Reconstitution of CD3ζ Coupling to Calcium Mobilization via Genetic Complementation*

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The integrity of the T cell receptor complex (CD3-TCR) transduction machinery is central to T cell development and to T cell effector function. Molecular dissection of the multimeric CD3-TCR complex revealed that at least two associated polypeptides, CD3ζ and CD3ε, autonomously couple antigenic recognition event to early and late events of the intracytoplasmic activation cascade. A 18-amino acid motif based on a tandem YXXL stretch, the activation receptor homology sequence 1 (ARH-1) motif, is necessary and sufficient to the transducing properties of both CD3ζ and CD3ε. Stimulation of chimeric molecules made of ecto- and transmembrane domains of various cell surface proteins and intracytoplasmic domains of CD3ζ or CD3ε leads to an increase in the intracellular Ca²⁺ concentration ([Ca²⁺]i) in Jurkat cells. We describe here that a similar CD25/ζ chimeric molecule was unable to induce a detectable [Ca²⁺]i rise upon CD25 cross-linking once expressed in the murine thymoma BW-. A Ca²⁺ influx could, however, be triggered in BW- cells by thapsigargin, i.e., following depletion of Ca²⁺ stores. Somatic cell hybrids made from BW- and either thymocytes or mature lymph node T cells reconstituted the coupling of CD3ζ to the Ca²⁺ signal via an ARH-1 motif-dependent pathway. However, pervanadate-induced Ca²⁺ mobilization, a phenomenon attributed to tyrosine phosphorylation, was impaired in BW- cells and reconstituted in hybridomas. In contrast to the Ca²⁺ response, IL-2 production was induced in both BW- and hybrid cells, which questions the functional relevance of [Ca²⁺i], augmentation in T cell activation. In conclusion, the properties of the BW- thymoma, which define a novel group of CD3ζ transduction cell mutants, as well as its complementation by somatic cell fusion demonstrate that this cell line represents a useful model to dissect the signaling pathway that couples CD3ζ to Ca²⁺ mobilization by genetic reconstitution.

The CD3-TCR complex is a multimeric cell surface receptor composed of a module responsible for antigen recognition, and

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§ The abbreviations used are: TCR, T cell receptor; ARH-1, activation receptor homology sequence 1; [Ca²⁺i], intracellular Ca²⁺ concentration; IP₃, inositol 1,4,5-trisphosphate; PTK, protein-tyrosine kinase; SH2, src homology domain 2; TG, thapsigargin; IL, interleukin; mAb, monoclonal antibody.

Materials and Methods

mAb and Reagents—Mouse anti-human CD25 mAb (B1.49.9, Immunotech, Marseille, France), hamster anti-mouse CD8α (H58.55.3), and hamster anti-mouse CD3ε (2C11) have been described previously (9): mAb used in stimulation assays were purified on protein A-Sepha-
rose prior to use. Rabbit anti-hamster Ig antiserum was obtained from Jackson Laboratories. Thapsigargin (TG) was from Sigma and was used to stimulate intracellular Ca\(^{2+}\) release.

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**RESULTS**

**Deficient Coupling of CD3\(\xi\) to Ca\(^{2+}\) Mobilization in BW Cells**

The effect of cross-linking CD25/\(\xi\) chimeric molecules upon induction of Ca\(^{2+}\) response was investigated in different T cell lines. A CD25/\(\xi\) chimeric molecule was expressed in BW- and in JK cells, and representative clones of BW-.CD25/\(\xi\) (MM16.12) and JK.CD25/\(\xi\) (FEV20.2) transfected cells were further analyzed (Fig. 1, A and B). BW-.CD25/\(\xi\) and JK,CD25/\(\xi\) transfectants were stimulated with immobilized anti-CD25 mAb (10 \(\mu\)g/ml), and [Ca\(^{2+}\)]i values were measured by flow cytometry using a flow cytometer modified with a calcium-sensitive dye. The results are shown as the average variation of [Ca\(^{2+}\)]i as a function of time for a population of 30-50 cells (continuous line) and for two representative individual cells (dotted lines).

**IL-2 Production**

Flat-bottomed 96-well plates were coated with 10 \(\mu\)g/ml of indicated mAb in phosphate-buffered saline for 2 h at room temperature and 1 h at 4 \(^\circ\)C. After three washes in phosphate-buffered saline, 1 x 10^5 cells/well were added for 24 h at 37 \(^\circ\)C. Serial dilutions of cell supernatants were then assayed for their content in IL-2, using the CTL-2 cell line. Arbitrary IL-2 units/ml were calculated according to Gillis et al. (18).
Immobilized anti-CD25

BW. CD25/ζ

L16.24

T16.19

[Ca\textsuperscript{2+}] (nm)

min.

0 4 8 12

0 500 1000 1500

[Ca\textsuperscript{2+}] (nm)

min.

0 4 8 12

0 500 1000 1500

[Ca\textsuperscript{2+}] (nm)

min.

0 4 8 12

0 500 1000 1500

[Ca\textsuperscript{2+}] (nm)

min.

0 4 8 12

0 500 1000 1500

Fig. 2. Ca\textsuperscript{2+} responses elicited by CD25 cross-linking in T cell lines and hybridomas. At time zero, the indicated cell lines were allowed to settle in dishes that had been precoated with 10 μg/ml anti-CD25 mAb. In each panel are shown the [Ca\textsuperscript{2+}] responses of two typical single cells (dotted lines) and the average [Ca\textsuperscript{2+}] response of 30–50 cells (continuous line). The specificity of anti-CD25-induced Ca\textsuperscript{2+} mobilization was demonstrated by the absence of [Ca\textsuperscript{2+}] rise upon stimulation of each cell line on anti-CD8α coated glass coverslips (data not shown).

a weak but significant increase of [Ca\textsuperscript{2+}], due to the release of Ca\textsuperscript{2+} from intracellular stores; subsequent addition of Ca\textsuperscript{2+} in the extracellular medium resulted in a massive influx of Ca\textsuperscript{2+} ions across the plasma membrane (data not shown). From these results, it appears that CD3ζ is coupled to the canonical Ca\textsuperscript{2+} signaling pathway in JK but not in BW- cells. Therefore, these data suggest that one element connecting CD3ζ to the Ca\textsuperscript{2+} signaling machinery is missing in BW- cells.

Reconstitution of CD3ζ Coupling to Ca\textsuperscript{2+} Mobilization in BW-Cells—To further delineate the nature of the deficiency in the coupling of CD3ζ to the induction of [Ca\textsuperscript{2+}], increase in BW- cells, we investigated whether this defect could be complemented via somatic cell fusion. Accordingly, hybridomas made of BW-.CD25/ζ transfected cells and of either thymocytes (T16 hybrids) or lymph node cells (L16 hybrids) were generated and then selected for the cell surface expression of the CD25/ζ chimeric molecule. Representative hybrid cell lines corresponding to each of the two series (Fig. 1C, L16.24 and T16.19), were kept for further analysis. As shown in Fig. 2, stimulation of both L16.24 and T16.19 hybridomas through the CD25/ζ chimeric molecule led to an increase in [Ca\textsuperscript{2+}]. Although in the two hybridomas this increase was due to both Ca\textsuperscript{2+} release and Ca\textsuperscript{2+} influx (data not shown), we observed differences in the Ca\textsuperscript{2+} response of the two hybridomas. Responses of individual L16.24 cells showed [Ca\textsuperscript{2+}] oscillations, which are particularly marked during the first 5 min of stimulation. As the oscillations were not synchronous in different cells, the average response level was well below the maximal [Ca\textsuperscript{2+}], rise reached in single cell measurements. In contrast, [Ca\textsuperscript{2+}], responses of T16.19 hybridomas showed a large initial transient that was not followed by oscillations. Regardless of these differences, these complementation experiments demonstrate that the deficient coupling of CD3ζ to the induction of a [Ca\textsuperscript{2+}], response in BW- cells appears to be the consequence of a genetic recessive phenotype. Since JK cells are much more effective than reconstituted hybrids to mobilize Ca\textsuperscript{2+} upon chimera cross-linking, it is likely that a recessive component of a multigenic system is involved in BW- cell defect.

Reconstitution of CD3ζ Coupling to the Induction of a Ca\textsuperscript{2+} Response Is ARH-1 Motif-dependent—We further investigated whether the integrity of the ARH-1 motifs was a necessary condition to reconstitute CD3ζ coupling to Ca\textsuperscript{2+} mobilization in hybrids derived from BW- cells. A CD25/3YF chimeric molecule, composed of CD25 ecto- and transmembrane domains fused to a mutated form of CD3ζ, was expressed in BW- cells (Fig. 1A, BW-.CD25/3YF). In this mutant cDNA, the first Tyr residues of each of the three CD3ζ ARH-1 motifs were mutated into Phe residues. Hybridomas were then generated by fusing BW-.CD25/3YF cells to lymph node cells (L17 hybrids), and a representative hybrid cell line (L17.2) was assayed for Ca\textsuperscript{2+} response following CD25 stimulation. As shown in Fig. 3, stimulation of the CD25/3YF chimeric molecule did not lead to any detectable [Ca\textsuperscript{2+}], increase, whereas CD3 cross-linking induced a strong increase in [Ca\textsuperscript{2+}],. This result indicates that the reconstitution of CD3ζ coupling to the Ca\textsuperscript{2+} pathway depends upon the integrity of the ARH-1 motif.

Pharmacological Dissection of the Ca\textsuperscript{2+} Pathway Present in BW-Cells—The sesquiterpene lactone TG, by inhibiting microsomal Ca\textsuperscript{2+}-transport ATPases, depletes intracellular Ca\textsuperscript{2+} stores and thus provides an IP\textsubscript{3}-independent way of inducing Ca\textsuperscript{2+} release in most cell types (20). BW-.CD25/ζ and JK,CD25/ζ transfected cells as well as L16.24 and T16.19 hybridomas were stimulated with 0.5 μM TG in the presence or absence of extracellular Ca\textsuperscript{2+} to distinguish between Ca\textsuperscript{2+} release and Ca\textsuperscript{2+} influx. As shown in Fig. 4, TG treatment led to similar intracellular Ca\textsuperscript{2+} release and Ca\textsuperscript{2+} influx in all cell lines tested.
Coupling of CD3\(\zeta\) to Ca\(^{2+}\) Mobilization

![Image](https://via.placeholder.com/150)

**FIG. 3.** Ca\(^{2+}\) responses elicited by CD25 or CD3 cross-linking in L17.2 murine T cell hybridoma. L17.2 cells were settled onto an anti-CD25 (20 \(\mu\)g/ml) coated coverlip at time zero. After 4 min, anti-CD3 mAb (10 \(\mu\)g/ml) was added (first arrow), and cross-linking was performed using rabbit anti-hamster antiserum (RAH) (10 \(\mu\)g/ml, second arrow). The [Ca\(^{2+}\)]\(_i\) responses of two typical single cells (dotted lines) and the average [Ca\(^{2+}\)]\(_i\) response of 30-50 cells (continuous line) are shown. Demonstrating that the control of transmembrane Ca\(^{2+}\) influx by intracellular store depletion is functional in BW- cells and derived hybridomas. Moderate but significant differences were observed in the amplitudes of these responses. In particular, the amplitude of the [Ca\(^{2+}\)]\(_i\) increase due to Ca\(^{2+}\) release was significantly larger in BW- than in L16.24 and T16.19 cells (\(p < 0.001\)); on the contrary, the [Ca\(^{2+}\)]\(_i\) increase due to Ca\(^{2+}\) influx was significantly smaller (\(p < 0.001\)) in BW- cells than in the complemented cells (see also Fig. 5 in which the response to TG was evoked in a Ca\(^{2+}\)-containing solution). The reason for these differences has not yet been identified.

Pervanadate has been shown to induce an augmentation of [Ca\(^{2+}\)]\(_i\), which has been attributed to PTK stimulation (21-23), and therefore constitutes another useful way of triggering a Ca\(^{2+}\) response while bypassing the requirement for cell surface receptor engagement. This property was used to examine in BW-.CD25/\(\zeta\) cells the integrity of the PTK system involved in the coupling of CD3/ARH-1 motif to [Ca\(^{2+}\)]\(_i\) mobilization. As expected, 100 \(\mu\)M pervanadate triggered a [Ca\(^{2+}\)]\(_i\) increase in JK-.CD25/\(\zeta\) cells (data not shown) and in L16.24 cells (Fig. 5A). When pervanadate was added to L16.24 cells in the absence of extracellular Ca\(^{2+}\), an intracellular Ca\(^{2+}\) release was observed (Fig. 5B). As the delay between pervanadate application and Ca\(^{2+}\) release was quite variable from cell to cell, the average Ca\(^{2+}\) response developed slowly compared to that elicited by TG (Fig. 4). Readmission of extracellular Ca\(^{2+}\), after pervanadate-induced store depletion, led to a large Ca\(^{2+}\) influx. In contrast to L16.24 cells, no increase in the [Ca\(^{2+}\)]\(_i\) was detected in BW-.CD25/\(\zeta\) cells stimulated with pervanadate (Fig. 5C).

If the unique cellular target of pervanadate was a set of PTK, these experiments would suggest that at least one PTK involved in the coupling of CD3\(\zeta\) to Ca\(^{2+}\) mobilization was defective in BW- cells. However, vanadate is also a classical inhibitor of ATPases, including Ca\(^{2+}\)-ATPases (24); therefore, we examined the possibility that an inhibition of these Ca\(^{2+}\) pumps could contribute to the [Ca\(^{2+}\)]\(_i\) increase observed in the presence of pervanadate. In TG-treated L16.24 cells, when Ca\(^{2+}\) influx was abolished by suppression of external Ca\(^{2+}\), [Ca\(^{2+}\)]\(_i\) rapidly decreased to the resting level due to the activity of the plasma membrane Ca\(^{2+}\)-ATPase, the only Ca\(^{2+}\) extrusion system in T cells (25). Upon readmission of Ca\(^{2+}\), [Ca\(^{2+}\)] increased again, and remained elevated after addition of pervanadate. However,
when external Ca²⁺ was removed 5–10 min after the application of pervanadate, [Ca²⁺]₀ decreased more slowly than before pervanadate treatment, as can be visualized on a different time scale (data not shown), and did not reach the resting level but even exhibited a delayed increase (Fig. 5D). This phenomenon was not a consequence of repetitive exposures to Ca²⁺-free solutions (data not shown). These results show that 100 μM pervanadate (or a fraction of the vanadate molecules not peroxidi- zed by H₂O₂) caused a partial inhibition of the plasma membrane Ca²⁺-ATPase and may have also caused a Ca²⁺ release from TG-insensitive stores. If the microsomal Ca²⁺-ATPase is similarly affected by pervanadate, part of the pervanadate-induced intracellular Ca²⁺ release could be due to this TG-like effect. Thus, the [Ca²⁺]₀ rise elicited in L16.24 by pervanadate appears to result not only from a PTK-dependent phospholipase Cγ-1 activation (21–23) but also from a partial inhibition of Ca²⁺-ATPases. Therefore, the absence of pervanadate-induced Ca²⁺ response in BW- cells could have more than one origin. However, Fig. 5E shows that pervanadate also caused a partial inhibition of Ca²⁺-ATPases in BW- cells as in the L16.24 hybridoma. Thus, even though PTK are not the unique targets of pervanadate, it appears that a mutation affecting an ARH-1 proximal PTK (or a PTK-dependent event) best explains the BW- functional defect observed in the cou-
The CD25/3YF chimeric molecule, indicating that the integrity were mutated in the CD25/3YF chimeric molecule, these motifs were present, and thus the transducing defect was located at some other point downstream in the activation cascade. In addition, once Ca2+ stores were emptied (e.g., after TG treatment), a normal Ca2+ response was observed in BW- cells, showing that the coupling between store depletion and Ca2+ influx was not affected and that the mutation affects a point upstream of Ca2+ store depletion.

The observation that pervanadate failed to elicit a Ca2+ response in BW- cells has important implications. We were first concerned by the fact that besides inhibiting protein-tyrosine phosphatases and thus increasing PTK-dependent phosphorylation, pervanadate (or nonoxidized vanadate) could also inhibit Ca2+-ATPases, a fact that has often been underestimated. We indeed observed that pervanadate did reduce the activity of plasma membrane Ca2+-ATPases, an effect that may amplify in L16.24 cells the Ca2+ response due to tyrosine-phosphatase inhibition. However, the same partial inhibition of plasma membrane Ca2+-ATPases by pervanadate was observed in BW-.CD25/3 cells. Thus, the absence of a pervanadate-induced Ca2+ response in BW-.CD25/3 cells cannot be explained by a low sensitivity of plasma membrane Ca2+-ATPases to pervanadate in these cells. Therefore this lack of response likely results from the fact that a PTK-dependent event is not induced by pervanadate in BW-.CD25/3 cells.

A provisional conclusion is that the mutation could affect either the set of ARH-1 proximal PTK responsible for phospholipase Cγ1 activation or another PTK-dependent machinery implicated in Ca2+ release. The functional absence of p56lck and/or p59Fyn src family PTK as well as ZAP70 and/or p72syk tandem SH2 PTK could result in the BW- mutant phenotype, since these PTK have been proposed to be involved in the initiation of intracellular events triggered by CD3γ cross-linking. Indeed, in BW-.CD25/3 cells, triggering through CD16/ZAP or CD16/syk chimeric molecules in JK cells suggests the involvement of these PTK in the Ca2+ pathway. In addition, it has been shown that oxidizing conditions (1 mM H2O2) induce Ca2+ mobilization through an indirect activation of tandem SH2 PTK, which occurs independently of src family kinases. The inability of such oxidizing conditions to induce Ca2+ mobilization in BW- cells (data not shown), supports the possibility of a deficient activation of ZAP70 and/or p72syk in these cells. However, Northern blot analysis demonstrated that p59Fyn, p56lck, and minute amounts of p72syk mRNA could be detected in BW- cells as well as in L16.24 and T16.19 hybrids and in JK cells (data not shown). These results show that the corresponding genes are transcribed, but they do not prove that the PTK are functional in BW- cells. If the BW- functional defect is due to a mutated PTK, its effect is not a drastic one, since CD25 cross-linking or to CD8 cross-linking in cells transfected with a CD8γ construct; induction of tyrosine phosphorylation and of downstream in the activation cascade. In addition, the Ca2+ stores were emptied (e.g., after TG treatment), a normal Ca2+ response was observed in BW- cells, showing that the coupling between store depletion and Ca2+ influx was not affected and that the mutation affects a point upstream of Ca2+ store depletion.

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As mentioned above, one should also consider the possibility that the mechanism of Ca2+ release itself is affected in BW-
cells. In line with this hypothesis is the recent observation that CD25 cross-linking in BW-CD25/ξ cells induces both phospholipase C-γ1 activation and IP3 generation, a phenomenon that would not have been expected if the primary defect involved the early PTK-dependent phospholipase C-γ1 activation. The fact that IP3 is produced but Ca2+ is not released would mean that in addition to IP3, Ca2+ mobilization requires an accessory signal. Similarly, cross-linking of the antigen receptor in Lyn−/− DT40 B cells has been shown to cause a large IP3 production, which only results in a delayed and slow Ca2+ mobilization (31). It has been recently documented by ionomycin experiments. The next challenges and despite allowing the activation of the IL-2 gene.

Another possibility is that depletion of Ca2+ stores necessitates not only the opening of IP3-dependent channels but also an ARH-1-dependent signaling pathway. An-

Finally, our results also indicate that the induction of IL-2 secretion can occur without requiring a detectable Ca2+ response, at least initially (long term Ca2+ responses were not performed). The Ca2+ dependence of IL-2 production is quite controversial; some reports have shown that IL-2 production is strictly dependent upon the existence of a sustained [Ca2+]i rise (38) whereas others have shown that IL-2 secretion can take place without prior Ca2+ mobilization (39). It has been recently shown that the amplitude of the Ca2+ response that is required for the expression of the IL-2 gene or for inducing T cell proliferation is much lower after CD3 cross-linking than when the secretion can occur without requiring a detectable Ca2+ response. The next challenges will be the characterization of this alternative pathway as well as the identification of the defective molecule that in BW- cells prevents the coupling between CD3ζ and Ca2+ mobilization despite allowing the activation of the IL-2 gene.

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