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Transcriptional activation of an anti-oxidant mouse Pon2 gene by dexamethasone

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Glucocorticoids regulate multiple physiological processes such as metabolic homeostasis and immune response. Mouse Pon2 (mPon2) acts as an antioxidant to reduce cellular oxidative stress in cells. In this present study, we investigated the transcriptional regulation of mPon2 by glucocorticoids. In the presence of glucocorticoid analogue dexamethasone, the expression of mPon2 mRNA in cells was increased, whereas the expression was inhibited by a transcription inhibitor actinomycin D. Glucocorticoid receptors bound to the putative glucocorticoid response elements located between -593 bp and -575 bp of the mPon2 promoter. Transcriptional activity was completely blocked when the putative element was mutated. Taken together, these results suggest that the expression of the mPon2 gene is directly regulated by glucocorticoid-glucocorticoid receptor complexes. [BMB reports 2009; 42(7): 421-426]

INTRODUCTION

Glucocorticoids secreted from the adrenal cortex regulate numerous physiological processes, including glucose homeostasis and modulation of protein and fat metabolism as well as anti-inflammatory and immunosuppressive actions (1-4). Glucocorticoid receptors (GRs) are activated by glucocorticoids in the cytoplasm. Hormone-bound GRs are translocated into the nucleus and directly interact with unique glucocorticoid response elements (GRE) (5-7). Activated glucocorticoid receptors associated with coactivator molecules such as CBP (CREB-binding protein), steroid-receptor coactivator-1 (SRC-1), and glucocorticoid receptor interacting protein-1 (GRIP-1 or SRC-2), regulate the transcriptional activation of the target gene by chromatin remodeling and RNA polymerase II (8, 9). SRC-1 coactivators interact with the AF-1 domain of a nuclear hormone receptor through the formation of a LXXLL motif (10). Moreover, these coactivators play a role on chromatin decompaction by histone modification (10, 11). In addition, glucocorticoids stimulate the expression of antioxidant enzyme-related genes to suppress oxidative processes (12-14). Rat macrophages stimulated by a synthetic glucocorticoid dexamethasone reduce hydrogen peroxide production (12, 15). In Kupffer cells, the production of reactive oxygen species is inhibited by dexamethasone (14).

The paraoxonase gene family consists of Pon1, Pon2 and Pon3. Pon2 encodes a cellular antioxidant enzyme that protects cells against oxidative stress. The exogenous expression of Pon2 in cells exposed to H_2O_2 decreases intracellular oxidative stress (16), by which Pon2 hydrolyzes oxidized phospholipids, leading to a reduction in cellular lipid peroxides (16-18). In addition, Pon2 functions to decrease cholesterol in blood and to suppress apoptotic cell death induced by endoplasmic reticulum stress (19-21). However, the relationship of Pon2 with glucocorticoids remains unclear. Therefore, in this study, we investigated that the transcriptional activation of Pon2 is regulated by glucocorticoid-GR complexes.

RESULTS

Expression of mPon2 mRNA in NIH3T3 cells treated with dexamethasone

Based on previous studies showing that glucocorticoids could increase antioxidant enzyme activity (12, 13), we measured the production of reactive oxygen species (ROS) in cells treated with a synthetic glucocorticoid dexamethasone. As shown in Fig. 1A, the value of ROS production in dexamethasone-treated cells after 1 h decreased significantly compared to that of the control.

Next, we examined the expression of the antioxidant enzyme mPon2 gene in NIH3T3 cells stimulated by dexamethasone. In the presence of 0.1 μM dexamethasone, the level of mPon2 mRNA increased after 6 h and peaked at 12 h (Fig. 1B). This increasing pattern of mPon2 mRNA was maintained at 0.01 μM-1 μM of dexamethasone, but at 10 μM, the levels decreased (Fig. 1C). Interestingly, the levels of glucocorticoid receptor mRNA remained constant regardless of the dexamethasone treatment. When the cells were treated with the transcription inhibitor actinomycin D under the same conditions, the expression of mPon2 mRNA was completely blocked (Fig. 1D). These results suggest that dexamethasone may upregulate

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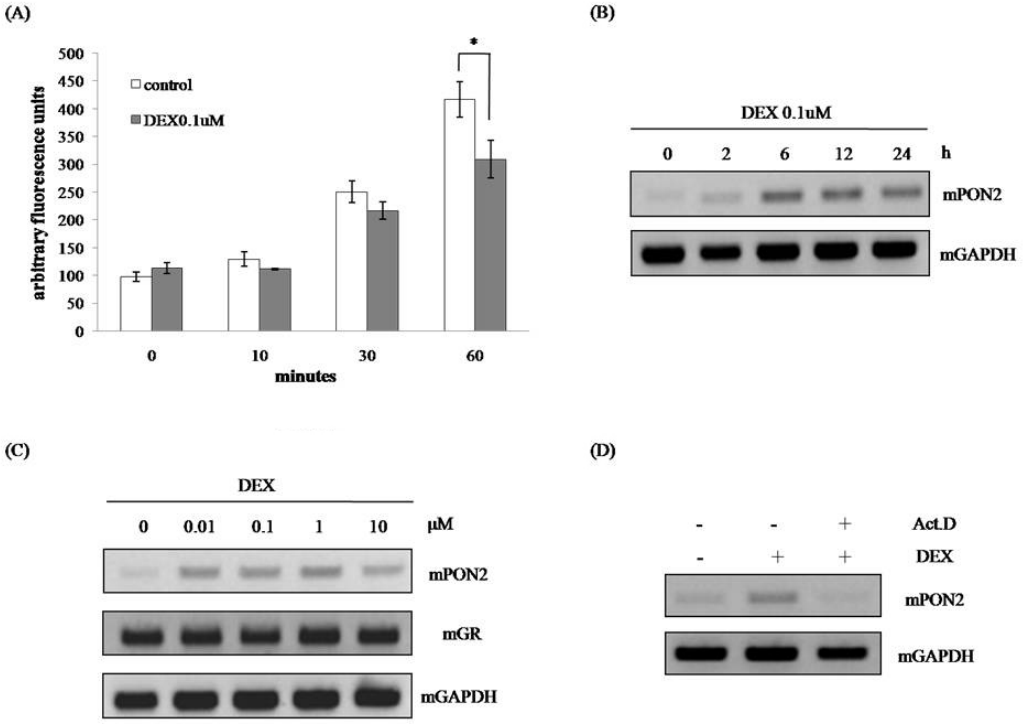

Fig. 1. The effect of dexamethasone on ROS production and mPon2 expression in NIH3T3 cells. (A) The production of ROS in NIH3T3 cells treated with dexamethasone (DEX). ROS production was quantified by the Amplex Red/peroxidase method in NIH3T3 cells treated with 100 nM dexamethasone for 12 h. ROS production was determined by spectrofluorimetry. An asterisk (*) indicates a statistically significant difference relative to the control ($P < 0.05$). (B) NIH3T3 cells were treated with 0.1 μM dexamethasone for the indicated time points. The induction of mPon2 mRNA was determined by reverse transcription-PCR. (C) NIH3T3 cells were treated with dexamethasone at concentrations of 0.01-10 μM for 12 h. Total RNA was extracted and analyzed by reverse transcription-PCR. Mouse GAPDH was used as a control. (D) Cells were treated with actinomycin D (50 ng/ml) for 1 h prior to the addition of 0.1 μM dexamethasone for 12 h. The level of mPon2 mRNA was determined by reverse transcription-PCR.

the transcription of mPon2 expression.

Identification of mPon2 as a glucocorticoid receptor target gene

By sequence analysis, we found that a putative GRE is located between -593 bp and -575 bp in the mPon2 promoter (Fig. 2A). To determine whether the GR binds to the mPon2 promoter in NIH3T3 cells, we conducted chromatin immunoprecipitation (ChIP) assays. As shown in Fig. 2B, the mPon2 proximal promoter was immunoprecipitated with the anti-GR antibody, whereas the non-specific IgG antibody was not. These results suggest that the putative GRE region located between -593 bp and -575 bp in the mPon2 promoter could be essential for binding to the glucocorticoid receptor.

The activity of the mPon2 promoter is regulated by dexamethasone through the GRE region

To determine that the transcriptional activity of mPon2 is induced by dexamethasone through the GRE region, luciferase reporter assays were performed. As shown in Fig. 3A, we

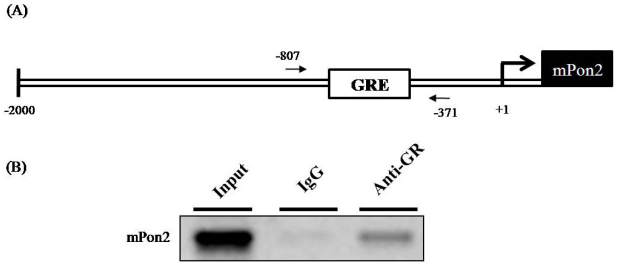

Fig. 2. Glucocorticoid receptors interact with the mPon2 promoter. (A) Schematic representation of the mPon2 construct. The putative glucocorticoid response elements (GRE) of the *mPon2* promoter are represented as white boxes. Arrows indicate the region of primers designed for PCR. (B) DNA-protein complexes from NIH3T3 cells were immunoprecipitated with anti-GR or anti-IgG antibodies. Samples were amplified by PCR reaction using primers flanking the proximal region (-807 bp to -371 bp) comprising the GRE of the mPon2 promoter.

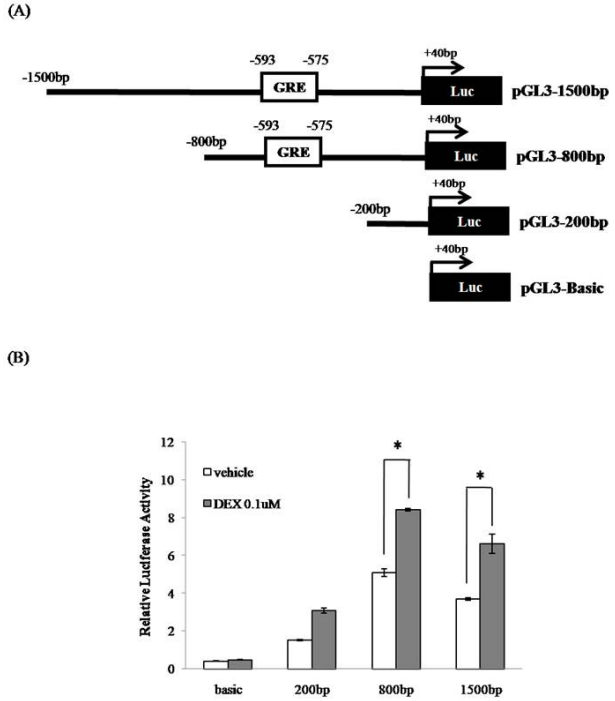

Fig. 3. The GRE region of the mPon2 promoter is required for the transactivation of glucocorticoids-GR complex. (A) Schematic representation of serial deleted mPon2 promoter constructs. pGL3-1,500 bp and pGL3-800 bp constructs include the putative GRE (-593 bp to -575 bp), while the pGL3-200 bp construct lacks the GRE region. (B) NIH3T3 cells were transiently transfected with the mPon2 fragment constructs, followed by the addition of 0.1 μM dexamethasone for 12 h. Cell lysates were assayed for luciferase activity and normalized to renilla luciferase activity. Error bars represent the standard error of the three independent experiments. Asterisks (*) indicate statistically significant differences for the dexamethasone treatment compared to the control ($P < 0.05$).

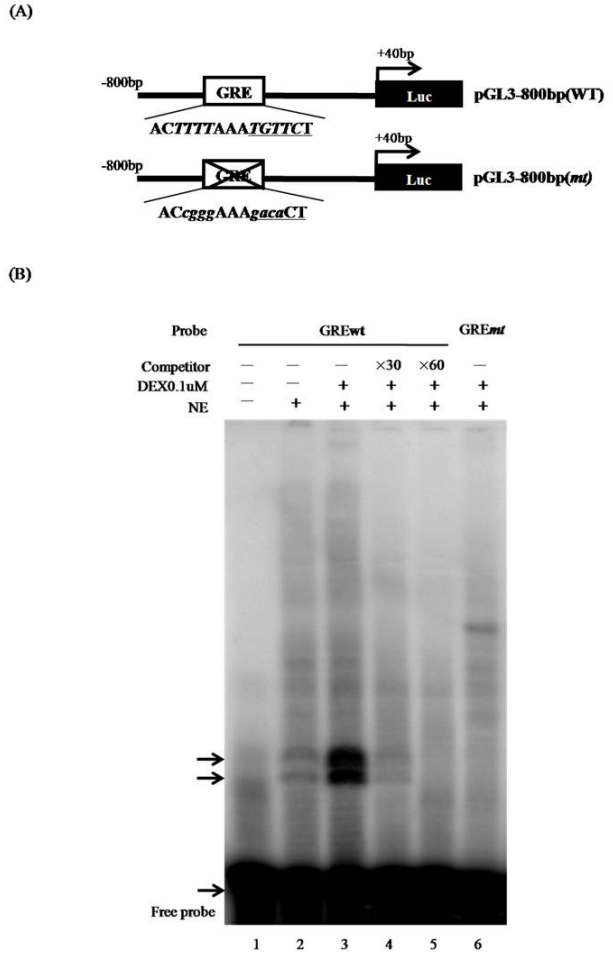

Fig. 4. The DEX-mediated mPon2 promoter activity is dependent on the GRE region. (A) Schematic representation of the GRE region within the mPon2 gene promoter. Putative GRE sequences are located between -593 bp to -575 bp in the 5'-flanking region. Mutated base pairs are indicated in italics. (B) Nuclear extracts of NIH3T3 cells were incubated with labeled oligonucleotide probes for EMSA. For competition assay, the reaction was incubated with 30-fold and 60-fold molar excess of unlabeled oligonucleotide probes. NE indicates nuclear extracts.

designed promoter constructs containing the putative GRE region (pGL3-1500 bp and pGL3-800 bp) as well as lacking the GRE (pGL3-200 bp). In the presence of dexamethasone, luciferase activity increased by approximately 2-fold in cells containing both pGL3-1500 bp and pGL3-800 bp compared to that in pGL3-200 bp (Fig. 3B). Luciferase activity in pGL3-800 bp cells stimulated by dexamethasone increased by 30% compared to the cells without a synthetic hormone. However, in the absence of dexamethasone, luciferase activity also increased in pGL3-800 bp and pGL3-1500 bp compared to that in pGL3-200 bp. It may occur through an indirect, possibly GR-ndependent mechanism. To confirm the real function of the GRE region in mPon2 promoter activation, we mutated the GRE region by site-directed mutagenesis (Fig. 4A). An electrophoretic mobility shift assay (EMSA) was conducted to determine the binding of the GR on the GRE region of the mPon2 promoter in the presence of dexamethasone. Nuclear extracts from NIH 3T3 cells stimulated by dexamethasone were bound to the oligonucleotides spanning the predicted GRE at positions of -593 bp to -575 bp (Fig. 4B, lane 3), which was effectively competed with excess unlabeled consensus sequences (Fig. 4B, lane 4-5). These observations were consistent with the results of the ChIP assay (Fig. 2B). EMSA also demonstrated that the mutated oligonucleotides bearing the putative GRE region did not bind with the nuclear protein. (Fig. 4B, lane 6). These results suggest that the transcriptional activation of the mPon2 gene is induced by glucocorticoid-GR complexes and could be mediated from binding through the

GRE region of the mPon2 promoter.

DISCUSSION

Glucocorticoid-GR complexes associate with coactivators on the promoter of target genes to regulate transcriptional activation. Coactivators bound to glucocorticoid receptors are known as SRC-1/CBP/p300/pCAF complexes, Rap46, RSP5/RPF1, and SWI/SNF family complexes (8, 22). Glucocorticoid-GR complexes bound to coactivators induce transcriptional activation of SULT1A1 and SULT1A3, cytosolic aryl sulfotransferases for detoxication as well as to regulate the expression of the Ucp3 gene in response to stress (23-25). In the present study, we show that the expression of mPon2 as an antioxidant enzyme against cellular oxidative stress is also induced by glucocorticoids. Previous reports and our data suggest that glucocorticoids play a role in the reduction of cellular oxidative stress by the transcriptional regulation of antioxidant enzymes and stress-response genes.

Pon2 affects various pathophysiological states including immunity, hyperlipidemia, atherosclerosis and cardiovascular diseases (17, 20, 26, 27). However, the transcriptional regulation of the Pon2 gene remains unclear. Recently, it has been reported that Pon2 expression is regulated by the transcription factor AP-1 in macrophages (28, 29). AP-1 binds to the AP-1 response element located 2 kb upstream of the transcription start site of the human Pon2 gene and transcriptionally activates Pon2 gene expression during macrophage differentiation (18, 28). Interestingly, glucocorticoids affect the transactivation of AP-1 with activated GRs (30). GRs activated by glucocorticoids bind to AP-1 and suppress AP-1 transactivation by inhibiting the CBP-associated HAT activity (31). However, the effect of glucocorticoids on AP-1 is excluded in our study, as the AP-1 response elements localize at -2 kb of the mouse Pon2 promoter. Therefore, we believe that the transcriptional activity of Pon2 in our study is directly regulated by glucocorticoid-GR complexes bound to the GRE region of the mPon2 promoter. The results from our study on glucocorticoid regulation of Pon2 expression elucidate an aspect of the molecular mechanism of antioxidant and anti-inflammatory responses mediated by glucocorticoids.

MATERIALS AND METHODS

Cell culture

NIH3T3 mouse embryonic fibroblast cells were obtained from the American Type Culture Collection (Manassas, USA) and cultured in DMEM (Dulbecco's Modified Eagle's Medium, GIBCO BRL) supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin at 37°C in 5% CO_2. At 70% confluency, the medium was changed to DMEM containing 5% dextran-coated charcoal-stripped serum and dexamethasone (Sigma-Aldrich, Inc., St. Louís, USA) for 12 h. Dexamethasone was dissolved in ethanol.

Reverse-transcription PCR

Total RNA was isolated using TRIzol reagent (Invitrogen Corp. Carlsbad, USA) and synthesized into cDNA using M-MLV reverse transcriptase (Promega Corp. Madison, USA). PCR analysis was performed at 95°C for 5 min, followed by 27 cycles of 95°C for 2 min, 60°C for 1 min and 72°C for 1 min. The primer sequences were as follows: mPon2 sense 5'- TAA CCC ACA TGG CAT TAG CA -3', antisense 5'- TAG TGG TCA TTG GTG GCG TA -3'; GR sense 5'- GAC CGA AAC AAA AGT GAT GG -3', antisense 5'- GTG AGT CTG GGG AAA CTC CT -3'; GAPDH sense 5'- AAC TTT GGC ATT GTG GAA GG -3', antisense 5'- ACA CAT TGG GGG TAG GAA CA -3'. Amplified fragments were analyzed on a 1.5% agarose gel.

Plasmid constructs

The mouse Pon2 core promoter fragment was generated by amplifying a region, -1500 bp to +40 bp, from a mouse Pon2 BAC clone (RP23-390N11, BACPAC Inc., Oakland, USA). PCR amplification was performed with the following primers: -1500 bp mPon2 sense 5' -AGG GGC CAG GTA CAA ACC GG -3', antisense 5'- ACC GAG CAG CTC CGG ACG CT -3'; -800 bp mPon2 sense 5'- AGA GAT GTC TCA GCA CTA AG -3', antisense 5'- ACC GAG CAG CTC CGG ACG CT -3'; -200 bp mPon2 sense 5'- GGG AGC TGA GCA GGT GCA GC -3', antisense 5'- ACC GAG CAG CTC CGG ACG CT -3'. PCR products were cloned in the pGL3 vector (Promega Corp. Madison, USA), and all constructs were confirmed by DNA sequence analysis.

Transient transfection and reporter assays

Cells were grown in 24-well plates at a density of 2 x 10^4 cells/well. After 24 h, cells were co-transfected with 1 μg/well of the pGL3 plasmid carrying the mPon2 promoter-driven firefly luciferase gene and 0.05 μg/well of the pRL-TK vector carrying the renilla luciferase gene using Exgen 500 transfection reagent (Fermentas UAB, Vilnius, Lithuania) according to the manufacturer's protocol. The pRL-TK vector was used as a control for normalization. At 12 h post-transfection, the culture medium was changed to a fresh medium containing 10% charcoal-stripped serum and the cells were treated with 100 nM dexamethasone for 12 h. After cell lysis, luciferase activity was analyzed with the Dual-Luciferase Assay System (Promega Corp., Madison, USA). Firefly and renilla luciferase levels were measured with a luminometer, respectively (Turner BioSystems, Inc., Sunnyvale, CA).

Site-directed mutagenesis

The QuickChangeTM Site-Directed Mutagenesis Kit (Stratagene Corp. La Jolla, USA) was used to generate mutations in the putative glucocorticoid response elements of the mPon2 promoter. Mutagenesis was performed using the pGL3-800 bp plasmid as a template according to the manufacturer's protocol. PCR conditions were 95°C for 30 s, followed by 15

cycles of 95°C for 30 s, 60°C for 1 min and 68°C for 10 min. PCR products were subsequently incubated with *Dpn* I to digest the parental DNA template, and the mutations were verified by sequencing.

Chromatin immunoprecipitation

NIH3T3 cells were cultured in 100 mm dishes. Following fixation, the cells were harvested and lysed with lysis buffer (50 mM Tris-Cl pH 8.1, 10 mM EDTA, and 1% SDS). Chromatin was sheared using a sonicator microprobe (GENEQ, Inc., Jarry East, Canada) and cleared by centrifugation at 15,000 rpm. The supernatant was transferred to fresh tubes containing ChIP dilution buffer (16.7 mM Tris-Cl pH 8.1, 167 mM NaCl, 1.2 mM EDTA, 1.1% Triton X-100 and 0.01% SDS), and the chromatin was immunoprecipitated with either anti-GR (Abcam Corp., Cambridge, UK) or anti-IgG antibodies. After conjugating to protein A agarose beads (Upstate, NY, USA), immunoprecipitated samples were eluted and digested with proteinase K (Roche, Basel, Switzerland) at 95°C for 10 min. DNA was purified using mini-columns (Bio-Rad Laboratories, Hercules, USA). The mPon2 promoter region was PCR- amplified using the following primers: sense 5'-CAT GCA AGC AAA ACA ACC ATG C-3', antisense 5'-GAA GCT AGG AAA CAA ACT CAG GAC-3'. Amplified fragments were analyzed on a 1.5% agarose gel.

Electrophoretic mobility shift assay

Nuclear extracts were prepared using NE-PER nuclear and cytoplasmic extraction reagents (Pierce, Rockford, IL) according to the manufacturer's protocol. The protein amount was quantitated using the Bradford Protein Assay (Bio-Rad Laboratories, Hercules, USA). The double-stranded oligonucleotides containing the putative GRE binding site was labeled with [α-^{32}P] CTP (3,000 Ci/mmol at 10 mCi/ml) by the Klenow fragment (Takara Bio Inc. Shiga, Japan). Nuclear extracts (5 μg) were incubated for 30 min at room temperature with 2 pM of a labeled oligonucleotide probe in 25 μl of binding buffer (100 mM Tris-Cl pH7.9, 500 mM KCl, 10 mM DTT) containing 20 μg of poly (dI:dC). Binding was electrophoresed through 5% gel (29:1 acrylamide/bisacrylamide) in 0.5X TBE buffer at 4°C. For competition assay, the reaction was preincubated with 30-fold and 60-fold molar excess of unlabeled oligonucleotide for 5 min before the addition of a labeled oligonucleotide probe. Radioactivity was quantitated using the Personal Molecular Imager FX (Bio-Rad Laboratories, Hercules, USA). The sequences of double-strand oligonucleotides as probes were as follows: GRE wild, 5'- GTTCAAAACAATAAGAACATTTAA AA GTACAATTTT-3'; GRE mutant, 5'-GTTCAAAACAATAA*GT GTC*TTT*CCCG*GTACAATTTT-3'. Consensus nucleotides are underlined. Mutated nucleotides are indicated in italics.

Measurement of ROS using amplex red assay

Cells plated on 96-well plates (2 x 10^4 cells/well) were incubated with DMEM medium containing charcoal-stripped serum with dexamethasone. Amplex Red (50 μM) and horseradish peroxidase (0.1 U/ml) were added in a final volume of 200 μl/well. Diphenylene iodonium was used at a concentration of 2.5 μM. Intracellular production of H_2O_2 was fluorometrically determined according to the manufacturer's instructions (Amplex Red, Molecular Probes Inc., Eugene, OR). The fluorescence spectrum was monitored at 37°C during 1 h with the SpectraMax Gemini EM Microplate Reader (Molecular Devices, Sunnyvale, CA) at an excitation wavelength of 544 nm and emission of 590 nm.

Statistical analysis

Data are presented as mean ± SE of three independent experiments. Comparisons were analyzed by a one-way ANOVA using the SPSS software package (SPSS Inc.). The value of $P < 0.05$ was considered for statistical significance.

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