Physical and Functional Association between Thymic Shared Antigen-1/Stem Cell Antigen-2 and the T Cell Receptor Complex*

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Thymic shared antigen-1 (TSA-1)/stem cell antigen-2 (Sca-2) is a glycosylphosphatidylinositol (GPI)-anchored antigen expressed on lymphocytes. We have previously demonstrated that a signal via TSA-1/Sca-2 inhibits T cell receptor (TCR)-mediated T cell activation and apoptosis. To elucidate a molecular mechanism for TSA-1-mediated modulation of the TCR-signaling pathway, we examined whether TSA-1 is physically coupled to the TCR in the present study. TSA-1 was clearly associated with CD3ζ chains in T cell hybridomas, activated T cells, and COS-7 cells transfected with TSA-1 and CD3ζ cDNA. The physical association was confirmed on the surface of T cells in immunoprecipitation and confocal microscopy. The analysis using stable and transient transfectants expressing a transmembrane form of TSA-1 revealed that the association of CD3ζ did not require the GPI anchor of TSA-1. Finally, tyrosine phosphorylation of CD3ζ chains was induced after stimulation with anti-TSA-1, suggesting that a functional association between these two molecules also exists. These results imply that the physical association to CD3ζ underlies a regulatory role of TSA-1/Sca-2 in the TCR-signaling pathway.

Thymic shared antigen-1 (TSA-1)/stem cell antigen-2 (Sca-2) is a Ly6-related differentiation antigen expressed on immature thymocytes and thymic epithelial cells (1–4). Recently, cDNA encoding human TSA-1 has been isolated, and it was shown that TSA-1 mRNA is expressed in human lymphoid tissues as well as various nonlymphoid tissues (5). Although TSA-1/Sca-2 is a useful marker in early T cell development and T cell activation and seems to play a regulatory role in thymocyte differentiation (6–8), functions of TSA-1/Sca-2 remain largely obscure.

In a previous study, we have analyzed a role of TSA-1 in mature T cells and demonstrated that it functions as a modulator of T cell receptor (TCR)-signaling pathway (6, 9, 10).

Anti-TSA-1 mAb inhibited tyrosine phosphorylation of CD3ζ chains and IL-2 production induced by anti-CD3 stimulation in T cell hybridomas (9), suggesting that a signal via TSA-1 regulates early and late events in TCR signaling. The findings observed in this in vitro study were further strengthened by the fact that in vivo injection of anti-TSA-1 mAb completely blocked anti-TCR/CD3-mediated apoptosis of thymocytes (10). Thus, TSA-1/Sca-2 seems to be an important cell surface molecule regulating T cell differentiation and activation by virtue of its ability for modulating TCR-mediated signal transduction. However, since TSA-1 is a glycosylphosphatidylinositol (GPI)-anchored membrane protein and thus does not have its transmembrane and cytoplasmic regions, it is not known how TSA-1 transmits signals into the cytoplasm of the cell.

In the present study, we addressed the above question by analyzing the molecular interaction between TSA-1 and the TCR. The data clearly demonstrated that TSA-1 is physically and functionally associated with CD3ζ chains of the TCR complex, and strongly suggested that the regulatory role of TSA-1 on TCR signaling is based on this intermolecular association.

EXPERIMENTAL PROCEDURES

Cell Lines and Hybridomas—2B4 is a murine T cell hybridoma that is specific for pigeon cytotome c plus I-EK (11). LK35.2 is a B cell hybridoma and used as accessory cells (12). Jurkat-derived transfectants expressing either GPI-anchored or a transmembrane form of TSA-1 have been established by us as described previously (9). All cell lines were maintained in RPMI 1640 medium supplemented with 10% fetal calf serum, penicillin (100 units/ml), and streptomycin (100 μg/ml) at 37 °C in a humidified atmosphere of 5% CO2. For maintaining Jurkat-derived transfectants, G418 (Life Technologies, Inc.) was added at a concentration of 1 mg/ml.

mAb and Reagents—The following monoclonal antibodies were used: 145-2C11 (13) and HMT3–1 (14), anti-CD3ε; H146-968 (15), anti-CD3ζ; A2B4 (11), anti-clonotypic antibody recognizing TCR-α of 2B4; M17/5.2 (16), anti-leukocyte function-associated antigen-1 (LFA-1); M1/42 (obtained from American Type Culture Collection, Rockville, MD), anti-major histocompatibility complex class I; D7 (17), anti-Ly-6A/E; E6 (18), anti-Ly-6C; G7 (19), anti-Thy-1.2; and PRST1 (6) and GR12, anti-TSA-1. GR12 is a rat mAb against TSA-1 that has been newly established by us, and the specificity of this mAb has been defined using Jurkat-derived transfectants expressing mouse TSA-1 (data not shown). No. 387, polyclonal antiserum against CD3ζ, was generously provided by Dr. Allan M. Weissman. Normal rat and hamster IgG were purchased from Cappel (Durham, NC).

Cell Preparations—T cells were enriched from spleen cells of C57BL/6 mice by immunomagnetic negative selection as described previously (9).

DNA Transfection—A total of 1 × 107 COS-7 cells was washed with Hepes-buffered saline and resuspended in 1 ml of ice-cold Hepes-buffered saline. Fifteen micrograms of plasmid DNA were added to the cell suspension in a cuvette (Gene Pulser Cuvette, Bio-Rad), and the electric pulse (250 V, 960 μF) was applied by a Gene Pulser (Bio-Rad). After 2 days of culture in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum, cells were harvested, washed twice with...
Association between TSA-1/Sca-2 and the TCR

RESULTS

Physical Association between TSA-1/Sca-2 and the CD3ζ—To elucidate a molecular mechanism for TSA-1/Sca-2-mediated modulation of the TCR signaling pathway, we examined whether TSA-1 could be physically associated with the TCR subunits. When 2B4, a T cell hybridoma which constitutionally expressed TSA-1, was solubilized with Triton X-100 lysis buffer, immunoprecipitated with anti-TSA-1 mAb, and immunoblotted with anti-CD3ζ mAb, no physical association between TSA-1 and CD3ζ was observed (Fig. 1B). However, when 2B4 cells were solubilized with digitonin lysis buffer, a clear band migrating at 16 kDa was observed in anti-CD3ζ immunoprecipitates (Fig. 1A). Although the amount of CD3ζ associated with TSA-1 was low, the result was very reproducible; we have observed the association in at least 10 independent experiments. The immunoprecipitation with M17/5.2, a mAb against mouse LFA-1, did not co-precipitate any CD3ζ, indicating that the association between TSA-1 and CD3ζ is specific.

We further examined whether the association of CD3ζ is detected in other GPI-anchored proteins. 2B4 cells were solubilized with digitonin lysis buffer, immunoprecipitated with various mAbs against other GPI-anchored and transmembrane surface proteins, and immunoblotted with anti-CD3ζ (Fig. 2). Again, the association of CD3ζ with TSA-1 was observed and this was confirmed using another rat mAb against TSA-1, GR12. However, none of mAbs against other GPI-anchored proteins such as Thy-1, Ly-6A/E, and Ly-6C, nor mAbs against transmembrane proteins such as LFA-1 and class I co-precipitated CD3ζ. Since these control mAbs were able to immunoprecipitate their corresponding surface Ags efficiently (data not shown), the result demonstrated a selectivity of CD3ζ chains for the ability to associate with TSA-1 among GPI-anchored molecules.

We next asked whether other chains of the TCR complex could be associated with TSA-1. The lysates of 2B4 cells were immunoprecipitated with anti-TSA-1, subjected to two-dimensional electrophoresis, and immunoblotted either with anti-CD3ζ or with anti-CD3ε. The result of Fig. 3 clearly demonstrated that TSA-1 was associated with CD3ε as well as CDζ. Since CD3ε was not bound to TSA-1 in the 2B4 mutant that lacks expression of CD3ζ, the association between TSA-1 and CD3ε seemed to be dependent on the existence of CD3ζ chains (data not shown).

To investigate the direct association of TSA-1 to CD3ζ, COS-7 cells were transiently transfected with TSA-1 cDNA, CD3ζ cDNA, or both, and analyzed for the association (Fig. 4). A band migrating at 16 kDa was detected in COS-7 cells trans-
Association between TSA-1/Sca-2 and CD3ε in Normal T Cells—We next examined whether TSA-1 can also be associated with CD3ε in normal T cells. Since mRNA and protein expression of TSA-1 cannot be detected in resting T cells (6), no association was observed when we used freshly isolated T cells for immunoprecipitation analysis (data not shown). However, a clear co-precipitation of CD3ε was detected in anti-TSA-1 immunoprecipitates from concanavalin A-activated T cells (Fig. 7). The association seemed to be specific, since the immunoprecipitation with anti-LFA-1 mAb did not co-precipitate any CD3ε (Fig. 7).

Physical Association between TSA-1/Sca-2 and the CD3ε in Jurkat-derived Transfectants Expressing a Transmembrane Form of TSA-1—We had established three types of Jurkat-derived transfected in our previous study (9). J2A11 cells were transfected with the expression vector alone, J6C4 cells with wild-type GPI-anchored TSA-1, and J4B1 cells with a transmembrane form of TSA-1, which consisted of the transmembrane and cytoplasmic portion of class I Db fused to the extracellular portion of TSA-1. The immunoprecipitation and immunoblotting analysis was performed using these transfecteds to determine whether the attachment to the plasma membrane via the GPI anchor is required for the physical association between TSA-1 and CD3ε. As shown in Fig. 8, CD3ε was bound to TSA-1 in J6C4 cells but not in J2A11 cells.
showing the ability of murine GPI-anchored TSA-1 to associate with human CD3ζ. In J4B1 cells, although the amount of CD3ζ bound to TSA-1 was small compared with that observed in J6C4 cells, the association of CD3ζ with a transmembrane TSA-1 was apparently identified. Moreover, in COS-7 cells transfected with a transmembrane TSA-1 together with CD3ζ, the association between these two molecules was clearly observed (Fig. 4D). Thus, the GPI anchor does not seem to be critical for the interaction between TSA-1 and CD3ζ, which is concordant with our previous data regarding the functional role of TSA-1 in TCR signaling.

**Functional Association between TSA-1/Sca-2 and the CD3ζ**—Finally, we assessed whether biochemical changes could be induced in CD3ζ by activating through TSA-1. To this end, we analyzed tyrosine phosphorylation of CD3ζ chains from 2B4 T cell hybridomas after stimulation with anti-TSA-1 in the presence with accessory cells. As shown in Fig. 9, stimulation of 2B4 cells with 2C11 resulted in tyrosine phosphorylation of CD3ζ. In contrast, stimulation with anti-LFA-1 or anti-class I did not induce any CD3ζ phosphorylation, indicating that engagement of any cell surface molecules by the mAb does not generally lead to the induction of CD3ζ phosphorylation. However, when 2B4 cells were stimulated through TSA-1 with GR12 or PRST1, the induction of tyrosine phosphorylation of CD3ζ was evident (Fig. 9). Interestingly, the amount of phosphorylated CD3ζ induced with PRST1 was much greater than that induced with GR12, although GR12 co-immunoprecipitated CD3ζ more efficiently than did PRST1 (see Fig. 2). The result suggested that the direct physical association of TSA-1 to CD3ζ leads to positive signaling events such as phosphorylation in CD3ζ chains when the signal was delivered to TSA-1 by cross-linking with the mAbs.

**DISCUSSION**

Many rodent and human GPI-anchored proteins have been implicated in regulation of T cell activation, since mAbs against these GPI-anchored proteins induce T cell activation as monitored by interleukin-2 production and proliferation. T cell activation induced by a signal through GPI-anchored proteins is dependent upon expression of the TCR; anti-Thy-1 and anti-Ly-6 mAbs fail to stimulate a TCR2 variant cell line, and the defect was able to be restored by TCR expression in these variant cell lines (22, 23). In addition to the positive regulation by GPI-anchored proteins in T cell activation, some GPI-anchored proteins transduce a negative signal that inhibits anti-CD3-mediated TCR signaling (24). We have previously demonstrated that a signal via TSA-1/Sca-2 inhibits TCR/CD3-mediated activation and apoptosis both in vitro and in vivo (6, 9, 10). Thus, the TCR seems to be an essential molecule in signaling pathway of GPI-anchored proteins at least in T cells.

A number of studies have indirectly suggested that there is a physical and/or functional association between some GPI-anchored proteins and the TCR. By using chemical cross-linkers,
protein and CD3 considered to be specific. Given that TSA-1 is a GPI-anchored protein, it was reported that CD45 is mutually associated with Thy-1 and the TCR, indicating that Thy-1 can physically interact with the TCR through CD45 (25). In another study, a T cell clone was stably transfected with antisense Ly-6A RNA (26). Cell surface expression of Ly-6A was markedly suppressed in this transfectant, but surprisingly surface expression of the TCR was greatly inhibited as well because of the reduction of TCR-β mRNA. The Ly-6A antisense transfectant was then transfected with TCR-β cDNA, and surface TCR expression was reconstituted without the expression of Ly-6A. However, TCR signaling was still impaired in this transfectant due to the absence of Ly-6A.

Despite these observations, it seems to be very difficult to demonstrate a direct association of the TCR to Thy-1, Ly-6, or other GPI-anchored proteins in immunoprecipitation analysis. Nonetheless, we are able to provide evidence that TSA-1/Sca-2 is physically associated with TCR in the present study. When TSA-1/Sca-2 expressed on the cell surface was stimulated with anti-TSA-1 mAbs, CD3ζ in the TCR complex was induced to be phosphorylated in its tyrosine residues (Fig. 9). This result indicates that a functional association also exists between these two molecules, and argues against the possibility that the interaction between TSA-1 and CD3ζ occurs merely during the process of solubilization and immunoprecipitation.

We do not know why we can successfully detect the physical association of the TCR to TSA-1 among many GPI-anchored proteins. Since none of mAbs against Thy-1, Ly-6A/E, and Ly-6C co-precipitated CD3ζ in an experiment in which both mAbs, PRST1 and GR12, against TSA-1 clearly co-precipitated CD3ζ (Fig. 2), the association between TSA-1 and CD3ζ is considered to be specific. Given that TSA-1 is a GPI-anchored protein and CD3ζ has a very short extracellular portion, the interaction between TSA-1 and CD3ζ could be mediated by an as yet undefined membrane protein, which could serve as a linker between these two proteins (27). This “linker” protein presumably functions not only in T cells but in COS-7 cells (Fig. 4). Moreover, the association between TSA-1 and the “linker” protein could not be dependent on the GPI anchor, but on primary sequence motifs of TSA-1. An effort should be made to identify the “linker” protein in biochemical analysis.

Alternatively, another possibility may account for the mechanism underlying the physical association between TSA-1 and CD3ζ. GPI-anchored proteins are known to be localized to caveolae, glycosphingolipid-rich areas in the cell membrane (28–30). Caveolae are also enriched in signal-transducing molecules, such as GTP-binding proteins, small G proteins, and nonreceptor-type tyrosine kinases (31). It has been proposed that some of the GPI-anchored proteins, such as Thy-1 and Ly-6A, can be localized to caveolae and interact with the TCR. This interaction could be mediated by a GPI-anchored protein “linker” that associates with both the TCR and the GPI-anchored protein. Further studies are needed to clarify the molecular basis of this association.
that caveolae could represent a specialized signaling compartment at the cell surface (32). Although lymphocytes do not have caveolae due to the lack of caveolin, there is the same membrane microdomain that are enriched in glycosphingolipids in lymphocytes (33). Thus, if the TCR complex or the CD3ζ may reside in this microdomain, GPI-anchored proteins could be associated with CD3ζ by lipid-protein interactions, thereby forming a signaling compartment at the surface of T cells. Stimulation of GPI-anchored proteins with mAbs results in the delivery of a signal through this signaling compartment. If this possibility is correct, TSA-1 is not special among other GPI-anchored proteins but our mAbs against TSA-1 could be specific among other mAbs against GPI-anchored proteins. Although we can not thus far explain whether and how a transmembrane TSA-1 could be localized in this signaling compartment, the above hypothesis is very attractive, given that most of GPI-anchored proteins have a signal-transducing ability when cross-linked with mAbs.

Although the mechanism is not fully understood, our previous finding that a signal through TSA-1 down-modulates the TCR signaling pathway could be explained by the physical association between TSA-1 and CD3ζ. Cross-linking of TSA-1 with the mAb induces the phosphorylation of tyrosine residues in CD3ζ chains (Fig. 9) through the activation of the Src family tyrosine kinases, which may subsequently cause recruitment of another protein tyrosine kinase, ZAP-70 (34). Thus, intracellular signal-transducing molecules could be sequestered from TCR-signaling pathways to the TSA-1/CD3ζ complex, resulting in down-modulation of TCR signaling. Studies are in progress to elucidate a molecular mechanism for the TSA-1-signaling pathway.

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