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Tyrosine Phosphorylation of Synthetic Peptides with Sequences Found in CD3-ζ and Ig-α Chains by Nonreceptor Protein Tyrosine Kinases, p59^{fyn} and p56^{lck}, Expressed with the Baculovirus System in Insect Cells

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ABSTRACT

Tyrosine [^{32}P]phosphorylated peptide probes for screening of cDNA expression libraries were prepared by phosphorylating synthetic peptides with [γ-^{32}P]ATP in an immune complex kinase reaction. The *Src* family kinases, p56^{lck} (Lck) and p59^{fyn} (Fyn), were expressed in insect cells from baculovirus vectors. The peptides that contain sequences found at the tyrosine-based activation motifs of the CD3-ζ chain and the Ig-α chain, a PDGF-receptor peptide, and angiotensin II were *in vitro* labeled with the kinases added as an immune complex with anti-Lck and anti-Fyn conjugated to Sepharose beads. The Lck and Fyn kinases expressed in insect cells were autophosphorylated on tyrosine and found to be fully active. In the immune complex kinase assay, more than 40% of the radioactivity added as [γ-^{32}P]ATP were incorporated into these peptides, which were isolated by HPLC after the reaction. In the kinase reaction with mixed substrates, some differences were found between Lck and Fyn in the preference of utilization of peptide substrates.

Key words: Protein tyrosine kinase, Baculovirus vector, CD3-ζ chain, Ig-α (MB-1) chain, Immune complex kinase assay

INTRODUCTION

Protein tyrosine phosphorylation is the earliest change found on stimulation of many receptors for growth factors and cytokines, and of multichain immune recognition receptors. Protein tyrosine phosphorylation acts as a molecular switch to create binding sites for the Src homology 2 (SH2) domain, a conserved protein module of about 100 amino acids found in a diverse group of cytoplasmic signaling proteins (1, 2). SH2 domains are compact, self-contained modules

with a deep pocket for binding of a phosphotyrosine (3-5). High affinity binding of an SH2 domain requires a specific amino acid sequence of about 4 residues immediately C-terminal to the phosphotyrosine (6). The ability of a given tyrosine-phosphorylated protein, which is often a protein tyrosine kinase, to bind specific SH2-containing proteins determines the intracellular signaling pathways selectively activated following the tyrosine phosphorylation (2, 7).

A method has been developed by Schlessinger *et al.* to clone cDNAs for proteins with SH2 domains. They used the tyrosine-[^{32}P]-phosphorylated C-terminal fragment of the epidermal growth factor (EGF) receptor to screen the λgtll library of human brain cDNA (8, 9). Using this method, it should be possible to clone cDNAs for new signaling proteins with unique SH2 domains and also to identify the SH2 proteins with specific affinity to the tyrosine-phosphorylated structure used in the screening of a library.

In this paper, we described a method for preparing tyrosine-[^{32}P]-phosphorylated peptides to clone the cDNAs encoding proteins with SH2 domains. Our method utilized the p56^{lyk} (Lck) and p59^{fyn} (Fyn) tyrosine kinases expressed by the baculovirus system in insect cells. The tyrosine kinases purified as immune complexes with anti-Lck/Fyn antibody conjugated to Sepharose beads were incubated with [γ-^{32}P]ATP and non-phosphorylated synthetic peptides with the sequences containing a known tyrosine phosphorylation site(s). The tyrosine-[^{32}P]phosphorylated peptides were isolated by HPLC and found to contain a large portion of ^{32}P-radioactivity added as [γ-^{32}P]ATP.

MATERIALS AND METHODS

Peptides and antibodies

[Val^5]-Angiotensin II, DRVYVHPF, monoclonal anti-phoshotyrosine antybody (clone PT-66, mouse IgG_1), monoclonal anti-rabbit immunoglobulins (clone RG-16, reacts with an epitope sensitive to reduction) conjugated with alkaline phosphatase, and goat anti-mouse immunoglobulins (Fc-specific) conjugated with alkaline phosphatase were purchased from Sigma. CD3-ζ peptides contained the sequences, GLYQGLSTA (zeta Y120) and DTYDALHMQ (zeta Y131), of the residues 118-126 and 129-136 of the human CD3-ζ chain (10). MB-1 (Ig-α) peptide contained the suquence, DENLYEGLNLDDCSMYEDISR, of the residues 154-174 of the human MB-1 (Ig-α) chain (11). Platelet-derived growth factor receptor (PDGF-R) peptide contained the sequence, ESDGGYMDMS, of the residues 735-744 of the human PDGF-R-β (12). These peptides were synthesized by a procedure of Fmoc chemistry with a Model 431A Peptide Synthesizer (Applied Biosystems), and purified to more than 95% purity by HPLC on a reverse-phase column (Waters, 5 μ, C18-300 Å, μ Bondasphere,

3.9×15 cm) developed by a gradient from 7% to 35% acetonitrile in 0.1% trifluoroacetic acid (TFA) over 40 min at a flow rate of 5 ml/min.

Preparations of antibody and antibody conjugates

A peptide consisting of residues 29-43 of human Fyn and an additional C-terminal cysteine, RYGTDPTPQHYPSFGC, was synthesized and joined to chicken serum albumin using the heterobifunctional crosslinking reagent, m-maleimidobenzoyl-N-hydroxysulfosuccinimide ester (Pierce).

Digestion of mouse lck cDNA (clone NT18 (13), kindly provided by Dr. R. M. Perlmutter) with StuI and BamHI restriction endonucleases generated a 474 bp fragment encompassing nucleotides 140 through 613 of the lck cDNA. This fragment, encoding residues -8 to 150 of Lck, was inserted into pATH1 vector (ATCC 37695) (14) doubly digested with SmaI and BamHI at the polylinker site. *E. coli* RRI (ATCC 31343) transformed by this construct were grown in Luria Broth supplemented with 20 μg/ml tryptophan and 40 μg/ml ampicillin and then induced to produce a *TrpE-Lck* fusion protein by reducing the tryptophan concentration to one-tenth and the addition of 10 μg/ml indole acrylic acid. The bacteria were lysed by sonication and the *TrpE-Lck* fusion protein was purified by SDS-PAGE. The fusion protein was electroblotted onto a PVDF membrane (Immobilon, Millipore) and located by staining with Ponsau S. The portion of membrane where the fusion protein was located was broken to powder in liquid nitrogen and used in preparing a water-in-oil emulsion with an adjuvant.

Polyclonal antibodies directed against Fyn and Lck were prepared by immunization of New Zealand White male rabbits with the antigens described above.

Antibody was purified by DEAE-cellulose (DE52, Whatman) column chromatography and covalently coupled to BrCN-activated Sepharose 4B at 5 mg protein/ml of beads.

Construction of recombinant baculovirus (15)

Transfer vector construction Mouse lck plasmid NT18 was digested with StuI restriction endonuclease, ligated with XbaI linkers, and cut with XbaI and DraI, resulting in an 1.6 kb fragment that codes for Lck. Human fyn plasmid pUC-Fyn (16) (obtained from Japanese Cancer Research Resources Bank) was also cut with restriction endonucleases NspI and EcoRI, filled in with T4 polymerase and Klenow, ligated with XbaI linkers, and digested with XbaI. Resulting 1.7 kb fragment coding for Fyn was isolated. Each of these two fragments was subcloned into the NheI site of the baculovirus transfer vector pBlueBac (Invitrogen). Recombinant transfer plasmids containing the inserts in the correct transcriptional orientation were designated pBB-lck and pBB-fyn.

Preparation of recombinant viruses Transfer of lck and fyn cDNA from the transfer vectors to the *Autographa californica* nuclear polyhedrosis virus (AcNPV) genome was achieved by cotransfection. *Spodoptera frugiperda* (Sf9) insect cells (2×10^6) in a 15 ml flask with 2 ml of Grace's medium containing 10% fetal calf serum (FCS) were transfected with the following mixtures: 2 μg of pBB-lck or pBB-fyn purified by CsCl-gradient centrifugation, 1 μg of AcNPV DNA, 950 μl of transfection buffer (137 mM NaCl, 6 mM D-glucose, 5 mM KCl, 0.7 mM Na_2HPO_4, 20 mM HEPES pH 7.1, and 15 μg/ml sonicated calf thymus DNA) and 50 μl of 2.5 M $CaCl_2$ added in this order. After 4 h of incubation at 27℃, cells were rinsed carefully and added with 5 ml fresh complete medium containing 10% FCS and 150 μg/ml X-gal. Five to ten days after transfection, the medium changed blue, showing the marker beta-galactosidase gene on pBlueBac was transferred to AcNPV and properly expressed. The blue medium was diluted to 10^2-10^5 folds and one ml of each dilution was inoculated to 2×10^6 SF9 cells on a 6 cm dish for 1 h. Top agar (1 × complete medium containing 10% FCS and 150 μg/ml X-gal with 3% Funakoshi LM-01 agarose, cooled down to 37℃) was then overlaid to the cells. After four to ten days of incubation at 27℃, blue plaques became visible. Several of occulusion-negative blue plaques were obtained by several cycles of plaque purification. Recombinant virus clones were checked for Lck or Fyn production by Western blotting.

Production of Fyn and Lck by baculovirus expression

Sf9 cells (1×10^7) were plated in a plastic dish (9×13 cm) and incubated until the cells attach to the dish. The medium was removed and the cells were washed with Grace's medium without serum. Recombinant AcNPV in 5 ml of Grace's medium was infected at a multiplicity of infection of 5 to 10. After 1 h of infection, the medium with serum was added and the culture was kept at 27℃ for 39 to 42 h.

The infected Sf9 cells were then harvested, washed three times with phosphate-buffered saline, and lysed on ice with 1 ml lysis buffer [1% NP-40, 20 mM Tris-HCl pH 7.5, 150 mM NaCl, 2.5 mM EDTA, 50 mM NaF, 1 mM Na_3VO_4, 20 μg/ml leupeptin, 100 units/ml aprotinin, 1 mM phenylmethylsulfonyl flouride (PMSF), and 10% glycerol.] The lysate was kept on ice for 1 h. The crude preparation of tyrosine kinases was obtained by centrifugation of the lysate at 12, 300 $\times g$ for 10 min at 4℃.

In vitro tyrosine phosphorylation of peptides by immune complex tyrosine kinases

Two hundred μl of the crude preparation of a tyrosine kinase, Lck or Fyn, were mixed with 10 μl of anti-Lck or -Fyn Sepharose beads at 4℃ for 2 h. Then the beads were washed once with the lysis buffer, once with 0.1 M Tris-HCl, pH

7.4, containing 0.5 M LiCl, once with washing buffer, which had the same ingredients as the lysis buffer except that the concentration of NP-40 was reduced to 0.1%, and once with the kinase assay buffer (17) [20 mM Tris-HCl, pH 7.5, and 10 mM $MnCl_2$.] The washed beads were suspended in 17-100 μl of the kinase assay buffer. Peptide (24-40 nmol in 2-6 μl) and [γ-^{32}P]ATP (10-100 μCi in 1-10 μl, 3,000-7,000 Ci/mmol, ICN Biochemicals Inc., CA, U. S. A.) were added to the suspended beads. Incubation was either at room temperature for 30 min or at 30℃ for 10 min. The incubated mixture was subjected to centrifugation at 1,370×g for 2 min to collect the supernatant. The Sepharose beads were washed twice with 100 μl each of 0.1% TFA in water. The supernatant and the washings were combined and the ^{32}P-labeled peptides were isolated by reverse-phase HPLC using Waters μ Bondasphere column (5 μ C18-300 Å, 0.39×15 cm) developed by a gradient of acetonitrile from 7% to 63% in 0.1% TFA over 40 min at a flow rate of 1 ml/min. Absorbance at 220 nm was monitored with L-4000 UV Detector (Hitachi) and ^{32}P-radioactivity was monitored with Model 171 Radioisotope Detector (Beckman) by Cerenkov radiation in a 1 ml flow cell. Effluent from the HPLC column was sequentially passed through a 17.7 μl flow-cell to monitor UV and a 1 ml loop to monitor radioactivity. The sensitivity of monitoring radioactivity was enhanced at the expense of resolution by the use of a large loop.

RESULTS AND DISCUSSION

Characterization of Lck and Fyn tyrosine kinases produced by recombinant baculoviruses

Lck and Fyn produced in Sf9 cells had kinase activity which was shown both by western blotting of lysates from recombinant-virus infected cells with anti-phosphotyrosine antibody (Fig. 1-A-d), and by *in vitro* kinase assay of immunoprecipitates. The recombinant Lck and Fyn immunoprecipitated from Sf9 lysates by anti-Lck and anti-Fyn were autophosphorylated on incubation with [γ^{32}-P]ATP in the kinase assay buffer (Fig. 1-B). The results shown in Fig. 1-A indicate that the anti-Lck used is not reactive with Fyn and the sensitivity of antigen detection was highest with anti-Lck, intermediate with antiphosphotyrosine, and lowest with anti-Fyn. The anti-Lck was produced in rabbit by immunizing an antigen containing the unique N-terminal domain, the SH3 domain, and a portion of the SH2 domain of mouse Lck.

In the infected cells, Fyn and Lck were autophosphorylated and tyrosine-phosphorylated many insect and/or viral proteins (data not shown). It is reported that in mammalian cells, these kinases are negatively regulated by phosphorylation of the C-terminal tyrosine residues (19, 20). A kinase, CSK, responsible

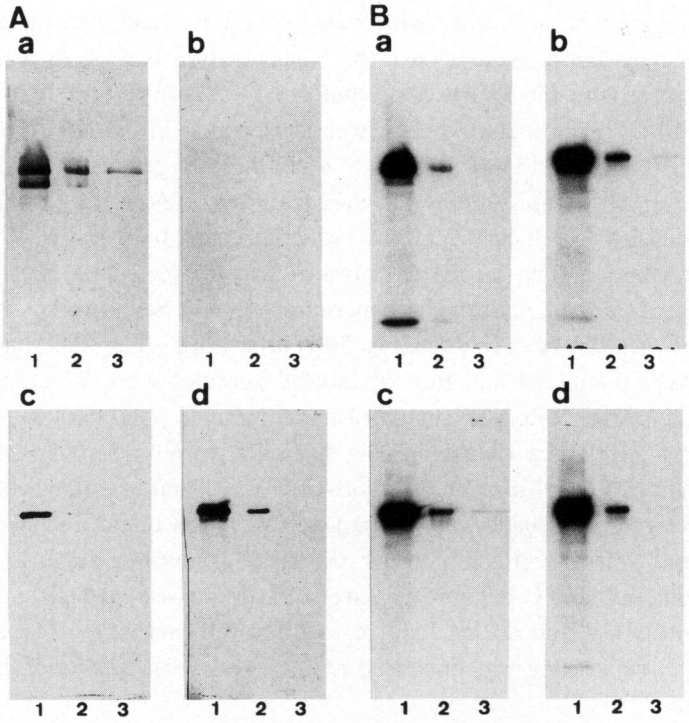

Fig. 1 Characterization of Lck and Fyn expressed with the baculovirus system. Lck (a) or Fyn (b, c, and d) in 200 μl of the recombinant baculovirus-infected Sf9 lysate was bound to 10 μl of anti-Lck (a) or anti-Fyn (b, c, and d) Sepharose beads. The beads were suspended in 19 μl of the kinase assay buffer. One μl of [γ-^{32}P]ATP (10 μCi, 3,000 Ci/nmol) was added to the beads and the mixture was incubated at 30℃ for 10min. The beads were washed twice with 100 μl each of the kinase assay buffer. Washed beads were suspended in 50 μl of SDS-sample buffer (18) containing 2% mercaptoethanol. The suspensions were heated at 100℃ for 2 min. Aliquots (10 μl in lanes 1, 1 μl in lanes 2, and 0.1 μl in lanes 3) of the heated samples were subjected to SDS-PAGE (18). The proteins separated on the gel were electroblotted onto PVDF membranes, which was subsequently blocked by incubation with bovine serum albumin (30 mg/ml) at 60℃ for 30min. Then, the blot was probed with antibody [to Lck (a and b), to Fyn (c), or to phosphotyrosine (d)] followed by alkaline phosphatase-coupled antibody to rabbit IgG (a, b, and c) or to mouse IgG (d). The blot was visualized by the addition of 5-bromo-4-chloro-3-indolyl phosphate (Sigma) and nitro blue tetrazolium (Sigma). After the immunoblotting, the blotted membranes were exposed to XAR film (Kodak) for 1 h. A, immunoblots; B, autoradiograms; a, anti-Lck immunoprecipitate; b, c, and d, anti-Fyn immunoprecipitates; a and b, immunoblotting with anti-Lck; c, immunoblotting with anti-Fyn; d, immunoblotting with anti-phosphotyrosine.

for such regulation was identified in mammalian cells (21), but the same activity against mammalian *Src*-family kinases is not seen in insect cells (22). This may explain the observed high activity of these kinases expressed in insect cells. The lower phosphorylation of the C-terminal tyrosine, however, is not verified so far. With SDS/PAGE analysis, Fyn was detected as 59K single band by rabbit anti-Fyn serum on Western blot, whereas Lck, by rabbit anti-Lck serum, as two major bands of 56K and 50K (Fig. 1-A-a). The band at 56K was phosphorylated *in vitro* much more prominently than the band at 50K (Fig. 1-B-a). The amount of Fyn and Lck produced in Sf9 cells was 1-2% of total proteins in cleared lysate as revealed by Commassie-blue stained SDS polyacrylamide gels. Although several clones for lck recombinant were isolated, we failed to get more than one clone of fyn recombinant in spite of purification of eighteen blue plaques. This can be explained if fyn product is toxic for insect cells. Morgan *et al.* (23) reported a potential toxicity of src kinase activity for host Sf9 cells. It is thus possible that the only clone obtained by us is somewhat defective, , though it has proper size of apparent 59 K, immunoreactivity against anti-Fyn serum, and kinase activity comparable to Lck produced by our lck clones.

Phosphorylation of CD3-ζ peptides, angiotensin II, and MB-1 peptide by immune complex Lck

The amino acid sequences of zeta Y120 and Y131 peptides and MB-1 (Ig-α) peptide represent the sequences found at the tyrosine-based activation motifs of the T cell receptor/CD3 complex and the B cell antigen receptor (24, 25). The peptides contain those tyrosine residues which are phosphorylated upon stimulation of these receptors (26-28). [Val^5]-Angiotensin II has been used as a substrate of the *Src* family protein tyrosine kinases (29).

Under the coditions used in the experiments shown in Fig. 2-a, -b, and -c, about 56%, 59%, and 50% of the ^{32}P-radioactivety eluted from the HPLC were recovered in the ^{32}P-labeled zeta Y120 peptide, zeta Y131 peptide, and angiotensin II, respectively. About 83-86% of the ^{32}P-radioactivity eluted from the HPLC was recovered in the ^{32}P-labeled MB-1 peptide (Fig. 2-d). The ^{32}P-labeled zeta Y120, zeta Y131, and MB-1 peptides were eluted from the reverse-phase column slightly ahead of the corresponding non-phosphorylated peptides, which were monitored in the HPLC by absorbance at 220 nm. About 6% of the ^{32}P-radioactivity added to the reaction mixture in Fig. 2-d was found in association with the immune complex. Almost all of the radioactivity in the immune complex was located at Lck on analysis by SDS-PAGE.

Differential utilization of peptide substrates in the phosphorylation reaction by Lck

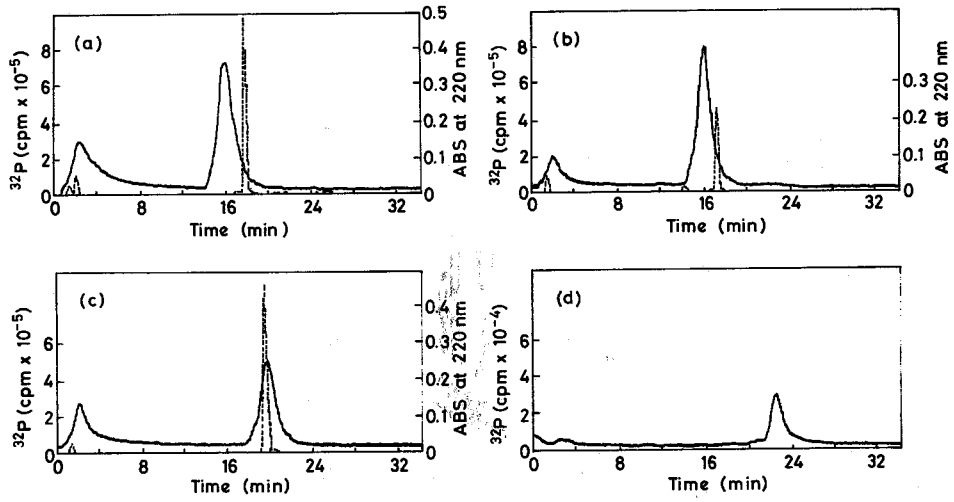

Fig. 2 Phosphorylation of CD3-ζ peptides [zeta Y120 (a) and zeta Y131 (b)], angiotensin II (c), and MB-1 peptide (d) by immune complex Lck. (a, b, and c) Lck in 200 μl of the recombinant baculovirus-infected Sf9 lysate was bound to 10 μl of anti-Lck Sepharose beads. The beads were suspended in 17 μl of the kinase assay buffer. Two μl of either 20 mM zeta Y120 peptide (a), 20 mM zeta Y131 peptide (b) or 20 mM angiotensin II (c) and 1 μl of [γ-^{32}P]ATP (10 μCi, 3,000 Ci/mmol) were added to the beads. The mixtures were incubated at 30℃ for 10 min. The ^{32}P-peptides were separated by HPLC as described in MATERIALS AND METHODS. ——, ^{32}P-radioactivity (^{32}P); ----, absorbance at 220 nm (ABS at 220 nm). (d) 200 μl of Lck lysate was bound to 10 μl of anti-Lck Sepharose beads. The beads were suspended in 100 μl of the kinase assay buffer. Six μl of 4 mM MB-1 peptide and 10 μl of [γ-^{32}P]ATP (100 μCi, about 4,500 Ci/mmol) were added to the beads, and the mixture was incubated for 30 min at room temperature. The peptide was used in the reaction after reduction with 20 mM dithiothreitol at 39℃ for 30 min. About one hundredth of the ^{32}P-peptide was analyzed by HPLC.

and Fyn

Possible difference between Lck and Fyn in the preference of peptide substrates was studied in the phosphorylation reaction with four peptides simultaneously added as substrates (Fig. 3). The peptides used were zeta Y120 peptide, zeta Y131 peptide, angiotensin II, and PDGF-R peptide. The PDGF-R peptide represents a portion of the PDGF-R kinase insert region which is tyrosine-phosphorylated on stimulation of cells with PDGF and recognized by the SH2 domain of the phosphatidylinositol 3-kinase p85 subunit (6, 30, 31). Both Lck and Fyn phosphorylated more zeta Y120 and Y131 peptides than PDGF-R peptide and angiotensin II (Fig. 3). It was not possible to determine individually the ^{32}P-

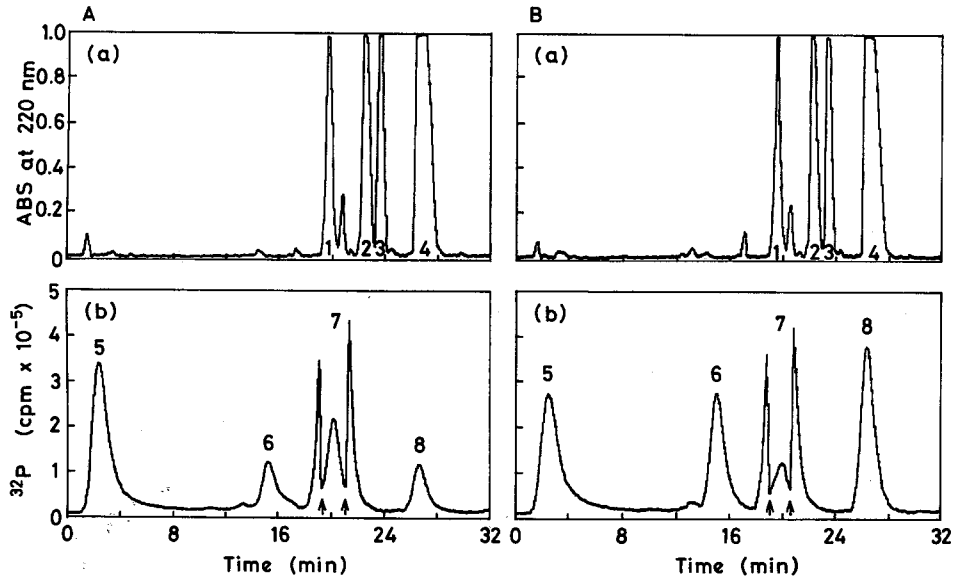

Fig. 3 Difference between Lck and Fyn in the preference of peptide substrates added as a mixture to the phosphorylation reaction. Lck (A) or Fyn (B) in 200 μl of the recombinant baculovirus-infected Sf9 lysate was bound to 10 μl of anti-Lck (A) or -Fyn (B) Sepharose beads. The beads were suspended in 79 μl of the kinase assay buffer. One μl of [γ-^{32}P]ATP (10 μCi, 3,000 Ci/mmol) and 5 μl each of 20 mM zeta Y120 peptide, 20 mM zeta Y131 peptide, 20 mM angiotensin II, and 20 mM PDGF-R peptide were added to the beads. The mixtures were incubated at 30℃ for 10 min. The peptides in the reaction mixtures were separated by HPLC using reverse-phase column (Waters μ Bondasphere, 5 μ C18-300 Å, 0.39×15 cm) developed by a gradient of acetonitrile from 7% to 28% in 0.1% TFA over 30 min at a flow rate of 1 ml/min. A, immune complex Lck was used as the enzyme; B, immune complex Fyn was used as the enzyme; (a), records of absorbance at 220 nm; (b), records of ^{32}P-radioactivity; peak 1, PDGF-R peptide; peak 2, zeta Y131 peptide; peak 3, zeta Y120 peptide; peak 4, angiotensin II; peak 5, [γ-^{32}P]ATP and its hydrolysates; peak 6, ^{32}P-phosphorylated PDGF-R peptide; peak7, ^{32}P-phoshorylated zeta Y131 and Y120 peptides, which were not recorded in (b) as separate peaks due to a low resolution in monitoring the radioactivity: peak 8, ^{32}P-phosphorylated angiotensin II. In peak 7, full-scale of the recording was automatically readjusted to a new scale multiplied by 10 when 90% full-scale was reached at the time indiacated by the first arrows in (b). Integrator recordings of ^{32}P-radioactivity revealed that peaks 5, 6, 7, and 8 in A-(b) contained 15.9%, 5.7%, 73.0%, and 5.4% of the total radioactyivity eluted from the HPLC column and those in B-(b) contained 16.2%, 11.7%, 57.4%, and 14.7%, respectively.

incorporations into phosphorylated zeta Y120 and Y131 peptides due to a low resolution of the radioactivity monitoring. Lck phosphorylated more zeta peptides as compared with the phosphorylation by Fyn, whereas Fyn phosphorylated more PDGF-R peptide and angiotensin II than Lck did (Fig. 3). These results suggest that Lck and Fyn are different in the preference of amino acid sequences of substrates near the tyrosine residue.

Use of ^{32}P-labeled peptides in screening cDNA libraries

The ^{32}P-labeled zeta Y120 and Y131 peptides were used in the screening of λgtll cDNA library for a human T cell leukemia line, Jurkat. The ^{32}P-labeled MB-1 peptide was used in the screening of λgtll cDNA library for human spleen cells. In both screenings, several independent clones were obtained for further characterization.

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