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PHOSPHORYLATION SITES OF MYELIN BASIC PROTEIN BY A CATALYTIC FRAGMENT OF NON-RECEPTOR TYPE PROTEIN-TYROSINE KINASE p72*syk* AND COMPARISON WITH THOSE BY INSULIN RECEPTOR KINASE

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SUMMARY: Myelin basic protein has been used as a model substrate for determination of substrate recognition motif of various protein kinases. In this report phosphorylated sites of bovine brain myelin basic protein were studied with a catalytic fragment of protein-tyrosine kinase p72*syk*. Three of four tyrosine residues in myelin basic protein were phosphorylated by this kinase. Major phosphorylated site was ^{134}Y and minor phosphorylated sites were ^{68}Y and ^{127}Y. As the phosphorylation site by p56*lck* was only ^{68}Y, the recognition motif of p72*syk* was quite different from that of p56*lck*. Furthermore, the fact that elution pattern on HPLC of the phosphopeptides obtained by insulin receptor kinase was different from that by p72*syk* suggested that the recognition motif of p72*syk* was also different from that of insulin receptor kinase. These results may suggest that each protein-tyrosine kinase has a specific substrate recognition motif.

We have recently isolated a clone which encodes a protein-tyrosine kinase from a porcine spleen (1). This clone had a 1884-base-pair-long open reading frame encoding 628-amino-acid polypeptide with a calculated molecular weight of 71,618. The deduced amino acid sequence did not contain a ligand binding or membrane spanning region but did a well conserved protein-tyrosine kinase domain and two *src* homology region 2 (SH2) domains. We designated this gene as *syk* after spleen tyrosine kinase and called this kinase as p72*syk* which was classified as one of the non-receptor type protein-tyrosine kinases. However little is known about the nature of the endogenous substrates for p72*syk*. In addition, the recognition sequence of both endogenous and exogenous substrates for this enzyme is not yet well defined. A previous report from this laboratory has shown that catalytic fragment of p72*syk* phosphorylated 3 of 5 tyrosine residues in H2B histone and neither acidic nor basic amino acids around these tyrosine residues are

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important for the recognition, but higher order structure may be important for the recognition of substrate proteins (2).

It has been shown that myelin basic protein, which is the major basic protein of the central nervous system myelin is phosphorylated at multiple sites by various protein serine/threonine kinases (3-6). Consequently this protein has been used as a good tool for determination of substrate recognition sites of various serine/threonine kinases (7). Although the physiological significance of tyrosine phosphorylation of bovine brain myelin basic protein containing 4 tyrosine residues is not clear at this time, this protein has been shown to be a good substrate for some protein-tyrosine kinases, such as $p72^{syk}$, $p56^{lck}$, insulin receptor kinase and acidic fibroblast growth factor receptor kinase (8-11). In these reports the phosphorylation site of myelin basic protein was only determined using $p56^{lck}$ and ^{68}Y was indentified as the only tyrosine residue which could be phosphorylated by this kinase (9). It seems to be interesting to know whether another non-receptor type protein-tyrosine kinase $p72^{syk}$ could phosphorylate the same site of this protein as recognized by $p56^{lck}$. In this paper we have demonstrated that the phosphorylation sites of bovine brain myelin basic protein by catalytic fragment of $p72^{syk}$ are ^{68}Y, ^{127}Y and ^{134}Y. Furthermore, it has been also shown that the substrate recognition of $p72^{syk}$ is also different from that of insulin receptor kinase judging from the elution profile of radioactive peptides on high performance liquid chromatography (HPLC).

EXPERIMENTAL PROCEDURES

Materials and chemicals

Bovine brain myelin basic protein was obtained from Sigma and was purified by a reverse-phase C18 column (0.39 x 15 cm, μ Bondasphere 5 μm C18-100A, Waters) and by a TSKgel SP-2SW (0.46 x 25 cm, TOSOH) on HPLC. Catalytic fragment of $p72^{syk}$ from porcine spleen was obtained by the method of Kobayashi *et al.* (12) and was employed as a catalyst because we did not succeed in the purification of this kinase with the molecular mass of 72KDa as yet. Insulin receptor kinase from human placenta was purified by the method of Roth *et al.* (13) and Yonezawa *et al.*(14). [γ-^{32}P]ATP was obtained from NEN. Lysylendopeptidase (Achromobactor lytics M 497-1) was obtained from Wako Pure Chemicals. Trypsin (bovine pancreas Type XIII) was purchased from Sigma. Other chemicals were obtained from commercial sources.

Phosphorylation of myelin basic protein

Typical protein phosphorylation reaction was carried out in 2 ml of the reaction mixture containing 50 mM Hepes-NaOH (pH 8.0), 0.75 mM [γ-^{32}P]ATP (about 1.5 x 10^8 cpm), 5 mM $MgCl_2$, 10 μM vanadate, 4.12 mg of myelin basic protein and 40 KDa kinase (catalytic fragment of $p72^{syk}$) at 30°C for over night (16-17 h). After incubation, the reaction was stopped by the addition of HCl at a final concentration of 10 mM. To remove [γ-^{32}P]ATP, the reaction mixture was applied to Dowex-1 (500-750 μl) which was equilibrated with 10 mM HCl. Then the column was washed with 5-7.5 ml of 10 mM HCl to elute myelin basic protein. The eluate was applied to a reverse-phase C18 column on HPLC and eluted at a constant flow rate of 1 ml/min, with a linear gradient of acetonitrile from 0 to 50 % in 0.2 % trifluoroacetic acid (pH 2.0) at room temperature. UV absorbancy at 214 nm and radioactivity were monitored. Radioactivity of ^{32}P was determined with an Aloka LSC-950 liquid scintillation counter by Cerenkov radiation. Phosphorylated myelin basic protein fractions were collected and concentrated by the lyophilizer.

The phosphorylation of myelin basic protein by insulin receptor kinase was as follows. After the preincubation of insulin receptor kinase with 3 mM ATP, 10 mM $MgCl_2$ and 3 mM $MnCl_2$ at 20°C for 15 min, 3 volume of the solution containing 20 mM Hepes-NaOH (pH 7.5), 10 mM $MgCl_2$, 3 mM $MnCl_2$, 10 mM 2-mercaptoethanol, 0.1 % Triton X 100, myelin basic protein (4.12 mg) and [γ-^{32}P]ATP (about 1.5 x 10^8 cpm) was added and incubated at 30°C for over night. Purification of phospho-myelin basic protein was as described above.

Digestion of phospho-myelin basic protein and separation of phospho-peptides

The concentrated materials as described above were dissolved in 0.5 ml of 0.1 M Tris-HCl (pH 9.3) and digested at 30 °C for 24 h by lysylendopeptidase (lysylendopeptidase : myelin basic protein = 1 : 250 in molar ratio). The mixture was applied to the reverse-phase C18 column on HPLC again and eluted under the condition described above. The radioactive phosphopeptides were further purified as indicated in each experiment.

Sequential Edman Degradation

Amino acid sequences of purified phosphopeptides were determined by automated Edman degradation using a gas-phase protein sequencer (Applied Biosystems, model 477A) equipped with an on-line reverse-phase chromatography system for identification of phenylthiohydantoin (PTH) amino acids.

RESULTS AND DISCUSSION

After incubation for 16-17 h with 40KDa kinase, 0.23-0.28 mol of phosphate was incorporated per mol of myelin basic protein judging from the acid-precipitable radioactivity. Then the phosphorylated myelin basic protein was digested with lysylendopeptidase. The elution profile of phosphopeptides from reverse-phase C18 column was presented on Fig. 1 and the radioactive peaks were indicated from left to right, as peaks I, II, III, IV and V, respectively.

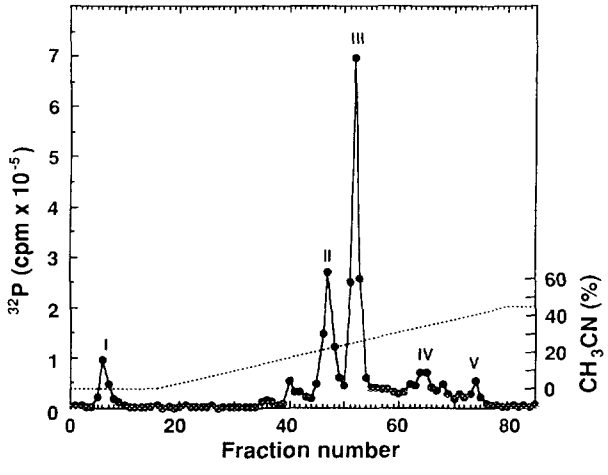

Fig.1. Separation of radioactive phosphopeptides by a reverse-phase column on HPLC. Lysylendopeptidase-treated phosphopeptides were injected into a reverse-phase C18 column as described under "EXPERIMENTAL PROCEDURES". Fractions of 0.5 ml each were collected and the radioactivity was determined. •—• and ---, radioactivity of phosphopeptide and concentration of acetonitrile, respectively.

The phosphoamino acid analysis of each peak by high voltage paper electrophoresis after acid hydrolysis (15) showed that the peak I was inorganic phosphate and the peaks II-V contained phosphotyrosine. As the eluting position of peak V was accordant with that of undigested phospho-myelin basic protein, this peak seemed to be non-digested or only slightly digested myelin basic protein.

When peak IV was further digested with lysylendopeptidase and applied to the same reverse-phase column, the radioactive peak was eluted at the position corresponding to that of peak III (data not shown). This result suggested that peak IV was produced by incomplete digestion of peptides containing peak III. Then we analyzed about two major peaks II and III.

In the next experiment the peak III in Fig. 1 was further purified by rechromatography on the reverse-phase column under slightly modified condition. The peak III was lyophilized and dissolved in water, and applied to the reverse-phase column on HPLC. The phosphopeptide was eluted with a linear gradient of acetonitrile from 0 to 18 % in 10 mM phosphate buffer (pH 4.2). The radioactive peak eluted at 14 % acetonitrile in this buffer was collected, lyophilized and dissolved in water. Then the radioactive peptide was further applied to the TSKgel SP-2SW column on HPLC and eluted with 25 ml of linear gradient from 0.1 M pyridine-acetate (pH 3.1) to 0.25 M pyridine-acetate (pH 3.25). Flow rate was 0.5 ml/min and 0.5 ml each fraction was collected. The radioactive fractions ranging from 40 to 42 were collected and lyophilized. Then this material was subjected to a sequential Edman degradation. The result on the sequence analysis of peak III was shown in Fig. 2. Comparing with the primary structure of myelin basic protein (16), the sequence of peak III corresponded to PGFG^{127}YGGRASD^{134}YK. As there was no peak in 12th cycle where PTH-tyrosine should be detected, we decided that phosphate was incorporated into ^{134}Y, because the peak of PTH derivative of phosphotyrosine was not detected in this sequencing system (17). By this reason, we judged that no phosphate was incorporated into ^{127}Y.

In the next experiment, peak II was analyzed by the same method described for peak III. However, this peak contained several phosphopeptides and we could not separate them by the same procedures. To change the charge of these peptides, we digested the phosphopeptides in peak II by trypsin (trypsin : peak III = 1 : 50 in w/w) at 30°C for 24 h in 0.3 M ammonium bicarbonate buffer (pH 8.5) and applied the peptides to the reverse-phase C18 column on HPLC. These phosphopeptides were eluted under the same conditions described under EXPERIMENTAL PROCEDURES. The three radioactive peaks were recovered from the column and were named peaks IIa (0 % acetonitrile), IIb (19 %) and IIc (21%), respectively. Each peak was collected and further purified by the TSKgel SP-2SW column and eluted with the same conditions described above. The peptide in each radioactive peak was subjected to the sequential Edman degradation.

Fig. 3 showed the sequence analysis of peak IIb. The sequence of peak IIb corresponded to TTH^{68}YGSLPQK and phosphate was incorporated into ^{68}Y. The results on the peptide sequencing of peaks IIc and IIa showed that these sequence were PGFG^{127}YGGR and ASD^{134}YK, respectively (data not shown). The sequence of peak

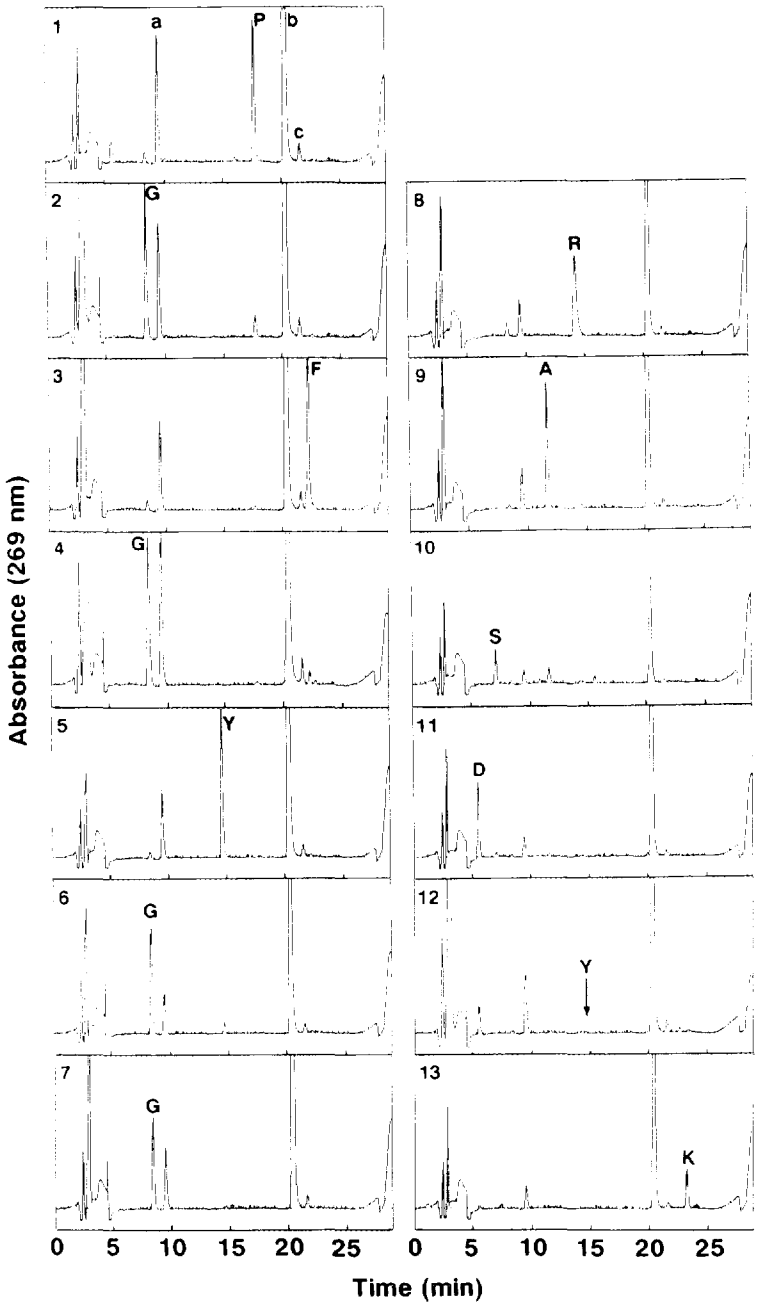

Fig. 2. Sequence analysis of peak III after separation by a reverse-phase column and SP-2SW column on HPLC.

Phosphopeptide III was redissolved in 100 % acetonitrile and analyzed with the gas phase sequencer. The figure shows reverse-phase chromatograms of PTH derivatives corresponding to the first 13 cycles. More than 50 % of phenylthiohydantoin derivative of serine was detected as its dithiothreitol adduct (DTT-S) (20). Other abbreviations: P, PTH-proline; G, PTH-glycine; F, PTH-phenylalanine; Y, PTH-tyrosine; R, PTH-arginine; A, PTH-alanine; S, PTH-serine; D, PTH-aspartic acid; K, PTH-lysine; a, b and c are dimethylphenylthiourea, diphenylthiourea and diphenylurea, respectively.

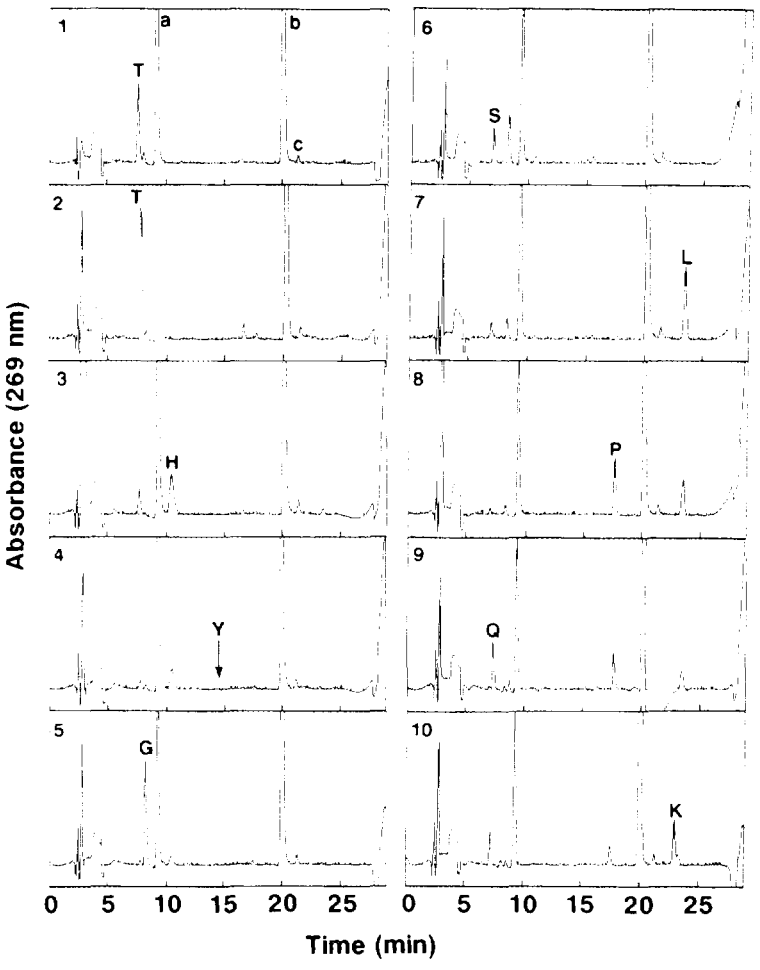

Fig. 3. Sequence analysis of peak IIb after rechromatography by a reverse-phase column and SP-2SW column on HPLC.

Phosphopeptide IIb was redissolved in 100% acetonitrile and analyzed with the gas phase sequencer. The figure shows reverse-phase chromatograms of PTH derivatives corresponding to the first 10 cycles. Abbreviations: T, PTH-threonine; H, PTH-histidine; L, PTH-leucine; Q, PTH-glutamine, respectively. Other abbreviations are same as legend to Fig. 2.

IIc corresponded to N-terminal side of peak III and ^{127}Y was the phosphorylated site, and that of peak IIa located to C-terminal side of peak III and ^{134}Y was the phosphorylated site.

These results were summarized in Fig. 4. The catalytic fragment of p72^{syk} could phosphorylate three tyrosine residues in myelin basic protein, namely ^{68}Y, ^{127}Y and ^{134}Y. Peak II seemed to consist of two peptides, probably ^{58}DGHHAARTTHYGSLPQK (69 %) and ^{123}PGFGYGGRASDYK (31 %). The latter sequence was same as that of peak III. The difference in the elution positions of the minor peptide in peak II and that in peak III in reverse-phase HPLC analysis seemed to depend on the number of phosphate in tyrosine residues. Namely the peptide in peak III

^{58}DGHHAARTTH^{68}YGSLPQKAQGH
II-b

^{117}GAEGQKPGFG^{127}YGGRASD^{134}YKSAHKGLKGH
II-c III II-a

Fig. 4. Sequence around the phosphorylated tyrosine residues in myelin basic protein by 40 KDa kinase.
Y with outline letter shows phosphotyrosine. Phosphopeptides of peaks IIa, IIb, IIc and III are underlined.

has only one phosphate and the main peptide in peak II has two phosphates. When peak II was digested with trypsin, the major peptide was divided into DGHHAAR and TTHYGSLPQK and minor peptide was divided into PGFGYGGR and ASDYK, respectively.

In order to know whether the mechanism of substrate recognition by p72^{syk} is different from that by receptor-type protein-tyrosine kinase, the phosphorylation sites of myelin basic protein were analyzed using insulin-receptor kinase. Myelin basic protein was fully phosphorylated by insulin receptor kinase and about 0.26 mol of phosphate was incorporated per mol of this protein. The radioactive protein was digested with lysylendopeptidase and applied to the reverse-phase column on HPLC under the same conditions indicated in Fig. 1. As shown in Fig. 5, the elution profile of the

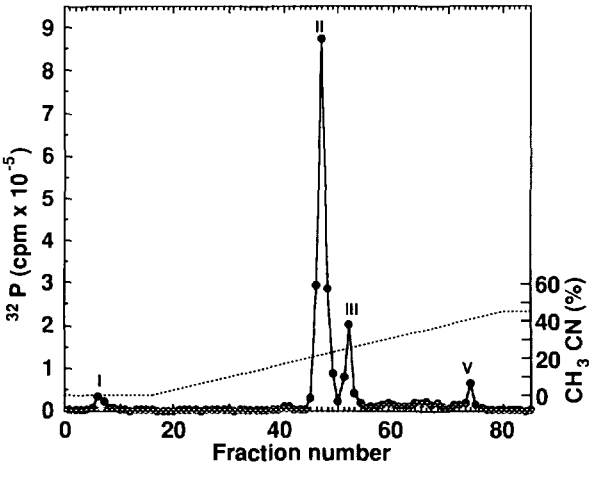

Fig. 5. Separation of radioactive phosphopeptides by a reverse-phase column on HPLC.
Myelin basic protein was phosphorylated by insulin-receptor kinase for 16-17 hr. Then phosphorylated myelin basic protein was treated with lysylendopeptidase and the phosphopeptides were injected into a reverse-phase C18 column as described under "EXPERIMENTAL PROCEDURES". Fractions of 0.5 ml each were collected and the radioactivity was determined. •—• and ---, radioactivity of phosphopeptide and concentration of acetonitrile, respectively.

phosphopeptides obtained by insulin-receptor kinase was apparently different from that by p72^{syk} (Fig. 1). Namely, peak II was much bigger than peak III. This result suggests that phosphorylation sites of myelin basic protein by insulin receptor kinase may be different from those by p72^{syk} and p56^{lck}.

It has been reported that brain myelin basic protein could be phosphorylated by several protein-tyrosine kinases (8-11). Although the physiological significance of this protein-tyrosine phosphorylation was not clarified, myelin basic protein seems to be good model substrate for comparison of the substrate recognition motif of each protein-tyrosine kinase. Phosphorylation site of myelin basic protein by protein-tyrosine kinases was first reported by Wang *et al.* (9) using p56^{lck} purified from bovine thymus. In this report they indicated that only ^{68}Y in the sequence -T-T-H-Y-G-S-L-P-K- found in bovine myelin basic protein was phosphorylated by this kinase. However they also indicated that p40 kinase from bovine thymus which was similar to a catalytic fragment of p72^{syk} (1,18), could not phosphorylate myelin basic protein (9).

In this paper, we have clarified the phosphorylation sites of myelin basic protein by p72^{syk} and three of four tyrosine residues in this protein were shown to be phosphorylated (Fig. 4). It has been reported that a cluster of acidic amino acids (especially glutamic acid) is usually important for the recognition of tyrosine residues by protein-tyrosine kinases (19). In fact ^{134}Y in myelin basic protein is identified as the major phosphorylated site and one aspartic acid is observed next to the phosphorylated tyrosine on the N-terminal side. However ^{68}Y and ^{127}Y are determined to be minor phosphorylated sites of myelin basic protein by this kinase, and both glutamic acid and aspartic acid were not detected near the phosphorylated tyrosine residues. We have previously reported that using H2B histone as a substrate, the recognition of tyrosine residues for phosphorylation by p72^{syk} was not related to the existence of acidic amino acids near tyrosine residues (2). The result in this report together with the previous one strongly suggested that the acidic amino acids around the tyrosine residue were not necessarily required for the recognition of phosphorylation sites by p72^{syk}.

By the analysis using Chou-Fasman program (20) the secondary structure such as β-turn or α-sheet was not also detected in the amino acid sequence around the major phosphorylation site ^{134}Y. This result together with previous analysis on H2B histone suggested that secondary structure itself was not critical for the recognition of tyrosine residues by p72^{syk}.

It is obvious that the phosphorylation sites of myelin basic protein by p72^{syk} were different from that by p56^{lck} (9). In addition, the phosphorylation sites of myelin basic protein by p72^{syk} and insulin receptor kinase also seemed to be different (Figs. 1 and 5). The result in Fig. 5 suggested that the major phosphorylated sites by insulin receptor kinase seemed to be ^{68}Y and/or ^{127}Y and ^{134}Y. It was also shown that similar amount of phosphate was distributed in each of latter two sites. The present results have suggested that each protein-tyrosine kinase has a different substrate recognition motif and performs different physiological function *in vivo*.

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