Proteome Analysis Reveals Caspase Activation in Hyporesponsive CD4 T Lymphocytes Induced in vivo by the Oral Administration of Antigen

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The oral administration of antigen can lead to systemic antigen-specific hyporesponsiveness, also known as oral tolerance. This phenomenon is a representative form of immune tolerance to exogenous antigen under physiological conditions. We have previously reported that long term feeding of dietary antigen to ovalbumin-specific T cell receptor (TCR) transgenic mice induced oral tolerance of peripheral T cells with impairment in their TCR-induced calcium-signaling pathway. In this study, we utilized two-dimensional electrophoresis to compare intracellular protein expression patterns of orally tolerant and unsensitized CD4 T cells. We detected 26 increased and 16 decreased protein spots and identified 35 of these by mass spectrometry. The results indicated that the expression of caspases was up-regulated and that the protein levels of intact proteins susceptible to caspase cleavage, such as Grb2-related adaptor downstream of Shc (GADS), were decreased in orally tolerant CD4 T cells. Western blotting experiments confirmed that expression of the active form of caspase-3 and the antiapoptotic factor, X-linked inhibitor of apoptosis, were both up-regulated in orally tolerant CD4 T cells, which were found to be nonapoptotic. We further demonstrated that orally tolerant CD4 T cells could not form normal TCR signaling complexes associated with GADS and showed down-regulated phospholipase C-γ1 activation, which is likely to contribute to the impairment of TCR-induced calcium signaling. Our findings indicate that orally tolerant CD4 T cells up-regulate caspase activation and show decreased levels of caspase-targeted proteins, including TCR signaling-associated molecules, while up-regulating antiapoptotic factors, all of which appear to contribute to their unique tolerant characteristics.

The oral administration of antigen (Ag) can lead to systemic Ag-specific hyporesponsiveness, also known as oral tolerance. This phenomenon is thought to prevent hypersensitivity to ingested antigen (1, 2) and is a representative form of peripheral tolerance to exogenous Ag under physiological conditions (3). In addition, it is believed to be an effective therapeutic tool for the treatment of autoimmune diseases (4, 5). As in the case of other forms of tolerance, oral tolerance is mediated at the T cell level (6, 7). Three mechanisms are well documented for oral tolerance induction: (a) Ag-specific T cell clonal deletion (8, 9), (b) functional unresponsiveness of T cells to Ags (anergy) (10–13), and (c) active suppression mediated by regulatory T cells that produce immunosuppressive cytokines, such as transforming growth factor-β and interleukin (IL)-10 (14–17). However, the precise molecular mechanisms underlying the induction of oral tolerance remain unclear.

Previous studies demonstrated that Ag-specific T cell hyporesponsiveness was caused by defects in intracellular signaling events from the T cell receptor (TCR) and co-stimulatory molecules, such as incomplete protein phosphorylation (18–21) and impaired calcium/nuclear factor of activated T cells (NFAT) signaling (22). Furthermore, genetic experiments, such as DNA array methods, have recently been performed to analyze hyporesponsive T cells entirely from multidirectional points (23–25). However, to date, there are no detailed studies dealing with tolerant T cells examining total specific protein expression levels. In the postgenomic era, proteome analysis is expected to be the bridge between the genomic sequence and protein characteristics underlying cellular behavior, since translated proteins can be posttranslationally modified (26–28). The development of two-dimensional electrophoresis (2-DE) provides the high resolution of complex protein mixtures with high reproducibility. In addition, the combination of 2-DE, modern mass spectrometry (MS) technology, and rapidly accumulating genomic sequence data permits accurate and speedy identification of cellular proteins. With these advances, proteome analysis can be utilized in the research of intracellular protein changes in cells following a certain stimulus.

We have previously reported that long term feeding of dietary Ag to ovalbumin (OVA)-specific TCR transgenic mice (OVA23-3 mice) induced oral tolerance of peripheral T cells (29). In this experimental model, orally tolerant T cells showed impaired calcium/NFAT signaling following TCR-mediated activation, despite normal activation of the mitogen-activated propidium iodide; XIAP, X-linked inhibitor of apoptosis; GADS, Grb2-related adaptor downstream of Shc; SLP-76, Src homology 2 domain-containing leukocyte protein of 76 kDa; LAT, linker for activation of T cells; PLC-γ1, phospholipase C-γ1; APC, antigen-presenting cell; IFN-γ, interferon-γ; IAP, inhibitor of apoptosis; FITC, fluorescein isothiocyanate; CHAPS, 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonic acid; Pipes, 1,4-piperazinediethanesulfonic acid; MALDI-TOF, matrix-assisted laser desorption/ionization time-of-flight.

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kinase pathway. In this study, we used oral tolerance-induced OVA23-3 mice to investigate the expression of intracellular proteins using 2-DE to analyze the specific characteristics of in vivo orally tolerized splenic CD4 T cells compared with unsensitized CD4 T cells.

EXPERIMENTAL PROCEDURES

Mice—8–10-week-old BALB/c male mice were purchased from Clea Inc. (Tokyo, Japan). Male homozygous for the OVA peptide 232–339/9-Aβ, restricted TCR transgene from the T cell clone 7-3-7 on the BALB/c background (OVA23-3 mice) were produced as previously described (30). All studies were performed according to the Guidelines for Animal Experimentation of the Faculty of Agriculture (University of Tokyo).

OVA-Specific Oral Tolerance Induction in OVA23-3 Mice—6–8-week-old OVA23-3 mice were orally administered with dietary Ag containing 20% hen egg white (egg white diet; Funabashi Farm, Funabashi, Japan) or 1.0 mg/ml OVA in flat bottom 96-well plates and 4 mouse TCR CD45R/B220 mAb (RA3-6B2), and phycoerythrin-conjugated anti-flow cytometric analyses, FITC- or allophycocyanin-conjugated anti-CD11c mAb, and biotinylated anti-mouse CD45R/B220 mAb. After washing and secondary labeling with anti-FITC microbeads, cells were subjected to positive enrichment via the magnetic column. After release of 2-DE, gels were silver-stained as previously described (32). Stained gels were imaged with Gel dock (Bio-Rad), and spot intensity was measured using ImageGauge software (Fuji Photo Film, Kanagawa, Japan). Normalized spot intensities were calculated from more than 10 spots of each gel.

Protein Identification by MALDI-TOF MS—In-gel tryptic digestions of excised protein spots were performed with modified protocols as previously described (33). Briefly, destained gels were reduced and alkylated, followed by tryptic digestion (Promega, Madison, WI). Digested peptides were eluted with 5% formic acid, 50% acetonitrile. Peptides were concentrated and desalted using ZipTipC18 (Millipore Corp., Bedford, MA) according to the manufacturer’s protocol. Purified peptides were mixed with 10 mg/ml α-cyano-4-hydroxycinnamic acid, 0.2% aqueous trifluoroacetic acid, acetonitrile (1:1) as a matrix. The mass spectra were recorded by Voyager-DE STR (Applied Biosystems, Foster City, CA). Internal calibration was performed using autoproto-lytic trypsin peptide fragments of 842.50 and 2211.1046 Da, and specific peaks were applied to the MS-fit program (available on the World Wide Web at protospect.ucsf.edu/ucafitml4.0/msfit.html). Proteins were identified with 50 ppm accuracy and a minimum of four matching peptides and 25% peptide coverage of total amino acids.

Stimulation of CD4 T Lymphocytes with Abs—Purified CD4 T cells were stimulated with cross-linking of TCR and CD4 molecules with Abs for the indicated times as previously described (28).

Immunoprecipitation and Western Blotting—For Western blots of the pro form and active form of caspase-3, harvested cells were lysed in CHAPS cell extract buffer (50 mM Pipes/KOH, pH 6.5, 0.5% CHAPS, 2 mM EDTA, 5 mM dithiothreitol, protease inhibitor), and detections were performed using an apoptosis sampler kit (Cell Signaling Technology, Beverly, MA) according to the manufacturer’s instructions. Purified proteins were eluted in 0.5% Nonidet P-40 lysis buffer for immunoprecipi- tation of stimulated CD4 T cells and in PLC lysis buffer (50 mM Heps- NaOH, pH 7.5, 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1.5 mM MgCl2, 1 mM EDTA, protease inhibitor) for Western blot of whole cell lysate. Western blot of phospho c-Jun was performed as described in Ref. 29. Other Western blots were performed as follows. Immunoblots were blocked with 5% skim milk in TBS-Tween for 1 h, followed by probing with primary antibody for 1 h at room temperature or overnight at 4 °C. After washing with TBS-Tween three times, immunoblots were incubated with secondary antibody for 1 h. Immunoblots were then washed with TBS-Tween three times, and detections were performed with ECL (Amersham Biosciences, Arlington Heights, IL).

Early Apoptotic and Dead Cell Detection by Fluorescence-activated Cell Sorting—Spleno-cytes were enriched for CD4-positive cells with anti-CD4 microbeads and magnetic separation columns, according to the manufacturer’s recommended protocol. Cells were then labeled with FITC-conjugated annexin V (Roche Applied Science), PE, and APC-conjugated anti-mouse CD4 mAb according to the manufacturer’s in- structions. Their staining profiles were analyzed by fluorescence-acti- vated cell sorting LSR and CellQuest software (BD Biosciences, Mountain View, CA).

DNA Fragmentation Assay—Triplicate 4 × 106 purified T cells were subjected to a DNA fragmentation assay with diphenylamine reagent as previously described in Ref. 34.

RESULTS

Orally Tolerant CD4 T Lymphocytes Are Induced by Long Term Ag Administration—To obtain orally tolerant splenic CD4 T cells, OVA23–339-specific TCR transgenic mice (OVA23-3) were fed with dietary Ag (20% egg white diet) for 4 weeks. As shown in our previous study (29), these CD4 T cells exhibited reduced proliferation and reduced IL-2, IL-4, and IFN-γ cytokine production when incubated with OVA and APCs in vitro (Fig. 1, A and B). These characteristics of CD4 T cells are evident after 4 consecutive weeks of oral administration of dietary Ag (data not shown). One known mechanism of tolerance induction is through the active suppression of regula-
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Fig. 1. Long term oral administration of egg white diet induces splenic CD4 T cell tolerance. Splenic CD4 T cells from control and egg white diet-fed OVA23-3 mice were stimulated in vitro with OVA in the presence of APCs, and proliferative responses and cytokine production were measured. A, proliferative responses between 48 and 72 h after stimulation were measured. The data are shown as the average from triplicate cultures ± S.D. B and C, culture supernatants were recovered and pooled from triplicate cultures after 24-h incubation for IL-2 and 48-h incubation for IL-4, IFN-γ, and IL-10. The detection limits of IL-2, IL-4, IFN-γ, and IL-10 were 156, 32, 540, and 63 pg/ml, respectively. Data represent the results from at least three independent experiments. *, not detected.

In Vivo Protein Expression Analysis of Orally Tolerant CD4 T Lymphocytes with 2-DE—To characterize protein expression levels of orally tolerant CD4 T cells, we compared these cells with unsensitized CD4 T cells obtained from control diet-fed OVA23-3 mice by 2-DE. We used whole cell lysates to represent the in vivo state of cellular proteins and to make an accurate comparative study. We also utilized narrow range immobilized pH gradient gels for the first dimension electrophoresis to obtain high resolution separation. Fig. 2A shows silver-stained two-dimensional separated gels from both control and orally tolerant CD4 T cells. The arrowheads show the up-regulated protein spots when the two gels were compared. We also used luminescent staining, which is known to deliver a wide quantitation range (35), but could not produce the same sensitivity as observed using silver staining (data not shown). In relation to the detected spots, similar results were obtained when comparing spot intensities between gels. We detected 42 changed spots between pH 4.5 and 6.7, of which 26 were increased and 16 decreased for orally tolerant CD4 T cells. Of the differentially expressed spots, we identified 35 by peptide mass fingerprinting. Table I shows the peptide mass fingerprint results, which contain experimental and theoretical pl and molecular weights, NCBI protein accession number, the number of matching peptides, and the amino acid coverage of the obtained peptides. The -fold induction of the protein spots (orally tolerant versus control CD4 T cells) are also shown. There were some unidentified spots that had no matching proteins in the data base to date, although we could identify specific digested peptide peaks, or in some cases, the amount of protein expressed was too little to be precisely identified by this method.

Caspase Activities in Orally Tolerant CD4 T Lymphocytes—Up-regulated proteins in orally tolerant CD4 T cells included both caspase-1 and caspase-3. The caspases are a family of cysteine aspartic acid proteases that play a central role in the regulation of apoptosis (36). Activation of members of the caspase family requires their cleavage; for example, the pro form of caspase-3 (32 kDa) is cleaved into its active form (17 or 20 kDa) (37). Furthermore, the effector caspases are responsible for the cleavage of target proteins. In our results of 2-DE gels, some of the proteins identified with decreased expression levels in orally tolerant CD4 T cells have been reported to undergo caspase-dependent cleavage: i.e. STE20-like kinase MST-1, which promotes apoptosis after cleavage (38, 39), and GADS (40, 41). In addition, actin is cleaved into 40-, 31-, 29-, and 15-kDa polypeptides by caspase-1 and -3 (42, 43), and we found that the 31- and 29-kDa cleaved forms of actin were increased in orally tolerant CD4 T cells (Table I). These findings also suggest that active caspase-3 or -1 was up-regulated in orally tolerant CD4 T cells, although we only detected the pro form of caspase-3 or -1 up-regulation in 2-DE gels. Therefore, we next analyzed caspase-3 cleavage by Western blotting. As expected, the pro form and active form of caspase-3 were increased in orally tolerant CD4 T cells (Fig. 3A). These results suggest that caspases play an important role in orally tolerant CD4 T cells.

Although the activation of caspases contributes to apoptosis, there are some reports that caspase-3 activation occurs in nonapoptotic T cells (44–46). We therefore examined whether orally tolerant CD4 T cells underwent apoptosis. As shown in Fig. 3B, very few apoptotic (annexin V-positive) and necrotic (PI-positive) cells were identified in both control and orally tolerant CD4 T cells. In addition, purified CD4 T cells that were subjected to 2-DE and Western blots exhibited negligible levels of DNA fragmentation (Fig. 3C). These results indicated that orally tolerant CD4 T cells, which were nonapoptotic, up-regulated caspase activation and suggested the possibility that orally tolerant CD4 T cells augmented the expression of anti-apoptotic proteins, such as caspase suppressors. One of the known endogenous caspase inhibitor protein families is the inhibitors of apoptosis (IAPs) protein family (47). XIAP is the most potent of the IAPs and is restricted to targeting caspase-9 (48). We therefore speculated that XIAP aids in maintaining the viability of orally tolerant CD4 T cells.

Caspases in TCR Signaling Impairment of Orally Tolerant CD4 T Lymphocytes—We previously reported that orally tolerant CD4 T cells in this experimental system exhibited impairment in the NFAT/calcium signaling pathway (29). Since the 2-DE studies of orally tolerant CD4 T cells showed degradation of intact GADS, which is involved in proximal TCR signaling (49), we proposed that the caspase-dependent cleavage of TCR signaling components contributed to signaling impairment. To confirm whether GADS was cleaved by caspase-3, Western blot analysis was performed. As predicted, the 26-kDa caspase-3-

[Image of graph and table]
dependent cleaved GADS (40) was detected in orally tolerant CD4 T cells (Fig. 4A). We then investigated GADS-associated molecules in stimulated CD4 T cells by immunoprecipitation with anti-GADS pAb. Fig. 4B shows the tyrosine phosphorylation of GADS-associated molecules. The TCR-induced calcium/NFAT pathway is critically initiated by SLP-76 and PLC-γ1.
Orally tolerant CD4 T cells and unsensitized CD4 T cells were lysed and subjected to 2-DE (Fig. 2). Each spot was identified by in-gel tryptic digestion and MALDI-TOF MS analysis. Protein spots were normalized with more than 10 spots of each gel, quantified, and then compared. Protein spot-fold induction was calculated as egg white diet-fed CD4 T cells/control diet-fed CD4 T cells. Spot numbers are shown in Fig. 2. Each identified protein shows protein spot-fold induction, name, NCBI protein accession number (NCBI accession no.), theoretical pl (Theor. pl), theoretical molecular weight (Theor. MW), experimental pl (Ex. pl), experimental molecular weight (Ex. MW), the number of peptide peak matches by MS (Peptide matches), and the percentage of amino acids coverage by matching peptides (A.A. coverage). Protein spot-fold inductions were shown at >1.30 and <0.76. Unidentified proteins showed no matched proteins on the data base to date or too little expression to be precisely identified.

### Table I

| Spot number | Protein spot-fold induction | Identified protein | NCBI accession no. | Theor. pl | Theor. MW | Ex. pl | Ex. MW | Peptide matches | A.A. coverage |
|-------------|---------------------------|--------------------|-------------------|-----------|---------|-------|-------|----------------|---------------|
| 1           | 4.44                      | Lipocortin 1 (Annexin I) | 113945            | 6.6       | 38,692  | 6.5   | 39    | 8             | 31            |
| 2           | 4.05                      | Caspase-3 (CPP-32)   | 2489327           | 6.5       | 31,475  | 6.4   | 32    | 7             | 31            |
| 3           | 3.99                      | Ela                 | 1284891           | 5.9       | 42,575  | 5.7   | 40    | 14            | 27            |
| 4           | 3.41                      | Annexin VI          | 113963            | 5.3       | 75,887  | 5.3   | 70    | 11            | 28            |
| 5           | 3.39                      | Unidentified        |                   |           |         |       |       |                |               |
| 6           | 2.96                      | Statmin (Phosphoprotein p19) | 1711560          | 5.8       | 17,275  | 5.4   | 17    | 5             | 26            |
| 7           | 2.79                      | Unidentified        |                   |           |         |       |       |                |               |
| 8           | 2.63                      | Secretory protein precursor (YM-1) | 11140877        | 5.4       | 44,459  | 5.4   | 45    | 10            | 32            |
| 9           | 2.56                      | Caspase-1 (Interleukin-1β-converting enzyme) | 266322           | 5.7       | 45,641  | 5.7   | 49    | 11            | 25            |
| 10          | 2.54                      | RIKEN cDNA 1110010E24 gene | 18043446          | 5.1       | 46,853  | 5.1   | 46    | 9             | 29            |
| 11          | 2.41                      | Protein for MGC:37456 | 1935398           | 5.2       | 36,084  | 5.2   | 45    | 15            | 52            |
| 12          | 2.38                      | Carbonyl dehydratase (EC 4.2.1.11) II | 19745184         | 6.5       | 29,035  | 5.8   | 29    | 9             | 37            |
| 13          | 2.35                      | Actinα              | 71619             | 5.3       | 41,737  | 5.3   | 31    | 5             | 27            |
| 14          | 2.04                      | Unidentified        |                   |           |         |       |       |                |               |
| 15          | 1.89                      | Δ-aminolevulinate acid dehydratase | 122834           | 6.3       | 36,024  | 6.1   | 36    | 6             | 28            |

**a** Actin is predicted caspase-cleaved fragment, described in (Ref. 43). Amino acid coverage means the coverage of predicted fragment.

PLC-γ1 activity is regulated by tyrosine phosphorylation (50) and association with LAT (51) and SLP-76 (52). We found that GADS-associated SLP-76 and GADS-LAT-SLP-76-associated PLC-γ1 (53) were decreased in both phosphorylation and association in orally tolerant CD4 T cells. Most notably, the association between GADS and SLP-76 was dramatically disrupted. In addition, the phosphorylation of TCR-ζ chain and LAT was reduced in orally tolerant CD4 T cells. We have previously described the reduced phosphorylation of TCR-ζ chain and PLC-γ1 independent of their abilities to associate with GADS (29), so we further analyzed the phosphorylation of SLP-76 and LAT. As shown in Fig. 4C, the phosphorylation of SLP-76 and LAT was reduced in orally tolerant CD4 T cells. The protein levels of LAT and PLC-γ1 were not altered in both populations (data not shown), whereas SLP-76 was slightly decreased in orally tolerant CD4 T cells (Fig. 4D). It has also been implied that SLP-76 undergoes caspase-dependent cleavage (40), so we investigated caspase-dependent cleavage of SLP-76. As shown in Fig. 4D, SLP-76 was cleaved in orally tolerant CD4 T cells. These results suggest that the caspase-dependent cleavage of GADS and SLP-76 inhibit the associations that are formed by these molecules, thus contributing to TCR signaling impairment in orally tolerant CD4 T cells.

**DISCUSSION**

Here we report the proteome analysis of CD4 T cells tolerated *in vivo* by the oral administration of Ag. Although there are many published reports aiming to clarify tolerant T cell characteristics using multiple experimental procedures, this is, to our knowledge, the first report of a proteome analysis of *in vivo* tolerant CD4 T cells. Our results are not totally consistent with results from previous studies of tolerant T cell gene expression and function.
obtained from DNA array analyses (23, 25). However, we have obtained new insights into the mechanisms of tolerance by studying post-translational protein processing in orally tolerant CD4 T cells.

In this experimental model of oral tolerance induced by long term feeding of dietary Ag, splenic CD4 T cells exhibited a lack of proliferative ability and reduced cytokine production (Fig. 1, A and B). These hyporesponsive CD4 T cells therefore resembled previously described anergic T cells. Since immunosuppressive cytokine production was not detected in this model (Fig. 1C) (29), the induction of oral tolerance under these conditions is not considered to be caused by active suppression but rather by clonal deletion or anergy. Recently, the suppressive function of CD25+ CD4 T cells has been demonstrated in oral tolerance (17, 54). The CD4 T cells from orally tolerant mice in our system contained CD25+ CD4 T cells (29); however, the decrease of these cells did not affect the response of CD4 T cells from the egg white diet-fed mice (29). Thus, the reduced response of CD4 T cells after Ag feeding was not mediated by CD25+ CD4 T cells. In our system, these CD25+ CD4 T cells themselves are in a hyporesponsive state (17, 54). The observation that we could not find any evidence of active suppression in our system is consistent with previous studies examining Ag dose-dependent oral tolerance (55, 56); low dose Ag preferentially induces tolerance mediated by regulatory T cells, whereas high dose Ag deletes or anergizes CD4 T cells (8, 11, 31, 35–39). Our two-dimensional gel electrophoresis studies (Fig. 2, Table I) reflected the in vivo state directly and revealed that caspase-dependent protein cleavages were frequently occurring in orally tolerant CD4 T cells. Although these findings suggest that orally tolerant CD4 T cells underwent clonal deletion by apoptosis, most of them were nonapoptotic when freshly isolated (Fig. 3, B and C). Our model thus explains the link between clonal anergy and clonal deletion. Previous studies suggest that caspase activation is involved in the early steps of nonapoptotic T cell activation (46). In cases when caspase activation does not result in apoptosis, antiapoptotic factors, such as IAPs, may rescue cells from apoptosis (60). We observed an increase of XIAP in orally tolerant CD4 T cells (Fig. 3D). These T cells may have acquired resistance to apoptosis and therefore survived deletion, most likely by activation-induced cell death, by expressing antiapoptotic factors, which result in an anergic-like state. Indeed, the number of splenic CD4 T cells in transgenic mice decreased significantly in the first 3 weeks of egg white diet feeding; however, the decrease from 3 weeks onward was slight, which suggests that extensive deletion occurs mainly in the early stages of Ag feeding (data not shown). Consequently, orally tolerant CD4 T cells appear to maintain the subtle balance between their survival and apoptosis by adjusting pro- and antiapoptotic proteins.

With regard to TCR signaling impairment of orally tolerant CD4 T cells, we have previously reported that the calcium/NFAT pathway is abnormal in these cells despite normal activation of the mitogen-activated kinase pathway (29). In this report, we showed intact GADS degradation and cleavage in orally tolerant CD4 T cells (Table I, Fig. 2, and Fig. 4A), whereas GADS mRNA levels are not significantly altered (data not shown). GADS is a hematopoietic adaptor protein and plays an important role in proximal TCR signaling by LAT and the SLP-76 (49, 61–64) and is shown to undergo caspase cleavage. Our results showed the appropriate molecular weight of the predicted GADS cleavage product in orally tolerant CD4 T cells (Fig. 4A). We also detected in the 2-DE comparative study another hematopoietic-restricted scaffold protein, Grb2-related adaptor protein (Grap) (65), which belongs to the Grb2 family and regulates TCR signaling as does GADS (64, 66) but contains no caspase-cleaved site (40). In contrast to GADS, Grap was expressed at the same level in both populations of CD4 T cells (data not shown). These results strongly suggest that degradation of intact GADS and SLP-76 molecules is caused by selective caspase-dependent protein cleavage. GADS is known to undergo caspase cleavage at amino acid sequences between its Src homology 2 domain, which associates with LAT, and C-terminal Src homology 3 domain, which associates with SLP-76 (40, 41). It has been reported that GADS caspase-3-cleaved products also have binding capacity to these molecules and perturb TCR signaling by SLP-76 and LAT, leading to impairment of NFAT activation (40, 41). Furthermore, SLP-76 cleavage products may also perturb TCR signaling. Thus, it is possible that GADS and SLP-76 cleavage products may inhibit TCR signaling in orally tolerant CD4 T cells.

Yankee et al. (41) demonstrated that FAS signaling leads to GADS cleavage, which subsequently leads to impairment in the GADS-SLP-76 complex. Our results clearly showed that the formation of the TCR signaling complex involving GADS was defective in in vivo induced nonapoptotic orally tolerant CD4 T cells, suggesting that caspase-dependent cleavage of GADS and SLP-76 is one of the causes (Fig. 4). These caspase-dependent defects in the assembly of TCR signaling molecules may cause the reduced phosphorylation of LAT, SLP-76, and PLC-γ1. However, it is also possible that the abilities of these molecules to undergo phosphorylation are altered independent of GADS association in orally tolerant CD4 T cells by other mechanisms (Fig. 4C). In any case, we demonstrated that PLC-γ1 activity is...
regulate caspase activation and show a selective decrease in the levels of caspase-targeted proteins and that antiapoptotic factors were concomitantly up-regulated. Furthermore, the cleavage of key TCR signaling molecules by caspases appears to result in defects in the TCR signaling. Conclusively, our results suggest that orally tolerant CD4 T cells undergo caspase activation to maintain their unique tolerant characteristics.

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