T Cell Receptor Complexes Containing FcεRIγ Homodimers in Lieu of CD3ζ and CD3η Components: A Novel Isoform Expressed on Large Granular Lymphocytes

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Summary

CD3ζ and CD3η form disulfide-linked homo- or heterodimers important in targeting partially assembled Tiα-β/CD3γδε T cell receptor (TCR) complexes to the cell surface and transducing stimulatory signals after antigen recognition. Here we identify a new TCR isoform expressed on splenic $CD2^+$, CD3/Tiα-β^+, $CD4^-$, $CD8^-$, $CD16^+$, $NK1.1^+$ mouse large granular lymphocytes (LGL), which are devoid of CD3ζ and CD3η proteins. The TCRs of this subset contain homodimers of the γ subunit of the high affinity receptor for IgE (FcεRIγ) in lieu of CD3ζ and/or CD3η proteins. The LGL display natural killer–like activity and are cytotoxic for B cell hybridomas producing anti-CD3ε and anti-CD16 monoclonal antibodies, demonstrating the signaling capacity of both TCR and CD16 in this cell type. These findings provide evidence for an additional level of complexity of TCR signal transduction isoforms in naturally occurring T cell subsets.

The TCR has been described as a multimolecular complex formed by three groups of transmembrane proteins: (*a*) the clonotype antigen/MHC recognition unit, termed Tiα-β (or Tiγ-δ) heterodimer (1–3); (*b*) the highly homologous CD3γ, CD3δ, and CD3ε subunits (4–8); and (*c*) the structurally distinct CD3ζ and CD3η subunits, alternatively spliced products of a common genetic locus (9–12). FcεRIγ, an essential component of FcεRIγ and the transmembrane type FcγRIII (CD16) (13), has significant structural homology to CD3ζ and CD3η (9, 10, 14, 15) and is encoded on the same chromosome (mouse chromosome 1), suggesting that CD3ζ/η and FcεRIγ are derived from a common ancestral gene (11, 12, 16, 17). In addition, CD3ζ can substitute for FcεRIγ to form a high affinity IgE receptor on *Xenopus* oocytes injected with mRNAs for FcεRIα, FcεRIβ, and CD3ζ in the absence of FcεRIγ (18). Moreover, in the CTLL cell line, CD3ζ, CD3η, and FcεRIγ genes are coexpressed and their proteins form atypical disulfide-linked dimers in the TCR complex of that cell (19). These in vitro results suggested to us that subunits other than CD3ζ/η might be incorporated into a functional TCR. To investigate this possibility and determine whether heterogeneity in TCR signal transduction subunits exists within physiologic cell populations, we focused our attention on a subset of T lymphocytes with unusual phenotypic and functional attributes and the morphology of LGL. The results identify among splenocytes a population of IL-2-responsive T cells expressing a "nonconventional" functional TCR isoform containing an FcεRIγ homodimer in lieu of CD3ζ and η subunits.

Materials and Methods

Flow Cytometric Analysis. LGL cells (2–3 × 10^6 cells/ml) were stained with RM2-1 (anti-CD2; 10 μg/ml), 2C11 (anti-CD3ε; 10 μg/ml), H57-597 (anti-Tiα-β; 10 μg/ml), 3A10 (anti-Tiγ-δ; 10 μg/ml), GK1.5 (anti-CD4; 10 μg/ml), ADH4 (anti-CD8; culture supernatant), 2.4G2 (FcγRII/III; 10 μg/ml), and PK136 (NK1.1; culture supernatant) followed by FITC-conjugated second antibodies and analyzed on an Epics V cell sorter. Percent reactivities were 17% for CD2, 92% for CD3, 88% for Tiα-β, 0% for Tiγ-δ and CD4, 7% for CD8, 83% for FcγRII/III, and 88% for NK1.1. Note that the percent CD2 reactivity is misleading since essentially all LGL express low levels of CD2. Although most LGL are $CD8^-$, a small fraction of the cells (5–15%) were found to be $CD8^+$.

Polymerase Chain Reaction Analysis. For PCR analysis, a cDNA copy was produced from 15 μg of total cellular RNA using an oligo(dT) primer and AMV reverse transcriptase (Molecular Genetics Resources, Tampa, FL). 10% of the product was used as a template for PCR using the sense amplimer 5'GGTGCCATAGCTGGAGGAAC3' located at base pairs 470–488 of FcγRIII and the

J. Exp. Med. © The Rockefeller University Press • 0022-1007/92/01/0203/07 $2.00
Volume 175 January 1992 203–209

antisense amplimer 5'GGAGGCACATCACTAGGGAG3' at base pairs 738–714 in the transmembrane region of FcγRIII (numbers are according to reference 20). The PCR product of FcγRIII is a 269-bp fragment. To identify FcγRIIb$_1$ and FcγRIIb$_2$ the same sense amplimer was used with the antisense amplimer 5'GCAGCTTCTTCCAGATCAGG3', which lies at base pairs 1232–1213 of FcRγIIb$_1$, 3' to the 138-bp insertion found in FcRγIIb$_1$ as compared with FcRγIIb$_2$. Amplification of FcRγIIb$_1$ and FcRγIIb$_2$ cDNAs produce DNA fragments of 484 and 345 bp, respectively. For PCR, the denaturing, annealing, and extension were performed at 94°C for 1 min, 60°C for 1 min, and 72°C for 0.5 min, respectively, on a Techne thermocycler using the Gene Amp Kit reagents (Perkin Elmer Cetus, Norwalk, CT) for 35 cycles. The products were run on a 2% agarose gel, blotted to Zeta-Probe membrane (Bio-Rad Laboratories, Richmond, CA), and hybridized to the oligonucleotide 5'GCCTGTCACCATCACTGTCC3' at base pairs 642–661 of FcγRIII and base pairs 921–940 of FcγRIIb$_1$ and FcγRIIb$_2$. The oligonucleotide was labeled by 5' phosphorylation using polynucleotide kinase and γ-[^{32}P]ATP. Hybridization was performed in 6× SSC, 5× Denhardt's, 10 μg/ml denatured salmon sperm DNA, and 0.1% SDS at 54°C. The blot was then washed for 20 min in 6× SSC-0.1% SDS at the hybridization temperature and exposed at −70°C to Kodak X-Omat AR x-ray film.

RNA Analysis. Total RNA was isolated from cell lines using guanidine isothiocyanate (21). RNA concentrations were measured spectrophotometrically and 10 μg of total RNA was run per lane on a 1% agarose gel containing 2.2 M formaldehyde and 1× MOPS buffer (21). The RNA was then transferred to nitrocellulose in 20× SSC and the RNA blot hybridized to a rat FcεRIγ-specific cDNA probe comprising the 3' BamHI/XbaI fragment (14). Hybridization was carried out in a solution containing 50% formamide, 5× SSC, 5× Denhardt's, 250 μg/ml denatured salmon sperm DNA, 50 mM $NaHPO_4$, pH 6.5, at 42°C for 16–20 h with 10^6 cpm/ml probe. Subsequently, the blot was washed in 2× SSC, 0.1% SDS for 15–30 min at room temp and 0.1× SSC, 0.1% SDS at 50°C for 30 min, and was exposed to Kodak X Omat AR x-ray film at −70°C for the indicated time.

For RNase protection, pBSΔ17 was derived from the mouse CD3η cDNA clone pBS17 (10) as follows: a portion of the CD3η cDNA, the StyI/SmaI fragment, containing the 3' 681 bp of exon 9 was excised from pBS17. The StyI 5' overhang was filled with Klenow and the linearized plasmid was ligated to obtain pBSΔ17. Antisense RNA was synthesized from pBSΔ17 linearized with AccI using T3 polymerase, Riboprobe Gemini System kits (Promega Biotech, Madison, WI) and 100 μCi of [^{32}P]UTP according to previously described methods (22). Antisense RNA probe (5×10^5 cpm) and total cell RNA were hybridized in 30 μl of a solution consisting of 80% formamide, 0.4 M NaCl, 1 mM EDTA, 40 mM Pipes, pH 6.7, at 45°C. After 18 h, the RNAs were digested for 1 h at 30°C by adding 300 μl of a solution consisting of 5 mM EDTA, 0.3 M NaCl, 10 mM Tris/HCl, pH 7.5, and 4 μg/ml RNase T1. After proteinase K digestion, phenol/chloroform: isoamylalcohol extraction, and ethanol precipitation, half of each sample was analyzed on a 5% denaturing polyacrylamide gel.

Labeling, Immunoprecipitation, and Two-dimensional Reducing/Nonreducing SDS-PAGE Analysis. For metabolic labeling, 3×10^7 cells were suspended in methionine- and cysteine-free RPMI 1640 supplemented with 100 U/ml rIL-2 and 10% FCS (ICN Biochemicals, Irvine, CA), which had been dialyzed against PBS and labeled with Trans-^{35}S-Label for 3 h followed by 1 h chasing in regular media consisting of RPMI 1640 supplemented with 10% FCS and 100 U/ml rIL-2. Cells were then washed with 150 mM NaCl, 20 mM Tris/HCl, pH 7.4 (TBS), and lysed in digitonin lysis buffer solution (1% digitonin in TBS supplemented with 0.24 TIU/ml aprotinin, 1 mM PMSF, 5 μg/ml leupeptin, and 10 mM iodoacetamide). For cell surface labeling, 10^7 LGL were surface iodinated with 1 mCi of ^{125}I by the lactoperoxidase method and lysed in digitonin lysis buffer solution. Postnuclear supernatant was subjected to immunoprecipitation with mAb-coupled Sepharose beads or protein A-Sepharose beads precoated with rabbit antibodies. After extensive washing, the immunocomplex was eluted by boiling in Laemmli's nonreducing sample buffer solution (23). Samples were resolved on two-dimensional (2-D)[1] nonreducing/reducing diagonal gels, and labeled proteins were detected by autoradiography or fluorography. Gels were exposed to X-Omat AR x-ray films for 4 d. Molecular weight markers were OVA (44K), carbonic anhydrase (28K), lactoglobulin (18K), lysozyme (13K), and bovine trypsin inhibitor (7K).

Cytotoxic Assay. Target cells were labeled with ^{51}Cr (100 μCi/10^6 cells) for 1 h at 37°C. Targets were then washed three times and added to V-bottomed microtiter plates at 5,000 cells/well in RPMI 1640 containing 10% FCS and 100 U/ml rIL-2. Effector cells were added at the indicated ratios in a final volume of 180 μl. Plates were centrifuged at 800 rpm for 5 min and then incubated for 4 h at 37°C. After recentrifugation at 2,000 rpm for 2 min, 90 μl was removed from each well for assay of gamma radioactivity. Percent specific lysis was calculated according to the formula: $100\times [(E - C)/(M - C)]$; where E is the experimental value in cpm, C is the control value, and M is the maximum release value. C was determined as the average release in control wells from which effector cells were omitted. M was determined as the average release in wells to which 1% NP-40 was added in place of effector cells. All determinations were performed in triplicate.

Results and Discussion

Splenocytes from B10.BR and C3H/HeJ mice cultured for several weeks in the presence of rIL-2 as described (24) express both Tiα-β and CD16. This result is of note since these two structures are mutually exclusive in cellular distribution with rare exception (25, 26). As shown in Fig. 1 *A*, IL-2-dependent LGL from B10.BR are CD2$^+$, CD3$^+$, Tiα-β$^+$, Tiγ-δ$^-$, CD4$^-$, CD8$^-$, FcγR$^+$, NK1.1$^+$. The phenotype of LGL from C3H/HeJ is identical except for the absence of the allelic NK1.1 marker, which is not expressed in the C3H/HeJ strain (27). Because antibody 2.4G2 does not distinguish between FcγRII and FcγRIII, PCR analysis was used. PCR with specific oligonucleotide amplimers shows that FcγR on the LGL are exclusively of the FcγRIII isotype, the mouse homologue of CD16 (Fig. 1 *B*). In contrast, S49 thymoma cells express both FcγRII and FcγRIII (Fig. 1 *B*) while Cl.MC/57.1 mast cells express readily detectable FcγRIII (Fig. 1 *B*) and, on longer exposures of the autoradiogram, FcγRII (data not shown). Flow cytometric analysis using mAbs against three different Vβ gene products showed that the LGL are polyclonal (Table 1). Consistent with this finding, we detected usage of multiple Jβ segments in both Vβ6$^+$ and Vβ8$^+$ populations using PCR analysis with amplimers for Vβ and Jβ regions (L. D'Adamio, manuscript submitted for publication) (data not shown). These results indicated that the LGL populations are not restricted to a

[1] *Abbreviation used in this paper:* 2-D, two-dimensional.

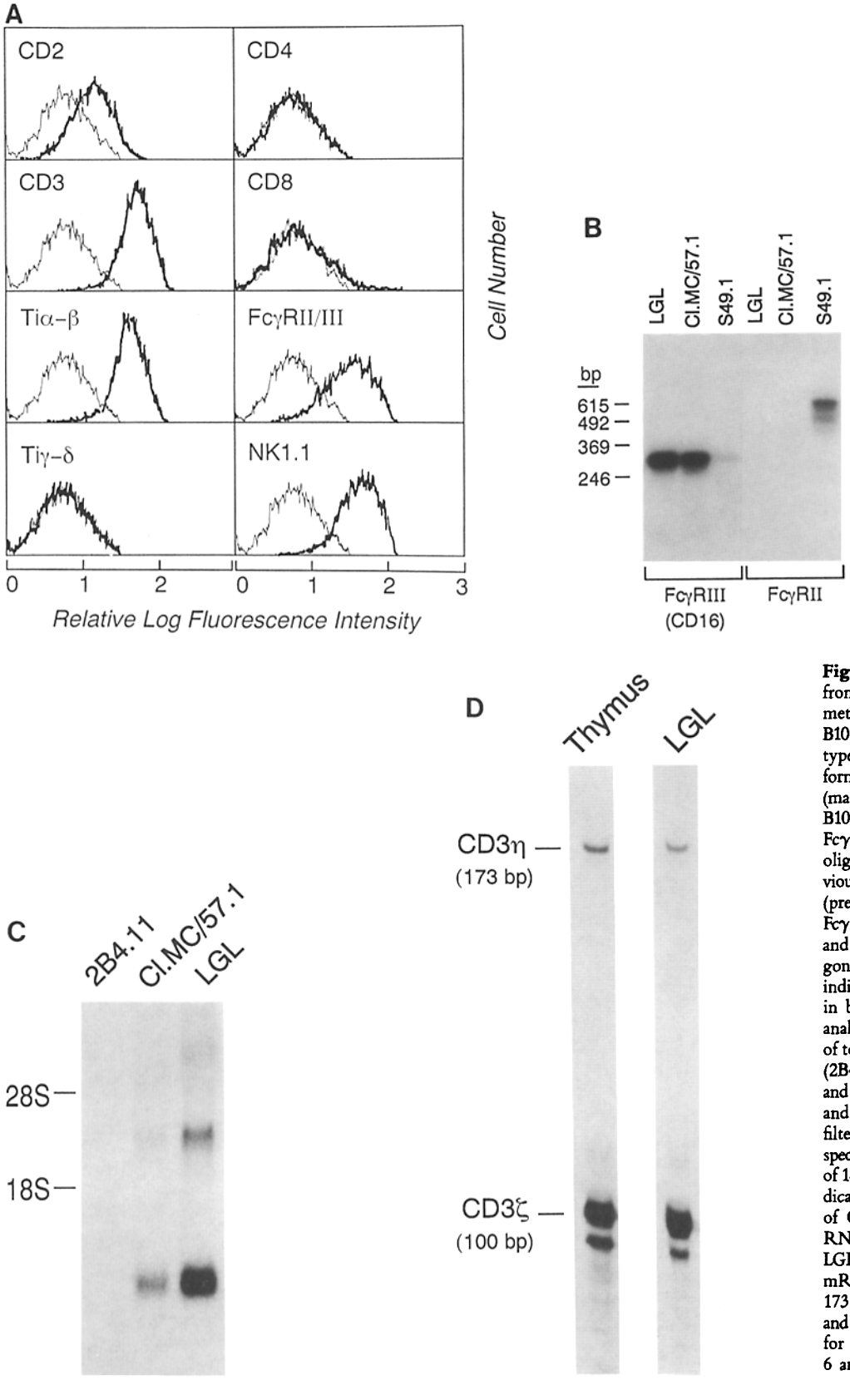

Figure 1. Characterization of LGL from B10.BR mice. (*A*) Flow cytometric analysis of surface antigens on B10.BR LGL. (*B*) Analysis of FcγR isotype by PCR. Reverse PCR was performed with RNAs from Cl.MC/57.1 (mast cell line; FcγRII^{low}, FcγRIII^{high}), B10.BR LGL, and S49 (T lymphoma; FcγRII^{high}, FcγRIII^{low}) using specific oligo primers to FcγRIII (CD16; previously denoted as FcγRIIa) or FcγRIIb (previously denoted as FcγRIIb1 and FcγRIIb2). PCR products were blotted and probed with specific internal oligonucleotides. Numbers on the left side indicate the molecular weight markers in base pairs. (*C*) Northern blotting analysis of FcεRIγ expression. 10 μg of total RNA from a T cell hybridoma (2B4.11), a mast cell line (Cl.MC/57.1), and B10.BR LGL were size fractionated and transferred onto a nitrocellulose filter. The filter was hybridized with a specific probe for FcεRIγ. The positions of 18S and 28S ribosomal RNAs are indicated. (*D*) RNase protection analysis of CD3ζ/η mRNA. 15 μg of total RNA from thymocytes and B10.BR LGL were analyzed for CD3ζ/η mRNAs by RNase protection analysis. 173 and 100 bp signals represent CD3η and CD3ζ, respectively. Exposure times for RNAs from thymus and LGL are 6 and 15 h, respectively.

Table 1. *Vβ Usage in LGL Determined by Flow Cytometry*

LGL	Vβ usage		
	Vβ8	Vβ6	Vβ3
		%	
B10.BR no. 1	77	<1	3
B10.BR no. 2	16	41	<1
C3H/HeJ	43	<1	<1

Cells were stained with mAbs F23.1 (anti-Vβ8), 44.22.1 (anti-Vβ6), and KJ25 (anti-Vβ3), and FITC-conjugated second antibody. The fraction of cells specifically expressing each Vβ was determined by flow cytometry using an irrelevant IgG mAb as a control.

unique clonotype. The LGL herein have a similar phenotype to a population of thymus-dependent cells distinct from NK cells described by Pardoll and colleagues (28) in both thymus and spleen. The high percentage of Vβ8 usage in the above LGL is consistent with the finding that the Vβ8 family is used at a greater frequency in the Tiα-β$^+$, CD4$^-$, CD8$^-$, NK1.1$^+$ subpopulation than in other T cells (28). Although not tested, it is likely that the previously described Tiα-β$^+$, NK1.1$^+$ population (28) expresses CD16 and represents <1% of total splenic T cells (28).

Because CD16 expression requires the FcεRIγ subunit (29), we examined LGL for FcεRIγ mRNA. As shown in Fig. 1 *C*, the amount of steady-state 0.7-kb FcεRIγ mRNA in the LGL is equivalent to or greater than that of the mast cell line C1.MC/57.1. To specifically address whether FcεRIγ might also be a component of the TCR on LGL, cells were

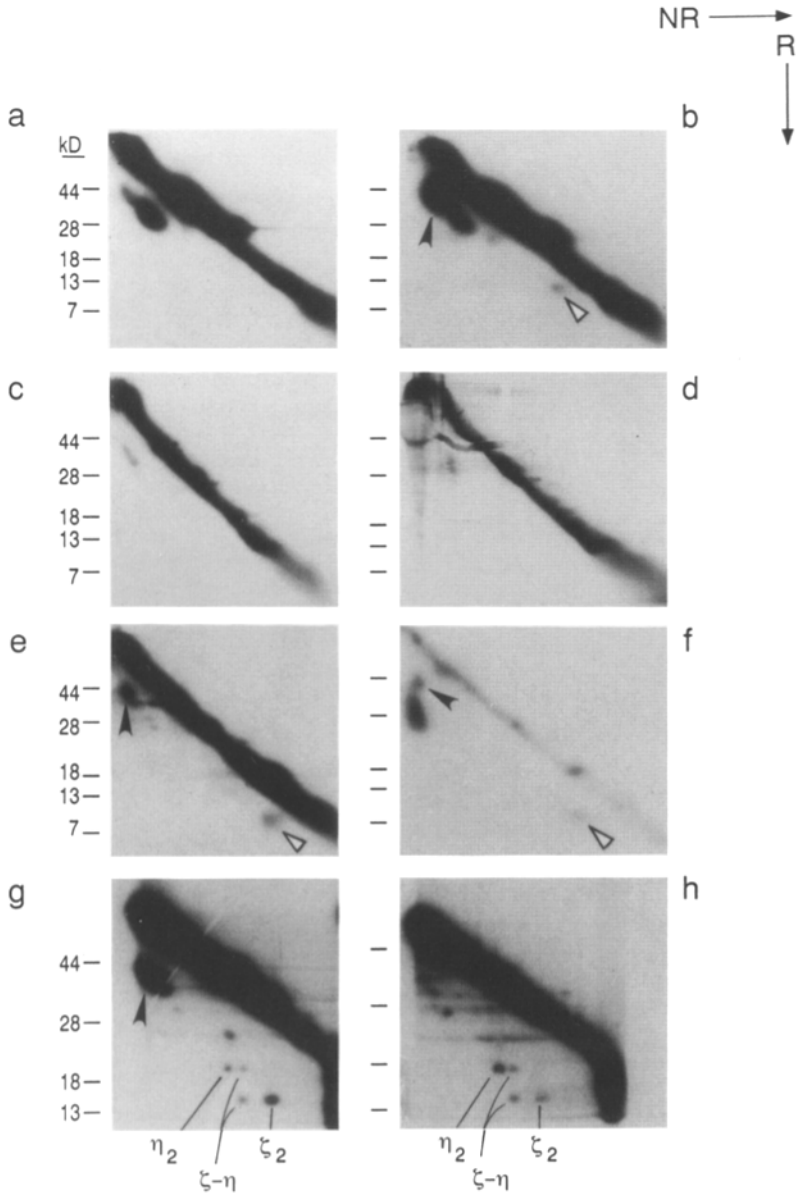

Figure 2. Characterization of the TCR complex expressed on LGL. Metabolically labeled (*a–e*) and surface iodinated (*f*) LGL and metabolically labeled MAζ-η301 (*g* and *h*) were lysed in digitonin lysis buffer solution and immunoprecipitated with (*a*) 3A10 (hamster mAb against Tiγ-δ), (*b* and *g*) 2C11 (hamster mAb against CD3ε), (*c*) normal rabbit serum, (*d* and *h*) rabbit antiserum no. 386 against CD3ζ/η (30), or (*e* and *f*) rabbit antibody against human FcεRIγ (19). Proteins were resolved in 2-D nonreducing/reducing SDS-PAGE followed by autoradiography (*a–e*, *g*, and *h*) or fluorography (*f*). Closed and open arrowheads indicate positions of Tiα-β and FcεRIγ, respectively.

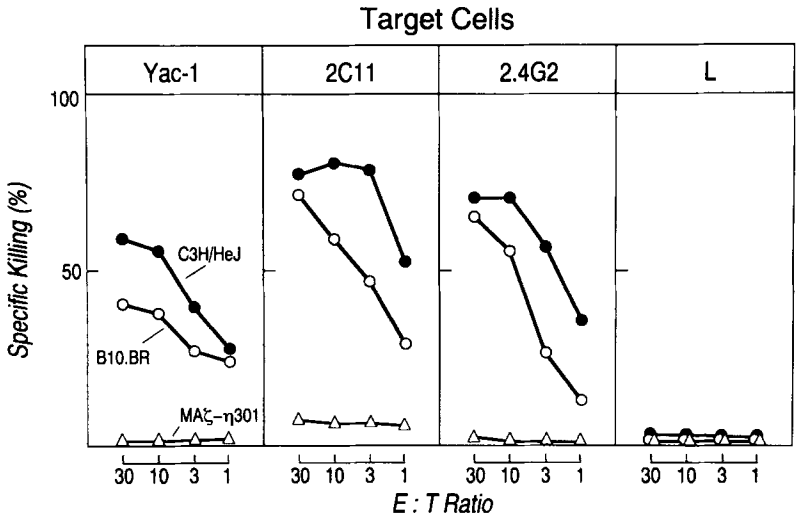

Figure 3. Cytotoxic activity of LGL. Cytotoxic activity of B10.BR LGL no. 2 (○), C3H/HeJ LGL (●), and MAζ-η301 (△) were analyzed by standard 4-h ^{51}Cr release assay with the indicated E/T ratios.

metabolically labeled with ^{35}S-methionine/cysteine and immunoprecipitation was performed with either the anti-CD3ε mAb, 2C11, anti-CD3ζ/η antibody (30), or anti-FcεRIγ antibody (19). As shown in Fig. 2, 2C11 precipitates the Tiα-β heterodimer as an off-diagonal spot in a nonreducing/reducing 2-D-diagonal gel. Surprisingly, however, no CD3ζ/η dimers (CD3ζ/ζ, CD3ζ/η, or CD3η/η) are observed in 2C11 or anti-CD3ζ/η immunoprecipitates (Fig. 2, *b* and *d*, respectively). This contrasts with the results from T cells expressing "conventional" TCR subunits containing CD3ζ/η homo- or heterodimers (Fig. 2, *g* and *h*). Instead of CD3ζ/η, we observe a disulfide-linked homodimer of molecular weight ~9,000 associated with TCR (Fig. 2 *b*). This low molecular weight structure represents FcεRIγ dimers as shown by the fact that rabbit anti-FcεRIγ antibody precipitated both the FcεRIγ homodimer and Tiα-β heterodimer (Fig. 2 *e*). Furthermore, the same dimers are precipitated from surface-iodinated LGL cells by the anti-FcεRIγ antibody (Fig. 2 *f*). Identical results are obtained with each of the LGL in Table 1. Thus, we conclude that LGL express on their cell surface a novel type of TCR complex in which an FcεRIγ homodimer substitutes for CD3ζ and/or CD3η dimers. The absence of CD3ζ/η proteins is not a consequence of a weak association between the TCR and CD3ζ/η dimers in these cells since direct immunoprecipitation with rabbit anti-CD3ζ/η antibody also failed to identify CD3ζ/η dimers (Fig. 2 *d*).

Of particular interest, RNase protection analysis identifies the presence of CD3ζ and CD3η mRNAs in the LGL (Fig. 1 *D*), and these mRNAs are of the appropriate size as judged by RNA blots (data not shown). This discordance between CD3ζ/η mRNA and protein expression is striking but not without precedent. We have previously shown that the level of CD3ζ/η proteins increases during T cell differentiation despite a decrease in the steady-state level of their mRNAs, demonstrating that expression is controlled, at least in part, by a posttranscriptional mechanism (12). The lack of detectable CD3ζ/η protein in LGL expressing CD3ζ/η mRNAs defines yet another likely posttranscriptional control mechanism. Given that CD3ζ-FcεRIγ and CD3η-FcεRIγ heterodimers but not FcεRIγ homodimers have been described in CTLL (19), the regulation of dimer expression among these subunits is likely to be complex.

To examine whether this novel TCR is functional in LGL, cells were analyzed for cytotoxic activity and IL-2 production after receptor crosslinking. As shown in Fig. 3, LGL show a strong cytotoxic activity against two B cell hybridomas, one expressing an anti-CD3ε mAb (2C11) and a second expressing anti-CD16 mAb (2.4G2). Unlike with 2C11 or 2.4G2 hybridomas, no significant killing of NS1 or PC61 producing an anti-IL-2Rα (p55) mAb was observed (data not shown). LGL also show spontaneous cytotoxic activity against the NK-sensitive target YAC-1 cells but failed to lyse L cells. In contrast, none of the targets are killed by a helper T cell hybridoma, MAζ-η301 (31), These results indicate that CD16 as well as the novel TCR containing FcεRIγ can transit signals leading to cytotoxic activity of LGL. However, unlike MAζ-η301, LGL produce no significant level of IL-2 when incubated in wells precoated with anti-CD3ε mAb (data not shown).

In conclusion, FcεRIγ homodimers can substitute for CD3ζ and CD3η homo- or heterodimers in targeting partially assembled Ti α-β/CD3γδε TCR complexes to the cell surface and transducing stimulatory signals after TCR triggering (31–36). Thus, TCRs can exist in multiple isoforms being comprised of various disulfide-linked dimers of the CD3ζ/η-FcεRIγ family (19, 32, 35, 36). Although it is not known whether the signal transduction properties of FcεRIγ-containing TCRs are distinct from conventional CD3ζ- and CD3η-containing TCRs, this is a likely possibility. It is also noteworthy that members of CD3ζ/η-FcεRIγ family can dimerize differentially in other receptor complexes. For example, human NK cells express CD3ζ as well as FcεRIγ in association with CD16 in the absence of other TCR components (Tiα, Tiβ, CD3γ, CD3δ, CD3ε) (37–39). It is now critical to ascertain the functional attributes of the various CD3ζ/η-FcεRIγ dimers.

We are grateful to Drs. J.-P. Kinet, R. D. Klausner, H. Yagita, R. Kubo, S. Tonegawa, K. Rock, H. Reiser, and J. Bluestone for mAbs. We also thank A. Diener, and B. Yandava for technical assistance and Dr. H.-R. Rodewald for PCR amplimers.

This work was supported in part by National Institutes of Health grants AI-19807, AI-21226, AI-31269, and AI-27336.

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Received for publication 20 June 1991 and in revised form 27 August 1991.

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