

studied to date for NO is extremely high, with binding constants on the order of 10^{11} M⁻¹ (9). Furthermore, the kinetics of dissociation are very slow: The rate constant for the release of bound NO from human hemoglobin is on the order of 10^{-5} s⁻¹ at 20°C and neutral pH (9). However, Fe(III) heme proteins bind NO with much less affinity, with binding constants in the range of 10^3 to 10^5 M⁻¹, and the kinetics of dissociation are faster ($k_{\text{off}} = 1$ to 40 s⁻¹) (10). Thus, an Fe(III) heme protein would be a much better carrier of NO if release of this effector after dilution is required. On the basis of the spectral changes observed (Figs. 1 and 2), it appears likely that an Fe(III) heme protein may be involved in the *Rhodnius* salivary vasodilator.

To further confirm the importance of the Fe(III) heme protein, we carried out experiments using electron paramagnetic resonance (EPR) spectroscopy. Fe(III) heme proteins have an odd number of electrons that exist in a high-spin form when pentacoordinated and typically give rise to EPR spectral features at spectroscopic splitting factors (g) of 6 and 2 (11, 12). Addition of NO to an Fe(III) heme protein produces an even-electron species that is EPR silent (12). The EPR spectra of *Rhodnius* salivary gland homogenates before and after exposure to an argon atmosphere and after subsequent exposure to NO are shown in Fig. 4 (13). Only a weak signal was observed for the homogenate before argon equilibration, but strong signals at $g = 6$ and $g = 2$ were observed after argon equilibration (Fig. 4, B and D). These signals disappeared after subsequent exposure of the homogenate to NO. The small signal at 3200 G ($g = 2.08$) that does not change with argon or NO equilibration is probably produced by a nonheme-based radical that accounts for ~5% of the intensity of the high-spin Fe(III) signal. The observation that the shape of this $g = 2.08$ signal does not change after NO equilibration eliminates a nitrosyliron(II) center from consideration because that odd-electron species gives rise to very characteristic EPR spectral features in precisely the same region of the EPR spectrum (14). This characteristic is shown in Fig. 4E, where dithionite has been added to the untreated homogenate solution to produce the Fe(II)NO species.

Chromatofocusing of whole-gland homogenates on a Mono P column yielded three major heme proteins, with isoelectric points (pI's) of 7.97, 7.53, and 6.94. The reduced proteins had apparent masses of 26.0, 23.1, and 23.4 kD and were present in amounts of 192, 57.6, and 44.0 pmol per gland pair, respectively (15). Taken together, these three proteins account for at least 90% of the total heme protein content of the glands. Preliminary results indicate that

all three proteins display identical NO-binding properties, which closely match the properties of the whole-gland homogenates. The summed content of these proteins (293 pmol per gland pair) also matches the total content of NO, 282 pmol per pair, measured in the absence of Hg²⁺.

Nitric oxide has recently been identified as an important signal molecule involved in the regulation of vascular tone in vertebrates (2); its presence in *Rhodnius* saliva suggests a remarkable case of convergent evolution. The *Rhodnius* heme proteins are well suited to enhance feeding success by unloading the vasodilatory NO as they are diluted in the host bloodstream; further, NO exhibits antiplatelet activity and may help inhibit the formation of the platelet plug (2). Although NO synthesis is related to a variety of functions in vertebrates (2), it has previously been reported only once in another invertebrate (16). It remains to be seen whether the ability to produce NO is a phylogenetically old trait or whether it has arisen independently in different lineages.

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Regulation of TCR Signaling by CD45 Lacking Transmembrane and Extracellular Domains

Siniša Volarević, Barbara B. Niklinska, Christopher M. Burns, Carl H. June, Allan M. Weissman, Jonathan D. Ashwell*

The CD45 protein is a transmembrane tyrosine phosphatase that is required for normal T cell receptor (TCR)-mediated signaling. A chimeric complementary DNA encoding the intracellular enzymatically active portion of murine CD45 preceded by a short amino-terminal sequence from p60^{c-src} was transfected into CD45⁻ T cells. Expression of this chimeric protein corrected most of the TCR signaling abnormalities observed in the absence of CD45, including TCR-mediated enhancement of tyrosine kinase activity and Ca²⁺ flux. Thus, the enzymatically active intracellular portion of CD45 is sufficient to allow TCR transmembrane signaling.

Stimulation of T cells through the antigen-specific receptor (TCR) initiates increases in tyrosine phosphorylation (1), phosphatidylinositol hydrolysis, and intracellular Ca²⁺ concentration ([Ca²⁺]_i) (2). Studies

of T cell lines that are deficient in expression of CD45 have established that this transmembrane molecule, whose intracytoplasmic domains have intrinsic tyrosine phosphatase activity (3, 4), participates in coupling the TCR to these activation events (5, 6). Studies of CD45-deficient variants of a murine T cell line, YAC-1,[†] have shown that CD45 expression is inversely related to spontaneous tyrosine phosphorylation of a number of substrates, including the ζ chain of the TCR. In contrast to the typical rapid [Ca²⁺]_i elevation exhibited by the YAC-1 wild-type

S. Volarević, B. B. Niklinska, C. M. Burns, J. D. Ashwell, Biological Response Modifiers Program, Laboratory of Immune Cell Biology, National Cancer Institute, National Institutes of Health, Bethesda, MD 20892.

C. H. June, Immune Cell Biology Program, Naval Medical Research Institute, Bethesda, MD 20889.

A. M. Weissman, Experimental Immunology Branch, National Cancer Institute, National Institutes of Health, Bethesda, MD 20892.

*To whom correspondence should be addressed.

(WT) cells, stimulation of the CD45⁻ cells with monoclonal antibodies (mAb) to CD3 resulted in delayed, asynchronous [Ca²⁺]_i oscillations (6).

Experiments were done to determine if expression of the intracellular portion of CD45 alone is sufficient to restore normal TCR-mediated signaling in CD45⁻ T cells. Since the *in vivo* biological activity of CD45 might be dependent upon its location in the plasma membrane, a cDNA that encoded a chimeric molecule [the NH₂-terminal first 15 amino acids of pp60^{v-src} (Src) and the enzymatically active intracellular portion of murine CD45] was created (myr-iCD45, Fig. 1A). The first 14 amino acids of Src constitute a sequence that results in its own myristylation, and can direct heterologous proteins to the plasma membrane (7). Two independent CD45⁻ mutants, N1 and N2, were transfected, and stable neomycin-resistant transfectants from both N1 (N1.S100) and N2 (N2.S1 and N2.S4) cells were obtained. Expression of the TCR, as judged by flow cytometry, was comparable in all of the cells (6, 8). Polymerase chain reaction (PCR) amplification with oligomers specific for the Src myristylation sequence (5') and COOH-terminal CD45-sequence (3') detected chimeric transcripts from N2.S1 and N1.S100, but not WT, N1, N2, or N2.S4 cells (8). To assess the amount of myr-iCD45 protein, detergent lysates of cells were analyzed by immunoblotting with an antiserum to the cytoplasmic domains of CD45 (4). Full-length CD45 was detected only in WT

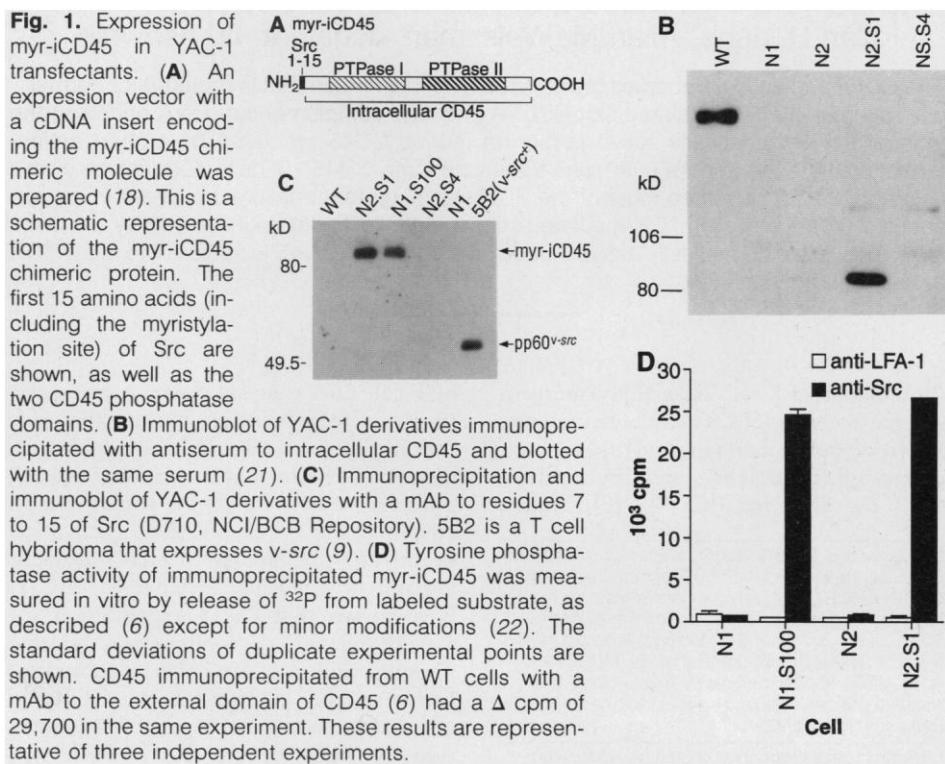
cells. In agreement with the PCR analysis, this antiserum detected a protein with the predicted molecular size of the myr-iCD45 chimeric molecule (~83 kD) in N2.S1, but not N1, N2, or N2.S4 cell lysates (Fig. 1B). A few nonspecific bands of larger molecular size were detected but did not correlate with expression of the chimeric molecule. A mAb to the NH₂-terminal portion of Src was used to immunoprecipitate and for immunoblotting and detected a molecule of the predicted size for myr-iCD45 in detergent lysates of N2.S1 and N1.S100 cells but not the untransfected cells or N2.S4 (Fig. 1C). Also shown is a control T cell hybridoma that was transfected with pp60^{v-src} (9); anti-Src detected pp60^{v-src} but not the higher molecular size band that represents myr-iCD45. Myr-iCD45 was also detected in total membrane preparations made from N2.S1 and N1.S100 cells, indicating that at least a portion of this molecule was directed to membranes (8), although the subcellular distribution of myr-iCD45 is not yet characterized. Finally, the mAb to Src specifically precipitated large and similar amounts of tyrosine phosphatase activity from N1.S100 and N2.S1 but not N1 or N2 cells, indicating that the myr-iCD45 chimera is enzymatically active (Fig. 1D).

Loss of CD45 expression in YAC-1 cells results in spontaneous tyrosine hyperphosphorylation of a number of proteins (6). To determine if the myr-iCD45 chimeric protein would substitute for WT CD45 protein in regulation of tyrosine phosphorylation,

constitutive tyrosine phosphorylation was analyzed (Fig. 2A). Immunoprecipitation and immunoblotting with a mAb to phosphotyrosine detected a number of hyperphosphorylated species in the CD45⁻ N1 cell and the G418-resistant N2.S4 cell that does not express the myr-iCD45 chimeric molecule. The most prominent species included a doublet at 21 and 23 kD, and bands at 38, 56, and 71 kD. In contrast, expression of myr-iCD45 resulted in a return of a phosphotyrosine profile similar to the WT cells, with the exception of the prominent band at 56 kD in N2.S1 cells. The reason for this difference between the two myr-iCD45-expressing cells is not known. The doublet at 21 and 23 kD migrated similarly to phosphorylated ζ chain of the TCR in YAC-1 cells, which is spontaneously hyperphosphorylated in the absence of CD45 (6). The state of ζ phosphorylation was directly determined by immunoprecipitation with serum to ζ and immunoblotting with anti-phosphotyrosine antibodies (Fig. 2B). No phospho- ζ was detected in WT cells, whereas the CD45⁻ N1 and N2.S4 cells expressed their characteristic phospho- ζ doublet. Although a small amount was detected, ζ phosphorylation was minimal in both of the myr-iCD45-expressing cells, N2.S1 and N1.S100 (Fig. 2B).

Fluctuations in [Ca²⁺]_i that can be induced by stimulation through the TCR were assessed. WT cells had an early increase in [Ca²⁺]_i after stimulation (6) (Fig. 3A). CD45⁻ N1 and N2.S4 cells responded with a small and delayed [Ca²⁺]_i increase, which reflects delayed and asynchronous Ca²⁺ oscillations in these cells after stimulation with mAbs to CD3 (anti-CD3) (6). The myr-iCD45⁺ N2.S1 and N1.S100 cells responded to anti-CD3 with kinetics similar to WT and with an even greater increase in mean [Ca²⁺]_i than seen in WT cells. This enhanced response by N2.S1 and N1.S100 cells was reproduced in three independent experiments and is due to an increase in the fraction of responding cells (60% of WT cells versus 80% of myr-iCD45 transfectants) (Fig. 3B) as well as higher [Ca²⁺]_i in the responding population. Whether the difference in the maximal stimulated [Ca²⁺]_i between WT and myr-iCD45-transfected cells was a reflection of some property that differs between whole and truncated CD45 requires the analysis of more transfectants.

Activation-induced tyrosine phosphorylation of the TCR ζ subunit, another early event in TCR signaling, was also examined (Fig. 4). Unstimulated WT cells had a small amount of spontaneous ζ tyrosine phosphorylation that was rapidly (within 5 min) enhanced by treatment with anti-CD3. TCR ζ from the CD45⁻ cells was already hyperphosphorylated and did



not exhibit any increase upon stimulation. Expression of the myr-iCD45 chimeric molecule resulted in spontaneous ζ tyrosine phosphorylation equivalent to WT cells, and stimulation with anti-CD3 once again increased phosphorylation. Similar results were obtained with N1.S100 cells (10).

Our data show that the intracellular portion of CD45, in the absence of the transmembrane and external domains, is sufficient to restore baseline tyrosine phosphorylation of most substrates and activation-induced Ca^{2+} flux and tyrosine kinase activity. The CD45 extracellular domain is 391 to 552 amino acids in length, depending on the isoforms generated by alternate mRNA splicing, and is heavily glycosylated (11). This variability in the extracellular domain, and the precise regulation of the pattern of isoform expression during lymphoid development and activation, implies a functional significance for this part of the molecule (12). The CD45 molecule may be a receptor protein tyrosine phosphatase that is regulated by ligand binding (13). The human B cell molecule CD22 binds the T cell CD45RO form (lacking exons 4, 5, and 6) (14). In addition, T cell surface CD45 interacts with surface molecules on the same cell, such as the TCR, Thy-1, CD2, and CD4 (15, 16). There may be some isoform selectively in these associations, in that CD4 cocaps with isoforms other than

CD45RB but not CD45RB itself (16). If antibodies are used to bring CD45 into proximity with these molecules, the resulting signals may be stimulatory or inhibitory, depending on the particular interactions (17). Whatever the ligand for CD45, its binding may regulate enzymatic activity or substrate interactions and hence directly affect signal transduction. In the case of YAC-1 cells, the transmembrane and external portions of CD45 appear to be "dispensable"; it is still possible, however, that they might participate in regulating the response to a more physiologic stimulus, such as antigen presented on the surface of an

antigen-presenting cell. Our results show that the transmembrane and extracellular portions of CD45 are not required to regulate tyrosine phosphorylation in the resting state or for transmembrane signaling that is induced by antibody perturbation of the TCR.

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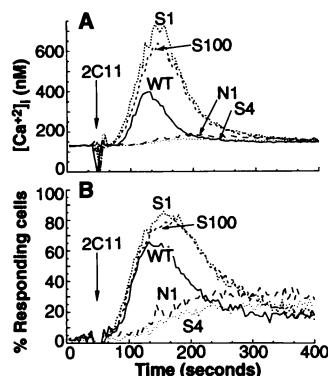


Fig. 3. TCR-mediated increases in $[Ca^{2+}]_i$. (A) Mean $[Ca^{2+}]_i$, and (B) percent of responding cells after stimulation with anti-CD3. The $[Ca^{2+}]_i$ was determined by flow cytometry according to the procedure of Rabinovitch and June (24). Briefly, YAC-1 cells were loaded with 2.5 μ M indo-1 (Molecular Probes, Junction City, Oregon) in HBSS for 25 min at 31°C. After establishing base-line values, purified mAb to CD3, 145-2C11 (25), was added to make a final concentration of 20 μ g/ml. Cells were analyzed for violet:blue fluorescence emission ratio (395 nm:500 nm) with an Ortho CytoFluorograph flow cytometer (Westwood, Massachusetts). A standard curve was used to convert this ratio to absolute $[Ca^{2+}]_i$. Cells with elevated $[Ca^{2+}]_i$ (>2 SD above base line) were taken as responding cells.

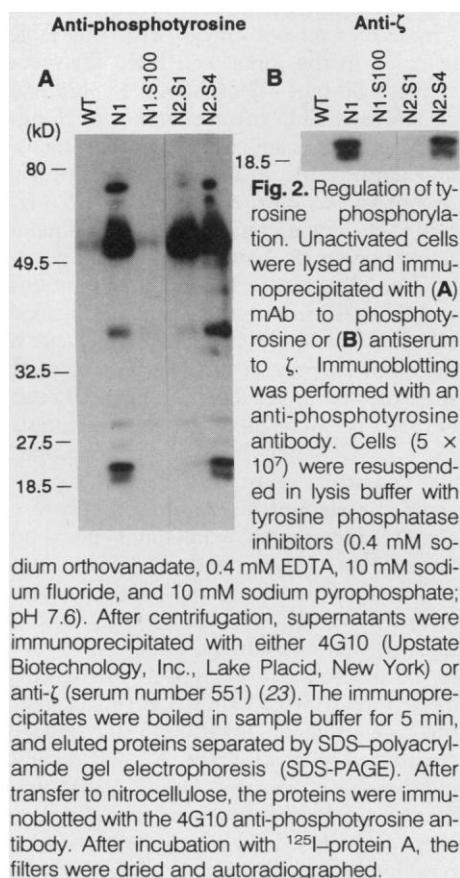


Fig. 2. Regulation of tyrosine phosphorylation. Unactivated cells were lysed and immunoprecipitated with (A) mAb to phosphotyrosine or (B) antiserum to ζ . Immunoblotting was performed with an anti-phosphotyrosine antibody. Cells (5×10^7) were resuspended in lysis buffer with tyrosine phosphatase inhibitors (0.4 mM sodium orthovanadate, 0.4 mM EDTA, 10 mM sodium fluoride, and 10 mM sodium pyrophosphate; pH 7.6). After centrifugation, supernatants were immunoprecipitated with either 4G10 (Upstate Biotechnology, Inc., Lake Placid, New York) or anti- ζ (serum number 551) (23). The immunoprecipitates were boiled in sample buffer for 5 min, and eluted proteins separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). After transfer to nitrocellulose, the proteins were immunoblotted with the 4G10 anti-phosphotyrosine antibody. After incubation with ¹²⁵I-protein A, the filters were dried and autoradiographed.

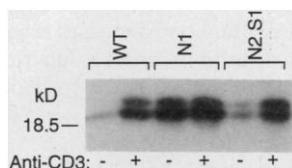


Fig. 4. Anti-CD3-induced tyrosine phosphorylation of the ζ chain of the TCR. WT (CD45⁺), N1 (CD45⁻), and N2.S1 (myr-iCD45⁺) cells were washed in ice-cold PBS and resuspended in complete medium at a density of 5×10^7 cells/ml with or without 30 μ g/ml of anti-CD3 (145-2C11) for 5 min at 37°C. The cells were then washed in ice-cold PBS and the cell pellets immediately frozen. Detergent lysates of these cells were immunoprecipitated with anti- ζ (serum number 551) and immunoblotted with anti-phosphotyrosine as described in Fig. 2.

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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. [^{32}P] angiotensin II (Sigma) was phosphorylated in vitro with p60^{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

Robin R. Hovis, Jerald A. Donovan, Michael A. Musci, David G. Motto, Frederick D. Goldman, Susan E. Ross, Gary A. Koretzky*

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- γ 1 (PLC- γ 1) 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

R. R. Hovis, J. A. Donovan, S. E. Ross, Department of Internal Medicine, University of Iowa College of Medicine, Iowa City, IA 52242.

M. A. Musci, University of Iowa College of Medicine, Iowa City, IA 52242.

D. G. Motto, Department of Physiology and Biophysics, University of Iowa College of Medicine, Iowa City, IA 52242.

F. D. Goldman, Department of Pediatrics, University of Iowa College of Medicine, Iowa City, IA 52242.

G. A. Koretzky, Departments of Internal Medicine and Physiology and Biophysics, University of Iowa College of Medicine, Iowa City, IA 52242.

*To whom correspondence should be addressed.