The CD3ζ Cytoplasmic Domain Mediates CD2-induced T Cell Activation

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Summary

CD2-mediated T lymphocyte activation requires surface expression of CD3-Ti, the T cell receptor (TCR) for antigen major histocompatibility complex protein. Given the importance of CD3ζ in TCR signaling, we have directly examined the ability of the CD3ζ cytoplasmic domain to couple CD2 to intracellular signal transduction pathways. A cDNA encoding a chimeric protein consisting of the human CD3ζ cytoplasmic domain (amino acid residues 31–142) fused to the CD8α extracellular and transmembrane domains (amino acid residues 1–187) was transfected into a $CD2^+CD3^-CD8^-$ variant of the human T cell line Jurkat. The resulting transfectants expressed the CD8α/CD3ζ chimeric receptor at the cell surface in the absence of other TCR subunits. Stimulation of these transfectants with anti-$T11_2$ + anti-$T11_3$ monoclonal antibodies (mAbs) initiated both a prompt cytosolic free calcium ($[Ca^{2+}]_i$) rise and protein tyrosine kinase activation. Stimulation with either intact anti-$T11_2$ + anti-$T11_3$ mAbs or purified $F(ab')_2$ fragments resulted in interleukin 2 (IL-2) secretion. In contrast, control cell lines transfected with a cDNA encoding wild-type CD8α, and thus lacking surface expression of the CD3ζ cytoplasmic domain, failed to show any $[Ca^{2+}]_i$ rise, protein tyrosine kinase activation, or IL-2 secretion after identical stimulation. These data directly establish the CD3ζ cytoplasmic domain as a necessary and sufficient component of the CD3-Ti complex involved in T lymphocyte activation through CD2. Moreover, they show that CD2 signaling can function in the absence of Fc receptors.

T lymphocytes can be activated via the TCR (CD3-Ti) or by triggering through a number of other surface structures, including CD2 in humans and Thy-1 in mice (1, 2). The same second messenger pathways are activated by stimulation via anti-CD2 mAbs or anti-TCR mAbs (3–6). In addition, CD2-mediated T lymphocyte activation requires the presence of CD3-Ti at the cell surface (7–9). Both the TCR dependence of CD2 triggering and the similarities in activation events triggered through CD2 or TCR imply a functional interaction between CD2 and the CD3-Ti complex. However, the role of the CD3-Ti subunits in coupling CD2 to signal transduction mechanisms is unknown (10). A substantial body of evidence has documented a central role for CD3ζ in TCR-mediated triggering (11, 12). More recent work has shown that a CD3ζ chimeric protein is sufficient by itself to trigger early and late T cell activation events (13–15). We have employed a similar strategy to directly investigate the role of CD3ζ in mediating CD2 signaling. Here we show that expression of the cytoplasmic domain of CD3ζ as a CD8α/CD3ζ chimera at the cell surface in the absence of other TCR or FcR components is both necessary and sufficient to allow CD2-mediated T cell activation.

Materials and Methods

Cell Lines. J77 is a $CD2^+CD3^+Ti^+CD8^-CD16^-$ derivative of the leukemic human T cell line Jurkat. 31-13 is a $CD2^+CD3^-Ti^-$ mutant of J77 lacking a functional Tiβ subunit (8). These lines were maintained at 37°C in culture medium (RPMI 1640 + 10% FCS, 1 mM sodium pyruvate, 2 mM L-glutamine, 50 U/ml penicillin-streptomycin) under 5% CO_2. WT-6, derived by transfection of 31-13 with J77 Tiβ cDNA, thereby restoring surface $CD3^+Ti^+$ expression (8), was maintained in culture medium supplemented with 0.5 mg/ml G418 (Gibco/BRL, Gaithersburg, MD).

Antibodies. The specificities, designations, and isotypes of the murine mAbs used in this study are as follows: anti-CD2: anti-$T11_1$, 3T4-8B5, IgG2a; anti-$T11_2$: 1Old2-4C1, IgG2; anti-$T11_3$: 1Mono2A6, IgG3; anti-CD3: 2Ad2A2, IgM, or RW28C8, IgG1; anti-CD4: 19Thy5D7, IgG2; anti-CD8: 21Thy2D3, IgG1. N23 is a rabbit anti-human CD3ζ antiserum raised against the human CD3ζ COOH-terminal peptide CDTYDALHMQALPPR conjugated to BSA using sulfo-MBS (Pierce Chemical Co., Rockford, IL) according to the supplier's directions and shown to immunoprecipitate and Western blot human CD3ζ. 4G10 antiphosphotyrosine murine mAb was kindly provided by Dr. Brian Druker (DFCI, Boston, MA) (15). Purified anti-$T11_2$ and anti-$T11_3$ $F(ab')_2$ fragments were prepared as described (16).

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Chimera Generation. Using PCR techniques, the complete coding region of human CD8α was amplified from clone pL2-M (17) (kindly provided by Dr. Jane Parnes, Stanford University, Stanford, CA) using oligonucleotides containing a 5′ BglII recognition sequence (CD8α 5′ sense oligonucleotide: nucleotides 88–108 of reference 17, 5′GCGAAGATCTTTGGCCATGGCCTTACCAGTGACCGCC3′; CD8α 3′ antisense oligonucleotide: nucleotides 773–795 of reference 17, 5′CGCGAGATCTTTAGACGTATCTCGCCGAAAGGC3′). After gel purification and digestion with BglII, the fragment was subcloned into the BamHI site of the expression vector pPink2 (18) creating a construct termed pCD8α/Pink2 for use in transfection (see below). Similarly, a chimeric cDNA encoding the NH_2-terminal 187 amino acids of CD8α fused to amino acid residues 31–142 of human CD3ζ clone was generated by annealing overlapping PCR fragments encompassing these regions amplified from CD8α (clone pL-2M) and CD3ζ (clone pGEM3zζ (19), kindly provided by Drs. Allan Weissman and Richard Klausner, National Institutes of Health, Bethesda, MD), respectively (CD8α chimera 5′ sense oligonucleotide identical to CD8α 5′ sense oligonucleotide listed above; CD8α/CD3ζ overlap antisense oligonucleotide: 5′GCGCTCCTGCTGAACTTCACTCTGTGGTTGCAGTAAAGGG3′; CD8α/CD3ζ overlap sense oligonucleotide: 5′GGTTATCACCCTTTACTGCAACCACAGAGTGAAGTTCAGCAGG3′; CD3ζ3′ antisense oligonucleotide nucleotides 545–566 from reference 19: 5′CGCGAGATCTTTAGCGAGGGGGCAGGGCCTGC3′). The two overlapping PCR fragments were annealed and filled in using the Klenow fragment of DNA polymerase, and 10% of the products from this reaction were subjected to PCR amplification with CD8α 5′ sense and CD3ζ3′ antisense oligonucleotides to yield the chimeric CD8α/CD3ζ PCR fragment. After gel purification and BglII digestion, the fragment was subcloned into the BamHI site of pPink2 resulting in a construct termed pCD8α/CD3ζ/Pink2. Identity of the resulting plasmids was verified by restriction enzyme analysis and DNA sequencing.

Transfections and Selection. pCD8α/CD3ζ/Pink2 and pCD8α/Pink2 were each transfected into 31-13 cells by electroporation using a cell porator system (Gibco/BRL) with a capacitance set at 330 μF. Transfectants were selected in culture medium supplemented with 1.0 mg/ml G418. Resistant clones were further selected for CD8 surface expression by indirect immunofluorescence analysis (16). Positive clones were maintained in culture medium containing 0.5 mg/ml G418.

Cell Surface Labeling, Immunoprecipitation, and Electrophoresis. 10^8 cells were washed four times in ice-cold PBS (0.9% NaCl, 1 mM KCl, 15 mM $NaPO_4$, pH 7.5), resuspended in 0.5 ml PBS, and labeled with 2 mCi $Na^{125}I$ (NEN/Dupont, Wilmington, DE) using the lactoperoxidase method. After washing three times with cold TBS (25 mM Tris, pH 7.5, 0.9% NaCl, 1 mM KCl), cells were lysed in 1% digitonin lysis buffer as described (20). Postnuclear supernatant from 2 × 10^7 cells was immunoprecipitated by rotating overnight at 4°C with 60 μl mAb-coupled protein A–Sepharose beads (~2–4 mg mAb/ml beads) in the case of CD2, CD3, CD4, and CD8, and 5 μl antiserum and 60 μl protein A–Sepharose beads in the case of anti-CD3ζ or nonimmune rabbit serum. Antigen-antibody-bead complexes were washed, solubilized, and analyzed by SDS-PAGE as described (20). Western blots were performed as described (20), except that alkaline phosphatase–conjugated goat anti-rabbit IgG (Bio-Rad Laboratories, Richmond, CA) was used as the second step. Bands were visualized after developing the blots according to the supplier's directions.

Protein Tyrosine Kinase Activation. Aliquots of 10^7 cells, washed in HBSS (Gibco/BRL) at 25°C, were resuspended in 0.5 ml HBSS at 37°C and stimulated with a 1:100 dilution of the indicated antibody at 37°C. Stimulation was quenched at various times by adding 0.5 ml ice-cold 2× lysis buffer (2% Triton X-100 in 50 mM Tris, pH 7.5, 20 mM EDTA, 10 mM NaF, 2 mM Na_3VO_4, 10 mM $Na_4P_2O_7$) for 25 min followed by centrifugation. Baseline (time 0) samples were obtained by adding stimulating antibody after quenching cell suspensions with 2× lysis buffer. 50 μl (10%) of the resulting postnuclear lysates was solubilized in 2× reducing Laemmli sample buffer and analyzed by 7.5–17.5% gradient SDS-PAGE and Western blotting with 4G10 antiphosphotyrosine mAb as described above. The remaining lysate material was immunoprecipitated with anti-CD3ζ, resolved by 7.5–17.5% gradient SDS-PAGE and analyzed by Western blotting with 4G10 as described (20), using biotinylated goat anti–mouse IgG (Sigma Chemical Co., St. Louis, MO) followed by streptavidin plus biotinylated alkaline phosphatase as a second step (Bio-Rad Laboratories).

Cytosolic Free Calcium ($[Ca^{2+}]_i$[1]) Measurement. After overnight incubation in G418-free culture medium, aliquots containing 2 × 10^6 cells were resuspended in 0.5 ml culture medium containing 2 μg/ml of the acetoxymethyl ester of indo-1 (Molecular Probes, Eugene, OR) for 45 min at 37°C. Subsequently, cells were pelleted and resuspended in 2 ml culture medium. Fluorescence measurements were made using a fluorimeter (SFM25; Kontron, Zurich, Switzerland) with excitation and emission wavelengths of 350 and 405 nm, respectively. Baseline fluorescence was monitored for 3 min, after which a 1:200 dilution of antibody was added and fluorescence monitored for 15 min. The cells were kept in suspension by constant stirring. At the end of each analysis, maximal fluorescence was determined by adding Triton X-100 to 0.05% and minimal fluorescence determined after addition of EGTA to 2.5 mM. $[Ca^{2+}]_i$ was calculated as described (21).

Determination of IL-2 Production. 10^5 cells were plated in 96-well round-bottomed plates (final volume, 0.2 ml) and stimulated at 37°C for 24 h in the presence of 5 ng/ml PMA with either anti-CD3 (2Ad2A2), anti-$T11_2$ (1Old2-4C1) plus anti-$T11_3$ (1Mono2A6), anti-CD8 (21Thy2D3), or calcium ionophore (A23187 at 1 μg/ml final concentration; Sigma Chemical Co.). All mAbs were used either as ascites at saturating concentration (1:200 final) or in purified form at 10 μg/ml final concentration. Serial dilutions of culture supernatants were then tested for their capacity to support proliferation of 10^4 CTLL-20 cells as described (22).

Results and Discussion

Transfection of the Jurkat $CD3^-$ mutant line 31-13 resulted in 12 independently derived surface CD8-expressing clones for each cDNA denoted 31-13.α for plasmid pCD8α/Pink2 or 31-13.αζ for pCD8α/CD3ζ/Pink2, respectively. Flow cytometric analysis of representative clones for each construct are shown in Fig. 1. Whereas J77, 31-13, and WT-6 (a 31-13 transfectant in which CD3-Ti surface expression has been restored after transfection of a wild-type Tiβ cDNA [8]) express no detectable CD8, 31-13 transfectants containing either full-length CD8α cDNA (31-13.α2) or chimeric CD8α/CD3ζ cDNA (31-13.αζ6, 31-13.αζ11) expressed readily detectable CD8 at the surface. The expression level of CD8 was reproducibly greater in the native CD8α-containing lines as compared with clones expressing CD8α/CD3ζ chi-

[1] *Abbreviation used in this paper:* $[Ca^{2+}]_i$, cytosolic free calcium.

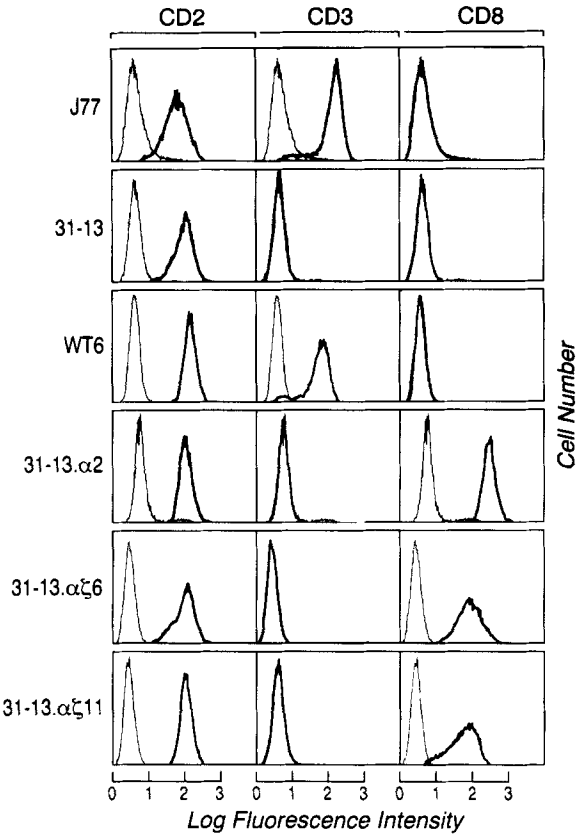

Figure 1. Surface phenotype of Jurkat derivatives and transfectants. Immunofluorescence analysis of Jurkat derivatives J77, 31-13 ($CD3^-/Ti^-$), WT-6 ($CD3^+/Ti^+$), 31-13.α2 ($CD3^-/Ti^-$,$CD8\alpha^+$), 31-13.αζ6 ($CD3^-/Ti^-$,CD8α/CD3ζ$^+$), and 31-13αζ11 ($CD3^-/Ti^-$,CD8α/CD3ζ$^+$). Histograms display reactivity with the indicated murine anti-human mAb (*thick line*) as compared with second step alone (*thin line*).

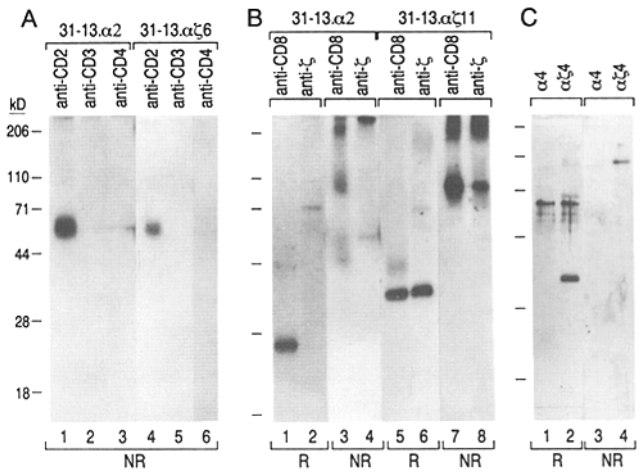

Figure 2. Biochemical characterization of 31-13 transfectants. Surface iodinated cells were lysed in digitonin containing lysis buffer and immunoprecipitated with the indicated antibody-conjugated beads (anti-CD2, anti-CD3, and anti-CD8) or antibody-coated protein A–Sepharose (anti-CD4, rabbit anti-CD3ζ). Immunoprecipitates were dissolved in Laemmli SDS sample buffer, and analyzed by SDS-PAGE (12.5% acrylamide) and subsequent autoradiography. Each lane contains material from ~10^7 cells. (*A*) Analysis of CD8α (31-13.α2)- and CD8α/CD3ζ (31-13.αζ6)-expressing transfectants under nonreducing conditions after immunoprecipitation with anti-CD2, anti-CD3, and anti-CD4. (*B*) Analysis of CD8α (31-13.α2) and CD8α/CD3ζ (31-13.αζ11) transfectants under reducing and nonreducing conditions after immunoprecipitation with anti-CD8 or rabbit anti-CD3ζ (antiserum N23) as indicated. (*C*) Western blot analysis of CD8α (31-13.α4)- and CD8α/CD3ζ (31-13.αζ4)-expressing transfectants. Anti-CD8 immunoprecipitates of 31-13.α4 and 31-13.αζ4 were transferred to nitrocellulose after SDS-PAGE and probed with the rabbit anti-CD3ζ antiserum N23 as described in Materials and Methods.

meric protein. Neither 31-13 nor any of the transfectant clones containing CD8α or chimeric CD8α/CD3ζ expressed surface CD3, while all lines expressed comparable levels of surface CD2 (Fig. 1).

To characterize the CD8α and CD8α/CD3ζ proteins expressed on the surface of the above transfectants, cells were immunoprecipitated with antibodies to CD8, CD3ζ, CD2, CD3ε, and, as a control, CD4 after surface iodination and digitonin lysis. Nonreducing SDS-PAGE analysis of anti-CD2 immunoprecipitates revealed the characteristic 55-kD band in both CD8α- and CD8α/CD3ζ-expressing clones (31-13.α2 and 31-13.αζ6; Fig. 2 *A*, lanes *1* and *4*). In contrast, CD3 and CD4 (as a negative control) were undetectable on these cell lines (Fig. 2 *A*, lanes *2*, *3*, *5*, and *6*). Immunoprecipitation of clone 31-13.α2 with anti-CD8 revealed a single band of 24 kD under reducing conditions (Fig. 2 *B*, lane *1*). This agrees with the predicted size for unglycosylated CD8α (17) and suggests that, in accordance with prior observations (13), Jurkat and its variants may be deficient in their ability to perform certain aspects of posttranslational modification of CD8α. Under nonreducing conditions, higher molecular mass multimers were observed, consistent with the known pattern for CD8α (31-13.α2; Fig. 2 *B*, lane *3*). A species of 33 kD, consistent with the size predicted for an unglycosylated CD8α/CD3ζ chimeric protein, was observed in anti-CD8 immunoprecipitates of CD8α/CD3ζ transfectants as shown for clone 31-13.αζ11 under reducing conditions (Fig. 2 *B*, lane *5*). Parallel analysis of immunoprecipitates of this clone with rabbit anti-CD3ζ revealed a 33-kD band with similar mobility to the band immunoprecipitated with anti-CD8α (Fig. 2 *B*, lane *6*). Immunoprecipitation analysis of clones 31-13.αζ4 and 31-13.αζ6 yielded results in agreement with those for 31-13.αζ11 (data not shown). The identity of the 33-kD species as the chimeric CD8α/CD3ζ protein was confirmed by Western blot analysis of anti-CD8α immunoprecipitates of 31-13.αζ4 with rabbit anti-CD3ζ (Fig. 2 *C*, lane *2*). Under nonreducing conditions, anti-CD8 and rabbit anti-CD3ζ immunoprecipitates of transfectants expressing CD8α/CD3ζ revealed a major species of ~100 kD (Fig. 2 *B*, lanes 7 and *8*), which would correspond to the size predicted for a homotrimer. This band was also apparent in anti-CD8 immunoprecipitates subjected to nonreducing SDS-PAGE and Western blot analysis with rabbit anti-CD3ζ (31-13.αζ4; Fig. 2 *C*, lane *4*). Note that the ~60-kD material seen under nonreducing conditions is nonspecific, as is evident in all nonreducing sample lanes (Fig. 2 *C*, lanes *1* and *2*; and data not shown). Given the absence of other specific

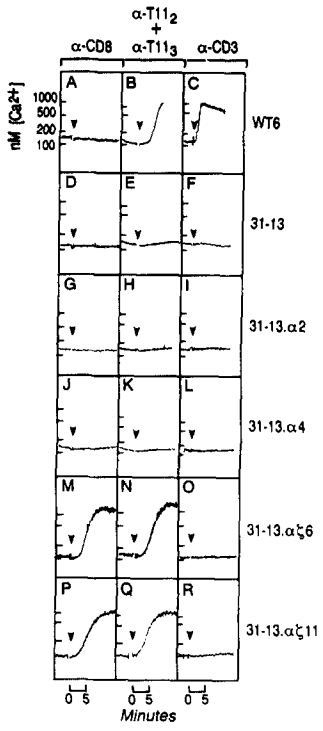

Figure 3. Calcium mobilization after receptor crosslinking in Jurkat derivatives and transfectants. Cell lines loaded with Indo-1 were stimulated (*arrowhead*) with 1:200 dilutions of ascites containing anti-CD8 (21Thy2D3), anti-CD2 (anti-T11$_2$ [1Old2-4C1] + anti-T11$_3$ [1Mono2A6]), or anti-CD3 (2Ad2A2) as indicated. Fluorescence emission at 405 nm was monitored continuously for >3 min before stimulation and for >12 min afterwards. $[Ca^{+2}]_i$ was determined as described in Materials and Methods. These results are representative of seven independently derived CD8α/CD3ζ chimeras expressing 31-13 transfectants and seven independently derived full-length CD8α-expressing 31-13 transfectants.

bands on one-dimensional analysis with anti-CD3ζ under reducing conditions (Fig. 2 *B*; and data not shown), we conclude that the higher molecular mass species seen under nonreducing conditions represent homo-multimers.

The ability of the chimeric protein to participate in transducing T cell activation signals was assessed by monitoring $[Ca^{+2}]_i$ mobilization as a function of surface receptor crosslinking. As shown for WT-6 ($CD2^+CD3^+CD8^-$), addition of an anti-CD3 antibody or a mitogenic combination of anti-CD2 antibodies results in a prompt rise in intracellular calcium (Fig. 3, *B* and *C*). As expected, anti-CD8 did not elicit a calcium rise in these cells (Fig. 3 *A*), even when rabbit anti-mouse Ig was added as a second step (data not shown). Moreover, stimulation of the $CD2^+CD3^-$ parental line 31-13 with anti-CD2, CD3, or CD8 mAbs failed to mobilize calcium (Fig. 3, *D*, *E*, and *F*). No calcium rise was observed in cell lines expressing the full-length CD8α protein after stimulation with anti-CD8 (Fig. 3, *G* and *J*). In contrast, after anti-CD8 treatment of CD8α/CD3ζ-expressing clones, a prompt rise in intracellular calcium was detectable within 3 min (Fig. 3, *M* and *P*). These results demonstrate the importance of the CD3ζ subunit in mediating signaling and are entirely consistent with previous observations (13–15).

We next determined whether the CD8α/CD3ζ structure could functionally substitute for the entire CD3-Ti complex and allow T cell activation through CD2. As shown in Fig. 3, CD2 crosslinking with anti-T11$_2$ plus anti-T11$_3$ resulted in a prompt increase in $[Ca^{2+}]_i$ in the case of CD8α/CD3ζ chimera–containing clones (shown for 31-13.αζ6 and 31-13.αζ11; Fig. 3, *N* and *Q*), similar to that seen in WT-6 (*B*). In contrast, no calcium rise was detected in transfectants expressing full-length CD8α (31-13.α2; Fig. 3, *H* and *K*). The lack of detectable calcium mobilization in response to anti-CD3 in 31-13 and all CD8α and CD8α/CD3ζ transfectants as well as the $CD3^-$ parental line (Fig. 3, *F*, *I*, *L*, *O*, and *R*) is consistent with the absence of surface CD3. Crosslinking with anti-CD4 failed to elicit a detectable calcium rise in any

Table 1. *IL-2 Production by CD8α/CD3ζ Chimera–expressing Cell Lines*

Exp.	Cells	IL-2 production					
		Media	Anti-CD3	Anti-CD8	Anti-T11$_{2+3}$	Ca ionophore	F(ab')$_2$ (anti-T11$_{2+3}$)
					U/ml		
1	WT-6	<2	32	<2	512	256	
	31-13	<2	<2	<2	<2	128	
	31-13.αζ6	<2	<2	32	32	128	
	31-13.αζ11	<2	<2	64	64	128	
	31-13.α2	2	<2	2	2	256	
2	WT-6	<2			256	128	128
	31-13.αζ6	2			64	128	32
	31-13.α2	2			2	64	<2

Cells were incubated for 24 h with various stimuli in the presence of 5 ng/ml PMA (final concentration). In Exp. 1, all antibodies were used as ascites at 1:200 final concentration. For Exp. 2, all antibodies and F(ab')$_2$ fragments were used at 10 μg/ml final concentration. Supernatants were harvested and tested in duplicate in serial dilution for their capacity to support the growth of the IL-2-dependent murine T cell line CTLL20. Results are expressed in arbitrary U/ml of IL-2 secreted. The last dilution able to maintain the viability of 10,000 CTLL-20 cells was defined as containing 1 arbitrary U/ml. Titration experiments performed in parallel with rIL-2 (Biogen, Cambridge, MA) revealed that 1 arbitrary U is equivalent to ~1.2 U of rIL-2. The data shown are representative of three (Exp. 1) or two (Exp. 2) independent experiments.

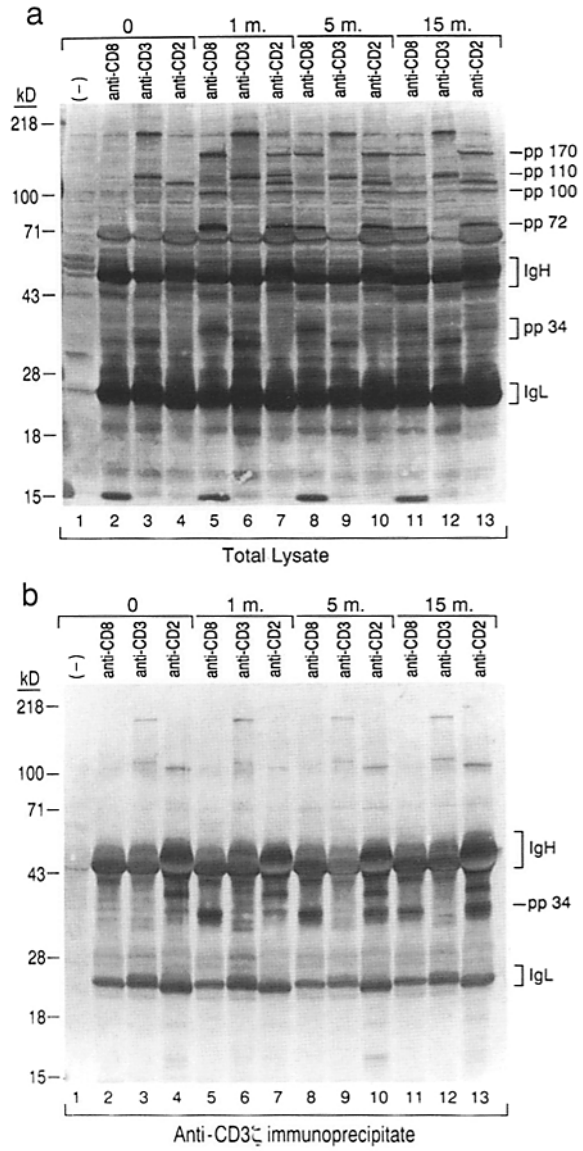

Figure 4. Protein tyrosine kinase activation of a CD8α/CD3ζ-expressing 31-13 transfectant by anti-CD2 or anti-CD8. Aliquots of 10^7 31-13.αζ6 cells were stimulated with the indicated antibodies at 1:100 dilution for various times in minutes as shown. 10% of the postnuclear lysate from each sample was directly subjected to SDS-PAGE (7.5–17.5% acrylamide), while the remaining 90% was immunoprecipitated with rabbit anti-CD3ζ antiserum N23, and then subjected to SDS-PAGE (7.5–17.5% acrylamide). After transfer to nitrocellulose, all samples were probed with antiphosphotyrosine mAb 4G10 and developed as described in Materials and Methods. (*a*) Tyrosine phosphorylation of proteins in total postnuclear lysates of 31-13.αζ6 cells stimulated with anti-CD8, anti-CD3, or anti-CD2 (antiT11$_2$ + anti-T11$_3$) as a function of time. Bands appearing as a result of stimulation are denoted pp170, pp110, pp100, pp72, and pp34. IgH and IgL denote antibody heavy chain and light chain, respectively. (*b*) Tyrosine phosphorylation of CD8α/CD3ζ chimeric protein from anti-CD3ζ immunoprecipitates of 31-13.αζ6 cells stimulated as in *a*. The CD8α/CD3ζ chimera is denoted pp34.

of the Jurkat derivatives used herein, confirming the specific nature of activation via CD3ζ (data not shown).

We further investigated the ability of the chimeric CD8α/CD3ζ to reconstitute CD2-stimulated IL-2 production in the absence of surface CD3/Ti. As expected, stimulation of CD8α/CD3ζ chimera–expressing cells with anti-CD8 resulted in IL-2 production (Table 1, Exp. 1). The magnitude of the IL-2 response from CD8α/CD3ζ chimera–expressing lines after CD8 triggering was consistently found to be quantitatively similar to the levels of IL-2 production from CD3/Ti$^+$ line WT-6 stimulated with anti-CD3. More importantly, stimulation of CD8α/CD3ζ chimera–expressing TCR$^-$ clones, of which 31-13.αζ6 and 31-13.αζ11 are representative, with anti-T11$_2$ plus anti-T11$_3$ for 24 h, resulted in significant levels of IL-2 production. As previously reported, stimulation of WT-6 through CD2 resulted in an IL-2 response exceeding that of anti-CD3 stimulation by an order of magnitude (8). Interestingly, CD2 stimulation of CD8α/CD3ζ chimera–expressing TCR$^-$ clones produced levels of IL-2 comparable in magnitude with CD8 triggering of these cells, but clearly less than the IL-2 response of CD2-stimulated WT-6 (Table 1, Exp. 1). CD2 stimulation of WT6 or 31-13.αζ6 using purified anti-T11$_2$ plus anti-T11$_3$ F(ab')$_2$ fragments resulted in levels of IL-2 production comparable with levels seen using intact IgG (Table 1, Exp. 2). As expected, the CD3$^-$ line 31-13 and transfectants expressing only full-length CD8α, of which 31-13.α2 is representative, failed to produce detectable amounts of IL-2 to any of the above stimuli. Nevertheless, since a combination of PMA and calcium ionophore induced IL-2 production from each cell line tested, lack of IL-2 production from 31-13 or 31-13.α2 is not a consequence of an intrinsic defect in IL-2 gene induction (Table 1, Exp. 1). The ability of purified anti-T11$_2$ plus anti-T11$_3$ F(ab')$_2$ fragments to elicit IL-2 production in the CD8α/CD3ζ chimera–expressing clone excludes a role for Fc receptors in mediating this effect.

To examine the role of the CD8α/CD3ζ chimera in CD2-stimulated protein tyrosine kinase activation, we compared the patterns of tyrosine phosphorylated proteins after stimulation through CD2 or the chimeric receptor. Western blot analysis of 31-13.αζ6 total cellular lysates using an antiphosphotyrosine mAb disclosed a number of substrates that became tyrosine phosphorylated as a result of either anti-CD2 or anti-CD8 stimulation when compared with the zero time points or stimulation with anti-CD3 (Fig. 4 *a*, lanes *1–4* and *5–13*). Interestingly, several substrates were phosphorylated in response to either anti-CD2 or anti-CD8 (denoted pp170, pp100, pp72), whereas at least one substrate (pp110) was evident only after CD2 stimulation (Figure 4*a*, compare lanes *4*, *7*, *10*, and *13* with lanes *2*, *5*, *8*, and *11*). In addition, the appearance of new bands in the 34-kD range suggested that the chimeric receptor was tyrosine phosphorylated as a result of either CD8 or CD2 stimulation (Fig. 4 *A*, lanes *5*, *7*, *8*, *10*, *11*, and *13*). To confirm this, anti-CD3ζ immunoprecipitates of part of the material analyzed in Fig. 4 *a* were subjected to SDS-PAGE and Western blotting using antiphosphotyrosine mAb. This analysis clearly demonstrates that the chimeric receptor (denoted pp34) is phosphorylated as a result of either anti-CD8 stimulation (Fig. 4 *b*, lanes *5*, *8*, and *11*) or anti-CD2 stimulation (Fig. 4 *b*, lanes *7*, *10*,

and *13*). It should be noted that the time to maximal CD8α/CD3ζ phosphorylation was much shorter for CD8 stimulation (~1 min) than for CD2-mediated activation (~15 min) (Fig. 4*b*, lanes *4* and *13*; and data not shown). The basis for this kinetic difference remains to be determined. As expected, stimulation of cell line 31.12.α2 with CD2, CD3 or CD8 failed to yield any specific tyrosine phosphorylated protein bands in Western blot analysis of either total cellular lysates or anti-CD3ζ immunoprecipitates (data not shown).

That the cytoplasmic domain of CD3ζ can function in lieu of the TCR complex (Tiα-β/CD3γδε[ζ-ζ or ζ-η]) in facilitating T cell activation through CD2 suggests that CD3ζ is a critical TCR component in T cells necessary for coupling CD2 to cytoplasmic second messenger pathways. This conclusion is particularly noteworthy given that in NK cells, in contrast to T cells, CD2 signaling activates second messenger pathways in the absence of TCR (23). Interestingly, NK cells express cell surface CD3ζ as a component of a molecular complex with the low affinity IgG Fc receptor CD16 (FcγRIII) (24). This observation suggests that in NK cells CD3ζ may also play a central role in CD2-mediated triggering. Consistent with this notion, we have recently shown that transfection of a $CD3^-$ $CD2^+$ Jurkat line with the transmembrane form of CD16 results in surface expression of CD16 in association with endogenous CD3ζ homodimers and restores CD2-mediated activation (16). While these results further underscore the importance of CD3ζ in mediating CD2 signaling, we can not exclude a functional role for other components of the TCR (e.g., CD3γδε) in this process. In fact, the quantitative difference in IL-2 production by CD2-stimulated CD8α/CD3ζ chimera–expressing lines as compared with TCR-expressing lines may reflect the participation of other TCR components in the CD2 signaling pathway. Although previous work has documented a role for Fc receptors in anti-CD2 mAb–mediated signaling under some experimental conditions (25, 26), the present findings with the FcR^- cell line Jurkat using purified $F(ab')_2$ fragments show that CD2 triggering can function independently of FcR subunits.

The biochemical basis of the functional dependence of CD2 signaling on CD3ζ is presently unknown. It is clear, however, that the cytoplasmic domains of both CD2 and CD3ζ are required for this functional interaction (16, 27), which implies that the relevant biochemical components are intracellular structures. The fact that T cell activation through the PI-linked surface structure Thy-1 has been shown to require cell surface CD3ζ (10) makes a direct physical association between this extracellular structure and CD3ζ unlikely. Similarly, the interaction between CD2 and CD3ζ, if not direct, may be indirect and/or transient and involve as yet unidentified cellular constituents. The ability of a single chain chimeric receptor to link accessory molecules to second messenger pathways should further facilitate molecular dissection of biochemical components and interactions important in mediating T cell activation through CD2 and other accessory structures.

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