Mapping of Functional Domains of Human High Molecular Weight and Low Molecular Weight Kininogens Using Murine Monoclonal Antibodies†

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ABSTRACT: Thirty-four monoclonal antibodies directed against human high molecular weight (HMW) and low molecular weight (LMW) kininogens and their derivatives were obtained, and the specificities of the antibodies were assayed by enzyme-linked immunosorbent assay (ELISA). By use of HMW kininogen, kinin-free HMW kininogen, kinin-free and fragment 1-2 (fr 1-2) free HMW kininogen, fr 1-2–light chain of HMW kininogen, LMW kininogen, kinin-free LMW kininogen, heavy chain of LMW kininogen, and light chain of LMW kininogen, the monoclonal antibodies were characterized and classified into four groups: (A) 20 monoclonal antibodies reacting with only the heavy chain, a common region of HMW and LMW kininogens; each of these monoclonal antibodies possessed the specificity to domain 1 (2 monoclonal antibodies), domain 2 (2 monoclonal antibodies), domain 3 (7 monoclonal antibodies), and both domains 2 and 3 (7 monoclonal antibodies) of the heavy chain; (B) 7 monoclonal antibodies reacting with fr 1-2, a unique histidine-rich region; (C) 5 monoclonal antibodies reacting with the light chain of HMW kininogen; (D) 2 monoclonal antibodies reacting with the light chain of LMW kininogen. Two monoclonal antibodies in the first group (group A), designated HKG H7 and H12, effectively suppressed the thiol proteinase inhibitor activity of HMW kininogen to papain and calpains and of LMW kininogen to papain, but the others did not affect it. Further, all the monoclonal antibodies which recognized the fr 1-2 or light chain of HMW kininogen (groups B and C) suppressed the clotting activity. By competition ELISA, fr 1-2 specific monoclonal antibodies were classified into five subgroups according to the difference of their recognition sites, and the light chain (HMW kininogen) specific monoclonal antibodies were also classified into three subgroups. Among them, HKG L2, which reacted specifically with the fr 1-2 region of HMW kininogen, neutralized about 70% of the clotting activity, and HKG L5, which recognized the light chain of HMW kininogen, neutralized more than 90% of it. Two light chain (LMW kininogen) specific monoclonal antibodies (group D) were found to recognize an identical antigenic determinant.

Human plasma contains two molecular forms of kininogens, high molecular weight (HMW)[1] kininogen and low molecular weight (LMW) kininogen, which are single-chain glycoproteins (Kato et al., 1981). The molecular structure of human HMW kininogen comprises four functional peptides: (1) the heavy chain, which contains three cystatin-type repeat sequences (domains 1, 2, and 3) (Salvesen et al., 1986) and possesses the thiol proteinase inhibitor (TPI) activity (Ohkubo et al., 1984); (2) the nonapeptide bradykinin, which induces the permeability of peripheral blood vessel (Holdstock et al., 1957); (3) fragment 1-2, the histidine-rich peptide, which is capable of binding to a negatively charged surface; and (4) the light chain of HMW kininogen, which is a binding domain with prekallikrein (Mandel et al., 1976; Tait & Fujikawa, 1986) and factor XI (Thompson et al., 1977). LMW kininogen is also composed of heavy chain, bradykinin, and light chain. The heavy chains and bradykinin moieties of HMW and LMW kininogens are identical, but their individual light chains are quite different in amino acid sequence and size (Takagaki et al., 1985).

Both kininogens are well-known to be a large potential source of kinin in human plasma. However, another functional role has been shown for HMW kininogen, acting as a cofactor in the contact activation of blood coagulation (Müller-Esterl & Fritz, 1984). The cofactor complexing with prekallikrein (Mandel et al., 1976; Tait & Fujikawa, 1986) or factor XI (Thompson et al., 1977) attaches to the negatively charged surface (Griffin & Cochrane, 1976b; Meier et al., 1977; Ikari et al., 1981), thereby increasing the activation rate of these zymogens by the adjacent factor XIIa molecule (Scott et al., 1984). HMW kininogen is susceptible to cleavage by human plasma kallikrein. The kallikrein releases bradykinin from HMW kininogen and converts an additional molecule of factor XII to factor XIIa, further amplifying the reaction (Kaplan, 1978).

On the other hand, thiol proteinase inhibitors (TPIs) with high molecular weight (M_r 60 000–170 000) (Sasaki et al., 1977; Ryley, 1979; Järvinen, 1979) and low molecular weight (M_r 10 000–14 000) (Grubb & Löfberg, 1982; Wakamatsu et al., 1982; Lenney et al., 1982) have been extensively isolated from plasma and various tissues. The physiological function

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[1] Abbreviations: HMW kininogen, high molecular weight kininogen; LMW kininogen, low molecular weight kininogen; TPI, thiol proteinase inhibitor; ELISA, enzyme-linked immunosorbent assay; DMEM, Dulbecco's modified Eagle's medium; Ig, immunoglobulin; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; HAT medium, hypoxanthine–aminopterin–thymidine medium; HT medium, hypoxanthine–thymidine medium; PMSF, phenylmethanesulfonyl fluoride; *p*-APMSF, (*p*-amidinophenyl)methanesulfonyl fluoride; PBS, phosphate-buffered saline; BSA, bovine serum albumin; Tween 20, poly(oxyethylene) sorbitan monolaurate; PBS–Tween, phosphate-buffered saline containing 0.05% Tween 20; PBS–TPB, phosphate-buffered saline containing 0.05% Tween 20, 2% poly(vinylpyrrolidone), and 0.2% BSA; FCS, fetal calf serum; β-ME, β-mercaptoethanol; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; FPLC, fast protein liquid chromatography; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid.

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of these TPIs is thought to be the regulation of thiol proteinases, such as cathepsins (B, H, L) and calpains (I, II). We have previously characterized three kinds of high molecular weight TPIs (α_1TPI, α_2TPI$_1$, and α_2TPI$_2$) from human plasma (Sasaki et al., 1981). Recently, the amino acid sequence of α_2TPI (M_r 64000) was deduced by analyzing the base sequence of the cDNA, and its identity with LMW kininogen was reported (Ohkubo et al., 1984). Moreover, Müller-Esterl et al. (1985), Sueyoshi et al. (1985), and Higashiyama et al. (1986a) have shown that both kininogens functionally inhibit the thiol proteinases.

More recently, Berrettini et al. (1986) produced monoclonal antibodies for HMW kininogen and reported that these monoclonal antibodies were useful for qualitative and quantitative probes for HMW kininogen in plasma samples. The antibodies specifically recognized the heavy chain and the light chain of HMW kininogen but did not affect the clotting activity of the HMW kininogen.

In the present study, we dealt with the production of murine monoclonal antibodies to human HMW and LMW kininogens and tried to carry out the mapping of functional domains of the kininogens by use of the antibodies. We further examined the effects of the monoclonal antibodies on the biological activities of the kininogens such as the TPI activity and clotting activity.

Materials and Methods

Materials

Dulbecco's modified Eagle's medium (DMEM) was purchased from Flow Laboratories, McLean VA. Medium NCTC-135 was obtained from Gibco Laboratories, Grand Island Biological Co., Grand Island, NY. Aminopterin, insulin, amphotericin B, and phenylmethanesulfonyl fluoride (PMSF) were purchased from Sigma Chemical Co., St. Louis, MO. Sodium pyruvate, oxaloacetic acid, hypoxanthine, thymidine, (*p*-amidinophenyl)methanesulfonyl fluoride (*p*-APMSF), *o*-phenylenediamine, poly(oxyethylene) sorbitan monolaurate (Tween 20), and poly(ethylene glycol) 1540 were obtained from Wako Pure Chemical Industries, Ltd., Osaka, Japan. Fetal calf serum (FCS) was purchased from Whittaker M. A. Bioproducts, Walkersville, MO. Protein A–Cellulofine and bovine serum albumin (BSA) were products of Seikagaku Kogyo Co., Ltd., Tokyo, Japan. Carbenicillin and streptomycin were purchased from Fujisawa Pharmaceutical Co., Ltd., Osaka, Japan, and Meiji Seika Kaisha, Ltd., Tokyo, Japan, respectively. 2,6,10,14-Tetramethylpentadecane (Pristane) was obtained from Aldrich Chemical Co., Inc., Milwaukee, WI. Biotin *N*-hydroxysuccinimide ester was purchased from Pierce Chemical Co., Rockford, IL. Peroxidase-conjugated IgG fraction of goat anti-mouse immunoglobulin antisera was obtained from Cappel Scientific Division, Cooper Biomedical, Malvern, PA. Purified rabbit anti-mouse class- and subclass-specific antisera to IgG$_1$, IgG$_{2a}$, IgG$_{2b}$, IgG$_3$, IgM, and IgA were purchased from Bio-Rad Laboratories, Richmond, CA. Rabbit antisera to mouse immunoglobulin κ and λ light chains were purchased from Litton Bionetics, Inc., Charleston, SC. A 96-well microtiter plate used for enzyme-linked immunosorbent assay (ELISA) was obtained from Nunc, Roskilde, Denmark. A 96-well tissue culture plate was purchased from Falcon, Becton Dickinson & Co., Oxnard, CA. Avidin–horseradish peroxidase was obtained from E-Y laboratories, Inc., San Mateo, CA. The BALB/cAnNCrj mouse was purchased from Charles River, Inc., Atsugi, Japan. Eleven-peptide (Ala-Arg-Val-*Gln-Val-Val-Ala-Gly*-Lys-Lys-Tyr) was kindly provided by Drs. P. W. Chou and K. Kurachi (Seattle, WA).

Culture Media. Dulbecco's modified Eagle's medium (DMEM) was supplemented with 10% FCS/20 mM glucose/10 mM *N*-(2-hydroxyethyl)piperazine-*N'*-2-ethanesulfonic acid (HEPES)/1.14 mM oxaloacetate/0.5 mM sodium pyruvate/200 units L^{-1} insulin/50 μM β-mercaptoethanol (β-ME)/260 μM carbenicillin/170 μM streptomycin/10 μM amphotericin B, pH 7.2, to make a complete DMEM (cDMEM). Hypoxanthine–aminopterin–thymidine (HAT) medium contained 100 μM hypoxanthine, 0.4 μM aminopterin, and 16 μM thymidine in cDMEM. Hypoxanthine–thymidine (HT) medium was identical with HAT without containing aminopterin.

The 8-azaguanine-resistant myeloma cell line NS-1 was generously gifted by Dr. T. Takahashi, Aichi Cancer Center Research Institute, Nagoya, Japan. Myeloma and hybridoma cells were maintained under a humidified atmosphere of 93% air/7% CO_2 at 37 °C. All cells were grown in cDMEM as described above.

Buffers. The buffers used in this experiment were as follows: phosphate-buffered saline (PBS), 20 mM phosphate buffer (pH 7.4)/150 mM NaCl; PBS–Tween buffer, PBS/0.05% Tween 20; and PBS–TPB buffer, PBS/0.05% Tween 20/2% poly(vinylpyrrolidone)/0.2% bovine serum albumin.

Methods

Purification of HMW Kininogen, LMW Kininogen, and Their Derivatives. HMW kininogen was isolated from fresh citrated human plasma by two-step column chromatographies on DEAE-Sephadex A-50 and Zn-chelate Sepharose 4B (Higashiyama et al., 1986a). Kinin-free HMW kininogen was purified from outdated human plasma by the same procedure as HMW kininogen (Higashiyama et al., 1986a). Kinin-free and fragment 1·2 free HMW kininogen (α_1TPI) was also purified from outdated human plasma by an improved procedure utilizing DEAE-cellulose, Zn-chelate Sepharose 4B, butyl-Toyopearl, HA-Ultrogel, and Red Sepharose (Ohkubo et al., 1987).

LMW kininogen was purified from fresh citrated human plasma by a developed procedure employing DEAE-Sephadex A-50, ammonium sulfate fractionation, DEAE-Sephacel, Red Sepharose, HA-Ultrogel and butyl-Toyopearl (unpublished results). Kinin-free LMW kininogens corresponding to α_2TPI$_1$ and α_2TPI$_2$ were purified as described previously (Ohkubo et al., 1984).

The fragment 1·2–light chain of HMW kininogen was isolated from kinin-free HMW kininogen by a procedure employing Zn-chelate Sepharose 4B and Sephacryl S-300 in the presence of β-ME (unpublished results). The heavy chain and light chain of LMW kininogen were prepared according to the method of Higashiyama et al. (1986a).

All purified proteins except for HMW kininogen migrated as a homogeneous single band on sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) in the absence of β-ME. HMW kininogen gave two bands, a major band of dimeric form (M_r 200000, ca. 90–95%) and a minor band of monomeric form (M_r 105000, ca. 5–10%), on the same gel electrophoresis as described previously (Higashiyama et al., 1986a).

Isolation of CB-1, Domain 2, and Domain 3 from Kinin-Free LMW Kininogen. The fragment which was composed of the amino-terminal region (1–160 amino acid sequence) of the heavy chain and light chain (372–409 amino acid sequence) of LMW kininogen held together with a disulfide bond, tentatively named CB-1, was obtained from kinin-free LMW kininogen (α_2TPI) by CNBr treatment as follows. Fifty

milligrams of kinin-free LMW kininogen was dissolved in 5 mL of 70% formic acid, and 50 mg of CNBr was added to the solution. Digestion was performed overnight in a sealed tube at room temperature. The reaction mixture was chromatographed by gel filtration on a Sephadex G-75 superfine column (3 × 142 cm) equilibrated with 20 mM Tris-HCl buffer, pH 7.5. The fractions containing CB-1 fragment were collected and further applied on a TSK gel DEAE-5PW column (Toyo Soda, Tokyo, Japan) using the Pharmacia fast protein liquid chromatography system (FPLC) (Pharmacia, Uppsala, Sweden).

Domain 2 (123–244 amino acid sequence) and domain 3 (245–362 amino acid sequence) of the heavy chain were isolated from kinin-free LMW kininogen according to the procedure of Salvesen et al. (1986). Fifty milligrams of kinin-free LMW kininogen was incubated with 100 μg of trypsin in 15 mL of 50 mM Tris-HCl buffer, pH 7.5, for 90 min at 30 °C. The digestion was stopped by the addition of *p*-APMSF to give a final concentration of 2 mM. The digested sample was chromatographed successively on a Sephadex G-75 superfine column and a TSK gel DEAE-5PW column. The tryptic peptide with molecular weight of 20000 was isolated as domain 3. The tryptic peptide with molecular weight of 40000 copurified with the above columns was further digested with chymotrypsin at a chymotrypsin to peptide weight ratio of 1:25 in 50 mM Tris-HCl buffer, pH 7.5, for 6 h at 30 °C, followed by the addition of PMSF to give a final concentration of 2 mM. Chymotrypsin-digested fragment, domain 2, was also isolated with a Superose column (Pharmacia, Uppsala, Sweden) using an FPLC system. The amino acid compositions and N-terminal amino acids of CB-1, domain 2, and domain 3 corresponded to those of the sequences 1–160 and 372–409, 123–244, and 245–362 of LMW kininogen, respectively. All purified proteins migrated as a homogeneous single band on SDS–PAGE in the absence of β-ME.

Purification of Calpain I from Human Erythrocytes and Calpain II from Human Kidneys. Calpain I, a low calcium dependent cysteine proteinase, was purified from human erythrocytes by employing column chromatographies on DEAE-cellulose, Ultrogel AcA34, Blue Sepharose, and DEAE Bio-Gel A according to the method described by Hatanaka et al. (1983).

Calpain II, a high calcium dependent cysteine proteinase, was purified from human kidneys on column chromatographies including DEAE-cellulose, Sephacryl S-300, DEAE Bio-Gel A, and Red Sepharose according to the method described by Ishiguro et al. (1987).

Determination of Protein Concentration. Protein concentrations were determined by employing an $E^{1\%}_{280nm}$ value of 7.3 for HMW kininogen and its derivatives and an $E^{1\%}_{280nm}$ value of 7.8 for LMW kininogens (Müller-Esterl & Fritz, 1984) and kinin-free LMW kininogen (α_2TPI$_1$ and α_2TPI$_2$).

Protein concentrations of CB-1, domain 2, and domain 3 of the heavy chain of kininogens were determined by the method of Lowry et al. (1951), using bovine serum albumin as standard. Protein concentrations of calpain I and calpain II were determined by employing an $E^{1\%}_{280nm}$ value of 7.2 (Higashiyama et al., 1986b). The concentration of purified monoclonal antibody was determined by assuming an $E^{1\%}_{280nm}$ value of 14.0 (Ey et al., 1978).

Immunization, Cell Fusion, and Growth of Clones. Purified human kininogens and their derivatives prepared as described above were used as antigen in this experiment. Twenty micrograms of purified HMW and LMW kininogens was emulsified in complete Freund's adjuvant, respectively, and injected subcutaneously into 2-month-old BALB/c mice. The mice received three to six subcutaneous booster injections of 10 μg of the proteins with complete Freund's adjuvant. A final intravenous injection of 10 μg of protein was given at 6–7 weeks after priming. Four days later, the spleen cells of the mice were harvested and fused with NS-1 mouse myeloma cells. Prior to the fusion, NS-1 myeloma cells were maintained in logarithmic growth phase between 1×10^5 and 5×10^5 cells per milliliter for at least 1 week. For cell fusion, the spleen was removed aseptically from the donor mouse and placed in a sterile petri dish containing cDMEM. A single cell suspension was obtained by dispersion with a Pasteur pipet. Spleen cells and NS-1 myeloma cells were collected separately by centrifugation at 400*g* for 10 min and washed 3 times with cDMEM.

Fusion of spleen cells and NS-1 myeloma cells was performed according to the technique of Köhler and Milstein (1975). Briefly, 2×10^7 myeloma cells and 1×10^8 spleen cells were mixed, centrifuged together, and fused by the addition of 1 mL of 50% poly(ethylene glycol) 1540 in DMEM, followed by slow dilution in 10 mL of DMEM. Then cells were centrifuged, resuspended in 30 mL of HAT medium, and dispersed into 96-well microtiter plates at 4×10^5 to 1×10^6 cells per well. Culture supernatants were harvested for screening 10 days after fusion. Positive colonies in wells for anti-kininogen activity or anti-light-chain activity by ELISA were expanded and then cloned by limiting dilution in the presence of thymocytes (5×10^5 cells/well) (Goding, 1980). Colonies derived from a single cell were assayed by ELISA, and those exhibiting anti-kininogen activity or anti-light-chain activity were expanded. Further, we repeated the limiting dilution 2 more times. Clones were either grown for antibody production or frozen in FCS containing 10% dimethyl sulfoxide and stored in liquid nitrogen. When substantial amounts of antibody were needed, the cloned cells (1×10^7 cell/mouse) were inoculated intraperitoneally in BALB/c mice pretreated with Pristane.

Screening of Antibodies against Kininogens by ELISA. Anti-human kininogen antibodies in hybridoma supernatants were detected by ELISA. Briefly, wells of 96-well microtiter plates were coated with 100 μL of kininogens (500 ng/mL) of their derivatives (500 ng/mL) in 20 mM Tris-HCl buffer, pH 7.5, for 2 h at room temperature or overnight at 4 °C. The wells were washed 3 times with PBS–Tween buffer and blocked with 150 μL/well of 1% BSA in 20 mM Tris-HCl buffer, pH 7.5, for 1 h at room temperature. The wells were washed 3 times with PBS–Tween buffer, and 50 μL of hybridoma supernatant and 50 μL of PBS–Tween buffer were added to the wells, and the plate was left for 2 h at room temperature. The wells were then washed 3 times with PBS–Tween buffer, and 100 μL of peroxidase-conjugated goat anti-mouse Igs (IgA, IgG, and IgM), which were diluted 10^4-fold in PBS–TPB buffer, was added to the wells. The plate was left for 2 h at room temperature. The wells were washed 3 times with PBS–Tween buffer, and 100 μL of *o*-phenylenediamine (0.4 mg/mL) containing 1.82 mM H_2O_2 in 0.1 M citrate–0.2 M phosphate buffer, pH 5.0, was added to the wells in the dark. The enzymatic reaction was stopped after 10 min by the addition of 50 μL of 2 N H_2SO_4. The optical density of the wells was determined by a two-wavelength microplate photometer, MTP-22 (Corona Electric Co., Ltd., Ibaragi, Japan), at 492 nm.

Class and Subclass Typing of Anti-Kininogen Monoclonal Antibodies. The immunoglobulin types of anti-kininogen monoclonal antibodies were determined by ELISA as described

above, using purified rabbit anti-mouse class- and subclass-specific antisera to IgG_1, IgG_{2a}, IgG_{2b}, IgG_3, IgM, and IgA, rabbit antisera to mouse immunoglobulin κ and λ light chains, and peroxidase-conjugated goat anti-rabbit Igs (IgA, IgG, and IgM).

Purification of Monoclonal Antibodies. IgG fractions of monoclonal antibodies were purified on a protein A–Cellulofine column. Five milliliters of mouse ascites fluid was dialyzed overnight against 20 mM Tris-HCl buffer, pH 8.6, containing 250 mM NaCl. The sample was centrifuged at 27000*g* for 40 min at 4 °C. The supernatant was applied to a protein A–Cellulofine column (1.7 × 4.0 cm) equilibrated with the same buffer. The column was washed with 100 mL of the same buffer, and the elution was performed at a flow rate of 10 mL/h at 4 °C with 0.5 M glycine hydrochloride buffer, pH 2.2, containing 250 mM NaCl. Fractions of 1 mL were collected, and the pH was neutralized with 1 mL of 1 M Tris-HCl buffer, pH 7.5. These preparations were dialyzed overnight against PBS at 4 °C.

Affinity-purified monoclonal antibodies were purified on HMW or LMW kininogen-coupled Sepharose 4B. Purified HMW or LMW kininogen (2 mg/mL of gel) was coupled to cyanogen bromide activated Sepharose 4B, and the IgG fraction of monoclonal antibodies obtained as described above was applied to the column (1.7 × 3.0 cm) equilibrated with PBS. The column was washed thoroughly with the same buffer, and IgG was eluted with 0.5 M glycine hydrochloride buffer, pH 2.2, containing 250 mM NaCl. These preparations were dialyzed overnight against PBS at 4 °C.

Assay of Thiol Proteinase Inhibitor Activity. The inhibition assays for HMW and LMW kininogens were performed by using papain (4.3 nanounits) as a target proteinase and *N*-benzoyl-L-arginine-*p*-nitroanilide as a substrate by the method described previously (Sasaki et al., 1981). One unit of activity was defined as the amount of inhibitor that inactivated papain to hydrolyze 0.5 μmol of *N*-benzoyl-L-arginine-*p*-nitroanilide min^{-1} mL^{-1} at 30 °C (Ohkubo et al., 1984).

The inhibitory activities of HMW kininogen to calpain I and calpain II were measured by using casein as substrate. HMW kininogen (50.4 pmol) and calpain I (126 pmol) or calpain II (99.7 pmol) were mixed in 375 μL of 0.48% casein and 300 μL of 20 mM Tris-HCl buffer, pH 7.5, containing 5 mM EDTA, 1 mM EGTA, and 5 mM β-ME. The mixture was preincubated for 5 min at 30 °C, and the reaction was started by the addition of $CaCl_2$ solution. The mixture was incubated for 20 min at 30 °C, and the reaction was stopped by addition of 750 μL of 10% trichloroacetic acid. The absorbance of the supernatant at 280 nm was measured with a Hitachi Model 228 spectrophotometer.

Assay of Clotting Activity of HMW Kininogen. Clotting activity of HMW kininogen was determined by the two-stage assay for actin-activated partial thromboplastin time using HMW kininogen deficient plasma (Griffin & Cochrane, 1976a). One unit of clotting activity is defined as the activity present in 1 mL of standard normal plasma.

Effects of Monoclonal Antibodies on Biological Activities of Kininogens. Assay 1. The effects of monoclonal antibodies on TPI activities of HMW and LMW kininogens to papain were examined. HMW kininogen (72.7 pmol) or LMW kininogen (78.1 pmol) was mixed with serial dilutions of affinity-purified monoclonal antibodies. After incubation at 30 °C for 2 h, the TPI activities of HMW and LMW kininogens were measured by the method described above.

Assay 2. The effects of monoclonal antibodies on TPI activities of HMW kininogen to calpain I and calpain II were examined. HMW kininogen (50.4 pmol) was mixed with serial dilutions of affinity-purified monoclonal antibodies. After incubation at 30 °C for 2 h, the TPI activities of HMW kininogen to calpain I and calpain II were measured by the method described above.

Assay 3. The effects of monoclonal antibodies on clotting activities of HMW kininogen were examined. HMW kininogen (6.72 nmol) was mixed with affinity-purified monoclonal antibodies (33.6 nmol). After incubation at room temperature for 4 h, the clotting activity of HMW kininogen was measured by the method described above.

Assay 4. The effects of monoclonal antibodies on clotting activities of HMW kininogen were also examined as follow: HMW kininogen (1.6 nmol) was mixed with serial dilutions of affinity-purified monoclonal antibodies. After incubation at 30 °C for 2 h, the clotting activity of HMW kininogen was measured by the method described above.

Biotinylation of Monoclonal Antibodies. Affinity-purified mouse anti-kininogen monoclonal antibodies were dialyzed overnight at 4 °C against 100 mM phosphate-buffered saline, pH 7.4. Two hundred microliters of biotin *N*-hydroxysuccinimide ester dissolved in dimethyl sulfoxide (1 mg/mL) was added to antibody solution (1 mg/mL). After a 4-h incubation at room temperature, the mixture was dialyzed overnight at 4 °C against PBS, pH 7.4. Aliquots of the antibody–biotin conjugate (100 μL) were transferred to Eppendorf microtest tubes and stored at −70 °C until use (Subba Rao et al., 1983).

Competition ELISA. Binding capacities of monoclonal antibodies labeled with biotin were assessed in the presence of increasing amounts of various unlabeled monoclonal antibodies; 96-well microtiter plates coated with HMW kininogen (25 ng/well) or LMW kininogen (10 ng/well) were incubated with biotinylated monoclonal antibodies at a final concentration of 1 μg/mL and unlabeled monoclonal antibodies at a final concentration of 10^2, 10, 1.0, 10^{-1}, or 10^{-2} μg/mL for 2 h at room temperature. The amount of biotinylated monoclonal antibodies bound to the solid-phase kininogen was estimated by further incubation with avidin–horseradish peroxidase (20 ng/mL), followed by the method as described in "ELISA" (Picard et al., 1986).

RESULTS

Hybridoma Production. Hybridomas for HMW and LMW kininogens were produced as described under Methods. From the results of ELISA using HMW kininogen and its derivatives or LMW kininogen and its derivatives, 75 hybridoma clones secreting anti-human kininogen antibodies were obtained. Each of the hybridomas was subcloned more than 3 times by limiting dilution. Finally, 34 hybridoma culture media containing monoclonal antibodies were obtained. The immunoglobulin subclass of each clone was determined by ELISA using specific antisera to each mouse immunoglobulin subclass. The anti-kininogen antibody-producing clones thus obtained were found to secrete IgG_1 (29 clones), IgG_{2a} (2 clones), and IgG_{2b} (3 clones) (Table I), and all 34 monoclonal antibodies had κ light chains. Each monoclonal antibody was isolated on protein A–Cellulofine column chromatography from mouse ascites fluid. These clones have been stable for 1–2 months in culture.

Characterization of Monoclonal Antibodies. The immunological reactivities of hybridoma culture media with kininogens and their derivatives were determined by ELISA. As shown in Table I, this result allowed us to classify the 34 monoclonal antibodies into 4 groups.

The first group (A) comprised 20 monoclonal antibodies

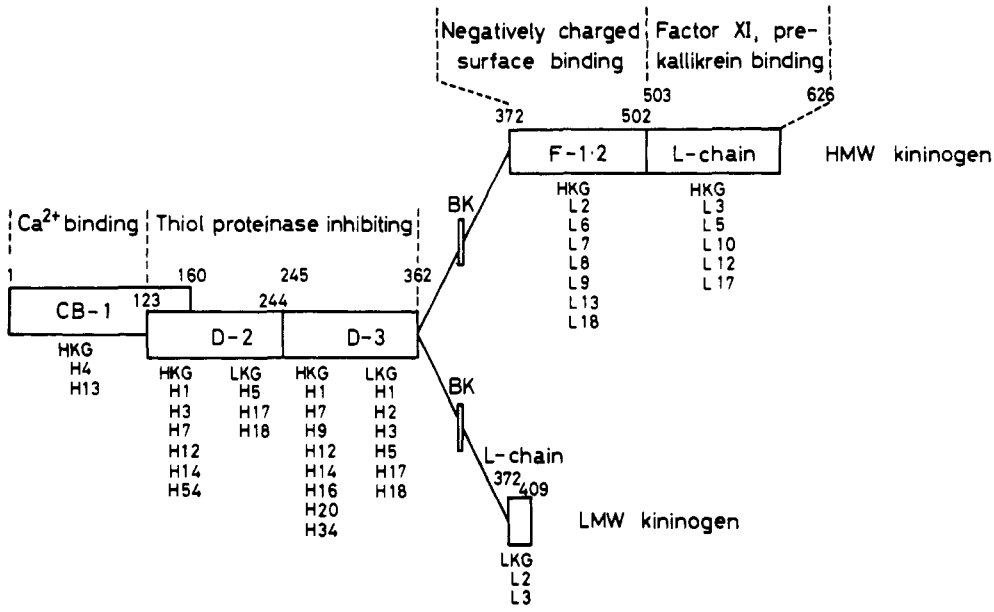

FIGURE 1: Linear model of functional domains recognized by monoclonal antibodies to HMW and LMW kininogens. The domains are shown as open horizontal bars with numbers indicating their amino acid residues. Functions are indicated above the bars, and monoclonal antibodies which recognize each domain are listed below the bars. The Ca^{2+} binding function of CB-1 is unpublished results. Abbreviations: D-2, domain 2; D-3, domain 3; BK, bradykinin; F-1·2, fragment 1·2; L-chain, light chain.

which recognized only the heavy chain of kininogens. The second group (B) composed of seven monoclonal antibodies was specific to the fragment 1·2, a unique histidine-rich region. The third group (C) included five monoclonal antibodies which recognized only the light chain of HMW kininogen. The last group (D) comprised two monoclonal antibodies which recognized only the light chain of LMW kininogen. These monoclonal antibodies as a whole covered all functional domains of both kininogens except for the kinin moiety.

Specificity of Monoclonal Antibodies to CB-1, Domain 2, Domain 3, and Heavy Chain. The immunological reactivities of hybridoma culture media with heavy chain and its three domains were determined by ELISA. As shown in Table II, the results allowed us to classify the 20 monoclonal antibodies to the heavy chain (group A) into 5 subgroups. The first subgroup comprised two monoclonal antibodies (HKG H4 and H13) which recognized only CB-1. The second subgroup included two monoclonal antibodies (HKG H3 and H54) which recognized only domain 2. The third subgroup comprised seven monoclonal antibodies (HKG H9, H16, H20, H34, LKG H1, H2, and H3) which recognized only domain 3. The fourth subgroup contained seven monoclonal antibodies (HKG H1, H7, H12, H14, LKG H5, H17, and H18) which recognized both domains 2 and 3. The last subgroup consisted of two monoclonal antibodies (HKG H8 and H9) which reacted with none of three domains but recognized only heavy chain. The specificity of these monoclonal antibodies to kininogens and their derivatives is shown in Figure 1.

Monoclonal Antibodies to Fragment 1·2 and Light Chains of Kininogens. To analyze the recognition sites of monoclonal antibodies on fragment 1·2 (group B) and light chains of kininogens (group C and group D), bindings of biotinylated monoclonal antibodies to HMW or LMW kininogen were examined in the presence of serially diluted unlabeled monoclonal antibodies.

Six monoclonal antibodies (HKG L7, L8, L12, L13, L17, and LKG L2) were satisfactorily labeled with biotin. Binding of biotinylated HKG L7 to solid-phase HMW kininogen (Figure 2A) was completely inhibited by unlabeled HKG L2 and L7 and effectively inhibited by HKG L18. Binding of biotinylated HKG L8 and L13, however, was not inhibited by

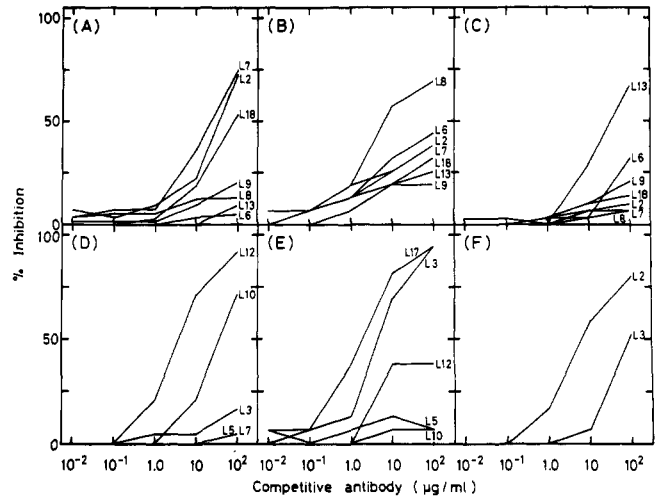

FIGURE 2: Competition binding assay of monoclonal antibodies to fragment 1·2 and light chains. Assay method of competitive inhibition is described under Methods. Binding of the biotinylated monoclonal antibodies (A) HKG L7, (B) HKG L8, (C) HKG L13, (D) HKG L12, (E) HKG L17, and (F) LKG L2 to solid-phase HMW kininogen (A–E) and LMW kininogen (F) was estimated in the presence of increasing concentrations of unlabeled monoclonal antibodies. Percent inhibition is shown on the ordinate as a function of unlabeled monoclonal antibody concentrations.

other unlabeled monoclonal antibodies (Figure 2B,C). As shown in Figure 2D, biotinylated HKG L12 was completely inhibited by unlabeled HKG L12 and effectively inhibited by HKG L10. Further, Figure 2E shows that biotinylated HKG L17 is completely inhibited by HKG L3 and L17 and moderately inhibited by HKG L12. Unlabeled HKG L5 does not inhibit the binding of biotinylated HKG L12 and L17 to solid-phase HMW kininogen.

These results indicate that three of seven monoclonal antibodies, designated HKG L2, L7, and L18, which recognize the fragment 1·2 of HMW kininogen, react with the same antigenic determinant or the close determinant and the others react with different antigenic determinant(s). Four of five monoclonal antibodies, designated HKG L10 and L12 and HKG L3 and L17, which recognize the light chain of HMW kininogen, react with the same antigenic determinant or the

Table I: Monoclonal Antibodies to Kininogens and Their Derivatives

monoclonal antibodies[c]	HMW-KG[d]	kinin-f HMW-KG	kinin-f and fr 1·2-f HMW-KG	H-chain	fr 1·2 and L-chain (HMW-KG)	LMW-KG I	LMW-KG II	kinin-f LMW-KG I	kinin-f LMW-KG II	L-chain[b] (LMW-KG)	sub-class	group[e]
HKG H1	1.60[a]	1.62	1.53	1.65	0.05	1.72	1.63	1.53	1.72	0.01	IgG_1	A
HKG H3	1.27	1.34	1.21	1.43	0.01	1.25	1.17	1.18	1.31	0.01	IgG_1	A
HKG H4	1.62	1.65	1.51	1.76	0.01	1.43	1.14	1.23	1.39	0.01	IgG_{2a}	A
HKG H7	1.30	1.31	1.14	1.32	0.05	1.29	1.17	1.17	1.24	0.01	IgG_{2b}	A
HKG H9	1.21	1.23	1.15	1.58	0.03	1.26	1.46	1.16	1.36	0.01	IgG_{2b}	A
HKG H12	1.47	1.48	1.41	1.52	0.06	1.52	1.46	1.44	1.49	0.01	IgG_1	A
HKG H13	1.37	1.23	1.16	1.37	0.01	1.04	0.78	0.98	1.16	0.01	IgG_1	A
HKG H14	1.53	1.49	1.49	1.58	0.03	1.57	1.46	1.48	1.48	0.01	IgG_1	A
HKG H16	1.53	1.60	1.54	1.58	0.04	1.57	1.57	1.53	1.67	0.01	IgG_1	A
HKG H20	1.64	1.60	1.50	1.71	0.05	1.66	1.53	1.58	1.65	0.01	IgG_1	A
HKG H34	0.67	0.55	0.53	0.60	0.01	0.49	0.55	0.56	0.64	0.05	IgG_{2b}	A
HKG H54	1.00	1.04	1.15	1.12	0.01	0.88	1.02	1.00	0.93	0.01	IgG_1	A
HKG L2	1.35	1.47	0.04	0.02	1.69	0.01	0.01	0.01	0.01	0	IgG_1	B
HKG L3	1.09	1.18	1.26	0.01	1.36	0.04	0.06	0.01	0.01	0.01	IgG_1	C
HKG L5	1.41	1.56	1.48	0.05	1.78	0.09	0.03	0.02	0.02	0.01	IgG_1	C
HKG L6	1.10	1.16	0.01	0.07	1.43	0.01	0.01	0.01	0.01	0.02	IgG_1	B
HKG L7	1.30	1.41	0.04	0.01	1.54	0.01	0	0.01	0.01	0.01	IgG_1	B
HKG L8	1.46	1.54	0.01	0.01	1.80	0	0.01	0.01	0	0	IgG_1	B
HKG L9	1.41	1.44	0.01	0.02	1.57	0.01	0	0	0	0	IgG_1	B
HKG L10	1.65	1.68	1.50	0.01	1.81	0.01	0	0.01	0.01	0	IgG_1	C
HKG L12	1.71	1.75	1.67	0.02	1.81	0.01	0.02	0.01	0.01	0	IgG_1	C
HKG L13	0.90	0.92	0	0.01	1.01	0	0	0	0	0	IgG_1	B
HKG L17	1.58	1.46	1.55	0.01	1.78	0.01	0.05	0.01	0.01	0	IgG_{2a}	C
HKG L18	1.36	1.39	0.02	0.01	1.50	0.01	0	0.01	0	0.01	IgG_1	B
LKG H1	0.90	1.11	1.09	1.07	0	0.82	0.96	1.07	1.02	0.01	IgG_1	A
LKG H2	0.92	1.11	1.08	1.05	0	0.91	1.07	1.05	1.02	0	IgG_1	A
LKG H3	0.95	1.05	1.07	1.01	0	0.94	1.04	1.05	1.02	0	IgG_1	A
LKG H5	0.93	1.07	1.04	1.00	0	0.84	1.01	1.01	1.00	0	IgG_1	A
LKG H8	0.78	0.82	0.82	0.85	0	0.62	0.81	0.74	0.63	0	IgG_1	A
LKG H9	0.82	0.83	0.85	0.82	0	0.68	0.81	0.77	0.66	0.01	IgG_1	A
LKG H17	1.08	1.15	1.11	1.08	0	0.83	1.03	1.06	1.02	0.01	IgG_1	A
LKG H18	1.04	1.05	1.04	1.05	0	0.77	1.04	0.96	0.93	0.01	IgG_1	A
LKG L2	0.06	0.08	0.04	0.05	0.02	1.14	0.70	0.24	0.36	0.80	IgG_1	D
LKG L3	0.05	0.07	0.01	0.04	0.02	1.02	0.68	0.26	0.41	0.77	IgG_1	D
control	0	0	0	0	0	0	0	0	0	0		

[a] Figures indicate absorbance at 492 nm. Antigens were bound to the microtiter plates at a concentration of 50 ng/100 μL per well overnight at 4 °C. Antibodies were used as culture medium containing 10% FCS. Optical density was measured at 10 min after the addition of substrate. [b] Light chain of LMW kininogen was bound to the microtiter plates at a concentration of 16.5 ng/100 μL per well overnight at 4 °C. [c] HKG, monoclonal antibodies produced by immunization of HMW kininogen. LKG, monoclonal antibodies produced by immunization of LMW kininogen. H, monoclonal antibodies which recognize heavy chain. L, monoclonal antibodies which recognize light chain or fragment 1·2. [d] HMW-KG, high molecular weight kininogen; kinin-f HMW-KG, kinin-free high molecular weight kininogen; kinin-f and fr 1·2-f HMW-KG, kinin-free and fragment 1·2 free high molecular weight kininogen; H-chain, heavy chain; L-chain, light chain; LMW-KG, low molecular weight kininogen; kinin-f LMW-KG, kinin-free low molecular weight kininogen. [e] Groups A, B, C, and D indicate the monoclonal antibodies specific to the heavy chain, fragment 1·2, the light chain of HMW kininogen, and the light chain of LMW kininogen, respectively.

closeby determinant, but HKG L5 appears to bind to a determinant different from that for the other four.

On the other hand, binding of biotinylated LKG L2 to solid-phase LMW kininogen was completely inhibited by LKG L2 and effectively inhibited by LKG L3 (group D) (Figure 2F). Further, binding of biotinylated LKG L3 was more strongly inhibited by LKG L2 than LKG L3 (data not shown). These data indicate that the monoclonal antibodies which recognize the light chain of LMW kininogen react with the identical antigenic determinant on the light-chain molecule.

Effect of Monoclonal Antibodies on Thiol Proteinase Inhibitor Activity. All 34 monoclonal antibodies were tested for the ability to suppress the TPI activity of HMW kininogen to papain. Measurement of the residual TPI activity after incubation of HMW kininogen with monoclonal antibody (IgG fraction) was performed in a monoclonal antibody to HMW kininogen molar ratio of 10:1. Two monoclonal antibodies disignated HKG H7 and H12 suppressed the TPI activity of HMW kininogen. The suppression patterns of TPI activities to papain after incubation of HMW or LMW kininogens with several concentrations of affinity-purified monoclonal antibodies (HKG H7 and H12) are shown in Figure 3A,B. In this experiment, one monoclonal antibody (HKG H12) suppressed the TPI activity of HMW and LMW kininogens to papain stronger than the other monoclonal antibody (HKG H7) at any molar ratios of antibody to kininogen.

Further, the suppression patterns of TPI activity of HMW kininogen to calpain I and calpain II by using the same monoclonal antibodies are shown in Figure 3C,D. Both monoclonal antibodies suppressed more than 90% of the TPI activity of HMW kininogen to calpain I, but they suppressed about 60% of its activity to calpain II. However, the molar ratio of monoclonal antibody to HMW kininogen to completely suppress the TPI activity was 0.4:1 for both calpains. Furthermore, we found that these monoclonal antibodies (HKG H7 and H12) did not react with 11-peptide containing the predicted reactive-site sequence (data not shown).

Effect of Monoclonal Antibodies on Clotting Activity. Fourteen monoclonal antibodies were tested for their ability to suppress the clotting activity of HMW kininogen in a monoclonal antibody to HMW kininogen molar ratio of 5:1 (Table III). Twelve monoclonal antibodies, which recognized the fragment 1·2 or light chain, suppressed the clotting activity, but the other monoclonal antibodies (HKG H7 and H13), which recognized the heavy chain, did not affect the clotting activity.

Table II: Specificity of Monoclonal Antibodies to CB-1, Domain 2, and Domain 3[a]

monoclonal antibodies	CB-1	domain 2	domain 3	heavy chain	reactive domain
HKG H1	0.01	0.59	0.19	0.50	2, 3
HKG H3	0.01	0.55	0.07	0.39	2
HKG H4	0.59	0.03	0.02	0.41	1
HKG H7	0.09	0.44	0.15	0.42	2, 3
HKG H9	0.05	0.01	0.49	0.42	3
HKG H12	0.11	0.63	0.30	0.60	2, 3
HKG H13	0.41	0.08	0.06	0.36	1
HKG H14	0.08	0.65	0.24	0.55	2, 3
HKG H16	0.07	0.06	0.65	0.65	3
HKG H20	0.09	0.06	0.76	0.70	3
HKG H34	0.03	0.01	0.20	0.22	3
HKG H54	0.02	0.24	0.08	0.22	2
LKG H1	0.02	0.01	0.63	0.53	3
LKG H2	0.01	0.01	0.67	0.57	3
LKG H3	0.03	0.02	0.62	0.55	3
LKG H5	0.02	0.71	0.29	0.56	2, 3
LKG H8	0.01	0.02	0.05	0.29	*b*
LKG H9	0.01	0.01	0.05	0.25	*b*
LKG H17	0.02	0.63	0.24	0.52	2, 3
LKG H18	0.02	0.56	0.19	0.46	2, 3
control	0.01	0.01	0.01	0.11	

[a] Antigens were bound to the microtiter plates at a concentration of 50 ng/100 μL per well overnight at 4 °C. Antibodies were used as the culture supernatant containing 10% FCS. Optical density was measured at 10 min after the addition of substrate. [b] Not reacted with individual three domains but with only heavy chain.

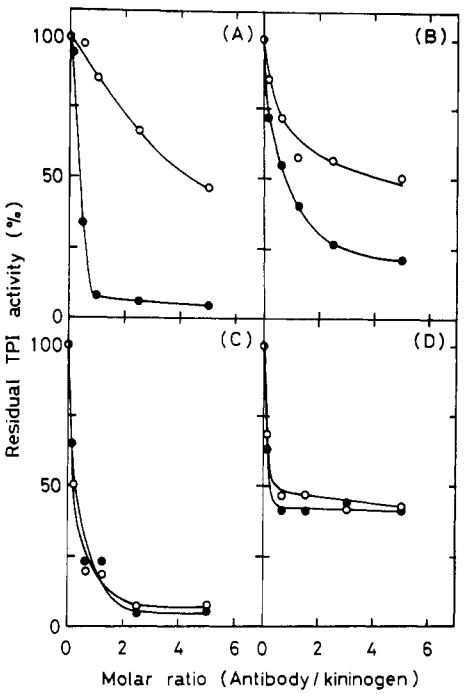

FIGURE 3: Effect of monoclonal antibodies on TPI activity of HMW and LMW kininogens. Suppression assays of TPI activity after 2-h incubation of purified HMW and LMW kininogens with various concentrations of monoclonal antibodies, HKG H7 (○) and HKG H12 (●), were performed as described under Methods (assays 1 and 2). Suppression patterns of HMW kininogen for papain (A), LMW kininogen for papain (B), HMW kininogen for calpain I (C), and HMW kininogen for calpain II (D) are shown. Residual TPI activity was indicated as the percentage of the full TPI activity when normal mouse IgG was added to the reaction mixture instead of these monoclonal antibodies.

The two monoclonal antibodies (HKG L2 and L5) were examined in detail for their effects on the clotting activity (Figure 4). HKG L5 neutralized more than 90% of the activity in a monoclonal antibody to HMW kininogen molar ratio of 11:1, while HKG L2 neutralized only about 70% of the activity in molar ratios of 5.5:1 and 11:1.

Table III: Effects of Monoclonal Antibodies on Clotting Activities of HMW Kininogen[a]

monoclonal antibodies	% inhibition	monoclonal antibodies	% inhibition
HKG L2	74.4	HKG L3	58.8
HKG L6	63.3	HKG L5	75.0
HKG L7	58.9	HKG L10	56.7
HKG L8	66.7	HKG L12	26.7
HKG L9	54.4	HKG L17	50.0
HKG L13	54.4	HKG H7	0.0
HKG L18	21.0	HKG H13	0.0
		normal mouse IgG	0.0

[a] HMW kininogen (6.72 nmol) was mixed with each monoclonal antibody (33.6 nmol) in an antibody to HMW kininogen molar ratio of 5:1. The mixtures were incubated at room temperature for 4 h. The clotting activity of HMW kininogen was measured by the method described under Methods (assay 3).

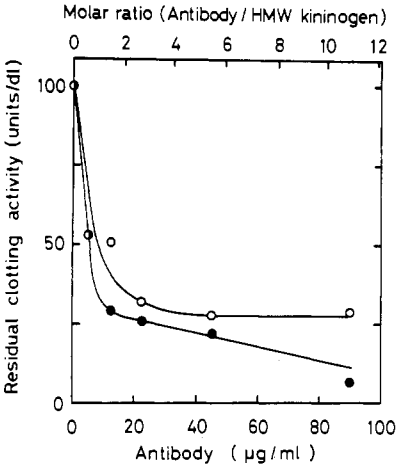

FIGURE 4: Effect of monoclonal antibodies on clotting activity of HMW kininogen. Residual clotting cofactor activity of kininogen was estimated after 2-h incubation of purified HMW kininogen and various concentrations of monoclonal antibodies: HKG L2 (○) and HKG L5 (●). For assay conditions, see Methods (assay 4).

DISCUSSION

In this report, we described the production and characterizations of 34 monoclonal antibodies to human HMW and LMW kininogens. These monoclonal antibodies specifically recognized the several peptide regions such as heavy chains, fragment 1-2, and light chains of HMW and LMW kininogens (Table I). Recently, Ohkubo et al. (1984) reported that the amino acid sequence of the heavy chain of α_2TPI contained two repeat sequences (104–225 and 226–347 amino acid sequences). Further, Salvesen et al. (1986) also reported that the heavy chain of LMW kininogen contained three homologous domains (domains 1, 2, and 3). They compared the amino acid sequence of three domains and showed that domain 2 and domain 3 were very similar to each other as compared to domain 1. These reports suggest that common antigenic sites may exist between domain 2 and domain 3. When monoclonal antibodies which reacted with the heavy chain of kininogens (group A) were characterized, 7 of the 20 monoclonal antibodies cross-reacted with both domain 2 and domain 3, but others specifically reacted with each fragment (CB-1, domain 2, and domain 3). On the other hand, in a comparison of the amino acid sequences between CB-1 and domain 2, the C-terminal region of CB-1 (123–160 amino acid sequence) partly overlapped with the N-terminal sequence of domain 2. However, two monoclonal antibodies (HKG H4 and H13), which recognized CB-1, reacted neither with domain 2 nor

with the light chain of LMW kininogen. Thus, these two monoclonal antibodies were thought to specifically react with domain 1. Among the heavy-chain-specific monoclonal antibodies, both LKG H8 and H9 did not react with CB-1, domain 2, and domain 3 (Table II). Further, these monoclonal antibodies did not react with domains 1·2 (1–244 amino acid sequence) (data not shown). The data suggest that the two monoclonal antibodies may recognize the connecting region between domain 2 and domain 3 or the tertiary structure of the heavy chain.

Most of the heavy-chain-specific monoclonal antibodies failed to react with S-carboxymethylated HMW kininogen. However, monoclonal antibodies which recognized fragment 1·2 and light chain of HMW kininogen could react with S-carboxymethylated HMW kininogen as well as native HMW kininogen (data not shown). The result suggests that most of the heavy-chain-specific monoclonal antibodies recognize the higher order structure, while the fragment 1·2 and light-chain-specific monoclonal antibodies react with the primary structure.

Ohkubo et al. (1984) reported that in a comparison of the amino acid sequence of α_2TPI with those of several different TPIs with low molecular weights, clear homologies were observed, and among these inhibitors the highly conserved amino acid sequence was Gln-Val-Val-Ala-Gly. They suggested that this sequence was the potential reactive site for these inhibitors. The monoclonal antibodies designated HKG H7 and H12 suppressed the TPI activity of HMW and LMW kininogens to papain (Figure 3A,B). However, the antibodies did not react with 11-peptide (Ala-Arg-Val-*Gln-Val-Val-Ala-Gly*-Lys-Lys-Tyr) containing the predicted reactive-site sequence (data not shown). Further, these two monoclonal antibodies were confirmed to recognize both domain 2 and domain 3 of the heavy chain, although the reactivity to domain 3 was weaker than that to domain 2 (Table II). It is speculated as a possible explanation that the antigenic determinant(s) recognized by these monoclonal antibodies may be located closely to the reactive site on the heavy chain and that the antibody molecules of HKG H7 and H12 may interfere with the inhibitory activity by steric hindrance or induction of conformational change. In this report, these two monoclonal antibodies completely suppressed the TPI activity of HMW kininogen to calpain I, but they suppressed only about 60% of its activity to calpain II (Figure 3C,D). Salvesen et al. (1986) described that calpain exhibited the affinity to bind only with domain 2 of the heavy chain of LMW kininogen. Further, we reported that the molar ratios of calpains to HMW kininogen to give complete inhibition of calpains were 1.4 for calpain I and 2.0 for calpain II (Ishiguro et al., 1987). The above data suggest that calpain I activity was inhibited by domain 2 of the heavy chain mainly and calpain II activity by both domain 2 and domain 3. On the basis of Figure 3, it is also suggested that the reactive site(s) of kininogens for papain and calpains is (are) located at the identical or closely related region on the heavy-chain molecule.

In the results of competitive assay (Figure 2A–C), monoclonal antibodies which recognized the fragment 1·2 (group B) were further classified into five subgroups for binding sites. This result indicates that fragment 1·2 possesses at least five different antigenic determinants, being located separately from each other. Light-chain-specific monoclonal antibodies (group c) were classified into three subgroups according to the difference of the binding site (Figure 2D,E). All monoclonal antibodies of both groups, however, suppressed the clotting activity as shown in Table III. In this regard, Sugo et al. (1980) and Scott et al. (1983) have reported that the fragment 1·2 moiety of HMW kininogen adsorbs to negatively charged surface and enhances the initiation of surface-activated blood coagulation. It is therefore likely that fragment 1·2 specific monoclonal antibodies, especially HKG L2, may interfere with the attachment of the fragment 1·2 moiety to the negatively charged surface (Table III, Figure 4). The light chain of HMW kininogen forms the complex with prekallikrein (Mandel et al., 1976) or factor XI (Thompson et al., 1977) in 1:1 stoichiometry and enhances the initiation of surface-activated blood coagulation. Tait and Fujikawa (1986) have recently reported that the binding site of HMW kininogen with plasma prekallikrein is located near the carboxyl terminus of the light chain. These reports suggest that the light-chain-specific monoclonal antibodies, especially HKG L5, may interfere with the binding of plasma prekallikrein and/or factor XI to the HMW kininogen light chain.

Obviously, the monoclonal antibodies, which suppress the TPI activity and the clotting activity of HMW kininogen, have proved the usefulness for the investigations of the relationship between the structure and function of kininogens. Further, these monoclonal antibodies would be useful for the clinical applications, namely, the qualitative and quantitative investigations of the kininogens in various pathological states such as congenital or acquired hemorrhagic disorders, inflammation, and cancers.

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Spinach Chloroplast Fructose-1,6-bisphosphatase: Identification of the Subtilisin-Sensitive Region and of Conserved Histidines†

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ABSTRACT: Chloroplast fructose-1,6-bisphosphatase (FbPase) is an essential enzyme in the photosynthetic pathway of carbon dioxide fixation into sugars. The properties of the chloroplast enzyme are clearly distinct from those of cytosolic gluconeogenic FbPases. Light-dependent activation via a ferredoxin/thioredoxin system and insensitivity to inhibition by AMP are unique characteristics of the chloroplast enzyme. However, preliminary amino acid sequence data (78 residues) have demonstrated that a significant degree of amino acid sequence similarity exists between spinach chloroplast and mammalian gluconeogenic fructose-1,6-bisphosphatase [Harrsch, P. B., Kim, Y., Fox, J. L., & Marcus, F. (1985) *Biochem. Biophys. Res. Commun. 133*, 520–526]. In the present study, we have identified two structural features of spinach chloroplast FbPase that appear to be common to all FbPases. These include (a) the presence of a protease-sensitive area located in a region equivalent to residues 51–71 of mammalian FbPases and (b) the recognition of two conserved histidine residues, equivalent to histidines-253 and -311 of the mammalian enzymes. In addition, we have obtained sequence information accounting for more than three-fourths of the primary structure of spinach chloroplast FbPase. The high degree of homology observed between the chloroplast enzyme and gluconeogenic FbPases suggests a common evolutionary origin for all fructose-1,6-bisphosphatases in spite of their different functions and modes of regulation.

Fructose-1,6-bisphosphatase (FbPase)[1] catalyzes the hydrolysis of fructose 1,6-bisphosphate to fructose 6-phosphate and inorganic phosphate. Because of its key role in gluconeogenesis, the enzyme has been extensively studied. Most of our knowledge on the subject comes from studies of the

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[1] Abbreviations: FbPase, fructose-1,6-bisphosphatase; SDS, sodium dodecyl sulfate; HPLC, high-performance liquid chromatography; FPLC, fast protein liquid chromatography; TFA, trifluoroacetic acid; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; PTH, phenylthiohydantoin; EDTA, ethylenediaminetetraacetic acid; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; NADP, nicotinamide adenine dinucleotide phosphate; PTH, phenylthiohydantoin.

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