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(54) Title: CHIMERIC CHAINS FOR RECEPTOR-ASSOCIATED SIGNAL TRANSDUCTION PATHWAYS

(57) Abstract

Chimeric proteins and DNA sequences encoding chimeric proteins are provided, where the chimeric proteins are characterized by an extracellular domain capable of binding to a ligand, a transmembrane domain and a cytoplasmic domain capable of activating a signaling pathway. The extracellular domain and cytoplasmic domain are not naturally found together. Binding of ligand to the extracellular domain results in transduction of a signal and activation of a signaling pathway in the cell, whereby the cell may be induced to carry out various functions relating to the signaling pathway. A wide variety of extracellular domains may be employed as receptors, where such domains may be naturally occuring or synthetic.

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CHIMERIC CHAINS FOR RECEPTOR-ASSOCIATED SIGNAL TRANSDUCTION PATHWAYS

INTRODUCTION

Technical Field

The field of this invention is the use of chimeric surface membrane proteins for signal transduction.

Background

Regulation of cell activities is frequently achieved by the binding of the ligand to a surface membrane receptor. The formation of the complex with the extracellular portion of the receptor results in a change in conformation with the cytoplasmic portion of the receptor undergoing a change which results in a signal being transduced in the cell. In some instances, the change in the cytoplasmic portion results in binding to other proteins, where the other proteins are activated and may carry out various functions. In some situations, the cytoplasmic portion is autophosphorylated or phosphorylated, resulting in a change in its activity. These events are frequently coupled with secondary messengers, such as calcium, cyclic adenosine monophosphate, inositol phosphate, diacylglycerol, and the like. The binding of the ligand results in a particular signal being induced.

There are a number of instances, where one might wish to have a signal induced by virtue of employing a different ligand. For example, one might wish to activate

particular T-cells, where the T-cells will then be effective as cytotoxic agents, or activating agents by secretion of interleukins, colony stimulating factors or other cytokines, which results in the stimulation of another cell. The ability of the T-cell receptor to recognize antigen is restricted by the nature of the host MHC molecules. Thus, the use of a chimeric T-cell receptor in which a non-MHC restricted ligand binding domain is linked directly to the signal transducing domain of the T-cell receptor would permit the use of the resulting engineered effector T-cell in any individual, regardless of MHC genetic background. In this manner, one may change the ligand which initiates the desired response, where for some reason, the natural agent may not be as useful.

There is, therefore, interest in finding ways to modulate cellular responses in providing for the use of ligands other than the normal ligand to transduce a desired signal.

Relevant Literature

The T-cell antigen receptor has a non-covalent association between a heterodimer and the invariant chains of CD3 (Weiss and Imboden (1987) Adv. Immunol., 41:1-38; Cleavers et al. (1988) Ann. Rev. Immunol., 6:629-662; Frank et al. (1990) Sem. Immunol., 2:89-97). Description of the zeta chain may be found in Ashwell and Klausner (1990) Ann. Rev. Immunol., 8:139-167.

The phosphatidylinositol-specific phospholipase C initiated activation by the T-cell receptor ("TCR") is described by Weiss et al. (1984) Proc. Natl. Acad. Sci. USA, 81:416-4173; and Imboden and Stobo (1985) J. Exp. Med., 161:446-456. TCR also activates a tyrosine kinase (Samelson et al. (1986) Cell, 46:1083-1090; Patel et al. (1987) J. Biol. Chem., 262:5831-5838; Chsi et al. (1989) J. Biol. Chem., 264:10836-10842, where the zeta chain is one of the substrates of the kinase pathway (Baniyash et al. (1988) J. Biol. Chem., 263:18225-18230; Samelson et al. (1986), supra). Fyn, a member of the src family of tyrosine kinases, is reported to coprecipitate with the CD3 complex, making it an excellent candidate for a TCR-activated kinase (Samelson et al. (1990) Proc. Natl. Acad. Sci. USA, 87:4358-4362).

The nature of the zeta chain in the TCR complex is described by Baniyash et al. (1988) J. Biol. Chem., 263:9874-9878 and Orloff et al. (1989) ibid., 264:14812-14817. The heterodimeric zeta and eta protein is described by Jin et al. (1990) Proc. Natl. Acad. Sci. USA, 87:3319-3323. Discussion of the homo- and heterodimers may be found in Mercep et al. (1988) Science, 242:571-574; and Mercep et al. (1989) ibid., 246:1162-1165. See also Sussman et al. (1988) Cell, 52:85-95. For studies of the role of the zeta protein, see Weissman et al. (1989) EMBO, J., 8:3651-3656; Frank et al. (1990) Science, 249:174-177; and Lanier et al. (1989) Nature, 342:803-805.

For discussion of the gamma subunit of the Fc_ϵ R1 receptor, expressed on mast cells and basophils and its homology to the zeta chain, see Bevan and Cunha-Melo (1988) Prog. Allergy, 42:123-184; Kinet (1989) Cell, 57:351-354; Benhamou et al., Proc. Natl. Acad. Sci. USA, 87:5327-5330; and Orloff et al. (1990) Nature, 347:189-191.

SUMMARY OF THE INVENTION

Intracellular secondary messenger activation is achieved by employing chimeric proteins having a cytoplasmic region associated with transduction of a signal and activation of a secondary messenger system, frequently involving a kinase, and an extracellular region capable of binding to a specific ligand and transmitting to the cytoplasmic region a signal upon the formation of a binding complex. Particularly, cytoplasmic sequences of the zeta and eta chains of TCR and the gamma chain of Fc_ϵ R1 are employed joined to other than the natural extracellular region by a trans-membrane domain. In this manner, cells capable of expressing the chimeric protein can be activated by contact with the ligand, as contrasted with the normal mode of activation for the cytoplasmic portion.

DESCRIPTION OF THE SPECIFIC EMBODIMENTS

Novel DNA sequences, such DNA sequences as parts of expression cassettes and vectors, as well as their

presence in cells are provided, where the novel sequences comprise three domains which do not naturally exist together: (1) a cytoplasmic domain, which normally transduces a signal resulting in activation of a messenger system, (2) a transmembrane domain, which crosses the outer cellular membrane, and (3) an extracellular receptor domain which serves to bind to a ligand and transmit a signal to the cytoplasmic domain, resulting in activation of the messenger system.

The cytoplasmic domain may be derived from a protein which is known to activate various messenger systems, normally excluding the G proteins. The protein from which the cytoplasmic domain is derived need not have ligand binding capability by itself, it being sufficient that such protein may associate with another protein providing such capability. Cytoplasmic regions of interest include the zeta chain of the T-cell receptor, the eta chain, which differs from the zeta chain only in its most C-terminal exon as a result of alternative splicing of the zeta mRNA and the gamma subunit of the $Fc_{\epsilon}R1$ receptor, and such other cytoplasmic regions which are capable of transmitting a signal as a result of interacting with other proteins capable of binding to a ligand.

A number of cytoplasmic regions or functional fragments or mutants thereof may be employed, generally ranging from about 50 to 500 amino acids, where the entire naturally occurring cytoplasmic region may be employed or only an active portion thereof. The cytoplasmic regions

of particular interest are those which may be involved with one or more secondary messenger pathways, particular pathways involved with a protein kinase, more particularly, protein kinase C (PKC).

Pathways of interest include the phosphatidylinositol-specific phospholipase involved pathway, which is normally involved with hydrolysis of phosphatidylinositol-4,5-bisphosphate, which results in production of the secondary messengers inositol-1,4,5-trisphosphate and diacylglycerol. Another pathway is the calcium mediated pathway, which may be as a result of direct or indirect activation by the cytoplasmic portion of the chimeric protein. Also, by itself or in combination with another pathway, the kinase pathway may be involved, which may involve phosphorylation of the cytoplasmic portion of the chimeric protein. One or more amino acid side chains, particularly tyrosines, may be phosphorylated. There is some evidence that fyn, a member of the src family of tyrosine kinases, may be involved with the zeta chain.

While usually the entire cytoplasmic region will be employed, in many cases, it will not be necessary to use the entire chain. To the extent that a truncated portion may find use, such truncated portion may be used in place of the intact chain.

The transmembrane domain may be the domain of the protein contributing the cytoplasmic portion, the domain of the protein contributing the extracellular portion, or

a domain associated with a totally different protein. For the most part, it will be convenient to have the transmembrane domain naturally associated with one or the other of the other domains, particularly the extracellular domain. In some cases it will be desirable to employ the transmembrane domain of the zeta, eta or $Fc_{\epsilon}R1$ gamma chains which contain a cysteine residue capable of disulphide bonding, so that the resulting chimeric protein will be able to form disulphide linked dimers with itself, or with unmodified versions of the zeta, eta, gamma or related proteins. In some instances, the transmembrane domain will be selected to avoid binding of such domain to the transmembrane domain of the same or different surface membrane protein.

The extracellular domain may be obtained from any of the wide variety of extracellular domains or secreted proteins associated with ligand binding and/or signal transduction. The extracellular domain may be part of a protein which is monomeric, homodimeric, heterodimeric, or associated with a larger number of proteins in a non-covalent complex. In particular, the extracellular domain may consist of an Ig heavy chain which may in turn be covalently associated with Ig light chain by virtue of the presence of CH1 and hinge regions, or may become covalently associated with other Ig heavy/light chain complexes by virtue of the presence of hinge, CH2 and CH3 domains. In the latter case, the heavy/light chain complex that becomes joined to the chimeric construct may

constitute an antibody with a specificity distinct from the antibody specificity of the chimeric construct. Depending on the function of the antibody, the desired structure and the signal transduction the entire chain may be used or a truncated chain may be used, where all or a part of the CH1, CH2, or CH3 domains may be removed or all or part of the hinge region may be removed. Various naturally occurring receptors may also be employed, where the receptors are associated with surface membrane proteins, such as CD4, CD8-alpha, or cytokine or hormone receptors. The receptor may be responsive to a natural ligand, an antibody or fragment thereof, a synthetic molecule, e.g., drug, or any other agent which is capable of inducing a signal.

Where a receptor is a molecular complex of proteins, where only one chain has the major role of binding to the ligand, it will usually be desirable to use solely the extracellular portion of the ligand binding protein. Where the extracellular portion may complex with other extracellular portions of other proteins or form covalent bonding through disulfide linkages, one may also provide for the formation of such dimeric extracellular region. Also, where the entire extracellular region is not required, truncated portions thereof may be employed, where such truncated portion is functional. In particular, when the extracellular region of CD4 is employed, one may use only those sequences required for binding of gp120, the HIV envelope glycoprotein. In the

case in which Ig is used as the extracellular region, one may simply use the antigen binding regions of the antibody molecule and dispense with the constant regions of the molecule (for example, the Fc region consisting of the CH2 and CH3 domains). The light and heavy regions may be fused together to form a variable region.

In some instances, a few amino acids at the joining region of the natural protein may be deleted, usually not more than 10, more usually not more than 5. Also, one may wish to introduce a small number of amino acids at the borders, usually not more than 10, more usually not more than 5. The deletion or insertion of amino acids will usually be as a result of the needs of the construction, providing for convenient restriction sites, ease of manipulation, improvement in levels of expression, or the like. In addition, one may wish to substitute one or more amino acids with a different amino acid for similar reasons, usually not substituting more than about five amino acids in any one domain. The cytoplasmic domain as already indicated will generally be from about 50 to 500 amino acids, depending upon the particular domain employed. The transmembrane domain will generally have from about 25 to 50 amino acids, while the extracellular domain will generally have from about 50 to 500 amino acids.

Normally, the signal sequence at the 5' terminus of the open reading frame (ORF) which directs the chimeric protein to the surface membrane will be the signal

sequence of the extracellular domain. However, in some instances, one may wish to exchange this sequence for a different signal sequence. However, since the signal sequence will be removed from the protein, being processed while being directed to the surface membrane, the particular signal sequence will normally not be critical to the subject invention. Similarly, associated with the signal sequence will be a naturally occurring cleavage site, which will also normally be the naturally occurring cleavage site associated with the signal sequence or the extracellular domain.

The chimeric construct, which encodes the chimeric protein according to this invention will be prepared in conventional ways. Since, for the most part, natural sequences may be employed, the natural genes may be isolated and manipulated, as appropriate, so as to allow for the proper joining of the various domains. Thus, one may prepare the truncated portion of the sequence by employing the polymerase chain reaction (PCR), using appropriate primers which result in deletion of the undesired portions of the gene. Alternatively, one may use primer repair, where the sequence of interest may be cloned in an appropriate host. In either case, primers may be employed which result in termini, which allow for annealing of the sequences to result in the desired open reading frame encoding the chimeric protein. Thus, the sequences may be selected to provide for restriction sites which are blunt-ended, or have complementary overlaps.

During ligation, it is desirable that hybridization and ligation does not recreate either of the original restriction sites.

If desired, the extracellular domain may also include the transcriptional initiation region, which will allow for expression in the target host. Alternatively, one may wish to provide for a different transcriptional initiation region, which may allow for constitutive or inducible expression, depending upon the target host, the purpose for the introduction of the subject chimeric protein into such host, the level of expression desired, the nature of the target host, and the like. Thus, one may provide for expression upon differentiation or maturation of the target host, activation of the target host, or the like.

A wide variety of promoters have been described in the literature, which are constitutive or inducible, where induction may be associated with a specific cell type or a specific level of maturation. Alternatively, a number of viral promoters are known which may also find use. Promoters of interest include the β-actin promoter, SV40 early and late promoters, immunoglobulin promoter, human cytomegalovirus promoter, and the Friend spleen focus-forming virus promoter. The promoters may or may not be associated with enhancers, where the enhancers may be naturally associated with the particular promoter or associated with a different promoter.

The sequence of the open reading frame may be obtained from genomic DNA, cDNA, or be synthesized, or

combinations thereof. Depending upon the size of the genomic DNA and the number of introns, one may wish to use cDNA or a combination thereof. In many instances, it is found that introns stabilize the mRNA. Also, one may provide for non-coding regions which stabilize the mRNA.

A termination region will be provided 3' to the cytoplasmic domain, where the termination region may be naturally associated with the cytoplasmic domain or may be derived from a different source. For the most part, the termination regions are not critical and a wide variety of termination regions may be employed without adversely affecting expression.

The various manipulations may be carried out in vitro or may be introduced into vectors for cloning in an appropriate host, e.g., E. coli. Thus, after each manipulation, the resulting construct from joining of the DNA sequences may be cloned, the vector isolated, and the sequence screened to insure that the sequence encodes the desired chimeric protein. The sequence may be screened by restriction analysis, sequencing, or the like. Prior to cloning, the sequence may be amplified using PCR and appropriate primers, so as to provide for an ample supply of the desired open reading frame, while reducing the amount of contaminating DNA fragments which may have substantial homology to the portions of the entire open reading frame.

The target cell may be transformed with the chimeric construct in any convenient manner. Techniques include

calcium phosphate precipitated DNA transformation, electroporation, protoplast fusion, biolistics, using DNA-coated particles, transfection, and infection, where the chimeric construct is introduced into an appropriate virus, particularly a non-replicative form of the virus, or the like.

Once the target host has been transformed, usually, integration, will result. However, by appropriate choice of vectors, one may provide for episomal maintenance. A large number of vectors are known which are based on viruses, where the copy number of the virus maintained in the cell is low enough to maintain the viability of the cell. Illustrative vectors include SV40, EBV and BPV.

The constructs will be designed so as to avoid their interaction with other surface membrane proteins native to the target host. Thus, for the most part, one will avoid the chimeric protein binding to other proteins present in the surface membrane. In order to achieve this, one may select for a transmembrane domain which is known not to bind to other transmembrane domains, one may modify specific amino acids, e.g. substitute for a cysteine, or the like.

Once one has established that the transformed host is capable of expressing the chimeric protein as a surface membrane protein in accordance with the desired regulation and at a desired level, one may then determine whether the transmembrane protein is functional in the host to provide for the desired signal induction. Since the effect of

signal induction of the particular cytoplasmic domain will be known, one may use the established methodology for determining induction to verify the functional capability of the chimeric protein. For example, TCR binding results in the induction of CD69 expression. Thus, one would expect with a chimeric protein having a zeta cytoplasmic domain, where the host cell is known to express CD69 upon activation, one could contact the transformed cell with the prescribed ligand and then assay for expression of CD69. Of course, it is important to know that ancillary signals are not required from other proteins in conjunction with the particular cytoplasmic domain, so that the failure to provide transduction of the signal may be attributed solely to the inoperability of the chimeric protein in the particular target host.

A wide variety of target hosts may be employed, normally cells from vertebrates, more particularly, mammals, desirably domestic animals or primates, particularly humans. The subject chimeric constructs may be used for the investigation of particular pathways controlled by signal transduction, for initiating cellular responses employing different ligands, for example, for inducing activation of a particular subset of lymphocytes, where the lymphocytes may be activated by particular surface markers of cells, such as neoplastic cells, virally infected cells, or other diseased cells, which provide for specific surface membrane proteins which may be distinguished from the surface membrane proteins on

normal cells. The cells may be further modified so that expression cassettes may be introduced, where activation of the transformed cell will result in secretion of a particular product. In this manner, one may provide for directed delivery of specific agents, such as interferons, TNF's, perforans, naturally occurring cytotoxic agents, or the like, where the level of secretion can be greatly enhanced over the natural occurring secretion. Furthermore, the cells may be specifically directed to the site using injection, catheters, or the like, so as to provide for localization of the response.

The subject invention may find application with cytotoxic lymphocytes (CTL), natural killer cells (NK), tumor-infiltrating-lymphocytes (TIL), neutrophils, basophils, T-helper cells, or other cells which are capable of killing target cells or are capable of secreting cytokines when activated. Thus, diseased cells, such as cells infected with HIV, HTLV-I or II, cytomegalovirus, hepatitis B or C virus, myobacterium avium, etc., or neoplastic cells, where the diseased cells have a surface marker associated with the diseased state may be made specific targets of the cytotoxic cells or the cytokine secreting cells. Alternatively, particular MHC proteins complexed with peptide ligands may be the targets recognized by the chimeric T-cell receptors described herein. By providing a receptor extracellular domain, e.g., CD4, which binds to a surface marker of the pathogen or neoplastic condition, e.g., gp120 for HIV, the cells

may serve as therapeutic agents. By modifying the cells further to prevent the expression or translocation of functional Class I and/or II MHC antigens, the cells will be able to avoid recognition by the host immune system as foreign and can therefore be therapeutically employed in any individual regardless of genetic background. Alternatively, one may isolate and transfect host cells with the subject constructs and then return the transfected host cells to the host.

Other applications include transformation of host cells from a given individual with retroviral vector constructs directing the synthesis of the chimeric construct. By transformation of such cells and reintroduction into the patient one may achieve autologous gene therapy applications.

For the antibody receptor, ligands of interest may include viral proteins, for example the gB envelope glycoprotein of human cytomegalovirus, and surface proteins found on cancer cells in a specific or amplified fashion, for example the HER-2 protein which is often amplified in human breast and ovarian carcinomas. For other receptors, the receptors and ligands of particular interest are CD4, where the ligand is the HIV gp120 envelope glycoprotein, and other viral receptors, for example ICAM, which is the receptor for the human rhinovirus, and the related receptor molecule for poliovirus.

The following examples are by way of illustration and not by way of limitation.

EXPERIMENTAL

Materials and Methods:

CD8/ζ chimera construction

The polymerase chain reaction, PCR (Mullis et al. (1986) Cold Spring Harbor Symposium on Quantitative Biology, LI, 263-273) was used to amplify the extracellular and transmembrane portion of CD8α (residues 1-187) from pSV7d-CD8α and the cytoplasmic portion of the human ζ chain (residues 31-142 from pGEM3zζ. DNA sequences are from (Littman et al. (1985) Cell 40:237-246; CD8), and (Weissman et al. (1988) Proc. Natl. Acad. Sci. USA, 85:9709-9713; ζ). Plasmids pSV7d-CD8α and pGEM3zζ were kindly provided by Drs. Dan Littman and Julie Turner (Univ. of CA, S.F.) and Drs. R.D. Klausner and A.M. Weissman (N.I.H.), respectively. Primers encoding the 3' sequences of the CD8 fragment and the 5' sequences of the zeta fragment were designed to overlap such that annealing of the two products yielded a hybrid template. From this template the chimera was amplified using external primers containing XbaI and BamHI cloning sites. The CD8/ζ chimera was subcloned into pTfneo (Ohashi et al. (1985) Nature, 316:606-609) and sequenced via the Sanger dideoxynucleotide technique (Sanger et al. (1977) Proc. Natl. Acad. Sci. USA, 74:5463-5467).

Antibodies

C305 and Leu4 mAbs recognize the Jurkat Ti β chain and an extracellular determinant of CD3 ε, respectively. OKT8, acquired from the ATCC, recognizes an extracellular epitope of CD8. The anti-ζ rabbit antiserum, #387, raised against a peptide comprising amino acids 132-144 of the murine ζ sequence (Orloff et al. (1989) J. Biol. Chem., 264:14812-14817), was kindly provided by Drs. R.D. Klausner, A.M. Weissman and L.E. Samelson. The anti-phosphotyrosine mAb, 4G10, was a generous gift of Drs. D. Morrison, B. Druker, and T. Roberts. W6/32 recognizes an invariant determinant expressed on human HLA Class 1 antigens. Leu23, reactive with CD69, was obtained from Becton-Dickinson Monoclonal Center (Milpitas, Ca.). MOPC 195, an IgG2a, (Litton Bionetics, Kensington, MD) was used as a control mAb in FACS analysis. Ascitic fluids of mAb were used at a final dilution of 1:1000 (a saturating concentra-tion) in all experiments unless otherwise stated.

Cell lines and Transfections

The human leukemic T cell line Jurkat and its derivative J.RT3-T3.5 were maintained in RPMI 1640 supplemented with 10% fetal bovine serum (FBS) glutamine, penicillin and streptomycin (Irvin Scientific). Chimera-transfected clones were passaged in the above medium with the addition of Geneticin (GIBCO, Grand Island, NY) at 2 mg/ml. Electroporation of pTfneo-CD8/ζ into Jurkat and

J.RT3-T3.5 was performed in a Bio-Rad Gene Pulser using a voltage of 250V and a capacitance of 960μF with 20μg of plasmid per 10^7 cells. After transfection, cells were grown for two days in RPMI before plating out in Geneticin-containing medium. Clones were obtained by limiting dilutions and screened for TCR and CD8/ζ expression by Flow Cytometry (see below). The Jurkat CD8 clone, transfected with the wild-type CD8 protein, was kindly provided by Drs. Julia Turner and Dan Littman.

Flow Cytometry

Approximately 1 x 10^6 cells/condition were stained with saturating concentrations of antibody, then incubated with fluorescein-conjugated goat anti-mouse Ab prior to analysis in a FACScan (Beckton Dickinson) as previously described (Weiss and Stobo 1984). Cells analyzed for CD69 expression were stained directly with fluorescein-conjugated Leu 23 (anti-CD69 mAb) or MOPC 195 (control mAb).

$[Ca^{+2}]_i$ Measurement by Fluorimetry

Calcium sensitive fluorescence was monitored as previously described (Goldsmith and Weiss 1987 Proc. Natl. Acad. Sci. USA, 84:6879-6883). Cells were stimulated with soluble mAb C305 and OKT8 at saturating concentrations (1:1000 dilution of ascities). Maximal fluorescence was determined after lysis of the cells with Triton X-100; minimum fluorescence was obtained after chelation of Ca^{+2}

with EGTA. $[Ca^{+2}]_i$ was determined using the equation $[Ca^{+2}]_i=K_d$ s $(F_{observed} -F_{Min})/(F_{max} -F_{observed})$, with K_d=250 nM as described (Grynkiewica et al. (1985) J. Biol. Chem., 260:3440-3448).

Inositol Phosphate Measurement

Cells were loaded with [^{3}H]myo-inositol (Amersham) at 40 μCi/ml for 3 hr. in phosphate buffered saline, then cultured overnight in RPMI 1640 supplemented with 10% fetal bovine serum. Cells were stimulated for 15 min. with the indicated antibodies at 1:1000 dilution of ascities in the presence of 10 mM LiCl to inhibit dephosphorylation of IP_1. The extraction and quantitation of soluble inositol phosphates were as described (Imboden and Stobo (1985) J. Exp. Med., 161:446-456).

Surface Iodinations

Cells were labeled with ^{125}I using the lactoperoxidase/glucose oxidase (Sigma) procedure as described (Weiss and Stobo (1984) J. Exp. Med., 160:1284-1299).

Immunoprecipitations

Cells were lysed at 2 x 10^7 cells/200 ml in 1% NP40 (Nonidet P40), 150 mM NaCl, and 10 mM Tris pH 7.8 in the presence of protease inhibitors, 1mM PMSF, aprotinin, and leupeptin. Lysis buffer for lysates to be analyzed for phosphotyrosine content was supplemented with phosphatase

inhibitors as described (Desai et al. 1990, Nature, VOL. 348:66-69). Iodinated lysates were supplemented with 10 mM iodoacetamide to prevent post-lysis disulfide bond formation. Digitonin lysis was performed in 1% Digitonin, 150 mM NaCl, 10mM Tris pH 7.8, 0.12% Triton X-100. After 30 min. at 4°C, lysates were centrifuged for 10 min. at 14,000 rpm., then precleared with fixed Staphylococcus aureus (Staph A; Calbiochem-Behring). Alternatively, lysates of cells stimulated with antibody prior to lysis were precleared with sepharose beads. The precleared lysates were incubated with Protein A Sepharose CL-4B beads which had been prearmed with the immuno-precipitating antibody. Washed immunoprecipitates were resuspended in SDS sample buffer +/- 5% ß-mercaptoethanol and boiled prior to electrophoresis on 11% polyacrylamide gels.

Stimulation of cells for assessment of phosphotyrosine content.

Cells were stimulated in serum free medium at 2 x 10^7 cells/ 200 µl with antibodies at 1:250 dilution of ascities. After 2 min. at 37°C, the medium was aspirated, and the cells lysed in 100 µl of NP40 lysis buffer. Lysates were precleared, then ultracentrifuged and samples resolved by SDS PAGE.

Immunoblots

Gels were equilibrated in transfer buffer (20 mM Tris-base, 150 mM glycine, 20% methanol) for 30 min. and transferred to nitrocellulose membranes in a Bio-Rad Western blotting apparatus run at 25 volts overnight. Membranes were blocked in TBST (10 mM Tris HCl [pH 8], 150 mM NaCl, 0.05% Tween 20) plus 1.5% ovalbumin, then incubated with either mAb 4G10 or rabbit anti-ζ antiserum (#387). The immunoblots were washed and incubated with a 1:7000 dilution of alkaline phosphatase-conjugated goat anti-mouse or goat anti-rabbit antibody. After 1-2 hours, the blots were washed and developed with nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate substrates as per manufacturer's instructions (Promega).

IL-2 Bioassay

For stimulation, cells were coated with the indicated antibodies at saturating concentrations (1:1000 dil. of ascites) for 30 min. at 4°C. After removal of unbound antibody, cells were spun onto 24-well tissue culture plates which had been precoated with rabbit anti-mouse Ig (Zymed Labs) and blocked with medium plus 10% FBS. Phorbol myristate acetate, PMA (Sigma) and ionomycin (Calbiochem) were added to final concentrations of 10 mg/ml and 1 mM, respectively. Cell-free supernatants were harvested after 20 hr. of culture and assessed for IL-2 content utilizing the IL-2 dependent CTLL-2.20 cell line

in the MTT colorimetric assay as described (Mosmann 1983, J. Immunol. Meth., 65:55-63.

RESULTS

Characterization of the CD8/ζ chimera in T cell receptor-positive and -negative Jurkat cells

The CD8/ζ chimeric construct described previously was transfected via electroporation into both the Jurkat human T cell leukemic line, yielding clone JCD8/ζ 2, and a Jurkat-derived mutant, JRT3.T3.5 deficient in full length Ti ß chain transcripts and protein, yielding Jß-CD8/ζ 14. Though JRT3.T3.5 expresses normal levels of Ti α and the CD3 subunits, its deficiency in Ti ß expression results in the absence of TCR expression on the cell surface (Ohashi et al. (1985) Nature, 316:606-609). Transfection of the chimera into this cell enabled assessment of ζ's signalling phenotype without the complication of the additional TCR chains. Levels of surface expression of the chimera and TCR in stably transfected clones were quantitated by flow cytometry using mAbs which recognize either CD8 (OKT8) or the CD3 ε subunit of the TCR (Leu 4). Fluorescence histograms of these clones which both express high levels of CD8/ζ was observed; this cell was used as a control in all of the experiments. The three clones express comparable levels of CD8 epitopes and T cell receptors with the exception of Jß-CD8/z 14, which fails to express surface TCR. Thus the CD8/ζ chimera can be

expressed on the cell surface in the absence of the TCR chains.

To characterize the structure of the CD8/ζ chimeric protein, cells were surface radioiodinated, lysed in 1% NP40, and subjected to immunoprecipitation with OKT8 or a normal rabbit antiserum raised against a cytoplasmic peptide sequence of murine ζ. Under reducing conditions, antibodies against either CD8 or ζ precipitate a single protein of 34-35kD from the chimera-transfected cell, while OKT8 precipitates a 29kD protein representing wild-type CD8 from Jurkat CD8. Although CD8 in its normal environment has an apparent molecular weight of 32-34kD, (Snow and Terhorst (1983), J. Biol. Chem., 258:14675-14681, preliminary experiments comparing CD8 in Jurkat and a CD8-positive line, HPB.ALL, suggest that the reduction in size of CD8 observed here results from a distinct pattern of glycosylation in the Jurkat host. Under non-reducing conditions a more complex pattern of proteins is seen in immunoprecipitates of both CD8 and the CD8/ζ chimera. This complexity is characteristic of CD8 precipitates since homomultimers and heteromultimers have been previously observed (Snow and Terhorst (1983), supra). The two prominent species immunoprecipitated from JCD8/ζ 2 migrating at approximately 70 and 100kD are likely to represent homodimers and homotrimers of the chimera. As there are no cysteine residues for the formation of disulfide linkages with the ζ portion of the chimera, any disulfide bonds formed in the chimera must occur through

CD8. Therefore, any protein forming a heterodimer with CD8/ζ is likely to form one with the wild-type CD8 and thus should not account for any signalling events specifically attributable to the CD8/ζ chimera.

Non-covalent association of the chimera with endogenous CD3 γ, δ, and ε may complicate the interpretation of signals transduced by the chimera. To determine whether removal of the extracellular and transmembrane domains of ζ is sufficient to result in its expression independent of the CD3 chains, cells were surface iodinated and lysed in digitonin, a detergent known to preserve the integrity of the TCR complex. Immunoprecipitates of the TCR in both Jurkat CD8 and the TCR-expressing chimera-transfectant JCD8/ζ2, show identical patterns characteristic of a CD3 (Leu 4) immunoprecipitate. Though TCR-associated ζ is not well iodinated, as its extracellular domain contains no tyrosine residues for labelling, ζ immunoblots of CD3 immunoprecipitates confirm its presence under these lysis conditions. A small quantity of labelled CD3 ε is seen in the Leu 4 immunoprecipitate of the TCR deficient cell despite the fact that this same mAb failed to stain this cell. The small amount of immunoprecipitated protein seen is likely due to radiolabelling of internal CD3 ε in a small number of permeabilized or non-viable cells during the labelling procedure. More importantly, no CD3 chains are detectable in precipitates of the CD8/ζ chimera in either TCR-position or -negative cells, nor is any chimera

apparent in the Leu 4 precipitate of JCD8/ζ 2. Intentional overexposure of the autoradiogram also fails to reveal TCR chains coprecipitating with the chimers. To further address the question of co-association of the chimera and TCR chains, the effect of antibody-induced down modulation of the TCR on chimera expression was assessed. Whereas overnight incubation of JCD8/ζ 2 with saturating amounts of C305, a mAb against an epitope of the Jurkat Ti ß chain, resulted in internalization of 94% of the TCR, surface expression of the CD8/ζ chimera was unaffected. By these two independent criteria, no discernible association exists between CD8/ζ and the CD3 γ, δ, and ε chains.

To determine whether a covalent link exists between endogenous ζ and the CD8/ζ chimera, ζ immunoblot analysis was performed comparing ζ and OKT 8 immunoprecipitates in Jurkat CD8 and JCD8/ζ 2. The anti-ζ antiserum immunoprecipitates both the chimera and ζ from JCD8/ζ 2, but only endogenous ζ from the Jurkat CD8 control. In contrast to the anti-ζ antiserum, OKT8 immunoprecipitates the chimera but not ζ in JCD8/ζ 2, while neither species is detected in Jurkat CD8. Collectively, the results from these experiments and those described above, argue against an interaction between the chimera and endogenous T cell receptor subunits.

Stimulation of CD8/ζ results in activation of the phosphatidylinositol and tyrosine kinase pathways

To determine whether binding of the extracellular domain of CD8/ζ would result in intracellular signalling events, the ability of OKT8 to elicit an increase in cytoplasmic free calcium ($[Ca^{+2}]_i$) in chimera-transfected cells was examined. A fluorimetry tracing obtained with JCD8/ζ 2 upon stimulation of its TCE with the anti-Ti ß monoclonal antibody C305 was obtained. With the addition of soluble OKT8, a substantial increase in calcium ($[Ca^{+2}]_i$) is seen, suggesting that the cytoplasmic domain of ζ is capable of coupling to signalling machinery which results in the activation of phospholipase C. The ability of the chimera to transduce a signal in cells lacking surface expression of the TCE chains was examined next. Stimulation of the TCR-negative Jß-CD8/ζ 14 with C305 results in no detectable increase in $[Ca^{+2}]_i$; however, OKT8 is still able to elicit a strong calcium response. The lack of significant increase in $[Ca^{+2}]_i$ with OKT8 stimulation in Jurkat CD8 demonstrates that the ζ portion of the chimera is required for the elicited $[Ca^{+2}]_i$ response.

Since the increase in $[Ca^{+2}]_i$ which occurs with TCR stimulation is attributed to increases in inositol phosphates, the ability of CD8/ζ to induce PIP_2 hydrolysis was tested by assessing changes in total soluble inositol phosphates following stimulation with OKT8. Stimulation of CD8/ζ with OKT8 resulted in the generation of inositol

phosphates in both chimera-expressing cells. In contrast, no inositol phosphates were noted with stimulation of the wild-type CD8 protein in Jurkat CD8. Stimulation of TCR in Jurkat CD8 and CD8/ζ2 induced increases in inositol phosphates, whereas in the TCR-deficient transfectant, Jß-CD8/ζ14, no such increase was observed upon TCR stimulation. These results are consistent with the calcium fluorimetry data and confirm the chimera's ability to activate phospholipase C even in the absence of endogenous cell surface TCR chains.

As stimulation of the T cell receptor activates a tyrosine kinase pathway in addition to inositol phospholipid pathway, it was important to determine whether chimera stimulation would result in tyrosine kinase activation. Western blots reveal a small number of tyrosine-phosphorylated proteins existing in all three clones prior to stimulation. Upon stimulation of Jurkat CD8 and JCD8/ζ2 with C305, (anti-Ti ß), the tyrosine kinase pathway is activated as demonstrated by the induction of tyrosine phosphorylation of a number of proteins. As expected, C305 has no effect in the TCR-negative transfectant, Jß-CD8/ζ14. Stimulation of the chimera on both JCD8/ζ2 nd Jß-CD8/ζ14 with OKT8 results in the appearance of a pattern of tyrosine-phosphorylated bands indistinguishable from that seen with TCR stimulation. In contrast, stimulation through wild-type CD8 in Jurkat does not result in induction of tyrosine phosphoproteins. Thus, the CD8/ζ chimera, in the absence

of Ti and CD3 γ, δ, and ε, is capable of activating the tyrosine kinase pathway in a manner analogous to that of an intact TCR.

Since JCD8/ζ2 expresses two discernible forms of ζ on its surface, -endogenous ζ and the CD8/ζ chimera-, each of which could be stimulated independently, the specificity of receptor-induced ζ phosphorylation was addressed. Immunoprecipitates of ζ derived from the three clones, either unstimulated, or stimulated with C305 or OKT8, were analyzed by western blotting with an anti-phosphotyrosine antibody. A small fraction of the ζ immunoprecipitates were blotted with ζ antiserum to control for differences in protein content between samples. Analysis of the lysate derived from TCR-stimulated Jurkat CD8 cells reveals a typical pattern of ζ phosphorylation with the multiplicity of bands from 16-21kD most likely representing the varying degree of phosphorylation of the seven cytoplasmic tyrosine residues of ζ. In this experiment, a small degree of constitutive ζ phosphorylation is detected in Jurkat CD8; however, this is not augmented by stimulation of the wild-type CD8 protein. Whereas phosphorylation of ζ is seen with stimulation of the TCR in JCD8/ζ2 though weaker than that seen in C305-stimulated Jurkat CD8, no induced phosphorylation of the chimera is apparent. Conversely, stimulation of the CD8/ζ chimeric receptor on both JCD8/ζ2 and Jβ-CD7/ζ14 results in a high degree of phosphorylation of the chimera exclusively, seen as an induced broad band

from 34-39kD. This result indicates that the receptor-activated kinase responsible for phosphorylation of ζ recognizes its substrate only in a stimulated receptor complex.

Stimulation of CD8/ζ results in late events of T cell activation

T-cell activation results from the delivery of receptor-mediated signals to the nucleus where they act to induce expression of specific genes. One such gene encodes the activation antigen CD69, whose surface expression is induced within hours of T cell receptor stimulation and appears to be dependent on activation of protein kinase C (Testi et al., J. Immunol., 142:1854-1860. Although the function of CD69 in T cell activation is not well understood, it provides a marker of distal signal transduction events. Flow cytometry reveals a very small degree of basal CD69 expression on unstimulated cells. Maximal levels are induced on all cells with phobol myristate acetate, PMA, an activator of protein kinase. Stimulation of the TCR results in induction of CD69 on Jurkat CD8 and JCD8/ζ2, but not on the TCR-negative clone, Jβ-CD8/ζ14. Moreover, stimulation of cells with OKT8 induces CD69 on both cells expressing the CD8/ζ chimera. Though a minimal degree of CD69 induction is apparent with stimulation of wild-type CD8 protein, this level is no higher than that observed with

stimulation of Jurkat CD8 with a Class I MHC antibody w6/32.

Perhaps the most commonly used criterion to assess late activation events is the production of the lymphokine, interleukin-2 (IL-2) (Smith (1986) Science, 240:1169-1176). The IL-2 gene is tightly regulated, requiring the integration of a number of signals for its transcription, making it a valuable distal marker for assessing signalling through the CD8/ζ chimera. Stimulation of Jurkat CD8 and JCD8/ζ 2 cells with TCR antibodies in the presence of PMA results in production of IL-2.

JCD8/ζ 2 and Jurkat CD 8 cells were stimulated with the indicated mAb or inomycin (1μm) in the presence of PMA (10ng/ml). IL-2 secretion was determined by the ability of culture supernatants of stimulated cells to support the growth of the IL-2 dependent CTLL-2.20 cells. Since PMA alone induces no IL-2 production in Jurkat, yet has a small direct effect on the viability of the CTLL 2.20 cells, values obtained with PMA alone were subtracted from each response value, yielding the numbers shown above Data from two independent experiments are presented.

Table 1. Induction of IL-2 Production

Treatment	IL-2 (Units/ml)			
	Jurkat CD 8		JCD8/ζ 2	
	Experiment #		Experiment #	
	#1	#2	#1	#2
Unstimulated	<0.1	<0.1	<0.1	<0.1
C305+PMA	13.5	9.1	3.7	2.1
OKT8+PMA	<0.1	<0.1	6.8	7.0
C305+OKT8+PMA	--	--	--	--
W6/32+PMA	<0.1	<0.1	<0.1	<0.1
Ionomycin+PMA	30.4	4.2	24.2	24.6

Importantly, while treatment with OKT8 on Jurkat CD8 induces no IL-2, similar treatment of JCD8/ζ results in levels of secreted IL-2 consistently higher than those produced in that cell with TCR stimulation. Jß-CD8/ζ 14 responded more weakly to all experimental stimuli in this assay, but the data were qualitatively similar in that this cell reproducibly secreted IL-2 in response to OKT8 but not to C305. These data confirm that in addition to early signal transduction events, later activation events occur upon stimulation of the CD8/ζ chimera, thus demonstrating its ability to couple to the relevant signal transduction pathways in a physiologic manner.

CD4-ζ CHIMERAS

Construction of CD4-zeta Chimeras

Plasmid pGEM3zeta bears the human zeta cDNA and was kindly provided by Dr. R.D. Klausner and Dr. S.J. Frank. The plasmid pBS.L3T4 bears the human CD4 cDNA, and was kindly provided by Dr. D. Littman and Dr. N. Landau. A BamHI-ApaI restriction fragment (app. 0.64 kb)

encompassing the entire human zeta chain coding sequence from residue 7 of the extracellular (EXT) domain, was excised from pGEM3zeta, and subcloned into the BamHI and ApaI restriction sites of the polylinker of pBluescript II SK (+) (pSK is a phagemid-based cloning vector from Stratagene, CA), generating pSK.zeta. Subsequently, a BamHI restriction fragment encompassing the entire CD4 coding sequence (app. 1.8 kb) was excised from pBS.L3T4, and subcloned into the BamHI site of pSK.zeta, generating pSK.CD4.zeta.

Single-stranded DNA was prepared from pSK.CD4.zeta (Stratagene pBluescript II protocol), and used as a template for oligonucleotide-mediated directional mutagenesis (Zoller, M. and Smith, M., Nucleic Acids Res. [1982] 10:6487-6500) in order to generate CD4-zeta chimeras with the desired junctions shown in Fig. 1 (and described below). CD4-zeta fusions 1, 2, and 3 were subsequently sequenced via the Sanger dideoxynucleotide technique (Sanger, F., Nicklen, S., and Coulson, A.R., PNAS [1977] 74:5463-5467), excised as EcoRI-ApaI restriction fragments, and cloned into the polylinker of expression vector pIK.1.1 or pIK.1.1.Neo at identical sites.

An EcoRI-BamHI restriction fragment (app. 1.8 kb) encompassing the entire coding region of CD4 was excised from pSK.CD4.zeta, and subcloned between the EcoRI and BglII sites of the pIK.1.1 or pIK.1.1.Neo polylinker.

The plasmid pUCRNeoG (Hudziak, et al., Cell [1982] 31:137-146) carries the neomycin gene under the transcriptional control of the Rous Sarcoma virus (RSV) 3' LTR. The RSV-neo cassette was excised from PURCNeoG as a HincII restriction fragment (app. 2.3 kb), and subcloned between the two SspI sites of pIK.1.1, generating pIK.1.1.Neo.

pIK.1.1 is a mammalian expression vector constructed by four successive cassette insertions into pMF2, which is created by inserting the synthetic polylinker 5'-HindIII-SphI-EcoRI-AatII-BglI-XhoI-3' into KpnI and SacI sites of pSKII (Stratagene), with loss of the KpnI and SacI sites. First, a BamHI-XbaI fragment containing the SV40 T antigen polyadenylation site (nucleotides 2770-2533 of SV40, Reddy, et al., Science [1978] 200:494-502) and an NheI-SalI fragment containing the SV40 origin of replication (nucleotides 5725-5578 of SV40) are inserted by three-part ligation between the BglII and XhoI sites, with the loss of the BglII, BamHI, XbaI, NheI, SalI and XhoI sites. These BamHI-XbaI and NheI-SalI fragments are synthesized by PCR with pSV2Neo (Southern and Berg, J. Mol. Appl. Gen. [1982] 1:327-341) as the template using oligonucleotide primer pairs 5'-GGTCGACCTGGATCCGCCATACCAC-ATTTGTAG-3', 5'-GCCGCGGCTCTAGAGCCAGACATGATAAGATAC-3', 5'-AAGCTTGTGCTAGCTATCCCGCCCCTAACTCCG-3', and 5'-CGAATTCGG-TCGACCGCAAAAGCCTAGGCCTCC-3', respectively, which incorporated BamHI, XbaI, NheI and SalI sites at their respective ends. Second, an SphI-EcoRI fragment

containing the splice acceptor of the human α1 globin gene second exon (nucleotides +143 to +251) is inserted between the SphI and EcoRI sites. This SphI-EcoRI fragment is synthesized by PCR with pπSVαHP (Treisman et al., PNAS [1983] 80:7428-7432) as the template using oligonucleotide primer pairs 5'-GTCTATAGCATGCTCCCCTGCTCCGACCCG-3' and 5'-GGTACCGAATTCTCCTGCGGGGAGAAGCAG-3', which incorporated SphI and EcoRI sites at their respective ends. Third, the synthetic polylinker 5'-EcoRI-BglII-ApaI-AatII-3' is inserted between the EcoRI and the AatII sites. Fourth, a HindIII-SacI fragment containing the CMV IE enhancer/promoter (nucleotides -674 to -19, Boshart et al., Cell [1985] 41:521-530) and a SacI-SphI fragment containing the CMV IE first exon/splice donor (nucleotides -19 to +170) are inserted by three-part ligation between the HindIII and SphI sites. The HindIII-SacI fragment is prepared by PCR with pUCH.CMV (M. Calos, Stanford Univ.) as the template using oligonucleotide primers 5'-CGCCAAGC-TTGGCCATTGCATACGGT-3' and 5'-GAGGTCTAGACGGTTCACTAAACGAGC-TCT-3', which incorporated HindIII and SacI sites at their respective ends. The SacI-SphI fragment is chemically synthesized.

RESULTS

Design of CD4-zeta Chimeras

The human CD4 molecule is currently the only well characterized high affinity receptor for HIV, and binds to the gp120 moiety of the HIV envelope glycoprotein, gp160.

The mature CD4 molecule comprises an extracellular (EXT) domain (residues 1-371 of the mature CD4 protein) for four tandem Ig-like V regions (V1-V4), a 24 aa transmembrane (TM) domain (residues 372-395 of the mature CD4 protein), and a 38 aa cytoplasmic (CYT) domain (residues 396-433 of the mature CD4 protein). Mutational analysis has shown that the determinants for high affinity binding of gp120 lie within the first 106 aa of CD4, V1.

The mature zeta molecule comprises a 10 aa EXT domain, a 20 aa TM domain, and a 112 aa CYT domain.

Three CD4-zeta chimeric receptors (F1, F2 and F3) were constructed from the extracellular (EC) and cytoplasmic (CYT) domains of CD4 and zeta respectively. The transmembrane (TM) domains of these CD4-zeta receptors are derived from zeta (F1, F2) or CD4 (F3). F2 and F3 possess all four V domains.

Specifically:

F2 retains the CD4 EXT domain comprising all four V regions (residues 1-370 of the mature CD4 protein), the TM domain of the zeta chain (residues 8-30 of the mature zeta chain) and the CYT domain of zeta (residues 31-142 of the mature seta chain).

F3 retains the CD4 EXT domain comprising all four V domains (residues 1-371 of the mature CD4 protein), the TM domain of CD4 (residues 372-395 of the mature CD4 chain), and the CYT domain of zeta (residues 31-142 of the mature zeta chain).

F1 retains only the V1 and V2 of the CD4 EXT domain (residues 1-180 of the mature CD4 protein), the TM domain of zeta (residues 8-30 of the mature zeta chain) and the cytoplasmic (CYT) domain of zeta (residues 31-142 of the mature zeta chain).

Transient Expression of CD4-zeta Receptors

Chimeric receptors F1, F2, and F3, and the native CD4 gene were introduced into an expression vector pIK.1.1 which directs transcription via the CMV promoter/enhancer (see below for further description). In order to evaluate the structural integrity and cell surface levels of expression of these chimeric receptors, a highly efficient transient expression system was employed. Constructs were introduced by electroporation into the human embryonic kidney cell line, 293, cells were harvested 24 hours later, and subsequently analyzed by FACS employing a FITC-coupled MAb specific for the V1 domain of CD4, OKT4A. It is apparent from the cell sorting that although similarly high levels of surface F2 and F3 are detected by OKT4A, the level of F1 detected by this antibody in the same transient assay is extremely low.

In order to address whether F1 was present in the membrane, and to assess the structure of the chimeric proteins, immunoprecipitation of radiolabelled proteins was carried out. 20 hours after electroporation of 293 cells with either F1, F2 or F3, cells were pulse-labelled with ^{35}S-methionine for four hours, lysed in 1% NP40, and

subjected to immunoprecipitation by either OKT4A (Ortho) or a rabbit antiserum raised against a cytoplasmic peptide of murine zeta (obtained from R. Klausner, NIH). The level of radiolabelled F1 relative to either F2 or F3 is significantly higher when antizeta antiserum instead of OKT4A is used as the immunoprecipitation agent. These results suggest that the F1 receptor may not present the necessary topology for efficient binding of V1-specific MAbs.

F1 and F2 Form Disulfide-Linked Homodimers; F3 is a Monomer

Native zeta exists as a disulfide-linked homodimer or as a heterodimer in which the zeta chain is associated with an alternatively spliced product of the same gene, Eta. F1 and F2 both possess the TM domain of zeta, and therefore should have the potential to form a homodimer (and possibly a heterodimer with native zeta) via the membrane proximal cysteine residue (position 11 of the mature zeta chain). In contrast, the TM domain of F3 is derived from CD4, and would therefore be expected to confer the native monomeric state of the native CD4 molecule to the F3 receptor.

In order to determine whether these receptors do form covalent linkages, immunoprecipitates of radiolabelled 293 cells which had been electroporated with each of the constructs under evaluation, were analyzed under reducing and non-reducing conditions. Under both reducing and non-

reducing conditions, a single protein of app. 70 kd is immunoprecipitated by OKT4A from 293 cells electroporated with F3. As expected, CD4 also gives rise to a single protein of app. 60 kd under both reducing and non-reducing conditions. In contrast, F1 and F2 give rise to proteins of app. 70 kd and 150 kd respectively under non-reducing conditions, approximately double that seen under reducing conditions (app. 35 kd and 70 kd respectively). These results demonstrate that F1 and F2, like native zeta, exist as disulfide-linked homodimers, whereas F3 exists as a ~~monomer as does native~~ CD4. ~~These~~ data do not rule out the ability of F3 to form a non-covalently associated dimer however.

Introduction of CD4-zeta Receptors into a Human T Cell Line

The chimeric receptor genes F1, F2, and F3, and the native CD4 gene, were introduced into a derivative of pIK.1.1 bearing a selective marker, pIK.1.1Neo. Each construct was stably introduced via electroporation into the human T cell leukemia line, Jurkat, and independent Jurkat clones obtained by limiting dilution and selection to G418. Cell surface expression of the chimeric receptor was assessed by FACS analysis of Jurkat clones employing FITC-coupled OKT4A. Although native Jurkat cells express a low level of CD4 on the cell surface, transfectants expressing high levels of F2 or F3 were readily identified due to the significantly higher levels of fluorescence

observed relative to untransfected cells. Similarly, stable clones expressing high levels of CD4 were also identified. In contrast, none of the clones isolated from cells electroporated with the F1 receptor construct revealed levels of OKT4A-specific fluorescence higher than that seen with native Jurkat cells (See above).

FACS analysis of over 100 Jurkat clones, revealed that the F3 receptor has the potential to be stably expressed in Jurkat cells at significantly higher levels (up to 50 fold) than the F2 receptor. The zeta TM domain is thought to be involved in assembly and cell surface expression of the TCR complex. Therefore it is possible that cell surface expression of receptors bearing the zeta TM domain is limited by the availability of "resident" TCR chains (Ti, CD3). Irving and Weiss Cell (1991) 64:891-901 demonstrated that a CD8-zeta receptor in which the TM domain of zeta was replaced by that of CD8, is expressed in Jurkat cells independently of the TCR complex. Presumably, the F3 receptor is similarly uncoupled from the resident TCR chains by virtue of the CD4 TM domain, and therefore is not subject to the putative constraint on surface expression levels experienced by the F2 receptor.

Induction of CD69 Expression Upon Stimulation of Native and Chimeric Receptors

CD69 (Leu-23) is an early human activation antigen present on T, B, and NK lymphocytes. CD69 is detected on the cell surface of T lymphocytes within 2 hours after

stimulation of CD3/TCR, reaching a maximal level by 18 to 24 hours. CD69 is therefore the first detectable cell surface protein induced in response to CD3/TCR-mediated signals, and represents a reliable marker of T cell activation. The ability of the CD4-zeta chimeric receptors to specifically mediate CD69 induction in the Jurkat T cell line, and selected representative Jurkat clones expressing either F2, F3, or CD4 for functional analysis was investigated.

Monoclonal antibodies specific for the Ti α/β or CD3 chains can mimic the effect of antigen and serve as agonists to stimulate signal transduction and T cell activation events. Cells were stimulated with immobilized MAbs specific for (a) the Ti β chain Jurkat, (C305), (b) the CD3 ϵ chain (OKT3), and (c) the V1 domain of CD4 (OKT4A). W6/32 recognizes an invariant determinant of human HLA class I antigens, and was used in some experiments as a negative control. CD69 expression was assayed by FACS analysis approximately 18 hours post-stimulation, employing FITC-coupled anti-Leu 23 MAb. The results were as follows. Unstimulated cells exhibit a very low level of basal CD69 expression, but upon stimulation with a pharmacological activator of protein kinase C, phorbol myristate acetate (PMA), maximal expression is induced. Stimulation of native Ti with the C305 MAb, or native CD3 with the OKT3 MAb, also results in induction to the CD69 marker. However, stimulation by OKT4A gives rise to a high level of CD69 expression only

for those transfectants expressing a chimeric CD4-zeta receptor. For a number of transfectants, particularly F3-derived, the level of CD69 induction observed upon stimulation is equal to that seen with PMA.

Stimulation of wild-type CD4 with OKT4A results in little or no induction of CD69, when assayed in a number of Jurkat CD4-transfectants. Similarly, treatment of transfectants with the class 1 antibody, W6/32, had no significant effect in this assay.

These results confirm and extend the work of Irving and Weiss (1991) supra involving the CD8-zeta chimeric receptor, and show that CD4 chimeric receptors possessing the cytoplasmic tail of zeta function effectively in initiation of T cell activation events. Specifically, chimeric CD4-zeta receptors bearing the CD4 TM domain (F3) mediate T cell activation more efficiently (at least with respect to CD69 induction) than those bearing the zeta TM domain (F2), despite the fact that the latter retains the homodimeric form of native zeta. It is possible that this difference in efficacy is due to the significantly higher levels of chimeric receptor present on the cell surface of Jurkat clones expressing F3 compared to F2, and/or the possibility that "uncoupling" of the receptor from the TCR complex augments signalling via this particular pathway at least.

F3 differs from F2 and native zeta, in that it does not exist in the form of a covalent homodimer. The CD8-zeta chimeric receptor described by Irving and Weiss

(1991), supra exists as a disulfide-linked dimer (as does native CD8), thus preserving the dimeric form of native zeta, despite the absence of the zeta TM domain. In contrast to CD8-zeta, the F3 receptor described here is unable to form a disulfide-linked homodimer as shown above. These data therefore demonstrate that covalent dimerisation of the chimeric receptor is not essential for initiation of T cell activation as measured by CD69 induction.

Following the procedure for detecting IL-2 expression, substantially as described above for the CD8-ζ chimera, IL-2 secretion was also observed in response to anti-CD4 antibody by Jurkat cells expressing the CD4-ζ chimera.

It is evident from the above results that one can provide for activation of various signalling pathways in a host cell by providing for expression of a chimeric protein, which may serve as a surface membrane protein, where the extracellular domain is associated with a ligand of interest, while the cytoplasmic domain, which is not naturally associated with the extracellular domain, can provide for activation of a desired pathway. In this manner, cells can be transformed so as to be used for specific purposes, where cells will be activated to a particular pathway by an unnatural ligand. This can be exemplified by using CD4 as the extracellular domain, where binding of an HIV protein can result in activation of a T-cell which can stimulate cytotoxic activity to

destroy infected cells. Similarly, other cells may be modified, so as to be more effective in disease treatment, or to immune effects and the like.

All publications and patent applications mentioned in this specification are herein incorporated by reference to the same extent as if each individual publication or patent application was specifically and individually indicated to be incorporated by reference.

The invention now being fully described, it will be apparent to one of ordinary skill in the art that many changes and modifications can be made thereto without departing from the spirit or scope of the appended claims.

WHAT IS CLAIMED IS:

1. A DNA sequence comprising in reading frame:

a sequence encoding a signal sequence;

a sequence encoding an extracellular surface membrane protein domain binding specifically to at least one ligand, by itself or in conjunction with a second extracellular domain;

a transmembrane domain; and

a cytoplasmic domain of the TCR associated zeta chain or eta chain, or the gamma chain of the $Fc_{\epsilon}R1$ receptor,

wherein said extracellular domain and cytoplasmic domain are not naturally joined together.

2. A DNA sequence according to Claim 1, wherein said cytoplasmic domain is the zeta chain.

3. A DNA sequence according to Claim 2, wherein said extracellular domain is the heavy chain of an Ig, by itself or in conjunction with a light chain, or truncated portions thereof.

4. A DNA sequence according to Claim 2, wherein said extracellular domain is CD8.

5. A DNA sequence according to Claim 2, wherein said extracellular domain is CD4.

6. A DNA sequence according to Claim 1, wherein said transmembrane domain is naturally joined to said extracellular domain.

7. A DNA sequence according to Claim 1, wherein said transmembrane domain is naturally joined to said cytoplasmic domain.

8. An expression cassette comprising a transcriptional initiation region, a DNA sequence according to Claim 1 under the transcriptional control of said transcriptional initiation region, and a transcriptional termination region.

9. An expression cassette according to Claim 8, wherein said transcriptional initiation region is functional in a mammalian host.

10. A cell comprising a DNA sequence according to Claim 1.

11. A cell according to Claim 10, wherein said cytoplasmic domain is the CD3 zeta chain.

12. A cell according to Claim 11, wherein said extracellular domain is the heavy chain of an Ig, by itself or in conjunction with a light chain, or truncated portions thereof.

13. A cell according to Claim 11, wherein said extracellular domain is CD8.

14. A cell according to Claim 11, wherein said extracellular domain is CD4.

15. A cell according to Claim 10, wherein said transcriptional initiation region is functional in a mammalian cell and said cell is a mammalian cell.

16. A cell according to Claim 15, wherein said mammalian cell is a human cell.

17. A protein comprising in the N-terminal to C-terminal direction:

an extracellular surface membrane protein domain binding specifically to at last one ligand, by itself or in conjunction with a second extracellular domain;

a transmembrane domain; and

a cytoplasmic domain of the CD3 zeta chain, the eta chain of CD3 or the gamma chain of the $Fc_{\epsilon}R1$ receptor,

wherein said extracellular domain and cytoplasmic domain are not naturally joined together.

18. A protein according to Claim 17, wherein said cytoplasmic domain is the CD3 zeta chain.

19. A protein according to Claim 17, wherein said extracellular domain is the heavy chain of an Ig, by itself or in conjunction with a light chain, or truncated portions thereof.

20. A protein according to Claim 17, wherein said extracellular domain is CD8.

21. A protein according to Claim 17, wherein said extracellular domain is CD4.

22. A mammalian cell comprising as a surface membrane protein, a protein according to Claim 17.

23. A mammalian cell according to Claim 22, wherein said extracellular domain is bound to a second protein to define a binding site.

24. A mammalian cell comprising as a surface membrane protein, a protein according to Claim 19, wherein said cell is a cytotoxic T lymphocyte.

25. A mammalian cell comprising as a surface membrane protein, a protein according to Claim 21, wherein said cell is a cytotoxic T lymphocyte.

26. A mammalian cell comprising as a surface membrane protein, a protein according to Claim 19, wherein said cell is substantially free of surface expression of at least one of Class I or Class II MHC.

27. A mammalian cell comprising as a surface membrane protein, a protein according to Claim 21, wherein said cell is substantially free of surface expression of at least one of Class I or Class II MHC.

28. A method for activating cells by means of a secondary messenger pathway, said method comprising:

contacting said cells with a ligand which binds to said extracellular domain and transduces a signal to said cytoplasmic domain,

whereby said secondary messenger pathway is activated.

29. A retroviral RNA or DNA construct comprising an expression cassette according to Claim 9.

INTERNATIONAL SEARCH REPORT

International Application No. PCT/US91/09431

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[3]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC (5): Please See Attached Sheet.
US CL : Please See Attached Sheet.

II. FIELDS SEARCHED

Minimum Documentation Searched[4]

Classification System	Classification Symbols
U.S.	435/7.22, 69.1, 69.7, 172.1, 172.3, 240.2; 424/93; 530/350; 536/27

Documentation Searched other than Minimum Documentation to the extent that such Documents are included in the Fields Searched[5]

Databases: Dialog (411)
Automated Patent System (Files USPAT 1971-1991)

III. DOCUMENTS CONSIDERED TO BE RELEVANT[14]

Category*	Citation of Document,[16] with indication, where appropriate, of the relevant passages[17]	Relevant to Claim No.[18]
Y	Cell, Vol. 40, issued February 1985, Littman et al., "The Isolation and Sequence of the Gene Encoding T8: A molecule Defining Functional Classes of T Lymphocytes", pages 237-246, see entire document.	1-29
Y	Proc. Natl. Acad. Sir. USA, Vol. 85, issued December 1988, Weissman et al, "Molecular Cloning and Chromasomal Localization of the Human T-Cell Receptor Chain: Distinction from the Molecular CD3 Complex", pages 9709-9713, see entire document.	1-29
Y	Cell, vol. 57, issued 05 May 1989, Kinet, "Antibody-Cell Interactions: Fc Receptors", pages 351-354, see entire document.	1-29
Y	Nature, vol. 342, issued 14 December 1989, Lanier et al, "Co-association of CD3 with a Receptor (CD16) for IgG Fc on Human National Killer Cells", pages 803-805, see entire document.	1-29

* Special categories of cited documents:[16]

"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier document but published on or after the international filing date
"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)
"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
"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
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step
"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
"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search[2]	Date of Mailing of this International Search Report[2]
11 FEBRUARY 1992	16 MAR 1992
International Searching Authority[1]	**Signature of Authorized Officer[20]**
ISA/US	GIAN WANG

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

Y	Nature, vol. 347, issued 13 September 1990, Orloff et al., "Family of Disulphide-Linked Dimers Containing the and claims of the T-cell Receptor and the chain of Fc Receptor", pages 189-191, see entire document.	1-29
Y	Nature, vol. 344, issued 12 April 1990, Byrn et al, "Biological Properties of a CD4 Immunoadhesin", pages 667-670, see entire document.	1-29

V. ☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE[1]

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☐ Claim numbers _, because they relate to subject matter (1) not required to be searched by this Authority, namely:

2. ☐ Claim numbers _, because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out (1), specifically:

3. ☐ Claim numbers _, because they are dependent claims not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. ☒ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING[2]

This International Searching Authority found multiple inventions in this international application as follows:

See attached sheet.

1. ☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application. (Telephone Practice)

2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Search Authority did not invite payment of any additional fee.

Remark on protest

☐ The additional search fees were accompanied by applicant's protest.

☐ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PREVIOUS SHEETS
(Not for publication)

I. CLASSIFICATION OF SUBJECT MATTER:
IPC (5):

C12Q 1/68; G01N 33/53; C12P 21/06; C12N 5/00; A01N 63/00; C07K 3/00, 13/00; C07H 15/12, 17/00

I. CLASSIFICATION OF SUBJECT MATTER:
US CL :

435/7.22, 69.1, 69.7, 172.1, 172.3, 240.2; 424/93; 530/350; 536/27

PCT/US 91/09431

Attachment to Form PCT/ISA/210

Observations where unity of invention is lacking

Detailed reasons for holding lack of unity of invention

The claims of the four groups have the characteristics of four distinct inventive concepts. Group I pertains to a DNA sequence, an expression cassette, a cell and a retroviral RNA or DNA construct. Group II pertains to a protein comprising in the N-terminal to C-terminal direction. Group III pertains to a mammalian cell comprising as a surface membrane protein. Group IV pertains to a method for activating cells by means of a secondary messenger pathway. Groups I-IV are separate and distinct inventions, and require materially different considerations and searches.

Itemized summary of claims groupings

I. Claims 1-16 and 29 are, drawn to a DNA sequence, an expression cassette, a cell and a retroviral RNA or DNA construct, classified in Class 435, subclass 240.2; Class 536, subclass 27.

II. Claims 17-21 are, drawn to a protein comprising in the N-terminal to C-terminal direction: an extracellular surface membrane protein, classified in Class 530, subclass 350.

III. Claims 22-27 are, drawn to a mammalian cell comprising as a surface membrane protein, classified in Class 435, subclass 240.2.

IV. Claim 28 is, drawn to a method for activating cells by means of a secondary messenger pathway, classified in Class 424, subclass 93.