Using a phage display library to identify basic residues in A-Raf required to mediate binding to the SH2 domains of the p85 subunit of phosphatidylinositol 3’-kinase

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Running Title:
Basic residues in A-Raf are required for p85 SH2 binding
Abstract

Src homology 2 (SH2) domains are found in a variety of cytoplasmic proteins involved in mediating signals from cell surface receptors to various intracellular pathways. They fold as modular units and are capable of recognizing and binding to short linear peptide sequences containing a phosphorylated tyrosine residue. Here we show that each of the SH2 domains of the p85 subunit of phosphatidylinositol 3’-kinase selects phage displayed peptide sequences containing the core L/I-A-R/K-I-R. The serine/threonine kinase A-Raf, containing the sequence LQRIRS, is associated with the p85 protein in both quiescent and growth factor stimulated cells. This suggests that p85 and A-Raf exist in a protein complex in cells and that complex formation does not require growth factor stimulation. We also show that p85 and A-Raf can bind directly to each other \textit{in vitro} and that this interaction is mediated in part by the p85 SH2 domains. Further, the p85 SH2 domains require at least one of four distinct basic-X-basic sequence motifs within A-Raf for binding. This is the first description of a phosphotyrosine-independent SH2 domain interaction that requires basic residues on the SH2 ligand.
Introduction

Phosphatidylinositol 3’-kinase (PI3 kinase) consists of an 85 kDa regulatory subunit (p85) and a 110 kDa catalytic subunit (p110), the latter of which is responsible for the phosphorylation of phosphatidylinositol lipids at the D3 position and serine phosphorylation of proteins (1-3). The p85 subunit contains a Src homology 3 (SH3) domain capable of binding to proline-rich sequences, a region homologous to the breakpoint cluster region (BCR) gene product, a p110 binding domain (110) and two SH2 domains. PI3 kinase activity increases in response to platelet-derived growth factor (PDGF) binding to its receptor, in large part because the p85:p110 complex is relocalized from the cytosol to the lipids at the plasma membrane, by p85 SH2 domains binding directly to tyrosine phosphorylated sites on the receptor (4,5).

The SH2 domains of p85 recognize and bind to proteins such as the PDGF receptor at sites that contain a pY-X-X-M sequence (pY = phosphotyrosine; X = any amino acid; M = methionine) in a phosphorylation-dependent manner. The residues within the p85 SH2 domain responsible for binding this sequence include a critical arginine residue that coordinates twice with the phosphate group of the phosphotyrosine residue and a hydrophobic pocket involved in methionine binding (6-8).

Over the past several years there have also been reports of SH2 domains binding to proteins via a phosphotyrosine-independent mechanism. These reports include several phosphoserine/phosphothreonine-dependent interactions (9-14). In addition, there have been a few reports that concluded that the SH2-mediated interaction was phosphotyrosine-independent but did not determine whether or not the interaction was instead dependent upon phosphoserine or phosphothreonine (15-18). In each instance, the precise amino acid residues involved in mediating these SH2 domain interactions both within the SH2 domain and on the SH2-bound
ligand have yet to be identified. There are also two reports of SH2 domains that can bind to unphosphorylated forms of the preferred tyrosine-containing peptides, albeit more weakly than their phosphorylated counterparts (19,20). The physiological significance of these low affinity interactions has yet to be demonstrated.

In this report, we describe a unique SH2 domain interaction involving the SH2 domains of the p85 subunit of PI3 kinase and the serine/threonine kinase A-Raf. This is the first description of an SH2 domain interaction that requires positively charged basic residues within the SH2 ligand (i.e. the A-Raf protein) to mediate binding to the p85 SH2 domains. Our results indicate that there are four distinct sites on the A-Raf protein, each of which is sufficient for p85 SH2 domain binding. Further, we also find that the p85 SH3 domain can bind A-Raf, suggesting that the p85:A-Raf complex is mediated by multiple domain interactions. We observe the presence of this p85:A-Raf complex in both quiescent and PDGF-stimulated cells, but do not find a complex between p85 and the more extensively characterized c-Raf kinase. These results suggest the possibility that p85 may act as an adapter protein for A-Raf, as it has been shown to do for the p110 catalytic subunit of PI3 kinase (5).
Experimental Procedures

Selection of SH2 domain binding phage – A phage display library (21), composed of a filamentous phage displaying random hexapeptides on its surface, was amplified. Selection of phage able to bind to the bait samples was carried out essentially as described (21) with the following changes. The TrpE fusion proteins used as bait were prepared (22,23) and immobilized using anti-TrpE antibodies and protein A sepharose beads (24). Throughout the selection, TrpE-bait samples remained immobilized on the beads and were recovered by centrifugation. Repeated rounds of selection used a fresh sample of immobilized TrpE-SH2 fusion protein. In addition, a control experiment using immobilized TrpE protein (no SH2) was also performed in order to control for sequences binding to non-SH2 components, such as the TrpE protein, TrpE antibodies, and protein A sepharose beads. Phage clones were randomly isolated after three rounds of selection for each TrpE-bait sample. The DNAs in their display regions were sequenced using the T7 DNA sequencing kit (Pharmacia) and a primer (5’-CCA GAC GTT AGT AAA TGA ATT TTC TGT AT-3’) which binds 45 nucleotides 3’ to the display region. Note that GST-fusion proteins should not be used unless the GST portion is removed since we observed a strong selection for the sequence RRWTWS with the GST protein alone and with several GST-fusion proteins each of which was immobilized on glutathione sepharose beads.

Immunoprecipitations and Immunoblots – Immunoprecipitations, immunoblotting and stripping methods have been described (25). The antibodies used were: preimmune (IgG purified from normal rabbit serum using a DEAE affi-gel blue column, Biorad, according to the manufacturers directions), A-Raf from Transduction Laboratories and c-Raf from Santa Cruz Biotechnology. The p85 antibodies used for immunoprecipitations were either from Transduction Laboratories (Fig 1A-B) or were rabbit p85 antibodies raised against residues 314-724 of bovine p85α (Fig.
These antibodies were affinity purified using a CNBr-activated sepharose column to which the protein antigen had been conjugated, according to the manufacturers instructions. The p85 antibodies used for immunoblotting were from Upstate Biotechnology. HA antibodies were from Roche Diagnostics (12CA5) or from Santa Cruz Biotechnology (Fig. 5). Data are representative of at least three independent experiments.

**Expression and Purification of His-A-Raf**– The cDNA encoding full length human A-Raf was amplified using polymerase chain reaction (PCR) and cloned into a *Bam*HI-*Eco*RI digested pBlueBacHis2A vector (Invitrogen). Subsequent steps to generate and express the histidine (His) tagged A-Raf protein were all carried out precisely as detailed in the Invitrogen MaxBac 2.0 transfection and expression manual. Briefly, the resulting plasmid was cotransfected with Bac-N-Blue DNA (Invitrogen) into Sf9 insect cells. Recombinant virus from the medium was harvested, diluted and used to infect fresh Sf9 cells. The infected Sf9 cells were overlayed with agarose containing 5-bromo-4-chloro-3-indolyl-β-D-galactoside to visualize the recombinant plaques. Recombinant virus from individual blue plaques were isolated, amplified in Sf9 cells and the viral DNA was isolated from a portion of the viral particles in the culture medium. A PCR analysis of the viral DNA allowed the identification of pure recombinant baculovirus that had undergone homologous recombination with our plasmid encoding His-A-Raf.

To obtain purified His-A-Raf protein, Sf9 cells were infected at a multiplicity of infection of 5, and harvested 4 days post infection. Sf9 cell pellets (10⁸ cells) were lysed in 50 mM sodium phosphate pH 7.0 / 300 mM NaCl by two freeze/thaw cycles. After removal of the cellular debris by centrifugation, the majority (~80-90%) of the soluble protein was His-A-Raf (Fig. 2C, lane 2). The sample was further purified on a Sephacryl S-200 HR column and the His-A-Raf-containing fractions were identified by SDS-PAGE and Coomassie blue staining (25).
His-A-Raf fractions were dialyzed against water, lyophilized and resuspended in 50 mM sodium phosphate pH 7.0 (Fig. 2C, lane 3).

Expression of HA-A-Raf – The cDNA encoding full length human A-Raf was amplified using PCR and cloned into a BglII-EcoRI digested HA3 vector. This vector is a modified form of pACTAG2 (26) that encodes three copies of a 9 amino acid repeat of the hemagglutinin (HA) tag prior to the multiple cloning site. The single EcoRI and BglII sites were removed from non-essential regions of this vector using separate digest and fill-in reactions. A pair of oligonucleotides (5’-AGC CGC AGA TCT AGA GTT AAC TCT GAG GGC C-3’ and 5’-CTC AGA ATT CGA GTT AAC TCT AGA TCT GC-3’) were then ligated into NotI-Apal digested vector to alter the multiple cloning site such that it now lacked a NotI site but instead contained the following unique sites: BglII, XbaI, HpaI, EcoRI and Apal. In vitro transcription of HA-A-Raf mRNA was carried out using linearized plasmid DNA as a template and the mMESSAGE mMACHINE™ kit (Ambion) according to the manufacturers directions. The HA-A-Raf mRNA was translated into protein using Retic Lysate IVT™ kit (Ambion) following the manufacturers protocol. Mutants were generated using the QuikChange site-directed mutagenesis method (Stratagene), according to the manufacturers directions. For site E, all four basic residues (KKKVK) were changed to alanine (AAAVA). The integrity of both wild type and mutant clones were verified by DNA sequencing of the entire coding region.

Cell Culture – NIH 3T3 cells (American Tissue Type Collection) were maintained as described (25). For PDGF stimulations, NIH 3T3 cells that were 80% confluent were serum starved in medium containing 0.5% fetal bovine serum for 2 days. They were then either stimulated for 5 minutes with 50 ng/mL PDGF BB or left unstimulated (25). HA-A-Raf samples were expressed transiently in COS-1 cells (American Tissue Type Collection) using a Lipofectamine delivery
system (Canadian Life Technologies) according to the manufacturers directions. Cells were lysed and samples were normalized after densitometric scanning of immunoblots of cell lysates (25) prior to their use in reconstitution experiments.

**GST-p85 proteins and Pull Down Experiments** – GST-p85 fusion proteins were generated by PCR amplification of the indicated p85 regions, and were cloned into pGEX2T (Pharmacia). The amino acids of bovine p85α present in each fusion protein are: SH3 (1-83), BCR (78-332), N-SH2 (314-446), 110 (431-623), C-SH2, (614-724), p85 (1-724), ΔSH3 (78-724). The sequence of each clone was verified by DNA sequencing. All inductions yielded proteins of the expected size as judged by Coomassie staining. Pull down experiments were carried out as described previously (23), using 10 µg of GST-fusion protein. A lysate control lane was included in each experiment and contained 10% of the amount of lysate present in the pull down mixture. Blots were stripped and reprobed with anti-GST antibodies (Santa Cruz) to ensure that comparable amounts of GST-p85 fusion proteins were present in each experiment (data not shown).
Results

Identification of SH2 domain-binding peptides that lack phosphotyrosine residues – We have taken a systematic approach to directly address the possibility that at least some SH2 domains may be able to bind to peptides/proteins in a phosphotyrosine-independent manner, and if so to identify the sequences responsible for mediating SH2 domain binding. Phosphotyrosine-dependent SH2 domain interactions have been described as high affinity interactions (4,27-30), therefore a system was chosen to look for possible phosphotyrosine-independent SH2 interactions in which phosphotyrosine residues would not be present. By using a phage displayed hexapeptide library, propagated in bacteria known to be devoid of tyrosine kinases (31-33), none of the peptides contained any phosphotyrosine residues. The phage were engineered to express a library of hexapeptide sequences on their surfaces as targets for SH2 domain binding, yet each phage contained only one hexapeptide sequence (21,34). Similar phage display libraries have been used previously to characterize the binding specifies of monoclonal antibodies and SH3 domains (35-39).

To confirm that the library contained a wide variety of hexapeptide sequences, 50 phage clones were randomly selected and the DNAs in their display regions were sequenced. The sequences obtained in this random sample of the library encoded a diverse assortment of hexapeptides (data not shown) and all were distinct from the sequences in Table 1. The individual SH2 domains of the p85 subunit of PI3 kinase, the GTPase activating protein of p21\textsuperscript{ras} (GAP) and phospholipase Cγ1 (PLCγ1) were expressed as TrpE-fusion proteins, collected using anti-TrpE antibodies and immobilized on protein A sepharose beads (22-24). Each was then used as bait to select phage displayed hexapeptides from the library which were capable of binding to the SH2 domain. In addition, a control experiment using immobilized TrpE
protein (no SH2) was also performed in order to control for sequences binding to non-SH2 components, such as the TrpE protein, TrpE antibodies, and protein A sepharose beads.

After three rounds of selection, each SH2 domain tested did show a preference for particular phage displayed hexapeptide target sequences and these were distinct from the TrpE-selected sequences (Table 1). The sequence GDYTLF was selected by each of the GAP and PLCγ1 SH2 bait proteins to differing degrees, while the strongest selection was demonstrated by the SH2 domains of p85, which preferentially bound phage displayed peptides with a core sequence of L/I-A-R/K-I-R. Note that there were no tyrosine residues in any of the p85 SH2 domain-selected hexapeptides. They did however contain conserved positively charged basic residues within the sequence motif basic-X-basic.

A-Raf contains a similar peptide sequence and is complexed with p85 in cells – Since the p85 SH2 domains demonstrated the strongest sequence selection, we searched the sequence data base with each of the p85 SH2 selected hexapeptide sequences in order to identify potential p85 SH2 binding proteins. One of the proteins identified in the search was the serine/threonine kinase A-Raf that contains the sequence LQRIRS (matching 5/6 amino acids of LARIRS).

To determine if p85 and A-Raf formed a complex in cells, a coimmunoprecipitation analysis was carried out. Both anti-p85 and anti-A-Raf immunoprecipitates were prepared from lysates of NIH 3T3 cells which had been serum starved and stimulated with PDGF, or left unstimulated. Samples were resolved by SDS-PAGE, transferred to nitrocellulose and the resulting Western blot was probed with anti-A-Raf antibodies. Bound antibodies were detected using a horseradish peroxidase conjugated secondary antibody, and visualized with chemiluminescence (Fig. 1A). The blot was then stripped and reprobed with anti-p85 antibodies (Fig. 1B). A-Raf protein was present in both anti-A-Raf and anti-p85 immunoprecipitates, but
not in the preimmune control lanes (Fig. 1A). Similarly p85 protein was observed in both anti-p85 and A-Raf immunoprecipitates, and not in preimmune control lanes (Fig. 1B). This suggests that A-Raf and p85 are constitutively associated in NIH 3T3 cells since the p85:A-Raf complex was observed both prior to and after PDGF stimulation. It is important to note that the p85:A-Raf complex was observed in untransfected NIH 3T3 cells expressing endogenous levels of these two proteins.

To determine if the related Raf kinase family member c-Raf was also able to form complexes with p85, a similar coimmunoprecipitation analysis was carried out. Anti-p85 immunoprecipitates did not contain detectable quantities of c-Raf protein (Fig. 1C) nor did the reciprocal anti-c-Raf immunoprecipitates contain p85 protein (Fig. 1D). These results show that p85 associates with A-Raf but not c-Raf, suggesting that the ability to bind to p85 is a unique feature of the A-Raf family member.

*p85 and A-Raf can bind directly to each other, an interaction mediated by p85 SH3 and SH2 domains* – We then went on to address whether the p85 and A-Raf proteins were binding directly to each other, since it was possible that other mammalian protein(s) could be mediating p85:A-Raf complex formation. The cDNA encoding the full length A-Raf protein was cloned into a vector that encodes three copies of a nine amino acid repeat of the hemagglutinin (HA) tag prior to the multiple cloning site. The HA-A-Raf protein was then synthesized using an *in vitro* transcription/translation system. Two products of similar size were generated (Fig. 2B), with the larger corresponding to HA-A-Raf. The smaller corresponds to A-Raf lacking the HA tag, likely as a result of initiation at the methionine residue at the beginning of the A-Raf sequence. Both full length p85 and the p85 C-terminal SH2 domain (C-SH2) were expressed in a bacterial system as glutathione S transferase (GST) fusion proteins (Fig. 2A). These GST-p85 fusion proteins
were collected on glutathione sepharose beads and tested in a pull down assay for their ability to bind the *in vitro* synthesized HA-A-Raf protein. Bound A-Raf was detected using a Western blot analysis. Both full length p85, as well as the individual p85 C-SH2 domain, but not the GST protein, were able to bind to *in vitro* synthesized A-Raf protein (Fig. 2B).

Further evidence in support of a direct interaction between p85 and A-Raf was provided using a similar pull down assay with isolated GST-p85 fusion proteins and purified baculovirus expressed His-tagged A-Raf (His-A-Raf). His-A-Raf was expressed in Sf9 insect cells using a baculovirus expression system and purified (Fig. 2C, lanes 2-3). Immobilized GST-fusion proteins containing full length p85, the N-terminal SH2 (N-SH2) and the C-SH2, but not GST alone, were capable of binding His-A-Raf (Fig. 2C, A-Raf blot). These results demonstrate a direct interaction between p85 and A-Raf, and show that an isolated p85 SH2 domain is sufficient to bind the A-Raf protein.

To determine if other domains of p85 were involved in A-Raf binding, similar pull down experiments were carried out (Fig. 2D) using a more extensive panel of GST-p85 fusion proteins (Fig. 2A). Several different domains of p85 were capable of independently binding to HA-A-Raf from COS-1 cell lysates, the N-SH2 domain, the C-SH2 domain, and the SH3 domain, as well as the full length p85 protein. In contrast, the BCR homology and p110 binding domains of p85 did not bind significant amounts of HA-A-Raf. This suggests that p85:A-Raf complex formation is mediated by multiple regions of the p85 protein.

*Specific basic-X-basic motifs on A-Raf are required for p85 SH2 domain binding* – Further experiments focused on the localization of the p85 SH2 binding sites on A-Raf. Based on the method used to identify A-Raf as a p85 binding protein, we predicted that the A-Raf sequence LQRIRS would be responsible for mediating p85 SH2 binding. Mutagenesis of both R209 and
R211 in the LQRIRS sequence (site R; Fig. 3A) to alanine, in the context of the full length HA-A-Raf protein, did not prevent its binding to the N-SH2 and C-SH2 of p85 (Fig. 3C-D). While it was possible that a different region of A-Raf may be responsible for p85 SH2 binding, it was also possible that there were multiple p85 SH2 binding sites on the A-Raf protein that could mediate the p85 SH2 domain interaction in the absence of the site R arginines.

To address the former possibility, a series of N-terminal and C-terminal deletion mutants of A-Raf were tested for their ability to bind to GST-p85 SH2 domains (data not shown). The results of these binding experiments were difficult to interpret. Some of the A-Raf mutants no longer bound to the p85 SH2 domains, yet some mutants with larger deletions appeared to restore binding. We interpreted these results to suggest that the three-dimensional structure of A-Raf was important to position key residues within A-Raf for productive p85 SH2 binding. Since the structure of the A-Raf protein is not known, it is not possible to assess the effects of such deletions in the overall folding of the A-Raf protein. We believe that the regained ability of some of the smaller A-Raf mutants to bind to the p85 SH2 domains may reflect alterations in the folding of the truncated A-Raf mutant proteins and/or the exposure of previously buried p85 SH2 binding motifs. Therefore in an attempt to maintain the structure of the A-Raf protein as much as possible, subsequent mutations were made in the context of the full length A-Raf protein.

The most striking feature of the p85 SH2 domain-selected hexapeptides was the two highly conserved basic residues spaced apart by one amino acid. There are a total of nine basic-X-basic sequences in A-Raf (sites R and A-H; Fig. 3A). Both basic residues were mutated to alanine at each of the other eight sites (A-H). The resulting mutant proteins were expressed in COS-1 cells and the level of mutant HA-A-Raf protein expression was assessed (Fig. 3B) and
normalized for subsequent pull down experiments. Each of these mutant HA-A-Raf proteins was still able to bind to the p85 SH2 domains (Fig. 3C-D). The fact that both p85 SH2 domains could bind A-Raf suggested that they might do so at distinct sites. This raised the possibility that multiple p85 SH2 binding sites may be present on A-Raf and that multiple site mutations may be required to prevent SH2 binding.

A-Raf and c-Raf share a high degree of sequence homology, yet c-Raf has been shown not bind p85 in cell lysates (Fig. 1C-D) or to p85 SH2 domains in vitro (11), therefore a sequence comparison between A-Raf and c-Raf was used to guide these mutagenesis experiments (Fig. 4A). Of the nine basic-X-basic sequences in A-Raf, three were identical (A, B and G), two were highly conserved allowing for substitutions of residues with similar properties (F and H), and four were considered to be divergent (C, R, D and E) between A-Raf and c-Raf. Mutation of these four divergent basic-X-basic sites (C, R, D and E; basic residues changed to alanine) was sufficient to prevent binding to each of the p85 SH2 domains (Fig. 4B). This C/R/D/E HA-A-Raf mutant was still able to bind to the full length GST-p85 fusion protein, likely as a result of a p85 SH3 domain-mediated interaction (Fig. 2C), suggesting that the folding of the C/R/D/E HA-A-Raf protein was not destroyed by these mutations. Add back mutants in which a single site was restored to the wild type basic residues were generated and tested in order to determine which of the four sites was actually required for p85 SH2 binding. Addition of any of the four sites (C, R, D or E) was sufficient to restore p85 SH2 binding (Fig. 4C-F). These results suggest there are four p85 SH2 binding sites on the A-Raf protein, each of which contains a basic-X-basic motif.

Experiments were then carried out to determine if the binding between the full length p85 protein and the C/R/D/E HA-A-Raf protein was in fact mediated by the p85 SH3 domain. Wild
type HA-A-Raf, the C/R/D/E mutant unable to bind to p85 SH2 domains, and each of the add back mutants were all able to bind to GST-p85 SH3, but not GST alone (Fig. 5A-F). This confirms that these mutations in HA-A-Raf do not perturb to p85 SH3 binding site on A-Raf.

A further GST-p85 fusion protein lacking only the SH3 domain (ΔSH3) was also generated and tested for its ability to bind wild type and mutant HA-A-Raf proteins (Fig. 5A-F). The GST-p85ΔSH3 fusion protein was also capable of binding to these HA-A-Raf proteins, including the C/R/D/E mutant, suggesting that addition contact(s) are made between the p85 and A-Raf proteins.
Discussion

These results demonstrate that a phage display library can be used to provide target peptide sequences devoid of phosphotyrosine residues, that are capable of binding to bait SH2 domains. Similar peptide sequences to those selected from the phage display library by the p85 SH2 domains were present in the serine/threonine kinase A-Raf and were required for p85 SH2 binding. The p85 and A-Raf proteins were found in the same protein complex within cells, indicating that they interact together in a biological setting, as well as in vitro. Therefore, this approach has facilitated the identification of a previously uncharacterized protein:protein interaction between p85 and A-Raf. The function of the p85:A-Raf complex is not known, however the fact that it is present in both quiescent and PDGF stimulated cells suggests that it is a constitutive association. A similar constitutive interaction has been described previously between p85 and p110 (5). This raises the possibility that p85 may act as an adapter protein for A-Raf, relocalizing it to activated growth factor receptors at the membrane, as p85 has been suggested to do for p110 (5). Since A-Raf has been shown to phosphorylate and activate MEK1 (40), this could provide a Ras-independent mechanism to activate the MAP kinase pathway.

The fact that c-Raf is not found in a similar complex with p85, suggests that different Raf family members may also play unique roles in signal transduction pathways. In support of this hypothesis, it has recently been reported that A-Raf but not c-Raf was detected in highly purified rat liver mitochondria (41). This report also demonstrates that A-Raf interacts specifically with hTOM and hTIM, human proteins with sequence similarity to mitochondrial outer and inner membrane protein-import receptors from lower organisms. The authors suggest that hTOM and hTIM may be involved in the mitochondrial transport of A-Raf. Interestingly, the p110 catalytic subunit of PI3 kinase has sequence homology to Vps34p, a yeast protein
involved in the sorting of proteins to the vacuole (42). This fact coupled with our identification of the p85 subunit of PI3 kinase as an A-Raf binding protein, raises the possibility that p85 may play a role in the subcellular localization of A-Raf.

Both the SH3 domain as well as each of the SH2 domains of p85 were found to be capable of binding independently to the A-Raf protein, suggesting that several distinct regions of each protein are involved in mediating binding. In addition, we find that a p85 fusion protein lacking only its SH3 domain still binds the C/R/D/E HA-A-Raf mutant (unable to bind to p85 SH2 domains) (Fig. 5B). This raises the distinct possibility that region(s) of p85 in addition to the SH3 domain, the N-SH2 domain and the C-SH2 domain are involved in mediating binding between p85 and A-Raf. The abilities of isolated SH3 and SH2 domains to fold as modular domains that retain their binding activities has been demonstrated using nuclear magnetic resonance, x-ray crystallography and functional binding assays (22,24,27,43-46). Whether or not the BCR homology and p110 binding regions of p85 are similarly able to fold appropriately when expressed in isolation is less clear. Therefore, although the BCR homology and p110 binding regions of p85 did not bind HA-A-Raf when expressed as isolated fragments of p85 fused to GST, they may contribute to A-Raf binding, when present in the context of the full length p85 protein. X-ray crystallography of the p85:A-Raf complex will be required to resolve this question.

We have characterized a novel interaction for the p85 SH2 domains that requires basic residues on A-Raf within the sequence motif basic-X-basic. Our results suggest that A-Raf contains four separate basic-X-basic sequences (designated C, R, D, E and containing the sequences LIKGRK, LQRIRS, EQRERK, DKKKVKNL respectively), each of which is capable
of binding to both the N-SH2 and C-SH2 of p85. This is the first report of a p85 SH2 ligand that lacks phosphotyrosine residues.

There have been several reports of phosphotyrosine-independent interactions for other SH2 domains. These reports include several phosphoserine/phosphothreonine-dependent interactions, such as those between: the breakpoint cluster region (BCR) protein, and the SH2 domains of Abl (9) and other proteins (10), the c-Raf kinase and the Fyn SH2 domain (11), the cyclin-dependent kinase homologue p130PITSLRE and the Blk SH2 domain (12), both the c-Raf and MEK1 kinases with the Grb10 SH2 domain (13), as well as the human immunodeficiency virus type 1 Nef protein and the Lck SH2 domain (14). In addition, there have been a few reports that concluded that the SH2-mediated interaction was phosphotyrosine-independent but did not determine whether or not the interaction was instead dependent upon phosphoserine or phosphothreonine. These interactions include: the ubiquitin binding protein p62 and the Lck SH2 domain (15), the SHC adapter protein and the Abl SH2 domain (16), and the Cbl adapter protein and the Fyn SH2 domain (17). One report of a phosphotyrosine-independent SH2-mediated interaction involved a protein expressed in activated lymphocytes, PAL, binding to the SH2 domain of SHC (18). Since the authors were unable to detect any phosphorylation of the PAL protein, they concluded that the interaction must be phosphotyrosine-independent, if not phosphorylation-independent. Curiously, mutation of the conserved arginine required for phosphotyrosine-dependent binding, within the SHC SH2 domain, prevented PAL interaction (18), suggesting that more experiments will be required to establish the basis for this interaction.

In each of these reports, the precise sequence of the ligand binding to the SH2 domain in a phosphotyrosine-independent manner was not determined. Identification of such sequences may be facilitated using the approach we describe here. That is, by screening a phage display library
with each of the SH2 domains involved and then searching the ligand for similar sequences. It is important to note that both of the SH2 domains of GAP and PLC\textgamma 1 also selected distinct sequences from the phage displayed hexapeptide library. Many of these sequences were specific for a particular SH2 domain, but each of these four SH2 domains selected a common sequence, GDYTLF. We therefore suggest that phage display libraries may be used to characterize the binding specificities of other SH2 domains, in addition to those of the p85 protein. This approach should facilitate the identification of novel SH2 binding proteins that may serve important functions in cell signaling pathways.

A-Raf is a very different p85 SH2 ligand compared to the typical pY-X-X-M-containing protein/peptide. SH2 domains in general are best known for their ability to bind proteins or peptides in a phosphotyrosine-dependent manner. The molecular details of many of these interactions have been elucidated using nuclear magnetic resonance and x-ray crystallography (7,29,30,47-49). SH2 domains have numerous highly conserved residues important for maintaining a common modular structure and for interaction with the phosphotyrosine portion of the ligand. The specificity of SH2 domain interactions is provided by the unique residues within the SH2 domain which contact residues on the ligand, usually C-terminal to the phosphotyrosine residue (50,51). For example, the unique regions within the p85 SH2 domains are responsible for its binding specificity for pY-X-X-M ligands.

A-Raf, on the other hand, requires one of several basic-X-basic motifs for binding to p85 SH2 domains. Given the fact that this newly identified p85 SH2 ligand is positively charged, while the previously characterized phosphotyrosine-containing p85 SH2 ligand is negatively charged and hydrophobic, it is unlikely that A-Raf binds to the same site on the p85 SH2 domains. Our results therefore, raise the interesting possibility that p85 SH2 domains may have
a second binding site; a phosphorylation-independent binding (PIB) site distinct from the phosphotyrosine-dependent binding (PDB) pocket.
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Acknowledgments. We thank U. Rapp and M. Waterfield for generously supplying cDNAs. Expert technical assistance was provided by T. Taylor. This work was supported by the Health Services Utilization and Research Commission of Saskatchewan, the Saskatchewan Cancer Agency and the Medical Research Council of Canada. E.S.M. is supported by an NSERC scholarship.
**FIG. 1.** **A-Raf, but not c-Raf, is constitutively associated with p85 in cells.**  
*A*, lysates from serum starved (-) or PDGF-stimulation (+) NIH 3T3 cells were immunoprecipitated with the indicated antibodies (PI = preimmune). Samples were resolved by SDS-PAGE, transferred to nitrocellulose and immunoblotted with an anti-A-Raf antibody. Bound antibody was detected with a horseradish peroxidase conjugated secondary antibody, and visualized using chemiluminescence. A control sample of cell lysate is shown on the left.  
*B*, the blot from *A* was stripped and reprobed with an anti-p85 antibody.  
*C*, cells were treated as in *A*, immunoprecipitated with the indicated antibodies, and immunoblotted with an anti-c-Raf antibody.  
*D*, the blot from *C* was stripped and reprobed with an anti-p85 antibody.

**FIG. 2.** **Multiple p85 domains can bind directly to A-Raf in vitro.**  
*A*, GST-p85 fusion proteins used in the pull down experiments.  
*B*, the indicated GST-p85 fusion proteins were used in pull down experiments with *in vitro* transcribed and translated HA-A-Raf. Bound HA-A-Raf was detected as described in Fig. 1A. A sample of the HA-A-Raf translation product is shown on the left.  
*C*, Coomassie stained gel (stain) of baculovirus expressed His-A-Raf in Sf9 lysates before (lane 2) and after column purification (lane 3). The sizes of the molecular weight standards (lane 1) are given in kilodaltons on the left.  
Anti-A-Raf immunoblot (Blot: A-Raf) of pull down experiment using the indicated GST-fusion proteins and purified His-A-Raf.  
*D*, the indicated GST-p85 fusion proteins were used in pull down experiments with COS-1 lysates containing HA-A-Raf protein. Bound HA-A-Raf proteins were detected using an anti-A-Raf antibody. A control lysate sample is present in the left most lane.
FIG. 3. Separate mutation of each of the nine basic-X-basic sequences in A-Raf does not prevent binding to the p85 SH2 domains. A, schematic representation of the domain structure of A-Raf. The locations of the nine basic-X-basic sequences within A-Raf are shown and have been designated as sites: R (containing the LQRIRS sequence), and A through H. B, COS-1 cell lysates containing the indicated HA-A-Raf basic-X-basic site mutants were immunoblotted with anti-HA antibodies to normalize the amounts used in subsequent pull down experiments. The indicated wild type (wt) or basic-X-basic site mutants (R, A-H) of HA-A-Raf were tested for their abilities to bind to immobilized GST-p85-N-SH2 (C) or GST-p85-C-SH2 (D) fusion proteins. In each case, the left-hand lane contains a control pull down sample using wt HA-A-Raf and immobilized GST protein. Bound HA-A-Raf proteins were detected as in B.

FIG. 4. Mutation of multiple basic-X-basic sequences in A-Raf are required to prevent binding to the p85 SH2 domains. A, a comparison of the basic-X-basic sequences in A-Raf with the corresponding sequences in c-Raf. Identical residues have been underlined. Conserved sequences have been defined as having amino acid residues with similar properties at analogous positions. B-F, pull down experiments using the indicated multiple basic-X-basic site HA-A-Raf mutants and GST-p85 fusion proteins. Bound mutant HA-A-Raf proteins were detected using an anti-HA antibody. Cell lysates (10% of what was present for each pull down sample) for each of these multiple mutants are shown in the left-hand lanes.

FIG. 5. The p85 SH3 domain and a p85 protein lacking only the SH3 domain (ΔSH3) can still bind the HA-A-Raf mutants, including C/R/D/E, defective in its ability to bind to the p85 SH2 domains. A-F, pull down experiments using the indicated wild type or multiple basic-
X-basic site HA-A-Raf mutants and GST-p85 fusion proteins. Bound mutant HA-A-Raf proteins were detected using an anti-HA antibody. Cell lysates (10% of what was present for each pull down sample) for each of these multiple mutants are shown in the left-hand lanes.
| Bait Protein* | Display Sequence† | Bait Protein* | Display Sequence† |
|--------------|------------------|--------------|------------------|
| no SH2       | TDITLF (18/20)   | GAP-N SH2    | GDYTLF§ (4/16)   |
| FHFYAF       | FAQWGS (3/16)    | GSQ2WGS      | (1/16)           |
| p85-N SH2    | AARNF (14/20)    | SFVAYA       | (1/16)           |
| WRVR         | YNVIQG         | HWFLRN       | (1/16)           |
| p85-C SH2    | AARIR (1/21)    | GTPNKH       | (1/16)           |
| LARRS        | (7/21)          | DISYRWD      | (1/16)           |
| FTHSLA       | (1/21)          | YPHANGL      | (1/16)           |
| PLCγ1-N SH2  | WJWANY (10/22)  | GAP-C SH2    | GDYTLF§ (5/20)   |
| LQSFY (3/22) | WYGWRW (6/19)   | GDYTLF§ (4/19)|                  |
| GDYTLF§ (2/22)| GQYTSF (3/19)   |               |                  |
| TRGYPN (1/22)| KHRGSP (3/19)   |               |                  |
| EPQRF (1/22) | RYFTYP (2/19)   |               |                  |
| SEQGFH (1/22)| ACQRYY (1/19)   |               |                  |
| AVGYV (1/22) | AMQFJT (1/19)   |               |                  |
| GRYYA (1/22) | VOYVWT (1/19)   |               |                  |
| RSHAF (1/22) | WNSGSP (1/19)   |               |                  |
| TWASGA (1/22) |               |               |                  |
| PLCγ1-C SH2  | GDYTLF§ (9/22)  | GAP-N+C SH2  | GDYTLF§ (5/20)   |
| (includes the SH3 domain) | RLRFY (4/20) |               |                  |
| RNYDD (2/22) | SGSBGG (1/20)   |               |                  |
| WLEVKN (1/22) | SAVRIL (1/20)   |               |                  |
| SGGXVR (1/22) | SAVONL (1/20)   |               |                  |
| QASRR (1/22) | SATRSL (1/20)   |               |                  |
| VRPVR (1/22) | RAYCIF (1/20)   |               |                  |
| RYQPNH (1/22) | FASAL (1/20)   |               |                  |
| FTAASA (1/22) | KVNPKG (1/20)   |               |                  |
| VISSQX (1/22) | ETELTY (1/20)   |               |                  |
| ALPFTL (1/22) | HGQVSV (1/20)   |               |                  |
| ITTHMH (1/22) | GAVVTT (1/20)   |               |                  |
| IYYFLS (1/22) | GLVSNL (1/20)   |               |                  |

* TrpE-fusion proteins immobilized on protein A sepharose beads using anti-TrpE antibodies.
† Amino acid sequence of the display region, as deduced from the DNA sequence. The number of phage clones identified with each sequence is given in parenthesis. All recurring sequences are encoded by identical nucleotide sequences, indicating that they were derived from a single clone.
‡ For these samples only 15 nucleotides encoding 5 amino acid residues were present in the display region of the phage.
§ The sequence GDYTLF is selected by each of the SH2 domains of GAP and PLCγ1.
A: Blot: A-Raf

B: Blot: p85

C: Blot: c-Raf

D: Blot: p85

- Lysate
- PI
- A-Raf
- PDGF

+  +  +  +  +
**A**  
p85 protein  
SH3  
GST  
SH3  
BCR  
GST  
BCR  
N-SH2  
GST  
N-SH2  
110  
GST  
110  
C-SH2  
p85  
GST  
SH3  
BCR  
GST  
N-SH2  
110  
GST  
110  
C-SH2

**B**  
HA-A-Raf  
GST  
p85  
C-SH2  
Blot: A-Raf  
*In vitro* translated HA-A-Raf

**C**  
1  2  3  
Stain  
M  lysate  pur  
116  97  84  66  55  45  
baculovirus expressed His-A-Raf  
GST  
N-SH2  
C-SH2  
p85  
Blot: A-Raf

**D**  
COS-1 expressed HA-A-Raf  
Blot: A-Raf
**A**

A-Raf

- CR1: B (K50, R52)
- CR2: C (K56, R68)
- CR3: D (R279, R281)
- CR4: E (K388-390, K392)
- CR5: F (R326, R328)

**B**

Cell lysates

- HA-A-Raf mutants

**C**

HA-A-Raf mutants + GST-p85-SH2

- +N-SH2

**D**

HA-A-Raf mutants

- +C-SH2

Blots: HA
| Site | A-Raf | c-Raf | Homology |
|------|-------|-------|----------|
| A    | PNKQRT| PNKQRT| Identical|
| B    | ALKVRG| ALKVRG| Identical|
| C    | LIKGRK| EHKGKK| Divergent|
| R    | LQRIRS| SQRORS| Divergent|
| D    | EORERK| AQRERA| Divergent|
| E    | DKKVKNIL| EKNKIRPR| Divergent|
| F    | YFRGRW| YYKKW| Conserved|
| G    | LRKTRH| LRKTRH| Identical|
| H    | TVKTRW| TVKSRW| Conserved|

Blots: HA-A-Raf mutants:

**B**

C/R/D/E

**C**

R/D/E, added back C

**D**

C/D/E, added back R

**E**

C/R/E, added back D

**F**

C/R/D, added back E

Blots: HA
Blots:

A: wild type HA-A-Raf
B: C/R/D/E
C: R/D/E, added back C
D: C/D/E, added back R
E: C/R/E, added back D
F: C/R/D, added back E
Using a phage display library to identify basic residues in A-Raf required to mediate binding to the SH2 domains of the p85 subunit of phosphatidylinositol 3’-kinase
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J. Biol. Chem. published online August 30, 2000

Access the most updated version of this article at doi: 10.1074/jbc.M004720200

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