Identification of the Penta-Peptide Constituting a Dominant Epitope Common to All Eukaryotic Hsp90 Molecular Chaperone

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Abstract We previously reported that, in human Hsp90 (hHsp90), there are 4 highly immunogenic sites, designated sites Ia, Ib, Ic, and II (Nemoto et al 1997). The present study was performed to further characterize their epitopes and to identify the epitope that is potentially common to all members of the Hsp90 family. Panning of a bacterial library carrying randomized dodecapeptides revealed that Glu_{251}-Ser-X-Asp_{254} constituted site Ia and that Pro_{295}-Ile-Trp-Thr-Arg_{299} did so site Ic. Site II (Asp_{701}-Pro_{717}) was composed of several epitopes. When 19 anti-hHsp90 monoclonal antibodies (mAbs) were subjected to immunoblotting against recombinant forms of 7 Hsp90-family members, 2 mAbs (K41110 and K41116C) that recognized site Ic bound to yeast Hsp90 with affinity identical to that for hHsp90; and one (K3729) that recognized Glu_{222}-Ala_{231} of hHsp90β could bind to human Grp94, an endoplasmic reticulum paralog of Hsp90. Among the 5 amino acids constituting site Ic, Trp_{297} and Pro_{295} were essential for recognition by all anti-site-Ic mAbs and Arg_{299} was important for most of them. The necessity of Ile_{296}, Thr_{298}, and Arg_{299}, which are replaced by Leu, Met/Leu, and Lys, respectively, in some eukaryotic Hsp90, was dependent on the mAbs, and K41110 and K41116C could react with Hsp90s carrying these substitutions. From these data taken together, we propose that the penta-peptide Pro_{295}-Ile-Trp-Thr-Arg_{299} of hHsp90 functions as an immunodominant epitope common to all eukaryotic Hsp90.

INTRODUCTION

Heat shock proteins (Hsps) exist essentially in all organisms. Reflecting their important and general roles as molecular chaperones, their amino acid sequences are highly conserved in every Hsp family even among eukaryotic and prokaryotic organisms. For instance, the amino acid sequences of Hsp60 (Venner et al 1990), Hsp70 (Hunt and Morimoto 1985), and Hsp90 (Hickey et al 1989; Rebbe et al 1987) of human cells are 48.2, 47.2, and 35.6% identical to their respective *E. coli* orthologs, GroEL (Hemmingsen et al 1988), DnaK (Bardwell and Craig 1984), and HtpG (Bardwell and Craig 1987).

The sequence homology of Hsps led to the hypothesis that the immune system could be triggered by bacterial antigens: Bacterial GroEL, for instance, shares a high degree of homology with host ortholog, which may result in an aberrant immune response and chronicity of inflammation (Ranney 1982; Tabeta et al 2000; Loesche et al 1985). However, it remains unknown whether or not the sequence homology of the Hsp family proteins is sufficient for all members of the family to share the same immunogenicity.

Hsp90 is composed of three domains, i.e. N-terminal, middle and C-terminal domains. The N-terminal domain (amino acids 1 – 220/230) carries an ATP/geldanamycin-binding site (Prodormou et al 1997a; Stebbins et al 1997). The N-terminal domain of human (Stebbins et al 1997) and yeast Hsp90 (Prodromou et al 1997a) and the middle domain of yeast Hsp90 (Meyer et al 2003) have been reported. Based on the results of crystallographic studies and the similarity of the structure, a molecular clamp model on the client binding has been proposed (Prodromou et al 1997b; Prodromou et al 2000; Meyer et al 2003). The C-terminal domain (c.a. 200 residues) is responsible for the dimerization (Minami et al 1994; Nemoto et al 1995). The three-dimensional structure of the C-terminal domain of Hsp90 remains unknown. Only the C-terminal half (amino acids 511-624) of the C-terminal domain of HtpG, a

bacterial ortholog of mammalian Hsp90, has been reported (Harris et al 2004). Recent studies indicate that the middle domain carries the client binding site (Meyer et al 2003), although an additional binding site might be present in the C-terminal domain (Young et al 1997: Scheibel et al 1998; Minami et al 2001; Harris et al 2004).

We previously produced 33 monoclonal antibodies (mAbs) against 2 isoforms of human Hsp90, *i.e.,* Hsp90α (hHsp90α) and Hsp90β (hHsp90β) (Nemoto et al 1997). Epitope mapping analysis by use of an enzyme-linked immunosorbent assay (ELISA) with deletion mutants and an octa-peptide multipin library demonstrated the existence of 4 highly immunogenic sites, designated sites Ia (amino acids 247-257); Ib (amino acids 263-270); Ic (amino acids 291-304); and II (amino acids 702-716). Sites Ia and Ib were localized within the highly charged region (amino acids 223-289) of hHsp90α; site Ic, C-terminally adjacent to the charged region; and site II, near the C-terminus. The amino acid sequences constituting sites Ia, Ib, and II vary between hHsp90α and hHsp90β. The highly charged region and the C-terminal one are dispensable for the growth of budding yeast (Borkovich et al 1989) and deleted in HtpG, the bacterial ortholog. In contrast, the amino acid sequence of site Ic is highly conserved in eukaryotic Hsp90, and is relatively conserved in all organisms (see Table 8).

We recently compared the immunogenic property of the Hsp90 molecular chaperone with polyclonal (pAbs) raised against various species of Hsp90-family member and monoclonal antibodies (mAbs) raised against human Hsp90 (hHsp90) (Kawano et al 2004). Among 33 mAbs developed against hHsp90α and hHsp90β, several mAbs that strongly reacted with yeast Hsc82 (yHsc82) recognized Asn_{291}-Ile_{304}, a conserved region of the family protein. The polyclonal antibody raised bacterial ortholog HtpG did not cross-react with hHsp90α and hHsp90β, human Grp94 (hGrp94) and human Trap1 (hTrap1), and *vice versa*. Hence, although mammalian Hsp90 shares some immunological reactivity with yHsc82, it is considerably distinct from its bacterial

ortholog, HtpG. from *Escherichia coli* (EcHtpG) and *Porphyromonas gingivalis* (PgHtpG) (Kawano et al 2004).

In the present study, we further characterized the epitopes of the anti-Hsp90 mAbs by a panning technique that selects the 12-amino acid sequences specifically recognized by the anti-Hsp90 mAbs. We found that 2 mAbs that recognized site Ic reacted with yHsc82 and all eukaryotic Hsp90s examined.

MATERIALS AND METHODS

Materials

The materials used and their sources were the following: Expression vector pQE9 and plasmid pREP4, from Qiagen Inc. (Chatsworth, CA, USA); expression vector pTrcHis TOPO and TOP10 competent cells, from Invitrogen (Carlsbad, CA, USA); CNBr-activated Sepharose 4B and low-molecular-weight markers from Amersham Biosciences (Piscataway, NJ, USA); Wizard genomic DNA purification kit, from Promega Corp. (Madison, WI, USA); kaleidoscope prestained 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); anti-Hsp90 mAb AC88/SPA-830 from Stressgen Biotech Corp. (Victoria, B.C., Canada); 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 form of hHsp90α, hHsp90β, hGrp94, EcHtpG, and PgHtpG, and plasmids carrying the N domain of EcHtpG (residues 1-336, designated EcHtpG-N) and hHsp90α (residues 1-400, hHsp90α-N) tagged with a histidine hexamer encoded by pQE9 were described previously (Kawano et al 2004; Nemoto et al 1996; Roi 1998; Nemoto et al 2001). Y1090[pREP4] was transformed with the plasmids and transformants were selected on Luria broth agar plates containing 50 μg/ml of ampicillin and 25 μg/ml of kanamycin.

Plasmids encoding full-length forms of yHsc82 (Borkovich et al 1989) and hTrap1

(Chen et al 1996; Song et al 1995) 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. For construction of yHsc82 and hTrap1, DNA fragments encoding the full-length forms were amplified by PCR, and then directly inserted into the pTrcHis TOPO expression vector in frame according to the manufacturer's recommendation. TOP10 cells were transformed with the plasmids and 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). The constructed plasmids were designated pTrcHis-yHsc82 and pTrcHis-hTrap1, respectively.

In vitro mutagenesis

Nucleotides ATC encoding Ile_{296}, TGG encoding Trp_{297}, ACC encoding Thr_{298}, and AGA encoding Arg_{299} of hHsp90α-N were substituted with CTC encoding Leu_{296}, GGG encoding Gly_{297}, ATG encoding Met_{298}, and GGA encoding Gly_{299}, respectively, by PCR-based site-directed mutagenesis in combination with *Dpn*I cleavage of a template. pQE9-hHsp90α-N (residues 1-400) was this template. As a result, pQE9-hHsp90α-N Trp297Gly, Thr298Met, Arg299Gly, and a double mutant Ile296Leu/Thr298Met were constructed. Mutagenesis was confirmed by DNA sequencing.

Expression and purification of recombinant proteins

After overnight cultivation of 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

with a Talon affinity column according to the manufacturer's protocol except that 10 mM imidazole and 10 μg/ml of leupeptin were added to the lysis buffer [20 mM Tris-HCl, pH 8.0, containing 0.1 M NaCl and 0.5 mg/ml lysozyme]. After extensive washing with washing buffer [20 mM Tris-HCl, pH 8.0, 0.1 M NaCl containing 10 mM imidazole] to remove non-adsorbed proteins, the bound proteins were eluted with 0.1 M imidazole (pH 8.0) containing 10% (v/v) glycerol. Purified proteins were used immediately or stored at -80^{o}C until used.

Production of polyclonal antibody (pAb)

Affinity-purified recombinant EcHtpG-N (amino acids 1-336) was further purified by electrophoresis on a 7.5% polyacrylamide gel of 0.5-cm thickness under nondenaturing conditions (Nemoto et al 1995). A separated protein was excised following visualization with a Gelcode silver snap kit (Pierce, Rockford, IL, USA). A piece of a polyacrylamide gel containing the recombinant protein was homogenized, and then injected into rabbits (Japan White) 5 times at 3-week intervals. The immunogen (approximately 1 mg) emulsioned with Freund's complete adjuvant was used for primary immunization, and subsequent booster injections were given with it in Freund's incomplete adjuvant. Polyclonal antibodies were purified by use of EcHtpG-N-conjugated CNBr-activated Sepharose (Amersham Biosciences) prepared according to the manufacturer's recommendation. Antisera and pH-neutralized, purified pAb were divided into small aliquots and stored in the presence of 0.03% NaN_3 at –80^{o}C. 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.

Anti-Hsp90 mAbs

K3700 and K41000 number series represent the mAbs produced from hybridoma cells of mice immunized with hHsp90β and hHsp90α, respectively (Nemoto et al 1997). By setting 1% as the threshold for the binding affinity to one hHsp90 isoform, we classified 10 (Fig. 1, indicated by bold letters) and 3 mAbs (indicated by bold italic letters) to be specific for hHsp90α and hHsp90β, respectively; and 19 mAbs reactive with both Hsp90 isoforms (indicated by non-bold letters). The mAb K3725A remained to be characterized because of its unusual behavior. The recognition sites of the 33 mAbs were described previously, which comprised 4 highly immunogenic sites, designated sites Ia, Ib, Ic, and II (Fig. 1 and Nemoto et al 1997).

Enzyme-linked immunosorbent assay (ELISA)

The binding affinity of the antibodies for the Hsp90-family proteins was determined by ELISA. Purified proteins (1 μg) coated on each well of a Maxisorp C96 titer plate (Nalge Nunc Int., Rochester, NY, USA) were incubated with various concentrations of purified mAbs in 0.1 ml of the blocking buffer [0.17 M H_3BO_4, pH 8.5, containing 0.12 M NaCl, 0.05% (v/v) Tween20, 1mM EDTA, and 0.05% (w/v) NaN_3 and 0.25% (w/v) bovine serum albumin] for 2 h at room temperature as reported previously (Hornbeck et al 1994). Following a 1-h incubation with the anti-mouse Ig(G+A+M) conjugated to alkaline phosphatase (1/4000 dilution) and washing, the alkaline-phosphatase activity was determined by measuring the absorbance at 655 nm after a 1-h incubation with Bluephos microwell phosphatase substrate (Kirkegaard and Perry Lab., Gaitherburg, MD, USA) at 30^{o}C.

Panning of the FliTrix peptide library

Amino acid sequences recognized by the antibodies were determined by using the FliTrix peptide library system (Invitrogen) according to the manufacturer's recommendation. Briefly, an aliquot of the amplified FliTrix peptide library ($2x10^{10}$ cfu) carrying randomized 12-amino acid sequences was screened by panning with anti-hHsp90 mAbs (20 μg/ml in 6-cm dish, Nalge Nunc Int., Roskilde, Denmark) or affinity-purified anti-EcHtpG-N pAb (40 μg/ml). Following 5 cycles of panning, selected bacteria were plated on RMG plates containing 0.1 mg/ml ampicillin. After induction of protein expression of the bacteria in IMC medium containing 0.1 mg/ml tryptophan for 6 h at 37°C, positive clones were selected by immunoblotting of bacterial lysates with respective antibodies used for panning. Finally, the 12-amino acid sequences of the clones that were reactive with the antibodies were deduced by DNA sequencing.

Extraction of Hsp90-family proteins from tissues and cells

Tissue extracts of male Wistar rat liver and leaves of rice *Oryza sativa* var. Taichung65 at seedling stage, an extract of *Paramecium caudatum* strain RB1 prepared as reported previously (Hirabayashi et al 1983), and cell pellets of budding yeast *Saccharomyces cerevisiae* W303-1A and fission yeast *Schizosaccharomyces pombe* $972h^-$ were each mixed with 3 volumes of SDS-sample buffer containing 8 M urea. After boiling for 5 min, aliquots (10 μl) were subjected to immunoblotting.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

Electrophoresis was performed in the presence of 0.1% SDS at a polyacrylamide concentration of 11% or 12.5%. Generally, 0.25-0.5 μg of purified proteins or 5 μl of lysates was loaded onto an SDS-PAGE gel. Separated proteins were stained with Coomassie brilliant blue or subjected to immunoblotting as reported previously (Nemoto et al 1997). Low-molecular-weight markers (Amersham Biosciences) and kaleidoscope prestained standards (Bio-Rad) were used as references for proteins stained with Coomassie brilliant blue and visualized by immunoblotting.

Immunoblotting analysis

Purified proteins or bacterial lysates containing recombinant proteins separated on SDS-PAGE gels were subjected to immunoblotting analysis as described previously (Nemoto et al 1997). Tissue and cellular extracts of rat liver, budding yeast, fission yeast, rice and *Paramecium* were prepared as reported previously (Hirabayashi et al 1983). First antibodies were used at 1-2 μg/ml for blotting of Hsp90 and at 4-8 μg/ml for panning clones in 0.1 M Tris-HCl, pH 7.5, containing 0.15 M NaCl, 0.05 % (v/v) each of Triton X100 and Tween20 (TBS-TX). Alkaline phosphatase-conjugated rabbit anti-mouse Ig(G+A+M) IgG was used as the second antibody at 0.2 μg/ml in 10 ml TBS-TX. Blots were finally visualized by incubation with 5-bromo-4-chloro-3-indolyl phosphate and nitro blue tetrazolium (Nakarai, Kyoto, Japan) at room temperature for 5-20 min.

Protein concentration

Protein concentrations were determined by the bicinchoninic acid method using bovine serum albumin as a standard (Pierce, Rockford, IL, USA).

RESULTS

Reactivity of anti-hHsp90 mAbs with the members of the Hsp90 family

Recently, the specificity of the anti-Hsp90 mAbs, which we developed against either hHsp90α or hHsp90β, has been characterized systematically with 7 recombinant Hsp90-family proteins, i.e., hHsp90α, hHsp90β, hGrp94, hTrap1, yHsc82, EcHtpG, and PgHtpG (Kawano et al 2004). Based on the binding profiles, we classified the 19 mAbs that could react with both of hHsp90α and hHsp90β into 4 categories: 4 mAbs that could blot hHsp90α, hHsp90β, and yHsc82, apparently with similar intensities; 5 mAbs that reacted with hHsp90s intensively and yHsc82 with a lesser intensity; 9 mAbs that reacted with hHsp90α and hHsp90β, but scarcely blotted other members; and finally, K3729, which could react with hGrp94 as well as with hHsp90α and hHsp90β (Kawano et al 2004).

Among the 9 mAbs that could blot yHsc82, 5 of them recognized site Ic and all of the 4 mAbs that blotted yHsc82 and hHsp90 with similar intensities recognized site Ic (mAbs with underlines in Fig. 1). To determine their binding affinities quantitatively, we performed ELISA with purified proteins as antigens (Fig. 2a). The bindings of K41110 and K41116C to yHsc82 were identical to those for hHsp90s (Fig. 2a). The binding affinities of K41122B and K41220 for yHsc82 were 1/30 and 1/3, respectively, of those for hHsp90s. Thus, the 8 anti-site-Ic mAbs were tentatively classified into 3 groups with respect to their binding to yHsc82: Group 1, 2 mAbs (K41110 and K41116C) bound to yHsc82 with the affinity identical to that for hHsp90s; Group 2, 3 mAbs (K41122B, K41220, and K41322) bound to yHsc82 with affinity lower than that for hHsp90s; and Group 3, 3 mAbs (K3720, K41331, and K41338) that did not bind to yHsc82. On the other hand, there were 4 mAbs (K3738, K41315, K41016, and

K41002) that could interact with yHsc82 and recognized the sites distinct from site Ic (Fig. 1). They recognized the sites distinct to each other, and therefore, they were not further characterized.

Considering the sequence similarity in site Ic between yHsc82 and hHsp90 (see Table 8), the reactivity of the anti-site Ic mAbs to yHsc82 was not surprising. Inversely, it is curious that the 8 anti-site-Ic mAbs bore a variety of reactivity toward yHsc82 from the potent binding of Group-1 mAbs to no binding of Group-3 mAbs. We will further investigate the reason for this later.

We also performed ELISA with K3729, which was the mAb solely reacted with hGrp94. It preferentially bound to hHsp90β over hHsp90α (Fig. 2b). Its binding affinity for hGrp94 was one-third of that for hHsp90β, but was still 3-fold higher than that for hHsp90α. Consistent with the results of the immunoblotting, K3729 did not react with other Hsp90-family members (Fig. 2b).

Essential amino acids constituting site Ia

We performed panning experiments using the FliTrix peptide library and anti-hHsp90 mAbs. We subjected 30 mAbs to the panning(indicated by marks attached to the names of mAbs in Fig 1). As a result, amino acids important for recognition were determined for 14 mAbs (indicated by asterisks); immunologically positive clones were not obtained for 9 mAbs (indicated by "-"); and immunologically positive clones were obtained for 7 mAbs, but they did not possess consensus sequences (indicated by "±") (see Tables 1, 2, 4-7). The epitope of 1 mAb (K41007) determined by use of a phage display system was reported previously (Nemoto et al 1997).

We first determined the amino acids essential for the immunogenicity of the anti-site Ia mAbs, i.e., K41102, K41116A, K41122A, and K41320 (Table 1). Because

K4000-series mAbs were derived from mice immunized with hHsp90α, the epitope sequences were homologous to the sequence of hHsp90α. In particular, Glu_{251}-Ser-X-Asp_{254} appeared to be important for the recognition. Even if amino acids were substituted: Glu_{251} was replaced by charged amino acids (Arg with K41102, #1 and Asp with K41102, #3). Ser_{252} was replaced by Gln in one clone (K41320, #2). Asp_{254} was replaced by amino acids with similar properties, *i.e.,* charged amino acid (Arg) and Asn.

Essential amino acids constituting site Ib

Five mAbs, K41241, K41315, K41020, K41107, and K41233 recognized the immunogenic site Ib, Ser_{263}-Asp-Glu-Glu-Glu-Glu-Lys-Lys_{270} (Nemoto et al 1997). The panning with K41020, K41107, and K41233 produced positive clones on immunoblotting, but amino acid sequences were not homologous to each other or to the sequence of site Ib (data not shown). The panning with the mAbs K41241 and K41315 failed to result in positive clones on immunoblotting.

Essential amino acids constituting site Ic

The presence of 8 anti-site Ic mAbs categorized as having a variety of bindings to yHsc82 (Fig. 2a and data not shown) strongly indicated the differential requirement of amino acids for their bindings. In order to evaluate it, we performed the panning of the peptide library by use of the mAbs with two typical binding characteristics to site Ic, i.e., K41110 and K41116C (Group-1 mAbs), which bound to site Ic with high affinity; and K3720, K41331, and K41338 (Group 3), which did not bind to it. As a result, we obtained 17 clones that interacted with 5 anti-site-Ic mAbs (Table 2). Among them, 15

dodecapeptides were aligned along with the sequence of site Ic without ambiguity. One clone (K41110, #3) could be reasonably aligned if a deletion was introduced. The alignment of the clone (K41116C, #2) was less confirmative, because there was little homology. However, the alignment seems to be reasonable if consider the chemical characteristics of the 5 amino acids, i.e., Thr-Met-Trp-Ser-Gly (Table 2, K41116C, #2) to refer to Pro_{295}-Ile-Trp-Thr-Arg_{299} (site Ic), as discussed in the following paragraphs.

Table 2 shows that the region of site Ic defined in the previous study (Asn_{291}-Ile_{304}) should be narrowed into 5 amino acids, i.e., Pro_{295}-Arg_{299}. The frequencies of respective amino acids appearing in the positive clones are summarized in Table 3. Trp_{297} was the sole amino acid appearing in all clones. Thereafter, Pro_{295} (76%) and Arg_{299} (47 %) were observed; Ile_{296} was observed at 35% frequency; and finally, Thr_{298} at 24%. Among the selected clones, Pro_{295}, Trp_{297}, and Arg_{299} existed simultaneously in 5 clones (29%). The combination of Pro_{295} with Trp_{297} was found at 47% and that of Arg_{299} with Trp_{297} was 18%. Taken together, Trp_{297} with Pro_{295} and/or Arg_{299} was present at 94%.

Table 3 further indicates the amino acids permissible at positions of Ile_{296} and Thr_{298}. At the position of Thr_{298}, Ala or Ser with properties similar to those of Thr was occasionally found. Thus, the percentage when Thr, Ala, or Ser was located at the position was 76% (Table 3). Similarly, in 7 clones (35%), hydrophobic amino acids, such as Val (5 clones), Ala (1 clone), Trp (1 clone), and Met (1 clone), were found at position 296. Hence, the percentage when Ile or other hydrophobic amino acids were located at the position of Ile_{296} was 82%.

Finally, Table 3 highlights the difference in amino acid preference between Group-1 and Group-3 mAbs. The frequencies of 5 amino acids emerging in the clones selected with Group-3 mAbs were always higher than those selected with Group-1 mAbs. In particular, it was noticeable that Ile_{296} was observed only in one clone (14%) reactive

against Group-1 mAbs, and in 5 clones (50%) against Group-3 mAbs. Thr_{298} was found in 1 clone (14%) against Group-1 mAbs, and in 3 clones (30%) against Group-3 mAbs. In contrast, the frequencies of Arg_{299} and Pro_{295} were comparable between the clones selected with the 2-group mAbs (Table 3). These findings strongly suggest that Ile_{296} and Thr_{298} are closely related to the specificity of anti-site-Ic mAbs. In fact, among the 5 amino acids of site Ic, Ile_{296} is replaced by Leu in yHsc82 (see Table 8), which reasonably explains the specificity of the anti-site-Ic mAbs. So, the substitution of Asp_{302} of hHsp90 to Ser in yHsc82 was not related to the binding of anti-site Ic mAbs.

Essential amino acids constituting site II

We estimated amino acids important for the recognition of anti-site II mAbs. As shown in Table 4, K41007 recognized Ala_{705}-Asp-Asp-Thr-Ser_{709} of hHsp90α (Nemoto et al 1997); K3705, Asp_{693}-Glu-Val-Pro-Ala_{697} of hHsp90β; and K3725, Pro_{705}-Asp-Glu-Ile-Pro_{709} of hHsp90β. Reflecting amino acids of site II altered between hHsp90α and hHsp90β, the sequences recognized by the mAbs resembled either one or the other hHsp90 isoforms (Table 4). Noticeably, distinct segments within site II were recognized by the mAbs. Thus, we concluded that sites Ia and Ic each, formed a single epitope, whereas site II was composed of several epitopes.

Amino acids essential for K3729

We were interested in K3729, because it cross-reacted with hGrp94 (Fig. 2b). Its epitope had remained ambiguous, because it bound to 4 separate sites at residues 212-312 of hHsp90α, i.e., residues 227-238, 258-270, 282-291, and 301-310 [see Table

5, hHsp90α (1)-(4) and Ref *11*]. Hence, we performed panning with K3729.

Although there were 4 potential epitope sites for K3729, it was precisely defined from 1 clone (Table 5, #1), which carried the sequence identical to Glu_{222}-X-X-X-Ser-Asp-Asp-Glu-Ala-Glu_{231} (E_{222}XXXSDDEAE_{231}) of hHsp90β (equivalent to Asp_{227}-Glu_{236} of hHsp90α). Taking other clones into consideration, we propose that Ser_{226}-Asp-Asp-Glu_{229} is the most important, in which Asp_{227} and Glu_{229} are indispensable. Ser_{226} could be substituted by Thr, another amino acid harboring a hydroxyl group or charged amino acids, such as Asp and His. Noticeably, Glu_{222} and Ile_{225} of hHsp90β, which are the amino acids altered between the hHsp90 isoforms, were mimicked in clones #1 and #2, respectively. Taking the preferential binding of K3729 to hHsp90β in mind, these amino acids should additionally contribute to the binding of K3729.

This assignment gave an answer as to why K3729 bound to 4 separate regions of hHsp90α in an earlier study (Nemoto et al 1997). Table 5 indicated that one (residues 227-238) of the 4 peptides corresponded to the true epitope of the mAb [hHsp90α(1)] but that the remaining ones [hHsp90α(2)-(4)] possessed similar sequences, i.e., Ser_{231}-Asp-(Glu)-Glu_{234}, Asp_{286}-(Gln)-Glu_{288}, or Asp_{301}-Asp-(Ile_{303}). Because we used a multi-pin peptide library covering amino acids 212-312 derived from hHsp90α (Nemoto et al 1997), the binding to the hHsp90α peptide was weaker that that to hHsp90β. Under the conditions used, the bindings to these 3 non-epitope sites [hHsp90α(2)-(4)] were overestimated.

This assignment also solved the question of why K3729 was capable of interacting with hGrp94 with an affinity higher than that for hHsp90α. hGrp94 possesses a sequence similar to the epitope of hHsp90β, and moreover, the sequence carries Glu_{291}, which is equivalent to Glu_{222} of hHsp90β (Table 5, underlined). Note that Glu_{222} of hHsp90β was partly involved in the binding of K3729 (Table 5, #1) and was altered to

Asp_{227} in hHsp90α.

Amino acids essential for K41218

The recognition site of K41218, which had been proved to exist within amino acids 48-196 (Nemoto et al 1997), was precisely determined to be Asp_{71}-Ser-Gly-X-Glu-Leu_{76}, in which Gly_{73} and Glu_{75} seemed to be essential and Asp_{71} and Leu_{76} were important (Table 6). Additionally, Leu_{70} and Ile_{78} may contribute to the binding.

Amino acids essential for K41002

The recognition site of K41002, which had been proved to exist within amino acids 290-312 (Nemoto et al 1997), was determined. As shown in Table 7, Gly_{310}-Glu-Phe-X-Lys-Ser_{315} was recognized by K41002. Charged amino acids, such as Arg and Asp, could be located at the position of Lys_{314}. The region defined in the present study (residues 310-315) is slightly shifted from the previous one (residues 290-312). However, this is inevitable, because we had defined the region by use of various truncated forms and the mAb could interact with hHsp90α 290-312 (**Nemoto et al 1997**).

Effect of the substitution of amino acids constituting site Ic

In order to evaluate the roles of the respective amino acids constituting site Ic, we expressed hHsp90α-N with mutated amino acids. When compared with the sequences corresponding to site Ic of eukaryotic Hsp90, Ile_{296} is substituted to Leu in *Candida*

albicans (Swoboda et al 1995), yHsc82 (Borkovich et al 1989), and heat-inducible Hsp82 (Farrelly and Finkelstein 1984) in budding yeast *S. cerevisiae* (Table 8). In plant cytoplasmic Hsp90, Thr_{298} is substituted to Met in bread wheat *Triticum aestivum* (NCBI accession, X98582) or to Leu in rice *Oryza sativa (japonica cultivar group)* (AB111810). In *Tetrahymena thermophilia* (AF151114) and *Toxoplasma gondii* (A344115), a double substitution to Leu_{296} and Met_{298} occurs. In a plastid-specific form of Hsp90, Thr_{298} is replaced by Met in *Secale cereale* (Schmitz et al 1996) and the double substitution to Leu_{296} and Met_{298} occurs in *Arabidopsis thaliana* (Milioni and Hatzopoulos 1997). In *Paramecium tetraurelia* (Frankel et al 2001), the substitution occurs simultaneously at 3 positions (Leu_{296}, Met_{298}, and Lys_{299}). With these substitutions in mind, we expressed hHsp90α-N with the mutations of Trp297Gly, Thr298Met, Ile296Leu/Thr298Met, and Arg299Gly. The recombinant proteins were purified by affinity chromatography (Fig. 4a). hHsp90α Arg299Gly appeared to be partially degraded (lane 5).

We first analyzed the bindings of anti-site-Ic mAbs to the mutants at positions 296, 297, and 298. As a positive control, we evaluated the binding of K41102, which recognized site Ia (Table 1). The mAb equivalently bound to hHsp90α-N irrespective of the substitutions (Fig. 3b). In contrast, the bindings of the anti-site Ic mAbs were drastically altered by the substitutions: None of the 8 mAbs bound to Trp297Gly even at the highest antibody concentration used (Fig. 3c-e). This finding faithfully reflected the appearance of Trp_{297} in all clones selected by panning (Table 2). In contrast, the bindings for Thr298Met and Ile296Leu/Thr298Met were dependent on the mAbs: The bindings of Group-1 mAbs to Thr298Met were scarcely affected (Fig. 4c); but the binding affinities for Thr298Met of the remaining 6 anti-site-Ic mAbs except for K41338 were decreased at least to one-third of those for the wild type. An additional substitution of Ile_{296} to Leu further decreased the binding affinities for anti-site Ic mAbs,

but the effect was little for Group-1 mAbs (Fig. 3c). Similarly, the effect was relatively small on 2 Group-2 mAbs (K41220 and K41322; Fig. 3d), but 1 Group-2 mAb (K41122B) and 2 Group-3 mAbs (K3720 and K41331) showed a significant reduction (<1/300) in binding affinity. The remaining Group-1 mAb (K41338) did not bind to the double mutant under the conditions employed (Fig. 3e). Therefore, we concluded that the binding affinities of Group-1 mAbs for the double mutant Ile296Leu/Thr298Met were slightly decreased (1/3 – 1/2) but that those of the remaining 6 mAbs were significantly reduced at least to one-tenth. In particular, the substitution of Ile_{296} to Leu significantly reduced the binding affinities of Group-3 mAbs. With these data taken together, we conclude that Ile_{296} and Thr_{298} were important for Group-3 mAbs (with the exception of K41338 for Thr_{298}), but were not critical for the recognition of Group-1 mAbs.

We then evaluated the effect of the substitution of Arg_{299} to Gly. The change slightly and reduced 10-fold, respectively, the bindings of K41116C and K41110 (Fig. 5b), and caused a significant reduction (<1/30) in those of the remaining 6 anti-site-Ic mAbs (Fig. 3c and d): Especially, K3720 never bound to Arg299Gly even at the highest antibody concentration (Fig. 3c). Thus, although Arg_{299} was one of the important determinants of site Ic for most anti-site-Ic mAbs, the effect was little on the Group-1 mAbs. In contrast, the mutation did not affect the binding of mAbs that recognized the sites other than site Ic (data not shown).

We also performed immunoblotting analysis. Group-1 mAbs could blot Thr298Met, Ile296Leu/Thr298Met, and Arg299Gly, but scarcely blotted Trp297Gly (Fig. 3a), in consistent to the results of ELISA (Fig. 3b and c).

Although we did not perform the mutation of Pro_{295}, this amino acid appeared to be important for antibody recognition, because: (i) the frequency (76%) of Pro_{295} appearing in the selected clones was higher than the 47% of Arg_{299} (Table 3); and (ii) EcHtpG, in

which Pro_{295}-Ile-Trp-Thr-Arg_{299} of eukaryotic Hsp90 is converted to Ala-Leu-Trp-Thr-Arg (Table 8), was not recognized by any site-Ic mAb.

Immunogenicity of the region corresponding to site Ic of EcHtpG

Although the sequences corresponding to site Ic of hTrap1, EcHtpG, and PgHtpG were partially homologous to that of hHsp90 (Table 8), none of the anti-site-Ic mAbs bound to them (Fig. 2a and data not shown). In view of the findings obtained by mutagenesis (Figs. 3 and 4), either one of Pro_{295} or Arg_{299} replaced in these 3 proteins should explain this phenomenon: Pro_{295} is replaced by Ala in EcHtpG and hTrap1; and Arg_{299} is replaced by Lys in PgHtpG. Alteration of Ile_{296} to Leu in EcHtpG and to Ala in PgHtpG, and that of Thr_{298} to Met in hTrap1, may additionally contribute to the phenomenon. On the other hand, these facts raised a new question: Is the region of other Hsp90-family members equivalent to site Ic of hHsp90 immunogenic? or is the triplet of Pro_{295}, Trp_{297}, and Arg_{299} essential for this role? To address this issue, we chose EcHtpG, which carries Ala at the position of Pro_{295} (Table 9). We performed panning of the peptide library with the pAb raised and purified with EcHtpG-N.

After 5 cycles of panning, all clones were positive on immunoblotting with the pAb. Then, we randomly selected 7 clones and sequenced the oligonucleotides of 36 bases. Among 7 clones, 5 clones (Table 9, #1-5) possessed 2 or 3 amino acids matching the site-Ic sequence of EcHtpG. Three clones (#2, #4, and #5) could be aligned in 2 ways. Interestingly, at the position of Arg_{236} of EcHtpG, equivalent to Arg_{299} of hHsp90α, charged amino acids (Lys and Glu) were located in 3 clones [#1, #2(1), and #5(2)]. On the other hand, the amino acids of both #6 and #7 were not similar to the site-Ic sequence, but they appeared to be partially homologous to a single site, Ile_{36}-Leu_{46} (Table 9). So, we conclude that the site of EcHtpG equivalent to site Ic of hHsp90α is

also immunogenic even though the sequence carries Ala at the position of Pro_{295}. In the 7 selected clones, we did not detect Trp at position 297. We do not know the reason for this at present.

Immunoblotting analysis of Hsp90 endogenously expressed in various species

The findings in Figs. 2b and 3c prompted us to examine the reactivity of Group-1 mAbs to endogenous forms of eukaryotic Hsp90-family members (Table 8, lower 4 species). K41110 and K41116C specifically recognized 80-90-kDa species from rat liver, budding yeast (*S. cerevisiae*), fission yeast (*Schizosaccharomyces pombe*) (Aligue et al 1994), rice (*Oryza sativa,* AB111810*),* and *Paramecium caudatum* (Fig. 5). To the contrary, K41220, which belonged to Group 2, did not bind to *P. caudatum* Hsp90 (lane 5) and scarcely bound to rice Hsp90 (lane 4). We also tested an anti-Hsp90 mAb AC88, which has been reported to react with Hsp90 from various species (Riehl et al 1985). Although it was developed against eukaryotic filamentous fungi *Achlya ambisexualis*, it efficiently recognized rat Hsp90 as previously reported (Chen et al 1997). It blotted *P. caudatum* Hsp90 as a faint band, and did not detect Hsp90 from other species (Fig. 5).

DISCUSSION

Recently we characterized the immunological cross-reactivity of the members of the Hsp90 family with a number of anti-hHsp90 mAbs. In combination with ELISA in the present study, we concluded that 2 mAbs reacted with yHsc82 with affinity identical to that for hHsp90, and 7 mAbs reacted with yHsc82 with affinities lower that that for hHsp90. Also, one reacted with hGrp94 with a moderate affinity. In the present study, we identified essential amino acids constituting various epitopes of hHsp90α and hHsp90β. In particular, 3 of the 4 highly immunogenic sites reported previously were further characterized in this study. As a result, we demonstrated that sites Ia and Ic each formed a single epitope, but showed that site II was composed of several epitopes. We could not define the amino acids essential for site Ib.

Among 11 amino acids constituting site Ia reported previously (Nemoto et al 1997), Glu_{251}-Ser-X-Asp_{254} of hHsp90α was most important for the recognition by the mAbs (Table 1). On the other hand, among 14 amino acids (Asn_{291}-Ile_{304}) of site Ic (Nemoto et al 1997), only 5 residues from Pro_{295} to Arg_{299} were required for the antibody recognition. The observations in this study indicate that the immunogenicity of Glu_{222}-Ala_{230} in hHsp90β is shared by Glu_{281}-Ala_{289} of hGrp94 and that that of site Ic in hHsp90 is shared by yHsc82.

The finding that hGrp94 shared immunogenicity with hHsp90β at the epitope for K3729 (Table 5) suggests the usefulness of this mAb for the detection of hGrp94. However, usefulness of K3729 for detection of Grp94 may be limited to mammals, because the sequence is conserved in mouse (Mazzarella and Green 1987), bovine (AB025193), pig (de Crom et al 1999) Grp94, but is altered in chick (Kulomaa et al 1986), barley (Walther-Larsen et al 1993), and *Arabidodopsis thaliana* (Ishiguro et al 2002).

Among 9 mAbs that reacted with yHsc82, 5 mAbs recognized site Ic. This may not be surprising in consideration of the sequence homology of site Ic (Table 8). However, it seems important to note that, among 8 anti-site-Ic mAbs, there were 3 anti-site-Ic mAbs that did not react with yHsc82. Among the 5 amino acids constituting the epitope of site Ic, Trp_{297} was essential; and Pro_{295} also appeared to be essential. The frequency of Arg_{299} appeared in the panning clones followed that of Pro_{295}: Arg_{299} was important for most site-Ic mAbs, but not essential for the immunoreactivity, because the mutant with Arg299Gly possessed potent reactivity toward 7 of the 8 anti-site-Ic mAbs (Fig. 4). Moreover, K41116C showed the binding to Arg299Gly comparable to that for an intact form and K41110 had considerable binding ability.

The frequencies of Ile_{296} and Thr_{298} that emerged in the panning clones were lower than that frequency of Arg_{299}. In fact, these 2 amino acids were less critical than Pro_{295}, Trp_{297}, and Arg_{299} were for the 2 Group-1 mAbs. It was not surprising that Group-1 mAbs did not require Ile_{296}, because we classified anti-site-Ic mAbs based on the reactivity to yHsc82, which carries Leu at position 296. However, it was noticeable that the 2 mAbs tolerated the substitution of Thr_{298} to Met.

The 5 amino acids of site Ic are completely conserved in most eukaryotic Hsp90s, such as those of mammals (Hickey et al 1989; Rebbe et al 1987; Moore et al 1989; Huang et al 1999), chick (Binart et al 1989; Meng et al 1993), fission yeast *S. pombe* (Aligue et al 1994), nematoda *Caenorhabditis elegans* (Massie et al 2003), and *Drosophila melanogaster* (Helmgren et al 1981). Moreover, Pro_{295} and Trp_{297} are completely conserved and Arg_{299} is highly conserved in eukaryotic Hsp90s.

The *in vitro* mutagenesis experiment combined with the immunoblotting analysis and ELISA demonstrated that K41110 and K41116C could interact with Pro_{295}-Ile(Leu)$_{296}$-Trp_{297}-Thr(Met)$_{298}$-Arg(Gly)$_{299}$. Rice Hsp90, which was recognized by the mAbs (Fig. 5), carries Leu instead of Thr_{298}, which further indicates that Leu at

position 298 could be blotted with the mAbs. Arg_{299} was also one of the important determinants for most of the anti-site-Ic mAbs. However, one of the Group-1 mAbs (K41116C) did not discriminate Arg299Gly from hHsp90α-N (wild type); and another (K41110) also bound to Arg299Gly, although the binding affinity was decreased to one-tenth. Actually, they could blot *P. caudatum* Hsp90 (Fig. 5), which potentially possesses Arg_{299}, as does *P. tetraurelia* Hsp90. Therefore, we conclude that anti-site Ic mAbs that belong to Group 1 recognize all eukaryotic Hsp90s.

The sequences of site Ic of hGrp94, hTrap1, EcHtpG, and PgHtpG are partially homologous to that sequence of hHsp90 (Table 7), but none of them were blotted with anti-site-Ic mAbs (Fig. 2b). Nevertheless, the binding affinities for the 4 recombinant proteins could be estimated by ELISA at higher antibody concentrations (Fig. 2a). PgHtpG could be partially recognized by Group-1 mAbs and K41122B (Fig. 2b). In contrast, hGrp94 was never recognized by Group-1 mAbs and was second least efficiently recognized by K41122B next to hTrap1 (Fig. 2b). The site-Ic sequence of PgHtpG is Pro-Ala-Trp-Thr-Lys, of which Ile_{296} and Arg_{299} are substituted to Ala and Lys, respectively. Thus, the change of Arg_{299} to Lys may primarily cause the loss of the binding. However, as we demonstrated in Figs. 5 and 6, the change of Arg_{299} had little effect on Group-1 mAbs, especially on K41116C. In view of this finding, we postulate that the second substitution of Ile_{296} to Ala additionally abrogated the binding. It may be not surprising that the change of Ile_{296} to Ala caused a more profound effect on the antibody binding than the change of Ile_{296} with respect to Leu on Group-1 mAbs.

Among the 7 recombinant proteins, hGrp94 was least efficiently bound by Group-1 mAbs on ELISA (Fig. 2b). However, the Pro_{295}, Trp_{297}, and Arg_{299} triplet and Ile_{296} as well is maintained in hGrp94 (Table 8). What does this apparent discrepancy imply? The sole amino acid substitution from Thr_{298} to Gln should be responsible for this phenomenon, for Gln located at the position of Thr_{298} prevented the antibody binding in

a dominant-negative manner. In order to address this issue, we compared the amino acids located at the position of Thr_{298}. Thr is located at position 298 in EcHtpG and PgHtpG identical to hHsp90α; and Met is present in hTrap1 (Table 8). In the panning experiment, Thr (4 clones), Ala (6 clones), and Ser (3 clones) were occasionally recovered; and each of Met, Leu, Val, and Asp, respectively, emerged in 1 clone (Table 2), which strongly suggests that Met and Leu were permitted to be located at this position but that Gln_{298} sterically interfered with the antibody binding. If this is true, inversely, the antibody raised against site Ic of hGrp94 would be expected to interact with hHsp90. In fact, we found that antibodies raised against site Ic of hGrp94 could interact with hHsp90s, although the affinity was decreased to one-tenth (Kawano et al 2004). This discussion concerning Ala at the position of Ile_{296} and Gln at the position Thr_{298} implies that Ile_{296} and Thr_{298} are also important for the recognition by Group-1 mAbs as well as by Group-3 mAbs, but that, principally, Group-1 mAbs less stringently recognize Ile_{296}, Thr_{298}, and Arg_{300} for their recognition than do Group-3 mAbs.

We propose that K41110 and K41116C can be used as potent tools for detecting all eukaryotic Hsp90. Historically, AC88/SPA830 has been used for the analysis of Hsp90, because it was the first-developed anti-Hsp90 mAb and interacts with Hsp90 from various species with the 2 isoforms of mammalian Hsp90 (Riehl et al 1985). However, its reactivity was limited in some groups of eukaryotes (Fig. 5 and Kawano et al 2004). Thus, although the contribution of AC88 in the previous studies should be admitted, K41110 and K41116C should be superior to AC88 for detection of eukaryotic Hsp90. Interestingly, Grp94 of some species, such as trypanozoma *Leishmania infantum* (Larreta et al 2000) and sea urchin *Strongylocentrotus purpuratus* (Smith et al 1996), possesses the sequence Pro-Ile-Trp-Thr-Arg identical to site Ic of hHsp90. Hence, Grp94 from these species could be recognized by all anti-site-Ic mAbs.

It was reported that antibodies against Hsp70 and Hsp90 were produced in patients

with lupus erythematosus (Minota et al 1988; Twomey et al 1993). It is important to evaluate whether HtpG of *P. gingivalis* as well as that of *Actinobacillus actinomycetemcomitans*, another implicated pathogen, is truly involved in the autoimmune responses in those patients. However, we demonstrated that, on immunoblotting analysis, 19 anti-hHsp90 mAbs tested could not recognize PgHtpG or EcHtpG even when they were anti-site Ic. Therefore, we suppose that that, even if antibodies against hHsp90 are produced in periodontitis, the antigen is unlikely to be derived from bacteria but rather is hHsp90 of host cells.

As clearly demonstrated in the present study, panning method of FliTrx peptide library is a powerful tool for determining the epitopes of mAbs. Moreover, this study further demonstrated that pAbs can be used for this technique. Serially truncated proteins and peptides produced by recombinant techniques, which are conventionally used for the identification of epitopes, could be substituted by this approach.

In addition to sites Ia and Ic, we defined essential amino acids of several anti-Hsp90 mAbs: K3705 recognized Asp_{693}-Ala_{697} of hHsp90β (Table 4); K3725B recognized Pro_{705}-Pro_{709} of hHsp90β (Table 4); K3729 primarily recognized Ser_{226}-Glu_{231} of hHsp90β (Table 5); K41218 recognized Asp_{71}-Leu_{76} (Table 6); and K41002 recognized Gly_{310}-Ser_{315} (Table 7). The information on the epitopes of these mAbs is useful for future investigation on the chaperone cycle of Hsp90.

The 3-dimensional structures of the N-terminal ATP-binding 220/230 amino acids of hHsp90 and yHsp82 and the middle domain (residues 273-528) of yHsp82 equivalent to residues 293-549 of hHsp90α have been determined (Stebbins et al 1997; Prodromou et al 1997a; Meyer et al 2003), which indicates that the 5 amino acids of site Ic form a loop exposed outside the domain. Moreover, we found that anti-site Ic mAbs (K41116C and K41220) could adsorb glucocorticoid receptor-Hsp90 complex from rat liver (J. Kishimoto and T. K. Nemoto, unpublished observation), which further

indicated that site Ic is exposed to outside of an Hsp90 molecule, and moreover, that site Ic is not directly involved in client binding.

We also mapped the epitope of K41002 (amino acids Gly_{310}-Ser_{315}) on the structure. The site formed an α-helix adjacent to site Ic; and the most important amino segment, Gly_{310}-Glu-Phe_{312}, was located to the outside of the domain (Prodromou et al 1997a). Similarly, the epitope of K41218 was mapped at the surface of the N-terminal domain, forming a loop connecting an α-helix and a β-strand structure (Stebbins et al 1997; Prodromou et al 1997a). Other epitopes defined in the present study may also be located on the surface of the Hsp90 molecule; however, they could not be mapped at present, because they were located in either the highly charged region or in the C-terminal region, the 3-dimensional structures of which remain unknown. Although the dimer-forming, C-terminal domain of HtpG of *E. coli* has been reported recently (Harris et al 2004), the C-terminal end of Hsp90 (amino acids 697-732), which carries immunogenic site II, can not be pictured yet, because the corresponding region is deleted in HtpG.

We previously reported that, even if residues 289-389 and its adjacent C-terminal region, residues 401-546 of hHsp90 were separately expressed, they formed a stable complex *in vitro* and *in vivo* (Tanaka et al 2001; Matsumoto et al 2002). With reference to the 3-dimensional structure (Meyer et al 2003), the interaction appears to be primarily maintained through the association of the 5 β-sheets strands (amino acids 324-392 in hHsp90α) and the longest α-helix structure (amino acids 410-428). We further propose as the activation mechanism of Hsp90 molecular chaperone that heat stress induces the liberation of the oligomerization/client-binding site of residues 311-350 by disrupting the intra-molecular interaction between residues 289-389 and 401-546 (Tanaka et al 2001; Matsumoto et al 2002). In fact, there are several lines of evidence that amino acids 289-389 are involved in client binding: A binding site of

hHsp90β for Ser/Thr kinase Akt/PKB was mapped to amino acids 327-340, equivalent to 335-348 of hHsp90α (Sato et al 2000); It has been proposed that amino acids 347-360 form a potential client-binding site (Meyer et al 2003). We recently mapped amino acids 311-350 as an essential region for self oligomerization/client binding (Nemoto et al 2004). Moreover, a similar β-sheet platform is involved in client binding of DnaK (Zhu et al 1996) and MHC I molecule (Fremont et al 1995). Therefore, it is interesting to investigate whether or not the accessibility of the antibodies to the 5 β-sheet strands as well as to the α-helix structure is modified coupled with the heat-induced activation process of the Hsp90 molecular chaperone. Such an investigation is now under way in our laboratory.

Previous studies demonstrated that the presence of antoantibodies to Hsp90 in systemic lupus erythematosus (Minota et al 1988). Although Hsp90 is a cytosolic protein, surface expression of Hsp90 on peripheral blood mononuclear cells was found in a quarter of the patients with systemic lupus erythematosus during active state (Erkeller-Yuksel et at 1992). The level of Hsp90 has been shown to be elevated in peripheral blood mononuclear cells of a subset of systemic lupus erythematosus patients, mainly caused by transcription activation of Hsp90β gene (Twomey et al 1993). Kenderov et al (2002) proposed that immunopathogenesis of lupus nephritis is associated with Hsp90 as an autoantigen and that the pathology is associated with altered idiotypic regulation of the anti-Hsp90 IgG autoantibodies. On the other hand, it remains unknown whether or not there are epitopes of Hsp90 specifically involved in autoimmune responses. Thus, it seems important to determine to the epitopes of Hsp90, such as those defined in the present study, are involved in responses of autoimmune diseases.

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FIGURE LEGENDS

Fig. 1. Epitope map of the mAbs developed against hHsp90α or hHsp90β - Recognition sites of 33 anti-hHsp90 mAbs are represented along with the 732 amino acid hHsp90α. N, M, and C represent N-terminal, middle, and C-terminal domains, respectively (Nemoto et al 1997). The hatched box (residues 223-289) represents highly charged region localized in the N-terminal domain. Isoform specificity of mAbs except for K3725A is indicated by their letters as follows: bold, hHsp90α specific; not modified, hHsp90α preferential; bold italic, hHsp90β specific; italic, hHsp90β preferential; and underlined, bound to both isoforms equivalently. The recognition sites of mAbs were identified as epitopes (indicated by boxes) or regions carrying the epitopes (indicated by bars). Highly immunogenic regions, sites Ia, Ib, Ic, and II correspond to residues 247-257, 263-270, 291-304, and 702-716, respectively. Thirty mAbs were tested for panning as described in "**MATERIALS AND METHODS**": Amino acids responsible for the recognition were identified for 14 mAbs (indicated by *); Nine mAbs did not yield positive clones on immunoblotting (-); Seven mAbs yielded clones positive on immunoblotting, but the sequences they recognized either resembled each other nor matched the sequence of hHsp90s (±). Two mAbs without marks were not subjected to the panning. [a]precisely defined by the panning experiment in this study (see Tables 5-8). [b]determined by phage display system in a previous study (Nemoto et al 1997 and see Table 4).

Fig. 2. ELISA of mAbs that could interact with yHsc82 or hGrp94 - (a) Bindings of the 4 mAbs that could blot yHsc82 as intensively-stained bands were quantified by ELISA. (b) The binding of K3729 was quantified by ELISA. Symbols for the 7 Hsp90-family proteins are the following: hHsp90α (open circle); hHsp90β (closed circle); hGrp94

(open square); hTrap1 (closed square); yHsc82 (open triangle); EcHtpG (closed triangle); and PgHtpG (closed inverted triangle).

Fig. 3. Effect of mutation at Ile_{296}, Trp_{297}, Thr_{298}, and Arg_{299} on the antibody binding – (a) Aliquots of purified hHsp90α-N wt (lane 1), Trp297Gly (lane 2), Thr298Met (lane 3), Ile296Leu/Thr298Met (lane 4), and Arg299Gly (lane 5) were separated on SDS-PAGE and then stained with Coomassie brilliant blue (CBB) or subjected to the immunoblotting with K41110 or K41116C. (b-d) ELISA was performed with hHsp90α-N wt (open circle), Trp297Gly (closed circle), Thr298Met (open square), and Ile296Leu/Thr298Met (open triangle) as antigens. Lane M, molecular markers. Groups 1-3 were categorized based on the reactivity toward yHsc82 (see "**RESULTS**").

Fig. 4. Effect of the mutation at Arg_{299} on the antibody binding – ELISA was performed with hHsp90α-N wt (open circle) and Arg299Gly (closed circle) as antigens by using 8 anti-site-Ic mAbs. Eight anti-site Ic mAbs were categorized into Groups 1 (a), 2 (b), and 3 (c) based on the reactivity toward yHsc82.

Fig. 5. Immunoblotting analysis of the Hsp90-family proteins endogenously expressed in eukaryotes - Tissue or cell pellets of several species were denatured in SDS-sample buffer, and aliquots (5 μl) were loaded onto SDS-PAGE gels. Separated proteins were immunoblotted with K41110, K41116C, K41220 or AC88/SPA-830 (Stressgen). Lane 1, rat liver; lane 2, budding yeast (*S. cerevisiae*); lane 3, fission yeast (*S. pombe*); lane 4, rice (*O. sativa*) and lane 5, *P. caudatum*. Lane M, kaleidoscope prestained standards.

Table 1. Epitope mapping with anti-site-Ia mAbs

hHsp90α		242 E E E K E <u>K E E K E S E D K P E</u> 257
hHsp90β		236 E K E E E D K D D E E K P 249
K41102	#1	G P M **E** I D R **S** G **D** V D
	#2	G G VS A **E** **S** L **D** H A W
	#3	R Q T**E** V D **S** G R D L **E**
K41116A	#1	R Q M V**E** **K** **E** **S** L **D** Q A
	#2	R N T W D Y W **E** **S** L **D** V
K41122A	#1	C G K R A L R **E** **S** L N R
K41320	#1	R P L G L Q E **E** **S** L **D** G
	#2	G V D G L D L P L **E** Q L

Site Ia is underlined. Amino acids identical to hHsp90α are indicated by bold letters.

Table 2. Epitope mapping with anti-site-Ic mAbs

hHsp90α		291 N K T K P I W T R N P D D I 304
hHsp90β		283 N K T K P I W T R N P D D I 296
Group 1		
K41110	#1	G **K** L A **P** T **W** **T** **R** Q S A
	#2	G D V R F S R V **W** M **R** G
	#3	F V P A E R Y **P** * **W** V D Q
	#4	L V G Q F H T **P** A **W** A E
	#5	T M R **P** **I** **W** A **R** S G A V
K41116C	#1	A V L M L **P** V **W** A C L S
	#2	G R **N** G K Q T M **W** S G G
Group 3		
K3720	#1	A A S S R V **P** **I** **W** S **R** R
	#2	V **W** **T** **R** A E L I G A F G
	#3	R G N V **P** V **W** **T** Q R S K
K41331	#1	Y M R F N T **P** **I** **W** A S T
	#2	V G A F G **P** W **W** S D H S
	#3	V E S V R A Y **P** **I** **W** A **R**
	#4	V **T** T **P** P **W** **T** A P K S R
K41338	#1	G G G G D I V **P** **I** **W** A **R**
	#2	G R G G A **I** **W** D **R** P E F
	#3	F M G F S L **P** V **W** L D P

*deletion. Site Ic is underlined. Amino acids identical to hHsp90 are indicated by bold letters.

Table 3. Amino acids that appeared at the 5 positions of site Ic in selected clones

	Frequency		
Amino acid	Total	Group 1	Group 3
Pro_{295}	13 (76%)	5 (71 %)	8 (80 %)
Ile_{296}	6 (35%)	1 (14 %)	5 (50%)
Ile/Val/Trp/Ala/Met_{296}	14 (82 %)	5 (71 %)	9 (90 %)
Trp_{297}	17 (100 %)	7 (100 %)	10 (100 %)
Thr_{298}	4 (24 %)	1 (14 %)	3 (30 %)
Thr/Ala/Ser_{298}	13 (76 %)	5 (71 %)	8 (80%)
Arg_{299}	8 (47 %)	3 (43 %)	5 (50 %)
Total	17 (100 %)	7 (100 %)	10 (100 %)

Table 4. Epitope mapping with anti-site-II mAbs

hHsp90α		701 D <u>D P T A D D T S A A V T E E M P</u> P L E G D D D 724
hHsp90β		693 D <u>E V A A E E P N A A V P D E I P</u> P L E G D E D 716
K41007[a]		W V **A** * **D T S** Y
K3705		**D E V** P **A** HV D G E L N
K3725B	#1	E **P D E** V A M E V R M G
	#2	V S T L I E L **P D E I P**
	#3	**D E I P** E P A V P C G G

*deletion. [a]reported in Nemoto et al 1997. Site II is underlined. Amino acids identical to hHsp90α (K41007) or hHsp90β (K3000 series) are indicated by bold letters.

Table 5. Epitope mapping with K3729

hHsp90α	227 D<u>KE</u>V<u>SDDEAE</u>EKEDKEEEKEKEEKESEDK 255
hHsp90β	222 <u>EKE I SDDEAE</u>EEKG***EKEEEDKDDEEK 247
hGrp94	267 TVEEPMEEEEAAKE*****<u>EKE</u>E<u>SDDEA</u>AV 291
yHsc82	215 KEVPIPEEEKKDEEKK****DEDDKKPKLE 240
K3729 #1	**E**VAE**SDDEAE**VY
#2	EPGL**I**D**DDE**RNT
#3	SPLQGST**DDE**SA
#4	RSDT**DDE**RSESG
#5	NWGQVES**D**L**E**F**E**
#6	SSLSDMH**D**C**E**SV
#7	AGVDT**D**W**E**PHGR
hHsp90α (1)	227 D**KE**V**SDDEAEEK** 238
hHsp90α(2)	258 I EDVG**SD**E**E**E**E**KK 270
hHsp90α(3)	282 **E**KI**D**Q**E**ELN 291
hHsp90α(4)	301 P**DD**ITN**EE**YG 310

*deletion. Amino acids of hHsp90β corresponding to the epitope of K3729 deduced from selected clones (#1 - #7) and those of hHsp90α and hGrp94 homologous to the epitope sequence of hHsp90β are underlined. Amino acids of positive clones and hHsp90α [(1)-(4)] identical to the sequence of hHsp90β are indicated by bold letters.

Table 6. Epitope mapping with K41218

hHsp90α		68 S K L D S * G K E L H I N L I 81
hHsp90β		63 S K L D S * G K E L K I D I I 76
K41218	#1	V **L** **D** * * **G** A **E** **L** K E R E N
	#2	A G N Y * **G** E **E** **L** L P V D
	#3	A L K * **G** A **E** **L** L **I** H G S
	#4	R R **D** **S** G **G** F **E** Q I W G
	#5	W **D** **S** * **G** F **E** **L** S G P G K

*deletion. Amino acids identical to hHsp90α are indicated by bold letters.

Table 7. Epitope mapping with K41002

hHsp90α	306 N E E Y G E F Y K S L T N D W 320
hHsp90β	298 Q E E Y G E F Y K S L T N D W 312
K41002 #1	D R **G E** G P R I N A R C
#2	A I M P K **E F** D **K S** E W
#3	A **G E F** G D L R R A R A

Amino acids identical to those of hHsp90α are indicated by bold letters.

Table 8. Comparison of site-Ic sequences of Hsp90 from various species

hHsp90α [a] /hHsp90β [a]	**NKTK PIWTRNPDDI**
hGrp94[a]	**N**D**I K PIW**Q**R**PSKE**I**
hTrap1[a]	**N**TLQA**IW**MMD**P**K**D**V
yHsc82 [a, b]/yHsp82[b]	**NKTK P**L**WTRNP**S**DI**
EcHtpG[a]	**NK**AQAL**WTRN**KSE**I**
PgHtpG [a]	IND**T**H**PA****WT**KK**PAD**L
rat Hsp90[b]	**NKTK PIWTRNPDDI**
Schizosaccharomyces pombe Hsp90[b]	**NKTK PIWTRNP**SEV
Oryza sativa Hsp90[b]	**NK**Q**K PIW**L**R**K**P**EE**I**
Paramecium tetraurelia Hsp90	**NK**N**K P**L**W**MKK**PDDI**

[a]Hsp90-family members subjected to the immunoblotting in Fig. 2.

[b]Hsp90-family members endogenously-expressed in cells subjected to the immunoblotting in Fig. 5. In the last line, the sequence of that of *P. tetraurelia* Hsp90 is shown instead of *P. caudatam* used in this study. Amino acids identical to those of hHsp90α/hHsp90β are indicated by bold letters.

Table 9. Epitope mapping of anti-EcHtpG-N pAbs

hHsp90α	291 N K T K P I W T R N P D D I 304
EcHtpG	228 N K A Q A L W T R N K S E I 241
#1	A G G G **A** V**W** N E V D L
#2(1)	L **K** **A** L G **L** N M E P V P
#2(2)	L K **A** **L** G L N M E P V P
#3	I G C I M **R** **N** A G T A N
#4(1)	T L Q **A** L K G C **T** **R** R Y
#4(2)	T L **Q** **A** **L** K G C T R R Y
#6(1)	**A** **L** E R K C G A L V E V
#6(2)	A L E R **K** C G **A** **L** V E V

hHsp90α	59 I S N S S D A L D K I R Y E 62
EcHtpG	36 I S N A S D A A D K L R F R 49
#5	**I** N V T L F V **A** **D** N **L** P
#7	T E T E V G L R **D** G **L** G

Three clones (#2, #4, and #6) are aligned in 2 ways. The 5 amino acids essential for site Ic are underlined (see Table 2). Amino acids identical to those of EcHtpG are indicated by bold letters.

FIGURE LEGENDS

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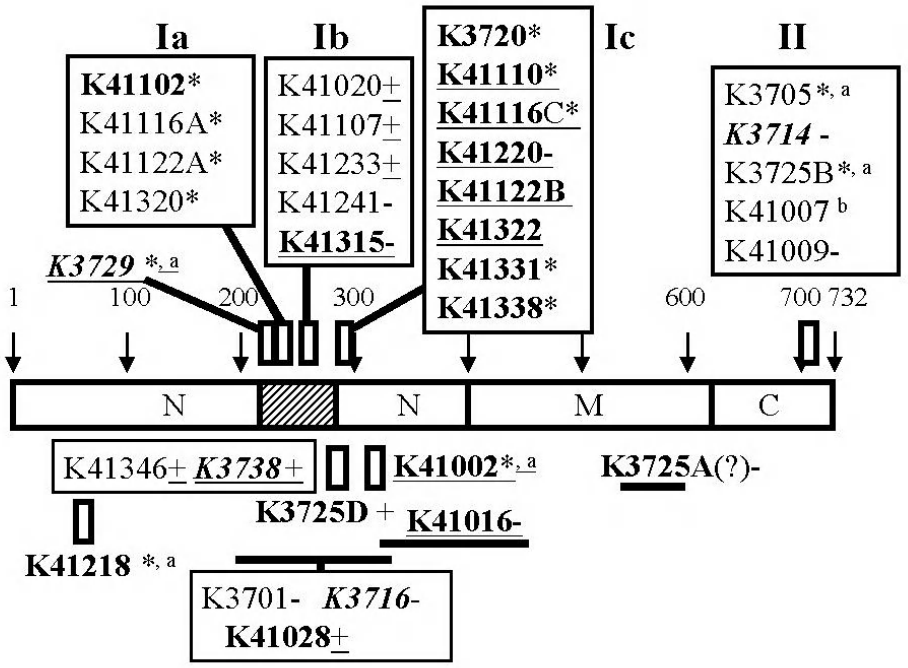
Fig. 1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K41315-
K3720*
K41110*
K41116C*
K41220-
K41122B
K41322
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K41338*
K3705*, a
K3714 -
K3725B*, a
K41007 b
K41009-
K3729 *, a
1
100
200
300
600
700 732
N
N
M
C
K41346± K3738±
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K3725D + K41016-
K41218 *, a
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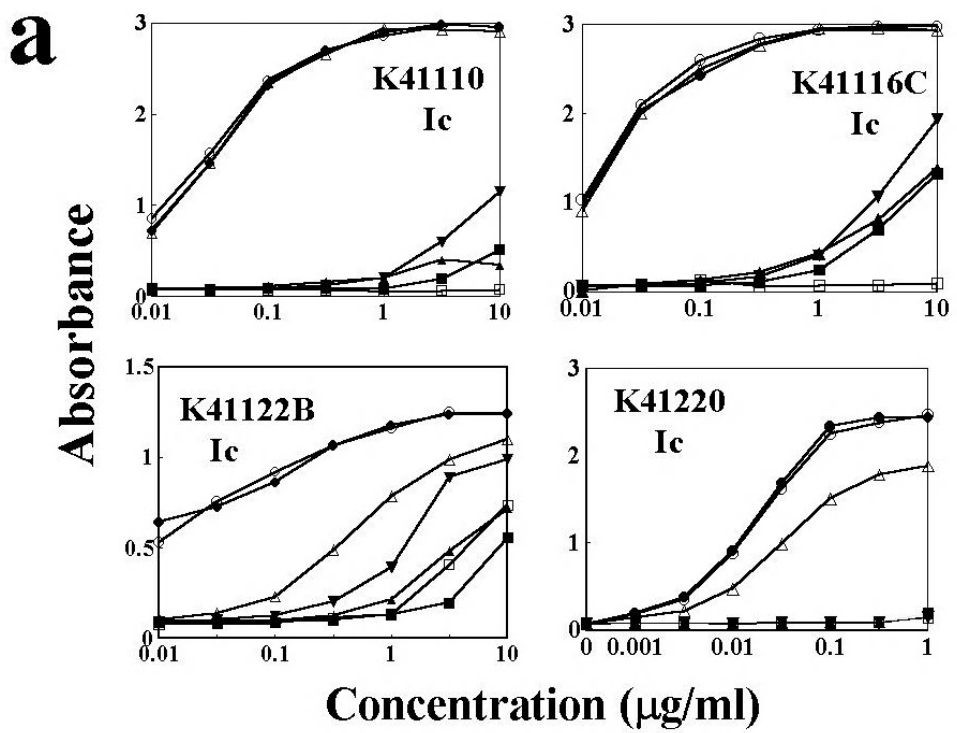
K41028±

Fig. 2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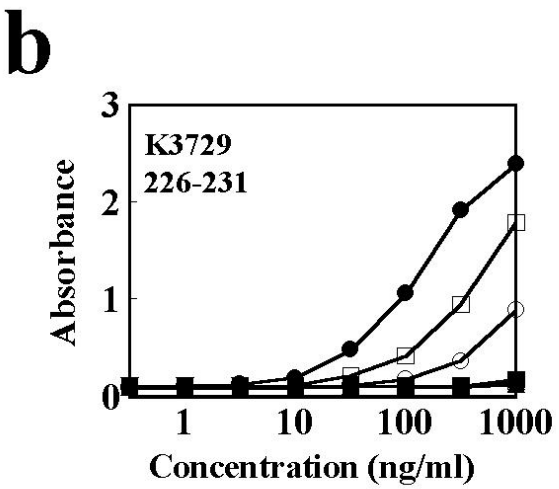
Ic
Concentration (μg/ml)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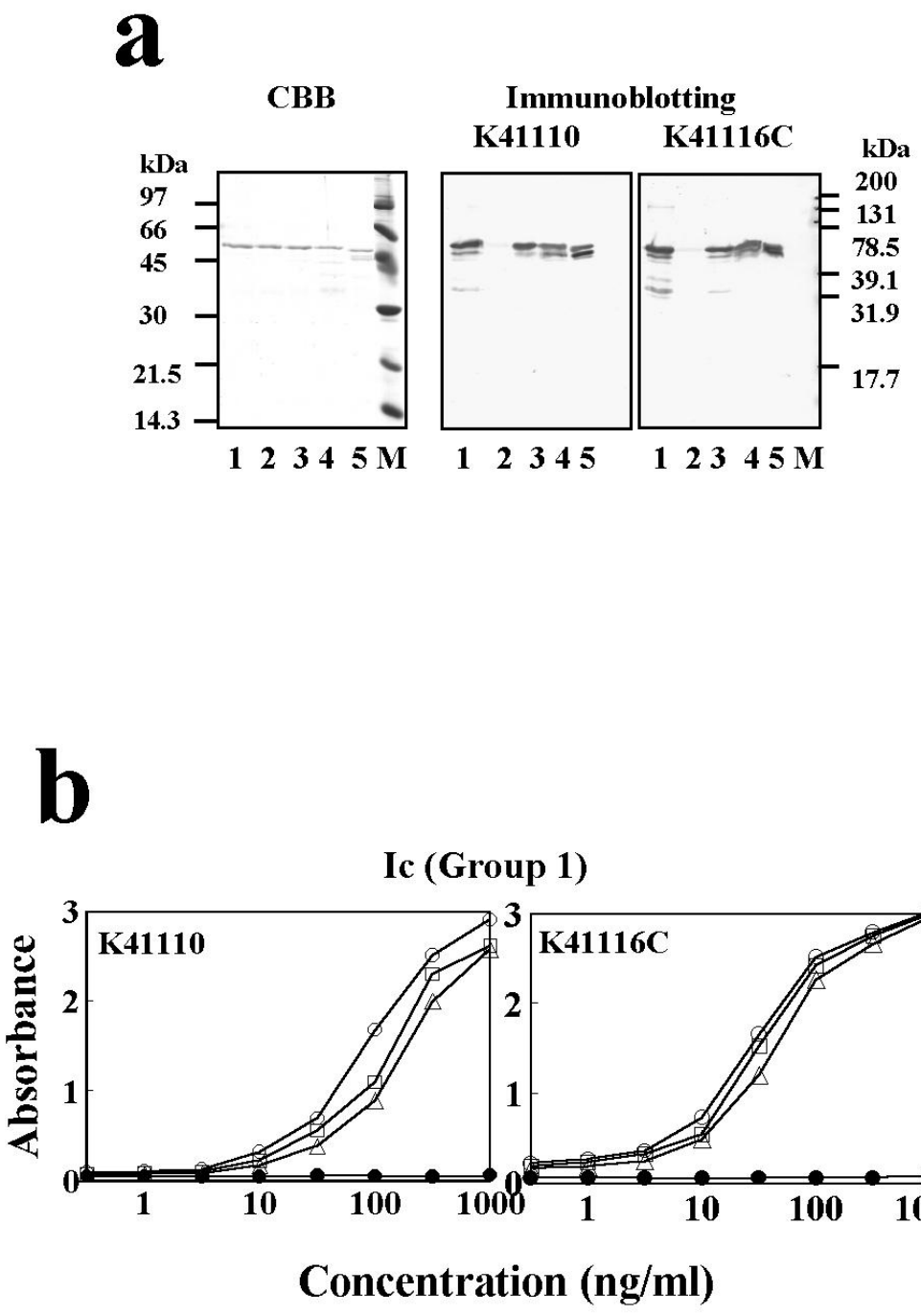
Concentration (ng/ml)

Fig. 3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kDa
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31.9
17.7
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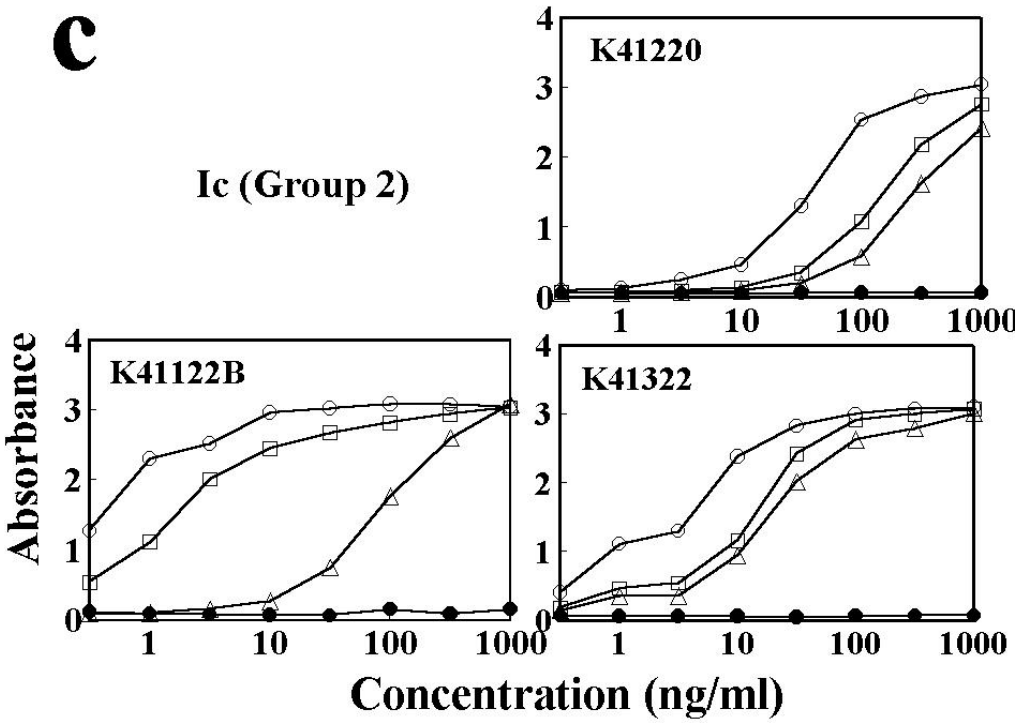
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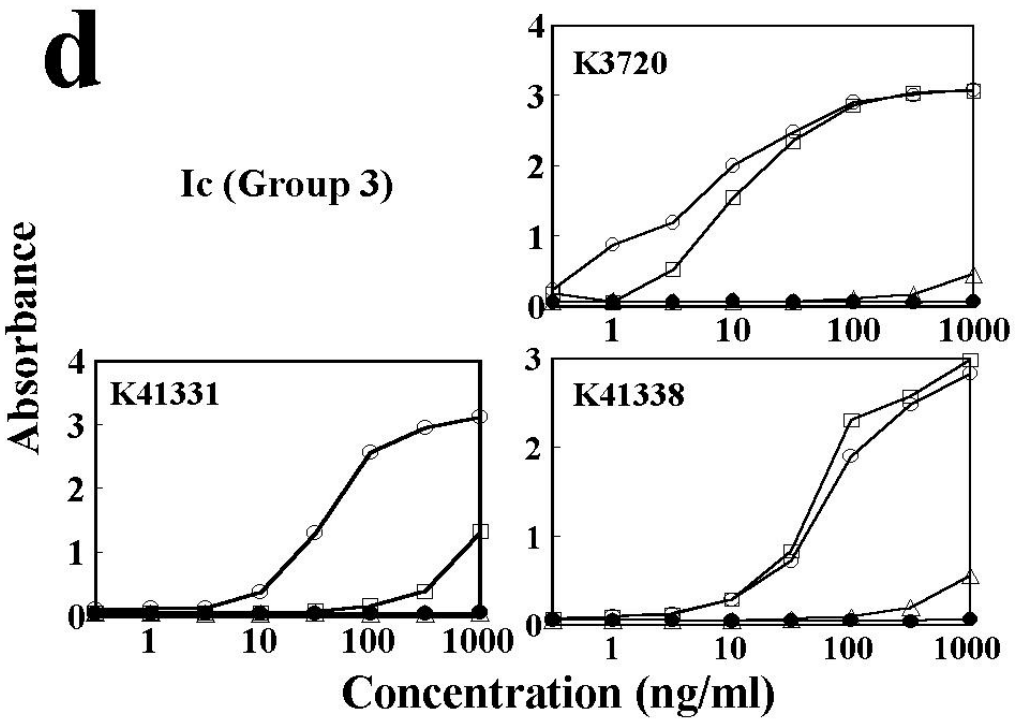
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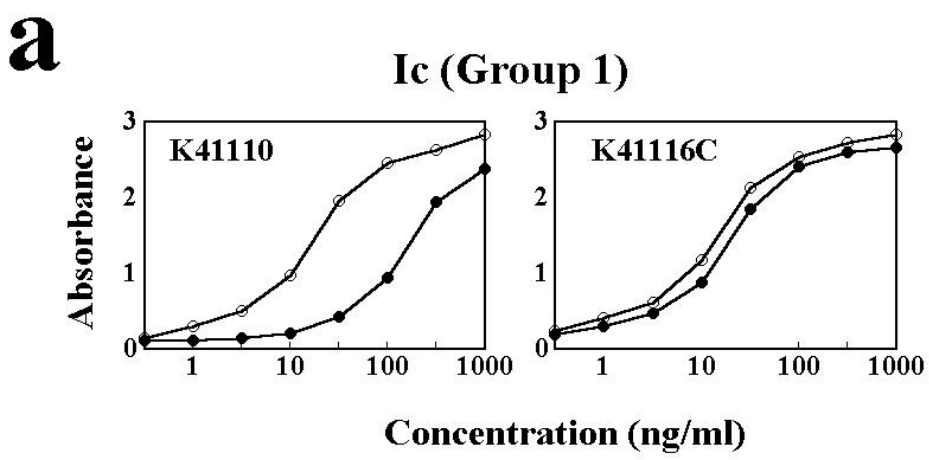
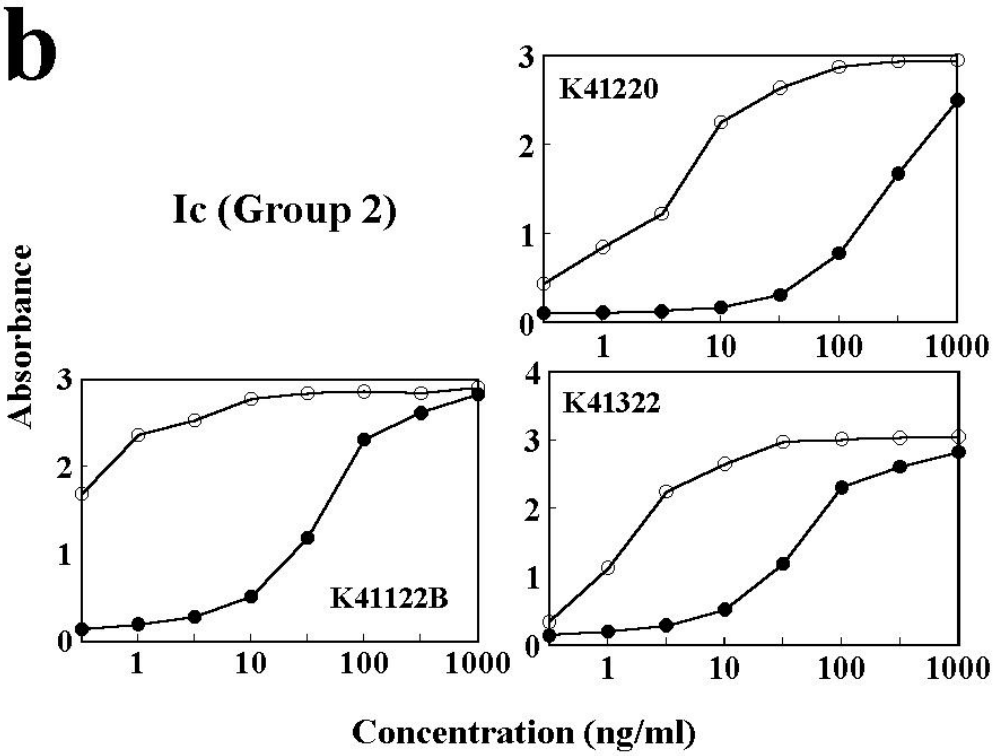
Ic (Group 3)
K3720
K41331
K41338
Absorbance
Concentration (ng/ml)
0
1
2
3
4
1
10
100
1000

Fig. 4


a
Ic (Group 1)
K41110
K41116C
Absorbance
Concentration (ng/ml)
0
1
2
3
1
10
100
1000


b
Ic (Group 2)
Absorbance
K41220
K41122B
K41322
Concentration (ng/ml)

c

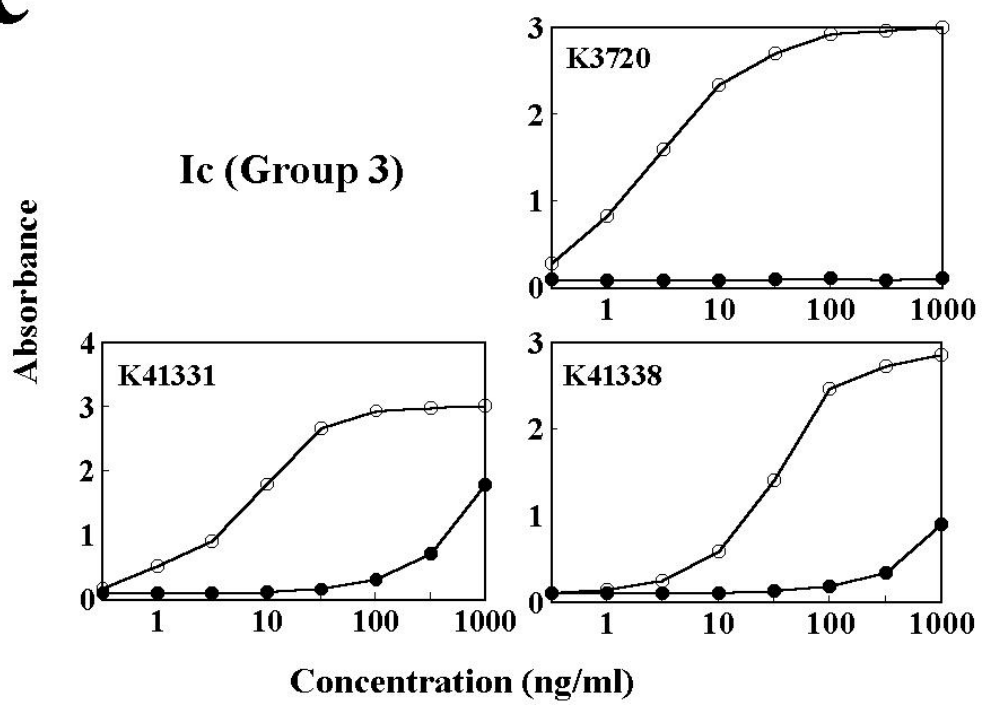
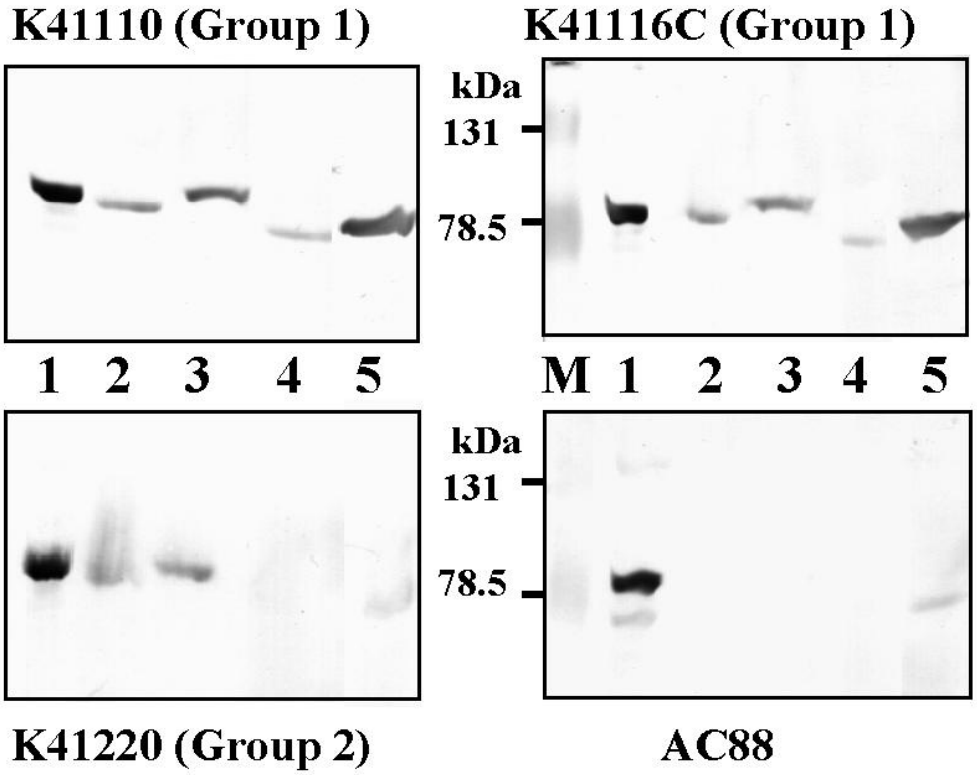

Ic (Group 3)
K3720
K41331
K41338
Absorbance
Concentration (ng/ml)
0
1
2
3
4
1
10
100
1000

Fig. 5


K41110 (Group 1)
K41116C (Group 1)
kDa
131
78.5
1 2 3 4 5
M 1 2 3 4 5
kDa
131
78.5
K41220 (Group 2)
AC88