Identification of the genes specifically expressed in orally tolerized T cells

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Abstract

Oral tolerance is the systemic immunological unresponsiveness that occurs after feeding protein antigens. Its physiological role is thought to be the prevention of hypersensitivity to food antigens, and its therapeutic use to treat inflammatory diseases has been suggested. Although it has been shown that CD4⁺ T cells mediate oral tolerance, the precise molecular mechanisms remain unclear. In the present study, we employed suppression subtractive hybridization and identified 10 genes specifically expressed in orally tolerized T cells. These included genes that were interesting in terms of their putative functions in the negative regulation of T cell activation, e.g. Culin 1, LAX, and Zfhx1b, as well as four genes that encoded unknown proteins. We further investigated the expression of these genes in hyporesponsive T cells induced *in vitro* (*in vitro* anergized T cells). We found that six of the 10 genes were highly expressed in these cells, and kinetic studies suggested that one was associated with the induction of anergy, while the other five were associated with the maintenance of anergy. The remaining 4 genes that were not expressed in *in vitro* anergized T cells are also of interest as they may play a specific role in *in vivo* T cell tolerance. Functional analysis of these genes should help to understand the complex mechanisms underlying the induction and maintenance of oral tolerance, and moreover, *in vivo* immune tolerance in general.

Abbreviations: Ag – antigen; APC – antigen presenting cell; CFA – complete Freund's adjuvant; IL – interleukin; mAb – monoclonal antibody; OVA – ovalbumin; TCR – T cell receptor

Introduction

Oral tolerance is the systemic immunological unresponsiveness that occurs after feeding protein antigens. The physiological role is thought to be prevention of hypersensitivity to food antigens (Ags), but also a number of models of experimental immunopathology can be inhibited by feeding Ags (Weiner 1997). Therefore, it is believed

that this approach could be used therapeutically to treat inflammatory diseases (Strobel and Mowat 1998; Faria and Weiner 1999; Garside and Mowat 2001). It has been shown that CD4⁺ T cells play an important role in oral tolerance. Three mechanisms have been proposed to be involved in the induction of tolerance: (1) unresponsiveness of T cells to Ags (anergy) (Whitacre et al. 1991; Malamed and Friedman 1993), (2) immune suppression

by regulatory T cells that produce immunosuppressive cytokines such as transforming growth factor β (TGF- β) or interleukin 10 (IL-10) (active suppression) (Chen et al. 1994; Tsuji et al. 2001), and (3) elimination of Ag-specific T cells *via* apoptosis (clonal deletion) (Chen et al. 1995; Garside et al. 1996). However, the precise molecular mechanisms underlying the induction of oral tolerance are still unclear.

Previous studies have demonstrated defects in intracellular signaling mediated by the T cell receptor (TCR) in hyporesponsive T cells. Upon TCR stimulation, these T cells show incomplete protein phosphorylation (Li et al. 1996; Fields et al. 1996; Utting et al. 2000) and impaired nuclear translocation of transcription factors for cytokine genes (Mondino et al. 1996; Sundstedt et al. 1996). We have studied characteristics of TCR-mediated signaling in orally tolerized T cells. We used ovalbumin (OVA)-specific TCR transgenic mice and induced tolerance of peripheral T cells by feeding high doses of OVA. In this model, orally tolerized T cells showed impaired calcium/NFAT signaling upon TCR stimulation, but normal activation of the mitogen-activated kinase pathway (Asai et al. 2002). Further, we have found that orally tolerized T cells up-regulate caspase activation and show decreased levels of caspase-targeted proteins, such as Grb2-related adaptor downstream of Shc (GADS), which is an important adaptor molecule in TCR signaling (Kaji et al. 2003).

In addition to such signaling studies, genetic analyses have recently been performed to identify the genes associated with the induction and maintenance of the hyporesponsive state of T cells. It has been reported that the expression levels of cyclin-dependent kinase inhibitors (p27^{Kip1} and p21^{Cip1}) are up-regulated in anergic T cells, and that anergic T cells are arrested in the G1 phase of the cell cycle (Boussiontis and Freeman 2000; Jackson et al. 2001). Tob is an anti-proliferative protein which associates with Smad2 and Smad4 and enhances their binding to DNA (Tzachanis et al. 2001). Tob is highly expressed in anergic T cells and inhibits IL-2 production by T cells. The inhibitory effect of Tob on IL-2 transcription is mediated by enhancement of Smad binding on negative regulatory element of the IL-2 promoter. Another report has shown that GRAIL, as an E3 ubiquitin ligase, is a key player in the induction of

the anergic phenotype of CD4⁺ T cells (Anandasabapathy et al. 2003). GRAIL is also expressed in anergic T cells and introduction of GRAIL into T cells dramatically inhibits IL-2 production. All of these genes are found to be expressed in *in vitro* anergized T cells. The relevance of these genes with *in vivo* tolerized T cells has not been addressed and few studies have explored gene expression profiles of *in vivo* tolerized T cells.

Thus, we employed suppression subtractive hybridization to identify the genes specifically expressed in orally tolerized T cells. In this report, we describe the identification of 10 genes that are specifically induced in orally tolerized T cells.

Materials and methods

Animals

BALB/c mice were purchased from Clea Japan (Tokyo, Japan). I-Ad-restricted OVA323-339 specific TCR transgenic mice (DO11.10) (Murphy et al. 1990) were maintained in our animal facility.

Peptide, Ab, and cytokine

OVA 323-339 (ISQAVHAAHAEINEAGR) was purchased from Mitotopes Pty. Ltd. (Clayton, Victoria, Australia). Anti-CD3 monoclonal antibody (mAb) (145-2C11), anti-IL4 mAb (11B11), and anti-clonotype mAb (KJ1.26; a mAb that recognizes the TCR encoded by the transgene of DO11.10 mice) were prepared from hybridoma-derived ascites and purified by protein G (Pharmacia, Uppsala, Sweden). Purified KJ1.26 was FITC-conjugated. Recombinant mouse IL-12 was purchased from R&D (Minneapolis, MN).

Induction of oral tolerance and immunization

DO11.10 mice were orally administered with dietary Ag containing 20% hen egg white (egg white diet; Funabashi Farm, Funabashi, Japan) for 28 days. In some experiments, oral tolerance was induced by an alternative method; i.e. OVA (100 mg/ml) (Wako, Tokyo, Japan) was administered to DO11.10 TCR transgenic mice in

their drinking water for 7 days. Control mice were immunized intraperitoneally with 0.1 mg of OVA emulsified in 0.1 ml of complete Freund's adjuvant (CFA) 10 days before sacrifice.

Preparation of T cells and antigen presenting cells (APCs)

CD4⁺ T cells were purified from spleens of DO11.10 mice by positive selection using MACS anti-CD4 microbeads (Miltenyi Biotech, Bergish Gladbach, Germany). Splenic CD4⁺ KJ1.26⁺ T cells were purified by positive selection using FITC-conjugated anti-clonotype mAb (KJ1.26), anti-FITC multisort kit (Miltenyi Biotech), and anti-CD4 microbeads (Miltenyi Biotech). Splenocytes of BALB/c mice were used as antigen presenting cells (APCs) after treatment with 50 µg/ml mitomycin C (Sigma–Aldrich, St. Louis, MO).

In vitro anergy induction

CD4⁺ T cells (1×10^6 /well) from spleens of DO11.10 mice were cultured with 0.5 µM OVA323-339 and APCs (2×10^6 /well) in the presence of anti-IL-4 mAb (5 µg/ml) and rIL-12 (2 ng/ml) in 24-well plates for 7 days to induce the development of Th1 cells. The collected cells (1×10^6 /well) were cultured with or without plate-coated anti-CD3 (10 µg/ml) in 24-well plates for 48 h. Cells were harvested and cultured for additional 48 h with medium alone.

Measurement of cytokine secretion and T cell proliferation

T cells (5×10^4 /well) were cultured with APCs (2×10^5 /well) in the presence or absence of 0.1 µM OVA323-339 in 96-well plates for 96 h. The culture supernatants were recovered after 24 h for assaying IL-2. The cytokine concentration was determined by measuring by ELISA as described previously (Hashiguchi et al. 2000). Proliferation was investigated by measuring the incorporation of [³H] thymidine (1 µCi/well) (ICN Pharmaceuticals, Costa Mesa, CA) during the final 24 h of culture.

Suppression subtractive hybridization

Preparation of total RNA was performed using ISOGEN (NIPPON GENE, Tokyo, Japan). cDNA synthesis was performed using SMARTTM PCR cDNA Synthesis Kit (Clontech, Palo Alto, CA). cDNA from orally tolerized CD4⁺ KJ1.26⁺ T cells was used as 'tester' and cDNA from control CD4⁺ KJ1.26⁺ T cells as 'driver' cDNA. Suppression subtractive hybridization was performed with the use of the PCR-select cDNA subtractive kit (Clontech, Palo Alto, CA), according to the manufacturer's protocol. Subtracted cDNAs were ligated into the pGEM-T Easy Vector (Promega, USA) and a subtracted cDNA library was obtained. Then, differential screening with the PCR-select differential screening kit (Clontech) was performed to eliminate false-positive clones. The confirmed positive clones were sequenced and examined for their differential expression by quantitative RT-PCR.

Quantitative RT-PCR

Preparation of total RNA and reverse transcription were performed as described previously (Ise et al. 2002). The Light Cycler PCR and real-time detection system (Roche Diagnostics GmbH, Mannheim, Germany) was used for amplification and quantification. The pairs of primers used were as follows: LAX sense, 5'-AGTAACCCAGC-CCCGCATTTCTTC-3'; LAX antisense, 5'-TTC-AGGAGTGCTTTCTATGC-3'; NAPG sense, 5'-CCCACTAAGAGTGTGAGCAG-3'; NAPG antisense, 5'-CGTCTCCTCAGTCAGACATC-3'; Emerin sense, 5'-AGCAGCTCTTACCAGGA-GTT-3'; Emerin antisense, 5'-AGCATTGCCTT-AGCTAGACC-3'; Zfp36 sense, 5'-GGCTCCC-ACAATACTATCCT-3'; Zfp-36 antisense, 5'-TTTAGGAGGGAAATTTGAGC-3'; Culin 1 sense, 5'-CGCAGTTATCAGCGCGATTTAC-3'; Culin 1 antisense, 5'-AGTGACTAGTTCCCC-TTTGG-3'; Zfhx1b sense, 5'-GAAGAGACTG-GGGATCACTC-3'; Zfhx1b antisense, 5'-CAT-GCCATCTTCCATATTGT-3'; unknown gene 1 sense, 5'-CTGGAGTCCTACCTGTCTGA-3'; unknown gene 1 antisense, 5'-ACCCTTACA-CAAAGGGCTTA-3'; unknown gene 2 sense, 5'-TCTGTAATAATAAGCATGGAAATGA-3'; unknown gene 2 antisense, 5'-CACTTTACAA-

ACAGCCCCTA-3'; unknown gene 3 sense, 5'-ATTTGCATCAAGAAAAAGCA-3'; unknown gene 3 antisense, 5'-GATGCTAGCGCTCTCA-GATA-3'; unknown gene 4 sense, 5'-AATCATG-TCAGCAGCTATGG-3'; unknown gene 4 antisense, 5'-GGCAGAGACACTGAAACAAC-3'.

Results

Induction of oral tolerance in OVA-specific TCR transgenic mice

In this study, our aim was to identify genes that are specifically expressed in orally tolerized T cells. To obtain orally tolerized T cells, OVA specific TCR transgenic mice (DO11.10) were orally administered with dietary Ag containing 20% hen egg white for 28 days. Control DO11.10 mice were immunized intraperitoneally with OVA emulsified in CFA 10 days before sacrifice. Antigen specific CD4⁺ T cells (CD4⁺KJ1.26⁺ T cells) were purified from splenocytes of OVA-fed or control mice. CD4⁺KJ1.26⁺ T cells from mice fed OVA exhibited reduced proliferation and IL-2 production upon antigenic stimulation, compared with those from control mice (Figure 1), suggesting that oral tolerance was induced in this experimental system.

Identification of genes highly expressed in orally tolerized T cells by means of suppression subtractive hybridization

RNA from orally tolerized and control CD4⁺KJ1.26⁺ T cells were isolated. cDNA was prepared and subjected to suppression subtractive hybridization to identify the genes specifically expressed in orally tolerized T cells. We established a subtracted cDNA library and 3300 clones were subjected to differential screening to eliminate false-positives. As a result, 110 clones were selected to be positives. The sequences of these clones were analyzed.

Finally, we performed quantitative RT-PCR for these 110 clones to confirm their differential expression. We obtained orally tolerized CD4⁺ T cells from splenocytes of DO11.10 mice fed OVA in their drinking water for 7 days. As a control, antigen-specific CD4⁺ T cells were

Figure 1. Induction of oral tolerance in DO11.10 mice. DO11.10 mice were orally administered with dietary Ag containing 20% hen egg white for 28 days. As a control, DO11.10 mice were immunized with 100 μg of OVA intraperitoneally 10 days before sacrifice. CD4⁺KJ1.26⁺ T cells were purified from spleens of these mice and cultured with 0.1 μM OVA323-339 in the presence of APCs for 96 h. (A) Proliferation was investigated by measuring incorporation of ³H-thymidine during the last 24 h of culture. (B) IL-2 content in the culture supernatant after 24 h was determined by ELISA.

purified from spleens of untreated DO11.10 mice. As a result of quantitative PCR, 10 clones were confirmed that they are highly expressed in orally tolerized T cells (Table 1). Among the 10 clones, four clones encoded transcripts of unknown proteins. Of the six known clones, Culin1 is an E3 ubiquitin ligase subunit (Sakamoto et al. 2001), NAPG has been reported to play an essential role in vesicular transport (Nishiki et al. 2001), Zfhx1b is a transcriptional repressor as a smad interaction protein (Verschuere et al. 1999) and Zfp36 is a Zinc finger protein (Taylor et al. 1995). LAX has been recently described as an inhibitor of TCR-mediated p38 MAPK activation and NFAT/AP-1 transcriptional activation (Zhu et al. 2002), and Emerin is a

Table 1. The genes identified in this study as oral tolerance-associated genes

Gene name	Fold induction ^a	Putative function
Unknown gene 3 ^b	4.4	
Unknown gene 2 ^b	2.8	
Unknown gene 4 ^b	2.4	
Culin1	2.3	The E3 ubiquitin ligase subunit
NAPG	2.2	Essential role in vesicular transport
Zfhx1b	2.2	Transcriptional repressor as a Smad interaction protein
Unknown gene 1 ^b	2.0	
Zfp36	1.9	Zinc finger protein
LAX	1.9	Inhibition of TCR mediated p38 activation and NFAT/AP-1 activation
Emerin	1.8	Lamin-binding nuclear membrane protein:relates to cell cycle

^aRatio of expression level in orally tolerized T cells to that in control T cells.

^bThe genes encode unknown proteins.

lamin-binding nuclear membrane protein (Fairley et al. 2002).

Expression of genes associated with oral tolerance in in vitro anergized T cells

Most studies to identify genes associated with the hyporesponsive state of T cells have been conducted in *in vitro* anergy-induction systems (Tzachanis et al. 2001; Anandasabapathy et al. 2003). There is a possibility that the mechanism underlying induction and maintenance of T cell hyporesponsiveness is different between *in vivo* and *in vitro* systems. Therefore, we investigated whether the genes highly expressed in orally tolerized T cells were also expressed in *in vitro* anergized T cells.

Naïve CD4⁺ T cells from DO11.10 mice were cultured with Ag and APCs in the presence of anti-IL-4 plus rIL-12 for 1 week to drive them into Th1 cells. Recovered Th1 cells were stimulated with plate-coated anti-CD3 for 48 h to induce anergy. Cells were harvested and rested for additional 48 h. Anergy was successfully induced since proliferation and IL-2 production upon Ag stimulation were significantly inhibited (Figure 2). RNA was prepared at 6, 24, and 48 h after anti-CD3 stimulation, and after resting culture (96 h). Then, quantitative RT-PCR was performed for the genes we had identified. The expression level was compared to untreated cells which were cultured with medium alone for the indicated periods.

As shown in Figure 3, expression of unknown gene 3, Culin1, Zfhx1b, unknown gene 1, Zfp36,

Figure 2. In vitro anergy induction for Th1 cells derived from DO11.10 mice. Naïve CD4⁺ T cells from spleens of DO11.10 mice were differentiated into Th1 cells by culturing with 0.5 μM OVA323-339 plus APCs in the presence of anti-IL-4 and rIL-12 for 7 days. Recovered Th1 cells were cultured with or without plate-coated anti-CD3 mAb for 48 h. Cells were cultured for an additional 48 h in culture medium without stimulation (resting culture). Viable cells were stimulated with 0.1 μM OVA323-339 in the presence of APCs for 96 h. Proliferation (A) and IL-2 production (B) were determined as described in Figure 1.

Figure 3. Expression of genes associated with oral tolerance in *in vitro* anergized T cells. Th1 cells were cultured with or without plate-coated anti-CD3 as described in Figure 2. Cells were harvested after 6, 24, 48 h, or after resting culture (96 h). RNA was extracted and converted into cDNA. Expression levels of the indicated genes were determined by quantitative PCR.

and Emerin were up-regulated in *in vitro* anergized T cells. Interestingly, the kinetics of expression of these genes were not the same. Zfhx1b was expressed rapidly and dramatically upon anergy-inducing stimulation with anti-CD3. The expression did decline after 48 h, but still remained at a relatively high level. Unknown gene 3, Culin 1, and Zfp36 were expressed at their maximum levels after resting culture. Unknown gene 1 and Emerin were expressed at maximum levels after 48 h of stimulation. On the other hand, significant enhancement of expression was not seen for

unknown gene 2, unknown gene 4, NAPG and LAX in *in vitro* anergized T cells.

Discussion

In the present study, we employed suppression subtractive hybridization and identified 10 genes specifically expressed in orally tolerized T cells. Most of the previous studies to analyze gene expression in hyporesponsive T cells have been conducted in *in vitro* anergy-induction systems. There is little information about gene expression profiles of *in vivo* tolerized T cells. Therefore, our work will help to understand the mechanisms underlying the induction and maintenance of *in vivo* immune tolerance, especially oral tolerance.

Among 10 genes we identified (Table 1), several genes were interesting in terms of their putative functions for the negative regulation of T cell activation. Culin 1 is an E3 ubiquitin ligase subunit (Sakamoto et al. 2001) and GRAIL, which was recently reported to be highly expressed in *in vitro* anergized-T cells, was also identified as an E3 ubiquitin ligase (Anandasabapathy et al. 2003) and inhibited cytokine gene expression when transduced in T cells. Although the precise mechanism of this inhibition remains unclear, it appears to be dependent upon intact endocytic trafficking. It can be speculated that Culin 1 functions in orally tolerized T cells to negatively regulate cytokine expression by its ubiquitin ligase activity. LAX is a membrane-associated adaptor protein, which was recently cloned (Zhu et al. 2002). Upon TCR stimulation, LAX is rapidly phosphorylated and interacts with some signaling molecules. LAX functions to negatively regulate signaling since overexpression of LAX inhibits TCR-mediated activation of p38 MAPK and NFAT/AP-1. Thus, LAX might contribute to the impaired signaling in orally tolerized T cells. Zfhx1b is a transcription repressor as a Smad interaction protein (Verschuere et al. 1999). It might inhibit IL-2 transcription by binding the IL-2 promoter in a manner similar to one of the anergy-associated genes, Tob, which is also a Smad-binding protein and negatively regulates IL-2 transcription (Tzachanis et al. 2001).

We investigated the expression of the genes we identified in *in vitro* anergized T cells (Figure 3). Six of the 10 genes were highly expressed in

anergized T cells, however kinetic differences in expression of these genes were observed. *Zfhx1b* was rapidly expressed upon anergy-inducing stimulation, but its expression level declined at 24 h and beyond. On the other hand, expression levels of the other five genes were up-regulated at later time points. Thus, it can be speculated that *Zfhx1b* is involved in induction, and the other five genes are associated with the maintenance of anergy. In contrast, four of the 10 genes were not expressed in *in vitro* anergized T cells. These four genes might play a specific role in *in vivo* T cell tolerance.

Functional analyses of the genes we have identified in this study are currently ongoing and will reveal the role of each in the hyporesponsive state of T cells. We believe that our study will help to understand the complex mechanisms underlying the induction and maintenance of oral tolerance.

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