Isolation and Characterization of Neutralizing Single-Chain Antibodies against *Xenopus* Mitogen-Activated Protein Kinase Kinase from Phage Display Libraries†

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ABSTRACT: MAP kinase kinase (MAPKK) is a dual specificity protein kinase that phosphorylates and activates MAP kinase *in vivo*. In this study, four mouse monoclonal single-chain Fv (scFv) antibodies (Y1−6, Y1−7, Y3−6, and Y3−11) that can specifically bind to *Xenopus* MAPKK were isolated from combinatorial scFv-displaying phage libraries. Three scFv clones (Y1−6, Y1−7, and Y3−6) were shown to efficiently inhibit MAPKK activity *in vitro*. Point mutation (D98K) at V_H-CDR3 of one (Y1−6) of these three clones markedly reduced its neutralizing activity. The wild-type scFv (Y1−6) inhibited the Mos-induced MAP kinase activation and germinal vesicle breakdown when injected into immature *Xenopus* oocytes, whereas the mutant scFv, Y1−6 (D98K), did not. The three neutralizing scFv clones (Y1−6, Y1−7, and Y3−6) were shown to bind to NH_2-terminal residues 1−23 of *Xenopus* MAPKK, whereas the epitope of a Y3−11 clone with no neutralizing activity was shown to lie between residues 33 and 67 of MAPKK. Furthermore, a synthetic peptide (the N16 peptide) corresponding to residues 2−17 of MAPKK suppressed the neutralizing activity of the wild-type Y1−6, and a rabbit polyclonal antibody against the N16 peptide was found to possess a strong neutralizing activity against MAPKK. These results demonstrate that the neutralizing antibodies characterized here inhibit the kinase activity of MAPKK by binding to the NH_2-terminal segment of MAPKK.

Mitogen-activated protein (MAP)[1] kinase is from a family of highly conserved serine/threonine kinases that are activated commonly by numerous extracellular signals in eukaryotic cells (Cobb et al., 1991; Sturgill & Wu, 1991; Pelech & Sanghera, 1992; Thomas, 1992). Activation of MAP kinase requires phosphorylation on both threonine and tyrosine residues, which is catalyzed by a dual specificity protein kinase, MAP kinase kinase (MAPKK) (Ahn et al., 1992; Nishida & Gotoh, 1993). MAPKK is, in turn, activated by phosphorylation on two serine/threonine residues, which is catalyzed by MAPKK kinases, such as Raf, Mos, and a group of structurally related kinases, including STE11 and MEKK (Marshall, 1995; Herskowitz, 1995; Xu et al., 1995). Mammalian MAPKK has recently been shown to play important roles in cellular responses such as proliferation, differentiation, and apoptosis (Cowley et al., 1994; Mansour et al., 1994; Xia et al., 1995).

MAPKK and MAP kinase are activated before germinal vesicle breakdown (GVBD) in the meiotic maturation of *Xenopus* oocytes triggered by progesterone (Ferrell et al., 1991; Gotoh et al., 1991a,b; Posada et al., 1991; Matsuda et al., 1992). GVBD is caused by the activation of the maturation-promoting factor (MPF), a complex of $p34^{cdc2}$ kinase and cyclin B that promotes transition from G_2 to M phase universally in eukaryotic cells (Masui & Clarke, 1979; Nurse, 1990; Murray & Hunt, 1993). Progesterone induces the synthesis of Mos protein, one of MAPKK kinases (Nebreda et al., 1993; Posada et al., 1993), which is necessary and sufficient for GVBD and the arrest at the second meiotic metaphase (Sagata et al., 1988, 1989; Yew et al., 1992).

Overexpression of dominant negative mutants in cells is one effective approach to elucidate physiological functions of signaling components. In fact, dominant negative MAPKK in which the two activating phosphorylation sites were replaced with alanine residues was recently shown to inhibit the NGF-induced differentiation of PC12 cells (Cowley et al., 1994). However, overexpression of this inactive MAPKK might inhibit not only the activation of endogenous MAPKK but also that of other potential substrates of MAPKK kinases that lie in distinct signaling pathways, since Raf-1 strongly binds to MAPKK in some systems (Huang et al., 1993; Moodie et al., 1993) and Mos interacts with MAPKK in the yeast two-hybrid system (Chen & Cooper, 1995). The need to express a sufficient amount of dominant negative mutants for a long time may also induce unexpected secondary effects.

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[1] Abbreviations: MAP, mitogen-activated protein; MAPKK, MAP kinase kinase; GVBD, germinal vesicle breakdown; MPF, maturation-promoting factor; scFv, single-chain Fv; mAb, monoclonal antibody; CDR, complementarity-determining region; FR, framework region; PCR, polymerase chain reaction; ELISA, enzyme-linked immunosorbent assay; GST, glutathione *S*-transferase.

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An alternative approach is to generate neutralizing antibodies and microinject them into cells (Mulcahy et al., 1985; Deshpande & Kung, 1987; Nebreda et al., 1995). Recently, we produced a neutralizing antibody (mouse polyclonal antibody) that can specifically and efficiently inhibit *Xenopus* MAPKK activity *in vitro* (Kosako et al., 1994a). Microinjection of this polyclonal antibody into immature oocytes inhibited (or delayed) progesterone- or Mos-induced GVBD, suggesting that MAPKK is required for initiating oocyte maturation (Kosako et al., 1994a). Furthermore, Mos-induced metaphase arrest in *Xenopus* embryos was shown to be prevented by coinjection of this anti-MAPKK neutralizing antibody (Kosako et al., 1994b). However, the inhibitory effect of this neutralizing antibody was not complete, and experiments using normal mouse IgG as a control or the antigen (bacterially expressed MAPKK) to cancel the neutralizing effect of the antibody were not necessarily ideal, sufficient control experiments. Furthermore, it was difficult to determine the epitope(s) recognized by this polyclonal antibody.

The display of antibody fragments (such as Fab or single-chain Fv) by fusion to the minor coat protein III on the surface of filamentous bacteriophage has offered a new way to obtain monoclonal antibodies [McCafferty et al., 1990; Barbas et al., 1991; Clackson et al., 1991; for reviews, see Lerner et al. (1992), Marks et al. (1992), and Winter et al. (1994)]. In this method, heavy (V_H) and κ light (V_κ) chain V genes are amplified by the polymerase chain reaction (PCR) and cloned into M13 surface expression vectors to make combinatorial libraries. Phage with antigen-binding activities can be selected by binding to and eluting from immobilized antigen. Interposing an amber codon between the antibody fragment and the coat protein allows the fragments to be secreted from infected bacteria. Furthermore, various antibody fragments against many different antigens have recently been isolated without immunization, from the same "single-pot" phage library constructed from genomic V segments (Nissim et al., 1994; Griffiths et al., 1994). The advantage of this method is that isolated antibodies can be mutated in V genes to change binding affinities.

In this study, we have isolated from phage display libraries scFv clones that can efficiently inhibit *Xenopus* MAPKK activity *in vitro*. Point mutation (D98K) at V_H-CDR3 of one of these clones markedly reduced its neutralizing activity. This mutant and wild-type scFv antibodies could be used to show the requirement of MAPKK in the Mos-induced oocyte maturation. This phage display system may provide useful tools for analyses of cellular functions of signaling components in general. Furthermore, we have demonstrated that the neutralizing scFv clones inhibit the kinase activity of MAPKK by binding to the NH_2-terminal segment of *Xenopus* MAPKK.

MATERIALS AND METHODS

Antibodies and Recombinant Proteins. Mouse anti-MAPKK monoclonal antibody (4A5) was produced by using bacterially expressed *Xenopus* MAPKK as an antigen. Rabbit anti-peptide antibody (anti-N16 antibody) was raised against the synthetic peptide corresponding to amino acids 2−17 of *Xenopus* MAPKK (Kosako et al., 1992). Rabbit anti-MAP kinase antibody was prepared as described previously (Gotoh et al., 1991b). Rat anti-mouse κ-chain-Sepharose 4B and horseradish peroxidase-conjugated rat anti-mouse κ-chain antibody were purchased from Zymed. Recombinant *Xenopus* MAPKK was prepared by cleaving GST−MAPKK with Factor Xa (Kosako et al., 1993) and by removing GST with a glutathione−agarose column. The malE−mos fusion protein (*Xenopus* c-*mos* protein kinase fused downstream of the maltose-binding protein of *Escherichia coli*) was prepared as described previously (Nebreda & Hunt, 1993; Kosako et al., 1994a). GST−KNMAPK, a GST fusion protein of the kinase-negative (KN) mutant of *Xenopus* MAP kinase (MAPK), was prepared as described previously (Kosako et al., 1993).

cDNA Synthesis from Immunized Mouse Spleen. A female BALB/c mouse was given intraperitoneal injections of recombinant *Xenopus* MAPKK (100 μg). Two booster injections (each 100 μg) were given at intervals of 3 weeks. Poly(A) RNA was isolated from the spleen 2 weeks after the last injection using the QuickPrep mRNA Purification Kit (Pharmacia). Then the first strand cDNA was synthesized using the SuperScript Preamplification System (BRL) with random hexamer.

PCR Primers. All the primers used for PCR are shown below. Restriction enzyme sites are underlined. Primer names precede the sequences: NT150, 5′-GCTGGATTGTTATTACTCGC-3′; NT151, 5′-AGTTCAGGCGGAGGCGGATCC-3′; NT152, 5′-CTCGCTTCCGCCACCTCCAGA-3′; NT266, 5′-AGGTGCAGCT(G/T)(C/A)AGGAGTCAGGACCT(A/G)G-3′; NT267, 5′-AGGTCCAGCTGCA(A/G)CA(A/G)TCTGGATCTGA-3′; NT268, 5′-AGGT(C/T)CA(A/G)CTGCAGCAG(C/T)CTGGGGC(A/T)GA-3′; NT269, 5′-AGGTGAAGCT(G/T)(G/C)T(G/C)GAGTCTGGAGG(A/T)GG-3′; NT270, 5′-AAGTG(A/C)AGCT(G/T)G(A/T)GGAGTCTGG(A/G)GGAGG-3′; NT271, 5′-AGGTGCAGCT(G/T)CTCGAGTCAGGACCT(A/G)G-3′; NT272, 5′-AGGTCCAGCTGCTCGAGTCTGGATCTGA-3′; NT273, 5′-AGGT(C/T)CA(A/G)CTGCTCGAG(C/T)CTGGGGC(A/T)GA-3′; NT274, 5′-AGGTGAAGCT(G/T)CTCGAGTCTGGAGG(A/T)GG-3′; NT275, 5′-AAGTG(A/C)AGCT(G/T)CTCGAGTCTGG(A/G)GGAGG-3′; NT276, 5′-GACATTGTGATG(A/T)CACAGTCTCCATC-3′; NT277, 5′-GATGTT(G/T)TGATGACCCAAACTCCACTC-3′; NT278, 5′-GACATTGTGCTGAC(A/C)CA(A/G)TCTCC(A/T)GC-3′; NT279, 5′-GAAA(A/T)TGT(G/T)CTCACCCAGTCTCCAGC-3′; NT280, 5′-GATATCCAGATCACACAGACTACATC-3′; NT281, 5′-GA(T/C)ATCAAGATGACCCAGTCTCCATC-3′; NT282, 5′-GACATTGTGATGACCCAGTCTCACAA-3′; NT283, 5′-CACGCAGAGCTCGTGATG(A/T)CACAGTCTCCATC-3′; NT284, 5′-CACGCAGAGCTC(G/T)TGATGACCCAAACTCCACTC-3′; NT285, 5′-CACGCAGAGCTCGTGCTGAC(A/C)CA(A/G)TCTCC(A/T)GC-3′; NT286, 5′-CACGCAGAGCTCGT(G/T)CTCACCCAGTCTCCAGC-3′; NT287, 5′-CACGCAGAGCTCCAGATGACACAGACTACATC-3′; NT288, 5′-CACGCAGAGCTCAAGATGACCCAGTCTCCATC-3′; NT289, 5′-CACGCAGAGCTCGTGATGACCCAGTCTCACAA-3′; NT290, 5′-CTGGACAGGGATCCAGAGTTCC-3′; NT291, 5′-CAGTCCAACTGTTCAGGACG-3′; NT292, 5′-GACTGAGGCACCTCCAGATG-3′; YA55, 5′-AGCATCAGATCTTTTGATTTCCAGCTTGGTGCCT-3′; YA56, 5′-AGCATCAGATCTTTTTATTTCCAGCTTGGTCCCC-3′; YA57, 5′-AGCATCAGATCTTTTTATTTCCAACTTTGTCCCC-3′; YA58, 5′-AGCATCAGATCTTTTCAGCTCCAGCTTGGTCCCA-

3′; YA59, 5′-CGTTGTGCTAGCTGAGGAGACGGTGAC-CGTGGT-3′; YA60, 5′-CGTTGTGCTAGCTGAGGAGACT-GTGAGAGTGGT-3′; YA61, 5′-CGTTGTGCTAGCTGCA-GAGACAGTGACCAGAGT-3′; YA62, 5′-CGTTGTGCT-AGCTGAGGAGACGGTGACTGAGGT-3′; HK4, 5′-CGC-GACCTGCCCATAGGCCCTCTTGCACAG-3′; HK5, 5′-GCGCACCTGCGAGGGCCTATGAAGTACT-GGGGTCAAGGAACC-3′.

Amplification of V_H and V_κ Genes. For the first PCR to amplify V_H-C_H1 and V_κ-C_κ fragments, the reaction mixture contained 1 μL of the cDNA sample, 50 μM each dNTP, an appropriate pair of primers (1 μM) (see below), and 2.5 u of Taq DNA polymerase in 50 μL of Taq buffer. The reaction was performed at 94 °C for 30 s, 57 °C for 30 s, and 72 °C for 30 s with 35 cycles for each of the 12 pairs of primers as below: NT266 × NT290 (for V_H1A and V_H1B) (second PCR; NT271 × YA59−62), NT267 × NT290 (for V_H2A) (second PCR; NT272 × YA59−62), NT268 × NT290 (for V_H2B, V_H2C, and V_H5) (second PCR; NT273 × YA59−62), NT269 × NT290 (for V_H3A and V_H3B) (second PCR; NT274 × YA59−62), NT270 × NT290 (for V_H3C and V_H3D) (second PCR; NT275 × YA59−62), NT276 × NT291 (for V_κ1) (second PCR; NT283 × YA55−58), NT277 × NT291 (for V_κ2) (second PCR; NT284 × YA55−58), NT278 × NT291 (for V_κ3) (second PCR; NT285 × YA55−58), NT279 × NT291 (for V_κ4) (second PCR; NT286 × YA55−58), NT280 × NT291 (for V_κ5a) (second PCR; NT287 × YA55−58), NT281 × NT291 (for V_κ5b) (second PCR; NT288 × YA55−58), NT282 × NT291 (for V_κ5c) (second PCR; NT289 × YA55−58).

For the second PCR to amplify V_H and V_κ fragments, 10 ng of each gel-purified V-C segment (about 500 bp) was mixed with each of the 48 pairs of primers as shown in parentheses above. The reaction was performed at 94 °C for 30 s, 57 °C for 30 s, and 72 °C for 30 s with 25 cycles.

Combinatorial Library Construction. Gel-purified V gene fragments (about 350 bp) were digested with *Xho*I and *Nhe*I (for V_H) or *Sst*I and *Bgl*II (for V_κ) and ligated with the correspondingly cut pScUAGΔcp3-M vector, a derivative of pScUAGΔcp3 (Akamatsu et al., 1993), in which the C region of human κ-chain was replaced with that of mouse. The ligated DNAs were electroporated into *E. coli* strain DH5α/F′IQ (BRL), and cells were grown overnight in 200 mL of terrific broth with 50 μg/mL ampicillin and 1% glucose. Phagemid DNAs purified from V_H (4×10^7 clones) and V_κ (3×10^7 clones) libraries were digested with *Nhe*I and *Eco*RI. V_H-containing fragments (4.3 kb) and V_κ-containing fragments (1.3 kb) were ligated and electroporated into DH5α/F′IQ. Cells were grown at 37 °C in 120 mL of 2YT broth with 50 μg/mL ampicillin and 1% glucose until the OD_{600} reached 1.0. Then cells (3×10^7 clones) were infected with VCSM13 helper phage (Stratagene) at a multiplicity of infection of 20, incubated at 37 °C for 15 min, and cultured overnight in 200 mL of 2YT broth with 50 μg/mL ampicillin and 75 μg/mL kanamycin. Phage particles were purified from the culture supernatant by two PEG precipitations and resuspended in 2.4 mL of HBS (25 mM HEPES-NaOH at pH 7.9 and 150 mM NaCl). The phagemid titer, measured as ampicillin-resistant colony-forming units (cfu), was 2.0×10^{12} mL^{-1}.

Isolation of MAPKK-Binding Clones. Phage (2.4×10^{12} cfu) were first loaded on an anti-mouse κ-chain-Sepharose column (0.2 mL). After being washed with HBS, phage were eluted with 3 mL of 0.1 M glycine hydrochloride (pH 2.1) and neutralized with 0.3 mL of 1.5 M Tris-HCl (pH 8.8). After the addition of 3 mL of HBS containing 0.2% bovine serum albumin, the phage suspension was loaded on a MAPKK−Toyopearl column (0.2 mL) prepared by coupling recombinant *Xenopus* MAPKK to AF-Tresyl Toyopearl 650M (Tosoh). Phage eluted with 0.1 M glycine hydrochloride (pH 2.1) were used to infect logarithmically growing DH5α/F′IQ, and cells were grown in 40 mL of 2YT broth with 50 μg/mL ampicillin and 1% glucose until the OD_{600} reached 1.0. Rescue of phagemid by VCSM13 superinfection and purification of phage particles by two PEG precipitations were carried out as described in the previous section. The phage suspension in 10 mL of HBS was used for the next round of selection. After three rounds of selection, an aliquot of phage eluted from a MAPKK−Toyopearl column was used to infect W3110/F′IQ, and cells were plated on an LB plate containing 50 μg/mL ampicillin and 1% glucose. Then 36 ampicillin-resistant colonies were picked and individually grown in 1 mL of 2YT broth with 1% glycerol at 30 °C. After the addition of 1 mM IPTG, cells were grown overnight at 30 °C. Aliquots (70 μL) of culture supernatants containing soluble scFv fragments were added to MaxiSorp plates (Nunc) that had been coated with 100 μL of 5 μg/mL recombinant *Xenopus* MAPKK in 0.2 M sodium carbonate buffer (pH 9.4). Bound scFv fragments were detected by horseradish peroxidase-conjugated anti-mouse κ-chain antibody and ImmunoPure ABTS (Pierce) as described by the supplier. Positive clones were stored in 2YT broth with 15% glycerol at −80 °C.

HaeIII Fingerprinting and Sequencing. Antigen-binding clones, toothpicked from frozen glycerol stocks, were amplified by PCR (94 °C for 30 s, 50 °C for 30 s, and 74 °C for 30 s with 25 cycles) using NT150 and NT292 primers. The products (about 850 bp) were purified using the QIAquick PCR Purification Kit (Qiagen), and aliquots (800 ng) were digested with *Hae*III and analyzed on a 2% agarose gel. Clones with different digestion patterns were sequenced by the dideoxy method using DyeDeoxy chain terminators (Applied Biosystems) and an Applied Biosystems 373A DNA sequencer. The purified PCR products (200 ng) were used as templates with the following primers: NT150 (5′ primer for V_H), NT151 (5′ primer for V_κ), NT152 (3′ primer for V_H), and NT292 (3′ primer for V_κ).

Production of a Mutant Clone. Phagemid DNA from Y1−6 was used as a template to amplify the DNA fragment encoding the NH_2-terminus of scFv (5′ fragment; 353 bp) by PCR (94 °C for 30 s, 50 °C for 30 s, and 74 °C for 30 s with 25 cycles) using the 5′ primer (NT150) and the mutagenic primer (HK4) and the DNA fragment encoding the COOH terminus (3′ fragment; 531 bp) using the mutagenic primer (HK5) and the 3′ primer (NT292). The 5′ or 3′ fragment was digested with *Xho*I and *Bsp*MI or *Bsp*MI and *Sst*I, respectively, and the DNA fragment (283 or 107 bp, respectively) was gel-purified. These two fragments were ligated with *Xho*I- and *Sst*I-digested phagemid DNA from Y1−6 to yield Y1−6 (D98K). The V_H sequence of this mutant Y1−6 was analyzed as described in the previous section to ensure that only the desired mutation had been introduced.

Three-Dimensional Modeling. Three-dimensional models for the binding domains of Y1−6 (wild type) or Y1−6 (D98K) were constructed with the help of the ABMOD and

ENCAD computer programs of Levitt (1983). ABMOD first aligns the sequence of the binding domains to be modeled to a structurally based multiple-sequence alignment of a set of binding domains of known three-dimensional structure. Then, the binding domains being built are divided into 28 segments (14 each in V_H and V_L). Each segment will have an optimal sequence match with a corresponding segment in one of the binding domains of known structure; the crystal structures of each of the optimally matched segments are combined to generate an initial model for the entire V_H-V_L structure. This model is then energy-refined by the program ENCAD which also carries out side chain substitutions and main chain insertions/deletions whenever required. Detailed descriptions of the combined ABMOD-ENCAD protocol for modeling of antibody binding domains appeared previously (Levy et al., 1989; Zilber et al., 1990; Glaser et al., 1992), and a critical evaluation of its results by comparing it to crystallographic data exists (Chothia et al., 1989).

Purification of scFv Fragments. Soluble scFv-expressing clones, toothpicked from frozen glycerol stocks, were grown overnight in 5 mL of 2YT broth with 50 μg/mL ampicillin and 1% glucose at 30 °C and then grown at 30 °C in 200 mL of 2YT broth with 50 μg/mL ampicillin until the OD_{600} reached 0.6. After the addition of 1 mM IPTG, cells were grown overnight at 30 °C. Each culture supernatant obtained by centrifugation at 90000*g* for 60 min at 2 °C was loaded on an anti-mouse κ-chain-Sepharose column (1.5 mL). After being washed with 3 mL of 20 mM Tris-HCl (pH 7.5), 1 M NaCl, 3 mL of 20 mM Tris-HCl (pH 7.5), 1% Triton X-100, and 3 mL of 20 mM Tris-HCl (pH 7.5) and 88 mM NaCl, scFv fragments were eluted with 0.1 M glycine hydrochloride (pH 2.4). The eluted scFv fragments were dialyzed overnight against 20 mM Tris-HCl (pH 7.5) and 88 mM NaCl at 4 °C, concentrated to about 5 mg/mL using a Centricon-30 apparatus (Amicon), and stored at −80 °C. Protein concentrations were determined by the method of Bradford (1976) using rabbit γ-globulin as a standard.

Immunoblotting. After SDS−polyacrylamide gel electrophoresis, proteins were transferred to poly(vinylidene difluoride) membranes (Immobilon-P, Millipore) in a solution containing 25 mM Tris, 192 mM glycine, and 20% methanol. After blocking with 3% bovine serum albumin (Sigma) in TBS-T [20 mM Tris-HCl (pH 7.6), 500 mM NaCl, and 0.1% Tween 20], membranes were incubated with rabbit anti-MAP kinase antibody, rabbit anti-N16 antibody, mouse 4A5 monoclonal antibody, or anti-MAPKK scFv fragments (this study) in TBS-T for 2 h. Immunoreactive bands were detected by horseradish peroxidase-conjugated second antibody (anti-rabbit Ig antibody, anti-mouse Ig antibody, or anti-mouse κ-chain antibody) and the ECL Western blotting detection system (Amersham).

Oocyte Microinjections. Stage VI *Xenopus* immature oocytes were selected by hand after dissected ovaries were treated with 2 mg/mL collagenase (Wako) in calcium-free modified Barth's solution [10 mM HEPES (pH 7.5), 88 mM NaCl, 1 mM KCl, 2.4 mM $NaHCO_3$, and 0.82 mM $MgSO_4$] for 2 h at 22 °C. Oocytes were microinjected with 50 nL of samples using an injector (Eppendorf Transjector 5246) in 50% Leibovitz-15 medium (Gibco). For assays of MAP kinase and histone H1 kinase, groups of 10 oocytes were homogenized at each time point in 200 μL of XB [20 mM Tris-HCl (pH 7.5), 60 mM β-glycerophosphate, 10 mM $MgCl_2$, 10 mM EGTA, 2 mM DTT, 1 mM Na_3VO_4, 1 mM PMSF, and 20 μg/mL aprotinin]. Homogenates were clarified by centrifugation at 15000*g* for 15 min at 2 °C.

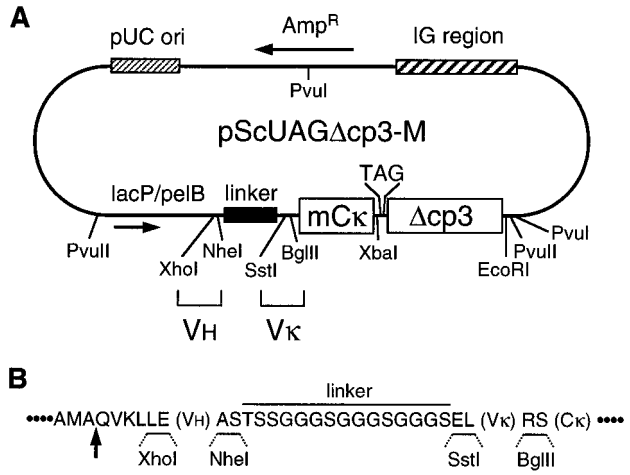

FIGURE 1: Structure of the phagemid vector pScUAGΔcp3-M. (A) A schematic diagram of the vector. Symbols used: IG region, replication origin of M13 phage; Amp^R, β-lactamase gene for ampicillin resistance; *pUC* ori, replication origin of pUC19; lacP, *E. coli lac* promoter; pelB, *pelB* leader peptide; linker, synthetic sequence encoding a short polypeptide linker to connect V_H and V_κ; *mCκ*, C region of mouse κ light chain gene; TAG, amber termination codon; and Δcp3, carboxyl-terminal domain of M13 gene III minor coat protein. Arrows indicate the direction of transcription. (B) The amino acid sequence surrounding the cloning sites for V_H and V_κ. Amino acids are shown in the single-letter code. An arrow indicates the cleavage site of the leader peptide.

Histone H1 Kinase Assay. Samples (5 μL of oocyte lysate) were incubated for 20 min at 25 °C with 10 μL of 3 mg/mL histone H1 (Sigma), 75 μM [γ-^{32}P]ATP (1 μCi), 30 mM Tris-HCl (pH 7.5), and 30 mM $MgCl_2$. The reaction was stopped by the addition of Laemmli's sample buffer and boiling. After electrophoresis, the gel was autoradiographed.

Epitope Mapping. The open reading frame of *Xenopus* MAPKK cDNA (Kosako et al., 1993) was amplified by PCR and cloned into the *Bgl*II site of pGEX-2T. Various deletion mutants truncated at the COOH and NH_2 termini were produced by using PCR and restriction enzyme sites (*Xba*I, *Pst*I, and *Ppu*MI). These proteins were expressed in *E. coli* strain BL21 (DE3) pLysS in the presence of 1 mM IPTG for 12 h and then purified by using glutathione−agarose columns as described previously (Kosako et al., 1993). After the addition of Laemmli's sample buffer and boiling, samples (each 0.5−1.5 μg) were analyzed by immunoblotting with various anti-MAPKK antibodies.

RESULTS

Selection of Clones with MAPKK Binding Activities. We used the polymerase chain reaction (PCR) to amplify the V_H and V_κ genes from the spleen mRNA of a mouse immunized with bacterially expressed *Xenopus* MAPKK. These V gene fragments obtained by the two-step PCR were digested with *Xho*I and *Nhe*I for V_H or *Sst*I and *Bgl*II for V_κ and cloned into the pScUAGΔcp3-M phagemid vector (Figure 1) (Akamatsu et al., 1993). Phagemid DNA purified from V_H and V_κ libraries was then digested with *Nhe*I and *Eco*RI. V_H and V_κ genes were combined in pScUAGΔcp3-M to produce single-chain Fv (scFv) fragments connected to mouse C_κ that is fused to the truncated M13 minor coat protein III. The ligated DNA was used to transform a suppressor strain of *E. coli* DH5α/F′IQ by electroporation,

VH sequences

	FR1	CDR1	FR2	CDR2	
Y1-6	LESGSEVVRPGVSVKISCKASGYAFS	NSWMN	WVKQRPGQGLEWIG	SINPSSGYTNYNQKFKD	
Y1-7	::::::L:K::A:::M:::::::T:T	SYV:H	::::K:::::::::	Y:::YNDG:T::E:::G	
Y3-6	::::GGL:K::G:L:L::A:::F:::	SYD:S	::R:T:EKR:::VA	Y:SSGG:N:Y:PDTV:G	
Y3-11	::::GGL:Q::G:L:L::A:::FT::	SYS:S	::R:T:EKR:::VA	Y:SNGG:T:Y:PDTV:G	

	FR3	CDR3	FR4	
Y1-6	KASLTADKSSSTAYMQLSSLTSEDSAVYYCAR	GPMDY-	WGQGTSVTVSSAS	(VH2A)
Y1-7	::T::S:::::::::E::::::::::::::::	:T:::-	::::::::::::::	(VH2A)
Y3-6	RFTISR:NAKN:L:L:M:::K:::T:M:::::	RYF:V-	::A::::::::::	(VH3)
Y3-11	RFTISR:NAKN:L:L:M:::K:::T:M:::V:	HGLLDY	:::::TL::::::	(VH3)

VK sequences

	FR1	CDR1	FR2	CDR2
Y1-6	ELLMTQTPLSLPVSLGDQASISC	RSSQSLVHSNGNTYLH	WYLQKPGQSPKLLIY	KVSNRFS
Y1-7	:::::::::::::::::::::::	:::::::::::::::::	:::::::::::::::::	:::::::
Y3-6	:::::::::::::::::::::::	:::::::::::::::::	:::::::::::::::::	:::::::
Y3-11	::V::::::T:S:TI:HP:::::	K:::::LD:D:K:::N	:L::R::::::R:::	L::KLD:

	FR3	CDR3	FR4	
Y1-6	GVPDRFSGSGSGTDFTLKISRVEVEDLGVYFC	SQSTYIPLT	FGAGTKLELKRS	(VK2)
Y1-7	:::::::::::::::::::::::A::::::::	::::HV:::	::::::::::::	(VK2)
Y3-6	::S::::::::::::::::::::A::::::::	P::::V:::	::::::::::::	(VK2)
Y3-11	::::::T:::::::::::::::::A::::::Y:	W:G:HF:Y:	::G:::::I:::	(VK2)

FIGURE 2: Amino acid sequences of V_H and V_κ of MAPKK-binding scFv fragments. Amino acids identical to Y1−6 are shown by colons. Classification of V subgroups and assignment of the framework regions (FR) and complementarity-determining regions (CDR) were according to Kabat et al. (1991).

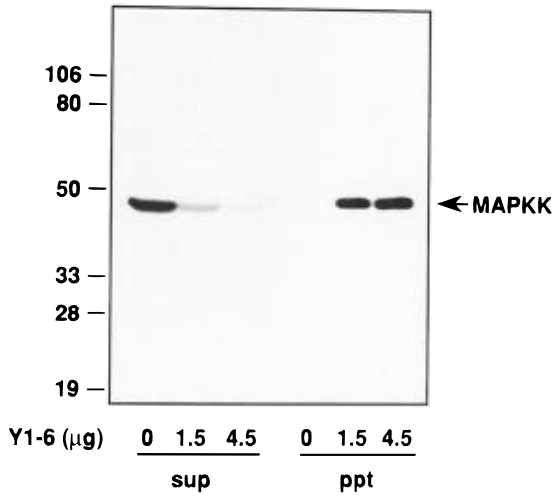

FIGURE 3: Immunodepletion of MAPKK from *Xenopus* oocyte extracts by Y1−6 scFv. Extracts from mature oocytes (20 μL, 10 mg/mL) were immunoprecipitated with 0.0, 1.5, or 4.5 μg of Y1−6 scFv that had bound to anti-mouse κ-chain-Sepharose. The supernatants (sup) and immunoprecipitates (ppt) were electrophoresed and immunoblotted with anti-N16 antibody raised against the N-terminal peptide of *Xenopus* MAPKK (Kosako et al., 1992).

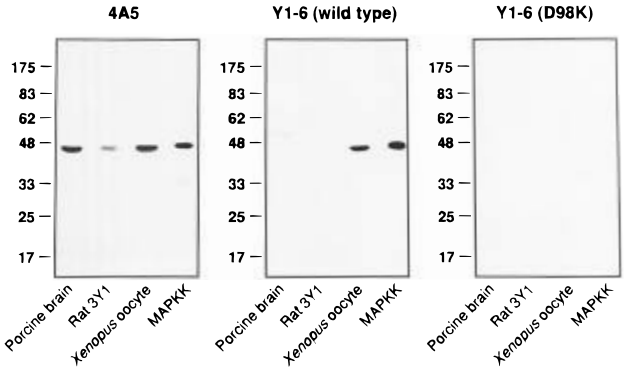

FIGURE 4: Reactivity of 4A5 mAb and Y1−6 scFv (wild type and D98K) in the immunoblot analysis. Extracts from porcine brain (40 μg), rat 3Y1 cells (20 μg) and *Xenopus* mature oocytes (40 μg), and bacterially expressed recombinant MAPKK (0.03 μg) were electrophoresed and immunoblotted with 0.4 μg/mL 4A5, the wild-type, or the mutant (D98K) Y1−6.

and phage particles were rescued from the obtained combinatorial library of 3×10^7 clones by superinfection of VCSM13 helper phage. To select phage carrying scFv connected to mouse C_κ on the surface, the rescued phage (2.4×10^{12} cfu) were passed down an anti-mouse κ-chain-Sepharose column, and bound phage were eluted with 0.1 M glycine hydrochloride (pH 2.1). The eluted phage were then selected by passing down a MAPKK−Toyopearl column. The bound phage were used to infect DH5α/F′IQ, and the library was again rescued by superinfection of the helper phage. The rescued phage were subjected to the next round of selection with anti-mouse κ-chain antibodies and bacterially expressed MAPKK. After three rounds of selection, an aliquot of phage eluted from a MAPKK−Toyopearl column was used to infect a nonsuppressor strain of *E. coli* W3110/F′IQ. Then 36 ampicillin-resistant colonies was randomly picked and induced with IPTG to produce soluble scFv fragments. Culture supernatants containing scFv were screened for binding to MAPKK by ELISA. We classified eight ELISA-positive clones into four independent clones (Y1−6, Y1−7, Y3−6, and Y3−11) by *Hae*III

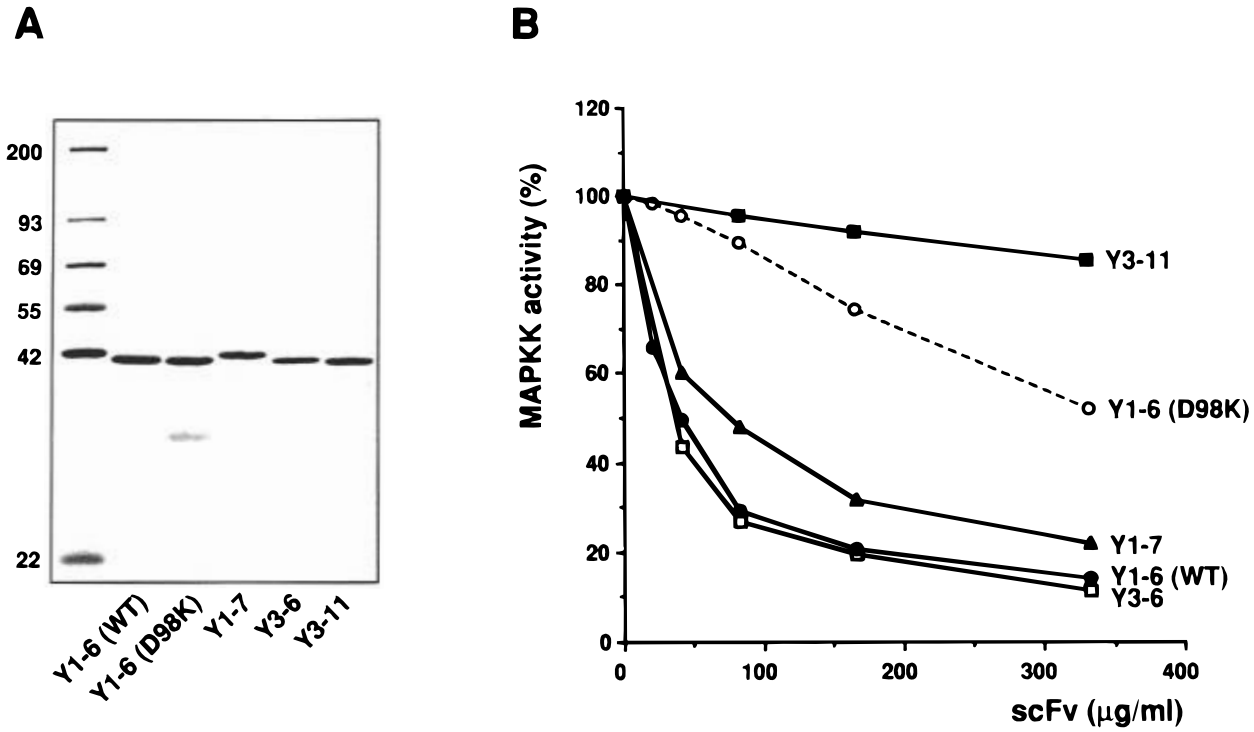

FIGURE 5: Comparison of neutralizing activity against MAPKK of five scFv clones. (A) Each scFv clone (1.5 μg) purified as described in Materials and Methods, and molecular weight standards were electrophoresed in a 10% acrylamide gel. The gel was stained with Coomassie blue. (B) Purified *Xenopus* MAPKK (6 μL, 15 μg/mL) was mixed with 4 μL of each scFv clone (0−5.0 μg) for 30 min at 0 °C. Then the mixture was incubated for 20 min at 30 °C with 100 μg/mL GST−KNMAPK, 50 μM [γ-^{32}P]ATP (2 μCi), 20 mM Tris-HCl (pH 7.5), and 20 mM $MgCl_2$ in a final volume of 15 μL. The reaction was stopped by the addition of Laemmli's sample buffer and boiling. After electrophoresis, the radioactivity of the GST−KNMAPK band was quantified using an image analyzer (FUJIX BAS2000).

fingerprinting. These four clones reacted specifically with the 45 kDa MAPKK band in total extracts from *Xenopus* mature oocytes in the immunoblot analysis (data not shown). Then nucleotide sequences of V_H and V_κ of these clones were determined, and the deduced amino acid sequences were aligned (Figure 2). Among them, three clones (Y1−6, Y1−7, and Y3−6) proved to possess the neutralizing activity against *Xenopus* MAPKK and bind to the same segment of MAPKK (see below).

Production of a Mutant Clone with Reduced Neutralizing Activity. Deduced sequences of V_H-CDR3 of the three clones with neutralizing activity comprised five amino acids, and aspartic acid at the fourth position was conserved (Figure 2). Then we produced a mutant of one of these clones (Y1−6) by changing this aspartic acid to lysine (D98K). A wild-type Y1−6 clone reacted specifically with MAPKK and could immunodeplete MAPKK from *Xenopus* oocyte extracts (Figure 3). A comparison by three-dimensional modeling between the binding domains of Y1−6 (wild type) and Y1−6 (D98K) suggested that the point mutation caused no great change in the whole structure (data not shown).

Soluble scFv fragments of the wild-type and the mutant Y1−6 were purified from culture supernatants of IPTG-induced W3110/F′IQ transformants by anti-mouse κ-chain column chromatography. Total extracts from porcine brain, rat fibroblastic 3Y1 cells and *Xenopus* mature oocytes, and bacterially expressed *Xenopus* MAPKK were immunoblotted with both scFv fragments at equal concentrations (0.4 μg/mL). The wild-type scFv specifically reacted with 45 kDa *Xenopus* MAPKK but hardly reacted with mammalian MAPKK (Figure 4). In contrast, the mutant scFv showed no reactivity (Figure 4). 4A5, a mouse monoclonal antibody

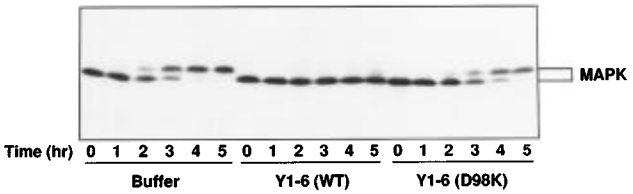

FIGURE 6: Effects of the wild-type and the mutant Y1−6 on MAP kinase activation in a cell-free system. Cell-free extracts (7.5 μL) obtained from *Xenopus* immature oocytes were mixed with 1.5 μL of buffer, the wild-type Y1−6 (5.0 mg/mL) or the mutant Y1−6 (5.0 mg/mL) for 30 min at 0 °C. Then, 1 μL of 50 μM okadaic acid, 10 mM ATP, 10 mM $MgCl_2$, 0.1 M creatine phosphate, and 0.5 mg/mL creatine kinase was added, and the incubation was performed at 22 °C for the indicated times. Samples (each 0.5 μL) were analyzed by immunoblotting with anti-MAP kinase antibody.

obtained by the hybridoma technique, reacted specifically with both *Xenopus* and mammalian MAPKK (MAPKK1 or MEK1; Figure 4).

To examine whether each scFv clone inhibits the kinase activity of purified MAPKK from mature oocytes *in vitro*, we then purified soluble scFv fragments of other three clones (Y1−7, Y3−6, and Y3−11) as above. Coomassie blue staining of each purified sample after SDS−PAGE made noticeable about 40 kDa scFv fragments as the major band (Figure 5A). After purified *Xenopus* MAPKK was mixed with increasing concentrations of each scFv clone, the mixture was assayed for the MAPKK activity by using the kinase-negative mutant of *Xenopus* MAP kinase as a specific substrate (Figure 5B). The results indicated clearly that Y1−6 (wild type), Y1−7, and Y3−6 efficiently inhibited the MAPKK activity in a dose-dependent manner. In contrast, Y3−11 hardly inhibited the MAPKK

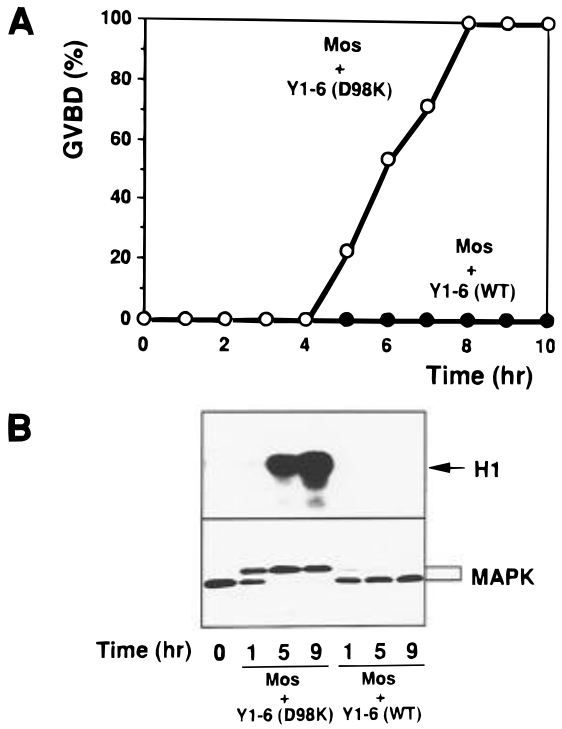

FIGURE 7: MPF and MAP kinase activities in oocytes injected with malE−mos and the wild-type Y1−6 or with malE−mos and the mutant Y1−6. (A) Oocytes were injected with a mixture of either malE−mos (0.15 mg/mL) and the wild-type Y1−6 (4.0 mg/mL) or malE−mos (0.15 mg/mL) and the mutant Y1−6 (4.0 mg/mL). Thirty oocytes were scored for the appearance of a white spot on the animal pole at the indicated times to measure GVBD. (B) Lysates prepared from each of the 10 oocytes in panel A at the indicated times were assayed for histone H1 kinase activity (upper) and analyzed by immunoblotting with anti-MAP kinase antibody (lower).

activity (Figure 5B). The inhibitory effect of the mutated Y1−6 was markedly weakened as compared with that of the wild-type Y1−6, and the concentration of this mutant scFv necessary to inhibit the MAPKK activity by 50% was approximately 9-fold higher than that of the wild-type scFv (Figure 5B).

Effects of scFv Clones in Cell-Free Extracts and in Cells. To examine whether scFv fragments of the wild-type or the mutant Y1−6 inhibit MAP kinase activation in a cell-free system, we prepared concentrated cell-free extracts from immature oocytes (Shibuya et al., 1992). After mixing with control buffer or the wild-type or the mutant scFv at 0 °C, okadaic acid (5 μM) was added to the extracts, and the incubation was performed at 22 °C. The okadaic acid treatment induced full activation of MAP kinase within 4 h in the buffer-treated extracts, as judged by the shift in electrophoretic mobility of the 42 kDa polypeptide recognized by anti-MAP kinase antibody (Figure 6). In contrast, in the wild-type scFv-treated extracts, no activation of MAP kinase occurred until 4 h and only slight activation occurred after 5 h, (Figure 6). In the mutant scFv-treated extracts, full activation of MAP kinase occurred after 5 h although delayed by about 1 h as compared with that of the buffer-treated extracts (Figure 6). Thus, there was a marked difference between the wild-type and the mutant scFv in the inhibitory effects on MAP kinase activation in a cell-free system.

It has been shown that microinjection of the malE−mos fusion protein into immature oocytes results in GVBD (Yew et al., 1992) and in rapid activation of MAP kinase followed by the activation of MPF (Posada et al., 1993). To examine the effects of the wild-type and the mutant scFv on Mos-induced activation of MPF and MAP kinase, we microinjected the malE−mos protein together with the wild-type or the mutant scFv into immature oocytes. In 100% of the oocytes injected with malE−mos and the mutant scFv, GVBD occurred by 8 h following injection, while GVBD was observed in no oocytes injected with malE−mos and the wild-type scFv (Figure 7A). Furthermore, marked elevation of histone H1 kinase activity and full activation of MAP kinase occurred within 5 h in oocytes injected with malE−mos and the mutant scFv (Figure 7B). In contrast, in oocytes injected with malE−mos and the wild-type scFv, no activity of histone H1 kinase was detected until 9 h following injection and activation of MAP kinase was extremely low (Figure 7B). Thus, Mos-induced activation of MPF and MAP kinase was inhibited by the wild-type scFv that can efficiently inhibit the MAPKK activity.

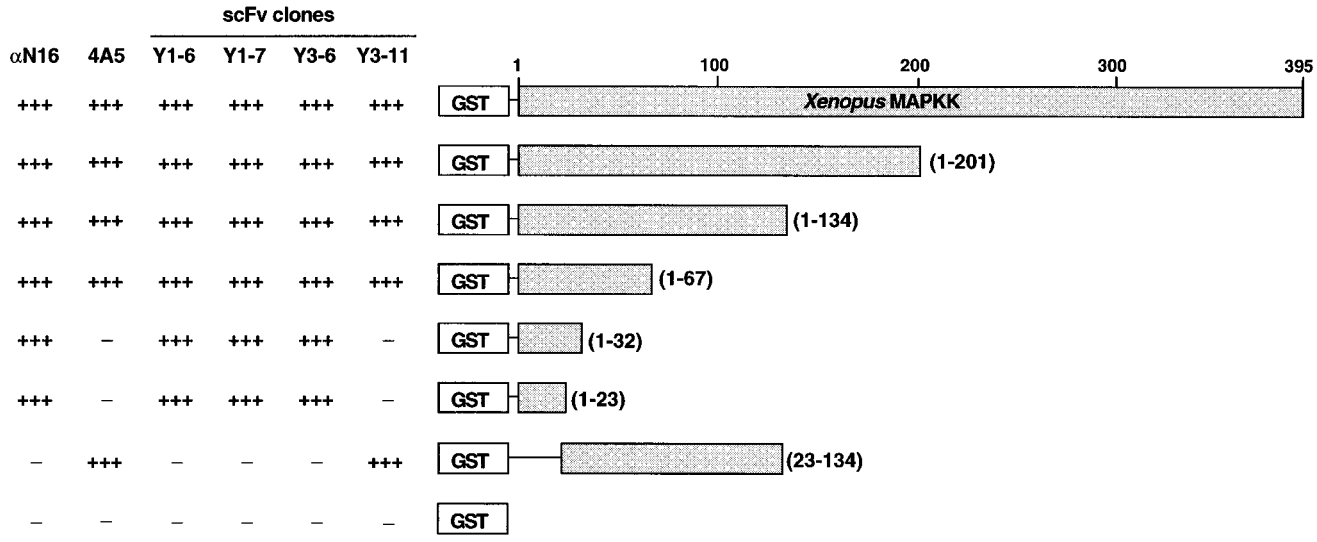

FIGURE 8: Mapping the epitopes in *Xenopus* MAPKK recognized by various anti-MAPKK antibodies. The figure shows the portions of *Xenopus* MAPKK fragments expressed in bacteria as GST fusion proteins and their reactivity with anti-N16 antibody, 4A5 mAb, and four scFv clones in immunoblotting. Strong reactivity is indicated by +++, and no reactivity is indicated by −.

Epitope Mapping of scFv Clones. To investigate the sites on MAPKK that bind to the above scFv clones, a series of *Xenopus* MAPKK proteins deleted at the COOH and NH_2 terminus was expressed in bacteria as GST fusion proteins. Immunoblotting of these MAPKK proteins revealed that the epitopes of the three scFv clones with neutralizing activity (Y1−6, Y1−7, and Y3−6) lie within the first 23 amino acids of *Xenopus* MAPKK (Figure 8). The epitopes of the Y3−11 scFv with no neutralizing activity and the 4A5 mAb (Figure 4) were shown to lie between residues 33 and 67 (Figure 8). Anti-N16 antibody, which was raised against the synthetic peptide (the N16 peptide) corresponding to amino acids 2−17 of MAPKK (Kosako et al., 1992), strongly recognized the NH_2-terminal 23 amino acids of the protein, like the three neutralizing clones (Figure 8).

To confirm that the three neutralizing scFv clones inhibit the MAPKK activity by binding to its NH_2-terminal segment, we examined whether the addition of the N16 peptide can suppress the neutralizing activity of the wild-type Y1−6. In the absence of the N16 peptide, the wild-type Y1−6 strongly inhibited the MAPKK activity *in vitro*, but this neutralizing activity of Y1−6 was efficiently suppressed by preincubation with the N16 peptide and the MAPKK activity was recovered (Figure 9A). Furthermore, the anti-N16 antibody was shown to possess the strong neutralizing activity against MAPKK (Figure 9B). These results demonstrated clearly that those antibodies that bind to the NH_2-terminal segment of MAPKK can inhibit its activity.

DISCUSSION

In this study, we have isolated three mouse scFv antibodies that can efficiently inhibit *Xenopus* MAPKK activity by making use of the phage display system. To our knowledge, these are the first examples of scFv fragments isolated from phage libraries that can neutralize enzymatic activity. When monoclonal antibodies are obtained by the phage display system, the antibody-encoding genes can also be obtained at the same time. Thus, it is possible to change binding affinities of the antibodies by mutagenizing these genes *in vitro*. In fact, the affinities of isolated antibodies have been improved by chain shuffling (Kang et al., 1991) and mimicking affinity maturation (Hawkins et al., 1992). In this study, we made a mutant scFv with reduced neutralizing activity against MAPKK. By using this mutant scFv as a control, we could strongly suggest that the MAPKK activity is required for Mos-induced initiation of oocyte maturation. A combination of the phage display system and the *in vitro* mutagenesis as above may be expected to provide an effective approach to analyze physiological functions of other proteins.

Recent studies from several laboratories demonstrated that activation of the MAPKK/MAP kinase cascade by injecting constitutively active MAPKK (Huang et al., 1995; Gotoh et al., 1995) or thiophosphorylated MAP kinase (Haccard et al., 1995) is sufficient for inducing GVBD in *Xenopus* oocytes in the absence of progesterone. In this study, we showed that microinjection of the neutralizing scFv against MAPKK inhibited Mos-induced GVBD (Figure 7A). This inhibitory effect was complete, in contrast to the partial inhibition of Mos-induced GVBD by injecting mouse polyclonal antibody against MAPKK (Kosako et al., 1994a). Thus, it is suggested that activation of the MAPKK/MAP kinase cascade is required and sufficient for initiating *Xenopus* oocyte maturation.

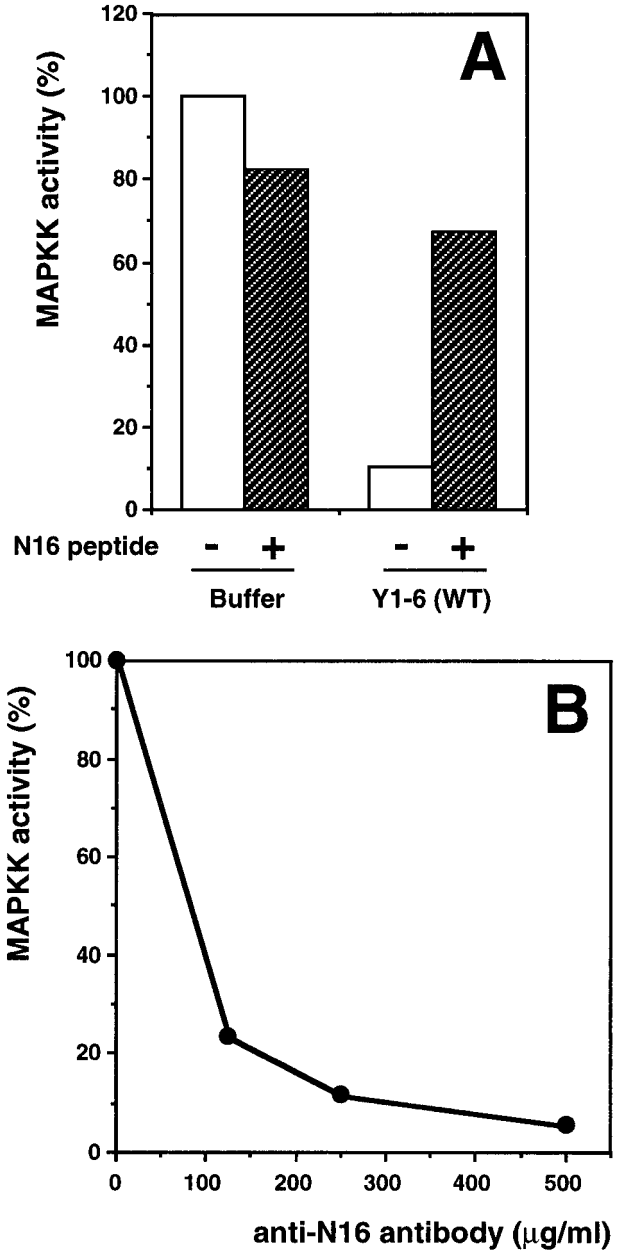

FIGURE 9: Effect of the N16 peptide on the neutralizing activity of the wild-type Y1−6 and the neutralizing activity of anti-N16 antibody. (A) Buffer or the wild-type Y1−6 (2 μL, 5.0 mg/mL) was mixed with 5 μL of the N16 peptide (0.0 or 0.3 mg/mL) for 30 min at 0 °C. Then the mixture (7 μL) was incubated with purified *Xenopus* MAPKK (3 μL, 15 μg/mL) for 30 min at 0 °C. After the addition of 5 μL of 150 μg/mL GST−KNMAPK, 150 μM [γ-^{32}P]-ATP (1 μCi), 60 mM Tris-HCl (pH 7.5), and 60 mM $MgCl_2$, the incubation was performed for 20 min at 30 °C. After electrophoresis, the radioactivity of the GST−KNMAPK band was quantified using an image analyzer. (B) The neutralizing activity of anti-N16 antibody against purified *Xenopus* MAPKK was measured as shown in Figure 5B.

Point mutation at V_H-CDR3 of one of the neutralizing scFv clones markedly reduced its neutralizing activity, but this mutant scFv retained weak neutralizing activity against MAPKK (Figure 5B). However, the mutant scFv showed no apparent reactivity with MAPKK in the immunoblot analysis conditions used (Figure 4), in which the mutant scFv might be washed out during the procedure due to its low affinity for MAPKK. In any case, mutated forms of scFv

for the use of control experiments should possess reduced binding affinity without a gross conformational change in the whole structure, and a design for such mutant forms of scFv can be facilitated by the three-dimensional modeling that has been successfully used for humanization of mouse monoclonal antibodies (Queen et al., 1989).

We have shown here that the scFv clones with neutralizing activity strongly bind to the NH_2-terminal 23 amino acids of MAPKK. Since the kinase domain of *Xenopus* MAPKK lies after about the 70th amino acid residue (Kosako et al., 1993), the mechanism by which the neutralizing scFv fragments inhibit the kinase activity of MAPKK may be of particular interest. Recently, Resing et al. (1995) reported that one of the intramolecular autophosphorylation sites on MAPKK was Thr-23, suggesting that the NH_2-terminal segment of MAPKK is located near the active center. Therefore, it is hypothesized that binding of the neutralizing scFv to the NH_2-terminal segment inhibits interaction between the active center and its substrate. In these NH_2-terminal 23 amino acids, there are three different amino acid residues between *Xenopus* (Asn-14, Glu-16, and Thr-18) and mammalian (Ala-14, Asp-16, and Ser-18) MAPKK, which might explain why the neutralizing scFv fragments against *Xenopus* MAPKK hardly bind to mammalian MAPKK (Figure 4). In any case, these neutralizing scFv fragments obtained here are expected to inhibit *Xenopus* MAPKK activity in a highly specific manner and thus to be useful for studying the roles of *Xenopus* MAPKK in various cellular responses.

Recent advances have permitted the intracellular expression and targeting of recombinant antibodies, such as scFv or Fab fused to the known targeting signals (*e.g*. nuclear localization signal sequences, mitochondrial presequences, or KDEL retention sequences). This 'intracellular immunization' has been successfully used to inhibit the functions of target proteins in a wide variety of nonlymphoid cells [Tavladoraki et al., 1993; Graus-Porta et al., 1995; Mhashilkar et al., 1995; for review, see Biocca and Cattaneo (1995)]. The spaciotemporal expression of the anti-MAPKK neutralizing scFv obtained here might reveal hitherto unidentified aspects of biological functions of the MAPKK/MAP kinase cascade.

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