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Detection of a Variety of Ser/Thr Protein Kinases Using a Synthetic Peptide with Multiple Phosphorylation Sites[1]

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A novel peptide with multiple phosphorylation sites, which we designated as multide, was developed to detect a wide variety of protein kinases in crude cell extracts. Multide, KKRKSSLRRWSPLTPRQMSFDC, has been designed to contain consensus sequences for various Ser/Thr protein kinases including cAMP-dependent protein kinase, protein kinase C, MAP kinases, and Ca^{2+}/calmodulin-dependent protein kinases in a single peptide. In-gel protein kinase assay using multide was found to be very useful for analyzing the activities of protein kinases that are altered in response to various extracellular stimuli. The substrate specificities of the protein kinases thus detected were further determined by using five multide analogs with different phosphorylation sites.

Key words: enzyme assay, in-gel assay, protein kinase, protein phosphorylation, signal transduction.

A wide variety of extracellular signals produce diverse physiological responses in cells by regulating the phosphorylation of specific target proteins. Therefore, the activities of various protein kinases in cell signaling pathways should be altered in response to various extracellular stimuli. Although increasing numbers of protein kinases have been identified as constituents of protein kinase cascades, such as MAPK pathways (*1*), many protein kinases remain to be identified. Hence, the development of simple methods for detecting the activities of a variety of protein kinases is useful for studies of the mechanisms of intracellular signaling.

In the past, efforts have been directed toward the development of highly specific peptide substrates with a single phosphorylation site to detect specific protein kinases in cells. In the present study, in contrast, a novel peptide (multide) with multiple phosphorylation sites, KKRKSSLRRWSPLTPRQMSFDC, was employed so as to detect a variety of protein kinases in a crude cell extract. Multide has been designed to contain five possible phosphorylation sites in a peptide comprising 22 amino acids on the basis of consensus sequences for Ser/Thr protein kinases such as CaMKII, CaMKIV, PKA, PKC, and MAPKs. We used an in-gel protein kinase assay, which we developed (*2-4*), which uses multide and its analogs as substrates, to detect the activities of a number of protein kinases in crude cell extracts.

MATERIALS AND METHODS

Materials—[γ-^{32}P]ATP (5,000 Ci/mmol) was obtained from Amersham. Poly(Lys) with an average molecular weight of 87,000, poly(Glu,Lys,Tyr) with an average molecular weight of 23,000, Kemptide (LRRASLG), and myelin basic protein were purchased from Sigma Chemicals. Recombinant mouse p42 MAPK fused with GST (inactive) and recombinant human MEK1 (active) were purchased from Upstate Biotechnology. K252a, a general protein kinase inhibitor, was purchased from Calbiochem. Multide and its analogs, shown in Fig. 1, and syntide-2 (PLARTLSVAGLPGKK) (*5*) were synthesized using a Shimadzu PSSM-8 automated peptide synthesizer, and purified by reverse-phase HPLC on a C_{18} column (ODS-80Tm, Tosoh). The identity and purity of the peptides were confirmed by fast atom bombardment-mass spectrometry. The peptides were conjugated to poly(Lys) or poly(Glu, Lys,Tyr) as described previously (*2*). CaM was prepared from rat testis by the method of Dedman *et al.* (*6*). CaMKII (*7*) and PKA (*8*) were prepared as described previously.

Preparation of Colo201 Extracts—Culture of Colo201, a non-adherent colon adenocarcinoma cell line, and treatment of the cells with K252a were carried out as described previously (*9*). The cells were suspended in 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 disrupted by five passages through a 25-gauge needle, and homogenized four times by sonication (Olympus UC100-D) for 30 s at 30-s intervals. The homo-

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Abbreviations: CaMK, Ca^{2+}/calmodulin-dependent protein kinase; MAPK, mitogen-activated protein kinase; MEK, MAPK or ERK kinase; PKA, cAMP-dependent protein kinase; poly(Lys), poly-L-lysine; poly(Glu,Lys,Tyr), poly(glutamic acid,lysine,tyrosine) containing 60% glutamic acid, 30% lysine, and 10% tyrosine.

genates were centrifuged at 15,000×g for 10 min, and the supernatants thus obtained were used as cell extracts. Protein concentrations were determined by the method of Bensadoun and Weinstein (*10*) using bovine serum albumin as a standard.

Activation of MAPK—Recombinant p42 MAPK (1 μg) was activated by incubating the enzyme in a reaction mixture at a final volume of 50 μl, containing 50 mM Tris-HCl (pH 7.4), 10 mM $MgCl_2$, 2 mM EGTA, 2 mM dithiothreitol, 50 μM ATP, and recombinant active MEK1 (80 ng) at 30°C for 30 min. The reaction was stopped by the addition of 10 mM EDTA or SDS sample buffer, and stored at −80°C.

In Vitro Protein Kinase Assay—The standard assay mixture for PKA comprised, in a final volume of 50 μl, 40 mM Hepes-NaOH (pH 8.0), 5 mM $Mg(CH_3COO)_2$, 0.1 mM EGTA, 2 mM dithiothreitol, 50 μM [γ-^{32}P]ATP (200–500 cpm/pmol), and 20 μM oligopeptide. The reaction was initiated by the addition of 0.01 μg of the catalytic subunit of PKA. After incubation for 1 min at 30°C, 40-μl aliquots were withdrawn and the incorporation of [^{32}P]phosphate into the substrate was determined by the phosphocellulose paper method (*11*). The assay of CaMKII was carried out as described above, except that 0.2 mM $CaCl_2$ and 1 μM CaM were added to the reaction mixture.

In-Gel Protein Kinase Assay—Polyacrylamide gel electrophoresis in the presence of SDS was carried out essentially according to the method of Laemmli (*12*) on slab gels consisting of a 10% acrylamide separation gel and a 3% stacking gel. Crude extracts (10 μg) from Colo201 cells were analyzed by in-gel protein kinase assay using 20 μM peptide conjugated to 20 μg/ml of poly(Lys) or 20 μg/ml of poly(Glu,Lys,Tyr) as a substrate essentially as described previously (*2, 3*). Autophosphorylation of protein kinases was detected using a polyacrylamide gel without any substrate. Phosphorylation was carried out at 25°C for 2 h with standard phosphorylation mixture comprising, in a final volume of 2.5 ml for a minigel (8.5×4.5×0.1 cm), 50 mM Tris-HCl (pH 7.4), 10 mM $MgCl_2$, 2 mM EGTA, 2 mM dithiothreitol, 50 μM [γ-^{32}P]ATP (10 μCi/ml). In the case where CaMKII was detected, a phosphorylation mixture containing 0.2 mM $CaCl_2$ and 1 μM CaM was used.

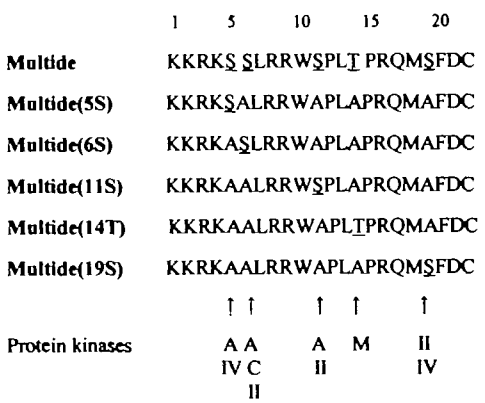

Fig. 1. **Amino acid sequences of multide and its analogs used as substrates in in-gel protein kinase assay.** Phosphorylatable residues are underlined, and possible phosphorylation sites for PKA (A), PKC (C), CaMKII (II), CaMKIV (IV), and MAPK (M) are shown by arrows.

RESULTS

Multide, KKRKSSLRRWSPLTPRQMSFDC, was designed to contain five possible phosphorylation sites for various Ser/Thr protein kinases such as CaMKII, CaMKIV, PKA, PKC, and MAPKs. In order to analyze the phosphorylation sites of multide, five multide analogs, in which four of the five phosphorylatable amino acid residues were replaced with Ala as shown in Fig. 1, were also synthesized.

The phosphorylation of multide and its analogs by PKA and CaMKII was compared with that of Kemptide and syntide-2 by *in vitro* kinase assay. Multide and multide(6S) were as efficient as substrates for PKA as Kemptide, which is a commonly used substrate for PKA (Table I). Multide-(5S), multide(11S), and multide(19S) were poor PKA substrates, while multide(14T) was not phosphorylated by PKA at all. Multide(19S) as well as multide served as good substrates for CaMKII. However, the efficiency of the phosphorylation of these peptides by CaMKII was several fold less effective than that of syntide-2.

In a previous study, we found that oligopeptides that are linked to amino acid polymers such as poly(Lys) or poly-(Glu,Lys,Tyr) are efficiently retained in the gel matrix and serve as substrates for protein kinases (*2, 3*). In this study, therefore, multide and its analogs were conjugated to poly(Lys) and used the in-gel assay. When purified preparations of PKA and CaMKII were analyzed by in-gel protein kinase assay using multide-poly(Lys) as a substrate, PKA showed an intense radioactive band corresponding to the position of the catalytic subunit of PKA (41 kDa) and CaMKII showed two bands corresponding to the α- (50 kDa) and β-isoforms (60 kDa) of CaMKII, as shown in Fig. 2. PKA gave a similar band in in-gel assay with multide(6S)-poly(Lys) as a substrate, suggesting that the major phosphorylation site of multide by PKA is Ser^6. Upon prolonged exposure, a dark band appeared in the same position in the in-gel assay with multide(5S)-, multide(11S)-, or multide(19S)-poly(Lys). The preference of PKA for multide(6S) is consistent with the results of the *in vitro* kinase assay as shown in Table I. CaMKII gave a dark radioactive band only when multide(19S) was used as a substrate and no positive band with the other four multide analogs. Although the content of the α-isoform is much greater than that of the β-isoform in purified CaMKII preparations (*13*), the β-isoform was observed as a more intense radioactive band than the α-isoform (Fig. 2B). The

TABLE I. **Comparison of the phosphorylation of multides and commonly used peptides by CaMKII and the catalytic subunit of PKA.** The activities of 0.01 μg PKA and CaMKII were determined using 20 μM peptide as described under "MATERIALS AND METHODS."

Substrates	Protein kinase activity (nmol/min/mg)	
	PKA	CaMKII
Multide	13,556	2,006
Multide(5S)	218	75
Multide(6S)	14,034	39
Multide(11S)	248	24
Multide(14T)	48	17
Multide(19S)	291	3,539
Kemptide	13,793	33
Syntide-2	546	9,450

reason for this phenomenon is not clear, but similar results were also obtained when the protein substrate was used for in-gel assay (*4*).

Next, inactive MAPK was activated by phosphorylation with MEK and used for the in-gel assay. Since the minimal consensus sequence for MAPK is known to be Ser/Thr-Pro (*14, 15*), both Thr^{14} and Ser^{11} may act as possible phosphorylation sites. However, when activated MAPK was analyzed by in-gel protein kinase assay, the enzyme activity was mainly detected when multide-poly(Lys) and multide-(14T)-poly(Lys) were used and only slightly detected with multide(11S)-poly(Lys) (Fig. 2C). This result is in good agreement with the previous report that Pro-Xaa-Ser/Thr-Pro is the best phosphorylation motif for MAPK (*16, 17*).

The successful use of in-gel assay with multide and its analogs as substrates for the differential detection of PKA, CaMKII, and MAPK led us to examine the possibility that this assay can be applied to the differential detection of various protein kinases in crude cell extracts. It has recently been demonstrated that treatment of Colo201 cells with K252a, a general protein kinase inhibitor, causes drastic morphological changes in the cells after 30-min (*9*), suggesting that the morphological changes might be triggered by the inhibition of a certain protein kinase. In order to identify changes in protein kinase activities in cells after treatment with K252a, the crude cell extracts were analyzed by in-gel assay using multide and its analogs as substrates, as shown in Fig. 3. Since the use of poly(Lys) to immobilize the oligopeptide substrates in the gel matrix produced radioactive bands in the PKA activity that were too intense to detect other protein kinase activities, poly-(Glu,Lys,Tyr) (*2*) was used instead of poly(Lys) in this experiment. In-gel assay using multide conjugate as a substrate revealed that the activities of at least three protein kinases with apparent molecular masses of 29, 50,

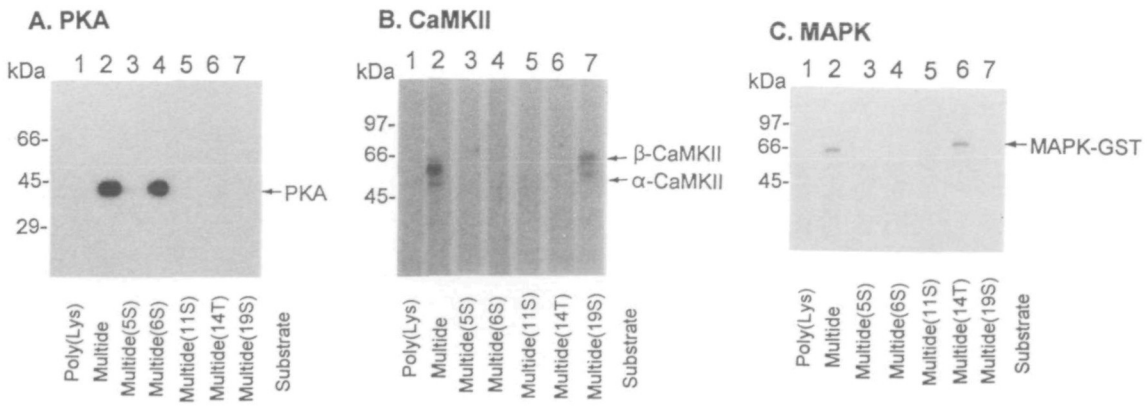

Fig. 2. **Detection of PKA, CaMKII, and MAPK by in-gel protein kinase assay.** Purified preparations of PKA (20 ng) (A), CaMKII (250 ng) (B), and activated MAPK-GST (20 ng) (C) were electrophoresed in an SDS-polyacrylamide gel containing 20 μg/ml poly(Lys) (lane 1), 20 μM multide-poly(Lys) (lane 2), multide(5S)-poly(Lys) (lane 3), multide(6S)-poly(Lys) (lane 4), multide(11S)-poly(Lys) (lane 5), multide(14T)-poly(Lys) (lane 6), and multide(19S)-poly-(Lys) (lane 7) as substrates. Inactive MAPK was activated by MEK and in-gel protein kinase assay was carried out as described under "MATERIALS AND METHODS."

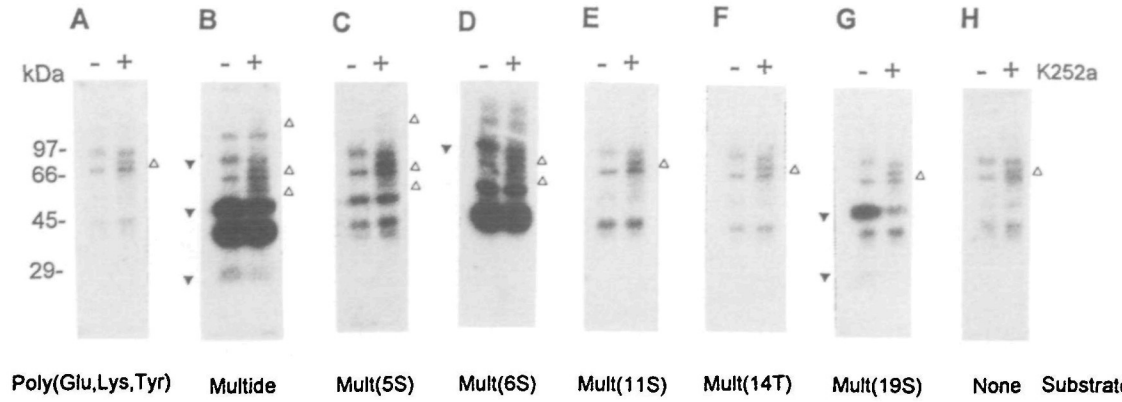

Fig. 3. **Protein kinase activities detected by in-gel assay of Colo201 cells before and after treatment with K252a.** Colo201 cells were treated with K252a for 30 min, washed with cold PBS, and harvested by centrifugation. Crude extracts (10 μg) from K252a-treated cells (+) or untreated cells (−) were electrophoresed in SDS-polyacrylamide gels containing 20 μg/ml poly(Glu,Lys,Tyr) (A), 20 μM multide-poly(Glu,Lys,Tyr) (B), multide(5S)-poly(Glu,Lys,Tyr) (C), multide(6S)-poly(Glu,Lys,Tyr) (D), multide(11S)-poly(Glu,Lys, Tyr) (E), multide(14T)-poly(Glu,Lys,Tyr) (F), and multide(19S)-poly(Glu,Lys,Tyr) (G) as substrates, or without substrate (H). In-gel protein kinase assay was carried out as described under "MATERIALS AND METHODS." The open triangles to the right of the gels indicate protein kinase activities that increased after treatment with K252a; closed triangles to the left of the gels indicate protein kinase activities that decreased after treatment.

and 90 kDa decreased after treatment (Fig. 3B). On the other hand, the activities of protein kinases with apparent molecular masses of about 60 (doublet), 76, and 150 kDa increased after treatment of the cells with K252a, an effect that might have been stimulated indirectly through different pathways. The 150-kDa kinase gave a dark band in in-gel assay with multide(5S)-poly(Glu,Lys,Tyr) (Fig. 3C), and the 60-kDa kinase gave positive bands in in-gel assay with multide(6S)-poly(Glu,Lys,Tyr) as well as with multide(5S)- poly(Glu,Lys,Tyr) (Fig. 3, C and D), indicating that the two protein kinases have different substrate specificities. In contrast, the 76-kDa kinase showed a positive radioactive band in the presence or absence of substrate (Fig. 3, A–H), suggesting that this protein kinase undergoes autophosphorylation. Among the three protein kinases whose activities decreased after treatment with K252a, the 90-kDa kinase showed a significant band with multide(6S)-poly(Glu,Lys,Tyr) in addition to multide-poly(Glu,Lys,Tyr) (Fig. 3, B and D), indicating that this kinase has a substrate specificity similar to that of PKA. The 50-kDa kinase activity was detected in in-gel assay with multide(5S)-poly(Glu,Lys,Tyr) (Fig. 3C), multide-(6S)-poly(Glu,Lys,Tyr) (Fig. 3D), and multide(19S)-poly-(Glu,Lys,Tyr) (Fig. 3G) in addition to multide-poly(Glu, Lys,Tyr) (Fig. 3B). The activities detected with multide-(5S)-poly(Glu,Lys,Tyr) and multide(6S)-poly(Glu,Lys, Tyr) were not significantly changed by treatment with K252a, but the activity detected with multide(19S)-poly-(Glu,Lys,Tyr) was strongly suppressed after treatment, indicating that at least two protein kinases with apparent molecular masses of 50 kDa are detected by multide. The 50-kDa kinase whose activity decreased strongly after treatment with K252a exhibited a substrate specificity similar to that of CaMKII (Figs. 2B and 3G).

DISCUSSION

In the present study, we developed a novel peptide with five potential phosphorylation sites, designated multide, in order to detect various protein kinases in crude cell extracts. Multide was designed to contain consensus phosphorylation sites for various Ser/Thr protein kinases, such as PKA, PKC, MAPKs, and CaMKs, in a synthetic peptide comprising 22 amino acids (Fig. 1). This peptide is expected to be especially useful for the detection of various protein kinases at the same time by in-gel assay. Purified preparations of PKA, CaMKII, and MAPK were analyzed by in-gel assay and all could be detected when multide conjugates were used as substrates (Fig. 2). CaMKII was detected with multide(19S)-poly(Lys) and MAPK was detected with multide(14T)-poly(Lys), as expected. Although multide contains three possible phosphorylation sites for CaMKII (Arg-Xaa-Xaa-Ser/Thr), only Ser^{19} was phosphorylated (Fig. 2B). This result, however, is reasonable because Arg-Gln-Met-Ser^{19}-Phe-Asp was designed to fit the most favorable phosphorylation sequence for CaMKII (*18*). In case of MAPK, the major phosphorylation site is Thr^{14}, not Ser^{11}, since the most favorable phosphorylation site for MAPK has been reported to be Pro-Xaa-Ser/Thr-Pro (*16, 17*). Unexpectedly, however, multide(6S)-poly(Lys) serves as much better substrate for PKA than multide-(11S)-poly(Lys), because the consensus sequence for phosphorylation by PKA has been reported to be Arg-Arg-Xaa-Ser/Thr (*19, 20*). The preference of PKA for multide(6S) over multide(11S) was also observed when PKA was assayed by the conventional method as shown in Table I. The reason why Arg-Arg-Trp-Ser^{11} serves as a poor phosphorylation site for PKA is not clear.

It is well known that a wide variety of extracellular signals produce diverse physiological responses in cells through changes in protein phosphorylation. Therefore, multide will provide a very useful tool for the detection of changes in the activities of various unknown protein kinases before and after treatment of cells with various stimuli. As an example, in this study, we used Colo201 cell extracts prepared before and after treatment with K252a, general protein kinase inhibitor that causes drastic morphological changes in cells (*9*). In this case, at least three protein kinase activities increased while more than three other protein kinase activities decreased after K252a treatment (Fig. 3). Furthermore, the substrate specificities of these enzymes could be determined using five multide analogs. At present, the identities of these enzymes are not clear, but it will be of great help in identifying enzymes if multide analogs are used to compare substrate specificities in addition to the molecular masses estimated by SDS-PAGE.

A similar experiment can be done using protein substrates with multiple phosphorylation sites such as myelin basic protein. Although different protein kinases were detected when myelin basic protein was used (data not shown), it is difficult to analyze the substrate specificity of each enzyme detected by in-gel assay.

In-gel assay using multide and its analogs as substrates as described in the present paper not only detects various protein kinases in cellular crude extracts but also gives valuable clues about their substrate specificities and molecular masses. The sensitivities of detection and patterns of protein kinases may change when different peptides are used. Multide is an example of a peptide substrate with multiple phosphorylation sites. An in-gel protein kinase assay method using an elegantly designed peptide with multiple phosphorylation sites and its analogs as substrates will be very useful for analyzing the activities of various protein kinases involved in signal transduction in cells.

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