FUNCTIONAL EXPRESSION OF THE MURINE Thy-1.2 GENE IN TRANSFECTED HUMAN T CELLS

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The stimulation of resting T lymphocytes to enter the cell cycle and to initiate transcription of a variety of genes encoding growth factors and immune mediators represents both an intriguing model system for exploring transmembrane signaling events and a topic of intrinsic interest to immunologists. Initiation of T cell proliferation and differentiation has been induced through the use of specific antigens, with plant lectins such as PHA or Con A, or via mAbs to a variety of cell surface molecules. Recent studies have indicated that all three types of stimuli may in fact operate through a common pathway involving the T3 (CD3) complex associated with the clonally distributed receptors on T cells (the T3-T cell antigen receptor [Ti][1] complex). Thus, it has been found that mAbs to either Ti on human T cells or to the T3 complex can mimic the effects of antigen and produce a rapid increase in intracytoplasmic calcium concentration ($[Ca^{2+}]_i$), IL-2 production, IL-2-R expression, and ultimately, T cell proliferation (1–3). Weiss and Stobo (4) have also shown an obligate requirement for the coexpression of T3 and the antigen receptor and observed that expression of these molecules appears to be required for the activation of a human T cell line by the lectin PHA.

On human T cells, two additional structures have been shown to be capable of transducing activation signals. A combination of two mAbs against distinct epitopes on the E-rosette receptor (T11) can stimulate human T cells (5). Others have shown that an mAb against a 44-kD glycoprotein (the 9.3 antigen), expressed on the majority of human peripheral T cells, can also fully activate human T lymphocytes (6) when combined with PMA. It has been proposed that triggering by the anti-T11 mAbs and the mAb against the 9.3 antigen is independent of the T3-Ti complex, even though antibody-induced modulation of the T3 complex resulted in the blockade of the T11 pathway of activation (5). The relationship of the T11 and 9.3 antigens to the activation produced by plant lectins has not been clearly defined. However, O'Flynn et al. (7) have shown that certain anti-T11 mAbs, which by themselves do not activate T cells, can selectively inhibit the rise in $[Ca^{2+}]_i$ induced by PHA, but not Con A or anti-T3 mAbs, and have concluded from this observation that PHA binds directly to

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[1] *Abbreviations used in this paper:* FMF, flow microfluorimetry; IP_3 inositol trisphosphate; Ti, T cell antigen receptor.

the T11 molecule, resulting in mobilization of Ca^{2+} and subsequent proliferation. Taken together, these results suggest the existence of multiple cell surface molecules capable of initiating intracellular T lymphocyte signaling events. The question of whether they belong to distinct activation pathways or converge on a common central mechanism remains unanswered.

Although mAbs against the murine equivalents of T3, T11, and 9.3 have not as yet been described, we and others (8–10) have shown that certain mAbs against the T cell differentiation antigen Thy-1 can activate T cells in a manner similar to that seen with plant lectins, leading to a polyclonal induction of IL-2 production, IL-2-R expression, proliferation, and a rapid increase in $[Ca^{2+}]_i$ (manuscript in preparation). The ability of Thy-1 to initiate murine T cell activation is of particular interest in light of its restricted tissue distribution (high on T cells, brain, and certain hematopoietic precursors [11, 12]), its pattern of ontogenetic expression (it appears on the earliest identified intrathymic precursors but not on their immediate progenitors [13]), and its structure (homologous to an immunoglobulin domain and apparently bound to the membrane via a glycolipid tail [14]). These features of Thy-1 suggest that this molecule may represent a broadly distributed receptor for stimulation of differentiation of a variety of cell types. An increased understanding of the relationship of Thy-1 structure to its ability to transduce extracellular signals, and of the biochemical basis for signal transmission, would therefore seem particularly valuable.

Our goals were to use mitogenic mAbs against Thy-1 in concert with transfection of a cloned Thy-1 gene, first to analyze the precise role of Thy-1 in T cell activation and then to determine the relationship between anti-Thy-1–induced activation, and that produced in response to plant lectins or to stimulation via the T3-Ti complex. Our results demonstrate that Thy-1 can be expressed at high levels following transfection of the human T cell line, Jurkat, and that mAbs to Thy-1 activate all Thy-1^+ transfectants in the absence of other murine cell surface molecules. The failure of one Thy-1^+ transfectant to produce IL-2 following stimulation via the T3-Ti complex despite a rise in $[Ca^{2+}]_i$, provides evidence for a multistep signaling process in T cell activation.

Materials and Methods

Cells and Cell Culture Conditions. The human T cell tumor line Jurkat (15) clone E6.1, was generously supplied by Dr. A. Weiss, University of California, San Francisco. EL4, a murine T lymphoma, was obtained from American Type Culture Collection, Rockville, MD. CTLL is an IL-2-dependent murine T cell line, used in a bioassay to measure IL-2 levels (16). All cells were maintained at 37°C, 7.5% CO_2 in DME with 4.5 g glucose/liter supplemented with nonessential amino acids (0.1 mM), sodium pyruvate (1 mM), glutamine (300 μg/ml), penicillin (100 U/ml), streptomycin (100 μg/ml), 2-mercaptoethanol (5×10^{-5} M), and 10–20% fetal calf serum; gentamicin (100 μg/ml) was added to spheroplast fusion cultures.

Construction of a Thy-1.2 Transfection Vector. A recombinant Charon 28 λ bacteriophage library derived from BALB/c DNA was screened with a mouse Thy-1.1 cDNA probe generously provided by S. M. Hedrick and M. M. Davis (17). The cDNA clone (~800 bp long) was confirmed to encode Thy-1.1 and to contain 5′ and 3′ untranslated regions by partial sequence analysis and restriction mapping. Three different Thy-1.2 genomic clones were isolated from the genomic library. For transfection experiments, an 8.1-kb Eco RI fragment derived from one genomic clone and known to contain a complete Thy-1.2 gene (18, 19) was ligated into the Eco RI site of the pSV2gpt vector (20) (Fig. 1).

The orientation of the Thy-1.2 gene within the vector was determined by restriction mapping.

Spheroplast Transfection. Spheroplasts were produced from *E. coli* strain HB101 bacteria harboring the pSV2gpt–Thy-1.2 vector as previously described (21, 22). Spheroplasts were fused to the E6.1 Jurkat line by the polyethylene glycol method and cultured at 10^5 Jurkat cells per well in 96-well microtiter culture dishes. After 48 h cultures were adjusted to the final concentration of selective medium (1.0 μg/ml mycophenolic acid, 250 μg/ml xanthine, and 15 μg/ml hypoxanthine). Cultures were fed weekly; growing colonies were detected 3–4 wk after fusion, expanded, and screened for Thy-1.2 expression by flow microfluorimetry (FMF) analysis. All Jurkat colonies that grew out of selection expressed Thy-1.2. These colonies were cloned at limiting dilution and reassayed for Thy-1.2 expression by FMF. All Jurkat clones have been phenotypically stable for Thy-1.2 expression, even when taken out of selective media for as long as 2 mo of continuous culture.

FMF Analysis. The cell population (10^6) selected for analysis was first incubated with an excess of either mAb culture supernatant or ascites for 30 min at 4°C, and then washed three times with HBSS containing 0.1% NaN_3 and 3% FCS. The cells were then stained with an excess of FITC-conjugated monoclonal mouse anti–rat κ chain antibody (MAR 18.5) (23) or FITC-conjugated goat anti–mouse Fab antisera (obtained from B. J. Fowlkes, National Institutes of Health). The stained cells were then analyzed on an EPICS V flow cytometer (Coulter Electronics, Inc., Hialeah, FL). Data are displayed as cell number (abscissa) vs. relative log fluorescence (ordinate).

RNA and DNA Blot Analysis. Cytoplasmic RNA and genomic DNA were prepared as previously described (24, 25). 10 μg of cytoplasmic RNA from each cell line to be analyzed were electrophoresed on a 1% agarose-formaldehyde gel, transferred to nitrocellulose, and hybridized to the nick-translated Thy-1.1 cDNA probe. For DNA blotting, 10 μg of genomic DNA from various cell lines were digested with Hind III restriction enzyme, electrophoresed on a 0.7% agarose gel, denatured, and transferred to nitrocellulose. The nitrocellulose-bound restriction fragments were then hybridized to nick-translated pBR322 plasmid DNA. Hybridization and washing conditions were as described previously (22). Autoradiographs were produced using intensifier screens at −70° for 24–48 h.

Monoclonal Antibodies. The properties of G7, a rat mAb to Thy-1 with T cell activating properties, have been previously described (9). A mouse cell line producing mAb to the human T3 (CD3) complex, OKT3, was obtained from American Type Culture Collection, Rockville, MD. AT15E is a rat mAb to Thy-1 (26) which has activating properties (8). I-22, III-5, and V-8 are mitogenic rat mAbs to Thy-1 (10) and were generously supplied by H. R. MacDonald, Laussane, Switzerland. C.305 is an mAb to the antigen receptor on Jurkat cells (4) and was kindly provided by A. Weiss, San Francisco CA. 2A3, a rat mAb to Thy-1 that was used in immunoprecipitation experiments, and 3A7 and 3H11, which were used in some activation experiments, are mAbs to Thy-1 that have been previously described (27). A mixture of anti-Thy-1 mAbs was used in some FMF experiments and in negative selection studies. It consisted of the following rat mAbs: G7, 3A7, 3H11, and 2A3 (described above), 2D4, and 4D11 (27). A mouse mAb to the Thy-1.2 allele (28) was used in one FMF experiment.

Cell Surface Labeling, Immunoprecipitation, and SDS-PAGE. 10^8 cells were labeled with $Na^{125}I$ by lactoperoxidase-catalyzed cell-surface iodination. Labeled membrane proteins were then extracted with 0.5% Triton X-100 in a 0.05 M Tris, and 0.3 M NaCl (pH 7.6) buffer containing 1 mM PMSF, 10 μg/ml leupeptin, and 10 mM iodoacetamide. For immunoprecipitation mAb 2A3 supernatant was reacted with MAR 18.5-Sepharose for 2 h at 4°C. These antibody-coupled Sepharose beads were then washed and reacted with the cell extract for 1 h at 4°C with continuous agitation. Immunoprecipitates were then washed three times in the same buffer used for membrane protein solubilization, except that the Triton X-100 concentration was reduced to 0.1%. The precipitated samples were then boiled for 5 min in buffer containing 5% SDS and 0.1 M DTT, then subjected to electrophoresis in 12.5% acrylamide slab gels containing 0.2% SDS. After electrophoresis,

the gel was fixed in 20% TCA, stained with Coomassie blue, destained, and dried; autoradiographs were produced using an intensifier screen at −70°C for 24 h.

Mutagenesis and Selection. A Thy-1.2-transfected Jurkat clone, 21C3.2F7, was cultured in ethyl methanesulfonate (200 μg/ml) for 24 h. Cells were taken out of selection media, cultured for four additional days, and then were negatively selected by treatment with a mixture of anti-Thy-1.2 mAbs (see above) at 1:200 dilution and rabbit complement at 1:5 dilution. Surviving cells were subsequently treated with the anti-Thy-1 mAb mixture and complement two additional times during the next 3 wk of culture. Cells not staining with the anti-Thy-1 mixture were sorted by preparative FMF and cloned by limiting dilution.

IL-2 Production Assay. Stimulation of Jurkat or transfected Jurkat cells was done in microtiter wells at 10^5 cells/well. Cells were stimulated with PHA at 1 μg/ml, the calcium ionophore A23187 at 100 ng/ml, OKT3 ascites at 1:1,000 dilution, C.305 culture supernatant at 1:50, anti-Thy-1 mAb ascites at 1:200, or anti-Thy-1 mAb culture supernatant at 1:10. In all cases PMA was present at 50 ng/ml. After 36 h of culture, IL-2 activity was assessed by measuring [^{3}H]TdR uptake of the IL-2-dependent line CTLL in response to a 25% concentration of the test supernatants. Means of triplicate determinations are given. The standard errors were usually <10% of the means. Neither PHA, A23187, PMA, nor any of the mAbs used in this study had inhibitory or stimulatory effects on CTLL.

Determination of Free Intracytoplasmic Calcium Concentration. In a modification of the method described by Tsien et al. (29), cells were loaded at 5×10^6/ml in DMEM containing 10% FCS with a final temperature of 10 μM Quin 2-AM (Sigma Chemical Co., St. Louis, MO) for 30 min. After being loaded, the cells were washed two times with Dulbecco's PBS, resuspended in the same buffer to a concentration of 5×10^6/ml, and used immediately after equilibration at 37°C for 5 min. Fluorescence intensity was measured with a fluorescence spectrophotometer (model LS-5; Perkin-Elmer Corp., Norwalk, CT) (excitation 339 nm, emission 492 nm). The cuvette chamber was maintained at 37°C, and the cell suspension was continuously stirred. After addition of $MnCl_2$ (5 μM final) to quench the fluorescence of any Quin 2 present in the extracellular compartment, a baseline was established. Antibody was added at a final concentration of 1:100 ascites. Minimum fluorescence (F_{min}) was determined by lysing the Quin-2 loaded cells with 0.1% Triton X-100 in the presence of 4 mM EDTA and sufficient Tris-buffer to raise the pH to 8.0 (to optimize chelation of Ca^{2+} by EDTA). Maximum fluorescence (F_{max}) was obtained after adding excess Ca^{2+} ($CaCl_2$, 10 mM final). Approximate values for cytoplasmic free Ca^{2+} were calculated by the formula: $[Ca^{2+}]_{ix} = 115 \text{ nM } (F_x - F_{min})/(F_{max} - F_x)$ (29).

Results

Expression of Thy-1.2 in Human Jurkat Cells. The human Jurkat line E6.1 was transfected by spheroplast fusion with the pSV2gpt–Thy-1.2 construct (Fig. 1). Expression of the *Eco-gpt* gene in transfected cells enabled selection and long-term maintainance of cells in the presence of mycophenolic acid, hypoxanthine, and xanthine. Individual growing colonies visible after ~3–4 wk were expanded, stained with a mixture of anti-Thy-1 mAb, and screened for Thy-1 expression by FMF. A cocktail of mAbs was used to reduce the probability of a false negative result in the screening assay, because of the possibility of selective epitope loss in transfected human cells. All colonies that grew out of selection medium showed strong membrane Thy-1 immunofluorescence. After cloning by limiting dilution, individual clones were again analyzed for Thy-1 expression by FMF (Fig. 2 illustrates the results of a representative experiment of this type). The E6.1 Jurkat clone and a transfected clone, 21C3.2F7, were each stained separately with antibodies against the human T3 complex and against the murine Thy-1.2 antigen. The anti-Thy-1.2 mAb did not react with the Jurkat line above back-

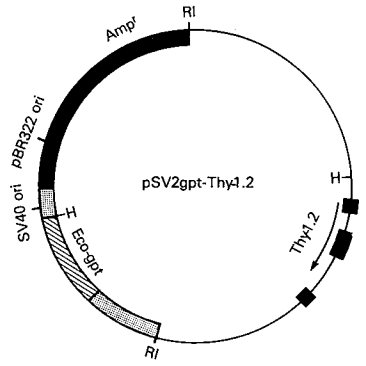

FIGURE 1. The pSV2gpt–Thy-1.2 transfection vector was constructed as described in Materials and Methods. Eco RI and Hind III restriction sites are indicated by RI and H, respectively. The 8.1-kb genomic Eco RI fragment containing the Thy-1.2 gene is indicated by the thin line, with dark boxes denoting the location of exons encoding Thy-1.2. The direction of transcription is indicated. The portion of the vector derived from pBR322 containing the ampicillin resistance gene is shown as a thick line. The portions of the vector derived from SV40 are shown as stippled. and the *Eco-gpt* gene is shown by crosshatching.

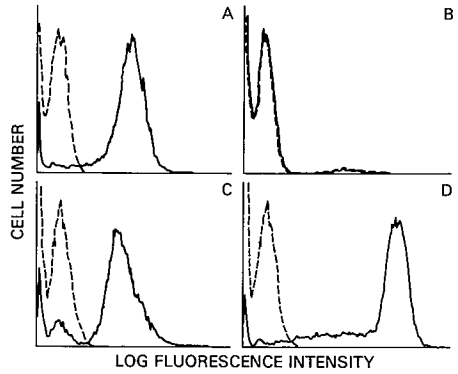

FIGURE 2. OKT3 and anti-Thy-1.2 staining of Jurkat and a Jurkat Thy-1.2 transfectant. The E6.1 clone of Jurkat (*A, B*) and a Thy-1.2-tranfected Jurkat clone, 21C3.2F7 (*C, D*), were either stained with OKT3 ascites (*A, C*) or an anti-Thy-1.2 ascites (*B, D*). FITC-conjugated second antibody in the absence of first antibody is shown as a control (-----).

ground levels, while it brightly stained the transfected clone 21C3.2F7 (Fig. 2, *B* and *D*). OKT3 staining was approximately equivalent for both the Jurkat and the Thy-1-transfected Jurkat clone. This maintenance of T3 expression was seen with all Thy-1 transfectants (data not shown). Although fluorescence levels of different mAbs are difficult to compare quantitatively, saturating amounts of mAbs were used in all cases and it appears that the transfected Thy-1.2 gene was being expressed at a greater level than the endogenous T3 protein product. This was also observed in all other transfected clones studied.

The expression of Thy-1.2 in Jurkat cells was further analyzed by RNA blotting (Fig. 3*A*) and by immunoprecipitation (Fig. 3*B*). In Fig. 3*A*, equal amounts of cytoplasmic RNA from the murine T cell line CTLL (lane *A*), the E6.1 Jurkat line (lane *B*), and a Thy-1.2-transfected Jurkat clone (lane *C*) were electrophoresed in formaldehyde agarose gels, transferred to nitrocellulose, and hybridized

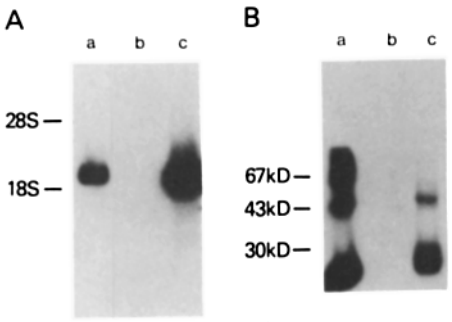

FIGURE 3. Thy-1 mRNA and protein expression in transfected Jurkat cells. (*A*) Cytoplasmic RNA (10 μg) from the CTLL line (lane *a*), the E6.1 Jurkat clone, (lane *b*), and a Thy-1-transfected Jurkat clone 21C3.2F7 clone (lane *c*) was electrophoresed on a 1% agarose-formaldehyde gel, transferred to nitrocellulose, and hybridized to nick-translated Thy-1.1 cDNA. The position of the 28 S and 18 S ribosomal RNA bands are indicated. (*B*) EL4 (lane *a*), Jurkat (lane *b*), and a Thy-1 transfected Jurkat clone, 21C3.2F7 (lane *c*) were surface labeled with ^{125}I and lysates prepared from equal number of cells subjected to immunoprecipitation with an anti-Thy-1 mAb bound to Sepharose beads, as described in Materials and Methods. Molecular mass markers (in kilodaltons) are shown on the left.

with a Thy-1.1 cDNA probe. Using stringent washing conditions after hybridization, RNA from the E6.1 nontransfected Jurkat line failed to hybridize, while strong hybridization was detected with RNA from the Thy-1.2 transfected cell. The Thy-1 message from the CTLL and 21C3.2F7 cell lines comigrated; however, there appeared to be a significantly greater amount of Thy-1 mRNA in the transfected Jurkat cells compared with CTLL.

To analyze the membrane expression of the Thy-1.2 molecule, cell surface proteins from a mouse T cell line, EL4, the E6.1 Jurkat line, and the Thy-1.2 transfectant 21C3.2F7, were iodinated, extracted with Triton X-100, immunoprecipitated with an antibody to Thy-1, and analyzed by SDS-PAGE (Fig. 3*B*). A broad band of ~25 kD was seen in precipitates from EL4 (lane *A*). In addition, a band at ~50 kD (p50) and other, less intense bands at higher molecular weights were immunoprecipitated. No bands were detectable in immunoprecipitates from Jurkat lysates (lane *B*). A major band of ~25 kD and a minor band of 50 kD were immunoprecipitated from the Thy-1.2-transfected Jurkat cell, both of which comigrated with similar bands precipitated from EL-4. The p50 band has been previously observed in anti-Thy-1 immunoprecipitates (9) and peptide mapping data indicate that p50 may be a dimer of the 25-kD Thy-1 molecule (Lögdberg, L., and K. C. Gunter, unpublished data).

Thy-1-transfected Jurkat Cells Express Some, But Not All, Thy-1 Epitopes. We have previously described an mAb against the Thy-1 molecule, G7, which could induce lymphokine production by and proliferation of resting murine T cells. The Jurkat line (E6.1) has been shown to produce IL-2 when reacted with mAbs against the T3 complex in combination with PMA (15). As an initial approach to the analysis of the function of Thy-1 in T cell activation, we intended to test whether the Thy-1.2-transfected Jurkat cell would secrete IL-2 when stimulated with the mitogenic mAb G7 plus PMA. However, when we analyzed a transfected Jurkat clone for G7 binding by FMF, no staining above background was seen (Fig. 4), although G7 reacted strongly with the murine T lymphoma, EL4 (data

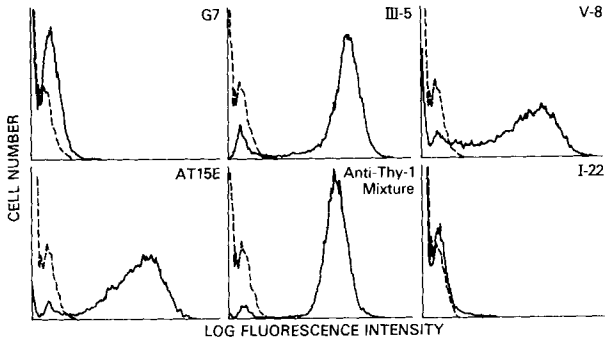

FIGURE 4. Staining of Thy-1.2-transfected Jurkat clone with a panel of mitogenic anti-Thy-1 mAbs. The indicated mAbs were reacted with the Thy-1.2-transfected Jurkat clone 21C3.2F7 followed by the second antibody, FITC MAR 18.5. FITC-conjugated second antibody in the absence of first antibody is shown as a control (----).

not shown). G7 has failed to bind to all Thy-1.2-transfected Jurkat clones tested (data not shown). Furthermore, the absence of the G7 epitope is not restricted to Jurkat transfectants, because when murine B cell lymphomas or macrophage lines were transfected with the same pSV2gpt–Thy-1.2 vector, Thy-1.2 molecules were expressed on the cell surface, but G7 failed to bind (manuscript in preparation).

The failure of the transfected cells to express the epitope recognized by mAb G7 prompted a more complete survey of transfected Jurkat lines with a panel of mitogenic anti-Thy-1 mAb (Fig. 4). Three out of four such mAbs bound to a Thy-1.2-transfected Jurkat cell, while mAb I-22 failed to bind. The mAbs V-8 and AT15E showed a broad distribution of fluorescence compared with III-5 or the cocktail of rat anti-Thy-1 mAbs. The basis for this apparent heterogeneity of binding is not known, although it varied from experiment to experiment. These anti-Thy-1 mAbs also have several differences in their activating properties compared with the prototype mAb, G7. G7 recognizes both the Thy-1.1 and Thy-1.2 alleles, and has been shown to stimulate IL-2 secretion from T cell hybridomas and normal spleen cells. Furthermore, the G7 mAb can stimulate proliferation of resting T cells from several strains of mice (10). The I-22, III-5, and V-8 mAbs have been shown to stimulate macrophage activating factor secretion from cytolytic T lymphocyte lines, but only one of these three mAbs (III-5) is capable of stimulating IL-2 production by and proliferation of normal spleen cells (10). The AT15E mAb does not stimulate resting T cell proliferation (9), but elicits IL-2 production from the EL4 line (8) and can stimulate macrophage activating factor secretion from at least one cytolytic T lymphocyte clone (10).

Functional Properties of the Thy-1-expressing Jurkat Cells. As a correlate to the binding experiment described above, one Thy-1.2-transfected Jurkat clone was tested for its ability to secrete IL-2 when stimulated with a panel of anti-Thy-1.2 mAbs plus PMA (Table I). As seen in this representative experiment, all the anti-Thy-1 mAbs that bound to the transfected Jurkat clone stimulated IL-2 secretion in the presence of PMA, but had no effect on the nontransfected E6.1 Jurkat clone. In addition, the combination of anti-Thy-1 mAbs 3A7 and 3H11 was

TABLE I
Anti-Thy-1–stimulated IL-2 Production from Jurkat and Thy-1.2-transfected Jurkat

Stimulus	Responding cell line	
	E6.1	21C3.2F7
Medium	949	1,008
PMA	2,743	2,555
III-5 + PMA	5,005	38,018
V-8 + PMA	3,924	34,520
I-22 + PMA	4,662	1,311
AT15E + PMA	3,872	16,141
G7 + PMA	2,941	3,401
3A7 + 3H11 + PMA	3,864	41,942

The Jurkat line (E6.1) and a Thy-1.2-transfected clone (21C3.2F7) were cultured with anti-Thy-1 mAbs (1:10 supernatant: III-5, V-8, I-22, and AT15E or 1:200 ascites: G7, 3A7, and 3H11) and/or PMA (50 ng/ml) as described in Materials and Methods. Supernatants were harvested and IL-2 content was determined by [^{3}H]TdR uptake of an IL-2–dependent cell line, CTLL. Data are expressed as mean cpm.

capable of stimulating IL-2 secretion from the transfected Jurkat cell. These two mAbs have been shown to act in a synergistic manner to stimulate normal mouse T cell activation in the presence of PMA, but neither mAb can activate by itself (manuscript in preparation). None of the anti-Thy-1 mAbs was capable of stimulating IL-2 secretion from the transfected cell in the absence of PMA (data not shown). In a similar fashion, OKT3 mAbs do not stimulate appreciable IL-2 secretion from Jurkat cells in the absence of PMA (15). The activation induced by the anti-Thy-1 mAbs was specific because no IL-2 production was observed when the transfected Jurkat line was stimulated by any of several mAbs against OKT11 in the presence of PMA (results not shown). Similarly, mAbs against T1 or HLA fail to stimulate the parental E6.1 Jurkat line (3).

Having established that the Thy-1.2 molecule retained its functional properties when expressed by the Jurkat cell, we were interested in determining how the expression of the introduced gene might affect the normal activation properties of the cell. To this end, a panel of five independent, cloned Thy-1.2-transfected Jurkat cell lines, derived from three separate spheroplast fusions, were tested with a variety of stimuli (Table II). As previously demonstrated (Table I), the combination of rat anti-Thy-1 mAbs, 3A7 and 3H11, was capable of stimulating all the Thy-1-bearing Jurkat cells in the presence of PMA. PHA, OKT3, and an anticlonotypic mAb (C.305) which had been previously shown to activate the Jurkat cell line (4) stimulated both the parental Jurkat cell and four of the five Thy-1.2 Jurkat transfectants. One transfectant (21C3.2F7) did not secrete IL-2 when stimulated with anti-T3 or -Ti mAbs; 21C3.2F7 also made very low to negligible levels of IL-2 in response to PHA, compared with Jurkat and other transfected clones. In contrast, 21C3.2F7 could produce IL-2 in response to the calcium ionophore A23187 plus PMA, indicating that it was still capable of IL-2 secretion. Furthermore, 21C3.2F7 produced IL-2 in response to anti-Thy-1 mAbs. These results imply that the IL-2 secretory mechanism of the clone is

TABLE II

IL-2 Production from a Panel of Thy-1.2-transfected Jurkat Cell Lines

Stimulus	Responding cell line					
	E6.1	21C3.2F7	21D6.2C5	J14C9	J19G4	J20F8
Medium	5,112	4,149	3,012	2,318	4,344	2,126
PMA	8,341	7,641	9,030	7,328	5,929	4,915
PHA + PMA	46,708	5,471	43,150	55,995	54,473	46,142
OKT3 + PMA	32,952	8,396	31,624	48,605	50,552	31,438
C.305 + PMA	43,485	9,249	44,830	40,082	32,520	24,418
3H11 + 3A7 + PMA	8,945	35,774	40,691	55,564	49,745	20,815
A23187 + PMA	35,530	33,482	36,438	48,963	31,838	28,751

The Jurkat line (E6.1) and five Thy-1.2-transfected Jurkat clones were cultured with PHA (1.0 μg/ml), OKT3 (1:1,000 ascites), the anti-Jurkat Ti mAb C.305 (1:50 sup), the anti-Thy-1 mAbs 3H11 and 3A7 (1:200 ascites), the calcium ionophore A23187 (100 ng/ml), and/or PMA (50 ng/ml). 21C3.2F7 and 21D6.2C5 are separate clones derived from the one spheroplast fusion; J19G4 and J20F8 were separate clones derived from a second fusion, while J14C9 was derived from a third fusion. IL-2 content of the stimulated supernatants was measured by [^{3}H]TdR uptake by an IL-2-dependent cell line, CTLL. Data are expressed as mean cpm.

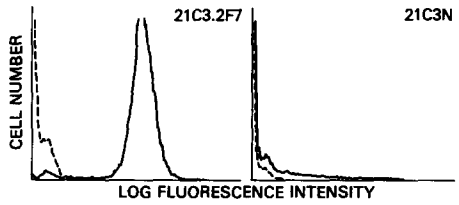

FIGURE 5. Anti-Thy-1 staining of a Thy-1-transfected Jurkat clone and a Thy-1$^-$ revertant. The Thy-1-expressing Jurkat transfectant, 21C3.2F7, and its Thy-1$^-$ derivative, 21C3N, were stained with a mixture of rat anti-Thy-1 mAbs (described in Materials and Methods) and MAR 18.5 (——) or with MAR 18.5 alone (-----).

intact. When stained with antibodies to T3 and Ti, the nonresponder 21C3.2F7 clone displayed equivalent levels of fluorescence compared with E6.1 Jurkat cells and other Thy-1.2-transfected Jurkat clones (Fig. 2, and data not shown). This result indicates that a quantitative defect in expression of the T3-Ti does not account for the failure of 21C3.2F7 to respond to receptor related ligands.

Derivation and Functional Properties of a Revertant Thy-1$^-$ Jurkat Clone. Although the data presented in Table II suggest that the Jurkat Thy-1 transfectant 21C3.2F7 was markedly deficient in its ability to secrete IL-2 in response to some cell surface ligand binding events, the possibility remained that the functional phenotype of this cell was merely representative of clonal variation within the parental Jurkat population and not due to the integration and expression of the foreign gene construct. To distinguish between these possibilities, we produced a Thy-1.2$^-$ revertant to this transfected Jurkat clone by chemical mutagenesis with ethyl methane sulfonate, negative selection using anti-Thy-1 mAbs and complement, and cell sorting. After cloning, one revertant (21C3N) was analyzed for surface expression of Thy-1. As shown in Fig. 5, the revertant clone did not stain above background levels with a mixture of rat mAbs to Thy-1. The staining of the Thy-1.2$^+$ Jurkat clone, from which 21C3N was derived, is shown for comparison.

To confirm that the 21C3N clone was derived from the nonresponder 21C3.2F7, equal amounts of genomic DNA from the two cell lines and the responder clone 21D6.2F6 were digested with Hind III restriction enzyme, electrophoresed on agarose gels, and transferred to nitrocellulose. The filters were then hybridized with nick-translated pBR322 plasmid DNA (Fig. 6). Multiple bands were present in all three cell lines, indicating that multiple copies of the construct were integrated into high molecular weight DNA. The hybridization patterns of DNA from the 21C3N (lane *C*) and 21C3.2F7 (lane *B*) clones were identical, indicating that (*a*) the 21C3N, Thy-1.2$^-$ revertant has not deleted the foreign gene from its genome, and (*b*) that 21C3N is derived from the nonresponder 21C3.2F7. The hybridization pattern of the responder clone 21D6.2F6 (lane *A*) shows less intense hybridization, indicating a reduced copy number for the Thy-1.2-containing construct. In addition, 21D6.2F6 lacks several of the bands seen in the other lanes, indicating that it is derived from a separate transfection event. There are common bands in all the digests. The major band of ~5.5 kb represents an intact internal segment of the pSV2gpt–Thy-1.2 construct. The other, less bright bands at varying molecular weights presumably represent junctional fragments derived from integration at sites within the Hind III fragment of the original construct DNA. Thus, this experiment indicates that the Thy-1.2$^-$ line, 21C3N, still contains pSV2gpt–Thy-1.2 in its genome, and furthermore is derived from the Thy-1.2$^+$ line, 21C3.2F7.

We next conducted experiments to determine if the loss of Thy-1.2 expression correlated with any changes in functional phenotype in 21C3N. In Table III, Jurkat, 21C3N, and 21C3.2F7 are compared in an IL-2 production assay. In this representative experiment, the Thy-1.2$^-$ 21C3N line, although derived from the nonresponder Thy-1.2$^+$ 21C3.2F7 line, responded normally to stimulation by

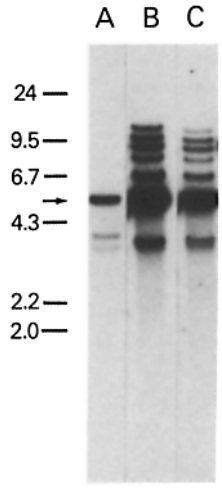

FIGURE 6. Analysis of pSV2gpt–Thy-1.2 derived DNA in transfectants. 10 μg of genomic DNA from the 21C3.2F7 Thy-1-transfected clone (lane *B*), its Thy-1 negative derivative, 21C3N (lane *C*), and a separate Thy-1 transfected Jurkat clone, 21D6.2F6 (lane *A*), were digested with Hind III, electrophoresed through an 0.7% agarose gel, transferred to nitrocellulose, and hybridized to a nick-translated pBR322 probe. Molecular size markers generated from phage λ digestion with Hind III are shown on the left.

TABLE III
IL-2 Production from a Thy-1.2$^+$ Transfectant and a Thy-1.2$^-$ Revertant Clone

Stimulus	Responding cell line		
	E6.1	21C3.2F7	21C3N
Medium	2,043	3,048	4,280
PMA	4,912	6,991	5,215
OKT3 + PMA	89,421	8,233	85,633
C.305 + PMA	75,632	7,554	78,491
3H11 + 3A7 + PMA	5,001	61,428	6,095
A23187 + PMA	80,643	68,113	60,235

The Jurkat line (E6.1), a Thy-1.2-expressing transfected line (21C3.2F7), and a derivative of 21C3.2F7 that no longer expresses Thy-1.2 (21C3N) were stimulated as indicated and as described in Table II. IL-2 content of the stimulated supernatants was measured by [^{3}H]TdR uptake by an IL-2-dependent cell line, CTLL. Data are expressed as mean cpm.

OKT3 and antireceptor mAb C.305. As would be expected, anti-Thy-1 antibodies had no effect on either the 21C3N or the E6.1 Jurkat clone. As before, the only stimuli capable of inducing IL-2 secretion from the 21C3.2F7 were anti-Thy-1 antibodies and ionophore. Thus, the 21C3N Thy-1.2$^-$ revertant line has a responder phenotype to stimulation via the T3-Ti complex.

Dissociation of Increases in [Ca^{2+}]$_i$ from Activation for IL-2 Production. Increases in [Ca^{2+}]$_i$ have been shown to accompany activation of T lymphocytes in response to mitogenic stimulation (30) and have also been seen in Jurkat cells stimulated with PHA, OKT3 mAbs, or mAbs against Ti (3). Since the 21C3.2F7 clone produced little if any IL-2 when stimulated with mitogenic mAbs other than anti-Thy-1, we wished to determine whether this clone would fail to show the usual increase in [Ca^{2+}]$_i$ that accompanies stimulation with the OKT3 mAb. Fig. 7*A* depicts the Quin 2 fluorescence changes induced by the OKT3 mAb on Jurkat cells (E6.1), Thy-1.2-transfected responder (21D6.2C5), Thy-1.2-transfected nonresponder (21C3.2F7), and the Thy-1.2$^-$ revertant responder line 21C3N. In all cases OKT3 was able to increase [Ca^{2+}]$_i$ from <100 nM to ~400 nM. Thus, both the 21C3.2F7 nonresponder cell line, as well as the transfectants that produced IL-2 in response to OKT3, showed the increase in [Ca^{2+}]$_i$ usually associated with complete cellular activation. In addition, mAbs against Thy-1 were also able to stimulate an increase in [Ca^{2+}]$_i$ in the Thy-1.2-transfected nonresponder (Fig. 7*B*) and responder (data not shown) Jurkat lines, indicating that increases in [Ca^{2+}]$_i$ are common to both the T3 and Thy-1 activation pathways. These findings suggest that significant differences in the mechanism of stimulation by anti-Thy-1 and anti-T3/Ti mAbs exist in that a functional dissociation is seen in 21C3.2F7 cells.

Discussion

The results reported here demonstrate the expression of murine Thy-1 by a transfected human T cell tumor, and document the ability of anti-Thy-1 mAbs to activate such cells. The Thy-1 gene was introduced into the human Jurkat cell line (E6.1), which secretes IL-2 in response to stimulation by PMA in combination

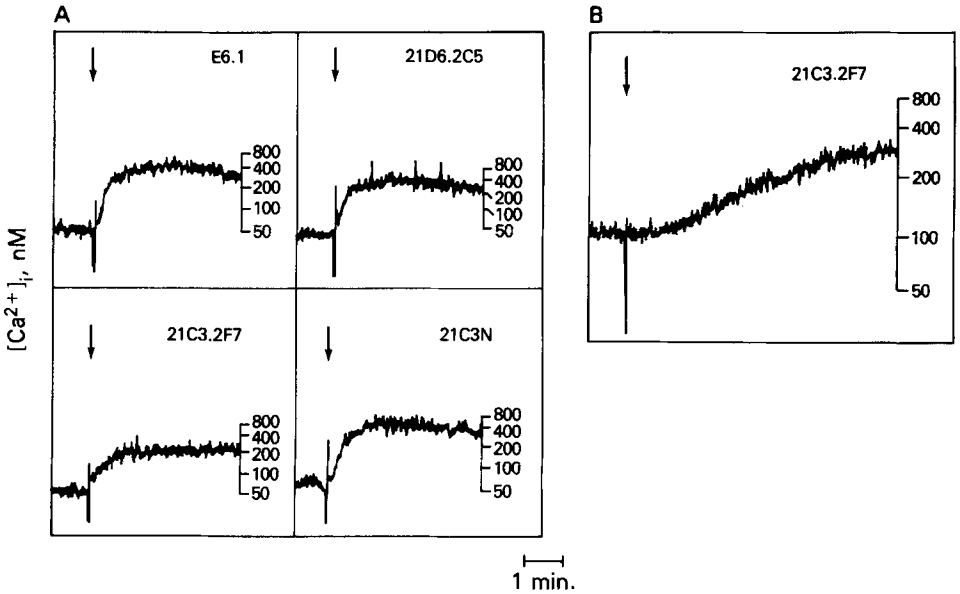

FIGURE 7. mAb-induced changes in intracytoplasmic calcium in Jurkat and Thy-1-transfected Jurkat. The indicated cell lines were loaded with Quin 2-AM, then stimulated with the OKT3 mAb (*A*) or 3A7, a mAb to Thy-1 (*B*). Approximate values for cytoplasmic free Ca^{2+} are given on the vertical axis.

with OKT3, clonotypic mAbs against its T cell receptor, PHA, or the calcium ionophore, A23187. All of these stimuli are also capable of producing a rapid rise in $[Ca^{2+}]_i$ in the absence of PMA. Our data demonstrate that high levels of Thy-1.2 protein, biochemically similar to that expressed on murine T cells, are present on the cell membrane of Jurkat after transfection with a Thy 1.2 genomic clone. Although the Thy-1.2 molecule on the transfected cells lacks certain serologically defined epitopes found on Thy-1^+ murine cells, several anti-Thy-1 mAbs previously shown to activate murine T cells could be shown to stimulate IL-2 production by the Thy-1^+ Jurkat transfectants when used together with PMA. One Thy-1^+ transfectant was unable to respond to stimulation by mAbs against T3 or the T cell receptor while it responded vigorously to stimulation by anti-Thy. Both the Thy-1^+ nonresponder line and a Thy-1 negative revertant which secreted IL-2 after stimulation by anti-T3 showed similar increases in $[Ca^{2+}]_i$ following anti-T3 binding, thus dissociating the rise in $[Ca^{2+}]_i$ from activation of the IL-2 gene.

Most mitogenic anti-Thy-1 mAbs (III-5, V-8, AT15E, and 3A7 and 3H11 in combination) bound to transfected Jurkat cells and were able to stimulate IL-2 production. Since none of these mAbs reacted with the parental Jurkat line, we can conclude that the stimulation of the transfected cells was mediated by the direct interaction of the antibody with Thy-1 and is not the result of a cross reaction between determinants on Thy-1 and determinants on the human T3 complex. Therefore, it is also likely that the stimulation of murine T cells by mitogenic anti-Thy mAbs is also mediated by a direct interaction with Thy-1 and not secondary to a cross reaction with determinants present on the murine

T3 homologue or the T cell receptor. However, we do not wish to conclude from these observations that binding of the anti-Thy mAbs is the only event necessary to trigger activation. It is possible that there are cell surface molecules present on both murine and human cells that must interact with Thy-1 after ligand binding to transmit an activation signal. Two mAbs able to activate murine T cells did not bind to Thy-1.2-transfected Jurkat cells. Although the explanation for the absence of the corresponding epitopes is unclear, the lack of these determinants did not prevent activation through sites recognized by distinct mAbs.

All Jurkat cells that expressed Thy-1.2 were stimulated by mitogenic mAbs to Thy-1. In contrast, one of these clones, 21C3.2F7, produced little IL-2 in response to anti-T3 or anti-Ti stimulation. The other transfected clones all responded normally to OKT3 and anti-Ti. The basis for the nonresponder phenotype is not readily apparent. It seems unlikely that the mere presence of Thy-1 interfered with T3 activity in 21C3.2F7 cells because the level of surface expression of Thy-1.2 was identical in responder and nonresponder transfectants. 21C3.2F7 has also been shown to express normal amounts of T3 and Ti molecules on the cell surface (Fig. 2, and data not shown). This clone can certainly produce IL-2, as judged by its response to ionophore and to anti-Thy stimulation. When a Thy-1$^-$ revertant of this clone was produced, it regained its normal responsiveness to OKT3. These results suggest that the responder/nonresponder phenotype is coregulated with Thy-1 expression in this clone.

Anti-T3 stimulation of the nonresponder clone led to a clear increase in $[Ca^{2+}]_i$ in a Quin 2 assay, indicating that some of the signals associated with receptor mediated activation are still occurring. Therefore, it is somewhat paradoxical that stimulation by the Ca^{2+} ionophore A23187 and PMA led to IL-2 production by the nonresponder Jurkat clone, whereas stimulation by OKT3, which resulted in an increase in $[Ca^{2+}]_i$ in the absence of a costimulator, failed to fully activate the cell in the presence of PMA. It is possible that ionophore induces pleiotropic effects in cellular cationic fluxes and that the change in $[Ca^{2+}]_i$ seen with OKT3 is insufficient to activate the cell. Alternatively, this particular clone may have a different threshold for responsiveness to $[Ca^{2+}]_i$, and ionophore may be enough stimulus to reach the threshold, whereas OKT3 is not.

Although the studies presented here, as well as earlier studies (9, 31, 32), conclusively demonstrate that antibodies to Thy-1 are capable of completely inducing the full program of T cell activation in vitro, the physiological role of this molecule in the ontogeny and function of T cells is not settled. At present we favor the view that Thy-1 plays a critical role as one of the alternative pathways of T cell activation. Thus, it would resemble in many respects the T11 and 9.3 antigens that have been described on human lymphocytes. It has been proposed (5) that T11 may represent a phylogenetically primitive pathway of cellular triggering which may have evolved before the antigen specific T cell receptor. T11 may be particularly critical in triggering thymocyte growth and differentiation. It has recently been shown that anti-T11 mAbs can trigger both T3$^+$ and T3$^-$ human thymocytes to express IL-2 receptors, but not to produce

IL-2 (33). It is conceivable that Thy-1 may play a similar role on mouse and rat thymocytes, but only remains as an alternative signalling mechanism on mouse peripheral T cells. It should be emphasized that Thy-1 is a member of the Ig superfamily and that the Ig V region–like domain on Thy-1 may play a receptor-like function for a ligand of similar structure on another cell type, particularly during the course of T cell differentiation in the thymus. Alternatively, Thy-1 may interact with itself, forming a V to V-like dimer, thereby either creating an active site for another transducing protein or forming a multisubunit Ca^{2+} ion channel (34).

Although the physiologic role of Thy-1 remains to be determined, the studies presented here demonstrate that a murine T cell membrane molecule can be expressed on human T cells with retention of functional properties associated with its expression in murine T cells. This system should prove useful in studying the structural requirements for activation via Thy-1 and for exploring the relationships among the various cell surface and intracellular molecules involved in T cell activation. Furthermore, our identification of a cell in which the sequence of events involved in normal receptor-mediated T cell triggering is reversibly interrupted may provide a valuable tool for the dissection of the cascade of events seen following antigen recognition.

Summary

The interaction of certain mAbs with the Thy-1 molecules of murine T lymphocytes leads to cell activation and proliferation. To examine the signal transduction mechanism underlying this process and to determine what, if any, relationship exists between Thy-1-dependent triggering and T cell activation mediated through the T3-antigen receptor (T3-Ti) complex, a genomic clone of murine Thy-1.2 was isolated and transfected into the human T cell tumor, Jurkat. The transfected gene was actively transcribed in these human cells and high levels of Thy-1.2 glycoprotein were found on the cell membrane. Although certain mAbs to Thy-1.2 failed to bind to the Thy-1 transfected Jurkat cells, several known mitogenic anti-Thy-1 mAbs did react, and in the presence of phorbol ester, induced IL-2 secretion. One Thy-1^+ transfectant out of five failed to produce IL-2 in response to anti-T3/Ti antibodies even though it retained the ability to increase intracytoplasmic calcium concentration ($[Ca^{2+}]_i$) in response to these ligands. A Thy-1 negative revertant of this cell regained anti-T3/Ti reactivity, suggesting a regulatory defect in signal transmission via T3/Ti in the original transfectant. These data confirm the ability of Thy-1 to act as an activation receptor for T cells. They reveal a potential role for changes in $[Ca^{2+}]_i$ in this process, in common with other pathways of T cell activation, but also indicate a more complex series of events is involved.

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