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BCR and RAF form a complex *in vivo* via 14-3-3 proteins

Sylvia Braselmann[1] and Frank McCormick

Onyx Pharmaceuticals Inc., 3031, Research Drive, Richmond, CA 94806, USA

[1]Corresponding author

In a yeast two-hybrid screen we identified a member of the 14-3-3 family of proteins that can bind to Bcr. 14-3-3β binds to the serine/threonine rich region B in the kinase domain encoded by the first exon. In this paper we show by co-immunoprecipitation that Bcr binds to Raf *in vivo* and we argue that this interaction is mediated by 14-3-3 dimers, based on the following findings. First, 14-3-3 isoforms bind to both Raf and Bcr. Second, Bcr does not bind to Raf directly in the two-hybrid system, but co-expression of 14-3-3β allows complex formation. Third, Bcr, 14-3-3 proteins and Raf co-elute in gel filtration and in sequential ion exchange chromatography and the three proteins can be co-immunoprecipitated from the separate fractions, indicating that they are present in a ternary complex. Moreover, ~10 times more Raf is bound to Bcr, and vice versa, in the membrane fraction (where Raf is activated) than in the cytosolic fraction. We suggest a new function for 14-3-3 proteins as a novel type of adaptor which acts by dimerization and binding to different proteins.

Keywords: Bcr/protein complexes/14-3-3 proteins/Raf

Introduction

The product of the Bcr gene is a 160 kDa phosphoprotein (Timmons and Witte, 1989) with a multidomain structure. The N-terminal first exon encodes a novel serine/threonine kinase activity (Maru and Witte, 1991), an oligomerization domain (McWhirter *et al.*, 1993) and a region that binds Src-homology 2 (SH2) domains in a phosphotyrosine-independent manner (Pendergast *et al.*, 1991). The C-terminus encodes a GTPase-activating function for the small GTP-binding protein Rac (Diekmann *et al.*, 1991). The middle part of the protein has a region of sequence similarity to guanine nucleotide exchange factors for the Rho family of GTP-binding proteins (the dbl homology; Ron *et al.*, 1991) and a pleckstrin homology domain (Musacchio *et al.*, 1993). Although these different domains suggest that Bcr is involved in different intracellular signalling pathways, little is known about its biological role.

More, however, is known about its role in the *bcr–abl* chimeric oncogene. The *bcr* gene is the site of two possible breakpoints in a reciprocal translocation to the c-*abl* proto-oncogene and the resulting Bcr–Abl fusion proteins are characteristic of certain leukemias (reviewed in Witte, 1993). The sequences in the first exon of *bcr*, which are fused upstream of the second exon of *abl*, are necessary for the activation of the Abl tyrosine kinase activity (McWhirter and Wang, 1991; Muller *et al.*, 1991), which is necessary for the oncogenic potential of the chimeric oncogene (Lugo *et al.*, 1990). A tyrosine (Tyr177) within the Bcr first exon becomes phosphorylated by the activated Abl kinase in Bcr–Abl and serves as a binding site for the SH2 domain of the Grb2 adaptor protein, thereby linking Bcr–Abl to the Ras signalling pathway (Pendergast *et al.*, 1993; Puil *et al.*, 1994).

To elucidate further the function of c-Bcr, we performed a yeast two-hybrid screen to identify proteins binding to Bcr. One of the proteins thus identified is a member of the 14-3-3 family of proteins. During the course of our investigation a report was published showing the binding of Bcr and Bcr–Abl to yet another member of the 14-3-3 family (Reuther *et al.*, 1994). These proteins are highly conserved and are found in a broad range of organisms and tissues. At least seven mammalian isoforms of 14-3-3 have been identified, and multiple isoforms are present in most cells (reviewed in Aitken, 1992). This family of proteins was originally reported to function as coregulators of tryptophan and tyrosine hydroxylases and inhibitors of protein kinase C (PKC) and they have been implicated in the stimulation of exocytosis from adrenal chromaffin cells (Aitken *et al.*, 1992 and references therein). Several recent findings, however, point to new roles for these proteins. The *Schizosaccharomyces pombe* homologs rad24 and rad25 are required for the DNA damage checkpoint and therefore have a role in determining the timing of mitosis (Ford *et al.*, 1994). In mammalian cells, 14-3-3 proteins have recently been shown to associate with the polyoma middle tumor antigen (Pallas *et al.*, 1994).

Several groups have reported an association between 14-3-3 proteins and Raf. The c-Raf protein is a component of the mitogen-activated protein (MAP) kinase pathway. Activation of this pathway is a central response of cells to mitogenic factors (Pages *et al.*, 1993). Binding of ligands to tyrosine kinase receptors leads to activation of Ras through an increase in the amount of the guanosine triphosphate (GTP)-bound form of Ras (reviewed in Hall, 1994), which leads to the activation of Raf. Raf then phosphorylates and activates the MAP kinase kinase or MEK [MAP kinase or extracellular signal-regulated kinase (ERK) kinase] (Dent *et al.*, 1992; Howe *et al.*, 1992; Kyriakis *et al.*, 1992), which in turn phosphorylates and activates the MAP kinases ERK1 and ERK2 (Crews *et al.*, 1992).

Activated Ras interacts directly with the N-terminal regulatory domain of Raf-1 (Moodie *et al.*, 1993; Vojtek *et al.*, 1993; Warne *et al.*, 1993; Zhang *et al.*, 1993), however, Ras binding alone does not activate Raf *in vitro*. Ras may function in the activation of Raf by recruiting it

to the membrane (Traverse *et al.*, 1993), since a modified form of Raf that contains a CAAX motif (C, cysteine; A, aliphatic amino acid; X, any amino acid; Hancock *et al.*, 1991) and is thereby directed to the plasmamembrane (Leevers *et al.*, 1994; Stokoe *et al.*, 1994) becomes activated without a requirement for activated Ras.

The mechanism of activation of Raf at the plasma membrane remains elusive so far, but 14-3-3 proteins may play a role (reviewed in Morrison, 1994). First, overexpression of the yeast homolog, BMH1, enhances the function of mammalian Raf in budding yeast and is required for Raf to be activated by Ras in this system (Irie *et al.*, 1994). Second, overexpression of mammalian 14-3-3 in yeast stimulates the biological activity of mammalian Raf (Freed *et al.*, 1994) and third, expression of 14-3-3 in *Xenopus* oocytes induces meiotic maturation, and Raf immunoprecipitated from these oocytes has increased kinase activity (Fantl *et al.*, 1994). However, when the activity was measured directly with bacculovirus expressed Raf and purified 14-3-3 *in vitro*, activation of Raf could not be shown reproducibly (Fu *et al.*, 1994). Moreover, 14-3-3 associates with Raf *in vivo* regardless of the subcellular localization or activation state of Raf, or whether Raf is bound to Ras (Freed *et al.*, 1994; Fu *et al.*, 1994). 14-3-3 may therefore be a cofactor in the activation of Raf, but so far it is difficult to conclude that it is the direct activator of Raf.

14-3-3 has been shown to exist as a dimer *in vivo* by several biochemical criteria. Therefore it seems likely that 14-3-3 proteins are able to bind to different proteins at the same time, thereby sequestering these proteins into one complex. In this report we show that Bcr and Raf are indeed found in a complex *in vivo*, bridged by the dimeric 14-3-3 protein.

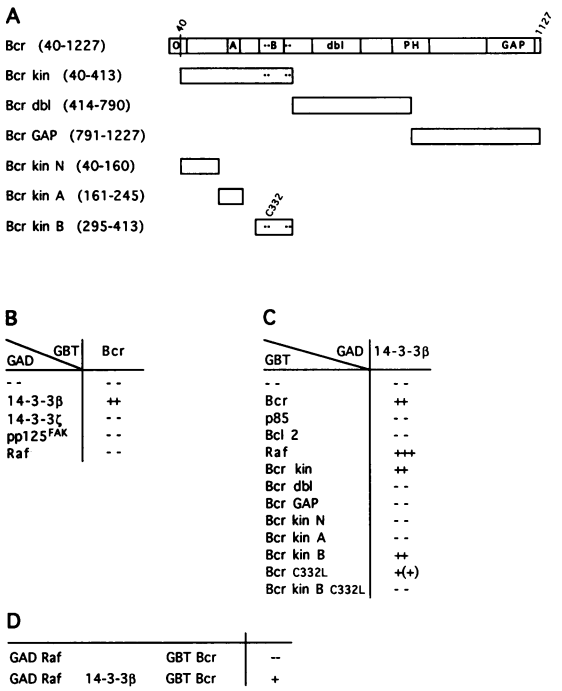


Fig. 1. Bcr binds to 14-3-3β in the two-hybrid system. (**A**) Structure of Bcr and description of the GBT–Bcr fusion proteins. O = oligomerization domain; A and B = serine/threonine rich regions in the kinase domain; dbl = dbl homology region; PH = pleckstrin homology domain; GAP = Rac GTPase activating domain. The cysteine pairs in the kinase domain of Bcr are symbolized by dots. (**B**, **C** and **D**) β-Galactosidase activity on a filter assay upon co-expression of the Gal4 DNA binding domain fusion protein (GBT) and Gal4 activation domain fusion protein (GAD) in the two-hybrid system (– – for no activity to +++ for strong activity). 14-3-3β in (D) is expressed as native protein.

Results

Bcr binds to 14-3-3β in the yeast two-hybrid system

To identify proteins that interact with Bcr, we performed a two-hybrid screen (Fields and Song, 1989; Chien, 1991; Fields and Sternglanz, 1994), using full-length Bcr fused to the Gal4 DNA-binding domain as bait and screening a HeLa cDNA library fused to the Gal4 activation domain. Interaction of two proteins in this system allows for growth on medium lacking histidine and for expression of β-galactosidase (β-Gal). 3.6×10^5 colonies were screened and of 18 different clones specific for Bcr one showed sequence identity to the β isoform of the 14-3-3 family of proteins. This clone was isolated three times independently. To address the specificity of these interactions we tested other Gal4 fusions. Different combinations of clones were cotransfected into the yeast YGH1 strain, plated on medium lacking tryptophan, leucine and histidine and after four days the colonies were subjected to a filter β-Gal assay (Figure 1B). Bcr binds specifically to 14-3-3β and does not bind to the empty vector pGADGH containing just the Gal4 activation domain, nor to $pp125^{FAK}$ (Focal adhesion kinase) or Raf in this vector. The ζ isoform of 14-3-3 has been shown to bind to Raf (Freed *et al.*, 1994), but it does not bind to Bcr in the two-hybrid system (Figure 1B). Conversely, 14-3-3β binds specifically to Bcr and not to the empty vector pGBT8 containing just the Gal4 DNA binding domain, nor to Bcl-2 or the phosphoinositol-3 kinase subunit p85 in this vector. However, 14-3-3β does not seem to bind as strongly to Bcr as it binds to Raf as judged by β-galactosidase activity.

As already described, Bcr has a multidomain structure and in order to determine which part of the Bcr protein binds to 14-3-3β, the kinase domain, the dbl homology domain and the Rac GAP domain were separately fused to the Gal4 DNA-binding domain in the GBT8 vector (Figure 1A). 14-3-3β does not bind to the dbl homology nor to the Rac GAP domain but binds specifically to the serine kinase domain encoded by the first exon of Bcr (Figure 1C). This correlates well with the data reported by Reuther and colleagues (Reuther *et al.*, 1994) and we attempted to define more closely the region of interaction. The kinase domain has two serine/threonine rich regions, the so-called A box (amino acid 197–239 in human Bcr) and the B box (amino acids 299–385) and further dissection in the two-hybrid system showed that 14-3-3β does not bind to the N-terminal part of the kinase domain nor to the A box, but binds specifically to the B box. This box also contains two pairs of cysteines and the second cysteine in the first cysteine pair (Cys322) has been shown to be essential for the serine kinase function of Bcr (Maru and Witte, 1991). In order to find out whether this cysteine is also essential for binding of 14-3-3 we tested a C332L mutation, which knocks out the serine kinase activity of

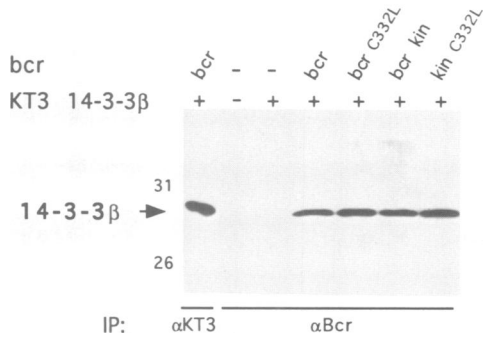


Fig. 2. Bcr binds to 14-3-3β *in vivo*. COS cells were electroporated with 3 μg KT3-tagged 14-3-3β and 5 μg bcr as indicated. Whole cell lysates were immunoprecipitated with the indicated antibodies, the proteins separated by SDS–PAGE on 12% gels (Novex), transferred to nitrocellulose and probed with α-KT3 antibodies. Roughly 20 times more of the α Bcr than of the α-KT3 immunoprecipitation was loaded. IP = immunoprecipitation.

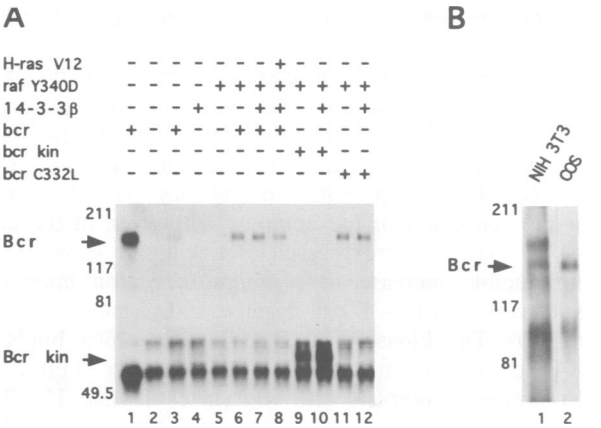


Fig. 3. Bcr binds to Raf *in vivo*. (**A**) Cos cells were electroporated with 5 μg bcr, 3 μg KT3-tagged 14-3-3β, 5 μg EE-tagged raf Y340D and 3 μg H-ras V12 as indicated. Whole cell lysates were prepared and immunoprecipitated as described. α-Bcr immunoprecipitate (Rb1) was loaded in lane 1 and α-Raf (C20) immunoprecipitate in lanes 2–12. The proteins were separated by SDS–PAGE on 8% gels (Novex), transferred to nitrocellulose and probed with α-Bcr antibody (TrpE-Bcr). (**B**) Two confluent 10 cm dishes of untransfected NIH3T3 and COS cells were lysed, immunoprecipitated with α-Raf antibodies and separated on SDS–PAGE and blotted as in (A).

bacculovirus produced Bcr (Maru and Witte, 1991). When this mutation is introduced into the full-length Bcr protein in the two-hybrid system, binding of 14-3-3β to Bcr is slightly reduced, whereas binding is abolished when this point mutation is introduced into the clone expressing just the B box, which is the smallest fragment of Bcr that is able to bind to 14-3-3β.

We also attempted to identify the region of binding within the 14-3-3β protein. However, none of the isolated domains of 14-3-3β was able to bind to Bcr in the two-hybrid system (data not shown).

In summary, we showed in the yeast two-hybrid system that the β isoform of 14-3-3 binds to the serine/threonine rich B box in the kinase domain of Bcr.

Bcr binds to 14-3-3β in vivo

We next investigated whether Bcr and 14-3-3β interact *in vivo* by transient transfection into COS cells. All DNAs used in the described experiments were cloned into a mammalian expression vector with a SV40 promoter/enhancer (EXV3). Human bcr cDNA was transiently transfected into COS cells together with 14-3-3β that had been tagged with the KT3 epitope (PPEPET) at the C-terminus. The cells were harvested after three days, lysed and immunoprecipitated with antibodies against the N-terminus of Bcr and the Western blots were probed with horseradish peroxidase-coupled antibodies against the KT3 tag on the 14-3-3β protein (Figure 2). Lane 1 shows 14-3-3β immunoprecipitated with α-KT3 antibodies as control. The level of endogenous Bcr in COS cells is very low, therefore the KT3-tagged 14-3-3β only becomes readily detectible after cotransfection of both proteins (lane 4) and only an overexposure of the blot will show a weak band of 14-3-3β immunoprecipitated via endogenous Bcr (lane 3). As in the two-hybrid experiment, the C332L mutation, which knocks out the serine kinase activity of Bcr, does not affect binding of 14-3-3β to Bcr. Moreover, the kinase domain of Bcr (corresponding to the first exon) is sufficient for binding and a C332L mutation in this kinase domain does not affect binding either (lane 6 and 7). The variations in the level of 14-3-3β immunoprecipitated in the complex with Bcr reflect the variation in the expression and immunoprecipitation of the different Bcr constructs (data not shown). In the complimentary experiment, Bcr complexed to KT3 tagged 14-3-3β can be immunoprecipitated with α-KT3 antibodies (data not shown).

We could therefore show that Bcr and 14-3-3β also interact *in vivo*, that the kinase domain of Bcr is sufficient for co-immunoprecipitation and that the C332L mutation, which abolishes kinase activity of Bcr, does not affect the interaction with 14-3-3β.

Bcr binds to Raf-1 in vivo

We have shown here that 14-3-3β binds to Bcr, and Freed and coworkers as well as others have shown that the β isoform and the ζ isoform of 14-3-3 also bind to Raf. 14-3-3 proteins have been shown to exist as dimers in their native state by several biochemical experiments, such as size exclusion chromatography, chemical crosslinking and sedimentation equilibrium analysis (Toker *et al.*, 1992 and references therein). Recently, a domain within the extreme N-terminal 26 residues of mammalian 14-3-3 has been identified as being responsible for dimerization (Jones *et al.*, 1995). Because of this dimerization potential of 14-3-3 we speculated that there might be a ternary complex between Raf and Bcr, bridged by 14-3-3 dimers. Bcr does not bind to Raf directly in the yeast two-hybrid system, but co-expression of 14-3-3β allows growth on selection plates lacking histidine, although it gives only little β-galactosidase activity (Figure 1D). We therefore proceeded to show complex formation between Bcr and Raf *in vivo*. We cotransfected the vectors containing raf, bcr and 14-3-3β in different combinations into COS cells, lysed the cells after 3 days and immunoprecipitated then with α-Raf antibodies. The immunoprecipitates were subjected to SDS–PAGE, blotted and probed with α-Bcr antibodies. Figure 3A shows that the overexpressed Bcr can be immunoprecipitated by endogenous Raf (lane 3). Not only is the kinase domain of Bcr sufficient for interaction with Raf *in vivo* (lanes 9 and 10), but even the serine/threonine

rich box B can be co-immunoprecipitated with α-Raf antibodies when expressed in COS cells (data not shown). Overexpression of 14-3-3β does not increase the complex formation between Raf and Bcr (compare lanes 6 and 7), nor does 14-3-3ζ or the combination of β and ζ (data not shown). This is likely due to the fact that 14-3-3 proteins are very abundant proteins, so that they are not limiting for the formation of the complex. However, in the case of the highly overexpressed Bcr kinase, a weak but reproducible increase in complex formation upon co-expression of 14-3-3β can be observed (compare lanes 9 and 10). The kinase-dead Bcr C322L, which binds to 14-3-3β just as efficiently as wild-type Bcr (Figure 2), also forms a complex with Raf (lanes 11 and 12). The converse experiment, i.e. immunoprecipitation with α-Bcr antibodies and Western blotting with α-Raf antibodies shows the same results (data not shown). The immunoprecipitation was done from cycling cells and there is no difference in complex formation when cells are starved or serum-stimulated (data not shown).

COS cells express very little endogenous Bcr, and transfection of 5 μg each of raf, bcr and the bcr kinase domain usually leads to a 5-, 20- and at least 50-fold expression over endogenous protein, respectively, as judged by immunoprecipitation and Western blot (data not shown). This overexpression makes the complexes readily visible in immunoprecipitation and Western blot.

Figure 3B shows the Raf–Bcr complex immunoprecipitated from untransfected COS cells and from NIH 3T3 cells, detectible in a long exposure. Likewise, Bcr can be co-immunoprecipitated with Raf in Jurkat T cells (data not shown). Thus, Raf and Bcr, both wild-type and the kinase-dead C332L mutation, form a complex *in vivo* and the serine/threonine rich region B in the kinase domain of Bcr is sufficient for interaction with Raf.

Bcr, 14-3-3 and Raf co-elute as a complex on ion exchange chromatography

Bcr binds to 14-3-3 and Raf binds to 14-3-3, and in order to show that all three proteins are present in the same ternary complex we transfected COS cells with expression vectors for bcr, raf and the KT3 tagged 14-3-3β, prepared S100 and subjected this extract to Mono Q column chromatography. The proteins were eluted with a salt gradient from 0 to 500 mM NaCl in 30 ml of solution and 1 ml fractions were collected. Fifteen microlitres of each fraction were loaded on gels, subjected to SDS–PAGE, blotted and probed with antibodies against Bcr, the KT3 tag in 14-3-3β and Raf. Figure 4A shows the fairly broad elution profile of the three proteins, whose elution peaks vary considerably. The elution peak of Bcr is in fractions 21 and 22 (~350 mM NaCl), the peak of 14-3-3β is fractions 20–22, whereas the elution of Raf peaks in fractions 23–25 (~380–410 mM NaCl). This peak also contains the majority of bandshifted Raf. It has been shown that in its native form the majority of Raf exists in a multiprotein complex containing the chaperone proteins hsp90 and p50 (Wartmann and Davis, 1994) and in this experiment the elution peak of Raf from the Mono Q column correlates exactly with the elution of hsp90 (data not shown).

We then proceeded to immunoprecipitate Raf from the separate fractions in order to see which proteins are

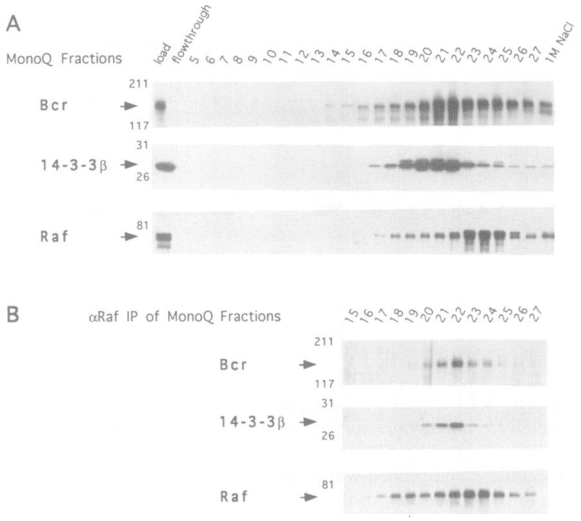


Fig. 4. Bcr, 14-3-3 and Raf co-elute on ion exchange chromatography. COS cells were electroporated with 5 μg bcr, 5 μg raf Y340D and 3 μg KT3-tagged 14-3-3β per dish, lysed after 3 days in hypotonic lysis buffer and S100 prepared as described. The pooled S100 fraction (25 mg protein total) was applied to a Mono Q ion exchange chromatography column and eluted with a gradient from 0 to 0.5 M NaCl followed by a final wash with 1 M NaCl. (**A**) Aliquots of each fraction were separated by SDS–PAGE, transferred to nitrocellulose and blotted with the indicated antibodies. (**B**) Aliquots of fractions 15–27 were adjusted to 450 mM NaCl and immunoprecipitated with α-Raf antibody (C-20). The Raf-immunoprecipitates were separated on SDS–PAGE, transferred to nitrocellulose and probed with antibodies as indicated.

actually bound to it. Figure 4B shows the α-Raf immunoprecipitation of fractions 15–27, which mirrors the original elution profile with its peak in fractions 23 and 24. Again, this correlates exactly with the profile of hsp90 in the Raf immunoprecipitation (data not shown). However, the pattern of 14-3-3 bound to Raf exactly mirrors the pattern of Bcr bound to Raf, with a pronounced peak in fraction 22. This strongly suggests that the same Raf molecules that are bound to 14-3-3 are also bound to Bcr, or in other words, that 14-3-3 bridges Raf to Bcr.

We then combined the lysate in fractions 21–23, the peak fractions of the Raf–14-3-3β–Bcr complex, adjusted the pH to 6.5 and subjected this pool to Mono S column chromatography, again eluting with a salt gradient from 0 to 500 mM NaCl. Most of Raf as well as a lot of 14-3-3 protein did not bind to the Mono S column, however, a substantial amount of Raf and 14-3-3 still co-eluted in the same fractions with Bcr (~240–300 mM NaCl), again strongly suggesting that these three proteins are present as one complex (data not shown). Likewise, we subjected cytosolic protein extract from untransfected Jurkat T cells directly to MonoS column chromatography and found the same profile of co-elution and co-immunoprecipitation of endogenous Raf, Bcr and 14-3-3 (data not shown).

When we separated the protein complexes of untransfected COS cells on a gel filtration column (HiPrep Sephracyl S300, Pharmacia), most of Raf and 14-3-3 eluted between the 67 000 and 230 000 kDa standards. However, a minor fraction of both 14-3-3 and Raf co-eluted with Bcr as a high molecular weight complex >670 000 kDa (data not shown).

Thus, Bcr, 14-3-3 and Raf co-elute in gel filtration and

in two sequential steps of ion exchange chromatography, and equivalent amounts of Bcr and 14-3-3 can be co-immunoprecipitated with Raf from separate fractions. From these data we cannot exclude the presence of other proteins in the complex, yet hsp90 elutes slightly differently.

More Bcr is bound to membrane-associated Raf than to cytosolic Raf

Upon activation of Ras, Raf becomes translocated to the membrane (Traverse *et al.*, 1993), where it gets activated by an as yet unidentified mechanism. It has been shown that 14-3-3 accompanies Raf to the membrane during this activation step (Freed *et al.*, 1994) and we therefore investigated whether Bcr would do the same by comparing the distribution of Bcr and Raf-associated Bcr between the soluble or supernatant fraction (S100) and the membrane or pellet fraction (P100). COS cells were transiently transfected with different combinations of bcr and raf, with or without the constitutively active H-ras V12. In this experiment we used the constitutively active Y340D mutation of Raf, which gave a higher level of expression, but exactly the same result can be achieved with wild-type Raf. Before harvesting, the cells were deprived of serum for 18 h. The cells were lysed in hypotonic lysis buffer, homogenized in a dounce homogenizer and cytosol and membranes separated by centrifugation at 100 000 *g*. The S100 and P100 fractions were split and one aliquot of each was immunoprecipitated with α-Bcr or α-Raf antibodies. The immunoprecipitates were separated by SDS–PAGE, transfered to nitrocellulose and the Western blots probed with the different antibodies as indicated.

Figure 5A shows the distribution of total Bcr between the cytosol and pellet fractions. Roughly 90% of Bcr is in the cytosol and ~10% is associated with membranes or other cell organelles (most obvious in lane S3 and S4 compared with P3 and P4, where Bcr is not overexpressed). Figure 5B shows the distribution of Raf, which is mainly present in the soluble fraction. The co-expression of active Ras leads to an increase in membrane association of Raf (compare lane P1 with P2, P3 with P4, P5 with P6).

Figure 5C shows the distribution of Bcr that is bound to Raf. Sixty to 70% of the Bcr co-immunoprecipitated with Raf is present in the pellet. The increase of Bcr in the pellet of Ras co-expressing cells reflects the increase of Raf in these pellets. Since it appears from the Western blot that only 5–10% of total Raf is in the P100 fraction (see Figure 4B), these results would indicate that a much higher percentage of Raf in the membrane is complexed to Bcr and show therefore that translocation of Raf to the membrane does not only lead to its activation, but also to increased complex formation with Bcr.

The activation of Raf can be shown in an *in vitro* coupled kinase assay by measuring the phosphorylation of myelin basic protein upon activation of MEK and ERK by Raf. Raf immunoprecipitated from the P100 of COS cells co-expressing active Ras activates the MAP kinase pathway at least 30-fold over Raf that has not been co-expressed with Ras. Co-expression of Bcr does not influence Raf activation under these conditions (data not shown). In an α-Bcr immunoprecipitation of the same cells ~10% of this Raf activity can be co-immunoprecipitated. In addition Raf immunoprecipitated by α-Bcr antibodies is

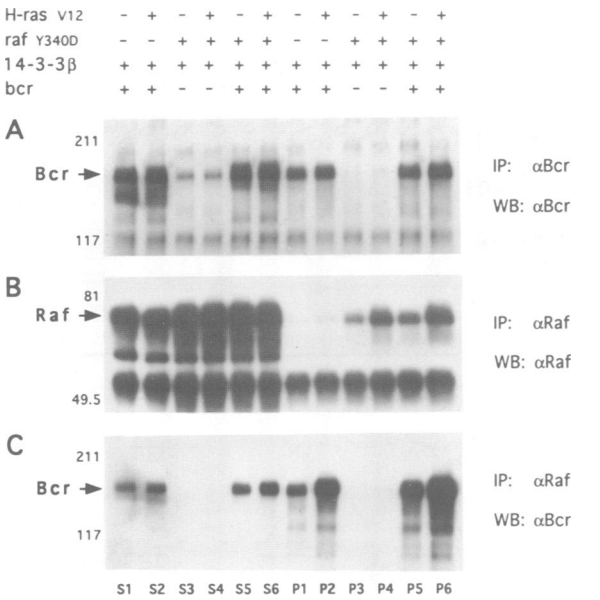


Fig. 5. Preferential binding of Bcr to membrane associated Raf. COS cells were electroporated with 10 μg bcr, 3 μg 14-3-3β, 5 μg rafY340D and 3 μg H-ras V12 as indicated. Before harvesting, the cells were serum-starved for 18 h, lysed in hypotonic lysis buffer and S100 and P100 prepared as described. Both S100 and P100 were split and one aliquot each immunoprecipitated with α-Bcr antibody (Rb1) and α-Raf antibody (C-20) as indicated (IP). The immunoprecipitates were separated by SDS–PAGE on 8% gels (Novex), transferred to nitrocellulose and probed with α-Bcr antibody (TrpE-Bcr) or α-Raf antibody (C-20) as indicated (WB). S1–S6 are immunoprecipitations of the S100 fractions, P1–P6 are immunoprecipitations of the corresponding P100 fractions. IP = immunoprecipitation, WB = Western blot. The lower band in (B) is the IgG heavy chain.

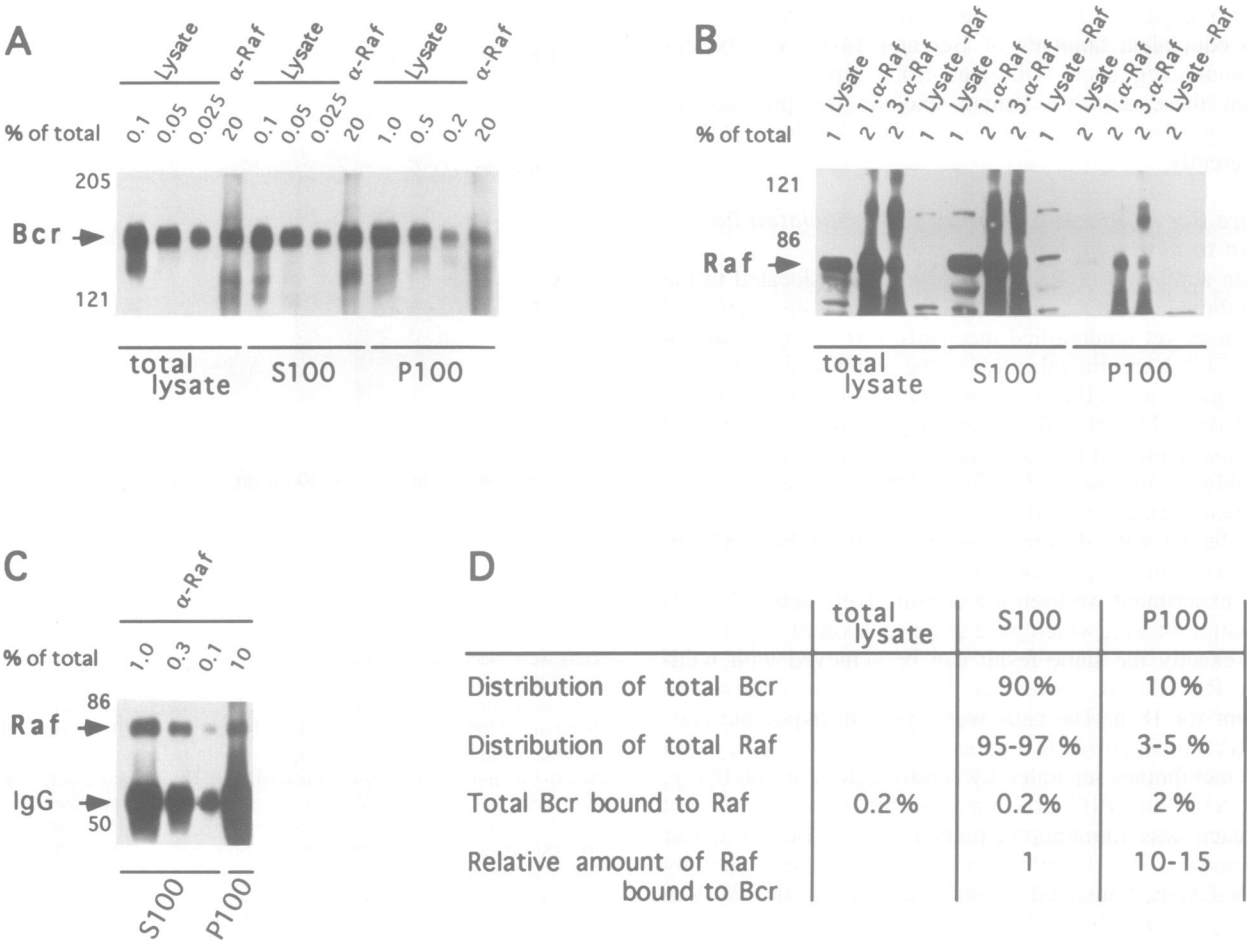
30-fold more active when co-expressed with active Ras (data not shown).

These results show that Bcr binds preferentially to activated, membrane associated Raf, however, this complex formation does not influence Raf activation of the MAP kinase pathway.

Stochiometry of the Raf–Bcr complex

In order to get a more precise impression of how much Bcr and Raf are in a complex *in vivo*, we depleted cell lysates of Raf by several consecutive immunoprecipitations and compared the amount of total Bcr present in the cell lysate with the amount of Bcr bound to Raf. We performed 10 independent experiments with untransfected COS cells as well as transient transfection of different combinations and amount of Bcr, wild-type Raf and the Y340D mutation of Raf that had been used in Figure 5. In each case the percentage of protein in the complex was in the same range, however, when Bcr was not overexpressed the signal was very weak compared with background.

Figure 6D shows the mean numbers calculated from the independent but very reproducible experiments. The numbers were derived by comparing the signal of Bcr in the α-Raf immunoprecipitate with the signal of total Bcr in a serial dilution of the lysate. Figure 6A–C shows representative blots, in this case the COS cells were transfected with 4 μg bcr and 4 μg wild-type raf. Total lysate and S100 and P100 fractions were prepared as described above. The cells were not starved before



D

	total lysate	S100	P100
Distribution of total Bcr		90%	10%
Distribution of total Raf		95-97 %	3-5 %
Total Bcr bound to Raf	0.2%	0.2%	2%
Relative amount of Raf bound to Bcr		1	10-15

Fig. 6. Stochiometry of the Raf–Bcr complex. COS cells were electroporated with 4 μg bcr and 4 μg raf. Before harvesting, the cells were stimulated for 30 min with 20% serum and whole cell lysates prepared (total lysate) of one aliquot and the other cells were lysed in hypotonic lysis buffer and S100 and P100 prepared as described. The lysates were consecutively immunoprecipitated three times for 2 h with fresh α-Raf antibody (C-20). Before the first and after the last immunoprecipitation an aliquot of the lysates was removed. Serial dilutions of the lysates and aliquots of the pooled immunoprecipitates were separated by SDS–PAGE on 8% gels (Novex), transferred to nitrocellulose and probed with α-Bcr antibody, TrpE-Bcr (**A**) or α-Raf antibody, C-12 (**B** and **C**). (**D**) shows the mean values calculated from 10 independent experiments.

harvesting but the cells used for the S100/P100 fractionation were stimulated with 20% serum for 30 min prior to harvesting in order to increase the membrane translocation of Raf. An aliquot of each lysate was taken off (Lysate) and the rest was immunoprecipitated three times with α-Raf antibody for 2 h with protein A–Sepharose beads added for the last 30 min. The immunoprecipitate was collected by centrifugation and the supernatant again immunoprecipitated with fresh α-Raf antibody. After the third round an aliquot of the lysate was taken off again (Lysate -Raf). Aliquots of the lysates and of the immunoprecipitates were separated by SDS–PAGE, transferred to nitrocellulose and the Western blots probed with the different antibodies as indicated.

Figure 6B shows that the C-20 α-Raf antibody very efficiently immunoprecipitates all Raf from the lysate, since there is no more Raf left in the lysate after three consecutive Raf immunoprecipitations (Lysate -Raf). Figure 6A is a Bcr Western blot showing that ~90% of total Bcr is in the soluble fraction, since the signal of 0.2–0.5% of the P100 lysate corresponds to 0.025–0.05% of the S100 lysate.

The signal of Bcr present in 20% of the pooled Raf immunoprecipitates corresponds roughly to the signal of Bcr in 0.025–0.05% of the total lysate. This would mean that all of Raf (100%) is complexed to ~0.2% of total Bcr (0.1–0.5% in nine independent experiments). The same amount of Bcr seems to be bound to Raf in the cytosolic or S100 fraction (0.125 to 0.5% in 10 independent experiments), whereas ~10 times as much (1–2.5% in 10 independent experiments) is complexed to Raf in the membrane or P100 fraction.

Unfortunately we were not able to do a similar calculation for the percentage of Raf bound to Bcr because the available α-Bcr antibodies are not good enough to deplete all Bcr from the lysate. However, we could determine the relative distribution of Raf between the S100 to the P100 by serial dilution of the α-Raf immunoprecipitate. Figure 6C shows that in this experiment ~3–5% of Raf is in the particulate fraction, or in other words, 20–30 times more Raf is in the S100 than in the P100. However, the amount of Bcr bound to total Raf in this experiment is only twice as much in the S100 than in the P100 (mean value of nine independent experiments, also see Figure 6A). This would mean that 10–15 times more Raf is bound to Bcr in the P100 than in the S100.

In summary, we have been able to determine that although in the soluble fraction only ~0.2% of total Bcr is complexed to Raf, the amount of complex is enormously increased when Raf is membrane-associated and active, since in the membrane fraction at least 10 times more Bcr is bound to Raf and 10–15 times more Raf is bound to Bcr.

Discussion

14-3-3β binds to the serine/threonine rich B box in the Bcr kinase domain

A member of the 14-3-3 family of proteins was isolated in a two-hybrid screen for proteins that are able to bind to Bcr. 14-3-3β binds specifically to the serine/threonine kinase domain encoded by the first exon of Bcr. This domain contains two serine/threonine rich regions, the so-called A box (amino acids 197–239 in human Bcr) with 28% serines, and the B box (amino acids 299–385) with 36% serines and threonines (Pendergast *et al.*, 1991). These two boxes are the binding sites for the Abl SH2 domain, which binds to Bcr in a phosphotyrosine independent manner, and they are necessary but not sufficient to render the Bcr–Abl chimeric oncoprotein transforming (Pendergast *et al.*, 1991). Further dissection of the kinase domain of Bcr in the two-hybrid system showed that 14-3-3β binds specifically to the B box. This box also contains two pairs of cysteines and the second cysteine in the first cysteine pair (Cys322) has been shown to be essential for the serine kinase function of Bcr (Maru and Witte, 1991). We therefore tested the effect of such a mutation on the interaction between Bcr and 14-3-3β. Although binding was abolished when this mutation was introduced into the smallest fragment of Bcr binding to 14-3-3β, namely the B box, binding was only slightly reduced when Cys332 was mutated in the context of full-length Bcr. It seems therefore, that C332 is not a direct determinant in 14-3-3 binding, but rather is important for proper structure or folding of the protein, as it might be involved in disulfide bridges. Interruption of these structures would probably be more deleterious in smaller fragments, explaining the loss of binding to the Bcr kin B C322L clone.

More likely, the overall structure and sequence context are important for binding. The sequences in Bcr that bind to 14-3-3β are very similar to regions in Raf and polyomavirus middle T, which all contain cysteine pairs in a serine rich context, and which all bind to proteins of the 14-3-3 family. For example, the sequences in box B of Bcr are 32% identical over an 87 amino acid stretch to the conserved region 2 (CR2) of Raf-1, with 17 serines and threonines conserved between Bcr and Raf-1. However, 14-3-3β binds less strongly to Bcr than to Raf and there is still specificity in binding to the different members of the 14-3-3 family. Reuther and colleages also found that the T-cell specific 14-3-3 protein (14-3-3τ) binds to Bcr (Reuther *et al.*, 1994). We have demonstrated that the ζ isoform, which binds to Raf, does not bind to Bcr in the two-hybrid system. This correlates well with the data of Reuther *et al.* (1994) who report that 14-3-3ζ is a poor substrate for the Bcr kinase activity. Moreover, protein kinase C (PKC) also contains these zinc finger-like structures and since it is regulated by 14-3-3 proteins, it seems more than likely that, under certain conditions, the two proteins will directly interact. However, we were not able to show binding of 14-3-3β or ζ to bovine PKCα in the two-hybrid system (not shown). This might indicate that 14-3-3 proteins as inhibitors only bind to activated PKC, or it might also indicate that PKCα and 14-3-3β and ζ are not the right combination of isoforms. In agreement with our results, Fu and colleagues also did not detect interaction between 14-3-3 proteins and PKCα *in vivo* (Fu *et al.*, 1994).

We attempted to identify the region in 14-3-3β responsible for the interaction with Bcr. All members of the 14-3-3 family contain two regions that could be involved in protein–protein interaction. One is a sequence homologous to the pseudosubstrate site in PKC (Aitken, 1992) and since 14-3-3 proteins are inhibitors of PKC, this might be a potential site of interaction. Another interesting feature is a sequence homology to the conserved C-terminus of Annexin proteins, and a peptide corresponding to this region is able to inhibit binding of activated PKC to its receptors (Mochly-Rosen *et al.*, 1991). Moreover, Annexin V has been shown to inhibit PKC (Schlaepfer *et al.*, 1992), and therefore this conserved region may likewise constitute a binding site for 14-3-3 to other proteins. We therefore made several fusion proteins of the different domains in the two-hybrid vectors. However, none of the isolated domains of 14-3-3β was able to bind to Bcr (data not shown). Either it is not possible to dissect further the small 30 kDa protein, or other features of the protein are essential for binding. 14-3-3 proteins are known to exist as dimers and during the preparation of this manuscript a report has been published which identifies a domain within the extreme N-terminal 26 residues of mammalian 14-3-3 as being responsible for dimerization (Jones *et al.*, 1995). Dimer formation might be essential for binding and will not be possible when the protein is expressed as smaller fragments.

14-3-3 is a new type of adaptor, linking Raf and Bcr into one complex in vivo

Recently, 14-3-3 proteins have been shown to be able to bind to a variety of cellular proteins. In our laboratory and in others it has been shown that the proto-oncogene Raf-1 binds to the β and ζ isoform of 14-3-3 both in the yeast two-hybrid system and *in vivo* (Freed *et al.*, 1994) and here we show that 14-3-3β binds to Bcr in the two-hybrid system and *in vivo*. Bcr also binds to the 14-3-3τ, the T-cell specific isoform (Reuther *et al.*, 1994).

In this paper we suggest that 14-3-3 dimers act as a new type of adaptor, linking Bcr and Raf into a specific complex. All results presented in this paper lead to this suggestion: first, both Bcr and Raf bind to specific isoforms of the 14-3-3 family of proteins. Second, Raf does not bind to Bcr directly in the yeast two-hybrid system, but co-expression of 14-3-3β results at least in a weak complex formation between Raf and Bcr, since it allows for growth on selection medium lacking histidine. Third, Raf and Bcr are indeed found in a complex *in vivo* and the same region of Bcr that binds to 14-3-3, namely the kinase domain or, more specifically, the serine/threonine rich region B in the kinase domain, is sufficient for complex formation between Bcr and Raf *in vivo*.

We have not been able to show a dependence on 14-3-3 in the complex formation between Bcr and Raf *in vivo*

because we could not find conditions in which there is no 14-3-3. All cells already express a lot of 14-3-3 proteins, so that overexpression of 14-3-3 in COS cells does not lead to an obvious and reproducible increase in complex formation. Even Raf protein expressed from a bacculovirus expression vector in Sf9 cells is already complexed to insect 14-3-3 (Freed *et al.*, 1994). The fact that both Raf and Bcr co-immunoprecipitate 14-3-3 proteins does not prove that the same 14-3-3 molecule or dimer is actually bound to Raf and Bcr at the same time, that is, in a ternary complex, since the population of 14-3-3 bound to and co-immunoprecipitated with Bcr could be distinct from the 14-3-3 molecules bound to Raf. However, when the three proteins were transiently co-expressed in COS cells and the cytosolic protein extracts were subjected to Mono Q ion exchange chromatography, all three proteins co-eluted in the same fractions. This chromatography also shows clearly that neither all of 14-3-3 nor all of Bcr is bound to Raf, since the peak of Raf does not correlate with, and only overlaps, the peak of the other two proteins. However, both 14-3-3 and Bcr could be co-immunoprecipitated with α-Raf antibodies from the separate fractions and the profile of Bcr bound to Raf now exactly matched the profile of 14-3-3 bound to Raf. When the fractions containing the peak of Raf-co-immunoprecipitable Bcr and 14-3-3 were pooled and resubjected to the differently charged Mono S chromatography column, Raf, 14-3-3 and Bcr still co-eluted, strongly suggesting that these three proteins indeed exist as a stable complex. Moreover, a minor proportion of Raf and 14-3-3 co-eluted with Bcr in a high molecular weight complex from a gel filtration column. The identical profile of 14-3-3 and Bcr in the different fractions of the Raf immunoprecipitation of the Mono Q fractions suggests that the same Raf molecules that bind 14-3-3 also bind Bcr. Since Bcr does not bind to Raf in the two-hybrid system it seems therefore more than likely that 14-3-3 acts as the adaptor between these two proteins.

The kinase domain of Bcr present in the Bcr–Abl oncogene has been shown to link Bcr–Abl to the Ras signalling pathway through phosphorylation of a tyrosine (Tyr177) in the Bcr kinase domain by the activated Abl kinase, which then serves as a binding site for the SH2 domain of the Grb2 adaptor protein (Pendergast *et al.*, 1993; Puil *et al.*, 1994). Moreover, it has been shown that Ras is required for transformation by Bcr–Abl (Sawyers *et al.*, 1995). The complex between Bcr and Raf reported here would be an additional link to the Ras and MAP kinase pathway. We have not investigated whether Bcr–Abl is also complexed to Raf, but assume that this is the case since Raf coprecipitates the kinase domain of Bcr, which is present in the Bcr–Abl fusion protein, and since it has been shown that 14-3-3τ also binds to Bcr–Abl (Reuther *et al.*, 1994).

Another point of convergence of Bcr and the Ras pathway is the GTPase activating domain for the small GTP binding protein Rac encoded by the C-terminus of Bcr. Rac1 has recently been shown to be involved in transformation by Ras, since it can inhibit transformation by active Ras, but not RafCAAX, with which it cooperates (Qiu *et al.*, 1995). The binding of Bcr containing a Rac GAP domain to active Raf might be of importance in this respect. The other domains of Bcr, the PH and the dbl domain, may also participate in cross-talk between signalling pathways involving Raf.

So far we have not seen any biological effect of the complex formation on Bcr or Raf. Overexpression of Bcr neither inhibits nor stimulates the Raf activation of the MAP kinase pathway. Raf on the other hand does not phosphorylate Bcr (data not shown). Bcr has been shown to phosphorylate 14-3-3τ (Reuther *et al.*, 1994), but the binding of at least 14-3-3β to Bcr does not seem to be involved in the kinase function of Bcr, since the C332L mutation in Bcr abolishes kinase function but not binding to 14-3-3β.

The observation that Bcr binds much more to membrane associated, and thereby activated, Raf makes it the first example of a protein preferentially bound to active Raf, since all other proteins that so far have been shown to form a complex with Raf, like hsp90/p50 and also 14-3-3, have been shown to bind to Raf regardless of activation status or membrane association (Wartmann and Davis, 1994). Recently it has been reported that 14-3-3 does not bind to activated Raf (Li *et al.*, 1995). Our own observations and published data (Freed *et al.*, 1994; Fu *et al.*, 1994) show that 14-3-3β binds to membrane associated Raf, although this binding might be of lower affinity. The increased complex formation between Bcr and active Raf is not due to increased binding of 14-3-3, but is probably due to increased dimerization or other effects of postranslational modification of 14-3-3, which can be phosphorylated by Bcr and possibly also by Raf. The need for modification of 14-3-3 could explain our finding that, when added to a lysate of COS cells, bacterially produced 14-3-3β does bind to both Raf and Bcr in this lysate, yet does not result in increased complex formation between Bcr and Raf in the same lysate (data not shown).

Although the percentage of the proteins involved in the complex is extremely small, the marked increase of complex formation in the membrane fraction is a strong indication for its physiological significance. An important effect could be the correct localization of Bcr and Raf, which are both kinases, in respect to each other and their substrates. The localization of Raf to the membrane is not only strictly regulated but is also the mechanism of its activation. Bcr is a multidomain protein and it is tempting to speculate that the interaction between Bcr and active Raf will modify some of these as yet unidentified activities.

The function of 14-3-3 proteins as a new type of adaptor leads to the expectation of a multitude of different protein complexes. The recent paper by Li and collegues (Li *et al.*, 1995) reporting that 14-3-3 proteins activate Raf kinase activity is not in contradiction to an adaptor function for 14-3-3, since the purified 14-3-3 protein does not activate Raf *in vitro* but needs mammalian cell extracts for this effect. To date, a number of proteins have been reported to be able to bind to specific 14-3-3 isoforms *in vivo*, and this list is likely to grow. Together with the growing family of 14-3-3 isoforms, a great variability, but at the same time specificity, can be envisaged through cell type specific expression of the isoforms, isoform specific dimerization and modification by the partner protein. A careful analysis of the 14-3-3 family of proteins will therefore be very important.

Materials and methods

Plasmids and antibodies

All plasmids were generated according to standard molecular biology procedures and sequenced to determine the proper reading frames of fusion proteins and mutations. The bcr cDNA and the mutated form was provided by Owen Witte, Howard Hughes Medical Institute, UCLA. The two-hybrid system fusion clones were generated by isolating the Bcr fragments as indicated in Figure 1A, the restriction sites blunted by filling-in with Klenow polymerase and ligated into the appropriate, blunted sites in the GAD vector to generate the correct reading frame. The control plasmids GAD-Raf, GBT-Raf, GBT-Bcl-2 and GBT-p85 encoded fusions to full-length proteins, GAD-pp125^{FAK} was N-terminally truncated at amino acid 94. The 14-3-3β clone was isolated in the two-hybrid screen and contained the full-length 14-3-3 protein in-frame with the Gal4 sequence, beginning at position −250 with a deletion from −97 to −3. The GBT-Raf and the 14-3-3ζ clone were kindly provided by Ellen Freed, GAD-Raf by Marcel Spaargaren and GBT-Bcl-2 by Maria J.Fernandez-Sarabia.

All cDNAs used in COS cells have been cloned into the mammalian expression vector EXV3. Full-length Bcr was cloned as an *Eco*RI fragment. The EE-RafY340D plasmid contains a GluGlu tag (EYMPME) at the C-terminus and was a kind gift of Susan Macdonald, Onyx. 14-3-3β has been tagged with a KT3 epitope (PPEPET) at the C-terminus and has been kindly provided by Ellen Freed.

Bcr immunoprecipitations were performed with the polyclonal Rb1 antibody directed against the 16 N-terminal amino acids of Bcr, the Western blots with a polyclonal antibody directed against a trypE fusion to amino acids 160–632 of Bcr (Timmons and Witte, 1989). Both antisera were generously provided by Daniel Afar and Owen Witte, Howard Hughes Medical Institute, UCLA. Raf immunoprecipitation and Western blots were done with the C20 or the C-12 antibody from Santa Cruz Biotechnology, and 14-3-3 immunoprecipitation and Western blots with the antibodies directed against the KT3 tag.

Yeast two-hybrid system

All yeast cultures were grown in standard liquid or on solid media, either based on rich YPD medium [1% (w/v) bacto-yeast extract, 2% (w/v) bacto-peptone, 2% (w/v) glucose], or minimal SC medium [0.67% yeast nitrogen base without amino acids (DIFCO), 0.075% complete supplement medium (lacking the indicated amino acids; BIO 101), 2% (w/v) glucose], both in the presence of 100 mg/l adenosine hemisulfate. For the yeast two-hybrid system the *Saccharomyces cerevisiae* strain YGH-1 (genotype: *MATa ura3-53 his3-200 ade2-101 lys2-801 trp1-902 leu2-3 Can^r gal4-542 gal80-538 LYS2::gal1$_{UAS}$-gal1$_{TATA}$-HIS3 URA3::gal1-lacZ*) was cotransformed with the different pGBT 8 and pGAD plasmid constructs (Hannon *et al.*, 1993). These yeast expression vectors contain the 2μ origin of replication trp1 (pGBT8) or leu2 (pGAD) gene for selection in yeast, and have under control of the strong ADH1 promoter either the GAL4 DNA-binding domain (amino acids 1–147; pGBT8) or the GAL4 activation domain (amino acids 768–881; pGAD), which are followed by a multiple cloning site for the in-frame generation of fusion proteins and a nuclear localization signal (Fields and Song, 1989; Hannon *et al.*, 1993). Transformation was performed by the lithium acetate method essentially as described in Schiestl and Giest (1989). Transformants were plated on solid SC medium lacking Trp and Leu for selection of double transformants and determination of transformation efficiency, and on SC plates lacking Trp, Leu and His to select for His$^+$ colonies. Qualitative determination of β-galactosidase activity was performed on His$^+$ yeast colonies or patches on SC–Trp/–Leu/–His plates by a filter assay using 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-Gal) as substrate, essentially as previously described (Hannon *et al.*, 1993).

COS cell transfection, immunoprecipitation and Western blot analysis

COS cells were electroporated (Huang *et al.*, 1993) with various plasmid combinations as indicated and plated onto 10 cm dishes. Seventy-eight hours later the cells were washed with PBS and 500 ml ice-cold lysis buffer was added [20 mM Tris–HCl (pH 8), 1% Triton X-100, 10% glycerol, 137 mM NaCl, 1.5 mM $MgCl_2$, 1 mM EGTA, 50 mM NaF, 200 μM Na_3VO_4, 1 mM PMSF, 20 μM leupeptin, 10 μg/ml aprotinin, 0.1 mM Pefabloc]. All further manipulations were done at 4°C. Cells were lysed for 20 min on a rocking plate, the lysates collected into Eppendorf tubes and cleared of nuclei and detergent insoluble material by centrifugation for 10 min at 14 000 r.p.m. in an Eppendorf microfuge. The lysates were precleared by incubating with protein A–Sepharose beads for 30 min on a wheel, centrifuged for 10 s at 14 000 r.p.m. in an Eppendorf microfuge and the supernatants were immunoprecipitated with different anibodies as indicated for 2 h with protein A–Sepharose beads added for the last 30 min. Immunoprecipitates were collected by centrifuging for 10 s at 14 000 r.p.m. in an Eppendorf microfuge and were washed twice with 1 ml of high salt (500 mM NaCl) lysis buffer and once with low salt (137 mM NaCl) lysis buffer. The drained beads were taken up in SDS–PAGE sample buffer and boiled for 5 min at 95°C. Equal amounts of lysate were separated by SDS–PAGE on 8% (for Raf and Bcr) or 12% (for 14-3-3) Tris–glycine gels (Novex). Separated proteins were transferred to nitrocellulose in a Bio-Rad transblot apparatus at 300 mA for 55 min at 4°C and the blots were blocked overnight in PBS with 5% milk and 0.1% Tween-20 at 4°C. Blots were washed once in TPBS (PBS with 0.05% Tween-20) and incubated with a 1/1000 dilution of the first antibody in TPBS with 3% BSA for 1 h at room temperature. The blots were washed three times with TPBS before incubating with goat-anti-rabbit second antibody coupled to horseradish peroxidase (1/15000 in TPBS–3%BSA, Bio-Rad) for 1 h at room temperature. After washing three times with TPBS, immunoreactive proteins were visualized by enhanced chemilumenescence (ECL, Amersham). The 14-3-3 blots were blocked overnight in I-block and incubated for 4 h with α-KT3 antibodies coupled to alkaline phosphatase at room temperature. After extensive washing the immunorective protein was visualized using the Western Light Chemiluminescent Detection System (Tropix, Inc.).

S100/P100 fractionation

COS cells were electroporated as described and after 60 h the cells were deprived of serum for 18 h. The cells were washed with PBS and scraped on ice into hypotonic lysis buffer (10 mM Tris pH 7.5, 5 mM $MgCl_2$, 1 mM EGTA, 1 mM diothiothreitol, 25 mM NaF, 100 μm Na_3Vo_4, 20 μM leupeptin, 10 μg/ml aprotinin and 0.1 mM Pefabloc). After 10 min on ice the cells were homogenized with 50 strokes in a Dounce homogenizer. Cell nuclei were removed by centrifugation and the supernatant was centrifuged at 100 000 *g*. The supernatant (S100) was taken off, brought to 1% Triton X-100 and antibody was added for immunoprecipitation as described. The sedimented fraction (P100) was rinsed briefly and resuspended carefully in 100 μl of hypotonic lysis buffer. The suspension was brought to 1% NP-40, vortexed and centrifuged for 10 min at 14 000 r.p.m. in an Eppendorf microfuge and antibody was added to the supernatant for immunoprecipitation as described.

Ion exchange chromatography

Sixteen 10 cm dishes of COS cells were electroporated with 5 μg Exv bcr, 5 μg Exv EE rafY340D and 3 μg Exv KT3 14-3-3β per dish. After 3 days the cells were harvested in hypotonic lysis buffer and cytosolic extract prepared as described above. The S100 fraction was filtered through a 2μ filter and 10 ml (2.5 mg/ml) were applied on a Mono Q column (HR5/5 Pharmacia LKB) equilibrated with buffer A (20 mM Tris pH7.5, 1 mM DTT, 20 μM leupeptin, 10 μg/ml aprotinin and 0.1 mM Pefabloc). The chromatography was performed on a column connected to a FPLC system at 4°C, flowrate of 1 ml/min, and 1 ml fractions were collected. After washing with buffer A, the proteins were eluted with a 30 ml gradient from 0 to 0.5 M NaCl and the collected fractions analyzed on SDS–PAGE followed by Western blotting. Three hundred and thirty microlitres of fractions 15–27 were brought to 1% Triton X-100, the salt concentration of each fraction adjusted to 450 mM NaCl and immunoprecipitated with αRaf antibody as described above.

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After the submission of this manuscript the crystal structure of the τ isoform dimer [Xiao,B. *et al.* (1995) *Nature*, **376**, 188–191] and of the ζ isoform dimer of 14-3-3 proteins [Liu,D. *et al.* (1995) *Nature*, **376**, 191–194] have been published. In both papers the authors refer to the large channel between the two proposed binding sites in the dimer, which would enable two target proteins to bind at the same time, and suggest therefore that 14-3-3 acts as a scaffold on which other proteins interact.