Bcr-Abl oncoproteins bind directly to activators of the Ras signalling pathway

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The cytosolic 185 and 210 kDa Bcr-Abl protein tyrosine kinases play important roles in the development of Philadelphia chromosome positive (Ph⁺) chronic myelogenous leukemia (CML) and acute lymphoblastic leukemia (Ph⁺ ALL). p185 and p210 Bcr-Abl contain identical *abl*-encoded sequences juxtaposed to a variable number of *bcr*-derived amino acids. As the mitogenic and transforming activities of tyrosine kinases involve stimulation of the Ras pathway, we analyzed Bcr-Abl oncoproteins for interactions with cytoplasmic proteins that mediate Ras activation. Such polypeptides include Grb2, which comprises a single Src homology 2 (SH2) domain flanked by two SH3 domains, and the 66, 52 and 46 kDa Shc proteins which possess an SH2 domain in their carboxy-terminus. Grb2 associates with tyrosine phosphorylated proteins through its SH2 domain, and with the Ras guanine nucleotide releasing protein mSos1 through its SH3 domains. mSos1 stimulates conversion of the inactive GDP-bound form of Ras to the active GTP-bound state. In *bcr-abl*-transformed cells, Grb2 and mSos1 formed a physical complex with Bcr-Abl. *In vitro*, the Grb2 SH2 domain bound Bcr-Abl through recognition of a tyrosine phosphorylation site within the amino-terminal *bcr*-encoded sequence (p.Tyr177-Val-Asn-Val), that is common to both Bcr-Abl proteins. These results suggest that autophosphorylation within the Bcr element of Bcr-Abl creates a direct physical link to Grb2-mSos1, and potentially to the Ras pathway, and thereby modifies the target specificity of the Abl tyrosine kinase. Bcr-Abl tyrosine kinases also phosphorylate Shc proteins on tyrosine, inducing the formation of an Shc-Grb2 complex that also has the potential to stimulate Ras. Bcr-Abl oncoproteins may therefore couple to the Ras pathway through the formation of multiple Grb2 complexes.

Key words: Bcr-Abl/Grb2/leukemia/Ras/Sos

Introduction

Many of the extracellular signals that normally regulate the proliferation and differentiation of hematopoietic cells activate transmembrane or receptor-associated cytoplasmic protein tyrosine kinases, which in turn transiently activate intracellular signalling pathways (Veillette *et al.*, 1989; Burkhardt *et al.*, 1991; Miura *et al.*, 1991; Rottapel *et al.*, 1991; Sherr, 1991; Grinstein and Furuya, 1992; Torigoe *et al.*, 1992; Wang *et al.*, 1992; Hanazono *et al.*, 1993; Kolanus *et al.*, 1993). Gain-of-function mutations within genes encoding tyrosine kinases can lead to constitutive activation of their products, and thereby induce aberrant proliferation and neoplastic transformation. A well-characterized example in the hematopoietic system involves the rearrangements of the *bcr* and *abl* genes in Philadelphia chromosome positive (Ph⁺) chronic myelogenous leukemia (CML) and acute lymphocytic leukemia (Ph⁺ ALL), resulting in the formation of chimeric *bcr-abl* oncogenes that are strongly implicated in the etiology of these leukemias (Konopka *et al.*, 1984; Shtivelman *et al.*, 1985; Mes-Masson *et al.*, 1986; Clark *et al.*, 1987; Daley *et al.*, 1987; Hermans *et al.*, 1987; Elefanty *et al.*, 1990; Heisterkamp *et al.*, 1990; Kelliher *et al.*, 1990; Gishizky *et al.*, 1993). Depending on the precise breakpoint within the *bcr* gene, fusion proteins of 210 kDa (p210) or 185 kDa (p185) are produced, which localize to the cytoplasm, and possess deregulated Abl tyrosine kinase activity (Konopka *et al.*, 1984; Mes-Masson *et al.*, 1986; Clark *et al.*, 1987; Hermans *et al.*, 1987; Lugo *et al.*, 1990; Dhut *et al.*, 1991; McWhirter and Wang, 1991). p210 and p185 Bcr-Abl contain identical *abl*-derived sequences but differ in the number of *bcr*-encoded amino acid residues, containing the amino-terminal 927 (or 902) or 426 amino acids of Bcr respectively. The tyrosine kinase activity of the Bcr-Abl proteins strongly correlates with their transforming potential in tissue culture, and is higher for the 185 kDa form which is characteristically associated with acute leukemias (McLaughlin *et al.*, 1989; Lugo *et al.*, 1990; Kelliher *et al.*, 1991).

Understanding the mechanisms by which Bcr-Abl proteins elicit their biological effects requires the identification of their intracellular targets. A large number of tyrosine kinase targets are characterized by conserved non-catalytic Src homology 2 (SH2) domains, sequences of ~95 amino acids that bind with high affinity to specific phosphotyrosine-containing sites such as those found on activated receptor tyrosine kinases, and thereby direct protein-protein interactions that regulate cytoplasmic signal transduction pathways (reviewed in Cantley *et al.*, 1991; Koch *et al.*, 1991; Pawson and Schlessinger, 1993). SH2 domains are often located adjacent to a distinct non-catalytic motif, the Src homology 3 (SH3) domain, which also functions in protein-protein interactions (reviewed in Musacchio *et al.*, 1992; Pawson and Gish, 1992; Pawson and Schlessinger,

1993). Some of these SH2/SH3-containing proteins also possess intrinsic catalytic domains which may be directly regulated by tyrosine phosphorylation while others lack obvious catalytic sequences and function as adaptor molecules to create multi-protein signalling complexes. In the latter category are two recently described proteins encoded by the *grb2* and *shc* genes (Lowenstein *et al.*, 1992; Pelicci *et al.*, 1992).

Grb2, the mammalian homolog of *Caenorhabditis elegans* Sem-5 and *Drosophila* Drk, is comprised entirely of a single SH2 domain flanked by two SH3 domains (Lowenstein *et al.*, 1992). Genetic evidence from *C. elegans* and *Drosophila* indicates that Sem-5 and Drk are required for activation of the Ras pathway by receptor tyrosine kinases (Clark *et al.*, 1992; Olivier *et al.*, 1993; Simon *et al.*, 1993). In mammalian cells, Grb2 directly couples a subset of phosphorylated receptor tyrosine kinases, which it binds with its SH2 domain (Lowenstein *et al.*, 1992; Rozakis-Adcock *et al.*, 1992; Buday and Downward, 1993) to mSos1, a Ras guanine nucleotide releasing protein (GNRP) (Bowtell *et al.*, 1992; Rozakis-Adcock *et al.*, 1992, 1993; Buday and Downward, 1993; Chardin *et al.*, 1993; Egan *et al.*, 1993; Li *et al.*, 1993). The SH3 domains of Grb2 associate with proline-rich motifs in the carboxy-terminal tail of mSos1 (Chardin *et al.*, 1993; Li *et al.*, 1993; Rozakis-Adcock *et al.*, 1993) allowing the association of mSos1 and Ras-GNRP activity with autophosphorylated epidermal growth factor (EGF) receptors (Gale *et al.*, 1993). These interactions apparently stimulate conversion of inactive, GDP-bound Ras to the active, GTP-bound form (Gale *et al.*, 1993). Although Grb2 associates with activated tyrosine kinases, it is not an efficient substrate for tyrosine phosphorylation in either *Drosophila* or mammalian cells (Lowenstein *et al.*, 1992; Rozakis-Adcock *et al.*, 1992; Olivier *et al.*, 1993). Hence, Grb2 function is apparently regulated by the inducible binding of its SH2 domain to phosphotyrosine sites.

The mammalian *shc* gene encodes three overlapping proteins of 46, 52 and 66 kDa which possess a carboxy-terminal SH2 domain, a more central glycine/proline-rich region and variable amounts of amino-terminal sequence (Pelicci *et al.*, 1992). p46^{shc} and p52^{shc} arise by differential use of translation initiation sites within a 3.4 kb *shc* mRNA, and are expressed in all mammalian cells that have been analyzed. A minor and variably expressed 66 kDa protein is encoded by a distinct *shc* transcript. Overexpression of p46^{shc} and p52^{shc} induces transformation of mouse fibroblasts (Pelicci *et al.*, 1992) and activates the Ras pathway in PC12 cells (Rozakis-Adcock *et al.*, 1992), suggesting that Shc proteins might normally be involved in mitogenic signalling. Shc proteins are phosphorylated on tyrosine in response to growth factors such as EGF and insulin (Pelicci *et al.*, 1992; Pronk *et al.*, 1993), and in cells transformed by the v-Src or v-Fps tyrosine kinases (McGlade *et al.*, 1992), and concomitantly bind the SH2 domain of the Grb2 protein. Grb2 and Shc are thus implicated in the control of Ras by tyrosine kinases in a number of different biological systems.

The activation of p21^{ras} appears critical for the induction of DNA synthesis and morphological transformation by oncogenic tyrosine kinases (Mulcahy *et al.*, 1985; Smith *et al.*, 1986; Feig and Cooper, 1988). Consistent with this notion, expression of p210 Bcr-Abl in murine myeloid cells induces the accumulation of GTP-bound Ras (Mandanas

et al., 1993). We have therefore analyzed Bcr-Abl proteins for interactions with cytoplasmic proteins that mediate Ras activation. Our data indicate that the amino-terminal, *bcr*-encoded sequence of Bcr-Abl contains a tyrosine phosphorylation site that binds Grb2, suggesting that Bcr sequences contribute to the interaction of Bcr-Abl proteins with their targets.

Results

Grb2 forms a stable complex with Bcr-Abl oncoproteins in vivo

Fibroblasts transformed by p185 or p210 Bcr-Abl possess a variety of phosphotyrosine-containing proteins (Lugo *et al.*, 1990) which might potentially associate with the Grb2 SH2 domain and thereby stimulate the Ras pathway. To test whether Grb2 interacts with phosphoproteins in *bcr-abl*-transformed cells, anti-Grb2 immunoprecipitates from Rat-1 (R1) fibroblasts transformed by expression of p185 Bcr-Abl (R1-p185) were immunoblotted with antibodies to phosphotyrosine. Multiple phosphoproteins were detected in association with Grb2 (Figure 1). One prominent phosphotyrosine-containing protein that co-precipitated with Grb2 had the same mobility as p185 Bcr-Abl, suggesting that Grb2 and Bcr-Abl proteins might form a stable complex *in vivo*. To investigate this possibility, anti-Grb2 immunoprecipitates from lysates of R1-p185 cells, or R1 fibroblasts expressing p210 Bcr-Abl (R1-p210), were probed with anti-Abl antibody. Both forms of Bcr-Abl were directly identified as co-precipitating with Grb2 (Figure 2A). In the reciprocal experiment, Grb2 was found to be associated with Bcr-Abl precipitated with anti-Abl antibodies (Figure 2B). Similar experiments using the CML K562 and the Ph⁺ ALL SUP-B15 cell lines, which express p210 and p185 Bcr-Abl respectively (Lozzio and Lozzio, 1975; Naumovski *et al.*, 1988), also demonstrated the co-precipitation of Grb2 and Bcr-Abl (data not shown). In repeated experiments, we have

Fig. 1. Grb2 associates with multiple phosphoproteins in *bcr-abl*-transformed cells. Lysates of R1 fibroblasts expressing p185 Bcr-Abl were immunoprecipitated with anti-Abl or anti-Grb2 antibodies as indicated, and the immune complexes were subsequently blotted with anti-phosphotyrosine antibodies. The positions of p185 Bcr-Abl and p52^{shc} are indicated by arrows, and the mobilities of size markers are shown.

not observed consistent differences in the fraction of Grb2 bound to either isoform of Bcr-Abl in R1 cells.

The association of Grb2 with the EGF receptor is strictly dependent on receptor autophosphorylation (Lowenstein *et al.*, 1992; Buday and Downward, 1993; Rozakis-Adcock *et al.*, 1993). To assess the potential role of Bcr-Abl autophosphorylation in the interaction with Grb2, anti-Grb2 immunoprecipitates from R1-p185 cells were treated with potato acid phosphatase, prior to immunoblotting with anti-Abl antiserum. Phosphatase treatment diminished the association of Bcr-Abl with Grb2, indicating that recognition of a Bcr-Abl phosphorylation site by Grb2 is likely to contribute to this interaction (data not shown).

The Grb2 SH2 domain is sufficient to bind Bcr-Abl oncoproteins *in vitro*

The region of Grb2 responsible for its association with p210 and p185 Bcr-Abl was further analyzed by reconstitution of these interactions *in vitro*. Full-length Grb2 was expressed in bacteria as a glutathione-S-transferase (GST) fusion protein, and isolated by chromatography on glutathione-Sephharose beads. Immobilized GST-Grb2 bound specifically to p210 Bcr-Abl (Figure 3) or p185 Bcr-Abl (data not shown) when incubated with lysates of R1-p210 or R1-p185 cells. By utilizing a GST fusion protein containing the isolated SH2 domain of Grb2, we found that the Grb2 SH2 domain alone was sufficient to form a stable complex with Bcr-Abl (Figure 3).

Co-precipitation of Bcr-Abl with Grb2 may be the consequence of a direct interaction between the two proteins, or may require an intermediary molecule. To determine

whether the Grb2 SH2 domain is capable of directly recognizing Bcr-Abl proteins, a filter binding assay was performed (Mayer *et al.*, 1991). Bcr-Abl and endogenous c-Abl proteins were immunoprecipitated from R1, R1-p185 and R1-p210 cells, subjected to SDS-PAGE and transferred to nitrocellulose. Replica filters were incubated either with a bacterial lysate containing the GST-Grb2 SH2 fusion protein or with GST alone. Binding of the GST proteins to the immobilized Abl proteins was detected by incubation of the filters with anti-GST antibodies followed by 125 I-labelled protein A. In this assay, GST-Grb2 SH2 bound to p210 and p185 Bcr-Abl but not c-Abl, whereas GST alone was incapable of binding to any of the Abl proteins (Figure 4). These results indicate that the SH2 domain of Grb2 directly binds to denatured Bcr-Abl proteins.

Tyr177 in Bcr-Abl is an autophosphorylation site recognized by the Grb2 SH2 domain

SH2 domains bind with high affinity to tyrosine phosphorylated ligands (Cantley *et al.*, 1991; Escobedo

Fig. 2. Bcr-Abl oncoproteins form complexes with Grb2 *in vivo*. Lysates of R1-p185 cells (lanes 1–3) or R1-p210 cells (lanes 4–6) were immunoprecipitated with anti-Abl or anti-Grb2 antiserum or normal rabbit serum (C). Duplicate samples were subsequently immunoblotted with anti-Abl antibodies (A) or anti-Grb2 antibodies (B). The positions of the Abl and Grb2 proteins are indicated with arrows.

Fig. 3. The SH2 domain of Grb2 binds Bcr-Abl *in vitro*. Bacterially expressed GST fusion proteins containing full-length Grb2, the Grb2 SH2 domain alone or either the amino- or carboxy-terminal Grb2 SH3 domains alone, were immobilized and incubated with lysates of R1-p210 cells. GST was included as a negative control. Proteins bound to the immobilized fusion proteins were recovered and immunoblotted with anti-Abl antibodies. An anti-Abl immunoprecipitate was included, and the position of p210 Bcr-Abl is depicted by an arrow. Association of Bcr-Abl with either Grb2 SH3 domain was detected on longer exposure.

Fig. 4. The Grb2 SH2 domain directly recognizes Bcr-Abl proteins but not c-Abl. Abl proteins were immunoprecipitated from R1, R1-p185 and R1-p210 cells, separated by gel electrophoresis and transferred to nitrocellulose. Replica filters were incubated with bacterial lysates containing GST alone (GST) or GST-Grb2 SH2 (Grb2 SH2) fusion proteins as indicated. The filters were subsequently incubated with anti-GST antibodies and probed with 125 I-labelled protein A. An identical filter was also blotted with anti-Abl antibodies to indicate the amount of c-Abl or Bcr-Abl protein in each sample. The positions of p145^{c-abl}, p185 Bcr-Abl and p210 Bcr-Abl are indicated.

Fig. 5. Tyr177 in p210 Bcr-Abl is an autophosphorylation site recognized by the Grb2 SH2 domain, and undergoes transphosphorylation in p160 Bcr. (a) Two-dimensional tryptic/V8 protease map of p210 Bcr-Abl. p210 Bcr-Abl was immunoprecipitated from K562 cells, labelled in an *in vitro* kinase assay using [γ - 32 P]ATP, and digested with trypsin followed by V8 protease. Peptides were separated by two-dimensional electrophoresis and chromatography. Previously reported tryptic phosphopeptides are identified by number (Liu *et al.*, 1993), and peptides produced by the action of both trypsin and V8 protease are identified by capital letters. The previously reported tryptic peptide #10 (Liu *et al.*, 1993) probably contains peptide L. (b) Depletion of peptides H and L by immobilized Grb2 SH2 fusion proteins. The trypsin/V8 double digest of p210 Bcr-Abl shown in (a) was mixed with immobilized GST fusion proteins containing the Grb2 SH2 domain, and peptides remaining in the supernatant were separated by two-dimensional electrophoresis and chromatography. Comparison with (a) indicates selective depletion of peptides H and L. (c) Two-dimensional tryptic/V8 protease map of p160 Bcr transphosphorylated by p210 Bcr-Abl. p160 Bcr immunoprecipitated from K562 cells was transphosphorylated *in vitro*, digested with trypsin and V8 protease, and the peptides were resolved by two-dimensional electrophoresis and chromatography. (d) Two-dimensional tryptic/V8 protease digest of p185 Bcr-Abl. p185 Bcr-Abl was immunoprecipitated from SUP-B15 cells, and analyzed as outlined in (a). (e and f) Elution positions of 32 P-labelled phosphotyrosine by manual Edman degradation of peptides H (e) and L (f). The counts corresponding to each cycle number are the c.p.m. remaining after the cycle.

et al., 1991; Songyang *et al.*, 1993). The specificity of ligand binding is largely dictated by the three residues immediately carboxy-terminal to the phosphotyrosine. These residues, together with the phosphorylated tyrosine, can make direct contact with the SH2 domain (Eck *et al.*, 1993; Waksman *et al.*, 1993). When employed to screen a degenerate phosphopeptide library, the Sem-5 SH2 domain showed a strong preference for Asn at the +2 position, two residues carboxy-terminal to phosphotyrosine (Songyang *et al.*, 1993). Indeed, the three known Grb2 SH2-binding sites, located on Shc, the EGF receptor and IRS-1, all have Asn at the +2 position (Buday and Downward, 1993; Pawson and Schlessinger, 1993; Skolnik *et al.*, 1993; J.McGlade, G.Pellicci, T.Pawson and P.G.Pellicci, unpublished results). Bcr-Abl proteins contain a single potential Grb2-binding site, Tyr177-Val-Asn-Val, identical to that found in the Shc protein. Remarkably, this site is located within the *bcr*-encoded sequence that is common to both Bcr-Abl proteins. Bcr-Abl has been previously shown to autophosphorylate *in vitro* and *in vivo* on tyrosine residues within the amino-terminal Bcr sequence (Liu *et al.*, 1993; Lu *et al.*, 1993). To test which Bcr-Abl phosphopeptides are recognized by Grb2, p210 Bcr-Abl was immunoprecipitated from K562 cells, autophosphorylated in an *in vitro* kinase assay using [γ - 32 P]ATP, and digested with trypsin followed by V8 protease. The proteolytic digest was then incubated with immobilized GST-Grb2 SH2 or Abl SH2 fusion proteins. The immobilized fusion proteins and any associated phosphopeptides were removed by centrifugation, and the remaining peptides were analyzed by two-dimensional electrophoresis and chromatography. Two phosphorylated peptides, H and L, were specifically depleted from the Bcr-Abl trypsin/V8 protease digest by incubation with immobilized GST-Grb2 SH2 fusion proteins (Figure 5a and b) but not by GST-Abl SH2 domains (data not shown). Phosphoamino acid analysis of peptides H and L indicated that they contained phosphotyrosine, but not phosphothreonine or phosphoserine (data not shown). Manual Edman degradation of peptides H and L (partial V8) placed the phosphotyrosine at cycle numbers 14 and 15 respectively (Figure 5e and f). In conjunction with the mobilities of the peptides in two-dimensional electrophoresis and chromatography, these results suggested the following peptide sequences: peptide H, GHGQPGADAEKP Fp.Y¹⁷⁷VNVE; peptide L, KGHGQPGADAEKPFp.Y¹⁷⁷VNVE. The underlined Glu (E) appeared resistant to cleavage by V8 protease. These results indicate that Tyr177 is indeed phosphorylated, and that peptides containing Tyr177 are specifically recognized by the Grb2 SH2 domain. Tyr177 is also transphosphorylated in p160 Bcr (Figure 5c) which forms stable complexes with Bcr-Abl in cells (Campbell *et al.*, 1990; Liu *et al.*, 1993; Lu *et al.*, 1993). p185 Bcr-Abl also utilizes Tyr177 as an *in vitro* autophosphorylation site (Figure 5d).

The Grb2 SH2 domain specifically interacts with the *bcr*-encoded sequence, p.Tyr177-Val-Asn-Val

The specificity of Grb2 binding to the Tyr177 autophosphorylation site on Bcr-Abl was investigated using biosensor technology (Felder *et al.*, 1993; Panayatou *et al.*, 1993; Payne *et al.*, 1993). A synthetic phosphopeptide (designated BCR) corresponding to the *bcr*-encoded residues 171–182 and containing phosphotyrosine at position 177, was

immobilized on the sensor chip of a Pharmacia BIAcore instrument; soluble full-length Grb2 or Grb2 SH2 fusion proteins bound to this immobilized phosphopeptide, as measured by surface plasmon resonance. The affinities of various phosphotyrosine-containing peptides for GST-Grb2 or GST-Grb2 SH2 were then assayed by their abilities to compete with this interaction. Soluble GST-Grb2 or GST-Grb2 SH2 was mixed with varying concentrations of the phosphopeptides under test, and injected across the sensor chip surface containing the immobilized BCR peptide. Addition of the soluble BCR phosphopeptide, or a phosphopeptide corresponding to the Grb2 binding site on Shc (Pellicci *et al.*, 1992), specifically inhibited the interaction between the immobilized BCR phosphopeptide and the Grb2 fusion proteins, whereas phosphopeptides corresponding to the GAP or the Syt recognition sites on the platelet-derived growth factor (PDGFR) receptor (Kashishian *et al.*, 1993; Kazlauskas *et al.*, 1993) did not (Figure 6). Substitution of Ala for Asn at the +2 position in the BCR peptide abolished the inhibition observed with the wildtype BCR phosphopeptide. The wildtype BCR peptide, but not the Asn→Ala BCR peptide, also inhibited the binding of p210 Bcr-Abl from R1-p210 cell lysates to an immobilized Grb2 SH2 fusion protein (data not shown). Taken together, these results indicate that one of the functions of the Bcr moiety of Bcr-Abl oncoproteins is to provide a site for tyrosine phosphorylation that can couple Bcr-Abl

Fig. 6. The Grb2 SH2 domain specifically interacts with the *bcr*-encoded sequence p.Tyr177-Val-Asn-Val. A BCR phosphotyrosine-containing synthetic peptide, corresponding to residues 171–182 of human Bcr, was immobilized on the sensor chip surface of a Pharmacia BIAcore instrument, and binding of GST-Grb2 fusion proteins to this peptide was monitored by surface plasmon resonance (Felder *et al.*, 1993; Panayatou *et al.*, 1993; Payne *et al.*, 1993). The affinities of various phosphotyrosine-containing peptides for GST-Grb2 (solid symbols) or GST-Grb2 SH2 (open symbols) were assayed by their abilities to compete with this interaction. Soluble GST-Grb2 or GST-Grb2 SH2 proteins were mixed with varying concentrations of the phosphotyrosine-containing peptides BCR (○), SHC (▽), Asn→Ala BCR (△), PDGFR 771 (□) or PDGFR 1009 (◇) and injected across the sensor chip surface containing the immobilized BCR peptide. The sequences of these synthetic phosphopeptides were: BCR, DAEKPFp.Y¹⁷⁷VNVEFK, corresponding to amino acids 171–182 of human Bcr, with a carboxy-terminal lysine added; Asn→Ala BCR, identical to the BCR peptide except for substitution of alanine for asparagine at the +2 residue carboxy-terminal to Tyr177; SHC, ELFDDPSp.Y³¹⁷VNVQNLDK corresponding to the Grb2 recognition site on the Shc protein (Pellicci *et al.*, 1992); PDGFR 771, SSNp.Y⁷⁷¹MAPYDNYK, corresponding to the GAP recognition site of the PDGF receptor (Kashishian *et al.*, 1992); PDGFR 1009, SSVLP.Y¹⁰⁰⁹TAVQP corresponding to the Syt recognition site on the PDGF receptor (Kazlauskas *et al.*, 1993). The signal obtained in the absence of competing peptide was assigned a value of 100%.

Fig. 7. Bcr-Abl oncoproteins complex with mSos1 *in vivo*. (A) Lysates of R1 or R1-p210 cells were immunoprecipitated with anti-Abl or anti-mSos1 antibodies, and the immunoprecipitated proteins subsequently immunoblotted with anti-Abl antibodies. Normal rabbit serum (C) was included as a negative control. (B) R1-p185 cells were lysed, immunoprecipitated with anti-Abl or anti-mSos1 antibodies, and immunoblotted with anti-Abl antibody. (C) Lysates of R1 or R1-p210 cells were immunoprecipitated with anti-mSos1 antibody or anti-Grb2 antibody, and immunoblotted with anti-mSos1 antibody. The positions of c-Abl, Bcr-Abl and mSos1 are indicated.

directly to Grb2 and hence to the Ras pathway, and that Asn at the +2 position is also critical for this interaction.

Recruitment of mSos1 into a complex with Bcr-Abl

The SH3 domains of Grb2 bind constitutively to proline-rich motifs within the carboxy-terminal tail of mSos1 (Li *et al.*, 1993; Rozakis-Adcock *et al.*, 1993). Upon stimulation with EGF, the mSos1–Grb2 complex binds to the activated EGF receptor at the inner plasma membrane, an event that may enable Sos to stimulate the dissociation of GDP from Ras (Buday and Downward, 1993). Since mSos1 is widely expressed in hematopoietic cells (Bowtell *et al.*, 1992), and thus may participate in Ras activation in leukemic cells, we investigated whether the association of Grb2 with Bcr-Abl proteins was accompanied by recruitment of mSos1 into this complex. R1 or R1-p210 cell lysates were immunoprecipitated with anti-mSos1 or anti-Abl antibodies, and immunoblotted with anti-Abl antibodies (Figure 7A). p210 Bcr-Abl, but not endogenous c-Abl, co-precipitated with mSos1. Similar results were obtained with p185 Bcr-Abl which co-precipitated with mSos1 from R1-p185 cell lysates (Figure 7B). The association of mSos1 with Bcr-Abl probably occurs through the Grb2 adaptor protein since mSos1 co-precipitated with Grb2 from lysates of both the parental R1 and the *bcr-abl*-transformed cells (Figure 7C). The Abl SH3 domain, which also binds proline-rich motifs (Ren *et al.*, 1993) and might potentially associate directly with mSos1, did not appear to bind efficiently to mSos1 *in vitro* (data not shown). Like Grb2, mSos1 was not significantly phosphorylated on tyrosine in *bcr-abl*-transformed cells (data not shown).

Shc proteins are phosphorylated on tyrosine and associated with Grb2 in cells transformed by p210 or p185 Bcr-Abl

Analysis of phosphotyrosine-containing proteins associated with Grb2 in *bcr-abl*-transformed cells suggests that Grb2 might bind phosphorylated ligands in addition to Bcr-Abl (Figure 1). A number of tyrosine kinases phosphorylate the SH2-containing Shc proteins, which apparently stimulate the Ras pathway through inducible association with Grb2–mSos1 (McGlade *et al.*, 1992; Pelicci *et al.*, 1992; Rozakis-Adcock *et al.*, 1992, 1993; Pronk *et al.*, 1993). K562 cells express three Shc isoforms, of 46, 52 and 66

Fig. 8. Shc proteins are tyrosine phosphorylated and associated with Grb2 in Ph⁺ leukemic cells. Lysates of the K562 cell line were immunoprecipitated with anti-Shc or anti-Abl antibodies, and the precipitated proteins immunoblotted with anti-phosphotyrosine, anti-Shc or anti-Grb2 antibodies as indicated. Normal rabbit serum was included as a negative control (C). The positions of p210 Bcr-Abl, the 66, 52 and 46 kDa Shc proteins and Grb2 are indicated. Prolonged exposure of this autoradiogram revealed a minor level of phosphotyrosine on p66^{shc}, and co-precipitation of p46^{shc}, as well as p52^{shc}, with Bcr-Abl (data not shown).

kDa, of which p52^{shc} is the predominant species (Figure 8). p52^{shc} and p46^{shc} were phosphorylated on tyrosine in K562 cells, and a minor fraction of these proteins was associated with Bcr-Abl (Figure 8). Shc proteins were also phosphorylated on tyrosine in the Ph⁺ ALL line, SUP-B15 (data not shown), and in R1-p185 and R1-p210 cells, but not in the parental R1 cells (Figure 9). Both isoforms of Bcr-Abl therefore elicit the phosphorylation of Shc proteins on tyrosine *in vivo*. To determine whether Grb2 binds to Shc proteins in cells expressing Bcr-Abl, anti-Grb2 immunoprecipitates were immunoblotted with anti-Shc antibodies. No Shc proteins co-precipitated with Grb2 from parental R1 cells. However, anti-Shc antibodies recognized proteins of 46 and 52 kDa in anti-Grb2 immunoprecipitates from lysates of R1-p185 cells (Figure 9). Furthermore, phosphotyrosine-containing proteins with these mobilities were detected in anti-Grb2 immunoprecipitates from R1-p185 cells (Figure 1). In the reciprocal experiment, co-precipitation of Grb2 by Shc antibodies was also observed (data not shown). The association of Shc and Grb2 was also detected in the Ph⁺ leukemic cell lines, and in R1-p210 cells (Figure 8 and data not shown). These results indicate that Bcr-Abl proteins induce the formation of a complex between tyrosine phosphorylated Shc and Grb2.

Fig. 9. The tyrosine phosphorylation of Shc proteins and their association with Grb2 in R1 fibroblasts is induced by expression of Bcr-Abl oncoproteins. (A) R1, R1-p185 and R1-p210 cell lysates were immunoprecipitated with anti-Shc antibodies and the immunoprecipitates immunoblotted with anti-Shc or anti-phosphotyrosine antibodies as indicated. The positions of the 66, 52 and 46 kDa Shc proteins are indicated. (B) Lysates of R1 and R1-p185 cells containing similar quantities of total protein, were immunoprecipitated with anti-Grb2, anti-Shc or normal rabbit serum (C), and the immune complexes blotted with anti-Shc antibodies.

Discussion

The rearrangements of the *bcr* and *abl* genes in CML and Ph⁺ ALL are striking examples of oncogene activation directly implicated in the development of human malignancy. Molecular analysis of chimeric *bcr-abl* oncogenes, and biochemical characterization of their products, has shown that the Bcr-Abl proteins are deregulated tyrosine kinases (Konopka *et al.*, 1984; Clark *et al.*, 1987; Lugo *et al.*, 1987; McWhirter and Wang, 1991; Muller *et al.*, 1991). These findings have suggested that Bcr-Abl proteins transform cells through the aberrant stimulation of tyrosine kinase targets that in turn constitutively activate mitogenic signalling pathways. However, the identities of the proteins that mediate the effects of Bcr-Abl have not been determined. Here, we have demonstrated that targets of Bcr-Abl include proteins that participate in Ras activation.

Bcr-Abl tyrosine kinases associate with Grb2 and mSos1

Potential targets of Bcr-Abl may be regulated directly by tyrosine phosphorylation, or indirectly by association with tyrosine phosphorylated proteins such as the autophosphorylated Bcr-Abl kinases themselves. In screening for proteins that associate with Bcr-Abl, we found that Bcr-Abl tyrosine kinases in both human leukemic cells and transformed rodent fibroblasts, are bound to Grb2, a polypeptide with the structure SH3-SH2-SH3 (Lowenstein

et al., 1992). Grb2 can function as a molecular adaptor by linking a subset of receptor tyrosine kinases and other phosphotyrosine-containing proteins to mSos1, a Ras-GNRP (Buday and Downward, 1993; Chardin *et al.*, 1993; Egan *et al.*, 1993; Gale *et al.*, 1993; Li *et al.*, 1993; Rozakis-Adcock *et al.*, 1993; Skolnik *et al.*, 1993). Genetic data from *C.elegans* and *Drosophila* suggest that the invertebrate homologs of Grb2 and mSos1 are crucial in coupling receptor tyrosine kinases to activation of the Ras pathway (Simon *et al.*, 1991; Clark *et al.*, 1992; Olivier *et al.*, 1993; Simon *et al.*, 1993).

These results suggested that Bcr-Abl might associate with an activator of Ras guanine nucleotide exchange and indeed, mSos1 was also found complexed with Bcr-Abl in *bcr-abl*-transformed cells. The simplest explanation for these findings is that Grb2 binds directly to Bcr-Abl oncoproteins through its SH2 domain, thereby recruiting mSos1, which is constitutively associated with the Grb2 SH3 domains, into a heterotrimeric Bcr-Abl-Grb2-mSos1 complex. The association of Grb2 with receptor tyrosine kinases is normally transiently induced by receptor autophosphorylation, which creates a specific binding site for the Grb2 SH2 domain (Lowenstein *et al.*, 1992; Buday and Downward, 1993; Pawson and Schlessinger, 1993; Rozakis-Adcock *et al.*, 1993). However, Grb2 only binds to a subset of receptor tyrosine kinases upon growth factor stimulation (Suen *et al.*, 1993), and has not been previously observed to bind stably to cytoplasmic tyrosine kinases. We have therefore investigated the mechanism by which Grb2 and Bcr-Abl might stably associate in *bcr-abl*-transformed cells.

Autophosphorylation within the bcr-encoded sequence of Bcr-Abl creates a Grb2 SH2-binding site

In vitro, the Sem-5 SH2 domain bound specifically to Bcr-Abl proteins. Previous work has shown that the Sem-5 SH2 domain binds preferentially to phosphotyrosine followed at the +2 position by an Asn (Songyang *et al.*, 1993). Examination of the sequences of p185 and p210 Bcr-Abl revealed a single potential binding site for the Grb2 SH2 domain (Tyr-Val-Asn-Val), centered on Tyr177 within the common amino-terminal *bcr*-encoded sequence of Bcr-Abl oncoproteins. We have demonstrated that Tyr177 undergoes autophosphorylation, and that the interaction of Bcr-Abl with the Grb2 SH2 domain is specifically inhibited by a soluble phosphotyrosine-containing peptide representing the Tyr177 phosphorylation site. Of interest, the ability of this peptide to bind with high affinity to the Grb2 SH2 domain, and thereby block the interaction with Bcr-Abl or the immobilized Bcr peptide, is dependent on Asn179 at the +2 position relative to phosphotyrosine.

These data strongly argue that the ability of Bcr-Abl oncoproteins to bind stably to Grb2, and hence to mSos1, results from autophosphorylation at Tyr177 which creates a specific binding site for the Grb2 SH2 domain. We have also observed binding of Bcr-Abl to Grb2 SH3 domains *in vitro*. However, since it is the Grb2 SH3 domains that apparently bind mSos1 *in vivo*, this is less likely to be of physiological relevance for Ras activation. The amino-terminal Bcr sequence is implicated in activation of the Abl tyrosine kinase domain (Muller *et al.*, 1991), has been proposed to interact with the Abl SH2 domain (Pendergast *et al.*, 1991; Muller *et al.*, 1992) and enhances the F-actin binding function of Abl (McWhirter and Wang, 1991). Our data suggest that Bcr sequences also contribute to interactions

of Bcr-Abl proteins with their targets by providing tyrosine autophosphorylation sites which allow Bcr-Abl to couple directly to SH2-containing signalling proteins. Consistent with the hypothesis that these Bcr-mediated interactions are important for Bcr-Abl transforming activity, a deletion mutant of p185 Bcr-Abl lacking residues 162–220 is impaired in its ability to transform primary bone marrow cultures (Muller *et al.*, 1991). Taken together, these results suggest that inhibiting the interactions of Grb2 with the phosphorylated Tyr177 site of Bcr-Abl is a potential approach to reversing the transformed phenotype of Ph⁺ leukemic cells.

Neither Grb2 nor mSos1 is significantly phosphorylated on tyrosine in *bcr-abl*-transformed cells, suggesting that their function is regulated by their physical association with phosphotyrosine-containing proteins. Since a fraction of Bcr-Abl localizes to F-actin filaments (McWhirter and Wang, 1991, 1993), binding of Grb2-mSos1 to Bcr-Abl may relocalize Ras-GNRP activity to the cortical cytoskeleton, providing access to Ras. Alternatively, mSos1 activity may be stimulated by interaction of Grb2–mSos1 with Bcr-Abl. p160 Bcr complexes with Bcr-Abl *in vivo* (Campbell *et al.*, 1990; Liu *et al.*, 1993) and undergoes transphosphorylation on Tyr177 *in vitro*, indicating that Bcr itself, which is expressed by the normal chromosome 22 in Ph⁺ leukemic cells, may also activate Grb2–mSos1.

Bcr-Abl induces the formation of Shc–Grb2 complexes

The *shc* gene products are apparently involved in Ras signalling, through an inducible interaction with Grb2 (Rozakis-Adcock *et al.*, 1992). The 46 and 52 kDa Shc proteins are phosphorylated on tyrosine in Ph⁺ leukemic cell lines, and in fibroblasts transformed by p210 or p185 Bcr-Abl. A 53 kDa phosphoprotein has been previously reported to associate with p210 Bcr-Abl in the K562 cell line, and in primary blast cells from patients with acute phase CML (Maxwell *et al.*, 1987; Li *et al.*, 1988). This protein may represent p52^{shc}, the predominant tyrosine phosphorylated Shc protein in Ph⁺ leukemic cells.

A minor fraction of Shc co-precipitated with Bcr-Abl proteins. This physical association of Shc polypeptides with Bcr-Abl is less striking than the Grb2–Bcr-Abl complex, but may serve to facilitate Shc phosphorylation. In *bcr-abl*-transformed cells, tyrosine phosphorylated Shc proteins associated with Grb2, as described previously in cells containing activated growth factor receptors and oncogenic tyrosine kinases. The multiple Grb2 complexes that are formed may act synergistically to enhance Ras activation and to enable Bcr-Abl oncoproteins to transform hematopoietic cells.

Materials and methods

Cell culture

The K562 cell line was obtained from the American Tissue Type Collection. The Ph⁺ ALL cell line SUP-B15 was generously provided by S. Smith (University of Chicago). K562 and SUP-B15 cells were maintained in RPMI medium supplemented with 10% fetal bovine serum (FBS). R1 fibroblasts were grown in Dulbecco's modified Eagle's medium (DMEM) containing 5% FBS. R1 cells stably expressing p185 or p210 Bcr-Abl (Lugo *et al.*, 1990) were maintained in DMEM supplemented with 5% FBS and 50 µg/ml G418.

Immunoprecipitations and Western blotting

Immunoprecipitations were carried out as previously described (Moran *et al.*, 1991). All cells were lysed in 1 ml of PLC lysis buffer [50 mM HEPES (pH 7.5), 150 mM NaCl, 10% (v/v) glycerol, 1% (v/v) Triton X-100, 1.5 mM MgCl₂, 1 mM EGTA, 10 µg/ml leupeptin, 10 µg/ml aprotinin, 1 mM sodium orthovanadate, 10 mM sodium pyrophosphate, 100 mM sodium fluoride and 1 mM phenylmethanesulfonyl fluoride]. Cell lysates were centrifuged for 15 min at 10 000 g and the supernatant was collected. In some experiments, protein concentrations of the clarified supernatants were determined and similar quantities of protein immunoprecipitated. Cell lysates were incubated and gently rocked at 4°C for 90 min with anti-Abl pEx5 antibody (Konopka *et al.*, 1984), anti-Grb2 50 antibody (Lowenstein *et al.*, 1992), anti-Shc antibody (Pelicci *et al.*, 1992) or anti-mSos1 antibody (Bowtell *et al.*, 1992) and 100 µl of 10% protein A–Sepharose. The immune complexes were washed three times with HNTG [20 mM HEPES (pH 7.5), 10% glycerol, 0.1% Triton X-100, 150 mM NaCl and 1 mM sodium orthovanadate] and heated in SDS sample buffer prior to gel electrophoresis and transfer to nitrocellulose with a semi-dry transfer apparatus at 0.8 mA/cm² for 60 min. In some experiments, dephosphorylation with potato acid phosphatase was performed as described by Moran *et al.* (1991) prior to resuspension in SDS sample buffer. For immunoblotting with anti-Abl, anti-Shc and anti-Grb2 antibodies, filters were blocked in Tris buffered saline (TBS) [20 mM Tris–HCl (pH 7.5), 150 mM NaCl] with 5% Carnation skim milk powder. Antibody incubations were carried out in TBS with 3% milk powder and 0.1% Tween. To detect Abl proteins, a mouse monoclonal antibody directed against the carboxy-terminal region of c-Abl was used at a concentration of 1 µg/ml (Schiff-Maker *et al.*, 1986). Grb2 55 antibodies raised against the entire Grb2 molecule were used for Western blotting at a 1/200 dilution (Lowenstein *et al.*, 1992). mSos1 immunoblotting was performed as previously described (Rozakis-Adcock *et al.*, 1993). For anti-phosphotyrosine immunoblotting, filters were blocked in TBS containing 5% bovine serum albumin and 1% ovalbumin and incubated with affinity purified anti-phosphotyrosine antiserum (Letwin *et al.*, 1988). Blots were then probed with ¹²⁵I-labelled protein A (35 µCi/µg, Amersham) in blocking solution and exposed to Kodak XAR-5 film. For anti-Abl blots, a secondary incubation was carried out with horseradish peroxidase conjugated anti-immunoglobulin antiserum (Sigma) prior to development with enhanced chemiluminescence reagents (Amersham) and exposure to Kodak XRP-5 film.

Bacterial fusion proteins

Regions of Grb2 and the Abl SH3 domain were isolated by the polymerase chain reaction (PCR) and subcloned into pGex expression vectors (Smith and Johnson, 1991). Full-length Grb2 and Grb2 SH2 fusion proteins have been described (Lowenstein *et al.*, 1992). The amino- and carboxy-terminal Grb2 SH3 fusion proteins contain portions of Grb2 corresponding to amino acid residues 1–58 and 159–217 respectively. The Abl SH3 fusion protein corresponds to amino acids 929–1020 of p210 Bcr-Abl. For the Abl SH2 fusion protein, the DNA encoding the amino-terminal region of type IV murine c-Abl was isolated by PCR, beginning with the *SnaI* restriction site and terminating at the 3' end of the SH2 domain. The Abl SH3 domain was subsequently deleted by PCR, and the fragment subcloned into a pGex vector. Bacterial cultures expressing the pGex vectors were grown in LB and 100 µg/ml ampicillin, and induced with 1 mM isopropyl thiogalactopyranoside (IPTG) for 4 h at 30 or 37°C. The induced bacteria were lysed by sonication in PLC LB, and the glutathione-S-transferase (GST) fusion proteins recovered from clarified lysates by glutathione–agarose (Pharmacia). The amount of each GST fusion protein was determined by comparison of bands on a Coomassie Blue stained gel with a dilution series of bovine serum albumin; similar quantities of fusion proteins were incubated with cell lysates for 90 min at 4°C. Proteins bound to the immobilized GST fusion proteins were recovered by centrifugation, washed three times in HNTG and resuspended in SDS buffer.

Two-dimensional trypsin/V8 protease maps of Bcr-Abl and Bcr proteins

For analysis of *in vitro* autophosphorylated sites, p210 and p185 Bcr-Abl were immunoprecipitated from K562 and SUP B15 cells respectively, by the p6D monoclonal antibody raised against a synthetic peptide, ABL, corresponding to amino acids 51–64 of human c-Abl (Li *et al.*, 1988). Proteins were labelled in an *in vitro* kinase assay using [γ -³²P]ATP, separated by SDS–PAGE and extracted from the gel as described by Liu *et al.* (1993). p160 Bcr was immunoprecipitated from K562 cells by an antibody raised against BCR, a synthetic peptide corresponding to amino acids 1256–1271 of the carboxy-terminal portion of Bcr, and transphosphorylated by p210 Bcr-Abl *in vitro* as previously described (Liu *et al.*, 1993). Performic acid oxidized proteins were first treated with *N*-

tosyl-L-phenylalanyl chloromethyl ketone (TPCK)-treated trypsin for 22 h and then with V8 protease (Glu-C) for 22 h under conditions that favor cleavage after Glu (pH 7.8 ammonium bicarbonate) (Liu et al., 1993). Peptides were separated by two-dimensional electrophoresis and chromatography, then sequenced by manual Edman degradation, and the elution position of 32 P-labelled phosphotyrosine was monitored (Sullivan and Wong, 1991). For analysis of peptides that bound to GST-Grb2 SH2, a trypsin/V8 protease digest of *in vitro* labelled p210 Bcr-Abl was mixed with immobilized GST-Grb2 SH2 or GST-Abl SH2 fusion proteins for 90 min at 4°C. Bound peptides were removed by centrifugation, and the remaining peptides analyzed by two-dimensional electrophoresis and chromatography.

BIACore analysis of phosphopeptides interacting with Grb2 SH2 or full-length Grb2

Phosphopeptides were synthesized by 9-fluorenyl methoxy carbonyl (Fmoc) chemistry according to published methods (Piccione et al., 1993). The sequences of these synthetic phosphopeptides were: BCR, DAEKPPF.Y¹⁷⁷.VNVEFK, corresponding to amino acids 171–182 of human Bcr, with a carboxy-terminal lysine added; Asn→Ala BCR, identical to the BCR peptide except for substitution of alanine for asparagine at the +2 residue carboxy-terminal to Tyr177; SHC, ELFDDPSp.Y³¹⁷VNVQNLDK corresponding to the Grb2 recognition site on the Shc protein (Pellicci et al., 1992); PDGFR 771, SSNP.Y⁷⁷¹MAPYDNYK, corresponding to the GAP recognition site of the PDGF receptor (Kashishian et al., 1992); PDGFR 1009, SSVLP.Y¹⁰⁰⁹TAVQP corresponding to the Syt recognition site on the PDGF receptor (Kazlauskas et al., 1993). For peptide immobilization to the sensor chip surface, a 0.5 mM solution of BCR phosphopeptide in 50 mM HEPES (pH 7.5) and 2 M NaCl was used following conditions previously described (Payne et al., 1993). GST fusion proteins containing full-length human Grb2 or the Grb2 SH2 domain were resuspended in 50 mM HEPES (pH 7.5), 150 mM NaCl and 3.0 mM EDTA, and concentrated using a Centricon 10 microconcentrator device (Amicon). Solutions (100 µl) containing 1 µM GST-Grb2 or 1 µM GST-Grb2 SH2 and the indicated concentration of soluble peptide in 50 mM HEPES, pH 7.5, 150 mM NaCl and 3 mM EDTA, were injected across the surface containing immobilized BCR phosphotyrosine peptide. The amount of GST-Grb2 or GST-Grb2 SH2 protein bound was estimated from the steady-state surface plasmon resonance signal at a fixed time following the end of the injection and the percentage bound relative to injection of GST-Grb2 or GST-Grb2 SH2 alone calculated (Ponzetto et al., 1993). After injection the peptide surface was regenerated using 2 M GuHCl.

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