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CLONING OF THE cDNA FOR THE DELETED SYK KINASE HOMOLOGOUS TO ZAP-70 FROM HUMAN BASOPHILIC LEUKEMIA CELL LINE (KU812)

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Summary. Syk kinase is one of the protein tyrosine kinases and forms a family with ZAP-70. We isolated two different sized cDNA clones of syk (Syk11 and Syk41) from a cDNA library of human KU812 (human basophilic leukemia cell line). The obtained two clones carried different messages, that Syk41 had a 69 bp-long insertion between the SH2 domain and the kinase domain compared with Syk11. Alignment of the two human syk predicted polypeptides with those of the porcine syk and ZAP-70 revealed that human syk was 5 amino acid longer than the porcine syk at the N-termini and that the insertion of the 23 amino acid found in Syk41 was present in the porcine syk and absent in ZAP-70. Reverse transcribed polymerase chain reaction targeting this region showed that both forms of the polyA RNA were expressed in Jurkat cells, human peripheral leukocytes and also KU812 cells and that the inserted form was dominant.

The aggregation of the IgE bound to the high affinity IgE receptor (FcεRI) on mast cells by multivalent antigens triggers the signal transduction pathway leading to the cellular degranulation. Several protein kinases were found to associate with FcεRI; $p56^{lyn}$ and $pp60^{c\text{-}src}$ in RBL-2H3 cells, and $p62^{c\text{-}yes}$ in mouse PT18 cells (1). A novel 72 kDa tyrosine kinase was also shown to be associated with FcεRI, and the phosphorylation of the PTK was observed immediately after aggregation of the receptor (2). In B cells, the 72 kDa PTK had been shown to be associated with the IgM and IgD receptor complexes (3) and was recently reported that the kinase would be identical to $p72^{syk}$ (4).

$p72^{syk}$ was first isolated from porcine liver and shown to be expressed in lymphocytes (5). The TCR ζ chain associated protein kinase, ZAP-70, was recently cloned and shown to be homologous to $p72^{syk}$ (6). CD3ζ and FcεRIγ have the structurally related features and can form the hetero-dimer in certain T cells (7).

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Abbreviations: FcεRI, high affinity receptor for IgE; PCR, polymerase chain reaction; PMA, phorbol 12-myristate 13-acetate; PTK, protein tyrosine kinase; RT-PCR, reverse transcribed polymerase chain reaction.

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In addition, the homodimer of the FcεRIγ was functionally replaceable with CD3ζ and CD3η (8). The FcεRIγ was found to be associated with FcγRI and FcγRIII (9), and p72syk was also observed to be with FcγRI and FcεRI (10, 11). Rat p72syk cDNA was recently cloned from RBL-2H3 and elucidated to associate with FcεRIγ upon FcεRI engagement (12).

We isolated syk cDNA from human basophilic leukemia cell line, KU812, and determined its primary structure. The comparison of the sequences of two isolates revealed that two forms of the syk mRNA were expressed in KU812. These two forms were also detected in Jurkat T cell and peripheral leukocytes.

Materials and Methods

Cells and cell culture. KU812 and Jurkat cells were cultured in RPMI1640 medium with 10% fetal calf serum. For activation of Jurkat cells, the cells were treated with phorbol 12-myristate 13-acetate and ionomycin for 16 hr (13). The peripheral leukocytes were isolated from the healthy human peripheral blood.

Isolation and characterization of human syk cDNA from human basophilic leukemia cell line (KU812). Two primers corresponding to the kinase domain of porcine syk gene were synthesized with a DNA synthesizer. Total RNA was extracted from KU812 cells by guanidine-isothiocyanate, and poly(A) RNA was purified with an oligo(dT) spin column (Clontech). cDNA was synthesized using an oligo(dT) primer. The part of syk cDNA was amplified by 25 cycle polymerase chain reaction (PCR) with the above primers. The PCR products was cloned into pGEM-T vector (Promega) by the dA-dT cloning method and sequenced by using a automated DNA sequencer (Applied Biosystems: Model 373A) with the fluorescence-dyed primers (Applied Biosystems Inc.). The cloned PCR primer was labeled with biotin-dUTP (New England Biolabs) by using random hexamer primers. The double strand cDNA primed with oligo(dT) was inserted into dephosphorylated ZAPII phage vector (Stratagene) after addition of EcoRI adopters (Pharmacia). In vitro packaged phages (total 7.5 x 10^5 independent clones) were infected to XL1-blue (Stratagene) and divided into 29 sub-libraries. The phage DNA was purified from each amplified sub-library, and PCR was carried out with above primers to screen sub-libraries containing syk cDNA. Plaque-hybridization was performed with one of the sub-libraries in a high-stringent condition. The hybridized plaques were visualized by using chemiluminescent detection reagents (New England Biolabs). Finally two positive phage clones were isolated. Phagemid pSyk11 and pSyk41 were excited in vitro from XL1-blue infected with the isolated phages and a helper phage, R408 (Stratagene). Both recovered phagemids were sequenced in both direction by using an automated DNA sequencer with the fluorescence-dye primers or terminators (Applied Biosystems Inc.).

Characterization of syk RNA by RT-PCR. Total RNAs were isolated from Jurkat cells and human peripheral leukocytes. cDNA was synthesized from the total RNAs with oligo(dT) primers. PCR was performed by using synthesized cDNA as templates with two primers having sequences as follows. F20: 5'-AAAGAAGTTCGACACGCTCTGG-3'. R19: 5'-CGTTCAAGACCGAGTATGCCT-3'. The PCR products were analyzed by southern hybridization with the biotinylated PCR fragment that was amplified from pSyk11 with the same primer set. The hybridized bands were visualized by chemiluminescent detection reagents.

Northern hybridization. Three μg of poly(A) RNA was fractionated by electrophoresis on 1 % agarose gel containing formaldehyde. RNA was transferred to a nylon membrane (BRL) by using a vacuum blotting apparatus (LKB/Pharmacia). The transferred membrane was hybridized with biotinylated syk cDNA prepared from pSyk41 in 5 x SSPE and 50 % formamide. After high-stringent wash (0.1 x SSC at 55 °C), hybridized probe was detected by chemiluminescent detection reagents.

Results

Cloning of the human syk cDNA. We first isolated the part of human syk cDNA from the RT-PCR (reverse transcribed polymerase chain reaction) product of KU812 poly(A) RNA by with primers synthesized according to porcine syk cDNA sequence (5). We screened out syk cDNAs from a ZAPII phage library constructed with oligo(dT) primed cDNA of KU812 poly(A) RNA by plaque hybridization using the random-primed biotinylated DNA of the PCR clone as a probe. Two independent clones, λsyk11

Figure 1. ***The nucleotide sequences of human syk cDNAs.*** The upper number of the pairs on the right refers to the nucleotide position, the lower one to amino acid position of Syk11. The region corresponding to Syk41 is bounded by arrows. The 69bp-long nucleotides (enclosed in a box) inserted at the position are indicated by a line.

and λsyk41, were obtained. Partial DNA sequence analysis and restriction endonuclease analysis of the phagemid DNAs (pSyk11 and pSyk41) recovered by *in vitro* excision suggested that two clones should carry the cDNA overlapped each other .

We determined the DNA sequences of the two isolates using fluorescence dye-primer or -terminator with the nested deletions constructed from the recovered phagemids. pSyk11 had a 2541 bp-long insert and a long open reading frame beginning at the 148 nucleotide and terminating at the 1987 nucleotide (Fig. 1). The molecular weight of the predicted polypeptide was 69 kDa. The other clone, pSyk41 carries a 2116 bp-long insert. Comparison of the both nucleotide sequences revealed that pSyk 41 have a 69bp insertion at the position of 995 bp in pSyk11. This insertion should be translated in-frame of that predicted in pSyk11 (Fig.1).

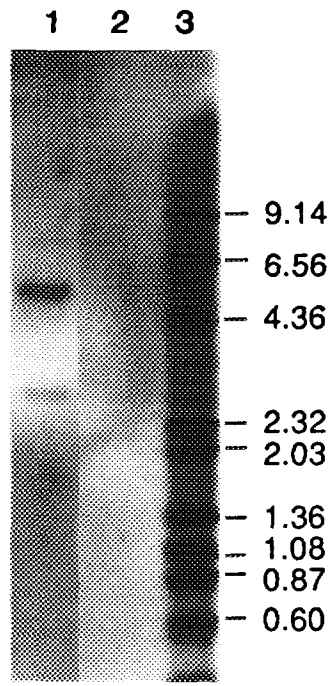

Figure 2. *Northern hybridization of KU812 poly (A) RNA.* 3μg KU812 poly(A) RNA (lane1), 5μg yeast total RNA (lane2) and the biotinylated DNA (lane3) were analyzed as described in Materials and Methods. The lengths of the biotinylated DNA are indicated at the right side of the blot.

Northern hybridization of poly(A) RNA from KU812 cells was performed with a EcoRI and BamHI fragment of pSyk41 as a probe (Fig. 2). Two RNA species were detected at the position of 5.2 kb and 2.8 kb.

Sequence comparison. As porcine syk, the primary amino acid sequence of human syk deduced from the cDNA sequence was shown to be homologous to those of the protein tyrosine kinases (PTKs). The amino acid sequence homology search revealed that there were two putative src homology 2 (SH2)-like domains and a C-terminal kinase domain found in the predicted amino acid sequence (Fig 3) like porcine syk and human ZAP 70. The degree of homologies of these predicted domains with other protein tyrosine kinases are summarized in Table 1. The most homologous kinase was human ZAP-70 (55 % , 46% and 64 % amino acid identity in SH2 (N), SH2 (C), and kinase domains, respectively). In SH2 domains, 29 to 38 % of amino acids were shared with other src family PTKs. 36 to 40 % of the amino acids in kinase domain was identical with other PTKs.

Fig. 3 shows the alignment of the amino acid sequences of human syk (Syk11), porcine syk (5), human ZAP-70 (6) and human syk that was isolated from a T-cell line (14). The deduced amino acid sequence of Syk11 was 5 amino acids longer than porcine syk and ZAP-70 at N-termini. Between the SH2 (C) and the kinase domains, 23 amino acid deletion in Syk11 was observed when compared with the porcine and the T-cell derived syk, and the insertion found in pSyk41 corresponded to this deleted region (see Fig. 1). Interestingly, the deletion of 24 amino acids was also observed in ZAP-70 at the same region.

Figure 3. ***Comparison of porcine syk, human ZAP-70 and human syk.*** Two src homology 2 (SH2) like sequences are indicated in boxes. Kinase domains are typed with bold letters.

The manner of the deletion between the SH2(C) and the kinase domains in human syk. To investigate whether 23 amino acid-deletion of syk is specifically occurred in KU812 cells, total RNA from Jurkat cells and human peripheral leukocytes were analyzed by RT-PCR. The used primers had sequences that correspond to nucleotide residue 860-883 and 1063-1041 of Syk11. The expected sizes of the PCR products were 182 bp and 251 bp from the short and the long type poly(A) RNA, respectively.

Table 1: Comparison of amino acid sequences

	SH2 (N)	SH2 (C)	Kinase
human syk SH2 (C)	31 %	-	-
porcine syk	91 %	94 %	97 %
human src	31 %	37 %	40 %
human yes	32 %	34 %	36 %
human hck	29 %	33 %	37 %
human lyn	38 %	28 %	36 %
human lck	32 %	33 %	41 %
human ZAP-70	55 %	46 %	64 %

Amino acid sequences of SH2 like regions and kinase domain in human syk gene were compared with the corresponding regions of the other protein kinases. porcine syk: (5), src; (15), yes: (16), hck: (17), lyn: (18), lck: (19), ZAP-70: (6).

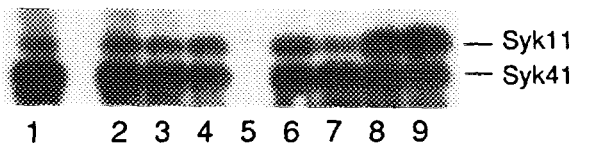

Figure 4. ***RT-PCR analysis of syk mRNA expressed in Jurkat cells, peripheral leukocytes and KU812 cells.*** RT-PCR products with F20 and R19 primers were fractionated by 2% agarose electorophoresis, transferred to nylone membrane, and detected as described in Materials and Methods (lane 1: Jurkat cells; lane 2: Peripheral leukocytes; lane3: PMA activated Jurkat cells; lane 4, 6: KU812 cells; lane 5: No template). Lane 7 to 9 were PCR products of the two syk cDNA isolates. Phagemid DNA of Syk11 and Syk41 (total 1ng) were mixed as follows; lane 7, 1:100; lane 8, 1:10; lane 9, 1:1. The positions of the PCR products from Syk11 and Syk41 are indicated at the right side.

Figure 4 shows the southern blot of the PCR products. KU821 cells expressed two types of RNA. The both types were also detected in the human T cell line, Jurkat cells, and peripheral leukocytes. To estimate relative amount of both RNAs, the competitive PCR was carried out using pSyk 41 and pSyk11 with same primer set. The comparison of the intensities of the both bands showed that about 1 to 10 % of the syk cDNA of the KU812 cells, peripheral leukocytes and PMA activated Jurkat cells were short type. In Jurkat cells, less than 1 % was short type. It seems that the activation of Jurkat cells by PMA might increase the amount of the short type poly(A) RNA.

Discussion

The feature of the deduced amino acid sequence of Syk11 differs from those of the porcine syk and human T-cell derived syk genes in two points. 1: Syk 11 should encode 5 amino acid longer product at the N-termini. 2: The 23 amino acid deletion was found between the SH2 (C) and the kinase domains. Since the T cell derived gene was cloned by PCR with specific primers corresponding to N and C terminal amino acid sequences (14), the 5 amino acids should be missed in the T cell derived gene. The direct amino acid sequence data of the human native syk kinase will reveal the true N-terminal amino acid sequence.

We suppose that the two forms of the syk mRNA should be generated by the alternative splicing pathway (under investigation). The most interesting point is whether the function of the both types differs from each other. The chimeric molecules of both the porcine and the T cell derived syk with CD16 showed kinase function *in vitro* (14), and ZAP-70, which has the similar structure of the deleted version of syk, showed the kinase activities (6), suggesting that the deleted syk must be functional.

The amino acid sequences of the syk were highly conserved between porcine and human. The deleted region was also well conserved, although the boundary amino acid sequence of the deleted region (aa 264-279 and 283-313 of the Syk11) was less conserved than the other regions (Fig. 3). These data suggest that the 23 amino acid-long region may have a function that is well conserved through evolution and that the deleted version of the syk kinase homologous to ZAP70 may also play a role in signal transduction. $p72^{syk}$ was recently cloned from RBL-2H3 (rat mast cell line) and shown to associated with FcεRIγ upon FcεRI engagement (12). By introducing Syk41 and Syk11 into FcεRI expressing transfectants, we are now investigating the role of $p72^{syk}$ kinase (especially of Syk11) in FcεRI mediated signal transduction.

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