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21. Cells (5×10^7) were lysed in buffer containing 0.5% Triton, immunoprecipitated with an antiserum to the intracellular portion of CD45, separated by SDS–polyacrylamide gel electrophoresis under reducing conditions, transferred to nitrocellulose, immunoblotted with same serum, and developed with ^{125}I–protein A (ICN, Costa Mesa, CA), as described (*21*).
22. [Val^5] angiotensin II (Sigma) was phosphorylated in vitro with p60$^{c\text{-}src}$ (Oncogene Science, Uniondale, NY) and purified with P81 Whatman filter paper. Triton X-100 lysates of 17.5×10^6 cells per point were immunoprecipitated with either a mAb to LFA-1 (anti–LFA-1) (FD441.8) or D710. Protein G–agarose (Gibco BRL, Gaithersburg, MD) or protein A–agarose, respectively, was the solid-phase reagent. After washing, enzymatic activity on the beads was measured by incubating them with 10 μl of labeled substrate for 30 min at 30°C and stopping the reaction by adding 550 μl of 5% activated charcoal in 20 mM Hepes buffer, pH 7.4. After centrifugation, the supernatant was counted in a scintillation counter.
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Rescue of Signaling by a Chimeric Protein Containing the Cytoplasmic Domain of CD45

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Surface expression of the CD45 tyrosine phosphatase is essential for the T cell antigen receptor (TCR) to couple optimally with its second messenger pathways. CD45 may be required to dephosphorylate a TCR-activated protein tyrosine kinase, which then transduces an activation signal from the TCR. A chimeric molecule that contained extracellular and transmembrane sequences from an allele of a major histocompatibility class I molecule and cytoplasmic sequences of CD45 restored TCR signaling in a CD45-deficient mutant T cell line. Thus, expression of the complex extracellular domain of CD45 is not required for the TCR to couple to its signaling machinery.

Surface expression of CD45, a transmembrane tyrosine phosphatase found on all nucleated hematopoietic cells (*1*), is required for efficient signaling through the TCR (*2–6*). Defects in both proximal and distal signal transduction events have been documented in several CD45-deficient mutants, although the precise signaling phenotype varies. Ligation of the TCR on wild-type cells results in the activation of a protein tyrosine kinase (PTK) followed by generation of phosphatidylinositol (PI) second messengers (*7*). Evidence suggests that these pathways are causally linked because tyrosine phosphorylation of phospholipase C–γ1 (PLC-γ1) is essential for activation of the PI pathway (*8*). One model for how CD45 regulates TCR signaling is that it interacts directly with a TCR-activated PTK, thought to be a member of the *src* family (*9*). Each *src* kinase possesses a COOH-terminal tyrosine that, when phosphorylated, inhibits its PTK function (*10*). A possible role of CD45 is to dephosphorylate this residue (*11*), allowing the PTK to interact effectively with the TCR to transduce an activation signal.

The features of CD45 required to promote TCR signaling remain unclear. Alternative splicing of the single gene encoding CD45 gives rise to a number of separate isoforms (*1*). These differ only in their extracellular sequences and have complete conservation of their cytoplasmic regions, which contain two tandem phosphatase domains. At least one isoform of CD45 interacts with CD22, a molecule expressed on the surface of B cells (*12*). Members of the CD45 family physically associate with several surface antigens on T cells (*13*), in an isoform-specific manner (*14*). The isoform specificity suggests that extracellular sequences of CD45 are essential for the protein-protein interactions, giving rise to the hypothesis that the extracellular regions of CD45 are required for its regulation of TCR signaling. An alternative hypothesis is that the conserved cytoplasmic domain of CD45, with its enzymatic activity, may be sufficient for TCR signaling.

We examined the requirement for expression of the extracellular and transmembrane domains of CD45 in TCR signaling by using J45.01 cells, a mutant derived from the human Jurkat T cell line, that have markedly diminished surface expression of CD45 (*4*). The TCR on this clone does not couple with either the PTK or PI second messenger pathways. Reconstitution of CD45 expression by gene transfer rescues the signaling defect (*15*). A chimeric molecule was made by overlap extension polymerase chain reaction (PCR) (*16*) that contained the extracellular and transmembrane domains of the HLA-A2 allele of the major histocompatibility complex (MHC) class I molecule and the cytoplasmic domain of CD45 (Fig. 1A). The A2 molecule was chosen for the chimera because it bears no homology with CD45, is not normally expressed on Jurkat cells, and antibodies are available for staining and immunoprecipitation. Additionally, we find that MHC class I molecules do not co-cap with the TCR complex in the Jurkat cell line. Transfection of wild-type A2 or A2-CD45 chimeric cDNA resulted in the appearance of clones expressing large amounts of protein immunoreactive with a monoclonal antibody (mAb) to A2 (anti-A2) on the cell (Fig. 1B) (*17*). These clones have maintained a stable phenotype while in culture for greater than 6 months. Immunoprecipitations with anti-A2 from biosynthetically labeled J45/CH11 cells (J45.01 transfected with the chimeric protein) revealed a protein of the expected molecular size (118 kD) seen on SDS–polyacrylamide gel electrophoresis (SDS-PAGE) that had tyrosine phosphatase activity in an in vitro assay. We compared the amount of tyrosine phosphatase activity present in membranes prepared from Jurkat, J45.01, and J45/CH11 (Fig. 1C); expression of the A2-CD45 chimera reconstituted phosphatase activity essentially to that of wild-type cells.

To determine if expression of the chimeric protein would allow for TCR signaling, we studied Jurkat, J45.01, J45.01 transfected with wild-type A2 and several independently derived clones expressing the A2-CD45 chimera. The first signaling event seen after TCR engagement on wild-type Jurkat was the rapid activation of a

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PTK resulting in multiple newly tyrosine phosphorylated species (Fig. 2). Stimulation of the TCR on J45.01 did not result in the appearance of these newly tyrosine phosphorylated proteins [Fig. 2 and (4)]. Similarly, there was no evidence for TCR-mediated PTK activation in J45/A2 (18). Expression of the A2-CD45 chimera, however, rescued this signaling defect in J45.01 by the appearance of tyrosine phosphorylated species with similar migration patterns on SDS-PAGE as seen in the wild-type cell following TCR stimulation (Fig. 2).

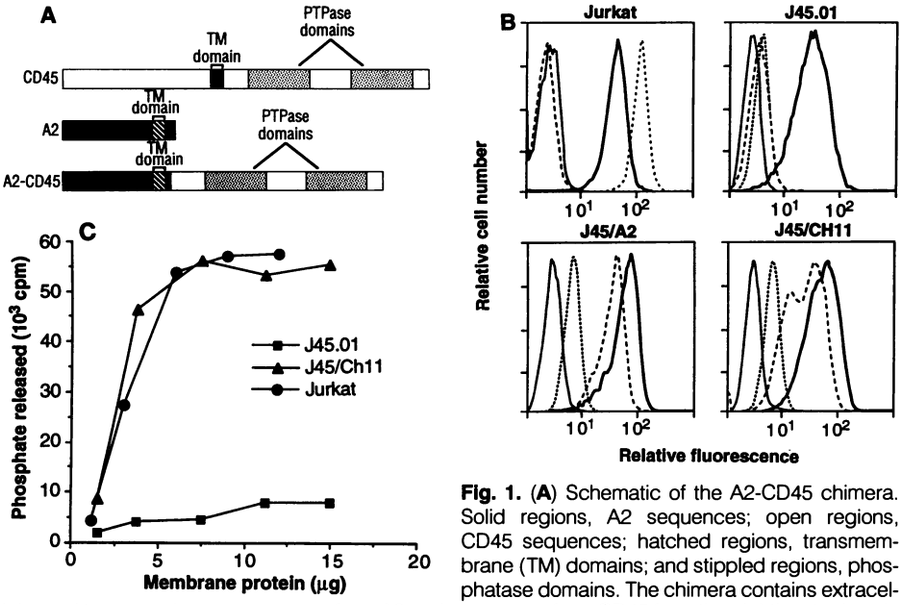

Fig. 1. (**A**) Schematic of the A2-CD45 chimera. Solid regions, A2 sequences; open regions, CD45 sequences; hatched regions, transmembrane (TM) domains; and stippled regions, phosphatase domains. The chimera contains extracellular and transmembrane sequences from A2 and sequences from CD45 from the eighth cytoplasmic amino acid through the "stop" (after cytoplasmic amino acid 707). (**B**) Expression of TCR, CD45, and A2 or the A2-CD45 chimera on various cell lines. Jurkat, J45.01, J45/A2 (J45.01 transfected with cDNA encoding full-length MHC class I A2), and J45/CH11 (J45.01 transfected with cDNA encoding the A2-CD45 chimera) were stained with a control antibody (MOPC 195, thin solid lines); anti-CD45 (mAb 9.4, dotted lines); anti-TCR (mAb OKT3, thick solid lines); or anti-HLA A2 (mAb CR11, dashed lines) and then analyzed on an Epics 753 flow cytometer (Coulter Electronics, Hialeah, Florida). Primary mAbs were from the American Type Culture Collection (Rockville, Maryland), except for CR11 from C. Lutz. Fluorescein isothiocyanate (FITC)–conjugated secondary Abs were from Cappel (West Chester, Pennsylvania). Transfections were done by electroporation (Gene Pulser, Bio-Rad, Richmond, California) at 250 V/966 μF (4). (**C**) Tyrosine phosphatase activity in membranes prepared from Jurkat, J45.01, and J45/CH11. Membranes were prepared (4) and aliquots were incubated for 5 min with 80,000 cpm of Raytide (Oncogene Science, Uniondale, New York) previously phosphorylated using immunoprecipitated Lck and [γ-^{32}P]ATP (Amersham, Arlington Heights, Illinois) (23). The amount of liberated [^{32}P]phosphate was quantified by liquid scintillation counting.

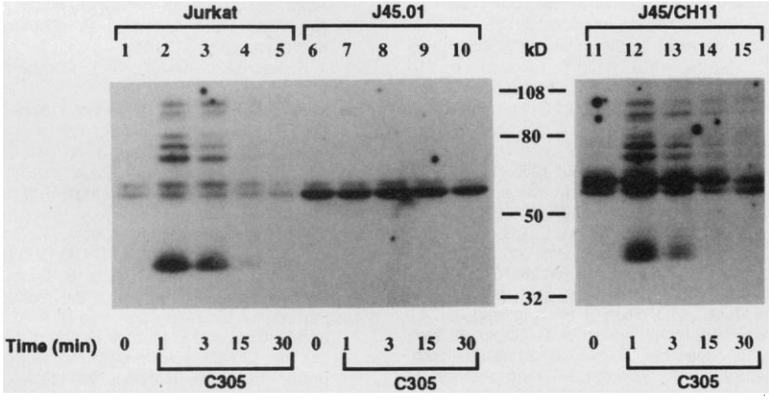

Fig. 2. Stimulation of the TCR on J45.01 transfected with the A2-CD45 chimera activated a PTK. Lysates were prepared from Jurkat, J45.01, and J45/CH11 (1×10^7 cell equivalents per lane) before and after stimulation for the times indicated with the mAb C305 [anti-TCR; (24)] and subjected to 10% SDS-PAGE. Gels were transferred to nitrocellulose and blotted with a mAb to phosphotyrosine (Oncogene Science, Uniondale, New York). Immunoreactive proteins were identified after incubation of the nitrocellulose with ^{125}I–protein A followed by autoradiography (3-day exposure).

The tyrosine phosphorylation of PLC-γ1, an early substrate of the TCR-activated PTK (19–21), is probably physiologically relevant because this event regulates the enzymatic activity of PLC-γ1 (8). We therefore examined the effect of TCR stimulation on PLC-γ1 tyrosine phosphorylation in the various clones. Jurkat, J45.01, J45/A2, and J45/CH11 each expressed equivalent amounts of PLC-γ1 (Fig. 3A, left). Stimulation of the TCR on Jurkat, but not J45.01, results in tyrosine phosphorylation of PLC-γ1 [Fig. 3A, right, and (20)]. Transfection with A2 did not change the response pattern of J45.01. However, expression of A2-CD45 on J45/CH11 resulted in tyrosine phosphorylation of PLC-γ1, albeit less than seen in the wild-type cell (Fig. 3A, right). Thus, expression of the cytoplasmic domain of CD45, in the context of the transmembrane and extracellular regions of an unrelated molecule, is sufficient for the TCR to couple to the PTK pathway resulting in the tyrosine phosphorylation of previously identified substrates.

Because tyrosine phosphorylation of PLC-γ1 should lead to increased generation of PI-derived second messengers, we examined the production of soluble inositol phosphates in the various cells after TCR stimulation (Fig. 3B). The J45.01 cells transfected with A2 still had a signaling defect, but stimulation of the TCR on J45/CH11 resulted in the production of soluble inositol phosphates, although less were generated than in the wild-type parent. We examined the integrity of the PI signaling pathway further by evaluating the ability of TCR stimulation to increase intracellular calcium (22) in the four cell lines. Spectrofluorimetric analysis of cells loaded with the calcium-sensitive dye, Indo-1, and then activated via the TCR showed a large, sustained increase in intracellular free calcium in Jurkat and J45/CH11 that was not seen in J45.01 or J45/A2 (Fig. 4).

Our data indicate that it is possible to rescue the entire cascade of known proximal events associated with TCR stimulation in our CD45-deficient cell by surface expression of A2-CD45, a chimeric protein containing no extracellular or transmembrane sequences derived from CD45. Thus, when expressed as a transmembrane protein, the cytoplasmic region of CD45, containing enzymatically active domains, is sufficient to support TCR signal transduction. However, in several of our assays of TCR activation, stimulation of the TCR on the clones transfected with the chimeric molecule resulted in a somewhat diminished signal as compared to stimulation of the TCR on wild-type Jurkat. This may be due to differences between in vivo enzymat-

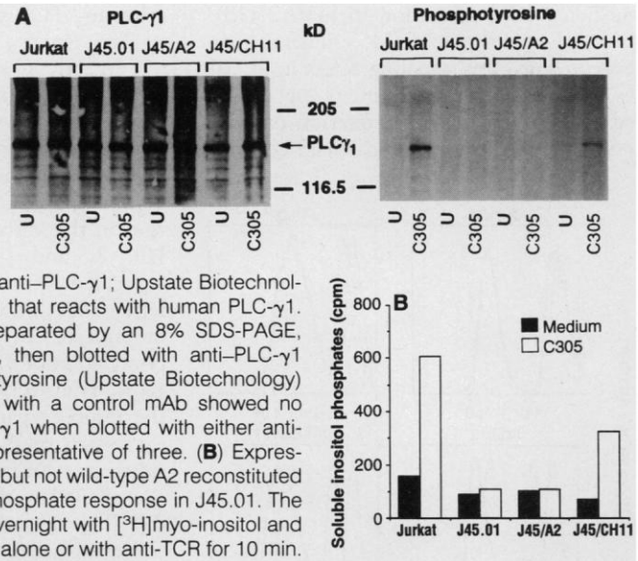

Fig. 3. (**A**) Stimulation of the TCR on J45.01 transfected with the A2-CD45 chimera resulted in tyrosine phosphorylation of PLC-γ1. The four cell lines were left unstimulated (U) or stimulated for 5 min with mAb, C305 (anti-TCR). Cells were lysed and proteins immunoprecipitated with mAb to bovine PLC-γ1 (anti–PLC-γ1; Upstate Biotechnology, Lake Placid, New York) that reacts with human PLC-γ1. Immunoprecipitates were separated by an 8% SDS-PAGE, transferred to nitrocellulose, then blotted with anti–PLC-γ1 (left) or a mAb to phosphotyrosine (Upstate Biotechnology) (right). Immunoprecipitation with a control mAb showed no bands in the region of PLC-γ1 when blotted with either antibody. This experiment is representative of three. (**B**) Expression of the A2-CD45 chimera but not wild-type A2 reconstituted the TCR-mediated inositol phosphate response in J45.01. The four cell lines were loaded overnight with [^{3}H]myo-inositol and then incubated with medium alone or with anti-TCR for 10 min. Soluble inositol phosphates were measured as described (*25*). The data reflect a mean of triplicates (SE <10% of the mean). This experiment is representative of eight.

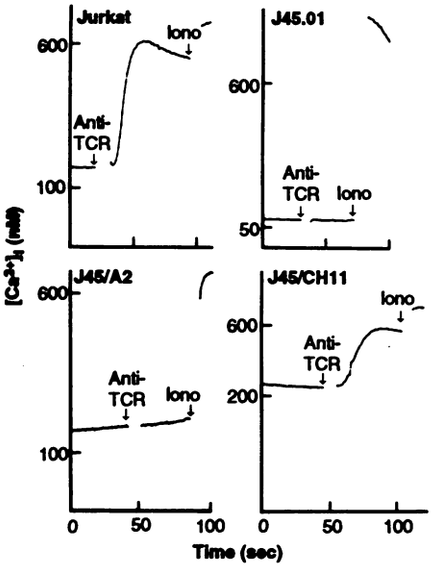

Fig. 4. Expression of the A2-CD45 chimera but not wild-type A2 reconstitutes the TCR-mediated calcium response in J45.01. Jurkat, J45.01, J45/A2, and J45/CH11 were loaded with the calcium-sensitive dye Indo-1, placed in a fluorimeter, and then stimulated with an activating mAb to the TCR (C305) (first arrow) as described (*15*). Real-time fluorescence was measured and values were converted to intracellular concentrations of free calcium. To ensure that cells were adequately loaded with Indo-1, ionomycin (Iono; second arrow) was added at the end of the experiment. The experiment shown is representative of three.

ic activity of the chimera compared with native CD45 or to less efficient coupling of the TCR with its signaling machinery in the absence of the extracellular domain of CD45. Thus, although not necessary for signaling, the extracellular region of CD45 may well participate in modifying the magnitude of the response either through directing interactions between CD45 and other molecules that participate in the signaling pathways or by affecting enzymatic activity of the phosphatase. Mutational analysis of the cytoplasmic domain as well as further study of the extracellular region of CD45 and its interactions with other molecules will help clarify the relative importance of regions of this phosphatase in the regulation of TCR signal transduction.

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