The EMBO Journal vol.10 no.5 pp.1201 - 1207, 1991

Association of CD8 with p56^{lck} is required for early T cell signalling events

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Communicated by R.A.Flavell

The human CD8 glycoprotein functions as a co-receptor during T cell activation by both binding to MHC class I and transducing a transmembrane signal. The ability of CD8 to transduce a signal is mediated in part by its association with the protein tyrosine kinase p56^{lck}. Using a panel of human CD8α mutants, we demonstrated that the presence of a functional p56^{lck} binding site is required for the early signalling events transduced by CD8, including increased $[Ca^{2+}]_i$ and protein tyrosine phosphorylation. In addition, our results demonstrate that wild-type and all mutant forms of CD8α have an inhibitory effect on signal transduction after CD3–CD3 or CD3–CD4 crosslinking when transfected into the ($CD3^+$, $CD4^+$, $CD8^-$) H9 T cell line, suggesting that intermolecular associations of CD8, independent of its association with p56^{lck}, are responsible for this effect. Signalling through CD4 or CD8 in a double positive thymocyte may therefore be different than in a single positive thymocyte or mature T cell.

Key words: CD8/p56^{lck}/PI pathway/T cell activation

Introduction

The T cell surface glycoproteins CD8 and CD4 play an important role in T cell development and activation. Several studies have demonstrated that these molecules may be capable of physically associating with the T cell receptor/CD3 (TcR/CD3) complex (Hoxie *et al.*, 1986; Minami *et al.*, 1987; Saizawa *et al.*, 1987; Takada and Engleman, 1987), suggesting that they may function as co-receptors during T cell activation. This association is due, at least in part, to the adhesion of CD8 and CD4 to MHC class I and II monomorphic domains respectively (Doyle and Strominger, 1987; Norment *et al.*, 1988). The co-receptor function of CD4 and CD8 is important in the regulation of signal transduction via the antigen-specific T cell receptor complex. Upon activation after antigen presentation or crosslinking the TcR with anti-CD3 antibodies, induction of tyrosine kinase activity occurs rapidly and may be required to regulate subsequent signals mediated by phosphoinositide hydrolysis, intracellular calcium flux ($[Ca^{2+}]_i$) and protein kinase C (PKC) translocation (Ledbetter *et al.*, 1990; June *et al.*, 1990a,b). Neither the molecules comprising the TcR/CD3 complex nor CD4 or CD8 contain intrinsic protein tyrosine kinase (PTK) activity. However, both CD8 and CD4 associate with a member of the src family of protein tyrosine kinases, p56^{lck} (Barber *et al.*, 1989; Rudd *et al.*, 1988; Veillette *et al.*, 1988) which is found predominantly in T lymphocytes (Marth *et al.*, 1985; Voronova and Sefton, 1986). Recently another member of the src family, *fyn*, was reported to be associated with the CD3 complex (Samelson *et al.*, 1990).

While the human CD4 molecule is expressed on the cell surface as a monomer, the human CD8 molecule is expressed on the surface as a dimer consisting of 32–34 kd monomers. CD8 also exists in higher mol. wt complexes. There are two forms of the dimer, either CD8α/α homomultimers or CD8α/β heterodimers (Terry *et al.*, 1989). Both the α and β chains are similar in size and are encoded by two closely linked genes (Gorman *et al.*, 1988). T cells expressing the α/β form of the TcR may express both CD8α/α and α/β on the same cell, although the co-expression of the two forms varies between individuals (Terry *et al.*, 1990). $CD8^+$ T cells expressing the γ/δ form of the TcR and $CD8^+$ natural killer cells express only the CD8α/α homodimer (Terry *et al.*, 1990; Baume *et al.*, 1990).

Upon interaction with the TcR, the association of CD8 and CD4 with p56^{lck} may regulate subsequent protein tyrosine phosphorylation. The formation of complexes between the TcR/CD3 and CD4 or CD8 may allow CD8/p56^{lck} or CD4/p56^{lck} to contact the proper substrates, beginning the signal transduction cascade. Phosphorylation of the CD3 zeta chain during T cell activation is one consequence of this interaction (Veillette *et al.*, 1989).

In addition to the stimulation of protein tyrosine kinase activity, the interaction of CD3 with CD4 or CD8 induced by CD3–CD8 or CD3–CD4 crosslinking, using mAbs conjugated to biotin and crosslinked with avidin on T cell lines, results in a >6-fold enhancement of the increase in $[Ca^{2+}]_i$ compared with that seen after crosslinking CD3, CD4 or CD8 alone (Ledbetter *et al.*, 1989). Such crosslinking mimics, to a certain extent, the interactions which occur between the TcR/CD3 complex and its co-receptors during the binding of these molecules to MHC class I or II and antigenic peptide. In both cases, the signals transduced result in increased $[Ca^{2+}]_i$ and protein tyrosine phosphorylation.

The cytoplasmic regions of CD8α and CD4 play a crucial role in their signal transducing functions. First, the cytoplasmic regions of human CD8α (28 amino acids) and CD4 (38 amino acids) both contain a short sequence motif containing two critical cysteine residues that mediates their interaction with the amino-terminal domain of p56^{lck} (Barber *et al.*, 1989; Turner *et al.*, 1990; Shaw *et al.*, 1990). The cytoplasmic domain of human CD8β does not contain this sequence and cannot bind p56^{lck} (Shaw *et al.*, 1990; Zamoyska *et al.*, 1989). Second, phosphorylation of the CD4 and CD8α cytoplasmic tails on serines regulates their expression upon activation. Following T cell triggering with either PMA, antigen presenting cells, anti-CD3 antibodies or a combination of anti-CD2 antibodies that recognize

different epitopes on CD2, some or all of the serine residues in the cytoplasmic tails of CD4 and CD8α are phosphorylated (Acres *et al.*, 1986, 1987; Blue *et al.*, 1987). Subsequently, both CD4 and the CD8α/β heterodimer are internalized, while the CD8α/α homodimer is not (Terry *et al.*, 1990). The CD8α cytoplasmic region is divided into two domains, C1 and C2, encoded by exons 5 and 6 respectively (Nakayama *et al.*, 1989). Interestingly, in the human gene, the $p56^{lck}$ binding site is found in the C1 domain, while the serine residues are found in the C2 domain.

Letourneur *et al.* (1990) and Zamoyska *et al.* (1989) showed that CD8α molecules which lack the cytoplasmic domain are deficient in antigen-induced IL-2 production. The murine CD8α occurs naturally in both full-length and 'tailless' forms due to alternate splicing (Zamoyska *et al.*, 1985). Letourneur *et al.* (1990) demonstrated that a murine T cell hybridoma transfected with TcR α and β genes from a murine $CD8^+$ cytotoxic clone specific for the MHC class I molecule $H\text{-}2K^b$ and murine CD8α (Lyt-2) lacking the cytoplasmic domain responded well to K^b splenocytes but not to K^b transfected L cells. However, when the hybridoma was transfected with the full length form of Lyt-2, the transfectants responded well to both. Using a similar gene transfer system, Zamoyska *et al.* (1989) demonstrated that cells expressing full-length CD8α molecules responded much better to antigen in the form of K^b-transfected L cells than did cells expressing the naturally occurring tailless murine CD8α molecules. Similarly, transfectants expressing a deletion mutant of the human CD4 molecule lacking a cytoplasmic tail were as efficient as those expressing the wild-type molecule at enhancing the response to antigen presenting cells, but were less efficient when challenged with antigen incorporated into liposomes (Sleckman *et al.*, 1988).The inability of truncated forms of CD4 or CD8α to induce cellular responses as well as their full-length counterparts is due at least in part to their inability to bind to $p56^{lck}$. However, since nearly the entire cytoplasmic domain is deleted in these tailless forms, it is not possible to differentiate between the importance of $p56^{lck}$ binding and the roles of other parts of the cytoplasmic domains.

To determine the role of the $p56^{lck}$ binding site, contained in the C1 domain of the CD8α cytoplasmic tail, we studied the early cell signalling events, protein tyrosine phosphorylation and intracellular calcium flux using human CD8α mutants expressed on the cell surface of the human ($CD4^+$, $CD3^+$, $CD8^-$) H9 T cell line. One mutant had a full-length cytoplasmic tail but the amino acids important for $p56^{lck}$ binding were altered, a second mutant lacked a tail and a third had a shorter tail which retained the $p56^{lck}$ binding site.

Results

CD8α mutants and transfectants

We constructed three CD8α mutants to analyze the specific role of the $p56^{lck}$ binding site and the C2 domain of the CD8α cytoplasmic tail (Figure 1). The CD8.2 mutant lacks the cytoplasmic domain, retaining only two cytoplasmic amino acids. The Δlck mutant retains the cytoplasmic domain, but is unable to bind $p56^{lck}$ because the two cysteines required for $p56^{lck}$ binding have been mutated to alanines. The CD8.12 mutant retains the first 12 amino acids in the cytoplasmic domain, which are encoded by exon 5 and comprise the C1 domain. It retains the $p56^{lck}$ binding site, but lacks the 16 amino acids encoded by exon 6, which comprise the C2 domain. The CD8.12 mutant is able to bind to and co-precipitate $p56^{lck}$ (Shaw *et al.*, 1990) while the Δlck mutant is not (data not shown).

Both wild-type and mutant forms of CD8α were subcloned into a mammalian expression vector (Margolskee *et al.*, 1988) and transfected into ($CD3^+$, $CD4^+$, $CD8^-$) H9 cells. Stable transfectants were selected by growth in hygromycin B and were FACS sorted to obtain populations which were 100% positive for surface CD8α expression at equivalent levels. Transfectants expressed 10-fold more surface CD8α than endogenous CD4. All transfectants expressed equivalent levels of CD8α, CD4, CD3 and CD45 (Figure 2). Using a radio-immune assay, we determined that the wild-type CD8α transfectants express 1.4×10^4 CD8α molecules, 1.8×10^4 CD3 molecules, 1.3×10^3 CD4 molecules, and 1.0×10^5 CD45 molecules. H9 transfectants containing vector alone, wild-type CD8α, the CD8.2 mutant, the CD8.12 mutant or the Δlck mutant were used to examine the ability of these forms of CD8α to transduce transmembrane signals.

Intracellular calcium flux

Previous studies have shown that simultaneous crosslinking of CD3 with CD8 or CD4, using mAbs conjugated to biotin and crosslinked with avidin on T cell lines, results in a >6-fold enhancement of the increase of $[Ca^{2+}]_i$ than that

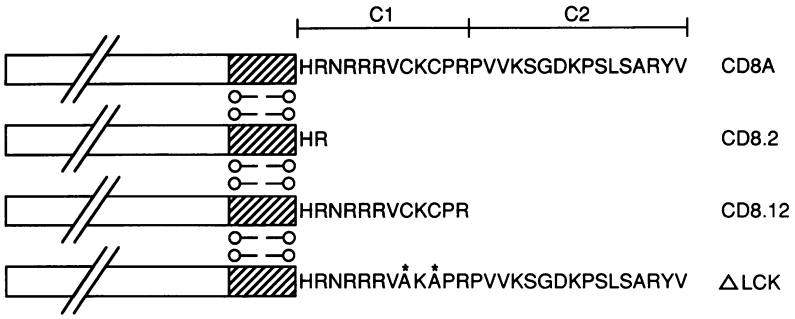

Fig. 1. Schematic depiction of CD8α mutant molecules. The cytoplasmic domain of CD8α was altered by oligonucleotide-directed mutagenesis to insert a stop codon (CD8.12 and CD8.2) or change individual amino acid residues (Δlck) in the coding region of the cytoplasmic domain. The predicted amino acid sequences of the cytoplasmic domain are given by the one-letter amino acid code. The C1 and C2 domains of the cytoplasmic region are encoded by exons 5 and 6 respectively.

seen after crosslinking CD3, CD8 or CD4 alone (Ledbetter *et al.*, 1987a). Similarly, heteroconjugate antibodies, such as CD3–CD8 and CD3–CD4, gained approximately two orders of magnitude in activity (Ledbetter *et al.*, 1989). A similar effect was observed in the parental H9 cells where CD3–CD3 crosslinking resulted in an increase in $[Ca^{2+}]_i$. This effect was enhanced upon crosslinking with the CD3–CD4 heteroconjugate.

We used conjugate antibodies to examine the responses of the transfectants to crosslinking CD3–CD3, CD3–CD8, CD3–CD4, CD4–CD4 and CD8–CD8. Each of the transfectants has been analyzed three times, with consistent results. While crosslinking with the homoconjugates CD4–CD4 and CD8–CD8 failed to induce a rise in $[Ca^{2+}]_i$ in parental H9 cells or any of the transfectants (data not shown), crosslinking with the homoconjugate CD3–CD3, and the heteroconjugates CD3–CD8 and CD3–CD4 led to increases in $[Ca^{2+}]_i$ in some or all of the transfectants. Crosslinking CD3 and CD8 resulted in increases in $[Ca^{2+}]_i$ only in the transfectants expressing wild-type CD8α and the CD8.12 mutant, both of which retain the ability to associate with p56^{lck} (Figure 3A). Both the wild-type CD8α and the CD8.12 transfectants show a 3- to 5-fold increase in $[Ca^{2+}]_i$ after CD3–CD8 crosslinking. In the vector alone, CD8.2 and Δlck transfectants, we were unable to detect an increase in $[Ca^{2+}]_i$ in response to CD3–CD8 crosslinking, demonstrating that a functional p56^{lck} binding site is required for activation of the PI pathway following the formation of a CD8α/TcR/CD3 complex.

While CD3–CD8 crosslinking required a functional p56^{lck} binding site to cause an increase in $[Ca^{2+}]_i$, all transfectants increased $[Ca^{2+}]_i$ in response to CD3–CD4 or CD3–CD3 crosslinking. However, when mutant or wild-type CD8α was present, the response to CD3–CD3 or CD3–CD4 crosslinking was inhibited compared with that observed in the vector-alone transfectant (Figure 3B and C). The vector-alone transfectant responded to CD3–CD3 crosslinking with a 5- to 7-fold increase in $[Ca^{2+}]_i$, reaching a plateau at 7 min. In the wild-type CD8α, CD8.12 and Δlck mutant transfectants, the rise in $[Ca^{2+}]_i$ began at the same rate observed in the vector-alone transfectant, but reached a plateau at 4 min, resulting in a 3- to 5-fold increase in $[Ca^{2+}]_i$. The response of the CD8.2 transfectant, however, proceeded more slowly and only attained a 2-fold increase in $[Ca^{2+}]_i$ (Figure 3B). CD3–CD4 crosslinking induced a 16-fold increase in $[Ca^{2+}]_i$ in the vector-alone transfectant. This response was decreased to a 5-fold rise in $[Ca^{2+}]_i$ in all CD8α transfectants (Figure 3C). The presence of the wild-type or any of the mutant forms of CD8α decreased the ability of the transfectants to respond to CD3–CD3 or CD3–CD4 crosslinking, demonstrating that this inhibition does not require binding to p56^{lck}.

Protein tyrosine phosphorylation

Because the activation of protein tyrosine kinase activity occurs before and may be required for $[Ca^{2+}]_i$ flux, we measured protein tyrosine phosphorylation in our

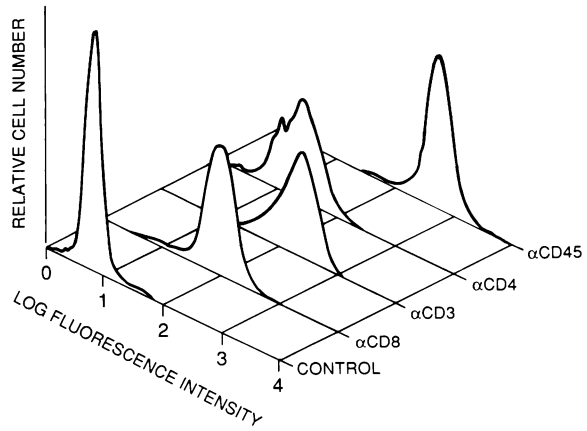

Fig. 2. Flow cytometry analysis of the H9 cells transfected with wild-type CD8α after staining with 1.0 μg/10^6 cells of an anti-CD8α antibody (G10-1), an anti-CD45 antibody (9.4), an anti-CD3 antibody (G19-4) or an anti-CD4 antibody (G17-2) followed by staining with 1.0 μg of a goat anti-mouse IgG antibody coupled to FITC (Southern Biotechnology Associates). Each fluorescence histogram is compared with a negative control histogram obtained after staining with a goat anti-mouse IgG–FITC antibody.

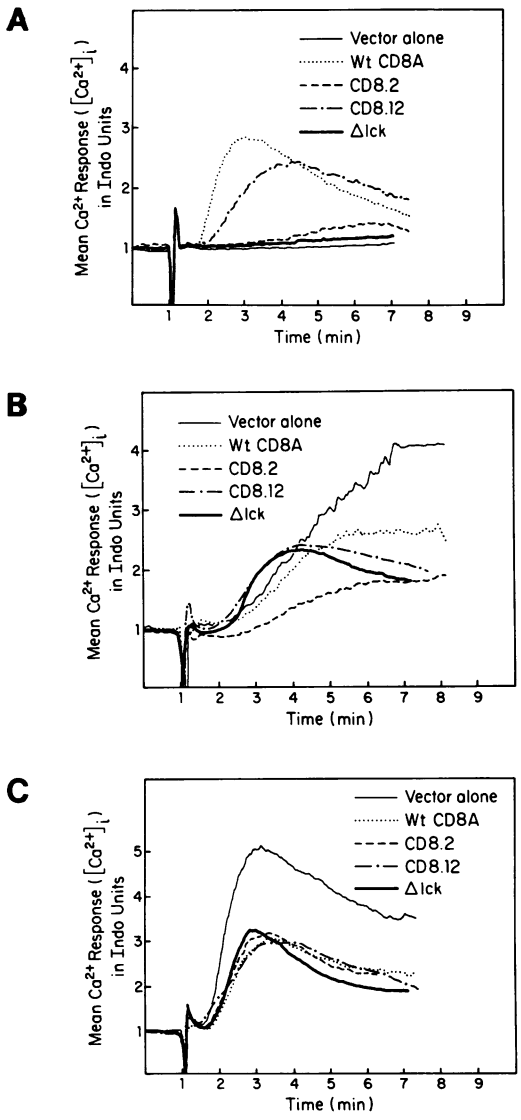

Fig. 3. Mobilization of $[Ca^{2+}]_i$ induced by crosslinking CD3–CD8, CD3–CD3 and CD3–CD4 with conjugate antibodies. Indo-1 loaded H9 transfectants were stimulated at 37°C at t = 1 min with conjugate antibodies at 10 μg/ml. One indo-1 unit is equivalent to 130 nM $[Ca^{2+}]_i$, two indo-1 units to 323 nM, three indo-1 units to 614 nM, four indo-1 units to 1005 nM, five indo-1 units to 2098 nM and six indo-1 units to 5189 nM. **A**, CD3–CD8 (G19-4/G10-1); **B**, CD3–CD3 (G19-4/G19-4); **C**, CD3–CD4 (G19-4/G17-2).

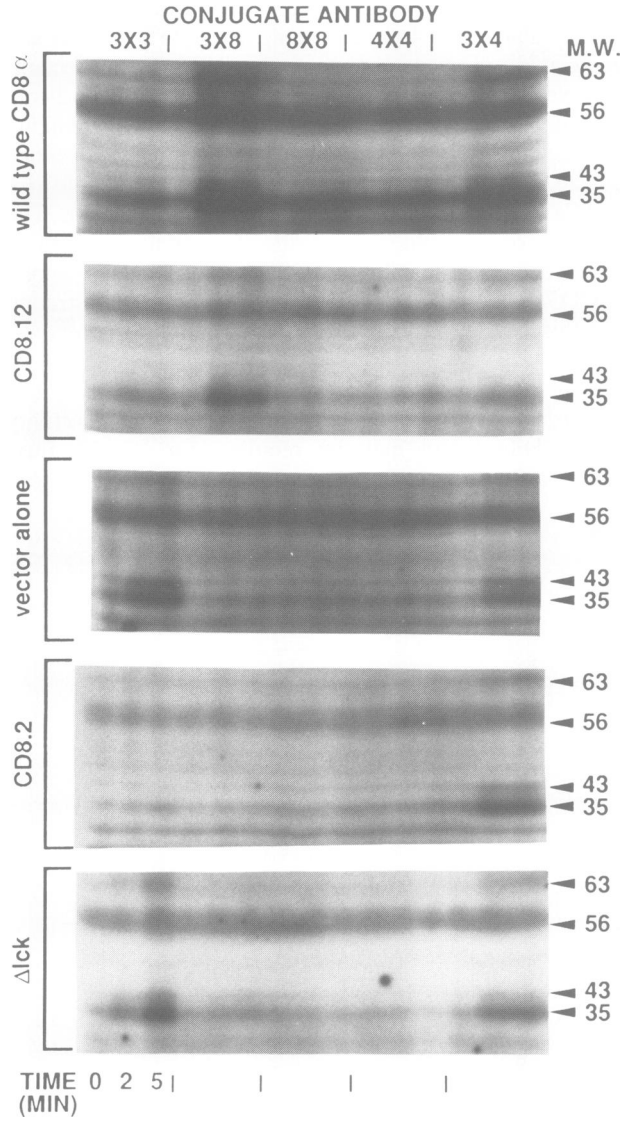

Fig. 4. Autoradiographs of 10% SDS−PAGE gels showing proteins phosphorylated on tyrosine residues in the wild-type CD8α, CD8.12, vector-alone, CD8.2 and Δlck transfectants after crosslinking with conjugate antibodies. 1×10^7 cells were stimulated at 37°C with 10 μg/ml conjugate antibodies (CD3−CD3, CD3−CD8, CD8−CD8, CD4−CD4, CD3−CD4) for 2 or 5 min, were pelleted and lysed with SDS sample buffer, boiled, separated on a polyacrylamide gel for transfer and immunoblotted. Rabbit anti-phosphotyrosine antibody was purified by hapten (phenyl phosphate) elution from phosphotyramine−Sepharose as described (June *et al.*, 1990a) and was used at 0.5 μg/ml, followed by [^{125}I]protein A.

transfectants. To determine whether crosslinking of cell surface molecules is able to activate PTK and to determine the role of different portions of the CD8α cytoplasmic domain in this activity, we stimulated the transfectants with the conjugate antibodies and measured tyrosine-phosphorylated proteins by immunoblotting with rabbit anti-phosphotyrosine antiserum followed by [^{125}I]protein A (Kamps and Sefton, 1988). Each of the transfectants has been analyzed three times with consistent results. Stimulation of the vector-alone transfectant with CD3−CD3 or CD3−CD4 crosslinking resulted in increased phosphorylation of several protein bands: 21 kd after 5 min, 35−43 kd, 56−59 kd, 63 kd and 79 kd after 2 and 5 min. Neither CD3−CD8 nor CD8−CD8 crosslinking caused an increase in protein

Table I. Quantitation of protein tyrosine phosphorylation of the 35 kd band after CD3−CD8 crosslinking

	CD3−CD8 crosslinking		
	Time (min)		
	0	2	5
Vector-alone	0.88 ± 0.40	0.68 ± 0.14	0.86 ± 0.42
CD8α	3.37 ± 0.88	9.32 ± 1.94	6.02 ± 1.02
CD8.12	2.18 ± 0.23	6.98 ± 0.66	4.34 ± 0.20
Δlck	2.12 ± 0.34	2.09 ± 0.63	2.19 ± 0.86
CD8.2	1.58 ± 0.89	1.55 ± 0.95	1.38 ± 0.67

Each value represents a ratio of the integrated intensity of the 35 kd band and the 30 kd band. The values expressed are the averages of measurements from two or three different experiments.

tyrosine phosphorylation in the vector-alone transfectant (Figure 4). Although the 0 time point lane for CD3−CD8 crosslinking appears lighter than the 2 and 5 min lanes, it is also lighter than the 0 time point lane for CD3−CD3, CD4−CD4 and CD8−CD8 crosslinking, indicating that less protein was loaded in this lane. Interestingly, CD4−CD4 crosslinking did not cause increased protein tyrosine phosphorylation of the 56 kd band, although this response would be predicted (Veillette *et al.*, 1989). Increased tyrosine phosphorylation of the 56 kd band was observed after CD4−CD4 crosslinking only in the CD8.2 mutant transfectant. This may be due to the high level of endogenous protein tyrosine phosphorylation in the H9 cells. This response is more clearly observed in other cell lines with lower endogenous levels of protein tyrosine phosphorylation such as the CEM cell line (J.Ledbetter, unpublished results).

Crosslinking CD3 and CD8 resulted in strong increases in protein tyrosine phosphorylation of the same set of proteins only in the transfectants expressing wild-type CD8α or the CD8.12 mutant. Both of these forms retain a functional p56^{lck} binding site. Following CD3−CD8 crosslinking, increased tyrosine phosphorylation occurred on several protein bands including those at 35−43, 56−59 and 63 kd (Figure 4). In addition, the wild-type CD8α and CD8.12 transfectants both showed increased tyrosine phosphorylation of the 56 kd band following CD8−CD8 crosslinking, suggesting that this band is p56^{lck} as previously reported (Veillette *et al.*, 1989) (Figure 4). Neither the vector alone nor the CD8.2 transfectant showed a detectable increase in protein tyrosine phosphorylation of these bands after CD3−CD8 or CD8−CD8 crosslinking (Figure 4). The phosphorylation of proteins was greatly diminished in the Δlck transfectant after CD3−CD8 crosslinking (Figure 4). To quantitate the response of the transfectants to CD3−CD8 crosslinking, we compared the integrated intensity of the 35 kd band with that of the 30 kd band, which does not increase in tyrosine phosphorylation in response to any of the crosslinking schemes used (Table I). These results confirm that a functional p56^{lck} binding site is required for activation of the PTK responsible for the strong increase in protein tyrosine phosphorylation of the 35−43, 56−59 and 63 kd bands following the formation of a CD8α/TcR/CD3 complex.

In contrast to CD3−CD8 crosslinking, all transfectants increased protein tyrosine phosphorylation of the 35−43 and 63 kd bands in response to CD3−CD4 crosslinking (Figure 4). All transfectants except the CD8.2 transfectant increased protein tyrosine phosphorylation in response to CD3−CD3

crosslinking. In the CD8.2 mutant, which lacks the CD8α cytoplasmic region, the signals induced by CD3–CD3 crosslinking were inhibited with the exception of a small increase in tyrosine phosphorylation of the 35 kd band. These results are consistent with those observed for $[Ca^{2+}]_i$ flux, in which the CD8.2 transfectant showed the smallest increase in $[Ca^{2+}]_i$ after CD3–CD3 crosslinking. These results support the hypothesis that the ability of CD8α to disrupt some signals does not require association with p56^{lck}.

Discussion

Our studies demonstrate that a functional p56^{lck} binding site is required for the induction of protein tyrosine phosphorylation and increased $[Ca^{2+}]_i$ caused by association of the CD8α/α homodimer and the TcR/CD3 complex. Only the H9 transfectants expressing wild-type CD8α or the CD8.12 mutant, which both retain the p56^{lck} binding site, were able to increase protein tyrosine phosphorylation of a set of protein bands or increase $[Ca^{2+}]_i$ after CD3–CD8 crosslinking. In contrast, the Δlck mutant, which has a complete cytoplasmic tail but contains mutations in the two cysteines required for p56^{lck} binding, was defective in these two early signalling events.

Protein tyrosine phosphorylation and increased $[Ca^{2+}]_i$ are caused by two different signal transduction pathways. A tyrosine kinase or kinases are responsible for protein tyrosine phosphorylation, while inositol phosphates and diacylglycerol are the second messengers responsible for mobilization of cytoplasmic free calcium and PKC activation. Stimulation of T cells with anti-TcR monoclonal antibodies activates both the tyrosine kinase and the phosphoinositol (PI) pathways, yet the relationship between them as well as their contribution to later T cell responses is not fully understood. Studies by Sussman *et al.* (1988), using variants of a murine hybridoma, suggested that the PI pathway may not be required for or involved in regulating interleukin-2 (IL-2) production. Studies by Desai *et al.* (1990) demonstrated that stimulation of the human muscarinic subtype-1 receptor (HMI) induces IL-2 production and IL-2 receptor α chain expression, when expressed in a T cell line. HMI elicits PI metabolism but does not induce the tyrosine kinase pathway, suggesting that the tyrosine kinase pathway is not required for later T cell activation responses. These studies do not preclude a role for the tyrosine kinase or other TcR induced signal transduction pathways in the regulation of IL-2 production.

The tyrosine kinase pathway may indeed regulate the PI pathway after T cell stimulation. Recent studies by June *et al.* (1990a,b) suggest that there is a link between the tyrosine kinase and PI pathways. They demonstrated both that induction of tyrosine kinase activity precedes PLC activation, and that herbimycin A, an inhibitor of substrate tyrosine phosphorylation, inhibits the PI hydrolysis and increased $[Ca^{2+}]_i$ observed after TcR stimulation, but not G-protein mediated PLC activation stimulated by the addition of aluminum fluoride. In addition, work by Nishibe *et al.* (1990) demonstrated that tyrosine phosphorylation of PLC-γ1 in intact cells or *in vitro* increased its catalytic activity, while treatment of activated PLC-γ1 with a phosphotyrosine phosphatase decreased its catalytic activity. These results suggest that tyrosine phosphorylation may be a regulatory event which links the TcR to the PI signalling pathway, as has been suggested with other non-lymphoid tyrosine kinase receptors (Wahl *et al.*, 1989). Our results support the hypothesis that tyrosine kinases regulate the PI pathway. Only forms of CD8α containing a functional p56^{lck} binding site are capable of causing increased protein tyrosine phosphorylation and $[Ca^{2+}]_i$ after CD3–CD8 crosslinking.

Despite the ability of some forms of CD8α to signal when crosslinked with CD3, the endogenous CD4 molecule signalled more strongly when crosslinked to CD3. Because p56^{lck} association is critical for the transduction of signals through CD8α and presumably CD4, differences in p56^{lck} activity associated with these two molecules may account for the differences in the strength of signalling we observed between CD8α and CD4. Interestingly, the transfectants expressed 10-fold more transfected CD8α than endogenous CD4. Recent reports have shown that the affinity of p56^{lck} for CD4 is greater than for CD8α (Turner *et al.*, 1990).

We compared signalling through TcR/CD3 and CD4 both before and after addition of wild-type or mutant forms of CD8α. All forms of CD8α inhibited the increase in $[Ca^{2+}]_i$ caused by CD3–CD3 or CD3–CD4 crosslinking regardless of their ability to bind p56^{lck}. There are two possible explanations for this inhibition. CD8α may interfere with CD3–CD3 or CD3–CD4 crosslinking through steric inhibition involving its extracellular domain. Alternatively, CD8α may inhibit signalling through CD3 and/or CD4 by competitive binding of other molecules involved in transmembrane signal transduction. This putative interaction with another molecule or molecules may involve either the transmembrane or extracellular portions of CD8α. Competition between CD4 and CD8 molecules has also been proposed in transgenic mouse studies in which the CD4 transgene is expressed at levels 15- to 30-fold higher than normal in all thymocyte subsets (Teh *et al.*, 1991). Positive selection of $CD8^+$ thymocytes was disrupted in doubly transgenic thymocytes expressing both the CD4 transgene as well as a transgenic class I-restricted TcR specific for the male H-Y antigen.

It is likely that the effects of overexpression of one co-receptor relative to the other are due to competition between CD4 and CD8 for association with other molecules involved in signalling. Candidate molecules include p56^{lck}, the phosphotyrosine phosphatase CD45, the TcR and other as yet unidentified transmembrane or membrane proteins. Our results demonstrate that competition for association with p56^{lck} is not the cause of the inhibition observed in the H9 transfectants, although competition between CD4 and CD8α for interaction with CD45 could influence activation signals in the H9 transfectants. Interaction with the phosphotyrosine phosphatase CD45 is thought to enhance activity of p56^{lck} by dephosphorylating tyrosine residues which are constitutively autophosphorylated (Ostergaard and Trowbridge, 1990).

Both CD4 and CD8α may physically associate with the TcR/CD3 complex during T cell stimulation (Hoxie *et al.*, 1986; Minami *et al.*, 1987; Saizawa *et al.*, 1987; Takada and Engleman, 1987) and enhance signal transduction (Ledbetter *et al.*, 1989; Emmrich *et al.*, 1986; Gabert *et al.*, 1987; Samstag *et al.*, 1988). Upon crosslinking with anti-CD3 antibody, co-association of either CD4 or CD8 is observed (Saizawa *et al.*, 1987; Takada and Engleman, 1987). During CD3–CD3 crosslinking in our H9 transfectants, CD4 and CD8α may be equally capable of associating with the TcR/CD3 complex. In the CD8α

transfectants CD4 and CD8α may both compete for association with CD3, causing less CD4 to enter the complex compared with vector alone ($CD4^+$, $CD8^-$) transfectants. Direct crosslinking of CD3 with CD4, however, would be expected to involve more CD4 than CD8α but may still be affected by cell surface expression of CD8α. Such cell surface associations may not be the same for all the CD8α mutants.

Associations of CD4 and CD8 with the TcR require the cytoplasmic tails of the co-receptors. Using fluorescence resonance energy transfer (FRET), Mittler *et al.* (1989) demonstrated that human CD4 transfected into a mouse hybridoma associated with murine CD3 after anti-CD3 antibody crosslinking whereas a truncated form lacking the cytoplasmic tail did not. The role of the CD8α cytoplasmic region in enabling the molecule to associate with the TcR/CD3 complex is unclear, although the lateral mobilities of both the full-length and tailless forms of murine CD8α are nearly identical (Letourneur *et al.*, 1990). Our studies using FRET suggest that competition for interaction with the TcR/CD3 complex is not the cause of CD8α inhibition of PI pathway signalling after CD3−CD3 or CD3−CD4 crosslinking. Preliminary results show that wild-type CD8α is capable of energy transfer with CD3 while the Δlck mutant is not, suggesting that a functional $p56^{lck}$ binding site is necessary for association between CD8α and CD3/TcR after CD3−CD3 crosslinking. These results indicate that inhibition of the increase in $[Ca^{2+}]_i$ observed in the $CD8\alpha^+$ H9 transfectants after CD3−CD3 and CD3−CD4 crosslinking is not due to competition for association with the CD3/TcR complex.

Our data demonstrate that a functional $p56^{lck}$ binding site is required for signalling through the CD8α/CD3/TcR complex via both the tyrosine kinase and the PI pathways. This result supports the hypothesis that a tyrosine kinase, in this case $p56^{lck}$, regulates signalling via the PI pathway. The inhibitory effects of CD8α on signalling through the CD3/TcR and CD3/TcR/CD4 complex, however, do not require $p56^{lck}$ binding.

Materials and methods

Plasmid constructions and mutagenesis

The human CD8α gene (Kavathas *et al.*, 1984) was subcloned into the *Hin*dIII−*Bam*HI site of Bluescript SK+ (Stratagene). Mutant DNAs were generated by oligonucleotide-directed mutagenesis with single-stranded phagemid DNA by the method described by Kunkel (1985). All oligonucleotides were synthesized by the Yale Medical School DNA synthesis facility. Sequences were determined using the dideoxy sequencing method (Sanger *et al.*, 1977). Wild-type and mutant forms of CD8α were subcloned into the *Hin*dIII−*Xba*I site of the expression vector EBOpLPP (Spickofsky *et al.*, 1990) for transfection into human cells.

Cell culture and transfections

The human T cell leukemia line H9 (Popovic *et al.*, 1984) was obtained from the laboratory of Dr Nancy Ruddle. H9 cells were grown in RPMI 1640 plus 10% fetal calf serum (FCS) (Whittaker M.A.Bioproducts). Cells were transfected via liposome mediated transfection. For transfection 35 mm dishes were precoated with Cell-Tak (Collaborative Research). Cell-Tak was diluted in an 0.1 M solution of sodium bicarbonate, pH 8.0, and dispensed at 3.5 μg/cm^2 surface area. Dishes were incubated at 37°C for 30 min and then washed twice with sterile H_2O. Five million H9 cells growing at a concentration of $3-4 \times 10^5$/ml were collected by centrifugation. The cells were washed twice with Opti-MEM (Gibco, Grand Island, NY), resuspended in 2 ml Opti-MEM and layered on the coated dishes. After 30 min at 37°C, the attachment of the cells was verified by inspection under the microscope. The Opti-MEM was then removed and the DNA/liposome mixture added. Plasmid DNA (10 μg) was added to 0.5 ml Opti-MEM, and 25 μl of lipofectin (Bethesda Research Labs) was added to an additional 0.5 ml Opti-MEM. The contents of the two tubes were mixed 15 min prior to addition to the cells. The cells were incubated with the DNA/liposome mixture for 12 h in a 37°C incubator, after which they were removed from the tissue culture plate by the addition of cold PBS and resuspended in 20 ml of RPMI 1640 plus 10% FCS. After 2 days, cells were grown in media containing 100 μg/ml hygromycin B (Calbiochem). After an additional 2 days, the concentration of hygromycin B was increased to 200 μg/ml. After 1 week the concentration of the hygromycin B was increased to 300 μg/ml at which time the control non-transfected H9 cells were 100% dead. Stably transfected populations of H9 cells were then assayed for CD8α surface expression by staining with the anti-CD8α mAb G10-1 (Ledbetter *et al.*, 1985) followed by a FITC-labeled goat anti-mouse IgG antibody (Southern Biotechnology Associates) and analyzed using a FACScan Flow Cytometer (Becton Dickinson).

Antibodies

Conjugate antibodies were constructed as described (Ledbetter *et al.*, 1989) using the CD3 antibody G19-4 (Hansen *et al.*, 1984), CD8 antibody G10-1 (Ledbetter *et al.*, 1985) and CD4 antibody G17-2 (Ledbetter *et al.*, 1987b). Analysis of the surface phenotype of the H9 transfectants utilized G10-1, G19-4, G17-2 and the CD45 antibody 9.4 (Ledbetter *et al.*, 1988). The FITC-conjugated goat anti-mouse IgG second step antibody was obtained from Southern Biotechnology Associates.

Measurement of $[Ca^{2+}]_i$

Cytoplasmic free calcium concentration was measured with the dye indo-1 (Molecular Probes) and a model 50 HH/2150 cell sorter (Ortho Diagnostics Systems) as described (Rabinovitch *et al.*, 1986). Cells (2×10^7/ml) were loaded with the acetoxymethyl ester of indo-1 by incubation for 45 min at room temperature. Prior to analysis, 1×10^6 cells were equilibrated at 37°C in RPMI 1640 with 10% FCS for 5 min. The baseline $[Ca^{2+}]_i$ was monitored for 1 min prior to addition of 10 μg of hetero- or homoconjugate antibodies. Laser light at 355 nm was used to excite indo-1, causing fluorescence emission in the violet spectrum to increase as Ca^{2+} is bound, while blue decreases as Ca^{2+} is bound. The violet/blue ratio is proportional to $[Ca^{2+}]_i$. The data were analyzed by computer programs which calculated the mean indo-1 violet/blue fluorescence ratio versus time. The percentage of responding cells versus time was analyzed by programs that first determined the values of the indo-1 ratio two standard deviations above the mean ratio of control cells and then plotted the percentage of cells above this threshold value versus time. One indo-1 unit is equivalent to 130 nM $[Ca^{2+}]_i$, two indo-1 units to 323 nM, three indo-1 units to 614 nM, four indo-1 units to 1005 nM, five indo-1 units to 2098 nM, and six indo-1 units to 5189 nM.

Anti-phosphotyrosine immunoblotting

Aliquots of 1×10^7 cells/ml were stimulated by addition of 10 μg conjugate antibodies at 37°C. Following stimulation, cells were lysed after 2 and 5 min. To lyse the cells, samples were pelleted for 8 s in a microcentrifuge, followed by aspirating the supernatant and adding 300 μl of SDS lysis buffer containing 50 μM sodium vanadate and 25 mM DTT. Samples were boiled for 5 min. The lysates were sheared by several passages through a 28 gauge needle and equivalent amounts of protein were loaded onto 10% polyacrylamide gels (Laemmli, 1970) and electrophoresed at 5 mA overnight. The proteins were transferred to Immobilon (Millipore Corp.) for 90 min at 4°C. Immunoblots were blocked overnight in saline/10 mM Tris/5% BSA/1% ovalbumin, and then incubated with 0.5 μg/ml anti-phosphotyrosine antiserum for 2 h, washed five times, incubated with 1.0 μCi/ml high specific activity ^{125}I-labeled protein A (ICN Biomedicals) and washed an additional five times. Prestained mol. wt markers (Bethesda Research Labs) were included on each gel.

Densitometry

Densitometry was carried out with a Visage 2000 imaging system (Bio Image, Millipore). The integrated intensity of the variable 35 kd band and of the constant 30 kd band was measured for the 0, 2 and 5 min time points after CD3−CD8 crosslinking, and the ratio of these two values was calculated. Measurements were taken of autoradiographs of two or three separate immunoblots of each of the H9 transfectants.

Acknowledgements

We are grateful to Drs David Stern and Andrey Shaw for a critical reading of the manuscript, to Derek Hewgill and Rocco Carbone for help with flow cytometry, to Phillipe Male for help with densitometry, and to Laura

Grosmaier and John Mennone for expert technical assistance. This work was supported by NIH grant CA 48115. P.K. is a scholar of the American Leukemia Society.

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Received on November 30, 1990; revised on February 11, 1991