The EMBO Journal Vol.17 No.22 pp.6551–6557, 1998

Functional dissection of the cytoplasmic subregions of the IL-2 receptor βc chain in primary lymphocyte populations

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The interleukin 2 (IL-2) receptor βc chain (IL-2Rβc) is known to regulate the development and function of distinct lymphocyte populations. Thus far, the functions of the IL-2Rβc cytoplasmic subregions have been studied extensively by using cultured cell lines; however, this approach has limitations with respect to their functions in distinct primary lymphocyte populations. In the present study, we generated mice each expressing a mutant form of an *IL-2Rβc* transgene, lacking the cytoplasmic A- or H-region, on an *IL-2Rβc* null background. We show that lack of the H-region, which mediates activation of the Stat5/Stat3 transcription factors, selectively affects the development of natural killer cells and T cells bearing the γδ T cell receptor. This region is also required for the IL-2-induced proliferation of T cells *in vitro*, by upregulating IL-2Rα expression. In contrast, the A-region, which mediates activation of the Src family protein tyrosine kinase (PTK) members, contributes to downregulation of the T cell proliferation function. The IL-2Rβc null mutant mice develop severe autoimmune symptoms; these are all suppressed following the expression of either of the mutants, suggesting that neither the Stats nor the Src PTK members are required. Thus, our present approach offers new insights into the functions of these cytoplasmic subregions of the IL-2Rβc chain.

Keywords: interleukin-2 receptor βc chain/lymphocyte development/natural killer cells/γδ T cells/T cell growth

Introduction

One of the characteristic features of cytokines is the functional pleiotropism that is mediated by their homologous receptors (Kishimoto *et al*., 1994; Paul and Seder, 1994; Taniguchi, 1995). One typical example is the interleukin-2 receptor (IL-2R) βc (IL-2Rβc) chain, which is responsible for mediating at least two cytokine signals, IL-2 and IL-15. In fact, the functional high-affinity IL-2R and IL-15R consist of three subunits: the IL-2Rα/IL-15Rα, IL-2Rβc and IL-2Rγc chains (Waldmann, 1989; Giri *et al*., 1994; Leonard *et al*., 1994; Taniguchi, 1995; Sugamura *et al*., 1996). The pleiotropic functions of IL-2Rβc in the development and regulation of lymphocytes have been underscored further by the observations made with mice carrying a null mutation in the *IL-2Rβc* alleles (IL-2Rβc-deficient mice). In fact, IL-2Rβc-deficient mice exhibit severe defects in the development of natural killer (NK) cells and intraepithelial lymphocytes (IEL) expressing the γδ T cell receptor (TCR) (γδ T cells). The development of peripheral T cells bearing αβ TCR (αβ T cells) is unaffected in the mutant mice; however, these T cells are activated spontaneously, resulting in plasma cell accumulation and high levels of autoantibodies (Suzuki *et al*., 1995, 1997a).

The cytoplasmic domain of human IL-2Rβc consists of three subregions, the S-, A- and H-regions, the structures of which are well conserved in the mouse (Hatakeyama *et al*., 1989b; Kono *et al*., 1990) (Figure 1A). It has been shown that the membrane-proximal S-region, which recruits the Jak1 protein tyrosine kinase (PTK), is essential to invoke receptor signalling (Leonard *et al*., 1994; Ihle, 1995; Taniguchi, 1995; Sugamura *et al*., 1996; O'Shea, 1997). Supporting this notion are the observations that any mutations leading to the loss of Jak PTK activation result in the total absence of IL-2 signalling *in vitro* and immunodeficiency *in vivo* (Leonard *et al*., 1994; Ihle, 1995; Taniguchi, 1995; Sugamura *et al*., 1996; Rodig *et al*., 1998). On the other hand, the functions of the latter two regions still remain obscure, although one may envisage that they may have biological roles in regulating the pleiotropic function of IL-2Rβc. In fact, several signalling molecules have been reported which interact with these regions, e.g. the A-region may interact with the Src family PTKs, Shc and the p85 subunit of PI3-kinase, and the H-region recruits the Stat5/Stat3 transcription factors (Hatakeyama *et al*., 1991; Horak *et al*., 1991; Cantrell *et al*., 1993; Leonard *et al*., 1994; Fujii *et al*., 1995; Ihle, 1995; Lin *et al*., 1995; Taniguchi, 1995; Friedmann *et al*., 1996; Ravichandran *et al*., 1996; Sugamura *et al*., 1996; Brennan *et al*., 1997; O'Shea, 1997).

Thus far, functional analyses of the IL-2Rβc chain have been carried out by conventional gene transfection assays in cultured cell lines *in vitro*, and important insights were gained as to the cytoplasmic subregions in proliferative signal transmission. In particular, the critical role of the cytoplasmic S-region in invoking signal transmission through activation of Jak1 PTK has been well documented

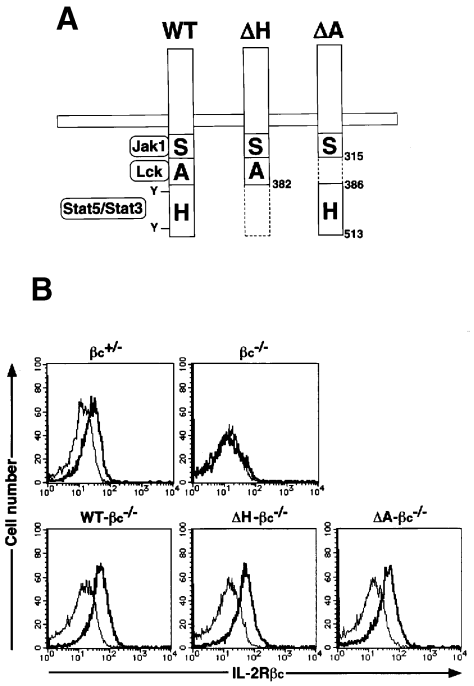

Fig. 1. Generation of mice expressing the mutant IL-2Rβc cDNA on an *IL-2Rβc* null background. (**A**) Schematic view of the murine IL-2Rβc chain and its mutants. IL-2 and IL-15 receptors are trimeric complexes each of which utilizes the specific α chain, in addition to the shared IL-2Rβc and IL-2Rγc chains (Waldmann, 1989; Giri *et al*., 1994; Leonard *et al*., 1994; Taniguchi, 1995; Sugamura *et al*., 1996). The cytoplasmic domain of the human IL-2Rβc chain consists of three regions, and these regions are well conserved between man and mouse (Hatakeyama *et al*., 1989b; Kono *et al*., 1990). The serine-rich region (S-region) of IL-2Rβc is necessary for binding of Jak1 PTK (Miyazaki *et al*., 1994; Russell *et al*., 1994). The acidic region (A-region) is required for physical association with and activation of $p56^{lck}$ and its family members (Taniguchi, 1995). The H-region is essential for Stat5/Stat3 activation (Fujii *et al*., 1995; Lin *et al*., 1995). The two indicated tyrosine residues in this figure, which provide the docking sites for the Stats, are conserved between the human and murine IL-2Rβc. In addition, several signalling molecules, such as Shc and PI3-kinases, have been reported to couple with the human IL-2Rβc in cultured cell lines (Hatakeyama *et al*., 1991; Horak *et al*., 1991; Cantrell *et al*., 1993; Leonard *et al*., 1994; Fujii *et al*., 1995; Ihle, 1995; Lin *et al*., 1995; Taniguchi, 1995; Friedmann *et al*., 1996; Ravichandran *et al*., 1996; Sugamura *et al*., 1996; Brennan *et al*., 1997; O'Shea, 1997). The ΔH- and ΔA-mutants of the murine IL-2Rβc chain lack the H-region (amino acid residues 383–513) or the A-region (residues 316–385), respectively. (**B**) Expression of IL-2Rβc on splenocytes. Splenocytes from 6-week-old Tg-$βc^{-/-}$ mice were cultured in the presence of 5 μg/ml concanavalin A (ConA) for 3 days, then stained with monoclonal antibodies (mAbs) to both IL-2Rβc and CD3. Data were gated on $CD3^+$ cells. Anti-IL-2Rβc unstained (fine line) and stained (bold line) profiles are shown in the histogram.

(Miyazaki *et al*., 1994; Russell *et al*., 1994; Ihle, 1995). However, it is not possible to study how the IL-2Rβc chain mediates its pleiotropic functions in the development and function of distinct lymphocyte populations by this approach. In addition, one may envisage that the functions of IL-2Rβc and the associated signalling molecules in cultured cell lines may not necessarily apply in primary cells (see Klingmuller *et al*., 1997; Wu *et al*., 1997).

In the present study, we took another approach to analyse the as yet obscure functions of the A- and H-regions of the IL-2Rβc chain, by generating mice each expressing a mutant form of an *IL-2Rβc* transgene, lacking one of these cytoplasmic regions, on an *IL-2Rβc* null background and comparing these with mice expressing the wild-type *IL-2Rβc* transgene. Our results clearly indicate that these two regions play distinct roles in lymphocyte development and function.

Results

Generation of mice expressing the mutant IL-2Rβc cDNA on an IL-2Rβc null background

In order to study the roles of the H-region or the A-region in the pleiotropic functions of IL-2Rβc, a transgene (Tg) encoding the mouse wild-type (WT) IL-2Rβc or mutants lacking either the H-region (ΔH-mutant) or the A-region (ΔA-mutant) (Figure 1A) was expressed using the human *CD2* promoter/enhancer (Zhumabekov *et al*., 1995), in order to ensure expression in cells of lymphoid lineage, in an *IL-2Rβc* null background (Suzuki *et al*., 1995) (Tg-$βc^{-/-}$ lines). As shown in Figure 1B, cell surface expression of transgene-encoded WT, ΔH-mutant or ΔA-mutant can be detected on stimulated splenocytes from each of the Tg-$βc^{-/-}$ lines (WT-$βc^{-/-}$, ΔH-$βc^{-/-}$ and ΔA-$βc^{-/-}$ mice, respectively) at levels slightly higher than those from heterozygous ($βc^{+/-}$) mice, but comparable with one another (Figure 1B). Experiments were performed on at least two different Tg founder lines with comparable surface expression in order to confirm the reproducibility of the data presented below.

Suppression of spontaneous αβ T cell activation by the IL-2Rβc cDNA expression

It has been shown that although the development of T cells (αβ T cells) in the thymus proceeds normally, spontaneous activation of these T cells in the periphery was observed in $βc^{-/-}$ mice, leading to the development of severe autoimmune symptoms (Suzuki *et al*., 1995). Typically, a dramatic reduction in B cell number was observed in $βc^{-/-}$ mice, presumably due to an exhaustive B cell differentiation by the activated T cells (Suzuki *et al*., 1995). When splenic T cells from Tg-$βc^{-/-}$ mice were analysed, the activation seen in $βc^{-/-}$ mice was not observed in any of the WT-$βc^{-/-}$, ΔH-$βc^{-/-}$ or ΔA-$βc^{-/-}$ mice, as revealed by the normal expression level of CD69 and by normal cell size (Figure 2A). In addition, the reduction in B cell number was not observed in these mice (Figure 2B). Other associated symptoms such as autoimmune haemolytic anemia, granulocytosis and splenomegaly found in $βc^{-/-}$ mice were not observed in these transgenic mice (data not shown). Thus, deletion of either the A-region or the H-region of the IL-2Rβc chain does not affect suppression of autoimmunity (see Discussion).

Regulation of the development and function of NK cells and γδ T cells by IL-2Rβc

The system employed in this study provides an opportunity to examine the roles of the H- and A-regions of the IL-2Rβc cytoplasmic domain in the development of two lymphocyte populations, which is impaired in $βc^{-/-}$ mice, i.e. NK cells and γδ T cells (Suzuki *et al*., 1997a). As shown in Figure 3A, expression of the WT or ΔA-mutant of IL-2Rβc restored the development of NK ($NK1.1^+$ $CD3^-$) cells, as revealed by flow cytometric analysis. In contrast, the ΔH-$βc^{-/-}$ mice failed to develop NK cells in the spleen (Figure 3A) and in peripheral blood (data not

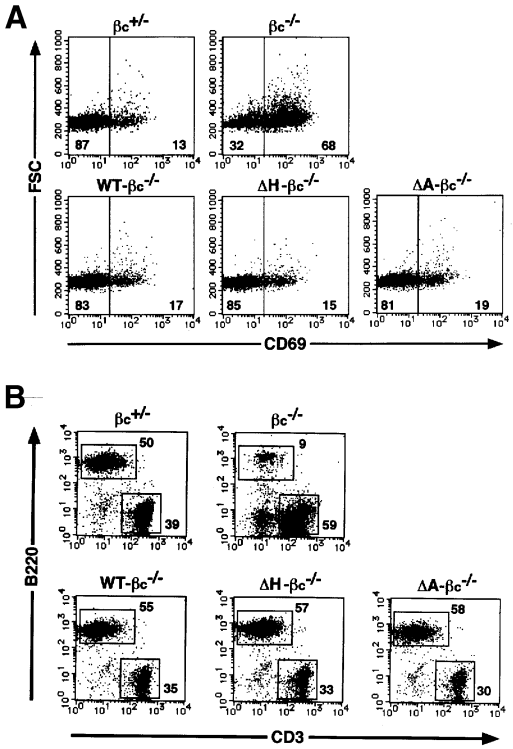

Fig. 2. Suppression of spontaneous αβ T cell activation by IL-2Rβc cDNA expression. (**A**) Rescue of abnormal T cell activation by transgenic expression of IL-2Rβc mutants in $\beta c^{-/-}$ mice. Cells from lymph nodes of 8-week-old mice were stained with mAbs to CD3 and to CD69 and gated on $CD3^+$ cells. (**B**) Splenocytes from mice at 8 weeks were stained with anti-CD3 and anti-B220 mAbs. The numbers represent the percentage of cells contained in each region. The average numbers of splenic B cells are as follows ($\times 10^6$ cells): $\beta c^{+/-}$, 32.2; $\beta c^{-/-}$, 0.5; WT-$\beta c^{-/-}$, 35.8; ΔH-$\beta c^{-/-}$, 34.6; and ΔA-$\beta c^{-/-}$, 37.1.

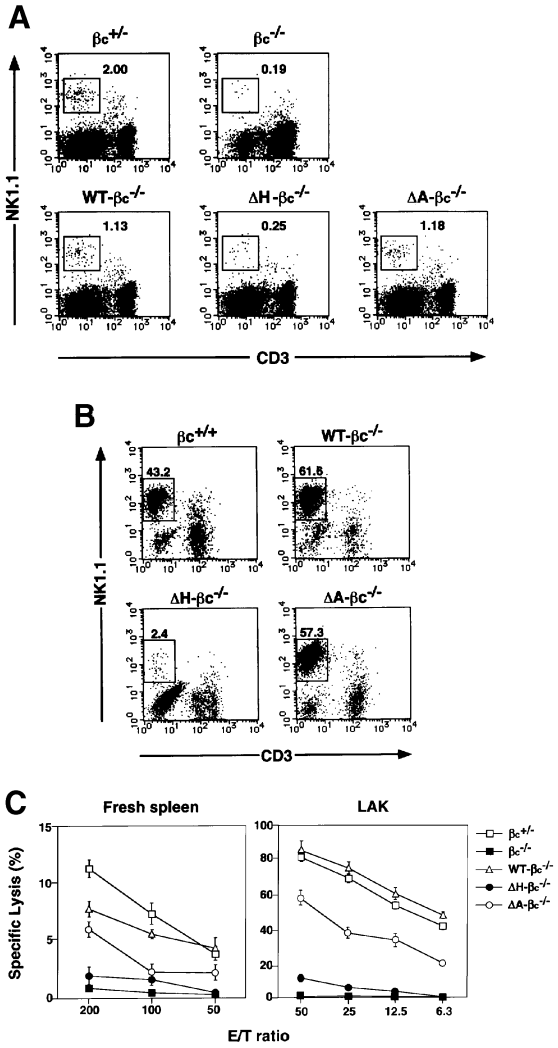

Fig. 3. Development of NK cells in mice expressing the wild-type or mutant IL-2Rβc. (**A**) Flow cytometric analysis of NK cells in Tg-$\beta c^{-/-}$ mice. Splenocytes were stained with anti-NK1.1 and anti-CD3 mAbs, and 10^4 viable lymphocytes were analysed. Numbers represent the percentage of $NK1.1^+$ cells in the gated population for a representative individual from each group. The experiments were performed several times and in different founder mice, and essentially the same results were obtained. (**B**) IL-15-induced development of NK cells from BM cells *in vitro*. BM cells were cultured in the presence of IL-15 (see Materials and methods) and cells subsequently recovered from cultures were analysed by flow cytometry. (**C**) NK cell-mediated cytotoxic activities of freshly isolated Tg-$\beta c^{-/-}$ splenocytes (left panel) or splenocytes activated *in vitro* with IL-2 (Georgopoulos *et al*., 1994) (right panel) (see Materials and methods for the details). Each symbol represents the mean and the SD of triplicate assays. Experiments were performed three times, and essentially the same results were obtained.

shown). Furthermore, NK cells were not generated *in vitro* from ΔH-$\beta c^{-/-}$ bone marrow (BM) cells when cultured in the presence of IL-15 (Figure 3B). As expected, the activation of Stat5/Stat3 by IL-15 (Lin *et al*., 1995) was also found to be completely abolished in ΔH-$\beta c^{-/-}$ BM cells (data not shown; see below), suggesting a potential contribution of these transcription factors to NK cell development.

Next, we examined the cell-mediated cytotoxicity function of NK cells in the spleens of these mice. As expected, expression of the WT IL-2Rβc chain restored NK cell-mediated cytotoxicity, albeit at a slightly reduced level compared with that of $\beta c^{+/-}$ splenocytes. On the other hand, splenocytes derived from ΔH-$\beta c^{-/-}$ mice showed virtually no cytotoxicity (Figure 3C, left panel). Interestingly, the cytotoxic activity in ΔA-$\beta c^{-/-}$ mice was significantly lower than that in WT-$\beta c^{-/-}$ mice (Figure 3C, left panel), despite the presence of a similar number of $NK1.1^+CD3^-$ cells (Figure 3A). In addition, this deficiency was observed consistently when these spleen cells were activated by IL-2 *in vitro* (Figure 3C, right panel), collectively suggesting that the IL-2Rβc A-region is not essential for the development of NK cells, but is required for the induction of their cytotoxic function.

Interestingly, the mice expressing the ΔH-mutant were also found to be defective in the development of γδ T cells in the IEL population (Figure 4). Taken together, these data reveal that the development of two lymphocyte populations is regulated commonly by the H-region of the IL-2Rβc chain.

Regulation of IL-2-induced T cell proliferation by the IL-2Rβc H- and A-regions

The roles of the A- and H-regions in the regulation of lymphocyte growth by IL-2 have been somewhat obscure in previous studies, in which different results were obtained by using different cultured cell lines (Fujii *et al*., 1995; Goldsmith *et al*., 1995; Friedmann *et al*., 1996). We thus examined the *in vitro* growth of the primary T cells from

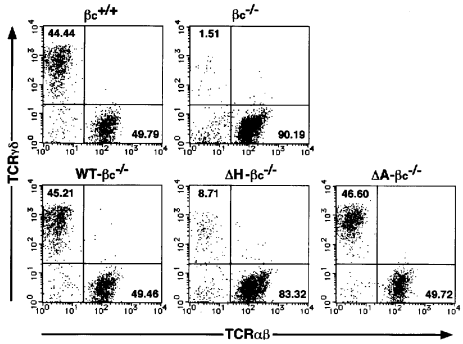

Fig. 4. Development of TCRγδ$^+$ IEL in mice expressing the wild-type or mutant IL-2Rβc. IEL isolated from Tg-βc$^{-/-}$ mice were stained with anti-TCRαβ and anti-TCRγδ mAb, and viable cells were analysed as described in Materials and methods. Numbers in the panel represent the relative percentage of TCRαβ$^+$ and TCRγδ$^+$ cells. Surface expression of transgenic IL-2Rβc was confirmed on IEL from Tg-βc$^{-/-}$ mice by flow cytometric analysis. The assay was performed several times, and essentially the same results were obtained.

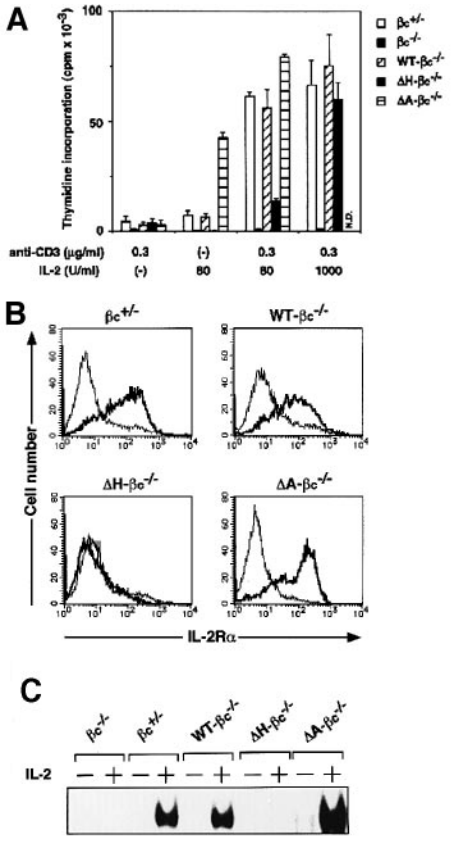

Fig. 5. Roles of the IL-2Rβc H-region in IL-2-induced T cell proliferation. (**A**) IL-2-dependent growth of splenic T cells from mice expressing the wild-type or mutant IL-2Rβc. Growth assays were performed in triplicate as described in Materials and methods. N.D., not determined. The assay was performed several times, and the results were highly reproducible. (**B**) Induction of IL-2Rα expression by IL-2 in T cells expressing the wild-type or mutant IL-2Rβc. Splenocytes were cultured with medium in the presence (bold line) or absence (fine line) of 80 U/ml IL-2 for 3 days in 6-well plates pre-coated with anti-CD3 mAb. Cells were stained with anti-CD3 and anti-IL-2Rα, and IL-2Rα expression is shown for the CD3$^+$ cells. (**C**) The IL-2-induced DNA-binding activity of Stat proteins in T cells expressing the wild-type or mutant IL-2Rβc.

these mice in response to IL-2. Interestingly, notable differences were observed between the splenic T cells from these mice. As shown in Figure 5A, splenocytes from ΔH-βc$^{-/-}$ mice failed to respond fully to IL-2 at a dose as high as 80 U/ml. However, this deficiency could no longer be detected when IL-2 was present at a higher concentration (1000 U/ml), at which the intermediate affinity IL-2R, consisting of IL-2Rβc and γc, can transmit the IL-2 signal (Nakamura *et al.*, 1994; Nelson *et al.*, 1994). In this context, IL-2-induced expression of IL-2Rα, which is critical for the formation of the high-affinity IL-2R (Waldmann, 1989), can be observed in βc$^{+/-}$, WT-βc$^{-/-}$ and ΔA-βc$^{-/-}$ T cells but not in ΔH-βc$^{-/-}$ T cells (Figure 5B). Thus, the failure of the ΔH-βc$^{-/-}$ T cells to respond to low concentrations of IL-2 is presumably due to the lack of IL-2Rα induction by IL-2. It is worth noting that induction of Stat5/Stat3 activities by IL-2 is impaired in splenic T cells from ΔH-βc$^{-/-}$ mice (Figure 5C). These observations are in agreement with those of the previous reports that Stat5/Stat3 is critical to the IL-2-induced activation of *IL-2Rα* gene transcription (Ascherman *et al.*, 1997; Nakajima *et al.*, 1997).

In contrast to ΔH-βc$^{-/-}$ T cells, ΔA-βc$^{-/-}$ T cells were found to show an enhanced growth response to IL-2 (Figure 5A), an observation suggesting a negative regulatory function of the A-region. In this respect, a notable difference was found in the kinetics of the IL-2-induced tyrosine phosphorylation of IL-2Rβc. In fact, receptor phosphorylation was barely detectable in WT-βc$^{-/-}$ T cells 60 min after IL-2 stimulation, but still remained high in ΔA-βc$^{-/-}$ T cells (Figure 6A, upper panel). Likewise, the IL-2-induced DNA-binding activity of the Stats (mostly Stat5; H.Fujii, unpublished data) also remained high in ΔA-βc$^{-/-}$ T cells (Figure 6A, middle panel). On the other hand, such a notable difference was not found in the kinetics of Jak3 phosphorylation, one of the earliest events after IL-2 stimulation, among these T cells (Figure 6A, lower panel).

The above results suggest that the A-region-mediated activation of a phosphotyrosine phosphatase(s) is involved in the negative regulation. Previously, it has been shown that the IL-2-induced activation of p56^{lck} is mediated by the A-region in cultured cell lines (Hatakeyama *et al.*, 1991), and this activation was also abolished in ΔA-βc$^{-/-}$ T cells (data not shown). In this context, one potential candidate is SHP-2, a cytoplasmic phosphotyrosine phosphatase, whose activation may be mediated by the Src family PTKs (Adachi *et al.*, 1997). In fact, the IL-2-induced tyrosine phosphorylation of SHP-2 is not detectable in ΔA-βc$^{-/-}$ T cells (Figure 6B), suggesting that the SHP-2 activation is impaired.

Discussion

It has been shown that the cytoplasmic S-region of IL-2Rβc is essential for association with and activation of Jak PTKs and, consequently, downstream IL-2 signal transmission. The IL-2Rβc chain also contains two additional subregions within the cytoplasmic domain, the A- and H-regions, which are not required for the Jak PTK activation but may modulate other important functions (Taniguchi, 1995; Sugamura *et al.*, 1996).

It was shown previously that the A-region is the site of interaction with p56^{lck} (or other Src family PTK members) (Hatakeyama *et al.*, 1991; Horak *et al.*, 1991; Taniguchi,

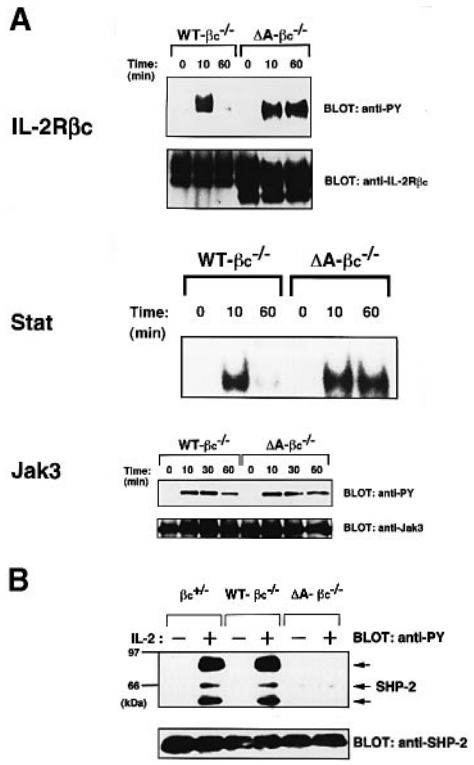

Fig. 6. Roles of the IL-2Rβc A-region in IL-2-induced T cell proliferation. (**A**) Kinetics of the IL-2-induced tyrosine phosphorylation of IL-2Rβc and Jak3, and Stat DNA-binding activity. IL-2-induced tyrosine phosphorylation of the IL-2Rβc chain remained high in ΔA-$\beta c^{-/-}$ T cells at 60 min, the time by which this phosphorylation is barely detectable in WT-$\beta c^{-/-}$ (upper panel) and $\beta c^{+/-}$ T cells (data not shown). The ΔA-mutant of IL-2Rβc contains two tyrosine residues at 395 and 498 within the H-region, and these residues are the 'docking' site for the Stats (Lin *et al*., 1995). The Stat activity induced by IL-2 is also prolonged in ΔA-$\beta c^{-/-}$ T cells (middle panel). In contrast, the kinetics of IL-2-induced Jak3 phosphorylation were similar in WT-$\beta c^{-/-}$ and ΔA-$\beta c^{-/-}$ T cells (lower panel). (**B**) Tyrosine phosphorylation of SHP-2 in $\beta c^{+/-}$, WT-$\beta c^{-/-}$ or ΔA-$\beta c^{-/-}$ T cells. After IL-2 stimulation for 10 min, cells were lysed, and the lysates were immunoprecipitated with anti-SHP-2 antibody and subjected to immunoblot analysis using anti-SHP-2 or anti-phosphyotyrosine antibody (4G10). The positions of SHP-2 and associated tyrosine phosphorylated proteins (see Adachi *et al*., 1997) are indicated.

1995), phosphatidylinositol 3-kinase (PI3-kinase) (Cantrell *et al*., 1993; Brennan *et al*., 1997) and Shc (Friedmann *et al*., 1996; Ravichandran *et al*., 1996), and that the H-region is essential for the recruitment and activation of Stat5/Stat3 (Fujii *et al*., 1995; Lin *et al*., 1995). However, the previous approach could not provide insights into the functions of the H- and A-regions in distinct lymphocyte populations. In the present study, we took an *in vivo* approach to study the functions of these regions. Our observations not only indicate the importance of these two regions in lymphocyte development and function, but also point to the limitations of the strategies used in cultured cell lines versus primary cells (Klingmuller *et al*., 1997; Wu *et al*., 1997).

It is interesting to note that loss-of-function mutations within the *IL-2*, *IL-2Rα* or *IL-2Rβc* genes commonly result in the development of autoimmunity (Sadlack *et al*., 1993; Kneiz *et al*., 1995; Suzuki *et al*., 1995; Willerford *et al*., 1995; Van Parjis *et al*., 1997; Rafaeli *et al*., 1998). It has been reported that the activation-induced cell death (AICD) in T cells is found to occur normally in *IL-2Rβc* null T cells (Suzuki *et al*., 1997b). Therefore, the mechanism by which IL-2Rβc suppresses autoimmunity still remains unclear at present. The present findings demonstrate that deletion of the A- or H-regions does not affect the suppressive function of IL-2Rβc for autoimmunity. One may therefore envisage that all the downstream signalling molecules whose activation is mediated by these regions, e.g. Stat5/Stat3 (Fujii *et al*., 1995; Lin *et al*., 1995), $p56^{lck}$/SHP-2 (Hatakeyama *et al*., 1991; Horak *et al*., 1991; Adachi *et al*., 1997), Shc/Ha-ras (Sato *et al*., 1992; Friedmann *et al*., 1996; Ravichandran *et al*., 1996) and PI3-kinase (Cantrell *et al*., 1993; Brennan *et al*., 1997), are not involved. On the other hand, these regions may be functionally redundant, so that deletion of both regions may affect the suppression of autoimmunity. It is also possible that the S-region-mediated activation of the Jak PTKs by these mutant receptors may be sufficient for the suppression. Hence, further work will be required to address this issue by generating mice expressing the IL-2Rβc lacking both the A- and H-regions, or the S-region.

Our results demonstrate the importance of the H-region for the development of two lymphocyte populations, the IEL γδ T and NK cells, both of which show extrathymic development. At present, the mechanism by which the H-region mediates this development is unclear. It is assumed that the IL-15 signal, rather than the IL-2 signal, is critical for the lymphocyte development (Mrozek *et al*., 1996; DiSanto, 1997; Ogasawara *et al*., 1998). We infer that the defect in ΔH-$\beta c^{-/-}$ mice may be attributed, at least in part, to the lack of Stat5/Stat3 activation. At present, it is unclear whether the H-region (and the Stats) is required for the development of these two lymphocyte populations from a common precursor.

The role of the H-region in T cell proliferation in response to IL-2 is clearer, and is in good agreement with the previous study demonstrating the Stat5/Stat3-mediated induction of the *IL-2Rα* gene (Ascherman *et al*., 1997; Nakajima *et al*., 1997). On the other hand, it is surprising that the T cells expressing the IL-2Rβc ΔA-mutant respond better in IL-2-mediated proliferation. In fact, several signalling molecules have been shown to be functionally linked to the A-region, such as the Src family members, Shc/Ha-ras and PI-3 kinase (Hatakeyama *et al*., 1991; Horak *et al*., 1991; Sato *et al*., 1992; Cantrell *et al*., 1993; Friedmann *et al*., 1996; Ravichandran *et al*., 1996; Brennan *et al*., 1997). In this regard, the role of this region in IL-2-induced cell proliferation was not firmly established with cultured cell lines, and different conclusions were drawn depending on the cell types used and/or the receptor expression systems (Fujii *et al*., 1995; Goldsmith *et al*., 1995; Friedmann *et al*., 1996). Our present results clearly indicate that the net result of the loss of the A-region results in the enhanced proliferation of primary T cells. This enhancement is accompanied by the prolonged phosphorylation of IL-2Rβc and the sustained DNA-binding activity of the Stat factors. Furthermore, the activations of $p56^{lck}$ PTK and SHP-2 phosphatase by IL-2 is dependent on the A-region. It has been shown that the $p56^{lck}$ PTK activation is dependent not only on the A-region, but also on the S-region which is crucial for the Jak PTK activation.

Collectively, we propose the operation of the following pathway of downregulation. Upon ligand stimulation, IL-2Rβc is activated by phosphorylation via Jak PTKs, and the $p56^{lck}$ PTK activation, possibly also mediated by the Jaks (Taniguchi, 1995), phosphorylates SHP-2 for its activation. Then, the phosphatase activity of SHP-2 downregulates the IL-2Rβc function. It is interesting that, in contrast to IL-2Rβc, the kinetics of Jak3 phosphorylation were not altered in ΔA-$βc^{-/-}$ T cells. This suggests that the action of SHP-2 is selective to the receptor, but not to Jak PTKs, whose negative regulation is mediated by another mechanism(s) (Endo *et al*., 1997; Naka *et al*., 1997; Starr *et al*., 1997). We have not yet studied the activation of other signalling molecules such as Ha-ras and PI3-kinase, the activation of which would rather promote cell proliferation. If these molecules were not activated in ΔA-$βc^{-/-}$ T cells, as previously shown in cultured cell lines, it would mean that these molecules are compensatory for the IL-2-induced proliferative response of T cells. Further work will be required to clarify this point.

Although further mechanistic insights will be required to assess how these cytoplasmic subregions of IL-2Rβc control lymphocyte development and function, our present work reveals the heretofore unknown roles of these subregions, and emphasizes the merit of the *in vivo* approach in dissecting the role of the various regions of the IL-2Rβc cytoplasmic domain in the regulation of the immune system.

Materials and methods

Construction of transgenes and generation of Tg mice

For the construction of the mouse IL-2Rβc ΔH-mutant, pMIL-2Rβ-10 (Kono *et al*., 1990) was amplified by PCR using the synthetic oligonucleotides 5′-ACCGAATTCTGAGATCTCTCCGCTG-3′ and 5′-TTAAAGCTTGGGCCCTAGTGGGGAGATCCCT-3′ as primers. The PCR product was digested with *Bgl*II and *Apa*I, and the resulting cDNA insert was introduced into the backbone DNA fragment of pMIL-2Rβ-10 (digested with *Bgl*II and *Apa*I) to generate pMIL-2RβΔH. For the construction of the mIL-2Rβc ΔA-mutant, pMIL-2Rβ-10 was amplified by PCR using synthetic oligonucleotides 5′-AATCTGCAGCCTCTGGCTGGAGAA-3′ and 5′-CCAAAGCTTAGATCTTTCTTCAGGGGC-3′ as primers. The PCR product was digested with *Pst*I and *Hin*dIII, then ligated with synthetic oligonucleotides corresponding to amino acids 298–315 of the mouse IL-2Rβc, and *Eco*RI–*Pst*I-digested pBluescript. The *Bgl*II fragment was isolated from the resulting plasmid and ligated into *Bgl*II-cleaved pMIL-2Rβ-10 to generate pMIL-2RβΔA. Coding sequences of the mIL-2Rβc wild-type, ΔH- and ΔA-mutants were excised from pMIL-2Rβ-10, pMIL-2RβΔH and pMIL-2RβΔA, respectively, by *Eco*RI and *Bam*HI digestion, and ligated into a human *CD2* expression cassette (Zhumabekov *et al*., 1995). All constructs were verified by DNA sequencing. Tg mice with the same background as the null mutant mice (i.e. C57BL/6) were generated, as previously described (Suzuki *et al*., 1996; Kimura *et al*., 1997). Each Tg mouse subsequently was mated with the null mutant mice to generate the desired mice. Generation and maintenance of the IL-2Rβ-deficient mice was described previously (Suzuki *et al*., 1995).

Flow cytometric analysis

Cells were incubated on ice with monoclonal antibodies (mAbs) for 30 min and analysed by flow cytometry (FACScalibur; Beckton Dickinson) using the CELLQuest software (Beckton Dickinson). The mAbs used in this study were as follows: anti-mouse CD3ε [fluorescein isothiocyanate (FITC)-conjugated or phycoerythrin (PE)-conjugated, clone 145-2C11], anti-mouse IL-2Rβc (PE-conjugated, clone TM-β1), anti-mouse IL-2Rα (FITC-conjugated, clone 7D4), anti-mouse CD69 (FITC-conjugated, clone H1.2F3), anti-mouse B220 (PE-conjugated, clone RA3-6B2), anti-mouse NK1.1 (PE-conjugated, clone PK136), anti-mouse TCRαβ (FITC-conjugated, clone H57-597) and anti-mouse TCRγδ (PE-conjugated, clone GL3). All mAbs were purchased from Pharmingen.

T cell growth assays

Growth assays were performed essentially as described previously (Suzuki *et al*., 1995). Splenocytes (1×10^5 cells) were cultured in triplicate in flat-bottomed 96-well plates, which were pre-coated with anti-CD3ε mAb (145-2C11, Pharmingen) for 12 h at 4°C, in 0.2 ml of RPMI-1640 medium supplemented with 10% fetal calf serum (FCS; Gibco-BRL), L-glutamine, sodium pyruvate, non-essential amino acids and 5×10^{-5} M 2-mercaptoethanol. Cells were pulsed with 0.5 μCi of [^{3}H]thymidine (Amersham) for the last 16 h of the 88 h culture period, and the [^{3}H]thymidine uptake levels were measured using a Top Count (Packard).

Immunoprecipitation and Western blot analysis

These analyses were done essentially according to the published procedure (Miyazaki *et al*., 1994). Briefly, splenocytes were incubated with 10 μg/ml ConA for 3 days. Cells were washed and starved for 16 h and stimulated with IL-2 or mock-stimulated. Cell lysates were immunoprecipitated with anti-IL-2Rβc (TM-β1) or anti-Jak3. Immunoprecipitates were separated by 7.5% SDS–PAGE and subsequently immunoblotted with anti-phosphotyrosine (4G10), anti-IL-2Rβc or anti-Jak3.

Electrophoretic mobility shift assay (EMSA)

The assay was carried out according to the published procedure (Fujii *et al*., 1995). Splenocytes were incubated with 10 μg/ml ConA for 3 days. Cells were washed and starved for 16 h and stimulated with IL-2 or mock-stimulated for 10 min and lysed. Whole cell extracts were subjected to EMSA assay (Fujii *et al*., 1995). The probe used in this assay contains the interferon-γ-activated site (GAS) sequence found in the promoter of the mouse *IRF-1* gene (Fujii *et al*., 1995).

Preparation of IEL

These lymphocytes were prepared essentially as described (Suzuki *et al*., 1997a). Briefly, whole small intestine was removed from mice, the Peyer's patches were excluded, and the lumen flushed with RPMI-1640 medium. The intestine was then cut into small pieces and rotated in RPMI medium at room temperature for 30 min. Displaced IEL were passed through nylon mesh to remove intestinal debris and centrifuged at 1300 r.p.m. for 5 min. Cells were resuspended in 5 ml of RPMI medium, overlaid onto a discontinuous gradient (75, 40 and 30%) of Percoll (Pharmacia), and centrifuged at 2500 r.p.m. for 30 min. The interface between 75% and 40% Percoll was recovered, washed, and resuspended in RPMI medium containing 10% FCS.

Preparation of NK cells and cytotoxicity assay

The NK cell preparation and cytotoxic assays were performed as previously described (Ogasawara *et al*., 1998). Lymphokine-activated killer assays were performed as described (Georgopoulos *et al*., 1994).

Development of NK cells from BM cells in vitro

The assay was carried out essentially as described (Ogasawara *et al*., 1998). BM cells were cultured in RPMI-1640 medium containing 10% FCS and simian rIL-15 (Genzyme, 40 ng/ml) for 10 days. Cells recovered from cultures were analysed by flowcytometry.

Acknowledgements

We thank Drs E.L.Barsoumian and M.S.Lamphier for their invaluable suggestions and advice. We also thank Drs D.Kioussis and M.Adachi for providing the VA hCD2 cassette and anti-SHP-2 antibody, respectively. This work was supported in part by the Research for the Future (RFTF) Program (96L00307) from the Japan Society for the Promotion of Science (JSPS) (to T.T.), and Grant-in-Aid for Scientific Research on Priority Areas No. 09273105 (to H.F and T.M.) from the Ministry of Education, Science and Culture of Japan. K.O. is a JSPS Research Fellow.

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Received August 11, 1998; revised and accepted September 24, 1998