Cytokine-independent Jak3 Activation upon T Cell Receptor (TCR) Stimulation through Direct Association of Jak3 and the TCR Complex*

Kazuhiro Tomita‡‡, Kaoru Saijo‡‡, Sho Yamasaki‡, Tomohiko Iida‡‡, Fubito Nakatsu‡, Hisashi Arase‡‡, Hiroshi Ohno‡ **, Takuji Shirasawa‡‡, Takayuki Kuriyama‡, John J. O'Shea‡‡§, and Takashi Saito‡‡¶

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Jak3 is responsible for growth signals by various cytokines such as interleukin (IL)-2, IL-4, and IL-7 through association with the common γ chain (γc) in lymphocytes. We found that T cells from Jak3-deficient mice exhibit impairment of not only cytokine signaling but also early activation signals and that Jak3 is phosphorylated upon T cell receptor (TCR) stimulation. TCR-mediated phosphorylation of Jak3 is independent of IL-2 receptor/γc but is dependent on Lck and ZAP-70. Jak3 was found to be assembled with the TCR complex, particularly through direct association with CD3δ via its JH4 region, which is a different region from that for γc association. These results suggest that Jak3 plays a role not only in cell growth but also in T cell activation and represents cross-talk of a signaling molecule between TCR and growth signals.

The function and fate of T cells after antigen recognition are determined by the quality and quantity of the combination of antigen-recognition signals through the T cell receptor (TCR) complex, co-stimulation signals through co-stimulation receptors such as CD28, and growth signals through cytokine receptors such as IL-2 receptor (R). The TCR complex is composed of clonotypic TCRαβ dimers recognizing the antigen-major histocompatibility complex as well as the CD3 complex responsible for signal transduction and is comprised of three dimers: γc, δε, and ζ homodimers (1, 2). All CD3 chains contain a common signaling motif, ITAM (immunoreceptor tyrosine-based activation motif), within the cytoplasmic region. The CD3γ, δ, and ε chains each contains one ITAM whereas CD3ζ possesses three. Antigen recognition by TCR induces tyrosine phosphorylation of ITAM of the CD3 chains, particularly the CD3ζ chain, by src family tyrosine kinases, Lck or Fyn, followed by recruitment of ZAP-70 to the phosphorylated ITAM (3). Subsequently, activated ZAP-70 induces downstream phosphorylation of various adaptor proteins including LAT, SLP-76, Vav, and PLCγ (4). On the other hand, growth signals in T cells are mediated through cytokine receptors such as IL-2R, IL-4R, or IL-7R. These cytokine receptor complexes contain a common γ chain (γc) as a component that has been shown to be crucial for signal transduction of cell growth (5). Functional mutation of γc was reported to result in X-linked severe combined immunodeficiency (6). Jak3 kinase is associated with γc (7, 8) and is responsible for transducing growth signals through the activation of STATs (signal transducers and activators of transcription) (9–11) and STAM (signal transducing adaptor molecule) (12). It has been shown in mouse and man that functional mutation of Jak3 also results in autosomal recessive severe combined immunodeficiency (13). We and others showed that Jak3-deficient mice revealed a similar severe combined immunodeficiency phenotype, characterized by a lack of B cells, NK cells, and by reduced numbers of most T cells (13–18). Nevertheless, despite the number of T cells being strongly reduced in these mice, the pattern of thymocyte development was almost normal, and T cells recovered with age in the periphery (17, 18).

During functional characterization of these peripheral T cells in Jak3-deficient mice, we found that these T cells from the knockout mice failed to exhibit not only the response to cytokines, which was expected, but also the early activation signals such as Cu2+ mobilization upon TCR stimulation (18). These analyses led us to assume the possible involvement of Jak3 in TCR signaling. Indeed, the present study demonstrates that Jak3 is directly associated with the TCR complex, particularly with the CD3ζ chain, and is phosphorylated upon TCR stimulation. We found that Jak3 utilizes a region for assembling with CD3ζ that is distinct from that with γc. These data suggest that Jak3 may be involved in TCR activation signals independently of γc that is involved in growth signals, and they imply the occurrence of cross-talk by a signaling molecule between pathways for antigen-recognition signals and growth signals.

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¶ Present address: Laboratory for Lymphocyte Signaling, Rockefeller Univ., 1230 York Ave., New York, NY 10021.
§§ Present address: Div. of Molecular Membrane Biology, Cancer Research Institute, Kanazawa University, 1-3-1 Takaramachi, Kanazawa 920-0984, Japan.
** Present address: Dept. of Microbiology and Immunology, Univ. of California, San Francisco, 152 Parnassus Ave., San Francisco, CA 94143.
†† To whom correspondence should be addressed. Tel.: 81-43-222-2198; Fax: 81-43-222-1791; E-mail: saito@med.m.chiba-u.ac.jp.

1 The abbreviations used are: TCR, T cell receptor; R, receptor; ITAM, immunoreceptor tyrosine-based activation motif; IL, interleukin; γc, γ chain; STATs, signal transducers and activators of transcription; STAM, signal transducing adaptor molecule; Ab(s), antibodies; mAb(s), monoclonal antibodies; GAH, goat anti-hamster Ig Ab; PAGE, polyacrylamide gel electrophoresis; GST, glutathione S-transferase; IFN, interferon.
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**EXPERIMENTAL PROCEDURES**

*Cells and Abs—* 23-1-8 is a KLH-specific T cell clone and is maintained by periodic antigen stimulation as previously described (19). 2B4 and D011.10 were widely used murine T hybridoma cells specific for cytochrome c and ovalbumin, respectively. E6.1 is a wild-type Jurkat cell line, and J.CaM1 (20, 21) and P16 (22) are Jurkat mutant cell lines deficient for Lck and ZAP-70, respectively (kindly provided by Dr. S. Weiss, California San Francisco, CA and Dr. R. Abrahm, Mayo Clinic, MN, respectively). Anti-Jak3 Abs (anti-peptide Asp169–Gly182 for Western blotting and anti-Jak3Pro498-Pro794 for Ab precipitation) were raised in rabbit as described (15). Rabbit antisera against the C and N terminal of human Jak3 were described (23). Anti-TCRβ mAb (H57-597) and anti-CD3ε mAb (H14-689A) were kind gifts from Dr. R. Kube and Dr. B. De Allergy and Immunology, San Diego, CA. Anti-γc mAbs (TUGm2, TUGm3) were previously described (5, 12) and provided by Dr. K. Sugamura (Tokohu Univ, Sendai). Anti-CD3ε mAb (145-2C11) was a gift from Dr. J. Bluestone (Chicago Univ., Chicago, IL). Anti-phosphotyrosine mAb (4G10) and anti-FLAG (M2) were purchased from Upstate Biotechnologies Inc., Lake Placid, NY and SIGMA, respectively, and goat anti-hamster Ig Ab (GAH) and sheep anti-mouse Ig Ab (SAM) were from Organen Teknica Corp.

**Plasmid Construction and Transfection—** To analyze the association of Jak3 and CD3ζ and the phosphorylation of Jak3, 1–3 × 10^6 293T cells were transiently transfected with the expressible constructs of Jak3 (pME-Jak3), CD3ζ (pME-ζ), and as well as with Lck and ZAP-70 using the LipofectAMINE®/Reagent (Life Technologies, Inc.) according to the manufacturer. The C- and N-terminal deletion mutants and the kinase-dead mutant Jak3 (K555A) were previously generated (23). ΔH4 deletion mutant of Jak3 lacking Phexε–Ser312 was constructed. Wild type and ΔH4 Jak3 were attached with a FLAG tag and cloned into pME18S. All the constructs were confirmed by DNA expression vector sequencing using Applied Biosystems PRISMS Dye Terminator Cycle Sequencing kit (PerkinElmer Life Sciences).

**Immunoprecipitation, Blotting, and Surface Biotinylation—** For biochemical analysis, T cells were stimulated by cross-linking with anti-CD3ε mAb (2C11) and GAH as described previously (24, 25). Stimulated T cells were lysed with a lysis buffer (1% digitonin, 1% Brij 97 or 1% Nonidet P-40/0.1% SDS (radioimmune precipitation buffer), 150 m NaCl, 5 mM EDTA, 10 mM NaF, 1 mM phenylmethylsulfonyl fluoride, leupeptin (10 μg/ml), aprotinin (10 μg/ml), antipain (25 μg/ml), chymostatin (25 μg/ml), pepstatin (10 μg/ml), and 10 mM iodoacetamide) at 4 °C for 40 min, and the cell lysates were immunoprecipitated with the indicated Abs followed by analysis on SDS-PAGE. For Western blotting the proteins were transferred onto a polyvinylidene difluoride membrane (Millipore), and the membrane was blotted with Abs and visualized using the ECL detection system (Amersham Pharmacia Biotech). For immunoprecipitation, surface labeling, cells were labeled by surface biotinylation as previously described (24, 25).

**In Vitro Binding Assay—** A construct of GST fusion protein containing the cytoplasmic domain of CD3ζ (GST-ζ) has been described (26). 35S-labeled, *in vitro* translated Jak3 probe containing the JH2–JH4 region (amino acids 281–783) was constructed by subcloning of SacI fragment of Jak3 into pCITE vector and was *in vitro* transfected using [35S]methionine. *In vitro* binding assay using the 35S-labeled Jak3 probe was performed as described previously (26, 27). Briefly, purified GST-ζ was adsorbed onto glutathione-Sepharose® 4B beads and incubated with *in vitro* translated Jak3 probe. Subsequently, Jak3 attached to the beads was eluted by 10–50 bead volumes of 20 mM glutathione and analyzed on SDS-PAGE.

**Measurement of Intracellular Ca**^2+**Response—** 1 × 10^6 purified splenic T cells were loaded with Indo-1 (Indo-1 AM, Molecular Probes, Eugene, OR) in the presence of P127 (Molecular Probes), washed, and incubated with anti-CD4-phycocerythrin and anti-CD8-fluorescein isothiocyanate (PharMingen, San Diego, CA) in the presence or absence of anti-CD3ε-biotin plus anti-CD4-biotin (RM4-5-biotin). The binding of RM4-5 to CD4 or CD8 molecules was not blocked by another anti-CD4 mAb (PL-172) (data not shown). After washing, T cells were stimulated by cross-linking with anti-CD3ε mAb (2C11) alone or with a mixture of anti-CD3 and anti-CD4 mAbs followed by co-cross-linking with streptavidin. Labeled and stimulated cells were subjected to Ca**^2+** analysis by FACScan (Becton Dickenson, Mountain View, CA). The Ca**^2+** flux was monitored for 512 s, and the results were analyzed with MULTITIME software (Phoenix Flow Systems, San Diego, CA).

**RESULTS**

**Impaired Early T Cell Activation Signal in Jak3-deficient T Cells—** We reported previously that splenic T cells from Jak3-deficient mice exhibited impaired early activation signals such as Ca**^2+** response upon cross-linking of the TCR complex with anti-CD3ε mAb (18). In contrast, Thomis et al. (28) described a similar analysis with a different conclusion, namely, that Jak3−/− peripheral T cells exhibited Ca**^2+** flux comparable with normal T cells upon TCR stimulation. The different results appear to be explainable by the different stimuli used in the two systems. We stimulated T cells by cross-linking with anti-CD3ε mAb alone, whereas Thomis et al. utilized stimulation by cross-linking of both CD3 and CD4 with biotin-conjugated anti-CD3 and anti-CD4 mAbs together with avidin. It is known that cross-linking of CD4 induces a severalfold increase of Lck activity (29), and further co-cross-linking of CD3 and CD4 with biotinylated Abs and avidin induced extremely strong activation signals for Lck activation and Ca**^2+** flux. We assumed that such strong signals might bypass the involvement of Jak3 for T cell activation. To examine this possibility, CD4^+ T cells from Jak3−/− mice were stimulated by cross-linking with either CD3 alone or CD3+CD4 in the biotin-avidin system, and Ca**^2+** responses were compared (Fig. 1). We found that Jak3−/− T cells failed to elicit Ca**^2+** flux upon CD3 cross-linking alone as we previously described (18), but these cells did respond at a level fairly similar to wild-type T cells upon cross-linking of CD3+CD4 in the biotin-avidin system. These results suggest that Jak3 is involved in early TCR activation signals. Therefore, we next tested whether Jak3 is phosphorylated upon TCR stimulation in normal T cells. Because T cells from Jak3-deficient mice did not proliferate at all and the cellularity was small, we could not use them for biochemical analysis, so we used T cell clones and hybridoma cells as described below.

**Induction of Jak3 Phosphorylation upon TCR Stimulation—** Jak3 is assembled with IL-2 receptor through the association with γc and is known to be phosphorylated upon IL-2 stimulation. Therefore, to avoid the involvement of IL-2 signaling in testing Jak3 phosphorylation upon TCR stimulation, a normal T cell clone 23-1-8 was cultured in the absence of exogenous IL-2 for 2 days, and then T cells in the resting stage were stimulated with immobilized anti-CD3ε mAb for 2–5 min. The cell lysates of stimulated T cells were immunoprecipitated with anti-Jak3 Ab and blotted with anti-phosphotyrosine mAb (4G10) and anti-Jak3 Ab. Tyrosine phosphorylation of Jak3 was induced as early as 2 min (Fig. 2A). Immunoprecipitation of Jak3 from IL-2 dependent T cell line CTL2-2 was used as a positive control for phosphorylated Jak3 (Fig. 2A, lane 6). It is unlikely that IL-2 secreted from the T cells upon TCR stimulation induced Jak3 phosphorylation during such a short period. When T cells were also stimulated in the presence of inhibitory mAbs against IL-2Ra and β chains to avoid the contribution of growth signals through IL-2R, Jak3 phosphorylation was similarly induced under such condition (data not shown).

**Although our results showed that IL-2 signals were not involved in TCR-induced Jak3 phosphorylation (Fig. 3), we found**
that T cell hybridoma cells do not express IL-2R α and β chains but do express γc on the cell surface (data not shown). Then, to avoid the possible involvement of γc in TCR-mediated Jak3 phosphorylation, T hybridoma cells were stimulated for 2 min in the presence of an anti-γc mAb that is known to inhibit γc-mediated signals. Despite the fact that all growth signals through IL-2, IL-4, and IL-7 were completely blocked under this condition (30, 31), Jak3 was similarly phosphorylated upon TCR stimulation (Fig. 3A). Furthermore, cross-linking of γc with anti-γc mAb and anti-rat Ig Ab did not induce Jak3 phosphorylation (Fig. 3A, lane 4), ruling out the possibility that γc cross-linking with Abs induced Jak3 phosphorylation. One other possible mechanism of the involvement of γc in inducing Jak3 phosphorylation upon TCR stimulation is that γc might be physically assembled in the TCR complex and that Jak3 is phosphorylated through γc upon T cell activation. To investigate this possibility, T hybridoma cells were stimulated by TCR cross-linking after surface biotinylation, and the cell lysates were immunoprecipitated with anti-CD3e mAb to analyze the TCR complexes on the cell surface. Although γc was clearly detected with anti-γc Ab, even if only one-tenth of the lysate was used (Fig. 3B, lanes 5 and 6), the band corresponding to γc was not detected by immunoprecipitation with anti-CD3e mAb (Fig. 3B, lanes 1–4), suggesting that γc was not involved in the TCR complex upon T cell activation. Collectively, these results indicate that TCR stimulation induces tyrosine phosphorylation of Jak3 independently of γc.

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**Jak3 Phosphorylation Is Mediated by Lck and ZAP-70**—We next addressed the question of how Jak3 is activated upon TCR stimulation. To determine the tyrosine kinase responsible for phosphorylating Jak3, we first analyzed Jak3 phosphorylation in Jurkat variant cell lines lacking either Lck (J.CaM1) (20) or ZAP-70 (P116) (22). The wild-type Jurkat cells induced Jak3 phosphorylation upon TCR cross-linking, but the Jurkat cell line lacking either Lck or ZAP-70 failed to induce the phosphorylation of Jak3, suggesting that Lck and ZAP-70 are involved in phosphorylation of Jak3 as upstream kinases in TCR activation (Fig. 4A).

Next, to elucidate how Lck and ZAP-70 contribute to the phosphorylation of Jak3, we analyzed by transfection with Jak3 and these kinases. We used a mutant Jak3 (K855A) bearing a point mutation at the ATP binding site as a substrate to avoid auto-phosphorylation. Transfection of either Lck or ZAP-70 induced Jak3 phosphorylation (Fig. 4B, lanes 2 and 3), and enhanced phosphorylation was observed when Lck and ZAP-70 were transfected together with Jak3 (Fig. 4B, lane 4). Collectively, these data demonstrated that Lck and ZAP-70 cooperatively phosphorylate Jak3.

**Direct Association of Jak3 with the TCR Complex**—The phosphorylation data suggested the possibility that Jak3 might
directly associate with the TCR complex. To test this possibility, cell lysates of normal T cell clones were immunoprecipitated with anti-CD3 Ab and blotted with anti-Jak3 Ab. As shown in Fig. 5A (top panel), both anti-CD3e and CD3ζ mAbs, which precipitate the TCR-CD3 complex, immunoprecipitated Jak3 whereas a control hamster mAb did not indicating that Jak3 is physically and constitutively associated with the TCR complex in normal T cells. On the other hand, Jak1 and Jak2 were not detected in the immunoprecipitation of the TCR complexes (Fig. 5A, middle and bottom panels, respectively). To examine which CD3 chain interacts with Jak3, we first tested the CD3ζ chain because anti-CD3ζ Ab clearly co-precipitated Jak3. The expressible cDNAs encoding CD3ζ and Jak3 were co-transfected into 293T cells and the cell lysates were immunoprecipitated with either anti-CD3ζ (Fig. 5B, left panel) or anti-Jak3 (Fig. 5B, right panel) Abs, followed by blotting with anti-Jak3 or CD3ζ mAbs, respectively. Jak3 was co-precipitated with CD3ζ in both experiments (Fig. 5B, lanes 1, 4), suggesting that Jak3 is directly associated with CD3ζ. The direct association between Jak3 and CD3ζ was further confirmed by in vitro binding analysis, where the binding capacity of in vitro translated 35S-labeled Jak3 protein corresponding to JH2-JH4 to a GST fusion protein containing the cytoplasmic tail of CD3ζ (GST-ζ) was analyzed. As shown in Fig. 5C, Jak3 specifically bound to GST-ζ but not to GST, whereas luciferase as a control did not bind to GST-ζ. These in vitro results strongly suggest that Jak3 directly associates with the TCR-CD3 complex, particularly with the CD3ζ, though the possibility of a similar association with CD3ε remains.

Association of Jak3 with CD3ζ via Jak3-JH4 Region—We next attempted to determine the binding region of Jak3 to
between Jak3 and CD3ζ, the JH1–3 domains were not required for the Jak3-CD3ζ interaction (Fig. 6A, lanes 1–4). However, the J4 mutant lacking JH1-JH4 as well as J5 lacking JH1-JH5 failed to bind to CD3ζ (Fig. 6A, lane 5), suggesting that the JH4 domain is a crucial region for Jak3-CD3ζ association. This conclusion was further supported by the analysis using N-terminal deletion mutants (Fig. 6B). Even ΔJ7-5 lacking JH7-JH5 was still co-precipitated with CD3ζ (Fig. 6B, lanes 1–3), indicating that the JH7-JH5 domains were not necessary for the CD3ζ binding. Finally, to confirm that the JH4 region is required for the association with CD3ζ, FLAG-tagged wild-type Jak3 and JH4-deletion mutant, ΔJH4, which lacks only the JH4 region, were constructed. As shown in Fig. 6C, ΔJH4 failed to associate with CD3ζ. Collectively, these data demonstrate that JH4 of Jak3 is the crucial domain of Jak3 for the association with CD3ζ.

**DISCUSSION**

In the present study, we demonstrated that Jak3 is activated upon TCR stimulation. Although Beadling et al. (32) reported that cytokine but not TCR stimulation induced the activation of Jak and STATs, they analyzed Jak1, Jak2, and various STATs but not Jak3 activation. On the other hand, Welte et al. (33) recently reported that STAT5 is activated upon TCR stimulation and assembles with the TCR complex. We have not observed significant induction of STAT5 phosphorylation upon TCR stimulation even by using Abs specific for phosphotyrosine of STAT5 in our system. A recent paper on STAT5-deficient mice (34) as well as the paper by Beadling et al. (32) also concluded that STAT5 is not involved in TCR activation signals. Considering these data that TCR stimulation does not induce STAT activation despite the fact that a minor population of STAT5 might be activated upon TCR stimulation, the activation of Jak3 and subsequent signaling through activated Jak3 may dominate the contribution to T cell activation. The STAT5 activation observed by Welte et al. (33) might be induced through TCR-activated Jak3, or alternatively, there may be another pathway to activate STAT5 independently of Jak3 activation. The recent report by Malaviya et al. demonstrating that Jak3 is phosphorylated in mast cells by FcεRI cross-linking (35) is consistent with our results in terms of γc-independent and ITAM-containing receptor-mediated activation of Jak3.

We started to analyze the involvement of Jak3 in TCR signaling from our previous analysis of Jak3-deficient mice (15, 16). We demonstrated that peripheral CD4+ T cells in Jak3−/− mice showed impaired early activation signals such as Ca2⁺ mobilization upon T cell activation. In the present study, we solved the discrepancy from Thomis et al. (28), which described that Jak3−/− peripheral T cells exhibited Ca2⁺ flux comparable with normal T cells, by analyzing stimulation conditions. When Jak3−/− T cells exhibited impaired Ca2⁺ response by CD3 cross-linking alone, strong aggregation of CD3+CD4+ T cells in the biotin-avidin system resulted in the induction of Ca2⁺ flux in Jak3−/− T cells comparable with normal T cells (Fig. 1). This result suggests that such strong signals may bypass some function of Jak3 for early T cell activation. A similar requirement has been described for CD4/CD8 co-receptors (36) in which strong activation such as super antigen skips the requirement of co-receptors but the same receptor needs a co-receptor for stimulation with a weak agonist. Such strong cross-linking may induce superactivation of Lck, which then bypasses the requirement of some signaling molecules such as Jak3 and induces strong activation of ZAP-70 and the downstream signaling pathway. Therefore, it is possible that under the physiological condition in vivo of stimulation with antigen/
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major histocompatibility complex or a suboptimal activation condition Jak3 may play a role in augmenting T cell activation signals. Because Jak3 is involved in both T cell activation and growth, these results provide evidence of the cross-talk of a signaling molecule between TCR signal and growth signal in the same T cells. Recently, similar cross-talk between cytokine signal and TCR signal has been reported in which IFN signals in T cells for growth arrest utilize TCR signal machinery including ZAP-70 and CD45 (37), whereas IFN signals in cells other than T cells utilize Jak-STAT pathways. In this case, the choice of TCR machinery versus Jak-STAT pathway by IFN-R appears to be determined in a cell type-specific manner. In our case, in contrast, Jak3 can be used in both TCR- and cytokine-signal pathways by assembling with distinct receptor components, CD3ζ and γc, in the same cells.

It is important to determine how TCR-stimulated Jak3 and IL-2R-stimulated Jak3 discriminate the downstream signaling pathway in the same cells without mixing the two activation signals. Physiologically, the association of Jak3 with both TCR complex and IL-2R complex indicates that Jak3 plays pivotal roles in regulating T cell activation/growth at two distinct time points in the sequence of T cell activation events. Jak3 contributes to initial TCR activation signals (on day 0), then T cells start to express cytokine receptors and produce cytokines, and thereafter Jak3 mediates growth signals upon cytokine binding on the receptors (on days 2–3). Thus, the constitutive associations of Jak3 to TCR and IL-2R induce T cell activation and cell growth, respectively. A possible mechanism for the differential activation of Jak3 by TCR and IL-2 signals may be based on the physical dissection of these two signaling complexes by membrane compartmentalization. Recent findings revealed that the glycolipid-enriched membrane compartment (GEM/raft) contains most of the phosphorylated signaling molecules such as phospho-CD3ζ, ZAP-70, LAT, Lck, and PLCγ and is crucial for T cell activation (38–41). It is possible to assume that TCR-activated Jak3 is localized within GEM/raft and may be involved in the activation of these TCR signaling molecules, whereas IL-2-activated Jak3 may not be located within GEM/raft.

The association of Jak3 with γc is mediated by the consensus sequence box1 (7, 8). CD40 has been reported to associate with Jak3 as a non-cytokine receptor (42). However, because CD40 contains a box1-like motif, the association appears to follow a similar rule to γc-associated cytokine receptors. In contrast, the CD3ζ chain does not contain such a box1-like motif. However, considering that the association of STAM with Jak3 has been shown to be mediated through the ITAM region within STAM (12) in the absence of its phosphorylation, it might be possible that Jak3 associates with CD3ζ through the non-phosphorylated ITAM. In this study, we determined the JH4 region of Jak3 to be the responsible region for the association with CD3ζ. The failure of CD3ζ binding by the specific JH4-deletion mutant of Jak3 supports this conclusion, although the possibility of an indirect structural alteration by JH4 deletion is not excluded. In addition, although we focused on CD3ζ as a responsible CD3ζ chain of Jak3-association, the possibility of a similar association with CD3ζ still remains because anti-CD3ζ Ab immunoprecipitated Jak3. In contrast, it has been demonstrated that γc associates with Jak3 through the N terminus region including JH7-6 (23). Therefore, the distinct association of
Fig. 6. Jak3 associates with CD3ζ through its JH4 region. A, JH4 of Jak3 is required for CD3ζ binding. A schematic representation of the wild-type and C-terminal deletion mutants of Jak3 (J1-J5) is shown in the upper panel. 3 × 10⁶ 293T cells were transfected with 12 μg of the indicated cDNAs together with CD3ζ. Lysates were immunoprecipitated with H146 and blotted with anti-Jak3 C-terminal Ab (middle left panel). Expression levels of various mutant Jak3 were analyzed by immunoblotting with anti-JAK3 C-terminal Ab (middle right panel) and H146 (lower panel). B, the JAK3 N terminus is not necessary for CD3ζ binding. A schematic representation of the Jak3 N-terminal deletion mutants is shown. 1 × 10⁶ 293T cells were transfected with 4 μg of wild-type or mutant Jak3 together with CD3ζ cDNAs. Lysates were immunoprecipitated with H146 and blotted with anti-Jak3 C-terminal Ab (upper panel). Expression levels of various mutants were analyzed by immunoblotting with anti-Jak3 C-terminal Ab (middle panel) and H146 (lower panel). C, JH4 is responsible for the binding of Jak3 with CD3ζ. FLAG-tagged wild-type or JH4-deletion mutants of Jak3 were transfected together with CD3ζ into 293T cells. Lysates were immunoprecipitated with H146 and blotted with anti-FLAG mAb (upper panel). Expression levels of wild type and the mutant Jak3 were analyzed by blotting with anti-FLAG mAb (middle panel) and H146 (lower panel). The arrow indicates FLAG wild-type Jak3.
Jak3 with CD3ζ and γc appears to be regulated through distinct regions of Jak3 as the binding sites (JH4 for TCR, JH7 for γc) as well as through different kinetics during T cell activation (day 0 for TCR stimulation, days 2–3 for IL-2R signaling). The precise mechanism and maps of the association between Jak3 and the TCR-CD3 complex may enable us to discriminate the in vivo contributions of Jak3 to activation and growth of T cells.

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REFERENCES

1. Klausner, R. D., Lippincott-Schwartz, J., and Bonifacino, J. S. (1990) Annu. Rev. Cell Biol. 6, 403–431
2. Malissen, B., and Schmitt-Verhulst, A. M. (1993) Curr. Opin. Immunol. 5, 324–333
3. Weiss, A., and Littman, D. R. (1994) Cell 76, 263–274
4. Wange, R. L., and Samelson, L. E. (1996) Immunity 5, 197–205
5. Takeshita, T., Asao, H., Ohtani, K., Ishii, N., Kumaki, S., Tanaka, N., Munakata, H., Nakamura, M., and Sugamura, K. (1992) Science 257, 379–382
6. Noguchi, M., Yi, H., Rosenblatt, H. M., Bonifacino, J. S., and Saito, T. (1995) Science 266, 1042–1045
7. Miyatake, S., Sakuma, M., and Saito, T. (1997) J. Exp. Med. 185, 351–356
8. Veillette, A., Bookman, M. A., Horak, E. M., Samelson, L. E., and Bolen, J. B. (1997) Immunity 7, 197–205
9. Ihle, J. N. (1996) Science 270, 331–334
10. Darnell, J. E., Jr. (1997) Cell 89, 1630–1635
11. O'Shea, J. J. (1997) Immunity 6, 1–11
12. Takeshita, T., Arita, T., Higuchi, M., Asao, H., Endo, K., Kuroda, H., Tanaka, N., Murata, K., Ishii, N., and Sugamura, K. (1997) Immunity 6, 449–457
13. Takeshita, T., Asao, H., Ohtani, K., Ishii, N., Kumaki, S., Tanaka, N., Munakata, H., Nakamura, M., and Sugamura, K. (1992) Science 257, 379–382
14. Noguchi, M., Yi, H., Rosenblatt, H. M., Bonifacino, J. S., and Saito, T. (1995) Science 266, 1042–1045
15. Park, S. Y., Saijo, K., Takahashi, T., Osawa, M., Arase, H., Hirayama, N., Miyake, K., Nakahashi, H., Shirasawa, T., and Saito, T. (1995) Immunity 3, 771–782
16. Russell, S. M., Tayebi, N., Nakajima, H., Riedy, M. C., Roberts, J. L., Aman, M. J., Migone, T. S., Noguchi, M., Markert, M. L., Buckley, R. H., et al. (1995) Science 270, 797–800
17. Thomis, C., Gurniak, C. B., Twel, E., Sharpe, A. H., and Berg, L. J. (1995) Science 270, 794–797
18. Saijo, K., Park, S. Y., Ishida, Y., Arase, H., and Saito, T. (1997) J. Exp. Med. 185, 351–356
19. Miyatake, S., Sakuma, M., and Saito, T. (1997) Eur. J. Immunol. 27, 1816–1823
20. Goldsmith, M. A., and Weiss, A. (1987) Proc. Natl. Acad. Sci. U. S. A. 84, 6879–6883
21. Strauss, D. B., and Weiss, A. (1992) Cell 70, 585–593
22. Williams, B. L., Schreiber, K. L., Zhang, W., Wange, R. L., Samelson, L. E., Leibson, P. J., and Abraham, R. T. (1998) Mol. Cell. Biol. 18, 1388–1399
23. Chen, M., Cheng, A., Chen, Y. Q., Hymel, A., Hanson, E. P., Kimmel, L., Minami, Y., Taniguchi, T., Changelian, P. S., and O'Shea, J. J. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 6910–6915
24. Takase, K., Wakiizaka, K., von Boehmer, H., Wada, I., Moriya, H., and Saito, T. (1997) J. Immunol. 158, 741–747
25. Wakiizaka, K., Masuda, Y., and Saito, T. (1998) Eur. J. Immunol. 28, 636–645
26. Nakano, H., Yamaizaki, T., Miyataye, S., Nozaki, N., Kikuchi, A., and Saito, T. (1998) J. Biol. Chem. 273, 6483–6489
27. Shiratori, T., Miyataye, S., Ohno, H., Nakaseko, C., Iseno, K., Bonifacino, J. S., and Saito, T. (1997) Immunity 6, 583–589
28. Thomis, D. C., Lee, W., and Berg, L. J. (1997) J. Immunol. 159, 4708–4719
29. Veillette, A., Bookman, M. A., Herak, E. M., Samelson, L. E., and Bolen, J. B. (1989) Nature 338, 257–259
30. Kondo, M., Takeshita, T., Ishii, N., Nakamura, M., Watanabe, S., Arai, K., and Sugamura, K. (1993) Science 262, 1874–1877
31. Kondo, M., Takeshita, T., Higuchi, M., Nakamura, M., Sudo, T., Nishikawa, S., and Sugamura, K. (1994) Science 263, 1453–1454
32. Streadling, C., Guschin, D., Witthuhn, B. A., Ziemiecki, A., Ile, J. N., Kerr, I. M., and Cantrell, D. A. (1994) EMBO J. 13, 5605–5615
33. Welle, T., Leitenberg, D., Dittel, B. N., al-Ramadi, B. K., Xie, B., Chinn, Y. E., Janeway, C. A., Jr., Bothwell, A. L. M., Bottomly, K., and Fu, X. Y. (1999) Science 283, 222–225
34. Mortizl, R., Scal, V., Pickorzi, R., Topham, D., and Ile, J. N. (1999) Immunity 11, 225–230
35. Malaviya, R., Zhu, D., Dibidik, I., and Uckun, F. M. (1999) J. Biol. Chem. 274, 7072–7073
36. Viola, A., Salio, M., Tuosto, L., Linkert, S., Acuto, O., and Lanavezach, A. (1997) J. Exp. Med. 186, 1775–1779
37. Petrich, E. C., III, Ho, S., Williams, B. L., Audet, S., Stancato, L. F., Gamero, A., Clause, K., Grimes, P., Weiss, A., Beeler, J., Finloum, D. S., Shores, E. W., Abraham, R., and Larner, A. C. (1997) Nature 390, 629–632
38. Friedrichson, T., and Kurzchalia, T. V. (1998) Nature 394, 802–805
39. Montixi, C., Langlet, C., Bernard, A. M., Thimonier, J., Dubois, C., Wurbel, M. A., Chauvin, B. J., Piersie, M., and He, H. T. (1998) EMBO J. 17, 5334–5348
40. Xavier, R., Brennan, T., Li, Q., McCormack, C., and Seed, B. (1998) Immunity 8, 723–732
41. Zhang, W., Tribble, R. P., and Samelson, L. E. (1998) Immunity 9, 239–246
42. Hanissian, S. H., and Geha, R. S. (1997) Immunity 6, 379–387