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A New Peptide Conjugate as a Highly Specific Substrate for MAP Kinase[1]

Isamu Kameshita, Atsuhiko Ishida, and Hitoshi Fujisawa[2]

Department of Biochemistry, Asahikawa Medical College, Asahikawa, Hokkaido 078

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A synthetic peptide (APRTPGGRC) cross-linked to poly-L-lysine through a carboxy-terminal cysteinyl residue was found to be a highly specific substrate for mitogen-activated protein (MAP) kinases. This peptide conjugate exhibited a much lower K_m value (74 μM) than the free peptide substrate (APRTPGGRR, $K_m > 1$ mM) previously used as a specific substrate for MAP kinases. Unlike myelin basic protein, which has been often used as a substrate for MAP kinases, this conjugate did not serve as substrate for cAMP-dependent protein kinase, protein kinase C, or multifunctional calmodulin-dependent protein kinases. Using the peptide conjugate as a substrate, MAP kinase activities in crude cell extracts were directly determined by *in vitro* assay and specifically detected by in-gel assay.

Key words: MAP kinase, PC12 cells, protein kinase, protein phosphorylation.

MAP kinases represent a family of protein serine/threonine kinases that are activated in response to various extracellular signals (*1-3*). In PC12 cells, this enzyme is rapidly activated upon stimulation of cells by growth factors such as NGF (*4-9*) and epidermal growth factor (*5, 6*), or by calcium influx caused by membrane depolarization (*9*), and the activation occurs through phosphorylation of both tyrosyl and threonyl regulatory sites in the enzyme (*4, 8-10*). Since MAP kinase activity changes drastically within a few minutes after stimulation, a quick and accurate assay method of MAP kinase is necessary for studying the physiological significance of this enzyme, but its development has been hampered by the lack of a specific and efficient substrate. Although MBP and MAP2 have been often used for *in vitro* assay of MAP kinase in crude extracts, they are known also to serve as substrates for such protein kinases as cAMP-dependent protein kinase, protein kinase C, and CaM-kinases (*11*). The phosphorylation site of MBP by MAP kinase was identified as Thr-97, and this site was reported to be specific for MAP kinase (*12*). The minimum consensus sequence for phosphorylation by MAP kinase was identified as a P-X-T/S-P motif (*13, 14*). Pelech and his associates employed a synthetic peptide (APRTPGGRR, called MBP peptide) modeled after the consensus sequence as a specific substrate for MAP kinase (*13*), but this peptide exhibited relatively high K_m and low V_{max} values as compared to MBP.

We recently found that peptides often became more efficient substrates when conjugated to amino acid polymers such as poly(Lys) (*15*). In the present study, therefore, two oligopeptides (CPRTPGGRR and APRTPGGRC) containing the PRTP motif, corresponding to the phosphorylation site of MBP by MAP kinase, were conjugated to poly(Lys) through their amino- or carboxy-terminal cysteinyl residues and their susceptibility to MAP kinase was examined. Since APRTPGGRC-poly(Lys) conjugate was the better substrate of the two, its properties were studied in detail. The results indicate that this substrate is very useful for not only *in vitro* assay but also in-gel assay of MAP kinases in crude cell extracts.

MATERIALS AND METHODS

Materials—[γ-^{32}P]ATP (5,000 Ci/mmol) was purchased from Amersham. Poly(Lys) with average molecular weights of 9,600, 23,000, and 87,000, Kemptide (LRRASLG), bovine serum albumin, MBP, and prestained molecular weight markers for SDS-polyacrylamide gel electrophoresis were obtained from Sigma Chemicals. Murine nerve growth factor (2.5 S) was purchased from Promega. Catalytic subunit of cAMP-dependent protein kinase was purified from bovine heart as described previously (*16*). Protein kinase C was purified from rat brain by the method of Woodgett and Hunter (*17*). CaM-kinase II (*18*) and CaM-kinase IV (*19*) were purified from rat brain as described previously.

Peptides (APRTPGGRR, CPRTPGGRR, and APRTPGGRC) containing the PRTP motif were synthesized using a Shimadzu PSSM-8 automated peptide synthesizer. The peptides containing cysteinyl residues were purified and conjugated to poly(Lys) by a heterobifunctional reagent through their amino- or carboxy-terminal cysteinyl residues (*15, 20*). Briefly, 0.4 mg of poly(Lys)$_{9.6}$, poly(Lys)$_{23}$, or poly(Lys)$_{87}$ was dissolved in 100 μl of 10 mM sodium

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[2] To whom correspondence should be addressed.

Abbreviations: CaM-kinase, calmodulin-dependent protein kinase; MAP kinase, mitogen-activated protein kinase; MAP2, microtubule-associated protein 2; MBP, myelin basic protein; NGF, nerve growth factor; poly(Lys), poly-L-lysine; poly(Lys)$_{9.6}$, poly(Lys)$_{23}$, and poly(Lys)$_{87}$, poly-L-lysine with average molecular weights of 9,600, 23,000, and 87,000.

phosphate buffer (pH 7.2) and mixed with 0.28 mg of *N*-(ε-maleimidocaproyloxy)succinimide (Dojindo Laboratories) dissolved in 11 μl of dimethyl formamide. The mixture was stirred for 30 min at room temperature, then extracted three times with equal volumes of dichloromethane to remove the unreacted reagent. Poly(Lys) solution (50 μl) thus obtained was mixed with 50 μl of 5 mM CPRTPGGRR or APRTPGGRC and stirred for 3 h at room temperature. If necessary, the ratio of the peptides to poly(Lys) was changed as indicated. The reaction was stopped by adding dithiothreitol in a final concentration of 5 mM, and the peptide conjugate solution was stored at −20°C in aliquots.

PC12 Culture and NGF Treatment—PC12 cells were cultured at 37°C in RPMI1640 supplemented with 10% horse serum and 5% new born calf serum in an atmosphere of 5% CO_2. PC12 cells attached to collagen-coated dishes (6.8×10^6 cells/90 mm dish) were treated with 100 ng/ml NGF at 37°C for 5 or 60 min, then washed three times with ice-cold PBS. The cells were removed with a scraper into 0.2 ml of an extraction buffer consisting of 20 mM Tris-HCl (pH 7.4), 20 mM NaCl, 1 mM sodium orthovanadate, 10 mM NaF, 5 mM sodium pyrophosphate, 0.5 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, 0.2 μM okadaic acid, and 10 μg/ml each of leupeptin, antipain, chymostatin, and pepstatin A at 0°C. The cells were suspended by five passages through a 25-gauge needle, and homogenized four times by sonication for 30 s with 30-s intervals. The homogenate was centrifuged at $15,000\times g$ for 10 min, and supernatant was used as a cell extract.

In Vitro Kinase Assay—The standard assay mixture for MAP kinase comprised, in a final volume of 25 μl, 50 mM Tris-HCl (pH 7.4), 10 mM $MgCl_2$, 2 mM EGTA, 2 mM dithiothreitol, 50 μM [γ-^{32}P]ATP (500-1,000 cpm/pmol), and indicated substrates. The reaction was initiated by the addition of PC12 extracts (10 μg) or protein kinases (0.1 μg). After incubation for 3 min at 30°C, the reaction was stopped by the addition of 25 μl of stop solution (100 mM ATP and 50 mM EDTA), 40-μl aliquots were withdrawn and the incorporation of [^{32}P]phosphate into the substrate was determined by the phosphocellulose paper method (*21*). The incorporation of [^{32}P]phosphate increased linearly for at least 3 min when a crude extract was used as an enzyme source.

In-Gel Kinase Assay—In-gel assay of protein kinases was performed essentially as described previously (*15, 22*). Protein samples were subjected to SDS-polyacrylamide gel electrophoresis on 10% polyacrylamide separation gel containing 25 μM peptide-conjugate or 0.5 mg/ml of MBP, and separated proteins were renatured *in situ*. The gel was incubated at room temperature for 1 h in the standard assay mixture for MAP kinase as described above, and analyzed by autoradiography (*22*).

Protein Determination—Proteins were determined by the method of Bensadoun and Weinstein (*23*) using bovine serum albumin as a standard.

RESULTS

Since it has been reported that MAP kinase activity in PC12 cells increases severalfold within few minutes after treatment with NGF and thereafter declines slowly (*5-9*), the crude extract of NGF-treated or untreated PC12 cells was used as a source of MAP kinase in the present study. Figure 1 shows the phosphorylation activities of the cell extracts toward MBP, MBP peptide (APRTPGGRR), APRTPGGRC conjugated to poly(Lys)$_{87}$, and Kemptide. The activities toward MBP, APRTPGGRR peptide,

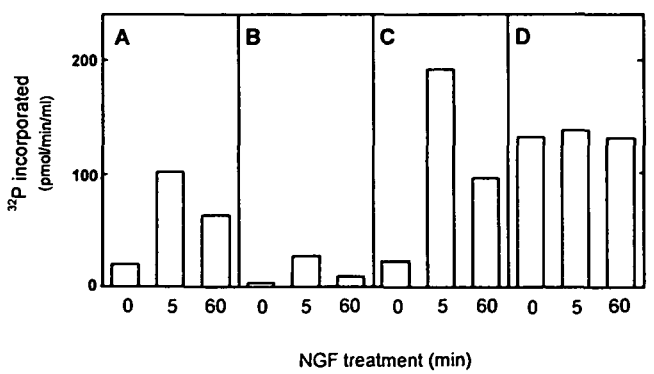

Fig. 1. **Phosphorylation of various substrates by the crude extracts of NGF-treated or untreated PC12 cells.** The kinase activities of approximately 10 μg of protein of the crude extracts of PC12 cells which had been treated with 100 ng/ml NGF at 37°C for the indicated times were measured using 0.2 mg/ml MBP (A), 1 mM MBP peptide (APRTPGGRR) (B), 0.2 mM APRTPGGRC conjugated to 0.16 mg/ml poly(Lys)$_{87}$ (C), or 80 μM Kemptide (D) as a substrate, as described under "MATERIALS AND METHODS."

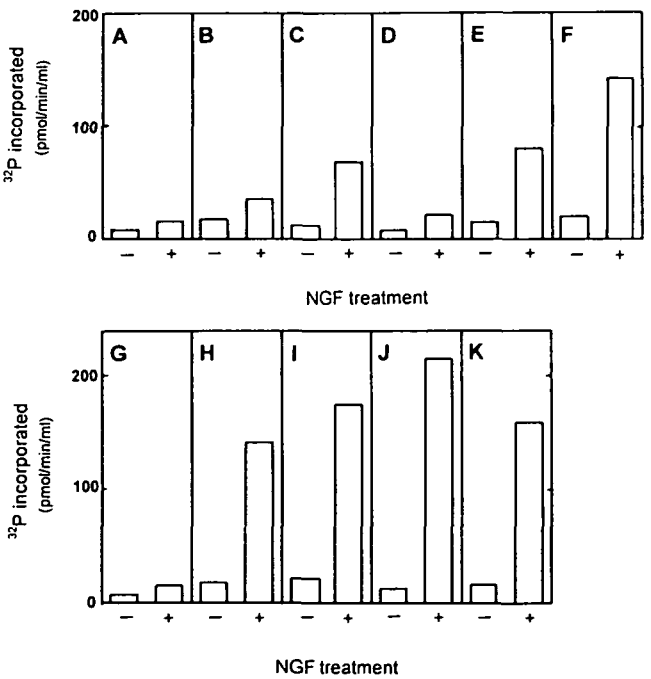

Fig. 2. **Phosphorylation of various peptide conjugates by the crude extracts of NGF-treated or untreated PC12 cells.** The kinase activities of approximately 10 μg of protein of the crude extracts of PC12 cells which were untreated (−) or had been treated with 100 ng/ml NGF at 37°C for 5 min (+) were measured using 0.1 mM CPRTPGGRR conjugated to 0.08 mg/ml of poly(Lys)$_{9.6}$ (A), poly(Lys)$_{23}$ (B), poly(Lys)$_{87}$ (C), and 0.1 mM APRTPGGRC conjugated to 0.08 mg/ml poly(Lys)$_{9.6}$ (D), poly(Lys)$_{23}$ (E), poly(Lys)$_{87}$ (F), or 0.032 mg/ml (G), 0.08 mg/ml (H), 0.16 mg/ml (I), 0.2 mg/ml (J), or 0.4 mg/ml (K) of poly(Lys)$_{87}$ as substrates, as described under "MATERIALS AND METHODS."

APRTPGGRC-poly(Lys)$_{87}$ increased severalfold at 5 min after NGF stimulation and declined at 60 min, suggesting that the activities toward these three substrates were attributable to MAP kinases. In contrast, the activity toward Kemptide, a known peptide substrate for cAMP-dependent protein kinase, was not significantly affected by NGF stimulation. The activity observed with 0.2 mM APRTPGGRC conjugated to poly(Lys)$_{87}$ as a substrate was much higher than that observed with 0.2 mg/ml of MBP or 1 mM APRTPGGRR, suggesting that the conjugated peptide possessing the PRTP motif was an efficient and specific substrate for MAP kinase. Figure 2 shows the phosphorylation of two PRTP motif-possessing peptides, CPRTPGGRR and APRTPGGRC, which were conjugated at their terminal cysteinyl residues to poly(Lys)s of three different sizes, poly(Lys)$_{9.6}$ (M_r 9,600), poly(Lys)$_{23}$ (M_r 23,000), and poly(Lys)$_{87}$ (M_r 87,000), for phosphorylation by MAP kinase. The peptide conjugated to poly(Lys)s at the carboxy-terminal cysteinyl residue showed higher activity than the peptide conjugated at the amino-terminal cysteinyl residue, and the peptide conjugated to poly(Lys)$_{87}$ showed higher activity than those conjugated to poly(Lys)$_{9.6}$ and poly(Lys)$_{23}$ (Fig. 2, A-F). When the effect of the ratio of APRTPGGRC to poly(Lys)$_{87}$ in the conjugate on the phosphorylation of the peptide conjugates by MAP kinase was examined (Fig. 2, G-K), the conjugate of 1 μmol of peptide to 2 mg of poly(Lys)$_{87}$ showed the highest activity. Table I shows the kinetic parameters of MAP kinase determined with MBP, MBP peptide, and the peptide conjugate as substrates. The estimated K_m value for MBP peptide was as high as 1.6 mM, but the K_m for the peptide conjugate was 74 μM, the value being close to the K_m for MBP (44 μM). The V_{max} for the peptide conjugate was 3- to 4-fold higher than that for MBP peptide, although it was a little lower than that for MBP. Thus, the peptide conjugate, APRTPGGRC-poly(Lys)$_{87}$, was a much better substrate for MAP kinase than MBP peptide, APRTPGGRR. The desired characteristics of a substrate for enzyme assay are its availability and specificity for the enzyme. Table II shows the specificities of MBP, MBP peptide, and the peptide conjugate for MAP kinase. Four representative second-messenger-responsive multifunctional protein kinases, cAMP-dependent protein kinase, protein kinase C, CaM-kinase II, and CaM-kinase IV, were examined for their ability to phosphorylate the three substrates. MBP, which has so far been most often used for the assay of MAP kinases in crude extracts, was very efficiently phosphorylated by cAMP-dependent protein kinase as well as the extract of the NGF-stimulated PC12 cells and also slightly phosphorylated by protein kinase C. In contrast, MBP peptide and the peptide conjugate were not significantly phosphorylated by any other protein kinase than the PC12 cell extracts under current assay conditions for MAP kinases. Although protein kinase C and CaM-kinases phosphorylated MBP under their optimal phosphorylation conditions, they did not phosphorylate MBP peptide or the peptide conjugate at all (data not shown). These results suggest that MBP peptide and its conjugate might be specific substrates for MAP kinases, although the peptide conjugate was the much better substrate. The addition of poly(Lys)$_{87}$ to the reaction mixture increased the activity toward MBP peptide about 4-fold (data not shown), in good agreement with the previous report that the activity of MAP kinase is stimulated by poly(Lys) (*24*), but much higher activation (about 30-fold) was observed when the peptide conjugate was used as a substrate (Table II), suggesting that efficient phosphorylation of the peptide conjugate was not attributable to the effect of poly(Lys) on MAP kinase activity. Thus, the peptide conjugate may be a potent and also specific substrate for MAP kinases.

We have developed a sensitive and selective method for detection of various protein kinases in crude tissue extracts in gels after SDS-polyacrylamide gel electrophoresis (in-gel assay) (*15, 22*). This method is inapplicable to oligopeptide substrates, which are eluted from the gel matrix during electrophoresis, but applicable to peptide conjugates or protein substrates, which are retained in the gel matrix (*15*). Figure 3 shows selective detection of MAP kinases in the crude extracts of NGF-stimulated PC12 cells by in-gel assay using the peptide conjugate as a substrate. As shown in Fig. 3C, approximately 10 μg of protein of PC12 cell extract gave an intense radioactive doublet corresponding in mobility to molecular weights of 40,000 to 45,000 on a gel containing the peptide conjugate as a substrate (the mobility coincided with 42- and 44-kDa MAP kinases), and the intensity increased severalfold at 5 min after NGF stimulation and declined at 60 min, in agreement with result of *in vitro* assay with the peptide conjugate (Fig. 1). When MBP was used as an immobilized substrate, 10 μg of protein of the extracts of the NGF-stimulated PC12 cells gave a doublet at 40,000 to 45,000, but 50 ng of catalytic subunit of cAMP-dependent protein kinase also gave an intense band at the same region (Fig. 3B). Since our previous results (*15*) suggest that 10 μg of protein of a PC12 cell extract contains about 10 ng of the catalytic

TABLE I. **K_m and V_{max} values of MAP kinase determined with MBP, MBP peptide, and the peptide conjugate.** The activities of 10 μg of protein of the crude extract of PC12 cells treated with NGF for 5 min were determined at various concentrations of the indicated substrates as described under "MATERIALS AND METHODS," and the K_m and V_{max} values were estimated from double reciprocal plots.

Substrate	K_m (μM)	V_{max} (pmol/min/ml)
MBP	44	1,190
APRTPGGRR	1,600	250
APRTPGGRC-poly(Lys)$_{87}$[a]	74	825

[a] Approximately 1 mM APRTPGGRC was conjugated to 1.6 mg/ml of poly(Lys)$_{87}$.

TABLE II. **Phosphorylation of MBP, MBP peptide, and peptide conjugate by various protein kinases.** The activities of approximately 0.1 μg each of the indicated purified enzyme and 10 μg of the PC12 cell extract untreated or treated with 100 ng/ml of NGF for 5 min were determined with the indicated substrates, as described under "MATERIALS AND METHODS."

Protein kinase	^{32}P incorporated (pmol/min/ml)		
	0.2 mg/ml MBP	0.2 mM APRTPGGRR	0.2 mM APRTPGGRC-poly(Lys)$_{87}$
cAMP-dependent protein kinase	310.6	0	0
Protein kinase C	2.2	0	0
CaM-kinase II	0	0	0
CaM-kinase IV	0	0	0
PC12 extract (−NGF)	22.0	0.8	23.3
PC12 extract (+NGF)	102.4	6.9	191.8

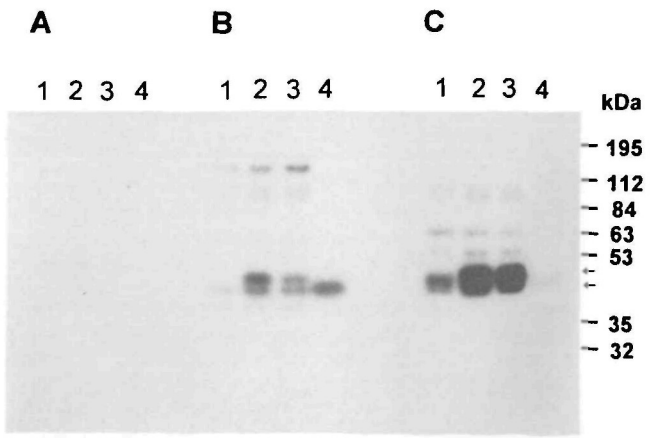

Fig. 3. **Detection of MAP kinase in crude cell extracts by in-gel assay.** Approximately 10 μg of protein of the crude extracts of PC12 cells which were untreated (lane 1) and had been treated with 100 ng/ml of NGF for 5 min (lane 2) or 60 min (lane 3), and 50 ng of catalytic subunit of cAMP-dependent protein kinase (lane 4) were electrophoresed on polyacrylamide gel containing no substrate (A), 0.5 mg/ml MBP (B), and 25 μM APRTPGGRC conjugated to 0.02 mg/ml poly(Lys)$_{87}$ (C), and analyzed by the in-gel protein kinase assay as described under "MATERIALS AND METHODS." The migration position of molecular weight markers is indicated on the right of the autoradiograph. The arrows indicate the positions of MAP kinases.

subunit of cAMP-dependent protein kinase, there may be a significant contribution of the catalytic subunit to the band observed with the PC12 cell extracts. The catalytic subunit showed no significant band on a gel containing the peptide conjugate (Fig. 3C, lane 4). Thus, the peptide conjugate can be used as a satisfactorily, if not absolutely, specific substrate for MAP kinases in in-gel assay as well as *in vitro* assay.

DISCUSSION

MAP kinases play important roles in signal transduction pathways, and alterations in the enzyme activities have been monitored by various methods, such as *in vitro* assay (*7, 24, 25*), immunocomplex assay (*9, 26*), in-gel assay (*5, 22*), gel mobility shift assay (*27, 28*), and detection of the activated enzyme by phosphotyrosine antibody (*29, 30*). For study of the physiological function of protein kinases, especially MAP kinases and others involved in intracellular signal transduction, it is necessary to determine the activity of the specific protein kinase in crude tissue or cell extracts. Simple and accurate assays for protein kinases in crude extracts by phosphorylation of protein or peptide substrates require efficient and specific substrates available in adequate quantity, but such specific substrates are not available for most protein kinases. In a few cases, synthetic peptide substrates have been successfully used for assays for specific protein kinases in crude extracts, for example, Kemptide for cAMP-dependent protein kinase (*31*), peptide-γ for CaM-kinase IV (*32*), and phospho-CREB peptide for glycogen synthase kinase 3 (*33*). For *in vitro* assay of MAP kinases, MAP2, MBP, and MBP peptide have so far been used as substrates, but MAP2 is known to serve as a substrate for many protein kinases (*34, 35*) and MBP was phosphorylated by cAMP-dependent protein kinase (Table II and Fig. 3). MBP peptide (APRTPGGRR), modeled after the phosphorylation site of MBP by MAP kinase, was not phosphorylated by cAMP-dependent protein kinase (Table II) but exhibited a very high K_m value (1.6 mM) (Table I), in agreement with the result of Pelech and his associates (*13*).

On the basis of the previous finding that linking of a peptide substrate to poly(Lys) sometimes increases efficiency of the peptide as a substrate for protein kinases (*15*), synthetic peptides similar to MBP peptide were conjugated to poly(Lys) and their availability for phosphorylation by MAP kinase was examined. A peptide, APRTPGGRC, conjugated to poly(Lys)$_{87}$ at the carboxy-terminal cysteinyl residue showed very low K_m (74 μM) and higher V_{max} value (Table I). In addition, in-gel assay revealed that the peptide conjugate could serve as a substrate that was highly, if not absolutely, specific for 42-/44-kDa MAP kinases. An additional faint radioactive band at 54 kDa also increased in the NGF-treated PC12 cell extract (Fig. 3C), suggesting that the conjugate may serve as a substrate for 54-kDa MAP kinase. Overall, the peptide conjugate is more suitable substrate for MAP kinases than MBP or MBP peptide, because it serves as a highly specific and efficient substrate for these enzymes. Other advantages of the peptide conjugate are that its strong positive charge serves to bind it to phosphocellulose paper in *in vitro* assay, and that its macromolecular nature enables in-gel assay of MAP kinases.

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