ENGAGEMENT OF CD4 AND CD8 EXPRESSED ON IMMATURE THYMOCYTES INDUCES ACTIVATION OF INTRACELLULAR TYROSINE PHOSPHORYLATION PATHWAYS

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One of the fundamental questions remaining in developmental immunology is how the repertoire of TCR specificities is selected. Accumulating data suggest that the target for selection is the large population of "immature" double-positive $CD4^+CD8^+$ thymocytes (1-3). However, double-positive thymocytes are generally considered to be functionally incompetent T cells (4-6). Indeed, despite the presence of a TCR-CD3 complex (7, 8), these cells are unable to respond to TCR complex stimulation by IL-2 secretion and IL-2-R expression (8, 9), although CD3 crosslinking has been shown to cause some degree of calcium mobilization (8, 9) and can result in thymocyte DNA degradation (10). Even in the absence of clear evidence for active signaling through the TCR of $CD4^+CD8^+$ thymocytes, it is indisputable that selection of the T cell repertoire involves interactions between this molecule and MHC antigens expressed in the thymus (2, 3, 11, 12). The demonstration that the CD4 and CD8 molecules expressed on double-positive thymocytes significantly contribute to selection events (11-13), and that CD4, upon engagement, causes modifications of TCR-CD3 expression (14), confirmed the earlier suggestions (15, 16) that these surface molecules may actually play active roles in the establishment of the mature T cell repertoire.

While studies of biochemical signals involved in immature T cell functions have focused primarily on pathways involving alterations in calcium or phosphatidyl-inositol metabolism, little is known about the possible involvement of tyrosine phosphorylation signals in these cells. Tyrosine protein phosphorylation has been widely implicated in a variety of processes, such as malignant transformation, the signal transduction of cell-to-cell communication, as well as cellular differentiation (for review, see reference 17). Interestingly, it has recently been observed that the CD4 and CD8 expressed on mature T cells are physically associated with the internal membrane tyrosine protein kinase $p56^{lck}$ (18-20) and, most importantly, that $p56^{lck}$ is enzymatically activated upon engagement of CD4 in these cells (21). These findings prompted

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the present study of tyrosine phosphorylation events coupled to engagement of accessory molecules in immature thymocytes. The data presented in this report demonstrate that crosslinking of surface CD4 and CD8 on immature thymocytes is associated with specific tyrosine protein phosphorylation signals. Further biochemical evidence for the existence of tyrosine phosphorylation pathways coupled to these accessory molecules is provided by the observations that the CD4 and CD8 antigens expressed on double-positive thymocytes are physically complexed to $p56^{lck}$ and that antibody-mediated aggregation of CD4 results in a rapid increase in the enzymatic activity of CD4-bound lck molecules. Taken together, these data provide a biochemical basis for the view that CD4 and CD8 play an active role in the selection of the mature T cell repertoire, and further suggest the importance of tyrosine protein phosphorylation in cellular differentiation.

Materials and Methods

Thymocytes. Adult thymocytes were prepared from 4-6-wk-old C57Bl/6 mice, while fetal thymocytes were obtained from timed pregnant C57Bl/6 mice. Cell suspensions were immediately placed upon removal in ice-cold HBSS and subsequently washed twice in this buffer. Whenever indicated, adult thymocytes were separated into double-positive ($CD4^+CD8^+$) cells and double-negative ($CD4^-CD8^-$)/single-positive ($CD4^+CD8^-$ and $CD4^-CD8^+$) cells using the following protocols. Enrichment for single-positive cells was obtained by two cycles of treatment with mAb M1/69 (22) plus complement in vitro. This treatment eliminates almost all double-positive thymocytes (23) and a subset of double-negative thymocytes (24). Resulting cells are 100% brightly positive for CD3 and contain <2% double-positive cells. Enrichment for $CD4^+CD8^+$ cells was obtained by three cycles of panning over plates coated with M1/69 or J11d (25) mAb and anti-rat IgG; only experiments in which double-positive cells represented >98% of the resulting population, as determined by flow cytometry analysis, are shown.

Antibody-mediated Cell Surface Antigen Crosslinking. Cells were incubated in serum-free media for 30 min on ice in the presence of saturating concentrations (>1 $\mu g/10^6$ cells) of various mAbs as described (19, 21). mAbs used were GK1.5 for CD4; 2.43 for CD8; 1.89.7 for T200 (CD45); 30H12 for Thy; and 2C11 for CD3ϵ. After this incubation period, cells were washed in cold media, resuspended in 37°C serum-free media alone, or in the presence of the second-step antibody (rabbit anti-rat [RAR][1] or rabbit anti-hamster [RAH] IgG) (Organon-Teknika, West Chester, PA) (19). This crosslinking step was performed for 1 min at room temperature and terminated by the addition of 2× Tris/NP-40/EDTA (TNE) lysis buffer (see below). Fab fragments of GK1.5 were used in saturating concentrations as described (21). For anti-phosphotyrosine immunoblotting, cells were lysed in boiling 2× SDS buffer (see below).

Immunoprecipitations, lck Immunoblotting, and Immune Complex Kinase Reactions. After lysing in TNE buffer (1× TNE=50 mM Tris, pH 8.0; 1% NP-40; 2 mM EDTA, pH 8.0) supplemented with protease and phosphatase inhibitors, lck proteins were immunoprecipitated from clarified cell lysates as described (19, 21). lck immunoblots and immune complex kinase reactions (either autophosphorylation or enolase phosphorylation reactions) were performed according to standard protocols (19, 21). CD4 and CD8 immunoprecipitates were obtained using the anti-CD4 mAb GK1.5 and anti-CD8 mAb 2.43, respectively (19). For depletion of CD4-associated lck, cell lysates were incubated with the anti-CD4 mAb GK1.5, followed by formalin-fixed *Staphylococcus aureus* protein A (Pansorbin; Calbiochem-Behring Corp., San Diego, CA) coupled to RAR IgG. After removal of the protein A-bound immune complexes, the non-CD4-associated lck $p56^{lck}$ was recovered by standard lck immunoprecipitation (19). Quantitation of the intensity of the bands was performed by cutting the bands from gels or nitrocellulose and counting in a ^{32}P or a gamma counter.

[1] *Abbreviations used in this paper:* D17, day 17 fetal; RAH, rabbit anti-hamster; RAR, rat anti-rat; TNE, 2× Tris/NP-40/EDTA.

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Antiphosphotyrosine Immunoblotting. After the appropriate treatment, 2–5 × 10^7 cells were lysed in boiling 2× SDS buffer (100 mM Tris, pH 8.0; 4% SDS; 40 mM EDTA, pH 8.0) supplemented with 200 μM sodium orthovanadate, 20 μg/ml each of leupeptin and aprotinin, and 100 mM sodium fluoride. After heating for 5 min at 100°C, DNA was sheared by passing through no. 20 and 25 needles. Sample buffer was then added to lysate volumes corresponding to equivalent cell numbers and resolved on 8% SDS-PAGE gels. Immunoblotting using the mouse antiphosphotyrosine mAb PY-20 (purchased from ICN Immunobiologicals, Lisle, IL) (26) was performed according to the manufacturer. The specificity of this antibody for phosphotyrosine has been extensively documented. For specific recovery of lck proteins from boiled SDS lysates, 4 vol of TNE buffer supplemented wtih protease and phosphatase inhibitors was added before lck immunoprecipitation to avoid SDS precipitation.

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Results and Discussion

To evaluate the possible involvement of tyrosine phosphorylation pathways in events related to selection of the T cell repertoire, we have examined the effects of antibody-mediated crosslinking of relevant cell surface antigens on the extent of tyrosine protein phosphorylation in murine thymocytes using antiphosphotyrosine immunoblotting (Fig. 1). Fresh thymocytes isolated from adult mice were found to contain a single major phosphotyrosine-containing protein migrating at ~56 kD on SDS-PAGE gels (Fig. 1 *A*, lane *1*). Crosslinking of CD4 (Fig. 1 *A*, lane *3*) (i.e., by anti-CD4 followed by anti-rat IgG) for 1 min resulted in a marked increase in tyrosine phosphorylation of products migrating at 56 (p56) and 110–120 (p120) kD. Smaller increases in the detectability of a series of products migrating between 68 and 72 kD (p72) were also observed. Similar alterations, although somewhat less prominent, were noticed after crosslinking of surface CD8 (Fig. 1 *A*, lane *4*). In contrast, no change in tyrosine protein phosphorylation could be detected after crosslinking of Thy-1.2 (Fig. 1 *A*, lane *5*) or T200 (CD45) (lane *7*), while CD3 crosslinking (lane *6*) was coupled to a tyrosine protein phosphorylation signal, which resulted in the enhanced phosphorylation of products migrating at 68–72 (p72) and 110–120 (p120) kD, without significantly altering the phosphorylation of the 56-kD substrate. The changes induced by crosslinking of CD4 are likely to be dependent on dimerization of this surface antigen since no change in tyrosine phosphorylation was observed after treatment with monovalent Fab fragments of the anti-CD4 mAb (data not shown). The modest increase in tyrosine protein phosphorylation seen after treatment with intact anti-CD4 alone (Fig. 1 *A*, lane *2*) is likely to be related to the ability of the bivalent antibody to induce a certain degree of crosslinking of surface CD4.

Since adult thymocytes contain a mixture of double-negative ($CD4^-/CD8^-$), single-positive ($CD4^+/CD8^-$ and $CD4^-/CD8^+$), and double-positive ($CD4^+/CD8^+$) thymocytes, we wished to address whether the evidence for signaling through CD4 and CD8 noted above actually reflected events in immature double-positive thymocytes. To this end, a similar analysis was performed on day 17 fetal (D17) thymocytes (which do not contain single-positive cells, references 4 and 5) (Fig. 1 *B*), demonstrating that CD4 (lane *2*) and CD8 (lane *3*) crosslinking of these cells induced the enhanced tyrosine phosphorylation of a similar set of cellular substrates as those detected in adult thymocytes (Fig. 1 *A*). Evidence for a tyrosine phosphorylation signal coupled with CD3 (but not p56) could also be seen in D17 thymocytes (Fig. 1 *A*, lane *4*), and future studies will address to what extent these changes involved the TCR-γ/δ-bearing cells and/or the immature TCR-α/β-expressing $CD4^+/CD8^+$

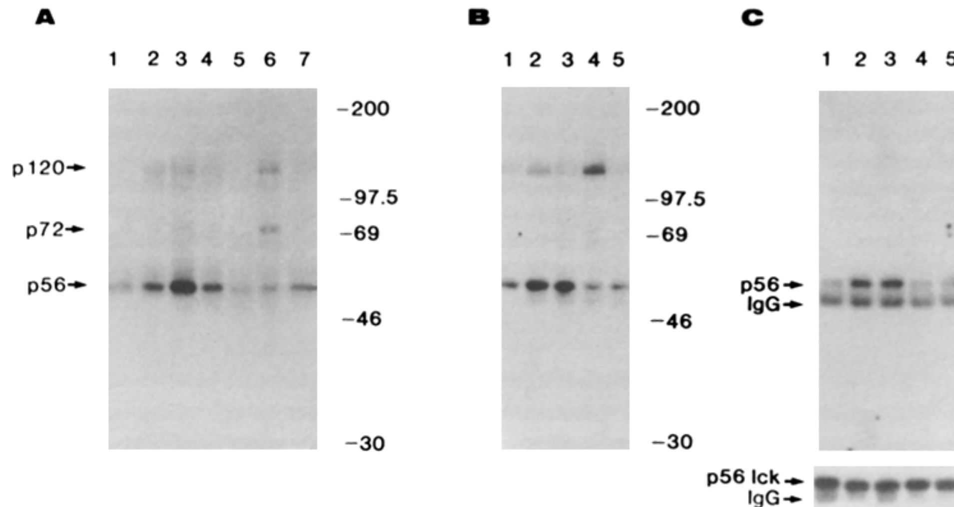

FIGURE 1. Effects of cell surface antigen crosslinking on tyrosine protein phosphorylation in thymocytes. (*A*) Adult thymocytes (4–6 wk old). Crosslinking with the appropriate antibodies was performed for 1 min as described in Materials and Methods. Cells were lysed in boiling SDS buffer and the phosphotyrosine-containing products present in these lysates detected by antiphosphotyrosine immunoblotting of 8% SDS-PAGE gels. Lane *1*, untreated control; lane *2*, anti-CD4 mAb GK1.5 alone; lane *3*, anti-CD4 mAb GK1.5 + RAR IgG; lane *4*, anti-CD8 mAb 2.43 + RAR IgG; lane *5*, anti-Thy-1.2 mAb 30-H12 + RAR IgG; lane *6*, anti-CD3 mAb 145-2C11 + RAH IgG; lane *7*, anti-T200 mAb M1.89.19.7 + RAR IgG. Exposure, 12 h. The positions of the 56-, 72-, and 120-kD substrates are indicated. (*B*) Fetal thymocytes (day 17). Same as in *A*. Lane *1*, untreated control; lane *2*, mAb GK1.5 + RAR IgG; lane *3*, mAb 2.43 + RAR IgG; lane *4*, anti-CD3 mAb 145-2C11 + RAH IgG; and lane *5*, anti-Thy-1.2 mAb 30-H12 + RAR IgG. Exposure, 12 h. (*C*) Fetal thymocytes (day 17). Lysate volumes corresponding to equal cell numbers of day 17 fetal thymocytes (the same ones as those used for the experiment depicted in *B*) were diluted with 4 vol of TNE buffer before immunoprecipitation of lck proteins with an lck-specific antisera. Subsequent analyses included antiphosphotyrosine immunoblotting (*top*) and anti-lck immunoblot (*bottom*). Lanes are as in *B*. Exposure, 12 h. The positions of $p56^{lck}$ and H chain of IgG are indicated.

thymocytes. It should be noted that, in marked contrast to observations in mature T cells (21, 27), crosslinking of CD4 or CD3 on immature thymocytes did not result in detectable tyrosine phosphorylation of the 21-kD ζ subunit of the TCR complex (Fig. 1, *A* and *B*); this finding is currently being further investigated.

Although the identities of the 68–72- and 110–120-kD tyrosine phosphorylation substrates remain to be defined, analysis of lck immunoprecipitates from D17 thymocytes by antiphosphotyrosine antibody immunoblotting (Fig. 1 *C*) showed that CD4 (lane *2*) and CD8 (lane *3*) crosslinking resulted in tyrosine phosphorylation of $p56^{lck}$, thus demonstrating that the prominent 56-kD substrate affected by CD4 and CD8 crosslinking was the *lck* gene product. Similar changes have been observed in mature murine lymphocytes (21). Further studies performed on these cells have established that CD4 crosslinking induces phosphorylation of at least two residues of $p56^{lck}$, tyrosine 505 (the major site of in vivo phosphorylation), and, to a lesser extent, tyrosine 394 (the major site of in vitro phosphorylation) (28). Taken together, these findings demonstrate that prominent tyrosine phosphorylation events can be

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induced by crosslinking CD3, CD4, and CD8 molecules on immature thymocytes, and that one of the phosphorylated substrates is $p56^{lck}$.

The distinct ability to regulate the state of tyrosine phosphorylation of $p56^{lck}$ by crosslinking of CD4 and CD8 on immature thymocytes indicates that these two surface receptors are coupled to specific and, most probably, related tyrosine phosphorylation pathways. Combined with the knowledge that the CD4 and CD8 molecules on mature T cells are physically associated with $p56^{lck}$ (19–21), these findings suggest that such CD4-lck and CD8-lck interactions may also exist in immature thymocytes. To test this possibility, we examined the levels of expression of $p56^{lck}$ as well as its interaction with CD4 and CD8 during thymic differentiation (Fig. 2). Using an lck-specific antiserum, immunoblotting of lck immunoprecipitates from fetal thymocytes revealed that $p56^{lck}$ is abundantly expressed as early as day 15 of fetal development (Fig. 2, lane *1*) and that relatively constant levels of this tyrosine kinase are maintained throughout thymic ontogeny (Fig. 2, lanes *4* and *7*). Examination of anti-CD4 and anti-CD8 immunoprecipitates for evidence of associated $p56^{lck}$ demonstrated a progressive increase in the extent of CD4-lck and CD8-lck interactions during development, being essentially absent in day 15 thymocytes (Fig. 2, lanes *2* and *3*) and reaching adult levels (lanes *11* and *12*) by day 19 (lanes *8* and *9*). These findings are consistent with the observation that CD4 and CD8 are not detectably expressed on the surface of murine thymocytes until day 16 of ontogeny (4, 5). Anti-Thy-1.2 and anti-T200 immunoprecipitates did not contain appreciable amounts of lck protein, in agreement with earlier reports on mature T cells (19) (data not shown).

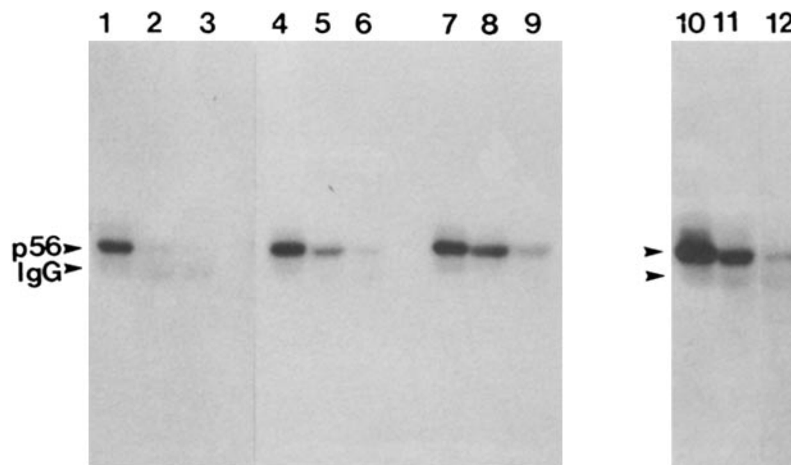

FIGURE 2. Expression of $p56^{lck}$ and its interaction with CD4 and CD8 in murine thymocytes. The relative levels of lck protein in various immunoprecipitates were examined by lck immunoblotting. Lanes *1–3*, day 15 thymocytes; lanes *4–6*, day 17 thymocytes; lanes *7–9*, day 19 thymocytes; and lanes *10–12*, adult thymocytes. Lanes *1*, *4*, *7*, and *10*, anti-lck immunoprecipitates; lanes *2*, *5*, *8*, and *11*, anti-CD4 (mAb GK1.5) immunoprecipitates; and lanes *3*, *6*, *9*, and *12*, anti-CD8 (mAb 2.43) immunoprecipitates. Exposure: lanes *1–3*, 24 h; lanes *4–9*, 6 h; lanes *10–12*, 24 h. Lanes *1–3* correspond to 2×10^6 thymocytes, whereas lanes *4–12* represent 10^7 cells. After correction for cell numbers, counting in a gamma counter of the ^{125}I bands represented in lanes *1–9* allowed us to establish that the relative levels of $p56^{lck}$ are constant over the period of ontogeny covered in this experiment (data not shown).

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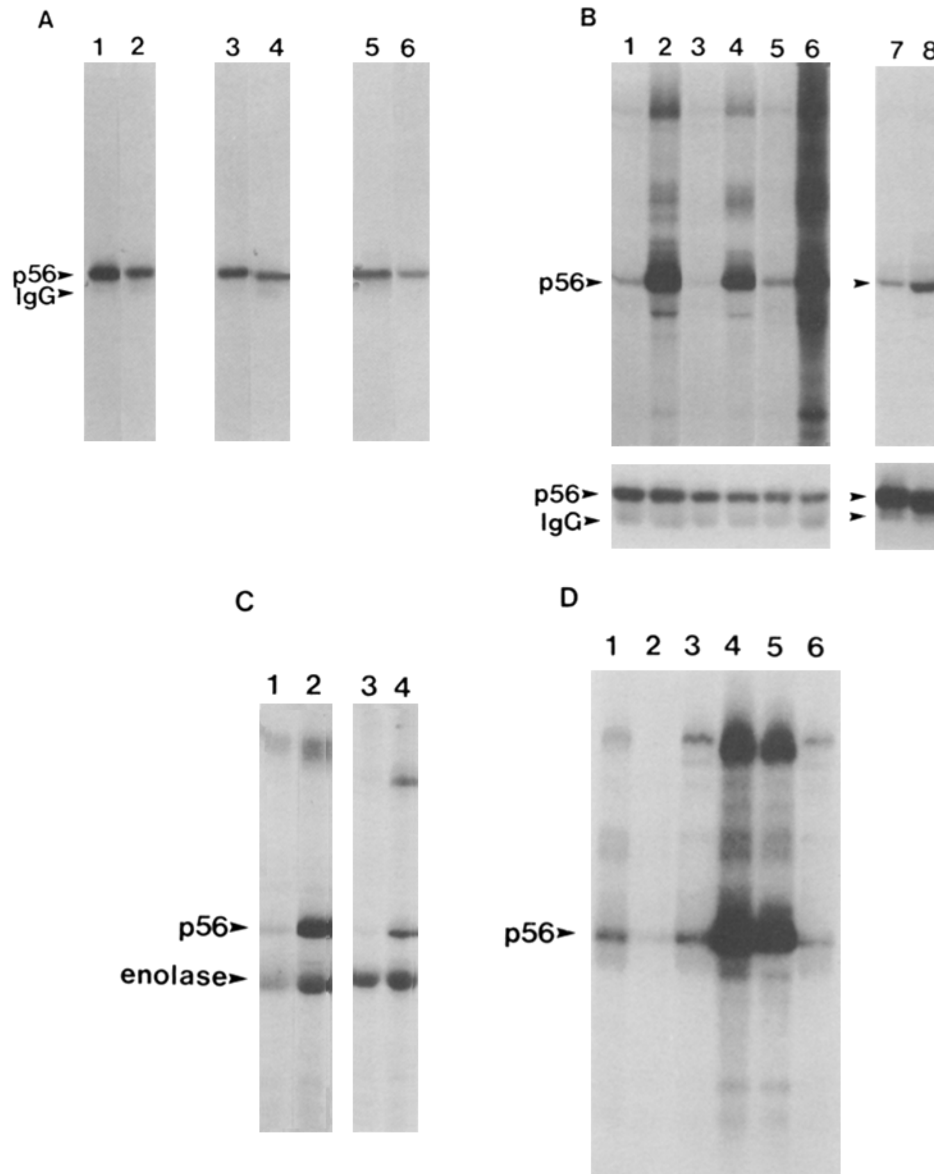

FIGURE 3. Analyses of $p56^{lck}$ in purified thymocyte subsets. (*A*) Expression of $p56^{lck}$ and its interaction with CD4 in thymocyte subsets. The relative levels of lck in various immunoprecipitates obtained from lysates of total or fractionated adult (4–6-wk-old) thymocytes were evaluated by lck immunoblotting. Lanes *1* and *2*, total adult thymocytes; lanes *3* and *4*, double-positive ($M1/69^+$) thymocytes; and lanes *5* and *6*, double-negative and single-positive ($M1/69^-$) thymocytes. Lanes *1*, *3*, and *5*, anti-lck immunoprecipitates; and lanes *2*, *4*, and *6*, anti-CD4 (mAb GK1.5) immunoprecipitates. Exposure, 12 h. (*B*) Effects of antibody-mediated crosslinking of CD4 on the tyrosine kinase activity of $p56^{lck}$ (autophosphorylation). Immune complex kinase reactions were performed on lck immunoprecipitates from untreated cells or cells incubated with anti-CD4 mAb GK1.5 and RAR IgG (crosslinking performed for 1 min). Reactions were conducted for 2 min at room temperature in the presence of γ-[^{32}P]ATP (12.5 μCi) and 1 μM cold ATP. Lanes *1* and *2*, total adult thymocytes; lanes *3* and *4*, $M1/69^+$ thymocytes; lanes *5* and *6*,

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These data suggest that signaling through CD4 and CD8 may be functional in immature thymocytes due to the conserved ability of these surface receptors to interact with p56^{lck}. To ascertain whether such interactions occur in immature T cell subsets, adult thymocytes were fractionated into double-positive (M1/69$^+$) and double-negative/single positive (M1/69$^-$) cells (see Materials and Methods), and the physical and functional interactions between CD4/CD8 and p56^{lck} were examined in each of these subsets. The experiments depicted in Fig. 3 *A* revealed that total thymocytes (lane *2*), double-positive thymocytes (lane *4*), and single-positive thymocytes (lane *6*) demonstrate an extensive degree of interaction between CD4 and p56^{lck}: ~50% of the lck protein expressed in double-positive thymocytes is associated with CD4. These findings, when combined with those obtained with D17 thymocytes (which contain only double-negative and double-positive thymocytes), allow us to clearly establish that the CD4 expressed on "immature" thymocytes physically interacts with p56^{lck}. Similar results have been obtained for CD8, although by comparison, the degree of CD8-lck interaction was consistently lower than that of CD4-lck (data not shown). We also wished to test whether, as is the case for mature T cells (21), engagement of accessory molecules in immature thymocytes is associated with the enzymatic activation of bound p56^{lck}. The results presented here concern exclusively the CD4-lck interactions, since antibody-mediated crosslinking of surface CD8 (in either thymocytes or mature T cells) has a more variable effect on the enzymatic properties of CD8-bound p56^{lck} in vitro (data not shown), and the basis for this difference is currently being investigated. Clearly, crosslinking of surface CD4 (Fig. 3 *B*) was found to result in a rapid (within 1 min) increase (3-10-fold) in the ability of p56^{lck} to phosphorylate itself in vitro in all adult thymocytes subsets (Fig. 3, lanes *2*, *4*, and *6*) as well as in D17 thymocytes (lane *8*). Increased phosphorylation of the exogenous substrate rabbit muscle enolase could also be demonstrated in these assays (Fig. 3 *C*). Additional experiments revealed that the induction of lck kinase activity seen after CD4 crosslinking was limited to CD4-bound lck molecules (Fig. 3 *D*).

The results presented in this report indicate that tyrosine protein phosphorylation pathways may play important functions in the physiology of developing thymo-

M1/69$^-$ thymocytes; and lanes *7* and *8*, total day 17 thymocytes. Lanes *1*, *3*, *5*, and *7*, untreated cells; and lanes *2*, *4*, *6*, and *8*, mAb GK1.5 + RAR IgG. (*Top*) Immune complex kinase reactions; (*bottom*) lck immunoblot. Exposure: kinase, 12 h; immunoblot: lanes *1-6*: 12 h; lanes *7* and *8*, 24 h. (*C*) Effects of CD4 crosslinking on the ability of p56^{lck} to phosphorylate the exogenous substrate rabbit muscle enolase. Kinase reactions were performed for 2 min at room temperature in the presence of γ-[^{32}P]ATP (12.5 μCi), 10 μM cold ATP, as well as an excess of rabbit muscle enolase. Lanes *1* and *2*, total adult thymocytes; and lanes *3* and *4*, total day 17 thymocytes. Lanes *1* and *3*, untreated cells; and lanes *2* and *4*, mAb GK1.5 + RAR IgG. Exposure 6 h. Quantitation of the enolase bands by densitometric analysis revealed that CD4 crosslinking induced three- and twofold increases in enolase phosphorylation in total adult thymocytes and day 17 thymocytes, respectively (data not shown). (*D*) Analyses of the effects of CD4 crosslinking on total, CD4-associated, and non-CD4-associated lck kinase activity in double-positive (M1/69$^+$) thymocytes. Lanes *1-3*, untreated cells; and lanes *4-6*, mAb GK1.5 + RAR IgG. Lanes *1* and *4*, total cellular lck; lanes *2* and *5*, CD4-associated lck; and lanes *3* and *6*, non-CD4-associated lck. Exposure, 12 h. The nature of the other phosphorylated species detected in these immunoprecipitates (especially the larger 120-130-kD products) remains to be established. Similar products are not detected in mature CD4$^+$ T cells in which a similar stimulation of CD4-associated lck kinase activity can be observed after CD4 crosslinking (21; unpublished data).

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cytes, providing mechanisms by which CD4, CD8, and possibly TCR/CD3 can transduce relevant intracellular signals implicated in selection of the mature T cell repertoire. Our data demonstrate that the following features pertain to CD4/CD8-mediated signaling events in immature thymocytes: (*a*) a physical association between the surface receptors and in the internal membrane tyrosine kinase $p56^{lck}$; (*b*) ability to stimulate the activity of bound lck molecules upon antibody-mediated crosslinking of CD4 (greater variability in the effects of CD8 crosslinking precludes a definitive statement on this issue and warrants further investigation); and (*c*) ability to stimulate the tyrosine phosphorylation of $p56^{lck}$ upon engagement of either CD4 or CD8. Moreover, crosslinking of CD4/CD8 was found to induce the tyrosine phosphorylation of additional cellular products (p72, p120), although these products appeared also to be substrates for other tyrosine phosphorylation signals, such as those induced upon CD3 crosslinking. Interestingly, the degree of p72 tyrosine phosphorylation observed after CD4/CD8 crosslinking is consistently less than that seen after CD3 stimulation, suggesting that the interaction of this product with CD4/CD8-activated signals transducers (most likely $p56^{lck}$) is less efficient. Although the mechanism and cell subset involved in tyrosine phosphorylation events observed after CD3 crosslinking remain to be established, our results provide strong evidence that CD4 and CD8 can also function as signal transducers in immature double-positive thymocytes, and that one way in which these events occur is through the ability of CD4 and CD8 to physically and functionally interact with $p56^{lck}$. Therefore, while uncertainty exists about the mechanism(s) involved in intracellular signal transduction after binding of antigen to the antigen receptor of immature thymocytes (8–10), the present study provides clear evidence for CD4- and CD8-mediated signaling through tyrosine phosphorylation. While the functional significance of these phosphorylation events in T cell differentiation are not known, our findings also provide formal evidence for the earlier made proposals (13–16) that CD4 and CD8 molecules participate as signaling molecules in T cell differentiation.

Summary

Accumulating data suggest that the target cells for selection events leading to establishment of the mature T cell repertoire are the functionally immature double-positive ($CD4^+/CD8^+$) thymocytes, and that the CD4 and CD8 antigens expressed on these cells play important roles in these processes. In an attempt to define the biochemical pathways implicated in these events, we have studied the effects of engagement of accessory molecules on tyrosine protein phosphorylation. The results of our experiments demonstrate that engagement of CD4 and CD8 expressed on double-positive thymocytes is coupled with a rapid tyrosine protein phosphorylation signal. Further analyses have revealed that these two surface molecules are physically associated with the internal membrane tyrosine protein kinase $p56^{lck}$ in immature thymocytes, and that the catalytic function of lck expressed in double-positive thymocytes is significantly enhanced upon engagement of CD4. These data provide evidence that tyrosine-specific protein phosphorylation pathways coupled to the CD4 and CD8 T cell surface antigens are functional in immature thymocytes, and therefore, formally prove that signaling functions of CD4 and CD8 molecules are operative in immature thymocytes.

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