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Immunology

Isolation and characterization of a T-lymphocyte somatic mutant with altered signal transduction by the antigen receptor

(calcium/phosphatidylinositol/transmembrane signaling/T-cell antigen receptor/modulation)

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ABSTRACT **We have developed an approach for deriving and characterizing antigen-receptor (CD3/Ti) signal-transduction mutants. This strategy combines receptor-mediated growth inhibition and fluorescence-activated cell sorting with the Ca^{2+}-indicator indo-1. Despite the expression of structurally normal CD3/Ti complexes, one such mutant (J.CaM1) fails to exhibit inositolphospholipid metabolism or Ca^{2+} mobilization in response to anti-CD3 or anti-Ti monoclonal antibodies and fails to produce lymphokines in response to these antibodies. Surprisingly, anti-Ti antibody retains its effectiveness as a stimulus for the down-regulation of CD3/Ti surface expression. These cells remain responsive to AlF_4^-, at least one anti-CD3 antibody, and some combinations of nonagonist anti-Ti and anti-CD3 antibodies. The mutation in J.CaM1 appears to lie in a proximal component of the signal-transduction apparatus.**

The T-cell antigen-receptor complex (reviewed in refs. 1 and 2), consisting of five to nine transmembrane proteins, is the principal structure responsible for recognizing antigen and initiating biochemical events that culminate in cellular activation. Little is known about the molecular basis of transmembrane signal transduction when ligands bind to the receptor complex. Since Ti, the antigen-specific heterodimeric receptor, is noncovalently associated with the CD3 (formerly T3) complex, a set of at least three nonpolymorphic proteins, it has been hypothesized that the CD3 complex transmits signals intracellularly when Ti binds antigen.

Although the proteins responsible for signal transduction are poorly defined, some of the associated biochemical events have been elucidated. The binding of ligands to the CD3/Ti complex results in increased cytoplasmic free Ca^{2+} concentration ($[Ca^{2+}]_i$) (3–8) via activation of the inositolphospholipid second-messenger pathway (5). These events may be triggered by a variety of ligands, including monoclonal antibodies (mAbs) reactive with Ti or CD3 epitopes, certain lectins, and appropriate antigens (reviewed in ref. 2). Many of the cellular processes of an activated T cell can be elicited by combinations of agents that increase $[Ca^{2+}]_i$ and activate protein kinase C (e.g., calcium ionophores and phorbol esters) (2).

We describe here a system for exploring further the molecules and events underlying transmembrane signaling by CD3/Ti. We have generated a somatic cell mutant, derived from the leukemic T-cell line Jurkat, that expresses a structurally normal, but functionally deficient, CD3/Ti complex. The coupling of this complex to the inositolphospholipid signaling pathway is altered, and these cells concomitantly exhibit functional defects in some, but not all, receptor-dependent processes.

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MATERIALS AND METHODS

Cell Line. The CD3/Ti-bearing leukemic T-cell line Jurkat, clone E6-1, was maintained as described (3).

mAbs. C305 is an anti-idiotype mouse mAb (IgM) specific for the Jurkat Ti (9). OKT3 (IgG2a; Ortho Diagnostics), anti-Leu-4 and anti-Leu-4 Fab fragments (IgG1), and 235 (IgM) (10) are mouse mAbs reactive with CD3 determinants.

Flow Cytometry. Cell surface immunofluorescence techniques were performed as described (9). For assessing $[Ca^{2+}]_i$ elevations, cells were loaded with the membrane-permeant pentakis(acetoxymethyl) ester of indo-1 (ref. 11; Molecular Probes, Junction City, OR) as described previously (12) for quin-2. Cells were exposed to the appropriate stimulus at 37°C and then analyzed in a FACS IV (Becton Dickinson). Cells were exposed to ultraviolet light (364 nm), and emission was detected through a 390-nm long-pass filter by photomultiplier tubes fitted with a 404-nm band-pass filter (near the maximal emission for Ca^{2+}-bound indo-1) or a 486-nm band-pass filter (near the maximal emission for Ca^{2+}-free indo-1), respectively. Shown in *Results* is the calculated ratio of emission at these two wavelengths (404 nm/486 nm) vs. relative cell number, with increasing ratio indicating increasing $[Ca^{2+}]_i$.

Inositol Phosphate Assay. Inositol phosphates were quantitated essentially as described (5).

$[Ca^{2+}]_i$ Measurement by Fluorimetry. Cells were loaded with indo-1, and $[Ca^{2+}]_i$ was determined in a Spex Fluorolog II spectrofluorometer (Spex Industries, Edison, NJ) with excitation at 334 nm and emission detection at 400 nm, using the equation $[Ca^{2+}]_i = K_d \times (F_{observed} - F_{min})/(F_{max} - F_{observed})$, where $K_d = 250$ nM (12).

Other Methods. Mutagenesis, cloning by limiting dilution, cell surface radioiodination and immunoprecipitation analysis, and interleukin 2 (IL-2) determinations were performed as described (9).

RESULTS

Selection Protocol: Phytohemagglutinin (PHA)-Resistant Cells. We observed that Jurkat cells ceased to proliferate in the presence of PHA (Table 1), a lectin that is mitogenic for normal peripheral blood lymphocytes and that can activate Jurkat cells in the presence of phorbol esters to produce IL-2. This growth arrest is dependent on the expression of a CD3/Ti complex, since a CD3/Ti-negative mutant of Jurkat was much less susceptible to the inhibitory effect of PHA. Previous work (3) had shown that the ability of PHA to stimulate mobilization of intracellular Ca^{2+} was likewise dependent on expression of CD3/Ti. We reasoned that receptor-bearing cells resistant to the growth-inhibitory ef-

Abbreviations: $[Ca^{2+}]_i$, cytoplasmic free Ca^{2+} concentration; IL-2, interleukin 2; mAb, monoclonal antibody; PHA, phytohemagglutinin; PMA, phorbol 12-myristate 13-acetate.

Table 1. Receptor-dependent growth inhibition of Jurkat cells by PHA

Cell line	CD3/Ti expression	PHA	Cell concentration, (cells per ml) × 10^{-3} Day 1	Day 3	Day 5
Jurkat	+	−	170	444	1856
		+	90	77	133
JRT-T3.1	−	−	140	470	1720
		+	110	367	1480

Jurkat and a CD3/Ti-negative mutant (JRT-T3.1) were plated at 10^5 cells per ml in the presence or absence of PHA (1 μg/ml) on day 0. Culture wells were then examined microscopically to determine the concentration of cells.

fect of PHA might also be deficient in transmembrane signaling via CD3/Ti.

To select for PHA-resistant cells, Jurkat cells were mutagenized with ethyl methanesulfonate, grown in the presence of PHA for 2 weeks, expanded, and then assessed by flow cytometry. Greater than 50% of the selected cells expressed little or no detectable surface CD3/Ti, consistent with the assumption that PHA selects against cells with functional CD3/Ti receptors. The cells with the greatest intensity of staining with anti-Ti mAb were sorted and then expanded for further selection.

Selection Protocol: Inability to Mobilize Ca^{2+}. It was necessary for our purposes to introduce a selection step to enrich for mutations in the proximal signaling events. Mobilization of intracellular Ca^{2+} is a biochemical event that is closely associated with receptor triggering, and one which may be analyzed in individual cells by flow cytometry. Therefore, PHA-resistant cells were loaded with indo-1 and subjected to flow cytometry. Wild-type cells responded uniformly to C305 mAb with an increased 404 nm/486 nm fluorescence ratio (Fig. 1*A*), indicating an increase in $[Ca^{2+}]_i$. The sorting window was gated on the PHA-resistant cells with the lowest indo-1 fluorescence ratio in the time interval from 1 to 3 min poststimulation with C305, and cells were sorted and expanded.

Subsequently, these cells were subjected to additional pairs of alternating sorts for Ca^{2+} nonmobilization and receptor expression. After four rounds of sorting, ≈50% of the cells failed to increase $[Ca^{2+}]_i$ in response to C305 (Fig. 1*B*); all of these nonresponsive cells were receptor-positive (data not shown). This mixed population was then subjected to limiting dilution for cloning.

Four of seven clones (Fig. 1*D*, a representative clone) failed to respond to C305 with significant elevations of $[Ca^{2+}]_i$, unlike the wild-type parent cell (Fig. 1*C*). Moreover, all four of these were receptor-positive by flow cytometry (Fig. 3 and data not shown). One clone, J.CaM1 (Jurkat-derived Ca^{2+} mutant no. 1), was subjected to more detailed analysis as described below.

CD3/Ti on J.CaM1 Is Inefficiently Coupled to the Inositolphospholipid Pathway. We first assessed the generation of inositolphospholipid-derived second messengers in response to anti-receptor mAb. Four- to six-fold elevations of the level of inositol phosphates (including the specific inositol trisphosphate fraction) were observed in the parent Jurkat, but not in J.CaM1, in response to C305 (anti-Ti) or OKT3 (anti-CD3) mAb (Table 2). Likewise, 4-fold increases in phosphatidic acid, a metabolite of the second messenger diacylglycerol, were observed in Jurkat but not in J.CaM1 in response to these mAbs (data not shown). Thus, the receptor complex in J.CaM1 appears to be inefficiently coupled to the inositolphospholipid pathway. This pathway is capable of being activated in J.CaM1, as demonstrated by the ability of AlF_4^-, a direct activator of guanine nucleotide-binding regulatory proteins (13) and of hydrolysis of phosphatidylinositol bisphosphate in hepatocytes (14), to stimulate elevation of $[Ca^{2+}]_i$ and generation of inositol phosphates in J.CaM1 (data not shown). These results imply that the defect in J.CaM1 resides in a proximal component of the signal-transduction apparatus.

J.CaM1 Expresses Grossly Normal Receptor-Complex Proteins. J.CaM1 and Jurkat cells were surface-radioiodinated and subjected to immunoprecipitation and $NaDodSO_4$/PAGE. No differences were detectable between the receptor proteins separated under either nonreducing or reducing

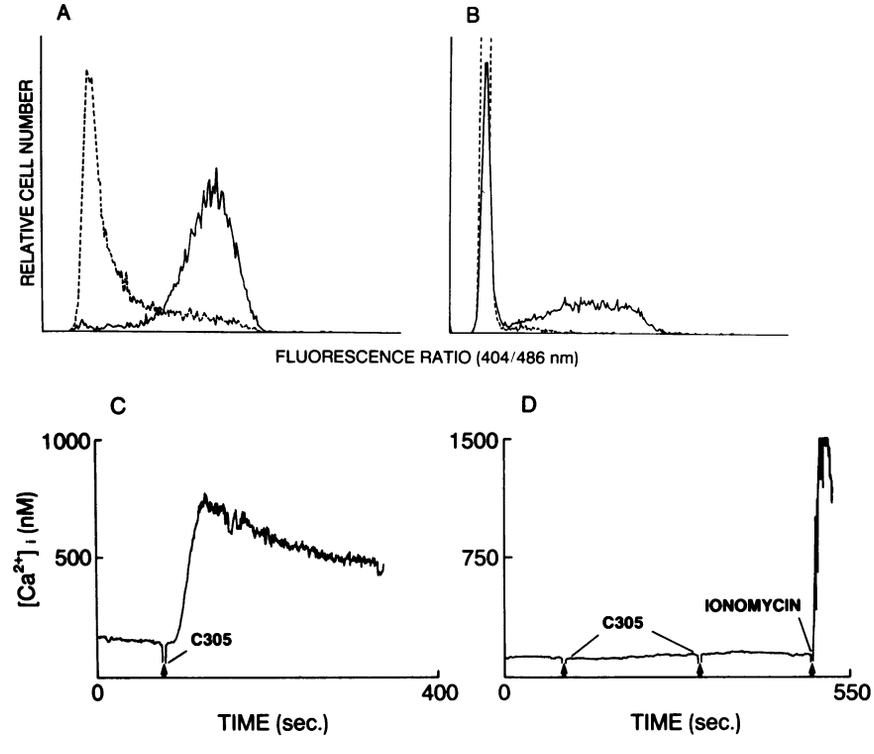

FIG. 1. Selection of Ca^{2+} mutants by flow cytometry and screening by fluorimetry. (*Upper*) Cells loaded with indo-1 were stimulated with mAb and analyzed by flow cytometry for $[Ca^{2+}]_i$. Increasing fluorescence ratio (404 nm/486 nm) correlates with increasing $[Ca^{2+}]_i$. Jurkat cells (*A*) stimulated for 1 min with mAb C305 (1:1000 dilution of ascites; solid line) are compared with unstimulated cells (dashed line). PHA-resistant cells sorted through four cycles exhibited a bimodal distribution after stimulation (*B*, solid line). (*Lower*) The responses of Jurkat cells (*C*) and of J.CaM1 cells (*D*) to increasing concentrations of C305 [arrow, 1:10,000 dilution for Jurkat; 1:10,000 and 1:1000 dilutions for J.CaM1; nonresponsiveness of J.CaM1 was also observed at 1:100 dilution (data not shown)] were compared by conventional Ca^{2+} fluorimetry. Ionomycin (1 μM, third arrow in *D*) demonstrated the presence of intracellular dye and the releasability of intracellular Ca^{2+} stores.

Table 2. Failure of anti-receptor antibodies to stimulate production of inositol phosphates in J.CaM1

	% control*			
	Jurkat		J.CaM1	
Stimulus	InsP_3	Ins$P$ + InsP_2	InsP_3	Ins$P$ + InsP_2
None	100 ± 27	100 ± 4	100 ± 5	100 ± 34
C305	397 ± 27	616 ± 3	126 ± 8	97 ± 6
OKT3	355 ± 14	502 ± 3	110 ± 4	104 ± 10

myo-[^{3}H]Inositol-labeled Jurkat and J.CaM1 cells were incubated with no mAb or with C305 or OKT3 mAb (ascites, 1:1000 dilution) for 10 min. Inositol mono- and bisphosphate (Ins$P$ + InsP_2) and inositol trisphosphate (InsP_3) were measured essentially as described in ref. 5.

*Mean ± standard error of three samples per condition, expressed as a percentage of the appropriate unstimulated control.

conditions (Fig. 2). In addition to the characteristic Ti and CD3 proteins, the recently described ζ-chain dimer (15) was detectable by two-dimensional nonreducing–reducing NaDodSO$_4$/PAGE after immunoprecipitation with an anti-ζ antiserum (data not shown). Finally, two-dimensional nonequilibrium pH gradient (NEPHGE)–NaDodSO$_4$/PAGE failed to reveal any differences in charge characteristics between the CD3/Ti proteins expressed by Jurkat and J.CaM1 (data not shown). Therefore, the receptor-associated proteins on J.CaM1 appear grossly normal.

A second way of assessing the physical condition of receptor proteins is flow cytometry with mAbs against multiple receptor epitopes. C305 (anti-Ti) and anti-Leu-4 (anti-CD3) stained J.CaM1 and Jurkat cells with comparable intensities (Fig. 3 *A* and *B*). Likewise, four other independent anti-receptor complex mAbs stained the two cell lines comparably (data not shown). Thus, the receptor proteins on J.CaM1 appear to express the same epitopes as do those on the parent cell line, implying no major loss of receptor structure.

J.CaM1 Has Selective Functional Deficits. We next examined two functional characteristics of the CD3/Ti complex: (*i*) its ability to mediate cell activation and (*ii*) its decreased concentration on the cell surface in response to receptor ligands (modulation). As previously described by our laboratory (16) anti-receptor mAbs in the presence of phorbol 12-myristate 13-acetate (PMA) stimulate the accumulation of IL-2 mRNA and the extracellular secretion of IL-2. As

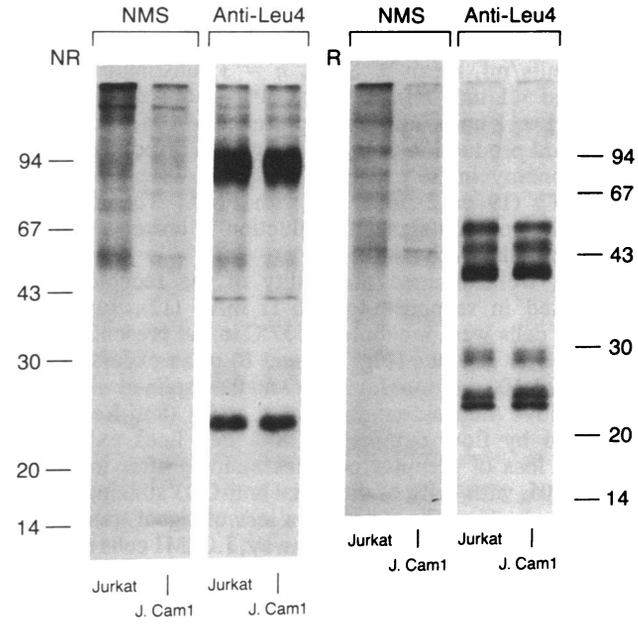

FIG. 2. Immunoprecipitation and NaDodSO$_4$/PAGE of receptor-complex proteins. Surface-radioiodinated cells were lysed and subjected to immunoprecipitation with either normal mouse serum (NMS) or anti-Leu-4 in the presence of 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS) to preserve CD3/Ti associations. Immunoprecipitates were analyzed under either nonreducing (NR) or reducing (R) conditions by NaDodSO$_4$/PAGE followed by autoradiography. Relative mobilities of standards are shown at left and right (kDa).

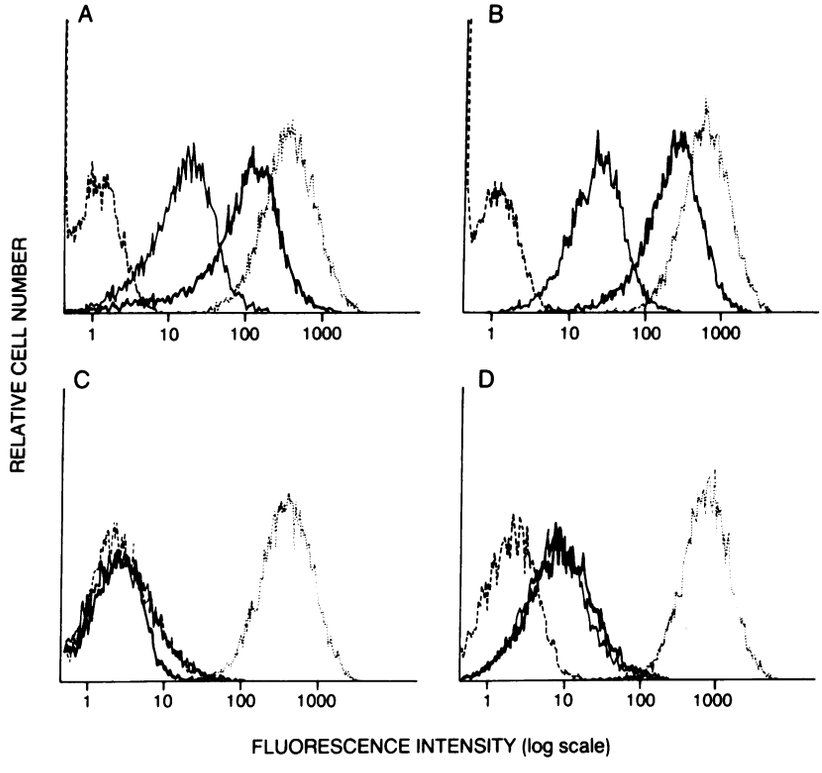

FIG. 3. CD3/Ti cell surface expression and modulation response assessed by flow cytometry. Jurkat (*A*) and J.CaM1 (*B*) cells were stained with fluorescein-conjugated mouse control IgG (dashed line), C305 (solid line), anti-Leu-4 (heavy solid line), or anti-β_2-microglobulin (dotted line). For modulation, Jurkat (*C*) and J.CaM1 (*D*) cells were incubated with C305 (1:100 dilution) at 37°C for 2.5 hr and then stained as above.

expected, while the ionophore ionomycin was able to synergize with PMA to stimulate IL-2 production by J.CaM1 (29 ± 15 units/ml, mean ± SEM, n = 3; unstimulated cells produced <1 unit/ml), neither C305 (1 ± 1 units/ml) nor OKT3 (1 ± 1 units/ml) in the presence of PMA stimulated significant production of IL-2. Jurkat cells responded well to either ionomycin (36 ± 6 units/ml), C305 (18 ± 1 units/ml), or OKT3 (19 ± 2 units/ml) with PMA. Therefore, the impaired proximal signal transduction is correlated with a loss of cellular activation.

Next, we examined the ability of the receptor to be modulated in response to anti-Ti mAb (17). Jurkat and J.CaM1 cells were incubated at 37°C in the presence (Fig. 3 *C* and *D*) or absence (Fig. 3 *A* and *B*) of an excess of C305 (ascites, 1:100 dilution) for 2.5 hr and then stained with a noncompetitive fluorescein-conjugated mAb (anti-Leu-4) and assessed by flow cytometry. Both cell lines exhibited a marked loss of receptor complex staining after incubation with C305, with ≈4% of the initial anti-CD3 staining remaining in each. Therefore, despite a loss of signal transduction via the inositolphospholipid pathway, J.CaM1 cells retain the modulation response to anti-receptor mAb.

The Receptor on J.CaM1 Is Not Entirely Devoid of Signaling Capability. We next explored combinations of stimuli to assess the potential of the J.CaM1 receptor complex to function in signal transduction. OKT3 (anti-CD3) was a nonagonist for Ca^{2+} mobilization when used alone, even at saturating levels (Fig. 4*A*), although it is a potent agonist in parental Jurkat cells (3). Surprisingly, when C305 (anti-Ti) was added subsequently, a prompt and substantial increase in $[Ca^{2+}]_i$ was observed (Fig. 4*A*). Likewise, when C305 was added first, no response was observed in J.CaM1, and a significant increase in $[Ca^{2+}]_i$ was seen following the subsequent addition of OKT3 (Fig. 4*B*). Increases in inositol phosphates were measurable following the stimulation with both mAb together (data not shown). Similar restoration of agonist potential was seen when either C305 or OKT3 was provided in the presence of a rabbit anti-mouse immunoglobulin antiserum (data not shown). That this restoration of agonist potential is not due entirely to a crosslinking (i.e., aggregation) phenomenon is suggested by the fact that monovalent Fab fragment of the mAb anti-Leu-4 also synergized with C305 to yield a substantial, although submaximal, mobilization of Ca^{2+} (Fig. 4*D*).

Finally, we were surprised to observe that the anti-CD3 mAb 235 (IgM isotype) alone was capable of eliciting a Ca^{2+} response (Fig. 4*C*). The potency of this mAb is unlikely to be due to immunoglobulin isotype since C305, also an IgM, is a nonagonist. Therefore, it appears that the receptor complex on J.CaM1 is inefficiently coupled to the signal-transduction apparatus but is capable of transducing some signals when subjected to appropriate perturbation.

DISCUSSION

We have described an approach for isolating somatic cell mutants that are deficient in signal transduction by the T-cell antigen-receptor complex. This approach involves lectin-mediated inhibition of proliferation, flow cytometry with cell surface immunofluorescence, and flow cytometry with the intracellular Ca^{2+} indicator indo-1. Here we identify and characterize one such mutant derived from the parental cell line Jurkat.

This mutant, J.CaM1, has a receptor complex that appears grossly normal by biochemical and immunofluorescence criteria. The CD3 and Ti proteins show no change in electrophoretic mobility or susceptibility to reduction and react with at least six independent mAbs directed against CD3/Ti epitopes. Therefore, the mutant phenotype is not due to detectable deletions or major conformation alterations of the receptor on the cell surface.

However, the antigen-receptor complex on J.CaM1 appears to be inefficiently coupled to the inositolphospholipid second-messenger pathway. Two mAbs against CD3 and Ti that are potent agonists in parental Jurkat cells fail to trigger generation of inositol phosphates or mobilization of intracellular Ca^{2+} in J.CaM1. Likewise, two other anti-Ti mAbs fail to elicit an increase in $[Ca^{2+}]_i$ in J.CaM1, despite their potency in parental Jurkat cells (data not shown). Therefore, J.CaM1 cells appear to have a mutation that affects the coupling of the receptor to hydrolysis of phosphatidylinositol bisphosphate.

Associated with this proximal signal-transduction defect is abrogation of receptor-mediated cell activation, as indicated by a loss of IL-2 production in response to anti-CD3/Ti mAb plus PMA. The generation of IL-2 is restored by stimuli that bypass the receptor complex. These results are consistent with the hypothesis that impairment of coupling to the inositolphospholipid pathway leads to an inability to transmit activation signals into the nucleus. Formally, it remains possible that an unrecognized second-messenger event is impaired in J.CaM1, contributing to a loss of cellular activation.

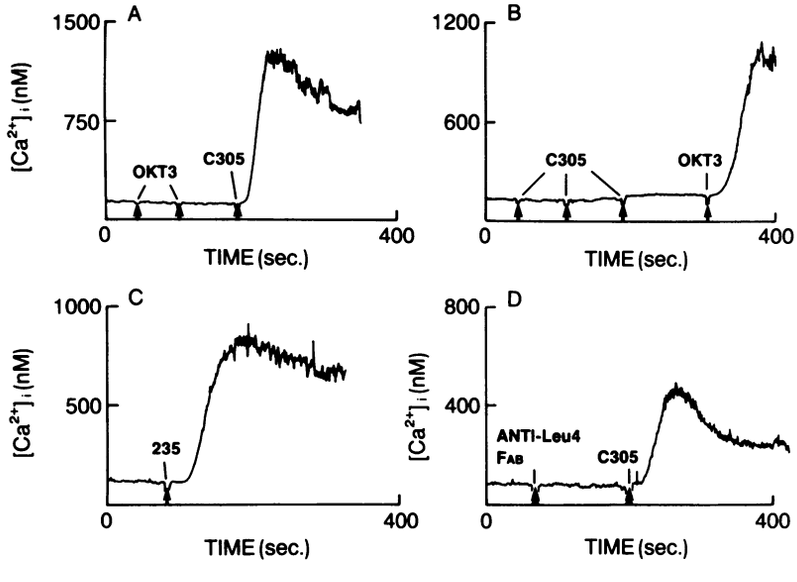

FIG. 4. Ca^{2+} fluorimetry of J.CaM1 with various anti-receptor-complex mAbs. Cells were stimulated with mAb OKT3 (arrows, 1:10,000 and 1:1000 dilutions) and then mAb C305 (1:1000 dilution) (*A*), with C305 (1:100,000, 1:10,000, and 1:1000 dilutions) and then OKT3 (1:1000 dilution) (*B*), with mAb 235 (1:1000 dilution) (*C*), or with Fab fragments (1 μg/ml) prepared from anti-Leu-4, followed by C305 (1:1000 dilution) (*D*). The anti-Leu-4 Fab fragments alone are potent agonists in wild-type Jurkat cells (data not shown). The purity of the Fab fragments was assessed by stimulating normal peripheral blood lymphocytes to proliferate. The preparation was judged by titration to exhibit <0.1% the activity of purified intact anti-Leu-4 mAb.

Despite loss of signaling via the inositolphospholipid pathway, the modulation response of J.CaM1 was intact. Like wild-type cells, J.CaM1 lost the surface expression of CD3/Ti within several hours of exposure to anti-Ti mAb. This finding implies strongly that the modulation response may be independent of transmembrane signaling by the inositolphospholipid pathway. Although phorbol esters induce phosphorylation of CD3 proteins and modulation of the receptor complex (18), the present observations suggest that binding of ligand to the receptor can induce modulation in the absence of protein kinase C-mediated phosphorylation of CD3.

Surprisingly, the receptor complex is not entirely devoid of responsiveness to some CD3/Ti mAbs. The combination of two nonagonist mAbs in J.CaM1 elicits inositolphospholipid metabolism and an increase in $[Ca^{2+}]_i$ even when one of the two mAbs is presented in the form of monovalent Fab fragments. Moreover, at least one anti-CD3 mAb, acting alone, retains agonist function in J.CaM1 as indicated by mobilization of intracellular Ca^{2+}. Previous work showed that there exist some cell surface Ti epitopes on HPB-ALL cells that are poorly responsive to mAbs (19). Here we have described a mutant cell line in which normally potent anti-Ti mAbs are nonagonists, but in which some combinations of nonagonist mAbs and at least one anti-CD3 mAb alone trigger acute signal transduction. Thus, while the Ti and CD3 molecules may be physically and functionally linked in their usual state, their functions in J.CaM1 are partially dissociated.

One hypothesis consistent with these findings is that the CD3 complex normally serves to amplify signal transduction, providing efficient communication between Ti and the intracellular signal-transduction enzymes (e.g., phospholipase C). J.CaM1, then, has a mutation in a proximal component of the cell surface signal-transduction apparatus that impairs this signal-transduction coupling process. This mutation effectively uncouples the antigen receptor from CD3, and therefore from the inositolphospholipid pathway, but allows more intense receptor stimuli to trigger inositolphospholipid metabolism. Whether the mutation resides in a receptor chain, a CD3 protein, or a more distal molecule (e.g., a guanine nucleotide-binding protein that couples to the receptor) is not known.

The selection and characterization strategy we have described should provide a mechanism for applying somatic cell genetics to the study of transmembrane signaling by the T-cell antigen-receptor complex or by any other receptor that is linked to Ca^{2+} mobilization. Analysis of a family of such mutants may provide insight into the molecular basis for signal transduction.

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