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Ly-6A is required for T cell receptor expression and protein tyrosine kinase fyn activity

Sang-Kyou Lee, Bing Su, Stephen E.Maher and Alfred L.M.Bothwell

Section of Immunobiology and Department of Biology, Yale University School of Medicine, New Haven, CT 06520-8011, USA

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To characterize the function of the Ly-6A antigen in T cell activation, antisense Ly-6 RNA was expressed in a stably transfected antigen-specific T cell clone. Reduced Ly-6A expression results in inhibition of responses to antigen, anti-TCR (anti-T cell receptor) crosslinking and concanavalin A plus recombinant interleukin 1 and causes impairment of *in vitro* fyn tyrosine kinase activity. More substantial reduction of Ly-6A results in reduction of TCR expression. Analysis of mRNA species indicates that the reduction is specific for the TCR β chain. These data demonstrate that Ly-6A may regulate TCR expression and may be involved in early events of T cell activation via regulation of fyn tyrosine kinase activity.

Key words: antisense transfectants/fyn tyrosine kinase/ Ly-6A/T cell activation/T cell receptor

Introduction

The murine Ly-6A antigen was first detected as an alloantigen on peripheral T cells. The presence of the Ly-6A antigen on cortisone resistant (medullary) thymocytes and absence on cortisone sensitive (cortical) thymocytes suggested that it is an antigen associated with the development of immunocompetence (McKenzie *et al.*, 1977). It is also expressed on $CD4^+$ T cells, lipopolysaccharide (LPS) stimulated splenic B cells, a small subpopulation of bone marrow cells (Spangrude *et al.*, 1988b; Rock *et al.*, 1989) and hematopoietic stem cells (Sca-1) (Spangrude *et al.*, 1988a; van de Rijn *et al.*, 1989).

T cell activation can be induced by crosslinking of various surface molecules including molecules that are anchored to the membrane by a glycosyl phosphatidylinositol (GPI) linkage. Crosslinking of the Ly-6A antigen results in an increase in intracellular calcium and activation of cells in the presence of phorbol myristate acetate (PMA) (Sussman *et al.*, 1988a). The GPI anchor is required for activation because a transmembrane form of Ly-6A antigen cannot mediate T cell activation (Su *et al.*, 1991). Expression of the TCR-CD3 complex is also required for the activation of T cells by this mechanism (Sussman *et al.*, 1988b). Stefanova *et al.* (1991) demonstrated that several GPI anchored cell surface antigens are complexed with the lck tyrosine kinase. The Thy-1 molecule is associated with the fyn tyrosine kinase in a murine T cell hybridoma and murine thymocytes (Thomas and Samelson, 1992). These biochemical studies suggest that a functional association exists between cell surface GPI anchored antigens and protein tyrosine kinases that are considered to be centrally involved in T cell signaling pathways.

A functional role for Ly-6 molecules in TCR-mediated responses was first suggested from studies of mutants of a T cell hybridoma (Yeh *et al.*, 1988). Mutants with reduced levels of Ly-6A exhibited impaired responses to antigen. In other experiments transient inhibition of Ly-6A with antisense oligonucleotides resulted in inhibition of antigen- and mitogen-driven T cell activation without affecting lymphokine responses or responses to PMA plus ionomycin (Flood *et al.*, 1990). While these studies indicated an important role for Ly-6A in T cell signaling they did not provide a suitable system for further studies of its functional role.

We have used a molecular approach to modify the levels of Ly-6A in order to evaluate its role in T cell signaling. Stable transfectants of the D10 T cell clone that have reduced levels of Ly-6A protein as a consequence of antisense Ly-6 RNA have been generated. These transfectants are functionally impaired in their responses to antigen, concanavalin A (ConA) plus recombinant interleukin 1 (rIL-1) and crosslinking with an anti-TCR monoclonal antibody (mAb). Reduced levels of Ly-6A antigen on the cell surface correlate with functional impairment. Transfectants with very low levels of Ly-6A also expressed very low levels of αβ TCR heterodimer as a consequence of reduced TCR β chain mRNA. Surprisingly, the expression of Ly-6A protein on these transfectants is necessary for optimal *in vitro* fyn tyrosine kinase activity irrespective of the expression of the TCR. This suggests that Ly-6A antigen may be involved in TCR-mediated signaling pathway through the fyn tyrosine kinase and a certain level of Ly-6A antigen is required for cell surface expression of the αβ TCR heterodimer.

Results

Generation of stable antisense Ly-6A transfectants of the D10 T cell clone

A plasmid was constructed that expresses Ly-6A antisense RNA and utilized to inhibit Ly-6A protein expression in the murine D10 T cell clone. This $CD4^+$ Th2 T cell clone is specific for conalbumin plus $I\text{-}A^k$ (Kaye and Janeway, 1984; Janeway *et al.*, 1988). The 770 bp *Eco*RI cDNA fragment encoding the entire Ly-6E protein (LeClair *et al.*, 1986) was ligated into the *Xho*I site of the pNeoSRαII vector in the orientation that results in transcription of antisense Ly-6A RNA. The cDNAs encoding Ly-6A and Ly-6E differ at only three nucleotides in the coding region and are considered allelic gene products (Palfree *et al.*, 1987). Expression of the antisense RNA is under the control of the SRα promoter which contains the SV40 promoter and HTLV-I long terminal repeat (LTR) enhancer. The resulting

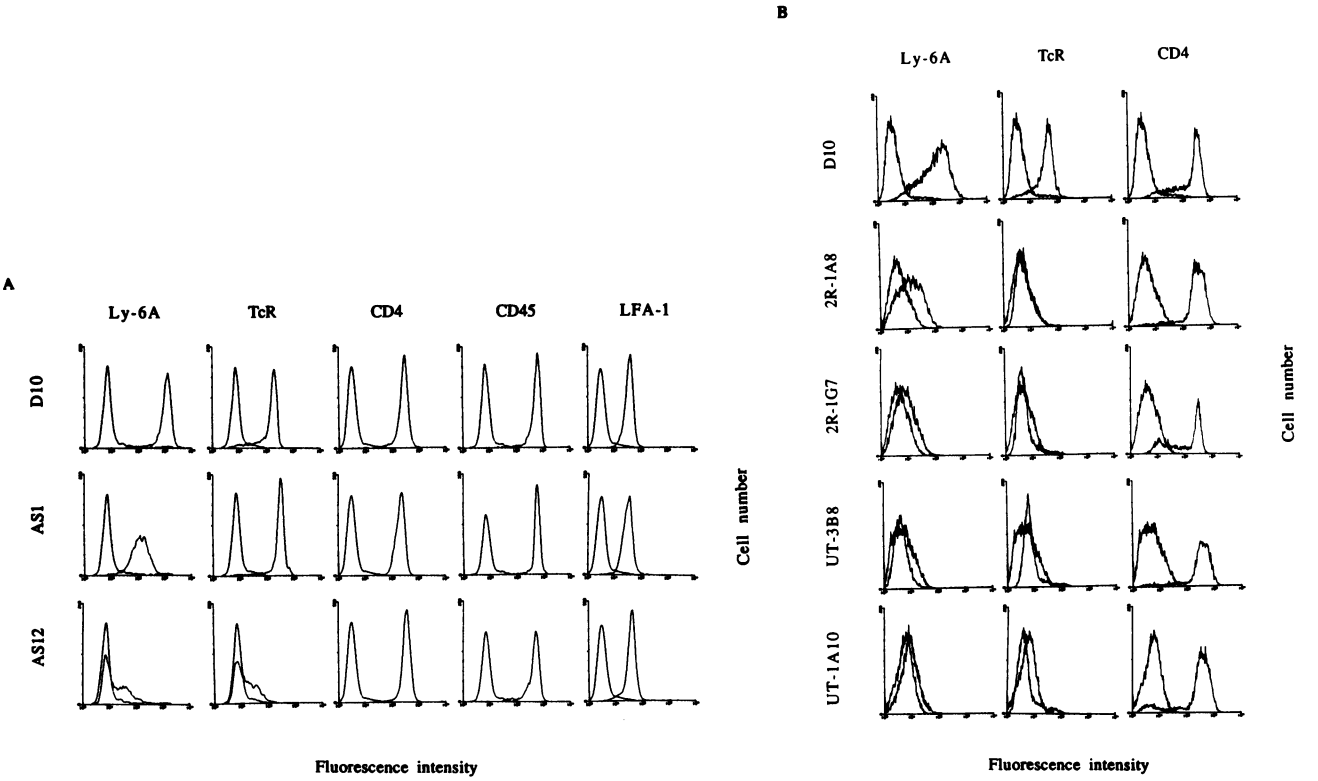

Fig. 1. Flow cytometric analysis of cell surface antigen on D10 and antisense transfectants. (**A**) Surface expression of Ly-6A, TCR heterodimer, CD4, CD45 and LFA-1 surface antigen on wild-type D10 cells and antisense transfectants AS1 and AS12 derived with pNeoSRα2R− construct. (**B**) Expression on antisense transfectants 2R-1A8 and 2R-1G7 (pNeoSRα2R−) and UT-3B8 and UT-1A10 (pNeoSRα3′UT−). Each histogram represents analysis of 10^6 viable T cells stained with mAb 34-11-3 (anti-Ly-6A), 3D3 (anti-TCR), GK1.5 (anti-CD4), TIB122 (anti-CD45) or m17/5.2 (anti-LFA-1) followed by FITC-conjugated anti-mouse or anti-rat IgG. Negative control was obtained by using rabbit anti-mouse or anti-rat FITC antibody alone.

Table I. Summary of transfectants

Transfectants	Construct	No. of transfectants screened	Phenotype
AS1, AS3	pNeoSRα2R−	2/62	Ly-6A^{med}TCR^{high}
AS12, 2R-1A8, 2R-1B11, 2R-1F9, 2R-1G7, 2R-2C2, 2R-2C3, 2R-2D10, 2R-2E11	pNeoSRα2R−	9/62	Ly-6A^{low}TCR^{low}
UT-1A10, UT-1D5, UT-2A9, UT-2H3, UT-3B8, UT-3G3	pNeoSRα3′UT−	6/31	Ly-6A^{low}TCR^{low}
TCR-1B4R, TCR-1D5, TCR-3B5	pPacSRαTCRβ+	3/32	Ly-6A^{low}TCR^{high}
6E-1C10, 6E-3F9	pPacSRαLy6E+	2/31	Ly-6A^{low}TCR^{high}

Total number of transfectants represents the number of transfectants derived from three independent transfections with pNeoSRα2R− or pNeoSRα3′UT− and two independent transfections with pPacSRαTCRβ+ or pPacSRαLy6E+.

plasmid, pNeoSRα2R−, was electroporated into D10 T cells as described by Su *et al.* (1991) and plated on irradiated NRK feeder layers. Stable transfectants were screened by fluorescence activated cell sorting (FACS) for Ly-6A expression using the 34-11-3 mAb.

From three independent transfections of D10 T cells with the pNeoSRα2R− construct, 11 out of 62 transfectants showed a significant reduction of Ly-6A surface expression (see Table I). Of these, nine transfectants express very low levels of Ly-6A, generally <5% of the level found on D10 cells. Two transfectants, AS1 and AS3, express ~10% of the amount of Ly-6A on wild-type D10 T cells or on vector-only (SRα) transfectants (Figure 1).

In order to examine whether this reduction is specific to the Ly-6A antigen, four clones, AS1, AS12, 2R-1A8 and 2R-1G7, were selected and analyzed by flow cytometry for expression of other cell surface antigens. As shown in Figure 1, CD4, CD45 and LFA-1 expression on these transfectants is equivalent to the levels on parental D10 cells. Cells transfected with the pNeoSRαII vector alone showed exactly the same pattern of expression as wild-type D10 cells (data not shown). AS1 cells have ~10% of the level of Ly-6A found on D10 cells and normal levels of TCR. Surprisingly, clones AS12, 2R-1A8 and 2R-1G7 express <5% of TCR heterodimer that is present on wild-type D10 cells. This is true for all transfectants which express <5% of Ly-6A protein present on wild-type D10 cells (see Table I).

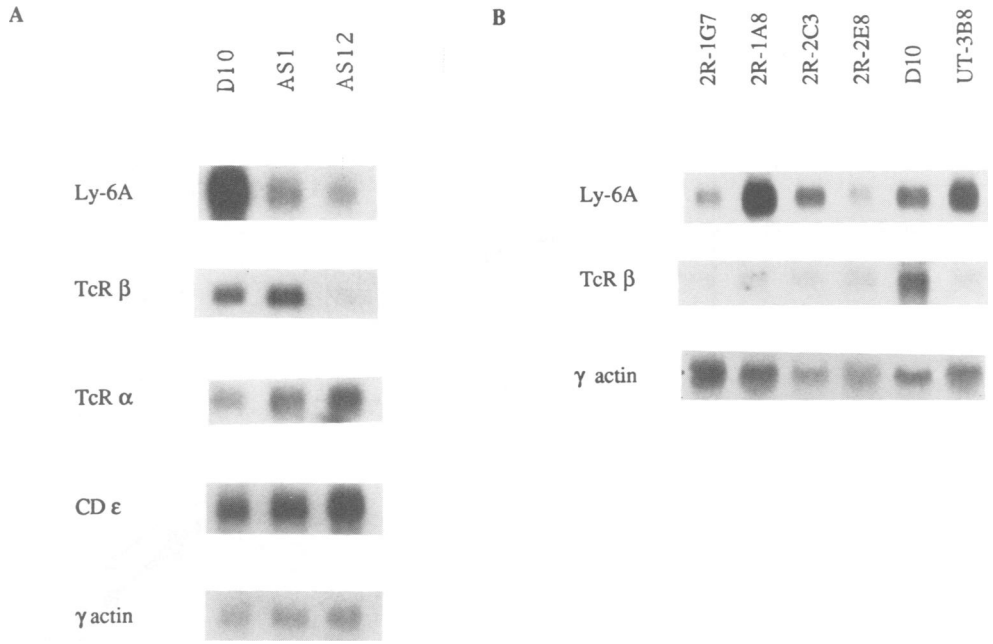

Fig. 2. Northern blot analysis of antisense transfectants. Ten micrograms of total RNA from D10, AS1 and AS12 cells (**A**), from 2R-1G7, 2R-1A8, 2R-2C3, 2R-2E8, D10 and UT-3B8 (**B**) was fractionated on an agarose gel. The filters were hybridized with the indicated probes and the single band of hybridization displayed. The size of the Ly-6A RNA is 1.1 kb, TCR α is 1.4 kb, TCR β is 1.4 kb, CD3 ϵ is 2.0 kb and γ-actin is 2.0 kb.

Antisense RNA of 3′ untranslated region efficiently inhibits Ly-6A expression

Antisense transfectants were also generated with 3′ untranslated (UT) sequences of Ly-6A cDNA. A 272 bp DNA fragment derived from the 3′UT sequences of the Ly-6A cDNA was amplified by PCR and cloned into the pNeoSRαII vector in the antisense orientation. This pNeoSRα3′UT− construct was linearized and electroporated into D10 T cells. Transfectants were grown on NRK feeder layers in the presence of G418 and surface expression of Ly-6A was examined by FACS analysis. Thirty-one transfectants were derived from three independent transfections. Six transfectants showed significant inhibition of Ly-6A expression (see Table I), and two clones, UT-1A10 and UT-3B8, were chosen for further analysis. These two transfectants also had very low levels of TCR which was similar to the phenotypes of transfectants derived from the pNeoSRα2R− construct (Figure 1B).

Absence of TCR β chain mRNA in Ly-6A^{low} transfectants

Northern blot analysis of mRNA expression in wild-type D10 and the Ly-6A antisense transfectants was performed to determine whether the reduced levels of Ly-6A and TCR protein expression observed were due to changes in RNA. In AS1 and AS12 transfectants there was a correlation between the amount of Ly-6A protein on the surface and Ly-6A RNA (Figure 2A). The level of endogenous Ly-6A mRNA in AS1 was significantly lower than that in D10 cells (~10% of the D10 mRNA level) and the AS12 showed the lowest Ly-6A mRNA level (~3% of the D10 mRNA level). The relative levels of RNA were determined by densitometric scanning of autoradiographs. However, the reduction in the level of endogenous Ly-6A mRNA was not observed in some transfectants. As shown in Figure 2B, the levels of endogenous Ly-6A mRNA in some transfectants remained the same as in wild-type D10, although the amount of Ly-6A antigen on the surface was <5% of that in D10 cells by FACS analysis. This result suggests that antisense RNA in some transfectants may inhibit the translation of mRNA without affecting the level of endogenous Ly-6A mRNA.

In all antisense Ly-6A transfectants the levels of the CD3 ϵ chain and TCR α chain mRNA were equivalent to that of wild-type D10 after normalization to γ-actin mRNA levels. Although the level of the TCR β chain mRNA is normal in the AS1 transfectant, surprisingly, all other antisense Ly-6A transfectants with low TCR on the surface showed very little TCR β chain mRNA. One possible explanation for the inhibition of TCR β chain expression is that there is some homology with the Ly-6A sequence and the TCR β chain RNA. Computer analysis of potential DNA sequence homology between Ly-6A and the TCR α, TCR β, CD4, CD45 and CD3 ϵ chain was performed and no significant homology was found. This strongly suggests that the reduction in TCR β mRNA in antisense Ly-6A transfectants is a consequence of reduced levels of the Ly-6A protein and not due to nonspecific inhibition by antisense Ly-6A RNA.

Functional analysis of antisense transfectants

The antisense Ly-6A transfectants were examined to ascertain their functional responsiveness to antigen, ConA + rIL-1, and crosslinking of the TCR with anti-clonotypic mAb. As shown in Figure 3A, the AS1 clone showed low responses to stimulation by antigen and crosslinking with anti-TCR mAb, and an impaired response to ConA + rIL-1 relative to levels of wild-type D10 cells. The AS12, 2R-1A8, UT-1A10 and UT-3B8 clones showed the lowest response to antigen plus I-A^k spleen cells and ConA + rIL-1 (Figure 3A and B). The immobilized clonotypic anti-TCR mAb could not activate these clones. However, all these transfectants had an equivalent response to PMA +

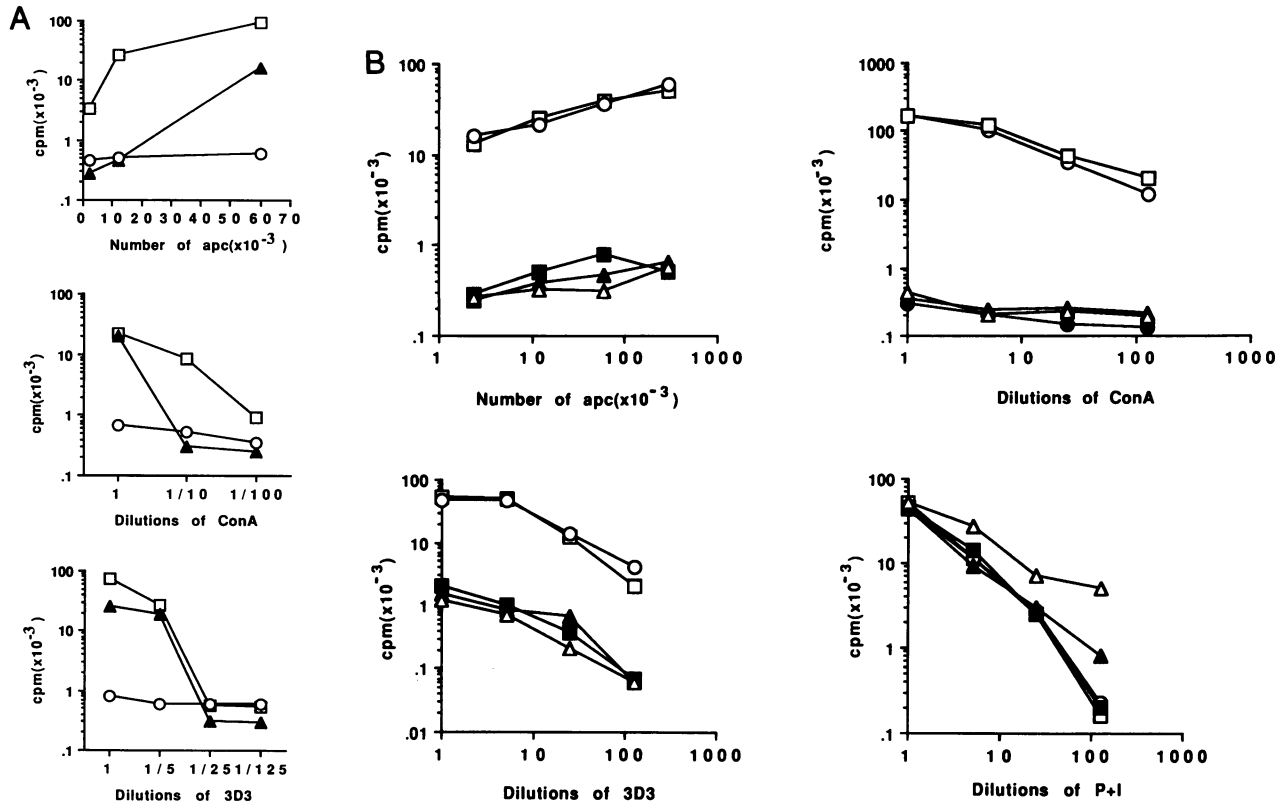

Fig. 3. Functional analysis of antisense transfectants. (**A**) AS-1 (▲), AS12 (○), D10 cells (□) and (**B**) 2R-1A8 (■), UT-1A10 (△), UT-3B8 (▲), D10 cells (○) and vector transfectant SRα (□) were incubated with different dilutions of conalbumin plus irradiated spleen cells from B10.BR, ConA (2.5 μg/ml) plus rIL-1 (3 pg/ml), immobilized 3D3 anti-TCR clonotypic antibody (10 μg/ml), or PMA (5 ng/ml) plus ionomycin (35 ng/ml). Proliferation was measured by [^{3}H]thymidine incorporation for 6 h at the end of 72 h of culture.

ionomycin which can bypass the TCR-mediated signaling pathway. The response of AS3 cells to these stimuli was very similar to that of AS1 (data not shown).

Expression of TCR β chain reconstitutes surface TCR molecules in Ly-6A^{low} TCR^{low} antisense transfectants

To assess the specificity and further characterize the phenotypes of our antisense Ly-6A transfectants, we reconstituted the TCR complex on AS12 transfectants by transfecting the TCR β chain cDNA in a vector that conferred puromycin resistance. A modified pSV2pac vector was used to transfect TCR β chain cDNA (pPacSRα-TCRβ+) into the G418 resistant AS12 transfectant. From two independent transfections 32 stable transfectants were generated and three clones showed significant TCR expression. Two clones, TCR-1B4R and TCR-1D5, were chosen for further analysis. As shown in Figure 4B, clone TCR-1B4R expressed ~70% of the TCR found on D10 cells and the level of TCR expression on clone TCR-1D5 was slightly higher than on D10 cells. CD4, CD45 and LFA-1 were present at normal levels on these TCR reconstitutants (data not shown). Most importantly, the level of Ly-6A on these transfectants remained as low as the parental AS12 transfectant. Thus transfection with the TCR β chain into the AS12 clone restored the expression of TCR heterodimer on the surface without changing the level of Ly-6A antigen.

Expression of Ly-6E can rescue TCR expression in Ly-6A^{low} TCR^{low} 3′UT antisense transfectants

The expression of Ly-6E protein was reconstituted on the 3′UT antisense transfectant, 3B8, by transfecting Ly-6E cDNA lacking the 3′UT region. By transfecting Ly-6E cDNA lacking the 3′UT region into 3′UT antisense transfectants competition between sense and antisense RNA can be avoided. Expression of the transfected Ly-6E antigen can be differentiated from endogenous Ly-6A expression by mAb. Thirty-one puromycin resistant transfectants were generated from two independent transfections. Two Ly-6E reconstitutants, 6R-1C10 and 6R-3F9, were identified and surface expression of various antigens was analyzed (Figure 4A). These transfectants had normal levels of surface CD4, CD45 and LFA-1. Although they expressed a high level of Ly-6E antigen on the cell surface, the level of Ly-6A remained low. The expression of Ly-6E antigen on the 3′UT antisense transfectant 3B8 restored the level of TCR to that of wild-type D10. These results demonstrates that Ly-6A/E antigen is essential for the expression of TCR on the cell surface in D10 cells.

Functional analysis of reconstituted transfectants

As shown in Figure 5, reconstitution of TCR expression by TCR β cDNA transfection on the AS12 antisense Ly-6 transfectant (TCR-1D5 and TCR-1B4R) could partially restore the responsiveness of these cells to antigen, ConA + rIL-1 and TCR crosslinking, but the level of stimulation

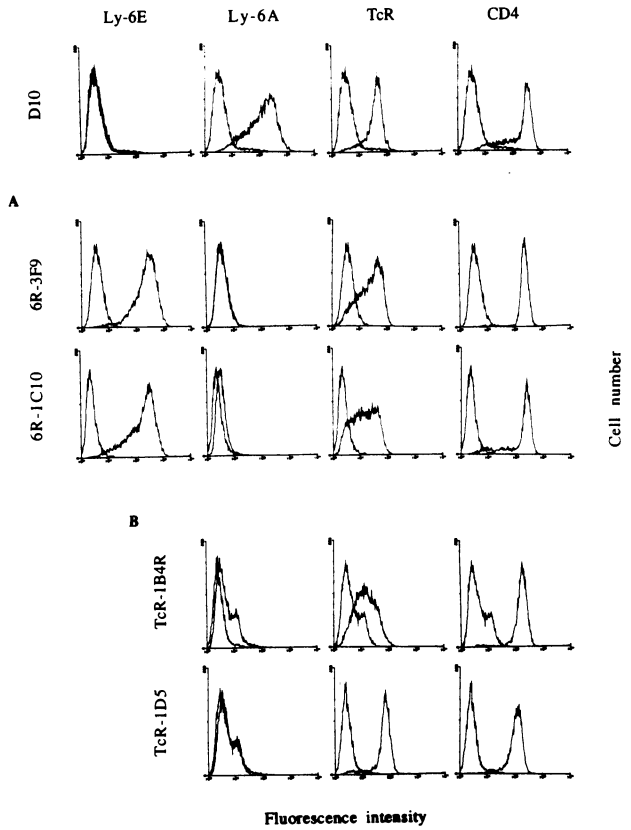

Fig. 4. Flow cytometric analysis of TCR and Ly-6E reconstitutants. Surface expression of Ly-6A, TCR, CD4 on Ly-6E reconstitutants, 6R-1C10 and 6R-3F9 (**A**) and TCR reconstitutants, TCR-1D5 and TCR-1B4R (**B**) was compared with that of D10 cells. Each histogram represents analysis of 10^6 viable cells stained with mAb SK70.94 (anti-Ly-6E), 34-11-3 (anti-Ly-6A), 3D3 (anti-TCR), GK1.5 (anti-CD4), followed by FITC-conjugated rabbit anti-mouse or anti-rat IgG. Negative control was obtained by using rabbit anti-mouse or anti-rat FITC antibody alone.

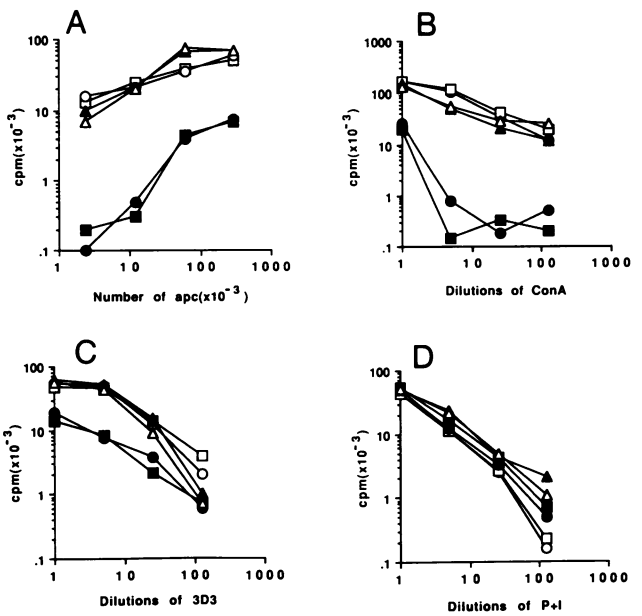

Fig. 5. Functional analysis of TCR or Ly-6E reconstitutants. TCR reconstitutants TCR-1D5 (●), TCR-1B4R (■), Ly-6E reconstitutants 6R-1C10 (△), 6R-3F9 (▲), wild-type D10 (○) and vector transfectant SRα (■) were incubated with different dilutions of (**A**) conalbumin plus irradiated spleen cells from B10.BR, (**B**) ConA plus rIL-1, (**C**) immobilized 3D3 anti-TCR clonotypic antibody and (**D**) PMA plus ionomycin. Proliferation was measured by [^{3}H]thymidine incorporation for 6 h at the end of 72 h of culture.

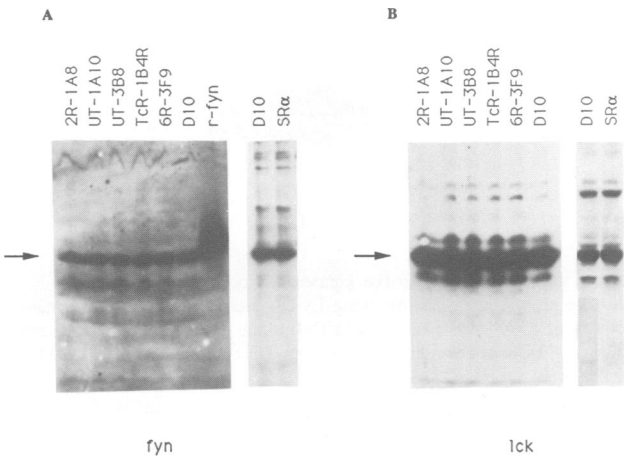

Fig. 6. Western blot analysis and *in vitro* kinase assay of p59^{fyn} and p56^{lck} in antisense Ly-6A transfectants and their reconstitutants. In panel A, the amount of p59^{fyn} and p56^{lck} protein was analyzed in antisense transfectant 2R-1A8, UT-1A10, UT-3B8, TCR reconstitutant TCR-1B4R, Ly-6E reconstitutant 6R-3F9, wild-type D10 and vector transfectant SRα. The amount of fyn protein was compared between D10 and SRα in a separate experiment. In panel A lane r-fyn represents 10 μl of recombinant fyn protein from HeLa cells transfected with fyn cDNA.

by PMA + ionomycin was comparable with that of wild-type D10 cells and vector transfectant SRα. This result indicates that the intracellular signaling pathway is intact, but that signaling through TCR is impaired due to the absence of the Ly-6A antigen. Therefore, Ly-6A antigen is required for TCR-mediated signal transduction in normal D10 cells. The response of Ly-6E reconstitutants (6R-1C10 and 6R-3F9) to antigen, ConA + rIL-1 and crosslinking with anti-TCR mAb is equivalent to that of wild-type D10 and vector transfectant SRα. They can also respond normally to PMA + ionomycin. These data clearly show that Ly-6E antigen can effectively substitute for the function of Ly-6A antigen in D10 T cells.

Functional association of Ly-6A with p59^{fyn} kinase activity

The Ly-6A antigen is capable of inducing the activation of normal T cells in the presence of PMA following crosslinking with anti-Ly-6A. The mechanism by which this occurs is unknown. Two protein tyrosine kinases, p56^{lck} and p59^{fyn}, are implicated as important regulators of early events in T cell activation. p56^{lck} is associated with the cytoplasmic portion of the CD4 and CD8 proteins and crosslinking of CD4 or CD8 molecules can induce *in vitro* kinase activity of p56^{lck} (Shaw *et al.*, 1989; Veillette *et al.*, 1989b; Turner *et al.*, 1990). p59^{fyn} is responsible for the kinase activity associated with the TCR complex (Samelson *et al.*, 1990). Crosslinking of CD3 in human T cells (Tsygankov *et al.*, 1992) or crosslinking of the TCR complex in the murine D10 T cell clone (S.-K.Lee, A.Shaw, S.E.Maher and A.L.M.Bothwell, submitted) increases the tyrosine kinase activity of p59^{fyn}. A recent study has shown association of protein tyrosine kinase fyn with several subcomponents of the CD3 complex including ζ chain (Timson Gauen *et al.*, 1992).

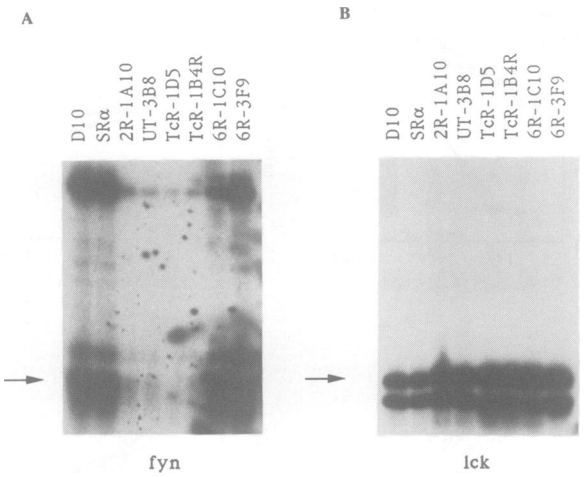

Fig. 7. *In vitro* kinase activity of fyn (**A**) and lck (**B**) in antisense Ly-6A transfectants and their reconstitutants. The fyn and lck *in vitro* kinase activities were analyzed in wild-type D10, vector transfectant SRα, antisense transfectants 2R-1A8, UT-3B8, TCR reconstitutants TCR-1D5, TCR-1B4R, Ly-6E reconstitutants 6R-1C10 and 6R-3F9.

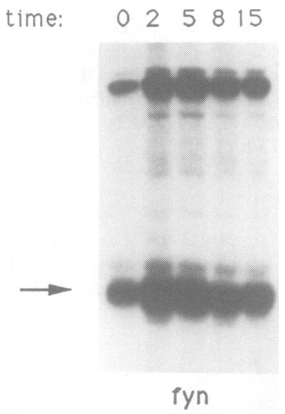

Fig. 8. Increase of *in vitro* fyn kinase activity and induction of tyrosine phosphotyrosine following Ly-6A crosslinking. *In vitro* kinase activity of $p59^{fyn}$ was examined in D10 clone at 0, 2, 5, 8 and 15 min following TCR crosslinking with an anti-TCR clonotypic mAb, 3D3.

In order to examine whether Ly-6A regulates either of these tyrosine kinase activities, the protein level and *in vitro* kinase activity of $p56^{lck}$ and $p59^{fyn}$ were examined in antisense Ly-6A transfectants and their reconstitutants. As shown in Figure 6, the level of $p56^{lck}$ and $p59^{fyn}$ in each transfectant was equivalent to wild-type D10 as judged by Western blot analysis. The size of $p59^{fyn}$ from the HeLa cell transfectants was slightly larger than seen in D10-derived transfectants. This is probably due to post-translational modification of $p59^{fyn}$ in HeLa cells. The amount of these two proteins in vector-only transfectants (SRα) was the same as in D10 cells. These results demonstrated that the level of $p59^{fyn}$ and $p56^{lck}$ protein was not influenced by the expression of antisense Ly-6A RNA.

To characterize the potential influence of reduced Ly-6 expression on early signaling events in T cells, lck and fyn *in vitro* kinase activity was examined. As shown in Figure 7, transfectants which have a low level of Ly-6A antigen on the surface (2R-1A8 and UT-3B8) showed <5% of fyn *in vitro* kinase activity in wild-type D10 cells and the SRα transfectant but normal levels of lck kinase activity.

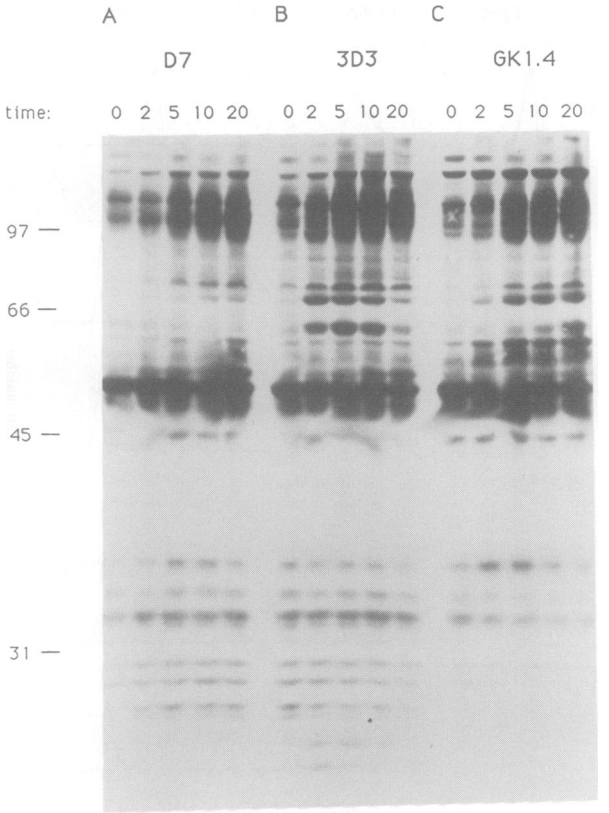

Fig. 9. Tyrosine phosphorylation of cellular substrates. D10 cells were examined at different time points following incubation with anti-Ly-6A (D7), anti-TCR (3D3) or anti-CD4 mAb (GK1.4) and crosslinking with goat anti-mouse or anti-rat Ab.

However, the *in vitro* kinase activity of $p59^{fyn}$ in TCR-reconstitutants (TCR-1D5 and TCR-1B4R) was still as low as that observed in antisense Ly-6A transfectants, 2R-1A8 and UT-3B8. The reconstitution of Ly-6E antigen on the surface of 3′UT antisense Ly-6A transfectants restored the fyn kinase activity to the level found in D10 cells. These results indicate that the majority of $p59^{fyn}$ may be associated directly with surface Ly-6A antigen or indirectly through other unknown intermediate molecules.

Induction of in vitro fyn kinase activity and tyrosine phosphorylation through Ly-6A antigen

Anti-receptor antibodies appear to mimic most, if not all, of the changes induced in response to the physiologic ligand. *In vitro* fyn kinase activity was examined at different time points following crosslinking of Ly-6A antigen on D10 T cells. The anti-Ly-6A mAb, D7, was bound to cells for 30 min at 4°C and crosslinked by goat anti-rat Ab at 37°C for given periods of time. As shown in Figure 8, *in vitro* fyn kinase activity was significantly increased from 2 to 5 min after crosslinking of the Ly-6A antigen. This observation suggests that Ly-6A antigen is functionally associated with fyn protein tyrosine kinase.

It is believed that the two protein tyrosine kinases, $p56^{lck}$ and $p59^{fyn}$, mediate discrete signaling mechanims in T cells (Straus and Weiss, 1992). Activation of lck tyrosine kinase by CD4 or CD8 crosslinking induces tyrosine phosphorylation on a different set of proteins than is observed following TCR stimulation (Veillette *et al.*, 1989a; Luo and Sefton,

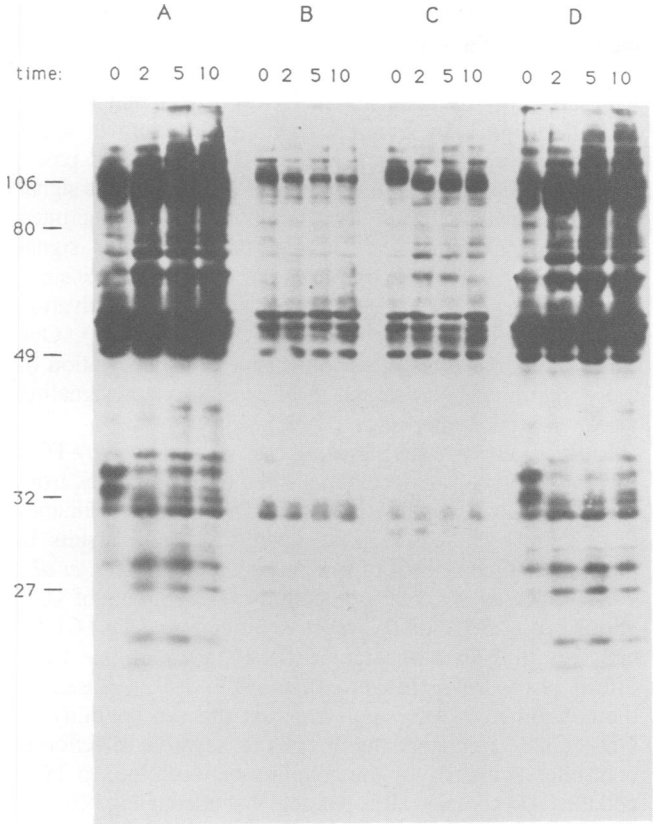

Fig. 10. Defect of tyrosine phosphorylation in antisense transfectants. The level of tyrosine phosphorylation on various cellular substrates was compared among D10 cells (**A**), antisense Ly-6 transfectant UT-3B8 (**B**), TCR reconstitutant TCR-1D5 (**C**) and Ly-6E reconstitutant 3F9 (**D**) at 0, 2, 5 and 10 min after TCR had been crosslinked by anti-TCR clonotypic mAb (3D3) and goat anti-mouse Ab.

1990). To determine whether activation signals through Ly-6A antigen are distinct from those mediated through CD4 or TCR, tyrosine phosphorylation patterns were examined following the crosslinking of Ly-6A, TCR complex and CD4 (Figure 9). Tyrosine phosphorylation of most substrates induced by TCR stimulation were also observed following Ly-6A antigen crosslinking, but was delayed. Induction of tyrosine phosphorylation on several proteins, including 70 and 64 kDa proteins, could not be detected. This finding suggests the possibility that the activation through surface Ly-6A antigen may use the same signaling pathway as activation through the TCR. However, the level of tyrosine phosphorylation induced by TCR crosslinking could not be obtained by Ly-6A crosslinking. A costimulatory signal necessary for complete activation of the T cell by Ly-6A crosslinking may be responsible for the tyrosine phosphorylation of 70 kDa and 64 kDa proteins.

Although CD4 crosslinking can induce tyrosine phosphorylation on most substrates similar to TCR crosslinking, the induction observed was not as rapid as that obtained by TCR stimulation. In addition, three novel substrates were found specific to CD4 crosslinking. First, a 180 kDa protein was dephosphorylated suggesting the possible activation of an unknown phosphatase by CD4 crosslinking. Tyrosine phosphorylation of two proteins, 58 and 54 kDa, were observed only upon crosslinking of CD4. The signaling events through CD4 may share certain signaling steps with those through TCR but there must be a distinct pathway unique to activation through CD4.

Defect of tyrosine phosphorylation in antisense Ly-6A transfectants and their TCR-reconstitutants

The pattern of tyrosine phosphorylation was examined following crosslinking of the TCR complex on wild-type D10 cells, antisense transfectants and reconstitutants. D10 and transfectants were incubated with anti-TCR clonotypic mAb, 3D3, on ice for 30 min followed by crosslinking with goat anti-mouse Ab for different periods of time. As shown in Figure 10A, in wild-type D10 cells phosphotyrosine-containing proteins with mobilities of 125, 105, 95, 70 and 64 kDa were readily apparent 2–5 min following stimulation of D10 cells. Tyrosine phosphorylation of these substrates was not observed in antisense Ly-6 transfectants (Figure 10B). In the TCR-reconstitutants, tyrosine phosphorylation on most of these proteins was not observed. Minor increases in tyrosine phosphorylation on 70 kDa and 64 kDa proteins were observed (Figure 10C). Reconstitution of Ly-6E antigen which restored the normal level of surface TCR can completely rescue the capability of these transfectants to be tyrosine phosphorylated upon TCR engagement (Figure 10D). These results demonstrate that Ly-6A antigen is critical in the tyrosine phosphorylation of all substrates induced by TCR crosslinking. The regulation of tyrosine phosphorylation in TCR-mediated signaling pathway by Ly-6A antigen may be achieved through the modulation of the fyn tyrosine kinase activity.

Discussion

The function of murine Ly-6A antigen in T cell activation was studied in the conalbumin-specific T cell clone D10 by expression of antisense Ly-6A RNA. Reduced expression of Ly-6A antigen on the cell surface correlated with functional impairment. Antisense Ly-6A transfectants were functionally impaired in their response to antigen, ConA plus rIL-1 and crosslinking with anti-TCR mAb. Transfectants with $<5\%$ of Ly-6A antigen on wild-type D10 cells expressed very low levels of the $\alpha\beta$ TCR heterodimer. Analysis of mRNA indicated that the reduction is specific for the TCR β chain. The signaling defect in these transfectants is closely associated with the TCR because these transfectants could be stimulated by PMA + ionomycin which activates cells by bypassing signals via the TCR.

Expression of surface TCR complex on the AS12 clone was reconstituted by transfecting the TCR β chain cDNA. Transfectants which showed increased levels of TCR but low levels of Ly-6A antigen were still impaired in their responses to antigen, ConA + rIL-1 and crosslinking of TCR, but showed normal responses to PMA + ionomycin. Ly-6E reconstitutants expressed high levels of Ly-6E antigen while the level of Ly-6A remained low and equivalent to that on the parental antisense transfectant UT-3B8. The expression of Ly-6E antigen instead of Ly-6A restored the level of TCR to the level of wild-type D10. These Ly-6E reconstitutants responded normally to antigen, ConA + rIL-1 and crosslinking with anti-TCR mAb. These results demonstrate that Ly-6A antigen is critical for the TCR-mediated signaling and a certain level of Ly-6A antigen is required for cell surface expression of the TCR.

The mechanism by which Ly-6A might regulate the levels of TCR β chain is unclear. It is possible that Ly-6A

expression may regulate a pathway that ultimately activates a transcription factor for the TCR β gene that could be in the promoter or the enhancer region. The promoters of Vβ gene segments contain a decanucleotide sequence that is required for optimal TCR β gene expression and is potentially regulatable by AP-1, ATF/CREB or related proteins (Anderson *et al.*, 1989). Alternatively, post-transcriptional regulation that might affect mRNA stability remains a possibility.

Our hypothesis is that Ly-6A/E or Ly-6C expression may play a very critical role in the development of thymocytes from double negative (DN) cells to mature thymocytes. Cells at the DN stage have been found to express IL-2Rα and possess thymic progenitor activity. These cells correspond to ~0.5–2% of total thymocytes. Interestingly, fetal thymic cells at d15 also display a very similar set of cell surface markers and the expression of transcription factors is very similar to that of activated mature T cells (Zuniga-Pflucker *et al.*, 1993). A major difference between the DN cells and activated mature T cells is the absence of TCR expression on the DN cells. Cells having the DN, IL-2Rα^+ phenotype accumulate in RAG2$^{-/-}$ mice. In this mouse sources of contaminating TCR bearing cells are eliminated. Cells having this DN, IL-2α^+ phenotype express significant levels of Ly-6A. However, the vast majority of cortical thymocytes are Ly-6A$^-$ or very low.

In the thymus DN cells progress to a CD4/8^{dull}, CD3-β/β^{low} stage followed by a double positive (DP), CD3-β/β^{low} stage followed by a DP, CD3-33 kDa/β^{low} stage followed by a DP, CD3α/β^{low} stage and finally a CD3^{high} single positive (SP) mature T cell (Groettrup *et al.*, 1993; Petrie *et al.*, 1993). During the transition between DP and SP T cells Ly-6A expression must be dramatically reduced. There must be strong inhibition of Ly-6A expression as the thymocytes begin to express CD4 and CD8. In peripheral T cells the Ly-6A/E antigen is primarily associated with CD4$^+$ T cells and the Ly-6C antigen is associated with CD8$^+$ T cells. The data obtained from our antisense Ly-6A transfectants suggest that the reduced levels of Ly-6A should provide a means to regulate the level of TCR β chain and perhaps other genes in these differentiating T cells. Several recent studies have emphasized the importance of the TCR β chain in regulating T cell development (Palmer *et al.*, 1993).

Analysis of tyrosine kinase activity of antisense Ly-6A transfectants and their reconstitutants revealed that the expression of Ly-6A is necessary for optimal *in vitro* fyn tyrosine kinase activity. These molecular genetic approaches substantiate the findings of others using biochemical approaches that there is an important interaction between GPI anchored cell surface proteins and src family tyrosine kinases. AS1 and AS3 transfectants (Ly-6A^{low} TCR^{high}) showed low fyn kinase activity. The AS12 transfectant demonstrated reduced *in vitro* fyn kinase activity even after reconstitution with the TCR β chain. Thus, the association of Ly-6A antigen and fyn tyrosine kinase activity is independent of the expression of TCR. The *in vitro* tyrosine kinase activity of lck in these transfectants is equivalent to that found in the wild-type D10 clone.

Functional association between Ly-6A and *in vitro* fyn kinase activity suggests that involvement of Ly-6A in TCR-mediated activation pathway might be accomplished via the activation of fyn tyrosine kinase. This possibility is strongly supported by the observation that fyn kinase activity is increased within 2 min following crosslinking of Ly-6A antigen in D10 cells. How does cytoplasmic p59^{fyn} associate with surface Ly-6A antigen? The Ly-6A antigen may mediate activation signals by interacting with other unknown signal transducing transmembrane proteins (Brown, 1993). p59^{fyn} may be associated with the cytoplasmic portion of this signal transducer or upon T cell activation p59^{fyn} may be recruited by this signal transducer. It is possible that this signal transducer might be a component of TCR complex since TCR-CD3 expression is required for T cell activation through the Ly-6 antigen (Sussman *et al.*, 1988b). One candidate is the ζ chain since tyrosine phosphorylation of the ζ chain has been shown to be necessary for signaling via Ly-6A (Wegener *et al.*, 1992).

Little is known of the signaling cascades initiated by TCR ligation in immature T cells but there are differences from mature T cells. Crosslinking of the TCR complex with anti-CD3 ϵ mAb increases intracellular Ca^{2+} and results in programmed cell death of immature T cells (Owen *et al.*, 1988; Finkel *et al.*, 1989). In contrast, crosslinking of TCR eliminates ~50% of the reactive immature CD4$^+$CD8$^+$ cells resulting in a population that cannot mobilize Ca^{2+} effectively *in vitro* (Finkel *et al.*, 1989, 1992). Based on these findings it was suggested that the susceptibility of CD4$^+$CD8$^+$ immature thymocytes to negative selection is controlled primarily by the coupling between the $\alpha\beta$ TCR and the CD3 complex. It is possible that uncoupling between the $\alpha\beta$ TCR and CD3 complex in cortical thymocytes might be due to the lack of Ly-6A antigen rendering these cells resistant to the cell death. The ligation of $\alpha\beta$ TCR coupled with CD3 complex in medullary thymocytes which express Ly-6A antigen might induce the Ca^{2+} mobilization and programmed cell death.

Tyrosine phosphorylation of multiple cellular substrates can be observed within minutes of TCR engagement (Samelson *et al.*, 1986; June *et al.*, 1990). In wild-type D10 cells, several substrates with molecular weights of 125, 105, 95, 70 and 64 kDa were tyrosine phosphorylated at 2 or 5 min after TCR crosslinking. Tyrosine phosphorylation of these substrates upon TCR crosslinking was significantly impaired in antisense Ly-6A transfectants which have low levels of Ly-6A antigen and TCR heterodimer. The impairment of tyrosine phosphorylation in these transfectants was not overcome by the rescue of TCR expression by transfection. Only complete reconstitution of TCR and Ly-6E molecules on the surface of antisense Ly-6 transfectants restored tyrosine phosphorylation of proteins following TCR stimulation by anti-TCR mAb. Failure to induce tyrosine phosphorylation by TCR crosslinking in antisense Ly-6A transfectants and TCR reconstitutants shows that Ly-6A antigen is essential for the induction of tyrosine phosphorylation through TCR. Functional association of Ly-6A antigen with fyn kinase activity indicates that tyrosine kinase fyn is the key regulator for tyrosine phosphorylation. This is consistent with our recent studies of D10 antisense fyn transfectants that have impaired fyn expression (S.-K.Lee, A.Shaw, S.E.Maher and A.L.M.Bothwell, submitted). Reduced levels of fyn result in reduced *in vitro* lck kinase activity but normal levels of lck protein are observed. Thus, the signal transduction pathway would first involve engagement of the TCR and Ly-6A followed by fyn and finally lck.

The pattern of tyrosine phosphorylation induced by Ly-6A crosslinking was very similar to that induced by TCR crosslinking. However, the kinetics of tyrosine phosphorylation were not as rapid as those observed after TCR crosslinking, and several proteins, including 70 and 64 kDa proteins, were not tyrosine phosphorylated. The similarity of the tyrosine phosphorylation patterns between crosslinking of either Ly-6A antigen or TCR in D10 cells suggests that the activation through Ly-6A antigen may share the same signaling pathway as activation through the TCR. Crosslinking of Ly-6A antigen itself may not be enough to induce a maximal level of tyrosine phosphorylation. The delayed kinetics and lack of tyrosine phosphorylation on several substrates may be due to the absence of costimulatory signal for activation of T lymphocytes by Ly-6A crosslinking. Crosslinking of CD4 induced tyrosine phosphorylation on several distinctive proteins in addition to most phosphotyrosine substrates observed after TCR crosslinking. The antisense transfectants we have created provide valuable tools to elucidate the function of Ly-6A/E antigen during T cell activation and T cell development.

Materials and methods

Cell culture

D10 cells were maintained in Click's EHAA (Irvine Scientific) medium containing 5% fetal calf serum (FCS) and 60 μM 2-mercaptoethanol. Initially, they were maintained in 5% Polyclone (Collaborative Research, Inc.). We now use supernatant (diluted 1:500) from the cell lines produced by Karasuyama and Melchers (1988) as a source of IL-2 and IL-4. D10 cells are regularly stimulated by conalbumin (100 μg/ml; Sigma Chemical Co.) and feeder cells (5×10^5 cells/ml) every 3 weeks.

Antibodies and reagents

The ascites of 34-11-3 (anti-Ly-6A) (Auchincloss *et al.*, 1981), HK1.4 (anti-Ly-6C) (Lancki *et al.*, 1984) and SK70.94 (anti-Ly-6E) (Kimura *et al.*, 1984) were prepared in nude mice. 3D3 (anti-clonotypic mAb for D10 TCR), GK1.5 (anti-CD4), m17/5.2 (anti-LFA-1) and RM2-4 (anti-CD2) were kindly provided by Dr C.Janeway (Yale University, New Haven). TIB122 (anti-CD45Ro) was a gift from Dr K.Bottomly (Yale University, New Haven). D7 (anti-Ly-6A/E) producing cells were kindly provided by Dr E.Shevach (NIH, Bethesda). Rabbit polyclonal antibodies specific to $p56^{lck}$ and $p59^{fyn}$ protein were kindly provided by Dr J.Bolen. 4G10 (anti-phosphotyrosine) and FITC-conjugated rabbit anti-mouse or anti-rat antibody were purchased from UBI (Lake Placid, NY) and Zymed (San Francisco, CA), respectively. Recombinant mouse IL-1α and ionomycin were purchased from Genzyme (Boston, MA) and Calbiochem, respectively. Concanavalin A and PMA were purchased from Sigma (St Louis, MO).

Plasmid constructs

The construction of the 3'UT plasmid was achieved by a PCR amplification of a 3' 272 bp DNA fragment. The 5' primer starts 5 bp downstream of the translation termination codon and the 3' primer starts at the *Eco*RI site located 95 bp upstream of the polyadenylation site. The primers are as follows: 5' primer: 5'-AACCGTCGACGAATTCCTCCCAATGACCTCC-ACC-3'; 3' primer: 5'-GGAACCCTCGAGGAATTCTGCCTCTTCAC-TGTG-3'. The primers contain *Sal*I and *Xho*I sites that were used to insert the PCR product into the *Xho*I site in the pNeoSRαII vector.

The pSV2pac vector (Vara *et al.*, 1986) was modified to create convenient restriction sites for cloning. A 600 bp *Sca*I–*Eco*RI fragment containing the SP72 polylinker was excised from pSP72 plasmid (Promega) and cloned into the unique *Sca*I and *Eco*RI sites of pSV2pac, resulting in pSV2pac72. This vector was used for the transfection of the AS12 clone and the 3'UT antisense transfectant 3B8.

For the TCR β chain reconstitution, a 2.6 kb *Sal*I fragment containing the D10 TCR β gene was used. Expression of the TCR β gene was driven by the SV40 promoter and HTLV-I enhancer. The combination of the SV40 promoter and HTLV-I enhancer was constructed in the pcDL-SRα296 plasmid described by Takebe *et al.* (1988). Here we use the name SRα when referring to this promoter. The insert was prepared from pSRαTCRβ (kindly provided by Dr Soon-Cheol Hong and Dr C.Janeway). This DNA fragment was cloned into the *Xho*I site of pSV2Pac72 vector by cohesive end ligation resulting in the DNA construct pPacSRαTCRβ+.

A 418 bp DNA fragment containing the coding sequence of the Ly-6E cDNA with convenient *Eco*RI restriction sites was amplified from the Ly-6E cDNA plasmid by PCR. The 5' primer is homologous to the Ly-6E beginning 10 bp 5' to the ATG translational start codon and the 3' primer starts 2 bp downstream of the translation termination codon. The primers for this reactions were as follows: 5' primer: 5'-GGAACCGTCGACGAATTCT-CTGAGGATGGACACTTC-3'; 3' primer: 5'-ACCCTCGAGGAATTC-CATCAGAGCAAGGTCTGC-3'. After digestion with *Eco*RI, the PCR fragment was cloned into the *Eco*RI site of pSRα to produce pSRαLy-6E+. A 2.0 kb *Sal*I fragment containing the SRα promoter and Ly-6E fragment was ligated into the *Xho*I site of pSV2pac72 resulting in pPacSRαLy-6E+.

PCR analysis

For each PCR, 1 ng of purified pSRαLy-6+ DNA digested with *Eco*RI was used as a template. Primers were synthesized by oligonucleotide synthesizer (Applied Biosynthesis) or ordered from the Department of Pathology, Yale University. PCRs were performed as recommended by USBC (Cleveland, OH). DNA template was mixed with 10 μl of 10× PCR buffer [100 mM Tris–HCl pH 8.3 (at 25°C), 500 mM KCl, 15 mM $MgCl_2$, 0.1% (w/v) gelatin], 200 mM of each dNTP, 0.2–1.0 μM of each primer and 2.5 units of ampli-Taq DNA polymerase (USBC). The volume was raised to 100 μl with distilled water and 100 μl of mineral oil was layered on top. The tubes were placed in thermal cycler (Coy Corporation, Ann Arbor, MI) for 40 cycles at 94°C for 1 min, 50°C for 1 min, and 72°C for 3 min. PCR products were analyzed on 1% agarose gels.

Transfection

D10 cells were transfected by electroporation as previously described (Su *et al.*, 1991). The plasmid DNA was linearized with *Sca*I and 25–50 μg of DNA was used to transfect 10^7 cells. Two days after electroporation the cells were plated in 96-well plates containing irradiated normal rat kidney (NRK) cells with regular growth medium containing 2.5 μg/ml ConA (Sigma Chemical Co.), 3 pg/ml rIL-1 (Genzyme) and 0.8 mg/ml G-418 (Gibco). NRK cells transfected with the *neo* gene (kindly provided by Dr P.Cresswell) were maintained in Bruff's medium with 10% FCS. When the cells were 80% confluent, they were trypsinized, plated into a flat-bottomed 96-well plate at a density of 5×10^2 cells/well and irradiated with 5280 rad using a cesium gamma irradiator. Transfectants were maintained in Click's medium containing 5% FCS and lymphokines.

Flow cytometric analysis

Viable cells (10^6) were washed and resuspended in 100 μl of PBS containing 5% FCS and 0.1% sodium azide and the expression of surface antigens was analyzed by immunofluorescence as described previously (Su *et al.*, 1991). Monoclonal antibodies used were 34-11-3 (anti-Ly-6A.2), 3D3 (anti-clonotypic mAb for D10 TCR), GK1.5 (anti-CD4), TIB122 (anti-CD45Ro), RM2-4 (anti-CD2) and m17/5.2 (anti-LFA-1). Binding was detected with fluorescein isothiocyanate (FITC)-conjugated rabbit anti-mouse or anti-rat secondary antibody.

Northern blot analysis

Total RNA was prepared and Northern blot analysis was performed as previously described (LeClair *et al.*, 1989). RNA samples were loaded on a single gel and filters were stripped and rehybridized with the specified probes. Ly-6 probe was 750 bp *Eco*RI cDNA fragment which encodes the entire Ly-6E protein (LeClair *et al.*, 1986). TCR α and β cDNA probes were 1.2 kb *Eco*RI fragment and 800 bp *Eco*RI–*Bgl*II fragment (Slanetz and Bothwell, 1991), respectively. For the CD3 ϵ chain probe, a 1.4 kb *Eco*RI fragment from pDL1 plasmid (Clevers *et al.*, 1988) was used. DNA fragments were labeled with [^{32}P]dCTP and [^{32}P]dATP (3000 Ci/mmol, Amersham) by random hexamer priming (Boehringer-Mannheim).

Crosslinking of surface antigen

Cells were washed with PBS and incubated with primary antibodies on ice for 30 min (100 μg/ml of 3D3, 1:10 dilution of D7 culture supernatant or 1:100 dilution of GK1.5 ascites). After incubation, cells were washed once with PBS and crosslinked with goat anti-mouse or rat Ab (30 μg/ml) for the indicated time period at 37°C in a CO_2 incubator. Following crosslinking cells were washed once with PBS at 4°C and lysed with KLB (see below) on ice for 30 min.

Functional assays

Wild-type D10 or antisense transfectants (3×10^4 cells/well) were dispensed into 96-well flat-bottomed tissue culture plates. Different dilutions

of antigen plus irradiated B10.BR spleen cells (3×10^5 cells/well), ConA (2.5 μg/ml) + rIL-1 (3 pg/ml), or PMA (5 ng/ml) + ionomycin (35 ng/ml) were added to each well as indicated. 3D3 mAb (10 μg/ml) was immobilized on the plate by overnight incubation at 37°C in a CO_2 incubator. Goat anti-rat Ab (30 μg/ml) was used to crosslink D7 mAb (culture supernatant from D7-producing cell line). The cells were cultured at 37°C for 72 h and then pulsed with [^{3}H]thymidine for an additional 6 h and harvested using an LKB Wallac 1295-001 cell harvester (LKB Instruments). The radioactivity incorporated was measured by scintillation counting. All data shown are the means of triplicate determinations.

Immunoblot analysis

Whole cell lysates from 5×10^6 cells were prepared by solubilization in KLB buffer [1% Triton, 150 mM NaCl, 20 mM Tris–Cl (pH 8.0), 100 mM Na_2VO_4, 25 mM NaF, containing protease inhibitor, 1 μg/ml each aprotinin and leupeptin] for 15 min on ice. Insoluble material was removed by centrifugation for 5 min at 12 000 *g*. Clarified lysates were boiled in sample buffer, fractionated by 10% SDS–PAGE, and transferred to nitrocellulose in Western transferring buffer (20 mM Tris, 150 mM glycine, pH 8) at 40 V overnight. The amount of protein in each lane was normalized using the Bradford assay (Bio-Rad). Transferred proteins were visualized by staining in Ponceau S solution (Sigma) for 10 min followed by destaining the filter with 5% acetic acid. The filter was incubated in blocking buffer, containing 3% BSA, then incubated with appropriate dilutions of respective antisera. Following incubation for 1 h at room temperature, the filters were rinsed in blocking buffer containing 0.05% Tween 20 and developed using anti-rabbit or anti-mouse secondary Ab conjugated to horseradish peroxidase (Hyclone) and ECL (Amersham).

Immune complex kinase assay

Cells were lysed in KLB (5×10^6 cells/ml) as described above. Cell lysates were precleared with Pansorbin (5 μl) (Calbiochem) for 30 min at 4°C followed by second preclearing with protein A–Sepharose for 30 min at 4°C. The tyrosine kinase proteins were immunoprecipitated with antisera specific for fyn (1 μl of antiserum) or lck (3 μl of antiserum) by rotating for 1 h at 4°C (Veillette *et al.*, 1988). After collection of the immune complexes with protein A–Sepharose (Sigma), they were extensively washed three times in KLB. Immune complex kinase assays were performed by adding kinase reaction buffer (25 mM HEPES pH 7.3, 100 μM Na_2VO_4, 3 mM $MnCl_2$, 3 mM $MgCl_2$) containing 10 μCi [γ-^{32}P]ATP (sp. act. 6000 Ci/mmol). Kinase reactions were then conducted for 90 s at room temperature and terminated by the addition of boiling sample buffer. The amount of protein in each sample was normalized as described above and the samples were analyzed on 10% SDS–polyacrylamide gel and radioactive bands were detected by autoradiography.

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