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Analysis of Ig-α – tyrosine kinase interaction reveals two levels of binding specificity and tyrosine phosphorylated Ig-α stimulation of Fyn activity

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The B cell antigen receptor complex (BCR) is composed of membrane Ig and heterodimers of Ig-α and Ig-β/γ. Recent findings indicate that Ig-α associates with Src-family kinases, including Fyn and Lyn, via an ~26 amino acid motif termed ARH1. Studies reported here (i) define two mechanisms whereby this motif binds Fyn and (ii) reveal an important functional consequence of binding, i.e. kinase activation. Mutational analysis indicates that specific low-affinity binding is determined by a short sequence, -DCSM-, in the motif and is not dependent on motif tyrosine residues. In contrast, the doubly tyrosine phosphorylated motif binds independently of DCSM and with high affinity. Importantly, this binding leads to Fyn activation. Taken together with studies which map low-affinity binding of Fyn or Lyn to the kinase's N-terminal unique region and high-affinity binding to the kinase's SH2 domain, these results suggest a mechanism of BCR activation in which the non-phosphorylated resting receptor is associated with Src-family kinases and, upon stimulation, tyrosine phosphorylation of Ig-α leads to reorientation and activation of receptor-associated kinases.

Key words: ARH1/B cell antigen receptor/Fyn/Ig-α/mIg/tyrosine phosphorylation

Introduction

B cells express a multimeric cell surface receptor complex (BCR) which transduces signals leading to cell activation, anergy or clonal deletion. These B cell biological responses are initiated by a BCR-triggered cascade of intracellular events involving, most proximally, the induction of protein tyrosine phosphorylation. Several tyrosine kinases have been found to be constitutively associated with the receptor, including Syk (Hutchcroft *et al.*, 1992) and the Src-family kinases Lyn, Blk, Lck and Fyn (Burkhardt *et al.*, 1991; Yamanashi *et al.*, 1991; Campbell and Sefton, 1992; Leprince *et al.*, 1992). These kinases are activated within 10 s of BCR stimulation (Campbell and Sefton, 1990; Gold *et al.*, 1990; Burkhardt *et al.*, 1991; Yamanashi *et al.*, 1992; Yamada *et al.*, 1993), and inhibition of their activity prevents subsequent BCR-induced responses (Cambier *et al.*, 1991; Padeh *et al.*, 1991). Tyrosine kinase activation leads, either directly or indirectly, to phosphorylation and/or activation of a variety of effectors, including phospholipase Cγ1 and 2 (PLCγ) (Carter *et al.*, 1991; Coggeshall *et al.*, 1992), VAV (Bustelo and Barbacid, 1992), mitogen-activated protein kinase II (MAPK II) (Casillas *et al.*, 1991; Pulverer *et al.*, 1991; Ettehadieh *et al.*, 1992), rasGTPase-activating protein (GAP) (Gold *et al.*, 1993), $p21^{ras}$ (Harwood and Cambier, 1993) and phosphatidylinositol-3 kinase (PI-3 kinase) (Pleiman *et al.*, 1994a). Some evidence implicates a pertussis toxin-sensitive GTP-binding protein in signaling by the BCR (Gold *et al.*, 1987; Klaus, 1990). These and other parallel or downstream effectors constitute an interrelated network of transducers which can be modified to drive various biological responses.

The complexity of the B cell response to antigen is paralleled by the receptor's structural complexity. The BCR is composed of a clonotypic antigen recognition substructure, membrane-bound Ig (mIg), which is non-covalently associated with disulfide-linked products of the B29 (Ig-β/γ) and mb-1 (Ig-α) genes that are inducibly tyrosine phosphorylated following receptor crosslinking (Hombach *et al.*, 1990; Campbell *et al.*, 1991; Gold *et al.*, 1991; Venkitaraman *et al.*, 1991). These latter chains are necessary for surface expression (for review see Reth *et al.*, 1991) and are capable of associating with Src-family tyrosine kinases (Lin and Justement, 1992) and activating calcium mobilization and protein tyrosine phosphorylation independently of mIg (Nomura *et al.*, 1991; Kim *et al.*, 1993; Sanchez *et al.*, 1993). The cytoplasmic tails of Ig-α and Ig-β contain a structural motif of ~26 amino acids which is found in components of a number of multi-subunit receptors, including Ig-α, Ig-β, TCRζ, CD3ε, CD3γ, CD3δ, FcεRIβ, FcεRIγ and hFcγRIIa (for review see Reth *et al.*, 1991; Cambier, 1992; Keegan and Paul, 1992). The motif which we refer to as the 'antigen receptor homology one' motif (ARH1), also termed ARAM (Weiss, 1993) and TAM (Klausner and Samelson, 1991), contains six precisely spaced conserved amino acids [D/E-$(X)_7$-D/E-$(X)_2$-Y-$(X)_2$-L/I-$(X)_7$-Y-$(X)_2$-L/I] (Reth, 1989). In CD3ε, and probably other ARH1 motifs, the two motif tyrosines residues are phosphorylated upon receptor crosslinking (Sancho *et al.*, 1993). Studies in which ARH1-containing cytoplasmic tails have been expressed as chimeras of irrelevant transmembrane proteins have revealed that ARH1 motifs carry sufficient information to mediate a variety of receptor functions, including tyrosine kinase activation, Ca^{2+} mobilization, cytolytic activity, IL-2 expression and endocytosis (Irving and Weiss, 1991; Letourneur and Klausner, 1991, 1992; Kolanus *et al.*, 1992; Romeo *et al.*, 1992; Vivier *et al.*, 1992; Wegener *et al.*, 1992; Kim *et al.*, 1993; Law *et al.*, 1993; Sanchez *et al.*, 1993).

Recently, we have shown that the cytoplasmic tails of Ig-α and Ig-β, each containing one ARH1 motif, possess sufficient

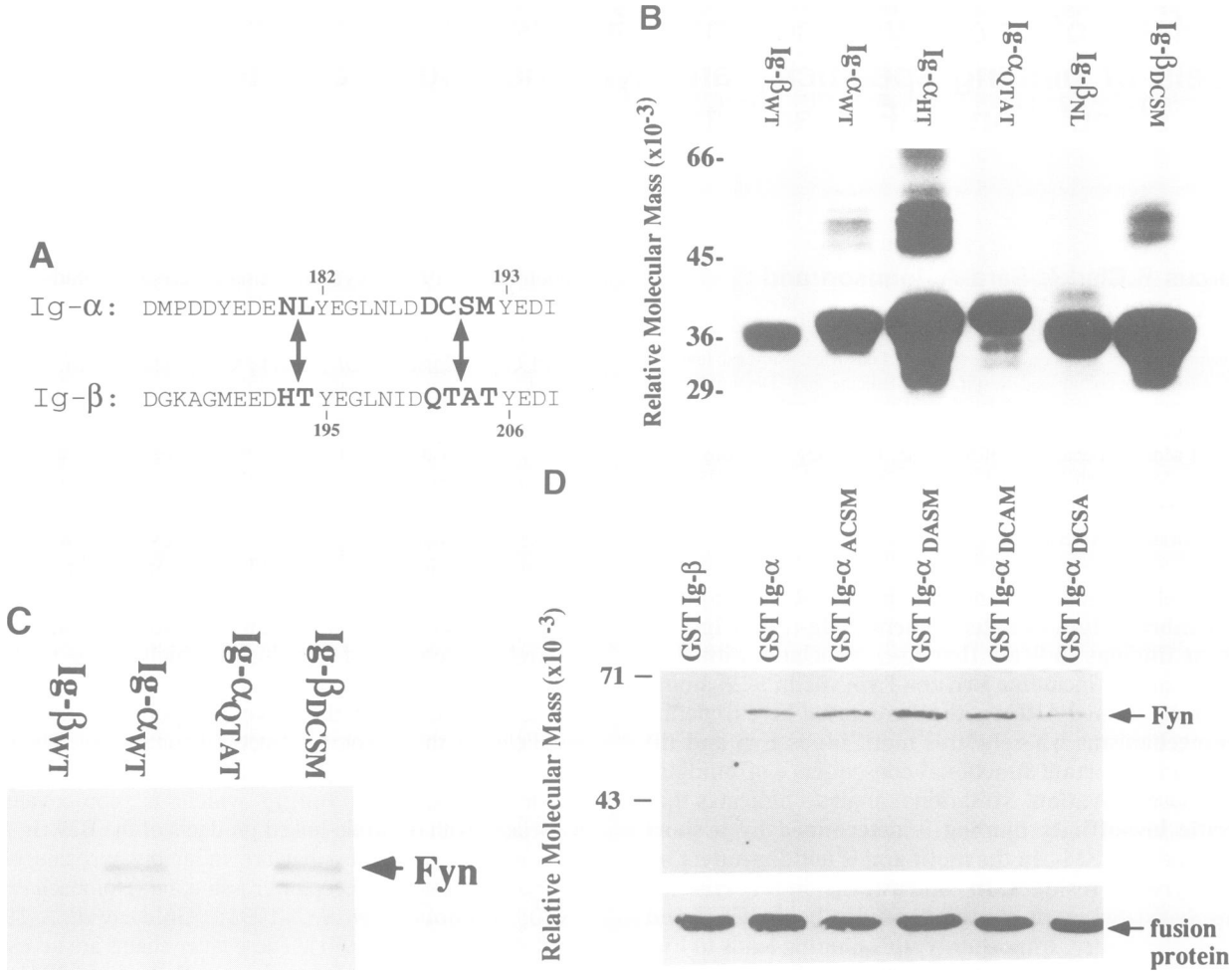

Fig. 1. The binding specificity of Ig-α and Ig-β is determined by four amino acids which lie between the conserved tyrosines in the ARH1 motifs. (**A**) Diagrammatic representation of Ig-α and Ig-β ARH1 motifs depicting the amino acid residues which where exchanged. Switch mutants were expressed as GST fusions. The positions of the conserved tyrosines of the ARH1 motif of each chain are indicated. (**B**) Comparison of proteins from lysates of the B cell lymphoma, K46, which bind to wild-type (WT) or Ig-α/Ig-β switch mutant cytoplasmic tails. Each switch mutant is denoted by its parent chain (Ig-α or Ig-β) and the amino acids which it contains from the other chain. Equal amounts of fusion protein (2 μg) were used to adsorb K46 cell lysates (2×10^7 cell equivalents/adsorption). Adsorbates were subjected to labeling by *in vitro* phosphorylation and analyzed by 10% reducing SDS–PAGE and autoradiography. (**C**) Immunoblot of ARH1 peptide (1 μg peptide/adsorption) adsorbates from Fyn$^+$NIH-3T3 cell lysates (3×10^6 cell equivalents/adsorption) with anti-Fyn antibodies. Adsorbates were subjected to 10% SDS–PAGE, transferred to nitrocellulose, then probed sequentially with anti-Fyn and alkaline phosphatase-labeled goat anti-rabbit antibodies. (**D**) Binding activity of point mutants of the Ig-α ARH1 motif. GST fusion proteins of the DCSM point mutants were cleaved with Factor Xa, coupled to Sepharose and used to adsorb Fyn$^+$NIH-3T3 cell lysates (as above). Adsorbates were fractionated as before and electrophoretic transfers were probed with anti-Fyn. Shown below are the results of anti-GST immunoblotting which document equivalent loading of each lane.

structural information to mediate receptor association with putative secondary effector molecules, including Lyn and Fyn (Ig-α), p38 (Ig-α), p40 (Ig-β) and p42 (Ig-β) (Clark *et al.*, 1992). Furthermore, the distinct binding capacity of each chain is invested within the chain's ARH1 motif. We now report that the binding specificity of the ARH1 motif of Ig-α for Fyn is determined by a four amino acid sequence (-DCSM- for Ig-α and -QTAT- for Ig-β) which lies between the conserved tyrosines of the motif. Although these tyrosines are not necessary for basal association of Ig-α with Src-family kinases, phosphorylation of both tyrosines increases, in a synergistic manner, binding of both Ig-α and Ig-β to Fyn. Complementary studies by Pleiman *et al.* (1994b) indicate that phosphotyrosine-independent 'resting' receptor binding involves a Fyn/Lyn unique region site and phosphotyrosine-dependent binding occurs in the kinase's SH2 domain. The specific activity of Fyn bound to phosphorylated Ig-α is ~3-fold higher than that bound to the unphosphorylated chain. *In toto*, these results suggest a novel mechanism whereby association of Src-family kinases with complex immune recognition receptors may occur by both phosphotyrosine-dependent and -independent mechanisms. Furthermore, phosphorylation of transducer chains such as Ig-α, which occurs rapidly following receptor ligation, may initiate reorientation and activation of receptor-associated Src-family kinases. Phosphorylation of other ARH1-containing subunits, such as Ig-β, may lead to recruitment and activation of kinases.

Results

The binding specificity of Ig-α and Ig-β is determined by four amino acids which lie between the conserved tyrosines of the ARH1 motifs

In previous studies we have shown that the cytoplasmic domains of Ig-α and Ig-β, each of which contains one ARH1

motif, bind to distinct sets of proteins. In particular, Ig-α specifically binds the Src-family kinases, including Lyn, Fyn and an unidentified molecule of 38 kDa, while Ig-β binds two unidentified proteins of 40 and 42 kDa (Clark *et al.*, 1992). Furthermore, we have shown that the binding activity was contained within the ARH1 motif of each tail. However, it was unclear from these studies which sequences within each motif determine binding specificity. We reasoned that because six of the 26 residues are conserved in all the ARH1 motifs and six additional residues are conserved between the Ig-α and Ig-β motifs, the 14 variant residues must determine the distinct binding activities of Ig-α and Ig-β. Therefore, we constructed a series of Ig-α and Ig-β switch mutants (Figure 1A) in which areas of divergent sequence were exchanged. When non-conserved amino acid residues N-terminal to the first conserved tyrosine (Ig-α:HT/Ig-β:NL) were exchanged, we observed no effect on the binding properties of either chain (Figure 1B). The apparent enhancement of phosphoprotein binding/labeling seen in the *in vitro* kinase reactions of Ig-α:HT precipitations was not seen reproducibly. In contrast, when the four non-conserved residues between the ARH1 tyrosines were exchanged (DCSM of Ig-α for QTAT of Ig-β), Ig-α failed to bind the cluster of proteins in the 50−60 kDa range which bind wild-type Ig-α. Likewise, the Ig-β switch mutant, containing the four amino acids (DCSM) of Ig-α, now bound this cluster of proteins. This cluster of molecules was shown previously to contain Src-family kinases Lyn and Fyn which are expressed in the K46 B cell lymphoma (Figure 1B).

To examine the specificity of Src kinase binding more quantitatively, we utilized synthetic peptides corresponding to either the wild-type, Ig-α (QTAT) or Ig-β (DCSM) fusion proteins to probe lysates of NIH-3T3 cells which overexpress Fyn (Fyn$^+$NIH-3T3) (Kawakami *et al.*, 1988), with the detection of Fyn by immunoblotting. As seen in Figure 1C, the ability to detect immunoreactive Fyn associated with the different peptides paralleled the ability of each motif to bind 50 and 59 kDa proteins detectable by *in vitro* kinase labeling (Figure 1B). The smaller immunoreactive band seen probably represents a degradation product of Fyn because it was only seen in occasional experiments and it increased over time in total cell lysates (data not shown).

The ability to transfer the Fyn binding activity from Ig-α to Ig-β by switching the DCSM sequence seems to contradict previous results in which point mutation in this area of the CD3ε ARH1 motif did not affect signaling function (Letourneur and Klausner, 1992). To address this apparent discrepancy, fusion proteins were made in which the aspartic acid, cysteine, serine and methionine were changed individually to alanine and their binding activity assessed. As shown in Figure 1D, none of these single amino acid changes, with the possible exception of the M to A switch, affected binding detectably. *In toto*, these results suggest that it is the overall structure of this region, and not the individual contribution of single amino acids, which determines binding and activation competency. Mutations involving a change or deletion of two or more amino acids may be needed to disrupt function.

In any case, it is clear that the ARH1 motif of Ig-α contains a sequence of four amino acids which determines binding specificity for Lyn and Fyn. The domain is flanked by two conserved tyrosines which in other ARH1-containing chains have been shown to be essential for the activation of signaling

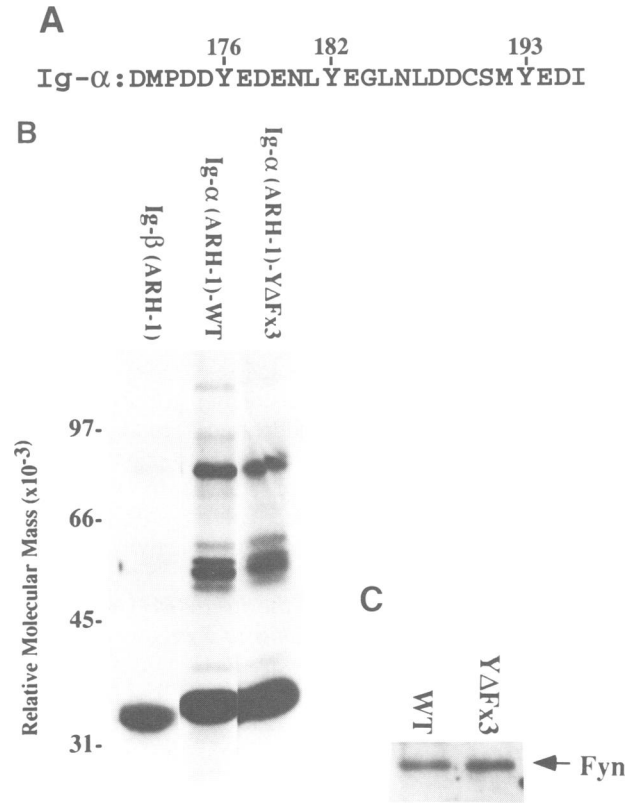

Fig. 2. The tyrosines of the ARH1 domain of Ig-α are not necessary for the binding of phosphorylatable proteins. (**A**) The amino acid sequence of Ig-α ARH1 is shown, with conserved (Y182, Y193) and non-conserved tyrosines (Y176) indicated. (**B**) Comparison of proteins from lysates of the B cell lymphoma, K46, which bind to wild-type and Ig-α ARH1 fusion proteins in which all three tyrosines had been changed to phenylalanine. Adsorbates were prepared as described in Figure 1B and incubated with [γ^{32}P]ATP in kinase buffer, washed and analyzed by 10% reducing SDS−PAGE and autoradiography. (**C**) Comparison, by anti-Fyn immunoblot, of the ability of wild-type and Ig-α tyrosine mutated fusion proteins to bind Fyn from the lysates of Fyn$^+$NIH-3T3 cells.

and endocytotic pathways, and to be phosphorylated upon receptor crosslinking (Letourneur and Klausner, 1992; Romeo *et al.*, 1992; Wegener *et al.*, 1992; Sancho *et al.*, 1993). Given the importance of these tyrosines for receptor-associated functions, we postulated that these residues, or their phosphorylation, may also play a role in effector binding.

The tyrosines of the ARH1 domain of Ig-α are not necessary for the binding to Fyn

To address formally the possibility that the observed basal binding of Fyn to Ig-α might depend on tyrosine phosphorylation of the motif, we mutated the two conserved ARH1 tyrosines of Ig-α and the third non-conserved tyrosine within this domain (Figure 2A) to phenylalanine and assayed the ability of this mutant to bind effector proteins as identified using *in vitro* kinase reactions. The results of a typical experiment are presented in Figure 2B. Substitution of phenylalanine for tyrosine at all of these positions did not significantly affect the ability of the Ig-α cytoplasmic domain to associate stably with previously identified and unidentified binding proteins. Additionally, fusion proteins of Ig-α in which the above tyrosines were mutated singly, or in various

combinations, bound as well as wild-type Ig-α (data not shown). When we examined Ig-α binding activity in lysates from Fyn+NIH-3T3 cells, no difference was seen in the amount of immunoreactive Fyn which bound the wild-type Ig-α ARH1 fusion protein or a fusion protein in which all three tyrosines were mutated to phenylalanine (Figure 2C). These data demonstrate that basal binding of Fyn to the ARH1 motif of Ig-α is independent of these tyrosines and thus could not be dependent on their phosphorylation.

Although the data discussed above indicate that non-phosphorylated receptors associate with effectors, activation of B lymphocytes via the antigen receptor leads to rapid tyrosine phosphorylation of Ig-α and Ig-β (Gold *et al.*, 1991) which could affect the receptor's binding activity. Although this question has not been addressed formally in BCR, crosslinking of TCR components leads to phosphorylation of ARH1 tyrosines (Sancho *et al.*, 1993). In view of these findings, we assessed the effect of Ig-α ARH1 tyrosine phosphorylation on its effector binding activity.

Tyrosine phosphorylation of the Ig-α ARH1 motif specifically increases its binding to Fyn

To produce phosphorylated Ig-α ARH1 motifs we cloned the catalytic domain of the tyrosine kinase Elk (Lhotak *et al.*, 1991; Rottapel *et al.*, 1991) into the expression vector pBC which utilizes a T7 polymerase promoter and a chloramphenicol resistance marker. This construct was transfected into the bacterial strain BL21/DE3 which contains a cDNA encoding T7 polymerase under control of the *lac* promoter. Subsequently, Ig-α ARH1 wild-type and Ig-α ARH1 tyrosine mutant containing fusion proteins were expressed and phosphorylated in these bacteria by induction with isopropylthio-β-D-galactoside. Cleavage of Ig-α ARH1 phosphorylated fusion protein with Factor Xa and subsequent SDS−PAGE and anti-phosphotyrosine immunoblotting demonstrated that the ARH1 tail, but not the GST fusion partner, was tyrosine phosphorylated. Anti-phosphotyrosine immunoblotting of the tyrosine mutants co-expressed with Elk demonstrated that the conserved tyrosines (Y182, Y193), and not the non-conserved tyrosine (Y176), of the Ig-α tail were phosphorylated (data not shown). The tyrosine phosphorylated fraction of the ARH1 wild-type fusion protein expressed as above was further purified using an anti-phosphotyrosine affinity column and then bound to glutathione−Sepharose beads. This fraction constituted only ~2−5% of the total fusion protein. Equal amounts of phosphorylated and non-phosphorylated fusion protein were then assayed for their ability to bind Fyn from the NP-40 lysates of Fyn+NIH-3T3 cells. As shown in Figure 3A, ~10-fold more immunoreactive Fyn associated with the phosphorylated Ig-α ARH1 motif than the non-phosphorylated motif.

To determine the relative role of the respective phosphotyrosines in the enhancement of Fyn binding, we synthesized peptides corresponding to the Ig-α ARH1 motif (residues 171−196) which were either non-phosphorylated or phosphorylated at tyrosine residues 182 or 193 or both. The use of synthetic phosphopeptides guaranteed stoichiometric phosphorylation at specific residues. These peptides were covalently coupled to Sepharose beads and used in adsorption of Fyn+NIH-3T3 cell lysates (Figure 3B). Fyn binding to all of the Ig-α peptides was detectable by immunoblotting. The amount of Fyn associated with the singly phosphorylated peptides was increased by 2- (Y182) and 1.6-fold (Y193) compared with the non-phosphorylated peptide (as determined using a PhosphorImager). Importantly, the amount of Fyn associated with the doubly phosphorylated peptide was increased by 24-fold. Further comparative analysis of adsorbates and cell lysates after adsorption revealed that the non-phosphorylated Ig-α peptide bound ~3−7% of the available Fyn, while the doubly phosphorylated peptide bound essentially all of the available Fyn present in Fyn+NIH-3T3 cell lysates under the conditions employed (data not shown).

To address further the possibility that new proteins bind to phosphorylated Ig-α, adsorbates from K46 lysates were analyzed by *in vitro* kinase reactions and SDS−PAGE. As shown in Figure 3C, there was no clear evidence of new phosphorylatable proteins binding the phosphorylated Ig-α peptide.

Both tyrosine phosphorylated Ig-α and Ig-β ARH1 motifs exhibit enhanced Fyn binding activity

To define the specificity of phosphotyrosine-dependent binding of ARH1 motifs to Fyn, we synthesized Ig-β and Ig-α ARH1 motifs which were phosphorylated at both of the ARH1 tyrosines. These peptides and non-phosphorylated Ig-α ARH1 peptides were coupled to Sepharose and used for analysis of Fyn binding. As before, phosphorylated Ig-α bound Fyn much more efficiently than non-phosphorylated Ig-α. Importantly, Ig-β bound Fyn as efficiently as phosphorylated Ig-α (Figure 4). These results suggest that the specificity of the phosphotyrosine-dependent interaction is not determined by the QTAT or DCSM motifs but, based on mapping studies by Songyang *et al.* (1993), probably by the residues immediately flanking the phosphotyrosines. These residues are conserved in Ig-α and Ig-β (see Figure 1), forming YEGL and YEDI sequences thought to constitute high-affinity binding sites for Src-family kinase SH2 domains.

The specific enzymatic activity of Fyn is increased upon binding to phosphorylated Ig-α

To determine the catalytic activity of the immunoreactive Fyn bound to the Ig-α peptides, we utilized a peptide-based *in vitro* kinase assay to measure activity in Ig-α ARH1 adsorbates of Fyn+NIH-3T3 lysates (Goldman *et al.*, 1994). When equivalent amounts of samples which had been tested for immunoreactive Fyn in Figure 3B were assayed for kinase activity (Figure 5A), the profile of enzymatic activity correlated with levels of immunoreactive Fyn with one exception. There was a discrepancy between the relative increase in immunoreactive Fyn (24-fold) and Fyn enzymatic activity (>60-fold) associated with doubly phosphorylated Ig-α, suggesting that Fyn bound to this peptide was more catalytically active than that bound to unphosphorylated Ig-α.

To quantitate this apparent enhancement in Fyn activity by phosphorylated Ig-α, we analyzed the ability of the Ig-α phospho-ARH1 peptide to activate Fyn *in vitro*. We immunopurified Fyn from Fyn+NIH-3T3 cell lysates and to constant amounts of Fyn we added increasing concentrations of doubly phosphorylated Ig-α or Ig-α in which the ARH1 tyrosine 182 or 193 had been changed to phenylalanine to prevent phosphorylation. We then assayed

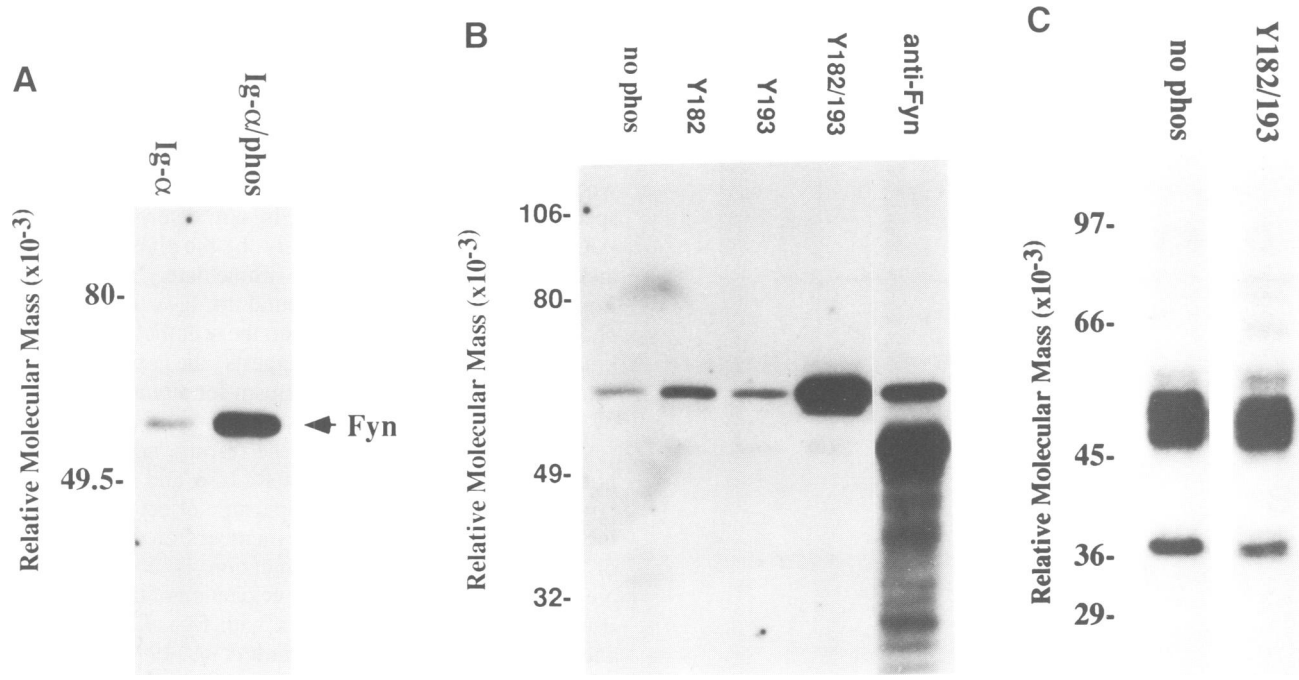

Fig. 3. Tyrosine phosphorylation of the Ig-α ARH1 motif specifically increases its binding of Fyn. (**A**) Anti-Fyn immunoblot of Fyn+NIH-3T3 cell proteins which bound to non-phosphorylated or phosphorylated ARH1 Ig-α fusion proteins. Washed adsorbates were separated by 10% reducing SDS−PAGE and transferred to nitrocellulose. The blots were then probed with rabbit anti-Fyn antiserum and [^{125}I]protein A (see Materials and methods). (**B**) Anti-Fyn immunoblot of proteins from Fyn+NIH-3T3 cell lysates which bound non-phosphorylated or phosphorylated Ig-α ARH1 peptides. In the lane labeled 'anti-Fyn', an anti-Fyn immunoprecipitate was analyzed as a positive control. Washed adsorbates were separated by 10% reducing SDS−PAGE and transferred to nitrocellulose. The blots were then probed sequentially with antibodies to Fyn and [^{125}I]protein A. Washed blots were subjected to autoradiography (see Materials and methods). (**C**) Similar spectra of phosphorylatable proteins bind to phosphorylated and non-phosphorylated Ig-α. Analysis of adsorbates from the lysates of K46 cells with non-phosphorylated or doubly tyrosine phosphorylated Ig-α ARH1 peptides. Adsorbates were subjected to *in vitro* phosphorylation and analyzed by 10% reducing SDS−PAGE and autoradiography.

the tyrosine kinase activity of the Fyn (Figure 5B). As shown, the specific activity of Fyn was increased almost 3-fold by incubation with phosphorylated Ig-α, but not by a corresponding Ig-α. The augmentation in Fyn activity was dose-dependent and saturable, and was detectable at low (5 μM) concentrations of peptide. When non-phosphorylated ARH1 was used in this assay, it became phosphorylated presumably by constitutively active kinase and this led to full activation of Fyn (data not shown).

Discussion

The ARH1 motif, a structural feature of several multi-subunit receptors expressed on cells of the hematopoietic lineage and certain viral proteins, has been implicated in transduction of signals across the plasma membrane. Cytoplasmic tails of Ig-α, Ig-β, TCRζ, CD3ε, FcεRIγ, hFcγRIIa, LMP2A and BLVgp30 have all been shown to function as transducers (Irving and Weiss, 1991; Kolanus *et al.*, 1992; Romeo *et al.*, 1992; Vivier *et al.*, 1992; Wegener *et al.*, 1992; Alber *et al.*, 1993; Kim *et al.*, 1993; Sanchez *et al.*, 1993; Beaufils *et al.*, 1994). In instances where it has been examined, i.e. CD3ε, TCRζ and hFcγRIIa, this motif alone has been found sufficient to mediate some or all transducer functions associated with the respective receptor (Kolanus *et al.*, 1992; Letourneur and Klausner, 1992; Romeo *et al.*, 1992; Wegener *et al.*, 1992). Data from a number of these studies indicate that signals transduced by these motifs in, for

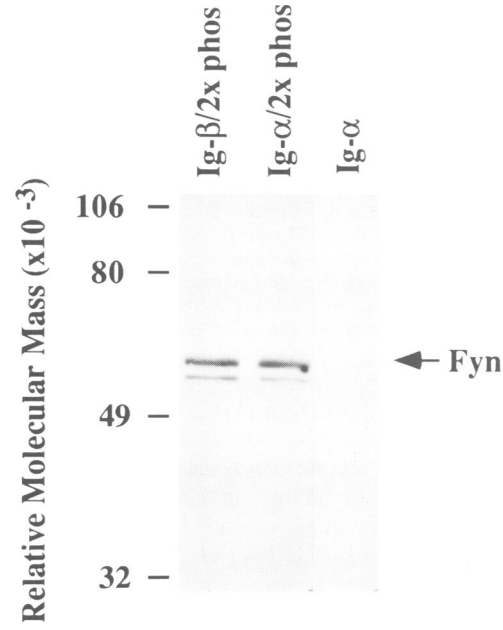

Fig. 4. Doubly phosphorylated Ig-β and Ig-α bind Fyn similarly. Synthetic doubly phosphorylated Ig-α and Ig-β ARH1 motif peptides and non-phosphorylated Ig-α ARH1 motif peptides were used to adsorb Fyn+NIH-3T3 cell lysates. Adsorbates were fractionated by SDS−PAGE, transferred and blotted with rabbit anti-Fyn and [^{125}I]protein A.

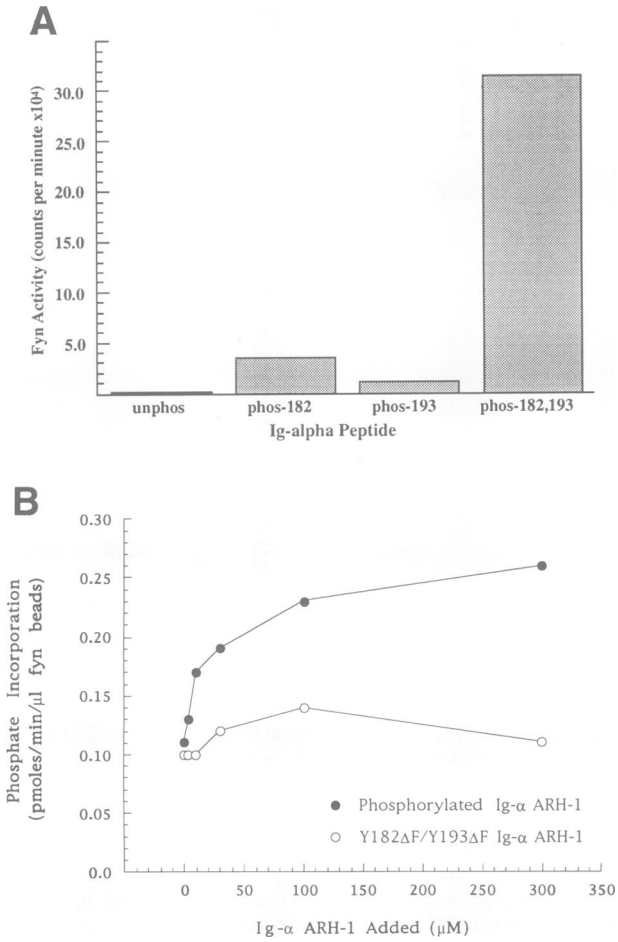

Fig. 5. The specific activity of Fyn is enhanced upon binding to phosphorylated Ig-α. (**A**) Fyn bound to phosphorylated Ig-α is catalytically active. Non-phosphorylated Ig-α or Ig-α in which peptide tyrosine 182, or tyrosine 193, or 182 plus 193 were phosphorylated, were used to adsorb lysates of Fyn⁺NIH-3T3 cells (~2 × 10^6 cells/sample). Adsorbates were then incubated with Fyn substrate peptide in the presence of [γ-^{32}P]ATP for 10 min at 30°C in kinase buffer. Reactions were stopped with TCA, blotted to phosphocellulose, washed extensively and counted by liquid scintillation (see Materials and methods). (**B**) The specific activity of Fyn is enhanced by the addition of phosphorylated Ig-α peptide. To anti-Fyn beads containing purified Fyn from Fyn⁺NIH-3T3 cells (2 × 10^6 cell equivalents/sample), increasing amounts of phosphorylated Ig-α peptide, or equivalent peptide in which tyrosine 182 and 193 were changed to phenylalanine, were added followed by incubation at 25°C for 1 h. Kinase activity was assayed as in panel A and Materials and methods.

example, Ig-α and Ig-β (Kim *et al.*, 1993; Sanchez *et al.*, 1993), differ qualitatively, suggesting that they may be coupled to distinct second messenger generating systems. Consistent with this possibility, in their non-phosphorylated state ARH1 motifs of Ig-α and Ig-β bind to distinct cytoplasmic effectors (Clark *et al.*, 1992). The distinct function of the motifs is presumably determined by this binding activity which is, in turn, determined by differences in the amino acid sequence of the respective motifs. To explore the structural basis of differential function, we conducted an analysis of the effects of motif mutation and phosphorylation on binding activity. We have defined two mechanisms by which Src-family kinases bind to the cytoplasmic domains of Ig-α. The first, an apparently low-affinity binding interaction, occurs independently of tyrosine phosphorylation of the motif. The specificity of this binding for Ig-α is determined by four amino acids, DCSM, in the context of the ARH1 motif, because replacement of the equivalent Ig-β sequence (QTAT) with DCSM makes it competent to bind Fyn. A second, high-affinity binding interaction, which occurs with both Ig-α and Ig-β, is seen when both of the ARH1 tyrosines are phosphorylated. The specificity of this interaction is not determined by the -DCSM- residues but, most likely, by the phosphotyrosines and the amino acids which immediately flank them. Importantly, when Fyn is bound to Ig-α by the phosphorylation-dependent mechanism the specific activity of the kinase is increased. This suggests the existence of a previously unrecognized mechanism for signal propagation by ARH1-containing receptors.

Our findings suggest that in the resting, non-ligated and non-tyrosine phosphorylated BCR, Ig-α and Ig-β are non-covalently associated with cytoplasmic effector molecules, including, in the case of Ig-α, members of the Src-family of tyrosine kinases. This contention is consistent with previous reports describing co-precipitation of the non-ligated B cell antigen receptor complex with Fyn, Lyn, Blk, Lck and Syk tyrosine kinases (Burkhardt *et al.*, 1991; Yamanashi *et al.*, 1991; Campbell and Sefton, 1992; Hutchcroft *et al.*, 1992; Leprince *et al.*, 1992). Although all of these effectors contain SH2 domains (Koch *et al.*, 1991), these domains can not mediate the 'resting receptor'−kinase interaction because the elimination of tyrosines from the Ig-α ARH1 does not destroy the binding activity. Similarly, SH3-mediated binding is unlikely because the only known binding motifs for SH3 domains are rich in proline (Olivier *et al.*, 1993; Ren *et al.*, 1993) and such sequences are not found in Ig-α or Ig-β. Recently, studies by Gauen *et al.* (1992) demonstrated that Fyn binds to TCRζ, CD3ε and CD3γ by virtue of its N-terminal unique region. Studies in our own laboratory (Pleiman *et al.*, 1994b) demonstrate that Lyn, Blk and Fyn bind non-phosphorylated Ig-α via an N-terminal unique region determinant. Taken together, these findings and the observation that the specificity of Fyn and Lyn binding is determined by a four amino acid sequence in the ARH1 motif, lead to the conclusion that a previously unrecognized mechanism of receptor−Src-family kinase interaction must be operative.

Our experiments did not examine if Src-family kinases, such as Fyn, bind directly to Ig-α or if an intermediate linker protein is required. To begin to address this question we have assayed the ability of synthetic non-phosphorylated or phosphorylated Ig-α ARH1 peptides to precipitate a GST−Fyn fusion protein (Fyn amino acids 1−255; Pleiman *et al.*, 1994a,b) from bacterial cell lysates. While phosphorylated Ig-α binds the fusion protein effectively and specifically, non-phosphorylated Ig-α binds weakly in some experiments and not at all in others (C.P.Pleiman, M.R.Clark and J.C.Cambier, unpublished observation). This could result from a lack of appropriate folding of these proteins or an absence of accessory 'linker' molecule. These results demonstrate clearly that the binding of Fyn to phosphorylated Ig-α is direct. However, the potential role of a linker in Fyn binding to unphosphorylated Ig-α cannot be excluded.

The functional importance of the observed ARH1−kinase unique region interaction extends beyond the association of Ig-α with tyrosine kinases of the Src-family. Preliminary

studies indicate that binding of another potential effector, i.e. p38, to Ig-α is also DCSM-dependent and Ig-β binding of p40 and p42 is QTAT-dependent (M.R.Clark and J.C.Cambier, unpublished observation). Thus, ARH1 binding to other effectors is apparently determined by the sequence in this area of the motif. Further, ARH1 motifs found in other receptor subunits vary greatly in sequence at the DCSM/QTAT equivalent position: TCRζa, RREE; TCRζb, KMAEA; TCRζc, TKDT; CD3γ, EYDQ; CD3δ, EDTQ; CD3ε, QRDL; FcεR1b, YSPI; and FcγRIIa, DKNI (for review see Cambier and Campbell, 1992). Based on results reported here, these differences in sequence would be expected to confer upon the respective resting receptor subunits the ability to interact with distinct sets of effector molecules. Furthermore, the repertoire of effectors bound and activated by the resting receptor may determine the biological outcome of receptor ligation.

In the case of the BCR, the importance of this sequence is supported by evidence from *in vivo* experiments. In two separate reports the cytoplasmic tail of Ig-α, expressed as a chimera with the extracellular domain of either μ or CD8, is capable of inducing tyrosine phosphorylation of a number of cytosolic proteins following ligation with monoclonal antibodies, while similar chimeras containing Ig-β instead of Ig-α are ineffective (Kim *et al.*, 1993; Sanchez *et al.*, 1993). These results are in apparent disagreement with those of Law *et al.* (1993), who found no differences in the signaling capacities of μ chimeras of Ig-α or Ig-β. However, in their study the polyclonal antibodies used for stimulation could have led to the formation of large receptor aggregates. Non-specific trapping of kinases within these aggregates may have led to spurious kinase activation.

Our results and the results of others indicate that in the resting state, the receptor is constitutively associated with several kinases, including Lyn, Fyn, Blk, Lck and Syk (Burkhardt *et al.*, 1991; Yamanashi *et al.*, 1991; Campbell and Sefton, 1992; Hutchcroft *et al.*, 1992). We postulate that receptor engagement activates one or more of these resident kinases leading to the phosphorylation of both of the Ig-α and Ig-β ARH1 motifs' tyrosines. These phosphorylative events enhance the recruitment and/or binding of Src-family kinases to Ig-α and Ig-β where activation occurs through one or more mechanisms.

Recruitment and activation of kinases by ligated BCR complexes has been difficult to demonstrate directly because the complex becomes insoluble after activation. However, if the constituent ARH1 motifs are expressed separately as chimeras, more kinase activity is precipitated with ligated than unligated chimeras (Law *et al.*, 1993). These recruited kinases include Syk, as appears to be the case for the closely related ZAP70 kinase and TCR components (Chan *et al.*, 1992; Straus and Weiss, 1993; Wange *et al.*, 1993), as well as Src-family members (Law *et al.*, 1993). Recent studies indicate that phosphorylated TCR components also express increased binding activity for Src-family kinases (Wange *et al.*, 1993).

The increased binding of Fyn to phosphorylated Ig-α reported here has been shown recently to involve the kinase's SH2 domain (Pleiman *et al.*, 1994b). This suggests a likely mechanism of Fyn activation following receptor-activated Ig-α phosphorylation. Phospho-ARH1 binding to the SH2 domains may displace the kinase's C-terminal phosphotyrosine, resulting in unfolding of the kinase, making its catalytic domain accessible to substrate and therefore more active. In support of this previously hypothesized 'derepression' mechanism (for review see Cooper and Howell, 1993; Sieh *et al.*, 1993), mutation of the SH2 domain of Src, which may prevent its interaction with the enzyme's C-terminal phosphotyrosine, increases the specific activity of the kinase ~3-fold (O'Brien *et al.*, 1990). Furthermore, displacing the Src C-terminal phosphotyrosine from the kinase SH2 domain with a high-affinity phosphopeptide increases the specific activity of the kinase (Liu *et al.*, 1993). Finally, it is noteworthy that the YEDI in Ig-α and Ig-β ARH1 is a consensus binding site for Src-family kinase SH2 domains (Songyang *et al.*, 1993). Once phosphorylated, this sequence is predicted to have higher affinity for the Fyn SH2 than the C-terminal phosphotyrosine-containing sequence of Fyn. Thus, such a mechanism of activation is very likely operative.

In conclusion, our data demonstrate that Fyn can bind to Ig-α by distinct phosphotyrosine-independent and -dependent mechanisms which may be operative in the resting and activated states of the BCR, respectively. Furthermore, phosphorylation-dependent binding of Ig-α may lead directly to propagation of signals by activation of Src-family kinases.

Materials and methods

Mutagenesis

Mutagenesis or truncation of the previously cloned cytoplasmic tails of Ig-α and Ig-β (Clark *et al.*, 1992) was accomplished with PCR. Oligonucleotide primers specific to each mutation (Figures 1A, 2A and 3A) were used in a standard reaction mixture containing 1.5 mM Mg^{2+} and cycled (94, 55 and 72°C, each for 1 min) 25–30 times. Constructs that involved either the exchange or point mutagenesis of internal nucleotides required the generation of DNA fragments in which the mutations were contained in the 5′ or 3′ regions of overlapping fragments. These fragments were knitted together and then amplified with complementary flanking primers. The Ig-α_{HT} and Ig-α_{QTAT} switch mutants and the Y182ΔF tyrosine mutant were generated by site-directed mutagenesis using a kit, as recommended by the manufacturer (Bio-Rad).

The oligonucleotide primers below were used for mutagenesis. For the final amplification of mutated or truncated cDNAs, primers which contained restriction sites (bold) were used to facilitate cloning.

Truncation mutants

Ig-α ARH1, 5′-GAGA**GGATCC**TGGACATGCCAGATGACTATGA-3′; 5′-GAG-A**GAATTC**GATGTCCTCATACATAGAACAGT-3′.

Ig-α flank, 5′-AGAC**GGATCC**TCAGGAAACGGTGGCAAAATGAG-3′; 5′-GGT-GCCCTGGAGTCCCCTGAACACCCCAAACTTCTCATT-3′; 5′-AATGAGA-AGTTTGGGGTGTCCAGGGGACTCCAGGGCACC-3′; 5′-AGAC**GAATTC**-TGGCTTTTCCAGCTGGGCATCT-3′.

Ig-β ARH1, 5′-AGAC**GGATCC**ATGACGGCAAGGCTGGGATGGA-3′; 5′-AGA-C**GAATTC**TATGTCTTCATAGGTGGCTGT-3′.

Ig-β flank, 5′-AGAC**GGATCC**TTGACAAGGATGTGACTCTTCGGACAGGGG-AGGTA-3′; 5′-AGAC**GAATTC**TTCCTGCCCTGGATGCTCTCCT-3′.

Ig-β/α/β, 5′-GAGA**GGATCC**TACTTGACAAGGATGACATGCCAGATGACT-ATGAAGAT-3′; 5′-CCCTGTCCGAAGAGTCACGATGTCCTCATACATAGA-ACA-3′; 5′-GTGACTCTTCGGACAGGGGAG-3′; 5′-AGAC**GAATTC**TTCCTG-CCCTGGATGCTCTCCT-3′.

Ig-α/β/α, 5′-GAGA**GGATCC**TCAGGAAACGGTGGCAAAATGAGAAGTTT-GGGGTGGACGGCAAGGCTGGGATGGA-3′; 5′-GCCCTGGAGTCCCCTGGA-TATGTCTTCATAGGTGGCTGTCTG-3′; 5′-TCCAGGGGACTCCAGGGC-3′; 5′-AGAC**GAATTC**TGGCTTTTCCAGCTGGGCATCT-3′.

Switch mutants

Ig-α_{HT}, 5′-AGAC**GGATCC**TCAGGAAACGGTGGCAAAATGAG-3′; 5′-CAGG-CCCTCATAGGTATGTTCATCTTCATA-3′; 5′-AGAC**GAATTC**TGGCTTTTC-CAGCTGGGCATCT-3′.

Ig-α_{QTAT}, 5′-AGAC**GGATCC**TCAGGAAACGGTGGCAAAATGAG-3′; 5′-GATGTCCTCATAGGTAGCAGTCTGATCAAGGTTCAG-3′; 5′-AGAC**GAA-TTC**TGGCTTTTCCAGCTGGGCATCT-3′.

Ig-β_{NL}, 5′-AGAC**GGATCC**TTGACAAGGATGTGACTCTTCGGACAGGGGA-GGTA-3′; 5′-GAAGCCCTCATAGAGGTTATCTTCCTCCAT-3′; 5′-ATG-GAGGAAGATAACCTCTATGAGGGCTTC-3′; 5′-AGAC**GAATTC**TTCCTGC-CCTGGATGCTCTCCT-3′.

Ig-β_{DCSM}, 5′-AGAC**GGATCC**TTGACAAGGATGTGACTCTTCGGACAGGGGAGGTA-3′; 5′-CACTATGTCTTCATACATGGAACAGTCGTCAATGTTCAAGCC-3′; 5′-GGCTTGAACATTGACCACTGTTCCATGTATGAAGACATAGTG-3′; 5′-AGAC**GAATTC**TTCCTGCCCTGGATGCTCTCCT-3′.

Tyrosine mutants

All tyrosine mutants were generated using the Ig-α ARH1 primers listed above in combination with the following:

Y176ΔF, 5′-GAGA**GGATCC**TGGACATGCCAGATGACTTTGA-3′; Y182ΔF, 5′-AAATCTCTTCGAGGGCCT-3′; and Y193ΔF, 5′-GAGA**GAATTC**GATGTCCTCAAACATAGAACAGT-3′.

Point mutants

All point mutants were generated using the Ig-α ARH1 primers listed above in combination with the following:

Ig-α_{ACSM}, 5′-GAGA**GAATTC**GATGTCCTCATACATAGAACAAGCATC-3′; Ig-α_{DASM}, 5′-GAGA**GAATTC**GATGTCCTCATACATAGAAGCGTCATC-3′; Ig-α_{DCAM}, 5′-GAGA**GAATTC**GATGTCCTCATACATAGCACAGTCATC-3′; and Ig-α_{DCSA}, 5′-GAGA**GAATTC**GATGTCCTCATAAGCAGAACAGTCATC-3′.

Cloning

PCR products were digested overnight at 25°C with *Bam*HI and *Eco*RI, then ethanol precipitated, resolved by electrophoresis through 3% NuSieve agarose (FMC BioProducts) and isolated with MERmaid (BIO 101). Purified DNA was ligated to pGEX-3X (Pharmacia) and transformed into the bacterium DH5α. Transformants, selected on ampicillin (100 μg/ml), were screened using PCR and plasmid DNA was purified from positive clones with tip-100 columns (Qiagen). Double-stranded DNA was sequenced directly using Sequenase Version 2.0 (USB) and a primer to pGEX-3X (GCATG-GCCTTTGCAGGG).

Fusion protein production

Confirmed clones were induced with 0.3 mM isopropylthio-β-D-galactoside and the glutathione-S-transferase (GST) fusion proteins were isolated by adsorption to glutathione−Sepharose 4B (Pharmacia) as described (Ausubel *et al.*, 1993). The relative amount of fusion protein bound per bead volume was then quantitated by reducing SDS−PAGE and staining with Coomassie blue. Some fusion proteins were subsequently cleaved with Factor Xa (Ausubel *et al.*, 1993) and coupled to CNBr-activated Sepharose 4B (Pharmacia) as recommended by the manufacturer.

Phosphorylated fusion protein production

The catalytic domain of the tyrosine kinase Elk was amplified from a cDNA generously provided by Dr Tony Pawson (University of Toronto, Canada), using PCR with the following oligonucleotide primers: 5′-AAGA-**GGATCC**GGTGGCCATGGAAGCTGTCCGGGAGTTTGC-3′ and 5′-AAGA**GAATTC**GAGTTCTCATGCCATTACCGACGG-3′. The PCR product was purified as described above, ligated to pBC (Stratagene) and transformed into the bacterium BL21/DE3. Transformants, selected on chloramphenicol (20 μg/ml) and induced with 0.3 mM IPTG, were screened for their ability to phosphorylate bacterial proteins by resolving total bacterial lysates by reducing SDS−PAGE, transferring to nitrocellulose and immunoblotting with the α-phosphotyrosine mAb, Ab-2 (Oncogene Science). A positive clone was chosen and subsequently transformed, by electroporation, with plasmids which encode Ig-α ARH1 (truncated wild-type) or Ig-α ARH1 tyrosine mutants. Double transformants were simultaneously selected on chloramphenicol (20 μg/ml) and ampicillin (100 μg/ml) and subsequently induced to express GST fusion protein, of which 2−5% was phosphorylated *in vivo* by Elk. To separate the phosphorylated product from non-phosphorylated product, ~40 mg of this mixed fusion protein was passed over an α-phosphotyrosine affinity column (IG2 at 14 mg/ml Sepharose, gift from Dr Ray Frackelton, Brown University, Providence, RI). The column was then washed with PBS [137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4 and 1.4 mM KH_2PO_4 (pH 7.4)] and the phosphorylated fusion protein eluted with 0.1 N acetic acid. The phosphorylated fusion protein eluate was neutralized with 1 M Tris (pH 9.0), dialyzed against PBS and incubated overnight at 4°C with glutathione−Sepharose. The relative amount of phosphorylated fusion protein bound per bead volume was then quantitated as described above.

Synthetic peptides

Synthetic peptides corresponding to Ig-α ARH1 or Ig-α ARH1 phosphorylated at either Y182 or Y193 or both were produced according to Kitas *et al.* (1989). Each peptide was then coupled to CNBr-activated Sepharose 4B (Pharmacia) at 5 mg peptide/ml beads.

Binding assay

The B cell lymphoma K46 (ATCC) or the fibroblast Fyn+NIH-3T3 (Kawakami *et al.*, 1988) were harvested by centrifugation (2×10^7 K46 or $1-3 \times 10^6$ Fyn+NIH-3T3 cells per sample) and lysed in 1 ml lysis buffer [0.5% NP-40, 150 mM NaCl, 10 mM Tris, 2 mM sodium orthovanadate, 10 mM sodium pyrophosphate, 0.4 mM EDTA, 10 mM NaF, 1 mM phenylmethylsulfonyl fluoride (PMSF) and 2 μg/ml of each aprotinin, leupeptin and α-1-antitrypsin] as described (Clark *et al.*, 1992). Cleared lysates were incubated with beads coated with fusion protein or synthetic peptide (5−10 μl of 50% slurry containing ~1 μg fusion protein or 2 μg peptide per μl slurry) for 4 h at 25°C or overnight at 4°C. Following adsorption, the beads were washed alternately with 1 ml lysis buffer or RIPA buffer [1% NP-40, 0.1% SDS, 0.5% deoxycholic acid, 50 mM Tris (pH 8.0) and 150 mM NaCl] three times. Washed adsorbates were then analyzed by a variety of methods (see below).

In vitro kinase assay

Adsorbates were washed twice in 1 ml kinase buffer [10 mM $MgCl_2$, 10 mM HEPES (pH 7.0), 2 mM sodium orthovanadate and 1 mM PMSF], pelleted, resuspended in 20 μl kinase buffer containing 10 μCi [γ-^{32}P]ATP (3000 Ci/mmol), and incubated for 10 min at 30°C. The samples were then washed in lysis buffer and resuspended in 30 μl reducing sample buffer. Proteins were separated by 10% SDS−PAGE and detected by autoradiography at −70°C for 1−2 h.

Assay of kinase activity

Fyn was immunoprecipitated from the lysates of Fyn+NIH-3T3 ($\sim 2 \times 10^6$ cell equivalents/sample) cells by sequential 1 h incubations at 25°C with rabbit anti-Fyn antibody (5 μg antibody/sample) (Clark *et al.*, 1992) and protein A Sepharose (Pharmacia). Equal amounts of bead-bound adsorbates (prepared as described above) or Fyn immunoprecipitates (after pre-incubation for 1 h with phosphorylated Ig-α peptide at concentrations from 1 μM to 1 mM) were washed twice in kinase buffer, pelleted, resuspended in 50 μl kinase buffer containing 2 mM exogenous substrate (RRGKGHDGLYQGL) corresponding to a portion of the TCRζ chain surrounding tyrosine 142, 10 μM ATP and 10 μCi of [γ-^{32}P]ATP (3000 Ci/mM), and incubated for 10 min at 30°C. Each reaction mixture was then quenched with 12 μl of 25% trichloroacetic acid and blotted onto Whatman P81 phosphocellulose. Blots were then washed several times in 75 mM phosphoric acid and dried with acetone. Dried blots were counted by liquid scintillation on a Beckman (model LS5801) beta scintillation counter. Data were analyzed as described previously (Goldman *et al.*, 1994).

Immunoblotting analysis

SDS−PAGE sample buffer-eluted adsorbates were resolved by reducing SDS−PAGE and transferred to nitrocellulose. Transfers were blocked with 3% non-fat dry milk in Tris buffered saline (TBS) [10 mM Tris (pH 8.0), 150 mM NaCl] for 4 h at 25°C, and probed with rabbit anti-Fyn diluted 1:500−1:1000 in milk−TBS for 2 h at 25°C. After incubation, membranes were washed several times alternately with TBS or TBS containing 0.05% Triton X-100, incubated with ^{125}I-labeled protein A for 1 h at 25°C, washed again, and the immunoreactivity visualized by autoradiography. Alternatively, radioactivity was quantitated using a Molecular Dynamic's PhosphorImager with ImageQuant version 3.22. Immunoblotting was also carried out using the α-phosphotyrosine mAb, Ab-2 (Oncogene Science). In these instances, 3% low-phosphate bovine albumin (ICN Biomedicals, Inc.) in TBS was utilized instead of non-fat dry milk. In some immunoblots alkaline phosphatase-conjugated goat anti-rabbit antibodies were used. These blots were developed in 100 mM Tris (pH 9.5) using a Vector II kit (Vector Laboratories Inc.).

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