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SS-A/Ro52, an Autoantigen Involved in CD28-Mediated IL-2 Production

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An autoantibody against SS-A/Ro52 (Ro52) is most frequently found in the sera of patients with Sjögren’s syndrome, systemic lupus erythematosus, and congenital heart block from anti-Ro52 Ab-positive mother. However, the physiological function of the autoantigen SS-A/Ro52 has not yet been elucidated. In this study, we describe the role of Ro52 protein in T cell activation. Overexpression of SS-A/Ro52 in Jurkat T cell resulted in enhanced IL-2 production following CD28 stimulation. Furthermore, transfection of anti-Ro52-specific small RNA duplexes partially blocked the expression of native and overexpressed Ro52 in Jurkat T cell, resulting in decreased IL-2 production via CD28 pathway in these cells. Finally, intracellular localization of Ro52 dramatically changed following CD28 stimulation. Our data reveal a novel function of Ro52 in CD28-mediated pathway, which eventually contributes to cytokine production and expression of the T cell biological programs. The Journal of Immunology, 2003, 170: 3653–3661.

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5 Abbreviations used in this paper: SS-J, Sjögren’s syndrome; CD28RE, CD28 response element; GFP, green fluorescent protein; EGFP, enhanced GFP; siRNA, small interfering RNA; mis-siRNA, missense-siRNA; PI, propidium iodide; RNAi, RNA interference; SLE, systemic lupus erythematosus; ss-siRNA, sense-siRNA; TR, Texas Red.

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enhancement of IL-2 production. In addition, IL-2 promoter activity assays involving these transfectants demonstrated that Ro52 is involved in CD28-NF-κB signaling pathway. Moreover, by inhibition of Ro52 expression using RNA interference (RNAi) with small interference RNA (siRNA) for Ro52 mRNA, IL-2 production is shown to be decreased following binding of anti-CD28 mAb alone, as well as anti-CD3 plus anti-CD28 costimulation. Our findings therefore reveal a novel function of Ro52 in CD28-mediated pathway that appears to be involved in the pathophysiology of autoimmune diseases such as SJS and SLE.

Materials and Methods

Abs and reagents

Anti-CD3 mAb, OKT3, has been previously described (11); anti-CD28 mAb (4B10) was developed by us (12); PE-conjugated anti-CD1, anti-CD2, anti-CD3, anti-CD4, anti-CD28, anti-CD154, anti-CD45RO, and anti-CD45RA, and PerCP-conjugated anti-CD3 were purchased from BD PharMingen (San Diego, CA); and FITC-conjugated anti-mouse Ig Ab was purchased from Sigma-Aldrich (St. Louis, MO). PMA, hygromycin, and poly-(l-lysine) were purchased from Sigma-Aldrich.

Establishment of Jurkat stable transfectants

NH2-terminal GFP-tagged form of Ro52 was generated by standard PCR method and subcloned into mammalian expression vectors pBMCQS-hygro. The enhanced green fluorescent protein (EGFP)-Ro52 construct was generated using cDNA from human peripheral lymphocytes as the template for Ro52 and the sense and antisense oligos 5’-CGGAATTCCGGCCATGGCTTCAGCAGCACGC-3’ and 5’-CGGATTCCTCCGGCATGGTTTGAGGA-3’, respectively. The resulting DNA fragment was subcloned into the EcoRI-KpnI sites of pEGFP-N2 vector (Clontech). pBMCQ5-Ro52-EGFP was obtained by subcloning the XhoI-NotI fragment of pEGFP-N2/Ro52 into the XhoI-NotI sites of pBMCQ5-hygro. The expression plasmid of EGFP, pBMCQ5/EGFP, was prepared by subcloning the XhoI-NotI fragment of pEGFP-N2/Ro52 into the XhoI-NotI sites of pBMCQ5-hygro. Authenticity of all DNA fragments generated by PCR was confirmed by DNA sequence analysis. Jurkat LT cells (parental J5) were transfected by X-treamGENE Q2 transfection reagent (Roche, Indianapolis, IN). Two days after transfection of indicated plasmids, the cells were selected for hygromycin (500 μg/ml) resistance for 4 wk. Then, single clone cells expressing EGFP (J5/EGFP transfectant) or Ro52-EGFP (J5/Ro52-EGFP transfectant) were selected using standard limiting dilution method.

Cell stimulation and measurement of IL-2 concentration

For solid-phase stimulation of cells, PBS alone or mAbs in PBS (CD3, OKT3, at 0.5 μg/ml, and/or CD28, 4B10, at 2.0 μg/ml, and/or CD154, TRAP1, at 2.0 μg/ml) were incubated in 96-well Costar plate (Corning, Corning, NY) for 8 h at 4°C. Cells (1 × 10^5) in 200 μl culture medium were then incubated for 48 h at 37°C. Cells were also stimulated with PMA (10 ng/ml) in anti-CD3-coated wells. After 24 h of incubation, culture supernatants were pooled from the triplicate wells and assayed for IL-2 content by sandwich ELISA, according to the manufacturer’s instruction (BD PharMingen).

Western blot analysis

After being harvested from wells, cells were washed with PBS and lysed in sodium dodecyl sulfate (SDS)-containing lysis buffer, consisting of 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 5 mM EDTA, 10 mM Tris-HCl (pH 7.4), 0.15 M NaCl, 1 mM PMSF, 0.5 mM NaF, 10 μg/ml aprotinin, and 0.02 mM NaVO4. After removal of precipitation by ultracentrifugation, cell lysates were then submitted to SDS-PAGE analysis on 5–20% gradient concentration gel under reducing condition using a mini-Protein II system (Bio-Rad Laboratories, Hercules, CA). For immunoblotting, the proteins were transferred to a polyvinylidene difluoride membrane (Immobilon-P; Millipore, Bedford, MA) in 25 mM Tris, 192 mM glycine, and 20% methanol, and the membrane was blocked for 1 h at room temperature in PBS with 0.05% Tween 20 containing 5% nonfat milk. Specific Ags were probed by the corresponding mAbs, followed by HRP-conjugated anti-mouse Ig (Amersham Pharmacia Biotech, Piscataway, NJ). Western blots were visualized by the ECL technique (NEW, Boston, MA).

Confocal laser microscopy

A total of 1.0 × 10^6 cells was attached to poly(t-lysine)-coated coverslip (Matsunami Glass, Tokyo, Japan), fixed with 4% paraformaldehyde. After washing in ice-cold PBS, cells were mounted onto glass slide (Matsunami Glass) with Prolong Antifade kit (Molecular Probes, Eugene, OR). The nuclei was stained with 1 μg/ml of propidium iodide (PI; Sigma-Aldrich) in PBS for 20 min, after treatment with RNase A (Sigma-Aldrich; 100 μg/ml in PBS) at 37°C for 30 min. Confocal microscopy was performed with an Olympus IX70 confocal microscope with 40 objective lenses (Olympus, Tokyo, Japan), using laser excitation at 490 and 568 nm.

Luciferase assay for IL-2 promoter activation

A total of 10^5 cells of Jurkat, EGFP-transfected Jurkat, and Ro52-EGFP-transfected Jurkat was transfected with 10 μg of pBR322–3×NF-AT-Luc vector, 3× NF-κB-Luc vector, 3× AP-1 luc vector, and 3× 28RE/AP-1-Luc vector (kindly provided by S. Watanabe, Institute of Medical Science, University of Tokyo, and S. Burakoff, Dana-Farber Cancer Institute, Harvard Medical School), and incubated for 24 h at 37°C. The cells were then stimulated as indicated with anti-CD3 Abs, anti-CD28 Abs, or PMA, as described above. After culturing overnight, control and treated cells were lysed in 100 μl of cell culture lysis reagent (Promega, Madison, WI) for 10 min on ice, and centrifuged. A total of 20 μl of the cleared supernatant was assayed for luciferase activity using the Promega luciferase assay kit with a CliniLux (Berthold Detection System, Oak Ridge, TN) luminometer. All treatments were performed in duplicate, and the results shown are mean ± SEs.

Flow cytometry

Cells were stained on ice for 30 min with the appropriate Abs at a concentration of 1 μg/ml. Using FACS Calibur (BD Biosciences, La Jolla, CA), which was equipped with an argon laser, cells were gated in the two-dimensional plot of forward scatter and side scatter. The fluorescence intensity of those Jurkat cells (FITC conjugation for EGFP and PE or PerCP for the cell surface marker) was detected. EGFP and Ro52-EGFP-positive cells were recognized and gated for further analysis of fluorescence intensity of PE or PerCP and generated a dual-parameter scattergram or single-parameter histogram.

siRNA against SS-A/Ro52

To design target-specific siRNA duplexes, we selected sequences of the type AA(N19) (N, any nucleotide) from the open reading frame of SS-A/ Ro52 mRNA (accession number = NM 003414) (13, 14). Moreover, we added the sequences to the 2nt 3′ overhangs of 2′-deoxythymidine, to generate a symmetric duplex with respect to the sequence composition of the sense and antisense 3′ overhangs. These symmetric 3′ overhangs are reported to help to ensure that the siRNAs are formed with approximately equal ratios of sense and antisense target RNA-cleaving siRNA (13). Therefore, we selected the target sequence from 113 to 133 downstream of the start codon of SS-A/Ro52 mRNA (sense siRNA (ss-siRNA): 5′-UCGAU CUCUCAGGGUUGGGATT). To visualize the efficiency of transfection, we also prepared Texas Red-conjugated ss-siRNA (ss-siRNA-TR). Moreover, miRNA silence ss-siRNA (ms-siRNA) at 4 nt was prepared to examine nonspecific effects of siRNA duplexes (mss-siRNA: 5′-UCGAACUCUGAGGUGGTT). These selected sequences were also submitted to a BLAST search against the human genome sequence to ensure that only one gene of the human genome was targeted. siRNAs were purchased from Japan Bio-service (Saitama, Japan). siRNA duplex formation (annealing) was performed as previously described (13–15). A total of 60 pmol of ss-siRNA duplexes was transfected into 0.5 × 10^6 cells, using HVJ-E vector (GenomeOne; kindly provided by Ishihara Sangyo Kaisha, Osaka, Japan). After 48 h of transfection, cells were prepared for examination.

Results

Establishment of Ro52-EGFP Jurkat stable transfectant

At first, we have established several stable Jurkat transfectants with Ro52-EGFP (JRG1–6) and EGFP (JG1–4). The expression of Ro52 and EGFP was demonstrated by Western blot analysis with anti-Ro52 and anti-EGFP mAbs (Fig. 1A). Parental Jurkat-LT (parental J5) and GFP vector-only transfectant (J5/EGFP) expressed equal amount of 52-kDa native Ro52 (filled arrowhead). In Ro52-EGFP-transfected J5 (J5/Ro52-EGFP), two distinct bands detected by anti-Ro52 mAb were observed: native Ro52 (filled arrowhead) and GFP-fused Ro52 (filled arrow). To confirm this observation, 

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the membrane was reprobed with anti-GFP Ab to detect GFP protein. Although J5/EGFP expressed the GFP protein at ~28 kDa, GFP-fused Ro52 protein was detected at ~80 kDa, similar to the anti-Ro52 mAb blot. In parental J5, no protein was detected by anti-GFP Ab. By immunofluorescence cytochemistry, Ro52-GFP and GFP were detected by immunofluorescence confocal microscopy. One representative clone of J5/EGFP-Ro52 (JRG3) (B) and J5/EGFP (JG1) (C) is shown. D, Cell surface Ag expression by flow cytometry. The data are shown as the mean fluorescence intensity ± SEs from four separate sets of independently generated clones.

**FIGURE 1.** Characterization of parental J5, J5-EGFP, and J5-EGFP/Ro52. A, Cell lysates were immunoblotted with anti-Ro52 and anti-GFP. Overexpressed Ro52-EGFP (filled arrow) was detected with anti-Ro52 blotting in J5/Ro52-EGFP (JRG3) (lane 3), compared with the endogenous Ro52 (filled arrowhead). EGFP-fused Ro52 (open arrow in lane 3) and EGFP in J5/EGFP (JG1) (open arrowhead in lane 3) were also detected with anti-GFP blotting. The same membrane was reprobed with anti-β-actin mAb to determine that each lane contained equal amount of proteins. The blots are representative of four sets of independently generated clones. B and C, Expression of Ro52-EGFP and GFP was detected by immunofluorescence confocal microscopy. One representative clone of J5/EGFP-Ro52 (JRG3) (B) and J5/EGFP (JG1) (C) is shown.

**Ro52 enhances IL-2 production via CD28 signaling**

To investigate the role of Ro52 in T cell function, we next examined IL-2 production by the Jurkat transfectants described above following treatment with such stimuli as anti-CD3, anti-CD28, anti-CD154, anti-CD3 plus anti-CD28, anti-CD3 plus anti-CD154, and anti-CD3 plus PMA. For this purpose, we randomly selected a representative Ro52-EGFP and EGFP Jurkat transfectants. J5/ Ro52-EGFP transfectant (JRG3) produced significantly higher level of IL-2 than parental J5 (J51) and EGFP vector-only-transfected Jurkat T cells (JG1) following CD3 plus CD28 or anti-CD3 plus CD154 costimulation (Fig. 2A). In parental (J51) and EGFP vector-only-transfected Jurkat T cells (JG1), incubation of the cells with anti-CD3 mAb alone, anti-CD28 alone, or anti-CD154 mAb alone did not result in significant IL-2 production, whereas stimulation with anti-CD28 alone induced significant IL-2 production in the Ro52-transfected Jurkat cells (JRG3). In contrast, stimulation with anti-CD154 mAb alone did not induce significant IL-2 production in the Ro52-transfected Jurkat cells. To avoid clonal variations, we next selected four parental cell lines, four EGFP vector-only transfectants, and six Ro52-EGFP transfectants that exhibited similar distribution of CD28 and/or CD3 Ag expression and analyzed the effect of CD28 stimulation on IL-2 production by each transfectant group (Fig. 2B). J5/Ro52-EGFP transfectants produced substantially more IL-2 than parental J5 cells and J5/EGFP transfectants. It should be noted that representative cell lines from each transfectant group produced similar levels of IL-2 after stimulation with PMA plus ionomycin, which bypassed membrane-signaling events (data not shown). These findings suggested that the observed difference seen in IL-2 production between J5/Ro52-EGFP and J5/EGFP transfectants was related to specific CD28-mediated triggering and not due to the overall intrinsic capacity of these cells to produce IL-2. The IL-2 gene promoter contains an enhancer known as the CD28RE (9), which functions
as an integrator of transcription factors activated through the TCR and CD28, and is essential for IL-2 transcription mediated through CD28. Studies by several laboratories suggested that members of the NF-κB, AP-1, and activating transcription factor-CREB transcription factor families bind to the CD28RE/AP-1 composite element (9, 16). To further determine the role of Ro52 in IL-2 production, parental cells and the Jurkat transfectants described above were transiently transfected with luciferase reporters containing either the IL-2 promoter or multimerized copies of NF-κB, NF-AT, or AP-1, and were stimulated with solid-phase anti-CD3 (0.5 μg/ml) plus anti-CD28 mAbs (2.0 μg/ml) for 48-h incubation. Luciferase assay for various IL-2 promoters of parental J5, J5/EGFP, and J5/Ro52-EGFP transfectants. Stimulation was conducted with solid-phase anti-CD3 (0.5 μg/ml) plus anti-CD28 mAbs (2.0 μg/ml) for 48-h incubation. The results shown are mean ± SEs.}

**FIGURE 2.** IL-2 production and IL-2 promoter activity assay following treatment with various stimuli. **A,** Parental J5 (J51), J5/EGFP (JG1), and J5/Ro52-EGFP (JRGG1) transfectants were incubated in anti-CD3 (0.5 μg/ml) alone, anti-CD28 (2.0 μg/ml) alone, anti-154 (2.0 μg/ml) alone, anti-CD3 plus anti-CD28 mAbs, or anti-CD3 plus anti-CD154-coated wells. Cells were also stimulated with PMA (10 ng/ml) in anti-CD3-coated wells. The data are shown as the mean ± SEs from four independent experiments. The asterisks indicate \( p < 0.001 \) by paired two-tailed t tests. **B,** IL-2 production by various clones (parental J5–4, EGFP vector-alone transfectants, JG1–4, and Ro52-EGFP transfectants, JRGG1–6) in the presence or absence of stimuli. Stimulation was conducted with solid-phase anti-CD3 (0.5 μg/ml) plus anti-CD28 mAbs (2.0 μg/ml) for 48-h incubation. **C,** Luciferase assay for various IL-2 promoters of parental J5, J5/EGFP, and J5/Ro52-EGFP transfectants. Stimulation was conducted with solid-phase anti-CD3 (0.5 μg/ml) alone, anti-CD28 (2.0 μg/ml) alone, or anti-CD3 plus anti-CD28 mAbs. All treatments were performed in triplicate, and the results shown are mean ± SEs.

Collectively, these results suggested that Ro52 is capable of potentiating several IL-2-related promoter activation events via the CD28 pathway in Jurkat T cells, particularly those involving NF-κB activity.

**Decrease of IL-2 production of Ro52-EGFP transfectant by RNA interference**

To more directly define the role of Ro52 in CD28 signaling in T cells, we performed studies to knock down the expression of Ro52 by RNAi with siRNA duplexes, and then assayed for IL-2 production via CD28 signaling. ss-siRNA was used to effectively knock down Ro52 expression, as determined by fluorescence confocal microscopy (Fig. 3, **A** and **B**), flow cytometry (**bottom panel** in Fig. 3C), and Western blot analysis (Fig. 3D). On the contrary, the expression level of GFP in J5/EGFP transfectant was not affected by siRNA transfection (**middle panel** in Fig. 3C). Moreover, siRNA treatment did not change the expression levels of CD3 or CD28 on the cell surface (Fig. 3E). Using these cells, we next examined the levels of IL-2 production under treatment conditions, as described in Fig. 2A (Fig. 3F). A fair amount of IL-2 production induced by anti-CD28 or anti-CD3 plus anti-CD28 stimulation was observed in J5/Ro52-EGFP in the presence or absence of transfection with mis-siRNA. However, following transfection with ss-siRNA, the level of IL-2 production was markedly decreased in J5/Ro52-EGFP treated with anti-CD28 or anti-CD3 plus anti-CD28.
FIGURE 3. Suppression of Ro52 in J5/Ro52-EGFP by siRNA leads to decreased IL-2 production. A, Ro52-EGFP in J5/Ro52-EGFP (JRG3) was detected as green fluorescence (right panel) using confocal laser microscopy. The nuclei, stained with PI, were merged with green fluorescence in right panel. The phase contrast view was shown in the left panel. B, After 48 h of ss-siRNA-TR incorporation into JRG3 cells, green fluorescence of Ro52-EGFP was diminished (right panel), when ss-siRNA-TR as red fluorescence was detected in cells (left panel, detecting red fluorescence merged with a phase contrast view). C, By flow cytometry, siRNAs showed no effect on EGFP fluorescence intensity in J5/EGFP transfectant, but ss-siRNA and ss-siRNA-TR decreased intensity of EGFP in J5/Ro52-EGFP transfectant. The histograms obtained for cells treated with or without ss-siRNA, ss-siRNA-TR, or mis-siRNA are superimposed. D, Detection of Ro52 protein in J5/Ro52-EGFP transfectant by Western blotting. The protein level of endogenous Ro52 (arrowhead) and Ro52-EGFP (arrow) was decreased by ss-siRNA (lane 2), compared with cells with transfection of mis-siRNA (lane 3), or without transfection of siRNA (lane 1). An equal amount of proteins in each lane was demonstrated by reproving with anti-/H9252-actin. E, The cell surface Ags CD3 and CD28 were detected by flow cytometry. After transfection with siRNA, cells were stained with anti-CD3 PerCP and anti-CD28. Transfection with siRNA did not change the surface level of CD3 and CD28 in J5/Ro52-EGFP transfectant. F, The inhibitory effect of siRNA on IL-2 production in J5/Ro52-EGFP. After 48 h of transfection with siRNA, cells were stimulated, and IL-2 was assayed with the same method as performed in Fig. 2A. The production level of IL-2 was decreased in ss-siRNA-transfected J5/Ro52-EGFP after stimulation with anti-CD28 alone or anti-CD3 plus anti-CD28. The asterisks indicate p < 0.001 by paired two-tailed t tests. All treatments were performed in triplicate, and the results shown are mean ± SEs.
FIGURE 4. Effect of siRNA on endogenous Ro52 in parental J5. A, After 48 h of transfection of ss-siRNA-TR, cells were attached to poly(l-lysine)-coated coverslip, fixed, and blocked with 3% goat serum in PBS (blocking buffer). After washing in ice-cold PBS, cells were incubated in 0.2% Triton X-100 in blocking buffer, then incubated with anti-Ro52 mAb (5 μg/ml) for 1 h at 4°C. After washing with blocking buffer, cells were incubated with FITC-conjugated anti-mouse Ig Ab (1:200 in blocking buffer) for 30 min at 4°C, and mounted onto slide glass with Prolong Antifade kit. The nuclei were stained with PI merged with green fluorescence in right panel. Ro52 in parental J5 (J51) was found diffusely throughout the cytoplasm (right panel), like J5/Ro52-EGFP transfectant. The left panel shows the phase contrast view. B, After 48 h of ss-siRNA-TR incorporation into J51 cells, green fluorescence of Ro52 by detection with anti-Ro52 and with FITC-conjugated anti-mouse Ig Ab was diminished (right panel), when ss-siRNA-TR as red fluorescence was detected in cells (left panel, detecting red fluorescence merged with a phase contrast view). C, Western blot analysis also showed the decreased level of endogenous Ro52 in parental J5 with ss-siRNA transfection (lane 2). The blots are representative of four separate experiments. D, The cell surface Ags CD3 and CD28 were detected by flow cytometry. Transfection with siRNA did not change the surface level of CD3 and CD28 in parental J5. E, The inhibitory effect of siRNA on IL-2 production in parental J5. After 48 h of transfection with siRNA, cells were stimulated with the indicated concentrations of solid-phase Abs, and IL-2 was assayed with the same method as performed in Materials and Methods. The production level of IL-2 was significantly decreased in ss-siRNA-transfected parental J5 after stimulation with mitogenic concentrations of anti-CD3 plus anti-CD28. The asterisks indicate *p < 0.001 by paired two-tailed t tests. All treatments were performed in triplicate, and the results shown are mean ± SEs.
CD28 (asterisks in Fig. 3F). Moreover, knockdown of Ro52 expression did not affect IL-2 production by J5/Ro52-EGFP cells stimulated by anti-CD3 plus PMA. These results hence suggested that the decreased level of Ro52 by siRNA affected only the CD28, but not the general CD3, pathway of activation.

Decrease of IL-2 production by suppression of native Ro52 with RNAi

To further define the role of Ro52 in CD28-mediated activation, we examined IL-2 production of parental J5 with its endogenous expression of Ro52 reduced by siRNA-mediated knockdown. The endogenous Ro52 protein was found mainly in the cytoplasm as detected by anti-Ro52 mAb with immunofluorescence confocal microscopy (Fig. 4A). This subcellular localization of Ro52 was similarly observed in J5/Ro52-EGFP transfec tant. Following ss-siRNA transfection in parental J5, Ro52 protein level was decreased, as determined by immunofluorescence confocal microscopy (Fig. 4B). The knockdown effect of ss-siRNA on endogenous Ro52 expression in parental J5 was also confirmed by Western blotting (Fig. 4C). In these cells, siRNA transfection did not have an effect on the expression of surface CD3 and CD28 (Fig. 4D). To produce IL-2 from parent Jurkat T cells, two signals from CD3 and CD28 are required, with optimal concentrations of anti-CD28 mAb being particularly necessary for this purpose. As shown in Fig. 4E, IL-2 production studies showed that parental J5 produced a fair amount of IL-2 in response to stimulation by anti-CD3 plus increasing concentration of anti-CD28. Transfection of mis-siRNA did not affect IL-2 production after anti-CD3 plus anti-CD28. In contrast, parental J5 cells with knockdown of Ro52 expression by ss-siRNA had decreased IL-2 production following stimulation by anti-CD3 plus anti-CD28, while this inhibitory effect of IL-2 production associated with knockdown of Ro52 expression was not observed following treatment with anti-CD3 plus PMA. More importantly, this inhibitory effect of IL-2 production by ss-siRNA was observed in stimulation with increasing mitogenic concentrations of anti-CD28 in the presence of anti-CD3. Together with the data presented earlier, our findings provided direct evidence that Ro52 plays an important role in CD28-mediated T cell activation pathway.

Subcellular localization of Ro52 in activated T cells

Because IL-2-related promoter activation was induced following anti-CD28 mAb stimulation, we hypothesized that this IL-2 promoter activation event would result from changes of subcellular localization of the Ro52 protein. For this purpose, we investigated the intracellular localization of Ro52-EGFP in cells before and after incubation with anti-CD28 mAb. In unstimulated cells, Ro52 was found to be mainly diffuse in the cytoplasm (Figs. 1b, 3A, and 4A), while aggregation in the cytoplasm appeared after anti-CD28-mediated stimulation (Fig. 5A, right panel). Moreover, a significant amount of Ro52 was detected as dot to needle-shape formation in cytoplasm (Fig. 5A, left panel). In contrast, after anti-CD3 stimulation, strong aggregation in the cytoplasm also occurred, unlike that in anti-CD28 stimulation (Fig. 5B, right and left panels). It should be noted that subcellular localization of EGFP in J5/EGFP did not change in the presence or absence of stimulation with anti-CD3 or anti-CD28 (Figs. 1C, 5C, and 5D). These results hence indicated that anti-CD28 stimulation resulted in dramatic changes in intracellular localization of Ro52 in the cytoplasm, suggesting that these changes may result in the enhancement of IL-2 production and NF-κB activation in Ro52 Jurkat transfectants after anti-CD28 stimulation.

Discussion

In the present study, we provided evidence that the autoantigen SS-A/Ro52 is involved in IL-2 production in T cells via the CD28 pathway.

Autoimmune diseases, including SjS, result from the failure of regulatory mechanisms that maintain self-tolerance. Although the
etiolologic agents that initiate autoimmunity are generally not defined, there is persistent infiltration of activated T cells at an established chronically inflamed site. Activated T cells serve as the trigger for a cascade of events that lead to amplification of the inflammatory process with the eventual resultant tissue damage at target site. In SjS or SLE, a coat-sleeve-like infiltration of CD4+ lymphocytes was infiltrated around the blood vessels, appendages, and secretory gland cells (4). The B7-CD28 pathway remains one of the most potent and well-characterized costimulatory interactions, and has a crucial role in T cell activation in autoimmune diseases. For example, abrogation of B7-CD28 interactions in vivo has been shown to block the development of several autoimmune diseases (17, 18). Our present results indicate that the Ro52 molecule appears to be directly involved in CD28-mediated T cell costimulation.

CD28/CTLA-4/B7 pathway plays an important role not only in T cell activation, but also in peripheral tolerance to autoantigens. In this respect, it is likely that in autoimmune disease states, triggering of T cells by unknown stimuli, such as a viral infection or environmental stress, may result in the overexpression of Ro52 in T cells. This development would lead to the subsequent up-regulation of the CD28 pathway and the potential breakdown in tolerance to autoantigens. Moreover, a common finding in all affected organs in patients with SjS or SLE is potentially progressive lymphocytic infiltration. Our findings that the up-regulation of Ro52 expression in T cells resulted in enhanced IL-2 production lead us to hypothesize that T cells expressing high levels of Ro52 infiltrate the focal region and produce Th1-type cytokines such as IL-2 and IFN-γ, thereby triggering and maintaining the local inflammatory processes. Our results show that overexpression of Ro52 in Jurkat T cells resulted in enhanced IL-2 production not only via CD3 plus CD28, but also via CD3 plus CD154 stimulation. In contrast, Ro52-overexpressed Jurkat T cells produced a significant amount of IL-2 when stimulated by CD28 mAb alone, but not CD154 mAb alone. These data support the notion that Ro52 is directly involved in the CD28 pathway. Because CD28 costimulation decreases the threshold of TCR/CD3-mediated T cell activation (19), it is reasonable that enhanced IL-2 production was observed by CD3 plus CD154 in Ro52-overexpressed Jurkat T cells.

Despite the powerful effect of anti-CD28 mAb on T cell responses, details of the molecular activation pathways induced by the CD28 receptor are incomplete at the moment. Early studies on CD28 signaling demonstrated that it provides a potent signal for the up-regulation of several cytokines, at the level of both transcription and mRNA stability (20). A potential connection between CD28 and transcription factors for IL-2 production was suggested by the identification of the proximal IL-2 promoters, which confer responsiveness to anti-CD28 Abs in conjunction with anti-CD3 mAb or phorbol ester or ionophore (9, 16, 21, 22). Despite several studies indicating that the NF-κB pathway is perhaps the most relevant biochemical or transcriptional target for the costimulatory activity of CD28, important questions remain to be elucidated, including: what exactly are the CD28-specific signals upstream of IκB kinase; is there recruitment of the IKK complex to CD28; and what are the protein kinase C-θ and Akt substrates in the NF-κB pathway via CD28?

Autoantibodies against components of the SS-A/Ro complexes include those recognizing a 60-kDa protein (Ro60), a 52-kDa protein (Ro52-α), and a 45-kDa protein (Ro52-β) (23–26). A molecular definition for these autoantigens has been provided by the isolation of cDNA clones (25, 27). The 60-kDa SS-A/Ro protein binds to hY RNA in an in vitro reconstitution assay (25), and has been proposed to function as part of a novel quality control of discard pathway for 5S ribosomal RNA (28). Ro52, one of the components of the SS-A/Ro complex, includes two isoforms, 52-α and 52-β. The 52-α form is ubiquitously expressed, whereas 52-β, lacking the central leucine zipper domain, has been detected at higher levels than 52-α in fetal heart aged 14–16 wk of gestation (29). The 52-β protein was shown to be present as a monomer, and to have promoted transcription activity in vitro (30). Therefore, the 52-β protein is considered to play a role in specific gene activation related to cardiac development, although the target gene(s) is not identified. In contrast, because the 52-α protein contains a classic zinc-finger DNA-binding domain, and its DNA-binding activity has been demonstrated (31), it is predicted to have regulatory function of unknown genes. However, the biological significance of this binding activity and the function of Ro52-α itself are still unclear. Neither the role of Ro52 in the SS-A/Ro complex nor the mechanism by which it participates in the pathogenesis of autoimmune diseases that express anti-SS-A Abs is presently established. Nevertheless, the above facts suggest that Ro52 might regulate gene transcription in T cell stimulation at inflammatory regions of SjS or SLE. Although Ro52 is present in both the nucleus and cytoplasm, our data showed that the majority of Ro52 in living Jurkat T cells transfected with Ro52-GFP was localized predominantly inside the cytoplasm, as previously described (32). Moreover, Ro52 strongly aggregated in the cytoplasm after CD28-mediated stimulation. These findings thus suggested that the interaction of Ro52 with other intracellular and/or nuclear structures appeared to be induced after stimulation with the CD28 pathway.

Although the in vivo Ro52-binding motif of DNA or RNA is unclear, the Ag has a high homology with a family of proteins containing both zinc finger and leucine zipper motifs, indicating a potential DNA-binding function (33). The C terminus of Ro52 shares homologies with two other zinc finger-bearing proteins, mouse regulatory protein T lymphocyte-1 and human ret finger protein, as well as with butyrophilin, a glycoprotein expressed in mammary tissues (27, 34). Therefore, our findings suggest that Ro52 might interact with genes in T cells and regulate the transcription of IL-2 as a transcriptional factor or regulator of other transcription factors.

Because a Ro52 knockout mouse model is not currently available, we used the RNAi method to analyze more precisely the function of overexpressed and native Ro52 in Jurkat cells. During the past several years, it is shown that RNAi is active in mammalian cells with sequence-specific, small (19–22 nt) dsRNAs (13, 14, 35). Although this approach helps to identify the mammalian gene function, one important limitation is that siRNA-based technology only provides a knockdown of the targeted protein, but not a knockout. The degree of depletion of protein by siRNA is dependent upon the abundance and the turnover of the targeted protein (13, 14, 35). Ro52 protein is expressed constitutively in parent Jurkat cells as well as other type of human cells, and treatment of parent Jurkat T cells and Ro52-transfected Jurkat cells with siRNA resulted in inhibition of IL-2 production following stimulation of anti-CD3 and/or anti-CD28 mAbs. Therefore, our findings strongly suggest that Ro52 is directly involved in CD28 activation pathway.

In summary, we have provided the first evidence for the involvement of Ro52 in CD28-induced production of IL-2, which is critical in several autoimmune diseases. Moreover, our findings of Ro52 as a novel component of the signaling pathways leading to CD28-mediated activation have provided new insights into the exquisite networks of signaling molecules for T cell activation, as well as into the development of novel molecular intervention for immune activation of T cells.
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References