Developmentally Regulated Expression of CD3 Components Independent of Clonotypic T Cell Antigen Receptor Complexes on Immature Thymocytes

By David L. Wiest, Kelly P. Kearse, Elizabeth W. Shores, and Alfred Singer

From Experimental Immunology Branch, National Cancer Institute, National Institutes of Health, Bethesda, Maryland 20892

Summary

CD3 signal transducing proteins are thought to be expressed on the surface of T cells only as part of clonotypic T cell receptor (TCR) complexes. Contrary to this paradigm, the present study describes surface expression of CD3 proteins independently of clonotypic TCR complexes, but only on immature thymocytes. Such novel clonotype-independent CD3 (CIC) complexes are composed primarily of CD3$\gamma\epsilon$ and secondarily of CD3$\delta\epsilon$ heterodimers that are independent of one another and are expressed on the cell surface in association with an unknown 90–100 kD protein termed CD3-associated protein (CD3AP). CIC complexes are expressed in normal mice on early thymocytes through the $CD4^+CD8^+$ stage of development, but not on mature peripheral T cells. Furthermore, CIC complexes are expressed by both TCR^- severe combined immunodeficiency (SCID) thymocytes and thymoma cell lines, in the absence of any clonotypic chains. The isolation and biochemical characterization of surface CIC complexes provides a structural basis for the signaling effects of anti-CD3ϵ antibody treatment in early thymocyte development.

The ability of the TCR complex to transduce signals after specific recognition of antigen resides in the invariant TCR-ζ and CD3 subunits (1–3). The CD3 subunits are only expressed on the surface of mature T cells in association with clonotypic chains of the TCR complex, but this has not been carefully examined for thymocytes early in development. Immature precursor cells enter the thymus as $CD4^-CD8^-TCR^-$ cells that are induced in the thymic microenvironment to enter the CD4/CD8 developmental pathway (4). However, thymocytes from mice that fail to rearrange the TCR-β gene locus fail to enter the CD4/CD8 developmental pathway and are arrested at the $CD4^-CD8^-$ stage (5–10). This developmental arrest can be overcome by treatment with anti-CD3ϵ mAbs which signal their differentiation into $CD4^+CD8^+$ cells (11, 12). Treatment with anti-CD3ϵ mAb has also been found to accelerate the differentiation of normal $CD4^-CD8^-$ thymocytes into $CD4^+CD8^+$ cells (13). These functional data suggest that immature $CD4^-CD8^-$ thymocytes do express on their cell surfaces CD3 components that are independent of clonotypic TCR chains and that are capable of transducing signals regulating thymic maturation. In this study, we provide a structural explanation for the effects of anti-CD3ϵ treatment on thymic maturation. We biochemically characterize a surface CD3 complex composed of independent CD3$\gamma\epsilon$ and CD3$\delta\epsilon$ heterodimers that are expressed separately from clonotypic TCR complexes on early fetal thymocytes through the $CD4^+CD8^+$ stage of maturation, but that are extinguished on mature peripheral T cells. Furthermore, we report that such CIC complexes are also expressed by TCR^- SCID thymocytes and TCR^- thymoma lines.

Materials and Methods

Animals. Young adult C57BL/6 and CB17 scid/scid (SCID) mice were obtained from the National Cancer Institute (Frederick, MD). FvBN fetal thymocytes, from mice at day 16 of gestation, were a gift of Dr. P. Love (National Institute of Child Health and Human Development, Bethesda, MD).

Antibodies and Cell Lines. Normal rabbit serum (NRS)[1] was obtained from bleeds of rabbits maintained in our facility. The mAbs used for immunoprecipitation and immunoblotting included: anti-TCR-β (H57-597) (14), anti-TCR-α (H28-710) (15), anti-TCR-δ (GL3) ([16]; Boehringer Mannheim Corp., Indianapolis, IN), anti-CD3ϵ (145-2C11) (17), anti-CD3ϵ (HMT3) (18), anti-CD3$\gamma\epsilon$ (7D6) (19), anti-CD3δ (20), and anti-TCR-ζ (551) (21). The BW5147 thymoma (22) and 2B4 hybridoma (23) were maintained in RPMI supplemented with 10% FCS.

[1] *Abbreviations used in this paper:* CD3AP, CD3-associated protein; CIC, clonotype-independent CD3; NEPHGE, nonequilibrium pH gradient electrophoresis; NRS, normal rabbit serum; pI, isoelectric point.

Cell Preparation and Culture. $CD4^+CD8^+$ thymocytes and splenic T cells from young adult C57BL/6 mice were prepared as described (24). Pools of thymocytes from adult CB17 scid/scid (SCID) mice were screened for the lack of CD4 and CD8 expression by flow cytometry as described (25).

Flow Cytometry. 10^6 $CD4^-CD8^-$ SCID thymocytes were washed in staining buffer (0.1% BSA and 0.1% NaN_3 in HBSS lacking phenol red) and then treated for 1 h at 4°C with either staining buffer alone, 2 μg anti-CD3ε (145-2C11), or 2 μg anti-TCR-β. After three washes in staining buffer, cells were stained with fluoresceinated anti-CD3ε (145-2C11) for 1 h at 4°C, washed three more times, and analyzed on a FACStar Plus ® (Becton Dickinson & Co., Mountain View, CA) using three decade logarithmic amplification.

Radiolabeling, Immunoprecipitation, and Electrophoresis. Where specified cells were treated with water soluble Bolton Hunter reagent (Pierce Chemical Co., Rockford, IL) and then iodinated with lactoperoxidase as described (26–28). Bolton Hunter reagent derivatizes both lysine residues and NH_2 termini of cell surface proteins with a structure that can then be radiolabeled with ^{125}I (26). Surface-labeled cells were extracted at 4°C using lysis buffer (1% digitonin/0.15 M NaCl/150 mM Tris, pH 7.4), immunoprecipitated, and electrophoresed under reducing conditions as described (27). Immune complexes to be resolved by nonequilibrium pH gradient electrophoresis (NEPHGE) were eluted in NEPHGE sample buffer for 1 h at 37°C, then resolved by NEPHGE followed by SDS-PAGE, as described (29). Gels were fluorographed using DMSO/2, 5-diphenyloxazole (PPO) (DuPont NEN, Boston, MA) by incubating two times for 1 h each in DMSO, followed by 2 h in 20% wt/vol PPO/DMSO, and 1 h in H_2O. After drying the gels were visualized either autoradiography at −70°C or by phosphoimagery, as indicated.

Ricin Fractionation. Digitonin extracts of ^{125}I surface-labeled $CD4^+CD8^+$ thymocytes were fractionated using ricin–Sepharose as described (30). Briefly, extracts were incubated with ricin beads for 16 h at 4°C, after which the beads were washed three times (lysis buffer containing 0.2% digitonin) and eluted for 4 h at 4°C with 200 mM β-lactose in wash buffer containing 0.5% digitonin.

Results

The present study was undertaken to determine if CD3 components were only expressed on the surface of T cells as part of clonotypic TCR complexes or whether they were also expressed on the surface of T cells independently of clonotypic TCR complexes. If CD3 signaling proteins were only expressed on T cells as components of TCR-α/β complexes, mAbs directed against CD3ε and TCR-β proteins should immunoprecipitate identical surface complexes. Consequently, we compared the molecular composition of surface complexes immunoprecipitated by anti-CD3ε and anti-TCR-β mAbs by SDS-PAGE under reducing conditions (Fig. 1 *A*). ^{125}I-labeled surface complexes immunoprecipitated from mature spleen T cells by anti-CD3ε and anti-TCR-β mAbs were essentially identical. In contrast, ^{125}I-labeled surface complexes immunoprecipitated from immature $CD4^+CD8^+$ thymocytes by anti-CD3ε mAbs contained four to fivefold more labeled CD3γ protein than surface complexes immunoprecipitated by anti-TCR-β mAbs (Fig. 1 *A*). To examine these surface complexes with greater resolution, we analyzed anti-CD3ε and anti-TCR-β immunoprecipitates by two-dimensional NEPHGE/SDS-PAGE (Fig. 1 *B*). Anti-CD3ε and anti-TCR-β immunoprecipitates from mature spleen T cells contained identical surface complexes (Fig. 1 *B*, *right*); however, this was not the case for immunoprecipitates from immature $CD4^+CD8^+$ thymocytes (Fig. 1 *B*, *left*). Anti-CD3ε immunorecipitates from detergent extracts of $CD4^+CD8^+$ thymocytes not only contained all of the subunits immunoprecipitated by anti-TCR-β, but also contained a large amount of surface CD3γ protein with a basic isoelectric point (pI) that was not present in anti-TCR-β immunoprecipitates and that was not present on spleen T cells (Fig. 1 *B*). This form of CD3γ was physically independent of clonotypic TCR-α/β complexes on immature $CD4^+CD8^+$ thymocytes as it was captured from the supernatant of anti-TCR-β immunoprecipitations by anti-CD3ε mAb, indicating association with CD3ε but not TCR-β chains (Fig. 1 *B*, *bottom left*). Indeed, preclearing of clonotypic TCR-α/β complexes by anti-TCR-β mAb and sequential immunoprecipitation with anti-CD3ε mAb (Fig. 1 *B*, *bottom left*) revealed surface complexes that contained: (*a*) CD3ε chains; (*b*) significant amounts of CD3γ protein with basic pI; and (*c*) small amounts of CD3δ; but did not contain clonotypic TCR-α, TCR-β, or invariant TCR-ζ chains. To more precisely analyze the composition of these TCR-independent CD3 complexes, we performed additional immunoprecipitation experiments (Fig. 1 *C*). Clonotypic TCR complexes were first removed by immunoprecipitation with anti-TCR-β mAb, and remaining surface CD3 subunits precipitated with antibodies specific for CD3ε, CD3γε, CD3δ, and TCR-ζ proteins. Surface CD3δ and CD3γ proteins that remained after removal of clonotypic TCR complexes existed as independent CD3δε and CD3γε heterodimers that were individually immunoprecipitated by either anti-CD3δ or anti-CD3γε antibodies (Fig. 1 *C*, *right*). Furthermore, these CD3 complexes were not associated with TCR-ζ chains as they were not immunoprecipitated by anti-TCR-ζ antibody (Fig. 1 *C*, *bottom right*). Thus, immature $CD4^+CD8^+$ thymocytes, but not mature spleen T cells, express surface CD3 proteins that are not part of clonotypic TCR-α/β complexes. We refer to these TCR-independent surface complexes as clonotype-independent CD3 (CIC) complexes, which consist primarily of CD3γε and secondarily of CD3δε heterodimers, and which account for 70–90% of the labeled CD3γ that is detected on the surface of immature $CD4^+CD8^+$ thymocytes.

It is unlikely that CIC complexes result from dissociation of intact TCR-α/β complexes by anti-TCR-β mAbs because: (*a*) appearance of CIC complexes was restricted to immature thymocytes and not mature T cells, and (*b*) because CD3γ chains in CIC complexes had a pI that was distinct from the more acidic pI of CD3γ chains in clonotypic TCR-α/β complexes. Nevertheless, we wished to detect CIC complexes on the surface of immature $CD4^+CD8^+$ thymocytes without anti-TCR-β immunoprecipitations. Because the basic pI of CD3γ chains in CIC complexes suggested incompletely processed N-linked sugars that lacked sialic acid and possibly galactose, we used an affinity matrix of the galactose-binding lectin *Ricin communis* agglutinin (31, 32) in an attempt to physically separate surface clonotypic TCR-α/β complexes from CIC complexes. Indeed, fractionation of cell lysates into

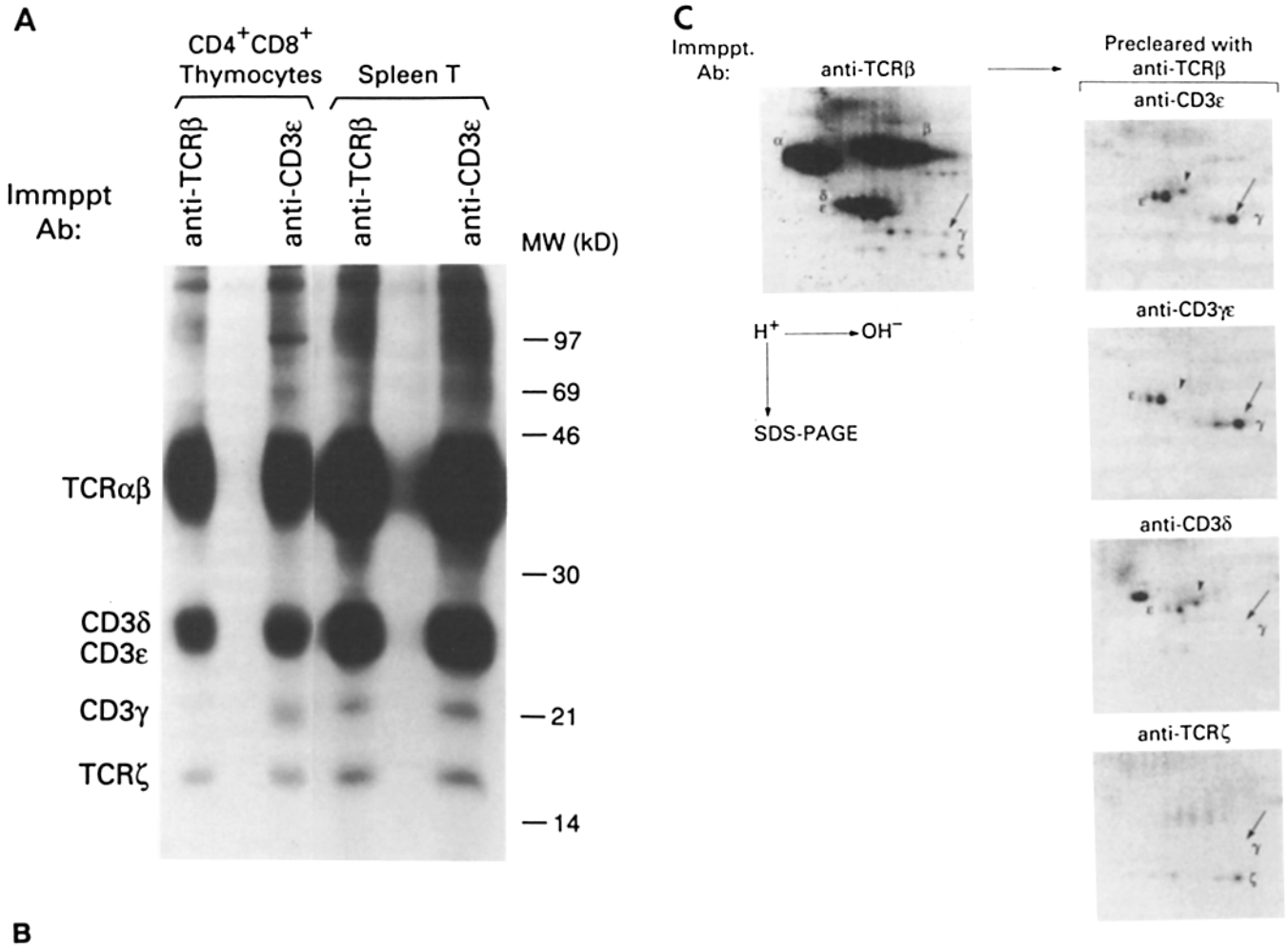

Figure 1. Immature CD4+CD8+ thymocytes, but not mature splenic T cells, express CD3 complexes that are physically distinct from TCR-α/β complexes. TCR complexes were isolated from digitonin extracts of Bolton Hunter–treated ^{125}I-surface–labeled immature CD4+CD8+ thymocytes and mature splenic T cells, using mAbs reactive with either CD3ϵ (145-2C11) or TCR-β. (*A*) More CD3γ is associated with CD3ϵ than with TCR-β in immature CD4+CD8+ thymocytes. The extracts were immunoprecipitated in parallel and resolved on one-dimensional SDS-PAGE gels. (*B*) CD3 that are physically distinct from the TCR-α/β are expressed by immature CD4+CD8+ thymocytes. Extracts were immunoprecipitated in parallel with either anti-CD3ϵ (145-2C11) or anti-TCR-β complexes (*top*) or sequentially with anti-CD3ϵ (145-2C11) after anti-TCR-β preclearing (*bottom*). Immune complexes were resolved using two-dimensional (NEPHGE × SDS-PAGE) gels. Discrimination between CD3δ and CD3ϵ proteins in these gels was accomplished by immunoblotting parallel gels with anti-CD3δ antibody (R9) and anti-CD3ϵ mAb (HMT3), and by the differential sensitivities of CD3δ and CD3ϵ proteins to endoglycosidase digestions. (*C*) CD3 complexes are composed as independent CD3$\delta\epsilon$ and $\gamma\epsilon$ heterodimers. After preclearing with anti-TCR-β, detergent extracts of iodinated CD4+CD8+ thymocytes were reprecipitated with either: (*a*) anti-CD3ϵ (145-2C11); (*b*) anti-CD3$\gamma\epsilon$ (7D6); (*c*) anti-CD3δ (R9); or (*d*) anti-TCR-ζ (551). Positions of the TCR components are indicated. *Arrows* indicate the position of CD3γ chains with a basic pI. *Arrowheads* indicate the position of CD3δ chains in the CIC complexes.

ricin-bound and ricin-unbound fractions revealed that all surface TCR-α/β complexes were contained within the ricin-bound fraction whereas all CIC complexes were contained within the ricin-unbound fraction (Fig. 2 *A*). The lack of ricin binding of CIC complexes demonstrates that they are biochemically distinct from TCR-α/β complexes expressed by $CD4^+CD8^+$ thymocytes. Thus, these data demonstrate that CIC complexes are expressed on the surface of immature $CD4^+CD8^+$ thymocytes in a form that is physically distinct from the clonotypic TCR-α/β surface complexes. Since the N-linked glycans of CIC complexes were immature, we verified that the surface labeling was indeed restricted to cell surface proteins as proteins known to be exclusively intracellular (i.e., ribophorin I, ribophorin II, tubulin, and $p56^{lck}$) were not ^{125}I-labeled (data not shown).

Since CIC complexes were physically distinct from clonotypic TCR-α/β complexes on $CD4^+CD8^+$ thymocytes that expressed both complexes, we asked if CIC complexes could be expressed on immature thymocytes that did not express clonotypic TCR complexes. Thymocytes from SCID mice are unable to productively rearrange genes encoding clonotypic TCR chains and so are arrested at the $CD4^-$

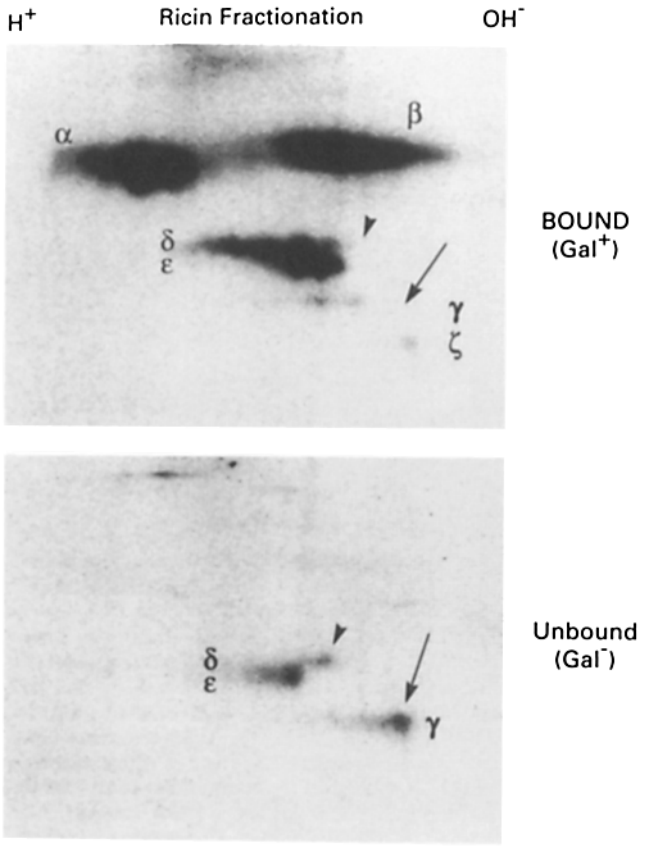

Figure 2. The CIC complexes are biochemically distinct from TCR-α/β complexes. Digitonin extracts of Bolton Hunter–treated ^{125}I-surface–labeled $CD4^+CD8^+$ thymocytes were fractionated using ricin-Sepharose. Both the ricin-bound and ricin-unbound fractions were immunoprecipitated with anti-CD3ε (145-2C11), after which the immune complexes were resolved on two-dimensional gels (NEPHGE × SDS-PAGE). Positions of TCR components are indicated. The position of the basic form of CD3γ is indicated by *arrows*. The migration of CD3δ is indicated by *arrowheads*.

$CD8^-TCR^-$ stage of development (5–8). We asked if these TCR^- SCID thymocytes expressed CIC complexes. Flow cytometric analysis of SCID thymocytes revealed a very low level of surface CD3ε protein whose staining could be specifically blocked by unlabeled anti-CD3ε mAb, but not by unlabeled anti-TCR-β mAb (Fig. 3 *A*), demonstrating that immature SCID thymocytes do express low levels of surface CIC complexes. To investigate the composition of the CD3 complexes expressed by the SCID thymocytes, surface-labeled SCID thymocytes were analyzed by immunoprecipitation. Consistent with the absence of clonotypic TCR complexes, anti-TCR-β mAb failed to specifically immunoprecipitate any surface labeled proteins from SCID thymocytes (Fig. 3 *B*, *left*). The faint bands that were present in some SCID anti-TCR-β immunoprecipitations were also nonspecifically precipitated by NRS (Fig. 3 *B*, *left*). Interestingly, however, immunoprecipitation of surface labeled proteins with anti-CD3ε mAb revealed CIC complexes on the surface of TCR^- SCID thymocytes that included CD3γ,δ, and ε chains. The presence of both CD3δ and CD3ε proteins in the 25-kD band of experiment 2 (Fig. 3 *B*, *left*) was confirmed by NEPHGE analysis (data not shown). In addition to these CD3 components, we also observed an unknown associated protein of 90–100 kD that we have termed CD3-associated protein (CD3AP). CD3AP contains no N-linked glycans (data not shown) and its further characterization is currently in progress. We then examined early (day 16) fetal thymocytes from normal mice for surface expression of CIC complexes. Although these early fetal thymocytes expressed little if any surface TCR-β proteins, sequential anti-CD3ε immunoprecipitations detected both CIC and clonotypic TCR-γ/δ complexes (Fig. 3 *B*, *right*). CIC surface complexes on fetal thymocytes could be isolated independently from clonotypic TCR-γ/δ surface complexes by preclearing cell lysates with anti-TCR-δ and anti-TCR-β mAbs, followed by sequential immunoprecipitation with anti-CD3ε mAb (Fig. 3 *B*, *right*). Thus, CIC complexes including CD3AP are expressed on the surface of both TCR^- SCID thymocytes and normal fetal thymocytes.

To further evaluate expression of CIC surface complexes on immature TCR^- thymocytes, we examined the immature TCR^- thymoma line BW5147. BW5147 cells did express surface CIC complexes consisting of CD3γ, ε, and CD3AP (Fig. 4, *top left*). The absence of CD3δ in surface CIC complexes on BW5147 cells reflects the absence of CD3δ protein expression in the BW5147 subline used (22). Importantly, because this BW5147 subline also fails to express TCR-β, the TCR-α chains remain unassembled within the endoplasmic reticulum and are not transported to the cell surface (data not shown). Indeed, significant amounts of intracellular TCR-α protein are present in BW5147 cells as demonstrated by protein immunoblots (Fig. 4, *bottom*). Despite the presence of a large intracellular pool of TCR-α chains, none of the TCR-α became iodinated during the surface labeling procedure (Fig. 4, *top left*). Taken together, these data demonstrate that ^{125}I-labeled CIC complexes were on the surface and were not internal protein complexes that had become ^{125}I-labeled at some point during the coupling reac-

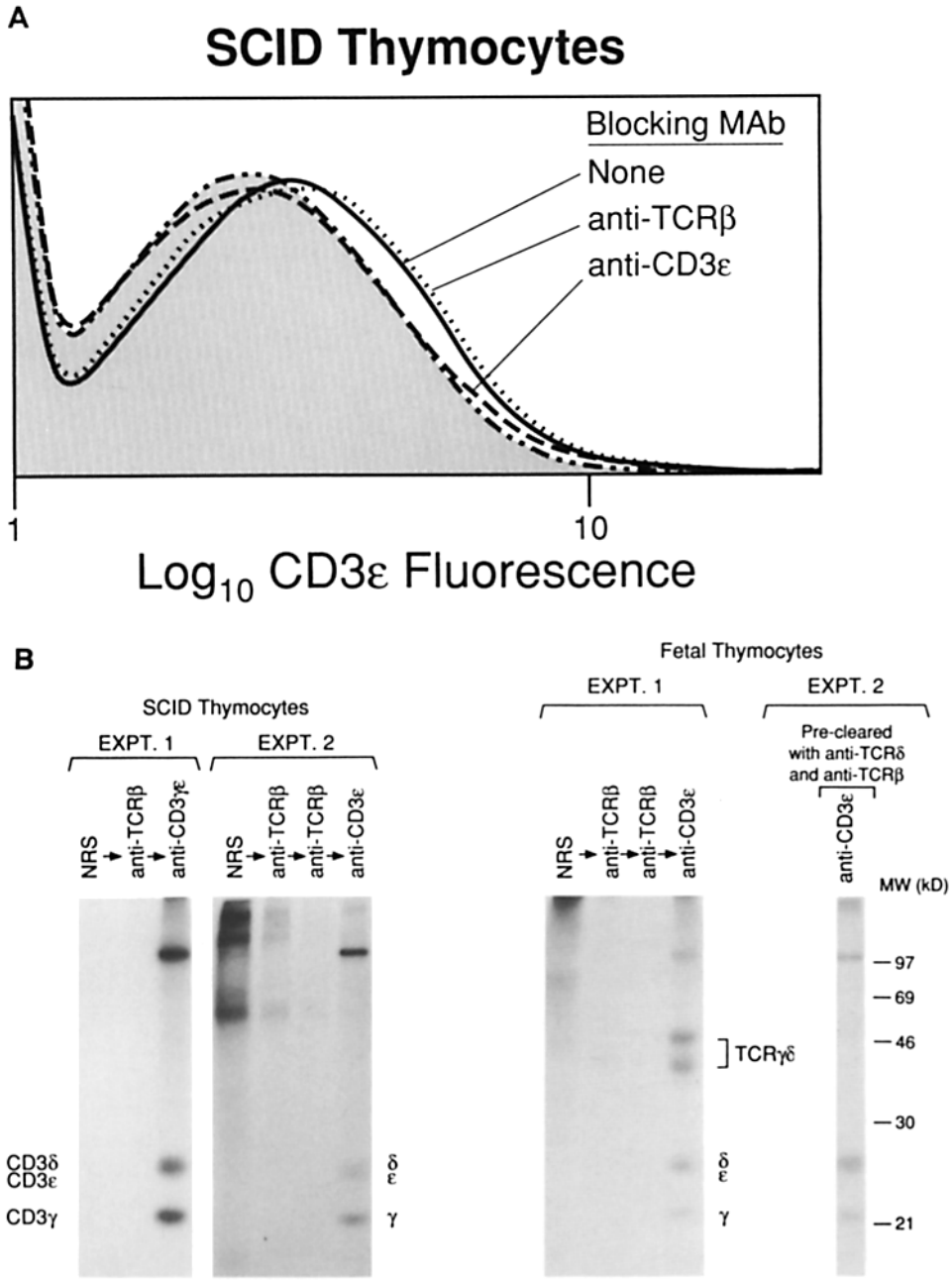

Figure 3. CIC complexes are expressed by TCR$^-$ SCID thymocytes and normal fetal thymocytes. (*A*) TCR$^-$ SCID thymocytes express low levels of CD3. CD4$^-$CD8$^-$ thymocytes from CB17 scid/scid mice were stained with fluoresceinated anti-CD3ε (145-2C11) after pretreatment with either staining medium, unlabeled anti-CD3ε (145-2C11), or unlabeled anti-TCR-β. (*B*) CIC complexes are expressed by TCR$^-$ immature thymocytes. Digitonin extracts of Bolton Hunter–treated ^{125}I-surface–labeled SCID thymocytes and fetal thymocytes were immunoprecipitated sequentially with the indicated mAbs and the resulting immune complexes resolved by SDS-PAGE under reducing conditions. The anti-CD3ε mAb used was 145-2C11. Positions of TCR components are indicated.

tion, and are consistent with the detection of isolated CD3 complexes on another thymoma cell line (33). Interestingly, even though we detected CIC complexes on BW5147 thymoma cells, we could not detect CIC complexes on the mature T cell hybridoma 2B4 that had been generated by fusion of a mature T cell with BW5147 thymoma cells (Fig. 4, *top right*). Thus, CIC complexes are expressed by an immature TCR$^-$ thymoma cell line but not expressed by a mature TCR$^+$ T cell hybridoma, mimicking the differential in vivo appearance of CIC complexes on immature thymocytes but not mature T cells.

Discussion

The present results demonstrate that immature thymocytes, but not mature T cells, express CD3 proteins independently of the clonotypic TCR complexes. CIC complexes consist primarily of CD3γε and secondarily of CD3δε heterodimers that are both physically and biochemically distinct from the CD3 chains that are part of clonotypic TCR complexes. In addition to CD3γ,δ, and ε proteins, CIC complexes are associated with an unknown surface protein termed CD3AP. CIC complexes may also contain other components that have

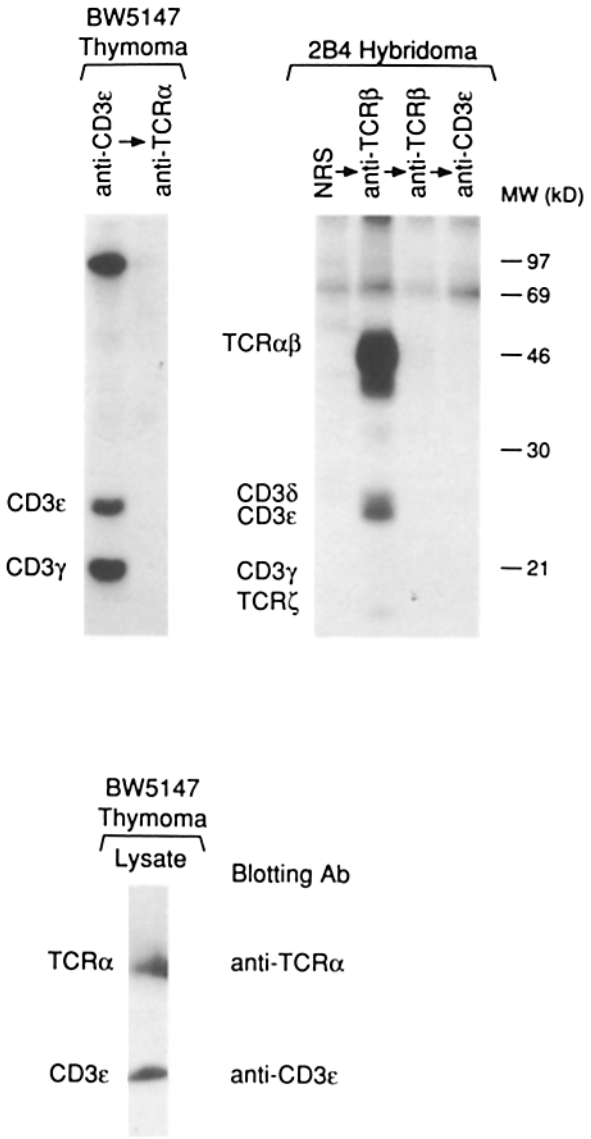

Figure 4. Expression of CIC complexes is developmentally regulated. Detergent extracts of 125I-surface-labeled BW5147 thymoma and 2B4 hybridoma cells were immunoprecipitated sequentially with the indicated antibodies (*top*). Digitonin lysates of unlabeled BW5147 thymoma cells were resolved by SDS-PAGE and immunoblotted with anti-TCR-α and anti-CD3ε (HMT3) antibodies. Positions of the TCR components are indicated.

not yet been identified. However, it is clear that CIC complexes are devoid of TCR-β chains and so are distinct from TCR-β–X dimeric complexes that have recently been described on immature thymocytes (7, 34).

The biochemical characterization of CIC complexes that are expressed on the surface of immature thymocytes required the direct analysis of surface-labeled membrane proteins. Previous attempts using metabolic labeling to analyze the structure of surface TCR complexes in immature thymocytes must necessarily be uninformative as metabolic labeling identifies newly synthesized proteins that may or may not be eventually expressed on the cell surface (10). This is a particular limitation of metabolic labeling experiments for examination of partial TCR complexes (10) that are largely, if not completely, retained intracellularly and not transported to the cell surface (1). In the present study, we exclusively used surface labeling procedures to identify CIC complexes; nevertheless, the observed immaturity of their N-linked glycans suggested that they had not been processed by glycosidases resident in the Golgi apparatus (31, 32) and so might not really have been expressed on the cell surface. Consequently, it was important to exclude the possibility that internal protein complexes had been artefactually labeled during the surface labeling reaction. In fact, we verified that the surface labeling was selective for cell surface proteins by looking for labeling of proteins known to be exclusively internal. No labeling of ribophorin I, ribophorin II, tubulin, or $p56^{lck}$ was observed in $CD4^+CD8^+$ thymocytes, nor did we observe any labeling of the abundant internal TCR-α chains expressed by BW5147 thymoma cells. Finally, we directly demonstrated by flow cytometry the presence of low levels of CD3ε expressed on the surface of SCID thymocytes. It is important to emphasize that a precedent exists for expression by immature thymocytes of proteins bearing incompletely processed N-linked glycans. For example, TCR-β chains existing as TCR-β–X heterodimers on the surface of immature thymocytes possess incompletely processed N-linked glycans (7, 34). The molecular explanation for this incomplete processing of sugars is unclear, but may be related to an inability of immature thymocytes to efficiently retain incompletely assembled TCR complexes within the endoplasmic reticulum.

Identification of CIC complexes on immature TCR^- thymocytes provides a structural basis for signaling in early thymocytes independently of clonotypic TCR complexes and provides a molecular basis for studies indicating functional effects of anti-CD3ε mAb on early thymocyte development (11–13). It is unclear why CIC complexes consist overwhelmingly of CD3γε, rather than CD3δε, complexes, but the predominance of CD3γε complexes has also been observed in $RAG\text{-}1^-$ thymocytes (12). The present results demonstrate that TCR^- early thymocytes express on their surfaces CIC complexes composed of proteins with the capability of transducing intracellular signals upon ligation by anti-CD3ε mAb (35). Thus, we think that CIC complexes are responsible for the ability of anti-CD3ε stimulation to promote the differentiation of $CD4^-CD8^-TCR^-$SCID and $RAG\text{-}1^-$ thymocytes into $CD4^+CD8^+$ cells (11, 12). In addition, we think that CIC complexes on early TCR^- thymocytes might play a role in inducing maturation of the thymic microenvironment (36).

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Address correspondence to Dr. David L. Wiest, Experimental Immunology Branch, National Cancer Institute, Building 10, Room 4B-17, 9000 Rockville Pike, Bethesda, MD 20892.

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