Table 2. ^{1}H (800 Mz)- and ^{13}C (200 MHz)-NMR Spectral Data[a] of Peracetylated Crocin (**1a**) and Neocrocin A (**2a**) (in $CDCl_3$)

Peracetylated crocin (**1a**)[b]			Peracetylated neocrocin A (**2a**) (α isomer)[b]					
No.	δ_H	δ_C	No.	δ_H	δ_C	No.	δ_H	δ_C
8, 8′		166.09	8		166.07	8′		166.92
9, 9′		124.57	9		124.55	9′		125.02
10, 10′	7.33 (d, 12.4)	141.45	10	7.32 (d, 11.4)	141.45	10′	7.20 (d, 12.4)	140.46
11, 11′	6.54 (dd, 11.7, 14.9)	123.66	11	6.53 (dd, 11.5, 15.1)	123.71	11′	6.50 (dd, 11.7, 14.9)	123.63
12, 12′	6.68 (d, 14.5)	145.42	12	6.68 (d, 15.2)	145.42	12′	6.60 (d, 15.1)	144.85
13, 13′		138.00	13		136.96	13′		136.85
14, 14′	6.42 (d, 10.1)	136.95	14	6.41 (d, 9.7)	136.33	14′	6.39 (d, 10.1)	136.02
15, 15′	6.73 (dd, 2.1, 8.0)	131.83	15	6.71 (br dd)	131.84	15′	6.72 (br dd)	131.67
19, 19′	1.98 (s)	12.87	19	1.97 (s)	12.70	19′	1.91 (s)	12.80
20, 20′	1.99 (s)	12.73	20	1.98 (s)	12.90	20′	1.98 (s)	12.90
Glucose A, C			Glucose A			Glucose C		
1	5.74 (d, 8.2)	92.08	1	5.74 (d, 7.8)	92.08	1	6.73 (d, 3.7)	88.99
2	5.20 (t, 9.0)	70.29	2	5.20 (t, 9.0)	70.29	2	5.07 (dd, 3.9, 10.3)	69.63
3	5.27 (t, 9.4)	72.90	3	5.26 (t, 9.6)	72.90	3	5.55 (t, 9.9)	70.08
4	5.01 (t, 9.6)	68.42	4	5.01 (t, 9.8)	68.38	4	5.03 (t, 10.1)	68.47
5	3.83 (ddd, 2.4, 6.4, 10.5)	74.01	5	3.82 (m)	74.01	5	4.05 (m)	70.99
6	3.59 (dd, 5.8, 11.7)	67.68	6	3.59 (dd, 5.8, 11.7)	67.68	6	3.53 (dd, 6.0, 11.0)	67.96
	3.93 (dd, 2.3, 11.4)			3.92 (1H, br d)			3.93 (br d)	
Glucose B, D			Glucose B			Glucose D		
1	4.55 (d, 8.2)	100.65	1	4.55 (d, 8.2)	100.65	1	4.53 (d, 7.8)	100.85
2	4.98 (dd, 7.8, 9.6)	70.99	2	4.97 (t, 8.9)	70.99	2	4.97 (t, 8.9)	70.99
3	5.17 (t, 9.4)	72.90	3	5.17 (t, 9.4)	72.90	3	5.19 (t, 9.4)	72.76
4	5.05 (t, 10.2)	68.42	4	5.05 (t, 9.4)	68.43	4	5.07 (t, 9.9)	69.03
5	3.63 (ddd, 2.3, 4.6, 10.1)	71.98	5	3.63 (m)	71.98	5	3.67 (ddd, 2.4, 4.5, 10.0)	72.02
6	4.09 (dd, 2.3, 12.4)	61.88	6	4.09 (dd, 1.8, 12.4)	61.88	6	4.11 (dd, 2.1, 12.2)	61.88
	4.24 (dd, 4.6, 12.4)			4.23 (dd, 5.0, 12.3)			4.26 (dd, 4.6, 12.4)	
Acetyl	2.00—2.07 (s)	20.65—20.80		1.98—2.06 (s)	20.65—20.94			
		169.45—170.73			169.36—170.79			

a) Chemical shifts are in ppm and coupling constants in Hz are in parentheses. b) Assignments were made using 1D-TOCSY, COSY, HMQC and HMBC.

cose A and B of **2a** were identical to those of **1a**, and the HMBC correlations of the H-1 (δ_H 5.74) of glucose A to C-8 (δ_C 166.07) of crocetin, and the H-1 (δ_H 4.55) of glucose B to C-6 (δ_C 67.68) of glucose A, suggested the existence of a gentiobiosyl moiety connected to crocetin at the anomeric position, similar to in **1a**. By contrast, the chemical shifts and coupling constants of glucose C suggested that the anomeric position of glucose C was in the α configuration, and the HMBC correlations of the H-2 (δ_H 5.07) of glucose C to C-8′ (δ_C 166.07) of crocetin, and the H-1 (δ_H 4.53) of glucose D to C-6 (δ_C 67.96) of glucose C, supported the notion that another gentiobiosyl moiety was connected to the crocetin *via* the number 2 position on glucose C. The ^{1}H- and ^{13}C-NMR data for **1a** and **2a** are summarized in Table 2 and key correlations of **2a** are shown in Fig. 2. These observations provided evidence that neocrocin A (**2**) possessed a unique binding system for sugars.

As neocrocin A (**2**) isolated from gardenia yellow was a mixture of isomers along with crocin (**1**), we initially suspected that neocrocin A (**2**) was an artefact formed by crocin (**1**) *via* the rearrangement of the acyl moiety during the isolation process. However, the LC profile of gardenia yellow, especially the peak height of neocrocin A (**2**), remained unchanged after dissolving again with ethanol and water under acidic and basic conditions. We thus concluded that neocrocin A (**2**) was an original constituent of the *G. jasminoides* fruits. To our knowledge, this is the first published evidence that the binding system for the sugars of a crocetin glycosyl ester is not limited to the anomeric position in nature. We are further investigating the possibility that other types of crocetin glycoside might also exist in this fruit through an ongoing study.

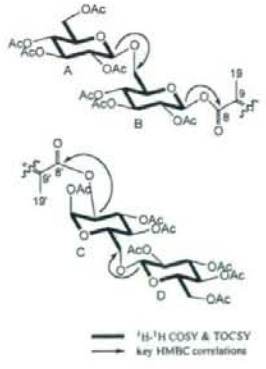

Fig. 2. Key Correlations of Peracetylated Neocrocin A (**2a**)

Experimental

General IR spectra were recorded on a JASCO FT/IR-4100. UV/Vis spectra were recorded on a JASCO UV-560. FAB-MS spectra were measured with JEOL JMS-700 system and ESI-MS spectra were measured with JEOL JMS-T100LC "AccuTOF". NMR spectra were recorded on JEOL JNM-ECA (800 MHz) and JNM-ECA (500 MHz). Diaion HP-20 was used for open-column chromatography. The preparative LC-MS system (Waters FractionLynx™MS auto purification system), which is described in our previous report,[6] was used for isolation of compounds on this research. The

solvents for LC-MS were purchased from Sigma-Aldrich Japan. All of the other chemicals were of reagent or HPLC grade and were used without further purification.

Plant Material The dried gardenia fruits, defined as the fruits of *Gardenia jasminoides*, were purchased from Uchida Wakanyaku Co., Ltd., Japan, in July 2004. The crude drug name was Gardeniae Fructus (Serial No.: VMAMQ), and the voucher specimen (GJ 72004) was deposited at Division of Food Additives in National Institute of Health Sciences.

Extraction and Isolation Gardenia yellow was extracted by stirring dried gardenia fruits (1 kg) with 50% aqueous ethanol at ambient temperature in the dark for 2 h. The part of extracted gardenia yellow (2.25 g) was subsequently fractionated on a Diaion HP-20 column (40 mm i.d.×250 mm) by successive elution with 0—100% MeOH. The 60—70% methanol eluate was then concentrated *in vacuo* and the residue was loaded into a preparative LC-MS system.[6] The conditions were as follows: column, Waters XTerra® Prep MS C_{18} (5 μm, 19 mm i.d.×100 mm); mobile phase, H_2O : MeOH = 58 : 42; flow rate, 10 ml/min; make-up liquid, MeOH 1.0 ml/min; injection volume, 200 μl; and detection and collection trigger, ESI (pos.) *m/z* 999.5. The electrospray source ran at a 3.0 kV capillary voltage, with 120 and 350 °C source and desolvation temperatures, respectively, and with 350 and 60 l/h desolvation and cone gases, respectively. The cone voltage was 50 V. Full-scan acquisition between *m/z* 100 and 2000 was performed at a scan speed of 0.3 s/scan, with a 0.1-s inter-scan delay. The solvent delivered to the electrospray interface was split in a 1 : 4 ratio, delivering around 200 μl/min to the interface. The peaks of crocin (**1**) (retention time (t_R) 15.3 min) and neocrocin A (**2**) (t_R 19.0 min) were each fractionated and concentrated *in vacuo* at <40 °C. The overall procedure was repeated approximately 25 times to afford 220 mg of crocin (**1**) and 18 mg of neocrocin A (**2**).

Crocin (1)[2] Red amorphous powder. IR (KBr) ν_{max} cm^{-1}: 1069, 1227, 1271, 1577, 1699, 2920, 3398. UV/Vis (MeOH) λ_{max} (log ε): 433 (4.49), 458 (4.46), 259 (3.65). HR-ESI-MS *m/z*: 999.3674 $[M+Na]^+$ (Calcd for $C_{44}H_{64}O_{24}Na$: 999.3685). ^1H (800 MHz)- and ^{13}C (200 MHz)-NMR spectral data: see Table 1.

Neocrocin A (2) Red amorphous powder. IR (KBr) ν_{max} cm^{-1}: 1023, 1227, 1270, 1578, 1702, 2918, 3375. UV/Vis (MeOH) λ_{max} (log ε): 432 (4.73), 457 (4.69), 258 (3.97). HR-ESI-MS *m/z* 999.3707 $[M+Na]^+$ (Calcd for $C_{44}H_{64}O_{24}Na$, 999.3685). ^1H (800 MHz)- and ^{13}C (200 MHz)-NMR spectral data: see Table 1.

Crocetin Dimethyl Ester (3) Methanolysis of **2** (10 mg) with 5% HCl methanol at room temperature for 50 h resulted in red amorphous powder (**3**, 3 mg) after purification by Silica gel column chromatography (EtOAc : hexane = 2 : 3). ESI-MS *m/z*: 327 $[M-H]^-$. ^1H-NMR ($CDCl_3$, 500 MHz) δ: 1.98 (6H, s, H-20, 20′), 1.99 (6H, s, H-19, 19′), 3.75 (6H, s, MeO×2), 6.35 (2H, br d, J=10.0 Hz, H-14, 14′), 6.53 (2H, dd, J=11.0, 15.0 Hz, H-11, 11′), 6.60 (2H, d, J=15.0 Hz, H-12, 12′), 6.70 (2H, dd, J=3.0, 8.0 Hz, H-15, 15′), 7.28 (2H, dd, J=1.4, 11.0 Hz, H-10, 10′); ^{13}C-NMR ($CDCl_3$, 125 MHz) δ: 12.87 (C-20, 20′), 12.99 (C-19, 19′), 51.92 (MeO×2), 123.88 (C-11, 11′), 126.48 (C-9, 9′), 131.40 (C-15, 15′), 135.43 (C-14, 14′), 136.79 (C-13, 13′), 138.97 (C-10, 10′), 143.84 (C-12, 12′), 169.02 (C-8, 8′).

Peracetylated Crocin (1a) and Neocrocine A (2a) Peracetylation of gardenia yellow (1.0 g) was carried out with Ac_2O/pyridine (cat. DMAP), affording peracetylated crocin (**1a**, 1.5 g) (t_R 26.0 min) and the a isomer of neocrocin A (**2a**, 7.4 mg) (t_R 27.9 min) using preparative LC/MS[6] under the following conditions: column, YMC J'sphere ODS-L80 (5 μm, 20 mm i.d.×250 mm); mobile phase, H_2O : MeCN = 42 : 58; flow rate, 10 ml/min; make-up liquid, MeOH 1.0 ml/min; injection volume, 200 μl; detection and collection trigger, ESI (positive mode), *m/z* 1587.5. The overall procedure was repeated approximately 30 times. The β isomer of peracetylated **2** could not be isolated because of overlapping with the peak of **1a**.

1a red amorphous powder. IR (KBr) ν_{max} cm^{-1}: 1063, 1222, 1371, 1615, 1751, 2944. UV/Vis (MeOH) λ_{max} (log ε): 436 (4.95), 462 (4.92), 260 (4.05): HR-ESI-MS *m/z*: 1587.5156 $[M+Na]^+$ (Calcd for $C_{72}H_{92}O_{38}Na$: 1587.5164). **2a** (an α isomer): red amorphous powder. IR (KBr) ν_{max} cm^{-1}: 1038, 1223, 1375, 1637, 1752, 2945. UV/Vis (MeOH) λ_{max} (log ε): 438 (4.81), 460 (4.77), 260 (4.05). HR-ESI-MS *m/z* 1587.5192 $[M+Na]^+$ (Calcd for $C_{72}H_{92}O_{38}Na$: 1587.5164); ^1H (800 MHz)- and ^{13}C (200 MHz)-NMR spectral data of **1a** and **2a**: see Table 2.

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TRAF6 distinctively mediates MyD88- and IRAK-1-induced activation of NF-κB

Masashi Muroi and Ken-ichi Tanamoto[1]

Division of Microbiology, National Institute of Health Sciences, Tokyo, Japan

Abstract: MyD88 and IL-1R-associated kinase 1 (IRAK-1) play crucial roles as adaptor molecules in signal transduction of the TLR/IL-1R superfamily, and it is known that expression of these proteins leads to the activation of NF-κB in a TNFR-associated factor 6 (TRAF6)-dependent manner. We found in this study, however, that a dominant-negative mutant of TRAF6, lacking the N-terminal RING and zinc-finger domain, did not inhibit IRAK-1-induced activation of NF-κB in human embryo kidney 293 cells, although the TRAF6 mutant strongly suppressed the MyD88-induced activation. The dominant-negative mutant of TRAF6 did not affect the IRAK-1-induced activation, regardless of the expression level of IRAK-1. In contrast, small interfering RNA silencing of TRAF6 expression inhibited MyD88-induced and IRAK-1-induced activation, and supplementation with the TRAF6 dominant-negative mutant did not restore the IRAK-1-induced activation. Expression of IRAK-1, but not MyD88, induced the oligomerization of TRAF6, and IRAK-1 and the TRAF6 dominant-negative mutant were associated with TRAF6. These results indicate that TRAF6 is involved but with different mechanisms in MyD88-induced and IRAK-induced activation of NF-κB and suggest that TRAF6 uses a distinctive mechanism to activate NF-κB depending on signals. *J. Leukoc. Biol.* 83: 000–000; 2008.

Key Words: *Toll-like receptor · IL-1 receptor · lipopolysaccharide*

INTRODUCTION

TLR/IL-1R family members share common intracellular signaling proteins including MyD88, the IL-1R-associated kinase (IRAK) family, and TNFR-associated factor 6 (TRAF6) [1, 2]. Ligand binding triggers the recruitment of MyD88 to the Toll/IL-1R (TIR) domain of TLR/IL-1R via a homophilic TIR–TIR interaction, which in turn, recruits IRAK-4 and IRAK-1 into the receptor complex. IRAK-4 does not bind IRAK-1 directly but is recruited into the complex through binding with MyD88. This allows IRAK-1 and IRAK-4 to come in close proximity, which induces IRAK-4 to phosphorylate IRAK-1 [3], probably triggering autophosphorylation of IRAK-1. Autophosphorylated IRAK-1 interacts with TRAF6 [4], leading to the activation of NF-κB [1].

MyD88 is known as a universal adaptor molecule that interacts with IL-1R and most of TLRs. MyD88 consists of an N-terminal death domain separated by a short internal linker from a C-terminal TIR domain, which is necessary for the interaction with the TIR domain of TLR/IL-1R. The death domain and the internal linker domain have been implicated in the interaction with IRAK-1 and IRAK-4, respectively [5]. IRAK-1 consists of an N-terminal death domain, which is involved in the binding of MyD88 [6], and a central serine/threonine kinase domain. The C-terminal region of IRAK-1 contains three potential TRAF6-binding sites, and mutation of the amino acids (Glu^{544}, Glu^{587}, Glu^{706}) in these sites to alanine greatly reduces activation of NF-κB [7]. The death domain and the internal domain between the death domain and the kinase domain of IRAK-1 are also involved in binding TRAF6. The N-terminal region (death domain and internal domain) and the first half of the C-terminal region are sufficient for IL-1-induced activation of NF-κB [8].

It is known that all of MyD88, IRAK-1, and TRAF6 are involved in TLR/IL-R signaling to activate NF-κB. However, it is still enigmatic how these molecules lead to the activation of NF-κB [9]. Polyubiquitination of TRAF6 is reportedly important for TLR/IL-1R signaling [10]. TRAF6 itself functions, in conjunction with the ubiquitin-conjugating enzyme complex Ubc13-Uev1A, as a ubiquitin ligase that catalyzes the formation of unique Lys^{63}-linked polyubiquitin chains [11, 12]. TRAF6 catalyzes Lys^{63}-linked polyubiquitination on TRAF6 itself, and the polyubiquitinated TRAF6 activates NF-κB signaling proteins by a proteasome-independent mechanism [11, 12]. On the other hand, it has also been reported that oligomerization of TRAF6 induces activation of NF-κB [12, 13]. However, the relationship between the polyubiquitination and the oligomerization is unknown, and the role of MyD88 and IRAK-1 in these events is still ambiguous. We report here for the first time in our knowledge that TRAF6 distinctively mediates MyD88-induced and IRAK-1-induced activation of NF-κB and that only IRAK-1 leads to oligomerization of TRAF6.

[1] Correspondence: Division of Microbiology, National Institute of Health Sciences, 1-18-1 Kamiyoga, Setagaya, Tokyo 158-8501, Japan. E-mail: tanamoto@nihs.go.jp

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MATERIALS AND METHODS

Cell culture and reagents

The human embryo kidney (HEK)293 cell line (obtained from the Human Science Research Resources Bank, Tokyo, Japan) was grown in DMEM (Invitrogen, Carlsbad, CA, USA), supplemented with 10% (v/v) heat-inactivated FCS (Invitrogen), penicillin (100 U/ml), and streptomycin (100 μg/ml). *Escherichi coli* 0111:B4 LPS was obtained from Sigma-Aldrich (St. Louis, MO, USA) and was repurified according to the method described by Hirschfeld et al. [14]. A stable cell population expressing FLAG-tagged TRAF6 and equine infectious anaemia virus (EIAV)-tagged TRAF6 was established as follows. After linearizing with *Bgl*II, expression plasmids encoding FLAG-tagged TRAF6 and EIAV-tagged TRAF6 were transfected into HEK293 cells by the calcium phosphate precipitation method. Transfected cells were selected for G418 resistance at a concentration of 1 mg/ml. An antiserum against the EIAV-tag epitope (amino acid sequence: ADRRIPGTAEE) was a kind gift from Dr. Nancy Rice (National Cancer Institute-Frederick Cancer Research and Development Center, Frederick, MD, USA). Antibodies against TRAF6 (H-274, Santa Cruz Biotechnology, Santa Cruz, CA, USA) and FLAG-epitope (M2, Sigma-Aldrich) were used. Anti-FLAG M2 affinity gel was from Sigma-Aldrich. A TRAF6 small interfering (si)RNA oligo (CGACGAAGAGAUAAUCGAUdTdT) [15] was synthesized by Qiagen (Valencia, CA, USA).

Plasmids

The coding regions of human MyD88 and IκB kinase β (IKKβ) were amplified by RT-PCR from total RNA prepared from human spleen (OriGene Technologies, Rockville, MD, USA) and THP-1 cells, respectively. The coding region of human TRAF6 was amplified from a human spleen cDNA library (Clontech, Palo Alto, CA, USA). A plasmid containing human IRAK-1 cDNA was obtained from the Mammalian Gene Collection. Deletions found in the IRAK-1 plasmid were corrected by PCR-mediated mutagenesis. The coding regions of all of these constructs were subcloned into mammalian expression vectors containing the N-terminal EIAV-tag and FLAG-tag epitope sequences. NF-κB-dependent luciferase reporter plasmid endothelial leukocyte adhesion molecule ligand (pELAM-L) was described previously [16]. All mutant plasmids were created by PCR-mediated mutagenesis, and mutations were confirmed by DNA sequencing.

NF-κB reporter assay, RNA interference, immunoprecipitation, and immunoblotting

The NF-κB-dependent luciferase reporter assay was performed as described elsewhere [17]. Briefly, HEK293 cells ($2–5\times10^5$ cells) were plated in six-well plates and transfected the following day by the calcium phosphate precipitation method with the indicated plasmids: 0.2 μg pELAM-L and 5 ng phRL-TK (Promega, Madison, WI, USA), for normalization. At 24–32 h after transfection, cellular extracts were prepared by adding a lysis buffer [10 mM HEPES-KOH, pH 7.9, 10 mM KCl, 5 mM EDTA, 40 mM β-glycerophosphate, 0.5% Nonidet P-40 (NP-40), 30 mM NaF, 1 mM Na_3VO_4, 100 nM okadaic acid] containing a protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany). Reporter gene activity was measured with a portion of the cellular extract, according to the manufacturer's (Promega) instruction. To another portion of the cellular extract, anti-FLAG M2-agarose (Sigma-Aldrich) was added, and the mixture was incubated at 4°C for 1 h. The agarose was washed three times with PBS containing 0.5% NP-40, and bound proteins were subsequently eluted from the agarose by incubating with 0.1% SDS. The resulting supernatant was subjected to SDS-PAGE. Proteins were transferred to a polyvinylidene difluoride membrane (Immobilon-P, Millipore, Bedford, MA, USA) and subjected to immunoblotting with the indicated antibodies. The signals were visualized by using an enhanced chemiluminescence system (GE Healthcare Bio-sciences, Piscataway, NJ, USA). For RNA interference, HEK293 cells ($1–3\times10^5$ cells) were plated in six-well plates and transfected the following day by the calcium phosphate precipitation method with the indicated amounts of a siRNA oligo. On the following day after the first transfection, reporter plasmids indicated expression plasmids, and the siRNA oligo were transfected further as described above. The transfected amount of siRNA oligo was normalized by supplementing an unrelated oligo. At 24–32 h after the second transfection, cellular extracts were prepared, and reporter activities were determined as above.

RESULTS

A dominant-negative mutant of TRAF6 inhibits MyD88-induced but not IRAK-1-induced activation of NF-κB

To explore the involvement of TRAF6 in MyD88- and IRAK-1-induced activation of NF-κB, we examined the effects of a dominant-negative mutant of TRAF6. It is well known that the deletion of the N-terminal RING and zinc-finger domain (aa 1–288) of TRAF6 abolishes the ability of TRAF6 to mediate IL-1- and LPS-induced activation of NF-κB [18] and that the N-terminal deletion mutant acts as a dominant-negative mutant [4]. Thus, this N-terminal deletion mutant of TRAF6 was expressed with MyD88 or IRAK-1 and measured NF-κB-dependent reporter activity in HEK293 cells (**Fig. 1**). As expected, the expression of MyD88 activated NF-κB, and the coexpression of the TRAF6 deletion mutant inhibited this activation in a dose-dependent manner. However, IRAK-1-induced activation of NF-κB was surprisingly unaffected by coexpression of the deletion mutant. On the other hand, MyD88- and IRAK-1-induced activation of NF-κB were inhibited by a kinase-dead mutant (K44A) of IKKβ, indicating that the activation induced by MyD88 and IRAK-1 is IKK-dependent. The expression levels of MyD88 and IRAK-1 were not affected by coexpression of the TRAF6 deletion mutant (Fig. 1, lower panels).

To confirm the inability of the TRAF6 deletion mutant to inhibit IRAK-1-induced activation of NF-κB, the effect of the deletion mutant was examined further by changing the expression levels of MyD88 and IRAK-1 (**Fig. 2**). The TRAF6 deletion mutant inhibited MyD88-induced activation of NF-κB (Fig. 2A, right), irrespective of the MyD88 expression level (Fig. 2B); however, IRAK-1-induced activation was not af-

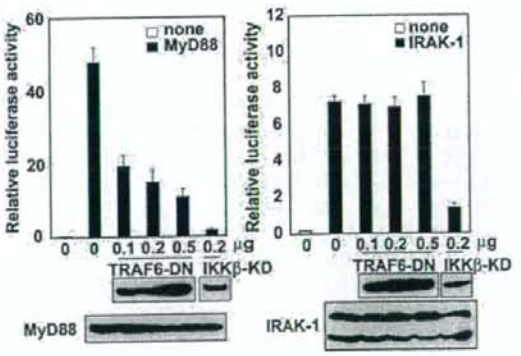

Fig. 1. A TRAF6 dominant-negative mutant inhibits MyD88-induced but not IRAK-1-induced activation of NF-κB. HEK293 cells were transiently transfected with a NF-κB-dependent luciferase reporter plasmid and an expression plasmid (0.1 μg) for MyD88 (left panel) or IRAK-1 (right panel) together with a kinase-dead (KD) mutant of IKKβ (K44A) or an increasing amount of a dominant-negative mutant plasmid for TRAF6 (TRAF6-DN; aa 289–522). After 30 h, cellular extracts were subjected to luciferase activity measurements and SDS-PAGE followed by immunoblotting. Values are means ± SEM from three independent experiments.

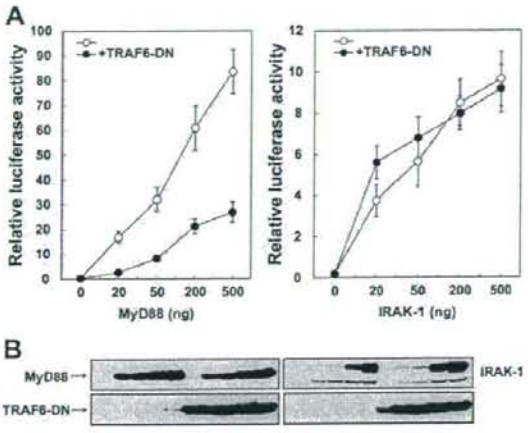

Fig. 2. A TRAF6 dominant-negative mutant did not affect IRAK-1-induced activation at any of the IRAK-1 expression levels. HEK293 cells were transiently transfected with a NF-κB-dependent luciferase reporter plasmid and an increasing amount of MyD88 (A) or IRAK-1 (B) expression plasmid in the absence (○) or presence (●) of a dominant-negative mutant plasmid (0.5 μg) for TRAF6 (TRAF6-DN; aa 289–522). After 30 h, cellular extracts were subjected to luciferase activity measurements and SDS-PAGE followed by immunoblotting. Values are means ± SEM from three independent experiments.

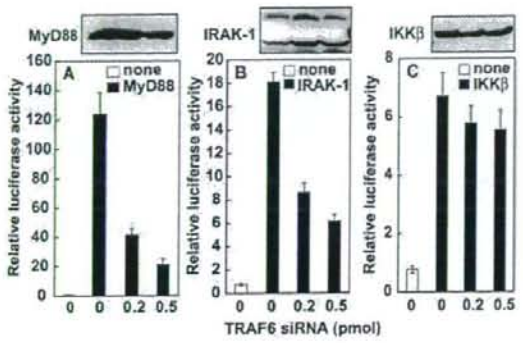

Fig. 3. A TRAF6 siRNA oligo inhibits MyD88-induced and IRAK-1-induced activation of NF-κB. HEK293 cells were transiently transfected with a NF-κB-dependent luciferase reporter plasmid and an expression plasmid (0.1 μg) for MyD88 (A), IRAK-1 (B), or IKKβ (C), together with an increasing amount of a TRAF6 siRNA oligo. After 30 h, cellular extracts were subjected to luciferase activity measurements and SDS-PAGE followed by immunoblotting. Values are means ± SEM from six independent experiments.

fected (Fig. 2A, left) at any of the IRAK-1 expression levels (Fig. 2B).

MyD88-induced and IRAK-1-induced activation of NF-κB require TRAF6

Results obtained with the TRAF6 dominant-negative suggest that TRAF6 is not involved in IRAK-1-induced activation of NF-κB. To confirm this finding, the effects of a TRAF6 siRNA were examined. An increasing amount of a TRAF6 siRNA oligo was transfected into HEK293 cells with MyD88, IRAK-1, or IKKβ, and NF-κB-dependent reporter activity was measured. Unexpectedly, IRAK-1-induced (**Fig. 3B**) as well as MyD88-induced (Fig. 3A) activation of NF-κB was inhibited by the transfection of the siRNA oligo in a dose-dependent manner. IKKβ-induced activation of NF-κB was not significantly affected by the siRNA oligo (Fig. 3C), indicating that the inhibition was not nonspecific. The expression levels of MyD88, IRAK-1, and IKKβ were not affected by the TRAF6 siRNA oligo (Fig. 3, upper panels). Furthermore, another TRAF6 siRNA oligo that targets different regions of TRAF6 mRNA also inhibited MyD88 and IRAK-1-induced activation of NF-κB (data not shown). These results indicate that although IRAK-1-induced activation was not inhibited by a TRAF6 dominant-negative mutant, MyD88- and IRAK-1-induced activation of NF-κB require TRAF6.

The N-terminal region of TRAF6 is believed to be important for signal transduction. However, as IRAK-1-induced activation of NF-κB was not affected by an N-terminal deletion mutant of TRAF6 but required TRAF6, the possibility that the C-terminal portion of TRAF6 is involved in IRAK-1-induced activation of NF-κB still remains. Thus, the effect of the TRAF6 N-terminal deletion mutant was evaluated after siRNA silencing of endogenous TRAF6 (**Fig. 4**). The transfection of a TRAF6 siRNA oligo did not affect basal NF-κB-dependent reporter activity. Coexpression of TRAF6, but not expression of the N-terminal deletion mutant, increased reporter activity (Fig. 4, left panel). The significant increase in reporter activity observed upon expression of MyD88 was inhibited by the transfection of the TRAF6 siRNA oligo as shown above. Under this condition, coexpression of TRAF6, but not the TRAF6 N-terminal deletion mutant, overcame the inhibition induced

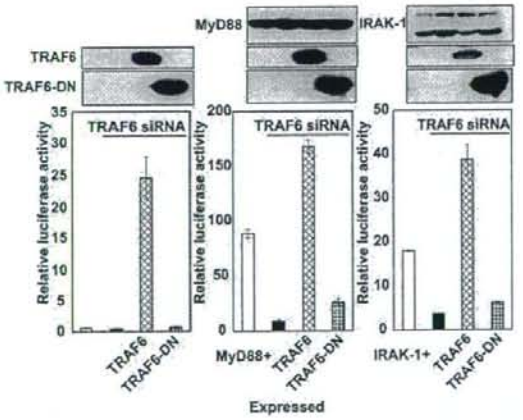

Fig. 4. C-terminal portion of TRAF6 is not involved in IRAK-1-induced activation of NF-κB. HEK293 cells were transiently transfected with a NF-κB-dependent luciferase reporter plasmid and a control vector (left panel), an expression plasmid (0.1 μg) for MyD88 (middle panel), or IRAK-1 (right panel) together with wild-type or a dominant-negative mutant plasmid (0.5 μg) for TRAF6 (TRAF6-DN; aa 289–522) in the absence (left bar) or presence (right three bars) of a TRAF6 siRNA oligo (0.5 pmol). After 30 h, cellular extracts were subjected to luciferase activity measurements and SDS-PAGE followed by immunoblotting. Values are means ± SEM from seven independent experiments.

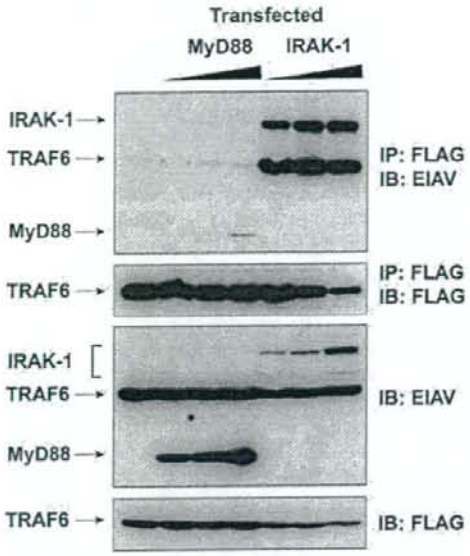

Fig. 5. IRAK-1 but not MyD88 induces oligomerization of TRAF6. HEK293 cells stably expressing FLAG-tagged TRAF6 and EIAV-tagged TRAF6 were transiently transfected with an expression plasmid for EIAV-tagged MyD88 or EIAV-tagged IRAK-1. After 30 h, cellular extracts were prepared, and FLAG-tagged TRAF6 was immunoprecipitated (IP). Precipitated, FLAG-tagged TRAF6 (second panel from top) and coprecipitated EIAV-tagged proteins (top panel) were detected by immunoblotting (IB). Part of each cell extract prepared above was subjected to the detection of EIAV-tagged proteins (second panel from bottom) and FLAG-tagged TRAF6 (bottom panel) by immunoblotting.

by TRAF6 siRNA (Fig. 4, middle panel). IRAK-1-induced activation of NF-κB was also inhibited by TRAF6 siRNA, and coexpression of TRAF6 again overcame the inhibition. However, coexpression of the N-terminal deletion mutant was not able to overcome the inhibition induced by TRAF6 siRNA (Fig. 4, right panel). TRAF6, the N-terminal deletion mutant of TRAF6, IRAK-1, and MyD88, was properly expressed (Fig. 4, upper panels). Thus, it is unlikely that the C-terminal portion of TRAF6 is capable of transmitting IRAK-1-induced activation of NF-κB.

IRAK-1 but not MyD88 induces oligomerization of TRAF6

The effects of the TRAF6 N-terminal deletion mutant differed between MyD88-induced and IRAK-1-induced activation of NF-κB. The fact that TRAF6 is required for both types of activation suggests that TRAF6 is differentially involved in the activation induced by these molecules. As it has been reported that TRAF6 oligomerization induces activation of NF-κB [12, 13], the oligomerization of TRAF6 was examined in response to the expression of MyD88 and IRAK-1. HEK293 cells stably expressing FLAG-tagged TRAF6, and EIAV-tagged TRAF6 were transiently transfected with an expression plasmid for EIAV-tagged MyD88 or EIAV-tagged IRAK-1. After preparing cell extracts, FLAG-tagged TRAF6 was immunoprecipitated (**Fig. 5**, second panel from the top) with anti-FLAG M2 affinity gel, and coprecipitated, EIAV-tagged proteins were detected by immunoblotting (Fig. 5, top panel). Upon expression of MyD88, a trace amount of MyD88, but no EIAV-tagged TRAF6, was coprecipitated with FLAG-tagged TRAF6. In contrast, EIAV-tagged TRAF6 as well as IRAK-1 were coprecipitated with FLAG-tagged TRAF6 when IRAK-1 was expressed. MyD88, IRAK-1, EIAV-tagged TRAF6 (Fig. 5, second panel from the bottom), and FLAG-tagged TRAF6 (Fig. 5, bottom panel) were properly expressed. Therefore, expression of IRAK-1 but not MyD88 induces oligomerization of TRAF6.

The effect of the TRAF6 N-terminal deletion mutant on IRAK-1-induced oligomerization of TRAF6 was next examined (**Fig. 6**). HEK293 cells stably expressing FLAG-tagged TRAF6 and EIAV-tagged TRAF6 were transiently transfected with an expression plasmid for EIAV-tagged IRAK-1 and an increasing amount of a plasmid expressing the EIAV-tagged TRAF6 N-terminal deletion mutant. After preparing cell extracts, FLAG-tagged TRAF6 was immunoprecipitated (Fig. 6, right part of the top panel) with anti-FLAG M2 affinity gel, and coprecipitated, EIAV-tagged proteins were detected by immunoblotting (Fig. 6, right part of the bottom panel). Upon expression of IRAK-1, EIAV-tagged TRAF6 as well as IRAK-1 were coprecipitated with FLAG-tagged TRAF6 as shown above. When the TRAF6 N-terminal deletion mutant was coexpressed, the mutant was also precipitated with FLAG-tagged TRAF6, and the amount of coprecipitated, EIAV-tagged TRAF6 was not affected by coexpression of the TRAF6 N-terminal deletion mutant. IRAK-1, EIAV-tagged and FLAG-tagged TRAF6, and the TRAF6 deletion mutant were expressed properly (Fig. 6, left panels). Thus, the expression of the TRAF6 N-terminal deletion mutant does not appear to inhibit the IRAK-1-induced oligomerization of TRAF6. Taken together, these data demonstrate that TRAF6 is differentially

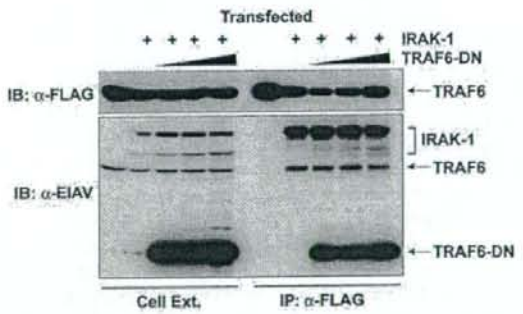

Fig. 6. IRAK-1 and a TRAF6 dominant-negative mutant were coprecipitated with TRAF6. HEK293 cells stably expressing FLAG-tagged TRAF6 and EIAV-tagged TRAF6 were transiently transfected with an increasing amount of a dominant-negative mutant plasmid for EIAV-tagged TRAF6 (TRAF6-DN; aa 289–522) together with an expression plasmid for EIAV-tagged MyD88 or EIAV-tagged IRAK-1. After 30 h, cellular extracts were prepared, and FLAG-tagged TRAF6 was immunoprecipitated. Precipitated, FLAG-tagged TRAF6 (right half of top panel) and coprecipitated EIAV-tagged proteins (right half of bottom panel) were detected by immunoblotting. Part of each cell extract (Cell Ext.) prepared above was subjected to the detection of EIAV-tagged proteins (left half of bottom panel) and FLAG-tagged TRAF6 (left half of top panel) by immunoblotting.

involved in MyD88-induced and IRAK-1-induced activation of NF-κB and that IRAK-1 but not MyD88 induces TRAF6 oligomerization.

DISCUSSION

In this study, we found that TRAF6 is differentially involved in MyD88- and IRAK-1-induced activation of NF-κB. MyD88 and IRAK-1 act as adaptor molecules in TLR/IL-1R signaling, and overexpression of each molecule leads to activation of NF-κB via their downstream signaling molecule TRAF6 (see ref. [1]). These observations were confirmed in the experiment in which TRAF6 siRNAs inhibited the activation of MyD88- and IRAK-1-induced NF-κB activation (Fig. 3). However, we found that a dominant-negative mutant of TRAF6 (N-terminal deletion of aa 1–288) inhibits only MyD88-induced activation (Figs. 1 and 2). It is unlikely that IRAK-1 activates NF-κB by using the C-terminal portion of TRAF6, as IRAK-1 failed to activate NF-κB when endogenous, wild-type TRAF6 was silenced by TRAF6 siRNA, and the N-terminal deletion mutant of TRAF6 was overexpressed (Fig. 4). We also found that expression of IRAK-1 but not MyD88 leads to oligomerization of TRAF6 (Fig. 5). It has been reported that oligomerization of TRAF6 induces activation of NF-κB [12, 13]. Thus, oligomerization of TRAF6 is probably involved in the IRAK-1-induced activation of NF-κB. The N-terminal deletion mutant of TRAF6 did not inhibit IRAK-1-induced TRAF6 oligomerization. Instead, the mutant formed a complex with the TRAF6 oligomer (Fig. 6), indicating that the TRAF6 oligomer consists of more than two molecules of TRAF6. This finding explains why the N-terminal deletion mutant of TRAF6 was not able to inhibit IRAK-1-induced activation of NF-κB.

Not only does IRAK-1 induce TRAF6 oligomerization, it is also associated with the TRAF6 oligomer (Fig. 5). We found that an IRAK-1 mutant (E544A/E587A/E706A), in which three putative TRAF6-binding sites were mutated, the mutation known to greatly impair the ability to activate NF-κB [7], did not induce oligomerization of TRAF6 (data not shown), suggesting that TRAF6 molecules form a complex through the binding to IRAK-1. Overexpression of IRAK-1 in HEK293 cells appears mainly as two forms on SDS-PAGE (see Fig. 1), with the slower migrating form recognized as the hyperphosphorylated form of IRAK-1 (see ref. [1]). Interestingly, the slower migrating form of IRAK-1 was predominantly coprecipitated with the TRAF6 oligomer (see Fig. 6). It has been reported that IL-1 stimulation leads to hyperphosphorylation of IRAK-1 by autophosphorylation and to association between phosphorylated IRAK-1 and TRAF6 [4]. Thus, it is likely that autophosphorylated IRAK-1 promotes TRAF6 oligomerization by binding to TRAF6.

Overexpression of MyD88 did not induce detectable TRAF6 oligomerization (Fig. 5), although expression led to a strong activation of NF-κB (Fig. 1). TRAF6 oligomerization in response to TLR/IL-1R stimulation has not been reported. There was also no detectable TRAF6 oligomerization in cells stably expressing FLAG-tagged TRAF6 and EIAV-tagged TRAF6 in response to IL-1, LPS, or Pam_3CSK_4 stimulation, when these cells were transiently expressed with the IL-1RI/IL-1R accessory protein, CD14/TLR4/myeloid differentiation protein-2 or TLR1/TLR2, respectively, although these stimulations induced a strong activation of NF-κB (data not shown). Thus, it is unlikely that TRAF6 oligomerization is required for the activation of NF-κB in response to TLR/IL-1R stimulation. TRAF6 reportedly functions, in conjunction with ubiquitin-conjugating enzyme complex Ubc13-Uev1A, as a ubiquitin ligase, and this ubiquitin ligase activity is involved in the activation of NF-κB [11, 12]. We found that transfection of a Ubc13 siRNA oligo into HEK293 cells inhibited MyD88-induced activation of NF-κB (data not shown). Therefore, the ubiquitin ligase activity of TRAF6 may be involved in the MyD88-induced activation. Fukushima et al. [19] reported that LPS-induced degradation of IκBα was severely impaired in macrophages and splenocytes isolated from heterozygous $Ubc13^{+/-}$ mice. However, Yamamoto et al. [20] reported that Ubc13-deficient B cells, bone marrow macrophages, and embryonic fibroblasts showed almost normal NF-κB activation in response to LPS, IL-1β, or a bacterial lipoprotein. Thus, further studies are needed to clarify the role of the ubiquitin ligase activity of TRAF6 in TLR/IL-1R signaling.

It is considered that IRAK-1 lies downstream of MyD88 in the TLR/IL-1R signaling processes. Thus, our result that a dominant-negative mutant of TRAF6 inhibited MyD88-induced, but not IRAK-1-induced, activation of NF-κB was surprising. There are two possible explanations for this finding. One is that IRAK-1 is not necessary for TLR/IL-1R signaling. The other is that the activation of NF-κB in response to IRAK-1 overexpression is qualitatively different from the activation induced physiologically in response to TLR/IL-1R stimulation. We are not able to exclude the second possibility. However, it has been reported that macrophages from IRAK-1 knockout mice showed only partial impairment of cytokine production and NF-κB activation in response to TLR4 stimulation [21]. In addition, Kawagoe et al. [22] recently found that the IRAK-1/IRAK-4 double-knockout did not affect macrophage activator lipoprotein peptide-2-induced activation of NF-κB and proposed the existence of a TLR-mediated, IRAK-1/IRAK-4-independent signaling pathway. It is possible that another IRAK member, such as IRAK-2, compensates for the lack of IRAK-1/IRAK-4 in this knockout mouse. However, we also found that IRAK-2-induced activation of NF-κB was not inhibited by a dominant-negative mutant of TRAF6 (data not shown). Therefore, IRAK-1 may not be involved in TLR/IL-1R signaling. This possibility remains to be studied.

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RESEARCH ARTICLE

The lipopolysaccharide-recognition mechanism in cells expressing TLR4 and CD14 but lacking MD-2

Takahiro Ohnishi, Masashi Muroi & Ken-ichi Tanamoto

Division of Microbiology, National Institute of Health Sciences, Tokyo, Japan

Correspondence: Ken-ichi Tanamoto, Division of Microbiology, National Institute of Health Sciences, 1-18-1 Kamiyoga, Setagaya, Tokyo 158-8501, Japan. Tel.: +81 3 3700 1141, ext.: 321; fax: +81 3 3700 9484; e-mail: tanamoto@nihs.go.jp

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LPS recognition; toll-like receptor signal transduction; cell activation.

Abstract

We analysed the lipopolysaccharide (LPS)-recognition mechanism in cells expressing TLR4 and CD14 but lacking MD-2. When TLR4 and CD14 were transiently expressed in HEK293 cells, cell-surface expression of TLR4 was observed, although the expression level was lower than that in cells coexpressing MD-2. We found that membrane CD14–TLR4 complexes were formed in these cells in response to LPS stimulation even in the absence of MD-2 expression, although NF-κB-dependent reporter activity was not induced. A strong activation of NF-κB was observed when these cells were stimulated with LPS followed by soluble MD-2 in this order, even when excess LPS was removed after formation of the CD14–TLR4 complex by washing cells prior to sMD-2 addition. From these results, we propose an additional LPS-recognition mechanism. In cells expressing TLR4 and CD14 but lacking MD-2, LPS is first transferred to membrane CD14 with the aid of LPS binding protein, which leads to the formation of the TLR4–CD14 complex. Then, the binding of soluble MD-2 to this complex triggers the transmembrane signal transduction. Cells expressing TLR4 and CD14 but lacking MD-2, such as airway epithelial cells, may be activated in response to LPS by this mechanism.

Introduction

Lipopolysaccharide (LPS) is a component of the outer membrane of Gram-negative bacteria and plays a major role in septic shock. LPS induces the production of various cytokines by the activation of transcription factors, including NF-κB and IRF-3 (Schletter *et al.*, 1995; Hatada *et al.*, 2000; Sakaguchi *et al.*, 2003). It is known that LPS binding protein (LBP), CD14, MD-2 and Toll-like receptor 4 (TLR4) are required for the recognition of LPS. Both CD14 and MD-2 exist in membrane-anchored and soluble forms, and it has been suggested that both forms are important for LPS recognition (Schromm *et al.*, 2001; Visintin *et al.*, 2001; Muroi *et al.*, 2002; Nagai *et al.*, 2002; Ohnishi *et al.*, 2003; Van Amersfoort *et al.*, 2003). How these factors recognize LPS on the cell surface has been discussed, and some models have been proposed. The most widely accepted model is as follows. LPS is transferred to membrane CD14 (mCD14) by LBP, and then the LPS–CD14 complex is recognized by the TLR4–membrane MD-2 (mMD-2) complex on the cell surface (da Silva Correia *et al.*, 2001; Gioannini *et al.*, 2004). This mCD14–TLR4–mMD-2 complex is able to transmit the activation signal of LPS to cytosol via the intracellular domain of TLR4.

The role of soluble CD14 (sCD14) in the LPS response has been widely studied. sCD14 exists in human plasma and its level increases on infection (Landmann *et al.*, 1995). It has been reported that, in cells expressing TLR4 and mMD-2, sCD14 confers responsiveness to LPS (Landmann *et al.*, 1996), indicating that sCD14 is able to be a substitute for mCD14. It has also been reported that artificially created LPS–sMD-2 complexes *in vitro*, with the assistance of sCD14, are able to activate cells expressing only TLR4 (Gioannini *et al.*, 2004). This suggests that sMD-2 is also able to substitute for mMD-2. In fact, it has been reported that cells expressing TLR4 and CD14 but lacking mMD-2 respond to LPS when sMD-2 is added (Jia *et al.*, 2004; Pugin *et al.*, 2004). However, how LPS is recognized in these cells has yet to be elucidated. In this paper, we investigate the LPS-recognition mechanism in cells expressing TLR4 and CD14 but lacking MD-2.

Materials and methods

Cells and reagents

The human embryonic kidney 293 cell line (obtained from the Human Science Research Resource Bank, Tokyo, Japan) was cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco BRL, Rockville, MD) supplemented with 10% (v/v) heat-inactivated fetal calf serum (FCS; Gibco BRL), penicillin (100 U mL^{-1}), and streptomycin (100 μg mL^{-1}). The anti-serum (no.1060) against the equine infectious anemia virus (EIAV)-tag epitope (amino acid sequence: ADRRIPGTAEE) was a kind gift from Dr Nancy Rice (NCI-Frederick Cancer Research and Development Center). Sulfosuccinimidyl-6-(biotinamido)-6-hexanamido hexanoate, sulfosuccinimidyl-2-[*p*-azidosalicylamido]ethyl-1,3′-dithiopropionate and immobilized streptavidin agarose came from Pierce (Rockford, IL). Anti-FLAG M2 agarose and anti-FLAG M2 antibody were obtained from Sigma-Aldrich Co. (St Louis, MO). *Escherichia coli* O111:B4 LPS (Sigma-Aldrich Co.) was re-purified according to Hirschfeld *et al.* (2000). Unless otherwise noted, all other chemicals were obtained from Wako Pure Chemical Industries, Ltd (Osaka, Japan).

Plasmid construction

cDNA encoding EIAV-tagged human CD14, TLR4 and MD-2 were separately cloned into a mammalian expression vector as previously described (Ohnishi *et al.*, 2001). A plasmid for FLAG-tagged TLR4 was constructed by inserting a coding sequence minus its predicted signal peptide sequences into the pFLAG-CMV-1 expression vector (Sigma-Aldrich Co.).

Biotinylation of cell-surface proteins

After plating 293 cells in 6 cm dishes, the cells were transfected with EIAV-tagged TLR4 (5 μg), EIAV-tagged CD14 (0.25 μg) and EIAV-tagged MD-2 (5 μg) expression plasmids using the calcium phosphate precipitation method. Twenty-four hours later, the cells were washed twice with PBS and treated with 2 mL of 0.5 mg mL^{-1} sulfosuccinimidyl-6-(biotinamido)-6-hexanamido hexanoate, a membrane-impermeable biotinylation reagent according to a previously described method (Ohnishi *et al.*, 2001). Cellular extracts were prepared using 200 μL of a lysis buffer (20 mM HEPES-KOH, 5 mM EDTA, 0.5% Nonidet P-40 and 10 mM KCl; pH7.9) containing a protease inhibitor mix (Roche Diagnostics, Basel, Switzerland). The cellular extracts were diluted with PBS containing 0.5% Nonidet P-40 to a final volume of 500 μL and then incubated with immobilized streptavidin agarose at 4 °C for 1 h. After washing three times with PBS containing 0.5% Nonidet P-40, the agarose was boiled in sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer. The resulting supernatant was subjected to SDS-PAGE and Western blot analysis. TLR4, MD-2 and CD14 were detected using a rabbit anti-EIAV antiserum.

Analysis of TLR4–CD14 association on a membrane surface

After plating 293 cells in 10 cm type I collagen-coated dishes (Corning Inc., Corning, NY), the cells were transfected with FLAG-tagged TLR4 (24 μg) and EIAV-tagged CD14 (0.2 μg) expression plasmids using the calcium phosphate precipitation method. Twenty-four hours later, the cells were washed three times with PBS and then stimulated with 0.01 μg mL^{-1} LPS in serum-free DMEM containing 0.1 μg mL^{-1} LBP at 37 °C for 3 h. After incubation, the cells were washed twice with PBS and collected into 1.5-mL microcentrifuge tubes. The cells were exposed to 1 mg mL^{-1} membrane-impermeable bifunctional cross-linking reagent, sulfosuccinimidyl-2-[*p*-azidosalicylamido]ethyl-1,3′-dithiopropionate in PBS, at 15 °C in the dark for 30 min with rocking. After UV irradiation at 25 °C for 1 min, the cells were washed with PBS, and the cell extracts were prepared with PBS containing 0.5% Nonidet P-40 and a protease inhibitor mix. SDS was added to the cell extracts to a final concentration of 1%. The cell extracts were boiled for 5 min and diluted to 5 mL with PBS containing 0.5% Nonidet P-40. The cell extracts were incubated with anti-FLAG M2 agarose at 4 °C for 1 h. The agarose was washed three times with PBS containing 0.5% Nonidet P-40 and incubated with 0.1 M glycine-HCl buffer, pH 2.5, for 5 min. The supernatants were subjected to SDS-PAGE and Western blot analysis. CD14 that cross-linked with TLR4 was detected using a rabbit anti-EIAV antiserum after the cross-linking reagent was cleaved with a reducing agent.

Electrophoresis and Western blotting

A discontinuous SDS-10% polyacrylamide gel (acrylamide/bisacrylamide ratio, 29 : 1) was prepared in an AE-6400 electrophoresis cell (ATTO Corp., Tokyo, Japan) according to the method of Laemmli (1970). Following the addition of Laemmli's reducing sample buffer, samples were boiled for 5 min and then electrophoresed. Electophoretically separated samples were transferred to a polyvinyliden difluoride membrane (Immobilon-P; Millipore Corp., Bedford, MA) with a semidry blot electrophoretic transfer system (EB-150; Toyo Roshi Kaisha Ltd, Tokyo, Japan). After blocking with 5% nonfat dry milk dissolved in TBST (10 mM Tris, 100 mM NaCl, 0.2% Tween 20; pH 7.5), the membrane was probed with the indicated antibody and washed with TBST three times. The membrane was then probed with a peroxidase-labelled second antibody and washed with TBST three times. The signals were visualized using an enhanced chemiluminescence plus system (Amersham, Arlington Height, IL).

Purification of recombinant human LBP, MD-2 and CD14

Human LBP was expressed in yeast and purified as previously described (Ohnishi *et al.*, 2001). The coding regions of human MD-2 and CD14 minus their signal sequences were subcloned into yeast expression vector pGAPZα (Invitrogen, Carlsbad, CA) with an N-terminal × 6 histidine tag sequence. MD-2 and CD14 were expressed in a Pichia expression system (Invitrogene) and purified with a Ni^{2+}-column (Novagen, Madison, WI) under denaturing conditions according to the manufacturer's recommendation.

NF-κB reporter assay

After plating 293 cells in 12-well plates (2×10^5 well^{-1}), the cells were transfected by the calcium phosphate precipitation method with the expression plasmids indicated in the figure, 0.1 μg of pELAM-L luciferase reporter plasmid, and 0.0025 μg of phRL-TK (Promega, Madison, WI) for normalization. After 24 h, cells were stimulated as indicated in the figure in serum-free DMEM. The reporter gene activity was measured according to the manufacturer's (Promega) recommendation.

Results

TLR4 was expressed on the cell surface in the absence of MD-2

To study the mechanism of LPS recognition in cells expressing TLR4 and CD14 but lacking MD-2, we used 293 cells transiently transfected with TLR4 and CD14. As it has been indicated that the association of TLR4 with MD-2 stimulates the expression of TLR4 on the cell surface (Nagai *et al.*, 2002), we first examined whether 293 cells express TLR4 on the cell surface in the absence of MD-2. We expressed TLR4 with/without CD14 and MD-2, and labelled the cell-surface proteins with a membrane-impermeable biotinylation reagent. The biotinylated proteins were collected with streptavidin-agarose, and electrophoresed by SDS-PAGE. Finally, cell-surface TLR4 was detected by Western blotting (Fig. 1). When cells were not treated with the biotinylation reagent, the expression of TLR4 on the cell surface was not detected, although a nonspecific band whose molecular weight was smaller than that of TLR4 was detected (Fig. 1, fourth lane from left). The cells transiently transfected with TLR4 and MD-2 strongly expressed TLR4 on the cell surface (Fig. 1, third and fifth lanes from left). Apparent cell-surface TLR4 expression was also observed in the cells transfected with both TLR4 and CD14 (Fig. 1, second lane from left), or with TLR4 alone (Fig. 1, first lane from left) at comparable levels, although the expression level was lower than that in cells expressing both TLR4 and MD-2.

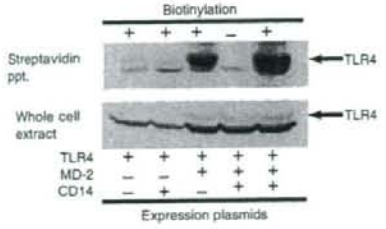

Fig. 1. Cell-surface expression of TLR4 in the absence or presence of MD-2. After plating 293 cells in 6 cm dishes, cells were transfected with the expression plasmids indicated (5 μg for EIAV-tagged TLR4 and MD-2, 0.25 μg for CD14). After 24 h, the cells either were left untreated or were treated with a membrane-impermeable biotinylation reagent, and cell extracts were prepared. The cell extracts were divided into two portions. One portion was analysed directly (lower panel) for TLR4 by Western blotting with an anti-EIAV tag antibody. Biotinylated proteins were collected from the second portion with streptavidin-agarose and boiled in SDS-PAGE sample buffer. The resulting supernatant (upper panel) was analysed for TLR4 by Western blotting with anti-EIAV tag antibody.

LPS induced the association of mCD14 with TLR4 in the absence of MD-2

Because cell-surface expression of TLR4 was observed even in the absence of MD-2, we next examined the formation of cell-surface TLR4–CD14 complexes in cells expressing TLR4 and CD14 but lacking MD-2. FLAG-tagged TLR4 and EIAV-tagged CD14 expression plasmids were transfected into 293 cells. After stimulation with LPS in the presence of LBP, cell-surface proteins were cross-linked with a membrane-impermeable bifunctional cross-linking reagent. After washing, cellular extracts were prepared and boiled in the presence of SDS to disrupt noncovalent protein–protein association. Following dilution, FLAG-tagged TLR4 was precipitated with anti-FLAG M2 agarose. The precipitated proteins were subjected to SDS-PAGE, and CD14 cross-linked with FLAG-tagged TLR4 was analysed by Western blot (Fig. 2). When cells were not treated with the cross-linking reagent, CD14 was not detectable, indicating that noncovalently associated CD14 was not coprecipitated with TLR4 under this experimental condition (Fig. 2, first and third lanes from left in upper panel). CD14 was also not precipitated with TLR4 in unstimulated cells (Fig. 2, second lane from left in upper panel). In contrast, when cells were stimulated with LPS and cell-surface proteins were cross-linked, CD14 was co-precipitated with TLR4 (Fig. 2, fourth lane from left in upper panel). The total expression levels of TLR4 and CD14 were not changed by treatment with LPS (middle and lower panels). These results indicate that LPS stimulation induces the formation of CD14–TLR4 complexes on the cell surface even in cells without MD-2 expression.

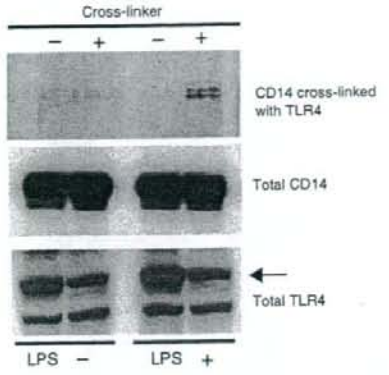

Fig. 2. LPS-induced formation of the cell-surface TLR4–CD14 complex in the absence of MD-2. After plating 293 cells in type I collagen-coated 10 cm dishes, cells were transiently transfected with FLAG-tagged TLR4 and EIAV-tagged CD14 expression plasmids (24 μg for TLR4 and 0.2 μg for CD14). After 24 h, the cells were washed three times with PBS, and the medium was replaced by a serum-free medium. The cells either were left unstimulated (left two columns) or were stimulated with LPS (0.01 μg mL^{-1}) in the presence of LBP (0.1 μg mL^{-1}) for 3 h (right two columns). The cells were then either left untreated or treated with a membrane-impermeable cross-linking reagent, and cell extracts were prepared. The cell extracts were divided into three portions. SDS was added to one portion of the extract to a final concentration of 1%, and the extract was boiled for 5 min. Then, FLAG-tagged TLR4 was precipitated with anti-FLAG M2 agarose, and CD14 that had been cross-linked with TLR4 was detected by Western blotting with an anti-EIAV tag antibody after the cross-linking reagent was cleaved with a reducing agent (upper panel). The other two cell-extract portions were analysed for CD14 (middle panel) and TLR4 (lower panel) by Western blotting with an anti-EIAV tag antibody and anti-FLAG M2 antibody, respectively.

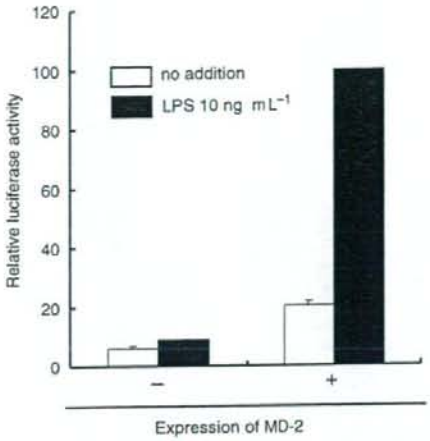

Fig. 3. Effect of MD-2 expression on LPS-induced activation of NF-κB in 293 cells. After plating 293 cell in 12-well plates, cells were transfected with TLR4 (0.02 μg), CD14 (0.02 μg) expression plasmids, pELAM-L (0.1 μg) and phRL-TK (0.0025 μg), and with/without MD-2 (0.02 μg) expression plasmid. After 24 h, the cells were washed three times with PBS, and the medium was replaced by a serum-free medium. The cells were treated for 6 h with LPS (0.01 μg mL^{-1}) in the presences of LBP (0.1 μg mL^{-1}), and luciferase activity was measured. Ordinate values are expressed relative to the normalized luciferase activity induced by LPS in 293 cells expressing MD-2. Values are means ± SE from at least three independent experiments.

The recognition of LPS in cells expressing TLR4 and CD14

Because CD14–TLR4 complex formation was observed in response to LPS in cells expressing TLR4 and CD14 but lacking MD-2, we studied whether LPS stimulation led to the activation of NF-κB (Fig. 3). When we stimulated these cells in the presence of LBP, no activation of NF-κB was observed (Fig. 3, left). In contrast, when the cells expressing TLR4, CD14 and MD-2 were stimulated with LPS, strong activation of NF-κB was observed (Fig. 3, right), as expected. These results indicate that, although LPS stimulation induces the formation of CD14–TLR4 complexes, intracellular signal transduction does not occur in the absence of MD-2.

We then stimulated 293 cells expressing TLR4 and CD14 with LPS in the presence of sMD-2 and LBP (Fig. 4). Surprisingly, no activation of NF-κB was observed, even in the presence of sMD-2 (Fig. 4a). We hypothesized that excess sMD-2 may inhibit LPS signal transduction by associating with LPS. We therefore first treated cells with LPS and LBP, and then added sMD-2 after 3 h. The delayed addition of sMD-2 strongly induced the activation of NF-κB (Fig. 4b). We also preincubated the cells with sMD-2 first, and after 3 h LPS and LBP were added to the medium (Fig. 5). No response to LPS stimulation was observed in this case (Fig. 5a). However, if sMD-2 was removed by washing prior to the addition of LPS and LBP, the cells responded strongly to LPS stimulation (Fig. 5b). These results indicate that the coexistence of LPS and sMD-2 leads to an inactivation of LPS signal transduction in cells. These results also suggest that, once LPS is transferred to mCD14 and the TLR4–CD14 complex is formed, the binding of sMD-2 to the TLR4–CD14 complex enables the activation of 293 cells expressing TLR4 and CD14. To confirm this, we preincubated 293 cells expressing TLR4 and CD14 with LPS in the presence of LBP, and after 3 h LPS and LBP were removed by washing. Cells were then treated with sMD-2, and reporter activity was measured. A strong activation of NF-κB was observed in the cells with the addition of sMD-2, even in the absence of LPS (Fig. 6).

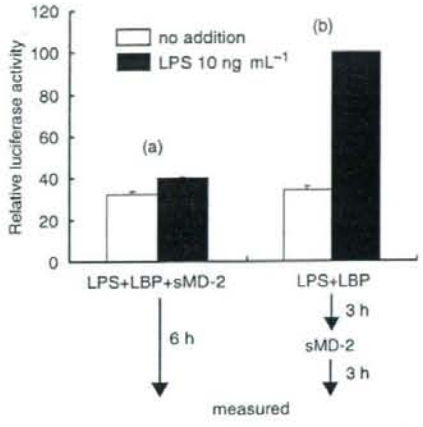

Fig. 4. Effect of the delayed addition of sMD-2 on LPS-induced activation of NF-κB in 293 cells expressing TLR4 and CD14. After plating 293 cells in 12-well plates, cells were transfected with TLR4 (0.02 μg) and CD14 (0.02 μg) expression plasmids, together with pELAM-L (0.1 μg) and phRL-TK (0.0025 μg). After 24 h, the cells were washed three times with PBS, and the medium was replaced by a serum-free medium. (a) The cells either were left untreated (open column) or were treated for 6 h with LPS (0.01 μg mL^{-1}, closed column) in the presence of sMD-2 and LBP (0.1 μg mL^{-1} each), and luciferase activity was measured. (b) The cells either were left untreated (open column) or were treated for 3 h with LPS (0.01 μg mL^{-1}, closed column) in the presence of LBP (0.1 μg mL^{-1}). sMD-2 was then added to a final concentration of 0.1 μg mL^{-1}. After incubation had been continued for a further 3 h, the luciferase activity was measured. Ordinate values are expressed relative to the normalized luciferase activity induced by LPS in the experiment (b). Values are means ± SE from at least three independent experiments.

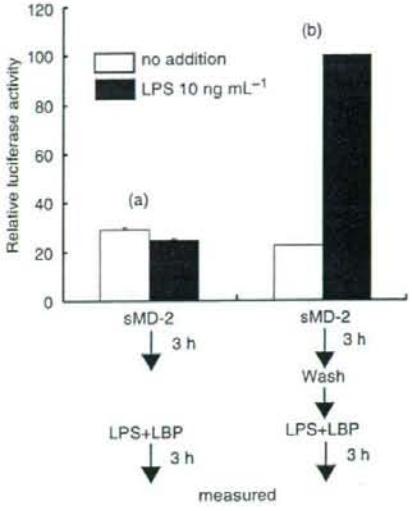

Fig. 5. Effect of the removal of sMD-2 prior to the addition of LPS and LBP on LPS-induced activation of NF-κB in 293 cells expressing TLR4 and CD14. After plating 293 cells in 12-well plates, cells were transfected with TLR4 (0.02 μg) and CD14 (0.02 μg) expression plasmids, together with pELAM-L (0.1 μg) and phRL-TK (0.0025 μg). After 24 h, the cells were washed three times with PBS, and the medium was replaced by a serum-free medium. Then, the cells were treated with sMD-2 (0.1 μg mL^{-1}) for 3 h. The cells either were left unwashed (a) or were washed three times with PBS (b), and then either left unstimulated (open column), or stimulated with LPS (0.01 μg m^{-1}, closed column) in the presence of LBP (0.1 μg mL^{-1}). After incubation for a further 3 h, the luciferase activity was measured. Ordinate values are expressed relative to the normalized luciferase activity induced by LPS in the experiment (b). Values are means ± SE from at least three independent experiments.

Discussion

In this study, we investigated the LPS-recognition mechanism in cells expressing TLR4 and CD14 but lacking MD-2. We used 293 cells transiently transfected with both TLR4 and CD14 expression plasmids. First, we studied the cell-surface expression of TLR4 in the absence of MD-2 expression. It has been demonstrated that the expression of MD-2 with TLR4 induces the translocation of TLR4 to the cell surface (Nagai *et al.*, 2002). Other studies have shown that MD-2 is necessary for the glycosylation of TLR4, which enhances the cell-surface expression of TLR4 (da Silva Correia & Ulevitch, 2002; Ohnishi *et al.*, 2003). In contrast, Viriyakosol *et al.* (2001) reported that TLR4 is translocated to the cell surface even in the absence of MD-2. Our results clearly demonstrated that the cell-surface expression of TLR4 is greatly enhanced by coexpression of MD-2, but apparent TLR4 expression was observed on the cell surface even in the absence of MD-2. It is therefore likely that MD-2 is not essential for the translocation of TLR4, although its expression strongly enhances the cell-surface expression of TLR4.

In cells expressing TLR4, CD14 and MD-2, it has been suggested that mCD14 and TLR4–mMD-2 complexes exist separately on the cell surface, and that the binding of LPS to mCD14 induces the formation of mCD14–TLR4–mMD-2 complexes (Jiang *et al.*, 2000). We examined the formation of TLR4–mCD14 complexes in cells expressing TLR4 and CD14 but lacking MD-2. Our results showed that the complex of TLR4 and mCD14 was formed by LPS stimulation even in the absence of mMD-2 (Fig. 2). This indicates that mMD-2 is not necessary for the formation of TLR4–mCD14 complexes, and that the binding of LPS to mCD14 can lead to the formation of TLR4–mCD14 complex without mMD-2. However, this complex formation did not lead to the activation of NF-κB, and MD-2 was required for activation (Fig. 3). In cells that expressed TLR4 and CD14 but lacked MD-2, it was thought that MD-2 was

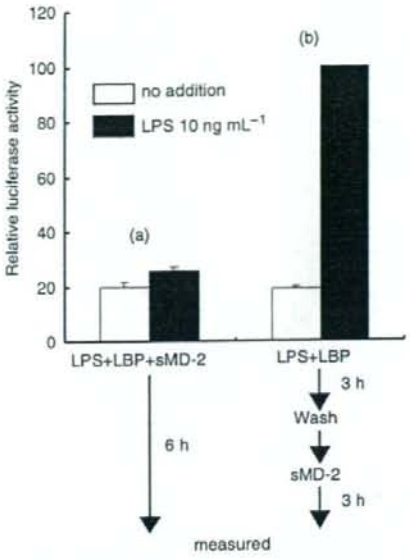

Fig. 6. Effect of the removal of LPS and LBP prior to the addition of sMD-2 on LPS-induced activation of NF-κB in 293 cells expressing TLR4 and CD14. After plating 293 cells in 12-well plates, cells were transfected with TLR4 (0.02 μg) and CD14 (0.02 μg) expression plasmids, together with pELAM-L (0.1 μg) and phRL-TK (0.0025 μg). After 24 h, the cells were washed three times with PBS, and the medium was replaced by a serum-free medium. (a) The cells either were left untreated (open column) or were treated for 6 h with LPS (0.01 μg mL^{-1}, closed column) in the presence of sMD-2 and LBP (0.1 μg mL^{-1} each), and luciferase activity was measured. (b) The cells either were left untreated (open column) or were treated with LPS (0.01 μg mL^{-1}, closed column) for 3 h in the presence of LBP (0.1 μg mL^{-1}). The cells were washed three times with PBS and then treated with sMD-2 (0.1 μg mL^{-1}). After incubation for a further 3 h, the luciferase activity was measured. Ordinate values are expressed relative to the normalized luciferase activity induced by LPS in the experiment (b). Values are means ± SE from at least three independent experiments.

supplied as sMD-2 (Jia *et al.*, 2004). In a situation in which sMD-2 exists at 0.1 μg mL^{-1}, it is expected that LPS will interact with sMD-2 (Viriyakosol *et al.*, 2001) before it interacts with mCD14 because of the overwhelming amount of sMD-2 compared with mCD14. LPS loses its activity if it interacts with sMD-2 before it interacts with CD14 (Fig. 5, and Viriyakosol *et al.*, 2001). The loss of activity probably depends on the formation of an inactive complex of aggregated LPS–sMD-2 (Gioannini *et al.*, 2004) or may partly depend on the increased aggregation of TLR4 in patches on the endosomes (Husebye *et al.*, 2006). There are thus two timings of addition of sMD-2 for the activation of cells expressing TLR4 and CD14 but lacking MD-2, namely before or after LPS interacts with CD14. When cells were pretreated with sMD-2 and excess sMD-2 was removed by washing prior to the addition of LPS and LBP, these cells responded strongly to LPS stimulation (Fig. 5). This result suggests that sMD-2 is transferred to TLR4, that the TLR4–sMD-2 complex is formed, and that finally the binding of LPS to mCD14 induces the formation of the TLR4–sMD-2–mCD14 complex. The formation of this complex leads to the activation of NF-κB. Because the TLR4–sMD-2 complex is formed first, this is a similar mechanism of LPS-recognition to that in cells expressing TLR4 and mMD-2. However, when cells were treated first with LPS in the presence of LBP, the strong activation of NF-κB was induced by the subsequent addition of sMD-2, even if LPS and LBP were removed by washing prior to the addition of sMD-2 (Figs 4 and 6). This result indicates that LPS is first transferred to mCD14 and that this transfer induces the formation of the TLR4–CD14 complex (Fig. 2); subsequently, the delayed binding of sMD-2 to the TLR4–CD14 complex is able to induce the activation of NF-κB.

We studied the activation of NF-κB as one of the activation markers for LPS signalling because the activation of NF-κB is the most common and well-studied type of signalling. It is also known that LPS stimulation leads to the activation of the MAPK kinase signalling pathway as well as to the activation of NF-κB (Kawai *et al.*, 1999). It would therefore be interesting to evaluate the role of MD-2 in the activation of the MAPK kinase signalling pathway.

The mechanism of LPS recognition on the cell surface has been discussed, and some models have been proposed. One widely accepted model is shown in Fig. 7a. The mCD14 molecule and the TLR4–mMD-2 complex exist separately on the cell surface (Jiang *et al.*, 2000). LPS is transferred to mCD14 by LBP, and the LPS–mCD14 complex is formed. The formation of this complex leads to the association of the LPS–CD14 complex with the TLR4–mMD-2 complex (Jiang *et al.*, 2000), and LPS is transferred to the TLR4–MD-2 complex (da Silva Correia *et al.*, 2001). Finally, the oligomerization of TLR4 occurs and the intracellular signalling is generated (Saitoh *et al.*, 2004). In contrast, the mechanism of LPS recognition in cells expressing TLR4 and CD14 but lacking MD-2 has not received much attention. Gioannini *et al.* have reported that the artificially created LPS–sMD-2 complexes with the assistance of sCD14 *in vitro* were able to activate cells expressing only TLR4 (Gioannini *et al.*, 2004), it is predicted that the transfer of LPS–sMD-2 complexes created in the medium to TLR4 may induce transmembrane signal transduction (Fig. 7b). In addition to these mechanisms, we propose a further LPS-recognition mechanism in these cells in the present study (Fig. 7c). mCD14 and TLR4 exist separately on the cell surface and LPS is transferred to mCD14 by LBP. This binding of LPS to mCD14 induces the association of mCD14 with TLR4, and the mCD14–TLR4

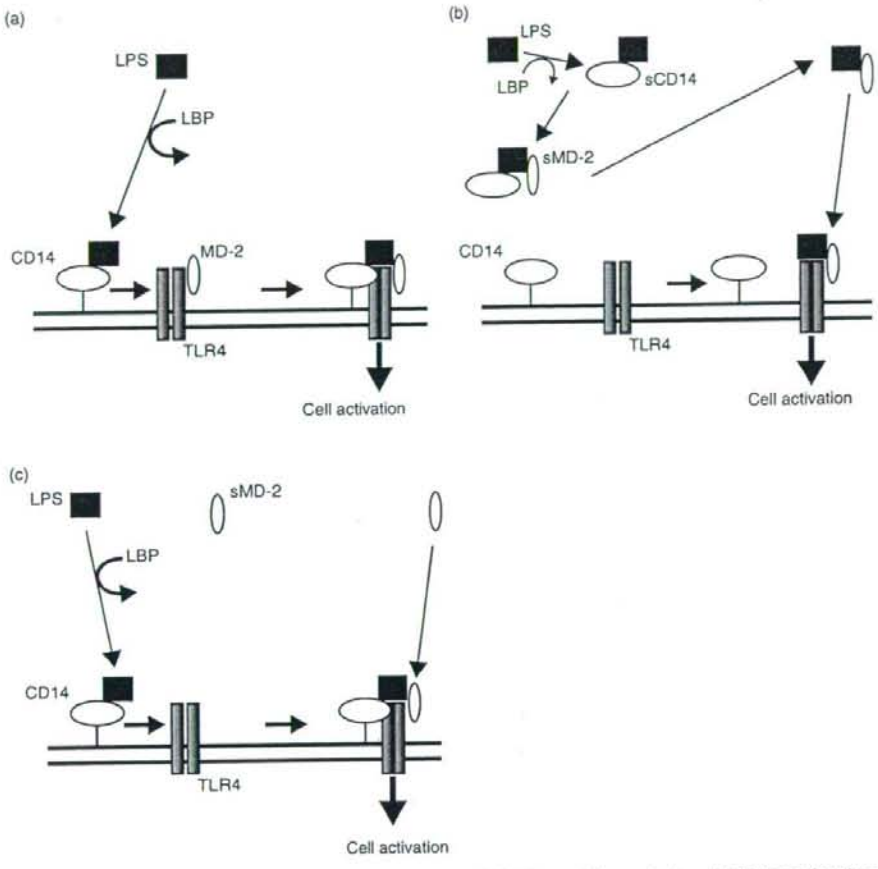

Fig. 7. Schematic representation of conventional (a, b) and proposed additional (c) LPS-recognition mechanisms. (a) LPS is transferred to mCD14 by LBP, and then the LPS–mCD14 complex is recognized by the TLR4–mMD-2 complex in cells expressing TLR4, MD-2 and CD14. (b) The LPS–sMD-2 complex is formed in the medium with the assistance of sCD14, and the LPS–sMD-2 complex is transferred to TLR4. (c) LPS is transferred to mCD14 by LBP, and this association induces the formation of the LPS–mCD14–TLR4 complex in cells expressing TLR4 and CD14. The binding of sMD-2 to this complex leads to the activation of the cells.

complex is formed. The binding of sMD-2 to this complex triggers the transmembrane signal transduction. The presence/absence and dynamics of sMD-2 in human plasma have been controversial. It has been reported that cells expressing TLR4 and CD14 but lacking MD-2 responded to LPS following the addition of plasma containing sMD-2 (Pugin *et al.*, 2004). We hypothesize that, if cells expressing TLR4 and CD14 but lacking mMD-2, such as airway epithelial cells, are exposed to LPS when the plasma sMD-2 level is already high, no activation of NF-κB may occur. However, if these cells are exposed to LPS followed by sMD-2 successively, such as in the case of infection (Pugin *et al.*, 2004), these cells will respond to LPS using our LPS-recognition mechanism.

Acknowledgements

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RESEARCH ARTICLE

MyD88-induced downregulation of IRAK-4 and its structural requirements

Fumihiko Hatao[1,2], Maya Yamamoto[2], Masashi Muroi[2], Michio Kaminishi[1] & Ken-ichi Tanamoto[2]

[1]Department of Metabolic Care and Gastrointestinal Surgery, Graduate School of Medicine, The University of Tokyo, Tokyo, Japan; and [2]Division of Microbiology, National Institute of Health Sciences, Tokyo, Japan

Correspondence: Ken-ichi Tanamoto, Division of Microbiology, National Institute of Health Sciences, 1-18-1 Kamiyoga, Setagaya, Tokyo 158-8501, Japan. Tel.: +81 3 3700 1141, ext. 272; fax: +81 3 3707 6950; e-mail: tanamoto@nihs.go.jp

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Toll-like receptor; IL-1 receptor; NF-κB.

Abstract

IRAK-4 plays an essential role in Toll-like receptor (TLR)/IL-1 receptor signaling. However, its signaling and regulation mechanisms have remained elusive. We have reported previously that stimulation of TLR2, TLR4 or TLR9, but not TLR3, leads to downregulation of IRAK-4 protein. Here, we show that expression of MyD88 leads to downregulation of endogenous as well as exogenously expressed IRAK-4 protein in HEK293 cells. Expression of TRIF did not cause IRAK-4 downregulation although it induced NF-κB activation. Expression of either a deletion mutant of MyD88 lacking its death domain or MyD88s, neither of which induced NF-κB activation, did not lead to IRAK-4 downregulation. MyD88-induced downregulation was observed in an IRAK-4 mutant lacking the kinase domain, but not in another mutant lacking the death domain. These results demonstrate that downregulation of IRAK-4 requires activation of the MyD88-dependent pathway and that the death domains of both MyD88 and IRAK-4 are important for this downregulation.

Introduction

Toll-like receptor (TLR)/IL-1 receptor (IL-1R) family members share common intracellular signaling proteins including MyD88, IL-1R-associated kinase (IRAK) family and TRAF6 (Fujihara *et al.*, 2003; Janssens & Beyaert, 2003). Ligand binding to TLR/IL-1R triggers the recruitment of adaptor proteins, such as MyD88 and TRIF, to the Toll/IL-1 receptor (TIR) domain of TLR/IL-1R via a homophilic TIR–TIR interaction, which in turn recruits IRAK-4 and IRAK-1 into the receptor complex. IRAK-4 does not bind IRAK-1 directly but is recruited into the complex through binding with MyD88. This allows IRAK-1 and IRAK-4 to come in close proximity, which induces IRAK-4 to phosphorylate IRAK-1 (Li *et al.*, 2002). The phosphorylated IRAK-1 interacts with TRAF6, leading to the activation of NF-κB (Cao *et al.*, 1996).

IRAK-4 plays an essential role in TLR/IL-1R signaling. Residual activation of NF-κB in response to IL-1 is still observed in IRAK-1-deficient cells (Kanakaraj *et al.*, 1998; Thomas *et al.*, 1999). In contrast, almost no response to TLR/IL-1R stimulation is observed in IRAK-4-deficient mice (Suzuki *et al.*, 2002) or in patients with IRAK-4 mutations (Picard *et al.*, 2003; Medvedev *et al.*, 2005). Although IRAK-4 is known to phosphorylate IRAK-1 (Li *et al.*, 2002), the requirement of its kinase activity is still controversial (Lye *et al.*, 2004; Qin *et al.*, 2004). In addition, although the internal regions of MyD88 located between its C-terminal TIR and N-terminal death domains have been reported to be necessary for the interaction with IRAK-4 (Burns *et al.*, 2003), the signaling mechanism and the regulation of IRAK-4 have remained elusive. We have reported previously that prolonged stimulation of TLR2, TLR4 or TLR9 leads to downregulation of IRAK-4 protein (Hatao *et al.*, 2004). In this study, we found that expression of MyD88 led to downregulation of IRAK-4 and analyzed the structural requirements of IRAK-4 for this downregulation.

Materials and methods

Cell culture and reagents

The HEK293 cell line (obtained from the Human Science Research Resources Bank, Tokyo, Japan) was grown in Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad, CA) supplemented with 10% (v/v) heat-inactivated fetal calf

serum (Invitrogen), penicillin (100 U mL^{-1}) and streptomycin (100 μg mL^{-1}). An antiserum against EIAV-tag epitope (amino acid sequence: ADRRIPGTAEE) was a kind gift from Dr Nancy Rice (NCI-Frederick Cancer Research and Development Center). An antibody against β-actin (AC-15) was obtained from Sigma-Aldrich (St Louis, MO).

Plasmids

The coding region of human MyD88 was amplified by reverse transcriptase (RT)-PCR from total RNA prepared from human spleen (OriGene Technologies, Rockville, MD). Plasmids containing mouse IRAK-4 (IMAGE: 3995220) and human TRIF (IMAGE: 5180098) were obtained from the Mammalian Gene Collection. Mutations found in the IRAK-4 and TRIF plasmids were corrected by PCR-mediated mutagenesis. The coding regions of all constructs described above were subcloned into mammalian expression vectors containing the N-terminal EIAV-tag or the FLAG-tag sequence. NF-κB-dependent luciferase reporter plasmid pELAM-L was described previously (Muroi *et al.*, 2002). All mutant plasmids were created by PCR-mediated mutagenesis and mutations were confirmed by DNA sequencing.

NF-κB reporter assay and immunoblotting

The NF-κB-dependent luciferase reporter assay was performed as described elsewhere (Muroi & Tanamoto, 2002). Briefly, HEK293 cells (2–5 × 10^5 cells) were plated in six-well plates and on the following day transfected by the calcium phosphate precipitation method with indicated plasmids, together with 0.2 μg of pELAM-L and 5 ng of phRL-TK (Promega, Madison, WI) for normalization. At 24–32 h after transfection, cellular extracts were prepared by adding a lysis buffer (10 mM HEPES–KOH, pH 7.9, 10 mM KCl, 5 mM EDTA, 40 mM β-glycerophosphate, 0.5% NP-40, 30 mM NaF, 1 mM Na_3VO_4, 1 mM DTT, 100 nM okadaic acid) containing a protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany). The reporter gene activity was measured using a portion of the cellular extract according to the manufacturer's (Promega) instruction. Another portion of the cellular extract was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were transferred to a polyvinylidene difluoride membrane (Immobilon-P; Millipore, Bedford, MA) and subjected to immunoblotting with the indicated antibodies. The signals were visualized using an enhanced chemiluminescence system (Amersham Biosciences, Piscataway, NJ).

Results

Expression of MyD88 leads to downregulation of IRAK-4

We have already reported that prolonged stimulation of TLR2, TLR4 or TLR9, but not TLR3, leads to downregulation of IRAK-4 protein (Hatao *et al.*, 2004). It is well known that signaling through these TLRs, with the exception of TLR3, involves the adaptor protein MyD88, and expression of MyD88 leads to the activation of NF-κB. Therefore, we transfected an increasing amount of MyD88 expression plasmid together with an NF-κB-dependent reporter plasmid in HEK293 cells and detected IRAK-4 protein (Fig. 1). The protein level of endogenous IRAK-4 decreased in proportion to the amount of MyD88 plasmid transfected (top panel) although the level of β-actin was not affected (third panel from the top). Expression of either a dominant-negative mutant (MyD88-DN; amino acids 135–296) or a splicing variant (MyD88s; lacks amino acids 110–154) of MyD88, neither of which induced NF-κB activation

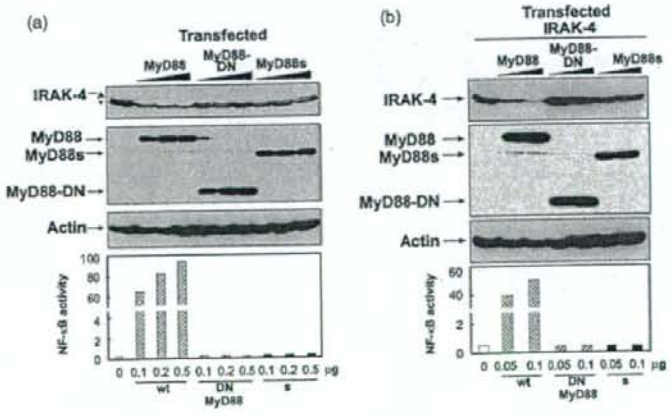

Fig. 1. MyD88-induced downregulation of IRAK-4. An increasing amount of expression plasmid for either EIAV-tagged MyD88, EIAV-tagged MyD88-DN (amino acids 135–296) or EIAV-tagged MyD88s (lacking amino acids 110–154) and an NF-κB-dependent luciferase reporter plasmid were transiently transfected without (a) or with (b) an expression plasmid for EIAV-tagged IRAK-4 into HEK293 cells. After 30 h, cellular extracts were subjected to luciferase activity measurements (bottom panels) and SDS-PAGE, followed by immunoblotting for the detection of endogenous IRAK-4 (a, top panel), EIAV-tagged IRAK-4 (b, top panel), EIAV-tagged MyD88 and its mutants (second panels) or endogenous β-actin (third panels). The asterisk in the top panel of (a) indicates a nonspecific band.

(bottom panel), did not lead to the decrease in IRAK-4 protein (top panel), although these proteins were expressed properly (second panel from the top). To confirm this, we expressed EIAV-tagged IRAK-4 together with an increasing amount of MyD88, MyD88-DN or MyD88s and detected EIAV-tagged IRAK-4 with an anti-EIAV antibody (Fig. 1b). Exogenously expressed IRAK-4 was also decreased by the expression of MyD88 but not by MyD88-DN or MyD88s (top panel). In this experiment as well, the level of β-actin was not affected (third panel), and MyD88 and all of its mutants were expressed properly (second panel) with the activation of NF-κB by wild-type MyD88 only (bottom panel).

Because stimulation of TLR3 did not lead to downregulation of IRAK-4 (Hatao *et al.*, 2004), and another adaptor protein TRIF is involved in TLR3-mediated signaling, we next asked whether expression of TRIF affects the level of IRAK-4 (Fig. 2). We transfected expression plasmids for EIAV-tagged IRAK4 and either MyD88 or TRIF together with an NF-κB-dependent reporter plasmid in HEK293 cells and detected IRAK-4 protein. Although expression of MyD88 and TRIF both activated NF-κB, only MyD88 induced downregulation of IRAK-4, indicating that downregulation of IRAK-4 requires the activation of the MyD88-dependent pathway. The level of β-actin was not affected by expression of these proteins.

Structural requirement of IRAK-4 for MyD88-induced downregulation

We next asked which domain of IRAK-4 is required to undergo MyD88-induced downregulation. IRAK-4 consists of an N-terminal death domain and a C-terminal kinase domain. We created deletion mutants lacking these domains and examined whether expression of MyD88 affects the level of these mutants (Fig. 3). Expression of MyD88 in HEK293 cells led to downregulation of wild-type IRAK-4 and the deletion mutant (dKD) of the kinase domain, indicating that the kinase domain is not necessary for downregulation. However, the IRAK-4 mutant (dDD) lacking the death domain did not undergo downregulation although the MyD88-induced activation of NF-κB was observed at a level

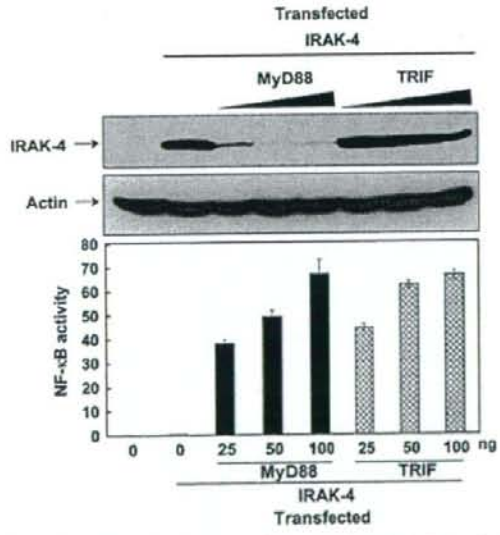

Fig. 2. TRIF does not induce downregulation of IRAK-4. An increasing amount of expression plasmids for either EIAV-tagged MyD88 or EIAV-tagged TRIF and an NF-κB-dependent luciferase reporter plasmid were transiently transfected with an expression plasmid for EIAV-tagged IRAK-4 into HEK293 cells. After 30 h, cellular extracts were subjected to luciferase activity measurements (bottom panel) and SDS-PAGE, followed by immunoblotting for the detection of EIAV-tagged IRAK-4 (top panel) or endogenous β-actin (middle panel). Values are means ± SEM from three independent experiments.

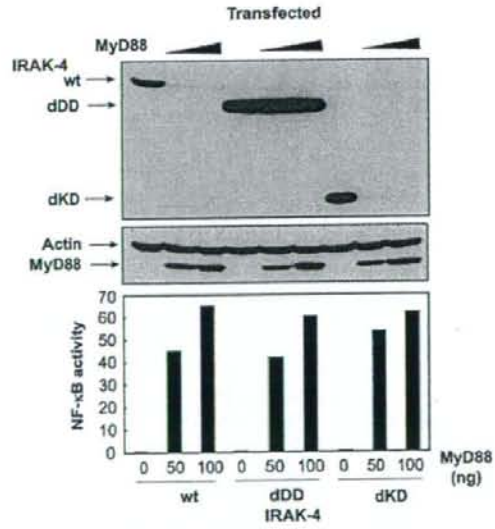

Fig. 3. Structural requirements for IRAK-4 for MyD88-induced downregulation. Each expression plasmid for wild-type (wt) IRAK-4, or an IRAK-4 deletion mutant lacking either death domain (dDD; deletion of amino acids 1–106) or kinase domain (dKD; deletion of amino acids 186–459) was transiently transfected with an increasing amount of EIAV-tagged MyD88 expression plasmid and an NF-κB-dependent luciferase reporter plasmid into HEK293 cells. After 30 h, cellular extracts were subjected to luciferase activity measurements (bottom panels) and SDS-PAGE, followed by immunoblotting for the detection of EIAV-tagged IRAK-4 as well as its mutants (top panel), EIAV-tagged MyD88 and endogenous β-actin (second panels).

comparable to the case when wild-type IRAK-4 was expressed (Fig. 3).

Discussion

We have reported previously that prolonged stimulation of TLR2, TLR4 or TLR9 led to downregulation of IRAK-4 protein whereas the stimulation of TLR3 did not affect the IRAK-4 level (Hatao *et al.*, 2004). MyD88 is involved in signaling through all of these TLRs except TLR3 (Fujihara *et al.*, 2003). Thus, we examined the effect of expression of MyD88 on IRAK-4 protein level and found that expression of MyD88 leads to downregulation of endogenous as well as exogenously expressed IRAK-4 protein (Fig. 1). Our result clearly demonstrates that stimulation of TLRs is not necessary but expression of MyD88 is enough for downregulation of IRAK-4. We used three different IRAK-4 expression plasmids, in which expression of IRAK-4 is controlled by cytomegalovirus (CMV), herpes simplex virus (HSV) thymidine kinase and GAPDH promoters, and IRAK-4 levels expressed through these plasmids were all downregulated by MyD88 (data not shown). In addition, expression of endogenous IRAK-4 is regulated by the promoter different from those described above. It is, therefore, unlikely that the downregulation of IRAK-4 was caused by a decrease in IRAK-4 transcription.

We have reported previously that downregulation of IRAK-4 induced by prolonged stimulation of TLR seems to be mediated through cleavage of IRAK-4 by a protease induced by the activation of NF-κB (Hatao *et al.*, 2004). In this study, MyD88-DN and MyD88s, neither of which induces NF-κB activation, did not lead to IRAK-4 downregulation. On the other hand, expression of TRIF induced a strong activation of NF-κB comparable to MyD88, but also did not cause downregulation (Fig. 2). Therefore, it appears that activation of NF-κB is not enough to induce downregulation of IRAK-4.

An IRAK-4 mutant lacking its death domain did not undergo downregulation, although strong activation of NF-κB was observed upon expression of MyD88 (Fig. 3). This may indicate that a protease that recognizes the death domain is responsible for the downregulation of IRAK-4. We have reported previously that downregulation of IRAK-4 in response to TLR stimulation was not inhibited by broad-spectrum caspase inhibitors (Z-VAD-FMK and A-Asp-CH_2-DCB), a serine protease inhibitor (E-64) and a cathepsin B inhibitor (CA-074 methyl ester), and that the proteasome was unlikely to be involved because a smaller molecular weight protein (*c.* 32 kDa), which appears to be a cleavage product of IRAK-4, was detected by an IRAK-4 antibody following the decrease in IRAK-4 protein (Hatao *et al.*, 2004). We also have not been able to find proteases that specifically recognize the death domain of IRAK-4 using a public protease database (PeptideCutter: http://www.expasy.org/tools/peptidecutter/). Thus, a novel protease or a protease whose recognition sequence has not been identified may be involved in the downregulation of IRAK-4.

An IRAK-4 mutant that lacks the entire kinase domain was downregulated by MyD88, indicating that its kinase domain is not necessary for the downregulation. The involvement of the kinase domain of IRAK-4 in TLR/IL-1R signaling is still controversial (Lye *et al.*, 2004; Qin *et al.*, 2004; Kawagoe *et al.*, 2007; Kim *et al.*, 2007; Koziczak-Holbro *et al.*, 2007). We have reported that downregulation of IRAK-4 in response to TLR stimulation occurred with slower kinetics than the activation of IRAK-1 (Hatao *et al.*, 2004). Thus, a feedback event induced by TLR stimulation seems to be involved in the downregulation. It is well known that prolonged stimulation of a TLR ligand induces a hyporesponsive state to subsequent challenge with ligands for different TLRs (Jacinto *et al.*, 2002; Sato *et al.*, 2002; Dobrovolskaia *et al.*, 2003; Yeo *et al.*, 2003). Because IRAK-4 is essential for signaling through most TLRs (Suzuki *et al.*, 2002), downregulation of IRAK-4 may be involved in the induction of this hyporesponsive state.

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