CAST, a Novel CD3ε-binding Protein Transducing Activation Signal for Interleukin-2 Production in T Cells*

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Tetsuo Yamazaki‡, Yuki Hamano‡, Hironori Tashiro, Katsuhiro Itoh, Hiroyasu Nakano§, Shoichiro Miyatake¶, and Takashi Saito†

From the Department of Molecular Genetics, Chiba University Graduate School of Medicine, 1-8-1 Inohana, Chuo-ku, Chiba 260-8670, Japan

Antigen recognition through T cell receptor (TCR)-CD3 complex transduces signals into T cells, which regulate activation, function, and differentiation of T cells. The TCR-CD3 complex is composed of two signaling modules represented by CD3ζ and CD3ε. Signaling through CD3ζ has been extensively analyzed, but that via CD3ε, which is also crucial in immature thymocyte development, is still not clearly understood. We isolated cDNA encoding a novel CD3ε-binding protein CAST. CAST specifically interacts in vivo and in vitro with CD3ε but not with CD3ζ or FeRγ via a unique membrane-proximal region of CD3ε. CAST is composed of 512 amino acids including a single tyrosine and undergoes tyrosine phosphorylation upon TCR stimulation. Overexpression of two dominant-negative types of CAST, a minimum CD3ε-binding domain and a tyrosine-mutant, strongly suppressed NFAT activation and interleukin-2 production. These results demonstrate that CAST serves as a component of preformed TCR complex and transduces activation signals upon TCR stimulation and represents a new signaling pathway via the CD3ε-containing TCR signaling module.

T cells recognize antigens (Ag)† presented by major histocompatibility complex on antigen presenting cells. The Ag recognition signal is then transmitted to the cytoplasm via the T cell receptor (TCR) complex, resulting in T cell activation leading to various cellular events such as proliferation, apoptosis, anergy, and differentiation as well as a variety of effector functions. The TCR complex is composed of eight polypeptides including clonally variant αβ or γδ chains and invariant CD3 chains that contain two noncovalently associated dimers γε and δε, and a disulfide-like ζ chain dimer (either ζ-ζ, ζ-η, or ζ-FeRγ) (1). Although αβ chains (γδ chains) are responsible for Ag recognition, CD3γ, δ, ε, and ζ chains play crucial roles in delivering the Ag recognition signal to the cytoplasm (2). Each CD3 chain possesses activation motifs in the cytoplasmic domain, termed ITAM (immune receptor tyrosine-based activation motifs). ITAM contains two YXXLXX sequences 7 or 8 amino acids apart. Whereas CD3γ, δ, ε, and FeRγ chains have one ITAM, ζ and η chains have three and two, respectively (3). Upon TCR stimulation, Src family protein-tyrosine kinases such as Lck and Fyn are activated to phosphorylate tyrosine residues within ITAMs, followed by recruitment of the second type of tyrosine kinase ZAP-70 (Syk) to phosphorylated ITAM (4, 5). Subsequently, such initial events lead to ZAP-70 activation and tyrosine phosphorylation of various intracellular proteins including phospholipase Cδ (6–8), Vav (9, 10), SLP-76 (11, 12), HS-1 (13), LAT (14–16), phosphatidylinositol 3-kinase (17), and Cbl (18), as well as Ca2⁺ mobilization, phosphatidylinositol hydrolysis, and the activation of mitogen-activated protein kinase cascade (2).

It has been shown that ITAM in not only CD3ζ but also CD3ε mediates activation signals, as demonstrated by experiments in which chimeric molecules composed of the extracellular domain of CD8 or CD25 with the cytoplasmic tail of CD3ε or CD3ζ such as CD8-ζ or CD25-ε can mimic the TCR signals by inducing various tyrosine phosphorylations of cytoplasmic proteins, Ca2⁺ mobilization, and phosphatidylinositol hydrolysis (19, 20). However, from experiments analyzing the function of T cells expressing ITAM-deficient CD3ζ chain, it has been shown that T cell activation through Thy-1 or Ly-6 depends on the presence of ITAM of the ζ chain and could not be replaced by ITAMs from other CD3 chains, indicating that the TCR-CD3 complex is composed of two functionally distinct signaling modules represented by CD3ζ and CD3ε (21, 22). Furthermore, physiological significance of such distinct signaling modules has also been demonstrated in immature thymocytes (23, 24). In addition to the fact that CD3ζ appears to associate with pre-TCR complex and clonotype-independent CD3 complex very weakly (25, 26), cross-linking of CD3ε induces differentiation of CD4⁺8⁻ thymocytes into CD4⁺8⁺ cells in RAG-2 knockout mice (27, 28). This implies that the signaling pathway through the module containing CD3ε plays an important role in controlling immature thymocyte development.

Phosphorylation-dependent downstream signals upon TCR stimulation, particularly molecules triggered by phosphorylation of ITAM, have been extensively investigated (29). However, signaling molecules, which constitutively associate with the TCR-CD3 complex, still remain to be elucidated. Such molecules should play a crucial role in T cell activation as a preformed signaling component of the TCR-CD3 complex. We therefore attempted to identify molecules, which constitutively associate with the CD3ε chain to clarify the preformed TCR signaling machinery as well as the downstream signaling path-
way of the activation module containing CD3ε as compared with CD3ζ. Utilizing the West-Western method (30), we identified a novel CD3ε-binding protein, CAST, as a CD3ε-associated signal transducer. In this report, we have analyzed its structure and function in T cell activation.

**EXPERIMENTAL PROCEDURES**

**Cells and Antibodies**—The ovalbumin-specific murine T cell hybridoma DO11.10 and Tαg-Jurkat cells, a Jurkat T cell line stably transfected with simian virus 40 large T antigen (providing by G. Crabtree, Stanford University, Stanford, CA), were maintained in RPMI 1640 (Life Technologies, Inc.) supplemented with 10% fetal calf serum, 5 mM glutamine, and 50 μM 2-mercaptoethanol (complete RPMI). Anti-CD3ε mAb, 145–2C11, was kindly provided by J. Bluestone (University of Chicago, Chicago, IL). Anti-α-HA mAb, 12CA5, and anti-phosphotyrosine mAb, 4G10, were purchased from Roche Molecular Biochemicals and Upstate Biotechnologies, Inc. (Lake Placid, NY), respectively. Anti-CAST antisera were prepared by immunizing New Zealand White rabbits (Japan SLC, Inc., Hamamatsu, Japan) subcutaneously with GST fusion protein that included a part of CAST corresponding to amino acids 1–489 used as the immunogen.

**Preparation of 32P-Labeled GST Fusion Proteins**—Constructs of GST fusion protein containing the cytoplasmic domain of CD3ε (GST-ε) and a fragment of 32P-labeled GST-ε as a screening probe were described previously (31). Briefly, purified GST-ε was adsorbed onto glutathione-Sepharose 4B beads (Amersham Pharmacia Biotech), which were then subjected to kinase reaction by the catalytic subunit of CAMP-dependent protein kinase (Sigma), and the labeled GST-ε was eluted by 10–50 beads volumes of 20 mM reduced glutathione. The probes were labeled to high specific activity (approximately 5–10 × 10^6 cpm/μg of protein).

**Screening of cDNA Library**—CD3ε-binding proteins were screened from a λgt11 library, which was constructed from mRNA from an human T-cell lymphophotrophic virus, type I transformed human T cell line, HAT109 (provided by Dr. M. Yoshida, University of Tokyo) as described previously (31). The total of 1.6 × 10^10 plaques were screened with 32P-labeled GST-ε. Positive phages were subsequently isolated, and the cDNA inserts were sequenced after subcloning into pBluescript SK II (pBSK/HK)

**Expressible Constructs and Transfection**—An expressible construct of CAST was constructed by inserting a full-length CAST cDNA into an expression vector, pBCMSGNeo (a gift of Dr. H. Karasuyama, Tokyo, Japan) or pEF-BOS (a gift of Dr. S. Nagata, Osaka, Japan). For Hα-tagged CAST, oligonucleotides corresponding to 5' and 3' sequences of HA were annealed and subcloned into the Xho I NotI fragment of pBlue-script SK II (pBSK/HK). A full-length cDNA of CAST was inserted into the EcoRI site of this vector (pBSK/HAKAST). Then a Xho I NotI fragment from pBSK/HAKAST was subcloned into the Xho I NotI fragment of pBCMSGNeo (pBCMSG/HK/A CAST).

For transient transfection, EcoR V-Smal fragment containing a full-length CAST cDNA was subcloned into a blunt-ended pEF-BOS (pEF-BOS/CAST). The minimal binding region (BR) of CAST to CD3ε, which corresponds to nucleotides 442–594, and a mutant CAST containing a mutation (Tyr → Phe) were prepared by polymerase chain reaction and subcloned into a blunt-ended pEF-BOS vector (pEF-BOS/BR, pEF-BOS/YF).

Stable transfectants of CAST into DO11.10 T cell hybridoma cells were obtained by electroperoration with 30 μg of pBCMSGNeo/HK/CAST using Gene Pulser II (Bio-Rad) with 310 V and 975 microfarads. For transient transfections, TAG-Jurkat T cells were co-transfected with 20 μg of NFAF-luciferase (a gift of Dr. G. Crabtree) and 40 μg of pEF-BOS, pEF-BOS/CAST, pEF-BOS/BR, and pEF-BOS/YF in serum-free RPMI 1640.

**In Vitro Binding Assays**—Specific interaction between CAST and CD3ε chains was analyzed using in vitro translated 35S-labeled CAST and its mutants and GST fusion protein of CD3ε and its truncated products as described previously (31). In vitro translation of a full-length or partial cDNAs of CAST subcloned into pCITE-4a (+) vector was performed with use of TNT^® coupled reticulocyte lysate systems (Promega, Madison, WI) in the presence of [35S]methionine. Preparation and purification of various GST fusion proteins were described previously (31). In vitro binding assay were performed in a binding buffer (1% Brij 97, 50 mM Tris, pH 7.6, 150 mM NaCl, 10 μM aprotinin, 12.5 μM antipain, 12.5 μM chymostatin, 50 μM leupeptin, 25 μM pepstatin A, 1 mM phenylmethylsulfonyl fluoride, 1 mM iodoacetamide) as described (31).

**Immunoprecipitation and Immunoblotting**—Cell lysates were pre-

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**Table:**

| Protein | Description |
|---------|-------------|
| CD3ε | CD3ε-binding Protein Mediating T Cell Activation |

**Fig. 1. Protein sequence of CAST.** The human CAST sequence was deduced from the nucleotide sequence of the CAST cDNA, starting at the first in-frame methionine residue. CAST consists of 512 amino acids. The minimal region required for binding with CD3ε as shown in Fig. 4 is boxed. A unique tyrosine residue located at position 82 is underlined. The human CAST nucleotide sequence will appear in the EBI and GenBank™ nucleotide data bases under the accession number AF017633.

**RESULTS**

**Identification of cDNAs Encoding CD3ε-associated Proteins**—To isolate cDNAs encoding CD3ε-associated proteins, a λgt11 expression library derived from human T cell line was screened with a 32P-labeled GST fusion protein containing the cytoplasmic domain of CD3ε. Eight positive clones were obtained as fusion proteins with β-galactosidase (31). Subsequent DNA sequencing of these clones revealed that they encoded five different proteins, and one of these clones encoded a novel protein, CAST (CD3ε-associated signal transmission). The entire coding sequence of cDNA and the deduced amino acid sequence of CAST are shown in Fig. 1. CAST is comprised of 512 amino acids including a single tyrosine residue (Tyr-82). Because Tyr-82 was followed by a leucine residue located two amino acids downstream, the sequence around Tyr resembles a half of ITAM or tyrosine signal motif for the interaction with the μ2

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chain of adapter complex AP-2 (32) (Fig. 1).

Association between CAST and CD3e in T Cells—CAST was identified as a CD3e-binding protein by West-Western method. To examine whether CAST interacts with CD3e under physiological conditions, we prepared the transfectants of a widely used ovalbumin-specific T cell hybridoma, DO11.10, which expressed CAST attached with HA tag at the N terminus (HA-CAST). HA-CAST was abundantly expressed in the transfectant (Fig. 2A, lane 1). When the cell lysate was immunoprecipitated with anti-CD3e mAb and immunoblotted with anti-HA mAb, a band corresponding to HA-CAST was clearly detected (Fig. 2A, lane 2). In contrast, no band was observed in the immunoprecipitates with a control hamster Ab. (Fig. 2A, lane 3). The specific interaction of CD3e and CAST was further confirmed for the endogenous CD3e and CAST in T cells. Human Jurkat cells were lysed in the 1% Brij97-containing buffer, and the lysate was immunoprecipitated with anti-CD3e mAb and blotted with anti-Cast Ab. As shown in Fig. 2B, CAST was co-precipitated with anti-CD3e mAb OKT3 (Fig. 2B, lane 2) but not with anti-Tac mAb 7G7 as a control (Fig. 2B, lane 3). The association was hardly detected in the cell lysates prepared in stronger detergent condition such as 1% Nonidet P-40 (data not shown), suggesting that the association between CD3e and CAST is weak. CAST was also detected by immunoprecipitation and blotting with anti-CAST Ab (Fig. 2B, lane 1). Because rabbit anti-CAST Ab does not precipitate CAST very well, the amount of CAST by immunoprecipitation with anti-CAST Ab was considerably underestimated. The specificity of the association of CAST with the TCR complex was further analyzed by immunoprecipitation with various Abs against other cell surface molecules on T cells. As shown in Fig. 2C, anti-CD3e mAb but not mAbs against CD2, CD45, or CD4 (not shown) co-precipitate CAST. These results demonstrate that CAST associates specifically and constitutively with the TCR complex under physiological condition.

Specific Association of CAST with CD3e but Not CD3ζ—We next analyzed the specificity of the binding of CAST with CD3e within the TCR complex. We examined particularly whether CAST interacted with CD3ζ family molecules (ζ, η, and FcRy), because these molecules contain ITAMs and constitute a different signaling module from CD3e in the TCR complex (21, 22). In addition, we investigated the CAST-binding region within CD3e by in vitro binding assays. [35S]methionine-labeled CAST was translated in vitro and precipitated with a variety of GST fusion proteins containing the entire or partial deletion mutants of the cytoplasmic domain of CD3e as well as the cytoplasmic tails of CD3ζ and FcRy chains (Fig. 3). CAST was co-precipitated with GST-CD3e (Fig. 3B, lane 7) but not with GST-CD3ζ or GST-FcRy (Fig. 3B, lanes 8 and 9). The specificity of the binding between CAST and CD3e was demonstrated by the fact that luciferase as a control was not precipitated with CD3e (Fig. 3B, lane 11). This result indicates that the association of CAST with the TCR complex is mediated through specific interaction with CD3e and implies that CAST functions in the downstream of the signaling module containing CD3e.

We determined the CAST-binding region within CD3e in the in vitro binding assay by co-precipitation with GST fusion protein containing various deletion mutants of CD3e. As shown in Fig. 3B, labeled CAST interacted with all GST fusion proteins (ζD1, ζD3, ζD5, and CD3e) except for GST-ζD4 (Fig. 3B, lanes 2–7). Because GST-ζD1 precipitated CAST, the proximal region to the transmembrane portion of CD3e composed of only 12 amino acids was found to be necessary and sufficient for the CAST/CD3e binding. Taken together, the results demonstrated that CAST interacts specifically with CD3e through its unique region proximal to the transmembrane domain.

Mapping of CD3e-binding Region in CAST—In addition to the CAST-binding region within CD3e, the CD3e-binding re-

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**Fig. 2. In vivo association between CAST and CD3e in T cells.**

**A.** Association of transfected CAST and endogenous CD3e. DO11.10 T cell hybridoma cells were transfected with HA-tagged CAST. The cell lysates were immunoprecipitated with anti-HA mAb (12CA5; lane 1), anti-CD3e mAb (145–2C11; lane 2), or a control Ab (hamster Ig (Ham Ig); lane 3) and immunoblotted (IB) with anti-HA mAb. The sample used for lane 1 originated from 2 × 10⁷ cells, and those for lanes 2 and 3 were derived from 2 × 10⁶ cells. The arrow indicates the position of HA-CAST. **B.** In vivo association between endogenous CAST and CD3e. 9 × 10⁶ human Jurkat cells were lysed in 1% Brij97-containing buffer, and the lysate was precipitated with anti-CAST Ab (lane 1), anti-CD3e mAb (OKT3; lane 2), or anti-Tac mAb as a control (7G7; lane 3) and blotted with anti-HA mAb. The sample used for lane 1 originated from 2 × 10⁷ cells, and those for lanes 2 and 3 were derived from 2 × 10⁶ cells. The arrow indicates the position of HA-CAST. **C.** In vivo association between CAST and CD3e. 6 × 10⁷ Jurkat cells were immunoprecipitated either with mAbs against CD2 (95–5–49), CD3 (OKT3), and CD45 (P10-89-4) and blotted with anti-HA mAb. The blots with anti-CAST Ab (not shown) showed similar results. As positive control of HA-CAST, whole cell lysate (WCL) was analyzed and blotted with anti-HA mAb (lane 1). Molecular size markers are indicated on the left. IP, immunoprecipitation; IB, immunoblot; IgH, immunoglobulin heavy chain.
region within CAST was also determined by in vitro binding assays. Serially truncated cDNAs of CAST (d1–d4) were subcloned into pCITE4a vector (Fig. 4A). [35S]Methionine-labeled in vitro translation products of these mutant CASTs were prepared. Then CAST and its truncated mutants were precipitated with GST-CD3e. As shown in Fig. 4B, d1, d2, and d4 bound strongly to CD3e, whereas d3 appeared to bind very weakly (Fig. 4B, lanes 5–8). Collectively, these results demonstrate that the region of amino acid residues 149–198 in the N-terminal half of CAST is responsible for the specific binding to CD3e. This region is boxed in Fig. 1.

**Tyrosine Phosphorylation of CAST upon TCR Stimulation**—Because CAST binds directly and constitutively to CD3e, it may have a function for signal transduction upon T cell activation. CAST contains a single tyrosine residue at amino acid position 82. If this unique tyrosine undergoes phosphorylation upon TCR stimulation, it is possible that phosphorylated CAST may function to transduce downstream signals. Therefore, we examined whether CAST undergoes tyrosine phosphorylation upon TCR stimulation. DO 11.10 T cell hybridomas overexpressing HA-CAST were prepared and stimulated for 2 min by cross-linking with anti-CD3e mAb (2C11), and the cell lysates were prepared in a buffer containing 1% digitonin. The lysates were immunoprecipitated with anti-HA mAb as a control and blotted with antiphosphotyrosine (anti-PY) mAb (4G10) (upper panel). The same membrane was rebotted with anti-CAST Ab to confirm that the phosphorylated band was indeed phosphorylated CAST (pCAST) and CAST. Molecular size markers are indicated on the left. IB, immunoblot.

**CAST Mediates Signals for NFAT Activation and IL-2 Production**—To investigate the function of CAST in T cell activation, we first analyzed the T cell hybridoma transfectants overexpressing CAST for IL-2 production. However, we failed to observe significant effect on IL-2 secretion (data not shown). Therefore, we decided to analyze the function of dominant-negative forms of CAST. For this purpose, two mutant forms of CAST.
CAST were prepared to serve as dominant-negative CASTs (Fig. 6A). One mutant was termed BR (binding region to CD3ε, amino acid residues 148–198), which was exclusively comprised of the minimal CD3ε-binding region of CAST as shown in Fig. 4. The other was a tyrosine mutant YF, in which a unique tyrosine (Tyr-82) was mutated to phenylalanine. Although BR and YF can bind to CD3ε in vitro binding assay (Fig. 4), it is particularly important to demonstrate the interaction in T cells to connect to the functional analysis.

Therefore, we confirmed the specific association of YF and BR with CD3ε and function as dominant-negative forms in T cells. The association of YF with CD3ε was analyzed in the transient transfection system. TAg-Jurkat cells (33) were transfected with YF and BR, and the cell lysates were immunoprecipitated with anti-CD3ε mAb and blotted with anti-CAST Ab. As shown in Fig. 6B, YF mutant was clearly co-immunoprecipitated with CD3ε (lane 6). Endogenous CAST was also immunoprecipitated with anti-CD3ε mAb (Fig. 6B, lane 2) as shown in Fig. 2B. Furthermore, the overexpression of BR induced the disappearance of the band corresponding to the endogenous CAST (Fig. 6B, lane 4), supporting the idea that BR competes with the CD3ε binding with endogenous CAST and thereby demonstrating that it functions as a dominant-negative form of CAST. Regarding the analysis of BR, because anti-CAST Ab unfortunately did not immunoprecipitate BR, T cell hybridoma cells transfected with BR were immunoprecipitated with anti-CD3ε Ab and blotted with anti-CAST Ab to investigate the association of BR and CD3ε. As shown in Fig. 6C, the specific precipitation of the BR protein with anti-CD3ε mAb (Fig. 6C, lane 9) but not with the control hamster Ig (Fig. 6C, lane 10) was detected as a 20-kDa band, although it was a very weak band because of the weak reactivity of anti-CAST Ab with BR even for blotting. Further evidence for competitive capability of BR to CD3ε binding by CAST was demonstrated with an in vitro binding assay. CAST and BR were in vitro translated with [35S]methionine, and the graded amount of BR was added to the mixture of CAST and GST-CD3ε. As shown in Fig. 6D, BR inhibited the CAST-CD3ε binding in a dose-dependent manner. Collectively, these results of biochemical analysis demonstrate that BR and YF indeed associate with CD3ε in T cells and function as dominant-negative forms of CAST by overexpression.

To analyze the function of these mutant CASTs in T cell activation, we first utilized a transient assay for NFAT activation by using NFAT-luciferase (33), a reporter construct containing multiple copies of the NFAT binding site of the IL-2 promoter fused to luciferase cDNA. We transiently transfected NFAT-luciferase in TAg-Jurkat T cells in combination with either empty vector, BR, YF, or the wild-type CAST. As endogenous control, sea pansy luciferase was also transfected into all cells and the NFAT-luciferase activity was normalized by sea pansy activity. No significant difference was observed in relation to luciferase activity when TAg-Jurkat T cells was transfected with anti-CAST Ab. As a control, whole cell lysate was also blotted (lane 1). The arrow indicates the position of CAST. C, the association between BR and CD3ε. Stable transfectants of DO-11-10 hybridoma expressing ha-BR-BR were lysed in 1% Brij97, and the cell lysate was immunoprecipitated with either anti-CD3ε mAb (2C11) (lane 9) or a control Ab (Hamster Ig (Ham Ig) (lane 10) and then blotted with anti-CAST Ab. Whole cell lysate was also blotted (lane 8). The arrow indicates the position of ha-BA-BR. D, BR competes with the CAST binding to CD3ε in vitro binding assay. [35S]Methionine-labeled in vitro translated products of CAST and BR were produced, and in vitro binding assay was performed with GST-CD3ε. The same amount of CAST (3 μl) was mixed with graded amount of BR (3 and 30 μl), and the complex with CD3ε was isolated and analyzed in SDS-PAGE. Molecular size markers are indicated on the left. IP, immunoprecipitation; IB, immunoblot; WCL, whole cell lysate.

**Fig. 6.** Dominant-negative forms of CAST and their association with CD3ε. A, schematic structures of the wild-type and two dominant-negative forms of CAST, BR and YF. BR consists exclusively of the binding region of CD3ε in CAST and is shown as a shaded box. YF has a tyrosine to phenylalanine mutation at amino acid residue 82. B, the YF mutant binds to CD3ε, and the BR can compete with the CD3ε binding with CAST. TAg-Jurkat cells were transiently transfected with the expressive constructs of BR (lanes 4 and 5) or YF (lanes 6 and 7). The cell lysates (equivalent to 5 × 10^7 cells) were immunoprecipitated with either anti-CD3ε mAb (OKT3) or a control mAb (9E10) and blotted.
fected with the empty vector or wild-type CAST (Fig. 7A). However, transfection with both BR or YF strongly reduced luciferase activity compared with those transfected with the empty vector (Fig. 7B), whereas the activities upon stimulation with phorbol myristate acetate plus ionophore are almost the same (see the legend of Fig. 7). NFAT activation was inhibited by BR in a dose-dependent manner (Fig. 7C). These functional data demonstrate that CAST exhibits signaling function for NFAT activation.

To analyze the effect on IL-2 production after NFAT activation, stable transfectants of DO11.10 hybridoma cells expressing BR were prepared. As shown in Fig. 7D, BR transfectants exhibited reduced levels of IL-2 production upon TCR stimulation. These results indicate that CAST mediates activation signals important for IL-2 production.

**DISCUSSION**

We described the identification and function of the novel CD3ε-associated molecule CAST in this study. Because CAST was cloned on the basis of specific binding to the cytoplasmic domain of CD3ε independently of its phosphorylation, it is expected to be a constitutive component of the preformed TCR-CD3 complex (34). As expected, CAST constitutively associates with CD3ε in T cells. CAST does not merely function as a component of the preformed TCR complex but also plays an important role in transducing activation signals in T cells despite the fact that CAST does not contain any particularly conserved functional domain structure.

The first evidence that CAST is important for T cell activation was demonstrated by its tyrosine phosphorylation upon T cell stimulation. Only a minor portion of CAST appeared to be phosphorylated upon stimulation. Preliminary results demonstrated that most of the phosphorylated CAST disappeared when cell lysates from anti-CD3ε-stimulated T cells were pre-cleared extensively, suggesting that the phosphorylated CAST was mostly associated with CD3ε on the cell surface.2 The

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2 T. Yamazaki, Y. Hamano, H. Tashiro, K. Itoh, H. Nakano, S. Miyatake, and T. Saito, unpublished observation.
second evidence was shown by demonstrating that dominant-negative forms of CAST inhibited NFAT activation. NFAT activation and IL-2 production was significantly reduced by overexpression of two dominant-negative mutants BR or YF. The structure of these two mutants provides insights for the mechanism of CAST function. Because BR is the minimal region of CAST for CD3e binding, BR inhibits NFAT activation by blocking the CD3e binding of endogenous CAST. Indeed, we demonstrated that BR competed with the binding with endogenous CAST in T cells. In addition, this result suggests that there is an important region(s) in CAST other than the CD3e-binding region, which is required for signaling function of CAST probably through association with other signaling molecules. Because the YF mutant also suppressed NFAT activation, it is likely that one of the CAST-binding molecules recognizes the unique phosphorylated Tyr-82 and then transduces downstream signals leading to NFAT activation. Because the YF mutant could not associate with such a downstream protein, its recruitment by CAST to the TCR complex on the cell surface was inhibited in YF-transfected cells. In contrast to the inhibitory effect of these two dominant-negative types of CAST mutants, overexpression of wild-type CAST had little effect on NFAT activation. This might be explained on the basis of preliminary results showing that CAST associated with the TCR complex on the cell surface, which is only a minor fraction of overexpressed CAST, is mainly phosphorylated to mediate signaling function. Alternatively, unidentified CAST-binding protein(s) may be limited.

It is noted that BR or YF did not suppress NFAT activation completely, and increasing the dose of BR cDNA for transfection did not result in further reduction. This is probably due to the presence of other pathways to induce NFAT activation upon TCR stimulation, which can not be blocked by dominant-negative CAST mutants. The TCR-CD3 complex is composed of two distinct signaling modules: one is composed of CD3e, and the other is composed of CD3ζ (21, 22). It is possible that distinct signaling molecules may be associated with the downstream signals of each module. CAST is the first signaling molecule that dissects functions of these two signaling modules by specific association with CD3e. Therefore, even if the pathway immediately downstream of the CD3e-containing module was blocked by the expression of dominant-negative CAST mutants, NFAT activation signals may be transduced via CD3ζ. This may explain why NFAT activation was not completely blocked by the overexpression of BR or YF and also is consistent with the observation that CAST binds specifically to CD3e but not to CD3ζ. Furthermore, this also suggests that CAST-mediated activation signal is not dispensable by the signal through the CD3ζ module. To confirm this hypothesis, we are currently analyzing NFAT activation in T cells expressing the CD3ζ chain lacking all ITAMs.

We determined that CAST-binding region within CD3ζ was located in the N-terminal 12 amino acids of the cytoplasmic domain of CD3ζ. This membrane-proximal region of CD3ζ is very unique because other signaling molecules such as Fyn and ZAP-70 are known to associate with CD3ζ via its ITAM (35, 36). CAST is the first molecule possessing the binding specificity to CD3ζ because other molecules associate with both CD3ζ and CD3e. Therefore, the membrane-proximal region of CD3ζ with which CAST associates may serve as a signaling motif and mediate ITAM-independent signaling upon TCR stimulation. This idea is supported by the fact that the first 13 amino acids of the cytoplasmic region of CD3ζ are completely conserved between mouse and human. Previous study on signaling function of the cytoplasmic tail of CD3ζ by Letourneur and Klausner (19) is also consistent with our results. This study demonstrated the crucial role of ITAM of CD3ζ for T cell activation. In addition, it revealed that CD3ζ in the presence of the membrane-proximal region induced severalfold higher IL-2 response than that of CD3ε lacking this region, indicating the important contribution of the membrane-proximal region of CD3ε for T cell activation.

Signaling pathways through CD3ε are important not only in mature T cells but also in immature thymocytes (27, 28, 37). The maturation of thymocytes in RAG-2-deficient mice is blocked at the transitional stage from CD4-8- thymocytes to CD4?8? cells (38). However, it has been demonstrated that this blockade in maturation becomes reversed upon stimulation of thymocytes by cross-linking of CD3ε (27, 28). Considering that pre-TCR complex has a very weak association with CD3ζ as compared with the TCR complex on mature T cells (25, 26, 39), these experiments suggest that signaling through the CD3ζ-containing module plays an important role in differentiation and selection of thymocytes and that CAST may contribute partly to this pathway by exhibiting function for downstream signaling of the TCR or pre-TCR complex. The in vivo function of CAST in thymocytes development is now being addressed by studies on transgenic mice expressing a dominant-negative CAST under a T cell-specific promoter.

When the expression of CAST in various tissues was investigated, it was found that CAST is expressed in a wide range of tissues with relatively high expression in heart, skeletal muscle, ovary, and small intestine (data not shown). This finding indicates diverse functions of CAST in various types of cells. Furthermore, it is noted that the C terminus of CAST is rich in Lys and Arg, which may represent a characteristic for nuclear localization. Indeed, preliminary results of the analysis of cellular localization of CAST demonstrated that CAST is located both in the cytoplasm and the nucleus (data not shown). Because it has been shown that CD3ζ also exist in the nucleus (31), CAST in the nucleus may be co-localized with the nuclear CD3ζ. Alternatively, these results suggest that CAST possess a distinct function other than mediating activation signals in the nucleus. Further analysis is required to elucidate such diverse functions of CAST.

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REFERENCES
1. Malissen, B., and Schmitt-Verhulst, A.-M. (1993) Curr. Opin. Immunol. 5, 324–333
2. Weiss, A., and Littman, D. R. (1994) Cell 76, 263–274
3. Rath, M. (1989) Nature 338, 883–884
4. Ishizaki, M., Irving, B. A., van Oers, N. N., Chan, A. C., and Weiss, A. (1994) Science 263, 1136–1139
5. Wange, R. L., Malek, S. N., Desiderio, S., and Samelson, L. E. (1993) J. Biol. Chem. 268, 19797–19801
6. Weiss, A., Koretzky, G., Schatzman, R., and Kadlecek, T. (1991) Proc. Natl. Acad. Sci. U. S. A. 88, 5484–5488
7. Sprent, J., and Pemberton, A. R., and Abraham, T. (1991) J. Biol. Chem. 266, 12115–12130
8. Park, D. J., Rho, H. W., and Rhee, S. G. (1991) Proc. Natl. Acad. Sci. U. S. A. 88, 5450–5456
9. Buvelo, X. R., Ledbetter, J. A., and Barabid, M. (1992) Nature 356, 68–71
10. Gubins, E., Coggshall, K. M., Baier, G., Katsav, S., Bunn, P., and Altman, A. (1993) Science 260, 822–825
11. Jackman, J., Motto, D., Sun, Q., Tanemoto, M., Turck, C., Peltz, G., Koretzky, G., and Findell, P. (1994) J. Biol. Chem. 270, 7029–7032
12. Motto, D. G., Ross, S. E., Wu, J., Hendricks-Taylor, L. R., and Koretzky, G. A. (1996) J. Exp. Med. 185, 1937–1943
13. Fusaki, N., Matsuda, S., Nishizumi, N., Umemori, H., and Yamamoto, T. (1996) J. Immunol. 156, 1369–1377
14. Buday, L., Egan, S. E., Vicana, P. R., Cantrell, D. A., and Downward, J. (1994) J. Biol. Chem. 269, 9019–9025
15. Sieh, M., Batsker, A., Schlessinger, J., and Weiss, A. (1993) Mol. Biol. Cell. 14, 4435–4442
16. Zhang, W., Sloan-Lancaster, J., Kitchen, J., Tribble, R. P., and Samelson, L. E. (1998) Cell 92, 83–92
17. Huang, X., Li, Y., Tanaka, K., Moore, K. G., and Hayashi, J. I. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 11618–11622
18. Donovan, J., Wange, R., Langdon, W., and Samelson, L. (1994) J. Biol. Chem.
Letourneur, F., and Klausner, R. D. (1992) Science 255, 79–82
Irving, B. A., and Weiss, A. (1991) Cell 64, 891–901
Aoe, T., Goto, S., Ohno, H., and Saito, T. (1994) Int. Immunol. 6, 1671–1679
Wegener, A.-M. K., Letourneur, F., Hoeveler, A., Brocker, T., Luton, F., and Malissen, B. (1992) Cell 68, 83–95
Levelt, C. N., and Eichmann, K. (1995) Immunity 3, 667–672
Shinkai, Y., Ma, A., Cheng, H.-L., and Alt, F. W. (1995) Immunity 2, 401–411
Punt, J. A., Kubo, R., Saito, T., Finkel, T. H., Kathiresan, S., Blank, K. J., and Hashimoto, Y. (1991) J. Exp. Med. 174, 775–783
Takase, K., Wakizaka, K., von Boehmer, H., Wada, I., Moriya, S., and Saito, T. (1997) J. Immunol. 158, 741–747
Levelt, C. N., Mombaerts, P., Iglesias, A., Tonegawa, S., and Eichmann, K. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 11401–11405
Shinkai, Y., and Alt, F. W. (1994) Int. Immunol. 6, 995–1001
Wargis, R. L., and Samelson, L. E. (1996) Immunity 5, 197–205
Skolnik, E. Y., Margoulis, B., Mohammadi, M., Lowenstein, R., Fischer, R., Drepps, A., Ulrich, A., and Schlesinger, J. (1991) Cell 65, 83–90
Nakano, H., Yamazaki, T., Miyatake, S., Nezaki, N., Kikuchi, A., and Saito, T. (1996) J. Biol. Chem. 271, 6483–6489
Ohno, H., Stewart, J., Fournier, M. C., Bosshart, H., Rhee, I., Miyatake, S., Saito, T., Gallusser, A., Kirchhausen, T., and Bonifacino, J. S. (1995) Science 269, 1872–1875
Northrop, J. P., Ullman, K. S., and Crabtree, G. R. (1993) J. Biol. Chem. 268, 2917–2923
Wienands, J., Larbolette, O., and Reth, M. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 7865–7870
Osman, N., Turner, H., Lucas, S., Reif, K., and Cantrell, D. A. (1996) Eur. J. Immunol. 26, 1063–1066
Gauen, L. K. T., Zhu, Y., Letourneur, F., Hu, Q., Bolen, J. B., Matis, L. A., Klausner, R. D., and Shaw, A. (1994) Mol. Cell. Biol. 14, 3729–3741
Malissen, M., Gillet, A., Ardonin, L., Bouvier, G., Trucy, J., Ferrier, P., and Vivier, E. (1995) EMBO J. 14, 4641–4653
Shinkai, Y., Rathbun, G., Lam, K.-P., Oltz, U. M., Stewart, V., Mendelsohn, M., Charron, J., Datta, M., Young, F., Stall, A. M., and Alt, F. W. (1992) Cell 68, 855–867
Kuwabara, I., Ohno, H., Punt, J. A., Hashimoto, Y., and Saito, T. (1994) J. Immunol. 152, 2148–2156