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DELETIONAL ANALYSIS OF THE N-TERMINAL HALF OF THE LYN GENE

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INDEXING WORDS

src-family; lyn; p56^{lyn}; modulatory region; protein-tyrosine kinase; B cell activation; signal transduction

SYNOPSIS

The products of the viral & cellular src gene, p60$^{v\text{-}src}$ and p60$^{c\text{-}src}$, appear to be composed of multiple functional domains. Highly conserved regions called src homology 2 and 3 (SH2 and SH3),comprising amino acid residues 88 to 250, are believed to modulate the protein-tyrosine kinase activity present in the carboxy-terminal halves of the src proteins. To explore the functions of these regions more fully, we have introduced deletion mutations within the amino-terminal half of p56^{lyn}, a member of the src-related family of the protein tyrosine kinases. These mutant alleles were expressed under the control of the Moloney murine leukemia virus long terminal repeat in the vector plasmid pZip-neo-SV(X) to examine the biochemical and biological properties of the mutant proteins after introduction into NIH3T3 cells and WEHI-231(immature B-cell line). No mutant proteins induced transformed foci in NIH3T3 transfectants. Deletional mutant affecting residues 163 to 234 (lyn-dC) impaired kinase activity severely and residues 35 to 60 (lyn-dV), to the lesser extent. In WEHI-231 transfectants, we could also found the inhibition of kinase activity with deletions of residues 163 to 234 (lyn-dC) and 35 to 60 (lyn-dV). WEHI-231 transfectant with lyn-dC, named WE-C was induced lesser growth arrest by crosslinking membrane IgM compared with wild type (wt) WEHI-231 cells. Down Regulation of the mutant protein stimulated with anti-IgM antibodies was detected in wt WEHI-231, but not in WEHI-231 transfectants, WE-V and WE-C. We show here the effect of the mutations in the regulatory domain on the kinase activity and the biological function of the lyn gene product, p56^{lyn}.

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INTRODUCTION

To date, more than 20 retroviral oncogenes are known to have the capacity to transform cells in vitro and form tumors in vivo. Of these retroviral oncogenes, the v-src gene of Rous sarcoma virus (RSV) has been studied most extensively since temperature-sensitive mutants of RSV first suggested the existance of an " oncogene ". All retroviral oncogenes (v-onc's) are derived from cellular counterparts (c-onc's) in the host genome. At present, seven src-related cellular genes, c-yes, c-fgr, fyn, lck, lyn[37], hck, and possibly tkl as well as c-src have been molecularly cloned[11]. These genes could encode tyrosine-specific protein kinases that are highly homologous with the c-src protein, p60$^{c\text{-}src}$[13]. Thus, src and these other genes comprise a gene-family called the " src-family "

I.*p56^{lyn} and sIgM*; Lyn (lck/yes related novel tyrosine kinase) was isolated from a human cDNA library with v-yes probe under relaxed condition[37]. The lyn product is membrane-associated 56-kilodalton(kDa) protein that carries protein-tyrosine kinase activity and preferentially expressed in hematopoietic cells of myeloid lineage (macrophages/monocytes and platelet) and of lymphoid lineage (B lymphocytes and T lymphocytes infected with HTLV-I)[38]. Recently, p56^{lyn} was found to be associated with surface immunoglobulin(sIg),IgM in B cells and may act as a signal transducer in association with surface receptors that lack an intracellular catalytic domain. This concept is strongly supported by recent findings that T lymphocyte specific src-like kinase p56^{lck} is physically and functionally associated with the T cell surface antigens CD4 and CD8 [28][32][33]. Crosslinking of these surface immunoglobulins(sIgs) of resting B cells by antigens or anti-Ig antibodies activate B cells to enter the G1 phase of the cell cycle, where they become susceptible to proliferative signals that are normally provided by helper T cells [7][18]. This biologically important responses are proceded by early biochemical events such as phosphatidyl-inositol(PI) turnover and eventually activation of protein kinase C and Ca^{2+} mobilization [10][25]. Cross-linking membrane IgM on the immature B-cell line WEHI-231, however, induces growth arrest. When B cells are stimulated with anti-IgM antibodies, p56^{lyn} expressed in B cells is down-regulated within 1 hr and slowly recovered to the original level within 24 hr. Part of the p56^{lyn} could be co-immunoprecipitated with IgM, but the extent of its co-precipitation was less than that of the co-precipitation of CD4 and p56^{lck}, suggesting that the association of p56^{lyn} with IgM may be indirect.[38].

II. *Functional Analysis of src-family kinases* ; c-src and seven src-related genes are classified in one gene family for the following reasons : (1) They encode 55-60 KDa protein-tyrosine kinases that lack a typical transmembrane stretch. (2) Their

products show extensive homology with each other a contiguous stretch of approximately 460 residues towards the carboxy-terminus (C-terminus). (3) So far as investigated, the positions of their exon/intron boundalies in coding regions are conserved. The src-family genes share four functional domains, an amino-terminal (N-terminal) unique region, a modulatory region (SH2, SH3 region), a kinase domain and a C-terminal regulatory region (Fig.1). The 60-90 residues after Gly-2 (glycine at position 2) are quite divergent. This " unique region (Variable region)" might contain the recognition sequences for interaction with other proteins, for example, regulatory proteins or extracellular receptors. It is also believed that the first seven amino-terminal residues of p60$^{c\text{-}src}$ are required for myristylation and consequent attachment to the cell membrane[14]. The sequences of about 150 amino acids following the unique region show moderate homology in different members of the src family. This region (residues 80-260), called " modulatory region ", includes the SH3 (or A) and SH2 (or B and C) areas. The kinase domain is considered to be src homology 1 (SH1). Residues 260-516 in p60$^{c\text{-}src}$ encompass the catalytic domain that exhibits tyrosine-specific protein kinases activity, called " kinase domain "[1][21]. Tyr-527 in p60$^{c\text{-}src}$ is conserved in members of the src family (C-terminal regulatory region) and the phospholylation of this tyrosine residue depresses tyrosine kinase activity[3][4]. In p60$^{c\text{-}src}$, SH3 includes residues 86 to 136 (region A : 88-137) and SH2 extends from 137 to 241 (region B : 148-187, region C : 220-230). These are conserved in other cytoplasmic tyrosine kinases[29], abl (SH3 and SH2) and fps (SH2), and are also conserved in a new class of oncogene products of crk (SH2 and SH3)[24], phospholipase CII (SH2 and SH3)[31], GAP (SH2)[8][35], α-spectrin (SH3) (Fig.1).

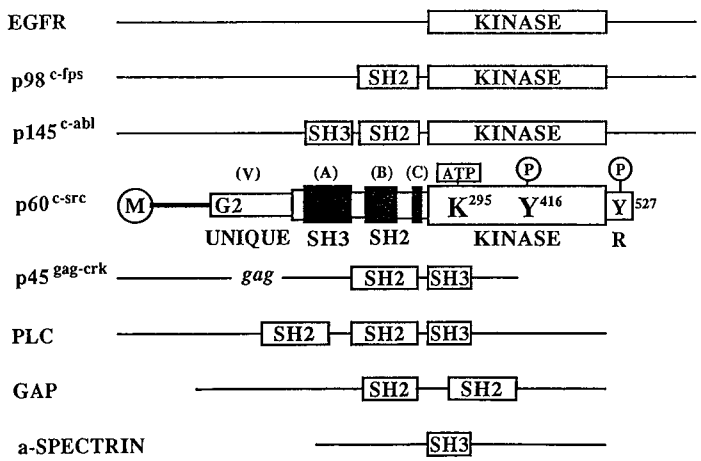

Fig.1 A model of functional domains of the src family tyrosine kinases. Additional proteins which have homology to the modulatory domain are illustrated. M : Myristylation site, G2 : Glycine at position 2, K : Lysine, Y : Tyrosine, R : Regulatory domain.

The function of this domain is still unknown, but is considered to be the site of binding to cytoplasmic protein(s) to modulate signals. The first evidence that the modulatory region could alter the function of $p60^{src}$ came from mutations in the SH2 region of $p60^{v\text{-}src}$ and mutated v-src protein inactivate its transforming activity or affect the morphology of transformed cells[2][5][6][17][26][36] . The mutants are transformation defective, incompletely transforming, or temperature sensitive. Various linker insertions and deletions in the SH2 region of fps produce host-dependent phenotypes or impaired kinase activity[29]. Recently, mutations of $p60^{v\text{-}src}$ have been found that also give a host-dependent transformation defective phenotype. It has been suggested that the SH2 region interacts with a host cell protein. Taken together, these results suggest that SH2 and SH3 may be important for regulation of kinase activity and for interaction with cellular proteins that serve as substrates or regulators of src-related kinases. To explore this possibility, we have constructed a set of lyn alleles that deleted in the unique region, and SH2 (B and C) region. By introducing the mutant alleles into NIH3T3 cells and WEHI-231 cells in a retrovirus vector, we have found that lesions in SH2 region have profound effect on the specific kinase activity of $p56^{lyn}$, with patterns that imply the existence of positive regulatory domains.

MATERIALS AND METHODS

Cell Culture : WEHI-231 cells (μ+, δ-,κ+) are murine B cell lymphomas. WEHI-231 cells, and WEHI-231 transfectants were maintained in RPMI1640 medium supplemented with 10% fetal bovine serum, 2mM L-glutamine, 50μg/ml streptomycin and $5x10^{-5}$M 2-mercaptoethanol. Murine NIN3T3 cells, and NIH3T3 transfectants were maintained in Dulbecco's modified Eagle's medium with 10% calf serum.

Plasmid Constructions : The 1686 base pair (nucleotides 264-1949) fragment of lyn cDNA which contains the entire lyn coding sequence, was cloned in the cloning vector, pUC118/MluI. After introduction of the deletional mutations using several restriction sites, the mutated lyn cDNA fragments were expressed under the control of the Moloney murine leukemia virus long terminal repeat(MoMLV-LTRs) in the vecter plasmid pZip-neo-SV(X) and the resulting plasmid was termed "pZip-neo-lyn".

Mutant Construction : All enzymes were obtained from TAKARA,Inc. pZip-neo-lyn/dV contains a deletion of sequences between the XhoII site(base pair[bp]403) after the Asp-35 codon and the XhoII site(bp477) after the Asp-60 codon. pZip-neo-lyn/dB contains a deletion of sequences between the BalI site(bp657) after the Ala-119 codon and the HindIII site(bp792) after the Ser-164 codon. pZip-neo-lyn/dC contains a deletion of sequences between the HindIII site(bp792) after the Ser-164 codon andthe NcoI site(bp1002) after the Pro-234 codon. pZip-neo-lynF^{508} has conversion of Tyr-508 in $p56^{lyn}$ to a phenylalanine residue by using oligonucleotide-directed mutagenesis[19]. After digestion with the appropriate

restriction enzymes, 5'-cohesive end was filled up with Klenow large fragment, 3'-cohesive end was flattened with T4-DNA polymerase, blunt ends were religated, regenerating the correct reading frame. (Fig. 2)

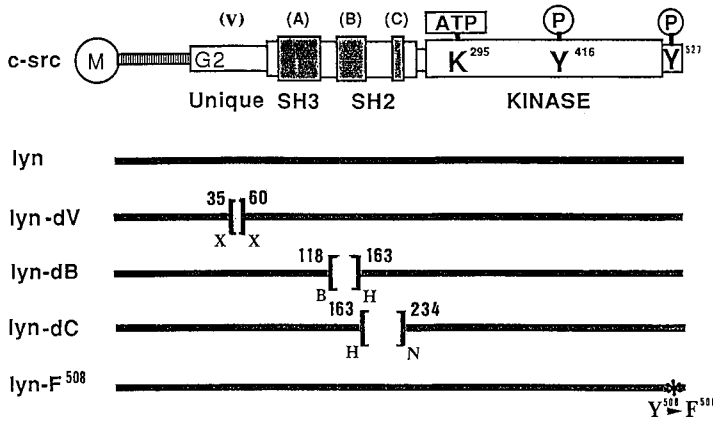

Fig.2 Schematic positions of deletion mutants. the positions of the restriction sites used in the constructs are shown. Parental lyn and all mutants. G2., Glycine at position 2 ; Unique., unique region ; Kinase., protein-tyrosine kinase domain., ; X., XhoII (or Sau3AI)site ; B., BalI site ; H., HindIII site ; N., NcoI site. Y : Tyrosine, F : Phenylalanine, K : Lysine. Amino acids are numbered according to the wt sequence

Production of Recombinant Retroviruses expressing mutated p56*lyn*: Helper-free virus stocks were derived from these reconstructed plasmids using the packaging cell lines ψ−2. DNA of pZip-neo-lyn and -mutated lyn was introduced into ψ−2 cells by CaPO4 transfection, and the cells were selected for resistence to G418(400μg/ml). Ecotropic helper-free virus from these cells was then used to infect NIH3T3 cells and WEHI-231 cells.

Introduction of mutated lyn cDNA into NIH3T3 cells and WEHI-231 cells : NIH3T3 cells and WEHI-231 cells were infected with high titer virus supernatant (plus polybrene[8μg/ml]). After 3 h at 37°C in 5% CO_2, the cells were rinsed and fresh medium was added. After incubation for 48h at 37°C in 5% CO_2, NIH3T3 cells were split 1/20 and G418(400μg/ml) resistent clones were selected . WEHI-231 transfectant clones were selected with the limiting dilutional method with G418(800μg/ml).

Antibody : A polyclonal anti-peptide antibody that recognizes amino acids Arg-25 to Ala-119 was obtained from Y.Yamanashi(Institute of Medical Science, University of Tokyo). The antibody (α-Lyn) was affinity-purified from the anti-Lyn antiserum by sequential chromatography on a LacZ-Sepharose column and purified Lyn-LacZ-Sepharose column[38].

Colony formation : Anchorage-independent growth was assayed by seeding cells in 0.33% agar containing minimal essential medium supplemented with 10% calf serum,1% chick serum,10% tryptose phosphate broth, and antibiotics. The plates were incubated at 37°C for 2 weeks and the number of colony formation was scored.

Cell lysates and Immunoprecipitation : Cells were lysed in RIPA buffer containing 150mM NaCl, 10mM Tris hydrochloride (pH 7.4), 1mM EDTA, 1% Triton-X100, 0.1% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 2mM phenylmethylsulfonyl fluoride. Lysates were reacted with an excess of the α-Lyn antibody and rotated at 4°C for 1 h. A 50μl sample of 1:3 slurry protein A-Sepharose CL-4B (Phrmacia) was added, and the lysates were rotated at 4°C for 1 h. Immunoprecipitates were washed three times with RIPA buffer.

In vitro kinase assays : Washed immunoprecipitates were split into two equal volumes. One portion was boiled in sample buffer and electrophoresed on a 8.5% SDS-PAGE gel. The proteins were then transferred to a nitrocellulose filter for Western blotting. The second portion was suspended in 30μl of kinase buffer containing 20mM Tris hydrochloride (pH 7.2), 5mM $MgCl_2$, and incubated with 10μCi of [γ-^{32}P]ATP (5000Ci/mmol) at 30°C for 15min. The product of the kinase reaction was subjected to SDS/8.5%-PAGE followed by autoradiography.

Immunoblotting : Cells were solubilized in RIPA buffer, and samples of the lysates containing 20μg of protein were subjected to SDS/8.5% PAGE. Protein blotting, sequential incubations with purified anti-Lyn antibodies and alkaline phosphatase-conjugated anti-rabbit IgG antibody, and the color reaction were performed as recommended by the supplier of the ProtoBlot system (Promega) except that 2% low-fat milk was used instead of bovine serum albumin.

Down Regulation of WEHI-231 transfectants with α-IgM antibody : WEHI-231 cells (1x10^{6}cells/ml) were incubated with goat anti-mouse IgM antibody (10μg/ml) and harvested at the time of 0min, 2min, 15min, 30min, 1h, 3h, and 6h. Each samples were suspended in 60μl sample buffer, boiled for five min and subjected to SDS/8.5% PAGE. The expression of the mutated lyn product was evaluated with Western blotting.

G1 arrest of WEHI-231 transfectants with α-IgM antibody : WEHI-231 cells and WEHI-231 transfectants (1x10^{5}cells/ml) were incubated with α-IgM antibody (3μg/ml and 10μg/ml) for two days and the extent of the growth arrest was evaluate.

RESULTS

1.Morphology of infected cells and anchorage-independent growth (Data not shown)

NIH3T3 cells infected with pZip-neo-lyn and overexpressing p56^{lyn} displayed refractile and spindle-shaped morphology but no anchorage-independent growth. NIH3T3 cells infected with viruses carrying the deletion mutants (including the lyn-F^{508}) displayed no remarkable morphological change compared with the cells infected with virus carrying pZip-neo-lyn. Anchorage-independent growth was examined by assaying the ability of the mutant lyn virus-infected cells to form colonies in soft agar. No colonies were formed by cells infected with our mutant series. Taken together these data demonstrated that these mutant lyn proteins at

least do not have the positive effect on the transformation activity.

2.Analysis of mutant lyn proteins in NIH3T3 transfectants (Fig.3)

To determine the expression level of the altered proteins , we lysed the NIH3T3 transfectants with sample buffer and subjected SDS-PAGE followed by Western blotting. The levels of protein after Western blotting varied. NIH3T3 transfectant clone with parental lyn , termed NLL-8 shows high level of p56^{lyn} expression, NLV-1 (NIH3T3 transfectant clone with lyn-dV) shows 1/3 , NLC-17 (NIH3T3 transfectant clone with lyn-dC) shows 1/5 of the level of NLL-8, and extremely small amount of expression was detected in NLF-3 (NIH3T3 transfectant clone with lyn-F) and NLB-3 (NIH3T3 transfectant clone with lyn-dB) (Fig.3a). Densitometric analysis showed the relative density of the bands of the mutated proteins. The density level of the p48$^{lyn\text{-}dC}$ expressed in NLC-17 and the p53$^{lyn\text{-}dV}$ expressed in NLV-1 is about 25% and 40% of the p56 ^{lyn} expressed in NLL-8,respectively. (Fig.3b). Mutated proteins expressed in NLF-3 and NLB-3 may have the toxic effect on the cell growth or have unstability at the mRNA level.

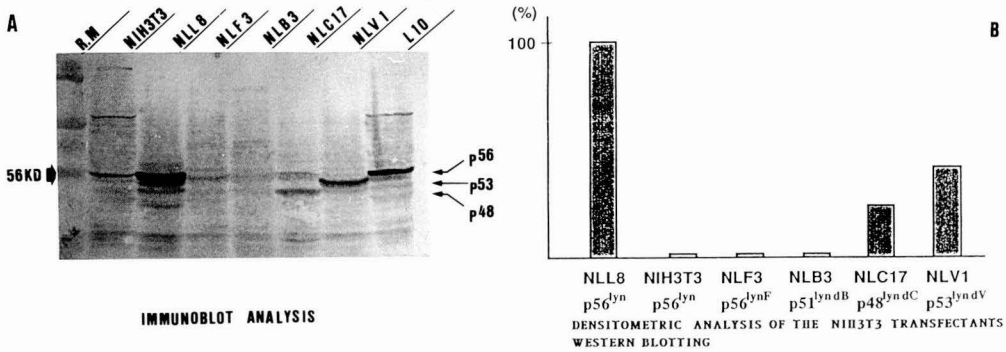

Fig.3 a. Expression of the altered proteins in NIH3T3 transfectants. NIH3T3 transfectants infected with parental(lyn) or mutant viruses were collected and lysed as described in Materials and Methods. NLL-8 and L10 : NIN3T3 transfectant clone with lyn, NLB-3 : NIN3T3 transfectant clone with lyn-dB, NLC-17 : NIN3T3 transfectant clone with lyn-dC, NLF-3 : NIN3T3 transfectant clone with lyn-F, NLV-1 : NIN3T3 transfectant clone with lyn-dV.The p56^{lyn} and its deleted form , p53$^{lyn\text{-}dV}$and p48$^{lyn\text{-}dC}$, were detected by transfer to nitrocellulose and binding to α-Lyn antibody, followed by immunoblotting. The expression of p56$^{lyn\text{-}F}$ and p51$^{lyn\text{-}dB}$ was so low and was not expressed enough to detect. b. Densitometric analysis of the NIH3T3 transfectants in Western Blotting.

3.Analysis of in vitro kinase activities of mutant lyn proteins in NIH3T3 transfectants (Fig.4)

In vitro kinase activities without exogenous substrates indicates an autophospholylation of the mutated lyn proteins themselves. The band at about 56KDa of the NLL-8 shows high level of autophospholylation. The band of NLV-1 at about 53KDa was almost 1/8 of NLL-8 and that of NLC-17 was almost undetectable at about 48KDa (Fig.4a). Densitometric analysis showed the level of the kinase activities of the p53$^{lyn\text{-}dV}$ expressed in NLV-1 and the p48$^{lyn\text{-}dC}$

expressed in NLC-17 is about 5% and 15% of the p56^{lyn} expressed in NLL-8 (Fig.4b). Compared with the ratio of protein expression, the relative kinase activities of NLV's and NLC's were lower than that of NLL-8 (Fig.4c). This suggests the possibility that the deletion of the region V (35-60) and C (163-243) have a negative effect on the protein-tyrosine kinase of the lyn product. So we wanted to see whether the mutated lyn product also have the negative effect on the biological activities of WEHI-231 cells, which expressed p56^{lyn} at high level. WEHI-231 transfectants were cloned and their mutated proteins expression and their kinase activities were examined.

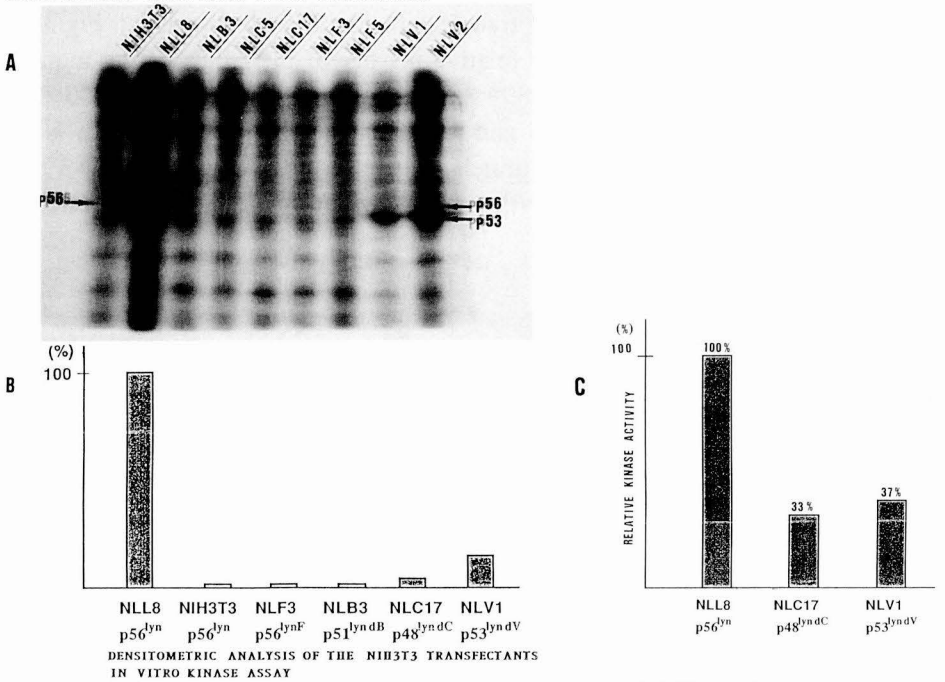

Fig.4 a. In vitro kinase assays of mutant lyn proteins in NIH3T3 transfectants. NLL-8 : NIN3T3 transfectant clone with lyn, NLB-3 : NIN3T3 transfectant clone with lyn-dB, NLC-5 and 17 : NIN3T3 transfectant clone with lyn-dC, NLF-3 and 5 : NIN3T3 transfectant clone with lyn-F, NLV-1 and 2 : NIN3T3 transfectant clone with lyn-dV. b. Densitometric analysis of the NIH3T3 transfectants in kinase assays. c. Relative kinase activity of the mutant proteins in NLL-8, NLC-17, and NLV-1. Results were averages of more than three experiments

4.Analysis of mutant lyn proteins in WEHI-231 transfectants (Fig.5)

The amount of the mutant lyn protein p48$^{lyn\text{-}dC}$ was so much in WEHI-dC(WE-C1-3 and 5).The level of the mutant lyn products may have the host-dependent manner. Deleted mutant proteins, p48$^{lyn\text{-}dC}$ and p53$^{lyn\text{-}dV}$ were detected with degradated form. The expression of the p48$^{lyn\text{-}dC}$ was as much as that of the endogenous lyn gene product. The p53$^{lyn\text{-}dV}$ was masked with endogenous p53^{lyn} and the expression level could not be exactly evaluated.

5.Analysis of in vitro kinase activities of mutant lyn proteins in WEHI-231 transfectants (Fig.6)

WEHI-231 transfectant clones with lyn-dB, dC, dV and F termed WE-B, WE-C, WE-V and WE-F, respectively, were analyzed. The band at about 56KDa was detected in WE-F1,2,WE-B1,2 WE-V1 and 2, however in WE-C1 and WE-C2, no band at about 56KDa was detected. Since the mutant lyn proteins in WEHI-231 transfectants except for WE-C1 and WE-C2 were not expressed so much, the band at about 56KDa indicated the kinase activities of the endogenous p56^{lyn}. WE-C1 and 2 have lost their kinase activities of the endogenous p56^{lyn}, in addition to their kinase activities of exogenous mutant lyn proteins. The mechanism that the lyn-dC product suppresses or neutralizes the endogenous lyn product was not yet analyzed. Increase of the kinase activity is, in general closely related to the biological response to the extracellular stimuli (eg. tramsmembrene signal transduction). Next we examined the effect of the lyn-dC protein on the signal transduction using the responsiveness of WEHI-231 cells to the crosslinking of the surface IgM.

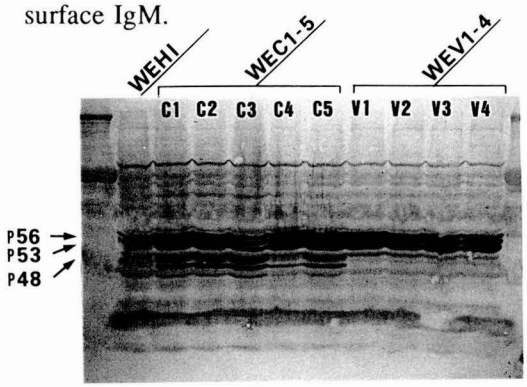

Fig.5 Exression of the altered proteins in WEHI-231 transfectants. Immunoblot analysis of the WEHI-dC and WEHI-dV transfectants are shown. WEHI-231 transfectants clone with lyn-dC and dV were termed WE-C (1-5) and WE-V (1-4), respectively.

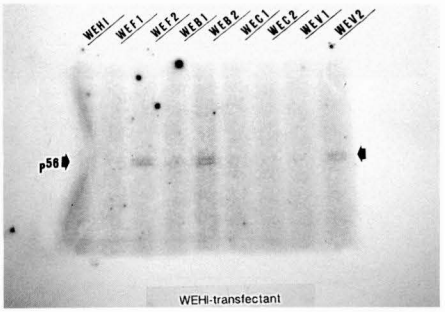

Fig.6 In vitro kinase assays of mutant lyn proteins in WEHI-231 transfectants. WE-F1 and 2 : WEHI-231 transfectant clone with lyn-F, WE-B1 and 2 :WEHI-231 transfectant clone with lyn-dB, WE-C1 and 2 : WEHI-231 transfectant clone with lyn-dC, WE-V1 and 2 : WEHI-231 transfectant clone with lyn-dV.

6.Down Regulation of WEHI-231 transfectants with α-IgM antibodies (Fig.7)

When the specific antigens or ligands bind to the receptor molecule, the complexes are aggregated and internalized to the cytoplasm, resulting in a decrease of the associated molecules. On cross-linking of surface IgM with anti-IgM antibodies, the src-like kinase p56^{lyn} expressed in B cells is down-regulated[38]. In addition, p56^{lyn} is co-immunoprecipitated with surface IgM from detergent lysate of cells. The cross-linking also induces rapid increase of tyrosine phosphorylation of at least three major proteins in B cells. Anti-IgM antibodies mimic the ligand

binding by crosslinking the surface IgMs. Down regulation of WE-C1 with α-IgM antibodies was examined (Fig.7a). Densitometric analysis of the results revealed that the early response was disturbed and the extent of the decrease of p56^{lyn} was impaired compared with the time course curve that was observed in wt WEHI-231 (Fig.7b). In this system, signal transduction via cell membrane was impaired by the expression of the mutated protein with low kinase activity. The lyn-dC protein may inhibits the pathway related to the protein-tyrosine kinase.

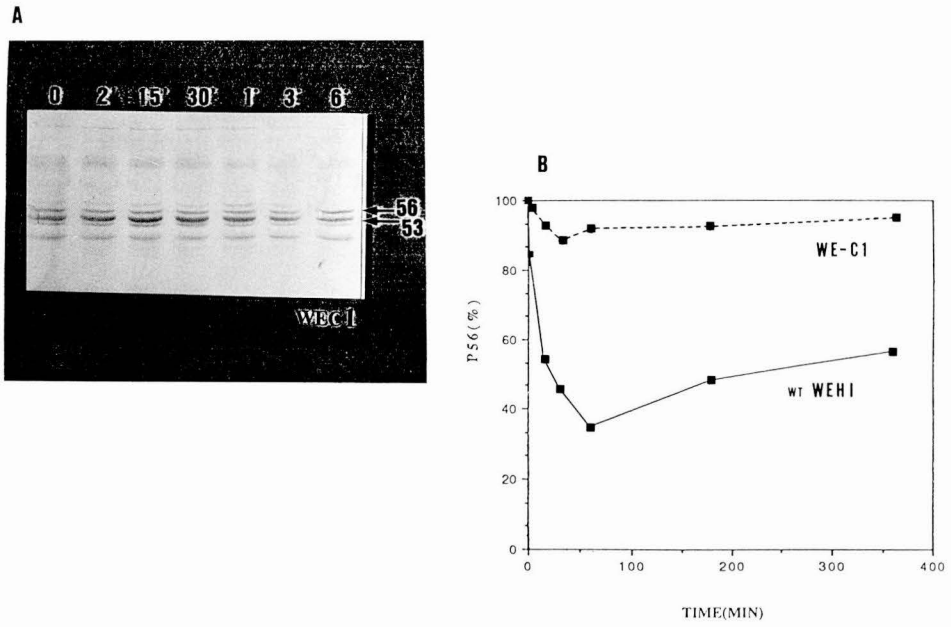

Fig.7 a. Down Regulation of WE-dC1 with α-IgM antibody. Cells were harvested at 0min, 1min, 2min, 15min, 30min, 1h, 3h, and 6h, each. b. Densitometric analysis of the Down Regulation. Plain line ; normal control of wt WEHI-231. Dotted line ; WEHI-C1.

7.G1 arrest of WEHI-231 transfectants with α-IgM antibody (Fig.8)

WEHI-231, WE-V1, and WE-C1 were incubated with α-IgM antibody(3 and 10μg/ml) for two days , and checked the cell count. There was no significant defference in G1 arrest between the two concentrations. Without α-IgM antibody, no difference was observed between WEHI-231 and WE-C1 and WE-V1, but two days after incubation with α-IgM antibody, WE-C1 and WE-V1 grew moderately (2.5 x 10^5, and 1.9 x 10^5, respectively), despite of no growth in wt WEHI-231(0.8 x 10^5). This indicated the inhibition or modification of the signal pathway by lyn-dC and lyn-dV protein.

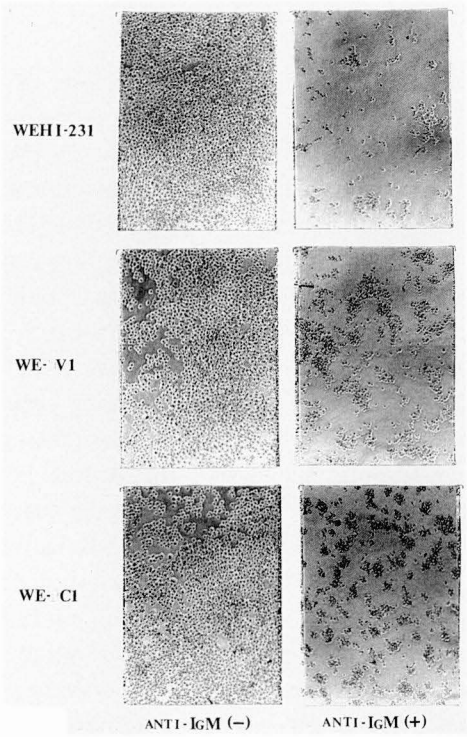

Fig.8 G1 arrest of WEHI-231 transfectants with α-IgM antibody (10μg/ml).

DISCUSSION

Residues 80 to 260 in p60$^{c\text{-}src}$, outside the kinase domain, are conserved in all members of the src family. These regions in p60$^{c\text{-}src}$ are now called regions A (88 to 138), B (148 to 190), and C (220-231), respectively. Several lines of evidence indicate that these regions have some effect on both tyrosine kinase activity and transforming ability. Deletions or point mutations in region A (almost equal to SH3) can change p60$^{c\text{-}src}$ into a transforming protein, although most of the mutant src proteins only induce a partially transformed phenotype. Kato et al.[15] demonstrated that a mutant c-src that contains amino acid substitutions at amino acids 63, 95, and 96 induces focus formation on CEF cells and confers anchorage-independent growth on the cell. As an extention of this finding, Potts et al[12]. showed that single amino acid substitutions at position 90, 92, or 95 or del(92-95) slightly enhance the tyrosine kinase activity of p60$^{c\text{-}src}$ and its oncogenic potential. These findings imply that region A in p60$^{c\text{-}src}$ has an inhibitory effect on transforming activity. The function of SH2 in p60$^{c\text{-}src}$ has not yet been analyzed in detail, but Nemeth et al[30].reported that deletions including region B and part of region C did not increase the tyrosine kinase activity or transforming activity of p60$^{c\text{-}src}$ and that c-src deletion mutants lacking residues 112 to 169, part of region A and B, displayed severely restricted phosphorylation

of substrates in vivo. Several mutations in the SH2 and SH3 regions of v-src render p60$^{v\text{-}src}$ either entirely, conditionally, or partially defective in transforming ability.[2][5][6][17][20][26][34][36] In particular, mutant src proteins containing deletions in region B [del(172)[34] and del(149-174)[6]] or insertions at position 225 or 228 in region C[6] displayed a host-dependent phenotype. Hirai et al.[12] showed that deletion mutants and some point mutations affecting residues 109 to 156 inhibited kinase and transforming functions, whereas deletions affecting residues 187 to 226 generally had positive effect on one or both functions. Semba et al.[30] reported that mutant p59^{fyn} with a small deletion including region C produces a transforming protein. Their observation appear to be contrary to a previous observation that v-src mutants containing insertion in region C showed a host-dependent transforming phenotype. This difference may be due to a difference in the mutations or on the genes analyzed. In the case of c-src, many oncogenic mutations are known to change p60$^{c\text{-}src}$ into a transforming protein. On the contrary, in the case of other src family kinases, no oncogenic mutations except removal or replacement of the tyrosine residue of fyn[16], lck[22], and hck[39] corresponding to Tyr-527 in p60$^{c\text{-}src}$ have been reported. In this work, we have demonstrated that deletions including region C or V in p56^{lyn} induce diminishment of protein kinase activity and biological activity in signal transduction. These results showed that the deletion of the region C has the negative effect on protein kinase activity in p56^{lyn}. The biological significances of regions A, B, and C are still unknown. One simple hypothesis is that common or similar cellular proteins can interact with proteins that share these regions and coordinately regulate them during signal transduction. Recently, Reynolds et al.[27] suggested that region B may be involved in the interaction of the activated src protein (in which Tyr-527 is replaced by Phe) with two phosphotyrosine-containing cellular proteins. From the study of p47$^{gag\text{-}crk}$, which is an oncogene product of the chicken CT10 virus, it was found that the p47$^{gag\text{-}crk}$ protein could specifically bind phosphotyrosine-containing proteins, including activated tyrosine kinases and the SH2/SH2' regions of p47$^{gag\text{-}crk}$ were necessary and sufficient for the association with phosphotyrosine-containing proteins. These results suggest that a function of the SH2/SH2' regions is to recognize the autophosphorylation of tyrosine kinases and to bind the SH2/SH2' containing molecules to the tyrosine kinases[23]. If src family kinases actually interact with cellular proteins through these regions, variants containing mutations in these regions may be helpfull for identifying these cellular components. With this concept, we introduced mutated lyn gene into WEHI-231 cells and checked their biological activity in signal transduction. WEHI-231 cells are murine immature B cell line that carry surface IgM (sIgM). Signaling by membrane immunoglobulin (mIg), the B-lymphocyte

antigen receptor, regulates B-cell maturation and activation. Crosslinking of mIg by antigen or by anti-Ig antibodies inactivates immature B cells, eliminating many of the B cells capable of producing auto-antibodies. By contrast, crosslinking of mIg promotes activation of mature B cells for clonal expansion and antibody production against foreign antigens. Crosslinking mIgM on the immature B-cell line WEHI-231 induces growth arrest. In our study, impairment of G1 arrest of WE-C1 revealed that the mutated lyn product may inhibit the kinase activity of itself and endogenous p56^{lyn} with cross talk. In general, the B-cell activation, like T-cell activation, has two different signal pathways, phosphoinositide hydrolysis and tyrosine phosphorylation.(9) Only tyrosine phosphorylation in WE-C1 was impaired, but not the phosphoinositide pathway. To examine possible involvement of p48$^{lyn\text{-}dC}$ in B-cell activation, we investigated the effect of sIgM crosslinking on p48$^{lyn\text{-}dC}$ by immunoblotting. On crosslinking of sIgM of wt WEHI-231 with goat anti-IgM antibodies, transient decrease of p56^{lyn} was observed (approximately 50-60% decrease 3 hours after sIgM crosslinking). However, in WE-C1, no down regulation of p56^{lyn} or p48$^{lyn\text{-}dC}$ was observed in response to anti-IgM antibody. These data support the conclusion that the mutated lyn product, p48$^{lyn\text{-}dC}$ inhibit the tyrosine-phosphorylation pathway (including endogenous p56^{lyn}) followed by the inhibition of down regulation and G1 arrest in response to anti-IgM antibody.

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