J. Biochem. **136**, 711–722 (2004)
DOI: 10.1093/jb/mvh178

A Comprehensive Study on the Immunological Reactivity of the Hsp90 Molecular Chaperone

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Received May 22, 2004; accepted September 13, 2004

Periodontitis is a chronic infectious disease, *Porphyromonas gingivalis* being the most implicated pathogen. In the present study, we investigated the role of *P. gingivalis* HtpG (PgHtpG), a bacterial ortholog of mammalian Hsp90, in the growth of *P. gingivalis* and also assessed the immunological cross-reactivity of the members of the Hsp90 family. Antiserum against rat liver Hsp90 potently reacted with yeast Hsp90, called Hsc82, and also weakly with human Hsp90 (hHsp90) and human mitochondrial paralog Trap1, but did not react with PgHtpG, *Escherichia coli* HtpG, or human endoplasmic reticulum paralog Grp94. Moreover, among 19 monoclonal antibodies raised against hHsp90, nine cross-reacted with yeast Hsc82, and one with human Grp94, but none bound to PgHtpG or *E. coli* HtpG. Among them, three mAbs that strongly reacted with yeast Hsc82 recognized Asn_{291}–Ile_{304}, a conserved region of the family protein. The polyclonal antibody raised against a peptide, Met_{315}–Glu_{328}, of human Grp94, which corresponded to the conserved region of hHsp90, cross-reacted with hHsp90, but not with other Hsp90-family members. Thus, although mammalian Hsp90 shares some immunological reactivity with yeast Hsc82, human Grp94, and human Trap1, it is considerably distinct from its bacterial ortholog, HtpG. Disruption of the *P. gingivalis htpG* gene neither affected bacterial survival nor altered the sensitivity of *P. gingivalis* to various forms of stress.

Key words: Grp94, Hsp90, HtpG, *P. gingivalis*, Trap1.

Abbreviations: Hsp(s), heat shock protein(s); Hsp90, 90-kDa heat shock protein; hHsp90, human Hsp90; hHsp90α and hHsp90β, α and β isoforms of hHsp90, respectively; hGrp94, human 94-kDa glucose-regulated protein/endoplasmic reticulum paralog of Hsp90; hTrap1, human Trap1/mitochondrial paralog of Hsp90; yHsc82, yeast 82-kDa heat shock cognate protein; EcHtpG, *Escherichia coli* ortholog of eukaryotic Hsp90; PgHtpG, *Porphyromonas gingivalis* HtpG; GroEL, bacterial ortholog of mammalian Hsp60; DHFR, dihydrofolate reductase; GST, glutathione *S*-transferase; mAb, monoclonal antibody; pAb, polyclonal antibody; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate; and ELISA, enzyme-linked immunosorbent assay.

Reflecting the importance of the roles played by heat shock proteins (Hsps) in all organisms, their amino acid sequences are highly conserved even among eukaryotic and prokaryotic organisms. For instance, the amino acid sequences of human Hsp60 (*1*), Hsp70 (*2*), and Hsp90 (*3*, *4*) are 48.2, 47.2, and 35.6% identical to the respective *E. coli* orthologs, *i.e.,* GroEL (*5*), DnaK (*6*), and HtpG (*7*). It is generally required to define the specificity when anti-Hsp antibodies are prepared. This information is useful not only for experimental use of the antibodies, but also for understanding of the roles of Hsps in autoimmune responses in infectious diseases such as periodontitis.

Periodontitis is a chronic infectious disease, *Porphyromonas gingivalis* being the most implicated pathogen (*8–11*). There is a line of evidence that microbial Hsps are immunodominant antigens in many microorganisms (*12*). Accordingly, it has been postulated that continuous exposure to Hsps from periodontal bacteria may cause cross-reactions with human Hsps. Among Hsp families, GroEL/chaperonin, the ortholog of mammalian Hsp60, is a major antigen in various bacterial infections (*13–17*). The number of periodontitis patients showing a positive humoral immune response to GroEL was higher than the number of periodontally healthy subjects (*14*). The roles of other Hsp families and their immunological cross-reactivity are poorly understood.

In the present study, in order to comprehensively understand the immunological cross-reactivity of the members of the Hsp90 family, we performed immunochemical analyses with monoclonal antibodies (mAbs) raised against human Hsp90 (hHsp90), and polyclonal antibodies (pAbs) raised against *P. gingivalis* HtpG (PgHtpG), rat liver Hsp90, human 94-kDa glucose-regulated protein Grp94 (hGrp94), and the N- and C-terminal domains of *Escherichia coli* HtpG (EcHtpG). Their immunoreactivity against recombinant forms of hHsp90, yeast

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Hsp90 (yHsc82), hGrp94, human mitochondrial paralog of Hsp90 (hTrap1), EcHtpG, and PgHtpG was compared by means of immunoblotting analysis and an enzyme-linked immunosorbent assay (ELISA). We found that most of the antibodies were specific for the Hsp90-family proteins used as antigens. Nevertheless, some of them cross-reacted with other members of the family, but none of the antibodies that recognized eukaryotic Hsp90-family members cross-reacted with PgHtpG, and *vice versa.*

Secondly, in order to determine the role of HtpG in *P. gingivalis*, we constructed a *P. gingivalis* mutant with a disrupted *htpG* gene and compared its growth under stress conditions. The results revealed no difference in growth under normal or stress conditions between the wild and mutant strains.

MATERIALS AND METHODS

Materials—The materials used and their sources were as follows: Expression vectors pQE9 and pQE30, and plasmid pREP4, from Qiagen Inc. (Chatsworth, CA, USA); expression vector pTrcHis TOPO and TOP10 competent cells, from Invitrogen (Carlsbad, CA, USA); expression vector pGEX4T-1, an alkphos-direct labeling kit, CDP-Star detection reagent, Sephacryl S300 superfine, and low-molecular-weight markers, from Amersham Biosciences (Piscataway, NJ, USA); kaleidoscope prestained molecular standards, from Bio-Rad (Richmond, CA, USA); restriction enzymes and DNA-modifying enzymes, from Nippon Gene (Tokyo, Japan); Talon metal affinity resin, from Clontech Laboratories Inc. (Palo Alto, CA, USA); a Wizard genomic DNA purification kit, from Promega Corp. (Madison, WI, USA); anti-Hsp90 mAb (AC88/SPA-830) and anti–*E. coli* GroEL mAb (SPS-870) from Stressgen Biotech Corp. (Victoria, B.C., Canada); alkaline phosphatase–conjugated goat anti-rabbit IgG, from EY Laboratories Inc. (San Mateo, CA, USA); and alkaline-phosphatase–conjugated rabbit anti-mouse Ig(G+A+M), from Zymed Laboratories Inc. (San Francisco, CA, USA).

Construction of Bacterial Expression Vectors—Construction of plasmids carrying the full-length forms of hHsp90α, hHsp90β, hGrp94, EcHtpG, and the N-terminal domain (residues 1–336, EcHtpG-N) and C-terminal domain (residues 337–624, EcHtpG-C) of EcHtpG tagged with a histidine hexamer encoded by pQE9 (Qiagen) was described previously (*18*–*21*). Y1090[pREP4] was transformed with the plasmids, and the transformants were selected on Luria broth agar plates containing 50 μg/ml of ampicillin and 25 μg/ml of kanamycin.

Plasmids encoding the full-length forms of yHsc82 (*22*) and hTrap1 (*23*, *24*) were generously provided by Drs. Y. Kimura (Tokyo Metropolitan Institute of Medical Science, Tokyo, Japan) and W.-H. Lee (the University of Texas, San Antonio, Texas, USA), respectively. Genomic DNA of *P. gingivalis* ATCC 33277 was purified by use of the Wizard genomic DNA purification kit (Promega). For construction of PgHtpG, yHsc82, and hTrap1, DNA fragments encoding the full-length forms were amplified by PCR with templates described above and the following primer sets: 5′-ATGAGTAAGAAAGGAACAATCGGG (underlined, first Met codon) and 5′-TCAGAGAAGACGCTGGCTG (underlined, stop codon) for PgHtpG (*25*); 5′-ATGGATCCGGCTGGTGAAACTTTTGAATTTC (underlined, Ala2 codon) and 5′-AGCTGCAGATCAACTTCTTCCATCTCGGTG (underlined, stop codon) for yHsc82 (*22*); and 5′-ATGGCGCGCGAGCTG (underlined, first Met codon) and 5′-TCAGTGTCGCTCCAGGGC (underlined, stop codon) for hTrap1 (*23*). Amplified DNA fragments were directly inserted into the pTrcHis TOPO expression vector according to the manufacturer's recommendation. The constructed plasmids were designated as pTrcHis-PgHtpG, pTrcHis-yHsc82, and pTrcHis-hTrap1, respectively. For construction of pTrcHis TOPO glutathione S-transferase (pTrcHis-GST), the DNA fragment encoding GST was amplified by PCR using pGEX4T-1 (Amersham Biosciences) as a template. The DNA fragment was directly inserted into pTrcHis TOPO as described above. TOP10 cells transformed with the plasmids were selected on Luria broth agar plates containing 50 μg/ml of ampicillin. The insertion and orientation of the DNA fragments were confirmed by conducting Hot Star PCR (Qiagen Inc., Chatsworth, CA, USA).

Expression and Purification of Recombinant Proteins—After overnight cultivation of the transformed bacteria at 37°C, recombinant proteins were expressed at 30°C for 4 h in the presence of 0.2 mM isopropyl-β-D-thiogalactopyranoside. They were purified by affinity chromatography on a Talon affinity column according to the manufacturer's protocol except that 10 mM imidazole was included in the lysis/washing buffer. After extensive washing out of non-adsorbed substances, bound proteins were eluted with 0.1 M imidazole (pH 8.0) containing 10% (v/v) glycerol. The histidine-hexamer-tagged form of GST, encoded by pTrcHis-GST, as well as dihydrofolate reductase (DHFR) encoded by pQE30 were expressed and purified by Talon affinity chromatography as above. The purified proteins were stored at –80°C until used.

Production of Antibodies—Affinity-purified recombinant proteins were further purified by electrophoresis on a 7.5% polyacrylamide gel of 0.5-cm thickness under non-denaturing conditions (*26*). Proteins except for HtpG-N each migrated to the position of the indicating dimeric form. HtpG-N migrated to a monomeric position (*21*). They were excised following visualization with a Gelcode silver snap kit (Pierce, Rockford, IL, USA). A piece of a polyacrylamide gel containing recombinant proteins (approximately 1 mg) was homogenized, and then injected into rabbits (Japan White) 4–6 times at 3-week intervals. The immunogen emulsified with Freund's complete adjuvant was used for primary immunization, and subsequent booster injections were given with it in Freund's incomplete adjuvant. The production of mAbs against hHsp90, and pAbs against rat liver Hsp90, recombinant hGrp94, and a peptide of hGrp94 (Met315–Glu328) was reported previously (*18*, *20*, *27*). Animal care and experimental procedures were conducted in accordance with the Guidelines for Animal Experimentation of Nagasaki University with the approval of the Institutional Animal Care and Use Committee. Antisera were divided into small aliquots and stored in the presence of 0.03% NaN_3 at –80°C.

Removal of the Antibodies Bound to the N-Terminal Tag Sequences of Recombinant Proteins—Recombinant proteins possessed either of two types of N-terminal tags, *i.e.*, 12 amino acids for hHsp90α, hHsp90β, hGrp94, EcH-

tpG, EcHtpG-N, and EcHtpG-C derived from vector pQE9, or 35 amino acids for hTrap1, yHsc82, and PgHtpG from pTrcHis TOPO. Hence, in order to eliminate the effect of the antibodies that might react with tag peptides, the pAbs were pre-treated with either TrcHis-GST, which carried the 35-amino acid tag, or DHFR, which carried the 12–amino acid tag. That is, the antiserum (1/300 dilution) prepared by immunization with PgHtpG was pre-incubated with TrcHis-GST (0.1 mg/ml) overnight at 4°C with constant rotation. Antisera (1/300 dilution) against hGrp94, EcHtpG-N, and EcHtpG-C were pre-incubated with DHFR as above. These antisera were then used for ELISA and immunoblotting. This treatment was sufficient to suppress the binding of the pAbs to the tag sequences (data not shown). Antiserum against Hsp90 from rat liver and mAbs against hHsp90 were used without any pre-treatment.

Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis (SDS-PAGE)—Electrophoresis was performed in the presence of 0.1% SDS at a polyacrylamide concentration of 12.5% unless otherwise stated. Generally, 0.5–1 μg of proteins or 5 μl of bacterial lysates was loaded onto an SDS-PAGE gel. The separated proteins were stained with Coomassie Brilliant Blue or subjected to immunoblotting as reported previously (*18*). Low-molecular-weight markers (Amersham Biosciences) or kaleidoscope prestained standards (Bio-Rad) were used as molecular markers.

Size-Exclusion Gel Chromatography—Size-exclusion gel chromatography was performed on a Sephacryl S300 superfine column (1 × 75 cm) equilibrated with 20 mM Tris-HCl (pH 8.0) and 1 mM EDTA containing 50 mM NaCl. Twenty-drop fractions (1 ml) were collected.

Immunoblotting Analysis—Aliquots (2 μl) of bacterial lysates containing recombinant proteins separated on SDS-PAGE gels were subjected to immunoblotting analysis as described previously (*18*). After transfer of the proteins to a polyvinylidene difluoride membrane (Millipore Corp., Bedford, MA, USA), the membrane was reacted with the first antibodies (1/2,000–1/30,000–diluted antisera or 1 μg/ml of mAbs) for 2h, washed 5 times, and then incubated with alkaline phosphatase–conjugated goat anti-mouse IgG or rabbit anti-mouse Ig(G+A+M) (0.1–0.2 μg/ml) for 1 h. Finally, bands were visualized by incubation with 5-bromo-4-chloro-3-indolyl phosphate and nitro blue tetrazolium (Promega, Madison, WI, USA). All incubations were carried out at room temperature.

Enzyme-Linked Immunosorbent Assay (ELISA)—The binding affinity of the antibodies for the Hsp90-family proteins was determined by ELISA. The purified proteins (1 μg) coated on the wells of a Maxisorp C96 titer plate (Nalge Nunc Int., Rochester, NY, USA) were incubated with various concentrations of antisera or purified mAbs in 0.1 ml of the blocking buffer [0.17 M H_3BO_4, pH 8.5, 0.12 M NaCl, 0.05% (v/v) Tween20, 1 mM EDTA, and 0.05% (w/v) NaN_3 containing 0.25% (w/v) bovine serum albumin] for 2 h at room temperature according to the method of Hornbeck *et al.* (*28*). Finally, the alkaline-phosphatase activity was determined by measuring the absorbance at 600 nm following 1-h incubation with Bluephos microwell phosphatase substrate (Kirkegaard and Perry Lab., Gaitherburg, MD, USA) at 30°C.

Bacterial Strain of P. gingivalis, Cell Culture, and Lysate Preparation—P. gingivalis (ATCC 33277) was grown at 37°C under an anaerobic atmosphere (10% CO2, 10% H2, and 80% N2) in enriched brain heart infusion (BHI) medium [37 g of BHI (Difco Laboratories, Detroit, Mich, USA), 5 g of yeast extract (Difco), 1 g of cysteine, 5 mg of hemin, and 1 mg of vitamin K_1/liter] and on enriched tryptic soy agar (TS agar) [40 g of Trypto-Soya agar (Nissui, Tokyo, Japan), 5 g of BHI, 1 g of cysteine, 5 mg of hemin, and 1 mg of vitamin K_1/liter], both in the presence of 0.1 mg/ml gentamycin (Sigma). A gene-disruption mutant of *P. gingivalis htpG* was cultured under identical conditions except that 10 μg/ml erythromycin (Sigma) was added. The cells were harvested by centrifugation. The pellet was suspended in 10 mM Tris-HCl (pH 7.5) containing 10 μg/ml leupeptin (Peptide Institute, Osaka, Japan). The cells were broken by sonication, and the supernatant obtained on centrifugation was saved as the bacterial lysate, as reported previously (*29*).

Disruption of htpG of P. gingivalis—The *htpG* gene of *P. gingivalis* was disrupted by insertion of an *ermF-ermAM* cassette by homologous recombination. Practically, a 2.2-kb *ermF-ermAM* cassette was amplified by PCR using a primer set (5′-GAAGATCTGCATGCCTGCAGCAGGTCG and 5′-GAAGATCTGACCATGATTACGAATTCGAGC: *Bgl*II sites, underlined) with pKD355 (*29*) as a template. This amplified cassette was cleaved with *Bgl*II and then ligated into the *Bgl*II site of pUC19-PgHtpG (see Fig. 6a). The *htpG* gene with the inserted *ermF-ermAM* cassette was again amplified by PCR with the primer set used for the amplification of *htpG* and then directly inserted into pCR-Blunt II-TOPO (Invitrogen). The resultant plasmid was linearized by *Not*I cleavage, and then introduced into *P. gingivalis* by electroporation for homologous recombination. Colonies were selected on TS plates containing 10 μg/ml erythromycin.

Agar Diffusion Assay—P. gingivalis cells were anaerobically grown in enriched BHI medium and spread on enriched TS plates, and then a sterile disk containing 4 μl of 3, 6 or 15% hydrogen peroxide was placed at the center of each plate, followed by anaerobic incubation at 37°C. After 4 days, the diameter of the circular area, in which *P. gingivalis* did not grow, was measured.

Southern Hybridization—A 583-bp fragment of *htpG* (see Fig. 6a) was amplified by PCR with a primer set (5′-ACCTCAATGTGGACTATCCGTTC; and 5′-AACTGACCATCCAACAGCATCAC) and pTrcHis-PgHtpG as a template. This fragment was labeled by use of the alkphos-direct labeling kit and used as a probe for Southern hybridization. Genomic DNA of *P. gingivalis* (4 μg) was cleaved with *Bam*HI, and then an aliquot (0.6 μg) was electrophoresed on a 0.8% agarose gel at a constant voltage of 30 V for 5 h. Following denaturation and transfer of the separated DNA to a membrane (Biodine Plus Membrane, Japan Genetics, Tokyo, Japan), hybridization was performed with the probe at 55°C overnight. After extensive washing of the membrane, the hybridization signals were detected with the CDP-Star detection reagent (Amersham Biosciences).

N-Terminal Amino Acid Sequencing—The N-terminal amino acid sequences of recombinant proteins were determined after separation of the recombinant proteins

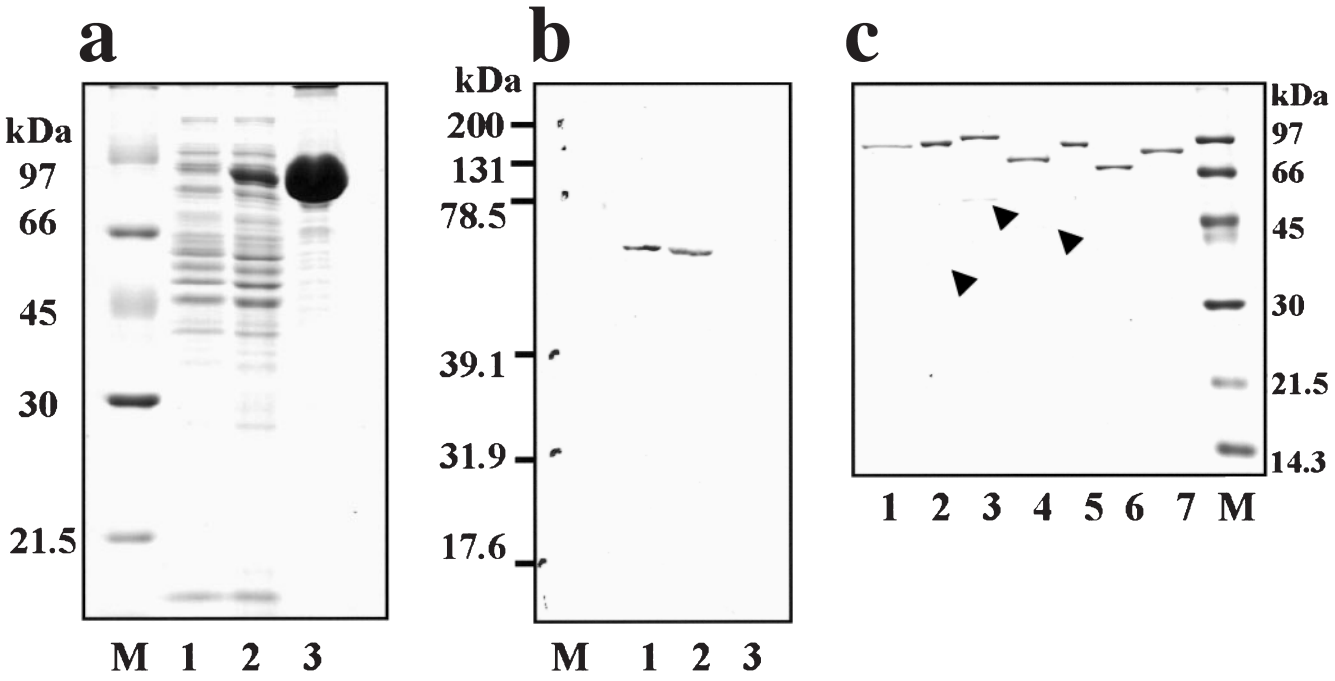

Fig. 1. **Expression and purification of Hsp90-family members.** Proteins (10 μg) were separated by SDS-PAGE, and then stained with Coomassie Brilliant Blue (a) or subjected to immunoblotting analysis against GroEL (b). Lane 1, bacterial lysate without induction of protein expression; lane 2, lysate with induction of PgHtpG; lane 3, purified PgHtpG; and lane M, molecular markers. An anti-GroEL mAb was used at 1:5,000 dilution. (c) Aliquots (0.5 μg) of purified samples were subjected to SDS-PAGE. Lane 1, hHsp90α; lane 2, hHsp90β; lane 3, hGrp94; lane 4, hTrap1; lane 5, yHsc82; lane 6, EcHtpG; lane 7, PgHtpG. Lane M, molecular markers. Arrowheads in panel "c" indicate major contaminants (see Table 1).

by SDS-PAGE and transfer to a polyvinylidene difluoride membrane (Bio-Rad). After having been stained with Coomassie Brilliant Blue, the bands were excised and directly sequenced with a model 477A protein sequencer (PE Biosystems).

Protein Concentrations—Protein concentrations were determined by the bicinchoninic acid method (Pierce, Rockford, IL, USA).

RESULTS

Expression and Purification of Hsp90-Family Proteins—We expressed the recombinant, full-length form of PgHtpG in *E. coli* (Fig. 1a). After the induction of protein expression, the 80-kDa band of PgHtpG was a major one for the bacterial lysate (lane 2), and hence this constituent was easily purified to homogeneity by one-step chromatography on a Talon affinity column (lane 3). The sample was completely free from lysate proteins of *E. coli*, such as GroEL, as shown on immunoblotting analysis: GroEL present in the lysate was eliminated at the affinity-purification step (Fig. 1b, lane 3). The N-terminus of the recombinant protein comprised Gly_{-34} (Table 1), indicating that the N-terminal Met at position –35 was processed from recombinant PgHtpG.

Similarly, six recombinant proteins of the Hsp90 family were expressed and purified by affinity chromatography (Fig. 1c). The preparations of Hsp90β, hGrp94, and hTrap1 reproducibly contained contaminants of 35, 53, and 44 kDa, respectively (indicated by arrows). As reported previously, the 53-kDa species was the C-terminal half of hGrp94 starting at Met_{315} (Table 1 and Ref. *20*). This species seemed to be co-purified with the full-length form, because it formed a dimer involving bridging to the intact protein at the middle and C-terminal domains (*19*, *20*). N-terminal sequencing demonstrated that the N-terminus of the 35-kDa species was the second Ala of the recombinant protein (Table 1). Thus, this species still retained the N-terminal tag, which could be adsorbed to the affinity resin. An attempt to determine the N-terminus of the 44-kDa species of hTrap1 was not successful, presumably because the band was of hetero-

Table 1. **N-terminal sequences of recombinant proteins.**

Protein	kDa	Detected amino acids	Corresponding sequence
PgHtpG	80	GGSHHHHHHGMSAM	g_{-34}gshhhhhhgmsam
hHsp90β	35	MRGSHHHHHH	m_{-12}rgshhhhhh
hGrp94	53	MNDIKPIWQ	M_{315}NDIKPIWQ
hTrap1	44	could not be determined	

Numbers represent amino acid residues. Amino acids in small letters are derived from vector pQE9 (hHsp90β) or pTrcHis TOPO (PgHtpG).

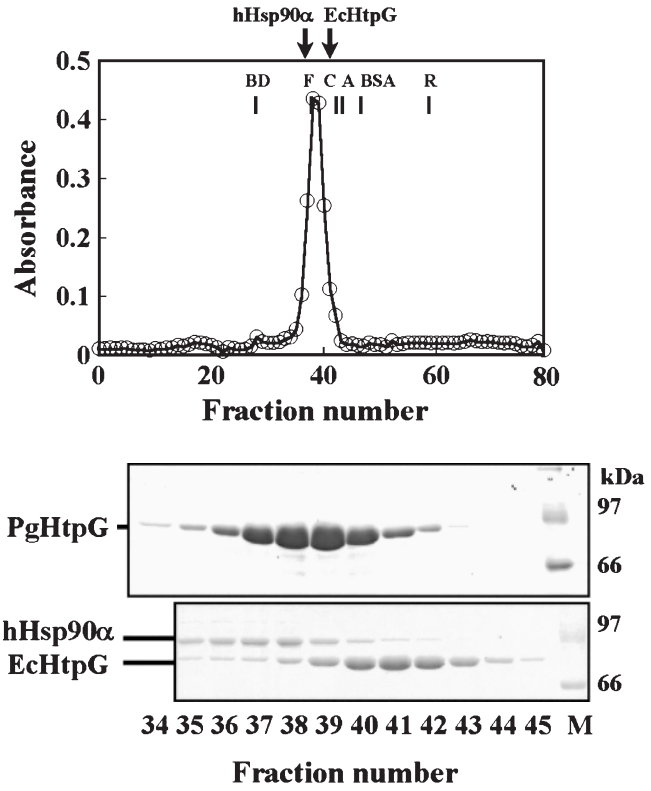

Fig. 2. **Size exclusion gel chromatography of PgHtpG.** Affinity-purified PgHtpG (1.6 mg) or a mixture of hHsp90α (0.5 mg) and EcHtpG (1 mg) was separated by size-exclusion chromatography on a Sephacryl S300 superfine column as described under "MATERIALS AND METHODS." Absorbance at 280 nm was measured. Molecular weight markers: Blue dextran 2000 (void volume, BD), ferritin (F, 440 kDa, Stokes' radius = 61 Å), catalase (C, 232 kDa, 61 Å), aldolase (A, 158 kDa, 52.2 Å), bovine serum albumin (BSA, 67 kDa, 35.5 Å), and ribonuclease A (R, 13.7 kDa, 16.4 Å). Following the chromatography, aliquots (16 μl) of the fractions were denatured and then separated by SDS-PAGE. The separated proteins were stained with Coomassie Brilliant Blue. M, molecular markers.

geneous components. We suspected that this species was also a truncated form. Taken together, although several preparations contained small amounts of truncated forms, the recombinant proteins appeared to be purified to near homogeneity.

On size exclusion gel chromatography on a Sephacryl S300 column, PgHtpG was eluted as a single peak (Fig. 2). A mixture of recombinant hHsp90α and EcHtpG was also separated on the column (Fig. 2). Reflecting their molecular masses, 90 kDa for hHsp90α, 80 kDa for PgHtpG, and 70 kDa for EcHtpG, they were eluted in this order from the column with Stokes radii of 69, 60, and 55 Å, respectively. The identity of the values for hHsp90α and EcHtpG to those previously reported (*30*, *31*) indicated that PgHtpG existed as a homodimer, as hHsp90α and EcHtpG did. Compared with ferritin (440 kDa), a spherical protein with a similar Stokes' radius (61 Å), PgHtpG (160 kDa as a dimer) as well as hHsp90α (180 kDa) and EcHtpG (140 kDa) had smaller molecular masses. This may be due to the highly elliptic structures of the dimmers, as demonstrated by electron microscopic studies (*32*, *33*).

Reactivity of the pAbs toward Members of the Hsp90 Family—To determine the specificity of the antisera obtained on immunization for the Hsp90 family proteins, we performed ELISA of the purified recombinant proteins (Fig. 1c) and immunoblotting analyses with bacterial lysates containing the seven proteins. ELISA with the antiserum against rat liver Hsp90 revealed the strongest binding to yHsc82 (Fig. 3a, left, open triangles), followed by that to hHsp90β (closed circles), hTrap1 (closed squares), and hHsp90α (open circles) in decreasing order. None of the others, i.e., hGrp94, EcHtpG and PgHtpG, were recognized under the conditions employed. Consistent with this finding, the immunoblotting analysis showed that the antiserum most potently reacted with 90-kDa yHsc82, and weakly with 90-kDa hHsp90s and 75-kDa hTrap1. Thirty-five- and 44-kDa bands blotted with the pAb under these conditions represented nonspecific binding (Fig 3a, right, arrowheads).

Identical examinations were carried out with the antisera against hGrp94 (Fig. 3b), PgHtpG (Fig. 3c), EcHtpG-N (Fig. 3d), and EcHtpG-C (Fig. 3e). ELISA demonstrated that, in all cases, the antisera specifically recognized the respective recombinant proteins used as immunogens. In agreement with this finding, the antiserum against hGrp94 specifically reacted with the 53-kDa truncated and 100-kDa full-length forms of hGrp94; whereas 80- and 90-kDa bands represented non-specifically bound species (arrowheads in Fig. 3b, right). The antiserum against PgHtpG specifically reacted with 78-kDa PgHtpG (Fig. 3c, right). The apparent molecular mass of endogenous PgHtpG was slightly smaller than that of the recombinant form (80 kDa) because of the 35-amino acid tag (compare Figs. 1a and 3c). The 70-kDa band was specifically blotted with anti-EcHtpG-N and EcHtpG-C sera (Fig. 3, d and e, right). Additionally, the small amount of a 70-kDa species present in all lanes (Fig. 3, d and e, right) was endogenous EcHtpG present in the *E. coli* lysate. Therefore, in contrast to in the previous study (*25*), we did not observe a specific band of PgHtpG on the immunoblotting with anti–EcHtpG-N (Fig. 3d) or anti–EcHtpG-C pAbs (Fig. 3e). Also, a band of EcHtpG was not detected with anti-PgHtpG pAb (Fig. 3c).

Reactivity of Anti-hHsp90 mAbs toward Members of the Hsp90 Family—In our previous study, we used anti-hHsp90 mAbs for the detection of yHsc82, because some of the mAbs cross-reacted with yHsc82 (*34*). However, the specificity of the mAbs had not been determined systematically, because the recombinant Hsp90 repertories were limited at the time. Hence, the expression of the seven Hsp90-family proteins (Fig. 1c) prompted us to investigate the cross-reactivity of the mAbs. Among 33 anti-hHsp90 mAbs developed, we here examined 19 mAbs that could bind to both hHsp90 isoforms, with the expectation of broader binding specificity.

Based on the immunoblotting profiles, we classified these 19 mAbs into four categories: Group 1, three mAbs that reacted with hHsp90α, hHsp90β, and yHsc82, apparently with identical intensities (Fig. 4a); Group 2, six mAbs (four mAbs shown in Fig. 3b; data not shown for K3738 or K41315) that reacted with hHsp90s preferentially but also reacted with yHsc82 weakly; Group 3, nine mAbs (four mAbs shown in Fig. 4c; data not shown for K3714, K3716A, K3725D, K41028, or K41102) that

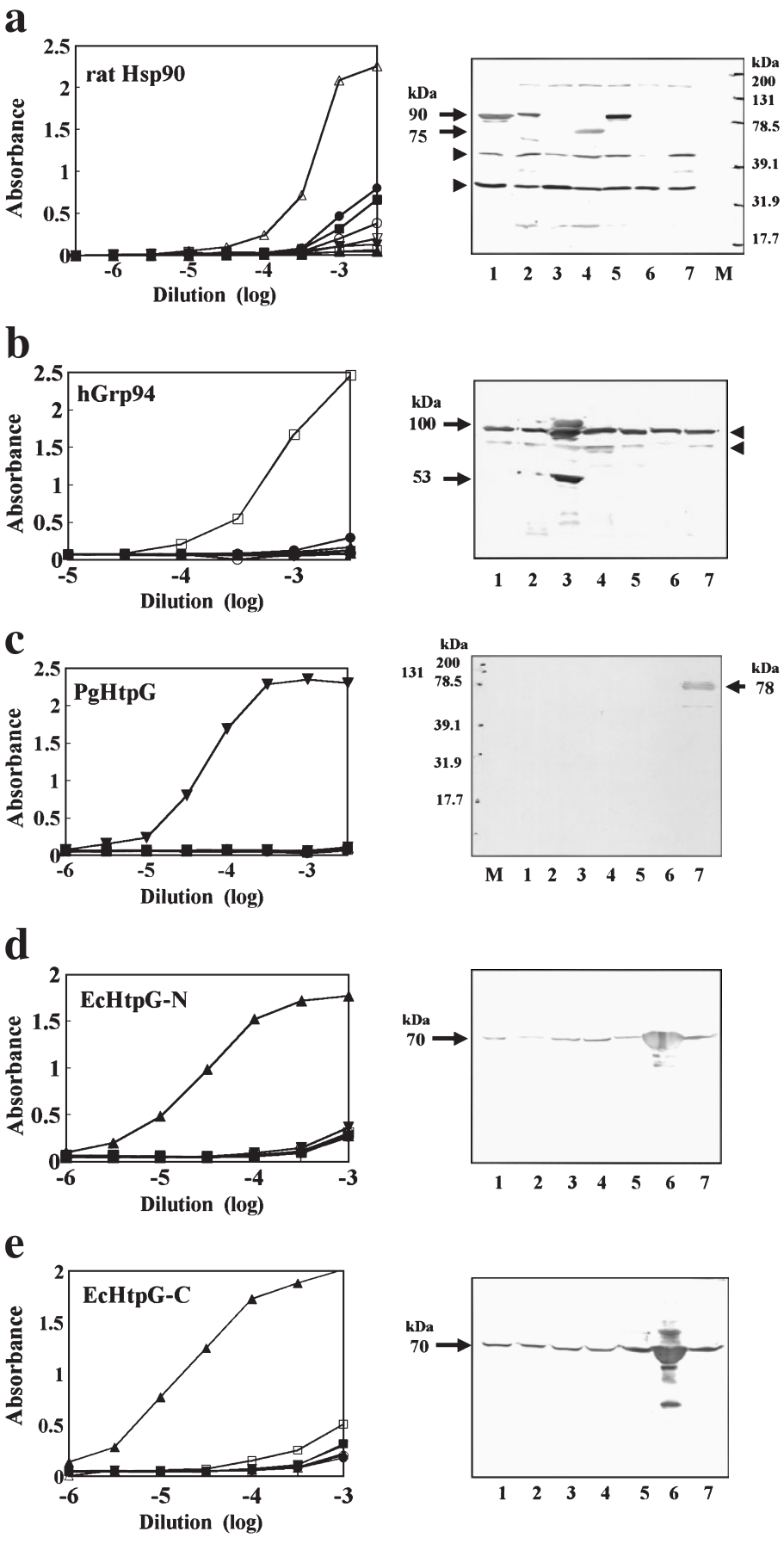

Fig. 3. **ELISA and immunoblotting of the Hsp90-family proteins with pAbs.** The immunological reactivity of antisera against Hsp90-family proteins was determined by ELISA with the purified Hsp90-faimily proteins or by immunoblotting with bacterial lysates (5 μl) containing the recombinant proteins. Antisera were raised against (a) rat liver Hsp90 (dilution rate of the serum = 1/2,000); (b) hGrp94 (1/2,000); (c) PgHtpG (1/3,000); (d) EcHtpG-N (1/30,000); and (e) EcHtpG-C (1/30,000). hHsp90α (open circles); hHsp90β (closed circles); hGrp94 (open squares); hTrap1 (closed squares); yHsc82 (open triangles); EcHtpG (closed triangles); and PgHtpG (closed inverted triangles). Lane numbers 1–7 are identical to those of Fig. 1. Lane M, kaleidoscope prestained standards. Arrowheads, nonspecific binding.

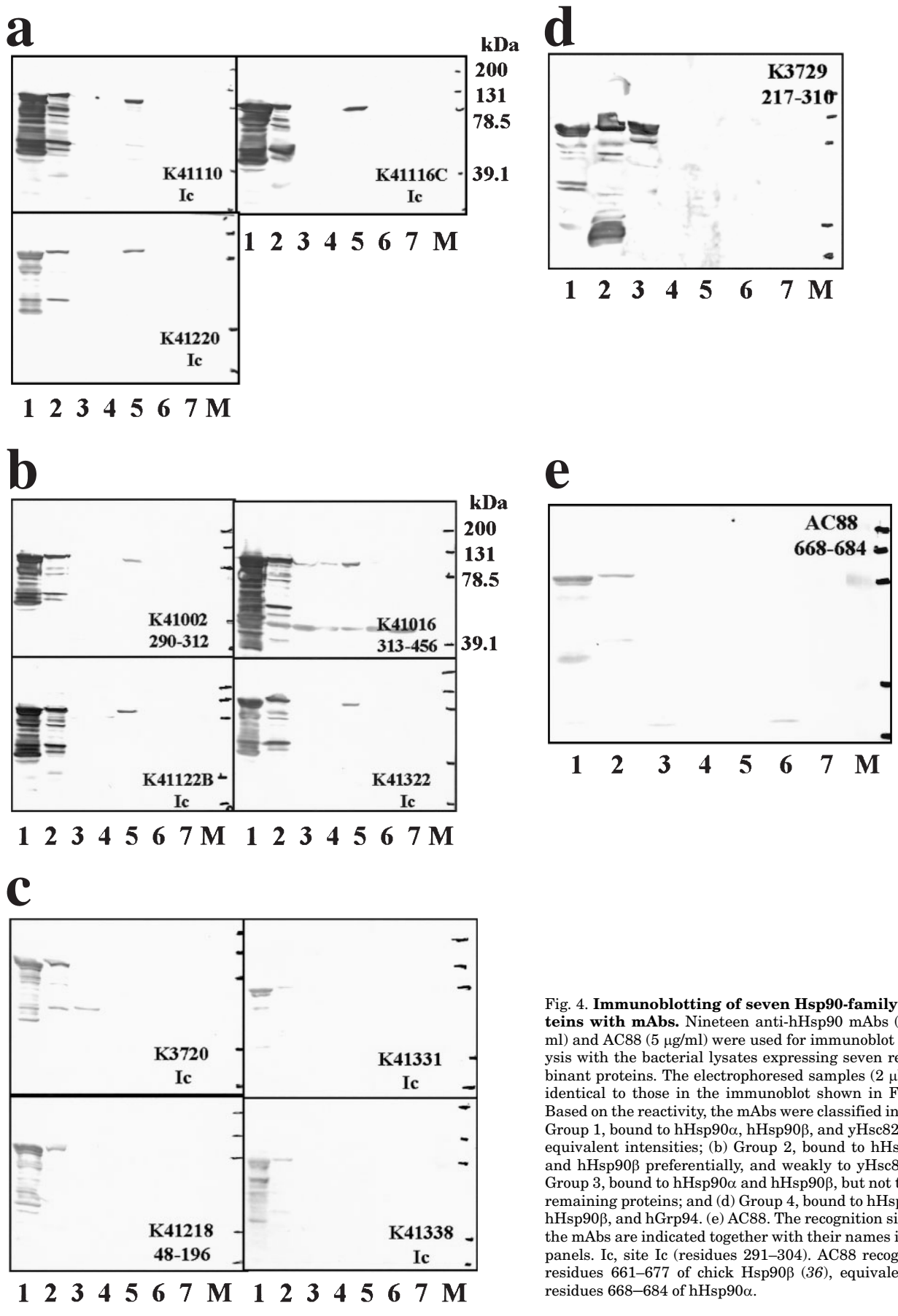

Fig. 4. **Immunoblotting of seven Hsp90-family proteins with mAbs.** Nineteen anti-hHsp90 mAbs (1 μg/ml) and AC88 (5 μg/ml) were used for immunoblot analysis with the bacterial lysates expressing seven recombinant proteins. The electrophoresed samples (2 μl) are identical to those in the immunoblot shown in Fig. 3. Based on the reactivity, the mAbs were classified into (a) Group 1, bound to hHsp90α, hHsp90β, and yHsc82 with equivalent intensities; (b) Group 2, bound to hHsp90α and hHsp90β preferentially, and weakly to yHsc82; (c) Group 3, bound to hHsp90α and hHsp90β, but not to the remaining proteins; and (d) Group 4, bound to hHsp90α, hHsp90β, and hGrp94. (e) AC88. The recognition sites of the mAbs are indicated together with their names in the panels. Ic, site Ic (residues 291–304). AC88 recognizes residues 661–677 of chick Hsp90β (*36*), equivalent to residues 668–684 of hHsp90α.

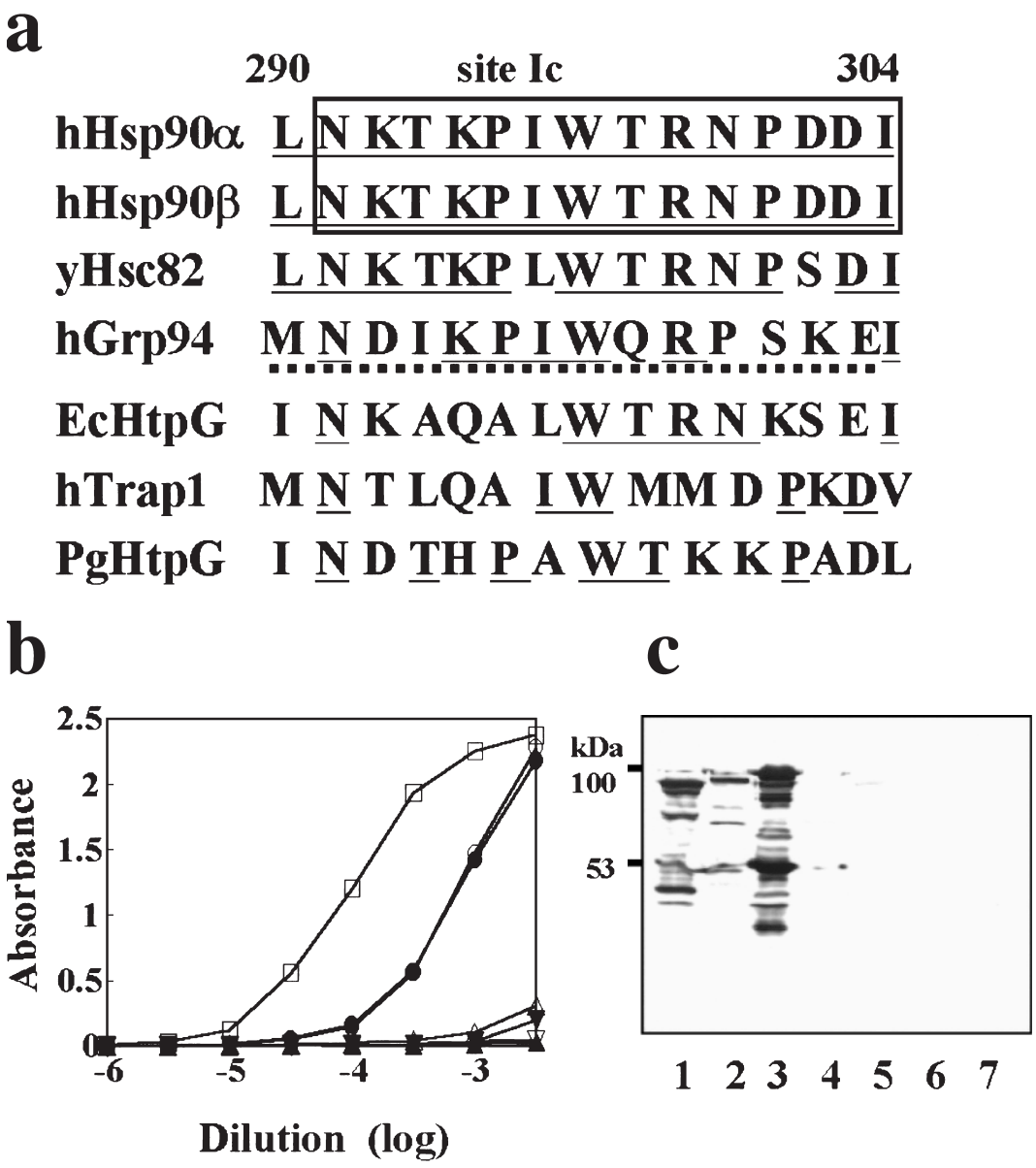

Fig. 5. **Immunoblotting and ELISA of seven Hsp90-family proteins with pAb raised against a peptide of hGrp94.** (a) The amino acid sequences of the seven Hsp90-family proteins at Asn_{291}–Ile_{304} (site Ic), *i.e.*, one of the most immunogenic sites of hHsp90, are compared. Amino acids constituting site Ic are boxed and those identical to in hHsp90α are underlined. The peptide sequence of hGrp94 used for the immunization is indicated by a dashed line. Amino acid numbers are those for hHsp90α. (b and c) ELISA (b) and immunoblot analysis (c) of seven recombinant proteins with a pAb against the hGrp94 peptide. The symbols and lanes 1–7 are identical to those in Fig. 3.

reacted with hHsp90α and hHsp90β, but scarcely reacted with other Hsp90-family members; and finally, Group 4, a single mAb, K3729, that reacted with hGrp94 as well as with hHsp90α and hHsp90β (Fig. 4d).

We also found that AC88, which is believed to possess binding capacity as to Hsp90 from a variety of species (*35*), could only detect two hHsp90 isoforms among the seven Hsp90-family proteins (Fig. 4e).

Reactivity of pAb Raised against a Highly Conserved Amino Site of the Hsp90-Family Proteins—Immunoblotting analysis with anti-Hsp90 mAbs demonstrated the cross-reactivity between hHsp90 and yHsc82 for nine mAbs (Fig. 4). Among them, three mAbs could blot yHsc82 as an intensively-stained band (Fig. 4a). Noticeably, the three mAbs recognized an identical site (Asn291–Ile304), corresponding to one (designated as site Ic in Ref. *18*) of the four highly immunogenic sites of hHsp90.

As expected, the amino acid sequence of site Ic (Fig. 5a, boxed) is highly conserved between hHsp90s and yHsc82, and is relatively conserved among all seven Hsp90-family members (Fig. 5a, underlined). Accordingly, we suspected that a pAb raised against site Ic might recognize several members of the Hsp90 family. To test this possibility, we developed a pAb against a 14–amino acid peptide of hGrp94, corresponding to site Ic of hHsp90 (Fig. 5a, dotted line). ELISA demonstrated that, as expected, the pAb

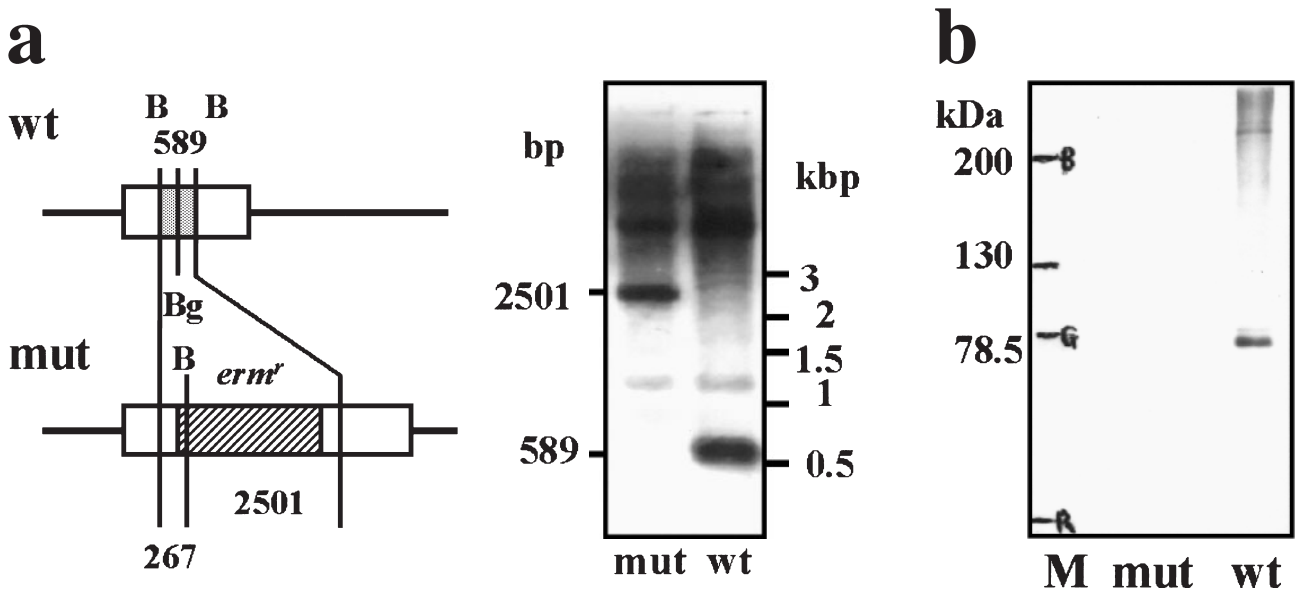

Fig. 6. **Southern hybridization of the HtpG disruption mutant of P.** gingivalis. (a) Genomic DNA digested with *Bam*HI was subjected to Southern blotting with an *htpG* probe (dotted square) as described under "MATERIALS AND METHODS." The hatched box represents the *ermF-ermAM* cassette (*erm*^{r}). Numbers represent base lengths between the *Bam*HI sites. B, *Bam*HI site; Bg, *Bgl*II site; wt, wild; mut, mutant. A one-kb ladder (New England Biolabs, Beverly, MA, USA) stained with ethidium bromide was used for reference. (b) Lysate proteins (12 μg) of the mutant and wild strains of *P. gingivalis* were separated by SDS-PAGE on a 7.5% polyacrylamide gel, and PgHtpG was detected by immunoblotting with anti-PgHtpG pAb (1/3,000 dilution) as in Fig. 3c. wt, wild; mut, mutant. Lane M, kaleidoscope prestained standards.

recognized hGrp94 with the highest affinity among the seven recombinant proteins. In addition, the pAb bound to hHsp90α and hHsp90β with tenfold lower affinity. However, yHsc82, hTrap1, EcHtpG, and PgHtpG were scarcely recognized by the pAb. Immunoblotting analysis faithfully reproduced the results of ELISA, except that yHsc82 was blotted as a faint band (Fig. 5c, lane 5). A 53-kDa band (lane 3) representing a truncated hGrp94 starting at Met315 was strongly stained with the pAb, because it still retained the antigen site. These results indicated that the immunogenicity of site Ic was partially shared by some members of the Hsp90 family among eukaryotic Hsp90-family members, but was substantially different between eukaryotic and prokaryotic members.

Effect of Disruption of htpG on the Growth of P. gingivalis—Disruption of the *P. gingivalis htpG* gene was achieved by homologous recombination with linearized DNA encoding *htpG* interrupted with an *ermF-ermAM* cassette, and was confirmed by Southern hybridization of genomic DNA of the mutant strain cleaved by *Bam*HI (Fig. 6a): The mutant strain gave a 2.5-kb fragment due to the insertion of the 2.2-kb *ermF-ermAM* cassette. Additionally, a 267-b fragment should be generated through cleavage at an endogenous *Bam*HI site of *htpG* and at a site derived from the pUC19 polylinker adjacent to the *ermF-ermAM* cassette (Fig. 6a). However, the fragment was too small to be observed under the conditions used. The 78-kDa PgHtpG was not immunologically detected in the mutant strain (Fig. 6b). Molecular species larger than 150 kDa observed in the wild strain seemed to be non-solubilized PgHtpG molecules.

To determine the role of HtpG in *P. gingivalis*, we examined the effects of several forms of stress. The growth rate at 37°C was not different between the wild and mutant strains, and was completely suppressed at 41°C in both strains (Fig. 7a). We also examined the effect of cold stress on the growth of the mutant strain. The growth was retarded at 30°C and stopped at 20°C, but there was again no difference between the wild and mutant strains (data not shown). The mutant, as well as the wild strain, was sensitive to 4% ethanol (Fig. 7b). Also, no difference was found in the sensitivity to 3, 6, and 15% of hydrogen peroxide, monitored as growth inhibition on TS plates (Fig. 7c).

DISCUSSION

The amino acid sequences of the seven Hsp90-family proteins are compared in Table 2 (*3*, *4*, *6*, *22*, *23*, *25*, *37*). It is reasonably postulated that the immunological cross-reactivity of proteins reflects their homology in amino acid sequence. However, the truth may be somewhat different, because the sequence homology is the sum of the results of comparison of whole sequences and the immunodominant sites are occasionally localized within restricted regions. In fact, the immunodominant sites of hHsp90 are predominantly localized within four restricted regions, as reported previously (*18*). Furthermore, the present study demonstrated that the difference in the immunoreactivity to an hGrp94 peptide between hHsp90 and yHsc82 did not reflect their overall similarity in site Ic (Fig. 5).

ELISA and the immunoblotting data demonstrated that the pAbs against PgHtpG, hGrp94, and the N- and C-terminal domains of EcHtpG solely reacted with the proteins used for immunization among the seven Hsp90-

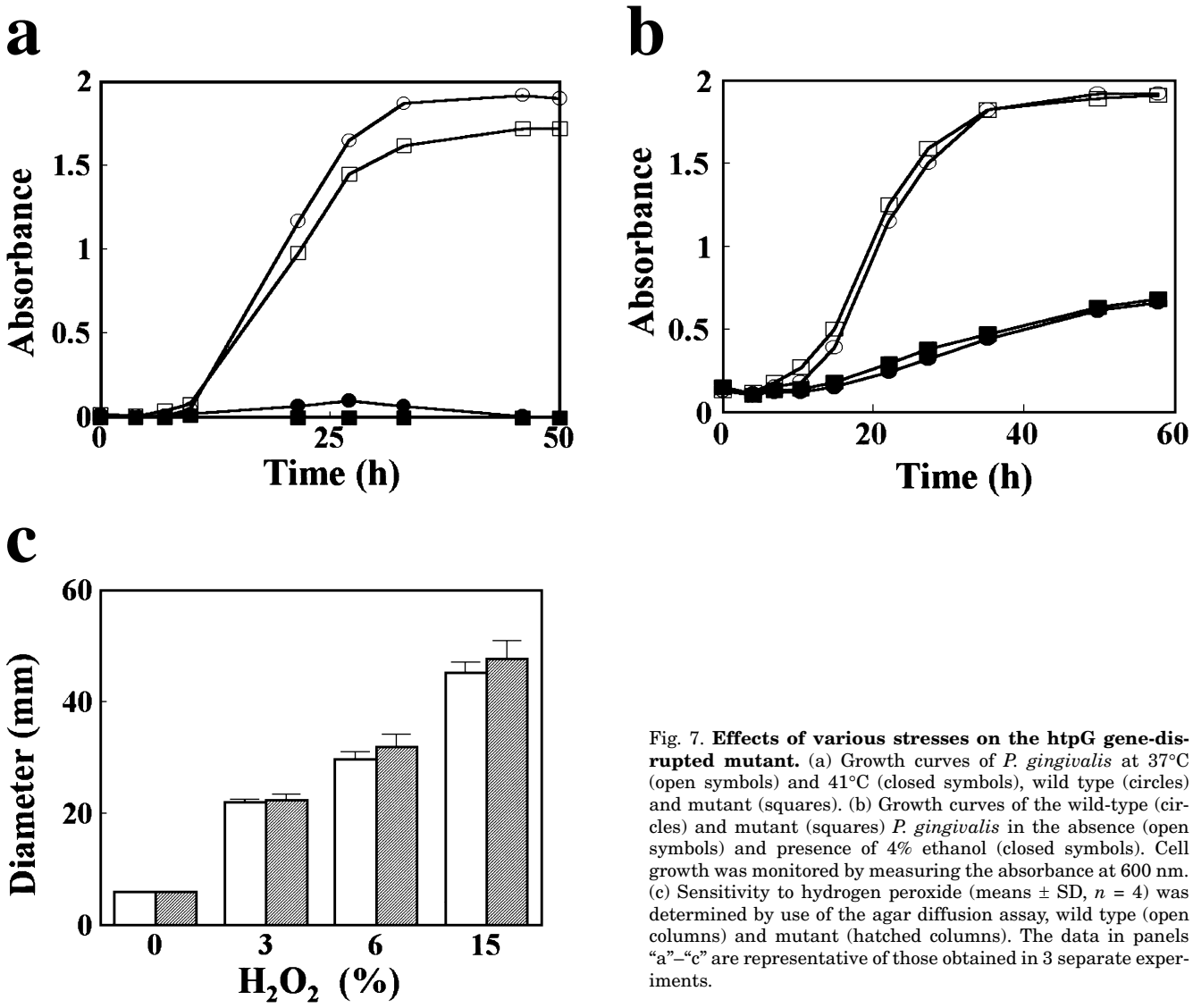

Fig. 7. **Effects of various stresses on the htpG gene-disrupted mutant.** (a) Growth curves of *P. gingivalis* at 37°C (open symbols) and 41°C (closed symbols), wild type (circles) and mutant (squares). (b) Growth curves of the wild-type (circles) and mutant (squares) *P. gingivalis* in the absence (open symbols) and presence of 4% ethanol (closed symbols). Cell growth was monitored by measuring the absorbance at 600 nm. (c) Sensitivity to hydrogen peroxide (means ± SD, n = 4) was determined by use of the agar diffusion assay, wild type (open columns) and mutant (hatched columns). The data in panels "a"–"c" are representative of those obtained in 3 separate experiments.

family members. However, the pAb against rat Hsp90 cross-reacted with yHsc82, and additionally with hHsp90α, hHsp90β, and hTrap1 with lower affinities. Three anti-hHsp90 mAbs among the 19 strongly bound to yHsc82, six mAbs bound to yHsc82 weakly and 1 even bound to hGrp94. The polyclonal antibody against site Ic of hGrp94 cross-reacted with hHsp90s. These results clearly demonstrate that some members of the Hsp90 family, at least, some specific sites, share immunological reactivity with each other. Nevertheless, it should be emphasized that the immunoreactivities of mammalian and bacterial Hsp90s are substantially distinct.

It is not surprising that all of the three anti–site-Ic mAbs that strongly reacted with yHsc82 (Fig. 4a) recognized a highly conserved region of hHsp90. In order to further investigate the properties of the immunogenic site, we raised a pAb by immunization with an hGrp94 peptide (Met315–Glu328). We initially assumed that the pAb raised against Met315–Glu328 of hGrp94 would react with yHsc82 and hHsp90 similarly, because immunoblotting analysis with mAbs indicated the kinship between yHsc82 and hHsp90 (Fig. 4, a and b), and the identity of 12 of 14 amino acids between hHsp90 and yHsc82 (Fig. 5a). However, this was not the case (Fig. 5b).

Table 2. **Sequence homology among members of the Hsp90 family.**

	hHsp90α	hHsp90β	yHsc82	hGrp94	EcHtpG	hTrap1	PgHtpG
hHsp90α	100	85.5	60.9	43.7	35.6	27.4	20.1
hHsp90β		100	61.4	43.6	35.2	27.2	21.0
yHsc82			100	43.5	37.0	27.2	21.0
hGrp94				100	32.0	26.4	22.3
EcHtpG					100	32.8	26.6
hTrap1						100	21.8

Values are percentages.

The most probable explanation is that the replacement of Ile296 of hHsp90 and hGrp94 with Leu in yHsc82 (Fig. 5a) significantly affected the immunoreactivity to the pAb. We further suppose that the dependence on Ile296 may be responsible for the difference in the recognition of yHsc82 by the eight anti–site-Ic mAbs (Fig. 4, a–c).

The sera of certain periodontitis patients were shown to contain anti-Hsp60 antibodies that reacted with the bacterial ortholog GroEL (*13*, *17*). Thus, at least, there are anti-GroEL antibodies that are capable of binding to Hsp60 from a variety of species. Moreover, a small population of periodontitis patients (2/30 subjects) showed antibodies reactive with human Hsp60, and the serum from one of the two patients specifically interacted with a synthetic peptide of *P. gingivalis* GroEL corresponding to the highly conserved region (*13*). Therefore, the cross-reactivity of the antibodies of patients should be carefully investigated for every Hsp family.

It was earlier reported that antibodies against Hsp70 and Hsp90 were produced in patients with lupus erythematosus (*38*, *39*). It is of interest to determine whether or not HtpG of *P. gingivalis* as well as *Actinobacillus actinomycetemcomitans*, another implicated pathogen, is truly involved in the autoimmune responses in those patients. However, we demonstrated that antibodies raised against PgHtpG as well as EcHtpG could not detect hHsp90α or hHsp90β. Even the mAbs and pAb targeting highly conserved and immunogenic site Ic of hHsp90 and hGrp94, respectively, could not detect bacterial ortholog HtpG. Therefore, we propose that, even if antibodies against hHsp90 are produced in periodontitis, the antigen is unlikely to be derived from bacteria, but is hHsp90 of host cells.

Lopatin *et al.* (*25*) reported that pAbs raised against EcHtpG and hHsp90, as well as mAb AC88 raised against *Achlya ambisexualis* Hsp90, could recognize PgHtpG, although neither our anti-PgHtpG pAb nor AC88 recognized it under our experimental conditions. They also showed that the antibodies commonly reacted with a 44-kDa protein of *P. gingivalis*. Furthermore, a 40-kDa species from this bacterium was detected with rabbit anti-hHsp90 pAb. In that study, the 44-kDa species was postulated to be a C-terminal fragment of the full-length form (*25*), considering the epitope site (amino acids 661–677 of chick hsp90β) recognized by AC88 (*36*). However, we did not find 40- and 44-kDa species in the lysate of *P. gingivalis,* when the pAb against PgHtpG was used for the immunoblotting (Fig. 5b). We suspect that the 40- and 44-kDa species are not related to PgHtpG, but are proteins nonspecifically bound to the antibodies, presumably due to the extremely high concentrations of the antibodies used.

We also found that AC88 could detect only two hHsp90 isoforms among the seven Hsp90-family proteins. In contrast, three mAbs belonging to Group 1., *i.e.,* K41110, K41116C, and K41220, which detected yHsc82, were superior to AC88 as tools for probing eukaryotic Hsp90. Detailed data on the mAbs in terms of binding affinity and species specificity are now accumulating in our laboratory.

The present study confirmed the previous finding by Sweier *et al.* (*40*) that *htpG* is dispensable for *P. gingivalis* and that an *htpG* gene disruption mutant has no apparent disadvantage in terms of stress responses. This situation is quite in contrast to the phenomenon observed for eukaryotic cells, the disruption of the two genes for cytosolic Hsp90 being lethal in yeast (*22*). It is well known that Hsp90 is abundantly expressed in the mammalian cytosol, representing 1–2% of all cytosolic proteins in brain and liver tissues; and hence, Hsp90 is one of the major proteinaceous constituents in the cytosol. However, this protein is not a major constituent in *P ginigivalis* (Kawano, T., Kikuchi, Y., Nakayama, K., and Nemoto, T. K., unpublished observation). Moreover, *htpG* is absent from *Mycoplasma*, *Streptococci*, and most species of *Archaea* (*41*). *E. coli* strains devoid of HtpG behave like wild-type strains and show no specific phenotypes, which further suggests a limited role for HtpG in bacterial organisms (*42*). However, the expression of *E. coli* HtpG was heat- and chemically-induced in complex media, but was not affected in a simple glucose minimal medium, indicating that HtpG is important for cells carrying a large pool of potential clients (*43*). On the other hand, protective effects of HtpG with respect to thermal stress management (*44*) and cold acclimation (*45*) were detected in cyanobacteria. Accordingly, HtpG in *P. gingivalis* might exert its function under certain conditions. Careful examination of stress conditions should be conducted to evaluate the role of HtpG in *P. gingivalis*.

We are very grateful to Dr. W.-H. Lee (the University of Texas, San Antonio, Texas, USA) for providing the plasmid for hTrap1. We also thank Drs. E. Tanaka (National Cancer Center, Tokyo, Japan), and K. Shibahara, K. Yamada, and S. Yamada (Nagasaki University, Nagasaki, Japan) for their contributions in the initial step of the present work. This work was supported by a Grant-in-Aid for Scientific Research on Priority Areas from the Ministry of Education, Culture, Sports, Science, and Technology of Japan (to T.K.N.).

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