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T Cells Expressing Receptors of Different Affinity for Antigen Ligands Reveal a Unique Role for p59^{fyn} in T Cell Development and Optimal Stimulation of T Cells by Antigen[1]

Oliver Utting, Soo-Jeet Teh, and Hung-Sia Teh[2]

Signaling from the TCR involves the protein tyrosine kinase p59^{fyn} (*Fyn*). Previous studies have shown that T cell development occurs normally in *Fyn*$^{-/-}$ mice. In this study, we investigated the requirement for *Fyn* in the development and function of T cells expressing either the transgenic 2C TCR, with high affinity for its Ag ligand, or the transgenic H-Y TCR, representative of a low affinity TCR. Although *Fyn* was not essential for positive selection of thymocytes expressing either the 2C or the H-Y TCR, it facilitated the down-regulation of the heat-stable Ag in positively selected CD4$^-$CD8$^+$ thymocytes in both 2C and H-Y mice. Negative selection of thymocytes expressing the H-Y TCR also occurs efficiently in *Fyn*$^{-/-}$ mice. However, in *Fyn*$^{-/-}$ mice, there was a preferential survival of thymocytes that expressed higher levels of the CD8 coreceptor and the transgenic TCR. Positively selected CD4$^-$CD8$^+$ thymocytes and peripheral T cells expressing either the 2C or the H-Y TCR differed in their requirement of *Fyn* for optimal proliferation responses to stimulation by antigenic ligands. Whereas 2C *Fyn*$^{-/-}$ or 2C *Fyn*$^{+/+}$ thymocytes and peripheral T cells responded optimally to stimulation by the specific Ag, H-Y *Fyn*$^{-/-}$ thymocytes and peripheral T cells were hyporesponsive compared with *Fyn*$^{+/+}$ cells. Significantly, in response to a defined low affinity ligand, both 2C *Fyn*$^{-/-}$ thymocytes and peripheral T cells required *Fyn* for optimal response to Ag stimulation. Thus, *Fyn* plays a role during thymocyte development and is required for optimal responses to low affinity/avidity ligands. ***The Journal of Immunology,*** **1998, 160: 5410–5419.**

Functional $\alpha\beta$ T cells are derived from bone marrow precursor cells. These precursor cells migrate to the thymus, where they differentiate and are subject to selection (reviewed in Ref. 1). To complete this process, double negative (DN)[3] (CD4$^-$CD8$^-$) precursor cells must successfully rearrange their TCR β-chain to allow for expression of the pre-TCR (2, 3) and progression to the double positive (DP) (CD4$^+$CD8$^+$) stage. A significant expansion of this population occurs before these cells rearrange their TCR α-chain and begin to express their TCRs (4). It is this population of immature DP TCR$^+$ cells that are subject to thymic selection (1, 5). These processes entail both the selection of T cells bearing receptors able to recognize peptides in the context of self-MHC (positive selection) and the deletion of autoreactive T cells (negative selection). Several hypotheses have been put forward to explain how the TCR can propagate signals for both positive and negative selection. In a qualitative model, the final outcome depends on signaling events that somehow differ from one another qualitatively (6, 7). In a quantitative model, the strength of the signals propagated by the TCR results in either deletion or selection (8, 9). Recent results in fetal thymic organ cultures have provided support for both the qualitative (10, 11) as well as the quantitative models (12, 13). However, despite differences in observations between these studies, the data are consistent with an affinity/avidity model for thymic positive and negative selection (1). Thus, increasing the overall avidity during the selection process by either increasing peptide concentrations (13) or by increasing the surface expression levels of CD8 (14–16) led to the conversion of a positively selecting thymus to a negatively selecting one.

Recent work has suggested that the efficiency of signaling from the TCR during development can influence the outcome of the positive and negative selection processes (17). In this study, Yamazaki et al. (17) demonstrated that with TCR signaling compromised by the deletion of the TCR ζ-chain, thymocytes expressing a transgenic male-specific TCR were able to develop in the male background, which would normally have been deleting (18). If deletion of the TCR ζ-chain allows for survival of autoreactive T cells in such a strong deleting background, then it is likely that signaling molecules associated with the TCR ζ-chain play a role in the signaling events during selection.

The *Fyn* protein tyrosine kinase (PTK) has been shown to be constitutively associated with the TCR ζ-chain (19). Despite its close link to the TCR ζ-chain, however, initial work with *Fyn*$^{-/-}$ mice showed that thymocytes in these mice appear to undergo normal development (20, 21). Thymocytes derived from these mice revealed no differences in CD4/CD8 staining profiles compared with control mice, and thymocyte yields were unaffected. Several lines of evidence do, however, indicate that *Fyn* plays a role in thymocyte development. Recent studies in *Lck*$^{-/-}$*Fyn*$^{-/-}$ mice demonstrate that $\alpha\beta$ T cell development was completely halted by the concurrent disruption of the *lck* and *fyn* genes (22, 23). As mice lacking a functional *lck* gene (24) or overexpressing a catalytically inactive form of *Lck* (25) display a substantial but

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[3] Abbreviations used in this paper: DN, double negative (CD4$^-$CD8$^-$); DP, double positive (CD4$^+$CD8$^+$); *Fyn*, p59^{fyn}; H-Y TCR, transgenic TCR specific for the male Ag presented by H-2D^b; *Lck*, p56^{lck}; PTK, protein tyrosine kinase; SP, single-positive (CD4$^-$CD8$^+$ or CD4$^+$CD8$^-$); 2C TCR, transgenic TCR specific for the p2Ca peptide presented by H-2L^d; PE, phycoerythrin; HSA, heat-stable Ag.

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not absolute reduction in single positive (SP) thymocyte numbers, these results suggest that *Fyn* is at least able to perform some of the signaling functions required for T cell development.

Examinations of SP thymocytes from $Fyn^{-/-}$ mice showed that although T cell development was apparently normal, SP thymocytes from $Fyn^{-/-}$ mice demonstrated functional defects in TCR- (20, 21) and Thy-1- (26) mediated responses. Proliferation responses, calcium ion fluxes, and IL-2 production were significantly depressed in $Fyn^{-/-}$ thymocytes. Studies of peripheral T cell responses have provided confusing results. Stein et al. (20) found that proliferation was largely normal but IL-2 secretion and calcium flux were decreased. In contrast, Appleby et al. (21) found that the proliferative response was decreased, as was calcium flux. Both groups found decreases in tyrosine-phosphorylated substrates in peripheral $Fyn^{-/-}$ T cells. It has been demonstrated that *Fyn* phosphorylates tyrosine residues within immunoreceptor tyrosine-based activation motifs on the TCR/CD3 chains upon TCR stimulation (27). *Fyn* has also been shown to phosphorylate Zap-70, leading to activation of this kinase (27). More recent studies have also shown that $Fyn^{-/-}$ thymocytes have a block in their ability to up-regulate CD25 (IL-2Rα) but are able to secrete normal levels of IL-2 (28).

Whether the role of *Fyn* is as a redundant partner to *Lck* or as a kinase able to perform a unique and essential role in TCR signaling has not been satisfactorily answered. To address the question of whether *Fyn* is required for the development of T cells bearing specific subsets of TCRs, we mated mice with the $Fyn^{-/-}$ mutation with mice transgenic for TCRs with differing affinities for their Ag ligands. The 2C TCR, whose ligand has been identified as the naturally processed peptide p2Ca (LSPFPFDL) presented by L^d MHC class 1 molecules (29) was chosen as an example of a high affinity TCR. The affinity of the 2C TCR for the p2Ca/L^d ligand is $\sim 2 \times 10^6\ M^{-1}$ (29). We also studied the H-Y TCR (18) as a representative of a low affinity TCR (30). Our results demonstrate that *Fyn* plays a role both in signaling during thymocyte development and during the response of thymic and peripheral T lymphocytes to stimulation by specific Ags. The function of *Fyn* is most evident in the response of T cells to low affinity ligands.

Materials and Methods

Mice

Breeders for the H-2^b 2C TCR transgenic mice (31, 32) were kindly provided by Dr. Denis Loh (Nippon Roche Research Center, Kamakura, Kanagawa, Japan). The H-2^b 2C TCR mice were in the seventh to eighth generation of backcross to C57BL/6 (H-2^b) mice. The 2C TCR is specific for the naturally processed peptide, p2Ca (LSPFPFDL), presented by L^d MHC class 1 molecules (29). The p2Ca peptide is derived from the mitochondrial protein 2-oxoglutarate dehydrogenase (33). H-2^b H-Y TCR transgenic mice were produced as previously described (34). The H-Y TCR transgenic mice have been backcrossed onto a C57BL/6 background. $Fyn^{-/-}$ mice, on a mixed 129 (H-2^b) and C57BL/6 background, were kindly provided by Dr. R. Perlmutter (Howard Hughes Medical Institute, University of Washington, Seattle, WA). TCR transgenic mice and $Fyn^{-/-}$ mice were mated to produce transgenic animals with the $Fyn^{-/-}$ mutation. C57BL/6 (B6), DBA/2(H-2^d), and B10.BR (H-2^k) mice were obtained from The Jackson Laboratory, Bar Harbor, ME. BDF_1 mice were F_1 mice obtained by mating B6 mice with DBA/2 mice. All animals were maintained in the animal facility at the University of British Columbia in the Department of Microbiology and Immunology.

Flow cytometric analysis of lymphocytes

Single cell suspensions of thymocytes and lymph node cells were prepared. Cells (1×10^5) were stained with mAb for 15 min on ice in 50 μl of FACS buffer (PBS with 2% FCS). Cells were washed and then incubated with secondary Ab for 15 min on ice in 50 μl of FACS buffer. Cells were then washed and resuspended in FACS buffer for analysis on a FACScan IV flow cytometer using LYSIS II software (Becton Dickinson, Mountain View, CA). Data were collected for 1×10^4 viable cells, as determined by forward and side light scatter analysis. mAbs used were T3.70 (anti-Vα3, specific for the α-chain of the H-Y TCR) (35, 36); F23.1 (37) (anti-Vβ8; specific for the β-chain of the H-Y or 2C TCR); 1B2 (anti-2C TCR Id) (38); 53.67 (anti-CD8α) FITC conjugate; anti-CD4 (GK1.5) phycoerythrin (PE) conjugate; and M1/69 (anti-mouse heat-stable Ag (HSA)). mAbs were biotinylated except where indicated. The hybridoma lines producing mAbs specific for CD4, CD8α, and HSA were obtained from the American Type Culture Collection, Manassas, VA.

Proliferation assays

Thymocytes and lymph node cells were harvested from transgenic mice and were used as responder cells in standard proliferation assays. Purification of CD8 SP cells was as previously described (39). Thymocytes were first depleted of $CD4^+$ cells by incubating with anti-CD4 (GK1.5) mAb followed by depletion of $CD4^+$ cells by anti-mouse Ig-coated Dyna beads (Dynal, Oslo, Norway). The nonadherent cells after this treatment contained $CD4^-CD8^-$ and $CD4^-CD8^+$ thymocytes. These nonadherent cells were then incubated with biotinylated anti-CD8β mAb and the $CD8^+$ cells positively selected with streptavidin-conjugated MicroBeads (Miltenyi Biotec, Auburn, CA). The purity of the positively selected cells was determined by staining the positively selected cells with fluoresceinated goat $F(ab')_2$ anti-mouse Ig Abs (Southern Biotechnology Associates, Birmingham, AL), which reacted with the anti-CD8β mAb, and phycoerythrin-conjugated anti-CD4 mAb. Thymocytes purified in this manner were >99% $CD4^-CD8^+$. Highly purified CD8 SP lymph node cells were isolated by the same method. Anti-CD3ϵ (2C11) mAb stimulations were done using 1 μg/ml of purified Ab and 20 U/ml of exogenous IL-2. Stimulator cells (5×10^5 cells/well) were irradiated splenocytes (2000 rad) from female or male B6 mice for H-Y TCR transgenic cells and BDF_1, B6, or B10.BR mice in the case of 2C TCR transgenic mice. The peptide transporter-deficient cell lines T2-L^d and T2-K^b (40) were derived by transfecting the human (T × B) hybridoma T2 with L^d or K^b. Cells were cultured in Iscove's modified Dulbecco's medium (Life Technologies, Burlington, Canada) supplemented with 5×10^{-5} M β-mercaptoethanol, 10% FCS (Life Technologies), and antibiotics. Cells were cultured in 96-well round-bottom plates. The stimulation period was 3 days for 2C TCR and 4 days for H-Y TCR thymocyte and lymph node cultures. A total of 1 μCi of [^{3}H]TdR was added to each culture well 16 h before harvest.

Results

Phenotypic analysis of thymocytes and lymph node cells from 2C/2C $Fyn^{-/-}$ *and H-Y/H-Y* $Fyn^{-/-}$ *mice*

Previous analyses of $Fyn^{-/-}$ mice have revealed these mice to have similar thymocyte subset distribution as their control littermates (20, 21). However, these analyses were done on normal mice with heterogeneous TCR repertoires. We sought to determine the role of *Fyn* in T cell development by following the developmental fate of $Fyn^{+/+}$ and $Fyn^{-/-}$ T cells that express a defined TCR. Using this approach, we hoped to identify conditions in $Fyn^{-/-}$ mice in which TCR signaling was not sustained by the activities of other *Src* kinases. We chose to study two TCRs with differing affinities for their Ag ligands: the 2C TCR, with high affinity for its Ag ligand, and the H-Y TCR, representative of a low affinity TCR. The H-Y TCR is specific for an undefined male (H-Y) peptide presented by the H-2D^b MHC class I molecule; this TCR is positively selected in female H-2^b mice and is negatively selected in male H-2^b mice (18, 41).

The CD4/CD8 phenotype of thymocytes from female H-2^b H-Y TCR transgenic mice with or without the $Fyn^{-/-}$ mutation is shown in Figure 1. The $Fyn^{-/-}$ mutation had several effects on thymocyte development in female H-2^b H-Y TCR transgenic mice. In $Fyn^{-/-}$ mice, the proportion of DP thymocytes was slightly increased. A corresponding reduction in the proportion of DN thymocytes compensated for this increase. The expression of the CD4 and CD8 coreceptor molecules was slightly down-regulated on DP thymocytes from $Fyn^{-/-}$ mice. Furthermore, the number of $Fyn^{-/-}$ DP thymocytes that expressed higher levels of the transgenic TCR β-chain (detected by the F23.1 mAb) and the transgenic TCR α-chain (detected by the T3.70 mAb) was also increased. Previous studies have shown that DP thymocytes that

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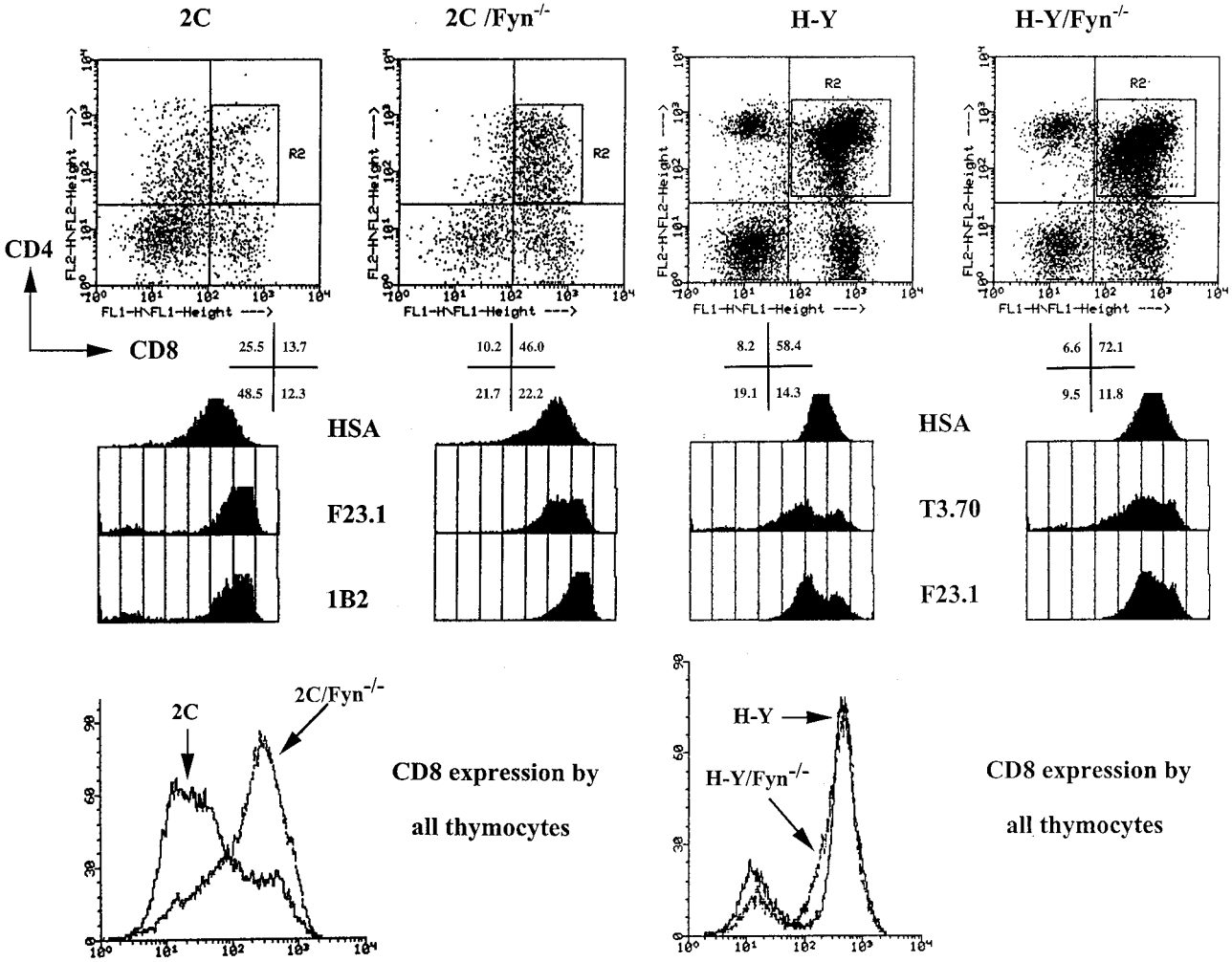

FIGURE 1. Increased CD8 expression and numbers of DP thymocytes in the 2C $Fyn^{-/-}$ but not H-Y $Fyn^{-/-}$ mice. Thymocytes from H-2^b 2C and female H-2^b H-Y TCR transgenic mice with or without the $Fyn^{-/-}$ mutation were triply stained with anti-CD4 PE, anti-CD8 FITC, and another cell surface molecule as indicated. DP cells were gated as indicated by the R2 gate. Histograms indicated the expression of HSA and transgenic TCR expression (Tricolor) shown for this cell population. The F23.1 and 1B2 mAb detected the TCR-β and Id of the 2C TCR, respectively. The TCR α- and β-chains of the H-Y TCR were detected by the T3.70 and F23.1 mAb, respectively. The percentages of thymocytes in each quadrant of the dot plots are indicated below each figure. The CD8 expression by all thymocytes from the indicated mouse line is indicated in the histograms at the *bottom* of the figure. Data from one representative experiment of three are shown.

have been positively selected by thymic MHC ligands up-regulate TCR expression levels (42, 43). If the lack of *Fyn* facilitated positive selection, this would have been accompanied by an increase in the production of CD8 SP thymocytes in $Fyn^{-/-}$ mice. However, this was not observed (Fig. 1). One explanation for this observation is that while *Fyn* is not required for the up-regulation of TCR levels on positively selected DP thymocytes, it may be required for the efficient differentiation of positively selected DP thymocytes into SP thymocytes. Thus, the lack of *Fyn* may result in the accumulation of DP thymocytes that expressed higher levels of the H-Y TCR.

The 2C TCR is positively selected in H-2^b mice and negatively selected in H-2^d mice (44). Previous studies have suggested that the 2C TCR is very strongly positively selected in H-2^b mice. Thus, the expression of a transgenic CD8 molecule led to the deletion of DP thymocytes in H-2^b 2C mice (15). In that same study, it was shown that the same level of transgenic CD8 expression in female H-2^b H-Y TCR transgenic mice did not lead to the deletion of DP thymocytes but instead enhanced positive selection (15). This was taken as evidence that the H-Y TCR was weakly positively selected in female H-2^b mice. Here we showed that the proportion of DP thymocytes in $Fyn^{-/-}$ H-2^b 2C TCR transgenic mice was significantly increased (Fig. 1, Table I). This increase in DP thymocytes in $Fyn^{-/-}$ mice was associated with an increase in the number of DP thymocytes that expressed high levels of CD8 in 2C $Fyn^{-/-}$ mice (Fig. 1). This result can be explained by assuming that *Fyn* is involved in the negative selection of thymocytes. In the absence of *Fyn*, thymocytes that would normally have been deleted will be able to survive. In H-2^b $Fyn^{-/-}$ 2C TCR transgenic mice, this translates into a higher proportion of DP thymocytes that retained high expression of the CD8 coreceptor molecule. This effect of *Fyn* is specific for H-2^b 2C TCR transgenic mice since the $Fyn^{-/-}$ mutation did not lead to an increase in CD8 expression in female H-2^b H-Y TCR transgenic mice (Fig. 1).

Since *Fyn* influenced the survival of DP thymocytes in H-2^b 2C TCR transgenic mice, we next determined whether *Fyn* can alter the course of negative selection in male H-2^b H-Y TCR transgenic mice. Negative selection in these male transgenic mice is associated with deletion of DP thymocytes, a greatly reduced thymocyte cell yield, and a preponderance of DN thymocytes that expressed

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Table I. *Effect of* Fyn *on the proportions of thymocyte subsets in TCR transgenic mice*[a]

Mutation	No. of Thymocytes $\times 10^{-7}$ (mean ± SEM)	% of Total Thymocytes (mean ± SEM)			
		$CD4^-8^-$	$CD4^+8^+$	$CD4^+8^-$	$CD4^-8^+$
H-Y	7.8 ± 1.0	18 ± 2.3	58 ± 3.4	8.5 ± 0.6	16 ± 1.4
H-Y/*Fyn*$^{-/-}$	12.7 ± 2.1	10 ± 1.7	67 ± 2.0	9.1 ± 0.6	14 ± 1.7
2C	1.7 ± 0.2	45 ± 3.6	22 ± 2.9	14.8 ± 2.2	18 ± 2.9
2C/*Fyn*$^{-/-}$	4.2 ± 0.6	21 ± 3.9	46 ± 3.2	6.0 ± 1.2	27 ± 2.3

[a] Six mice (6–12 wk old) were analyzed per group. Thymocytes from H-2^b 2C TCR transgenic mice with or without the *Fyn*$^{-/-}$ mutation or female H-2^b H-Y TCR transgenic mice with or without the *Fyn*$^{-/-}$ mutation were stained with PE-labeled anti-CD4 and FITC-labeled anti-CD8 mAbs and analyzed in the FACScan flow cytometer. The total number of viable thymocytes recovered from these transgenic lines is indicated.

the H-Y TCR (18, 45). Thus, if *Fyn* is the major tyrosine kinase mediating negative selection, we would expect to see an increase in the survival of DP thymocytes that expressed the H-Y TCR. However, we found that the yield of thymocytes from either male *Fyn*$^{+/+}$ or male *Fyn*$^{-/-}$ H-Y TCR transgenic mice was not significantly different ($\sim 2 \times 10^7$ cells from either *Fyn*$^{+/+}$ or *Fyn*$^{-/-}$ mice), and the surviving population in *Fyn*$^{+/+}$ or *Fyn*$^{-/-}$ mice was predominantly of the DN phenotype (Fig. 2). These results indicate that *Fyn* is not essential for efficient negative selection in these mice, though *Fyn* did have a subtle effect in male H-Y transgenic mice. We found that the surviving thymocytes in *Fyn*$^{-/-}$ mice differed from *Fyn*$^{+/+}$ mice in the expression levels of CD8 and TCR (Fig. 2). In *Fyn*$^{-/-}$ male mice, there were more thymocytes that expressed higher levels of the CD8 coreceptor and the H-Y TCR (Fig. 2). Thus, the lack of *Fyn* enables the survival of thymocytes that expressed slightly higher levels of CD8 and the H-Y TCR.

Functionally immature SP thymocytes are characterized by the expression of high levels of HSA (46, 47). To assess whether the *Fyn*$^{-/-}$ mutation may have subtle effects on the development of CD8 SP cells, we examined expression of this cell surface molecule on DP and SP thymocytes in *Fyn*$^{+/+}$ and *Fyn*$^{-/-}$ mice. We found that although *Fyn* did not affect the expression of HSA on DP thymocytes from either female H-2^b H-Y or H-2^b 2C TCR transgenic mice (Fig. 1), it did affect the expression of HSA in CD8 SP thymocytes from these mice (Fig. 3). In *Fyn*$^{+/+}$ H-Y TCR transgenic mice, the majority of CD8 SP thymocytes maintained high HSA expression, and a minority of these cells had down-regulated HSA. However, almost all *Fyn*$^{-/-}$ CD8 SP thymocytes from these mice maintained high expression of HSA. Similarly, in *Fyn*$^{+/+}$ 2C TCR transgenic mice, the majority of CD8 SP thymocytes had down-regulated HSA. However, the majority of CD8 SP thymocytes in 2C *Fyn*$^{-/-}$ mice maintained high expression of HSA. This delayed down-regulation of the HSA maturity marker may reflect a role for *Fyn* in the development of CD8 SP thymocytes. No significant differences in transgenic TCR α- or β-chain expression were detected in CD8 SP thymocytes of H-2^b 2C and female H-2^b H-Y TCR transgenic mice with the *Fyn*$^{-/-}$ mutation (Fig. 3).

The expression of the transgenic TCRs and HSA was also determined for peripheral CD8 SP T cells. We found that CD8 SP cells from the lymph nodes of 2C TCR transgenic mice exclusively expressed the 2C TCR, and the *Fyn*$^{-/-}$ mutation did not affect the expression of the transgenic TCR in these cells (Fig. 4). By contrast, CD8 SP cells from the lymph nodes of female H-2^b H-Y TCR transgenic mice showed a very heterogeneous expression of the TCR α-chain. Only a minority of these CD8 SP cells continued to express high levels of the transgenic TCR α-chain (Fig. 4). The predominant expression of endogenous TCR α-chains in peripheral CD8 SP cells has previously been reported and attributed to the preferential expansion of CD8 SP cells that express endogenous TCR α-chains in these mice (48). Significantly, the *Fyn*$^{-/-}$ mutation did not alter the TCR repertoire of peripheral CD8 SP cells in H-Y TCR transgenic mice. This result suggests that the positively selected CD8 SP cells in *Fyn*$^{+/+}$ or *Fyn*$^{-/-}$ mice, regardless of whether they expressed transgenic or endogenous TCR α-chains, were expanded in a similar fashion in peripheral lymphoid organs. In contrast to CD8 SP thymocytes, the vast majority of CD8 SP lymph node cells expressed low levels of HSA (Fig. 4). This likely reflects the more functionally mature status of CD8 SP lymph node cells and suggests that *Fyn* is not essential for the functional maturation of peripheral CD8 SP cells.

CD8 SP thymocytes and lymph node cells with the Fyn$^{-/-}$ *mutation are hyporesponsive to low affinity antigenic ligands*

We next sought to determine the responsiveness of cells with or without the *Fyn*$^{-/-}$ mutation to stimulation by anti-CD3ϵ mAb. To ensure that any responses we observed were strictly those of CD8 SP cells, we first depleted cell populations of CD4 SP, DP, and DN cells as previously described (39). Once highly purified (>99% CD8 SP) cell populations were obtained, the responsiveness of CD8 SP thymocytes and lymph node cells to anti-CD3ϵ (2C11) mAb stimulation in the presence of 20 U/ml of exogenous IL-2 was determined. As seen in Figure 5, thymocytes from female H-2^b H-Y TCR transgenic mice with the *Fyn*$^{-/-}$ mutation were hyporesponsive to stimulation by anti-CD3ϵ mAb. This functional defect was not observed in CD8 SP lymph node cells, since no significant difference in proliferative response was observed between the *Fyn*$^{+/+}$ and *Fyn*$^{-/-}$ populations. This result agrees with the previously published data (20, 21) in that only *Fyn*$^{-/-}$ thymocytes, but not *Fyn*$^{-/-}$ lymph node cells, were refractive to stimulation by anti-TCR mAb. Similar experiments with CD8 SP thymocytes and lymph cells expressing the 2C TCR provided different results, however. 2C11 stimulation of 2C CD8 SP thymocytes and lymph node cells (Fig. 5) revealed comparable proliferation in both the *Fyn*$^{-/-}$ and *Fyn*$^{+/+}$ populations. This difference in responsiveness between H-Y and 2C CD8 SP thymocytes to stimulation by 2C11 likely reflects differences in the functional maturity of these thymocytes.

We next determined the effect of *Fyn* on the proliferative responses of CD8 SP thymocytes and lymph node cells from female H-2^b H-Y and H-2^b 2C TCR transgenic mice to stimulation by their physiologic ligands. In this situation, the affinity of the TCR for its Ag ligand is not circumvented by the direct stimulation of the CD3ϵ-chain. Therefore, this approach provides a more critical evaluation of the requirement of *Fyn* for the activation of T cells by their cognate ligands. Purified CD8 SP thymocytes and lymph node cells were stimulated with irradiated splenocytes in a standard proliferation assay. Cells transgenic for the H-Y TCR were stimulated with syngeneic (C57/BL6) male splenocytes, whereas 2C TCR cells were stimulated with BDF_1 (C57/BL6 × DBA/2) splenocytes. As seen in Figure 6, both *Fyn*$^{-/-}$ CD8 SP thymocytes

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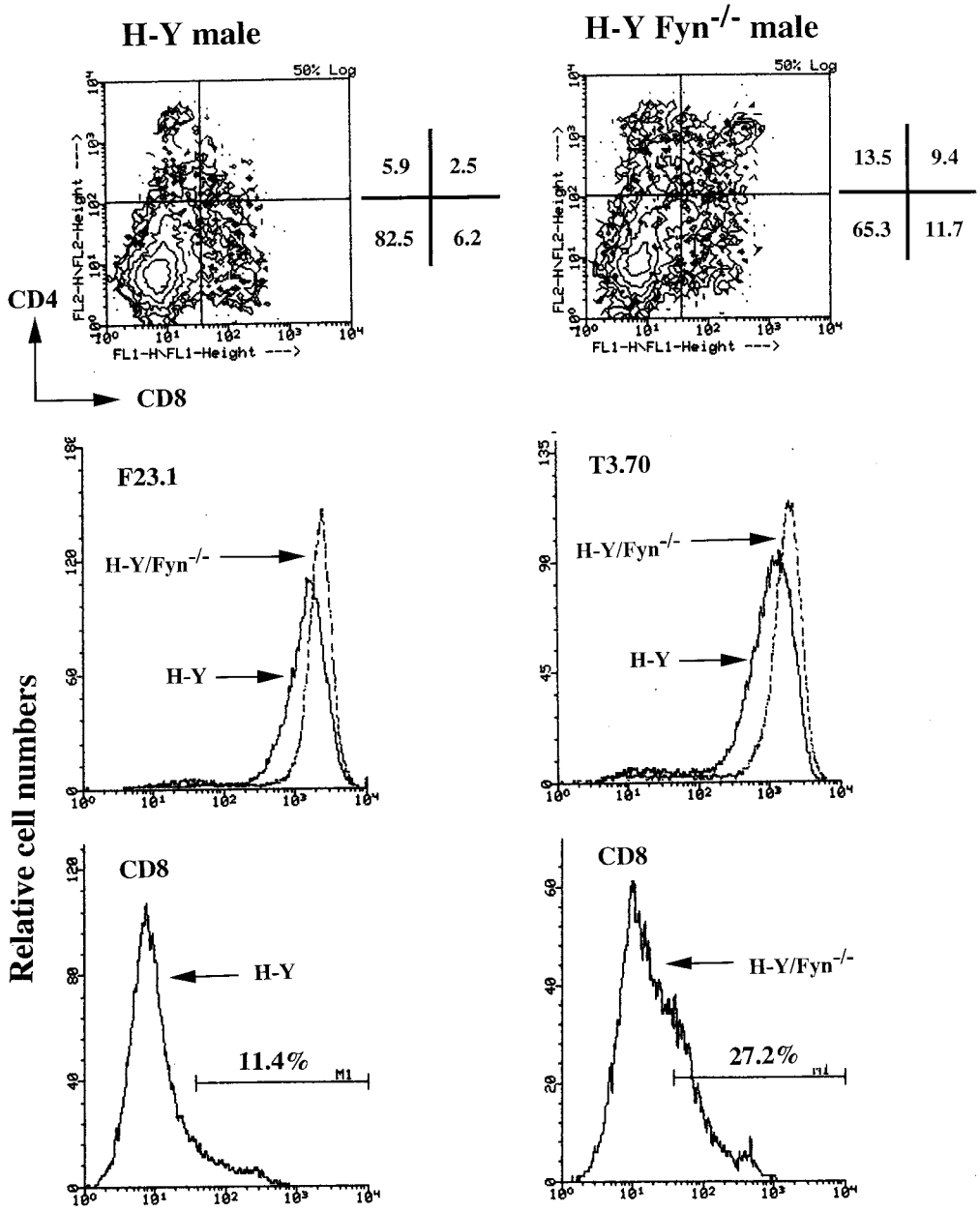

FIGURE 2. Increased CD8 and H-Y TCR expression by thymocytes from male H-2^b H-Y TCR transgenic mice with the *Fyn*$^{-/-}$ mutation. Thymocytes from male H-2^b H-Y TCR transgenic mice with or without the *Fyn*$^{-/-}$ mutation were triply stained with anti-CD4 PE, anti-CD8 FITC, and biotinylated mAb to either the transgenic TCR α- (T3.70) or β- (F23.1) chain of the H-Y TCR. Binding of the biotinylated mAb was detected by streptavidin-tricolor conjugate. The percentages of thymocytes in each quadrant of the dot plots are indicated beside each figure. The F23.1, T3.70, and CD8 expression levels by all thymocytes from the indicated mouse line are indicated in the histograms. The percentages of thymocytes expressing high levels of the CD8 molecule are indicated. Data from one representative experiment of three are shown.

and lymph node cells transgenic for the H-Y TCR were hyporesponsive to the male Ag. The response of this TCR to its cognate ligand was ~10-fold lower than the *Fyn*$^{+/+}$ cells. Neither the *Fyn*$^{+/+}$ nor the *Fyn*$^{-/-}$ cells were able to respond to female B6 splenocytes, indicating that the observed response was indeed male specific.

In contrast to these results, *Fyn*$^{-/-}$ CD8 SP thymocytes or lymph node expressing the 2C TCR showed only slightly reduced proliferative responses to stimulation by BDF_1 splenocytes relative to their *Fyn*$^{+/+}$ counterpart (Fig. 7). These CD8 SP cells did not respond to B6 or B10.BR splenocytes, indicating that the response was indeed specific for H-2^d. Thus, depending on the transgenic TCR, CD8 SP thymocytes or lymph node cells display differential requirement for *Fyn* in optimal proliferative response to stimulation by their specific Ag.

One hypothesis to account for the differential requirement for *Fyn* by CD8 SP thymocytes or lymph node cells expressing either the H-Y or the 2C TCR for optimal responses to stimulation by their specific Ag is that the H-Y TCR is a low affinity TCR and the 2C TCR is a high affinity TCR. We reasoned that a low affinity TCR is more dependent on *Fyn* than a high affinity TCR for optimal stimulation by Ag. To test this hypothesis we took advantage of the fact that the affinity of the 2C TCR for various antigenic ligands has been determined. We used human T2 cells transfected with either the L^d or K^b molecules as APCs (40). Human T2 cells are deficient in peptide transport and the L^d or K^b molecules

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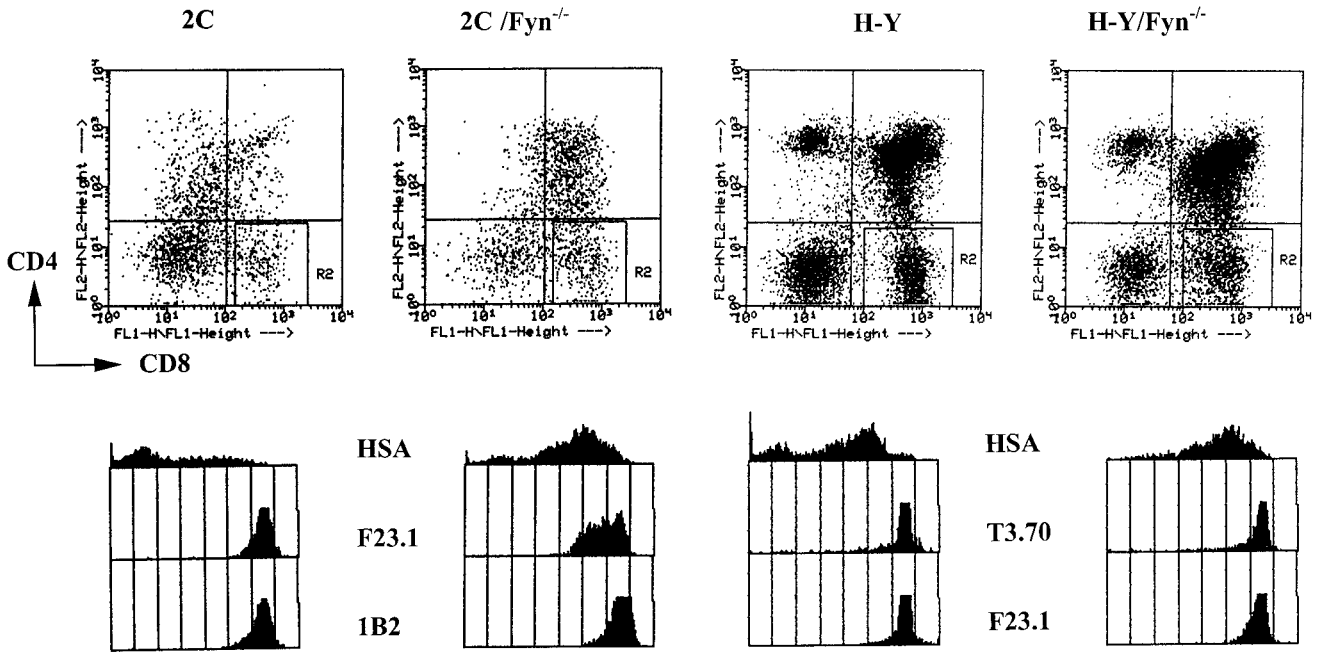

FIGURE 3. CD8 SP *Fyn*$^{-/-}$ thymocytes maintained elevated expression of HSA levels. Thymocytes from H-2^b 2C and female H-2^b H-Y TCR transgenic mice with or without the *Fyn*$^{-/-}$ mutation were stained as described in Figure 1. The percentages of thymocytes in each quadrant of the dot plots are as indicated in Figure 1. The expression of the indicated cell surface molecule by gated CD8 SP thymocytes are depicted in the histograms below the dot plots. Data from one representative experiment of three are shown.

expressed by T2 cells can be loaded with a specific peptide, which in our case is p2Ca. The density of the L^d/p2Ca or the K^b/p2Ca ligand on T2 cells can be varied by exposing these APCs to different concentrations of p2Ca. The relative affinity of the 2C TCR for the L^d/p2Ca and the K^b/p2Ca ligand has been determined to be $\sim 2 \times 10^6$ M^{-1} and $\sim 3 \times 10^3$ M^{-1}, respectively (29). This approach therefore enabled us to determine whether CD8 2C TCR$^+$ SP thymocytes or lymph node cells can display differential requirements for *Fyn* in their response to a high affinity (L^d/p2Ca) or a low affinity (K^b/p2Ca) ligand. As shown in Figure 8, 2C *Fyn*$^{-/-}$

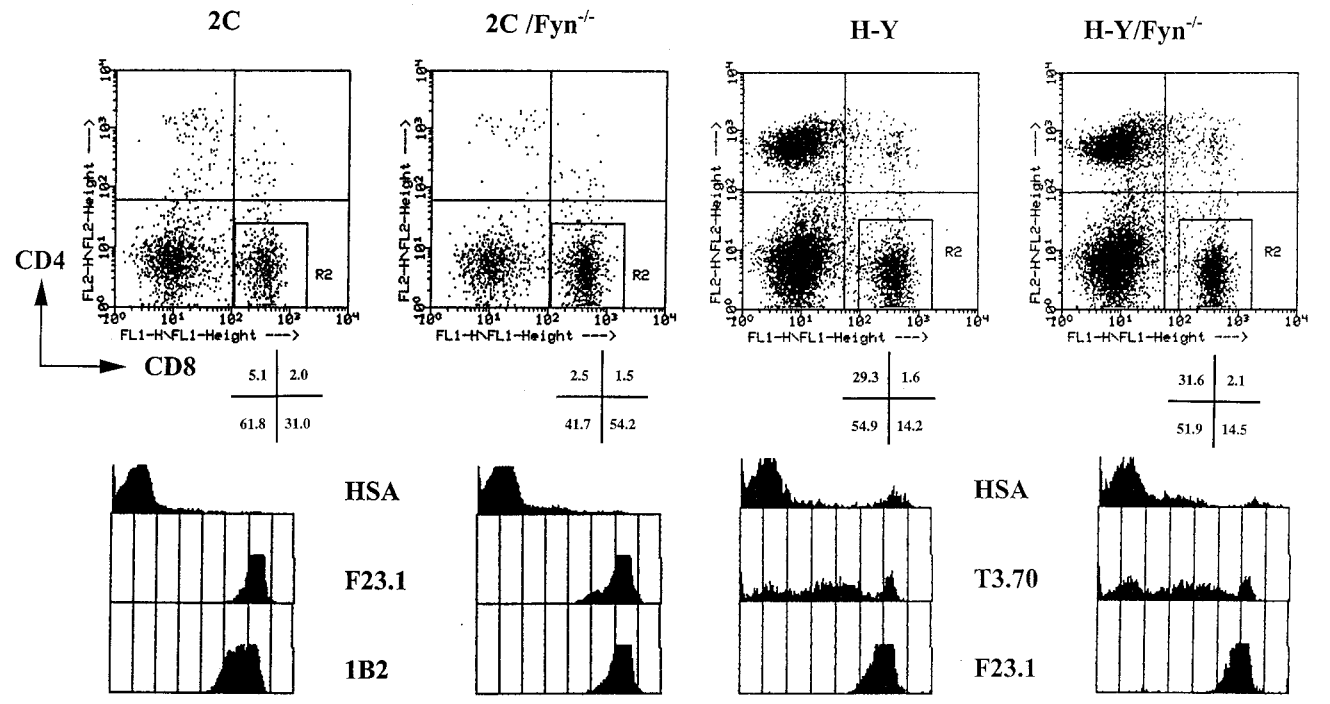

FIGURE 4. Down-regulation of HSA by CD8 SP peripheral T cells from *Fyn*$^{-/-}$ mice. Lymph node cells from H-2^b 2C and female H-2^b H-Y TCR transgenic mice with or without the *Fyn*$^{-/-}$ mutation were stained as described in Figure 1. The percentages of cells in each quadrant of the dot plots are indicated below each plot. The expression of the indicated cell surface molecule by gated CD8 SP thymocytes is depicted in the histograms below the dot plots. Data from one representative experiment of three are shown.

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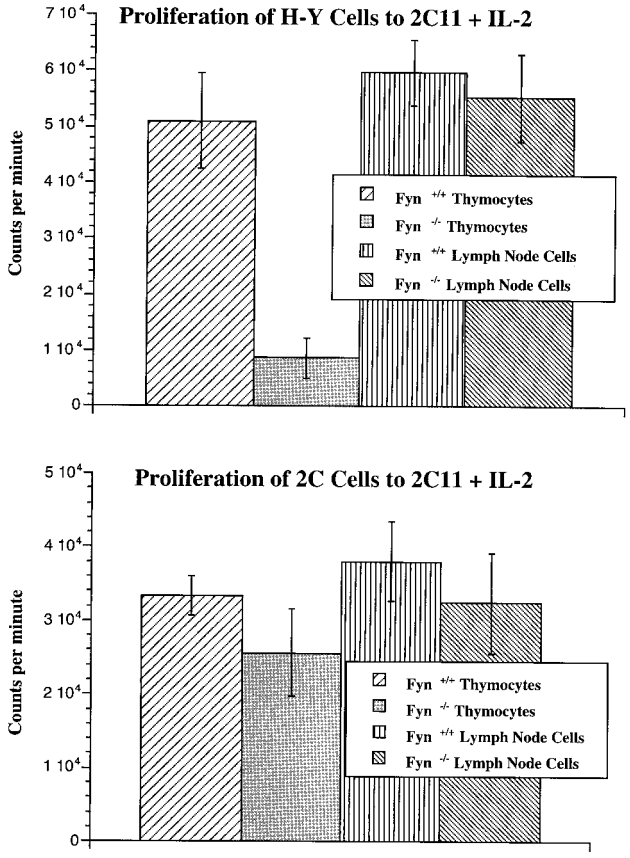

FIGURE 5. *Fyn*$^{-/-}$ thymocytes expressing the H-Y TCR are hyporesponsive to CD3ε stimulation. Purified CD8 SP thymocytes or lymph node cells (1×10^4; see *Materials and Methods*) from female H-2^b H-Y TCR transgenic mice were stimulated with 1 μg/ml of 2C11 (anti-CD3ε mAb) in the presence of 20 U/ml of murine rIL-2. Cultures were set up in triplicates. Each culture was incubated with 1 μCi of [^{3}H]TdR/well for the final 16 h before harvest. Data from one representative experiment of three are shown.

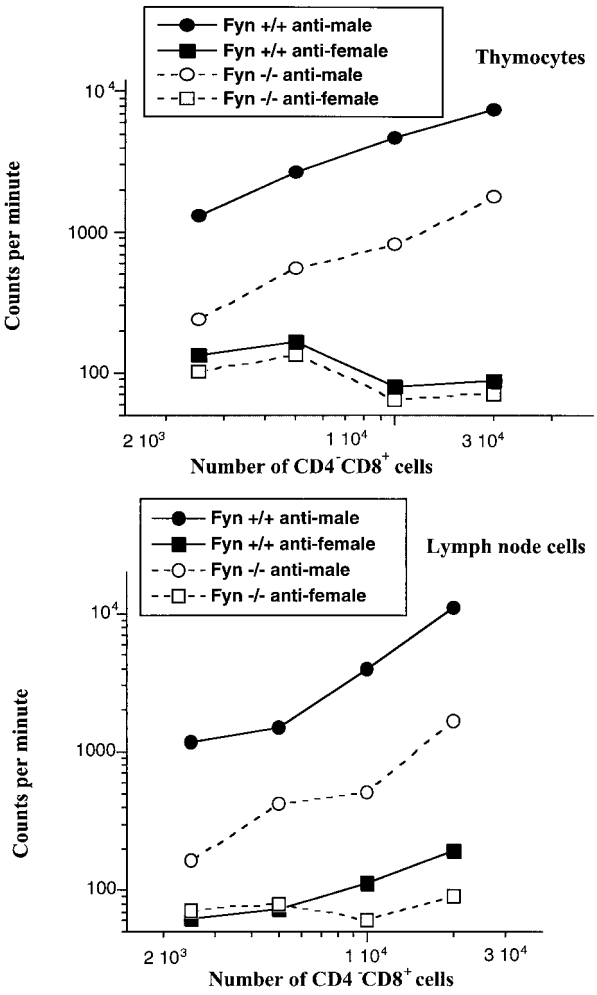

FIGURE 6. H-Y *Fyn*$^{-/-}$ thymocytes and lymph node cells are hyporesponsive to anti-male stimulation. The indicated numbers of purified CD8 SP thymocytes or lymph node cells from female H-2^b H-Y TCR transgenic mice were cultured in triplicate with 5×10^5 irradiated male splenocytes in 96-well plates. Irradiated female splenocytes were used as a negative control. Cultures were incubated with 1 μCi of [^{3}H]TdR/well for the final 16 h before harvest. Data from one representative experiment of three are shown.

thymocytes required 100- to 1000-fold more p2Ca peptide to respond to the same extent as the *Fyn*$^{+/+}$ thymocytes when stimulated with the T2-L^d cells and exogenous p2Ca peptide. This response was p2Ca specific since no proliferative response was observed in the presence of another L^d-binding peptide (YPH FMPTNL) (29) (data not shown). Thus, even for a high affinity ligand, CD8 SP thymocytes required a relatively high ligand density for optimal proliferation to the specific Ag. These results indicate that the lowering of the avidity of the T cell/APC interaction revealed a requirement for *Fyn* by CD8 SP thymocytes expressing a high affinity TCR for its specific Ag. By contrast, only minor differences were evident between CD8 SP *Fyn*$^{-/-}$ and *Fyn*$^{+/+}$ lymph node cells in their response to the L^d/p2Ca ligand, even at the lowest peptide concentrations studied (Fig. 8). This independence of *Fyn* by CD8 SP lymph node cells even at low ligand density likely reflects the more functionally mature status of the lymph node cells relative to thymocytes.

The results examining the role of *Fyn* in the response of 2C CD8 SP lymphocytes and lymph node cells to the low affinity K^b/p2Ca ligand are shown in Figure 9. As a result of such a low affinity interaction between T cells and APCs, it was necessary to add exogenous IL-2 to these cultures to detect a proliferative response to the K^b/p2Ca ligand. As seen in Figure 9, even in the presence of exogenously added IL-2, proliferative responses were only observed at higher peptide concentrations. Significantly, both *Fyn*$^{-/-}$ CD8 SP thymocytes and lymph node cells were hyporesponsive to stimulation by the K^b/p2Ca ligand relative to their *Fyn*$^{+/+}$ counterparts. Not only were the proliferative responses by *Fyn*$^{-/-}$ cells substantially reduced, the responses attained by the *Fyn*$^{-/-}$ cells did not approach that of *Fyn*$^{+/+}$ cells, even at high ligand density. These observations emphasized the importance of *Fyn* in optimizing the responses of T cells expressing low affinity TCRs to stimulation by their specific Ag.

Discussion

This study was aimed at establishing whether the *Fyn* PTK has an essential and unique role in TCR signaling during T cell development and T cell activation. This was done using mice transgenic for TCRs with differing affinity for their antigenic ligands and with the *Fyn* null mutation. Our results indicate that *Fyn* plays a role in TCR signaling during both thymic development and the proliferation responses of mature T cells and is differentially required for high and low affinity/avidity interactions.

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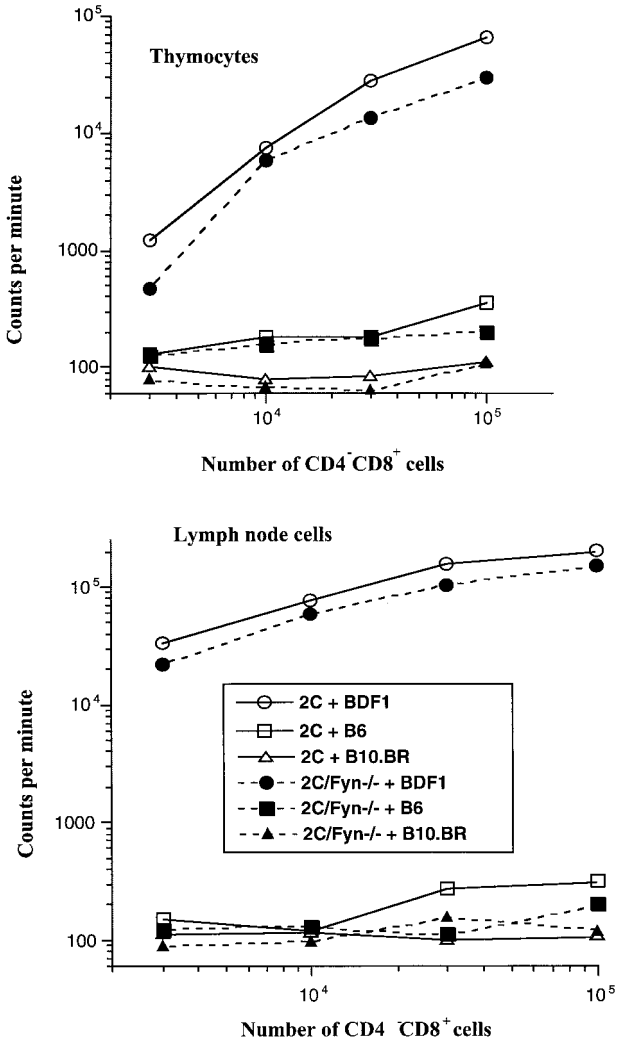

FIGURE 7. 2C *Fyn*$^{-/-}$ thymocytes and lymph node cells exhibit normal proliferative responses to stimulation by BDF_1 spleen cells. The indicated numbers of purified CD8 SP thymocytes or lymph node cells from female H-2^b H-Y TCR transgenic mice were cultured in triplicate with 5×10^5 irradiated BDF_1 splenocytes in 96-well plates. Irradiated B6 and B10.BR splenocytes were used as negative controls. Cultures were incubated with 1 μCi of [^{3}H]TdR/well for the final 16 h before harvest. Data from one representative experiment of three are shown.

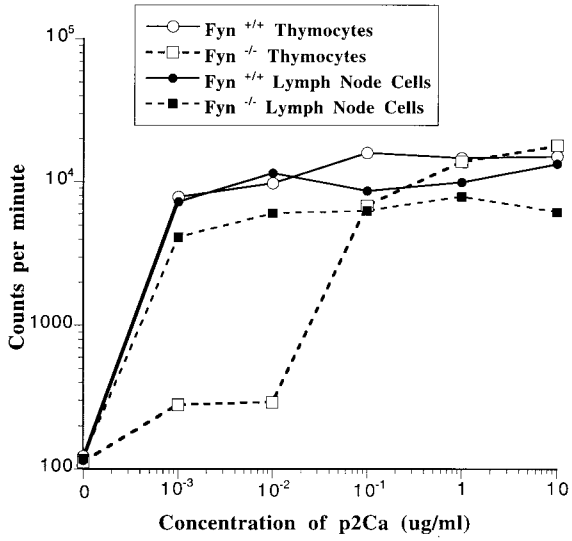

FIGURE 8. 2C *Fyn*$^{-/-}$ thymocytes are hyporesponsive to stimulation by the high affinity L^d/p2Ca ligand at low ligand density. Purified CD8 SP thymocytes or lymph node cells from H-2^b 2C TCR transgenic mice were cultured in triplicate with mitomycin C-treated T2-L^d cells. The indicated concentrations of the p2Ca peptide were added. Cultures were incubated with 1 μCi of [^{3}H]TdR/well for the final 16 h before harvest. Data from one representative experiment of three are shown.

A previous study suggests that the positive selection of CD8 SP thymocytes expressing high levels of the H-Y TCR occurs normally even in the absence of *Fyn* expression (49). Our results are consistent with this report in that we also observed normal production of CD8 SP thymocytes expressing high levels of the H-Y TCR in female H-2^b *Fyn*$^{-/-}$ H-Y TCR transgenic mice. Our study extended this finding by demonstrating that *Fyn* does have subtle effects on positive selection. Our results suggest that although *Fyn* is not essential for the positive selection of the H-Y TCR, it may facilitate the transition of positively selected DP thymocytes into SP thymocytes. Furthermore, we found that *Fyn* facilitated the down-regulation of HSA on positively selected CD8 SP thymocytes. Previous reports have provided evidence for the 2C TCR being strongly selected in H-2^b 2C mice (31), with increases in CD8 expression leading to deletion (14, 15). With a recent report indicating that the efficiency of TCR signaling during T cell development is greatly reduced by the abrogation of TCR-ζ-derived signals (17), it is reasonable to hypothesize that other molecules that contribute to TCR signaling may also influence these processes.

Deletion of *Fyn* is an ideal means to study partial disruption of TCR-ζ signaling. The *Fyn* PTK is constitutively associated with the TCR ζ-chain and has been shown to phosphorylate it upon TCR stimulation, allowing for the association of Zap-70 with the ζ cytoplasmic tail (reviewed in Ref. 50). Murine Zap-70$^{-/-}$ thymocytes arrest at the DP stage (51), suggesting that Zap-70 signaling is essential for T cell development. As *Fyn* phosphorylates

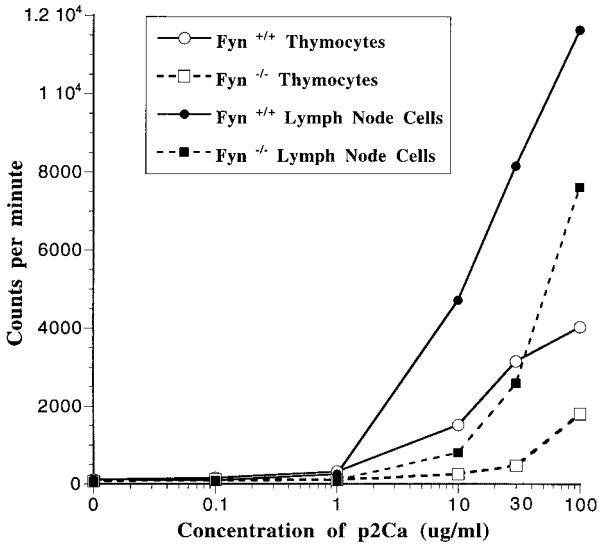

FIGURE 9. 2C *Fyn*$^{-/-}$ thymocytes and lymph node cells are hyporesponsive to a low affinity ligand. Purified CD8 SP thymocytes or lymph node cells from H-2^b 2C TCR transgenic mice were cultured with mitomycin C-treated T2-K^b cells. The indicated concentrations of the p2Ca peptide were added. Cultures were incubated with 1 μCi of [^{3}H]TdR/well for the final 16 h before harvest. Data from one representative experiment of three are shown.

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and activates Zap-70 (27), it may play a role during thymocyte development. However, this situation is complicated by the finding that another PTK, namely *Lck*, can also phosphorylate Zap-70 (27). Thus, the question of whether *Fyn* is a redundant kinase for T cell development and function has not been resolved.

In this study, we found that although *Fyn* is not essential for negative selection, it alters the cell surface phenotype of surviving thymocytes in either a very strongly positively selecting MHC background (H-2^b 2C mice) or a negatively selecting MHC background (male H-2^b H-Y mice). In H-2^b 2C TCR transgenic mice, introduction of the $Fyn^{-/-}$ mutation led to a twofold increase in the number of DP thymocytes. In male H-2^b H-Y TCR transgenic mice, the $Fyn^{-/-}$ mutation enables the survival of thymocytes that expressed higher levels of the CD8 coreceptor and the H-Y TCR. These observations are consistent with the hypothesis that the $Fyn^{-/-}$ mutation may reduce the signaling efficiency of the TCR/CD3 complex during the negative selection process. This hypothesis agrees with previous findings showing that lowering the TCR signaling efficiency by altering the number of ζ-chains or by the deletion of the ζ-chain can allow for the survival of thymocytes that would have been expected to have been deleted (17, 52).

If the $Fyn^{-/-}$ mutation lowers the effective signaling capabilities of the TCR/CD3 complex and allows for higher avidity interactions before deletion, then one would anticipate higher levels of coreceptor expression on $Fyn^{-/-}$ cells. This was indeed the case in the 2C $Fyn^{-/-}$ thymocyte population, where CD8 expression was higher than that of $Fyn^{+/+}$ thymocytes. The reduced deletion and increased CD8 expression in the $Fyn^{-/-}$ 2C system demonstrate that *Fyn* does play a role in TCR signaling during thymocyte development. This effect of *Fyn* was not observed in female H-2^b H-Y TCR transgenic mice. This can be explained by postulating that the H-Y TCR is positively selected by relatively low affinity/avidity thymocyte/selecting cell interactions in female transgenic mice. This level of interaction leading to the positive selection of H-Y thymocytes in female mice is presumed to be well below the deletion threshold, but above the minimum positive selection threshold. Under these conditions, we do not expect *Fyn* to have a discernible effect on the survival of DP thymocytes, and this is observed.

We have shown previously that high cell surface expression of the HSA molecule on CD8 SP thymocytes correlates with positive selection by low affinity/avidity ligands and that these cells are functionally immature (39). Here, we have observed that HSA levels in CD8 SP thymocytes from both H-Y $Fyn^{-/-}$ and 2C $Fyn^{-/-}$ thymocytes are elevated relative to those that expressed *Fyn*. This observation also argues for the selection of these thymocytes by weaker selecting signals and further implicates a role for *Fyn* in the positive selection process. However, we noted that although $Fyn^{-/-}$ thymocytes from both TCR transgenic backgrounds had increased HSA expression, only the H-Y $Fyn^{-/-}$ thymocytes were hyporesponsive to stimulation by anti-TCR Abs. This observation may be explained by the hypothesis that the H-Y TCR is positively selected with low efficiency in the first place. The further lowering of this efficiency by introducing the $Fyn^{-/-}$ mutation will lead to the production of CD8 SP thymocytes that are functionally less mature. We have recently shown that the 2C TCR, when selected by very weak selecting ligands in an H-2^k thymus, also led to the production of CD8 SP HSA^{high} thymocytes that were hyporesponsive to stimulation by anti-TCR Abs (39). That only minimal differences can be observed in the 2C thymocytes can be explained by the same hypothesis. In this situation, the selecting ligand for these cells is of sufficiently high affinity/avidity that the $Fyn^{-/-}$ mutation does not lower the effective signal enough to slow the development of these cells as significantly as in the H-Y system. Hence, these cells are able to proliferate when stimulated with 2C11 and IL-2.

Stimulation of CD8 SP $Fyn^{-/-}$ cells with their physiologic ligands provided evidence that *Fyn* is indeed involved in the proliferation responses of both thymocytes and lymph node cells. As these stimulation protocols do not circumvent the natural affinity of the TCR for its ligand, these results provide clear evidence for a role for *Fyn* in signaling from low affinity TCRs. Using purified CD8 SP cell populations, we saw that both the thymocyte and lymph node cell populations from H-Y $Fyn^{-/-}$ mice were hyporesponsive in an anti-male response. This observation suggested that the low affinity H-Y response was compromised by the *Fyn* mutation. However, the response of CD8 SP 2C thymocytes or lymph node cells to naturally processed ligands on BDF_1 splenocytes did not reveal a role for *Fyn* in this response. We attribute this to the high affinity of the 2C TCR for the L^d/p2Ca ligand. Thus, *Fyn* is not essential for an optimal response by CD8 SP thymocytes or lymph node cells, which expressed a high affinity TCR for its cognate ligand. Importantly, we found that we can reveal a role for *Fyn* in 2C TCR signaling by lowering the affinity/avidity of the 2C/APC interaction by using T2-K^b or T2-L^d cells as APCs and varying the concentrations of the p2Ca peptide. Under these conditions, we observed a role for *Fyn* in the response of CD8 SP 2C thymocytes to the high affinity L^d/p2Ca ligand at low ligand density. In the case of the low affinity K^b/p2Ca ligand, we were able to demonstrate a role for *Fyn* even at high ligand density. These observations underscore the importance of *Fyn* in optimizing responses to low affinity ligands and provide independent evidence supporting the conclusion that the H-Y TCR is indeed a low affinity TCR.

The results presented here provide evidence of a role for *Fyn* in TCR signaling during both thymocyte development and the activation of positively selected thymocytes and peripheral T cells. That the role of *Fyn* was not as evident in previous reports can be explained by the heterogeneous TCR backgrounds studied. We propose that *Fyn* is an important player in the positive selection of T cells by low affinity/avidity ligands and in the activation of positively selected T cells by low affinity/avidity ligands. This function of *Fyn* appears not to be compensated for by other PTKs and argues against *Fyn* being a redundant kinase in T cell development and T cell activation.

Acknowledgments

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