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The Squamous Cell Carcinoma Antigen 2 Inhibits the Cysteine Proteinase Activity of a Major Mite Allergen, Der p 1*

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Yasuhisa Sakata,[a] Kazuhiko Arima,[a] Toshiro Takai,[b] Wataru Sakurai,[c] Kiyonari Masumoto,[a] Noriko Yuyama,[d] Yoshinori Suminami,[e] Fumio Kishi,[f] Tetsuji Yamashita,[g] Takeshi Kato,[b] Hideoki Ogawa,[b] Kazuma Fujimoto,[h] Yo Matsuo,[c] Yuji Sugita,[d] and Kenji Izuhara[a,i,j]

From the [a]Division of Medical Biochemistry, Department of Biomolecular Sciences, the [h]Division of Gastroenterology, Department of Internal Medicine, and the [i]Division of Medical Research, Center for Comprehensive Community Medicine, Saga Medical School, Saga, 849-8501, the [b]Atopy (Allergy) Research Center, Juntendo University, Tokyo, 113-8421, the [c]Computational Proteomics Team, Protein Research Group, RIKEN Genomic Sciences Center, Yokohama, 230-0045, [d]Genox Research, Inc., Tokyo, 112-8088, [e]Onoda Municipal Hospital, Onoda, 756-0094, the [f]Department of Microbiology and Immunology, Kagoshima University Dental School, Kagoshima, 890-8544, and [g]Mitsubishi-Kagaku BCL, Tokyo, 174-8555, Japan

The squamous cell carcinoma antigens 1 (SCCA1) and SCCA2 belong to the ovalbumin-serpin family. Although SCCA1 and SCCA2 are closely homologous, these two molecules have distinct properties; SCCA1 inhibits cysteine proteinases such as cathepsin K, L, and S, whereas SCCA2 inhibits serine proteinases such as cathepsin G and human mast cell chymase. Although several intrinsic target proteinases for SCCA1 and SCCA2 have been found, the biological roles of SCCA1 and SCCA2 remain unknown. A mite allergen, Der p 1, is one of the most immunodominant allergens and also acts as a cysteine proteinase probably involved in the pathogenesis of allergic diseases. We have recently shown that both SCCA1 and SCCA2 are induced by two related Th2-type cytokines, IL-4 and IL-13, in bronchial epithelial cells and that SCCA expression is augmented in bronchial asthma patients. In this study, we explored the possibility that SCCA proteins target Der p 1, and it turned out that SCCA2, but not SCCA1, inhibited the catalytic activities of Der p 1. We furthermore analyzed the inhibitory mechanism of SCCA2 on Der p 1. SCCA2 contributed the suicide substrate-like mechanism without formation of a covalent complex, causing irreversible impairment of the catalytic activity of Der p 1, as SCCA1 does on papain. In addition, resistance to cleavage by Der p 1 also contributed to the inhibitory mechanism of SCCA2. These results suggest that SCCA2 acts as a cross-class serpin targeting an extrinsic cysteine proteinase derived from house dust mites and that it may have a protective role against biological reactions caused by mites.

The squamous cell carcinoma antigens 1 (SCCA1: SERPINB3)[1] and SCCA2 (SERPINB4) belong to the ovalbumin-serpin (serine proteinase inhibitors) family and are 91% identical at the amino acid level (1). Both genes locate at 18q21.3 very closely, suggesting that either gene could arise from the other by gene duplication (2). SCCA1 was originally purified from squamous cell carcinoma of uterine cervix (3), and it turned out that SCCA1 and SCCA2 are co-expressed broadly in normal tissues: the epithelium of tongue, tonsil, esophagus, uterine cervix, vagina, and the conducting airways; Hassall's corpuscles of the thymus; and some areas of the skin (4). Although SCCA1 and SCCA2 are very homologous, these two molecules have distinct properties; SCCA1 inhibits cysteine proteinases such as cathepsin K, L, S, and papain, whereas SCCA2 inhibits serine proteinases such as cathepsin G and human mast cell chymase (1, 5, 6). The specificities of SCCA1 and SCCA2 are due to the difference in the reactive site loop (RSL) sequences because only 7 amino acid residues among 13 (54%) were identical in the RSL regions (P7 to P6′) of these proteins (7). Although target proteinases for most serpins are the chymotrypsin family, the serpin inhibiting cysteine proteinases is defined as a cross-class inhibitor. Cytokine response modifier A (CrmA) derived from cowpox virus and proteinase inhibitor 9 (PI9, SERPINB9) inhibits both a serine proteinase (granzyme B) and a cysteine proteinase (caspase proteins) (8–11). So thus far, SCCA1, CrmA, and PI9 are all obvious cross-class serpins (12). Although several intrinsic target proteinases for SCCA1 and SCCA2 have been found, the biological roles of SCCA1 and SCCA2 remain unknown. We have recently shown that expression of both SCCA1 and SCCA2 is up-regulated by two related Th2-type cytokines, IL-4 and IL-13, in bronchial epithelial cells and that SCCA expression is augmented in bronchial lesions and in peripheral blood of bronchial asthma patients (13). It is well known that IL-4 and IL-13 are involved in the pathogenesis of bronchial asthma (14, 15), predominantly expressed in the lesions of asthma patients (16–18). These findings raise the possibility that SCCA1 and SCCA2 may perform their activities in the lesions of bronchial asthma.

Der p 1 and Der f 1, group I allergens derived from house dust mites, *Dermatophagoides pteronyssinus* and *Dermatophagoides farinae*, respectively, are major components of mites (10–20%), and their presence is closely correlated with development of bronchial asthma, atopic dermatitis, and allergic rhinitis (19–22). It has been reported that more than half of anti-mite allergen antibodies and 10–20% of total IgE in aller-

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[j] To whom correspondence should be addressed. Tel.: 81-952-34-2261; Fax: 81-952-34-2058; E-mail: kizuhara@med.saga-u.ac.jp.

[1] The abbreviations used are: SCCA, squamous cell carcinoma antigen; RSL, reactive site loop; CrmA, cytokine response modifier A, PI9, proteinase inhibitor 9; PAR-2, protease-activated receptor 2; MALDI-TOF, matrix-associated laser desorption ionization time-of-flight; PEO-M, polyethylene oxide-maleimide.

TABLE I
Comparison of k_{cat} and K_m for BSA-treated and SCCA2-treated Der p 1

	k_{cat}	K_m	k_{cat}/K_m
	s^{-1}	μM	s^{-1}/M^{-1}
BSA-treated (n = 3)	0.444 ± 0.0173	248 ± 16.2	1790 ± 46.3
SCCA2-treated (n = 3)	0.0155 ± 0.00260	290 ± 71.0	54.2 ± 4.40[a]

[a] Statistically significant difference *versus* BSA-treated at p = 0.0000003.

gic patients are anti-Der p 1 antibodies, which indicates that Der p 1 is one of the most immunodominant allergens (19, 21). Der p 1 is a 25-kDa cysteine proteinase; its amino acid sequence conserves 3 critical amino acids (Cys-34, His-170, and Asn-190) comprising the catalytic triad as other cysteine proteinases (23). The structure of Der p 1 has been modeled based on the crystal structure of papain, suggesting that Der p 1 is composed of two domains separated by a cleft, where the active site with the catalytic triad locates (24). It has been reported that Der p 1 cleaves several proteins such as occludin (25), protease-activated receptor 2 (PAR-2) (26), CD23 (27), CD25 (28), and CD40 (29) *in vitro*. Although the precise role of the catalytic activity of Der p 1 *in vivo* has not been elucidated, the following results indicate that the catalytic activity of Der p 1 would be important for the pathogenesis of bronchial asthma, in addition to its antigenicity. 1) Der p 1 disrupts tight junctions by cleaving occludin, increasing the permeability of the bronchial epithelial barrier (25). 2) Der p 1 causes secretion of inflammatory cytokines such as IL-6, IL-8, granulocyte-macrophage colony-stimulating factor, and RANTES in bronchial epithelial cells by activating PAR-2 (26, 30, 31). 3) Der p 1 induces the Th2 subset by cleaving CD25 on T cells (28) or CD40 on dendritic cells (29).

We hypothesized that SCCA proteins induced by IL-4 and IL-13 target extrinsic proteinases derived from house mites in the lesions of bronchial asthma. To explore this possibility, we examined whether SCCA1 and SCCA2 inhibit the catalytic activities of group I mite allergens in this study. It turned out that SCCA2 inhibited the catalytic activities of both Der p 1 and Der f 1 and that SCCA2 performed its inhibitory activity by irreversibly impairing the catalytic activity of Der p 1 and by being resistant to the cleavage by Der p 1. These results suggest that SCCA2 acts as a cross-class serpin targeting extrinsic cysteine proteinases, Der p 1 and Der f 1, and that SCCA2 may have a protective role against mite-caused biological reactions.

EXPERIMENTAL PROCEDURES

Materials—Papain, E-64, cathepsin G, cathepsin L, and human mast cell chymase were purchased from Sigma, Peptide Institute Inc. (Osaka, Japan), Calbiochem, Athens Research & Technology (Athens, GA), and Cortex Biochem (San Leandro, CA), respectively.

Generation of Plasmids and Recombinant Proteins—SERPINB3 and *SERPINB4* cDNA incorporated into pGEX(-KG)-4T (Amersham Biosciences) were prepared as reported before (32). SCCA2 mutants were generated by oligonucleotide-directed mutagenesis using two complemented primers with mutations. Standard PCR amplification was performed using the SCCA2 cDNA as a template and a mixture of primers. DNA fragments with mutations were ligated into pGEX-KG-SCCA2 plasmid. The RSL-replaced mutants of SCCA1 and SCCA2 were similarly generated by digestion and ligation into the StuI/XbaI site of them.

GST-fused SCCA1 and SCCA2 proteins were expressed in an *Escherichia coli* strain, BL21, and isolated by using glutathione-Sepharose 4B beads (Amersham Biosciences). Purity of the generated proteins was greater than 95%, as estimated by Coomasie staining of an SDS-PAGE gel. Concentrations of the proteins were determined by Protein Assay (Bio-Rad).

Generation of the Der p 1 and Der f 1 Protein—Recombinant Der p 1 and Der f 1 proteins were generated as described before (33, 34). Briefly, proforms of four recombinant house dust mite group 1 allergens, Der p 1-N52Q, Der p 1-WT, Der f 1-N53Q, and Der f 1-WT, were secreted into the culture supernatant of transfectant cells of *Pichia pastoris* and converted to the mature forms with prosequences removed by dialysis against an acidic buffer. The mature forms were purified with anion exchange column chromatography. The purity was more than 95%, as estimated by SDS-PAGE. The protein concentration was determined by Protein Assay. Der p 1-N52Q and Der f 1-N53Q were used for most experiments.

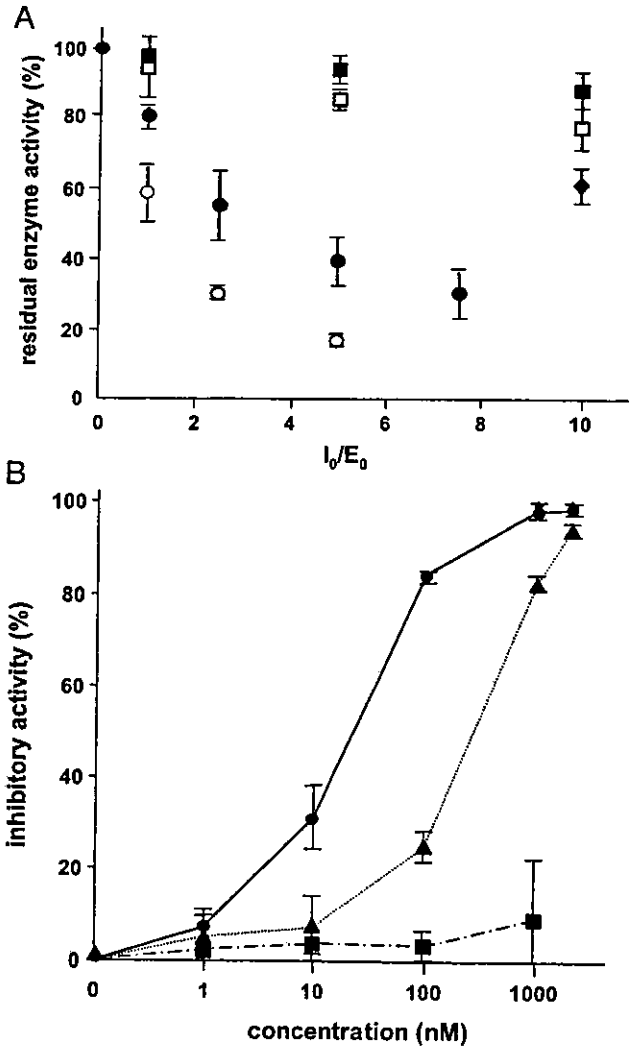


FIG. 1. **Inhibitory effects of SCCA molecules on the catalytic activities of group I house mite allergens.** In *A*, SCCA1 (*square*) or SCCA2 (*circle*) or E-64 (*rhombus*) was incubated with 10 nM preactivated Der p 1 (*closed*) or Der f 1 (*open*) at the indicated I_0/E_0 ratio for 30 min at 25 °C. Residual enzyme activities are depicted. In *B*, SCCA1 (*closed triangles*) or SCCA2 (*closed circles*) or GST alone (*closed squares*) was incubated with the indicated concentrations of preactivated Der p 1 at the I_0/E_0 ratio = 1 for 30 min at 25 °C. Inhibitory activities are depicted.

Enzyme Assays—Enzyme assays of Der p 1 and Der f 1 were performed as described before (33, 35). The substrate used for enzyme assays was butyloxycarbonyl-Gln-Arg-methylcoumarin (Boc-Gln-Ala-Arg-MCA), purchased from Peptide Institute Inc. The indicated concentrations of Der p 1 or Der f 1 preactivated with 2 mM dithiothreitol for 10 min at 25 °C were incubated with the indicated concentrations of GST-fused SCCA proteins for 30 min at 25 °C in activity-measuring buffer (50 mM sodium phosphate, pH 7.0, 1 mM EDTA, 2 mM dithiothre-

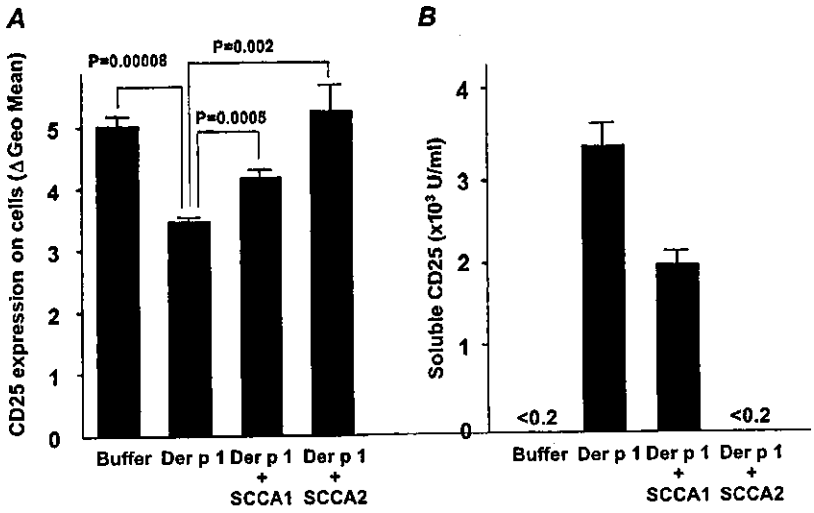


FIG. 2. **Inhibitory effects of SCCA molecules on the cleavage of CD25 by Der p 1.** One μM SCCA1 or SCCA2 was incubated with 2 μM preactivated Der p 1 for 30 min at 25 °C. Then, stimulated Jurkat T cells were incubated with a mixture of Der p 1 and SCCA proteins with final concentrations 0.6 and 0.3 μM, respectively, for 2 h at 37 °C. Expression of CD25 on the cell surface (*A*) and amount of soluble CD25 (*B*) are depicted.

itol, and 0.001% BSA). Upon addition of 100 μM substrate to the reaction mixture, the residual enzyme activity was measured by continuous monitoring, using excitation and emission wavelengths of 380 and 460 nm, respectively.

Cleavage Assay of CD25—Procedures of cleavage assay of CD25 were performed as described before (28). A human acute T cell leukemia cell line, Jurkat T cells, was stimulated with 100 nM PMA (Sigma) and 1 μM ionomycin (Sigma) for 30 h in RPMI 1640 medium containing 10% fetal calf serum followed by suspension with serum-free AIM V medium (Invitrogen). Two μM Der p 1 preactivated with 2 mM dithiothreitol was incubated with 1 μM SCCA proteins for 30 min at 25 °C. Then, CD25 cleavage was performed by incubating Jurkat cells with the mixture of Der p 1 and SCCA proteins with the final concentrations of Der p 1 and SCCA proteins 0.6 and 0.3 μM, respectively, for 2 h at 37 °C. Expression of CD25 on cell surface was analyzed by flow cytometry using anti-CD25 antibody (Beckman Coulter), and soluble CD25 in the supernatant was immunoassayed in the Mitsubishi Kagaku BCL laboratories by means of a commercial kit.

Matrix-associated Laser Desorption Ionization Time-of-Flight (MALDI-TOF) Mass Spectrometry—Five μM Der p 1 and 10 μM SCCA proteins were mixed in phosphate reaction buffer for 2 h at 4 °C, and then the reactive samples were applied to Voyager RP MALDI-TOF mass spectrometry (PerSeptive Biosystems, Framingham, MA).

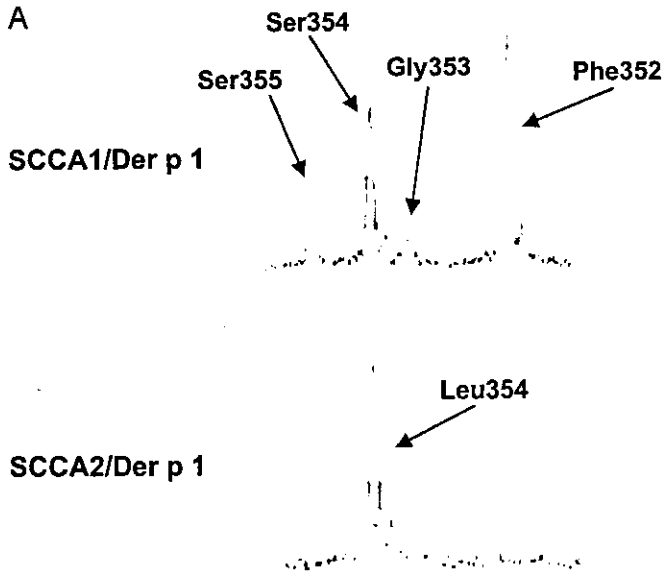
Separation of the Incubated SCCA Proteins and Der p 1—After 2 μM Der p 1 and SCCA proteins were incubated in phosphate reaction buffer for 30 min at 25 °C, the reaction mixture was applied to the high pressure liquid chromatography system equipped with ProteinPak 300SW (Waters, Milford, MA). Then, the subjected samples were eluted with phosphate buffer (50 mM sodium phosphate, pH 7.0), and each fraction was subjected to SDS-PAGE or the enzyme assay. The gels were stained with silver using 2D-SILVER STAIN II "Daiichi" (Daiichi Pure Chemicals, Tokyo, Japan). The quantities of the proteins on the gels were determined by SYPRO Ruby staining (Molecular Probes, Eugene, OR)

Chemical Modification of the Cysteine Residues of Der p 1—The fractionated Der p 1 was incubated with 0.4 mM EZ-Link PEO-maleimide activated biotin ((+)-biotinyl-3-maleimidopropiomamidyl-3, 6-dioxaoctanediamine; PEO-M-biotin, Pierce) for 2 h at 25 °C. The samples were applied to SDS-PAGE and then transferred to polyvinylidene difluoride membranes. The membranes were blotted with horseradish peroxidase-conjugated streptavidin (Zymed Laboratories Inc., South San Francisco, CA).

Synthesized Peptides—Synthesized peptides used for inhibition assays were Thr-Ala-Val-Val-Gly-Phe-Gly-Ser-Ser-Pro-Thr-Ser-Thr (SCCA1–13 mer), Thr-Ala-Val-Val-Val-Val-Glu-Leu-Ser-Ser-Pro-Ser-Thr (SCCA2–13 mer), and Thr-Ala-Val-Val-Val-Val-Gly-Leu-Ser-Pro-Thr-Ser-Thr (SCCA2 tm-13 mer), all purchased from Peptide Institute Inc. The peptides were dissolved in dimethyl sulfoxide (Wako, Osaka, Japan).

RESULTS

Expression and Purification of Functional SCCA1 and SCCA2—To perform functional analyses of SCCA1 and SCCA2, we expressed and purified recombinant proteins of GST-fused SCCA1 and SCCA2. We confirmed that SCCA1



B

position	14	13	12	11	10	9	8	7	6	5	4	3	2	1	1'	2'	3'	4'	5'	6'
SCCA1	G	A	E	A	A	A	A	T	A	V	V	G	F	G	S	S	P	T	S	T
SCCA2	G	V	E	A	A	A	A	T	A	V	V	V	V	E	L	S	S	P	S	T

FIG. 3. **Identification of the cleaved sites of SCCA molecules by Der p 1.** Ten μM SCCA1 or SCCA2 and 5 μM Der p 1 were incubated for 2 h at 4 °C, and the reactive samples were applied to MALDI-TOF mass spectrometry. The detected peaks (*A*) and the identified cleavage sites (*B*) are depicted. The *arrows* represent the peptides from the indicated residues to the C terminus (*A*).

inhibited the cysteine protease activities of papain and cathepsin L but not the serine protease activities of cathepsin G and human mast cell chymase, whereas SCCA2 showed the opposite effects, as reported previously (32). These results demonstrated that purified SCCA1 and SCCA2 proteins were functional.

Inhibition of Catalytic Activities of Der p 1 and Der f 1 by SCCA2—We first analyzed whether SCCA1 or SCCA2 inhibited catalytic activities of Der p 1 and Der f 1. The k_{cat} and K_m values of Der p 1 used in the experiments were estimated as 0.444 ± 0.0173 s^{-1} and 248 ± 16.2 μM, respectively (Table I). An irreversible inhibitor for cysteine proteinase, E-64, displayed only 39% of inhibition at a 10:1 ratio at 10 nM Der p 1 or

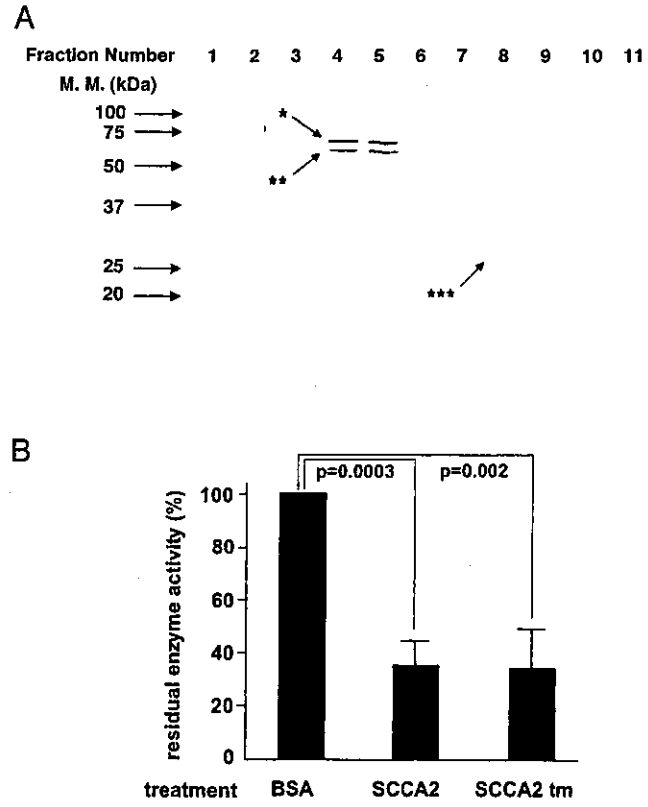


FIG. 4. **Elution profile of the mixture of SCCA molecules and Der p 1 by gel-filtration column and the catalytic activity of SCCA-treated Der p 1.** Two μM SCCA proteins was incubated with 2 μM preactivated Der p 1. In *A*, the samples incubated for 30 min were applied to the gel-filtration column. Elution profiles stained with silver are depicted. The *arrows* represent the intact (*) and truncated (**) SCCA2 and Der p 1 (***), respectively. *M. M.*, molecular mass. In *B*, the catalytic activities of the fractionated Der p 1 in the presence of SCCA2 or BSA are depicted. In *C*, fractionated Der p 1 in the presence of SCCA2 or BSA was incubated with PEO-M-biotin followed by blotting with horseradish peroxidase-conjugated streptavidin or was stained by SYPRO Ruby. The incorporation of PEO-M-biotin and their representative data are depicted.

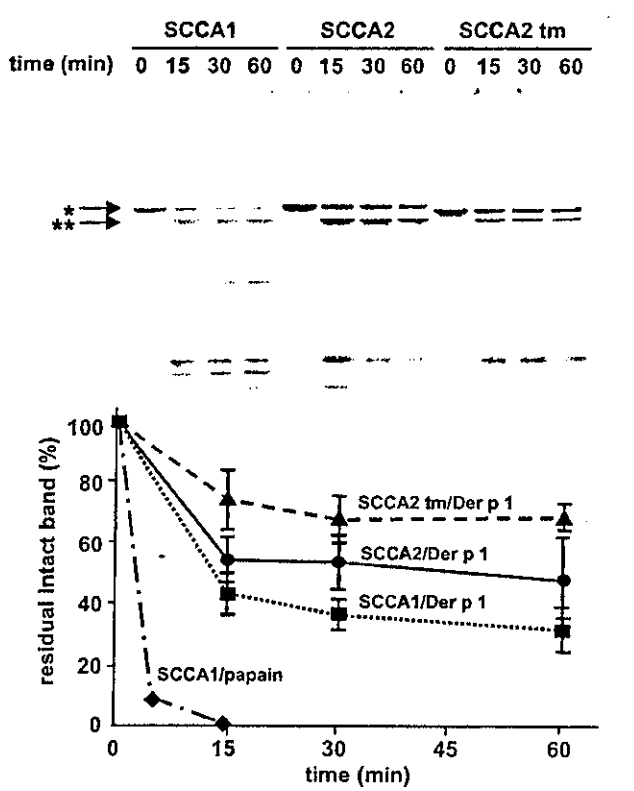


FIG. 5. **Cleavage profile of SCCA molecules by Der p 1.** SCCA2 proteins were incubated with Der p 1 or papain, as shown in Fig. 4. The samples incubated for the indicated times followed by SYPRO Ruby staining and the amounts of intact SCCA proteins at the indicated time are plotted. The *arrows* represent the intact (*) and truncated (**) SCCA proteins.

Der f 1 (Fig. 1*A*). Therefore, active site titration of Der p 1 or Der f 1 was impossible, so we analyzed dose-dependent effects of SCCA1 and SCCA2 on the catalytic activity of Der p 1 and Der f 1. When the concentration of Der p 1 or Der f 1 was fixed at 10 nM, SCCA2 inhibited catalytic activities of both Der p 1 and Der f 1 in a dose-dependent manner, whereas SCCA1 showed only 10% inhibition at 10:1 ratio (Fig. 1*A*). The inhibitory effects of SCCA2 were independent of glycosylation of Der p 1 and Der f 1 (data not shown). When the ratio of Der p 1 and SCCA1 or SCCA2 was fixed at 1:1, the inhibitory effect of SCCA2 increased dependent of the concentration, reaching almost 100% at 1 μM (Fig. 1*B*). SCCA1 showed a weaker inhibitory effect when compared with SCCA2. GST alone had no inhibitory effect. These results demonstrated that SCCA2, and to a lesser extent SCCA1, targeted Der p 1 and that SCCA2 is a cross-class serpin that can inhibit both serine and cysteine proteinases as well as CrmA, PI9, and SCCA1.

Inhibitory Effects of SCCA Proteins on CD25 Cleavage by Der p 1—To examine whether SCCA2 shows its inhibitory effect on Der p 1, when Der p 1 targets not only the synthetic peptide, but also an intact protein as a substrate, we analyzed the inhibitory effects of SCCA proteins on CD25 cleavage by Der p 1. Incubation of Der p 1 with activated Jurkat cells caused the cleavage of CD25 (Fig. 2*A*, 31 ± 2.0%, $n = 3$). The presence of SCCA2 completely restored expression of CD25, and so did SCCA1 to some extent. Furthermore, existence of the soluble CD25 in the supernatant was observed in parallel with the cleavage of CD25 on Jurkat cells (Fig. 2*B*). These results meant that SCCA2 also exerts its inhibitory effects on the interaction of Der p 1 with CD25.

Determination of the Cleavage Sites in SCCA1 and SCCA2 by Der p 1—We next identified the cleavage sites in RSLs of SCCA1 and SCCA2 by Der p 1, using MALDI-TOF mass spectrometry. Analysis of Der p 1-cleaved peptides in SCCA1 by MALDI-TOF mass spectrometry showed the existence of four

TABLE II
Alignment of RSLs of SCCA proteins and their inhibitory activities

Position	Proximal hinge							Reactive site loop												Distal hinge	Inhibition
	14	13	12	11	10	9	8	7	6	5	4	3	2	1	1′	2′	3′	4′	5′	6′	11′	
SCCA2	G	V	E	A	A	A	A	T	A	V	V	V	V	E	L	S	S	P	S	T	C	+
SCCA1	·[a]	A	·	·	·	·	·	·	·	·	·	G	F	G	S	·	P	T	·	·	H	−
SCCA2 RSL1	·	A	·	·	·	·	·	·	·	·	·	G	F	G	S	·	P	T	·	·	H	−
SCCA1 RSL2	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	+
SCCA2 mut1 (V351G)	·	·	·	·	·	·	·	·	·	·	·	G	·	·	·	·	·	·	·	·	·	−
SCCA2 mut2 (V352F)	·	·	·	·	·	·	·	·	·	·	·	·	F	·	·	·	·	·	·	·	·	−
SCCA2 mut4 (L354S)	·	·	·	·	·	·	·	·	·	·	·	·	·	·	S	·	·	·	·	·	·	−
SCCA2 mut3 (E353G)	·	·	·	·	·	·	·	·	·	·	·	·	·	G	·	·	·	·	·	·	·	++
SCCA2 E353A	·	·	·	·	·	·	·	·	·	·	·	·	·	A	·	·	·	·	·	·	·	++
SCCA2 E353Q	·	·	·	·	·	·	·	·	·	·	·	·	·	Q	·	·	·	·	·	·	·	++
SCCA2 mut5 (S356P, P357T)	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	P	T	·	·	·	++
SCCA2 tm (E353G, S356P, P357T)	·	·	·	·	·	·	·	·	·	·	·	·	·	G	·	·	P	T	·	·	·	++++
SCCA2 tm P356A	·	·	·	·	·	·	·	·	·	·	·	·	·	G	·	·	A	T	·	·	·	++++

[a] ·, the same amino acid as SCCA2.

peaks corresponding to the peptides from Phe-352, Gly-353, Ser-354, and Ser-355 to the C terminus, whereas the analyses with SCCA2 displayed only one peak, corresponding to the peptide from Leu-354 to the C terminus (Fig. 3). These results indicated that Der p 1 would interact with SCCA2 firmly, generating a serpin-proteinase complex as other serpins do. In contrast, the interaction between Der p 1 and SCCA1 would not be tight, and Der p 1 would cleave non-specifically the RSL of SCCA1.

Non-covalent Binding of SCCA2 with Der p 1—Although it is well known that a serpin and its target proteinase form an acyl-enzyme intermediate linked by an oxy-ester bond, stable for hydrolysis (36), we have recently demonstrated that SCCA1 inhibited the catalytic activity of papain without forming a covalent bond (32). We next analyzed the association manner of SCCA2 and Der p 1. To retain the native association between SCCA2 and Der p 1, we employed a gel-filtration system. We used a mixture of Der p 1 and SCCA2 in which the catalytic activity of Der p 1 was completely inhibited. Subsequently, SCCA2 and Der p 1 were eluted according to their molecular masses (Fig. 4*A*). These results demonstrated that SCCA2 interacted with Der p 1 by non-covalent binding as well as the interaction between SCCA1 and papain.

Irreversible Inhibition of Der p 1 by SCCA2 Treatment—We next investigated how SCCA2 inhibited the catalytic activity of Der p 1 without forming a covalently bound complex. To study the effects of the interaction between SCCA2 and Der p 1, we analyzed the catalytic activity of the SCCA2-treated Der p 1 eluted by the gel-filtration column. Although the catalytic activity of Der p 1 was completely inhibited in the solution containing 2 μM Der p 1 and SCCA2, which was applied to the column, it turned out that the catalytic activity of fractionated Der p 1 decreased, but still existed, when compared with the solution before fractionation (36 ± 9.4%, n = 3, Fig. 4*B*). The k_{cat}/K_m value of SCCA2-treated Der p 1 was significantly less than that of BSA-treated Der p 1 (54.2 ± 4.40 *versus* 1790 ± 46.3 s^{-1} M^{-1}, p = 0.0000003, Table I), which confirmed the impairment of the catalytic activity.

These results raised the possibility that SCCA2 treatment caused a conformational change of Der p 1, down-regulating its catalytic activity. To explore this possibility, we compared chemical modification of SCCA2-treated or BSA-treated Der p 1 by biotin-conjugated maleimide, a cysteine residue-modifying reagent. Modification of SCCA2-treated Der p 1 by biotin-conjugated maleimide was reduced when compared with the level of BSA-treated Der p 1 (56 ± 14%, n = 3, Fig. 4*C*). These results suggest that down-regulation of the catalytic activity of Der p 1 may be due to its conformational change, although we cannot exclude the possibility that the cysteine residue of the active center was unexpectedly modified by SCCA2.

Resistance of SCCA2 against Cleavage by Der p 1—Although the irreversible conformational change of Der p 1 contributed to the inhibition mechanism of SCCA2, it could not fully explain it because fractionated Der p 1 still showed catalytic activity at the reducing level (Fig. 4*B*). As only intact serpin sustains its inhibitory activity in the suicide substrate-like mechanism, it would be possible that the difference of susceptibility to cleavage by the target proteinase could affect the inhibitory activity of serpin. To explore this possibility, we analyzed the digestion profile of SCCA2 in the presence of Der p 1 in a time-dependent manner. It turned out that SCCA2 was resistant to digestion by Der p 1 when compared with SCCA1 and that 53% of SCCA2 still existed, intact, after 30 min of incubation (Fig. 5). When SCCA1 was incubated with papain, intact SCCA1 immediately began to decrease and was completely lost within 15 min. These results demonstrated that resistance against the cleavage by the target proteinase was a unique property of SCCA2, and it could at least partially explain why SCCA2 exerted its potent inhibitory activity on Der p 1 when compared with SCCA1.

Preference of Amino Acids in the RSL Sequences of SCCA2 and SCCA1 for Inhibitory Effect on Der p 1—The distinct properties of SCCA1 and SCCA2 regarding the inhibitory effects on Der p 1 are assumed to be due to the difference of their RSL sequences. Actually, swapping the RSL of SCCA1 for that of SCCA2, or *vice versa*, revealed that the inhibitory effect on Der p 1 was dependent on the RSL of SCCA2 (Table II, SCCA1 RSL2, SCCA2 RSL1). We then exchanged each amino acid specific for the RSL of SCCA2 with that corresponding to SCCA1 and analyzed its inhibitory effect on Der p 1 (Table II). When Val-351, Val-352, or Leu-354 was replaced with Gly, Phe, or Ser, respectively, all of the mutated types attenuated the inhibitory effect (SCCA2 mut1, SCCA2 mut2, SCCA2 mut4), demonstrating that these residues were critical. Surprisingly, when Glu-353 or both Ser-356 and Pro-357 were exchanged with Gly or Pro and Thr, respectively, the inhibitory effect was augmented when compared with native SCCA2 (SCCA2 mut3, SCCA2 mut5). Furthermore, when Glu-353, Ser-356, and Pro-357 were all replaced with Gly, Pro, and Thr, respectively, the inhibitory action was dramatically up-regulated (Fig. 6, *A* and *B*, SCCA2 tm). Although Glu-353 was exchanged with Ala or Gln instead of Gly, the inhibitory effect was more enhanced than native SCCA2 (SCCA2 E353A, SCCA2 E353Q), indicating that removal of the ionic strength of Glu-353 would be important for up-regulation of the inhibitory effect. Furthermore, as switching of Pro-356 of SCCA tm with Ala did not influence the activity, Pro-356 in SCCA2 tm would not be critical, but removal of Pro-357 from native SCCA2 would be important (SCCA2 tm P356A). Finally, we confirmed that swapping of the RSL of SCCA tm for that of SCCA1 still sustained its potency,

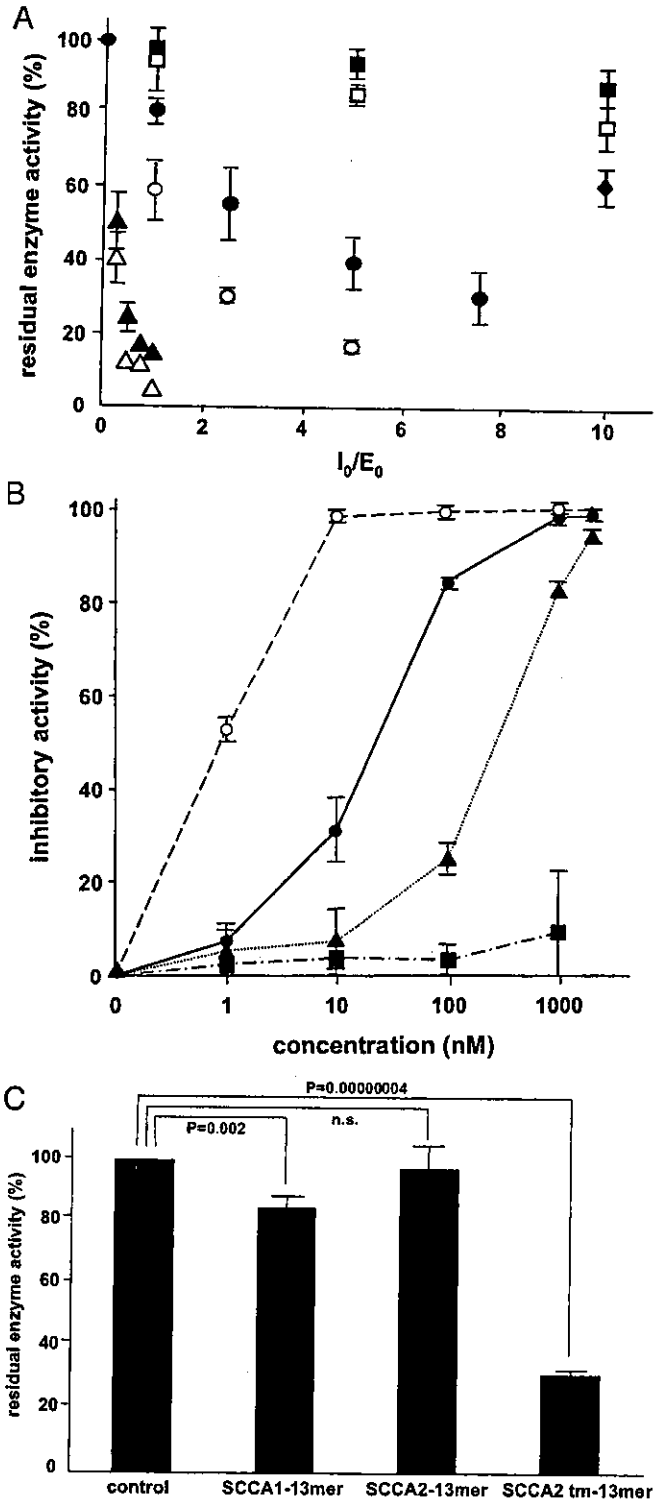


FIG. 6. **Inhibitory effects of SCCA2 tm on the catalytic activity of Der p 1.** In *A*, SCCA2 tm (*triangle*) was incubated with 10 nM Der p 1 (*closed*) or Der f 1 (*open*) at the indicated I_0/E_0 ratio, as shown in Fig. 1. In *B*, SCCA2 tm (*open circles*) was incubated with the indicated concentrations of Der p 1 at the I_0/E_0 ratio = 1, as shown in Fig. 1. The results of Fig. 1, *A* and *B*, are superimposed. In *C*, 10 nM Der p 1 was incubated with a 1:1000 ratio of each synthetic peptide for 30 min at 25 °C. Residual enzyme activities are depicted.

demonstrating the importance of the amino acid sequence of the RSL (SCCA1 RSL2 tm, data not shown).

To validate the importance of the amino acid sequence of the RSL in SCCA2 tm, we generated synthetic peptides corresponding to the RSLs of SCCA1, SCCA2, and SCCA2 tm, and then analyzed their inhibitory effects. At 1:1000 ratio, the peptide corresponding to SCCA2 tm, but not the peptides corresponding to SCCA1 and SCCA2, inhibited the catalytic activity of Der p 1 (Fig. 6*C*), again demonstrating the critical role of the sequence of the RSL in SCCA proteins.

Characterization of SCCA2 tm—We then compared the biochemical characteristics of SCCA2 and SCCA2 tm. It turned out that the biochemical properties of SCCA2 tm were almost the same as SCCA2. 1) It blocked the cleavage of CD25 by Der p 1 (data not shown). 2) It was cleaved by Der p 1 between Gly-353 and Leu-354 (data not shown). 3) It did not form a covalent complex with Der p 1 (data not shown). 4) SCCA2 tm caused irreversible inhibition on the catalytic activity of Der p 1 (33 ± 17%, n = 3, Fig. 4*B*), and incorporation of maleimide decreased to the same level as SCCA2 (48 ± 16%, n = 3, Fig. 4*C*). However, SCCA2 tm was more resistant to cleavage by Der p 1 than SCCA2 (67.5 ± 7.3% *versus* 53.1 ± 8.8% at 30 min, 68.0 ± 4.1% *versus* 47.7 ± 13.8% at 60 min, Fig. 5). These results demonstrated that the difference of resistance against cleavage by Der p 1 contributed to the different inhibitory activities of SCCA2 and SCCA2 tm.

DISCUSSION

In this study, we demonstrated that SCCA2 inhibited the cysteine proteinase activity of group I mite allergens, Der p 1 and Der f 1. Although it had been thought that SCCA2 targeted only serine proteinases, SCCA2 inhibits both serine and cysteine proteinases belonging to the cross-class serpin family, as well as CrmA, PI9, and SCCA1. It has been reported that Der p 1 performs various biological activities correlated with allergic reactions such as disruption of tight junction by cleaving occludin (25), secretion of inflammatory cytokines in bronchial epithelial cells by activating PAR-2 (26, 30, 31), and induction of Th2 subset by cleaving CD25 or CD40 (28, 29) *in vitro*. Although the precise pathological role of the catalytic activity of Der p 1 *in vivo* remains unclear, it is assumed that the cysteine proteinase activity of Der p 1 acts as a trigger or a worsening factor of bronchial asthma, based on the *in vitro* data. If this were the case, SCCA2 might play a protective role against group I mite allergens in the lesions of bronchial asthma, although IL-4 and IL-13, which induce expression of SCCA2 in bronchial epithelial cells, are themselves known to be involved in the pathogenesis of bronchial asthma (14, 15). The interaction between SCCA2 and group I mite allergens may help us elucidate the complexity of bronchial asthma. Alternatively, SCCA proteins might play a protective role against cysteine proteinases derived from parasites because it is known that some cysteine proteinases derived from *Leishmania mexicana* affect its virulence (37) and that IL-4/IL-13 protect against various parasites (38).

We next examined the inhibitory mechanism of SCCA2 on Der p 1, and the following events occurred. 1) SCCA2 was cleaved at the predicted site in its RSL (Fig. 3). 2) SCCA2 and Der p 1 did not form a complex with a covalent binding (Fig. 4*A*). 3) Interaction with SCCA2 partially impaired the catalytic activity of Der p 1, probably by irreversible conformational change (Fig. 4, *B* and *C*). The serpins employ a suicide substrate-like inhibitory mechanism in which the exposed RSL of the serpin is recognized by the proteinase, and then a "bait" peptide bond (P1-P1′) that mimics the normal substrate of the proteinase is attacked by the active serine residue of the proteinase (12, 39). Upon the interaction, a standard serpin forms an acyl-enzyme intermediate with a serine proteinase linked by an oxy-ester bond. In its cleaved form, the P side of the RSL inserts into the body of the protein, which dramatically changes the conformations of the serpin and the proteinase, rendering it impossible for the ester bond to hydrolyze (36). We

have recently shown that the inhibitory mechanism of another cross-class serpin, SCCA1, is unique among the serpin superfamily in that SCCA1 performs its inhibitory activity in two ways: contributing the suicide substrate-like mechanism without formation of a covalent complex and causing irreversible impairment of the catalytic activity of papain (32). The biochemical events occurring in SCCA2 and Der p 1 described above indicate that this mechanism would be common among cross-class serpins. It is speculated that the thiol-ester bond between a cross-class serpin and its target cysteine proteinase was unstable or that the distorted ester bond located not so far from catalytic partners, enabling the ester bond to hydrolyze. Concomitantly, the interaction may induce conformational change of the target proteinase, which irreversibly loses its catalytic activity. The reports that other cross-class serpins, CrmA and PI9, do not form SDS-resistant complexes with caspase proteins, although they do so with a serine proteinase, granzyme B (8–11), may indicate the same properties.

We also found that SCCA2 was resistant to cleavage by Der p 1 when compared with SCCA1 (Fig. 5). This is a unique property of SCCA2 contributing to the inhibition mechanism against Der p 1, different from the interaction of SCCA1 with papain. The analyses of substitution of each amino acid suggested that Leu-354 at SCCA2 would be critical for resistance against Der p 1 because SCCA2 mut4, in which Leu-354 was replaced with Ser, was susceptible to cleavage by Der p 1, diminishing the inhibitory activity against Der p 1 (Table II and data not shown). It is assumed that Leu-354 would block the nucleophilic reaction of Cys-34 at Der p 1 toward the P1 residue (Glu-353), based on the homology modeling of the interaction between SCCA2 and Der p 1.[2] In addition to the comparison between SCCA1 and SCCA2, by substituting each amino acid in the RSL of SCCA2, we unexpectedly succeeded in generating a very potent inhibitor (SCCA2 tm) when compared with native SCCA2 (Fig. 6). The analyses of amino acid replacement suggested that removal of ionic strength in Glu-353 would stabilize the interaction of the RSL and the cleft of Der p 1, leaving the SCCA molecule more resistant to the cleavage by Der p 1 (Table II and Fig. 5). Analysis of the comparison between SCCA2 and SCCA2 tm also supported the notion that enhancement of the resistance against cleavage by Der p 1 would lead to enhancement of the inhibitory activity of the SCCA2 molecule.

Although the precise role of the catalytic activity of Der p 1 in the pathogenesis of allergic diseases remains unclear, it is hoped that an inhibitor against its catalytic activity has the potential to be developed into a therapeutic reagent for allergic diseases arising from mite allergens. Actually, it has been reported that a peptide-based inhibitor, PTL11028, showed inhibitory effects for the catalytic activities of group I mite allergens and improved airway hyperreactivity, inflammation, and systemic sensitization induced by Der p 1 in rats (40, 41). Structural analyses of the interaction between SCCA/Der p 1 would give us a hint as to how to develop a novel low molecular weight compound to block the catalytic activity of group I mite allergens.

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Research Communication

Induction of Drug-metabolizing Enzymes and Transporters in Human Bronchial Epithelial Cells by Beclomethasone Dipropionate

Y. Kuzuya,[1] T. Adachi,[2] H. Hara,[2] A. Anan,[1] K. Izuhara[3] and H. Nagai[1]

[1]Department of Pharmacology, Gifu Pharmaceutical University, 5-6-1 Mitahora-higashi, Gifu 502-8585, Japan
[2]Laboratory of Clinical Pharmaceutics, Gifu Pharmaceutical University, 5-6-1 Mitahora-higashi, Gifu 502-8585, Japan
[3]Division of Medical Biochemistry, Department of Biomolecular Sciences, Saga Medical School, 5-1-1 Nabeshima, Saga, 849-8501, Japan

Summary

Inhaled steroids are the most potent anti-inflammatory therapy commonly used in bronchial asthma. There are, however, a small number of asthmatic patients who do not respond to inhaled steroid-treatment. The stimulation of metabolism and excretion of inhaled drugs at bronchial tissues might lead to a decrease in the effect of the drugs, although the molecular mechanism of this resistance is unclear. In this study, we found that beclomethasone dipropionate (BDP) stimulated the expression of mRNAs for uridine 5′-diphosphate glucuronosyl transferase 2B4 and 2B11, and transporters such as multidrug resistance P-glycoprotein, multidrug resistance-associated protein 1 and 2 in cultured bronchial epithelial cells. It is possible that the individual differences of expression of drug metabolizing enzymes and transporters and their enhancement with BDP are implicated in the individual differences of reactivity over steroid medical treatment.

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Keywords Uridine 5′-diphosphate glucuronosyl transferase; multidrug resistance P-glycoprotein; multidrug resistance-associated protein; beclomethasone dipropionate; bronchial epithelial cells.

INTRODUCTION

Recent studies have demonstrated the importance of airway inflammation and immune activation in the pathogenesis of bronchial asthma (*1, 2*). Steroids are the most potent anti-inflammatory therapy commonly used for this disease (*3*). They improve airflow obstruction and decrease the accelerated decline in lung function associated with asthma. Inhaled steroids have become established as a cornerstone therapy to increase the concentration of drug at impaired bronchial tissues and reduce the incidence of systemic side-effects associated with oral steroid treatment. There are, however, a small number of asthmatic patients who do not respond to inhaled steroid-treatment (*4, 5*). Although the molecular mechanism of this resistance is unclear, some possible mechanisms have been reported such as decrease of glucocorticoid receptor (*6, 7*), dysfunction of glucocorticoid receptor (*8, 9*), and increase of expression of transcription factors implicated in inflammation such as activator protein-1 (AP-1) (*10*). Moreover, it is possible that the stimulation of metabolism and excretion of inhaled drugs at bronchial tissues leads to a decrease of the effect of the drugs. However, the mechanism by which inducers of drug metabolism and drug transport exert their effects in human is not sufficiently defined, especially in peripheral tissues.

Tissues encounter a wide range of foreign compounds including drugs using drug metabolizing enzymes and transporters through three steps: As the phase I metabolizing step, cytochrome P450 (CYP) proteins hydrolyze foreign drugs. The principal CYP isoforms, CYP1A2, 2C9 and 3A4 are well known to be expressed in the liver, and contribute to first-pass metabolic excretion of commonly prescribed drugs (*11*). Previous studies using immunostaining and assay of catalytic activity revealed the presence of CYPs in human lungs (*12, 13*). Nevertheless, the involvement of respiratory tissues in the drug metabolism system has received relatively little attention in comparison with major target sites such as hepatic and duodenal counterparts, because of their low metabolic ability. As the phase II metabolizing step, conjugation with glucuronic acid is responsible for deactivation and elimination of exogenous compounds including CYP-metabolized drugs. Moreover, the glucuronization is

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Address correspondence to: T. Adachi, Laboratory of Clinical Pharmaceutics, Gifu Pharmaceutical University, 5-6-1 Mitahora-higashi, Gifu 502-8585, Japan. Tel: + 81-58-237-3931. Fax: + 81-58-237-5979. E-mail: adachi@gifu-pu.ac.jp

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also implicated in the elimination of endobiotics such as hormones, vitamins, bilirubin and glycolipids. This broad range of glucuronidation activity can be attributed to the heterogeneity of uridine 5'-diphosphate glucuronosyl transferases (UGTs) (*14*). Based upon differences in sequence homology and substrate specificity, two families of UGT, UGT1 and UGT2, have been identified. A recent study has demonstrated that UGTs in the lung play an important role in the detoxication of nicotine-derived tobacco-specific nitrosamine and its metabolites (*15*). Finally, ATP-dependent transmembrane proteins such as multidrug resistance P-glycoprotein (*MDR1*, P-gp), and multidrug resistance-associated proteins 1 (MRP1) and 2 (MRP2) transport numerous compounds including drugs out of cells as the phase III metabolizing step. P-gp was identified as an important factor in multidrug resistance of human tumors and acts as an ATP-dependent efflux pump that exports anticancer agents (*16*). It appears that numerous hydrophobic molecules including steroids and other physiological substrates are also transported by P-gp (*17*). On the other hand, MRPs are known to transport relative hydrophilic molecules such as glutathione S-conjugated drugs, glucuronate conjugated drugs and structurally diverse conjugated organic anions (*18*). Recently, P-gp and MRP1 have been detected in bronchiolar epithelial cells (*16, 19*). The human respiratory epithelium is in direct contact with inhaled drugs. Therefore, the induction of drug metabolizing enzymes and transporters in the epithelial cells could modulate drug clearance. In the present study, we investigated the stimulation of CYPs, UGTs and transporters in bronchial epithelial cells by beclomethasone dipropionate (BDP) an inhaled steroid.

MATERIALS AND METHODS

Cell Culture

Normal human bronchial epithelial cells (NHBE) and normal human hepatocytes (NhHep) were purchased from Sanko Junyaku Co. Ltd, (Tokyo, Japan) and were cultured in small airway epithelial cell growth medium (SAGM, Sanko Junyaku) and hepatocyte culture medium (HCM, Sanko Junyaku), respectively. Human Calu-3 bronchial epithelial cells and human hepatoma cells (HepG2) were obtained from American Type Culture Collection and cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, 100 units/ml penicillin and 100 μg/ml streptomycin as medium. BEAS-2B human bronchial epithelial cells were cultured in LHC-9 medium (Wako Pure Chemical Industries, Ltd., Osaka, Japan). They were kept under an atmosphere of 5% CO_2/95% air at 37°C. The cells (2×10^5 cells in a 10-cm dish) were cultured to confluence, and then the medium was replaced with fresh medium containing reagents and cultured for the indicated times. The cells were washed with 5 ml of cold phosphate buffered saline and used for mRNA assay.

Assay of mRNA

Total RNA was extracted from the treated cells with TRIzol reagent (Invitrogen, Carlsbad, CA, USA). First-strand cDNA was generated from 4 μg of total RNA. Reverse-transcription was carried out for 10 min at 25°C followed by 50 min at 42°C and 15 min at 70°C in a 20-μl volume containing 50 mM Tris-HCl, pH 8.3, 75 mM KCl, 3 mM $MgCl_2$, 10 mM dithiothreitol, 0.5 mM deoxynucleoside triphosphate (dNTP), 0.6 μg random primer, 0.5 units of RNase inhibitor and 200 units of reverse transcriptase Superscript II (Invitrogen). Aliquots of the reverse-transcription reaction mixture (1 μl) were amplified with primers specific for human CYPs, UGTs and transporters. For amplification of these, the 25-μl PCR reaction mixtures contained 25 mM Tris-HCl, pH 8.4, 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM of each dNTP and 1 unit of Takara EX Taq™ (Takara Bio, Otsu, Japan) and PCR was carried out under the optimum conditions. The linear range of the PCR amplification products was established by gradually increasing the cycle numbers. Aliquots of the PCR mixture were separated on agarose gels and stained with ethidium bromide. Densitometric analysis of the PCR products was performed with NIH Image. Data were analyzed using the Student's *t*-test and *P* values less than 0.05 were considered significant.

RESULTS AND DISCUSSION

Firstly, we investigated the expression of mRNAs for CYPs, UGTs and transporters in bronchial epithelial cell lines. The expressions of CYP1A2, 2C9, 3A4 mRNA in bronchial epithelial cell lines were lower than those in hepatocytes. In UGTs, mRNA for UGT1A, 2B4, 2B11 and 2B17 were highly expressed in both hepatocytes and bronchial epithelial cell lines, while expression of UGT2B10 mRNA was observed only in hepatocytes. On the other hand, expressions of mRNA for transporters such as MDR1, MRP1 and MRP2 were observed both in hepatocytes and in bronchial epithelial cell lines. MRP1 mRNA was highly expressed in bronchial epithelial cell lines (Table 1).

The effects of BDP on expression of mRNAs for enzymes and transporters in bronchial epithelial cell lines were investigated. However, addition of BDP (1×10^{-9} to 1×10^{-6} M) did not affect the CYP2C9 mRNA and CYP3A4 mRNA expression in NHBE cells and BEAS-2B cells (data not shown). On the other hand, BDP at 1×10^{-7} M increased the expression of UGT2B4 mRNA in BEAS-2B cells, and the expression UGT2B11 mRNA in NHBE cells and BEAS-2B cells, as shown in Fig. 1. Moreover, BDP at 1×10^{-6} M significantly stimulated the expression of mRNA for MDR1, MRP1 and MRP2 in BEAS-2B cells. MDR1 mRNA were well induced with BDP at 1×10^{-7} M as shown in Fig. 2A. In NHBE cells, addition of BDP (1×10^{-6} M) stimulated the expression of mRNA for transporters (Fig. 2B).

Table 1
Expression of mRNAs for CYPs, UGTs and transporters in hepatocytes and bronchial epithelial cells

	Hepatocytes		Bronchial epithelial cells		
	NhHep	HepG2	NHBE	BEAS-2B	Calu-3
CYP1A2	+ + +	+ +	+	+	+
CYP2C9	+ +	+ +	+ +	+	+ +
CYP3A4	+ +	+ + + +	+ + +	+	+
UGT1A	ND	+ + +	+ + + +	+	+ + + +
UGT2B4	ND	+ + +	+	+ + +	+
UGT2B10	ND	+ +	−	−	−
UGT2B11	ND	+ + +	+	+ + +	+ +
UGT2B17	ND	+ + +	+ + +	+ + + +	+ + +
MDR1	+ +	+ + + +	+	+ + +	+ + + +
MRP1	+	+ + + +	+ + +	+ + +	+ + +
MRP2	+ + +	+ + + +	+ +	+	+

+ + + +, very high intensity; + + +, high intensity; + +, medium intensity, +, low intensity; −, not detected; ND, not determined.

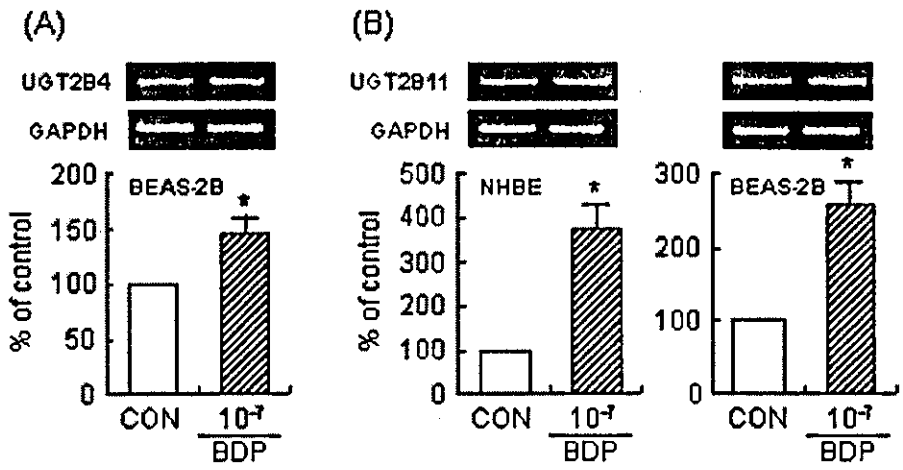

Figure 1. Effects of beclomethasone dipropionate on the expression of mRNA for UGT2B4 (A) and UGT2B11 (B) in human bronchial epithelial cells. NHBE and BEAS-2B cells were incubated for 24 h with or without BDP (indicated final concentration). Aliquots (4 μg) of total RNA were subjected to RT-PCR and products were separated by agarose gel electrophoresis. Each mRNA level was normalized against GAPDH mRNA level. Values (mean ± SE, $n = 3$) are expressed as percentages relative to untreated cells (CON). Significant differences ($^*P < 0.05$) compared with control were analyzed using the Student's t-test.

The present study demonstrates for the first time that a common inhaled steroid BDP induced the expression of UGTs and transporters in bronchial epithelial cells. Aerosols are routinely delivered to the bronchial regions of the airways for the treatment of acute or chronic lung diseases such as asthma or cystic fibrosis. The respiratory epithelium is in direct contact with drugs in inhaled air. Therefore, the activities of drug-metabolizing and transferring proteins in this epithelium could modulate the activity and toxicity of administered drugs. Bronchial epithelial cells are known to express CYPs (*20*), UGTs (*21*) and transporters (*22, 23*). Expressions of metabolic enzymes and transporters have been induced by administered drugs in the liver (*24, 25*), and this might result in the reduction of effectiveness of drug therapy, whereas the changes of metabolic enzymes in peripheral tissues are obscure.

To date, UGT subfamily members have been shown to be induced by various chemical substances (*26*), and several transcription factors such as AP-1 and nuclear factor kappa B

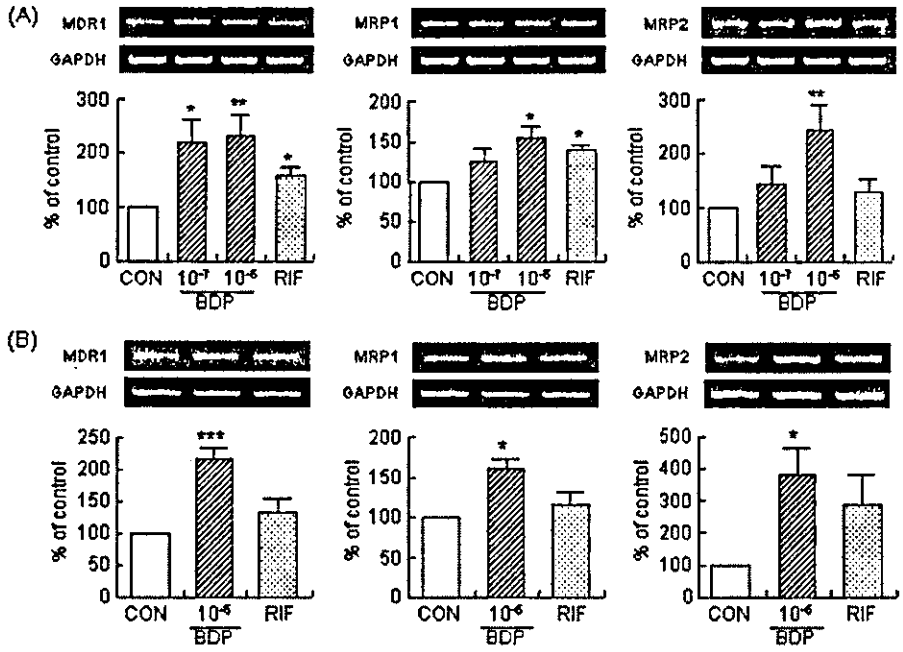

Figure 2. Effects of beclomethasone dipropionate on the expression of mRNA for MDR1, MRP1 and MRP2 in BEAS-2B human bronchial epithelial cells (A) and NHBE human bronchial epithelial cells (B). BEAS-2B or NHBE cells were incubated for 24 h with or without BDP (indicated final concentration) or rifampicin (RIF, 10^{-5} M). Aliquots (4 μg) of total RNA were subjected to RT-PCR and products were separated by agarose gel electrophoresis. Each mRNA level was normalized with GAPDH mRNA level. Values are expressed as percentages relative to untreated cells (CON). Significant differences ($^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.005$) compared with control were analyzed using the Student's *t*-test.

(NF-κB) are reportedly involved in the regulation of these expressions (*27, 28*). On the other hand, glucocorticoids have been shown to increase or inhibit gene expression through a transcription process implicated with AP-1 and NF-κB (29). From these results, it is possible that BDP regulates transcription of UGT gene through an activation of these and other transcription factors.

It has been reported that transporters such as MRP1 and MDR are expressed in mast cells (*30*) and macrophages (*31*). These transporters have also been implicated in the excretion of leukotriene C_4 (LTC_4) (*30, 32*). Wijnholds et al. reported that mice lacking MRP showed decreased secretion of LTC_4 (*33*). Since bronchial epithelial cells also express LTC_4 (*34*), the stimulation of transporter expression by BDP might enhance the LTC_4 secretion, resulting in the exacerbation of immune reaction accompanied by steroid resistance.

Inhaled steroids have been established as an essential therapy. However there are a small number of asthmatic patients who show steroid-resistance (*4, 5*). Steroid resistance poses a therapeutic problem and may result in the use of high doses of steroids, leading to serious side effects. It is possible that the stimulation of metabolism and excretion of inhaled drug at bronchial tissues leads to a decrease in the effect of the drug, although the molecular mechanism of this resistance is unclear. In this study, we have demonstrated that BDP stimulates the expression of UGT, MDR and MRPs in bronchial epithelial cells *in vitro*. It is thus possible that the individual differences of expression of drug metabolizing enzymes and transporters, and their transactivation by BDP, might be implicated in the individual differences of reactivity to steroid medical treatment.

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IL-4-Induced GATA-3 Expression Is a Time-Restricted Instruction Switch for Th2 Cell Differentiation[1]

Noriyasu Seki,*† Mayumi Miyazaki,* Wataru Suzuki,* Katsuhiko Hayashi,‡ Kazuhiko Arima,§ Elmarie Myburgh,‖ Kenji Izuhara,§¶ Frank Brombacher,‖ and Masato Kubo[2]*#

An initial activation signal via the TCR in a restricted cytokine environment is critical for the onset of Th cell development. Cytokines regulate the expression of key transcriptional factors, T-bet and GATA-3, which instruct the direction of Th1 and Th2 differentiation, through changes in chromatin conformation. In this study, we investigated the kinetics of IL-4-mediated signaling in a transgenic mouse, expressing human IL-4R on a mouse IL-4αR-deficient background. These experiments, allowing induction with human IL-4 at defined times, demonstrated that an IL-4 signal was required at the early stage of TCR-mediated T cell activation for lineage commitment to Th2, along with structural changes in chromatin, which take place in the conserved noncoding sequence-1 and -2 within the IL-4 locus. At later times, however, IL-4 failed to promote efficient Th2 differentiation and decondensation of chromatin, even though GATA-3 was clearly induced in the nuclei by IL-4 stimulation. Moreover, IL-4-mediated Th2 instruction was independent from cell division mediated by initial TCR stimulation. The role of IL-4 signaling may have a time restriction during Th2 differentiation. In late stages of initial T cell activation, the chromatin structure of the IL-4 locus retains condensation state. These results demonstrate that IL-4-induced GATA-3 expression is time-restriction switch for Th2 differentiation. *The Journal of Immunology,* **2004, 172: 6158–6166.**

Helper T cells exhibit cytokine expression patterns that divide them into at least two functionally distinct subsets. Th1 cells secrete IL-2, IFN-γ, and TNF-α, which promote cellular immune responses against intracellular pathogens and viruses, mediate delayed-type hypersensitivity responses, and may lead to organ-specific autoimmune diseases. Th2 cells produce IL-4, IL-5, IL-6, IL-10, and IL-13, which promote humoral immune responses mainly against extracellular pathogens. IL-4 and IL-5 regulate the immune response via mast cells and eosinophils, especially in atopic and allergic conditions. The development of Th cells is determined by cytokines present at the early stages of T cell activation, upon encounter with Ag on APCs. The most critical role for the instruction of Th1 and Th2 development is played by cytokines such as IL-12 and IL-4, which act through the STAT4 pathway, or through the STAT6 signaling pathway, respectively (1–4).

Differential cytokine production within Th1 and Th2 cells is controlled at the level of gene transcription. During differentiation, IL-4 transcription is amplified by TCR stimulation in the presence of IL-4. TCR stimulation regulates the expression of a *trans* activator of the IL-4 promoter, *c-maf*, and also regulates the activation of NF-AT and AP-1 family members (5–7). IL-4R signaling controls expression of GATA-3, a Th2-specific transcription factor that regulates lineage commitment to Th2 (8, 9). TCR-mediated signaling, together with CD28 stimulation, augments GATA-3 expression through activating NF-κB (10). GATA-3 protein induces further GATA-3 expression in an autocrine manner, leading to massive up-regulation of GATA-3 transcription (4, 11, 12). In the absence of IL-4-mediated STAT6 activation, ectopic overexpression of GATA-3 inhibits IL-12Rβ2 expression (8) and chromatin remodeling at the IL-4 locus, as well as Th2 cytokine gene expression (12–14). Therefore, in Th2 differentiation, the role of the IL-4 signal is thought to be the initial induction of GATA-3.

Decondensation of chromatin is characterized by hyperacetylation of histones H3 and H4, as well as by increased accessibility to restriction enzymes, DNase I, and transcription factors (15–17). During Th2 differentiation, the chromatin structure in the IL-13/IL-4 locus changes, allowing transcription of the Th2 cytokine genes. In Th cells, TCR stimulation in the presence of IL-4 elicits a cluster of DNase I-hypersensitive sites (18, 19) and histone acetylation (20). Agarwal et al. (21, 22) have found six Th2-specific hypersensitive sites, named HS-I to -V and HS-Va, in the region spanning the IL-4 promoter to the KIF3 locus. Takemoto et al. (23) found additional sites, named HSS1 to HSS3, in the noncoding sequences between the IL-13 and IL-4 genes. The sequences of HSS1–3 and HS-Va are highly conserved between humans and mice, and are designated as conserved noncoding sequence-1 and -2, respectively (24). The deletion of these sequences either in a human YAC transgene or in the endogenous mouse locus reduces secretion of Th2 cytokines during restimulation (24–26). In both Th1 and Th2 lineages, core histone acetylation seems to occur within the first 48 h in the promoter, HS-Va, or conserved

*Research Institute for Biological Sciences, Tokyo University of Science, Yamazaki, Noda City, Chiba, Japan; †Research and Development Division, Mitsubishi Pharma, Aoba-ku, Yokohama, Japan; ‡Department of Molecular Embryology, Research Institute Osaka Medical Center for Maternal and Child Health, Izumi-shi, Osaka, Japan; §Division of Medical Biochemistry, Department of Biomolecular Sciences, and ¶Division of Medical Research, Center for Comprehensive Community Medicine, Saga Medical School, Saga, Japan; ‖Division of Immunology, University of Cape Town, Groote Schuur Hospital, Cape Town, South Africa; and #Signal/Network Team, RIKEN Research Center for Allergy and Immunology, RIKEN Yokohama Institute, Suehiro-cho, Tsurumi, Yokohama, Kanagawa, Japan

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[2] Address correspondence and reprint requests to Dr. Masato Kubo, Division of Immunobiology, Research Institute for Biological Sciences, Tokyo University of Science, 2669 Yamazaki, Noda City, Chiba 278, Japan. E-mail address: mysolfc@rs.noda.tus.ac.jp

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noncoding sequence-1 and -2. The IL-4 signal then further regulates Th2-specific acetylation, subsequently leading to chromatin remodeling (20, 27). HSS1–3, HS-II, intronic enhancer (IE),[3] and HS-Va have all the consensus sequence for GATA-3 binding. Indeed, in Th2 cells, the HS-Va region is precipitated by Ab against GATA-3 (20, 22). This suggests that GATA-3 binding to conserved noncoding sequence-1 and -2 could regulate Th2-specific histone acetylation and chromatin remodeling. However, the mechanisms of how GATA-3 regulates these structural changes remain unclear.

IL-2 and IFN-γ mRNA are induced within 6 h in G_1 to S phase, while IL-4 mRNA is induced after 48 h at a time point when T cells have undergone more than three cell cycles. This indicates that cell division might be needed to induce Th2 cytokine transcription (28). However, other reports using a cell cycle blocker and IL-4 withdrawal to control early IL-4R signaling showed that IL-4 instructed IL-4 production in the first S phase (29, 30). The coordination of IL-4R and TCR signaling regulates IL-4 gene transcription (31, 32). Nevertheless, the individual roles of IL-4R signaling and TCR signaling remain unclear, because both occur around the same time, and their effects are experimentally difficult to separate.

To overcome this problem, we established a transgenic (Tg) mouse model, expressing human IL-4Rα (hIL-4Rα), under the control of an IE from the Ig H chain Eμ locus, to allow specific expression in lymphocytes only. The chimeric IL-4R molecule, composed of the hIL-4Rα chain and the mouse common γ-chain is responsive to hIL-4, therefore allowing us to control IL-4R signaling independent of endogenous IL-4 in lymphocytes only. In this study, we found that IL-4 signaling regulates the competence of effector cytokine production during a restricted phase of initial T cell activation, irrespective of progressive cell divisions. We discuss the importance of the timing of IL-4-mediated GATA-3 expression on Th2-specific chromatin remodeling and on the Th2 lineage commitment.

Materials and Methods

Mice

Tg constructs for the hIL-4R were expressed under the control of an IE of Ig H chain locus (Eμ) promoter. A Tg line that expressed hIL-4R on T and B cells at similar level to endogenous mouse (m)IL-4R (hIL-4RαTg) was selected for this study. The hIL-4R Tg mice were backcrossed to BALB/c genetic background for more than 10 generations. OVA-specific TCR (DO11.10) Tg mice on BALB/c background were kindly provided by K. Murphy (Washington University, St. Louis, MO). IL-4Rα-deficient (mIL-4Rα knockout (KO)) mice were generated on a BALB/c genetic background (33) and crossed with hIL-4RαTg mice. BALB/c mice were purchased from Sankyo (Tokyo, Japan).

Preparation of Th cell

Single spleen cell suspensions were incubated with anti-CD8 mAb for 30 min on ice, and $CD8^+$ cells and B cells were eliminated with rabbit anti-mouse Ig-coated dishes. The enriched $CD4^+$ T cells were suspended in RPMI 1640 medium containing 10% (v/v) FCS, 10 mM HEPES-KOH, pH 7.4, 2 mM L-glutamine, and 50 μM 2-ME, and were stimulated with a combination of plate-coated anti-TCR mAb (H57-597) and soluble anti-CD28 mAb (PV-1), as previously described (34). For DO11.10 Tg T cells, cells were stimulated with 1 μM $OVA_{323-339}$ and irradiated APCs. After 48 h, 30 U/ml IL-2 was added, and cells were cultured another 5 days. Cells from BALB/c and mIL-4Rα KO mice were stimulated in the presence of anti-IL-4 mAb (11B11). Th2 development in hIL-4Rα Tg was conducted with rhIL-4 (10 U/ml) (PeproTech, London, U.K.). Th1 and Th2 cells were prepared with the induction culture by the addition of either 10 U/ml rIL-12 and anti-IL-4 mAb (11B11) or 100 U/ml rIL-4 and anti-IL-12 mAb (C15.6 and C17.8; The Wister Institute, Philadelphia, PA), respectively.

Intracellular cytokine staining

The activated $CD4^+$ T cells were restimulated with anti-TCR mAb for 6 h in the presence of 2 μM monensin (Sigma-Aldrich, St Louis, MO). The cells were fixed with 4% paraformaldehyde and permeabilized with 0.5% Triton X-100. After blocking with 3% BSA-PBS, cells were stained with anti-IFN-γ (XMG1.2) FITC and anti-IL-4 (11B11) PE Abs (34). Flow cytometric analysis was conducted on a FACSort and analyzed by CellQuest software (BD Biosciences, San Diego, CA).

Proliferation assay

$CD4^+$ T cells were stimulated with either mouse or hIL-4 in the presence of PMA for 48 h. The cells were pulsed with [^{3}H]thymidine (0.5 μCi/well) for the last 12 h, and the incorporation of [^{3}H]thymidine was measured by a beta counter.

Analysis of cell division and cytokine production. The enriched $CD4^+$ T cells were labeled with CFSE (Molecular Probes, Eugene, OR) by incubating 1×10^7 cells/ml in PBS with 10 μM CFSE for 8 min at room temperature. The labeling process was stopped with the addition of one aliquot FCS and subsequent three washing steps. Cell divisions were analyzed by FACSort with intracellular cytokine staining after restimulation with anti-TCR mAb, as described above.

Northern blot analysis

Total cytoplasmic RNA was isolated from cells using a TRIzol reagent (Life Technologies, Rockville, MD). Two micrograms of RNA was separated on a 1% (w/v) agarose gel containing 2.2 M formaldehyde. Transfer of RNA onto a Hybond N membrane (Amersham Pharmacia Biotech, Piscataway, NJ), hybridization, and washing were performed according to the procedure supplied by the manufacturer (Roche). The probes used were digoxigenin-labeled antisense riboprobes transcribed from the cDNA template of *IL-12Rβ2*, T-bet, *GATA-3*, and *G3PDH*. Signals were visualized using an alkaline phosphatase-conjugated, anti-digoxigenin Ab (Roche, Mannheim, Germany).

Separation of nuclear and cytoplasmic fraction and Western blot analysis

The nuclear and cytoplasmic fractions were separated by nuclear and cytoplasmic extraction reagent (Pierce, Rockford, IL). The nuclear and cytoplasmic protein (25 μg) were loaded on SDS-PAGE and transferred to polyvinylidene difluoride membrane. The membranes were blotted with anti-GATA-3 mAb (HG3-35) (Santa Cruz Biotechnology, Santa Cruz, CA), anti-NF-AT1 mAb (4G6-G5) (Santa Cruz Biotechnology), anti-NF-AT2 mAb (7A6) (Alexis, San Diego, CA), and anti-c-*maf* rabbit serum (M-153) (Santa Cruz Biotechnology). The blots were visualized with HRP-conjugated goat anti-mouse or anti-rabbit Ig (DAKO, Glostrup, Denmark).

Restriction enzyme accessibility assay

Enriched $CD4^+$ T cells (5×10^6 cells) were used for each condition. Nuclei were prepared, as described previously (20), and resuspended in buffer F (100 mM NaCl, 50 mM Tris (pH 8.0), 5 mM $MgCl_2$, 1 mM EGTA, and 1 mM 2-ME). *Xho*I and *Hga*II (100 U; Toyobo, Osaka, Japan) were added and incubated at room temperature for 1 h. Purified DNA was digested with either *Eco*RI or *Hind*III, ethanol precipitated, and resuspended in TE buffer, and the concentration was measured at absorbance of 260 nm. The digested DNA (5 μg) was transferred onto a Hybond N membrane (Amersham Pharmacia Biotech), and blotted with digoxigenin-labeled appropriated probes.

Retrovirus infection

The protocol for retroviral infection has been previously described in detail (12). Briefly, the murine *GATA-3* cDNA was inserted into pMX-GFP (green fluorescent protein) vector (pMX-GFP-GATA-3). To ensure that GATA-3 and GFP were translated bicistronically, an internal ribosomal entry site was ligated upstream of the GFP. The pMX-GFP-GATA-3 and pMX-GFP control plasmid were transfected into a packaging cell line, PLAT-E, using FuGENE6 (Roche), and, after incubation for 24~48 h, the culture supernatant was harvested and condensed as a viral stock. $CD4^+$-enriched T cells were stimulated with anti-TCR and anti-CD28 mAbs and infected with a viral stock at the indicated time point after primary stimulation. The viral infected $CD4^+$ T cells were restimulated with anti-TCR mAb for 6 h in the presence of 2 μM monensin, and subsequent intracellular staining was conducted.

[3] Abbreviations used in this paper: IE, intronic enhancer; GFI, growth-factor independent; GFP, green fluorescent protein; hIL, human IL; KO, knockout; mIL, mouse IL; Tg, transgenic.

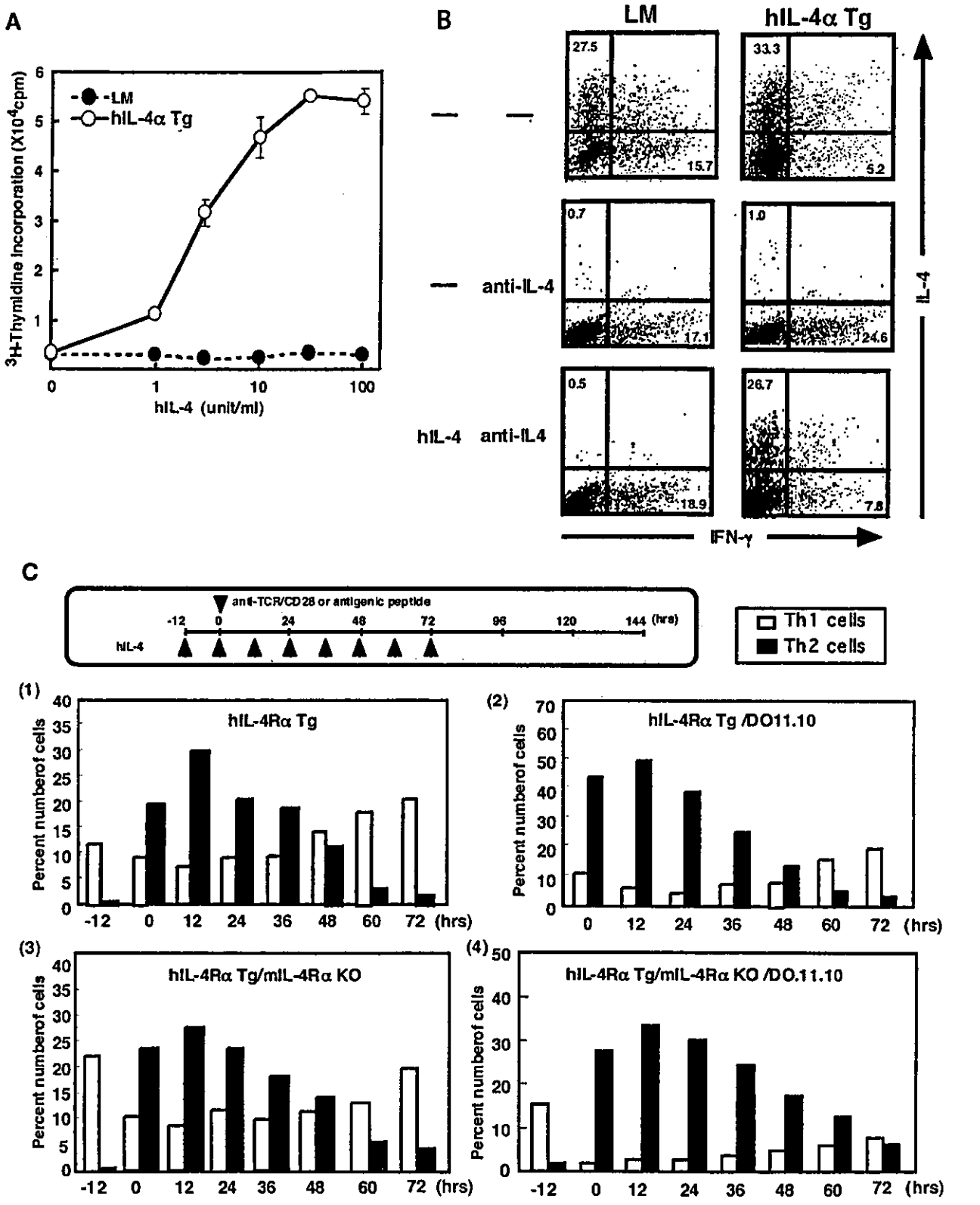
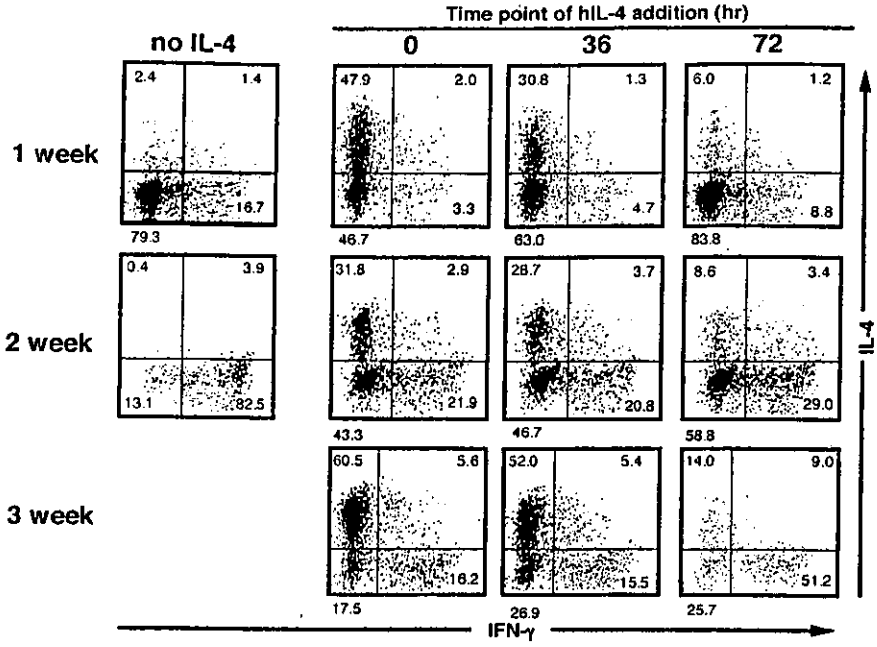
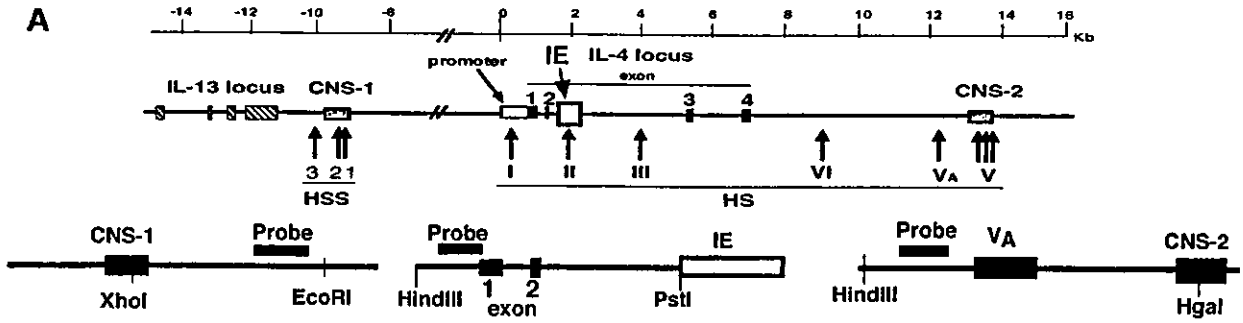
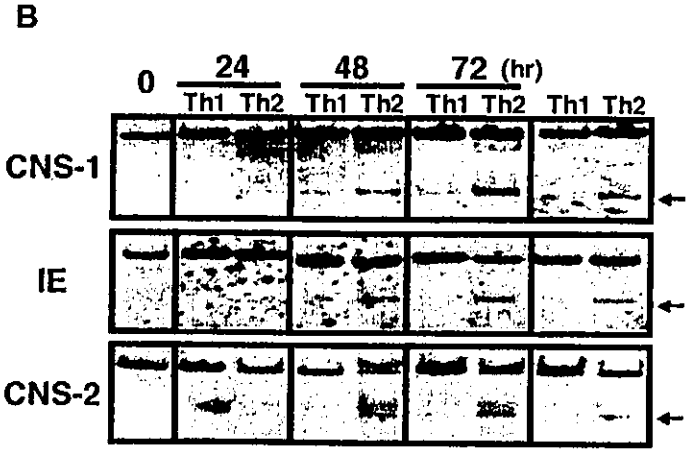


FIGURE 1. *A*, Enriched CD4$^+$ T cells, obtained either from control BALB/c littermates (●) or Tg animals containing hIL-4R (hIL-4R Tg) (○), were stimulated with hIL-4 in the presence of PMA (50 ng/ml) and anti-mIL-4 mAb for 48 h. The proliferative response was measured by the incorporation of [^{3}H]thymidine. *B*, Th cell differentiation was induced with a combination of anti-TCR and anti-CD28 mAbs in the presence or absence of anti-mIL-4 mAb and/or hIL-4. After 7 days, cells were restimulated with anti-TCR mAb and stained for intracellular cytokine. *C*, CD4$^+$ T cells from hIL-4Rα Tg (1) and hIL-4Rα Tg/mIL-4R KO (3) were stimulated with the combination of anti-TCR and anti-CD28 mAbs at time point 0. For CD4$^+$ T cells from hIL-4Rα Tg/DO11.10Tg mice (2) and hIL-4Rα Tg/mIL-4Rα KO/DO11.10 mice (4), cells were stimulated with OVA peptide in context of APCs at time point 0. For hIL-4Rα Tg (1) and DO11.10/hIL-4Rα Tg (2), endogenous IL-4 was neutralized with anti-mIL-4 mAb. hIL-4 (10 U/ml) was added at different time points before or after initial T cell activation (−12 and 0–72 h). For time point −12 h, after 7 days from time point 0, cells were restimulated with anti-TCR mAb, and intracellular cytokine staining was conducted. The numbers represent the percentage of either IL-4- or IFN-γ-producing cells. The experiment was independently performed three times with similar results.







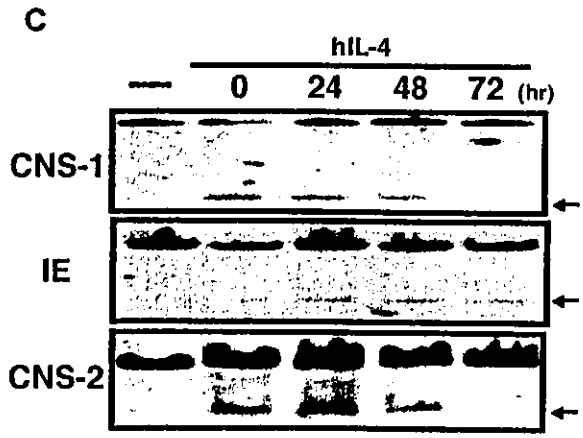


FIGURE 2. $CD4^+$ T cells from hIL-4R Tg were stimulated by anti-TCR and anti-CD28 mAbs in the presence of anti-mIL-4 mAb. hIL-4 was added 0, 36, and 72 h after initial activation. These precultured cells were repeatedly stimulated with anti-TCR and anti-CD28 mAbs in the presence of hIL-4 at weekly intervals. Cells were restimulated with anti-TCR mAb, and the cytokine production profile was assessed by intracellular cytokine staining. The experiment was independently performed three times with similar results.

FIGURE 3. *A*, Position of conserved noncoding sequence-1, IE, and conserved noncoding sequence-2 region on the IL-4 locus (*upper panel*). Restriction enzyme site and probe for the detection of conserved noncoding sequence-1, IE, and conserved noncoding sequence-2 (*lower left*). HSS1~3 and HS I~V indicate DNase I-hypersensitive site. *B*, Nuclei were prepared from naive $CD4^+$ T, Th1, and Th2 cells (5×10^6 cells) and digested with *XhoI* for conserved noncoding sequence-1, *PstI* for IE, or *HgaI* for conserved noncoding sequence-2. DNA was purified and digested with either *Eco*RI for conserved noncoding sequence-1 or *Hind*III for IE and conserved noncoding sequence-2 and analyzed by Southern blotting, using probes for conserved noncoding sequence-1 or conserved noncoding sequence-2. The arrow indicates *XhoI*, *PstI*, and *HgaI* sites on conserved noncoding sequence-1, IE, and conserved noncoding sequence-2. $CD4^+$ T cells from DO11.10 Tg mice were stimulated with OVA peptide and APCs in Th1- and Th2-skewing condition. Nuclei were prepared at different time points after initial T cell activation and the restriction enzyme assay was conducted, as described in *A*. *C*, $CD4^+$ T cells from hIL-4R/mIL-4R KO/DO11.10 mice were stimulated with OVA peptide in the context of APCs. hIL-4 was added at different time points, and cells were further cultured until 7 days after the initial activation. Nuclei were prepared from the precultured cells, and the restriction enzyme assay was conducted. The experiment was independently performed three times with similar results.

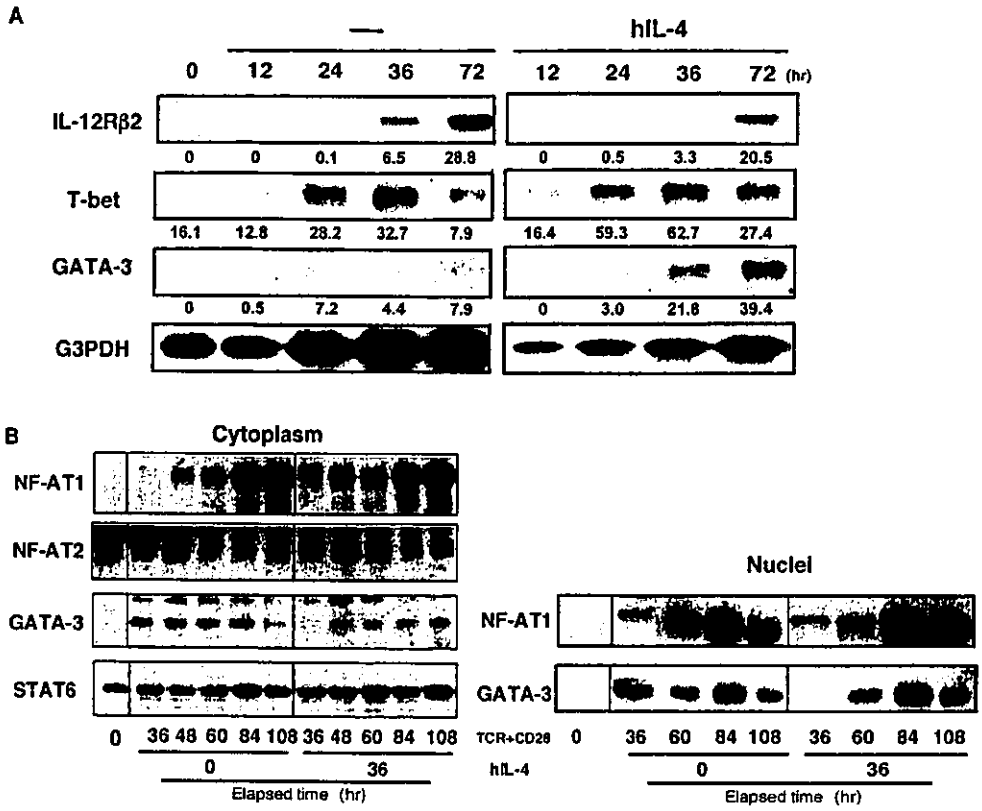


FIGURE 4. *A*, Enriched hIL-4R Tg/IL-4KO $CD4^+$ T cells were stimulated with anti-TCR and anti-CD28 mAbs in the presence or absence of hIL-4. Cells were harvested at the indicated time points after the initial activation, and the expression of IL-12β2, T-bet, GATA-3, and G3PDH mRNAs was measured by Northern blotting analysis. The number indicates the analyzed time points. *B*, Enriched hIL-4R Tg cells were stimulated with anti-TCR and anti-CD28 mAbs in the presence of anti-mIL-4 mAb. hIL-4 was added at time 0 or 36 h after initial activation, and protein expressions of NF-AT1, NF-AT2, GATA-3, and STAT6 in cytoplasmic (*left*) or nuclear (*right*) fraction were examined by Western blotting. The levels of the transcript were quantitated with densitometry and normalized with G3PDH.

Results

hIL-4R substitutes for the function of the mIL-4R

We established a series of Tg mouse lines expressing hIL-4Rα chain under the control of the Ig H chain promoter, and selected lines in which T cells apparently expressed hIL-4Rα chain for backcrossing to a BALB/c genetic background. As expected, T (Fig. 1*A*) and B (data not shown) cells from Tg mice, but not from control littermates, responded to hIL-4 in a proliferation assay. Furthermore, splenic $CD4^+$ T cells from the Tg animals, stimulated with exogenous hIL-4 in the presence of anti-mIL-4 mAb, showed Th2 differentiation at levels comparable to those seen with endogenous IL-4 (Fig. 1*B*). These results suggest that hIL-4 is able to fully substitute for endogenous mIL-4, resulting in Th2 polarization.

To study the kinetics of IL-4-mediated Th2 instruction, T cells from hIL-4Rα Tg and DO11.10/hIL-4α Tg were stimulated with anti-TCR and anti-CD28 mAbs and OVA antigenic peptide in the presence of BALB/c APCs. To eliminate the effect of endogenous IL-4, cells were cultured in anti-IL-4 mAb. To exclude signaling via endogenous IL-4 completely, hIL-4Rα Tg mice were also crossed with mIL-4Rα KO mice on a BALB/c genetic background (hIL-4Rα Tg/IL-4Rα KO). Time 0 was the initial stimulation with either anti-TCR and anti-CD28 mAb, or OVA peptide, and 10 U/ml hIL-4 was added at different times (−12 to 72 h; Fig. 1*C*, *top panels*).

First, we defined the kinetic relationship between initial TCR signaling and IL-4 signaling. Before T cell activation though TCR, $CD4^+$ T cells of hIL-4Rα Tg were cultured in the presence of hIL-4 for 12 h, and then after hIL-4 withdrawal, cells were stimulated with either anti-TCR and anti-CD28 mAbs, or Ag. The IL-4-pretreated T cells failed to induce IL-4-producing cells upon restimulation with anti-TCR mAb (Fig. 1*C*). When T cells from hIL-4Rα Tg, hIL-4Rα Tg/DO11.10 double Tg, or hIL-4Rα/mIL-4Rα KO/DO11.10 mice were initially stimulated with TCR cross-linking or OVA in the absence of hIL-4, T cells differentiated no or few Th2 cells. Th2 cells first appeared when hIL-4 was added between 0 and 36 h after initial TCR stimulation (Fig. 1*C*). However, their frequency rapidly declined thereafter. At 60~72 h after initial TCR stimulation, the frequency of IL-4-producing Th2 cells was drastically reduced in both initial TCR cross-linking and antigenic stimulation (Fig. 1*C*). These results demonstrate that TCR activation is needed before IL-4 signaling to induce Th2 differentiation, and that the IL-4 signal is effective 0~48 h after initial TCR activation.

However, we cannot exclude the possibility that an unskewed population, which has remained at day 7, could subsequently differentiate into Th2 cells. To investigate this possibility, hIL-4Rα Tg $CD4^+$ T cells that had been treated with hIL-4 at 0, 36, or 72 h were constitutively restimulated with Ag and hIL-4 at weekly intervals, and their differentiation profile was determined after 1, 2, or 3 wk. In cells that had been treated with hIL-4 at 0 or 36 h, the frequency of Th2 cells increased during the 3-wk restimulation (Fig. 2). In contrast, in cells that had been treated with hIL-4 at 72 h, Th2 differentiation was impaired, and consecutive Ag and hIL-4 restimulation did not restore it (Fig. 2), although these T cells retained hIL-4 responsiveness in a proliferation assay (data not shown). These results suggest that $CD4^+$ T cells lost their capability to differentiate into Th2 cells during initial TCR activation.

Kinetics of Th2-specific chromatin remodeling at conserved noncoding sequence-1 and -2 on IL-4 locus was related to the requirement of the IL-4 signal

A conformational change in chromatin structure to allow access to the transcriptional factors is critical for cell lineage commitment (21, 23). To understand the behavior of chromatin at the IL-4 locus during Th2 differentiation, nuclear fractions were collected at different times after primary stimulation, and treated with restriction

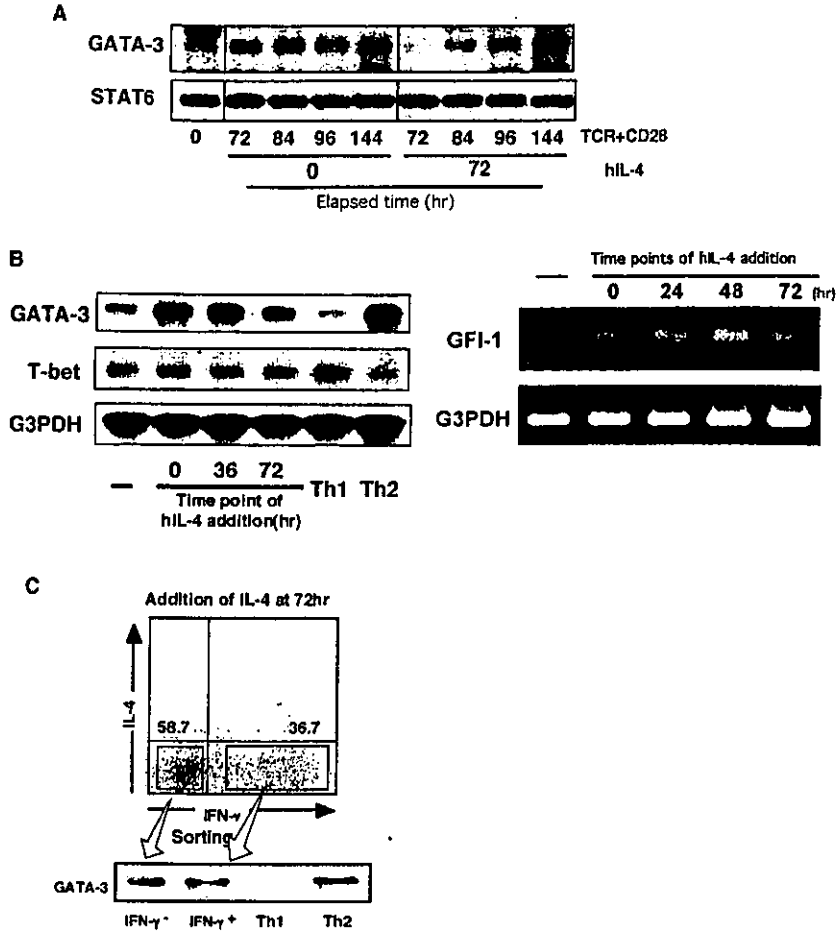


FIGURE 5. *A*, Enriched hIL-4R Tg cells were stimulated with anti-TCR/CD28 mAbs (time point 0) in the presence of anti-mIL-4 mAb. hIL-4 was added at 0 or 72 h, and cytoplasmic GATA-3 and STAT6 were assessed by Western blotting. The experiment was performed three times independently with similar results. *B*, Enriched hIL-4R Tg/mIL-4R KO $CD4^+$ T cells were stimulated with anti-TCR and anti-CD28 mAbs. hIL-4 was added at 0, 36, and 72 h, and expression of T-bet, GATA-3, and G3PDH mRNA was analyzed by Northern blotting. Expression of GF-1 was analyzed by RT-PCR. *C*, TCR-activated $CD4^+$ T cells from hIL-4R Tg/mIL-4R KO mice were stimulated with hIL-4 at 72 h. After 5 days, cells were restimulated with anti-TCR mAb, and intracellular cytokine staining was conducted. IFN-γ-producing and nonproducing cells were sorted, and expression of GATA-3 was examined by Western blotting.

enzymes that cut in each regulatory region, the conserved noncoding sequence-1, conserved noncoding sequence-2, and IE (Fig. 3*A*). $CD4^+$ T cells from DO11.10 Tg mice were stimulated with OVA in the presence of APCs, and 48 h later, Th2-specific alterations in the chromatin structure were observed in the conserved noncoding sequence-1 and -2 regions and IE (Fig. 3*B*).

We next examined whether the requirement for an IL-4 signal correlated with the kinetics of Th2-specific chromatin remodeling at conserved noncoding sequence-1 and -2. $CD4^+$ T cells from hIL-4Rα/mIL-4Rα KO/DO11.10 mice were stimulated with Ag, and hIL-4 was added at different time points. Seven days after the initial stimulation, chromatin structure was examined as above. When hIL-4 was given 0–48 h after initial stimulation, Th2-specific remodeling was seen in the conserved noncoding sequence-1 and -2 regions; but when hIL-4 was given 72 h after initial stimulation, remodeling was absent (Fig. 3*C*). However, the IE that mainly acts on mast cells showed distinct profile in the remodeling. Decondensation was observed even when hIL-4 was given 72 h after initial stimulation (Fig. 3*C*). These results indicated that the requirement for IL-4 signaling for the competence to secrete IL-4 in restimulation strictly corresponds with the alteration of the chromatin structure at conserved noncoding sequence-1 and -2 regions.

Influence of the IL-4 signal on the expression of transcriptional factors regulating IL-4 gene expression

GATA-3 is a master regulator controlling the lineage commitment of Th2 cells (12, 14), and its expression is tightly regulated by the IL-4-mediated STAT6 activation pathway (11). Therefore, selective expression of GATA-3 occurs in committed Th2 cells only. We examined the kinetics of *GATA-3*, *T-bet*, and *IL-12Rβ2* mRNA expression. $CD4^+$ T cells from hIL-4Rα Tg/mIL-4Rα KO mice were stimulated with anti-TCR/CD28 mAbs. In the absence of hIL-4, T cells predominantly differentiated into Th1 cells and no GATA-3 expression was found. When hIL-4 was added at time 0, GATA-3 expression clearly appeared from 24 h after initial TCR/CD28 stimulation (Fig. 4*A*). These results demonstrate that initial GATA-3 expression was regulated by the IL-4 signal. In contrast,

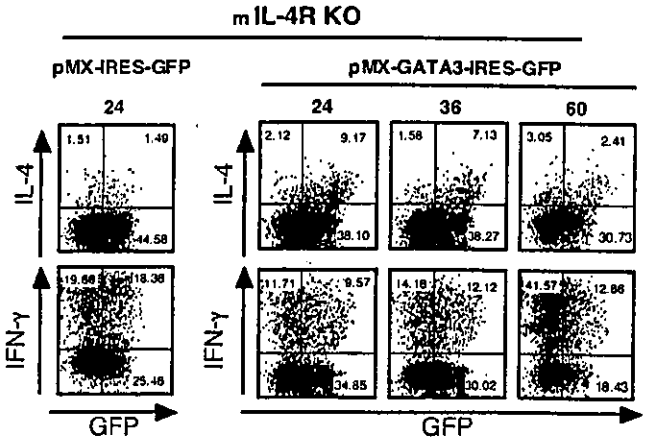


FIGURE 6. $CD4^+$ T cells from mIL-4R KO were stimulated with anti-TCR and anti-CD28 mAbs, and infected with the retrovirus vector, pMX-GATA3-IRES-GFP, at different time points. At day 7 after the initial activation, cytokine production was examined by intracellular staining. The experiment was performed three times independently with similar results.

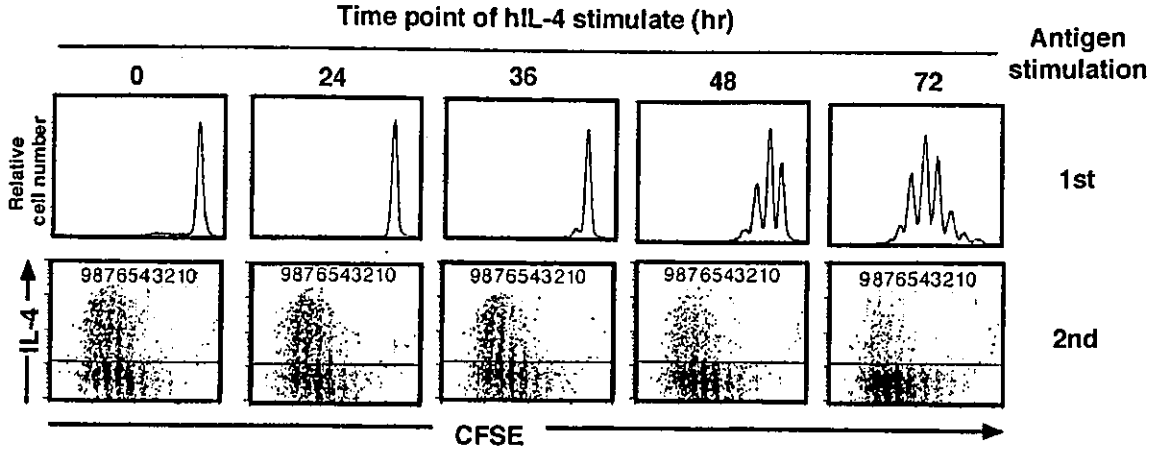


FIGURE 7. CFSE-labeled DO11.10 CD4$^+$ T cells were stimulated with OVA peptide in the presence of BALB/c APCs, and cell division was assessed by the intensity of green fluorescence at indicated time point after initial T cell stimulation (1st). The *x*-axis represents the intensity of green fluorescence, with progression of cell division moving *right* to *left* owing to dilution of intracellular dye. To examine IL-4 production ability in recall response, cells were restimulated with anti-TCR mAb (2nd) after 7 days, and IL-4-producing cells were detected, as described for Fig. 1. 0–9, Indicates the number of cell divisions. The experiment was performed three times independently with similar results.

T-bet and IL-12Rβ2 expression kinetics were not affected by the IL-4 signal (Fig. 4*A*).

Other transcriptional factors or coactivators, NF-AT and c-*maf*, are also involved in commitment to the Th2 lineage (35–38). Thus, we examined IL-4 responsiveness on the expression of these transcriptional factors. The hIL-4 Tg CD4$^+$ T cells expressed a small amount of NF-AT2, and no NF-AT1, c-*maf*, or GATA-3. Following TCR stimulation, expression of NF-AT2 increased rapidly for 48 h and then declined independently of IL-4. Similarly, NF-AT1 expression increased following TCR stimulation, however, in later time points (Fig. 4*B*). IL-4 delayed the induction of NF-AT1 expression in the cytoplasm, and c-*maf* expression was not detected in the nuclear fraction (data not shown). Furthermore, IL-4 made no difference to TCR-induced dephosphorylation of NF-AT1 or its translocation to the nucleus (Fig. 4*B*). Together, these results suggest that the IL-4 signal did not influence the kinetics of NF-AT1 and -2 expression, or of TCR-induced nuclear localization.

Administration of hIL-4 at 0 or 36 h after initial TCR stimulation rapidly induced GATA-3 protein in cytoplasm and nuclei (Fig. 4*B*), again confirming IL-4-dependent GATA-3 expression at early stages of initial activation. We next studied whether the hIL-4R-mediated signal was able to induce GATA-3 expression even later, as IL-4R failed to transduce the signal in Th1 cells. Thus, we analyzed expression of GATA-3 at 72 h, in which most T cells were differentiating into Th1 cells. We also examined the expression of growth-factor independent 1 (GFI-1), which was induced by IL-4-STAT6 signaling. Both GATA-3 mRNA and protein as well as GFI-1 mRNA clearly appeared at this late time point (Fig. 5, *A* and *B*).

Next, we studied whether GATA-3 protein is only expressed in the residual uncommitted population of T cells. Subsequent GATA-3 expression in Th1-committed and uncommitted T cells was examined when hIL-4 was added at 72 h. At day 7, cells were restimulated with anti-TCR mAb, IFN-γ-producing or nonproducing subpopulations were sorted, and their GATA-3 protein was analyzed by Western blotting. Both Th1-committed and uncommitted T cells expressed GATA-3 to a similar extent (Fig. 5*C*). These results indicate that IL-4 signaling through the hIL-4R induced GATA-3 expression, even in a Th1-skewing condition.

Timing of GATA-3 expression regulates the efficiency of Th2 development

Ectopic expression of GATA-3 can mimic the function of IL-4 signal, by inducing Th2 cytokines and Th2-specific chromatin remodeling, but our results address that timing may be critical. To further investigate this, we examined whether ectopic expression of GATA-3 late in the differentiation of CD4$^+$ T cells could result in efficient development of Th2 cells. GATA-3 and GFP were coexpressed in T cells by a bicistronic retrovirus construct (pMX-GATA3-GFP), and the proportion of IL-4-producing GFP-positive population was assessed. The mIL-4R KO T cells were stimulated with anti-TCR/CD28 mAbs, and then infected with pMX-GATA3-GFP after 24, 36, or 60 h. Infection at 24 h resulted in 9% GATA-3- and IL-4-coproducing T cells, which corresponds to ~20% of the total GFP$^+$ cells (Fig. 6). Infection at 60 h reduced the proportion of T cells producing IL-4, to ~7% of total GFP$^+$ cells (2.4% GFP$^+$IL-4$^+$ from 33.1% GFP$^+$ cells). These results confirm that the timing of GATA-3 expression is critical for determining lineage commitment. Nevertheless, the presence of some IL-4$^+$/GFP^{high} cells after infection at 60 h indicates that even at this late stage, high levels of GATA-3 expression can induce commitment to the Th2 lineage.

Relationship between cell division and lineage-specific IL-4 gene expression

Previous reports concluded that lineage-specific IL-4 gene expression is regulated by the number of cell divisions. Thus, we studied the relationship between cell division and IL-4 signaling required for Th2 lineage commitment. CFSE-labeled hIL-4Rα Tg/mIL-4Rα KO/DO11.10 CD4$^+$ T cells were stimulated with OVA peptide. Cell division was profiled in a time kinetic (Fig. 7, *upper panel*), and the proportion of IL-4-producing cells following restimulation with anti-TCR mAb was examined at day 7 (Fig. 7, *lower panel*). Consistent with previous observations (26), IL-4-producing cells appeared after five generations (Fig. 7, *lower panel*). At 36 h poststimulation, in which hIL-4 efficiently induced Th2 differentiation (Fig. 1*C*), most T cells remained at the stage before undergoing into first cell division, while at 72 h poststimulation, all cells entered into successive cell division (Fig. 7, *upper panel*). These results suggest that IL-4 signaling may be able to

instruct Th2 lineage commitment before progression to cell division.

Discussion

This study investigated the relevance of IL-4 signaling for the regulation of Th2 differentiation. We established a Tg mouse model allowing us to separate IL-4 signaling from TCR-mediated stimulation. IL-4-mediated signaling regulates chromatin structure at the IL-4 locus and thereby influences the competence to secrete effector cytokines during Th2 differentiation. In this study, we show evidence that the initial 48 h is a critical period for Th2 commitment. This commitment depended on IL-4-induced GATA-3 expression, as we demonstrated that Th2 cells differentiated from naive T cells only, when GATA-3 was expressed at appropriate times. Furthermore, we found that IL-4-mediated lineage commitment was independent of cell division.

During lineage commitment, TCR and IL-4 signaling act synergistically on Th2-specific alterations of chromatin structure, by hyperacetylation of core histones H3 and H4 (20). TCR stimulation causes the formation of BAG or Brm associated factor (BAF) complexes with the nuclear matrix, leading to decondensation of heterochromatin (39). However, the regulatory regions of the IL-4 locus are acetylated equally in the first 24 h in both Th1- and Th2-skewing conditions (20), suggesting that initial TCR signaling promotes the decondensation of heterochromatin and the early increase of histone acetylation. Th2-specific decondensation in the regulatory regions of the IL-4 locus became visible only in a time window between 36 and 48 h (Fig. 3*C*). These observations suggest that if the chromatin remains condensed for long times, it eventually becomes incapable of Th2-type condensation. The major role of IL-4 signaling is to induce GATA-3 expression, and the induced GATA-3 then initiates the Th2-specific chromatin remodeling, subsequently leading to competence to secrete IL-4 upon restimulation (12). Therefore, the timing of GATA-3 expression may be critical for permissive chromatin alteration. This notion is supported by evidence that the timing of Th2-specific chromatin remodeling in the conserved noncoding sequence-1 and -2 regions matches the kinetics of the IL-4-mediated GATA-3 expression.

Previous work has examined whether cell division was necessary for the instruction of IL-4 production upon restimulation (28). It has been reported that T cells have to complete a certain number of cell cycles to become competent to secrete relatively high amounts of IL-4 upon restimulation. We confirmed this as five cell divisions were needed until substantial IL-4 production upon restimulation (Fig. 7, *lower panel*). However, the previous report did not clearly indicate the timing of IL-4 signaling during Th2 differentiation (28), because it ignored the possible involvement of IL-4 secreted from naive T cells. Richter et al. (29) argued that cell division is not critical for instruction into IL-4-producing cells upon restimulation, because use of L-mimosine, which blocks the cell cycle before S phase, did not affect IL-4-induced Th2 differentiation. Furthermore, they showed that coordination of TCR and IL-4R signaling is necessary for the recall production of IL-4, and that this coordination is able to control Th2 commitment for at least 1 day. Their results resemble our data using hIL-4R Tg mice, as IL-4 was able to induce a substantial number of Th2 cells, even before the cells undergo cell division. Taken together, our data suggest that the timing of GATA-3 expression, rather than cell division, is the important factor in the acquisition of competence to secrete IL-4.

The recent discovery of GFI-1 provides an alternative explanation for the role of IL-4 signaling, namely that IL-4 simply selects a subpopulation that responds to the IL-4-STAT6 signaling, rather than instructing the entire population into Th2 differentiation (4, 40). Previous reports have demonstrated that IL-4R signaling is selectively impaired in Th1-committed cells (41–43). This selective defect causes the selective expansion of IL-4-responding Th2 cells. However, in this study, we showed that the IL-4 signal introduced through hIL-4R is able to induce GATA-3 and GFI-1 expression, even in Th1-committed cells. In these cells, the expressed GATA-3 and GFI-1 expression dose not promote Th2 differentiation, suggesting that IL-4 signaling may act by instruction, rather than selection. However, further investigation will be required to clarify the role of IL-4 signaling on Th2 lineage commitment.

Acknowledgments

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