PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : A61K 38/03, 38/10, 38/17, 38/43, C12N 5/06, C12Q 1/68 — A1

(11) International Publication Number: **WO 95/24915**

(43) International Publication Date: 21 September 1995 (21.09.95)

(21) International Application Number: PCT/US95/03438

(22) International Filing Date: 17 March 1995 (17.03.95)

(30) Priority Data:
215,116 17 March 1994 (17.03.94) US

(60) Parent Application or Grant
(63) Related by Continuation
US 215,116 (CIP)
Filed on 17 March 1994 (17.03.94)

(71) Applicant *(for all designated States except US)*: NATIONAL JEWISH CENTER FOR IMMUNOLOGY AND RESPIRATORY MEDICINE [US/US]; 1400 Jackson Street, Denver, CO 80206 (US).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: CAMBIER, John, C. [US/US]; 2216 Ivanhoe Street, Denver, CO 80207 (US). PLEIMAN, Christopher, M. [US/US]; Apartment 704, 4801 East 9th Avenue, Denver, CO 80220 (US). CLARK, Marcus, R. [US/US]; Apartment 2007, 400 North McClurg Court, Chicago, IL 60611 (US).

(74) Agents: KOVARIK, Joseph, E. et al.; Sheridan Ross & McIntosh, Suite 3500, 1700 Lincoln Street, Denver, CO 80203 (US).

(81) Designated States: AU, CA, FI, JP, KR, NO, US, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

Published
With international search report.

(54) Title: PRODUCT AND PROCESS FOR REGULATING SIGNAL TRANSDUCTION PATHWAYS

(57) Abstract

Products and processes are disclosed for regulating signal transduction pathways in cells. One aspect of the invention relates to a peptide having a YXXLXXXXXXXYXXψ amino acid motif that is useful in regulating the activity of tyrosine kinases, lipid kinases and adaptor molecules. A separate aspect of the present invention relates to a product and process for inhibiting signal transduction pathways in cells involving a peptide capable of binding to an SH3 domain of a tyrosine kinase, thereby blocking the binding and activation of an effector by the tyrosine kinase. Both the above compound and peptide composition can be useful in the treatment of medical disorders such as allergic responses, autoimmune diseases, inflammatory responses, cancer, immunodeficiency diseases, immunoproliferative diseases and diseases caused by viruses, such as EBV and BLV.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	**GB**	United Kingdom	**MR**	Mauritania
AU	Australia	**GE**	Georgia	**MW**	Malawi
BB	Barbados	**GN**	Guinea	**NE**	Niger
BE	Belgium	**GR**	Greece	**NL**	Netherlands
BF	Burkina Faso	**HU**	Hungary	**NO**	Norway
BG	Bulgaria	**IE**	Ireland	**NZ**	New Zealand
BJ	Benin	**IT**	Italy	**PL**	Poland
BR	Brazil	**JP**	Japan	**PT**	Portugal
BY	Belarus	**KE**	Kenya	**RO**	Romania
CA	Canada	**KG**	Kyrgystan	**RU**	Russian Federation
CF	Central African Republic	**KP**	Democratic People's Republic of Korea	**SD**	Sudan
CG	Congo			**SE**	Sweden
CH	Switzerland	**KR**	Republic of Korea	**SI**	Slovenia
CI	Côte d'Ivoire	**KZ**	Kazakhstan	**SK**	Slovakia
CM	Cameroon	**LI**	Liechtenstein	**SN**	Senegal
CN	China	**LK**	Sri Lanka	**TD**	Chad
CS	Czechoslovakia	**LU**	Luxembourg	**TG**	Togo
CZ	Czech Republic	**LV**	Latvia	**TJ**	Tajikistan
DE	Germany	**MC**	Monaco	**TT**	Trinidad and Tobago
DK	Denmark	**MD**	Republic of Moldova	**UA**	Ukraine
ES	Spain	**MG**	Madagascar	**US**	United States of America
FI	Finland	**ML**	Mali	**UZ**	Uzbekistan
FR	France	**MN**	Mongolia	**VN**	Viet Nam
GA	Gabon				

PRODUCT AND PROCESS FOR REGULATING SIGNAL TRANSDUCTION PATHWAYS

This invention was made in part with government support under USPHS Grants AI21768, AI20519, AI29903 and DK47121, all awarded by the National Institutes of Health. The government has certain rights to this invention.

CROSS-REFERENCE TO RELATED APPLICATIONS

The present application is a continuation-in-part of Serial No. 08/215,116 for "Product and Process for Regulating Signal Transduction Pathways", filed March 17, 1994, which is incorporated by reference herein in its entirety.

FIELD OF THE INVENTION

The present invention relates to a product and process for regulating a signal transduction pathway in a cell using peptides capable of regulating the activity of tyrosine kinases, lipid kinases and adaptor molecules.

BACKGROUND OF THE INVENTION

In all multicellular organisms, cell to cell communication coordinates the growth, differentiation and metabolism of the multitude of cell types contained in an organism. Communication between cells over long distances is facilitated by extracellular products (ligands) which act as signal molecules. Signal molecules travel through the organism to specific cells, where the signals induce a specific response only on those target cells that have

receptors capable of binding those signal molecules. Binding of a signal molecule to a receptor initiates complex intracellular reactions within the cell bearing the receptor, triggering modifications of molecules present in the cell or altering patterns of gene expression, resulting in alteration of biological functions of the cell. This process is called signal transduction.

Despite a long-felt need to understand and discover methods for regulating signal transduction networks in cells, the complexity of such networks has precluded development of products and processes for regulating signal transduction networks involved in disease. Binding of a protein to a target molecule can result in a variety of physiological events. A protein can modify a target molecule by, for example: allosteric alteration of the target molecule; by phosphorylating the target molecule; or by translocating the target molecule to another region of a cell. Because binding between a protein and a target molecule can be, for instance, serendipitous, responsible for translocation of a target molecule, responsible for activation of a target molecule, responsible for inhibitory actions etc., it is difficult to predict what particular steps of a signal transduction network are ultimately responsible for particular biological functions taking place.

As such, there remains a need to elucidate how particular cellular pathways operate and what molecules and/or functional elements of such molecules are

responsible for triggering and/or regulating such cellular pathways. In particular there is a need for products and processes that permit the effective regulation of specific steps of a signal transduction network. Regulation of specific steps of a signal transduction network permit the implementation of predictable controls of signal transduction in cells, thus enabling the treatment of various diseases that are caused by aberrations to a cell's signal transduction pathways.

SUMMARY OF THE INVENTION

The present invention generally relates to the regulation of signal transduction pathways and is more particularly directed to two particular portions of a cell's transduction pathways. The heretofore unappreciated and unrecognized interactions between particular molecules in a signal transduction pathway has resulted in the elucidation of products and processes that permit regulation of cells such as B cells, T cells, macrophages, dendritic cells and pluripotent stem cells. Moreover, the present invention permits regulation and treatment of various medical disorders including allergic responses, autoimmune diseases, inflammatory responses, cancer, immunodeficiency diseases, immunoproliferative diseases, and viral diseases including, but not limited to, caused by Epstein Barr Virus (EBV) infection (e.g., chronic fatigue syndrome or infectious mononucleosis) and Bovine Leukemia Virus (BLV) infection (e.g, B lymphocyte transformation).

One aspect of the present invention includes a method to regulate signal transduction, comprising contacting a cell with an effective amount of a compound comprising a peptide having an YXXLXXXXXXXYXXΨ amino acid motif, or a mimetope thereof. The present invention also relates to therapeutic compositions containing such compounds, and a kit utilizing such compounds in conjunction with a tyrosine kinase and a means for measuring the activity of the tyrosine kinase.

Another apsect of the present invention includes a method to regulate signal transduction, comprising contacting a cell with an effective amount of a compound a peptide, or a mimetope thereof, capable of binding to an SH3 domain of a tyrosine kinase, thereby blocking the binding of an effector to the tyrosine kinase. Also included in the present invention are therapeutic compositions containing such compounds, and a kit containing such compounds, together with a tyrosine kinase and a means for measuring the activity of the tyrosine kinase.

Other aspects and embodiments of the present invention will become obvious to one of ordinary skill in the art after consideration of the drawings and detailed description provided below.

BRIEF DESCRIPTION OF THE FIGURES

Fig. 1 is a schematic diagram of signal transduction pathways in a cell.

Fig. 2 is a schematic diagram of intracellular reactions before and after receptor ligation.

Fig. 3 is a diagrammatic representation of Ig-α and Ig-β ARH1 motifs switch mutants.

Fig. 4 depicts protein binding to wild type or Ig-α/Ig-β switch mutants.

Fig. 5 depicts protein binding to ARH1 peptide.

Fig. 6 depicts the binding activity of point mutants of the Ig-α ARH1 motif.

Fig. 7 depicts protein binding to wild type and Ig-α ARH1 fusion proteins in which three tyrosines were altered to phenylalanine.

Fig. 8 depicts binding of Fyn protein to wild type and Ig-α tyrosine mutated fusion proteins.

Fig. 9 illustrates Fyn binding to tyrosine phosphorylation of Ig-α ARH1 motifs.

Fig. 10 depicts protein binding to non-phosphorylated or phosphorylated Ig-α ARH1 peptides.

Fig. 11 depicts Fyn binding to phosphorylation of Ig-α and Ig-β peptides.

Fig. 12 depicts Fyn binding to doubly phosphorylated Ig-α and Ig-β. Fig. 13 illustrates the activity of Fyn protein when bound by phosphorylated Ig-α protein.

Fig. 14 illustrates the specific activity of Fyn protein bound to phosphorylated Ig-α peptide.

Fig. 15 depicts stimulation of phosphorylation of two proline-rich regions by Lyn and Fyn protein.

Fig. 16 depicts the binding of Fyn and Lyn protein to p85 peptides.

Fig. 17 depicts binding of SH3 domain fusion proteins to p85 peptides.

Fig. 18 illustrates stimulation of activity of PI-3K by SH3 domain fusion proteins.

Fig. 19 illustrates stimulation of PI-3K activity by p85 peptides.

Fig. 20 illustrates the immunoprecipitation of Shc protein using an Ig-α ARH1 motif.

Fig. 21 illustrates the immunoprecipitation of Shc protein using a variety of phosphorylated and nonphosphorylated ARH1 motifs.

Fig. 22 illustrates the immunoprecipitation of Shc protein using phosphorylated and nonphosphorylated Ig-α and Ig-ß ARH1 motifs.

Fig. 23 illustrates the differential binding of PI-3K, Syk, Lyn and Shc protein to a variety of biphosphorylated ITAM peptides.

DETAILED DESCRIPTION

The present invention relates to a novel product and process for regulating signal transduction pathways in a cell. As used herein, "signal transduction" refers to the intracellular chemical reactions that enable a cell to modify its biological functions based on the environment outside of the cell. The present invention includes two novel compounds capable of regulating signal transduction

pathways in a cell. The first novel compound of the present invention includes a peptide capable of regulating the activity of a src-family tyrosine kinase, a syk-family kinase, a Shc molecule and/or PI-3K, such peptide including an amino acid sequence represented by an YXXLXXXXXXXYXXΨ amino acid motif, in which X can be any amino acid and Ψ can be either leucine or isoleucine, or a mimetope thereof. The second novel compound of the present invention is a proline-rich peptide (Pro-rich peptide) or a mimetope thereof, capable of binding to an SH3 domain of a tyrosine kinase, thereby blocking the binding of a kinase to an effector of the kinase. According to the present invention, any amino acid sequence comprising such a peptide or nucleic acid sequence encoding such a peptide as defined herein includes a mimetope of such sequence.

The invention is particularly advantageous in that it provides novel compounds for regulating a wide variety of cellular functions by regulating different stages of signal transduction in a cell and provides for methods to identify additional compounds capable of regulating signal transduction in a cell.

Signal transduction pathways are used by cells to enable a cell to adjust to its environment. A signal transduction pathway transmits signals received outside of a cell, across the cell's plasma membrane, through the cytoplasm of the cell and into the nucleus. Signals transmitted in this manner typically result in alteration of gene expression in the cell. Multiple cellular elements

can be responsible for regulation of signal transduction in a cell. Such elements can include initiator molecules, transducer molecules, amplifier molecules, primary target molecules and second messenger molecules. As used herein, "molecule" can refer to a protein or a lipid. A schematic representation of proposed signal transduction pathways is shown in Fig. 1.

Initiator molecules are capable of initiating signal transduction in a cell. An example of an initiator molecule is an extracellular ligand capable of binding to a transducer molecule comprising a membrane-bound receptor on the surface of a cell. As used herein, a "ligand" refers to a molecule that donates electrons when a molecule associates with a second molecule. Extracellular ligands can include, for example hormones, growth factors, antigens, other differentiation agents, and other cell type specific mitogens. Following binding to an initiator molecule, a transducer molecule transmits a signal across the plasma membrane of a cell to intracellular amplifier molecules. Transducer molecules can include any cell surface receptor having cytoplasmic regions capable of interacting with intracellular proteins involved in signal transduction. Transducer molecules can include a kinase domain or be associated with an intracellular kinase. Examples of transducer molecules include, for example tyrosine kinase receptors, alpha and beta adrenergics, G protein-linked receptors, and other receptors involved in the immune response.

Receptors can be comprised of multiple proteins referred to as subunits, one category of which is referred to as a multisubunit receptor is a multisubunit immune recognition receptor (MIRR). MIRRs include receptors having multiple noncovalently associated subunits and are capable of interacting with src-family tyrosine kinases. MIRRs can include, but are not limited to, B cell antigen receptors, T cell antigen receptors, Fc receptors and CD22. One example of an MIRR is an antigen receptor on the surface of a B cell. The MIRR on the surface of a B cell comprises membrane-bound immunoglobulin (mIg) associated with the subunits Ig-α and Ig-β or Ig-γ, which forms a complex capable of regulating B cell function when bound by antigen. An antigen receptor can be functionally linked to an amplifier molecule in a manner such that the amplifier molecule is capable of regulating gene transcription.

A transducer molecule can interact with an amplifier molecule to further extend transmission of a signal received at the cell surface into the cytoplasm of a cell. An amplifier molecule is typically an enzyme capable of modifying a primary target molecule in such a manner that intracellular physiological changes occur resulting in alteration of gene transcription. Amplifier molecules can modify primary target molecules by, for example: allosteric alteration of a primary target molecule; by phosphorylating a primary target molecule; or by translocating a primary target molecule to another region of a cell. A primary target molecule can include, but is not limited to, a

target molecule or an effector. As used herein, a "target molecule" is a molecule which is modified by a particular enzyme by phosphorylation. As used herein, an "effector" is a molecule which is modified by a particular enzyme by allosteric alteration. Examples of amplifier molecules involved in signal transduction include, but are not limited to, a kinase receptor and a non-receptor kinase, such as a src-family kinase or a syk-family kinase.

Src-family tyrosine kinases are enzymes capable of phosphorylating tyrosine residues of a target molecule. Typically, a src-family tyrosine kinase contains one or more binding domains and a kinase domain. A binding domain of a src-family tyrosine kinase is capable of binding to a target molecule and a kinase domain is capable of phosphorylating a target molecule bound to the kinase. Members of the src family of tyrosine kinases are characterized by an N-terminal unique region followed by three regions that contain different degrees of homology among all the members of the family. These three regions are referred to as src homology region 1 (SH1), src homology region 2 (SH2) and src homology region 3 (SH3). Both the SH2 and SH3 domains are believed to have protein association functions important for the formation of signal transduction complexes. The amino acid sequence of an N-terminal unique region, varies between each src-family tyrosine kinase. An N-terminal unique region can be at least about the first 40 amino acid residues of the N-terminal of a src-family tyrosine kinase.

Syk-family kinases are enzymes capable of phosphorylating tyrosine residues of a target molecule. Typically, a syk-family kinase contains one or more binding domains and a kinase domain. A binding domain of a syk-family tyrosine kinase is capable of binding to a target molecule and a kinase domain is capable of phosphorylating a target molecule bound to the kinase. Members of the syk-family of tyrosine kinases are characterized by two SH2 domains for protein association function and a tyrosine kinase domain.

A primary target molecule is capable of further extending a signal transduction pathway by modifying a second messenger molecule. Primary target molecules can include, but are not limited to, phosphatidylinositol 3-kinase (PI-3K), $P21^{ras}$GAPase-activating protein and associated P190 and P62 protein, phospholipases such as PLCγ1 and PLCγ2, MAP kinase, Shc and VAV. A primary target molecule is capable of producing second messenger molecule which is capable of extending molecules include, but are not limited to diacylglycerol and inositol 1,4,5-triphosphate (InsP3). Second messenger molecules are capable of initiating physiological events which can lead to alterations in gene transcription. For example, production of InP3 can result in release of intracellular calcium, which can then lead to activation of calmodulin kinase II, which can then lead to serine phosphorylation of a DNA binding protein referred to as ets-1 proto-onco-protein. Diacylglycerol is capable of activating the signal

transduction protein, protein kinase C which affects the activity of the AP1 DNA binding protein complex. Signal transduction pathways can lead to transcriptional activation of genes such as c-fos, egr-1, and c-myc.

According to the present invention, Shc is an adaptor molecule. An adaptor molecule comprises a protein that enables two other proteins to form a complex (e.g., a three molecule complex). Shc protein enables a complex formation including Grb2 and SOS. Shc comprises an SH2 domain that is capable of associating with the SH2 domain of Grb2.

Molecules of a signal transduction pathway can associate with one another using recognition sequences. According to the present invention, recognition sequences enable specific binding between two molecules. Recognition sequences can vary depending upon the structure of the molecules that are associating with one another. A molecule can have one or more recognition sequences, and as such can associate with one or more different molecules.

Signal transduction pathways are capable of regulating the biological functions of a cell. Such functions can include, but are not limited to the ability of a cell to grow, to differentiate and to secrete cellular products. Signal transduction pathways can regulate the biological functions of specific types of cells involved in particular responses by an animal, such as immune responses, inflammatory responses and allergic responses. Cells involved in an immune response can include, for example, B cells, T cells, macrophages, dendritic cells, natural

killer cells and plasma cells. Cells involved in inflammatory responses can include, for example, basophils, mast cells, eosinophils, neutrophils and macrophages. Cells involved in allergic responses can include, for example mast cells, basophils, B cells, T cells and macrophages. In addition, such responses by an animal can be regulated by signal transduction pathways of pluripotent progenitor cells which are capable of developing into mature cells involved in the aforementioned responses and other functions of an animal. Abnormal biological functions of cells can result in disease. As such, modulation of signal transduction pathways to correct such abnormalities can be useful in the treatment of disease. In addition, a variety of medical procedures, such as transplantation of organs and skin, require regulation of the biological function of specific cell types. As such, modulation of signal transduction pathways in such cells can be useful in particular medical procedures.

It is known by those of skill in the art that binding between a protein and a target molecule can result in a variety of biological events. For example, binding between a protein and a target molecule can result in modification of the conformation of the target molecule, thereby enabling the target molecule to be bound by a second protein, or alternatively, to prevent typical binding to occur, thereby modulating regulation of systems in which such molecules are involved. In another example, binding between a protein and a target molecule can result in the

translocation of the target molecule to bring the target molecule within the proximity of other proteins capable of binding the target molecule. In yet another example, binding between a protein and a target molecule can result in the modification of the target molecule in such a manner that the activity of the target molecule is activated, stimulated or inhibited. The activity of the target molecule can include, for example, enzymatic activity or binding activity. It is also known by those of skill in the art that the binding between a protein and a target molecule can be dependent upon specific recognition sequences on the protein and on the target molecule. According to the present invention, recognition sequences are sequences that enable a protein to bind to a target molecule in a specific manner. A single protein can bind to different target molecules using different recognition sequences included in the sequence of the protein. Conversely, a single target molecule can be bound by different proteins using different recognition sequences included in the sequence of the target molecule. The complexity of molecular interactions between proteins and target molecules limits the ability of one of skill in the art to understand and predict particular interactions.

In one embodiment, a compound of the present invention is capable of regulating signal transduction in a cell by regulating the activity of a tyrosine kinase, preferably the activity of the tyrosine kinases Fyn, Lyn, Blk, Syk, Yes, Lck, Btk, Hck, Src and Zap70. In another embodiment,

a compound of the present invention is capable of regulating regulating the activity of an adaptor molecule, preferably Shc. In yet another embodiment, a compound of the present invention is capable of regulating the activity of a lipid kinase, preferably PI-3K. In yet another embodiment, a compound of the present invention is capable of regulating the activity of proteins including GAP, CD22 and MAPK.

In another embodiment, the compound of the present invention comprises an isolated peptide (or a mimetope thereof) that has an amino acid motif of YXXLXXXXXXXYXXΨ. According to the present invention, an isolated, or biologically pure, peptide, is a peptide that has been removed from its natural milieu. As such, "isolated" and "biologically pure" do not necessarily reflect the extent to which the protein has been purified. An isolated compound of the present invention can be obtained from a natural source or produced using recombinant DNA technology or chemical synthesis. As used herein, an isolated peptide can be a full-length protein or any homolog of such a protein in which amino acids have been deleted (e.g., a truncated version of the protein), inserted, inverted, substituted and/or derivatized (e.g., acetylated, glycosylated, carboxymethylated anchored by myristoylated, prenylated or palmitoylated amino acids) such that the peptide is capable of regulating the activity of a tyrosine kinase, an adaptor molecule and/or a lipid kinase.

In accordance with the present invention, a "mimetope" refers to any compound that is able to mimic the ability of an isolated compound of the present invention. A mimetope can be a peptide that has been modified to decrease its susceptibility to degradation but that still retain regulatory activity. Other examples of mimetopes include, but are not limited to, protein-based compounds, carbohydrate-based compounds, lipid-based compounds, nucleic acid-based compounds, natural organic compounds, synthetically derived organic compounds, anti-idiotypic antibodies and/or catalytic antibodies, or fragments thereof. A mimetope can be obtained by, for example, screening libraries of natural and synthetic compounds for compounds capable of regulating the activity of a tyrosine kinase as disclosed herein. A mimetope can also be obtained, for example, from libraries of natural and synthetic compounds, in particular, chemical or combinatorial libraries (i.e., libraries of compounds that differ in sequence or size but that have the same building blocks). A mimetope can also be obtained by, for example, rational drug design. In a rational drug design procedure, the three-dimensional structure of a compound of the present invention can be analyzed by, for example, nuclear magnetic resonance (NMR) or x-ray crystallography. The three-dimensional structure can then be used to predict structures of potential mimetopes by, for example, computer modelling. the predicted mimetope structures can then be produced by, for example, chemical synthesis, recombinant

DNA technology, or by isolating a mimetope from a natural source (e.g., plants, animals, bacteria and fungi).

In one embodiment, a compound of the present invention includes an isolated ARH1 peptide that has an amino acid sequence which enables the peptide to be bound by a src-family tyrosine kinase in a manner such that the peptide is capable of regulating the activity of the src-family tyrosine kinase. An ARH1 peptide of the present invention is of a size and nature that enables the peptide to be bound by at least one binding site of a src-family tyrosine kinase. In particular, an ARH1 peptide of the present invention is capable of being bound by an N-terminal unique region, an SH2 domain, and an SH3 domain of a src-family tyrosine kinase. Preferably, an ARH1 peptide of the present invention is capable of being bound by an N-terminal unique region and an SH2 domain of a src-family tyrosine kinase.

In another embodiment, a compound of the present invention includes an isolated ARH1 peptide that has an amino acid sequence that enables the peptide to be bound by a syk-family kinase in a manner such that the peptide is capable of regulating the binding of a syk-family tyrosine kinase to a target molecule (e.g., an MIRR). An ARH1 peptide of the present invention is of a size and nature that enables the peptide to be bound by associating with at least one binding site of a syk-family tyrosine kinase. In particular, an ARH1 peptide of the present invention is capable of being bound by an SH2 domain of a syk-family kinase, preferably both SH2 domains of a syk-family kinase.

In yet another embodiment, a compound of the present invention includes an isolated ARH1 peptide that has an amino acid sequence that enables the peptide to be bound by Shc in a manner such that the peptide is capable of regulating the binding of a Shc molecule to a target molecule (e.g., Grb2 or SOS). An ARH1 peptide of the present invention is of a size and nature that enables the peptide to be bound by at least one binding site of a Shc molecule. In particular, an ARH1 peptide of the present invention is capable of being bound by an SH2 domain of a Shc molecule.

In yet another embodiment, a compound of the present invention includes an isolated ARH1 peptide that has an amino acid sequence that enables the peptide to be bound by PI-3K in a manner such that the peptide is capable of regulating SP-6 kinase activation. An ARH1 peptide of the present invention is of a size and nature that enables the peptide to be bound by at least one binding site of a PI-3K molecule. In particular, an ARH1 peptide of the present invention is capable of being bound by an SH2 domain of a PI-3K molecule.

A compound of the present invention includes an ARH1 peptide comprising at least about 4 amino acid residues, preferably about 15 amino acid residues, more preferably about 17 amino acid residues, though the peptide may be at least about 26 amino acid residues. In one embodiment, an ARH1 peptide of the present invention has a size of about 3kD and has about 26 amino acid residues.

In one embodiment, an amino acid sequence of an ARH1 peptide of the present invention includes an ARH1 motif having at least 2 tyrosine residues and at least two leucine and/or isoleucine residues. As used herein, "motif" refers to a series of amino acid residues contained within a protein having a particular function. Preferably, an ARH1 motif of the present invention includes tyrosine, leucine and/or isoleucine residues having a spatial arrangement represented by an YXXLXXXXXXXYXXΨ amino acid motif using standard one letter amino acid code, and in which X can be any amino acid, "Ψ" can be either leucine or isoleucine.

In one embodiment, an ARH1 peptide can include a sequence capable of regulating the activity of a src-family tyrosine kinase in B cells. Preferably, src-family kinase-specific ARH1 peptides include YXXLXXXDCSMYXXΨ, YXXLXXXQTATYXXΨ, YXXLXXXYSPIYXXΨ or YXXLXXXNQETYXXΨ, more preferably an ARH1 motif substantially similar to the ARH1 motif of Ig-α, Ig-ß, FcεRIß or FcεRIγ protein, and even more preferably YXXLXXXDCSMYXXΨ. A particularly preferred ARH1 peptide of the present invention comprises the amino acid sequence YEGLNLDDCSMYEDI, with the amino acid sequence NLYEGLNLDDCSMYEDI being even more preferred.

In another embodiment, an ARH1 peptide can include a sequence capable of regulating the activity of a syk-family tyrosine kinase. Preferably, syk-family kinase-selective ARH1 peptides include an amino acid motif YXXLXXXTKDTYXXΨ, YXXLXXXQRDLYXXΨ, YXXLXXXYSPIYXXΨ or YXXLXXXNQETYXXΨ, more preferably an ARH1 motif substantially similar to the ARH1

motif of TCRςc, CD3ϵ, FcϵRIγ or FcϵRIß protein, and even more preferably an amino acid motif YXXLXXXTKDTYXXΨ or YXXLXXXQRDLYXXΨ. A particularly preferred syk-family kinase-selective ARH1 peptide of the present invention comprises the amino acid motif YQGLSTATKDTYDAL or YEPIRKGQRDLYSGL, with the amino acid motifs DGLYQGLSTATKDTYDAL or NPDYEPIRKGQRDLYSGL being even more preferred.

A syk-family kinase-selective peptide of the present invention can be either phosphorylated or not phosphorylated. A non-tyrosine phosphorylated syk-family kinase-selective peptide preferably comprises an ARH1 motif comprising the amino acid motif YXXLXXXDCSMYXXΨ, more preferably an ARH1 motif substantially similar to an Ig-α ARH1 motif, and even more preferably an ARH1 motif comprising the amino acid motif NLYEGLNLDDCSMYEDI. A tyrosine phosphorylated syk-family kinase-selective peptide preferably comprises an ARH1 motif comprising the amino acid motif pYXXLXXXTKDTpYXXΨ, pYXXLXXXQRDLpYXXΨ, pYXXLXXXYSPIpYXXΨ or pYXXLXXXNQETpYXXΨ, more preferably an ARH1 motif substantially similar to the ARH1 motif of TCRςc, CD3ϵ, FcϵRIγ or FcϵRIß protein in which the peptide is phosphorylated, and even more preferably the amino acid motif pYXXLXXXTKDTpYXXΨ or pYXXLXXXQRDLpYXXΨ.

In yet another embodiment, an ARH1 peptide can include a sequence capable of binding to a Shc adaptor molecule. Preferably, Shc-selective ARH1 peptides include YXXLXXXDCSMYXXΨ, YXXLXXXQTATYXXΨ, YXXLXXXYSPIYXXΨ or

YXXLXXXNQETYXXΨ, more preferably an ARH1 motif substantially similar to the ARH1 motif of Ig-α, Ig-ß, FcεRIγ or FcεRIß protein, and even more preferably an YXXLXXXDCSMYXXΨ ARH1 motif. A particularly preferred ARH1 peptide of the present invention comprises the amino acid motif YEGLNLDDCSMYEDI, with the amino acid motif NLYEGLNLDDCSMYEDI being more preferred.

A Shc-selective peptide of the present invention can be either tyrosine phosphorylated or not tyrosine phosphorylated. A non-tyrosine phosphorylated Shc-selective peptide preferably comprises an ARH1 motif YXXLXXXDCSMYXXΨ, more preferably an ARH1 motif substantially similar to an Ig-α ARH1 motif, and even more preferably an ARH1 motif comprising the amino acid sequence NLYEGLNLDDCSMYEDI. A tyrosine phosphorylated Shc-selective peptide preferably comprises an pYXXLXXXQTATpYXXΨ, pYXXLXXXYSPIpYXXΨ or pYXXLXXXNQETpYXXΨ ARH1 motif, more preferably an ARH1 motif substantially similar to an Ig-ß, FcεRIγ or FcεRIß protein, and even more preferably an ARH1 motif comprising the amino acid motif DHTYEGLNIDQTATYEDI, DRLYEELNHVYSPIYSEL or DAVYTGLNTRNQETYETL, in which the peptide is phosphorylated.

In yet another embodiment, an ARH1 peptide can include a sequence capable of binding to PI-3K. Preferably, PI-3K-selective ARH1 peptides comprise an amino acid motif YXXLXXXDCSMYXXΨ, YXXLXXXQTATYXXΨ, YXXLXXXYQRDLYXXΨ, YXXLXXXYYSPIYXXΨ or YXXLXXXNQETYXXΨ, more preferably an ARH1 motif substantially similar to the ARH1 motif of Ig-α, Ig-ß, CD3ε, FcεRIß or FcεRIγ protein, and even more

preferably an YXXLXXXNQETYXXΨ ARH1 motif. A particularly preferred ARH1 peptide of the present invention comprises the sequence DAVYTGLNTRNQETYETL. In a preferred embodiment, a PI-3K-selective peptide of the present invention is tyrosine phosphorylated.

Particularly preferred mimetopes of an ARH1 peptide of the present invention can include, but are not limited to the amino acid sequences shown in Table 1.

TABLE 1

Consensus	D	X	X	X	-	X	X	X	X	D/E	X	X	Y	X	X	L/I	X	X	X	X	-	-	-	-	-	X
mouse MB-1	D	M	P	D	-	D	Y	E	D	E	N	L	Y	E	G	L	N	L	D	D	-	-	-	-	-	C
human MB-1	D	A	G	D	-	E	Y	E	D	E	N	L	Y	E	G	L	N	L	D	D	-	-	-	-	-	C
mouse B29	D	G	K	A	-	G	M	E	E	D	H	T	Y	E	G	L	N	I	D	Q	-	-	-	-	-	T
human B29	D	S	K	A	-	G	M	E	E	D	H	T	Y	E	G	L	D	I	D	Q	-	-	-	-	-	T
mouse TCR-ζ/ηa	E	T	A	A	N	L	Q	D	P	N	Q	L	Y	N	E	L	N	L	G	R	-	-	-	-	-	R
mouse TCR-ζ/ηβ	K	Q	Q	R	R	R	N	P	Q	E	G	V	Y	N	A	L	Q	K	D	K	M	-	-	-	-	A
mouse TCR-ζc	E	R	R	R	G	K	G	H	-	D	G	L	Y	Q	G	L	S	T	A	T	-	-	-	-	-	K
mouse TCR-ηc	E	R	R	R	G	K	G	H	-	D	G	L	Y	D	S	H	F	Q	A	V	Q	F	-	-	-	G
mouse CD3-γ	D	K	Q	T	-	L	L	Q	N	E	Q	L	Y	Q	P	L	K	D	R	E	-	-	-	-	-	Y
mouse CD3-δ	E	V	Q	A	-	L	L	K	N	E	Q	L	Y	Q	P	L	R	D	R	E	-	-	-	-	-	D
mouse CD3-ε	N	K	E	R	P	P	P	V	P	N	P	D	Y	E	P	I	R	K	G	Q	-	-	-	-	-	R
chicken TII.15	D	R	Q	N	-	L	I	A	N	D	Q	L	Y	Q	P	L	G	E	R	N	-	-	-	-	-	D
mouse FcεRI-γ	A	A	I	A	S	R	E	K	A	D	A	V	Y	T	G	L	N	T	R	N	-	-	-	-	-	Q
mouse FcεRI-β	E	L	E	S	K	K	V	P	D	D	R	L	Y	E	E	L	N	H	V	Y	-	-	-	-	-	S
human FcγRIIa	E	T	N	N	D	Y	E	T	A	D	G	G	Y	M	T	L	N	P	R	A	P	T	D	D	D	K
BLV gp30a	P	E	I	S	L	T	P	K	P	D	S	D	Y	Q	A	L	L	P	S	A	-	-	-	-	-	P
BLV gp30b	D	Y	Q	A	L	L	P	S	A	P	E	I	Y	S	H	L	S	P	V	-	-	-	-	-	-	K
EBV LMP2	D	P	Y	W	G	N	G	D	R	H	S	D	Y	Q	P	L	G	T	Q	D	-	-	-	-	-	Q
EBV EBNA2	-	-	-	-	-	-	-	-	-	M	P	T	F	Y	L	A	L	H	G	-	-	-	-	-	-	G

In another embodiment, an ARH1 peptide of the present invention is capable of stimulating the specific activity of a src-family tyrosine kinase. As used herein, the term "specific activity" refers to the rate at which a src-family tyrosine kinase can modify a primary target molecule. The rate of specific activity can be measured by, for example, the rate at which a src-family tyrosine kinase phosphorylates a primary target molecule. Specific activity can also refer to the rate of autophosphorylation of a src-family tyrosine kinase. Preferably, an ARH1 peptide of the present invention is capable of stimulating the specific activity of a src-family tyrosine kinase at least about 2-fold and up to about 70-fold, and more preferably from about 3-fold to about 60-fold. Stimulation of specific activity is seen when the peptide is incubated with immunoprecipitates of a src-family tyrosine kinase.

An ARH1 peptide of the present invention capable of stimulating the activity of a src-family tyrosine kinase includes at least two phosphorylated tyrosine residues. Preferably, a stimulatory ARH1 peptide includes an ARH1 motif having at least two phosphorylated tyrosine residues spatially arranged in the manner described herein. Preferably, a stimulatory ARH1 peptides of the present invention comprises the amino acid motif pYXXLXXXDCSMpYXXΨ, pYXXLXXXQTATpYXXΨ, pYXXLXXXYSPIpYXXΨ or pYXXLXXXNQETpYXXΨ in which pY refers to a phosporylated tyrosine residue, more preferably a ARH1 motif substantially similar to the ARH1 motif of Ig-α, Ig-ß, FcεRIß, or FcεRIγ protein in

which the tyrosine residues are phosphorylated, and even more preferably an pYXXLXXXDCSMpYXXΨ ARH1 motif. A particularly preferred ARH1 peptide of the present invention comprises the sequence pYEGLNLDDCSMpYEDI, with the sequence NLpYEGLNLDDCSMpYEDI being more preferred.

Without being bound by theory, it is believed that a src-family tyrosine kinase can non-covalently associate with an ARH1 motif of a membrane-bound receptor. First, the N-terminal unique region of a src-family tyrosine kinase associates with the ARH1 motif of a receptor and remains unstimulated until a ligand binds to the extracellular domain of the receptor. Upon ligand binding, the receptor undergoes a conformational change that initiates phosphorylation of the tyrosine residues contained in the ARH1 motif, e.g. the tyrosines are phosphorylated by the bound kinase. The SH2 domain of the bound kinase then binds to the phosphorylated ARH1 motif. It is believed that the second association event derepresses the activity of the bound kinase by modulating the position of a c-terminal phosphorylated tyrosine in such a manner that the kinase domain of the bound kinase becomes available to modify a primary target of the kinase. Thus, the phosphorylated ARH1 motif is capable of derepressing the bound kinase by removal of elements actively inhibiting the activity of the kinase. Without being bound by theory, Fig. 2 represents interactions that can take place between a portion of a receptor complex (Ig-α) and intracellular molecules. In particular, Fig. 2

illustrates the conformational change of a tyrosine kinase (kin.) that can occur following ligation of a receptor by ligand.

According to the mechanism described above, an ARH1 peptide containing an ARH1 motif is not an enzyme. Rather, the peptide associates with a signal transduction molecule and induces conformational changes of the signal transduction molecule. It is also believed that non-phosphorylated or partially phosphorylated tyrosine residues of the ARH1 motif can inhibit binding of a N-terminal unique region of a src-family tyrosine kinase to an ARH1 peptide.

An inhibitory src-family tyrosine kinase ARH1 peptide of the present invention can include an ARH1 motif having two tyrosine residues, wherein only one of the tyrosine residues is phosphorylated or neither tyrosine residue is phosphorylated. Preferably, the tyrosine residues of an inhibitory ARH1 peptide of a src-family tyrosine kinase is not phosphorylated. A preferred src-family tyrosine kinase inhibitory ARH1 peptide of the present invention includes an amino acid motif FXXLXXXXXFXXI, more preferably the motif FXXLXXXDCSMFXXΨ, FXXLXXXQTATFXXΨ, FXXLXXXYSPIFXXΨ or FXXLXXXNQETFXXΨ, and even more preferably the motif FXXLXXXDCSMFXXΨ. A particularly preferred src-family tyrosine kinase inhibitory ARH1 peptide of the present invention comprises includes an amino acid motif FEGLNLDDCSMFEDI, with the amino acid motif DMPDDYEDENLFEGLNLDDCSMFEDI being more preferred.

According to the present invention, a signal transduction regulatory compound of the present invention can comprise an amino acid sequence containing one or more ARH1 motifs of the present invention. For example, a compound of the present invention that is capable of regulating the activity of a syk-family kinase can include the amino acid sequence YXXLXXXTKDTYXXΨ, covalently associated with the amino acid sequence YXXLXXXQRDLYXXΨ, YXXLXXXYSPIYXXΨ or YXXLXXXNQETYXXΨ. Alternatively, the amino acid sequence YXXLXXXTKDTYXXΨ can be covalent linked to the amino acid sequence YXXLXXXQRDLYXXΨ, which itself is covalently linked to YXXLXXXQRDLYXXΨ, YXXLXXXYSPIYXXΨ or YXXLXXXNQETYXXΨ. Preferably, a multiple ARH1 motif containing compound of the present invention comprises the amino acid sequence YQGLSTATKDTYDAL, covalently associated with the amino acid sequence YEPIRKGQRDLYSGL, and more preferably comprises the amino acid sequence DHTYQGLSTATKDTYDAL, covalently associated with the amino acid sequence NPDYEPIRKGQRDLYSGL.

A separate aspect of the present invention relates to the recognition that one can regulate the activity of particular enzymes, predicated upon the appreciation that proline-rich regions of such enzymes can be targeted to hinder normal binding patterns, thus allowing one to modify enzymatic activities in the signal transduction pathway.

Previous investigators have shown that the enzyme PI-3K can act as a target molecule for up to five enzymes, e.g. platelet derived growth factor receptor (PDGFR), $pp60^{v-}$

^{src}, pp60$^{c\text{-}src}$, insulin receptor, IL-4 receptor and the CSF-1 receptor (Otsu et al., pp. 91-104, 1991, *Cell.*, Vol. 65). PDGFR was shown to bind to the SH2 domain of PI-3K using a recognition sequence comprising a phosphopeptide having a YXXM amino acid motif, wherein X is any amino acid and the Y residue is phosphorylated (*ibid.*). Such binding was shown to activate PI-3K function. Thus, one of skill in the art would be led to believe that the mechanism for activating PI-3K is through binding of PI-3K to the YXXM motif of a protein. In addition, pp60$^{v\text{-}src}$ and pp60$^{c\text{-}src}$ were shown to bind to PI-3K by their SH3 domains (*ibid.*). Such binding was not shown to result in activation of PI-3K. Thus, enzyme binding to PI-3K can occur using different recognition sequences and limited activation of the PI-3K can be achieved upon binding of a phosphopeptide sequence YXXM.

In one embodiment, a compound of the present invention is capable of inhibiting the binding of the SH3 domain of a tyrosine kinase to a primary target molecule, thereby inhibiting the activation of the target molecule. Suitable target molecules of the present invention include proteins having proline-rich sequences capable of being bound by a tyrosine kinase. Preferably, target molecules of the present invention include, but are not limited to, effector molecules such as PI-3K, ras-GAP, 3BP1 and 3BP2.

According to the present invention, a compound of the present invention comprises an isolated Pro-rich peptide capable of regulating the activity of receptor and/or non-

receptor tyrosine kinases which can act as amplifiers in a signal transduction pathway. As used herein, a Pro-rich peptide also refers to an isolated Pro-rich peptide. A Pro-rich peptide of the present invention is particularly capable of regulating signal transduction pathways originating from ligand binding to a receptor capable of associating with a src-family tyrosine kinase by regulating the activity of a src-family tyrosine kinase. Suitable receptors of the present invention include, but are not limited to a B cell antigen receptor, a T cell antigen receptor, an Fc receptor, MHC class II, an IL-4 receptor and CD40. Suitable src-family tyrosine kinases of the present invention include, but are not limited to Fyn, Lyn, Lck, Blk, Syk, Yes, Btk, Hck, Zap70 and Src.

A Pro-rich peptide of the present invention is capable of regulating a tyrosine kinase by interfering with the association of the kinase with a primary target molecule. A Pro-rich peptide of the present invention comprises a sufficient size to enable the peptide to bind to a tyrosine kinase in such a manner that the peptide interferes with the association of the kinase with a primary target molecule. Preferably, a Pro-rich peptide of the present invention has at least about 5 amino acid residues, more preferably from about 7 amino acid residues to about 40 residues, and even more preferably from about 10 amino acid residues to about 30 amino acid residues. A Pro-rich peptide of the present invention can be of a size as great as about 85kD and as small as 1kD.

A Pro-rich peptide of the present invention comprises an amino acid sequence that enables the peptide to bind to a tyrosine kinase in such a manner that the peptide interferes with the association of the kinase with a primary target molecule. Preferably, a Pro-rich peptide of the present invention comprises an amino acid sequence capable of binding to an SH3 domain of a tyrosine kinase, more preferably to an SH3 domain of a src-family tyrosine kinase, and even more preferably to a proline-rich sequence binding site included in an SH3 domain of a src-family tyrosine kinase.

In one embodiment, the amino acid sequence of a Pro-rich peptide of the present invention includes a proline-rich motif having from about 20% proline residues to about 100% proline residues, more preferably from about 30% proline residues to about 90% proline residues, and even more preferably from about 40% proline residues to about 80% proline residues. The proline residues can be spatially arranged in a manner appropriate to enable a Pro-rich peptide of the present invention to be bound by a proline-rich binding site on a tyrosine kinase. Such a spatial arrangement can be represented by a PPXPXPXPPXPXP amino acid motif or a PXPXXPPXPPXP amino acid motif, wherein X represents any amino acid residue. Proline residues are symbolized by P.

In a preferred embodiment, a Pro-rich peptide of the present invention comprises the proline-rich domain of PI-3K. PI-3K is a heterodimeric protein composed of a non-

catalytic p85 subunit (85kD) and a catalytic p110 subunit (110kD). PI-3K is capable of phosphorylating inositol lipids on the D-3 hydroxyl position. Contained within the p85 subunit are two proline-rich domains. A first proline-rich domain of PI-3K extends from about residue 80 to about residue 104 and can include the amino acid motif KKISPPTPKPRPPRPTPVAPGSSKT. A second proline-rich domain of PI-3K extends from about residue 299 to about residue 318 and can include the amino acid motif NERQPAPATPPKPPKPTTVA. Preferably, a Pro-rich peptide of the present invention includes at least one amino acid motif comprising KKISPPTPKPRPPRPTPVAPGSSKT or NERQPAPATPPKPPKPTTVA. According to the present invention, K=lysine, R=arganine, V=valine, and Q=glutamine.

A Pro-rich peptide, or a mimetope thereof, of the present invention is capable of inhibiting the activation of a target molecule by a tyrosine kinase, due to the Pro-rich peptides prior binding to the tyrosine kinase, thus preventing the binding, and hence activation, of the target molecule. Preferably, a Pro-rich peptide of the present invention is capable of inhibiting the binding of a tyrosine kinase to a target molecule by about 70%, more preferably by about 80% and even more preferably by about 90%. In addition, a Pro-rich peptide of the present invention is capable of substantially inhibiting binding (between 90% and 100%) between a tyrosine kinase and a target molecule when the peptide is present at a 200:1 ratio of peptide to kinase, more preferably at a 100:1

ratio of peptide to kinase, and even more preferably at a 20:1 ratio of peptide to kinase.

In a preferred embodiment, a Pro-rich peptide of the present invention is capable of inhibiting the activation of PI-3K when the peptide is introduced into a cell containing PI-3K. Preferably, such PI-3K activation is inhibited by at least about 80% and more preferably by at least about 90%.

According to the present invention, a mimetope of the compounds of the present invention described in detail herein is capable of crossing a lipid bilayer, such as a plasma membrane of a cell. Mimetopes capable of crossing a lipid bilayer can be organic molecules, in particular carbohydrate-based molecules. Also according to the present invention, a compound of the present invention can include concatomers, wherein one or more ARH1 peptides or one or more Pro-rich peptides are linked together.

Another aspect of the present invention relates to an isolated nucleic acid molecule that encodes a compound of the present invention as herein disclosed. According to the present invention, references to nucleic acids also refer to nucleic acid molecules. In accordance with the present invention, an isolated nucleic acid molecule is a nucleic acid molecule that has been removed from its natural milieu (i.e., that has been subject to human manipulation). As such, "isolated" does not necessarily reflect the extent to which the nucleic acid molecule has be purified. An isolated nucleic acid molecule can include

DNA, RNA, or hybrid or derivatives of either DNA or RNA. Nucleic acid molecules of the present invention can include a regulatory region that controls expression of the nucleic acid molecule (e.g., transcription or translation control regions), full-length or partial coding regions, and combinations thereof. Any portion of a nucleic acid molecule the present invention can be produced by (1) isolating the molecule from its natural milieu; (2) using recombinant DNA technology (e.g., PCR Amplification, cloning); or (3) using chemical synthesis methods. A nucleic acid molecule of the present invention can include functional equivalents of natural nucleic acid molecules encoding a compound of the present invention including, but not limited to, natural allelic variants and modified nucleic acid molecules in which nucleotides have been inserted, deleted, substituted, and/or inverted in such a manner that such modifications do not substantially interfere with the nucleic acid molecules' ability to encode a compound of the present invention capable of regulating signal transduction. Preferred functional equivalents include nucleic acid sequences that are capable of hybridizing under stringent conditions, to: at least a portion of a nucleic acid molecule encoding an ARH1 peptide derived from an Ig-α, Ig-ß, TCRςc, CD3ϵ, FcϵRIγ or FcϵRIß protein; or at least a portion of a nucleic acid molecule encoding a Pro-rich peptide derived from a PI-3K protein. Stringent hybridization conditions are described in Sambrook et al, Molecular Cloning: A Laboratory Manual,

Cold Spring Harbor Labs Press, 1989, which is incorporated herein by reference in its entirety). As guidance in determining what particular modifications can be made to any particular nucleic acid molecule, one of skill in the art should consider several factors that, without the need for undue experimentation, permit a skilled artisan to appreciate workable embodiments of the present invention. For example, such factors include modifications to nucleic acid molecules performed in a manner so as to maintain particular functional regions of the encoded compound of the present invention including: an ARH1 motif capable of being bound by a tyrosine kinase, an adaptor molecule or a lipid kinase in such a manner that the motif is capable of regulating the activity of the kinase or adaptor molecule; or a proline-rich motif capable of binding to the SH3 domain of a tyrosine kinase, thereby altering the activity or binding ability of the kinase. Functional tests of these various characteristics (e.g., binding studies, kinase assays, lipid phosphorylation assays) allows one of skill in the art to determine what modifications to the nucleic acid molecules would be appropriate and which would not.

In one embodiment, an isolated nucleic acid molecule of the present invention includes a nucleic acid sequence that encodes an ARH1 peptide of the present invention, examples of such peptide being disclosed herein. An ARH1 peptide nucleic acid molecule of the present invention can include a nucleic acid sequence encoding an ARH1 motif having an amino acid sequence YXXLXXXXXXXYXXΨ or a mimetope

thereof. Preferred nucleic acid molecules encoding ARH1 peptides include nucleic acid sequences encoding the peptide sequence YXXLXXXDCSMYXXΨ, YXXLXXXQTATYXXΨ, YXXLXXXYSPIYXXΨ, YXXLXXXNQETYXXΨ, YXXLXXXTKDTYXXΨ, YXXLXXXQRDLYXXΨ, or a mimetope thereof. More preferred nucleic acid molecules encoding ARH1 peptides include nucleic acid sequences encoding the peptide sequence NLYEGLNLDDCSMYEDI, DHTYEGLNIDQTATYEDI, DRLYEELNHVYSPIYSEL, DAVYTGLNTRNQETYETL, DGLYQGLSTATKDTYDAL, NPDYEPIRKGQRDLYSGL, or a mimetope thereof. An even more preferred nucleic acid molecule comprises the sequence: 5'ATG GGG AGT AGC AAG AGC AAG CCT AAG GAC CCC AGC CAG CGC CGG GAC TAC AAG GAC GAC GAT GAC AAG GAC ATG CCA GAT GAC TTT GAA GAT GAA AAT CTC TTT GAG GGC CTG AAC CTT GAT GAC TGT TCT ATG TTT GAG GAC ATC 3'; or 5' ATG GGG AGT AGC AAG AGC AAG CCT AAG GAC CCC AGC CAG CGC CGG GAC TAC AAG GAC GAC GAT GAC AAG GAC GGC AAG GCT GGG ATG GAG GAA GAT CAC ACC TTT GAG GGC TTG AAC ATT GAC CAG ACA GCC ACC TTT GAA GAC ATA 3'.

At least a portion of a nucleic acid molecule encoding an ARH1 peptide can be covalently associated (using standard recombinant DNA methods) to any other sequence that encodes for at least a portion of a distinct component (e.g., control element) to produce an ARH1 peptide of the present invention. The sequences can be attached in such a manner so that the sequences are transcribed in-frame, thereby producing a functional ARH1 peptide capable of regulating the activity of a src-family tyrosine kinase, a syk-family kinase, a Shc molecule and a PI-3K.

In a separate aspect and embodiment of the present invention, an isolated nucleic acid molecule comprises a nucleic acid sequence that encodes for at least one Pro-rich peptide of the present invention, examples of such peptides being disclosed herein. An Pro-rich peptide nucleic acid molecule of the present invention includes a nucleic acid sequence encoding a proline-rich motif having an amino acid sequence capable of binding to the SH3 domain of a tyrosine kinase. Preferred nucleic acid molecules encoding Pro-rich peptides include nucleic acid sequences encoding the peptide sequences PPXPXPXPPXPXP or PXPXXPPXPPXP. At least a portion of a nucleic acid molecule encoding a Pro-rich peptide can be covalently associated (using standard recombinant DNA methods) to any other sequence that encodes for at least a portion of a distinct component (e.g., control element) to produce a Pro-rich peptide of the present invention. The sequences can be attached in such a manner so that the sequences are transcribed in-frame, thereby producing a functional Pro-rich peptide capable of regulating the activity of a tyrosine kinase.

In other embodiments, a nucleic acid sequence is used that encodes for a signal or leader segment that is capable of promoting secretion of a compound of the present invention from the cell that produces the compound. Nucleic acid sequences encoding the leader or signal segments are covalently associated (by base pair linkage) to the 5' end (amino terminal end) of a nucleic acid molecule. The leader

or signal segments can be segments which naturally are associated with a protein of the present invention, or can be heterologous segments. To obtain membrane-bound embodiments, nucleic acid sequences are used that contain at least one transmembrane segment capable of anchoring a compound of the present invention to a lipid-containing target molecule, such segments including at least a portion of a transmembrane domain and at least a portion of a cytoplasmic domain. A nucleic acid sequence encoding a transmembrane segment is covalently associated (by base pair linkage) to the 3' end (carboxyl end) of a nucleic acid molecule encoding a compound of the present invention. A nucleic acid molecule encoding a compound of the present invention capable of being membrane-bound contains at least one nucleic acid sequence encoding a segment ligated to the 3' end of an a nucleic acid sequence encoding an extracellular domain in a manner such that the transmembrane encoding sequences are transcribed in-frame. Preferred signal or leader segments are segments naturally associated with an Ig-α, Ig-ß, TCRςc, CD3ϵ, FcϵRIγ or FcϵRIß protein or a PI-3K protein. Preferred transmembrane segments include segments that are naturally associated with an Ig-α, Ig-ß, TCRςc, CD3ϵ, FcϵRIγ or FcϵRIß protein or a PI-3K protein.

Another embodiment of the present invention relates to a fusion protein that includes a domain containing a compound of the present invention attached to a fusion segment. Inclusion of a fusion segment as part of a

compound of the present invention can enhance the stability of the compound during production, storage and/or use. Furthermore, a fusion segment can function as a tool to simplify purification of a compound of the present invention, such as to enable purification of the resultant fusion protein using affinity chromatography. A suitable fusion segment can be a domain of any size that has the desired function (e.g., increased stability and/or purification). One or more fusion segments can be joined to amino and/or carboxyl terminals of the claimed compound and linkages between a fusion segment and a compound can be made to be susceptible to cleavage to enable straight-forward recovery of the compound. Fusion proteins are preferably produced by culturing a recombinant cell transformed with a fusion nucleic acid sequence encoding a protein that includes a fusion segment attached to either the carboxyl and/or amino terminal end of the compound.

Fusion proteins of the present invention include a nucleic acid molecule encoding a compound of the present invention attached (by base pair linkage) to a nucleic acid molecule encoding a glutathione S transferase fusion segment capable of being cleaved by Factor X or thrombin, preferably Factor X. In one embodiment, an ARH1 fusion protein of the present invention includes a nucleic acid molecule encoding the amino acid sequence YXXLXXXXXXXYXXT attached (by base pair linkage) to a nucleic acid molecule encoding a glutathione S transferase fusion segment capable of being cleaved by Factor X. Preferably, an ARH1 fusion

protein includes a nucleic acid molecule encoding at least a portion of an Ig-α, Ig-ß, TCRζc, CD3ε, FcεRIß, FcεRIγ protein, and more preferably includes a nucleic acid molecule encoding the amino acid sequence NLYEGLNLDDCSMYEDI, DHTYEGLNIDQTATYEDI, DRLYEELNHVYSPIYSEL or DAVYTGLNTRNQETYETL, DGLYQGLSTATKDTYDAL or NPDYEPIRKGQRDLYSGL, attached (by base pair linkage) to a nucleic acid molecule encoding a glutathione S transferase fusion segment capable of being cleaved by Factor X. A particularly preferred ARH1 fusion protein of the present invention includes a nucleic acid molecule encoding the amino acid sequence DMPDDYEDENLYEGLNLDDCSMYEDI, DHTYEGLNIDQTATYEDI, DRLYEELNHVYSPIYSEL, DAVYTGLNTRNQETYETL, DGLYQGLSTATKDTYDAL or NPDYEPIRKGQRDLYSGL.

A separate aspect and embodiment of the present invention relates to a Pro-rich fusion protein comprising the amino acid sequences PPXPXPXPPXPXP or PXPXXPPXPPXP attached (by base pair linkage) to a nucleic acid molecule encoding a glutathione S transferase fusion segment capable of being cleaved by Factor X. Preferably, a Pro-rich fusion protein includes a nucleic acid molecule encoding at least a portion of a PI-3K protein, and more preferably includes a nucleic acid molecule encoding the amino acid sequence KKISPPTPKPRPPRPTPVAPGSSKT or the amino acid sequence NERQPAPATPPKPPKPTTVA attached (by base pair linkage) to a nucleic acid molecule encoding a glutathione S transferase fusion segment that is capable of being cleaved by Factor X.

In yet another embodiment, an SH3 fusion protein of the present invention includes a nucleic acid molecule encoding at least a portion of an SH3 domain of a tyrosine kinase attached (by base pair linkage) to a nucleic acid molecule encoding glutathione S transferase fusion segment capable of being cleaved by Factor X. Preferably, an SH3 fusion protein includes a nucleic acid molecule encoding at least a portion of an SH3 domain derived from src-family tyrosine kinases including Fyn, Lyn, Btk, Lck, Syk, Yes, Hck, Src or Zap70, more preferably Lyn, attached (by base pair linkage) to a nucleic acid molecule encoding glutathione S transferase fusion segment that is capable of being cleaved by Factor X.

The present invention also includes a recombinant molecule comprising a nucleic acid molecule encoding a compound of the present invention operatively linked to a vector capable of being expressed in a host cell. As used herein, "operatively linked" refers to insertion of a nucleic acid sequence into an expression vector in such a manner that the sequence is capable of being expressed when transformed into a cell. As used herein, an "expression vector" is an RNA or DNA vector capable of transforming a host cell and effecting expression of an appropriate nucleic acid molecule, preferably replicating within the host cell. An expression vector can be either prokaryotic or eukaryotic, and typically is a virus or a plasmid.

Construction of desired expression vectors can be performed by methods known to those skilled in the art and

expression can be in eukaryotic or prokaryotic systems. Suitable prokaryotic systems are bacterial strains, including, but not limited to various strains of *E.coli* various strains of *bacilli* or various species of *Pseudomonas*. In prokaryotic systems, plasmids are used that contain replication sites and control sequences derived from a species compatible with a host cell. Control sequences can include, but are not limited to promoters, operators, enhancers, ribosome binding sites, and Shine-Dalgarno sequences. Expression systems useful in eukaryotic host cells comprise promoters derived from appropriate eukaryotic genes. Useful mammalian promoters include early and late promoters from SV40 or other viral promoters such as those derived from baculovirus, polyoma virus, adenovirus, bovine papilloma virus or avian sarcoma virus. Expression vectors of the present invention include any vectors that function (i.e., direct gene expression) in recombinant cells of the present invention including bacterial, yeast, other fungal, insect, plant mammalian cells. An expression system can be constructed from any of the foregoing control elements operatively linked to the nucleic acid molecules of the present invention using methods known to those skilled in the art (see, for example, Sambrook, et al. *ibid.*).

Host cells of the present invention can be: cells naturally capable of producing a compound of the present invention; or cells that are capable of producing a compound of the present invention when transfected with a

nucleic acid molecule encoding a compound of the present invention. Host cells of the present invention include, but are not limited to, bacterial, fungal, insect, plant and mammalian cells. More preferred host cells include *Escherichia*, *Bacillus*, *Saccharomyces*, SF9 and Drosophila.

A recombinant cell is preferably produced by transforming a host cell with one or more recombinant molecules, each comprising one or more nucleic acid molecules of the present invention operatively linked to an expression vector containing one or more transcription control sequences. A recombinant molecule of the present invention is a molecule that can include at least one of the nucleic acid molecules heretofore described, operatively linked to at least one of any transcription control sequence capable of effectively regulating expression of the nucleic acid molecule(s) in the cell to be transformed.

It may be appreciated by one skilled in the art that use of recombinant DNA technologies can improve expression of transformed nucleic acid molecules by manipulating, for example, the number of copies of the nucleic acid molecules within a host cell, the efficiency with which those nucleic acid molecules are transcribed, and the efficiency with which the resultant transcripts are translated, and the efficiency of post-translational modifications. Recombinant techniques useful for increasing the expression of nucleic acid molecules of the present invention include, but are not limited to, operatively linking nucleic acid molecules

to high-copy number plasmids, integration of the nucleic acid molecules into one or more host cell chromosomes, addition of vector stability sequences to plasmids, substitutions or modifications of transcription control signals (e.g., promoters, operators, enhancers), substitutions or modifications of translational control signals, modification of nucleic acid molecules of the present invention to correspond to the code on usage of the host cell, deletion of sequences that destabilize transcripts, and use of control signals that temporarily separate recombinant cell growth from recombinant enzyme production during fermentation. The activity of an expressed recombinant peptide of the present invention may be improved by fragmenting, modifying, or derivitizing nucleic acid molecules encoding such a peptide.

In accordance with the present invention, recombinant cells of the present invention can be used to produce a compound of the present invention by culturing such cells under conditions effective to produce such a compound, and recovering the compound. Effective conditions to produce a compound of the present invention include, but are not limited to, appropriate media, bioreactor, temperature, pH and oxygen conditions that permit peptide production. An appropriate medium refers to any medium in which a cell of the present invention, when cultured, is capable of producing a compound of the present invention. An effective medium is typically an aqueous medium comprising assimilable carbohydrate, nitrogen and phosphate sources,

as well as appropriate salts, minerals, metals, and other nutrients, such as vitamins. The medium may comprise complex nutrients or may be a defined minimal medium. The medium may also contain chemical reagents which select for expression of particular recombinant molecules. Such reagents include, but are not limited to, neomycin, ampicillin, tetracycline, chloramphenicol and mycophenolic acid.

Depending on the vector and host system used for production, resultant compounds of the present invention may either remain within the recombinant cell; be secreted into the fermentation medium; be secreted into a space between two cellular membranes, such as the piroplasmic space in *E.coli*; or be retained on the outer surface of a cell or viral membrane. The phrase "recovering the compound" refers simply to collecting the whole fermentation medium containing the compound and need not imply additional steps of separation or purification. A compound of the present invention can be purified using a variety of standard protein purification techniques, such as, but not limited to, affinity chromatography, ion exchange chromatography, filtration, electrophoresis, hydrophobic interaction chromatography, gel filtration chromatography, reverse phase chromatography, concanavalin A chromatography, chromatafocusing, differential solubilization, and immunoprecipitation. A compound of the present invention is preferably retrieved in "substantially pure" form. As used herein, "substantially pure" refers to

a purity that allows for the effective use of the compound as a therapeutic composition or in an assay system. A substantially pure compound of the present invention, for example, should be capable of regulating the activity of a tyrosine kinase, a Shc molecule, a lipid kinase, a PI-3K or a tyrosine kinase effector in a cell without exhibiting substantial toxicity to such cell.

One embodiment of the present invention is a therapeutic composition that, when administered to an animal in an effective manner, is capable of regulating signal transduction in the cells of that animal. A therapeutic composition of the present invention is useful for the treatment of any disease caused in part by abnormal signal transduction in a cell. Such diseases include cancer, autoimmune disease, immunodeficiency diseases, immunoproliferative diseases, allergic responses, and inflammatory responses. A therapeutic composition of the present invention is also useful in the regulation of an immune response during medical treatments, such as transplantation of organs or skin. Autoimmune diseases can include, for example, systemic lupus, myasthenia gravis, rheumatoid arthritis, insulin dependent diabetes mellitus and experimental allergic encephalomyelitis. Immunodeficiency diseases can include, for example, human AIDS, severe combined immunodeficiencies and hypogammaglobulinemia. Immunoproliferative diseases can include, for example, lymphomas and leukemias. In addition, a therapeutic composition of the present invention is

useful for the treatment of all forms of cancer, including tumor formation.

A therapeutic composition of the present invention is also useful for the treatment of viral diseases such as those caused by herpesviruses such as Epstein-Barr virus (EBV). Such diseases include for example infectious mononucleosis, chronic fatigue syndrome and B-lymphoproliferative disorders in immunocompromised hosts. B lymphocytes can become latently infected with EBV. The EBV are not susceptible to attack by the host immune system while in a latent state in the B cell. The EBV remains latent if the B cell is resting (i.e., not producing antibody to be secreted) because EBV gene replication requires proteins involved in host cell gene replication and resting B cell transcription is minimal. Upon activation of a resting B cell, EBV proliferates because the host cell gene transcription machinery is increased. A therapeutic composition of the present invention can protect an animal from an EBV-derived disease by stimulating activation of signal transduction in resting B cells, thereby causing the EBV virus to replicate and leave its latent state in such cells. The proliferating EBV is then susceptible to attack by the host immune system.

LMP2A is an integral membrane protein which regulates reactivation from latency. LMP2A can interact with Lyn and Syk via its ARH1 motif, thereby stimulating the enzymatic activity of Lyn or Syk resulting in increased reactivation of EBV from latency. Thus, ARH1 peptides derived from LMP2A

can be useful in protecting an animal from EBV-derived disease.

EBV is also implicated in post-transplant lymphoproliferative disorders (PLPD) that can be exacerbated by immunosuppressive drug treatment. Lack of expression of the EBV nuclear protein EBNA2 is implicated in the etiology of solid tumor formation. Thus, ARH1 peptides derived from EBNA2 can be useful in protecting an animal from tumor formation by stimulating expression of EBV, thereby increasing suceptibility of the EBV to attack by the host immune system.

A therapeutic composition of the present invention is also useful for the treatment of tumors caused by transformation of cells infected with viruses, such as Bovine Leukemia Virus (BLV). Host animals infected with BLV produce antibodies to the BLV protein gp30. Upon expression of gp30 on the surface of BLV infected B lymphocytes, such antibodies can bind and cross-link the surface-bound gp30, thereby activating the B lymphocytes. Activation of the B lymphocyte can lead to transformation of the lymphocyte to a malignant cell. A therapeutic composition of the present invention can inhibit activation of resting B cells by gp30 cross-linking, thereby inhibiting B cell transformation.

A therapeutic composition includes a compound of the present invention associated with a suitable carrier. As used herein, a "carrier" refers to any substance suitable as a vehicle for delivering a compound of the present invention to a suitable *in vitro* or *in vivo* site of action.

As such, carriers can act as an excipient or formulation of a therapeutic composition containing a compound of the present invention. Preferred carriers are capable of maintaining a compound of the present invention in a form that is capable of associating with a tyrosine kinase. Examples of such carriers include, but are not limited to water, phosphate buffered saline, Ringer's solution, dextrose solution, serum-containing solutions, Hank's solution and other aqueous physiologically balanced solutions. Aqueous carriers can also contain suitable auxiliary substances required to approximate the physiological conditions of the recipient, for example, by enhancing chemical stability and isotonicity. Suitable auxiliary substances include, for example, sodium acetate, sodium chloride, sodium lactate, potassium chloride, calcium chloride, and other substances used to produce phosphate buffer, Tris buffer, and bicarbonate buffer. Auxiliary substances can also include preservatives, such as thimerosal, m- and o-cresol, formalin and benzol alcohol. Preferred auxiliary substances for aerosol delivery include surfactant substances non-toxic to a recipient, for example, esters or partial esters of fatty acids containing from about six to about twenty-two carbon atoms. Examples of esters include, caproic, octanoic, lauric, palmitic, stearic, linoleic, linolenic, olesteric, and oleic acids. Therapeutic compositions of the present invention can be sterilized by conventional methods and/or lyophilized.

Carriers of the present invention can also include adjuvants, including, but not limited to, Freund's adjuvant; other bacterial cell wall components; aluminum-based salts; calcium-based salts; silica; polynucleotide; toxoids; serum protein; viral coat protein; and other bacterial-derived preparations.

Useful carriers for a compound of the present invention include any artificial or natural lipid-containing target molecule, preferably cells, cellular membranes, liposomes, and micelles. Preferably, therapeutic compositions of the present invention are administered in the form of liposomes or micelles if a transmembrane segment is associated with a compound of the present invention. Liposome and micelles of the present invention are capable of delivering a compound from the extracellular space of a cell to the intracellular space of a cell. Concentrations of a compound of the present invention combined with a liposome or a micelle include concentrations effective for delivering a sufficient amount of the compound to a cell such that signal transduction in such cell is regulated.

A therapeutic composition of the present invention comprises at least one of the compounds of the present invention as described above and may also include at least one additional therapeutic composition capable of regulating signal transduction. As described in more detail above, an ARH1 peptide of the present invention is capable of regulating early events in a signal transduction

pathway. An ARH1 peptide of the present invention can regulate the activity of a src-family tyrosine kinase or a syk-family kinase, which regulates the activity of membrane-associated molecules. As also described above, an ARH1 peptide (Shc-selective peptide or a PI-3K-selective peptide) or a Pro-rich peptide of the present invention is capable of regulating steps in the signal transduction pathway that do not directly involve interaction with a membrane-bound receptor. As such, an ARH1 peptide or Pro-rich peptide regulates signal transduction events later in the pathway. Thus, one of skill in the art can appreciate that the compounds of the present invention can be used in any combination to enable dual regulation of signal transduction in a cell. For example, a therapeutic composition of the present invention can contain at least one ARH1 peptide of the present invention and at least one Pro-rich peptide of the present invention. Preferably, a therapeutic composition of the present invention can contain at least one phosphorylated ARH1 peptide capable of stimulating signal transduction in a cell and at least one Pro-rich peptide capable of inhibiting signal transduction in a cell, or at least one non-phosphorylated ARH1 peptide capable of inhibiting signal transduction in a cell and at least one Pro-rich peptide capable of inhibiting signal transduction in a cell.

Therapeutic compositions of the present invention can be administered to any animal, preferably to mammals, and even more preferably to humans. Acceptable protocols to

administer therapeutic compositions of the present invention in an effective manner include individual dose size, number of doses, frequency of dose administration, and mode of administration. Modes of delivery can include any method compatible with prophylactic or treatment of a disease. Modes of delivery include, but are not limited to, parenteral, oral, intravenous, topical or local administration, such as by aerosol or transdermally.

The present invention also includes a kit to identify a compound capable of inhibiting stimulation of activation of a src-family tyrosine kinase. Such kits include: (1) a tyrosine kinase; (2) a compound of the present invention capable of stimulating the activity of the tyrosine kinase; and (3) a means for measuring the activity of the tyrosine kinase. It is within the skill of one in the art to modify a kit of the present invention to identify inhibitory compounds by, for example, adding to the kit a primary target molecule capable of being modified by the src-family tyrosine kinase and a means for detecting such modification. The activity of a tyrosine kinase can be measured by, for example, measuring autophosphorylation or modification of a primary target molecule by the kinase.

In one embodiment, a kit of the present invention comprises: (1) a src-family tyrosine kinase (2) and a stimulatory ARH1 peptide of the present invention; and (3) an amount of γP^{32}-ATP sufficient to provide desired phosphorylation. Preferably, a kit of the present invention can include, but is not limited to the src-family tyrosine

kinases Fyn, Lyn, Lck, Blk, Syk, Yes, Btk, Hck, Src, Zap70 or any combination thereof, more preferably Fyn or Lyn or any combination thereof, and even more preferably Fyn. A suitable stimulatory ARH1 peptide of the present invention includes a phosphorylated ARH1 peptide as disclosed herein. Preferably, a kit of the present invention includes a phosphorylated ARH1 peptide having the sequence YEGLNLDDCSMYEDI, with the sequence NLYEGLNLDDCSMYEDI being more preferred. Any means to detect incorporation of the γP^{32}-ATP into the src-family tyrosine kinase can be included in the kit, such as means for phosphocellulose blotting. In another embodiment, a kit of the present invention can further comprise any peptide having a tyrosine residue. Preferably, such a peptide comprises the amino acid sequence RRGKGHDGLYQGL.

In another embodiment, a kit of the present invention can include antibodies specific for phosphotyrosine residues to be used as a means to detect autophosphorylation or phosphorylation of a primary target molecule. A phosphorylated or non-phosphorylated target molecule can be bound to a surface, such as nitrocellulose or an ELISA plate prior to or following addition of an anti-phosphotyrosine antibody. A target molecule can also remain unbound after addition of an antibody if antibody binding is to be analyzed by, for example, fluorescence activated cell sort (FACS) analysis. A kit of the present invention can further include a "development component" to detect binding of the antibody to the target molecule. The

development component can include a compound that binds to the antibody, the compound being coupled to a detectable tag. A binding compound can include, but is not limited to, a secondary antibody that is capable of binding to the primary antibody when the primary antibody is bound to the target molecule; a bacterial surface protein that binds to antibodies, such as Protein A or Protein G; a biotin-streptavidin or biotin-avidin coupled detection system; a cell that interacts with antibodies, such as a T cell or B cell or macrophage; a eukaryotic cell surface protein that binds to antibodies, such as an FC receptor; and a complement protein. A variant of detectable tags can be used, including, but not limited to radioactive, enzymatic, and fluorescent labels. Detection of the tag can be accomplished using a variety of well-known techniques, depending on the assay. For example, an enzymatic assay, (e.g., use of alkaline phosphatase or horseradish peroxidase) often yields a colorimetric product that can be detected visually or by an instrument such as a densitometer or a spectrophotometer. A kit of the present invention can further include reagents for conducting immunoprecipitation and immunoblot analysis.

In a separate aspect and embodiment of the present invention, a kit comprises: (1) a tyrosine kinase (2) a proline-rich protein; and (3) a primary target molecule. Preferably, a kit of the present invention can include, but is not limited to the src-family tyrosine kinases Fyn, Lyn, Lck, Blk, Syk, Yes, Btk, Hck, Src, Zap70 or any combination

thereof, more preferably Fyn, Lyn, Blk, Lck, Hck, Src, Yes or any combination thereof, and even more preferably Lyn or Fyn or any combination thereof. Suitable proline-rich proteins include, but are not limited to PI-3K and p85 [80-104]. Preferably, primary target molecules include protein or lipid molecules capable of being phosphorylated, more preferably lipid molecules, and even more preferably, phosphotidylinositol, phosphatidylinositol 4-phosphate, phosphatidylinositol 4,5-biphosphate or a combination thereof. Any means to detect phosphorylation of the target molecule can be included in the kit, such as means for thin layer chromatography analysis. Such a kit is useful in discovering suitable compounds of the present invention that are capable of competitively inhibiting the activity of the tyrosine kinase/target molecule associations.

A kit of the present invention can be used in an automated system or a manual system requiring each step to be carried out by a laboratory technician. The test kit can also include all appropriate reaction buffers required to maintain the enzymatic activity of a tyrosine kinase.

A kit of the present invention is particularly useful for the identification of therapeutic compositions capable of regulating signal transduction in cells involved in an immune response, in particular, in B cells and T cells. Particularly useful compounds include those compounds which bind to and/or activate tyrosine kinases, adaptor molecules or lipid kinases, with high specificity that permits the compounds to be administered in low dosages.

Another embodiment of the present invention is a method to identify compounds capable of inhibiting signal transduction in a cell. In one embodiment of the present invention, an inhibitory compound can be identified by a method including the steps of: (a) contacting a tyrosine kinase with a putative inhibitory compound to form an reaction mixture; (b) contacting the reaction mixture with a stimulatory ARH1 peptide of the present invention; and (c) determining the ability of the putative inhibitory compound to inhibit autophosphorylation of the tyrosine kinase. Preferably, a tyrosine kinase used in the method can include, but is not limited to the src-family tyrosine kinases Fyn, Lyn, Lck, Blk, Syk, Yes, Btk, Hck, Src, Zap70, or any combination thereof, more preferably Fyn, Lyn or Btk, or any combination thereof, and even more preferably Btk or Fyn, or any combination thereof. A suitable stimulatory ARH1 peptide includes a phosphorylated ARH1 peptide as disclosed herein.

In another embodiment, an inhibitory compound can be identified by contacting a tyrosine kinase with a target molecule capable of being phosphorylated by the kinase to form a reaction mixture. The reaction mixture is then contacted with a putative inhibitory compound to form an assay mixture. A stimulatory ARH1 peptide of the present invention is then added to the assay mixture and the ability of the putative inhibitory compound to inhibit phosphorylation of the target molecule by the tyrosine kinase is measured.

With respect to a separate aspect and embodiment of the present invention, an inhibitory compound can be identified by a method including the steps of: (a) contacting a tyrosine kinase with a putative inhibitory compound to form a reaction mixture; (b) contacting the reaction mixture with a proline-rich protein to form an assay mixture; (c) contacting the assay mixture with a primary target sequence capable of being modified by the tyrosine kinase; and (d) determining the ability of the putative inhibitory compound to inhibit modification of the target molecule by the tyrosine kinase. Suitable tyrosine kinases include src-family tyrosine kinases, preferably including, but not limited to the kinases Fyn, Lyn, Lck, Blk, Syk, Yes, Btk, Hck, Src, Zap70 or any combination thereof, more preferably Fyn, Lyn or Btk or any combination thereof, and even more preferably Btk or Fyn. Preferably, a proline-rich protein useful in the method of the present invention includes, but is not limited to PI-3K. Preferably, primary target molecules include protein or lipid molecules capable of being phosphorylated, more preferably lipid molecules, and even more preferably phosphatidylinositol, phosphatidylinositol 4-phosphate, phosphatidylinositol 4,5-phosphate, or a combination thereof. Methods to detect modification of the primary target molecule can include, for example, phosphocellulose blotting or thin layer chromatography.

Another aspect of the present invention includes a method to identify compounds capable of regulating signal

transduction using a cell-based assay. In one embodiment, a cell-based assay of the present invention useful for the identification of compounds capable of stimulating signal transduction includes the steps of: (a) contacting a cell with a putative stimulatory compound in such a manner that the compound is capable of entering the cell; (b) incubating the compound and the cell so as to allow association of the compound with appropriate intracellular molecules; (c) lysing the cell; and (d) determining the state of phosphorylation of an intracellular molecule bound to the compound. Suitable cells include, but are not limited to, mammalian cells. Suitable intracellular molecules include, but are not limited to, src-family tyrosine kinase, in particular Lyn, Fyn, Lck, Blk, Syk, Yes, Btk, Hck, Src, and Zap70. Methods to detect the state of phosphorylation of an intracellular molecule include ELISA assays or FACS analysis. Preferably, a lysed cell can be contacted with an antibody specific for an intracellular molecule bound to an ELISA plate. The plates can be washed and then contacted with an anti-phosphotyrosine antibody bound by a tag as described in detail above. The amount of anti-phosphotyrosine antibody bound to the ELISA plate can be measured to determine the extent of phosphorylation and, therefore stimulation, of an intracellular molecule by the putative stimulatory compound. Another method for determining the state of phosphorylation of the intracellular molecule includes contacting an antibody specific for an intracellular molecule with the lysed cells

in solution. An anti-phosphotyrosine antibody can be added to the solution and analyzed by FACS analysis. The anti-phosphotyrosine antibody can have a fluorescent tag or a second antibody having a tag can be added to the solution.

In another embodiment, a cell-based assay of the present invention is useful for the identification of a compound capable of inhibiting signal transduction. A cell-based assay of the present invention can include the steps of: (a) contacting a cell with a putative inhibitory compound; (b) incubating the compound with the cell for so as to allow the compound to bind intracellular molecules; (c) ligating membrane-bound receptors on the surface of the cell; (d) lysing the cell; and (e) determining the state of phosphorylation of an intracellular protein bound to the putative inhibitory molecule. Suitable cells include, but are not limited to, mammalian cells. Suitable intracellular molecules can include, but are not limited to Lyn, Fyn, Lck, Blk, Syk, Yes, Btk, Hck, Src, and Zap70. The state of phosphorylation of the intracellular protein can be detected using the methods described in detail immediately above.

In yet another embodiment, a cell-based assay of the present invention can include the steps of: (a) contacting a cell with a putative inhibitory compound; (b) incubating the compound with the cell so as to allow the compound to bind intracellular molecules; (c) ligating membrane-bound receptors on the surface of the cell; and (d) determining the ability of a putative inhibitory molecule to inhibit

calcium mobilization in the cell. Suitable cells include, but are not limited to, mammalian cells. Suitable putative regulatory molecules of include mimetopes of Syk-selective, Shc-selective or PI-3K-selective peptides of the present invention. Calcium mobilization can be measured using the methods generally described in Justement et al., *J. Immunol.* 143:881-886, 1989.

In yet another embodiment, a cell-based assay of the present invention can include the steps of: (a) contacting a cell with a putative inhibitory compound; (b) incubating the compound with the cell so as to allow the compound to bind intracellular molecules; (c) ligating membrane-bound receptors on the surface of the cell; and (d) determining the ability of a putative inhibitory molecule to inhibit phosphorylated phosphoinositide formation in the cell. Suitable cells include, but are not limited to, mammalian cells. Formation of phosphorylated phosphoinositides can be measured using the method generally described in Ransom et al. (*J. Immunol.* 137:708-715, 1986).

In a preferred embodiment, the cells used in a cell-based assay of the present invention are permeabilized prior to contacting the cells with a putative inhibitory compound. Techniques to permeabilize cells for use in an assay of the presetn invention are described below in the Examples.

The following examples are provided for the purposes of illustration and are not intended to limit the scope of the present invention.

Examples

Example 1

This example illustrates that the binding specificity of Ig-α and Ig-ß can be determined by four amino acids which lie between the conserved tyrosines of the ARH1 motifs.

A series of Ig-α and Ig-ß switch mutants were constructed in which areas of divergent sequence were exchanged (Fig. 3, shown by bold lettering). Mutagenesis or truncation of the cytoplasmic tails of Ig-α and Ig-ß was accomplished with polymerase chain reaction (PCR) amplification using cDNA templates of Ig-α and Ig-ß. Oligonucleotide primers specific to each mutation were used in a standard PCR reaction mixture containing 1.5 mM Mg^{2+} and cycled (94°, 55°, 72°C, each for 1 minute) 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 and Y193ΔF tyrosine mutants were generated by site-directed mutagenesis using a kit as recommended by the manufacturer (Biorad).

The oligonucleotide primers listed below were used for mutagenesis. For the final amplification of mutated or truncated cDNAs, primers which contained restriction sites

(underlined) were used to facilitate cloning. Truncation mutants were created using the following primers:

Ig-α ARH1: 5'-GAGAGGATCCTGGACATGCCAGATGACTATGA-3';
5'-GAGAGAATTCGATGTCCTCATACATAGAACAGT-3'

Ig-α flank: 5'-AGACGGATCCTCAGGAAACGGTGGCAAAATGAG-3';
5'-GGTGCCCTGGAGTCCCCTGAACACCCCAAACTTCT CATT-3';
5'-AATGAGAAGTTTGGGGTGTCCAGGGGACTCCAGGGC ACC-3';
5'-AGACGAATTCTGGCTTTTCCAGCTGGGCATCT-3'

Ig-ß ARH1: 5'-AGACGGATCCATGACGGCAAGGCTGGGATGGA-3';
5'-AGACGAATTCTATGTCTTCATAGGTGGCTGT-3'

Ig-ß flank: 5'-AGACGGATCCTTGACAAGGATGTGACTCTTCGGACA GGGGAGGTA-3';
5'-AGACGAATTCTTCCTGCCCTGGATGCTCTCCT-3'

Ig-ß/α/ß: 5'-GAGAGGATCCTACTTGACAAGGATGACATGCCAGATGACTA TGAAGAT-3';
5'-CCCTGTCCGAAGAGTCACGATGTCCTCATACATAG AACA-3';
5'-GTGACTCTTCGGACAGGGGAG-3';
5'-AGACGAATTCTTCCTGCCCTGGATGCTCTCCT-3'

Ig-α/ß/α: 5'-GAGAGGATCCTCAGGAAACGGTGGCAAAATGAGAAGTTTG GGGTGGACGGCAAGGCTGGGATGGA-3';
5'-GCCCTGGAGTCCCCTGGATATGTCTTCATAGGTGGCTG TCTG-3';
5'-TCCAGGGGACTCCAGGGC-3';
5'-AGACGAATTCTGGCTTTTCCAGCTGGGCATCT-3'

Switch mutants were created using the following primers:

Ig-α_{HT}: 5'-AGACGGATCCTCAGGAAACGGTGGCAAAATGAG-3';
5'-CAGGCCCTCATAGGTATGTTCATCTTCATA-3';
5'-AGACGAATTCTGGCTTTTCCAGCTGGGCATCT-3'

Ig-α_{QTAT}: 5'-AGACGGATCCTCAGGAAACGGTGGCAAAATGAG-3';
5'-GATGTCCTCATAGGTAGCAGTCTGATCAAGGTTCAG-3';
5'-AGACGAATTCTGGCTTTTCCAGCTGGGCATCT-3'

Ig-β_{NL}: 5'-AGACGGATCCTTGACAAGGATGTGACTCTTCGGACAGGG GAGGTA-3';
5'-GAAGCCCTCATAGAGGTTATCTTCCTCCAT-3';
5'-ATGGAGGAAGATAACCTCTATGAGGGCTTC-3';
5'-AGACGAATTCTTCCTGCCCTGGATGCTCTCCT-3'

Ig-β_{DCSM}: 5'-AGACGGATCCTTGACAAGGATGTGACTCTTCGGACAGGG GAGGTA-3';
5'-CACTATGTCTTCATACATGGAACAGTCGTCAATGTTCA

AGCC-3';
5'-GGCTTGAACATTGACCACTGTTCCATGTATGAAGACAT
AGTG-3';
5'-AGACGAATTCTTCCTGCCCTGGATGCTCTCCT-3'

Tyrosine mutants were created using the Ig-α ARH1 primers listed above in combination with the following primers:

Y176ΔF: 5'-GAGAGGATCCTGGACATGCCAGATGACTTTGA-3'

Y182ΔF: 5'-AAATCTCTTCGAGGGCCT-3'

Y193ΔF: 5'-GAGAGAATTCGATGTCCTCAAACATAGAACAGT-3'

Point mutants were created using the Ig-α ARH1 primers listed above in combination with the following primers:

$Ig\text{-}\alpha_{ACSM}$: 5'-GAGAGAATTCGATGTCCTCATACATAGAACAAGCATC-3'

$Ig\text{-}\alpha_{DASM}$: 5'-GAGAGAATTCGATGTCCTCATACATAGAAGCGTCATC-3'

$Ig\text{-}\alpha_{DCAM}$: 5'-GAGAGAATTCGATGTCCTCATACATAGCACAGTCATC-3'

$Ig\text{-}\alpha_{DCSA}$: 5'-GAGAGAATTCGATGTCCTCATAAGCAGAACAGTCATC-3'

PCR products encoding the Ig-α and Ig-ß mutants listed above were digested overnight at 25°C with BamHI and EcoRI, resolved by electrophoresis through 3% NuSieve agarose (FMC BioProducts) and isolated with MERmaid (BIO 101). Fusion proteins of each Ig-α and Ig-ß mutant was produced by ligating the BamHI and EcoRI digested PCR products into BamHI/EcoRI digested pGEX-3X (Pharmacia) and transforming the resulting recombinant molecules into the bacteria DH5α. Transformants, selected on ampicillin (100μg/ml), were screened using PCR and plasmid DNA was purified using tip-100 columns (QIAGEN). Double-stranded DNA was sequenced directly using Sequenase Version 2.0 (USB) and a primer to pGEX-3X (5'-GCATGGCCTTTGCAGGG-3').

Transformed cells producing an appropriate Ig-α and Ig-ß fusion protein were used to prepare fusion proteins by inducing 1 liter of log phase cells (O.D. 595 = 0.375) with 0.2 mM isopropyl ß-D-thiogalactopyranoside (IPTG) for 3 hours. Cultures were collected and cells pelleted by centrifugation. The cell pellets were resuspended in 5 ml of phosphate buffered saline (PBS) containing 1% Triton X-100, lysed by sonication, and the lysate cleared by centrifugation. Cleared lysates were incubated with 500μl of a 50:50 slurry of glutathione-Sepharose beads overnight at 4°C and then washed thoroughly with PBS containing 1% Triton X-100 and 0.02% sodium azide. The relative amount of fusion protein bound per bead volume was quantitated by reducing SDS-PAGE and staining with Coomassie blue. Some fusions proteins were subsequently cleaved with 30μg of Factor Xa (Boehinger Mannheim) in 150 mM NaCl and 1 mM $CaCl_2$ overnight at 4°C. The cleaved peptides were washed from the beads in a total volume of 4 ml, concentrated and directly coupled to 400 μl of a 50:50 slurry of cyanogen bromide activated Sepharose beads (Pharmacia) maintaining an equal ratio of moles of peptide to bead volume for all cleaved peptides (2 hours at room temperature). Remaining reactive groups were blocked by incubating the beads in 0.2 M glycine for another 2 hours at room temperature. The beads were washed extensively to remove unbound peptide and stored in lysis buffer containing 0.02% sodium azide.

The ability of the various Ig-α and Ig-ß mutant fusion protein to bind to a src-family tyrosine kinase was tested

using binding and *in vitro* kinase assays. The B lymphoma cells, K46, or the fibroblast cells, Fyn^{+}NIH-3T3, were harvested by centrifugation [$2X10^{7}$ (K46) or 1 to $3X10^{6}$ (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 2μg/ml of each aprotinin, leupeptin, and α-1-antitrypsin]. Cleared lysates were incubated with beads coated with whole fusion protein or peptide (5-10μl of 50:50 slurry) for 4 hours at 25°C or overnight at 4°C. Following adsorption, the beads were washed with 1 ml lysis buffer three times and analyzed by a variety of methods.

The ability of the Ig-α and Ig-ß mutant fusion proteins to bind a protein having tyrosine kinase activity, *in vitro* kinase assays were performed. In the *in vitro* kinase assay adsorbates (prepared as described above) 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 20μl of kinase buffer containing 10μCi of γ-^{32}P ATP, 3000 Ci/mm and incubated for 10 minutes at 30°C. The samples were then washed in lysis buffer, and resuspended in 30μl reducing sample buffer. Equivalent amounts of proteins were separated by 10% SDS-PAGE and detected by autoradiography at -70°C for 1 to 2 hours. The results are shown in Fig. 4. Each switch mutant is denoted by its parent chain (Ig-α or Ig-ß) and the amino acids which it contains from the other chain. When non-conserved

amino acid residues amino-terminal to the first conserved tyrosine (Ig-α:HT/Ig-ß:NL) were exchanged, no effect was observed on the binding properties of either chain (Fig. 4). When the four nonconserved residues between the ARH1 tyrosines where exchanged (DCSM of Ig-α for QTAT of Ig-ß), Ig-α failed to bind the cluster of proteins in the 50-60 kD range which bind wild type Ig-α. Likewise, the Ig-ß switch mutant, containing the four amino acids (DCSM) of Ig-α now bound this cluster of proteins.

To further examine the specificity of src-family tyrosine kinase binding to Ig-α and Ig-ß, synthetic peptides corresponding to either the wild type, Ig-α (QTAT) or Ig-ß (DCSM) switch mutants were used to probe lysates of NIH-3T3 cells which overexpress Fyn (Fyn^{+}NIH-3T3).

Fyn immunoblotting analysis was performed as follows. Adsorbates prepared as described above were eluted with SDS-PAGE sample buffer and 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) and 150 mM NaCl] for 4 hours at 25°C, and hybridized with rabbit anti-Fyn antibody diluted 1:500 to 1:1000 in milk-TBS for 2 hours 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 hour at 25°C, washed again, and 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 monoclonal, α-phosphotyrosine antibody, 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.).

As seen in Fig. 5, 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 (Fig. 4). The smaller immunoreactive band represents a degradation product of Fyn. Fusion proteins were also made (as described above) in which the aspartic acid, cysteine, serine and methionine of Ig-α were changed individually to alanine and their binding activity assessed. GST fusion proteins of the DCSM point mutants were cleaved with factor Xa, coupled to Sepharose and used to adsorb Fyn+NIH3T3 cell lysates as described in Example 1. Adsorbates were fractionated as before and electrophoretic transfers were probed with anti-Fyn. As shown in Fig. 6, none of these single amino acid changes affected binding.

Taken together, the results indicate that it is the overall structure of the ARH1 region, and not the individual amino acids, which contribute to binding of an ARH1 motif to Fyn and the activation of Fyn. Mutations involving a change or deletion of two or more amino acids

should be needed to disrupt function. In addition, the ARH1 motif of Ig-α contains a sequence of four amino acids which determines binding specificity for Lyn and Fyn.

Example 2

This example demonstrates that the tyrosines of the ARH1 domain of Ig-α are not necessary for the binding to Fyn.

As described above, the two conserved ARH1 tyrosines of Ig-α (at position 182 and 193) and the third non-conserved tyrosine (at position 176) were mutated to phenylalanine using methods described in Example 1. Positioning of the tyrosines are as follows:

```
          176         182                 193
D M P D D Y E D E N L Y E G L N L D D C S M Y E D I
```

The ability of these mutants to bind to Fyn protein was assayed using the *in vitro* kinase assay described in Example 1. Adsorbates were prepared as described in Example 1 and incubated with [γ^{32}P]-ATP in kinase buffer, washed and analyzed by 10% reducing SDS-PAGE and autoradiography.

The results of a typical experiment are presented in Fig. 7. Substitution of phenylalanine for tyrosine at all of these positions did not affect the ability of the Ig-α cytoplasmic domain to stably associate with Fyn. Comparison was made between the ability of wild type and Ig-α tyrosine mutated fusion proteins to bind Fyn immunoprecipitated from the lysates of 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 (Fig. 8). 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.

Example 3

This example demonstrates that tyrosine Phosphorylation of the Ig-α ARH1 motif specifically increases its binding to Fyn.

To produce phosphorylated Ig-α ARH1 motifs the catalytic domain of the tyrosine kinase Elk was cloned 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-ß-D-galactoside.

The catalytic domain of the tyrosine kinase Elk was amplified from a cDNA provided by Dr. Tony Pawson (University of Toronto), using PCR amplification using with the following oligonucleotide primers: 5'-AAGAGGATCCGGTGGCCATGGAAGCTGTCCGGGAGTTTGC-3' and 5'-AAGAGAATTCGAGTTCTCATGCCATTACCGACGG-3'. The PCR product was purified as described above in Example 1, ligated to pBC (Stratagene), and transformed into the bacteria BL21/DE3. Transformants, selected on chloramphenicol (20μg/ml) and induced (as described above) with 0.2 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 (as described in Example 1) with the α-phosphotyrosine antibody, 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 (20μg/ml) and ampicillin (100μg/ml) and subsequently induced to express GST-fusion protein of which 2 to 5% was phosphorylated *in vivo* by Elk. In order to separate the phosphorylated product from the non-phosphorylated product, approximately 40 mg of this mixed fusion protein was passed over an α-phosphotyrosine affinity column (IG2 at 14 mg/ml Sepharose, gift of Dr. Ray Frackelton, Brown University). The column was then washed with phosphate buffered saline [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 phosphate buffered saline, 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 in Example 1.

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 were tyrosine

phosphorylated. 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 about 2 to 5 percent of the total fusion protein. Equal amounts of phosphorylated and nonphosphorylated fusion protein were then assayed for their ability to bind Fyn from the NP40 lysates of Fyn$^+$NIH-3T3 cells (as described in Example 1). Anti-Fyn antibody was used to immunoprecipitate Fyn from Fyn$^+$NIH-3T3 cells. The immunoprecipitated Fyn was incubated with 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. As shown in Fig. 9, approximately 10-fold more immunoreactive Fyn associated with the phosphorylated Ig-α ARH1 motif than the nonphosphorylated motif.

To determine the relative role of the respective phosphotyrosines in the enhancement of Fyn binding, peptides were synthesized (using Applied BioSystems model 430A) corresponding to the Ig-α ARH1 motif (residues: 171-196) which were either nonphosphorylated or phosphorylated at tyrosine residues 182 or 193 or both. Peptide sequence and purity was verified by amino acid hydrolysis analysis (Waters Associates Pico-Tag system). The use of synthetic phosphopeptides guaranteed stoichiometric phosphorylation at specific residues. These peptides were covalently

coupled to Sepharose beads (as described above for fusion protein derived peptides) and used in adsorption of Fyn$^+$NIH-3T3 cell lysates. An anti-Fyn immunoprecipitate was analyzed as a positive control (Fig. 10, "anti-Fyn" label). 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. The results shown in Fig. 10 indicate that 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 2.0 (Y182) and 1.6 (Y193) fold compared to the nonphosphorylated peptide (as determined using a PhosphorImager). The amount of Fyn associated with the doubly phosphorylated peptide was increased by 24-fold.

To further address the possibility that new proteins bind to phosphorylated Ig-α, adsorbates from K46 lysates were analyzed by *in vitro* kinase reactions and SDS-PAGE. Adsorbates were subjected to *in vitro* phosphorylation and analyzed by 10% reducing SDS-PAGE and autoradiography according to methods described in Example 1. As shown in Fig. 11, there was no clear evidence of new phosphorylatable proteins binding the phosphorylated Ig-α peptide.

Example 4

This example demonstrates that both tyrosine phosphorylated Ig-α and Ig-ß ARH1 motifs exhibit enhanced Fyn binding activity.

Ig-ß and Ig-α ARH1 motifs peptides which were phosphorylated at both of the ARH1 tyrosines were synthesized as described in Example 1. These phosphorylated peptides and additional non-phosphorylated Ig-α ARH1 peptides were coupled to Sepharose and used for analysis of Fyn binding. Synthetic doubly phosphorylated Ig-α and Ig-ß ARH1 motif peptides and non-phosphorylated Ig-α ARH1 motif peptides were used to adsorb Fyn+NIH3T3 cell lysates. Adsorbates were fractionated by SDS-PAGE transferred and blotted with rabbit anti-Fyn and I^{125} protein A. As shown in Fig. 12, phosphorylated Ig-α bound Fyn more efficiently than nonphosphorylated Ig-α. Ig-ß bound Fyn as efficiently as phosphorylated Ig-α. These results suggest that the specificity of the phosphotyrosine dependent interaction is not determined by the QTAT or DCSM motifs.

Example 5

This example demonstrates that 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, a peptide based *in vitro* kinase assay was used to measure activity in Ig-α ARH1 adsorbates of Fyn^{+}NIH-3T3 lysates (prepared as described in Example 1). Nonphosphorylated Ig-α or Ig-α in which peptide tyrosine 182, or tyrosine 193, or 182 plus 193 were phosphorylated were used in the experiment. Fyn was immunoprecipitated from the lysates of Fyn+NIH-3T3 (~$2x10^6$ cell equivalents/sample) cells by sequential 1 hour

incubations at 25°C with rabbit anti-Fyn antibody (5μg antibody/sample) and protein A Sepharose (Pharmacia). Equal amounts of bead bound adsorbates (prepared as described above) or Fyn immunoprecipitates (after pre-incubation for 1 hour with phosphorylated Ig-α peptide at concentrations from 1 μM to 1 mM) were washed twice in kinase buffer, pelleted, resuspended in 50μl kinase buffer containing 2 mM exogenous target molecule (RRGKGHDGLYQGL) corresponding to a portion of the TCR-ζ chain surrounding tyrosine 142, 10μM ATP and 10μCi of [γ-^{32}P] ATP (3000 Ci/mM), and incubated for 10 minutes at 30°C. Each reaction mixture was then quenched with 12μl 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.

When equivalent amounts of samples which had been tested for immunoreactive Fyn were assayed for kinase activity, the profile of enzymatic activity correlated with levels of immunoreactive Fyn with one exception (see Fig. 13). 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, the ability of the Ig-α

phospho-ARH1 peptide to activate Fyn *in vitro* was analyzed. Immunopurified Fyn from Fyn$^+$NIH-3T3 cell lysates (prepared as described in Example 1) was added in constant amounts to 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. To anti-Fyn beads containing purified Fyn from Fyn$^+$NIH-3T3 cells (2 x 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 hour. Kinase activity was then assayed. As shown in Fig. 14, the specific activity of Fyn was increased almost three-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 (5μM) concentrations of peptide.

Taken together, the results described in Examples 1 through 5 indicate that Fyn is capable of binding to an ARH1 motif containing the sequence DCSM. In addition, phosphorylation of tyrosine residues flanking the DCSM sequence are important for Fyn stimulation.

Example 6

This example demonstrates the ability of peptides stimulating reflecting the two proline-rich regions within PI-3K to inhibit binding of Lyn and Fyn to PI-3K.

Two peptides were synthesized having the proline-rich sequences of KKISPPTPKPRPPRPTPVAPGSSKT (p85 α-subunit [80-

104]) and NERQPAPATPPKPPKPTTVA (p85 α-subunit [299-318]). The peptides were synthesized on an Applied BioSystems model 430A. Peptide sequence and purity was verified by amino acid hydrolysis analysis (Waters Associates Pico-Tag system, Single letter amino acid code abbreviations: K, Lysine; I, isoleucine; S, serine, P, proline; T, threonine; R, arginine; V, valine; G, glycine; N, asparagine; E, glutamic acid; Q, glutamine). $p56^{lyn}$ ([Lyn 1-131]) and $p89^{fyn}$ ([Fyn 1-144]) peptides, that contain the respective SH3 domains of the kinases, were coupled to cyanogen bromide activated Sepharose beads (Pharmacia) and used to probe detergent lysates of the K46 B lymphoma cells that contain PI-3K in the presence or absence of p85 [80-104] and p85 [299-318]. These directly coupled beads were produced as follows. DNA fragments encoding the SH3 domains of $pS9^{fyn}$ and $p56^{lyn}$ were amplified with the polymerase chain reaction (PCR) from K46 cDNA templates using the following primers:

Fyn (1-144)

5': 5'-ATATCAGGATCCTGATGGGCTGTGTGCAATGTAAG-3'

3': 5'-CAGTCAGAATTCGATGGAGTCAACTGGAGCCA-3'

Lyn (1-131)

5': 5'-ATATCAGGATCCTGATGGGATGTATTAAATCA-3'

3': 5'-CAGTCAGAATTCGAACCACTCTTCAGTTTC-3'.

Each primer encodes a unique restriction site (underlined, BamHI: GGATCC; EcoRI: GAATTC) that facilitated subsequent cloning. The PCR products were then digested with either BamHI or EcoRI and ligated into pGEX-3X (Pharmacia). DNA sequence analysis was performed to confirm the accuracy of the PCR generated products (Sequenase 2.0, U.S. Biochemical Corp.). The pGEX-3X constructs were transfected into *Escherichia coli* strain DH5a(BRL-Gibco) and fusion proteins prepared by inducing 1 liter of log phase cells (O.D. 595 = 0.375) with 0.2 mM isopropyl ß-D-thiogalactopyranoside (IPTG) for 3 hours. Cultures were collected and cells pelleted by centrifugation. The cell pellets were resuspended in 5 ml of phosphate buffered saline (PBS) containing 1% Triton X-100 and .02% sodium azide and fusion protein was isolated from the cells.

Fusion protein was then bound to beads (2 ml of a 50:50 slurry) and cleaved with 30μM of factor Xa (Boehinger Mannheim) in 150 mM NaCl and 1 mM $CaCl_2$ overnight at 4°C. The cleaved peptides were then washed from the beads in a total volume of 4 ml, concentrated by Speed-Vac to 2 ml, and then directly coupled to 400μl of a 50:50 slurry of cyanogen bromide activated Sepharose (Pharmacia)

maintaining an equal ratio of moles of fusion protein to bead volume for all fusion proteins (2 hours at room temperature). Remaining reactive groups were then blocked by incubating the beads in 0.2 M glycine for another 2 hours at room temperature. The beads were then washed extensively to remove unbound peptide and stored in lysis buffer containing 0.02% sodium azide).

Binding and peptide inhibition experiments were performed as follows. Clarified lysates [1% NP-40, 150 mM NaCl, 10 mM tris-HCI (pH 7.4), 2.0 mM sodium orthovanadate, 10 mM sodium pyrophosphate, 0.4 mM EDTA, 10mM NaF, 1mM phenylmethylsulfonyl fluoride, aprotinin (2 mg/ml), leupeptin (2 mg/ml), and α-1-antitrypsin (2 mg/ml)] of K46 cells ($1x10^7$ cell equivalents per sample) were prepared and incubated with beads coated with either Fyn [1-144] or Lyn [1-131] (10 ml of a 50:50 slurry) fusion protein. Proline-rich peptides from p85 were added into adsorptions in concentrations 200, 20, and 2 times that of Lyn and Fyn fusion protein on beads and incubated overnight. Directly coupled glutathione S Transferase (GST) peptide bead adsorptions were performed as a control. The beads were then washed five times in lysis buffer, resuspended in sample buffer, and eluate separated by SDS-PAGE (10%). Proteins were then transferred to Immobilon (Millipore) and probed with antibodies to p85 (Upstate Biotechnology, Inc.). Clarified lysate from solubilized cells ($1x10^6$ cell equivalents) was included as a control (WCL). Membranes were thoroughly washed in tris buffered saline (TBS)

containing 0.02% Triton X-100 and subsequently incubated with goat antibody to rabbit IgG conjugated with alkaline phosphatase (Fisher). The membranes were washed again and developed with alkaline phosphatase target molecule (Vector Labs). Fig. 15A illustrates p85 peptide binding to Fyn [12-144] and Fig. 15B illustrates p85 peptide binding to Lyn [27-131]. When present at twice the concentration of the fusion protein, p85 [80-104] inhibited PI-3K binding to Fyn by approximately 90% (as determined by scanning densitometry). Both the p85 [80-104] and p85 [299-318] peptide completely blocked the association with PI-3K when the peptides were present at concentrations 20 and 200 times that of the Lyn or Fyn fusion protein. The p85 [299-318] peptide inhibited PI-3K association with Fyn when the concentration of the peptide was at 200 times that of the Fyn fusion protein, but showed no detectable inhibition of binding at lower concentrations (Fig. 15A). Similar results were observed using beads coupled to the SH3 of Lyn except that inhibition by both p85 [80-104) and p85 [299-318] was weaker (Fig. 15B), suggesting that Fyn may have a lower affinity for p85 than Lyn. These findings indicate that recognition of the 80-104 sequence within p85 is necessary for SH3 binding to PI-3K.

To determine the sufficiency of the proline-rich p85 sequences in mediating the association of PI-3K with SH3 domains of Lyn and Fyn, p85 [80-104] and p85 [299-318] peptides were coupled to CnBr-activated Sepharose 4B beads and used to probe lysates of K46 cells (as described

above). Proteins bound by the peptides on the beads were eluted by boiling in sample buffer, subjected to SDS-polyacrylamide electrophoresis (PAGE), transferred to nylon membranes and immunoblotted with an anti-Fyn antibody and an anti-Lyn antibody. Fig. 16A shows the binding of Fyn to p85 [80-104] and p85 [299-318]. Fig. 16B shows the binding of Lyn to p85 [80-104] and p85 [299-318]. Binding of p85 [80-104] to Lyn and Fyn was easily detectable. The p85 [299-3181 peptide exhibited no detectable binding activity. Immunoreactive Lyn or Fyn did not bind to a control peptide containing residues 1-18 of the mouse Csk protein. Thus, the proline-rich region spanning residues 80 to 104 of PI-3K contains sufficient information to mediate Src-family kinase SH3 binding to p85.

To determine if binding of p85 [80-104] to SH3 domains of Lyn and Fyn is a direct interaction, detergent lysates of bacteria expressing truncated Lyn- and Fyn-GST fusion proteins were probed with beads coupled to p85 [80-104] and p85 [299-318]. Lyn [1-27], Lyn [27-131), Lyn [131-243], and Fyn [1-27] fusions with GST were prepared as similarly as described above for Lyn [1-131] and Fyn [1-I44]. DNA fragments encoding the $p59^{fyn}$ and $p56^{lyn}$ peptide fragments were amplified with the polymerase chain reaction (PCR) from K46 CDNA using the following primers pairs:

Lyn [1-27]:

5': 5'-ATATCAGGATCCTGATGGGATGTATTAAATCA-3'

3': 5'- CAGTCAGAATTCTTCAGTATTACGTACTGGTTG-3'

Lyn [27-131]:

5': 5'-AATCAGGATCCTGGACCGAACTATTTATGTGAGA-3'

5': 5'- CAGTCAGAATTCGAACCACTCTTCAGTTTC-3'

Lyn [131 -243]:

5': 5' - ATATCAGGATCCTGTGGTTCTTCAAGGACATAACAAGG-3'

3': 5' - CAACTTAATGGACTCCCGGG-3'

Fyn [1-27]:

5': 5'-ATATCAGGATCCTGATGGGCTGTGTGCAATGTAAG-3'

3': 5' - CAGTCAGAATTCCCCAGAGCTCTGGTTCAGGCT-3'

Each primer encodes a unique restriction site (underlined, BamHI: GGATCC; EcoRI.; GAATTC; and Sma 1: CCCGGG) that facilitated subsequent cloning into pGEX-3X. *Escherichia coli* DH5α cells expressing respective GST-fusion proteins were collected from 2 ml cultures. Bacterial lysates containing Glutathione-S-transferase (GST) fusion proteins (Lyn [1-27], Lyn [27-131], Lyn [131-243], Fyn [1-27], and Fyn [1-144]) were adsorbed with (10 μl of a 50:50 slurry) p85 [80-104] and p85 [299-318] peptides were coupled to Sepharose 4B beads at a ratio of 2 mg per ml of activated beads following instructions provided by manufacturer. Adsorbates were washed thoroughly with lysis buffer, resuspended in sample buffer and fractionated by SDS-PAGE (10%). Proteins were visualized by coomassie blue staining. The p85 [80-104] peptide bound only the Lyn and Fyn fusion proteins that contained SH3 domains as determined by coomassie staining of gels after SDS-PAGE (Lyn [27-131], Lyn [1-131], and Fyn [1-144]; Fig. 17C). The p85 [299-318] peptide bound the same proteins as p85 [80-104] (Fig. 17D). Adsorptions with glutathione-

Sepharose beads were done to establish that each bacterial lysate contained equivalent amounts of GST fusion proteins (Fig. 17B). Control adsorptions with glycine-Sepharose beads confirmed that non-specific binding to Sepharose beads did not occur (Fig. 17A). These data indicate that the proline-rich regions within the p85 subunit of PI-3K can mediate selective and direct association with the SH3 domains of Lyn and Fyn.

Example 7

This example demonstrates the effect of SH3 binding on PI-3K activity.

Antibodies to the p85 subunit (1 μl of whole antiserum, Upstate Biotechnology, Inc.) were added to lysates of clarified K46 cells ($1x10^7$ cells per sample). Immune complexes were then precipitated with 40 μl of (50:50 slurry) protein A beads (Pharmacia). Adsorbates were washed and divided into equal portions and added to lysis buffer containing various concentrations of Lyn [27-131], Fyn [1-144], or Blk [1-108] peptides that were prepared by cleavage of GST-fusion proteins with Factor Xa as described in Example 6. After incubation overnight at 4°C, adsorbates were thoroughly washed in PAN buffer [100 mM NaCl, 20 mM PIPES pH 7, and 2 mg/ml aprotinin]. Then, 5 μl of a 50:50 slurry of washed beads was incubated for 15 minutes at 30° C in 10 μl containing 0.2 mg/ml sonicated phosphatidylinositol (Sigina or Avanti Polar Lipids), 20 mM Hepes (pH 7.2), 5 μCi of [γ^{-32}P] ATP, and 10 μM ATP. The reactions were terminated by the addition of 100 μl of 1M

HCl, the phospholipids were extracted with 200 μl of $CHCl_3$ - methanol (1:1), the organic phase was washed with 80 μl of HCl - methanol (1:1), and lyophilized to dryness. The phospholipids were then dissolved in 2x5 μl of $CHCl_3$-methanol (1:1), spotted onto a Silica Gel 60 plate (Sigma) that had been presoaked with 1% potassium oxalate, and resolved by ascending thin layer chromatography in a buffer containing $CHCl_3$-methanol-4M NH_4OH (9:7:2). Phospholipid standards (Sigma) were run on the same plate. The radiolabeled lipids were detected by autoradiography and quantitated with a PhosphorImager scanner and ImageQuant software (Molecular Dynamics).

The results are shown in Fig. 18. SH3 containing polypeptides derived from both Lyn and Fyn increased PI-3K activity by approximately 7- and 5-fold, respectively, whereas Blk, which does not associate with PI-3K, had no effect. This activation was half-maximal in the presence of approximately 10 μM peptide and was blocked by the addition of 100 μM p85 [80-104]. In control experiments, fragments of the N-terminal unique region of Lyn and Fyn failed to stimulate PI-3K activity. These data indicate that SH3 domains of Lyn and Fyn can directly stimulate PI-3K activity, presumably by binding to proline-rich regions within the p85 subunit. This activation is independent of both p85 subunit phosphorylation and binding of phosphotyrosine-containing peptide to p85. The data suggest that the reported antigen receptor stimulation induced increase in precipitability of p85 with antibodies to anti-

phosphotyrosine may reflect increased association of PI-3K with phosphorylated srcfamily kinases and/or CD19, rather than PI-3K phosphorylation per se.

Example 8

This example demonstrates the importance of SH3 - p85 [80-104] interaction in B cell antigen receptor mediated PI-3K activation. PI-3K was immunoprecipitated from purified resting B cells (resting mouse B cells (p>1.062) were isolated by Percoll density gradient sedimentation) that were unstimulated or stimulated with antibody specific for B cell antigen receptor. The activity of the immunoprecipitated PI-3K was then assessed. Antigen receptor ligation led to an approximately 7-fold increase in PI-3K activity that was maximal at 1 min (Fig. 19). Resting B cells were permeabilized to allow entrance of p85 [80-104] and p85 [299-318] peptide into the cells, and then stimulated using antibody specific for antigen receptors (anti-IgM and anti-IgD) for 1 min. Cells ($5x10^7$ cells per sample) were permeabilized in Mire's Buffer [10mM $MnCl_2$, 2 mM EGTA and 20 mM $CaCl_2$ (Ca^{+2} buffered to 30 nM), 1 mM 2-mercaptoethanol, 40 mM HEPES (pH 7.4), and 285 μg/ml α-lysophosphatidylcholine, palnmitoyl] either alone, or containing 1.4 mM p85 [80-104] and p85 [299-318] on ice for 1 min. Cells were warmed for 2 min at 37° C prior to incubation for 5 minutes at 37° C in the presence of either phosphate buffered saline alone (open bars) or with 50 μg/ml monoclonal anti-IgD (JA12) and anti-IgM (b-7-6) (solid bars). Cells were then collected by centrifugation,

lysed in 1% NP-40 containing buffer (0.5 ml) and incubated for 30 min. on ice. Nuclei were removed by centrifugation (14,000 g for 15 min). To each sample was added 5 μl (50:50 slurry) of protein A-Sepharose (Pharmacia) and 4 μl of antiserum to p85 (Upstate Biochemcals Inc.) and incubated overnight at 4°C. Adsorbates were washed five times with 1% NP-40 lysis buffer and twice with PAN buffer. Portions (5 μl of a 50:50 slurry) of each precipitate were then removed and assayed for PI-3K enzymatic activity.

In the presence of p85 [80-104], stimulation of PI-3K activity by anti-Ig was completely blocked (Fig. 19). The p85 (299-318] peptide had no detectable effect on receptor mediated activation of PI-3K activity. Essentially identical results were obtained when the B cell lymphoma line, K46μml7, was used. Immunoblotting confirmed that equivalent amounts of p85 were present in each precipitate. Probing of duplicate blots with antibodies to phosphotyrosine (Ab-2, Oncogene Science) revealed no detectable phosphorylation of p85. This suggests that the observed increase in PI-3K activity that follows BCR stimulation is independent of tyrosine phosphorylation of p85. PI-3K activation induced by anti-CD3 stimulation is also independent of tyrosine phosphorylation. These findings suggest that PI-3K activation occurs via direct Src-family kinase SH3 domain binding following antigen receptor ligation.

Example 9

This example demonstrates that Shc binds to Ig-α and Ig-ß ARH1 motifs.

A. Shc binds phosphorylated Ig-α and Ig-ß ARH1 and to nonphosphorylated Ig-α ARH1 motifs

Full cytoplasmic domains and ARH-1 motifs peptides were obtained by cleavage from bacterially expressed GST fusion proteins as described in Clark et al. (*EMBO J.* 13:1911-1919, 1994). In some cases peptides were phosphorylated using a truncated recombinant form of the B subunit of the Insulin Receptor Kinase (BIRK) (described in Herrera et al., *J. Biol. Chem.* 263:5560-5568, 1988). GST-peptides were adsorbed to glutathione Sepharose beads, which were washed in kinase buffer (50mM Hepes [pH 7.4], 5mM $MnCl_2$, 2mM $MgCl_2$, 2mM Na_3Vo_4) and incubated in kinase buffer (0.5 ml) containing 0.5mM ATP, (1 μCi [γ-^{32}P] ATP as a tracer) and 0.1 unit of BIRK (1 unit defined as the amount of kinase required to incorporate 1 nmole phosphate/min into the PLCγ1 based peptide RRNPGFYVEANPMP). The reaction was allowed to proceed for 12-16 hr at 30°C. Subsequently, peptides were cleaved from the GST using Factor Xa (Boehringer Mannheim, Germany) and coupled directly to Sepharose beads (Pharmacia, Stockholm, Sweden) in equimolar amounts. Stoichiometry of phosphorylation was typically 20-25%. Alternatively, synthetic phosphorylated and nonphosphorylated peptides were purchased from Macromolecular Resources Inc. (Fort Collins, CO). Sequences of ARH1 peptides used are:

Ig-α, DMPDDFEDENL(p)YEGLNLDDCSM(p)YEDI;

Ig-ß, DGKAGMEEDHT(p)YEGLNIDQTAT(p)YEDI;
CD-ϵ, NPD(p)YEPIRKGQRDL(p)YSGL; and TCR-ζ,
DGL(p)YQGLSTATKDT(p)YDAL. Phosphorylated tyrosines are indicated by (p).

B lymphoma K46J, J558Lμm3 plasmacytoma, wild type NIH-3T3 and Fyn+ NIH-3T3 fibroblast cell lines were cultured in IMDM supplemented with 50 U/ml penicillin, 50 μg/ml streptomycin and 5% heat inactivated fetal calf serum (FCS). K46J expressing an IgM anti-H2k^k were grown in presence of 1 mg/ml G418. J558Lμm3 expressing a nitrophenyl (NP)-specific receptor were cultured in presence of 1 μg/ml mycophenolic acid, 15 μg/ml hypoxanthine, 250 μg/ml xanthine.

Stimulated cells were prepared as follows. K46J cells (50 x 10^6/ml) were stimulated with anti-μ (b-7-6) mAb (50 μg/ml) for 1 min at 37°C. Log phase J558Lμm3 plasmacytoma cells (50 x 10^6/ml) were stimulated for 1 min at 37°C with NP_{12}-BSA (50 μg/ml) and immediately lysed. Stimulated and unstimulated K46J and J558Lμm3, and NIH-3T3 cells were lysed in 1% NP-40 lysis buffer: 1% NP-40, 10mM Tris, 150mM NaCl, 1mM EDTA, 1mM PMSF, 2 μg/ml each of aprotinin, leupeptin, α-1 antitrypsin, 10mM NaF, 2mM Na_3Vo_4 and incubated for 15 min on ice. Lysates (0.5 ml) were centrifuged in eppendorf tubes for 5 min at x14000 rpm.

Ig-α protein was immunoprecipitated from the cleared lysates described above using anti-Ig-α antibody (10 μg) directly coupled to CNBr activated Sepharose beads (20 μl of 50% $^v/_v$) (Pharmacia, Sweden). The immunoprecipitation

mixtures were incubated for 30 min at 4°C. The co-immunoprecipitated proteins were first eluted with 20mM p-NPP (0.1 ml) for 30 min on ice and Ig-α was subsequently eluted with Laemmli sample buffer.

Adsorptions were also performed by incubating cleared lysates with: Sepharose beads with no peptide (Con), Ig-α or Ig-ß full cytoplasmic domain (FCD), CD3-ϵ, TCR-ζ, or nonphosphorylated and phosphorylated (pIg-α) ARH1 motif peptides. Cell lysates (20 x 10^6 cells in 0.5 ml) were precipitated for 2 hr at 4°C using GST or GST/Grb2 full length fusion proteins (10 μg) bound to glutathione Sepharose beads (20 μl of 50% $^{v}/_{v}$) (Pharmacia), or various synthetic and bacterially expressed peptides (20 μg) directly coupled to CNBr activated Sepharose beads (20 μl of 50% $^{v}/_{v}$) (Pharmacia). Adsorbates were quickly washed with lysis buffer and eluted with Laemmli sample buffer. Precipitation of GST/SHC-SH2 fusion protein was performed using nonphosphorylated and phosphorylated ARH1 peptides (10 μg) coupled Sepharose beads (6 μl of 50% $^{v}/_{v}$) which were incubated 1 hr at 4°C with bacterial lysates containing GST/SHC-SH2 domain fusion protein (0.5 ml).

The immunoprecipitates and absorbates were fractionated by 10% SDS-PAGE. The fractionated proteins were transferred on Immobilon-P membranes (Millipore) and blocked using Tris Buffered Saline containing 5% Bovine Serum Albumin for 2 hr. The blots were then incubated with the following antibodies: monoclonal anti-phosphotyrosine (Ab2; Oncogene Science), anti-p85, rabbit anti-SHC (UBI)

and rabbit anti-Ig-α. Immunocomplex formation using the anti-SHC and anti-Ig-α antibodies was detected using an alkaline phosphatase goat anti-rabbit-Ig (SBA). Immunocomplex formation using the anti-pTyr antibody was detected using horseradish peroxidase goat anti-mouse-Ig (Biorad, CA).

The results shown in Fig. 20 indicate that the p-NPP eluates were fractionated equivalently and immunoblotted with anti-SHC. The results shown in Fig. 21 indicate that Shc binds to tyrosine phosphorylated Ig-α cytoplasmic domain (Ig-αFCD and pIg-αFCD) and to the ARH1 motif (Ig-αARH1 and pIg-αARH1). Shc bound to tyrosine phosphorylated Ig-α and Ig-β, and to nonphosphorylated Ig-α. The association was found to be specific because no binding was detected to nonphosphorylated Ig-β, CD3ϵ and TCR-ς ARH1 peptides. In addition, the p85 subunit of PI-3K to nonphosphorylated Ig-α ARH1 peptide was not detected. Thus, the data indicates that Shc associates with phosphorylated Ig-α and Ig-β ARH1 peptide, but not to non-phosphorylated Ig-β, CD3ϵ and TCR-ς ARH1 peptides.

The ability of Shc to bind to nonphosphorylated Ig-α ARH1 peptide was confirmed by absorbing Shc using a mutated Ig-α ARH1 peptide in which the tyrosine residues were substituted with phenylalanine residues (described in Clark et al., *ibid.*) and immunoblotting with anti-Shc antibody. The results indicate that an equivalent amount of Shc associated with the mutated peptide compared with wild type peptide.

B. Shc associates to the Ig-α ARH1 motif by two distinct mechanisms

Cell lysates were prepared as described in Section A. Adsorptions were performed using the resulting cleared lysates by incubating the lysates with tyrosine phosphorylated (p) and nonphosphorylated Ig-α and Ig-ß ARH1 peptide Sepharose beads in absence (-) or presence (+) of 50mM p-NPP on lysates from K46J cells or bacterial lysate containing GST/SHC-SH2 domain fusion protein. The GST/SHC-SH2 domain fusion was adsorbed on glutathione Sepharose beads (Glut). The absorbed protein was eluted, fractionated and immunoblotted as described in Section A using anti-SHC (Fig. 22a) or anti-phosphotyrosine (anti-pTyr, Fig. 22b) antibodies or stained with Comassie brilliant Blue (Fig. 22c).

The results shown in Fig. 22 indicate that Shc binding to nonphosphorylated Ig-α ARH1 peptide was not blocked by p-NPP (Fig. 22a), indicating phosphotyrosine independence in the interaction. Conversely, p-NPP inhibited Shc binding to phospho-Ig-ß ARH1 peptide (Fig. 22a). Referring to Fig. 22b, equivalent amounts of Shc was found to associate with the phospho-Ig-α ARH1 peptide in lysates of stimulated and unstimulated cells (right panel), but phospho-Ig-α ARH1 associated tyrosine phosphorylated Shc was detected only in lysates of stimulated cells (left panel). Thus, Shc tyrosine phosphorylation has no detectable effect on its ability to bind to the phospho-Ig-α ARH1 peptide. Referring to Fig. 22c, tyrosine phosphorylated Ig-α and Ig-ß both

bound to the GST/SHC-SH2 in a p-NPP sensitive manner, but they did not bind GST alone. Lack of additional Comassie Blue stained bands in GST/SHC-SH2 containing lanes, indicates that this association is direct.

C. Association of Shc with the nonphosphorylated Ig-α ARH1 motif is dictated by the amino acid sequence DCSM within the ARH1 motif, and does not require ARH1 motif tyrosine phosphorylation and binding of src-family kinases

Switch mutants of Ig-α and Ig-ß, in which DCSM of Ig-α was substituted with QTAT of Ig-ß and vice versa were prepared using the method disclosed in Clark et al., *ibid*. In addition, mutants containing the amino acid sequences ACSM, DASM, DCAM and DCSA were produced according to the method disclosed in Clark et al., *ibid*. The results indicate that the four amino acid sequence DCSM conferred specificity for nonphosphorylated Ig-α ARH1 peptide binding to Shc. In addition, the single amino acid sequence modifications to the DCSM sequence did not disrupt binding of the peptide to Shc. Thus, the DCSM sequence in the Ig-α ARH1 motif binds to Shc.

Taken together, the foregoing data indicates that Shc associates with an Ig-α ARH1 motif via a DCSM sequence. The data further indicates that the phosphorylation state of Ig-α and Ig-ß ARH1 motifs can effect the ability of Shc to bind to the peptide.

Example 10

This example demonstrates the differential ITAM binding of src-family tyrosine kinases, syk-family tyrosine kinases, adaptor molecules and lipid kinases.

A. Biphosphorylated ITAMs induce tyrosine phosphorylation in permeabilized B cells

Synthetic peptides corresponding to nonphosphorylated ITAMs in which the motif tyrosines were changed to phenylalanine or biphosphorylated ITAMS, were produced using an f-moc chemistry with HBTU active esters (Macromolecular Resources, Colorado State University). Peptide sequences corresponding to wildtype ITAMS are: ENLYEGLNNLDDCSMYEDI (mIgα); DHTYEGLNIDQTATYEDI (mIgß); DGLYQGLSTATKDTYYDAL (mTCRζc); NPDYEPIRKJQRDLYSGL (mCD3ϵ); DRLYEELNHVYSPIYSEL (mFcϵRIß); DAVYTGLNTRNQETYETL (mFcϵRIγ). To construct phosphorylated peptides, phosphotyrosine (Nova Biochem) was substituted for tyrosine at each position. Peptides were deprotected by incubation for 90 minutes in 90% TFA, 2.5% aminole, and 2.5% ethane dithiol, purified by high performance liquid chromatography (HPLC) on a C18 column, and analyzed by mass spectrometry to assure predicted mass. As necessary, peptides were coupled to cyanogen bromide activated Sepharose 4B (Pharmacia) per manufacturer's instructions at 2 mg peptide per milliliter packed beads. In all cases, coupling efficiency, based on HPLC analysis of the coupled gel effluent, was >90%.

J558Lμm3 cells, propagated in Iscove's modified Dulbecco's medium (IMDM) supplemented with 5% fetal calf serum (Hyclone), Streptomycin, Penicillin, 2-

mercaptoethanol, and L-glutamine at 37°C in 7.5% CO_2, were harvested by centrifugation, washed in IMDM medium without supplements, and permeabilized as previously described by Campbell et al. (*Proc. Natl. Acad. Sci. USA.* 88:3982-3986, 1991). Briefly, cells (2 x 10^7 cells per sample) were incubated in 500 µl Mire's Buffer (10mM $MnCl_2$, 10mM MgOAc, 2mM EGTA and 296µM $CaCl_2$ [Ca^{+2} buffered to 30nM], 1mM 2-mercaptoethanol, 40mM HEPES [pH 7.4], and 285 µ/ml α-lysophosphatidylcholine, palmitoyl) either alone or containing various concentrations of ITAM peptides (1µM to 1mM) for 5 minutes at 37°C. Cells were then collected by centrifugation, lysed in 1% NP-40 lysis buffer (0.5 ml) and nuclei removed by centrifugation (12,000 x g for 10 min).

Increasing concentrations of biphosphorylated pITAM peptides and nonphosphorylated ITAMs were added to the permeabilized cells. The concentrations included 0, 1µM, 10µM, 100µM and 1mM peptide. The cells were incubated with the peptide for varying amounts of time, including 0, 15 sec., 30 sec., 45 sec., 1 min., 2 min. and 5 min. Induction of protein tyrosine phosphorylation was analyzed by immunoblotting using the method described in Example 9.

The results indicated that all of the pITAMs were able to induce protein tyrosine phosphorylation in the permeabilized J558Lµm3 cells. Induction was not observed in lysed cells. Measurable inductive phosphorylation was observed at 100µM or greater concentrations of pITAM and was detectable at 15 sec. Nonphosphorylated ITAMs had no detectable effect on total cellular protein tyrosine

phosphorylation. Taken together, these results indicate that all phosphorylated ITAMs can initiate cellular protein tyrosine phosphorylation, while nonphosphorylated peptides can not.

B. Biphosphorylated ITAMs activate Lyn, but not Syk, in permeabilized B cells

Permeabilized J558Lμm3 cells were prepared using the method described in Section A. Such cells were then stimulated for 5 min. with 1mM CDε pITAM peptide. Lysates of the stimulated cells were prepared by harvesting the cells by centrifugation, washing them in IMDM medium without supplements, and resuspending them in lysis buffer (1% NP-40, 150mM NaCl, 10mM Tris [pH 7.5], 2mM sodium orthovanadate, 1mM phenylmethylsulfonyl flouride (PMSF), 0.4mM EDTA, 10mM NaF, and 1 μg/ml each of aprotinin, leupeptin, and α-1antitrypsin). The lysates were incubated on ice for 10 minutes and cleared of particulate nuclear/ cytoskeletal components by centrifugation at 12,000 x g for 10 minutes.

Lyn and Syk protein was then immunoprecipitated from the cleared lysates by incubating the lysates from 2 x 10^7 cells with 50 μg anti-Lyn antibody or 50 μg anti-Syk antibody for 1 hour at 0°C to form immunocomplexes and then recovering the immunocomplexes by incubating the lysates with 5 μl of protein-A sepharose (Pharmacia) pre-adsorbed with 5 μl of antisera for 2 hours at 4°C with constant mixing by inversion. Adsorbates were then washed 3 times with 1 ml ice cold lysis buffer and eluted by resuspension

in 50 μl reducing SDS-PAGE sample buffer. Samples were boiled for 5 minutes prior to SDS-PAGE (8 or 10% gels). SDS-PAGE gels were then transferred to PVDF membranes using a semi-dry blotting apparatus following conditions recommended by the manufacture (Millipore).

Membranes containing electrophoretically transferred proteins were blocked using Tris buffered saline (TBS) containing either 5% non-fat dry milk or phosphate-free bovine serum albumin overnight at 4°C, and subsequently probed first with mouse IgG_1 monoclonal α-phosphotyrosine (1:1000; AB2, Oncogene Science) antibodies. Membranes were then washed several times alternately with TBS or TBS containing 0.05% Triton X-100, incubated with dilute Protein A (Amersham) or rat anti-mouse IgG_1 (Jackson Research Labs) conjugated to horseradish peroxidase and washed again. Immunoreactive proteins were visualized by an enhanced chemiluminescence (ECL) detection system (Amersham). The membranes were them stripped in buffer containing 100mM 2-mercaptoethanol, 2% SDS, and 62.5mM Tris (pH 6.7) for 30 minutes at 56°C prior reprobed with α-Lyn (1:500) or α-Syk (1:500) antibodies.

The results indicate that anti-phosphotyrosine immunoblotting of the Lyn and Syk immunoprecipitates revealed inductive phosphorylation of Lyn but not Syk. Subsequent immunoblotting using anti-Lyn and anti-Syk antibodies indicated that the amount of Lyn or Syk present in the anti-phosphotyrosine blotted samples were equivalent.

The kinase activity of immunoprecipitated Lyn or Syk was then tested using the following kinase assay. The immunoprecipitated Lyn and Syk were washed twice in *in vitro* kinase buffer (10mM $MgCl_2$, 10mM Hepes [pH 7.0], 2mM sodium orthovanadate, and 1mM PMSF), and resuspended in 25 μl of kinase buffer containing 2mM exogenous substrate corresponding to a portion of the Lck kinase autophosphorylation site, RRLIEDAEYAARG, 10μM ATP and 10μCi [γ-32P] ATP (New England Nuclear-Dupont), and incubated for 10 minutes at 30°C. Alternatively, 5μl of SF9-Lyn lysate was used instead of affinity-purified Lyn. As necessary, Sf9-Lyn lysates were pre-incubated with ITAM peptides (concentrations varied from 1μM to 1 mM) for 1 hour at 4°C prior to the addition of the substrate/ATP containing reaction mix. Each reaction was quenched with trichloroacetic acid (final concentration of 5%) and blotted onto Whatman p81 phosphocellulose. Blots were washed four times with an excess of 75mM phosphoric acid, dried with acetone, and quantitated by liquid scintillation spectrometry. Determination of the Sf9-Lyn K_m and V_{max} required that the substrate concentration (present in the reaction mix) be varied from 100μM to 10mM.

The results indicate that the catalytic activity of immunoprecipitated Lyn increased 3-fold in permeabilized cells stimulated with CD3ϵ pITAM peptide, while nonphosphorylated CD3ϵ ITAM peptide had no effect. Permeabilization alone had no detectable effect on Lyn activity when compared to Lyn immunoprecipitated from

resting J558Lμm3 cell lysates. Syk activation was not detected in the presence of CD3ϵ ITAM peptide. This result indicates that pITAM is capable of stimulating a src-family kinase.

C. Direct activation of Lyn by biphosphorylated ITAMs

pITAMs were tested for their ability to directly regulate src-family kinase activity by adding pITAM peptides to baculovirus SF9 cell expressed Lyn protein. DNA spanning the entire coding region of mouse $p56^{lyn}$ was amplified with the polymerase chain reaction (PCR) from K46 cDNA using the following primers:

5'-AGATACGGATCCGGATGTATTAAATCAAAAAGG-3', and

5'-AGATCATGAGATCTGCGGCCGCCTACGGTTGCTGCTGATAC-3'.

Production of first strand cDNA and PCR reactions were performed as previously described (Pleiman et al., *Proc. Natl. Acad. Sci. USA* 91:4268-4272, 1994). The PCR product was then cleaved with Bam H1 and Not 1 restriction endonucleases, subcloned into pSK Bluescript (Stratagene, La Jolla, CA), and sequence confirmed by DNA sequence analysis. The Bam H1/Not 1 fragment encoding Lyn was then subcloned into the baculovirus expression vector pAcGP67B (Pharmingen). This construct was transfected into Sf9 cells using the Baculogold tranfection kit following manufacturer's instructions (Pharmingen). Cells were maintained in Sf900 II serum-free medium (Gibco) and virus propagated as per manufacturer's protocol (Pharmingen). To generate Lyn protein (Sf9-Lyn), 6 x 10^6 Sf9 cells were infected with recombinant virus at a multiplicity of

infection of 5. Infected Sf9 cells were grown for 72 hours prior to lysis in 1 % NP-40 lysis buffer.

Lyn (5 μl of whole cell lysate of 1 x 10^6 cells/ml) were pre-incubated with pITAMs and assayed for the ability of lyn to phosphorylate the autophosphorylation site of Lck, RRLIEDAEYAARG using the kinase described above in Section B. The results indicate that activation of Lyn by Ig-α pITAM was both dose dependent and saturable, while saturating doses of nonphosphorylated Ig-α ITAM had little effect on Lyn activity. Significant activation was seen using 6.7 μM Ig-α pITAM. A 2-fold increase in the Vmax of Lyn, from 0.0742 pmole/minute/μl lysate to 0.137 pmole/minute/μl lysate, while the K_m remained unchanged.

The pITAMs Ig-α, Ig-β, TCRζc, CD3ε, FcεRIγ and FcεRIβ were then tested using 300 μM of each peptide. The ability the pITAMs to activate Lyn varied. Ig-α pITAM increased Lyn activity 5.1-fold, FcεRIβ pITAM increased Lyn activity 3.9-fold, Ig-β pITAM increased Lyn activity 3.4-fold and FcεRIγ increased Lyn activity 3.1-fold, over their nonphosphorylated counterpart.

D. Biphosphorylated ITAMs exhibit differential effector binding activity

The binding specificity of the various pITAMs to PI-3K p85 subunit, Syk, Lyn and Shc were tested by incubating 2 mg peptide/ml pITAM peptide coupled beads with NP-40 lysates prepared from 2 x 10^7 K46 B lymphoma cells as described in Section A. After extensive washing in lysis buffer, pITAM binding proteins were eluted from the beads

using reducing SDS-PAGE sample buffer, fractionated by electrophoresis, transferred to PVDF membranes and immunoblotted using whole rabbit antisera α-Lyn (1:500), α-Syk (1:500), and α-PI3-k (p85 subunit, 1:1000, UBI), and affinity purified rabbit antisera α-Shc (1:500, UBI) antibodies.

The results shown in Fig. 23 indicate that Ig-α, Ig-β and FcεRIß pITAMs bind preferentially to Lyn, while TCRςc, CD3ε and FcεRIγ bind preferentially to Syk. The pITAM-Shc binding pattern was similar to that of Lyn, while PI-3K showed strong binding to both FcεRIγ and Ig-α pITAMs. This comparative analysis indicates that specificity of interaction between different pITAM sequences and effector molecules.

While various embodiments of the present invention have been described in detail, it is apparent that modifications and adaptations of those embodiments will occur to those skilled in the art. It is to be expressly understood, however, that such modifications and adaptations are within the scope of the present invention, as set forth in the following claims:

What is claimed is:

1. A method for regulating signal transduction comprising contacting a cell with an effective amount of a compound comprising a peptide having an amino acid motif of YXXLXXXXXXXYXXΨ, or a mimetope thereof.

2. The method as set forth in Claim 1, wherein said amino acid motif comprises a sequence selected from the group consisting of YXXLXXXDCSMYXXΨ, YXXLXXXQTATYXXΨ, YXXLXXXTKDTYXXΨ, YXXLXXXQRDLYXXΨ, YXXLXXXYSPIYXXΨ, YXXLXXXNQETYXX, or a mimetope thereof.

3. The method as set forth in Claim 1, wherein said amino acid motif comprises a sequence selected from the group consisting of NLYEGLNLDDCSMYEDI, DHTYEGLNIDQTATYEDI, DRLYEELNHVYSPIYSEL, DAVYTGLNTRNQETYETL, DGLYQGLSTATKDTYDAL, NPDYEPIRKGQRDLYSGL, or a mimetope thereof.

4. A method of any one of Claims 1, 2 or 3, in which the amino acid motif is phosphorylated at both Y residues.

5. A method of any one of Claims 1, 2, 3 or 4, in which the amino acid motif binds to a cellular component selected from the group consisting of a src-family tyrosine kinase, a lipid kinase and a signal transduction adapter molecule.

6. A method of any one of Claims 1, 2, 3, 4 or 5 in which the amino acid motif binds to a cellular component selected from the group consisting of Fyn, Lyn, Blk, Syk, Yes, Lck, Btk, Hck, Src, and Zap70.

7. A method to identify compounds capable of inhibiting stimulation of activation of a src-family tyrosine kinase, said method comprising:

(a) contacting a src-family tyrosine kinase capable of autophosphorylating with a putative inhibitory compound to form a reaction mixture;

(b) contacting said reaction mixture with a stimulatory compound comprising an YXXLXXXXXXXYXXΨ amino acid motif, or a mimetope thereof, wherein the tyrosine residues in said motif are phosphorylated; and

(d) assessing the ability of said putative inhibitory compound to inhibit activation of said src-family tyrosine kinase by said stimulatory compound by determining autophosphorylation of said src-family tyrosine kinase.

8. The method of Claim 7, wherein said src tyrosine kinase is selected from the group consisting of Fyn, Lyn, Blk, Syk, Yes, Lck, Btk, Hck, Src, and Zap70.

9. A method to regulate signal transduction comprising contacting a cell with an effective amount of a compound comprising a peptide, or a mimetope thereof, capable of binding to an SH3 domain of a tyrosine kinase, thereby blocking the binding of an effector to said tyrosine kinase.

10. A method as set forth in one of Claim 9, wherein said proline-rich domain is selected from the group consisting of KKISPPTPKPRPPRPTPVAPGSSKT and NERQPAPATPPKPPKPTTVA.

11. A therapeutic composition for regulating signal transduction in a cell, said composition comprising a peptide having an YXXLXXXXXXXYXXΨ amino acid motif, or a mimetope thereof, and a phramaceutically acceptable excipient.

12. A therapeutic composition for regulating signal transduction in a cell, said composition comprising a peptide, or a mimetope thereof, capable of binding to an SH3 domain of a tyrosine kinase, thereby blocking the binding of an effector to said tyrosine kinase.

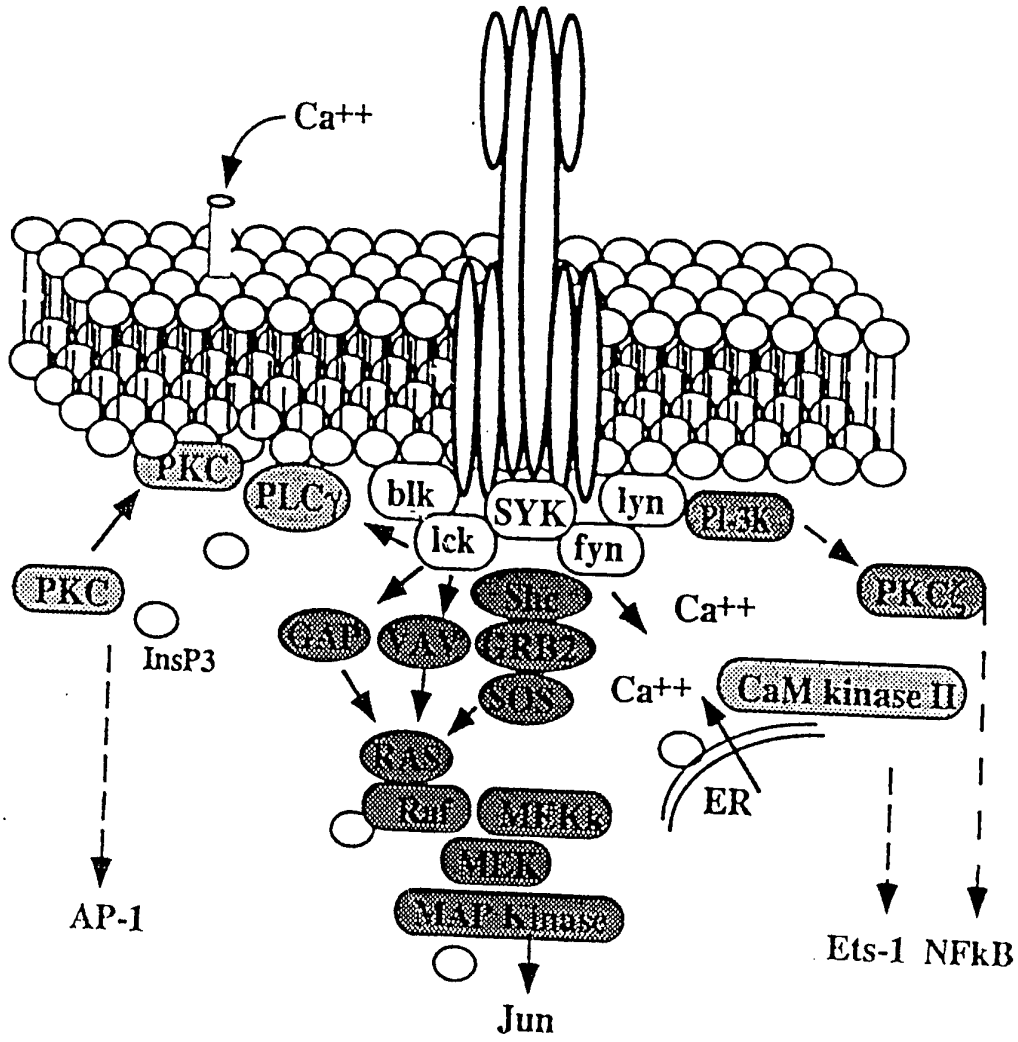
Ca++
PKC
PLCγ
blk
SYK
lyn
PI-3K
lck
fyn
PKC
InsP3
Shc
GAP
VAV
GRB2
SOS
Ca++
Ca++
CaM kinase II
PKCζ
RAS
Raf
MEKK
MEK
MAP Kinase
ER
AP-1
Jun
Ets-1
NFkB

FIG. 1

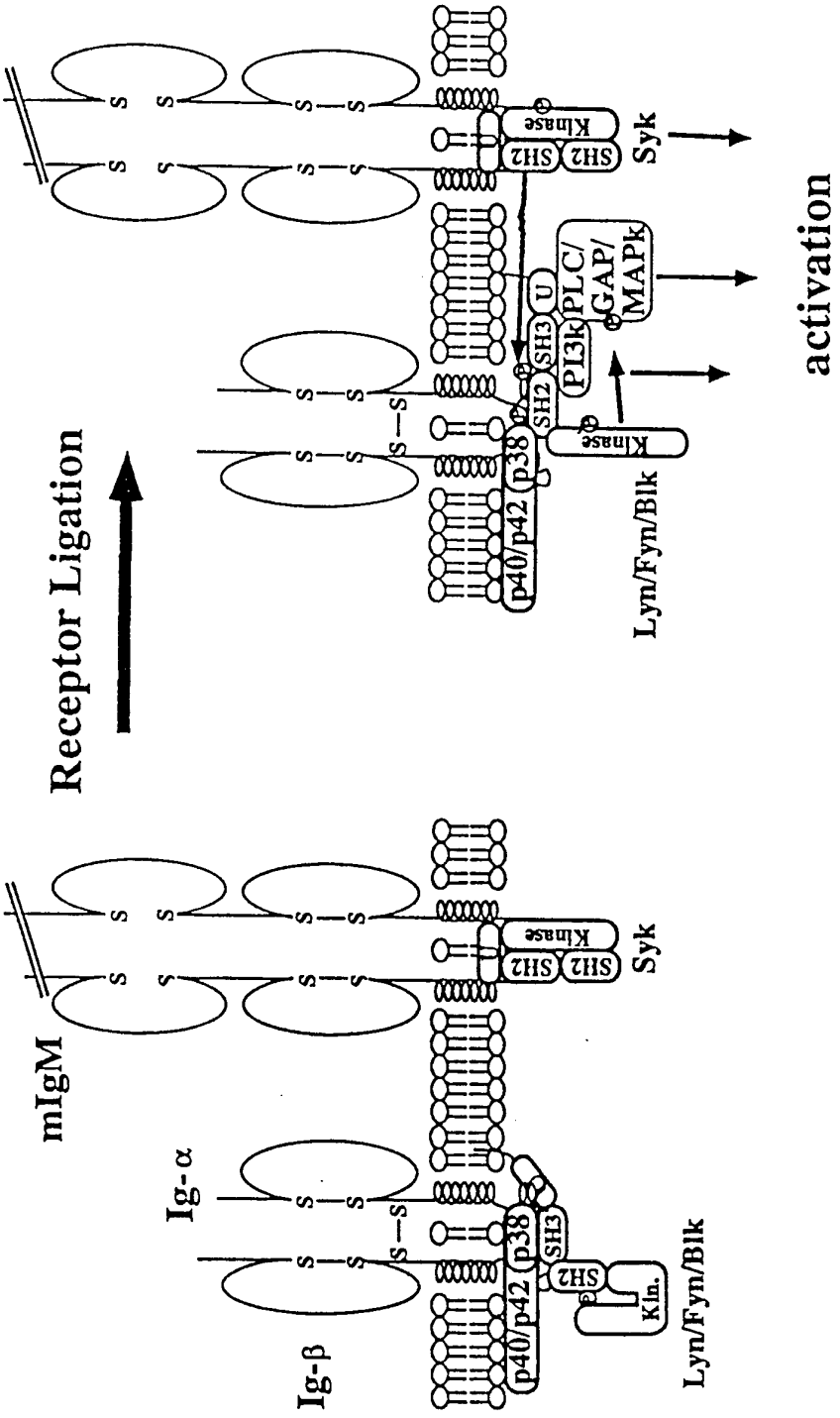
mIgM
Ig-α
Ig-β
Receptor Ligation
Syk
Klnase
SH2
SH3
PI3k
PLC/
GAP/
MAPk
p40/p42
p38
Lyn/Fyn/Blk
activation

FIG. 2

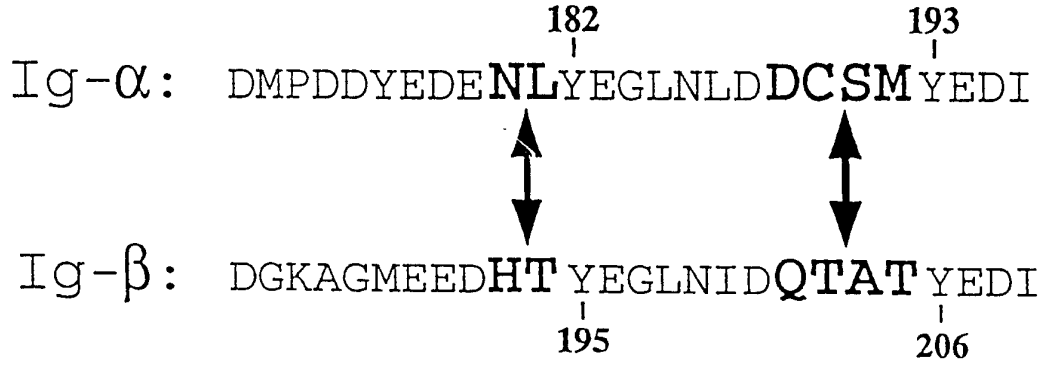
182
193
Ig-α: DMPDDYEDENLYEGLNLDDCSMYEDI
Ig-β: DGKAGMEEDHTYEGLNIDQTATYEDI
195
206

FIG. 3

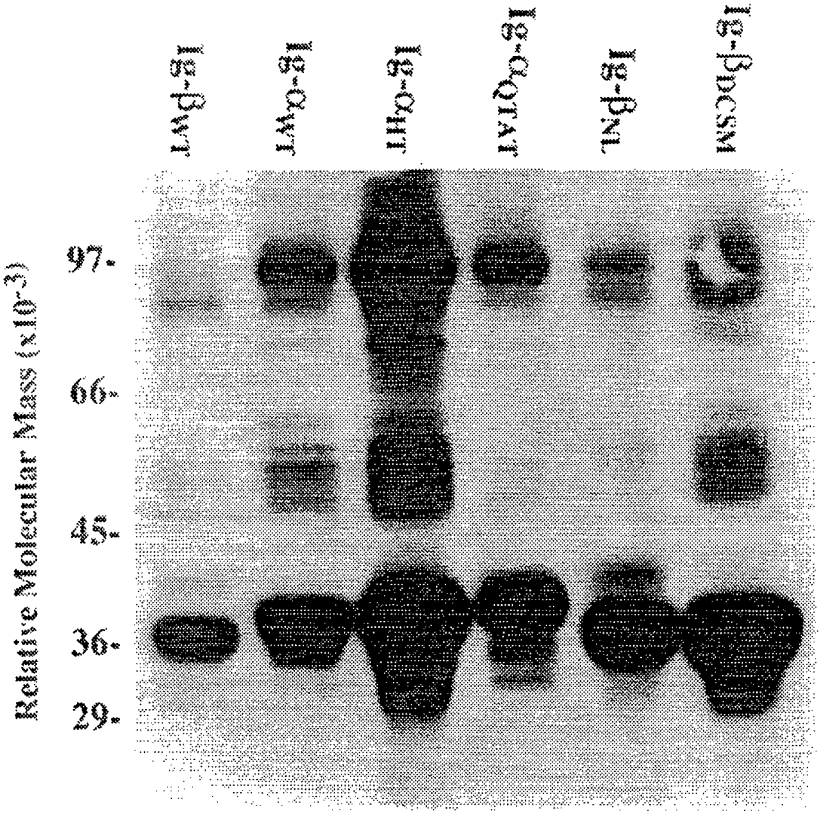
Ig-βWT
Ig-αWT
Ig-αHT
Ig-αQYAT
Ig-βNL
Ig-βDCSM
Relative Molecular Mass (x10-3)
97-
66-
45-
36-
29-

FIG. 4

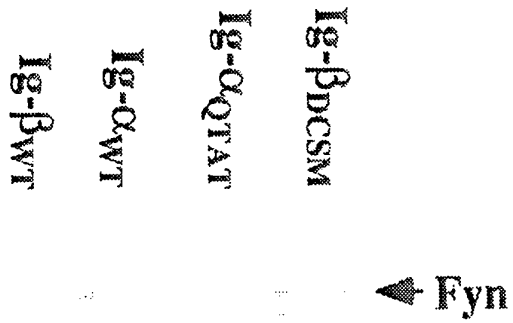

FIG. 5

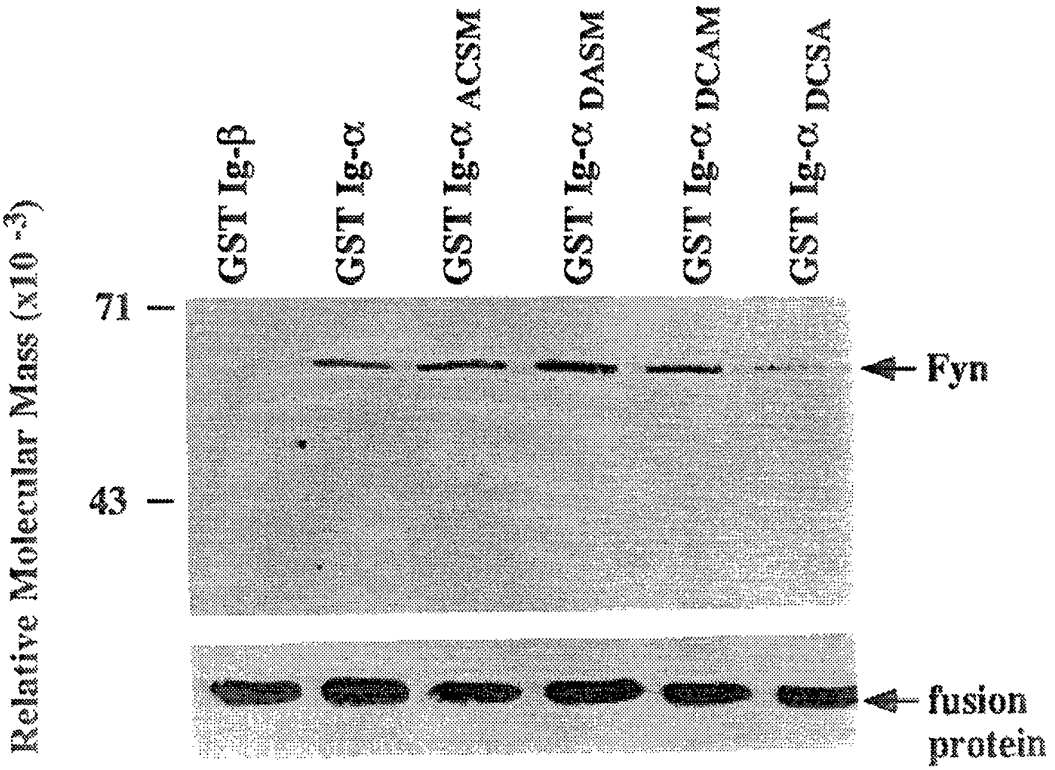
GST Ig-β
GST Ig-α
GST Ig-α ACSM
GST Ig-α DASM
GST Ig-α DCAM
GST Ig-α DCSA
Relative Molecular Mass (x10 -3)
71
43
Fyn
fusion protein

FIG. 6

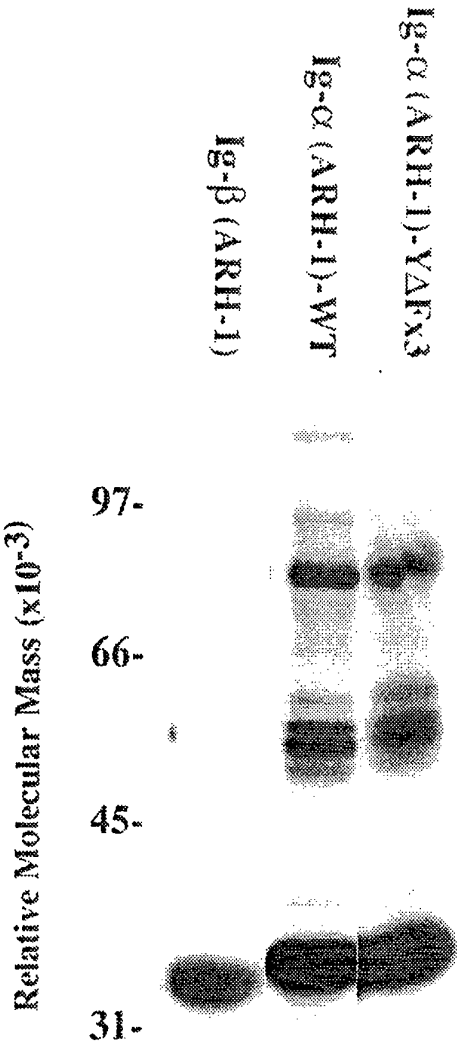
Ig-α (ARH-1)-YΔFx3
Ig-α (ARH-1)-WT
Ig-β (ARH-1)
Relative Molecular Mass (x10-3)
97-
66-
45-
31-

FIG. 7

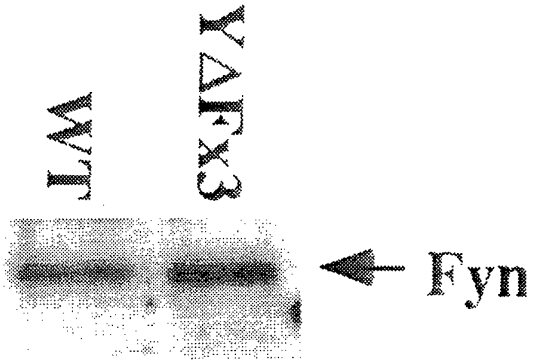

FIG. 8

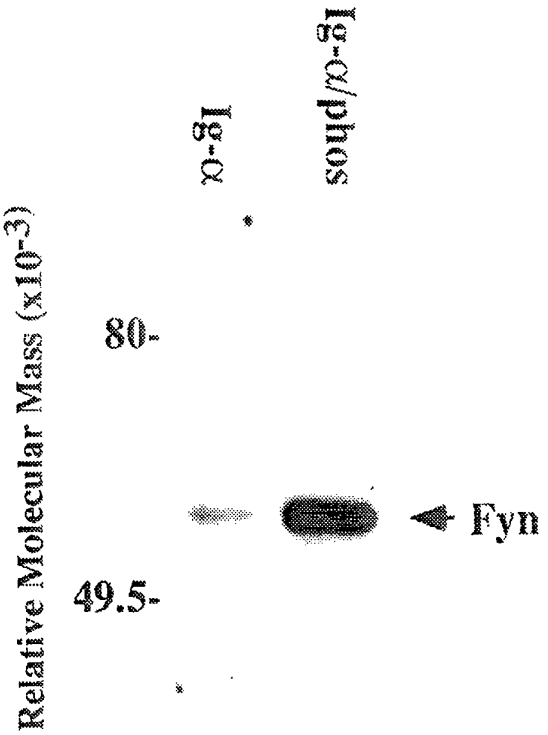

FIG. 9

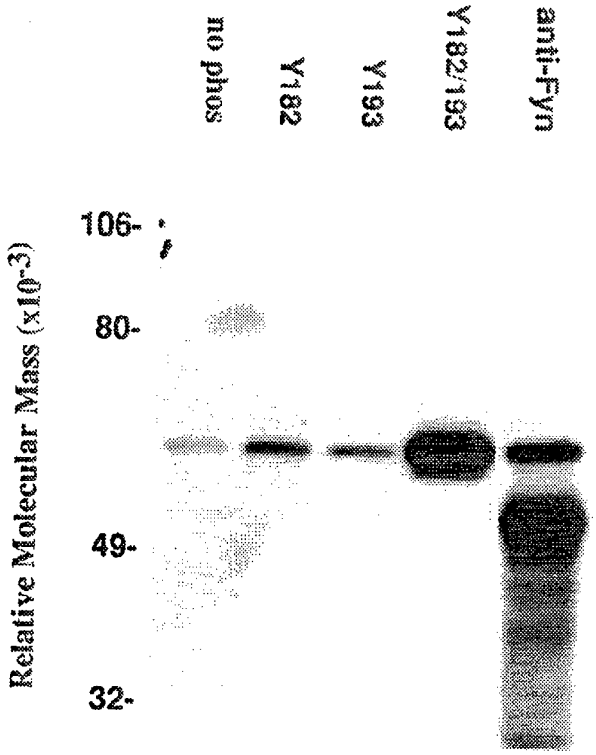
no phos
Y182
Y193
Y182/193
anti-Fyn
106-
80-
49-
32-
Relative Molecular Mass (x10-3)

FIG. 10

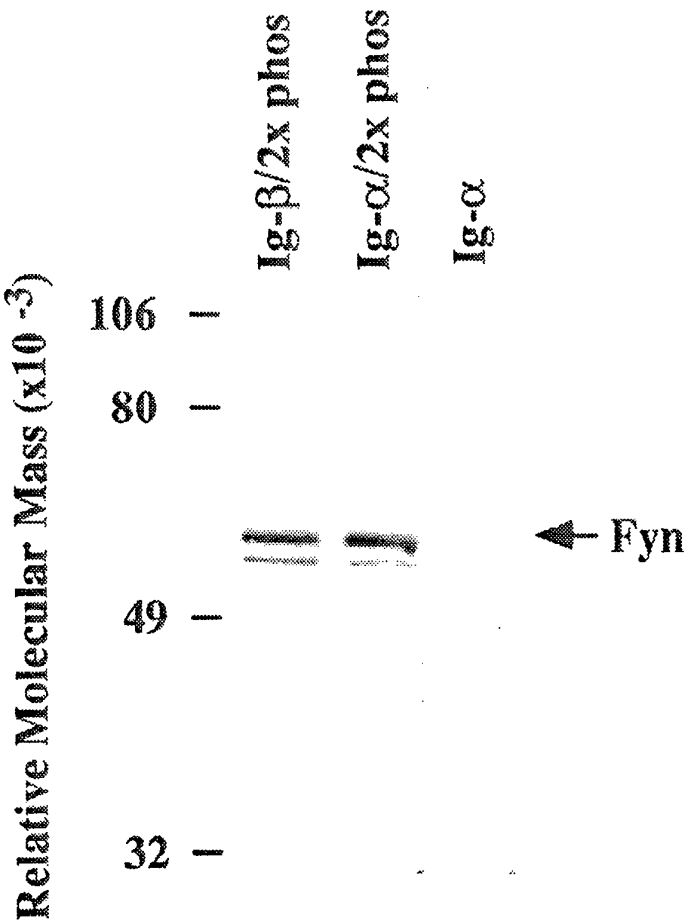
Ig-β/2x phos
Ig-α/2x phos
Ig-α
Relative Molecular Mass (x10 -3)
106
80
49
32
Fyn

FIG. 11

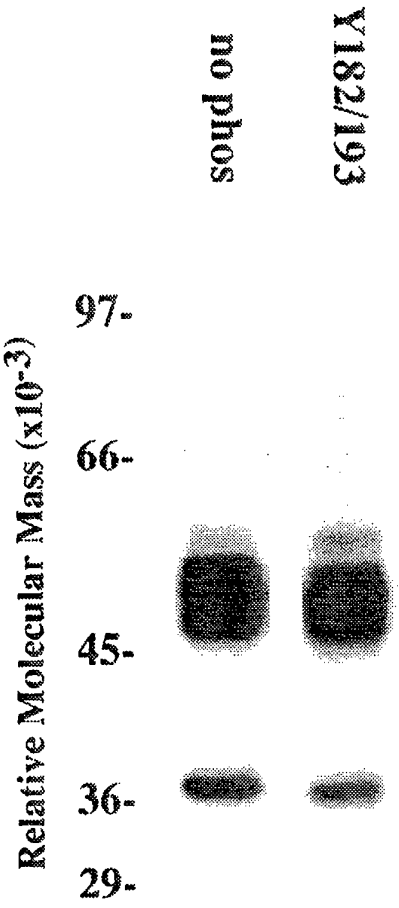

FIG. 12

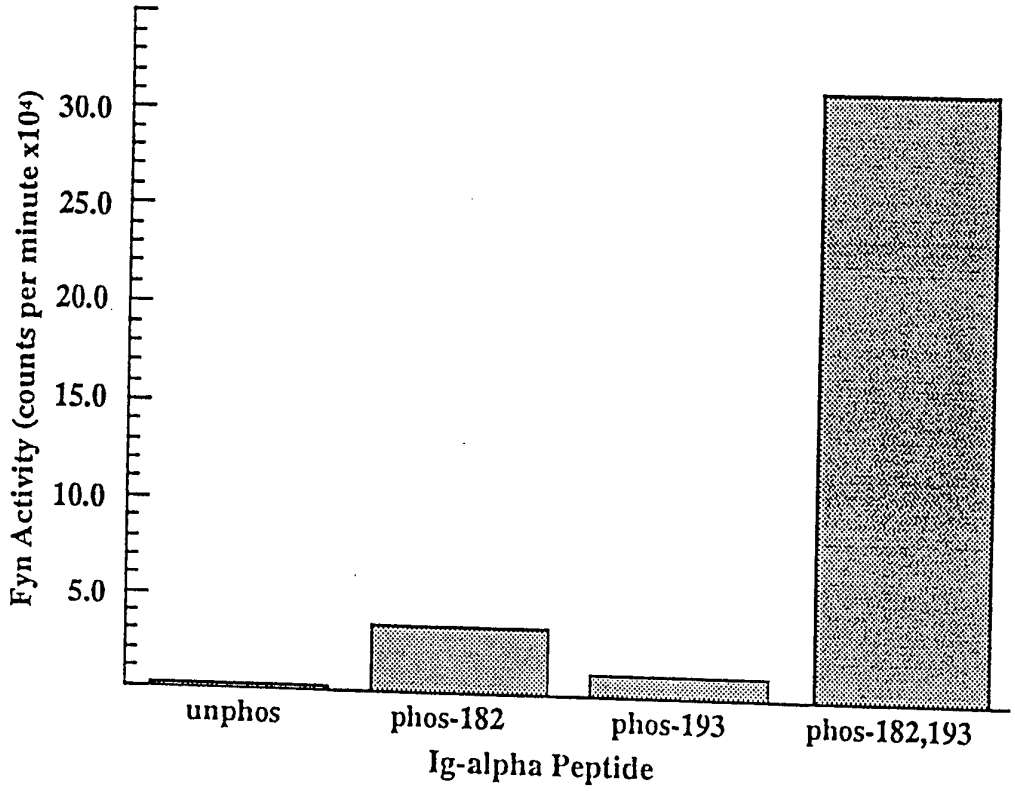
Fyn Activity (counts per minute x10⁴)
30.0
25.0
20.0
15.0
10.0
5.0
unphos
phos-182
phos-193
phos-182,193
Ig-alpha Peptide

FIG. 13

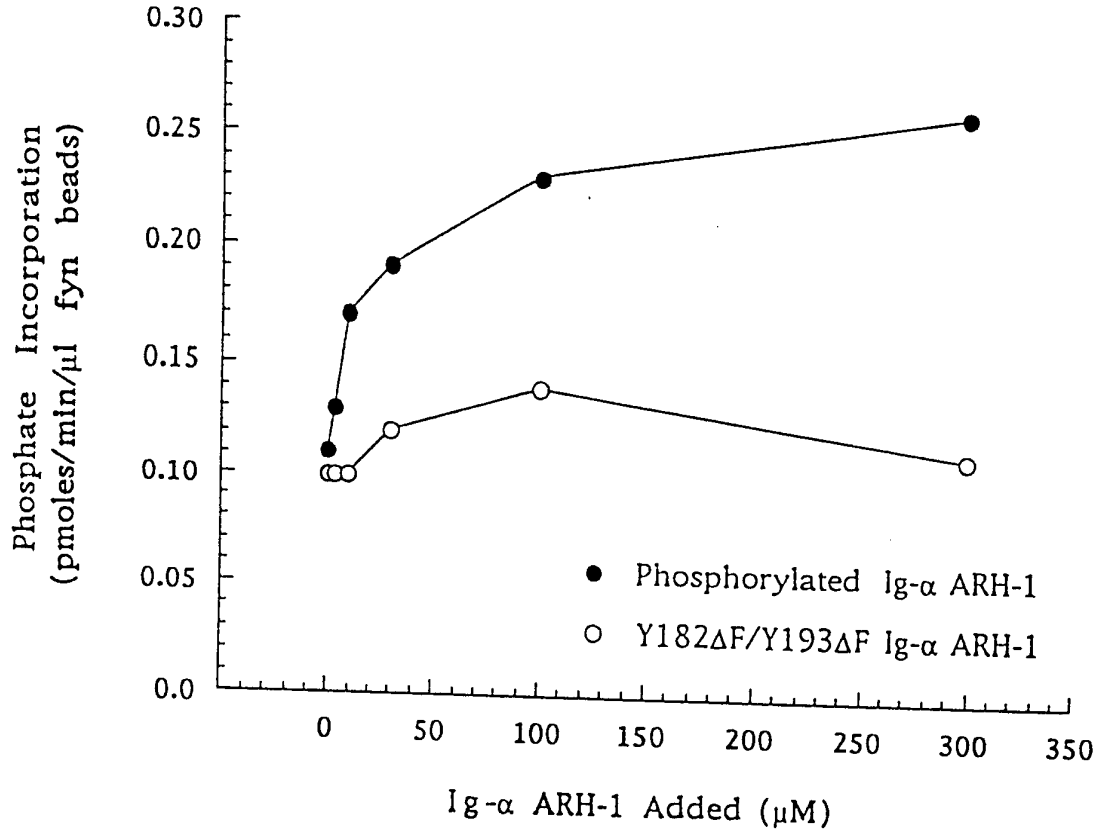
Phosphate Incorporation (pmoles/min/µl fyn beads)
0.30
0.25
0.20
0.15
0.10
0.05
0.0
0
50
100
150
200
250
300
350
Ig-α ARH-1 Added (µM)
Phosphorylated Ig-α ARH-1
Y182ΔF/Y193ΔF Ig-α ARH-1

FIG. 14

A.

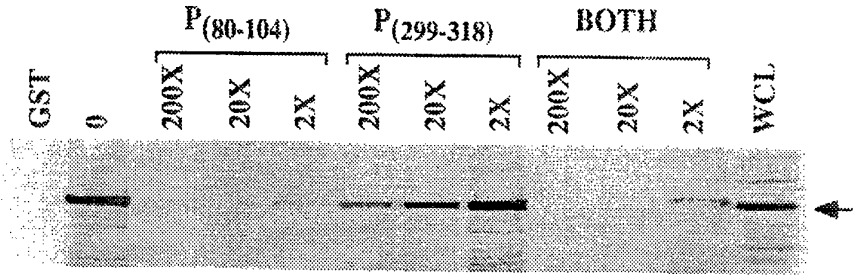

FIG. 15A

B.

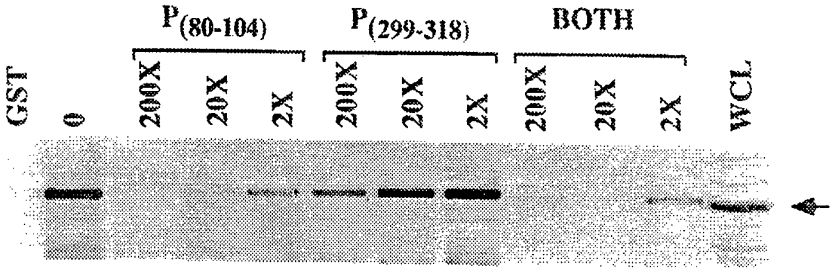

FIG. 15B

A.

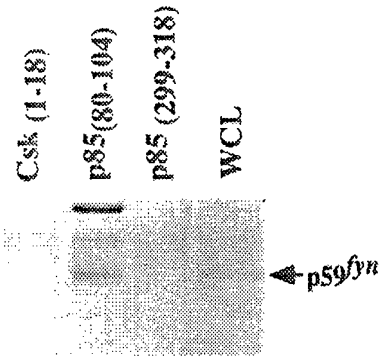

FIG. 16A

B.

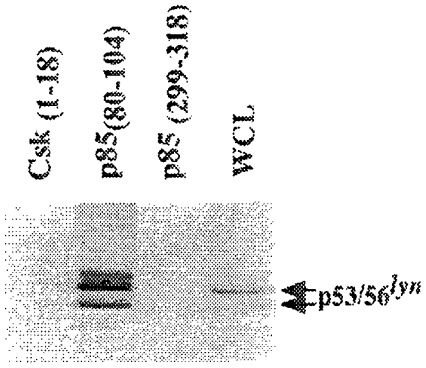

FIG. 16B

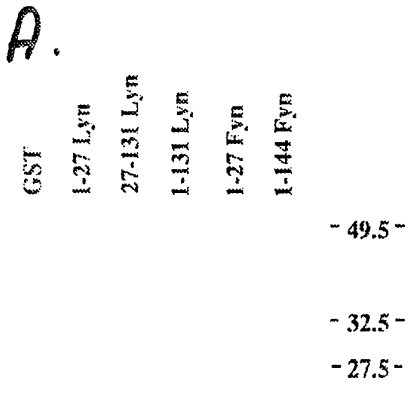

FIG. 17A

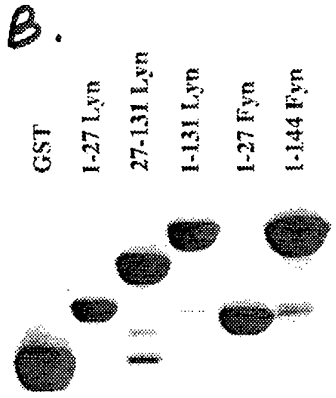

FIG. 17B

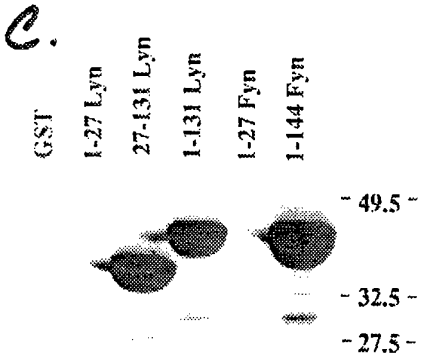

FIG. 17C

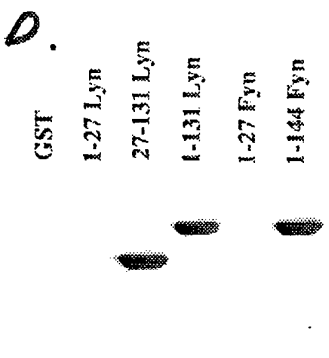

FIG. 17D

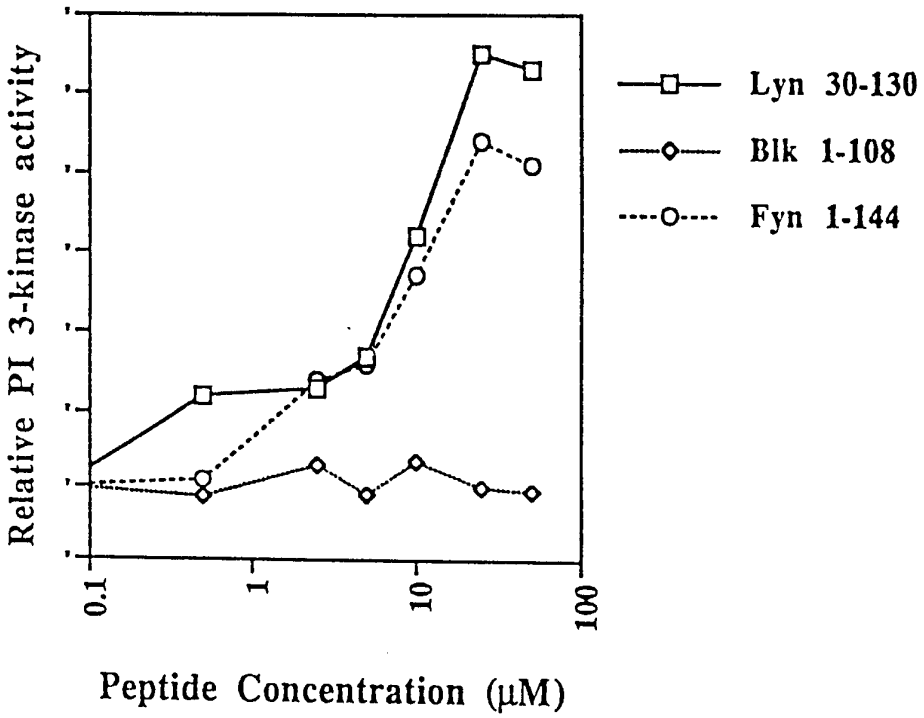
Relative PI 3-kinase activity
0.1
1
10
100
Peptide Concentration (μM)
Lyn 30-130
Blk 1-108
Fyn 1-144

FIG. 18

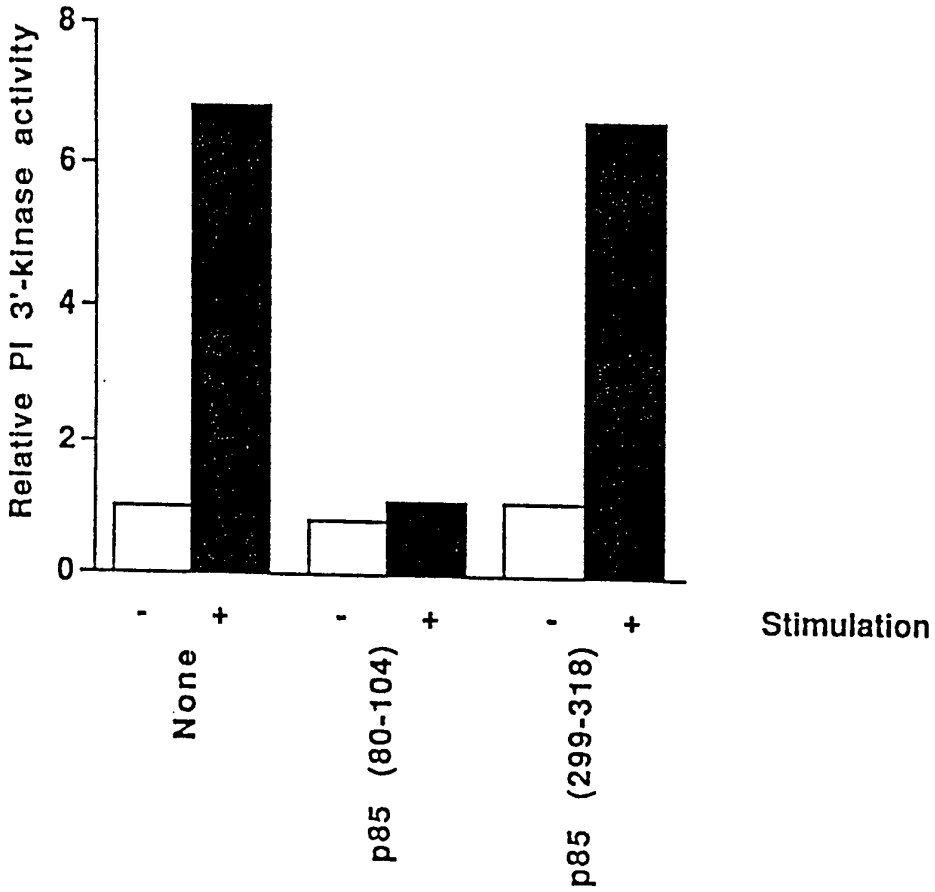
Relative PI 3'-kinase activity
8
6
4
2
0
- + - + - +
Stimulation
None
p85 (80-104)
p85 (299-318)

FIG. 19

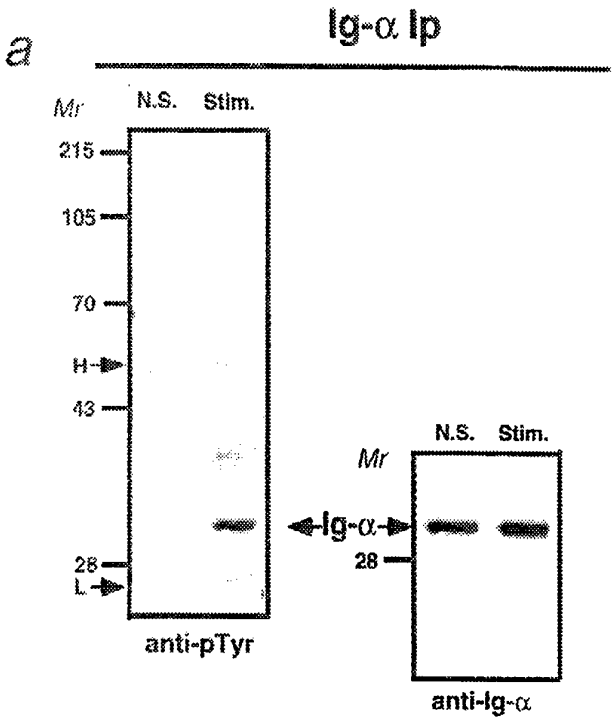

FIG. 20A

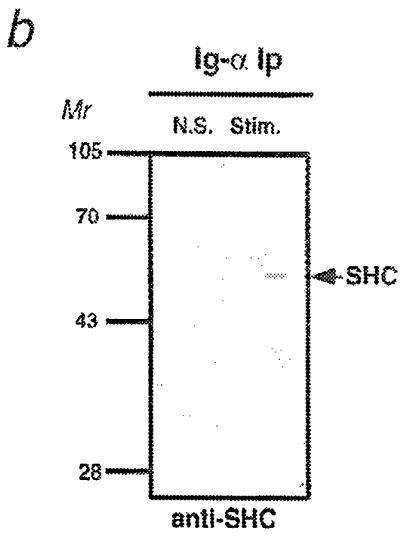

FIG. 20B

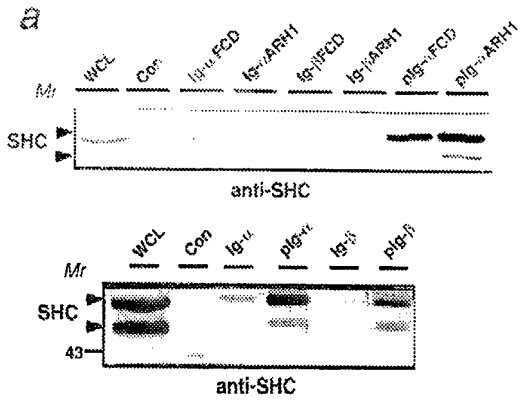

FIG. 21A

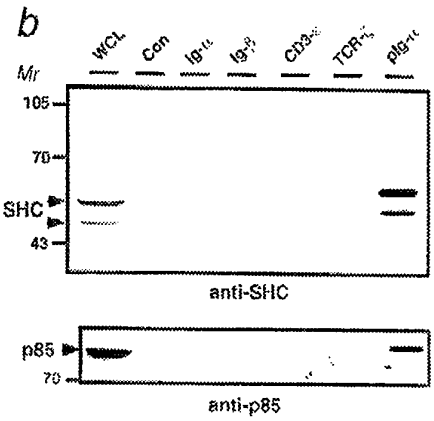

FIG. 21B

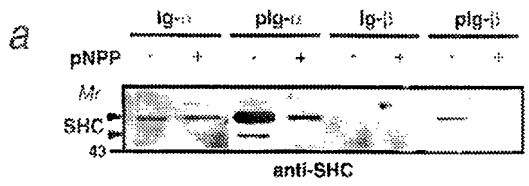

FIG. 22A

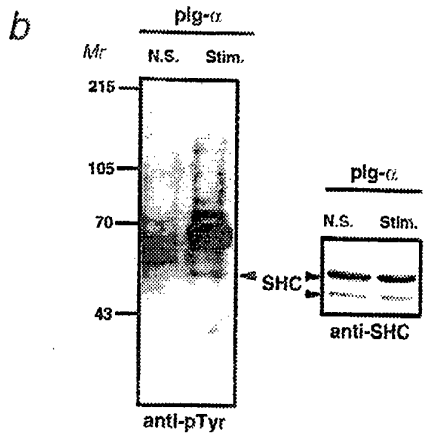

FIG. 22B

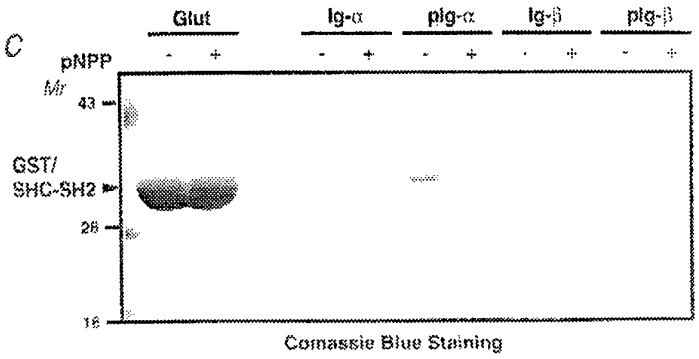
C
Glut
Ig-α
pig-α
Ig-β
pig-β
pNPP
Mr
43
GST/
SHC-SH2
28
16
Comassie Blue Staining

FIG. 22C

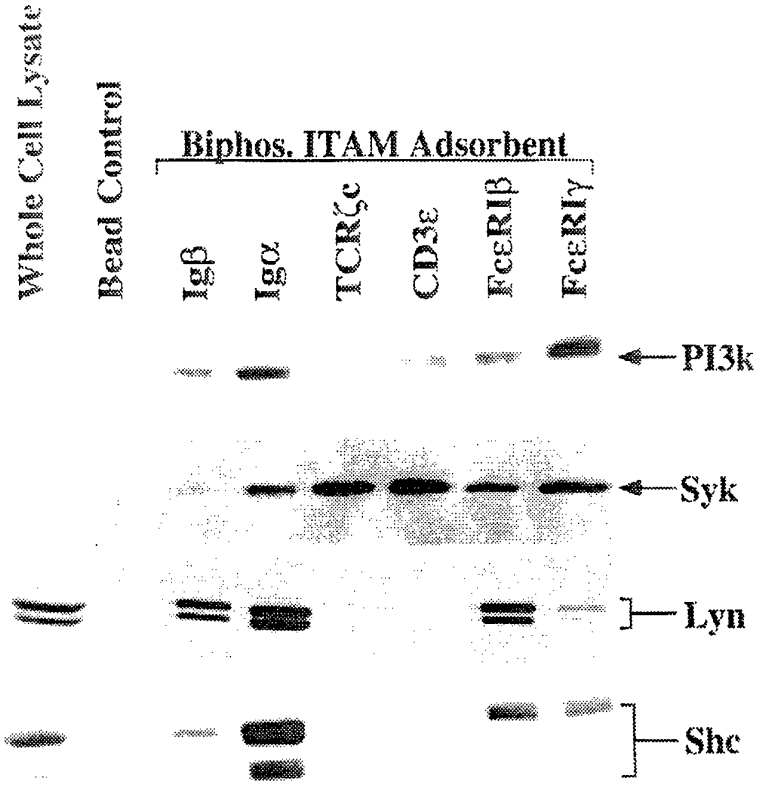
Whole Cell Lysate
Bead Control
Biphos. ITAM Adsorbent
Igβ
Igα
TCRζc
CD3ε
FcεRIβ
FcεRIγ
PI3k
Syk
Lyn
Shc

FIG. 23

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US95/03438

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :Please See Extra Sheet.
US CL :Please See Extra Sheet.
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : Please See Extra Sheet.

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Immunology Today, Volume 13, No. 2, issued 1992, Keegan et al., "Multichain immune recognition receptors: similarities in structure and signaling pathways", pages 63-68, see Figure 2.	1-8, 11
Y	Proceedings of the National Academy of Sciences, Volume 88, issued May 1991, Campbell et al., "IgM antigen receptor complex contains phosphoprotein product of B29 and mb-1 genes", pages 3982-3986, see Figure 4.	1-8, 11
Y	Science, Volume 258, issued 02 October 1992, Clark et al., "The B Cell Antigen Receptor Complex: Association of Ig-α and Ig-β with Distinct Cytolasmic Effectors", pages 123-126, see Figure 1.	1-8, 11

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 05 MAY 1995	Date of mailing of the international search report 07 JUN 1995
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer JAMES KETTER Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US95/03438

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Nature, Volume 338, issued 30 March 1989, Reth, "Antigen receptor tail clue", pages 383-384, see the figure.	1-8, 11
Y	Molecular and Cellular Biology, Volume 11, No. 2, issued February 1991, Escobedo et al., "A Phosphatidylinositol-3 Kinase Binds to Platelet-Derived Growth Factor Receptors through a Specific Receptor Sequence Containing Phosphotyrosine", pages 1125-1132, see page 1131.	9, 10, 12
Y	Science, Volume 257, issued 07 August 1992, Cicchetti et al., "Identification of a Protein that Binds to the SH3 Region of Abl and Is Similar to Bcr and GAP-rho", pages 803-806, see Figure 2.	9, 10, 12
Y	Cell, Volume 65, issued 05 April 1991, Escobedo et al., "cDNA Cloning of a Novel 85 kd Protein That Has SH2 Domains and Regulates Binding of PI3-Kinase to the PDGF β-Receptor", pages 75-82, see entire document.	9, 10, 12
Y	Cell, Volume 65, issued 05 April 1991, Skolnik et al., "Cloning of PI3 Kinase-Associated p85 Utilizing a Novel Method for Expression/Cloning of Target Proteins for Receptor Tyrosine Kinases", pages 83-90, see entire document.	9, 10, 12
Y	Cell, Volume 65, issued 05 April 1991, Otsu et al., "Characterization of Two 85 kd Proteins that Associate with Receptor Tyrosine Kinases, Middle-T/pp60c-src Complexes, and PI3-Kinase", pages 91-104, see entire document.	9, 10, 12
Y	Journal of Virology, Volume 66, No. 4, issued April 1992, Wages et al., "Mutations in the SH3 Domain of the src Oncogene Which Decrease Association of Phosphatidylinositol 3'-Kinase Activity with pp60v-src and Alter Cellular Morphology", pages 1866-1874, see entire document.	9, 10, 12
Y	Cell, Volume 69, issued 01 May 1992, Fantl et al., "Distinct Phosphotyrosines on a Growth factor Receptor Bind to Specific Molecules that Mediate Different Signaling Pathways", pages 413-423, see entire document.	9, 10, 12

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US95/03438

A. CLASSIFICATION OF SUBJECT MATTER:
IPC (6):

A61K 38/03, 38/10, 38/17, 38/43; C12N 5/06; C12Q 1/68

A. CLASSIFICATION OF SUBJECT MATTER:
US CL :

514/7; 435/15, 240.2

B. FIELDS SEARCHED
Minimum documentation searched
Classification System: U.S.

514/7; 435/15, 240.2

B. FIELDS SEARCHED
Electronic data bases consulted (Name of data base and where practicable terms used):

APS, MEDLINE