Isolation of Mutant T Lymphocytes with Defects in Capacitative Calcium Entry

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Summary
Calcium and calcium-binding proteins play important roles in the signaling cascade leading from the initial engagement of TCRs on T cells to the fully activated state. To undertake a molecular dissection of this cascade, we first isolated a Jurkat T cell line derivative containing the NF-AT promoter element driving transcription of the diptheria toxin A chain gene (dipA), resulting in rapid cell death. Selecting viable cells that fail to activate NF-AT-dependent transcription, we isolated two independent cell lines possessing defects in capacitative Ca2+ entry. NF-AT-dependent transcription can be restored in these cells by expression of a constitutively active calcineurin, but not by overexpression of the Ca2+ regulatory protein CAML, which can normally replace the Ca2+ signal. The defect in these cell lines probably lies between CAML and calcineurin in the T cell activation cascade.

Introduction
T lymphocytes play a pivotal role in both the initiation and the regulation of the immune response. Physiological triggering of the T cell antigen receptor (TCR/CD3) results in a number of distinct intracellular biochemical events, including activation of tyrosine kinases, an increase in the concentration of intracellular calcium ([Ca2+]i), activation of protein kinase C (PKC), and activation of ras gene products (Altman et al., 1990; Weiss and Littman, 1994). Together, these intracellular events are coordinated to activate the orderly expression of at least 100 independent gene products, leading to T cell activation, proliferation and, ultimately, differentiation and the acquisition of immune function (Crabtree, 1989; Ullman et al., 1990). T lymphocyte proliferation is principally regulated in an auto-
crine fashion by the T cell growth factor interleukin-2 (IL-2) (Cantrell and Smith, 1984). The IL-2 gene is transcriptionally activated in response to TCR/CD3 stimulation, and the signaling requirements for IL-2 gene expression reflect precisely those required for T cell commitment to DNA synthesis (Cantrell and Smith, 1984; Ullman et al., 1990). Accordingly, the regulation of the IL-2 gene has served as a paradigm for inducible T cell-specific gene expression. A number of trans-acting factors have been identified that bind to the IL-2 enhancer and are responsible for the T cell activation-dependent expression of this element. These include NF-AT, NFIL-2A (GAP/Oct-1), CD28RE, AP-1, and NF-kB (Crabtree, 1989; Ullman et al., 1990). We have focused our attention primarily on NF-AT, since it plays a major role in the regulation of the IL-2 gene (Durand et al., 1988; Shaw et al., 1988); it has the same signaling requirements for its activation as IL-2 gene expression (Hivroz-Burgaud et al., 1991; Shaw et al., 1988); and, like the IL-2 gene, its activity is inhibited by the immunosuppressant drugs cyclosporin A (CsA) and FK506 (Emmel et al., 1989; Flanagan et al., 1991). Recent studies have indicated that NF-AT is composed of at least two components (Flanagan et al., 1991): a nuclear component (NF-ATc) that is synthesized de novo in response to PKC or Ras activation and can be replaced by high level expression of AP-1 family (Jain et al., 1992; Northrop et al., 1993), and a preexisting cytoplasmic subunit (NF-AT, NF-AT2) that is translocated to the nucleus in response to increased [Ca2+]i (Flanagan et al., 1991), whereupon it interacts with NF-AT, and subsequently binds to its cognate DNA recognition site (Flanagan et al., 1991). NF-AT, was recently defined and is encoded by a dispersed gene family of at least four members, designated NF-ATC1 (Northrop et al., 1994), NF-ATC2 (Jain et al., 1993; Northrop et al., 1994), NF-ATC3 (Ho et al., 1995; Hoey et al., 1995; Masuda et al., 1995), and NF-ATC4 (Ho et al., 1995; Hoey et al., 1995). Strong evidence suggests that the effects of increased [Ca2+]i on NF-AT, translocation are mediated by the action of the calcium/calmodulin-dependent phosphatase calcineurin. However, a precise molecular description of these events is lacking at present. In addition, the molecular pathway underlying mitogenic Ca2+ influx across the T cell plasma membrane has not yet been defined. To identify steps in the T cell signal transduction cascade, we have used the diptheria toxin A (dipA) chain gene under the control of NF-AT binding sequences (NFATdipA). Activation of cells stably transfected with the NFATdipA construct results in the expression of the dipA gene, whose action completely inhibits eukaryotic protein synthesis (Pappenheimer, 1977) and consequently results in rapid cell death. Using this strategy as a selection scheme, we initiated a genetic screen for mutations in steps leading to NF-AT-dependent gene transcription. Of the six independent mutants that have been isolated, two are described here in detail. Both mutants are profoundly

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defective in mitogen-induced Ca²⁺ influx and expression of the endogenous IL-2 gene.

Results

Experimental Rationale
To select mutants in the T cell activation pathways, we established a derivative of the Jurkat NFATC-1 cell line (Fiering et al., 1990) that is stably transfected with the plasmid pNFATAP.neo (NZdipA) (Figure 1A). This plasmid places dipA under the transcriptional control of NF-AT. DipA, the active fragment of the two-chain diphtheria toxin, catalyzes the ADP-ribosylation of a critical histidine residue in elongation factor-2 (EF-2) (Pappenheimer, 1977), thereby inhibiting EF-2, blocking protein synthesis, and consequently causing rapid cell death. NF-AT-dependent transcription is essentially undetectable in unstimulated T cells, whereas it is rapidly induced by stimulation with the combination of the calcium ionophore, ionomycin, and phorbol myristate acetate (12-O-tetradecanoyl-phorbol13-acetate; PMA) (Durand et al., 1988; Fiering et al., 1990; Mattila et al., 1990; Shaw et al., 1988; Verweij et al., 1990). While unstimulated NZdipA cells remain perfectly viable, treatment with ionomycin and PMA results in the NF-AT-dependent expression of the dipA gene and the subsequent rapid death of >99% of the cell population. CSA, which is known to potently inhibit NF-AT-dependent transcription by inhibiting calcineurin (Clipstone and Crabtree, 1992), effectively blocked the ability of ionomycin and PMA to induce the death of NZdipA cells (Figure 1B).

We have used the NZdipA cell line to screen for mutations in the components of the signaling pathway that lead to NF-AT-dependent transcription. The experimental strategy is outlined in Figure 1C. To increase the potential recovery of mutations in the signaling pathway, the NZdipA clone, 1.5.22, was exposed to 200 rads cesium-source γ irradiation prior to stimulation (>70% of the cells remained viable after this treatment). Irradiated cells were then subjected to three successive rounds of stimulation (24 hr) with ionomycin and PMA to isolate cells with defects. Surviving cells fell into two classes: cis mutants and trans mutants. Cis mutants did not contain bona fide mutations in the NF-AT signaling pathway, since ionomycin and PMA were still able to induce expression of the endogenous NF-AT(2) reporter gene in these cells. Rather, these cells presumably harbored mutations in the dipA gene locus.
or suppressor mutations at the EF-2 locus, making them insensitive to the effects of diphtheria toxin. Such mutants were eliminated from the pool of surviving cells by using the fluorescence activated cell sorter (FACS) to exclude those cells that activated the NF-ATlacz gene (Fiering et al., 1990). Trans mutants did not express the endogenous NF-ATlacz gene, and therefore presumably possessed mutations in critical components of the signaling cascade leading to the activation of NF-AT-dependent transcription. To determine whether the resistance of the selected clones was the result of a mutation or represented the presence of naturally occurring epigenetic variants, we attempted to select resistance cells without mutagenesis. Subjecting NZdiP cells to selection with immunycin and PMA did not yield any resistant clones, indicating that the defects in the resistant clones were the result of a low frequency-induced mutation.

Selection and Characterization of Cells Harboring Mutations in the Signalling Pathway Leading to NF-AT Activation

Using the approach described above, we have isolated six independent trans-mutant clones that have survived the selection protocol outlined in Figure 1C. Two of these clones, NZdiP-TS 101 and NZdiP-TS 108 (referred to hereafter as M101 and M108) have been characterized in detail. We transfected a cDNA plasmid, pGHED7-1, which encodes a histidine mutation in EF-2 and, hence, confers diphtheria toxin resistance into both M101 and M108 (Nakarnishhi et al., 1988). As can be seen in Figure 2A, in marked contrast with control Trunc.4 cells, both M101 and M108 fail to activate NF-AT-dependent β-galactosidase activity in response to immunycin and PMA. Furthermore, RNase protection analysis revealed that, unlike control cells, M101 and M108 did not express the endogenous IL-2 gene in response to immunycin and PMA stimulation (Figure 2B).

Nuclear Translocation of the Cytoplasmic Subunit of NF-AT is Defective in M101 and M108

Following T cell activation and the concomitant increase in [Ca²⁺], NF-AT, translocates from the cytosol to the nucleus, whereupon it combines with the newly synthesized NF-AT, and binds to its cognate receptor elements (Flanagan et al., 1991). Flanagan et al. (1991) have demonstrated that NF-AT DNA binding activity and transcriptional activity can be reconstituted in vitro by mixing cytoplasmic extracts from unstimulated Jurkat cells together with nuclear extracts from PMA-stimulated Jurkat cells. In this in vitro complementation assay was used to investigate the integrity of both NF-AT, and NF-AT, in the mutant cell lines. Cytoplasmic extracts from control cells stimulated with both immunycin and PMA show little or no reconstituted NF-AT DNA binding when mixed together with nuclear extracts from PMA-stimulated Jurkat cells, since under these conditions NF-AT, has translocated from the cytosol to the nucleus and is no longer present in the cytoplasmic fraction (Figure 3). In marked contrast, NF-AT, was readily detectable in the cytoplasmic extracts of immunycin- and PMA-stimulated M101 (Figure 3; compare lanes 1 and 2 with 3 and 4) and M108 cells (data not shown), indicating that NF-AT, had failed to translocate to the nucleus in the mutant cells in response to immunycin and PMA stimulation. Taken together, these results indicate that NF-AT, fails to translocate efficiently in M101 and M108, thereby explaining the reduced levels of NF-AT DNA binding activity in the nuclear fractions of both mutants.

M101 and M108 Exhibit a Selective Defect in Calcium-Dependent Transcription

To determine whether this apparent defect was specific to NF-AT or also affected other transcription factor complexes, we examined the transcriptional activity of NF-κB and NFIL-2A (OAP-Oct-1) reporter gene constructs in the mutant cells. Like NF-AT, NFIL-2A-dependent transcription requires both an increase in intracellular calcium and a PKC-dependent signal (Ulliman et al., 1990), which can be mimicked pharmacologically by immunycin and PMA, respectively. Whereas NFIL-2A-directed transcription was readily apparent in stimulated control Trunc.4 cells, it was undetectable in both M101 and M108 cells stimulated with immunycin and PMA (Figure 4A, data not shown). NFκB-dependent transcription is induced by PMA alone (Leandro and Baltimore, 1989), but can be further increased with immunycin stimulation (Mattila et al., 1990) (Figure 4B). Interestingly, the extent of PMA-induced NFκB-depen-
Control (Trunc4) | M101
---|---
Cytosolic Extracts | NS | + | +
Pf 1 | + | + | +

PMA-stimulated Control
Nuclear Extracts | + | + | + | +

Figure 3. M101 is Defective in the Nuclear Translocation of NF-AT.

Cytosolic (C) or nuclear (N) extracts prepared from control Trunc4 (lanes 1-2) and M101 (lanes 3-4) cells after the indicated stimulation conditions were assayed for NF-AT binding activity. Translocation of NF-AT was monitored by mixing nuclear extracts prepared from PMA-stimulated control cells with cytosolic extracts prepared from control Trunc4 cells (lanes 1, 2) or M101 cells (lanes 3, 4) after 6 hr incubation in the presence (lanes 2, 4) or absence (lanes 1, 3) of 2 μM ionomycin and 10 ng/ml PMA. Arrows indicate the migration of the NF-AT DNA-protein complex for all lanes.

Figure 4. M101 is Defective in Calcium-Dependent Transcriptional Events

Trunc4 and M101 cells transfected with either OAP/Oct1-CAT (A) or NF-κB-CAT (B) reporter constructs were treated with the indicated agents: medium alone (ND), 10 ng/ml PMA alone, or 2 μM ionomycin and 10 ng/ml PMA. Cells were assayed for chloramphenicol acetyltransferase activity as described in Methods. The data represent the mean of duplicate transfections and are representative of three independent experiments.

Expression of a Calcium-Independent Constitutively Active Mutant Calcineurin Can Overcome the Signaling Defect in M101 and M108

The failure of NF-AT to translocate to the nucleus following stimulation of M101 and M108 (see Figure 3) and the selective blockade of Ca"+"-dependent signaling is highly reminiscent of the effects of the immunosuppressants cyclosporin A (CSA) and FK506. CSA and FK506 bind to immunophilins efficiently forming complexes that block the nuclear translocation of NF-AT. (Flanagan et al., 1991). NF-AT and NFIL-2A (OAP/Oct1) translocation, as well as the Ca"+"-dependent component of NF-κB translocation, by inhibiting the calcium/calmodulin-regulated serine/threonine phosphatase calcineurin (Clipstone and Crabtree, 1993; Liu et al., 1991; O'Keefe et al., 1992). The similarity between the effects of CSA/FK506 and the signaling defect in the mutant cells raised the possibility that M101 and M108 harbored mutations in their endogenous calcineurin genes. To test this possibility, we determined whether expression of the wild-type calcineurin A (CNA) or B (CNB) subunit in the mutant cell lines was able to complement the signaling defect. Neither CNA nor CNB were able to reconstitute ionomycin- and PMA-induced NF-κB-directed transcription in M101 and M108 (Figure 5A; data not shown for M108), indicating that a mutation in the endogenous calcineurin genes does not underlie the signaling defect in these cells.

Calcineurin has previously been established as an essential element in the T cell signal transduction cascade and as a major effector of the Ca"+" signal (Clipstone and Crabtree, 1992, 1993; O'Keefe et al., 1992). We therefore utilized a Ca"+"-independent constitutively active mutant of calcineurin (CNUMUT2B) to localize the site of the signaling defect in M101 and M108 relative to calcineurin. Expression of CNUMUT2B in wild-type cells synergizes with PMA to activate NF-AT-dependent transcription (Figure 5B) in a
capacitance are defective in the mutant cells. These results localize the signaling defect in both M101 and M108 as residing upstream of calcium in the signal transduction cascade.

Recently, a Ca\(^{2+}\) regulatory protein (CAML) was identified in Jurkat cells for its ability to interact with cyclolipins that have access to the T lymphocyte receptor signaling pathway (Bram et al., 1993). Remarkably, overexpression of this protein will replace the requirement for calcium ionophore in the activation of either NF-AT or IL-2 transcriptions (Bram and Crabtree, 1994). We therefore tested the ability of CAML to activate the M108 clone by transfection with CAMI and assaying the levels of NF-AT-dependent transcription (Figure 5D). CAML is unable to activate M108 (data not shown for M101), implying that the defect in this cell line is between CAML and calcium in the T cell activation pathway.

**M101 and M108 Exhibit Defects in Mitogen-Stimulated Calcium Influx**

Ca\(^{2+}\)-independent manner (Clipstone and Crabtree, 1993). Figure SC shows that CNMUT2B also synergizes with PMA to activate NF-AT-dependent transcription in M101 (data not shown for M108), indicating that neither NF-AT nor the intrinsic signaling pathway downstream of calcium in the mutant cells. These results localize the signaling defect in both M101 and M108 as residing upstream of calcium in the signal transduction cascade. Recently, a Ca\(^{2+}\) regulatory protein (CAML) was identified in Jurkat cells for its ability to interact with cyclolipins that have access to the T lymphocyte receptor signaling pathway (Bram et al., 1993). Remarkably, overexpression of this protein will replace the requirement for calcium ionophore in the activation of either NF-AT or IL-2 transcriptions (Bram and Crabtree, 1994). We therefore tested the ability of CAML to activate the M108 clone by transfection with CAML and assaying the levels of NF-AT-dependent transcription (Figure 5D). CAML is unable to activate M108 (data not shown for M101), implying that the defect in this cell line is between CAML and calcium in the T cell activation pathway.
Figure 6. Ionomycin Evokes an Abnormally Small \([\text{Ca}^{2+}]_i\) Rise in M101 and M108. Responses to 2 \(\mu\)M ionomycin plus 20 ng/ml PMA (added at the arrow) are shown for control Trunc.4 cells and mutant cells. Each graph is the average response of 100–200 cells in a single experiment. The initial transient and particularly the sustained phase of the \([\text{Ca}^{2+}]_i\) increase are diminished in both mutants.

Figure 7. Ionomycin in the Presence of High Levels of Extracellular Ca\(^{2+}\) Activate NF-AT-Directed Transcription in Mutant Cells. (A) Ionomycin (15 \(\mu\)M) and 10 mM added \(\text{Ca}^{2+}\) in complete medium elicits a sustained \([\text{Ca}^{2+}]_i\) increase in M101 (right) similar to that observed in control cells under standard stimulation conditions (left). The average responses of 100–200 cells are shown. (B) lacZ expression in control, M101, and M108 cells. Cells were stimulated for 8 hr at 37°C in complete medium supplemented where indicated with 10 ng/ml PMA, 2 \(\mu\)M ionomycin, or 15 \(\mu\)M ionomycin and 10 mM \(\text{Ca}^{2+}\). NF-AT-directed transcription in M108 is restored to wild-type levels, while NF-AT-directed transcription is partially restored in M101 under the conditions shown in (A).

The calcium signaling defect was further examined using the endoplasmic reticulum (ER)–Ca\(^{2+}\)–ATPase inhibitor, thapsigargin (TG). By blocking \(\text{Ca}^{2+}\) uptake, TG unmasks a constitutive leak of \(\text{Ca}^{2+}\) from the ER and thereby depletes intracellular stores (Lytton et al., 1991; Thastrup et al., 1990). In T cells and many other cells, \(\text{Ca}^{2+}\) store depletion is known to trigger \(\text{Ca}^{2+}\) influx through plasma-membrane \(\text{Ca}^{2+}\) channels by a process referred to as capacitative \(\text{Ca}^{2+}\) entry (Gouy et al., 1990; Putney, 1990; Mason et al., 1991; Sarkadi et al., 1991; Zweißlach and Lewis, 1993). As expected, treatment of control cells with TG resulted in a large sustained increase in \([\text{Ca}^{2+}]_i\) (Figure 8A). In contrast, TG evoked only a small \(\text{Ca}^{2+}\) transient in M101 and M108 with a greatly reduced plateau phase. The contribution of \(\text{Ca}^{2+}\) influx to these responses was determined by stimulating with TG in the absence of extracellular \(\text{Ca}^{2+}\). Under these conditions, TG evokes a small \(\text{Ca}^{2+}\) transient in wild-type as well as mutant cells, a result of unopposed \(\text{Ca}^{2+}\) leakage from intracellular stores followed by \(\text{Ca}^{2+}\) extrusion across the plasma membrane (Figure 8B). The similarity between mutant and control responses in a \(\text{Ca}^{2+}\)-free solution demonstrates that TG is able to deplete \(\text{Ca}^{2+}\) stores in the mutants. Subsequent addition of media containing 2 mM \(\text{Ca}^{2+}\) to control cells evokes a substantial \([\text{Ca}^{2+}]_i\) increase due to influx through depletion-activated \(\text{Ca}^{2+}\) channels in the plasma membrane (Figure 8B). In contrast, the addition of media containing 2 mM \(\text{Ca}^{2+}\) evoked only a small \([\text{Ca}^{2+}]_i\) increase in M101 and M108, indicating a relative lack of \(\text{Ca}^{2+}\) entry across the plasma membrane. These results provide direct evidence that both mutant cell lines exhibit a profound defect in the capacitative \(\text{Ca}^{2+}\) entry pathway normally coupled to the depletion of intracellular \(\text{Ca}^{2+}\) stores.

To assess further the nature of the two mutants, we carried out heterokaryon fusion analysis. Transient heterokaryons formed between control Trunc.4 cells and either M101 or M108, were assayed for changes in \(\text{Ca}^{2+}\) influx in response to TG. In each case, fusion of Trunc.4 to either mutant cell line partially complemented the signaling defect, resulting in a level of \(\text{Ca}^{2+}\) influx roughly midway between that of each of the donor cell populations (Figure 9A). These results indicate that the mutations in M101 and M108 are not able to suppress normal \(\text{Ca}^{2+}\) responses completely. In contrast, fusion of M101 and M108 to each other did not rescue the defect (Figure 9B), suggesting that the two mutations defined by M101 and M108 cannot complement one another in this assay. These cell fusion experiments must be interpreted with caution, since it is possible that organelles or protein complexes may have to intermix between the donor cells in order to permit assembly of the capacitative \(\text{Ca}^{2+}\) entry mechanism, and we do not know the time needed for this process.

Discussion
To gain insights into the mechanisms that transmit signals from the TCR to control gene transcription, we have iso-
Figure 8. M101 and M108 Are Defective for Capacitive Ca\(^{2+}\) Influx

(A) Ca\(^{2+}\) measured in control and mutant cells in response to addition of 1 μM TG in Ringer's solution (2 mM Ca\(^{2+}\), arrow). The steady-state response is greatly diminished in both mutants. (B) Intracellular Ca\(^{2+}\) release and Ca\(^{2+}\) influx measured in TG-stimulated control and mutant cells. As indicated at the arrows, cells were washed with Ca\(^{2+}\)-free Ringer's, 1 μM TG in Ca\(^{2+}\)-free Ringer's, or 1 μM TG in normal Ringer's (2 mM Ca\(^{2+}\)). The Ca\(^{2+}\) transient under Ca\(^{2+}\)-free conditions is relatively normal in the mutants, but the subsequent response to Ca\(^{2+}\) addition is much smaller than control. Thus, the coupling between store depletion and activation of Ca\(^{2+}\) entry across the plasma membrane is defective in the mutants.

Figure 9. The Mutations in M101 and M108 Appear to Be Codominant and Do Not Complement Each Other

Transient heterozygous fusion between control Trunc 4 cells and the indicated mutant cell lines were made and the Ca\(^{2+}\) responses of individual fused cells were observed. Cells were treated for 450 s with 1 μM TG in Ca\(^{2+}\)-free Ringer's solution. After the 450 s timepoint, the cells were treated with normal Ringer's solution (2 mM Ca\(^{2+}\)).

(A) The average responses of 38 M101/Trunc 4 heterozygous (solid line) fall between those of 24 self-fused Trunc 4 cells (dotted line) and 23 self-fused M101 cells (dashed line). This suggests that the mutation in M101 is codominant or recessive. (B) M101 and M108 do not complement each other. The averaged Ca\(^{2+}\) response of 26 M101/M108 heterozygous (solid line) is similar to the Ca\(^{2+}\) responses of the same 23 self-fused M101 cells (dashed line) shown above or 45 self-fused M108 cells (dotted line).

Labeled mutants in the human Jurkat T cell leukemia line that harbor defects in the signal transduction cascade leading to transcription factor activation. Thus, we established a stable cell line (Jurkat NZdipA) containing dipA under the transcriptional control of NF-AT. Stimulation of Jurkat NZdipA with ionomycin and PMA induces NF-AT-directed dipA expression, resulting in the rapid death of the cells. By isolating cells that survive this selection protocol, we have obtained two independent mutant cell lines (M101 and M108) that exhibit defects in the T cell signal transduction cascade leading to activation of NF-AT-dependent transcription. These mutants appear to be allelic, recessive (or at least not completely dominant), and are not restricted to the activation of NF-AT. Rather, both mutants exhibit a more pleiotropic signaling defect, including other Ca\(^{2+}\)-regulated transcription factors, such as NF-κB and NF-IL-2A (OAP/CTC-). Analysis of the Ca\(^{2+}\) signal in fura-2-loaded cells demonstrated that M101 and M108 are deficient for mitogen-stimulated Ca\(^{2+}\) influx.

The selection regimen was designed to take advantage of two points. First, mutation fixation requires DNA replication, which occurs during S phase (Hartwell and Weinert, 1989). Since it takes approximately 4 hr to go from the end of S to G2 to finally M, it is very unlikely that M101 and M108 are siblings, as only a 3 hr incubation was done before the bulk population was divided equally into ten flasks. Secondly, γ irradiation induces a 3 hr mitotic arrest, which would further delay any cellular replication (Kao and Puck, 1969; Weinert, 1992). These two points taken together suggest a 7 hr window before any of the irradiated cells would replicate. The ancestral cells of M101 and M108 were separated well before this window of time had ended. Furthermore, M101 and M108 are phenotypically different in their responses to extracellular Ca\(^{2+}\). Based on these considerations, the mutations in M101 and M108 are probably independent.

Calcium plays a pivotal role in the regulation of T cell activation. Stimulation of the TCR/CD3 complex with antigen or monoclonal antibodies activates phospholipase C
to produce inositol 1,4,5-trisphosphate (IP₃), which trig-
ggers a dramatic increase in [Ca²⁺] by releasing calcium
from intracellular stores and evoking sustained influx of
calcium across the plasma membrane (Goldsmith and
Weiss, 1988; Imboden and Stobo, 1985; Nisbet-Brown et
al., 1985; Weiss et al., 1984a). Intracellular Ca²⁺ chelators
such as BAPTA, which effectively buffer the increase in
[Ca²⁺] that results from intracellular stores but do not di-
nimish the increase in [Ca²⁺] due to influx across the
plasma membrane, do not inhibit IL-2 gene expression
(Gelland et al., 1988). Conversely, blockade of Ca²⁺ influx
by chelating extracellular Ca²⁺ completely prevents TCR/
CD3-induced IL-2 gene expression (Weiss et al., 1994b).
Finally, Ca²⁺ ionophores such as ionomycin, in the pre-
sence of phorbol ester, act as potent T cell mitogens (Mastro
and Smith, 1983). Taken together, these data indicate that
sustained mitogen-induced Ca²⁺ influx across the PM is
essential for the initiation of IL-2 gene transcription.

In striking contrast with control cells, treatment of both
M101 and M108 with ionomycin failed to produce a signif-
icantly sustained rise in [Ca²⁺]. Several lines of evidence
suggest that this finding accounts for the failure of iono-
mycin and phorbol ester to activate NF-AT-dependent
transcription in the mutants. First, concentrations of iono-
mycin and Ca²⁺ sufficient to elevate [Ca²⁺], in M101 and
M108 to levels found in stimulated wild-type cells, suc-
cceeded in activating NF-AT-mediated IScZ transcription
(Figure 7). Second, the PKC-mediated arm of the mito-
genic signaling pathway appears to be intact, as phorbol
ester induced normal levels of both NF-κB (Figure 4B)
and AP-1 (data not shown) activity and synergized with
constitutively active calcineurin to activate NF-AT (Figure
5B). Finally, the ability of constitutively active calcineurin
to promote NF-AT activity demonstrates that other ele-
ments of the signaling pathway, such as NF-AT and the
nuclear translocation machinery, are functional in the mu-
tant cells. Thus, the signaling defect in these cells appears
to reside in the mechanism that generates the sustained
increase in [Ca²⁺]. Consistent with this interpretation is
the finding that the calcium regulatory protein CAML
(Bram and Crabtree, 1994) is unable to rescue the defect
in M108 and M101 (Figure 5D), implying that the defect
in these clones is TCR-receptor distal to CAML and recep-
tor-proximal to calcineurin.

Much recent evidence suggests that antigen and mito-
gen elicited a sustained [Ca²⁺] rise in T cells through a
process known as capacitative Ca²⁺ entry. According to this
hypothesis, the depletion of the ER Ca²⁺ stock that
results from IP₃-triggered Ca²⁺ release generates a second
signal that opens Ca²⁺ channels in the plasma mem-
bane (Putney, 1990; Putney and Bird, 1993). The opera-
tion of this transduction mechanism has been demonstrated in
a great variety of cells by the ability of store-depleting agents
such as TG to activate profound Ca²⁺ influx without sig-
ificantly affecting IP₃ levels (Putney, 1990; Putney and
Bird, 1993). Two types of evidence indicate that mitogen-
regulated Ca²⁺ channels in T cells are activated by the
depletion of Ca²⁺ stores. First, TCR stimulation fails to
increase [Ca²⁺] in cells pretreated with a maximal dose of
TG (Mason et al., 1991; Sarkadi et al., 1991). Second,
shinma et al., 1994). The observation that two independent genetic approaches have produced mutations that affect intracellular calcium regulation, resulting in an inability to activate distal nuclear transcription events, underscores the critical role that calcium plays in T cell activation.

The molecular mechanisms underlying capacitative Ca<sup>2+</sup>-entry have not yet been clearly defined. A large variety of mediators have been proposed, including a novel diffusible messenger and small G proteins, as well as direct contact between proteins in the ER and plasma membranes (Fasolato et al., 1994; Putney and Bird, 1993). Further studies of calcium channels that mediate capacitative Ca<sup>2+</sup>-entry have yet to be isolated, largely as a result of their unique properties (Hoth and Penner, 1992; McDonald et al., 1993; Zweifach and Lewis, 1993) and a lack of high affinity blockers to serve as biochemical probes for channel purification (Fasolato et al., 1994; Lewis and Cahalan, 1995). These studies should significantly further our understanding of the mechanism by which calcium influx is regulated during the activation of T lymphocytes and other nonexcitable cells.

**Experimental Procedures**

**Cells and Cell Culture**

Jurkat cells were maintained in complete RPMI 1640 (GIBCO) supplemented with 10% (v/v) heat-inactivated (30 min at 56°C) fetal calf serum (Gemini Bioproducts), 100 U/ml penicillin, 50 U/ml streptomycin, 2mM HEPES (pH 7.2) (growth medium) in a 7% CO<sub>2</sub>, 93% air atmosphere. Clones transfected with pHFAT4Dpa.neo or pHFAT4DpaAurinc.neo were periodically cycled for 1 week intervals in the above media with 300 μg/ml hygromycin B (Calbiochem) and 1 μg/ml G418 (Calbiochem).

pHFAT4Dpa.neo Plasmid Construction

The plasmid pcDNA2x was used to prepare the vector for the Dpa construct. pcDNA2x was digested with HindIII and BamHI to construct a vector containing the NFAT1-controlled regulation unit and the polyadenylation motif. The ends were blunted with T4-DNA polymerase, phosphatase-treated, and gel purified. From the plasmid pD4A2x, a NcoI-BglII fragment was isolated and blunt-ended with Klenow. This fragment was ligated to the HindIII-BstXI pcDNA2x fragment to generate pcDNA2xDpa. A 2.7-kb fragment from a BamHI partial digest of pcDNA2xDpa was isolated and gel purified. The 2kb minimal promoter driving the neomycin (neo) resistance gene was isolated from the plasmid pBS2a.neo by digesting the plasmid with BamHI. The BamHI fragments from both pcDNA2xDpa and pBS2a.neo were ligated and the sense-oriented pHFAT4Dpa.neo plasmid was isolated. The orientation of the plasmid was confirmed by restriction digest. The pHFAT4DpaAurinc.neo plasmid was deleted by deletion of a 1.4 kb BamHI fragment containing the Dpa gene. The sense-oriented pHFAT4DpaAurinc.neo plasmid was isolated and the orientation of this plasmid was also confirmed by restriction digest.

**Transfection and Selection of G418-Resistant Jurkat NZDpa Clones**

The pHFAT4Dpa.neo and the pHFAT4DpaAurinc.neo plasmids were linearized by digesting with PvuI and gel-purified. Jurkat NFATZ cells were centrifuged and resuspended at a concentration of 10<sup>6</sup> cells/ml in growth media containing approximately 20 μg/ml of either of the purified DNA fragments (300 μg/ml) of the cells were electroporated in a Bio Rad GenePulser at 250 V with 900 μF capacitance. The electroporated cells were resuspended in 24 ml of growth media and 1 ml was seeded into a 24-well cell culture plate. The media were brought to 1 mg/ml with G418 24 hr after the initial plating of the cells, and the neomycin-containing media were changed periodically until resistant cells grew out of that selected population. The pHG418-1 DNA plasmid (Nakanishi et al., 1996), which confers resistance to dihydropterycin, was linearized with EcoRI and gel-purified (as above). This linearized DNA was electroporated into all mutant cell line isolated (as above). The pHG418-1-transfected mutants were grown in complete RPMI containing 100 μg/ml complete dihydropterycin toxin (List Biological Laboratories, Campbell, Calif.) every 24 hr after electroporation. Approximately 3-4 weeks after growing the pHG418-1-transfected mutants in the presence of dihydropterycin, viable cells were cloned utilizing the FAC5. The clones were expanded in RPMI containing 100 μg/ml dihydropterycin toxin and grown in this media for an additional month to verify that the mutants were now resistant to dihydropterycin toxin.

**5-Bromo-2'-Deoxyuridine Assays**

The FAC5-Gal assays were carried out as described previously (Flaing et al., 1990, 1991; Nolen et al., 1988). Between 10<sup>4</sup> and 10<sup>5</sup> cells were resuspended in 50 μl 0.1% sodium dodecyl sulfate (SDS) and 50 μl 0.1 M sodium hydroxide buffer in 0.5 M sodium phosphate buffer, pH 7.2. After 1 hr at 37°C, the absorbance was determined at 595 nm in a Beckman DU 640 spectrophotometer. Each sample was assayed in triplicate. The percentage of untransfected Jurkat cells transfected with 10<sup>5</sup> cells was calculated.

**Mutant Selection Procedure**

Log phase growing Jurkat cells (2 × 10<sup>5</sup>) (Jurkat cells normally divide every 16-24 hr under optimum conditions) were exposed, at room temperature, to 200 nM of γ irradiation. This level of irradiation allowed 70-75% survival of the cells. This was done to limit the number of "hit" or mutation events in each cell to an average of just one mutation event per cell, because multiple mutations would be very hard to characterize and to clone (but the chances of hitting the same pathway is very small). Immediately after mutagenesis, the 2 × 10<sup>5</sup> cells were returned to the cell culture incubator. The 2 × 10<sup>5</sup> cells were divided equally into ten 24-well plates at a cell density of 6 × 10<sup>5</sup> (or 2 × 10<sup>5</sup> cells/well/3 hr after mutagenesis and subsequent incubation). M101 and M101 came from flasks labeled 5X001-1 and 5X002-2, respectively. Cells in all flasks were allowed to recover from the mutagenesis (returning to log phase growth) and reach a cell density of approximately 1 × 10<sup>5</sup> cells/ml. When the cells grew to a density of 1 × 10<sup>5</sup> cells/ml (this level of cell growth, 200 nM of γ irradiation, was usually achieved after 3 days), the dead cells and debris were removed using standard Ficol-Hypaque method. (This is validated by the recovery of all cells from the medium, comparison of the number of viable cells from the original population to the final cell yield.)
NFαTαβδαγ construct. A small percentage of cell death could also be attributed to the nonspecific but toxic effects of PMA and ionomycin (data not shown).

Fresh RPMI was added to all flasks every 3-5 days in order to encourage cell growth. Usually 2-3 weeks after the first stimulation, the cells in these and other flasks grew back to 104 total cells. When the total cells in the flasks reached this approximate number, the stimulation protocol mentioned above was repeated. This stimulation protocol was repeated for a total of 3-5 times for each flask of cells. After each stimulation, a small number of cells was harvested along with control cells and the levels of γ-galactosidase activity were determined using the FACS-Gal assay. This was done to monitor how the γ-galactosidase activity in the various flasks of cells changed after each stimulation. When the results of the γ-galactosidase activity were analyzed utilizing the FACS-Gal assay, the cells were stimulated for 8 hr and the FACS-Gal assay was performed following standard procedures. The 8 hr time point immediately preceding the FACS-Gal assay was determined as the selection strategy. To ensure that the change in phenotype was not due to induction of cell mutations at the dpα locus or suppressor mutations that could have occurred during the FACS-Gal assay, the cells were also cloned based only on viability using the FACS and the resulting clones were later analyzed for γ-galactosidase activity and cell death.

Electrophoretic Mobility Shift Assays (EMSA)

Jurkat, NFAT2, and NZDpα cells were stimulated for 2 hr with 10 ng/ml PMA and 2 μM ionomycin at 37°C in complete medium. NF-AT binding activity was quantitatively constituted from unstimulated nuclear and cytoplasts fractions of Jurkat cells. Nuclear extracts were made as described previously (Fiering et al., 1990; Ohnstein and Edlund, 1986). Cytoplastic extracts were made from the same cells as the nuclear extracts (Planagan et al., 1991).

EMSA were done essentially as described (Fiering et al., 1990; Planagun et al., 1991). Total protein used in each binding reaction was 10 μg in a solution consisting of 10 mM Tris-HCl (pH 7.5), 50 mM NaCl, 0.5 mM EDTA, 5% glycerol, and 1.5 mg poly(dI-dC). The protein solutions were incubated for 20 min at 37°C in complete medium. NF-κB was derived from the human IL-2 enhancer (-285 to -255 bp). The oligonucleotide sequence is 5′-CCGAGAGAAAAACCTGTCCATACAGAAGCGCTT-3′. The samples were electrophoresed on 4% polyacrylamide gels.

Ribonuclease Protection Assays

Ribonuclease protection assays were done as described (Fiering et al., 1990; Melton et al., 1990). A probe was used that is capable of revealing transcription of both αεZ and endogenous IL-2 (Iwata et al.). Total RNA was purified by the guanidinium isothiocyanate/cesium trifluoroacetaate (CTFA) isopycnic centrifugation technique (Pharmacia). RNA (10 μg) was used for each experimental point. For mapping and sequence identification of the IL-2 mRNA, a 5′-labeled RNA probe was transcribed from the p3-digested pBluescript. Hybridization was done at 42°C, and the samples were digested with 5 mg/ml RNase A and 200 U/ml RNase T1 at 37°C for 1 hr. The digested samples were electrophoresed on a 6% denaturing polyacrylamide gel.

Heterokaryon Fusions

Cells were treated for 30 min at 22°C-25°C with 0.5 mM calcium/AM (which fluoresces green; Molecular Probes) to label the cytoplasm or with 1.6 μM 1,1-dioctadecyl-3,3,3,3-tetrachloroethylenecarbonyl perchlorate (dil, which fluoresces red-orange; Molecular Probes) to label the plasma membrane. After three washes in RPMI 1640, cells were resuspended at 2 x 106/ml and placed in wells of a 24-well plate with 2 x 104 of each fusion partner well per well. Cells were centrifuged (7 min at 400 x g), supernatant was aspirated, and cells were fused at 22°C-25°C essentially as described previously (Goldsmith and Weis, 1986), but in the presence of 0.1% xylitol (54% polyethylene glycol (PEG-1000; Electron Microscopy Sciences), 4% RPMI 1640, and 25 μl/ml of 7.5% sodium bicarbonate. After fusion, cells were returned to normal growth conditions for 37°C for at least 1 hr, after which they were loaded with 1 μM fura-2/AM for Ca2+ imaging. Imaging experiments were performed as described below. Following each Ca2+ imaging experiment, cells were observed using fluorescent and rhodamine filter sets (Chroma Technology Corporation) to determine which cells contained both calcine and dill, indicating a fused pair. Approximately 9% of the cells were double-labeled in each fusion experiment. [Ca2+]i values from fused cells were averaged using Igor Pro software (Wavemetrics).

Reporter Assays

Cells were transiently transfected, as described previously (Citronelle and Crabtree, 1992), with 5 μg of either OAP/CAT, NF-κB-CAT, or IκB-CAT. After washing, cells with fresh experiments, cells were transiently co-transfected with 10 μg of either pBBS, pBS-INA, pBBS-Nch, or the constitutively active pBBS-CMUT2B and 5 μg NF-AT-CAT. These plasmids have been described previously (Citronelle and Crabtree, 1992). The cells were stimulated for 24 hr after infection with the indicated agents at 37°C in complete medium for 20 hr. Ionomycin and PMA were used at final concentrations of 2 μM and 10 ng/ml, respectively. Following stimulation for 20 hr, the cells were harvested and assayed for chloramphenicol acetyl transferase activity according to established procedures (Gorman et al., 1989). The OAP/CAT-, NF-κB-CAT, and IκB-CAT data represent the mean of determinations from duplicate transfections and are representative of four independent experiments. The ON CAT data represent the mean of determinations from duplicate transfections and are representative of three independent experiments. For the CMU overexpression experiments, cells were transiently co-transfected with 5 μg of either pBBS-CMUT2B and 5 μg NF-AT-CAT. The CMU overexpression plasmid (CLUT12), and 5 μg NF-AT-CAT and SE-Fu Luc reporter plasmids described previously (Bram and Crabtree, 1994). Cells were incubated at 37°C in complete medium for 40 hr, and treated with the indicated agent(s). Ionomycin and PMA were used at final concentrations 0.5 μM and 25 ng/ml, respectively. The NF-AT-specific SEAP activity was then determined as described previously (Bram et al., 1993).

Video Microscopic Measurements of Intracellular Calcium ([Ca2+]i)

Cells were incubated at a density of 104/ml in culture medium containing 1 or 3 μM fura-2/AM (Molecular Probes) for 30 min at 37°C. After washing twice with fresh medium, loaded cells were allowed to settle onto poly-l-lysine-coated coverslips chambers. The chambers were placed on the stage of an inverted microscope (Nikon Diaphot). Experiments with ionomycin and PMA were performed in the presence of complete medium containing 10% fetal calf serum, and incubated temperature was maintained at 35°C-37°C by a thermostatically controlled stream of warm air directed at the point of contact between the microscope objective (40X CF Fluor, NA 1.3, Nikon) and the chamber. TG experiments were conducted at room temperature in a modified mammalian Ringer's solution containing: 150 mM NaCl, 4.5 mM KCl, 0 or 2 mM CaCl2, 1 mM MgCl2, 10 mM glucose, and 5 mM HEPES (pH 7.4 with NaOH). Cells were illuminated alternately at 350 ± 5 nm and 380 ± 10 nm using a 75 W xenon lamp and interference filters (Omega Optical) in a computer-controlled filter wheel (Lambda-10, Sutter Instruments). Emitted fluorescence was passed through a 480 nm long-pass filter to an intensified CCD camera (Hamamatsu Photonic). A VideoProbe image processor (ETM Systems) was used to digitize, average, and background-correct fluorescence images, and to divide each 350/380 image pair pixel-by-pixel to calculate ratio images. Bleaching of fura-2 was minimized by alternating use of light sources by a factor of 100 and by using an electronic shutter to restrict the illumination to periods of data collection. [Ca2+]i was estimated from ratio images using the relation (Ca2+)i = K′ (R - R0) / (1/2 - R0)), where K′, R0, and R are determined using an in situ calibration method as described previously (Lewis and Cahalan, 1989).
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Note Added in Proof