Harding HP, Novoa I, Zhang Y, Zeng H, Wek R, Schapira M *et al.* (2000). *Mol Cell* **6**: 1099–1108.
Hong M, Li M, Mao C, Lee AS. (2004). *J Cell Biochem* **92**: 723–732.
Hoshino T, Tsutsumi S, Tomisato W, Hwang HJ, Tsuchiya T, Mizushima T. (2003). *J Biol Chem* **278**: 12752–12758.
Hu PJ, Yu J, Zeng ZR, Leung WK, Lin HL, Tang BD *et al.* (2004). *Gut* **53**: 195–200.
Huang YC, Chuang LY, Hung WC. (2002). *Mol Pharmacol* **62**: 1515–1521.
Huo R, Zhu YF, Ma X, Lin M, Zhou ZM, Sha JH. (2004). *Cell Tissue Res* **316**: 359–367.
Husain SS, Szabo IL, Tamawski AS. (2002). *Am J Gastroenterol* **97**: 542–553.
Jamora C, Dennert G, Lee AS. (1996). *Proc Natl Acad Sci USA* **93**: 7690–7694.
Jiang HY, Wek SA, McGrath BC, Lu D, Hai T, Harding HP *et al.* (2004). *Mol Cell Biol* **24**: 1365–1377.
Johnson AJ, Hsu AL, Lin HP, Song X, Chen CS. (2002). *Biochem J* **366**: 831–837.
Kao JP, Harootunian AT, Tsien RY. (1989). *J Biol Chem* **264**: 8179–8184.
Kaufman RJ. (2002). *J Clin Invest* **110**: 1389–1398.
Kawai S, Nishida S, Kato M, Furumaya Y, Okamoto R, Koshino T *et al.* (1998). *Eur J Pharmacol* **347**: 87–94.
Kismet K, Akay MT, Abbasoglu O, Ercan A. (2004). *Cancer Detect Prev* **28**: 127–142.
Koki AT, Masferrer JL. (2002). *Cancer Control* **9**: 28–35.
Koomagi R, Mattern J, Volm M. (1999). *Anticancer Res* **19**: 4333 4336.
Kulp SK, Yang YT, Hung CC, Chen KF, Lai JP, Tseng PH *et al.* (2004). *Cancer Res* **64**: 1444–1451.
Kurisu J, Honma A, Miyajima H, Kondo S, Okumura M, Imaizumi K. (2003). *Genes Cells* **8**: 189–202.
Landry SJ. (2003). *Biochemistry* **42**: 4926–4936.
Leahy KM, Ornberg RL, Wang Y, Zweifel BS, Koki AT, Masferrer JL. (2002). *Cancer Res* **62**: 625–631.
Lee AS. (2001). *Trends Biochem Sci* **26**: 504–510.
Lim JW, Kim H, Kim KH. (2001). *Lab Invest* **81**: 349–360.
Lopez-Parra M, Claria J, Titos E, Planaguma A, Parrizas M, Masferrer JL *et al.* (2005). *J Hepatol* **42**: 75–81.
Luo S, Baumeister P, Yang S, Abcouwer SF, Lee AS. (2003). *J Biol Chem* **278**: 37375–37385.
Mima S, Tsutsumi S, Ushijima H, Takeda M, Fukuda I, Yokomizo K *et al.* (2005). *Cancer Res* **65**: 1868–1876.
Morris JA, Dorner AJ, Edwards CA, Hendershot LM, Kaufman RJ. (1997). *J Biol Chem* **272**: 4327–4334.
Okada K, Sugihara H, Bamba M, Bamba T, Hattori T. (2000). *Cancer Genet Cytogenet* **118**: 99–107.
Patel MI, Subbaramaiah K, Du B, Chang M, Yang P, Newman RA *et al.* (2005). *Clin Cancer Res* **11**: 1999–2007.
Piazza GA, Rahm AL, Krutzsch M, Sperl G, Paranka NS, Gross PH *et al.* (1995). *Cancer Res* **55**: 3110–3116.
Reddy RK, Mao C, Baumeister P, Austin RC, Kaufman RJ, Lee AS. (2003). *J Biol Chem* **278**: 20915–20924.
Riendeau D, Charleson S, Cromlish W, Mancini JA, Wong E, Guay J. (1997). *Can J Physiol Pharmacol* **75**: 1088–1095.
Ristimaki A, Honkanen N, Jankala H, Sipponen P, Harkonen M. (1997). *Cancer Res* **57**: 1276–1280.
Ron D. (2002). *J Clin Invest* **110**: 1383–1388.
Roybal CN, Yang S, Sun CW, Hurtado D, Vander Jagt DL, Townes TM *et al.* (2004). *J Biol Chem* **279**: 14844–14852.
Schroeder CP, Yang P, Newman RA, Lotan R. (2004). *Cancer Biol Ther* **3**: 847–852.
Shen J, Chen X, Hendershot L, Prywes R. (2002a). *Dev Cell* **3**: 99–111.
Shen Y, Meunier L, Hendershot LM. (2002b). *J Biol Chem* **277**: 15947–15956.
Smalley WE, Ray WA, Daugherty JR, Griffin MR. (1995). *Am J Epidemiol* **141**: 539–545.
Song MS, Park YK, Lee JH, Park K. (2001). *Cancer Res* **61**: 8322–8330.
Sorensen HT, Friis S, Norgard B, Mellemkjaer L, Blot WJ, McLaughlin JK *et al.* (2003). *Br J Cancer* **88**: 1687–1692.
Tanaka K, Tomisato W, Hoshino T, Ishihara T, Namba T, Aburaya M *et al.* (2005). *J Biol Chem* **280**: 31059–31067.
Tomisato W, Tanaka K, Katsu T, Kakuta H, Sasaki K, Tsutsumi S *et al.* (2004a). *Biochem Biophys Res Commun* **323**: 1032–1039.
Tomisato W, Tsutsumi S, Hoshino T, Hwang HJ, Mio M, Tsuchiya T *et al.* (2004b). *Biochem Pharmacol* **67**: 575–585.
Tomisato W, Tsutsumi S, Rokutan K, Tsuchiya T, Mizushima T. (2001). *Am J Physiol Gastrointest Liver Physiol* **281**: G1092–G1100.
Tsutsumi S, Gotoh T, Tomisato W, Mima S, Hoshino T, Hwang HJ *et al.* (2004). *Cell Death Differ* **11**: 1009–1016.
Tsutsumi S, Tomisato W, Takano T, Rokutan K, Tsuchiya T, Mizushima T. (2002). *Biochim Biophys Acta* **1589**: 168–180.
Wang JL, Lin KL, Chen JS, Lu YC, Jiann BP, Chang HT *et al.* (2004). *Biochem Pharmacol* **67**: 1123–1130.
Williams CS, Watson AJ, Sheng H, Helou R, Shao J, DuBois RN. (2000). *Cancer Res* **60**: 6045–6051.
Wooden SK, Li LJ, Navarro D, Qadri I, Pereira L, Lee AS. (1991). *Mol Cell Biol* **11**: 5612–5623.
Xu W, Liu L, Charles IG, Moncada S. (2004). *Nat Cell Biol* **6**: 1129–1134.
Yoshida H, Okada T, Haze K, Yanagi H, Yura T, Negishi M *et al.* (2000). *Mol Cell Biol* **20**: 6755–6767.
Yu M, Haslam RH, Haslam DB. (2000). *J Biol Chem* **275**: 24984–24992.
Zhang X, Morham SG, Langenbach R, Young DA. (1999). *J Exp Med* **190**: 451–459.
Zweifel BS, Davis TW, Ornberg RL, Masferrer JL. (2002). *Cancer Res* **62**: 6706–6711.

Functional Gene Cloning and Characterization of MdeA, a Multidrug Efflux Pump from *Staphylococcus aureus*

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Received November 3, 2005; Accepted January 5, 2006; published online January 10, 2006

A DNA fragment conferring drug resistance was cloned from the chromosomal DNA of *Staphylococcus aureus* N315 using a drug hypersensitive *Escherichia coli* KAM32 as the host. Although *E. coli* KAM32 cells were sensitive to many antimicrobial agents, transformed cells harboring a recombinant plasmid carrying the DNA region became resistant to several structurally unrelated antimicrobial agents, such as tetraphenylphosphonium chloride, Hoechst 33342 and norfloxacin. These results suggest that the cloned DNA fragment carries a gene(s) encoding a multidrug efflux pump. We partially determined the nucleotide sequence of the cloned DNA and found the *mdeA* gene within it. The *E. coli* cells transformed with the *mdeA* gene showed efflux activity of Hoechst 33342. On the other hand, *S. aureus* cells transformed with *mdeA* showed elevated resistance to doxorubicin, daunorubicin, tetraphenylphosphonium chloride, Hoechst 33342, ethidium bromide and rhodamine 6G. Elevated energy-dependent efflux of ethidium was observed with transformed *S. aureus*. We found that the *mdeA* gene was expressed under normal growth conditions in *S. aureus* N315.

Key words MdeA; multidrug efflux pump; drug resistance; *Staphylococcus aureus*

Infectious diseases pose a great threat for human beings. After World War II, many antibiotics have been discovered, developed, and used for the treatment of patients infected with pathogenic bacteria. It had been expected that infectious diseases might be well controlled by using the antibiotics. However, drug resistant bacteria appeared soon after the use of antibiotics. Nowadays, bacteria that show resistance against multiple drugs appeared and are called multidrug resistant bacteria. It is very difficult to treat patients infected with multidrug resistant bacteria. Only a few antimicrobial agents are effective on the multidrug resistant bacteria, such as methicillin-resistant *Staphylococcus aureus* (MRSA), vancomycin-resistant enterococci (VRE) and so on. Thus, emergence and widespread occurrence of multidrug resistant bacteria are big problems for the health of human beings.

There are several mechanisms of drug resistance in bacteria; 1) inactivation of the drug by degradative- or modification-enzymes, 2) alteration of the drug target, 3) appearance of alternative enzymes that are not inhibited by the drug, 4) reduced membrane permeability of the drug, and 5) active extrusion of the drug from cells. Among these mechanisms, drug extrusion has been recognized as the major mechanism for multidrug resistance. Multidrug efflux pumps extrude structurally unrelated multiple drugs. Thus, multidrug efflux pumps are most important for bacterial escape from the toxicity of compounds of various structures.

In order to successfully control multidrug resistant bacteria, extensive knowledge of the properties is required of the multidrug efflux pumps and of the molecular mechanisms underlying microbial antibiotic resistance. MRSA occupies the highest percentage of bacterial isolates that are drug resistant in hospitals.[1]

Although only five multidrug efflux pumps encoded by the chromosomal DNA of *S. aureus* have been reported,[2—10] there are many (more than 30) genes for putative multidrug efflux pumps judging from the genome sequence data.[11] The five reported multidrug efflux pumps are, NorA,[2—5] SepA,[6] MdeA,[7] NorB[8] and MepA.[9,10] The NorA pump has been well characterized.[2—6] However, the properties of the other pumps are not as well elucidated. We have been trying to clone as many genes for the multidrug efflux pumps as possible from the chromosome of *S. aureus* in order to gain insight into the complete picture of the multidrug efflux pumps in this microorganism. We employed two strategies for the gene cloning of the multidrug efflux pumps; 1) functional cloning using the drug hyper-susceptible *Escherichia coli* strain as a host, and 2) PCR cloning of all possible genes for multidrug efflux pumps using the *S. aureus* drug susceptible strain as a host. It is advantageous to use a strain of *S. aureus* for which the genome sequence is available as a source of chromosomal DNA.[11] Here we report the functional cloning of a gene for a multidrug efflux pump, which was identified as MdeA, and the characterization of the pump in cells of both *E. coli* and *S. aureus*.

MATERIALS AND METHODS

Bacteria and Growth *S. aureus* N315 (an MRSA strain), of which the genome project has been completed and the genome information available,[11] was used as a source of chromosomal DNA. *Escherichia coli* strain KAM32[12] (a drug hyper-susceptible strain) lacking two multidrug efflux pumps AcrAB and YdhE, and *S. aureus* RN4220 (a methicillin sensitive strain), were used as hosts. *S. aureus* N315 cells and RN4220 cells were grown in a nutrient medium (NISSUI Co.), and *E. coli* KAM32 cells were grown in L medium (1% polypepton, 0.5% sodium chloride, 0.5% yeast extract) at 37 °C. Growth of cells was monitored turbidimetrically at 650 nm.

Gene Cloning and Sequencing Chromosomal DNA was prepared from *S. aureus* N315 by the method of Berns and Thomas.[13] The DNA was partially digested with a re-

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striction enzyme *Sau*3AI, and the fragments ranging in size from 4 to 10 kbp were separated by sucrose density gradient centrifugation. Plasmid pBluescript II KS (+) (TOYOBO Co.) was digested with *Bam*HI, dephosphorylated with bacterial alkaline phosphatase and ligated with the chromosomal DNA fragments by using a Ligation Kit Ver. 2 (TaKaRa Co.). Competent cells of *E. coli* KAM32 were transformed with recombinant plasmids and were spread onto agar plates containing L broth, 9 μg/ml of tetraphenylphosphonium chloride (TPPCl), 100 μg/ml ampicillin and 1.5% agar. The plates were incubated at 37 °C for 24 h. Candidate colonies were replica-plated, and plasmids were isolated from each of the candidates. Plasmids were reintroduced into *E. coli* KAM32 cells and spread onto the same type of plate. The plasmids were then isolated from each of the transformants that appeared on the plate. One of the candidate plasmids possibly carrying a gene responsible for TPPCl resistance was designated as pBTP3. For sub-cloning, the insert in the pBTP3 plasmid was digested with *Nsp*I and blunt ended with T4 polymerase (TaKaRa Co.) and ligated to the *Hinc*II site of pSTV28, a vector. The resulting recombinant plasmid, designated as pSTP2, was introduced into *E. coli* KAM32 cells, and the transformants were tested for sensitivity or resistance to TPPCl. The pSTP2 plasmid was digested with *Eco*RI and *Pst*I, and ligated to the corresponding sites of pRIT5,[14] a shuttle vector between *E. coli* and *S. aureus*. The resulting recombinant plasmid was introduced into *S. aureus* RN4220 cells.[15] The resulting plasmid was designated as pRTP2.

The nucleotide sequence was determined by the di-deoxy chain termination method[16] using a DNA sequencer (ALF Express, Pharmacia Biotech.).

Drug Susceptibility Testing The minimal inhibitory concentrations (MICs) of various drugs were determined in Mueller-Hinton (MH) broth (Difco) containing different drugs at various concentrations as reported previously.[17] Cells were incubated in the medium at 37 °C for 24 h, and the growth was examined by visual inspection.

Hoechst 33342 Efflux Assay in *E. coli* Cells Cells of *E. coli* KAM32 harboring control plasmid or recombinant plasmid were grown in 10 ml of L broth. After harvesting, the cells were washed with modified Tanaka buffer,[18] in which sodium salts were replaced with potassium salts, and then resuspended in the same buffer containing 1 μM of Hoechst 33342 and 5 mM 2,4-dinitrophenol (DNP) and incubated at 37 °C for 10 h. Cells were washed with 0.1 M Mops-tetramethylammonium hydroxide (pH 7.0) containing 2 mM $MgSO_4$, and resuspended in the same buffer. Changes in fluorescence intensities of Hoechst 33342 were measured with a fluorescent spectrometer at excitation and emission wavelengths of 355 nm and 457 nm, respectively.[19] The cell suspension was incubated at 37 °C for 5 min, and then glucose was added as an energy source to the cells.

Ethidium Efflux Assay in *S. aureus* Cells Cells were harvested at the late exponential phase of growth and washed twice with 20 mM HEPES–NaOH buffer (pH 7.0). Cells were then suspended in HEPES–NaOH buffer containing carbonyl cyanide *m*-chlorophenylhydorazone (CCCP) at 20 μM and ethidium bromide at 5 μM, followed by incubation at 37 °C for 30 min. The cells were centrifuged, washed, and resuspended in the HEPES–NaOH buffer, and changes in fluorescence intensities were measured with a fluorescent spectrometer at 530 nm (excitation) and 600 nm (emission) wavelengths.[20]

RT–PCR Analysis Cells of *S. aureus* N315 were harvested at the exponential phase of growth. Total cellular RNA was isolated from the cells using the Qiagen RNeasy Mini Kit (Qiagen Inc., U.S.A.), treated with RNase-free DNase (Promega, U.S.A.) (1 U of enzyme/μg RNA for 2 h at 37 °C) and re-purified using the same kit. A 1 ng sample of DNase-treated RNA was used as a template for RT-PCR with the OneStep RT-PCR Kit (Qiagen Inc., U.S.A.) according to the manufacturer's protocol. Primer pairs specific for and internal to *mdeA*, *norA* or *rpsL* (encoding a constitutively expressed gene for ribosomal protein; a control) genes were used for RT-PCR. Primers used for *mdeA* were GCCATTGATGATGACAACGA (forward) and GGAGCGACAACATGGAAAAG (reverse), for *norA* were CTGGTATGGTAATGCCTGGTG (forward) and TCGCTGACATGTAGCCAAAG (reverse), and for *rpsL* were CACCACAAAAACGTGGTGTATG (forward) and TGTTGTTTGATAAACGCACACG (reverse). The reaction mixtures were incubated for 30 min at 50 °C, followed by 15 min at 95 °C, and 28 cycles of 30 s at 94 °C, 30 s at 60 °C and 30 s at 72 °C. A 5 μl sample of each reaction product was analyzed by Agarose X (3% w/v) (Wako Pure Chemicals) gel electrophoresis for the presence of expected RT-PCR products (for *mdeA* 168 bp, for *norA* 90 bp, and for *rpsL* 102 bp).

RESULTS

We cloned a gene responsible for resistance to TPPCl from the chromosomal DNA of *S. aureus* N315. Introduction of a plasmid pBTP3 carrying the gene into *E. coli* KAM32 cells that originally showed hyper-susceptibility to many antimicrobial agents conferred elevated resistance to (higher MIC of) TPPCl. We found that there were 3 open reading frames (ORFs) derived from the chromosome of *S. aureus* in the recombinant plasmid pBTP3. We sub-cloned the ORFs and found that ORF *SA2203* was responsible for the elevated resistance. The deduced amino acid sequence of the protein encoded by the *SA2203* showed 99% identity with MdeA of *S. aureus* Buttle strain.[7] Therefore we conclude that the ORF *SA2203* in *S. aureus* N315 corresponds to *mdeA* of the Buttle strain. Some difference in the deduced amino acid sequences might be due to differences in the two strains. The MdeA protein has been reported to be a multidrug efflux pump belonging to the MF family with 14 predicted transmembrane segments.[7] Thus, it seems that MdeA of N315 also possesses 14 predicted transmembrane segments.

We constructed a plasmid pSTP2 which carries the *mdeA* gene (*SA2203*) and its putative promoter, and analyzed the properties of MdeA in *E. coli* cells. We determined the MIC values of 20 antimicrobial agents: ampicillin, erythromycin, streptomycin, tetracycline, norfloxacin, ciprofloxacin, ofloxacin, nalidixic acid, acriflavine, ethidium bromide, rhodamine 6G, 4′,6-diamidino-2-phenylindole, benzalkonium, chlorhexidine, sodium dodecyl sulfate, doxorubicin, daunorubicin, triclosan, TPPCl and Hoechst 33342. Cells of *E. coli* KAM32/pSTP2 showed elevated MICs of Hoechst 33342 (16-fold), TPPCl (8-fold) and norfloxacin (2-fold) compared with cells of *E. coli* KAM32 (Table 1). No change in the MIC value was observed with 17 other antimicrobial

Table 1. Increase in MIC Values of Several Antimicrobial Agents in *E. coli* Cells Carrying *mdeA*

Drugs	MIC (μg/ml)	
	KAM32/pSTV28 (control)	KAM32/pSTP2 (carrying *mdeA*)
Hoechst 33342	0.25	4
TPPCl	4	32
Norfloxacin	0.03	0.06

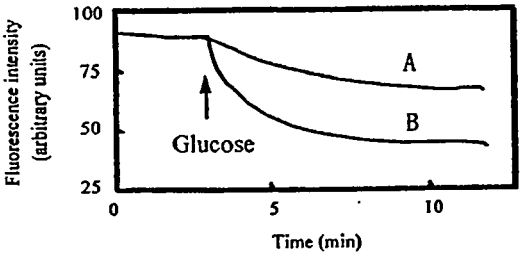

Fig. 1. Efflux of Hoechst 33342 Due to MdeA in *E. coli* Cells

Energy-starved cells of *E. coli* KAM32/pSTV28 (A) (control; not possessing *mdeA*) or *E. coli* KAM32/pSTP3 (B) (possessing *mdeA*) were loaded with Hoechst 33342. At the time point indicated by an arrow, glucose (final concentration, 20 mM) was added to energize the cells. The fluorescence of Hoechst 33342 was monitored with a fluorescence spectrometer at 37 °C over time. The downward deflection indicates the efflux of Hoechst 33342 from the cells.

agents. The *mdeA* gene is located in the multicloning site of vector pSTV28 in the opposite direction to the *lac* promoter. We also constructed a plasmid in which the *mdeA* gene is located downstream from the *lac* promoter in the same direction. We observed no difference in the MIC values between the two types of cells harboring either one of the plasmids (data not shown). Thus, we conclude that the putative promoter present upstream from the *mdeA* gene can function as a promoter in *E. coli* cells.

We investigated efflux activity of MdeA. We prepared energy-starved cells and loaded with Hoechst 33342. Addition of glucose as an energy donor elicited a large efflux of Hoechst 33342 in cells of *E. coli* KAM32/pSTP2 (Fig. 1). Only a little efflux was observed with control cells *E. coli* KAM32/pSTV28 (Fig. 1), which seems to be due to residual efflux pumps in *E. coli* KAM32. Thus, we conclude that the MdeA is really an energy-dependent drug efflux pump.

We cloned the *mdeA* gene using *E. coli* cells as the host, because *E. coli* is a very convenient host for gene cloning, and analyzed some properties in these *E. coli* cells. However, since *mdeA* is a gene from *S. aureus*, it is important to analyze the properties of MdeA in cells of *S. aureus*. We constructed a plasmid pRTP2 which carries the *mdeA* gene using pRIT5 as a shuttle vector between *E. coli* and *S. aureus*,[14] and introduced the resulting plasmid pRTP2 into cells of *S. aureus* RN4220. We determined the MIC values of the 20 antimicrobial agents described above with *S. aureus* RN4220/pRTP2 (carrying the *mdeA* gene) and RN4220/pRIT5 (control). Cells of RN4220/pRTP2 showed elevated MICs of doxorubicin (8-fold), daunorubicin (4-fold), ethidium bromide (4-fold), Hoechst 33342 (4-fold), TPPCl (4-fold), ciprofloxacin (2-fold), acriflavine (2-fold) and rhodamine 6G (2-fold) compared with cells of RN4220/pRIT5 (Table 2). These increases in the MICs were reproducible. The *mdeA* gene is located downstream from the

Table 2. Increase in MIC Values of Several Antimicrobial Agents in *S. aureus* Cells Introduced with *mdeA*

Drugs	MIC (μg/ml)	
	RN4220/pRIT5 (control)	RN4220/pRTP2 (carrying *mdeA*)
Doxorubicin	32	256
Daunorubicin	8	32
Ethidium bromide	8	32
Hoechst 33342	4	16
TPPCl	32	128
Ciprofloxacin	0.5	1
Acriflavine	32	64
Rhodamine 6G	4	8

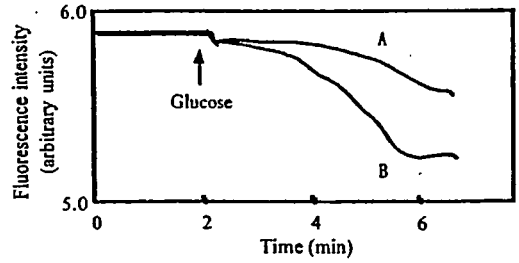

Fig. 2. Efflux of Ethidium from Cells of *S. aureus*

Energy-starved cells of *S. aureus* RN4220/pRIT5 (A) (control) or *S. aureus* RN4220/pRTP2 (B) (*mdeA*-introduced cells) were loaded with ethidium bromide. At the time point indicated by an arrow, glucose (final concentration, 10 mM) was added to energize the cells. The fluorescence of ethidium was monitored with a fluorescence spectrometer at 37 °C over time. The downward deflection indicates the efflux of ethidium from the cells.

spa promoter of *S. aureus* in the opposite direction in the pRTP2 plasmid. We also constructed a plasmid in which the *mdeA* gene is located in the downstream region of the *spa* promoter with the same direction. However, we observed no difference in MIC values between cells harboring either one of the plasmids (data not shown). Therefore we conclude that the *mdeA* gene is expressed from its own promoter, and the *spa* promoter gave no significant effect on the expression of the *mdeA* gene in the plasmid.

We measured ethidium efflux activity in *S. aureus* RN4220 cells harboring pRTP2 carrying the *mdeA* gene. We observed elevated energy-dependent efflux of ethidium from cells of *S. aureus* RN4220/pRTP2 (Fig. 2). Little efflux was observed with cells of RN4220/pRIT5 (control), which would be due to efflux pumps present in the RN4220 strain. Thus, it is clear that MdeA is a functional drug efflux pump in cells of *S. aureus*.

Although it became clear that MdeA is functional in *S. aureus* cells when *mdeA* is introduced in a multicopy-number plasmid, it is important to test whether *mdeA* is expressed from the chromosome in the original N315 cells. Therefore, we measured expression of the *mdeA* gene in cells of *S. aureus* N315 grown in a nutrient medium by the RT-PCR method. We found that *mdeA* is indeed expressed in *S. aureus* N315 (Fig. 3). It seemed that the expression level was roughly similar to that of *norA*, a gene for a multidrug efflux pump, NorA, a major multidrug efflux pump in *S. aureus*.

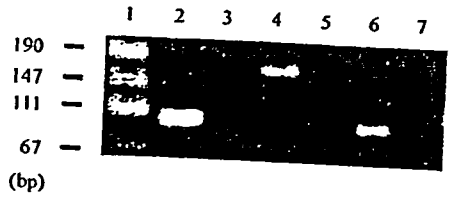

Fig. 3. RT-PCR Analysis

RT-PCR products indicating the expression of the *rpsL* (lane 2, lane 3), *mdeA* (lane 4, lane 5), and *norA* (lane 6, lane 7) genes of *S. aureus* N315 are shown. A 5 μl aliquot of each reaction product was analyzed by Agarose X (3% w/v) gel electrophoresis. Plasmid pUC19 digested with the restriction enzyme *Msp*I was used as a molecular weight standard (lane 1). Samples without reverse transcriptase reaction were used as a negative control (lane 3, lane 5, lane 7).

DISCUSSION

Among the 20 antimicrobial agents tested, Hoechst 33342, TPPCl and norfloxacin were found to be substrates for MdeA produced in *E. coli* cells. On the other hand, doxorubicin, daunorubicin, ethidium bromide, Hoechst 33342, TPPCl, ciprofloxacin, acriflavine and rhodamine 6G were found to be substrates for MdeA in *S. aureus* cells. The MIC values in *S. aureus* RN4220 cells were higher than those in *E. coli* KAM32 cells. Although we do not know the exact reason for this discrepancy in substrate specificity, there are several possibilities. The presence of the outer membrane in *E. coli*, but not in *S. aureus*, may affect this property of the pump, or differences in membrane component(s) (or cellular component) in the two bacteria may affect the property. A difference in substrate specificity when expressed in *S. aureus* and in *E. coli* has not been reported with NorA[2—5] or with SepA.[6] Thus, a factor which modulates the property of MdeA may be present in cells of *S. aureus* or *E. coli*.

Huang *et al.* reported[7] that benzalkonium chloride was a substrate for MdeA in the *S. aureus* Buttle strain. However, we were unable to repeat this result. We noticed that there were some differences in the deduced amino acid sequences (5 residues among 479 residues) in the MdeAs derived from *S. aureus* Buttle strain[7] and *S. aureus* N315 strain. The different residues are as follows; T81S in putative trans-membrane segment 3 (TMS3), F159L in putative loop region between TMS5 and TMS6, I234V in TMS8, A235V in TMS8, and F338I in TMS11. These differences may affect the substrate specificity in MdeA. They also reported that fluoroquinolone was not a substrate for MdeA. However, we observed some changes in MIC values of fluoroquinolones between *S. aureus* RN4220 and RN4220 harboring a plasmid carrying the *mdeA* gene, and between *E. coli* KAM32 and KAM32 harboring a plasmid carrying *mdeA*. Thus, it seems that fluoroquinolones are moderate substrates for MdeA.

We found that the gene *mdeA* was expressed in cells of *S. aureus* N315 to a certain level. The expression level of *mdeA* was roughly similar to that of *norA*. It has been reported that disruption of the *norA* gene of *S. aureus* resulted in a decrease in the MIC values of several antimicrobial agents.[2—5] These results indicate that NorA is involved in the intrinsic resistance of *S. aureus* against several antimicrobial agents. Also, it has been reported that over-expression of *norA* resulted in elevated resistance against several drugs.[3] Thus, it is likely that MdeA is also involved in intrinsic resistances against multiple antimicrobial agents in *S. aureus* N315, and further elevated expression due to mutation in the promoter region of *mdeA* will result in elevated resistances against multiple drugs. Since there are many genes for putative multidrug efflux pumps in *S. aureus*,[11] over-expression of one or some of such gene(s) should result in more elevated MIC values of multiple antimicrobial agents. This suggests that super MRSAs that show much higher resistance to many antimicrobial agents may emerge. Therefore we believe that analyses of whole multidrug efflux pumps in *S. aureus* are important.

Acknowledgements We thank Drs. K. Hiramatsu of Juntendo University Medical School and T. Ohta of Tsukuba University Medical School for providing *S. aureus* N315 and vector pRIT5, respectively. We also thank Dr. M. Varela of Eastern New Mexico University for critical reading of the manuscript. This research was supported by a grant from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

REFERENCES

1) Henry F. C., *Emerging Infectious Diseases*, 7, 178—181 (2001).
2) Yoshida H., Bogaki M., Nakamura S., Ubukata K., Konno M., *J. Bacteriol.*, 172, 6942—6949 (1990).
3) Schmitz F. J., Kohrer K., Scheuring S., Verhoef J., Fluit A., Heinz H. P., Jones M. E., *Clin. Microbiol. Infect.*, 5, 287—290 (1999).
4) Fournier B., Truong-Bolduc Q. C., Zhang X., Hooper D. C., *J. Bacteriol.*, 183, 2367—2371 (2001).
5) Yu J. L., Grinius L., Hooper D. C., *J. Bacteriol.*, 184, 1370—1377 (2002).
6) Narui K., Noguchi N., Wakasugi K., Sasatsu M., *Biol. Pharm. Bull.*, 25, 1533—1536 (2002).
7) Huang J., O'Toole P. W., Shen W., Amrine-Madsen H., Jiang X., Lobo N., Palmer L. M., Voelker L., Fan F., Gwynn M. N., McDevitt D., *Antimicrob. Agents Chemother.*, 48, 909—917 (2004).
8) Truong-Bolduc Q. C., Dunman P. M., Strahilevitz J., Projan S. J., Hooper D. C., *J. Bacteriol.*, 187, 2395—2405 (2005).
9) Kaatz G. W., McAleese F., Seo S. M., *Antimicrob. Agents Chemother.*, 49, 1857—1864 (2005).
10) McAleese F., Petersen P., Ruzin A., Dunman P. M., Murphy E., Projan S. J., Bradford P. A., *Antimicrob. Agents Chemother.*, 49, 1865—1871 (2005).
11) Kuroda M., Ohta T., Uchiyama I., Baba T., Yuzawa H., Kobayashi I., Cui L., Oguchi A., Aoki K., Nagai Y., Lian J., Ito T., Kanamori M., Matsumaru H., Maruyama A., Murakami H., Hosoyama A., Mizutani-Ui Y., Takahashi N. K., Sawano T., Inoue R., Kaito C., Sekimizu K., Hirakawa H., Kuhara S., Goto S., Yabuzaki J., Kanehisa M., Yamashita A., Oshima K., Furuya K., Yoshino C., Shiba T., Hattori M., Ogasawara N., Hayashi H., Hiramatsu K., *Lancet*, 357, 1225—1240 (2001).
12) Chen J., Morita Y., Huda M. N., Kuroda T., Mizushima T., Tsuchiya T., *J. Bacteriol.*, 184, 572—576 (2002).
13) Berns K. I., Thomas C. A. J., *J. Mol. Biol.*, 11, 117—120 (1965).
14) Nilsson B., Abrahmsen L., Uhlen M., *EMBO J.*, 4, 1075—1080 (1985).
15) Schenk S., Laddaga R. A., *FEMS Microbiol. Lett.*, 73, 133—138 (1992).
16) Sanger F., Nicklen S., Coulson A. R., *Proc. Natl. Acad. Sci. U.S.A.*, 74, 5463—5467 (1977).
17) Japan Society of Chemotherapy., *Chemotherapy* (Tokyo), 38, 103—105 (1990).
18) Tanaka S., Lerner S. A., Lin E. C. C., *J. Bacteriol.*, 93, 642—648 (1967).
19) van Veen H. W., Margolles A., Muller M., Higgins C. F., Konings W. N., *EMBO J.*, 19, 2503—2514 (2000).
20) Frank R. S., Peter L., Jeanne N. T., Lauren A. Z., Kim L., *Proc. Natl. Acad. Sci. U.S.A.*, 97, 1433—1437 (2000).

THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 281, NO. 44, pp. 33422–33432, November 3, 2006

Heme Oxygenase-1 Protects Gastric Mucosal Cells against Non-steroidal Anti-inflammatory Drugs*

Received for publication, March 6, 2006, and in revised form, June 14, 2006 Published, JBC Papers in Press, August 31, 2006, DOI 10.1074/jbc.M602074200

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Gastric mucosal cell death by non-steroidal anti-inflammatory drugs (NSAIDs) is suggested to be involved in NSAID-induced gastric lesions. Therefore, cellular factors that suppress this cell death are important for protection of the gastric mucosa from NSAIDs. Heme oxygenase-1 (HO-1) is up-regulated by various stressors and protects cells against stressors. Here, we have examined up-regulation of HO-1 by NSAIDs and the contribution of HO-1 to the protection of gastric mucosal cells against NSAIDs both *in vitro* and *in vivo*. In cultured gastric mucosal cells, all NSAIDs tested up-regulated HO-1. In rats, orally administered indomethacin up-regulated HO-1, induced apoptosis, and produced lesions at gastric mucosa. An inhibitor of HO-stimulated NSAID-induced apoptosis *in vitro* and *in vivo* and also stimulated NSAID-produced gastric lesions, suggesting that NSAID-induced up-regulation of HO-1 protects the gastric mucosa from NSAID-induced gastric lesions by inhibiting NSAID-induced apoptosis. Indomethacin activated the *HO-1* promoter and caused nuclear accumulation of NF-E2-related factor 2 (Nrf2), a transcription factor for the *HO-1* gene. Examination of phosphorylation of p38 mitogen-activated protein kinase (MAPK) and experiments with its inhibitor strongly suggest that the nuclear accumulation of Nrf2 and resulting up-regulation of HO-1 by NSAIDs is mediated through NSAID-dependent activation (phosphorylation) of p38 MAPK. This is the first report showing the protective role of HO-1 against irritant-induced gastric lesions.

Non-steroidal anti-inflammatory drugs (NSAIDs)[2] are a useful family of therapeutics, accounting for nearly 5% of all prescribed medications (1). The anti-inflammatory actions of NSAIDs are mediated through their inhibitory effects on cyclooxygenase (COX) activity. COX is an enzyme essential for the synthesis of prostaglandins (PGs), which have a strong capacity to induce inflammation. On the other hand, NSAID use is associated with gastrointestinal complications (2), with about 15–30% of chronic users of NSAIDs suffering from gastrointestinal ulcers and bleeding (3, 4).

Although PGs have a strong protective effect on gastrointestinal mucosa, the inhibition of COX by NSAIDs is not the sole explanation for the gastrointestinal side effects of NSAIDs (5). We have recently demonstrated that NSAIDs induce apoptosis in primary cultures of gastric mucosal cells in a manner independent of COX inhibition (6–9). As for the molecular mechanism governing this apoptosis, we recently proposed that permeabilization of cytoplasmic membranes by NSAIDs stimulates Ca^{2+} influx which in turn induces production of the C/EBP homologous transcription factor (CHOP), and activates calpain, a Ca^{2+}-dependent cysteine protease, both of which have apoptosis-inducing ability (6). Furthermore, we suggested that both COX-inhibition and NSAID-induced cell death (such as apoptosis) in gastric mucosa are required for production of NSAID-induced gastric lesions *in vivo* (10). Cellular factors that suppress NSAID-induced apoptosis are therefore important for protection of gastric mucosa from NSAID-induced gastric lesions.

When cells are exposed to various stressors, including NSAIDs, they induce a number of proteins, so-called stress proteins, in order to protect themselves against such stressors. Molecular chaperons are representative stress proteins. Their up-regulation in cells confers resistance to various stressors as the chaperons re-fold or degrade denatured proteins produced by stressors (11). It has been shown that cytosolic molecular chaperones (such as heat shock proteins (HSPs)) and endoplasmic reticulum (ER) molecular chaperons (such as glucose-regulated proteins (GRPs)) are up-regulated by NSAIDs and make cells resistant to NSAIDs (12, 13). Furthermore, geranylgeranylacetone (GGA), the leading anti-ulcer drug on the Japanese market, has been reported to induce HSPs at

* This work was supported by grants-in-aid for scientific research from the Ministry of Health, Labor, and Welfare of Japan, as well as by Japan Science and Technology Agency, the Daiwa Securities Health Foundation, the Mochida Memorial Foundation for Medical and Pharmaceutical Research, the Suzuken Memorial Foundation, and the Japan Research Foundation for Clinical Pharmacology. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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[2] The abbreviations used are: NSAIDs, non-steroidal anti-inflammatory drugs; AMC, aminomethylcoumarin; BAPTA-AM, 1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′*N*′-tetraacetic acid; β-NA, β-nicotinamide adenine dinucleotide phosphate; CHOP, C/EBP homologous transcription factor; CO, carbon monoxide; COX, cyclooxygenase; EGFP, enhanced green fluorescent protein; ER, endoplasmic reticulum; ERK, extracellular signal-regulated kinase; FBS, fetal bovine serum; GRP, glucose-regulated protein; HE, hematoxylin and eosin; HO-1, heme oxygenase-1; HSP, heat shock protein; IL, interleukin; JNK, c-jun N-terminal kinase; MAPK, mitogen-activated protein kinase; MTT, 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl tetrazolium bromide; Nrf2, NF-E2-related factor 2; PG, prostaglandin; PI3K, phosphatidylinositol 3-kinase; SnMP, Sn(IV) Mesoporphyrin; TUNEL, TdT-mediated dUTP-biotin end-labeling; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid; PIPES, 1,4-piperazinediethanesulfonic acid.

gastric mucosa that protect gastric mucosal cells against NSAIDs and other gastric irritants (14–17).

Heme oxygenase-1 (HO-1) is another type of stress protein. Not only its substrate, heme, but also various stressors such as oxidative stressors, ultraviolet irradiation, inflammatory cytokines, and heavy metals, have been reported to induce HO-1 production (18–20). HO-1 degrades heme to carbon monoxide (CO), free iron, and biliverdin. Biliverdin is subsequently converted into bilirubin by biliverdin reductase (18–20). Bilirubin and biliverdin are potent antioxidants and CO has anti-apoptotic activity. Therefore, up-regulation of HO-1 in cells makes cells resistant to apoptosis induced by various stressors (19–21).

HO-1 is also known as HSP32; however, the mechanism governing regulation of its expression is different from that of other HSPs (22). HO-1 is a phase II drug detoxifying enzyme. Such enzymes are regulated in a coordinated manner through a consensus *cis*-element and transcription factors, such as NF-E2-related factor 2 (Nrf2). HO-1-inducing stressors, such as reactive oxygen species, translocate Nrf2 from the cytoplasm into the nucleus where it binds to the consensus *cis*-element (Maf-recognition element (MARE)) to stimulate the transcription of genes located downstream (23–25). A number of kinases are involved in this activation (translocation) of Nrf2. They are mitogen-activated protein kinases (MAPKs) (extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK) and p38 MAPK) and phosphatidylinositol 3-kinase (PI3K). It has been suggested that the kinases involved in HO-1 up-regulation are different from each other depending on stressor and cell species (26–28).

It was recently reported that certain NSAIDs up-regulate HO-1 production in some types of cells (29–33). In this study, we show that all NSAIDs tested up-regulate HO-1 in cultured gastric mucosal cells, possibly through the p38 MAPK-dependent nuclear accumulation of Nrf2. The results of experiments with a specific inhibitor of HO (Sn(IV) Mesoporphyrin, SnMP) suggest that this up-regulation contributes to the suppression of NSAID-induced apoptosis and NSAID-induced gastric lesions.

EXPERIMENTAL PROCEDURES

Chemicals, Plasmids, and Animals—RPMI 1640 medium was obtained from Nissui Pharmaceutical Co. Pronase E and type I collagenase were purchased from Kaken Pharmaceutical Co. and Nitta Gelatin Co., respectively. Pluronic F127, fluo-3/AM, 1,2-bis(2-aminophenoxy)ethane-*N,N,N'N'*-tetraacetic acid (BAPTA-AM) was obtained from Dojindo Co. Flurbiprofen was from Cayman Chemicals and Loxoprofen was kindly provided by Sankyo Co. Fetal bovine serum (FBS), heme, β-nicotinamide adenine dinucleotide phosphate (β-NADP), glucose-6-phosphate dehydrogenase, glucose 6-phosphate, diclofenac, anysomycin, ibuprofen, paraformaldehyde, probenecid, proteinase K, and 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) were obtained from Sigma. SP600125, indomethacin and aspirin were obtained from Wako Co. Mayer's hematoxylin, 1% eosin alcohol solution and Malinol were from MUTO pure chemical Co. Terminal deoxynucleotidyl transferase (TdTase) was from TOYOBO Co. Biotin 14-ATP, Alexa Fluor 488 goat anti-rabbit immunoglobulin G, Alexa Fluor 488 conjugated with streptavidin and Lipofectamine (TM2000) were from Invitrogen. VECTASHIEL was from Vector Laboratory. SnMP was from Frontier Scientific Inc. Celecoxib was from LKT Laboratories Inc. Antibodies against HSP72, Nrf2, lamin B, GRP78 and actin were purchased from Santa Cruz Biotechnology Inc. Antibodies against HO- and p38 MAPK were from Stressgen and Cell Signaling Technology Inc., respectively. Acetyl-DEVD-methylcoumarinamide was from Peptide Institute Inc. O.C.T. compound was from Sakura Fintechnical. PD98059, SB203580, LY294002, and the Dual Luciferase Assay System, including a control plasmid harboring the *Renilla reniformis* luciferase gene (pRL-SV40), were from Promega. A plasmid containing the *Photinus pyralis* luciferase gene under control of the *HO-1* gene promoter (pHO15luc) (34) was a gift kindly donated by J. Alam (Alton Ochsner Medical Foundation). This plasmid contain 15 kbp of mouse *HO-1* 5′-flanking region. A plasmid expressing enhanced green fluorescent protein (EGFP) (pEGFP-C1) was obtained from Clontech Laboratories Inc. Male guinea pigs weighing 200–300 g and male Wistar rats weighing 160–200 g were purchased from Kyudo Co. The experiments and procedures described here were carried out in accordance with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the National Institute of Health, and were approved by the Animal Care Committee of Kumamoto University.

Gastric Damage Assay—Gastric damage assays were performed as described previously (10). Rats, which had been fasted for 24 h, were intraperitoneally injected with SnMP (dissolved in 0.1 N NaOH, adjusted to pH 7.6 with HCl). One hour later, indomethacin in 1% methylcellulose was orally administered. Three hours after the oral administration, the rats were sacrificed by decapitation under light anesthesia with ethyl ether, and the stomachs were removed and scored for hemorrhagic damage by an observer unaware of the treatment the rats had received. Calculation of the scores involved measuring the area of all lesions in millimeters squared and summing the values to give an overall gastric lesion index.

Cell Culture, Transfection, and Cell Viability Assay—Gastric mucosal cells were isolated from guinea pig fundic glands, as described previously (17, 35). Isolated gastric mucosal cells were cultured for 12 h in RPMI 1640 containing 0.3% v/v FBS, 100 units/ml penicillin, and 100 μg/ml streptomycin in type-I collagen-coated plastic culture plates in 5% CO_2, 95% air at 37 °C. After removing non-adherent cells by washing with RPMI 1640, cells that were attached to the plate at ~50% confluence were used. Guinea pig gastric mucosal cells prepared under these conditions have been previously characterized, with the majority (about 90%) of such cells being identified as pit cells (17, 35).

Human gastric carcinoma cells (AGS) were cultured in RPMI1640 medium supplemented with 10% FBS, 100 units/ml penicillin and 100 μg/ml streptomycin in 5% CO_2, 95% air at 37 °C. Unless otherwise noted, cells (0.8×10^4 cells per well in 24-well plates, 4×10^4 cells per well in 6-well plates, 6×10^5 cells in 100-mm plates) were cultured for 24 h and then used in the experiments. Transfection of cells with plasmid was carried

out using Lipofectamine (TM2000) according to the manufacturer's instructions. Transfected cells were used for experiments after a 24-h recovery period. Transfection efficiency was determined in parallel plates by transfection of cells with the pEGFP-C1 control vector. Transfection efficiency was more than 80% in all experiments.

NSAIDs were dissolved in Me_2SO or Na_2CO_3 (for indomethacin only) and control experiments (without NSAIDs) were performed in the presence of the same concentrations of Me_2SO or Na_2CO_3. Cells were exposed to NSAIDs by changing the medium. Cell viability was determined by the MTT method.

Immunoblotting Analysis—Whole cell extracts and nuclear extracts were prepared as described previously (36, 37). The protein concentration of samples was determined by the Bradford method. Samples were applied to 8% (HSP72 and GRP78), 10% (lamin B, Nrf2, p38 MAPK, and actin) or 12% (HO-1) polyacrylamide SDS gels, subjected to electrophoresis, and proteins then immunoblotted with appropriate antibodies.

Luciferase Assay—The luciferase assay was performed as described previously (7). Cells were transfected with 0.375 μg of each of the *P. pyralis* luciferase reporter plasmids (pHO15luc or its vector) and 0.125 μg of the internal standard plasmid bearing the *R. reniformis* luciferase reporter (pRL-SV40). *P. pyralis* luciferase activity in cell extracts was measured using the Dual Luciferase Assay System and then normalized for *R. reniformis* luciferase activity.

Histological and Immunohistochemical Analysis—Gastric tissue samples were fixed in 4% buffered paraformaldehyde, embedded in O.C.T. compound and cryosectioned. Sections were stained first with Mayer's hematoxylin and then with 1% eosin alcohol solution for histological examination (hematoxylin and eosin (HE) staining). Samples were mounted with Malinol and inspected using microscopy (Olympus IX70).

For immunohistochemical analysis, sections were blocked with 2.5% goat serum for 10 min and then incubated for 12 h with antibody against HO-1 (1:500 dilution) in the presence of 2.5% bovine serum albumin, and finally incubated for 1 h with Alexa Fluor 488 goat anti-mouse immunoglobulin G. Samples were mounted with VECTASHIELD and inspected using fluorescence microscopy (Olympus IX70).

TdT-mediated dUTP-biotin End-labeling (TUNEL) Assay—Gastric tissue samples were fixed in 4% buffered paraformaldehyde, embedded in O.C.T. compound and cryosectioned. Sections were first incubated with protenase K (10 μg/ml) for 15 min at 37 °C, then with TdTase and biotin 14-ATP for 1 h at 37 °C and finally with Alexa Fluor 488 conjugated with streptavidin for 1 h. Samples were mounted with VECTASHIELD and inspected using fluorescence microscopy (Olympus IX70).

Measurement of HO Activity—Enzymatic activity of HO was determined as described previously (38), with some modifications.

Sample preparation from cultured cells: Cells were lysed by freeze-thawing and sonication in the 0.1 M potassium phosphate buffer (pH 7.4) and centrifuged at 1000 × *g* for 10 min. The supernatants were applied to the HO assay system (see below).

Sample preparation from gastric mucosa: Gastric mucosal cells prepared from rats were homogenized in the 0.1 M potassium phosphate buffer (pH 7.4) containing 0.25 M sucrose, and centrifuged at 15,000 × *g* for 10 min. The supernatants were further centrifuged at 105,000 × *g* for 60 min. The precipitates were resuspended with the 0.1 M potassium phosphate buffer (pH 7.4) containing 0.15 M KCl and applied to the HO assay system (see below).

HO Assay System—After determination of the protein concentration, samples were incubated for 60 min at 37 °C in the dark with the following reagents: heme (17 μM), rat liver cytosol (10 mg/ml), $MgCl_2$ (2 mM), glucose-6-phosphate dehydrogenase (4 units), glucose 6-phosphate (0.85 mM), and β-NADP (2 mM) in 0.6 ml of 0.1 M potassium phosphate buffer (pH 7.4). The reaction was stopped by placing the tubes on ice. The amount of bilirubin generated was estimated with a scanning spectrophotometer and was defined as the difference between 452 and 530 nm. The HO activity is expressed as pmol of bilirubin per milligram of protein per hour.

Caspase Activity Assay—The activity of caspase-3 was determined as described previously (39). Briefly, cells were collected by centrifugation and suspended in extraction buffer (50 mM PIPES (pH 7.0), 50 mM KCl, 5 mM EGTA, 2 mM $MgCl_2$, and 1 mM dithiothreitol). Suspensions were sonicated and centrifuged, after which the supernatants were incubated with fluorogenic peptide substrates (acetyl-DEVD-methylcoumarin amide) in reaction buffer (100 mM HEPES-KOH (pH 7.5), 10% sucrose, 0.1% CHAPS, and 1 mg/ml bovine serum albumin) for 15 min at 37 °C. The release of aminomethylcoumarin (AMC) was determined using a fluorescence spectrophotometer. One unit of protease activity was defined as the amount of enzyme required to release 1 pmol of AMC/min.

Measurement of the Intracellular Ca^{2+} Level—Intracellular Ca^{2+} levels were monitored as described (6). Briefly, cells were incubated with 4 μM fluo-3/AM in assay buffer supplemented with 0.1% bovine serum albumin, 0.04% Pluronic F127 and 2 mM probenecid, for 40 min at 37 °C. After washing twice with assay buffer, cells were suspended in assay buffer supplemented with 2 mM probenecid. Cells were transferred to a water-jacketed cuvette and the fluo-3 fluorescence was then measured with a HITACHI F-4500 spectrofluorophotometer. The intracellular Ca^{2+} level was calculated according to the equation $[Ca^{2+}]_i = K_d(F - F_{min})/(F_{max} - F)$, where K_d is the apparent dissociation constant (400 nM) of the fluorescent dye-Ca^{2+} complex.

Statistical Analysis—All values are expressed as the mean ± S.D. One-way analysis of variance (ANOVA) followed by Scheffe's multiple comparison test was used for evaluation of differences between groups. The Student's *t* test for unpaired results was used for the evaluation of differences between two groups. Differences were considered to be significant for values of $p < 0.05$.

RESULTS

NSAIDs Up-regulate HO-1—Up-regulation of HO-1 production by NSAIDs was examined in primary cultures of guinea pig gastric mucosal cells. This type of cell has been used as an *in vitro* model for physiological and pathological studies of gastric mucosa, because various characteristic features of gastric mucosal cells *in vivo* (such as vigorous secretion of mucin) are

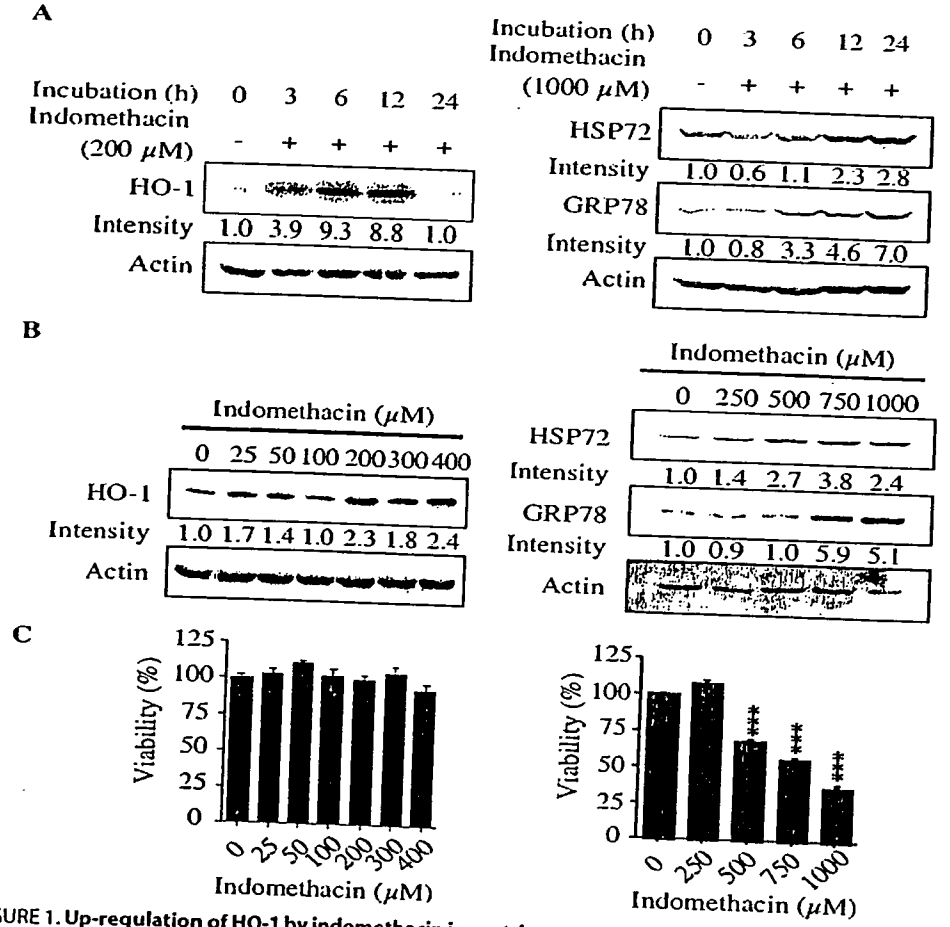

FIGURE 1. **Up-regulation of HO-1 by indomethacin in gastric mucosal cells in primary culture.** Guinea pig gastric mucosal cells in primary culture were incubated with the indicated concentrations of indomethacin for indicated periods (*A*), 6 h (HO-1 in *B* and *C*) or 24 h (HSP72 and GRP78 in *B* and *C*). Whole cell extracts were prepared and analyzed by immunoblotting with an antibody against HO-1, HSP72, GRP78, or actin. The band intensity was determined and expressed relative to the control (*A* and *B*). Cell viability was determined by the MTT method. Values shown are relative to the control (in the absence of indomethacin) and are given as the mean ± S.D. ($n = 3$). ***, $p < 0.001$ (*C*).

reproduced in this system (17). As shown in Fig. 1*A*, treatment of cells with indomethacin up-regulated HO-1 very rapidly (within 3 h of the addition of indomethacin) and transiently (HO-1 levels returned to pre-treatment levels 24 h after the addition). Indomethacin is known to up-regulate other stress proteins (HSPs and GRPs) (12, 13). The results in Fig. 1*A* show that up-regulation of HO-1 by indomethacin occurs prior to that of HSP72 and GRP78. Fig. 1*B* shows the effects of different doses of indomethacin on HO-1 up-regulation. Up-regulation of HO-1 was just apparent at 25–50 μM indomethacin and was distinct at 200–400 μM indomethacin. These concentrations of indomethacin did not affect cell viability (Fig. 1*C*), showing that up-regulation of HO-1 by indomethacin is not the result of indomethacin-induced cell damage. On the other hand, up-regulation of HSP72 and GRP78 required much higher concentrations of indomethacin (Fig. 1*B*); in other words, up-regulation of these proteins occurs simultaneously with cell damage (Fig. 1*C*).

We also examined up-regulation of HO-1 by other NSAIDs (diclofenac, ibuprofen, aspirin, flurbiprofen, celecoxib, and loxoprofen). All of the NSAIDs tested up-regulated HO-1 (Fig. 2) at concentrations that did not affect cell viability (data not shown). As was the case for indomethacin, some NSAIDs (diclofenac and flurbiprofen showed two peaks in their dose response profile of HO-1 up-regulation (Fig. 2). COX exists as two subtypes, COX-1 and COX-2, for which celecoxib and flurbiprofen are COX-2-selective in their action. Results in Fig. 2 show that all NSAIDs tested increased cellular HO-1, irrespective of their COX-2 specificity. IC_{50} values for COX inhibition of each NSAID (40–42) are not related to the concentration required for HO-1 up-regulation (Figs. 1 and 2). Furthermore, loxoprofen is a pro-drug, meaning that its active metabolite but not itself has COX inhibitory activity (43). Therefore, it seems that NSAIDs up-regulate HO-1 independently of COX inhibition (see Fig. 6*B*).

Contribution of HO-1 Up-regulation by NSAIDs to Protection of Gastric Mucosal Cells in Vitro and in Vivo—Because up-regulation of HO-1 in cells protects cells against various stressors (19, 21), it is possible that up-regulation of HO-1 by NSAIDs protects gastric mucosal cells against NSAIDs. To test this idea, we examined the effect of an inhibitor of HO on NSAID-induced cell death *in vitro*. SnMP is a representative inhibitor of HO, which inhibits the enzymatic activity of HO by acting as a substrate analogue (44). As shown in Fig. 3*A*, SnMP stimulated cell death in the presence of various concentrations of NSAIDs (indomethacin, diclofenac, and ibuprofen), lowering the concentrations of NSAIDs required for induction of cell death. The concentration of SnMP used in the experiments pertaining to Fig. 3*A* did not affect cell viability in the absence of NSAIDs (Fig. 3*A*). Based on a previous report (45), the concentration used is enough to specifically inhibit HO activity. In fact, we measured the activity of HO under the same conditions as in Fig. 3*A* and confirmed that the activity of HO was stimulated by treatment of cell with indomethacin and this stimulation was diminished by simultaneous treatment with SnMP (Fig. 3*C*). Cell death, as highlighted in Fig. 3*A*, appears to be mediated by apoptosis given that we observed NSAID-dependent activation of caspase-3 under the same experimental conditions as in Fig. 3*A* (data not shown) and treatment of cells with SnMP stimulated the activity of caspase-3 in the presence of each NSAID (Fig. 3*B*). On this basis, the results in Fig. 3 show that SnMP stimulates NSAID-induced apoptosis and, therefore, suggest that up-regulation of HO-1 by NSAIDs contributes to protection of gastric mucosal cells from NSAID-induced apoptosis.

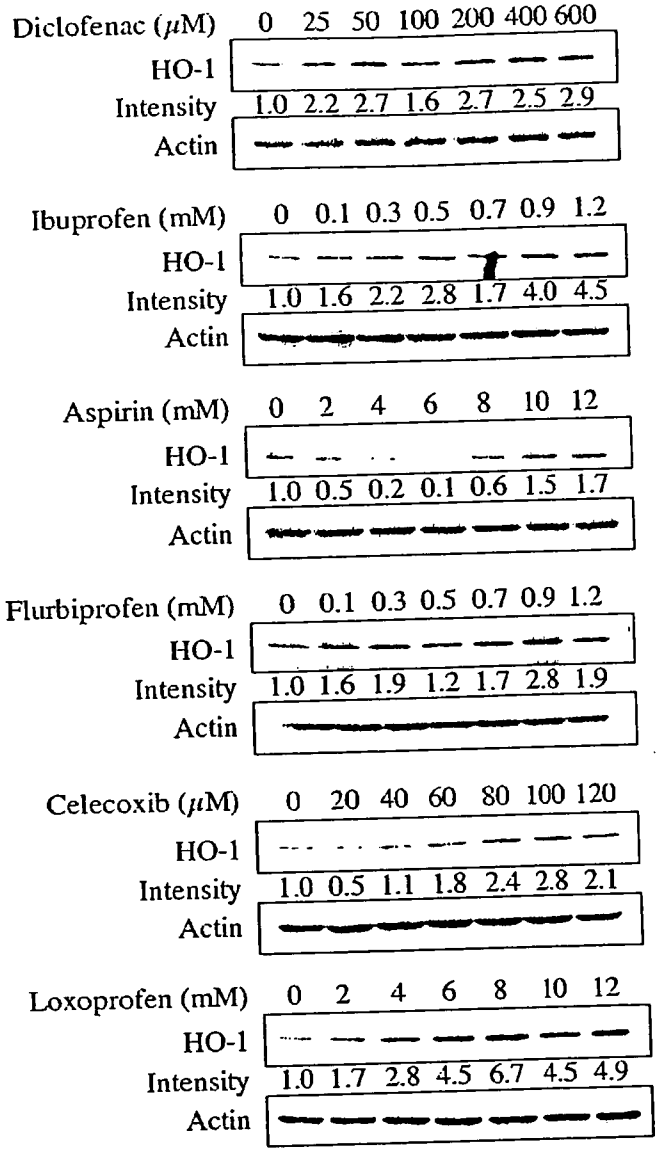

FIGURE 2. **Up-regulation of HO-1 by various NSAIDs.** Guinea pig gastric mucosal cells in primary culture were incubated with the indicated concentration of each NSAID for 6 h. Up-regulation of HO-1 was monitored as described in the legend to Fig. 1.

To address the *in vivo* relevance of the *in vitro* result (HO-1 up-regulation by NSAIDs), we tested whether orally administered NSAIDs up-regulate HO-1 in the gastric mucosa of rats. Oral administration of 10 mg/kg indomethacin produced gastric lesions in rats (see Fig. 5*A*) as described previously (10). Sections were prepared from the gastric tissues of these rats and were subjected to histological and immunohistochemical analysis. HE staining showed the presence of lesions in the gastric mucosa of indomethacin-administered rats but not in that from vehicle-administered rats (Fig. 4*A*). Furthermore, immunohistochemical analysis with an antibody against HO-1 showed that HO-1 is up-regulated in the gastric mucosa of indomethacin-administered rats relative to that from vehicle-administered rats (Fig. 4*A*).

We also examined effect of indomethacin on the level of apoptosis at gastric mucosa that was monitored by TUNEL assay. Accompanying the production of gastric lesions, an increase in

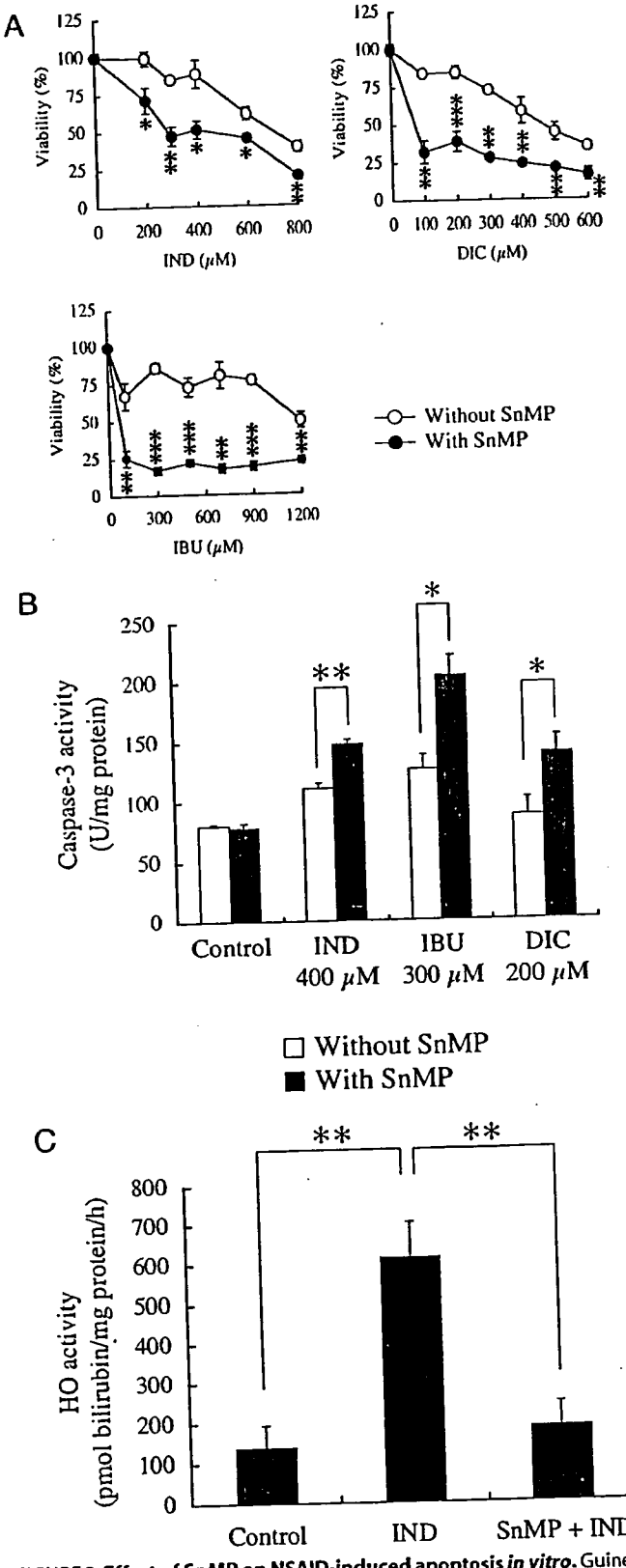

FIGURE 3. **Effect of SnMP on NSAID-induced apoptosis *in vitro*.** Guinea pig gastric mucosal cells in primary culture were incubated with the indicated concentrations of indomethacin (*IND*), diclofenac (*DIC*), or ibuprofen (*IBU*) in the presence or absence of 50 μM SnMP for 16 h (*A* and *B*) or 6 h (*C*), as indicated. Cell viability was determined using the MTT method and shown are relative to the control (in the absence of both NSAIDs and SnMP) (*A*). Activities of caspase-3 (*B*) or HO (*C*) in cells were measured and expressed as described under "Experimental Procedures" (*B* and *C*). Values are given as mean ± S.D. ($n = 3$). ***, $p < 0.001$; **, $p < 0.01$; *, $p < 0.05$.

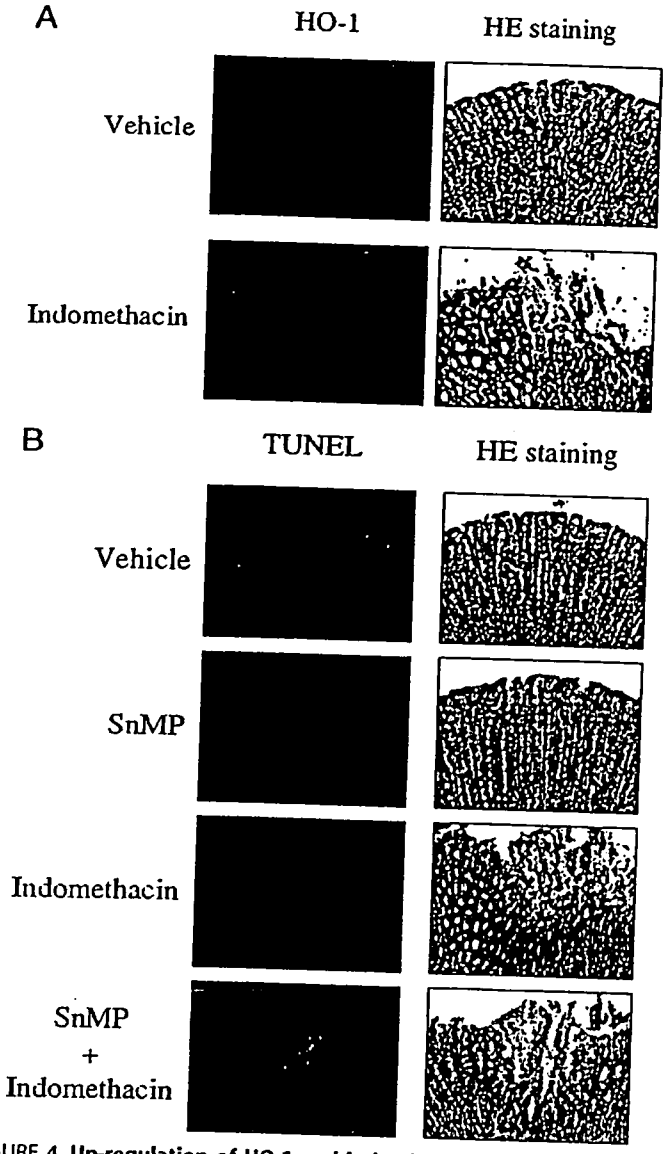

FIGURE 4. **Up-regulation of HO-1 and induction of apoptosis by indomethacin at gastric mucosa *in vivo*.** Rats were intraperitoneally pre-administered with 1 μmol/kg SnMP or vehicle 1 h before the administration of indomethacin (*B*). Rats were orally administered with 10 mg/kg indomethacin (*A* and *B*). After 4 h, sections of gastric tissues were prepared and subjected to histological examination (HE staining) and immunohistochemical analysis with an antibody against HO-1 (*A*) or TUNEL assay (*B*).

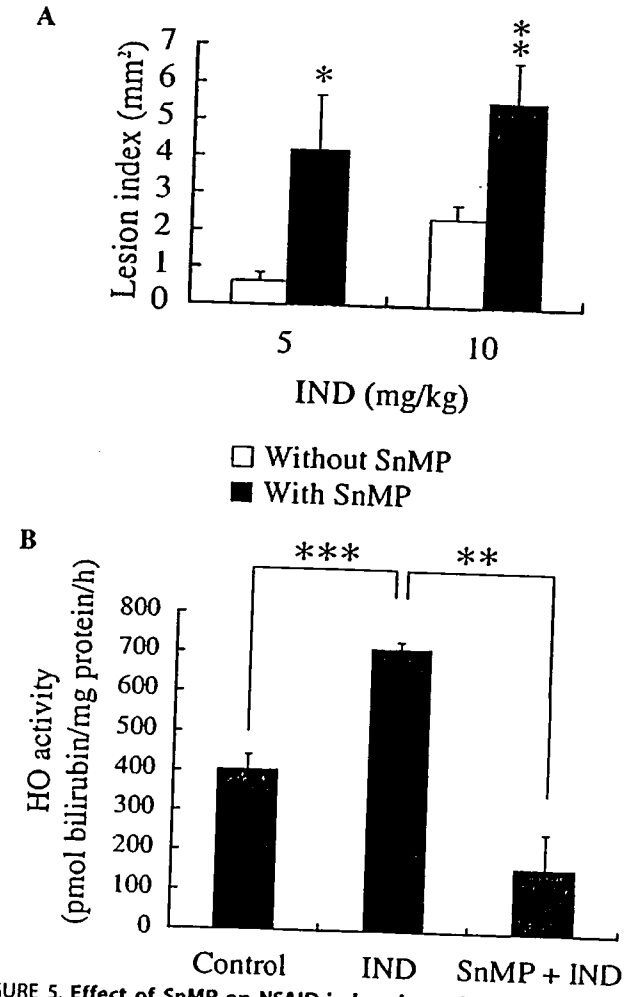

FIGURE 5. **Effect of SnMP on NSAID-induced gastric lesions.** Rats were intraperitoneally administered with 1 μmol/kg SnMP or vehicle. After 1 h, animals were administered orally with the indicated doses of indomethacin. After 3 h, the stomach was removed. The stomach was scored for hemorrhagic damage (*A*). Activities of HO in the stomach were measured and expressed as described under "Experimental Procedures" (*B*). Values are given as mean ± S.D. ($n = 3$–6). ***, $p < 0.001$; **, $p < 0.01$; *, $p < 0.05$.

TUNEL-positive cells (apoptotic cells) was observed with the indomethacin administration (Fig. 4*B*). Furthermore, pre-administration of SnMP stimulates indomethacin-induced apoptosis whereas this pre-administration did not induce apoptosis without subsequent indomethacin administration (Fig. 4*B*). These results suggest that up-regulation of HO-1 by indomethacin contributes to protection of gastric mucosal cells from NSAID-induced apoptosis also *in vivo*.

To examine the role of this NSAID-dependent HO-1 up-regulation in gastric mucosa, we examined the effect of SnMP on NSAID-induced gastric lesions in rats. As shown in Fig. 5*A*, pre-administration of SnMP (1 μmol/kg, intraperitoneally) stimulated the production of gastric lesions following oral administration of indomethacin. This administration of SnMP did not produce gastric lesions unless it was followed by the oral administration of indomethacin (data not shown). Based on a previous report (46), the concentration used should be adequate to specifically inhibit HO activity. In fact, we measured the activity of HO under the same conditions as in Fig. 5*A* and confirmed that the activity of HO at gastric mucosa was stimulated by the oral administration of indomethacin and this stimulation was diminished by the intraperitoneal pre-administration of SnMP (Fig. 5*B*). These results strongly suggest that the indomethacin-induced up-regulation of HO-1 in gastric mucosa contributes to the protection of gastric mucosa from the formation of indomethacin-induced gastric lesions.

Mechanism for Indomethacin-induced Up-regulation of HO-1—To investigate the molecular mechanism governing the up-regulation of HO-1 by NSAIDs, instead of using guinea pig gastric mucosal cells in primary culture, we used AGS cells in which various molecular biology techniques can be used. First, we reproduced HO-1 up-regulation by indomethacin in AGS cells (Fig. 6*A*). In this cell type, the slight up-regulation of HO-1 seen at relatively low concentrations of indomethacin in primary cultures of guinea pig gastric mucosal cells (Fig. 1) was not

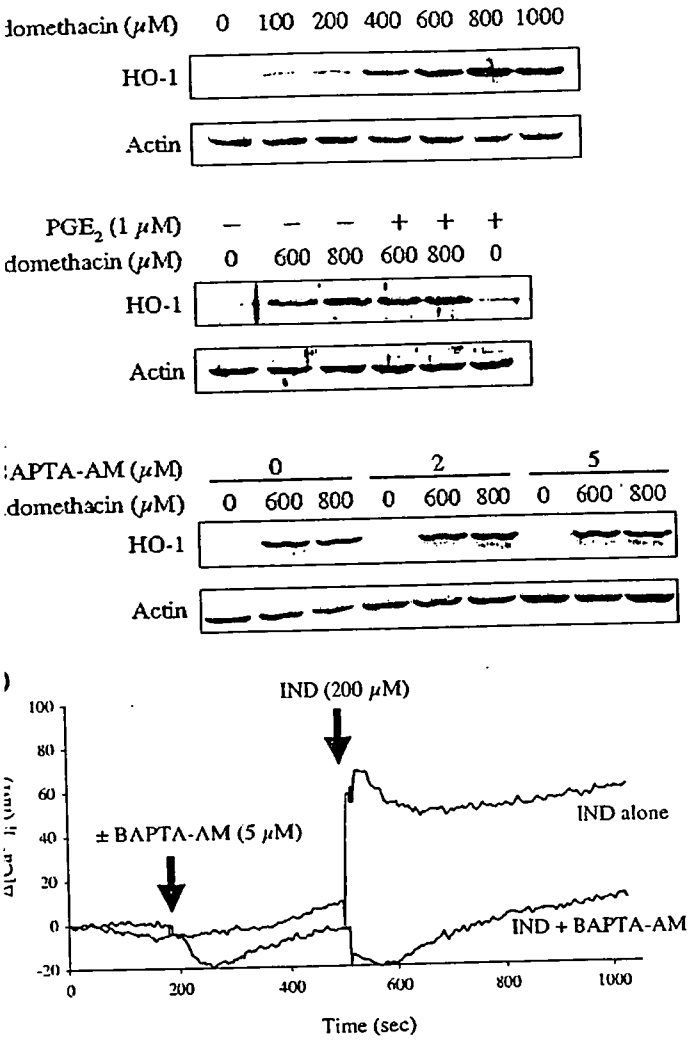

FIGURE 6. **Up-regulation of HO-1 by indomethacin in AGS cells.** AGS cells were preincubated with the indicated concentrations of BAPTA-AM for 1 h (*C*). Cells were incubated with the indicated concentrations of indomethacin for 6 h in the absence (*A* and *C*) or presence of 1 μM PGE_2. Up-regulation of HO-1 was monitored as described in the legend of Fig. 1. The intracellular Ca^{2+} level was monitored using a fluo-3/AM assay system. Fluo-3/AM-loaded cells were first treated with or without BAPTA-AM and then with indomethacin (*IND*). The time course of fluo-3 fluorescence change was monitored and increase in the intracellular Ca^{2+} level ($\Delta[Ca^{2+}]_i$) is shown (*D*).

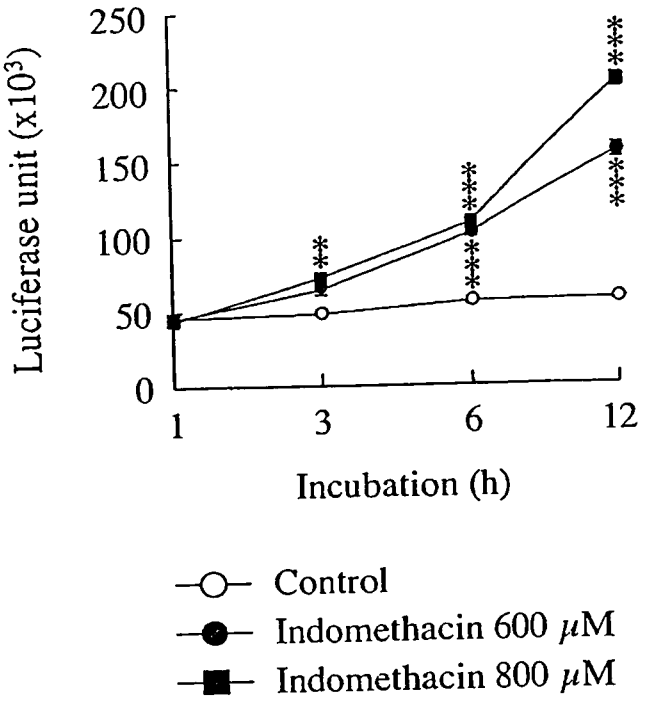

FIGURE 7. **Activation of the promoter activity of the *HO-1* gene by indomethacin.** AGS cells were co-transfected with pRL-SV40 (internal control plasmid carrying the *R. reniformis* luciferase gene) and pHO15luc (the promoter sequences of the *HO-1* gene were inserted upstream of the *P. pyralis* luciferase gene) or its vector. After 24 h, cells were incubated with the indicated concentrations of indomethacin for the indicated periods. *P. pyralis* luciferase activity was measured, and normalized for *R. reniformis* luciferase activity. Values were calculated by subtracting the background level (values from vector control experiments). Values are mean ± S.D. ($n = 3$). ***, $p < 0.001$; **, $p < 0.01$.

observed. We next examined the effect of exogenously added PGE_2, a major PG in gastric mucosa, on indomethacin-induced up-regulation of HO-1. As shown in Fig. 6*B*, the addition of 1 μM PGE_2 to the culture medium did not attenuate the indomethacin-induced up-regulation of HO-1. We previously determined the level of PGE_2 in the culture medium of AGS cells to be about 10 nM (47). Therefore, inhibition of PGE_2 synthesis by indomethacin (COX inhibition) does not seem to be involved in the up-regulation of HO-1 by indomethacin. We recently reported that various NSAIDs, including indomethacin, increase intracellular Ca^{2+} levels and that this increase is responsible for NSAID-dependent up-regulation of some proteins, such as claudin-4, GRP78, and CHOP (6, 13, 48). Here, we tested the contribution of this increase in the intracellular Ca^{2+} level to HO-1 up-regulation by indomethacin. As shown in Fig. 6*C*, an intracellular Ca^{2+} chelator (BAPTA-AM) did not affect HO-1 up-regulation by indomethacin. The concentrations of BAPTA-AM used in this experiment have been shown to inhibit the up-regulation of claudin-4 and GRP78 in AGS cells (13, 48) and we confirmed that the concentration of BAPTA-AM completely inhibited the indomethacin-dependent increase in the intracellular free Ca^{2+} level (Fig. 6*D*) (because clear increase in the intracellular Ca^{2+} level was not observed with 600 or 800 μM of indomethacin (maybe because of its inhibitory effect on fluo-3 fluorescence), we used 200 μM of indomethacin). Results suggest that increases in intracellular Ca^{2+} levels are not involved in indomethacin-induced up-regulation of HO-1.

Up-regulation of HO-1 by heme and various other stressors is due to activation of its transcription; in other words, *cis*-elements in the promoter of the *HO-1* gene and its specific transcription factors, such as Nrf2, are important for the up-regulation (23, 24, 49). We measured the activity of the *HO-1* gene promoter using a reporter plasmid where the promoter sequence of the *HO-1* gene was inserted upstream of the luciferase gene (34). As shown in Fig. 7, treatment of cells with indomethacin-stimulated luciferase activity in cells in both a dose- and incubation period-dependent manner, suggesting that the up-regulation of HO-1 by indomethacin is regulated at the level of transcription.

We then examined the effect of indomethacin on the amount of nuclear Nrf2. Nuclear fractions were prepared from indomethacin-treated or control AGS cells and the amount of Nrf2

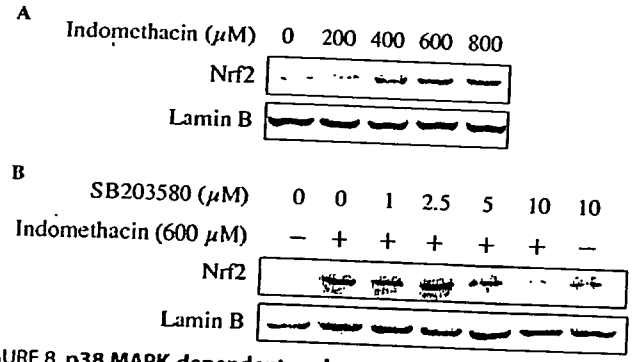

FIGURE 8. **p38 MAPK-dependent nuclear accumulation of Nrf2.** AGS cells were preincubated with or without an inhibitor of p38 MAPK (SB203580) for 0.5 h (*B*). Cells were incubated with the indicated concentrations of indomethacin for 3 h (*A* and *B*). Nuclear extracts were prepared and were analyzed by immunoblotting with an antibody against Nrf2 and lamin B.

was monitored by immunoblotting analysis. As shown in Fig. 8*A*, indomethacin increased the amount of Nrf2 in nuclear fractions, suggesting that indomethacin stimulated the translocation of Nrf2 from the cytoplasm into the nucleus.

Various kinases have been reported to be involved in HO-1 up-regulation and Nrf2 nuclear accumulation (26, 27, 50). In this study, we tried to identify the kinase involved in the NSAID-induced up-regulation of HO-1 using a specific inhibitor for each kinase (PI3K and MAPKs (ERK, JNK, and p38 MAPK)). As shown in Fig. 9, an inhibitor of p38 MAPK (SB203580), but not inhibitors for other kinases (PI3K, ERK, and JNK), suppressed the up-regulation of HO-1 by indomethacin. SB203580 also suppressed indomethacin-dependent nuclear accumulation of Nrf2 (Fig. 8*B*). It is known that p38 MAPK is activated by its phosphorylation (51). We found that as well as anysomycin, an activator of p38 MAPK, indomethacin increased levels of the phosphorylated form of p38 MAPK in cells (Fig. 10). Furthermore, this phosphorylation was almost completely inhibited by SB203580 but not by SnMP (Fig. 10). SB203580 did not affect the expression of HO-1, the nuclear accumulation of Nrf2 or the phosphorylation of p38 MAPK in the absence of indomethacin (Figs. 8–10). None of these inhibitors used in experiments pertaining to Figs. 8–10 affected cell viability at the concentrations used (data not shown) which, based on previous reports (52–56), would have been sufficient to inhibit each target molecule specifically.

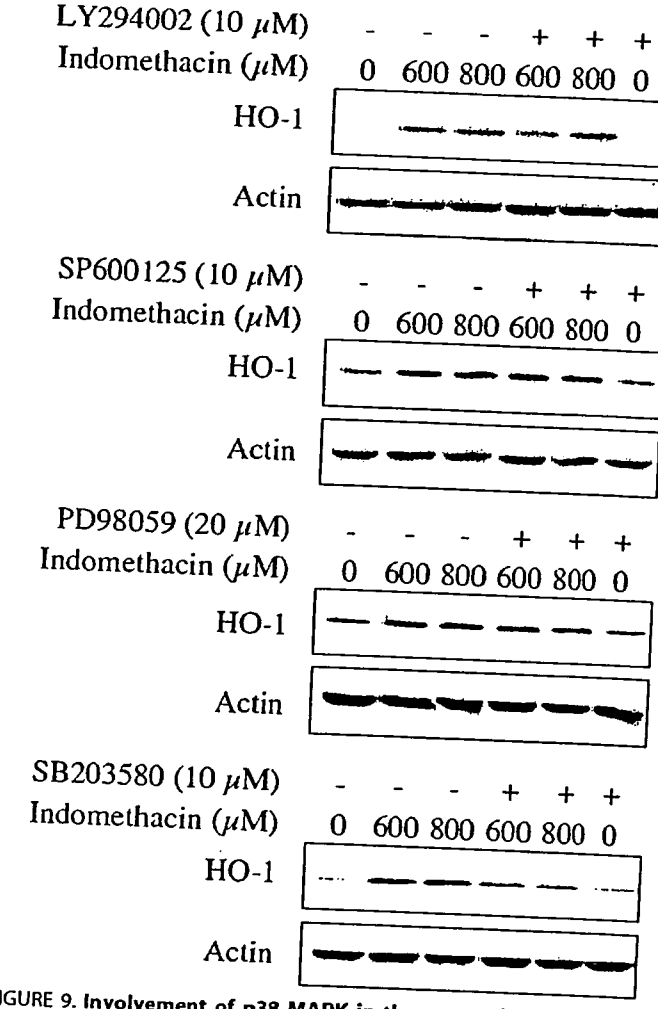

FIGURE 9. **Involvement of p38 MAPK in the up-regulation of HO-1 by indomethacin.** AGS cells were preincubated with indicated concentrations of each inhibitor (LY294002 for PI3K, SP600125 for JNK, PD98059 for ERK, SB203580 for p38 MAPK) for 0.5 h and further incubated with the indicated concentrations of indomethacin for 6 h. Up-regulation of HO-1 was monitored as described in the legend of Fig. 1.

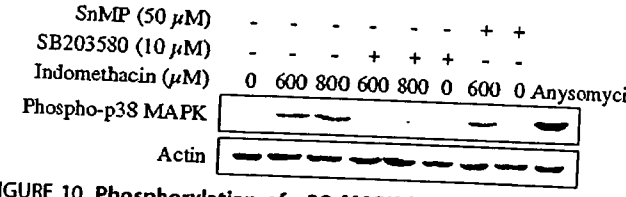

FIGURE 10. **Phosphorylation of p38 MAPK by indomethacin.** AGS cells were preincubated with the indicated concentrations of SB203580 or SnMP for 0.5 h and further incubated with the indicated concentrations of indomethacin or anysomycin (25 μg/ml) for 6 h. Whole cell extracts were prepared and analyzed by immunoblotting with an antibody to the phosphorylated form of p38 MAPK.

DISCUSSION

In this study we found that all of the NSAIDs tested up-regulate HO-1 in primary cultures of guinea pig gastric mucosal cells. Because the concentrations of NSAIDs and incubation periods required for the up-regulation of HO-1 were relatively low and short, respectively, when compared with that of HSPs and GRPs, the NSAID-induced up-regulation of HO-1 seems to be important for the pharmacological actions of NSAIDs *in vivo*. In fact, we have shown that orally administered indomethacin up-regulates HO-1 at gastric mucosa at doses that cause production of gastric lesions in rats.

Using a specific inhibitor for HO, SnMP, we examined the physiological role of NSAID-induced up-regulation of HO-1 both *in vitro* and *in vivo*. HO inhibition by SnMP stimulated NSAID-induced apoptosis both *in vitro* and *in vivo* and also stimulated NSAID-induced production of gastric lesions *in vivo*. Taking previous observations into consideration, we speculate that both of these phenomena (*in vitro* and *in vivo*) are related to each other. NSAIDs induce not only necrosis but also apoptosis in primary cultures of gastric mucosal cells (9). Furthermore, we suggested that both COX inhibition at the gastric mucosa and direct gastric mucosal cell damage (such as induction of apoptosis) by NSAIDs are required for the production of gastric lesions by NSAIDs *in vivo*; gastric lesions developed in a manner that depends on both an intravenously administered

ɔw dose of indomethacin (inhibition of COX activity at the ;astric mucosa without direct gastric mucosal cell damage) and ın orally administered cytotoxic COX-2-selective NSAID direct gastric mucosal cell damage without inhibition of COX ıt gastric mucosa) (10). We consider that NSAID induced up-·egulation of HO-1 contributes to the protection of gastric nucosa from the formation of gastric lesions by suppressing NSAID-induced apoptosis of gastric mucosal cells. From this ɔoint of view, we propose here that non-toxic HO-1 inducers are therapeutically beneficial as anti-ulcer drugs, by analogy to the non-toxic HSP inducer, GGA (clinically used anti-ulcer drug).

As for the mechanism of NSAID-induced up-regulation of HO-1, we have shown using a luciferase reporter assay that up-regulation occurs at the level of transcription and that the transcription factor for the *HO-1* gene, Nrf2, is accumulated in the nucleus in the presence of indomethacin. These results show that the mechanism is similar for NSAIDs and other HO-1 inducers (19). Because the kinase involved in Nrf2 activation and the resulting HO-1 up-regulation differ according to the stressor and cell species, we tried to identify the kinase responsible, using specific inhibitors of various kinases. An inhibitor of p38 MAPK (SB203580) suppressed not only indomethacin-dependent HO-1 up-regulation but also nuclear accumulation of Nrf2, strongly suggesting that the nuclear accumulation of Nrf2 and resulting up-regulation of HO-1 by indomethacin is mediated through the activation (phosphorylation) of p38 MAPK.

Although HO-1 was reported to activate p38 MAPK through CO production (57), the idea that the activation of p38 MAPK by NSAIDs is the result of the HO-1 up-regulation was not supported by the observation that SnMP did not suppress the NSAID-stimulated phosphorylation of p38 MAPK (Fig. 10).

At present, it is unclear how NSAIDs activate p38 MAPK. That is to say, the direct target of NSAIDs that leads to HO-1 up-regulation has not been defined. COX is a target of NSAIDs, which accounts for their anti-inflammatory activity, because PGs, such as PGE_2, have a strong capacity to induce inflammation. Because the capacity of each NSAID to up-regulate HO-1 did not correlate with their ability to inhibit COX and given that exogenously added PGE_2 did not suppress the up-regulation of HO-1 by NSAIDs, COX does not seem to be involved in NSAID-induced up-regulation of HO-1. We recently proposed that the cytotoxicity of NSAIDs results from the interaction of these molecules with cell membranes. The ability of each NSAID to result in membrane permeabilization correlated well with their cytotoxicity. NSAIDs increase the intracellular level of Ca^{2+} by stimulating Ca^{2+} influx through permeabilization of cytoplasmic membranes, and BAPTA-AM, an intracellular Ca^{2+} chelator, suppressed NSAID-induced apoptosis (6). However, membrane permeabilization and the resulting increase in the intracellular Ca^{2+} level also do not appear to be involved in NSAID-induced up-regulation of HO-1. Higher concentrations of NSAIDs are required for membrane permeabilization and increased intracellular Ca^{2+} levels than are required for HO-1 up-regulation (6), and BAPTA-AM did not suppress the HO-1 up-regulation by indomethacin. It was recently reported that thapsigargin, a specific inducer of the ER stress response, up-regulates HO-1 (58). Moreover, we reported that NSAIDs induce the ER stress response (7). However, the idea that NSAID-dependent HO-1 up-regulation is mediated through the ER stress response was not supported by our observations. In particular, the time course and dose response properties of HO-1 up-regulation did not correlate with those of the ER stress response (up-regulation of GRP78), and BAPTA-AM suppressed the NSAID-induced ER stress response (up-regulation of GRP78) (13) but not the up-regulation of HO-1.

HO-1 up-regulation has been suggested to play a protective role in inflammation. HO-1 deficiency in humans is associated with susceptibility to inflammation (59) and HO-1 knock-out mice show higher mortality rates after exposure to endotoxin than wild-type mice (60). Furthermore, HO-1 up-regulation inhibits or stimulates production of tumor necrosis factor (TNF) α (a pro-inflammatory mediator) or interleukin (IL)-10 (an anti-inflammatory mediator), respectively, through CO production, and inhibits microvascular endothelial cell-leukocyte adhesion through bilirubin production (61–63). Therefore, up-regulation of HO-1 by NSAIDs may be involved in not only the protection of gastric mucosa from NSAID-induced gastric lesions but also in the anti-inflammatory activity of NSAIDs.

NSAIDs show various pharmacological activities other than their anti-inflammatory action (such as chemopreventive activity and anti-Alzheimers disease activity). The HO-1 up-regulation by NSAIDs may also be involved in these activities. Epidemiological studies have shown that prolonged use of aspirin or other NSAIDs reduces the risk of Alzheimers disease (64), although the mechanism for this activity is not fully understood. Tau protein plays a major role in the development of Alzheimers disease and high levels of reactive oxygen species are believed to promote the development of Alzheimers disease (65). Overexpression of HO-1 was reported to result in decreased levels of tau protein in cells (66). We consider that the NSAID-induced up-regulation of HO-1 is involved in the anti-Alzheimers disease activity of NSAIDs resulting from a decrease in the level of tau protein and reactive oxygen species. On the other hand, based on epidemiological and animal studies, it was proposed that inducers of phase II drug detoxifying enzymes, that include HO-1, can be useful as chemopreventive drugs for cancer, because they can metabolize (detoxify) endogenous and environmental carcinogens (67). Furthermore, in *nrf2*-disrupted mice the chemopreventive effect of dithiolethiones (an inducer of phase II drug detoxifying genes) was lost because of a defect in the expression of phase II drug detoxifying genes (68). Based on these observations, we consider that up-regulation of HO-1 by NSAIDs is involved in their chemopreventive activity through the induction phase II drug detoxifying enzymes such as HO-1.

Acknowledgment—We thank Dr. J. Alam (Alton Ochsner Medical Foundation) for the pHO15luc plasmid.

REFERENCES

1. Smalley, W. E., Ray, W. A., Daugherty, J. R., and Griffin, M. R. (1995) *Am. J. Epidemiol.* 141, 539–545
2. Hawkey, C. J. (2000) *Gastroenterology* 119, 521–535

3. Barrier, C. H., and Hirschowitz, B. I. (1989) *Arthritis Rheum.* **32**, 926–932
4. Fries, J. F., Miller, S. R., Spitz, P. W., Williams, C. A., Hubert, H. B., and Bloch, D. A. (1989) *Gastroenterology* **96**, 647–655
5. Lichtenberger, L. M. (2001) *Biochem. Pharmacol.* **61**, 631–637
6. Tanaka, K., Tomisato, W., Hoshino, T., Ishihara, T., Namba, T., Aburaya, M., Katsu, T., Suzuki, K., Tsutsumi, S., and Mizushima, T. (2005) *J. Biol. Chem.* **280**, 31059–31067
7. Tsutsumi, S., Gotoh, T., Tomisato, W., Mima, S., Hoshino, T., Hwang, H. J., Takenaka, H., Tsuchiya, T., Mori, M., and Mizushima, T. (2004) *Cell Death Differ.* **11**, 1009–1016
8. Tomisato, W., Tanaka, K., Katsu, T., Kakuta, H., Sasaki, K., Tsutsumi, S., Hoshino, T., Aburaya, M., Li, D., Tsuchiya, T., Suzuki, K., Yokomizo, K., and Mizushima, T. (2004) *Biochem. Biophys. Res. Commun.* **323**, 1032–1039
9. Tomisato, W., Tsutsumi, S., Rokutan, K., Tsuchiya, T., and Mizushima, T. (2001) *Am. J. Physiol. Gastrointest Liver Physiol.* **281**, G1092–G1100
10. Tomisato, W., Tsutsumi, S., Hoshino, T., Hwang, H. J., Mio, M., Tsuchiya, T., and Mizushima, T. (2004) *Biochem. Pharmacol.* **67**, 575–585
11. Mathew, A., and Morimoto, R. I. (1998) *Ann. N. Y. Acad. Sci.* **851**, 99–111
12. Gehrmann, M., Brunner, M., Pfister, K., Reichle, A., Kremmer, E., and Multhoff, G. (2004) *Clin. Cancer Res.* **10**, 3354–3364
13. Tsutsumi, S., Namba, T., Tanaka, K. I., Arai, Y., Ishihara, T., Aburaya, M., Mima, S., Hoshino, T., and Mizushima, T. (2006) *Oncogene* **25**, 1018–1029
14. Tomisato, W., Takahashi, N., Komoto, C., Rokutan, K., Tsuchiya, T., and Mizushima, T. (2000) *Dig. Dis. Sci.* **45**, 1674–1679
15. Takano, T., Tsutsumi, S., Tomisato, W., Hoshino, T., Tsuchiya, T., and Mizushima, T. (2002) *Dig. Dis. Sci.* **47**, 1546–1553
16. Tomisato, W., Tsutsumi, S., Tsuchiya, T., and Mizushima, T. (2001) *Biol. Pharm. Bull.* **24**, 887–891
17. Hirakawa, T., Rokutan, K., Nikawa, T., and Kishi, K. (1996) *Gastroenterology* **111**, 345–357
18. Ponka, P. (1999) *Am. J. Med. Sci.* **318**, 241–256
19. Maines, M. D. (1997) *Annu. Rev. Pharmacol. Toxicol.* **37**, 517–554
20. Tenhunen, R., Marver, H. S., and Schmid, R. (1969) *J. Biol. Chem.* **244**, 6388–6394
21. Brouard, S., Otterbein, L. E., Anrather, J., Tobiasch, E., Bach, F. H., Choi, A. M., and Soares, M. P. (2000) *J. Exp. Med.* **192**, 1015–1026
22. Chou, Y. H., Ho, F. M., Liu, D. Z., Lin, S. Y., Tsai, L. H., Chen, C. H., Ho, Y. S., Hung, L. F., and Liang, Y. C. (2005) *Int. J. Biochem. Cell Biol.* **37**, 604–615
23. Igarashi, K., Kataoka, K., Itoh, K., Hayashi, N., Nishizawa, M., and Yamamoto, M. (1994) *Nature* **367**, 568–572
24. Ishii, T., Itoh, K., Takahashi, S., Sato, H., Yanagawa, T., Katoh, Y., Bannai, S., and Yamamoto, M. (2000) *J. Biol. Chem.* **275**, 16023–16029
25. Cho, H. Y., Jedlicka, A. E., Reddy, S. P., Kensler, T. W., Yamamoto, M., Zhang, L. Y., and Kleeberger, S. R. (2002) *Am. J. Respir. Cell Mol. Biol.* **26**, 175–182
26. Elbirt, K. K., Whitmarsh, A. J., Davis, R. J., and Bonkovsky, H. L. (1998) *J. Biol. Chem.* **273**, 8922–8931
27. Lee, J. M., Hanson, J. M., Chu, W. A., and Johnson, J. A. (2001) *J. Biol. Chem.* **276**, 20011–20016
28. Buckley, B. J., Marshall, Z. M., and Whorton, A. R. (2003) *Biochem. Biophys. Res. Commun.* **307**, 973–979
29. Alcaraz, M. J., Habib, A., Creminon, C., Vicente, A. M., Lebret, M., Levy-Toledano, S., and Maclouf, J. (2001) *Biochim. Biophys. Acta* **1526**, 13–16
30. Hou, C. C., Hung, S. L., Kao, S. H., Chen, T. H., and Lee, H. M. (2005) *Ann. N. Y. Acad. Sci.* **1042**, 235–245
31. Nascimento-Silva, V., Arruda, M. A., Barja-Fidalgo, C., Villela, C. G., and Fierro, I. M. (2005) *Am. J. Physiol. Cell Physiol.* **289**, C557–563
32. Grosser, N., Abate, A., Oberle, S., Vreman, H. J., Dennery, P. A., Becker, J. C., Pohle, T., Seidman, D. S., and Schroder, H. (2003) *Biochem. Biophys. Res. Commun.* **308**, 956–960
33. Cantoni, L., Valaperta, R., Ponsoda, X., Castell, J. V., Barelli, D., Rizzardini, M., Mangolini, A., Hauri, L., and Villa, P. (2003) *J. Hepatol.* **38**, 776–783
34. Alam, J., Wicks, C., Stewart, D., Gong, P., Touchard, C., Otterbein, S., Choi, A. M., Burow, M. E., and Tou, J. (2000) *J. Biol. Chem.* **275**, 27694–27702
35. Tomisato, W., Hoshino, T., Tsutsumi, S., Tsuchiya, T., and Mizushima, T. (2002) *Dig. Dis. Sci.* **47**, 2125–2133
36. Schreiber, E., Matthias, P., Muller, M. M., and Schaffner, W. (1989) *Nucleic Acids Res.* **17**, 6419
37. Tsutsumi, S., Tomisato, W., Takano, T., Rokutan, K., Tsuchiya, T., and Mizushima, T. (2002) *Biochim. Biophys. Acta* **1589**, 168–180
38. Tenhunen, R., Marver, H. S., and Schmid, R. (1968) *Proc. Natl. Acad. Sci. U. S. A.* **61**, 748–755
39. Hoshino, T., Tsutsumi, S., Tomisato, W., Hwang, H. J., Tsuchiya, T., and Mizushima, T. (2003) *J. Biol. Chem.* **278**, 12752–12758
40. Schroeder, C. P., Yang, P., Newman, R. A., and Lotan, R. (2004) *Cancer Biol. Ther.* **3**, 847–852
41. Ben-Chetrit, E., Fischel, R., Hinz, B., and Levy, M. (2005) *Rheumatol. Int.* **25**, 332–335
42. Kawai, S., Nishida, S., Kato, M., Furumaya, Y., Okamoto, R., Koshino, T., and Mizushima, Y. (1998) *Eur. J. Pharmacol.* **347**, 87–94
43. Tanaka, Y., Nishikawa, Y., and Hayashi, R. (1983) *Chem. Pharm. Bull. (Tokyo)* **31**, 3656–3664
44. Valaes, T., Petmezaki, S., Henschke, C., Drummond, G. S., and Kappas, A. (1994) *Pediatrics* **93**, 1–11
45. Ghattas, M. H., Chuang, L. T., Kappas, A., and Abraham, N. G. (2002) *Int. J. Biochem. Cell Biol.* **34**, 1619–1628
46. Nakahira, K., Takahashi, T., Shimizu, H., Maeshima, K., Uehara, K., Fujii, H., Nakatsuka, H., Yokoyama, M., Akagi, R., and Morita, K. (2003) *Biochem. Pharmacol.* **66**, 1091–1105
47. Tsutsumi, S., Mima, S., Tomisato, W., Hoshino, T., Tsuchiya, T., and Mizushima, T. (2003) *Exp. Biol. Med. (Maywood)* **228**, 1089–1095
48. Mima, S., Tsutsumi, S., Ushijima, H., Takeda, M., Fukuda, I., Yokomizo, K., Suzuki, K., Sano, K., Nakanishi, T., Tomisato, W., Tsuchiya, T., and Mizushima, T. (2005) *Cancer Res.* **65**, 1868–1876
49. Itoh, K., Wakabayashi, N., Katoh, Y., Ishii, T., Igarashi, K., Engel, J. D., and Yamamoto, M. (1999) *Genes Dev.* **13**, 76–86
50. Kietzmann, T., Samoylenko, A., and Immenschuh, S. (2003) *J. Biol. Chem.* **278**, 17927–17936
51. Lee, J. C., Kassis, S., Kumar, S., Badger, A., and Adams, J. L. (1999) *Pharmacol. Ther.* **82**, 389–397
52. Young, P. R., McLaughlin, M. M., Kumar, S., Kassis, S., Doyle, M. L., McNulty, D., Gallagher, T. F., Fisher, S., McDonnell, P. C., Carr, S. A., Huddleston, M. J., Seibel, G., Porter, T. G., Livi, G. P., Adams, J. L., and Lee, J. C. (1997) *J. Biol. Chem.* **272**, 12116–12121
53. Dudley, D. T., Pang, L., Decker, S. J., Bridges, A. J., and Saltiel, A. R. (1995) *Proc. Natl. Acad. Sci. U. S. A.* **92**, 7686–7689
54. Gong, P., Cederbaum, A. I., and Nieto, N. (2003) *J. Biol. Chem.* **278**, 29693–29700
55. Kiemer, A. K., Bildner, N., Weber, N. C., and Vollmar, A. M. (2003) *Endocrinology* **144**, 802–812
56. Rockwell, P., Martinez, J., Papa, L., and Gomes, E. (2004) *Cell Signal* **16**, 343–353
57. Zhang, X., Shan, P., Otterbein, L. E., Alam, J., Flavell, R. A., Davis, R. J., Choi, A. M., and Lee, P. J. (2003) *J. Biol. Chem.* **278**, 1248–1258
58. Linden, T., Doutheil, J., and Paschen, W. (1998) *Neurosci. Lett.* **247**, 103–106
59. Yachie, A., Niida, Y., Wada, T., Igarashi, N., Kaneda, H., Toma, T., Ohta, K., Kasahara, Y., and Koizumi, S. (1999) *J. Clin. Investig.* **103**, 129–135
60. Poss, K. D., and Tonegawa, S. (1997) *Proc. Natl. Acad. Sci. U. S. A.* **94**, 10925–10930
61. Hayashi, S., Takamiya, R., Yamaguchi, T., Matsumoto, K., Tojo, S. J., Tamatani, T., Kitajima, M., Makino, N., Ishimura, Y., and Suematsu, M. (1999) *Circ. Res.* **85**, 663–671
62. Lee, T. S., and Chau, L. Y. (2002) *Nat. Med.* **8**, 240–246
63. Lee, T. S., Tsai, H. L., and Chau, L. Y. (2003) *J. Biol. Chem.* **278**, 19325–19330
64. Akiyama, H., Barger, S., Barnum, S., Bradt, B., Bauer, J., Cole, G. M., Cooper, N. R., Eikelenboom, P., Emmerling, M., Fiebich, B. L., Finch, C. E., Frautschy, S., Griffin, W. S., Hampel, H., Hull, M., Landreth, G., Lue, L., Mrak, R., Mackenzie, I. R., McGeer, P. L., O'Banion, M. K., Pachter, J.,

Pasinetti, G., Plata-Salaman, C., Rogers, J., Rydel, R., Shen, Y., Streit, W., Strohmeyer, R., Tooyoma, I., Van Muiswinkel, F. L., Veerhuis, R., Walker, D., Webster, S., Wegrzyniak, B., Wenk, G., and Wyss-Coray, T. (2000) *Neurobiol. Aging* **21**, 383–421
65. Klein, J. A., and Ackerman, S. L. (2003) *J. Clin. Investig.* **111**, 785–793
66. Takeda, A., Perry, G., Abraham, N. G., Dwyer, B. E., Kutty, R. K., Laitinen, J. T., Petersen, R. B., and Smith, M. A. (2000) *J. Biol. Chem.* **275**, 5395–5399
67. Kwak, M. K., Wakabayashi, N., and Kensler, T. W. (2004) *Mutat. Res.* **555**, 133–148
68. Ramos-Gomez, M., Kwak, M. K., Dolan, P. M., Itoh, K., Yamamoto, M., Talalay, P., and Kensler, T. W. (2001) *Proc. Natl. Acad. Sci. U. S. A.* **98**, 3410–3415

Biochem. J. (2007) 402, 397–403 (Printed in Great Britain) doi:10.1042/BJ20060841

Mechanism for the degradation of origin recognition complex containing Orc5p with a defective Walker A motif and its suppression by over-production of Orc4p in yeast cells

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Orc5p is one of six subunits constituting the ORC (origin recognition complex), a possible initiator of chromosomal DNA replication in eukaryotes. Orc5p contains a Walker A motif. We recently reported that a strain of *Saccharomyces cerevisiae* having a mutation in Orc5p's Walker A motif (*orc5-A*), showed cell-cycle arrest at G_2/M and degradation of ORC at high temperatures (37 °C). Over-production of Orc4p, another subunit of ORC, specifically suppressed these phenotypes [Takahashi, Yamaguchi, Yamairi, Makise, Takenaka, Tsuchiya and Mizushima (2004) J. Biol. Chem. 279, 8469–8477]. In the present study, we examined the mechanisms of ORC degradation and of its suppression by Orc4p over-production. In *orc5-A*, at high temperatures, ORC is degraded by proteasomes; either addition of a proteasome inhibitor, or introduction of a mutation of either *tan1-1* or *nob1-4* that inhibits proteasomes, prevented ORC degradation. Introduction of the *tan1-1* mutation restored cell cycle progression, suggesting that the defect was due to ORC degradation by proteasomes. Yeast two-hybrid and co-immunoprecipitation analyses suggested that Orc5p interacts preferentially with Orc4p and that the *orc5-A* mutation diminishes this interaction. We suggest that this interaction is mediated by the C-terminal region of Orc4p, and the N-terminal region of Orc5p. Based on these observations, we consider that ATP binding to Orc5p is required for efficient interaction with Orc4p and that, in *orc5-A*, loss of this interaction at higher temperatures allows proteasomes to degrade ORC, causing growth defects. This model could also explain why over-production of Orc4p suppresses the *orc5-A* strain's phenotype.

Key words: DNA replication, origin recognition complex (ORC), Orc4p, Orc5-Ap, Walker A motif, yeast.

INTRODUCTION

The initiation of chromosomal DNA replication must be tightly regulated, and co-ordinated with cell division, to replicate the genome just once per cell cycle. In *Escherichia coli*, adenine nucleotides bound to the DnaA protein, the initiator of chromosomal DNA replication, play a major role in this regulation. DnaA has a high affinity for both ATP and ADP. The ATP–DnaA complex is active for DNA replication both *in vivo* and *in vitro*, but the ADP–DnaA complex is inactive [1–3]. DnaA has intrinsic ATPase activity and thus inactivates itself, suppressing over-initiation [4,5]. Acidic phospholipids interact with DnaA via conserved basic amino acid residues, and this re-activates the ADP–DnaA complex, and suppresses over-initiation of DNA replication [6–10].

In eukaryotes, ORC (origin recognition complex), a possible initiator of chromosomal DNA replication, binds to ATP and ADP [11–14]. ORC was originally identified as a six-protein complex that specifically binds to *Saccharomyces cerevisiae* origins of chromosomal DNA replication [11]. Homologues have been found in various eukaryotes, including humans [15]. In *S. cerevisiae*, ORC's ATP-binding activity has been well characterized *in vitro*. ORC has two subunits (Orc1p and Orc5p), which bind ATP. Orc1p, but not Orc5p, has ATPase activity [12]. Orc5p, but not Orc1p, can bind to ADP [13]. ATP binding to Orc1p, but not to Orc5p, is essential for specific binding of ORC to origin DNA [12,14]. ATP binding to Orc5p increases the affinity of Orc1p for ATP [14]. In contrast with these biochemical studies, ATP binding to ORC has not been examined extensively by genetic techniques, and therefore its role *in vivo* remains unknown.

We previously reported that a strain of *S. cerevisiae* having a mutation in the Walker A motif (consensus sequences for ATP-binding protein) of Orc5p (*orc5-A*) showed, at high temperatures, decreased levels of ORC, cell cycle arrest at G_2/M, and a growth defect [16]. In the present study, experiments using a proteasome inhibitor, and a mutation (*tan1-1* or *nob1-4*) that inhibits proteasomes, suggested that the decreased ORC level is mediated by degradation by proteasomes. Since the *tan1-1* mutation also suppressed the defects in cell cycle progression and cell growth in *orc5-A*, we presume these are also due to increased ORC degradation.

In *orc5-A*, over-production of Orc4p, another subunit of ORC, can also suppress the defects seen at high temperature [16]. This suppression was specific to *orc5-A*: Orc4p did not restore function in *orc5-1*, another temperature-sensitive strain that has a mutation outside the ATP-binding domain [16]. Furthermore, the suppression was specific to Orc4p; over-production of each of the other subunits (Orc1p, Orc2p, Orc3p and Orc6p) did not restore function [16]. These results revealed that Orc4p and ATP binding to Orc5p are closely linked. One possibility is that Orc5p can preferentially interact with Orc4p via its Walker A motif domain. In the present study, we used yeast two-hybrid analysis to suggest that Orc5p's interaction with Orc4p depends on the Walker A motif of Orc5p. We therefore consider that ATP binding to Orc5p is important for Orc5p to interact with Orc4p, which in turn is important for the stability of ORC in cells, and hence DNA

Abbreviations used: AD, activation domain; BD, binding domain; HA, haemagglutinin; MG-132, carbobenzoxy-L-leucyl-L-leucyl-leucinal; ONPG, *o*-nitrophenyl β-D-galactopyranoside; ORC, origin recognition complex; ORF, open reading frame; SC, synthetic complete.

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Table 1 Yeast strains

Strains	Genotypes	Reference
EGY48	*MATα trp1 ura3 his3 6lexAop-LEU2*	[18]
FY24	*MATα trp1Δ63 ura3-52 leu2Δ1*	[17]
tan1-1	*MATα ura3-52 leu2Δ1 tan1-1*	[17]
W303-1A	*Mata ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1*	[19]
YY411	*Mata ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 orc5-A*	[16]
YY412	*Mata ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 orc5-A orc2-3HA::HIS3 orc6-3HA::TRP1*	The present study
Y138	*Mata ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 nob1-4::LEU2*	[20]
NT411	*MATα trp1Δ63 ura3-52 leu2Δ1 orc5-A*	The present study
NT412	*MATα trp1Δ63 ura3-52 leu2Δ1 orc5-A tan1-1*	The present study
NT422	*Mata ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 orc5-A nob1-4::LEU2*	The present study

replication and cell growth. We also suggested that the Orc4p–Orc5p interaction is mediated by the C-terminal region of Orc4p and the N-terminal region of Orc5p that contains the Walker A motif.

EXPERIMENTAL

Strains, plasmids and medium

S. cerevisiae strains are listed in Table 1 [16–20]. Cells were cultured in SC (synthetic complete) medium.

The *orc5-A* gene (*orc5K43E*) was introduced into pRS406 to create pRS406-*orc5-A* [16]. This plasmid was transformed into FY24 and *tan1-1* strains and transformed cells were selected on SC agar plates lacking uracil. The resultant strains were transferred to plates containing 5-fluoro-orotic acid (the two-step gene replacement method) [21]. After checking by PCR, the resultant strains were named NT411 (FY24, *orc5-A*) and NT412 (FY24, *tan1-1*, *orc5-A*) respectively. We introduced the *nob1-4* mutation into the *orc5-A* mutant to construct NT422 strain (W303-1A, *orc5-A*, *nob1-4*).

Modifications of *ORC2* and *ORC6* genes with 3 × HA (haemagglutinin) were performed as described previously [22]. PCR was performed using pFA6a-3HA-TRP1 or pFA6a-3HA-His3MX6 plasmid as a template and primers containing DNA sequences for the C-terminal region of *ORC6* or *ORC2* gene respectively. The amplified DNA was transformed into YY411. The construct of the resultant strain (YY412) was confirmed by the colony PCR method.

FACS analysis of cellular DNA content

Samples were prepared as previously described [23,24], with the following modifications. Cells were pelleted by centrifugation, washed with sterilized water, and fixed in 70% (v/v) ethanol for 12 h. Cells were again pelleted, resuspended in 50 mM sodium citrate, sonicated for 1 min, treated with 0.25 mg/ml RNaseA for 1 h at 50 °C, and then with 1 mg/ml proteinase K for 1 h at 50 °C. DNA was stained with 50 μg/ml of propidium iodide, and 20 000 cells from each sample were scanned with a FACSCalibur instrument (Becton Dickinson).

Assessment of ORC subunits in yeast chromatin

Yeast spheroplasts were lysed with Triton X-100, and samples were processed into soluble (supernatant) and chromatin (insoluble precipitate) fractions by centrifugation, as previously described [24]. Equivalent amounts (total protein) of chromatin fractions were subjected to electrophoresis on 7.5 or 10% polyacrylamide gels containing SDS, transferred to a PVDF membrane, and probed with monoclonal antibodies against Orc3p (SB3), Orc5p (SB5) and HA (12CA5) [24,25].

Yeast two-hybrid analysis

Plasmids pSH18-34 (a reporter plasmid in which the *lacZ* gene is located downstream of a 6-fold repeated operator of the *lexA* gene), pEG202 [a plasmid to express BD fusions (binding domain fusions) under the control of the *ADH* promoter] and pJG4-5 [a plasmid to express AD fusions (activation domain fusions) under the control of a *GAL1* promoter] were purchased from OriGene Technologies. In pJG4-5, the ampicillin resistance gene was replaced by a chloramphenicol resistance gene, to construct pJG4-5Cm^R. DNA fragments containing intact or partial ORF (open reading frame) for each ORC subunit were prepared by PCR and inserted into pEG202 or pJG4-5Cm^R.

EGY48 cells were transformed with pSH18-34, a pEG202 derivative and a pJG4-5Cm^R derivative.

β-Galactosidase assay: cells were collected by centrifugation, suspended with 1 ml of Z buffer (100 mM phosphate buffer, pH 7.0, 10 mM KCl, 1 mM $MgSO_4$ and 50 mM 2-mercaptoethanol) to which were added three drops of $CHCl_3$ and one drop of 0.1% SDS. After mixing and incubation for 5 min at 37 °C, the β-galactosidase reaction was started by addition of 0.2 ml of ONPG (*o*-nitrophenyl β-D-galactopyranoside) and stopped by addition of 0.5 ml of 1 M Na_2CO_3. After centrifugation, the absorbance at 420 nm of supernatant was determined. One unit of β-galactosidase hydrolyses 1 μmol of ONPG per minute.

Co-immunoprecipitation assay

Yeast whole cell extract was prepared by the standard glass beads method in the presence of protease inhibitors. Whole cell extract was incubated with Dynabeads Protein G (Dynal Biotech) conjugated with an antibody against HA (12CA5) for 1 h at 4 °C with rotation. After centrifugation, precipitates were washed and finally suspended with SDS sample buffer (150 mM Tris/HCl, pH 6.8, 50% glycerol, 1.4 M 2-mercaptoethanol, 5% SDS and 0.0025% bromophenol blue). Samples were analysed by immunoblotting with an antibody against HA or LexA.

RESULTS

In *orc5-A*, ORC degradation depends on the proteasome system

Most intracellular proteins are degraded, in a controlled way, by the ubiquitin–proteasome system. Polyubiquitinated proteins are degraded by 26 S proteasomes, which are composed of a 20 S proteasome core and a 19 S regulatory subunit [26–29]. We here examined whether the proteasome system degrades ORC in *orc5-A*

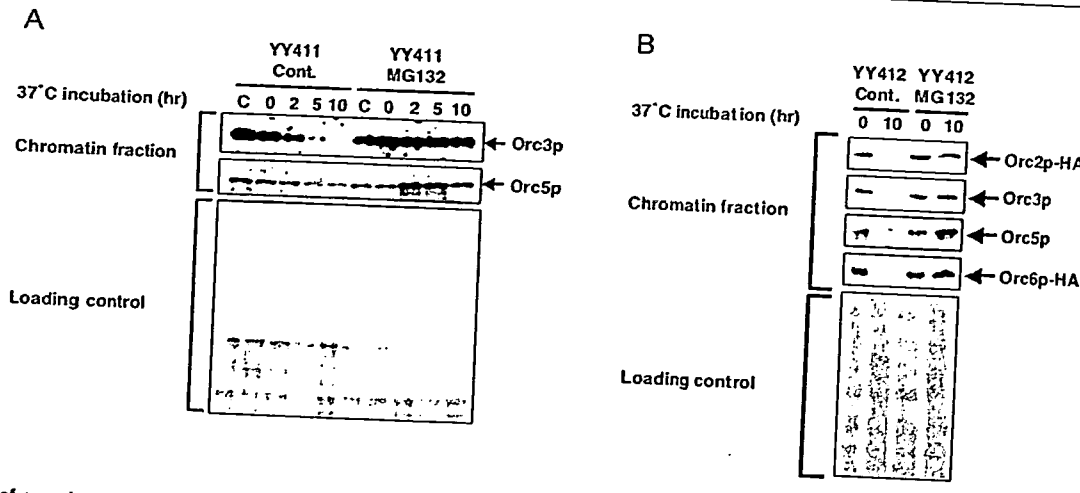

Figure 1 Effect of a proteasome inhibitor on ORC degradation in *orc5-A* cells

YY411 and YY412 (in which Orc2p and Orc6p are tagged with HA) cells were cultured in SC medium to exponential phase at 24 °C and further incubated in the presence or absence of MG-132 (10 μg/ml) for 1 h. Then the incubation temperature was changed to 37 °C, and the culture was sampled as shown. Chromatin fractions were prepared and analysed by immunoblotting using monoclonal antibodies specific for Orc3p, Orc5p or HA. As loading controls, samples were stained with silver (**A**, **B**). Control samples (shown as 'C') were taken before the MG-132 treatment (**A**).

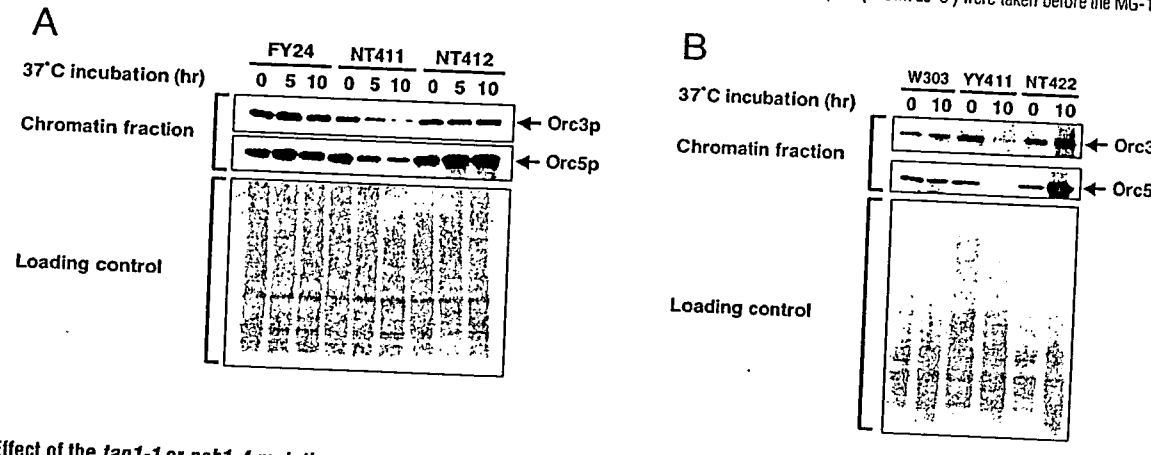

Figure 2 Effect of the *tan1-1* or *nob1-4* mutation on ORC degradation caused by the *orc5-A* mutation

FY24 (wild-type), NT411 (*orc5-A*) and NT412 (*orc5-A*, *tan1-1*) (**A**) or W303-1A (wild-type), YY411 (*orc5-A*) and NT422 (*orc5-A*, *nob1-4*) (**B**) cells were cultured in SC medium and sampled, and chromatin fractions were prepared and analysed by immunoblotting, as described for Figure 1.

(YY411) at high temperatures. After temperature shift-up (from 24 to 37 °C), both Orc3p and Orc5p disappeared from *orc5-A* cells (Figure 1A), as described previously [16]. Pre-incubating cells with a proteasome inhibitor [MG-132 (the proteasome inhibitor carbobenzoxy-L-leucyl-L-leucyl-leucinal)] [30,31] completely suppressed this disappearance (Figure 1A). We also examined the state of ORC subunits other than Orc3p and Orc5p under these conditions. As shown in Figure 1(B), Orc2p and Orc6p were decreased in *orc5-A* cells at 37 °C and this phenotype was suppressed by pre-incubation of cells with MG-132. Although we could not detect Orc1p and Orc4p by immunoblotting even in the wild-type strain, we presume that all ORC subunits disappear from *orc5-A* cells at 37 °C.

The intensity of the Orc5p band increased after addition of MG-132 (Figure 1A). This increase was diminished by pre-incubation of cells with cycloheximide (an inhibitor of protein synthesis; results not shown), suggesting that this increase is due to the stimulation by MG-132 of the expression of Orc5p.

To test further whether the proteasome system was responsible for the disappearance of ORC, we examined the effect of a mutation which disrupts the proteasome system. The *tan1-1* mutation is located in the *PRE8* gene, which encodes one of the subunits of the 20 S proteasome core [17]. We introduced the *orc5-A* mutation into the *tan1-1* mutant, and into its corresponding wild-type strain (FY24), to construct strains NT412 and NT411 respectively (Table 1). Similar to experiments using the W303-1A genetic background, in NT411, the *orc5-A* mutation caused ORC disappearance at high temperatures (Figure 2A). However, ORC did not disappear from NT412 (FY24, *orc5-A*, *tan1-1*) cells, indicating that *tan1-1* stabilized ORC5-A (ORC containing the protein product of the *orc5-A* gene). Results in Figures 1(A) and 2(A) strongly suggest that in the *orc5-A* strain, ORC disappearance at high temperatures is mediated by the proteasome system. We confirmed that 37 °C is a semi-permissive temperature for the *tan1-1* mutant; the mutant could grow at 37 °C (Figure 4) but not at 38.5 °C (results not shown) and results in Figure 2(A) suggested that the proteasome system is partially inactivated at 37 °C in the mutant, this being consistent with results in a previous paper [17].

The *nob1-4* is a mutation in the gene encoding a protein that binds to the 19 S regulatory subunit and is essential for the ubiquitin–proteasome system. Furthermore, this mutation causes a temperature-sensitive growth phenotype [20]. We introduced the *orc5-A* mutation into the *nob1-4* mutant to construct strain NT422. As shown in Figure 2(B), both Orc3p and Orc5p were decreased

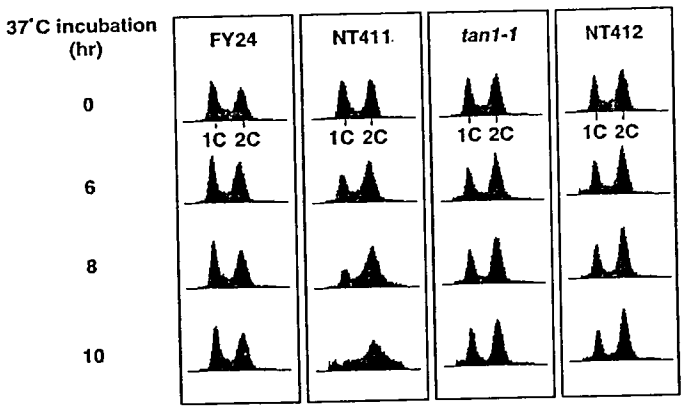

Figure 3 FACS analysis, showing the effect of the *tan1-1* mutation on cell cycle arrest caused by the *orc5-A* mutation

FY24 (wild-type), NT411 (*orc5-A*), the *tan1-1* strain and NT412 (*orc5-A*, *tan1-1*) cells were cultured in SC medium to exponential phase at 24 °C, and then the temperature was increased to 37 °C. The culture was sampled as indicated, and cellular DNA content was analysed by FACS.

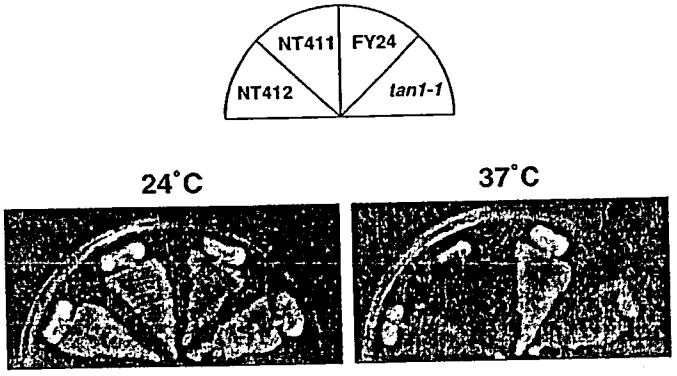

Figure 4 The effect of the *tan1-1* mutation on temperature-sensitive growth caused by the *orc5-A* mutation

NT412 (*orc5-A*, *tan1-1*), NT411 (*orc5-A*), FY24 (wild-type) and the *tan1-1* strain were incubated on SC agar plates at 24 °C or 37 °C for 2 days.

in YY411 (W303-1A, *orc5-A*) but not in NT422 (W303-1A, *orc5-A*, *nob1-4*), confirming that the ORC disappearance at high temperatures in the *orc5-A* strain is mediated by the ubiquitin–proteasome system.

We next examined the effect of *tan1-1* on other characteristics of *orc5-A*. In a W303-1A genetic background, *orc5-A* caused cell cycle arrest at G_2/M phase at high temperatures [16]. FACS analysis of NT411 showed that cells with 2C DNA content (G_2/M phase) accumulated after temperature shift-up (Figure 3), which is consistent with results from YY411 (W303-1A, *orc5-A*) cells [16]. On the other hand, in NT412 (FY24, *orc5-A*, *tan1-1*) cells, populations of both G1 and G_2/M cells (1C and 2C respectively) were observed even 10 h after temperature shift-up (Figure 3). In the *tan1-1* single mutant, cell cycle progression was not so affected, when compared with the wild-type strain, FY24 (Figure 3). Thus, in NT412, the *tan1-1* mutation suppressed cell cycle arrest at G_2/M phase. We therefore conclude that in *orc5-A*, cell cycle arrest is probably due to the proteasome-dependent degradation of ORC.

We also examined the effect of *tan1-1* on temperature-sensitive growth in *orc5-A* cells. As shown in Figure 4, cells containing the *orc5-A* mutation (NT411) could not grow at 37 °C, as described previously [16]. Interestingly, the *tan1-1* mutation completely restored ability to form colonies at 37 °C (Figure 4), and NT412 cells showed a growth curve similar to that of the wild-type strain (results not shown). Cell growth of the *tan1-1* single mutant was similar to that of the wild-type strain (results not shown). All these results strongly suggest that in the *orc5-A* strain, the growth defect is due to proteasome-dependent degradation of ORC.

Yeast two-hybrid analysis of the interaction between Orc5p and Orc4p *in vivo*

Our previous studies showed that Orc5p interacts with Orc4p via the Walker A motif region [16]. We have used a yeast two-hybrid system to test this idea. The ORF encoding wild-type Orc5p, or Orc5-Ap, was fused to a *lexA* DNA-binding domain to construct BD fusions. The ORF encoding each of the ORC subunits (Orc1p–Orc6p) was fused to a B42-HA transcriptional activation domain, to construct AD fusions. Plasmids containing the AD fusion genes and the BD fusion genes are introduced into cells; if the fusion proteins interact, they activate expression of reporter genes, β-galactosidase and the *LEU2* gene product, which allows growth on SC agar plates without leucine [32].

First, we studied wild-type Orc5p. Co-expression of a BD fusion of *ORC5* (BD-*ORC5*) and an AD fusion of *ORC4* (AD-*ORC4*) caused a much higher level of the β-galactosidase activity than the vector control, suggesting that Orc5p and Orc4p interact with each other (Figure 5A). Co-expression of BD-*ORC5* and AD-*ORC2* also caused high level of β-galactosidase activity, showing that Orc5p and Orc2p interact with each other (Figure 5A). Co-expression of BD-*ORC5* and AD fused to *ORC6* or other *ORC* genes (*ORC1*, *ORC3*) caused little or negligible respectively, stimulation for β-galactosidase activity, suggesting that Orc5p does not interact strongly with these subunits (Figure 5A). We confirmed, by immunoblotting analysis, that all AD and BD fusion proteins were expressed approximately equally (results not shown). Therefore, in cells, Orc5p seems to have the high affinity for Orc4p. It also interacts with itself (Figure 5A).

We next studied the *orc-5A* mutant. Co-expression of BD-*orc5-A* and AD-*ORC4* caused much the same level of β-galactosidase activity as the vector control (Figure 5A), suggesting that the mutation destroys Orc5p's ability to interact with Orc4p. We confirmed that BD-*ORC5* and BD-*orc5-A* were expressed approximately equally (results not shown), and conclude that the interaction between Orc4p and Orc5p requires the ATP-binding domain of Orc5p. Results in Figure 5(A) also suggest that the interaction between Orc5p with itself, and with Orc2p, also requires the ATP-binding domain of Orc5p.

For biochemical confirmation of the interaction between Orc5p and Orc4p, we performed co-immunoprecipitation experiments by use of crude whole cell extracts. Whole cell extracts were prepared from yeast expressing both AD-*ORC4* (HA–Orc4p) and BD-*ORC5* (LexA–Orc5p) or BD-*orc5-A* (LexA–Orc5-Ap), immunoprecipitated with an antibody against HA and detected by immunoblotting with an antibody against HA or LexA. As shown in Figure 5(B), approximately equal amounts of HA–Orc4p and LexA–Orc5p or LexA–Orc5-Ap were expressed in each strain and approximately equal amounts of HA–Orc4p were precipitated using the antibody against HA, suggesting that co-immunoprecipitation experiments worked well under the conditions. A significant amount of LexA–Orc5p was co-precipitated with HA–Orc4p, showing that Orc5p physically interacts with Orc4p under the conditions. A smaller amount of LexA–Orc5-Ap than of LexA–Orc5p was co-precipitated with HA–Orc4p, showing that the interaction of Orc4p with Orc5-Ap is weaker than that with LexA–Orc5p. These results suggest that the physical