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Chimeric Protein of CD8α Extracellular Domain and CD4 Transmembrane and Cytoplasmic Domain Binds More Efficiently to p56lck than CD8α

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$p56^{lck}$ (lck), a cytoplasmic protein tyrosine kinase of the src family, is noncovalently associated with the cell surface coreceptors CD4 and CD8, which are expressed on thymocytes and mature T cells. The coreceptor protein plays an important role during the differentiation of thymocytes and the activation of T cells. DNA constructs were designed to study the roles of CD4 and CD8 during the differentiation of thymocytes. One is a chimeric cDNA which consists of coding regions for the extracellular domain of CD8α and the transmembrane and cytoplasmic domain of CD4. The other is the same chimeric cDNA but with a point mutation converting Cys to Ala in the lck-binding site to disrupt the association. We confirmed that the CD8α/CD4 chimeric molecule bound to lck more efficiently than the wild type CD8α protein. However, the chimeric protein with the Cys→Ala mutation did not associate with lck. The results suggest a possibility that the CD8α/CD4 chimeric protein may behave like a CD4 protein in associating with lck and that it may deliver a signal inside the cell in a similar manner. Analysing effects of the mutant CD8α/CD4 chimeric protein expression in developing thymocytes will elucidate the role of lck during the determination of CD4/CD8 cell lineages.

Mature T cells express either CD4 or CD8 on their surface. Helper T cells express a CD4 coreceptor which binds to the class II major histocompatibility complex (MHC) protein and cytotoxic T cells express a CD8 coreceptor which binds to the class I MHC protein. This propensity to express coreceptors suitable to each T cell lineage is very strong and it suggests that T cell lineage commitment is a strictly regulated process. Recently, the question of how immature thymocytes make the correct choice between CD4 and the CD8 T cell lineage in the thymus has been of particular interest (Fung-Leung et al., 1991; Basson et al., 1998; Saito and Watanabe, 1998).

Although it is clear that TCR, with CD4 or CD8, recognizes self-MHC proteins (plus peptide) during the positive selection of thymocytes (Blue et al., 1988; Fung-Leung et al., 1991; Luescher et al., 1995), the mechanism of how the decision for differentiation into CD4/CD8 cell lineages is made is still under intense investigation. One possibility has been suggested that T cells receive a signal, generated upon MHC recognition, directing the lineage commitment (Seong et al., 1992; Parnes and Seong 1994; Chan et al., 1998). In contrast, the initial decision may be made independent of MHC recognition, and subsequent cellular selection serves to eliminate those cells that express an inappropriate combination of TCR and coreceptors (Crump et al., 1993; Lucas et al., 1995). Recently, it was suggested that both mechanisms may be operating in developing thymocytes (Lucas et al., 1995; Matechak et al., 1996; Chan et al., 1998). However, it is still not clear whether there exists any signal and, if there is any difference in the signals generated by CD4 and CD8 after their coengagement with TCR to either class I or class II MHC molecules during the diffentiation process. It was shown that the specificity of TCR determines the lineage of thymocytes to CD4 or CD8 single positive (SP) T cells. It was reported that the cytoplasmic domain of CD4 can transduce a signal for developing thymocytes to differentiate into the CD4 lineage (Seong et al., 1992; Parnes and Seong, 1994; Itano et al., 1996). However, it was reported that the coreceptors function just to enhance the interaction between TCR and MHC proteins, rather than delivering any specific signals during CD4/CD8 lineage commitment (Ravichandran and Burakoff, 1994; Smith et al., 1996).

Since $p56^{lck}$ (lck) is associated with CD4 and CD8

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coreceptors and plays a critical role in delivering the activation signal in mature cells (Straus and Weiss, 1993; van Oers et al., 1996; Bachmann et al., 1999), it is important to determine whether lck also plays any critical role during the cell lineage commitment. It was already shown, using knockout mice, that lck is required for double negative thymocytes to develop into the double positive stage (Molina et al., 1992; Levin et al., 1993; Groves et al., 1996; Nicolai et al., 1996). However, it is not clear, in the same knockout mice model, whether lck is operating during the CD4/CD8 lineage commitment. On the other hand, it was reported that CD45, a tyrosine phosphatase which activates lck during the T cell activation process, is necessary for the differentiation of double positive thymocytes into mature CD4 and CD8 cells (Stone et al., 1997; Seavitt et al., 1999). It is likely that lck may also be the target of CD45 during the differentiation process, and probably also during lineage commitment (Veillette et al., 1988, 1990; Shaw et al., 1989; Turner et al., 1990; Irie et al., 1995, 1998; Wallace et al., 1995; Basson et al., 1998).

To investigate these possibilities, we intend to analyze the effects of the transgenic expression of chimeric CD8α/CD4 and its mutant which cannot associate with lck. It has not been proven yet whether CD8α/CD4 chimeric protein associates with lck more efficiently, like a CD4 protein, than CD8α, and, therefore, behaves like CD4 in delivering signals through lck. In addition, to test whether lck is critical during the CD4/CD8 lineage commitment, the mutant form of the CD8α/CD4 chimeric protein will be very useful. Here, we show that both the cell surface expressed CD8α and the CD8α/CD4 protein bind to lck and the chimeric protein binds much more efficiently than CD8α. In addition, we confirmed that the CD8α/CD4 mutant (CD8α/CD4-Ala) protein does not bind to the lck protein.

Materials and Methods

Antibodies

Anti-lck rabbit serum, AP (Alkaline phosphatase) conjugated anti-rat antibody and AP conjugated anti-rabbit antibody were purchased from Promega. Anti-CD8α (53.6.72) antibody was purified and conjugated with FITC as described previously (Coligan et al., 1991).

Construction

cDNAs for murine CD8α (Johnson-Tardieu et al., 1996) and murine CD8α/CD4 chimera containing the extracellular domain of CD8α and the transmembrane and cytoplasmic domain sequences of CD4 (Seong et al., 1992) were previously described. Using a primer (5′-G-AAG-AAG-ACC-TGC-CAG-GCA-CCC-CAC-CGG-ATG-CAG-3′ and 5′-CTG-CAT-CCG-GTG-GGG-TGC-CTG-G CA-GGT-CTT-CTT-C-3′), cysteine 420 (TCA) to alanine (GAC) mutation (C420A) in the cytoplasmic domain of CD8α/CD4 were generated by an overlap polymerase chain reaction with pfu Taq polymerase (Veillette et al., 1990). After PCR, *DpnI* was treated to remove the DNA template of PCR. *DpnI* treated DNA was transformed into DH5a. Point mutation introduced in to the CD8α/CD4 chimeric cDNA (CD8α/CD4-Ala) was confirmed by sequencing. The three cDNAs were cloned into pRcCMV with *Bam*HI and their expressions were driven by the CMV-promoter. pBJneo-lck, a plasmid DNA used for lck expression, was described previously (Miceli et al., 1991).

Stable Transfection

L cells, seeded at 1.0×10^6 in a 60-mm dish containing DMEM (Gibco) supplemented with 10% FBS, were transfected with 10 µg of pBJneo-lck by calcium phosphate precipitation (Di Nocera and Dawid, 1983). G418 (1 mg/ml) was added at 48 h of transfection for selection. Selected L cells were confirmed for lck expression by Western blotting. The cells expressing lck were transfected additionally with CD8α, CD8α/CD4, and CD8α/CD4-Ala (9 ug of each DNA), together with 1 µg of a plasmid DNA (pRetro-On vector) containing a puromycin resistance gene, and selected with puromycin (2 mg/ml). After 2-3 weeks, clones were divided into 24-well plates and analysed for their surface expression of the proteins.

Analysis of the expression of CD8α, CD8α/CD4, and CD8α/CD4-Ala proteins

Dishes of transiently transfected L cells were washed twice with phosphate buffered saline (PBS), and harvested by scraping with PBS containing 0.1 mM EDTA. Five percent of harvested cells from each dish was used for quantitation of the surface expression of proteins using 1 µg of FITC-conjugated anti-CD8α antibody (53.6.72) at room temperature for 10 min. After washing, cells were resuspended in 200 µl of PBS containing 0.2 µg/ml propidium iodide (PI), and were analyzed on a FACStar Plus (Becton Dickinson).

Co-immunoprecipitation

Cells expressing equivalent levels of surface CD8 were used for lysate preparation. Cells were lysed in 100 µl of TNE (137 mM NaCl, 20 mM Tris-HCl [pH 8.0]) with 1% NP-40, 10% glycerol, and protease inhibitor (aprotinin) and rocked at 4℃ for 20 min. Lysate was cleared by centrifugation at 12,000 x g for 15 min, and protein concentration was determined by the Bradford assay (Bio-Rad). Each lysate was then used for immunoblotting and immunoprecipitation. One hundred micrograms of protein extract was used for immunoblot analysis and 500 µg of the extract was used for immunoprecipitation. Five hundred micrograms of protein was incubated with 20 µl of 50% suspensions of protein G Sepharose for preclearing at 4℃ for 1 h. The beads were pelleted and the supernatant was incu-

bated for 3-4 h with 4 μg of 53.6.72 antibody. After 1 h incubation with 50 μl of the protein G Sepharose, the immune complexes were pelleted and washed three times with TNE buffer. Washed immune complexes were resuspended in 2X SDS-PAGE loading buffer and released by boiling for 5 min.

Immunoblotting

Immunoblot analysis was carried out as previously described (Veillette et al., 1990). Briefly, 100 μg of whole cell extracts and eluted immune complexes were subjected to 15% SDS-PAGE and transferred to a nitrocellulose membrane. The membrane was incubated in blocking solution [3% non fat dry milk, 150 mM NaCl, 50 mM Tris-HCl, pH 7.5, 0.05% Tween 20] for 1 h at room temperature. After incubating with the anti-lck antiserum and anti-CD8α antibody (53.6.72), specific bands were detected with anti-rabbit IgG or anti-Rat IgG conjugated with alkaline phosphatase in buffer solution (100 mM Tris-Cl, pH 9.5, 100 mM NaCl, 10 mM $MgCl_2$) containing 165 mg/ml BCIP and 330 mg/ml NBT.

Results

Construction of CD8α/CD4-Ala

Cystein residue of the CD4 cytoplamic domain (at 420

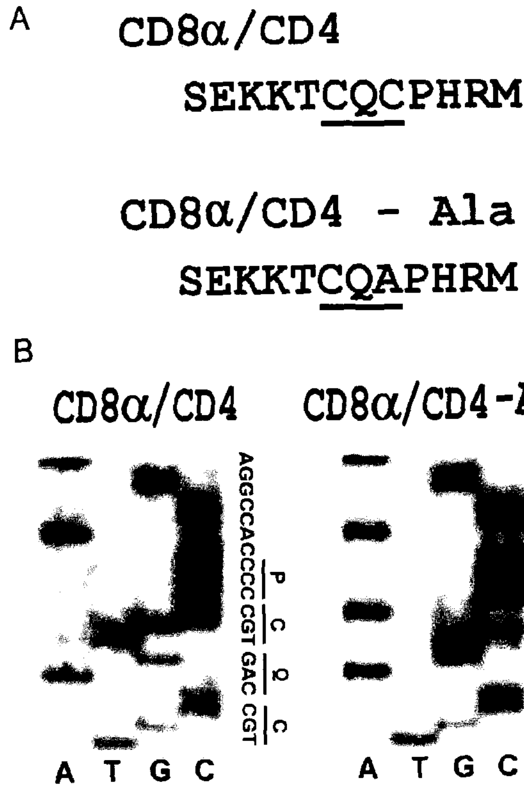

Fig. 1. Sequence comparison between lck binding sites of CD8α/CD4 and those of CD8α/CD4-Ala. A, Cys-X-Cys motif is essential for lck binding. B, DNA seqeuncing of the lck binding sites.

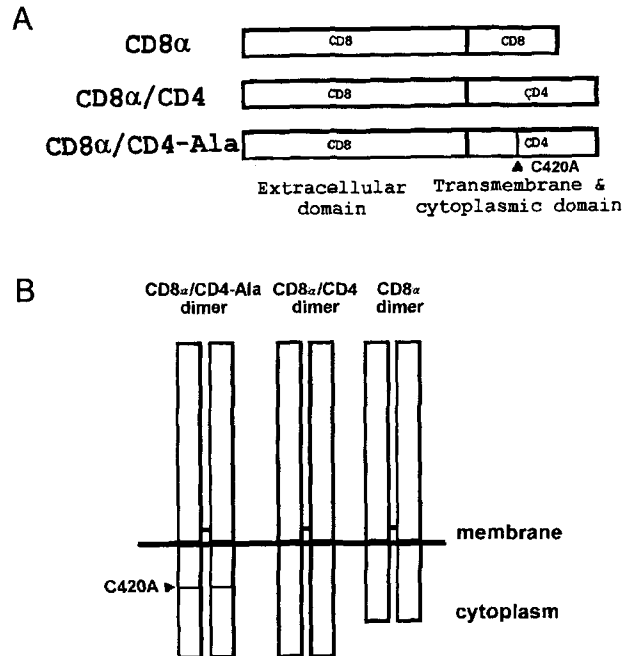

Fig. 2. Three cDNA constructs used for transfection analysis. A, CD8 cDNA expresses native CD8α protein; CD8α/CD4 cDNA encodes a chimeric protein consisting of the CD8α extracellular and CD4 transmembrane and cytoplasmic domain; CD8α/CD4-Ala cDNA encodes a point-mutated chimeric protein in the lck binding region. Arrowhead indicates the position of the mutated lck binding site. B, All three proteins have a cystein residue in the CD8α extracellular domain for dimerization for homodimer formation on the cell surface.

aa) was converted to alanine using synthesized oligonucleotides covering the sequence. Two Cys residues (at 418 and Cys) of CD4 have been shown to be significant in its binding to lck using the zinc ion (Huse et al., 1998; Lin et al., 1998). A mutation in one of the two Cys residues is enough to disrupt the association of CD4 with lck (Turner et al., 1990; Veillette et al., 1990; Huse et al., 1998; Lin et al., 1998). CD8α also has two Cys residues in the cytoplasmic tail, similarly to CD4, however, mutations in both Cys residues were required to disrupt its association with lck (Turner et al., 1990). Thus, we designed to mutate Cys (420) of the CD8α/CD4 hybrid protein to Ala in order to abolish the association between CD8α/CD4 and lck. The point mutation introduced into chimeric cDNA was confirmed by DNA sequencing (Fig. 1).

Expression of CD8α, CD8α/CD4 and CD8α/CD4-Ala

The cDNAs were linked to the human CD2 promoter and also to the CMV promoter. DNA constructs with the human CD2 promoter were made to generate transgenic mice expressing the proteins in a T-cell specific manner. The DNA constructs expressed the proteins in mouse L cells after transfection, however, the expression levels were too low to analyze the differences in the amount of lck association among the proteins. DNA constructs driven by the CMV promoter were also transfected, by calcium phosphate precipitation, into

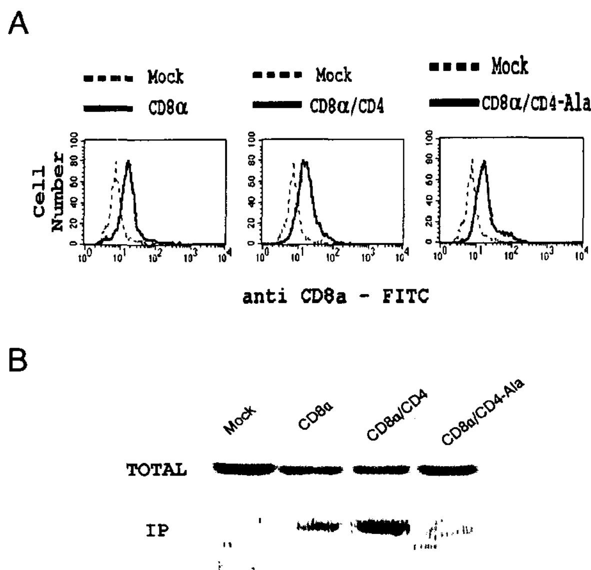

Fig. 3. Surface expression pattern of three proteins and those association with lck. A, Surface expression level of three proteins. Dotted line is for vector transfectant. Solid line is for cDNA transfects. Expression level is shown as a relative fluorescence intensity. All three panels show the difference in fluorescence intensity by about two-fold. B, Western blot analysis of lck. In the upper lane, lck expression was detected in whole cell lysate (100 ug). In lower lane, coimmunoprecipitated lck with anti-CD8α antibody (53.6.72) was detected. CD8α/CD4 binds to lck more efficiently than CD8α by about three-fold. CD8α/CD4-Ala does not bind to lck.

mouse L cells which had been transfected with lck and confirmed for their expression (Fig. 3B). CD8α was generally expressed more efficiently than the other proteins. One possibility for this different efficiency in the surface expression of the proteins may be due to the steric hindrance of large cytoplasmic domain of CD4 during homo-dimerization of each CD8α/CD4 and CD8α/CD4-Ala (Fig. 2). Therefore, to compare the binding activity of CD8α and CD8α/CD4 with lck, we made stable cell lines which express CD8α, CD8α/CD4 and CD8α/CD4-Ala at similar levels. Among 144 selected clones, each clone for CD8α, CD8α/CD4 and CD8α/CD4-Ala expressing similar levels of protein were chosen. Their fluorescence intensities were higher than vector transfected control cells by at least two-fold (Fig. 3A).

Co-immunoprecipitation of lck with CD8α, CD8α/CD4, and CD8α/CD4-Ala

Mouse L cell clone transfected with pBJneo-lck were tested for the expression of lck (Fig. 3B) and further used for the transfection of the CD8α, CD8α/CD4, and CD8α/CD4-Ala expressing vectors. The selected clones showed some variations in the amount of lck proteins when whole cell lysates were analysed by Western blot, however, clones expressing CD8α and the CD8α/CD4 chimeric protein showed similar expression levels of the lck protein (Fig. 3B). Since CD8α, CD8α/CD4 and CD8α/CD4-Ala proteins share the CD8α extracellular domain, the proteins can be detected by and immunoprecipitated with 53.6.72. Immunoprecipitation of the cell lysates with 53.6.7.2 also brought down lck (Fig. 3B). Amount of lck coimmunoprecipitated with CD8α/CD4 was much higher (about 3 fold) than that with CD8α. This result suggests that the CD8α/CD4 protein can bind to lck more efficiently than CD8α by about three fold. It was reported that 50-80% of lck in the CD4 T cell and about 25% of lck in CD8 T cell is associated with each coreceptor and that CD4 binds to lck more efficiently than to CD8 by about 2-3 fold (Veillette et al., 1988). Therefore, the binding affinity of CD8α/CD4 seems to be similar to a native CD4 protein. On the other hand, the mutant CD8α/CD4-Ala protein did not coimmunoprecipitate the lck protein even though the clone expressing the mutant protein showed higher expression of lck than the other clones. Therefore, CD4 transmembrane and cytoplasmic domain of the CD8α/CD4 chimeric protein behave similarly to CD4 in, at least, its lck binding characteristics and perhaps also in the way of delivering a signal inside a cell.

Discussion

Thymocytes mature to express various phenotypic and functional properties during their developmental process. This is usually determined by whether the TCR binds to class I MHC (CD8 cytotoxic T cells) or class II MHC (CD4 helper T cell) (Fung-Leung et al., 1991). One of the most intriguing aspects during this process is whether the divalently associated changes, such as the expression of class I (class II) MHC-restricted TCR and CD8 (CD4), are controlled by any specific signal delivered from the cell surface or whether they are just the result of selective survival of cells that express an appropriate pair of TCR and CD4/CD8 coreceptors (Saito and Watanabe, 1998).

Do developing thymocytes have any mechanism to distinguish their TCR interactions with class I or class II MHC proteins? If they do, what molecule(s) is responsible for it? Since TCR is produced by random gene rearrangements and there is no known difference in TCR complex recognizing class I or class II MHC proteins, it is unlikely that TCR itself delivers any specific signals for the lineage commitment. CD4 and CD8 act as coreceptors and deliver signals necessary for the activation process in mature T cells when they recognize antigens presented on self-MHC proteins. Although coreceptor-independent clones were identified, it is very rare and, therefore, they are exceptional cases (Paterson et al., 1994; Muller and Kyewski, 1995). So, it is possible that the coreceptors also play an important role in the lineage commitment by delivering a necessary signal(s). Antibody blocking experiment and coreceptor knockout mice experiment (Fung-Leung et al., 1991) suggest that the corecptor proteins are necessary for the development of CD4 and CD8 T cells.

Since both CD4 and CD8 are associated with lck and share the signal transduction pathway, it is uncertain whetehr the coreceptors can discern the difference in interaction between class I or II MHC and TCR through lck alone (Basson et al., 1998; Killeen et al., 1998). It has been shown that lck bound to CD4 has much more active kinase activity than that bound to CD8 (Veillette et al, 1988, 1990) and this may be one of the major differences which may decide the cell fate during the maturation step (Ravichandran and Burakoff, 1994). However, it was reported that there was not any special difference in the differentiation of thymocyte in transgenic mice expressing native CD8α or mutant CD8α molecules containing a disrupted lck binding site (Smith et al., 1996). Therefore, it is not obvious if lck is actually working in the lineage commitment.

We have previously reported that transgenic expression of chimeric protein consisted of the CD8α extracellular domain and the CD4 transmembrane and cytoplasmic domain drove thymocytes expressing class I restricted transgenic TCR into CD4 cell lineage (Seong et al., 1992). The results were interpreted in a way that the CD4 transmembrane and cytoplasmic domain delivers a signal that drive cells to differentiate into CD4 lineage even though the cells expressed class I MHC-restricted TCR. If it is the case, lck is likely to play a key role in this process. One way to prove is to see the effects of transgenic expression of CD8α/CD4-Ala, as shown in this report, which does not associate with lck. According to the differential avidity model in which CD4 lineage commitment needs a stronger signal than CD8α lineage, there is a possibility that CD8α/CD4 leads thymocytes expressing class I MHC-restricted TCR to develop into CD4 lineage because the cytoplasmic tail of CD8α/CD4 can produce a stronger signal than CD8α. On the other hand, because CD8α/CD4-Ala cannot produce a proper signal through lck, it may not be able to lead thymocytes expressing class I MHC-restricted TCR into CD4 cell lineage. However, it has not been clearly shown yet whether the CD8α/CD4 chimeric protein is able to associate with lck just like CD4, that is, in a more efficient way than CD8. Our results in this report clearly showed that CD8α/CD4 is associated with lck more efficiently than CD8α and suggest that the cytoplasmic domain of the chimeric protein may act just like CD4 in delivering a signal inside a cell.

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