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Itk and Fyn Make Independent Contributions to T Cell Activation

By X. Charlene Liao,* Dan R. Littman,‡ and Arthur Weiss*

From the *Department of Microbiology and Immunology, and Department of Medicine, Howard Hughes Medical Institute, University of California, San Francisco, California 94143; and ‡The Skirball Institute of Biomolecular Medicine, Howard Hughes Medical Institute, New York University Medical Center, New York, New York 10016

Summary

Itk is a member of the Btk/Tec/Itk family of nonreceptor protein tyrosine kinases (PTKs), and has been implicated in T cell antigen receptor (TCR) signal transduction. Lck and Fyn are the Src-family nonreceptor PTKs that are involved in TCR signaling. To address the question of how these members of different families of PTKs functionally contribute to T cell development and to T cell activation, mice deficient for both Itk and either Lck or Fyn were generated. The Itk/Lck doubly deficient mice exhibited a phenotype similar to that of Lck-deficient mice. The phenotype of the Itk/Fyn doubly deficient mice was similar to that of Itk deficient mice. However, the Itk/Fyn doubly deficient mice exhibited a more severe defect in TCR-induced proliferation of thymocytes and peripheral T cells than did mice deficient in either kinase alone. These data support the notion that Itk and Fyn both make independent contributions to TCR-induced T cell activation.

Materials and Methods

Generation of Itk/Fyn and Itk/Lck Doubly Deficient Mice. Itk-deficient mice have been previously reported (5). Fyn-deficient mice were obtained from Paul L. Stein and Philippe Soriano (10) at Fred Hutchinson Cancer Research Center (Seattle, WA). Lck-deficient mice were obtained from Tak W. Mak (Amgen Institute, Toronto, Canada reference 8). Itk/Fyn and Itk/Lck doubly deficient mice were obtained by breeding Itk-deficient mice with Fyn-deficient mice and Lck-deficient mice, respectively. Geno-
typing of these mice was carried out using PCR. The oligonucleotides for genotyping the itk locus are as follows: Itk knockout (KO): 5′ AGCTCGCATCTACGCTGGTCGGTTGAC 3′; A sne− Mice, Additional PCR reactions using the Itk KO-specific oligos were performed to distinguish itk−/− and itk+/- in certain genotypes.

The oligonucleotides for genotyping the itk locus are as follows:

| Genotype | Source | Total cells | Percentage of T cells | Total T cells|
|----------|--------|-------------|-----------------------|--------------|
| +/- (n = 12)* | Thymus | 10.2 ± 4.3 | 10^−7 | 2.1 ± 0.8 | 85.7 ± 4.5 | 9.0 ± 3.5 | 3.2 ± 1.3 | 3.0 ± 1.0 |
| fyn−/− (n = 3)* | Thymus | 17.2 ± 3.3 | 10^−7 | 2.0 ± 1.2 | 88.6 ± 1.9 | 6.9 ± 2.6 | 2.6 ± 0.3 | 2.6 ± 0.7 |
| ttk−/− (n = 16)* | Thymus | 8.7 ± 1.8 | 10^−7 | 3.2 ± 1.2 | 80.7 ± 8.0 | 7.1 ± 2.6 | 8.9 ± 6.2 | 1.0 ± 0.5 |
| fyn−/− ttk−/− (n = 7)* | Thymus | 12.3 ± 4.2 | 10^−7 | 3.7 ± 1.5 | 84.7 ± 4.5 | 6.7 ± 2.8 | 5.0 ± 1.5 | 1.4 ± 0.5 |
| +/- (n = 12)* | Lymph nodes | 6.1 ± 1.4 | 10^−7 | 77.8 ± 8.0 | 4.7 ± 1.1 | 3.3 ± 1.5 | 1.2 ± 0.9 | 47.4 ± 9.2 | 26.0 ± 2.4 | 1.8 ± 0.4 |
| fyn−/− (n = 3)* | Lymph nodes | 6.6 ± 1.6 | 10^−7 | 74.8 ± 6.5 | 5.0 ± 1.6 | 1.9 ± 1.0 | 1.4 ± 1.9 | 47.0 ± 4.7 | 24.4 ± 3.0 | 2.0 ± 0.3 |
| ttk−/− (n = 16)* | Lymph nodes | 6.1 ± 2.3 | 10^−7 | 60.7 ± 9.2 | 3.6 ± 1.4 | 5.2 ± 2.3 | 1.1 ± 1.9 | 27.2 ± 5.5 | 27.2 ± 7.9 | 1.2 ± 0.7 |
| fyn−/− ttk−/− (n = 7)* | Lymph nodes | 5.9 ± 2.0 | 10^−7 | 54.8 ± 7.6 | 3.2 ± 1.0 | 5.8 ± 3.7 | 0.7 ± 0.4 | 25.7 ± 3.2 | 22.6 ± 5.1 | 1.2 ± 0.4 |

Data are expressed as mean ± standard deviation.
* Mice, 7-12 wk old; numbers of animals studied are shown in parentheses.
† Cervical, brachial, axillary, superficial inguinal, and mesenteric.
‡ The number of T cells in the lymph nodes was calculated based on the percentages of cells stained positive with anti-CD3e.
Results

T Cell Development in the Absence of Both Itk and Fyn.

Maturation of thymocytes takes place in an ordered sequence of developmental events (13). Early immature thymocytes do not express either the CD4 or the CD8 coreceptors (DN). As a result of pre-TCR signaling events, the DN cells acquire expression of both CD4 and CD8 to become DP cells. Subsequently, expression of one of the coreceptors is extinguished, and the thymocytes become mature CD4+ and CD8+ single positive T cells. Mice lacking Fyn, an Src family nonreceptor PTK have normal numbers of thymocyte and T cell subpopulations as well as normal levels of surface expression of the TCR chains (Table 1 and Fig. 1), consistent with previous reports (9, 10). On the other hand, mice deficient in Itk have a partial block in T cell development (Table 1 and Fig. 1) in agreement with our previous studies (5). Itk-deficient mice have reduced numbers of mature lymph node T cells as well as a reduced ratio of CD4 to CD8 single positive cells in the thymus and the periphery.

To address whether Itk and Fyn might functionally interact, we examined the developmental consequences of a defect in both of these kinases. Mice deficient for both Fyn and Itk expression were derived by breeding. Analysis of the Itk/Fyn doubly deficient mice demonstrated similar thymic and peripheral T cell phenotypes as the Itk-deficient mice (Table 1 and Fig. 1). Hence, the absence of Fyn did not further enhance the developmental consequences of Itk-deficiency.

A few mice deficient in both Lck and Itk were also obtained. The developmental consequences of this breeding revealed that mice deficient in both Itk and Lck had thymic phenotypes indistinguishable from Lck-deficient mice (data not shown). A partial developmental arrest at the DN to DP transition and a 10-fold reduction in thymocyte number were observed, consistent with studies of the Lck-deficient mice previously reported (8). As observed in the Lck-deficient mice, only a few peripheral T cells were detectable in the spleens and lymph nodes of the Itk/Lck doubly deficient animals. Thus, the absence of Itk does not further augment the already severe phenotype observed in the Lck-deficient mice.

Thymocyte Proliferation in the Absence of Both Itk and Fyn.

To address whether Itk and Fyn make independent contributions to the processes that lead to T cell activation, thymocytes and splenocytes were prepared from Itk/Fyn doubly deficient mice and compared with those from mice lacking either kinase alone in TCR-mediated proliferative responses. Thymocytes lacking either Fyn or Itk consistently showed a reduction in proliferation to anti-CD3 mAb over a broad dose range when compared to control Itk+Fyn+ thymocytes (Fig. 2 A), consistent with previous studies (9, 10). Interestingly, thymocytes lacking both Itk and Fyn exhibited a much more severe proliferative defect, detectable over all doses of anti-CD3 mAb used. These results suggest that both Fyn and Itk contribute to the proliferative response induced by TCR stimulation in thymocytes.

Proliferation of Mature T Cells in Response to Anti-TCR and Allogeneic MHC Stimulations. To examine the response of mature peripheral T cells, splenocytes from Itk/Fyn doubly deficient mice as well as from mice deficient in either kinase alone or control mice were stimulated with anti-CD3 mAb in the presence or absence of PMA. As has been previously reported (9, 10), the proliferation of Fyn—splenocytes was similar to that of wild-type (Itk+Fyn+) splenocytes. On the other hand, splenocytes lacking Itk showed a proliferative response of 15–30% of that of the Itk+Fyn+ cells (Fig. 2 B and data not shown). Consistent with the observation using thymocytes, Itk—Fyn— splenocytes had a much more profound reduction in their proliferative response. Taken together, these results suggest that Itk and Fyn both make at least some independent contributions to TCR-regulated pathways that lead to T cell proliferative responses.

To examine the impact of these kinases on antigen-dependent responses, mature T cells from mutant mice were also examined in MLR. Consistent with the results with anti-CD3 induced proliferation, Itk—Fyn— splenocytes responded very poorly to allogeneic MHC stimulation, whereas Itk-deficient splenocytes had an intermediate level of proliferation (Fig. 3). On the other hand, splenocytes lacking Fyn exhibited near normal alloantigen-induced proliferation (data not shown), consistent with previous studies (9, 10). These results support the notion that both...
Itk and Fyn functionally contribute to T cell responses to alloantigens.

Discussion

We describe here the characterization of Itk/Fyn doubly deficient mice in T cell development and activation. Our data failed to reveal an additive effect upon thymocyte and T cell development in mice that lack both Fyn and Itk. On the other hand, we observed a synergistic loss of proliferative responses of these cell populations in the absence of both Itk and Fyn. The severe defect in proliferative responses induced with TCR stimulation suggests that these kinases provide at least some independent functions in the events leading to T cell and thymocyte proliferation.

Itk-deficient mice and Fyn-deficient mice have distinctive phenotypes. Mice lacking Itk have a reduced ratio of CD4 to CD8 single positive thymocytes (5). The absolute numbers of mature CD4+ T cells are also reduced by about twofold compared to wild-type mice expressing Itk (Table I). Fyn-deficient mice, on the other hand, do not exhibit an obvious developmental phenotype (9, 10). The main immune system defect of Fyn-deficient mice is in mature thymocytes that have diminished proliferative responses as well as reduced tyrosine phosphorylation of cellular proteins upon TCR stimulation (9, 10). Overall, the developmental consequences resulting from a lack of either Itk or Fyn is quite modest.

Itk/Fyn doubly deficient mice resembled Itk-deficient mice in their T cell developmental phenotypes, suggesting that even in the absence of Itk, Fyn is not critically required for T cell development, nor does it play a function that is redundant with Itk. In contrast, thymocytes and mature T cells in Itk/Fyn doubly deficient mice exhibited a severe defect in proliferative responses upon TCR stimulation. This defect was much more pronounced than would be predicted by an additive effect of lacking either kinase alone. These data suggest that both Itk and Fyn are important in T cells activation and are likely to play partially distinct functions.

To address whether Fyn is the only specific and functional partner of Itk in T cell activation, we also generated mice deficient for both Itk and Lck, another Src family member expressed in T cells. The developmental phenotypes of Itk/Lck double-deficient mice resembled those of Lck-deficient mice (data not shown; reference 8). There was an early block of thymocyte maturation resulting in reduced numbers of CD4+CD8+ double-positive thymocytes.
and very few mature T cells. Because of this early block in development, it is difficult to assess whether Lck deficiency exacerbates the later defect due to the absence of Itk. The phenotype of Itk/Lck-deficient mice is also distinct from Fyn/Lck doubly deficient mice (11, 12), which have a complete lack of double-positive thymocytes. The results of Fyn/Lck doubly deficient mice indicate that there is a certain degree of functional redundancy between Fyn and Lck in promoting transition of thymocytes from double-negative to double-positive stages. The results also suggest that Lck-deficiency provides a good platform to reveal Fyn’s contribution to thymocyte development. The lack of similarity between Itk/Lck double-deficient mice and Fyn/Lck doubly deficient mice, however, suggests that Itk is not the sole molecule mediating signals from Fyn at these specific stages of thymocyte maturation. This interpretation is consistent with our studies of Itk/Fyn doubly deficient mice demonstrating that both Itk and Fyn contribute to T cell activation; lack of either kinase alone is not sufficient to abolish T cell activation.

In B lymphocytes, Btk is thought to have functions similar to those of Itk in T cells. Mice deficient in Btk exhibit defects in B cell development and in BCR-mediated proliferative responses. The Src-family PTK Lyn has been shown to regulate the activity of Btk, and mice lacking Lyn have decreased proliferative responses. In T cells, Lck and Fyn may have functions similar to Lyn in B cells. Thus, these PTKs may be involved in activating Itk. The finding that the absence of Fyn exacerbates the proliferative defect of Itk-null T cells suggests that Fyn is additionally involved in pathways distinct from those involving Itk. At least one of these Fyn-specific pathways may involve yet another cytoplasmic PTK. Recent studies have shown that in T cells, Fyn specifically regulates TCR-mediated activation of Pyk2, a PTK homologous to the focal adhesion kinase (14). Thus, Fyn and Itk are likely to regulate at least some distinct events involved in the proliferative response resulting from TCR stimulation. Further work will be required to define these distinct events.

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Address correspondence to Dr. Arthur Weiss, UCSF, Box 0724, 3rd and Parnassus, San Francisco, CA 94143. Phone: 415-476-1291; FAX: 415-502-5081; email: aweiss@itsa.ucsf.edu

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