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Immunology

Subcellular localization of T-cell receptor complexes containing tyrosine-phosphorylated ζ proteins in immature CD4+CD8+ thymocytes

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ABSTRACT **The T-cell antigen receptor (TCR) is a complex of at least six different proteins (α, β, γ, δ, ε, and ζ) that is assembled in the endoplasmic reticulum (ER) and transported to the cell surface. Unlike mature T cells, most immature CD4+CD8+ thymocytes retain within the ER and degrade greater than 90% of some of the TCR components they synthesize, resulting in low surface expression of TCR complexes. The few surface TCR complexes that most immature CD4+CD8+ thymocytes do express are only marginally capable of transducing signals mobilizing intracellular calcium. The inverse relationship with TCR expression and function suggested that phosphorylated ζ (*P*-ζ) molecules might function in CD4+CD8+ thymocytes either as an ER retention signal for newly synthesized TCR complexes or as a negative regulatory modification of TCR complexes present on the cell surface. The present study sought to evaluate these two possibilities by determining the subcellular location of TCR complexes containing *P*-ζ chains. We found that, unlike unmodified ζ chains, all *P*-ζ chains in CD4+CD8+ thymocytes existed in assembled TCR complexes and that all TCR complexes containing *P*-ζ molecules had undergone carbohydrate processing events indicative of transit through the Golgi apparatus. These results demonstrate that *P*-ζ chains are exclusively associated with mature TCR complexes, excluding the possibility that *P*-ζ serves as an ER retention signal in immature thymocytes. Although we could not directly determine the representation of *P*-ζ chains among surface TCR complexes, we found that 60–70% of surface TCR complexes on immature CD4+CD8+ thymocytes were associated with tyrosine-phosphorylated protein(s) and that this percentage was inversely correlated with their signaling competence. These results support the concept that tyrosine phosphorylation serves as a negative regulatory modification of certain TCR-associated proteins.**

The T-cell antigen receptor (TCR) is a multisubunit transmembrane complex composed of at least six different proteins (α, β, γ, δ, ε, and ζ) (1). The TCR proteins can be divided into three families: the clonotypic αβ chains, the CD3 components (γδε), and the ζ-associated family of proteins, which exist either as ζζ homodimers or heterodimers in association with η or Fcγ proteins (2). TCR ζ chains play a unique role in the intracellular transport and signaling capability of TCR complexes. In studies using T-cell hybridomas, it was established that partial (αβγδε) TCR complexes lacking ζ chains are targeted to lysosomes for degradation, and only fully assembled (αβγδεζ) TCR complexes are efficiently transported to the cell surface (3, 4). Truncation of up to 40% of the cytoplasmic tail of ζ does not affect its assembly with other TCR proteins, but it greatly impairs the signal-transducing capability of surface TCR complexes (5). Indeed, chimeric transmembrane proteins unrelated to the TCR/CD3 complex except that they contain the intracytoplasmic tail of ζ are capable of stimulating TCR-associated signal-transduction pathways indistinguishable from those generated by surface TCR complexes (6, 7). These data, together with the finding that ζ may physiologically function with the Fcγ receptor (2, 8, 9), suggest that ζ proteins couple receptor engagement to intracellular signaling pathways.

The cytoplasmic region of TCR ζ contains six tyrosine residues that are phosphorylated after TCR or CD4 crosslinking, presumably by activation of the tyrosine kinases fyn or lck (10). In CD4+CD8+ thymocytes undergoing thymic differentiation and selection a significant fraction of ζ chains are phosphorylated, a consequence of intrathymic engagement of either CD4 or TCR molecules expressed on developing CD4+CD8+ thymocytes and subsequent activation of intracellular tyrosine kinases (11). Immature CD4+CD8+ thymocytes whose CD4 molecules are engaged *in vivo* retain and selectively degrade most newly synthesized TCR chains within the endoplasmic reticulum (ER) for reasons that are not yet understood (12), and the few surface TCR complexes that are transported to the plasma membrane are only marginally capable of signal transduction (13, 14). Release from intrathymic CD4 engagement results in TCR ζ dephosphorylation and is paralleled by increased TCR expression and improved TCR signaling (15–17). Improvement of TCR signaling in "released" CD4+CD8+ thymocytes occurs even when increases in surface TCR number are experimentally prevented (16, 17). Thus, surface expression and signaling ability of TCR in CD4+CD8+ thymocytes are both inversely correlated with the phosphorylation status of TCR ζ chains.

To evaluate the potential role(s) of phosphorylated ζ (*P*-ζ) molecules in regulating TCR transport and/or function in CD4+CD8+ thymocytes, it was necessary to identify the subcellular compartment in which *P*-ζ chains are located, as the subcellular compartment containing *P*-ζ is not yet known for any cell type.

MATERIALS AND METHODS

Animals and Cell Preparation. C57BL/6 (B6) mice 6–8 weeks of age were obtained from The Jackson Laboratory. CD4+CD8+ thymocytes were isolated by their adherence to plastic plates coated with anti-CD8 monoclonal antibody (mAb) (83-12-5) and were typically >96% CD4+CD8+ as described (11). 2B4 T-hybridoma cells were maintained as described (3).

Antibodies. The following mAbs were used in this study: anti-CD3ε 145-2C11 (18), anti-TCRβ H57-597 (19), and anti-phosphotyrosine (*P*-Tyr) 4G10 (Upstate Biotechnology, Lake Placid, NY). The following antisera were used: anti-CD3δ R9 (20) and anti-ζ 551 (21).

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Abbreviations: Endo H, endoglycosidase H; ER, endoplasmic reticulum; mAb, monoclonal antibody; *P*-Tyr, phosphotyrosine; *P*-ζ, phosphorylated ζ; RCA, *Ricin communis* agglutinin; TCR, T-cell antigen receptor; WGA, wheat germ agglutinin.

Surface Labeling and Immunoprecipitation. $CD4^+CD8^+$ thymocytes were surface iodinated with 2.5 mCi (1 Ci = 37 GBq) of ^{125}I in the presence of lactoperoxidase (12). Cells were lysed in 1% digitonin (Wako Pure Chemical, Osaka) and immunoprecipitation was performed as described (12). Endoglycosidase H (Endo H) digestion was carried out as previously described (12).

Lectin-Affinity Chromatography. Cells were lysed in 1% digitonin and diluted with buffer to yield a final detergent concentration of 0.5%. Half of the sample was added to 500 μl of *Ricin communis* agglutinin (RCA)-conjugated beads (EY Laboratories), which had been washed three times in phosphate-buffered saline (PBS) and once in 0.5% digitonin lysis buffer, and incubated for 16 hr at 4°C. Samples were centrifuged, and supernatants were removed and placed on ice. RCA-bound material was eluted by the addition of 200 mM β-lactose (Sigma) in digitonin wash buffer, for 3–4 hr at 4°C. Samples were centrifuged, supernatants were removed, and binding proteins were immunoprecipitated.

Elution of Anti-*P*-Tyr-Bound Material. Material immunoprecipitated with anti-*P*-Tyr mAb was washed three times, resuspended in wash buffer containing 150 mM phenyl phosphate (Sigma) and incubated for 3–4 hr at 4°C. Samples were centrifuged, and supernatants were removed (elute fraction) and transferred to a new tube. Elution was checked by examining material remaining on beads after addition of phenyl phosphate, and it was found to be complete at the end of the incubation period.

Gel Electrophoresis and Immunoblotting. Samples were subjected to SDS/PAGE and Western blotting was performed as described (16). Multiple autoradiographs of different exposures were scanned to ensure linearity.

RESULTS

All *P*-ζ Chains Are Assembled into TCR Complexes. In our initial series of experiments, we were interested in determining if *P*-ζ proteins exist in thymocytes exclusively as a component of TCR complexes or if *P*-ζ proteins also exist independently. To do this, we performed sequential immunoprecipitations of digitonin lysates of thymocytes in which ζ chains that had been assembled into TCR complexes were immunoprecipitated with anti-TCRβ mAb and then the remaining non-TCR-associated ζ chains were immunoprecipitated with anti-ζ antiserum (Fig. 1). The immunoprecipitates were resolved by SDS/PAGE under reducing conditions (to break disulfide bonds between ζ dimers), transferred to nitrocellulose, and blotted with an anti-ζ antiserum (551) which detects both unmodified and phosphorylated forms of ζ protein. These two forms of ζ protein are distinguishable by their distinct electrophoretic mobilities (corresponding to 16 and 21 kDa) (Fig. 1). Whereas unmodified ζ chains were present in thymocytes either associated or unassociated with TCRβ molecules, all phosphorylated ζ chains were associated with TCRβ, as no "free" *P*-ζ chains remained to be immunoprecipitated by anti-ζ antiserum (Fig. 1*A*, lanes 1 and 3).

This finding was confirmed in the second part of the experiment, in which *P*-ζ chains present in digitonin lysates of thymocytes were first immunoprecipitated with anti-*P*-Tyr mAb (Fig. 1*B*, lane 1). Anti-*P*-Tyr mAb immunoprecipitated essentially all *P*-ζ chains, as there was little remaining *P*-ζ to be subsequently precipitated by either anti-TCRβ or anti-ζ reagents (Fig. 1*B*, lanes 2–4). In addition to *P*-ζ chains, anti-*P*-Tyr immunoprecipitates also contained some unmodified ζ chains that were associated with *P*-ζ (Fig. 1*B*, lane 1). The anti-*P*-Tyr-precipitated material was then eluted from the anti-*P*-Tyr mAb and sequentially reprecipitated with anti-TCRβ mAb (to capture TCR-associated *P*-ζ chains) followed by anti-ζ antiserum (to capture unassociated *P*-ζ chains) (Fig. 1*C*). It can be seen that all *P*-ζ and unmodified ζ proteins present in the anti-*P*-Tyr immunoprecipitate were assembled into TCR complexes, as they were all immunoprecipitated by anti-TCRβ mAb, with no ζ chains left to be precipitated by anti-ζ antiserum (Fig. 1*C*, lanes 1–3). It might be noted that these results also demonstrate that immunoprecipitation of digitonin lysates with anti-*P*-Tyr mAb does not disrupt TCR complexes containing *P*-ζ.

Thus, unlike unmodified ζ proteins, *P*-ζ proteins exist in thymocytes only as part of assembled TCR complexes, and TCR complexes containing *P*-ζ chains are a subset of all TCR complexes.

Characterization of Oligosaccharide Side Chains on *P*-ζ-Associated Glycoproteins. To localize *P*-ζ-containing TCR complexes within the cell, we examined the sensitivity of *P*-ζ-associated TCR chains to digestion with Endo H, an enzyme specific for immature oligosaccharides which have not been processed by Golgi-specific glycosidases and glycosyltransferases (22, 23). Because ζ itself is not glycosylated, we examined the associated glycoprotein CD3δ, which has three asparagine-linked oligosaccharide chains (24, 25). During transit through the Golgi complex, one of the three carbohydrate side chains of CD3δ is processed to a complex (mature) oligosaccharide; the other two remain as oligomannose (immature) structures. After Endo H digestion, Golgi-processed (Endo H-resistant) CD3 δ chains migrate at 20 kDa,

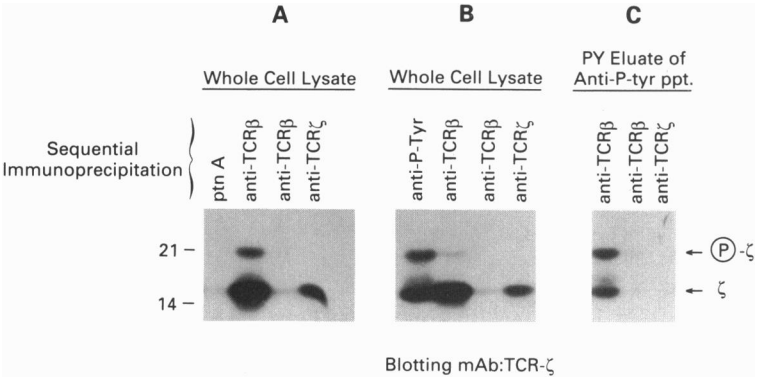

FIG. 1. All *P*-ζ chains are associated with assembled TCR complexes. Digitonin lysates were sequentially immunoprecipitated as indicated, and material was resolved on SDS/PAGE under reducing conditions, transferred to nitrocellulose, and probed with anti-ζ antiserum (551). Anti-ζ antiserum 551 detects both unmodified and phosphorylated forms of TCR ζ chains, each of which is indicated. Positions of molecular mass markers (kDa) are on the left. (*A*) Lysates were sequentially immunoprecipitated with protein A beads alone, followed by two rounds of anti-TCRβ mAb immobilized on protein A beads, followed by anti-ζ (anti-TCRζ) antiserum immobilized on protein A beads. (*B*) Lysates were sequentially immunoprecipitated with anti-*P*-Tyr mAb immobilized on protein A beads, followed by two rounds of anti-TCRβ mAb immobilized on protein A beads and finally by anti-ζ antiserum immobilized on protein A beads. (*C*) Material precipitated by anti-*P*-Tyr mAb was eluted by the addition of 150 mM phenyl phosphate (PY) and then sequentially immunoprecipitated with anti-TCR reagents as indicated.

whereas pre-Golgi (Endo H-sensitive) CD3 δ proteins migrate at 17 kDa.

In these experiments, CD3 δ chains from $CD4^+CD8^+$ thymocyte lysates were sequentially immunoprecipitated with anti-*P*-Tyr mAb (to capture all *P*-ζ-containing TCR complexes) followed by anti-CD3ε mAb (to capture remaining TCR complexes) (Fig. 2). Unlike TCR complexes assembled with unmodified ζ chains that contained both Endo H-resistant and -sensitive CD3 δ chains (Fig. 2, lane 4), *P*-ζ-containing TCR complexes contained only Endo H-resistant CD3 δ chains (Fig. 2, lane 2). Thus, *P*-ζ-containing TCR complexes can be localized in $CD4^+CD8^+$ thymocytes to the medial Golgi apparatus or beyond (Fig. 2, lane 2).

Localization of *P*-ζ by Lectin Separation of TCR Glycoproteins. To confirm that *P*-ζ chains in thymocytes are present only in TCR complexes that have already reached the medial Golgi apparatus or beyond, we used lectin affinity chromatography to physically separate mature from immature TCR complexes. The lectin RCA binds terminal galactose residues, and, to a lesser extent, internal galactose residues capped by sialic acid (26), both of which are added in the trans-Golgi compartment of the cell (27). In this experiment, thymocyte lysates were exposed to an RCA affinity matrix and separated into unbound and bound fractions; bound material was eluted from the RCA affinity matrix with β-lactose. CD3 δ chains from each lysate fraction were subsequently immunoprecipitated with anti-CD3ε mAb and assessed for Endo H resistance (Fig. 3). It can be seen that the RCA affinity matrix had effectively separated immature from mature TCR complexes, since RCA-unbound material contained Endo-H-sensitive CD3 δ chains, whereas RCA-bound material contained Endo-H-resistant CD3 δ chains (Fig. 3 *Left*). Most importantly, probing the same anti-CD3ε immunoprecipitates with anti-ζ antiserum to detect TCR-assembled ζ chains revealed that *P*-ζ chains were present only in the RCA-bound fraction (Fig. 3 *Right*).

These findings confirm that TCR complexes containing *P*-ζ have reached at least the trans-Golgi compartment of the cell. Interestingly, TCR complexes containing the unphosphorylated form of ζ are also primarily found in the RCA-bound fraction, suggesting that fully assembled TCR complexes containing ζ ($\alpha\beta\gamma\delta\varepsilon\zeta$) are present predominantly as mature complexes.

Cell Surface TCR Complexes. Since the TCR complexes that contained *P*-ζ chains had transited at least to the trans-Golgi compartment, we wished to determine if some were also expressed at the cell surface. Unfortunately, ζ labels poorly, if at all, by surface labeling procedures because it expresses only nine extracellular amino acids. Nevertheless, we reasoned that it should be possible to immunoprecipitate surface TCR complexes containing *P*-ζ chains with anti-*P*-Tyr mAb. In this experiment, lysates from $CD4^+CD8^+$ thymocytes that had been surface labeled with ^{125}I were immunoprecipitated with either anti-*P*-Tyr mAb (to capture *P*-Tyr-containing surface TCR complexes) or anti-TCRβ mAb (to capture all surface TCR complexes) (Fig. 4). Many surface proteins were immunoprecipitated by anti-*P*-Tyr mAb (Fig. 4 *Left*); to determine if anti-*P*-Tyr mAb had immunoprecipitated any surface TCR complexes, we eluted the precipitated material off the anti-*P*-Tyr beads and reprecipitated the eluate with anti-TCRβ mAb to specifically recapture TCR complexes (Fig. 4 *Center*). It is clear that anti-*P*-Tyr mAb had precipitated surface TCR complexes, as revealed by the presence of both ^{125}I-labeled TCR α and β

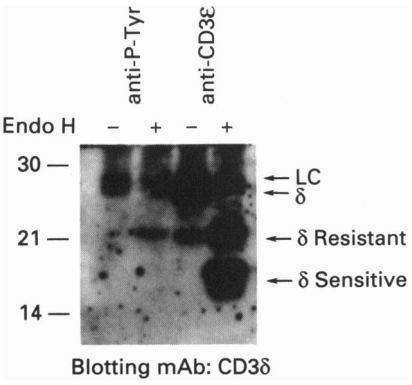

FIG. 2. Oligosaccharide side chains on *P*-ζ-associated TCR proteins. Digitonin lysates of $CD4^+CD8^+$ thymocytes were sequentially immunoprecipitated with anti-*P*-Tyr mAb followed by anti-CD3ε mAb. Immunoprecipitated material was either mock treated or digested with Endo H, subjected to SDS/PAGE under reducing conditions, transferred to nitrocellulose, and probed with antiserum raised against CD3δ. Immature (Endo H-sensitive) and mature (Endo H-resistant) forms of δ are indicated. Immunoglobulin light chain (designated LC) was also present in control precipitates of mAb without lysate (data not shown).

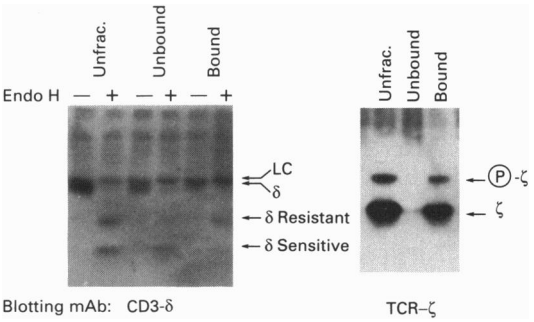

FIG. 3. Separation of mature and immature TCR complexes by RCA matrix. Lysates of B6 thymocytes were subjected to lectin affinity chromatography and were either unfractionated or separated into unbound and bound material. Samples were then immunoprecipitated with anti-CD3ε mAb, mock treated or digested with Endo H, subjected to SDS/PAGE under reducing conditions, transferred to nitrocellulose, and probed with antiserum specific for either CD3δ or TCRζ, as indicated. Immature (Endo H-sensitive) and mature (Endo H-resistant) forms of δ are indicated, as are the phosphorylated and unphosphorylated forms of ζ. Immunoglobulin light chain (designated LC) was also present in control precipitates of mAb without lysate (data not shown).

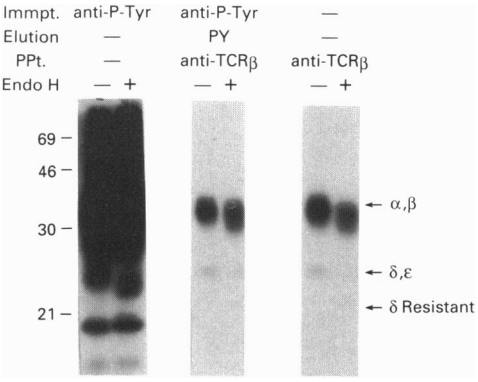

FIG. 4. Immunoprecipitation of cell surface TCR complexes from $CD4^+CD8^+$ thymocytes by anti-*P*-Tyr mAb. Purified $CD4^+CD8^+$ thymocytes were surface labeled with ^{125}I and lysed in 1% digitonin, and lysates were immunoprecipitated with either anti-*P*-Tyr mAb (*Left* and *Center*) or anti-TCRβ mAb (*Right*). In *Center*, material immunoprecipitated by anti-*P*-Tyr mAb was eluted with 150 mM phenyl phosphate (PY) and reprecipitated with anti-TCRβ mAb. Immunoprecipitated material was either mock treated or digested with Endo H to further resolve CD3 δ and ε chains. Samples were subjected to SDS/PAGE under reducing conditions. Densitometric scanning of autoradiographs was performed at multiple exposures to ensure that quantitation of band intensities was within the linear range.

chains and CD3 δ and ε chains (Fig. 4 *Center*). Because CD3 δ and ε chains are difficult to resolve, the immunoprecipitates were also treated with Endo H to selectively reduce the electrophoretic mobility of CD3δ without affecting CD3ε, which is not glycosylated. Quantitative comparisons of surface TCR α and β and CD3 δ and ε chains present in anti-*P*-Tyr eluates (Fig. 4 *Center*) versus total anti-TCRβ immunoprecipitates (Fig. 4 *Right*) indicate that 60–70% of TCR complexes on the surfaces of $CD4^+CD8^+$ cells are associated with tyrosine-phosphorylated protein(s).

Finally, we wished to determine if the signaling competence of surface TCR complexes on $CD4^+CD8^+$ thymocytes correlated inversely with the fraction of surface TCR complexes associated with tyrosine-phosphorylated protein(s). Removal of $CD4^+CD8^+$ thymocytes from their intrathymic environment and placement in short-term single-cell suspension culture at 37°C is known to result in spontaneous dephosphorylation of ζ (11) and increased signaling ability of surface TCR complexes (16). In contrast, neither ζ phosphorylation nor signaling competence changes in $CD4^+CD8^+$ thymocytes cultured at 4°C (11, 16). Consequently, we assessed the relative numbers of surface TCR complexes that could be immunoprecipitated by anti-*P*-Tyr mAb from $CD4^+CD8^+$ thymocytes that had been cultured overnight at either 4°C or 37°C (Table 1). In these experiments, digitonin lysates of ^{125}I surface-labeled cells were immunoprecipitated by anti-*P*-Tyr mAb. The precipitated material was then eluted and reprecipitated with anti-CD3ε mAb to specifically recapture surface TCR complexes that were associated with tyrosine-phosphorylated proteins; the material not originally immunoprecipitated by anti-*P*-Tyr mAb (supernatant fraction) was also precipitated with anti-CD3ε mAb to recapture surface TCR complexes that were not associated with tyrosine-phosphorylated proteins. As we had found in fresh $CD4^+CD8^+$ thymocytes (Fig. 4), 60–70% of surface TCR on $CD4^+CD8^+$ thymocytes cultured overnight at 4°C were associated with tyrosine-phosphorylated proteins (Table 1). In contrast, only 35% of surface TCR complexes on $CD4^+CD8^+$ thymocytes cultured overnight at 37°C were associated with tyrosine-phosphorylated proteins. Western blotting of lysates confirmed that dephosphorylation of TCRζ had occurred in cells cultured at 37°C (Table 1 legend). To further examine the correlation with *P*-ζ, we examined unstimulated 2B4 T-hybridoma cells, whose TCR complexes signal effectively and do not contain any detectable *P*-ζ chains on Western immunoblots (data not shown). Interestingly, surface TCR of unstimulated 2B4 T-hybridoma cells fail to be precipitated by anti-*P*-Tyr mAb (Table 1), indicating that the association of surface TCR complexes with tyrosine-phosphorylated proteins parallels the presence of *P*-ζ and is a feature of surface TCR complexes on immature $CD4^+CD8^+$ thymocytes but is not a feature of TCR complexes on all T cells. These results demonstrate that most surface TCR complexes on $CD4^+CD8^+$ thymocytes are associated with tyrosine-phosphorylated proteins and that the relative number of surface TCR complexes associated with tyrosine-phosphorylated proteins is inversely correlated with their signaling competence.

Table 1. Immunoprecipitation of cell surface TCR complexes by anti-*P*-Tyr mAb

In vitro culture		Relative amount of surface TCRαβ after anti-*P*-Tyr immunoprecipitation, %	
Cells	Temp., °C	Supernatant	Phenyl phosphate eluate
$CD4^+CD8^+$ thymocytes	4	34	66
$CD4^+CD8^+$ thymocytes	37	65	35
2B4 T-hybridoma cells	37	100	0

Purified $CD4^+CD8^+$ thymocytes were cultured in single-cell suspension for ≈14 hr at either 4°C or 37°C and then surface labeled with ^{125}I. Digitonin lysates were immunoprecipitated with anti-*P*-Tyr mAb (4G10). The nonprecipitated surface material (designated Supernatant) and the phenyl phosphate eluate of the immunoprecipitated surface material were reprecipitated with anti-CD3ε mAb as described for Fig. 4. The relative amounts of surface TCRαβ molecules were determined by densitometric scanning and are expressed in terms of percent TCRαβ in each fraction. In the same experiment, total amounts of unphosphorylated ζ (16 kDa) and phosphorylated ζ (21 kDa) chains in cultured thymocytes were independently determined by Western blotting and normalized to amounts present in $CD4^+CD8^+$ thymocytes cultured at 4°C, and were found to be as follows: unphosphorylated ζ, 4°C = 1.0 and 37°C = 2.0; *P*-ζ, 4°C = 1.0 and 37°C = 0.12. No *P*-ζ chains were detected in lysates of unstimulated 2B4 T-hybridoma cells.

DISCUSSION

Most thymic selection is thought to result from TCR signals induced in developing $CD4^+CD8^+$ thymocytes. Paradoxically, most $CD4^+CD8^+$ thymocytes express low numbers of surface TCR complexes that are of limited competence. The molecular basis for low TCR number and competence in such thymocytes is not completely understood, but both TCR number and competence are actively regulated in $CD4^+CD8^+$ thymocytes by intrathymically generated CD4 signals, one consequence of which is tyrosine phosphorylation of TCR ζ chains. In this report we demonstrate that *P*-ζ chains in $CD4^+CD8^+$ thymocytes are entirely associated with mature TCR complexes that have transited to the trans-Golgi compartment or beyond. These results exclude the possibility that *P*-ζ is a signal responsible for retaining newly synthesized TCR molecules within the ER of $CD4^+CD8^+$ thymocytes. Rather, the observation that 60–70% of TCR complexes on the surface of $CD4^+CD8^+$ thymocytes are associated with tyrosine-phosphorylated protein(s) is consistent with tyrosine phosphorylation of TCR-associated proteins functioning as a negative modification contributing to the blunted ability of TCR to mobilize intracellular calcium in $CD4^+CD8^+$ thymocytes.

The present study utilized two different experimental approaches to demonstrate that *P*-ζ molecules in $CD4^+CD8^+$ thymocytes were associated with mature TCR complexes that had transited the Golgi apparatus and were not associated with immature TCR complexes in the ER. First, we examined the processing of carbohydrate side chains on CD3 δ proteins in TCR complexes that were associated with *P*-ζ molecules, and we found that all TCR complexes containing *P*-ζ molecules had undergone carbohydrate processing events indicative of transit to at least the medial Golgi compartment. Second, we physically separated immature and mature TCR complexes by affinity chromatography with the lectin RCA, which primarily binds terminal galactose residues, and we found that *P*-ζ proteins were present exclusively in the RCA-bound material that had transited to the trans-Golgi compartment and beyond.

The failure to detect *P*-ζ molecules in the ER of $CD4^+CD8^+$ thymocytes effectively excludes tyrosine phosphorylation of ζ proteins as responsible for the retention within the ER of newly synthesized TCR proteins in $CD4^+CD8^+$ thymocytes. Therefore, the mechanism responsible for blocked egress of newly synthesized TCR proteins from the ER remains unresolved. However, the present study contains two unexpected observations which may ultimately bear on the mechanism responsible for low TCR expression in $CD4^+CD8^+$ thymocytes. First, we found that virtually all TCR complexes containing ζ chains (either unmodified or phosphorylated) were predominantly bound by RCA, indicating that most fully assembled TCR complexes in $CD4^+CD8^+$ thymocytes had transited the trans-Golgi compartment. We had expected that most fully assembled TCR complexes would be found

within the ER of $CD4^+CD8^+$ thymocytes (12). Second, we found that unmodified ζ chains were present in significant amounts in $CD4^+CD8^+$ thymocytes as non-TCR-associated ("free") proteins. This contrasts with what has been observed in T-hybridoma cells, where ζ is limiting and rapidly assembled into TCR complexes (1). There exist at least two alternative explanations for these observations: it is possible that fully assembled TCR complexes containing ζ chains rapidly leave the ER of $CD4^+CD8^+$ thymocytes as they do in T-hybridoma cells, but the number of such fully assembled complexes is severely limiting because of a cryptic assembly defect in $CD4^+CD8^+$ thymocytes; alternatively, it is possible that TCR complexes are efficiently assembled with ζ but are retained and rapidly disassembled within the ER of $CD4^+CD8^+$ thymocytes. The presence in $CD4^+CD8^+$ thymocytes of "free" ζ chains then either reflects ζ chains awaiting assembly with other TCR components or reflects ζ chains released from disassembled TCR complexes. Detailed investigation of TCR assembly and disassembly in $CD4^+CD8^+$ thymocytes should resolve this question.

With regard to TCR complexes on the surface of $CD4^+CD8^+$ thymocytes that are impaired in their ability to mobilize intracellular calcium (17), we found that 60–70% of such complexes are associated with tyrosine-phosphorylated protein(s). Importantly, we also found that the fraction of surface TCR complexes associated with tyrosine-phosphorylated protein(s) markedly declined during experimental maneuvers that lead to ζ chain dephosphorylation and to improved TCR signaling ability, namely disengagement of surface CD4 molecules by removal of $CD4^+CD8^+$ thymocytes from their intrathymic environment and placement in short-term suspension cultures that are devoid of Ia^+ cells expressing the physiologic ligand for CD4. It might be tempting to conclude from these observations that *P-*ζ chains are responsible for the impaired signaling competence of surface TCR complexes on $CD4^+CD8^+$ thymocytes because it is known that (*i*) CD4 crosslinking induces tyrosine phosphorylation of TCR-associated ζ chains (11); (*ii*) *P-*ζ is the major phosphotyrosine-containing protein that is detected by immunoblotting of TCR precipitates from $CD4^+CD8^+$ thymocytes (11); (*iii*) most tyrosine-phosphorylated proteins, other than *P-*ζ, do not spontaneously dephosphorylate during suspension culture of $CD4^+CD8^+$ thymocytes (11); and (*iv*) ζ chain phosphorylation has been suggested to restrict TCR signaling to the γ, δ, and ε proteins of the TCR complex (28). Nevertheless, it is important to emphasize that other tyrosine-phosphorylated proteins are known to be associated with TCR in T-hybridoma or T-leukemia cells, especially after TCR crosslinking, such as fyn (29) and ζ-associated protein 70 (30). Therefore, the possibility exists that one of these other tyrosine-phosphorylated proteins might be the relevant molecule for regulating TCR signaling competence in immature $CD4^+CD8^+$ thymocytes.

In conclusion, the present study has excluded the possibility that *P-*ζ functions as an ER retention protein in immature $CD4^+CD8^+$ thymocytes. Instead, we found that an unexpectedly high fraction of surface TCR complexes on $CD4^+CD8^+$ thymocytes are associated with tyrosine-phosphorylated protein(s) and that the relative number of surface TCR complexes associated with tyrosine-phosphorylated protein(s) is inversely correlated with their signaling competence. These results support the concept that tyrosine phosphorylation serves as a negative regulatory modification of certain TCR-associated proteins.

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