The B Cell Antigen Receptor Complex: Association of Ig-α and Ig-β with Distinct Cytoplasmic Effectors

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The B cell antigen receptor complex is a hetero-oligomeric structure composed of antigen binding, membrane immunoglobulin, and transducer-transporter substructures. The transducer-transporter substructure is composed of disulfide-linked dimers of immunoglobulin (Ig)-α and Ig-β/γ subunits that are products of the mb-1(α) and B29 (β/γ) genes. Although the receptor complex associates with Src family kinases that are activated after receptor ligation, the site of interaction of these and other cytoplasmic effector molecules with receptor subunits is unknown. The cytoplasmic tails of Ig-α and Ig-β chains were found to associate with distinct sets of effector molecules. The Ig-α chain cytoplasmic domain bound to the Src family kinases Lyn and Fyn, phosphatidylinositol-3 kinase (PI-3 kinase), and an unidentified 38-kilodalton phosphoprotein; the cytoplasmic tail of Ig-β bound PI-3 kinase and unidentified 40- and 42-kilodalton phosphoproteins. Binding activity was found to occur within a 26–amino acid sequence of Ig-α and Ig-β that contains a motif [(Asp or Glu)-(any amino acid)$_7$-(Asp or Glu)-Tyr-(any amino acid)$_3$-Leu-(any amino acid)$_7$-Tyr-(any amino acid)$_2$-(Leu or Ile)] previously implicated in signal transduction via other receptors including the Fc$_\epsilon$ receptor I and the T cell antigen receptor. These findings indicate that the subunits act independently to activate distinct second messenger pathways.

Engagement of the B cell antigen receptor complex initiates signaling mechanisms that can lead to immune cell proliferation and differentiation (*1*). Signal transduction by this receptor causes rapid activation of receptor-associated Src family tyrosine kinases including Lyn, Fyn, and Blk (*2*). These kinases act directly or indirectly to phosphorylate and activate multiple effectors including phospholipase C-γ1 and phospholipase C-γ2 (PLC-γ1 and PLC-γ2 (*3*), PI-3 kinase (*4*), and guanosine triphosphatase activating protein (GAP) (*5*). The site or sites of interaction between the antigen receptor complex and its immediate effectors is unknown.

The B cell antigen receptor complex is composed of an antigen recognition substructure, membrane immunoglobulin (mIg), that is noncovalently associated with a second substructure involved in receptor transport and signal transduction. This substructure consists of two heterodimers composed of Ig-α chains disulfide-linked to Ig-β or Ig-γ chains (*6*, *7*). These mIg-associated chains contain within their cytoplasmic domains a conserved motif of six precisely spaced amino acids, the antigen receptor homology 1 motif (ARH1) (*8*), which is found in a number of other signal transducer chains including the T cell antigen receptor (TCR) ζ, η, γ, δ, and ε chains, Fc$_\epsilon$ receptor I β and γ chains, and human Fc$_\gamma$ receptor IIA. Studies in which the cytoplasmic domains of ε or ζ have been expressed as chimeras of irrelevant transmembrane proteins suggest that even a single cytoplasmic domain of 22 amino acids that contains an ARH1 motif carries sufficient structural information to activate signaling pathways (*9*).

ARH1 domains are expressed in multiple copies, as many as ten, in antigen receptor complexes. This multiplicity may allow receptors to couple to divergent signal transduction pathways. This hypothesis predicts that cytoplasmic domains containing ARH1 motifs in distinct amino acid contexts may bind different cytoplasmic effector molecules.

We tested whether the cytoplasmic domains of the putative B cell antigen receptor signal transduction molecules, Ig-α and Ig-β, each of which has one ARH1 motif, contain sufficient structural information to form stable associations with secondary effectors of signal transduction. We expressed these domains as fusion proteins with glutathione-S-transferase (GST) (Fig. 1) and coupled these proteins to glutathione Sepharose beads (Pharmacia). We used these constructs as probes to identify molecules that can bind the receptor complex and performed a series of adsorptions with NP-40 lysates of the B cell lymphoma, K46. The adsorbed proteins were subjected to an in vitro kinase assay with [α-^{32}P]ATP (adenosine triphosphate) and analyzed by reducing SDS–polyacrylamide gel electrophoresis (SDS-PAGE) and autoradiography. Minimal kinase activity was precipitated with beads coupled to GST (27 kD) or GST fused to the COOH-terminal 169 amino acids of the platelet-derived growth factor receptor (PDGFR) (54 kD) (residues 939 to 1108) (*10*). In contrast, fusion proteins that contained the cytoplasmic domains of Ig-β (36 kD) or Ig-α (39 kD) precipitated one or more protein kinases as shown by their intense phosphorylation in subsequent in vitro kinase assays (Fig. 2). This phosphorylation was inhibited by incubation of adsorbates with 100 μM 5′-*p*-fluorosulfonyl benzoyladenosine, which inhibits kinase activity (*11*). No phosphorylation of the fusion proteins was seen in the absence of cell lysates, which indicates that the fusion proteins themselves have no kinase activity. Also, preadsorption of lysates with an excess of GST Sepharose resulted in no qualitative alteration in the precipitation of kinase activity by Ig-α or Ig-β (*11*).

Several kinase substrates from K46 lysates bound to Ig-α and Ig-β fusion proteins. Two, with molecular sizes of 76 and 85 kD, associated with both fusion proteins. The other substrates preferentially associated with one or the other cytoplasmic tail. In particular, several species with sizes of 50 to 59 kD preferentially associated with Ig-α, whereas Ig-β selectively bound two species of 40 and 42 kD. Nearly identical results were obtained when we used lysates from murine splenic B cells (*12*). Stimulation of K46 cells before lysis with antibodies to IgM or AlF_4^- did not alter the pattern of phosphoprotein binding (*13*). Furthermore, no qualitative differences were found in phosphoprotein binding among beads of varying fusion protein densities (64-fold range).

In order to define the submolecular specificity of fusion protein phosphorylation, we cleaved the Ig-α and Ig-β fusion proteins with Factor Xa after adsorption and phosphorylation (*14*) to yield a 27-kD fragment containing GST and 12- or 10-kD fragments containing the Ig-α or Ig-β cytoplasmic domains, respectively. Only those proteolytic fragments that contained cytoplasmic domains were detectably phosphorylated. These data indicate that the cytoplasmic domains of Ig-α and Ig-β can mediate the selective association with several kinase substrate proteins and that the cytoplasmic domains of these fusion pro-

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teins are substrates for the associating kinase or kinases.

To further localize the phosphoprotein binding domains of Ig-α and Ig-β, we expressed the ARH1 motif alone as fusion proteins (Fig. 1) and used these constructs as probes in vitro kinase reactions. The pattern of phosphoprotein binding was essentially identical to that seen with the entire cytoplasmic domains (Fig. 2), and the smaller size of the fusion proteins allowed the identification of an additional band of 38 kD that associated exclusively with the Ig-α ARH1 motif.

Src family tyrosine kinases, including $p56^{lck}$, p53 and $p56^{lyn}$, and $p59^{fyn}$ and $p56^{blk}$, associate with mIgM in digitonin lysates of B cells (*2*). The multiple bands between 50 and 59 kD precipitated preferentially by Ig-α are reminiscent of these kinases and suggest that Ig-α may mediate their association with the B cell antigen receptor complex. To examine this possibility, we performed in vitro kinase assays on fusion protein adsorbates from two cell lines that preferentially express one Src family kinase: LSTRA cells, a T cell lymphoma line that overexpresses Lck (*15*), and NIH 3T3–Fyn cells (4.14.37), which are NIH 3T3 cells that overexpress Fyn (*16*). We compared adsorbates from K46 cells, which express Lck, Lyn, and Fyn (*17*), to adsorbates from each of the above cell lines (Fig. 3A). Precipitations from K46 cells yielded multiple bands between 50 and 59 kD with prominent species of 53 and 56 kD. The clearing of K46 lysates, before adsorption by Ig-α, with antibodies to Lyn (anti-Lyn) deleted the 53- and 56-kD species (Fig. 3B). Furthermore, these bands could be reprecipitated from solubilized Ig-α adsorptions with anti-Lyn (*18*). In contrast, when we probed lysates of LSTRA cells with the Ig-β and Ig-α fusion proteins, we detected no phosphoprotein with a size appropriate for $p56^{lck}$. To confirm that $p56^{lck}$ was indeed absent, we probed LSTRA adsorbates with antibodies to $p56^{lck}$ (Fig. 3C). Although the antibody readily detected $p56^{lck}$ in total LSTRA cell lysates, no immunoreactive proteins were detected in any of the fusion protein adsorbates. Similar results were obtained from ECH408 cells, a B cell lymphoma that expresses Lck (*19*). We also examined the association of $p59^{fyn}$ with the Ig-α and Ig-β cytoplasmic domains by probing lysates of NIH 3T3–*fyn* cells. A 59-kD phosphoprotein was bound almost exclusively by Ig-α (Fig. 3A). In some experiments, this phosphoprotein was bound, although to a much lesser degree, by Ig-β (Fig. 3D). When Ig-α adsorbates were solubilized and then immunoprecipitated with antibodies to $p59^{fyn}$, a single 59-kD phosphoprotein was detected (Fig. 3D). These data suggest that the cytoplasmic domain of Ig-α can mediate the association of the B cell antigen receptor complex with some, but not all, Src family kinases.

Although Lyn and Fyn appear to associate preferentially with Ig-α, both Ig-α and Ig-β are phosphorylated in in vitro kinase

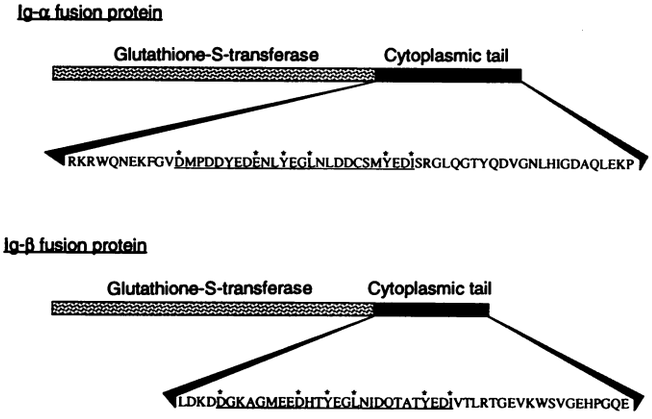

Fig. 1. The cytoplasmic domains of Ig-α and Ig-β expressed as fusion proteins to GST. Abbreviations for the amino acid residues are: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr. The amino acids that comprise each cytoplasmic domain are shown; residues of the ARH1 motif are marked with an asterisk, and the amino acids expressed in the ARH1-containing truncations are underlined. DNA fragments that encoded the cytoplasmic domains of Ig-α and Ig-β were amplified with the polymerase chain reaction (PCR) from K46 cDNA with the following primers: Ig-α: 5′-AGACGGATCCTCAGGAAACGGTGGCAAAATGAG-3′ and 5′-AGACGAATTCTGGCTTTTCCAGCTGGGCATCT-3′; Ig-β: 5′-AGACGGATCCTTGACAAGGATGACGGCAAGGCT-3′ and 5′-AGACGAATTCTTCCTGCCCTGGATGCTCTCCT-3′. For amplification of the ARH1-containing truncations, the following primers were used: Ig-α: 5′-GAGAGGATCCTGGACATGCCAGATGACTATGA-3′ and 5′-GAGAGAATTCGATGTCCTCATACATAGAACAGT-3′; Ig-β: 5-AGACGGATCCATGACGGCAAGGCTGGGATGGA-3′ and 5′-AGACGAATTCTATGTCTTCATAGGTGGCTGT-3′. Each primer encoded restriction sites (underlined) that facilitated subsequent cloning. Thirty cycles were completed in a buffer that contained 2.0 mM $MgCl_2$ with an annealing temperature of 55°C. The PCR product was cloned into pCR1000 (Invitrogen) as recommended by the manufacturer and sequenced. The cDNA fragments were then ligated into pGEX-3X (Pharmacia) at the Bam HI and Eco RI sites and transfected into *Escherichia coli* DH5α. To induce and isolate the fusion protein, we stimulated 1 liter of cells in log phase with 0.3 mM isopropyl β-D-thiogalactopyranoside. After 3 hours, the cells were pelleted and lysed in 1% Triton X-100 in 5 ml of phosphate-buffered saline (PBS) by repeated cycles of freezing and thawing. The cleared supernatant was incubated with packed glutathione-Sepharose beads (1.0 ml) (Pharmacia) for 30 min and then washed with 1% Triton X-100 in PBS. We confirmed the fidelity of fusion protein translation by resolving portions of protein bound to beads by SDS-PAGE, transferring them to nitrocellulose, and probing them with antibodies to peptides from the cytoplasmic tails of Ig-α and Ig-β (*6*). Approximately equal amounts (~5 μg) of Ig-β and Ig-α fusion proteins were used for each adsorption as judged by Coomassie blue staining of SDS-PAGE gels.

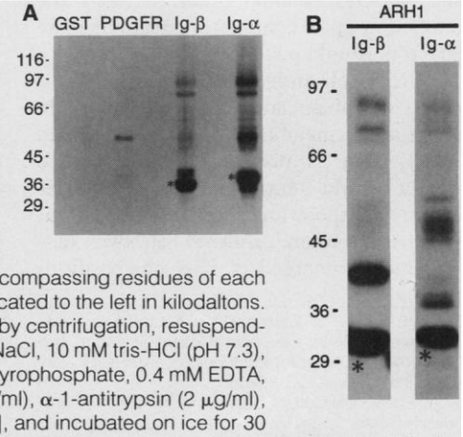

Fig. 2. Specific association of several phosphoproteins with the cytoplasmic domains of Ig-α and Ig-β. In vitro kinase assays performed on adsorbates prepared as in Fig. 1 revealed that each fusion protein adsorbed kinase activity that phosphorylated the fusion proteins (position of each fusion protein marked with an asterisk). No kinase activity was associated with GST alone or with the COOH-terminal tail of the PDGFR. (**A**) Results obtained with the entire cytoplasmic domains of Ig-α and Ig-β. (**B**) Fusion proteins that contained only the ARH1-encompassing residues of each cytoplasmic tail. Molecular size markers are indicated to the left in kilodaltons. K46 cells (20 × 10^6 per sample) were collected by centrifugation, resuspended in lysis buffer (1.0 ml) [0.5% NP-40, 150 mM NaCl, 10 mM tris-HCl (pH 7.3), 2.0 mM sodium orthovanadate, 10 mM sodium pyrophosphate, 0.4 mM EDTA, 10 mM NaF, aprotinin (2 μg/ml), leupeptin (2 μg/ml), α-1-antitrypsin (2 μg/ml), and 1 mM phenylmethylsulfonyl fluoride (PMSF)], and incubated on ice for 30 min. After centrifugation, the supernatant from ~2 × 10^7 cells was incubated overnight at 4°C with packed beads (5 to 20 μl) coated with the fusion protein (5 to 10 μg). The beads were then washed four times in lysis buffer, once in kinase wash buffer (150 mM NaCl, 10 mM tris, 2 mM sodium orthovanadate, and 1 mM PMSF) and once in kinase buffer [10 mM $MgCl_2$, 10 mM Hepes (pH 7), 2 mM sodium orthovanadate, and 1 mM PMSF]. Washed beads were resuspended in kinase buffer (20 μl) that contained 10 μCi [γ-^{32}P]ATP and incubated for 10 min at 30°C. The samples were then washed three times with lysis buffer. Proteins were resuspended in sample buffer, separated by SDS-PAGE under reducing conditions, and detected by autoradiography.

assays. To further define this kinase activity, we performed in vitro kinase assays on proteins adsorbed from K46 lysates and then conducted phosphoamino acid analysis on the labeled fusion proteins (Fig. 4). The Ig-α fusion protein was phosphorylated on Tyr (39% of total phosphorylation) to a greater extent than the Ig-β fusion protein (4%). In contrast, Ig-β was phosphorylated primarily on Thr (73%). Thr (30%) and Ser (31%) phosphorylation of Ig-α was also detected. These data are consistent with the observation that some Src kinases preferentially associate with Ig-α. Furthermore, these data indicate that one or more Ser-Thr kinases also associate with the B cell antigen receptor complex through interaction with the cytoplasmic domains of Ig-α and Ig-β. This observation is consistent with data that show that mIgM-associated Ig-α and Ig-β are constitutively phosphorylated in vivo on Ser and Thr (*20*).

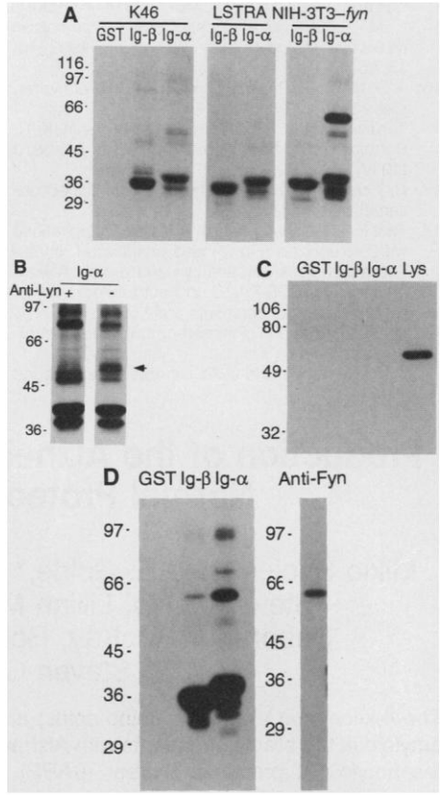

Fig. 3. Preferential association of the cytoplasmic domain of Ig-α with p53^{lyn}, p56^{lyn}, and p59^{fyn}. (**A**) Cell lines that preferentially express p56^{lck} (LSTRA) or p59^{fyn} (NIH 3T3–*fyn*) were assayed (Fig. 2) and compared to precipitations from K46 cells. (**B**) Preclearing of p53^{lyn} and p56^{lyn} with anti-Lyn. (**C**) Immunoblot for p56^{lck} in Ig-α precipitations from LSTRA (20×10^6 cells per sample). (**D**) Preferential binding of a 59-kD species from the lysates of NIH 3T3–*fyn* (2×10^6 cells per sample) of Ig-α. The 59-kD species was reimmunoprecipitated from solubilized Ig-α precipitations to p59^{fyn} antibodies (right lane). Rabbit antibodies were raised to p59^{fyn} (residues 29 to 48), p56^{lck} (residues 476 to 509), or Lyn (residues 1 to 130 expressed in bacteria and cleaved from GST) and affinity-purified. The method used for protein blotting has been described (*6*). Briefly, precipitations or total cell lysates (2×10^6 cells per sample) resolved by SDS-PAGE were transferred to nitrocellulose. The membrane was blocked with 3% bovine serum albumin (BSA) in 10 mM tris (pH 8) and 150 mM NaCl [tris-buffered saline (TBS)] and probed with rabbit antibodies (2 μg/ml) in BSA-TBS. After washing in alternating cycles of TBS with and without 0.05% triton, the membranes were incubated with ^{125}I-labeled protein A, washed again, and subjected to autoradiography. To immunoprecipitate Lyn, lysates were incubated with 20 μg of antibody and then precipitated with protein A. To reimmunoprecipitate p59^{fyn}, Ig-α adsorbate from NIH 3T3–*fyn* lysates was subjected to an in vitro kinase reaction, boiled in 0.5% SDS, 20 mM tris (pH 8.0), and 1 mM dithiothreitol (200 μl), and then diluted with lysis buffer (800 μl). The mixture was centrifugated, and antibodies to p59^{fyn} (10 μg) and protein A were added and incubated for 4 hours at 4°C. After four washings, the immunoprecipitates were analyzed by SDS-PAGE. Molecular size markers are indicated to the left in kilodaltons.

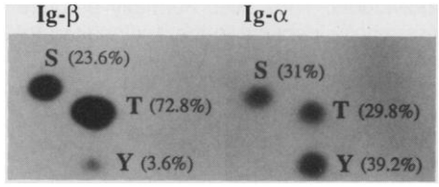

Fig. 4. Phosphoamino acid analysis of the cytoplasmic domains of Ig-α and Ig-β. We performed in vitro kinase assays on adsorbates of K46 cell lysates as described in Fig. 2. After SDS-PAGE, the fusion proteins were transferred to Immobilon-P (Millipore) and subjected to phosphoamino acid analysis essentially as described (*29*). The location of the phosphoserine (S), phosphothreonine (T), and phosphotyrosine (Y) residues are indicated along with the percent of the total radioactivity constituted by each residue. Quantitation of radioactivity in phosphoamino acids was performed with PhosphorImager scanner and ImageQuant software (Molecular Dynamics).

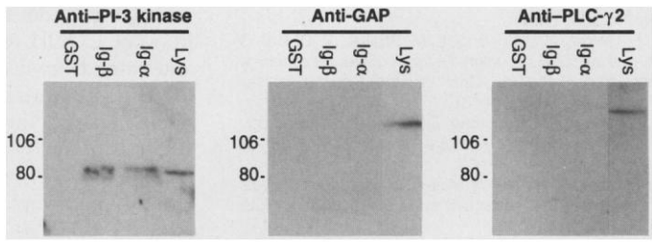

Fig. 5. Association of PI-3 kinase but not GAP or PLC-γ2 with Ig-α and Ig-β. GST, Ig-α, or Ig-β adsorbates from K46 lysates (20×10^6 cells per sample) or whole cell lysates (2×10^6 per sample) were subjected to SDS-PAGE, transferred to nitrocellulose, and probed with antibodies to PI-3 kinase (Upstate Biologicals, Lake Placid, New York), PLC-γ2, or GAP (rabbit polyclonal antisera prepared against a GST fusion protein that contained amino acids 171 to 448 of human GAP) (Fig. 3). Molecular size markers are indicated to the left in kilodaltons.

Ligation of antigen receptors on B and T cells leads to the activation of a variety of effector enzymes including the PLC-γ isoforms, Ras-GAP, and PI-3 kinase (*3–5*). To examine if any of these molecules associate with Ig-α or Ig-β, we probed proteins from K46 cells adsorbed to the fusion proteins by immunoblotting using antibodies to PLC-γ2, GAP, and PI-3 kinase (Fig. 5). Antibodies to the p85 subunit of PI-3 kinase recognized an 85-kD protein in Ig-α and Ig-β adsorbates. In contrast, we observed no reactivity of antibodies to PLC-γ2 or GAP with proteins that bound to Ig-α or Ig-β. This association is not surprising because PI-3 kinase activity is immunoprecipitated with antibodies to phosphotyrosine and p56^{lyn} from B cells stimulated by antigen receptor ligation (*4*). Because neither Ig-α nor Ig-β contains a PI-3 kinase consensus binding sequence (*21*), it is unlikely that PI-3 kinase binds directly to either molecule. Phosphorylation of the fusion proteins was apparently not necessary for PI-3 kinase or for any of the above mediators to bind. Indeed, preliminary experiments in which the binding to Tyr-phosphorylated ARH1 motifs or motifs in which tyrosines have been mutated to phenylalanines indicate that phosphorylation is not only unnecessary for binding but in fact mediates the dissociation of the Src kinases and PI-3 kinase from the receptor complex (*22*).

Our data indicate that three types of kinases that mediate divergent pathways of signal transduction—PI-3 kinase, Src family tyrosine kinases, and one or more Ser-Thr kinases—interact with isolated components of the B cell antigen receptor complex. The pp40 and pp42 subunits are not reactive with antibodies to mitogen-activated protein (*23*). Each chain of the Ig-α–Ig-β heterodimer can interact differentially with these molecules. The consensus ARH1 motifs found in multiple copies in Fc_ϵ receptors and lymphocyte antigen receptors may not be redundant but might mediate the coupling of these receptors to different pathways of cell activation. Our observations and this contention are sup-

ported by two findings. First, Ig-α–CD8 chimeric heterodimers but not Ig-β–containing chimeras are able to transduce signals leading to interleukin-2 production in the B cell lymphoma, A20 (*24*). Second, stimulation through isolated CD3 ε or TCR ζ chains results in distinct patterns of phosphorylation (*9*) and Ca^{2+} mobilization responses (*25*).

The observation that Ig-α and Ig-β can bind to different effector molecules may have implications for the function of other oligomeric receptors that can utilize alternate ARH1-containing chains to assemble different signal transduction complexes. For example, TCRs that contain δ-ε heterodimers may use different signal transduction pathways than those that contain γ-ε heterodimers (*26*). Differences in lymphocyte responses to antigen, which occur as a function of maturation, could be determined by such expression of alternative signal transduction complexes (*27*).

The Src family tyrosine kinases and pp40 and pp42 have different patterns of association with the ARH1 motifs of Ig-α and Ig-β, which suggests that they may interact with distinct sites within the cytoplasmic domains. These interactions, which are stable in NP-40, are reminiscent of other kinase-linked receptors such as the PDGFR, in which discrete regions mediate the receptor's association with distinct effector molecules (*21, 28*). Presumably, chain-specific residues, in the context of the antigen receptor's ARH1 motifs, determine the specificity of these interactions.

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Production of the Alzheimer Amyloid β Protein by Normal Proteolytic Processing

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The 4-kilodalton (39 to 43 amino acids) amyloid β protein (βAP), which is deposited as amyloid in the brains of patients with Alzheimer's disease, is derived from a large protein, the amyloid β protein precursor (βAPP). Human mononuclear leukemic (K562) cells expressing a βAP-bearing, carboxyl-terminal βAPP derivative released significant amounts of a soluble 4-kilodalton βAPP derivative essentially identical to the βAP deposited in Alzheimer's disease. Human neuroblastoma (M17) cells transfected with constructs expressing full-length βAPP and M17 cells expressing only endogenous βAPP also released soluble 4-kilodalton βAP, and a similar, if not identical, fragment was readily detected in cerebrospinal fluid from individuals with Alzheimer's disease and normal individuals. Thus cells normally produce and release soluble 4-kilodalton βAP that is essentially identical to the 4-kilodalton βAP deposited as insoluble amyloid fibrils in Alzheimer's disease.

In the brains of patients with Alzheimer's disease (AD), amyloid is deposited in senile plaques (*1, 2*) and, in many cases, in the walls of cerebral and meningeal blood vessels (*3*). The amyloid fibrils in the brains of patients with AD are composed of a 39– to 43–amino acid amyloid β protein (βAP) (*4–8*) that is derived from a set of 695– to 770–amino acid precursor proteins (*7, 9–11*) collectively referred to as the amyloid β protein precursor (βAPP). In each of the 695- to 770-residue βAPPs, the βAP is an internal peptide that begins 99 residues from the COOH-terminal end of the βAPP and extends from the extracellular-intraluminal region into the middle of the hydrophobic, membrane-spanning domain. The βAPP is normally processed in constitutive secretory (*12–15*) and endosomal-lysosomal (*16–20*) pathways. In the secretory pathway, the βAPP is cleaved within the βAP (*21, 22*) to produce a large secreted derivative and a small membrane-associated fragment, neither of which can produce amyloid because they do not contain the entire

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