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Immunology

A general method for facilitating heterodimeric pairing between two proteins: Application to expression of α and β T-cell receptor extracellular segments

(antigen recognition/leucine zipper/protein engineering)

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ABSTRACT **Generation of soluble T-cell receptor (TCR) molecules by a variety of genetic engineering methods has been hampered by inefficient pairing of α and β subunits in the absence of their respective transmembrane regions and associated CD3 components. To overcome this obstacle, we have added 30-amino acid-long segments to the carboxyl termini of α and β extracellular domains via a cleavable flexible linker. These peptide segments (BASE-p1 for α and ACID-p1 for β) have been previously shown to selectively associate to form a stable heterodimeric coiled coil termed a leucine zipper. Homodimeric structures are not permitted due to electrostatic repulsion among amino acid side chains. Expression of a representative TCR–leucine zipper fusion protein in a baculovirus expression system results in production of αβ TCR heterodimer at 0.6–1.4 mg/liter. This yield is 5- to 10-fold greater than that of the TCR expressed in the absence of the synthetic leucine zipper sequence. The structure of the TCR component of the fusion heterodimer was judged to be native when probed with a panel of 17 mAbs specific for α and β constant and variable domains. A mAb specific for the isolated BASE-p1/ACID-p1 coiled coil was also generated and shown to react with the TCR fusion protein. The above technology should be broadly useful in the efficient production and purification of TCRs as well as other heterodimeric proteins.**

The T-cell receptor (TCR) complex consists of multiple transmembrane polypeptide chains on the surface of T lymphocytes (1–3). The disulfide-linked αβ heterodimer (Ti) is the clonally unique component that possesses a recognition site for antigen in the context of the major histocompatibility complex (MHC), whereas the invariant CD3 components (γ, δ, ε, ζ, and η) are involved in signal transduction. Because of the intimate membrane association of this complex, the attributes of TCR recognition have been studied indirectly.

Given the importance of understanding in explicit molecular terms the process by which T cells recognize pathogens and autoantigens, recent efforts have focused on production of recombinant soluble forms of the TCR heterodimer. Prokaryotic expression has yielded substantial quantities of TCR protein, which, unfortunately, comprise a very low fraction of native or correctly refolded material (4–6). In eukaryotic expression systems, immunoglobulin chimeras with TCR α and β extracellular segments have been produced (7, 8). In addition, glycosyl-phosphatidylinositol-linked chimeras (9, 10) released from the cell surface by phosphatidylinositol phospholipase C, as well as α and β chimeras fused to CD3ζ transmembrane and cytoplasmic segments using a thrombin-cleavable linker, have been generated (11). A major problem with each of these strategies is the inefficient pairing between α and β subunits. Monomers as well as homodimers, in addition to the desired heterodimers, are produced. This mispairing complicates protein purification and leads to a substantial reduction in useful protein yield.

Here, we describe a method to facilitate TCR pairing through the use of synthetic peptide sequences that create a heterodimeric coiled coil (12). A TCR αβ heterodimer derived from the mouse N15 cytotoxic T lymphocyte (CTL) clone specific for a well-characterized vesicular stomatic virus octapeptide in the context of the K^b MHC class I molecule was chosen for the model (13, 14). This approach offers a general method to express heterodimeric proteins.

MATERIALS AND METHODS

Construction and Transfection of N15αζ and N15βζ Chimeras. The cDNAs encoding the N15 TCR were isolated by priming total RNA from the N15 CTL clone with oligonucleotides specific for the 3′ untranslated region of constant region (C) α subunit and $C_\beta 2$. cDNA was generated by reverse transcription and amplified by standard PCR technology. The full-length N15α and N15β cDNAs were subcloned into the pCRII vector (Invitrogen) for sequence analysis and further genetic manipulation. Two pairs of N15αζ and N15βζ plasmids were engineered. The first pair, p2N15αζ and p2N15βζ, was created by replacing the variable (V) and C regions of 2B4α and 2B4β in the previously described constructs (11) with PCR-generated DNA fragments of N15α and N15β V and C regions. The second pair of plasmids was termed pcN15αζ and pcN15βζ. pcN15αζ was generated by ligation of an *Eco*RI–*Hin*dIII fragment of the N15α cDNA to a *Hin*dIII–*Eco*RI fragment encoding a segment of C_α fused to a linker sequence (SSADLVPRGSTTAPS) connecting C_α to the murine ζ-chain transmembrane region and cytoplasmic domain. The *Hin*dIII–*Eco*RI fragments were made using a combination of restriction site-containing oligonucleotides and PCR and subcloned into the *Eco*RI-digested pCRII vector. The N15βζ construct was generated in a similar fashion by ligation of a 5′ *Eco*RI–*Nco* I fragment from the N15β cDNA with a *Nco* I–*Eco*RI fragment encoding a segment of C_β, the same linker and ζ domains. The cDNA fragments of αζ and βζ constructs were then subcloned into

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Abbreviations: TCR, T-cell receptor; sTCR, soluble TCR; mAb, monoclonal antibody; MHC, major histocompatibility complex; V, variable; C, constant; CTL, cytotoxic T lymphocytes; SP, single positive.

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pcDL-SRα296 (15) to yield pcN15$\alpha\zeta$ and pcN15$\beta\zeta$, respectively, and sequenced.

RBL-2H3 cells (11) were maintained in Dulbecco's modified Eagle's medium/10% fetal calf serum/2 mM glutamine/penicillin at 50 units per ml/streptomycin at 50 μg/ml/50 μM 2-mercaptoethanol. The RBL-2H3 cells were harvested by trypsinization and resuspended in phosphate-buffered saline for electroporation. Twenty micrograms of linearized p2N15$\alpha\zeta$ and p2N15$\beta\zeta$ plus 2 μg of pSV2neo were used to transfect RBL-2H3 cells by electroporation (250 V, 800 μF). The cells resistant to G418 at 0.4 mg/ml were screened by cell cytometry with H57-597 (H57) monoclonal antibody (mAb) for C_β expression (16). By sorting for mAb H57 reactivity, a cell line, termed 2N15$\alpha\zeta+\beta\zeta$, expressing high levels of reactivity, was obtained. The 2N15$\beta\zeta$ cell line was generated by cotransfection of p2N15$\beta\zeta$ plus pSV2neo as above. The cN15$\alpha\zeta+\beta\zeta$ cell line was made in an identical manner to 2N15$\alpha\zeta+\beta\zeta$, except that pcN15$\alpha\zeta$ and pcN15$\beta\zeta$ plasmids were used. For immunoprecipitation studies, cells were surface labeled with ^{125}I, lysed in 1% Triton X-100, and immunoprecipitated with 1ζ3A1 (anti-CD3ζ) (17) coupled to protein A-Sepharose beads (3 mg/ml).

Baculovirus Constructs. Constructs for expressing N15 soluble TCR (sTCR) in the baculovirus system were generated by using PCR to introduce a termination codon 1 amino acid after the extracellular membrane proximal cysteine residue of the α and β C regions with specific 3′ primers for the 2N15$\alpha\zeta$ and 2N15$\beta\zeta$, respectively, in conjunction with 5′ primers derived from the relevant 5′ untranslated regions. After PCR, the DNA fragments were then subcloned individually into the *Bam*HI site of baculovirus expression vector pVL1392 (PharMingen) downstream of the polyhedrin promoter to yield psTCR$_{N15}\alpha$ and psTCR$_{N15}\beta$. To next generate constructs containing the TCR fused to leucine zipper components (ACID-p1 for β and BASE-p1 for α) carboxyl terminal to the membrane-proximal cysteine, two DNA fragments encoding a linker sequence and leucine zipper peptide were generated by using synthetic oligonucleotides and PCR. The DNA fragments were then subcloned into the *Bam*HI–*Eco*RI site of the pCRII vector for DNA sequencing analysis and further manipulation. The TCR–leucine zipper cDNAs for sTCR$_{N15LZ}\alpha$ and sTCR$_{N15LZ}\beta$ were generated by ligation of *Eco*RI–*Bam*HI fragments from the pcN15$\alpha\zeta$ and pcN15$\beta\zeta$ constructs to the *Bam*HI–*Eco*RI fragments of BASE-p1 and ACID-p1 DNA, respectively. The sTCR$_{N15LZ}\alpha$ and sTCR$_{N15LZ}\beta$ cDNAs were individually subcloned into the *Eco*RI site of pVL1392 to yield psTCR$_{N15LZ}\alpha$ and psTCR$_{N15LZ}\beta$. sTCR$_{N15}$ and sTCR$_{N15LZ}$ α and β constructs were sequenced and found to be error-free.

Production, Purification, and Analysis of sTCR using a Baculovirus Expression System. psTCR$_{N15}\alpha$, psTCR$_{N15}\beta$, psTCR$_{N15LZ}\alpha$, and psTCR$_{N15LZ}\beta$ were each cotransfected with wild-type baculoviral DNA and recombinant virus stocks obtained from the resulting plaques (18, 19). The stocks were used for production of sTCR by infecting High 5 cells in serum-free medium SF900II (GIBCO). For production of sTCR$_{N15}$ protein, sTCR$_{N15}\alpha$ and sTCR$_{N15}\beta$ viruses were used at a multiplicity of infection of 10:1 and 5:1, respectively. For protein production, cells were infected at a density of 1×10^6/ml, and supernatants were harvested at 72 hr. An identical strategy was used for production of sTCR$_{N15LZ}$ protein, except that sTCR$_{N15LZ}\alpha$ and sTCR$_{N15LZ}\beta$ baculovirus stocks were used. Protein production was scaled up from 100 ml in spinner flasks to 8 liters with an oxygen overlay and "sparging" (20). For affinity purification, immunoaffinity matrices were prepared by covalently coupling mAb H57 to protein A-Sepharose beads (Repligen) at a concentration of 5 mg/ml using dimethylpimelimidate as described (21). For preclearing, murine anti-human CD8 mAb 21Thy5D7 was used and coupled in an identical fashion. The mAb H57 affinity matrix was eluted with 0.1 M glycine·HCl, pH 3.0. The eluted samples were immediately neutralized by collection in tubes containing 1.5 M Tris·HCl, pH 8.9.

mAb Production and Analysis. The mAbs reactive with the N15 TCR were generated by immunizing a male Wistar rat with 2×10^7 cN15$\alpha\zeta+\beta\zeta$ cells at 2-week intervals. After five i.p. immunizations, the rat was boosted with 20 μg of Triton X-100-solubilized N15$\alpha\zeta+\beta\zeta$ protein bound to 1ζ3A1 protein A-Sepharose. Four days later, splenocytes were fused with NS1 cells by using standard methods (17). Ten days after fusion, the supernatants were assayed for reactivity with the N15 TCR dimers on cN15$\alpha\zeta+\beta\zeta$ cells by cell ELISA and cell cytometry analysis. For production of mAbs to the synthetic leucine zipper sequence, a BALB/c mouse was immunized i.p. with 50 μg of the HPLC-purified disulfide-linked ACID-p1/BASE-p1 dimer in complete Freund's adjuvant and boosted several times with antigen in incomplete Freund's adjuvant. Hybridomas were produced as above but screened by ELISA using the immunizing peptide coated to Immulon-II plates (Dynatech) at 2–5 μg/ml. The reactivity of sTCR$_{N15LZ}$ with mAbs was detected by a sandwich ELISA procedure. In brief, H28, H57, or MR9.4 at 5 μg/ml were coated on Immulon-II plates at room temperature for 2 hr and blocked with 1% bovine serum albumin in borate-buffered saline at room temperature for 2 hr. Subsequently, sTCR sTCR$_{N15LZ}$ was added at 0.5 μg/ml on each well at 4°C overnight; then plates were treated with various hybridoma cell supernatants and developed with horseradish peroxidase-conjugated secondary antibodies. For cell cytometry analysis of rat mAbs, 200,000 cells of the N15 CTL, N15$\alpha\zeta+\beta\zeta$, single-positive (SP) thymocytes or N15$\alpha\beta$58 [an N15α and β cDNA transfectant of the 58$\alpha^-\beta^-$ variant of BW5147 (22)] were examined on a FACScan.

RESULTS AND DISCUSSION

TCR Homodimers and Heterodimers Produced by $\alpha\zeta$ and $\beta\zeta$ Transmembrane Fusion Proteins. α and β cDNAs encoding the N15 murine TCR specific for a well-characterized vesicular stomatitis virus octapeptide/H-2K^b complex (13, 14) were isolated by PCR, subcloned, and sequenced. Analysis of the TCR sequences indicates that the α subunit is encoded by V_α8, joining $(J)_\alpha$5, and C_α DNA, whereas the β subunit is encoded by V_β5.2, diversity (D)2, J_β2.6, and C_β2 DNA (23). To produce soluble TCR protein in sufficient quantities for functional and structural studies, we initially used the recently described TCR-ζ fusion system (11). $\alpha\zeta$ and $\beta\zeta$ cDNAs were subcloned into pcDL-SRα296 (11) and subsequently cotransfected separately or together along with

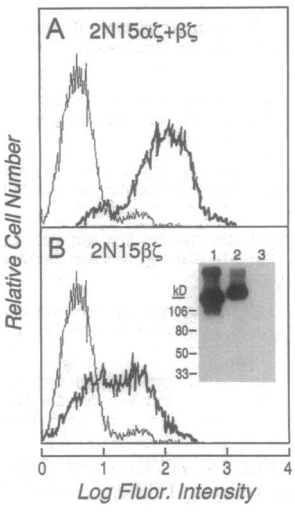
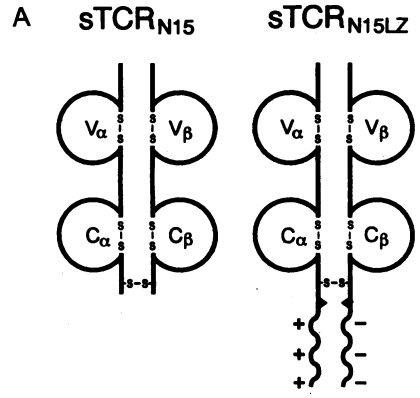


FIG. 1. Homodimers and heterodimers formed on the surface of TCR-ζ transfectants. Flow cytometric analysis of 2N15$\alpha\zeta+\beta\zeta$ (*A*) transfected with $\alpha\zeta$ and $\beta\zeta$ cDNA as well as 2N15$\beta\zeta$ cells (*B*) transfected with the $\beta\zeta$ cDNA alone stained with mAb H57 (dark line) or control mAb (light line) followed by fluorescein-conjugated anti-hamster antibody. (*Inset*) Chimeric molecules precipitated from 10^7 cells with 1ζ3A1 (anti-CD3ζ) mAb from 2N15$\alpha\zeta+\beta\zeta$ cells (lane 1) and 2N15$\beta\zeta$ cells (lane 2) analyzed by SDS/12.5% PAGE under nonreducing conditions. Control immunoprecipitation from 2N15$\beta\zeta$ with mAb H28 (lane 3) shows the specificity of the band detected. Molecular mass markers are phos-phorylase B (106 kDa), bovine serum albumin (80 kDa), ovalbumin (50 kDa), and carbonic anhydrase (33 kDa). Fluor., fluorescence.

pSV2neo into RBL-2H3 cells. After G418 selection, cells expressing high levels of anti-TCR C_β mAb reactivity (H57) were sorted by fluorescence-activated cell sorting. As shown in Fig. 1*A*, the double transfectant 2N15$\alpha\zeta$+$\beta\zeta$ is reactive with mAb H57. However, Fig. 1*B* shows that the single transfectant 2N15$\beta\zeta$ is also H57 reactive. The latter results suggest that β segment may be expressed on the surface of RBL-2H3 transfectants as either a $\beta\zeta$-$\beta\zeta$ homodimer or $\beta\zeta$ monomer. To examine this possibility, 2N15$\beta\zeta$ and 2N15$\alpha\zeta$+$\beta\zeta$ cells were surface labeled with ^{125}I, lysed, and immunoprecipitated with the 1ζ3A1 anti-CD3ζ mAb (17) or as a control, with the anti-C_α mAb H28-710 (H28) (24). As shown in Fig. 1*B* (*Inset*), a 120-kDa band consistent with the size of a $\beta\zeta$-$\beta\zeta$ homodimer was specifically immunoprecipitated. The mobility of this band is slightly slower than that of the predominant band seen in the 1ζ3A1 immunoprecipitate from 2N15$\alpha\zeta$+$\beta\zeta$ lysates. On the basis of sequential immunoprecipitation studies with mAbs H57 and H28, the 2N15$\alpha\zeta$+$\beta\zeta$ lysates apparently contain $\alpha\zeta$-$\beta\zeta$ heterodimers, as well as $\beta\zeta$-$\beta\zeta$ homodimers (data not shown). Thus, fusion of the TCR α and β extracellular segments to the transmembrane and cytoplasmic domain of CD3ζ does not exclusively give rise to heterodimers. Similar results were seen with a TCR $\beta\zeta$ fusion protein derived from the 2B4 cytochrome C/I-E^k-restricted TCR (data not shown).

Inefficient Pairing of Secreted α and β TCR Extracellular Segments. Both the lack of selectivity in dimer formation afforded by the TCR-ζ chimeric expression system and the requirement for enzymatic cleavage of proteins from cell lysates hinder large-scale purification of the TCR. We therefore chose to use a baculovirus expression system (18, 19) for production of a secreted $\alpha\beta$ TCR as described in *Materials and Methods*. TCR α and β constructs were generated by introducing a termination codon 1 amino acid carboxyl terminal to the membrane-proximal cysteine of each subunit (Fig. 2*A*). Fig. 3*A* shows a Coomassie-stained gel of immunoprecipitates from culture supernatants of High 5 cells coinfected 3 days previously with sTCR$_{N15}\alpha$ and sTCR$_{N15}\beta$ baculovirus stocks and analyzed by SDS/PAGE. As shown, under nonreducing conditions (lanes 1–3), the majority of α and β proteins are present as monomers (two predominant α monomers of 29 and 31 kDa and multiple β monomers of 30–35 kDa) and, thus, do not associate with each other. Furthermore, sequential immunoprecipitation studies with mAbs H57 and H28 show that a significant fraction of 60-kDa material represents $\alpha\alpha$ and $\beta\beta$ homodimers (data not shown).



B

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ATTCGGCTTC CAAGTGTCAT TTCCACC ATG AAC ATG CGT CCT GTC ACC TCC TCA GTT
                               MET Asn MET Arg Pro Val Thr Ser Ser Val
                               <-  -   -   -   -   -   - Leader peptide

CTC GTG CTC CTC CTA ATG CTC AGA AGG AGC AAT GGA GAC TCC GTG ACC CAG ACA
Leu Val Leu Leu Leu MET Leu Arg Arg Ser Asn Gly Asp Ser Val Thr Gln Thr
 -   -   -   -   -   -   -   -   -   -   -   -><-   -   -   -   -   -

GAA GGC CTG GTC ACT GTC ACC GAG GGG TTG CCT GTG AAG CTG AAC TGC ACC TAT
Glu Gly Leu Val Thr Val Thr Glu Gly Leu Pro Val Lys Leu Asn Cys Thr Tyr
 -   -   -   -   -   -   -   -   -  N15  Vα   -   -   -   -   -   -

CAG ACT ACT TAT TTA ACT ATT GCC TTT TTC TGG TAT GTG CAA TAT CTC AAC GAA
Gln Thr Thr Tyr Leu Thr Ile Ala Phe Phe Trp Tyr Val Gln Tyr Leu Asn Glu
 -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -

GCC CCT CAG GTA CTC CTG AAG AGC TCC ACA GAC AAC AAG AGG ACC GAG CAC CAA
Ala Pro Gln Val Leu Leu Lys Ser Ser Thr Asp Asn Lys Arg Thr Glu His Gln
 -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -

GGG TTC CAC GCC ACT CTC CAT AAG AGC AGC AGC TCC TTC CAT CTG CAG AAG TCC
Gly Phe His Ala Thr Leu His Lys Ser Ser Ser Ser Phe His Leu Gln Lys Ser
 -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -

TCA GCG CAG CTG TCA GAC TCT GCC CTG TAC TAC TGT GCT CTG AGT GAA GGA GGA
Ser Ala Gln Leu Ser Asp Ser Ala Leu Tyr Tyr Cys Ala Leu Ser Glu Gly Gly
 -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -

AAC TAC AAA TAC GTC TTT GGA GCA GGT ACC AGA CTG AAG GTT ATA GCA CAC ATC
Asn Tyr Lys Tyr Val Phe Gly Ala Gly Thr Arg Leu Lys Val Ile Ala His Ile
 -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -><-   -

CAG ..... GTT CCC TGT TCG TCA GCA GAC CTG GTT CCG CGC GGA TCC ACT ACA
Gln       Val Pro Cys Ser Ser Ala Asp Leu Val Pro Arg Gly Ser Thr Thr
 -   Cα    -   -  -><- Linker  -><- Thrombin site  -><-   -
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C

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GCT CCA TCA GCT CAG TTG AAA AAG AAA TTG CAA GCA CTG AAG AAA AAG AAC GCT
Ala Pro Ser Ala Gln Leu Lys Lys Lys Leu Gln Ala Leu Lys Lys Lys Asn Ala
Linker  -><-  -   -   -   -   -   -   -   -   -   - Basic peptide

CAG CTG AAG TGG AAA CTT CAA GCC CTC AAG AAG AAA CTC GCC CAG TAG GATTCA
Gln Leu Lys Trp Lys Leu Gln Ala Leu Lys Lys Lys Leu Ala Gln  .
 -   -   -   -   -   -   -   -   -   -   -   -   -   -   ->
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C

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AATTCGGCTT GAAGAAGC ATG TCT AAC ACT GTC CTC GCT GAT TCT GCC TGG GGC ATC
                    MET Ser Asn Thr Val Leu Ala Asp Ser Ala Trp Gly Ile
                    <-  -   -   -   -   - Leader peptide-  -   -   -

ACC CTG CTA TCT TGG GTT ACT GTC TTT CTC TTG GGA ACA AGT TCA GCA GAT TCT
Thr Leu Leu Ser Trp Val Thr Val Phe Leu Leu Gly Thr Ser Ser Ala Asp Ser
 -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -> <-  -

GGG GTT GTC CAG TCT CCA AGA CAC ATA ATC AAA GAA AAG GGA GGA AGG TCC GTT
Gly Val Val Gln Ser Pro Arg His Ile Ile Lys Glu Lys Gly Gly Arg Ser Val
 -   -   -   -   -   -   -   -   - N15 Vβ  -   -   -   -   -   -   -

CTG ACG TGT ATT CCC ATC TCT GGA CAT AGC AAT GTG GTC TGG TAC CAG CAG ACT
Leu Thr Cys Ile Pro Ile Ser Gly His Ser Asn Val Val Trp Tyr Gln Gln Thr
 -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -

CTG GGG AAG GAA TTA AAG TTC CTT ATT CAG CAT TAT GAA AAG GTG GAG AGA GAC
Leu Gly Lys Glu Leu Lys Phe Leu Ile Gln His Tyr Glu Lys Val Glu Arg Asp
 -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -

AAA GGA TTC CTA CCC AGC AGA TTC TCA GTC CAA CAG TTT GAT GAC TAT CAC TCT
Lys Gly Phe Leu Pro Ser Arg Phe Ser Val Gln Gln Phe Asp Asp Tyr His Ser
 -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -

GAA ATG AAC ATG AGT GCC TTG GAA CTG GAG GAC TCT GCT ATG TAC TTC TGT GCC
Glu MET Asn MET Ser Ala Leu Glu Leu Glu Asp Ser Ala MET Tyr Phe Cys Ala
 -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -

AGC TCT CTC CGC TGG GGG GAT GAA CAG TAC TTC GGT CCC GGC ACC AGG CTC ACG
Ser Ser Leu Arg Trp Gly Asp Glu Gln Tyr Phe Gly Pro Gly Thr Arg Leu Thr
 -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -

GTT TTA GAG GAT CTG ..... GCA GAC TGT TCG TCA GCA GAC CTG GTT CCG CGC
Val Leu Glu Asp Leu       Ala Asp Cys Ser Ser Ala Asp Leu Val Pro Arg
 -   -><-  -   -   Cβ2  -   -    -><- Linker  -><- - Thrombin

GGA TCC ACT ACA GCT CCA TCA GCT CAG CTC GAA AAA GAG CTC CAG GCC CTG GAG
Gly Ser Thr Thr Ala Pro Ser Ala Gln Leu Glu Lys Glu Leu Gln Ala Leu Glu
site -><   - - Linker -><-  -   -   - Acidic peptide -   -   -

AAG GAA AAT GCA CAG CTG GAA TGG GAG TTG CAA GCA CTG GAA AAG GAA CTG GCT
Lys Glu Asn Ala Gln Leu Glu Trp Glu Leu Gln Ala Leu Glu Lys Glu Leu Ala
 -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -

CAG TAG GAATAG
Gln  .
 - ->
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FIG. 2. sTCR$_{N15}$ and sTCR$_{N15LZ}\alpha$ and β sequences. (*A*) Schematic shows V and C domains of sTCR$_{N15}\alpha$ and β extracellular segments and the related sTCR$_{N15LZ}\alpha$ and β subunits, which are joined to BASE-p1 and ACID-p1 peptides, respectively, via a flexible linker including a thrombin cleavage site (▲). The cDNA sequences of sTCR$_{N15LZ}\alpha$ (*B*) and sTCR$_{N15LZ}\beta$ (*C*) are given with the boundaries of each domain shown. ACID-p1 and BASE-p1 sequences were designed with codon use in mind. Arrowheads indicate positions at which sequences of sTCR$_{N15}$ and sTCR$_{N15LZ}$ subunits diverge. Note that the majority of C_α and C_β sequences are not given.

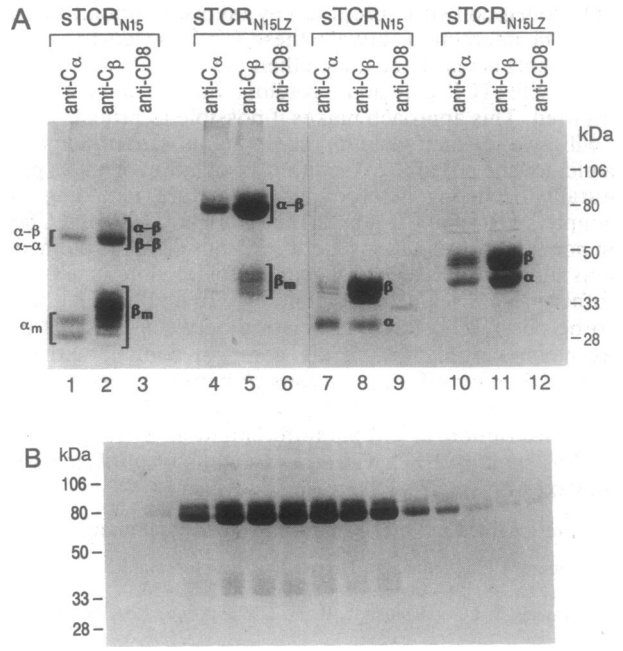

FIG. 3. Heterodimeric nature of sTCR$_{N15LZ}$ isolated from baculovirus supernatants. (*A*) SDS/PAGE analysis of sTCR$_{N15}$ and sTCR$_{N15LZ}$ proteins precipitated directly from culture supernatants (5 ml) of baculovirus-infected High 5 cells using 10 μl of protein A-Sepharose beads coupled at 3–5 mg/ml with anti-C$_\alpha$ (mAb H28), anti-C$_\beta$ (mAb H57), and anti-CD8 (mAb 21Thy5D7). Samples were run on SDS/10% PAGE under nonreducing (lanes 1–6) or reducing (lanes 7–12) conditions, and gels were stained with Coomassie blue. Positions of indicated monomers (α_m, β_m) or dimers ($\alpha\beta$, $\alpha\alpha$, $\beta\beta$) are shown under nonreducing conditions. Although not labeled, noncovalently associated $\alpha\beta$ heterodimers migrate to the same position in the gel as β monomer (β_m; lanes 2 and 5). (*B*) Similar analysis under nonreducing conditions of aliquots of mAb H57-affinity-purified peak fractions derived from 8 liters of supernatants from High 5 cells infected with sTCR$_{N15LZ}\alpha$ and β baculoviruses.

Only a small amount of 60-kDa sTCR$_{N15}\alpha\beta$ heterodimer is present in either anti-C$_\alpha$ (mAb H28) or anti-C$_\beta$ (mAb H57) immunoprecipitates. Consistent with this result is the finding that under reducing conditions (lanes 7–9), mAb H28 preferentially immunoprecipitates the α subunit, whereas mAb H57 immunoprecipitates >30 times more β than α subunit. Note that mAb H57 always immunoprecipitates more protein than mAb H28 due to a higher affinity. Analysis of baculovirus-infected supernatants at earlier or later times after infection did not alter these results (data not shown). Thus, in the absence of the other native TCR components, the secreted α and β TCR segments remain largely unassociated or in homodimeric form. In light of the propensity for TCR-ζ fusion proteins to form both homodimers and heterodimers in RBL-2H3 cells, as well as the poor association between secreted TCR α and β extracellular segments here, it would appear that the intrinsic affinity of recombinant N15α and N15β subunits for one another is low.

Design of a Secreted $\alpha\beta$ TCR Using a Heterodimeric Coiled Coil. As a strategy to promote preferential pairing of TCR α and β extracellular segments to form TCR heterodimers, we fused two related acidic and basic leucine zipper peptide sequences known to form unique heterodimeric coiled coils (12) to the carboxyl termini of the TCR extracellular segments. BASE-p1 and ACID-p1 peptides were appended to α and β segments, respectively. These peptides were previously shown to have a 10^5-fold greater propensity for heterodimer formation over homodimer formation, largely as a result of electrostatic interactions mediated by amino acid residues at the e and g positions of the synthetic leucine zipper structure. Fig. 2 provides both a schematic structure and the sequences of N15α-BASE-p1 and N15β-ACID-p1 constructs, which encode 5′ → 3′ the TCR V and C domains and the linker sequence, including a thrombin cleavage site and the leucine zipper sequence.

Production and Purification of sTCR$_{N15LZ}$ in a Baculovirus Expression System. In contrast to the products resulting from sTCR$_{N15}\alpha$ and β baculovirus infection, the secreted TCR protein after coinfection with sTCR$_{N15LZ}\alpha$ and sTCR$_{N15LZ}\beta$ baculoviruses is heterodimeric (Fig. 3*A*). Under nonreducing conditions (lanes 4–6), a single, major 80-kDa band is found in both anti-C$_\alpha$ mAb (H28) and anti-C$_\beta$ mAb (H57) immunoprecipitates. The small percentage of lower-molecular-mass material in the anti-C$_\beta$ mAb immunoprecipitates represents sTCR$_{N15LZ}\beta$ monomers or noncovalently associated dimers. Under reducing conditions (lanes 10–12), 45-kDa and 40-kDa bands are detected in anti-C$_\alpha$ and anti-C$_\beta$ mAb immunoprecipitates. The intensities of the faster migrating sTCR$_{N15LZ}\alpha$ band and more slowly migrating sTCR$_{N15LZ}\beta$ bands are nearly equivalent.

After blotting to poly(vinylidene difluoride) and Coomassie staining for visualization, the 80-kDa band was cut out and loaded onto a gas-phase protein sequenator (model 470A; Applied Biosystems). Equivalent picomole yields of α and β sequences were obtained using the 03RPTH program. Although 70% of the sTCR$_{N15LZ}\beta$ sequence began at the predicted N terminus (aspartate), 30% began at the +3 position (glycine), indicating a second cleavage site for the signal peptidase. Sequencing of the 45-kDa and 40-kDa presumptive β and α bands, respectively, from poly(vinylidene difluoride) blots of SDS/PAGE samples run under reducing conditions confirmed their derivation. The disparity between subunit sizes in SDS/PAGE and theoretical molecular weights (α = 27,516 Da and β = 32,174 Da) is likely due to N-linked glycan additions (four and three potential N-linked glycosylation sites for N15β and α subunits, respectively), as well as anomolous migration in SDS/PAGE, perhaps as a result of the large number of charged amino acids. Furthermore, the heterogeneous size of the β subunit (Fig. 3*A*, lane 10) is reflective of several glycoforms.

To next determine whether large-scale preparation of sTCR$_{N15LZ}$ could be achieved, 8 liters of supernatant from High 5 cells coinfected with sTCR$_{N15LZ}\alpha$ and sTCR$_{N15LZ}\beta$ baculoviruses were purified on an H57 immunoaffinity matrix. As shown by the Coomassie stained gel of peak fractions (Fig. 3*B*), sTCR$_{N15LZ}$ heterodimer could be purified to >90% in a one-step procedure as a set of closely associated bands. Amino acid sequencing identified the same TCR α and β sequences in each component (data not shown). With this method, minor contaminants include β monomers as well as higher-molecular-mass TCR aggregates. The yield of TCR$\alpha\beta$ heterodimer from the procedure ranges from 0.6 to 1.4 mg/liter (n = 3) of supernatant when sampled 72 hr after infection. This result contrasts with the 5- to 10-fold lower yield of sTCR$_{N15}$ heterodimer (0.08–0.4 mg/liter, n = 10) from High 5 cells coinfected under identical conditions with sTCR$_{N15}\alpha$ and sTCR$_{N15}\beta$ baculoviruses.

Native Configuration of the TCR Domain Structure in sTCR$_{N15LZ}$. To next assess whether the sTCR$_{N15LZ}$ protein was native, we used a series of 17 mAbs reactive with the transmembrane TCR on the N15 CTL clone. Three of these antibodies, MR9.4 (anti-Vβ5.1.2), H57, and H28, were previously derived by others (16, 24, 25). In addition, we produced 14 additional mAbs reactive with the N15 CTL, as well as the N15$\alpha\beta$58 cell transfectant expressing the native N15 TCR. On the basis of cell cytometry reactivity patterns with SP thymocytes from C57BL/6 animals, these antibodies fall into four groups. Three groups represented by N15R4, N15R8, and N15R53 stain the V regions of N15, whereas the fourth group (N15R13, R39, and R43) is C$_\beta$ specific. The

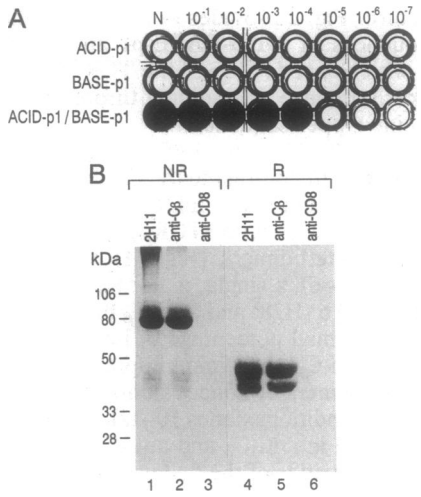

FIG. 4. Analysis of a mAb specific for a heterodimeric coiled coil peptide sequence. mAbs were produced against disulfide-linked versions of the purified heterodimeric peptide. (*A*) mAb 2H11, when tested by ELISA undiluted (N) or at serial dilutions (10^{-1}–10^{-7}) for reactivity against individual components (ACID-p1 or BASE-p1) of the coiled coil alone or in heterodimeric form (ACID-p1/BASE-p1). (*B*) Comparison of the ability of mAb 2H11 vs. mAb H57 (anti-Cβ) to immunoprecipitate $sTCR_{N15LZ}$ protein from High 5 cell supernatants. Immunoprecipitation conditions and analysis were as in Fig. 3, except that mAb 2H11 was coupled to GammaBind Plus (Pharmacia). NR, nonreduced; R, reduced.

$V_\beta 5$-specific mAbs N15R4 and the related mAbs R7, R15, R22, R28, R34, R35, R46, and R54, as well as R8, react with 2N15$\beta\zeta$ cells and a fraction of SP thymocytes. In contrast, N15R53 is N15 TCR specific (clonotypic) and, hence, not reactive with SP thymocytes. All 12 V region-specific antibodies react with $sTCR_{N15LZ}$ by ELISA ($\geq$0.2 OD unit). On the basis of reactivity with mAbs of clonotypic specificity, V_β specificity and C_α and C_β specificity, we judge the $sTCR_{N15LZ}$ protein to be native in configuration.

Production of a mAb Specific for the ACID-p1/BASE-p1 Heterodimeric Coiled Coil. Unlike its component ACID-p1 and BASE-p1 peptides, the heterodimeric coiled coil is a stable structural element (12). Thus, we reasoned that it should be possible to produce mAbs specific for epitopes of the leucine zipper and yet unreactive with the relatively unstructured component peptides, thereby generating generic reagents capable of selecting heterodimers in the expression system. To test this notion, BALB/c mice were immunized with 50 μg of a disulfide-linked dimeric version of the ACID-p1/BASE-p1 peptide (12), and mAbs were produced. One such antibody termed 1Velcro2H11 (mAb 2H11) was found to react with the peptide immunogen as well as $sTCR_{N15LZ}$ by ELISA. As shown in Fig. 4*A*, mAb 2H11 is unreactive with individual ACID-p1 or BASE-p1 peptides but reacts strongly with the ACID-p1/BASE-p1 heterodimer, measurable to an ascites dilution of 10^{-5}–10^{-6}. Furthermore, mAb 2H11 readily immunoprecipitates $sTCR_{N15LZ}\alpha\beta$ heterodimers from $sTCR_{N15LZ}\alpha$ plus $sTCR_{N15LZ}\beta$-coinfected High 5 cell supernatants in a manner equivalent to mAb H57 (Fig. 4*B*). The small amount of lower-molecular-mass material in the 2H11 immunoprecipitate likely represents secreted $\alpha\beta$ heterodimers that are not disulfide-linked.

Implications. A protein engineering methodology using recombinant DNA techniques to greatly increase the efficiency in heterodimer formation between TCR α and β subunits has been developed. This approach involves appending related but distinct peptide sequences (BASE-p1 and ACID-p1) to the carboxyl termini of the α and β subunits, respectively. BASE-p1 and ACID-p1 peptides generate a stable coiled coil structure, thereby favoring subunit association. The interaction between these synthetic leucine zipper components is restricted due to their electrostatics such that, unlike with TCR-ζ fusion proteins, homodimers are not permitted. This approach makes it possible to bring together at will two distinct subunit components. In principle, it should now be possible to facilitate association of any type of naturally occurring heterodimeric structure including, for example, MHC class II α and β subunits or CD8α and CD8β components. Associations between individual protein domains such as TCR V_α and V_β can be fostered in the absence of other protein segments (C_α and C_β). In addition, it should also be possible to force association between proteins that may never or only transiently come in contact with one another, thereby offering a means to better understand regulatory events affecting cellular activation, cell cycle control, gene transcription, or cellular differentiation. The availability of mAbs such as 2H11 will offer a means of purifying such complexes on small- or large-scale levels.

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