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Mouse monoclonal antibodies that specifically recognize an amino-terminal epitope of p56^{lck} protein tyrosine kinase

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p56^{lck} is a T cell-specific protein tyrosine kinase of the src family of proto-oncogenes which has been implicated in T cell signal transduction. Here we describe the production of mouse monoclonal antibodies directed against recombinant human p56^{lck} purified from an *E. coli* expression system. The antibodies were characterized by ELISA, RIA and immunoprecipitation of p56^{lck} from T cell lysates. A specific epitope was revealed at the aminoterminus of the p56^{lck} molecule by using Western blotting of deletion mutants and distinct domains of p56^{lck} expressed in *E. coli*. Potential applications of the results obtained are discussed.

Protein tyrosine kinase; Monoclonal antibody; p56^{lck}; Epitope mapping; Signal transduction

1. INTRODUCTION

Growth regulation, proliferation and differentiation of cells are controlled by extracellular signals that ultimately lead to alterations in gene expression. Protein tyrosine kinases are crucially involved in these processes. They are subdivided into two groups: cell surface receptor protein tyrosine kinases and non-receptor protein tyrosine kinases. Particular attention was drawn to the src family of proto-oncogenes, cytoplasmic, non-receptor protein tyrosine kinases which have been found associated with several cell surface receptors [1–5]. They are key elements of many signal transduction pathways transducing the signals received at cell surface receptors to the cell interior [6].

The protein tyrosine kinase p56^{lck} is a typical member of the src family of protein tyrosine kinases expressed at high levels in T lymphocytes [7]. In vivo p56^{lck} is myristoylated at the aminoterminal glycine residue [8,9]. This modification directs p56^{lck} to the cytoplasmic face of the cell membrane, where it has been demonstrated to be physically associated with CD4 and CD8 cell surface receptors [3–5]. The individual kinases are quite conserved among species and there is a high sequence identity among the various family members. The homology of p56^{lck} to p60$^{c\text{-}src}$ is especially high within the kinase domain. The homologies within the src homology regions 2 and 3 (SH2 and SH3) are lower but still significant. Targeted mutation of the *lck* locus by homologous recombination in mice showed severe deficiencies of the immune system [10]. These and other findings indicate that p56^{lck} is of crucial importance in the activation of mature T lymphocytes and the selection of maturing T cells in the thymus.

The catalytic activity of p56^{lck} is regulated through phosphorylation/dephosphorylation of the tyrosine residue at position 505 by phosphotyrosine phosphatase CD45 [2,11,12] and the protein tyrosine kinase p50^{csk} [13–16]. Excessive or deregulated activity of p56^{lck} leads to malignant transformation [17,18]. p56^{lck} has also been shown to modulate the activities of several proteins involved in signal transduction pathways [19], including rasGAP [20], MAPK [21], PI-3-kinase [22], and Vav [23].

In this paper we report the production and characterization of mouse monoclonal antibodies (mAb's) directed against human recombinant p56^{lck} purified from an *E. coli* expression system. In order to determine the epitopes recognized by the individual mAb's several different deletion mutants and single domains of p56^{lck} were constructed, expressed in *E. coli*, and probed in Western blotting experiments together with the mAb's.

2. MATERIALS AND METHODS

2.1. *Immunization and production of mouse monoclonal antibodies*

Human p56^{lck} was overexpressed in *E coli* using pDS expression vectors [24] and purified to homogeneity. Female Balb/Cj mice (6 weeks old) were immunized with 20 μg of purified p56^{lck} emulsified in 1 vol. of complete Freund's adjuvant (Difco). The antigen dose was given partly subcutaneously (s.c., 4 × 50 μl) and partly intraperitoneously (i.p., 100 μl). The immunization was repeated i.p. with the same dose/mouse in incomplete Freund's adjuvant at 30 day intervals. Small amounts of blood were taken from the tail vein 14 days after the boosts and tested for antibody titer by Enzyme-linked immunosorbant assays or radio-immunoassays (ELISA or RIA, see below). After reaching

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high enough serum titers the mice were boosted once more i.p. without adjuvant and sacrificed 4 days later. The spleen was aseptically removed, teased and a monocellular suspension of splenocytes was fused with the PAI myeloma cell line [25]. Hybridoma cell populations were grown in selective media and their supernatants were examined for specificity by ELISA. The best reacting hybridomas were recloned and expanded. The mAb's they produced were further analyzed for isotype and specifity using Western blotting techniques. Five such mAb's were selected, produced in large quantities and purified on protein-A columns. The purified mAb's were all of the IgG2a subclass and were designated as 8F12-G8, 2C12-C7, 2C12-E9, 6C1-B11 and 1D5-G1.

2.2. *ELISA/RIA*

Microtiter plates were coated with purified $p56^{lck}$ at 2 μg/ml in PBS. To reduce unspecific binding the plates were then saturated with 1% BSA followed by overnight incubation with 100 μl of hybridoma supernatant or diluted serum. Reacted antibodies were revealed with phosphatase-linked or peroxidase-linked anti-mouse IgG followed by the appropriate substrate. Alternatively ^{125}I-labelled anti-mouse IgG antibodies were also used as detecting agents.

2.3. *Plasmid constructions*

All *lck*-encoding sequences were derived from the plasmid pUC12/YT16 [26]. pUC12/YT16 was digested with *Nco*I, the resulting 5' overhang was filled in followed by digestion with *Hin*dIII. The resulting 1,837 bp fragment was ligated into the plasmid pDS56RBSII(−1) [24] which had been cut with *Hin*dII and *Hin*dIII. The resulting plasmid encodes the protein MRDPS-$p56^{lck}$. All different variants of $p56^{lck}$ were cloned by PCR. The plasmid encoding MRDPS-$p56^{lck}$ was used as a template in PCR reactions using the primers shown in Table I.

The PCR fragments encoding amino-terminal deletion mutants of $p56^{lck}$ were digested with *Nco*I and *Hin*dIII and cloned into pDS56RBSII,*Nco*I [24] which had been digested with *Nco*I and *Hin*dIII. The PCR-fragments encoding the SH3+SH2, the SH3 and the SH2 domain were digested with *Bam*HI and cloned into the *Bam*HI site of the vector pDS56RBSII(−2) [24].

All expression plasmids were transformed into the *E coli* strain M15 [27] containing the repressor plasmid pREP4 [24]. Single colonies were picked and the presence of *lck*-encoding sequences was verified by restriction enzyme digestion of mini-prep DNA. Colonies that contained *lck*-encoding plasmids were cultured in LB medium to an optical density of 0.6 and the expression of the recombinant $p56^{lck}$-variants was then induced by addition of IPTG to 2 mM. After 4 h of induction the *E coli* cells were pelleted by centrifugation and the pellet was dissolved in sample buffer.

2.4. *Protein electrophoresis and Western blotting*

Proteins were separated by reducing SDS gel electrophoresis on 12.5%(w/v) polyacrylamide gels [28]. The gels were either stained with Coomassie blue or electrophoretically transferred onto nitrocellulose membranes. The nitrocellulose membranes were then probed with mouse monoclonal antibodies or with rabbit polyclonal antiserum specific for $p56^{lck}$. After washing, the membranes were reacted with peroxidase-labelled goat anti-mouse or goat anti-rabbit antibodies. The signal was developed by using the ECL-system (Amersham) as described by the manufacturer

2.5. *Immunoprecipitation and kinase assays*

Immunoprecipitations and in vitro kinase assays were performed as described by Amrein et al. [20].

3. RESULTS

We have raised mouse mAb's specific for the protein tyrosine kinase $p56^{lck}$ by following the immunization and selection procedures described in Material and Methods. Five mAb's were chosen and further analyzed in immunoprecipitation experiments. Detergent lysates

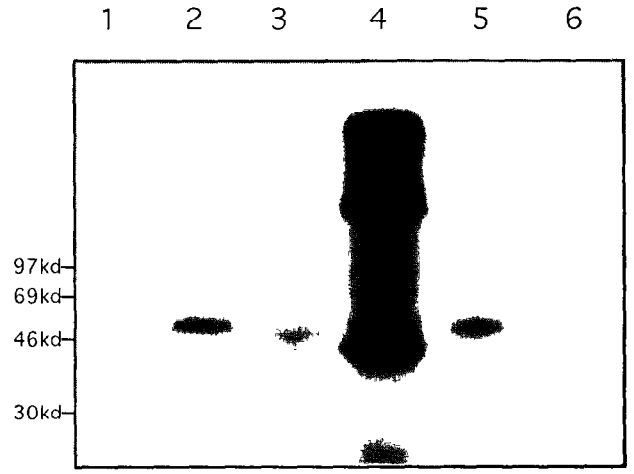

Fig. 1. Immunoprecipitation of $p56^{lck}$ from T cell lysates and in vitro kinase assays (Autoradiogramm). Lane 1, preimmuneserum; lane 2, polyclonal rabbit anti-$p56^{lck}$ antiserum; lane 3, mAb 2C12-C7; lane 4, mAb 1D5-G1; lane 5, mAb 6C1-B11; lane 6, mAb 8F12-G8.

of T lymphocytes were probed with the mAb's and immunecomplexes formed were subjected to in vitro kinase reaction experiments. Results show that all five mAb's specifically precipitate a phosphoprotein of the apparent M_r of 56 kDa (Fig. 1, and data not shown). Rabbit polyclonal antibodies revealed the same protein whereas pre-immune sera did not precipitate any detectable phosphoprotein (Fig. 1). Thus, we conclude that each of the five mAb's tested specifically recognizes and immunoprecipitates $p56^{lck}$.

To determine the epitope recognized by the individual mAb's several variants of $p56^{lck}$ (Fig. 2) were produced in *E. coli*. The expression of the $p56^{lck}$ variants was demonstrated by SDS-polyacrylamide gel electrophoresis of *E. coli* cell extracts followed by Coomassie blue staining of the gels. All variants of $p56^{lck}$, except MRDPS-$p56^{lck}$, $p56^{lck\Delta 1\text{-}30}$ and the SH2 domain of $p56^{lck}$, gave a visible band (Fig. 3).

Table I

Primers used in PCR reactions

*lck*Δ1-9	5'agctcacccatggaagatgac3'
	5'gggcctctcaagctttcctctcaa3'
*lck*Δ1-30	5'atagtccccatggatggcaag3'
	5'gggcctctcaagctttcctctcaa3'
*lck*Δ1-48	5'cgggaccccatggttacctac3'
	5'gggcctctcaagctttcctctcaa3'
*lck*Δ1-60	5'gcttcccccatggaagacaac3'
	5'gggcctctcaagctttcctctcaa3'
lck-kinase	5'ccagaagcccatggagccgtgg3'
domain	5'gggcctctcaagctttcctctcaa3'
lck-SH3+SH2	5'ccggatcccactgcaagacaacctggttat3'
domain	5'taggatccgggcttctgggtctggcagg3'
lck-SH3	5'ccggatcccactgcaagacaacctggttat3'
domain	5'tag-gatccgggttcgggctccaggctgttc3'
lck-SH2	5'ccggatcccctggttcttcaagaacctgagcc3'
domain	5'taggatccgggcttctggtctggcagg3'

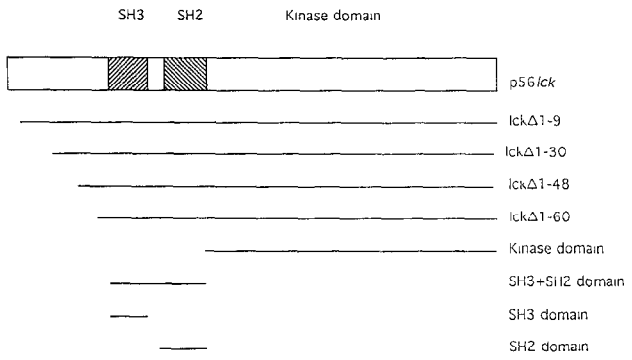

Fig. 2. Schematic structure of p56^{lck} and its variants.

Western blotting experiments using polyclonal rabbit anti-p56^{lck} antisera confirmed that all variants of p56^{lck} were expressed in *E. coli*, although at different levels (Fig. 4a). In these experiments the SH2 and SH3 domains were detectable only after prolonged exposure of the blots (data not shown).

To identify the epitope(s) recognized by the five different mAb's directed against p56^{lck} Western blotting experiments were performed. Four out of the five mAb's tested reacted with MRDPS-p56^{lck} but neither of them recognized any of the amino-terminal p56^{lck} deletion mutants, nor did they react with any of the isolated domains of p56^{lck} (Fig. 4b). Fig. 4b shows the immunoblot obtained using the mAb 8F12-G8. The same results were obtained using the mAb 6C1-B11, 2C12-E9 and 1D5-G1, whereas the mAb 2C12-C7 did not react

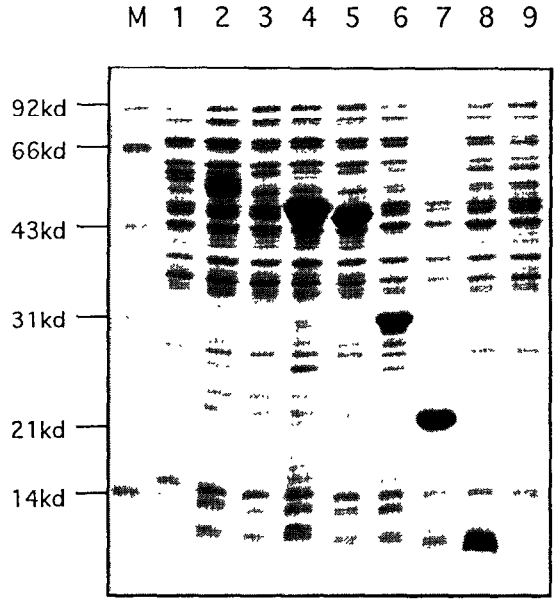

Fig. 3. Over-expression of p56^{lck} variants in *E. coli* 12% (w/v) SDS-polyacrylamide gel stained with Coomassie blue. Lane 1, MRDPS-p56^{lck}; lane 2, p56$^{lck\Delta 1\text{-}9}$; lane3, p56$^{lck\Delta 1\text{-}30}$; lane4, p56$^{lck\Delta 1\text{-}48}$; lane 5, p56$^{lck\Delta 1\text{-}60}$; lane 6, p56^{lck}-kinase domain; lane 7, p56^{lck}-SH3+SH2 domain; lane 8, p56^{lck}-SH3 domain; lane 9, p56^{lck}-SH2 domain.

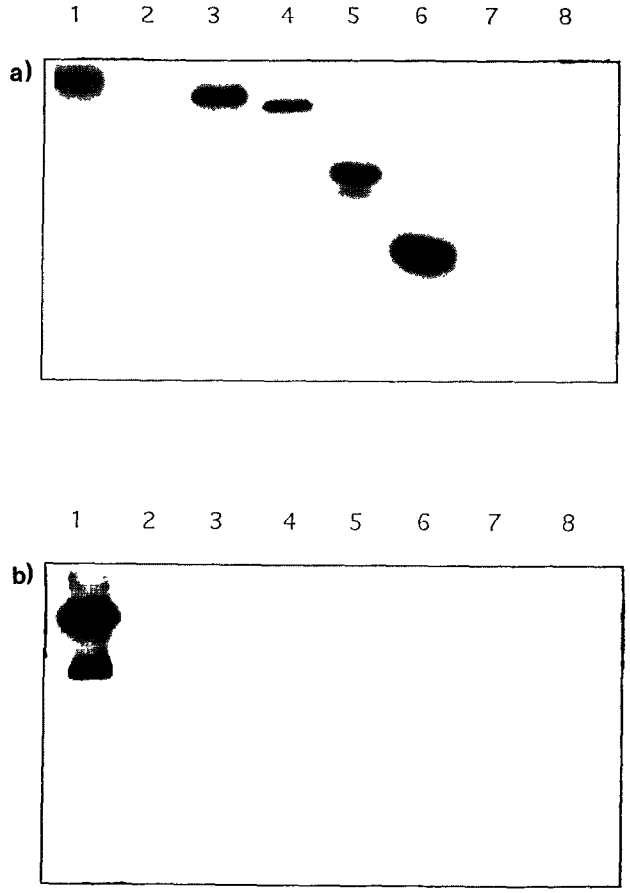

Fig. 4. Immunoblot with (a) rabbit polyclonal anti-p56^{lck} antisera, and (b) mouse mAb 8F12-G8. Lane 1, MRDPS-p56^{lck}; lane 2, p56$^{lck\Delta 1\text{-}30}$; lane 3, p56$^{lck\Delta 1\text{-}48}$; lane 4, p56$^{lck\Delta 1\text{-}60}$; lane 5, p56^{lck}-kinase domain; lane 6, p56^{lck}-SH3+SH2 domain; lane 7, p56^{lck}-SH3 domain; lane 8, p56^{lck}-SH2 domain.

with any of the p56^{lck} variants tested in these experiments. These results demonstrate that an amino-terminal deletion of 30 amino acids of p56^{lck} is sufficient to destroy the epitope recognised by four different mAb's. Moreover, additional studies using the amino-terminal deletion mutant p56$^{lck\Delta 1\text{-}9}$ demonstrated that the epitope recognized by the four mAb's lies within the very amino-terminus of p56^{lck} (data not shown).

To verify that the first few amino-terminal amino acids of p56^{lck} constitute an epitope recognized by the mAb's, MRDPS-p56^{lck} was blotted onto nitrocellulose and Western blotting experiments were performed in the presence or absence of two synthetic peptides corresponding to amino acids 1–14 (peptide no. 11'861-B6) and 6–17 (peptide no. 11'861-B10) of p56^{lck}. Both peptides completely blocked the recognition of MRDPS-p56^{lck} by mAb 8F12-G8 if present in a 1,000-fold excess over p56^{lck} (Fig. 5). The peptide corresponding to amino acids 1–14 of p56^{lck} blocked the reaction of MRDPS-p56^{lck} with the mAb partially even if present in equal amounts (Fig. 5a).

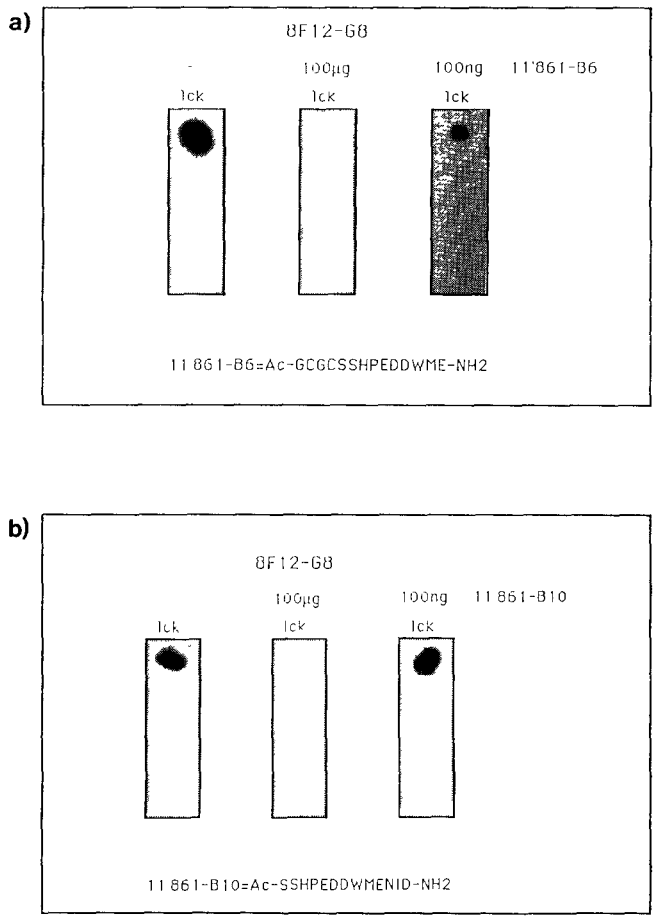

Fig. 5 Immunoblot with mouse mAb 8F12-G8 in the absence (−) or presence of 100 ng or 100 µg of the two peptides: (a) peptide no 11'681-B6 (amino acids 1-14) and (b) peptide no 11'861-B10 (amino acids6–17). 100 ng of purified MRDPS-p56^{lck} were spotted in each case.

4. DISCUSSION

In this study we have described the production and characterization of five mouse mAb's raised against the human protein tyrosine kinase p56^{lck}. Four of the mAb's map to the extreme amino-terminus of p56^{lck} and thus reveal a common and specific epitope on p56^{lck}. The fifth monoclonal antibody (2C12-C7) seems to recognize a conformational epitope since it does not react with p56^{lck} in Western blotting experiments, but is able to immunoprecipitate p56^{lck} from T cell lysates. The finding of a specific recognition of an epitope displayed at the amino-terminus of p56^{lck} by our mAb's is not unique since mouse mAb's raised in an other laboratory are also preferentially directed to the amino-terminus of p56^{lck} (personal communication).

The fact that our mAb's react only with the amino-terminus of p56^{lck} could indicate that lymphocyte clones recognizing other sequences of p56^{lck} are eliminated during thymic selection. Indeed mouse p56^{lck} is highly homologous to human p56^{lck} (96.3% overall homology) whereas within the amino-terminus the homology is lower (80% homology in the first 10 amino-terminal amino acids). This lower homology of the amino-terminal p56^{lck} sequences might allow a preferential selection of T cell clones recognizing such an epitope.

In a recent publication Rouer and Benarous reported the discovery of a splice variant of p56^{lck} in which the first 35 amino-terminal amino acids of p56^{lck} are replaced by 10 residues starting at an alternative AUG start codon [29]. The mAb's described in this publication could be used to discriminate between the two variants of p56^{lck} and therefore might provide an ideal tool to study the expression and tissue distribution pattern of the p56^{lck} variants. Moreover, they could be used in a variety of biochemical as well as cell biological studies, in vitro and in vivo, to analyze the properties and functions of the two splice forms.

In a series of additional experiments we have used the peptide corresponding to the epitope recognized by the mAb's to elute p56^{lck} from a mAb-immunoaffinity column (data not shown). Results indicate that, using this peptide, p56^{lck} can be eluted specifically from the column in a biologically active form. This will allow the affinity purification of p56^{lck} under mild conditions, thereby avoiding the harsh treatments that are normally used to disrupt antigen–antibody interactions.

Another application might be to use the epitope sequence recognized in this paper as a tag for other proteins. By DNA cloning the epitope sequence could be linked to any recombinant protein in such a way that it can serve both as a signal for myristoylation and as a tag for the detection and the purification of the recombinant protein. This combination of epitope tag, monoclonal antibody and synthetic peptide, thus offers the possibility of purifying recombinant proteins under conditions expected to maintain full biological activity. Moreover, any tagged protein could easily be visualized in intact cells using the mAb's in immunofluorescence studies.

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