,P.E. and Veillette,A. (1993) *Mol. Cell. Biol.*, **13**, 7112–7121.

Gout,I. *et al.* (1993) *Cell*, **75**, 25–36.

Koch,C.A., Anderson,D., Moran,M.F., Ellis,C. and Pawson,T. (1991) *Science*, **252**, 668–674.

Kruh,G.D., Perego,R., Miki,T. and Aaronson,S.A. (1990) *Proc. Natl Acad. Sci. USA*, **87**, 5802–5806.

Laemmli,U.K. (1970) *Nature*, **227**, 680–685.

Lehmann,J.M., Riethmuller,G. and Johnson,J.P. (1990) *Nucleic Acids Res.*, **18**, 1048.

Li,N., Batzer,A., Daly,R., Yajnik,V., Skolnik,E., Chardin,P., Bar-Sagi,D., Margolis,B. and Schlessinger,J. (1993) *Nature*, **363**, 85–88.

Liu,X., Brodeur,S.R., Gish,G., Songyang,Z., Cantley,L.C., Laudano,A.P. and Pawson,T. (1993) *Oncogene*, **8**, 1119–1126.

Lowenstein,E.J., Daly,R.J., Batzer,A.G., Li,W., Margolis,B., Lammers,R., Ullrich,A., Skolnik,E.Y., Bar-Sagi,D. and Schlessinger,J. (1992) *Cell*, **70**, 431–442.

Matsuda,M., Mayer,B.J., Fukui,Y. and Hanafusa,H. (1990) *Science*, **248**, 1537–1539.

Matsuda,M., Mayer,B. and Hanafusa,H. (1991) *Mol. Cell. Biol.*, **11**, 1607–1613.

Matsuda,M., Reichman,C.T. and Hanafusa,H. (1992a) *J. Virol.*, **66**, 115–121.

Matsuda,M., Tanaka,S., Nagata,S., Kojima,A., Kurata,T. and Shibuya,M. (1992b) *Mol. Cell. Biol.*, **12**, 3482–3489.

Matuoka,K., Shibata,M., Yamakawa,A. and Takenawa,T. (1992) *Proc. Natl Acad. Sci. USA*, **89**, 9015–9019.

Mayer,B.J. and Baltimore,D. (1993) *Trends Cell Biol.*, **3**, 8–13.

Mayer,B.J. and Hanafusa,H. (1990a) *J. Virol.*, **64**, 3581–3589.

Mayer,B.J. and Hanafusa,H. (1990b) *Proc. Natl Acad. Sci. USA*, **87**, 2638–2642.

Mayer,B.J., Hamaguchi,M. and Hanafusa,H. (1988) *Nature*, **332**, 272–275.

Musacchio,A., Gibson,T., Lehto,V.-P. and Saraste,M. (1992) *FEBS Lett.*, **307**, 55–61.

Olivier,J.P., Raabe,T., Henkemeyer,M., Dickson,B., Mbamalu,G., Margolis,B., Schlessinger,J., Hafen,E. and Pawson,T. (1993) *Cell*, **73**, 179–191.

Pendergast,A.M. *et al.* (1993) *Cell*, **75**, 175–185.

Potts,W.M., Olsen,M., Boettiger,D. and Vogt,V.M. (1987) *J. Gen. Virol.*, **68**, 3177–3182.

Prasad,K.V.S., Janssen,O., Kappeller,R., Raab,M., Cantley,C.L. and Rudd,C.E. (1993) *Proc. Natl Acad. Sci. USA*, **90**, 7366–7370.

Reichman,C.T. (1992) Ph.D. Thesis. The Rockefeller University, New York.

Reichman,C.T., Mayer,B.J., Keshav,S. and Hanafusa,H. (1992) *Cell Growth Differen.*, **3**, 451–460.

Ren,R., Mayer,B.J., Cicchetti,P. and Baltimore,D. (1993) *Science*, **259**, 1157–1161.

Rosenberg,N. and Witte,O.N. (1988) *Adv. Virus Res.*, **35**, 39–81.

Roussel,R.R., Brodeur,S.R., Shalloway,D. and Laudano,A.P. (1991) *Proc. Natl Acad. Sci. USA*, **88**, 10696–10700.

Rozakis-Adcock,M., Fernley,R., Wade,J., Pawson,T. and Bowtell,D. (1993) *Nature*, **363**, 83–85.

Sabe,H., Knudsen,B., Okada,M., Nada,S., Nakagawa,H. and Hanafusa,H. (1992a) *Proc. Natl Acad. Sci. USA*, **89**, 2190–2194.

Sabe,H., Okada,M., Nakagawa,H. and Hanafusa,H. (1992b) *Mol. Cell. Biol.*, **12**, 4706–4713.

Simon,M.A., Dodson,G.S. and Rubin,G.M. (1993) *Cell*, **73**, 169–177.

Smith,D.B. and Johnson,K.S. (1988) *Gene*, **67**, 31–40.

Songyang,Z. *et al.* (1993) *Cell*, **72**, 767–778.

Tanaka,S. *et al.* (1994) *Proc. Natl Acad. Sci. USA*, in press.

ten Hoeve,J., Morris,C., Heisterkamp,N. and Groffen,J. (1993) *Oncogene*, **8**, 2469–2474.

Tsuchie,H., Chang,C.H.W., Yoshida,M. and Vogt,P.K. (1989) *Oncogene*, **4**, 1281–1284.

Tybulewicz,V.L.J., Crawford,C.E., Jackson,P., Bronson,R.T. and Mulligan,R.C. (1991) *Cell*, **65**, 1153–1163.

Van Etten,R.A., Jackson,P. and Baltimore,D. (1989) *Cell*, **58**, 669–678.

Waksman,G. *et al.* (1992) *Nature*, **358**, 646–653.

Wang,J.Y.J. (1993) *Curr. Opin. Genet. Dev.*, **3**, 35–43.

Wang,J.Y.J. and Baltimore,D. (1985) *J. Biol. Chem.*, **260**, 64–71.

Wasenius,V.-M., Saraste,M., Salven,P., Eraemaa,M., Holm,L. and Lehto,V.-P. (1989) *J. Cell Biol.*, **108**, 79–93.

Weng,Z., Taylor,J.A., Turner,C.E., Brugge,J.S. and Seidel-Dugan,C. (1993) *J. Biol. Chem.*, **268**, 14956–14963.

Received on December 15, 1993; revised on February 15, 1994