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Monoclonal Antibody NM2 Recognizes the Protein Kinase C Phosphorylation Site in B-50 (GAP-43) and in Neurogranin (BICKS)

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Abstract: Mouse monoclonal B-50 antibodies (Mabs) were screened to select a Mab that may interfere with suggested functions of B-50 (GAP-43), such as involvement in neurotransmitter release. Because the Mab NM2 reacted with peptide fragments of rat B-50 containing the unique protein kinase C (PKC) phosphorylation site at serine-41, it was selected and characterized in comparison with another Mab NM6 unreactive with these fragments. NM2, but not NM6, recognized neurogranin (BICKS), another PKC substrate, containing a homologous sequence to rat B-50 (34–52). To narrow down the epitope domain, synthetic B-50 peptides were tested in ELISAs. In contrast to NM6, NM2 immunoreacted with B-50 (39–51) peptide, but not with B-50 (43–51) peptide or a C-terminal B-50 peptide. Preabsorption by B-50 (39–51) peptide of NM2 inhibited the binding of NM2 to rat B-50 in contrast to NM6. NM2 selectively inhibited phosphorylation of B-50 during endogenous phosphorylation of synaptosomal plasma membrane proteins. Preabsorption of NM2 by B-50 (39–51) peptide abolished this inhibition. In conclusion, NM2 recognizes the QASFR peptide in B-50 and neurogranin. Therefore, NM2 may be a useful tool in physiological studies of the role of PKC-mediated phosphorylation and calmodulin binding of B-50 and neurogranin. **Key Words:** Epitope mapping—GAP-43—Monoclonal antibody—Protein kinase C—Phosphorylation site of rat B-50.
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Recently, a set of mouse monoclonal antibodies (Mabs; NM1–8) raised against bovine B-50 [also termed GAP-43 (growth-associated protein-43), F1, neuromodulin, and PP46 (Skene, 1989)] has been partially characterized (Mercken et al., 1992). All B-50 Mabs recognize B-50 in bovine, human, and rat brain and do not distinguish between phosphorylated and dephosphorylated rat B-50 on western blot. In this study, we characterized one Mab in order to obtain a defined immunochemical tool to interfere with the suggested functions of B-50, such as Ca^{2+}-induced neurotransmitter release (De Graan et al., 1991).

An impressive body of evidence suggests that nervous tissue-specific B-50 has an important role, not yet clarified, in a number of related physiological processes, such as neuronal development, neuritogenesis, growth cone function, neuroplasticity (nerve regeneration, long-term potentiation), and neurotransmission (for reviews, see Skene, 1989; Coggins and Zwiers, 1991; De Graan et al., 1991; Gispen et al., 1991; Strittmatter et al., 1991, 1992).

Phosphorylation of B-50 by protein kinase C (PKC) (Aloyo et al., 1983), as has been shown to occur in vitro and in vivo (Dekker et al., 1989; Dent and Meiri, 1992; Spencer et al., 1992), is thought to be one of the crucial reactions in these physiological processes. B-50 dephosphorylation has been shown to be mediated in vitro by various protein phosphatases (Liu and Storm, 1989; Han and Dokas, 1991). Rat B-50 (226 amino acids, 23.6 kDa) contains one unique conserved PKC phosphorylation site at serine-41 (Coggins and Zwiers, 1989; Apel et al., 1990; Nielander et al., 1990; Spencer et al., 1992).

Other posttranslational modifications of B-50

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Abbreviations used: ASP, ammonium sulfate precipitate of B-50-enriched fraction; BICKS, bovine B-50-immunoreactive C-kinase substrate; IgGs, immunoglobulins of class G; IR, immunoreactivity; Mab, monoclonal antibody; OD, optical density; PKC, protein kinase C; SAP, *S. aureus* V8 protease; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; SPM, synaptosomal plasma membranes; TBS, Tris-HCl–buffered saline.

(Zwiers et al., 1985; Skene and Virag, 1989) and association with specific proteins (Liu and Storm, 1990; Strittmatter et al., 1990; Sudo et al., 1992) have also been implicated in the physiological effects of B-50. Calmodulin binding to dephosphorylated B-50 at the amino acid sequence 39–56 (Alexander et al., 1988; De Graan et al., 1990; Chapman et al., 1991) at intracellular resting Ca^{2+} levels is of particular relevance for an epitope mapping study. Association of calmodulin inhibits phosphorylation of B-50 at serine-41 by PKC. In addition, calmodulin (Apel et al., 1991) inhibits the phosphorylation of B-50 by casein kinase II at serine residues different from those phosphorylated by PKC. A rise in the intracellular Ca^{2+} concentration (e.g., by membrane depolarization or receptor-activated Ca^{2+} influx) dissociates calmodulin from B-50 and releases the calmodulin inhibition of PKC phosphorylation of B-50 (Alexander et al., 1988).

The rat B-50 amino acid sequence 34–52 (Nielander et al., 1987) is homologous to a stretch of 15 amino acids in the neuron-specific protein neurogranin (formerly termed P17, 76 amino acids, 7,837 Da; Baudier et al., 1989, 1991). However, B-50 and neurogranin differ in all other parts of their primary structure. Neurogranin is identical to BICKS (bovine B-50-immunoreactive C-kinase substrate; Coggins et al., 1991) and RC3 (Watson et al., 1990). As reported for B-50, neurogranin (BICKS) binds to calmodulin-Sepharose in the absence of Ca^{2+} (Coggins et al., 1991) and is phosphorylated by PKC, yielding a single phosphopeptide with sequence IQASFR after tryptic digestion (Baudier et al., 1991; Chen et al., 1993). The physiological function of neurogranin is still obscure. In previous studies, we have applied polyclonal rabbit B-50 antibodies to study the physiological role of PKC-mediated phosphorylation of B-50 (Oestreicher et al., 1983; Dekker et al., 1989, 1991). The B-50 antibodies inhibited B-50 phosphorylation and Ca^{2+}-induced noradrenaline release in permeated synaptosomes, establishing a causal relation between B-50 and noradrenaline release and suggesting the involvement of B-50 phosphorylation (Dekker et al., 1989, 1991). To substantiate that B-50 is essential for Ca^{2+}-induced neurotransmitter release, it would be useful to select B-50 antibodies that recognize one single epitope interfering with B-50 function. For this purpose, we have examined NM1–8, compared NM2 and MM6 in epitope mapping, and selected NM2.

MATERIALS AND METHODS

Materials

Two B-50 peptides were synthesized and kindly provided by Dr. Van Nispen at Organon (Oss, The Netherlands): B-50 (39–51) peptide (39–51; *N*-acetyl-Q-A-S-F-R-G-H-I-T-R-K-K-L-COOH) and B-50 (43–51) peptide (43–51; H_2N-R-G-H-I-T-R-K-K-L-COOH). A synthetic B-50 (214–226) peptide (214–226; H_2N-E-G-K-E-D-P-E-A-D-Q-E-H-A-COOH) was a gift of Dr. F. Margolis (Roche Institute of Molecular Biology, Roche Research Center, Nutley, NJ, U.S.A.).

S. aureus V8 protease (SAP) was purchased from ICN, Immuno-Biologicals (Zoetermeer, The Netherlands); α-chymotrypsin was obtained from bovine pancreas (Boehringer, Mannheim, F.R.G.).

Antibodies

Mouse Mabs to B-50, NM2, NM3, NM4, and NM6, were obtained as ascites from Innogenetics (Ghent, Belgium). Mabs were characterized previously by Mercken et al. (1992). Polyclonal rabbit B-50 antibodies were raised and characterized as reported previously (Oestreicher et al., 1983; Oestreicher and Gispen, 1986; Mercken et al., 1992). Immunoglobulins of class G (IgGs) were purified by affinity chromatography, using for the polyclonal antibodies a B-50 column and for the ascites a protein G column (Pharmacia, Uppsala, Sweden), by a procedure similar to that described by Oestreicher et al. (1983). IgGs were dialyzed extensively against twice distilled water, freeze-dried, and stored at −80°C.

Purification of rat B-50 and neurogranin

B-50 was isolated from adult rat brain (Wistar rats, 120–140 g; TNO, Zeist, The Netherlands) according to the procedure of Zwiers et al. (1985), with the modification that further purification of B-50 from the ammonium sulfate precipitate of the B-50–enriched fraction (ASP) was performed on a calmodulin-Sepharose column (Alexander et al., 1988). Neurogranin was isolated from bovine brain as reported by Coggins et al. (1991).

Digestion of B-50 by proteases

Rat B-50 was phosphorylated by PKC and digested by SAP (5 ng/μl) at 30°C for 20 min in buffer A, which contained 10 mM $MgCl_2$, 0.1 mM $CaCl_2$, and 10 mM Tris-HCl at pH 7.4. Digestion of PKC-phosphorylated B-50 by chymotrypsin (5 ng/μl) was carried out at 30°C for 30 min in buffer A. These procedures were a modification of those described by Oestreicher et al. (1989) and Han and Dokas (1991). Digestions were terminated by heating for 10 min at 100°C. Proteolysis products were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on 15% acrylamide running gels, autoradiography, and western immunoblot.

Western blotting

Purified neurogranin (BICKS, 0.5 μg/lane) was analyzed by SDS-PAGE on a 15% acrylamide running gel. Proteins from 11% and 15% acrylamide gels were transferred electrophoretically to nitrocellulose and immunoincubated as previously reported (Mercken et al., 1992). In order to reduce loss of neurogranin from the blot during the various steps of the immunoincubation, blots of neurogranin were fixed in 2.5% glutaraldehyde for 20 min at room temperature. Prior to the reaction with the antibodies, reactive aldehyde groups on the blot were blocked with 10% skimmed milk proteins and 2% normal goat serum in Tris-buffered saline (TBS; 0.15 M NaCl, 0.05 M Tris-HCl, pH 7.4).

ELISA

Microtiter plates (96 wells, Maxisorp-F96; Nunc, Roskilde, Denmark) were coated by incubation overnight at 37°C in 100 μl of antigen solution in 0.05 M $NaCO_3$, pH 9.6, or in 0.01 M Tris-HCl, pH 8.5, containing 10 mM NaCl and 10 mM NaN_3. After removal of the coating solution,

the plates were washed and blocked by a blocking buffer containing TBS, 10% skimmed milk proteins, and 2% normal goat serum. In some experiments, prior to the blocking, the coated antigen was fixed to the well by incubation with 2.5% glutaraldehyde in TBS for 20 min at room temperature. Antibodies in the ascites were diluted in 100 μl of 10% blocking buffer, added to the antigen-coated wells, and incubated for 1 h at 37°C. Wells were washed three times with 100 μl of TBS-T (TBS containing 0.5% Tween). Antigen antibody reaction was detected by incubation for 1 h at 37°C with horseradish peroxidase conjugated to rabbit anti-mouse antibodies (Jackson, Immunoresearch, West Grove, PA, U.S.A.) diluted in TBS-T. After the plates were rinsed three times with TBS-T (3 min) and TBS (3 min), the color reaction was developed using *o*-phenylenediamine (2 mg/ml in 0.1 *M* citric acid, pH 4.5) and 0.02% hydrogen peroxide as substrates. The color reaction was stopped by addition of 50 μl of 2 *M* H_2SO_4 after 5–20 min (final volume 150 μl). The reaction time varied in different experiments and was selected in such a way that, within one experiment, the readings were distributed within the optical density (OD) range of 0–2,500 (OD × 1,000). OD was determined as the light absorption difference at 492 nm and 690 nm in an Easy Reader (EAR.400, Labinstruments, Austria). In competition assays, NM2 or NM6 was preabsorbed with 2 or 0.8 μg of peptides by addition to ascites fluids (diluted 1:5,000) and incubation for 2 h at 37°C.

Protein phosphorylation

Synaptosomal plasma membranes (SPM) were prepared from rat forebrain (Dekker et al., 1991). Aliquots (10 μg of protein) of SPM were preincubated for 5 min at 30°C in 20 μl of buffer A (consisting of 10 m*M* Tris-HCl, 10 m*M* $MgCl_2$, 0.1 m*M* $CaCl_2$, pH 7.4). Phosphorylation of SPM was initiated by addition of 5 μl of [γ-^{32}P]ATP (7.5 μ*M*, 2 μCi, 3,000 Ci/mmol; Amersham, Buckinghamshire, U.K.) and was stopped after 15 s by addition of 12.5 μl of denaturation buffer. Preincubation (5 min at 30°C) and phosphorylation (15 s at 30°C) of SPM were carried out in the presence and absence of 0.025 μg (1.6×10^{-13} mol) of IgGs of NM2 with 0.8 μg (5×10^{-10} mol) of B-50 (39–51) peptide or with 0.6 μg (6×10^{-10} mol) of B-50 (43–51) peptide. Each of the peptides was also tested in the absence of NM2. Proteins were separated by SDS-PAGE using 11% acrylamide running gels, followed by autoradiography of the gel. The radiolabelling of individual phosphorylated protein bands was analyzed by quantitative densitometry of the autoradiograms (Dekker et al., 1991; Hens et al., 1993*a*).

Other measurements

Protein content was determined by the method of Bradford (1976) using bovine serum albumin as the standard. Data are presented as the means ± SEM, with n being the number of independent analyses. Statistical analysis was performed with the two-tailed Student's *t* test. A value of $p < 0.05$ is considered significant.

RESULTS

Immunoreactivity (IR) of proteolytic fragments of rat B-50

In earlier studies (Mercken et al., 1992), we have shown that NM2 and NM6 react with purified rat B-50 in either phosphorylated isoform or dephosphorylated isoform and do not distinguish between these isoforms of B-50. Because preliminary evidence had suggested that the epitope recognized by NM2 was located close to the PKC phosphorylation site in B-50, we examined by western blot whether NM2 and NM6 would bind to phosphorylated fragments of B-50 produced by limited protease digestion. Previous studies had revealed that digestion by SAP of rat B-50 phosphorylated by PKC (Oestreicher et al., 1989; Nielander et al., 1990) and by chymotrypsin (Han and Dokas, 1991) generates specific phosphorylated fragments of B-50 (Fig. 1; see also Fig. 6). After investigation of the time course of the enzyme digestions of B-50 phosphorylated by PKC, we selected one condition and made western blots of the gels on which the proteins of the digests were separated. As shown by the autoradiogram of a part of the gel, limited digestion by SAP of purified B-50 phosphorylated by PKC produced two phosphorylated fragments, the 28-kDa SAP1 and 15-kDa SAP2 (Fig. 1A, lane 2). Limited digestion by chymotrypsin generated primarily 12-kDa phosphorylated fragments (CH1; Fig. 1A, lane 3). Each of the phosphorylated fragments, SAP1 [B-50 (1–132)], SAP2 [B-50 (1–65)], and CH1 [B-50 (1–42)], contains the unique PKC phosphorylation site in rat B-50 located on the serine residue at position 41 (Coggins and Zwiers, 1989; Apel et al., 1990; Nielander et al., 1990). NM6 recognized only the original intact B-50 (Fig. 1B, lanes 1 and 3). Figure 1C demonstrates that NM2 immunoreacted with B-50, SAP1, and SAP2, but did not bind to CH1 (Fig. 1B, lane 4). Neither NM2 nor NM6 bound to CH1 (Fig.

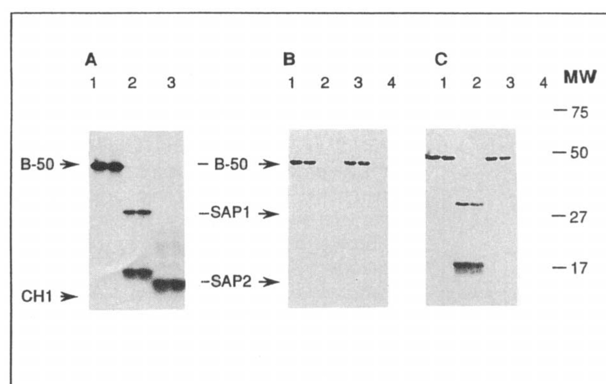

FIG. 1. Autoradiogram and immunodetection on western blots of B-50 phosphorylated by PKC, the phosphorylated fragments SAP1 and SAP2, produced by SAP, and the phosphorylated fragment CH1 formed by chymotrypsin. Digestions were carried out as reported in Materials and Methods. **A:** An autoradiogram of the gel. **B** and **C:** Immunostaining with NM6 and NM2 (diluted 1:4,000), respectively. Samples of 0.5 μg of phosphorylated B-50 in lanes 1 (A, B, and C) and lanes 3 (B and C) have not been digested; in lanes 2 (A, B, and C), they were digested with SAP (5 μg/ml) for 20 min. The samples of digestion with chymotrypsin for 30 min (5 μg/ml) are shown in lane 3 (A) and lane 4 (B and C). NM2 bound to B-50, SAP1, and SAP2 (C), whereas NM6 only recognized B-50 (B). Neither NM2 nor NM6 bound to CH1.

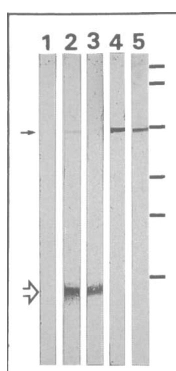

FIG. 2. Cross-reaction of NM2, but not of NM6, with neurogranin as demonstrated by western blot. Neurogranin was immunostained by NM2 and NM3 (diluted 1:2,000; strips 2 and 3), but not stained by NM4 and NM6 (diluted 1:500; strips 4 and 5). The Mabs also detected bovine B-50 as a contaminant of the neurogranin preparation. Strip 1 is the control in which no primary antibody was added. At the right side, the positions of the molecular mass markers are indicated, from top to bottom: 94, 67, 43, 30, and 20.1 kDa. At the left side, neurogranin (open arrow) and B-50 (small arrow) are indicated.

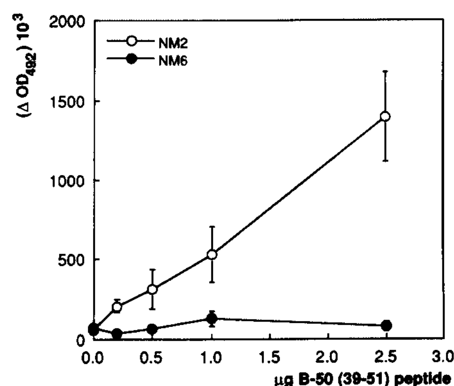

FIG. 4. Effect of the dose of synthetic B-50 (39–51) peptide on the IR of NM2 and NM6 using this peptide as the coating antigen of the microtiter plate. The ELISA was performed with a 1:5,000 dilution of NM2 and a 1:1,000 dilution of NM6. Student's t test was used for testing significance; data for NM2 were significantly different ($p < 0.01$) from the control without peptide. Data for NM6 were not significantly different from the control.

1B and C, lanes 4). Autoradiography showed that the absence of IR of some of the fragments was not caused by failure of the fragments to remain attached to the nitrocellulose carrier. Increasing the amount of the chymotryptic B-50 fragment by loading threefold more on the gel did not result in detection on the immunoblot by NM2 or NM6. NM1 and NM3 behaved in a similar manner as NM2 in this test, whereas NM4 behaved like NM6 (data not shown).

IR of NM2 with neurogranin

After we had found that the epitope for NM2 may be located near the PKC phosphorylation site of B-50, we examined whether the Mabs bind to neurogranin. For it is known that neurogranin has a consensus sequence of the PKC phosphorylation site similar to that of B-50 and a conserved calmodulin binding domain (Baudier et al., 1991). Figure 2 shows a western blot of semipurified neurogranin immunoreacted with diluted NM2, NM3, NM4, and NM6. NM2 and NM3 recognized neurogranin (17 kDa, indicated by open arrow). NM4 and NM6 did not detect neurogranin, although these Mabs were used in a fourfold higher concentration in this test. All Mabs revealed the presence of bovine B-50 (small arrow in Fig. 2) as a contaminant of the neurogranin preparation. The IR of NM2 was also documented by ELISA. Figure 3 shows the IR of NM2 as a function of the dilution of the antibody and the dose of neurogranin used as the coating antigen. The OD signal of the ELISA was proportional to the concentration of antibody in the solution of the diluted primary antibody and the concentration of neurogranin in the coating solution. Because it was found that glutaraldehyde fixation of neurogranin was necessary to detect this protein at low amounts (e.g., <0.5 μg) by immunoblot, we also examined whether glutaraldehyde fixation following coating of the microtiter plate with neurogranin altered the IR of NM2. Aldehyde fixation reduced IR of NM2 20-fold (data not shown).

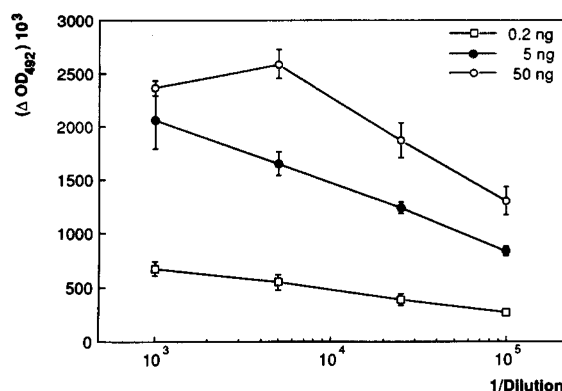

FIG. 3. Effect of the dilution of NM2 antibodies and concentration of coating neurogranin on the IR of NM2. IR is expressed in OD units of the assay (means ± SEM, n = 4).

IR of NM2 with the B-50 domain containing the PKC phosphorylation site

Because of our interest in the PKC phosphorylation site of B-50, we tested two synthetic peptides comprising this domain in ELISA. Figure 4 shows the results of an ELISA for NM2 and NM6 with a coat of various amounts of B-50 (39–51) peptide (see Materials and Methods). The IR of NM2 increased with increasing doses of this peptide. In contrast, the IR signal for NM6 was approximately equal to the background signal and independent of the dose of the coated peptide, though NM6 was applied in a fivefold higher concentration than NM2. Table 1 shows a qualitative comparison of limiting dilutions (titers) of NM2 with NM6 in ELISAs with coats of different proteins and polypeptides at different doses. NM2 and NM6 did not recognize B-50 (43–51) peptide. The lack of IR was not due to failure of this nonapeptide to absorb onto the microtiter plate well, because a polyclonal antiserum raised to this peptide produced a positive signal. As shown, NM2 immunoreacted very well with B-50 present in ASP (Zwiers et al., 1985). NM2 detected neurogranin very well in ELISA. NM6 recog-

TABLE 1. *Comparison of the titer of IR of NM2 and NM6, using an ELISA with B-50, B-50 peptides, and neurogranin as the antigen coat*

Antigen	Titer	
	NM2	NM6
B-50[a]	2.5×10^5	5×10^6
ASP[b]	10^7	10^8
B-50 (39–51) peptide[c]	2×10^4	Neg
B-50 (43–51) peptide[c]	Neg	Neg
Neurogranin[a]	6×10^5	Neg

Titer is defined as the reciprocal of the limiting dilution that resulted in a signal (OD) of 0.100 above background signal. Negative (Neg) means that the signal was not different from background.
[a] 5 ng of protein.
[b] Enriched in 25 ng of B-50.
[c] 500 ng of peptide.

nized only purified rat B-50 and B-50 in ASP. The titer of NM6 was more than 10-fold higher than the titer of NM2; this was also found using equal amounts of protein of the affinity-purified IgGs of NM2 and NM6 in an ELISA with a coat of B-50 in ASP (data not shown).

We tested whether preabsorption of NM2 by B-50 peptides would affect the IR of NM2 with respect to different antigen coats. Table 2 shows that the used dose (0.8 μg) of B-50 (39–51) peptide (abbreviated to 39–51) inhibited the IR of NM2 on coats of purified B-50 (Exp. 1), ASP (Exp. 2), and B-50 (39–51) peptide (Exp. 4). Following preabsorption with B-50 (43–51) peptide (abbreviated to 43–51), NM2 was not inhibited when using a coat of purified B-50 (Exp. 1) or B-50 (39–51) peptide (Exp. 4), and inhibited a little with a coat of ASP (Exp. 2). The IR of NM2 for B-50 was not influenced by preabsorption with B-50 (214–226) peptide (Exp. 3). This C-terminal peptide of B-50 has been used previously to raise a polyclonal B-50 antiserum which was applied in developmental studies of the B-50 expression in the embryonic mouse nervous system (Biffo et al., 1990). A higher dose (2 μg) of the peptides in the preabsorption test did not alter the results qualitatively.

When the B-50 (39–51) and (43–51) peptides were used as competitors for the antigen coat of neurogranin, only the B-50 (39–51) peptide reduced the IR of NM2. Using neurogranin (BICKS, 0.8 μg) as the competitor, the IRs of NM2 for the antigen coat of rat B-50 and of B-50 (39–51) peptide were reduced (data not shown). The IR of NM6 for B-50 was not influenced by preabsorption with either of the three synthetic B-50 peptides used in Table 2 or with neurogranin (data not shown).

Inhibition of the endogenous B-50 phosphorylation of SPM by NM2 and prevention by preabsorption with B-50 (39–51) peptide

B-50 antibodies of NM2 were tested for their inhibitory action on the endogenous phosphorylation of B-50 in SPM that is mediated by PKC (Aloyo et al., 1983). IgGs of NM2 were purified by affinity chromatography on a protein G column in order to avoid interference in the phosphorylation assay by constituents of the ascites fluid other than the B-50 antibodies. The phosphorylation assay was carried out as described under Materials and Methods and analyzed by quantitative densitometry of the obtained autoradiograms. The autoradiogram of Fig. 5 demonstrates that affinity-purified IgGs of NM2 (three lanes numbered 4) selectively inhibited endogenous B-50 phosphorylation (inhibition 36.7 ± 2.5%, $p < 0.001$) with respect to control (100%, three lanes numbered 1 and 7). The extent of inhibition was dependent on the dose of IgGs. Endogenous phosphorylation of B-50 in SPM (control, 100%, three lanes numbered 1 and 7) was not affected by the addition of B-50 (39–51) peptide (three lanes numbered 2) or B-50 (43–51) peptide (three lanes numbered 6). However, preabsorption of the NM2 antibodies by a large excess of B-50 (39–51) peptide (three lanes numbered 3) prevented the inhibition of B-50 phosphorylation (94.5 ± 7.5%, not significantly different from control) by NM2 IgGs. Preabsorption of NM2 by B-50 (43–51) peptide (three lanes numbered 5; in large molar excess) was less effective (59.7 ± 6.5%, $p < 0.001$) in prevention of the NM2-induced inhibition.

DISCUSSION

Characterization of B-50 Mabs: NM2 versus NM6

The aim of this study was to select and characterize a Mab that inhibits PKC-mediated phosphorylation

TABLE 2. *Effect of preabsorption of NM2 on the IR in an ELISA using different antigen coats*

Experiment no.	Antigen coat	Preabsorbed with:	OD × 10^3	%
1	B-50	None	436 ± 62	100
	B-50	39–51	1	0.5
	B-50	43–51	390 ± 13[a]	90
2	ASP	None	2,612 ± 134	100
	ASP	39–51	162 ± 28[b]	6
	ASP	43–51	2,088 ± 102[c]	80
3	ASP	None	834 ± 90	100
	ASP	214–226	804 ± 123[a]	96
4	39–51	None	2,724 ± 95	100
	39–51	39–51	0	0
	39–51	43–51	2,695 ± 114[a]	99

IR is expressed in units of OD × 1,000 (means ± SEM, n = 4). Student's *t* test was used to determine significance. Each experiment was carried out three times. NM2 was diluted $1{:}10^4$, $1{:}10^6$, $1{:}2.5 \times 10^5$, and $1{:}6 \times 10^3$ for the respective antigen coats of the four experiments: 5 ng of purified B-50 (Exp. 1), ASP containing approximately 25 ng of B-50 (Exps. 2 and 3), and 500 ng of B-50 (39–51) peptide (Exp. 4). The preabsorption was carried out with 0.8 μg of the indicated peptide added to the diluted antibody as described in Materials and Methods.
[a] $p > 0.05$, not significant; [b] $p < 0.001$; [c] $p < 0.01$.

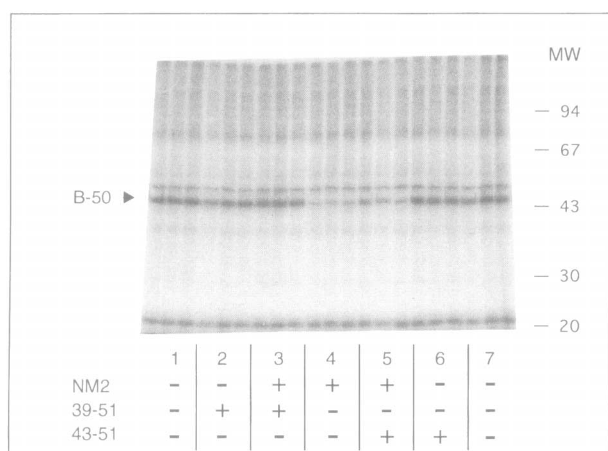

FIG. 5. Effect of preabsorption of NM2 with B-50 (39–51) peptide and B-50 (43–51) peptide on the NM2-induced inhibition of the B-50 phosphorylation in SPM. As described in Materials and Methods, aliquots of SPM were preincubated with no addition (lanes 1 and 7), with added B-50 (39–51) peptide (lanes 2) or added B-50 (43–51) peptide (lanes 6), with diluted affinity-purified NM2 antibodies (lanes 4), or with NM2 antibodies together with B-50 (39–51) peptide (lanes 3) or B-50 (43–51) peptide (lanes 5). Phosphorylation was started by addition of ATP and stopped 15 s later. Protein phosphorylation (n = 3) was analyzed by SDS-PAGE and autoradiography. The endogenous B-50 phosphorylation in SPM under various conditions is indicated by an arrowhead (B-50) in the autoradiogram. NM2 antibodies (lanes 4 and 5) inhibited selectively B-50 phosphorylation. Preabsorption with B-50 (39–51) peptide prevented the inhibition by NM2, whereas preabsorption with B-50 (43–51) peptide reduced the inhibition of NM2.

of B-50, suggested to be crucial for the physiological role of B-50 (De Graan et al., 1991). This study maps the epitope recognized in B-50 and neurogranin by the monoclonal NM2, one of the set of mouse B-50 Mabs NM1-8 (Mercken et al., 1992). These B-50 Mabs recognize B-50 in bovine, human, and rat brain and do not distinguish between phosphorylated and dephosphorylated rat B-50 on western blot. The B-50 Mabs were selected from hybridoma clones secreting antibodies raised to a bovine brain extract (Mercken et al., 1992). Polyclonal B-50 antibodies have been raised in rabbits against rat B-50 (Oestreicher et al., 1984, 1989). Antibodies from both sources cross-react with B-50 of other vertebrates. This potential can be understood from the findings that the primary structure of B-50 is highly conserved in vertebrate evolution; particularly close homology is present in the first 60 amino acids containing the N-terminal domain of membrane attachment and the PKC phosphorylation site (LaBate and Skene, 1989).

Here we have documented that NM2 recognizes an epitope located in the 39–51 domain of rat B-50 containing the unique PKC phosphorylation site. This identification is based on the following findings (Fig. 6):

(a) NM2 recognizes the SAP1 fragment [B-50 (1–132)] and the SAP2 fragment [B-50 (1–65)], but not the CH1 fragment [B-50 (1–42)]. As NM2 does not recognize the CH1 [B-50 (1–42)], it appears that the epitope for NM2 does not reside in the N-terminal part of the phosphorylated fragments SAP1 and SAP2.

(b) NM2 reacts with B-50 (39–51) peptide and with neurogranin containing a nonapeptide, KIQASFRGH, which is identical to the amino acid sequence 37–45 of rat B-50.

(c) NM2 does not react with the B-50 (43–51) peptide, which lacks the amino acid sequence QASF. Some binding activity has been found in preabsorption tests.

(d) NM2 does not bind the synthetic C-terminal B-50 (214–226) peptide.

(e) During endogenous phosphorylation of native proteins of SPM, NM2 inhibits selectively the B-50 phosphorylation that occurs at serine-41, the unique phosphorylation site of PKC (Coggins and Zwiers, 1989; Apel et al., 1990; Nielander et al., 1990). Preabsorption of NM2 by B-50 (39–51) peptide prevents this inhibition.

Therefore, we suggest that NM2 recognizes the native and denatured epitope in rat B-50 that contains at least the sequence QASFR, including the unique PKC phosphorylation site at serine-41. We have not been able to test if this sequence represents the minimum epitope that NM2 recognizes. It is remarkable that a cut in this domain (e.g., by chymotrypsin) abolishes the IR of NM2. Also, we cannot exclude the possibility that the epitope in rat B-50 may extend a little more N-terminally to include the KI at positions 37–38, and C-terminally into the adjoining GHxxRKK peptide (Fig. 6). The cross-reactivity with neurogranin may be suggestive of this. Results obtained by ELISA on plates coated with similar amounts (nanograms) of rat B-50 and neurogranin (molecular ratio B-50/neurogranin = 1/3) suggest that NM2 recognized purified B-50 and neurogranin with approximately the same affinity. However, the coating efficiency and the availability of specific domains of the coated proteins could be different. The small, but significant, effect of preabsorption of NM2 by B-50 (43–51) peptide could indicate that part of the epitope is located in this domain.

No cross-reaction was found with another well known PKC substrate MARCKS (myristoylated alanine-rich C kinase substrate). This protein can be phosphorylated by PKC and binds calmodulin in an amino acid sequence domain quite different from that of B-50 and neurogranin (Aderem, 1992).

For the other Mab NM6, the epitope in rat B-50 is clearly different from that of NM2. We may only roughly indicate the epitope of NM6 as located somewhere in the C-terminal (132–214) domain of rat B-50.

B-50 antibodies to elucidate the physiological role of B-50

As demonstrated, NM2 is a reproducible and defined antibody recognizing the PKC phosphorylation

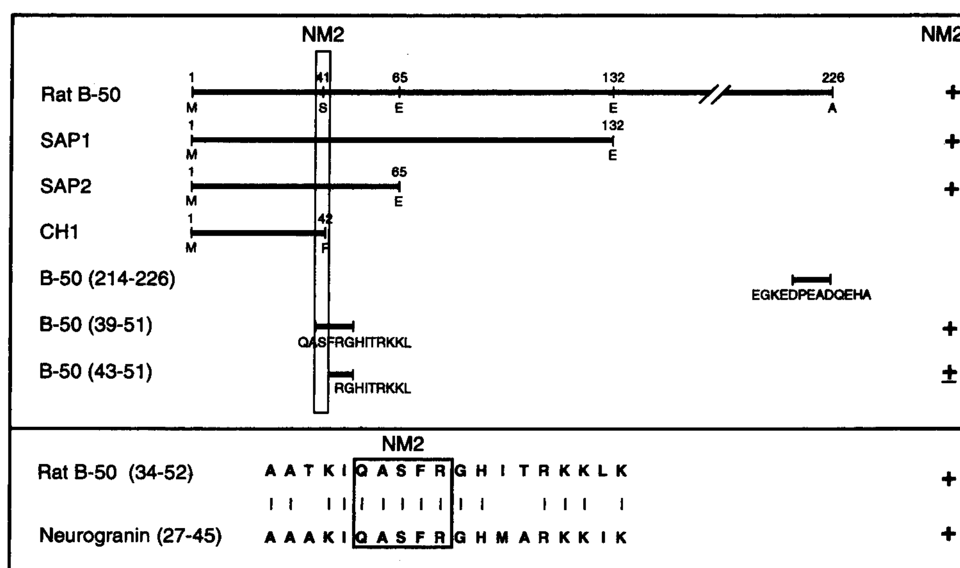

FIG. 6. Schematic representation of the primary structure of rat B-50, proteolytic phosphorylated fragments (Nielander et al., 1990), peptides, and neurogranin (Baudier et al., 1991), showing the homology of amino acid sequences, PKC phosphorylation site, the epitope, and IR of NM2.

site of B-50 and neurogranin. Because this antibody inhibits PKC-mediated phosphorylation of native B-50, it may be used to interfere with this reaction to study its physiological consequences.

Previously, we have reported that polyclonal affinity-purified B-50 antibodies inhibit B-50 phosphorylation in SPM and increase simultaneously the formation of phosphatidylinositol 4,5-bisphosphate (Oestreicher et al., 1983), suggesting that B-50 phosphorylation is associated with this second messenger pathway (Van Dongen et al., 1985). Introduction of polyclonal B-50 antibodies into permeated synaptosomes inhibits Ca^{2+}-induced noradrenaline release and B-50 phosphorylation (Dekker et al., 1989, 1991; Hens et al., 1993*b*). This evidence indicates that the function of B-50 and neurotransmitter release from nerve terminals may be causally related. In recent experiments, selective blocking of the PKC phosphorylation domain in B-50 by NM2 has been tested in our study of the involvement of B-50 in stimulated neuropeptide release. Application of NM2 in permeated synaptosomes resulted in inhibition of Ca^{2+}-induced cholecystokinin-8 release (Hens et al., 1993*a*).

More possibilities are open. Studies are currently in progress to find out whether in vitro other biochemical reactions of B-50 and neurogranin are affected by the presence of NM2, e.g., association and dissociation of calmodulin and dephosphorylation of the phosphorylated PKC site by protein phosphatases. Other applications would be measurement of the relative content of B-50 and neurogranin in tissues and cell cultures by either semiquantitative western blot or ELISA. In in vivo studies, immunoprecipitation of the proteins by NM2 using protein G beads could be applied to estimate changes in B-50 and neurogranin phosphorylation in the hippocampal slice model of long-term potentiation (Gianotti et al., 1992).

The expression and immunocytochemical distribution of B-50 (Jacobsen et al., 1986; Oestreicher and Gispen, 1986; Benowitz et al., 1988; McGuire et al., 1988) and neurogranin (Represa et al., 1990) in the nervous system are different with regard to development and regional and subcellular localization. B-50 is highly expressed during neuronal differentiation, neurite outgrowth, and axonal regeneration and is down-regulated on synaptogenesis. After neuronal polarization, B-50 becomes restricted to the presynaptic axonal domain (Van Lookeren Campagne et al., 1992). Neurogranin is expressed much later in development and is mainly located postsynaptically in dendrites and cell bodies of certain brain areas (Represa et al., 1990).

The high B-50 expression during development of the nervous system and axonal regeneration has been explained by the suggestion that B-50 has a decisive role in neuritogenesis (e.g., Strittmatter et al., 1992). To test the role of B-50 in neurite outgrowth, Shea et al. (1991) introduced polyclonal B-50 antibodies into developing neuroblastoma cells in culture. They found that this resulted in the arrest of neurite elongation, suggesting that B-50 must be available for continued neuritogenesis. Using NM2 in this paradigm, it is now possible to examine whether cyclic phosphorylation by PKC and dephosphorylation are required for persistent neurite outgrowth.

In immunocytochemical studies, Meiri et al. (1991) have used a well defined B-50 Mab (2G12/G7) that recognizes exclusively the peptide IQA(S-PO_4)FR,

thus detecting specifically the PKC-phosphorylated site at serine-41 in B-50. By means of this Mab and another undefined Mab that does not distinguish between phosphorylated and dephosphorylated B-50, Meiri et al. (1991) showed that PKC-mediated phosphorylation of B-50 is not manifested during initial neurite outgrowth in vitro and in vivo. By using these Mabs, Dent and Meiri (1992) reported further on the regulation of B-50 phosphorylation in individual growth cones in neuronal cell cultures. These authors suggested that B-50 phosphorylation by PKC is not required for neurite outgrowth per se, but it may be involved in discriminatory translation of extracellular signals to various intracellular responses.

Schreyer and Skene (1991) characterized B-50 Mabs immunocytochemically and applied these to study the transport of B-50 in the rat dorsal column axons after induction of B-50 synthesis by peripheral nerve injury. The authors compared the patterns of the B-50 immunostaining in rat spinal cord for various Mabs and showed that the observed differences were related to the ability of these Mabs to bind to various isoforms of posttranslationally modified B-50.

In conclusion, we have characterized NM2 as a Mab that recognizes a common epitope (QASFR) in B-50 and neurogranin. NM2 can indeed interfere selectively, in an in vitro model, with one of the posttranslational modifications of B-50, namely, endogenous B-50 phosphorylation by PKC in SPM. This suggests that NM2 can be used to study specifically the effects of interactions of other proteins with this epitope in either B-50 or neurogranin. Studies using NM2 are now in progress, investigating the significance of B-50 phosphorylation/dephosphorylation and B-50 binding to calmodulin in neurotransmitter release from permeated synaptosomes.

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